The Role of the Environment in Transmission of Healthcare Associated Infection

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I, Elaine Anne Cloutman-Green confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed ……..

Date ……..

This thesis is partly based upon on work from the following jointly-authored publications:

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ABSTRACT

Infectious diseases are the current leading cause of human death and within this category nosocomial infections remain the most frequent complication of hospitalization. A range of infection prevention and control activities are employed to combat the selection and spread of these organisms. The principle components of which are: early identification of carriage/infection, patient isolation, improved hand hygiene, environmental control and good antimicrobial stewardship. In order to properly focus these interventions, it is essential to know how and when cross transmission has occurred.

There is an ongoing debate about the role of the environment in the spread of healthcare associated infections and to what extent if any it acts as a potential vector for transmission. Within the healthcare setting patients spend a substantial amount of time surrounded by equipment and environmental surfaces that may be contaminated with microorganisms. In order to establish what role the environment could play, tracking the spread of organisms by molecular typing is key. The current methods used to do this are complex and often are only available at reference laboratories. This means that turnaround times are slow and only provide retrospective confirmation of cross-transmission events.

Infection control interventions that can be used prior to receiving results play an important role. The selection and effectiveness of these interventions are often poorly supported by research studies, leading to problems with the introduction of evidence based practice and thus difficulty in selecting the most appropriate response to suspected cross transmission.

This thesis aims to explore the role of the environment in cross transmission of infection by developing sampling methodologies to permit environmental surveillance, validating and developing typing techniques in order to establish epidemiological links between patients and environmental contamination and to evaluate infection control interventions to aid in prevention of cross transmission events.

CONTENTS

FIGURES

TABLES

ABBREVIATIONS

ACH	air changes per hour
AFLP	amplified fragment length polymorphism
ATPase	adenylpyrophosphatase triphosphatase
ATP	adenosine triphosphate
BHI	brain heart infusion
bp	base pairs
BS6	bed space 6
BS7	bed space 7
BSA	bovine serum albumin
CDC	Centre for Disease Control
CDI	*Clostridium difficile* infection
cDNA	complementary DNA
CFU	colony forming units
CICU	Cardiac Intensive Care Unit
COSHH	control of substances hazardous to health
CRE	carbapenem resistant Enterobacteriaceae
CT	cycle threshold
DMEM	Dulbecco's Modified Eagles Medium
DMHP	dry mist hydrogen peroxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxynucleotide tri phosphates
DoH	Department of Health
DTT	Dl-dithiothreitol

EAEC	enteroaggregative e.coli
E.coli	escherichia coli
ECM	extracellular matrix
eDNA	extracellular deoxyribonucleic acid
EHEC	enterohemorrhagic pathogenic e.coli
EPEC	enteropathogenic pathogenic e.coli
EPIC	European Prevalence of Infection in Intensive Care
EPS	extracellular polymeric substance
ESBL	extended spectrum β-lactamase
EtBr	ethidium bromide
ETEC	enterotoxigenic pathogenic e.coli
ExPEC	Extraintestinal pathogenic e.coli
FBS	foetal bovine serum
FCS	foetal calf serum
GAM	generalised additive models
GOSH	Great Ormond Street Hospital
H	hours
HAI	hospital acquired infection
HCAI	healthcare associated infection
HCW	healthcare workers
HDU	high dependency unit
HEPA	high efficiency particulate air
HODU	haematology/oncology day unit
HP	hydrogen peroxide
HPV	hydrogen peroxide vapour

HSCT	hematopoietic stem cell transplantation
HSCTU	hematopoietic stem cell transplantation unit
HTS	high throughput sequencing
HVR-7	hyper variable region number 7
IIU	Infectious Disease and Immunology Unit
IPC	internal positive control
IR	incidence ratios
ISO	International Organisation for Standardisation
ITU	intensive care unit
IV	intravenous
KB	kilo-base
kDa	kilo-Dalton
KL	Kullback-Leibler
MALDI-TOF	matrix-assisted laser desorption/ionisation time-of-flight
MDR	multi drug resistant
mg	milligram
ml	millilitre
min	minutes
MITU	medical intensive care unit
MLST	multi locus sequence typing
mM	micro molar
MMLV	Moloney Murine Leukaemia ~Virus
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectrometry
NCBI	National Center for Biotechnology Information,
NCTC	national collection of type cultures

ng	nanograms
NHNN	National Hospital of Neurology and Neurosurgery
NHS	National Health Service
NIBSC	National Institute for Biological Standards and Control
NICU	neonatal intensive care unit
nm	nanometers
NPV	negative predictive value
nt	nucleotide
ORF	open reading frame
PBS	phosphate buffered saline
PC	Pearson Correlation Co-efficient
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PGM	personal genome machine
PHE	Public Health England
PICU	paediatric intensive care unit
PIP	prior inpatient colonisation
pmol	picomole
PPE	personal protective equipment
PPV	positive predictive value
RAPD	random amplified polymorphic DNA
REP	repetitive extragenic palindromic sequence
REP-PCR	repetitive extragenic palindromic sequence PCR
RLU	relative light unit
RNA	**r**ibonucleic acid
RNase	ribouclease

S	seconds
SAB	Sabouraud agar
SCC	Staphylococcal cassette chromosome
SITU	surgical intensive care unit
SNV	single nucleotide variant
SPP	species
ST	sequence type
TSA	tryptone soya agar
TVC	total viable count
UK	United Kingdom
USA	United States of America
UTI	urinary tract infections
UV	ultraviolet light
VNTR	multi-locus variable number tandem repeat
VRE	vancomycin resistant Enterococci
v/v	volume to volume
WGS	whole genome sequence
WHO	World Health Organisation
w/v	weight to volume
˚C	degrees Celsius
µl	microlitres
µM	micro molar
µg	micrograms

Chapter 1 INTRODUCTION

1.1 THE IMPORTANCE OF HEALTHCARE ASSOCIATED INFECTIONS

Infectious diseases are the current leading cause of human death and within this category nosocomial infections remain the most frequent complication of hospitalization.[1] The United Kingdom (UK) Department of Health (DoH) defines healthcare associated infection (HCAI) as "any infection by any infectious agent acquired as a consequence of a person's treatment by the UK National Health Service (NHS) or which is acquired by a health care worker in the course of their NHS duties".[2]

HCAI may lead to poor clinical outcomes and death.[3] In high income countries HCAI affects approximately 5 – 15% of patients, whereas figures from low income countries indicates that prevalence rates are in the region of 15 – 19%.[4] Within the United States (US) in 2002 there were ~1.7 million HCAIs of which ~100000 resulted in death, of which a significant proportion were antimicrobially resistant.[5] Within the United Kingdom (UK) HCAI affect >6% of hospitalised patients, with those patients in intensive care units (ITUs) being fourfold more likely to develop an infection.[6] Therefore different ward types and specialities differ in the associated risk of HCAI.[7]

Microbes within the healthcare environment can be transmitted by inhalation, contaminated hands or surfaces, faecal-oral routes or bodily fluids.[7] Patients that are particularly susceptible to HCAI include those with severe underlying disease, long hospital stays, old age, and admission to ITU.[7] It is estimated that of the HCAIs developed within the ITU, 40 – 60% are due to endogenous flora, 20 – 40% are due to the hands of contaminated healthcare workers (HCW), 20 - 25% are due to antibiotic driven change and 20% is potentially due to environmental contamination.[8] However the role of the environment in these figures is contentious.[8]

Despite a range of infection prevention and control activities being employed to combat HCAI, the overall prevalence estimation within England has only fallen from 8.2% to 6.4% from 2006-2011.[1] Yet up to a third of HCAIs are believed to be preventable, with only 37% of HCAIs the result of directly attributable transmission from other patients.[9, 10] It is however difficult to differentiate a true nosocomial transmission from unrelated cases and without epidemiological typing assumptions about outbreaks may be falsely drawn.[11] This is partly because HCAIs present a unique challenge due to the

difficulties in establishing when patients are asymptomatically colonised, not just symptomatically infected; which complicates determining transmission dynamics.[11]

1.1.1 The Importance of Paediatric Infection Control

Infection control is particularly important within the paediatric environment due to the diverse needs and susceptibilities of these patients.[12] HCAI among children differ from those observed in adult populations, by both the sites and patterns of distribution.[12] Children interact closely with their environment, through toys and other objects, and so environmental contamination poses a unique risk. [12], [13] They also utilise shared ward areas more frequently than adult patients and due to the movement of both patients and their families through hospital environments, it is postulated that they are much more likely to be involved in person to person transmission of infection.[14]

Many viral infections are first acquired in childhood and may be associated with prolonged infectivity and increased viral load.[12] It was recently demonstrated that 12.2% of paediatric patients developed HCAIs with respiratory or gastrointestinal viruses.[15] Additionally children who become colonised with multi-drug resistant (MDR) bacteria may be colonised for prolonged periods with continuous self-contamination. Both cases act as a means by which children become an important source of environmental contamination and a reservoir for other forms of transmission.

1.2 Organisms Commonly Responsible for Healthcare Associated Infection

1.2.1 *Staphylococcal* species

Staphylococcal species (spp.), which are Gram-positive cocci belonging to the Staphylococcaceae, were first described in 1882 by Sir Alexander Ogstoni.[16] *Staphylococcus epidemidis* in a normal constituent of skin flora and is predominantly a cause of nosocomial infection in catheterized patients, as well causing infective endocarditis.[17] *Staphylococcus aureus*, which was named for its golden pigment by Friedrich J. Rosenbach, is a common human pathogen and causes skin and soft tissue infections, and pneumonia, as well as infections linked to invasive devices within all age groups.[16, 18, 19] In addition to infection it can also cause toxin related illness, such as toxic shock and food intoxications.[18]

Staphylococcus aureus is a leading human pathogen associated with HCAI worldwide, and can cause both colonisation and infection.[18] *Staphylococcus aureus* is isolated

from the anterior nares in about 30% of the population and nasal carriage often precedes infection.[18] It also colonises other sites such as the throat, perineum and axillae, but the anterior nares is the site most commonly associated with colonisation.[19, 20] Children are thought to act as vectors for *Staphylococcus aureus* spread both within the community and hospital setting.[19] Within the UK there were 8767 reported incidents of *Staphylococcus aureus* bacteraemia in 2012 with an associated mortality rate of 50% in patients with complicated infection, and 30% in other groups.[21] *Staphylococcus aureus* can survive for long periods within the environment, but the main source of transmission within the healthcare setting is believed to be person to person via the hands of HCW, although it is believed the environment may play a role.[22]

The main form of clinically significant resistance in *Staphylococcus aureus* is resistance to β-lactam agents including methicillin. The mechanism for this resistance resulting in methicillin-resistant *Staphylococcus aureus* (MRSA) was first identified in 1981 and involves the expression of transpeptidase (PBP2a) encoded for by the chromosomal *mecA* gene, which is located on a mobile genetic element known as the *Staphylococcal* cassette chromosome (SCC).[16] This resistance has led to MRSA being identified as a major cause of preventable nosocomial infections, with a mortality from bacteraemia of 20% at seven days and 38% at 30 days.[23, 24]

MRSA infection is a leading cause of morbidity and mortality in inpatients, with the incidence of infections increasing twofold/threefold since the late 2000s worldwide.[25] MRSA prevalence rates in the US increased from 46.3/1000 inpatients in 2006 to 66.4/1000 inpatients in 2010, and within the EU there were ~170000 patients detected as MRSA positive annually.[26, 27] MRSA strains now account for 20% - 40% of *Staphylococcus. aureus* detected in hospitals where strains are endemic, such as those within the UK.[28]

Transmission of MRSA, similar to methicillin sensitive *Staphylococcus aureus*, is via the hands of HCWs, although other modes of spread, such aerial dispersal, have been noted in the literature.[29] MRSA environmental contamination has been demonstrated to be linked with the level of nasal carriage in patients.[20, 30, 31] 5% of HCWs caring for MRSA positive patients become long term colonised with MRSA of which 5% go onto develop clinical infection. Short term colonisation is thought to be much more frequent, however HCWs carrying MRSA have rarely been demonstrated to be the source of outbreaks.[7] The exact mode of acquisition is therefore unknown but there have also

been links to seasonality in *Staphylococcus aureus* infections, with more being detected in summer months.[25]

Legislation and national infection control interventions were introduced within England in an effort to decrease rates of MRSA within hospitals. These included targeted screening of at risk patients in 2006 and active surveillance of all patients in 2010 (as discussed in section 1.5.2.).[32] These were introduced as it was acknowledged that the spread of MRSA presented a significant threat to public health.[33] This has led to a reduction in rates of MRSA by 85% between April 2003 and March 2011.[32] However the focus has been on MRSA rather than sensitive *Staphylococcus aureus*, due to the associated treatment complications. The spread of *Staphylococcus aureus* is clonal, whether sensitive or resistant, and in recent years there has been an increase in community infections with both sensitive and resistant *Staphylococcus aureus* leading to skin and soft tissue infections in otherwise healthy young adults.[34]

1.2.2 *CLOSTRIDIUM DIFFICILE*

Clostridium difficile is a Gram-positive anaerobe that was first isolated in stool in 1935.[35] *Clostridium difficile* causes disease through the production of enterotoxin A and cytotoxin B which represent the major virulence factors for *Clostridium difficile*.[35, 36] However not all strains of *Clostridium difficile* carry the toxin genes. *Clostridium difficile* infection (CDI) is due to the presence of a toxin competent strain of *Clostridium difficile* in faeces. CDI may be associated with no disease (colonisation) or with a spectrum of *Clostridium difficile* associated disease, ranging from mild diarrhoea to pseudomembranous colitis and toxic megacolon.[37]

Within the US a fourfold increase in rates of CDI has been observed since 1993, with levels reaching 346,800 cases and ~30,000 deaths in 2010. Within the US CDI is 21% more common than MRSA infections (not including colonisation).[35] In 2009 the rate of CDI in Scotland was 0.71/1000 occupied bed days in those aged over 65.[37] Mortality rates depend on whether the infecting *Clostridium difficile* strain is a toxin hyper producer or not, but can be as high as 30%.[36, 38] Mortality rates are highest amongst elderly patients who have underlying medical conditions.[39, 40] Risk factors for developing CDI include prior antibiotic use, especially clindamycin, cephalosporins and/or fluoroquinolones.[37] 25% of cases of CDI occur in paediatric patients; the majority of cases occur in haematology/oncology patients, but the source of these infections is unclear.[14]

Patients with CDI excrete between $10*10^4$ and $10*10^7$ of *Clostridium difficile*/gram of faeces.[39] Contamination of the environment is common due to aerosolization of spores during diarrhoeal episodes.[39] This can lead to high levels of environmental contamination, especially in areas such as toilets and side rooms, as well as on pieces of equipment.[39, 41] *Clostridium difficile* spores are resistant to disinfectants and can survive for months or years on surfaces.[39] This has led to the opinion that while the main route of transmission is via the hands of HCW, the environment acts as a possible reservoir.[35]

Within the NHS a surveillance system was established in 1990, with mandatory surveillance instigated in 2004. Clinical interventions were also introduced in the form of high impact interventions or care bundles.[42] Strategies for controlling CDI have focussed on hand hygiene compliance, antimicrobial stewardship and environmental control.[43] This has led to a decrease in cases in the >64 age group, however the incidence in the 2 – 64 age group only decreased by 9%.[42] Other strategies to control CDI have included early diagnosis, staff education and use of appropriate isolation strategies.[36]

1.2.3 *KLEBSIELLA* SPECIES

Klebsiella spp. are Gram-negative non-motile organisms first described in 1882 and belonging to the Enterobacteriaceae.[44-46] A distinguishing feature of the genus is production of a thick polysaccharide coat which when hyperproduced, and along with the *megA* gene, is thought to be associated with virulence.[47, 48] The genus occupies a diverse range of ecological niches including: soil, water and warm blooded mammals.[44] In 1984 the genus *Klebsiella* was classified as containing five species: *K. pneumoniae*, *K. oxytoca*, *K. terrigena*, *K. ornithinolytica* and *K. planticola*. *K. pneumoniae* is comprised of three subspecies: *K. pneumoniae sub sp pneumoniae*, *K. pneumoniae sub sp. ozenae* and *K. pneumoniae sub sp. rhinoscleromatis*.[45]

Klebsiella pneumoniae is the most important of the species and accounts for 95% of all *Klebsiella* spp. isolated from clinical samples.[49] The species is characterised by its inability to grow at 10^{o}C or failure to utilize L-sorbose as a carbon source unlike the other *Klebsiella* spp.[45] *Klebsiella pneumoniae* is an important nosocomial pathogen, causing 15% of Gram-negative infections within intensive care units (ITUs) and is the second most frequent cause of Gram-negative sepsis, and is also known to cause pneumonia, urinary tract and wound infections.[50-54] *Klebsiella pneumoniae* infections primarily affect immunocompromised patients and have an associated mortality rate of

>50%. This is in part due to the capsule; which is considered to be a significant virulence factor, with certain capsular types being linked to invasive disease e.g. K1, K2, K45 and K57.[51, 55]

Klebsiella spp. are frequently found within the human gastrointestinal tract, but have also been found to colonise both the skin and upper respiratory tract of hospitalised patients.[54] Colonisation with microorganisms is known to be a potential source for cross transmission and *Klebsiella pneumoniae* is capable of causing asymptomatic colonisation of patients; this can lead to an unrecognised reservoir.[51, 56, 57] Colonisation with *Klebsiella pneumoniae* and/or *Escherichia coli* has been noted to be more likely in patients that are also colonised with MRSA. This is likely due to similar risk factors for colonisation.[57] For infants the median length of colonisation after discharge from hospital was 12.5 months and in adults the longest length of carriage was 40 months, meaning that these patients continue to act as reservoirs in the community after they have left the healthcare environment.[58-60] Carriage length was extended with prior or further antibiotic use, and four negative samples were required before a patient could be considered clear of colonisation.[58-60]

Isolates associated with nosocomial infection are often linked with antibiotic resistance including carbapenemases such as *Klebsiella pneumoniae* carbapenemase (KPC) and Oxacillinase-type β-lactamas.[55] These antibiotic resistance mechanisms combined with other virulence factors such as siderophores and adhesins, mean that *Klebsiella pneumoniae* is capable of causing outbreaks both in children and adult hospital units.[44] Worldwide the non-uniform spread of extended spectrum β-lactamase (ESBL) positive *Klebsiella pneumoniae* has led to prevalence rates as high as 45.4%.[44, 61, 62] ESBLs are plasmid borne and result in resistance to broad spectrum cephalosporins and other antibiotics, meaning they are more difficult and expensive to treat.[52, 63] As *Klebsiella* spp. are closely related to other members of the Enterobacteriaceae horizontal exchange of plasmids and insertion elements is facilitated. This means that the intra species strain variation within *Klebsiella pneumoniae* strains is considerable, with many strain specific genes and genomic arrangements.[47]

It is believed that *Klebsiella* spp. are primarily acquired from environmental sources in addition to other patients.[64] *Klebsiella* spp. are particularly problematic within paediatric environments where 77% of children are reported to be colonised in their stool, 19% in the pharynx and 42% have contamination on their hands.[65]

1.2.4 *ENTEROBACTER* SPP.

Enterobacter spp. are Gram-negative Proteobacterium belonging to the Enterobacteriaceae.[66] The genus forms a sub-clade with *Klebsiella* spp. and represent saprophytic organisms that constitute a normal part of the human digestive tract.[67] The main pathogenic species within this genus are *Enterobacter cloacae*, *Enterobacter aerogenes* and *Chronobacter sakazakii* (formerly *Enterobacter sakazakii)*.[68] Within the *Enterobacter* spp., *Enterobacter cloacae* actually consists of a large complex consisting of 12 genetic clusters, including *E. asburiae*, *E. cancerogenius*, *E. hormaechei*, *E. ludwigii*, *E. kobei*.[69, 70] The *Enterobacter cloacae* complex includes an extremely diverse group of bacteria that are associated with plants, soil and humans.[66] All members of the complex can reproduce rapidly at room temperature and remain viable for several days on surfaces.[71]

Enterobacter cloacae rarely causes primary disease in healthy adults; however it is frequently isolated from non-stool samples in hospitalised patients.[72] Within hospitalised patients it is an important opportunistic pathogen that has been responsible for urinary tract infections (UTIs), sepsis, pneumoniae and bronchopneumonary dysplasia in hospitalised neonates.[66, 67, 73, 74] Increasingly outbreaks have been detected associated with *Enterobacter cloacae* mortality rates of 27% - 61%. These have been linked to equipment such as contaminated infusions, door knobs improperly disinfected digital thermometers, and contaminated blood gas machines.[69, 71, 75-77] Outbreaks often continue for months and require interventions such as improvements in hand hygiene practices, admission restriction and ward closures or changes in antibiotic policy to bring them under control.[72, 78] Due to the genetic diversity within the *Enterobacter cloacae* complex it is often difficult to know whether outbreaks are due to a single source or whether the situation is actually due to polyclonal endemic infections, due to different isolates within the *Enterobacter cloacae* complex.[69]

Infections due to *Enterobacter cloacae* are complicated due to the presence of intrinsic resistance to: penicillins, first, second and third generation cephalosporins and amoxicillin/clavulanic acid, as they produce a chromosomal AmpC β-lactamase.[75-77] Centre for Disease Control (CDC) ITU surveillance data demonstrates that within this setting 31% of *Enterobacter* infections were caused by *Enterobacter* spp. resistant to third generation cephalosporins.[79] These resistant species have been associated with increased mortality, hospital stays and hospital charges.[80]

Cronobacter sakazakii (formerly *Enterobacter sakazakii*) is mostly associated with outbreaks due to contaminated infant formulae.[81-86] It is also an opportunistic pathogen and a rare cause of meningitis, necrotizing enterocolitis and sepsis. [85, 87]

Most *Enterobacter cloacae* infections are thought to be endogenous, but when exogenous acquisition occurs it is thought to be via contaminated equipment or associated with transmission by hand carriage and hand transfer.[88]

1.2.5 *ESCHERICHIA COLI*

Escherichia coli (*E. coli*) is the major constituent of the resident facultative anaerobic microbiota in the healthy human digestive tract.[89-91] It is a Gram-negative bacillus and member of the Enterobacteriaceae with the population composed of five major phylogenetic groups designated A, B1, B2, D and E.[89, 92, 93] Up until the 1940s it was considered that *E. coli* was non-pathogenic. However phenotypic and genotypic studies have now demonstrated that pathogenic *E. coli* strains exist and belong to only a few subgroups or clonal groups and these have been divided into pathotypes. Pathotypes are based upon the diseases they cause and the virulence factors they possess, as well as their host of isolation.[94, 95]

- Enterotoxigenic pathogenic *E. coli* (ETEC) – common cause of travellers' diarrhoea and is estimated to cause ~40,000 cases a year worldwide.[96] In addition it causes diarrhoea in neonates and young children within developing countries causing ~1 billion episodes per year, and is an important cause of diarrheal deaths in developing countries.[94, 97] Disease is facilitated by the production of both heat labile toxin and heat-stable toxin, and toxin detection is required for disease confirmation.[94, 98, 99]

- Enteropathogenic pathogenic *E. coli* (EPEC) – causes persistent diarrhoea in children under five years of age in developing countries and is characterised by the ability to attach to intestinal epithelial cells and create lesions.[94, 100]

- Enterohemorrhagic pathogenic *E. coli* (EHEC) – results in haemorrhagic colitis and haemolytic uremic syndrome.[94]

- Enteroaggregative *E. coli* (EAEC) - emergent diarrhoeal pathotype implicated in travellers' diarrhoea and infection in immunocompromised children in developing countries.[94]

- Extraintestinal pathogenic *E. coli* (ExPEC) - colonises the extraintestinal compartment of animals and humans, and can result in UTIs, meningitis, peritonitis and sepsis.[94] Most extra-intestinal infections are caused by pylogenetic groups B2 and D.[101]

In contrast to commensal strains, different pathotypes contain specific virulence genes, these virulence factors include: adhesins and fimbriae, toxins, siderophores, polysaccharide coatings and invasins.[89, 94, 102]

Pathogenic *E. coli* infections are responsible for significant social and economic costs for both community and hospital healthcare.[103, 104] *E. coli* is the most common aetiological agent causing UTI infection and is believed to be responsible for 90% of community UTIs and 50% of nosocomial UTIs with infection especially common within elderly patients.[105-108] It has been estimated that 150 million cases of *E. coli* UTI occur globally per year, and cost about 6 billion dollars for national health resources.[103] Within the US alone UTI treatments in 2000 amounted to costs of ~$2.47 billion.[109]

In addition to causing UTIs, *E. coli* is a frequent cause of other infections including intra-abdominal infection, meningitis and sepsis.[50, 54, 107, 110, 111] It is the leading cause of Gram-negative bacteraemia and is a serious threat in immunocompromised patients, with a case fatality of 5 – 30%.[90, 108, 110-116]14

E. coli is the leading cause of bacteraemia within the UK.[117] Between 2002 and 2009 reported bloodstream infections caused by *E. coli* have increased by 71% (from 688 to 18240), over the same time period a significant decline in antimicrobial susceptibility has also been noted.[54] The most appropriate therapeutic options for *E. coli* are usually β-lactams or fluoroquinolones; however increased resistance has been reported in both invasive and non-invasive isolates.[118, 119] MDR CTX-M-15 producing strains of *E. coli* have emerged worldwide as an important community and hospital pathogen, constituting a serious public health concern.[103, 120]

The mechanism of spread within healthcare settings is not fully understood, especially outside of outbreak situations, although person-person spread is believed to be most likely.[107, 121] It is possible to be colonised with more than one strain of *E. coli* and the levels of colonisation in healthy patients with resistant isolates is increasing, with one study finding a tenfold increase between 2006 and 2010.[108, 122] This is significant as *E. coli* is known for its propensity to exchange genetic material and for its mobile genome, despite its clonal population structure, with its accessory genome being

extremely heterogeneous.[123, 124] Therefore plasmids and other resistance determinants could be exchanged, especially as colonisation with resistant strains can last for up to 178 days.[125]

1.2.6 *ACINETOBACTER* SPP.

The genus *Acinetobacter* consists of 33 organisms of which 18 have been named and described as species.[126, 127] All *Acinetobacter* spp. are non-fermentative Gram-negative coccobacilli belonging to the family *Moraxellaceae*, and with the exception of *Acinetobacter baumannii*, they are widely distributed in soil and water.[128-130] *Acinetobacter baumannii* species is actually a complex comprised of *Acinetobacter baumannii* and its close genomic relatives species 2 and 13TU.[126] The members of this complex are very difficult to distinguish accurately and therefore reports both within this thesis and the literature should be assumed to refer to the complex.[126] *Acinetobacter baumannii* is rarely detected from natural environments whereas it is frequently identified in healthcare environments.[127, 131]

Infections caused by non *Acinetobacter baumannii* species are relatively unusual and are usually linked to catheter related sepsis.[126] *Acinetobacter baumannii* in contrast is becoming increasingly recognised as a cause of nosocomial infections, including pneumonia, sepsis, wound infections, UTIs and post neurosurgery meningitis.[132] Outbreaks often implicate the hospital environment, where *Acinetobacter baumannii* can survive for long periods on surfaces and survive desiccation and spread via contaminated hands.[128, 132-135] *Acinetobacter* spp. were the 7th most common isolates recovered from critically ill patients during the European Prevalence of Infection in Intensive Care (EPIC) study, accounting for between 8 and 10% of all cases of bacteraemia and pneumoniae respectively.[133, 136] Outside of the ITUs, burns and high dependency units experience the highest rates of infection, infections are associated with adverse clinical outcomes including: high rates of morbidity and mortality, prolonged hospital stay and substantial healthcare expense.[126, 128, 131]

Infections with antibiotic resistant *Acinetobacter baumannii* have been reported, including organisms resistant to all available antimicrobials.[128] UK surveillance data show that resistance to carbapenems within *Acinetobacter baumannii* has increased from <0.5% in 1990 – 24% in 2007.[126] Carbapenem antibiotics are usually the therapy of choice and carbapenemases resistance is associated with high levels of morbidity and mortality. Accordingly controlling dissemination of resistant *Acinetobacter baumannii* is an infection control priority.[132, 134] Carbapenemase resistance in

Acinetobacter baumannii is mainly due to the expression of OXA-β-lactamases, whilst non-OXA meditated carbapenemase resistance is rare.[137] Risk factors for infection with resistant Acinetobacter baumannii include: prior antibiotic exposure, length of stay in an ITU, mechanical ventilation, trauma.[132]

1.2.7 ADENOVIRUS

Adenovirus is a double stranded DNA virus that is associated with respiratory, ocular and gastrointestinal disease, especially in children. Adenoviruses consist of a linear DNA genome, which is 30-38kbp. The genome codes for between 30 and 40 genes and there is 70-95% homology within adenovirus species and 5-20% homology between species.[138, 139] Genes inherited by all modern adenovirus from their common ancestor are centrally located in the genome, consisting of 16 shared genes, that are termed genus defining. Additional niche-specific genes in each lineage are located mostly near the genome termini E1, and E4.[138]

There are seven species of adenovirus (A through G) with 52 serotypes or subgroups, roughly one third of which are associated with human diseases.[111] Historically, species definition has been based on a complex range of immunological, biological and biochemical characteristics (e.g. haemaglutination patterns, tissue tropism or oncogenicity in newborn hampsters).[139] Serotype (subgroup) is defined by quantitative neutralization with hyperimmune sera. Certain species of the virus, mainly species C, have demonstrated the ability to become latent post primary infection; this is significant as it can allow the virus to reactivate in a host with a lowered immune system.[140]

Adenovirus infection is common, causing between 2 and 8% of childhood respiratory infections.[140] It has a worldwide distribution and is endemic in paediatric populations, with most primary infection occurring between the ages of 6 months and 5 years.[139, 141] However adenovirus is a cause of infection in all age groups and is often transmitted from children to adult household contacts.[142, 143] Infection is normally self-limiting and presentations include: upper and lower respiratory tract infection, gastroenteritis and acute conjunctivitis.[139] Site of disease is linked to the adenovirus subgroup causing the infection and route of transmission i.e. respiratory, faecal-oral or conjunctival routes.[144]

Severe infection can occur in both immunocompetent and immunocompromised patients, leading to hospitalization, with infection being more severe the younger the

patient.[139, 140] The most prevalent cause of adenovirus infection is species C within the civilian population (which is important for reactivation) and species B and E in military outbreaks. Acquisition or reactivation of adenovirus during hematopoietic stem cell transplantation (HSCT) can lead to high morbidity and mortality in this patient group. Presentations include pneumonia, hepatitis, hemorrhagic cystitis, colitis, pancreatitis, meningoencephalitis and disseminated disease, although asymptomatic infection has also been recorded. The rate of adenovirus infection in this patient group ranges from 5 to 47%, with the number being highest within the paediatric setting and mortality rates of 76% recorded in disseminated disease.[140, 145] Co-infection with more than one species or subgroup is known to be much more likely in immunocompromised patients than immunocompetent ones, 30% compared to 5% in immunocompetent.[139] However beyond this little is known about the specific disease association of individual adenovirus subgroups in terms of disease severity or initial presentation.[146] Children with underlying diseases who undergo prolonged inpatient stays are at high risk of severe adenovirus infection and represent a source for nosocomial transmission.[141]

Adenovirus is a hardy virus that is likely to survive well in the environment with sustained infectivity on a nonporous surface having been demonstrated for up to 35 days after inoculation.[142] Nosocomial outbreaks have high secondary attack and mortality rates requiring rapid infection control measures.[141] In one paediatric outbreak study secondary attack rates were 46% with 53% of contacts having severe clinical outcomes.[141] Not only do adenovirus outbreaks have significant clinical impacts, they also have substantial financial ones.[147]

1.2.8 NOROVIRUS

Norovirus (formerly known as Norwalk like virus) belongs to the family *Caliciviridae*.[148] Norovirus has a positive sense RNA genome with an average length of 7.5kbp. The genome is highly variable and is classified into five groups GI GII GIII GIV and GV, three of which are found in humans (GI, GII and GIV).[148] Gastric flu was first described in 1928 and norovirus is the single most common cause of gastroentertitis with an estimated 21 million cases annually worldwide.[149] Within the period 2002 – 2003 alone it cost the NHS an estimated £115 million through lost bed days, cancelled operations and staff absence.[150-152]

Norovirus gastroenteritis is typically a mild self-resolving illness with an incubation period of 24 - 48 hours and a symptomatic period 12 – 60 hours, although they can be longer in children, the immunocompromised and the elderly.[149, 153, 154] Symptoms start

with sudden onset nausea, followed by projectile vomiting and watery diarrhoea.[154] Within nosocomial cases there can be additional sequelae, which include gastro-intestinal bleeding, protein malnutrition and higher than expected cause related mortality.[149, 155]

Patients with norovirus infection can excrete viral loads of 10^8 viral copies/g in stools and projectile vomiting is associated with $3*10^7$ virus particles emitted as an aerosol.[156, 157] The infectious dose for norovirus is between 10 and 100 viral copies and exposure can be due to contaminated fomites or inhalation of infectious particles from vomitus aerosols.[150, 158]

Norovirus particles retain infectivity on surfaces and are resistant to a variety of disinfectants.[159] Due to this stability and infectivity, environmental contamination has been implicated in successional outbreaks in numerous scenarios; this is purportedly responsible for the 30% attack rates seen in close contacts of norovirus patients.[148, 160-162] Norovirus outbreaks are also difficult to control, and management is based up on rapid identification of cases and isolation or cohorting infected patients and sometimes ward closures.[153] Norovirus outbreaks are seasonal with the peak occurring in the winter months. Due to the number of community cases, it is almost impossible therefore to prevent cases being introduced into the hospital environment within the winter months.[153, 163, 164] Management of norovirus outbreaks is further complicated by a lack of herd immunity due to the antigentic diversity present in norovirus isolates and the fact that norovirus can be shed for a prolonged period after symptoms have ceased, up to 182 days. In one study it was found that in 30% of volunteers virus was shed without symptoms ever being present, indicating that a subclinical reservoir within the clinical environment is possible.[154, 165]

Infection control measures that have been used to try to control outbreaks include: emphasising the need for hand washing with soap and water both before and after contact with ill patients, affected staff remaining off work until 48 hours after their symptoms have resolved, restricting staff movements between affected and unaffected patients and restricting visitors. However evidence that these measures are effective is poor.[165]

1.2.9 *PSEUDOMONAS AERUGINOSA*

Pseudomonas aeruginosa are Gram-negative aerobic coccobacilli, which are ubiquitous, inhabiting water, soil, plants and humans.[166, 167] The growth of

Pseudomonas aeruginosa in water is not directly linked to the organic content of that water and therefore it can exist, reaching relatively high numbers, within the clinical environment in water and on moist surfaces.[167] It is an opportunistic pathogen, causing 10 – 20% of nosocomial infections. It is particularly in the cystic fibrosis population where it can lead to a steady decline in lung function.[168] Other patients at risk of *Pseudomonas aeruginosa* infection are neutropenic patients and those with severe burns or foreign devices.[167, 169] *Pseudomonas aeruginosa* outbreaks have been linked to contaminated tap water and contaminated medical devices and equipment.[169]

1.2.10 VANCOMYCIN RESISTANT *ENTEROCOCCI*

Enterococcal spp. are Gram-positive organisms, two of which are common commensals of the human digestive tract, *E. faecalis* and *E. faecium*. Vancomycin resistant enterococci (VRE) was first described in 1988 and has gone on to become an important nosocomial pathogen worldwide.[170] During a survey of the National Healthcare Safety Network in 2009 – 2010 *Enterococcal* spp. and VRE caused (13%) and (3%) respectively of HCAI.[171] Within the US in 2003 30% of *Enterococcal* spp. isolates were resistant to vancomycin, up from 0.3% in 1989.[170, 172] Asymptomatic colonisation exceeds infection tenfold, but VRE infection are linked to increased morbidity, mortality and healthcare costs.[170]

Risk factors for VRE acquisition include: disease severity, length of hospital stay, and prior antibiotic exposure. Vancomycin resistance does not arise de novo in vancomycin susceptible *Enterococcal* spp. isolates via spontaneous mutation; instead susceptible patients acquire VRE exogenously, in the context of antibiotic selective pressure.[170] It is thought therefore that VRE acquisition is often via the hands of HCW. However it is difficult to separate the risk of hand transfer from that of environmental contamination, because of this controversies remain about the most efficacious ways to reduce the rate of spread.[170, 171]

1.3 THE IMPACT OF THE ENVIRONMENT ON HEALTHCARE ASSOCIATED INFECTION

There is an ongoing debate about the role of the environment in HCAI and to what extent, if any, it acts as a potential vector for transmission.[1] In 1987 the CDC stated that nosocomial infections were not related to environmental contamination.[161] However within the acute healthcare setting patients spend a substantial amount of

time surrounded by equipment, devices and environmental surfaces that may be contaminated with microorganisms.[1] This is because microorganisms are ubiquitous within the environment including indoor air, outdoor air and surface contamination. Contamination does not necessarily represent a health hazard as non-critical equipment is not required to be sterile, and detection of organisms does not necessarily indicate a significant finding.[88, 173] Some of the controversy may depend upon the number of variables involved in both detection and interpretation as most data is not collected in a systematic way and whether organisms present a hazard is likely to depend upon: individual patients, the nature of the pathogen, and the concentration of that pathogen. Cut-offs for all of these data are hard to establish.[88]

1.3.1 Previous Studies on the Role of the Environment in Healthcare Associated Infection

A number of studies have been undertaken examining contamination within the clinical environment. Most of them have looked for the presence of a single organism during a snap shot study, often linked to an outbreak or the introduction of an infection control intervention. Long term environmental sampling studies are rare, especially any that focus jointly on viruses, and bacteria. The findings of these studies are discussed in greater detail in Chapter 3.

1.3.2 Organism Environmental Survival

Reports of bacterial and viral survival on surfaces are inconsistent, with a wide range reported (see Table 1-1). Survival may depend on the state of the organism within the environment i.e. whether in spore form, vegetative state, biofilm, or in organic matter.[8] Other variations are likely to be due to the test conditions (as discussed further in Chapter 3) and may be due to variations in: species and strain, inoculum size, humidity, suspension medium and surface material tested.[174-176]

What is important for determining HCAI risk is that the longer an organism can persist on a surface the longer it can present a risk, and the more of an organism that is present the greater the chance of survival and reaching a susceptible host.[177, 178] Even if environmental survival is poor, low infectious doses for some organisms may mean that they are present in sufficient numbers to still cause infection.[179]

Organism	Infectious Dose (if known)	Length of Survival on Surfaces
Staphylococcus aureus	<15CFU/10^6 (oral dose)	7 days – >1 year

Clostridium difficile	1CFU (in mouse models)[174]	5 months
***Klebsiella* spp.**	No experimental evidence	<1 hour – 30 months
E. coli	10 CFU[180]	<1 hour – 16 months
Acinetobacter spp.	No experimental evidence	3 days - 5 months
Adenovirus	<150 viral copies	7 days – 3 months
Norovirus	10 – 100 viral copies	Norovirus (including Feline *Calicivrus*) 8 hours – 14 days
Pseudomonas aeruginosa	10^8 (oral dose)	6 hours – 16 months
VRE	No experimental evidence	5 days – 4 months

Table 1-1 Length of survival of common nosocomial pathogens on surfaces and associated infectious dose, where known.[175, 181]

1.4 MONITORING OF ORGANISMS WITHIN THE HEALTHCARE ENVIRONMENT

Historically, environmental contamination by infectious organisms was thought to have a negligible impact on the incidence of nosocomial infection.[182] This view is now challenged with opinion growing that the environment may act as an intermediary reservoir for pathogens, resulting in HCAI.[183, 184] Screening of both patients and the environment may play an important role in controlling HCAI as prompt recognition of outbreaks, in order to instigate interventions, is key to controlling cross transmission of infection.[185] This recognition requires both efficient environmental and patient sampling techniques as well as epidemiological typing in order to determine whether the cases are from a common source, and therefore whether infections are linked to cross transmission.[185]

1.4.1 ENVIRONMENTAL SCREENING APPROACHES

Monitoring of the environment can be undertaken for two main reasons: to monitor hygiene standards or to detect specific pathogens.[186] However cleanliness is difficult to define and there is little consensus about what constitutes a 'clean' surface.[187]

The selection of methods for sampling the environment may therefore be affected by the reason for undertaking sampling. Decisions linked to sampling method may be affected by the surfaces that require sampling. Optimal methods for performing either microbial hygiene monitoring or specific pathogen detection have not been agreed.[188, 189]

Most hospital centres currently use a visually clean assessment, meaning that surfaces are free of dirt, dust and debris. However a recent study using this method demonstrated that when 82% of ward sites were visually clean, only 30% of sites were considered bacteriologically clean.[184] Consequently it has been proposed that hospitals should monitor the level of microbial contamination within the environment either through aerobic colony counts, using adenylpyrophosphatase triphosphatase (ATPase) as a surrogate for microbial contamination or the presence of indicator organisms such as MRSA.[8, 190, 191] Another alternative cleaning monitoring method is to use fluorescent markers which are applied to surfaces prior to cleaning, and then checking whether those markers are removed.[8] When this method has been used to monitor cleaning in healthcare settings 50% of rooms were considered 'clean', a level that rose to 82% post education intervention with cleaning staff.[192]

Three main methods of sampling for viable bacteria are available within the healthcare setting:

- Contact plates
- Swabbing with direct plating
- Swabbing with enrichment

Contact Plates

Contact plates provide a quantitative method for detection of bacteria from the environment. They can be placed on any flat surface and allow rapid recovery of bacteria.[190] Contact plates can underestimate bacterial counts on surfaces and have been demonstrated to have a recovery sensitivity of between 10.5% – 54% of

Staphylococcal spp. from a surface.[190, 193, 194] Recovery limits for contact plates have been recorded as 27.6 colony forming units (CFU)/100 cm^2 for adsorbed cells and 31.4 CFU/cm^2 for unadsorbed cells.[188]

Contact plates can underestimate the number of bacteria upon a surface due to clumping of bacteria cells. In addition, they can be ineffective when high levels of contamination on surfaces mean that individual colonies cannot be counted or when sampling wet surfaces where individual colonies are not distinct.[190]

Variability in detection by contact plates may be linked to variation in surface pressure during sampling. A surface pressure of 20-25g/cm^2 is recommended, but this is difficult to monitor when sampling in a clinical environment and as such is subject to interpretation.[195] Further variation in contact plate sensitivity may be due to the length of time the plate is held on the surface. Optimal contact time to recover 100% of *E. coli* from a stainless steel surface was 4hours; however this duration allows for bacteria multiplication.[196] Other studies have suggested a contact time of 60 seconds.[197]

Swab Sampling

Swab samples allow for recovery from hard to reach surfaces such as bed rails, taps, and drains.[188, 198] Swab sampling sensitivity has been shown to be affected by a number of factors including: pre-moistening of the swab, swab type, swabbing technique, swabbing area and use of enrichment.

Recovery limits for swab samples using direct sampling onto blood agar for adsorbed cells has been demonstrated as 26CFU/100cm^2 and for unadsorbed cells 8.8 CFU/100cm^2.[188] Sensitivity of swab sampling has been shown to be anywhere between 1.5% and 87% although surface areas sampled in these studies are not consistent.[174, 199-206] Swab sampling provides a qualitative result as release from the swab onto the plate may not be consistent. Release of microorganisms from the swab is dependent upon the type of swab utilised. A number of different swabs have been evaluated and utilised in published studies with the main ones being cotton, rayon, and flocked swabs.

The recovery from surfaces using nylon swabs has been shown to be 58%.[207] The design of flocked nylon swabs, where each individual fibre is inserted into a plastic shaft, has increased the yield of bacteria from a surface by up to 60% compared to a standard nylon swab with a release rate into the sampling fluid of >80%.[208] Cotton swabs have a recovery sensitivity of $1.1*10^3$/cm^2 MRSA, if the swab is moistened

before sampling.[186] Other studies have demonstrated that cotton swabs have a recovery of 71.2%.[209] Rayon swabs have a similar sensitivity to cotton swabs at 71.9%.[209]

Enrichment of swabs has demonstrated increased sensitivity from 61.3% to 80% in one study and from 70% - 74% in another.[209, 210] Pre-moistening of the swab before sampling has been demonstrated to increase recovery from 57.5 – 83.4%.[209] When pre-moistening and enrichment were combined, one study demonstrated 100% recovery.[209]

When using swab sampling, it is important to sample a sufficient area. This varies considerably in the literature from $5cm^2$ – $25cm^2$.[207, 211] Sampling technique is also a factor with sensitivity increased with consistent application and swab rotation.[190] One study had recommended that initial swabbing should be undertaken with a pre-moistened dry flocked swab followed by swabbing the same area again with a dry flocked swab in order to soak up any residual fluid.

Swab/Contact Plate Comparison

Studies comparing the use of swabs and contact plates for recovery of bacteria from the environment have found conflicting results. Obee et al. (2007) found that contact plates demonstrated higher recovery for adsorbed cells than swabbing.[188] Other studies have found that swabbing techniques and contact plates offered comparable levels of recovery.[212] Finally, studies using enrichment found that swabbing was more sensitive for detection than the use of contact plates.[213]

Decisions about whether to use contact plates or swabs may depend upon the organism that is the target for recovery. Lemmen et al. (2001) detected 94/174 (54%) clinical surfaces positive for Gram-positive cocci using swabs vs 121/174 (69.5%) using contact plates.[197] In the same study they recovered Gram-negative rods from 66/89 (74.2%) of clinical surfaces screened using swabs vs 38/89 (42.7%) screened using contact plates. This indicates that contact plates may be more sensitive for Gram-positive bacteria than Gram-negative surfaces from clinical environments, but that swabs may be better for Gram-negative bacteria. This may be linked to how bacteria survive within the environment, as Gram-negative organisms are more likely to be present within biofilms and therefore require agitation to remove from the surface.

One of the key advantages of using swabs is that they can be used for the recovery of viruses as well as bacteria and that the sample can be used for molecular techniques

as well as culture methods. Swabs are also cheaper than using contact plates and are less likely to leave residue on surfaces. For these reasons swabbing techniques are more appropriate than contact plates.[199]

1.4.2 WATER SCREENING

Several factors make hospital buildings a suitable site for colonisation by bacteria and moulds. They contain large and complex water systems with low flow, which predisposes them to biofilm formation, and water temperatures are often optimal for bacterial growth.[214] Although these conditions exist in other buildings, the occupants of hospitals are more susceptible to infection and or colonisation caused by exposure to microorganisms within the healthcare environment.[214] The mode of transmission for waterborne infection include: direct contact, indirect contact, inhalation of dispersed aerosols from water sources such as sinks, and aspiration of contaminated water.[88] Healthcare environments within the UK undertake regular sampling for the presence of *Pseudomonas aeruginosa* on wards where high risk patients are located, such as ITUs. Healthcare centres in both the US and UK screen for *Legionella* spp. as part of routine infection control.

1.4.3 TYPING STRATEGIES

Identifying colonised and or infected patients is a key factor in limiting HCAI.[215] There is no standard definition to assess when patient to patient transmission has occurred; however cross-transmission of bacteria is suspected when isolates are found to be of identical species and have the same antibiotic sensitivity pattern.[216] Viral cross-transmission is suspected when in-patients develop symptoms after being within the hospital environment for over 48 hours and test positive for the same virus.[216] In order to monitor and evaluate the effectiveness of infection control measures, rapid identification and typing of organisms is essential.[185, 217]

Bacterial diversity is exploited across typing schemes in order to investigate isolates.[218] Both phenotypic and molecular typing techniques are widely used, but they are based on different principles.[219] It is assumed that phenotype correlates to genotype, but it is possible that there is little correlation between these two and that additional differences exist due to the typing methods themselves.[219] Whichever methods are used they need to offer a suitable level of discrimination for the setting, with higher levels of discrimination required for single outbreak investigations, than is needed for country wide surveillance.[219] Additionally typing techniques should be reproducible, rapid and easy to use.[108] Many typing techniques require that samples

are sent away for typing; this results in retrospective typing occurring and delays of days to weeks.[185, 220] Rapid and portable in-house typing techniques are required in order to determine relatedness and enable organism containment.[69, 185, 216, 220-222]

1.4.3.1 PHENOTYPIC TYPING

Primary typing of cultured bacterial isolates is undertaken by phenotypic typing techniques.[223, 224] This initial typing takes into account colony morphology, Gram-staining and macroscopic features.[185, 224, 225] For viruses grown in cell culture primary phenotypic typing to determine species is based upon cytopathic effect observed in cell line, in conjunction with time taken to grow (see Figure *1-1* A – Monolayer within Vero cell line B - adenovirus cytopathic effect in Vero cell lines.Figure 1-1).

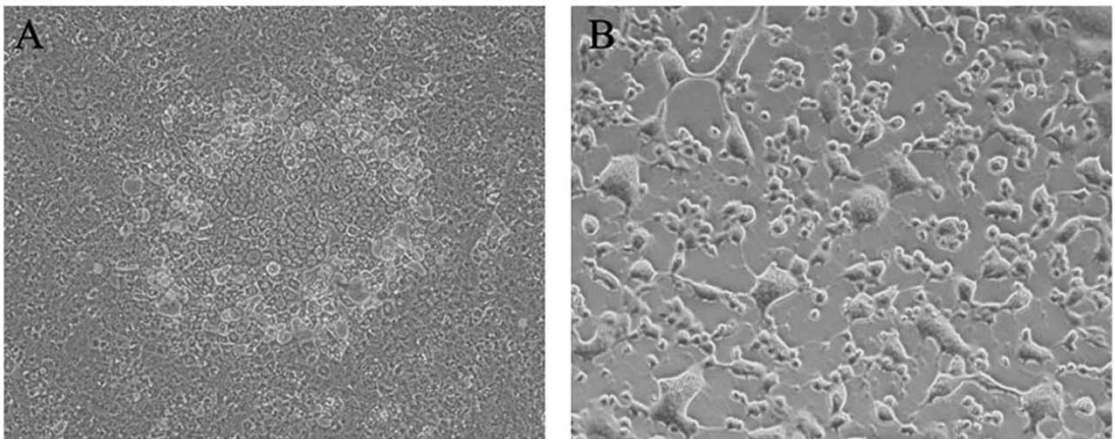

Figure 1-1 A – Monolayer within Vero cell line B - adenovirus cytopathic effect in Vero cell lines.[226]

Selected bacterial isolates then undergo a panel of biochemical reactions which are used to identify commonly isolated bacteria.[224] These panels have good discriminatory power for most clinical isolates but are often less discriminatory for environmental bacteria and they cannot differentiate beyond the species level. The major disadvantage of these techniques is that both culture and biochemical tests require time.[185]

Bacterial identification utilising biochemical means may be imprecise for some genera in assigning species, and molecular techniques for identification are expensive.[224] For some Gram-negative species such as *Enterobacter cloacae* and *Klebsiella* spp. identification even to species level can be problematic using biochemical techniques.[69, 227] Increasingly matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS) is being used to for identification of bacteria using

spectral fingerprints, that are based upon the molecular weight of protein components, and demonstrate increasing discrimination for some of these species.[224]

Antimicrobial Resistance

Antimicrobial resistance is a global issue and is linked to public health.[228] Antimicrobial resistance testing is one the main typing methods that permits discrimination within species within routine laboratory settings. However it is limited due to its dependence on the diversity, stability and detectability of acquired resistance mechanisms.[225]

The most widely utilised methods for characterising isolates are: disc diffusion methods, antimicrobial gradient methods (Etest strips) (BioMerieux, Mary l'Etoile, France) and commercially available automated systems, such as the Phoenix and Vitek.[229] These systems are all highly standardized, but the process although more rapid than sending isolates away for reference laboratory typing, still takes 24 – 72 hours.[229]

Errors can occur in phenotypic resistance testing linked to inoculum preparation and culture conditions. Errors occur at between 0 and 8% depending upon the laboratory and testing systems.[230] Phenotypic typing using antimicrobial resistance remains important even in light of the use of molecular typing techniques, as in a recent study *Acinetobacter baumanii* genotypic resistance was in discordance with phenotypic resistance profiles in 79% of isolates.[231] This has implications for clinical therapy.

Phenotypic Subtyping

The accurate identification of pathogens beyond the species level is crucial to the facilitation of epidemiological investigations.[52] Viral isolates undergo further phenotypic typing to determine viral subtype via serotyping. Serotyping is also used along with biotyping for bacterial sub-typing of organisms such as *Klebsiella pneumoniae* and *E. coli*.[55, 218, 224, 232] Serotype is defined by quantitative neutralization with hyperimmune sera. Serotyping is frequently not available as it requires the use of culture and supply of neutralizing antibodies, usually raised in rabbits, and often of low titre.[233] Serotyping is also slow, laborious, expensive and species assignments can be imprecise, with non-typable results in 30% of cases and cross reactivity in 18%.[224, 233-235] For this reason serotyping is usually now undertaken using molecular means based on the genes responsible for differences in serotype.[55]

One of the main issues with using phenotypic sub-typing is that it is assumed that phenotypic characterization is a true expression of the organisms genotype, and there is an increasing recognition that is not the case.[236] It has been observed for *E. coli* that phenotypically indistinguishable isolates are genetically divergent.[237] In addition it is has been noted that virulence is not a uniform property of any given pathogen and its expression is dependent upon a number of factors, and is likely to differ during the course of infection, between hosts and dependent on the environment. As a result testing at different points could yield different results.[238] Phenotypic typing therefore cannot reliably discriminate between isolates.[239]

1.4.3.2 GENOTYPIC TYPING

Rapid and discriminatory genotypic methods are useful for undertaking source identification and epidemiology.[223] They are especially useful for undertaking typing when outbreaks are clonal and isolates cannot be distinguished using phenotypic techniques.[11, 63] The role of genotypic typing techniques is to render a judgement about whether isolates of an infectious agent are epidemiologically linked.[236] Genotypic methods assess these epidemiological links using variation in organism genomes, rather than variation in expression. Variation is brought about by genome composition i.e. the presence or absence of plasmids, overall structure, or nucleotide variation.[225] As these differ between bacteria the same typing scheme may not be suitable for all organisms.[236]

The evolution of DNA sequences in natural populations that leads to this variation is described by parameters such as recombination, mutation, growth and selection rates.[221] Most typing techniques utilise loci under neutral genetic variation to track organisms, and so the selection of loci depends upon the biology of individual species.[221, 238, 240, 241] The resolution of typing methods is determined by the quality and quantity of mutation events they are able to detect.[242] Every genome from every isolate is unique due to the errors made by DNA polymerase during replication.[225] Mutations in non-coding DNA regions may not effect phenotype or be detected using low resolution approaches.[242] However the level of discrimination provided by detection of these single nucleotide errors is not always necessary.[225]

Knowledge of the frequency and relative weight of mutations and recombination events in evolution is essential for understanding microorganisms and interpreting typing data.[243] Genetic diversity within a species is usually estimated in relation to the recombination rate.[244] Organisms range from truly clonal such as *Mycobacterium* spp. or *Staphylococcus aureus* to organisms where recombination is typical such as

Neisseria gonorrheae.[243] However this view is becoming increasingly challenged with the increasing use of whole genome sequencing (WGS). This is apparent for organisms like *E. coli* that have previously been considered clonal with little recombination. Instead of clonality the situation is now understood to be that although recombination affects ~10% of the core genome, recombination within the accessory genome is frequent, though often under 2kb in size.[95]

Epidemiological time periods must also be considered when analysing genotypic results, especially where the evolutionary mutation rate of the species is known.[225] In outbreaks that occur within healthcare for limited time periods, small amounts of detected variation may prove significant. If the same results are found when comparing against a library with isolates over a six month period the same result may be less significant.[245, 246] Results can only be interpreted taking clinical and infection control information into consideration and this must be done locally.

1.4.3.3 PULSE FIELD GEL ELECTROPHORESIS

Pulse field gel electrophoresis (PFGE) represents the current gold standard for most bacterial typing and it is widely used within reference centres.[247, 248] PFGE uses restriction endonucleases to create macro restriction fragments from the whole genome that are then separated using electrophoresis. Separation of these large fragments is possible due to periodic alteration of the direction of the electric field during the running of an agarose gel.

The choice of restriction enzyme is a critical variable in the PFGE process.[236] In general there is an inverse relationship between the length of recognition sequence of the restriction enzyme and the frequency of their occurrence in the genome.[236] The number of restriction sites is also affected by the G+C content of the bacterial genome.[236] Macro restriction fragments are made with a rare cutter enzyme that recognise between 10 and 30 sites, ranging in size between 20 and 600kpb.[225]

Four main categories of change are involved in genomic evolution leading to bacterial diversity: insertions, deletions, re-arrangements and substitutions.[236] Theoretically insertions and deletions would both affect migration of restriction fragments, depending on the size of the alteration.[236] Substitutions and re-arrangements outside of a restriction site would not necessarily be detectable using PFGE.[236] If a genetic event occurred that directly affected the restriction site this might have a greater effect on the PFGE fingerprint than a similar change that did not affect the restriction site.[236] For

this reason isolates with the same banding pattern are referred to as indistinguishable rather than identical.

PFGE fingerprint interpretation is as follows: isolates with 0 band differences are considered to be indistinguishable, isolates with between 1 and 3 band differences are interpreted as closely related and are usually thought to represent a single mutational change (usually consider 95 – 100% similarity). 4 - 6 band differences are considered to be linked to 2 genomic changes and are considered to be possibly related and potentially part of the outbreak (80 – 95% similarity). ≥7 band differences equate to more than three mutational events and are considered to not be part of the outbreak.[225, 246]

Decisions linked to interpretation of PFGE fingerprints are due to the expectation that during patient to patient spread a single genetic mutation event leading to a change in banding pattern could occur and it is possible for two mutation events to happen and for the isolates to still be part of the same outbreak.[236] Although the criteria are based upon predicting band differences from mutational events there are circumstances were single genetic events could lead to a four band pattern difference whereas two genetic events could theoretically lead to maintaining identical PFGE fingerprints (see Figure 1-2.).

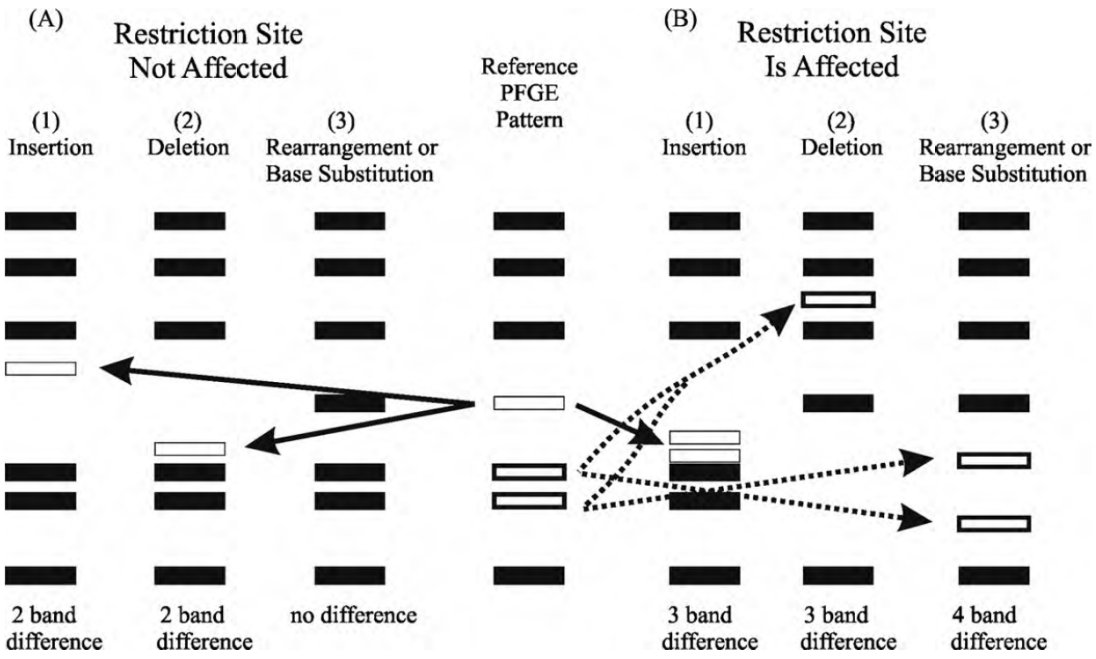

Figure 1-2 PFGE restriction-fragment patterns resulting from different genetic events.[236]

PFGE despite its popularity as a typing method does have some limitations. It is very labour intensive and cannot provide results in real time as the results take 2 - 4 days and the process also requires expensive equipment.[108, 225, 249] In order for PFGE to be reproducible both within and between laboratories requires computational analysis to prevent subjectivity and intensive quality control.[248, 250, 251]

PFGE is able to discriminate macroscopic genomic changes within the genome, but as no specific DNA sequence information is collected, discrimination can be limited.[252] Plasmids might appear on a PFGE analysis as a single difference in band pattern.[236] As the process depends upon gel electrophoresis it is subject to the limitations associated with that process. Artefacts can be mis-identified as bands, and fragments differing by less than 5 – 10% in size co-migrate. Finally fragments less than 50bp in size cannot be visualised using gel electrophoresis.[109, 236]

PFGE is the most widespread typing approach for enteric pathogens. However PFGE does not always provide maximum discrimination for closely related clonal isolates within this group.[253-255] *XbaI* is used as the restriction endonuclease for *E. coli* and *Enterobacter* spp. and *SmaI* used for *Klebsiella* spp.[87, 256] It therefore remains to be determined if PFGE is the most appropriate method, given recent molecular typing developments in terms of both discrimination and reproducibility.[257]

1.4.3.4 Single Locus Sequence Typing

Fredrick Sanger developed DNA sequencing technologies in 1977 based on the chain termination method.[258] Sanger sequencing techniques are the most commonly used methods of sequence based typing.[223] Analysis of a single locus provides limited information compared to that provided by schemes that examine the entire genome, however if a suitably variable locus is selected then typing can be discriminatory. Single locus sequencing requires a defined target and data quality is affected if there is either too much or too little DNA present. Read lengths are limited to ~1000 bases and so to sequence an entire genome in order to find single nucleotide variants from the whole genome (SNV's) or other information requires months if not years of work.[223]

DNA sequencing based methods have important advantages over PFGE as they have shorter assay times and permit fully comparable data between sites but they are expensive to use routinely.[259] The most common single locus sequence type methods are genotyping of *Streptococcus pyogenes* using the *emm* locus, and typing of *Staphylococcus aureus* using the *spa* gene. Single locus sequencing typing is also

used for multi-locus sequence typing (MLST), where housekeeping genes are individually sequenced and the results then combined to provide typing information.[223]

Multi-locus Sequence Typing

Multi-locus sequence typing (MLST) was first introduced in 1998 and detects variation within housekeeping genes, each usually 400 – 500bp. The use of seven housekeeping genes is suggested as a minimum and they should be evenly distributed across the genome, in order to provide sufficient coverage and to reduce the influence of hot spots for mutation and recombination.[218, 260, 261] Although seven loci are suggested, some schemes use between five and eight loci.[217] Housekeeping genes are selected as targets for sequencing as they are considered to be under neutral pressure selection.[262] Each locus is given a number in relation to other isolates at that loci within a database and the combination of numbers forms a numerical code for a sequence type (ST).[103] The use of a numerical code permits the genetic relatedness of isolates to be compared across sites, as it is both portable and reproducible.[103, 249]

MLST is a reliable typing method, but it is expensive, time consuming and requires a high level of technical skill.[219] MLST although highly discriminatory for establishing patterns of long term evolution, is not sufficiently discriminatory to distinguish closely related isolates that come from single centre outbreaks.[248, 263] It also does not give much insight into recent genetic history or acquisition of mobile genetic elements.[263]

The routine use of MLST is considered to be the reference standard for examining *E. coli* isolates; however despite reproducibility there is little consensus on processing methodology, which could affect sequencing results and there are three different databases for analysing *E. coli* alone.[89]

***Spa* Typing**

The population structure of *Staphylococcus aureus* is dominated by a few prevalent clones, with clonal complexes CC20 and CC30 accounting for >95% of UK hospital MRSA cases.[264] *Spa* types are based upon a single gene and are usually genetically distinct, a single base pair change can produce two different but highly related *spa* types.[264, 265] *Spa* typing has a low discriminatory power compared to PFGE but is more portable, it has comparative resolution to that seen with MLST.[21]

Adenovirus Typing

The *hexon* gene encodes for the adenovirus capsid and is the source of viral serotype as the capsid determines reaction in neutralisation assays. Molecular typing is targeted at the *hexon* gene, within which there are 7 hyper variable regions, 6 lying in loop 1 and 1 lying in loop 2.[266] Little is known of the level of divergence within subgroups outside of the 7 hypervariable regions within the *hexon* gene.[146] Sequencing of the entire Adenovirus genome where available suggests that if typing is carried out outside of the *hexon* gene the genetic split of the virus may no longer relate to its current acknowledged serotype/subgroup. However whole genome sequence is not available for all adenovirus species.

1.4.3.5 MULTIPLE-LOCUS VARIABLE NUMBER TANDEM REPEAT TYPING

Multiple-locus variable number tandem repeats (VNTRs) were discovered in 1982 in *Mycobacterium tuberculosis* and represent genetic polymorphisms that can be used for species specific typing.[87] Most VNTR loci are <200bp and are evenly distributed throughout bacterial genomes.[225] VNTR loci represent some of the most diverse genomic loci within bacterial populations and are therefore suitable markers for assessing genome diversity.[218] Each repeat unit or motif consists of DNA elements that are repeated in tandem, the sequence of which is maintained within species. [87, 267] Repeats vary between strains in copy number due to indel mutations, resulting from DNA polymerase slippage or unequal cross over events, leading to slipped strand mispairing.[267] These events lead to the loss or insertion of repeats at a locus, depending upon whether the error occurs on the nascent strand (insertion) or template strand (deletion).[87, 141, 267] Recombination can also play a role, especially if mutations involve large numbers of repeat units.[267]

Most VNTRs have no phenotypic effect and for the most part are undergoing neutral genetic selection, however some VNTR loci can alter important biological function.[267] In *Neisseria meningitidis*, *Haemophilus influenza* and *Mycoplasma hyorhinis*, homopolymeric or dinucleotide repeats located between – 35 and – 10 regions of the promoter differentially affect transcription of downstream genes, dependent upon the number of repeated sequence units. In other cases VNTR sites affect the actual amino acid sequence of proteins, rather than affecting transcription levels.[267] If a decision is made to use VNTR loci that are located within or near a gene then validation must include assessing the locus for signs of altered phenotypic or selective effects.[267]

Designing of VNTR schemes requires the availability of whole genome sequence data for the target species, in order to identify the repeat motifs. Primers are then designed to target the flanking sequence of the motifs of interest and amplification is undertaken using PCR.[268] Amplicons are separated utilising electrophoresis and the repeats sized and given a score for each locus. Differences in amplicon size are assumed to be due to variation in repeat copy number at the target loci.[141] VNTR analysis can resolve otherwise indistinguishable isolates and is particularly good for distinguishing between recently evolved isolates.[251, 253]

Six to ten loci are usually suggested for developing VNTR schemes, which should be distributed across the genome and ideally be located within non-coding regions of the chromosome. The advantage of utilising VNTR schemes is that they are fast, non-labour intensive and offer discrimination that may make them a suitable alternative to both PFGE and MLST.[269, 270]

1.4.3.6 REPETITIVE EXTRAGENIC PALINDROMIC BASED PCR TYPING

Repetitive extragenic palindromic based PCR (REP-PCR) is based on PCR amplification of non-coding repetitive extragenic palindrome (REP) DNA sequences that are located throughout the genome.[239, 271] REPs comprise a distinct group of genomic repeats that occur in a high abundance (>100 copies) within enteric bacteria.[272] These sequences are highly conserved inverted repeats that contain palindromes within their sequence and are thought to be involved in binding of DNA polymerase and chromosome organisation, although their true function is unknown.[272, 273] In some cases REP sequences appear as targets for transposition and recombination events. ISKpn1 insertion sequences insert into the REP sequence of *Klebsiella pneumoniae* and IS1397 and IS621 insert into REP sequences of *E. coli*. [274]

REP-PCR utilises primers that target these non-coding REP sequences and the PCR produces multiple amplicons of different sizes that form a DNA fingerprint when separated by gel electrophoresis. The fragment sizes are then processed to generate REP-PCR profiles for each isolate and can then be used for strain delineation.[89, 275] Analysis of REP-PCR typing results is complicated by the subjectivity of the analysis parameters and the need to decide on those parameters for different bacteria.[260] Diversilab (BioMerieux, Mary l'Etoile, France) is a commercial system that utilises REP-PCR amplification, followed by a microfluidics detection system using the Agilent 2100 bioanalyzer and computational analysis. The sequences of the actual primers used are not supplied with this commercial assay. However the advantage of using a commercial system is that it is rapid and semi-automated.[120]

1.4.3.7 WHOLE GENOME SEQUENCING AND HIGH THROUGHPUT SEQUENCING

High throughput sequencing (HTS) technologies are different from Sanger sequencing in the fact that they undertake massively parallel sequencing, permitting high throughput.[258] In comparison to single locus sequencing the basic steps of HTS solid phase sequencing are: library production, bridge/emulsion PCR, pyro sequencing and data analysis. Both the bridge/emulsion PCR and pyro sequencing steps have similarities to conventional single locus sequencing, although the volumes and chemistries per reaction are significantly altered. Library production enables PCR to be undertaken with no defined target, DNA is sheared and adapters ligated onto the ends enabling PCR to be undertaken of de novo targets.

HTS technology is rapidly evolving and new platforms are frequently being released, there are nearly 20 different HTS platforms on the market and each of them has their own advantages and disadvantages (see Table 1-2).[223, 276] There are four commonly used systems: Ion torrent Personal Genome Machine (PGM) (Life Technologies, Paisley, UK), Illumina Systems (Ilumina, San Diego, US) SOLiD system (Applied Biosystems, Warrington, UK) and 454 System (Roche, Burgess Hill, UK).[277] One of the major differences between platforms is in their read lengths, ranging between 35bp and 700bp.[278] Sequencing runs range in time from two hours on the Ion Torrent PGM (Life Technologies, Paisley, UK) to 14 days on the Genome Analyzer IIx (Ilumina, San Diego, US).[223]

HTS allows acquisition of Gigabases of data by producing millions of >35bp segments from a single input, with over 100fold redundancy in the number of segments generated.[223, 258] When applied to bacteria whole genomes these segments or contigs are then overlaid using sequence analysis technology to form intact whole genome sequences. The sequencing error rate using this type of system can be <1% because many versions of the whole genome sequence are produced, giving a high level of redundancy (see *Table 1-2*.).[223] The accuracy of whole genome sequencing (WGS) therefore using high throughput platforms is comparable to Sanger sequencing (error rates of 0.03 – 0.07%) when assembling multiple overlapping reads.[223] These whole genome sequences can then be compared further preventing errors.[279]

Unlike other none total chromosome methods WGS can provide a considerably more in depth genomic view rather than a genomic snapshot.[280] Emerging data about the differences between core and accessory genomes indicates that they may still not provide a complete genomic data set.[236, 280]

The declining cost of HTS has led to a number of studies being undertaken to explore the application of sequencing entire bacterial genomes for epidemiological typing.[281] *E.coli* is second only to *Staphylococcus aureus* in the number of sequences that have been undertaken. Even so this represents only a small fraction of available genomic information as the data on its accessory genome is still being accumulated.[281] Other organisms that have undergone WGS in order to begin collecting data for epidemiological typing include *Clostridium difficile* that was used to demonstrate that 45% of cases which had been presumed to be cases of cross transmission were in fact independently acquired.[282] Ramos et al. (2014) utilised WGS to demonstrate that genome plasticity occurred at all levels of *Klebsiella pneumoniae* from whole genome to individual nucleotide levels, and that this might be important due to *Klebsiella pneumoniaes'* diverse lifestyle.[44] Finally Petty et al. (2014) utilised WGS to show that the global *E. coli* clone ST131 possesses both shared clonal attributes and a variable complement of virulence genes.[283]

Use of high throughput sequencing platforms for typing requires information on genomic features linked to specific organism such as; polymorphism distribution, intergenic region sizes and positively selected loci ratios, since these will impact on mutation and recombination rates differently, and could result in non-convergent and incongruent phylogenies.[281] Although standard typing techniques suffer from not having the resolution of WGS, it is possible that WGS may provide a level of information that makes it difficult to interpret typing data without a greater understanding of the bacterial genomics.[215, 216]

SNVs are either synonymous or nonsynonymous and can occur in genes that encode proteins. Non synonymous SNVs result in amino acid replacement and provide a substrate for evolutionary selection. Synonymous SNVs do not alter the structure of proteins and are therefore functionally neutral. SNVS are easy to detect and can be used for phylogenetic studies. WGS has been utilised to study the mutation rates using SNVs within *Staphylococcus aureus* and it was determined that mutation rates vary between 2 and $3.4*10^{-6}$ mutations per site per year. This equates to 5.6 – 9.5 mutations per year across the genome or 1 SNV every five weeks.[264] This represents the number of changes in a highly clonal organism where recombination events are thought to be rare and it is therefore unlikely to represent the situation found in more diverse organisms, such as *Klebsiella* spp.[281]

HTS for targeted genomic sequencing represents a single, potentially rapid, approach as it can provide epidemiological, virulence and resistance information based upon a single test.[230] In order to provide it in a more accessible format for clinical use companies are making commercial kits that will provide information on organism identification, virulence and resistance. One of these is the Pathogenica HAI Biodetection kit (Pathogenica, Boston, US) run on the Ion Torrent PGM (Life Technologies, Paisley, UK).[280] Limitations of both whole genome and targeted genome sequencing on high throughput platforms are currently linked to the cost of each genome to be sequenced and the amount of data produced, analysis of which requires significant bioinformatics input.[258, 276, 284]

Platform	Illumina MiSeq	Ion Torrent PGM	Illumina GAIIx	Illumina HiSeq 2000
Instrument cost	$128 k	$80 k	$256 k	$654 k
Sequence yield/run	1.5-2Gb	20-50Mb (314 chip) 100-200Mb (316 chip) 1Gb (318 chip)	30Gb	600Gb
Run time	27 hours	2 hours	10 days	11 days
Reported accuracy	Mostly >Q30	Mostly Q20	Mostly >Q30	Mostly >Q30
Observed raw error rate	0.8%	1.71%	0.76%	0.26%
Read length	Up to 150 bases	~200 bases	Up to 150 bases	Up to 150 bases
Paired reads	Yes	Yes	Yes	Yes
Insert size	Up to 700 bases	Up to 250 bases	Up to 700 bases	Up to 700 bases
Typical DNA requirement	50 – 1000ng	100 – 1000ng	50 – 1000ng	50 – 1000ng
Sequencing cost per Gb	$502	$1000 (318 chip)	$148	$41

Table 1-2 Technical specifications of high throughput sequencing platforms.[276]

HTS is a term that refers to the collection of data on all high throughput platforms. Collection of this data can include both whole genome sequences (WGS) and targeted

genomic sequence collection. Within Chapter 4 of this thesis in order to differentiate, the term WGS is used to refer to sequencing undertaken on the MiSeq platform (Ilumina, San Diego, US), after which the whole genome sequence of the bacteria was analysed. Whereas HTS refers to targeted genomic sequencing, where although data representing the whole genome may have been collected, data analysis was only undertaken on targeted genomic sections,

1.5 APPROACHES TO CONTROLLING HEALTHCARE ASSOCIATED INFECTION

For many organisms carriage and colonisation precedes infection. It is important therefore to prevent not only infection with microorganism but also carriage and colonisation.[264] The principle components of infection prevention and control interventions are: early identification of carriage/infection, patient isolation, eradication of carriage if appropriate, improved hand hygiene, environmental control and good antimicrobial stewardship.[29] All of these interventions aim to control the spread of organisms reducing the risk of individual patient acquisition or endogenous infection and have been shown to be useful in ending outbreaks.[30, 285] However the effectiveness of these policies varies significantly across hospital settings, and when interventions are introduced they are rarely introduced singly, leading to challenges in pursuing evidence based practice.[24]

One example of this was the bundle of infection control interventions brought in within the NHS in order to reduce MRSA transmission. The bundle included: hand hygiene, isolation of MRSA positive patients, suppression/decolonisation therapy and screening for asymptomatic carriers.[286] A similar bundle was introduced in order to control *Clostridium difficile* rates.[287] The lack of evidence base in this area makes it difficult to assess the success of individual intervention components and in establishing which component of the bundle is the most effective. Despite the need to improve the evidence base within infection control, prospective head to head comparison studies are both prohibitively expensive and represent ethical challenges and are thus rarely undertaken.[287]

1.5.1 HAND HYGIENE

The importance of hand hygiene has been recognised since Semmelweis in the 1840s.[288] Within the modern healthcare environment it is still considered one of the most efficient and cost effective ways of preventing cross transmission of

microorganisms, to remove contaminants from the hands of HCWs.[289, 290] HCWs contaminate their hands by touching the environment and patients during routine care activities, and thus if hand hygiene practices are sub-optimal then microbial transmission can occur.[291] Effective hand hygiene includes the application of adequate amounts of hand hygiene agent, be that soap or alcohol gel, adequate duration of hand hygiene with suitable mechanical action, coverage of all hand surfaces, and adequate drying.[292]

A methodology for prompting hand hygiene at critical points was developed to support the undertaking of hand hygiene within clinical areas.[6] The 'Clean Care is Safer Care' initiative was launched in 2005 by the World Health Organisation (WHO) with the aim of reducing HCAI and a consensus guideline on hand hygiene was published in 2009, which outlined the WHO 'Five Moments for Hand Hygiene' (see Figure 1-3.).[293, 294] A hand hygiene compliance of 90% when auditing 200 observations is commonly used for determining compliance with hand hygiene guidance.[295]

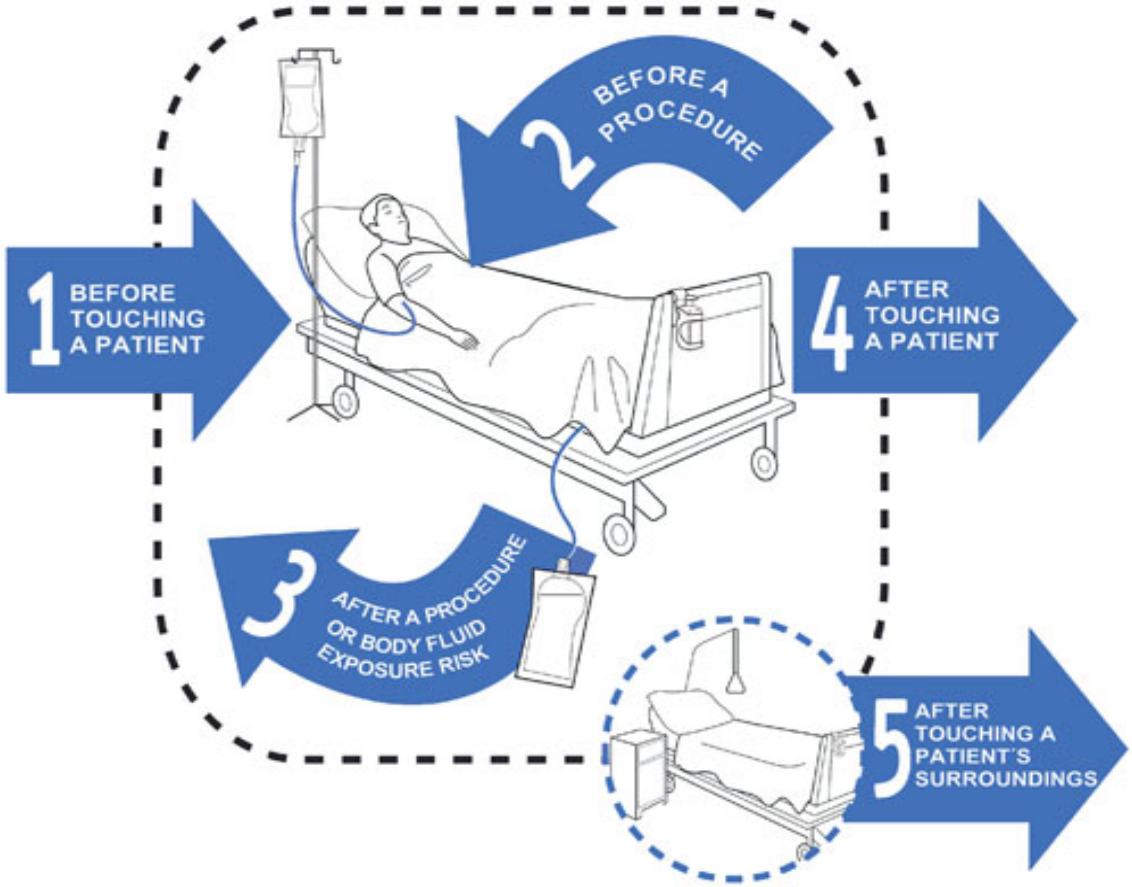

Figure 1-3 WHO 'Five Moments for Hand Hygiene'.[296]

Hand Hygiene Compliance

Despite widespread agreement with the principle that effective control of HCAI requires both regular hand hygiene and decontamination of high touch sites within the clinical environment, compliance varies widely with both components.[21, 295] Compliance for hand hygiene varies widely even within groups of HCWs, and the average observed compliance was only 50%.[7, 295, 297] Mathematical modelling has demonstrated that hand hygiene compliance of >50% is required to interrupt VRE transmission.[298] In another clinical modelling study a compliance of >70% was suggested as the threshold.[299]

Hand hygiene compliance is often therefore seen to be a key issue when attempting to reduce HCAI, however compliance may be influenced by the number of episodes required. For instance within ITU environments, which are complex, with multiple patient-nurse interactions throughout the day, hand hygiene is required on up to 60 occasions throughout the shift for nursing staff.[300] There are therefore many occasions during which failure to undertake hand hygiene could potentially lead to patient harm, however undertaking that number of hand hygiene activities requires a substantial resource input from HCWs.

Measurement of Hand Hygiene Compliance

Although it is acknowledged that hand hygiene compliance is essential the measurement of that compliance is difficult to achieve.[301] Direct observation is the most common method, although other methods such as product utilization surveys have been assessed.[301] All methods have issues with either practicality or validity, an issue that is only made more difficult by the increasing use of single rooms within the NHS inhibiting lines of sight.[301] However direct observation in itself could alter results, due to the Hawthorne effect, where an individuals behaviour changes when observed.[301] Despite the issues with direct observation it is still considered the reference standard, but it is highly labour intensive and subject to observer bias.[295] Observation validity is affected by the work load of the unit, physical factors linked to the unit and where the observations are undertaken.[295] The largest flaw with most observational audit tools is that they do not audit the WHO ‘Five Moments for Hand Hygiene’ which is the standard that is actually being set for clinical members of staff and thus compliance may be overestimated.[301]

Evidence for Hand Hygiene Impact on HCAI

It is intuitive that hand hygiene should affect transmission rates as studies have shown that 44% of nurses have yielded Gram-negative bacteria using finger imprinting methods.[302] Hand hygiene is effective at reducing microbial load as one study has shown that the average CFU count on HCWs hands were higher on those not undertaking patient care than those in direct patient contact, indicating that increased hand hygiene was managing their microbial loads.[303]

The introduction of the 'Cleanyourhands' campaign within the UK in 2004 was followed by a decline in both MRSA and *Clostridium difficile* rates over the next four years, however as previously mentioned this was introduced in concert with other intervention measures.[293] The one prospective study that has investigated the rate of improved hand hygiene compliance on *Clostridium difficile* acquisition also found that it substantially affected *Clostridium difficile* acquisition rates.[287]

Alcohol Gel vs Soap and Water

The use of alcohol gel instead of hand washing results in less damage to hands, saves time and for most bacteria and viruses is as efficacious.[304, 305] The time required to undertake hand hygiene with alcohol gel is less than that with soap and water, 12.7 seconds (s) vs 21.1s respectively.[305] Alcohol gel solutions containing 70 – 80% alcohol are preferred, although solutions with alcohol content as low as 50% can be efficacious.[305] The placement of alcohol gel is a key factor in its use, but education is also important so that when dealing with organisms such as norovirus and *Clostridium difficile*, hand hygiene is undertaken with soap and water instead.[306]

Issues with Hand Hygiene

There is some evidence that the law of diminishing returns applies to hand hygiene with the greatest benefits occurring in the first 20% of compliance.[288, 307] Therefore solely focussing on hand hygiene may reach the point where it has little further effect on decreasing HCAI rates. In addition as only roughly 4% of hand hygiene opportunities are observed using the direct observation method, it is possible audit results could potentially bear little resemblance to actual compliance.[288]

Even when hand hygiene is appropriately undertaken one study has demonstrated that 4.4% of bacteria loaded onto hands can still be recovered from hands post hand hygiene activity, emphasising the importance of undertaking efficient hand hygiene.[308]

In another study (with students) who washed their hands with soap and water, higher levels of microbial loads were recovered after hand washing than before, indicating that hand washing when undertaken must also be undertaken appropriately to be effective.[309]

1.5.2 ACTIVE SURVEILLANCE

Active surveillance of patients in order to detect 'silent' colonisation with nosocomial pathogens is championed in both the UK and the US for specific organisms.[299, 310] The CDC recommends active screening for detection of carbapenemase resistant Enterobacteriaceae and it was recently advised for high risk patients in the UK.[310, 311] Within the UK active surveillance is undertaken for MRSA, whereas targeted surveillance based on certain patient groups is undertaken for *Clostridium difficile*. However the evidence for active surveillance is not always straight forward, and there are drawbacks to this approach which include: financial burden, nursing and laboratory workload, and delayed patient throughput.[299]

Active screening as an approach is based upon the concept that for control of target (usually antibiotic resistant) organisms early identification and isolation of infected or colonized patients is key, with asymptomatic carriage being a reservoir for ongoing transmission.[312] However even when undertaking active screening optimal testing strategies are not well defined and the implementation of such screening varies between centres.[313]

This may be why the evidence to support active screening is mixed. Within the UK targeted screening used to be undertaken for MRSA. This was introduced in 2006 and mandatory screening was undertaken in 'high risk' areas such as haematology oncology. In 2010 active screening was introduced for all elective admissions apart from paediatrics, maternity and some day cases, on the basis of reduction observed through screening and other interventions that had previously been introduced.[286] Although it is hard to weigh the impact of each intervention due to this bundled approach, one study examining the change in screening approaches found that by using active screening rather than targeted screening, they identified 45% more MRSA carriers.[314] This contrasts with another study that found that the introduction of active surveillance had no effect on MRSA rates, however they were looking at infection rather than colonisation.[315]

Screening policies for both patients and HCWs are likely to depend upon the organism and whether the situation is endemic or epidemic; it has been suggested that a lack of active surveillance may be the reason for the growing numbers of circulating ESBLs now detected.[7, 316] Finally this approach relies on the presence of sufficient isolation or cohorting facilities to allow for patient isolation.

1.5.3 CONTACT PRECAUTIONS

The principle behind contact precautions is that they are a group of procedures that reduce the risk of transmission of infection through direct and indirect patient contact. It includes the use of personal protective equipment (PPE) (masks, gloves, gowns/aprons and eye protection) as well as the use of single rooms for isolation of patients, to provide a physical barrier.[317] These are applied in addition to standard (initially called universal) precautions; which are a way of reducing the risk of blood borne pathogens and those infections linked to bodily secretions.[10] The introduction of standard precautions was important for infection prevention and control as it introduced the widespread routine use of gloves.[6]

Contact precautions require HCWs to adhere to hand hygiene upon entering and exiting patient rooms as well as prior to donning of PPE and after removal of PPE. It also requires staff to either use patient designated equipment or to undertake equipment cleaning between patients.[5] This can lead to perceptions of decreased time with patients.[5]

Personal Protective Equipment

There is little controversy within the literature related to the use of gloves, gowns/aprons and masks when undertaking contact precautions.[215] This does not however mean that their use is without issue.

Gowns are used as it has been found that contamination can be recovered on 63% of HCWs uniforms, and there is a correlation between levels of uniform contamination and the level of hand contamination.[10] The use of gowns/apron attempts to control this transfer of contamination from patients to clothing and interrupt the potential spread of contamination from that clothing back onto other patients.[10, 318] The wearing of gowns has been demonstrated to be protective against VRE transmission in a study that examined interventions.[319]

As gowns/aprons also become contaminated during use their regular change and the use of gloves to do so is key in controlling spread, however incorrect glove use increases the risk of cross transmission.[6, 7] Gloves become rapidly contaminated and compliance for appropriate use of gloves and gowns is between 22 – 79% as some HCWs believe that the benefits have not been proven and correct use of PPE takes time.[5] Compliance with PPE use can also be limited by availability; one study found that depending on the size of glove needed availability of gloves in isolation rooms was between 49.4% - 72.1%.[320] Potentially of greater concern is that, in a study examining the removal of gloves, 78% of participants contaminate their hands with whatever contamination is present on the gloves, which reaffirms the need to undertake hand hygiene post glove removal.[321] Unfortunately the use of gloves has been observed to decrease compliance with hand hygiene.[322]

Isolation

Isolation precautions are used to disrupt the chain of transmission by separating infectious patients from those who are neither colonised or infected.[323] The most effective form of isolation is the use of private rooms, where along with geographical isolation staff are also cohorted to deal with either infected or uninfected patients.[215] In addition to reducing the risk of cross transmission, single occupancy rooms can result in an increase in patients comfort through increased privacy and fewer room to room transfers.[324]

A systematic review of practices for MRSA control recommends that isolation of patients with MRSA should be a priority as it can have a substantial effect on MRSA acquisition rates.[315] There is also evidence that the use of isolation rooms reduces *Clostridium difficile* acquisition.[39] However two studies found that no conclusions could be drawn about the effectiveness of isolation precautions for preventing the transmission of MRSA and ESBLs.[315, 316]

Within the NHS three quarters of patient isolation requirements are due to either MRSA or *Clostridium difficile*.[325] Failure to isolate patients can be because of the fact that single rooms are taken up by patients for non-infectious reasons, no isolation rooms being available or specific patient factors, such as dependency requirements.[325] In hospitals where isolation facilities constituted >30% of the available bed spaces, only 1 isolation failure was recorded across sites.[325] For MRSA a correlation has been shown linking isolation failures to MRSA incidence.[325] This reinforces the concept that sufficient isolation facilities should be available at all times.[326]

Within the US the CDC recommends contact precautions for MRSA as well as carbapenemase producing Enterobacteriaceae; however for other organisms such as VRE and other multidrug resistant Gram-negatives individual judgement is advised.[327] This results in variation across centres as when to isolate, with most centre isolating for MRSA, VRE and carbapenemase producing Enterobacteriaceae, but only 20% isolation for multiply drug resistant *Pseudomonas aeruginosa* and *Acinetobacter* spp.[327] In addition decisions about when to end isolation and contact precautions varied with no consensus available.[327]

Recently it has been demonstrated that 12.2% of paediatric patients developed HCAIs with respiratory or gastrointestinal viruses.[328] Another factor with isolation in the paediatric setting is that isolation can be a problem for young children as in long term inpatients it can affect developmental milestones.[329] In adults in the United States isolation has also been associated with increased levels of anxiety and depression whilst not found to be effectively preventing the spread of MRSA.[329] Other consequences for patients are a decrease in staff time, increased perceived concerns over care and an increase in both pharmacy and prescribing errors.[215, 293, 323] Patients undergoing barrier precaution in a single room average 39% fewer interactions per hour than non-isolated patients and those contacts are not extended in order to compensate for the reduction in visitation numbers.[330]

In terms of staffing negative consequences of isolation can be decreased staff satisfaction due to the strain of dealing with high acuity illness and being isolated from colleagues who are not caring for infectious patients.[215, 323] Compliance with all components of contact precautions is on average 28.9%, but as the number of patients in contact precautions increases the compliance decreases, with 40% of patient being in contact precautions representing the tipping point.[5] Finally isolation has significant associated costs and failure to discontinue isolation when appropriate in one hospital was found to cost $141,000 dollars for one year.[323]

1.5.4 VENTILATION

Ventilation refers to the process of introducing and distributing outdoor and properly treated air into a building or room.[331] The amount of that air circulated per hour in relation to the room volume is the ventilation rate (in air changes per hour). The air changes per hour affects dilution of infectious agents within isolation rooms as well as the movement of air within a physical space.[331] Air movement can be affected by both physical barriers within a building space and or the movement of people.[331] Most

ventilation is designed for the comfort of building inhabitants, and therefore maintaining temperature and minimise odours.[331]

Airborne transmission refers to the passage of microorganisms through aerosols or droplets, resulting in the infection or colonisation of a person. Different particle sizes present different risks to those in the vicinity patients.[332] Aerosols are a suspension of solid or liquid particles in a gas with a particle size from 0.001 to over 100 µm. Aerosols remain in suspension and may travel far from the patient. A droplet nucleus is the airborne residue of a potentially infectious aerosol from which most of the liquid has evaporated.[331] Particles of different sizes pose different risks.[332, 333] Airborne aerosols require additional measures to reduce cross transmission compared to larger droplets.

To prevent transmission of airborne infection masks are utilised, primarily to prevent the transmission of viruses, especially during aerosol generating procedures. However data has shown that mask use alone does not prevent viral transmission and that eye protection must be used in addition. Incorrect mask removal can present a contamination risk to the HCWs face as well as gloves.[334] All of these factors mean that ventilation plays an important role in prevention of airborne transmission.

Natural cross ventilation was popular in many hospitals to control infection until the 1970s. In modern day hospitals mechanical ventilation is used to control air changes and air flow.[332] Negative pressure isolation rooms are required to house patients with aerosol transmissible agents and work to prevent aerosol escape using air pressure differentials combined with a high air change rate to rapidly remove airborne contaminants.[335] Positive pressure rooms combined with the use high efficiency particulate air (HEPA) filters, on the incoming outdoor air, are designed to protect immunocompromised patients.[335] This is because 1 CFU of *Aspergillus* spp. can result in infection.[336]

Certain organisms such as *Mycobacterium tuberculosis*, varicella zoster virus, and measles are transmitted by aerosol and therefore mechanically ventilated negative pressure rooms are necessary to prevent cross transmission.[7] For other organisms such as viral haemorrhagic fevers and pandemic influenza, although not transmitted by true aerosol, the need for containment is such that mechanical ventilation is considered necessary. There is little evidence for many conditions about when airborne contact precautions can be stopped and in addition to placing patient within mechanically ventilated rooms, contact precautions must be undertaken.[332]

Despite the obvious need for these rooms the design of mechanically ventilated isolation rooms is not evidence based, and if badly designed can place HCWs at risk.[335] There is also evidence that the equipment associated with mechanical ventilation, such as ventilation grills, can themselves be the source of outbreaks and so must be closely monitored.[331, 337, 338]

1.5.5 Environmental Control

In 1968 E. H. Spaulding proposed three categories of cleaning requirements in relation to clinical risk.[339] Surfaces were divided into noncritical, semi critical and critical. Environmental surfaces within clinical environments are considered to be non-critical items, as they only come into contact with intact skin, which is a barrier to disease acquisition, and are thus considered at low risk of transmitting disease to patients.[339] The impact of the role of environmental disinfection in reducing infection rates remains a controversial issue despite many reports suggesting a link between the environment and specific microbial outbreaks.[340]

Despite this, in recent years both the CDC and DoH have issued guidance on the frequency of cleaning and the standard that that cleaning must reach.[341] This is because the types of contamination present on a surface are unknown, and in contrast to the 1960s when the guidelines were first established, pathogens now vary widely in their susceptibility to antimicrobials.[21] Guidelines for the cleaning of surfaces within hospitals need to take into account parameters that are relevant to prevention of transmission of nosocomial pathogens; such as ward type (likelihood of patients being carriers etc), expected frequency of hand contact with the surface and susceptibility of the patients population.[88]

Biofilms

In nature most microorganisms exist primarily within biofilms, attaching to both living and inanimate surfaces.[342] The development of biofilms is recognised as an important driver for persistent infections linked to clinical equipment, such as central venous catheters.[343] There is an increasing recognition that biofilms also play an important role within the clinical environment and on surfaces.[342, 343] This is because the adaption of lifestyle within biofilms permits survival within hostile environments.[343]

The first stage of biofilm formation is adherence to the surface, within Gram-negative bacteria this is facilitated by flagella and pili and within Gram-positive bacteria by surface proteins.[343] Once attachment has occurred the biofilm proliferates and

extracellular polymeric substances (EPS) are produced, forming a three dimensional structure. EPS contains extracellular DNA (eDNA), polysaccharides and proteins.[344] eDNA facilitates the initial stage of binding to biomaterials and forms the structural backbone of the biofilm promoting aggregation.[344] eDNA is formed by the release of bacterial genomic DNA, mostly by cell lysis, but also by active excretion into the matrix.[344] Twitching motility then mediates biofilm expansion and active colonisation of surfaces.[345] Finally a dispersion phase occurs, where bacteria are released from the biofilm to permit colonisation of new clinical surfaces by hand or other transfer (see Figure 1-4.).[343]

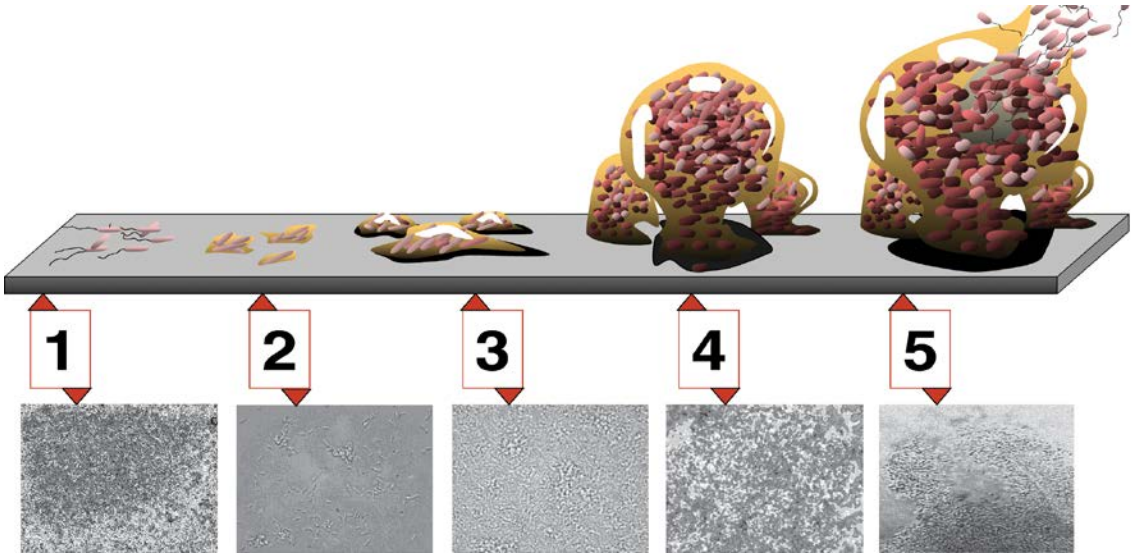

Figure 1-4 Five stages of Biofilm Development - Stage 1, initial attachment; Stage 2, irreversible attachment; Stage 3, maturation I; Stage 4, maturation II; Stage 5, dispersion.[346]

1.5.5.1 ENVIRONMENTAL CLEANING

Cleaning of all surfaces should aim to remove epidemiologically important pathogens.[347] It has been estimated that between 5 – 70% of microorganisms in patients rooms are there as a result of ineffective surface disinfection, mainly because the disinfectant has not reached all areas.[348] Environmental cleaning to control this contamination is considered an underutilized intervention in order to prevent HCAI.[288] This argument is countered by those who say that whilst expenditure on cleaning is justified from an aesthetic point of view, it is far from clear that disinfection of the environment is an important infection control intervention.[349]

Throughout healthcare, guidance exists that governs the cleanliness of healthcare premises in order to try and deliver both a suitable aesthetic and control risk.[350] However guidance for when routine cleaning should occur is often confusing, stating

that, depending on risk, cleaning should be undertaken either daily/ three times a week/ or when surfaces are visibly soiled as well as after the patient is discharged.[351] Part of the reason for this is that governmental guidance on disinfection lags behind scientific evidence and the role of nosocomial pathogens is changing.[352] Interpreting this guidance means that cleaning should be tailored to clinical risk and targeted cleaning may be an important factor for controlling HCAI.[189, 341]

Cleaning guidance does not cover division of labour; however in general routine hospital cleaning is undertaken by cleaners who manually apply either disinfectant or detergent to surfaces, whereas additional intermittent cleaning is undertaken by nursing staff.[288] In order to undertake thorough cleaning education and training in the correct protocols has been shown to improve both cleaning performance and infection rates.[189]

In addition there are many biocide specific factors to consider when examining environmental cleaning including: disinfectant efficacy, environmental impact, correct and practical use, safety, effects of the presence of soil and compatibility with surfaces.

Product Efficacy

Minimum efficacy of products used for environmental disinfection are determined by European Test standards, based upon International Organization for Standardization (ISO) 5725-Parts 1-3 (1994).[353] Testing situations and minimum efficacy requirements are described in Table 1-3. Most tests are suspension based tests, where organisms specified are suspended within the test solution.

European Test standards (EN)	Test Organism	Test Conditions	Test Requirements
EN 1040	*Staphylococcus aureus* *Pseudomonas aeruginosa*	Suspension-based study used as a presumptive test	5 $\log_{10}$ reduction in ≤5 minutes
EN 1275	*Candida albicans* *Aspergillus brasiliensis*	Suspension-based study used as a presumptive test	4 $\log_{10}$ reduction in ≤15 minute
EN 1276	*Staphylococcus aureus* *Pseudomonas aeruginosa*	Suspension-based study used to formally	5 $\log_{10}$ reduction in

	Enterococcus hirae *Escherichia coli*	evaluate bactericidal activity	≤5 minutes
EN 13697	*Staphylococcus aureus* *Pseudomonas aeruginosa* *Enterococcus hirae* *Escherichia coli* *Candida albicans* *Aspergillus brasiliensis*	Carrier-based study used to formally evaluate bactericidal and fungicidal activity on non-porous surfaces	4 $\log_{10}$ reduction of bacteria in ≤5 minutes and 3 log reduction of fungi in ≤15 minutes
EN 1650	*Candida albicans* *Aspergillus brasiliensis*	Suspension-based study used to formally evaluate fungicidal activity	4 $\log_{10}$ reduction in ≤15 minutes
EN 13624	*Candida albicans* *Aspergillus brasiliensis*	Suspension-based study used to formally evaluate fungicidal activity of products that are used in the medical area for disinfecting instruments by immersion	4 $\log_{10}$ reduction in ≤60 minutes
EN 13727	*Staphylococcus aureus* *Pseudomonas aeruginosa* *Enterococcus hirae*	Suspension-based study used to formally evaluate bactericidal activity of products that are used in the medical area (e.g. hygienic handrub)	3-5 $\log_{10}$ reduction in 1-5 minutes depending on claim
EN 14347	*Bacillus subtilis* *Bacillus cereus*	Suspension-based study used as a presumptive test	4 $\log_{10}$ reduction in ≤120 minutes
EN 14348	*Mycobacterium avium* *Mycobacterium terrae*	Suspension-based study used to formally evaluate mycobactericidal activity	4 $\log_{10}$ reduction in ≤60 minutes

Table 1-3 European Test standards for testing of chemicals to be used in surface and equipment decontamination, based upon ISO 5725-Parts 1-3.[353]

Surface Decontamination

The aim of disinfection is to eliminate most microbes and typically involves the use of disinfecting agents, rather than detergent. The degree of organism destruction is based upon their sensitivity to chemical agents, with spores and *Mycobacteria* being the most resistant, followed by Gram-negative organisms, Gram-positive organisms and fungal organisms.[181, 354]

Biocides at high concentrations cause massive cellular damage, but they have different modes of action and therefore although the physiological outcomes are similar the biochemical actions may be quite different.[355] This is only true at high concentrations of the biocide and so the concentration within the product formulation is key.[354] As high concentrations of biocide can impact upon both user health and surfaces integrity, efficacy must be balanced against toxicity and environmental degradation.[354]

Three types of solutions are used for cleaning: detergents which remove organic matter and suspend grease and oil, disinfectants which rapidly kill or inactivate infectious particles and detergent-disinfectants which achieve both organic matter removal and organism kill.[181] Traditional cleaning methods use either detergents or disinfectants plus detergents.[347]

Within disinfectants there are three classifications of action. High level disinfectants are capable of inactivating/destroying spores while intermediate level disinfectants are able to inactivate resistant viruses, such as adenovirus and poliovirus, as well as *Mycobacterial* spp. Finally low level disinfectants are able to destroy vegetative bacteria.[356] Disinfectants active at intermediate and low levels are not capable of inactivating spores.[356]

No specific guidance on which of these cleaning solutions should be used within the clinical environment for routine cleaning is given in guidance.[181] However DoH guidance indicates that sporicidal or chlorine releasing agents should be used for terminal cleaning post discharge of a patient infected with *Clostridium difficile*.[357]

Detergents vs Chlorine

Surface detergents containing aldehydes or quaternary ammonium compounds are used much more frequently in Germany and other European countries outside of

England and Italy. They are also used less widely than chlorine based compounds in the US.[358] However such compounds are not active against either spores or resistant viruses such as adenovirus and norovirus.[8] Within countries where aldehydes/quaternary ammonium compounds are not frequently used chlorine releasing agents are considered the gold standard.[35]

The bactericidal activity of disinfectants is inversely proportional to the degree of soiling upon surfaces and therefore including a detergent within the solution is important for full activity.[352] Hypochlorites are the most common chlorine disinfectants as they possess broad spectrum high level disinfectant activity, do not leave toxic residues, are unaffected by water hardness, comparatively inexpensive, fast acting, and capable of removing both dried or fixed organisms biofilms from surfaces.[359] However some professionals believe that the indiscriminate use of disinfectants within the healthcare environment is not justified due to the fact that they are expensive, corrosive to the local environment and potentially toxic to the wider environment when disposed of in waste water.[341, 357, 360]

Evidence for Disinfection over Detergents

Detergents have been shown to become contaminated during the cleaning process and thus in themselves pose a contamination risk. In one study 22 detergents used for cleaning of floors were sampled prior during and post use, Gram-negative bacteria were found in 10/22 freshly prepared samples, 20/22 samples after 30 minutes of cleaning and 21/22 samples at discard.[361] Another study on cleaning solutions for floors found that CFU/ml counts rose to 24,000 in detergent solutions vs 20CFU in disinfectant solutions.[339] Finally Barker et al. (2004) found that cleaning with detergent instead of disinfectant resulted in an increase in norovirus cases.[157, 361, 362]

Problems with Chlorine Cleaning Chemistries

In addition to surface degradation chlorine based products can produce irritation in skin, nose and lungs and may act a sensitizer, resulting in a potential increases in occupational risk of asthma and respiratory disease.[363, 364] Despite this many nurses and cleaners are unaware of the exposure risk; which can continue for up to 20 minutes after the completion of cleaning tasks. This can also result in patient and visitor exposure if chlorine based agents are used for routine cleaning as well as terminal disinfection.[363] Additionally chlorine gas can be produced by accidental combination of cleaning products, resulting in potential staff illness.[365]

1.5.5.2 EQUIPMENT CLEANING

Regular cleaning by domestic staff needs to be supported by intermittent cleaning based on clinical activity within bed spaces.[341] In addition to intermittent cleaning most equipment cleaning, especially within ITUs, is undertaken by nursing staff using wipes.[189] Use of wipes is important because equipment and other sites can become rapidly contaminated without being linked to high numbers of interactions.

There are a number of factors that can affect the efficiency of cleaning with wipes. Contact times between wipes and surfaces should be at least 60 seconds in order to optimize bacterial killing.[354] Even if this contact time is adhered to it is substantially less than the contact time that is usually required between a biocide and a surface due to the decreased level of biocide remaining on the surface post wipe.[360]

Nurses unlike cleaners are often not educated about the most appropriate way to undertake cleaning, in terms of contact times, surface differences and the need for appropriate mechanical action.[21, 288] As a result the cleaning is often not undertaken as per protocol.[366] Despite issues with cleaning being undertaken in real life situations, a simulation has shown that the use of intermittent decontamination using wipes is more effective than routine cleaning once a day at maintaining decreased levels of surface contamination.[288]

Surface contamination is rarely uniform and if sufficient decontamination does not rapidly occur then residual organisms can be moved from the initial location of high contamination across a wider surface area, especially if wipes are used to clean more than a single object.[21, 288] Pathogens can also be transferred between wipes and hands, and also potentially onto wipe containers.[21]

Although decontamination of equipment and appropriate clinical surfaces is a nursing responsibility their main priority will always be patient care. Accordingly cleaning will be reduced when nursing staff are busy, this is exactly the time when microbial contamination is likely to increase, due to increases in workload and additional traffic and so cleaning is required more than during less busy periods.[367]

1.5.5.3 NOVEL DECONTAMINATION TECHNOLOGIES

Effective cleaning relies upon operators to correctly distribute and apply cleaning agents.[368] In order to aid terminal disinfection of rooms a number of new automated decontamination technologies have been developed in order to support the cleaning process.[368] All non-touch automated systems require cleaning prior to their use, to bring rooms to a suitable aesthetically clean standard.[368]

The two main types of technologies are those using ultra violet (UV) light and those using hydrogen peroxide technologies.

UV is virucidal and bactericidal and acts by targeting the nucleic acids as well as modifying capsid and proteins. Single stranded RNA and DNA viruses are more susceptible than bacteria and double stranded DNA viruses.[369-372]

H_2O_2 (hydrogen peroxide) was first discovered by Louis Thénard in 1818, decomposes into non-toxic bi-products of water and oxygen. Vaporised H_2O_2 is typically used at lower concentrations compared to its use as a liquid disinfectant and it has been suggested that the disinfectant properties of the gaseous form may be distinct from those of the liquid. H_2O_2 is an oxidising agent that reacts with proteins, lipids and nucleic acids.[373, 374]

Two platforms are currently available for use of hydrogen peroxide (HP) in room decontamination. One (Glossair) uses aerosolized H_2O_2 delivered via a pressure generated aerosol. Aerosolized droplets are introduced via a unidirectional nozzle with a particle size of 8-10um. It delivers a solution of 5-6% H_2O_2 and <50ppm silver. Following exposure the aerosol decomposes naturally without active aeration of the room being necessary.[368] The other platform (Bioquell) delivers a heat generated vapour of 30-35% w/w aqueous H_2O_2 through a high velocity air stream, to achieve a homogeneous distribution. HP is delivered until the air is saturated and then the aeration unit catalyses the breakdown into oxygen and water.[368]

The one study that has utilised HP routinely as part of the terminal cleaning process notes that the risk of acquisition of MDR organisms in rooms where patients were admitted post HP treatment was 64% less than in those rooms that had been terminally cleaned with detergent. For rooms that had been occupied by a previous patient with VRE acquisition rates were reduced by 80% in rooms treated by HP compared to those rooms that were untreated.[375]

1.5.6 The Role of Design

Establishing a reliable evidence base to inform approaches to infection control has been problematic. This is due to the multitude and complexity of factors influencing the spread of infection, and particularly those that affect patient interactions with people and the environment.[376]

Buildings are complex ecosystems that house trillions of microorganisms that interact with each other and the humans occupying the environment. Building attributes such as sources of ventilation, airflow rates, and relative humidity correlate with microbiome composition.[376] Despite this acknowledged impact, architects use a comfort model for designing indoor spaces, with little understanding of how these affect human health and microbial transmission.[332, 376] The built environment can therefore play an equal role in either supporting or inhibiting efficient, high quality healthcare.[324]

There is an increasing focus on the need for a scientific evidence base to be introduced into hospital design.[376] Within the US the build environment is recognised as having a profound effect on human health, and there is a requirement in healthcare facility design that building should 'first do no harm'.[336] Within the UK the health benefits of well-planned architecture have been recognised since Florence Nightingale, who introduced the concept of natural ventilation into hospital premises.[376] This meant that for many years hospitals were designed with south facing windows to allow sunlight in and cross ventilation to dilute airborne infection.[332]

Infection control measures in modern hospitals can be supported by architecture i.e. the provision of sufficient space to treat patients, sufficient isolation rooms and hand hygiene facilities.[326, 336] However many infection control requirements of the built environment are expensive, non-evidence based and not based in regulation, and it can accordingly be difficult for hospitals to support the investment.[326, 377] Inappropriate hospital design can result in increasing in HCAI, especially when ventilation is inappropriate, or washing of equipment leads to exposure of patients, due to inappropriate sink location or water contamination.[324]

Due to the very different healthcare facilities that are available worldwide it is very difficult to make comparisons between them.[326] Two studies that observed changes in HCAI when moving to new hospitals, which were supposed to be better designed in order to support infection control, found that this on its own was not sufficient to result in improvement of HCAI levels.[326, 378]

1.5.7 Environment Composition

There is a need to support infection control within hospitals by designing surfaces that are easy to clean, resistant to moisture, safe for patients, capable of resisting degradation due to cleaning products and if possible have a topography that is resistant to the formation of biofilms.[336, 342, 348] A lot of focus has recently been placed

upon environmental sustainability both for entire building systems, such as ventilation, but also for individual surface components, such as avoiding the use of volatile organic compounds in materials.[336]

Surfaces should be made of non-porous material, free from fissures and crevices that prevent removal of dirt, have rounded corners, and not be made from cloth.[336, 352] The use of stainless steel should be carefully considered as although good for avoiding microbial growth; they can also be difficult to clean with many disinfectants.[336, 359]

In addition to these requirements there are now hundreds of products on the market that contain low concentration biocides: including fabrics and surfaces.[354] Among these are surfaces that contain heavy metals, such as silver and copper, and surfaces that are impregnated with germicides such as quaternary ammonium compounds, or triclosan.[379] For all of these surfaces the presence of organic matter and contamination can inhibit any antimicrobial activity and so routine cleaning is still required. Their use is also associated with an increase in financial resource requirements.[359]

1.6 RESEARCH OBJECTIVES

The specific objectives were as follows:

Chapter 3:

Work undertaken as part of Chapter 3 aims to develop sampling methodologies for the detection of both bacteria and viruses within the clinical environment. These sampling methodologies will then be applied to a number of units within both adult and paediatric settings in order to determine levels of environmental contamination outside of infection control outbreaks.

Chapter 4:

To facilitate infection control investigations typing techniques will be developed and validated against the current reference standard for Gram-negative bacteria (*Enterobacter species*, *E. coli* and *Klebsiella* species). These typing techniques will then be applied to isolates detected within Great Ormond Street Hospital over a two year period (2011 – 2012), some of which will be linked to outbreak investigations, in order to determine if there was a link between those organisms found within the environment, and those found within patients.

Chapter 5:

This chapter evaluates the use of available infection control interventions that could prevent either the accumulation of potentially pathogenic organisms within the clinical environment, or aim to prevent transfer of those organisms from the environment to patients. These interventions include both cleaning and environmental decontamination and the role of design.

1.7 Flow Diagram of Thesis Structure

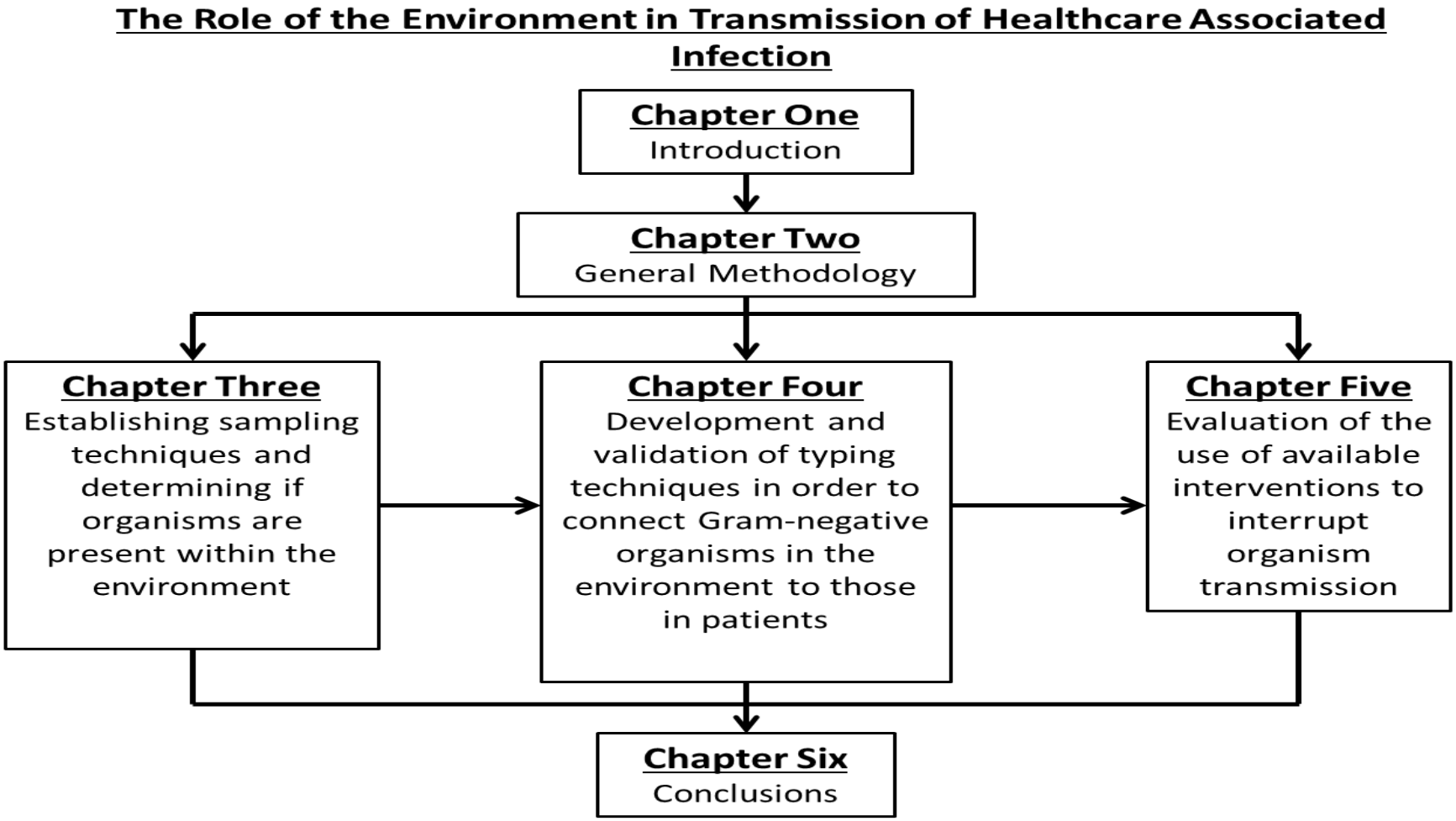

Figure 1-5 Flow diagram of thesis structure.

Chapter 2 MATERIALS AND METHODS

All laboratory work within this thesis was undertaken using good laboratory practice, aseptic techniques and in accordance with localised health and safety protocols, risk assessment and following Control of Substances Hazardous to Health (COSHH) regulations.

2.1 CELL CULTURE METHODS

2.1.1 CONTINUOUS CELL CULTURES

Cell culture was undertaken in green African monkey cell line (VERO) (European Collection of Cell Culture, Salisbury, UK) for adenovirus serotypes. All cells were maintained at 37°C with 5% CO_2.

VERO cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Gillingham, UK) supplemented with 10% (v/v) foetal bovine serum (FBS) (Sigma-Aldrich, Gillingham, UK), 1% (v/v) Hepes buffer (Sigma-Aldrich, Gillingham, UK), 0.05% (v/v) ciprofloxacin and 0.2% (v/v) penicillin.

Cell lines were passaged using trypsin EDTA (Sigma-Aldrich, Gillingham, UK) for normal cell maintenance.

2.1.2 CELL CULTURE FROM INOCULATION EXPERIMENTS

VERO cells were cells were transferred into inoculation media for sample culture containing Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Gillingham, UK) supplemented with 2% (v/v) foetal bovine serum (FBS) (Sigma-Aldrich, Gillingham, UK), 1% (v/v) Hepes buffer (Sigma-Aldrich, Gillingham, UK), 0.05% (v/v) ciprofloxacin and 0.2% (v/v) penicillin.

250µl of adenovirus type strains (C1 NCTC 0011051v/C2 NCTC 0108051v/A31 NCTC 0011265v) or inoculation test sample was inoculated into VERO cell culture and grown for up to 7 days, and checked daily. Positive results were detected when cells demonstrate a characteristic cytopathic effect, visualised using a light microscope. If an indeterminate cytopathic effect was noted the sample was passaged after the initial 7 day period. Viral quantification was undertaken using methods described in 2.2.5.4.

2.2 MOLECULAR BIOLOGICAL METHODS

2.2.1 EXTRACTION

2.2.1.1 DEOXYRIBONUCLEIC ACID EXTRACTION

Deoxyribonucleic acid (DNA) extractions were undertaken using kits where the following stages were undertaken:

- Chemical lysis and protein removal
- Mechanical lysis
- Ethanol precipitation
- Binding of DNA to a silica membrane under high salt conditions
- Removal of residual contaminants
- Elution of bound DNA, under low salt conditions and elution in a DNA stabilising buffer

For clinical virology samples; 10µl of internal positive control (IPC) was added to 200µl of the sample. The deoxyribonucleic acid (DNA) was extracted using the Qiagen mini kit (Qiagen, Crawley, UK) and eluted in 100µl of buffer AE, as described in the manufacturer's instructions.

For clinical bacteriology samples; Suspensions were heated to 95^{o}C for 10 minutes and bead beated with lysing matrix B for 40 seconds at 6.5m/s in the FastPrep instrument (MP Biomedicals, Illkirch, France), The DNA was extracted using the Qiagen mini kit (Qiagen, Crawley, UK) and eluted in 100µl of buffer AE, as described in the manufacturer's instructions.

For environmental samples; swabs were vortexed for 30 seconds within the vials to release virus particles and 200µl of the solution transferred to a clean tube containing 10µl of IPC. The DNA was extracted using the Qiagen mini kit (Qiagen, Crawley, UK) and eluted in 100µl of buffer AE, as described in the manufacturer's instructions.

For bacterial isolates; Suspensions were heated to 95^{o}C for 10 minutes and a tenfold dilution in buffer AE was bead beated with lysing matrix B (MP Biomedicals, Illkirch, France) for 40 seconds at 6.5m/s in the FastPrep instrument (MP Biomedicals, Illkirch,

France), centrifuged at 20,000 * g for 2 minutes and supernatant removed to a fresh tube.

For bacterial isolates (*Enterobacter cloacae* multi-locus variable number tandem repeat typing); suspensions were heated to 95^{o}C for 10 minutes and a tenfold dilution in buffer AE was centrifuged at 20,000 * g for 2 minutes and supernatant removed to a fresh tube.

For bacterial isolates (Repetitive extragenic palindromic based polymerase chain reaction); 1µl loop of a fresh overnight culture suspended in 300µl of microbead solution was extracted using the Mo Bio extraction kit (Mo Bio Laboratories, Carlsbad, America) following the manufacturer's instructions, eluting in 35µl of buffer MD5. The DNA was quantified using the Nanodrop (Thermo Scientific Waltham, UK) and adjusted to a concentration of 35ng/µl (see section 2.2.4.).

For bacterial isolates (High Throughput Sequencing): Suspensions were heated to 95^{o}C for 10 minutes and bead beated with lysing matrix B for 40 seconds at 6.5m/s in the FastPrep instrument (MP Biomedicals, Illkirch, France), The DNA was extracted using the Qiagen mini kit (Qiagen, Crawley, UK) and eluted in 100µl of buffer AE, as described in the manufacturer's instructions. The DNA was quantified using the Qubitt (Life Technologies, Paisley, UK) and adjusted to a concentration of 0.5ng/µl or 0.2ng/µl depending upon the needs of further processing (see section 2.2.4.).

2.2.1.2 RIBONUCLEIC ACID EXTRACTION

Swabs were vortexed within the vials for 30 seconds and 200µL was aliquoted into clean vials for nucleic acid extraction. 10µL of mouse genomic DNA was added as an internal control. Nucleic acids were extracted using the Qiagen RNeasy mini kit (Qiagen, UK) as per the manufacturer's instructions using the following stages:

- Cell lysis in the presence of RNase inhibitors and RNA stabilization
- RNA binding to a silica membrane under high salt conditions
- Contaminant removal
- Elution under low salt conditions and elution in a RNA stabilising buffer

2.2.2 Co-Extraction (DNA/RNA)

Swabs were vortexed within the vials for 30 seconds and 200µL was aliquoted into clean vials for nucleic acid extraction. 10µL of mouse genomic DNA was added as an internal control. Nucleic acids were extracted using the Qiagen RNeasy mini kit (Qiagen, UK) with the addition of a 56°C 10 minute heat step prior to extraction; subsequent procedures were carried out according to the manufacturers protocol.

2.2.3 First-strand Complementary DNA synthesis

Samples extracted for ribonucleic acid (RNA) were subject to a complementary DNA (cDNA) step. 21.2µL of nucleic acid extract was added to 0.24µg/µL random primers (Life Technologies, UK), and 0.02mM deoxynucleotide triphosphates (dNTPs) (Bioline, UK), heated at 65°C for 5 minutes then 4°C for 2 minutes. 1× First strand buffer (Life Technologies, UK), 0.05mM DL-Dithiothreitol (DTT) (Life Technologies, UK), 2 units/µL of RNase out (Life Technologies, UK), and 15 units/µL of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV) (Life Technologies, UK) were added per sample and the mixture heated at 37°C for 30 minutes then 70°C for 15 minutes. Samples were stored at -80°C until semi-quantitative polymerase chain reaction (PCR) was performed as described in section 2.2.5.3.

2.2.4 Measurement of DNA concentration & Purity

The concentration of DNA used for use in repetitive extragenic palindromic based PCR (REP-PCR) was estimated using a NanoDrop1000 spectrophotometer (Thermo Scientific, Waltham, UK) where Abs. 1.0 at 260nm = 50mg/ml. The purity of DNA used in this study was indicated by the ratio of OD260/280 lay between 1.7 and 2.0.

The purity of DNA for use with whole genome sequencing (WGS) and high throughput sequencing (HTS) was assessed using fluorometric-based methods on the Qubit (Life Technologies, Paisley, UK) and was indicated by the ratio of OD260/280 and lay between 1.7 and 2.0. The DNA concentration for use with HTS was estimated by using either the High Sensitivity dsDNA assay (Life Technologies, Paisley, UK) or the Broad Range dsDNA assay (Life Technologies, Paisley, UK) dependent on predicted DNA concentration.

2.2.5 REAL-TIME PCR

All samples were extracted with an inhibition control (10ul of mouse muscle cell culture). Real-time PCR was run in parallel with the target PCR using the primers listed in Table 2-1 to determine the presence of sample inhibition. Samples identified as inhibited were diluted 1:10 and re-tested.

Primer Name	Primer Type	Sequence	Reference
Mus-F	Forward	5' – GGA CAC TAT GCC CCT CCT TAG A – 3'	(380)
Mus-R	Reverse	5' – AGC TCC AAA CTC CGT CTC TGT AA – 3'	(380)
Mus-Probe	Probe	5' NED – TTG GGA ACA AAA CAC CCA – MGBNFQ 3'	(380)

Table 2-1 Mouse muscle cell (Mus) internal positive control primers and probe.

2.2.5.1 *STAPHYLOCOCCUS AUREUS* PCR

Real-time PCR detection of *Staphylococcus aureus coa* gene was carried out in the following reaction mixture: 14µl 2X QuantiTect multiplex mastermix (Qiagen, Crawley, UK), 0.1µM primer Sa-3-f, 0.1µM primer Sa-3-r, 0.1µM coa-probe, 0.1µl Mus-F, 0.1µl Mus-R, 0.1µl Mus-Probe, 10µl DNA extract and molecular grade water to give a final volume of 28µl (see *Table 2-1* and *Table 2-2*).

The reactions were cycled on the ABI prism 7300 real-time PCR system (Applied Biosystems, Warrington, UK) as follows: 95˚C for 10 minutes followed by 45 cycles of 95˚C for 15 seconds and 60˚C for 60 seconds.

Or:

Real-time PCR detection of Enterobacteriaceae *dnaK* gene was carried out in the following reaction mixture: 14µl 2X QuantiFast multiplex mastermix (Qiagen, Crawley, UK), 0.1µM primer Sa-3-f, 0.1µM primer Sa-3-r, 0.1µM coa-probe, 0.1µl Mus-F, 0.1µl Mus-R, 0.1µl Mus-Probe, 10µl DNA extract and molecular grade water to give a final volume of 28µl (see Table 2-1 and Table 2-2).

The reactions were cycled on the ABI prism 7500 real-time PCR system (Applied Biosystems, Warrington, UK) as follows: 95˚C for 20 seconds followed by 45 cycles of 95˚C for 3 seconds and 60˚C for 30 seconds.

Primer Name	Primer Type	Sequence
Sa-3-f	Forward	5' – GTA GAT TGG GCA ATT ACA TTT TGA AGG – 3'
Sa-3-r	Reverse	5' – CGC ATC TGC TTT GTT ATC CCA TGT A – 3'
coa-probe	Probe	5' FAM – TAG GCG CAT TAG CAG TTG CAT A – BHQ1 5'

Table 2-2 *Staphylococcus aureus coa* gene real-time PCR primers and probe.

2.2.5.2 ENTEROBACTERIACEAE PCR

Real-time PCR detection of Enterobacteriaceae *dnaK* gene was carried out in the following reaction mixture: 14µl 2X QuantiTect multiplex mastermix (Qiagen, Crawley, UK), 0.2µM primer Ent-dnak-f, 0.2µM primer Ent-dnak-r, 0.2µM Ent-dnak-probe, 0.1µl Mus-F, 0.1µl Mus-R, 0.1µl Mus-Probe, 10µl DNA extract and molecular grade water to give a final volume of 28µl (see Table 2-1 and Table 2-3).

The reactions were cycled on the ABI prism 7300 real-time PCR system (Applied Biosystems, Warrington, UK) as follows: 95˚C for 10 minutes followed by 45 cycles of 95˚C for 15 seconds and 60˚C for 60 seconds.

Or:

Real-time PCR detection of Enterobacteriaceae *dnaK* gene was carried out in the following reaction mixture: 14µl 2X QuantiFast multiplex mastermix (Qiagen, Crawley, UK), 0.2µM primer Ent-dnak-f, 0.2µM primer Ent-dnak-r, 0.2µM Ent-dnak-probe, 0.1µl Mus-F, 0.1µl Mus-R, 0.1µl Mus-Probe, 10µl DNA extract and molecular grade water to give a final volume of 28µl (see Table 2-1 and Table 2-3).

The reactions were cycled on the ABI prism 7500 real-time PCR system (Applied Biosystems, Warrington, UK) as follows: 95˚C for 20 seconds followed by 45 cycles of 95˚C for 3 seconds and 60˚C for 30 seconds.

Primer Name	Primer Type	Sequence
Ent-dnak-F	Forward	5’ – ACC TGG GTA CWA CCA ACT CTT GTG T – 3’
Ent-dnak-R	Reverse	5’ – GTC ACT GCC TGA CGT TTA GC – 3’
Ent-dnaK-Probe	Probe	5’ FAM – AGG ATG GTG AAA CTC TGG TWG GTC AGC C – BHQ1 3’

Table 2-3 Enterobacteriaceae *dnaK* gene real-time PCR primers and probe.

2.2.5.3 NOROVIRUS PCR

Real-time PCR detection of the norovirus GI and GII open reading frame genes was performed. The reaction was as follows: 10µL 2X Fast Universal PCR mastermix (Applied Biosystems, Warrington, UK), 0.1µM of COG1F, 0.1µM COG1R, 0.05µM RING1(a)-TP, 0.05µM RING1(b)-TP, 0.1µM QNIF2, 0.1µM COG2R, 0.1µM QNIFS, 10µL cDNA and molecular grade water to give a final volume of 25 µl (see *Table 2-4*).

The reactions were cycled on the ABI prism 7500 real-time PCR system (Applied Biosystems, Warrington, UK) as follows: 95˚C for 20 seconds followed by 45 cycles of 95˚C for 3 seconds and 60˚C for 30 seconds.

Primer Name	Primer Type	Sequence	Reference
COG1F	Forward	5'–CGYTGGATGCGNTTYCATGA–3'	(381)
COG1R	Reverse	5'–CTTAGACGCCATCATCATTYAC–3'	(381)
RING1(a)-TP	Probe	5'-FAM-AGATYGCGATCYCCTGTCCA-BHQ1-3'	(381)
RING1(b)-TP	Probe	5'-FAM-AGATCGCGGTCTCCTGTCCA-BHQ1-3'	(381)
QNIF2	Forward	5'–ATGTTCAGRTGGATGAGRTTCTCWGA–3'	(382)
COG2R	Reverse	5'–TCGACGCCATCTTCATTCACA–3'	(381)
QNIFS	Probe	5'-JOE–AGCACGTGGGAGGGCGATCG–BHQ1-3'	(382)

Table 2-4 Norovirus GI and GII open reading frame gene real-time PCR primers and probes.

2.2.5.4 ADENOVIRUS PCR

Real-time PCR detection of the adenovirus *hexon* gene was carried out in the following mixture: 10µl 2X QuantiFast multiplex mastermix (Qiagen, Crawley, UK), 0.1µM of Adeno Forward, 0.1µM Adeno Reverse, 0.1µM Adeno probe, 0.1µl Mus-F, 0.1µl Mus-R, 0.1µl Mus-Probe, 10µl DNA extract and molecular grade water to give a final volume of 25µl (see Table 2-1 and Table 2-5).

The reactions were cycled on the ABI prism 7300 real-time PCR system (Applied Biosystems, Warrington, UK) as follows: 95˚C for 10 minutes followed by 45 cycles of 95˚C for 15 seconds and 60˚C for 60 seconds.

Or:

Real-time PCR detection of the adenovirus *hexon* gene was carried out in the following mixture: 10µl 2X Fast Universal PCR mastermix (Applied Biosystems, Warrington, UK), 0.1µM of Adeno Forward, 0.1µM Adeno Reverse, 0.1µM Adeno probe, 0.1µl Mus-F, 0.1µl Mus-R, 0.1µl Mus-Probe, 10µl DNA extract and molecular grade water to give a final volume of 25µl (see Table 2-1 and Table 2-5).

The reactions were cycled on the ABI prism 7500 real-time PCR system (Applied Biosystems, Warrington, UK) as follows: 95˚C for 20 seconds followed by 45 cycles of 95˚C for 3 seconds and 60˚C for 30 seconds.

Primer Name	Primer Type	Sequence	Reference
Adeno Forward	Forward	5' – GCC ACG GTG GGG TTT CTA AAC TT – 3'	(383)
Adeno Reverse	Reverse	5' – GCC CCA GTG GTC TTA CAT GCA CAT C -3'	(383)
Adeno Probe	Probe	5' FAM – TGC ACC AGA CCC GGG CTC AGG TAC TCC GA – BHQ1 3'	(383)

Table 2-5 Adenovirus hexon gene real-time PCR primers and probe.

2.2.6 ADENOVIRUS TYPING

PCR detection of hyper variable region 7 (HVR-7) of the Adenovirus *hexon* gene was carried out in the following mixture: 1x Bioline Buffer (Bioline, London, UK), 1.5mM $MgCl_2$ (Bioline, London, UK), 0.25µM of HVR-7 Forward, 0.25µM HVR-7 Reverse, 1mM dNTP's (Bioline, London, UK), 2.5 units of Bioline Taq (Bioline, London, UK), 10µl DNA extract and molecular grade water to give a final volume of 50µl (see Table 2-6.).

The reactions were cycled on a thermocycler as follows: 95˚C for 10 minutes followed by 40 cycles of 95˚C for 60 seconds, 51˚C for 60 seconds and 72^{o}C for 60 seconds and a final elongation 72^{o}C for 3 minutes.[384]

Primer Name	Primer Type	Sequence	Reference
HVR-7 Forward	Forward	5'- CTG ATG TAC TAC AAC AGC ACT GGC AAC ATG GG - 3'	(384)
HVR-7 Reverse	Reverse	5'- GCG TTG CGG TGG TGG TTA AAT GGG TTT ACG TTG TCC AT – 3'	(384)

Table 2-6 Sequence based adenovirus typing using *hexon* gene PCR primers.

The cycling conditions and reagent proportions of this assay were modified in order to enable amplification from clinical extracts as well as extracted cell culture. PCR detection of HVR-7 of the adenovirus *hexon* gene was carried out in the following mixture: 1x Accuprime PCR Buffer II (Invitrogen, Paisley, UK), 0.2µM of HVR-7 Forward, 0.2µM HVR-7 Reverse, 2.5 units of Accuprime Taq (Invitrogen, Paisley, UK),10µl DNA extract and molecular grade water to give a final volume of 50µl. The reactions were cycled on the as follows: 94˚C for 2 minutes followed by 40 cycles of 94˚C for 30 seconds, 60˚C for 30 seconds and 68^{o}C for 60 seconds and a final elongation 68^{o}C for 3 minutes.

Amplified products were electrophoresed through a 2% (w/v) agarose gel with 12.5% (v/v) ethidium bromide for staining and bands were visualised by UV transillumination. Bioline hyper ladder IV (Bioline, London, UK) was run alongside the amplicons.

Amplified products were then sequenced and analysed as described in section 2.2.7.

2.2.7 SANGER DNA SEQUENCING

Adenovirus typing PCR products were purified using Microspin columns which contain pores that allow molecules of differing sizes to pass through at different times. Larger molecules are excluded first into an eluate which is the purified PCR product (GE Healthcare Life Sciences, Little Chalfont, UK).

Sequencing PCR for adenovirus typing was carried out in the following mixture: 1µl Big Dye 3.1 PCR buffer (Invitrogen, Paisley, UK), 0.02µM of HVR-7 Forward, 0.02µM HVR-7 Reverse, 2µl Big Dye 3.1 reaction mix (Invitrogen, Paisley, UK), and 4.5µl amplified product to give a final volume of 12.5µl. The reactions were cycled as follows: 25 cycles of 96˚C for 20 seconds, 50˚C for 5 seconds and 60^{o}C for 4 minutes. Amplified sequencing products were then cleaned up using ethanol precipitation and pellets re-suspended in 5µl Hi-Di formamide (Life Technologies, Paisley, UK) prior to sequencing on an ABI 3130 capillary sequencer (Applied Biosystems, Warrington, UK) as per manufacturer's instructions.

Sequencing data was analysed using the Lasergene Suite version 12.0.0. (DNAStar, Madison, USA) and sequencing were uploaded for comparison on NCBI nucleotide BLAST available at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

2.2.8 WHOLE GENOME SEQUENCING ON THE MISEQ PLATFORM

47 *Klebsiella* species isolates and 1 *Enterobacter* species isolate extracted as listed in methods section 2.2.1.1 were adjusted to 0.2ng/ul using the Qubit DNA High Sensitivity assay (see methods section 2.2.4.). Library preparation was performed using the Nextera XT kit (Illumina, San Diego, USA) kit as per manufacturer's instructions as described below.

Enzymic DNA fragmentation and tagmentation was undertaken at 55^{o}C for 5 minutes followed by PCR amplification of the tagmented DNA. The amplification reactions were cycled on as follows: 72^{o}C for 3 minutes, 95^{o}C for 30 seconds followed by 12 cycles of 95^{o}C for 10 seconds, 55^{o}C for 30 seconds and 72^{o}C for 30 seconds and a final elongation 72^{o}C for 5 minutes.

The resulting amplified barcoded PCR products were normalised using 0.5x Agencourt AMPure XP (Beckman Coulter, High Wycombe, UK) to allow for size selection of >250bp amplicons. The resulting libraries were then pooled and run on the MiSeq platform (Illumina, San Diego, USA) using the 500 cycle V2 cartridge (Illumina, San Diego, USA) as per manufacturer's instructions.

2.2.9 PATHOGENICA HIGH THROUGHPUT SEQUENCING ON THE ION TORRENT PLATFORM

47 *Klebsiella* species isolates and 1 *Enterobacter* species isolate extracted as listed in methods section 2.2.1.1 were adjusted to 0.5ng/ul using the Qubit DNA High Sensitivity assay (see methods section 2.2.4.). Library preparation was performed using the HAI Biodetection kit (Pathogenica, Boston, USA) kit as per manufacturer's instructions as described below.

The hybridisation and ligation reactions to attach barcodes to the target DNA were cycled on as follows: 94^{o}C for 10 minutes, ramp to 60^{o}C at 0.1^{o}C/second followed by 60^{o}C for 10 minutes. This was followed by a capture reaction at 94^{o}C for 2 minutes, then 37^{o}C for 30 minutes. Preparations then underwent an exonuclease digestion to remove unwanted DNA at 94^{o}C for 15 minutes.

The resulting barcoded DNA fragments were normalised using 0.8x Agencourt AMPure XP (Beckman Coulter, High Wycombe, UK) to allow for size selection of >200bp DNA fragments. The Ion Torrent PGM sequencing platform (Life Technologies, Paisley, UK) was used to sequence the DNA fragments. Template preparation was carried out with the Ion PGM Template OT2 200 Kit (Life Technologies, Paisley, UK), according to manufacturer's instructions.

2.2.10 MULTI-LOCUS VARIABLE NUMBER TANDEM REPEAT TYPING

2.2.10.1 *ENTEROBACTER CLOACAE* MULTI-LOCUS VARIABLE NUMBER TANDEM REPEAT TYPING

Fifty five target loci were selected utilising Tandem Repeat Finder (http://tandem.bu.edu/trf/trf.html) using the whole genome sequence of *Enterobacter cloacae* (accession no. CP001918.1) as a reference sequence.

Eighteen of these fifty five loci were selected for primer design based on the following criteria: indel score of 0, percentage matches of over 90% and where possible whole number copy repeat sizes. Primers were designed using NCBI primer design programme available at www.ncbi.nlm.nih.gov. A maximum of fifty base-pairs can be amplified on either side of the target sequence and out of these eighteen potential targets, fifteen loci had flanking sequences that were suitable for primer set design.

Two different master mixes were used depending upon how the PCR was to be setup. If automated setup was performed using the QiAgility (Qiagen, Crawley, UK), Platinum Taq (Invitrogen, Paisley, UK) was substituted for the Core Taq (Qiagen, Crawley, UK)

used during manual setup. This was because PCR setup with the QiAgility took ~45 minutes and therefore a hot start Taq polymerase was required.

QiAgility PCR Setup: PCR detection of *Enterobacter cloacae* multi-locus variable number tandem repeat (VNTR) loci was carried out in the following mixture: 2.5µl 10x Coral Buffer (Qiagen, Crawley, UK), 0.2µM of locus forward primer, 0.2µM locus reverse primer, 1mM dNTPs, 5 units of Platinum Taq (Invitrogen, Paisley, UK), 2µl DNA extract and molecular grade water to give a final volume of 25µl (see *Table 2-7*.).

The reactions were cycled on as follows: 94˚C for 3 minutes followed by 35 cycles of 94˚C for 30 seconds, 57˚C for 30 seconds and 72^{o}C for 45 seconds and a final elongation 72^{o}C for 10 minutes.

Manual PCR Setup: PCR detection of *Enterobacter cloacae* VNTR loci was carried out in the following mixture: 1x Coral Buffer (Qiagen, Crawley, UK), 1mM dNTPs, 0.2µM of Locus Forward primer, 0.2µM Locus Reverse primer, 5 units of Core Taq (Qiagen, Crawley, UK), 2µl DNA extract and molecular grade water to give a final volume of 25µl (see Table 2-7.).

The reactions were cycled on a thermocycler as follows: 94˚C for 3 minutes followed by 35 cycles of 94˚C for 30 seconds, 57˚C for 30 seconds and 72^{o}C for 45 seconds and a final elongation 72^{o}C for 10 minutes.

Amplified products were electrophoresed through a 1.5% (w/v) agarose gel overlayed with Gel Red solution (Thermo Fisher Scientific, Waltham, USA) for staining and bands were visualised by ultra violet (UV) transillumination. Bioline hyper ladder II (Bioline, London, UK) was run alongside the amplicons. Amplified products were also examined using the QiAxcel automated electrophoresis system (Qiagen, Crawley, UK).

Primer Name	**Primer Type**	**Sequence**
Locus 1	Forward	5’ – ACC GTT ACG CAT CAG CAG CGG – 3’
Locus 1	Reverse	5’ – GCC CCT GCG CCA CAG GCT TA – 3’
Locus 2	Forward	5’ – TCT GCG TGA AAT GCC CGG TGG – 3’
Locus 2	Reverse	5’ – CAA CAG CGC CGG GTT TTG CCG – 3’
Locus 3	Forward	5’ – ACG AAC CCT CAC GTG CAA AAT CA – 3’
Locus 3	Reverse	5’ – CCC GCC CGC GAT GAC AGA A – 3’
Locus 4	Forward	5’ – CTT GGC CGG AAC CGC AAA GC – 3’
Locus 4	Reverse	5’ – AGC GTC CGC TTG CCG ACT TT – 3’
Locus 5	Forward	5’ – CGG CCA GCT GTT CTG CTG CT – 3’
Locus 5	Reverse	5’ – GAT CCG TCT CGT CTG GCG GC – 3’

Locus 6	Forward	5’ – GTT TAT TGC CGC CGC GCT GG – 3’
Locus 6	Reverse	5’ – ACA CGC CTG TGA AAC AAA AGG GA – 3’
Locus 7	Forward	5’ – CGC TCA ACC TCG CCA GAA TGA CC – 3’
Locus 7	Reverse	5’ – CCA CGT TCA CCA GGC TTT TCA GC – 3’
Locus 8	Forward	5’ – ACC GTC TCG GTC TGA CCC GC – 3’
Locus 8	Reverse	5’ – GGC AAA ATT GAG GTA AAT TCG CCC G – 5’
Locus 9	Forward	5’ – CCC GCG GGG AAG GCA AAA CC – 3’
Locus 9	Reverse	5’ – CGA CAG CGA AGC GTC CGG AC – 3’
Locus 10	Forward	5’ – GCC GTT ATC ATT GCC GCC GC – 3’
Locus 10	Reverse	5’ – GGT GAT AAC ACC GAC AAC GGT GG – 3’
Locus 11	Forward	5’ – ACC TGC CTA AAG GCG ACG CG – 3’
Locus 11	Reverse	5’ – GAG GTA GCG CCC AAC GCC TG – 3’
Locus 12	Forward	5’ – AGA TTT CGC TGG CTG GCT TTG T – 3’
Locus 12	Reverse	5’ – AAG GTG GTG TTG CCG CCT GG – 3’
Locus 13	Forward	5’ – TCG CGC AGA AAG AGA CCG GC – 3’
Locus 13	Reverse	5’ – GGT TGC TGC TCT TTG CCA CGG – 3’
Locus 14	Forward	5’ – AGC GTG TTG AGG TTC TCA TCC GC – 3’
Locus 14	Reverse	5’ – CAA CGG TTG CGT AAG CTT CCG – 3’
Locus 15	Forward	5’ – GGT GTC GGG TGA TCT CGC CC – 3’
Locus 15	Reverse	5’ – GCT GGT CAG TGT GAA CGT CAG CA – 3’

Table 2-7 *E. cloacae* VNTR loci primers.

2.2.10.2 *KLEBSIELLA PNEUMONIAE* MULTI-LOCUS VARIABLE NUMBER TANDEM REPEAT TYPING

PCR detection of *Klebsiella pneumoniae* VNTR loci was carried out in the following mixture: 2.5µl 10x Buffer (Invitrogen, Paisley, UK), 0.2µM of locus forward primer, 0.2µM locus reverse primer, 1mM dNTPs, 5 units of Platinum Taq (Invitrogen, Paisley, UK), 3µl DNA extract and molecular grade water to give a final volume of 25µl (see Table 2-8.).

The reactions were cycled on a thermocycler as follows: 94˚C for 2 minutes followed by 35 cycles of 94˚C for 30 seconds, 58˚C for 30 seconds and 72^{o}C for 60 seconds and a final elongation 72^{o}C for 10 minutes.

Primers were modified to permit analysis on the ABI 3130 capillary sequencer (Applied Biosystems, Warrington, UK) by the addition of fluorescent dyes to the forward primers.

Primer Name	Primer Type	Sequence	Reference
Locus A	Forward	5’ FAM - AGC GTA TCT GCC ATT GCC	(55)
Locus A	Reverse	5’- CAG CAT GGC CAG TTT GTC	(55)
Locus E	Forward	5’ HEX - CCA AATCCG GGT ATT TAT CG	(55)
Locus E	Reverse	5’- TTC GAT ACC CAT CCG GAA G	(55)

Locus H	Forward	5’ NED - ATG ACC AAG GAA GAA CCC G	(55)
Locus H	Reverse	5’- CTT TAC CTG GCA TGC GAA CG	(55)
Locus J	Forward	5’ FAM - ACC GGA TTA AGC GCT ATT CC	(55)
Locus J	Reverse	5’- TTC CTC GCC CAC GGA TAG	(55)
Locus K	Forward	5’ HEX - GAG CTG GCG GCT GGA ATA	(55)
Locus K	Reverse	5’- GCA ATC TGC CCG GAA ATA	(55)
Locus D	Forward	5’ NED - GCA GGT CTC GTC TTC ATT CC	(55)
Locus D	Reverse	5’- TGA CCA TCG AAG AGG CG	(55)
Locus N1	Forward	5’ FAM - CAT CAG GTG CAA GAT TCA A	UP
Locus N1	Reverse	5’- TGA GCG ATT GCT GGC CTA	UP
Locus N2	Forward	5’ HEX - GAT GCG GCA AGC ACC AC	UP
Locus N2	Reverse	5’- ACG CCC TGA CCA TTA TGC	UP
Locus N4	Forward	5’ NED - GTG CGG TGA TTG TGA TGG	UP
Locus N4	Reverse	5’- CTG ACA ACG TCG ATG TGG	UP

Table 2-8 *Klebsiella pneumoniae* VNTR loci primers (UP = unpublished primers).

Amplified PCR products underwent AFLP analysis on an ABI 3130 capillary sequencer (Applied Biosystems, Warrington, UK) using MapMarker 1500X-Rhodamine size standards (Bioventures, Murfreesboro, United States of America). Additionally amplified products were electrophoresed through a 1.5% (w/v) agarose gel stained with 12.5% ethidium bromide and bands were visualised by UV transillumination. Bioline 1Kb ladder (Bioline, London, UK) was run alongside the amplicons.

2.2.11 Repetitive Extragenic Palindromic Based PCR

Repetitive extragenic palindromic based PCR (REP-PCR) primer sequences are not listed as the typing is performed using a commercial kit and primer sequences are not disclosed.

2.2.11.1 *Klebsiella* and *Enterobacter* Repetitive Extragenic Palindromic Based PCR

REP-PCR detection for Klebsiella and Enterobacter species was carried out in the following mixture: 1x GeneAmp PCR buffer (Applied Biosystems, Warrington, UK), 2µl Diversilab primer mix (BioMerieux, Mary l’Etoile, France), 18µl Diversilab REP-PCR mastermix (BioMerieux, Mary l’Etoile, France), 2.5 units AmpliTaq (Applied Biosystems, Warrington, UK), and 2µl DNA extract to give a final volume of 25µl (no reagents concentration listed as part of kit details).

The reactions were cycled on a thermocycler as follows: 94˚C for 2 minutes followed by 35 cycles of 94˚C for 30 seconds, 55˚C for 30 seconds and 70^{o}C for 90 seconds and a final elongation 70^{o}C for 3 minutes.

Amplified products were visualised via a microfluidics detection system using the Agilent 2100 bioanalyzer and results uploaded onto the Diversilab web system (BioMerieux, Mary l'Etoile, France). Computational analysis was performed using the either the Pearson Correlation Co-efficient or the Kullback-Leibler analysis to calculate pairwise similarities between all samples tested. REP-PCR traces were flagged as low intensity for traces where no detected peaks for that amplified product was greater than 100 relative fluorescent units. Low intensity traces were only included in the analysis if no acceptable trace could be detected after six sample repeats.

2.2.11.2 *E. COLI* REPETITIVE EXTRAGENIC PALINDROMIC BASED PCR

REP-PCR detection for *E. coli* was carried out in the following mixture: 1x GeneAmp PCR buffer (Applied Biosystems, Warrington, UK), 2µl Diversilab primer mix (BioMerieux, Mary l'Etoile, France), 18µl Diversilab REP-PCR mastermix (BioMerieux, Mary l'Etoile, France), 2.5 units AmpliTaq (Applied Biosystems, Warrington, UK), and 2µl DNA extract to give a final volume of 25µl (no reagents concentration listed as part of kit details).

The reactions were cycled on a thermocycler as follows: 94˚C for 2 minutes followed by 35 cycles of 94˚C for 30 seconds, 50˚C for 30 seconds and 70^{o}C for 90 seconds and a final elongation 70^{o}C for 3 minutes.

Amplified products were visualised via a microfluidics detection system using the Agilent 2100 bioanalyzer and results uploaded onto the Diversilab web system (BioMerieux, Mary l'Etoile, France). Computational analysis was performed using the either the Pearson Correlation Co-efficient or the Kullback-Leibler analysis to calculate pairwise similarities between all samples tested. REP-PCR traces were flagged as low intensity for traces where no detected peaks for that amplified product was greater than 100 relative fluorescent units. Low intensity traces were only included in the analysis if no acceptable trace could be detected after six sample repeats.

2.3 ENVIRONMENTAL SAMPLING

2.3.1 VIROLOGICAL SURFACE SAMPLING

A cotton tipped swab was lightly moistened in a vial of 1ml molecular grade water. The sample area was as described in *Table 2-9* and *Table 2-10*. The surface was swabbed by rotating the swab and moving it first horizontally across the sampling area, then vertically and finally diagonally. The cotton tip of the swab was then broken off into the fluid. One additional vial of molecular grade water was produced to act as a negative control. Swabs were stored at 2-4^{o}C for a maximum of 48 hours before further processing as described in sections 2.2.5.4 and 2.2.5.3.

2.3.1.1 INFECTION CONTROL VIROLOGICAL SCREENING SITES

Table 2-10 describes the sites used by the GOSH infection control team as part of their routine screening (discussed in Chapter 5). This list of sites is used to monitor levels of virus within cubicles both pre and post clean. Post clean screening is undertaken for all bone marrow transplant cubicles where an adenovirus positive patient has been present. Other screening is undertaken for both norovirus and adenovirus as required by infection control.

Swab No.	Site	Area to be Swabbed
1	Floor outside known negative patient room	10 cm^2
2	Filing cabinet	10 cm^2
3	Floor outside known positive patient room	10 cm^2
4	Nurse's station	10 cm^2
5	Sluice/medication room door handle	Entire handle
6	Floor by main exit doors	10 cm^2
7	Notes trolley	10 cm^2
8	PC keyboards	Every key and surface on the right 50% (~10 cm^2)
9	Telephone on nurse's station	Entire key and handset
10	Ward exit door handle	Entire handle

Table 2-9 Sampling sites for virology ward screening at GOSH.

Swab No.	Site	Area to be Swabbed
1	Floor under sink	10cm^2
2	Clinical waste bin inner rim (under lid)	Entire rim
3	Chair with arms (right)	Where hands rests (10cm^2)
4	Door handle into patient bathroom (cubicle side)	Entire handle
5	Telephone keypads	Entire keypad
6	Taps in Patient Bathroom	Entirety of both taps
7	Mattress top	10cm^2
8	Bed/Cot frame under bed	10cm^2
9	Trolley surface (in ante room if present)	10cm^2
10	Side window sill (right hand side)	10cm^2
11	Cubicle room exit door handle (cubicle side)	Entire handle
12	Corridor floor outside of cubicle/ante room entrance	4 inch^2/10cm^2
13	Negative	N/A

Table 2-10 Sampling sites for virology cubicle screening at GOSH.

2.3.1.2 NOROVIRUS/ADENOVIRUS ROUTINE MONITORING SITES

Environmental screening for adenovirus and norovirus was undertaken weekly on two wards at GOSH over a 6 months period from January – June 2011. Twelve sites were sampled within the shared ward area on each ward with a total of 144 swabs taken (Table 2-9). Ward one was an 11 bedded in-patient hematopoietic stem cell transplantation unit (HSCTU) where all beds were located within single occupancy rooms with en-suite facilities. The second ward was a 10 bedded Immunology/Infectious Disease Unit (IIU), where all beds were located within ante-chambered single occupancy rooms with en-suite facilities. Sampling was undertaken at similar times of day in relation to cleaning throughout the sampling period.

2.3.1.3 HAEMATOLOGY/ONCOLOGY DAY UNIT VIROLOGICAL SCREENING SITES

A one off screen was undertaken on a haematology/oncology day unit (HODU) to establish the level of environmental contamination present (Table 2-11).

Swab No.	Site	Area to be Swabbed
Day Procedure Bay		
1	Chair arm (right)	Where hand rests
2	Trolley	10cm^2
3	Bed rail	10cm^2
4	Nurses base	10cm^2

5	Window ledge	10cm^2
Recovery		
6	Emergency door	Entire bar
7	Trolley	10cm^2
8	Chair seat	10cm^2
9	Notes container	10cm^2
10	Bed rail	10cm^2
11	Top of portable TV	10cm^2
Procedure Room		
12	Keyboard	Every key and surface on
13	Prep surface	10cm^2
14	Trolley	10cm^2
15	Clinical waste bin inner rim (under lid)	Entire rim
16	Bed rail	10cm^2
Assisted Toilet 1		
17	Baby change	10cm^2
18	Exit door handle	Entire handle
Assisted Toilet 2		
19	Baby change	10cm^2
20	Exit door handle	Entire handle
21	Toilet seat	10cm^2
Cubicle ensuite (pre level two clean)		
22	Chair arm (right)	Where hand rests
23	Fan	Fan blades
24	Toy	10cm^2
25	Bed rail	10cm^2
26	Trolley	10cm^2
Bay 1		
27	Bed rail	10cm^2
28	Chair arm (right)	Where hand rests
29	Toy	10cm^2
30	Fan	Fan blades
31	Window ledge	10cm^2
Corridor		
32	Chair arm (right)	Where hand rests
33	Nurses station phone	Entire key and handset
34	Crash trolley	10cm^2
Bay 2		
35	Toy	10cm^2
36	Chair arm (right)	Where hand rests
37	Bed rail	10cm^2
38	Trolley	10cm^2
39	Childs table	10cm^2
Bay 3		
40	Dolls house	10cm^2
41	Table	10cm^2
42	Bed rail	10cm^2
43	Chair arm (right)	Where hand rests

44	Fan	Fan blades
Height & Weight room		
45	Keyboard	Every key and surface on
46	Nappy change	10cm^2
47	Weighing seat	10cm^2
48	Exit door handle	Entire handle
49	Window ledge	10cm^2
50	Clinical waste bin inner rim (under lid)	Entire rim
Reception		
51	Notes trolley	10cm^2
52	Keyboard	Every key and surface on
53	Desk	10cm^2
54	Window ledge	10cm^2
55	Phone	Entire key and handset
Reception Seating		
56	Toy van	10cm^2
57	Chair arm (right)	Where hand rests
58	TV	10cm^2
59	Window ledge	10cm^2
60	Apron dispenser	10cm^2
Cubicle 1		
61	Toilet exit door handle	Entire handle
62	Bed rail	10cm^2
63	Clinical waste bin (toilet)	Entire rim
64	Clinical waste bin inner rim (under lid)	Entire rim
65	Trolley	10cm^2
Cubicle 2 (pre level two clean)		
66	Exit door handle	Entire handle
67	Bed rail	10cm^2
68	Pillow	10cm^2
69	Clinical waste bin inner rim (under lid)	Entire rim
70	Trolley	10cm^2
Treatment Room 1		
71	Teddy	10cm^2
72	Trolley	10cm^2
73	Drug trolley	10cm^2
74	Bench	10cm^2
75	Exit door handle	Entire handle
Treatment Room 2		
76	Toy	10cm^2
77	Bench	10cm^2
78	Computer trolley	10cm^2
79	Trolley	10cm^2
80	Clinical waste bin inner rim (under lid)	Entire rim
Treatment Room 3		
81	Trolley	10cm^2
82	IV bench	10cm^2
83	Keyboard	Every key and surface on

84	Clinical waste bin inner rim (under lid)	Entire rim
85	Drip stand	$10cm^2$
Treatment Room 4		
86	Bench	$10cm^2$
87	Computer	$10cm^2$
88	Clinical waste bin inner rim (under lid)	Entire rim
89	Computer trolley	$10cm^2$
90	Window ledge	$10cm^2$

Table 2-11 Virological screening sites on the haematology/oncology day unit.

2.3.2 BACTERIAL SAMPLING METHODS

Detection methods included:

- Direct sampling using contact plates with selective or non-selective agar (see section 2.3.2.1.)
- Direct sampling using swabs that are then plated onto selective agar plates (see section 2.3.2.2.)
- Swab sampling with an enrichment step with subsequent plating onto selective agar (see section 2.3.2.3.)
- Commercially available swab systems that combine swab and enrichment (see section 2.3.2.4.)

Swabs were taken to the right and contact plates to the left, when sampling identical sites. Where swabs were taken in the centre of a surface, contact plates taken directly above.

2.3.2.1 DIRECT SAMPLING USING CONTACT PLATES WITH SELECTIVE OR NON-SELECTIVE AGAR TOTAL VIABLE COUNTS

Total viable counts (TVC) sampling was carried out with 5.5 cm diameter contact plates (24 cm^2). Sampling utilised Tryptone Soya Agar (TSA) contact plates pressed onto surfaces for 10 seconds and then incubated aerobically at 37^oC for 48 hours.

Additional identification was undertaken via the MALDI Biotyper platform (Bruker UK, Coventry, UK) or API (BioMerieux, Mary l'Etoile, France) as required (see section 2.3.2.5).

2.3.2.2 DIRECT SAMPLING USING SWABS THAT ARE THEN PLATED ONTO SELECTIVE AGAR PLATES

This method was used where it was felt that organisms would be present in sufficient quantities that the reduced sensitivity of not using an enrichment step was countered

by being able to deliver a rough numerical count. Swabs were used instead of contact plates in circumstances when multiple primary agars were required for growth and identification. Direct plating was utilised to give semi-quantitative data (range: 0 to 3) in conjunction with chromogenic agar for primary identification of isolates.

Cotton tipped swabs were lightly moistened in a vial of sterile water. The surface was swabbed by rotating the swab and moving it first horizontally across the sampling area, then vertically and finally diagonally, before being replaced in the charcoal transportation media. Swabs were stored at 2-4^{o}C for a maximum of 48 hours before processing.

Charcoal swabs were inoculated onto selective and non-selective agar plates within 48 hours of samples being taken. Plates utilised included methicillin resistant *Staphylococcus aureus* (MRSA) Chromogenic Agar, Braziers Agar and Urine Chromagenic Agar (Oxoid, Basingstoke, UK). Non-selective plates utilised throughout the project included Blood Agar and MacConkey Agar (Oxoid, Basingstoke, UK). Braziers Agar was incubated anaerobically for 37^{o}C for 48 hours, all other agars were incubated aerobically at 37^{o}C for 48 hours.

Additional identification was undertaken via the MALDI Biotyper platform (Bruker UK, Coventry, UK) or API (BioMerieux, Mary l'Etoile, France) as required (see section 2.3.2.5.).

2.3.2.3 SWAB SAMPLING WITH AN ENRICHMENT STEP WITH SUBSEQUENT PLATING ONTO SELECTIVE AGAR

A cotton tipped swab was lightly moistened in a vial of sterile water. The surface was swabbed by rotating the swab and moving it first horizontally across the sampling area, then vertically and finally diagonally, before being replaced in the charcoal transportation media. Swabs were placed into a selective broth for 48 hours in order to increase test sensitivity before being plated onto selective and non-selective agars for identification. Testing is qualitative with no quantitative comparison possible. Swabs were stored at 2-4^{o}C for a maximum of 48 hours before processing.

For Gram-negative bacteria during surveillance of Great Ormond Street Hospital (GOSH) Paediatric Intensive Care Unit (PICU) swabs were placed into Brain Heart Infusion (BHI) enrichment media (Oxoid, Basingstoke, UK) and incubated for 48 hours at 37^{o}C. After 48 hours the broth was sub-cultured onto Urine Chromagenic Agar, Violet Red Bile Glucose Agar and MacConkey agar (Oxoid, Basingstoke, UK) plus a Colistin disk (BioMerieux, Mary l'Etoile, France).

During sampling at the National Hospital for Neurology and Neurosurgery (NHNN) swabs were placed in salt broth (Oxoid, Basingstoke, UK) to enrich for *Staphylococcus aureus* and sub-cultured onto MRSA Chromogenic Agar (Oxoid, Basingstoke, UK). Additional swabs were also taken and placed in Robertson's Cooked Meat (Oxoid, Basingstoke, UK) and sub-cultured onto Braziers Agar (Oxoid, Basingstoke, UK) for *C. difficile*. Swabs were also taken for *Acinetobacter* species and enriched using AVMA broth before being sub-cultured onto Urine Chromogenic Agar (Oxoid, Basingstoke, UK). Braziers Agar was incubated anaerobically for 37^{o}C for 48 hours, all other agars were incubated aerobically at 37^{o}C for 48 hours.

Additional identification was undertaken via the MALDI Biotyper platform (Bruker UK, Coventry, UK) or API (BioMerieux, Mary l'Etoile, France) as required (see section 2.4.2.5.).

2.3.2.4 MICROSNAP ENTEROBACTERIACEAE SWABS

Microsnap Enterobacteriaceae swabs (Hygiena International, Watford, UK) combine enrichment and detection in a two-stage process. Swabbing was undertaken in an identical fashion to that listed in 2.3.2.3. with the Microsnap system that contained a pre-moistened swab. BHI broth was released from the bulb in the swab post sampling and the swabs were incubated in the broth for seven hours at 37^{o}C. 100µl of the enrichment broth was mixed with the detection substrate for 30 minutes at 37^{o}C. Relative light units (RLU) were then recorded and the remaining broth plated onto Urine Chromagenic Agar, Violet Red Bile Glucose Agar and MacConkey Agar (Oxoid, Basingstoke, UK) plus a Colistin disk (BioMerieux, Mary l'Etoile, France) and incubated for 48 hours at 37^{o}C.

Additional identification was undertaken as described in section 2.3.2.5.

2.3.2.5 BACTERIAL IDENTIFICATION

Bacterial isolates were initially typed using phenotypic features (colony morphology, colour and macroscopic features). Selected isolates then underwent Gram staining and either biochemical identification using API kits (BioMerieux, Mary l'Etoile, France) or matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) analysis on the MALDI Biotyper system (Bruker UK, Coventry, UK) as per manufacturer's instructions. Additional antibiotic sensitivity testing using the disk diffusion method on Iso-sensitest agar (Oxoid, Basingstoke, UK) and E-test (BioMerieux, Mary l'Etoile, France) was undertaken as required.

2.3.2.6 BACTERIAL QUANTIFICATION

Bacterial suspensions linked to survival and decontamination studies, as well as for standard creation, were quantified using the Miles and Misra method.[385] Inoculation suspensions (~ 5 Macfarlane units for control suspensions) were quantified using the following plate counting method: serial tenfold dilutions were made from this suspension in sterile water and 5 drops (20µl) of the 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} dilutions were pipetted onto pre-warmed blood agar plates and incubated at 37^{o}C for 24 hours. Bacterial counts were determined from the blood agar dilution plates that demonstrated discreet colonies and values calculated to colony forming units per ml. Bacterial recovery swabs were quantified in an identical fashion but 10^{-1}, 10^{-2}, 10^{-3} from the starting sample were used for quantification.

2.3.2.7 BACTERIAL SWABBING FOR INOCULATION EXPERIMENTS

Cotton tipped swab was lightly moistened in a vial of 1ml molecular grade water. The surface was swabbed by rotating the swab and moving it first horizontally across the sampling area, then vertically and finally diagonally. The cotton tip of the swab was then broken off into the fluid. One additional vial of molecular grade water was produced to act as a negative control. Swabs were stored at 2-4^{o}C for a maximum of 48 hours before further processing as described in sections 2.2.5.1 and 2.2.5.2.

2.3.3 BACTERIAL SAMPLING SITES

2.3.3.1 GRAM-NEGATIVE SURVEILLANCE ON THE PAEDIATRIC INTENSIVE CARE

The PICU at GOSH was sampled on twenty occasions over five months using both TVCs as described in 2.3.2.1 and Enterobacteriaceae swabs as described in sections 2.3.2.3 and 2.3.2.4. Sampling focused on two adjacent bed spaces and the shared ward area (see Table 2-12 and Table 2-13).

Swab No.	Site	Area to be Swabbed
1	Floor near sink	10cm^2
2	Bin	10cm^2
3	Chair arm (right)	Where hand rests (~10cm^2)
4	Bed rails (right)	10cm^2
5	Suspended shelf surface	10cm^2
6	Mouse	Entire surface
7	Sink rim	Entire back rim
8	Sink bowl	Entire bowl
9	Trolley	10cm^2
10	PC Keyboard	Every key and surface on the right 50% (~10cm^2)
11	Lamp	10cm^2
12	Soap dispenser	Entire of dispensing surface

Table 2-12 Gram-negative surveillance bed space sampling locations on the GOSH Paediatric Intensive Care Unit.

Swab No.	Site	Area to be Swabbed
1	Telephone on nurses station	Entire key and handset
2	PC keyboards	Every key and surface on the right 50% (~10cm^2)
3	Nurses station	10cm^2
4	Notes trolley	10cm^2
5	Corridor floor outside of bed space 7	10cm^2
6	Corridor floor outside of bed space 10	10cm^2
7	Medication board	10cm^2
8	Dirty utility door handle	Entire handle
9	Desk by nurses station	10cm^2
10	Crash trolley	10cm^2
11	Floor by main exit doors	10cm^2
12	Exit door button	Entire button

Table 2-13 Gram-negative surveillance ward sampling locations on the GOSH Paediatric Intensive Care Unit.

2.3.3.2 GREAT ORMOND STREET HOSPITAL INTENSIVE CARE UNIT BACTERIAL SINK SAMPLING

Data was collected in three intensive care unit (ICUs) within GOSH including: the cardiac intensive care unit (CICU), PICU and neonatal intensive care unit (NICU). Sampling was undertaken in parallel with the observational data collection, see methods section 2.5.2. Swab samples were collected from eight sinks spread over the three units, both at the beginning (after morning cleaning with detergent) and at the end of observation days (before evening cleaning daily) then once a week for the next three weeks subsequently. Samples were collected from seven different locations on each sink at each sampling occasion: faucet, left sink back - lip, right sink back - lip, left side of the sink bowl, right side of the sink bowl, depression pad on the gel dispenser and depression pad on the soap dispenser) in each of the eight sinks. Samples were processed as described in methods section 2.3.2.2. and plated onto Urine Chromagenic Agar, Blood Agar and MacConkey agar (Oxoid, Basingstoke, UK).

2.3.3.3 NATIONAL NEUROLOGICAL HOSPITAL BACTERIAL SAMPLING

Sampling was carried out over a three month validation period (each month referred to as a sampling period i.e. 1, 2 or 3) to identify sampling methodologies. A total of 1408 samples were taken in an adult surgical and medical ICU in order investigating the best ways of measuring surface microbial flora. Sampling included TVCs and specific pathogen quantification (MRSA, *Clostridium difficile* and antibiotic resistant *Acinetobacter* species). Samples were processed as described in methods section 2.3.2.1 and 2.3.2.3. Surfaces were categorised as low (under 2 feet in height), medium (2 – 4 feet) or high (greater than 4 feet). In addition surfaces were categorised as to whether they were high touch or not (see Table 2-14).

Ward sites included:

Alcohol hand gel and hand cream dispensers, domestic waste bin, nurses station, nurses station keyboard, nurses station phone, dispensing trolley, medicines fridge door handle, medicines fridge (top), curtain rail, floors by all doors and door plates/handles at all exits, sinks and taps, X-ray box (top), nurses station chair arms, crash trolley, storage trolley.

Bed space sites included:

Sites (Surgical Intensive Therapy Unit)	Sites (Medical Intensive Therapy Unit)
Bed rails	Bed rails
Storage unit (top)	Storage trolley
Storage unit (shelf)	Ops computer (top)
Floor under bed	Floor under bed
Clinical waste bin	Bed side table
Ops computer (top)	Clinical waste bin
Bed wheels	Bed wheels
Bed side table	Alcohol gel dispenser
Alcohol gel dispenser	
Apron dispenser	

Table 2-14 Bacterial sampling sites at the National Neurological Hospital.

2.3.3.4 HAEMATOLOGY/ONCOLOGY DAY UNIT SAMPLING SITES

A one off screen was undertaken on HODU, to establish the level of environmental contamination present using methods described in sections 2.3.2.1, 2.3.2.3 and 2.3.2.7 using sites described in Table 2-15.

Swab No.	Site	Area to be Swabbed
Day Procedure Bay		
1	Chair arm (right)	Where hand rests
2	Trolley	10cm^2
3	Bed rail	10cm^2
4	Nurses base	10cm^2
5	Window ledge	10cm^2
Recovery		
6	Emergency door	Entire bar
7	Trolley	10cm^2
8	Chair seat	10cm^2
9	Notes container	10cm^2
10	Bed rail	10cm^2
11	Top of portable TV	10cm^2
Procedure Room		
12	Keyboard	Every key and surface on
13	Prep surface	10cm^2
14	Trolley	10cm^2
15	Clinical waste bin inner rim (under lid)	Entire rim
16	Bed rail	10cm^2
Assisted Toilet 1		
17	Baby change	10cm^2
18	Exit door handle	Entire handle
Assisted Toilet 2		
19	Baby change	10cm^2
20	Exit door handle	Entire handle
21	Toilet seat	10cm^2
Cubicle ensuite (pre clean after isolation of an MRSA positive patient)		

22	Chair arm (right)	Where hand rests
23	Fan	Fan blades
24	Toy	10cm^2
25	Bed rail	10cm^2
26	Trolley	10cm^2
Bay 1		
27	Bed rail	10cm^2
28	Chair arm (right)	Where hand rests
29	Toy	10cm^2
30	Fan	Fan blades
31	Window ledge	10cm^2
Corridor		
32	Chair arm (right)	Where hand rests
33	Nurses station phone	Entire key and handset
34	Crash trolley	10cm^2
Bay 2		
35	Toy	10cm^2
36	Chair arm (right)	Where hand rests
37	Bed rail	10cm^2
38	Trolley	10cm^2
39	Childs table	10cm^2
Bay 3		
40	Dolls house	10cm^2
41	Table	10cm^2
42	Bed rail	10cm^2
43	Chair arm (right)	Where hand rests
44	Fan	Fan blades
Height & Weight room		
45	Keyboard	Every key and surface on
46	Nappy change	10cm^2
47	Weighing seat	10cm^2
48	Exit door handle	Entire handle
49	Window ledge	10cm^2
50	Clinical waste bin inner rim (under lid)	Entire rim
Reception		
51	Notes trolley	10cm^2
52	Keyboard	Every key and surface on
53	Desk	10cm^2
54	Window ledge	10cm^2
55	Phone	Entire key and handset
Reception Seating		
56	Toy van	10cm^2
57	Chair arm (right)	Where hand rests
58	TV	10cm^2
59	Window ledge	10cm^2
60	Apron dispenser	10cm^2
Cubicle 1		
61	Toilet exit door handle	Entire handle

62	Bed rail	10cm^2
63	Clinical waste bin (toilet)	Entire rim
64	Clinical waste bin inner rim (under lid)	Entire rim
65	Trolley	10cm^2
Cubicle 2 (pre clean after isolation of antibiotic resistant Gram-negative patient)		
66	Exit door handle	Entire handle
67	Bed rail	10cm^2
68	Pillow	10cm^2
69	Clinical waste bin inner rim (under lid)	Entire rim
70	Trolley	10cm^2
Treatment Room 1		
71	Teddy	10cm^2
72	Trolley	10cm^2
73	Drug trolley	10cm^2
74	Bench	10cm^2
75	Exit door handle	Entire handle
Treatment Room 2		
76	Toy	10cm^2
77	Bench	10cm^2
78	Computer trolley	10cm^2
79	Trolley	10cm^2
80	Clinical waste bin inner rim (under lid)	Entire rim
Treatment Room 3		
81	Trolley	10cm^2
82	IV bench	10cm^2
83	Keyboard	Every key and surface on
84	Clinical waste bin inner rim (under lid)	Entire rim
85	Drip stand	10cm^2
Treatment Room 4		
86	Bench	10cm^2
87	Computer	10cm^2
88	Clinical waste bin inner rim (under lid)	Entire rim
89	Computer trolley	10cm^2
90	Window ledge	10cm^2

Table 2-15 Bacterial screening sites on the haematology/oncology day unit.

2.3.3.5 BACTERIAL AIR SAMPLING

Air sampling was performed using a Sampl'air lite (BioMerieux, Mary l'Etoile, France), sampling 1m^3 of air onto a blood agar plate (Oxoid, Basingstoke, UK). Air sampling occurred at similar times to the surface sampling, separated in time by at least 1 hour to minimize user contamination. Air samples were taken from the top right hand corner of each bed space at floor level (see Figure 2-1). In addition samples were taken from the nurses station and doorways/Gates (see methods section 2.5.1.). 228 TVC samples were taken, 40 of which were taken using TSA and SAB Agar as well as Blood Agar (Oxoid, Basingstoke, UK) to determine which gave the most

comprehensive result. All plates were read and colony forming units (CFU) were counted per plate giving total viable counts (TVC) that was recorded at 48hrs after incubation at 37°C.

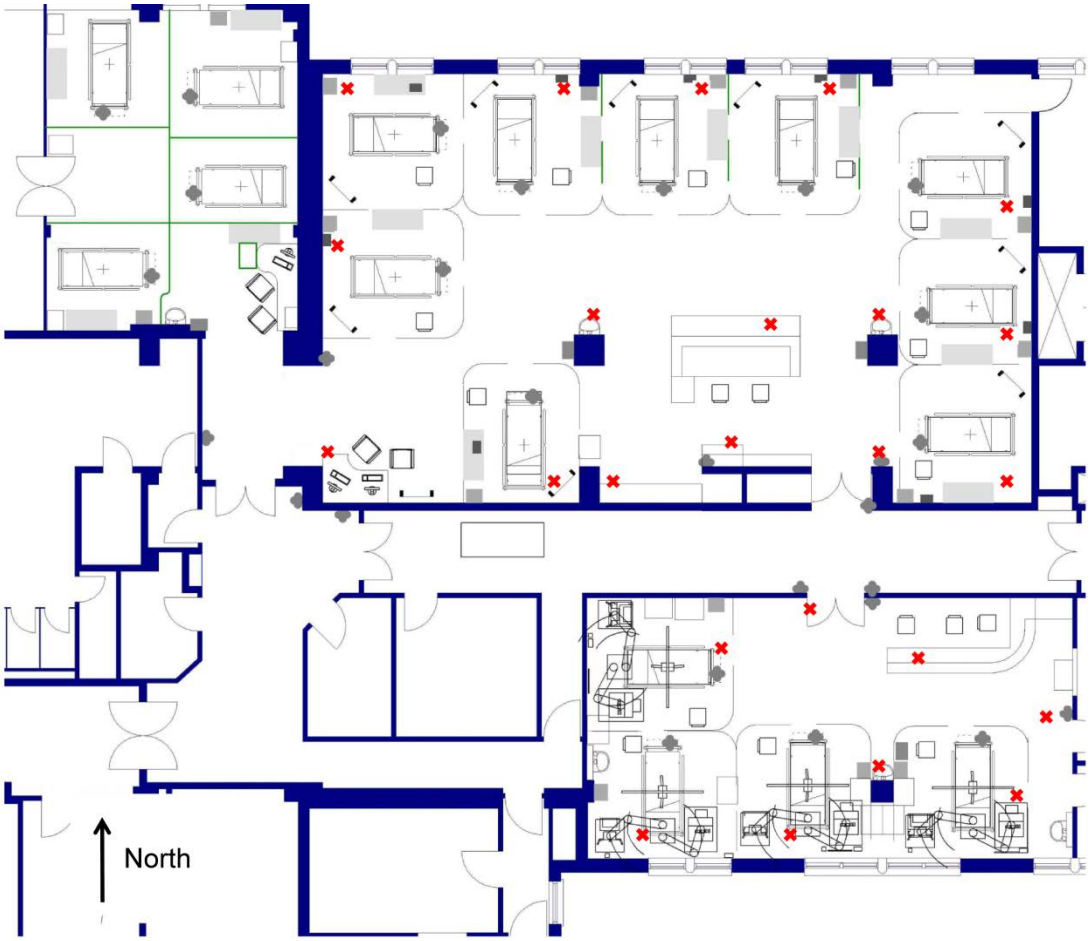

Figure 2-1 Floor plans with red x's used to identify air sampling points of the surgical intensive care unit, medical intensive care unit and high depency unit at the NHNN.

2.3.3.6 DOOR HANDLE SAMPLING

Microbiological surveillance data were collected at the same time as handle usage using contact plates as described in section 2.3.2.1. Door handles were cleaned thoroughly with 70% isopropyl alcohol wipes immediately before the start of the movement observations and swabs taken to ensure the handles and plates were free from bacteria. Sampling was undertaken at the start of the observation period and repeated at the same sites following a 150 minute observation (see section 2.5.1.). Observation periods were repeated twice a day to straddle both morning ward rounds and afternoon visits by relatives for 3 days.

2.4 SURFACE INOCULATION METHODS

Inoculation experiments were used to test the effectiveness of decontamination methods as well as to conduct survival studies. For each test organism and control sample, 250µl of the microbial/viral suspension or extracted DNA was inoculated in 50µl droplets onto a 5x5 cm area of glazed ceramic tile surface with a minimum of 1 duplicate, and allowed to dry for two hours at room temperature. Swabbing of the test surface was undertaken as described in methods section 2.3.2.7.

Figure 2-2 Ceramic tiles incoculated with 250µl of susepnsion per replicate in 50µl droplets, with four replicates per tile.

2.4.1 SURVIVAL STUDIES

A 43 m^3 chamber, with adjustable air changes per hour (ACH), temperature, and relative humidity was used as a stable environment to test the long term viability of *Klebsiella pneumoniae* (NCTC 13368), *Staphylococcus aureus* (NCTC 65711), and adenovirus serotype 1 (NCTC 0011051v). DNA extracted as described in methods

section 2.2.1.1. of *Klebsiella pneumoniae* (NCTC 13368), *Staphylococcus aureus* (NCTC 65711), and adenovirus serotype 1 (NCTC 0011051v) was also inoculated. The air supply was high efficiency particulate air (HEPA) filtered. Temperature and relative humidity were set to 16°C and 43% respectively.

Each time point had four replicates and samples were taken at point 0, 24h, 24 h, 72 h, 1 week, 2 weeks, 3 weeks, 4 weeks and 3 months. Viability testing was undertaken as described in methods section 2.1.2 and 2.3.2.6. DNA detection was by real-time PCR as described in methods sections 2.2.5.1, 2.2.5.2. and 2.2.5.4.

2.4.2 Decontamination Studies

After inoculation as described in section 2.4. the decontamination method was then performed on the test sample, with the control being maintained in conditions that were as similar as possible. A ceramic surface was selected for inoculation experiments in order to carry out experimentation on a surface that would enable maximum recovery.

For viability bacterial suspensions were selected that allowed recovery of a minimum of 10^6 colony forming units/ml of microorganism (McFarland No. 5; >1.0 optical density at 620 nm). Adenovirus was inoculated from cell culture for viability testing or extracted cell culture for DNA exposure experiments ~10^9 viral genome copies/ml as determined using adenovirus standards). See sections 2.1.2 and 2.3.2.6.

For molecular detection and DNA degradation studies molecular detection of viruses and bacteria were undertaken using real-time PCR as described in sections 2.2.5.1, 2.2.5.2 and 2.2.5.4

2.4.2.1 Ultra Violet Light Equipment Decontamination

The use of ultraviolet light as delivered by the Nanoclave cabinets (Nanoclave, London, UK), which uses short wave ultraviolet light (UV-C 280 – 100nm) targeted from 360^o around an object placed within in order to decontaminate object it.

Viral testing involved the use of adenovirus species A31 (NCTC 0011265v) in VERO cell culture medium inoculated onto surfaces as described in section 2.4.from a stock suspension with a viral genome concentration of approximately 2.9^{10} viral copies/ml (as determined by real-time PCR using standards provided by National Institute for Biological Standards and Control (NIBSC)). During this analysis viral detection was undertaken by PCR as described in methods section 2.2.5.4 for DNA degradation and by tissue culture as described in methods section 2.1.2 for viability.

Surface Study

A ceramic surface was selected for inoculation experiments in order to carry out experimentation on a surface that would enable maximum recovery. 250 µl of the adenovirus A31 suspension was inoculated in 50 µl droplets onto a 5x5 cm area of glazed ceramic tile surface with a minimum of one duplicate, and allowed to dry for two hours at room temperature.

Medical Device Decontamination Testing

Four medical devices were tested at two sampling points, one after a three minute UV exposure and one after a six minute UV exposure. The medical devices included metallic and plastic surface types. There was insufficient space on the device for a control inoculation.

Devices submitted to preliminary testing and initial results:

1. A remote control with a plastic surface

2. A saturation monitor with a plastic surface

3. A Dinomap (automated blood pressure device) with a plastic surface

4. A piece of dialysis equipment was tested with a metal plate

2.4.2.2 HYDROGEN PEROXIDE VAPOUR ROOM DECONTAMINATION

The effectiveness of hydrogen peroxide vapour (HPV) technology was assessed by comparing two commercially available HPV technologies, Glosair 400 (ASP, Wokingham, UK) and Bioquell Q10 (Bioquell, Andover, UK).

A 43 m^3 chamber, with adjustable air changes per hour (ACH), temperature, and relative humidity was used to test the effect of hydrogen peroxide vapour on organism viability and DNA. The air supply was High Efficiency Particulate Air (HEPA) filtered. Temperature and relative humidity were set to 20°C and 40% respectively 1 hour before the experiment. During the experiment the chamber was sealed and at negative pressure compared to adjoining rooms. Measured ambient temperature and relative humidity ranged from 20 - 23°C and 38 – 40% for the test inside the chamber respectively, for this particular experiment the chamber ACH was off and fan on to achieve a well-mixed environment. Control sample tiles for bacteria and virus for were left outside the chamber at room temperature (25°C).

Equipment	Experiment	Cycle numbers	Cycle dosage	Contact time
Bioquell Viable and DNA exposure	1	1	10g/m^3	15 min
Glosair Viable bacteria	1	1	6ml/m^3	2hours
DNA exposure	2	1	9ml/m^3	2 hours
DNA exposure	3	3 consecutive	12ml/m^3	2 hour
DNA exposure	4	3 consecutive	6ml/m^3	2 hour
DNA exposure	5	3 consecutive	6ml/m^3	30 min
DNA exposure	6	3 consecutive	6ml/m^3	15 min

Table 2-16 Hydrogen peroxide cycling conditions for Glossair and Bioquell systems for both viability and DNA denaturation experiments.

Organisms tested with Glosair 400 (ASP, Wokingham, UK) included *Klebsiella pneumoniae* (NCTC 13368), *Staphylococcus aureus* (NCTC 65711), and adenovirus serotype 1 (NCTC 0011051v). Viability was determined as described in methods section 2.1.2 and 2.3.2.6. DNA degradation was assessed by real-time PCR as described in methods sections 2.2.5.1, 2.2.5.2 and 2.2.5.4.

2.4.2.3 CLEANING AGENT COMPARISON

1000ppm sodium hypochlorite (NaClO) (Sigma-Aldrich, Gillingham, UK), 1000ppm sodium dichloroisocyanurate (NaDCC) (Chlor clean) (Guest Medical, Aylesford, UK) and chlorine dioxide (ClO_2) (Tristel, Snailswell, UK) were compared to determine both their ability to render *Klebsiella pneumoniae* (NCTC 13368), *Staphylococcus aureus* (NCTC 65711), and adenovirus species C serotype 1 ((NCTC 0011051v) non-viable and to establish the effect on DNA.

Organisms were exposed to each cleaning agent for 10 minutes, 60 minutes and 120 minutes by wiping the ceramic tile with a single use cloth saturated with the agent for 10 seconds. Sampling was undertaken as described in method section 2.3.1 and 2.3.2.7. Viability was determined as described in methods section 2.1.2 and 2.3.2.6. DNA degradation was tested after extraction (see methods section 2.2.1.1) by real-time PCR as described in methods sections 2.2.5.1, 2.2.5.2 and 2.2.5.4.

2.5 SPACE SYNTAX

2.5.1 GATE COUNTING

Gates were defined as those thresholds across which individuals travel i.e. doorways with and without doors. People were watched as they moved across these gates with a single movement defined as one individual crossing the threshold of any gate as defined above. Movements through all gates in the SITU and MITU at the National Hospital were monitored for three days on a daily basis from 10:30 to 13:00 and from 14:30 to 17:00. Individuals were assigned to one of several groups, namely staff local to the ward, other hospital staff, patients, and their visitors. A "run-in" period of sham observation of three weeks was undertaken in order to minimise any bias which the observation process itself might trigger. Gate counting was undertaken by a team of researchers to enable all gates to be observed.

Microbiological samples were collected in parallel as described in methods section 2.3.4.6.).

2.5.2 SINK OBSERVATION

The usage of 24 hand wash basins within the three different wards (cardiac intensive care unit (CICU), PIC and neonatal intensive care unit (NICU)) was monitored for three consecutive days, each day consisting of three 1.5 hour periods from eight in the morning until six in the evening, totalling 13.5 hours per sink. Sinks observed were all in the open ward environment and had identical fixtures and fittings (see Figure 2-3.). The observation data included the number of times each hand wash basin was used to undertake a hygienic hand wash, the length of time spent per hand washing event and bed space occupation. Observer fatigue was countered by the use of multiple observers with periodic observation cross checking to ensure consistency. Sink observation was undertaken by a team of researchers to enable all sinks to be observed.

Microbiological samples were collected in parallel as described in methods section 2.3.4.2).

Figure 2-3 Floor plans for CICU, NICU and PICU at GOSH with sink locations labelled and numbered in red.

2.5.3 MOVEMENT STUDIES

In order to determine whether user behaviour inside the PICU contributed to bacterial contamination and thus potential spread of infection, an observational study was performed in two bed spaces, henceforth referred to as BS6 and BS7. Data was collected during three weekday days, 6 hours per day totalling 30 hours per bed space. The resulting data was coded in a spreadsheet as a string of contacts grouped by individual round of observations. Each round starts when the main nurse or another person (doctor, visitor, other medical specialist and non-medical staff) entered the bed space and concluded the moment they leave the bed space. Each contact was recorded with a unique number.

Eight contact categories were selected: washing hands, throwing waste in a bin, gloves on, gloves off, pick up an object from, leave an object onto, cleaning and other (general contacts). 21 objects were selected to record contacts: clinical waste bin, domestic waste bin, chair with arms, bed rails, bed, suspended shelf surface, mouse, chair, sink rim, sink bowl, trolley surface, small trolley surface, keyboard, gel dispenser, paper, panels, gloves, apron dispenser, soap dispenser, lamp, domestic waste bin.

The observational study was done at the same time as testing for bacterial contamination via TVC (as described in methods section 2.3.2.1.) and was undertaken by Space Syntax Ltd.

2.5.4 VISIBILITY

Plans of the three intensive care units (PICU, CICU, NICU) were analysed to quantify the visibility of each hand wash basin in terms of floor area i.e. the number of square meters of the floor area from which that hand wash basin was visible.[386] Each bed space had a dedicated hand wash basin. The visibility score were calculated for three conditions:

Visible area: Observer standing, when all curtains of bed spaces are drawn open.

Limited visible area: Sitting, when all curtains of bed spaces are drawn open.

Curtained area: When the curtained to the closest bed space to a specific sink is drawn closed.

The visibility of the sink was then related to the sink usage data collected as described in methods section 2.6.2.

2.6 STATISTICAL ANALYSIS

With the exception of the analysis detailed in sections 2.6.1 and 2.6.2., results were analysed using SPSS 16.0 and 22.0 for Windows as detailed in within each chapter.

2.6.1 SPATIAL CONTAMINATION ANALYSIS

In order to compare the different types of surfaces and furniture involved sampled in section 2.3.4.3., TVCs were analyzed using a Poisson mixed model with canonical log link function, quantifying association between bacterial counts and objects. Explanatory variables for models included: 'Object', 'Bedside' (including bed occupancy), and 'Ward'. Diagnostic plots were examined to assess the quality of the model fit. The 'Object' variable had eleven classes which were bed rails (reference class), floor, alcohol hand gel pump, bedside table, bed wheels, chair, clinical waste bin, storage trolley and unit top and shelf, top of computer. For the variable 'Ward', the reference class was the MITU.

Spatial analysis used to analyze the spatial variability of microorganisms in the air and on surfaces from samples taken as described in methods sections 2.3.4.3 and 2.3.4.5. Because numerous surface samples were taken at each location, mean TVCs were used. The spatial distribution of microbiological results, both surface and air samples, were analyzed using Generalized Additive Models (GAM).[387] This regressive approach was used to model the counts of microorganisms growing on TSA plates,

with a Poisson distribution model (using the log canonical link), adjusted on Ward (MITU and SITU), sample site situation (bedside or not), and Occupancy of the bed (if sample performed at a bedside). The locations of each sample were referenced using Cartesian coordinates, which where modeled using thin plate splines.[388] The model selection was based on analysis of covariance for nested models and UBRE-score. The conditions of use were checked using classical graphical means.

The statistical analysis was performed using the software R 2.10.1 and the mgcv package developed by Simon Wood.[389] Maps were performed using the geographic information system ArcGIS. All p-values were compared to the classical α-threshold of 0.05.

2.6.2 SINK USE ANALYSIS

To analyse observation data collected as described in section 2.5.2., standard linear regression analysis was used. Explanatory variables were 'visibility' and 'occupation' of the bed space and observed effect on sink usage and length of hand washing, in turn.

The microbial data collected as described in section 2.3.4.2. had a three-level hierarchical structure as repeated measurements were taken at the beginning and at the end of the observation day (1st level) from seven different locations (2nd level) within eight sinks (3rd level). Therefore, data was analysed with multilevel regression using linear mixed effects model using ML and Bayesian estimation using Markov Chain Monte Carlo (MCMC) simulations. The model accounts for the correlation within each cluster. In the model, random intercepts were included for each cluster and the model checks through diagnostic plots to look for violation of the model assumptions. The explanatory variables were defined as 'usage' of the sink and the 'locations' within the sink. The variable 'location' had three categories, where the categories were formed according to their risk of transmission of bacteria through contamination (i.e. sink bowls, back of sink - lips, faucet/gel-soap dispenser). In addition, to investigate whether the effect of wash basin usage differed between sink locations, the interaction between 'location' and 'usage' was included in the analysis. The baseline category was defined as the bowl of the sink. All the explanatory variables were defined to have fixed effects.

All the analysis was made through MLwiN version 2.20 which is a specialized software for fitting multilevel models.[390]

2.6.3

HOSPITAL ADENOVIRUS MONITORING

To analyse data collected as described in section 2.3.1.1 a standard linear regression analysis was used. Explanatory variables were ‘object’, ‘season’, ‘cleaning number’ and ‘room’, in turn.

In detail, the factors of interest are given as follows:

`Object' which had twenty levels (sink, mattress, door handle, phone, TV, waste, window, bathroom, trolley, floor, bed, chair, sluice, macerator, fan, table, light, scales, CD player, computer). `Room' included: ward areas (HSCUT and IIU), communal ward areas (HSCUT and IIU) and cubicles (eleven HSCTU cubicles, five IIU cubicles). ‘Season' has four levels (winter, spring, summer and autumn) and `Cleaning' was the number of times a surface was cleaned and took values one to six.

Akaike's Information Criterion (AIC) was used in combination with Analysis of Deviance to test whether each variable had an effect on the outcome. All the analysis was made through MLwiN version 2.20 which is a specialized software for fitting multilevel models.[390]

2.7 EPIDEMIOLOGICAL ANALYSIS

Interpretation of molecular typing results was informed where necessary by epidemiological information. This was only utilised for isolates that had not been selected on the basis of suspected cross transmission.

Isolates were determined to be potentially linked if patients had been present in the same location (ward) at the same time or within a two week period to allow for an environmental reservoir.

2.8 BIOINFORMATIC ANALYSIS

With the exception of the analysis detailed in sections 2.8.1, 2.8.2, 2.8.3 and 2.8.4 sequencing data was analysed using the Lasergene Suite version 12.0.0. (DNAStar, Madison, USA).

2.8.1 MISEQ ANALYSIS ONE

The genomes produced from DNA extracts processed as described in section 2.2.8. were assembled by Piklu Bhattacharya at the ithree institute (University of Technology, Sydney, Australia) using the in-house bioinformatics genome assembly and analysis

pipeline.[391] Briefly, genomes were assembled using the A5-miseq pipeline, which can process reads up to 500 nucleotides (nt) long and constructs the de Bruijn graphs with k-mers up to 500nt.[392]

Based on the initial phylogeny based on alignments of marker genes, the *Klebsiella pneumoniae* MGH78578 genome sequence was selected to draw a reference-based whole genome alignment of the *Klebsiella pneumoniae* isolates included in this study. MGH78578 is a completely-closed reference genome sequence freely available in the GenBank database (accession number CP000647). The phylogenetic analysis was carried out using an established reference-based whole genome alignment pipeline available at the bioinformatics facility of the ithree institute at the University of Technology, Sydney – Australia (*Roy Chowdhury et al – manuscript in preparation*). Reference-based consensus sequences of each isolate were combined into a multiple sequence alignment and alignment columns containing only unresolved nucleotides were removed, leaving only the differential site patterns. RaxML v7.2.6 was used subsequently to construct the Maximum Likelihood based phylogenetic tree.[393] The confidence in each clade of the Maximum Likelihood tree was estimated using RAxML's rapid bootstrap procedure with automatic extended majority-rule criterion (NN bootstraps) and the resulting tree and bootstrap confidence estimates were visualized using FigTree v1.4.0.[394]

All analyses were performed without knowledge of the species determination, resistance phenotype or pulse field gel electrophoresis results.

2.8.2 MiSeq Analysis Two

Reads from the MiSeq run described in section 2.2.8.were aligned by Alex Rolfe against the *Klebsiella pneumoniae* MGH78578 genome sequence (Genbank accession number CP000647) using the Bowtie2 aligner (verson 2.1.0) in paired end mode with parameters " --sensitive --minins 10 --maxins 1400" and a "--rg-id" parameter to store the sample name in the alignment output. An average of ~224,000 reads were mapped per sample (median ~172,000, range 98,440 to 645,734 reads, with median 64.87% aligned concordantly at least 1 time). Output files were then sorted and merged to generate a single BAM file with all aligned reads and then FreeBayes (version 0.9.9.2-14) was used on that file to generate a VCF file of SNV (single nucleotide variants) calls. SNV calls were filtered to include only those with 10 reads of coverage and at least 5 reads supporting the call. If SNVs could be called in some samples then the other samples were included as "-" for missing data. FASTA and PHYLIP files

were then generated using only genomic loci at which at least one sample showed a SNV. The PHYLIP file was used as input to RaxML to generate the WGS phylogenetic trees with parameters "-T 16 -p $$ -f a -s ${project}.phylip -n ${project}.raxml-gtrgamma.tree -m GTRGAMMA -x $$ -N autoMRE -k". Trees were generated both for the full set of samples and for a subset of more closely related samples (*Klebsiella* species and a *Klebsiella pneumoniae* tree). Due to the input format, which contained only variant genomic loci, the tree distances do not measure a true distance (i.e., SNVs / genome size) but rather a relative value (i.e. SNVs/number of variant loci). Trees were optimized for display using the same optimal leaf ordering as was used for the HAI BioDetection trees.

All analyses were performed without knowledge of the species determination, resistance phenotype or pulse field gel electrophoresis results.

2.8.3 PHYLOSHIFT ANALYSIS OF MISEQ SEQUENCING DATA

The genomes produced from DNA extracts processed as described in section 2.2.8. were assembled by Piklu Bhattacharya at the ithree institute (University of Technology, Sydney, Australia) using the in-house bioinformatics genome assembly and analysis pipeline.[391] Briefly, genomes were assembled using the A5-miseq pipeline, which can process reads up to 500 nucleotides (nt) long and constructs the de Bruijn graphs with k-mers up to 500nt.[392]

A phylogenetic tree was constructed using PhyloSift.[395] PhyloSift works by identifying homologs of universally conserved 37 single copy elite markers (which accounts for approximately1% of any bacterial genome) from any given draft bacterial genome. For the purposes of this study, PhyloSift was used to create a concatenated multiple alignment of the marker genes from all draft *Klebsiella* species genomes and an *Escherichia coli* (55989) and a *Vibrio cholerae* (N16961) were included as outgroups. From this alignment, a phylogeny was inferred using FastTree2. The two outgroup isolates were removed from the final phylogenetic tree in order to display relationships among isolates at a higher resolution. Internal tree branches were annotated with the support value for each of the clades.

All analyses were performed without knowledge of the species determination, resistance phenotype or pulse field gel electrophoresis results.

2.8.4 PATHOGENICA HAI BIODETECTION

Sequencing data collected as described in section 2.2.9. were analyzed on the Ion Torrent server (Life Technologies, Paisley, UK) using the Pathogenica Ion PGM Sequencer analysis plug-in of Pathogenica's HAI BioDetection software v1.2 (Pathogenica, Boston, USA). This software used 103 to 106 sequencing reads to determine the genomic sequence of all of the loci targeted by the assay for each organism present in the sample. RAxML was used to generate a phylogenetic tree for the samples.[393]. A shell-scripting interface to the software (rather than the Ion Torrent Server interface) was used to create the tree, whereby the probe weight and partition values were identical to those present in software v1.2. After tree construction, each tree's leaves were ordered using an optimal leaf ordering algorithm to make clusters easier to identify by eye.[396]

Chapter 3 DETECTION OF MICROORGANISMS WITHIN THE CLINICAL ENVIRONMENT

3.1 INTRODUCTION

Historically, environmental contamination by pathogenic microorganisms was believed to have a negligible impact on the rate of nosocomial infections and that the evidence base for environmental surveillance was negligible.[182, 328, 397] Inanimate surfaces within the clinical environment are considered non-critical as the item only comes into contact with intact skin, and intact skin is considered a barrier to disease transmission. It was therefore determined that non-critical items or contact with them carried little risk of transmitting pathogens to patients.[177, 362] This view is now challenged with increasing evidence that the environment acts as an intermediary reservoir for pathogens causing healthcare associated infection (HCAI).[183, 184]

Microorganisms that cause HCAI must have the capacity to both contaminate the environment and survive within it.[300, 341] Organisms enter this environmental niche by being released as droplets through respiratory secretions or diarrhoea and vomiting, via dust, or deposition of skin scales.[341] If pathogenic organisms can survive within a niche and survive beyond the stay of one patient, they have the potential to pose a risk to subsequent patients.[341] In order to cause HCAI, there must be direct or indirect transmission from this niche to a susceptible host. To prove that the environment was the source of HCAI is difficult. Proving that the organism originated from the environment requires molecular tagging, distinct antibiograms or whole genome sequencing. Therefore demonstration of an impact on HCAI by affecting a niche is used as an indirect measure of an environmental source.[300]

The aim of cleaning within healthcare environments is accordingly to reduce levels of microorganisms so that they do not pose a cross-transmission risk to patients. The only official UK Department of Health guideline in this area is the assessment of environmental cleanliness through visual inspection and regular disinfection of high contact surfaces.[398] However one study has demonstrated that even when 82% of ward sites were visually clean, only 30% of sites were considered clean by microbiological sampling.[399] Visual inspection was therefore shown not to a reliable assessment of environmental cleanliness.[341] As a result it has been proposed that hospitals should monitor the level of microbial contamination within the environment, either through total viable counts/aerobic colony counts or the presence of indicator

organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA).[191] Griffith et al. (2003) have suggested that a surface should be considered to have failed cleaning and thus be a risk to patients if the number of colony forming units (CFU) detected is in excess of 2.5CFU/cm^2 on an agar contact plate (60CFU/plate).[400] However Dancer (2004) proposed a higher cut-off limit of 5CFU/cm^2 (120CFU/plate) with failures leading to bed space closures and repeat cleaning.[191] These recommendations do not however currently extend to encompass viruses.

There is a requirement to develop testing methods that can be applied to the clinical setting in order to determine if there are pathogenic organisms within the clinical environment. If organisms are present, we aim to evaluate how the proposed 'standards' relate to contamination and if results can be interpreted in order to determine risk to patients.

3.2 CHAPTER AIMS

The aim of this chapter was to develop methods for sampling the clinical environment for both bacteria and viruses and to then apply these methods to both adult and paediatric units to determine the presence of environmental contamination. Methods were also established for inoculation of surfaces that could then be utilised in further decontamination and survival studies. The specific aims are listed below:

1. To develop an environmental sampling methodology that could be applied to clinical settings by testing available sampling techniques and commonly used sites.

2. To develop a model for undertaking inoculation and survival/decontamination studies in order to evaluate the capacity of pathogens to survive in environmental niches.

3. Application of the developed sampling methodology to clinical settings to determine the levels of environmental contamination present within those environments.

4. To use levels of environmental contamination to draw conclusions about cleanliness and risk of HCAI.

3.3 RESULTS

3.3.1 VALIDATION OF INOCULATION EXPERIMENT METHODOLOGY (SWAB SAMPLING)

In order to establish the sensitivity of swabbing as a recovery technique and to establish a method of testing decontamination techniques, seven organisms were tested for recovery by cotton tipped swab from ceramic tiles (see method sections 2.4.1 and 2.4.). Inoculation experiments were undertaken using sterile water for the suspension. This demonstrated a 1 – 2 $\log_{10}$ loss between the inoculum and recovered counts with recovery rates of between 89% – 92% (see Table 3-1). Phosphate buffered saline (PBS) and 0.3% bovine serum albumin (BSA) were also tested; as organisms within the clinical environment are often associated with proteinaceous material. Under these test conditions, loss of viability appeared to be minimal (see Table 3-1). Despite the increased recovery seen under proteinaceous conditions, decontamination testing was undertaken using suspensions made in sterile water as this reagent would not interfere with the decontamination approaches being studied (see Chapter 5).

Organism	Condition	Sterile Water CFU/ml	Sterile 1 * PBS CFU/ml	Sterile 0.3% BSA CFU/ml
Pseudomonas aeruginosa **NTCC 12903**	Inocula	$1.09 * 10^{11}$	$2.00 * 10^{09}$	$3.5 * 10^{08}$
	Recovered	$6.6 * 10^{9}$	$1.53 * 10^{09}$	$2.13 * 10^{08}$
	% Recovery	88.97	98.75	97.48
Acinetobacter baumannii **(clinical strain)**	Inocula	$1.4 * 10^{11}$	$4.00 * 10^{09}$	$4.00 * 10^{09}$
	Recovered	$9.8 * 10^{9}$	$6.68 * 10^{09}$	$3.38 * 10^{09}$
	% Recovery	89.64	102	99.24
Enterococcus faecalis **ATCC 29212**	Inocula	$1.19 * 10^{11}$	$2.50 * 10^{09}$	$5.25 * 10^{08}$
	Recovered	$1.82 * 10^{10}$	$1.60 * 10^{09}$	$3.38 * 10^{08}$
	% Recovery	92.64	97.94	97.81
MRSA (clinical strain)	Inocula	$2.00 * 10^{11}$	$4.10 * 10^{09}$	$1.09 * 10^{11}$
	Recovered	$2.00 * 10^{10}$	$1.20 * 10^{09}$	$2.82 * 10^{10}$
	% Recovery	91.2	94.45	94.68
Escherichia coli **(clinical strain)**	Inocula	$1.5 * 10^{11}$	$3.60 * 10^{08}$	$5.00 * 10^{08}$
	Recovered	$1.98 * 10^{10}$	$3.78 * 10^{08}$	$2.88 * 10^{08}$

	% Recovery	92.13	100	97.25
Klebsiella pneumonia **(NCTC 13368)**	Inocula	$1.5 * 10^{11}$	$8.90 * 10^{09}$	$1.00 * 10^{09}$
	Recovered	$5.40 * 10^{09}$	$2.32 * 10^{09}$	$2.56 * 10^{08}$
	% Recovery	87.08	94.13	93.42
Staphylococcus aureus **(NCTC 6571)**	Inocula	$6.0*10^{9}$	$1.30 * 10^{09}$	$4.90 * 10^{10}$
	Recovered	$3.5*10^{8}$	$3.35 * 10^{08}$	$1.23 * 10^{10}$
	% Recovery	87.38	93.54	94.38

Table 3-1 Recovery of bacterial species after drying, inoculated onto ceramic tiles and recovered using cotton tipped swabs. Inoculums suspended in sterile water, phosphate buffered saline or bovine serum albumin. CFU = colony forming units.

3.3.2 Estimating Survival of Bacteria and Viruses Within the Environment

To establish a better understanding of how three organisms linked to HCAI would behave in the longer term under experimental conditions, a survival experiment was undertaken as described in the methods section 2.4.1. Organisms were inoculated onto ceramic tiles and organism recovery was tested at 24 hours, 48 hours, 72 hours and at one week, two weeks, three weeks, four weeks and twelve weeks. *Staphylococcus aureus*, *Klebsiella pneumoniae* and adenovirus all demonstrated viability even after twelve weeks in dry conditions (see Table 3-2).

Staphylococcus aureus **(NCTC 6571)**					
Sampling Time	**Control CFU/ml**	**Test CFU/ml**	**Log_{10} Control**	**Log_{10} Test**	**Log_{10} Reduction**
24 hrs	$1.87*10^{09}$	7.75E+05	9.27	5.89	3.38
48 hrs	$1.87*10^{09}$	3.31E+04	9.27	4.52	4.75
72 hrs	$1.87*10^{09}$	2.52E+04	9.27	4.40	4.87
week 1	$1.87*10^{09}$	1.06E+03	9.27	3.03	6.25
week 2	$1.87*10^{09}$	7.43E+03	9.27	3.87	5.40
week 3	$1.87*10^{09}$	5.44E+03	9.27	3.74	5.54
week 4	$1.87*10^{09}$	8.20E+03	9.27	3.91	5.36
week 12	$1.87*10^{09}$	1.37E+02	9.27	2.14	7.14
Klebsiella pneumoniae **(NCTC 13368)**					
Sampling Time	**Control CFU/ml**	**Test CFU/ml**	**Log_{10} Control**	**Log_{10} Test**	**Log_{10} Reduction**
24 hrs	$1.05*10^{09}$	$8.06*10^{04}$	9.02	4.91	4.11

48 hrs	$1.05*10^{09}$	$2.83*10^{04}$	9.02	4.45	4.57
72 hrs	$1.05*10^{09}$	$9.00*10^{03}$	9.02	3.95	5.07
week 1	$1.05*10^{09}$	$1.06*10^{03}$	9.02	3.03	5.99
week 2	$1.05*10^{09}$	$7.75*10^{02}$	9.02	2.89	6.13
week 3	$1.05*10^{09}$	$8.75*10^{02}$	9.02	2.94	6.08
week 4	$1.05*10^{09}$	$3.69*10^{02}$	9.02	2.57	6.45
week 12	$1.05*10^{09}$	$2.00*10^{01}$	9.02	1.30	7.72

Adenovirus C2 (NCTC 0108051v)		
Sampling Time	**Control**	**Test**
24 hrs	Positive	Positive
48 hrs	Positive	Positive
72 hrs	Positive	Positive
week 1	Positive	Positive
week 2	Positive	Positive
week 3	Positive	Positive
week 4	Positive	Positive (3/4 replicates required passaging)
week 12	Positive	Positive (4/4 replicates required passaging)

Table 3-2 Recovery of *Klebsiella pneumoniae*, *Staphylococcus aureus* and adenovirus over a three month period inoculated onto ceramic tiles and recovered using cotton tipped swabs. Four replicants were inoculated for each species for each time point, for adenovirus after week three some of the replicants required additional passaging in VERO cells to permit detection. CFU = colony forming units.

3.3.3 Sampling Methodology Development

The surfaces selected for sampling at the adult units within the National Hospital of Neurology and Neurosurgery (NHNN) during the three month pilot phase of this study offered an opportunity for comparison between the common ward areas within the units studied (e.g. nurses' station) as well as between bed spaces and ward types surgical intensive care unit (SITU) and medical intensive care unit (MITU). Sampling in all areas was carried out in a systematic way in order to allow comparison between surfaces and locations. Surface types within the clinical environment were also considered and

included materials such as varnished wood, linoleum flooring, stainless steel, and textured plastics.

In the following sections a number of sampling techniques and culture conditions are described. These were trialled to develop a sampling methodology that would form the basis of the rest of this project (see sections 3.3.4 and 3.3.5.).

In studies undertaken at Great Ormond Street Hospital (GOSH), the surfaces selected were those that have been shown to be linked to contamination within the environment, and sites that have been linked to outbreaks at GOSH, such as sinks.

3.3.4 Air Sampling

228 total viable count air samples were taken (see methods section 2.3.2.1.), 40 of which were taken using both tryptone soya agar (TSA) and Sabouraud agar (SAB) plates in parallel in order to determine which gave the most comprehensive result. The SAB plates provided greater consistency of low level fungal isolate detection but the units were not high efficiency particulate air (HEPA) filtered and so this was considered to be of lesser importance. TSA plates were selected as the agar of choice for the remaining sampling as they consistently provided higher bacterial counts than the SAB plates. Analysis of these results is presented in Table 3-3.

3.3.5 Surface Sampling for Specific Organism Detection

During the pilot phase of this research, both swab sampling and contact plate methods were evaluated for detection of specific organisms.

3.3.5.1 Comparison Between Swab Sampling and Contact Plate Methods for Specific Organism Detection

Having established the optimal method for swab sampling during development of inoculation experiment methodology (section 3.3.1.), a comparison was made between swab sampling and contact plates at two hospitals: NHNN (adult hospital) and GOSH (paediatric hospital).

National Hospital of Neurology and Neurosurgery

Over a three month period, 1020 samples were taken from 127 sites using contact plates, direct plating and enrichment sampling as per methods section 2.3.4.3.

Over a three month period, 381 samples were collected from 127 sites for detection of MRSA. Three of these samples (0.8%) were positive, all detected via enrichment

sampling only. The positive sites included the storage unit top and bed wheels within an isolation bed space and the apron dispenser in the neighbouring empty bed space post clean.

Sampling for *Acinetobacter* species (spp.) involved taking 258 samples from 86 sampling sites using contact plates, direct plating and enrichment sampling as per methods section 2.3.2. All were *Acinetobacter* spp. negative by all sampling techniques.

Further sampling was undertaken for *Clostridium difficile* using contact plates, direct plating and enrichment sampling as per method section 2.3.2. Over a three month period 381 samples were collected from 127 sites all of which were negative by all sampling techniques.

Great Ormond Street Hospital

A one off sampling validation was undertaken where infection control information could be accessed to determine patient status; this was not the case at NHNN. This targeted sampling session involved taking samples from 20 sites on the Immunology and Infectious Disease unit (IIU) for MRSA in a cubicle prior to cleaning that had been used for MRSA isolation (see methods section 2.3.2.). Of the 60 samples taken, all the selective agar contact plates were negative for MRSA. One sample was positive on direct plating (5%), the window sill. Two swabs (10%) were positive using enrichment for MRSA, the windowsill and the room heater.

3.3.6 Relationship Between Spatial Layout and Bacterial Contamination

For estimating bacterial contamination (not specific organisms), TVCs were determined as described in methods section 2.3.2.1. Sampling was carried out over an initial three month period with each month referred to as a sampling period i.e. 1, 2 or 3. A total of 228 air samples and 426 surface samples were taken using non selective contact plates in both SITU and MITU in order to test the hypothesis that organisms could be cultured from the clinical environment (see Figure 3-4.). Samples were taken from a range of surfaces including bed frames, trolleys and bed wheels as described in methods section 2.3.4.3. Surfaces were categorised as level 0 (<0.6m in height), level 1 (0.6m-1.2m) or level 3 (>1.2m). In addition, surfaces were categorised as to whether they were high touch or not using previous studies in the literature.[174, 191]

TVCs were averaged between all sampling sessions and assigned arbitrary colours based on number of colonies recovered, see Figure 3-1, Figure 3-2 and Figure 3-3. Green areas had a mean of less than 50CFUs. Yellow areas had a mean of 50 -100 CFUs. Pink areas had a mean of between 101 and 200 CFUs. Finally red areas had over 200 CFUs.

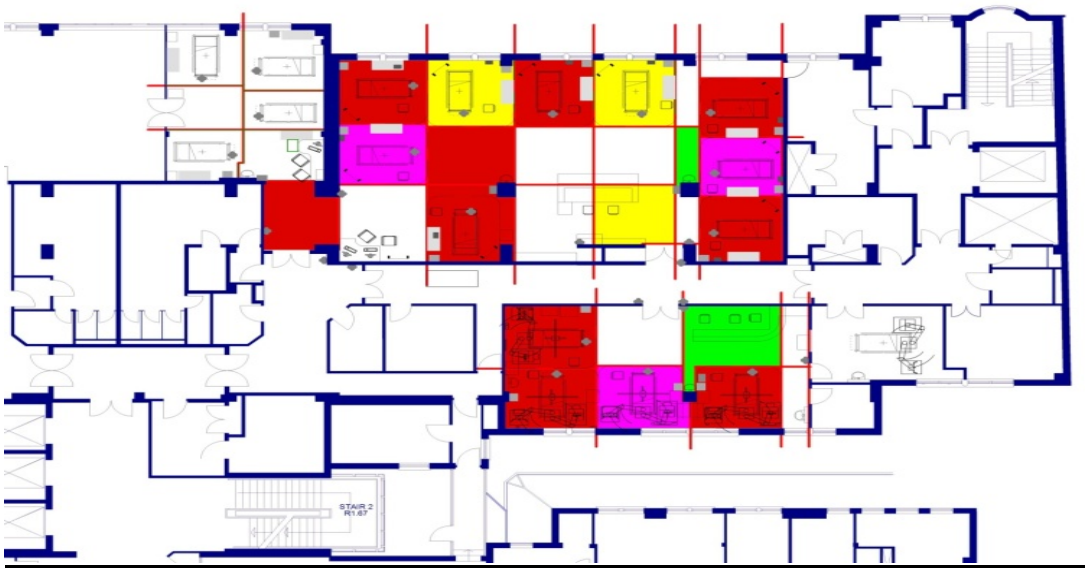

Figure 3-1 Mean number of CFUs detected at level 0 in bed spaces and SITU and MITU ward areas over a 3 month period on sampling sites under 0.6m (green areas <50 CFUs, yellow areas 50 -100 CFUs. pink areas 101 and 200 CFUs, red areas over 200 CFUs).

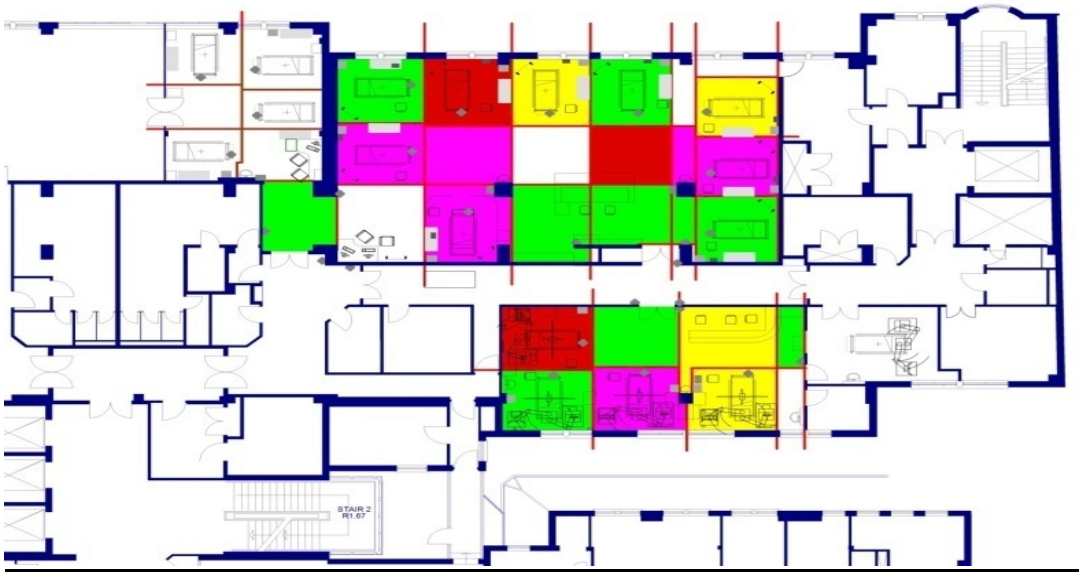

Figure 3-2 Mean number of CFUs detected at level 1 in bed spaces and SITU and MITU ward areas over a three month period on sampling sites 0.6m-1.2m high

(green areas <50 CFUs, yellow areas 50 -100 CFUs. pink areas 101 and 200 CFUs, red areas over 200 CFUs).

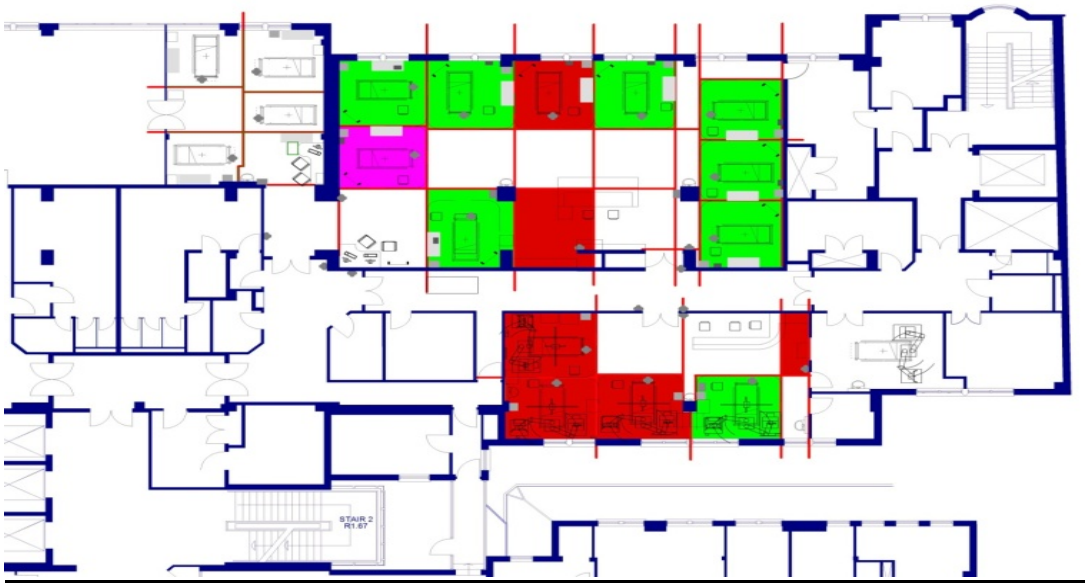

Figure 3-3 Mean number of CFUs detected at level 2 in bed spaces and SITU and MITU ward areas over a three month period on sampling sites >1.2m high (green areas <50 CFUs, yellow areas 50 -100 CFUs. pink areas 101 and 200 CFUs, red areas over 200 CFUs).

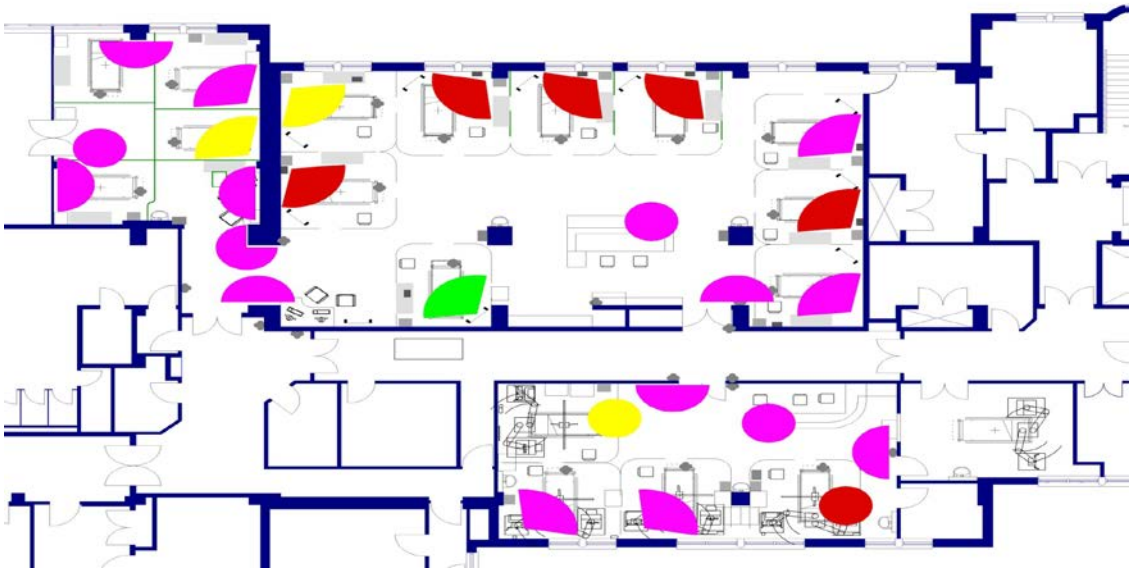

Figure 3-4 Mean number of CFUs detected at by air sampling in bed spaces and SITU and MITU ward areas over a three month period. Coloured areas represent the proportion of area within the location sampled by the air sampler (green areas <50 CFUs, yellow areas 50 -100 CFUs. pink areas 101 and 200 CFUs, red areas over 200 CFUs).

These data showed that the main determinants of microbial density were height, with mid-levels surfaces associated with touch often being less contaminated (see Table 3-3.). Air contamination did not appear to always correlate with levels of surface contamination. Contamination levels did not appear to be linked to specific bed spaces for surface sampling but may be for air sampling.

To test this theory, the data was analysed using Generalized Additive Models (GAM) to determine the spatial variability of microorganisms in the air and on surfaces and so assess the link between contamination, height, bed spaces/shared ward and occupation, see Figure 3-5.[387] Because numerous surface samples were taken at each location, mean CFUs were used. The locations of each sample were referenced using Cartesian coordinates, which were modelled using thin plate splines (see methods section 2.7.1).

Unit	Height level	Sampling Period 1			Sampling Period 2			Sampling Period 3		
		Median	No. Samples	CFU Range	Median	No. Samples	CFU Range	Median	No. Samples	CFU Range
SITU	Level 0 <0.6m	182	15	40-350	107	16	17-350	350	16	18-350
	Level 1 0.6m-1.2m	71.2	33	2-269	34.8	36	1.5-350	64.5	36	3-350
	Level 2 >1.2m	79.2	13	5-350	29.5	15	2.5-197	14.5	15	1-350
	Air	98.5	12	60-167	60	11	27-158	164	11	39-419
MITU	Level 0 <0.6m	150.3	7	6-623	350	9	2-350	205	9	4-350
	Level 1 0.6m-1.2m	34.4	24	238.3	28.7	25	2-126	55.4	25	9.7-350
	Level 2 >1.2m	200	5	27-350	27	5	6-350	350	5	22-350
	Air	103	7	99-217	88	7	50-167	182	7	51-213

Table 3-3 Median and range of CFUs per 0.24m² plates (55cm diameter) detected at levels 0, 1 and 2 and during air sampling in bed spaces and within shared ward areas in SITU and MITU over a three month sampling period (periods 1, 2 and 3).

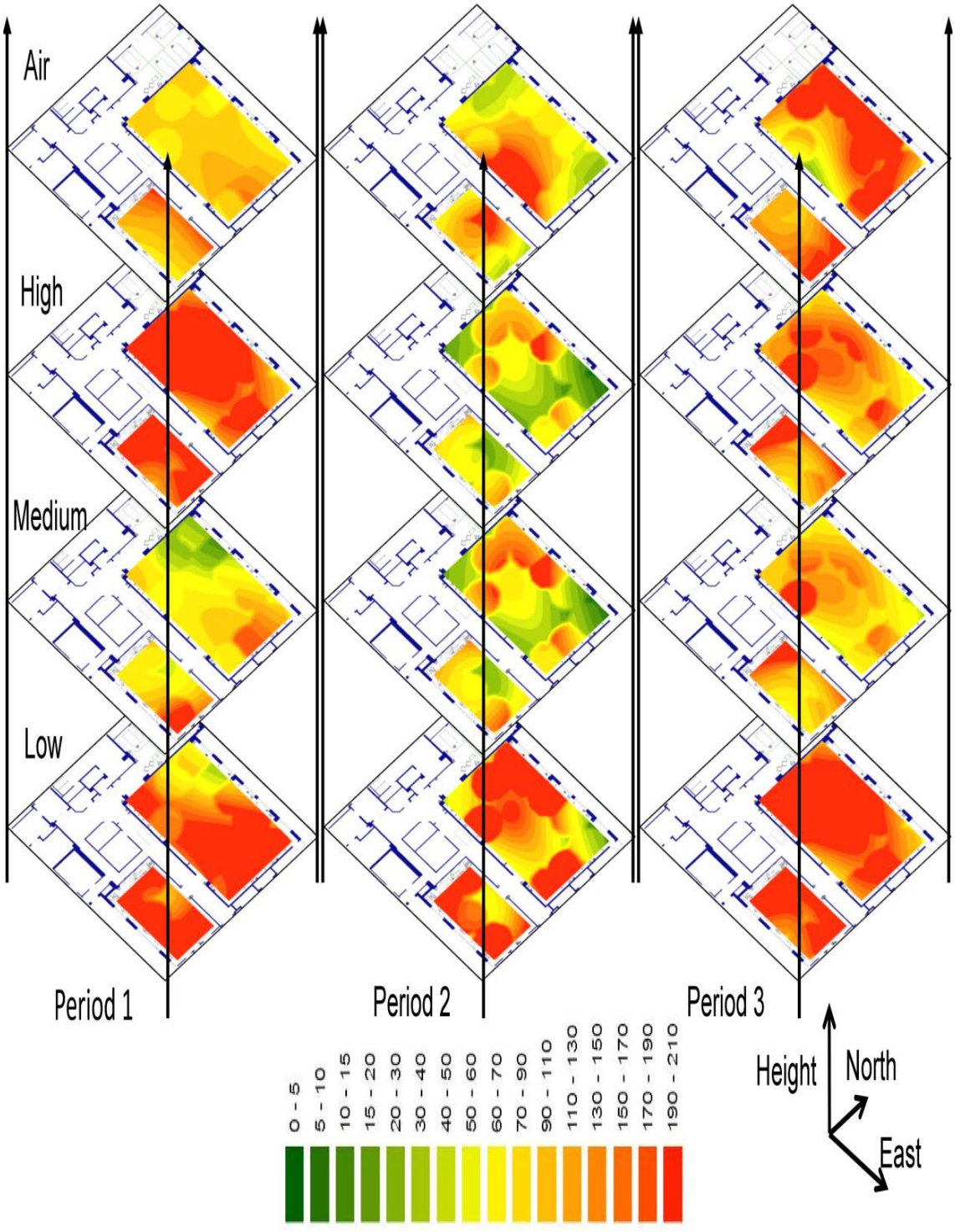

Figure 3-5 Modelling estimation of microorganism CFUs over a three month sampling period within the MITU and SITU at the NHNN. Results were adjusted on bedside, bed occupancy, level (for surface analysis), Ward and location. CFU estimations for the three sampling periods at different height levels and within air samples were included for each location. The coloured scale equates colours with CFU values.

Sampling Period (% explained deviance - n§)	Co-factors		Incidence Ratio (IR) [CI95%]	p-value
Period 1				
(81.5% - n=19)	**Bedside**	Non Bedside*	1	-
		Non occupied Bedside	0.74[0.58;0.93]	0.007**
		Occupied Bedside	1.11[0.91;1.35]	0.26
	Ward	MITU*	1	-
		SITU	0.33[0.2;0.57]	<0.001**
Period 2				
(85.7% - n=19)	**Bedside**	Non Bedside*	1	-
		Non occupied Bedside	0.71 [0.52;0.97]	0.02**
		Occupied Bedside	0.6[0.47;0.76]	<0.001**
	Ward	MITU*	1	-
		SITU	14.34 [8.08;25.44]	<0.001**
Period 3				
(49.6% - n=19)	**Bedside**	Non Bedside*	1	-
		Non occupied Bedside	1.92 [1.54;2.39]	<0.001**
		Occupied Bedside	1.96 [1.66; 2.31]	<0.001**
	Ward	MITU*	1	-
		SITU	0.07 [0.04;0.11]	<0.001**

Table 3-4 Spatial analysis of air samples from MITU and SITU using a Generalized Additive Mix Model.

Risk factors were assessed each day by using Generalized Additive Mix Model, adjusted on Bedside (occupied or not), Ward and Location.

§ n: number of locations

*reference class

**p<0.05

Sampling Period (% explained deviance - n[§])	Co-factors		Incidence Ratio (IR) [CI95%]	p-value
Period 1				
(38% - n=49)	**Bedside**	Non Bedside*	1	-
		Non occupied Bedside	0.78 [0.67;0.89]	0.0002**
		Occupied Bedside	1.72 [1.54;1.91]	<0.001**
	Ward	MITU*	1	-
		SITU	0.77[0.59;1[	0.05**
	Height	Level 0 <0.6m	1	-
		Level 1 [0.6-1.2m]	0.39 [0.37;0.42]	<0.001**
		Level3 >1.2m	0.59 [0.56;0.64]	<0.001**
Period 2				
(56% - n=57)	**Bedside**	Non Bedside*	1	-
		Non occupied Bedside	6.96 [5.54;8.73]	<0.001**
		Occupied Bedside	3.14 [2.84;3.48]	<0.001**
	Ward	MITU*	1	-
		SITU	0.18 [0.13;0.24]	<0.001**
	Height	Level 0 <0.6m *	1	-
		Level 1 [0.6-1.2m]	0.39 [0.37;0.42]	<0.001**
		Level3 >1.2m	0.31 [0.29;0.33]	<0.001**
Period 3				
(27% - n=57)	**Bedside**	Non Bedside*	1	-
		Non occupied Bedside	2.24 [2.04;2.46]	<0.001**
		Occupied Bedside	1.5 [1.39;1.61]	<0.001**
	Ward	MITU*	1	-
		SITU	0.09 [0.07;0.12]	<0.001**
	Height	Level 0 <0.6m *	1	-
		Level 1 [0.6-1.2m]	0.47 [0.44;0.49]	<0.001**
		Level3 >1.2m	0.6 [0.57;0.64]	<0.001**

Table 3-5 Spatial analysis of surface samples from MITU and SITU using a Generalized Additive Mix Model.

Risk factors were assessed each day by using Generalized Additive Mix Model, adjusted on Bedside (occupied or not), Ward, height and Location. § n: number of locations, *reference class, **p<0.05

Roughly 120 samples were collected during each sampling period in order to obtain TVCs. Results for surface and air samples showed large differences, with the second sampling period demonstrating the lowest levels of contamination for both MITU and

SITU. Both air and surface contamination levels varied, but globally, MITU showed higher contamination levels than SITU, see Table 3-4. Within MITU the lowest height level (<0.6m) was the most contaminated for sampling period 2 (median TVC 350), but samples taken above 1.2m were the most contaminated for sampling periods 1 and 3 (median TVCs 200 and 320 respectively). Mid-level surfaces sampled (between 0.6 and 1.2m), mostly representing high touch surfaces, demonstrated the lowest levels of contamination during all three sampling periods on MITU (median TVC resp. 34.4, 28.7, 55.4). Within the SITU the lowest height level (<0.6m) was the most contaminated for sampling periods 1, 2 and 3 (median TVCs were 182, 107 and 350), but the height level with lowest contamination varied between mid and high level samples depending on sampling period.

Contamination varied within occupied/unoccupied bed spaces and the shared ward area both within and across sampling periods, see Table 3-5. During sampling periods 1 and 2, there were four and five beds occupied respectively for MITU and SITU. During sampling period 3, three and six beds occupied respectively for MITU and SITU. Bed spaces whether occupied or not were consistently more contaminated than the shared ward area for surface samples. For air samples unoccupied bed spaces were less contaminated during sampling period 1 and both occupied and unoccupied bed spaces were less contaminated than the shared ward area by air sampling during sampling period 2. Unoccupied bed spaces were more contaminated by surface sampling than occupied bed spaces for sampling periods 2 and 3, but not during sampling period 1.

Cofactors		**Incidence Ratio (IR) [CI95%]**	**p-value**
Surface types	**Bed rails***	1	-
	Floor	1.18 [0.76;1.83]	0.46
	Alcohol hand gel pump	0.27 [0.1;0.79]	0.02**
	Bed side table	0.087 [0.01;0.74]	0.03**
	Bed wheels	1.97 [1.21;3.21]	0.01**
	Chair (seat)	0.45 [0.09;2.24]	0.32
	Clinical waste bin	0.61 [0.29;1.28]	0.19
	Storage trolley	0.41 [0.18;0.91]	0.03**
	Storage unit - shelf	0.62 [0.21;1.84]	0.39

	Storage unit - top	0.48 [0.25;0.89]	0.02**
	Top of computer	1.06 [0.61;1.84]	0.83
Ward	**MITU***	1	-
	SITU	0.89 [0.64;1.24]	0.49

Table 3-6 Analysis of the association between bacterial counts and object type for the different surfaces sampled at the NHNN.

The adjusted incidence ratios (IR) are presented with their 95% confidence intervals.
*reference class
**p<0.05

In order to compare the different types of surfaces and furniture involved in HCAI, TVCs were analyzed using a Poisson mixed model with the canonical log link function, quantifying association between bacterial counts and objects (see methods section 2.7.1.. Results showed that bedside tables (p=0.03), storage trolleys (p=0.03), alcohol hand gel pumps (p=0.02), and top of the storage unit - top (p=0.02) were significantly less contaminated when compared to bed rails, see Table 3-6. Bed wheels were significantly more contaminated than the bed rails (p=0.01). There was no significant difference between porous and non-porous surface types.

3.3.7 Long Term Sampling (Paediatric Intensive Care Unit Bacterial Sampling)

Sampling at the NHNN during the pilot study occurred once a month over a three month period. Due to the size of the units studied it was not possible to sample all bed spaces equally on every occasion. In order to study bed spaces in greater depth and explore the difference between an adult intensive care unit (ITU) and a paediatric ITU, sampling was undertaken in two bed spaces within the paediatric intensive care unit (PICU) at GOSH, referred to as Bed Space 6 (BS6) and Bed Space 7 (BS7) as well as the shared ward area. Sampling occurred weekly over a five month period as described in methods section 2.3.4.1.

Table 3-7. illustrates the wide range of TVCs throughout the given time period by surface. Among the most contaminated objects in both bed spaces were the sinks (bowls and rims), the floor under the sinks and the chairs arms. Overall, BS7 had less contamination than BS6. There was considerable variation in terms of TVC on all objects except the lamp which had a range of only 0 – 9 for BS6 and 0 – 30 for BS7.

Site sampled		BS6 n = 20	BS7 n = 20
Sink bowl	Mean	128	93
	Median (Range)	64.5 (0 – 596)	75 (0 – 320)
Floor under sink	Mean	121	111
	Median (Range)	112 (4 – 268)	104 (0 – 296)
Chair arms	Mean	114	61
	Median (Range)	90 (0 – 388)	42.5 (0 – 210)
Sink rim	Mean	64	76
	Median (Range)	50 (0 – 284)	76 (0 – 228)
Keyboard	Mean	64	25
	Median (Range)	33 (0 – 445)	12.5 (0 – 98)
Clinical waste bin	Mean	46	46
	Median (Range)	31 (0 – 117)	24.5 (0 – 228)
Soap dispenser	Mean	39	25
	Median (Range)	9.5 (0 – 322)	5.5 (0 – 222)
Computer mouse	Mean	30	28
	Median (Range)	12 (0 – 168)	12 (0 – 138)
Bed rails	Mean	16	29
	Median (Range)	2 (0 – 112)	4.5 (0 – 244)
Suspended shelf	Mean	16	6
	Median (Range)	0 (0 – 184)	0 (0 – 121)
Lamp	Mean	14	3
	Median (Range)	3.5 (0 – 9)	0 (0 – 30)
Trolley surface	Mean	13	7
	Median (Range)	0 (0 – 160)	0 (0 – 110)

Table 3-7 Mean, median and range of TVCs on objects within bed space 6 and bed space 7 on PICU over the five month study period.

Bed spaces within the PICU at GOSH do not have high level objects; this was seen as a significant difference between the ITU bed spaces in NHNN and GOSH. This meant that it was not possible to do a spatial analysis similar to that done for the NHNN data as almost all sampling points were located within the medium height level.

It was decided instead to cluster the data into areas within the immediate bed area (bed rails, lamp, and suspended shelf), the wider bed space (chair arms, keyboard,

clinical waste bin, computer mouse, and trolley surface) and the sink area (sink bowl, sink rim, soap dispenser) with and without the floor.

Bed Space	Zone	Mean TVC	Median TVC	TVC Range
6	Near bed (n = 60)	15	1	0 – 184
6	Bed space (n = 100)	53	23	0 – 445
6	Sink area (with floor) (n = 80)	88	50	0 – 596
6	Sink area (without floor) (n = 60)	77	43	0 – 596
7	Near bed (n = 60)	13	0	0 – 244
7	Bed space (n = 100)	33	15	0 – 228
7	Sink area (with floor) (n = 80)	76	66	0 – 320
7	Sink area (without floor) (n = 60)	64	43	0 - 320

Table 3-8 Mean, median and range of CFUs on objects within bed space 6 and bed space seven on PICU clustered into those objects within the sink area (sink bowl, sink rim, soap dispenser) with and without the floor, bed space area (chair arms, keyboard, clinical waste bin, computer mouse, and trolley surface) and the near bed space area (bed rails, lamp, and suspended shelf) over the five month study period.

When bed spaces were split into zones it became clear that within both bed spaces the sink area was the most contaminated, even excluding the floor near the sink, see Table 3-8. Within BS6 when zones were analysed using a t-test there was no significance difference between the sink zone with and without floor, but the sink zone (including floor) was significantly more contaminated than both the bed space and the near bed space ($p=0.03$ and $p=0.00003$) respectively. If samples for the floor by the sink were removed the sink zone itself was still significantly more contaminated than the near bed space ($p=0.002$). For BS7 there was a significant difference in the level of contamination between the sink zone and the sink zone excluding the floor ($p=0.003$). The sink zone excluding floor was significantly more contaminated than both the bed space and the near bed space ($p=3*10^{-5}$ and $p=1.4*10^{-9}$) In BS6 the median TVC

was 43 compared to 1 for the near patient area, and in BS7 the median for the sink zone was 43 with a median of 0 for the near patient zone. The ranges were still broadly even when surfaces were grouped with this zoning approach with the near bed zone for BS6 equalling 0 – 184 and for BS7 equalling 0 – 244. This indicates that although the near bed zone was generally less contaminated there were still sampling periods when contamination is high.

3.3.8 The Use of Total Viable Counts to Evaluate Contamination Levels

Two standards have been suggested in order to use TVCs to monitor contamination levels within the clinical environment. Griffith et al. (2009) have suggested a cut off of 2.5CFU/cm^2 on an agar contact plate (60CFU/plate).[400] Whereas Dancer (2004) has proposed a higher cut-off limit of 5CFU/cm^2 (120 CFU/plate), To evaluate the use of these suggested standards, TVCs from two bed spaces and the shared ward area were sampled (n = 720) on PICU and analysed using the proposed cut offs.

Throughout the PICU bed space sampling period only one bed space on one sampling occasion (BS7 period 2) had no TVCs over 59. Only on seven occasions out of 40 would either bed space have passed the Dancer criteria (BS6 periods 3, 4, 13, 18 and BS 7 3, 13, 15). For BS6 sampling period 9 demonstrated the highest levels of contamination with 8/12 sites failing using the Griffith standard, see Table 3-9. BS7 had three sampling periods where 6/12 sampling sites failed by the Griffith standard (9, 18, 20). In the shared ward area sampling, six sampling periods had contamination levels where 6/12 sampling sites failed by the Griffith standard (4, 5, 6, 9, 15, 17). All sites that failed by the Dancer criteria also failed by the Griffith criteria.

Within BS6, the floor under the sink and sink bowl had mean CFUs which failed the Dancer criteria. The chair arms, sink rim and keyboard had mean CFUs that failed the Griffith criteria. For BS7, all objects passed on mean CFUs according to the Dancer criteria. The floor under the sink, chair arms, sink bowl and sink rim all failed according to the Griffith criteria. Four objects within the shared ward area had means that would fail the Griffith criteria: the floor by BS7 and 10, medication board and the exit door panel, see Table 3-10.

Of the sites sampled within the shared ward area, 20 sites failed across the sampling period by the Dancer criteria, with an additional 30 failing by the Griffith criteria, see Table 3-11. BS6 had 29 sites that failed by the Dancer criteria with an additional 12

sites failing by the Griffith criteria and during the sampling of BS7, 23 sites failed by the Dancer criteria with an additional 21 sites failing by the Griffith criteria.

BS 6 Site sampled	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Average
1.Floor under sink	59	46	4	5	240	36	216	180	176	83	174	148	112	100	112	268	150	111	132	68	121
2. Clinical waste bin	11	116	57	64	69	100	23	2	96	24	4	0	17	62	117	18	33	29	11	64	46
3. Chair with arms	120	64	92	26	92	76	128	88	388	240	37	111	23	268	0	106	300	15	47	55	114
4. Bed rails	1	90	0	0	8	8	19	30	3	0	31	0	0	0	0	0	0	3	112	23	16
5. Suspended shelf	0	0	0	0	9	184	0	0	0	1	28	0	0	42	2	2	0	51	0	4	16
6. Mouse	22	11	4	0	28	112	13	6	168	4	99	0	6	33	30	13	2	37	2	3	30
7. Sink rim	55	35	72	106	47	1	60	53	86	45	134	16	26	46	66	0	31	3	130	284	65
8. Sink bowl	55	176	33	43	248	20	0	74	75	286	176	92	12	50	31	108	47	2	596	436	128
9. Trolley surface	0	0	0	0	34	160	0	0	0	1	1	0	0	0	0	0	0	70	0	0	13
10. Keyboard	53	1	6	11	27	5	92	13	445	40	106	0	98	56	126	84	106	5	10	3	64
11. Lamp	13	4	4	0	15	74	11	3	0	0	1	0	0	22	0	12	14	96	0	2	14
12. Soap dispenser	0	4	6	20	208	49	10	5	67	0	322	0	0	19	9	5	24	21	15	4	39
Average	32	46	23	23	85	69	48	38	125	60	93	31	25	58	41	51	59	37	88	79	56
No. of sites in BS that failed 60cfu standard	0	3	2	2	5	4	2	2	4	1	2	2	2	1	3	3	2	3	1	2	41
No. of sites in BS that failed 120cfu standard	1	1	0	0	3	2	2	1	4	2	4	1	0	1	1	1	1	0	3	2	29

Table 3-9 TVCs of colonies cultured using contact plates from the surfaces of BS6 over a 5 month period (20 sampling periods). Bed space failures are listed across the sampling period number with failures by the Dancer standard coloured red, and failure by the Griffith standard coloured orange, all other results for bed spaces are coloured green. The number of sampling sites within each sampling period that failed are listed and coloured orange if the TVC failed by the Dancer standard and red if the TVC failed by the Griffith standard. Individual sampling sites are coloured white if they are <60CFU, blue if TVC is between 60 and 119 and pink if >119CFU.

BS 7 Sites sampled	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Average
1.Floor under sink	296	59	55	121	130	0	102	180	65	74	100	240	112	136	116	132	47	91	106	66	111
2. Clinical waste bin	10	9	24	41	27	228	55	2	88	0	3	0	17	92	25	7	123	65	92	3	46
3. Chair with arms	65	34	42	43	106	37	21	88	39	0	210	28	23	45	24	30	54	86	110	126	61
4. Bed rails	7	23	2	0	0	10	2	30	2	4	0	244	0	70	0	20	2	75	77	5	29
5. Suspended shelf	0	1	0	0	0	1	0	0	121	0	0	0	0	0	0	0	3	0	0	0	6
6. Mouse	0	3	18	6	21	14	27	6	57	0	4	10	6	66	19	8	5	138	128	15	28
7. Sink rim	85	33	28	106	81	62	96	53	94	0	80	0	26	31	72	172	138	124	12	228	76
8. Sink bowl	126	10	117	150	61	68	320	74	40	84	76	94	12	107	42	204	58	0	73	135	93
9.Trolley surface	0	0	0	0	24	0	0	0	110	0	0	0	0	2	0	0	1	0	0	0	7
10. Keyboard	0	7	12	17	7	62	20	13	6	3	36	9	98	0	17	44	82	0	0	63	25
11. Lamp	0	7	0	0	30	0	5	3	0	0	13	0	0	0	0	1	0	8	0	2	3
12. Soap dispenser	2	6	5	0	23	43	0	5	122	222	3	21	0	15	10	0	0	7	7	2	25
Average	49	16	25	40	43	44	54	38	62	32	44	54	25	47	27	52	43	50	50	54	42
No. of sites in BS that failed 60cfu standard	2	0	1	1	3	3	2	2	6	2	3	1	2	4	2	0	1	4	5	2	44
No. of sites in BS that failed 120cfu standard	2	0	0	2	1	1	1	1	0	1	1	2	0	1	0	3	2	2	1	3	23

Table 3-10 TVCs of colonies cultured using contact plates from the surfaces of BS7 over a 5 month period (20 sampling periods). Bed space failures are listed across the sampling period number with failures by the Dancer standard coloured red, and failure by the Griffith standard coloured orange, all other results for bed spaces are coloured green. The number of sampling sites within each sampling period that failed are listed and coloured orange if the TVC failed by the Dancer standard and red if the TVC failed by the Griffith standard. Individual sampling sites are coloured white if they are <60CFU, blue if TVC is between 60 and 119 and pink if >119CFU.

Ward Sites sampled	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Average
1. Phones	27	55	89	208	65	36	22	72	65	17	23	23	43	53	56	21	102	20	90	3	55
2. Keyboard	43	27	17	38	23	100	52	31	88	30	34	78	93	45	128	8	21	19	24	37	47
3. Nurses station	57	17	6	61	88	76	45	79	39	4	35	3	3	48	68	0	192	52	22	43	47
4. Notes trolley	48	32	65	154	75	8	4	37	2	22	186	28	24	42	33	0	67	1	2	1	42
5. Floor outside BS 7	39	68	49	124	101	184	204	106	121	42	80	216	52	188	89	164	161	51	56	31	106
6. Floor outside BS 10	80	84	15	137	83	112	53	51	57	226	154	152	28	0	83	29	0	52	60	168	81
7. Medication board	181	144	134	32	175	1	0	66	94	22	108	39	96	0	18	44	0	176	4	18	68
8. Utility door handle	83	0	8	21	5	20	2	39	40	5	6	7	17	308	73	48	13	8	4	61	38
9. Desk	50	176	20	44	39	160	101	25	110	4	34	23	42	59	0	0	62	41	60	49	55
10. Crash trolley	7	21	4	4	0	5	3	28	6	0	9	0	2	47	0	0	5	0	6	0	7
11. Exit doors	98	41	21	47	17	74	32	56	0	63	48	19	240	190	0	300	108	84	52	94	79
12. Exit door button	35	17	75	87	15	49	97	34	122	15	81	53	69	75	140	72	31	20	13	33	57
Average	62	57	42	80	57	69	51	52	62	38	67	53	59	88	57	57	64	44	33	45	57
No. of sites in ward that failed 60cfu standard	3	2	3	3	5	4	2	4	4	1	3	1	3	1	4	1	4	1	3	2	50
No. of sites in ward that failed 120cfu standard	1	2	1	3	1	2	1	0	2	1	2	2	1	3	2	2	2	1	0	1	30

Table 3-11 TVCs of colonies cultured using contact plates from the surfaces of shared ward space over a 5 month period (20 sampling periods). Failures of the shared ward area are listed across the sampling period number with failures by the Dancer standard coloured red, and failure by the Griffith standard coloured orange, all other results for bed spaces are coloured green. The number of sampling sites within each sampling period that failed are listed and coloured orange if the TVC failed by the Dancer standard and red if the TVC failed by the Griffith standard. Individual sampling sites are coloured white if they are <60CFU, blue if TVC is between 60 and 119 and pink if >119CFU.

As the analysis of the PICU data using the Griffith/Dancer criteria suggested numerous failures of both sites and bed spaces; additional data from the pilot study (MITU and SITU (n = 426), and two paediatric outpatient clinics (n = 148) were analysed. A total of 1174 samples were taken. See Table 3-12 below.

Location	Bed Space Failures at 120CFU Standard	Bed Space Failures at 60CFU Standard	Sampling Site Failures at 120CFU Standard	Sampling Site Failures at 60CFU Standard
PICU bed space	31 (78%) (n = 40)	39 (98%) (n = 40)	52 (11%) (n = 480)	137 (28%) (n = 480)
PICU shared ward area	N/A	N/A	30 (13%) (n = 240)	80 (33%) (n = 240)
Outpatients	N/A	N/A	29 (20%) (n = 148)	62 (42%) (n = 148)
MITU bed space	10 (83%) (n = 12)	12 (100%) (n = 12)	38 (33%) (n = 114)	51 (45%) (n = 114)
MITU shared ward area	N/A	N/A	5 (11%) (n = 45)	13 (29%) (n = 45)
SITU bed space	16 (70%) (n = 25)	20 (86%) (n = 25)	52 (27%) (n = 192)	82 (43%) (n = 192)
SITU shared ward area	N/A	N/A	9 (20%) (n = 75)	24 (32%) (n = 75)
Total	57 (74%) (n = 77)	71 (92%) (n = 77)	201 (17%) (n = 1174)	512 (44%) (n = 1174)

Table 3-12 Summary of ward and outpatient TVC screening results using 60CFU and 120CFU suggested standards listed by site or bed space.

Results shown in Table 3-12 indicate that when using the suggested standard of 2.5CFU/cm^2 (60CFU), 92% of bed spaces and 44% of sites sampled failed screening. Using the suggested standard of 5CFU/cm^2 (120CFU), over half the bed spaces (74%) would have required the bed space to be closed and re-cleaned and 17% of sites would have failed. Results were broadly similar across ward types, with a range of 11 – 27% failing when using the 120CFU standard and 28 – 45% with the 60CFU standard. Colony counts for bed spaces were similar for the 60CFU standard with a range of 86 – 100% failing screening. Using the 120CFU standard, the range was similar for bed spaces with between 70 – 83% failing screening.

3.3.9 Microbiological Surface Sampling of the Environment for Specific Organisms

Due to the large number of site failures when judging contamination using proposed TVC standards, analysis of environmental surveillance data was undertaken for MRSA, Enterobacteriaceae, adenovirus and norovirus as an alternative strategy for assessing contamination.

3.3.9.1 MRSA Bacterial Surveillance Screening

Over a three month period at the NHNN 381 sites were sampled using enrichment swabs (see methods section 2.3.4.3.) of these three samples were positive (0.8%). The positive sites included the storage unit top and bed wheels within an isolation bed space and the apron dispenser in the neighbouring empty bed space post clean.

A one off sampling for MRSA that was undertaken at GOSH involved taking samples from 20 sites on the IIU for MRSA in a pre-clean cubicle used for MRSA isolation. Of the 20 samples taken for enrichment swabs, two were positive (10%). For direct plating 20 samples were taken and one was positive (5%).

During an intensive screen of haematology/oncology day unit (HODU) 90 samples swab were taken, these were plated by direct culture as described in methods section 2.3.4.4. Of the 90 samples taken three were positive for MRSA (3%), all of which were taken from a room currently being used to isolate an MRSA positive patient, 3/5 (60%).

3.3.9.2 Enterobacteriaceae Bacterial Surveillance Screening of Paediatric Intensive Care Unit at Great Ormond Street Hospital

Screening of two bed spaces (BS6 and BS7) and the shared ward area on PICU at GOSH was undertaken over a four month period using enrichment screening, samples were taken using MicroSnap swabs (Hygiena International, Watford, UK) (as outlined in the method section 2.3.2.4.) in order to determine the levels of Enterobacteriaceae present.

Over the sampling period a number of Enterobacteriaceae positive samples were detected. 432 Samples were taken over the sampling period of which eleven sites were positive (3%). Species cultured and identified included six *Pantoea* species, two *Klebsiella* species and three *Enterobacter* species (see methods section 2.3.2.5.). Sites detected as positive included the floors by the sink, trolley surface and suspended shelf surface within the bed space, and the nurses' station, phone and crash trolley within the shared ward area.

Table 3-13 shows the mean relative light unit (RLU) counts that correspond to the presence of coliforms for each object tested; an RLU of 10 is considered a positive result.

Site Sampled		**BS6 (n = 144)**	**BS7 (n = 144)**
Floor under the sink	Mean RLU	45.85	48.31
	Median RLU	4	6
	(RLU Range)	(0-256)	(0-349)
Clinical waste bin	Mean RLU	2.23	2.39
	Median RLU	1	2
	(RLU Range)	(<10)	(<10)
Chair arms	Mean RLU	62.46	2.46
	Median RLU	2	2
	(RLU Range)	(0-585)	(<10)
Bed rails	Mean RLU	2.23	17.46
	Median RLU	1	2
	(RLU Range)	(<10)	(0-150)
Suspended shelf	Mean RLU	93.85	2.62
	Median RLU	3	1
	(RLU Range)	(0-1157)	(<10)
Computer mouse	Mean RLU	4.31	12.23
	Median RLU	3	2
	(RLU Range)	(0-25)	(0-125)
Sink rim	Mean RLU	2.77	5.08
	Median RLU	3	3
	(RLU Range)	(<10)	(0-24)
Sink bowl	Mean RLU	15.38	35.23
	Median RLU	5	4
	(RLU Range)	(0-156)	(0-403)
Trolley surface	Mean RLU	4.46	2.77
	Median RLU	2	2
	(RLU Range)	(0-21)	(<10)
Keyboard	Mean RLU	16.08	4.92
	Median RLU	3	3
	(RLU Range)	(0-171)	(0-20)

Lamp	Mean RLU	3.78	34.54
	Median RLU	3	3
	(RLU Range))	(0-11)	(0-398)
Soap dispenser	Mean RLU	4	5.15
	Median RLU	3	4
	(RLU Range)	(<10)	(0-20)

Table 3-13 Mean and range of RLU counts over the five month sampling period on PICU. Enterobacteriecae presence detected using MicroSnap swabs. RLUs >9 were taken as positive indicators for the presence of Enterobacteriecae.

The sink bowl and floor underneath the sink were identified as being sites that were likely to harbour Enterobacteriaceae in both bed spaces. Other objects had variable Enterobacteriaceae detection varying between bed spaces and over time. The clinical waste bins in both bed spaces never demonstrated positive numbers of RLUs.

On the majority of occasions it was not possible to identify the species of Enterobacteriaceae from the brain heart infusion (BHI). Frequently organisms cultured were other potential Gram-negative pathogens such as *Pseudomonas aeruginosa*, or other environmental organisms such as *Stenotrophomonas* spp. BHI is a non-selective broth and these may represent overgrowth of other bacteria.

Site Sampled	TVC	RLU	Species Cultured
Floor under sink	176	10	
Keyboard	45	10	
Floor outside BS7	188	10	
Lamp	0	11	
Nurses station	48	11	
Floor outside BS10	28	11	
Bed rails	70	12	
Floor under sink	136	13	
Floor outside BS10	0	13	
Nurses station	3	14	
Floor under sink	240	15	
Lamp	0	15	
Nurses station	192	15	
Exit doors	56	16	
Keyboard	128	16	
Floor outside BS7	89	16	

Floor under sink	150	16	*Klebsiella species*
Sink bowl	107	19	
Soap dispenser	10	20	
Medication board	108	20	
Sink rim	72	24	
Floor under sink	90	27	
Crash trolley	9	30	
Suspended shelf surface	2	37	
Exit doors	0	43	*Klebsiella species*
Floor under sink	112	58	
Phones	43	60	
Exit doors	108	61	
Medication board	66	80	
Chair with arms	106	96	
Mouse	19	125	
Exit doors	0	144	*Enterobacter cloacae*
Bed rails	0	150	
Sink bowl	12	156	
Floor under sink	180	186	*Pantoea* species
Floor under sink	116	349	
Sink bowl	94	403	
Suspended shelf surface	42	1157	*Enterobacter cloacae*

Table 3-14 Enterobacteriecae detection using MicroSnap swabs and the Enterobacteriaceae cultured from the broth over the five month sampling period on PICU, compared to the numbers of CFUs detected from TVCs collected at the same time from the same site. TVCs failing the Dancer standard are coloured red, and failures by the Griffith standard are coloured orange, all other results are green. RLUs >9 indicate the presence of Enterobacteriaceae within the sample.

Sampling was undertaken using MicroSnap swabs (Hygiena International, Watford, UK) and TVCs taken in parallel. The TVCs taken in parallel vary from 0 – 240, with most being under 60CFUs (see Table 3-14.). Statistical correlation tests were performed to determine whether there was a correlation between levels of contamination (in TVCs) and the levels of Enterobacteriaceae as detected in RLU's no significant relationship was identified. This indicates that the number of organisms found on a surface using TVCs cannot be correlated to levels of Enterobacteriaceae on

the same surface. Low TVCs can therefore be linked to the detection of high RLUs on the same surface taken at the same time.

3.3.9.3 PAEDIATRIC INTENSIVE CARE UNIT/CARDIAC INTENSIVE CARE UNIT/ NEONATAL INTENSIVE CARE UNIT SINK SCREENING

In addition to the bed space and ward sampling undertaken on PICU at GOSH, sink sampling was undertaken over three ITUs at GOSH (PICU, cardiac intensive care unit (CICU) and neonatal intensive care unit (NICU) Sinks were selected as potential sentinel sites due to their association with high levels of TVCs, RLUs, and indicator organism isolation. Microbial samples were collected from eight sinks spread over three months at GOSH as described in methods section 2.3.4.2.). The samples underwent direct plating and the degree of contamination was calculated and classified according to a scale of 0 (no growth) to 3 (greatest). Enterobacteriaceae were detected at all sites, but particularly high levels of contamination were detected on the lips of sinks (see Figure 3-6).

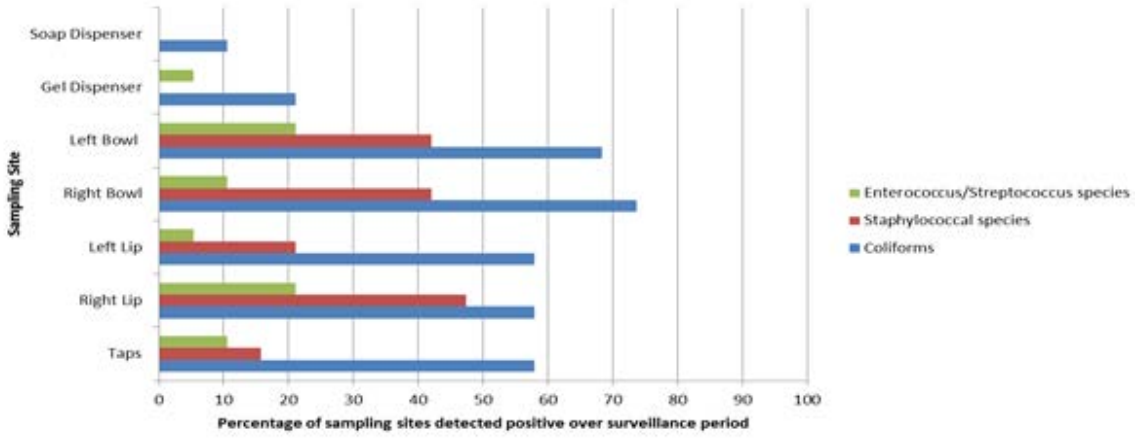

Figure 3-6 Percentage of sampling occasions where organism were cultured from sampling locations within three ITUs and GOSH over a three month period.

3.3.9.4 VIROLOGICAL SURVEILLANCE SCREENING

The environment in communal ward areas on a IIU and hematopoietic stem cell transplantation unit (HSCTU) was screened for adenovirus and norovirus using real-time PCR as described in methods sections 2.2.5.3 and 2.2.5.4. Screening was also undertaken within the cubicles of adenovirus positive patients during routine occupation on the IIU. Screening of these two areas sought to establish the risk of viral escape from cubicles where patients were isolated in order to be able to risk assess this transmission route.

Hematopoietic Stem Cell Transplantation Unit and Immunology/Infectious Disease Units

Over a 6 month period 539 samples were taken on the HSCTU and IIU as described in methods section 2.3.1.2. of which 13 (2.4%) were positive (Table 3-15). Ten positive samples were found on HSCTU and three on IIU; four were positive for adenovirus and nine for norovirus GII. All positive norovirus samples were found on HSCTU.

Ten of the positive samples were floor swabs with six recovered from the floor by the main ward exit doors. Other sites with multiple positive samples were the floor outside a known negative and known positive patient's room. Positive samples were also recovered from a filing cabinet, sluice door handle and notes trolley.

Cubicles

Screening of cubicles occupied by adenovirus positive patients before post-discharge cleaning was performed demonstrated that significant levels of contamination occur within these bed spaces (Table 3-15). A total of 36 samples were taken within IIU cubicles occupied by adenovirus positive patients before post-discharge cleaning, of these 33 were detected as being positive (92%). All locations were positive for adenovirus on at least one occasion (Table 3-15). The floor under the sink, patient bathroom taps and corridor floor outside of the cubicle entrance were positive on two out of three samples rather than three out of three. Importantly, the exit door handle from the cubicles was always contaminated.

Haematology/Oncology Day Unit

Ninety samples were taken on one day on HODU. Eleven were adenovirus positive (12%) and one was positive for norovirus GI (1%) (Table 3-15). The norovirus positive site was the clinical waste bin lid within an isolation cubicle. Adenovirus positive sites included the surfaces of toys (dolls house, teddy bear and toy bricks), two chair arms, two exit door handles, one fan, two clinical surfaces (a trolley and an intravenous bench (IV) bench) and a clinical waste bin. All but one of the positive samples were recovered from bays and rooms where patients are weighed or treated. Locations with multiple positive samples were bays 2 and 3 and treatment room 1.

HSCTU and IIU				
No.	**Location**		**Adenovirus**	**Norovirus**
1	Floor outside known	HSCTU	0 *(n = 25)*	1 *(n = 25)*

	negative patient room	IIU	1 *(n = 24)*	0 *(n = 24)*
2	Filing cabinet	HSCTU	0 *(n = 25)*	1 *(n = 25)*
		IIU	0 *(n = 24)*	0 *(n = 24)*
3	Floor outside known positive patient room	HSCTU	0 *(n = 25)*	1 *(n = 25)*
		IIU	1 *(n = 24)*	0 *(n = 24)*
4	Nurse's station	HSCTU	0 *(n = 25)*	0 *(n = 25)*
		IIU	0 *(n = 24)*	0 *(n = 24)*
5	Sluice/medication room door handle	HSCTU	0 *(n = 25)*	1 *(n = 25)*
		IIU	0 *(n = 24)*	0 *(n = 24)*
6	Floor by main exit doors	HSCTU	1 *(n = 25)*	4 *(n = 25)*
		IIU	1 *(n = 24)*	0 *(n = 24)*
7	Notes trolley	HSCTU	0 *(n = 25)*	1 *(n = 25)*
		IIU	0 *(n = 24)*	0 *(n = 24)*
8	PC keyboards	HSCTU	0 *(n = 25)*	0 *(n = 25)*
		IIU	0 *(n = 24)*	0 *(n = 24)*
9	Telephone on nurse's station	HSCTU	0 *(n = 25)*	0 *(n = 25)*
		IIU	0 *(n = 24)*	0 *(n = 24)*
10	Ward exit door handle	HSCTU	0 *(n = 25)*	0 *(n = 25)*
		IIU	0 *(n = 24)*	3 *(n = 24)*
11	Chair arm/crash trolley	HSCTU	0 *(n = 25)*	0 *(n = 25)*
		IIU	0 *(n = 24)*	0 *(n = 24)*

Adenovirus Positive Patient Cubicle (Pre-clean)			
No.	**Location**	**Adenovirus**	**Norovirus**
1	Floor under sink	2 *(n = 3)*	Not done
2	Clinical waste bin inner rim (under lid)	3 *(n = 3)*	
3	Chair with arms (right)	3 *(n = 3)*	
4	Door handle into patient bathroom (cubicle side)	3 *(n = 3)*	
5	Telephone keypads	3 *(n = 3)*	
6	Taps in patient bathroom	2 *(n = 3)*	
7	Mattress top	3 *(n = 3)*	
8	Bed/Cot frame under bed	3 *(n = 3)*	
8	Trolley surface (in ante room if present)	3 *(n = 3)*	
10	Side window sill (right hand side)	3 *(n = 3)*	
11	Cubicle room exit door handle (cubicle side)	3 *(n = 3)*	

12	Corridor floor outside of cubicle/ante room entrance	2 *(n = 3)*	
HODU			
No.	**Location**	**Adenovirus**	**Norovirus**
1	Day procedure bay	0 *(n = 5)*	0 *(n = 5)*
2	Recovery bay	0 *(n = 6)*	0 *(n = 6)*
3	Procedure room	0 *(n = 5)*	0 *(n = 5)*
4	Assisted toilet 1	0 *(n = 2)*	0 *(n = 2)*
5	Assisted toilet 2	0 *(n = 3)*	0 *(n = 3)*
6	En-suite cubicle	0 *(n = 5)*	0 *(n = 5)*
7	Bay 1	1 *(n = 5)*	0 *(n = 5)*
8	Corridor	1 *(n = 3)*	0 *(n = 3)*
9	Bay 2	2 *(n = 5)*	0 *(n = 5)*
10	Bay 3	2 *(n = 5)*	0 *(n = 5)*
11	Height and weight room	1 *(n = 6)*	0 *(n = 6)*
12	Reception	0 *(n = 5)*	0 *(n = 5)*
13	Reception seating	0 *(n = 5)*	0 *(n = 5)*
14	Cubicle 2	0 *(n = 5)*	0 *(n = 5)*
15	Cubicle 2 (pre-clean)	0 *(n = 5)*	1 *(n = 5)*
16	Treatment room 1	2 *(n = 5)*	0 *(n = 5)*
17	Treatment room 2	1 *(n = 5)*	0 *(n = 5)*
18	Treatment room 3	0 *(n = 5)*	0 *(n = 5)*
19	Treatment room 4	1 *(n = 5)*	0 *(n = 5)*

Table 3-15 Number of positive samples within the IIU, HSCTU, HODU found for adenovirus and norovirus for each sampling strategy. (n = total number of sampling points)

3.3.9.5 TOTAL VIABLE COUNTS IN RELATION TO INDICATOR ORGANISMS

A number of potentially pathogenic organisms were isolated from the environment during the sampling undertaken. Where possible these indicator organisms were compared to TVCs taken at the same sampling site at the same time.

For sampling undertaken on the HODU, of the 16 sites that were positive for indicator organisms, none would have failed using the Dancer criteria for TVCs. Of the sites positive for indicator organisms, 4/16 would have failed if using the Griffith criteria for environmental monitoring (see Table 3-16.). For the 16 sites sampled, 3/16 sites were positive for pathogenic bacteria by culture, and 1/16 was positive for

Enterobacteriaceae by PCR (see methods sections 2.2.5.2, 2.2.5.3, 2.2.5.4 and 2.3.2.3. The other 12 sites were PCR positive for viruses.

Site Name	TVC	Norovirus (PCR)	Adenovirus (PCR)	Ent DNA (PCR)	MRSA (Culture)
Fan	16				Pos
Bed rail	6				Pos
Trolley	59				Pos
Toy	41		Pos		
Chair arm	81		Pos		
Chair arm	55		Pos		
Trolley	1		Pos		
Dolls house	14		Pos		
Fan	3		Pos		
Exit door handle	119		Pos		
Clinical waste bin	2			Pos	
Clinical waste bin	38	Pos			
Teddy	73		Pos		
Exit door handle	17		Pos		
Clinical waste bin	94		Pos		
Bench	0		Pos		

Table 3-16 Sampling sites positive on the HODU for norovirus, adenovirus Entobacteriaceae or MRSA compared against CFUs present on TVC samples taken from the same surface at the same time analysed by the Griffith criteria. Orange colour coded sites failed by the Griffith criteria (>59CFUs) green colour coded sites were <60CFU and therefore were determined to pass the Griffith criteria.

During sampling undertaken for Enterobacteriaceae on PICU, 11 sites were detected as positive, six for *Pantoea* species, three for *Enterobacter* species and two for *Klebsiella* species (see Table 3-17.). Of the 11 sites positive, four would have failed to meet the Dancer criteria and five the Griffith criteria. Six of the 11 positive sites were negative for a positive RLU result, only two of which overlapped with TVC failures using either standard. The sites that failed most frequently were the floor under the sink and the exit door panel.

Site Sampled	TVC	RLU	Species Cultured
Crash trolley	6	0	*Pantoea* species
Exit doors	0	144	*Enterobacter cloacae*
Exit doors	0	43	*Klebsiella* species
Floor under sink	148	4	*Pantoea* species
Floor under sink	150	16	*Klebsiella* species
Floor under sink	180	186	*Pantoea* species
Floor under sink	188	4	*Enterobacter* species
Nurses station	39	3	*Pantoea* species
Phones	65	4	*Pantoea* species
Suspended shelf surface	42	1157	*Enterobacter cloacae*
Trolley surface	0	0	*Pantoea* species

Table 3-17 Sampling sites positive on GOSH PICU for Entobacteriaceae species compared against CFUs present on TVC samples and RLUs detected from MicroSnap swabs taken from the same surface at the same time analysed by either RLU or the Griffith and Griffith criteria. Orange colour coded sites failed by the Griffith criteria (>59CFUs) , red colour coded sites failed by the Dancer criteria (>119CFUs) green colour coded sites were <60CFU and therefore were determined to pass the Griffith criteria. Blue colour coded sites are those not positive by RLU for Enterobacteriaceae and yellow colour coded sites are those positive for Enterobacteriaceae by RLU (>9 RLUs).

3.4 DISCUSSION

3.4.1 ENVIRONMENTAL SAMPLING METHODOLOGY

Method Selection for Environmental Sampling

The sampling methodology developed for this work took into account the sampling methods evaluated in previous studies.[174, 197] Cotton swabs for molecular based testing were selected for sampling. Although they may have reduced sensitivity compared to flocked nylon swabs they can be broken into test vials due the shaft of the swabs being wooden rather than plastic.[174, 199, 201-204] Cotton swabs were also selected for culture based testing as these enable comparison with molecular testing and were the standard type validated within GOSH, with the sample stabilised within charcoal transport medium (see Appendix 1).

A sampling size of 10cm^2 was utilised as this is a common size in the relevant literature and was determined to be of sufficient size to detect organisms within the environment as long as sufficient sites were sampled.[401, 402] Sampling surfaces larger than this were found to dry out the swab and therefore negatively affect sampling efficiency. Due to the increased detection offered by pre-moistening, swabs were wetted with molecular grade water before surface sampling was undertaken.[209]

Experimental validation of the direct inoculation protocol demonstrated that using sterile water as the moistening and sampling reagent the recovery varied between 87.08% - 92.64% (see section 3.3.1.). This is higher than demonstrated in some of the published comparisons but other variables such as humidity, temperature, recovery surface, and inoculation technique were not comparable and were likely to affect recovery percentage.[174-176] The bacterial inoculum was higher in this study than that used in some of the published work (10^8 – 10^{11} vs 10^3 – 10^6) and this has been shown to affect recovery.[174] In addition, we tested a greater variety of organisms and the strain types were not identical to those tested in the literature. This has been shown by other studies to affect survival and therefore potentially recovery.[403, 404] The presence of protein improved recovery of organisms from the surface and appeared similar if BSA or PBS was used (see section 3.3.1.). The reasons for this are unclear but may due to enhanced bacterial survival, perhaps through prevention of desiccation.

Comparison of direct inoculation, enrichment and selective contact plates within the clinical environment in a pre-cleaned isolation cubicle at GOSH demonstrated a 10% MRSA positivity rate using enrichment, 5% using direct plating and 0% using selective

agar contact plates. Within the clinical environment at NHNN, 2% of sites were positive by enrichment with no other sampling methods detecting MRSA (see section 3.3.5.1.).

After the pilot phase of the study, direct inoculation of swabs onto plates was undertaken of samples where organisms were believed to be of sufficient levels to be detected and a level of crude quantification was required (level 1, 2 or 3 depending on how far the inoculum grew on the plate). Where estimates of levels were lower, enrichment was used in order to increase test sensitivity by up to 18.7%.[210]

In this study we used non-selective contact plates to quantify bacterial contamination on surfaces in order to capture all viable bacterial types. However issues (specifically in terms of quantification) were observed when sampling was undertaken of moist surfaces or heavily contaminated ones.[405] Application of contact plates to the surface in this study was undertaken for 10 seconds. While some studies have increased the length of this time, this proves impractical when sampling within a clinical environment where large numbers of samples are required.[406] Many articles within the literature do not state contact time between the contact plate and the surface, but when listed, contact times vary from 5 seconds – 60 seconds.

3.4.2 INOCULATION AND SURVIVAL MODEL

Opinion used to be that environmental survival of organisms was short term with Gram-negative bacilli believed to rapidly disappear from dry surfaces and the half-life of *Staphylococcus aureus* being less than 24 hours.[407] Although these survival times have been adjusted in recent years, there is still a failure to form a consensus related to whether Gram-positive or Gram-negative bacteria demonstrate longer environmental survival. Galvin et al. (2012) found that Gram-positive bacteria such as *Staphylococcus aureus* and *Enterococcal* spp. survive for longer in dry environments than Gram-negative bacteria, with the exception of *Acinetobacter baumannii*.[190] In comparison Kramer et al.(2006) found that Gram-negative bacteria survive in the environment for longer than Gram-positive bacteria, with the exception of *Staphylococcus aureus* at low humidity.[175]

Within the literature, *Klebsiella* spp. have been shown to survive anywhere from two hours – thirty months, *Staphylococcus aureus* from seven days – seven months and adenovirus from seven days – three months.[175]

The survival experiments undertaken in this study have demonstrated that viable *Staphylococcus aureus*, *Klebsiella pneumoniae* and adenovirus can be recovered from

non-porous dry environments after being kept for three months at mean humidity of 43% and 16^{o}C temperature, although the $\log_{10}$ reduction from the initial inoculum was substantial, 7.14 of *Staphylococcus aureus*, and 7.72 for *Klebsiella pneumoniae* (see section 3.3.2.). Survival of microorganisms on surfaces is dependent upon the bacterium/viral species, inoculum size, humidity, temperature, suspension medium and the material tested.[174-176] Variations in these factors may explain the wide range of results found when examining organism environmental survival. Surface type used in survival experiments can have a significant impact as viruses can be trapped within the matrix especially if the surface is porous. Other studies have demonstrated that even if the same type of test material is used, other variations in conditions cause the material to reveal inconsistent results.[175] For this reason, we always inoculated our experiments in an identical manner with distribution of organisms on the surface being kept consistent along with surface type, humidity, temperature, suspension medium, strain type and as far as possible inoculum level. This enabled results across multiple experiments to be compared and the results demonstrated consistency. These results are described in more detail in Chapter 5.

One of the potential short comings of the survival and inoculum experiments undertaken is that the same type strains and species were used throughout. This was done to enable across experiment comparison; however, in addition to variation between species under experimental conditions, work has shown that there is variation in survival within species. Survival of all *Staphylococcus aureus* strains is not identical under the same survival conditions.[404] The survival of biofilm forming strains of *Acinetobacter baumannii* has been shown to be longer than that for non-biofilm producing strains, 36 vs 15 days.[403] There is also some evidence that resistant strains may survive longer than sensitive ones. Outbreak strains of MRSA survived longer on surfaces than non-outbreak strains and isolates of *Pseudomonas aeruginosa* collected from hospital surfaces were more likely than isolates from clinical specimens to be β-lactamase producers, 95% vs 36%.[190] It is therefore possible that experimental results captured will only reflect the strain type tested rather than the entire species. Additionally, growth of viruses in cell culture for survival experiments means that they may not always represent the clinical situation.[175]

Although the levels of viable bacteria recovered after the survival experiments was low, the longer an organism persists on a surface, the longer it can present a risk within the healthcare environment. Even if environmental survival is poor, a low infectious dose can mean that viruses/bacteria persist in sufficient numbers to be infectious for several

days, weeks or in some cases months after contamination.[179, 408] The results of the survival experiments demonstrate that, within conditions that can be found in clinical environments, *Staphylococcus aureus*, *Klebsiella pneumoniae* and adenovirus could survive for >3 months and therefore would present a potential risk to patients and staff.

3.4.3 Application of Sampling Methodology to Clinical Settings

Surface Type

When analysing data on surfaces for the pilot study it was decided to use bedrail contamination as the reference standard for use in a GAM (see section 3.3.6.). The bed rails were selected as the reference standard due to published data demonstrating that bed rails are linked with the highest levels of contamination within bed spaces.[184, 408-410] Compared to bed rails, bed wheels were the most contaminated objects sampled with a mean TVC 1.97 times higher than on the bed rail. Bedside alcohol gel dispensers were 0.27 times less contaminated than bed rails. The bedside table was less contaminated than the bed rail, although in previous studies these have been linked with similar levels of contamination.[405, 411] Both the storage trolley and the top of the storage unit were also less contaminated than bed rails. Within sampling undertaken at GOSH, bed rails were found to demonstrate relatively low levels of contamination within the ITU with mean of 16CFU/24cm^2 for bed space 6 and 6CFU/24cm^2 for bed space 7. A bed rail was detected as positive for MRSA during the screening of the HODU in a cubicle utilised to isolate MRSA patients prior to cleaning. This was associated with a TVC count of 6CFU/24cm^2 from the same surface.

Some investigators have reported that material has no influence on survival whilst others have described longer survival on plastic or steel.[88] Higher survival of viruses has been noted on non-porous material for most viruses with the exception of adenovirus; which has demonstrated higher persistence on porous material such as paper towels.[161] Other authors have found differences in survival between Gram-negative bacteria, with isolates surviving longer on synthetic plastics compared to cotton and terry fabrics, despite the later demonstrating increased surface area.[176, 412] Despite this, when a comparison of TVCs between porous and non-porous materials was undertaken it showed no significant differences (see section 3.3.6.). Therefore surface type alone does not explain the differences in levels of detection of TVCs between objects.

Surface Height

Within the study undertaken at NHNN surface types were divided into high >1.2m, medium 0.6m-1.2m and low <0.6m zones to see if that affected TVCs. Results varied between wards and sampling occasions, but for the SITU the lower height levels was most contaminated on all sampling occasions (see section 3.3.6.). This finding is similar to that of Best et al. (2014) who demonstrated those locations close to and including the floor remained the least clean.[413] This may be may be as a result of cleaners focussing on more frequently touched middle and high site areas, but may also be because lower sites, such as bed wheels, are more difficult to clean. Within MITU the lowest height level was the most contaminated on one sampling period, but for the other two the highest height level was the most contaminated. It therefore appears that surface height has more of an impact than surface material in determining contamination, with the floors and items in contact with them being particularly contaminated.

Floors are known to become rapidly contaminated with pathogens in the hospital environment.[183] In our work floors were consistently highly contaminated. The floors represented the site where most Gram-negative organisms were detected outside of the sink during surveillance on the PICU at GOSH. The floor by the sink grew *Pantoea* spp. *Klebsiella* spp. and *Enterobacter* spp. from samples, all of which were associated with high TVCs. The floor was also the most commonly positive site for detection of norovirus and adenovirus. Furthermore, the highest contamination levels occurred on bed wheels and objects at the lowest height level, indicating that these surfaces are hard to reach during cleaning procedures or that they are the objects that get most rapidly re-contaminated (see section 3.3.6.).

Despite this, some studies have dismissed floor contamination as not high risk as most adult patients will have limited contact with the floor.[328, 414] Some authors have even called for a reduction in focus on floor cleaning as there is little evidence between floor cleaning and reduction in HCAI rates, calling on a focus of cleaning high touch sites.[191, 192, 409, 415] Within the paediatric setting, children outside of the ITUs will interact with the floor on a regular basis as many will play with toys and will be learning to walk/crawl.[12] Even within the adult setting, conceptually the passage of trolleys and other equipment over the floor to patient's cubicles may transfer the viruses/bacteria into a new environment. Staff and visitors place objects such as bags and boxes onto floors; this provides a vehicle for the transfer of microorganisms from the floor to other

surfaces.[416] Given the low infectious dose of viruses/bacteria such as norovirus and *E. coli* and the potential for ready transfer between surfaces, it is therefore possible that this contamination represents a real risk of infection transmission.[415]

The medium height level (0.6m-1.2m) item that was most associated with high levels of contamination were the door handles. Door handles have been found to be a source of contamination within the clinical environment.[417] During cubicle screening of rooms where patients were isolated for adenovirus, exit door handles were universally contaminated (see section 3.3.9.4.). Therefore even if current infection control guidance in terms of personal protective equipment (PPE) were adhered to, staff and visitors hands would be contaminated on exit of the room and provide a route of contamination to the shared ward area or other patient cubicles.[296] The exit door panel from the shared ward area on PICU has a mean CFU over the sampling period that would fail by the Griffith standard, with 9/20 individual sampling point having over 60CFUs.[399] The exit doors also had raised RLUs on four occasions, one of which was associated with growth of *Enterobacter cloacae* and once for *Klebsiella* spp (see section 3.3.9.2.). The sluice room door handle on the HSCTU was positive for norovirus on 1/25 occasions and the ward exit door handle on the IIU was positive for norovirus 3/24. Touching of door handles could provide a means of hand contamination and transfer both within and between clinical areas, this is explored further in Chapter 5.[417]

Objects Linked to Frequent Interactions

Within the shared ward areas the item that has been identified in previous studies as being the most likely to be contaminated were the computer keyboards.[184, 418, 419] High level contamination level of keyboards was not seen during sampling undertaken at the NHNN, probably because of ease of access for cleaning as they were in the shared ward areas and because they use wipable keyboards on the unit.[420] The results from the NHNN are similar to those found in a study by Dancer et al. (2006).[409] Computer keyboards at GOSH in clinical areas are also wipable and, within the PICU, demonstrated variability between bed space 6 and 7. BS7 had a lower mean and median than BS6. The two bed spaces are identical in terms of design and patient mix. The keyboards within the bed space are mainly accessed by the nurses covering shift and so contamination is unlikely to be due to visitors. It is therefore likely that variations in contamination are linked to the variations in practice by nursing staff.

Keyboards were not linked to detection of any potential indicator organisms throughout the sampling period, despite the higher TVCs detected on keyboards in BS7.

Toys are potentially high risk items that are unique to paediatrics and therefore were not studied outside of GOSH. Within the HODU, three toys were positive for adenovirus out of the ninety surfaces sampled, a dolls house, teddy bear and some toy bricks (see section 3.3.9.4.). No toys were sampled within the other areas as within the clinical ward environment toys are linked to particular patients and are not shared. The presence of the teddy bear breached the hospital toy policy as it was a soft toy and not cleanable. The other two toys conformed with hospital guidance. Teddy bears have been shown to be contaminated within one week of being present in a hospital ITU with bacteria and fungi, such as *Klebsiella* spp., *E. coli*, *Staphylococcus aureus* and *Candida* spp.[421] In other studies, soft toys have been shown to be contaminated between 90% - 92% of the time, both in general practitioners waiting areas and in NICUs.[422, 423]

Even hard toys have been demonstrated as having a 13.5% level of contamination within the general practitioner setting. Hard toys can be cleaned and decontaminated, but in our study the doll's house was unvarnished which made decontamination difficult as the surface was porous.[422] Toys may therefore present a risk as, by their very nature they come into close contact with patients.[12] If patients bring their own toys to hospital, they are likely to become contaminated and may present a recontamination risk to the local environment, to the patient and home environment.[422]

Within all the studies undertaken on the GOSH ITUs, sinks were not only associated with high levels of TVCs, indicating general bacterial contamination, but also detection of specific pathogens, both within and around the sink. Enterobacteriaceae including *Klebsiella* spp. and *E. coli* were particularly prevalent on the sink lips (see section 3.3.9.3.). Several studies have found that Gram-negative contamination centres on sinks and moist areas such as baths.[419, 424-427] In a children's hospital many of the patients are in nappies and it is to be expected that faecal flora will be prevalent, including in sink bowls, due to hand washing post nappy changing. However the presence of Enterobacteriaceae in and around sinks presents a potential clinical risk.

Air Sampling vs Surface Sampling

Air sampling was undertaken at the NHNN on three occasions over three months on two units (SITU, MITU) that had a mixed ventilation system (see section 3.3.4.). Air

sampling was not undertaken after the pilot study undertaken at the NHNN as the air data would be more difficult to interpret at GOSH, where the ITUs, IIU and HSCTU have HEPA filtrated air. The differences in ventilation design between these units would have prevented cross comparison between units and affected data interpretation.

The results of the investigation at the NHNN showed differences between air and surface samples; these could be interpreted as a lack of relationship between surface and aero-contamination (see section 3.3.6.). Air contamination was variable but generally showed less contamination at unoccupied bedsides, clustering around the nurses' station with more contamination at occupied bedsides. We observed surfaces that were heavily contaminated in areas of low aero-contamination suggesting that air sampling provides a measure of the transient aero-contamination and enables a snap-shot of general contamination levels to be gathered. King et al. (2013) demonstrated that bioaerosols can be deposited across a room at different distances from a source, due to air movement, that can be modified by furniture and people's behaviour.[428] Other studies looking at *Clostridium difficile* transmission have noted that aero-contamination could be detected but it was linked to periods of activity, such as bed changing and ward rounds. These aero-contamination events were linked to dissemination of the bacteria on surfaces in some studies but not in others. This suggests that contamination events for air and surfaces were often not temporally linked and could depend on the virus/bacteria.[39, 429, 430]

Surface sampling is affected by general contamination as some particles eventually settle on surfaces but surface samples are also highly affected by human behaviours within the clinical environment such as object touching. Our results are similar to other studies that have investigated surface contamination variability and transmission by health professionals. In one study that inoculated cauliflower mosaic virus DNA onto a phone within an NICU cubicle, the virus was spread to 58% of the sampling sites within that cubicle over a seven day period. It was also spread to five of five other cubicles and to shared ward locations where up to 80% of sites were positive; overall, 23% of collected samples were positive over the seven day sampling period. There was also a temporal link to the spread of surface contamination with the areas within the initial cubicle having highest positivity rates at four and eight hours post inoculation sampling and other sites becoming positive later, with door handles on other cubicles becoming positive first.[431] This work means that, for some viruses and bacteria, surface

contamination depends more on human behaviours and cleaning procedures than aero-contamination.

Location Differences

Surfaces that were located within occupied bed spaces at the NHNN were always more contaminated than surfaces located within the shared ward area. TVC contamination was highest within bed spaces as has been demonstrated in other studies. This may be due to access for cleaning and lack of patient source.[183, 405]

In contrast, surfaces within the PICU at GOSH which were all close to the bed space, demonstrated lower levels of contamination than those within the shared ward area (see section 3.3.9.3.). Those surfaces closest to the patient were less contaminated than those further away within the bed-space and within the sink area. This may be explained by the fact that the bed spaces within GOSH ITUs had limited non-touch surfaces, which showed significant contamination within the MITU and SITU. In addition, cleaning was undertaken by nursing staff as well as domestic staff within bed spaces (see Chapter 5 for further details).

Whether a bed space was occupied was not consistently related to an increase TVCs compared to unoccupied bed spaces (see section 3.3.6.). Although empty bed spaces should be easier to clean and access should not be a problem, increased contamination was noted on some occasions indicating that these bed spaces are not always contamination free. One of the MRSA positive samples detected within the SITU was detected in a cleaned bed space. Bed spaces are cleaned after a patient is discharged but are then left empty until the next patient is admitted. During the time that the bed space is vacant, contamination can arise from the air as well as from contaminated hands.[367, 428] As bed spaces within PICU were consistently occupied, a comparison with the NHNN could not be made.

Differences existed not only between shared ward areas and bed spaces but between ward areas. Surfaces sampled at SITU were always significantly less contaminated than surfaces sampled at MITU. Differences in contamination between SITU and MITU can also be explained by differences in patient health status. SITU patients are short term stay, most are ventilated and require high intensity support from staff. Visitation is limited to two people per bedside and is only allowed during certain hours. Due to the severity of illness on this ward, visitors have limited interaction with patients and the bedside environment. In comparison, MITU patients are usually long stay and are

undergoing long-term recovery, these patients have much of their care, such as feeding, undertaken by visitors and are capable of interacting with their bedside environment to a much greater extent than those on SITU. Due to the long stay nature of these patients, they have more of their own possessions within the bed space and visitation is encouraged, tends to be regular and prolonged. Furthermore, MITU is a smaller unit and includes less staff than SITU.

3.4.4 USE OF ENVIRONMENTAL CONTAMINATION DETECTION TO MAKE CLINICAL RISK ASSESSMENT

Assessment of Surfaces Using Total Viable Counts

In order to assess the clinical environment for both effective cleaning and risk to patients, both Dancer (2004) and Griffith et al. (2003) have suggested TVC cut-offs and this has been adopted by other authors for further work.[191, 400, 418, 432] Despite the suggestion of standards linked to TVC cut-offs, there is no consensus on useful actions in response to sampling results, sampling methods or limits of acceptable risk.[184]

Of the 1174 TVC samples taken over the study period, 17% of sites failed when using the original Dancer criteria of 5CFU/cm^2 and 44% of sites failed when using the Griffith criteria, 2.5CFU/cm^2.[191, 399] When using the criteria to assess bed spaces, 74% would have failed at least one site if using the original Dancer criteria and 92% would fail using the Griffith criteria (see section 3.3.8.). When examining surfaces rather than bed spaces, one other study demonstrated 32% of sites would have failed by the Dancer criteria in an acute stroke unit, 23% of sites in an ITU, and 27.5% in a medical day unit, figures similar to those seen within the SITU and MITU at the NHNN.[409] Another study found that 7.7% of sites failed with the Dancer criteria which was lower than any of the areas sampled within our study, although it is similar to the PICU bed spaces failures at 11%. Studies using the Griffith criteria have demonstrated failure rates of between 23% - 52% with one study identifying an average CFU of 28 times higher than the Griffith criterion.[177, 401, 433] The failure rate of sites using the Griffith criteria in this study was between 28% and 45%; this is similar to the range identified in these other studies.

Within the original Dancer (2004) paper, there is also a standard for the detection of indicator organisms.[191] This paper suggests that indicator organism such as *Staphylococcus aureus*, *Clostridium difficile*, vancomycin resistant enterococci (VRE), *Salmonella* spp. and multiply resistant Gram-negative bacilli could be undertaken.

Levels of indicator organism should be <1CFU/cm^2, which equates to <24CFU per contact plate.

The Dancer standard for monitoring the environment for indicator organisms includes the monitoring of hand touch sites for multiply resistant organisms. Utilising this standard, 2.5% (1/40) of bed spaces would have failed. If applying a multiple resistant standard, no bed space failures would be registered (see section 3.3.9.5.). One failing hand touch site was the suspended shelf surface. The other hand touch site was the trolley surface, which was positive for a Gram-negative bacilli. However, the TVC was 0 and the sample was only detected by enrichment and so it would not qualify as a failure. If the same standard was used for all surface sites sampled, 0.4% (3/750) would fail, again depending on the definition of multiply resistant Gram-negative bacteria. The nurses station phone, nurses station and suspended shelf would fail with TVCs of 39, 65, 42 respectively, but the other sites positive for Gram-negative bacilli were either floor sites (and so would not be counted) or had TVCs of 6, 0, 0, and 0. Al-Hamad et al. (2008) conducting a clinical validation of the Dancer criteria found that both a cabinet and a handle that had TVCs ranging from 0.1 – 6CFU/cm^2 were persistently contaminated with MRSA indicating that TVCs were not necessarily linked to the presence of pathogens on the surface.[184]

Assumptions of the Dancer Criterion

In the publication that sets out the Dancer criterion a number of assumptions are made that facilitate the use of TVCs to make risk assessments about clinical risk to patients:[191]

- An increased microbial burden suggests insufficient cleaning
- A heavy microbial burden may mask the chances of finding a pathogen
- A heavy level of contamination implies an increased chance of finding an epidemiologically related pathogen

Although cleaning is acknowledged to be important in the control of microorganisms within the hospital environment, it has been demonstrated that surfaces become rapidly re-contaminated, reaching their original levels within four hours of cleaning.[434] As the Dancer standard does not indicate when sampling should be performed in relation to cleaning, it is difficult to know when such sampling should be undertaken.[191] The Griffith standard has been consistently utilised in his publications with screening

undertaken both before cleaning and 10 minutes after cleaning to determine its effect.[190, 399] Galvin et al. (2012) has suggested that microbial assessments utilising TVCs are only useful if they are linked directly to cleaning monitoring as cleaning of the environment will only temporally change the numbers of infectious agents present, especially when TVCs are used to look at contamination that is mostly derived from skin flora.[190] Data within this study has demonstrated that there are differences in TVC contamination between ward types, even in the same hospital, and with the same cleaning regimen.

Work done within this study on comparing detection of microorganisms within the environment has demonstrated that low levels of microorganisms can be detected if enrichment is used (see section 3.3.3.). Contamination of pathogens within the clinical environment is usually present at considerably lower numbers of microorganisms than would be found in clinical specimens and they are probably in a different physiological state.[190] In general, nosocomial pathogens are present in the range of 1 – 100CFU/cm^2 and so enrichment is required in order to detect them.[174] Although viable, these microorganisms may be more difficult to culture.[190] There is no sampling method for detection of indicator organisms presented as part of the Dancer criteria. TVCs may not detect pathogens present in low levels as they are likely to be masked by more dominant skin flora. However, this can be countered by selection of alternative sampling methods over the use of contact plates as microbial monitoring for pathogenic organisms does not require enumeration; presence or absence is sufficient.[190]

The final assumption presented by S. J. Dancer (2004) is that pathogens are present in the environment in proportion to the level of overall contamination.[191] In one study it has been found that there was a significant association with TVCs over 2.5CFU/cm^2 and MRSA detection from the same site ($p=0.001$).[405] Other studies like this one have found no correlation between TVCs and detection of MRSA as an indicator organism.[184, 190, 197, 401, 406] Risk is more likely to be related to the frequency and level of surface contamination with specific pathogens in combination with touch and hand hygiene practice. It is therefore difficult to relate the detection of TVCs to infection risk.[406]

It has been suggested as a consequence that data from microbial hygiene assessment should be used to provide trend analysis and monitor efficiency over time. This is contrary to the measures suggested in the Dancer criterion of closing bed spaces and

instigating deep cleaning after resampling.[190] It has also been suggested that using hygiene assessment as part of continuous monitoring both standardization and optimization of a method would be required regarding when sampling should be done in relation to cleaning, and whether the presence of any CFUs would be acceptable directly after cleaning.[190] Hygiene assessments utilised within the literature are usually performed sporadically; usually at the start and end of an outbreak or as part of defined piece of research rather than a continual assessment of cleaning; it is accordingly hard to truly evaluate their usefulness within the clinical setting.[190]

Within this study we believe that we have demonstrated that within a dynamic hospital environment, a large proportion of sites screened for bacterial contamination would fail if using the criteria suggested by previous authors. This could lead to the closure of wards or bed spaces, increased costs and decreased patient care. Surfaces were frequently contaminated, even with routine cleaning (see section 3.3.8.). Despite raised TVCs, no evidence has been found to link raised TVCs to transmission of HCAIs either in this or other studies. Therefore the suggested criterion for assessing clinical risk are not practical and probably not very accurate. Monitoring of cleaning using TVC sampling may prove useful in order to improve cleaning efficiency as part of continuous assessment but to do so would require the implementation of continued use and a standardized method. Without such a method and practice, microbial counts give little indication of when a surface was last cleaned, how well it was cleaned and with what.[190]

Use of Indicator Organisms

Sampling of environmental sites was undertaken in shared ward and clinical areas during non-outbreak settings and in occupied rooms of infected patients to assess the ability to detect pathogens from surface sites in order to determine risk of transmission.

Viruses within the Clinical Environment

Relatively few studies have been conducted looking at levels of norovirus within the environment and only two have examined the contamination with adenovirus outside of the outbreak setting. Of the studies that have looked for detection of norovirus, one also looked for rotavirus and astrovirus but did not separate out the data for individual viruses. Data from this study showed that swabs taken four years apart on two paediatric wards had contamination rates of 20% in 2004 falling to 6% in 2008.[435, 436] The other norovirus study only found 1/176 samples (0.8%) to be positive.[437] Within

an adenovirus outpatient study at GOSH, 37% of sites were detected as positive for adenovirus DNA.[438] In the only other published adenovirus study, based within in a general surgery unit, virus was only detected in 1/176 sites (0.8%).[437]

Data collected within our study showed that over a six month period, 539 samples were taken on the HSCTU and IIU of which 13 (2.4%) were positive, 0.7% (4/539) for adenovirus and 1.7% (9/539) for norovirus (see section 3.3.9.4.). These numbers are substantially lower than demonstrated in some of the other studies, but are similar to those seen in the study by Carducci et al. (2011).[437] Within the HODU, 1% (1/90) of sites were positive for norovirus and 12% (11/90) were positive for adenovirus. This situation is similar to our adenovirus outpatient study although the levels are still lower (12% vs 37%).[438] The ward is cleaned with chlorine due to the high risk nature of the patients in contrast to outpatients where only detergent is used. This may explain the reduced number of positive sites (see Chapter 5).

Within the IIU, 36 samples were taken from cubicles occupied by adenovirus positive patients before post-discharge cleaning of which 33 were positive (92%) (see section 3.3.9.4.). This demonstrates that adenovirus positive patients highly contaminate their environment. This is likely to be due to the prolonged admission periods, sometimes for up to two years, in the ward setting. Furthermore, these patients bring personal items with them which both interfere with cleaning as cleaners will not move them and act as a source of re-contamination. Finally, the interaction of patients with the environment through toys, walking and/or crawling increases the risk of acquiring microorganisms from the environment.[12]

One explanation for the variability in viral detection rates between studies is that surface detection is affected by a number of factors. These include viral species (due to virus size, variability within and between genera), low concentration of virus, presence of inhibitors, and limits of detection.[161] As PCR was used to detect the viruses within this study, viability could not be determined and so may over estimate levels of infectious virus contamination (see Chapter 5).

Bacteria within the Clinical Environment

Within this study, MRSA was found in 10% of sites within one isolation cubicle at GOSH before cleaning and in 60% of sites in another. Within an occupied isolation bed space in the NHNN SITU 20% of sites were positive for MRSA and, interestingly, were still positive in 10% of sites after cleaning in an adjacent empty bed space.

Within the literature, positive sites for MRSA vary from 3.3% to 73%.[29, 197, 210, 401, 414, 439, 440] Variations in positivity rates do exist depending on whether patients were colonised/infected. Boyce et al. (2007) found that 69% of environmental samples were positive for patients who were colonized vs 73% for patients who were infected.[441] Another study found 34.1% of environmental samples positive in rooms occupied by colonized patients.[439] Schabrun et al. (2006) pooled MRSA TVC contamination rate data from the literature and found that they ranged between 0CFU - >500CFU, with a mean of 82.1CFU/cm^2.[442] The wide variations in detection rates seen in our study are therefore are consistent with the published literature. Clearly any differences in sampling methodologies, type and number of personnel, activity, moisture, materials and air filtration will impact on detection rates.

We and others have observed that MRSA can be detected in bed spaces and cubicles not occupied by MRSA positive patients. In one study, MRSA was isolated from the air in the absence of MRSA positive patients on 7.6% of sampling occasions. In another study, MRSA was cultured from 43% of beds from non MRSA colonized patients.[174]

Gram-negative species were detected using enrichment in 15% (6/40) of bed spaces, each time at a rate of 8% per (1/12) bed space. Enterobacteriaceae were detected within the sink bowl on over 70% of occasions with sink lips contaminated over 50% of the time. In the HODU, 1% (1/90) of samples were positive for Enterobacteriaceae by PCR, but no sinks were sampled. Other studies have generally focussed on antibiotic resistant Gram-negative bacteria from the environment such as extended spectrum β-lactamase producers, and not on the species. In such studies, detection rates range between 3.8% – 30%.[174, 197, 203, 211, 419, 424, 440]

Most of the sites detected as positive in this and other studies were those linked to moisture i.e. sinks or baths.[424] Contaminated surfaces in a similar study included baths (8 times), bed rails (2), remote (1), and baby scales (2). Of the 18 positive samples, 15 of the isolates were *Klebsiella* spp., two were *E. coli* and one isolate was *Citrobacter freundii*. Of the 18 isolates, 10 were related to patient colonising organisms, 9 of which were *Klebsiella* spp. The organisms found in the environment by this study were *Pantoea* spp. 6/11, *Enterobacter* spp. 3/11 and *Klebsiella* spp. 2/11. The sites in which Gram-negative organisms were detected differed to those usually colonised with MRSA that tend to be high touch sites. It is therefore possible that even if the same sites are sampled for both MRSA and Gram-negative bacteria, there may

be an underestimation of Gram-negative organisms within the environment if these do not include areas such as sinks.

Variation in detection of Gram-negative organisms was not as wide as that seen with MRSA. This is probably due to ease of culture, (i.e. biofilm), the degree of patient shedding (dependent on diarrhoea), sampling/culture methodology, difficulty of cleaning, and ongoing outbreaks.[174]

In conclusion, this study has shown that extensive distribution of bacterial and viral contamination occurs outside of outbreaks. Sampling of these sites has been shown to be effective with the sampling methods used. Extensive contamination exists within isolation cubicles and could present a source of cross transmission to other patients if transferred out of the cubicle environment. Specific species isolation rates within this study are in line with others studies published in the literature but sampling strategies should be based upon the target species in order to avoid underestimation of pathogen contamination.

Evidence of the Link between Environmental Contamination and HCAI

Historically it has been stated that organisms within the inanimate hospital environment (particularly on surfaces and in the air) contribute negligibly to HCAI; however there now exists a general consensus that environmental cleanliness is important in controlling HCAI.[182] During this study, we have demonstrated that microorganisms are able to survive within the environment both by experimental studies and by the use of TVCs and specific pathogen detection (see sections 3.3.2, 3.3.9.2 and 3.3.9.4.). Pathogenic organisms have been demonstrated both within this study and within the literature to be detected even in rooms where there is no obvious source, indicating survival beyond the length of one patient stay or transference of contamination from other patient rooms.[170] There remains a need to prove that the environment acts as a source of HCAI by demonstrating a correlation between levels of bacterial contamination and HCAI levels.

Wilson et al. (2011) utilized a randomized cross over trial within ITUs in two hospitals to demonstrate that enhanced cleaning reduced levels of environmental contamination and hand carriage of staff with *Staphylococcus aureus*.[434] However, this reduction in environmental load (as measured by TVCs) did not impact on the acquisition of MRSA. Two other studies found that cleaning with disinfectant rather than detergent had no effect on HCAI rates. However, both studies mainly focused on floor contamination.[326,

[443] Wilcox et al. (2003) undertook a cross over study using hypochlorite vs neutral detergent on two elderly care medicine wards looking for detection of *Clostridium difficile*. 35% of 1128 environmental samples grew *Clostridium difficile*.[201] Both wards were identical in design and had similar case mix but had different nursing and cleaning staff. Both wards were cleaned with disinfectant. However whilst there was a significant decrease in cases on ward X from 8.9 – 5.3 cases/100 admissions (p=<0.05), this was not seen in ward Y. The reasons for this are unclear. One study has demonstrated that a reduction in HCAI MRSA infections with the use of an additional cleaner on the unit alongside a reduction in TVCs.[183] It is however difficult to compare studies that discuss the use of cleaning in relation to HCAI rates as there are differences not only in cleaning products, cleaning staff and cleaning techniques, but also in sampling methodologies. The multi-faceted nature of clinical interactions means that it is difficult to prove a correlation between reduction in environmental contamination and cross transmission.

Another method for determining the relationship between the environment and cross transmission of infection is the use of statistical methods to determine whether entering a cubicle that has previously been occupied by a positive patient, and therefore potentially contaminated, affects acquisition risk. The averages of eight studies related to hospital transmission including VRE, *Acinetobacter baummannii*, *Clostridium difficile* and MRSA showed an average 73% increased chance of acquisition if the previous patients occupying the room had been colonised/infected.[192]

Studies have demonstrated that patients admitted to rooms previously occupied by colonised/infected patients with VRE had increased the chance of acquisition by between 37.1% and 87.5%.[170, 444-446] Patients entering a room previously occupied by someone with *Acinetobacter baumannii* were 71.8% more likely to be colonised with *Acinetobacter baumannii*. The same study demonstrated a 41.7% increased chance of acquiring *Pseudomonas aeruginosa*. Environmental contamination with MRSA has been found to be a key component of MRSA colonisation pressure.[447] Finally one study determined that previous room occupancy by an MRSA positive patient increased the likelihood of MRSA acquisition by the current occupant by 28.8%.

These studies demonstrate that factors that influence environmental contamination can impact of the risk of HCAI. However, the data is mixed, largely due to the complex relationship between environmental contamination and human disease.

3.5 CONCLUSIONS

In this study, we have developed a sampling methodology that we have then applied to the healthcare environment. As part of this work, we have analysed the environmental variability of microorganisms with respect to object type at different height levels and in different zones around the patient bed. The results demonstrate that organisms can be detected from the environment using the methods developed and that relationships between organisms and the environment are complex. Changes occur within the distribution of TVCs both within wards from place to place and time to time, although some sites such as floors and sinks are more likely to have high levels of contamination.

Specific pathogens were screened to determine the relationship between TVCs and detection of pathogenic species. Our data demonstrates that in a dynamic hospital environment, a large proportion of sites screened for bacterial contamination would fail if using hygiene criteria suggested by previous authors, particularly those sites closest to the patients. Paradoxically, many sites that did not have potentially pathogenic bacteria would fail while sites that were contaminated with potentially pathogenic bacteria would pass. It may be more cost effective to use a pathogen specific panel for surveillance with the use of sentinel sites rather than TVCs at multiple sites with the ward environment.

The importance of controlling specific pathogens is highlighted by the growing evidence of cross transmission, especially where patients are admitted to rooms of a previously positive patient. In this context, HCAI prevention requires appropriate use of bed spaces and adequate discharge cleaning (see Chapter 5). Importantly, even in wards with a high proportion of single rooms, the shared areas will also be contaminated and must also be considered as potential sources of pathogens.

The work presented in this chapter emphasises the importance of evidence based infection control policies. These should not only include good hand hygiene practices, but should consider ward design and use of space as will be discussed in Chapter 5.

Chapter 4 EPIDEMIOLOGICAL TYPING OF CLINICAL AND ENVIRONMENTAL ENTEROBACTERIACEAE ISOLATES

4.1 INTRODUCTION

Great Ormond Street Hospital (GOSH) is a paediatric hospital with over 300 beds. Within GOSH the hospital has a policy of active screening for resistant Gram-negative bacterial carriage and infection of all patients both on admission and in high risk patients (such as those on the haematopoietic stem cell transplantation unit (HSCTU)). GOSH defines multi-drug resistant (MDR) Gram-negative bacteria as being: Gentamicin resistant plus resistant to at least one other antibiotic of the following: Amikacin, Ciprofloxacin, Ceftazidime or Piptazobactam; or resistance to Amikacin or the Carbapenems irrespective of Gentamicin resistance.[448] Once a patient is identified as carrying an MDR Gram-negative, their record is tagged and the clinical isolate undergoes both phenotypic and molecular typing. If the resistant species is detected more than 48 hours after admission, the patient is classified as having a healthcare associated infection (HCAI) and further infection control investigations are undertaken to identify a possible source. These investigations are informed by the molecular typing results of that and other patient isolates.

Of the antibiotic resistant Gram-negative bacterial species frequently detected within GOSH, the Enterobacteriaceae are the most common. The family Enterobacteriaceae is comprised of a heterogeneous group of bacterial species. *Enterobacter cloacae* for instance may actually represent a large complex of over 12 genetic clusters and *Klebsiella pneumoniae* is comprised of three closely related sub species.[45, 69, 70, 449] Due to this diversity, typing accuracy is problematic which has important implications for epidemiological surveillance.[215, 221]

Outbreaks caused by *Enterobacter* species (spp.), *E. coli* and *Klebsiella* spp. are often clonal in nature. Therefore when outbreaks occur it is important to identify whether clusters of infections are due to a shared source, or due to polyclonal isolates of the same species.[69, 215] Different techniques for typing of clonally linked isolates are available, with molecular techniques extensively exploited in recent years.

Detection of diversity within closely related isolates requires typing schemes to have a high level of discrimination.[242] Discrimination is especially important for outbreak investigations as both the parent and their descendent strains are often included in the same sample set.[69, 221, 450-452] In addition to discrimination, techniques should be

accessible to clinical laboratories, reproducible, cost effective, easy to use, with easily interpretable results.[87, 255]

In a survey of 154 clinical microbiology laboratories who routinely type isolates, the top four methods for typing were:[453]

- Pulsed field gel electrophoresis (PFGE), used in 32% of centres
- DNA sequencing used in 22% of centres. This includes multi-locus sequence typing (MLST) and other capillary sequencer methods such as multi-locus variable number tandem repeat (VNTR) typing
- Random amplified polymorphic DNA (RAPD), repetitive extragenic palindromic sequence PCR (REP-PCR) or other fragment length typing schemes; used in 16% of centres
- Phenotypic methods; used in 2.2% of laboratories

All of the above molecular typing schemes look at specific areas of the genome, whether through housekeeping genes or specific genomic sites (restriction sites, tandem repeats, repetitive extragenic palindromic sequences) and assume they are representative of entire genome diversity.[454] However the genetic discrimination of bacterial isolates provided by these techniques may be limited and lack sufficient resolution to allow accurate inference of transmission events.[69, 220]

To improve discrimination there has been a trend for strain typing methods to becoming increasingly species specific.[455] This is partly because no single core of universal genes has been evaluated for typing for all pathogens. Within the Enterobacteriaceae, difficulties with phenotypic identification make it advantageous to use a typing technique capable of crossing species boundaries. With the advent of high throughput sequencing (HTS) for microorganisms new typing schemes are being developed based on either whole genome sequence (WGS) data or by targeted simultaneous sequencing of a number of genes (such as ribosomal genes).[241, 278, 395, 456] These have yet to be extensively evaluated for outbreak typing within the Enterobacteriaceae.

Once data are acquired, there is variability in what different clinical microbiology laboratories use to define epidemiological relatedness. This variability is greater with some schemes than others, and is a particular issue within newly developed typing techniques.[457]

4.1.1 CHAPTER AIMS

The aim of this chapter was to develop and evaluate methods for undertaking molecular based typing of Enterobacteriaceae for routine use specifically with *Klebsiella* spp, *Enterobacter* spp and *E. coli*. Once methods for typing were undertaken they were applied to an outbreak investigation occurring at GOSH in order to draw conclusions about which methods would prove suitable for continued use. The specific aims are listed below:

1. To develop a multi-locus variable number tandem repeat typing scheme for *Enterobacter cloacae* and to modify a published VNTR typing scheme for *Klebsiella pneumoniae*.

2. To evaluate repetitive extragenic palindromic sequence PCR for typing of *Enterobacter* spp., *Klebsiella* spp, and *E. coli*.

3. Undertake high throughput sequencing/whole genome sequencing using two platforms via two different methods on a group of 48 isolates comprising three species (the platforms and analysis of which are listed in section 4.2.4.)

4. To compare data from aims 1, 2 and 3 in order to draw conclusions about how to undertake routine clinical typing to investigate outbreaks.

4.2 RESULTS

4.2.1 ISOLATE SELECTION

4.2.1.1 TYPING COMPARISON

Forty eight isolates that were initially identified as *Klebsiella pneumoniae* were investigated by all techniques (PFGE, VNTR, REP-PCR, Pathogenica, Phyloshift and whole genome sequencing (WGS)) and the results for typing methods compared in section 4.2.5. The isolates were temporally linked and detected from both the environment and patients during 2011 and 2012. They represented the main PFGE types detected within that period at GOSH. Isolates selected comprised a single large PFGE cluster of 25 indistinguishable isolates (GREA14KL-7) that were believed to be linked to the outbreak described in section 4.2.5.1. Three other small clusters of identical PFGE types were chosen, one where cross transmission was believed possible on the cardiac intensive care unit (CICU) but had not been confirmed (GREA14KL-3) and two where there was no crossover of patients and so the isolates were considered unconnected (GREA14KL-4 and GREA14KL-9). Other isolates were selected as they were unique by PFGE. In addition to the single ward where an outbreak was believed to have occurred (HSCTU), isolates came from a number of different wards, mostly surgical and private patient wards. One isolate was from a sample was collected within outpatients from patient 3 (isolate no. 3). Environmental isolates originated from samples processed from two wards (HSCTU and CICU) where cross transmission may have occurred.

As details of these samples was sent for external analysis these 48 isolates are referred to as isolates 1 – 48 in sections 4.2.4 and 4.2.5. The additional isolate numbers for those isolates used to validate both REP-PCR and VNTR analysis are also listed in Table 4-1 along. Cluster numbering has been kept consistent between analysis of typing techniques for these isolates where possible. On subsequent identification via MALDI-ToF post typing analysis four of the isolate tested were identified as *Klebsiella oxytoca* and one was identified as *E. asburiae*, this was tested but removed from HTS and WGS analysis to permit better cluster resolution.

4.2.1.2 REPETITIVE EXTRAGENIC PALINDROMIC SEQUENCE PCR VALIDATION

In addition to the 48 eight isolates investigated by all typing techniques a further 11 isolates detected from environmental samples during 2011 and 2012 were included in the REP-PCR typing analysis for both comparison with PFGE and determination of analysis similarity cut-offs, see Table 4-1. Of these seven three were identified by biochemical analysis as *Enterobacter cloacae* and four as *Klebsiella oxytoca* (see methods section 2.3.2.5). One of the *Klebsiella* oxytoca isolates was subsequently identified as *Klebsiella pneumonia* by MALDI-ToF identification (sample S20262). Additionally one of the *Enterobacter cloacae* isolates was subsequently identified as *Klebsiella pneumoniae* by MALDI-ToF (sample S67498). These isolates were included in this typing technique validation to explore the way that closely related Enterobacteriaceae performed.

Intra-laboratory reproducibility was investigated for both *E. coli* and *Klebsiella pneumoniae* using nine isolates for each; which represented the first resistant isolate of patients newly diagnosed with MDR Gram-negative flora, either as carriage or causing infection during 2008 (see section 4.2.3.2.).

Intra-laboratory reproducibility for *Enterobacter* spp. was validated using 18 isolated detected during 2000 – 2001. These isolates were selected as they occurred during a period of frequent *Enterobacter* spp. detection within GOSH and thus permitted sufficient numbers to be processed to permit analysis to be undertaken (see section 4.2.3.2.)

Inter-laboratory reproducibility was tested using a panel of *E. coli* and *Klebsiella pneumoniae* isolates provided as part of an international study (see section 4.2.3.3.).

4.2.1.3 MULTI-LOCUS VARIABLE NUMBER TANDEM REPEAT VALIDATION

In addition to the 48 eight isolates investigated by all typing techniques a further six of the additional isolates that underwent REP-PCR typing also underwent VNTR typing, see Table 4-1. These included environmental isolates identified as *Klebsiella oxtoca* and *Enterobacter cloacae* species. The *Klebsiella pneumoniae* VNTR scheme validated was specific for *Klebsiella pneumoniae* and so isolates of other species were included to see how this typing scheme would perform.

Isolate No.	Isolate Source	PFGE	VNTR	REP-PCR	Pathogenica	Phyloshift	MiSeq	MiSeq
1	Patient 1	Unique	X	X	X	X	X	X
2	Patient 2	Unique	X	X	X	X	X	X
3	Patient 3	Unique	X	X	X	X	X	X
4	Patient 4	Unique	X	X	X	X	X	X
5	Patient 5 *	GREA14KL-4	X	X	ND	ND	ND	ND
6	Patient 6	Unique	X	X	X	X	X	X
7	Patient 7	GREA14KL-7	X	X	X	X	X	X
8	Patient 8	GREA14KL-3	X	X	X	X	X	X
9	Patient 5	GREA14KL-3	X	X	X	X	X	X
10	Patient 25	Unique	X	X	X	X	X	X
11	Patient 23	GREA14KL-3	X	X	X	X	X	X
12	Patient 24	Unique	X	X	X	X	X	X
13	Patient 9	Unique	X	X	X	X	X	X
14	Patient 10	Unique	X	X	X	X	X	X
15	Patient 11	Unique	X	X	X	X	X	X
16	Environmental	Unique	X	X	X	X	X	X
17	Environmental #	Unique	X	X	X	X	X	X
18	Environmental	GREA14KL-4	X	X	X	X	X	X
19	Patient 12	GREA14KL-7	X	X	X	X	X	X
20	Environmental	GREA14KL-7	X	X	X	X	X	X
21	Patient 13	GREA14KL-7	X	X	X	X	X	X
22	Patient 14	GREA14KL-7	X	X	X	X	X	X
23	Patient 12	GREA14KL-7	X	X	X	X	X	X
24	Patient 12	GREA14KL-7	X	X	X	X	X	X
25	Patient 15	GREA14KL-	X	X	X	X	X	X
26	Patient 16	GREA14KL-7	X	X	X	X	X	X
27	Patient 14	GREA14KL-7	X	X	X	X	X	X
28	Patient 16	GREA14KL-7	X	X	X	X	X	X

29	Patient 17	GREA14KL-7	X	X	X	X	X	X
30	Patient 13	GREA14KL-7	X	X	X	X	X	X
31	Patient 18	Unique	X	X	X	X	X	X
32	Patient 19	GREA14KL-7	X	X	X	X	X	X
33	Patient 18	GREA14KL-7	X	X	X	X	X	X
34	Patient 20	GREA14KL-7	X	X	X	X	X	X
35	Environmental #	GREA14KL-9	X	X	X	X	X	X
36	Environmental	GREA14KL-9	X	X	X	X	X	X
37	Environmental #	GREA14KL-7	X	X	X	X	X	X
38	Patient 17	GREA14KL-7	X	X	X	X	X	X
39	Patient 21	GREA14KL-7	X	X	X	X	X	X
40	Patient 22	GREA14KL-7	X	X	X	X	X	X
41	Patient 15	GREA14KL-7	X	X	X	X	X	X
NCTC 13368	*Klebsiella* spp. type strain	Unique	X	X	X	X	X	X
43	Environmental	GREA14KL-7	X	X	X	X	X	X
44	Environmental	GREA14KL-7	X	X	X	X	X	X
45	Environmental	GREA14KL-7	X	X	X	X	X	X
46	Environmental #	Unique	X	X	X	X	X	X
47	Environmental	GREA14KL-7	X	X	X	X	X	X
48	Patient 15	GREA14KL-7	X	X	X	X	X	X
S67498	Environmental	ND	X	X	ND	ND	ND	ND
S20245	Environmental #	ND	X	X	ND	ND	ND	ND
S20253	Environmental #	ND	X	X	ND	ND	ND	ND
S50381	Environmental 𝒇	ND	ND	X	ND	ND	ND	ND
S50380-1	Environmental 𝒇	ND	ND	x	ND	ND	ND	ND
S50380-2	Environmental 𝒇	ND	ND	X	ND	ND	ND	ND
S50404	Environmental 𝒇	ND	ND	X	ND	ND	ND	ND
S50403	Environmental 𝒇	ND	ND	X	ND	ND	ND	ND
S20255	Environmental 𝒇	ND	X	X	ND	ND	ND	ND

S20260	Environmental	ND	X	X	ND	ND	ND	ND
S20262	Environmental ℵ	ND	X	X	ND	ND	ND	ND

Table 4-1 Isolates selected for typing comparison study along with PFGE type where available. Table lists which tests were undertaken, study number and sample number, as well as the source of the sample. ℵ = *E. cloacae* # = *Klebsiella oxytoca*, * = *Enterobacter asburiae*. Boxes coloured green = typing technique undertaken on isolate, boxes coloured with technique not done on isolate. NA – not applicable, ND – not done.

4.2.2 VARIABLE NUMBER TANDEM REPEAT TYPING SCHEME DEVELOPMENT AND VALIDATION AGAINST PULSE FIELD GEL ELECTROPHORESIS

4.2.2.1 ENTEROBACTER CLOACAE

Enterobacter cloacae was selected as the target organism for initial VNTR design as there was no published VNTR scheme available, and along with *Klebsiella* spp. it represented the species most frequently linked with detection from sinks during sampling within GOSH (see Chapter 3).

The initial validation of 15 potential loci was performed on 24 isolates from Public Health England (PHE) and GOSH as well as two type strains (NCTC 10005 and ATCC 700323) (see methods section 2.2.10.1). Of the 15 loci, four were identified as potentially discriminatory, containing variable numbers of repeats and little or no primer cross reactivity with other sites within the *Enterobacter cloacae* genome (see Table 4-2.). Several of the isolates tested failed to amplify at any loci indicating they were not correctly identified phenotypically as *Enterobacter cloacae* while the type strains did (see Figure 4-1.)

Locus	Outcome
Locus 7	Nucleotide position 3162518 – 3162545, repeat size of 7 bases demonstrated minimal variation with only 2 differences in repeat number.
Locus 10	Nucleotide position 835605 – 835645, repeat size of 18 bases demonstrated at least 3 different numbers of repeats,
Locus 12 (Figure 4-1)	Nucleotide position 4521760 – 4522318, repeat size of 189 bases demonstrated at least 5 different numbers of repeats
Locus 14	Nucleotide position 4687849 – 4687879, repeat size of 9 bases demonstrated at least 4 different repeat numbers

Table 4-2 *E. cloacae* VNTR discrinatory loci validation results.

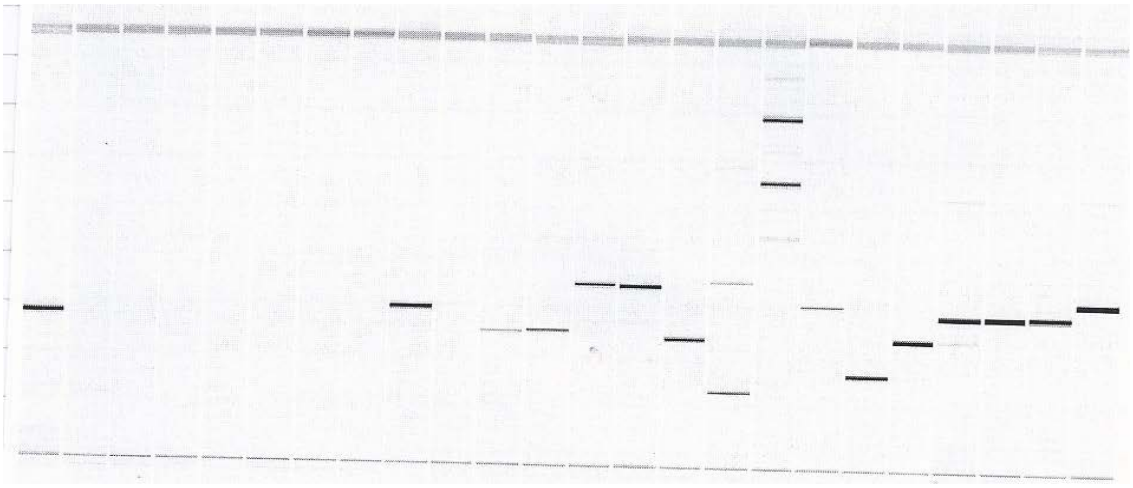

Figure 4-1 QiAxcel automated electrophoresis gel of 24 isolates amplified by Enterobacter cloacae VNTR locus 12 PCR primers.

4.2.2.2 KLEBSIELLA PNEUMONIAE

54 isolates were used to validate a modified version of the VNTR scheme published by Turton et al. (2010) as described in methods section 2.2.10.2. [(55)] The sample included seven isolates identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS) as *Klebsiella oxytoca* and one isolate of *Enterobacter asburiae* as well as 46 of *Klebsiella pneumoniae* (see methods section 2.3.2.5.). Nine loci were selected, six from the published assay, plus loci N1, N2, and N4 which are utilised for in-house VNTR typing by the reference laboratory (Figure 4-2). PFGE had been performed in 48 of the 54 isolates.

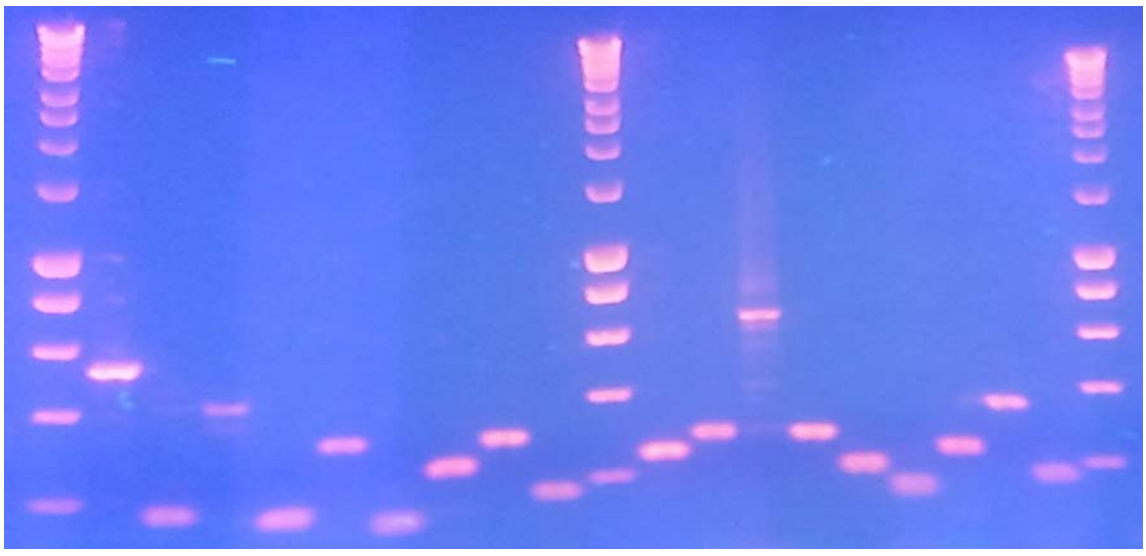

Figure 4-2 Isolates 14 and 33 respectively amplified using nine loci *Klebsiella pneumoniae* VNTR primers (A, E, H, J, K, D, N1, N2 and N4) visualised on a 1.5% agarose gel stained with ethidium bromide.

Isolates were allocated to the same cluster if the number of repeats were the same across all nine loci or differed by only one repeat at one locus. Using this definition three clusters were apparent for the isolates tested, cluster 3 (purple), cluster 4 (blue) and cluster 5 (pink) (see Table 4-3). Cluster 4 was comprised of two sub clusters, 4a (mid blue), cluster 4b (light blue). When epidemiological information was included cluster 5 was re-classified as part of cluster four, forming a third sub cluster, 4d (see methods section 2.7). With the exception of isolate no. 44, all isolates within cluster 4d were identical. 22 of the isolates were determined to be unique by VNTR (green). Including all but one of the non *Klebsiella pneumoniae* isolates, the exception being S20245. Cluster 4b was the predominant sub-cluster within cluster 4.

	VNTR Locus								
Isolate No.	**A**	**E**	**H**	**J**	**K**	**D**	**N1**	**N2**	**N4**
15	8	4	-	-	2	2	3	3	1
10	6	4	0	1	2	2	1	4	1
1	6	2	1	1	2	1	3	3	1
16	5	5	-	19	2	2	4	3	1
13	5	5	0	-	2	2	4	3	1
4	5	4	0	-	2	2	4	3	1
18	3	5	2	-	2	1	3	4	1
6	3	5	2	14	2	1	3	4	1
11	3	5	2	14	2	1	3	4	1
8	3	5	2	14	2	1	3	4	1
9	3	5	2	14	2	1	3	4	1
14	3	1	2	0	1	1	1	3	1
S20260	2	-	-	-	-	-	-	4	1
2	2	3	5	1	1+	2	4	2	1
3	2	3	1	0	1	1	4	1	1
NCTC 13368	1	-	-	-	-	1	1	3	3
5 EA	1	-	-	-	0	2	1	4	1
12	1	3	1	14	2	2	2	3	1
24	1	2	4	1	0	3	1	4	1
26	1	2	4	1	0	3	1	4	1
29	1	2	4	1	0	3	1	4	1
45-	1	2	4	1	0	3	1	4	1
S20245 KO	1	2	4	1	0	3	1	4	1

7	1	2	4	1	0	2	1	4	1
19	1	2	4	1	0	2	1	4	1
23	1	2	4	1	0	2	1	4	1
22	1	2	4	1	0	2	1	4	1
S67498	1	2	4	1	0	2	1	4	1
20	1	2	4	1	0	2	1	4	1
21	1	2	4	1	0	2	1	4	1
25	1	2	4	1	0	2	1	4	1
30	1	2	4	1	0	2	1	4	1
28	1	2	4	1	0	2	1	4	1
27	1	2	4	1	0	2	1	4	1
33	1	2	4	1	0	2	1	4	1
32	1	2	4	1	0	2	1	4	1
34	1	2	4	1	0	2	1	4	1
36	1	2	4	1	0	2	1	4	1
47	1	2	4	1	0	2	1	4	1
S20253	1	2	4	1	0	2	1	4	1
40	1	2	4	1	0	2	1	4	1
43	1	2	4	1	0	2	1	4	1
41	1	2	4	1	0	2	1	4	1
44	1	2	4	1	0	3	1	2	1
48	1	2	4	1	0	2	1	2	1
38	1	2	4	1	0	2	1	2	1
39	1	0	0	0	1	1	3	3	3
S20255 KO	0	-	-	-	-	-	-	12	1
37 KO	0	-	-	-	-	2	1	12	-
35 KO	0	-	-	-	-	2	1	12	1
46 KO	0	-	-	-	1	1	3	-	3
S20262 KO	0	-	-	-	0	2	1	12	1
31	-	-	-	1	0	1	1	-	1
17 KO	-	-	-	-	-	-	-	-	1

Table 4-3 VNTR typing results with nine loci for 54 isolates. Isolates were clustered if they were had identical VNTR types or altered by one repeat at one. Clusters are colour coded: unique = green, cluster 3 = purple, cluster 4 = blue/pink (4a = mid blue, VNTR 4b = light blue, VNTR cluster 4d/5 = pink) EA = *E. asburiae*, KO = *K. oxytoca*.

Loci Variation

Locus J had the largest number of repeats present of all the loci, with up to 19 repeats detected. Locus A had the greatest variety of repeat numbers detected, with eight different numbers of repeats detected, including no amplification of the locus. This suggests that Locus A has the highest discriminatory power of all loci. Within the outbreak strain (cluster 4) at Locus D, 22 isolates had two repeats and six isolates had three repeats. At Locus N2, 25 isolates had four repeats and three isolates had two repeats. One isolate (44) had alterations from the 1,2,4,1,0,3,1,4,1 VNTR profile at two locations, D and N2 (see Table 4-3). Locus N4 demonstrated the lowest level of tandem repeat diversity, with only four isolates having a repeat size other than one. One isolate (37) failed to amplify at this locus and the other three isolates had three repeats (samples 39, 46 and NCTC 13368).

Inter-Laboratory Validation

Thirteen of the isolates also underwent VNTR typing at the PHE. Amplicons at the PHE were visualised using gel electrophoresis. These results were compared to GOSH study VNTR results where analysis was undertaken using capillary electrophoresis. Except for Locus J where visualisation was undertaken with both gel and capillary electrophoresis due to the size of the repeat (see Table 4-4.). Comparative results indicate that it is possible to differentiate repeat sizes using the two methods. However five of the thirteen VNTR types differed by at least one repeat at one locus between analysis at the two centres, only one sample differed by more than one repeat at more than one locus. Isolates 17 and 33 had differences in Locus N4, when using the two different analysis methods. Isolate 11 had a repeat difference of one (13 vs 14) at Locus J despite them both being visualised using gel electrophoresis. Isolate 5 had different repeat sizes at 7/9 loci indicating an alternative isolate may have been tested. Isolate 16 had an insertion sequence (IS) detected in Locus J, there are no consensus rules for determining this.

Isolate No.	PHE VNTR	Study VNTR
5	**3,5,2,-,2,1,3,4,1**	**1,-,-,-,0,2,1,4,1**
6	3,5,2,14,2,1,3,4,1	3,5,2,14,2,1,3,4,1
8	3,5,2,14,2,1,3,4,1	3,5,2,14,2,1,3,4,1
9	3,5,2,14,2,1,3,4,1	3,5,2,14,2,1,3,4,1
11	**3,5,2,13,2,1,3,4,1**	**3,5,2,14,2,1,3,4,1**

16	**5,5,-,IS,2,2,4,3,1**	**5,5,-,19,2,2,4,3,1**
17	**No amplicons**	**,-,-,-,-,-,-,-,1**
19	1,2,4,1,0,2,1,4,1	1,2,4,1,0,2,1,4,1
20	1,2,4,1,0,2,1,4,1	1,2,4,1,0,2,1,4,1
23	1,2,4,1,0,2,1,4,1	1,2,4,1,0,2,1,4,1
27	1,2,3,1,0,2,1,4,1	1,2,4,1,0,2,1,4,1
29	1,2,4,1,0,2,1,4,1	1,2,4,1,0,3,1,4,1
33	**1,2,4,1,0,2,1,4,-**	**1,2,4,1,0,2,1,4,1**

Table 4-4 Comparison of 13 VNTR sample resuts detected via gel electrophoresis (PHE) and capillary electrophoresis (GOSH) IS = insertion sequence.

Comparison with Pulse Field Gel Electrophoresis Results

PFGE typing was performed on 48 isolates by the PHE, which is considered to be the reference standard. This was done as part of the routine clinical service and was compared to VNTR and the results are shown in Table 4-5.[55, 236] For 41/48 (85%) of these isolates clustering was identical between the two techniques. Nineteen of the 48 isolates were unique when typed using VNTR, of these 15 were also unique when typed using PFGE. When the VNTR data was compared to the available PFGE data all of the isolates within cluster 3 had a PFGE profile of GREA14KL-3, apart from isolate 6 which was previously considered unique. This was the earliest isolate within this cluster and so may have been considered to have a unique PFGE profile when initially tested. Isolates 5 and 18 were considered to be linked by PFGE forming the cluster GREA14KL-4, but were unique when using VNTR. By PFGE isolate 37 was determined to be part of the GREA14KL-7 cluster, but this isolate had a unique profile by VNTR. This isolate was initially identified as a *Klebsiella pneumoniae* by biochemical phenotypic identification, but during this study the isolate was subsequently identified as *Klebsiella oxytoca* by MALDI-ToF (see methods section 2.3.2.5.). Similarly isolate 36 was originally identified as *Klebsiella oxytoca,* but the isolate cultured and processed for this study was identified using MALDI-ToF MS as *Klebsiella pneumoniae*. The initial PFGE profile for this isolate clustered it with isolate 35; which was taken from the same surface at the same time. The VNTR result on this isolate links it with cluster 4 as part of PFGE group GREA14KL-7 rather than GREA14KL-9. With the above noted exceptions all isolates typed by PFGE as GREA14Kl-7 clustered within VNTR cluster 4 (see Table 4-5.).

Isolate No.	PFGE	VNTR
1	Unique	Unique
2	Unique	Unique
3	Unique	Unique
4	Unique	Unique
5	GREA1L-4	Unique
6	Unique	3
7	GREA14KL-7	4a
8	GREA14KL-3	3
9	GREA14KL-3	3
10	Unique	Unique
11	GREA14KL-3	3
12	Unique	Unique
13	Unique	Unique
14	Unique	Unique
15	Unique	Unique
16	Unique	Unique
17	Unique	Unique
18	GREA14KL-4	Unique
19	GREA14KL-7	4a
20	GREA14KL-7	4a
21	GREA14KL-7	4a
22	GREA14KL-7	4a
23	GREA14KL-7	4a
24	GREA14KL-7	4b
25	GREA14KL-7	4a
26	GREA14KL-7	4b
27	GREA14KL-7	4a
28	GREA14KL-7	4a
29	GREA14KL-7	4b
30	GREA14KL-7	4a
31	Unique	Unique
32	GREA14KL-7	4a

33	GREA14KL-7	4a
34	GREA14KL-7	4a
35	GREA14KL-9	Unique
36	GREA14KL-9	4a
37	GREA14KL-7	Unique
38	GREA14KL-7	4d
39	GREA14KL-7	Unique
40	GREA14KL-7	4a
41	GREA14KL-7	4a
NCTC 13368	Unique	Unique
43	GREA14KL-7	4a
44	GREA14KL-7	4d
45	GREA14KL-7	4b
46	Unique	Unique
47	GREA14KL-7	4a
48	GREA14KL-7	4d

Table 4-5 Comparison of PFGE and VNTR results for 48 isolates. VNTR clusters are colour coded: purple = cluster 3 and blue = cluster 4 (light blue = 4b, mid blue = 4a and dark blue = 4d).

4.2.3 Repetitive extragenic palindromic Sequence PCR Validation Against Pulse Field Gel Electrophoresis

4.2.3.1 Determining Similarity Cut-Offs for *Klebsiella* Species Using Repetitive Extragenic Palindromic Sequence PCR

59 isolates detected from both patient and environmental samples during 2011 and 2012 were processed by REP-PCR as described in methods section 2.2.11.1. Isolates including 24 environmental samples and 35 patient samples from 23 patients on seven wards as described in 4.2.1.

No consensus was available for interpretation of isolate relatedness using REP-PCR based linked to percentage similarity. In order to determine a suitable analysis cut-off for outbreak isolates results were analysed using both available analysis algorithms, and different similarity cut-offs (see methods section 2.2.11.1.).

All isolates were interpreted with either Pearson Correlation Co-efficient (PC) or the Kullback-Leibler (KL) analysis using 98%, 95%, 93% and 91% similarity cut-offs to determine clustering. See Table 4-6.

Sample No.	PC 98	PC 95	PC 93	PC 91	KL 98	KL 95	KL 93	KL 91
1	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
2	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
3	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
4	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
5	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
6	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
7	4e	4e	4e	4b	4e	4e	4b	4b
8	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
9	Unique	3	3	3	Unique	3	3	3
10	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
11	Unique	3	3	3	Unique	3	3	3
12	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
13	Unique	Unique	1	1	Unique	Unique	Unique	Unique
14	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
15	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
16	Unique	Unique	1	1	Unique	Unique	Unique	Unique
17	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
18	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
19	4e	4e	4e	4b	4e	4e	4b	4b
20	4d	4d	4d	4d	Unique	4d	4d	4d
21	Unique	4a	4a	4a	Unique	4a	4a	4a
22	4e	4e	4e	4b	4e	4e	4b	4b
23	Unique	Unique	4a	4a	Unique	Unique	Unique	Unique
24	Unique	4a	4a	4a	Unique	4a	4a	4a
25	Unique	4c	4c	4c	Unique	Unique	1	1
26	4e	4e	4e	4b	4e	4e	4b	4b
27	4e	4e	4e	4b	4e	4e	4b	4b
28	4d	4d	4d	4d	4d	4d	4d	4d
29	4d	4d	4d	4d	4d	4d	4d	4d
30	4e	4e	4e	4b	4e	4e	4b	4b
31	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
32	Unique	4e	4e	4b	Unique	4e	4b	4b
33	4f	4b	4b	4b	4f	4b	4b	4b

34	4f	4b	4b	4b	4f	4b	4b	4b
35	8	8	8	8	8	8	8	8
36	Unique	4b	4b	4b	Unique	4b	4b	4b
37	8	8	8	8	8	8	8	8
38	Unique	4b	4b	4b	Unique	4b	4b	4b
39	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
40	5	5	5	5	5	5	5	5
41	4d	4d	4d	4d	Unique	4d	4d	4d
42	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
43	4b	4b	4b	4b	4b	4b	4b	4b
44	Unique	Unique	Unique	4a	Unique	4g	4g	4g
45	Unique	4d	4d	4d	Unique	4d	4d	4d
46	8	8	8	8	Unique	8	8	8
47	Unique	4b	4b	4b	Unique	4g	4g	4g
48	Unique	4e	4e	4b	Unique	4e	4b	4b
S67498	Unique	4a	4a	4a	Unique	4a	4a	4a
S20245	4b	4b	4b	4b	4b	4b	4b	4b
S20253	Unique	4c	4c	4c	Unique	Unique	1	1
S50381	2	2	2	2	2	2	2	2
S50380-1	2	2	2	2	2	2	2	2
S50380-2	2	2	2	2	2	2	2	2
S50404	2	2	2	2	2	2	2	2
S50403	2	2	2	2	Unique	2	2	2
S20255	5	5	5	5	5	5	5	5
S20260	5	5	5	5	5	5	5	5
S20262	5	5	5	5	5	5	5	5

Table 4-6 59 isolates detected in 2011 and 2012 processed by REP-PCR and interpreted with both the Pearson Correlation Co-efficient and the Kullback-Leibler analysis using 98%, 95%, 93% and 91% similarity cut-offs to determine clustering. Isolates where changing analysis type or similarity cut-off altered isolate clustering are coloured red. Isolates with only low intensity traces available are coloured green (samples 31 and 45).

For 39/59 isolates results were unaltered by changing the analysis algorithm or similarity cut-off, as long as sub-clusters of cluster 4 were only counted at the main cluster 4 level (see Table 4-6.). Two isolates 31 and 45 only had low intensity traces available, which may affect data reliability (see methods section 2.2.11.1.)

Two isolates (13 and 16) were determined to be identical from cluster 1 by PC analysis at cut-offs 93% and 91%. Both were however determined to be unique by all KL analysis. Isolate 23 was determined to be unique by KL analysis and was also classed as unique utilising PC analysis with 98% and 95% cut-offs. Isolate 44 was clustered as part of cluster 4 using KL analysis at 95%, 93% and 91% similarity cut-offs, and was considered to be unique when analysed by PC analysis at all thresholds.

For PC analysis, results varied for 13 isolates between the 98% and 95% cut-offs, all of which went from being called unique to being part of a cluster. If the similarity cut-off was adjusted from 95% to 93% isolates 13 and 16 were clustered together instead of being called unique. Finally if a 91% cut-off was used, isolate 44 was considered as part of cluster 4, instead of being considered unique.

When using the KL analysis 16 sample results varied between the 98% and 95% cut-offs, all of which went from being called unique to being part of a cluster. Two results would be altered using a similarity cut-off of 93% instead of 95%, samples 25 and S20243 would cluster together as a new cluster. No results would change by using a 91% threshold instead of a 93% for KL analysis.

When combined with epidemiological information it was felt that using a either a 93% or a 91% cut-off using PC analysis would present an accurate epidemiological picture and therefore the more conservative 93% cut-off was used for data analysis see methods section 2.7.

4.2.3.2 INTRA-LABORATORY REPRODUCIBILITY

Enterobacter spp.

The use of REP-PCR for typing of *Enterobacter* spp. was evaluated through retrospective typing of 18 MDR *Enterobacter* spp. isolates that were detected at GOSH in 2000 and 2001. Samples were processed as discussed in the methods section 2.2.11.1. To determine intra-laboratory reproducibility all extracts were run in at least duplicate. All isolates had also been processed for PFGE by the PHE, which was considered to be the reference standard.[87, 236]

All samples processed attained the minimum levels of amplification required to be included in analysis (see methods section 2.2.11.1.) which was undertaken using the Pearson Correlation Co-efficient with a 93% similarity cut-off (as decided in section 4.2.3.1.).

REP-PCR results for *Enterobacter* spp. demonstrated good reproducibility when replicates of samples were tested, see Figure 4-3 and Figure 4-4. However sample 00B61229a did not cluster with either 00B61229b or 00B61229c.

Reproducibility did not appear to vary as a result of PFGE type. Additionally on the whole samples with the same PFGE group did cluster together by REP-PCR analysis. However, there were a few exceptions: 00B59823 (a and b) and 00B31316 (a and b) clustered within PFGE group B, but by REP-PCR they clustered with one sample linked with PFGE type E (00B4586), and another sample linked to PFGE type C (00B34811b).

REP-PCR could not discriminate between PFGE types: A/D, E or G as demonstrated by the single cluster seen by REP-PCR analysis. PFGE types H and K also clustered together by REP-PCR with the exception of sample 00B38661 (a and b); which although part of PFGE type K clustered separately from all other samples.

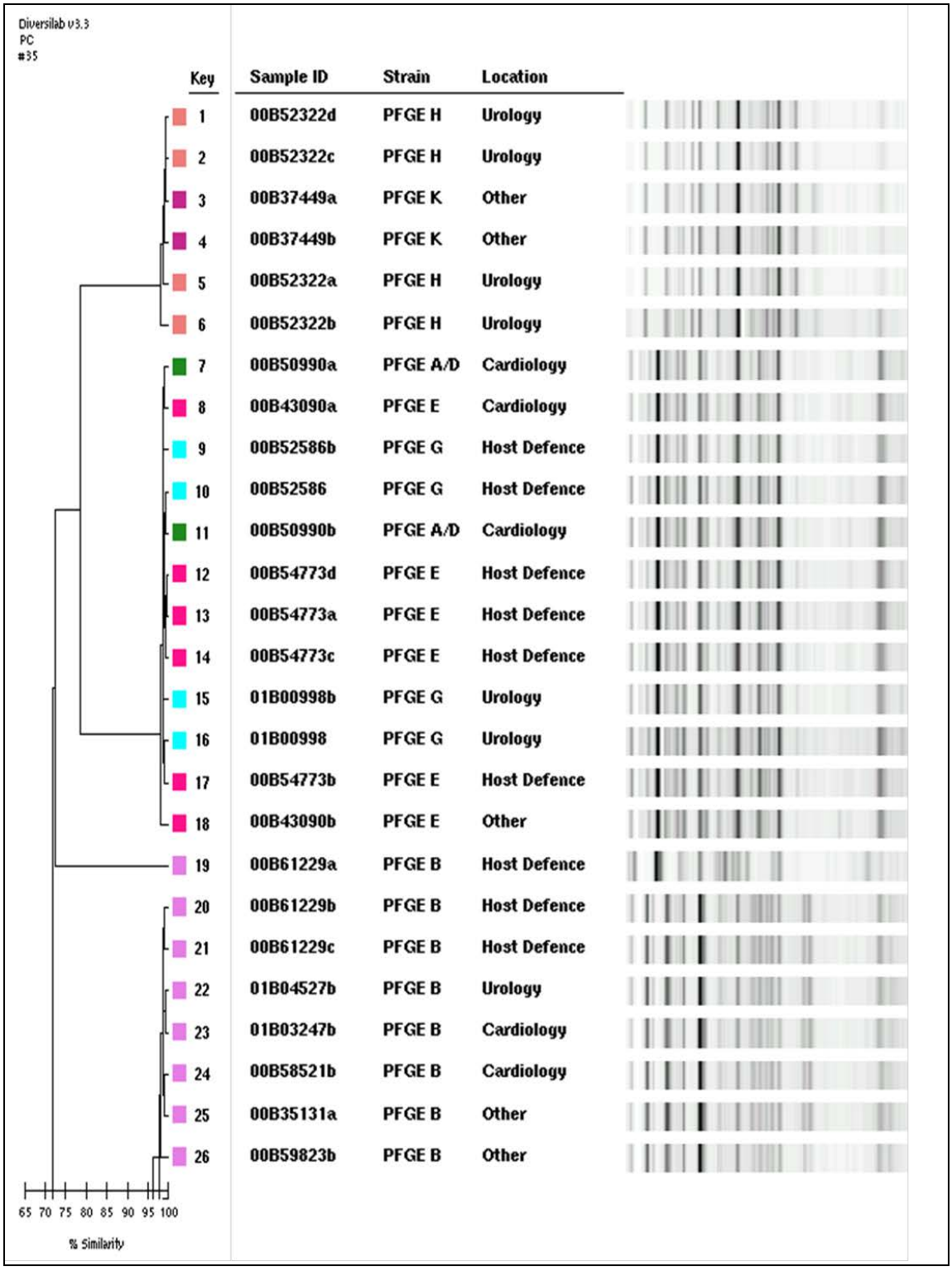

Figure 4-3 Dendrogram demonstrating REP-PCR results for 18 *Enterobacter* spp. isolates from 18 patients detected between 2000-2001 along with with PFGE results. Processing was performed in duplicate and analysed by Pearson Correlation Co-efficient. Colour coding: PFGE type H = peach, PFGE type K = dark pink, PFGE type A/D = green, PFGE type E = bright pink, PFGE type G = aqua, PFGE type B = purple and PFGE type B = light pink (continued on next page).

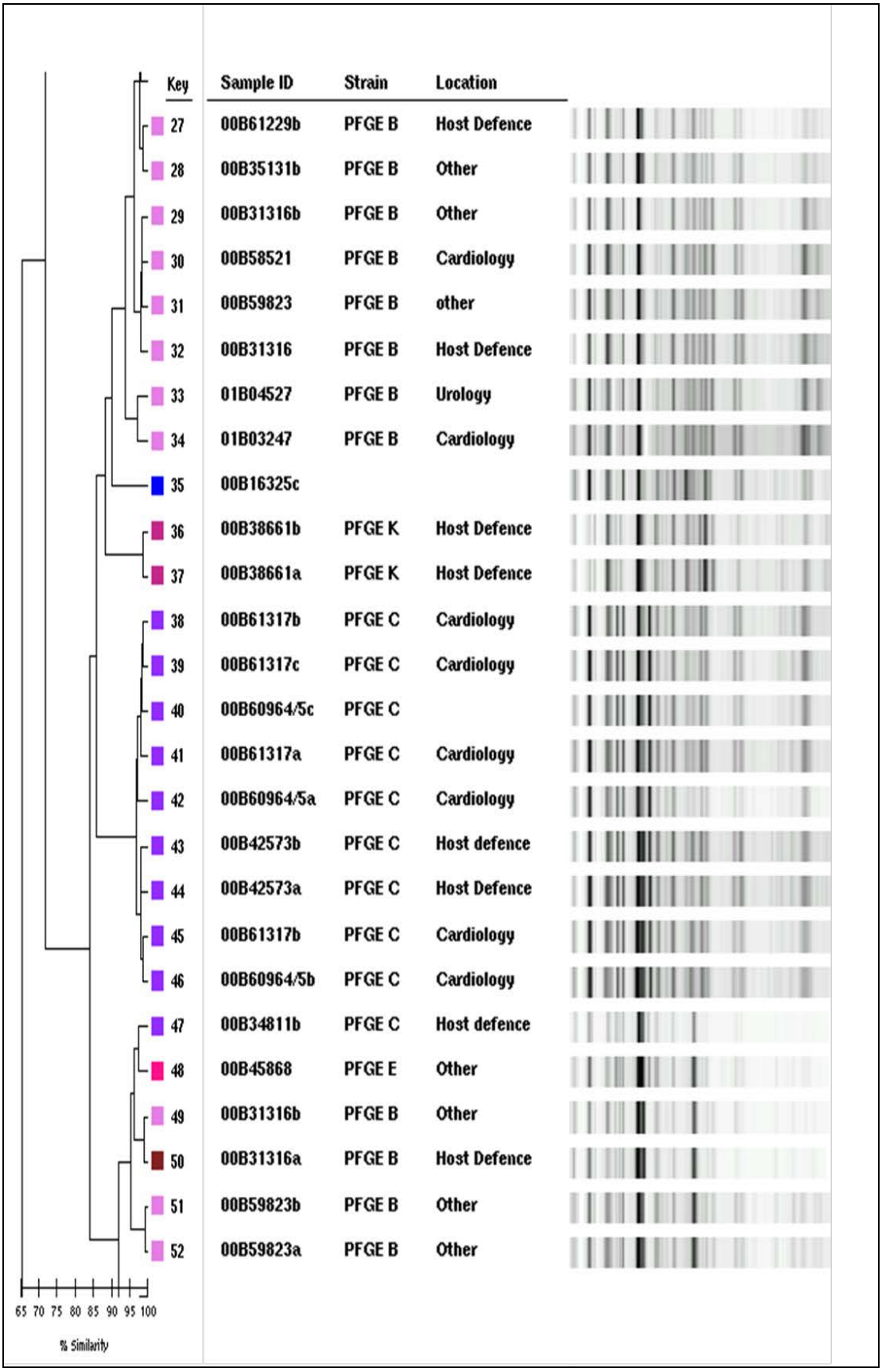

Figure 4-4 Continued from Figure 4-3 above.

Klebsiella Species

The use of REP-PCR for typing of *Klebsiella* spp. was evaluated through retrospective typing of nine isolates. These isolates represented the first resistant isolate of patients newly detected with MDR Gram-negative flora in 2008 as identified within methods section 2.3.2.5., either as carriage or causing infection. To determine intra-laboratory reproducibility all isolates were run in duplicate and analysed using the Pearson Correlation Co-efficient using a >93% similarity cut-off.

All isolate duplicates grouped in pairs at >98% similarity when analysed using the Pearson Correlation Co-efficient, indicating that they were indistinguishable, except for 08B56878 (see Figure 4-5). The fingerprints for 08B56878 and 08B56878A clustered at ≥95% and were identical when overlaid, with one band of 08B56878 demonstrating increased intensity over those seen in 08B56878A. This led to the two samples appearing to be less closely related, but were still considered indistinguishable using 93% similarity as a cut-off. Two unrelated individual samples (08B17954 and 08B0262) were indistinguishable. Further investigation revealed that these isolates related to patients who had not been epidemiologically linked as in-patients, but had attended the same outpatient unit, haematology oncology day unit (HODU), on the same day.

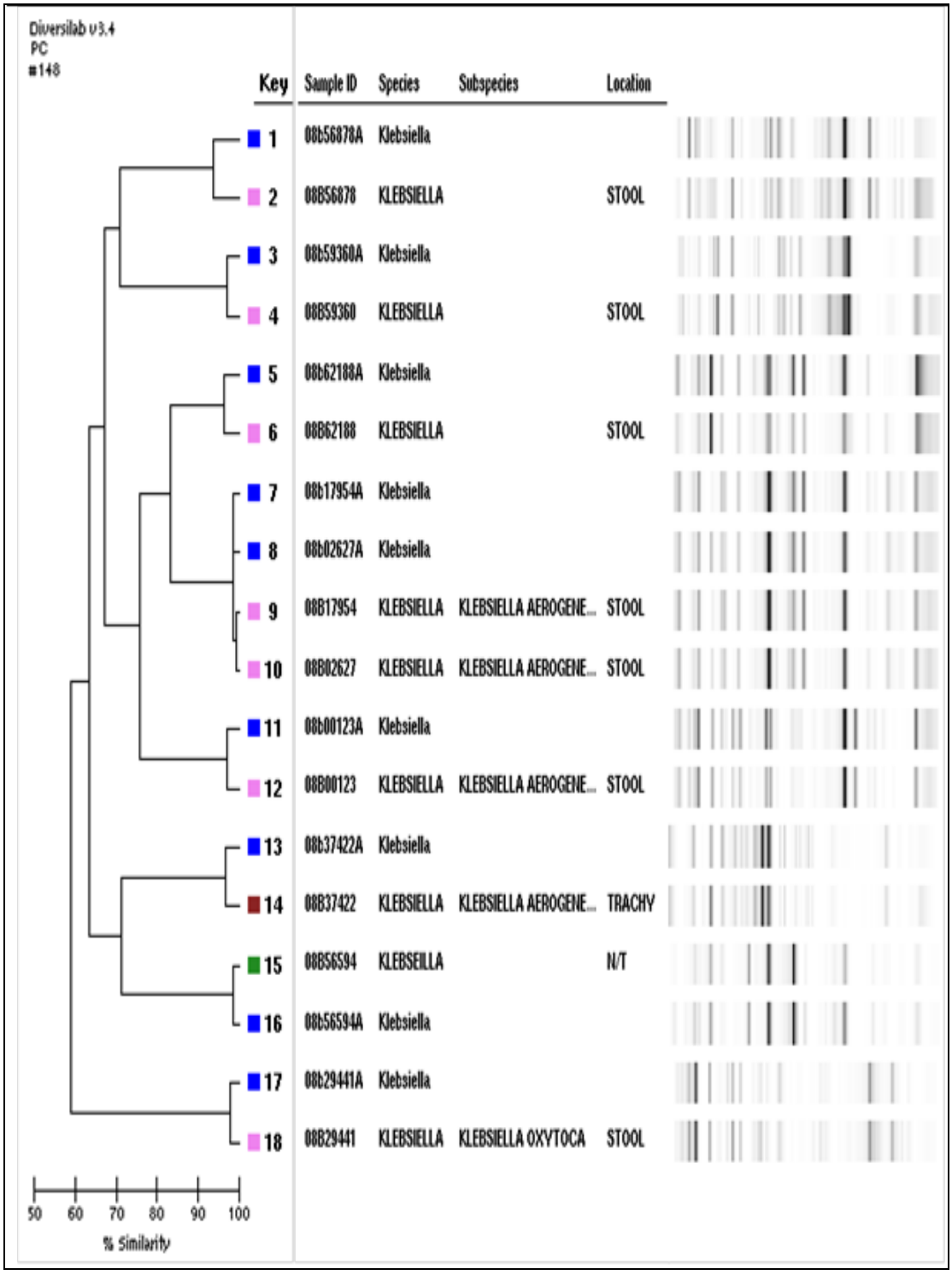

Figure 4-5 Dendrogram demonstrating REP-PCR results of nine *Klebsiella* species isolates from nine patients performed in duplicate and analysed by Pearson Correlation Co-efficient.

E.coli

The use of REP-PCR for typing of *E. coli* was evaluated through retrospective typing of nine isolates. These isolates represented the first resistant isolate of patients newly diagnosed with MDR Gram-negative flora in 2008 as identified within methods section 2.2.11.1. To determine intra-laboratory reproducibility all isolates were run in duplicate and analysed using the Pearson Correlation Co-efficient using a >93% similarity cut-off.

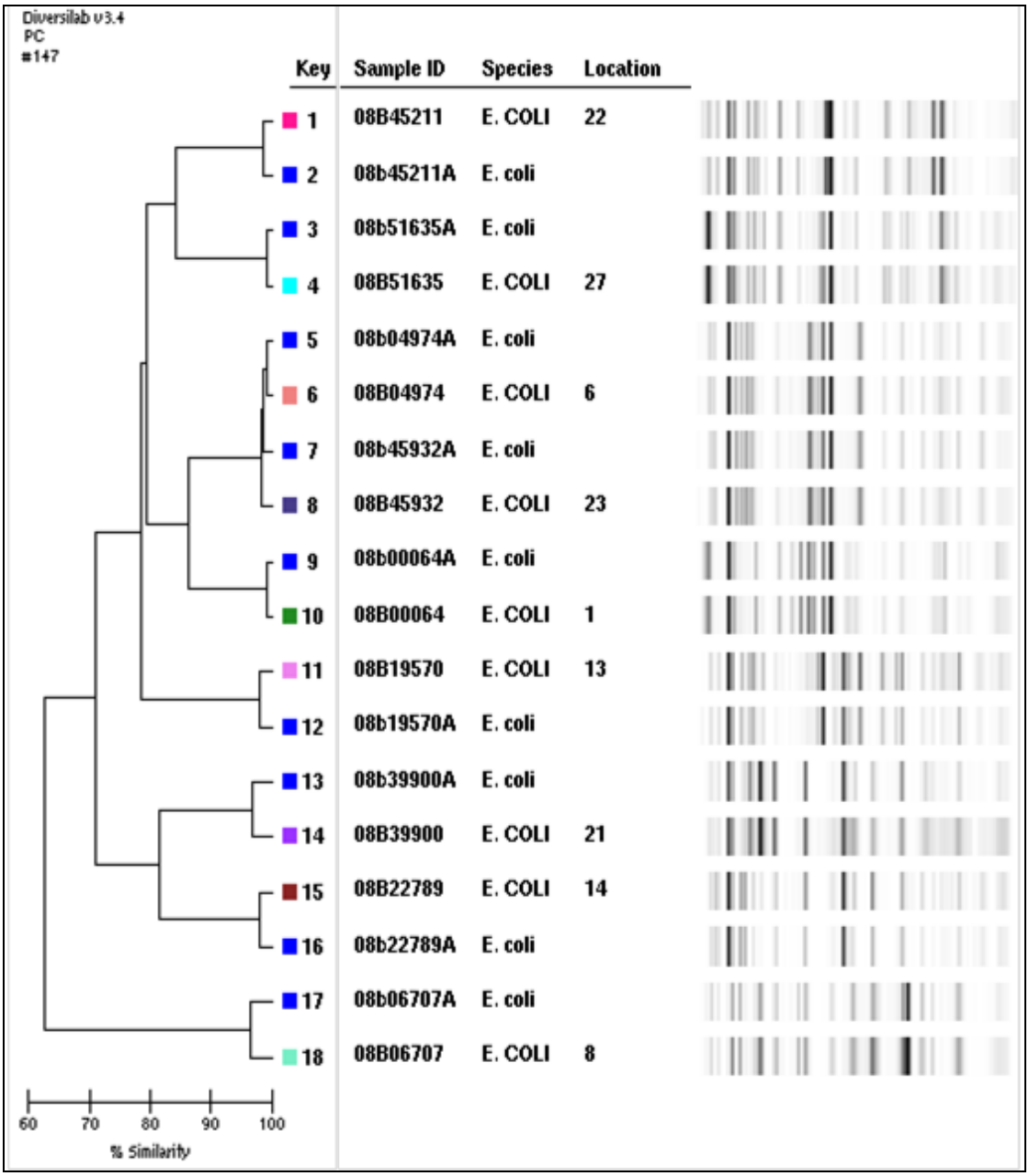

Figure 4-6 Dendrogram demonstrating REP-PCR results of nine *E. coli* isolates from nine patients performed in duplicate and analysed by Pearson Correlation Co-efficient

All *E. coli* duplicates grouped in pairs at >98% similarity when analysed using the Pearson Correlation Co-efficient, indicating that they were indistinguishable using a >93% cut-off (Figure 4-6). In addition two individual samples (08B04974 and 08B45932) were indistinguishable despite having no previously known epidemiological links. Further investigation revealed that these isolates related to patients who had not been epidemiologically linked as in-patients, but had attended the same outpatient unit (HODU) on the same day. These were different patients to those investigated within the above *Klebsiella* REP-PCR section.

4.2.3.3 INTER-LABORATORY COMPARISON

To understand inter-laboratory reproducibility of the REP-PCR typing system, work was undertaken as part of an international study involving 11 participating centres in six countries across Europe (Austria, England, Germany, Spain, The Netherlands) and Canada.[458] In total 39 *E. coli* isolates and 39 *Klebsiella* spp. that had been previously typed by PFGE were selected from the collection of the Hospital Hygiene Department in Bronovo Hospital, Netherlands and were sent out to the 11 centres. The centres were anonymised to the PFGE clustering.

Isolates were processed as discussed in methods section 2.2.11.1. as per the shared study protocol. Data was analysed using the Pearson Correlation Co-efficient with Isolates demonstrating a similarity of <95% considered different and isolates with a similarity >98% considered indistinguishable. Isolates between 95% and 98% analysed by looking at individual peaks to determine relatedness.

Low intensity traces (see methods section 2.2.11.1.) were only used if no satisfactory traces could be acquired after six repeats being processed. In total typing data was available for analysis for 425 of 429 *E. coli* and 422 of 429 *Klebsiella* spp. Four *E. coli* isolates were not processed or determined non-typable (<1%) and one showed no amplification in one of the centres (<1%) and six of the *Klebsiella* spp. samples (1%) were not processed. The analyses by the individual centres were confirmed by the central laboratory.

Klebsiella pneumoniae Comparison Results

PFGE was used as a reference standard to permit comparison of results between centres. PFGE identified one cluster of six isolates, three clusters of three isolates, three clusters of two isolates, and 18 unique isolates. In comparison, our study identified four clusters: 1 containing five isolates, 2 containing two isolates, 3 containing two isolates, 4 containing two isolates plus 28 unique isolates for *Klebsiella* spp. (see Table 4-7). Compared to results seen at other centres our results demonstrated increased levels of unique isolate interpretation for *Klebsiella* spp., although our isolate clustering broadly agreed with other centres.

After six repeat PCRs, four samples failed to demonstrate amplification that met the minimum analysis criteria, i.e. low intensity traces. These isolates (26K, 31K, 33K and 36K) had the most acceptable low intensity trace included in the analysis. All four low intensity traces failed to cluster with any other isolates (see Table 4-7).

Of the isolates that were called unique: sample 33K was called unique by 2/11 centres with one additional centre finding it non-typable. Within our centre, 33K demonstrated low intensity amplification, and may therefore have clustered appropriately with 02K if sufficient amplification had occurred. 5/11 centres identified 02K as unique, and 4/11 centres clustered 33K with another isolate unrelated by PFGE. 31K also failed to amplify sufficiently to permit cluster identification; this isolate by PFGE should have clustered with 26K; which also had low intensity amplification, and 05K which was subsequently determined to be unique in our analysis. 10/11 centres found 05K to be unique by REP-PCR and 6/11 centres found that 26K and 31K clustered together. 36K should have clustered with 09K and 23K according to PFGE typing. 36K demonstrated low intensity amplification; however both 09K and 23K were identified as being unique despite sufficient peak sizes. 4/11 centres found all three of these isolates to be unique, with one centre reporting no growth from these isolates, thus preventing typing.

Finally our centre identified clustering that was not seen with PFGE. Cluster 2 contained 03K and 19K, this finding was repeated by 9/11 centres. Cluster 4 contained isolates 21K and 28K, this clustering was also identified by five other centres. One cluster, 3, contained 38K and 15K, this finding was not repeated in any other centre. Three other centres clustered 38K with 21K, while 7/11 found this isolate to be unique, thus matching the PFGE type.

	PFGE	Centre 8	Centre 9	Centre 4	Centre 1	Centre 10	Centre 11	Centre 6	Centre 2	Centre 3	Centre 7	Centre 5
01K												
04K												
08K												
12K												
20K												
18K												
15K												
27K												
34K												
23K								NV				
09K								NV				
36K								NV				
02K												
33K											NT	
06K												
28K												
05K												
26K												
31K												
11K												
39K												
19K								NV				
03K												
37K												
35K											NT	
21K												
38K												
10K											NT	
29K						NT						
16K												
17K												
24K								NV				
30K						NT						
07K												
13K												
14K								NV				
22K												
25K												
32K												

Table 4-7 Comparison of the local clustering of *Klebsiella* spp. and the clustering of the isolates using PFGE. Isolates belonging to one cluster according to local analysis or PFGE are indicated by the same colour. Isolates left blank were considered unique isolates according to central analysis. GOSH results were those in centre 5. NT: Non-typeable. NV: Non-viable.[458]

E. coli Comparison Results

PFGE was used as a reference standard to permit comparison of results between centres. Overall there was little correlation between PFGE type and REP-PCR type among any of the test centres (see Table 4-8.).

PFGE identified one cluster of six isolates, three clusters of three isolates, three clusters of two isolates, and 18 unique isolates. In comparison within our study eight clusters were identified: 1 containing two isolates, 2 containing three isolates, 3 containing two isolates, 4 containing six isolates, 5 containing seven isolates, 6 containing two isolates, 7 containing 2 isolates and 8 containing two isolates plus 13 unique isolates. Compared to results seen at other centres our results demonstrated that the largest cluster found by three other centres formed two clusters in our centre. Apart from this, the isolate clustering in our centre agreed broadly with the other centres.

After six repeat PCRs, four samples failed to demonstrate amplification that met the minimum analysis criteria, i.e. low intensity traces. These isolates (02E, 15E, 28E and 37E) had the most acceptable low intensity trace included in the analysis. Three of the four isolates with low intensity failed to cluster with any other isolates, the fourth 37E clustered with 18E. 02E and 28E were unique by PFGE, but 15E should have clustered with 33E, 23E, 12E, 20E and 29E. Within this PFGE cluster 20E was also determined to be unique by REP-PCR in our centre, this finding was not repeated in the other testing centres.

The isolates determined to be unique by PFGE included 31E, 32E, 18E, 37E, 14E, 24E, 25E, 13E, 11E, 35E, 07E, 27E, 02E, 04E, 08E, 10E, 21E and 28E. Isolates 31E and 32E clustered together by REP-PCR in 9/11 centres. 18E and 37E clustered by REP-PCR in 7/11 centres. Although determined to be unique by PFGE, isolates 14E, 24E and 25E were identified as identical by 8/11 centres. Two further centres found 14E and 24E to be the same and another determined 24E and 25E to be linked. Isolate 11E was found to be unique by only 1/11 centre. Our centre (Centre 5) found that it clustered with 12E and 13E, with three other centres repeated this finding. Other centres clustered 11E with a number of different isolates (01E, 16E, 38E, 36E, 09E). One unique isolate (07E) by PFGE was clustered with 34E, 03E, 33E, 29E, 30E, 17E, 01E, 16E, 38E, 36E. This finding was not repeated in other centres.

	PFGE	Centre 1	Centre 2	Centre 3	Centre 4	Centre 5	Centre 6	Centre 7	Centre 8	Centre 9	Centre 10	Centre 11
31E												
32E											NT	
22E							NA					
39E											NP	
18E												
37E												
14E												
24E												
25E												
34E												
06E												
03E											NT	
26E												
19E												
05E												
13E												
33E												
23E												
12E												
20E												
15E												
29E												
30E												
17E												
09E												
01E												
16E												
38E											NP	
36E												
11E												
35E												
07E												
27E												
02E												
04E												
08E												
10E								NP				
21E												
28E												

Table 4-8 Comparison of the local clustering of *E. coli* and the clustering of the isolates using PFGE. Isolates belonging to one cluster according to local analysis or PFGE are indicated by the same color. Isolates left blank were considered unique isolates according to central analysis. GOSH results were those in centre 5. NT: Non-typeable. NP: Not processed. NA = No amplification.[(458)]

4.2.3.4 COMPARISON WITH PULSE FIELD GEL ELECTROPHORESIS RESULTS

PFGE was performed on 48 of the 59 *Klebsiella spp.* isolates as described in section 4.2.1 by PHE, which is the reference standard. [236, 459], Of the 48 samples typed using both methods 40/48 (83%) of results were in concordance by the two methods when using a 93% cut-off and PC analysis (see Figure 4-8). 18 isolates were determined to be unique by REP-PCR of which 14 were also considered unique by PFGE.

Of those samples that were discrepant, isolate 8 clustered as part of GREA14KL-3 by PFGE along with samples 9 and 11; however when using REP-PCR analysis this sample was identified as unique.

Isolate 16 was determined to be unique by PFGE, but clustered with isolate 13 by REP-PCR as part of cluster 1.

Isolate 44 was counted as part of cluster GREA14KL-7 by PFGE, but was determined to be unique by REP-PCR.

Isolate 25 was also counted as part of cluster GREA14KL-7 by PFGE, but clustered with sample 20253 (PFGE not performed) by REP-PCR.

Isolate 39 clustered with GREA14KL-7 by PFGE, but was unique by REP-PCR.

Three isolates (35, 37, and 46) clustered together (cluster 8) and were identified as *Klebsiella oxytoca* rather than *Klebsiella pneumoniae* by subsequent MALDI-ToF MS identification. These isolates were originally identified by PFGE as GREA14KL-9, GREA14KL-7 and unique.

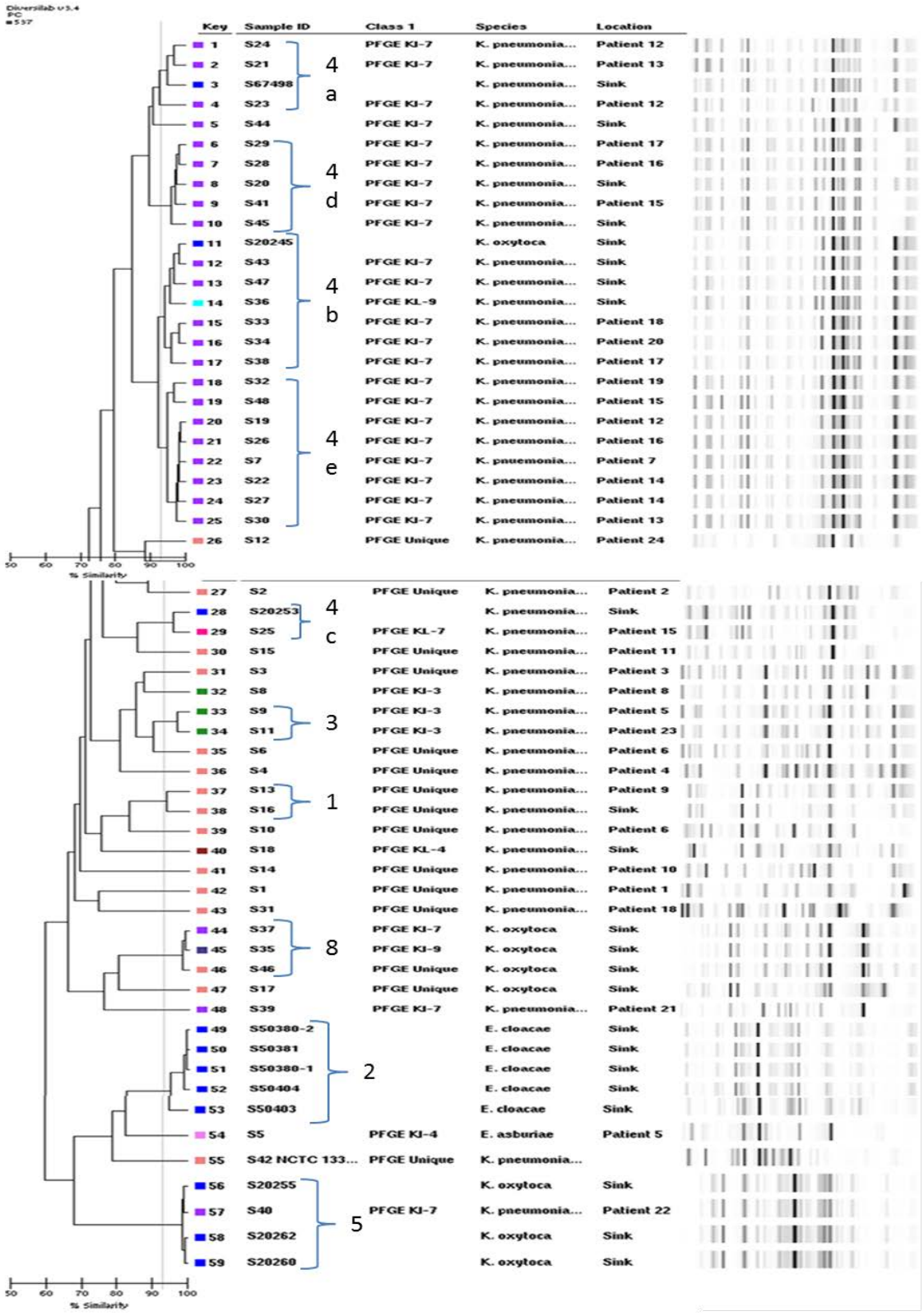

Figure 4-7 REP-PCR clustering of 59 isolates of four species using the Pearson Correlation Co-efficient at a 93% cut-off demonstrating six clusters main clusters (1, 2, 3, 4, 5, 8) with cluster 4 consisting of 5 sub-clusters (4a – 4e). PFGE were colour coded: PFGE KL-7 = purple, PFGE KL-4 = pink, PFGE KI-3 = green, PFGE KL-9 = aqua, PFGE unique = peach and no PFGE results available = blue.

4.2.4 High Throughput Sequencing Validation Against Pulse Field Gel Electrophoresis

4.2.4.1 Pathogenica Typing Analysis Validated Against Pulse Field Gel Electrophoresis

Typing was undertaken using the Pathogenica HAI Biodetection typing kit (Pathogenica, Boston, USA) (as described in the methods sections 2.2.9 and 2.8.4.) on 48 *Klebsiella pneumoniae* isolates that were linked to both patients and the environment, see section 4.2.1. One isolate (5) was subsequently identified as *Enterobacter cloacae* by the Pathogenica kit and *Enterobacter asburiae* by MALDI-ToF and so this was not included in the analysis. Six main clusters were identified (1, 2, 3, 4, and 7). Cluster numbering was selected to maintain consistency across typing technique analysis, see Figure 4-8. The main cluster (Cluster 4) included the large PFGE cluster GREA14KL-7, which it sub-clustered into five groups (4a – 4e). In addition there was a more distant cluster, cluster 8; which contained isolates later identified by MALDI-ToF MS identification to be *Klebsiella oxytoca* (see methods section 2.3.2.5.).

Comparison of PFGE and Pathogenica results demonstrated concordance in 33/47 (70%) of results. Five isolates were considered unique by both Pathogenica (isolates 1, 3, 4, 14, 15), and by PFGE. Pathogenica clustered three isolates which were unique by PFGE (2, 10, and 12) within a new cluster, cluster 2. Another unique isolate, isolate 6, clustered with isolates 8, 9 and 11, all of which havehad the PFGE type GREA14KL-3. Samples 13 and 16 had unique PFGE types, but cluster together by Pathogenica. Three unique isolates by PFGE (31, 39, and 42) formed cluster 7 by Pathogenica. Isolate 36 was identified by PFGE as GREA14KL-9, but was linked to PFGE cluster GREA14KL-7 by this HTS analysis.

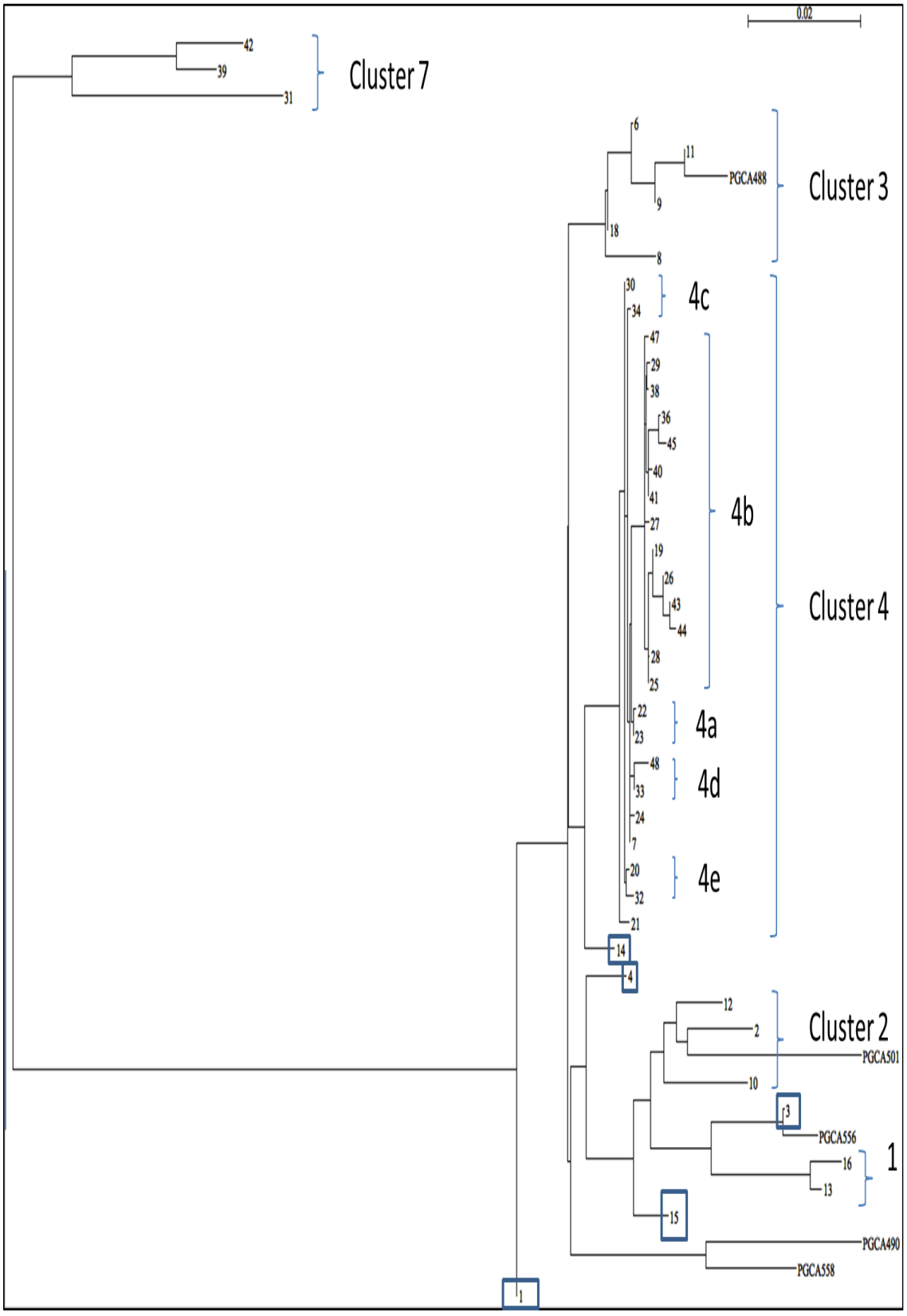

Figure 4-8 Pathogenica typing of 43 *Klebsiella pneumoniae* isolates, identifying five clusters (1, 2, 3, 4, and 7) with sub-clustering identified within cluster 4 (4a – 4e). Blue boxes indicate unique isolates by Pathogenica analysis.

Four isolates (17, 35, 37, and 46) clustered together within cluster 8 and were identified as *Klebsiella oxytoca* rather than *Klebsiella pneumoniae* on subsequent identification (see Figure 4-9.). These isolates were originally identified by PFGE as GREA14KL-9, GREA14KL-7 and unique.

Figure 4-9 Pathogenica typing cluster 8 demonstrating the relationship between four *Klebsiella oxytoca* isolates.

4.2.4.2 MiSeq Data Analysis Validated Against Pulse Field Gel Electrophoresis

48 isolates underwent whole genome sequencing (WGS) on the Miseq platform (Illumina, San Diego, USA) as described in methods section 2.2.8. The 48 isolates consisted of samples linked to both the environment and patients on two wards, with additional patient samples from five other wards. These represented one large and three small clusters of isolates identified as indistinguishable by PFGE and isolates identified as unique (see section 4.2.1.). Data from the MiSeq run was then sent to two different bioinformatics groups for phylogenetic analysis and the results subsequently compared.

Full single nucleotide variant (SNV) analysis was not undertaken between samples as insufficient published information exists to determine the molecular clock within *Klebsiella* spp. and therefore the significance of individual SNVs.[51, 460] However numbers of SNVs within clusters was used as a measure of cluster diversity.

Four of these 48 isolates were identified as *Klebsiella oxytoca* on fresh identification by MALDI-ToF MS analysis (see methods section 2.3.2.5.). These were included in MiSeq analysis one, but not in MiSeq analysis two. The isolate from sample 5 was identified as *Enterobacter asburiae* by MALDI-ToF MS and WGS data and was therefore not included in the phylogenetic analysis.

Inter-Laboratory Analysis Comparison

MiSeq WGS on the 48 isolates was analysed independently by two separate centres to identify analysis reproducibility. One blinded analysis was undertaken by Alex Rolfe (MiSeq analyais one) and the other by Piklu Bhattacharya (MiSeq analysis two) as described in methods sections 2.8.1 and 2.8.2.

MiSeq Data Analysis One

MiSeq analysis one identified five clusters of related isolates (2, 3, 4, 7 and 8), with cluster 8 representing *Klebsiella oxytoca,* see Figure 4-10.

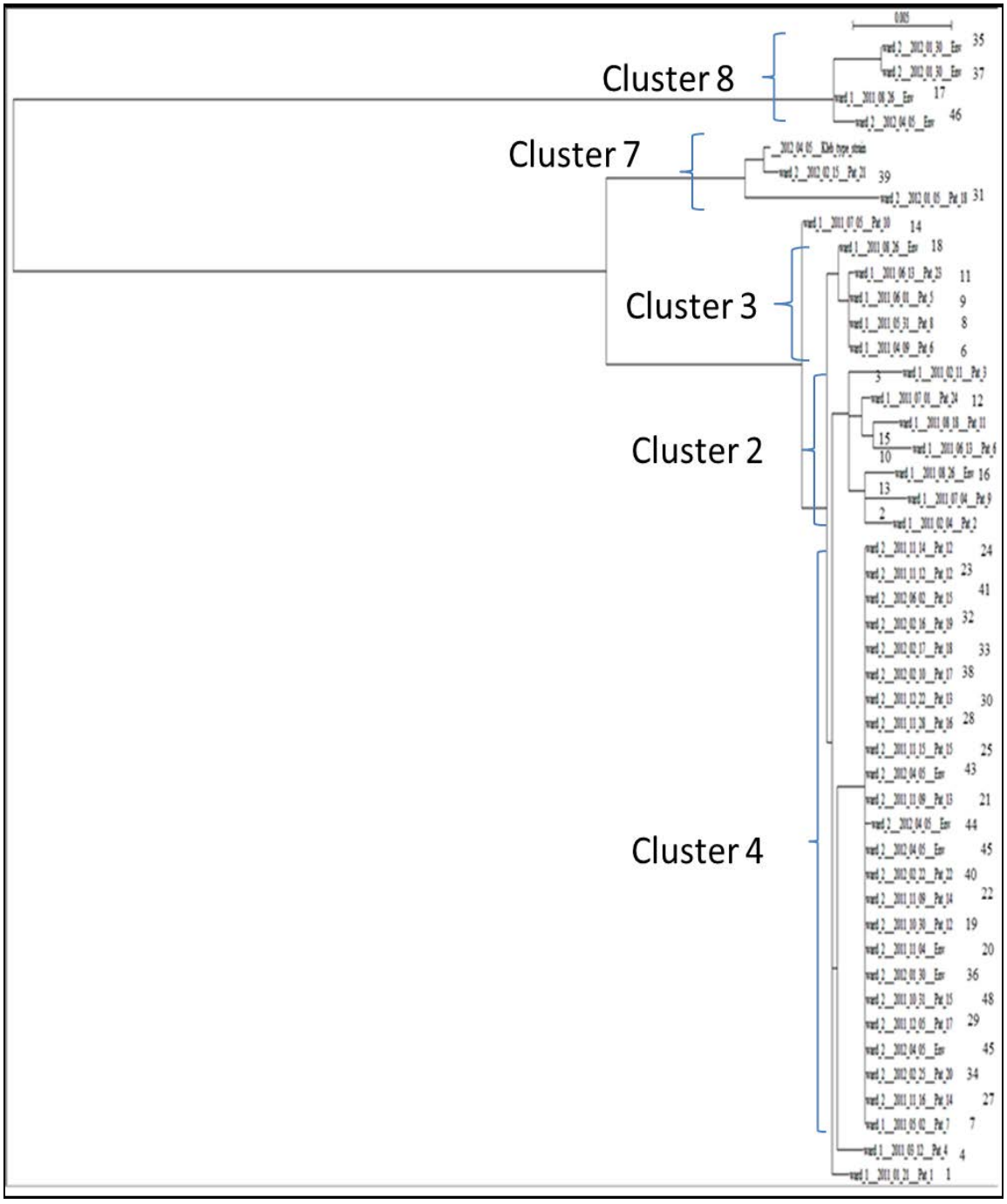

Figure 4-10 Analysis of MiSeq sequencing data by Alex Rolfe including 47 *Klebsiella* spp. isolates demonstrating five clusters (2, 3, 4, 7, and 8). Cluster 8 consists of *Klebsiella oxytoca* isolates, PFGE cluster GREA14KL-7 is represented by cluster 4.

Comparison of the analysed WGS data to PFGE results showed concordance in 31/47 (66%) of isolates. Three isolates were identified as unique during the analysis, all of which were also unique by PFGE. The other isolates formed five clusters (2, 3, 4, 7 and 8), the largest of which was cluster 4, which had no sub-clusters. Cluster 4 contained isolates identified as GREA14KL-7 by PFGE. Of the results that did not match with PFGE isolates 2, 3, 10, 12, 13 and 15 all formed one cluster, all of which were unique by PFGE. Cluster three consisted of five isolates (6, 18, 8, 9 and 11). Isolates 8, 9 and 11 were part of the PFGE GREA14KL-3 cluster, isolate 6 was unique and isolate 18 was typed as GREA14KL-4. The four isolates identified as *Klebsiella oxytoca* formed cluster 8 and had been identified by PFGE as either unique, GREA14KL-9 or GREA14KL-7. Three unique isolates S31, S39, S42 by PFGE formed the novel cluster 7. Finally isolate 36 which had been identified as GREA14KL-9 by PFGE was placed within cluster 4, along with isolates typed as GREA14KL-7 by PFGE.

MiSeq Data Analysis Two

As the four *Klebsiella oxytoca* isolates were excluded from this analysis, comparison was done on the remaining 43 *Klebsiella pneumoniae* isolates, see Figure 4-11. Identical clustering was seen for 28/43 (65%) of isolates with both PFGE and WGS.

Samples fell into six clusters (1, 2, 3, 4, 5 and 7), with one unique isolate (14) that was also unique by PFGE. Of the remaining unique isolates by PFGE, isolates 1, 3, and 15 formed cluster 2. Isolates 2 and 4 formed cluster 5 and isolates 31, 39 and 42 formed cluster 7. Isolate 39 had been typed by PFGE as GREA14KL-7, other isolates of which formed cluster 4. All the other isolates in cluster 7 were typed as unique by PFGE. Cluster 1 was formed of isolates 10, 13 and 16 all of which were unique by PFGE. Sample 18, which was identified as GREA14KL-4 by PFGE, joined samples 6, 8, 9, and 11 to form cluster 3. Sample 6 had previously been classified as unique. Sample 36 was identified by PFGE as GREA14KL-9, but was identified as part of cluster 4 alongside isolates PFGE typed as GREA14KL-7 in this analysis.

Samples within cluster 4 appeared identical with no sub-clusters identified

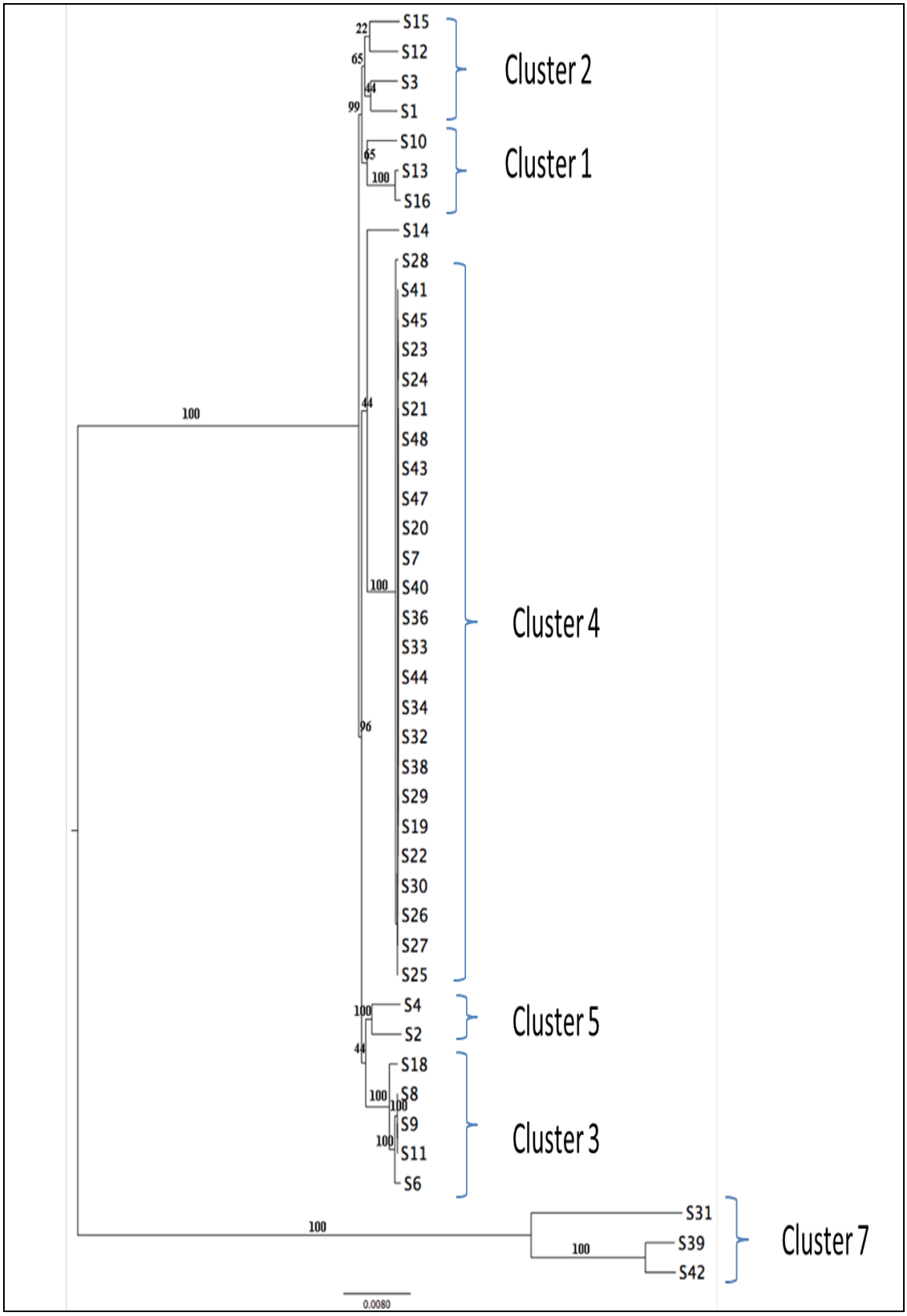

Figure 4-11 Analysis of MiSeq sequencing data by Piklu Bhattacharya including 43 *Klebsiella pneumoniae* isolates. demonstrating six clusters (1, 2, 3, 4, 5, and 7). PFGE cluster GREA14KL-7 is represented by cluster 4.

Cluster Number	Total SNVs Within the Cluster
1	11,842
2	19,313
3	3727
4	1139
5	11,168
7	60,012

Table 4-9 SNV analysis of the total number of SNVs within clusters 1, 2, 3, 4, 5 and 7 identified by MiSeq analysis two.

Within the clusters there was variation in the number of SNVs detected (see Table 4-9.). Within clusters 3 and 4 there were considerably fewer SNVs than within the other clusters. Clusters 3 and 4 were examined further with the addition of epidemiological information and were considered to be isolates detected linked to *Klebsiella pneumoniae* cross transmission events on two wards (see methods section 2.7.).

MiSeq Analysis Final Results

Six results varied between MiSeq analysis one and two, isolates 1, 2, 4, 10, 13 and 16 (see Table 4-10.). These altered results were re-analysed with further epidemiological information in order to produce a set of final MiSeq results for further typing comparison

Isolate No.	MiSeq Analysis One	MiSeq Analysis Two	MiSeq Final Analysis
1	Unique	2	Unique
2	2	5	2
3	2	2	2
4	Unique	5	Unique
5	ND	ND	ND
6	3	3	3
7	4	4	4
8	3	3	3
9	3	3	3
10	2	1	2
11	3	3	3
12	2	2	2
13	2	1	2
14	Unique	Unique	Unique
15	2	2	2
16	2	1	Unique
17	8	ND	8
18	3	3	3
19	4	4	4
20	4	4	4

21	4	4	4
22	4	4	4
23	4	4	4
24	4	4	4
25	4	4	4
26	4	4	4
27	4	4	4
28	4	4	4
29	4	4	4
30	4	4	4
31	7	7	7
32	4	4	4
33	4	4	4
34	4	4	4
35	8	ND	8
36	4	4	4
37	8	ND	8
38	4	4	4
39	7	7	7
40	4	4	4
41	4	4	4
42	7	7	7
43	4	4	4
44	4	4	4
45	4	4	4
46	8	ND	8
49	4	4	4
48	4	4	4

Table 4-10 Comparison of MiSeq anlaysis one and MiSeq analysis two results, with a final results informed by epidemiological analysis. Green colour coding = results that differ between MiSeq analysis one and MiSeq analysis two. ND = not done.

The six results that varied between MiSeq analysis one and two had been selected for typing validation studies as they were all unique by PFGE. As such no epidemiological investigation had been undertaken of the isolates in under to determine if they were cases of cross transmission between patients. In order to interpret the two differing sets of results in order to determine a final MiSeq result isolates 1, 2, 4, 10, 13 and 16 were investigated further and results combined with epidemiological information (see section 2.7) .

Isolate 1 was from a sample collected from patient 1; this patient did not cross over as an inpatient with any other patients that were identified as having isolates as part of cluster 2. The sample was thus determined to be unique.

Isolate 2 was from sample 2 collected from patient 2 and could form part of either cluster 2 or 5. This patient did not cross over as an inpatient with any other patients

that were identified as having isolates as part of cluster 2. This patient also did not overlap with the other patient (patient 4) that was determined to be part of cluster 5. This patient was in two weeks before patient 9 (isolate 13) and therefore it was determined that the isolate should cluster within cluster 2, to allow for the possibility of environmental transmission.

Isolate 10 was from a sample taken from patient 25 and was determined to part of either cluster 1 or cluster 2. Patient 25 overlapped with other patients that were part of cluster 2 but not those in cluster 1 and so was considered to be part of cluster 2.

Isolate 13 belonged to patient 9 and was analysed to be part of either cluster 2 or cluster 1. Although this patient did not have any overlapping inpatient stays with other patients, they had been an inpatient two weeks after patient 2. This isolate was thus determined to be part of cluster 2, in order to allow for environmental cross transmission,

Isolate 16 was cultured from an environment sample taken from the cardiac intensive care unit (CICU) where cross transmissions identified in cluster 3 occurred. None of the other patients that formed part of cluster 1 or 2 had ever been an inpatient on the unit. This isolate was the sole remaining isolate in cluster 1 and so this isolate was determined to be unique.

4.2.4.3 PHYLOSHIFT DATA ANALYSIS OF MISEQ DATA VALIDATED PULSE FIELD GEL ELECTROPHORESIS

Phyloshift data analysis based on 37 genes was undertaken on MiSeq WGS data of *Klebsiella pneumoniae* 47 isolates as described in methods section 2.8.3. [461] The analysis revealed seven clusters (1, 2, 3, 4, 5, 7 and 8) and one unique sample, isolate 28 (see Figure 4-12). 26/47 (55%) of isolates clustered identically with PFGE with Phyloshift, Subsequent to sample processing, four isolates of the 47 were identified as *Klebsiella oxytoca* by MALDI-ToF analysis (2.3.2.5.).

There were no isolates that were determined to be unique by both PFGE and Phyloshift and analysis. The unique sample by Phyloshift was isolate 28; which had a PFGE profile of GREA14KL-7. All other PFGE profiles for GREA14KL-7 clustered within cluster 4, except isolate 37 which was subsequently identified as being a *Klebsiella oxytoca*.

Phyloshift clustered isolates 1, 3, 10 and 12 together into cluster 2, all of which were unique by PFGE. Isolates 2, 8, 9, 11, and 18 formed cluster 3. Within cluster 3, isolate 2 was unique by PFGE, isolate 18 was GREA14KL-4 and the others were identified as forming cluster GREA14KL-3.

Isolates 4 and 6 were unique by PFGE but comprised cluster 5 in this analysis. Cluster 1 was formed of isolates 13 and 16, both of which were unique by PFGE. Other unique PFGE isolates (42, 39, and 31) formed a single cluster, cluster 7.

Cluster 8 was comprised of *Klebsiella oxytoca* isolates 35, 46, 37 and 17. Isolates 17 and 46 were unique by PFGE. Isolate 35 was grouped as GREA14KL-9 by PFGE and isolate 37 was part of GREA14KL-7. The only other isolate not to match with PFGE type was sample 36 which had a PFGE type of GREA14KL-9, but typed using Phyloshift as part of the GREA14KL-7 cluster.

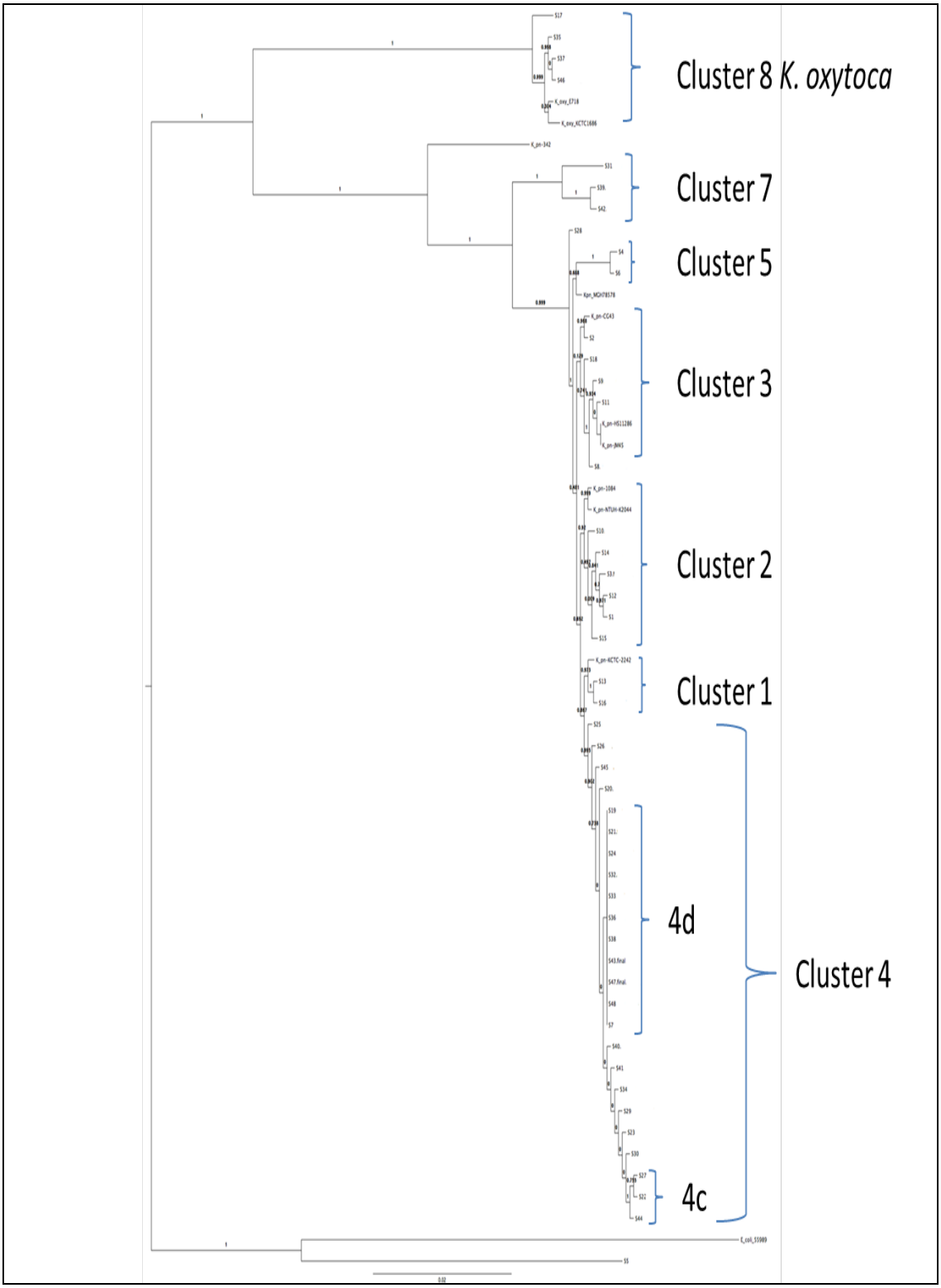

Figure 4-12 Analysis of MiSeq sequencing data by Phyloshift analysis including 47 *Klebsiella* species isolates. demonstrating seven clusters (1, 2, 3, 4, 5, 7 and 8). PFGE cluster GREA14KL-7 is represented by cluster 4, with sub-clustering present. Cluster 8 is comprised of Klebsiella *oxytoca* isolates.

4.2.5 Comparison of Outbreak Investigation Typing Results for Fragment Analysis Techniques, High Throughput Sequencing and Whole Genome Sequencing

In order to compare the typing techniques validated in sections 4.2.2, 4.2.3 and 4.2.4 48 isolates were typed using all techniques (VNTR, REP-PCR, HTS and WGS). Of these 48 isolates 25 were related to the outbreak described in section 4.2.5.1 and were tested retrospectively and compared see how typing techniques would perform in an outbreak investigation.

4.2.5.1 Klebsiella pneumoniae Outbreak

Twelve patients over eight months had a first detection of presumed acquired *Klebsiella pneumoniae* on the HSCTU at GOSH, detected as part of routine admission/weekly surveillance screens in 2011 and 2012 (see Table 4-11). All isolates were extended spectrum β-lactamase (ESBL) positive and resistant to Ceftazidime, Ciprofloxacin, Gentamicin and Piptazobactam. All isolates were sent for typing by PFGE and were determined to be indistinguishable (designated strain identification GREA14KL-7). Patients had been admitted to the HSCTU for between 0 and 299 days with a mean admission prior to acquisition of 51 days. Two patients were positive for the outbreak strain on day 0, both of these patients had overlapped with discharged carriers of the same strain on the HODU prior to admission.

The timeline of the outbreak is shown in Table 4-11. Patient 7 was admitted to the HSCTU on the 8/10/10 and remained until the 27/07/11. During this time he was first detected as positive for the outbreak strain on the 02/05/2011. He was then re-admitted to the unit on the 08/08/11 until the 18/10/11. During this second admission period, one other patient (patient 23) was detected as positive in September, three patients in October and two patients in November. One patient was admitted with the outbreak strain in December (patient 17) and another was admitted with the outbreak strain in January (patient 18). In January two additional patients also acquired the outbreak strain whilst on the unit. No further cases were detected until May, at which point there was one further patient acquisition (patient 22) prior to the end of the outbreak.

Seven patients (patients 12, 13, 14, 15, 16, 17 and 18) had multiple isolates typed during the outbreak period. Positive samples included stool and urine samples. Time from detection of the resistant strain to receiving a typing result for these isolates by PFGE was three weeks on average.

In addition to patient screening, 151 swabs were taken from both cubicles of positive patients and the shared ward area. Of the initial 21 swabs taken from the shared ward area, only one site was positive for *Klebsiella pneumoniae*, the intravenous (IV) medication room sink, where clinical equipment was cleaned.

Figure 4-13 Image of the intravenous medication room equipment sink on the HSCTU where the outbreak strain of *Klebsiella penumoniae* was detected in both 2011 and 2012.

During the subsequent environmental screen post infection control interventions, 130 swabs were taken (discussed in Chapter 5). Twenty sites were sampled using the methods developed in Chapter 3 within each of five cubicles and a further 30 swabs were taken from the shared ward area. No sites within the cubicles of known carriers were positive despite long admissions within the same cubicle, one patient had been positive within the same cubicle for over three months. Within the shared ward areas three sites were positive for *Klebsiella pneumoniae* including: the IV room sink and lips, the medication room sink back and the entrance sink plug hole. In addition to *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Enterobacter* spp. were isolated from these swabs. These isolates demonstrated identical antibiograms to the outbreak strain and the *Klebsiella pneumoniae* isolates shared the same PFGE type.

Interventions linked to this outbreak related to cleaning and design will be discussed further in Chapter 5.

Patient	Admissions with Date of Detection												
Patient 7	admitted to ward 08/08/11 already positive - 299 PIP												
Patient 23		detected 13/09/2011 - 94 days PIP											
Patient 13			detected 17/10/2011 - 20 PIP										
Patient 15			detected 20/10/2011 - 21 PIP										
Patient 12			detected 30/10/2011 - 27 days PIP										
Patient 16				detected 07/11/2011 - 112 PIP									
Patient 14				detected 09/11/2011 - 15 PIP									
Patient 17					detected 05/12/2011 - 0 PIP								
Patient 18						detected 05/01/2012 - 0 PIP							
Patient 19						detected 16/01/2012 - 13 PIP							
Patient 20						detected 25/01/2012 - 8 PIP							
Patient 22										detected 22/05/2012 - 42 PIP			
	08/2011	09/2011	10/2011	11/2011	12/2011	01/2012	02/2012	03/2012	04/2012	05/2012	06/2012	07/2012	08/2012

Table 4-11 *Klebsiella pneumoniae* on the HSCTU outbreak timeline by month with the number of consecutive inpatient days prior to colonisation (PIP) within GOSH per patient. Green = un-colonised period of admission to HSCTU, Red = colonised period of admission to HSCTU.

4.2.5.2 COMPARISON OF TYPING TECHNIQUE RESULTS

Forty eight isolates were investigated using PFGE, VNTR, REP-PCR, Pathogenica HTS, Phyloshift data analysis and WGS and the results for typing methods compared, see Table 4-12. The 48 samples consisted of temporally linked isolates detected from both the environment and patients during 2011 and 2012. Isolates were selected on the basis of PFGE type with a single large cluster of 25 isolates that were identical by PFGE (GREA14KL-7) and were believed to be linked to the outbreak described in section 4.2.5.1. Three other small clusters of identical PFGE types were chosen, one where cross transmission was believed possible on the cardiac intensive care unit (CICU) but had not been confirmed (GREA14KL-3) and two where there was no crossover of patients and so the isolates were considered unconnected (GREA14KL-4 and GREA14KL-9). Additional isolates were selected as they were unique by PFGE. In addition to the single ward where an outbreak was believed to have occurred (HSCTU), isolates came from a number of different wards, mostly surgical and private patient wards. One isolate was from a sample collected within outpatients from patient 3 (isolate no. 3). Environmental isolates originated from samples processed from two wards (HSCTU and CICU) where cross transmission may have occurred.

PFGE	VNTR	REP-PCR (93% PC)	Pathogenica	Phyloshift	MiSeq Final Analysis	Isolate No.	Isolate Source
Unique	Unique	Unique	7	7	7	42	*Klebsiella* spp. type strain
Unique	Unique	Unique	7	7	7	31	Patient 18
Unique	Unique	Unique	Unique	5	Unique	4	Patient 4
Unique	Unique	Unique	Unique	2	Unique	1	Patient 1
Unique	Unique	Unique	Unique	2	2	3	Patient 3
Unique	Unique	Unique	2	3	2	2	Patient 2
Unique	Unique	Unique	2	2	2	10	Patient 25
Unique	Unique	Unique	2	2	2	12	Patient 24
Unique	Unique	Unique	Unique	2	Unique	14	Patient 10
Unique	Unique	Unique	Unique	2	2	15	Patient 11
Unique	Unique	1	1	1	2	13	Patient 9
Unique	Unique	1	1	1	Unique	16	Environmental
Unique	Unique	Unique	8	8	8	17	Environmental #
Unique	Unique	8	8	8	8	46	Environmental #
Unique	3	Unique	3	5	3	6	Patient 6
GREA14KL-4	Unique	Unique	ND	ND	ND	5	Patient 5 *
GREA14KL-4	Unique	Unique	3	3	3	18	Environmental
GREA14KL-9	Unique	8	8	8	8	35	Environmental #
GREA14KL-9	4a	4b	4b	4d	4	36	Environmental
GREA14KL-3	3	Unique	3	3	3	8	Patient 8
GREA14KL-3	3	3	3	3	3	9	Patient 5
GREA14KL-3	3	3	3	3	3	11	Patient 23
GREA14KL-7	Unique	Unique	7	7	7	39	Patient 21
GREA14KL-7	Unique	8	8	8	8	37	Environmental #
GREA14KL-7	4a	Unique	4b	4	4	25	Patient 15
GREA14KL-7	4a	Unique	4b	4	4	40	Patient 22
GREA14KL-7	4d	Unique	4b	4c	4	44	Environmental
GREA14KL-7	4a	4d	4b	Unique	4	28	Patient 16
GREA14KL-7	4a	4c	4	4d	4	7	Patient 7
GREA14KL-7	4a	4c	4b	4d	4	19	Patient 12
GREA14KL-7	4a	4d	4e	4	4	20	Environmental

GREA14KL-7	4a	4a	4	4d	4	21	Patient 13
GREA14KL-7	4a	4c	4a	4c	4	22	Patient 14
GREA14KL-7	4a	4a	4a	4	4	23	Patient 12
GREA14KL-7	4b	4a	4	4d	4	24	Patient 12
GREA14KL-7	4b	4c	4b	4	4	26	Patient 16
GREA14KL-7	4a	4c	4b	4c	4	27	Patient 14
GREA14KL-7	4b	4d	4b	4	4	29	Patient 17
GREA14KL-7	4a	4c	4c	4	4	30	Patient 13
GREA14KL-7	4a	4c	4e	4d	4	32	Patient 19
GREA14KL-7	4a	4e	4d	4d	4	33	Patient 18
GREA14KL-7	4a	4e	4c	4	4	34	Patient 20
GREA14KL-7	4d	4e	4b	4d	4	38	Patient 17
GREA14KL-7	4a	4d	4b	4	4	41	Patient 15
GREA14KL-7	4a	4b	4b	4d	4	43	Environmental
GREA14KL-7	4b	4d	4b	4	4	45	Environmental
GREA14KL-7	4a	4b	4b	4d	4	47	Environmental
GREA14KL-7	4d	4c	4d	4d	4	48	Patient 15

Table 4-12 Comparison of 48 isolates from both environmental and patient samples by PFGE, VNTR, REP-PCR, Pathogenica, Phyloshift and MiSeq typing techniques. Results organised by PFGE result, with difference between fragment analysis techniques (PFGE, VNTR and REP-PCR) colour coded in purple and differences between sequence based techniques (Pathogenica, Phyloshift and MiSeq final analysis) colour coded in green. * = *E. asburiae* # = *K. oxytoca*.

Overall there was good agreement with typing of the outbreak strain GREA14KL-7, but agreement between other schemes about isolates unique by PFGE was less consistent.

When comparing samples across all techniques 22/43 (51%) of isolates clustered identically across all typing methods. All consensus isolates were either GREA14KL-7 or GREA14KL-3 by PFGE. Unique isolates were detected more infrequently in sequence based analysis techniques, with only 10 samples being identified as unique across all three analysis types (Pathogenica, Phyloshift, MiSeq final analysis). This compared to 53 isolates determined as unique using fragment analysis techniques. No isolates were unique across all typing methods.

Typing of Multiple Isolates on the Same Patient

Patients 12, 13, 14, 15, 16, 17 all had multiple samples submitted for typing during the outbreak on HSCTU, all of which typed as linked to the outbreak strain. Typing included isolates (48 and 41) from one patient (patient 15) taken seven months apart, with consistent results. Only one patient, patient 18, had different typing results for the same species between samples. One isolate grown from a urine sample on the 5th January 2012 was typed by PFGE, VNTR and REP-PCR as unique and by other methods as part of cluster 7. The second isolate from this patient was cultured from a stool sample taken on the 17th February 2012 and typed as part of the outbreak strain GREA14KL-7 or cluster 4.

Clusters of Isolates Not Associated with the HSCTU Outbreak

A further apparent outbreak, potentially linked to environmental cross transmission, was detected by these typing methods. Isolates from patients 5, 8 and 23 (CICU) were previously linked by PFGE typing as part of GREA14KL-3. One further isolate from patient 6, had a unique PFGE profile (isolate 6). However this isolate was subsequently typed as cluster 3 by MiSeq final analysis, Pathogenica and VNTR, along with the other patient isolates. In addition an environmental sink isolate from the clinical hand wash basin on the unit; which had previous typed as GREA14KL-4 by PFGE (18), was also placed in cluster 3 by all HTS typing techniques.

Samples 25, 28, 36, 40, and 44 had only one typing scheme that disagreed with the others. Of these disagreements four occurred within the clustering by REP-PCR, and one by PFGE.

Isolate 1 was determine to be unique by fragment analysis techniques as well as Pathogenica and MiSeq final analysis, but formed part of cluster 2 for Phyloshift analysis.

Isolate 2 was unique by all fragment analysis techniques, but formed part of cluster 2 by both Pathogenica and MiSeq final analysis. This sample clustered differently with Phyloshift, forming part of cluster 3 with samples 8, 9, 11, and 18.

Isolates 3 and 12 were unique by all fragment analysis techniques as well as by Pathogenica, but formed part of cluster 2 by all other techniques.

Isolate 10 was unique by all fragment analysis techniques and formed part of cluster 2 for all sequence based techniques.

Isolates 13 and 16 were unique by VNTR, PFGE and MiSeq final analysis, but formed part of cluster 1 by targeted sequence analysis.

For isolate 14 all techniques determined it to be unique, except for Phyloshift which clustered it as part of cluster 2.

Isolate 15 was determined to be unique by fragment analysis techniques and Pathogenica, but other techniques included it as part of cluster 2.

Isolate 6 was unique by PFGE and REP-PCR, but formed part of cluster 3 in all other techniques, except Phyloshift.

Isolate 8 was part of cluster 3 by all techniques except REP-PCR; which identified it as being unique.

Isolate 4 was unique by all fragment analysis techniques as well as Pathogenica and MiSeq final analysis, but formed part of cluster 5 by Phyloshift.

Isolates 31, 39 and 42 were all unique by fragment analysis techniques and formed part of cluster 7 by all sequence based techniques.

The isolates that were subsequently identified as being *Klebsiella oxytoca* (17, 35, 37, 46) formed part of cluster 8 by all sequence based methods. Isolate 17 was considered unique by all fragment analysis methods. Isolates 35, 37 and 46 were included as part of cluster 8 by REP-PCR and considered unique by VNTR. PFGE clustering varied and included GREAK-7, GREA14KL-9 and unique.

Diagnostic Test Specifications Using Either PFGE or MiSeq as Reference Standards

PFGE is considered to be the current reference standard for typing within the Enterobacteriaceae. In order to compare typing methods a comparison of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) was undertaken, using both the current reference standard and using WGS data. A result was considered a true positive when it matched the reference standard in assigning an isolate to the same isolate cluster. A result was considered a true negative when it matched the reference standard in assigning a unique type. False positives were where a cluster was assigned when the isolate was considered unique by the reference standard and a false negative consisted of the method assigning a unique identification when the reference standard had clustered the isolate.

When compared to PFGE, VNTR had a sensitivity of 84% and a specificity of 88%, with a PPV for typing agreement of 93% and a NPV of 74%. If using the MiSeq consensus analysis, VNTR had a sensitivity of 66% with a specificity of 100%. The PPV was 100% with an NPV of 21%.

When PFGE was used as the reference standard the sensitivity of REP-PCR was 77% and the specificity was 67%, with a PPV of 79% and an NPV of 63%. Using the MiSeq consensus analysis sensitivity was 64% and specificity 60%. The PPV for matched typing results was 93% and the NPV 17%.

For MiSeq analysis, if MiSeq analysis 1 was compared with PFGE as the reference standard, the sensitivity was 100% with a specificity of 15%. The PPV was 61% and the NPV was 100%. For MiSeq analysis 2 the sensitivity when compared to PFGE was 100% and the specificity was 7%, with a PPV of 67% and a NPV of 100%. When the data analysis was combined to form a consensus the sensitivity of WGS versus PFGE was 100% with a specificity of 25%. The PPV was 72% and the NPV 100%.

The sensitivity of Pathogenica when PFGE was used as the reference standard was 100%, with a specificity of 26.32%. The PPV was 67% and the NPV was 100%. When Pathogenica was compared to MiSeq final analysis as a reference standard the sensitivity was 95% and the specificity 60%. The PPV was 95% and the NPV 60%.

When comparing Phyloshift against PFGE the sensitivity was 96% with a specificity of 0%. For MiSeq final analysis the sensitivity was 98% and specificity 0%. As the Phyloshift identified an isolate as unique that was not identified by MiSeq or PFGE the

NPV for Phyloshift was 0% and the PPV 84% for MiSeq and the PPV was 57% and the NPV 0%.for PFGE.

Finally PFGE with MiSeq consensus as the reference standard had a specificity of 68%, a sensitivity of 44%, a PPV of 84% and an NPV of 25%.

VNTR typing therefore had the highest PPV when compared against both MiSeq final analysis and PFGE of the fragment analysis techniques.

4.3 DISCUSSION

Prospective molecular typing undertaken during this study was performed using PFGE. PFGE results took an average three weeks from sample collection to result. PFGE represents the current reference standard for bacterial typing, it does however have limitations. PFGE is very labour intensive and as demonstrated by the turnaround time in this study cannot provide results in real time. The PFGE process requires expensive equipment and considerable expertise and so was not able to be conducted in-house at GOSH.[225] In addition although PFGE analysis is undertaken using the Dice coefficient and rules established by Tenover et al. (1995), a recent review demonstrated that PFGE studies had definitions which varied from 67 – 100% similarity requirements for relatedness.[246, 457] In order to investigate whether other typing techniques represented a viable alternative to PFGE typing for routine use VNTR, REP-PCR, HTS and WGS typing were investigated.

4.3.1 VARIABLE NUMBER TANDEM REPEAT (VNTR) TYPING SCHEMES FOR *ENTEROBACTER CLOACAE* AND *KLEBSIELLA PNEUMONIAE*

Designing of Multi-locus Variable Number Tandem Repeat Typing

Two different VNTRs studies were undertaken as part of the typing evaluation (see sections 4.2.2.1 and 4.2.2.2.). The first involved the development of a VNTR scheme for *Enterobacter cloacae*. Four loci were identified all of which showed some level of tandem repeat variation (section 4.2.2.1.). The only other published VNTR scheme for this genus is for *Enterobacter sakazakii* and this scheme also uses four loci. Comparison of this published *Enterobacter sakazakii* VNTR scheme to PFGE analysis demonstrated that it compared favourably across 112 isolates representing 16 subgroups, although PFGE was shown to be more discriminatory.[87] It is crucial when selecting loci for VNTR that they are optimised in terms of tandem repeat variation. If they are too unstable then they may not reflect the 'real' distribution of bacterial genotypes and if they are too stable then they may be not be discriminatory enough making interpretation difficult.[462] Seven loci are normally selected for VNTR typing schemes, although this may be less organism specific and more to do with the number of loci usually used for the design of MLST schemes. It is however possible that a smaller number of loci may be adequate for typing of *Enterobacter cloacae*.

Designing of VNTR schemes requires the availability of whole genome sequence data for the target species, in order to identify the repeat motifs.[141] The current number of

genomes sequences for species is usually limited to a few strains, plus the type strain.[70] Genome sequencing so far has demonstrated considerable diversity within species, especially in species such as *Klebsiella* and *Enterobacter*.[66, 69, 70, 449, 463, 464] For example eight complete whole genome sequences of *Enterobacter* spp. are currently available on Genbank four of which belong to *Enterobacter cloacae* and only one of which is linked to human infection.[66] Too few sequences from the same organism exist and so many VNTR scheme, like the one in this study, are designed based on only one whole genome sequence. They are thus based on the assumption that the available sequence will represent all strains within that species.

The Effect of Culture on Multi-locus Variable Number Tandem Repeat Typing

Some of the failed amplification of loci within both VNTR schemes is likely to be due to poor phenotypic identification, and/or increased genetic diversity when compared to the reference genome.[87] For example within this study one isolate (S20245) identified by MALDI-ToF MS and API 20E (Bio-Merxieux, Mary l'Etoile, France) as *Klebsiella oxytoca*, when typed, amplified at all loci, whereas the type strain NCTC 13368 failed to amplify at 4/9 loci (see section 4.2.2.2.).

Comparison of VNTR data with PFGE typing demonstrated consensus in the majority of cases (85%). VNTR identified both isolates within cluster GREA14KL-4 as unique and added an additional, previously unique, sample to the GREA14KL-3 cluster. All isolates of *Klebsiella oxytoca* and *Enterobacter* spp. were identified as unique, bar one which was clustered with the outbreak strain. One other sample (36) was identified as GREA14KL-9 along with its partner isolate 35 by PFGE; the difference in result by VNTR type is probably due to an initial mixed culture.

Other Factors Affecting Multi-locus Variable Number Tandem Repeat Typing

One of the advantages of VNTR schemes is that they are portable.[221] Despite this, a comparison undertaken by the Centre for Disease Control (CDC) of a number of centres, identified differences between fragment sizing between two types of capillary sequencers, suggesting a need for external quality control when establishing a VNTR scheme.[465] To test intra-laboratory performance, comparison of VNTR results between two centres (GOSH and the PHE) was undertaken, and was shown to be comparable in 8/13 samples (section 4.2.2.2.). One sample had completely unrelated VNTR types and is likely to be due to mixed colonies on the initial isolation plate. Three samples varied by one repeat size at a single locus. This is likely to be due to

differences in sizing repeats between the use of a capillary sequencer and the use of agarose gel, and is another reason for allowing some variation in loci sizing when interpreting results. One sample size difference between centres was identified at Locus J, where one centre classed this as an insertion sequence, and the other called it a large tandem repeat. This has been noted in the literature as another evaluation that is required to produce an interpretative algorithm, as the size difference in a locus may not always reflect the real number of tandem repeats due of the presence of insertions.[466]

Loci for the *Klebsiella pneumoniae* VNTR were selected as suitable for VNTR as they had a relatively high mutation rate.[55] Hypermutation of tandem repeats has been shown to be on average 10^{-2} – 10^{-3} per generation; with *E. coli* demonstrating $7.0*10^{-4}$ mutations/generation for a single loci and $6.4*10^{-4}$ mutations/generation across 28 loci.[267, 463] Rapid mutation rates are thought to be a result of compounding effects of various factors intrinsic to loci, including repeat copy number, repeat unit size, and functionality of the mismatch repair system. Repeat copy number effects appear to apply both across loci and within an individual locus. In a mutation study undertaken on *E. coli* by Vogler et al. (2006) it was shown that mutation rates were high and varied across loci.[267] Of 186 mutations detected 74.7% were single repeat changes (either insertions or deletions). The remaining 47 (25.3%) involved multiple repeat changes ranging from 2 – 20 repeats. When mutations occurred in large repeat copy numbers they were more likely to be due to deletions than insertions. [267]

Within the loci tested for *Klebsiella pneumoniae* Locus J was the largest along with Locus H (both the largest number of base pairs) (see section 4.2.2.2.). The smallest locus tested was locus D; which consisted of 14 base pair repeats with a 119 base pair flanking sequence. No evolution of repeats was noted in Locus J in repeat samples from patients, although it did represent the greatest variation in repeat number present, as well as the largest repeat size. Locus D was one of the two loci that demonstrated deviation from the main outbreak VNTR type while the other was loci N2 (a 57 base pair repeat). Locus N2 changed by a double repeat in isolate 44 and a single repeat in Locus D.

The main pitfall suggested of VNTR schemes is that two identical clones may appear different because selection has led to the generation of two variants that have differences in one or more VNTR loci. This is due to the inherent instability of tandem repeats and has been suggested as a limitation of its use as a typing scheme.[463, 466,

[467] One study that compared high throughput amplified fragment length polymorphism (AFLP) and VNTR when looking at genomic variation in functionally constrained genomic regions, and regions of hypermutability, has however demonstrated that although enhanced variability occurred in the repeat regions, the clustering of strains remained traceable.[250] Within patients in this study the VNTR type did not alter in repeat samples, even when repeated samples were taken for up to seven months apart.

The one sample that appeared to be related to but different from the outbreak strain was an environmental sink sample (isolate 44). Variation within the environmental sample did not match that of an additional isolate grown on the same plate from the same swab, which matched the main outbreak type. This sample was included as part of the outbreak cluster, but only when epidemiological information was included as part of the analysis. Little is known about the factors that affect mutation rates of tandem repeat loci and this is particularly relevant when the isolates are from different niches or hosts.[267, 463, 468] Environmental isolates are likely to be subject to different selection pressures to those present within human hosts, especially as growth is likely to occur in biofilms.

Interpretation of Multi-locus Variable Number Tandem Repeat Typing

In the published version of the *Klebsiella pneumoniae* VNTR scheme no interpretative guidance is given.[55] Some publications have used a similar interpretative algorithm as used in this study, i.e. +/- 1 or difference in only one locus, with or without additional epidemiological information.[249] This is the same interpretation which is used in-house by the PHE (personal communication J. Turton). Other publications have used different guidelines for clustering, some of which used the MLST cluster defining rule that differences of ≤2 alleles were grouped as a cluster, with the most common type being chosen to be cluster defining.[251] Another study defined isolates as related if they varied at fewer than four loci.[469] [470] There is a need to define what related means across the literature, although this is complicated by variations in the number of loci used and a lack of thorough mutation studies to understand the factors affecting mutation rates in all species.[465]

Christiansson et al. (2011) undertook a study where up to 20 isolates were taken from 30 patients in two long term care facilities. They found that colonisation with more than 1 VNTR type was found in eight individuals.[108] Of the eight colonised patients with more than one VNTR type, three differed by only one repeat unit at only one locus.

These isolates would be identified as identical using the algorithm used for this study. The remaining five differed by 2 – 6 loci, with differences occurring between different sites (perineum and urine) and may therefore represent different strains. It has also been suggested that intra-loci differences occur one tandem repeat at a time during outbreaks while unrelated isolates are more likely to differ by more than one tandem repeat.[108] This was not supported by sampling of isolates within this study, as part of cluster 4 varied by 2 repeats, and thus was only included when epidemiological information was included within the analysis (section 4.2.2.2.). It may also be that a different criterion may be required for interpretation of environmental isolates.[249]

4.3.2 Repetitive Extragenic Palindromic Sequence PCR for typing of *Enterobacter* species, *Klebsiella* species, and *E. coli*

The use of REP-PCR was evaluated for *Enterobacter* spp, *Klebsiella* spp. and *E. coli* as part of this study. REP-PCR judges the possible relatedness of isolates based on the number of different fragments obtained; which are the result of insertions, deletions, inversions of DNA or mutation of the repetition.[459] For the detection of clonal outbreaks of *Enterobacter* spp. and *Klebsiella* spp. PFGE is considered to be the reference standard.[69]

Use of REP-PCR for typing has a number of advantages over other typing techniques. REP-PCR targets have been shown to be stable as targets for typing in patients over a prolonged period of time, with the same profile being found in a patient sampled at regularly intervals for a period of one year.[471] As the technique is less affected by species identification as the same primers are used for both *Enterobacter* spp. and *Klebsiella* spp. it is less adversely affected by organism mis-identification.[459] It has also been able to type a number of strains that were not typable by PFGE, either due to mis-identification or other reasons.[459, 472] The main drawback of the Diversilab REP-PCR system is that the primer sequences used in PCR reactions are proprietary, thus limiting the potential for trouble shooting or assay development by the user in response to specific needs. The system also has significant cost implications compared to the other fragment analysis typing schemes.

Comparison of Repetitive Extragenic Palindromic Sequence PCR and Pulse Field Gel Electrophoresis Results

Study comparisons between PFGE and REP-PCR have varied in their outcomes. Consensus between REP-PCR and PFGE has varied between 60% and 97%.[69, 120]

Some studies have found that REP-PCR differentiates better than PFGE, dividing within PFGE types. However it could be that this is an over-estimation of relatedness, rather than true increased discrimination.[69, 120, 472] Other studies have identified that REP-PCR is not as discriminatory as PFGE.[103, 223, 458, 467] Some of these studies demonstrated that discrimination was species specific, with *Enterobacter* spp. and *Klebsiella* spp. linked to increased discrimination in comparison to PFGE, and *E. coli* clustering into larger clusters than shown by PFGE.[458, 459, 472]

PFGE for *E. coli*, *Enterobacter* spp. and *Klebsiella* spp. in this study confirmed that the paediatric patients admitted to GOSH during this period had a mixture of resistant isolate detections of an unknown source and multiple cross-transmissions with several clusters of PFGE types. For the outbreak study PFGE and REP-PCR demonstrated a concordance in 83% of samples (section 4.2.3.4.). For *Enterobacter* spp. REP-PCR could not distinguish between some PFGE clusters and other samples clustered by PFGE were split by REP-PCR clusters. This matches the findings described in the literature.[69, 120, 472] Within both the outbreak study and the international centre comparison *Klebsiella* spp. isolates that were linked by PFGE were sometimes found to be unique. In comparison, and in line with published studies on *E. coli*, REP-PCR was found not to be as discriminatory as PFGE but it did split some PFGE clusters (sections 4.2.3.3 and 4.2.3.4.).[458, 459, 472]

Interpretation of Repetitive Extragenic Palindromic Sequence PCR Typing

One of the reasons for discrepancies found between studies in terms of agreement with PFGE may be due to the lack of agreements between authors on either the correct analysis algorithm or the percentage cut-off used to determine isolate similarity. This has been noted in a number of publications to be one of the major limitations of the system.[459, 467, 473]

Analysis of REP-PCR profiles can utilise either the Pearson Correlation Co-efficient or the Kullback-Leibler analysis to calculate pairwise similarities among all samples tested. If using the Pearson Correlation Co-efficient, isolate similarity is judged based on the number of different fragments obtained and the intensity of the bands present; and band presence is weighted over band intensity. The Kullback-Leibler analysis is based on both the number of different fragments obtained and the intensity of the bands present, with band intensity weighted over band presence. As both interpretations are based on the number of bands and amplification intensity the analysis outcome may be equivalent.[474]

A number of publications have used the Pearson Correlation Co-efficient with a similarity cut-off of ≥98% similarity as indistinguishable and of less than ≤95% similarity as different. For isolates that have similarity between 95 and 98% digital overlays of the isolate results were compared.[458, 459] Others have used a cut-off of ≥97% similarity as indistinguishable and of ≤95% similarity as different, with overlays or number of band differences used for the intervening analysis.[77, 103, 474] Other studies using Pearson Correlation Co-efficient have utilized a wide variety of cut-offs including: ≥95%,[73, 89, 120, 467, 475-477] ≥93%,[478] ≥92%,[56] or a ≥90%.[76] Some of these authors varied cut-offs between the above figures for *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. as they found that no one figure provided sufficient discrimination.

When using the Kullback-Leibler analysis most studies have used either ≥95% similarity or ≥97% similarity as indistinguishable and of less than ≤95% similarity as different with overlays or number of band differences used for the intervening analysis.[474, 479, 480] In the two studies that compared results using both, one found that results were identical whereas the other found that Kullback-Leibler was less discriminatory.[474, 479]

When analysing the produced dendogram a similarity cut-off must be imposed to determine isolate clusters. If a cut-off is set to be too discriminatory, potential association between isolates could be missed and the outbreak would be believed to be multi-clonal. If however the discrimination method is too low it could lead to the conclusion that outbreaks are monoclonal, leading to unnecessary infection control interventions. It is therefore important to be aware of the genetic evolution of the organism investigated in order to be able to draw correct conclusions about genetic relatedness and to always include epidemiological information in result interpretation.[473]

Within this study a cut-off of ≥93% using Pearson Correlation Co-efficient analysis, was used. This was felt to the most appropriate cut-off when informed by epidemiological information (section 4.2.3.1.). The only exception to the use of a ≥93% using Pearson Correlation Co-efficient analysis was during the intra-laboratory comparison where a ≥95% - ≥98% cut-off was set by the study co-ordinator to fit in with their previous work.[458, 459]

Sources of Variation in Repetitive Extragenic Palindromic Sequence PCR

Another reason for variability between studies may be due to the variation both within and between centres performing the assay. As part of the multi-centre comparison study considerable variation was noted between centres and this was identified as being possibly due to: variance between people performing the assays, inconsistent amplification, inconsistent quality/quantity of DNA and/or variation between equipment.[458]

A number of isolates in our study failed to amplify sufficiently even when DNA was quantitated within the acceptable study range; however issues exist with quantitation of DNA using the nanodrop platform. In a validation study undertaken on the DNA from this study results from nanodrop quantification varied when performed on different days and did not correlate to DNA as quantified by other methods (data not shown). This may account for some of the variability between samples.

Healy et al. (2005) in the initial publication of the Diversilab version of the REP-PCR assay found that reproducibility between *E. coli* grown on different culture agars was 97%; which is problematic if using a 98% cut-off for studies.[455] No other papers discuss the culture medium used for their assays and it was not a component of the protocol for the mutli-centre comparison study.[458] However this could impact on DNA quantification as the level of extracellular polysaccharide material excreted varies dependent on the culture media utilised for growth. Extracellular polysaccharide can not only affect extraction efficiency but can also contain DNA. Highly mucoid colonies may contain non chromosomal DNA excreted as part of the extracellular polysaccharide, this would be included in DNA quantification, but which would not be suitable for amplification in the assay.[344, 345, 481-484] This may be especially true for environmental organisms.

Amplification efficiency and the variations linked to it may be due to the thermocyclers used. Healy et al. (2005) validated the assay on five different thermocyclers and found a reproducibility of >96%.[455] This would again affect analysis undertaken using a ≥98% cut-off. To counter this within this study a single validated thermocycler was used for all amplification; however some amplicon variation was still noted.

A number of studies have noted problems with reproducibility of results in addition to the multi-centre comparison undertaken by Voets et al. (2013).[458] These studies have noted that different extractions have led to different clustering, especially but not

exclusively, when initial low level amplification was detected.[467] Inconsistency was also noted between extracts taken from the same isolate and between amplification runs.[467] Within this study REP-PCR typing has been demonstrated to be reproducible as long as stringent quality control measures are undertaken, as poor data is not always apparent (section 4.2.3.2.). This has included always running isolates in duplicate on separate amplification runs in order to compare results and if necessary using different extracts. All of these measures have cost implications.

4.3.3 HTS For Investigation of a group of 48 isolates of three species

A number of different high through put sequence based typing techniques were evaluated in this study. These included the Pathogenica system; which undertakes typing based on twelve 10,000 base pair sequenced fragments per isolate (see methods section 2.8.4). Other data analysis was undertaken on the same raw data produced by MiSeq sequencing and included: Phyloshift which analysed 37 genes from the core bacterial genome, and WGS typing, based on assembly of the whole genome using a reference sequence (see methods sections 2.8.1 and 2.8.2).

Comparison of High Throughput Sequence Typing and Pulse Field Gel Electrophoresis

Findings in published studies comparing HTS based typing approaches and PFGE have found variable results. One study found that clusters that were indistinguishable by PFGE were not linked when analysed using WGS comparison, implying that PFGE inappropriately clustered isolates when there was no epidemiological relationship.[220] Other studies have found that PFGE was not discriminatory enough and so failed to cluster isolates sufficiently when compared to WGS.[220, 223] Typing results in our study when compared to PFGE were in line with the findings from the later published studies (sections 4.2.4.1, 4.2.4.2 and 4.2.4.3.). Pathogenica demonstrated a 70% consensus with PFGE, MiSeq analysis one a 66% match, MiSeq analysis two 65% similarity and Phyloshift 55%. Typing based on sequence data demonstrated increased clustering between isolates and fewer isolates classified as unique.

Sources of Variation in High Throughput Sequence Typing Analysis

Both targeted sequencing and WGS approaches are affected by issues linked to the typing of the core or accessory genome. The core genome represents the genes present in all strains of a species while the pan genome consists of a core and all genes within the accessory gene pool.[70, 95, 464] The accessory gene pool consists of

genes that vary between strains of the same species and is thought to be an important source of variability gained through lateral gene transfer, to permit adaptation to specific niches.[464, 485] This raises questions about what genes should be used for defining genome similarity and whether it should include the core genome, the accessory genome, or both.[70]

Data analysis of WGS data requires the use of a reference genome. The current number of sequenced genomes for many species is normally limited to a few strains plus the type strain genome.[70] Although the amount of WGS being undertaken is increasing, many available genomes are incomplete as finishing a sequence requires both gap closure and resolution of sequencing errors this is time and resource intensive.[464] When using one reference genome it is possible to miss genes present within the accessory genome

Eight complete whole genome sequences of *Enterobacter* spp. are currently available on Genbank. *Enterobacter cloacae* spp. are diverse and includes bacteria associated with plants, soil and humans.[66] *E. cloacae* genomes share 3540 core genes with 645 – 825 genes currently identified within their accessory genome. Four *Klebsiella pneumoniae* species are currently available; these have a core genome of 4269 genes, 98.8% of which are chromosomally located, The core genes formed 65 – 75% of the total predicted genome; accessory plus core genome, with most of the accessory genes located on plasmids.[44, 47] For *E. coli* the core genome is 2200 genes, forming roughly half of the gene complement. The pan genome is ~13,000 genes, with the trend for continual increase with each newly sequenced genome.[486] When a reference assembly is undertaken, data included in the analysis is likely to be biased towards inclusion of the core genome of a species, with the inclusion of some accessory genes dependent on those contained within the reference sequence. In the case of *Klebsiella* and *Enterobacter* genomes, a number of plasmids are included alongside core genomes in reference databases as they contain antibiotic resistance determinants; this expands the inclusion of accessory genes within the analysis.

Due to these issues it is important to choose the correct reference when undertaking assembly, especially in the Enterobacteriaceae, as they are so diverse.[464] Single nucleotide variant (SNV) detection is a problem if there are only a few reference genomes available, especially for the Enterobacteriaceae. This is even more of a problem when trying to look at strains within a species, as many more samples are needed to capture representative genomes.[51, 221]

Studies have found that targeted sequencing of either the core alone or core plus the accessory genome can be of use to accurately determine the evolutionary history of organisms.[221, 278, 280, 461, 464] Both the Pathogenica system and Phyloshift focus on the use of core genes within the genome in order to undertake typing. In the case of Pathogenica this is supplemented by analysis of the accessory genome for identification of antibiotic resistance; which are often carried on plasmids.[278, 395, 456] Both systems make the assumption that isolates demonstrating the same strain type using targeted sequencing approaches will share other unmeasured genetic characteristics.[487] It has been suggested that the most important criteria for loci selection in the case of targeted genome typing, should be selection of targets based on nucleotide diversity rather than gene function.[488] Despite this, one experimental study has shown that including positively selected genes did not hinder typing, and non-coding regions also yielded similar results, suggesting that the use of core genes alone for typing is a reliable approach.[243] Both targeted sequence based techniques in this study appear to provide results that closely resemble WGS (section 4.2.5.2.).

Interpretation of High Throughput Sequence Typing

In order to investigate the use of WGS for prospectively typing it is necessary to understand the population structure of the significant pathogenic species of interest. HTS typing has so far demonstrated considerable diversity within species and has demonstrated the need to develop clear criteria in order to group strains within a species.[485] Lineages need to be studied both on a national scale but also within individual patients and specimens, to determine how frequently SNVs arise, and how these affect evolutionary fitness.[489] This is needed to provide the necessary context for interpretation of typing results.[220]

SNVs are often relatively evolutionarily stable and can be identified within the core genome.[51, 221, 464, 485] However using SNVs for clustering of strains presents a problem as there is no standardised criteria that can used to determine whether patient to patient transmission has occurred.[457] Some studies have used exclusively molecular criteria to determine genetic relatedness and then used genetic relatedness alone to suggest patient to patient cross transmission. Other studies have required genetic relatedness along with clinical relatedness: such as overlapping hospital stay or patient proximity.[457] It is also possible that separate molecular clocks may be required for separate genes as synonymous substitutions are likely to occur at different rates as different genes are under different types of selective pressure.[490] This work has

currently been undertaken for some Gram-positive organisms such as *Staphylococcus aureus* where a cut off of 40 SNVs was determined to be suitable. This detail is currently missing for the Enterobacteriaceae and additional sequencing studies are required to capture the genetic diversity of clinical strains.[51, 220]

For this reason within this study data interpretation was based on phylogenetic cluster rather than number of SNVs, with the number of SNVs within clusters used to guide interpretation. If the number of SNVs within WGS clusters is taken into account the low number of SNVs in clusters 4 and 3 suggest that these are true cases of possible cross transmission (section 4.2.4.2.). The large number of SNVs within the other clusters suggested much greater diversity and that isolates are related but not linked to direct cross transmission. This interpretation was confirmed with the addition of epidemiological analysis. This indicated that although the data is not currently available to determine directional isolate transmission SNVs can be used to determine when clusters are linked to cross transmission events due to differences in isolate diversity.

To study isolate stability over time within patients carrying Enterobacteriaceae one study looked at carriage of an outbreak strain over an 18 month period, during which 11 SNVs were acquired.[460] Stability of patient isolates appears to be considerable, in that the patient isolates sampled within this study remained stable for up to seven months as has been described in other studies.[52] However there is variation with patients at different sites. One study that involved sequencing of seven isolates from a patient over a four week period revealed the production of seven SNVs. Four SNVs were present in all four of the urine isolates, groin and BAL isolates shared three SNVs. Throat isolate shared three SNV that were also seen in the urine sample.[51] Within this study variation was seen between urine and faecal isolates in one patient taken two days apart, suggesting that similar but unrelated isolates are carried in different anatomical sites (section 4.2.5.2.).

Possible Differences between Environmental and Clinical Isolates

Microbial species investigated so far using HTS have been markedly skewed towards invasive isolates of clinically significant pathogens. Host shifts or changes in transmission pathways can impose strong evolutionary pressures on pathogen populations to adapt to a new niche.[486, 491] Intra-strain variation at the nucleotide level may therefore relate to life style adaptations.[44] In studies that may contain an environmental source it is crucial to understand how these differences in selective

pressure may cause two related isolates to evolve differently within a short period of time, to ensure accurate typing investigation.[66]

Environmental organisms present in the clinical environmental may interact with pathogenic species through horizontal gene transfer when in close proximity. This exchange may have implications for infection control as environmental organism have been shown to have large accessory genomes that may act as genetic sinks for virulence genes and antibiotic resistance.[77, 457, 468] Several studies have underlined the environmental resistome as a source of resistance genes. Within the clinical environment many antibiotics are excreted unchanged and can persist they may thus lead to selection pressure for microorganisms present on sinks or other surfaces.[468, 492] It is particularly important therefore that studies are undertaken to understand how these factors affect environmental isolates, as this can affect the typing results received.[51, 70, 464]

4.3.4 Routine Clinical Typing For Outbreak investigation

Within this study two outbreaks were identified (clusters three and four), both linked to the presence of outbreak strains isolated from sinks within the relevant clinical environment. In the outbreak on the HSCTU the outbreak was only controlled when the sink environment was altered to remove the source of the outbreak, the equipment sink (see Chapter 5). The finding of the outbreak strains was initially by antibiogram, but subsequently supported by PFGE typing. These findings identify environmental contamination as not just a potential risk, but a likely cause of clinical disease.

During the initial outbreak investigation on the HSCTU identification of isolates was undertaken by the API 20E system (Bio-Merxieux, Mary I'Etoile, France) (see methods section 2.3.2.5). Five of the outbreak isolate initially identified as *Klebsiella pneumoniae* using this system were subsequently shown to be either *Klebsiella oxytoca*/*Enterobacter asburiae*/*Enterobacter cloacae*. *Enterobacter asburiae* was the identification given for isolate 5 by MiSeq analysis one and two and by MALDI-ToF MS (see methods section 2.8.1, 2.8.2 and 2.3.2.5). Isolate 5 was identified as *Enterobacter cloacae* by the Pathogenica system (see methods section 2.8.4). All four isolates (35, 37, 46, 17) identified as *Klebsiella oxytoca* were consistently identified by all none API methods. This has also been found in a study undertaken by Giammanco et al. (2011) where the API 20E system (Bio-Merxieux, Mary I'Etoile, France) mis-identifed 5/30 isolates.[493] Inappropriate identification can significantly impact typing as

schemes such as VNTR are species specific and so can lead to either a non-typable or aberrant typing result.

Within the fragment analysis based typing schemes evaluated in this study VNTR appeared to give best concordance with HTS data, although a number of isolates were identified as unique in comparison (section 4.2.5.2.). A number of those isolates identified as unique formed part of the *Klebsiella oxytoca* cluster and therefore, if correctly identified would not be included in the *Klebsiella pneumoniae* VNTR scheme for typing. VNTR correctly identified both clusters of outbreak strains (clusters 4 and 3), with the exception of the environmental isolate (18) linked to cluster 3. REP-PCR failed to identify 3/5 isolates within the second outbreak cluster (cluster 3), finding them to be unique. This indicated that VNTR is probably the most appropriate scheme to use as an in-house typing scheme until HTS can be undertaken in a routine fashion.

There were some isolates that clustered variably between MiSeq analysis one and two, Phyloshift and Pathogenica this makes data interpretation more complicated. Within MiSeq analysis two clusters 1 and 2 could be considered to be one cluster dependent on the analysis criteria used. This would bring samples 10, 13 and 16 in line with analysis from MiSeq analysis one. Other isolates that showed a lack of consensus between the different analyses of the MiSeq data were isolates 1, 2, and 4 (section 4.2.4.2.).

Isolate 1 was considered to be unique in both the Pathogenica and MiSeq analysis one. However it was considered to form part of cluster 2 by MiSeq analysis two and Phyloshift analysis. Isolate 2 was clustered as part of cluster 2 by both the Pathogenica and MiSeq analysis one, but both Phyloshift and MiSeq analysis two clustered it separately. For sample 4 MiSeq analysis one and Phyloshift analysis clustered the sample as part of cluster 5, while both MiSeq analysis two and Pathogenica considered the isolate to be unique. These comparisons suggest that the analysis performed for MiSeq analysis one and Pathogenica are more similar, while MiSeq analysis two and Phyloshift are more comparable for these samples. All three of these isolates were considered to be unique by fragment length typing techniques. The difficulty in interpreting the results of these three samples, demonstrates that no typing scheme can be interpreted without routine epidemiological information to inform clinical decision making.

MiSeq analysis one and two varied by three results, if cluster 1 was considered to be shared with cluster 2. If these three samples are excluded, as they demonstrate a lack

of consensus across sequencing typing schemes; then Phyloshift differed from the MiSeq WGS analysis by a further three samples and Pathogenica by one result. This suggests that the Pathogenica analysis more closely resembles the data derived from WGS analysis.

Although the WGS sequence data is assumed to offer the greatest discrimination for isolates it may find links between unconnected isolates due to the high level of clustering detected.[69, 451, 452] Studies using a purely molecular HTS approach without inclusion of epidemiological information are likely to report falsely high patient to patient transmission rates, since patients months or years apart may have genetically similar isolates and are unlikely to represent true person to person transmission.[457] However this could be due to intermediate sources such as the environment, or the presence of institutional strains, possibly carried by staff. As a consequence more rigorous definitions that require genetic relatedness along with clinical criteria might miss transmission events if they do not allow for intermediate sources.[457]

In addition to data interpretation issues discussed in each of the typing scheme sections, there remain some other scientific barriers to implementation of HTS for infection control typing. One study has reported that multiple variants of a strain could persist in an infected individual/population without any one becoming dominant. As HTS studies have not been validated for detection limits it is unclear whether this data would be captured or would be available through the currently data analysis programmes available.[494] There remains considerable contention about how to sample colonies for typing, specifically whether to take multiple single colonies or a sweep from a plate in order to capture diversity.[495] Greater validation needs to be undertaken to answer these issues before routine implementation is undertaken.[486]

There are also a number of implementation barriers to the introduction of HTS based typing within the routine clinical setting. First, there is a need to streamline bioinformatics and for access to readily accessible curated databases, this is easier to do with kit based systems such as Pathogenica, but comes at an increased cost.[278, 456] Outside of kit based systems there are a number of bioinformatics programmes becoming available that provide either WGS analysis or targeted analysis of both the core and accessory genomes, all of which still require a high level of bioinformatics knowledge.[464] Secondly, it requires a considerable IT infrastructure, especially if the data is to be kept and shared between centres as clinically relevant.[223, 496] Thirdly

linked to increased oversight in order to achieve accreditation, universal use of HTS would require a level of standardization and consistency not currently attained.[185]

As typing data does not currently provide sufficient information to predict phenotypic expression of antibiotic resistance, even if the data is captured, there is still a need for good phenotypic identification and processing. This is especially true for those typing schemes that are species specific, such as VNTR.[230] Additionally although HTS and other typing schemes can identify the source of transmission, they do not always explain the mode of spread and therefore both targeted sampling as discussed in Chapter 3 and interventions as discussed in Chapter 5 are essential for outbreak control.[215] Finally HTS cannot truly be utilised for routine infection control typing until it ceases to be limited by a lack of real time deployment. For this reason fragment based typing schemes are likely to continue to be used, at least in the short term.[284]

4.4 CONCLUSIONS

This study has demonstrated that sequence based techniques, whether targeted or whole genome have the capacity to identify cross transmission between patients and the environment. They may however provide a level of discrimination that could make interpretation difficult for infection control teams without the support of better information. This information would need to include data on molecular clocks for both invasive, non-invasive and environmental isolates and definitions for how these should be interpreted linked to cross transmission in order to permit their use in outbreak investigations. The criteria for the definition of relatedness must be independently investigated for each species.[457]

Until HTS methods are common place and standard practice, rapid typing methods are still required.[461] From the analysis in this study VNTR provides a tool that is capable of discriminating at a level that could detect transmission between patients, at least within isolates that do not have an environmental source. VNTR is therefore the most appropriate tool for current use in-house as it is rapid, inexpensive and portable between centres.

There remains a difficulty with all the typing schemes, except PFGE, that there are no definitions of relatedness that can be universally applied, even for single species. Long term carriage studies of patients colonised in multiple sites, with and without exposure to antibiotics are needed. This should be done in addition to sampling of environmental isolates where work should be done examining mutation rates given different environmental exposures e.g. to cleaning agents and mixed biofilms.[264]

Another barrier to the implementation of HTS in routine practice is the amount of data produced. This requires a large amount of information technology infrastructure. Additionally finding a way to deliver the large amounts of data to infection control teams and other clinicians in a context driven, epidemiologically linked fashion is a major challenge.[284]

Finally as most of infection control typing continues to be retrospective and therefore does not immediately inform outbreak control, prevention of cross transmission events will continue to rely on infection control interventions. Optimal interventions may depend upon the organism targeted.[215, 457] For this reason they are discussed in more detail in Chapter 5.

Chapter 5 INFECTION CONTROL INTERVENTIONS

5.1 INTRODUCTION

Published studies as well as those described in Chapters 3 and 4 have demonstrated that potentially pathogenic organisms are present within the healthcare environment.[438, 497, 498] In order to aid in the prevention of healthcare associated infection (HCAI), the environment should be considered as a possible source of infection and targeted appropriately.[183, 341, 368]

Kleypas et al. (2011) suggest that the best way of reducing HCAIs after hand hygiene is environmental control, which should be upon based on good cleaning, thorough removal of organisms by mechanical action and by disinfection.[21, 300, 360] Routine cleaning within Great Ormond Street Hospital (GOSH) is undertaken with Tristel Fuse (Tristel, Snailwell, UK); which contains chlorine dioxide (ClO_2). This is used except for those areas deemed to be at high risk for environmental contamination (determined based on work undertaken in Chapter 3) or containing particularly susceptible patients.[499] In these high risk areas ChlorClean (Guest Medical, Aylesford, UK) which contains sodium dichloroisocyanurate (NaDCC) is utilised for all routine cleaning, and areas cleaned in this way include the hematopoietic stem cell transplantation unit (HSCTU) and the infectious diseases and immunology unit (IIU) as well as the other haematology/oncology units. In contrast cleaning during studies undertaken as part of this work at the National Hospital for Neurology and Neurosurgery (NHNN) was undertaken with microfibre cloths and water.

GOSH also undertakes 'deep clean' post discharge for patients colonised/infected with pathogens such as viruses, methicillin resistant *Staphylococcus aureus* (MRSA), *Clostridium difficile*, resistant Gram-negative flora and vancomycin resistant *Enterococci* (VRE).[500] These are referred to as level 2 cleans (level 1 cleans being standard discharge cleans) and involve removal of all linen and clinical equipment from the cubicle; these then undergo a separate cleaning protocol. The remaining items, floor, and walls to hand height are cleaned using NaDCC and disposable cloths and mops.

The most extensive cleans performed within GOSH are referred to as level 3 cleans and are undertaken on discharge in cubicles where patients have been positive for CRE, MRSA with a skin shedding condition, *Mycobacterium tuberculosis* or norovirus with vomiting.[500] Additionally level 3 cleans are undertaken in the HSCTU and IIU

when patients have been adenovirus positive. Level 3 cleans are the same as level 2 cleans, but include the cleaning of all walls and ceilings.

As chlorine based cleaning chemistries such as (NaDCC) can impact the fabric of the environment and the efficiency of standard cleaning techniques has been questioned there has become an increasing focus on the use of novel decontamination technologies.[501-503] Technologies such as those utilising hydrogen peroxide (HP) and ultraviolet light (UV) are becoming more widely utilised as they are less reliant on user efficiency in order to decontaminate the healthcare environment.[504-506]

As much as decontamination of the environment has been identified as a corner stone for environmental control, healthcare environments are complex, especially within intensive care units, and it is likely that there will be multiple healthcare worker-patient and healthcare worker-environment interactions.[300] With increased interactions there is increased potential for the environment to become contaminated in between decontamination episodes, it has therefore it has been suggested by a number of authors that there is a role for the built environment in controlling transmission of HCAI.[324, 332, 336] Despite this suggestion, little work has been undertaken, with the exception of air ventilation, to investigate how building design and use can affect microbial contamination. This study aims to investigate ways of managing the environment to minimise transfer organisms within it through cleaning, movement modifications and design.

5.2 CHAPTER AIMS

The aim of this chapter was to utilise the sampling techniques and inoculation studies developed in Chapter 3, to test both current and novel techniques for decontamination of clinically relevant pathogens. On the basis of these studies a quality algorithm linked to the standards discussed in Chapter 3 was developed for use with routine monitoring. In addition, this study aimed to identify how the use of clinical environments, and thus levels of microbial contamination could be affected by design and to identify potential interventions that could be used for improvement. The specific aims of this work were to:

1. Establish the effectiveness of current cleaning chemistries (NaDCC, ClO_2, and sodium hypochlorite (NaClO)).

2. Undertake a study to understand the effectiveness of cleaning in practice and to develop a monitoring algorithm to assess cleaning quality.

3. Investigate the use of novel technologies for surface and room decontamination.

4. Investigate the effect of hospital design and staff movement on contamination of the clinical environment.

5.3 RESULTS

5.3.1 SELECTION OF ORGANISMS FOR STUDY WITHIN INTERVENTION STUDIES

5.3.1.1 *KLEBSIELLA* PNEUMONIAE

Klebsiella pneumoniae was selected as species for inoculation studies as it had been demonstrated, during experiments discussed in Chapter 3, to be able to survive within the environment for a number of months as well as being a potential source of outbreaks. As *Klebsiella* spp. are capable of forming biofilms and as such produce a high level of extracellular polymeric substance (EPS) they may respond differently to cleaning and were important to include. The type strain NTCC 13368 was investigated as part of the outbreak study discussed in Chapter 4, and although not representative of the outbreak strain, it was included as the *Klebsiella* spp. strain within this study as it is a well characterised strain.

The stability of DNA from the type strain NCTC 13368 was explored as described in methods section 2.4.1. Extracted DNA from the type strain was demonstrated to be stable on a ceramic surface at a mean temperature and humidity of 16^{o}C and 43% respectively for >2 months.

5.3.1.2 ADENOVIRUS

Human adenovirus consists of six common clinical species. These species are diverse and so in order to select a species to use for inoculation studies it was important to establish which species was most commonly represented in the patients that were admitted to GOSH. To achieve this, all primary adenovirus isolates received between 2009 and 2011 from patients within GOSH were typed using sequencing of the *Hexon* gene using single locus sequence based typing (see methods section 2.2.6.).

83 samples from 60 patients were successfully amplified. Some samples failed to amplify due to insufficient volume or viral loads of fewer than 50,000 copies/ml (see Figure 5-1.). Sequence data from type strains (available from Genbank database) were also included and analysed as described in methods section 2.8. There was only one result that did not cluster as expected with the other sequences and that is a sequence downloaded from Genbank AF542119, the provenance of which is unknown.

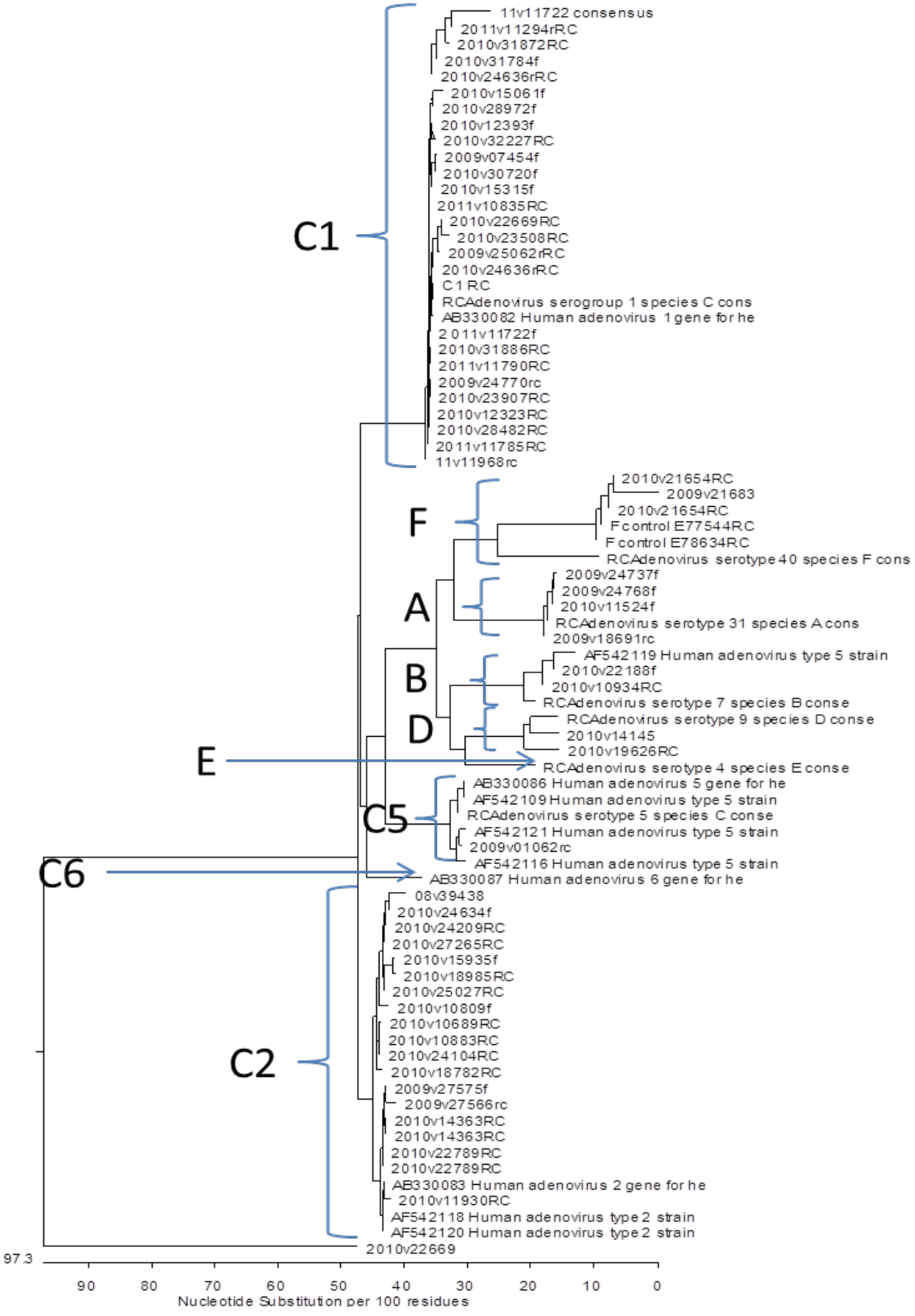

Figure 5-1 Adenovirus *hexon* gene typing results aligned by ClustalW analysis for adenovirus isolates received between 2009 and 2011 from patients within GOSH, with additional Genbank reference sequences for adenovirus serotypes.

Patients within the HSCTU and the IIU were mainly infected with adenovirus species C, with a small minority of patients positive for adenovirus species A and F. Other species were detected in non-immunocompromised patients located outside of these two units. Typing results from patients located within the HSCTU and IIU were of particular interest, as patients within those areas were at risk of severe clinical infection when exposed to viruses. This is particularly pertinent because adenovirus was detected within the environments of these units as discussed in Chapter 3.

As a result of these typing results adenovirus species C was selected as the species for inoculation studies. To establish how genetically similar these serotypes were a whole genome sequence alignment was produced from available sequences taken from the Genbank database as described in methods section 2.8.

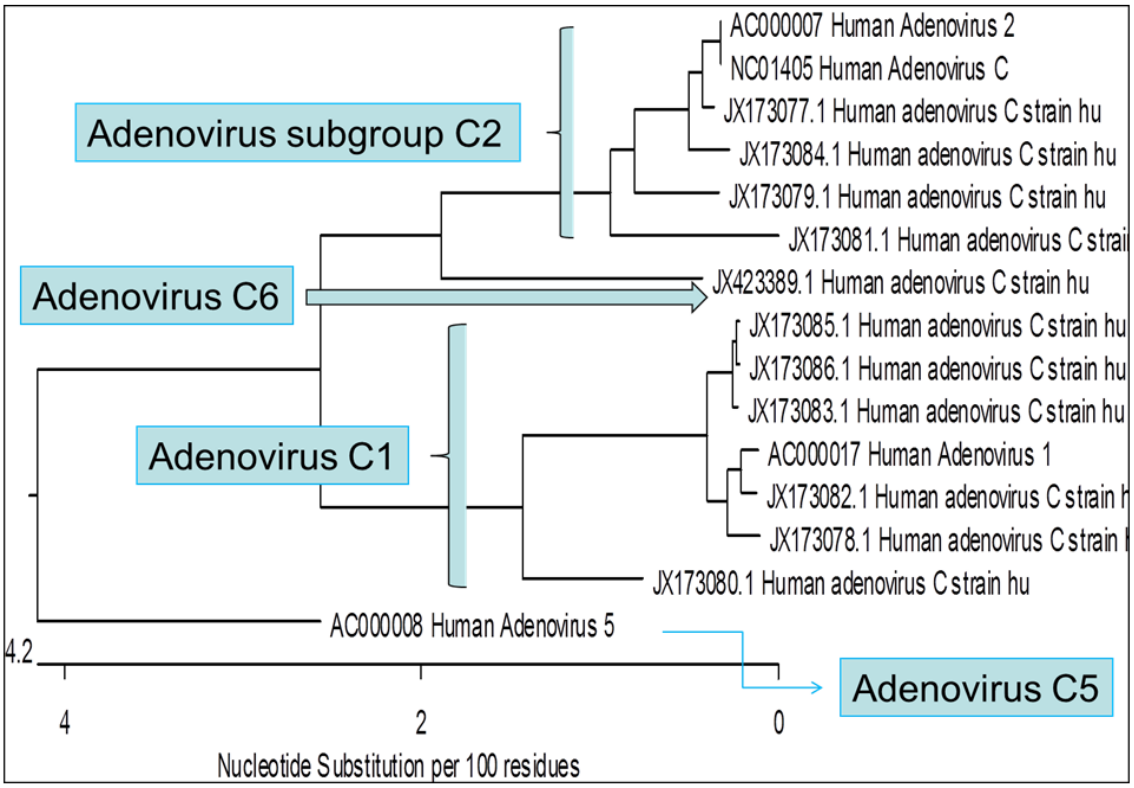

Figure 5-2 Whole genome ClustalW alignment of Genbank reference sequences.

Adenovirus species C demonstrates a highly conserved genome, see Figure 5-2. Genotype 5 was rarely detected from patient samples and no adenovirus species genotype 6 was detected. The C1 cluster of patients was marginally larger than the C2 cluster. Because of these studies adenovirus C1 was selected as type strain to be utilised for inoculation studies.

The stability of DNA from the type strain C1 (NCTC 0011051v) was explored as described in methods section 2.4.1. Extracted DNA from the type strain was demonstrated to be stable on a ceramic surface at a mean temperature and humidity of 16^{o}C and 43% respectively for >2 months.

5.3.1.3 STAPHYLOCOCCUS AUREUS

Staphylococcal spp. were the most common isolates detected throughout the sampling undertaken for Chapter 3. Within the Staphylococcus genus *Staphylococcus aureus* is the species most frequently associated with HCAI and has been shown to survive within the environment for >3 months by studies undertaken as part of this work. For this reason it was decided to use NCTC 65711 within the inoculations studies.

The stability of DNA from the type strain NCTC 65711 was explored as described in methods section 2.4.1. Extracted DNA from the type strain was demonstrated to be stable on ceramic surface at a mean average temperature and humidity of 16^{o}C and 43% respectively for >2 months.

5.3.2 ESTABLISHING THE EFFECTIVENESS OF CURRENT CLEANING CHEMISTRIES

5.3.2.1 EXPERIMENTAL STUDY

In order to investigate how current cleaning chemistries utilised at GOSH rendered organisms non-viable, inoculation studies were undertaken. The impact of these cleaning chemistries on the integrity of DNA was also determined with a view to assessing if DNA degradation was sufficient to permit molecular surveillance of organisms in the environment. Three cleaning chemistries were investigated:

- NaCIO (Sodium Hypochlorite) (Sigma–Aldritch, Gillingham, UK) which has been determined in the literature to degrade DNA,
- CIO_2 (Tristel Fuse Chlorine Dioxide) (Tristel, Snailwell, UK) which is utilised for routine cleaning in non-high risk areas at GOSH
- NaDCC (ChlorClean Sodium dichloroisocyanurate) (Guest Medical, Aylesford, UK) which is used for level 2 and 3 cleans as well as routine cleaning in high risk areas at GOSH.

Both NaDCC and NaCIO had 1000ppm available chlorine; Tristel Fuse does not list available CIO_2.

Klebsiella pneumoniae (NCTC 13368), *Staphylococcus aureus* (NCTC 65711) and adenovirus C1 (NCTC 0011051v) were inoculated onto ceramic tiles and exposed to NaClO, NaDCC and ClO_2 for 10 minutes, 60 minutes and 120 minutes as described in methods section 2.4.2.3. Samples were then processed for viability and for DNA amplification as described in methods sections 2.2.5.1, 2.2.5.2, 2.2.5.4, 2.3.2.6 and 2.1.2.

Data analysis was undertaken looking at the mean colony forming units (CFU/ml) log_{10} reduction between four replicates for viability studies. This is standard practice within the published literature and is in line with ISO standards (see Chapter 1).[21] For molecular studies DNA degradation was assessed by examining the mean cycle threshold value (CT) change within test samples compared to controls using real-time PCR amplification, this is also standard practice within the literature.[507, 508] No quantification was performed using PCR standards because, as discussed in Chapter 3, swabbing is not a quantitative sampling method as DNA extraction from cotton swabs is not 100% efficient. However a CT value increase of 3.3 CTs equates to a theoretical 1 log_{10} reduction in detectable genomes within the input extract. A PCR value of 40 equates to a negative clinical result and a CT result >45 equates to undetectable as this is the end point of the assay.

Bacterial Viability

Klebsiella pneumoniae was determined to be non-culturable after 60 minutes of exposure to all cleaning agents. *Staphylococcus aureus* remained viable after 120 minutes of exposure to ClO_2. Both NaDCC and NaClO produced negative culture results at 60 minutes of exposure, see Table 5-1.

Bacterial Suspension	Exposure Time (minutes)	Inoculum CFU/ml	NaClO CFU/ml	NADCC CFU/ml	ClO_2 CFU/ml	Control CFU/ml
***S. aureus* 10-1**	10	$6*10^9$	845	370	2340	$3.5*10^8$
***S. aureus* 10-1**	60	$6*10^9$	ND	ND	745	$4*10^9$
***S. aureus* 10-1**	120	$6*10^9$	ND	ND	5	$3.5*10^8$
K. pneumoniae	10	$1.2*10^{10}$	705	225	905	$5*10^8$

10-1						
***K. pneumoniae* 10-1**	60	$1.2*10^{10}$	ND	ND	ND	$5*10^{8}$
***K. pneumoniae* 10-1**	120	$1.2*10^{10}$	ND	ND	ND	$5*10^{8}$

Table 5-1 Effect on *Klebsiella pneumoniae* and *Staphylococcus aureus* viability when exposed to NaClO, NaDCC and ClO_2. for either 10, 60 or 120 minutes. ND = not detected.

Bacterial DNA Degradation (Viable Suspension Inoculated and Exposed)

Bacterial suspensions in saline were inoculated onto ceramic tiles as described in methods section 2.4. with four replicates run for each 10^{-1}, 10^{-2}, and 10^{-3} test and control dilutions. DNA degradation was inferred by an increase in mean test CT when compared with mean control CT (ΔCT) when tested by real-time PCR.

Klebsiella pneumoniae DNA demonstrated degradation with all three cleaning chemistries, see Figure 5-3. The highest levels of degradation were observed in the 10^{-1} dilutions. The combined ΔCT (all ΔCT differences from controls combined for each cleaning agent) as a measure of DNA degradation was greatest for NaClO (97) followed by NaDCC (79) and ClO_2 (68).

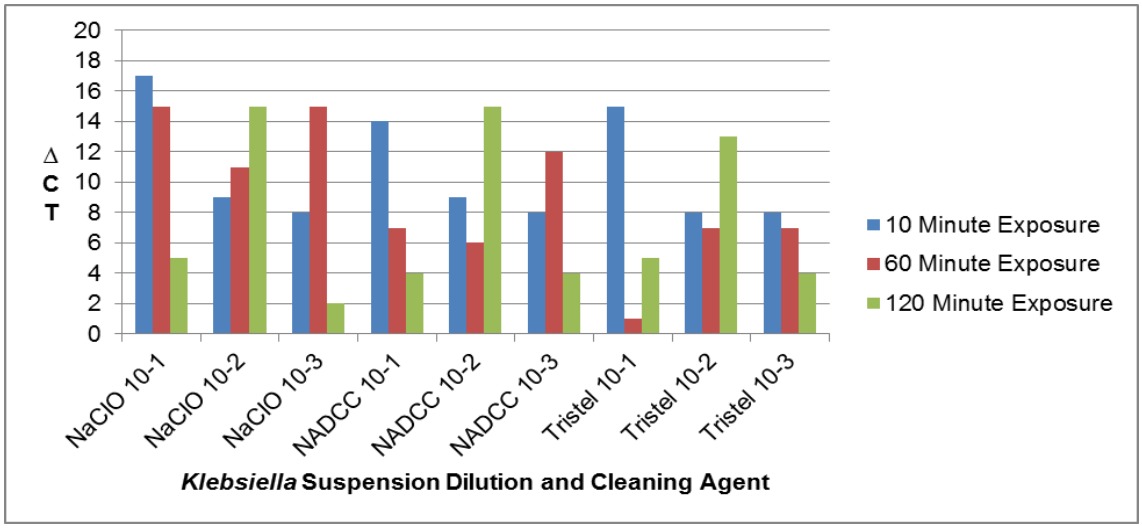

Figure 5-3 Alteration in CT detection of DNA in comparison to controls for *Klebsiella pneumoniae when* exposed to NaClO, NaDCC and ClO2. for either 10, 60 or 120 minutes.

When exposed to NaClO 7/9 averaged replicates became undetectable by PCR and so were assigned a CT value of 45. For NaDCC 4/9 averaged replicates were undetectable by PCR and for ClO_2 3/9. Interestingly, at 60 minutes, all cleaning

chemistries led to decrease in CT detected, indicating increased availability of DNA in the starting sample.

Samples with a CT greater than 40 would be considered negative for clinical samples. All averaged replicate results, at all time points, when exposed to NaCIO would be considered negative using a cut-off of 40 CTs, 7/9 would be negative when exposed to NaDCC and 6/9 for ClO_2 exposed DNA.

Staphylococcus aureus DNA degraded at all dilutions for both NaCIO and NaDCC, but failed to demonstrate degradation in comparison to the control for 2/3 dilutions when exposed to ClO_2. The highest levels of degradation were observed in the 10^{-1} dilutions, see Figure 5-4. The exposure time with the greatest number of amplified samples was 60 minutes, where all samples had detectable levels of DNA present. The combined ΔCT change was greatest for NaCIO (80) followed by NaDCC (66) and ClO_2 (18).

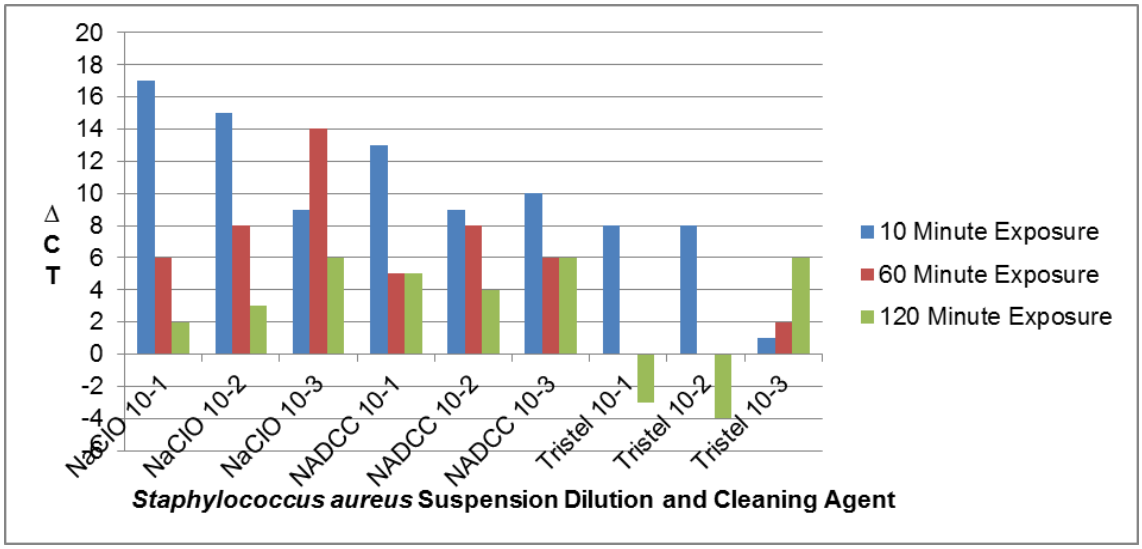

Figure 5-4 Alteration in CT detection of DNA in comparison to controls for *Staphylococcus aureus* when exposed to NaCIO, NaDCC and ClO_2. for either 10, 60 or 120 minutes.

When the inoculated *Staphylococcus aureus* suspension was exposed to NaCIO 1/9 averaged replicates became undetectable by PCR, 3/9 averaged replicates became negative when exposed to NaDCC and 1/9 exposed to ClO_2.

Samples with a CT greater than 40 would be considered negative for clinical samples. When exposed to NaCIO all averaged replicates (9/9) would be considered negative using a greater than 40 CT cut-off. Of samples exposed to NaDCC 7/9 averaged replicates were considered negative and 6/9 averaged replicates exposed to ClO_2.

Virus Viability

Adenovirus tissue culture at a concentration of $4.75*10^8$ copies/ml was inoculated onto ceramic tiles as described in methods section 2.4 with four replicates run for each 10^{-1}, 10^{-2}, 10^{-3} 10^{-4}, 10^{-5}, and 10^{-6} test and control dilutions. Adenovirus C1 tissue culture samples were non-viable post 120 minute exposure to NaClO, NaDCC and ClO_2 when grown in tissue culture for 6 days as described in methods section 2.1.2.

Virus DNA Degradation

Viable adenovirus C1 tissue culture was inoculated onto ceramic tiles as described in methods section 2.5.2.3. DNA degradation was observed with all three cleaning chemistries when tested by PCR as described in methods section 2.2.5.4. The exposure time with the lowest CT (indicating greatest available DNA) within amplified samples was after 10 minutes exposure for all cleaning agents, except ClO_2; which demonstrated the lowest CTs after 120 minutes of exposure. The combined ΔCT change was greatest for NaClO (51) followed by NaDCC (46) and ClO_2 (44) (see Figure 5-5). No averaged replicate (mean of four individual test replicates) became undetectable or negative using a >40CT cut-off.

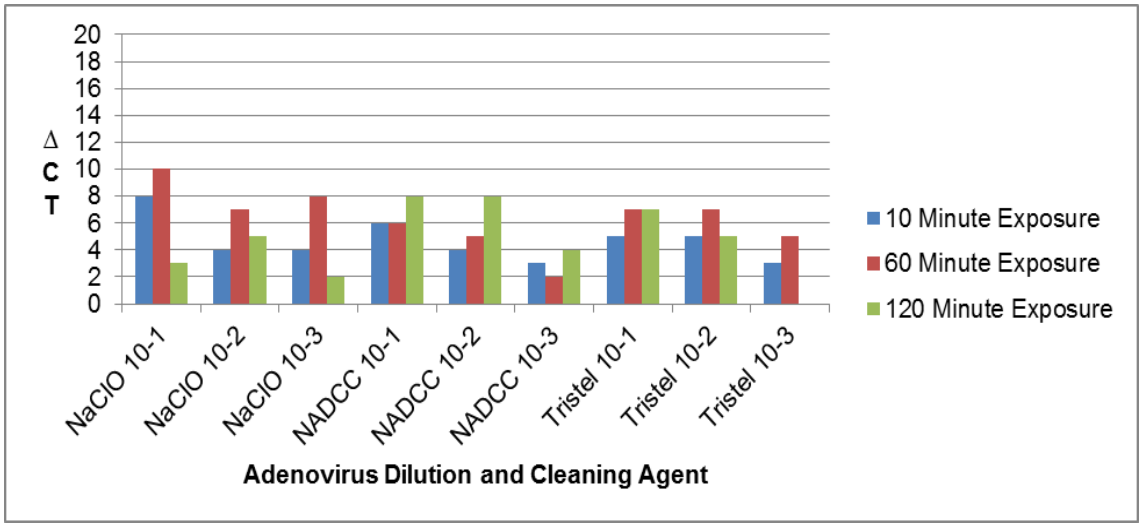

Figure 5-5 Alteration in CT detection of DNA in comparison to controls for adenovirus C1 tissue culture when exposed to NaClO, NaDCC and ClO_2. for either 10, 60 or 120 minutes.

Adenovirus C1 extracted DNA was inoculated as described in methods section 2.4. to determine if the presence of protein within the tissue culture altered results for DNA degradation. DNA inoculated samples were exposed to NaDCC for 120 minutes and results compared to those when tissue culture was inoculated.

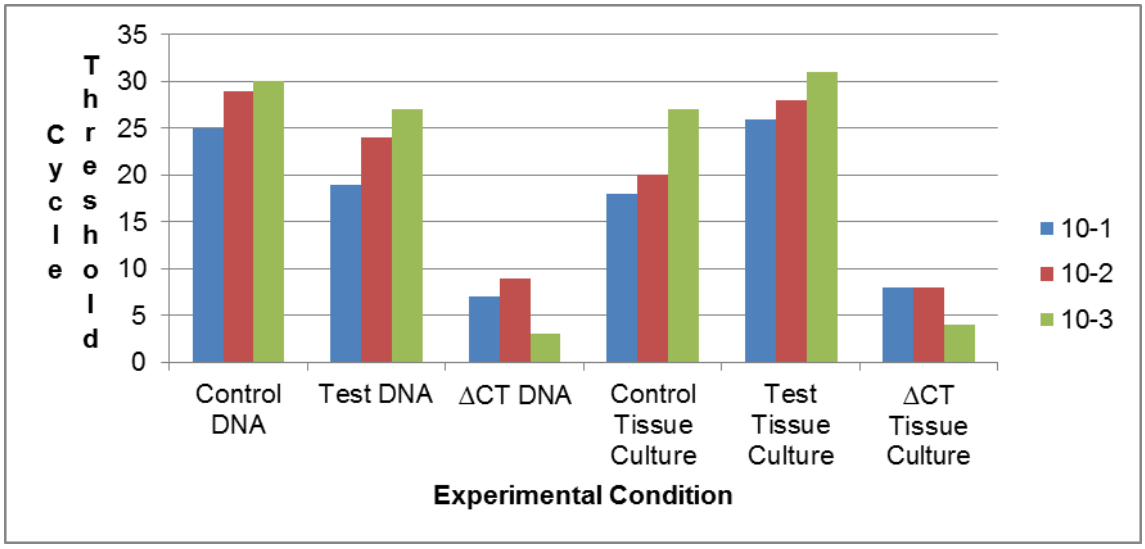

Figure 5-6 Alteration in CT detection of DNA inoculation versus tissue culture inoculation at three dilutions for adenovirus C1 when exposed to NaDCC for 120 minutes.

No averaged replicates became undetectable or negative using a >40CT cut-off for positivity. In the presense of protein there was a ΔCT reduction range of 4 – 8 CTs, for DNA there was a ΔCT reduction range of 3 – 9 CTs, see Figure 5-6. Cumulative ΔCT changes were similar, with 19 for DNA inoculations and 20 for tissue culture.

5.3.2.2 HOSPITAL APPLICATION STUDY

As NaDCC had been demonstrated to degrade DNA during the experimental study described in 5.3.2.1., screening of cubicles by PCR that had been occupied by adenovirus positive patients was undertaken within the HSCTU and IIU after cleaning with NaDCC. Data was collected using environmental screening methods as discussed in Chapter 3 and methods section 2.3.1.1. The data collected and analysed was the result of infection control environmental surveillance over a 5 year period with 794 surfaces screened in 48 cubicles from 2005 to 2009. For the first three years surveillance data was collected as part of routine infection control surveillance, rather than an experimental study and so the selection of sites screened varied slightly during this period.

Of the total sites sampled during the screening period 28% of surfaces were detected as positive for adenovirus DNA. In one instance it was found that sinks, clinical waste bins, phones and floors were still contaminated after six consecutive cleans. In another bathroom taps, bed frames, mattresses, taps, trolleys, window sills, exit door handles and chair arms were only decontaminated after five consecutive cleans.

Further analysis was undertaken during the final twelve months of the screening period when a new screening protocol was introduced as per methods section using linear regression techniques (see methods section 2.6.). Statistical modelling demonstrated that objects present in cubicles such as: bed frames, mattresses, telephones, bathroom taps, exit door handles, and chair arms, were the objects most likely remain contaminated, with chair arms being significantly linked to contamination (p=0.008). These items were identified as difficult to clean and/or are linked to high levels of parent/patient contact.

The number of surfaces found to be adenovirus DNA free per cubicle during this period ranged from 60% - 100%; however of the total 585 sites cleaned once only, 23% of sites remained positive. Of the 48 cubicle screens analysed, twelve (25%) had at least one site with a CT value of <34 or at least two sites with samples containing adenovirus detected at between 34 and 38 cycles.

Second and fourth cleans were significantly less effective at removing adenovirus DNA than the initial clean, or third and fifth cleans (p=<0.01). After a second clean all but one contaminated cubicles remained positive for adenovirus DNA. Following a third clean, only three cubicles remained contaminated, and this reduced to two after a fourth clean. One cubicle remained positive after a fifth clean and no cubicles had adenovirus DNA detected after a sixth clean. Table 5-2 shows the results for a cubicle that required six consecutive cleans to have no adenovirus DNA detected. This cubicle was screened by both trained and untrained personal which produced different results on identical sites screened within two hours of each other and no other members of staff entering the cubicle. The possible reasons for this variation are discussed in more detail in Chapter 3.

Site	Post 1st L3C (CT)	Post 2nd L3C (CT)	Post 3rd L3C (untrained) (CT)	Post 3rd L3C (rescreen - trained) (CT)	Post 4th L3C (CT)	Post 5th L3C (CT)
Floor under sink	34	38	40	ND	ND	ND
Clinical waste bin	35	39	ND	33	37	ND

Chair arms	35	41	38	35	34	38
Bathroom door handle	ND	44	ND	38	42	ND
Telephone	33	36	ND	35	34	ND
Bathroom taps	ND	ND	ND	ND	37	ND
Mattress top (patient)	ND	37	40	38	34	36
Bed frame (parent)	39	IS	ND	35	34	ND
Trolley	ND	41	ND	IS	39	ND
Window sill	41	ND	ND	36	39	ND
Exit door handle	ND	41	ND	40	39	ND
Corridor floor	43	37	45	36	34	ND

Table 5-2 Adenovirus real-time PCR results on the same cubicle after 5 level three cleans (L3C) with no inpatient admission inbetween screening and cleaning. Screening undertaken by both trained and untrained personal. IS insufficient ND not detected.

Cubicles sampled during the summer months had significantly less adenovirus DNA detected than those sampled during the winter months (p=<0.01). There was no link between individual cubicles for detection of contamination.

5.3.3 EVALUATION OF NOVEL DECONTAMINATION TECHNIQUES

As demonstrated in section 5.3.2.2. and in Chapter 3 interventions utilising current cleaning chemistries are not always successful at reducing environmental contamination and so two newly available technologies were evaluated for decontamination; either for use on clinical equipment or rooms.

Data analysis was undertaken looking at the mean colony forming units (CFU/ml) $\log_{10}$ reduction between four replicates for viability studies. This is standard practice within the published literature and is in line with ISO standards (see introduction).[21] For molecular studies DNA degradation was assessed by examining the mean cycle threshold value (CT) change within test samples compared to controls using real-time PCR amplification, this is also standard practice within the literature.[507, 508] No quantification was performed using PCR standards because, as discussed in Chapter 3, swabbing is not a quantitative sampling method as DNA extraction from cotton swabs in 100% efficient. However a CT value increase of 3.3 CTs equates to a theoretical 1 $\log_{10}$ reduction in detectable viral genomes within the input extract. A PCR value of 40 equates to a negative clinical result and a CT result >45 equates to undetectable as this is the end point of the assay.

5.3.3.1 OBJECT DECONTAMINATION

The use of ultraviolet light (UV) for decontamination of clinical equipment was investigated using UV cabinets (Nanoclave Technologies, London, UK). Cabinets use short wave UV light (UV-C) targeted from all directions in order to decontaminate an object. An inoculation experiment was undertaken as described in methods section 2.4.2.1. During the inoculation experiment adenovirus species A (serotype 31) was used, as work was undertaken prior to the adenovirus typing described in section 5.3.1.2.

Virus Viability

Viable adenovirus A31 was inoculated from tissue culture onto tiles as described in methods section 2.4. Tissue culture contained ~$2.9*10^{10}$ copies/ml of adenovirus and dilutions 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were used. A six minute exposure to UV rendered all replicates at all dilutions of the virus non-detectable by tissue culture at six days.

Virus DNA Degradation on Simple Surfaces

Viable adenovirus A31 was inoculated in duplicate from tissue culture onto tiles as described in methods section 2.1.2. The effect of UV on adenovirus DNA was determined utilising adenovirus PCR as described in methods section 2.2.5.4.

		Exposure time					
	Control	1 min	2 min	3 min	4 min	5 min	6 min
Mean CT (n=4)	18	22	27	27	34	ND	ND

Table 5-3 CT values of adenovirus PCR performed on swabs from inoculated test areas of ceramic material after accumulative exposure to UV light in the Nanoclave cabinet. Not detected (ND) = CT>45.

After a 3 minute UV exposure a ΔCT of 9 was detected and after 5 minutes DNA was undetectable by real-time PCR, see Table 5-3.

Virus DNA Degradation on Complex Surfaces

Five medical devices were tested at two sampling time points (as described in methods section 2.4.2.1.), one after a three minute UV exposure and another after a six minute UV exposure. The medical devices consisted of metallic and plastic surface types. As there was inadequate space for a control, control data was used from experiments run in parallel on ceramic tiles in order to calculate the $\log_{10}$ reduction (control CT at 18).

As multiple versions of the same object were not available multiple sites were inoculated onto the same equipment, 50% were sampled after three minutes of exposure to UV light and the other 50% were sampled after six minutes of exposure.

Both the remote control and pulse oximetry monitor consisted of plastic surfaces and demonstrated a ΔCT of 11 and 10 respectively after a six minute exposure. A single piece of dialysis equipment was tested, where the sample area consisting of a metal plate. It demonstrated a ΔCT of 7 after a three minute exposure and DNA was undetectable after a six minute exposure. The electronic blood pressure gauge consisted of a plastic surface and demonstrated a ΔCT of 5 after a six minute exposure. There was no difference in CT detection between sampling at time points three minutes and at six minutes (see Table 5-4.)

	Remote Control (Mean CT)	Pulse Oximetry Monitor (Mean CT)	Blood Pressure Gauge (Mean CT)	Dialysis Equipment (Mean CT)
3 minute exposure	20	32	23	25
6 minute exposure	29	28	23	ND

Table 5-4 CT values of adenovirus PCR performed on swabs from inoculated test areas on medical devices after accumulative exposure to UV light in the Nanoclave cabinet. Not detected (ND) = CT>45.

Position of the surface tested appeared to impact on adenovirus DNA detection, with objects containing complex surface sites that were partially obscured, such as the electronic blood pressure gauge, demonstrating a lower CT (increased DNA detected) after a six minute exposure. This may also explain the discrepancy in results for the pulse oximeter, as some of the inoculation sites may have been exposed to higher levels of UV than others, due to the complex nature of the surface.

5.3.3.2 ROOM DECONTAMINATION

Hydrogen Peroxide (HP) H_2O_2 has been suggested as an effective additional decontamination method to support standard surface cleaning. In this study, we assessed the ability of HP treatment to eliminate viable bacteria and viruses and degrade DNA. Two different HP–based instruments were investigated as described in methods section 2.4.2.2: the Bioquell Q10 (Bioquell, Andover, UK) which used hydrogen peroxide vapour (HPV) and the GLOSSAIR™ 400 (Advanced Sterilization Products, Wokingham, UK) which utilises a dry mist hydrogen peroxide (DMHP) system. The Bioquell system produces a vapour from 30% H_2O_2 using heat evaporation. In contrast, the Glossair system produces a fine dry mist by aerosolizing a solution containing 5% v/v hydrogen peroxide, with silver cations at less than 50ppm. HP cycles were undertaken as described in Table 5-5 and methods section 2.4.2.2.

Equipment	Experiment No.	Cycle No.	Cycle Dosage	Contact Time
Bioquell Viable and DNA exposure	E1	1	$10g/m^3$	15 min
Glosair Viable bacteria and DNA exposure	E1	1	$6ml/m^3$	2hours

DNA exposure	E2	1	9ml/m^3	2 hours
DNA exposure	E3	3 consecutive	12ml/m^3	2 hour
DNA exposure	E4	3 consecutive	6ml/m^3	2 hour
DNA exposure	E5	3 consecutive	6ml/m^3	30 min
DNA exposure	E6	3 consecutive	6ml/m^3	15 min

Table 5-5 Hydrogen peroxide cycling conditions for Glossair and Bioquell systems for both viability and DNA denaturation experiments.

Bacterial Viability

Klebsiella pneumoniae (NCTC 13368) and *Staphylococcus aureus* (NCTC 65711) suspensions were made in each of 1 * phosphate buffered saline (PBS) and 0.3% bovine serum albumin (BSA) and inoculated onto ceramic tiles. Tiles were placed on a surface at waist height within an environmentally controlled chamber and exposed to the manufacturers' recommended HP cycle (methods section 2.4.2.2.). *Klebsiella pneumoniae* was determined to be non-culturable after exposure to a standard cycle of the Bioquell HP instrument in both suspensions made up in PBS and BSA, representing a >7 $\log_{10}$ reduction, see Table 5-6. The inoculum used with the standard Glossair cycle was greater (10^9 vs 10^{10}) and *Klebsiella pneumoniae* remained viable even though Glossair achieved >8 $\log_{10}$ reduction under both suspension conditions. *Staphylococcus aureus* had residual viability under all test conditions. The lowest log kill was achieved by the Bioquell cycle with a suspension in PBS while Glossair achieved a >9 $\log_{10}$ reduction. Log kills for Glossair and Bioquell were similar for BSA suspensions with both demonstrating a >8 $\log_{10}$ reduction.

	1 * Phosphate Buffered Saline Bacterial Suspension			0.3% Bovine Serum Albumin Bacterial Suspension		
Organism and HP Technology	**Control CFU/ml**	**Test CFU/ml**	**Log Reduction**	**Control CFU/ml**	**Test CFU/ml**	**Log Reduction**
Bioquell *S. aureus*	$3.35*10^{8}$	$2.63*10^{2}$	6.11	$1.23*10^{10}$	25	8.69
Glossair *S. aureus*	$4.49*10^{10}$	10	9.65	$1.73*10^{9}$	10	8.24
Bioquell *K. pneumoniae*	$2.32*10^{9}$	ND	8.37	$2.56*10^{8}$	ND	7.41
Glossair *K. pneumoniae*	$4.5*10^{10}$	10	9.65	$2.44*10^{9}$	10	8.39

Table 5-6 Comparative effectiveness at reducing bacterial viability of Bioquell and Glossair hydrogen peroxide decontamination methods using standard cycling conditions.

Virus Viability

Viable adenovirus C1 was inoculated from tissue culture onto tiles as described in methods section 2.5.2.2. Tissue culture contained $4.16*10^{8}$ copies/ml of adenovirus and dilutions 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were used. Post exposure to HP via standard cycles of Bioquell and Glossair all replicates at all dilutions of the virus were rendered non-detectable by tissue culture at six days.

Bacterial DNA Degradation

Staphylococcus aureus (NCTC 13368) and *Klebsiella pneumoniae* (NCTC 65711) DNA was inoculated onto ceramic tiles as described in methods section 2.4. with four replicates run for each test and control dilution. Tiles were placed on a surface at waist height within an environmentally controlled chamber and exposed to the manufacturers' recommended HP cycle. Additional cycles were undertaken using the Glossair technology with variations in terms of contact time and HP dosage as described in the methods section 2.4.2.2.

DNA degradation was not consistent, with only cycle Glossair E2 (9ml/m^3 cycle dose) showing reductions in DNA amplification at all dilutions when compared to the control, see Figure 5-7. 4/18 dilutions across experiments when exposed to HP by all Glossair cycles demonstrated no change in CT relative to the control; 10^{-1} E1, 10^{-3} E1, 10^{-1} E4 and 10^{-3} E5 (see table 5.6. for cycle differences). 2/18 dilutions across experiments when exposed to HP by Glossair demonstrated an increase in amplification when CT was compared to controls, tests 10^{-1} E3 and 10^{-3} E6, indicating greater DNA amplification. 12/18 tests showed between 1 and 15 CT increases between control and test CTs, indicating DNA degradation. For Bioquell exposed samples 2/6 tests demonstrated an increase in amplification relative to control sample with both the 10^{-1} and 10^{-2} dilutions (decrease in CT). Of the other four dilutions that were exposed to the HP cycle no difference between control and test CTs was noted.

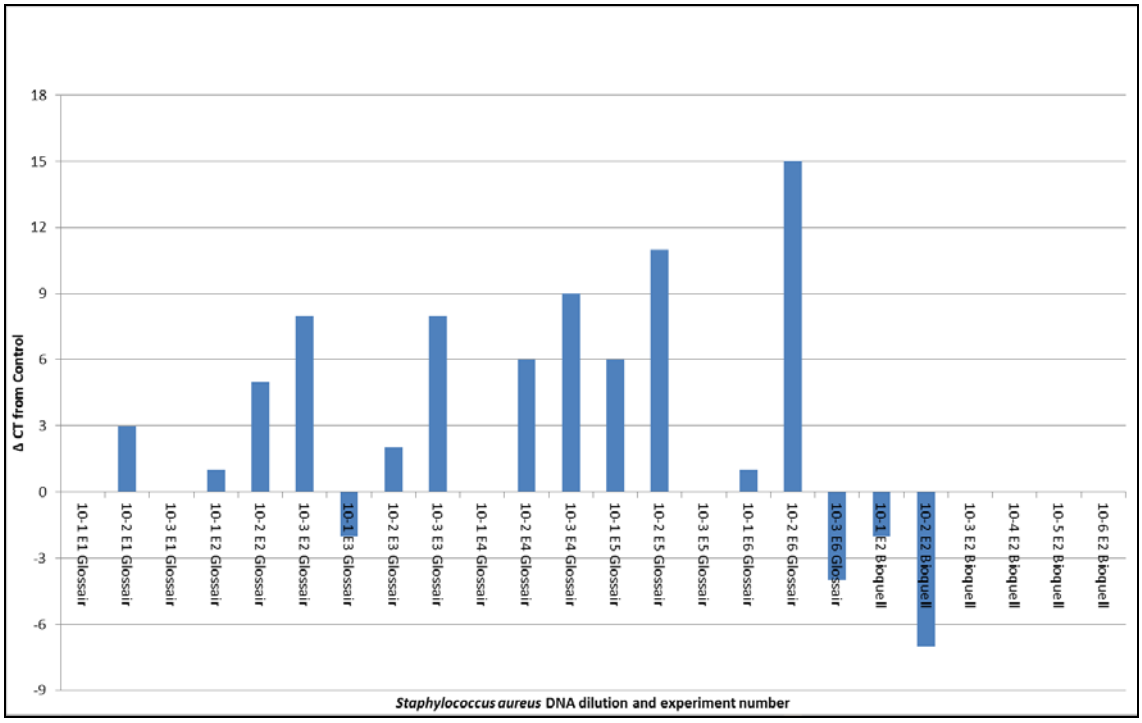

Figure 5-7 *Staphylococcus aureus* real-time PCR results for Glossair and Bioquell experiments comparing CTs between test conditions and control (Δ CT).

Klebsiella pneumoniae DNA degradation was demonstrated with all six Glossair HP cycles at all but two test dilutions, see Figure 5-8. CT increases between 1 and 14 CTs occurred in 16/18 tests between control and test CTs, indicating DNA degradation. One test, 10^{-1} dilution in cycle E1, demonstrated no change between control and test CTs. One further test, 10^{-2} cycle E2, demonstrated a 1 CT decrease between test and control, indicating increased DNA amplification. All tests exposed to Bioquell HP

demonstrated a decrease in CT between test and control, with CTs decreasing in the test between 1 and 8 CTs, indicating increased DNA amplification.

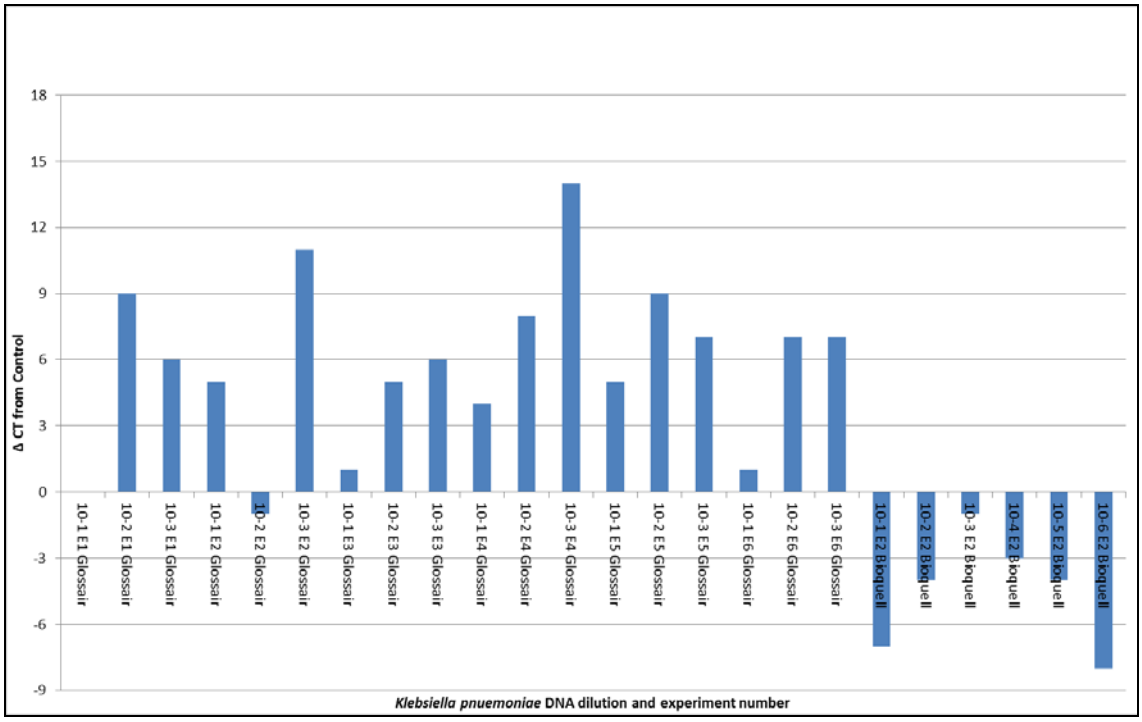

Figure 5-8 *Klebsiella pneumoniae* real-time PCR results for Glossair and Bioquell experiments comparing CTs between test conditions and control (Δ CT).

Viral DNA Degradation

Tests for adenovirus DNA degradation were initially run on the ABI 7500 Fast real-time PCR machine (Applied Biosystems, Warrington, UK) using fast amplification conditions and master mix (Fast Universal PCR mastermix (Applied Biosystems, Warrington, UK)) as described in methods section 2.2.4.4. Amplification for tests exposed to Glossair cycles was limited, but this was not seen for controls (see Figure 5-9). Tests were re-run using the same PCR machine, but using slow amplification conditions and master mix (QuantiTect mastermix (Qiagen, Crawley, UK)) as described in methods section 2.2.4.4. Amplification was then observed in almost all test samples (see Figure 5-10). A similar affect was not observed for Bioquell exposed samples processed at the same time.

Figure 5-9 Fast amplification of adenovirus DNA exposed to the Glossair platform.

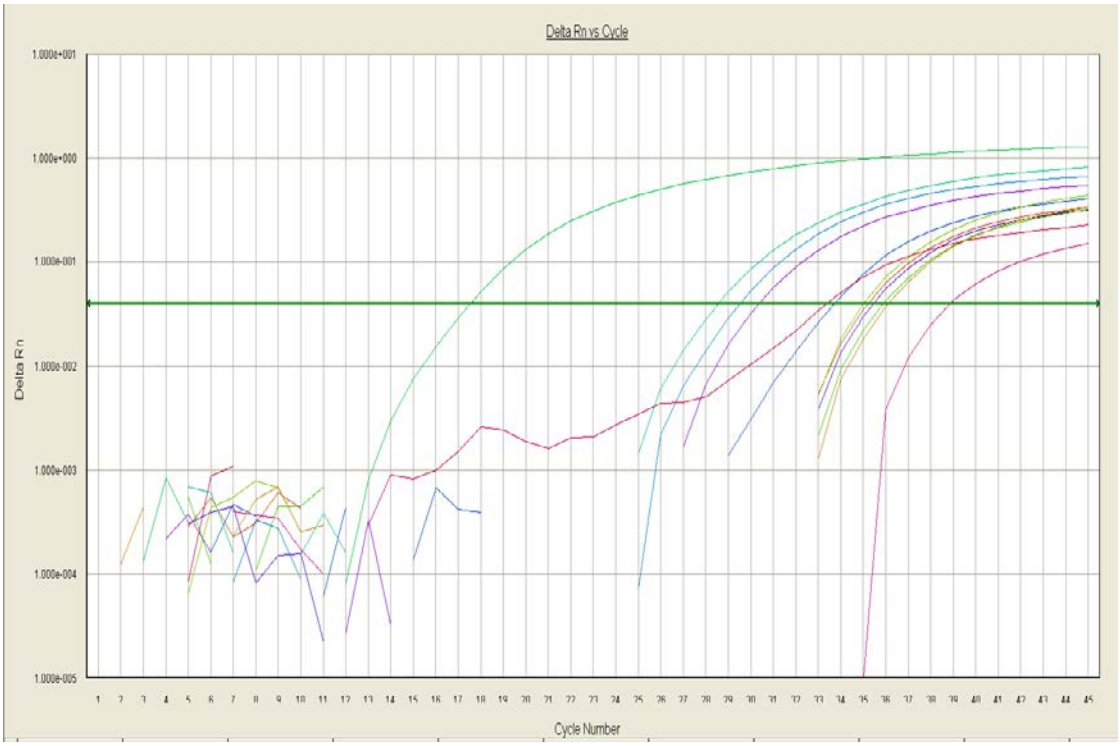

Figure 5-10 Slow amplification of adenovirus DNA exposed to the Glossair platform.

Adenovirus C1 DNA was inoculated onto ceramic tiles as described in methods section 2.5. with four replicates run for each 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} test and control dilutions. Tiles were placed on a surface at waist height within an environmentally controlled chamber and exposed to the manufacturers' recommended HP cycle. Additional cycles were undertaken using the Glossair technology with variations in terms of contact time and HP dosage as described in the methods section 2.4.2.2. and Table 5-5.

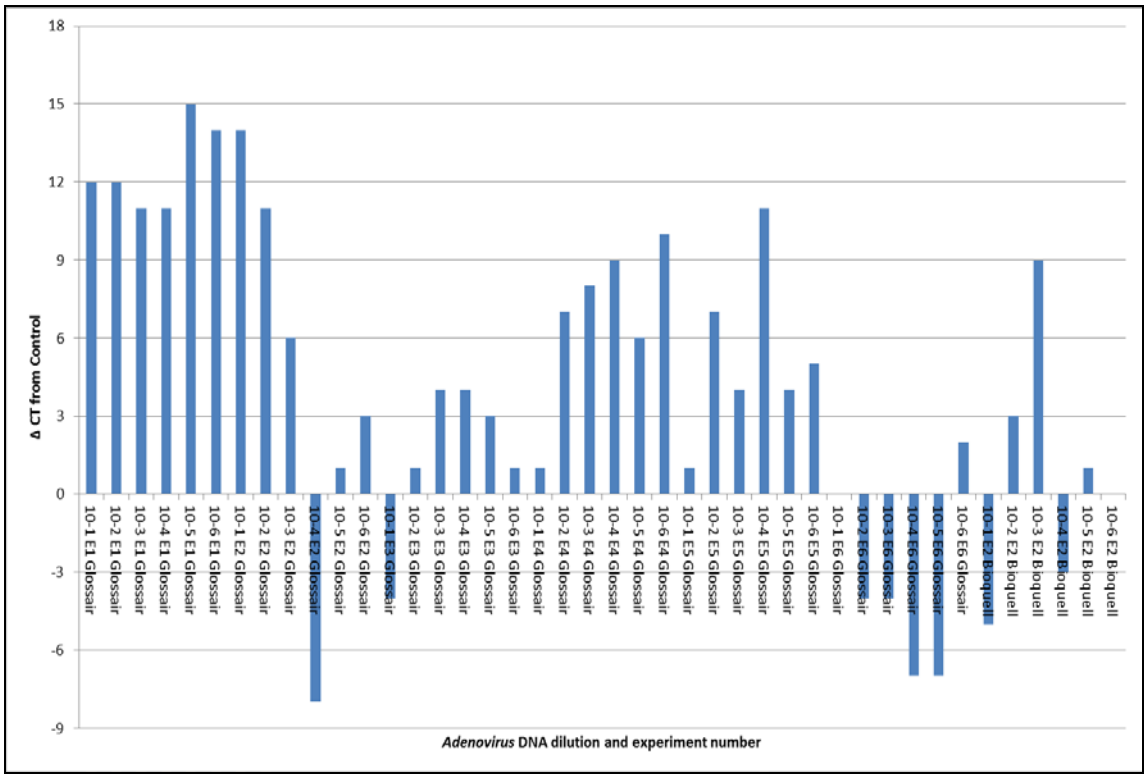

Figure 5-11 Adenovirus real-time PCR results for Glossair and Bioquell experiments comparing CTs between test conditions and control (Δ CT).

Adenovirus DNA degradation was demonstrated with all six Glossair HP cycles and at all, but seven test dilutions (10^{-4} E2, 10^{-1} E3, 10^{-1} E6, 10^{-2} E6, 10^{-3} E6, 10^{-4} E6 and 10^{-5} E6). Bioquell HP exposure resulted in a reduction in CT for dilutions 10^{-1} and 10^{-4} and an increase in CT for dilutions 10^{-2}, 10^{-3} and 10^{-5}, with no change versus control seen for the 10^{-6} dilution. Changes of >3 logs (increase of >11 CTs) was demonstrated for Glossair experiment 1 between control and test samples for all dilutions.

No single HP cycle degraded DNA reproducibly across both bacterial and viral DNA (Figure 5-7, Figure 5-8 and Figure 5-11). The standard Glossair cycle caused the highest level of DNA degradation, but for both *Staphylococcus aureus* and *Klebsiella pneumonia* there were still dilutions with no change from controls.

5.3.4 SINKS

Sinks have been demonstrated to be a potential source of outbreaks within the healthcare setting (see Chapter 4). The outbreak described continued for some months despite the practice of cleaning with NaDCC which was validated as effective in section 5.2.2.1. Outside of outbreak situations and in spite of routine cleaning using the techniques validated in section 5.3.2.1., high CFU counts and pathogens were detected on sinks within GOSH (as discussed in Chapters 3). A number of studies were undertaken both at GOSH and at the NHNN in order to better understand why sinks were contaminated, why that contamination persisted within the HSCTU, and why it was not consistent between sampling occasions in other areas.

5.3.4.1 RELATIONSHIP BETWEEN VISIBILITY AND SINK USAGE

Pilot work was undertaken on an adult ITU based at the NHNN to understand the relationship between sink visibility and sink use. Within the adult ITU the bed space:sink ratio was higher than at GOSH 1.3:1 compared to 1:1. Sink visibility was analysed as described in methods section 2.6.2. Figure 5-12 demonstrates the visibility of some sinks was influenced when the curtains were drawn around the bed space.

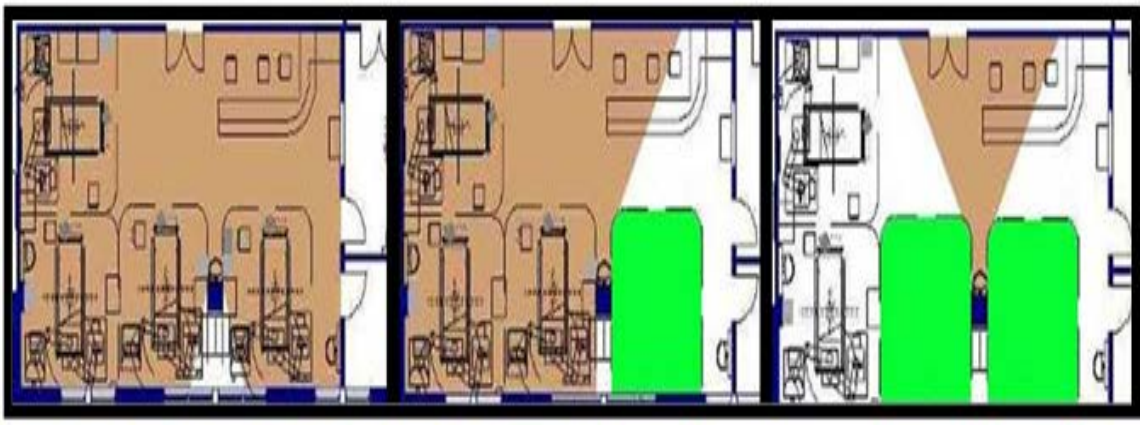

Figure 5-12 The effect of curtain closure on the visibility of sink 5 within medical intensive care unit at the NHNN (sink in the centre of the image). The sink is visible within the brown area and the green area is the area blocked when curtains are closed.

When no curtains were drawn the central sink was visible from the entirety of the unit; when the curtain on the right hand bed space was closed the sink remained visible from most locations within the unit; however when the curtains were drawn in both the adjacent bed spaces the sink was visible only from the main entrance gate and the nurses station.

In the medical intensive care unit (MITU) ward sink visibility was identified as being related to sink usage as is demonstrated in Table 5-7.

Sink No. in MITU	Number of Time Utilised During a 3 Hour Observation	% Visibility	
		Curtains Opened	Curtains Closed
Sink 4 (left hand sink in Figure 5-12)	11	89	15
Sink 5 (central sink in Figure 5-12)	34	97	68
Sink 6 (right hand sink in Figure 5-12)	1	77	13

Table 5-7 Sink usage in the MITU related to visibility based upon curtains open/closed as monitored over a three hour observation period (two 1.5 hour sessions) on a single day.

5.3.4.2 SINK SCREENING AND SINK LOCATION STUDY AT GOSH

Following the pilot at NHNN, which indicated that visibility may play a role in sink usage, further investigations exploring the links between sink visibility and sink use was undertaken at GOSH on the paediatric intensive care unit (PICU), cardiac intensive care unit (CICU) and neonatal intensive care unit (NICU). The visibility of hand wash basins was calculated on the basis of the ward plans as described in methods section 2.5.4. Within GOSH, the bed space:sink ratio was 1:1; however because of the ward layout not all sinks were equally visible, with sinks in central bed spaces usually demonstrating higher levels of visibility, see Figure 5-13. In addition as sinks were mostly situated on pillars at the end of bed spaces some were more visually obstructed than others, depending upon bed space position.

Sink usage was observed as described in the methods section 2.5.2. and analysed as described in methods sections 2.6.2. using linear regression analysis.

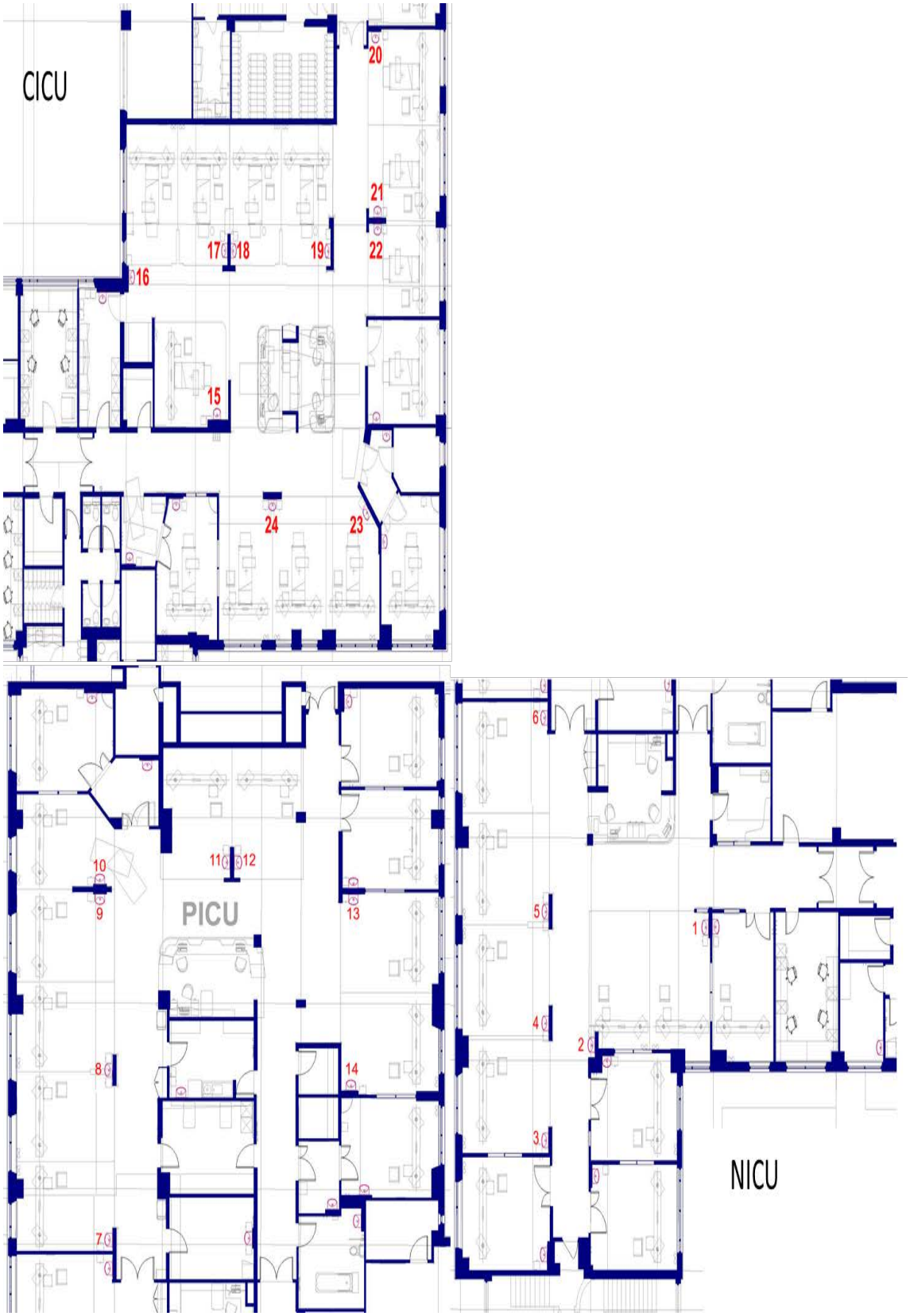

Figure 5-13 GOSH floor plans for areas studied (PICU, CICU and NICU) showing numbered sink locations.

It was found that as the proportion of visible area increased, the number of hand washing episodes also increased (p=0.007). The more visible the sink the more frequently it was utilized for hand washing. Sink usage was then assessed in relation to bed space occupancy. Visibility remained the main determinant of sink usage; however the average time spent per hand washing episode was influenced by bed occupancy. When the bed space was occupied and visible, people washed their hands for longer than when the bed space was not occupied (p=<0.001).

The mean length of hand washing per sink ranged from 9.28 – 22.13 seconds. The shortest hand washing time observed was 4.83 seconds, and the longest was 65.28 seconds, with an overall mean of 14.59 seconds based on 1063 observations.

Although hand washing compliance was not specifically monitored as part of these observations, GOSH undertakes monthly hand washing on all three units with an average compliance during the observation period of 93%, based on over 7000 observations using the National Patient Safety Agency audit tool.[509] Hand washing compliance within the individual units averaged at 97% for CICU, 85% for PICU and 87% for NICU during the period of the study.

Determinants of Sink Contamination

Bacterial swab samples were collected from eight sinks located in the three units at GOSH (PICU, NICU, and CICU) in conjunction with observational studies of the levels of hand washing undertaken.

Microbial data was collected daily during the hand washing observation period in both the morning and afternoon and then once a week for three weeks subsequently as described in methods section 2.3.4.2. The aim of this work was to model the effect of hand washing on the change of bacterial growth levels in the sinks and to gain an understanding of bacterial sink contamination as a potential transmission route. Microbial growth in relation to sink use was modelled using multilevel regression using linear mixed effects models as described in methods section 2.6.2.

All sinks and associated soap and alcohol dispensers had some bacterial contamination over the sampling period. The major organisms identified were *Staphylococcal* spp, followed by Enterobacteriaceae and *Enterococcal* spp. Greater sink usage was associated with higher levels of bacterial contamination within the bowl of the sink. In contrast, compared to the bowl of the sink, the contamination of the sink lips and soap/alcohol dispensers were inversely related to sink usage ((sink) p=0.018,

(gel dispenser) p=0.049)). Enterobacteriaceae were detected at all sites except for soap and alcohol dispensers, but were particularly high on the lips of sinks; *Staphylococcal* spp. were detected at all sites.

During sampling it was observed that although against hospital policy, a number of items were stored in the sinks and personnel and professional equipment was placed on the sink edges, whilst undertaking hand hygiene and procedures (see Figure 5-14).

Figure 5-14 Image of inappropriate sink use on the GOSH PICU.

5.3.5 MOVEMENT STUDIES

Having determined that utilisation of sinks impacted on bacterial contamination (see section 5.3.4.2.) it was decided to investigate how user behaviour inside the PICU at GOSH and intensive care units (ITUs) at the NHNN contributed to bacterial contamination within bed spaces and at other points of focus within the wards, i.e. doorways.

5.3.5.1 PAEDIATRIC INTENSIVE CARE UNIT BED SPACE STUDY

Work was undertaken as an observational study described in methods section 2.5.3. The activity of staff and visitors, mostly focused on nurses, was recorded over three days in two bed spaces on PICU (BS6 and BS7). This observational study was done at the same time as the final three days of testing for bacterial contamination, as discussed in Chapter 3. Figure 5-15 shows the layout of the two bed spaces where both the observations and testing for contamination were performed.

The most contact in both bed spaces was focussed on the nurse's trolley surfaces: 113 contracts in BS6 and 115 in BS7. In BS6, there were also a number of contacts with the clinical equipment panels (92), whilst in BS7, the bed rails (76 for rail to the left and 82 for rail to the right) were frequently contacted. 48% of the objects had between 50 and 100 total contacts over the three days of observation.

Hand hygiene constituted 43% of all movements (see methods section 2.5.3.). Hand washing was slightly more popular than decontamination using alcohol gel with 162 versus 149 events. When entering the bed spaces nursing staff undertook hand hygiene, either with hand washing or alcohol gel, on 41% of occasions. Upon exiting the bed space nursing staff under took hand hygiene, either with hand washing or alcohol gel, on 9.6% of occasions.

Gloves were used in 43% of the movements within bed spaces. Of those movements that included glove use, they were changed on average 2.2 times. Fresh gloves when donned were most frequently taken from glove dispensers within the sink zone, rather than those present by the trolley in the bed space area.

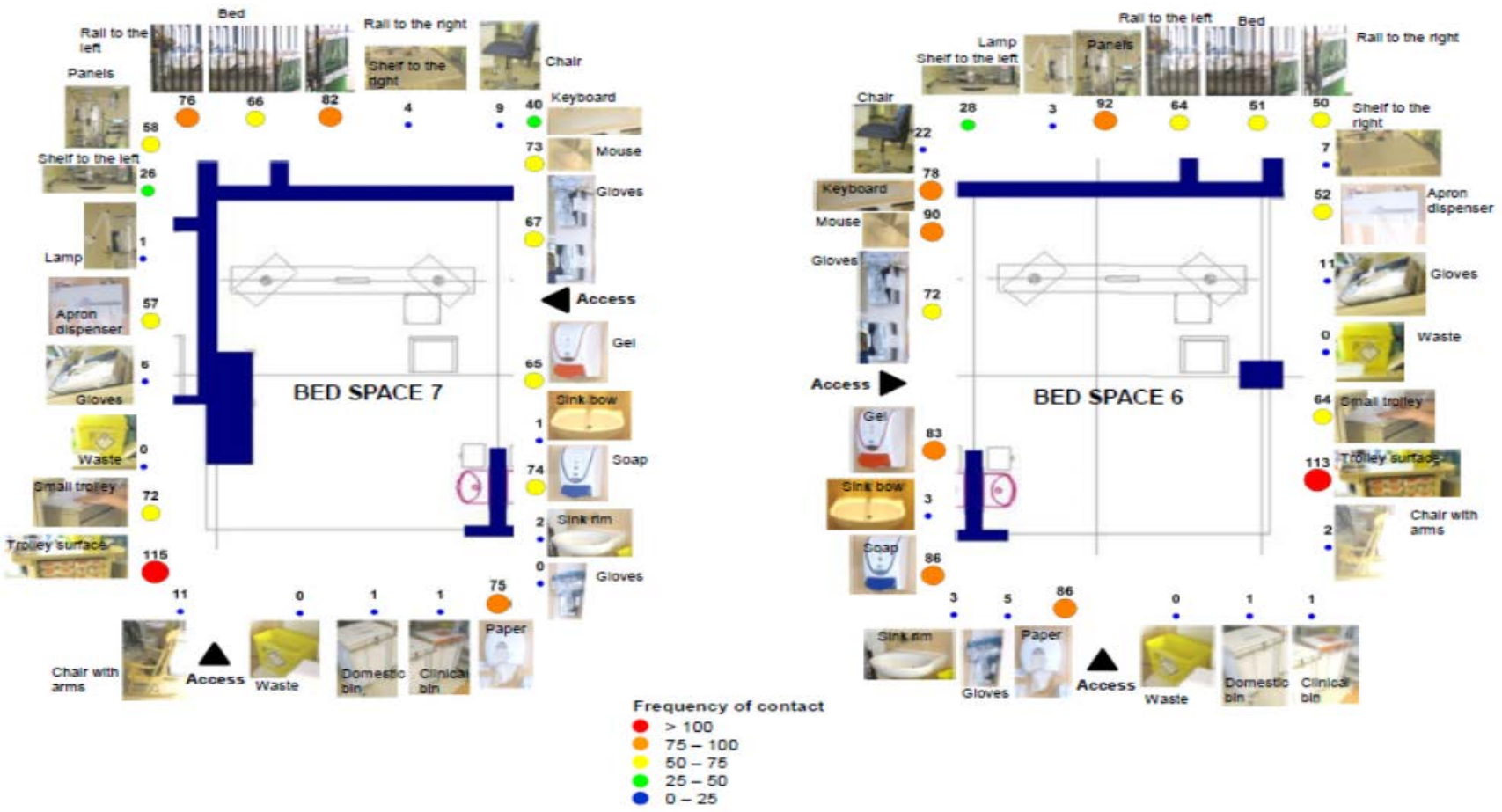

Figure 5-15 Frequency of contact with objects within bed space 6 and bed space 7 on PICU over a three day observation period Circle size and colour indicate frequency of contact.

Object	Bed Space 6		Bed Space 7	
	Total CFUs	Total. No of Contacts	Total CFUs	Total No. of Contacts
Sink Bowl	1034	3	208	1
Sink Rim	417	3	364	2
Bed Rails	138	114	157	158
Chair Arms	117	2	322	11
Clinical Waste Bin	104	0	160	0
Lamp	98	3	10	1
Trolley Surface	70	113	0	115
Suspended Shelf Surface	55	35	0	30
Computer Mouse	42	90	281	73
Soap Dispenser	40	86	16	74
Keyboard	18	78	63	40

Table 5-8 Total number of CFUs detected on contact plates over three days along with the total number of contacts observed during the same three day period in bed spaces 6 and 7 on PICU per object.

Table 5-8 compares the CFU counts and the frequency of contacts over the three days observation period. There was no statistically significant correlation between contamination and frequency of contact. The sink area was determined to be the most contaminated in both bed spaces as determined by CFUs, as well as being linked to contamination with pathogens in 5.3.4.2. During the observational study, activity was recorded as movements between two objects. It was decided to investigate whether there was a link between areas of contamination and areas activity. Table 5-9. shows the ten most common movements between two objects.

Movement From	Movement To	Number of Trips
Sink Bowl	Soap Dispenser	156
Paper Towels	Domestic Waste Bin	151
Soap Dispenser	Paper Towels	151
Clinical Bin	Clinical Bin	136
Bed Rails	Clinical Bin	85
Clinical Bin	Sink Bowl	56
Trolley Surface	Clinical Bin	51
Glove Dispenser	Trolley Surface	50

Clinical Bin	Trolley Surface	39
Clinical Bin	Gel Dispenser	35

Table 5-9 Ten most frequent trips made between two objects during a three day observation period on GOSH PICU.

The three most common movements made were between objects required for hand washing: sink bowl – soap, paper towel – domestic bin, soap – paper towel. In addition, six of the ten most common movements involve the clinical bin, which is also among the more contaminated objects in both bed spaces.

Data on movements was examined again for BS6 based on zoning so that three clustered areas were considered: the near bed area (n = 4)), the wider bed space (n = 9) and the sink area (n = 13).

Zone	Number of Trips Within Zone	Number of Trips Between Zones	Number of Trips Between Zones
Near bed	138	Near bed to sink area	195
Sink area	571	Bed space to sink area	482
Bed space	727	Near bed to bed space	562

Table 5-10 Total number of trips within BS6 between objects located within the sink, near bed and wider bed space zones as well as the number of trips between zones.

The number of trips from within the bed space (both near the bed space and wider bed space area) to the sink area was 677 and this represented the greatest number of directional trips. There were also a large number of trips within the bed space, with all movements combined indicating 1248 trips involving the sink zone. In comparison there were 1289 trips that involved the wider bed space, either within the wider bed space itself or trips from the near bed space to the wider bed space. The area with the fewest within zone movements was the near bed space with only 138, indicating that the highest levels of activity during the observation study were not based around contacts with the patient bed side (see Table 5-10.). However this study did not capture direct nurse patient interactions as it focussed on contact with objects within the clinical environment.

In addition to routine cleaning within the bed spaces nurses undertook cleaning with Clinell Universal surface wipes (GAMA Healthcare, London, UK). Cleaning of surfaces was observed in 16% of movement rounds and in 1.6% of all contacts. The objects most frequently cleaned were trolley surfaces within the bed spaces.

5.3.6 INTERVENTIONS TO REDUCE CONTAMINATION OF SINKS

Having established the potential for sinks to act as a reservoir for the spread of potential pathogens, a number of interventions were undertaken on the HSCTU during the outbreak described in Chapter 4 to try and reduce the risk. These included:

- Level 2 cleans of all shared ward areas with NaDCC
- Improved sink use - no items to be stored near sinks or in sinks, no items left to dry on sinks
- Improved sink cleaning in medication room (post environmental screening) – all hand washing sinks and equipment sinks to be cleaning with NaDCC every two hours
- Door handles to be cleaned six times a day

Utilisation of sinks was identified as providing a potential route of transmission from the contaminated sink environment into patients' rooms and this into patients. Trays for intravenous infusion were left to dry on the sink surface as were tablet crushers (see Figure 5-16). The installed sink was a stainless steel unit with draining board inserted into a covered wooden countertop, with under sink storage and no splash back present on the wall.

Figure 5-16 Photograph of the equipment sink within the HSCTU.

Despite two hourly cleaning for three months *Klebsiella* spp. and *Enterobacter* spp. continued to be detected on sink surfaces. The number of sites detected as positive increased during the second environmental screen.

After three months of cleaning with NaDCC the sink sealant appeared as shown in Figure 5-17.

Figure 5-17 Image of the sealant breakdown on the equipment sink within the HSCTU.

The damaged sealant was replaced but despite this the sink areas continued to remain positive for the outbreak strain of *Klebsiella pneumoniae* described in Chapter 4, and other pathogens. Additionally new cases continued to be detected despite the above interventions and so In July 2012 a new sink design was installed within the unit.

The newly designed and installed sink unit was stainless steel with no drainage area (see Figure 5-18). Sealant was above the height of taps where present and less sealant was utilised as the unit was self-contained. The back of the sink was raised to form a metal splash back. No under sink storage was included. After the sink was replaced no further cases were detected and environmental screens linked to the sink were negative.

Figure 5-18 Replacement equipment sink fitted on the HSCTU at GOSH.

5.3.7 ARE DOOR HANDLES A POTENTIAL SOURCE OF ENVIRONMENTAL CONTAMINATION?

Door handles are frequently associated with high levels of both bacterial and viral contamination (as discussed in Chapter 3). To understand how both the traffic through a door and door handle design affect the levels of contamination a study was undertaken at the NHNN within a Surgical Intensive Care Unit (SITU) (with an attached high dependency unit (HDU)) and MITU. Gates were studied which represent access point to the unit, both with and without doors. Gates shown in Figure 5-19 are those with doors and thus permit microbial sampling of the associated door handles

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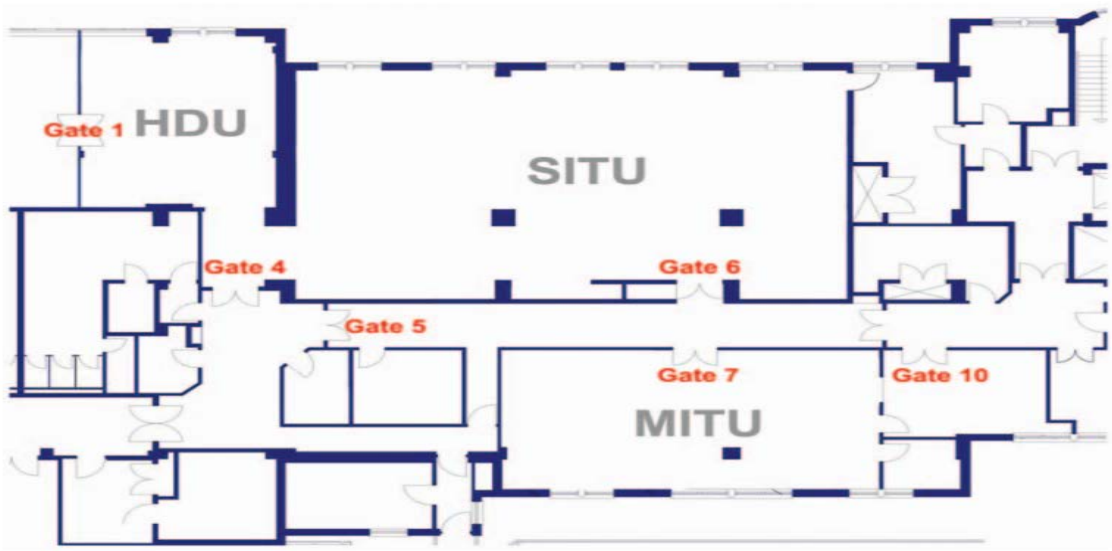

Figure 5-19 Floor plan of the SITU, MITU and HDU at the NHNN showing gates with associated door at the NHNN.

A parallel study was not undertaken at GOSH as no door handle variation was present within the ITUs. Traffic was observed as described in methods section 2.5.1. for three consecutive days.

During observation periods there was variation in the number of patients admitted; with HDU having between zero and four patients, SITU having five to seven patients; and MITU three to four patients. The highest number of movement recorded in a single 150 minute observation period was 241 movements within SITU when six out of nine beds were occupied. Ward staff were responsible for 50% of all movements observed through that gate. Table 5-11 displays the total number of movements according to category of building user over a three day observation period. Doorways were denoted as Gates as not all doorways had doors. For this study only Gates with doors were included. Movements through the main entrances to the ITUs (Gates number 4 and 5), constituted almost 51% of all movements. 87% of movement through all gates were made by staff members, of which 54% belonged to the ward observed. Patient and visitors utilised gates much less frequently, at 1% and 12% respectively. These data demonstrate large variation in traffic across doorways, which were related to location and time, but not direction. Ward and hospital staff generated the majority of these events.

	Ward Staff		**Other Staff**		**Visitors**		**Patients**		**Total Movements Through Gate**
Gate No.	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	
1	41	66	21	34	0	0	0	0	62
4	381	50	262	31	146	18	8	1	797
5	249	36	332	57	109	16	6	1	696
6	295	51	219	38	58	10	3	1	575
7	580	57	102	39	37	4	5	0	724
10	36	73	13	27	0	0	0	0	49
Total	1582	54	949	33	350	12	22	1	2903

Table 5-11 Movement of each type of person per gate, both absolute number and proportion of the total movement through that gate.

There were three types of door handle design present on the units studied: a flat push plate, pull handles and lever handles (see Figure 5-20).

Figure 5-20 Different door handle types present on doors within MITU, SITU and HDU at the NHNN.

Figure 5-21 demonstrates that there was considerable variation between doors in terms of the mean CFUs detected; samples were collected as described in methods section 2.3.4.6. Confluent or near confluent bacterial growth on door handles was sometimes detected in the context of low levels of traffic (Gates 1 and 10). These exceptions may be explained by less frequent contact with highly contaminated hands. When confluent samples were excluded, as they prevented quantification, a significant correlation existed between movement density and CFUs when data was analysed by Spearman's Rho Product Moment test ($p=<0.01$). Low traffic density was associated with low CFUs for Gates 1 and 10 and the more heavily used doors at Gates 4, 5, 6 and 7 were more contaminated.

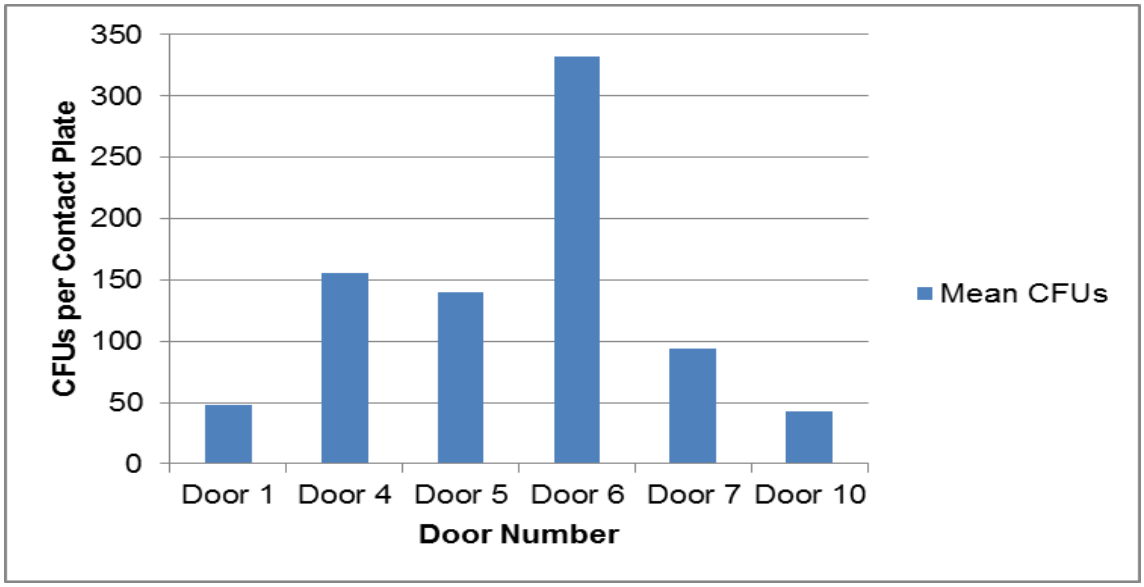

Figure 5-21 Mean number of CFUs detected by doorway during the study period on the SITU, MITU and HDU at the NHNN.

Gate 6 was the only gate to exhibit fluctuating levels of contamination based upon time of day, where the afternoon samples were consistently found to be greater than 300CFU/plate or were confluent. Gate 6 was not as heavily used as Gate 7 and had similar proportions of staff groups entering and exiting the unit. Gate 6 represented the main route to and from the sluice room and timing of contamination may have been linked to removal of clinical waste prior to the end of the domestic workers day.

In order to investigate further the differential patterns of contamination across gates analysis was undertaken linked to door handle type.

Gate No.	Movements In	CFU/ Movement In	Movements Out	CFU /Movements Out	CFU/ Movement
1	Handle	0.43	Handle	8.56	4.63
4	Pull	1.82	Push	0.49	1.18
5	Pull	2.63	Push	1.29	1.97
6	Pull	5.44	Push	0.99	3.27
7	Push	0.62	Pull	0.76	0.69
10	Handle	1.57	Handle	14.52	8.56

Table 5-12 Ratio of CFU/Movement for each type of handle.

Traffic density heading either in or out of the doors was balanced and not influenced by door handle design (Table 5-12.). However analysis of individual and mean CFUs for

each type of door handle, revealed that bacterial load on pull handles was consistently higher than those on the push plates located on the other side of the door, although not statistical significant when analysed using a one way ANOVA test (p=0.053). Further analysis relating to handle type revealed that lever handles had the highest ratio (6.38 CFUs/movement), followed by pull handles (2.24 CFUs/movement), which were in turn were nearly double that of the push plates (1.20 CFUs/movement) (see Table 5-12). A representation of why this might occur is shown in Figure 5-22.

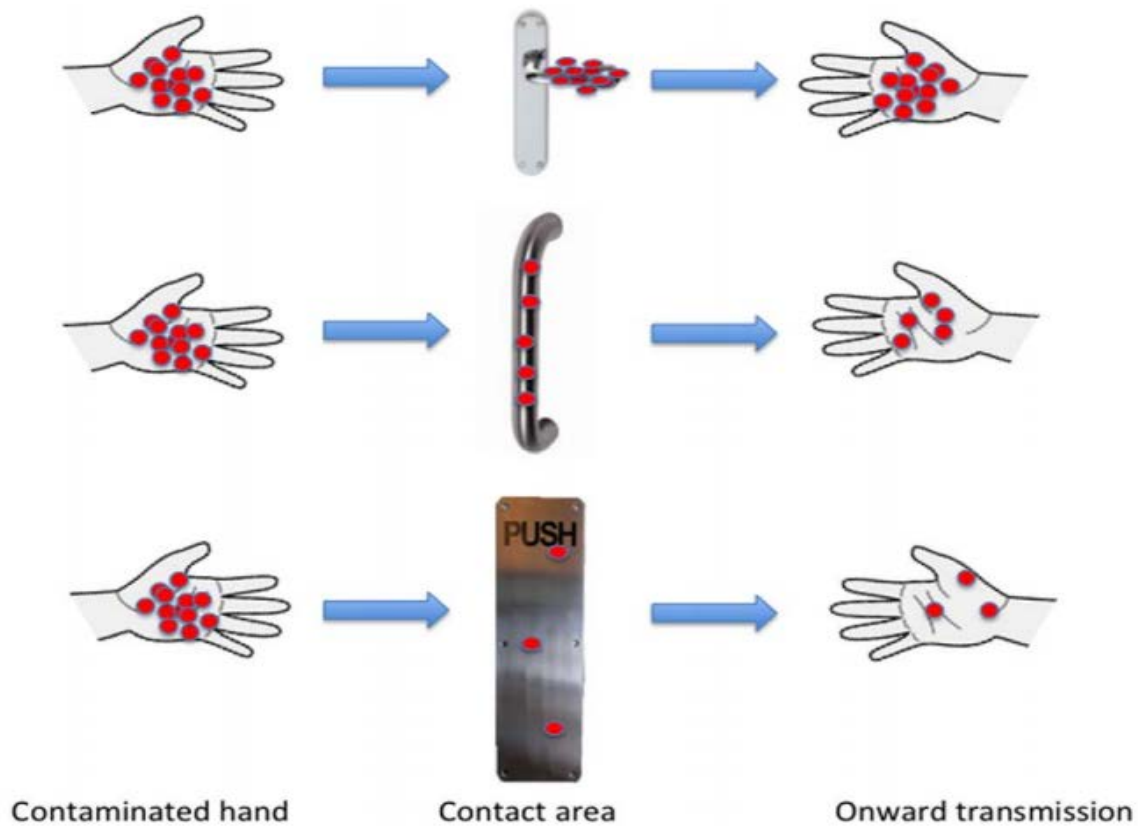

Figure 5-22 ***Representation of the distribution of microbes on door handle types with the same starting level of hand contamination.***[417]

5.4 DISCUSSION

5.4.1 TO ESTABLISH THE EFFECTIVENESS OF CURRENT CLEANING CHEMISTRIES.

Selection of Cleaning Chemistries

Cleaning must be undertaken with the most appropriate cleaning agents, in an appropriate fashion, if it is to make a difference to the risk of environmental cross transmission.[189] Chlorine based products are considered to be the cheapest and easiest form of environmental disinfectants and result in the irreversible lysis of bacterial cells.[189, 357, 359] In this chapter, the effectiveness of three disinfectants were investigated, particularly assessing the impact of concentration and formulation (see section 5.3.2.1.).

In addition to disinfectant factors those factors relating to the microorganism tested are also important and were therefore also examined.[189] Bacteria commonly associated with both environmental contamination and HCAI were tested. Adenovirus was also investigated as an indicator virus for surface disinfection, as well as water and air decontamination.[333] Along with polioviruses, adenoviruses are considered to be model viruses and surrogates for testing virucidal efficacy of all human pathogenic viruses.[508] However viruses, even from the same family, are not characterized by a uniform response to disinfectants, therefore within the literature often adenovirus species C serotypes 2 or 5 are utilised.[508] Within this study adenovirus species C serotype 1 was selected, as this represented the serotype that infected the majority of patients within high risk areas at GOSH (see section 5.3.1.2.). Sauerbrei at al. (2004) demonstrated that differences in adenovirus species sensitivity are probably due to adenovirus genomes, as they are non-enveloped viruses.[508] We demonstrated that the genomes of adenovirus species C are very similar and so the use of adenovirus C1 for disinfectant studies should reflect results that would have been achieved with either of the virus serotypes used more frequently within the literature.[508]

Finally application factors such as the type of surface, type of soil, mode of application, contact time, humidity and temperature affect the efficiency of disinfection.[189] For these reasons unlike standard ISO disinfectant testing protocols, as discussed in the introduction, testing was undertaken as per standard environmental conditions found within GOSH, (temperature, humidity) and applied as per the GOSH cleaning protocol (single use disposable cloths, one per surface wiped for 10 seconds). This permitted testing of bacteria and viruses in circumstances where they are less susceptibility to

disinfection, i.e. dried onto a surface rather than in hydrated suspension.[510] This more accurately reflects real world conditions where surface disinfection is a two stage process of rehydration followed by disinfection.[510]

Effectiveness of Chlorine Cleaning in Laboratory Testing

For registration as an environmental surface disinfectant a 3 – 5 $\log_{10}$ reduction should be achieved in the viability of test organisms.[21] Hospital surface contamination ranges from a mean <10CFU/cm^2 to 300/cm^2 depending on the study; this maximum level is in part likely to be linked to maximum quantitation of contact plates as discussed in Chapter 3.[8, 21] Viral loads are likely to be considerably higher, with norovirus contaminating surfaces at up to 30 million viral copies/ml.[157]

Within this study there were differences in response to NaDCC, ClO_2 and NaClO between *Staphylococcus aureus* and *Klebsiella pneumoniae* for viability. *Klebsiella pneumoniae* demonstrated a 6 $\log_{10}$ kill for NaDCC and a 5 $\log_{10}$ kill for ClO_2 and NaClO after 10 minutes, with no *Klebsiella pneumoniae* recovered for any agent after a 60 minute exposure. All agents met the standard of a >5 $\log_{10}$ kill of *Staphylococcus aureus.* However, it remained viable after 10 minutes exposure to all cleaning agents, and was still detected after 120 minutes of exposure to ClO_2, despite achieving a 7 $\log_{10}$ kill. No viable *Staphylococcus aureus* was recovered after a 60 minute exposure for either of the other agents (see section 5.3.2.1.).

The Centre for Disease Control (CDC) discusses a requirement for a one minute contact time for non-critical equipment and environmental surfaces.[511] These data demonstrate that it is unlikely that a 5 $\log_{10}$ reduction would occur within the amount of contact time suggested. A 6 $\log_{10}$ kill is often used as the required cut-off for examination of disinfectant activity in laboratory studies. For ClO_2 this would require a 60 minute contact time to achieve.[512] Additionally viable colonies of *Staphylococcus aureus* were recoverable after a 120 minute exposure and although the number of colonies recovered was small, they could still present a potential for harm to at risk patients.

The finding that NaClO and NaDCC have similar activity has been identified in other studies, and the delayed killing of *Staphylococcus aureus* also noted.[356, 513] While ClO_2 showed reduced activity, its use for disinfection of *Clostridium difficile* spores found was to be more effective than NaClO in one study undertaken within a ward environment.[514] One reason for this may be because of the levels of proteinacious

material present within clinical environments. Chlorine based agents react with organic matter and so the antimicrobial activity is proportionally decreased based on the level of organic material present, but the level at which they are affected varies between agents.[515] It is therefore necessary to have sufficiently high levels of available chlorine to oxidise cellular components, which may explain why NaClO is more active at low concentrations compared to NaDCC. In spite of high levels of protein within the adenovirus tissue culture, all agents rendered 785 million viral copies/ml of adenovirus non-viable after 120 minutes of exposure.[356] Therefore the recommended levels of 0.1% (1000ppm) available chlorine are suitable to attain high level disinfection with GOSH hospital cleaning protocols.

Chlorine DNA Degradation

Monitoring of DNA degradation utilising molecular methods, such as real-time PCR, is widely recognised as both sensitive and specific.[507, 508] Molecular changes within the genome results in loss of PCR amplification of specific target regions and is therefore one of the most suitable tools to evaluate the effect of disinfectants on both bacterial and viral DNA.[508]

As molecular detection for evaluation of environmental contamination is increasingly common, additional testing was undertaken to explore the effect of chlorine based agents on DNA. Chlorine is an oxidative biocide which due to its low molecular weight should be able to pass through cell walls and membranes in order to react with cellular components such as DNA, resulting in single or double strand breaks.[355, 516]

There is little available information within the literature on the effect of chlorine on DNA in solution and none on the effect of chlorine based agents on DNA on surfaces.[516] Of the studies that have been undertaken on the effects of chlorine on DNA within solution for both viruses and bacteria, all demonstrated that chlorine based agents are capable of degrading DNA.[157, 369, 507, 517] However both contact time and size of DNA fragment had an effect on degradation and this was especially true for ClO_2.[369]

DNA degradation in this study was greatest for all three organisms with NaClO, followed by NaDCC and ClO_2 (see section 5.3.2.1.). All bacterial DNA samples would be considered negative using clinical cut offs after 120 minute exposures to NaClO, indicating greatest DNA degradation occurred with this agent. However for adenovirus there was substantially less DNA degradation detected despite the much smaller adenovirus genome. This is a similar finding to that of Page et al. (2010) who found

that in solution adenovirus amplification was only reduced by 50% in the presence of chlorine.[507] This is most likely due to the fact that in comparison with bacterial suspensions there was a substantial amount of non-adenovirus DNA present in the inoculum. This is because adenovirus is grown in tissue culture and over 50% of the DNA present originates from the Green African Monkey cell line (data not shown). As a result the available chlorine will be binding to non-adenovirus as well as adenovirus DNA, in both the extracted and non-extracted tissue culture, resulting in decrease in detectable degradation. This may not necessarily reflect the true situation with environmental chlorine exposure.

5.4.2 To undertake a study to understand the effectiveness of cleaning in practice and to develop a monitoring algorithm to assess cleaning quality.

Cleaning has rarely been investigated as a scientific entity, mostly due to the difficulty in controlling so many interactions; however most of the investigations undertaken have been to investigate terminal cleaning.[341] Terminal disinfection aims to ensure that a room or bed space can be used safely for future patients, without posing a risk of infection.[189] Thorough cleaning of all surfaces is often not feasible prior to patient discharge.[288] The persistence of adenoviral contamination indicates how difficult it can be to decontaminate occupied rooms (as discussed in Chapter 3). The efficiency of terminal disinfection was investigated in this study as even when the most effective disinfectant product is used the degree of efficiency of the cleaning staff will ultimately determine its success since they determine both wiping action and contact time.[21] Adenovirus was selected for this work as it is a non-enveloped virus which is considered to be resistant to infection.[518] As described in this chapter in section 5.3.2.2., 28% of surfaces remained positive for adenovirus post terminal cleaning with NaDCC. As NaDCC was capable of denaturing DNA and making adenovirus non-viable; detection of virus was considered to be a cleaning failure supporting a view that cleaning with effective agents is only 90% effective due to human error.[514]

Viability vs DNA Detection

PCR cannot differentiate between viable and non-viable virus. It has been demonstrated that the effect of disinfectants on the adenovirus genome does not always correlate with the speed at which the virus is rendered non-viable.[508] Living cells demonstrate a higher susceptibility to chlorine than DNA, most likely due to the

presence of multiple targets rather than just DNA molecules, thus leading to an over estimation of the virological risk to patients.[157, 508]

25% of the cubicles tested post terminal cleaning with NaDCC had adenovirus DNA detected and required at least one re-clean to render it PCR negative, and one cubicle required cleaning five times for no adenovirus DNA to be detected (see section 5.3.2.2.). Viral DNA may remain intact when the virus has been rendered non-infectious. For practical purposes, positive PCR results are frequently used to indicate the presence of infectious viral particles as for a virus to be considered truly inactivated, viral nucleic acids must be destroyed.[157] Tuladhar et al. (2012) noted that there was a 10fold difference between the level of viable virus and the level of PCR detected DNA and so this was taken into account during algorithm development, as discussed below.[508, 519]

Monitoring Algorithm

Routine auditing of the environment using the environmental screening methods developed as part of Chapter 3 offered a way to compare cleaning results over time. Results from the applied hospital study in 5.3.2.2. were analysed along with epidemiological information on cross transmission events. These data were considered in the light of clinical risk assessment strategies discussed in Chapter 3 and utilised to establish a cleaning monitoring algorithm to decide when cubicles were sufficiently clean to permit further in-patient admissions. The algorithm was as follows:

- The cubicle is opened with no further cleaning required if no site has an adenovirus CT of <39.
- If the cubicle has two sites with CTs of no lower than 34, then those sites are re-cleaned twice using NaDCC and the cubicle can be re-opened, as long as the sites positive to do not include the floor inside the room. If the floor inside the cubicle is positive than the cubicle undergoes a repeat ‘deep clean’ and is re-screened.
- If the cubicle has more than two sites with a CT of 34 – 38 or if any one site has a CT of lower than 34, then the entire room must have a repeat ‘deep clean’ and be re-screened in full before opening.

- If the same objects fails to become negative after three cleans and screens, that object if possible is replaced within the cubicle and disposed of appropriately.

This algorithm was applied to all environmental screens from 2010 onwards (see Environmental Screening policy, Appendix 1). As a result of the data analysis demonstrating that only a small number of cubicles fail two cleans, and that if they fail twice then they are likely to continue to fail, an amendment to the policy was introduced.[500] This was based on mounting evidence that persistent cleaning failures are due to human error. For example no correlation between the amount of time taken to clean a surface and the effectiveness of cleaning was noted in one study.[413]

Another study demonstrated that the thoroughness of cleaning did not equate to hospital size, case mix or geographical location.[348] Therefore it is important that terminal disinfection is delivered with not only a set of standards and adequate training, but audit and monitoring to ensure delivery of efficient cleaning.[189] The change to the algorithm was that if a cubicle fails two screens then it triggers a meeting to investigate cleaning methods and a review of any new adenovirus infections on the unit. These meetings include cleaners, ward staff and infection control and are used in order to recognise cleaning issues that had occurred.[500] In combination these measures are considered to support and facilitate the use of environmental screening to monitor discharge cleaning efficiency.

5.4.3 To investigate the use of novel technologies for surface and room decontamination.

Equipment cleaning as well as terminal cleaning is based upon manual application of existing cleaning chemistries. In spite of interventions to improve cleaning, studies note that only 85% efficacy is possible.[351] Due to this reliance on manual application of cleaning processes it has been suggested that novel cleaning technologies could be used to support both equipment cleaning and room decontamination.[413, 520]

Novel decontamination technologies often come with substantial capital equipment costs as well as the being limited to terminal disinfection due to the need to remove both personnel and patients from the room. Therefore their use needs to be carefully evaluated.[351, 521]

The Use of UV-C for Equipment Decontamination

Adenovirus is one of the most resistant organisms to UV-C radiation (280 – 100nm); resistance is in part due to the double stranded nature of the genome.[335, 506] UV irradiation breaks the molecular bonds of DNA via cyclobutyl-pyrimidine dimerization and the affect is as a function of light intensity and exposure time.[332, 379, 512, 516]

The studies described in this chapter in section 5.3.3.1 demonstrated that UV exposure via the Nanoclave cabinet on flat surfaces led to the degradation of adenovirus DNA, inoculated from viable culture material, such that it became undetectable by PCR. As adenovirus is likely to become non-viable before DNA becomes non-detectable by PCR, the exposure time required for reduction in viable organisms may be less than used in these experiments. No other equipment decontamination studies have been published, but when UV light has been validated for room decontamination it has been shown to decrease vegetative bacteria viability by 4 $\log_{10}$ indicating that the level of decontamination achieved in this study was higher.[379, 522] This is likely due to higher light intensity exposure due to its close proximity to the surface. The intensity of the radiation will decrease proportionate to the square of the distance between the source and the object.

As demonstrated by the object decontamination results (section 5.3.3.1.), this process worked best for flat surfaces where all test locations get equal exposure to UV light. These results are similar to those found by other studies that demonstrated reduced decontamination efficiency when objects were not directly exposed due to shading, with efficiency decreased by >2 $\log_{10}$.[379, 413, 512, 520, 522]

There are potential drawbacks with these technologies. Objects require cleaning in order to remove soiling and allow adequate penetration by UV light; this process may not be more efficient than chlorine cleaning alone.[379, 523] Haas et al. (2014) noted that manual cleaning efficiency actually decreased when the use of UV for room decontamination was introduced as cleaners were aware that an additional cleaning step was to be undertaken post clean.[522] Additionally UV radiation can reduce the life span of pieces of equipment made of plastic or fabric.[512]

Hydrogen Peroxide Room Decontamination

Hydrogen peroxide is effective against a wide range of bacteria and viruses and may provide a new and efficient cleaning method.[373] The chemical breaks down to insignificant amounts of oxygen and water, and has the best safety profile of the

gaseous decontamination methods available. Killing occurs in two modes. One occurs at low H_2O_2 concentrations and is due to DNA damage (mode one), and the second occurs at higher concentrations and is dependent on the presence of oxygen and hydroxyl radicals and therefore only effective under aerobic conditions (mode two).[373, 413, 524] Under experimental conditions it has been demonstrated that 100% of nucleic acids showed extensively fragmented DNA when exposed to liquid H_2O_2 in both the exponential phase and the stationary phase.[373]

Adenovirus at a concentration of 416 million viral copies/ml was rendered tissue culture negative by exposure to both HP systems (see section 5.3.3.2.). Bacterial viability was decreased by >6 $\log_{10}$ for both systems, with the lowest log kill seen in *Staphylococcus aureus* using the Bioquell system. These results are similar to those seen in other studies, apart from one study by Fu et al. (2012) which identified a reduced kill for the Glossair system.[413, 510, 519, 525-527] This study was however funded by Bioquell and contained a number of their employees as authors.

Bacterial and Viral DNA Degradation by Hydrogen Peroxide

As well as being effective against viable microorganisms, it has been suggested that HP could be used as a DNA degradation agent to eliminate nucleic acids. As cleaning efficiency in hospitals is increasingly evaluated using PCR, detection of non-viable DNA may lead to cleaning failure and re-cleaning of the area. If effective against DNA, HP treatment may prevent over-estimation of cleaning failure rates.

The Glossair system utilised 5-6% HP in comparison to the Bioquell system that utilises 30-35% HP. The level of DNA degradation observed in all organisms tested was less with the Bioquell system. This may be related to the higher H_2O_2 concentration used by Bioquell, possibly resulting in mode two killing versus mode one killing, as seen with the Glossair system. In this investigation the recovered DNA for Bioquell tests was frequently greater than that of the control (section 5.3.3.2.). This is probably due to the increased level of moisture produced during the chemical breakdown of the H_2O_2 with the Bioquell system. This may enable greater recovery of DNA. Degradation of DNA was greater for adenovirus genomes than for bacterial genomes, unlike that seen for chlorine based disinfectant.

The level of DNA degradation was greater for Glossair, although inconsistent. This is a potential problem with molecular environmental monitoring using HP. In addition the Glossair system contains <50ppm cationic silver ions. Silver is believed to bind to DNA

potentially impairing both DNA replication and also PCR amplification due to its effect on double stranded DNA.[516, 528, 529] This may be the reason for the issues with the detection of DNA degradation when fast cycling conditions were used as it may have prevented the DNA denaturation step from occurring thus impairing amplification. The only other publication to examine DNA degradation caused by cationic silver as detected by PCR ran samples using slow cycling conditions on the same PCR platform as this study.[529] Knowing that the silver binding interferes with PCR amplification is crucial as environmental monitoring by PCR could appear falsely negative.

Surface Decontamination with HP

HP was shown in published studies to work differently on different surfaces, with the longest time to reduction occurring on stainless steel. Unfortunately chlorine based products cannot be used to clean stainless steel, making it a difficult material to clean within the clinical environment.[525] The fastest decrease in viability occurred on linoleum flooring, with intermediate times observed on glass, plastic and ceramic tiles (15 minutes for a 4 $\log_{10}$ reduction).[525] Our study took place on ceramic tiles and results should therefore give a good general indication of the effect of HP for decontamination of microorganisms.

One of the main advantages of HP is that it can be used on electrical equipment, which would be damaged by the use of biocides. It can also reach locations which are difficult to clean manually.[413, 530] However neither HP system works well against biofilms or on deteriorated fabric.[374] Additionally since these systems work by breaking down into water and oxygen they are unlikely to work well against surfaces that are already moist, such as sinks, showers and baths. This might have a significant impact on its effectiveness in routine use as many organisms will exist within the clinical environment in biofilms, and HP does not have the mechanical action used with chlorine cleaning to aid in biofilm disruption.

Surfaces still require cleaning before the use of HP as H_2O_2 reacts with proteins, lipids and nucleic acids within the environment oxidising them, thereby reducing its efficacy.[351, 373, 374, 531] Within this study reductions in viability were ~1 $\log_{10}$ less in the presence 0.3% BSA when compared to PBS alone. The extra cleaning lasts 90 – 120 minutes and is in addition to the time for the HP cycle.[532] This can offer logistical challenges, especially when the use of chlorine alone can achieve a >5 $\log_{10}$ kill, as has been demonstrated in this study (see section 5.3.2.1.). For this reason the use of

chlorine disinfection without the use of HP in health economic analysis is more favourable than either HP plus chlorine or HP alone.[357]

Other challenges involved with the routine use of HP are the staff time needed to transport equipment, and safety concerns due to the interference with both mechanical ventilation and fire alarm systems.[351, 413] In addition the Bioquell system is large as it requires both a HP unit and an aeration unit and so it may not be suitable for small isolation rooms.[533] However due to the $\log_{10}$ reductions observed within this study it is indicated that HP could be a useful adjunct to routine cleaning if these logistical issues can be overcome.

5.4.4 To investigate the effect of hospital design and staff movement on contamination of the clinical environment.

The design of the healthcare environment is increasing recognised for its impact on healthcare quality and outcomes.[376, 431] However architects use a 'comfort' model where the design of indoor spaces have sufficient temperature, humidity and air flow provide comfortable surroundings.[376] Increasingly there is the need for scientific studies to be undertaken to understand the effect of design on the utilisation of these complex spaces and on the microbiome that exists within them.[376] Microorganism transmission can occur due to inappropriate design of both equipment within that environment and the environment itself.[336]

To our knowledge there was no previous coordinated study of how people's behaviour is influenced by the built environment and how this relates to microbial spread.[534] In this study we combined microbiological and observational approaches with statistical modelling to assess the use of sinks and doorways to demonstrate how this could be influenced by the ward environment.

Hand Contamination

Contamination of hand contact surfaces may be a significant factor in the nosocomial spread of microorganisms, both during and after hand washing events.[400] Hand hygiene has a strong modifying effect on hand contamination. If performed perfectly, in an uncontaminated sink environment, contamination would never be present on health care workers hands. However real world impediments include imperfect compliance, and cross contamination from the hands of healthcare workers to the environment and vice versa.[288]

Healthcare workers touch surfaces within the clinical environment with greater frequency than they touch patients and have demonstrated decreased hand hygiene compliance when they do so, both within this study and others.[288] Additionally transmission of microorganisms can occur within the setting of appropriate hand hygiene when hands become contaminated during the hand washing episode itself, from contaminated sink lips, drain or bowls.[214] Contamination of all of these sites was observed within the ITUs in this study (section 5.3.4.2.) and the objects included within the highest number of movements were the sink bowl, soap dispenser, paper towel dispenser and the domestic waste bin. All of these were located within the sink zone of the bed space and demonstrated high levels of CFUs.

As demonstrated in this study and others soap and alcohol dispensers have been found to be contaminated with fomites.[535] It would appear that the alcohol gel and dispensers are not contaminated prior to clinical use.[536] Therefore the source of contamination is exogenous and may be due to aerosols generated by sink use or from contaminated hands.[289] The latter is likely to explain the contamination on the lever of the dispenser, which was the most contaminated area sampled within this study, that has most contact with the users hands, and is the most difficult to clean.[535]

Fingerpads of nursing staff have been found to be contaminated with coliforms, especially after washing patients.[302] As with the organisms found on sinks within this study, the most frequent pathogens isolated from the hands of healthcare workers were *Acinetobacter baumanii*, *Klebsiella pneumoniae* and *Staphylococcus aureus*.[303] Not only were fingerpads found to be contaminated but these pathogens were found to transfer from the hands to fabric and from hands to the environment.[302]

The frequency of hand washing affects the type, and number of microorganism on hands, as well as whether hand hygiene was undertaken with alcohol gel or soap and water.[290] In order to prevent carriage of organisms on hands, especially Gram-negative organisms, skin needs to be in good condition.[7, 537] In addition to impacting on organism carriage skin condition is one of the factors that is used to explain low compliance to hand hygiene.[537]

In addition to hand hygiene glove use is a major way of preventing hand contamination. Gloves were used in 43% of movements within this study, roughly equal to the amount of hand hygiene actions undertaken (see section 5.3.5.1.). Gloves were changed frequently, but were most often taken from the glove dispenser located within the sink area. The gloves within this area are as likely to be as contaminated as objects such

as the soap and alcohol gel dispensers. Gloves are often perceived as being protective for the member of staff wearing them, rather than protecting the patient and incorrect glove use has been observed within the clinical environment, where 37% of cases were inappropriate.[6] In this case gloves could be contaminated and then utilised for patient interactions, increasing the likelihood of transfer.

The T-zone includes the mucous membranes within the eyes, nose and mouth. It has been noted that even within healthcare setting members of staff engage in frequent face touching, with one study noting that healthcare workers touched the T-zones a mean number of 19 times over a two hour period, which may place healthcare workers at risk of organism acquisition/transfer. . Additionally organisms could survive on the skin for minutes to hours and thus present a source of hand contamination when touched in the future, with a possible spread to patients and surfaces.[538]

Hand Hygiene Compliance

Effective control of HCAI requires both regular hand hygiene and decontamination of high touch sites within the clinical environment.[21, 295] Despite the widespread agreement with this principle, compliance varies widely, even within groups of healthcare workers, with compliance often averaging only 50%.[7, 295] Mathematical modelling has demonstrated that hand hygiene compliance of >50% is required to interrupt vancomycin resistant Enterococci (VRE) transmission.[298] The hand hygiene compliance on the units studied during data collection was >80%.

ITU environments are complex with multiple patient-nurse interactions throughout the day resulting in up to 60 hand hygiene opportunities/shift for nursing staff.[300] Most hand hygiene opportunities are undertaken by nursing staff, at 77%, compared to 8% undertaken by physicians and therefore observations within this study focussed upon this group.[330]

Nurses have identified work flow, access to supplies and deficiencies within the built environment as obstacles to good quality patient care.[324] In addition the built environment has been identified as key to facilitating improved hand hygiene and avoiding cases of HCAI by placing the correct number of sinks in the correct place within the clinical environment.

The CDC and World Health Organisation (WHO) have identified that sink location and sink number are often a contributing factors to sub-optimal hand hygiene.[290, 324] Studies that have looked at the amount of time that hand hygiene compliance requires

have noted that full compliance to hand hygiene can take between 60 and 240 minutes per shift.[539] This is partly because even without undertaking hand hygiene for the recommended 60 seconds it took nursing staff over a minute to walk to a sink and the return to the patient's bedside.[324, 539, 540]

Hand Washing Duration

The WHO recommend that hand washing duration should be between 40 and 60 seconds.[4] This work was supported by a study that demonstrated it required 60 seconds to undertake effective hand washing, where all hand hygiene steps were completed.[538] Healthcare workers often take considerably less time to wash their hands than the times proposed, with one study finding that 22% of workers took less than 10 seconds to undertaken hand hygiene and another demonstrating that workers took between 6.6 and 30 seconds to undertake hand hygiene.[4, 538] During this study mean hand washing duration ranged from 9.28 seconds – 22.13 seconds depending upon the sink (see section 5.3.4.1.). The shortest hand washing duration observed was 4.83 seconds and the longest was 65.25 seconds. This indicates that although hand hygiene compliance was high on the units studied, hand hygiene efficiency is unlikely to have been high. This was identified in another study where although hand hygiene compliance was high, more than 90% of hand washes were inadequate.[292]

One of the proposed reasons why hand washing compliance reduces when units relocate to new environments with a 1:1 bed space to sink ratio is that staff feel a reduction in perceived risk to themselves.[541, 542] Risk is one of the major drivers for hand hygiene and the perception of what is clean and what is dirty in terms of equipment and body fluids, meaning that the elective component of hand hygiene are driven by perception rather than a set of healthcare rules.[290]

This may explain our observation that hand washing duration increased at sinks within occupied bed spaces (see section 5.3.4.1.). When nursing staff are undertaking direct patient care they have a greater perception of risk, both to themselves and to their patients. This leads them to undertake hand washing with greater efficiency and thus increases hand hygiene duration. Environments are still not perceived as being 'risky' and so there is not an equivalent perception driven hand washing response.

This concept is supported by one study that demonstrated 42% of hand hygiene activity was focussed prior to contact with a patient and 39% directly after contact with a patient.[330] Although our study did not focus on direct patient contact and thus the

WHO's 'five moments', observations were made that nursing staff only undertake hand hygiene 9.6% of the time when they exit bed spaces, despite frequent contact with the environment; whereas hand hygiene upon entry was 41%. This has been seen in other centres undertaking hand hygiene compliance audits.[189]

Sink Visibility

In view of the importance of hand washing, numerous studies have examined ways of improving hand washing compliance. Some studies have demonstrated an increase in hand hygiene compliance when wards have moved from a shared sink to a 1:1 sink bed space ratio.[298, 326] Others have not found an increase in compliance; however the majority have not directly examined the impact of sink visibility.

It may be possible to explain some of the published data by considering sink location as hand hygiene compliance varies widely dependent upon the built environment. For example, even when sink numbers are increased, their location at the entrance of patient rooms may impede visibility, which may explain the lack of apparent improvement in hand washing frequency.[378, 543-545] Indeed in one study that examined sink position in terms of distance from the bed space, a 26% reduction in HCAI was noted in those patients whose bed space was closest to the sink.[540] Our findings support the observations of Boyce at al. (2001) who demonstrated an increase in compliance when moving to new units with an improved bed space to sink ratio. In this facility, care was taken to ensure that sink access was not obstructed by medical equipment, which could have affected lines of sight (see section 5.3.4.1.).[540]

One of the reasons that visibility may play a key role in hand hygiene compliance is that hand hygiene is not an entirely rational behaviour.[286] The hospital environment is both rich and attention seeking with multiple demands being placed upon the attention of healthcare workers.[546] Attention is an ongoing cognitive process which requires the gathering of information from the environment. Divided attention is the cognitive process of sharing attention between multiple sources. It can be considered that a failure to process visual cues to enable appropriate hand hygiene may be classed as a failure of divided attention leading to an error in omission of undertaking an action which had been intended.[286, 546]

We have shown that hand wash basin usage is directly related to their visibility. When sinks are more visible, people wash their hands more frequently. This is true even when their usage is controlled for bed occupancy and bed space:sink ratio, accounting

for perceived clinical risk. By making sinks more visible they provide a richer visual cue in order to support healthcare worker attention. This was shown in one other published study with the use of alcohol gel where improved line of sight increased compliance from 37% - 66%.[546] Sink location may be particularly important when staff/ward relocation occurs due to the unfamiliar environment and reduced awareness of sink position.

Sink Design and Sink Contamination

During long term environmental screening, human movements have been found to have a considerable effect on the bacterial flora of sinks and the spread of that flora to adjacent spaces.[547] A study that examined sink flora during the move into a new hospital building identified that the composition of flora changed and stabilised when patients were introduced into clinical areas and the species found altered from being water borne commensals to species linked with opportunistic clinical infection, with *Enterobacter cloacae* and *Serratia marcescens* predominating.[547] As discussed in Chapter 3 Gram-negative bacteria have been frequently linked to isolation from sinks in this study. In both Chapter 4 and previous studies it has been demonstrated that patients become colonized and sometimes infected by bacteria from sinks, although these results indicate that sinks may also become 'infected' from patients.[548]

Multiple hospital room design elements, including sink placement and design, can impact on nosocomial transmission of infection.[549] Testing of in use hand wash sinks with fluorescent and other markers has demonstrated that water can create aerosols that travel for upwards of one meter from the sink and thus potentially contaminate nearby surfaces with organisms.[549] Additionally when hands are dried with paper towels ballistic droplets are produced contaminating up to 2m from the dispenser, although the distance that the contamination travelled depended upon volunteers, skin condition, technique and body temperature.[289, 336, 550] These contaminated droplets may contribute to the high levels of surface contamination detected during this study and help explain why the sink area is most contaminated region. This indicates that not only the sink but the sink area needs to be the focus of a design approach.[189]

Our multilevel analysis showed that hand washing is an important factor directly relating to levels of bacterial contamination on sinks and around the sink area (see section 5.3.4.2.). Sink usage was inversely related to levels of bacterial contamination on the outside of the sink bowl area, including taps, gel/soap dispensers and lips of the sink. This has implications in the face of increasing use of single rooms and 1:1 bed

space ratios as depending upon occupancy rates, certain sinks may routinely get less use than others, increasing their likelihood of contamination.[336] Inactivity may be a risk for spreading bacterial via contact with taps and sink lips.

In contrast bacterial levels in the sink bowls increased as the number of hand washing actions increased. Increased usage may increase the risk of spreading microorganisms via sink bowls as these are frequently used for inappropriately storing/placing bowls and IV bags. Shallow basin depths can result in the splashing of the contaminant to surfaces adjacent to the hand washing sink or if the patient is close directly onto the patients, thus potentially being a source of HCAI.[549] These findings indicate that patterns of sink usage are important determinants of microbial contamination on and around the sinks.

Positioning of sinks in order to encourage frequent use and therefore prevent the establishment of high levels of bacterial contamination may therefore impact upon the risk of HCAI within the clinical environment. Placement of all sinks must be considered not just hand washing sinks, especially in relation to patients and clean equipment. The sink involved with the *Klebsiella pneumoniae* outbreak described in Chapter 4 was non-compliant with a number of suggested design features meant to prevent HCAI.[324] The sink was designed with under sink storage, the sink area was not designed to prevent splashing and contamination of the sink area and finally the sink was not well sealed which permitted biofilm development (see section 5.3.6.).

Hota et al. (2009) described a HCAI outbreak linked to the placement of sinks within 1m of patient heads, that resulted in an outbreak of *Pseudomonas aeruginosa* due to aerosolisation from sinks.[549] Not only is placement considered to be important for avoiding HCAI but also there is a need to consider the design of those sinks, the material they are composed of and the ease with which cleaning of them can be performed.[189]

Biofilms on Sink Surfaces

Once a sink becomes contaminated with microorganisms permanent removal can be problematic. The materials utilised within the built environment for sink and room design, play a role in the community composition of the environmental microbiome.[88] *Pseudomonas aeruginosa* and other clinically important pathogens such as *Enterobacter* spp. have been isolated in high numbers from wet surfaces, such as sinks, both in this study and in others.[88] Environmental disinfection policies should be

based upon risk assessments for different surface types with specific guidance for different types of cleaning.[189] This is especially true for sinks and other moist surface that have been linked to high levels of contamination within this study. Due to the nature of the surface disinfectants are likely to be diluted on application and cleaning efficiency affected by the presence of biofilms.[189]

The ability of bacteria to adhere to surfaces and form biofilms is one of the main factors resulting in the inefficiency of disinfecting agents, despite there being proven in vitro activity, such as that demonstrated in 5.3.2.1.[551] Disinfection with chlorine without disruption of the biofilms present will only offer low level organism control because the remaining viable bacteria will continue to multiply until previous levels of organisms are re-established.[341, 548] Biofilms that contain MDR organisms have been found within the clinical environment despite chlorine cleaning on high contact items such as doors and curtains.[189, 552]

Biocides such as chlorine based agents have lower activity towards sessile bacteria, a state in which most organisms within biofilms are found.[504, 551] This is because most disinfectants are developed and tested again planktonic bacteria. Microorganisms in biofilms can be up to 1000 times more resistant to toxic substances than those in suspension.[551] Cleaning studies within this work were also undertaken against planktonic bacteria, but the chlorine studies were undertaken in the presence of mechanical action; this would be crucial in the disruption of biolfilms on surfaces.

It has been suggested that organisms within the environment will therefore be exposed to sub lethal doses of biocides, which could potentially induce resistance.[88, 189] Although there is evidence that some biocides such as Ticlosan, quaternary ammonium compounds and silver ions can select for biocide resistance, there is no evidence for resistance linked to the use of chlorine based disinfectants.[553, 554] It is important when using these agents that lethal concentrations of the agent are rapidly delivered in order to avoid selection of resistance.[189]

Although there is no link between chlorine use and resistance it has been demonstrated that non-lethal concentrations of biocides can actually promote biofilm formation. Additionally mixed biofilm formation as detected on the sinks in both the HSCTU and ITUs at GOSH are also linked with both increased biofilm production and increased resistance to antimicrobials.[344, 555] Despite the in vitro sensitivity of organism to chlorine, flushing, as attempted for the outbreak intervention linked to the HSCTU, cannot be completely effective.[548] Also as seen in the Figure 5-17 of the sink

area increased cleaning with chlorine can instead make the situation worse by degrading the fabric of the sink and sink area.

Once significant biofilm has developed on the sink, it may be, as observed in the outbreak on the HSCTU, that the only intervention that will end cross transmission is the replacement of the sinks themselves.[548] It must be noted that unless an intervention in terms of sink design or placement occurs, this may not be a permanent solution as the practice that led to the initial contamination event is unlikely to have changed long term.[548]

Door handles

There are very limited data on door handles and their potential for microbial transmission. In a study looking at surrogate markers of nosocomial pathogen transmission, door handles were highlighted as one site that rapidly became contaminated within the context of a neonatal intensive care setting.[431] A recent study has shown that it is possible to reduce bacteria on door handles provided they are regularly cleaned; however even with regular cleaning, bacteria were detected on more than 20% of handles.[556]

The presence of bacteria on hands and surfaces increases the risk of transmission from hands to the environment and vice versa during hand manipulations.[88] Some surfaces within the clinical environment are only cleaned approximately 30% of the time, with door handles within the clinical environment being one of the least frequently cleaned surfaces.[557] One study demonstrated that once a door handle was contaminated, the subsequent 14 people who used that handle had contaminated hands after use.[157]

We have demonstrated that within a number of different healthcare environments, door handles are regularly contaminated with both bacteria and viruses (see Chapter 3 and section 5.3.7.). Furthermore the design, frequency, and manner in which they are operated are independently related to contamination density and this in turn may impact on the potential for microbial spread. We also identified a relationship between how often and how many people cross door thresholds and the number of bacteria deposited on door handles. This finding supports the requirement for hand hygiene whenever hospital thresholds are crossed, as per WHO guidance.[296]

Cleaning, both of hands and the environment, has been widely accepted as an important factor in curbing the spread of pathogens in hospitals.[558] Our data indicate

that, while cleaning is important, it is not always practical, as in some cases a single touch by a contaminated hand was sufficient to result in a confluent plat. Other approaches may therefore be required. This could include recent design innovations linked to no touch door opening or handles that release antimicrobials either by gel or other means.[559]

5.5

CONCLUSIONS

Decontamination within the clinical environment is an important way of reducing microbial contamination on surfaces. The efficiency with which decontamination is undertaken is driven by not only the biocide used but also their application. Therefore cleaning efficiency studies that evaluate in use cleaning are important. Decontamination of the cleaning environment can be supported by the use of novel technologies; however these cannot replace routine cleaning and can only be used as an adjunct to terminal disinfection. Cleaning undertaken by nursing staff may be the key to reducing environmental contamination on high risk sites, but only if those sites are perceived as a clinical risk and the decontamination of them supported even at times of high clinical work load.

Application of cleaning techniques in high risk areas requires auditing of cleaning standards in order to ensure patient safety and the introduction of monitoring algorithms to evaluate cleaning should be considered. The use of molecular techniques to undertake auditing is acceptable, but only if the disinfectant is known to affect DNA to permit interpretation of cleaning data. In addition, items that are known to be difficult to clean due to biofilms or high levels of use should be targeted for additional cleaning and as the subject of education so that cleaning is undertaken in a way that ensure adequate contact time and concentration of the applied biocide.

Chapter 6 CONCLUSIONS AND FUTURE AVENUES

This project aimed to fill three major gaps in the available literature:

1. To develop methods for undertaking bacterial and viral sampling of the clinical environment and to apply them in order to determine levels of environmental contamination.

2. To develop and validate different typing techniques for Gram-negative bacteria (*Enterobacter species*, *E. coli* and *Klebsiella* species) and apply them in order to determine if there was a link between those organisms found within the environment, and those found within patients.

3. To validate available infection control interventions for their effect on environmental contamination.

6.1 SUMMARY OF MAIN FINDINGS

6.1.1 TO DEVELOP METHODS FOR UNDERTAKING BACTERIAL AND VIRAL SAMPLING OF THE CLINICAL ENVIRONMENT AND TO APPLY THEM IN ORDER TO DETERMINE LEVELS OF ENVIRONMENTAL CONTAMINATION.

A combination of culture and molecular techniques were validated to optimise sensitivity for recovery of organisms present in the clinical environment. Sampling reported in this thesis demonstrated that organisms can be recovered from throughout the healthcare environment. Differences were observed in the location of different bacteria and viruses, leading to the development of different screening algorithms for bacteria and viruses to enable detection. Particularly high levels of organisms were detected within patient rooms where patients were isolated for viruses and in outpatients and day unit wards.

Certain items within the healthcare environment were demonstrated to be more likely to be contaminated with microorganisms. Sink areas including the sink itself, but also the area immediately around the sink were found to harbour Gram-negative pathogens, especially within the paediatric setting. Gram-positive bacteria were more frequently detected in high touch areas and on sites, such as fans, that were more likely to be contaminated by skin scales.

Both bacteria and viruses were found to survive for prolonged periods at room temperature. This finding is in line with the published literature for *Staphylococcus*

aureus, but is novel for *Klebsiella pneumoniae*. This prolonged survival for more than 3 months indicates that if these pathogens are present within the healthcare environment, and if not removed by cleaning, could represent a potential risk to patients.

Little correlation was detected between the presence of specific pathogenic organisms and the level of total viable counts upon surfaces. Although the use of contact plates facilitates quantitative measurement of flat surfaces and may therefore be useful for audit purposes, they lack the overall sensitivity necessary to rule out the presence of pathogens within the clinical setting. For this reason it is proposed that specific pathogen detection constitutes the preferred investigation to underpin risk assessments of the clinical environment.

6.1.2 To develop and validate different typing techniques for Gram-negative bacteria (*Enterobacter* species, *E. coli* and *Klebsiella* species) and apply them in order to determine if there was a link between those organisms found within the environment, and those found within patients.

Identification of potential sources of outbreaks and determining whether cross transmission events have occurred is crucial to enable selection of appropriate interventions. This is far from simple. Chapter 4 discussed the potential role for environmental pathogens as the source of an outbreak. For Gram-negative species such as *Enterobacter* and *Klebsiella*, even primary identification can prove problematic. A number of typing schemes such as multi-locus variable number tandem repeat typing (VNTR) rely upon accurate identification of isolates through phenotypic typing before molecular typing can occur. Other techniques such as pulse-field gel electrophoresis can only be undertaken at reference laboratories, resulting in a delay to receive appropriate typing information.

All typing techniques have advantages and disadvantages. The most appropriate technique will depend upon a number of variables such as the length of time over which the outbreak occurs, whether results need to be transferred to other centres, and the level of discrimination required (this will depend whether investigations are local or national). One of the universal problems with all the typing techniques evaluated was the need to develop and validate interpretation criteria, and the need for these to be informed by isolates from closely defined epidemiological cohorts as well as molecular variation.

This study demonstrated that whole genome and high throughput sequencing methods could be used to provide a suitable typing scheme. However these techniques require further information if they are to be used routinely. Specifically data on differences in molecular clocks of individual species and individual strains and an understanding of how these differ between patients who are colonised or infected and those found within the environment. Use of these techniques is currently limited by cost, data handling, interpretation of the results and the delay all of these cause.

In lieu of comprehensive genomic coverage (whether of the core genes or core plus accessory genes) VNTR appeared to provide the most accessible, rapid and discriminatory technique for typing of these organisms.

6.1.3 To validate available infection control interventions for their effect on environmental contamination.

As pathogens are present within the environment and linked to cross transmission, as demonstrated within Chapters 3 and 4, then their removal via cleaning is an important infection control intervention. As contamination levels have been shown to differ between wards and between different locations on wards then knowledge of both patient susceptibility and of organism distribution may be key in making decisions about cleaning interventions in order to prevent cross transmission. Detection of pathogens within this work has demonstrated that current cleaning standard of visually free of dirt, dust and debris does not mean that the environment will be free of pathogens. As such organisms that remain after cleaning may be a risk to patients. Retrospective typing has shown that cross transmission was occurring on the haematology oncology day unit. Although these patients may be at relatively lower risk and are exposed to normal community microorganisms, the transmission of multidrug resistant organisms within the unit has the potential for significant patient impact. As such, interventions in terms of alteration of the cleaning product should be considered, despite the increase in expense.

Cleaning may be modified by a number of techniques, such as introduction of chlorine based cleaning products or delivery through an airborne system, such as hydrogen peroxide. If cleaning is modified to order to reduce risk then it is crucial to be able to monitor cleaning to ensure that it is being delivered effectively. Monitoring should be appropriate to the cleaning technique. In the case of the adenovirus study undertaken as part of this work 25% of terminal cleans with NaDCC failed to successfully remove potential pathogens. Therefore without monitoring there is a risk that the additional

investment in cleaning will be unsuccessful in controlling cross transmission. Total viable counts may have a role to play in sampling directly after cleaning in order to measure levels of bacterial contamination; however the turnaround of this technique is 48 hours and therefore will not give rapid enough information to allow direct intervention. PCR was determined to be more effective as a method of environmental monitoring. This is due to its speed and that co-screening could be undertaken for both viruses and bacteria at the same time. Although PCR is likely to overestimate the levels of viable contamination, if appropriate cleaning chemistries are used it is an appropriate technique to assess their efficacy.

Cleaning has also been shown within the *Klebsiella pneumoniae* outbreak, discussed in both Chapters 4 and 5, to be potentially ineffective in removing biofilms within clinical environments and damaging. Chemistries such as NaDCC that are able to affect biofilm can lead to degradation of infrastructural materials, which results in changes in surface topography leading to increased resilience to cleaning.

As surfaces become rapidly re-contaminated after cleaning (as was shown in the door handle study) cleaning by itself is unlikely to be able to ensure that the environment is free from pathogens. In order to help in controlling contamination by containing it to certain points, facilitating cleaning or by changing user behaviour, design may have an important role to play.

If the environment can be acknowledged to be a source of risk to patients, as demonstrated in this work, a logical plan can be instituted to limit cross transmission. This could include better environmental design coupled with efficient hand hygiene. When planning clinical environments, infection control teams should be involved in making designs that can support infection control activities such as hand washing and also impact on critical moments that are recognised as targets for high impact interventions. These include not only the number and types of sinks required, but the placement of those sinks within the new unit. In addition the location of doors and the types of handles should be considered as our results indicate that both traffic density and door location impact on contamination.

This strategy is reflected in the WHO 5 moments of hand hygiene, where ‘changing environment’ i.e. when entering and leaving a bed space, is a requirement for performance of hand hygiene. However, poor compliance is still linked to patient contact and more needs to be done to raise awareness of the environment as an infection risk and to encourage appropriate behaviour.

6.2 LIMITATIONS OF THE STUDY

This study has five main limitations:

Firstly, it is assumed that the specific pathogens that were included in the sampling algorithm are representative of the nosocomial pathogens that are frequently associated with healthcare associated infection (HCAI). This may not be the case. The specific pathogens tested for (norovirus, adenovirus, *Staphylococcus aureus* and Gram-negative bacteria) represented those organisms, which are important for HCAI within paediatric settings in England, and were therefore determined to be clinically significant. In different settings other species may be more important. In adult units for example, *Clostridium difficile* may play a larger role and in other countries vancomycin resistant *Enterococci* constitutes more of a problem.

Secondly, the organisms used to represent infection caused by Gram-positive, Gram-negative bacteria and viruses in this study (adenovirus, *Klebsiella pneumoniae* and *Staphylococcus aureus*) may not actually be representative. The individual strains selected were also assumed to be representative of their species. This assumption was made in order to facilitate testing due to resource limitations, but also because these were the most clinically significant organisms within those groups in terms of cross transmission events occurring at Great Ormond Street Hospital. However it's still an assumption and not all strains will be representative of the organisms causing clinical infection.

Thirdly, viability testing was not undertaken for viruses recovered from the environment and it was assumed that a positive PCR result represented a risk to patients. The use of tissue culture delays results and would require an idea of which viruses could be present prior to inoculation, whereas PCR permits a panel approach.

Fourthly, it was not possible when undertaking typing analysis to determine directional transmission of *Klebsiella pneumoniae*, in order to truly confirm whether the environment acted as a source. This is because there was insufficient genomic data available on Klebsiella pneumoniae to be certain about data interpretation using single nucleotide variants. Epidemiological information however strongly indicates a continuous environmental source leading to cross transmission of *Klebsiella pneumoniae* to patients.

Finally this work has not demonstrated that interventions such as terminal cleaning with monitoring have an impact on levels of HCAI within Great Ormond Street Hospital.

This is in part because of the way that data is recorded, meaning that bed spaces are not listed and so it is not possible to associate cases of HCAI with specific rooms. In addition although active surveillance and typing is undertaken for a number of resistant bacteria, typing is not undertaken routinely for viruses and so it is often difficult to link cases. Additionally although levels of HCAI are decreasing as interventions are introduced, interventions are rarely introduced singly, with a bundle approach being more common. Therefore it is difficult to determine the level at which the infection control interventions introduced as part of this work have contributed to decreases in HCAI levels.

6.3 FUTURE WORK

- Work needs to be done to understand how host colonisation, immune evasion, virulence, transmission within and between host and the environment can change phenotype and or genotype. This data is required for interpretation of whole genome sequence typing data. This work may involve:
    - Following up colonised patients over a period of twelve months to see how isolates vary and looking at co-colonisation
    - Pairing isolates from carriage samples and those in infected sites and undertaking whole genome sequencing to look at variation
    - Examining isolates from patients colonised in multiple sites to determine within host variation
    - Sequencing isolates from an immune competent host as they become immunocompromised and immunocompromised hosts as they reconstitute to identify the impact of immune function
    - Investigation of environmental isolates and further active surveillance to determine directional transmission
- As microbial resistance becomes an increasing problem work should be undertaken to better understand how genomic resistance relates to phenotypic resistance, and how testing for such resistance should be undertaken.
- Work on development of an *Enterobacter cloacae* VNTR scheme will be continued as better phenotypic identification is now available, which may improve results.

6.4 CONCLUSIONS

In summary, this thesis and the resulting publications have contributed to the understanding of the distribution of organisms within the clinical environment, the effect of interventions - such as cleaning and design - that can impact upon them and typing of those organisms in order to identify reservoirs and aid outbreak control.

The data acquired has led to changes within infection control at Great Ormond Street Hospital. New policies and standard operating procedures were formulated, informed by the evidence collected. The resulting policies have been made available to other centres.

Design based changes have ensured that staff and visitors are able to exit areas of high contamination, such as cubicles, without touching the door and also access hand hygiene directly afterwards. Sink environments, where possible, have been modified to provide shelf space so that items are not stored on potentially contaminated sink surfaces. New sinks being introduced now follow design principles, such as splash backs, that reduce sink contamination.

Cleaning standards have been adjusted on the basis of this work, with NaDCC now used for cleaning throughout the hospital. Certain clinical environments are monitored for high risk organisms, such as adenovirus, using molecular techniques instead of visual standards. For antibiotic resistant bacterial species, such as carbapenemase producing Enterobacteriaceae, additional cleaning steps have been introduced, including the use of hydrogen peroxide vapour decontamination in addition to chlorine.

Identification of different likely reservoirs for Gram-negative and Gram-positive bacteria and viruses during this work has led to the development of screening algorithms. These are now utilised whenever potential cross transmission is detected to identify environmental reservoirs when present so that interventions can be targeted. This has resulted in decreased time to introduce appropriate interventions, such as sink control, for Gram-negative bacteria. Finally, undertaking of in-house typing using VNTR has decreased the time to outbreak confirmation and thus improved outbreak response.

Many of the potential interventions identified in this study are low cost. As such they can be introduced in centres without substantial financial and scientific resources. As such, developing this evidence based approach to understanding which interventions are effective is key to correctly allocating resources to prevent HCAI.

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Chapter 8 APPENDIX

Great Ormond Street Hospital for Children NHS Foundation Trust

Microbiological Screening of Environment Policy

LEAD DIRECTOR: Liz Morgan, supported by John Hartley, DIPC

POLICY APPROVED BY: John Hartley

DATE POLICY APPROVED:

IMPLEMENTATION DATE:

REVIEW DATE:

	Document Control Sheet
Policy Title	Microbiological Screening of Environment Policy
Purpose of Policy/ Assurance Statement	To reduce the risk of acquisition and cross transmission with potentially pathogenic microorganisms this policy describes - when microbiological screening of the environment - how sampling is performed and the expected standard results are interpreted against.
Target Audience (Policy relevant to)	All members of Infection Prevention and Control Team Lead nurses, Heads of Nursing, Ward managers or equivalent Cleaning Managers, floor managers and Supervisors All members of clinical staff Commissioning and Engineering Team CSP's Occupational Health
Lead Executive Director	John Hartley
Name of Originator/ author and job title	Elaine Cloutman-Green - Clinical Scientist Infection Control
Version (state if final or draft)	Final
Date reviewed	March 2013
(Previous review dates)	
Circulated for Consultation to (*Please list Committee/Group Names*):	Infection Control Committee Microbiology and Virology departments
Amendments:	
Links to other policies or relevant documentation	Major outbreaks, microbiological screening instructions to wards Microbiological Specimen Collection Policy Policy for the Control of MRSA Infection Cleaning Policy Infection Clean Protocol Ventilation Policy Legionella Control Policy laboratory protocol BSOP0058
If draft	
Draft Number	
Comments to	
By	

1. Table of contents

2. Introduction

A range of Trust policies are designed to reduce the risk of patients acquiring potentially pathogenic microorganisms by cross transmission from other patients, staff or visitors, or directly from the environment. Standard infection prevention and control precautions and environmental cleaning are adequate to reduce risk in most situations.

Policies are present as clinical guidelines:
http://www.gosh.nhs.uk/clinical_information/clinical_guidelines?category=I

However, some situations arise where additional controls are implemented for which microbiological monitoring is required to validate the control has been successfully applied.

3. Aims and objectives

The aim of this policy is to help reduce the risk of patients or staff acquiring potentially pathogenic microorganisms from the environment.

The objective of the policy is to define

3.1 When microbiological screening of the environment is required

3.1.1 Environmental clearance for specific organisms:

Where a specific microorganism or clinical infections is recognised to lead to greater risk through environmental contamination, whether or not a cross transmission has occurred assurance is required that adequate cleaning has taken place beyond that demonstrated by the satisfactory standard checks (e.g. visual) after appropriate cleaning has been completed and before re-occupation of area.

Organisms and procedure listed below.

3.1.2 Confirmation of protective isolation ventilation:

Where increased patient susceptibility is present due to a severe immunodeficiency (congenital or acquired) and local risk assessment has designated requirement for a protective environment including HEPA filtered air. (Procedure below - Appendix 5: Environmental air screening protocol for critical ventilation systems supplying clinical areas**;** Schedule of sampling is in Estates Ventilation Policy).

Operating theatre environment – Validation of microbiological air standard is required at commissioning and annually as part of the planned preventative maintenance described in Estates Ventilation Policy (Testing procedure below)

Water quality – microbiological quality of water is monitored routinely for legionella and *Pseudomonas aeruginosa* (as described in Estates Legionella policy) and as required when investigations of specific organisms suggests water may be involved in the transmission.

3.1.3 How sampling is performed and the expected standard results are interpreted against

Procedures and standards are shown below in appendices

3.2 This policy does NOT cover

- Monitoring of air volumes and pressures as part of the validation of specialist ventilation Units (schedule and parameters in Ventilation policy)
- Schedule for testing for legionella (see legionella control policy) and Pseudomonas aeruginosa in water (to be included in Estates document in preparation)
- Microbiological monitoring of water in dialysis, Decontamination department, hydrotherapy Pool where separate policies exist
- Environmental monitoring in Pharmacy suites, gene or cell therapy, SSD, mortuary, laboratories or other research areas where separate polices exist

4 Duties and responsibilities

Infection prevention and control (IPC) team – to update policy and undertake procedure. To teach other to undertake procedures as required by clinical areas and keep records.

Staff at ward level – to liaise with IPC, ensure area clean and ready; to undertake screening if trained

External contracted companies – to comply to these procedures

5 Procedure or guideline

5.1 Environmental clearance for specific organisms

Screening will only take place after appropriate adequate cleaning has been completed and signed off according to normal standards (Infection Clean Protocol GOS-EAF-PRO-10587)

Organisms Screened For:

Individual risk assessment is performed by the Infection Prevention and Control Team (IPC Team) to grade risk associated with any particular organism. The need to perform screening is dependent on the organism, any current outbreak/transmission and susceptibility of individuals who may be exposed.

Examples of organisms which may be screened for include:

Methicillin resistant *Staphylococcus aureus* (MRSA)
Multi-resistant gram negative species
Clostridium difficile
Vancomycin resistant enterococci (VRE)
Norovirus
Adenovirus
Carbapenem resistant Enterobacteriaceae and *Acinetobacter* species

Other organisms as determined by IPC Team

5.2 Preparation of bed spaces and patient areas

Bed spaces may be screened post a level 2 or 3 clean carried out by the cleaning contractor.

Bed spaces should be left for at least 2 hours post clean to allow all surfaces to dry adequately.

Bed spaces need to have been checked by a cleaning supervisor and ward representative (as per cleaning SLA) before screening takes place and the bed space checking form must be signed to say that a check has taken place before screening is undertaken. Bed spaces must be visibly clean; if they are not then screening must be suspended until the room has been re-cleaned.

Bed spaces must be free of all disposable equipment, clinical equipment and linen. If this is not the case screening cannot be carried out until these are removed. Please note that the room is unlikely to be organism free if these items have not been removed pre-clean as they impede cleaning. It may be advisable to request the room be cleaned again before screening.

If the room has not been properly prepared or is still visibly dirty this must be flagged both to the cleaning contract supervisor and the senior ward staff member, as well as the Infection Prevention and Control Team and the member of Facilities in charge of the cleaning contract.

If this situation is found an incident form must be completed.

Screening should be carried out by a trained member of staff. This may be a member of IP&C Team or a ward designated staff member.

After screening the bed space will be sealed until the results are back and a decision as to whether or not to open the room made.

Screening samples may be processed by a number of methods with availability of results differing between 1 and 5 days.

The decision to open the room must be made in conjunction with ward manager (who is expected to liaise with consultant staff) and IP&C and an individual risk assessment.

If a bed space is found to have two consecutive cleaning failures due to microbial detection after consultation with the IP&C Team then a meeting must be called to include the contract manager, the Mitie supervisor and appropriate ward staff and an action plan developed. (Infection Cleaning Policy/document library)

5.2.1 General Ward/Clinic Areas

General ward areas may be screened in response to cross transmission or outbreaks. Ward areas will be screened by a member of the Infection Control Team and may be carried out in relation to a requested level 2/3 cleaning instead of the routine ward clean.

5.2.2 When is Screening Necessary:

The risk assessment considers the organisms, underlying condition of host, potentially susceptible individuals, current transmissions, prior experience with this organism and any clinical consequences of delay in bed opening. Screening may be requested for specific very high risk

organisms (highly transmissible or highly resistant) in any situation, or other organisms when involved in outbreaks.

5.2.3 MRSA

Experience has shown certain strains and patient conditions lead to increased risk of cleaning failure (despite visually satisfactory inspection). For some children, the IP&C team are able to alert these children on PIMS as carrying highly transmissible strains. Screening will be carried out post discharge of patients with a highly transmissible strain of MRSA after a level 3 clean. Screening may also be necessary with other strains in an outbreak situation.

Screening results will take 72 hours (longer if processed over a weekend)

Criteria for bed space reopening - all sites free from MRSA

5.2.4 Multi-resistant Gram Negative Species

A large number of children are present in the trust colonised with multiresistant bacteria and routine trust screening, isolation and cleaning protocols are adequate to control most of these.

However, certain highly resistant bacteria may be present for which screening should be carried out post discharge. The alert that this is a highly resistant strain of gram negative microorganism should be included on the alert in PIMS. A level 3 clean is usually required pre screen.

Screening results will take 72 hours (longer if processed over a weekend)

Examples include Enterobacteriaceae (Klebsiella, Enterobacter and Escherichia) or *Pseudomonas aeruginosa* carrying genes for transmissible carbapenemases, or multiresistant *Acinetobacter baumanii* (MRAB), which have caused major outbreaks in other London hospitals.

Criteria for bed space reopening - all sites free from Multi-resistant Gram Negative organisms

5.2.5 Clostridium difficile

Screening may be requested in response to outbreaks or in relation to specific patient factors.

C. difficile cases are usually sporadic in this trust, but the organism produces very resistant spores and outbreaks frequently occur in many health care settings. Screening will not be carried our routinely following detection of a case, but may be carried out in response to a request from the Infection Control Team/Consultant Microbiologist.
Screening results will take up to 7 days

Criteria for bed space reopening - all sites free from *C. difficile*

5.2.6 Vancomycin Resistant Enterococcus faecium or Enterococcus faecalis (VRE)

Screening may be requested in response to outbreaks or in relation to specific patient factors.

Detection of VRE is uncommon in GOSH, but the faecally carried organism has been responsible for outbreaks in the past, usually felt to be propagated through environmental contamination

despite cleaning. Screening will be considered post discharge of any patient colonised or infected with VRE, this should be included on the alert in PIMS and would require a level 3 clean pre screen.

Screening results will take 72 hours (longer if processed over a weekend)

Criteria for bed space reopening - all sites free from VRE

5.2.7 Norovirus

Norovirus and other enteric viruses survive well in the environment and have a low infectious dose. Environmental decontamination is paramount for control. Risk assessment must consider that certain immunocompromised individuals will excrete high levels of virus for long periods and environmental contamination may be high.

Screening will be carried out in response to a request from the Infection Control Team/Consultant Microbiologist. Screening may also be requested:
in response to outbreaks and
in rooms where the next admitted patient is likely to be immunosuppressed (e.g. Lion, Giraffe, Elephant and wards)

Screening results will take up to 72 hours (longer if processed over a weekend)

Criteria for bed space reopening - all sites free from Norovirus

5.2.8 Adenovirus

Adenovirus may survive well in the environment and are excreted long term in large numbers in some immunocompromised children. Epidemiological data suggests cross transmission may occur in the hospital setting.

All cubicles occupied by Adenovirus excreting children which will next have severely immuncompromised children in (currently usually Fox, Robin and Butterfly BMT cubicles) must be screened after the level 3 clean post discharge.

Screening may also be requested
in response to outbreaks and
in rooms where the next admitted patient is likely to be immunosuppressed (e.g. Lion, Giraffe, Elephant and wards)

Screening results will take up to 72 hours (longer if processed over a weekend)

Criteria for re-opening: Decision to open room before results will be assessed with respect to the risk in next room occupant.

5.3 Criteria for bed space reopening:

The cubicle is opened with no further cleaning required if no site has an Adenovirus positive CT result of lower than 39.

If the cubicle has 2 sites positive with Adenovirus at CT's of no lower than 34, then those sites are recleaned twice using chlorine and the cubicle can be re-opened, as long as the sites positive

to do include the floor inside the room. If the floor inside the cubicle is positive than the cubicle undergoes a repeat 'deep clean' and is re-screened.

If the cubicle has more than 2 sites with an Adenovirus positive CT of 34 – 38 or if any one site has a CT of lower than 34, then the entire room must have a repeat 'deep clean' and be re-screened in full before opening.

If the same objects fails to become negative after 3 cleans and screens, that object if possible is replaced within the cubicle and disposed of appropriately.

6 Step by step guide and flowchart

6.1 How to Carry Out Environmental clearance for Specific organisms

A set of predefined areas should be screened according to the attached protocols.

See appendix 1 – bacterial screening protocol
See appendix 2 – bacterial screening form
See appendix 3 – viral screening protocol
See appendix 4 – viral screening form

6.2 Ward and Other Environmental Screening

Ward Screening

General ward areas may be screened in response to cross transmission or outbreaks. Ward areas will be screened by a member of the Infection Control Team and may be carried out in relation to a requested level 2/3 cleaning instead of the routine ward clean. Screening results will take up to 7 days depending on the organism screened for.

Swab No.	Site	Area
1	Corridor floor outside of cubicle/ante room entrance of a known positive	10cm^2
2	Corridor floor outside of cubicle/ante room entrance of a known negative	10cm^2
3	Storage trolley surface outside of the room of known positive	10cm^2
4	Storage trolley surface outside of the room of known negative	10cm^2
5	Sluice work surface	10cm^2
6	Sluice room door handle (exit to corridor)	Entire handle
7	Macerator lid	Round the entire seal
8	Nappy weighing scales	Entire top surface 10cm^2
9	PC keyboards	Every key and surface on the right 50% (~10cm^2)
10	Nurses station	10cm^2
11	Nurses station phone	Entire Keypad and handle
12	Notes trolley	10cm^2

6.3 Monitoring of areas with critical mechanical ventilation systems (providing protective HEPA filtered environment or operating theatre standard air quality)

Screening will be carried out by the IPC team, or a company that fulfils the screening criteria under direction of estates, under the following conditions:

- During commissioning
- As part of the annual maintenance schedule annual verification

- In areas where work has been undertaken on mechanical ventilation units providing protective HEPA filtered environment or operating theatre standard air quality where air quality may be altered (work on filter or down stream of filter)
- In areas supplied by mechanical ventilation where the fabric of the room has been breached.

All maintenance work or renovation in the clinical areas must be discussed with the Infection Control Team prior to implementation. Screening will be undertaken using the protocol set out in appendix 5 and form appendix 6:

appendix 5 – Environmental air screening protocol for critical ventilation systems supplying clinical areas

appendix 6 – Screening of critical ventilation systems - form

Screening will involve the use of settle plates (blood agar alone is adequate) and air sampling (carried out prior to the placement of settle plates) onto blood agar. Sampling will be undertaken post level 3 clean and the room will be closed to entry throughout the sampling time. Screening time will depend on the area to be screened, but will take a minimum of three hours.
Screening results will take 72 hours (longer if processed over a weekend)

Plates are incubated according to laboratory protocol BSOP0058

6.3.1 Specifications for microbiological air sampling (BSOP0058)

Test types:

1 cubic metre air tests (also called active air test or air test): collected using a calibrated air sampling device, 1 cubic metre of air will be sampled on to a suitable agar plate. In house we use blood agar, although other non-selective agar is suitable.

Incubation: 18-24 hours at 35 -37C with initial 1 day cfu report
Then 2 additional days at room temperature to give final report.

Reading: Plates are read for colony count (fungi are identified to genus level)

Report: Bacterial count in cfu/m3
Fungal count in cfu/m3 (ensure report no Fungi grown, if none, and any growth is identified to genus level)

Settle plates: 9 cm blood agar plates left for 2 hours.

Incubation: 18-24 hours at 35 -37C with initial 1 day cfu report
Then 2 additional days at room temperature to give final report.

Reading: Plates are read for colony count (fungi are identified to genus level)

Report: Bacterial count in cfu/ plate
Fungal count in cfu/plate (ensure report no Fungi grown, if none, and any growth is identified to genus level)

6.3.2 Criteria for satisfactory validation – depends on the standard the area has been built and designed to e.g. HTM2025 or HTM 03-01 (or replacement documents);

Theatres:
HTM 03-01 modified standards:
none HEPA filtrated area -10 or less cfu/m3 total count
HEPA filtrated area -10 or less cfu/m3 with no fungal colonies

HTM 2025 modified standards:
none HEPA filtrated area -35 or less cfu/m3 total count
HEPA filtrated area -35 or less cfu/m3 with no fungal colonies

Requirement for each area should be described in the Estates produced schedule but in summary:

Theatres:
Theatres are currently operating to 2025 (to which they were designed) but we aim to achieve 03-01 where possible, so theatre results that do not meet 03-01 total count need to be reviewed.

Protective isolation rooms and ward areas:
Newer PPVL rooms should meet HTM 03-01, with no Fungi detected

Older rooms and wards were built with HTM2025 standards.
If 03-01 standard is not meet the area needs review and individual decision made considering commissioning and annual verification records of what was achieved.

6.3.3 Areas requiring specialised ventilation screening:

- Operating theatres
- Clinical areas with HEPA filtration – Robin and Fox wads; Butterfly Transplant Suite
- PICU, NICU, CICU, Angio Suite, HSDU
- All individual rooms providing protective isolation (e.g. the positive pressure ventilated lobby rooms)

Other critical ventilation systems, shown below, are **NOT** covered by this policy
- Pharmacy: Cytoxic Suite, TPN Suite, Sterile Unit
- SSD
- Gene and cell therapy suites
  Areas should comply with good manufacturing clean room standards, administered by Pharmacy or research sponsors.
- Pathology laboratories. Covered by Pathology documents.
- Mortuary

6.4 Requirements for microbiological sampling

- Investigations will be undertaken by the Infection Control Team / Microbiology Department during routine laboratory working hours only or by delegated competent staff employed by Estates or GOSH staff trained by IPC
- Any problems requiring urgent attention must be discussed with the Infection Control Team.
- Notification of the cleaning programme must be sent in advance, in writing, by the project leader concerned to the Infection Control Department
- Confirmation that the programme is on time, must be made by phone, by the project leader to the Infection Control Team (CNS or Infection Control Clinical Scientist) or Infection Control Doctor
- Appropriate arrangements for removal and storage of furniture, sterile and non sterile stores must be made prior to commencement of any work.

6.4.1 Procedures to be undertaken prior to microbiological sampling

- Air flow and pressure parameters must be confirmed as meeting standards by Estates prior to any microbiological testing - Ventilation validation reports MUST be sent to Infection Control Team / Microbiology Department in time for these to be checked prior to sampling
- Level 3 clean (Infection Clean Protocol GOS-EAF-PRO-10587)
- Check that all appropriate windows and doors are closed
- Check that air conditioning is switched on. It must be ensured that both main and backup systems are fully functional.
- The area must be locked and left empty for a minimum of 2 hours prior to air sampling.
- It is the responsibility of the project leader concerned to inform the Infection Control Team that the area is ready for sampling.

6.5 Communication of results

- If testing is performed by an external company employed by Estates, result must be sent to Infection Control Team as well
  Results will be made available by the Medical Microbiologist, or member of the Infection Control Team, who will phone and email the appropriate manager / project leader
- Where a microbiological failure is documented, individual advice on re-cleaning and sampling will be given by the Infection Control Team or Medical Microbiologist

7 Local arrangements for implementation

7.1 Who to Contact

Infection Control Team bleep 0640 ext 5284 and either the Virology or Microbiology lab as appropriate.

7.2 Who Should Carry Out Screening

This should be performed by a member of the Infection Control Team or a member of staff trained by the Infection Control Team; or company appointed by Estates

7.3 Distribution of Screening Results

The member of staff in charge of the ward will be contacted with the environmental screening results. If the cubicle/ward area is passed as clean the area is then available for use.

If the cubicle/ward area fails the first environmental screen the Infection Control team will discuss with the member of staff in charge what subsequent tasks need to be undertaken. The cleaning contract liaison and the cleaning supervisor will also be informed of any screening failure (as per the Infection Clean Protocol GOS-EAF-PRO-10587).

If repeat cleaning and screening is undertaken as a result of a failure the reporting process is the same. However if a cubicle/ward area fails its environmental screening more than twice a meeting will be called as detailed in the cleaning policy to evaluate why failures are occurring and how the situation can be resolved.

7.3.1 Archiving of screening results

Samples will be booked in to the laboratory computer system. Paper copies of results are not sent to clinical areas but will be archived in IP&CT office in a folder called Environmental Screening for future reference.

8 Training arrangements

Staff carrying out environmental screening should be trained and signed off as competent by a member of the Infection Control Team. A training update should be carried out annually and a training record maintained. Members of staff being trained to carry out environmental screening need to be trained to carry out both bacterial and viral sampling; as these procedures are different.

The training will be carried out by the IP&C team and record maintained locally by the ward or theatres.

9 Auditing and monitoring

Annual audit against Estates maintenance records to confirm microbiological air tested when appropriate

Annual audit against level 3 clean list to check screening was requested when appropriate

Annual audit training records for update

Audit that all external companies have followed our procedure

10 Appendices

Appendix 1: Screening Protocol

Bacterial Environmental Screening Method (MRSA and Multi-Resistant Gram Negative Organisms)

Introduction

Bacteria can be a source of contamination when a patient who is infected/colonised is present in a cubicle. These bacteria can also survive for long periods in the environment and so when a patient, with an alert requiring a level three clean, is discharged or moves room, we need to know that the cubicle is free from bacteria before placing another patient inside. The Infection Control team may ask that you sample (swab) a room after cleaning to make sure that all the bacteria have been removed.

What you need

Supplies needed on ward

- Permanent marker pen (to write on tubes)
- Charcoal swabs (kept in a clean dry location)
- Gloves
- Disposable aprons (white)
- Polythene specimen bag to put your samples in once taken
- Sterile water (from the clean utility)

Supplies provided upon request by bacteriology (phone bacteriology each time):

- **Bacteria Environmental Screening Form** (1 per cubicle - to be completed during screening by the person swabbing)

What you do

When you have been asked to screen a cubicle by the Infection Control Team:

Call bacteriology and ask for the number **Bacterial Environmental Screening Forms** (1 per cubicle). Tell them where you are screening and for which organism/alert.
Find out from the person in charge the information you need to fill out the **Bacterial Environmental Screening Form** and complete it.
When you have everything from the **what you need section** including gloves and aprons (taken from the clean utility room) put everything in a clean suitable place outside of the cubicle i.e. a fold table, **or** use a clean plastic tray which you can take into the room.
Wash your hands thoroughly with soap and water.
Put on a disposable plastic apron and gloves.
Enter the cubicle
Write on the **Bacterial Environmental Screening Form** in the **Swab No.** column the number of the swab you are taking (i.e. 1 – 12) and write in the **Site Swabbed** column which area you are going to sample (use the sites to be included table on the back of the form). Label the swab you are going to use with the swab number, site, cubicle number, ward and date. Only label one swab at a time!

Once your swab is labelled and the form written take a clean swab from its pack, being careful not to touch the cotton end.

Lightly moisten the swab in the sterile water.

Swab the area (use the **Area to be Swabbed** description in the table on the back of the form to help you)

Put the cotton end of the swab back into the tube containing the charcoal transport media.

Put the tube in the clean sample bag.

Collect the plastic caps to be thrown away in a clinical waste bin when you have finished taking all the swabs.

Repeat steps 7 and 8 with each swab to be taken.

If at any point you think you may have got something on your gloves you **must** change them for a clean pair.

If the room does not appear clean or the appropriate items (curtains etc) have not been removed report this to the nurse in charge or contact the Infection Control Team.

The swab remains and all gloves and aprons **must** go in a clinical waste bin. If you have used a tray make sure it is thoroughly washed with soap and water.

Make sure that you wash your hands thoroughly with soap and water.

Fill in the form on the cubicle door to say that sampling has been carried out.

Send the completed form and samples to bacteriology using the chute system.

Tell the person in charge that the screening has been completed.

Swab No.	Site	Area to be Swabbed
1	Floor under sink	4 $inch^2$/10cm^2
2	Bathroom door handle	Entire handle
3	Chair with arms	Both arms (where hands sit) ~4 $inch^2$/10cm^2 on each
4	Oxygen outlet (above bed)	Entire surface
5	Telephone keypads	Entire keypad
6	Taps in Patient Bathroom	Entirety of both taps
7	Mattress top	4 $inch^2$/10cm^2
8	Bed/Cot frame under bed	4 $inch^2$/10cm^2
9	Trolley surface (in ante room if present)	4 $inch^2$/10cm^2
10	Side window sill (right hand side)	4 $inch^2$/10cm^2
11	Cubicle room exit door handle (cubicle side)	Entire handle
12	Corridor floor outside of cubicle/ante room entrance	4 $inch^2$/10cm^2

Additional sites should be swabbed (samples 13+) if there are any areas that look dusty or unclean. This should be reported back to the Infection Control Team.

Contact Numbers

Infection Control: 5284
Bacteriology lab 5280/8661
Consultant Microbiologist: 7930/5237/8594

Appendix 2: Screening Form

Bacterial Environmental Screening Form

Cubicle Tested: _____ **Ward**: _____

Patients ID: _____ **Patient discharge date**: _____

Date of Cleaning: _____ **Level of Cleaning**: _____

Bacteria: _____

Date: **Time**:

Laboratory No.	Swab No.	Site Swabbed	Culture Results
	1	Floor under sink	
	2	Bathroom door handle	Entire handle
	3	Chair with arms	
	4	Oxygen outlet (above bed)	
	5	Telephone keypads	
	6	Taps in Patient Bathroom	
	7	Mattress top	
	8	Bed/Cot frame under bed	
	9	Trolley surface (in ante room if present)	
	10	Side window sill (right hand side)	
	11	Cubicle room exit door handle (cubicle side)	
	12	Corridor floor outside of cubicle/ante room entrance	

Please inspect the room prior to screening. If the room is visibly dirty do not screen and inform the infection control team on ext 5284/bleep 0640. Out of hours inform the PEC on duty. If any of the sites are not present select another site and list it on the form**.**
Always screen 12 sites.

Name: _____ Signature:_____

Sites to be Included In Environmental Swabbing

Swab No.	Site	Area to be Swabbed
1	Floor under sink	10cm^2
2	Bathroom door handle	Entire handle
3	Chair with arms	Both arms (where hands sit) ~5cm^2 on each
4	Oxygen outlet (above bed)	Entire surface
5	Telephone keypads	Entire keypad
6	Taps in Patient Bathroom	Entirety of both taps
7	Mattress top	4 inch^2/10cm^2
8	Bed/Cot frame under bed	4 inch^2/10cm^2
9	Trolley surface (in ante room if present)	10cm^2
10	Side window sill (right hand side)	4 inch^2/10cm^2
11	Cubicle room exit door handle (cubicle side)	Entire handle
12	Corridor floor outside of cubicle/ante room entrance	10cm^2

If any of the items on the above list are not there (i.e. mattress) or an anteroom is not present, please take at least 12 swabs. Choose which extra places/items to swab from the suggested list below. Please swab any areas which are visibly dirty and report them to the Infection Control Team. Make a note of where each extra sample was taken from on the **Bacterial Environmental Screening Form** in the space provided.

Suggested Additional Areas

Angle poise lamps
Television
Monitoring equipment
Bed lockers
Radiator grills

Contact Numbers

Infection Control: 5284
Bacteriology lab 5280/8661
Consultant Microbiologist: 7930/5237/8594

Appendix 3: Screening Protocol

Enteric Viruses Environmental Screening Method (Noro and Adeno Viruses)

Introduction

Enteric viruses (e.g. Norovirus and Adenovirus) can be a source of contamination when a patient who is infected is present in a cubicle. These viruses can also survive for long periods in the environment and so when a patient with Adeno or Norovirus is discharged or moves room, we need to know that the cubicle is free from virus before placing another patient inside. The Infection Control team may ask that you sample (swab) a room after cleaning to make sure that all the virus particles have been removed.

What you need

Supplies needed on ward (call virology when running low on swabs or pens):

- Permanent marker pen (to write on tubes)
- Sterile cotton wool swabs (kept in a clean dry location)
- Gloves
- Disposable aprons (white)
- Polythene specimen bag to put your samples in once taken

Supplies provided upon request by virology (phone virology each time):

- Batches of tubes of sterile water (12 per cubicle to be swabbed)
- **Enteric Virus Environmental Screening Form** (1 per cubicle - to be completed during screening by the person swabbing)

What you do

When you have been asked to screen a cubicle by the Infection Control team:

Call virology and ask for the number **Enteric Virus Environmental Screening Forms** (1 per cubicle) and tubes of sterile water (12 per cubicle) you need.
If out of hours call bacteriology for tubes - see contact numbers at the bottom of the sheet
Find out from the person in charge the information you need to fill out the **Enteric Virus Environmental Screening Form** and complete it.
When you have everything from the **what you need section** including gloves and aprons (taken from the clean utility room) put everything in a clean suitable place outside of the cubicle i.e. a fold table, **or** use a clean plastic tray which you can take into the room.
Wash your hands thoroughly with soap and water.
Put on a disposable plastic apron and gloves.
Write on the **Enteric Virus Environmental Screening Form** in the **Swab No.** column the number of the swab you are taking (i.e. 1 – 12) and write in the **Site Swabbed** column which area you are going to sample (use the sites to be included table on the back of the form). Label the tube you are going to use with the swab number, label both the lid of the tube and the side of the tube. Only label one tube at a time!
Once your tube is labelled and the form written take a clean swab from the pack, being careful not to touch the cotton end.
Enter the cubicle to be screened, open the tube and lightly moisten the swab in the water.
Swab the area (use the **Area to be Swabbed** description in the table on the back of the form to help you)
Put the cotton end of the swab back into the water in the tube and break off the wooded end so that you can do up the lid.

Put the tube in the clean sample bag.
Collect the broken wooden ends to be thrown in a sharps bin when you have finished taking all you swabs.
Repeat steps 6 and 7 with each swab to be taken.
If at any point you think you may have got something on your gloves (i.e. water) you **must** change them for a clean pair.
Throw away the swab remains in the sharps bin and all gloves and aprons **must** go in a clinical waste bin. If you have used a tray make sure it is thoroughly washed with soap and water.
Make sure that you wash your hands thoroughly with soap and water.
Fill in the form on the cubicle door to say that sampling has been carried out.
Send the completed form and samples to virology using the chute system.
Tell the person in charge that the screening has been completed.

Swab No.	Site	Area to be Swabbed
1	Floor under sink	4 inch^2/10cm^2
2	Bedside Lamp controls	10cm^2
3	Chair with arms	Both arms (where hands sit) ~4 inch^2/10cm^2 on each
4	Door handle into patient bathroom (cubicle side)	Entire handle
5	Telephone keypads	Entire keypad
6	Taps in Patient Bathroom	Entirety of both taps
7	Mattress top	4 inch^2/10cm^2
8	Bed/Cot frame under bed	4 inch^2/10cm^2
9	Trolley surface (in ante room if present)	4 inch^2/10cm^2
10	Side window sill (right hand side)	4 inch^2/10cm^2
11	Cubicle room exit door handle (cubicle side)	Entire handle
12	Corridor floor outside of cubicle/ante room entrance	4 inch^2/10cm^2

Contact Numbers

Infection Control: 5284
Virology Lab: 8507
Bacteriology lab (out of hours only): 5280/8661
Consultant Microbiologist: 7930/5237/8594

Appendix 4: Screening form

Enteric Viruses Environmental Screening Form (Noro/Adeno)

Cubicle Tested: _____ **Ward**: _____

Patients ID: _____ **Patient discharge date**: _____

Date of Cleaning: _____ **Level of Cleaning**: _____

Virus: _____

Date: **Time**:

Laboratory No.	Swab No.	Site Swabbed	Culture Results
	1	Floor under sink	
	2	Bedside Lamp controls	
	3	Chair with arms	
	4	Door handle into patient bathroom (cubicle side)	
	5	Telephone keypads	
	6	Taps in Patient Bathroom	
	7	Mattress top	
	8	Bed/Cot frame under bed	
	9	Trolley surface (in ante room if present)	
	10	Side window sill (right hand side)	
	11	Cubicle room exit door handle (cubicle side)	
	12	Corridor floor outside of cubicle/ante room entrance	

Please inspect the room prior to screening. If the room is visibly dirty do not screen and inform the infection control team on ext 5284/bleep 0640. Out of hours inform the PEC on duty. If any of the sites are not present select another site and list it on the form**.**
Always screen 12 sites.

If the room is being screened for Adenovirus (BMT patients):

- Any pillows should have been thrown away prior to the room being cleaned. Please indicate if this was the case _____

- The clinical waste bin should have been sent to MEDU prior to the room being cleaned.

Please indicate if this was the case _____
Name: _____ Signature: _____

Sites to be Included In Environmental Swabbing

Swab No.	Site	Area to be Swabbed
1	Floor under sink	10cm^2
2	Bedside Lamp controls	10cm^2
3	Chair with arms	Both arms (where hands sit) ~5cm^2 on each
4	Door handle into patient bathroom (cubicle side)	Entire handle
5	Telephone keypads	
6	Taps in Patient Bathroom	Entirety of both taps
7	Mattress top	
8	Bed/Cot frame under bed	
9	Trolley surface (in ante room if present)	10cm^2
10	Side window sill (right hand side)	4 inch^2/10cm^2
11	Cubicle room exit door handle (cubicle side)	Entire handle
12	Corridor floor outside of cubicle/ante room entrance	10cm^2

If any of the items on the above list are not there (i.e. mattress) or an anteroom is not present, please still take 12 swabs. Choose which extra places/items to swab from either the suggested list below, or areas which are visibly dirty. Make a note of where each extra sample was taken from on the **Enteric Viruses Environmental Screening Form** in the space provided.

Suggested Additional Areas

Angle poise lamps
Television
Monitoring equipment
Bed lockers

Contact Numbers

Infection Control: 5284
Virology Lab: 8507
Bacteriology lab (out of hours only): 5280/8661
Consultant Microbiologist: 7930/5237/8594

Appendix 5: Environmental air screening protocol for critical ventilation systems supplying clinical areas

Method for Environmental Air Screening of Critical Ventilated Clinical Areas

Introduction

Microbiological air sampling of mechanically ventilated clinical areas designated 'critical' is required at commissioning and regular validation verification, as stipulated in the Ventilation policy.
Each critically ventilated area will have a 'schedule' (currently under protection by Estates) describing the design and validation standards.
Non - HEPA filter supplied areas will usually be designed to 'Theatre standard' (</= 10 or less cfu/m3 if commissioned against HTM 03, </= 35 cfus if HTM 20:25), while those areas with HEPA filtration (protective isolation rooms, ward common areas, or HEPA filtered theatres) will be expected to provided standard theatre air plus additionally be free of detectable fungus.

Test selection and number of samples:

Routine theatre standard air testing - Routine air testing will be carried out with a validated air sampling device, (currently the Sampl'air air sampler is available to Infection Control), but other validated devices are suitable as long as they check minimum of $1m^3$.

One $1m^3$ air test will be performed per room eg operating theatre, preparation room, patient bedroom, room antechamber; multiple tests in large areas eg one per bed space in open HEPA filtered unit. The device will be located in centre of area where patient usually sited.

Additional fungal testing in HEPA filtered areas - Additional tests for bacterial and fungal growth will be performed by use of settle plates.

4 plates per operating theatre or room; two per smaller areas e.g. prep or antechamber.

Before testing ensure ventilation is to standard and area is clean
Do not test a commissioned or validated area unless Estates (or designated company) have confirmed the mechanical ventilation if performing to standard volume, flow and pressure regimen.

What you need:

Supplies required:

- Permanent marker pen (to write on agar plates)
- Gloves x 2
- Disposable overshoes x 2
- Disposable theatre cap x2
- Scrubs
- Surgical mask x 2
- Sterile/disposable gown x2
- Polythene specimen bag to put your samples in once taken
- Rubber bands (to band your plates together for safe transportation)
- Agar plates
- One blood agar plate for each air sample
- Four blood plates for each bedspace or theatre area sampled

- Two blood plates for each antechamber sampled
- Sterile filter heads for the air sampler
- Sampl'air Lite air sampler
- Tape (to tape up the room whilst settle plates are down)
- Notice (to say keep out sampling underway)
- **Environmental Screening of Mechanically Ventilated Rooms Form** (1 per area - to be completed during screening by the person carrying out the screening)
- Smoke testing equipment pack
- Tray/autoclave bag to hold equipment

What you do:

1) Charge Sampl'air lite overnight and ensure that filter heads have been autoclaved
2) Before agreeing to sample ventilation performance should be confirmed as working to specification with the measurement details sent to estates and to the IP&CT and a level 3 clean has been carried out (wait at least 60 minutes after cleaning before sampling to let the chlorine dry thoroughly)
3) Before sampling inform the microbiology lab that there will be environmental samples collected for processing
4) Collect equipment into a tray or autoclave bag and change into scrubs
5) When you arrive at the room ensure there is a cleaning sign off sheet on the door indicating that a level 3 clean has been carried out
6) Wash hands with soap and water (where available), alcohol if not
7) Don overshoes, cap, gown, mask and gloves in that order
8) Visually inspect the room for cleanliness and for breaches in the building fabric. Ensure all clinical equipment and fabric items such as curtains and pillows have been removed. If room has not been cleaned or there is anything that would affect sampling **DO NOT SAMPLE!** Check with estates and IP&CT team
9) Undertake smoke testing of the room to ensure the direction of air flow is as expected. If not as expected **DO NOT SAMPLE!** Check with estates and IP&CT team
   Break the smoke tube so that smoke is released being careful not to inhale
   Attach the bulb and press to produce smoke along the door frames, plug sockets and other sources of ingress/egress of air
10) Label blood agar plates using a permanent pen as written on the **Environmental Screening of Mechanically Ventilated Rooms Form**

a) Include date and time of sampling, room sampled, air sample, initials of person sampling

11) Take a labeled blood agar plate and fit it to the Sampl'air lite

a) Remove the protective cover from the top of the air sampler
b) Remove the lid of the agar plate
c) Clip the base of the agar plate into place on the top of the air sampler
d) Place the lid of the agar plate onto the opened sterile filter pack (see step 12)

12) Attach the sterile filter head to the Sampl'air Lite:

a) Open the sterile pack without touching the inside of the pack
b) Lift the filter from the pack by its sides without touching the top filter section
c) Attach the filter head onto the top of the Sampl'air Lite, covering the attached agar plate without touching it

13) Press the on button on the Sampl'air Lite this will bring up a screen saying SAMPLE

a) Press the green arrow button, this brings up a message saying 10Min
b) Press the right arrow button which changes the 10Min message to 1000L
c) Press the green arrow button again, this bring up a message saying START 01:00 (indicating the sampler will start with a 1 minute delay)

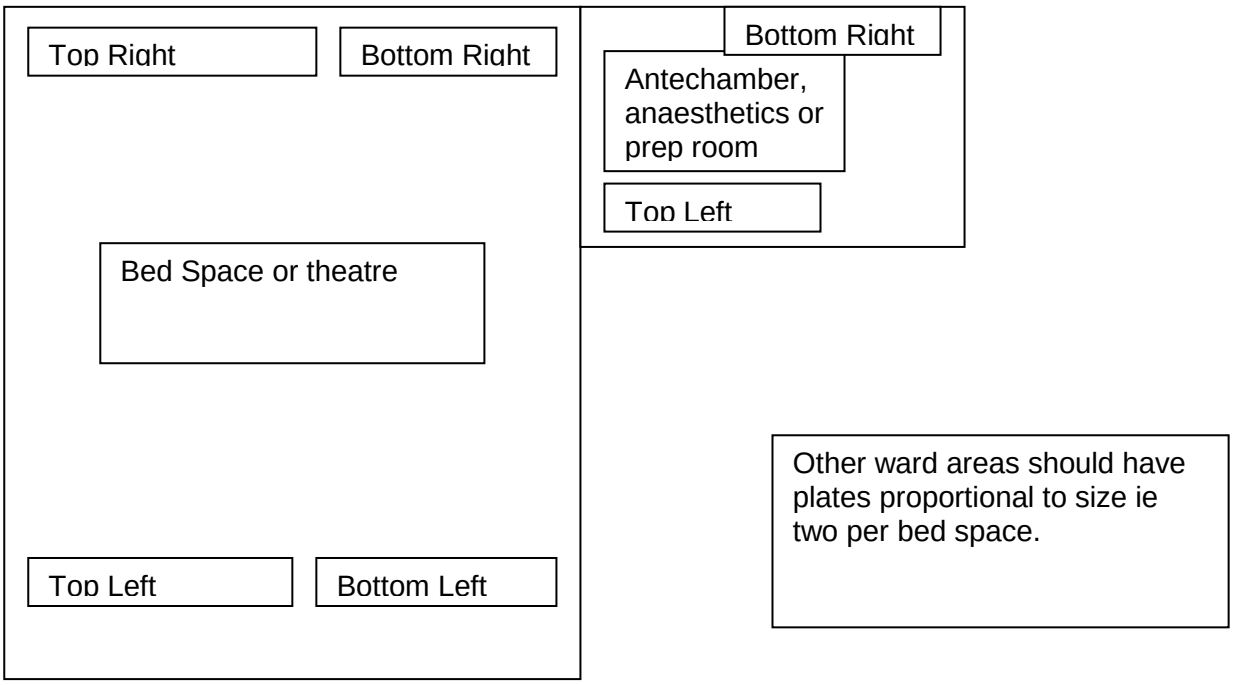
14) Enter the first room to sample (always start in the room with the cleanest air and work your way backwards e.g. theatre prep, main theatre, anaesthetic room. Place the sampler in the centre of the room, if possible at waist height and press the green arrow button to start the sampler. You will then have 1 minute to vacate the space before the sampling starts. (you

can also start the sampler with a remote from the doorway if you can get a good angle to the sampler)

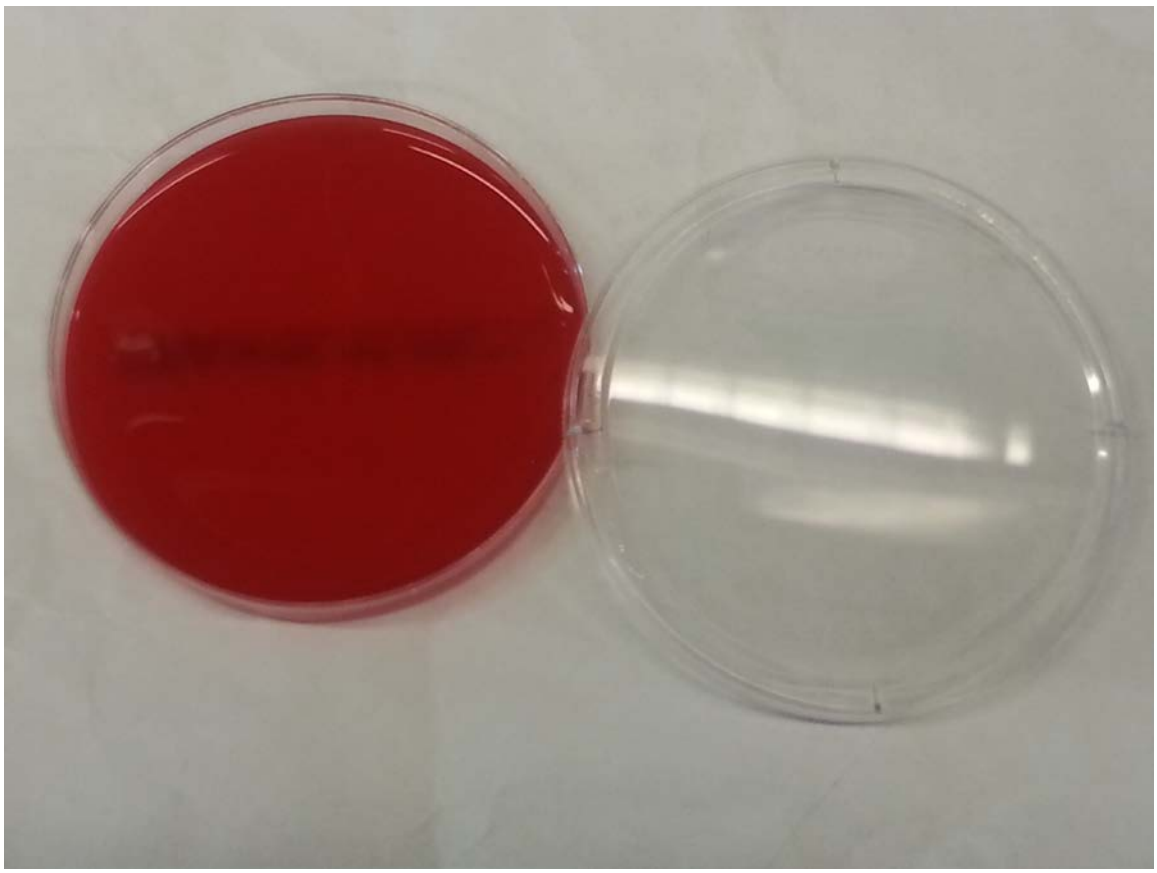
15) You must remove yourself from the space that you are sampling completely and make sure that all doors are closed. Time roughly ten minutes. When approaching the doorway to the sampling space you should be able to hear the sampler beeping if it has finished. If there is an observation window the sampler has a bar that fills and a counter that clocks up as sampling is completed
16) When the sampler has finished collect the sampler being sure not to touch the filter section on the top.
17) Remove the filter section by holding the sides, ensuring you do not touch the top section. Place the filter clean side down on the sterile wrapping (e.g. with the top filter section raised above the paper) and place the lid of the agar plate onto the agar plate before removing it and placing in a specimen bag
18) Repeat this process for each room to be sampled. Change filter heads if you ever suspect a contamination event might have occurred or after every 10 air samples taken
19) When all rooms have had an air sample taken label up settle plates for each room. Settle plates should be labelled as per the Environmental Screening of Mechanically Ventilated Rooms Form

a) Labelling should include date and time of sampling, room sampled, position within the room and initials of person sampling

20) Plates should be positioned in the room as follow:



a. Plates should be positioned with the lid of the plate balanced against the edge of the agar plate itself (see picture below)



b. In a room with antechamber, settle plates should be placed in the room farthest from exit first, being careful not to contaminate them via touch or aerosolized droplets. Plates should then be placed in the antechamber before exit. In a theatre plats should be placed in prep room, then theatre.

21) When settle plates have been placed leave the sampling area and tape up the entrance to prevent others accessing the sampling area, put up notice saying Infection Control Sampling Underway. Use an elastic band to collect together air sampling plates and remove PPE

22) Give air sampling plates and form to bacteriology for processing (plates will be incubated at 37^{o}C for 48 hours and will be read for total viable counts at 24 and 48 hours)

23) Settle plates must be left for a minimum of two hours before collection. When collecting settle plates appropriate PPE must again be worn, remove tape and signage. Sign and date the Mitie form to say sampling has been undertaken.

24) Give settle plates to bacteriology for processing (plates will be incubated at 37^{o}C for 24 and room temperature for a further 48 hours. Plates will be read for total viable counts and fungal growth at 24, 48 and 72 hours.

25) Email the appropriate project team to say that sampling has been undertaken and with a date that the expected results are due.

26) When microbiology results are available phone the projects team to let them know whether the room has passed or failed and confirm by email (sent to the IP&CT, projects team and estates and facilities)

Appendix 6: Screening Form

Environmental Screening of critical ventilated clinical areas: Form

Area Tested: _____ **Date of Cleaning**: _____

Level of Cleaning: _____ **Smoke testing as expected (circle)**: ☒ ☑

Reason for screening: _____

Date: **Time**:

Laboratory No.	Sample Type (air or settle plate)	Site	Culture Results

Please inspect the room prior to screening. If the room is visibly dirty do not screen and inform the infection control team on ext 5284/bleep 0640. Out of hours inform the floor manager.

Name: _____ Signature:_____

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PLOS ONE

Healthcare Environments and Spatial Variability of Healthcare Associated Infection Risk: Cross-Sectional Surveys

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Abstract

Prevalence of healthcare associated infections remains high in patients in intensive care units (ICU), estimated at 23.4% in 2011. It is important to reduce the overall risk while minimizing the cost and disruption to service provision by targeted infection control interventions. The aim of this study was to develop a monitoring tool to analyze the spatial variability of bacteriological contamination within the healthcare environment to assist in the planning of interventions. Within three cross-sectional surveys, in two ICU wards, air and surface samples from different heights and locations were analyzed. Surface sampling was carried out with tryptic Soy Agar contact plates and Total Viable Counts (TVC) were calculated at 48hrs (incubation at 37°C). TVCs were analyzed using Poisson Generalized Additive Mixed Model for surface type analysis, and for spatial analysis. Through three cross-sectional survey, 370 samples were collected. Contamination varied from place-to-place, height-to-height, and by surface type. Hard-to-reach surfaces, such as bed wheels and floor area under beds, were generally more contaminated, but the height level at which maximal TVCs were found changed between cross-sectional surveys. Bedside locations and bed occupation were risk factors for contamination. Air sampling identified clusters of contamination around the nursing station and surface sampling identified contamination clusters at numerous bed locations. By investigating dynamic hospital wards, the methodology employed in this study will be useful to monitor contamination variability within the healthcare environment and should help to assist in the planning of interventions.

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Introduction

Notable progress has been made in the last 15 years in developing and implementing systems to reduce the risk of healthcare associated infections (HCAI) but with only a moderate reduction on the overall prevalence of HCAI in England from 8.2% (2006) to 6.4% in 2011 [1]. In the same period, HCAI has increased in patients in intensive care units (ICU) (23.4% in 2011). Some infections, such as *Staphylococcus sp.* and *Enterobacteriaceae* remain problematic within England representing 21.3% of the reported HCAI and 32.4% respectively [1]. Similar rates are seen worldwide e.g. Brazil (12.6% [2]), or Estonia (26% [2]) clearly demonstrating that HCAI rates are a continuing concern internationally [3,4,5].

The reasons why HCAI remain high in the face of universal infection control precautions may be because of the demanding environments required for patients with severe and complex pathologies, such as in ICUs and in facilities caring for high densities of immunocompromised patients. It is becoming increasingly apparent that the environment itself can be an important intermediary reservoir for potentially pathogenic

microbes [6]. Surfaces, ward design, hand washing, staff behaviours, and ward management all contribute to pathogen behaviour [7] and the risk of HCAI [8]. How best to monitor and manage these environments is, however, still controversial [9,10].

If the spatial variability of microbiological contamination can be effectively evaluated, it could facilitate targeted infection control interventions to reduce the role of the environment as an intermediary source of cross transmission. The objective of this study was to develop a methodological approach to assess the spatial and temporal variability of bacteriologic contamination, both distant to and at bedsides in an ICU setting. By investigating a dynamic hospital ward, such an approach aimed to identify area of high levels of consistent contamination.

Methods

Data Collection

The screening procedure included 24 different sampling locations (five samples at each location), 8 in a four bedded medical intensive care unit (MITU) and 16 in a nine bedded surgical intensive care unit (SITU) (Figure 1) at the University College London Hospital (UCLH). Ward sampling was carried out with Tryptic Soy Agar (TSA) contact plates (5.5cm diameter i.e. 24cm^2) in order to provide a quantitative measure on a non-selective growth medium, which would enable growth of skin and environmental flora [11,12,13]. Surfaces were sampled at these different locations in each bed space and distant to bed (door pressure panels or handles, nursing station etc). Samples were always taken from the same site on all locations (e.g. the centre panels of doors, the centre of the floor space, the centre of bed rails, centre of the bottom left bed wheel, above the bed head). Sites varied slightly depending on what furniture occupied the bed space at the time. Surface sampling included surfaces at different heights, low (<0.6 m), medium (0.6 to 1.2 m, including high touch surfaces) and high (>1.2 m). Air samples were taken from MITU and SITU in each bed space at the back right hand corner, around the nurse's station and at access points onto the units. Air sampling was performed using a Sampl'air lite (Aes Chemunex), sampling 1m^3 of air onto a blood agar plate [14]. Air sampling occurred at similar times to the surface sampling, separated in time by at least 1 hour to minimize user contamination. All plates were read and colony forming units (CFU) were counted per plate (5.5cm diameter, i.e. 24cm^2) giving Total Viable Counts (TVC) that was recorded at 48hrs after incubation at 37°C [11].

In order to evaluate changes through time, the screening procedure (surface and air sampling) was replicated through three cross-sectional surveys at different times over a period of three months. The interval between each cross-sectional survey was of 1 month: it allowed comparison between the short stay SITU and the longer stay MITU as patient length of stay in SITU was less than a month whereas patient length of stay in MITU was over a month.

As many factors are involved in spatial distribution of bacterial contamination, the screening procedure also took into account some of these factors. Firstly, we recorded whether the bed was occupied or unoccupied and if the bedridden patient had been ascribed an infection control alert. Secondly, in each sampling location, surface types were selected for a different surface type and material at each location. Surface types included bed rails, floor, alcohol hand gel pump, bedside table, bed wheels, chair, clinical waste bin, storage trolley and unit top and shelf, top of computer, and surface material (porous and non-porous) are described in Table S1. Thirdly, samples were taken at the same time at each cross-sectional survey, without alteration to ventilation or cleaning regime, in order to ensure data comparability. Cleaning procedure was undertaken adhering to Department of Health Guidelines [15]: at UCLH, routine cleaning was undertaken using water and microfibre with no detergent, twice a day (8 am and 3 pm). Samples were taken three hours after routine morning cleaning in an attempt to standardize procedures. In addition, ITU cleaning does take place throughout the day as nurses actively wipe down surfaces with alcohol within bed spaces. The area studied had windows that did not open and air conditioning.

Statistical analysis

Common specifications. In order to compare the different risk factors (type of surfaces, furniture, bedside, bed occupancy, height level and locations) involved in HCAI, TVC was analyzed using Generalized Additive Mixed Model (GAMM) [16,17]. This regressive approach was allowed to model the counts of micro-organisms growing on TSA plates, with a Poisson distribution model (using the log canonical link), adjusted on risk factors. The model selection was based on analysis of covariance for nested models and the Un-Biased Risk Estimator (UBRE) score. Diagnostic plots were examined to assess the quality of the model fit, according to Augustin et al. [18]. For each factor, incidence ratios (IRs) were calculated as the exponential of retrieved parameter estimates, comparing each class to the reference class. With an incidence ratio significantly higher than 1, a surface type was considered to be at risk of being more contaminated than the reference class, with a higher TVC, whatever the TVC of the reference class. A contrario, with an incidence ratio significantly lower than 1, a surface type was considered to have a lower TVC than the reference class. The statistical analysis was performed using the software R 2.10.1 (The R Foundation for statistical computing, Vienna, Austria), and the mgcv 1.7-22 package developed by Simon Wood [19]. Maps were performed using the geographic information system ArcGIS (Environmental Systems Research Institute, Redlands, California). All p-values were compared to the classical α-threshold of 0.05.

GAMM for surface types. Associations between bacterial counts and surface types were assessed using a GAMM model including the following risk factors: 'Surface types' and 'Ward' (MITU or SITU). The 'Surface types' variable had eleven classes, which were bed rails (reference class), floor, alcohol hand gel pump, bedside table, bed wheels, chair, clinical waste bin, storage trolley and unit top and shelf, top of computer. For the variable 'Ward', the reference class was MITU. The location according to beds and bed occupancy (non-bedside, unoccupied bedside, occupied bedside) and the cross-sectional survey date were modelled as random effect. In

Figure 1. Map of the studied units. The red crosses represent the sampling locations. MITU: medical intensive care unit; SITU: surgical intensive care unit; b: bed; w: clinical waste bin; n: nurse's station.
doi: 10.1371/journal.pone.0076249.g001

addition, a comparison of TVC between porous and non-porous materials and between different materials was provided by using Kruskall-Wallis ranking test.

Spatial GAMM. Spatial analysis was performed to assess the spatial variability of micro-organisms in the air and on surfaces using GAMM. Because numerous surface samples were taken at each location, mean TVCs were used. The risk factors, included in the regression model were: 'Bedside' including occupancy of beds (non bedside, unoccupied bedside, occupied bedside), 'Ward' (MITU and SITU), 'Height level' (<0.6m, [0.6m-1.2m], >1.2m) and locations. The reference classes were respectively non bedside, MITU, height level <0.6m. The locations of each sample were referenced using Cartesian coordinates, which where modelled using thin plate splines [20]. The selected models were also used for mapping the TVCs from the surface environment and from the air environment, using gridded coordinates. Bedside was specified according to the distance to a bed, and the occupancy was specified according to the current occupancy for each cross-sectional survey.

Results

TVCs were obtained for a total of 370 samples, i.e. between 120 and 130 samples for each cross-sectional survey. During the first and second cross-sectional surveys, all of the bed spaces on MITU (100%) and 5 of the 9 beds on SITU (55,6%) were occupied. During the third cross-sectional survey, 3 of the

Table 1. Total viable counts (TVC) per 24cm^2 plates (5.5cm diameter).

Care unit	Height level	First cross-sectional survey	Second cross-sectional survey	Third cross-sectional survey
SITU	Level 0	182 (n=15)	107 (n=16)	350 (n=16)
		(40-350)	(17-350)	(18-350)
	Level 1	71.2 (n=33)	34.8 (n=36)	64.5 (n=36)
		(2-269)	(1.5-350)	(3-350)
	Level 2	79.2 (n=13)	29.5 (n=15)	14.5 (n=15)
		(5-350)	(2.5-197)	(1-350)
	Air	98.5 (n=12)	60 (n=11)	164 (n=11)
		(60-167)	(27-158)	(39-419)
MITU	Level 0	150.3 (n=7)	350 (n=9)	205 (n=9)
		(6-623)	(2-350)	(4-350)
	Level 1	34.4 (n=24)	28.7 (n=25)	55.4 (n=25)
		(12.3-238.3)	(2-126)	(9.7-350)
	Level 2	200 (n=5)	27 (n=5)	350 (n=5)
		(27-350)	(6-350)	(22-350)
	Air	103 (n=7)	88 (n=7)	182 (n=7)
		(99-217)	(50-167)	(51-213)

Median, sample size (n) and (min, max), are presented for each cross-sectional survey, at each height level and care unit.

Level 0: <0.6m; Level 1: [0.6m-1.2m]; Level 2: >1.2m

SITU: surgical intense care unit MITU: medical intensive care unit

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Table 2. Spatial analysis of Air samples.

Cross-sectional surveys (% explained deviance -n§)	Cofactors		IR [CI95%]	p-value
First cross-sectional survey				
(81.5% - n=19)	Bedside	Non Bedside*	1	-
		Non occupied Bedside	0.74[0.58;0.93]	0.007**
		Occupied Bedside	1.11[0.91;1.35]	0.26
	Ward	MITU*	1	-
		SITU	0.33[0.2;0.57]	<0.001**
	Spatial location***		-	<0.001**
Second cross-sectional survey				
(85.7% - n=19)	Bedside	Non Bedside*	1	-
		Non occupied Bedside	0.71 [0.52;0.97]	0.02**
		Occupied Bedside	0.6[0.47;0.76]	<0.001**
	Ward	MITU*	1	-
		SITU	14.34 [8.08;25.44]	<0.001**
	Spatial location***		-	<0.001**
Third cross-sectional survey				
(49.6% - n=19)	Bedside	Non Bedside*	1	-
		Non occupied Bedside	1.92 [1.54;2.39]	<0.001**
		Occupied Bedside	1.96 [1.66; 2.31]	<0.001**
	Ward	MITU*	1	-
		SITU	0.07 [0.04;0.11]	<0.001**
	Spatial location***		-	<0.001**

Risk factors were assessed each day by using Generalized Additive Mixed Model, adjusted on Bedside (occupied or not), Ward and location.

§. n: number of locations

*. reference class

**. p<0.05

***. spatial location was modelled by thin plate splines not providing unique IR.

IR: incidence ratio SITU: surgical intensive care unit MITU: medical intensive care unit

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4 beds on MITU (75%) and 6 of the 9 beds on SITU (66.7%) were occupied. Organisms were identified to genus level, and the predominant genus identified was *Staphylococcus*.

Crude counts are presented in Table 1 and Incidence Ratios in Tables 2, 3, 4. The lower height level (<0.6m) was consistently the most contaminated within the SITU (median TVCs between the first, second and third cross-sectional surveys were 182, 107 and 350 per 24cm^2 respectively). In contrast the pattern of contamination in MITU was more variable between cross-sectional surveys. For example the lower height level (<0.6m) was most contaminated for the second cross-sectional survey (median TVC 350 per 24cm^2), but with the highest level (>1.2m) most contaminated for the first and third cross-sectional surveys (median TVCs 200 and 350 per 24cm^2 respectively). Mid-level surfaces sampled (between 0.6 and 1.2m) did however demonstrate consistently lower levels of contamination during all the three cross-sectional surveys (median TVCs 34.4, 28.7, 55.4 per 24cm^2 respectively).

To enable the impact of location and bed occupancy on contamination to be assessed, TVCs were analysed in a statistical model as described in the methods. As Tables 2-4 demonstrate, there was a highly significant variation in contamination throughout the units analysed. When TVCs were adjusted for ward (SITU or MITU), bed occupancy and sample location, mid and high-level surface samples were significantly less contaminated than samples taken from locations under 0.6 m (Incidence Ratios -IR- respectively at 0.39, 95% Confidence interval [0.37; 0.42], and 0.59 [0.56; 0.64] (first cross-sectional survey), 0.39 [0.37; 0.42] and 0.31 [0.29; 0.33] (second cross-sectional survey) and 0.47 [0.44; 0.49] and 0.6 [0.57; 0.64] (third cross-sectional survey)).

Table 3. Spatial analysis of Surface samples.

Cross-sectional surveys	Cofactors		IR [CI95%]	p-value
(% explained deviance -n§)				
First Cross-sectional survey				
(38% - n=49)	Bedside	Non Bedside*	1	-
		Non occupied Bedside	0.78 [0.67;0.89]	0.0002**
		Occupied Bedside	1.72 [1.54;1.91]	<0.001**
	Ward	MITU*	1	-
		SITU	0.77[0.59;1]	0.05**
	Height	Level 0 <0.6m	1	-
		Level 1 [0.6-1.2m]	0.39 [0.37;0.42]	<0.001**
		Level 3 >1.2m	0.59 [0.56;0.64]	<0.001**
	Spatial location***		-	<0.001**
Second cross-sectional survey				
(56% - n=57)	Bedside	Non Bedside*	1	-
		Non occupied Bedside	6.96 [5.54;8.73]	<0.001**
		Occupied Bedside	3.14 [2.84;3.48]	<0.001**
	Ward	MITU*	1	-
		SITU	0.18 [0.13;0.24]	<0.001**
	Height	Level 0 <0.6m *	1	-
		Level 1 [0.6-1.2m]	0.39 [0.37;0.42]	<0.001**
		Level 3 >1.2m	0.31 [0.29;0.33]	<0.001**
	Spatial location***		-	<0.001**
Third cross-sectional survey				
(27% - n=57)	Bedside	Non Bedside*	1	-
		Non occupied Bedside	2.24 [2.04;2.46]	<0.001**
		Occupied Bedside	1.5 [1.39;1.61]	<0.001**
	Ward	MITU*	1	-
		SITU	0.09 [0.07;0.12]	<0.001**
	Height	Level 0 <0.6m *	1	-
		Level 1 [0.6-1.2m]	0.47 [0.44;0.49]	<0.001**
		Level 3 >1.2m	0.6 [0.57;0.64]	<0.001**
	Spatial location***		-	<0.001**

Risk factors were assessed each day by using Generalized Additive Mixed Model, adjusted on Bedside (occupied or not), Ward, height and location.

§. n: number of locations

*. reference class

**. p<0.05

***. spatial location was modelled by thin plate splines not providing unique IR.

IR: incidence ratio SITU: surgical intensive care unit MITU: medical intensive care unit

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Table 4. Effects on bacterial counts for the different surface types sampled.

Cofactors		IR [CI95%]	p-value
Surface types	Bed rails*	1	-
	Floor	1.18 [0.76;1.83]	0.46
	Alcohol hand gel pump	0.27 [0.1;0.79]	0.02**
	Bed side table	0.087 [0.01;0.74]	0.03**
	Bed wheels	1.97 [1.21;3.21]	0.01**
	Chair (seat)	0.45 [0.09;2.24]	0.32
	Clinical waste bin	0.61 [0.29;1.28]	0.19
	Storage trolley	0.41 [0.18;0.91]	0.03**
	Storage unit - shelf	0.62 [0.21;1.84]	0.39
	Storage unit - top	0.48 [0.25;0.89]	0.02**
	Top of computer	1.06 [0.61;1.84]	0.83
Ward	MITU*	1	-
	SITU	0.89 [0.64;1.24]	0.49

The adjusted incidence ratios (IR) are presented with their 95% confidence intervals.

*. reference class

**. p<0.05

SITU: surgical intensive care unit MITU: medical intensive care unit

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Levels of contamination on the surface types studied are displayed in Table 4. Contamination is presented as adjusted incidence ratios (IR) related to TVC on the bed rails (reference class). Bed wheels, bedside table, storage trolley, alcohol hand gel pump, and top of the storage unit, were all more contaminated than bed rails. Bed wheels were most contaminated with a mean TVC 1.97 times higher than on the bed rail (95% Confidence Interval 95%CI [1.12; 3.21]). Alcohol hand gel (wall mounted) and alcohol hand gel pump (patient bed side), (IR=0.27 [0.1; 0.79]), bedside table (IR=0.087 [0.01; 0.74]), storage trolley (IR=0.41 [0.18; 0.91]) and the top of the storage unit (IR=0.62 [0.25; 0.89]) were all less contaminated than bed rails. The comparison of TVC between porous and non-porous materials and between different materials showed no significant differences (p=0.53 and p=0.198, respectively).

Surfaces located at occupied bedsides were always more contaminated than surfaces located away from bed spaces (IR at 1.72, [1.54; 1.91] -first cross-sectional survey-, 3.14 [2.84; 3.48] -second cross-sectional survey- and 1.5 [1.39; 1.61] -third cross-sectional survey). Apart from the first cross-sectional survey, surfaces located at unoccupied bedsides were also more contaminated (IR at 0.78, [0.87; 0.89] -first cross-sectional survey-, 6.96 [5.54; 8.73] -second cross-sectional survey- and 2.24 [2.04; 2.46] -third cross-sectional survey). Surfaces sampled at SITU were always significantly less contaminated than surfaces sampled at MITU (IR at 0.77 [0.59; 0.99] -first cross-sectional survey-, 0.18 [0.13; 0.24] -second cross-sectional survey-, and 0.09 [0.07; 0.12] - third cross-sectional survey).

Air contamination was variable, showing less contamination at unoccupied bedsides during the first cross-sectional survey, a cluster around the nurse's station during the second cross-sectional survey, and more contamination at bedsides during the third cross-sectional survey (air samples IRs at unoccupied and occupied bedsides were 0.74 [0.58; 0.93] and 1.11 [0.91;

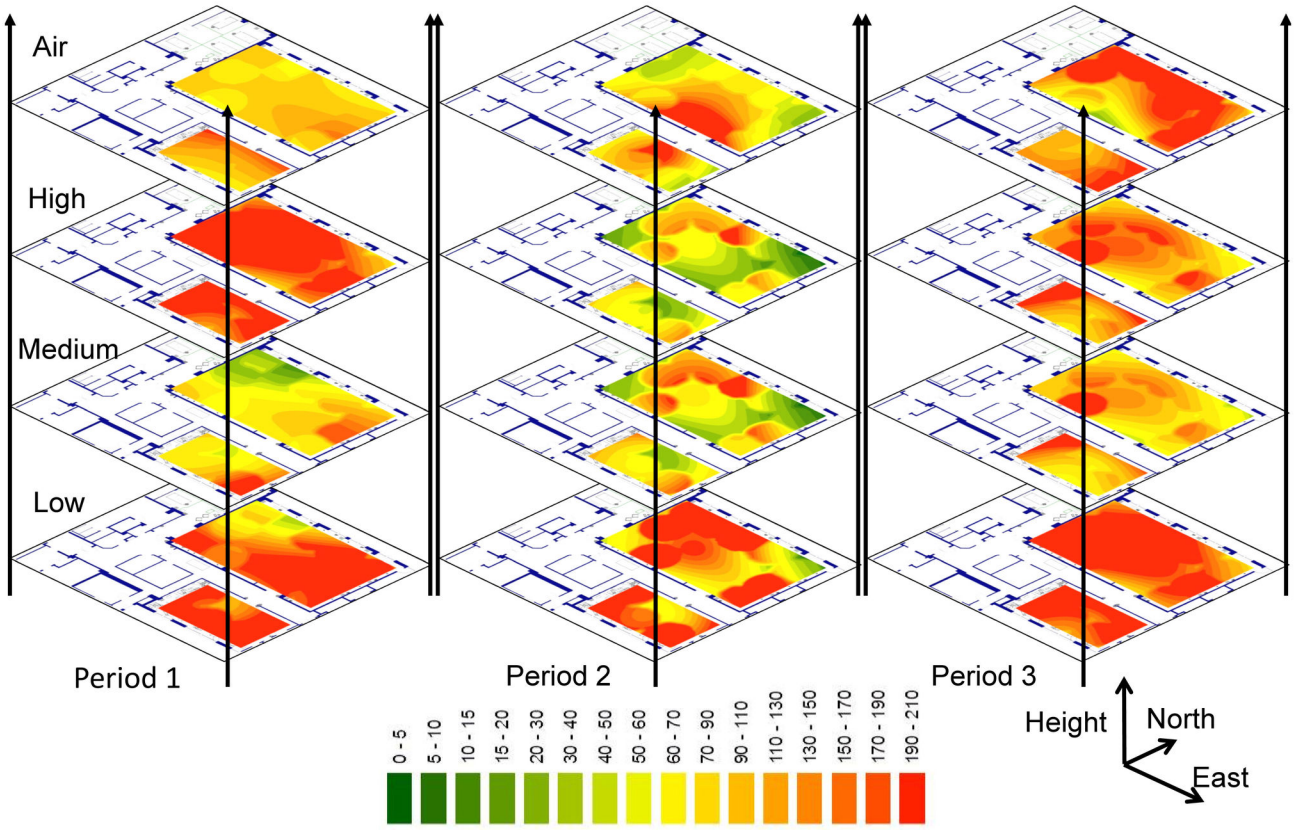

Figure 2. Estimation of the counts of micro-organisms. Results were adjusted on bedside, bed occupancy, height level (for surface analysis), Ward and location. Total Viable Count (TVC) estimations for the three cross-sectional surveys at the different height levels including air sampling are presented at each location. The coloured scale showed the values of TVC.

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1.35] respectively -first cross-sectional survey-, 0.71 [0.52; 0.97] and 0.6 [0.47; 0.76] respectively -second cross-sectional survey-, and 1.92 [1.54; 2.39] and 1.96[1.66; 2.31] respectively -third cross-sectional survey). Figure 2 shows clusters of contamination predominantly around bed locations but, during the second cross-sectional survey, in the air around the nurse's station.

The regression models developed for this study, which utilise TVC data, location, and occupancy, were found to be accurate at the first and second cross-sectional surveys (with percentages of explained deviance at 81,5 and 85,7% respectively). However these models were less accurate for the third cross-sectional survey. Sample location remained a significant factor in predicting colony counts ($p<0.0001$) for all cross-sectional surveys.

Discussion

In this study we have analyzed the environmental variability of micro-organisms within an ICU healthcare environment, by measuring TVCs on surfaces and in air. We aimed to establish an approach to facilitate monitoring and analysis of microbial contamination, which could be applied to healthcare settings, even if our approach did not evaluate precisely all the factors involved in the contamination variability (such as healthcare worker behaviour, patient status, modification of air-conditioning, people-traffic). The results showed that, for this particular environment, while contamination relationships were complex, some patterns emerged that could be modelled and used to estimate the distribution of microbial contamination. In this particular setting hospital design per se could not be the sole determinant of contamination. Staff behaviours, cleaning procedures and the nature and severity of a patient's condition may also have been important contributors to levels of bacteria. Such factors are amenable to mitigation by changes in ward layout to influence staff behaviour, improving accessibility to cleaning staff and by changes in healthcare components design such as use of easy clean surfaces.

Occupation of bed spaces and illness severity appeared to be consistent predictors of contamination. Air and surface TVCs were generally higher in MITU where patients are usually long stay and require care, such as feeding, in which there is substantial interaction with their bedside environment and particularly with relatives. These long-stay patients have more of their own possessions within the bed space and visitation is encouraged, tending to be regular and prolonged. SITU

patients are usually short-stay, ventilated and require high intensity support from staff. Visitation is limited to 2 people per bedside and is only allowed during certain hours. Visitors, due to the severity of illness on this ward, have limited interaction with patients and the bedside environment. Prolonged stay in ICU has been shown to increase the risk of acquiring an HCAI [21].

Colony counts in air and on surfaces varied between locations, height, bedside location and bed occupancy. There were differences between air and surface samples, indicating that the source of microbes may differ. Air sampling provides both a measure of transient aero-contamination and a snap shot of more widespread microbial levels. Surface sampling is affected by aero-contamination, as some particles will eventually settle on surfaces. King et al. [22] showed that bioaerosols can be deposited across a room at different distances from a source, due to air movement, which can be modified by furniture and people behaviour. Organisms that have settled may then be transferred to other sites by touch, and by air eddy currents generated by human traffic. Surface samples are highly affected by human behaviours within the ward environment, and particularly by touch. Our results show that the air and surfaces within bed spaces were consistently highly contaminated. In contrast, the aero-contamination at the SITU nurse's station during the second cross-sectional survey, occurred in the context of low surface TVCs. We also observed surfaces, which were heavily contaminated in areas of low aero-contamination. Surfaces within the middle height range were generally less contaminated. Even though the cleaning regime wasn't comprehensively assessed, cleaning was probably also an important factor in determining microbial levels.

Our results, in combination with other studies [6,23,24], support continuous environmental monitoring and not only in response to outbreaks. Continuous monitoring will permit the establishment of baseline data for units that can be used to target interventions. Sampling will also permit the identification of surfaces that are linked with higher levels of contamination where a design solution may be sought. It is important not only to evaluate the hotspots within a ward, such as bed spaces and nurses stations, but also to evaluate more globally what the HCAI risks are of a 'functional unit'. Such data can be used to inform benchmarking as a means of evaluating cleaning regimes [25,26,27]. Numerous studies meticulously describe surface cleanliness as these relate different cleaning procedures, including detergent use, design, behaviour [12,28,29,30,31], and production and distribution of aerosols [13,32,33]. Our work did not evaluate cleaning procedures nor the factors involved in distribution of aerosols. ITU cleaning does take place throughout the day as nurses actively wipe down surfaces with alcohol within bed spaces. Therefore we cannot state with certainty what impact cleaning would have had on our results. It was not our intention to ascertain the quality of cleaning but to measure 'real life' microbial levels within a hospital environment. Better cleaning protocols and systems would doubtless have great impact at those sites identified as high risk. Our work does however provide information that could promote easier, more effective cleaning for less effort by better design and use of space.

HCAI has not been eliminated despite increased interventions [15,34,35] and it is not known which component of the control process is most effective nor which to pursue further. Levels of micro-organisms within the environment may be related to healthcare design and/or processes. A scientific approach to studying the relationship between healthcare design and HCAI risk has been problematic due to the multitude of factors that affect a patient's interaction with both people and environment. Healthcare environments, hospital design and healthcare behaviours contribute to the risk of HCAI, in addition to the health status of each patient. These components include numerous variables including cleaning regimes, ventilation, bed locations and occupancy, windows and doors, material, staff and patient movements and height level. The complex interactions that do and can occur within the healthcare setting should be borne in mind when monitoring contamination. In this study we assessed the spatial variability of contamination, using a monitoring approach based on surface and air sampling at different location, height and time. Even when the main covariates were taken into account (distance to bed, bed-occupancy, care unit, location, height level, surface type), spatial variability still remained. This indicates that other cofactors were influencing contamination. This approach however can be used to assess the spatial variability of contamination over the working day, or before and after a specific intervention or event (e.g. an intervention in the ventilation system or a major contamination). By identifying areas of high levels of consistent contamination, the methodology employed in this study will be useful to monitor contamination variability within the healthcare environment and should help to assist in the planning of interventions.

Supporting Information

Table S1. Sampled surface: type of material and porosity.
(DOCX)

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Author Contributions

Conceived and designed the experiments: NK ECG SG JCH. Performed the experiments: ECG ND. Analyzed the data: JG SG. Contributed reagents/materials/analysis tools: JG ECG VG ND SG JCH NK. Wrote the manuscript: JG ECG NK. Interpreted the results: JG ECG SG NK. Supervised all stages of the study: NK.

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Hospital Door Handle Design and Their Contamination with Bacteria: A Real Life Observational Study. Are We Pulling against Closed Doors?

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Abstract

Objective: To determine whether microbial contamination of door handles in two busy intensive care units and one high dependency unit was related to their design, location, and usage.

Design: Observational study of the number of viable bacteria on existing door handles of different design at defined entry/exit points with simultaneous data collection of who used these doors and how often.

Setting: Two busy specialised intensive care units and one high dependency unit in a tertiary referral NHS neurological hospital.

Main outcome measures: Surface bacterial density on door handles with reference to design, location, and intensity of use.

Results: We found a significant correlation between the frequency of movements through a door and the degree to which it was contaminated ($p = <0.01$). We further found that the door's location, design and mode of use all influenced contamination. When compared to push plate designs, pull handles revealed on average a five fold higher level of contamination; lever handles, however, displayed the highest levels of bacterial contamination when adjusted for frequency of use. We also observed differences in contamination levels at doors between clinical areas, particularly between the operating theatres and one of the ICUs.

Conclusions: Door handles in busy, "real life" high acuity clinical environments were variably contaminated with bacteria, and the number of bacteria found related to design, location, mode and frequency of operation. Largely ignored issues of handle and environmental design can support or undermine strategies designed to limit avoidable pathogen transmission, especially in locations designed to define "thresholds" and impose physical barriers to pathogen transmission between clinical areas. Developing a multidisciplinary approach beyond traditional boundaries for purposes of infection control may release hitherto unappreciated options and beneficial outcomes for the control of at least some hospital acquired infections.

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Introduction

Healthcare Acquired Infections (HCAIs) continue to threaten the quality of patient care. The human and financial cost to individuals, healthcare organisations and society is considerable, approximating to £1.5bn per annum in the UK alone [1]. Governments and healthcare providers have intervened with a variety of measures, guidelines and regulations designed to control HCAIs [2]. Accordingly, much progress has been achieved with interventions relating to hand hygiene, strict infection control monitoring and cleaning regimes. Further progress is likely to follow from the identification of other potentially important contributors to HCAI, such as the design of the hospital itself and how this determines people's movement and behaviour within it [3]. There is increasing interest in the design of healthcare establishments, driven by issues of efficiency in both primary and secondary care facilities [4]. Hospital design is even more relevant for maintaining care quality in the face of space constraints, higher patient acuity, shorter lengths of inpatient stay and financial pressures. The operational challenges set by these agendas are substantial, and consideration should also be given to how these design variables might present, or prevent, opportunities for transmission of pathogenic organisms. Little data exist to inform how hospital design might impact on the potential for HCAI transmission [5]. With this in mind, built-environment experts, clinicians, microbiologists, and statisticians came together to

examine possible relationships between defined elements of hospital design, behaviour and environmental contamination.

Specifically, we sought to generate data relating to microbial contamination on door handles and how this might be related to factors relating to their design and use. We selected three high acuity environments for study as these are known to act as hotspots for HCAI transmission [6]. Finally, we suggest using relevant findings as evidence to generate novel strategies for infection control.

Methods

This was an observational study of a nine-bedded surgical intensive therapy Unit (SITU), a newly refurbished four-bedded medical intensive therapy unit (MITU) with a side room, and a four-bedded high dependency unit (HDU), all located in close proximity to each other on one floor of a busy urban hospital. We obtained waivers from our ethics Committees for the work as the study neither involved patient contact, nor was disruptive to patient care. Studies were carried out in a six month period between 2008 and 2009. We gathered information relating to ward layout, which way the doors into, out from, and within the units opened, how often they were used, by whom, the door handle design, and finally contamination density by potentially harmful microorganisms.

Figure 1 shows a plan of the units. Gates were defined as those thresholds across which individuals travel. Gate numbers were not consecutive, as some gates had no doors. Gates and doors (when present) were numbered using the same numbering system. Gate 1 identified the door connecting the HDU to the operating theatres zone; Gate 4 the main entrance to the SITU and HDU; Gate 5 the doorway to the main corridor separating SITU from MITU; Gate 6 the second entrance into the SITU; Gate 7 the main entrance to MITU, and Gate 10 one of the entrances to the only side room of MITU which opens directly into the main corridor. This side room could also be accessed through MITU.

Doors with push plates always had a fixed pull handle on the other side. The direction of push or pull varied from door to door. Gates 4 and 6 were furnished with a pull handle to enter the unit, whereas Gates 5 and 7 used a pull handle to leave the unit. We observed staff and visitors for at least three days for all six gates. The doors at gates 1 and 10 had lever handles while the other four (Gates 4, 5, 6 and 7) were double leaf doors designed to be pushed on one side and pulled on the other. Accordingly, the doors we studied had three different designs: flat rectangular metal plates on the push side of the double doors, longitudinal fixed door handle bars on the pull side of the double doors and a short horizontal lever handle on both sides of gates 1 and 10. These different designs are shown in figure 2.

Observing people's movement

We watched where people moved to and from and recorded our observations. We were careful to allow a "run-in" period of sham observation of three weeks in order to minimise any bias which the observation process itself might trigger. A single movement was defined as one individual crossing the threshold of any gate as defined above and the locations of which are illustrated in Figure 1. We monitored all movements through all gates in the three units on a daily basis from 10:30 to 13:00 and from 14:30 to 17:00. Individuals were assigned to one of several groups, namely staff local to the ward, other hospital staff, patients, and their visitors.

Microbiology

Microbiological surveillance data were collected at the same time as handle usage using Tryptone Soy Agar (TSA) Rodac impression plates with a surface area of 16.7 cm^2. We chose Rodac plates rather than a swabbing technique as it reduces variation relating to swab material type and swabbing technique. The plates were read after 48 hours' incubation for Total Viable Counts (TVCs). We sampled both door handles and door plates. These were cleaned thoroughly with 70% isopropyl alcohol wipes immediately before the start of the movement observations and

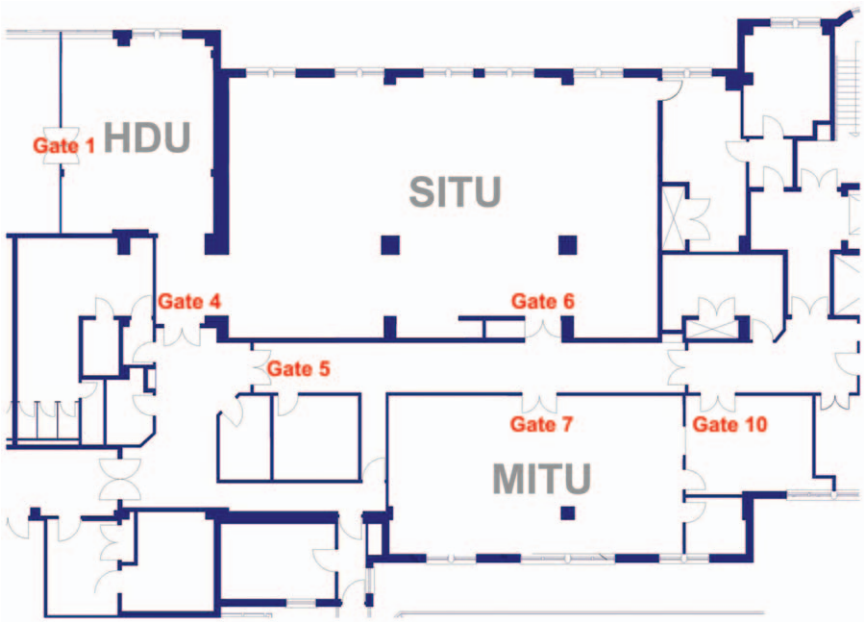

Figure 1. Plan of the units. Gates were defined as those thresholds across which individuals travel. Gate numbers were not consecutive, as some gates had no doors. Gates and doors (when present) were numbered using the same numbering system.
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Figure 2. Images of the door handle types.
doi:10.1371/journal.pone.0040171.g002

swabs taken to ensure the handles and plates were free from bacteria. We repeated the sampling at the same sites following a 150 minute observation period. This was found to be sufficient for observing substantial door usage whilst practical for continuous observation by a single worker. We developed consistent sampling techniques whereby we sampled a 100 cm^2 area at the centre of the door push plates, or a rotation of the Rodac impression plates around the vertical centre of the fixed vertical door handles. This was repeated twice a day to straddle both morning ward rounds and afternoon visits by relatives, and for three days.

Statistical analysis

Data were analysed using SPSS 16.0 for Windows. Initial data analysis demonstrated the data distribution to be non-Gaussian. Accordingly, we used Spearman's Rho Product Moment test to determine the relationship, if any, between movements through various doors and microbial densities. We used one way ANOVA for least significant difference analysis to establish the significance of any difference between means. After correction for extreme values, we used the Pearson Product Moment test parametric analysis. We expressed results as means ± standard deviation. Values were considered significant for p values of less than 0.05.

Results

Bed Occupancy and Movements

We observed ward traffic for periods of seven consecutive days, during which there were no to four patients present in the four bedded HDU; five to seven patients in the nine bedded SITU; and three to four patients in the four bedded MITU. We recorded up to 241 movements across a gate in 150 minutes at a time when only six out of nine beds were occupied. Staff based on that ward were responsible for 50% of all movements through this particular gate. Accordingly, various staff members had to exit and/or enter the unit about 120 times over a two and a half hour period. Table 1 displays the total number of movements according to category of building user over a seven day observation period. These data demonstrate large variations of traffic across doorways, which were related to location and time, but not direction. Ward and hospital staff generated the majority of these events. Movements through the main entrances to the ITUs (Gates number 4 and 5), constituted almost 47% of all movements.

Door Handle Design, Movement density and Microbial Growth

Microbial growth from Gate 6 was on many occasions either confluent, or too numerous to count, as was one sample from Gate 5. There was little effect of sample timing on TVCs apart from Gate 6, where the afternoon samples were consistently found to be greater than 300 or were confluent.

Figure 3 shows the considerable range of average TVCs retrieved from both sides of each door. We occasionally detected confluent or near confluent bacterial growth on door handles in the context of low levels of traffic (Gates 1 and 10). These exceptions can only be explained by less frequent contact with highly contaminated hands. When these heavily contaminated samples were excluded, a significant correlation between movement density and TVCs emerged (<0.01). Low traffic density was associated with low TVCs for Gates 1 and 10 and the more heavily used doors at Gates 4, 5, 6 and 7 were more contaminated. Further analysis of the pattern of contamination in the more heavily used doors indicated that other factors were contributing to microbial contamination.

Traffic density heading either in or out of the doors was balanced and was not influenced by the door handle design. Analysis of individual and average TVCs for each type of door handle, however, revealed that bacterial load on pull handles was consistently higher than that on the push plates located on the other side of the door. This narrowly failed to reach statistical significance ($p = 0.053$). Further analysis relating to handle type revealed that lever handles had the highest ratio (6.38 TVCs/movement), followed by Pull handles (2.24 TVCs/movement), which were in turn nearly double that of the Push plates (1.20 TVCs/movement). Interestingly, the ratio of TVCs/movements on the lever handles located on the inside of the doors used to exit from the side room and HDU was much higher than the corresponding handle on the other side of the door (Table 2). The table also shows that pull handles had a higher ratio of TVC per movement than the push handles.

Discussion

We found a relationship between how often and how many people cross door thresholds and the number of bacteria deposited on door handles. This finding supports the requirement for hand hygiene whenever hospital thresholds are crossed [7]. These critical moments in potential microbial transmission are increas-

Table 1. Various Types of Users Passing Through Each Gate.

Door No	Ward Staff		Other Staff		Visitor		Patient		Total
	No.	%	No.	%	No.	%	No.	%	
1	41	66%	21	34%	0	0%	0	0%	62
4	381	50%	262	31%	146	18%	8	1%	797
5	249	36%	332	47%	109	16%	6	1%	696
6	296	51%	219	38%	58	10%	3	1%	576
7	580	57%	402	39%	37	4%	5	0%	1024
10	36	73%	13	27%	0	0%	0	0%	49

No. denotes the number of individuals moving through a gate. This is then expressed as a percentage broken down by their reason for being on the ward.
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ingly recognised as targets for high impact interventions. We found that much traffic arose from the need to access the sluice room, offices, rest rooms, and separate equipment and storage areas.

Our results indicate that door location had an impact on contamination. For example, the handle used to exit the HDU via Gate 1, to access the operating theatres, was far more contaminated than the handle used the other way when adjusted for frequency of movement. This may be an indicator of ward activity, hand hygiene, or handle design. As expected, we observed a consistently high level of hand hygiene in the operating theatres and this may be the reason for the low level of contamination on the handle used to enter the HDU. In contrast, staff entering the theatre from the HDU (''out'' handle) will likely have come into direct contact with high acuity patients in a less controlled environment and may have found it more difficult to maintain such high levels of hand hygiene compliance. This however may not be the full story. The average contamination per movement was highest at this gate and also at Gate 10, which connects a MITU side room with the corridor. This may relate to door handle design, as both gates were operated by lever handles.

Door handle design may also have contributed to the TVC/movement results for Gates 4,5 and 6. While the hand hygiene facilities were identical on both sides of these three gates, and the activity within the SITU would clearly be greater than outside the SITU, we always observed greater contamination on the ''in'' pull handle than the ''out'' push plate. Accepting the variables relating to activity, as discussed above, it is plausible that pull handles ''capture' more organisms than push plates. We suggest that this relates to ''skin to metal ratio'' as illustrated in Figure 4. It would seem logical that door handles that either ''capture'' a larger proportion of whatever hand contamination is present, concentrate what is captured onto a smaller surface area or both, is a reasonable explanation for our data. The pull handles require grabbing at some point along the vertical bar of the fixed handle, focusing the contact point on the handle and thus reducing the area and concentrating contamination to a small surface. The potential for concentrating microorganisms was even greater on lever handles, where the length of the handle bar is less than one quarter of that of the vertical fixed handle, thereby acting as a smaller lens focussing the microorganisms left behind on contact. Whilst a logical explanation for our findings, we cannot dismiss the possibility that door handle design had no influence on contamination and that sole determinants of contamination were ward activity and hand hygiene.

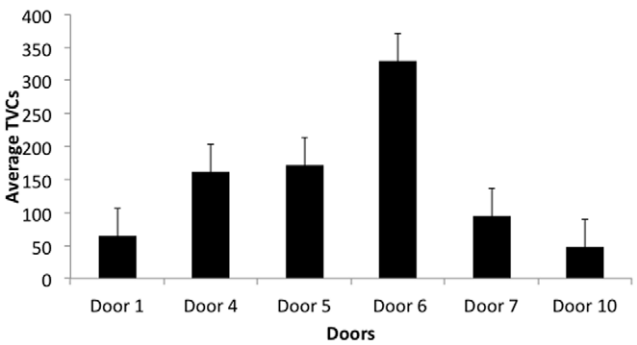

Figure 3. Average Total Viable Counts +/−1 Standard deviation retrieved from both sides of each door.
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The design of the healthcare environment is increasing recognised for its impact on health care quality and outcomes [8–9]. To our knowledge there is no coordinated study of how people's behaviour is influenced by the built environment and how this relates to microbial spread [10]. We show here that a multidisciplinary approach both reveals the true complexity of microbial spread and the challenge this sets for effective strategies for its control. In the absence of a more 'intelligently designed' built environment, recent focus on the near patient space [7] and alcohol based gels has been of great benefit. The WHO recommends undertaking hand hygiene when entering the patient environment. However as staff compliance with hand hygiene is routinely less than 100% [11], introduction of microbes into bed spaces is still a risk. Accordingly, optimising ward design to limit the risk of contamination, is still of value.

Optimising ward design to limit microbial spread is not straightforward and will be determined by many factors such as the existing building if not a new build, limitations on space, and use. In the setting described in this manuscript, we observed that closer, more accessible storage and supply rooms would have resulted in less time spent fetching, carrying and performing mandated handwashing. Closer storage would likely have limited the opportunities for cross contamination and releasing time for direct patient care. In some settings, closer storage of some ward related items may facilitate contamination with patients' flora and this could be undesirable. Whatever the physical and financial constraints and activity demands, we would advocate an informed approach to ward design/modification, to at least consider the implications for the potential for microbial spread. Of particular importance is the area within and around the sluice. We noted high contamination levels on Gate 6, which controlled access to the sluice room. This study did not set out to identify the bacterial species recovered from the door handles. We cannot therefore

Table 2. Ratio of TVC/Movement for Each Type of Handle.

Gate No.	Going In	TVC/Movement in	Going out	TVC/Movement out	TVC/Movement
1	Handle	0.43	Handle	8.56	4.63
4	Pull	1.82	Push	0.49	1.18
5	Pull	2.63	Push	1.29	1.97
6	Pull	5.44	Push	0.99	3.27
7	Push	0.62	Pull	0.76	0.69
10	Handle	1.57	Handle	14.52	8.56

doi:10.1371/journal.pone.0040171.t002

state whether these organisms were skin commensals, such as coagulase negative staphylococci, transiently carried *S aureus*/MRSA, or faecal organisms such as *E coli*. If the latter were predominant, it would indicate that the high levels of contamination emanated from the sluice. The sluice room represents a potentially problematic area where a door is desirable to help limit the spread of faecal organisms while also providing surfaces, such as the handles, which could facilitate organism transmission.

There are very limited data on door handles and their potential for microbial transmission. In a study looking at surrogate markers of nosocomial pathogen transmission, door handles were highlighted as one site that rapidly became contaminated within the context of a neonatal intensive care setting [12]. A recent study has shown that it is possible to reduce bacteria on door handles provided they are regularly cleaned. Even with regular cleaning, bacteria were detected on more than 20% of handles [13].

Cleaning, both of hands and the environment, has been widely accepted as an important factor in curbing the spread of pathogens in hospitals [14]. Our data indicate that, while cleaning is important, it is not always practical, as in some cases a single touch by a contaminated hand was sufficient to result in a confluent plate. A potentially innovative approach to limiting environmental contamination is the use of spontaneously antimicrobial surfaces. Of these, copper-based microfibre cleaning systems [15] or copper furnishings look particularly promising, although the latter are expensive and still in need of regular cleaning [16].

The layout of the units, variably and constantly contaminated by the sick patients they contain, can therefore support or undermine policies designed to limit the spread of infection as well as enabling healthcare staff to work more effectively. The use of automatic doors or the elimination of doors altogether could be a solution to reducing the dissemination of microorganisms acquired from door handles, although should be weighed up against the potential for airborne transmission and the importance of visually defined thresholds, themselves prompting hand hygiene. Our findings offer a possible explanation for Cepeda *et al's* surprising findings that side room use in the context of ICUs failed to reduce the rate of MRSA cross-infection [17]. This, however, is only one of a number of healthcare design features that could be considered

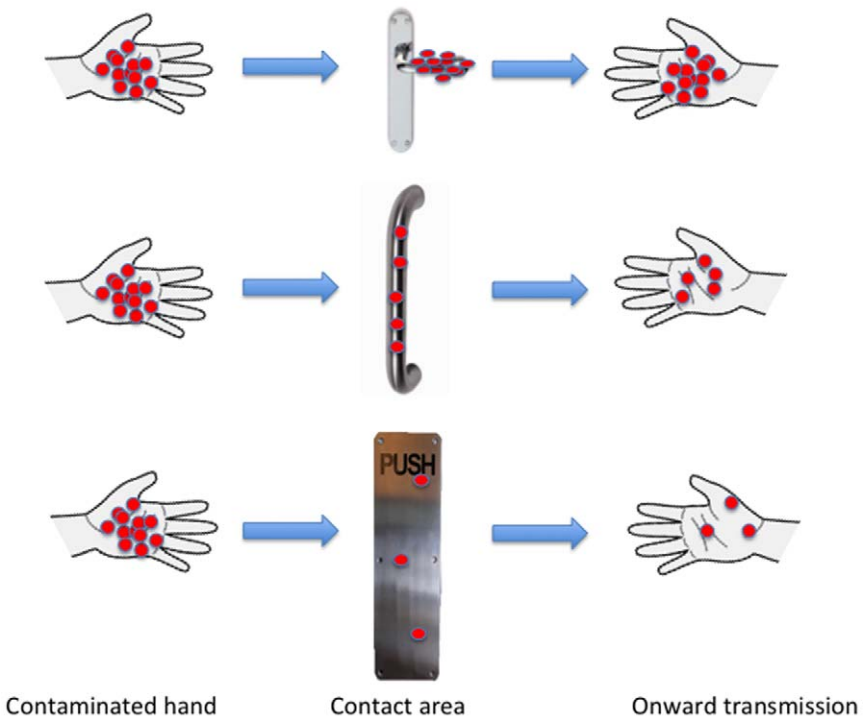

Figure 4. Transmission potential in relation to door handle type.
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to optimise effective delivery of care and control of healthcare associated infections.

Architects may not have the necessary information or knowledge available to inform optimal healthcare design as regards the spread of infection. Whilst door handle design may appear trivial at the design stage and largely ignored, it is one of many "trivial" design features that might silently undermine microbial transmission control. Novel door handles are being developed and may prove to be more 'resistant' to microbial contamination than existing designs. The multidisciplinary approach taken in this study could serve as a paradigm for future healthcare design. A network of architects, engineers, microbiologists, nurses doctors and hospital administrators working together at multiple stages of the design process could achieve those efficiencies seen in car and kitchen design and manufacturing. These synergies between providers of healthcare and those responsible for the buildings in which it is delivered would seem essential for better, evidence based and optimal healthcare building design.

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Author Contributions

Conceived and designed the experiments: VG NK HW EC-G. Performed the experiments: HW EC-G CK. Analyzed the data: HW CK EC-G VG NK. Contributed reagents/materials/analysis tools: HW CK EC-G VG NK. Wrote the paper: HW VG NK CG. Conceived the study and pulled together the multidisciplinary team: VG NK.

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Appendix 8.8

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

Use of UV-C radiation to disinfect non-critical patient care items: a laboratory assessment of the Nanoclave Cabinet

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Abstract

Background: The near-patient environment is often heavily contaminated, yet the decontamination of near-patient surfaces and equipment is often poor. The Nanoclave Cabinet produces large amounts of ultraviolet-C (UV-C) radiation (53 W/m^2) and is designed to rapidly disinfect individual items of clinical equipment. Controlled laboratory studies were conducted to assess its ability to eradicate a range of potential pathogens including *Clostridium difficile* spores and Adenovirus from different types of surface.

Methods: Each test surface was inoculated with known levels of vegetative bacteria (10^6 cfu/cm^2), *C. difficile* spores (10^2-10^6 cfu/cm^2) or Adenovirus (10^9 viral genomes), placed in the Nanoclave Cabinet and exposed for up to 6 minutes to the UV-C light source. Survival of bacterial contaminants was determined via conventional cultivation techniques. Degradation of viral DNA was determined via PCR. Results were compared to the number of colonies or level of DNA recovered from non-exposed control surfaces. Experiments were repeated to incorporate organic soils and to compare the efficacy of the Nanoclave Cabinet to that of antimicrobial wipes.

Results: After exposing 8 common non-critical patient care items to two 30-second UV-C irradiation cycles, bacterial numbers on 40 of 51 target sites were consistently reduced to below detectable levels (≥ 4.7 $\log_{10}$ reduction). Bacterial load was reduced but still persisted on other sites. Objects that proved difficult to disinfect using the Nanoclave Cabinet (e.g. blood pressure cuff) were also difficult to disinfect using antimicrobial wipes. The efficacy of the Nanoclave Cabinet was not affected by the presence of organic soils. *Clostridium difficile* spores were more resistant to UV-C irradiation than vegetative bacteria. However, two 60-second irradiation cycles were sufficient to reduce the number of surface-associated spores from 10^3 cfu/cm^2 to below detectable levels. A 3 $\log_{10}$ reduction in detectable Adenovirus DNA was achieved within 3 minutes; after 6 minutes, viral DNA was undetectable.

Conclusion: The results of this study suggest that the Nanoclave Cabinet can provide rapid and effective disinfection of some patient-related equipment. However, laboratory studies do not necessarily replicate 'in-use' conditions and further tests are required to assess the usability, acceptability and relative performance of the Nanoclave Cabinet when used *in situ*.

Keywords: Ultraviolet radiation, Surface disinfection, Nosocomial pathogens, Adenovirus

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Background

Important nosocomial pathogens such as methicillin-resistant *Staphylococcus aureus*, *Clostridium difficile* and vancomycin-resistant enterococci are often present on inanimate surfaces within the local environment of infected patients [1-3]. Many of these surfaces (e.g. blood-pressure cuffs, bed rails, bedside furniture) only come into contact with a patient's intact skin – a highly effective barrier against microbes. Consequently, such surfaces are considered "non-critical" and rather than being returned to a central sterilising services department for re-processing, can be decontaminated *in situ* [4,5].

Routine cleaning of the near-patient environment has been associated with a reduction in surface contamination [2]. However, cleaning of near-bedside equipment and furniture is not always adequate, especially if it is a nursing responsibility and they are busy [5,6]. Inadequate cleaning allows microbial contaminants to survive and persist on environmental surfaces and whilst non-critical surfaces pose little direct risk to patients [4], they can act as a source from which healthcare workers can contaminate their hands and may serve as vectors for cross-transmission.

Ultraviolet-C (UV-C) radiation has been used for many years to disinfect water and its bactericidal effects, due mainly to its inactivation of microbial DNA, have been well documented [7]. More recently, UV-C has been used to disinfect hospital rooms [8-10] and its ability to reduce the number of healthcare–associated pathogens within the near-patient environment has been demonstrated [9-11]. However, for microorganisms to be destroyed they must be directly exposed to UV-C irradiation; any surface not in the direct path of the UV-C rays will not be effectively disinfected [8].

The Nanoclave Cabinet (Nanoclave Technologies LLP, London, UK) produces large amounts of UV-C light. Its purpose is to rapidly disinfect clinical equipment, furniture and electronic devices. Inside the Cabinet are 48 UV-C lamps (32×30 W and 16×25 W) mounted, in banks of eight, to each of the six internal surfaces, including the door. Angled mirrored reflectors help minimise shadowing by directing and concentrating the UV-C rays onto the item to be disinfected. This six-sided emission of UV-C light means any item placed in the cabinet is subjected to a dosage of 53 W/m^2.

The aim of this study was to assess, under controlled laboratory conditions, the ability of the Nanoclave Cabinet to effectively disinfect a range of artificially contaminated non-critical patient care items.

Methods

The Nanoclave Cabinet

The Nanoclave Cabinet is made from stainless steel and can be manufactured in a range of different dimensions. The Cabinet supplied for use during this investigation measured 129 cm × 94 cm × 89 cm (l × w × h) and was mounted on a base which raised the height of the unit to 1.6 m (Figure 1). To ensure that all the UV-C lamps were working correctly, a device controller measured the power consumption of the lamps during operation. Any significant drop in power resulted in the failure and cessation of the cycle. The Nanoclave Cabinet also incorporates a data logging feature which, for additional safety, is independent from the device controller. Current meters monitor the current drawn by each bank of lamps and UV-C sensors monitor the actual UV-C output of the lamps. These data are collected onto an SD card and can be printed using a thermal printer.

Effectiveness of the Nanoclave Cabinet against a range of pathogenic bacteria

Test organisms

Testing involved a range of potential nosocomial pathogens: methicillin-sensitive *Staphylococcus aureus* (MSSA; NCTC 10788), methicillin-resistant *Staphylococcus aureus* (MRSA; EMRSA-15 variant B1 (environmental isolate)), *Enterococcus hirae* (NCTC 12367), vancomycin-resistant *Enterococcus faecalis* (VRE; clinical isolate), *Escherichia coli* (NCTC 10418), multi-resistant *Acinetobacter baumannii* (MRAB; OXA-23 clone 1 (clinical isolate)), extended spectrum beta-lactamase (ESBL) producing *Klebsiella pneumoniae* (environmental isolate) and *Pseudomonas aeruginosa* (NCTC 6749).

Clinical isolates were taken from clinical specimens and stored in the microbiology laboratory. Only the isolated microorganisms and not the specimens (e.g. urine; sputum; faecal samples) were stored. Clinical isolates were fully anonymised and testing was only to assess the effectiveness of the Nanoclave Cabinet. The organisms

Figure 1 The Nanoclave Cabinet.

were not tested to reveal any additional information and there was no way to link them to individual patients. Thus, ethics consideration was not deemed necessary by UCLH Research and Development.

Ability of the Nanoclave Cabinet to disinfect non-critical patient care items

Preparation of test items Eight items of near-bedside equipment of the type and in the condition of those likely to be found in the ward environment were included in the study; a blood pressure gauge, a patient call button, an infusion pump, a tympanic thermometer, an oximeter base unit, a computer keyboard (and mouse), a TV remote control and a blood pressure cuff. Each surface was marked with up to nine individual sample points.

Prior to each experiment, each test surface was cleaned using a (hand hot) damp microfibre cloth, left to air-dry under ambient conditions and disinfected using 70% alcohol spray. The efficacy of this cleaning protocol was assessed using agar contact plates and residual microbial numbers were consistently reduced to below detectable levels.

Exposure of test items to UV-C radiation A single colony of MRSA, VRE, MRAB or *Kleb pneumoniae* was aseptically transferred into 10 ml sterile nutrient broth (Oxoid, Basingstoke, UK). A stationary-phase culture (~10^8 cfu/ml) was obtained by incubating the bacteria at 37°C for 18 h. After incubation, the culture was transferred to a sterile universal container and centrifuged at 1500 × g for 10 min. The supernatant was discarded and the pellet re-suspended in 10 ml sterile ¼-strength Ringer's solution (an isotonic salt solution; Oxoid).

For each test item, 10 μl of bacterial suspension (containing approximately 10^6 cfu) was inoculated onto each sample point and, rather than being left as a droplet, spread over a 1 cm^2 test area. Immediately after inoculation, the test item was placed in the Nanoclave Cabinet on a stainless steel lattice rack and exposed for 30 sec to the UV-C light source.

Although the base of the item (i.e. the surface facing the rack) was exposed to UV-C light emitted from the base of the cabinet, any sample point in direct contact with the lattice bars remained protected from the rays. Thus, after irradiation, to ensure the entire surface area was exposed to a UV-C dose of at least 1,590 J/m^2 (53 W/m^2 × 30 sec exposure), the positioning of the object within the Cabinet was altered and the irradiation cycle repeated.

After exposure, a pre-moistened cotton-tipped swab was used to sample each sample point. Each swab was placed in 1 ml ¼-strength Ringer's solution and vortexed to release the bacteria. One hundred microlitres of the resulting suspension was plated onto a pre-poured blood agar plate (Oxoid) and incubated at 37°C for 24 hours.

Non-exposed control samples Test items were inoculated as previously described. Immediately after inoculation, each test area was sampled using a pre-moistened cotton-tipped swab. Each swab was placed in 9 ml ¼-strength Ringer's solution and vortexed to release the bacteria. The resulting suspension was diluted 100-fold and 100 μl of the diluted sample plated onto blood agar. Plates were incubated at 37°C for 24 hours.

Comparative performance of antimicrobial wipes

To compare the efficacy of the Nanoclave Cabinet with that of an antimicrobial wipe, three patient care items (blood pressure cuff, tympanic thermometer, patient call button) were inoculated with a representative organism (*Acinetobacter baumannii*). Selected test areas were cleaned 'poorly' (one wiping stroke), 'moderately well' (two wipes) or 'thoroughly' (four wipes) using an antimicrobial wipe (VWR International Disinfectant Wipes: active ingredients: peroxides, benzalkonium chloride; VWR International, Lutterworth, UK). Each test area was sampled with a pre-moistened swab which, prior to plating, was vortexed within 1 ml of neutralising solution (phosphate buffered saline incorporating 3% Tween 80 (w/v), 0.3% lecithin (w/v), 0.1% sodium thiosulphate (w/v)).

Effect of organic soiling on the efficacy of the Nanoclave Cabinet

A stationary-phase culture of MSSA, *E. hirae, E. coli* or *P. aeruginosa* was prepared as previously described. After centrifugation, cell pellets were re-suspended in either 0.03% bovine serum albumin (BSA; w/v) sterilized by membrane filtration or, to represent heavy soiling, 0.3% BSA (w/v) and 0.3% "packed" sheep erythrocytes (v/v), which were prepared as follows. Three millilitres of sterile defribinated sheep blood (TCS Biosciences Ltd, Buckingham, UK) was centrifuged at 800 × g for 10 minutes. The supernatant was discarded and the pellet re-suspended in a balanced salt solution. This process was repeated until the supernatant was colourless. The packed erythrocytes were then re-suspended and added to a sterile solution of BSA (3.0 g bovine albumin (fraction V), 0.1 g tryptone, 0.85 g sodium chloride, 97 ml distilled water). The resulting suspension was diluted 10-fold.

Sterile stainless steel discs (1 cm in diameter) were inoculated with 20 μl bacterial suspension (~ 10^6 cfu) and allowed to dry for 80 minutes at 30°C. Two discs were then attached to each surface of a plastic cube, placed in the Nanoclave Cabinet and exposed for 60 sec to the UV-C light source. Thus, each of 12 discs was positioned either vertically or horizontally and exposed to a UV-C dose of 3,180 J/m^2. After exposure, each disc

was aseptically transferred to 10 ml tryptone soya broth containing sterile glass beads and vortexed for 1 min. The resulting suspension was diluted 10-fold and 100 μl of the diluted sample plated onto tryptone soya agar. Control discs were inoculated and incubated as described but were cultured without having been exposed to UV-C. All plates were incubated at 37°C for 24 hours.

Analysis of results

For each test surface, the number of colonies recovered from each irradiated or wiped test area was subtracted from the number of colonies recovered from the corresponding control sample. The results were used to calculate the mean log reduction in microbial viability and, thus, the efficacy of the Nanoclave Cabinet or antimicrobial wipes. Data analysis was performed using Microsoft Excel 2007. Statistical significance was determined by use of t tests and was at a level of $P < 0.05$.

Effectiveness of the Nanoclave Cabinet against *Clostridium difficile* spores

Spore suspensions of *Clostridium difficile* were prepared as previously described [12] and stored in a 1:1 solution of alcohol (70%) and phosphate buffered saline.

A stainless steel sheet was cleaned using a (hand hot) damp microfibre cloth, left to air-dry under ambient conditions and disinfected using 70% alcohol spray. A spore suspension of *C. difficile* 027 (clinical isolate) was centrifuged at 1500 × g for 10 min and re-suspended in 10 ml sterile ¼-strength Ringer's solution. A 10 μl aliquot (containing approximately 10^6 cfu) was inoculated onto the steel surface and spread over a 1 cm^2 test area. Immediately after inoculation, the sheet was placed in the Nanoclave Cabinet and exposed to two 60 sec UV-C cycles. After exposure, the test surface was sampled using a pre-moistened cotton-tipped swab which was transferred to 1 ml ¼-strength Ringer's solution and vortexed to release the spores. One hundred microlitres of the resulting suspension was plated onto a pre-poured Brazier's agar plate (Oxoid) and incubated under anaerobic conditions at 37°C for 48 hours. Experiments comprised a minimum of three replicate samples and were repeated to incorporate lower inoculum levels and longer exposure times. The effect of organic soiling was investigated by re-suspending spores of *C. difficile* NCTC 11209 (ribotype 001) in 0.03% BSA and inoculating sterile stainless steel discs as described previously.

Effectiveness of the Nanoclave Cabinet against Adenovirus

A stainless steel sheet and a ceramic tile were cleaned and disinfected as previously described. Adenovirus species (serotype 31) was grown in a Vero cell line. A 10 μl aliquot (containing approximately 10^9 viral genomes) was inoculated onto the test surface and spread over a 5 cm^2 test area. After being allowed to air-dry (ambient conditions) for 2 h, the sheet (or tile) was placed in the Nanoclave Cabinet and exposed to two 30 sec UV-C cycles. After exposure, the test surface was sampled using a pre-moistened cotton-tipped swab which was transferred to 0.5 ml molecular grade water and vortexed to release the virus particles. Viral nucleic acid was extracted from 200 μl of the resulting suspension using a DNA Miniprep Kit (Qiagen, Crawley, UK) and eluted into 100 μl UV irradiated buffer. Ten microlitres of the extract was processed using a semi-quantitative Adenovirus real time polymerase chain reaction (PCR) [13]. All PCR's were run with a negative extraction, as well as negative and positive controls; the latter to monitor assay performance across runs. Experiments comprised four replicate samples and were repeated to incorporate longer exposure times.

Results

Ability of the Nanoclave Cabinet to disinfect non-critical patient care items

Effectiveness of the Nanoclave Cabinet against vegetative bacteria

Fifty-one individual sample sites associated with eight near-bedside items of clinical equipment and furniture were inoculated with MRSA, VRE, MRAB and *Klebsiella pneumoniae*. Loss in microbial viability varied depending on surface type (Table 1) but exposing 40 of the 51

Table 1 Ability of the Nanoclave Cabinet to disinfect non-critical patient care items

	minimum and maximum log_{10} reduction after exposure to two 30-second UV-C cycles [a]															
	Blood pressure gauge (n = 8)		Patient call button (n = 8)		Infusion pump (n = 5)		Tympanic thermometer (n = 9)		Oximeter (base unit) (n = 7)		Computer keyboard/mouse (n = 4)		TV remote control (n = 4)		Blood pressure cuff (n = 6)	
Pathogen [b]	min	max	min	max	min	max	min	max	min	max	min	max	min	max	min	max
MRSA	4.40	>5.29	>4.74	>5.17	>4.94	>5.08	2.16	>5.45	>5.25	>5.48	>4.97	>5.10	>5.05	>5.32	1.93	>5.13
VRE	>5.11	>5.23	>5.05	>5.21	>4.93	>5.30	1.49	>5.44	>4.91	5.27	4.28	>5.03	4.93	>5.16	2.13	>5.00
A. baumannii	3.44	>5.54	5.32	>5.59	>5.39	>5.56	2.29	>5.64	>5.13	>5.48	4.90	>5.74	>5.33	>5.75	3.46	>5.39
Kleb. pneum	2.76	>5.19	>4.84	>5.25	4.04	5.07	1.02	>5.11	>4.33	>5.08	>5.11	>5.21	>5.05	>5.12	3.24	>5.34

[a] initial inoculum: 10^6 cfu/cm^2.
[b] test organism suspended in ¼-strength Ringer's solution.

target sites (78%) to two 30-second UV-C irradiation cycles consistently reduced the number of contaminating organisms by at least 4.7 $\log_{10}$ values and/or to below detectable levels (10 cfu).

The Nanoclave Cabinet was less effective when used to disinfect the tympanic thermometer and the blood pressure cuff (Table 1). Although two 30-second UV-C cycles reduced bacterial numbers on some sites to below detectable levels, on others, bacterial numbers were reduced by less than 2 $\log_{10}$ values.

Comparative performance of the Nanoclave Cabinet and antimicrobial wipes

'Thoroughly' cleaning the tympanic thermometer with an antimicrobial wipe (four wiping strokes) reduced the number of bacteria on most sample points to below detectable levels (Table 2). A single wiping motion (defined as a 'poor' clean) was less effective than the Nanoclave Cabinet in reducing contamination levels on the display panel but more effective when used to disinfect the probe receptor and earpiece holder. When used to disinfect the infra-red sensor neither antimicrobial wipes nor the Nanoclave Cabinet were particularly effective in reducing bacterial numbers. Whilst two 30-second UV-C cycles achieved a 2.30 $\log_{10}$ reduction, cleaning with an antimicrobial wipe only reduced bacterial numbers by 2.14 $\log_{10}$ values (Table 2).

When used to disinfect a blood pressure cuff, the Nanoclave cabinet reduced the number of bacteria on the pump and pump tubing by more than 5 $\log_{10}$ values (Table 2). 'Thorough' cleaning using an antimicrobial wipe achieved a similar log reduction but less effective wiping reduced bacterial numbers by between 2.38 and 3.94 $\log_{10}$ values. Antimicrobial wipes were least effective when used to disinfect the inner cuff surface and either side of the velcro fastening. The Nanoclave Cabinet was comparatively more effective and reduced the number of bacteria contaminating these surfaces by between 3.46 and 4.28 $\log_{10}$ values (Table 2).

When used to disinfect the patient call button, the Nanoclave Cabinet reduced the number of bacteria on all target sites to below detectable levels (> 5.3 $\log_{10}$ values). Cleaning using an antimicrobial wipe was equally effective although a 'poor' wiping technique allowed organisms to persist on the rear panel and rubber grip.

Effect of organic soiling on the efficacy of the Nanoclave Cabinet

In the presence of low level soiling (0.03% BSA), two 30-second UV-C irradiation cycles reduced MSSA, *Enterococcus hirae*, *Escherichia coli* and *Pseudomonas aeruginosa* numbers to below detectable levels and achieved at least a 5.8 $\log_{10}$ reduction in microbial viability. Increasing the organic challenge had little effect upon the efficacy of the Nanoclave Cabinet which, in the presence of BSA (0.3%) and red blood cells reduced MSSA and *P. aeruginosa* numbers to below detectable levels (i.e. achieved a 6 $\log_{10}$ reduction) within 60 seconds (Table 3).

Effectiveness of the Nanoclave Cabinet against Clostridium difficile spores

The Nanoclave Cabinet was less effective against *C. difficile* spores, particularly those of the clinical strain. Two 30-second cycles achieved a 3.55 $\log_{10}$ reduction in *C. difficile* NCTC 11209 spore numbers (Table 3). In comparison, two 60-second cycles reduced the number of 027 spores by just 1.14 $\log_{10}$ values (Figure 2). A 2.18 $\log_{10}$ reduction was

Table 2 Comparative performance of the Nanoclave Cabinet and antimicrobial wipes when used to disinfect patient care items

	Mean (± SD) $\log_{10}$ reduction [a] (n = 3)			
	Nanoclave	Antimicrobial wipes		
	(2 × 30 sec)	'poor' wiping	'moderate' wiping	'thorough' wiping
Tympanic thermometer				
display panel	>5.49	4.45 ± 1.03	5.04 ± 0.60	>5.39
infra-red sensor	2.29 ± 0.93	1.94 ± 0.03	1.96 ± 0.25	2.14 ± 0.14
plastic lid	>5.64	>5.40	>5.40	>5.40
probe receptor	4.55 ± 1.07	5.16 ± 0.17	>5.48	>5.48
earpiece holder	3.44 ± 0.13	5.25 ± 0.40	5.28 ± 0.35	>5.49
Blood pressure cuff				
Velcro (hook)	3.60 ± 0.98	1.91 ± 0.07	2.42 ± 0.14	2.66 ± 0.09
Velcro (loop)	4.28 ± 0.96	1.50 ± 0.03	2.26 ± 0.19	2.67 ± 0.06
inner cuff surface	3.46 ± 1.47	1.73 ± 0.09	2.65 ± 0.08	2.30 ± 0.08
pump	>5.39	2.38 ± 0.15	2.90 ± 0.08	>5.60
pump tubing	>5.07	2.75 ± 0.40	3.94 ± 1.22	>5.33

[a] initial inoculum: 10^6 cfu/cm^2.

Table 3 Effect of organic soiling on the efficacy of the Nanoclave Cabinet

	Mean (± SD) log_{10} reduction	
	Light soiling (n = 36) 0.03% BSA	**Heavy soiling (n = 12) 0.3% BSA + 0.3% sheep erythrocytes**
MSSA	> 7.18	6.19 ± 0.76
P. aeruginosa	> 6.12	>5.99
E. coli	> 5.84	not tested
E. hirae	> 6.15	not tested
C. difficile spores (ribotype 001)	3.55 ± 0.47	not tested

achieved after a total exposure time of 5 min (i.e. two 150-second cycles). Increasing the cycle time further had no significant effect ($P > 0.05$; Figure 2). However, when the initial inoculum was ≤ 10^4 cfu/cm^2 two 60-second UV cycles were sufficient to reduce the number of *C. difficile* 027 spores to below detectable levels (Figure 3).

Effectiveness of the Nanoclave Cabinet against Adenovirus species A

Viability assays were not available for Adenovirus, so persistence of viral DNA following inoculation of viable cell culture, detected by polymerase chain reaction (PCR), was used as a surrogate marker. PCR may detect both viable and non-viable virus, depending upon the integrity of the DNA present on the surface. UV-C degrades DNA, so there will be loss of viability before total loss of detectable DNA by PCR, but the point at which all viable virus is lost is not known. The levels of retrievable viral genomes are recorded as a function of the PCR assay using the Cycle Threshold (CT) values. The CT is the number of doubling cycles required before the assay became positive. A small CT represents a higher starting load and each 3.3 CT increase between samples equates to a 1 log_{10} reduction in detectable viral genome. A CT value of 45 is the assay end-point and DNA considered 'undetectable'.

The ability of the Nanoclave Cabinet to degrade Adenovirus DNA was only tested on smooth metal or ceramic surfaces. On these surfaces the UV-C is shown to degrade Adenovirus DNA by the increase in CT value following successive exposures (Table 4). Regardless of test surface, six 30-second cycles (3 minutes) increased the mean CT between 9 and 10 CT values. Thus, a total exposure time of 3 minutes resulted in a 3 log_{10} reduction in detectable viral DNA. After an exposure time of 6 minutes, viral DNA was undetectable on both the stainless steel and ceramic test surface (i.e. a 6 log_{10} reduction had been achieved).

Discussion

The routine cleaning and disinfection of the near-patient environment is often inadequate and many items of near-patient equipment and furniture have been identified as potential bacterial reservoirs [3,6,14-18]. The efficacy of many traditionally used products and practices has been questioned as has their human and ecological safety [19]. Such concerns have prompted an increasing interest in the use of additional or alternative surface disinfectants, for example, self-disinfecting surfaces [20], hydrogen peroxide vapour [21] and ultraviolet light.

Ultraviolet irradiation is considered an acceptable and environmentally friendly means of disinfecting surfaces in healthcare settings [10]. The Tru-D Rapid Room Decontamination device (Lumalier Corporation) can eliminate vegetative bacteria and *C. difficile* spores from

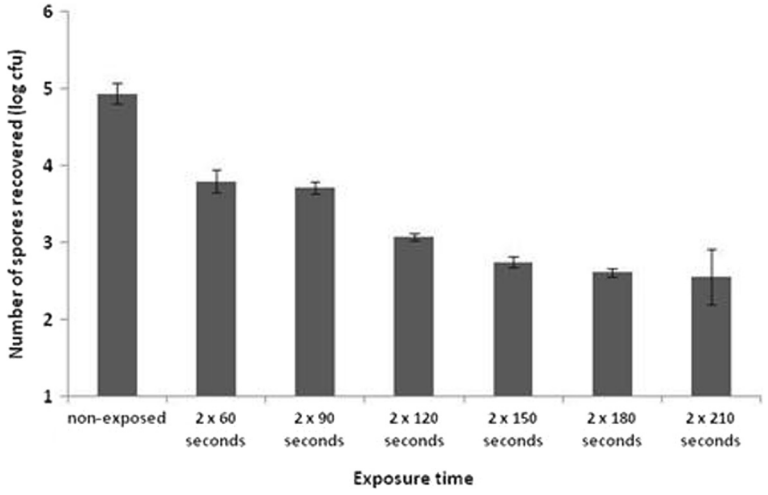

Figure 2 The effect of cycle duration upon the mean number of *Clostridium difficile* 027 spores recovered from a stainless steel surface (n = 5; error bars indicate the standard deviation).

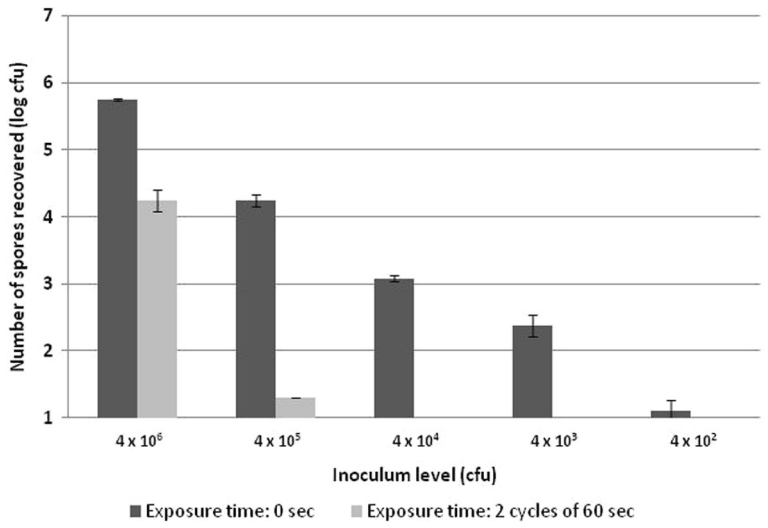

Figure 3 Efficacy of the Nanoclave Cabinet against the spores of *Clostridium difficile* ribotype 027: the effect of inoculum level (n = 3; error bars indicate the standard deviation).

contaminated surfaces within 15 min and 50 min respectively [9,10]. However, such UV-C devices cannot be used when the room is occupied and a lengthy cycle time is impractical if a rapid turn-over of beds is required.

The Nanoclave Cabinet is used to disinfect individual patient-care items. Any item placed in the Cabinet is subjected to six-sided emission of UV-C light both directly and via angled mirror reflectors. During the current study, the Nanoclave Cabinet was used to disinfect a variety of non-critical patient care items and the UV-C light caused no observable damage. However, the range of surface materials tested was by no means exhaustive. Not all materials are suitable. The Medicines and Healthcare products Regulatory Agency (MHRA) has advised that the outer coating of flexible endoscopes may be damaged by direct exposure to ultraviolet light [22]. UV-C light is also injurious to soft contact lens polymers albeit at a dose much higher (250 mW/cm^2) than that generated by the Nanoclave Cabinet (5.3 mW/cm^2) [23].

Table 4 Effect of cycle duration upon the degradation of Adenovirus DNA

Exposure time	Mean Cycle Threshold (CT) value [a] (n = 4)	
	Stainless steel sheet	Ceramic tile
0 min (control)	17	18
1 min	22	22
2 min	25	27
3 min	27	27
4 min	33	34
5 min	31	45
6 min	45	45

[a]A 3.3 CT increase equates to a 1 log reduction in detectable viral genome. A CT value of 45 is the assay end-point and when DNA is considered undetectable.

Each patient-care item was irradiated for 30 sec. The surface was rotated to expose those areas initially in contact with the rack, and the irradiation cycle repeated. Two irradiation cycles ensured the entire surface area was exposed to a UV-C dose of 1,590 J/m^2 (53 W/m^2 × 30 s exposure time) and that much of the surface was subjected to twice this dose (3,180 J/m^2; 53 W/m^2 × 60 s exposure time). This was sufficient to reduce the number of vegetative contaminants on the majority of sample points by at least 4.7 $\log_{10}$ values. However, not all test points demonstrated the same reduction. Poor penetration of the UV-C rays and/or significant shadowing, enabled bacteria to persist on the tympanic thermometer and the blood pressure cuff (Table 2). It was possible to reach the deep recesses associated with the thermometer (e.g. probe receptor; earpiece holder) with an antimicrobial wipe and wiping reduced the number of contaminating organisms to below detectable levels. In contrast, although surface contamination decreased as the thoroughness of wiping increased, antimicrobial wipes were less effective than the Nanoclave Cabinet in disinfecting the blood pressure cuff, particularly the Velcro fastener.

The Nanoclave Cabinet was less effective on surfaces contaminated with *C. difficile* spores. Previous studies have also found *C. difficile* spores to be more resistant to UV-C radiation than vegetative bacteria [9,10]. Exposing a highly contaminated surface (10^6 cfu/cm^2) to a total dose of 6,360 J/m^2, achieved a small (1.14) but significant $\log_{10}$ reduction in *C. difficile* 027 spore numbers. Exposing a less contaminated surface (10^5 cfu/cm^2) to

the same UV-C dose achieved a 3 $\log_{10}$ reduction. The clumping of high numbers of spores may inhibit the penetration of UV-C rays. Spores within a clump may be shielded and protected by those directly exposed. Nonetheless, when dried onto a surface, high numbers of *C. difficile* NCTC 11209 spores (10^6 cfu/cm^2) were reduced by 3.55 log values within 60 seconds (i.e. after a comparatively lower dose of 3,180 J/m^2; Table 3). Spores of wild type variants of *C. difficile* have also been shown to be more resistant to chemical disinfectants than those of laboratory strains [24]. Additionally, spore size can vary both within and between strains [25] and an increased spore diameter may reduce the ability of the UV-C rays to penetrate the various spore layers [26].

There are no European Standard sporicidal surface tests for the medical area; current standards are suspension tests which require a 3 or 4 $\log_{10}$ reduction within 30, 60 or 120 minutes [27]. When used to disinfect a surface contaminated with *C. difficile* spores at levels equating to 10^3 cfu/cm^2, the Nanoclave Cabinet achieved a 3 $\log_{10}$ reduction within 2 minutes – a shorter, more relevant exposure time than those specified by current standards. However, the manufacturers of the Nanoclave stipulate that the Cabinet should only be loaded with one item at a time (as illustrated in Figure 4). Thus, in contrast to whole room decontamination devices, the total time required to disinfect a number of items using the Nanoclave Cabinet could be high, particularly if the cabinet is used for viral disinfection.

Adenovirus is associated with respiratory, ocular and gastrointestinal disease, especially in children. Once excreted, it can survive and remain infectious within the environment for up to 35 days. As a double stranded DNA virus, Adenovirus is particularly resistant to UV irradiation [28]. During the current study, the Nanoclave Cabinet rendered high levels of Adenovirus DNA (10^9 viral genomes), on flat stainless steel sheets or ceramic tiles, undetectable by a sensitive PCR. However, to achieve this level of degradation (> 6 $\log_{10}$ reduction in detected viral DNA) it was necessary to expose the test surfaces to twelve 30-second UV cycles (i.e. a total dose of 19,080 J/m^2). A lower exposure time may be required to achieve a 6 $\log_{10}$ reduction in viable virus as Adenovirus is likely to become non-viable before DNA becomes non-detectable by PCR.

Figure 4 Placing an item to be disinfected inside the Nanoclave Cabinet.

It is also stated, both in the technical specifications document and the instructions for use of the Nanoclave Cabinet, that *"items to be disinfected must be physically clean before irradiation"*. Removal of visible soil is important both aesthetically and chemically. Organic soils are known to react with disinfectant molecules reducing their bioavailability. UV-C is also absorbed by organic materials [8] and whilst the Nanoclave Cabinet is not intended to be used to decontaminate heavily soiled invasive items, some non-critical patient care items may be difficult to manually clean. As with other UV-C irradiation devices [9], the efficacy of the Nanoclave Cabinet was not reduced by bovine serum albumin or red blood cells. However, it is acknowledged that the soiling experiments were only carried out using flat stainless steel discs and whilst the positioning of the discs within the Cabinet did not influence the reductions obtained, the presence of organic materials within recesses and/or areas of significant shadowing may effect the ability of the Nanoclave Cabinet to rapidly and effectively disinfect patient-care items.

Conclusions

There are no standard test methods or acceptance requirements for equipment such as the Nanoclave Cabinet. During the current study, the test requirements for chemical disinfectants were used as the basis for the acceptance criteria. These stipulate that a bactericidal and sporicidal product should achieve a 5 $\log_{10}$- and a 3 $\log_{10}$ reduction respectively. The Nanoclave Cabinet effectively reduced the numbers of a range of potential pathogens including *Clostridium difficile* spores and Adenovirus from most, but not all, test surfaces and patient-care items. High level bacterial and viral disinfection (> 5 $\log_{10}$ reduction) was achieved within 1 and 6 minutes respectively suggesting that the Nanoclave Cabinet could be used to provide rapid and effective disinfection of patient-related equipment. However, bacteria did persist on some test sites; these areas may have been 'in shadow' due to individual item shape and other decontamination methods may be required. Furthermore, the Nanoclave Cabinet can only be loaded with one item at a time and the real life practicability of such a system

was not assessed as part of this investigation. Laboratory studies do not necessarily replicate 'in-use' conditions and further studies are required to assess the acceptability and usability of the Nanoclave Cabinet within the clinical environment and its performance if Standard Operating Procedures are not adhered to.

Competing interests

Nanoclave Technologies LLP (London, UK) provided temporary loan of the Nanoclave Cabinet and financial support to cover the costs of consumables. However, Nanoclave Technologies did not contribute to the study design nor the writing or editing of the manuscript.

Authors' contributions

SA, CB and MW carried out the bacterial investigations. EC-G carried out the Adenovirus study. GM drafted the manuscript. APRW conceived of the study. APRW, AF, CB and JH critically reviewed the manuscript. All authors participated in the design and co-ordination of the study and read and approved the final manuscript.

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