Investigations of Carbapenem-resistant Klebsiella species and associated clinical considerations

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INVESTIGATIONS OF CARBAPENEM-RESISTANT *KLEBSIELLA* SPECIES AND ASSOCIATED CLINICAL CONSIDERATIONS

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ABSTRACT

The use of many antibiotics to treat infections has become limited in the last decade. *Enterobacteriaceae*, especially *Klebsiella* spp., have acquired resistance to quinolones, aminoglycosides, cephalosporins and carbapenems. Resistance to β-lactams is mediated via extended-spectrum β-lactamases, AmpC type β-lactamases and carbapenemases combined with porin loss. Carbapenems are the antibiotics of last resort. The emergence of carbapenemase-producing organisms (CPOs) has led to Public Health England introducing a national toolkit to limit their spread. As part of this requirement, the Charing Cross microbiology laboratory of the Imperial College Healthcare NHS Trust revised its screening programme for the detection of CPOs. This improved the detection and isolation of CPOs, and highlighted *Klebsiella* spp. were more of a problem with respect to multidrug resistance than previously thought.

Thirty-nine carbapenem-resistant *Klebsiella* strains were characterised. Phenotypic tests identified the strains as *Klebsiella pneumoniae* (n = 36) and *Klebsiella oxytoca* (n = 3). Detailed whole-genome sequence (WGS) analyses showed the *K. oxytoca* were *Klebsiella michiganensis* and one of the *K. pneumoniae* strains to be *Klebsiella variicola* subsp. *variicola*.

The *K. michiganensis* strains were all of sequence type 138. They were predicted to encode the β-lactamases bla_{GES-5}, bla_{SHV-66}, bla_{TEM-1}, bla_{OXA} and $bla_{CTX-M-15}$, and the 12-gene operon of the kleboxymycin biosynthetic gene cluster. This gene cluster encodes for tilimycin and tilivalline, enterotoxins previously thought only to be carried by *K. oxytoca* strains.

Incorporation of antimicrobial resistance and virulence gene data showed hypervirulent, multidrug-resistant *K. pneumoniae* strains encoding both aerobactin and *rmpA* (the regulator of mucoid phenotype) or colibactin are present in West London Hospitals. These are a cause for concern, as they have the potential to cause outbreaks that are untreatable.

WGS analyses yield more accurate and comprehensive data compared with phenotypic testing, enabling exact identification of clinically important strains, detailed outbreak investigations and molecular characterisation of antibiotic resistance and virulence genes in clinical settings.

Thirty-two bacteriophages were isolated from sewage water and found to infect one or more of the clinical *Klebsiella* isolates. Some phages with broad host ranges (i.e. they infected *K. pneumoniae*, *K. michiganensis*, *K. variicola* and *K. grimontii* strains) were identified, which may have use in clinical therapeutics against multidrug-resistant infections. These bacteriophages remain to be characterised in detail.

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ACKNOWLEDGEMENTS

I dedicate this thesis to my son, his struggle with health at a young tender age has inspired me to contribute to research and science to better patient care and treatment. And to my family, my husband, Mum and friends who are like my family (Beatrice & Paulette) for their continual support and unconditional love. Also to my faith, which kept me going even in the darkest of times.

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AUTHOR'S DECLARATION

I declare that all the material contained in this thesis is my own work, unless otherwise acknowledged in individual chapters.

LIST OF ABBREVIATIONS

AAHC, antibiotic-associated haemorrhagic colitis

ACN, acetonitrile

AST, antimicrobial susceptibility testing

BSAC, British Society for Antimicrobial Chemotherapy

CFU, colony-forming unit

CIM, Carbapenem Inactivation Method

CXH, Charing Cross Hospital

CPE, carbapenem-producing *Enterobacteriaceae*

CPO, carbapenemase-producing organism

CRO, carbapenem-resistant organism

EMA, European Medicines Agency

ESBL, extended-spectrum β-lactamase

FDA, US Food and Drug Administration

FN, false negative

FP, false positive

GI, gastrointestinal

HAI, healthcare-associated infections

HH, Hammersmith Hospital

mHT, modified Hodge test

ICHNT, Imperial College Healthcare NHS Trust

IMP, imipenemase-type metallo- β -lactamase

KPC, *Klebsiella pneumoniae* carbapenemase

LOD, limit of detection

MALDI-TOF, matrix-assisted laser desorption/ionization time of flight

MBL, metallo-β-lactamase

MDR, multidrug-resistant

MIC, Minimum Inhibitory Concentration

NDM, New Delhi metallo-β-lactamase

OXA, oxacillinase

OXA-48, carbapenem-hydrolysing oxacillinase-48

PFGE, pulsed-field gel electrophoresis

PHE, Public Health England

PT, phage therapy

SMH, St Mary's Hospital

TM, tilimycin

TN, true negative

TP, true positive

TV, tilivalline

UK, United Kingdom

VIM, Verona integron-mediated metallo-β-lactamase

VNTR, variable number tandem repeats

WGS, whole-genome sequencing/whole-genome sequence

CHAPTER 1: INTRODUCTION

The global spread of carbapenemase-producing *Enterobacteriaceae* (CPE), *Pseudomonas aeruginosa* and *Acinetobacter* species has significantly increased in many parts of the world especially in South America, USA, Asia and Europe and has become a critical medical and public health issue (WHO, 2014). The concern is because these bacteria are generally resistant to all β-lactam antibiotics and also show co-resistance to multiple classes of other antimicrobial agents, leaving very few treatment options (Livermore, 2012a). Controlling the spread of carbapenemase-producing organisms (CPOs) is not easy due to the diversity of carbapenem-hydrolysing enzymes that have emerged and the ability of the resistance genes to spread among multiple bacterial species (Curran and Otter, 2014; Grundmann *et al.*, 2010). Whole-genome sequence (WGS) data are being used increasingly to study CPOs within academic and Public Health England (PHE) laboratories, but there is little opportunity for clinical laboratory workers to learn how sequence data can be used to better characterise the antibiotic-resistance genes carried by CPOs. Bacteriophage (phage) therapy has been suggested as an alternative or adjunct treatment option in CPO-associated infections (Inal, 2003). The following will introduce the carbapenemases and provide background information on *Klebsiella* spp. (the focus of my studies). A review that I contributed to describing *Klebsiella* phages and their uses in treatment of clinical infections caused by *Klebsiella* spp. is also provided (Appendix 1).

1.1 *KLEBSIELLA PNEUMONIAE* AND THE CLINICALLY RELEVANT RELATED SPECIES *KLEBSIELLA OXYTOCA*

The genus *Klebsiella* belongs to the family *Enterobacteriaceae*, named after Edwin Klebs a 19th century German microbiologist. *Klebsiella* are non-motile, Gram-negative, lactose-fermenting facultatively to strictly aerobic bacteria. Members of the genus *Klebsiella* express two types of antigens on their cell surface that contribute to their pathogenicity; the lipoploysaccharide O antigen and a capsular polysaccharide K antigen whose structure varies. This in turn allows *Klebsiella* species to be classified into various serotypes. Until recently it was thought that there were 77 K antigens and 9 O antigens of *Klebsiella pneumoniae*.

However, using WGS analyses Wyres *et al*. (2016) were able to identify a total of 134 distinct K-loci in *Klebsiella* spp.

Klebsiella species are naturally found in the environment. Within the genus *Klebsiella* the two species that cause the majority of human infections are *Klebsiella pneumoniae* and *Klebsiella oxytoca*. These species are widely considered as opportunistic pathogens asymptomatically colonising the skin, mouth, respiratory and gastrointestinal (GI) tract of healthy humans (Holt *et al*., 2015). WGS of 300 human-derived isolates of *K. pneumoniae* showed the existence of >150 lineages of *K. pneumoniae* with numerous multidrug-resistant (MDR) or hyper-virulent clones (Holt *et al*., 2015). The genome of some *K. pneumoniae* strains encodes virulence factors associated with invasive community-acquired diseases (Holt *et al*., 2015). Antimicrobial resistance genes are generally more common in human carriage isolates causing hospital-acquired infections (HAIs) than the community-carriage isolates. So far no studies have been carried out in the UK to determine GI carriage of *K. pneumoniae* and closely related species and their relationship to infection.

Studies conducted by Conlan *et al*. (2012a), Farida *et al*. (2013) and Dao *et al.* (2014) from healthy volunteers have shown the carriage rate of *Klebsiella* spp. to be more prevalent from mouth, nose and skin samples (~10%) than from stool samples (3.8%). Feldman *et al*. (2013) showed that most HAIs with carbapenemase-resistant *K. pneumoniae* are caused by clonal group ST258 or the hyper-virulent type belonging to clonal group ST23 (which causes pyogenic liver abscesses) carried within the GI tract of the patient. Hypervirulent *K. pneumoniae* is more virulent than classical *K. pneumoniae* and mainly infects individuals in the community and is now occurring globally. The genetic factors (related to capsule and aerobactin production) that confer *K. pneumoniae*'s hypervirulent phenotype are present on a large virulence plasmid. Similar to classical *K. pneumoniae*, hypervirulent *K. pneumoniae* is becoming increasingly resistant to antimicrobials due to acquisition of mobile elements carrying resistance genes. New hypervirulent strains emerge when extensively drug-resistant classical strains acquire hypervirulent-specific virulence determinants, resulting in nosocomial infections leading to invasive disease in individuals (Russo and Marr, 2019).

Martin *et al*. (2016) showed an increased risk of infection with *K. pneumoniae* was associated with GI colonisation, ~5.2% in colonised patients versus 1.3% in non-colonised patients. Gorrie *et al.* (2017) demonstrated *K. pneumoniae* to be a normal member of the human GI microbiota and the GI carriage of the organism with patients on their admission to intensive care was associated with subsequent infection rather than from upper-respiratory-tract carriage. Using genome data, these authors showed a match between GI carriage and infection isolates in most patients.

In the community *K. pneumoniae* causes lung diseases, typically community-acquired pneumonia in older populations with debilitating diseases such as alcoholism, diabetes or chronic broncho-pulmonary diseases after the host aspirates colonising organisms into the lower respiratory tract (Holt *et al*., 2015). In the hospital setting, *K. pneumoniae* accounts for approximately 8% of all HAIs. Carbapenem-resistant *K. pneumoniae* are ranked among the recently published World Health Organisation list of antibiotic-resistant “priority/critical” pathogens, for nosocomial infections for which research and development of new antibiotics are required (WHO, 2017a).

K. pneumoniae causes nosocomial infections of the urinary tract, lower respiratory tract and surgical wound sites of patients, particularly those in intensive care units. Here the route of entry is endogenous, i.e. due to colonisation (especially from the GI tract), or exogenous from the healthcare environment and contamination of invasive devices, respiratory support equipment or urinary catheters. Excessive use of antibiotics also contributes to the increased likelihood of nosocomial infection (Holt *et al.,* 2015).

Similar to *K. pneumoniae*, *K. oxytoca* has a prominent polysaccharide capsule which provides resistance against host defence mechanisms (Trivedi *et al*., 2015). It is a Gram-negative bacterium found in the environment (Hoffman *et al*., 2010). *K. oxytoca* has been associated with neonatal septicaemia among premature infants in neonatal intensive care units: here the route of infection is due to colonisation of an infant’s GI tract during delivery and also from the hospital environment (Moles *et al.*, 2015). Mode of delivery, invasive procedures such as enteral feeding using nasogastric tubes, and bacterial contamination of milk and the hands of healthcare workers can contribute to infant colonisation with *K. oxytoca* (Berthelot *et al*., 2001). *K. oxytoca* can also be detected in the GI tract of

~2-10% of healthy humans (Schlenker and Surawicz, 2009), and is considered to be part of the normal gut microbiota, with the potential to become an opportunistic pathogen. The bacterium has been shown to be a causative agent of antibiotic-associated haemorrhagic colitis (AAHC), a disease caused by the overgrowth of *K. oxytoca* with the release of cytotoxin when the indigenous GI microbiota is disturbed during antibiotic treatment (Zollner-Schwetz *et al*., 2008; Herzog *et al*., 2014). AAHC is usually preceded by antibiotic treatment with penicillins, which are frequently prescribed for paediatric patients (Herzog *et al*., 2014).

1.2 TREATMENT OF *KLEBSIELLA* INFECTIONS

Klebsiella infections are mainly treated with cephalosporins, carbapenems and aminoglycosides but there is now emerging resistance to these antibiotics because *Klebsiella* spp. produce extended spectrum β-lactamases (ESBLs) or carbapenemases that confer resistance to cephalosporins and carbapenems (Sekyere *et al.*, 2016). By acquiring a combination of resistance mechanisms to these antimicrobials *Klebsiella* spp. can become MDR: the carbapenem group of antibiotics is the last line of defence against Gram-negative infections that are resistant to other antibiotics. Consequently, MDR *Klebsiella* infections are treated with combination therapy comprising a β-lactam and aminoglycosides or using colistin and tigecycline as a monotheraphy (Sekyere *et al.*, 2016).

The emergence of MDR strains of *K. pneumoniae* has become an urgent threat to human health because of their association with outbreaks of HAIs and community-acquired infections globally (Holt *et al*., 2015). Since carbapenemases are transferred from species via plasmids, GI carriage of *Enterobacteriaceae* (members of the normal gut microbiota) serves as a reservoir and vector of CPEs and also promotes cross-transmission of resistance in healthcare settings (Viau *et al*., 2016).

1.3 CARBAPENEMASES

Carbapenemases are a diverse group of bacterial enzymes that vary in their ability to hydrolyse carbapenems and other β-lactam antibiotics (Queenan and Bush, 2007). Carbapenem antibiotics include ertapenem, meropenem, imipenem and doripenem, and are β-lactam antibiotics with broad-spectrum

antibacterial activity. Carbapenemases can be acquired via transferable plasmids, or be chromosomally encoded, and are widely distributed in Gram-negative bacteria (Ambler, 1980; Jeon *et al*., 2015). The Ambler classification system is used to characterise carbapenemases, and differentiates β-lactamases into four classes (A, B, C and D) based on their amino acid sequences (Ambler, 1980; Hall and Barlow, 2005).

Class A enzymes inactivate the β-lactam ring by means of a catalytically active serine residue in the enzyme active site, e.g. serine-based penicillinases (Jeon *et al*., 2015). Class A enzymes include *K. pneumoniae* carbapenemase (KPC) determinants, and are associated with successful clonal lineages of bacteria (e.g. *K. pneumoniae* ST258). Four of these lineages have a selective advantage in hospital settings, where antimicrobial use is high and the environment provides numerous opportunities for transmission of organisms, with further dissemination of the resistance genes via transmissible plasmids and integrons (Curiao *et al*., 2010). *K. pneumoniae* ST258 has given rise to several epidemics globally, especially in the United States and Israel (Livermore, 2012a; Grundmann *et al.*, 2010). Recently the isolation of another hypervirulent clone, the MDR *K. pneumoniae* ST23 reported in Korea, Vietnam, China and Brazil, has become a cause for concern as this sequence type is associated with severe invasive human diseases (Cejas *et al*., 2014).

Plasmid-encoded GES-type β-lactamase also belongs to the class A enzymes and is an ESBL discovered in 2000 in *K. pneumoniae* clinical isolates from French Guiana, and subsequently named "Guiana extended spectrum" (Bonnin *et al.*, 2017). GES-type β-lactamases are slow carbapenem hydrolysers with increased hydrolysis of cephamycin and ceftazidime. To date, 31 variants of GES have been identified, most commonly in *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli* and *K. pneumoniae*. All GES-carrying *Enterobacteriaceae* have carbapenem-hydrolysing activity due to a replacement of glycerine at position 170 by an asparagine or a serine. The GES-5 variant has also been found in *K. oxytoca* due to plasmid or intergron transfer in UK hospitals (Diene and Rolain, 2014). The GES-5 variant has now disseminated quite widely, being reported both in nosocomial and environmental settings in South America, Korea and Africa due to the selective pressure resulting from the overuse of carbapenems (Nordmann and Poirel, 2014).

Class B enzymes are known as metallo-β-lactamases (MBLs) and can hydrolyse carbapenems effectively. They require zinc as a metal co-factor for their catalytic activity (Hall and Barlow, 2005; Palzkill, 2013). MBLs include the Verona integron-mediated metallo-β-lactamase (VIM), which has been a concern in Europe for several years, and the imipenemase (IMP) class, which is more prevalent in Japan and the Asian subcontinent and now spreading globally (Livermore and Woodford, 2006). MBLs are detected usually in *P. aeruginosa*, and members of the family *Enterobacteriaceae* (Livermore and Woodford, 2006). The reason there is a threat of carbapenem resistance within the UK in our healthcare settings is due to the above-mentioned organisms acquiring an additional resistance gene that encodes for the New Delhi metallo-β-lactamase (NDM) and now passed on to *K. pneumoniae*. NDM *K. pneumoniae* has been introduced into the UK by individuals who have visited India or Pakistan, and NDM strains are a major cause of outbreaks in the high-dependency units of west London hospitals (Johnson and Woodford, 2013; Nordmann *et al*., 2011). The discovery of a *K. pneumoniae* ST14 strain harbouring the NDM carbapenemase gene, from a Swedish patient who received healthcare in India, is becoming a major threat worldwide producing pockets of outbreak situations in healthcare settings (Giske *et al*., 2012).

Class C enzymes also have a serine at their active site and are rare chromosomally encoded cephalosporinases (Ambler class C/AmpC) produced by *Enterobacteriaceae* and may possess slightly extended activity towards carbapenems, but their clinical significance remains unclear (Queenan and Bush, 2007).

Class D oxacillinases (OXA) have an active serine residue and hydrolyse carbapenems weakly; these enzymes belong to the OXA family and are most commonly produced by *Acinetobacter* species but have also been identified in some *P. aeruginosa*, *E. coli* and *K. pneumoniae* strains. OXA-48, which often mediates low-level carbapenem resistance but not resistance to the extended spectrum β-lactams, is now spreading rapidly in Europe and is also a cause for concern in UK hospitals (Potron *et al*., 2013a; Glasner *et al.,* 2013).

Current resistance status of CPOs since the detection of the first strain in 1980 indicates that CPOs have spread rapidly worldwide with epidemiological

studies showing that different carbapenemases predominate in different areas of the world. NDM carbapenemase is more prevalent in the Indian subcontinent, while KPC is endemic in the United States. OXA-48 carbapenemase producers are more prevalent in Turkey and North Africa (Suay-García and Pérez-Gracia, 2019).

1.4 CLINICAL ASPECTS OF INFECTIONS CAUSED BY CARBAPENEM-RESISTANT GRAM-NEGATIVE BACTERIA

In the UK, CPOs - especially *K. pneumoniae* (KPC, NDM) and *Acinetobacter* (OXA-48) species - are a real challenge to infection control in both hospital and community settings (Figure 1.1 and Figure 1.2): firstly, due to their ability to spread and cause outbreaks with different species of *Enterobacteriaceae* with the same mobile resistance gene (Grundaman *et al*, 2010); secondly, CPOs inhabit the GI tract and decolonisation therapy only reduces the number of CPOs without elimination (Curran and Otter, 2014). The majority of people are colonised with these organisms with no further complications but, in immunocompromised patients, colonisation can lead to endogenous infection, which can be a cause of cross-infection (Curran and Otter, 2014; Patel *et al*., 2007). Falagas *et al.* (2014) have shown that patients with invasive CPO infections have a twofold higher number of deaths than those infected with antibiotic-sensitive strains.

Infections caused by CPEs include urinary tract infections, peritonitis, septicaemia, pulmonary infections, soft-tissue infections and device-associated infections with no gender preference and most of the cases are in adults in high-dependency units (Freeman *et al*., 2015). Carbapenem-resistant *K. pneumoniae* strains carrying a virulence gene (*magA*) responsible for the production of capsular type K1 and causing community-acquired pyogenic liver abscess are on the increase globally (Lin *et al*., 2013). *A. baumannii* carbapenemase OXA-23, OXA-48 and OXA-51 strains account for most (30%) of the imipenem resistance in southern England (Livermore, 2012a; Azimi *et al*., 2015). CPOs are extremely MDR (resistant to carbapenems, penicillins and cephalosporins) and often carry genes that confer resistance to other antimicrobials (aminoglycosides and quinolones) leading to limited therapeutic options in infections caused by these organisms (Livermore and Woodford, 2006).

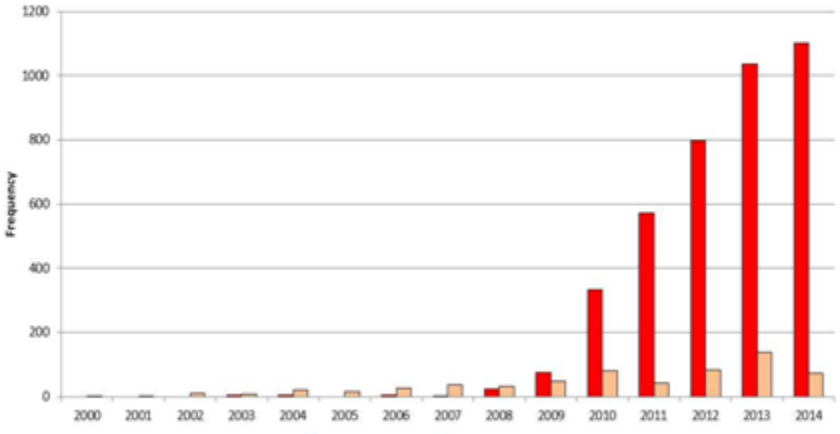


Figure 1.1. The number of confirmed CPO isolates referred from UK laboratories. There has been a rapid increase in CPOs in the UK between 2000 and 2014, from five per year from 2003 to 2007 to as high as 1,000 in 2013 (Curran and Otter, 2014). Most CPOs in the UK are *Enterobacteriaceae*. Red bars, *Enterobacteriaceae*; orange bars, non-fermenters. Data are from the PHE Antibiotic Resistance Monitoring and Reference Laboratory (AMRHAI). Figure taken from Livermore (2012a).

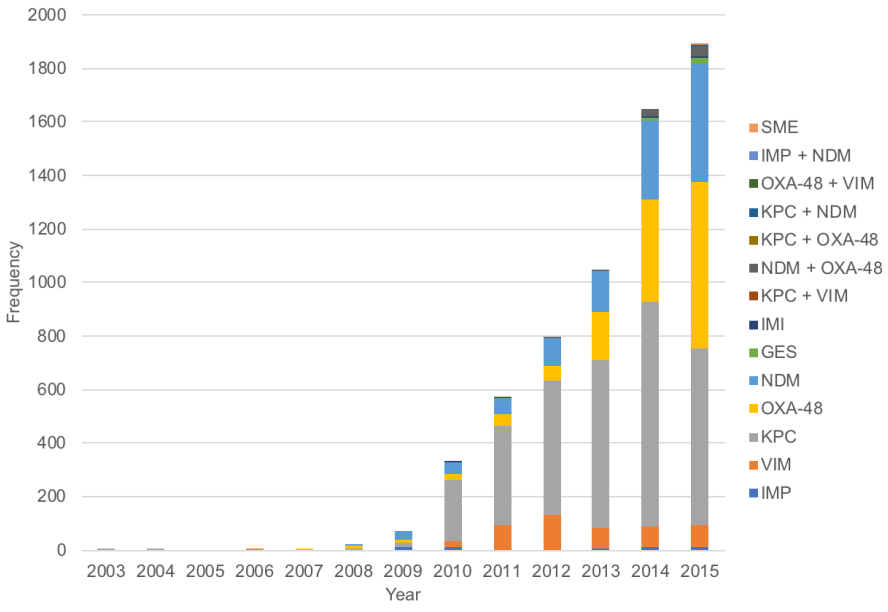


Figure 1.2. Carbapenemase-producing *Enterobacteriaceae*: laboratory-confirmed cases from UK laboratories 2003 to 2015 (last updated 10 October 2016). Data used to produce the graph taken from https://bit.ly/2miStRD (accessed 26 March 2018).

1.5 CURRENT THERAPEUTIC TREATMENT OPTIONS AND THE CAUSE OF THE RISE OF CPOS

For many years, oxyimino-cephalosporins such as cefuroxime and cefotaxime were the "workhorse" antibiotics of UK hospitals and worldwide (Livermore and Woodford, 2006). The wide use of these antibiotics as a primary therapy for the treatment of urinary, respiratory and abdominal infections has led to bacteria acquiring resistance against this class of antibiotics through the production of ESBLs. This has led to the use of carbapenems, often used as the antibiotics of last resort against MDR organisms, which in turn has given rise to bacteria that have acquired the carbapenemase-resistance gene (Livermore, 2012a). In addition, excessive usage and bad stewardship of antibiotics in healthcare settings has brought about selective pressure for antibiotic resistance in organisms that cause HAIs (Freeman *et al.*, 2015). Development of new antibiotics is scarce, and pharmaceutical companies shying away from investment in the development of new antibiotics (because of financial, licensing and regulatory concerns) compacts today's crisis caused by MDR strains (Theuretzbacher, 2012). Costs and complexity associated with conducting clinical trials together with the fact that CPOs are diverse with respect to their production of resistance enzymes, the species involved and epidemiology further add to the problems associated with development of new antibiotics/antimicrobials/inhibitors. In the past two decades only three new classes of antibiotics have been developed with a narrow spectrum and they are only active towards Gram-positive bacteria (Theuretzbacher, 2012; Gupta and Nayak, 2014; Ling *et al.*, 2015). Butler *et al*. (2017) recently listed 37 new antibiotics that would possibly be effective towards both Gram-negative and Gram-positive bacteria. Two β-lactamase/β-lactam combinations mainly for Gram-negative bacteria are in the pipeline and being evaluated in clinical trials (running since 2015). The outcome of these trials is awaited to see if these combination therapies can be of clinical use. Another report showed that as of May 2017, only 8 out of 51 antibiotics in the clinical pipeline belong to new classes, again stressing the lack of novel antibiotic development (WHO, 2017b). Recent literature shows there are a few antibiotics which have been approved for therapeutic usage and have been used successfully: they are ceftazidime/avibactam, meropenem/vaborbactam, plazomicin and eravacycline, but therapeutic failure is already developing with

some of them (Suay-García and Pérez-Gracia, 2019). To date, treatment options for the newly emerging CPOs are limited, with isolates remaining susceptible to colistin (although resistance is on the rise) and intermediate to tigercycline with significant drawbacks in terms of their therapeutic viability which can lead to resistance and with colistin usage limited due to its neurotoxicity and nephrotoxicity (Carmeli *et al*., 2010; Livermore, 2012a). Therefore, a need for an alternative therapy is inevitable. In the current climate phage therapy (PT) could be an effective alternative for the treatment of resistant bacterial infections.

1.6 CHARACTERISATION OF *KLEBSIELLA* SPP. IN THE GENOMIC ERA

Whole genome sequencing is the generation of the complete nucleotide sequence of a genome from DNA extracted from an organism and allows clinical isolates of, for example, *Klebsiella* spp. to be compared with one another and with reference sequences which are deposited in public repositories such GenBank and EMBL. The high resolution (e.g. differentiating strains at the single nucleotide level) offered by WGS has revolutionised our understanding and management of *Klebsiella*-associated infections. WGS allows accurate characterisation of transmission events and outbreaks, and provides information concerning the genetic basis of phenotypic characteristics, including antibiotic susceptibility and virulence (Van Belkum, 2003; Relman, 2011; Ladner *et al.*, 2019; Yu *et al*., 2019).

With respect to nosocomial infections, it is the responsibility of the clinical microbiology laboratory to identify the population of bacteria causing an infection. At present, this depends heavily on phenotypic methods such as growth on differential media, API 20E and MALDI-TOF. The time lag and low positive culture rate of traditional pathogen diagnosis make it difficult to rapidly identify many pathogens. In recent years, the increasing number of suspected infections and rapid increase in the rate of transmission have brought severe challenges to the diagnosis and treatment of infectious diseases. Therefore, successful identification of the microbe(s) responsible for an infection requires the rapid diagnosis of clinical infectious agents, and there is now a move to using ‘culture-independent’ methods such as WGS to increase the speed with which infectious bacteria can be identified, and the accuracy with which they can be characterised. For example, the Mycobacterial Reference Service of PHE now routinely uses WGS for identification of *Mycobacterium* sp. isolates (PHE, 2019).

Development of third-generation sequencing methods such as nanopore technology may allow point-of-care WGS in the near future. Nanopore sequencing has already been used to study an Ebola outbreak in real time (Quick *et al.*, 2016), and is beginning to be used to monitor virulence plasmids of nosocomial isolates of "high-risk" clones of *K. pneumoniae* ST15, ST48, ST101, ST147 and ST383 (Turton *et al.*, 2019). This has the advantage of being faster, more accurate and high-throughput than short-read sequencing methods that are widely available, and will play an increasingly important role in the rapid and accurate diagnosis of infectious diseases. With the continuous development of sequencing technologies and improvement in databases of genomes of pathogenic microorganisms, detection of infectious diseases and epidemic investigations can occur in real time, greatly improving the treatment of critical patients with severe infections (Van Belkum, 2003; Relman, 2011; Ladner *et al.*, 2019; Yu *et al*., 2019).

The emergence of MDR *Klebsiella* spp. is not only a global health problem but it is also becoming a threat in individual health organisations, as has been experienced in the Imperial College Healthcare NHS Trust (ICHNT). It is relatively inexpensive to generate WGS data. For example, microbesNG https://microbesng.com/) provides WGS of bacteria for as little as £50 per genome. This relative cheapness has allowed large-scale studies of *Klebsiella* genomes to be undertaken. The first of which was by Holt *et al.* (2015), which showed *K. pneumoniae* represented three distinct species: KpI (*K. pneumoniae*), KpII (*K. quasipneumoniae*) and KpIII (*K. variicola*). As more *K. pneumoniae* genome sequences have become available, it has become apparent that *K. pneumoniae* is more diverse than first thought, comprising seven distinct phylogenetic lineages: *K. pneumoniae*, *K. quasipneumoniae* subsp. *quasipneumoniae*, *K. quasipneumoniae* subsp. *similipneumoniae*, *K. variicola* subsp. *variicola*, *K. variicola* subsp. *tropica*, *K. quasivariicola* and *K. africana* (Rodrigues *et al.*, 2019). Holt *et al.* (2015) also showed *K. pneumoniae* comprised more than 150 distinct deeply branching lineages (clonal groups) that included numerous multidrug-resistant or hypervirulent clones, and that antimicrobial resistance genes were common among *K. pneumoniae* isolates associated with human carriage and HAIs.

Individual *K. pneumoniae* genomes contain approximately 5500 genes, including <2000 common genes shared among most strains and 3500 drawn from

an extensive pool of ≥30,000 protein-coding sequences with hundreds of antimicrobial resistance (AMR) genes (Holt *et al.*, 2015). A small number of globally distributed *K. pneumoniae* clonal groups are acquiring AMR genes at a rapid rate (Wyres *et al.*, 2016). The most widely recognised AMR clone is ST258, associated with the dissemination of the KPC gene. Genome analyses have shown ST258 has extensive capsular antigen and AMR gene diversity, so too do ST14/15, ST43 and ST147 – which have each disseminated from a particular area globally; ST14/15 from Asia, ST43 from United Arab Emirates and ST147 from Europe. It is now routine for WGS to be used in research laboratories to characterise key elements of the *Klebsiella* spp. genomic framework: namely identification of STs, AMR determinants including acquired genes and common mutations, known virulence genes and alleles, plasmids, and capsular and O antigen loci (Wyres *et al.*, 2016).

Using WGS, Gorrie *et al.* (2017) showed the GI microbiota is a source of *K. pneumoniae* infections in ICU patients. From a clinical perspective screening patients before admission using the various genomic databases to understand the nature of the gut colonisation and subsequent isolation of carriers could help end outbreaks and prevent future ones. Also, advanced knowledge of the antimicrobial susceptibility profiles of *K. pneumoniae* carried in a patient's gut before admission to hospital could guide the choice of prophylactic and therapeutic antimicrobial treatment. Screening of patients when transferred from tertiary referral hospitals to specialised hospitals using available genomic databases could be valuable to prevent hospital-to-hospital spread of AMR genes for the management or prevention of MDR infections (Gorrie *et al.*, 2018).

K. pneumoniae ST307 (closely related to ST258) has been around for the last 20 years but its importance went unnoticed before the availability of WGS (Wyres *et al.*, 2019). Its resistance and virulence traits have become clinically significant, as ST307 has the ability to acquire and disseminate carbapenemases. With this knowledge clinical laboratories can enhance their surveillance programmes to monitor ST307 alongside other known clones and detect emerging MDR threats and stop outbreaks.

Lam *et al.* (2018) used WGS to identify ST23, a hypervirulent *K. pneumoniae* that carries the virulence factor colibactin, a genotoxin encoded by the synthesis locus *clb* (*pks*) and responsible for promoting gut colonisation and

the mucosal invasion responsible for pyogenic liver abscess, which has been shown to be a significant risk factor for colorectal cancer. Lam et al. (2018) noted only those ST23 strains that encoded the *clb* locus caused disease. This information is important from a clinical perspective, as once known the patient can be treated and monitored to prevent further complications. The additional knowledge provided can also aid the clinical reference laboratory to update their characterisation tools. For example, to date UK clinical reference laboratories only monitor for the presence of *rmpA* and *wcaG*, capsule-associated virulence genes linked to pyogenic liver abscess formation in hypervirulent strains of *K. pneumoniae*. The findings of Lam *et al.* (2018) suggest reference laboratories may also need to include the *clb* locus and other genes which may be linked to invasive diseases in their routine screening.

Another notable development from the study of Lam *et al.* (2018) was the genotyping tool Kleborate. This has been combined with Kaptive (Wyres *et al.*, 2016) and made available as an online tool (http://kaptive.holtlab.net) allowing those with limited bioinformatics expertise to capsule and O antigen type *Klebsiella* WGS data. The results can be downloaded in a graphical interface, presenting data in an easily interpretable manner that can be understood by anybody without a bioinformatic background (Wick *et al.*, 2018). Tools such as this are very important to clinical microbiologists such as myself, who recognise WGS is going to become a routine tool in clinical laboratories but do not have any training in bioinformatics analyses. Online tools such as the Institut Pasteur *Klebsiella pneumoniae* MLST website (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html) allow sequence type and other information (e.g. antibiotic resistance genes, virulence genes) to be easily generated from WGS data. The *Klebsiella oxytoca* MLST website (https://pubmlst.org/koxytoca/) allows only sequence typing, but is still useful to those with limited bioinformatics training.

If WGS is the future of clinical microbiology, then it is paramount that staff working in clinical laboratories are trained or at least have a basic understanding of the tools available to characterise WGS data. During my Professional Doctorate studies I intend to gain experience in using these and other bioinformatics tools to better understand how WGS can benefit the clinical microbiology laboratory. There is now widespread recognition of the great potential for genomics to enhance surveillance and tracking of specific pathogens, and of AMR more generally, and

to aid infection control and outbreak investigations. At present, biomedical scientist training is based on phenotypic culture methods, but maybe it is time for training programmes to include genomics and bioinformatics as core components if we are to fully utilise these developing technologies.

1.7 PHAGES TO TREAT ANTIBIOTIC-RESISTANT INFECTIONS

Lytic phages are viruses that infect and kill bacteria, and are among the most abundant entities on Earth, with viruses being present at an estimated number of ~10^{31} particles (Breitbart and Rohwer, 2005). Phages are ubiquitous and are found anywhere there are bacteria: e.g. in water sources, sewage, soil and the intestines of animals (Hoyles *et al.*, 2014). More than 6000 different (lytic and lysogenic) phages have been discovered and described morphologically; this includes 6196 bacterial and 88 archaeal viruses (Ackermann and Prangishvilli, 2012). Most of these viruses are tailed with a small proportion being filamentous and all are classified according to their morphology and genetic content (i.e. DNA vs RNA) and the host they infect (Whittebole *et al*., 2014). Phages are obligate intracellular parasites of bacteria with different life cycles. For PT, lytic phages are recommended due to their lethal activity. Appendix 1 represents a review I contributed to, outlining what is known about phages infecting *Klebsiella* spp. and how these are being tested for use in PT, as alternatives or adjuncts to antibiotic therapy. The review has been made available as a preprint (Herridge *et al.*, 2019) and is currently under review by the *Journal of Medical Microbiology*. I believe it should be possible to isolate phages against MDR *Klebsiella* strains, and these phages could be used in the future to treat MDR infections or decolonise patients when they are admitted to hospital, to reduce the likelihood of outbreaks and/or the incidence of MDR infections.

The information obtained from the studies referenced in the review was useful for the current study, highlighting that many phage studies rely on *in vitro* assays. While a few phages have been tested in animal models with successful results, future research needs to be concentrated on clinical trials in animal models and humans to show the efficacy and safety of these treatments before they can be accepted as safe for use in humans. The current study will not be able to address animal or human studies due to lack of time and funding.

1.8 AIMS

To control the spread of CPOs, improved clinical laboratory methods are needed to increase the range of bacteria detected and the diverse range of carbapenem-hydrolysing enzymes that have emerged. These methods can include isolation, phenotypic and genomic methods. Clinical laboratories rely on simple isolation approaches, antimicrobial susceptibility testing, MALDI-TOF and PCR-based methods to characterise clinical isolates, but WGS is becoming routine in academic and PHE reference laboratories, and is likely to be used routinely in clinical laboratories in the next 5-10 years. In addition, alternatives or adjuncts to antibiotic therapies are needed to combat the increasing number of antibiotic-resistant infections. Therefore, the aims of my Professional Doctorate studies are to:

1. Develop an improved screening method for detection of CPOs in the West London Hospitals covered by the Imperial College Healthcare NHS Trust.
2. Characterise a collection of carbapenem-resistant *Klebsiella* isolates, to understand how simple bioinformatics approaches can be used to identify and better understand resistance genes in clinically relevant bacteria.
3. Isolate lytic phages active against clinically relevant carbapenem-resistant *K. pneumoniae* and *K. oxytoca* from sewage samples, and begin to characterise them in terms of their phenotypic and genomic properties.

The objectives of the project are to:

1. Undertake an extensive literature review on available infection control and screening programmes, and to use the information gathered to select the best products for testing for the isolation of CPOs in the Microbiology Laboratory of Charing Cross Hospital.
2. Plan and conduct a study with the products identified in objective 1, and identify which commercial medium is best for isolating a wide range of CPOs.
3. Screen sewage samples, known to be abundant sources of lytic phages active against Gram-negative bacteria, to isolate phages active against a collection of antibiotic-resistant strains of *Klebsiella* spp. isolated in West London hospitals since 2010.
4. Propagate the phages from objective 3 to purity and screen them against a panel of clinical isolates to determine their host ranges.

5. Isolate DNA from the purified phages (objective 4) to allow restriction profiles to be generated. Phages with unique restriction profiles will have their whole genomes sequenced and their genes annotated and functions assigned. They will also be characterised phenotypically, by assessing their tolerance to stresses (e.g. temperature, pH, chloroform), their adsorption rates, burst sizes and one-step growth curves.

CHAPTER 2: EVALUATION OF FOUR DIFFERENT CHROMOGENIC MEDIA FOR THE DETECTION OF CARBAPENEMASE-PRODUCING ORGANISMS IN WEST LONDON HOSPITALS

2.1 PERSONAL CONTRIBUTION TO THIS STUDY

This project was carried out while I worked as a Senior Biomedical Scientist in the Microbiology laboratory of Charing Cross Hospital. I was tasked with identifying an improved screening procedure for detection of carbapenemase-producing organisms (CPOs) from patient samples. I conducted an extensive literature review on available infection control and screening programmes. I also had meetings with product manufacturers, studied the different products on the market and selected the best products for the isolation of CPOs. Based on my findings I produced a project plan to study which of four different commercially available media was best for isolating a wide range of CPOs. The study was carried out under my supervision, and I assisted with the laboratory work. I also educated the team on antibiotic resistance, how to use the selected products, helped with the interpretation of results and research outcomes, and wrote up the work for publication (Shibu *et al.*, 2016; Appendix 2).

2.2 INTRODUCTION

Carbapenems are used as last-resort antibiotics for the treatment of infections caused by multidrug-resistant (MDR) *Enterobacteriaceae* (*Escherichia*, *Klebsiella* and *Enterobacter* spp.) and pseudomonads (*Acinetobacter* and *Pseudomonas* spp.). During the last decade carbapenem resistance has been increasingly reported and CPOs have become a growing challenge in healthcare settings (Grundmann *et al*., 2010; Freeman *et al*., 2015). In the UK, CPOs – especially *Klebsiella pneumoniae* CPOs – are a challenge to infection control in hospital settings: firstly, due to their ability to spread and cause outbreaks with different species of *Enterobacteriaceae* with the same mobile resistance gene (Grundaman *et al.*, 2010); secondly, as CPOs inhabit the gastrointestinal (GI) tract, decolonisation therapy only reduces the number of CPOs without elimination (Curran and Otter, 2014). The majority of people are colonised with these organisms with no further complications but in immunocompromised patients colonisation can lead to endogenous infection, which can be a cause of cross-

infection (Curran and Otter, 2014; Patel *et al*., 2007). Furthermore, studies have shown patients with invasive CPO infections have a two-fold higher number of deaths than those infected with antibiotic-sensitive strains (Falagas *et al.*, 2014).

CPOs are extremely MDR (resistant to carbapenems, penicillins and cephalosporins) and often carry genes that confer resistance to other antimicrobials (aminoglycosides and quinolones) leading to limited therapeutic options for infections caused by these organisms (Livermore and Woodford, 2006). CPOs may express a number of different genes which encode different types of β-lactamases capable of hydrolysing carbapenems. These enzymes include the class A carbapenemases (*Klebsiella pneumoniae* carbapenemase (KPC) types), the class B or metallo-β-lactamases (MBLs) (imipenem resistant metallo-β-lactamase (IPM), New Delhi metallo-β-lactamase (NDM), and Verona integron-encoded metallo-β-lactamase (VIM) types), and the class D oxacillinases (OXA) (e.g. OXA-48-like enzymes) (Livermore, 2012a). CPOs are associated with high rates of morbidity and mortality, particularly among critically ill patients with prolonged hospitalisation (Curren and Otter, 2014; Osei Sekyere *et al*., 2016). They are usually resistant to broad-spectrum β-lactam antibiotics (encompassing cephalosporins, carbapenems, penicillins, monobactams, and cephamycins) and β-lactam–β-lactamase inhibitor combinations (ampicillin-sulbactam, amoxicillin-clavulanate, ticarcillin-clavulanate, and piperacillin-tazobactam), oxyimino-cephalosporins (e.g. ceftriaxone, ceftazidime, and cefotaxime), aztreonam and carbapenems (e.g. meropenem, ertapenem, imipenem, and doripenem). They are also frequently resistant to antibiotics used in combination with β-lactam antibiotics (e.g. aminoglycosides, quinolones, and tetracyclines) because of additional types of resistance genes carried by *Enterobacteriaceae*, making them even more of a concern for the health sector since the treatment options are very restricted (Viau *et al*., 2016).

To date, CPOs generally remain susceptible to colistin (although resistance is on the rise) and intermediate to tigecycline. However, there are significant drawbacks in terms of the therapeutic viability of tigecycline which can lead to resistance, and colistin usage is limited due to its neurotoxicity and nephrotoxicity (Carmeli *et al*., 2010; Livermore, 2012a). As such, tigecycline and colistin are used as the antibiotics of last resort to treat CPOs. Increased use of these antibiotics clinically (Freeman *et al.*, 2014) and in animal husbandry (Zdziarski *et al*., 2003)

has led to the emergence of bacteria with resistance to these antibiotics, and resistant strains have been reported during therapy (Rodríguez-Avial *et al*., 2012; Osei Sekyere *et al*., 2016). In addition, carbapenemase-encoding genes harboured by CPOs are mostly transposon- and/or integron-encoded determinants that can easily disseminate to other enterobacterial strains and species (Johnson and Woodford, 2013; Osei Sekyere *et al*., 2016), which means cross-infection among patients is of great concern within hospital settings. GI carriers of CPOs are thought to be the main reservoir of cross-transmission in healthcare settings (Curran and Otter, 2014; Patel *et al*., 2007). Consequently, there is a need to implement adequate preventive measures, including active surveillance, in clinical laboratories to contain the spread of pathogenic CPOs (Viau *et al.*, 2016). Several studies have highlighted the need for such programmes, and how they may help reduce transmission of GI CPOs in combination with control programmes.

During 2006, most hospitals in Israel faced a clonal outbreak of carbapenem-resistant *Klebsiella pneumoniae* that was not contained by local control measures. This led the Ministry of Health to issue guidelines in March 2007 that required individual hospitals to isolate patients who were suspected of carriage of resistance organisms with a dedicated nursing staff together with improved early detection methods within the Microbiology laboratory. Prompt feedback alerting infection control of all new carbapenemase-producing *Enterobacteriaceae* (CPE) isolates eventually resulted in the containment of the outbreak (Schwaber *et al*., 2011). Enfield *et al*. (2014) showed strict implementation of the CDC 2012 CRE Toolkit resulted in controlling an outbreak of CPE in intensive care units (ICUs) of hospitals in the United States. The CDC 2012 CRE Toolkit comprised enhanced infection control measures including staff education, improved hand hygiene, strict isolation, thorough environmental hygiene and assessment, and antibiotic stewardship. The drawback with this study was that screening for CPEs was only done once a week and not for each patient on their admission to hospital, so it did not provide the true number of CPE carriers admitted to hospital. Sypsa *et al*. (2012) isolated CPEs on patient admission, kept CPE-colonised patients together and away from non-colonised patients, and implemented improved hand hygiene and contact precautions in the surgical unit of a tertiary-care hospital in Greece. This provided a better surveillance system and led to a notable reduction of the spread of CPE within 8-12 weeks of

implementation. A study carried out in the ICU ward of New York Downstate Medical Center, Brooklyn, documented a significant reduction in CPO carriage (the mean number (± SD) of new patients per 1,000 patient-days per quarter with cultures yielding carbapenem-resistant *K. pneumoniae* decreased from 9.7 ± 2.2 before the intervention to 3.7 ± 1.6 after the intervention (P<0.001)) within the first year of implementation of a screening and infection control programme (Kochar *et al*., 2009). Success of the programme relied on screening of GI carriage of carbapenem-resistant *Klebsiella pneumoniae* and *Acinetobacter baumannii* by culturing rectal swabs and isolating patients in curtain-partitioned cubicles while results were pending; if results were positive, the ward was closed for 2 days and cleaned with isopropanol and an ammonium-based compound. Availability of a quick screening test would likely have led to even greater reduction of carriage rates (Viau *et al.*, 2016).

Kwong *et al*. (2018) showed rapid identification of CPE using real-time PCR methods combined with epidemiological and genomic investigations (i.e. whole genome sequencing (WGS) of the resistant bacteria) was critical for identifying and controlling transmission of CPE in Australian hospitals. The results of the study suggest that routine WGS of isolates would allow centralised comparison of CPEs between different hospitals that would aid rapid identification and location of the source of infection, and the potential to control further transmission. The main drawback of this study was the high cost, although WGS technologies are getting cheaper; albeit still rather prohibitive for routine screening use in the clinical setting.

Colonisation rates for CPEs have been on the increase in the UK over the past decade. In 2014, Public Health England (PHE) produced guidelines to prevent or reduce the spread of CPEs in health and residential care settings (PHE, 2014). They recommended risk-factor-based screening of all patients on admission to acute healthcare settings (PHE, 2013). This included the following measures.

1. Determining whether the patient had recently been admitted to any hospital in countries/regions with reported high prevalence of healthcare-associated CPE (e.g. North Africa and the Middle East, Bangladesh, China, Cyprus, Greece, India, Ireland, Israel, Italy, Japan, Malta, Pakistan, South East Asia, South/Central America, Turkey, Taiwan and the USA).

2. Determining whether the patient had recently been admitted to a hospital in areas of the UK where problems associated with CPE had been reported (the North West (especially Manchester) and London).

3. Following the patient admission flow chart for infection prevention and control of CPE (Figure 2.1).

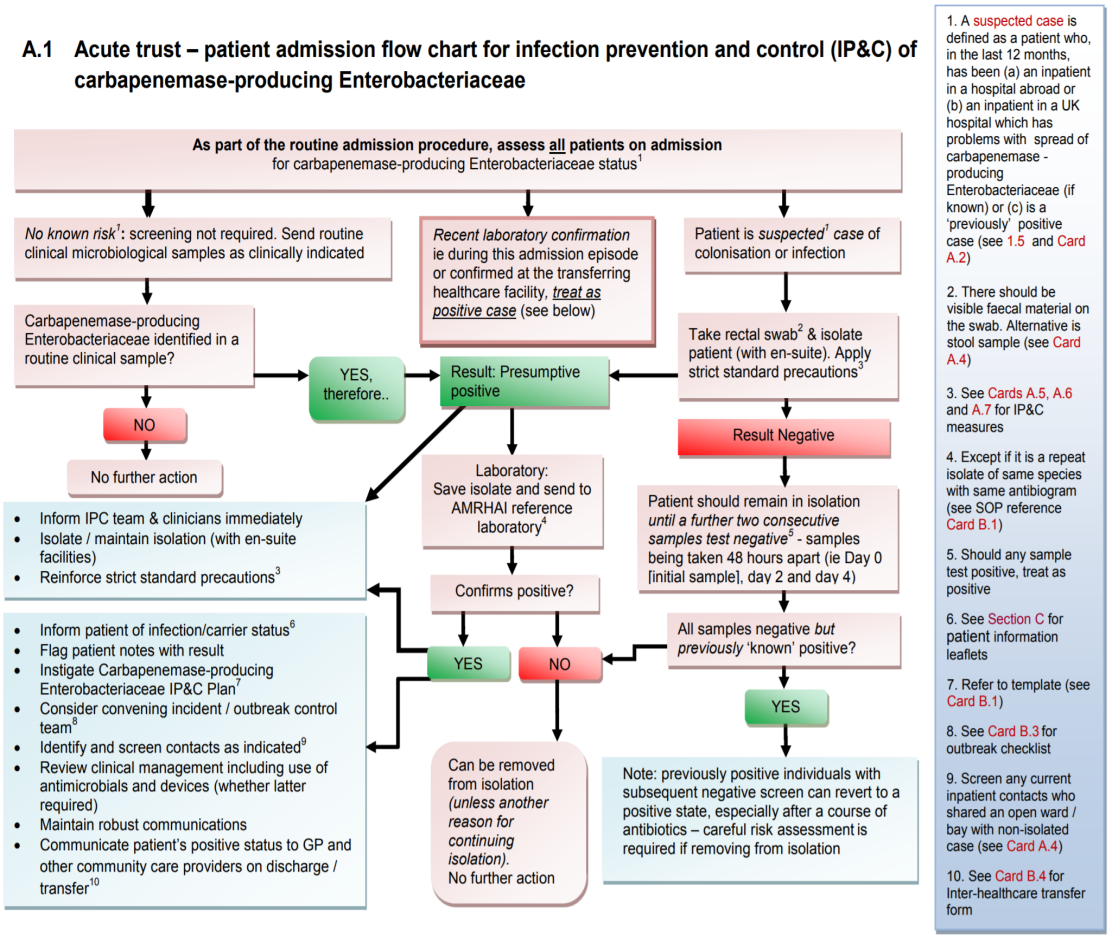


Figure 2.1. Acute trust patient admission flow chart for infection prevention and control (IP&C) of CPE including pseudomonads. Image reproduced from PHE (2013) under the terms of the Open Government Licence v2.0.

The threat of carbapenem resistance within the UK's healthcare system is further increased because the above-mentioned organisms can rapidly acquire an additional resistance gene that encodes for the New Delhi metallo-β-lactamase (NDM). The resistance genes are carried on plasmids which also harbour other

antibiotic resistance genes with rapid dissemination to other species of *Enterobacteriaceae*. This resistance (i.e. NDM) is now highly prevalent in *Klebsiella pneumoniae*. NDM *Klebsiella pneumoniae* was introduced into the UK by individuals returning from visits to India or Pakistan, where the NDM variant is endemic (Johnson and Woodford, 2013; Nordmann *et al*., 2011). This led to NDM strains causing outbreaks in the high-dependency units of west London hospitals.

2.2.1 Prevalence of carbapenemase resistance in UK

The first confirmed UK detected carbapenemase was an *Acinetobacter* isolate with IMP-1 in 2000, isolated from a hospitalized patient with a history of travel from an endemic area. From 2008, the numbers of confirmed CPE increased dramatically in the UK: 23 in 2008, 73 in 2009, 333 in 2010, and 561 in 2011 (Figure 2.2). These were seen more in *Klebsiella* spp. (80%, mainly *Klebsiella pneumoniae),* followed by *Escherichia coli* (10%) and *Enterobacter* spp. (8%), with the remaining 2% comprising occasional isolates of *Citrobacter*, *Morganella*, *Providencia*, *Raoultella and Serratia* spp*.* The enzymes produced included KPC (62%), NDM (14%), VIM (12%), OXA-48-like (9%) and IMP (2%) types, each from a different hospital around north western side of UK. In 2008, Hammersmith Hospital (HH), part of the Imperial College Healthcare NHS Trust (ICHNT) in the London area, experienced the first carbapenemase outbreak in the renal unit. This was caused by *Klebsiella pneumoniae* with OXA-48; which was a dominant outbreak strain, but with horizontal transfer of an OXA-48-encoding plasmid to other strains (Thomas *et al.*, 2013).

From 2008, the southern area of UK started experiencing a wider distribution of *Enterobacteriaceae* with NDM and by the end of 2011 genes encoding NDM enzymes had been detected in 138 isolates of *Enterobacteriaceae* and 11 *Acinetobacter baumannii* isolates. Multiple species with NDM enzymes were isolated from several patients, which is consistent with either *in vivo* transfer of a resistance plasmid or initial colonisation or infection by more than one species. The epidemiological follow-up for these cases showed all these patients with NDM-positive isolates had travelled to or had healthcare contact in India or Pakistan.

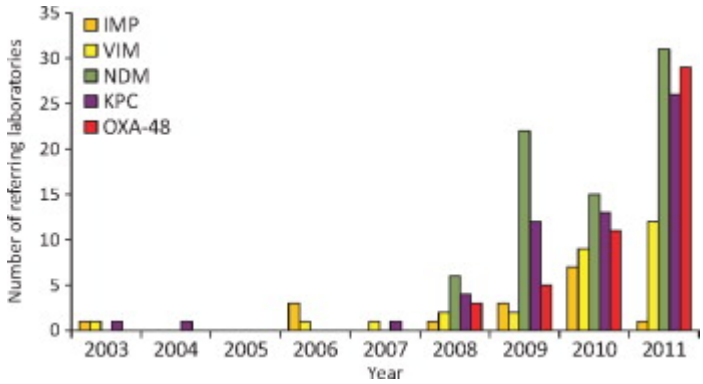


Figure 2.2. Numbers of UK laboratories referring at least one carbapenemase-producing *Enterobacteriaceae* (CPE) isolate to the Antibiotic Resistance Monitoring and Reference Laboratory (Health Protection Agency). Additionally, in 2010 and 2011, two and one laboratories referred IMI-producing CPE isolates, respectively. In 2011, one laboratory referred at least one CPE isolate producing both KPC and VIM enzymes (Cantón *et al*., 2012).

2.2.2 CPE history at the Imperial College Healthcare NHS Trust

The first isolation of CPE (OXA-48) in the West London Hospitals belonging to the ICHNT was in 2008 in the renal unit of HH from a routine wound culture. PHE was notified of its isolation. Subsequent years showed an increase in OXA-48 CPE within the renal unit and other wards within HH. A general increase of these CPE in UK hospitals was also noticed by the PHE surveillance unit at Colindale, which responded to the need to control their spread by the introduction of the CPE tool kit in 2014 (PHE, 2013).

Between 2014 and 2015 a CPE outbreak of NDM CPE in the renal unit at HH and the vascular unit at St Mary's Hospital (SMH) was identified, and 40 patients were confirmed with NDM *Klebsiella pneumoniae* on screening. The CPE toolkit (PHE, 2013) was only being used as a guide to screen selected high-risk patients at that time. As such, NDM *Klebsiella pneumoniae* was not being monitored in the ICHNT, as it was not a recognised problem within the Trust at that time. The outbreak was traced to a renal patient who had travelled abroad for surgery, returning to the UK with their gut colonised with NDM *Klebsiella pneumoniae*. The patient was admitted to both HH and SMH during the timeframe consistent with being the source of cross-transmission of the resistant CPE among

patients in this outbreak. The ICHNT response to this was to recognize the need for a continuous widespread screening campaign that was cost-effective, rapid, accurate, reproducible and able to cope with the large volume of samples expected each month (~4000 patients/month), with good sensitivity and specificity for all CPE and not only OXA-48. The Microbiology laboratory at Charing Cross Hospital (CXH) worked closely with the infection control team to implement such a large-scale screening programme, dealing with samples from all five hospitals within the West London ICNHT. The objective of this study was, therefore, to identify carbapenem-resistant organisms (CROs) using routinely collected local microbiology data, to describe the epidemiology of carbapenem resistance among clinical *Enterobacteriaceae* and its spread within five West London Hospitals. In order to manage the high laboratory demand for a large screening programme contributing to preventing or reducing the spread of CPE within ICHNT, a trial of different media and in-house testing was performed for one week.

Before implementation of a screening programme it is important to consider certain factors, i.e. patient type at admission, identifying the high-risk group, the baseline prevalence and type of resistance mechanism within the locality. First, the infection control team worked out a screening protocol to identify high-risk patients according to the CPE toolkit (PHE, 2013) and appropriate in-house considerations. All high-risk general medicine admissions to the five hospitals within the Trust were screened for CPE for one week. In addition, all patients within the renal unit, the vascular unit, and all private patients, paediatric ICU patients and cancer patients were screened for CPE for one week. This was done to identify those at risk of CPE infections, and to gather baseline information regarding what CPEs were in circulation within the Trust.

The Microbiology laboratory concurrently conducted a study to assess and validate four different chromogenic media (Brilliance CRE; chromID Carba; CXmSCARBA-1; and CXmSCARBA-2) designed for the isolation of CPEs including those producing OXA-48-like enzymes. These media incorporate antimicrobials for the inhibition of other micro-organisms and biomarkers to differentiate species using chromogenic substrates with a pH indicator. The overall aim was to find the most rapid, cost-efficient screening method with high sensitivity and specificity for the detection of CPEs for use in the West London ICHNT. They were compared with the standard method in use at the time, which comprised

inoculating samples (mainly rectal/perineum swabs) onto a MacConkey plate with ertapenem and meropenem discs (10 µg) and further characterisation of any isolates that showed resistance to these antibiotics. Namely, phenotypic identification of isolates (using matrix-assisted laser desorption/ionization time of flight; MALDI-TOF), testing with additional antibiotics and presumptive CPOs being sent to PHE for typing to identify the resistance gene(s).

As the work on CPE screening was started at ICHNT, van der Zwaluw *et al.* (2015) published a new method (CIM method) to detect carbapenemase activity in Gram-negative rods within 8 h. This method reported high concordance with results obtained by PCR to detect genes coding for the carbapenemases IMP, KPC, NDM, OXA-23, OXA-48, and VIM. It was said to allow reliable detection of carbapenemase activity encoded by various genes in species of *Enterobacteriaceae* (e.g. *Klebsiella pneumoniae, Escherichia coli* and *Enterobacter cloacae*), but also in the non-fermenters *Pseudomonas aeruginosa* and *Acinetobacter baumannii.* The CIM method was reported to be a cost-effective and highly robust phenotypic screening method that could reliably detect carbapenemase activity. The medical team thus requested that the efficacy of this method be tested in a limited number of samples initially (due to limited resources). Accordingly, 18 positive controls and four negative controls were accessed by this method.

2.3 MATERIALS AND METHODS

2.3.1 Samples

Clinical samples

Rectal, perineal and/or throat swabs were taken for CRO screening from 236 patients hospitalised on ten different wards during an outbreak (mainly in the high-dependency units, including the renal dialysis unit) at HH and SMH between March and April 2015.

Positive and negative controls

The positive controls comprised a collection of 20 CROs obtained from clinical samples (previously isolated from patients during routine screening; with all positive isolates stored in viabank cryogenic storage beads (ProLab) at -70°C). The isolates had been defined at the molecular level by the reference laboratory,

as part of standard operating procedure. The resistance mechanisms were determined by multiplex PCR (Poirel *et al*., 2011a), which detects various genes that encode for acquired carbapenemases. The positive controls comprised three isolates with NDM-1 (*Escherichia coli*, *n* = 1; *Klebsiella pneumoniae*, *n* = 1; and *Proteus mirabilis*, *n* = 1), two with IMP (*Escherichia coli*, *n* = 1; and *Pseudomonas aeruginosa*, *n* = 1), five with KPC (*Klebsiella pneumoniae*, *n* = 5), five with OXA-48 (*Acinetobacter baumannii*, *n* = 1; *Escherichia coli*, *n* = 1; *Klebsiella pneumoniae*, *n* = 2; and *Serratia marcescens*, *n* = 1), and five with VIM (*Enterobacter cloacae*, *n* = 1; *Klebsiella oxytoca*, *n* = 1; *Klebsiella pneumoniae*, *n* = 1; and *Pseudomonas aeruginosa*, *n* = 2).

The negative controls comprised 20 isolates of non-CROs (taken from a collection of PHE characterised patient isolates stored in viabank cryogenic storage beads at -70°C) showing a diverse range of resistance mechanisms consisting of extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases with porin loss (Table 2.1). These were characterised by the standard operating protocols for susceptibility testing conforming to British Society for Antimicrobial Chemotherapy (BSAC) guidelines (BSAC, Version 12, 2015). Isolate identification was performed by MALDI-TOF MS (Biotyper, Bruker).

2.3.2 Culture of bacteria from clinical swabs

Each clinical swab was suspended in 0.5 ml of sterile saline (0.85% in water) to generate a homogeneous suspension and processed using the in-house screening protocol (Figure 2.3). After vortexing, an aliquot of this suspension (50 µl) was used to inoculate each chromogenic medium (Wilkinson *et al*., 2012). In the limited number of cases that multiple swabs (from different sites) were obtained from the same patient, a single suspension was prepared using all swabs (rectal, perineal and/or throat). Samples were plated on to each of four different chromogenic media: Brilliance CRE (PO1226A, Oxoid Ltd, UK), chromID Carba (bioMérieux, France), CXmSCARBA-1 (Chromagar, France) and CXmSCARBA-2 (Chromagar, France) provided by E&O laboratories (Figure 2.4).

Table 2.1: Negative controls used in this study.

Control	Strain	Characteristics*
1	*Enterobacter cloacae* M22	AmpC positive
2	*Klebsiella pneumoniae* W88	ESBL positive with porin loss
3	*Enterobacter aerogenes* W31	AmpC positive
4	*Enterobacter* spp. X34	AmpC + ESBL positive
5	*Klebsiella pneumoniae* W68	ESBL positive
6	*Escherichia coli* H97	ESBL positive
7	*Klebsiella pneumoniae* F44	ESBL positive
8	*Klebsiella pneumoniae* H32	ESBL positive with porin loss
9	*Klebsiella pneumoniae* T10	ESBL positive with porin loss
10	*Klebsiella pneumoniae* T73	ESBL positive with porin loss
11	*Enterobacter* spp. H88	AmpC positive with porin loss
12	*Klebsiella pneumoniae* F22	CTX M ESBL positive with porin loss
13	*Enterobacter cloacae* W46	AmpC positive
14	*Klebsiella pneumoniae* M68	AmpC + ESBL positive
15	*Klebsiella pneumoniae* X15	ESBL positive
16	*Pseudomonas aeruginosa* M30	Porin loss + efflux
17	*Enterobacter cloacae* F09	AmpC positive
18	*Klebsiella pneumoniae* H03	ESBL positive
19	*Pseudomonas aeruginosa* S89	Porin loss
20	*Enterobacter cloacae* W66	ESBL + AmpC positive

*AmpC, AmpC-type β-lactamase, ESBL, extended-spectrum β-lactamase; CTX M ESBL, cefotaxime-resistant ESBL.

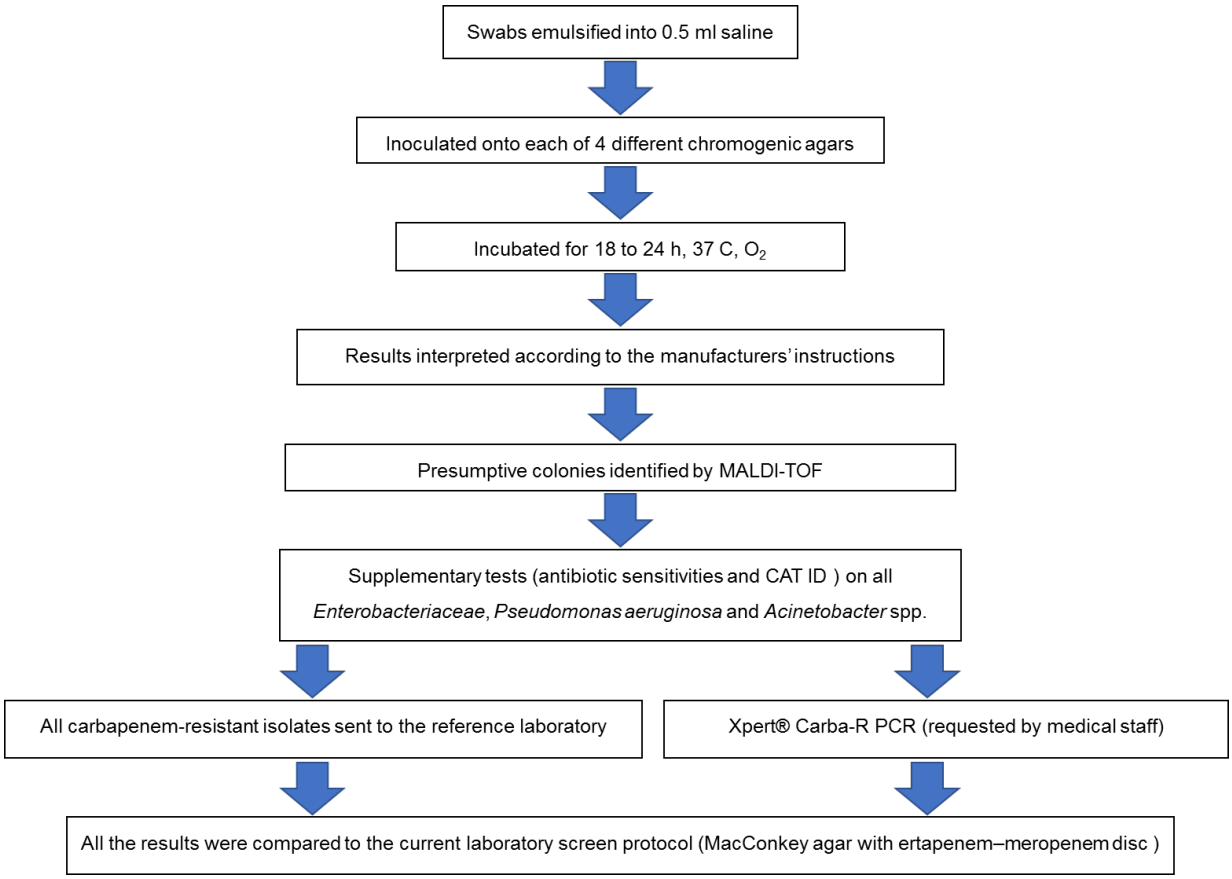


Figure 2.3. Protocol used in the initial CRO screening study in ICHNT.

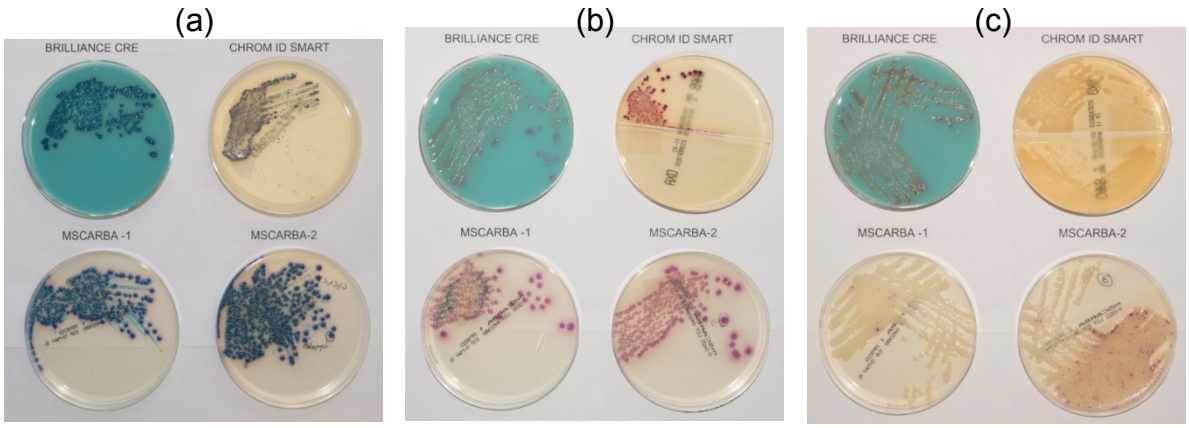


Figure 2.4. Examples of culture growth on the four different chromogenic media. (a) *Klebsiella pneumoniae*; (b) *Escherichia coli*; (c) *Acinetobacter baumanii.* Note: Chrom ID Smart = ChromID Carba.

The inoculum was streaked to obtain individual colonies and all media were incubated aerobically at 37 °C for 18-20 h. Both coloured and white colonies were regarded as presumptive CROs in accordance with the manufacturers' instructions (Figure 2.4). Strains of *Escherichia coli* produced red colonies on all four chromogenic media, whereas *Klebsiella pneumoniae* produced blue colonies on Brilliance CRE, CXmSCARBA-1 and CXmSCARBA-2, and green colonies on chromID Carba. *Pseudomonas aeruginosa* and *Acinetobacter baumannii* both formed colourless/white colonies on all four chromogenic agars. *Pseudomonas* spp. and *Acinetobacter* spp. were distinguished by performing an oxidase test (Oxoid), the former being oxidase positive and the latter oxidase negative.

The clinical suspensions were also plated on to MacConkey agar (Oxoid) with ertapenem and meropenem discs (10 µg; Oxoid), the standard method at the time – which followed the standard operating protocols of the national guidelines (UK Standards for Microbiology Investigations, 2013) with susceptibility testing conforming to BSAC guidelines (Version 12, 2013; http://bsac.org.uk/stewardship-surveillance/susceptibility/methodologylatestversion/). Here any *Enterobacteriaceae* with zones of inhibition (<27 mm) for ertapenem and/or meropenem were regarded as presumptive CROs. All presumptive CRO colonies were stored in cryobank cryogenic storage beads at -70°C and subcultured on nutrient agar slopes for sending to the PHE reference for laboratory identification. The culture media with a pure culture displaying colonies with morphological characteristic of interest were identified to species level using MALDI-TOF MS. If the culture plate were mixed or did not show enough growth (i.e. only one or two colonies in total), the colonies were subcultured on to blood agar (Oxoid) and identified the following day. All identified *Enterobacteriaceae* isolates from each patient were subcultured on to blood agar and incubated overnight to perform phenotypic testing.

2.3.3 Culture of positive and negative controls

Each isolate stored in cryovial beads at -70 °C was thawed and subcultured onto non-selective blood agar and incubated aerobically for 24 h at 37 °C. Colonies of fresh pure cultures of each positive control were then suspended in sterile saline and adjusted to McFarland 0.5 (~10^8 CFU/ml) with the aid of a

densitometer (Densimat, bioMérieux).The adjusted suspension was then ten-fold serially diluted to 10^5 CFU/ml (Wilkinson *et al*., 2012). Aliquots (10 µl) of each dilution were inoculated onto each of the four chromogenic agar plates. For the negative controls, a 10 µl aliquot of an undiluted 0.5 McFarland suspension was inoculated onto each of the four chromogenic agar and checked for growth following incubation. All culture plates were incubated for 18-20 h at 37 °C.

The sensitivity and specificity for all methods were calculated using the formulae:

Sensitivity = TP/(TP + FN)

Specificity = TN/(FP + TN)

Positive predictive value = TP/(TP + FP)

Negative predictive value = TN/(FN + TN)

TP = true positive; FP = false positive; TN = true negative; FN = false negative.

To ascertain the limit of detection (LOD) of the four chromogenic media, control organisms displaying the five different resistant mechanisms were selected and a fresh pure culture of each was suspended in saline and adjusted to the density of McFarland 0.5 (~10^8 CFU/ml), serially diluted to 10^{-8} (i.e. to 1 CFU/ml), and the different dilutions spread plated onto the four chromogenic media. The LOD was determined based on the minimal colony count allowing detection on the respective screening plates.

2.3.4 Carbapenem Inactivation Method (CIM)

A suspension of 10 µl (loop full) of organism grown on blood agar was prepared in 400 µl of water for each control strain. Subsequently, a disc containing 10 µg meropenem (Oxoid) was immersed in the suspension and incubated for a minimum of 2 h at 37 °C. Similarly, discs containing ertapenem and imipenum (10 µg; Oxoid) were used separately. The disc was removed from the suspension using an inoculation loop, placed on a Mueller-Hinton agar plate inoculated with a sensitive indicator strain of *Escherichia coli* (ATCC 29522; using 0.5 McFarland suspension) and incubated at 37 °C for 18-24 h. If the control strain produced carbapenemase, the meropenem/ertapenem/imipenum in the disc was inactivated and thus allowed uninhibited growth of the susceptible indicator strain. Discs

incubated in suspensions that did not contain carbapenemases yielded a clear inhibition zone, due to the antibiotics remaining intact.

2.3.5 Identification of bacteria and susceptibility testing

All isolates that grew on CPE selective media were identified by MALDI-TOF MS, whether presumptive CROs or not, to gain some insight into the selectivity of the media. All isolates identified as *Enterobacteriaceae, Psuedomaonas* spp. and *Acinetobacter* spp. were screened for possible carbapenemase production following the standard operating procedures in accordance with the UK national guidelines (PHE, 2013). This involved sensitivity testing against 18 antimicrobials following the BSAC disc diffusion method, with the inclusion of MAST CAT ID discs for the presumptive CPO isolates showing resistance to meropenem. CAT ID discs consist of an indicator carbapenem (faropenem; MAST group Ltd, UK) and were tested for their sensitivity and specificity for detecting carbapenemase-producers (Figure 2.5).

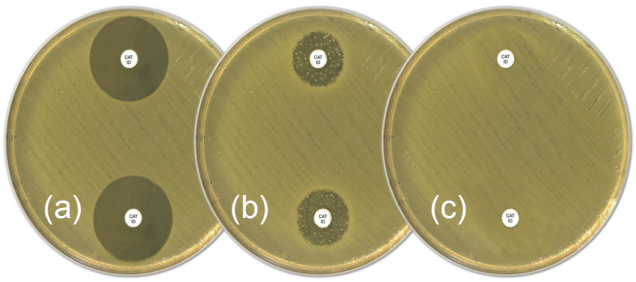


Figure 2.5. Image illustrating interpretation criteria for CAT ID discs. (a) Organism sensitive to CAT ID = negative (non-CPO). (b) Organism showing micro colonies within the zones was interpreted as an OXA-48 type CPO. (c) Organism resistant to CAT ID = positive (CPO).

The results were interpreted using BSAC break points (http://bsac.org.uk/stewardship-surveillance/susceptibility/methodologylatestversion/). Any organisms showing resistance to one of the two selected carbapenem discs (ertapenem and metropenem) were further tested by E-strip (Launch) to ascertain the Minimum Inhibitory Concentration (MIC). Isolates showing phenotypic evidence of

carbapenemase production (i.e. MIC to ertapenem ≤0.5 µg/ml or to meropenem ≤2 µg/ml) were sent to the PHE reference laboratory at Colindale, UK, for typing through Variable Number Tandem repeats (VNTR) analysis and resistance mechanisms sort by multiplex PCR which detects various genes that encode for acquired carbapenemases including class A (GES, IMI, KPC, NMC, and SME), class B (AIM, GIM, IMP, KHM, NDM, SIM, SPM, TBM, and VIM) and class D (OXA-48-like) carbapenemases.

2.3.6 Validation of Cepheid's GeneXpert System (in-house molecular detection of carbapenemases)

Cepheid's GeneXpert System provides a molecular platform claimed to deliver faster, more accurate results that improve patient outcomes, operational efficiencies, and reduce the overall cost of care. The Xpert Carba-R PCR is able to detect the five prevalent carbapenemases (IMP-1, KPC, NDM, OXA-48, and VIM) and can be performed directly from rectal swabs or from culture. The PCR test is quick and simple to use with results available within <1 h. Due to limited resources (only 30 cartridges were provided free by Cephid) to validate the Xpert® Carba-R PCR test, and the priority to test any presumptive isolate that needed an urgent result due to infection control purposes and better patient management, only those isolates identified by the medical microbiology consultants on daily benchrounds were targeted for evaluation. Of the 49 presumptive CPO isolates from patients, 23 isolates were tested together with four positive controls (*Klebsiella pneumoniae* ATCC 1705: KPC positive; *Klebsiella pneumoniae* NCTC 13442: OXA 48 positive; *Klebsiella pneumoniae* NCTC 13443: NDM positive; and *Pseudomonas aeruginosa* NCTC 13437, VIM positive; Oxoid) using Xpert® Carba-R PCR following the manufacturer's instructions. The primers and probes in the Xpert® Carba-R assay detect proprietary sequences for the $bla_{\text{IMP-1}}$ (IMP-1), bla_{KPC} (KPC), bla_{NDM} (NDM), $bla_{\text{OXA-48}}$ (OXA-48), and bla_{VIM} (VIM) gene sequences associated with carbapenem-resistance in Gram-negative bacteria.

2.4 RESULTS

During the screening period, a total of 236 clinical samples (perineum, rectum or throat swabs) were examined for CPOs using the standard method at

that time and to the four chromogenic agars. 236 samples from 236 patients yielded 267 colonies of interest that were subcultured to purity on blood agar and their identification confirmed using MALDI-TOF. These included *Acinetobacter baumannii, Citrobacter koseri, Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Morganella morganii, Pseuodomonas aeruginosa, Pseuodomonas citronellolis* and *Pseuodomonas* spp. In some cases (n = 26; patients) the target organisms were isolated on all five agars and in other cases (n = 264) they were only isolated on some of the media. Colonies which exhibited similar morphological characteristics to the target bacterial groups (for example, correct colour and shape but smaller size) were also checked by Gram staining and any Gram-negative organisms identified by MALDI-TOF. This was done to assess the performance of the chromogenic agars. In all such cases, the isolates were shown to either be Gram-positive cultures (n = 100) or identified as *Enterococcus faecium* (n = 22) or *Stenotrophomonas maltophilia* (n = 9). For the remaining clinical samples (187 patients), no colonies of interest were observed on any of the agars.

A representative isolate of each organism of interest per clinical sample was selected for phenotypic characterisation (n = 85); either that originally isolated by the standard method (n = 31) or the healthiest looking subculture from the chromogenic agars if not found using the standard method for a particular clinical sample (n = 54 samples). Forty-nine of the representative isolates displayed phenotypic evidence of carbapenemase and were further characterized by PCR at the PHE reference laboratory. Thirty-three isolates were confirmed as CPOs (i.e. positive for genes that encode acquired carbapenemases; each isolated from a different patient) and 16 were identified as non-CPOs (showed phenotypic evidence of carbapenemase but PCR confirmed the resistance was due to ESBL/AmpC activities; isolated from 15 different patients, i.e. two of the 16 non-CPOs were isolated from one patient).

The standard screening method isolated 23 of the 33 CPOs, but failed to detect nine NDM and one OXA-48 producer (Table 2.2). Brilliance CRE isolated 32/33 CPOs, failing to detect one NDM (*Klebsiella pneumoniae* 118; which was detected by chromID Carba and CXmSCARBA-2); chromID Carba isolated 31/33 CPOs, failing to detect two NDM (*Klebsiella pneumoniae* 159 and *Klebsiella pneumoniae* 173; both of which were only isolated on Brilliance CRE); whilst both CXmSCARBA-1 and CXmSCARBA-2 isolated 29/33 CPOs and failed to detect

four NDM (three of which were not detected by either of the CXmSCARBA plates) (Table 2.2). Of the 30 presumptive CPOs isolated using the standard method, seven were found to be negative by the reference laboratory PCR (i.e. non-CPOs; false positives) (Table 2.2, Table 2.3). Brilliance CRE isolated the most presumptive CPOs (n = 45), with 13 of these being false positives. Of the 37 presumptive CPOs isolated on Chrom ID CARBA, six were false positives (non-CPOs), whilst seven out of 36 presumptive CPOs isolated on CXmSCARBA-1 were false positive and five false positives were obtained using CXmSCARBA-2 (from 34 presumptive CPOs).

Among the 33 CPO isolates, 32 were confirmed as *bla*$_{NDM}$ carbapenemase producers by both phenotypic testing and PHE reference laboratory PCR. *Klebsiella pneumoniae* was the most prevalent NDM CPO and was isolated from 30 patients. *Escherichia coli bla*$_{NDM}$ carbapenemase producer was isolated from two patients, and *Acinetobacter baumannii bla*$_{OXA-48}$ carbapenemase producer was isolated from one patient.

Table 2.2: Results showing comparison of four different chromogenic culture media for detection of CPOs in clinical samples compared to the standard screening method.

	Medium				
Clinical samples (n = 236)	**Standard method**	**Brilliance CRE**	**chromID Carba**	**CXm SCARBA-1**	**CXm SCARBA-2**
Negative* (n = 188)	206	191	199	200	202
Positive** (n = 48)	30	45	37	36	34
(CPO)*** (n = 33)	23	32	31	29	29
Non-CPO**** (n = 15)	7	13	6	7	5

*****Organisms sensitive to carbapenemase but grew on the plate.

**Presumptive CPOs.

***Organisms with confirmed carbapenemase resistance gene.

****Organisms showing resistance to carbapenemase due to ESBL/AmpC or porin loss.

Table 2.3: Evaluation results for screening of four different chromogenic agar with 236 clinical samples.

	Standard method	Brilliance CRE	chromID Carba	CXm SCARBA-1	CXm SCARBA-2
TP	23	32	31	29	29
TN	196	190	197	196	198
FP	7	13	6	7	5
FN	10	1	2	4	4
Sensitivity	0.6970	0.9697	0.9394	0.8788	0.8788
Specificity	0.9655	0.9360	0.9704	0.9655	0.9754
Positive predictive value	0.7667	0.7111	0.8378	0.8056	0.8529
Negative predictive value	0.9515	0.9948	0.9899	0.9800	0.9802

TP = True positive, TN = True negative, FP=False positive, FN = False negative.

2.4.1. Comparison of the performance of the chromogenic media and standard screening method to detect CPOs

Clinical swab samples from 236 patients were assessed for CPOs using four different chromogenic agars in parallel with the standard method. Forty-nine presumptive CPOs were found from 48 clinical samples, of which 33 were confirmed to be CPOs by the Reference laboratory (32 NDM and one OXA48). In general, the more recently developed commercial chromogenic agars were better for determining which clinical samples in this screening study contained CPOs than the standard method (Table 2.3). All four chromogenic media displayed higher sensitivity and better negative predictive values than the standard method. Three of the chromogenic media also provided better positive predictive values (98.0–99.5%) and specificity as good as (96.6%; CXm SCARBA-1) or better (97.0% and 97.5%; chromID Carba and CXm SCARBA-2, respectively) than the standard method (76.7% positive predictive value and 96.6% specificity) (Table 2.3). The specificity (93.6%) and positive predictive value (71.1%) of Brillance CRE were the lowest of all methods (as a result of the higher number of false positives), but its negative predictive value (99.5%) was the highest.

2.4.2. Investigation of the LOD for the four chromogenic media

The LOD of the four chromogenic media was tested using 20 CPO controls, with different serial dilutions. In general, the positive controls grew well on the four chromogenic agars, with similar colony counts seen for the plated dilutions of positive control strains (~10^8, 10^5 and 10^3 CFU/ml) on the chromogenic plates as obtained using blood agar. However, three of the OXA-48 carbapenemase control strains (*Escherichia coli* S780063, *Klebsiella pneumoniae* NCTC 13442 and *Klebsiella pneumoniae* S315414) showed poor growth on chromID Carba; *Proteus mirabilis* F1266228 (NDM-1) did not grow on chromaID Carba and displayed poor growth on the other three chromogenic media, compared to blood agar counts; and *Klebsiella oxytoca* X1299295 (VIM) did not grow on Brilliance CRE (data not included).

To determine the LOD of each chromogenic agar, a representative positive control strain for each carbapenemase group was re-examined with serial dilution of the McFarland 0.5 suspension to 10^{-8} and plating of undiluted suspension, 10^{-3}, 10^{-5}–10^{-8} dilutions. Overall, CXmSCARBA-1 chromogenic medium was observed to provide the best LOD of carbapenemase-producing bacteria of the four chromogenic media (Table 2.4).

2.4.3. Investigation of the specificity of the four chromogenic media

The different chromogenic media displayed different levels of specificity, based on inhibition of growth of negative controls (n = 20) (Table 2.5). All 20 negative controls displayed heavy growth on blood agar (control plates) (data not included). None of the chromogenic media tested inhibited the growth of all negative controls, and all four media displayed heavy growth for controls 2, 5 and 9 (*Klebsiella pneumoniae* W1945888, W1741068 and T1490010, respectively). In addition, all four chromogenic media afforded growth (weak in some cases) of all nine *Klebsiella pneumoniae* negative control strains. Only five of the 20 negative controls were unable to grow on any of the chromogenic media (3, 4, 6, 13 and 17; five of the seven *Enterobacter* strains tested). Overall, CXmSCARBA-2 inhibited the growth of the negative controls better than the other chromogenic media (Table 2.5).

Table 2.4: Limit of detection of each chromogenic agar determined using carbapenemase-producing positive controls with known resistance mechanisms (n = 20).

Data are presented as CFU/ml.

Medium	NDM* (n = 3)	OXA-48 (n = 5)	KPC (n = 5)	VIM (n = 5)	IMP (n = 2)
Brilliance CRE	10^2	10^2	10^2	10^2	10^3
ChromID Carba	10^2	10^5-10^8	10^2-10^3	10^2	10^3
CXmSCARBA-1	10^2	10^2	10-10^2	10-10^2	10^2
CXmSCARBA-2	10^2	10^3	10^3	10^3	10^3
Blood agar	1-10^2	10^2-10^3	10-10^2	10-10^2	10^2

*NDM, New Delhi metallo-β-lactamase; OXA-48, OXA (oxacillinase) group of β-lactamases (Class D); KPC, *Klebsiella pneumoniae* carbapenemase; VIM, Verona integron-mediated metallo-β-lactamase; IMP, IMP-type carbapenemases (metallo-β-lactamases) (Class B).

2.4.4. Examination of CAT ID disc test for detecting carbapenemase-producers

Due to the constraints in the number of discs that were provided by the manufacturers to validate the product, only the first 38 presumptive CPE isolates (chronologically) were tested with CAT ID to detect the presence of carbapenemases. In general, CAT ID showed good correlation with the PHE results (Table 2.6), apart from two isolates, *Klebsiella pneumoniae* 7 and *Escherichia coli* 22 (CAT ID resistant [indicating presence of carbapenemase] but the PHE results were negative for carbapenemase genes). A possible explanation for this discrepancy may be that these bacteria had porin loss, which can result in a positive CAT ID result. The inaccuracy of CAT ID for carbapenemase detection of *Pseudomonas aeruginosa*, which is intrinsically resistant to faropenem, was also highlighted (Table 2.6). Overall, however, CAT ID was able to detect all of the five common carbapenemases with 85.7% specificity, 96.88% positive predictive value and 100% negative predictive value.

Table 2.5: Examination of the growth of non CPOs (negative controls) on four test chromogenic agars.

Control	Brilliance CRE	chromID Carba	CXmSCARBA-1	CXmSCARBA-2
1	NG*	NG	+/+++	NG
2	+++	+++	+++	+++
3	NG	NG	NG	NG
4	NG	NG	NG	NG
5	+++	+++	+++	+++
6	NG	NG	NG	NG
7	+++	NG	+	NG
8	+++	+	+++	+++
9	+++	+++	+++	+++
10	+++	+	+++	+++
11	NG	+++	+++	+++
12	+++	+	+++	+++
13	NG	NG	NG	NG
14	+++	+	+++	+++
15	+++	+	+++	+
16	+++	+++	+++	+++
17	NG	NG	NG	NG
18	+++	+/+++	+++	+
19	+++	+++	+++	+++
20	+++	+	+	NG
NG	7	7	5	8
+	0	6 or 7	2 or 3	2
+++	13	7 or 6	11 or 12	10

*NG, No growth; +, poor growth; +++, heavy growth.

Table 2.6: Validation of CAT ID using 38 presumptive carbapenemase-producers.

Strain	CAT ID*	Reference laboratory
Acinetobacter baumannii 83	R	OXA 58/51
Escherichia coli 5	R	NDM
Escherichia coli 22	R	Negative
Escherichia coli 176	S	Negative
Escherichia coli 179	R	NDM
Klebsiella pneumoniae 7	R	Negative
Klebsiella pneumoniae 10	R	NDM
Klebsiella pneumoniae 39	R	NDM
Klebsiella pneumoniae 81	R	NDM
Klebsiella pneumoniae 82	R	NDM
Klebsiella pneumoniae 112	R	NDM
Klebsiella pneumoniae 113	R	NDM
Klebsiella pneumoniae 114	R	NDM
Klebsiella pneumoniae 115	R	NDM
Klebsiella pneumoniae 116	R	NDM
Klebsiella pneumoniae 117	R	NDM
Klebsiella pneumoniae 118	R	NDM
Klebsiella pneumoniae 119	R	NDM
Klebsiella pneumoniae 120	R	NDM
Klebsiella pneumoniae 121	R	NDM
Klebsiella pneumoniae 144	R	NDM
Klebsiella pneumoniae 159	R	NDM
Klebsiella pneumoniae 162	R	NDM
Klebsiella pneumoniae 173	R	NDM
Klebsiella pneumoniae 174	R	NDM
Klebsiella pneumoniae 177	R	NDM
Klebsiella pneumoniae 178	R	NDM
Klebsiella pneumoniae 180	S	Negative
Klebsiella pneumoniae 190	R	NDM
Klebsiella pneumoniae 191	R	NDM
Klebsiella pneumoniae 192	R	NDM
Klebsiella pneumoniae 216	R	NDM
Klebsiella pneumoniae 218	R	NDM
Klebsiella pneumoniae 223	R	NDM
Klebsiella pneumoniae 235	S	Negative
Pseudomonas aeruginosa 83	R	Negative

Strain	CAT ID*	Reference laboratory
Pseudomonas aeruginosa 175	R	Negative
Pseudomonas aeruginosa 221	R	Negative
Pseudomonas aeruginosa 236	R	Negative

*R, resistant – indicating presence of carbapenemase; S, sensitive – indicating no carbapenemase.

2.4.5. Susceptibility testing of presumptive CPOs

Of the three antibiotics included in CIM test, imipenem displayed the best detection of carbapenemase activity with a positive result obtained for all of the CPOs tested (irrespective of resistance gene) (Table 2.7). In addition, negative results were seen with imipenem for the four non CPOs included. Ertapenem and meropenem also gave negative results for the four non CPOs. However, meropenem also gave negative results for three CPOs, whilst ertapenem performed worst of the three antibiotics in the CIM test (with 5 CPOs identified as negative) (Table 2.7).

2.4.6. Investigation of in-house PCR detection of carbapenemase-producers

The in-house Xpert® Carba-R PCR performed in this screening study showed 99.9% agreement with the PCR results from the reference laboratory (Table 2.8). But detected a false positive NDM carbapenemase gene for *Klebsiella pneumoniae* X96, which the reference laboratory identified was negative (Table 2.8). The discrepancy may be due to poor laboratory technique. It was also noted that three of the samples physicians requested be tested were actually Gram-positive organisms (not one of the target organisms). This was due to clinical considerations and the fact these PCRs were set up for these strains (which were also sent to PHE reference lab) before isolate identification was completed. Whilst this was a relatively limited investigation (24 presumptive CPOs, three Gram positive organisms and four control cultures), the findings suggest that Xpert® Carba-R PCR could be a useful in-house method for the rapid detection of carbapenemase activities.

Table 2.7: Investigation of CIM test to accurately detect carbapenemase activity of presumptive CPOs.

Strain	Resistance gene	CIM test (10 µg)*		
		Imipenem	Ertapenem	Meropenem
Acinetobacter baumannii 18	OXA-58/51	+	-	-
Enterobacter cloacae 9	VIM	+	+	+
Escherichia coli 7	NDM & OXA-48	+	+	+
Escherichia coli 16	OXA-48	+	+	+
Escherichia coli 43	ESBL	-	-	-
Klebsiella oxytoca 14	VIM	+	+	+
Klebsiella pneumoniae 1	KPC	+	+	+
Klebsiella pneumoniae 3	OXA-48	+	-	-
Klebsiella pneumoniae 4	NDM	+	-	-
Klebsiella pneumoniae 11	NDM	+	-	+
Klebsiella pneumoniae 12	NDM	+	+	+
Klebsiella pneumoniae 13	OXA-48	+	+	+
Klebsiella pneumoniae 15	NDM	+	+	+
Klebsiella pneumoniae 44	ESBL & porin loss	-	-	-
Klebsiella pneumoniae 45	ESBL	-	-	-
Klebsiella pneumoniae 46	ESBL & porin loss	-	-	-
Proteus mirabilis 2	NDM	+	+	+
Pseudomonas aeruginosa 6	VIM	+	-	+
Pseudomonas aeruginosa 8	VIM	+	+	+
Pseudomonas aeruginosa 17	VIM	+	+	+
Serratia marcescens 5	OXA-48	+	+	+
Serratia marcescens 10	NDM	+	+	+

*+, Positive; -, negative.

Table 2.8: Evaluation of Xpert® Carba-R PCR to detect resistance genes harboured by presumptive CPOs and carbapenemase-positive controls.

Strain	Xpert® Carba-R PCR	PHE results
Klebsiella pneumoniae X30	NDM	NDM
Klebsiella pneumoniae F14	NDM	NDM
Klebsiella pneumoniae H84	NDM	NDM
Escherichia coli F81	Negative	Negative
Klebsiella pneumoniae M72	NDM	NDM
Escherichia coli S95	Negative	Negative
Klebsiella pneumoniae H26	NDM	NDM
Gram positive rod W15	Negative	Negative
Klebsiella pneumoniae X30	NDM	NDM
Klebsiella pneumoniae W77	OXA-48	OXA-48
Escherichia coli H03	NDM	NDM
Klebsiella pneumoniae H97	Negative	Negative
Gram positive cocci W73	Negative	Negative
Klebsiella pneumoniae W26	NDM	NDM
Escherichia coli W08	Negative	Negative
Escherichia coli H79	Negative	Negative
Klebsiella pneumoniae W64	Negative	Negative
Gram positive cocci F04	Negative	Negative
Klebsiella pneumoniae W31	NDM	NDM
Escherichia coli F36	Negative	Negative
Klebsiella pneumoniae X96	NDM	Negative
Klebsiella pneumoniae X95	NDM	NDM
Klebsiella pneumoniae S84	NDM	NDM
Klebsiella pneumoniae M19	NDM	NDM
Klebsiella pneumoniae H17	NDM	NDM
Klebsiella pneumoniae H96	NDM	NDM
Klebsiella pneumoniae S62	NDM	NDM
Klebsiella pneumoniae NCTC 13422	OXA-48	OXA-48
Klebsiella pneumoniae ATCC 1705	KPC	KPC
Escherichia coli NCTC 13476	IMP	IMP
Pseudomonas aeruginosa NCTC 13437	VIM	VIM

2.5 DISCUSSION

CPOs are of concern as they are associated with high rates of morbidity and mortality, particularly among critically ill patients with prolonged hospitalisation. CPOs are MDR pathogens, which makes them even more of a concern for the health sector as the treatment options are very restricted. It is also noted that the carbapenemase genes harboured by CPOs are mainly transposon- or integron-encoded determinants that can easily disseminate to other enterobacterial strains and species (Johnson and Woodford, 2013). This means cross-infection is of great concern within hospital settings. These facts suggest the need to implement adequate preventive measures, including active surveillance, to contain the spread of these pathogens. Since GI carriers of CPOs are thought to be the reservoir of cross-transmission in healthcare settings, surveillance is necessary.

There is currently no accepted 'gold standard' method for the detection of CPOs from clinical samples (Nass *et al*., 2013). The purpose of this study was to evaluate screening and confirmation methods that, when paired, would provide a streamlined workflow for the detection of CPOs in the clinical microbiology laboratory.

The first part of this study was evaluation of the suitability of four available chromogenic media that have been recommended for isolation of CPOs, compared to the standard method, to select the appropriate media for isolating CPOs in such surveillance programmes. Secondly, evaluation of different identification/characterization tests available at the time was performed to confirm carbapenemase production of the presumptive CPOs isolated. During outbreaks in clinical settings, it is necessary to determine the carbapenemase gene class of the isolated CPOs. Therefore, evaluation of a multiplex PCR method was also incorporated into the work, as an optimal endpoint of this workflow – designed to achieve the maximum sensitivity and specificity for detection of CPOs, with consideration for the cost and turnaround time for results.

All four chromogenic media were shown to be better at isolating CPOs than the standard method. The standard method performed equally well as the chromogenic agar at inhibiting the growth of TN but performed poorly in relation to FN (i.e. did not pick up all TP). The overall sensitivity of the standard method was 70% with 77% positive predictive value (PPV), much lower than the other media

examined. The standard method did, however, perform comparatively well for specificity (97%) and negative predictive value (95%).

Brilliance CRE isolated the most TP CPOs (32/33) of all media tested, with the least FN. However, this medium also provided more FP and, therefore, was not specific enough to be selected as the screening medium. This was in agreement with previous findings, which showed that Brilliance CRE agar isolated non-CPOs, ESBLs and AmpCs, thus lacked specificity and required further confirmation tests of presumptive CPOs isolated using this medium (Cohen Stuart *et al.*, 2013). The ChromeID Carba performed well in relation to TP and TN, and was also the best medium out of the four in relation to FN, with 94% specificity and 97% sensitivity. However, it showed poor or no growth of OXA-48 control strains and poor growth of *Proteus mirabilis* F1266228 (NDM-1) strains. These results agreed with those of Girlich *et al.* (2013), who demonstrated ChromID Carba had limited efficacy in an OXA-48-dominant setting.

CXm SCARBA-1 recovered less TP than Brilliance CRE and ChromeID Carba, with TN, FP and FN comparatively similar to ChromeID Carba; with 88% sensitivity and 97% specificity. On the LOD experiment CXm SCARBA-1 recovered all the five carbapenemases at a low inoculum of 10^2 CFU/ml. CXm SCARBA-2 recovered less TP than Brilliance CRE and ChromeID Carba, but showed the best TN, FP and FN of the media tested, including the standard method, with 88% sensitivity and 98% specificity. On the LOD experiment CXm SCARBA-2 recovered all five carbapenemase types at a low inoculum of 10^3 CFU/ml, thus did not perform as well as CXm SCARBA-1.

CXm SCARBA-1 and CXm SCARBA-2 displayed the best LODs of the chromogenic media tested for the detection of the five most common CPEs (KPC, NDM, VIM, IMP and OXA-48). Both CXm SCARBA media showed the same sensitivity (87%), but CXmSCARBA-2 showed slightly higher specificity (98%, compared to 97% for CXm SCARBA-1). Whilst both CXm SCARBA media were capable of isolating bacteria with the five carbapenemases, they failed to detect four NDM CPOs from the clinical samples. Although our results showed CXm SCARBA-2 to be more specific in the detection of carbapenemases than the other media, CXm SCARBA-1 was selected for our screening medium because colonies on the agar were more defined and showed better colouration, and so were easier to interpret than those on CXm SCARBA-2. CXm SCARBA-1 was also better in

isolating the non-lactose-fermenting CPOs (i.e. *Pseudomonas* and *Acinetobacter* spp.) and had a lower LOD ($10–10^2$ CFU/ml) for KPC and VIM CPOs than the other media (Table 2.4). The lack of colouration and definition, with weak growth seen using CXm SCARBA-2 made it more difficult to interpret results from this medium than for CXm SCARBA-1.

When selecting a screening chromogenic culture method, it is important to select a medium where the target organisms grow happily, with clear definition and precision of colour (according to manufacturer's instructions) since the experience/expertise of users may vary. With this in mind, the colonies on CXm SCARBA-1 were easier for all biomedical staff with various levels of experience to interpret the results with more accuracy than for CXm SCARBA-2. Accordingly, CXm SCARBA-1 was chosen as the screening medium for clinical CPO surveillance procedures.

A number of CPO characterization tests were investigated for efficacy and consideration of accurate and timely identification of carbapenemase type in the clinical setting. MAST CAT ID uses the carbapenem faropenem in a single paper screening disc compliant with EUCAST, and was shown to be a highly effective and easy-to-use tool in the confirmation of CPOs including OXA-48. However, all 4 *Pseudomonas aeruginosa* presumptive CPOs, 22 *Escherichia coli* and 7 *Klebsiella pneumoniae* displayed resistant CAT ID results (indicating carbapenemase), despite being confirmed as non-CPOs by Reference laboratory (PCR). *Pseudomonas* and *Acinetobacter* spp. are known to be intrinsically resistant to faropenem (Schurek *et al.*, 2007), which may account for these results. In addition, resistance to the CAT ID test may be due to porin loss rather than carbapenemase production. Accordingly, CAT ID would reduce the number of presumptive CPOs needing to be sent to reference laboratory for further gene-based testing, but still potentially include false positives (i.e. non-CPOs) being sent and thus unnecessary costs.

The CIM test relies on the enzymatic hydrolysis of carbapenem antibiotics to detect the presence of carbapenemases. The efficacy of the CIM test was determined using 18 reference laboratory-confirmed CPOs and eight negative controls. Of the three antimicrobials tested, the carbapenem imipenem (10 µg disc) provided 100% agreement with PCR results from the reference laboratory followed by meropenem (10 µg disc) and ertapenem (10 µg disc). van der Zwaluw

et al. (2015) reported one limitation of the CIM was its inability to detect low-level carbapenemase activity among *Acinetobacter baumannii* isolates when using meropenem/ertapenem, which they were able to overcome by the usage of imipenem (10 µg disc). In this study, one *Acinetobacter baumannii* OXA-58/51 strain was included and was consistent with van der Zwaluw and colleagues' observations (i.e. negative for both meropenem and ertapenem, but positive for imipenem). Our results also showed that the CIM was capable of detecting carbapenemase production in *Enterobacteriaceae* (Table 2.7). Therefore, its performance superseded CAT ID. Indeed, the CIM test identified a new carbapenemase-producing isolate with unknown carbapenemase-encoded genes (not currently identified by standard reference laboratory PCRs) during a pilot screening study which ran for six months following the current research work, using the recommended workflow for screening CPEs (i.e. output of this work).

Xpert® Carba-R PCR was able to detect the five most common carbapenemases (KPC, NDM, VIM, OXA-48, IMP-1), with the added benefit that this test can be performed directly using rectal swabs or from bacterial cultures. In addition, in-house use of Xpert® Carba-R PCR affords results for presumptive CPOs within hours, rather than the one to two weeks for PHE reference laboratory results to be returned. Clearly, there are potential clinical benefits from in-house application of Xpert® Carba-R PCR (whether on clinical samples or presumptive CPOs isolated from them). Our results showed good agreement between in-house Xpert® Carba-R PCR results and the reference laboratory resistance mechanism data (Table 2.8).

In this study, the Xpert® Carba-R test was performed from cultures and proved to be a quick and simple confirmative tool for the detection of CPOs (<1 h) for known resistance genes. The only limitations of this assay were that new carbapenemase families or new variants of known families may not be detected and the relatively high cost (approx. £30 per isolate/sample). Initially, any presumptive CPOs identified using the standard method were confirmed by the modified Hodge test (mHT) (PHE, 2013). However, the mHT lacked specificity and produced FP results for organisms harbouring ESBL/AmpC or loss of permeability. This resulted in a large volume of FP isolates (non-CPOs) being sent to the reference laboratory, which increased costs. In addition, the mHT is very labour-intensive, so not feasible for high numbers of samples/isolates. The findings of our

study demonstrate that mHT should be replaced with either Xpert® Carba-R PCR or the CIM method. However, whilst the Xpert® Carba-R PCR carbapenemase gene verification results showed high sensitivity and specificity, the costs and technical difficulties of this multiplex PCR assay are not ideal for routine use as a confirmatory test for CPOs. As such, our clinical recommendation was that Xpert® Carba-R be used when it is clinically relevant to confirm carbapenemase activity and gene class (i.e. including for infection, prevention and control investigation, and/or when the clinical situation requires rapid confirmation). It should also be noted that the CIM method requires extensive training of staff before use and is, thus, not as user-friendly across clinical laboratories as the CAT ID test.

Taking everything into consideration (including performance (sensitivity, specificity, timeliness), ease of use by all technical staff and cost of the screening and confirmatory tests), the proposed workflow for CPO screening in clinical settings was to use CXmSCARBA-1 medium followed by the CAT ID (which was easier to perform than CIM) to reduce the number of non-CPOs sent for PCR testing. Xpert® Carba-R PCR should then be used to confirm positive carriers rapidly, limiting the unnecessary prolonged isolation of newly admitted patients and enabling the rapid isolation of those who are carriers, providing better management of patients and guiding the appropriate antibiotic therapy, thus reducing costs to the infection control programme and preventing outbreak situations. However, using Xpert® Carba-R PCR as a universal screening tool for rectal samples may not be desirable or affordable due to its high cost (£30 per sample). It can also be used as a tool for screening high-risk patients such as those returning from areas of endemicity, those who have been transferred from long-term care facilities or those who are known to have been in contact with a carrier.

The MAST CAT ID proved to be a good indicator for the presence of carbapenemases in *Enterobacteriaceae* but was not suitable for *Pseudomonas* spp. As it is not specifically designed to detect OXA-48-like β-lactamases (interpretation of the disc is subjective to the observation of micro colonies within the zone; Figure 2.5) and their derivatives which are the main cause of carbapenemase resistance in *Acinetobacter* spp., this test is also not advisable for confirming *Acinetobacter* CPOs. However, high-level resistance to temocillin is a

presumptive indicator for the presence of OXA-48 in *Acinetobacter* spp. (Glupcznski *et al*., 2012), so could be used in such cases.

2.5.1 Impact of the screening study on protocols/standard procedures for surveillance programmes and subsequent observations/findings

Based on the initial screening study the infection control team at ICHNT implemented a risk-based admission screening programme taking into account the prevalence of carbapenemase resistance within the community, travel patterns and demographics of the population and the level of care provided by the hospital. The laboratory protocol below was implemented to deal with the screening specimens received (Figure 2.6), with additional weekly screens for one month on wards housing patients with carbapenemase-positive samples.

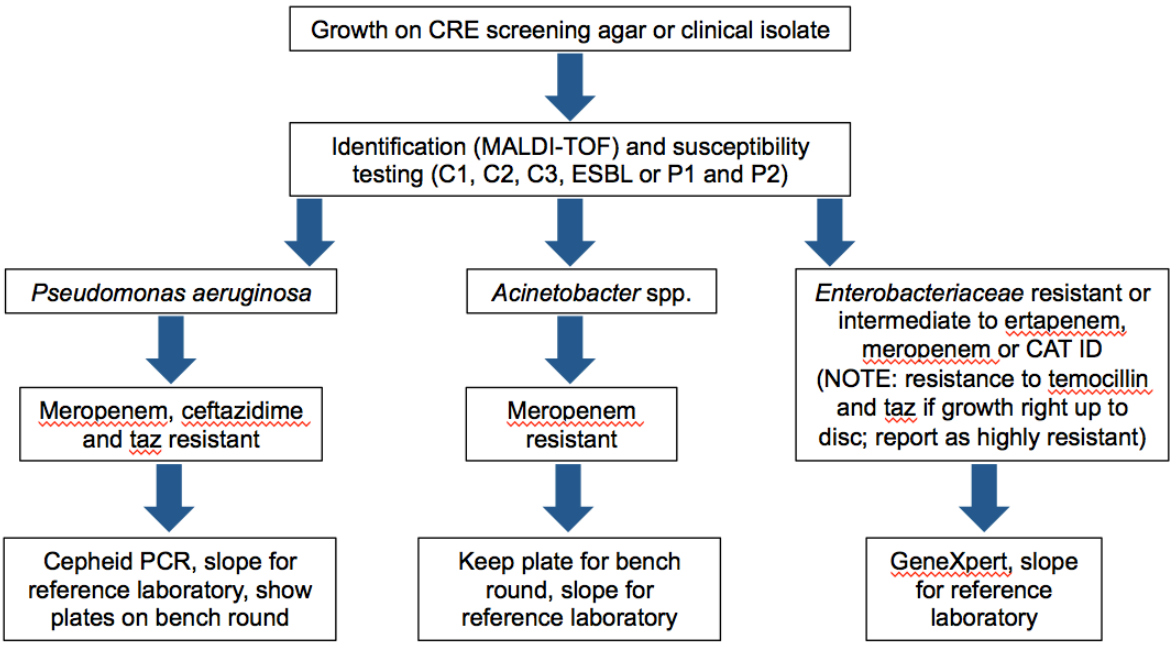


Figure 2.6. Flow chart for screening programme put together for the West London Hospitals belonging to the ICHNT. Average cost per screen £8.

The “Guiana extended spectrum" (GES)-type plasmid-encoded β-lactamase belongs to the class A enzyme and is an ESBL discovered in 2000 in *Klebsiella pneumoniae* clinical isolates from French Guiana (Naas *et al*., 2016; Bonnin *et al.*, 2017). This carbapenemase slowly hydrolyses carbapenems due to

a replacement of glycine at position 170 by asparagine/serine. GES-5-positive *Klebsiella oxytoca* have been detected in UK hospitals, with their emergence thought to be due to plasmid or intergron transfer among related bacteria (Diene and Rolain, 2014). The optimised workflow was able to isolate such *Klebsiella oxytoca* GES-5 strains, previously not found in the ICHNT environment. (These strains will be discussed further in Chapter 3.) This also shows the importance of using a culture method as an initial platform to detect the presence of 'new and emerging' carbapenemases before they are characterised at the genomic level.

Benefits noted since the introduction of the surveillance programme in March 2015 include improved infection control, although compliance with sample collection varied across wards/hospitals within the ICHNT (Figure 2.7). There was also improved record-keeping with respect to carbapenemase-positive samples, which enabled detection of 'potential' outbreaks (Figure 2.8). In addition, analysis of the data gathered (both from the routinely collected microbiology data and reference laboratory) enabled greater understanding of the epidemiology of carbapenem resistance in the ICHNT. This led to recognition of the increasing issue with carbapenem resistance within inpatient healthcare settings in the West London Hospitals (Figure 2.8). The prevalence of different CPOs and resistance mechanisms were found to change over time and more CPEs were isolated during the winter months. This could be due to increased respiratory infections in vulnerable cohorts in the community during the winter months (December–February) with complications that require hospitalization.

The question remains as to whether we are still missing any potential CPO carriers even with the screening programme. It is difficult to assess the sensitivity and specificity of the screening programme, but case studies have highlighted two instances of "silent carriers" within a bay in a ward of the renal unit of HH that were missed by the screening programme. All screened cases in the bay indicated no further spread of the CPE but typing of the two CPE isolates (from "silent carriers") were consistent with that of an outbreak three months previously. One contact had been negative on previous routine screening and their positive CPO from subsequent screening was consistent type with that of an outbreak three months prior to their positive screen. The second case was a patient found to be CPO-positive on ward weekly screening but who had been negative on six previous routine screenings. The typing of the isolate confirmed a match with a previous

contact of a CPO case nine months earlier (personal communication, Francis Davies).

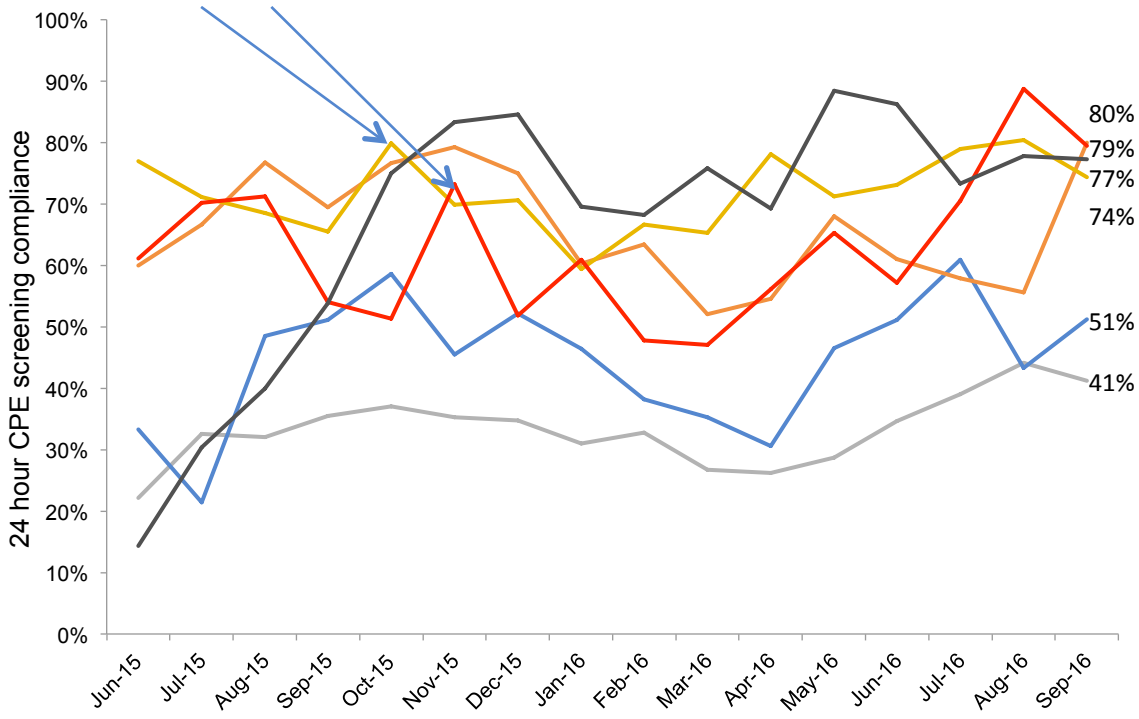


Figure 2.7. Compliance with screening policy in ICHNT. Arrows indicate peaks representing outbreaks of NDM in the vascular and renal units in 2015. Grey, all trust; light blue, private patients; orange, adult & paediatric ICU; yellow, renal unit; red, vascular unit, black, haematology (image produced by the statistics department of the ICHNT).

Although ICHNT managed to introduce a successful screening method, the FP rate was relatively high. Based on observations, ~40% of the presumptive CPOs tested with the in-house Xpert® Carba-R PCR were negative but very few of these were confirmed as CPO by the reference laboratory (due to other resistance mechanisms, as stated above). It is plausible that inclusion of the CIM confirmatory test (of presumptive CPOs) may reduce the number of FPs being sent to reference laboratory thus reducing cost. In an effort to further improve specificity, evaluation of different methods of isolation and molecular characterisation were – at the time of writing – ongoing within the ICHNT.

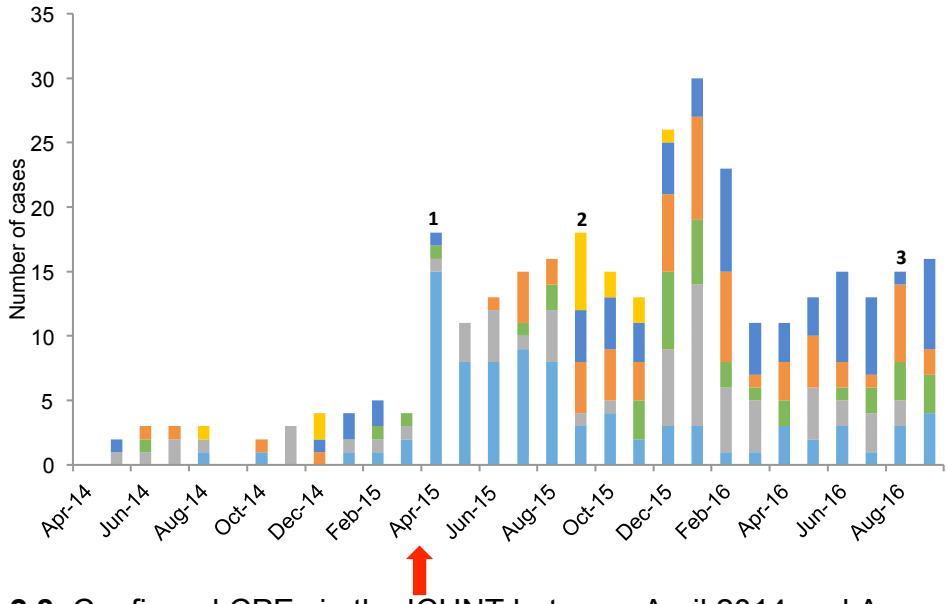


Figure 2.8. Confirmed CPEs in the ICHNT between April 2014 and August 2016. Yellow, *Klebsiella* sp. GES-5 outbreak; dark blue, *Enterobacteriaceae* (other); orange, *Escherichia coli* OXA-48; green, *Escherichia coli* NDM; grey, *Klebsiella pneumoniae* OXA-48; light blue, *Klebsiella pneumoniae* NDM. Red arrow indicates introduction of new screening programme; the 6-month screening study ran between March and September 2015. 1, *Klebsiella pneumoniae* was the bacterium isolated most frequently and was responsible for an NDM-associated outbreak in April 2015. 2, An outbreak caused by GES-5-positive *Klebsiella oxytoca* was detected with the improved screening method. 3, An outbreak of *Escherichia coli* OXA-48 detected – slightly higher in number compared to preceding months, similar to that in December 2015 to February 2016.

The limitation of this study was the scarcity of products to test due to financial constraints and lack of time to carry out experiments; most of the work was conducted during routine working hours. However, strengths of the study were that it was conducted in real time using multiple chromogenic media and was the first study to evaluate the CXm SCARBA-1 and CXm SCARBA-2 media. This study also resulted in the implementation of the first carbapenemase screening programme in the ICHNT. This allowed additional infection control measures to target CPE to be launched in April 2015: enhanced screening (including renal outpatients), contact precautions for known CPE carriers, enhanced chlorine

disinfection of the environment, labelling of electronic case notes for identification of readmission, regular teleconference calls internally and with PHE, and enhanced antibiotic stewardship.

This study also gained a lot of publicity as it was presented orally to clinicians/infection control and technical staff at CXH. A scientific poster presentation of the study also took place at the European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Amsterdam, Netherlands 9-12 April 2016 (Shibu *et al.*, 2016; Appendix 2). The poster was also being used in promotional material by E & O Laboratories Ltd., for their growth media. The screening study led to implementation of a surveillance programme that satisfied PHE recommendations. Dissemination of my work led staff at other hospitals to contact me to provide guidance on organising screening studies within their hospitals. In addition, PHE acknowledged how well I ran the study (personal communication). Since the introduction of the new screening programme brought about a reduction of spread of CPOs in the ICHNT, the infection control team was very appreciative of my work and in return partly funded my fees for my Professional Doctorate. A summary of the work was published in the October 2016 edition of *The Biomedical Scientist*.

2.6 SUMMARY

Screening for GI carriage of CPOs is significant for the development of infection control strategies for these organisms. When selecting a screening programme, adopting a universal surveillance has greater potential for detection and prevention of outbreaks but comes at a cost. This study showed chromogenic culture media are useful as screening tools for the isolation of antimicrobial-resistant CPOs when compared with the standard method at that time (namely, MacConkey agar with 10 μg ertapenem/10 μg meropenem). The potential disadvantage of using chromogenic media in clinical laboratories is the increased cost and variable interpretation between different technicians (due to different levels of experience). However, the benefits regarding infection control are clear. The results of the current study showed no one chromogenic media topped all categories (specificity, sensitivity, PPV, NPV, LOD). Indeed, the medium selected for the surveillance programme had the best LOD of the four chromogenic media

tested, but may lack sensitivity for carbapenemase-producers that confer low-level resistance. The Xpert® Carba-R PCR performed well for in-house confirmation of CPOs, allowing earlier and more accurate information to be passed to infection control teams and clinicians, but is restricted to five genes. Laboratory detection and confirmation remains challenging, as there is phenotypic overlap with ESBL/AmpC hyper-production with permeability. This led to delay in the recognition of the GES-5-producing isolates in the follow-up surveillance period. The developed screening programme was a vast improvement on previous protocols and has been applied to support widespread surveillance of CPOs in clinical settings. This has directly led to better infection control and managing CPO carriage/transfer within the patient population.

2.7 UPDATE BASED ON CURRENT RECOMMENDATIONS (AS OF JUNE 2019)

Since the study detailed in this Chapter was conducted and written up, PHE guidelines for the screening of MDR *Enterobacteriaceae* have remain unchanged and no changes have been made to the PHE tool kit (this is currently under review, and an updated version will be published in summer 2019). CPE remain a major concern as the number of positive isolates detected on a yearly basis is increasing throughout the UK. Since the introduction of the tool kit by PHE in 2015, many hospitals in the UK have adopted it and are using it to set up their own surveillance and screening systems. In complying with the recommendations, the ICNHT came up with the surveillance and screening protocol described above, which brought about an increase in and better method of CPE detection within the Trust. Since 2015, the CXH laboratory has continued to improve its screening methods; the changes are not mentioned here as I moved jobs and have changed Trust.

There has been a marginal increase in the number of hospital-acquired infections caused by *Klebsiella* spp., highlighted by the introduction of laboratory surveillance of *Klebsiella* spp. bacteraemia in England, Wales and Northern Ireland (PHE, 2017). Between 2015 and 2016 the total number of cases of *Klebsiella* spp. bacteraemia in England, Wales and Northern Ireland increased by 15% (from 7,746 to 8,944 episodes), an increase of 13.0 to 15.0 per 100,000 population.

The surveillance report (PHE, 2017) also showed antibiotic susceptibility trends from 2012 to 2016 in England and Northern Ireland for blood culture isolates. Among *Klebsiella* spp. the most common mechanism of resistance to third-generation cephalosporins (cefotaxime or ceftazidime) was plasmid-mediated ESBL production. The analysis for *Klebsiella* spp. isolates (all species) showed that resistance to cefotaxime and ceftazidime remained stable between 2012 and 2016, mostly at 10% over the five-year period for each antibiotic. For *Klebsiella pneumoniae,* resistance to cefotaxime and ceftazidime was stable throughout the five-year period remaining at 12% in 2016 for each antibiotic, whereas *Klebsiella oxytoca* showed a lower level of resistance to these agents compared to *Klebsiella pneumoniae.* The proportion of isolates reported resistant to piperacillin/ tazobactam increased gradually over the five-year period for *Klebsiella pneumoniae* and *Klebsiella oxytoca* (from 13% in 2012 to 17% in 2016), with a marginal increase in resistance to ciprofloxacin and gentamycin. Resistance to carbapenems (meropenem and ertapenem) remained low between 2012 and 2016, with ≤2% of isolates reported as resistant. PHE (2017) also highlighted that *Klebsiella* spp. are the commonest hosts of carbapenemases belonging to the KPC, OXA-48-like, NDM, VIM or IMP families; other types of carbapenemase, such as GES-encoded enzymes, also occur and have caused outbreaks in some UK hospitals. The ESPAUR report in 2018 (PHE, 2018) also highlighted an increased number of IMP-positive CPE identified in 2017 compared with previous years due to an outbreak of IMP-positive *Klebsiella pneumoniae* in a London hospital, and also highlighted these bacteria are becoming more widespread.

As resistance to carbapenems warrants close vigilance, in May 2015 PHE launched the electronic reporting system (ERS) for the enhanced surveillance of carbapenem resistance in Gram-negative bacteria to better understand the epidemiology of these organisms (PHE, 2016a). The web-based ERS enhanced surveillance website was designed to enable laboratories in NHS Trusts in England to capture specimen, demographic, healthcare setting and risk factor details as part of the core and enhanced dataset as CPE pose significant treatment and public health challenges (PHE, 2018). However, the website was withdrawn in April 2019. No reason has been given.

From April 2017, due to the notable increase in *Klebsiella* spp. infections, the Government's response was to extend the enhanced surveillance of

bacteraemias caused by Gram-negative organisms to include *Klebsiella* spp. (https://www.gov.uk/government/statistics/klebsiella-species-bacteraemia-monthly-data-by-location-of-onset). This supports the ambition to reduce infections by 50% by 2021. To this end all NHS hospitals in UK are to report monthly patient-level data of any *Klebsiella* spp. bloodstream infections to Public Health England (https://www.gov.uk/government/statistics/klebsiella-species-bacteraemia-annual-data). Data for 2017 to 2019 for 148 hospitals show an average of 850.84 isolates per month across the reporting hospitals (Figure 2.9).

In April 2019, a survey was introduced by PHE for all tertiary hospitals to collect data on carbapenem resistance among *Klebsiella* spp. isolated from sterile sites. This survey will run for six months, with the results due to be issued later in 2019. The survey was introduced in response to the ESPAUR report 2018 (PHE, 2018), which showed the estimated burden of resistant bloodstream infections caused by *Klebsiella pneumoniae* in England in 2017. Isolates resistant to both carbapenems and colistin, 0.4%; resistant to colistin (but not carbapenems), 2.8%; resistant to carbapenems (but not to colistin), 0.8%; resistant to third-generation cephalosporins (but not to colistin or carbapenems), 12.6%. Data for *Klebsiella oxytoca* were also reported: resistant to both carbapenems and colistin, 0.0%; resistant to colistin (but not carbapenems), 1.8%; resistant to carbapenems (but not to colistin), 0.2%; resistant to third-generation cephalosporins (but not to colistin or carbapenems), 0.2%.

When comparing results from PHE with the report of Public Health Wales (Public Health Wales, 2018), in Wales *Klebsiella* spp. remain the 3rd commonest cause of bloodstream infections with resistance patterns remaining almost the same as those reported by PHE (PHE, 2017).

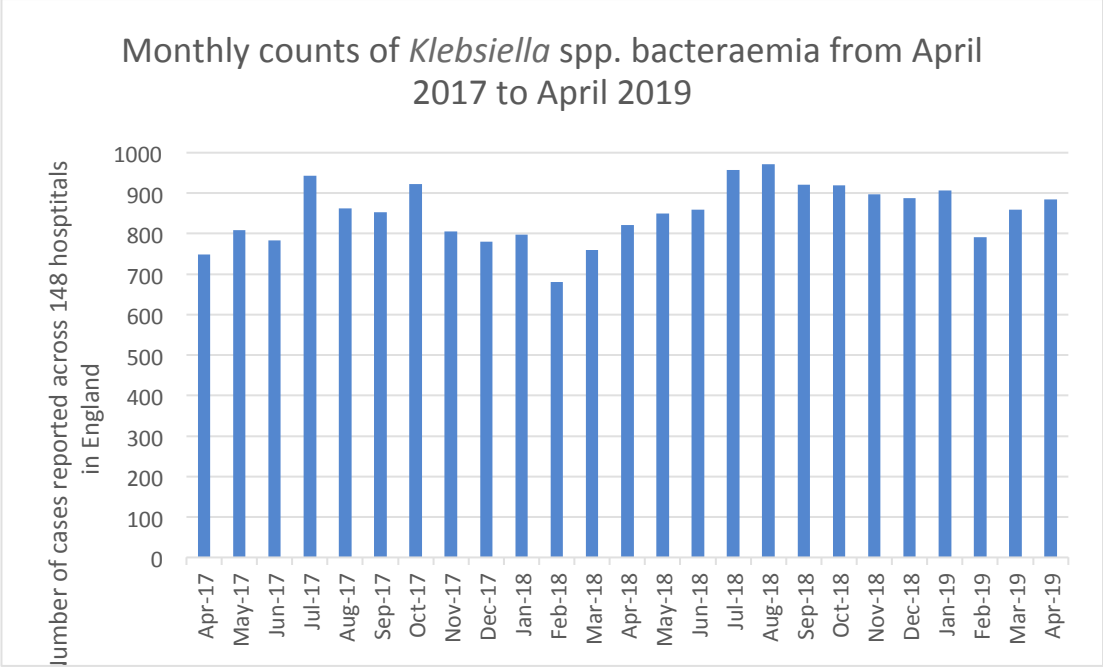


Figure 2.9. Monthly count of total cases of *Klebsiella* spp. bacteraemia reported by 148 hospitals in the UK. Data retrieved from https://www.gov.uk/government/statistics/klebsiella-species-bacteraemia-monthly-data-by-location-of-onset on 18 June 2019.

CHAPTER 3: GENOMIC CHARACTERISATION OF ANTIBIOTIC-RESISTANT ISOLATES ORIGINALLY IDENTIFIED AS *KLEBSIELLA OXYTOCA*, AND ISOLATION OF LYTIC BACTERIOPHAGES THAT INFECT THEM

3.1 INTRODUCTION

Around 3 to 7% of all HAIs are caused by *Klebsiella* spp. (Khan *et al.*, 2015). While much attention has been focused on the clinical relevance of *Klebsiella pneumoniae*, *Klebsiella oxytoca* has received very little attention but is being detected with increasing frequency in clinical laboratories (Chapter 2; Singh *et al.*, 2016; Moradigaravand *et al*., 2017; Chen *et al.*, 2019). This is due to a combination of improved screening methods (Chapter 2; Cheng *et al.*, 2012) and use of molecular methods, including WGS, to characterise isolates (Moradigaravand *et al*., 2017; Chen *et al.*, 2019). The English Surveillance Programme for Antimicrobial Utilisation and Resistance (Public Health England, 2017) showed the two commonest species among blood culture isolates of the genus *Klebsiella* were *K. pneumoniae* (81%) and *K. oxytoca* (17%). Similar to *K. pneumoniae*, *K. oxytoca* strains are acquiring various antimicrobial resistance genes (Moradigaravand *et al*., 2017) and are detected in hospitals across the UK; consequently, *K. oxytoca* is now emerging as the second most important pathogen within *Klebsiella* species.

3.1.1 Carriage of *K. oxytoca* and contribution to disease

K. oxytoca is a member of the family *Enterobacteriaceae*, and is a Gram-negative, non-motile, indole-positive encapsulated rod-shaped bacterium. It is found in the environment (Hoffman *et al*., 2010) at low levels and in the nasopharynx and the gastrointestinal tract of humans, where it is considered part of the normal gut microbiota of some individuals. Carriage rates vary from study to study, but are generally higher in stool samples from healthy subjects (5 to 38% (Thom, 1970), 1.6% (Högenauer *et al.*, 2006), ~2 to 10% (Schlenker and Surawicz, 2009), 2.1 to 2.9% (Cheng *et al.*, 2012)) than the nasopharynx (1 to 6%; Rosenthal and Tager, 1975).

Like *K. pneumoniae*, *K. oxytoca* is an opportunistic pathogen in humans, and is becoming increasingly associated with nosocomial infections, particularly in immunocompromised patients and preterm infants with underlying conditions that

require prolonged stays in hospitals, and community-acquired infections, and is responsible for high morbidity among patients (Broberg *et al*., 2014; WHO, 2016; Chen *et al*., 2019). *K. oxytoca* is a causative agent of *C. difficile*-negative antibiotic-associated haemorrhagic colitis (AAHC) and diarrhoea, which can occur after treatment with penicillin, ampicillin, amoxicillin, first-generation cephalosporins or pristinamycin (Beaugerie *et al.*, 2003; Högenauer *et al.*, 2006; Herzog *et al*., 2014). Symptoms include sudden onset of bloody diarrhoea with severe abdominal cramps 4-10 days after taking antibiotics and are resolved on cessation of antibiotic therapy (Yamada *et al*., 2014). *K. oxytoca* overgrows other members of the gut microbiota in the presence of antibiotics, and strains release cytotoxins that cause disease (Zollner-Schwetz *et al*., 2008; Herzog *et al*., 2014; Unterhauser *et al*., 2019). Colitogenic strains encode the non-ribosomal peptides tilvalline and tilimycin (kleboxymycin; Tse *et al.*, 2017), which are enterotoxic metabolites, but there is no information available on the prevalence of the relevant genes in strains (Unterhauser *et al*., 2019). These are encoded on a secondary metabolite biosynthetic gene cluster (Tse *et al*., 2017; Unterhauser *et al*., 2019), and both metabolites are present in the human intestine during active colitis. Tilimycin is a genotoxin that interacts with DNA to activate damage repair mechanisms that cause DNA strand damage that results in lesions in mouse caecal enterocytes, with the same thought to happen in human enterocytes (Unterhauser *et al*., 2019). Tilvalline binds tubulin and stabilises microtubules leading to mitotic arrest and loss of barrier integrity. Both enterotoxic metabolites induce apoptosis (cell death), and it is thought they work together in the pathogenicity of colitis (Unterhauser *et al*., 2019). *K. oxytoca* has also recently been linked to jejunal necrosis and antibiotic-associated fulminant pseudomembranous enterocolitis in a 79-year-old man with a history of antibiotic therapy, but it is not known whether enterotoxic metabolites were involved in the disease (Nagamura *et al*., 2019). In addition to causing colitis in some individuals, enteric carriage of *K. oxytoca* may play a role in the transmission of antibiotic resistance genes to other bacteria and act as a source of nosocomial infections (Molton *et al.*, 2013).

K. oxytoca has been detected in the faeces of a subset of preterm infants via cultivation or shotgun metagenomics, but its association with preterm-associated infections such as late-onset sepsis or necrotising enterocolitis is

unknown (Chen *et al.*, 2019). In this cohort, several factors contribute to colonisation of an infant's gastrointestinal tract with one or more *Enterobacteriaceae*: these include the significant number of antibiotics (prophylactic and treatment) these infants receive in the first days and weeks post birth, an immature immune system, an unstable microbiome, mode of delivery, invasive procedures such as enteral feeding using nasogastric tubes, bacterial contamination of milk and the hands of healthcare workers, and the hospital environment (Berthelot *et al*., 2001; Moles *et al.*, 2015; Chen *et al.*, 2019). *K. oxytoca* was detected in the faeces of 24/146 US preterm infants based on a taxonomic analysis of shotgun metagenomic data (Chen *et al.*, 2019). A nationwide multicentre observation study in France between 2006 and 2016 showed that *K. oxytoca* caused meningitis in a small number of children (0.22%; 4/1859), with *K. pneumoniae* responsible for 0.48% of cases (Carrie *et al*., 2019).

Three cases of arthritis caused by *K. oxytoca* appear in the literature (Ménard *et al*., 2010; Silvagni-Gutiérrez *et al*., 2017; Hertting *et al.*, 2018). The first case involved community-acquired spontaneous arthritis in a 30-month-old girl, and was caused by a bacterium identified as *K. oxytoca* using API 20E and 16S rRNA gene sequencing (Ménard *et al.*, 2010). Silvagni-Gutiérrez *et al*. (2017) reported a case related to septic arthritis caused by *K. oxytoca.* Hertting *et al*. (2018) reported a case of septic arthritis in a 4-week-old infant, with *K. oxytoca* identified as the causative agent by MALDI-TOF. It is believed the infant acquired the infection through vein puncture when being treated in hospital during the first 3 weeks of life. Hospital- or community-acquired endocarditis is sometimes caused by *K. oxytoca* (Table 3.1). Similar to arthritis, this is very rare as *Klebsiell*a spp. account for only about 10% of endocarditis cases, which are predominantly healthcare-acquired (Hauser *et al.*, 2017). Endocarditis due to *K. oxytoca* appears to be mainly community-acquired (Table 3.1).

Table 3.1: Hospital-acquired (HA) and community-acquired (CA) *K. oxytoca* endocarditis.

HA or CA?	Affected individual(s)	Reference
CA	Elderly woman with high fever	Chen *et al*. (2006)
CA	Drug addict	Geisenberger *et al*. (2015)
CA	Intravenous drug user	Hauser *et al.* (2017)
CA	Elderly patient with nephrolithiasis	Memon *et al*. (2018)
CA	Intravenous drug user	Mohamed *et al*. (2016)
HA	Haemodialysis patient	Ruiz-Zorrilla López *et al*. (2009)
HA	Elderly man with stroke	Ullah *et al.* (2016)
HA	Elderly man after transurethral resection	Watanakunakom (1985)

3.1.2 Nosocomial infections and antibiotic resistance associated with *K. oxytoca*

Nosocomial infections are putting huge pressure on the healthcare system globally, both financially and with respect to morbidity and mortality rates. They also promote antimicrobial resistance. Because of these factors, nosocomial infections are considered a quality index of healthcare facilities (Bouza *et al.*, 2019). The EPINE study, which has documented nosocomial infections in Spain for the past 25 years (Bouza *et al.*, 2019), reported the prevalence of these infections to be between 7.7 and 8.5%, remaining relatively stable over the reporting period. Across the European Union, nosocomial infections affect approximately 4.1 million patients each year (Bouza *et al.*, 2019).

K. oxytoca-associated nosocomial infections are reported infrequently. Singh *et al.* (2016) reported 23/654 *Klebsiella* isolates recovered from Indian clinical samples were *K. oxytoca*, with more than half these isolates resistant to imipenem, meropenem, gentamicin, amikacin, ceftazidime and ceftriaxone. Singh *et al.* (2016) also reported *K. oxytoca* is now being isolated more frequently from neonatal intensive care units and adult critical units than in the past.

The English surveillance programme for antimicrobial utilisation and resistance (ESPAUR) report from a 5-year surveillance programme showed the

proportion of *K. oxytoca* bloodstream isolates resistant to different classes of antibiotics over time has increased (ESPAUR, 2017). Resistance to gentamicin, ciprofloxacin, third-generation cephalosporins and carbapenems was seen in ≤7% of isolates throughout the surveillance period. An increase in resistance to piperacillin/tazobactam and co-amoxiclav was seen (10-15% and 13-20%, respectively) in *K. oxytoca* isolates, with the proportion of resistant isolates higher in 2016 compared with 2012 (ESPAUR, 2017).

K. oxytoca are acquiring a range of resistance mechanisms, including ESBLs and AmpC β-lactamases, leading to increased usage of broad-spectrum antibiotics including carbapenems to treat *K. oxytoca* infections, which has led to the increase observed in carbapenem resistance (D'Angelo *et al*., 2016). Eades *et al.* (2016) and Kabir *et al.* (2016) reported the first instances of GES-5-associated carbapenem resistance. Resistance to the carbapenems imipenem and meropenem is on the rise among *K. oxytoca* isolates worldwide, especially in the United States, and is also appearing in the UK (ESPAUR, 2017).

In the UK, Public Health England (PHE) confirmed the presence of at least one carbapenemase in 2,595 *Enterobacteriaceae* that was classed within the 'big 5' carbapenemase families (KPC, OXA-48-like, NDM, VIM, IMP). Within the UK, *K. oxytoca* harbouring the 'big 5' carbapenemases are seen infrequently compared with other countries (ESPAUR, 2017). In the United States, *K. pneumoniae* carbapenemase-1 (KPC-1) is the most common; this is a class A β-lactamase capable of hydrolysing carbapenems (Yigit *et al*., 2003). In an Indian setting, Singh *et al*. (2016) showed 58% of *K. oxytoca* isolates were KPC producers, as compared to 30% of *K. pneumoniae* isolates. Similar results were observed in an Austrian study that reported meropenem resistance to be up to 58% (Hoenigl *et al*., 2012) and described a nosocomial outbreak of KPC-producing *K. oxytoca*, highlighting the clinical importance of infection with this bacterium. Although *K. oxytoca* carriage may remain asymptomatic, the bacterium is still considered to be an opportunistic pathogen of clinical significance with its association with infections in hospitalised patients, including children and neonates. Herruzo *et al.* (2017) described a VIM-linked *K. oxytoca* infection outbreak in a neonatal unit in Spain, where the source of the outbreak was other patients. *K. oxytoca* harbouring VIM-1 were isolated from community-onset infections among patients attending the outpatient department of a Greek hospital

(Tsakris *et al*., 2011). Separate case studies reported the first isolation of *K. oxytoca* strains producing IMP-1 metallo-β-lactamases and NDM-1 in China (Hagiya *et al*., 2015; Wang *et al*., 2017).

3.1.3 Clinical characterisation of *K. oxytoca* isolates

Studies by Holt *et al.* (2015) and Wyres *et al*. (2016) have shown the potential for genomics to enhance surveillance and tracking of specific pathogens, especially *Klebsiella* spp., and AMR strains to aid infection control and outbreak investigations. Holt *et al*. (2015) used genomics on over 300 strains to aid investigations of AMR *Klebsiella* in hospitals through the detection of polyclonal outbreaks resulting from transmission of plasmids encoding acquired AMR genes as well as identification of AMR clones. The key elements of the *Klebsiella* genomics consist of identification of core genes, AMR determinants including acquired genes and common mutations, known virulence genes and alleles, plasmids and capsular and O antigen loci. Current resources available for characterising *Klebsiella* genomes include:

1. ***Klebsiella pneumoniae/oxytoca* BIGSdb.** An online database and integrated set of tools for analyses of genome assemblies (Bialek-Davenet *et al*., 2014). The *K. pneumoniae* MLST (multilocus sequence typing) database, core genome MLST, virulence and AMR gene databases are available through this single resource, which also hosts a searchable repository of *K. pneumoniae, K. variicola and K. quasipneumoniae* genomes. As of June 2016, the database included 2328 distinct STs (sequence types). Available at https://bigsdb.pasteur.fr/klebsiella.
2. **Kaptive.** A database of complete sequences of *Klebsiella* capsule loci and accompanying tool for identification and typing of capsule loci from genome assemblies (Wyres *et al.*, 2016; Wick *et al.*, 2018). Available at https://kaptive-web.erc.monash.edu/.
3. **NCBI Pathogen Detection Resources.** Curated databases of AMR genes and genomes of AMR bacterial pathogens. As at June 2016, the databases include 3275 AMR gene nucleotide sequences and 2391 annotated genomes drawn from GenBank. Genome-wide phylogenetic analyses, precomputed at the species level, can also be accessed. Available at https://www.ncbi.nlm.nih.gov/pathogens.

Our understanding of the phenotypic and genomic traits of *K. oxytoca* is limited when compared to *K. pneumoniae*. Molecular studies have revealed that *K. oxytoca* can be separated into three related but distinct phylogroups (Ko1, Ko2 and Ko6) according to house-keeping genes and 16S rRNA gene analysis. Herzog *et al.* (2014) showed that Ko2 bacteria were prevalent in stool samples, while Ko1 and Ko6 were more associated with the respiratory tract. These phylogroups have subsequently been found to represent distinct species: *Klebsiella michiganensis* (Ko1), *K. oxytoca* (Ko2) and *Klebsiella grimontii* (Ko6) (Brisse and Verhoef, 2001; Drancourt *et al.*, 2001; Passet and Brisse, 2018). These species can only be split using genomic data, not phenotypic tests such as MALDI-TOF and API 20E strips routinely used in clinical laboratories (Moradigaravand *et al*., 2017; Chen *et al.*, 2019). *K. michiganensis* and *K. oxytoca* are distinguishable based on $bla_{OXY\text{-}1}$ and $bla_{OXY\text{-}2}$ genes, respectively (Brisse and Verhoef, 2001), while *K. grimontii* accommodates Ko6 strains based on *rpoB*, *gyrA* and *rrs* gene sequence analyses (Passet and Brisse, 2018).

Despite the growing clinical importance of *K. oxytoca*, there is limited genomic information available for the population structure and epidemiology of antimicrobial-resistant *K. oxytoca*. A small pangenome study by Moradigaravand *et al*. (2017) showed that UK clinical isolates of *K. oxytoca* represent a highly diverse population, composed of several distinct clades corresponding to the three above-mentioned phylogroups (plus Ko5, a sublineage of Ko1 (Fevre *et al.*, 2005)). Although the *K. oxytoca* genomes were distinct from one another and *K. pneumoniae* genomes, they shared genes with *K. pneumoniae*, indicating recombination between the two species and further evidenced by sharing of antimicrobial resistance and virulence genes. However, Moradigaravand *et al*. (2017) also showed that the different *K. oxytoca* clades have acquired antimicrobial resistance and virulence genes independent of *K. pneumoniae*. *K. oxytoca* is acquiring resistance mechanisms involved in development of multidrug resistance. These include production of extended-spectrum β-lactamases (ESBLs), AmpC lactamases, *Klebsiella pneumonia* carbapenemase (KPC) and aminoglycoside-modifying enzymes (Moradigaravand *et al*., 2017).

Initially identified as *K. oxytoca* using API 20E, the five *Klebsiella* isolates recovered by Chen *et al.* (2019) from faeces of preterm infants were found, on genomic analyses, to represent three strains of *K. grimontii* and two strains of *K.*

michiganensis. In addition, detailed analyses of shotgun metagenomic data from US infants with a curated dataset of *K. oxytoca*, *K. michiganensis* and *K. grimontii* genomes showed that samples initially found to harbour *K. oxytoca* based on a crude taxonomic analysis more often than not contained *K. michiganensis* rather than *K. oxytoca*. The discrepancy between initial results and the more-refined analyses was due to poor annotation of publicly available datasets and taxonomic tools (Chen *et al.*, 2019). Therefore, there is the potential that *K. michiganensis* and *K. grimontii* may be more clinically relevant than *K. oxytoca*, and poor characterisation of isolates in the past has overlooked these opportunistic pathogens.

3.1.4 Aims of this Chapter

In Chapter 2, I showed that the improved screening method I helped implement in the clinical laboratory increased the detection of *K. oxytoca* from clinical samples and detected an outbreak caused by GES-5-positive *K. oxytoca* at the Haematopoietic Stem Cell Transplant (HSCT) unit of Charing Cross Hospital. Eades *et al.* (2016) subsequently presented findings on the outbreak at the 26th European Congress of Clinical Microbiology and Infectious Diseases. During routine laboratory screening a *K. oxytoca* strain isolated from a urine sample from a neutropenic HSCT recipient was identified as a presumptive carbapenemase producer, but in-house PCR was negative for carbapenemases. Consequently, the strain was sent to PHE for determination of the resistance mechanism. PCR confirmed the presence of the first *bla*$_{GES-5}$-positive *K. oxytoca* isolate associated with a clinical infection. Within a subsequent 18-month period of screening, 430 additional isolates of *K. oxytoca* were identified, 14 of which harboured the GES-5-encoding gene (Eades *et al.*, 2016). I had access to three of the strains (PS_Koxy1, PS_Koxy2 and PS_Koxy4) that I had determined the antimicrobial susceptibility profiles for. Molecular results from PHE showed PS_Koxy1 to have a unique pulsed-field gel electrophoresis (PFGE) profile, while PS_Koxy2 and PS_Koxy4 had PFGE profiles SMAR45KL-5 and VNTR profile 3,8,24,46,53,32,48 (Figure 3.1).

Whole-genome sequence data (unavailable to me) generated by Eades *et al.* (2016) were used only to show PS_Koxy1 (isolated from a throat swab from patient 3 on 18/12/2014), PS_Koxy2 (isolated from a urine sample from patient 5

on 13/8/2015) and PS_Koxy4 (isolated from a rectal swab from patient 4 on 2/9/2015) were *K. oxytoca* of sequence type (ST) ST138. No details were provided regarding how species identification was confirmed using genomic data. Presence of bla_{GES-5} – a relatively rare Amber class A carbapenemase among *Enterobacteriaceae* – and the ESBL CTX-M-15 was confirmed by PCR. The genome sequence data were not interrogated for presence of other antibiotic-resistance determinants. Therefore, the first aims of this Chapter were to fully characterise the isolates at the genomic level and compare their antimicrobial susceptibility profiles with their genomic profiles.

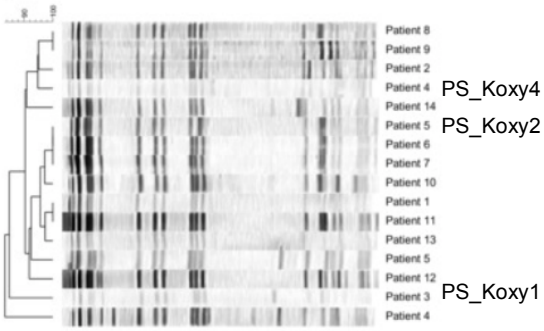


Figure 3.1. PFGE profiles of *Xba*I-digested DNA from GES-5-positive *K. oxytoca* strains. The image is taken from Eades *et al.* (2016). All the strains were described as being highly similar based on their PFGE profiles. Strains studied in further detail in this Chapter are shown.

Brown *et al.* (2017) proposed that phages active against *K. oxytoca* could be used to modulate the gut microbiome and growth of *K. oxytoca* in clinical settings. I believe bacteriophages – viruses that infect bacteria – may be used to decontaminate patients colonised with antibiotic-resistant *K. oxytoca*, and could contribute to infection control programmes. Therefore, the final aim of this Chapter was to isolate and begin to characterise lytic phages that infected GES-5-positive clinical strains of *K. oxytoca*. This represents the first study to isolate lytic phages against these bacteria.

3.2 MATERIALS AND METHODS

Note: the materials and methods detailed here apply to Chapters 3 and 4.

3.2.1 Collection and characterisation of isolates recovered from patients

Rectal and perineal swabs were taken for 236 patients hospitalised during an outbreak (mainly the high dependency unit including the renal dialysis unit) at the Hammersmith and Saint Mary's Hospital between March and April 2015 to screen for carbapenem-resistant organisms (CROs).

Each swab was suspended in 0.5 ml of 0.85% saline to generate a homogeneous suspension of material. After vortexing an aliquot of this suspension (50 µl) was used to inoculate Colorex™ mSuper CARBA™ (E&O Labs Chromagar, France) agar provided by E&O laboratories. Plates were incubated aerobically at 37 °C for 18-20 h. Coloured colonies (metallic blue, *K. pneumoniae* and *K. oxytoca*) were regarded as presumptive isolates of carbapenem-resistant *Klebsiella* spp. in accordance with manufacturer's instructions. All *Klebsiella* isolates were preserved on Cryobank cryogenic beads and stored at -70 °C.

3.2.2 Phenotypic identification and antibiotic susceptibility testing

Matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry: Recovered isolates were identified by MALDI-TOF mass spectrometry (Bruker, Coventry, UK). MALDI-TOF is a form of mass spectrometry that produces a protein spectrum (fingerprint) for an organism. A laser is fired at the test organism, which is co-crystallised with a matrix onto a metal target. The matrix absorbs the laser energy and protects the test organism such that whole proteins are desorbed from the target. The matrix also acts as a proton donor to ionise the proteins. When high voltage is applied, the ionised proteins are accelerated and then travel up the flight tube to a detector. The mass-to-charge ratio of the proteins is proportional to the square of the time taken to reach the detector. By measuring the time of flight, the mass/charge ratio can be calculated. Proteins from 2 kDa to 20 kDa can be detected with the Bruker Microflex LT instrument. The associated MALDI Biotyper software compares the protein spectrum obtained from the test organism with that of a library of spectra from well characterised organisms to provide 'best match' information. A score is assigned to each match to assist in interpretation.

From an overnight culture of each strain grown on Colorex^{TM} mSuper CARBA^{TM} (E&O Labs), a single colony was selected using a sterile wooden stick. The biological material was smeared as a thin film directly onto the available spots on the MALDI target plate (consisting of 96 target spots in total). Once dry, the biological materials on the target plate were overlaid with 1 µl of α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (BRUKER) within 1 h and allowed to dry at room temperature. The matrix solution was prepared as follows. To a 1.5 ml Eppendorf tube were added 500 µl of Chromasolv acetonitrile (ACN) (Fluka), 475 µl of Chromasolv water (Fluka) and 25 µl trifluoroacetic acid (Sigma Aldrich). The liquid was mixed well by inversion, ensuring the cap of the Eppendorf was always tightly closed as ACN evaporates quickly. 250 µl of the solution was added to a vial of HCCA (stored in the fridge at 2 to 8 °C). The HCCA was dissolved by scratch-mixing and vortexing at room temperature until the solution was clear, ensuring there was no yellow pellet left at the bottom of the vial. The resulting HCCA solution was stored in the dark and used within 1 week of making.

Each target plate was quality controlled by spotting it with control strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213. The dried plates were loaded onto the MALDI-TOF Biotyper for identification of isolates. Organisms placed on the MALDI-TOF Biotyper were identified and given a score relating to the confidence of the identification. The scoring range is: 0.000-1.699 = no reliable ID (red); 1.7-1.999 = probable genus ID (yellow); 2.0-2.299 = secure genus ID, probable species ID (green); 2.3-3.000 = highly probable species ID (green). Scores of 2.0 and above are acceptable. Any organism giving a score of less than 2.0 should be investigated and possibly identified using other phenotypic or genotypic methods. Repeating the smearing of the organism onto new spots may give a clearer result. Alternatively, the extended direct smearing method or the formic acid extraction method could be used to gain a more reliable score, which was not needed in this case as all identifications fell within 2.0-2.299 range.

API 20E: All isolates identified as *K. pneumoniae* and *K. oxytoca* by MALDI-TOF were further identified using the API (Analytical Profile Index) 20E (bioMérieux) kit, a biochemical panel of phenotypic tests for identification and differentiation of members of the family *Enterobacteriaceae* (Figure 3.2). For each isolate, a strip was set up according to the manufacturer's instructions and incubated at 37 °C for

18 to 24 h. Most results can be read after 24 h but for TDA, IND and VP, a drop of ferric chloride, a drop of Kovacs reagent, and a drop of 40% KOH plus a drop of α-naphthol, respectively, have to be added and left at room temperature for 10 min before results are recorded. Appearance of each chamber is compared to the API Reading Scale and marked positive or negative based on appearance. A seven-digit code is then obtained based on the total of positive scores (Figure 3.3). This code is used to identify the isolate under study.

Antimicrobial susceptibility testing: Isolates confirmed as *K. pneumoniae* or *K. oxytoca* using phenotypic tests were screened for possible carbapenemase production following UK national guidelines (PHE, 2013). This involved testing isolates against 18 antimicrobials (amikacin, amoxicillin, augmentin, aztreonam, cefotaxime, cefoxitin, ceftazidime, cefuroxime, ciprofloxacin, colistin, ertapenem, gentamicin, meropenem, tazocin, temocillin, tigecycline, tobramycin and trimethoprim) following the EUCAST disc diffusion method with the inclusion of MAST CAT-ID discs consisting of faropenem (MAST group LTD, UK). Control organisms used to monitor test performance of the antimicrobials were *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213.

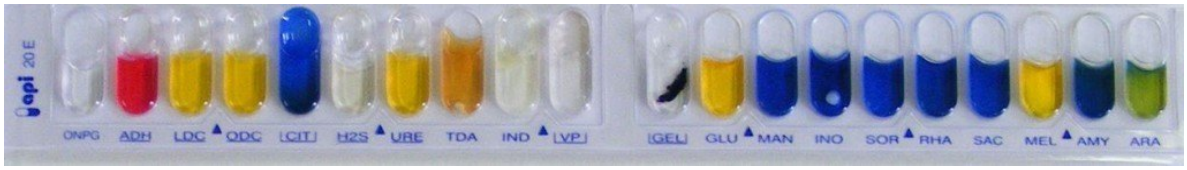


Figure 3.2. API 20E strip for identification of *Enterobacteriaceae*. The API 20E strip consists of 20 mini-test chambers containing dehydrated media with chemically defined compositions for each test. ONPG, tests for β-galactosidase activity by hydrolysis of the substrate *o*-nitrophenyl-β-D-galactopyranoside; ADH, decarboxylation of the amino acid arginine by arginine dihydrolase; LDC, decarboxylation of the amino acid lysine by lysine decarboxylase; ODC, decarboxylation of the amino acid ornithine by ornithine decarboxylase; CIT, use of citrate as sole carbon source; H_2S, production of hydrogen sulphide; URE, test for urease activity; TDA, detection of tryptophan deaminase activity (has to have reagent ferric chloride added); IND, indole test - production of indole from tryptophan by the tryptophanase (indole is detected by addition of Kovac's reagent); VP, the Voges-Proskauer test for the detection of acetoin (acetyl methylcarbinol) produced by fermentation of glucose by bacteria using the butylene glycol pathway; GEL, test for gelatinase (gelatin liquification); GLU, fermentation of glucose (hexose sugar); MAN, fermentation of mannose (hexose sugar); INO, fermentation of inositol (cyclic polyalcohol); SOR, fermentation of sorbitol (alcohol sugar); RHA, fermentation of rhamnose (methyl pentose sugar); SAC, fermentation of sucrose (disaccharide); MEL, fermentation of melibiose (disaccharide); AMY, fermentation of amygdalin (glycoside); ARA, fermentation of arabinose (pentose sugar).

	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	Oxidase
Reaction	+	-	+	-	+	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	-
Point	1	0	4	0	2	0	0	0	4	1	0	4	1	2	4	1	2	4	1	2	0
Add	**5**			**2**			**4**			**5**			**7**			**7**			**3**		
7-digit code 5245773																					

Figure 3.3. Recording of API 20E results to identify an isolate. Positive scores are given points. The total value of the points is used to generate the seven-digit code to identify an isolate. The code can be entered into the API web (https://apiweb.biomerieux.com/servlet/Authenticate?action=prepareLogin) to identify the isolate. 5245773 = *K. oxytoca*, ID 97.8%; possibility of *Raoultella planticola*, ID 1.6%.

Antimicrobial sensitivities were determined by preparing inoculum suspensions with several morphologically similar colonies (when possible) from overnight growth (16–24 h of incubation) preferably from a non-selective medium (blood agar). Using a sterile loop the colonies were suspended in sterile saline (0.85% NaCl, w/v, in water) to the density of a McFarland 0.5 standard, corresponding to approximately 1–2x10^8 CFU/mL for *Escherichia coli*. The density of the suspension was measured with a densitometer that had been calibrated with a 0.5 McFarland standard according to the manufacturer's instructions. It was important to carefully prepare the inoculum so that it produced semi-confluent growth after overnight incubation. A denser inoculum results in reduced zones of inhibition and a lighter inoculum can have the opposite effect. All inoculum suspensions were used within 15-60 min of preparation.

A sterile cotton swab was dipped into the inoculum suspension and the excess fluid removed by turning the swab against the inside of the tube to avoid over-inoculation of plates. The inoculum was spread evenly over the entire surface of the agar plate by swabbing in three directions or by using an automatic plate

rotator on to a Muller-Hinton agar plate (4 mm depth with +/- 0.5 mm maximum variation). Antimicrobial discs were handled and stored according to the manufacturer's instructions, and were applied firmly on the agar surface within 15 min of inoculation of the plates. It is important that zone diameters can be reliably measured and the maximum number of discs on a plate depends on the size of the plate, so that unacceptable overlapping of zones can be avoided. In this instance a maximum of six discs was used per plate. Discs were applied to plates using a disc dispenser and plates were incubated within 15 min of application of antimicrobial discs. The plates were inverted and incubated at 35 °C for 16–20 h.

In any disc diffusion test, the reading of zones is the most difficult variable to standardise. Reading of zones includes measuring the zone diameter, inspecting the zone edge and the detection of colonies within the inhibition zone. The zone sizes were read using calibrated callipers and interpreted as sensitive or resistant by referring to the EUCAST breakpoint guidelines (EUCAST, 2015). Carbapenemase confirmatory tests were performed on isolates found to be resistant or to have reduced susceptibility to the indicator carbapenems (i.e. ertapenem and meropenem). Meropenem offers the best compromise between sensitivity and specificity in terms of detecting carbapenemase producers. Ertapenem shows high sensitivity but low specificity in terms of detecting carbapenemase producers, and so is not recommended for routine use. However, ertapenem can be used for detection in specific instances such as in investigating an outbreak known to have been caused by an organism that is ertapenem-resistant but appears to be susceptible to meropenem, or where increased sensitivity is required. Another drawback with the use of ertapenem is the cost implications.

All organisms showing borderline or complete resistance to the indicator carbapenemases were further tested with the ertapenem and meropenem E-strips to ascertain the Minimum Inhibitory Concentration (MIC). Any organisms with MIC >0.12 µg/mL to both carbapenemases were sloped and sent to the PHE reference laboratory at Colindale for PCR and further testing to understand resistance mechanisms.

Gradient MIC testing (E-test®): E-test® is a well-established method for antimicrobial resistance testing in microbiology laboratories around the world. E-

test® consists of a predefined gradient of antibiotic concentrations on a plastic strip and is used to determine the MIC of antibiotics. Fresh colonies of an isolate grown on blood agar were transferred to saline to produce a suspension of 0.5 McFarland standard. A swab dipped in the suspension was used to inoculate a Mueller Hinton agar plate. E-strips (Launch) were applied and plates incubated for 24 h at 30-35 °C. Antimicrobials tested included: 3GC, third-generation cephalosporin; AMN, amikacin; AZT, aztreonam; CAZ, ceftazidime; COL, colistin; CPD, cefpodoxime; CRO, ceftriaxone; CTX, cefotaxime; ETP, ertapenem; FOX, cefoxatin; GEN, gentamicin; MER, meropenem; TEM, Temocillin; and TOB, tobramycin. After incubation the MIC gradients were read against the EUCAST breakpoint guidelines. Those isolates showing MICs >0.12 µg/mL for meropenem and ertapenem were considered resistant (EUCAST, 2015).

Also, organisms showing resistance to ETP, MER and TEM were considered presumptive carbapenemase-producers. Organisms could also show resistance to AZT, CAZ, COL, CPD, CRO, CTX and FOX.

3.2.3 Genotypic characterisation of bacteria

PHE characterisation: The isolates were characterised by PHE using a rapid and reliable multiplex PCR-based technique that was able to detect genes encoding carbapenemases belonging to different classes. Primers used in the PCR target 11 genes: *bla*$_{AIM}$, *bla*$_{BIC}$, *bla*$_{DIM}$, *bla*$_{GIM}$, *bla*$_{IMP}$, *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{OXA-48}$, *bla*$_{SIM}$, *bla*$_{SPM}$ and *bla*$_{VIM}$ (Poirel *et al*., 2011a). A real-time TaqMan multiplex PCR assay was also used to detect genes encoding the rare types of serine carbapenemases: GES, IMI/NMC and SME (Swayne *et al*., 2011). Variable number tandem repeat (VNTR) analysis, in which isolates are characterised by their repeat numbers at multiple loci, was also used to characterise the bacteria. The 34 *K. pneumoniae* isolates were further tested by a multiplex PCR which allowed identification of some common capsular types (K1, K2, K5, K54 and K57), which are those most associated with invasive disease or pathogenicity, a further capsular type (K20), the presence or absence of two putative virulence factors (*rmpA* and *wcaG*) and the 16S–23S internal transcribed spacer unit of *K. pneumoniae*, facilitating identification of this organism (Turton *et al*., 2010). Use of the multiplex PCR coupled with the VNTR analysis provides a rapid means of characterisation and typing of isolates.

Whole-genome sequencing of *Klebsiella* isolates: Due to phage specificity, *Klebsiella* phages often have a limited host range and can only infect strains of *Klebsiella* spp. with particular capsule types (Hoyles *et al.*, 2015). Therefore, it was important to accurately determine the capsule types of *Klebsiella* spp. isolated in the west London hospitals, beyond the limited scope of the multiplex PCR used by PHE to type only five capsule types.

Isolates were plated onto MacConkey agar, and passaged three times before DNA was extracted using the Gentra PureGene Qiagen DNA extraction kit (Qiagen). DNA quality was assessed by using agarose gel electrophoresis. Extracted DNA was frozen at -20 °C and sent to the Quadram Institute Bioscience, Norwich for library preparation and sequencing. Samples were run on an Illumina Nextseq500 instrument using a Mid Output Flowcell (NSQ® 500 Mid Output KT v2 (300 CYS); Illumina Catalogue FC-404-2003) following Illumina's recommended denaturation and loading procedures, which included a 1% PhiX spike-in (PhiX Control v3; Illumina Catalogue FC-110-3001). Data were uploaded to Basespace, where the raw data were converted to eight fastq files for each sample (four for R1, four for R2). Tentative identities had been attributed to genomes using Kraken 1.0 with the raw sequence data by Dr Lesley Hoyles (Wood and Salzberg, 2014), and this information was used to inform further analyses. Sequence data were quality checked, trimmed, assembled and annotated by Dr Lesley Hoyles, who provided me with Prokka-annotated files, with which I performed all additional bioinformatics analyses (except PhyloPhlAn and CheckM, which were done by Dr Hoyles using servers belonging to the UK MEDical BIOinformatics partnership (UK Med-Bio; Medical Research Council grant number MR/L01632X/1)). FastANI and BLASTP analyses were performed on the CLIMB (CLoud Infrastructure for Microbial Bioinformatics) server (Connor *et al.*, 2016).

Briefly, paired read data returned by the sequencing provider for each genome were concatenated (per genome: four files each for R1, four files each for R2) and checked using fastqc v0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Data were trimmed using trimmomatic 0.39 (SLIDINGWINDOW:5:20 MINLEN:50) (Bolger *et al.*, 2014), and paired reads retained. Genomes were assembled using the trimmed paired reads with SPAdes v3.13.0 (default settings) (Bankevich *et al.*, 2012). Gene

predictions and annotations were completed using Prokka v.1.12 (default settings) (Seemann, 2014). Completeness and contamination of genomes was assessed using CheckM v1.0.18 (Parks *et al.*, 2015). Average nucleotide identity of genomes with their closest relatives and type strains of species was assessed using FastANI (Jain *et al.*, 2018). PhyloPhlAn 0.99 (Segata *et al.*, 2013) was used to determine phylogenetic placements of strains within species. *K. oxytoca*-related genomes were uploaded to the *Klebsiella oxytoca* MLST website (https://pubmlst.org/koxytoca/) sited at the University of Oxford (Jolley *et al.*, 2018) on 6 September 2019 to determine allele number against previously defined house-keeping genes (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB* and *tonB*). *K. pneumoniae* genomes were analysed using the Institut Pasteur MLST database (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html) on 10 September 2019. Kleborate (Lam *et al.*, 2018; Wick *et al.*, 2018) and Kaptive (Wyres *et al.*, 2016) (http://kaptive.holtlab.net) were used to identify capsular type and O antigen type. Presence of antibiotic-resistance genes within strains was determined by BLASTP analysis of amino acid sequences of predicted genes within genomes against the Comprehensive Antibiotic Resistance Database (CARD) (downloaded 27 July 2019; protein homolog dataset) (Jia *et al.*, 2017).

Virulence factors encoded in the *K. pneumoniae* genomes were determined by BLASTP searches against proteins included in the Virulence Factor Database (VFDB) (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi; core dataset downloaded on 27 July 2019; Liu *et al.*, 2019). Results are reported for >70% identity and >90% query coverage (Chen *et al.*, 2019).

Checking for presence of the kleboxymycin gene cluster in the genomes of the *K. oxytoca* isolates. To determine whether the *K. oxytoca* isolates harboured the kleboxymycin biosynthetic gene cluster (Tse *et al.*, 2017), the proteins encoded in the genomes of the *K. oxytoca* strains were searched against those of the reference sequence (GenBank accession number MF401554; Tse *et al.*, 2017). This operon encoded all 12 genes of the biosynthetic gene cluster. I used the protein sequences of the 12 genes to create a BLASTP database, against which I compared the clinical strains.

I used the following commands to carry out the blast search:

```
makeblastdb -in MF401554.faa -dbtype 'prot' -out tilimycin
```

```
blastp -query PSKoxy1.faa -db tilimycin -outfmt '6 qseqid sseqid pident qlen qstart qend' -
max_target_seqs 1 -out PS_Koxy1_kleboxymycin.txt
blastp -query PSKoxy2.faa -db tilimycin -outfmt '6 qseqid sseqid pident qlen qstart qend' -
max_target_seqs 1 -out PS_Koxy2_kleboxymycin.txt
blastp -query PSKoxy4.faa -db tilimycin -outfmt '6 qseqid sseqid pident qlen qstart qend' -
max_target_seqs 1 -out PS_Koxy4_kleboxymycin.txt
```

The data were imported into Excel and I calculated the coverage of the query sequence for each BLASTP result, examining only hits with >90% coverage and 95% identity with database sequences, to avoid detection of false positives. I determined coverage using the following equation with each of the BLASTP outputs.

((Query end-Query start)/Query length) x 100 = Percent coverage

The Prokka-generated GenBank files for PS_Koxy1, PS_Koxy4 genomes and the GenBank file for MF401554 were imported into Geneious Prime v2019.2.1. Mauve was used to align each genome against MF401554 to identify contigs encoding genes of MF401554. Those contigs that had matches to MF401554 were extracted and a second Mauve alignment was produced; the alignment view was exported as an image to visualise the arrangement of genes in the genomes compared with MF401554.

3.2.4 Sewage samples

Mixed-liquor sewage wastewater samples were collected from five tanks at the Mogden Water Treatment Works and four at Ascot Sewage Treatment Works (both Thames Water) and stored at 4 °C. Aliquots were centrifuged at 5000 *g* for 20 min to pellet debris. The supernatant was filter-sterilised by passing it through a 0.45 µm cellulose acetate filter (Millipore) and stored at 4 °C until needed.

3.2.5 Isolation of lytic phages active against the *Klebsiella* isolates

For each isolate a colony grown on nutrient agar (Oxoid Ltd) was used to inoculate overnight cultures (aerobic, 37 °C) in 5 mL nutrient broth (Oxoid Ltd). Aliquots (9 mL) of filter-sterilised sewage samples were mixed with 1 ml of sterile 10x concentrated nutrient broth containing 50 mM $CaCl_2$ and 50mM of $MgCl_2$, and 200 µl of the overnight culture were added. The culture was incubated aerobically for 6 h at 37 °C. Samples were then centrifuged at 10,000 rpm for 5 min. Aliquots (200 µl) of the overnight cultures of the isolates were used to inoculate 0.2% agar overlays (kept molten at 45 °C) which were then poured over nutrient agar plates.

Once the overlays had set, aliquots (100 μl) of the centrifuged supernatants were spotted onto the set overlay. Plates then were incubated aerobically overnight at 37 °C. Presence of phages in supernatants was indicated by zones of clearing (lysis) or individual plaques on overlays.

3.2.6 Purification of phages

The isolated phages were then purified against their host strain (Hoyles *et al*., 2015). Supernatants were diluted (10^{-1} to 10^{-9}) in nutrient broth. An aliquot (100 µl) of each dilution was added to 3 ml of 0.2% agar overlay (kept molten at 45 °C) with 200 µl of an overnight culture of the host strain and poured over nutrient agar plates, which were incubated aerobically at 37 °C overnight. The following day individual plaques were picked and suspended in 1 ml nutrient broth for a minimum of 1 h. A second dilution series was prepared, and the above process repeated. Again, individual plaques were picked and suspended in nutrient broth. In total, the process was repeated at least three times to generate pure phage stocks, which were stored at 4 °C.

3.2.7 Preparation of bulk stocks of isolated phages

Once each phage was purified, it needed to be bulked up to allow isolation of sufficient DNA for analyses. To 100-200 ml of sterile nutrient broth containing 5 mM $CaCl_2$ and 5 mM of $MgCl_2$ was added 1 ml of an overnight broth culture of the phage's host strain. The culture was grown to mid-exponential phase, then it was inoculated with 100 µL of pure phage stock and incubated at 37 °C until the medium became clear (i.e. the bacteria were lysed by the phage). The lysate was poured into 50 mL Falcon tubes and centrifuged at 5000 rpm for 30 min. The supernatant was filter-sterilised using a 0.45 µm cellulose acetate filter (Millipore) into a sterile Falcon tube. To precipitate the phages, 10% (w/v) polyethylene glycol 8000 (PEG)/6% (w/v) NaCl was added to the filter-sterilised supernatant, with gentle mixing to dissolve all the PEG and NaCl. The sample was then incubated at 4 °C for 16 h (Hoyles *et al*., 2015). Then, the samples were centrifuged at 5000 rpm for 30 min at 4 °C. Pellets were resuspended in 250 μl of SM buffer (50 mM Tris/HCl, pH 7.5; 100 mM NaCl, 8 mM $MgCl_2.7H_2O$; http://cshprotocols.cshlp.org/content/2006/1/pdb.rec8111.full?text_only=true). PEG

was removed from samples by adding an equal volume of chloroform. Samples were mixed then centrifuged at 13,000 rpm for 5 min inside a safety cabinet. Once centrifuged the aqueous layer was removed without disrupting the PEG interface. The aqueous layer containing the purified phage particles was stored at 4 °C until DNA extraction.

3.2.8 Extraction of DNA from the isolated phages, and restriction enzyme profiling

DNA was extracted from phages using the method of Murphy *et al.* (2013). To the purified phages 250 μL of DNAse (20 mg/ml) and 5 μL RNAse (2 mg/ml) in 200 μL of SM buffer were added to remove bacterial (host) DNA. The mixture was left on a heating block at 37 °C for 30 min, then incubated at 80 °C for another 10 min to break up the phages to release proteins and DNA. After 10 min the mixture was placed into ice and transferred to a fume cupboard. Phage Disruption Buffer was made up by adding 7.2 μL of 2-mercaptoethanol (Sigma) to 1 mL of GTC stock solution consisting of 22.5 mL of 6 M guanidium thiocyanate solution (Sigma), 6.8 mL of H_2O, 1.76 mL of sodium citrate (pH 7) and 2.64 mL of 10% sarkosyl. This was mixed with 1 mL of DNase- and RNase-treated bacteriophage lysate and left to incubate for 10 min at room temperature. An equal volume of phenol:chloroform:isoamyl alcohol (Sigma) and 500 μL of Tris EDTA were then added, with the sample vortexed and centrifuged at 13,000 rpm for 5 min inside the fume cupboard. After centrifugation, the top (aqueous) layer was transferred to a sterile microcentrifuge tube and a second phenol extraction was carried out to remove all proteins. The DNA present in the aqueous phase was precipitated by adding 2.5 volume of 96% ice-cold ethanol and 1 volume of sodium acetate to ~500 μL of the sample. The sample was mixed gently and placed in ice for 2 min to help precipitate the DNA, then centrifuged for 15 min at 13,000 rpm at 10 °C. The supernatant was tipped off, and the DNA was left to air dry. The air-dried DNA was incubated for a few minutes at 65 °C to drive off excess ethanol, then the pelleted DNA was resuspended and dissolved in 50 μL of sterile water.

Agarose gel electrophoresis was used to assess the quality of DNA isolated from each phage. Agarose gels were prepared by adding 0.24 g of agarose to 30 mL of TAE buffer, which was heated for 1 min to dissolve all the agarose. After

cooling to ~50 °C, 5 μL of ethidium bromide was added to the molten agarose. The molten agarose was poured into a gel cast and a well comb was added. The gel was left for 20 min to set. The agarose gel was placed in a gel tank and covered in TAE buffer. A 1 kb DNA ladder was run with all DNA samples for 60 min at 50 mA. DNA in gels was visualised under UV light. The purity/quantity of DNA in samples was measured using a Nanodrop. Aliquots of DNA were digested with different restriction enzymes as recommended by the manufacturer (Thermo Scientific Fast digest): *Eco*R1 (digest time 5 min at 37 °C), *Eco*321 (digest time 5 min at 37 °C), *Hin*dIII (digest time 10 min at 37 °C) and *Nco*1 (digest time 5 min at 37 °C). An aliquot (30 μL) of each reaction mixture was loaded on to the gel with 3 μL of loading dye and run for 2.5 h at 50 V, 100 mA. The gel was then stained with ethidium bromide (300 μL TAE + 0.5 μL of ethidium bromide) for 20 min and viewed under UV light. Restriction enzyme profiling produces fingerprints of phages allowing the identification of novel phages (i.e. those with unique fingerprints) (Kęsik-Szeloch *et al.*, 2013).

3.2.9 Determination of host ranges for isolated phages

The host ranges of the purified phages were tested against a panel of strains available in the laboratory at the University of Reading, using agar overlays inoculated with 200 μL overnight culture of the test bacterium. For each phage, 10 μL of the purified phage was spotted onto the overlay, then left to dry for 10 min. Once dried, the plates were incubated at 37 °C overnight and checked the next day for the presence of bacterial lysis (i.e. clearing) and potential depolymerase activity (i.e. haloes). This 'spot testing' is a rapid and efficient method for determining the host ranges of phages in a large collection of bacteria (Clokie and Kropinski, 2009). The host ranges of the purified phages were tested against all strains described in Chapters 3 and 4, and a selection of *Klebsiella* strains described by Chen *et al.* (2019).

3.3 RESULTS

3.3.1 Phenotypic and genomic characterisation of the *K. oxytoca* isolates

PS_Koxy1 was isolated from the throat of a 75-year-old male, PS_Koxy2 was isolated from the urine of a 47-year-old female, and PS_Koxy4 was isolated

from a rectal swab of a 65-year-old male in a high-dependency unit (Renal Dialysis Unit of a group of west London hospitals). All three isolates were identified by MALDI-TOF and API 20E (profile 5245773, 97.8%) as *K. oxytoca*.

Antimicrobial sensitivities were determined. The strains were resistant to amoxicillin (zone diameter <14 mm), augmentin (<19 mm), aztreonam (<21 mm), cefotaxime (<17 mm), cefoxitin (<19 mm), ceftazidime (<19 mm), cefuroxime (<18 mm), ciprofloxacin (<19 mm), ertapenem (<22 mm), gentamicin (<14 mm), tazocin (<17 mm), temocillin (<19 mm), tobramycin (<14 mm) and trimethoprim (<14 mm), and sensitive to amikacin (>18 mm), colistin (MIC <2 µg/mL), meropenem (>22 mm) and tigecycline (>18 mm). Ertapenem resistance was confirmed by E-test (had an MIC > 0.12 µg/mL), as ertapenem is used as an indicator antibiotic for the detection of carbapenemase enzyme. The isolates were sloped and sent to the PHE reference laboratory at Colindale for PCR and further testing to understand resistance mechanisms.

It is not possible to differentiate *K. oxytoca* from closely related species *K. michiganensis* and *K. grimontii* using phenotypic tests (Saha *et al*., 2013; Passet and Brisse, 2017; Chen *et al.*, 2019), though it can be distinguished from *Klebsiella huaxiensis* on the basis of its Voges-Proskauer reaction (Hu *et al*., 2019). From recent studies (Moradigaravand *et al.*, 2017; Chen *et al.*, 2019), it is clear that *K. michiganensis* and *K. grimontii* are clinically relevant but under-reported because of inadequate genomic characterisation of isolates.

For the assignment of strains to species using whole-genome sequence data, ANI values should be calculated with all phylogenetically closely related species and ideally using the genome sequences of the type strains of species (Chun *et al.*, 2018; Table 3.2). ANI values generated represent a measurement of how similar two genome sequences are to one another. If the genomes of two strains share >95% ANI, they are considered to belong to the same species (Chun *et al*., 2018).

Determination of ANI with genomes of authentic *K. oxytoca*, *K. grimontii*, *K. michiganensis* (Chen *et al*., 2019), '*Klebsiella pasteurii*', '*Klebsiella spallanzanii*' and *K. huaxiensis* strains showed PS_Koxy1, PS_Koxy2 and PS_Koxy4 all represented isolates of *K. michiganensis*, not *K. oxytoca*, following the recommendations of Chun *et al.* (2018). They shared 98.74, 98.70 and 98.71%

ANI, respectively, with the type strain of *K. michiganensis*, and 99.12 to 100.00% ANI with each other (Table 3.2).

Classification should also be confirmed with at least one method of phylogenetic analysis in which at least 30 genes are included (Chun *et al.*, 2018). Phylogenetic analysis of sequences of the 400 most conserved proteins in a dataset of authentic *K. oxytoca*, *K. michiganensis*, *K. grimontii* and related genomes (Chen *et al.*, 2019; Hu *et al.*, 2019; Merla *et al.*, 2019) with the three strains confirmed PS_Koxy1, PS_Koxy2 and PS_Koxy4 were *K. michiganensis*, with the three clustering together in the phylogenetic tree confirming they were highly similar to one another (Figure 3.4).

3.3.2 Genomic characterisation of the *K. michiganensis* isolates

Whole-genome sequences of each of the isolates were uploaded to the *Klebsiella oxytoca* MLST website (https://pubmlst.org/koxytoca/), to confirm the results of Eades *et al.* (2016). All three strains were ST138. None of the genomes included in Figure 3.4 were of this sequence type (Chen *et al.*, 2019). No further data are available on this ST. Sequence data were uploaded to Kaptive (http://kaptive.holtlab.net) to see whether capsule and O antigen types of the isolates could be determined. All three isolates were of unknown capsule (K) (Figure 3.5) and O antigen (Figure. 3.6) types. The K types of the three strains were most closely related to KL68: PS_Koxy1 – low confidence match, 93.06% coverage, 87.65% identity, 17/18 genes matched; PS_Koxy2 and PS_Koxy4 – low confidence match, 93.06% coverage, 87.41% identity, 16/18 genes matched). All three strains were of an O antigen type related to O1v1: low confidence match, 93.33% coverage, 73.12% identity, 4/7 genes matched).

The protein sequences of the genes encoded by the isolates were compared against the latest release of CARD (Jia *et al.*, 2017), a "*curated collection of characterized, peer-reviewed resistance determinants and associated antibiotics, organized by the Antibiotic Resistance Ontology (ARO) and AMR gene detection models*". Presence of the β-lactamase with carbapenemase activity GES-5 (100% identity, bit-score 591 – perfect CARD match) was confirmed, so too was that of the ESBL CTX-M-15 (100% identity, bit-score 593 – perfect CARD match) (Figure 3.7; Eades *et al.*, 2016). GES-5 was not detected in any of the

other strains included in Figure 3.4, nor has it been reported by CARD as being encoded by *K. oxytoca*. PS_Koxy1, PS_Koxy2 and PS_Koxy4 also encoded SHV-66 (99.65% identity, bit-score 580 – strict CARD match; Figure 3.7), an ESBL not reported by CARD (Table 3.3) as being present in *K. oxytoca* and related species. In this study, SHV-66 (99.65% identity, bit-score 580 – strict CARD match) was also found in *K. michiganensis* strains E718 (Liao *et al.*, 2012), GY84G39 (unpublished), K1439 (unpublished) and 2880STDY5682598 (Moradigaravand *et al.*, 2017) (accession numbers GCA_000276705, GCA_001038305, GCA_002265195 and GCA_900083915, respectively), which had been included in the phylogenetic analysis (Figure 3.4).

Table 3.2: ANI values determined using FastANI for PS_Koxy1, PS_Koxy2 and PS_Koxy4 against each other and type strain whole-genome sequences.

Query sequence	Reference sequence (GenBank accession number)	ANI (%)
PS_Koxy1	PS_Koxy2	99.12
PS_Koxy1	PS_Koxy4	99.99
PS_Koxy2	PS_Koxy4	100.00
PS_Koxy1	*K. oxytoca* ATCC 13182^{T} (GCA_900977765)	92.59
PS_Koxy1	'*K. pasteurii'* SB3355 (GCA_901563825)	94.27
PS_Koxy1	*K. grimontii* 06D021^{T} (GCA_900200035)	93.91
PS_Koxy1	'*K. spallanzanii'* SB3356 (GCA_901563875)	89.26
PS_Koxy1	*K. huaxiensis* WCHKl090001^{T} (GCA_003261575)	88.25
PS_Koxy1	*K. michiganensis* W14^{T} (GCA_901556995)	98.74
PS_Koxy2	*K. michiganensis* W14^{T} (GCA_901556995)	98.70
PS_Koxy4	*K. michiganensis* W14^{T} (GCA_901556995)	98.71

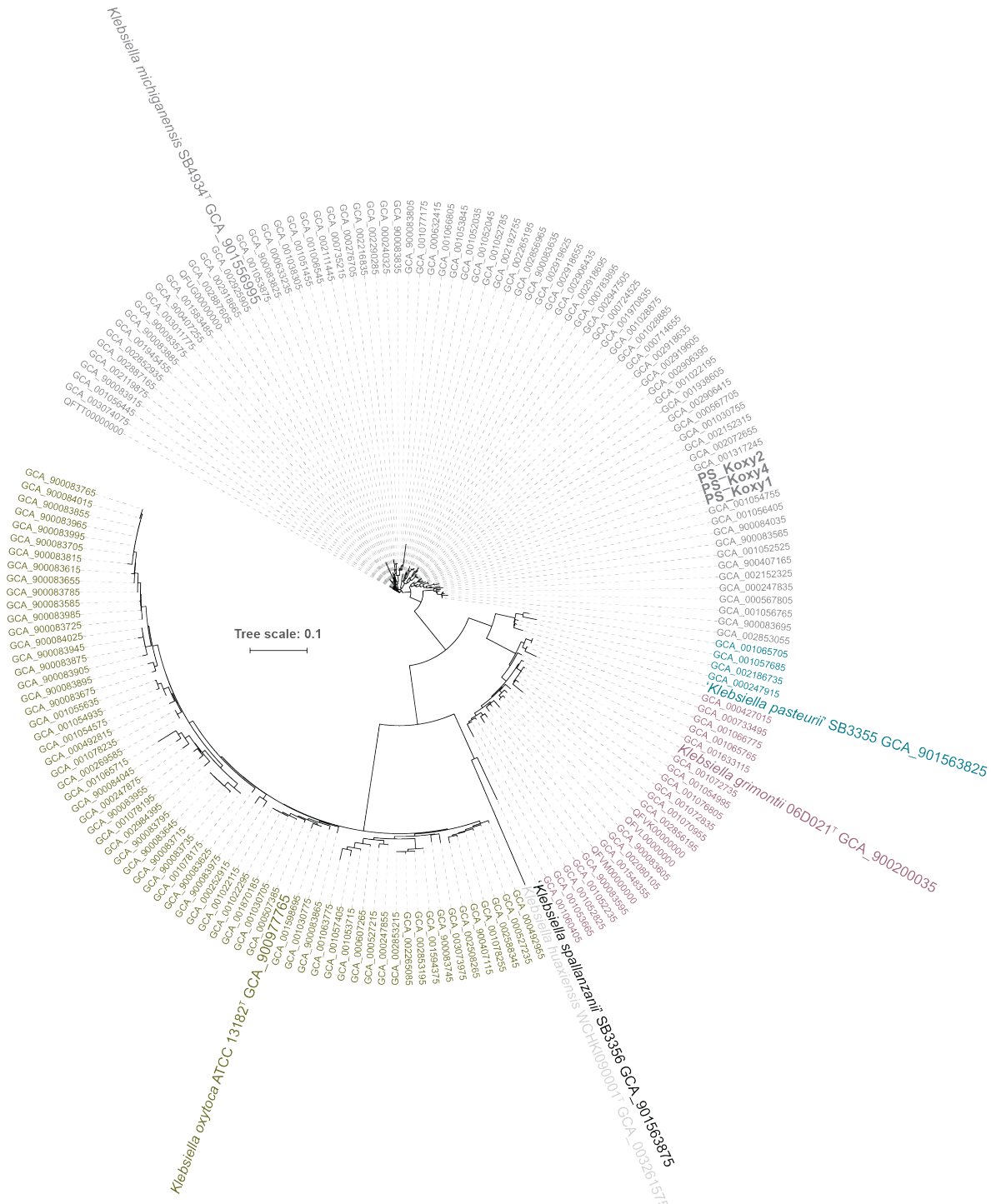
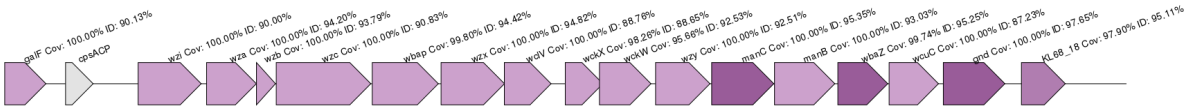
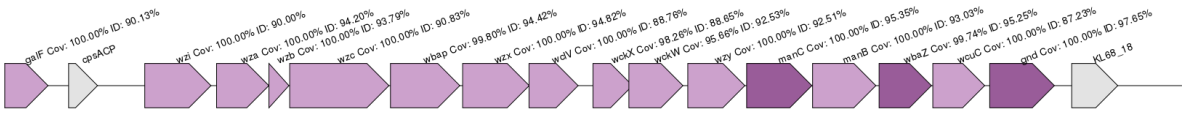


Figure 3.4. Phylogenetic analyses of whole-genome sequences confirming PS_Koxy1, PS_Koxy2 and PS_Koxy4 represent isolates of *K. michiganensis*. The phylogenetic tree was produced using PhyloPhlAn 0.99 (Segata *et al.*, 2013) implementing FastTree, which generates approximately maximum-likelihood trees for large alignments. The tree visualised using iTOL (https://itol.embl.de) and coloured using Adobe Illustrator. Scale bar, normalised fraction of total branch length. Analyses carried out and image produced by L. Hoyles.

PS_Koxy1



PS_Koxy2



PS_Koxy4

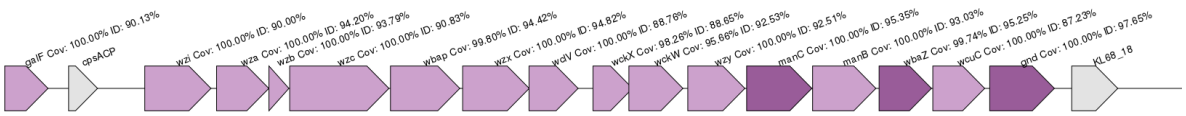


Figure 3.5. Capsule (K) typing of *K. michiganensis* PS_Koxy1, PS_Koxy2 and PS_Koxy4 as determined using the online version of Kaptive. Gene images were downloaded from Kaptive.

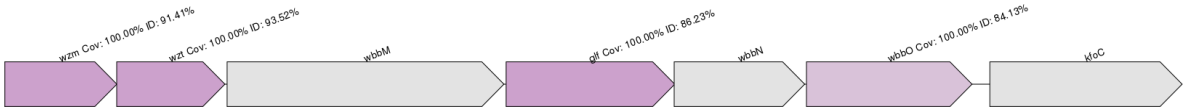


Figure 3.6. O antigen typing of *K. michiganensis* PS_Koxy1, PS_Koxy2 and PS_Koxy4 as determined using the online version of Kaptive. The gene image was downloaded from Kaptive.

In addition to the β-lactamases GES-5, CTX-M-15 and SHV-66, PS_Koxy1, PS_Koxy2 and PS_Koxy4 encoded several other antibiotic-resistance genes (Figure 3.7), some reported as rare (e.g. *acrB*, *acrD*, *mdtB*, *mdtC*) in *K. oxytoca* genomes by CARD while others were common (e.g. *baeR*, *emrA*, *emrB*, *fosA5*, *marA*) (Table 3.3). Antimicrobial profiling had shown the strains to be resistant to 14 of the antibiotics they were tested against (section 3.3.1). The strains' resistance to amoxicillin and augmentin (amoxicillin/clavulanic potassium) can be explained by them carrying OXA-1, a β-lactamase found in *K. oxytoca*. All the strains encoded CTX-M-15, which is an ESBL responsible for cephalosporin resistance (i.e. cefotaxime, cefoxitin, cefuroxime). *E. coli ampH* a class C AmpC β-

lactamase also confers cefoxitin resistance. AAC(6')-Ib7, *aadA*, APH(3'')-Ib, APH(6)-Id and AAC(3)-IIe genes encoded by the strains are responsible for aminoglycoside resistance (gentamicin, tobramycin). The isolates were sensitive to the aminoglycoside amikacin, which could be used therapeutically because of its reliable activity against Gram-negative aerobes; even with increased usage there is low incidence of resistance (Luis and Spencer, 1998). The presence of GES-5, SHV-66 and TEM-1 is responsible for the strains' resistance to the carbapenems amoxicillin, temocillin, ertapenem and tazocin; however, the strains were sensitive to meropenem. OXA-1 also confers resistance to tazocin, which would otherwise inhibit SHV and TEM β-lactamases. Ciprofloxacin resistance was due to the presence of the QnrB1gene together with *oqxA* and *oqxB* RND efflux pump. Trimethoprim resistance was conferred by *dfrA14* (Sköld, 2010).

3.3.3 Detection of the kleboxymycin biosynthetic gene cluster in the genomes of PS_Koxy1, PS_Koxy2 and PS_Koxy4

Tse *et al.* (2017) described the 12-gene operon encoding the kleboxymycin biosynthetic gene cluster. They deposited the annotated sequence of the cluster in GenBank (accession number MF401554). I used the protein sequences of the 12 genes encoded by the reference sequence to create a BLASTP database against which the proteins encoded in the genomes of PS_Koxy1, PS_Koxy2 and PS_Koxy4 were compared. Results were ordered by descending order of query coverage and sequence identity, to identify proteins in the three isolates showing high (>95%) identity with genes encoded within the kleboxymycin gene cluster. All three isolates encoded all 12 genes of the biosynthetic gene cluster, in consecutive genes (Table 3.4). PS_Koxy4 encoded the genes in the same orientation as MF401554, while PS_Koxy1 and PS_Koxy2 encoded the genes in the reverse orientation.

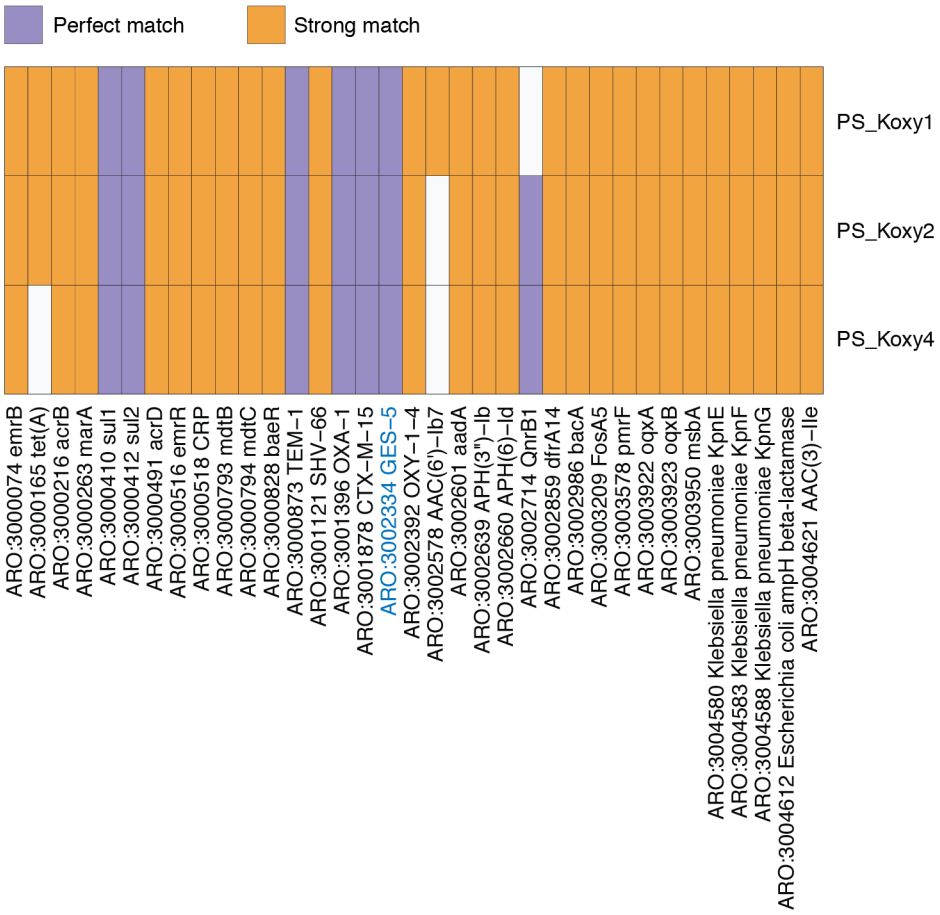


Figure 3.7. Comparison of genome data for the three *K. michiganensis* isolates with sequences in The Comprehensive Antibiotic Resistance Database (Jia *et al.*, 2017). Strict CARD match, not identical but the bit-score of the matched sequence is greater than the curated BLASTP bit-score cut-off; perfect CARD match, 100% identical to the reference sequence along its entire length. Loose matches are not shown to avoid presenting false positives based on sequences with low homology and bit-scores below CARD BLASTP cut-off recommendations. BLASTP analyses carried out by me and image produced by L. Hoyles.

Table 3.3: Summary of information for CARD genes found in *K. michiganensis* PS_Koxy1, PS_Koxy2 and PS_Koxy4.

CARD data from analyses of 107 *K. oxytoca* genomes (https://card.mcmaster.ca/prevalence). CARD Prevalence 3.0.5 is based on sequence data acquired from NCBI on 31 July 2019, analysed using RGI 5.0.0 (DIAMOND homolog detection) and CARD 3.0.3.

ARO: accession	Name	Definition	Prevalence (%) in *K. oxytoca**
3000074	*emrB*	Translocase in the *emrB*-TolC efflux protein in *E. coli*. It recognises substrates including carbonyl cyanide m-chlorophenylhydrazone, nalidixic acid and thioloactomycin.	99.19
3000165	*tet*(*A*)	Tetracycline efflux pump found in many species of Gram-negative bacteria.	3.25
3000216	*acrB*	Protein subunit of AcrA-AcrB-TolC multidrug efflux complex. AcrB functions as a heterotrimer which forms the inner membrane component and is primarily responsible for substrate recognition and energy transduction by acting as a drug/proton antiporter.	1.63
3000263	*marA*	In the presence of antibiotic stress, *E. coli* overexpresses the global activator protein MarA, which besides inducing MDR efflux pump AcrAB, also down-regulates synthesis of the porin OmpF.	95.33
3000410	*sul1*	Sulfonamide resistant dihydropteroate synthase of Gram-negative bacteria. It is linked to other resistance genes of class 1 integrons.	28.79
3000412	sul2	Sulfonamide resistant dihydropteroate synthase of Gram-negative bacteria, usually found on small plasmids.	6.54
3000491	*acrD*	Aminoglycoside efflux pump expressed in *E. coli*. Its expression can be induced by indole, and is regulated by *baeRS* and *cpxAR*.	1.87
3000516	*emrR*	EmrR is a negative regulator for the EmrAB-TolC multidrug efflux pump in *E. coli*. Mutations lead to EmrAB-TolC overexpression.	99.07
3000518	CRP	CRP is a global regulator that represses MdtEF multidrug efflux pump expression.	100
3000793	*mdtB*	MdtB is a transporter that forms a heteromultimer complex with MdtC to form a multidrug transporter. MdtBC is part of the MdtABC-TolC efflux complex.	0.93
3000794	*mdtC*	MdtC is a transporter that forms a heteromultimer complex with MdtB to form a multidrug transporter. MdtBC is part of the MdtABC-TolC efflux complex. In the absence of MdtB, MdtC can form a homomultimer complex that results in a functioning efflux complex with a narrower drug specificity.	0.93
3000828	*baeR*	BaeR is a response regulator that promotes the expression of MdtABC and AcrD efflux complexes.	98.13
3000873	TEM-1	TEM-1 is a broad-spectrum β-lactamase found in many Gram-negative bacteria. Confers resistance to penicillins and first	13.08

ARO: accession	Name	Definition	Prevalence (%) in *K. oxytoca**
		generation cephalosphorins.	
3001121	SHV-66	SHV-66 is an extended-spectrum β-lactamase found in *K. pneumoniae*.	ND
3001396	OXA-1	OXA-1 is a β-lactamase found in *E. coli*.	2.80
3001878	CTX-M-15	CTX-M-15 is a β-lactamase found in the family *Enterobacteriaceae*.	1.87
3002334	GES-5	GES-5 is a β-lactamase found in the family *Enterobacteriaceae*.	ND
3002392	OXY-1-4	OXY-1-4 is a β-lactamase found in *K. oxytoca*.	2.80
3002578	AAC(6')-Ib7	AAC(6')-Ib7 is a plasmid-encoded aminoglycoside acetyltransferase in *Enterobacter cloacae* and *Citrobacter freundii*.	4.67
3002601	*aadA*	ANT(3")-Ia is an aminoglycoside nucleotidyltransferase gene encoded by plasmids, transposons, integrons in *Enterobacteriaceae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Vibrio cholerae*.	21.50
3002639	APH(3'')-Ib	APH(3")-Ib is an aminoglycoside phosphotransferase encoded by plasmids, transposons, integrative conjugative elements and chromosomes in *Enterobacteriaceae* and *Pseudomonas* spp.	9.35
3002660	APH(6)-Id	APH(6)-Id is an aminoglycoside phosphotransferase encoded by plasmids, integrative conjugative elements and chromosomal genomic islands in *K. pneumoniae*, *Salmonella* spp., *E. coli*, *Shigella flexneri*, *Providencia alcalifaciens*, *Pseudomonas* spp., *V. cholerae*, *Edwardsiella tarda*, *Pasteurella multocida* and *Aeromonas bestiarum.*	11.21
3002714	QnrB1	Plasmid-mediated quinolone resistance protein found in *K. pneumoniae*.	0.93
3002859	*dfrA14*	Integron-encoded dihydrofolate reductase found in *E. coli.*	13.08
3002986	*bacA*	BacA recycles undecaprenyl pyrophosphate during cell wall biosynthesis, which confers resistance to bacitracin.	1.87
3003209	*fosA5*	Fosfomycin resistance gene isolated from clinical strain of *E. coli* E265. It is susceptible to amikacin, tetracycline and imipenem, and resistant to sulphonamide, cephalosporins, gentamicin, ciprofloxacin, chloramphenicol and streptomycin.	87.85
3003578	*pmrF*	Required for the synthesis and transfer of 4-amino-4-deoxy-L-arabinose to Lipid A, which allows Gram-negative bacteria to resist the antimicrobial activity of cationic antimicrobial peptides and antibiotics such as polymyxin.	0.93
3003922	*oqxA*	RND efflux pump conferring resistance to fluoroquinolone.	91.59
3003923	*oqxB*	RND efflux pump conferring resistance to fluoroquinolone.	2.80
3003950	*msbA*	Multidrug resistance transporter homolog from *E. coli* and belongs to a superfamily of transporters that contain an adenosine triphosphate (ATP) binding cassette (ABC) which is also called a nucleotide-binding domain (NBD). MsbA is a member of the MDR-ABC transporter group by sequence homology. MsbA transports lipid A, a major component of the bacterial outer cell membrane, and is the only bacterial ABC transporter that is essential for cell viability.	97.20

ARO: accession	Name	Definition	Prevalence (%) in *K. oxytoca**
3004580	*K. pneumoniae* KpnE	KpnE subunit of KpnEF resembles EbrAB from *E. coli*. Mutation in KpnEF resulted in increased susceptibility to cefepime, ceftriaxon, colistin, erythromycin, rifampin, tetracycline, and streptomycin as well as enhanced sensitivity toward sodium dodecyl sulfate, deoxycholate, dyes, benzalkonium chloride, chlorhexidine and triclosan.	97.20
3004583	*K. pneumoniae* KpnF	KpnF subunit of KpnEF resembles EbrAB from *E. coli*. Mutation in KpnEF resulted in increased susceptibility to cefepime, ceftriaxon, colistin, erythromycin, rifampin, tetracycline, and streptomycin as well as enhanced sensitivity toward sodium dodecyl sulfate, deoxycholate, dyes, benzalkonium chloride, chlorhexidine and triclosan.	98.13
3004588	*K. pneumoniae* KpnG	KpnG consists of ~390 residues and resembles EmrA of *E. coli*. Disruption of the pump components KpnG-KpnH significantly decrease resistance to azithromycin, ceftazidime, ciprofloxacin, ertapenem, erythromycin, gentamicin, imipenem, ticarcillin, norfloxacin, polymyxin-B, piperacillin, spectinomycin, tobramycin and streptomycin.	97.20
3004612	*E. coli ampH*	AmpH is a class C ampC-like β-lactamase and penicillin-binding protein identified in *E. coli*.	98.13
3004621	AAC(3)-IIe	Plasmid-encoded aminoglycoside acetyltransferase in *E. coli*.	2.80

*ND, no data.

Table 3.4: Results of BLASTP analyses comparing the protein sequences encoded within the kleboxymycin operon with those of *K. michiganensis* PS_Koxy1, PS_Koxy2 and PS_Koxy4.

Gene annotations for the PS_Koxy strains are from Prokka.

Encoding gene	PS_Koxy1	PS_Koxy2	PS_Koxy4	Identity (%)	Coverage (%)
mfsX	PSKoxy1_03008	PSKoxy2_03101	PSKoxy4_04877	96.10	99.76
uvrX	PSKoxy1_03007	PSKoxy2_03100	PSKoxy4_04878	97.21	99.87
hmoX	PSKoxy1_03006	PSKoxy2_03099	PSKoxy4_04879	99.21	99.80
adsX	PSKoxy1_03005	PSKoxy2_03098	PSKoxy4_04880	96.00	99.85
icmX	PSKoxy1_03004	PSKoxy2_03097	PSKoxy4_04881	97.62	99.52
dhbX	PSKoxy1_03003	PSKoxy2_03096	PSKoxy4_04882	97.70	99.62
aroX	PSKoxy1_03002	PSKoxy2_03095	PSKoxy4_04883	96.92	99.74
npsA	PSKoxy1_03001	PSKoxy2_03094	PSKoxy4_04884	95.06	99.80
thdA	PSKoxy1_03000	PSKoxy2_03093	PSKoxy4_04885	92.11	98.68
npsB	PSKoxy1_02999	PSKoxy2_03092	PSKoxy4_04886	96.43	99.93
npsC	PSKoxy1_02998	PSKoxy2_03091	PSKoxy4_04887	94.62	98.47
marR	PSKoxy1_02997	PSKoxy2_03090	PSKoxy4_04888	99.39	99.39

To determine the alignment of the genes encoded by the *K. michiganensis* strains with MF401554, all sequence data were imported into Geneious Prime v2019.2.1 in annotated GenBank format. For each genome, progressiveMauve alignments were done to determine which contig encoded the kleboxymycin biosynthetic gene cluster. Once the contigs had been identified, they were extracted from the GenBank files. The sequences of PS_Koxy1 and PS_Koxy2 sequences were reversed and complemented, and all three sequences were aligned against MF401554 using progressiveMauve. The gene alignment showed the *K. michiganensis* strains encoded the entire biosynthetic gene cluster (Figure 3.8).

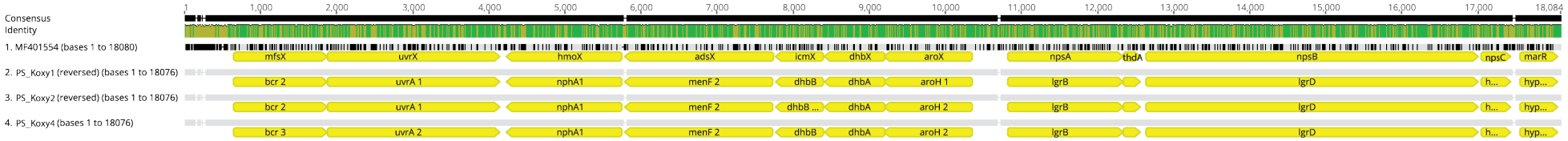


Figure 3.8. Demonstration that PS_Koxy1, PS_Koxy2 and PS_Koxy4 encode the kleboxymycin biosynthetic gene cluster. All strains encode the same genes as the reference sequence, MF401554 (Tse *et al.*, 2017). The image was produced by me using Geneious Prime 2019.2.1, from a progressiveMauve alignment of the reference sequence and the regions of the PS_Koxy1, PS_Koxy2 and PS_Koxy4 genomes encoding the gene cluster.

3.3.4 Isolation of lytic phages against the *K. michiganensis* isolates

The three isolates encoding GES-5 were screened against filter-sterilised sewage samples collected from four different mixed-liquor tanks (A–D) at the Mogden Sewage Treatment Works. After addition of 9 ml filter-sterilised sewage water to 10x concentrated sterile nutrient broth, samples were inoculated with 200 µl of overnight culture of each strain and incubated for 6 h at 37 °C. Aliquots (200 µl) of the centrifuged spent medium were used in spot assays to determine whether cultures were positive for lytic phages (i.e. whether they produced zones of clearing or individual plaques in overlays). One of each different plaque type was propagated to purity. In total, seven phages were enriched for in the sewage samples (Table 3.5).

Table 3.5: Phages propagated to purity from sewage water against GES-5-positive strains.

Strain	Phage isolated from tank			
	A	B	C	D
PSKoxy 1	1Ai			
PSKoxy 2		2B	2C	2Di
PSKoxy 4		4B		4Di
				4Dii

Host ranges of the phages were determined against all three strains, to see if the isolated phages were able to recognise a receptor common among these different strains and thus displayed broad-spectrum activity (Table 3.6).

Host ranges of the phages against a range of antibiotic-resistant *K. pneumoniae* and *Acinetobacter* spp. (6 *Acinetobacter baumannii*, 1 *Acinetobacter pittii*), and isolates recovered from preterm infants (Chen *et al.*, 2019) were also determined. None of the phages had any effect on the *Acinetobacter* spp. tested. However, some of the phages were able to infect one or more *K. pneumoniae* strains (Table 3.7).

Table 3.6: Host ranges of the lytic phages against the GES-5-positive isolates.

Strain	1Ai	2B	2C	2Di	4B	4Di	4Dii
PS_Koxy1	+ d	–	–	–	–	–	–
PS_Koxy2	+ d	+	+	–	+	–	–
PS_Koxy4	–	–	–	+	+	+	+

+, Lytic; d, depolymerase activity (haloes formed round plaques); –, no activity.

Table 3.7: Host ranges of the lytic phages against clinically relevant *Klebsiella* isolates.

Results are only shown for those phages that showed activity against one or more of the test strains.

Species	Strain	MLST	K, O types	2B	2Di	4Dii	Reference
K. pneumoniae	PS_Kpn32	ST35	K22, O1v1	+ d	–	–	Chapter 4
K. pneumoniae	PS_Kpn39	ST14	K64, O1v1	–	–	+ d	Chapter 4
K. pneumoniae	PS_Kpn41	ST15	K112, O1v1	–	–	+ d	Chapter 4
K. variicola	PS_misc5	*	K81, *	–	–	+	Chapter 4
K. pneumoniae	P010F	ST253	*, O1v2	–	+ d	–	Chen *et al.* (2019)
K. pneumoniae	P057K W	*	K11, O3/O3a	–	+ ?d	–	Chen *et al.* (2019)
K. grimontii	P079F P	ST76	*, O1v1	–	+ d	+ d	Chen *et al.* (2019)
K. pneumoniae	P080S T	ST2211	K10, O1v2	–	–	+	Chen *et al.* (2019)
K. pneumoniae	P008E	ST461	K10, O1v2	–	–	+	Chen *et al.* (2019)
K. grimontii	P038I	*	*, *	–	+ d	+	Chen *et al.* (2019)

+, Lytic; d, depolymerase activity (haloes formed round plaques); ?d, may be depolymerase activity (requires further study); –, no activity.

*, Unknown type.

3.4 DISCUSSION

3.4.1 Genomic characterisation of the GES-5-positive strains reveals them to be *K. michiganensis*

Strains PS_Koxy1, PS_Koxy2 and PS_Koxy4 had been isolated from a throat swab, urine sample and rectal swab, respectively, from patients hospitalised during an infection outbreak mainly associated with the high-dependency units

including the renal dialysis unit at the Hammersmith and Saint Mary's Hospitals between March and April 2015. They were collected for screening for carbapenem-resistant organisms using the screening protocol described in Chapter 2. The blue colonies on the culture medium (Colorex^{TM} mSuper CARBA^{TM}; E&O Labs Chromagar, France) were identified by MALDI-TOF and API 20E (profile 5245773, 97.8%) as *K. oxytoca*.

When received in the laboratory for my work, I confirmed the identity of the strains as *K. oxytoca* by API 20E testing. Eades *et al.* (2016) had previously reported the strains to be *K. oxytoca* based on whole-genome sequence data. They had also found the strains to be of ST138. As the whole-genome sequence data generated by Eades *et al.* (2016) were not publicly available, new sequence data were generated for the strains. Analysis of the genomes via the *Klebsiella oxytoca* MLST website confirmed the strains to be ST138. Following the recommendations of Chun *et al.* (2018) regarding using ANI between genomes to determine species affiliations, the three strains were found to be *K. michiganensis* (Table 3.2), with phylogenetic analyses confirming this affiliation (Figure 3.4). In addition, the strains were found to represent unknown capsule and O antigen types (Figure 3.5, Figure 3.6).

Antimicrobial susceptibility testing showed the isolates to be resistant to amoxicillin, augmentin, aztreonam, cefotaxime, cefoxitin, ceftazidime, cefuroxime, ciprofloxacin, ertapenem, gentamicin, tazocin, temocillin, tobramycin and trimethoprim, and sensitive to amikacin, colistin, meropenem and tigecycline. The genotypic testing of the strains using the in-house Xpert® Carba-R PCR failed to detect any resistance genes, thus agreeing with Dortet *et al*. (2016). This is why I (while working in the Charing Cross microbiology laboratory) had originally submitted the strains to the PHE reference laboratory at Colindale for PCR and further testing to understand resistance mechanisms.

As discussed below, bioinformatics analysis of the whole-genome sequences of the strains showed them to encode a number of antibiotic-resistance genes not reported by Eades *et al.* (2016), and a biosynthetic gene cluster known to produce enterotoxins found in intestinal contents of patients suffering from AAHC.

3.4.2 Clinical significance of *K. michiganensis*

K. michiganensis was originally proposed to describe an isolate closely related to *K. oxytoca* recovered from a toothbrush holder (Saha *et al.*, 2013). The bacterium is now recognised as an emerging pathogen, with this recognition due to improved genomic characterisation of clinical isolates that would have previously been described as *K. oxytoca* based on simple phenotypic tests (Zheng *et al.*, 2018; Pedersen *et al.*, 2018; Chen *et al.*, 2019; Seiffert *et al.*, 2019). Reports specific to *K. michiganensis* have only recently begun to appear in the literature.

A carbapenemase-producing *K. oxytoca* isolate was recovered from the stool sample of an immunocompromised patient hospitalized with acute diarrhoea in Fahzu, China (Zheng *et al.*, 2018). On analysis of genomic data, the isolate was found to harbour the carbapenemase-encoding genes $bla_{\text{KPC-2}}$, $bla_{\text{NDM-2}}$ and $bla_{\text{NDM-5}}$. ANI analysis of the isolate's genome showed it shared 100% ANI with the type strain of *K. michiganensis*. This study was the first to isolate *K. michiganensis* carrying these genes in China. A $bla_{\text{KPC-2}}$-encoding isolate of *K. michiganensis* recovered from a blood sample has also been reported in the US (Hazen *et al.*, 2018).

During a study of multidrug-resistant *Enterobacteriaceae* recovered from in-patients in 10 private hospitals in Durban, South Africa, Pedersen *et al*. (2018) isolated a strain of *K. michiganensis* belonging to ST170 and encoding $bla_{\text{NDM-1}}$. In addition, a $bla_{\text{OXA-181}}$- and $bla_{\text{NDM-1}}$-encoding strain of *K. michiganensis* isolated from a cancer patient in KwaZulu-Natal Province, South Africa has been reported (Founou *et al.*, 2018).

Seiffert *et al.* (2019) reported on a bloodstream infection caused by *K. michiganensis* in an immunocompromised patient in St Gall, Switzerland. Initially, the isolate was identified by MALDI-TOF as *K. oxytoca* but whole-genome sequence analysis identified it as *K. michiganensis*. Susceptibility testing revealed the isolate was resistant to first- to fourth-generation cephalosporins and the carbapenem ertapenem, and sensitive only to amikacin and colistin. Genomic analyses showed the strain to encode genes conferring resistance to β-lactamases ($bla_{\text{TEM-1B}}$, $bla_{\text{OXY-1-1}}$), aminoglycosides (*aac*(*3*)-*IId*, *aph*(*3'*)-*Ia*, *aadA5*, *strA*, *strB*), macrolides (*mph*(A)), sulphonamides (*su11, su12*), tetracycline (*tet*(*B*)) and trimethoprim (*dfrA17*). *K. pneumoniae* carbapenemase (KPC)3 gene was

encoded on plasmid IncFII$_{K2}$-FIB. This was the first study to identify *bla*$_{KPC}$ in *K. michiganensis* in Europe. A *bla*$_{KPC-3}$-encoding isolate of *K. michiganensis* has also been reported in the US (Hazen *et al.*, 2018).

During a study to characterise *Klebsiella* spp. recovered from preterm infants, Chen *et al*. (2019) showed ~10% of faecal samples harboured *Klebsiella* isolates, of which 1.8% were *K. michiganensis*. Once again, API 20E data had initially identified the isolates as *K. oxytoca*. ANI and phylogenetic analyses of whole-genome sequence data revealed the strains to be *K. michiganensis*. Phenotypic testing showed the *K. michiganensis* isolates had intermediate resistance to both benzylpenicillin (encoded FosA5) and meropenem (encoded OXY-1-2), β-lactamases which may prove problematic when selecting treatment regimens for necrotising enterocolitis or sepsis. Results from this study also highlighted refined analyses with curated sequence databases are needed to differentiate *K. oxytoca* and closely related species.

Similar to the above-mentioned studies, the three *K. michiganensis* strains characterised in this Chapter were isolated from immunocompromised patients with underlying conditions and carried a range of antibiotic-resistance genes (Table 3.3).

BLASTP of the proteins encoded in the strains' genomes against CARD confirmed the presence of the carbapenemase-encoding gene *bla*$_{GES-5}$ (discussed below) and the ESBL CTX-M-15, along with the β-lactamases *bla*$_{TEM-1}$ and *bla*$_{SHV-66}$. SHV-66 was also encoded in the genomes of *K. michiganensis* strains E718, GY84G39, K1439 and 2880STDY5682598 (included in the phylogenetic analysis, Figure 3.4). SHV-66 has previously only been reported in a minority of β-lactamase-producing *K. pneumoniae* in Guangzhou, China (Zuo *et al.*, 2006). Moradigaravand *et al.* (2017) noted that 2880STDY5682598 encoded a *bla*$_{SHV}$ gene but did not document its type nor indicate its novelty.

3.4.3 The "Guiana extended spectrum" (GES)-type β-lactamase

GES-type β-lactamase is an Ambler Class A plasmid-encoded ESBL. It was discovered in 2000 in *K. pneumoniae* clinical isolates from French Guiana (Naas *et al*., 2016; Bonnin *et al*., 2017). This carbapenemase slowly hydrolyses carbapenems due to a replacement of glycine at position 170 by

asparagine/serine. Over time, GES β-lactamases have been identified in other Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Enterobacter cloacae*, and are increasingly being identified in *Acinetobacter baumannii* (Chihi *et al*., 2016). One of the worrying features that GES enzymes share, as compared to other β-lactamases, is their ability to evolve into carbapenem-hydrolysing enzymes via single point mutations. Since GES-1 was first detected in 2000, numerous GES-types have been found in several countries, with 31 variants recognised (Bonnin *et al*., 2017). GES-associated outbreaks have also been reported (Smith *et al*., 2012). GES-1 variants that have emerged have the ability to hydrolyse carbapenems with increased affinity. More concerning, they are difficult to detect in clinical laboratories and available clinical data on outbreaks are scarce (Weldhagen, 2006).

The first GES-5 carbapenemase (glycine to serine substitution) was found in 2004, in *K. pneumoniae* recovered from patients at Bundang CHA Medical Center, Republic of Korea (Jeong *et al*., 2005). Since then, GES-5 has been reported in various Gammaproteobacteria (predominantly *P. aeruginosa* and *K. pneumoniae*) around the world (Table 3.8).

Eades *et al.* (2016) reported the GES-5-positive *K. oxytoca*-associated outbreak in UK hospitals, with the emergence of this genotype thought to be due to plasmid or intergron transfer among related bacteria (Diene and Rolain, 2014). It was not the first report of GES-5 in *K. oxytoca*: that appeared in Kabir *et al.* (2016), but is likely to reference one of the strains reported by Eades *et al.* (2016) as both were PHE publications.

Many laboratories in the UK and other countries find it difficult to isolate GES-5-positive carbapenemase-producing organisms as they remain sensitive to meropenem (i.e. it shows a different sensitivity pattern to other carbapenemase-producing organisms (CPOs)), which is one of the indicator carbapenems used for detection of CPOs. In the microbiology laboratory, if we had not used the indicator carbapenem ertapenem, which is more sensitive than meropenem in picking up carbapenemase activity, coupled with the optimised workflow outlined in Chapter 2, we would not have isolated the three GES-5-positive *K. michiganensis* strains discussed in this Chapter. Therefore, this study has also shown the importance of

using a culture method as an initial platform to detect the presence of 'new and emerging' carbapenemases before they are characterised at the genomic level.

Table 3.8: Worldwide detection of GES-5-positive strains in clinical settings.

Species	Isolated from	Country	Reference(s)
P. aeruginosa	Burn wound, clinical isolates	China	Wang *et al.* (2006); Wang *et al.* (2010)
K. pneumoniae	Sputum, clinical isolates	South Korea	Jeong *et al.* (2005); Bae *et al.* (2015)
P. aeruginosa	Bloodstream infections	Brazil	Picão *et al.* (2009)
K. pneumoniae	Rectal swab	Brazil	Picão *et al.* (2010)
P. aeruginosa	Clinical isolates	Spain	Viedma *et al.* (2009); Juan *et al.* (2013); Recio *et al.* (2018)
E. coli	Bed sore specimen, clinical isolate	South Korea	Kim *et al.* (2011); Bae *et al.* (2015)
P. aeruginosa	Nosocomial infections	Canada	Mataseje *et al.* (2012)
P. aeruginosa	Clinical isolates	Mexico	Castillo-Vera *et al.* (2012)
P. aeruginosa	Clinical isolate	Turkey	Iraz *et al.* (2014)
P. aeruginosa	Urine	India	Maurya *et al.* (2014)
K. pneumoniae	Clinical isolates	Portugal	Papagiannitsis *et al.* (2015); Aires-de-Souza *et al.* (2019)
P. aeruginosa	Clinical isolate	Lithuania	Mikucionyte *et al.* (2016)
K. oxytoca	Urine, wound, blood, rectal swab, bronchoalveolar lavage	UK	Eades *et al.* (2016)
A. baumannii	Clinical isolate	Saudi Arabia	Al-Agamy *et al.* (2017)
S. marcescens	Ascitic fluid	Brazil	Nodari *et al.* (2017)
K. pneumoniae	Rectal swab	France	Bonnin *et al.* (2017)
E. coli	Hospital wastewater	Thailand	Runcharoen *et al.* (2017)
P. aeruginosa	Clinical isolates	Czech Republic	Papagiannitsis *et al.* (2017)
E. cloacae	Wound	Czech Republic	Chudejova *et al.* (2018)
P. aeruginosa	Clinical isolate	Australia	Sherry *et al.* (2018)
K. pneumoniae	Clinical isolates	South Africa	Pedersen *et al.* (2018)
P. aeruginosa	Clinical isolates	Estonia	Telling *et al.* (2018)
P. aeruginosa	Clinical isolates	Japan	Hishinuma *et al.* (2018)
P. aeruginosa	Clinical isolates	Dubai	Ayoub Moubareck *et al.* (2019)
P. aeruginosa	Clinical isolates	Indonesia	Saharman *et al.* (2019)

This study also highlighted the difficulty in identifying novel carbapenemases by relying on routine clinical laboratory tests: the three *K. michiganensis* strains gave negative phenotypic carbapenemase test results – they were sensitive to meropenem in routine testing and produced a negative result with the in-house carbapenemase PCR (Ribeiro *et al*., 2014; Spyrakis *et al*., 2019). This misrepresentation is due to the fact that, at present, several subclasses of β-lactamases breach the barrier between classical ESBLs and carbapenemases. For example, the GES enzymes that possess carbapenemase activity (e.g. GES-5) are still reported in the literature as ESBLs, and many laboratories are recording them under the ESBL subgroup and this misclassification can also lead to lack of direct appropriate treatment. Although GES-5 belongs to the ESBL Ambler Class A plasmid-encoded β-lactamases exhibiting ESBL properties, changes in its active site enhance its activity conferring the ability to slowly hydrolyse carbapenems (Naas *et al*., 2008; Jeong *et al*., 2005); therefore, GES-5 should be classified as belonging to the carbapenemase group (Lee *et al*., 2012; Bae *et al*., 2007) and thereby the correct identification tools can be used to help to isolate GES-5-carrying strains in the clinical laboratory.

3.4.4 The three strains of *K. michiganensis* all encode the kleboxymycin gene cluster

It has been shown the *K. oxytoca* gut colonisation is linked with AAHC (Beaugerie *et al*., 2003). The disease is caused by the overgrowth of cytotoxin-producing *K. oxytoca* secondary to use of antibiotics such as penicillin or amoxicillin resulting in the presence of diffuse mucosal oedema and haemorrhagic erosions (Beaugerie *et al*., 2003). This type of colitis is distinct from the more common form of antibiotic-associated diarrhoea caused by toxin-producing *Clostridium difficile*, which usually give rise to watery diarrhoea resulting in mild to moderate disease.

Schneditz *et al*. (2014) showed TV, a pyrrolobenzodiazepine (PBD) derivative produced by *K. oxytoca*, is one of the enterotoxins responsible for causing AAHC. This toxic product is encoded by the heterologous expression of the kleboxymycin (also known as tilimycin (TM); Unterhauser *et al.*, 2019)

biosynthetic gene cluster consisting of 12 genes (Tse *et al*., 2017). *In vitro* assays showed that both kleboxymycin and TV are linked with cytotoxicity, with kleboxymycin showing a higher cytotoxicity than TV. Tse *et al.* (2017) also suggested that TV is not the main cytotoxin produced by AAHC-associated *K. oxytoca* strains. During biosynthesis of TV, the enterotoxin gene cluster produces three distinct secondary metabolites, two of which (TM, TV) are cytotoxic to human cells. TV is not produced directly by the non-ribosomal peptide synthetase encoded by the biosynthetic gene cluster. Instead an *N*-acylprolinal, which reacts spontaneously with indole, produces TV from the secondary metabolites TM and culdesacin. TM and TV belong to the PBD family of natural products, which exhibit antibacterial and anticancer activity by alkylating DNA. TM damages DNA while TV stabilises microtubules. Both enterotoxins trigger apoptosis (i.e. cell death) characteristic for the colonic epithelium in AAHC.

In this study, I used the protein sequences of the kleboxymycin gene cluster (Tse *et al.*, 2107) to determine whether the three *K. michiganensis* strains encoded the gene cluster. PS_Koxy1, PS_Koxy2 and PS_Koxy4 all encoded the complete kleboxymycin biosynthetic gene cluster (Table 3.4, Figure 3.8), suggesting they may be virulent in addition to displaying multidrug resistance. Further study needs to be done to confirm the isolates are actively producing the cytotoxin. This could be achieved by following the method used by Tse *et al*. (2017), who examined culture supernatants of *K. oxytoca* MH43-1 and compared them to supernatants from non-toxigenic strains. They confirmed the production of TV and kleboxymycin (TM) by MH43-1, and the presence of the gene cluster in the strain's genome. The virulence of strains that produce the enterotoxins could also be tested in mice (Schneditz *et al.*, 2014).

Schneditz *et al.* (2014) reported *npsA*/*npsB* were functionally conserved in six sequenced strains of *K. oxytoca*, based on a BLASTP analysis. All the genomes included in the study of Schneditz *et al.* (2014) were compared with those of the type strain of *K. oxytoca* and related species to confirm their species affiliations (Table 3.9). While some of the *npsA*/*npsB*-positive strains were *K. oxytoca*, others belonged to *K. grimontii* and '*K. pasteurii*'. Examining the genomes of the strains to determine if they carried the full kleboxymycin biosynthetic gene cluster, it was found that *K. michiganensis* 10–5242, E718 and KCTC 1686 did not encode homologues associated with the kleboxymycin biosynthetic gene cluster.

K. oxytoca 10–5245 encoded almost-complete homologues of four genes (EHS96696.1 (*marA*) 98.79% identity, 99.39% coverage; EHS96697.1 (*npsC*) 95.38% identity, 99.23% coverage; EHS96698.1 (*mfsX*) 96.68% identity, 99.87% coverage; EHS96699.1 (*uvrX*) 94.88% identity, 99.76% coverage) in contig JH603137.1. *K. oxytoca* 10–5243, '*K. pasteurii*' 10–5250, *K. oxytoca* 11492-1, *K. grimontii* SA2, *K. oxytoca* 10–5248 and *K. grimontii* M5a1 encoded the whole kleboxymycin biosynthetic gene cluster. All genes in all matches shared greater than 90% identity across greater than 99% query coverage.

Table 3.9: Genomes included in analyses conducted by Schneditz *et al.* (2014) with corrected species affiliations.

Assembly accession	Strain	Species	ANI with shown genome*	*npsA*/*npsB*
GCA_000240325.1	KCTC 1686	*K. michiganensis*	98.69%, GCA_901556995.1	–
GCA_000247835.1	10–5242	*K. michiganensis*	97.58%, GCA_901556995.1	–
GCA_000247855.1	10–5243	*K. oxytoca*	99.31%, GCA_900977765.1	+
GCA_000247875.1	10–5245	*K. oxytoca*†	99.13%, GCA_900977765.1	–
GCA_000247895.1	10–5246	*R. ornithinolytica*	99.21%, GCA_001598295.1	–
GCA_000247915.1	10–5250	'*K. pasteurii*'	99.29%, GCA _901563825.1	+
GCA_000252915.3	11492-1	*K. oxytoca*	99.15%, GCA_900977765.1	+
GCA_000276705.2	E718	*K. michiganensis*	98.37%, GCA_901556995.1	–
GCA_000427015.1	SA2	*K. grimontii*	99.33%, GCA_900200035.1	+
GCA_001078235.1	10–5248	*K. oxytoca*	99.25%, GCA_900977765.1	+
GCA_001633115.1	M5a1	*K. grimontii*	99.40%, GCA_900200035.1	+

*GCA_901556995.1 = *K. michiganensis* W14^{T}; GCA_900200035.1, *K. grimontii* 06D021^{T}; GCA_900977765.1 = *K. oxytoca* ATCC 13182^{T}; GCA_001598295.1 = *R. ornithinolytica* NBRC 105727^{T}; '*K. pasteurii*' SB3355 GCA _901563825.1.

Taking the results from my analysis of the three *K. michiganensis* strains and those from the reanalysis of the data of Schneditz *et al.* (2014), I have been able to show that the kleboxymycin biosynthetic gene cluster is not unique to *K. oxytoca*. Chen *et al.* (2019) showed that faecal carriage of *K. michiganensis* may be more clinically important than *K. oxytoca.* Therefore, it is possible that *K. michiganensis* is also a causative agent of AAHC. If *K. michiganensis* gut colonisation is linked with AAHC, laboratories may need to screen for presence of

toxigenic *K. michiganensis* (and *K. oxytoca*) isolates. Otherwise there is the potential for AAHC to be a silent killer if undetected from a clinical perspective. Further study is needed to investigate possible linkage between cytotoxin production and adverse clinical outcomes in *K. michiganensis* and *K. oxytoca* infections. Current knowledge on cytotoxin-producing *K. oxytoca* and its link to disease is severely limited by the lack of an accessible diagnostic test and little knowledge of the virulence of toxigenic strains. In the clinical laboratory, *Clostridium difficile* toxin is detected by screening stool samples from at-risk patients (i.e. in-patient, 3 days of hospitalisation, >64 years of age and displaying signs of diarrhoea) using an enzyme-linked immunoassay, a membrane-bound assay and in-house Gene Xpert PCR (Cepheid). Similar tests are not available commercially for detection of *Klebsiella*-associated toxins in samples from at-risk patients. From my experience, between 3 and 5 from 70 samples received per day in the clinical laboratory test positive for *C. difficile* toxins. Some diarrhoea cases may be caused by *Klebsiella*-associated toxins. Therefore, it would be worth clinical laboratories sending *K. oxytoca* isolated from stool samples of patients suffering from AACH to reference laboratories to check for the presence of toxin genes or production of metabolites.

3.3.5 Isolated phages infect GES-5-positive *K. michiganensis*, and clinically relevant strains of *K. grimontii* and *K. pneumoniae*

Kęsik-Szeloch *et al.* (2013) screened 48 clinical isolates of *K. oxytoca* from their own collection against a range of phages they had isolated from environmental and sewage samples collected in Poland. All eight of their *Myoviridae* phages (KP9, KP15, KP23, KP24, KP25, KP27, KP28, KP29), 4/8 of their *Siphoviridae* phages (KP1, KP17, KP18, KP19) and 9/16 of their *Podoviridae* phages (KP1, KP2, KP4, KP12, KP13, KP20, KP21, KP31, KP32) infected one or more of the 48 clinical *K. oxytoca* isolates tested. No genomic information was provided for the *K. oxytoca* isolates, but it is unlikely that *K. michiganensis* and *K. grimontii* isolates were not included in the collection given what we now know about the diversity of clinically relevant *K. oxytoca* strains (Moradigaravand *et al.*, 2017; Chen *et al.*, 2019).

Complete genome sequences of 109 *Klebsiella* phages have been deposited in GenBank/RefSeq as of 17 July 2019 (Herridge *et al.*, 2019). They all

belong to the double-stranded DNA (dsDNA) *Caudovirales*, comprising the families *Myoviridae*, *Siphoviridae* and *Podoviridae*. Of these, only PKO111 (*Myoviridae*, *Tevenvirinae*, *Jd18virus*, *Klebsiella virus PKO111*) and KOX1 (*Siphoviridae*, *Tunavirinae*, *Webervirus*; Herridge *et al.*, 2019) are known to infect *K. oxytoca* (Park *et al.*, 2017; Brown *et al.*, 2017). PKO111 with a genome of 168,758 bp was isolated from a sewage sample. It had a high efficiency of plating against *K. oxytoca* ATCC 43863 and KCTC 1686 (1.00 and 0.89, respectively) and very low efficiency of plating with *K. pneumoniae* KCTC 2242 ($3x10^{-7}$) and *Citrobacter sakazakii* ATCC 29544 ($1x10^{-7}$). KCTC 1686 was shown to be *K. michiganensis* not *K. oxytoca* by Chen *et al.* (2019), so PKO111 is the first phage known to infect *K. michiganensis*. Bioinformatics analyses showed the genome carried no human virulence-related genes (Park *et al.*, 2017), so it could potentially be used therapeutically against *K. oxytoca* and *K. michiganensis*.

Phage KOX1 (genome size 50,526 bp) was isolated from wastewater collected in Bendigo, Australia and was active against five of six *K. oxytoca* strains tested, including the type strain ATCC 13182^{T}, but was inactive against *K. pneumoniae*. No detailed genomic analyses of the *K. oxytoca* strains was undertaken, so it is possible one or more of the additional four strains could be *K. michiganensis* or *K. grimontii*. Brown *et al.* (2017) intended to collect lytic bacteriophages against *K. oxytoca* that could eventually be formulated into dosage forms that could be used to modulate the gut microbiome and growth of *K. oxytoca*. This would have the potential to avert or treat AAHC and diarrhoea caused by *K. oxytoca* during antibiotic therapy. Phage viability was minimally affected by bile salts but was reduced by two orders of magnitude when exposed to gastric acid (pH 2.5). Therefore, Brown *et al.* (2017) proposed that the phage should be co-administered with a proton pump inhibitor, such as omeprazole, to enhance phage viability and therapeutic efficacy.

Few other *K. oxytoca*-infecting phages have been reported. Karumidze *et al*. (2013) isolated a lytic bacteriophage, vB_Klox_2 (*Podoviridae*), active against *K. oxytoca* from sewage-contaminated river water in Georgia. No genome sequence data are publicly available for the phage, though it was reported that, based on restriction fragment profiling, the genome of vB_Klox_2 was 45 kb in size and a 1.2 kb fragment of DNA shared 99% identity with *Klebsiella* phage KP32. In addition, the phage was reported to infect 5–10% of the 123 *Klebsiella*

spp. it was tested against, but no details were given with respect to species so it has to be assumed that it infects both *K. oxytoca* and *K. pneumoniae* in the absence of additional information. More recently, Amiri Fahliyani *et al*. (2018) isolated and identified four novel lytic bacteriophages (phage-1, *Myoviridae*; phage-2, *Myoviridae*; phage-3, *Podoviridae;* phage-4, *Podoviridae*) from Isfahan public wastewater with activity against *K. oxytoca* ABG-IAUF-1, isolated from contaminated milk samples of Isfahan dairy herds suffering from mastitis. phage-2 infected *Enterobacter aerogenes* ATCC 13048 in addition to *K. oxytoca* ABG-IAUF-1. The bacteriophages were proposed as therapeutic agents to eradicate the pathogenic bacteria responsible for bovine mastitis in Iran.

I isolated seven phages with lytic activity against GES-5-positive *K. michiganensis* isolates (Table 3.5). These greatly increase the number of phages known to infect *K. michiganensis* and closely related species. I was able to purify the phages and test their host ranges against a collection of clinically relevant *Klebsiella* isolates. Although not reported in the Results section, I attempted to isolate DNA and use restriction enzyme profiling to characterise the phages, but did not produce any usable data. Consequently, the phages are currently being further characterised by L. Hoyles at Nottingham Trent University and will be reported on in the future.

Phage 1Ai infected PS_Koxy1, phages 2B, 2C and 2Di infected PS_Koxy2, and phages 4B, 4Di and 4Dii infected PS_Koxy4. The seven isolated phages were screened against 36 carbapenemase-resistant strains of *K. pneumoniae* (which will be described in more detail in Chapter 4) and some of the strains from Chen *et al*. (2019), which had been isolated from the faeces of preterm infants. The seven phages had different host profiles from one another (Table 3.6, Table 3.7). Several of the phages showed depolymerase activity against one or more of the *K. michiganensis*, *K. pneumoniae* and *K. grimontii* strains (Table 3.6, Table 3.7). Detection of depolymerase activity against test isolates means the phages are expressing polysaccharide depolymerases, enzymes which play a role in degrading the capsule surrounding *Klebsiella* spp. Depolymerases can destroy biofilms and increase the susceptibility of bacteria to antibiotics, phage infection and the immune system (Kęsik-Szeloch *et al*., 2013). Phage depolymerase action can be observed in the laboratory with the production of ‘haloes’ around clear

zones of lysis on bacterial culture plates after infection of bacteria with phage particles.

Taking phage 4Dii as an example, this phage showed lytic activity against its original *K. michiganensis* host strain, PS_Koxy4, and towards several test strains (Table 3.7). When considering use of phages as alternatives to antimicrobial therapies, those that reliably employ the lytic life cycle to reproduce are most suitable given lytic phages completely destroy the host cell (Herridge *et al.*, 2019).

Phage 4Dii also showed depolymerase activity towards some of the clinical strains it infected (Table 3.7). These depolymerases may have therapeutic uses, and should be investigated further when the genome sequences of the phages are available. Majkowska-Skrobek *et al*. (2016) identified, cloned and expressed a phage-derived capsule depolymerase, depoKP36, which produced haloes on lawns of *K. pneumoniae* in agar spot-tests. They also tested the ability of depoKP36 to treat infection caused by *K. pneumoniae* in an insect model of infection (*Galleria mellonella*) and found that 100% died without treatment, up to 40% survived when treated with depoKP36, and depoKP36 treatment of bacteria prior to infection resulted in only a 23% death rate. These results suggest that the decapsulating action of depoKP36 on *K. pneumoniae* produced a decreased ability of the bacterium to resist the host immune response. Pan *et al*. (2015) discovered nine polysaccharide depolymerases expressed by phage ΦK64-1, each of which demonstrated activity against a different capsular type of *K. pneumoniae* and could be used to generate cocktails of recombinant enzymes that could target a wide range of *K. pneumoniae* strains.

The use of phages to control bacterial pathogens is complicated because of the high degree of phenotypic diversity within populations of both phages and bacteria. Therefore, individual strains of target bacteria may be more or less susceptible or even resistant to different co-occurring phages. When considering therapeutic applications, it is necessary to understand in detail the phage and host interaction, which is affected by both biological and physical factors. The biological aspect is related to bacterial resistance, whereas temperature and pH are the main physical factors affecting phage adsorption and bacterial growth (Shende *et al.,* 2017). To understand phage-host interactions, it is important to identify which family phages belong to (e.g. *Myoviridae*, *Podoviridae*, *Siphoviridae*; as done for

Klebsiella-infecting phages by Herridge *et al.*, 2019). Phage characterisation can be achieved phenotypically and genomically. Phenotypic characterisation can be used to assign phages to families based on morphology as determined via transmission electron microscopy. It can also determine desirable traits such as resistance to chloroform (useful for determining appropriate DNA extraction methods), and to extremes of pH and temperature (Kęsik-Szeloch *et al.*, 2013), with pH tolerance being particularly relevant to use of phages to treat intestinal infections. Whole-genome sequencing will define the size and genetic content of phages, and allow identification of any virulence or antibiotic resistance genes encoded in genomes. Phages carrying such genes would not be used therapeutically as the genes may be introduced into the host bacterium during phage infection, especially if the phage has the potential to integrate into the bacterium's genome (i.e. is a lysogenic phage). Lysogenic phages may transfer genes into the host that can confer toxin production and antibiotic resistance traits to the bacterium, thus making the infection more virulent and difficult to treat (Harper *et al*., 2018; Herridge *et al*., 2019). Characterisation of both the host bacterium and the phages is also necessary to understand the specific receptors on the surface of the host bacterium that the phage's tail structure can attach to and identify susceptible bacteria and position itself for injecting its genetic material into the host cell. This can help in identifying novel therapeutic targets in bacteria.

There are several considerations to be made when selecting phages suitable for use as therapeutic agents. The phages must be effective in killing the target micro-organism(s), e.g. *Klebsiella* spp. During phage characterisation, *in vitro* assessments of phage lysis and burst size need to be carried out on cultures of host strains. Phages that produce rapid lysis of a bacterium and release large numbers of phage particles will produce large clear plaques. Moreover, phages with a broad host range are generally considered more useful than those with narrow host range so that multiple strains may be targeted at once. Lytic phages, due to the nature of their life cycle, clear bacteria quickly and efficiently compared to lysogenic phages, which integrate their genetic information into the host genome and remain dormant for an unspecified amount of time.

Phenotypic characterisation of the phages I isolated is required, including determination of burst size, and sensitivity to pH, temperature and chloroform. Once this is achieved the isolated phages must be tested *in vivo* using *Galleria* or

mouse models, to identify the therapeutic potential of the phages and whether they affect the immune response of the host. Hsu *et al*. (2019) showed that infection with lytic phages caused an increase in phage resistance (28% to 68%) in a known bacterial population common to the human gut microbiota. Quantitative shifts in sensitive and non-sensitive strains were seen, highlighting the system-level effect of phage infection. Phage infection did not necessarily clear the target species but instead modulated the ecosystem towards a more stable gut environment. As I would like to use my phages to treat intestinal infections or decolonise patients carrying multidrug-resistant bacteria on their admittance to hospital, it is important to know phage effects on the gut environment. By following the method of Gu *et al*. (2012) it should be possible to generate a phage cocktail (i.e. a combination of phages that have different but overlapping host specificities) to produce fewer phage-resistance variants which are effective in treatment of, for example, AAHC.

3.5 SUMMARY

In this Chapter, I characterised three of the *K. oxytoca* isolates collected from the outbreak 2015 collection reported on by Eades *et al.* (2016). Whole-genome sequence analyses showed these isolates to be *K. michiganensis* carrying a GES-5 carbapenemase gene previously not seen in *K. michiganensis* and a range of other antibiotic-resistance determinants not described by Eades *et al.* (2016). Further analyses showed the isolates encoded the 12-gene kleboxymycin biosynthetic gene cluster (Tse *et al.*, 2017) associated with the production of the cytotoxins TM and TV, causative agents of the cell damage experienced in AAHC-affected patients. In addition, the gene cluster was found in '*K. pasteurii*' and *K. grimontii* strains. The gene cluster had previously been associated only with *K. oxytoca*. Therefore, there is the potential that species other than *K. oxytoca* may contribute to AAHC, and this requires further investigation. I isolated seven lytic phages active against *K. michiganensis* PS_Koxy1, PS_Koxy2 and PS_Koxy4. These phages also infected clinically relevant *K. pneumoniae* and *K. grimontii* strains, showing they have a broad host range. The phenotypic and genomic characteristics of these phages will be determined in future work, as these phages could be used for other therapeutic effects (i.e. treating other *Klebsiella* infections or decolonisation) and not just in AAHC treatment.

CHAPTER 4: GENOMIC CHARACTERISATION OF CLINICAL STRAINS OF CARBAPENEM-RESISTANT *KLEBSIELLA PNEUMONIAE* FROM WEST LONDON HOSPITALS, AND ISOLATION OF LYTIC BACTERIOPHAGES THAT INFECT THEM

4.1 INTRODUCTION

Klebsiella pneumoniae belongs to the family *Enterobacteriaceae*. It is a Gram-negative, lactose-fermenting, gas-producing, facultatively anaerobic non-motile bacterium about 1 to 2 μm long and 0.5 to 0.8 μm wide. It possesses a thick polysaccharide capsule that is produced in great amounts in carbohydrate-rich media. The organism grows well at 37 °C and is killed by moist heat at 55 °C for 30 min (Gupta, 2002). *K. pneumoniae* is ubiquitous, commonly found in the natural environment (e.g. water and soil) and is a commensal of the human microbiota. The bacterium colonises the skin, mouth, and respiratory and GI tracts of healthy humans, but is also an opportunistic pathogen of immunocompromised individuals (Holt *et al*., 2015). The carriage rate of *K. pneumoniae* varies from study to study, but in healthy humans is ~10% in the mouth and nose and on the skin, 1-6% in the nasopharynx and 3.8-38% in stool samples; skin carriage is considered transient (Rose and Schreier, 1968; Podschun and Ullmann, 1998; Conlan *et al*., 2012b; Farida *et al*., 2013; Dao *et al.*, 2014). Carriage rates for hospitalised patients increase with the length of stay to 77% in the stool, 19% in the nasopharynx and 42% on the hands of patients. The high level of colonisation in these patients is associated with their increased exposure to antibiotics as part of treatment regimens (Rose and Schreier, 1968; Podschun and Ullmann, 1998).

4.1.1 GI carriage of *K. pneumoniae* and exogenous sources contribute to disease

Martin *et al*. (2016) suggested GI colonisation with *K. pneumoniae* was directly linked with extraintestinal infection. They screened 1765 patients for rectal carriage of *K. pneumoniae* then collected extraintestinal (respiratory, urinary or bloodstream) isolates from the same patients for a 3-month period. Almost a quarter of patients were colonised with *K. pneumoniae*, and 5.2% (21/406) of colonised patients developed an extraintestinal infection compared with 1.3% (18/1359) of non-colonised patients. Extraintestinal isolates matched rectal

isolates from the same patient based on capsular typing, MLST and WGS analyses. Based on their findings, Martin *et al.* (2016) suggested a possible means of infection control: isolate the colonising strain from a patient upon their admission to hospital as part of a risk assessment, determine the strain's antibiotic susceptibility profile and use this information to guide the treatment regimen if the patient became acutely ill. Gorrie *et al.* (2017) confirmed *K. pneumoniae* to be part of the normal gut microbiota of humans, and in agreement with Martin *et al.* (2016) showed GI carriage of the organism by the patient rather than throat carriage on admission to intensive care was associated with subsequent infection, confirming gut-colonising and infection isolates were the same in 80% of isolate pairs. Approximately 50% of *K. pneumoniae* infections resulted from the patients' own microbiota, and Gorrie *et al.* (2017) supported Martin *et al.* (2016) with respect to screening patients for colonisation on admission to reduce the risk of infection in colonised and other patients.

The healthcare environment, contamination of invasive devices (e.g. respirators, catheters) and excessive use of antibiotics also contribute to likelihood of nosocomial infections (Woldu, 2015; Holt *et al.*, 2015). In the hospital setting, *K. pneumoniae* accounts for approximately 8% of all HAIs. The organism causes HAIs of the urinary tract, lower respiratory tract, and burn and surgical wound sites of immunocompromised patients, particularly those in intensive care units. It is also associated with late-onset sepsis and necrotising enterocolitis in preterm infants (Chen *et al.*, 2019). Patients with KPC infections (typically caused by ST258 isolates) or PLA (caused by hypervirulent ST23 isolates) have been shown to carry their infecting strain in their GI tract for between 30 days (≤74%) and 6 months (<30%) following discharge from hospital (Feldman *et al*., 2013). Recent use of antimicrobials is not associated with persistent carriage of carbapenemase-producing *K. pneumoniae* (Feldman *et al.*, 2013).

4.1.2 Phenotypic and genomic characterisation of *K. pneumoniae*

Traditionally, clinical laboratories have used phenotypic (API 20E, antibiotic susceptibility testing, MALDI-TOF) or PCR-based assays (antibiotic resistance and virulence genes) to characterise *K. pneumoniae* strains, while reference laboratories have used PFGE, PCR-based assays (VNTR profiling, limited capsule typing; Turton *et al.*, 2010) and, more recently, some WGS analyses (for species

identification and MLST). MLST has been used to identify ST258 strains, which carry the KPC gene and other AMR gene(s) and have been responsible for hospital infection outbreaks around the world (Grundmann *et al.*, 2010; Livermore, 2012a). More recently, WGS has demonstrated ST258 is a recombinant strain that has undergone capsular exchange since its emergence (Holt *et al.*, 2015).

The availability of cheap WGS has seen huge changes in the ways *K. pneumoniae* can be characterised and it is being used more frequently to understand AMR and infection outbreaks. The first major study to make use of high-throughput WGS was by Holt *et al.* (2015). They analysed the genomes of 328 human and animal isolates of *K. pneumoniae* collected around the world, and confirmed *K. pneumoniae* was genomically complex, representing three species (*K. pneumoniae* (Kp1), *Klebsiella quasipneumoniae* (Kp2) and *Klebsiella variicola* (Kp3)) and phylogroup Kp2-B (Kp4), all capable of causing disease in humans. *K. pneumoniae* (Kp1) comprised more than 150 lineages with numerous multidrug-resistant or hypervirulent clones, and was more frequently associated with human carriage. The *K. pneumoniae* strain population studied had a large accessory genome (i.e. genes found in <95% of strains) of 29,886 protein-coding genes, including virulence genes associated with invasive community-acquired diseases.

Hospital-acquired strains encoded more AMR genes and fewer virulence genes (siderophores – aerobactin, yersiniabactin, salmochelin; toxin – colibactin) than community-acquired strains (Holt *et al.*, 2015). SHV, OKP-2 and LEN β-lactamases were core chromosomal genes of Kp1, Kp2 and Kp3, respectively, while genes conferring low-level resistance to fosfomycin and quinolones were core to all three species. (OKP-B has subsequently been found to be a core chromosomal gene of Kp4 (Rodrigues *et al.*, 2019)). Other AMR genes were distributed unevenly throughout the species, and were referred to as "*acquired AMR genes*" to differentiate them from the core AMR genes. These acquired AMR genes were commonly found in human-associated strains of Kp1 and Kp2, with ESBL alleles of SHV found in 40% of human isolates.

As more WGS data have become available for *K. pneumoniae* strains, it has become clear that the complex is genetically far more diverse than recognised by Holt *et al.* (2015). There are now seven phylogroups recognised within the *K. pneumoniae* complex, each corresponding to a different species: Kp1, *K.*

pneumoniae; Kp2, *K. quasipneumoniae* subsp. *quasipneumoniae*; Kp3, *K. variicola* subsp. *variicola*; Kp4, *K. quasipneumoniae* subsp. *similipneumoniae*; Kp5, *K. variicola* subsp. *tropica*; Kp6, '*Klebsiella quasiivariicola*'; Kp7, *Klebsiella africana* (Rodrigues *et al.*, 2019). Their clinical significance is unclear as Kp1 to Kp7 strains are often misidentified as *K. pneumoniae* or *K. variicola* in clinical laboratories using standard laboratory methods (Rodrigues *et al.*, 2018; 2019). Recently, protein markers that allow MALDI-TOF to differentiate the species have been described for Kp1 to Kp6, but they have yet to be adopted widely in clinical laboratories and reference MALDI-TOF spectra databases (Rodrigues *et al.*, 2018).

4.1.3 Virulence factors of *K. pneumoniae*

K. pneumoniae expresses two types of antigens on its cell surface that contribute to its mucosal colonisation and development of infections: the lipopolysaccharide O antigen and the capsular polysaccharide K antigen (Evrard *et al.*, 2010). The structures of these components vary among strains, and both are essential to the virulence of *K. pneumoniae*. The capsular material forms thick bundles of fibrillous structures that cover the bacterium's surface, protecting the bacterium from phagocytosis by polymorphonuclear granulocytes and preventing its killing by bactericidal serum factors via the complement-mediated cascade (Evrard *et al.*, 2010). The O antigen, along with lipid A and the core oligosaccharide, makes up lipopolysaccharide, and is involved in inactivating dendritic cells (Evrard *et al.*, 2010). Fimbriae and adhesins, together with *K. pneumoniae*'s ability to scavenge iron (using siderophores) from its surrounding environment, also serve as virulence factors (Broberg *et al.*, 2014).

Variation in the capsular K antigen allows the species to be classified into various serotypes. Seventy-eight K antigens and 12 distinct O antigens have been described based on serological data and are essential to the virulence of *K. pneumoniae*, but many clinical strains remain non-typeable (Shon *et al.*, 2013; Fang *et al.*, 2016). The capsular genes are encoded on a 10- to 30-kbp region of the genome known as the K locus (Wick *et al.*, 2018). The key O antigen genes are encoded on the O (*rfb*) locus, but some genes (*wbbY*, *wbbZ*) that contribute to O antigen variation are found outside the O locus (Wick *et al.*, 2018). For many years, capsule typing was restricted to five K types (K1, K2, K5, K54 and K57)

identified using PCR (Turton *et al.*, 2010). PCR-based O-genotyping has also become possible, but is not used by PHE (Fang *et al.*, 2016). By analysing protein-encoding genes in WGS data, Wyres and Holt (2016) found there to be 134 distinct capsular loci in *K. pneumoniae*, suggesting there are at least 134 distinct capsule types. It is now possible to use WGS data to K and O type *K. pneumoniae* genomes via Kaptive Web (http://kaptive.holtlab.net/) (Wick *et al.*, 2018).

The *rmpA* and *rmpA2* ("*regulator of mucoid phenotype*") genes are uncommon in strains characterised to date, but they are important virulence factors associated with hypermucoidy, as they upregulate capsule production (Broberg *et al.*, 2014). *rmpA* and *rmpA2* are associated with invasive human infections caused by ST23 isolates, which also typically carry three siderophore systems (aerobactin, salmochelin and yersinibactin) and/or a toxin (colibactin) (Holt *et al.*, 2015). These invasive infections, particularly pyogenic liver abscess (PLA), affect healthy and immunocompromised individuals (Lee *et al.*, 2017). *rmpA* has also been found in ST43 (along with yersiniabactin, salmochelin and aerobactin) and ST36 (along with yersiniabactin, colibactin and aerobactin), both linked to bacteraemia (Holt *et al.*, 2015). *rmpA2* upregulates K2 *cps* gene expression (Lai *et al.*, 2003). There has been an increase in the number of cases of community-acquired PLA due to the hypervirulent *K. pneumoniae* ST23 K1 serotype. Two small studies reported that *K. pneumoniae* was the responsible pathogen in 36% and 41% of cases; 70% of affected patients were Asian, but no information was available on travel histories or close contact with recent travellers to the Asian Pacific (Rahimian *et al*., 2004; Pastagia and Arumugam, 2008). Other infections caused by hypervirulent *K. pneumoniae* are endophthalmitis, infections of the central nervous system (including meningitis and brain abscess), osteomyelitis, septic arthritis and urinary tract infection (Fang *et al.*, 2000; Chang *et al*., 2001; Shon *et al*., 2013). Endemic spread of hypervirulent strains is currently confined to Asian countries (Lee *et al.*, 2017). While still rare, clinical isolates of hypervirulent, carbapenem-resistant *K. pneumoniae* have been reported, suggesting severe, untreatable infections could come to pose a significant threat to healthy individuals in the future (Lee *et al.*, 2017).

4.1.4 Treatment of *K. pneumoniae* infections

K. pneumoniae infections are mainly treated with cephalosporins, carbapenems and aminoglycosides. However, there is now emerging resistance to these antibiotics due to the bacterium producing ESBLs or carbapenemases that confer resistance to cephalosporins and carbapenems (Pitout *et al*., 2015; Sekyere *et al*., 2016). By acquiring a combination of resistance mechanisms, *K. pneumoniae* can become MDR as the carbapenem group of antibiotics is the last line of defence against Gram-negative infections that are resistant to other antibiotics. As a result, MDR *K. pneumoniae* infections are treated with combination therapy comprising β-lactams and aminoglycosides, or colistin and tigecycline as monotherapy (Woldu, 2015; Sekyere *et al*, 2016).

The emergence of MDR strains of *K. pneumoniae* has become an urgent threat to human health due to their association with outbreaks of HAIs and community-acquired infections globally (Holt *et al.*, 2015). Since carbapenemases are transferred from species via plasmids, GI-associated CPE and non-CPE organisms can serve as reservoirs and vectors (Viau *et al.*, 2016). It is established that GI carriage contributes to *K. pneumoniae* infections (Martin *et al.*, 2016; Gorrie *et al.*, 2017), thereby contributing to carbapenem resistance and promoting cross-transmission of CPE in healthcare settings (Viau *et al*., 2016). In the UK, *K. pneumoniae* is a real challenge to infection control in both hospital and community settings: 1) because of its ability to spread and cause outbreaks; and 2) because decolonisation therapy only reduces levels of the bacterium without elimination (Curran and Otter, 2014). Most people are colonised with these organisms with no further complications but, in immunocompromised patients, colonisation can lead to endogenous infection, which can be a cause of cross-infection (Patel *et al*., 2007; Curran and Otter, 2014). Bacteriophage (phage) therapy has been suggested as an alternative or adjunct treatment option in CPO-associated infections (Hoyles *et al*., 2014), and – as discussed in Chapter 3 – has been proposed as a means of decolonising patients upon admission to hospital.

4.1.5 Aims of this Chapter

In Chapter 2, I showed that the improved screening method I helped implement in the clinical laboratory increased the detection of *K. pneumoniae* from

clinical samples. In Chapter 3, I gained experience in analysing WGS data to characterise a small number of strains. I wished to extend my studies in this area by analysing a larger set of clinical isolates and focussing on *K. pneumoniae*, which is far more complex and has greater clinical impact than *K. oxytoca* and related species.

Therefore, the first aims of this Chapter were to fully characterise a selection of *K. pneumoniae* isolates I collected during my time at Charing Cross at the genomic level and compare their antimicrobial susceptibility profiles with their genomic profiles, and to understand how genomic data could be used to identify several virulence genes in genomic data.

Phages active against *K. pneumoniae* have been isolated from sewage, environmental and GI samples, and shown good therapeutic potential when tested in mice (reviewed in Herridge *et al.* (2019), Appendix 1). However, few lytic phages active against carbapenem-resistant *K. pneumoniae* have been isolated and characterised to date. In addition, many of the published studies do not characterise the bacterial strains infected by phages, making it impossible to know which phages could be used to treat *K. pneumoniae* infections in a clinical setting, or they do not adequately characterise the phage genomes, limiting the clinical application of phage gene products. As covered in Chapter 3, I believe phages may be used to decontaminate patients colonised with antibiotic resistant *Klebsiella* spp. and could contribute to infection control programmes. Therefore, the final aim of this Chapter was to isolate and begin to characterise lytic phages that infected carbapenem-resistant strains of *K. pneumoniae*.

4.2 MATERIALS AND METHODS

Refer to Chapter 3, section 3.2.

4.3 RESULTS

4.3.1 Initial characterisation of isolates included in my study

Between March and April 2015 during the evaluation of new CPO screening methods (Chapter 2) in the microbiology laboratory of Charing Cross Hospital, a total of 49 different isolates were identified and sent to the PHE reference laboratory for further characterization. Of these, 36 isolates identified as *K. pneumoniae* by API 20E (n = 26 - profile number 5205773, 81.8 % *K. pneumoniae*;

n = 10 - 5215773, 97.3% *K. pneumoniae*) and MALDI-TOF (score of 2.3, highly probable species identification) were confirmed by PCR to carry a range of carbapenemases (Table 4.1). Twenty-two were NDM-positive, 10 were OXA-48-positive, two were KPC-positive, and two were both NDM- and OXA-48-positive. Eight isolates were resistant (MIC 32 mg/L) to colistin, the last-resort antibiotic for treatment. Thirty-four of the isolates were resistant to the carbapenem indicator antibiotics ertapenem and temocillin, with an MIC greater than 32 mg/L for ertapenem. No data were available for two isolates (PS_Kpn4 and PS_Kpn16) as I was unable to test these in the clinical laboratory after moving jobs. Strains had variable resistance to meropenem: 28 were resistant (MIC greater than 32 mg/L), three had intermediate results, while two were sensitive (no data available for three isolates: PS_Kpn4, PS_Kpn16 and PS_Kpn27).

4.3.2 Identification of isolates using WGS analyses

As in Chapter 3, the WGSs of the 36 isolates identified by phenotypic methods as *K. pneumoniae* were generated. ANI values were determined for the isolates using reference strains for the seven phylogroups (species) of *K. pneumoniae* (Rodrigues *et al*., 2019). ANI values generated represent a measurement of how similar two genome sequences are to one another. If the genomes of two strains share >95 % ANI, they are considered to belong to the same species (Chun *et al.*, 2018). PS_misc5 shared 98.97% ANI with *Klebsiella variicola* subsp. *variicola* Kp342 (Figure 4.1). All other strains were *K. pneumoniae*, sharing between 98.42% and 99.04% ANI with *K. pneumoniae* ATCC 13883^{T} (Figure 4.1). Though it should be noted strains that shared >98.5 % ANI with *K. pneumoniae* shared >95 % ANI with *K. africana* (Figure 4.1). Chun *et al.* (2018) suggested strains belonging to different subspecies, and by extension closely related species, should be genomically coherent and form distinguishable clades by phylogenomic treeing. Phylogenetic analysis of the isolates with representative strains of the seven species of the *K. pneumoniae* complex (Rodrigues *et al.*, 2019) confirmed the species affiliations identified using ANI (Figure 4.2).

Table 4.1: Phenotypic information for *K. pneumoniae* isolates included in this study.

Strain	API 20E*	Source	Ward†	Resistance gene	Antibiotic‡																
					GEN	CIP	CFX	TAZ	AMO	TEM	CFT	ERT	TRI	CEF	CFN	AUG	COL	TIG	AZT	TOB	MER	AMI
PS_Kpn1	1	Perineum	HJW	NDM	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R
PS_Kpn2	1	Rectum	ZCO	NDM	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R
PS_Kpn3	1	Rectum	ORDU	OXA-48	S	R	R	S	R	R	S	R	R	S	R	R	S	R	R	S	R	R
PS_Kpn4	1	Cross-infection*f*	CWOP	OXA-48/NDM	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PS_Kpn7	1	Rectum	ZCO	NDM	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R
PS_Kpn9	2	Rectum	HJW	NDM	R	R	R	R	R	R	R	R	R	R	R	R	S	I	R	R	R	R
PS_Kpn10	1	Rectum	ZCO	NDM	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R
PS_Kpn11	1	Urine	NU	NDM	R	R	R	R	R	R	R	R	R	R	R	R	S	I	R	R	R	R
PS_Kpn12	1	Rectum	ZCO	NDM	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R
PS_Kpn13	1	Rectum	ZCO	NDM	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R
PS_Kpn14	1	Rectum	ZCO	NDM	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R
PS_Kpn15	1	Rectum	HDU	OXA-48	S	I	R	R	R	R	R	R	R	R	R	R	ND	R	R	S	I	S
PS_Kpn16	1	Rectum	AU	OXA-48	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PS_Kpn24	1	Rectum	ZCO	NDM	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R
PS_Kpn25	1	Rectum	ZCO	NDM	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R
PS_Kpn26	1	Rectum	ICU	NDM	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R
PS_Kpn27	2	Urine	CWOP	KPC	S	R	ND	R	ND	R	R	R	R	R	ND	R	S	ND	ND	ND	ND	ND
PS_Kpn28	1	Urine	RENAL	OXA-48	R	R	ND	R	ND	R	R	R	R	R	ND	R	S	R	ND	ND	R	S
PS_Kpn29	1	Urine	KERW	NDM	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R
PS_Kpn30	2	Mouth	HA7	NDM	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R
PS_Kpn31	1	Perineum	ICU	NDM	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R
PS_Kpn32	1	Drain fluid	DWU	OXA-48	R	R	R	R	R	R	R	R	R	R	R	R	S	I	R	R	S	R
PS_Kpn33	1	Urine	ZCO	NDM	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R
PS_Kpn35	1	Urine	ZCO	NDM	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R
PS_Kpn36	1	Urine	HEU	OXA-48	S	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	S
PS_Kpn37	2	Wound	HEU	NDM	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R
PS_Kpn38	1	High vaginal swab	CWOP	NDM	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R
PS_Kpn39	1	Wound	AE	NDM	R	R	R	R	R	R	R	R	R	R	R	R	S	I	R	R	R	R
PS_Kpn40	1	Wound	OPD	KPC	S	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	R	I
PS_Kpn41	1	Leg	GICU	NDM	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R
PS_misc2	2	Groin	ZCO	OXA-48/NDM	S	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	R	I
PS_misc3	2	Rectum	DWU	OXA-48	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R
PS_misc5	2	Rectum	ZCO	OXA-48	I	R	R	ND	R	R	R	R	R	R	R	R	ND	R	R	R	R	R
PS_misc6	2	Rectum	ZCO	NDM	S	I	R	ND	R	R	ND	R	R	S	S	R	ND	S	R	R	I	ND
PS_misc7	2	Rectum	DWU	OXA-48	S	I	R	R	R	R	R	R	R	R	R	R	ND	I	R	S	I	S
PS_misc8	2	Perineum	ZCO	OXA-48	S	R	R	R	R	R	R	R	R	R	R	R	ND	I	R	S	R	S

*1, profile number 5205773, 81.8 % *K. pneumoniae*; 2, profile number 5215773, 97% *K. pneumoniae*.

†HJW, John Humphrey Ward; ZCO, Zachary Cope Ward; ORDU, Outpatients Renal Dialysis Unit; CWOP, Chelsea and Westminster Outpatients; NU, Neptune Unit; HDU, High Dependency Unit; AU, Acute Unit; ICU, Intensive Care Unit; RENAL, Renal Unit; KERW, Kerr Ward; HA7, Hematology Ward; DWU, De Wardener Ward; HEU, Hemodialysis Unit; AE, Accident and Emergency; OPD, Outpatients Department; GICU , Geriatric Intensive Care Unit.

‡GEN, gentamicin; CIP, ciprofloxacin; CFX, cefuroxime; TAZ, tazocin; AMO, amoxycillin; TEM, temocillin; CFT, cefotaxime; ERT, ertapenem; TRI, trimethoprim; CEF, ceftazidime; CFN, ceftoxitin; AUG, augmentin; COL, colistin; TIG, tigecycline; AZT, azteonam; TOB, tobramycin; MER, meropenem; AMI, amikacin. R, resistant; S, sensitive; I, intermediate; ND, no data.

fNose, axilla and groin swabs.

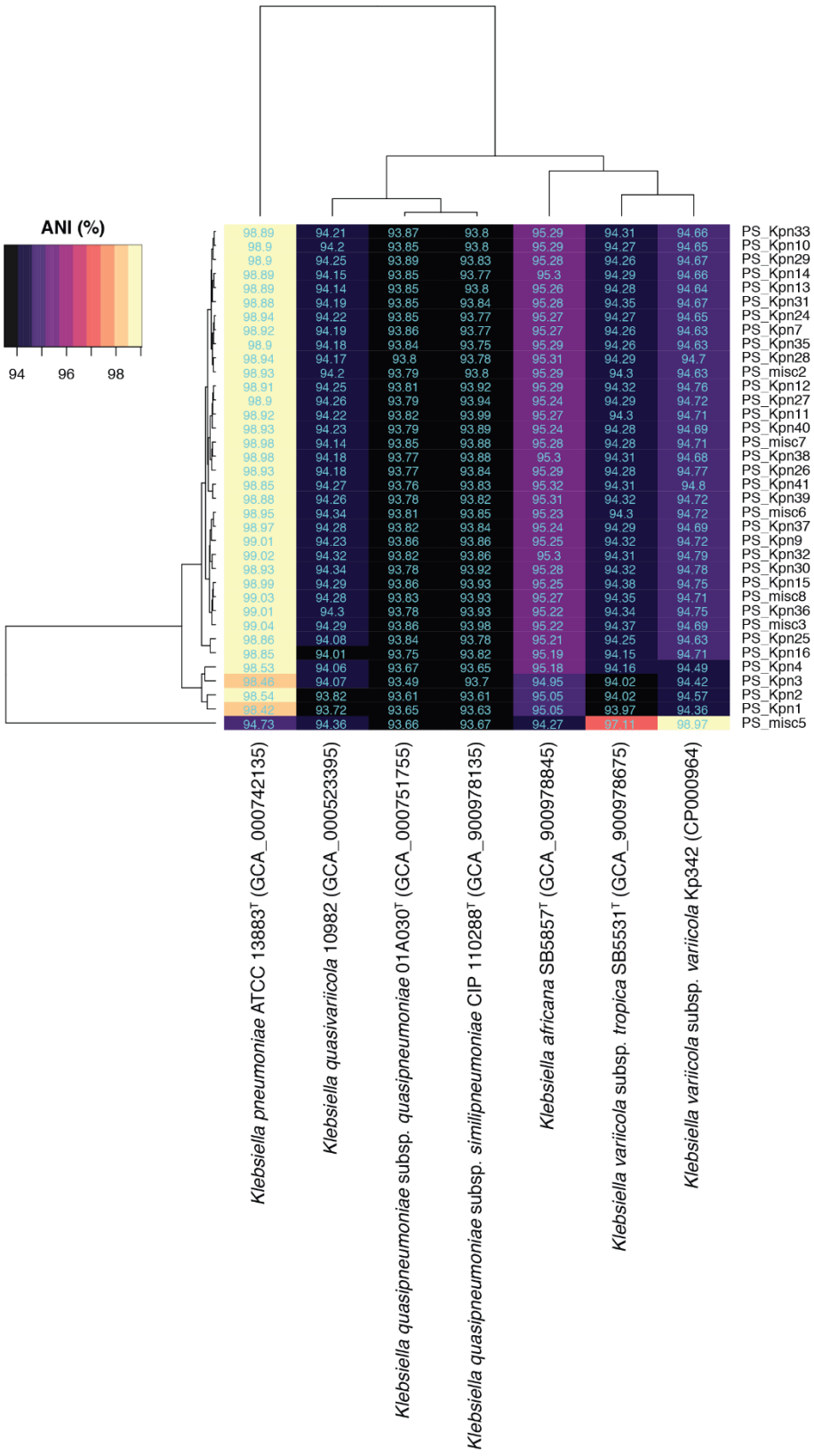


Figure 4.1. ANI values for the 36 clinical isolates when compared against *K. pneumoniae* complex reference strains (Rodrigues *et al.*, 2019). ANI data were generated using FastANI. The heatmap was generated from FastANI outputs by Dr L. Hoyles.

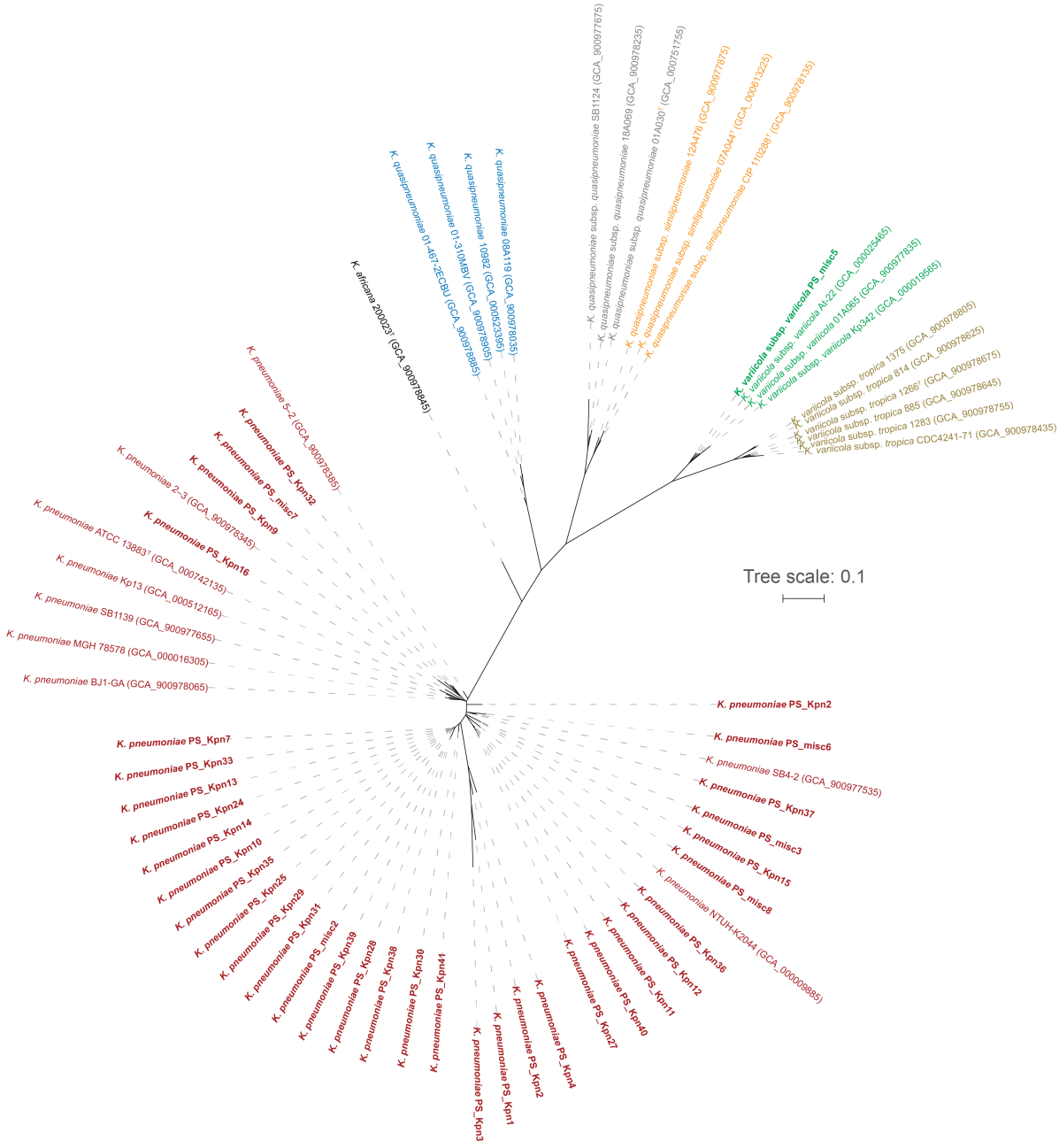


Figure 4.2. Unrooted phylogenetic tree showing the relationships of the clinical isolates with representatives of the seven species of the *K. pneumoniae* complex. Genomes included in the study of Rodrigues *et al.* (2019) were included in the analysis. The phylogenetic analysis was conducted by Dr L. Hoyles using PhyloPhlAn 0.99, which used 379 proteins common to each genome in its analysis. FastTree, which generates approximately maximum-likelihood trees for large alignments, was used by PhyloPhlAn. The tree was visualised using iTOL (https://itol.embl.de) and coloured using Adobe Illustrator. Scale bar, normalised fraction of total branch length.

4.3.3 Capsular (K), O antigen and multilocus sequencing typing of isolates using WGS data

I uploaded the WGSs of the 36 isolates to the Institut Pasteur MLST database (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html) to determine their MSLT sequence types (Table 4.2). K and O antigen types for the isolates were determined using Kaptive Web (Table 4.2). All but four of the strains could be assigned a ST. ST14 had greatest representation (n = 15), followed by ST15 (n = 3), ST45 (n = 3), ST11 (n = 2) and ST258 (n = 2); all other STs (ST16, ST35, ST101, ST147, ST294, ST512 and ST716) were represented once. Twelve of the ST14 isolates were K2:O1v1, while three were K64:O1v1. All the ST45 isolates were K52:OL101, and both ST258 isolates were K107:O2v2. The remaining STs were represented by a range of K and O types (Table 4.2).

PHE provided some MLST, K type, PFGE and VNTR data for 15 NDM-positive *K. pneumoniae* strains that had been linked to an outbreak that occurred between July 2014 and March 2015 in two hospitals with spread occurring due to frequent contact between the two hospitals, but WGS data were not available from PHE (Table 4.3). In March 2015, two clinical cultures of a carbapenem-resistant *K. pneumoniae* were identified in Hospital B mainly from vascular inpatient wards prompting screening of all patients on the ward (Otter *et al.*, 2017). In April 2015, screening in Hospital A mainly from renal inpatient wards identified one case that was epidemiologically linked to cases from July and December 2014. The spread occurred due to frequent contact between the hospitals, with 11 of the patients involved in the outbreak having contact with both Hospitals A and B in the three months before or after their date of first acquisition of CPE (Otter *et al.*, 2017).

Clusters of the outbreak strains shared the same, characteristic VNTR profiles, i.e. 9 isolates had the VNTR profile 634011211 (profile A (Hospital A) of Otter *et al.*, 2017), and three had VNTR profile 634011311 (profile B (Hospital B) of Otter *et al.*, 2017), while three strains had unique VNTR profiles (Table 4.3). Profiles A and B differed from each other by one repeat unit at one locus (underlined in the profile codes).

Table 4.2: K type, O antigen and MLST data for the strains included in this study.

Strain	K type	O antigen	MLST
PS_Kpn11	K107	O2v2	ST258
PS_Kpn27	K107	O2v2	ST258
PS_Kpn40	K107	O2v2	ST512
PS_misc6	K110	O2v1	ST716
PS_Kpn30	K112	O1v1	ST15
PS_Kpn41	K112	O1v1	ST15
PS_Kpn12	K15	O4	ST11
PS_Kpn16	K17	O1v1	ST101
PS_Kpn9	K18	O2v1	*
PS_Kpn1	K2	O4	*
PS_Kpn10	K2	O1v1	ST14
PS_Kpn13	K2	O1v1	ST14
PS_Kpn14	K2	O1v1	ST14
PS_Kpn24	K2	O1v1	ST14
PS_Kpn25	K2	O1v1	ST14
PS_Kpn28	K2	O1v1	ST14
PS_Kpn29	K2	O1v1	ST14
PS_Kpn31	K2	O1v1	ST14
PS_Kpn33	K2	O1v1	ST14
PS_Kpn35	K2	O1v1	ST14
PS_Kpn38	K2	O1v1	ST14
PS_Kpn7	K2	O1v1	ST14
PS_Kpn32	K22	O1v1	ST35
PS_Kpn36	K24	O2v1	ST11
PS_misc7	K30	O1v1	ST294
PS_Kpn37	K51	O3b	ST16
PS_Kpn15	K52	OL101	ST45
PS_misc3	K52	OL101	ST45
PS_misc8	K52	OL101	ST45
PS_Kpn3	K64	O1v1	*
PS_Kpn2	K64	O1v1	ST14
PS_Kpn39	K64	O1v1	ST14
PS_misc2	K64	O1v1	ST14
PS_Kpn26	K64	O1v1	ST147
PS_Kpn4	K64	O1v2	ST15
PS_misc5	K81	OL101	*

*, Unknown (no match in database).

Table 4.3: Genotypic data provided by PHE for outbreak strains compared with results determined in this study.

Strain	MLST	K type	VNTR profile	PFGE profile	This study		
					K type	O antigen	MLST
PS_Kpn1	ND	K2	634011311	ND	K2	O4	*
PS_Kpn29	ST14	K2	634011311	SMAR45	K2	O1v1	ST14
PS_Kpn31	ND	ND	634011311	SMAR45	K2	O1v1	ST14
PS_Kpn30	ND	ND	333011411	ND	K112	O1v1	ST15
PS_Kpn9	ND	ND	3-2521331	ND	K18	O2v1	*
PS_Kpn11	ND	ND	321321331	ND	K107	O2v2	ST258
PS_Kpn10	ND	K2	634011211	ND	K2	O1v1	ST14
PS_Kpn13	ND	ND	634011211	ND	K2	O1v1	ST14
PS_Kpn14	ND	ND	634011211	ND	K2	O1v1	ST14
PS_Kpn2	ND	K2	634011211	ND	K64	O1v1	ST14
PS_Kpn24	ND	K2	634011211	ND	K2	O1v1	ST14
PS_Kpn25	ST14	K2	634011211	SMAR45	K2	O1v1	ST14
PS_Kpn33	ST14	K2	634011211	SMAR45	K2	O1v1	ST14
PS_Kpn35	ND	ND	634011211	ND	K2	O1v1	ST14
PS_Kpn7	ST14	K2	634011211	ND	K2	O1v1	ST14

ND, No data provided by PHE.

*, Unknown type.

4.3.4 Genomic characterisation of AMR genes

The protein sequences of the genes encoded by the isolates were compared against the latest release of CARD (Jia *et al.*, 2017). No protein sequences were found to have a perfect CARD match for all 36 isolates (Figure 4.3). However, *KpnE* and *KpnF* had perfect/strict CARD match for all 36 isolates. In addition, a strict CARD match was seen for all 36 isolates for five other genes (*acrD*, *emrR*, *CRP*, *KpnG* and *KpnH*). Strict CARD match was also seen for 35/36 isolates for *acrB* (all but PS_Kpn1), *baeR* (all but PS_Kpn25), *marA* (all but PS_Kpn1), *msbA* (all but PS_Kpn3), *mtfA* (all but PS_Kpn1) and *pmrF* (all but

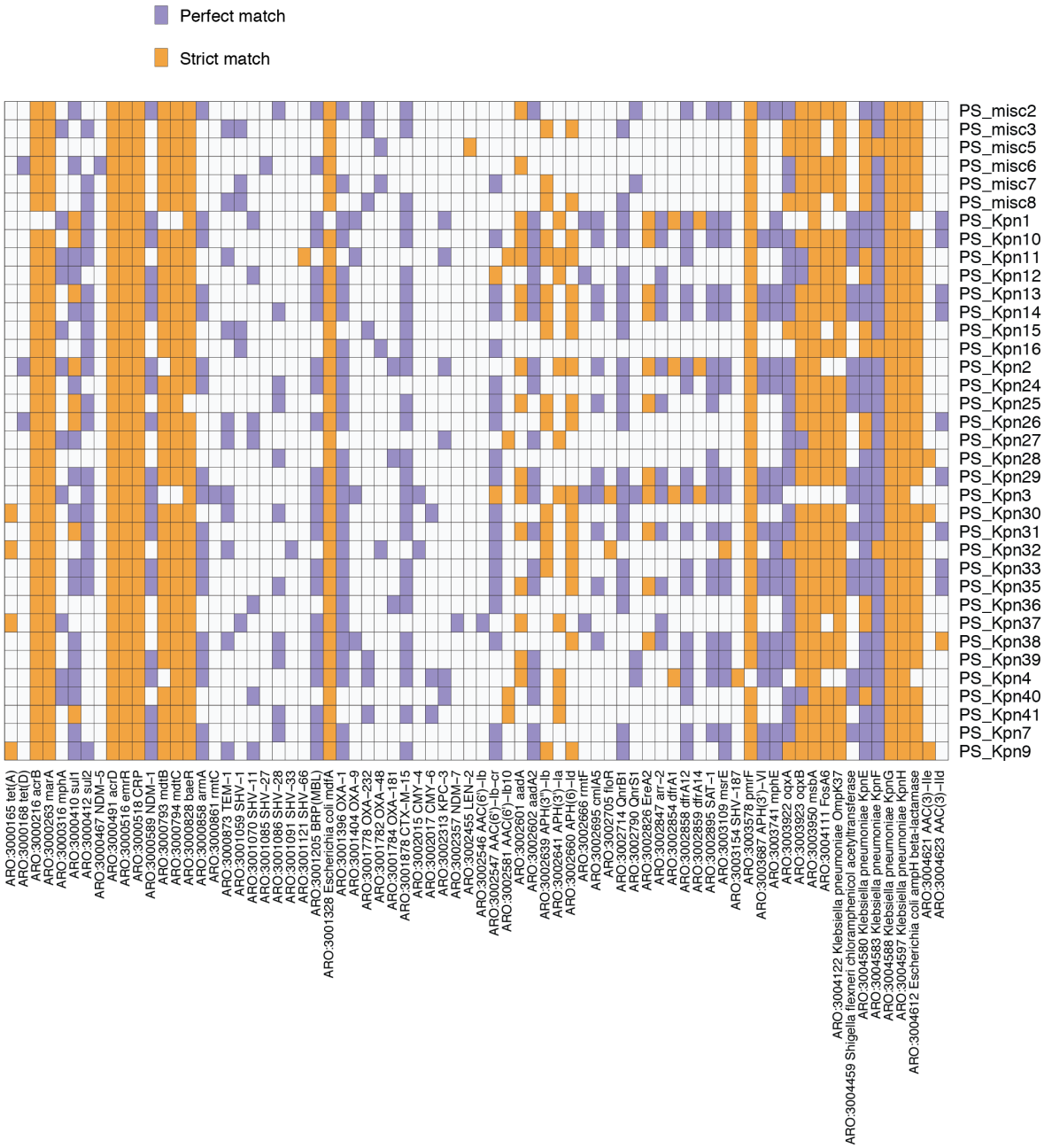


Figure 4.3. Comparison of genome data for the 36 (35 *K. pneumoniae*, one *K. variicola*) isolates with sequences in The Comprehensive Antibiotic Resistance Database (CARD) (Jia *et al.*, 2017). Strict CARD match, not identical but the bit-score of the matched sequence is greater than the curated BLASTP bit-score cut-off; perfect CARD match, 100% identical to the reference sequence along its entire length. Loose matches are not shown to avoid presenting false positives based on sequences with low homology and bit-scores below CARD BLASTP cut-off recommendations. BLASTP analyses carried out by me and image produced by Dr L. Hoyles.

PS_Kpn4); whilst strict CARD match was shown for 34/36 isolates (exceptions PS_Kpn1 and PS_Kpn3) for *mdtC*, for 33/36 isolates (exceptions PS_Kpn1, PS_Kpn3 and PS_Kpn4) for *ampH β-lactamase*, and for all isolates except PS_Kpn1, PS_Kpn2, PS_Kpn3 and PS_Kpn4 for *mdtB*.

Presence of *OXA-1*, *SAT-1*, *oqxA*, *KpnE* and *KpnF* was predicted for all ST14 strains (perfect CARD match) (Figure 4.3). In addition, all ST14 strains encoded (strict CARD match) *oqxB* and *msbA*; as well as the five genes listed above which were encoded by all 36 isolates, and seven of the nine genes encoded by most (≥ 32) of the isolates (*baeR* and *mdtB* being the exceptions). Interestingly, *AAC(6')-Ib-cr* was predicted (perfect CARD match) for all ST14 K2:O1v1 isolates but not encoded by the three ST14 K64:O1v1 strains (PS_Kpn2, PS_Kpn39 and PS_misc2). The presence of a number of other AMR genes was predicted (perfect CARD match) for the majority of ST14 strains: *APH(3')-V1*, *armA*, *BRP(MBL)*, *dtrA-12*, *msrE* and *mPHE* (not PS_Kpn25 and PS_Kpn28); *CTX-M-15* (not PS_Kpn7 and PS_Kpn24); and *acd2* (not PS_Kpn25, PS_Kpn28 and PS_Kpn38). Furthermore, *sul1* (perfect/strict CARD match for all but PS_Kpn28), *FosA6* and *OmpK37* (strict CARD match for all but PS_Kpn2) were also encoded by most ST14 strains.

Presence of *BRP(MBL)*, *CTX-M-15*, *CMY-6*, *KpnE* and *KpnF* genes was predicted (perfect CARD match) for all three ST15 strains, which also encoded (strict CARD match) *oqxB* and the previously listed genes encoded by all/majority of 36 isolates.

ST45 strains (n = 3) were predicted to encode *CTX-M-15*, *KpnF*, *OXA-232*, *QnrB1*, *SHV-1*, *sul-2* and *TEM-1* genes (perfect CARD match), as well as encoding (strict CARD match) *APH(3')-Ib*, *APH(3')-Id*, *oqxA*, *oqxB*, *OmpK37* and remaining 15 genes listed above (encoded by all/majority of 36 isolates).

ST11 strains were both predicted to encode *CTX-M-15*, *KpnF*, *oqxA*, *OXA-1*, *QnrB1* and *SHV-11* genes (perfect CARD match), as well as encoding (strict CARD match) *FosA6*, *oqxB*, *OmpK37* and remaining 15 genes listed above (encoded by all/majority of 36 isolates).

ST258 strains were both predicted to encode *aadA2*, *KPC-3*, *KpnF*, *mphA*, *oqxA*, *oqxB*, *sul1* and *TEM-1* genes (perfect CARD match), as well as encoding

(strict CARD match) *AAC(6')-Ib10*, *APH(3')-Ia*, *FosA6*, *OmpK37* and remaining 15 genes listed above (encoded by all/majority of 36 isolates).

All 36 isolates were confirmed to encode at least one NDM-, OXA- or KPC-gene (perfect CARD match), with numerous isolates predicted to encode more than one such gene (Table 4.4). No strict CARD matches were seen for these genes (Figure 4.3). Only four isolates (PS_Kpn16, PS_Kpn32, PS_misc5 and PS_misc7) were predicted to encode *OXA-48*, despite eight other isolates also having positive OXA-48 PCR data from the PHE reference laboratory. The presence of *KPC-3* was confirmed for the two isolates (PS_Kpn27 and PS_Kpn40) identified by PCR to carry the KPC resistance gene, along with four other isolates (all of which also had NDM-/OXA- genes) – three NDM strains and one OXA-48/NDM strain (PCR data). Three (PS_Kpn11, PS_Kpn25 and PS_Kpn38) of the 22 isolates characterised as carrying the NDM resistance gene by the PHE reference laboratory PCR were negative for CARD matches for NDM- genes (i.e. below CARD BLASTP cut-off recommendations for strict and perfect matches). Although they were each confirmed to encode *OXA-1* and/or *OXA-9* (Table 4.4). The presence of *NDM-1*, *OXA-1* and *OXA-232* was confirmed for both PS_Kpn4 and PS_misc2, characterized as OXA-48/NDM resistant by the reference laboratory.

Protein sequences associated with colistin resistance genes were also of particular interest in this study as eight clinical isolates (including 7 outbreak strains) displayed colistin resistance phenotypically. Colistin resistance is inherently seen in some Gram-negative bacteria such as *Serratia, Brucella* and *Burkholderia* species (Ah *et al.,* 2014). Acquired colistin resistance mainly occurs: 1) due to mutational changes in many endogenous genes involved in lipopolysaccharide synthesis involving the *mgrB* gene and upregulation of PhoP/PhoQ, these are the two sensor-regulator system which influence the synthesis of lipopolysaccharides (Poirel *et al*., 2014); and 2) through horizontal acquisition of genes as shown by the recent discovery of plasmid-encoding *mcr* genes (Liu *et al*., 2016). However, no CARD matches were seen for known colistin resistance genes (i.e. below CARD BLASTP cut-off recommendations for strict and perfect matches).

Table 4.4: Comparison of PCR and WGS resistance gene data.

Strain	Resistance gene	NDM-1	NDM-5	NDM-7	OXA-1	OXA-9	OXA-48	OXA-181	OXA-232	KPC-3
PS_Kpn1	NDM	+			+	+				+
PS_Kpn2	NDM	+			+			+		+
PS_Kpn3	OXA-48	+			+	+				
PS_Kpn4	OXA-48/ NDM	+			+				+	+
PS_Kpn7	NDM	+			+					
PS_Kpn9	NDM	+			+					
PS_Kpn10	NDM	+			+					
PS_Kpn11	NDM					+				+
PS_Kpn12	NDM	+			+					
PS_Kpn13	NDM	+			+					
PS_Kpn14	NDM	+			+					
PS_Kpn15	OXA-48								+	
PS_Kpn16	OXA-48				+		+			
PS_Kpn24	NDM	+			+					
PS_Kpn25	NDM				+					
PS_Kpn26	NDM	+			+					
PS_Kpn27	KPC									+
PS_Kpn28	OXA-48				+			+		
PS_Kpn29	NDM	+			+					
PS_Kpn30	NDM	+			+					
PS_Kpn31	NDM	+			+					
PS_Kpn32	OXA-48				+		+			
PS_Kpn33	NDM	+			+					
PS_Kpn35	NDM	+			+					
PS_Kpn36	OXA-48				+			+		
PS_Kpn37	NDM			+	+					
PS_Kpn38	NDM				+	+				
PS_Kpn39	NDM	+			+				+	
PS_Kpn40	KPC									+
PS_Kpn41	NDM	+							+	
PS_misc2	OXA-48 /NDM	+			+				+	
PS_misc3	OXA-48								+	
PS_misc5	OXA-48						+			
PS_misc6	NDM		+							
PS_misc7	OXA-48				+		+			
PS_misc8	OXA-48								+	

+, Positive by CARD (BLASTP) analysis.

4.3.5 Genomic characterisation of virulence genes

BLASTP searches were conducted against protein sequences included in VFDB (Liu *et al.*, 2019), with results confirmed by comparison with searches made through the VFDB VFanalyzer web portal (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi).

All strains were predicted to encode enterobactin (siderophore, linked to iron acquisition), AcrAB, RcsAB, type 1 fimbriae and *Escherichia coli* common pilus, and a range of capsule genes (as expected from results presented in Table 4.2). All strains except PS_Kpn41 were predicted to encode type 3 fimbriae (associated with biofilm formation). Twenty-nine of the strains (PS_Kpn1, PS_Kpn2, PS_Kpn3, PS_Kpn4, PS_Kpn7, PS_Kpn10, PS_Kpn11, PS_Kpn12, PS_Kpn13, PS_Kpn14, PS_Kpn15, PS_Kpn16, PS_Kpn24, PS_Kpn25, PS_Kpn28, PS_Kpn29, PS_Kpn30, PS_Kpn31, PS_Kpn32, PS_Kpn33, PS_Kpn35, PS_Kpn36, PS_Kpn38, PS_Kpn39, PS_Kpn41, PS_misc2, PS_misc3, PS_misc7 and PS_misc8) were predicted to encode the siderophore yersiniabactin. None of the strains encoded the siderophore salmochelin. Two strains (PS_Kpn4 and PS_Kpn41) were predicted to encode the siderophore aerobactin, and *rmpA*. PS_Kpn9 was the only strain to encode the allantoin utilisation operon. PS_misc7 encoded the complete colibactin gene cluster, while two strains (PS_Kpn1 and PS_Kpn11) encoded 14/16 genes of the cluster.

4.3.6 Isolation of phages that infect the clinical isolates, and their host ranges

Lytic phages were isolated for nine of the 36 clinical isolates (Tables 4.5 and 4.6), seven *K. pneumoniae* OXA-48 and two *K. pneumoniae* NDM. In total, 25 phages were isolated from Mogden wastewater samples (tanks A–E); none were isolated from Ascot wastewater samples tested. One phage each was isolated from all five tanks (A–E) for both PS_Kpn32 and PS_misc5. Four morphologically distinct phages (in terms of plaque morphologies) were isolated for PS_Kpn3 from tank C, as well as one PS_Kpn3 phage from tank D. One PS_misc6 phage each from tanks B–E, one PS_misc3 phage each from tanks B and E, one PS_Kpn15 phage and one PS_Kpn36 phage from tank A, and one PS_Kpn37 phage and one PS_misc7 phage from tank E.

The ability of the 25 purified phages to infect a panel of clinical *Klebsiella* isolates (Table 4.5, Table 4.6) was determined using the spot assay. Variation was seen across the isolated lytic phages, in relation to both depolymerase activity (haloes observed around plaques) and host range. However, depolymerase activity needs further investigation for some phages due to faint/possible haloes (unclear) being recorded. Bacteriophage PS_Kpn32_D displays the broadest host

range, showing activity against all three *K. michiganensis* strains, 21 *K. pneumoniae* isolates (including 14 of the 15 outbreak strains; PS_Kpn30 was not tested due to resuscitation issues) and the single *K. variicola* isolate tested (Table 4.5). Although all PS_Kpn phages displayed host ranges including more than one *Klebsiella* type (e.g. included strains from multiple *Klebsiella* species and/or multiple *K. pneumoniae* ST). In addition, PS_Kpn phages were successfully isolated for *K. pneumoniae* stains harbouring each of the three major resistance genes of NDM, OXA-48 and KPC. PS_misc phages displayed narrower host ranges (only one or two *Klebsiella* isolates) than PS_Kpn phages, with the exception of bacteriophage PS_misc7_E (two *K. michiganensis* strains and 20 *K. pneumoniae* isolates) and PS_misc6_C (two *K. michiganensis* strains, one *K. pneumoniae* isolate (PS_misc6) and the *K. variicola* isolate) (Table 4.6).

Ongoing phage work includes further phenotypic characterisation of the phages, and WGS analysis. Once completed, a report will be given to IBMS and a paper published in an IBMS journal for me to satisfy the conditions of the funding provided for this work.

Table 4.5: Host ranges of the lytic PS_Kpn phages against clinically relevant *Klebsiella* isolates.

Results are only shown for those phages that showed activity against one or more of the test strains.

Species	Strain	MLST	K, O types	3Ci	3Ciia	3Ciib	3Ciic	3D	15A	32A	32B	32C	32D	32E	36A	37E
K. michiganensis	PS_Koxy1	*	*, *	+	+	+	+			+	+	+	+	+		+
K. michiganensis	PS_Koxy2	*	*, *		+	+				+	+	+	+	+		+
K. michiganensis	PS_Koxy4	*	*, *							+ ?d	+	+	+	+		
K. pneumoniae	PS_Kpn1	*	K2, O4										+			
K. pneumoniae	PS_Kpn2	ST14	K64, O1v1			+				+	+		+ d			+
K. pneumoniae	PS_Kpn3	*	K64, O1v1	+	+	+	+	+ d							+	+
K. pneumoniae	PS_Kpn4	ST15	K64, O1v2	+	+	+	+									
K. pneumoniae	PS_Kpn7	ST14	K2, O1v1							+	+		+ d			+
K. pneumoniae	PS_Kpn10	ST14	K2, O1v1		+	+							+ d			+
K. pneumoniae	PS_Kpn11	ST258	K107, O2v2							+ d	+ d	+ d	+ d	+ d		
K. pneumoniae	PS_Kpn12	ST11	K15, O4							+	+	+	+	+		+
K. pneumoniae	PS_Kpn13	ST14	K2, O1v1	+	+	+	+						+ d			+
K. pneumoniae	PS_Kpn14	ST14	K2, O1v1							+			+ d			
K. pneumoniae	PS_Kpn15	ST45	K52, OL101						+							
K. pneumoniae	PS_Kpn16	ST101	K17, O1v1	+		+										
K. pneumoniae	PS_Kpn24	ST14	K2, O1v1										+ d			
K. pneumoniae	PS_Kpn25	ST14	K2, O1v1		+	+							+ d			+
K. pneumoniae	PS_Kpn27	ST258	K107, O2v2							+ d	+ d	+ d	+ d	+ d	+ d	
K. pneumoniae	PS_Kpn28	ST14	K2, O1v1							+ d	+ d	+ d	+ d	+ d		
K. pneumoniae	PS_Kpn29	ST14	K2, O1v1										+ d			
K. pneumoniae	PS_Kpn31	ST14	K2, O1v1										+ d			
K. pneumoniae	PS_Kpn32	ST35	K22, O1v1					+ d		+ d	+ d	+ d	+ d	+ d	+ d	
K. pneumoniae	PS_Kpn33	ST14	K2, O1v1										+ d			
K. pneumoniae	PS_Kpn35	ST14	K2, O1v1										+ d			
K. pneumoniae	PS_Kpn36	ST11	K24, O2v1												+	
K. pneumoniae	PS_Kpn37	ST16	K51, O3b							+	+	+	+	+		+
K. pneumoniae	PS_Kpn38	ST14	K2, O1v1							+	+	+	+ d	+		+ d
K. pneumoniae	PS_Kpn39	ST14	K64, O1v1												+	+ d

Species	Strain	MLST	K, O types	3Ci	3Ciia	3Ciib	3Ciic	3D	15A	32A	32B	32C	32D	32E	36A	37E
K. pneumoniae	PS_Kpn40	ST512	K107, O2v2							+ d	+ d	+ d	+ d	+ d	+	
K. pneumoniae	PS_Kpn41	ST15	K112, O1v1													+ d
K. pneumoniae	PS_misc2	ST14	K64, O1v1												+	
K. pneumoniae	PS_misc3	ST45	K52, OL101	+ d	+ d	+ d	+ d	+ d								
K. variicola	PS_misc5	*	K81, OL101						+ d				+		+	
K. pneumoniae	PS_misc6	ST716	K110, O2v1								+ d	+ d				
K. pneumoniae	PS_misc7	ST294	K30, O1v1							+	+	+	+	+		
K. pneumoniae	PS_misc8	ST45	K52, OL101						++							
K. pneumoniae	P010F	ST253	*, O1v2												+ d	
K. pneumoniae	P057K W	*	K11, O3/O3a												+ d	+ d
K. grimontii	P038I	*	*, *												+ d	

+, Lytic; d, depolymerase activity (haloes formed round plaques); ?d, may be depolymerase activity (requires further study); –, no activity.

*, Unknown type.

Table 4.6: Host ranges of the lytic PS_misc phages against clinically relevant *Klebsiella* isolates.

Results are only shown for those phages that showed activity against one or more of the test strains.

Species	Strain	MLST	K, O types	3B	3E	5A	5B	5C	5D	5E	6B	6C	6D	6E	7E
K. michiganensis	PS_Koxy1	*	*, *									+			+
K. michiganensis	PS_Koxy2	*	*, *									+			+
K. michiganensis	PS_Koxy4	*	*, *				+ d	+ d		+ d					
K. pneumoniae	PS_Kpn1	*	K2, O4												+
K. pneumoniae	PS_Kpn2	ST14	K64, O1v1												+
K. pneumoniae	PS_Kpn3	*	K64, O1v1	+	+ d										
K. pneumoniae	PS_Kpn7	ST14	K2, O1v1												+
K. pneumoniae	PS_Kpn10	ST14	K2, O1v1												+
K. pneumoniae	PS_Kpn12	ST11	K15, O4												+
K. pneumoniae	PS_Kpn13	ST14	K2, O1v1												+
K. pneumoniae	PS_Kpn14	ST14	K2, O1v1												+
K. pneumoniae	PS_Kpn15	ST45	K52, OL101												+
K. pneumoniae	PS_Kpn24	ST14	K2, O1v1												+ d
K. pneumoniae	PS_Kpn25	ST14	K2, O1v1												+ d
K. pneumoniae	PS_Kpn28	ST14	K2, O1v1												+ d
K. pneumoniae	PS_Kpn29	ST14	K2, O1v1												+ d
K. pneumoniae	PS_Kpn31	ST14	K2, O1v1												+ d
K. pneumoniae	PS_Kpn33	ST14	K2, O1v1												+ d
K. pneumoniae	PS_Kpn35	ST14	K2, O1v1												+ d
K. pneumoniae	PS_Kpn38	ST14	K2, O1v1												+
K. pneumoniae	PS_misc3	ST45	K52, OL101	+ d	+ d										
K. variicola	PS_misc5	*	K81, OL101			+ ?d	+ ?d	+ ?d	+ ?d	+ ?d		+ d			
K. pneumoniae	PS_misc6	ST716	K110, O2v1								+ d	+ d	+ d	+ d	+ d
K. pneumoniae	PS_misc7	ST294	K30, O1v1		+			+							+

+, Lytic; d, depolymerase activity (haloes formed round plaques); ?d, may be depolymerase activity (requires further study); –, no activity.

*, Unknown type.

4.4 DISCUSSION

4.4.1 WGS identified a clinical strain of *K. variicola* subsp. *variicola*

As with the *K. michiganensis* isolates characterised in Chapter 3, WGS analyses were more accurate than phenotypic tests (API 20E, MALDI-TOF) for characterisation of *K. pneumoniae* complex isolates, with one strain (PS_misc5) found to represent *K. variicola* subsp. *variicola* and not *K. pneumoniae* (Figure 4.1, Figure 4.2). Detection of the β-lactamase LEN-2 in its genome (Figure 4.3) confirmed PS_misc5's affiliation to *K. variicola*, as this is a core gene unique to the species (Holt *et al.*, 2015). Long *et al.* (2017) highlighted the inability of MALDI-TOF to differentiate Kp1, Kp2 and Kp3 strains from one another in a study of ESBL-producing *K. pneumoniae* isolated from clinical infections. They used WGS to allow species differentiation, and also characterised the first *K. variicola* strains to carry KPC or NDM genes. It is recommended that reference MALDI-TOF spectra databases in clinical and reference laboratories are updated to include data from Rodrigues *et al.* (2018), who have identified protein biomarkers that allow differentiation of six of the seven species of the *K. pneumoniae* complex. This will allow greater recognition of the role of non-*K. pneumoniae* (Kp1) isolates in infection.

The strain I characterised here (PS_misc5 (unknown ST, K81:OL101), isolated from a rectal swab) was resistant to 15 of the 18 tested antibiotics (Table 4.1). PCR testing had shown it to encode the β-lactamase OXA-48, which was confirmed by comparison of WGS data with CARD (Figure 4.3; Table 4.4). There are only two other reports of OXA-48-encoding *K. variicola* in the literature, one strain in each (Haldorson *et al.*, 2018; Potter *et al.*, 2018). PS_misc5 also encoded a range of AMR factors (crAB, MdtBC, CRP, BaeR, MsbA, MdfA, OqxAB, KpnEF, KpnGH) associated with multidrug efflux pumps, and resistance to polymyxin (PmrF), cefotaxime and cefoxitin (OmpK37), and β-lactams (*E. coli* ampH β-lactamase), and virulence genes (AcrAB, enterobactin, RcsAB, type 1 and 3 fimbriae, *E. coli* common pilus) linked to immune evasion, iron uptake, upregulation of capsule synthesis, adherence and biofilm formation, and colonisation of epithelial cells, respectively. Potter *et al.* (2018) demonstrated pilus-encoding *K. variicola* established higher titres than *K. pneumoniae* in murine models of bladder infection, leading them to suggest *K. variicola* should be

characterised to species level to improve patient outcomes during infection. Maatallah *et al.* (2014) described *K. variicola* as a frequent cause of bloodstream infections in Sweden, associated with higher mortality than *K. pneumoniae*. However, there are few other data available with respect to the role of this bacterium in infections. A colistin-resistant hypermucoid strain of *K. variicola* encoding *rmpA*, *rmpA2*, aerobactin, salmochelin and yersiniabactin has been isolated from the blood of patient in China (Lu *et al.*, 2018). The colistin resistance was associated with the presence of a D150G substitution in PhoP, part of the PhoP-Q two-component system, which is known to mediate colistin resistance. An outbreak in a Bangladeshi neonatal unit was recently reported, associated with hypervirulent MDR *K. variicola* ST771 (Farzana *et al.*, 2019).

Results from this Chapter and Chapter 3 have shown *Klebsiella* spp. can only be reliably identified based on WGS. But WGS or the bioinformatics expertise necessary to process and interpret sequence data are not available within clinical laboratories. Therefore, clinical laboratories often rely on PCR-based methods to differentiate species that cannot be split using phenotypic methods. Garza-Ramos *et al*. (2015) designed a multiplex-PCR probe system able to differentiate *K. variicola* from *K. pneumoniae* with high accuracy using the unique genes in the species' genomes. M-PCR-1 was used to assay a collection of MDR (n = 503) and antimicrobial-sensitive (n = 557) *K. pneumoniae* clinical isolates. *K. variicola* was detected with a prevalence of 2.1% (23/1,060), with13/23 isolates MDR, and 10/23 isolates antimicrobial sensitive. *rpoB* gene sequence analysis of the *K. variicola*-positive isolates confirmed the multiplex-PCR results. Implementation of this PCR in the clinical laboratory may allow better recording of *K. variicola* in clinical laboratories and facilitate epidemiological studies.

4.4.2 Antibtiotic resistance of *K. pnuemoniae*

For the OXA-48 strains, two isolates (PS_Kpn32 and PS_Kpn36) were sensitive to meropenem with a zone size <25mm and E-test MIC < 0.12mg/L. As OXA strains may not show resistance to all carbapenemase indicator antibiotics, i.e. can give very low level carbapenem resistance without cross-resistance to cephalosporins, they often remain susceptible to meropenem. However, resistance can be picked by ertapenem, in addition to showing high levels of resistance to temocillin and tazobactam. Ertapenem is a more sensitive indicator

for detecting carbapenem resistance than meropenem, but it is also the analogue most affected by porin-mediated mechanisms and so is the least specific (PHE, 2016b). KPC enzymes and MBLs tend to confer broader effects on the β-lactam resistance profile of the host strain.

Note: Since 2015 the screening cut-off point recommended by EUCAST (European Committee on Antimicrobial Susceptibility Testing) for zone size for meropenem has changed to <28mm (MIC remains the same >0.125 mg/L). Temocillin zone size and MIC were also under consideration by EUCAST, as temocillin was also used as an indicator antibiotic for the detection of CPE especially OXA-48 (Woodford *et al*., 2013) and used as an alternative treatment to urinary tract infection with bacteria producing ESBLs in a clinical setting. I had to do a verification for temocillin that zone sizes of ≥ 20mm and ≥ 12mm correspond to MIC of ≤ 8mg/L and 32 ≤mg/L, respectively (as recommended by Vanstone *et al.,* 2018). Once the verification was approved, the recommendation of Vanstone *et al.* (2018) was used to interpret susceptibility to temocillin. Another change to consider was that at the time of my study disc diffusion testing using colistin 10 mg disc was done to detect colistin susceptibility, but no zone size interpretation was available in EUCAST guidelines. Thus, any isolates with reduced zone size to colistin were tested with colistin e-test to determine the MIC (MIC colistin susceptibility was interpreted against EUCAST criteria; R >2 mg/L). When these isolates were referred to PHE's Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit for characterisation and susceptibility testing (PHE used agar dilution method), the reports came back with differences in colistin susceptibility compared to my data. This made me aware that disc diffusion for colistin can give rise to false susceptibility results and currently EUCAST (EUCAST, Version 9.0, 2019) or NCCLS (National Committee for Clinical Laboratory Standards) documents do not provide interpretive criteria for the testing of colistin. EUCAST now recommends that colistin MIC determination should be performed using broth microdilution (Gales *et al*., 2001; Tan and Ng, 2007). To this end I am currently evaluating the broth microdilution method at Wexham Park Hospital.

This discrepancy between the clinical laboratory and PHE results was also highlighted in the study carried out at the time of the outbreak at West London ICHNT (Otter *et al*., 2017). In this study colistin resistance was detected in isolates

from 25/38 (65.8%) patients for which colistin susceptibility was determined by PHE; the median colistin MIC was 8 mg/L. Only 8/25 (32.0%) isolates identified as colistin-resistant by PHE were reported as colistin resistant by our hospital clinical laboratory, meaning that colistin resistance was not detected until late in the outbreak in July 2015. This did not affect patient management at the time as colistin was not used for therapeutic purposes.

Two clusters of colistin-resistant isolates, one on the renal wards in Hospital A within VNTR sub-clone A, and one on the vascular wards at Hospital B within VNTR sub-clone B were found in the study of Otter *et al.* (2017). PHE WGS analysis identified three separate mechanisms of colistin resistance: two due to different mutations that each caused an early stop codon in the *mgrB* gene, and one due to a L/Q substitution at amino acid position 396 in *phoQ*. These are consistent with Olaitan *et al*. (2014) who reported evidence of widespread colistin-resistant *K. pneumoniae* due to inactivation of *mgrB*, a *PhoP*/*PhoQ* regulator. Giani *et al.* (2015) reported a large hospital outbreak of 93 bloodstream infections caused by colistin-resistant KPC-producing *K. pneumoniae* which was mostly explained by clonal expansion of a single *mgrB* deletion mutant. Taken together, this suggests that the clonal spread of *K. pneumoniae* with mutational colistin resistance may be a more important clinical threat than resistance through plasmid-mediated genes, and underlines the need for robust infection control interventions to prevent the clonal spread of resistance determinants (Otter *et al.*, 2017). Although colistin resistance is seen more in clinical settings, understanding of the clinical and epidemiological implications of the various types of colistin resistance is limited, with very few data on the frequency of emergence, fitness impact and strain variation (Ah *et al.,* 2014), and advice on how to limit the emergence of colistin resistance through better antibiotic stewardship is also lacking (Otter *et al*., 2017).

4.4.3 Carbapenemase gene analyses of *K. pneumoniae*

The PHE reference laboratory used a multiplex-PCR-based technique for detection of genes encoding carbapenemases belonging to different classes. Primers were designed to amplify the following genes: class A (KPC, IMI, NMC, GES AND SME), class B (IMP, VIM, GIM, NDM, KHM, TMB, DIM, SIM AND SPM)

and class D (OXA-48-like) carbapenemases. Three different multiplex reaction mixtures were defined and evaluated for the detection of all these genes.

K. pneumoniae is an important nosocomial pathogen, known to cause pneumonia, urinary tract, wound and blood infections (Brisse *et al*., 2009). It is often encapsulated, with over 100 known capsule types now recognised (Wyres and Holt, 2016). The capsule is an important virulence factor, and some capsular types, particularly K1 and K2 but also K54 and K57 (Fang *et al*., 2007), are associated with a community-acquired invasive PLA syndrome. In particular, the virulent clone ST23, referred to as CC23 (K1) or the K1 cluster, identified by MLST and PFGE (Turton *et al*., 2007) carries additional factors that increase its virulence: i.e. plasmid-borne *rmpA* (regulator of mucoid phenotype A) and *wcaG* (encoding capsular fucose synthesis), which together enhance the ability of the bacterium to evade phagocytosis by macrophages (Fang *et al*., 2007). The capsular type together with the putative virulence factors (*rmpA* and *wcaG*) were also included in the multiplex-PCR, with serotype-specific targets for capsular types K1, K2, K5 and K57. The remaining targets sought to detect the 16S-23S internal transcribed spacer region in *K. pneumoniae* as both *K. pneumoniae* and *K. pneumoniae* subsp. *ozaenae* give a band and can be distinguished on the basis of their malonate and Vogues-Proskauer reactions.

The reference laboratory confirmed that 22/36 clinical isolates were NDM-positive, 10 were OXA-48-positive, two were KPC-positive, and two were positive for both NDM and OXA-48 resistance genes. Eight of the 22 *K. pneumoniae* NDM clinical isolates were positive for the K2 capsular type common in the UK, and all 22 *K. pneumoniae* NDM were negative for *rmpA*/*wcaG* virulence factors.

Since the population of *K. pneumoniae* isolates belonged to an outbreak situation in a nosocomial setting, it was important to identify possible cases of transmission. This was performed by comparison of DNA fingerprints using PFGE, which provides an excellent method for this purpose together with VNTR analysis. Identification of these loci is useful for epidemiological investigations, since they have a relatively high mutation rate, the addition or deletion of sequence units usually occurring as a result of slipped strand mispairing during replication (Yazdankhah and Lindstedt, 2007). Using these methods, the isolates were described by a series of numbers and the profiles compared. Here we describe eight VNTR loci for *K. pneumoniae*, which provide discrimination at the same level

as using multiplex-PCR coupled with VNTR analysis, providing a rapid means of characterisation and typing isolates.

The *K. pneumoniae* NDM outbreak strains, ST14 with K2 capsular type, isolated in this study were related to the ones isolated in previous studies and belonged to the clone of outbreak strain dissimilated from the Indian subcontinent (Giske *et al*., 2012). The first-reported NDM-positive isolates (Yong *et al.*, 2009) were two isolates from Oman (Poirel *et al.*, 2010) and seven from Nairobi, Kenya (Poirel *et al.*, 2011b). All belonged to ST14, a member of the large CC292 complex and are clonal, suggesting that some strains could potentially cause outbreaks, which our data has corroborated. Woodford *et al.* (2011) described *K. pneumoniae* ST14 as a host lineage for the NDM-1 enzyme and a frequent host of CTX-M enzymes. In addition, ST14 is a single locus variant of ST15, which frequently carries CTX-M ESBLs. The ST14 isolates with NDM-1 carbapenemase are extremely MDR, as seen with this study: they are known to also harbour ESBLs and acquired AmpC enzymes besides the NDM MBL, thus conferring resistance to all β-lactams; they tend to also produce 16S methylases, often ArmA or RmtC, which confer resistance to all clinically useful aminoglycosides. Most remain susceptible to polymyxins, although resistance has been observed in some isolates (Kumarasamy *et al.*, 2010) as was the case in this study with 8 NDM strains (some belonging to the ST14 cluster) resistant to colistin. Worryingly, some of the ST14 isolates in this study carried genes determining serotype K2, which is one of the *K. pneumoniae* serotypes associated with invasive disease similar to those isolated from a study by Giske *et al*. (2012).

4.4.4 MLST of strains

MSLT analysis of the 36 isolates identified 15 ST14 isolates, 12 capsular type K2 and three K64, all O1v1. Eleven of the ST14 isolates were part of the outbreak, isolated from patients from the same ward and sharing the same antibiotic resistance genes (*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{NDM-1}}$, *bla*$_{\text{SHV-28}}$), with a few having an additional resistance gene (either *bla*$_{\text{OXA-181}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{OXA-232}}$, *bla*$_{\text{OXA-48}}$ or *bla*$_{\text{KPC-3}}$). The ST14 isolates also shared similar genes encoding for aminoglycoside with quinolone resistance gene. Common genes for efflux pump activation, which also contributes to various antibiotic resistances, were also identified (all 36 clinical isolates). Two ST258 isolates were identified, both harboured *bla*$_{\text{KPC-3}}$ together

with *bla*$_{SHV}$ and *bla*$_{TEM}$ β-lactamases. Both also shared the same capsular antigen (K107) and O antigen (O2v2) together with various genes for quinolone and aminoglycoside resistance. Three isolates belonged to ST45, harbouring *bla*$_{OXA-232}$ together with *bla*$_{SHV}$ and *bla*$_{TEM}$ β-lactamases, and shared the same capsular antigen (K52) and O antigen (OL101) together with various genes for quinolone and aminoglycoside resistance. The remaining isolates belonged to either ST11, ST15, ST16, ST35, ST101, ST147, ST294, ST512 or ST716 (each with distinct K types) with some having a common O antigen (O1v1 being most common). Of the 36 isolates, for 22 carbapenem resistance was due to the presence of *bla*$_{NDM-1}$, for four it was due to the presence of *bla*$_{OXA-48}$, for five it was due to *bla*$_{OXA-48}$ derivatives *bla*$_{OXA-232}$ and *bla*$_{OXA-181}$, and for three it was due to *bla*$_{KPC-3}$.

K. pneumoniae strains are intrinsically resistant to ampicillin due to the presence of the SHV β-lactamase (Class A). A derivative of *bla*$_{SHV}$ is *bla*$_{LEN}$ (Tärnberg *et al*., 2009), with the two not easily differentiated using PCR. Both class A β-lactamases were present in some of the *K. pneumoniae* isolates in this study. Most of the 36 clinical isolates carried several AMR genes, the majority of which are acquired via horizontal plasmid transfer (Holt *et al.*, 2015).

4.4.5 *K. pneumoniae* OXA-48

In general, the results of my WGS AMR analyses were consistent with the reference laboratory PCR findings for the 36 clinical isolates, with a few disparities. Of particular interest was the identification of the OXA-48 carbapenamse genes. The reference laboratory identified 12 OXA-48-producers, whereas WGS detected only four (the other eight being OXA-48 variants that are not differentiated by the generic PCR, namely OXA-181 and OXA-232). Potron *et al*. (2013b) characterised OXA-232, differing from OXA-181 by one amino acid substitution, leading to a weaker capacity to hydrolyse imipenem than OXA-181 and OXA-48. *K. pneumoniae* OXA-232 strains are able to hydrolyse all penicillins except temocillin, which has been reported to be a poor substrate of OXA-232. This has led to using temocillin as a valuable phenotypic tool for the presumptive detection of OXA-48 in clinical laboratories. However, results from this study showed that temocillin should be used carefully to detect OXA-48-like-producers, since some OXA-48 variants, such as OXA-232, possess a weak capacity to hydrolyse this β-lactam.

The *bla*$_{OXA-232}$ gene was identified in three enterobacterial isolates recovered from patients who had a link with India, further confirming the spread in the Indian subcontinent of OXA48-like carbapenemases in hospital settings.

CARD results (Figure 4.3) showed PS_Kpn25 harboured a CTX-M-15 ESBL β-lactamase, *bla*$_{OXA-1}$ and *bla*$_{SHV-28}$, whereas the reference laboratory identified it as carrying an NDM carbapenemase, with colistin resistance.

4.4.6 Genomic identification of virulence factors of *K. pneumoniae* using VFDB

Hypervirulent *K. pneumoniae* have been found to have more active siderophores, encoded by multigene operons that are included in the VFDB (Liu *et al.*, 2019). These siderophores facilitate iron transport across bacterial cell membranes, with iron essential for almost all life processes. Enterobactin along with one or both of aerobactin and yersiniabactin are associated with hypervirulent strains, with salmochelin rarely detected (Holt *et al.*, 2015). Yersiniabactin is the most common virulence factor associated with human *K. pneumoniae* infections (Liu *et al.*, 2019). Enterobactin was predicted to be encoded by all 35 *K. pneumoniae* strains, while yersiniabactin was predicted to be encoded by 29/35 strains (i.e. they encoded the full complement of genes for the operons of both siderophores). Strains producing enterobactin (with or without yersiniabactin production) essentially belong to hypervirulent *K. pneumoniae,* which are undergoing a global dissemination from the Asian Pacific region (Shon *et al.*, 2013) and are known to cause life-threatening infections both in young and healthy individuals from the community.

Two ST15 strains (PS_Kpn4 and PS_Kpn41) were predicted to encode the siderophore aerobactin (the most common siderophore secreted by hypervirulent *K. pneumoniae*; Liu *et al.*, 2019) and the "*regulator of mucoid phenotype*" *rmpA*. ST23 isolates with this phenotype are considered hypervirulent (Broberg et al., 2014), suggesting PS_Kpn4 and PS_Kpn41 are hypervirulent strains. PCR done by PHE for *rmpA* did not pick up this gene, suggesting the primers they use lack specificity. So far hypervirulent *K. pneumoniae* are relatively sensitive to most antibiotics, but as my study clearly shows MDR hypervirulent *K. pneumoniae* are now a concern in the West London Hospitals.

Additional virulence factors included in VFDB cover efflux pumps (AcrAB), antiphagocytosis (capsule), toxins (colibactin), capsule regulation (RcsAB), nutrition (allantoin utilisation), adherence and biofilm formation (type 1 and 3 fimbriae, *E. coli* common pilus), and immune evasion (capsule).

All 35 *K. pneumoniae* strains encoded AcrAB, a multidrug efflux pump that may also represent a novel virulence factor required for *K. pneumoniae* to resist innate immune defense mechanisms of the lung, thus facilitating the onset of pneumonia (Padilla *et al.*, 2010). They also all encoded RcsAB, responsible for upregulation of capsule (*cps*) expression (Liu *et al.*, 2019), and type 1 fimbriae and *Escherichia coli* common pilus (both associated with adherence and biofilm formation; Liu *et al.*, 2019). All strains except PS_Kpn41 were predicted to encode type 3 fimbriae (associated with biofilm formation).

One strain (PS_Kpn9) encoded the allantoin utilisation operon, a nutritional factor known to increase nitrogen availability during infection, and associated with hypervirulent *K. pneumoniae* strains that cause PLA (Chou *et al.*, 2004; Liu *et al.*, 2019).

PS_misc7 encoded the genotoxic microbial metabolite colibactin, which causes breaks in double-stranded DNA and is associated with development of chronic inflammation and bacterially induced colorectal cancer (CRC) (Lai and Lin, 2010; Wilson *et al.*, 2019). PS_Kpn1 and PS_Kpn11 encoded 14/16 genes required to produce colibactin. Whether the toxin is produced by these strains would need to be confirmed *in vitro*. Lai and Lin (2010) showed that PLA caused by *K. pneumoniae* significantly increased the risk of CRC. The carriage of the colibactin gene cluster may thus serve as a molecular basis underlying the epidemiological link between *K. pneumoniae* and CRC. More than 25% of *K. pneumoniae* strains isolated from Taiwan carried colibactin genes, and 66% belonged to the K1 type which is responsible for the development of invasive diseases such as PLA (Lai *et al*., 2014). The detection of the colibactin gene cluster in my isolates is of concern as different types of virulent *K. pneumoniae* are circling in the hospital environment and in the community of the West London area and if not checked may cause an outbreak.

4.4.7 *K. pneumoniae* phages and their therapeutic potential

The phage results in this study demonstrated the ability to isolate lytic phages for AMR *Klebsiella* clinical isolates harbouring carbapenemase resistance genes KPC, NDM and OXA-48, including colistin-resistant strains (a rare occurrence). PS_Kpn phages showed broad host ranges, against different *Klebsiella* types, with depolymerase activity often shown. Previous studies have shown that the phage-encoded enzymes (depolymerase) can counteract the resistance of encapsulated *K. pneumoniae* to key mechanisms of the innate host defense such as complement-mediated lysis and phagocytosis by macrophages (Lin *et al*., 2014; Pan *et al*., 2015; Majkowska-Skrobek *et al*., 2018; Chanishvili and Aminov, 2019). In addition, depolymerases are reportedly highly specific for a particular capsule serotype, as demonstrated by Lin *et al.* (2014) who used bacteriophage NTUH-K2044-K1-1 (that infects *K. pneumoniae* NTUH-K2044, capsular type K1) for diagnosis and treatment of K1 *K. pneumoniae* infections in mice. However, my data showed a number of *Klebsiella* bacteriophages with depolymerase activity against multiple K types (Table 4.5, Table 4.6).

The broad-spectrum activities of the phages in this study suggest they could be used to produce an effective cocktail consisting of several different phages, which would reduce the likelihood of phage-resistant bacteria developing during therapy (Oh *et al*., 2019). This study also shows the scope for using phages to control the dissemination of *K. pneumoniae*. In addition, bacteriophages could also be used for decolonisation purposes, leading to the reduction of the load of *K. pneumoniae* clones and thus reducing the chances of their transmission. Due to their relatively high specificity, phages could be used in a targeted manner, having little to no impact on the human gut microbiota compared to when conventional antibiotics are used. These features make bacteriophages potential alternatives to antibiotic therapies for clinical applications in infections caused by *K. pneumoniae*.

4.5 SUMMARY

In this Chapter the initial phenotypic and genotypic results obtained for the 36 MDR *K. pneumoniae* clinical isolates from the microbiology laboratory of Charing Cross Hospital were further investigated by WGS analyses, using a number of different genomic databases. While the majority of the strains were confirmed to be *K. pneumoniae* by WGS data, one was *K. variicola*, highlighting

the need for more refined characterisation methods in clinical laboratories. Some differences were seen in the number of carbapenemases detected for each strain and in relation to potential mechanism for colistin resistance. The genomic database CARD provided a wider range of protein sequences (for genes and regulators) to identify the different genes involved in carbapenem resistance. It aided my understanding of the various genes the bacteria harboured and that may be activated by external forces, thereby potentially guiding clinical application of antibiotics (i.e. identifying ones that should not be administered in order to reduce potential for increased resistance; as was the case regarding colistin resistance). It also highlighted that reference laboratories need to look closely at bacterial WGS data before setting targets for clinically relevant markers. From a clinical viewpoint, the in-depth knowledge obtained by WGS may not be necessary as all clinicians are interested in is whether a bacterium carries a resistance gene (or not) and speed of detection is paramount.

Similar to Chapter 3, this Chapter highlighted MALDI-TOF identification of bacteria used by the clinical laboratories is unable to differentiate closely related species. The identification of strains encoding both aerobactin and *rmpA*, and strains encoding colibactin along with their MDR phenotypes highlights hypervirulent, MDR *K. pneumoniae* strains are in circulation in West London Hospitals and are a cause of concern with respect to potential outbreaks.

Lytic phages, which may provide an alternative therapeutic option (including bacteriophage cocktails) to antibiotics, were successfully isolated for clinically relevant MDR *Klebsiella* strains. These require additional characterisation and warrant further investigation of their potential applications in clinical settings.

CHAPTER 5: GENERAL DISCUSSION

Antibiotics are becoming a limited resource due to the rise in *Enterobacteriaceae* presenting with resistance to quinolones, aminoglycosides (due to change in target site or enzymatic breakdown) and cephalosporins because these bacteria have acquired ESBLs and AmpC-type β-lactamases combined with porin loss. This has resulted in the increased use of carbapenems in hospitals, either as regimen escalation or as first-line empirical agents in settings where multidrug resistance is endemic (Livermore, 2012b). More worryingly, the increased use of carbapenems has resulted in the emergence of *Enterobacteriaceae* with carbapenem-hydrolysing β-lactamases (carbapenemases), rendering them resistant to antibiotics such as meropenem and ertapenem. Carbapenemases include MBLs of the NDM, IMP and VIM families, as well as non-MBLs such as KPC and OXA-48 (Livermore and Woodford, 2000).

The emergence of carbapenemases is of great concern as these enzymes provide a far more efficient and stable mechanism of resistance to carbapenems than combinations of ESBLs and impermeability. Moreover, acquired carbapenemases have the potential for horizontal spread between different species of clinically relevant *Enterobacteriacae* (i.e. *Escherichia*/*Shigella*, *Klebsiella*, *Enterobacter*, *Citrobacter* and *Raoultella* spp.), *Pseudomonas* spp. and *Acinetobacter* spp. Carbapenems are one of the most important last-resort antibiotics. In response to a small number of CPO outbreaks in hospitals in England in 2013, PHE developed a toolkit of guidance to promote the early detection, management and control of CPO colonisation and infections in acute hospitals (PHE, 2015). An NHS England Patient Safety Alert was issued with the CPO toolkit in March 2014, requesting all acute trusts to have a CPE plan by June 2014 (NHS, 2014).

In every hospital it is the diagnostic microbiology laboratory that plays an important role in the detection of CPOs, to aid in therapy and to improve infection control. GI carriage of CPOs is an important source of transmission. Isolation of carriers is one strategy that can be used to limit the spread of these bacteria.

At the request of PHE to promote early detection of CPOs, as a senior Biomedical Scientist and antibiotic lead in the microbiology laboratory at Charing

Cross Hospital, my part in a team response was to develop, implement and test a screening programme (Chapter 2) that was cost-effective, with a quick turnaround time and showing maximum sensitivity with reasonable specificity, ease of use and able to detect multiple types of carbapenemases. Other factors that I needed to consider when selecting and implementing the most suitable screening programme for our Trust were the prevalence of resistant bacteria with resistance determinants within the geographical region the Trust was covering together with the patient population. As detailed in Chapter 2, the chromogenic agar Colorex™ mSuperCARBA™ 1 (E&O) was found to be the best medium to screening isolates due its high limit of detection and CPO colonies grew much better on this medium so it was easy for staff to read results. Culture-based methods may not be optimal for infection control practices because of their long turnaround time and the fact presumptive identification of CPOs by cultivation still has to be confirmed by other methods. In addition to the reasons given previously, the rationale behind selecting a culture-based chromogenic agar for the initial screening was due to the fact culture-based testing was easier to implement, as the necessary equipment and knowledge are already present in the routine microbiology laboratory. These tests also have the potential to detect reduced susceptibility to carbapenems caused by newly emerging mechanisms even when the turnaround time is still more than 24 h. Chromogenic medium was selected over routine medium because chromogenic agars incorporate enzyme substrates that release a pigment when hydrolysed by bacterial enzymes (Bedernjak, 2010). Adding antibiotics to the medium makes it selective for a particular resistance trait. The pigmentation of the colonies makes it easier for laboratory staff to spot the CPOs and the presence of antibiotics cuts off the growth of the commensal microbiota within the sample. This is especially important when screening for CPOs from stool or rectal samples, as these sample types inherently have a high bacterial load, with CPOs representing a small proportion of the total bacteria in the majority of samples. Therefore, this cultivation-based approach provides specificity and convenience at a relatively low cost and with minimal to no staff training required.

In the clinical laboratory, suspected CPOs were subsequently identified to species level using MALDI-TOF MS (Bruker), with selection of resistant organisms assisted by CAT ID (Mast) with a panel of selected antibiotics. Resistant organisms were then referred to the PHE reference laboratory for identification of

resistance mechanisms and typed to the strain level using PCR- or WGS-based approaches. Xpert® Carba-R PCR (Cephid) was only used to confirm positive carriers, their contacts and those patients who have returned from travels from CPO-prevalent countries. Active CPO surveillance using this assay, supplemented with immediate patient isolation, proved to be an effective measure to limit the spread of CPO in our Trust.

Numerous studies have proved WGS testing to be a promising approach for screening for carriage of CPOs, as analyses of WGS data offer faster availability of results and increased sensitivity compared with traditional testing methods. In our screening programme, nucleic acid amplification testing (NAAT) or WGS are not used for screening rectal samples due to various anticipated limitations. These included the cost of the test and the fact that most of these tests have been designed to cover only the most common carbapenemases together with the inability to detect new or unanticipated carbapenemases. The concern about detecting new resistant carbapenemases together with newly emergent variants of previously characterised genes will need to be addressed by constant vigilance in updating targets in a chosen assay. Another challenge for nucleic-acid-based testing direct from samples (rather than isolating pure cultures of bacteria) DNA is extracted from stool, as faeces often contains PCR-inhibiting substances, and poor results may be obtained due to excessive shearing of DNA (Claassen *et al.,* 2013).

A major limitation of the work outlined in Chapter 2 is the lack of variety of individual carbapenemase classes tested in real time. At the time of the evaluation study, NDM was the most prevalent type of carbapenem resistance in *K. pneumoniae* across the five hospital sites covered by the Charing Cross microbiology laboratory. Consequently, many of the isolates used to evaluate the performance of the culture plates were NDM strains. One of the things I would do differentently in a new evaluation study would be to include clinical isolates of *K. pneumoniae* with a greater range of resistance mechanisms to validate and verify the different phenotypic and genotypic methods.

A strength of this work was its real-world environment. The evaluation was carried out using bacterial isolates that were representative of the population and showed a distribution of resistance mechanisms within the bacterial populations within the five hospitals. This was highlighted by the data generated by PHE, and

also through the genomic studies I carried out in Chapters 3 and 4. Another strength of the study was that the screening method was trialled on 19,644 CPO screens from 9,514 patients. The data were collected over a 6-month period from a diverse patient group across three hospital sites from central and north-west London, the inclusion of patients spanning the CPO risk spectrum, including patients from high-risk specialties including ICU and renal units, and robust statistical analysis accounting for repeated measures. This allowed the infection control team of the Trust to test an important national recommendation related to performing three sequential admission screens for CPOs, with the conclusion that the potential benefit did not outweigh the operational cost. The findings of this follow-on study have been published recently (Mookerjee *et al*., 2018).

In addition, the screening study (Chapter 2) was the first to evaluate the Colorex™ mSuperCARBA™ 1 (E&O) in a London hospital to identify the *K. pneumoniae* NDM outbreak, with some isolates showing colistin resistance (Otter *et al.*, 2017). It was also the first study to discover a novel $bla_{\text{GES-5}}$ carbapenemase gene in *K. oxytoca*, which through WGS analyses was confirmed to be *K. michiganensis* (Chapter 3).

With the introduction of improved screening programmes for CPOs, I believe as resistance to carbapenems is increasingly reported, the diagnostic microbiology laboratory has an important role to play in the rapid detection of CPOs in order to aid in therapy selection and improve infection control. Although techniques used to detect CPOs are diverse and abundant their sensitivities and specificities remain inconclusive. Genotypic techniques are used to confirm presence of carbapenemase genes, and are mostly used in reference laboratories such as those of PHE. Vanstone *et al*. (2018) evaluated the EntericBio CPE real-time PCR assay (Serosep Ltd) for the detection of the common carbapenemase resistance genes NDM, KPC, OXA-48-like, VIM, IMP and GES along with a variety of the less common genes with 100% sensitivity and specificity, improving turnaround time but still with the limitation of not being able to detect unknown resistance. However, this could offer an improvement and upgrading of the Xpert® Carba-R PCR (Cephid) for our workflow. But beyond that, I strongly believe culture-based methods are still essential and crucial for the initial detection of CPOs known and unknown. Chromogenic media are a new generation of media which can be considered as a truly rapid culture-based method for

carbapenemase detection and offer a sensitive, convenient, and relatively low-cost method for identifying CPOs. I also believe that evaluation and improvement of the screening methods does not stop with this study or that of Mookerjee *et al*. (2018), but with increased knowledge on carbapenemases, screening methods should be updated regularly due to ongoing technical developments and the emergence of new carbapenemase variants (Hammoudi *et al*., 2014).

During the outbreak screening period in 2015 using the new algorithm described in Chapter 2, while collecting isolates for my research I came across a few isolates that showed resistance to ertapenem and a low MIC to meropenem that grew on the new selective medium. MALDI-TOF identified the isolates as *K. oxytoca*. In-house PCR was negative for the five common carbapenemase genes. As part of an outbreak study by Eades *et al.* (2016), the isolates were identified by PHE as *K. oxytoca*, carrying a novel $bla_{\text{GES-5}}$ gene. MLST analysis with WGS data (not available to me) showed the isolates to be ST138. WGS analyses conducted in Chapter 3 showed these isolates to be *K. michiganensis*. This highlights our current reference MALDI-TOF databases lack discriminative power, which results in misidentification of pathogens. This to me raises concerns as to the accuracy of our reports regarding *K. oxytoca*-associated infection samples and the true prevalence of *K. michiganensis* from an epidemiological perspective.

Detailed WGS analysis of the *K. michiganensis* isolates (Chapter 3) confirmed they carried $bla_{\text{GES-5}}$ and were ST138, but also showed they encoded $bla_{\text{SHV-66}}$, $bla_{\text{TEM-1}}$ and ESBL $bla_{\text{CTX-M-5}}$. Because these strains were isolated from the gut of hospitalised patients who were immunocompromised and on various antibiotics, and because I wanted to learn more about basic bioinformatics, using the information provided by Tse *et al.* (2017), I looked at whether the strains encoded the kleboxymycin biosynthetic gene cluster associated with production of enterotoxins (tilimycin, tilivalline) known to be causative agents of non-*C. difficile* AAHC. All three strains encoded the complete 12-gene operon for the cluster. Further analyses showed the cluster to also be encoded by *K. grimontii* and '*K. pasteurii*' isolates (Chapter 3). Previously, the cluster was thought to be unique to *K. oxytoca*. Faecal carriage of *K. michiganensis* may be more clinically important than *K. oxytoca* (Chen *et al.*, 2019). Therefore, it is possible that *K. oxytoca* is not the only *Klebsiella* causative agent of AAHC, and laboratories may need to screen for presence of toxigenic *Klebsiella* isolates. Otherwise this could be a silent killer

if undetected from a clinical perspective. Further study is needed to investigate possible linkage between cytotoxin production and adverse clinical outcomes in *K. michiganensis* and *K. oxytoca* infections. From a clinical laboratory perspective, the only screening we do related to AAHC is for *C. difficile* toxin. Out of ~100 samples screened each day, on average four to five will be confirmed *C. difficile* toxin-positive. It is plausible that some of the *C. difficile*-negative samples may harbour toxigenic *K. oxytoca*, *K. michiganensis* or related bacteria. I am hopeful that from the information generated in this study, clinical microbiologists and the infection control team review their policies to increase the range of organisms to screen in AAHC (and antibiotic-associated diarrhoea) samples. If so, a rapid method for screening samples, such as PCR kits or enzyme-linked immunoassays, would need to be developed for detection of the enterotoxins.

Chapter 4 was dedicated to *K. pneumoniae*, as during the 2015 screening period, the majority of the *Enterobacteriaceae* isolated were shown by MALDI-TOF to be *K. pneumoniae*. Thirty-six of the isolates were characterised genomically. Thirty-five of the strains were found to be *K. pneumoniae*, while one was *K. variicola* subsp. *variicola*. This reinforces my comments in relation to the findings from Chapter 3: reference MALDI-TOF databases used in clinical laboratories need to be updated to provide accurate species identification and epidemiological data.

Many studies have shown *K. pneumoniae* to be among the most frequent organisms causing HAIs worldwide. It is represented by the ‘K’ in the ESKAPE pathogens, the six most significant and dangerous causes of drug-resistant hospital infections identified by the Infectious Diseases Society of America (Pendleton *et al*., 2013). It has also been recognised by the World Health Organization, Centers for Disease Control and Prevention, European Union, and other organisations as a significant threat to global public health as many strains are MDR (WHO, 2014), causing hospital outbreaks that are especially associated with the carbapenem-resistant clones. *K. pneumoniae* is also the organism in which the most new MDR genes are being discovered, before becoming widespread in other Gram-negative bacterial pathogens. These include ESBL, with its derivatives SHV and CTX-M, the carbapenemases KPC and NDM, and most recently *mcr-1*, the first plasmid-borne gene to be associated with colistin resistance.

In this study, I characterised the *K. pneumoniae* population circulating in our Trust (clinically important phylogenetic lineage) using MLST, and analyses of AMR and virulence genes. ST14 was the most common strain type, which is unsurprising as a number of the strains in my collection came from an outbreak (Otter *et al.*, 2017). The majority of ST14 (the cluster of outbreak strain at the West London Hospital Trust) belonged to the CG ST14/15 group capsular type K2, O antigen O1v1 with common resistance gene *blaNDM-1*, co-harbouring ESBL *blaCTX-M-15* type, other β-lactamases *blaSHV28, blaTEM-1* with quinolone and aminoglycoside resistance. Most of the outbreak cluster isolates also carried the virulence factor enterobactin or enterobactin with yersiniabactin, associated with PLA, belonging to the hypervirulent *K. pneumoniae* which are undergoing a global dissemination from the Asian Pacific. In fact, all 36 clinical isolates including the *K. variicolla* possessed all the virulence factors described by De Jesus *et al*., (2015) putting them under the hypervirulent strain category. Two isolates carrying *blaKPC-3 gene* belonging to the popular global outbreak CG ST258 and its derivative ST512 common in the United States were also amongst the collection together with ST43 (common in Europe) and ST147 (common in the middle east). This variation of strain types highlights the population diversity within the London area together with worldwide mobility (travel) bringing about carriage and spread of resistance. Other common carbapenemase resistance genes in this collection of clinical isolates were the *blaOXA-48* with its variants *blaOXA-232* and *blaOXA-181*, the tests we employed at our laboratory were unable to detect the variants. Using the genomic database CARD, I was able to identify the presence of the *pmrF* operon and the KpnEF efflux (in all 36 isolates) which has been linked to colistin resistance and/or the potential to acquire colistin resistance, as well as identification of the genotoxin colibactin gene cluster in three of the *K. pneumoniae* isolates and its potential link to chronic inflammation and colorectal cancer in humans.

This study using various genomic databases has allowed me to collect a wealth of information on *Klebsiella* spp. that are present in the West London area of UK. With this information a clinician can provide the best patient care and management which in turn can help infection control.

This study has clearly shown WGS is able to yield unambiguous data that enables a complete analysis of the whole genome, including MDR and virulence

genes as well as enabling more accurate characterisation. I expect WGS may ultimately become a highly powerful tool for both outbreak investigations and molecular characterisation of antibiotic resistance genes in routine clinical settings. However, WGS is still relatively expensive and requires an automated data interpretation system and a publicly available database. As such, routine implementation of WGS in clinical laboratories may not occur for several years but if this is the future then genomics and bioinformatics must be included as part of a Biomedical Scientists training.

A part of my research was to isolate bacteriophages against these very MDR *Klebsiella* spp. from sewage water. To this end I isolated seven *K. michiganensis* phages with lytic activity against the three toxigenic GES-5-positive *K. michiganensis* isolates which showed wide host range with both lytic and depolymerase activities against a collection of clinically relevant *Klebsiella* isolates. Twenty four *K. pneumoniae* phages and one *K. variicolla* phage were also isolated and demonstrated broad host range against different MDR *Klesiella* clinical isolates (*K. pneumoniae, K. grimontii, K.variicolla* and *K. michiganensis*). Initial attempts were also made to isolate DNA of sufficient quantity and quality for restriction enzyme profiling and WGS to characterise the phages, with mixed results. Accordingly, said genetic characterisation of the phages from this study is ongoing, together with further phenotypic characterisation, as they may have therapeutic uses in AAHC treatment regimens and can potentially be used in a cocktail format to eradicate gut colonisation of MDR *Klebsiella* spp.

Currently faecal microbiota transplantation (FMT) is used to restore normal gut microbiota in patients with *C. difficile* infection. Further studies are looking into whether FMT can be used to decolonise MDR organisms and prevent recurrent MDR infections (Saha *et al.,* 2019). This study has shown the potential for phages to be used therapeutically for gut decolonisation. However, further research is required to investigate such applications, including *in vivo* (animal and human) trials of phage or phage cocktails. With the current antibiotics crisis there is an increased interest in phage research and discussion of its usage and introduction of rules are being talked about at the Committee for Medicinal Products for Human Use at the European Medicines Agency (EMA). This may lead to EMA introducing a new regulatory framework for phage therapy. At these discussions there is also a possibility of introducing policies regarding adaptive licensing to allow limited

clinical use of phage therapy (Nilsson, 2014). Due to the lack of effective strategies to address the current unmet medical need for treatment of antibiotic-resistant infections, and in the absence of approved alternatives, some clinicians are turning to empirical ones, such as phage therapy, for compassionate treatment which is approved by the Food and Drug Administration (McCallin *et al*., 2019).

CHAPTER 6: PERSONAL REFLECTION

As I reflect on my journey while studying on the Professional Doctorate Programme (PDP), I also need to include the journey commencing before the course. The journey takes me back to 2005, when I attended a conference about bacteriophage (phage) and learned how one day these viruses would be useful to humankind. This subject really fascinated me, and I kept all the related documents in a secure place and thought to myself "I will come to this one day". At work I always expressed my interest in phage therapy to colleagues explaining that one day these bacteria-killing viruses may be our long-awaited solution to antibiotic resistance.

Then one day in October 2013, the departmental training officer at Charing Cross Hospital asked me if I wanted to study further, and without hesitation I expressed my interest to enrol in the PDP. She agreed that I should enrol for the course which would allow me to carry out research on phage. That seemed appropriate at the time with so much antibiotic resistance in bacteria, phages could be the long-awaited solution to treating antibiotic-resistant infections. I did not waste any time and enrolled on to the PDP at the University of Westminster, knowing deep within my very being this was a divine intervention.

I knew this was a big project I was taking on with being in full time employment at a senior level of great responsibility and with a family to take care of; I embarked on this great adventure with an inquisitive mind.

6.1 Remaining persistent amid challenging situations/and how these challenges were overcome

First, for any individual their main reason to embark on a PDP journey should be based on their true passion for research (in an area of interest) and academia and not based on any other interest. As when the rubber hits the road and when facing challenges, it is the passion for the research subject which will provide the fundamental driving force for you to carry on and not give up. This was true for me many times, as situations at work and changes in personal circumstances have been major challenge for me over the past 4 years. It has been incredibly difficult for me to get time off to complete my research. As I work for a very busy department with staff shotages it was difficult for my employers to

give me study time: both at the West London NHS Healthcare Trust (Imperial College London) and from my current employers (Frimley Health Trust).

In addition to my work-place challenges, I have had a very difficult time over the past 18 months due to the ill health of my husband. In October 2017 he underwent a triple bypass heart operation and is still recovering from it. I have had to juggle my research with caring for him and working full time (in a new environment). All this has taken its toll on me and it was the passion for the research subject that kept me going. Another thing that helped me to overcome my challenges was building good relationships with my supervisors; which become an important factor that shaped the rest of my academic and personal life during PDP research. Dr McCartney allowing me to have space in her laboratory at the University of Reading (from September 2016) to do my work has been a great help to me. Without this I would not have been able to do my practical work nearer to home; so also saving time travelling. My work place does not have the necessary expertise or equipment that are necessary for the phage work.

Another thing that caused concerns in the early years of the PDP was, although I knew clearly what my research aims and objectives were, I did not have all the necessary practical skills to carry out my work; i.e. methods of isolation of phages or the different kind of molecular methods, DNA extraction, genome sequencing and bioinformatics were all very new to me. But I was able to overcome these challenges by finding my supervisors, Dr Hoyles and Dr McCartney, were experts in these areas and we shared research interests. They provided me with the training and resources I needed to successfully carry out my research. In this way I was very fortunate to find a team of supervisors that matched and complimented my strengths and weaknesses as an individual. Especially as PDP taught modules were catered more towards the social sciences rather than clinical sciences, and the second year of the programme concentrated on research and various research methodologies aimed for social sciences. This period allowed me to begin to recognise and understand the various research methods but none of these methodologies were useful for my research. During this period, I relied heavily on my supervisors for support and guidance.

Another challenge I had to overcome was how would I find the funding to cover my tuition fees and funding for the research expenses. I reflected on this and how I had felt at the time using the Gibbs Reflective Cycle (1988), my feelings

at this time were quite hopeless. On evaluation of the situation, I was already working full time and there were no funds to put aside for the studies. I presented my research ideas to colleagues and connections outside of work with no results. My breakthrough came when my employers (West London Imperial College Healthcare NHS Trust) approval of my Professional Doctorate; one of the conditions was that my project had to meet a need for the Trust. To this end, and as part of the Microbiology team at Charing Cross Hospital, I was asked to organise and run a large screening programme to identify a streamlined way of quickly identifying carbapenemase-resistant *Enterobacteriaceae* (CRE) present in patients admitted to acute wards (renal, paediatric intensive care) and all immunocompromised hospitalised patients treated within the five hospitals of the Trust. This involved screening patient samples on different growth media. The reason for needing a rapid, cost-effective, streamlined isolation procedure was because the Trust was experiencing an increase in antibiotic-resistant organisms (especially those resistant to carbapenems) and the Trust needed to satisfy surveillance requirements of Public Health England (PHE) for multidrug-resistant organisms. The successful completion of this task allowed the infection control team of the Trust to fund half of my tuition fee for the first two years of the PDP.

In addition, before I could start the second part of my research to isolate phage that killed the collection of confirmed carbapenem isolates from the initial study, I had to find funding to cover research expenses. I started applying to different charities. When application forms were rejected there was a feeling of despair but success came when I was awarded funding by the Institute of Biomedical Science (IBMS) for my phage work (Appendix 3), and a summary of my work was published in the October 2016 edition of *The Biomedical Scientist* which bought about recognition of my work to a wider audience.

As I embarked on the phage side of my research, changes had to be made to the original aims due to time restraints.

Another milestone to overcome was in late 2017. I found a job nearer to home and moved to the Frimley Health Trust, so needed to get ethics approved to continue using my bacterial isolates after I had left West London Imperial College Healthcare NHS Trust. To my surprise approval was granted by the Tissue Bank at Imperial College without any opposition but one clause was that I must

acknowledge Imperial Trust Microbiology for the isolates in my thesis and any publications resulting from it.

As I reflect on my PDP journey and how it has shaped me as a researcher, I can say at the end of it all I have produced a coherent 40,000+-word thesis with likely publication of my work in scientific journals. The research has produced results which are very promising and further work could bring about a therapy for carbapenem-producing organisms (CPOs) and will have succeeded in answering the initial research question; could phage therapy be used to eradicate the carriage of CPOs in the gut of healthy humans?

On a personal level, the PDP has stretched me intellectually. During the past four years I have not just learned about my research topic but have also learned many core skills that I can apply to my job. It takes practice to become a good writer and communicator. My PDP student years have included much practice in writing funding applications (Appendix 3), poster presentation at conference (Appendix 2), reports and journal manuscripts, and also writing up the dissertation. The feedback I have received from my supervisors and peer reviewers has helped to improve my communication skills. And I am still learning and improving in this area. Research skills are valuable even in my field of work. As a trained researcher, I am now able to determine the best approach to a question, find relevant data, design a way to analyse it and make sense of my findings. My job also requires a great degree of project management. Finishing my dissertation requires me to design a project, make a realistic timeline, overcome setbacks, and manage stakeholders, which requires strong organisational and leadership skills. The PDP also helped me to improve my critical thinking skills as it trained me to approach problems systematically, see the links between ideas, evaluate arguments, and analyse information to come up with my own conclusions. PDP also allowed me to improve my skills in working with other people as doing tasks successfully requires knowing how to divide up a task and delegate, get along with others, communicate effectively (both in teams and also public speaking in the form of presentation), and resolve conflict.

The emotional stretching came with the various demands on my life and the separate roles that I must fulfil (as a Senior Biomedical Scientist, a part time PDP student and as a family woman). With all this I still sustained my enthusiasm and stimulus by selecting a highly interesting and timely topic. My balanced

relationship with my supervisors and the clearly defined expectations and focus on the target, self-motivation, discipline, a good work ethic and time management helped me to achieve what I thought the impossible.

One thing I have discovered within these four years about myself is that I am a researcher, I enjoyed the research aspects of the PDP. When looking in to the future, my deepest desire would be to continue with this research and succeed in completing to find a final answer for the research question. This may involve a change of career and looking for opportunities such as a postdoctoral research position in this area or similar.

APPENDIX 1

Preprint version follows, which is available from *PeerJ Preprints*.

Herridge, W.P., Shibu, P., O'Shea, J., Brook, T.C., Hoyles, L. (2019). Bacteriophages of *Klebsiella* spp., their diversity and potential therapeutic uses. PeerJ Preprints. 7, e27890v1 https://doi.org/10.7287/peerj.preprints.27890v1.

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Bacteriophages of *Klebsiella* spp., their diversity and potential therapeutic uses

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***Klebsiella* spp. are commensals of the human microbiota, and a leading cause of opportunistic nosocomial infections. The incidence of multi-drug resistant (MDR) strains of *Klebsiella pneumoniae* causing serious infections is increasing, and *K. oxytoca* is an emerging pathogen. Alternative strategies to tackle infections caused by these bacteria are required as strains become resistant to last-resort antibiotics such as colistin. Bacteriophages (phages) are viruses that can infect and kill bacteria. They and their gene products are now being considered as alternatives or adjuncts to antimicrobial therapies. Several *in vitro* and *in vivo* studies have shown the potential for lytic phages to combat MDR *K. pneumoniae* infections. Ready access to cheap sequencing technologies has led to a large increase in the number of genomes available for *Klebsiella*-infecting phages, with these phages heterogeneous at the whole-genome level. This review summarises our current knowledge on phages of *Klebsiella* spp. and highlights technological and biological issues relevant to the development of phage-based therapies targeting these bacteria.**

INTRODUCTION

Klebsiella spp. belong to the family *Enterobacteriaceae* and are non-motile, capsulate, Gram-negative bacilli. *Klebsiella pneumoniae* is a commensal bacterium found in the gastrointestinal and respiratory tracts, and on the skin of healthy individuals. It is also ubiquitous in the environment. It is an opportunistic pathogen capable of causing a wide range of community-acquired and nosocomial infections such as urinary tract infections (UTIs), respiratory tract infections, and infections of wounds and soft tissue [1]. It has, in recent years, become one of the world's leading causes of nosocomial infections with an increasing mortality rate, particularly in immunocompromised individuals, neonates and the elderly. It is also increasingly implicated in severe community-acquired infections such as pneumonia and meningitis [2]. Due to its widespread distribution and genetic make-up, *K. pneumoniae* has rapidly become a global threat to public health [3]. Similar to *K. pneumoniae*, *Klebsiella oxytoca* is an opportunistic pathogenic in humans, and is becoming increasingly associated with nosocomial infections, particularly in immunocompromised patients [4]. It is also acquiring antimicrobial resistance genes and is detected throughout the UK [5, 6]. Consequently, it is now considered the second most clinically important pathogen of the genus *Klebsiella* [4].

Given the reduction in the effectiveness of antimicrobial therapeutics to treat *Klebsiella*-associated infections, alternative strategies must be developed in response. This literature review will focus on bacteriophages (phages) of *Klebsiella* spp. and their potential for use as alternative antimicrobial agents.

Antibiotic resistance and *Klebsiella* spp.

Antibiotic resistance is defined as the ability of a bacterium, such as *K. pneumoniae*, to resist the effects of antimicrobial drugs that it was previously sensitive to. The development of antibiotic resistance is a result of the evolutionary process of natural selection, by which pathogenic bacteria are able to overcome the selection pressure applied to them during antimicrobial treatment, rendering the drug less effective [7]. The first antibacterial agents were discovered between 1910 and 1935 [8–10] with the most famous being the discovery of penicillin by Alexander Fleming in 1929. Hailed as 'magic bullets' in the fight against infection, these first antibacterial agents paved the way for the discovery of almost all classes of antibiotics in use today [11].

Despite early evidence of the possibility for future antibiotic resistance [12] and warnings that unrestricted use could reduce their effectiveness [13], antimicrobials have been taken for granted. The continued overuse of antibiotics in both healthcare and agricultural

settings over the course of the last century has contributed to the evolution and emergence of antibiotic-resistant strains of *Klebsiella* spp. and other Gram-negative bacteria.

K. pneumoniae strains are frequently resistant to extended-spectrum β-lactams such as penicillins and cephalosporins. Extended-spectrum β-lactamase (ESBL)-producing *K. pneumoniae* are able to target the β-lactam ring structure within antibiotic compounds, neutralising their antimicrobial activity [14]. Pathogenic ESBL-producing *K. pneumoniae* derive their antibiotic resistance enzymes most commonly from acquired genes such as SHV-5 and CTX-M-15 [14, 15]. Ever since ESBL-producing *K. pneumoniae* were first described by Knothe et al. [16] in Germany, *K. pneumoniae* carrying CTX-M-15 have spread throughout the world and are associated with a steadily increasing incidence of both nosocomial infections and, more recently, community-acquired infections with an increasing mortality rate [17–20].

ESBL-producing *K. pneumoniae* strains remain susceptible to the carbapenem class of antibiotics, which includes imipenem and meropenem. However, there is increasing incidence of *K. pneumoniae* infections caused by strains that have become resistant to even carbapenems. These multi-drug resistant (MDR) organisms are thought to have evolved in response to the increased use of carbapenems against ESBL-producing *K. pneumoniae*, with several independently evolved genetic elements conferring carbapenem resistance. *Klebsiella pneumoniae* carbapenemase (KPC) was first discovered in the United States [21] and has since spread to all other parts of the world [22].

In Europe, KPC was found to be the most common carbapenemase resistance gene in *K. pneumoniae* hospital-acquired infections (45%), followed by oxacillinase-48 (OXA-48-like) (37%), New Delhi metallo-β-lactamase (NDM) (11%), and Verona integron-encoded metallography-β-lactamase (VIM) (8%) [23]. In the UK, confirmed cases of KPC, OXA-48-like, NDM, and VIM rose from 0 to 1 cases in 2007 to 661, 621, 439 and 86 cases, respectively, in 2015 [24]. The spread of OXA-48-like *K. pneumoniae* strains has occurred mostly in the Mediterranean and Northern Africa and is primarily spread via ST101 strains as a result of travel in the regions, whereas ST395 is associated with clonal outbreaks throughout Europe [25].

NDM carbapenemase-producers originated in India, primarily in strains of *Escherichia coli* and *K. pneumoniae*, and have spread throughout the world as a direct result of travel to

and from the Indian subcontinent [26, 27]. Nordmann et al. [27] showed that more than half of NDM isolates from the UK were from patients with a history of travel to India or Pakistan. The UK appears to have the highest concentration of NDM isolates in Europe currently [28].

While MDR *K. pneumoniae* is itself a problem, its ubiquitous presence in both animal and human hosts, as well as in the environment, combined with its ability to acquire and maintain antimicrobial resistance plasmids and to pass those plasmids on to other Gram-negative bacteria, puts it into a relatively unique position to be able to amplify the spread of antimicrobial resistance genes throughout the world [29]. The contribution of *K. pneumoniae* to the antimicrobial resistance crisis is difficult to quantify. However, a recent population genomics study has shown that within- and between-hospital spread of carbapenem-resistant *K. pneumoniae* is the major driver of expansion of these bacteria within Europe, with carbapenemase-resistant isolates concentrated in clonal lineages ST11, ST15, ST11 and ST258/ST512 and their derivatives [30]. *K. pneumoniae* (and likely *K. oxytoca*) will continue to play a key role in the development of antimicrobial resistance and is, therefore, a prime target for novel antibacterial therapeutics [29].

Risk factors for *Klebsiella* infections

Primarily an opportunistic pathogen prevalent in the hospital setting, *K. pneumoniae* has become a common cause of hospital-acquired infections, such as UTIs and bloodstream infections (BSIs), in which antibiotic-resistant strains are becoming more difficult to treat and are associated with an increased mortality rate. Perhaps the most ubiquitous risk factors for all forms of hospital-acquired *K. pneumoniae* colonisation and infection are patient exposure to antibacterial agents and the length of hospital stay. Indeed, there consistently appears to be a positive correlation between the length of time a patient is required to stay in hospital and the chance of acquiring a *K. pneumoniae* infection simply due to the increased exposure to healthcare-associated pathogens with time [31–33]. Moreover, a considerable number of studies aimed at identifying risk factors associated with such infections recognise previous antibiotic treatment as an important factor, particularly the widespread use of cephalosporins in the case of ESBL-producing *K. pneumoniae* infection [34], and carbapenems, fluoroquinolones, glycopeptides and aminoglycosides for infections caused by carbapenemase-producing *K. pneumoniae* [31].

Not surprisingly, invasive procedures, such as surgical intervention and catheterisation, are also strongly associated with the acquisition of *K. pneumoniae* infection. Patients who are subject to invasive procedures such as the installation of a central venous catheter, for

example, are likely to be immunocompromised individuals who have been hospitalised for a severe underlying health condition. These patients are, therefore, particularly susceptible to opportunistic infections which could lead to a BSI, in the aforementioned example, soft tissue and wound infections in patients subject to surgical procedures, or even severe cases of pneumonia or meningitis in neonates [35, 36].

Clinical features of disease may also be an important risk factor in the development of *K. pneumoniae* infection. Meatherall et al. [37] identified chronic liver disease and cancer as being the most significant factors involved in the development of *K. pneumoniae* bacteraemia; several studies have evidenced a link between diabetes mellitus and invasive *K. pneumoniae* infection as a result of poor glycaemic control and subsequent bacterial resistance to phagocytosis [34, 38, 39]. Nouvenne et al. [32] suggested an association between cardiovascular, respiratory, renal and neurological diseases, and colonisation and infection by carbapenem-resistant *K. pneumoniae*.

K. oxytoca is the causative agent of paediatric antibiotic-associated haemorrhagic colitis, caused by overgrowth of the bacterium with the release of cytotoxin when the intestinal microbiota is disturbed by antibiotic treatment [40, 41]. Likely due to a combination of improved detection methods [6], increased international travel [5], contaminated hospital equipment [5], increasing numbers of immunocompromised patients and more complex treatment regimens, *K. oxytoca* is being isolated more frequently from neonatal intensive care units than in the past, and is now also being found in a range of clinical samples from adult patients admitted to critical care centres. *K. oxytoca* is showing multidrug resistance and appears to have higher drug resistance compared with *K. pneumoniae*, though this requires further study [42].

Virulence factors of *Klebsiella* spp.

K. pneumoniae, despite being considered an opportunistic pathogen, possesses an arsenal of virulence factors that enable the bacterium to both infect its host and resist the host immune response allowing it to cause severe disease. The most studied virulence factors associated with *K. pneumoniae* are the capsule, lipopolysaccharide (LPS), fimbriae and siderophores.

The capsule is an extracellular matrix made up of strain-specific polysaccharides that surrounds the bacterium forming a thick fibrous structure. The capsular polysaccharides produced by *K. pneumoniae* are called K antigens and, given that the polysaccharide produced depends on the strain of *K. pneumoniae*, they have been traditionally used to

identify the strain using serological techniques [43]. The role of the capsule in human disease has been studied extensively and it is thought to have a defensive role by providing protection against phagocytic immune cells, blocking complement-mediated lysis and reducing levels of proinflammatory cytokines [44, 45]. Indeed, the virulence of *K. pneumoniae* is greatly reduced in the absence of a capsule, as shown by infection of mice with acapsular mutants [46], and greatly increased in so-called hypervirulent strains which produce more capsular material resulting in a hypermucoviscous phenotype [2].

The LPS is composed of an O antigen, an oligosaccharide core and lipid A, and protrudes from the bacterial membrane [47]. The primary role of LPS in *K. pneumoniae* infection is protection from the complement-mediated lysis of bacterial cells by binding of the complement component C3b away from the bacterial membrane, preventing the formation of the membrane attack complex C5b-9. This is carried out by the O antigen of the LPS which, when absent, makes *K. pneumoniae* more susceptible to complement-mediated bacterial lysis [48].

K. pneumoniae expresses fimbriae which are membrane-adhesive protrusions involved in the adhesion of the bacterium to host surfaces facilitating its invasion. Two main types of fimbriae are exhibited by *K. pneumoniae*: type 1 fimbriae which are filamentous, and type 3 fimbriae which are helix-like in shape [49]. Moreover, the expression level of each type varies depending on the surface to which the bacteria attach. Type 1 fimbriae are expressed in the urinary tract and the bladder, but not in the gastrointestinal tract or the lungs [50]. Struve et al. [50, 51] speculate that the downregulation of type 1 fimbriae may be because it reduces the ability of *K. pneumoniae* to penetrate the intestinal mucus layer in the gastrointestinal tract, as is seen with *E. coli*; whereas in the lungs, selection against fimbriated cells occurs due to rapid elimination by phagocytes. Type 3 fimbriae bind to extracellular matrices and medical devices, and are important for the development of biofilms [51].

Finally, *K. pneumoniae* must acquire iron from the environment to grow and multiply. There is very little free iron to be found in mammalian hosts, so the bacterium must express siderophores. These are molecules that have a higher affinity for iron than mammalian iron transport molecules, such as transferrin, enabling the bacteria to obtain iron for rapid growth and subsequent invasion. The primary siderophore expressed by *K. pneumoniae* is enterobactin, expressed in the majority of pathogenic strains; however, salmochelin, yersiniabactin, colibactin and aerobactin can also be expressed. Indeed, hypervirulent strains of *K. pneumoniae* are able to express multiple siderophores and are

particularly associated with the expression of salmochelin, yersiniabactin, colibactin and/or aerobactin [52].

Genetic diversity of clinically relevant *Klebsiella* spp.

In keeping with the diversity of its virulence factors, antibiotic resistance mechanisms and clinical presentations, strains of *K. pneumoniae* also possess highly diverse and flexible genomes capable of producing considerable phenotypic variation. Indeed, the diversity of *K. pneumoniae* is such that the species is widely accepted to exist as four distinct phylogroups: KpI, KpII-A, KpII-B and KpIII, which have been suggested to have diverged into three distinct species: *K. pneumoniae* (KpI), *K. quasipneumoniae* (KpII) and *K. variicola* (KpIII) [52].

In their whole-genome sequencing and pangenome-wide association study, Holt et al. [52] found that severe community-acquired infections were more often caused by phylogroup KpI that expressed siderophores and *'regulators of mucoid phenotype genes' rmpA* and *rmpA2*, which regulate capsule production. Moreover, their study also confirmed the presence of SHV, OKP and LEN β-lactamases as core chromosomal genes of all phylogroups, whereas acquired antibiotic-resistance genes were more commonly found in KpI and KpII commensal isolates compared to either hospital-acquired or community-acquired infection isolates, suggesting antibiotic resistance plays more of a role in opportunistic hospital-acquired infections caused by commensal *K. pneumoniae*, whereas more severe community-acquired infections are caused by strains enriched with virulence factors such as siderophores and increased capsular production.

Hypermucoviscous strains of *K. pneumoniae* – i.e. those that exhibit virulence genes such as yersiniabactin and *rmpA* – were first described in Southeast Asia and are commonly associated with community-acquired pyogenic liver abscess [53]. These hypervirulent strains very rarely exhibit the antibiotic resistance gene profiles commonly associated with opportunistic hospital-acquired infections, and until recently have remained treatable with antibiotics [54]. However, *K. pneumoniae* isolates with combined hypervirulence and antibiotic resistance are emerging. Given the highly diverse genome of the species, and the increasing selective pressures being applied to them in the form of antibiotics, hypervirulent antibiotic-resistant *K. pneumoniae* is threatening to become untreatable [52, 54].

A pangenome study of *K. oxytoca* strains isolated from bloodstream infections the UK and Ireland showed that *K. oxytoca* has a highly diverse population, composed of several

distinct phylogroups (KoI, KoII, KoV, KoVI) [5]. It shares numerous antimicrobial genes and mechanisms with *K. pneumoniae*. *K. oxytoca* has been far less studied than *K. pneumoniae*, and extensive studies of its global epidemiology are required [5].

PHAGES OF *KLEBSIELLA* SPP.

A phage is a virus that infects bacteria and, as such, is found in all environments where bacteria would normally thrive. Viruses were initially suggested as a possible cause of clear zones on bacterial culture plates by William Twort in 1915, and in 1917 Felix d'Herelle confirmed this discovery, coining the term 'bacteriophage' [55, 56]. Prior to the discovery of the first antimicrobial agents, phages were considered the cure for bacterial infections and d'Herelle performed the first experimental phage therapy using an oral phage solution to treat dysentery [57]. However, after the discovery of antimicrobial compounds such as penicillin, the therapeutic uses of phages were largely left alone due to the subsequent success of the antibiotic era. Phages remained useful, however, for scientific research as tools to improve our understanding of molecular biology, horizontal gene transfer and bacterial evolution, and as diagnostic tools [58]. More recently though, given the rise in the number of MDR infections caused by bacteria such as *K. pneumoniae*, the use of phages has again come to the forefront as a potential alternative to current antimicrobial chemotherapies.

Life cycles

Phages primarily have two distinct life cycles they are able to adopt in order to reproduce: the lytic cycle and the lysogenic cycle. Both life cycles begin with the attachment of a phage to the surface of the bacterial host, followed by the subsequent injection of the phage's genetic material into the cell. In the lytic life cycle, the viral genome produces proteins that initiate the degradation of the bacterial genome, allowing the viral genetic material to take control of the host cellular machinery for the sole purpose of replicating the viral genome, synthesising viral proteins and assembling those proteins into viable phage particles that are released from the bacterial cell in large numbers, destroying the host. The phages that are released are then able to continue infecting bacteria nearby. In the lysogenic life cycle, the viral genetic material is incorporated into the bacterial DNA, forming a prophage, and is replicated passively upon replication of the bacterial genome without destroying the host. Prophages in the lysogenic cycle are able to enter the lytic cycle under certain conditions (e.g. in the presence of environmental stressors), and begin actively replicating and producing viable phages at the expense of the host [59].

Although the lytic/lysogenic phage life cycle is a well-established concept in phage biology, we now know there are multiple phage life cycles. Pseudolysogeny is the process by which the phage genome enters a bacterial host but neither stably establishes itself as a prophage nor initiates a destructive replicative response, remaining inactive and possibly awaiting more desirable environmental conditions for viral replication [60]. Chronic infection, resulting in the shedding of phage particles over long periods of time without destruction of the host cell, can occur with infection of filamentous phages in *Mycoplasma* [47]. Finally, the carrier state life cycle occurs when a heterogeneous population of bacteria, containing individuals both sensitive and resistant to a given lytic phage, leads to the destruction of sensitive bacteria and the survival of resistant bacteria creating a stable equilibrium between viral and bacterial propagation [60].

In the context of using phages as a therapeutic alternative to antimicrobial chemotherapy, those that reliably employ the lytic life cycle to reproduce are most suitable given that the end result is the destruction of bacterial host cells. Additionally, phages that are able to switch between multiple life cycles may not make reliable treatment options due to the possibility of dormancy and subsequent re-establishment of bacterial infection. This is just one aspect of comprehensive phage characterisation that is an important consideration when choosing appropriate phage treatments.

Phage characterisation

Phages of *K. pneumoniae* have been isolated from a variety of sources worldwide including wastewater, sewage, seawater and human intestinal samples, and belong to one of three families of the order *Caudovirales* (**Table 1**). These families make up the entirety of the order and are described as non-enveloped, tailed phages, with icosahedral heads containing double-stranded DNA: *Myoviridae* are characterised by long, straight, contractile tails; *Siphoviridae* by long, flexible, non-contractile tails; and *Podoviridae* by short, non-contractile tails [61].

Genomic comparisons of lytic *K. pneumoniae* phages of the order *Caudovirales* highlight a variety of useful similarities and differences. The expression of polysaccharide depolymerases, for example, has been observed in several recently discovered phages of *K. pneumoniae* [62–64] and these enzymes have a role in the degradation of the capsule surrounding the exterior of the bacterium. The breakdown of the capsule by phage depolymerases has been purported to combat *K. pneumoniae* biofilms [65] and increase the susceptibility of the bacterium to antibiotics, phage infection and the immune system [64]. Additionally, phage depolymerase action can be observed in the laboratory with the

production of ‘haloes’ around clear zones of lysis on bacterial culture plates after infection of *K. pneumoniae* with phage particles. This has become the basis for important laboratory methods used in the characterisation of novel phages, revealing phage specificity and host range [66].

Moreover, differences observed among *Myoviridae*, *Podoviridae* and *Siphoviridae* can be useful for preliminary identification. For example, sequence analysis reveals that *Myoviridae* tend to have a much larger genome size and a smaller GC content compared to *Podoviridae* and *Siphoviridae* (**Table 1**). Restriction analysis, which uses bacterial restriction enzymes to digest phage DNA, can also help to estimate the size of the phage genome in addition to identifying those that are already known to science prior to extensive characterisation, and analysis by transmission electron microscope is able to reveal morphological characteristics such as phage tail structures [64]. Phylogenetic analyses show several *Klebsiella* phages belong to accepted genera within the *Siphoviridae*, *Podoviridae* and *Myoviridae*, while others belong to new lineages with – as yet – no standing in viral taxonomy (**Figure 1**, **Supplementary Material**).

Specificity and host range

To infect its host, a lytic phage must first attach itself to a susceptible bacterial cell. It achieves this by recognising and binding a specific receptor on the surface of the host cell. This interaction between the phage tail structure and host receptor allows the phage to both identify susceptible bacteria and position itself for injecting its genetic material into the cell. Adsorption to the host can occur via any external structure depending on the phage and host, but in Gram-negative bacteria, such as *K. pneumoniae*, these can include the capsule, pili, outer membrane proteins, sugar moieties or LPS [67]. This process, therefore, determines host range, i.e. the breadth of hosts that any given phage can infect.

D'Andrea et al. [68] showed that their newly discovered lytic phage φBO1E was able to specifically target KPC-producing *K. pneumoniae* of the pandemic clonal group 258 (CG258) clade II lineage, but not those of the closely related clade I lineage, due to the recognition and targeting of specific capsular polysaccharides present on strains belonging to clade II. In contrast, Verma et al. [69] found that the lytic phage KPO1K2, specific for *K. pneumoniae* B5055, could infect multiple strains of *K. pneumoniae* as well as some strains of *E. coli* and, therefore, has a relatively broad host range compared to the clade-specific phage φBO1E.

It is generally considered, in the context of their therapeutic use, that lytic phages with a broad host range (e.g. at genus or species level) are more beneficial in combatting bacterial infection than those with a narrow host range (e.g. at strain level). Phages with a narrow host range are inappropriate for presumptive or prophylactic treatment, for example, and would rely on identification of an infective agent prior to treatment. Additionally, even phages considered to have a broad host range would generally have a narrower spectrum of activity compared to antibiotics [70]. Therefore, efforts to increase the spectrum of activity of phage treatment has led to the development of phage cocktails, to increase the host range by using multiple phages in a single treatment [71], and even the hybridisation of phage tail structures to increase the host range artificially [72].

THERAPEUTIC POTENTIAL OF *KLEBSIELLA PNEUMONIAE* PHAGES

There are a number of considerations to be made when selecting phages suitable for use as therapeutic antimicrobial agents. Firstly, phages must be effective in killing *K. pneumoniae*. During phage characterisation, *in vitro* assessments of phage lysis and burst size are carried out on cultures of *K. pneumoniae*. Phages that produce rapid lysis of a bacterium and release large numbers of phage particles will produce large clear plaques. Moreover, phages with a broad host range are generally considered more useful than those with narrow host range so that multiple strains may be targeted at once [73]. Secondly, lytic phages, due to the nature of their life cycle, clear bacteria quickly and efficiently compared to lysogenic phages, which integrate their genetic information into the host genome and remain dormant for an unspecified amount of time. In addition, lysogenic phages may transfer genes into the host that can confer toxin production and antibiotic resistance traits to the bacterium, thus making the infection more virulent and difficult to treat [73].

In vivo experimentation

Following *in vitro* investigations, the safety and effectiveness of any new therapeutic candidate must be measured in a suitable animal or insect model prior to human trials. In the case of *K. pneumoniae* phage research, mouse models have been used to investigate the effect of phage treatment against wound and soft tissue infections [74], pneumonia [66], liver abscesses [76] and bacteraemia [77], closely mirroring the spectrum of disease caused by the bacterium in humans. More recently, *Galleria mellonella* larvae have been used to test the efficacy of lytic phages and phage-encoded products to clear *K. pneumoniae* infections [78–80].

Kumari and colleagues have carried out a series of murine-based experiments aimed at identifying the therapeutic potential of the *K. pneumoniae* phage Kpn5. Isolated as one of five phage candidates (Kpn5, Kpn12, Kpn13, Kpn17, and Kpn22) from samples of sewage [81], Kpn5 was found to be the most effective, compared to the other four, when used to treat burn wound infections caused by *K. pneumoniae* B5055 in BALB/c mouse models [82]. When administered by intraperitoneal injection, Kpn5 produced an average 96.66% survival rate compared to the negative controls which had a survival rate of 0% [83]. Additionally, when compared to topical treatments with both natural products (honey and aloe vera gel) [84] and antimicrobial agents (silver nitrate and gentamicin) [85], Kpn5 was found to be superior in both cases, providing a higher level of protection and reduced mortality rates. However, despite the promising results that this research group has produced, the authors note the possibility of *K. pneumoniae* forming resistance to Kpn5, as highlighted in their *in vitro* experiments, and provide no data on phage host range, having used only a single strain of *K. pneumoniae* throughout their studies.

The delivery method of phage treatment is also an important consideration. For example, intraperitoneal injection is rarely used in human treatment given the relative ease of intravenous injection in most cases. In experiments carried out to treat murine lobar pneumonia, Cao et al. [75] determined that intranasal delivery of phage 1513 was able to produce a survival rate of 80% in the Swiss-Webster mouse model, compared to 0% of negative controls, 2 h after nasal inoculation of MDR *K. pneumoniae* 1513 as well as visibly reduced lung injury, in comparison to negative controls. Chhibber et al. [83] demonstrated that intraperitoneal injection of phage SS administered immediately after intranasal inoculation of *K. pneumoniae* B5055 into BALB/c mice resulted in complete clearance of bacteria in 5 days, compared to 10 days in untreated mice, although the authors state that even a short delay of 6 h post inoculation rendered treatment ineffective. However, Singla et al. [86] found that phage KPO1K2, encased in a liposome, was effective in treating lobar pneumonia induced in BALB/c mice by intranasal inoculation of *K. pneumoniae* B5055*,* even when phage treatment was delayed by up to 3 days.

Although there is a difference in the choice of phage in these published reports, and so studies cannot be compared directly, it does highlight the importance of investigating differing delivery methods of phage treatment, not only in a logistical sense but also in elucidating the most efficient method of delivery according to the type of infection and the length of incubation prior to treatment. Moreover, these studies have each measured the *in vivo* effect of phage treatment against only one strain of *K. pneumoniae*, providing no

information regarding phage host range. Further experiments should, therefore, seek to determine whether the host range(s) of their respective phages are broad enough to be considered useful for therapeutic purposes.

While several studies have reported successful use of *K. pneumoniae* phages to clear infections in murine and *Galleria* models, the effects of phage infection on the microbiome (i.e. microbiota, metabolome) must now be considered when assessing phages (individually or as phage cocktails) as a viable treatment or patient decontamination measure. Hsu et al. [87] showed that infection with lytic phages caused an increase in phage resistance (28% to 68%) in a known bacterial population common to the human gut microbiota. Quantitative shifts in sensitive and non-sensitive strains were seen, highlighting the system-level effect of phage infection. Phage infection did not necessarily clear the target species but instead modulated the ecosystem towards a more stable gut environment. Phages inducing simultaneous knockdown of *Enterococcus faecalis* and *Bacteroides fragilis* populations had little effect on the microbiota compared with *Escherichia coli* and *Clostridium sporogenes* phages, which caused significant decreases (10^6 per gram stool) in *Bacteroides vulgatus*, *Proteus mirabilis* and *Parabacteroides distasonis* populations, and 10^8 per gram stool decreases in *Akkermansia muciniphila* and *Bacteroides fragilis* populations. Perturbation of the microbiota by phages also affected the metabolome. Abundance of 17% of examined compounds was altered significantly in the presence of phages. During initial phage infection, Hsu et al. observed a 10-, 17-, and 2-fold reduction in tryptamine, a microbiome-associated metabolite known to play a role in accelerating gastrointestinal transit in mice [88]. This led them to suggest phage infection could be used to modulate the microbiome in a targeted manner to influence systemic health.

Combination therapy

A number of *in vitro* experiments have identified the possibility of bacterial resistance arising as a result of phage therapy [71, 75, 81, 89, 90]. To reduce the emergence of phage-resistant strains of *K. pneumoniae* during treatment, research has begun to explore combination therapy either by using phage cocktails or combining phage treatment with antibacterial drugs.

Gu et al. [71] generated a phage cocktail (i.e. a combination of phages that have different but overlapping host specificities) made up of three lytic phages (GH-K1, GH-K2 and GH-K3) specific to *K. pneumoniae* strain K7. The authors found that co-culture of K7 with the phage cocktail produced fewer phage-resistant variants of K7 and a more efficient

reduction in bacterial load compared to cultures treated with a single phage. Moreover, when treating bacteraemic mice, produced by intraperitoneal injection of K7, the phage cocktail produced a significantly lower blood bacterial count and enhanced mouse survival rates compared to mice treated with individual phages. A similar phenomenon was seen by Chadha et al. [89], who aimed to resolve *K. pneumoniae* B5055 burn-wound infections in BALB/c mice and found that their phage cocktail (made up of Kpn1, Kpn2, Kpn3, Kpn4 and Kpn5) induced a greater decrease in bacterial load compared to treatment with individual phages and a complete bacterial clearance in a shorter time.

Finally, in combining a lytic phage with ciprofloxacin against *K. pneumoniae* biofilms, Verma et al. [91] demonstrated a reduction in the development of both phage-resistant and ciprofloxacin-resistant *K. pneumoniae* strains, as well as having an enhanced effect against bacterial biofilms compared to individual treatments.

Human trials

The progression of phage research from *in vivo* experimentation to clinical trials involving humans has generated some friction among regulatory bodies in Western countries. However, countries in Eastern Europe and the former Soviet Union have routinely used phages in their healthcare systems for many years [92]. For example, the Eliava Institute of Bacteriophages, Microbiology and Virology in Georgia, and the Hirszfeld Institute of Immunology and Experimental Therapy in Poland both produce and supply phage therapeutic products specifically for routine human use [93].

In the West, regulatory issues surrounding the use of phages as therapeutic agents has hindered progress somewhat. It is not that there are specific regulations that prevent the use of phages in this way, but rather a lack of regulation that has placed limitations on progress. The unique nature of phages compared to traditional therapeutic agents, as evolving and self-replicating biological entities, requires them to have new rules and regulations regarding their safety, production and use. It is this lack of regulation in the EU and the UK, combined with a lack of interest from pharmaceutical companies, and the concept of personalised medicine often associated with phage therapeutics, which in itself is a new method of infection control, that makes approval for human trials a lengthy and difficult process [94]. However, it should be noted that the Belgian government has introduced a pragmatic framework that facilitates tailored phage therapy (magistral phage regulatory framework) allowing non-authorized phage products to be prepared by a pharmacist, for a given patient in line with a prescription from a physician and complying with relevant standards [95]. Phages are very occasionally and only under exceptional

circumstances used therapeutically in the wider EU under the umbrella of Article 37 (Unproven Interventions in Clinical Practice) of the Declaration of Helsinki [95].

Despite these regulatory hurdles, a limited number of human trials have been carried out in relation to phage therapy, although none have specifically targeted *K. pneumoniae*. Rhoads et al. [96], based in the USA, carried out a phase I clinical trial on 42 patients with chronic venous leg ulcers to investigate the safety of a phage preparation specific to *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *E. coli*. The authors reported no adverse effects of phage treatment. In the same year, Wright et al. [97], based in the UK, carried out a phase I/II clinical trial to determine the safety and efficacy of their phage product targeting *P. aeruginosa* in chronic otitis. Their study involved 24 patients with chronic otitis and showed a reduction in *P. aeruginosa* counts and, again, no adverse effects of phage treatment. Although consisting of a small sample size, the apparent success of these first human trials did little to prompt changes to the regulatory obstacles currently associated with phage therapy.

Future directions

Phage therapy shows promise as a potential response to the continued development and spread of MDR *K. pneumoniae*. *In vitro* and *in vivo* studies have confirmed the potential for phages to be used individually, as phage cocktails and in combination with current antimicrobial chemotherapeutic drugs. Moreover, the routine use of phage therapy in Eastern Europe, and the results from the small number of human trials that have been carried out in the West, suggest that phages are generally considered safe for use in humans. However, the lack of progress toward amending EU and UK regulations to account for phage therapy has hampered progress. The focus of future direction in the area of phage research must be to overcome this obstacle.

USING PHAGE-DERIVED GENE PRODUCTS

Another avenue of phage research aimed at finding therapeutic solutions to MDR *K. pneumoniae* is the potential to use specific phage gene products rather than phages themselves to combat infection. This kind of treatment could be advantageous in that it would be easier and quicker to gain clinical approval of a recombinant protein product over the direct use of phages. Indeed, phage-derived recombinant proteins may be used to directly combat infections caused by bacteria such as *K. pneumoniae,* or as part of a combinatory approach to complement or enhance current antimicrobial regimes.

Phage proteins

In the lytic life cycle of an infecting phage particle, there are a number of proteins that the phage can use to ensure successful adsorption, infection, replication and release of progeny. In terms of potential antimicrobial agents against *K. pneumoniae*, there are a number of biologically interesting proteins to consider. Peptidoglycan hydrolases and polysaccharide depolymerases are normally present on the tail spikes of a phage particle and are involved in successfully infecting a bacterium after adsorption. Polysaccharide depolymerases degrade the macromolecular carbohydrates that make up the capsule surrounding the bacterial cell wall, whereas peptidoglycan hydrolases break down the peptidoglycan layer to penetrate the cell wall and access the cytoplasm to allow the phage to deposit its genetic material [98].

Holins, endolysins and spanins are proteins that are produced after the infection of a bacterium, and are involved in the process of cell lysis whereby assembled phage particles 'burst' from the cell in order to spread and continue the infection cycle. Holins are hydrophobic transmembrane proteins that mediate the permeabilisation of the inner cell membrane. This cannot independently cause cell lysis; however, it allows endolysins and spanins to translocate from the cytoplasm, where endolysins degrade the peptidoglycan layer in between the inner and outer cell membranes, and spanins disrupt the outer cell membrane present on Gram-negative bacteria. This is followed by bacterial cell lysis via osmolysis [98].

Polysaccharide depolymerases

The capsule of *K. pneumoniae* is an important virulence factor and allows the bacterium to avoid phagocytosis and complement-mediated lysis. It is, therefore, a prime target for recombinant phage-derived proteins and has been studied extensively. For example, tail tubular protein A (TTPA), a structural tail protein of phage KP32, was shown to have additional polysaccharide depolymerase activity. Pyra et al. [99] cloned and expressed TTPA in *E. coli* and determined its enzymatic activity by agar spot tests on lawns of *K. pneumoniae* PCM2713, which produced translucent zones of reduced growth. Subsequent microscopic analysis of treated and untreated *K. pneumoniae* revealed cells treated with TTPA were stripped of their capsules. In a similar process of cloning, expression and agar spot-testing, Pan et al. [100] discovered nine polysaccharide depolymerases expressed by phage ΦK64-1, each of which demonstrated activity against a specific capsular type of *K. pneumoniae* which corresponded to the broad host range of the phage itself. This is interesting because not only does it confirm the role of enzymes such as polysaccharide depolymerases in the determination of phage-host specificity, but

it also lends the idea of artificially generated cocktails of recombinant enzymes that can target a wide range of *K. pneumoniae* strains.

A number of *in vivo* experiments have also been carried out investigating the effect of polysaccharide depolymerases on *K. pneumoniae* infection. Majkowska-Skrobek et al. [80] identified, cloned and expressed a KP36-derived capsule depolymerase, depoKP36, which produced haloes on lawns of *K. pneumoniae* in agar spot-tests. The authors tested the ability of depoKP36 to treat infection caused by *K. pneumoniae* in *Galleria mellonella* and found that 100% of the larvae died without treatment, up to 40% survived when treated with depoKP36 post infection, and depoKP36 treatment of bacteria prior to infection resulted in only a 23% death rate. These results suggest that the decapsulating action of depoKP36 on *K. pneumoniae* produced a decreased ability of the bacterium to resist the host immune response. This was confirmed in subsequent research [101].

Endolysins

Endolysins have been studied extensively for use against Gram-positive bacteria, due to the absence of an outer cell membrane found in Gram-negative bacteria such as *K. pneumoniae* which would normally hinder the action of the enzyme in the absence of spanins. However, recent research has produced some promising results regarding the use of endolysins against Gram-negative bacteria also. Maciejewska et al. [102] produced a recombinant endolysin from the *K. pneumoniae* phage KP27 and analysed its peptidoglycan-degrading activity on a range of Gram-negative bacteria, including strains of *K. pneumoniae*, *P. aeruginosa*, *Salmonella enterica* and *E. coli*, by co-incubation of bacteria and endolysin. The recombinant enzyme successfully lysed all strains of bacteria that were tested. However, the outer membrane of bacteria was permeabilised prior to endolysin treatment. This suggests that any potential endolysin-based infection control agents require mixing with outer-membrane-permeabilising agents to be effective against *K. pneumoniae* [102].

To overcome the need for additional outer-membrane-permeabilising agents during treatment of Gram-negative bacterial infections, artificial lysins (Artilysins) have been developed by the fusion of a phage endolysin with an outer-membrane-destabilising peptide [103]. Artilysins specific for *K. pneumoniae* have yet to be developed, but they have been successfully created for use against *P. aeruginosa* [104] and *Acinetobacter baumannii* [105]. This technology opens up the possibility of developing artificial endolysins for use in human therapy against not only MDR *K. pneumoniae* but also MDR Gram-negative infections.

Further research

Recombinant polysaccharide depolymerases and artificial endolysins have the potential to be used as therapeutic agents in the fight against MDR *K. pneumoniae*. Polysaccharide depolymerases are able to degrade the capsule, an essential virulence factor of *K. pneumoniae*, which could find uses such as boosting the host immune response against the bacterium, and breaking down biofilms to allow current antibiotic regimes to more easily access bacterial cells. Artificial endolysins have the potential to work against infection as an independent antimicrobial agent. Further research is required in this area to fully realise the potential of such phage-derived recombinant proteins, and in doing so the mechanisms by which they are able to inhibit bacterial growth and/or eliminate infection may lead to new breakthroughs. Importantly, an obvious advantage over phage therapy is that recombinant protein products for use in humans have well-defined and established rules and regulations regarding their production, safety and use in the EU and UK, whereas phage therapy does not.

CONCLUDING REMARKS

The increasing incidence of hospital-acquired and community-acquired infections caused by MDR *K. pneumoniae* and hypervirulent *K. pneumoniae*, respectively, is rapidly becoming a global threat to public health. The emergence of strains that are both MDR and hypervirulent is even more of a concern. *K. pneumoniae* is becoming as much of a threat today as its non-resistant counterparts were over a century ago prior to the discovery of antimicrobial compounds such as penicillin. In response, research efforts have begun to look back in time at a once-abandoned approach to bacterial infection, namely phage therapy. It is becoming increasingly clear that there is potential for phages and their gene products to become novel sources of antimicrobial strategies against MDR bacteria for which current treatment regimens are simply becoming ineffective at countering. However, the field of phage therapy is still very much in its infancy and is fraught with difficulties, both novel and familiar.

Safety

One of the major obstacles facing phage therapy are the novel safety implications regarding the use of self-replicating biological entities in humans. For example, it is evident that phages are capable of carrying antibiotic resistance [106] and toxin-encoding [107] genes that could be transferred to the target bacterium via the process of transduction. Proper characterisation is, therefore, important when considering phages for therapeutic uses, and the presence of potentially harmful genes is commonly screened for

during this process. However, the absence of harmful genes does not guarantee phage safety.

For example, the nature of a lytic phage is to increase its number at the expense of bacterial hosts. While this is the primary aim of phage therapy, little research has been conducted regarding the potential side-effects of this phenomenon. This is an important consideration because phages with a broad host range, or those within a phage cocktail, are often considered more appropriate for phage therapy. It is evident from the recent work of Hsu et al. [87] that introduction of even a single phage into the mouse microbiota can have effects on the microbiome. What effect might therapeutic use of phages have on the normal microbiota of a human? Might it be safer to use individual phages, with a narrow host range, to minimise disruption of the commensal microbiota? If so, phage therapy will rely on very specific identification of infecting bacteria, and having the correct phage available for treatment. Or perhaps this particular side effect may be deemed acceptable, as is the case with current antibiotic regimens. Additionally, the number of clinical trials that have assessed the safety of phage therapy in humans is limited, and those that have occurred have consisted of small sample sizes and often rely on patient-generated data [93].

Practicality

The second barrier that must be overcome are the practical issues associated with phage therapy in the EU and UK. As discussed earlier, the regulations required to govern the safety, production and use of virus-based infection control mechanisms do not currently exist. The last attempt at tackling these regulatory hurdles came in the form of a phase II clinical trial funded by the European Commission. *"Launched in 2013 and achieved in 2017, PhagoBurn was the world first prospective multicentric, randomised, single blind and controlled clinical trial of phage therapy ever performed according to both Good Manufacturing (GMP) and Good Clinical Practices (GCP)"* [108]. Although the project attempted to define appropriate practices for phage therapy during its assessment of efficacy and tolerability of phage-treated burn-wound infections [109], only temporary allowances were made. While recommendations for subsequent clinical trials were given, no further regulatory improvements have been attempted.

Moreover, if regulations are updated to account for phage therapy, where would producers of phage products stand in relation to intellectual property? Can naturally occurring biological entities be patented and sold, or would this be reserved for phage cocktails and phage–drug combinations that exhibit 'unnatural' antimicrobial properties?

Indeed, in terms of personalised medicine, phage cocktails may require production within the healthcare setting to suit a specific patient's needs. In this case, would the ingredients of a phage cocktail need to be individually patented and sold, or could cocktails be developed with the pliability for patient-specific modifications later? In the absence of profitable, patented technology, pharmaceutical companies may be reluctant to fund the research and development of such treatments.

Phage resistance

Finally, it could be argued that the issues surrounding phage therapy may be abrogated by using phage gene products instead. Being more akin to conventional antimicrobial therapeutics, they would be subjected to the well-established drug development processes and standards of production and safety that are currently in place. However, the use of both phages and their gene products against bacterial infection may still be subject to the age-old problem of bacterial resistance. Indeed, some of the studies outlined in this literature review suggest, or provide evidence of, the possibility of resistance against phage therapy, although this phenomenon has yet to be observed *in vivo*.

The first warnings regarding the development of antibiotic resistance [12, 13] went unheeded, resulting in the spread of MDR bacteria such as *K. pneumoniae*, and are the grounds upon which phage therapy has become a renewed topic of research. The development of novel antimicrobial agents is, therefore, not enough to combat infection and bacterial resistance in the long term. Strategies regarding the use of any novel antimicrobial treatments must be developed to minimise the risk of the development of resistance. In terms of phage therapy, such strategies might involve using combination treatments: for example, phage–drug combinations or complex phage cocktails designed to minimise the selection pressures applied against bacteria during treatment.

Prevention should be the primary focus of healthcare-associated infection control procedures. The implementation or improvement of policies aimed at reducing the risk of patients developing bacterial infections must be concurrent with the development of novel antibacterial therapeutics to minimise the spread of resistance to treatment. Such procedures may include hand and environmental decontamination, safe installation and maintenance of medical devices, prompt removal of medical devices that are no longer needed, screening and decolonisation programmes, and cautious use of antimicrobial agents.

Future research

The future of phage research is a promising one. Phages are perhaps the most numerous of all biological entities on the planet and as such could be the most valuable source of therapeutic solutions. As we further elucidate the interactions between phage and bacterium, as predator and prey, advances in our understanding of the molecular mechanisms defining such interactions may afford us new information and ideas that can be applied to infection control. Indeed, phage research has already led to the development of artificial phage-derived antibacterial proteins – Artilysins [103] – and the artificial alteration of phage host range to infect a greater range of bacteria than is naturally possible is just beginning to come to fruition [72].

Furthermore, recent technological advances have seen next-generation sequencing (NGS) become increasingly used in phage research, providing a more robust platform from which to launch detailed phage characterisation, screening of harmful genes and evaluating potentially useful gene products [110]. Further technological advancements, and categorisation of information attained from methods such as NGS can only lead us onwards, providing new solutions to old problems.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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Table 1. Known phages with that infect one or more strains of *Klebsiella*

Phage	Family	RefSeq/GenBank accession no.	Genome size (bp)	Source	Reference
vB_KpnM_KpV477	*Myoviridae*	NC_031087	168272	Clinical sample	[112]
ZCKP1	*Myoviridae*	MH252123	150925	Freshwater	[65]
KP15	*Myoviridae*	NC_014036	174436	Irrigated fields	[64]
JD001	*Myoviridae*	NC_020204	48814	Seawater	[113]
0507-KN2-1	*Myoviridae*	NC_022343	159991	Sewage	[114]
Matisse	*Myoviridae*	NC_028750	176081	Sewage	[115]
Miro	*Myoviridae*	KT001919	176055	Sewage	[116]
PKO111	*Myoviridae*	NC_031095	168758	Sewage	[117]
PMBT1	*Myoviridae*	LT607758	175206	Sewage	[118]
vB_KpnM_KB57	*Myoviridae*	NC_028659	142987	Sewage	Volozhantsev et al.*
vB_Kpn_F48	*Myoviridae*	MG746602	170764	Sewage	[119]
GH-K2	*Myoviridae*	Not Available	Unknown	Sewage	[71]
Kpn1	*Myoviridae*	Not Available	Unknown	Sewage	[89]
Kpn2	*Myoviridae*	Not Available	Unknown	Sewage	[89]
Kpn3	*Myoviridae*	Not Available	Unknown	Sewage	[89]
Kpn4	*Myoviridae*	Not Available	Unknown	Sewage	[89]
vB_KpnM_BIS47	*Myoviridae*	KY652726	147443	Sewage plant	[120]
JD18	*Myoviridae*	NC_028686	166313	Unknown	Fan et al.*
vB_KleM-RaK2	*Myoviridae*	NC_019526	345809	Unknown	[121]
Mineola	*Myoviridae*	MH333064	166130	Unknown	Boeckman et al.*
May	*Myoviridae*	MG428991	159631	Unknown	Nguyen et al.*
Menlow	*Myoviridae*	MG428990	157281	Unknown	Newkirk et al.*
vB_KpnM_KpV79	*Myoviridae*	MF663761	47760	Unknown	Komisarova et al.*
vB_KpnM_KpV52	*Myoviridae*	KX237516	47405	Unknown	Komisarova et al.*
1611E-K2-1	*Myoviridae*	MG197810	47797	Unknown	Lin et al.*
KP179	*Myoviridae*	MH729874	162630	Unknown	Kozlova et al.*
KP1	*Myoviridae*	MG751100	167989	Unknown	Kim*
3 LV-2017	*Myoviridae*	KY271397	35100	Unknown	[122]
4 LV-2017	*Myoviridae*	KY271398	33540	Unknown	[122]
Kpn112	*Myoviridae*	KJ021043	35560	Unknown	Chandekar et al.*
K64-1	*Myoviridae*	NC_027399	346602	Untreated water	[123]
KPV15	*Myoviridae*	KY000080	167034	Wastewater	[124]
KP27	*Myoviridae*	NC_020080	174413	Wastewater plant	[64]
KP34	*Podoviridae*	NC_013649	43809	Cesspool holding tank	[125]
vB_KpnP_KpV475	*Podoviridae*	NC_031025	42201	Clinical sample	[126]
vB_KpnP_KpV74	*Podoviridae*	KY385423	44094	Clinical sample	[126]
vB_KpnP_KpV48	*Podoviridae*	KX237514	44623	Clinical sample	[126]
KP32	*Podoviridae*	NC_013647	41119	Roadside ditch	[64]
P13	*Podoviridae*	Not Available	45976	Sewage	[127]
vB_KpnP_KpV41	*Podoviridae*	NC_028670	44203	Sewage	[126]
vB_KpnP_KpV71	*Podoviridae*	NC_031246	43267	Sewage	[126]
vB_KpnP_KpV766	*Podoviridae*	KX712071	41283	Sewage	[126]
vB_KpnP_KpV767	*Podoviridae*	KX712070	40395	Sewage	[126]
vB_KpnP_KpV763	*Podoviridae*	KX591654	40765	Sewage	[126]
K5-2	*Podoviridae*	KY389315	41116	Sewage	[128]
K5-4	*Podoviridae*	KY389316	40163	Sewage	[128]
KPO1K2	*Podoviridae*	Not Available	~42000	Sewage	[69]
Kpn5	*Podoviridae*	Not Available	~24000	Sewage	[81]
Kpn12	*Podoviridae*	Not Available	~24000	Sewage	[81]
Kpn13	*Podoviridae*	Not Available	~24000	Sewage	[81]
Kpn17	*Podoviridae*	Not Available	~24000	Sewage	[81]
Kpn22	*Podoviridae*	Not Available	~24000	Sewage	[81]
phiNK5	*Podoviridae*	Not Available	~29000	Sewage	[76]
Phage SS	*Podoviridae*	Not Available	Unknown	Sewage	[83]
Henu1	*Podoviridae*	MK203841.1	40352	Sewage	[129]
vB_KpnP_BIS33	*Podoviridae*	KY652725	41697	Sewage plant	[120]
vB_KpnP_IL33	*Podoviridae*	KY652724	41335	Sewage plant	[120]
vB_KpnP_PRA33	*Podoviridae*	KY652723	40605	Sewage plant	[120]
F19	*Podoviridae*	NC_023567	43766	Unknown	Chen et al.*
K11	*Podoviridae*	NC_011043	41181	Unknown	Savalia et al.*
Pylas	*Podoviridae*	MH899585	70408	Unknown	Powell et al.*
vB_KpnP_IME321	*Podoviridae*	MH587638	39906	Unknown	[130]
SH-Kp 152234	*Podoviridae*	KY450753	40578	Unknown	Zhi et al.*
KP8	*Podoviridae*	MG922974	73679	Unknown	Bokovaya et al.*
SH-Kp 152410	*Podoviridae*	MG835568	40945	Unknown	Xu et al.*
KP-Rio/2015	*Podoviridae*	KX856662	43557	Unknown	Meira et al.*
KN4-1	*Podoviridae*	LC413195	41219	Unknown	[131]
KN3-1	*Podoviridae*	LC413194	41059	Unknown	[131]
KN1-1	*Podoviridae*	LC413193	40236	Unknown	[131]
kpssk3	*Podoviridae*	MK134560	40539	Unknown	Shi et al.*
phiKpS2	*Podoviridae*	KX587949	44024	Unknown	[132]
myPSH1235	*Podoviridae*	MG972768	45135	Unknown	[79]
2044-307w	*Podoviridae*	MF285615	40048	Unknown	Zhao*
6 LV-2017	*Podoviridae*	KY271400	19260	Unknown	[122]
vB_KpnP_IME205	*Podoviridae*	KU183006	41310	Unknown	Bai et al.*
vB_Klp_5	*Podoviridae*	Not Available	Unknown	Unknown	[133]

Phage	Family	RefSeq/GenBank accession no.	Genome size (bp)	Source	Reference
vB_Klp_6	*Podoviridae*	Not Available	Unknown	Unknown	[133]
vB_KpnP_KpV289	*Podoviridae*	NC_028977	41054	Untreated sewage	[134]
NTUH-K2044	*Podoviridae*	NC_025418	43871	Untreated water	[135]
K5	*Podoviridae*	NC_028800	41698	Wastewater	Schneider et al.*
phiBO1E	*Podoviridae*	KM576124	43865	Wastewater	[68]
KPV811	*Podoviridae*	KY000081	42641	Wastewater	[124]
vB_Kp1	*Podoviridae*	NC_028688	40114	Wastewater plant	Alvez et al.*
vB_Kp2	*Podoviridae*	NC_028664	43963	Wastewater plant	Alvez et al.*
vB_KpnP_SU503	*Podoviridae*	NC_028816	43809	Wastewater plant	[136]
vB_KpnP_SU552A	*Podoviridae*	NC_028870	43595	Wastewater plant	[136]
AltoGao	*Podoviridae*	MF612071	43012	Wastewater plant	[137]
SopranoGao	*Podoviridae*	MF612073	61644	Wastewater plant	[137]
KLPN1	*Siphoviridae*	NC_028760	49037	Human caecum	[138]
KPP5665-2	*Siphoviridae*	MF695815	39241	Mastitis milk	[139]
1513	*Siphoviridae*	NC_028786	49462	Sewage	[75]
PKP126	*Siphoviridae*	NC_031053	50934	Sewage	[117]
Sushi	*Siphoviridae*	NC_028774	48754	Sewage	[140]
vB_KpnS_KpV522	*Siphoviridae*	KX237515	51099	Sewage	Komisarova et al.*
TSK1	*Siphoviridae*	MH688453	49861	Sewage	[90]
IME207	*Siphoviridae*	NC_031924	47564	Sewage	[141]
vB_KpnS_GH-K3	*Siphoviridae*	MH844531.1	49427	Sewage	[71, 142]
48ST307	*Siphoviridae*	KY271402	52338	Unknown	[122]
KPN N98	*Siphoviridae*	MG835858	59214	Unknown	Jeon et al.*
YMC16/01/N133_KPN_BP	*Siphoviridae*	MF476925	58387	Unknown	Jeon et al.*
YMC15/11/N53_KPN_BP	*Siphoviridae*	MF476924	59100	Unknown	Jeon et al.*
KPN N54	*Siphoviridae*	MF415413	59100	Unknown	Jeon et al.*
KPN N141	*Siphoviridae*	MF415412	49090	Unknown	Jeon et al.*
KPN U2874	*Siphoviridae*	MF415411	59087	Unknown	Jeon et al.*
KPN N137	*Siphoviridae*	MF415410	59100	Unknown	Jeon et al.*
Seifer	*Siphoviridae*	MH817999	58197	Unknown	Salazar et al.*
SH-Kp 160016	*Siphoviridae*	KY575286	49170	Unknown	Zhi et al.*
Sugarland	*Siphoviridae*	MG459987	111103	Unknown	Erickson et al.*
vB_Kpn_IME260	*Siphoviridae*	KX845404	123490	Unknown	Xing et al.*
NJR15	*Siphoviridae*	MH633487	49468	Unknown	Hao et al.*
NJS3	*Siphoviridae*	MH633486	49387	Unknown	Hao et al.*
NJS2	*Siphoviridae*	MH633485	50132	Unknown	Hao et al.*
TAH8	*Siphoviridae*	MH633484	49344	Unknown	Hao et al.*
phiKO2	*Siphoviridae*	NC_005857	51601	Unknown	[143]
NJS1	*Siphoviridae*	MH445453	49292	Unknown	Zhu et al.*
JY917	*Siphoviridae*	MG894052	37655	Unknown	Hao et al.*
vB_KpnS_IME279	*Siphoviridae*	MF614100	42518	Unknown	Zhao et al.*
1 LV-2017	*Siphoviridae*	KY271401	29880	Unknown	[122]
2 LV-2017	*Siphoviridae*	KY271396	44400	Unknown	[122]
2b LV-2017	*Siphoviridae*	KY271395	44279	Unknown	[122]
5 LV-2017	*Siphoviridae*	KY271399	47014	Unknown	[122]
vB_Kp3	*Siphoviridae*	KT367887	48493	Unknown	Alvez et al.*
phiKp-lyy15	*Siphoviridae*	Not Available	Unknown	Unknown	[144]
vB_Klp_1	*Siphoviridae*	Not Available	Unknown	Unknown	[133]
vB_Klp_3	*Siphoviridae*	Not Available	Unknown	Unknown	[133]
vB_Klp_4	*Siphoviridae*	Not Available	Unknown	Unknown	[133]
KOX1	*Siphoviridae*	KY780482	50526	Wastewater	[145]
phage Z	*Siphoviridae*	Not Available	Unknown	Wastewater	[63]
KP36	*Siphoviridae*	NC_029099	49818	Wastewater plant	[64]
MezzoGao	*Siphoviridae*	MF612072	49807	Wastewater plant	[137]
GH-K1	Unknown	Not Available	Unknown	Sewage	[71]
PBKP05	Unknown	Not Available	30240	Unknown	[146]
Kpp95	Unknown	Not Available	~175000	Unknown	[147]

*No paper associated with the RefSeq/GenBank record(s).


Morganella phage vB_MmoP_MP2 (NC_031115) [39,616 nt]
Morganella phage MmP1 (NC_011085) [38,457 nt]
Erwinia phage vB_EamP-L1 (NC_019510) [39,282 nt]
Includes Caudovirales, Podoviridae, Autographiinivirinae, Tesetptimavirus
Yersinia phage vB_YenP_AP10 (NC_028655) [39,235 nt]
Enterobacter phage phiEap-1 (NC_028772) [39,133 nt]
Klebsiella phage vB_KpnP_IME205 (KU183006) [41,310 nt]
Klebsiella phage kpssk3 (MK134560) [40,539 nt]
Klebsiella virus Kp1 (NC_028688) [40,114 nt]
Klebsiella phage SH-Kp 152410 (MG835568) [40,945 nt]
Klebsiella phage SH-Kp 152234 (KY450753) [40,578 nt]
Klebsiella phage KN4-1 (LC413195) [41,219 nt]
Klebsiella phage vB_KpnP_IME321 (MH587638) [39,906 nt]
Klebsiella phage KN1-1 (LC413193) [40,236 nt]
Klebsiella phage 2044-307w (MF285615) [40,048 nt]
Klebsiella phage K5-4 (KY389316) [40,163 nt]
Klebsiella phage K5-2 (KY389315) [41,116 nt]
Klebsiella phage Henu1 (MK203841) [40,352 nt]
Klebsiella phage KN3-1 (LC413194) [41,059 nt]
Klebsiella virus K11 (NC_011043) [41,181 nt]
Klebsiella phage vB_KpnP_KpV763 (KX591654) [40,765 nt]
Klebsiella phage vB_KpnP_KpV767 (KX712070) [40,395 nt]
Klebsiella virus K5 (NC_028800) [41,698 nt]
Klebsiella virus KP32 (NC_013647) [41,119 nt]
Escherichia virus K30 (NC_015719) [40,940 nt]
Klebsiella virus KpV289 (NC_028977) [41,054 nt]
Klebsiella phage vB_KpnP_KpV766 (KX712071) [41,283 nt]
Klebsiella vB_KpnP_PRA33 (KY652723) [40,605 nt]
Klebsiella phage vB_KpnP_BIS33 (KY652725) [41,697 nt]
Klebsiella phage vB_KpnP_IL33 (KY652724) [41,335 nt]
Caudovirales, Podoviridae, Autographiinivirinae, Przondovirus
Includes Caudovirales, Podoviridae, Autographiinivirinae, Phimunavirus
Vibrio phage VP93 (NC_012662) [43,931 nt]
Pantoea virus LIMElight (NC_019454) [44,546 nt]
Caudovirales, Podoviridae, Autographiinivirinae, Phikmvvirus
Proteus phage PM 75 (NC_027363) [41,480 nt]
Proteus phage PM16 (NC_027342) [41,268 nt]
Enterobacter phage phiKDA1 (JQ267518) [43,804 nt]
Klebsiella phage KPV811 (KY000081) [42,641 nt]
Klebsiella phage AltoGao (MF612071) [43,012 nt]
Klebsiella phage vB_KpnP_KpV74 (KY385423) [44,094 nt]
Klebsiella virus F19 (NC_023567) [43,766 nt]
Klebsiella phage phiBO1E (KM576124) [43,865 nt]
Klebsiella virus Kp2 (NC_028664) [43,963 nt]
Klebsiella virus SU503 (NC_028816) [43,809 nt]
Klebsiella virus KP34 (NC_013649) [43,809 nt]
Klebsiella virus KpV41 (NC_028670) [44,203 nt]
Klebsiella virus K244 (NC_025418) [43,871 nt]
Klebsiella virus KpV71 (NC_031246) [43,267 nt]
Klebsiella virus KpV475 (NC_031025) [42,201 nt]
Klebsiella phage myPSH1235 (MG972768) [45,135 nt]
Klebsiella phage vB_KpnP_KpV48 (KX237514) [44,623 nt]
Klebsiella phage KP-Rio/2015 (KX856662) [43,557 nt]
Klebsiella phage phiKpS2 (KX587949) [44,024 nt]
Klebsiella virus SU552A (NC_028870) [43,595 nt]
Caudovirales, Podoviridae, Autographiinivirinae, Drulisvirus
Ruegeria phage DSS3-P1 (NC_025428) [59,601 nt]
Loktanella phage pCB2051-A (NC_020853) [56,958 nt]
Xylella virus Salvo (KF626668) [55,601 nt]
Xylella virus Sano (KF626665) [56,147 nt]
Caudovirales, Siphoviridae, Sanovirus
Achromobacter phage phiAxp-2 (NC_029106) [62,220 nt]
Burkholderia virus AH2 (NC_018283) [58,065 nt]
Caudovirales, Siphoviridae, Ahduovirus
Burkholderia virus BcepNazgul (NC_005091) [57,455 nt]
Caudovirales, Myoviridae, Nazgulvirus
Proteus phage pPM_01 (NC_028812) [58,546 nt]
Providencia phage Redjac (NC_018832) [58,104 nt]
Includes Caudovirales, Siphoviridae, Chivirus
Klebsiella phage Seifer (MH817999) [58,197 nt]
Klebsiella phage KPN N98 (MG835858) [59,214 nt]
Klebsiella phage KPN U2874 (MF415411) [59,087 nt]
Klebsiella phage YMC15/11/N53_KPN_BP (MF476924) [59,100 nt]
Klebsiella phage KPN N54 (MF415413) [59,100 nt]
Klebsiella phage KPN N137 (MF415410) [59,100 nt]
Shewanella sp. phage 1/44 (NC_025463) [49,640 nt]
Pseudoalteromonas phage HS6 (KF302035) [35,330 nt]
Salmonella phage 9NA (NC_025443) [52,869 nt]
Rhizobium phage vB_RglS_P106B (NC_023566) [56,024 nt]
Agrobacterium phage 7-7-1 (NC_019519) [69,391 nt]
Pectobacterium phage ZF40 (NC_019522) [48,454 nt]
Iodobacteriophage phiPLPE (NC_011142) [47,453 nt]
Vibrio phage vB_VchM-138 (NC_019518) [44,485 nt]
Vibrio phage CP-T1 (NC_019457) [44,492 nt]
Vibrio phage 24 (KJ572844) [44,395 nt]
Edwardsiella phage PEi21 (NC_021342) [43,378 nt]
Edwardsiella phage MSW-3 (NC_020082) [42,746 nt]
Klebsiella phage 1611E-K2-1 (MG197810) [47,797 nt]
Klebsiella virus KpV52 (KX237516) [47,405 nt]
Klebsiella phage vB_KpnM_KpV79 (MF663761) [47,760 nt]
Klebsiella virus JD001 (NC_020204) [48,814 nt]
Klebsiella phage Kpn112 (KJ021043) [35,560 nt]
Caudovirales, Myoviridae, Jedunavirus
Salmonella virus SEN34 (NC_028699) [40,740 nt]
Caudovirales, Myoviridae, Brunovirus
Klebsiella phage IME207 (NC_031924) [47,564 nt]
Klebsiella phage 6 LV-2017 (KY271400) [19,260 nt]
Klebsiella phage 2 LV-2017 (KY271396) [44,400 nt]
Klebsiella phage 2b LV-2017 (KY271395) [44,279 nt]
Cronobacter phage ENT47670 (NC_019927) [47,611 nt]
Cronobacter phage ES2 (JF314845) [22,162 nt]
Salmonella phage SPN3UB (NC_019545) [47,355 nt]
Enterobacteria phage ES18 (NC_006949) [46,900 nt]
Salmonella phage vB_SosS_Oslo (NC_018279) [49,116 nt]
Yersinia phage PY54 (NC_005069) [46,339 nt]
Escherichia virus N15 (NC_001901) [46,375 nt]
Caudovirales, Siphoviridae, Ravinvirus
Klebsiella phage 48ST307 (KY271402) [52,338 nt]
Klebsiella phage phiKO2 (NC_005857) [51,601 nt]
Includes Caudovirales, Siphoviridae, Rogunavirus
Cronobacter virus Esp2949-1 (NC_019509) [49,116 nt]
Includes Caudovirales, Siphoviridae, Tunavirinae (Tunavirus, Tlsvirus)
Enterobacteria phage vB_EcoS_NBD2 (NC_031050) [51,802 nt]
Klebsiella virus PKP126 (NC_031053) [50,934 nt]
Klebsiella phage KOX1 (KY780482) [50,526 nt]
Klebsiella phage vB_KpnS_KpV522 (KX237515) [51,099 nt]
Klebsiella virus KLPN1 (NC_028760) [49,037 nt]
Klebsiella phage TSK1 (MH688453) [49,861 nt]
Klebsiella virus KP36 (NC_029099) [49,797 nt]
Klebsiella phage SH-Kp 160016 (KY575286) [49,170 nt]
Klebsiella phage vB_KpnS_GH-K3 (MH844531) [49,427 nt]
Klebsiella virus 1513 (NC_028786) [49,462 nt]
Klebsiella phage JY917 (MG894052) [37,655 nt]
Klebsiella virus Sushi (NC_028774) [48,754 nt]
Klebsiella phage NJS1 (MH445453) [49,292 nt]
Klebsiella phage MezzoGao (MF612072) [49,807 nt]
Klebsiella phage KPN N141 (MF415412) [49,090 nt]
Klebsiella phage NJS2 (MH633485) [50,132 nt]
Klebsiella phage NJR15 (MH633487) [49,468 nt]
Klebsiella phage NJS3 (MH633486) [49,387 nt]
Klebsiella phage TAH8 (MH633484) [49,344 nt]
Caudovirales, Siphoviridae, Tunavirinae, Webervirus
Tree scale: 0.1

Figure 1. Phylogenetic placement of dsDNA *Klebsiella* phages within the order *Caudovirales*. Placement of 109 genomes (**Table 1**) within ViPTree version 1.9 [148] was checked on 6 August 2019. Those sequences (*n* = 84) that clustered together in groups of three or more were analysed with their nearest phylogenetic relatives using ViPTreeGen v1.1.2 (--ncpus 8 --method 'bioinj') and a non-redundant set of genomes (fasta file of input sequences and newick-format file available in **Supplementary Material**) to generate the tree shown (annotated using https://itol.embl.de and Adobe Illustrator). Taxonomy of phages was checked via https://talk.ictvonline.org/taxonomy/ (release 2018b); accepted species names are written in italics. A phylogenetic tree showing the placement of the remaining 25 *Klebsiella* genomes within ViPTree version 1.9 is available in **Supplementary Material**.

APPENDIX 2

Poster presented at European Congress of Clinical Microbiology and Infectious Diseases, Amsterdam (April 2016).

Evaluation of different media for introduction of a CPE screening program at a UK hospital.

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1. Background

Carbapenems are effectively last-line antibiotics for the treatment of infections due to multidrug-resistant *Enterobacteriaceae*. During the last decade, carbapenem resistance has been increasingly reported and carbapenemase-producing *Enterobacteriaceae* (CPE) is emerging as a growing challenge in healthcare settings (Grundmann *et al.*, 2010). The resistant organisms produce different types of β-lactamases capable of hydrolysing carbapenems. These enzymes include the class A carbapenemases (KPC and GES types), the class B or metallo-β-lactamases (MBLs) (VIM, IPM, and NDM types), and the class D oxacillinases (e.g., OXA-48-like enzymes) ,(Livermore, 2012). It is also noted that the carbapenemase genes harboured by CPE are mostly transposon- and/or integron-encoded determinants that can easily spread to other *Enterobacteriaceae* strains and species, which means cross infection is of great concern within the NHS Hospitals (Johnson and Woodford, 2013). There is, therefore, a need to implement adequate preventive measures, including active surveillance, in order to contain the spread of these pathogens. Since gastrointestinal carriers of CPE are thought to be the reservoir of cross-transmission in health care settings, surveillance is necessary. Public Health England (PHE) recommend risk factor-based screening on admission to all acute hospital Trusts. In order to manage the laboratory demand for a large screening program, a trial of different media and in-house testing protocols was performed. The chosen screening method was then introduced for the first 6 months of patient screening.

2. Material/methods

236 CPE screening rectal swabs (including positive controls confirmed by Polymerase Chain Reaction (PCR)) were emulsified in 0.5ml saline and plated onto 4 chromogenic media – Brilliance CRE (Oxoid), ChromID carba (Biomerieux), CXmSCARBA-1, CXmSCARBA-2 (E&O), and also onto MacConkey agar with ertapenem and meropenem discs (current method). Plates were incubated for 18 hrs and interpreted according to manufacturer's instructions (Figure 2). Presumptive colonies were identified by MALDI-TOF, and disc susceptibility with the inclusion of MAST CAT-ID discs (MAST group Ltd, UK) performed and interpreted using the EUCAST (European Committee on Antimicrobial Susceptibility Testing) guideline (EUCAST,2015). Xpert Carb-R-PCR (Xpert® Carba-R 1,2014) was performed on 29 ertapenem and / or meropenem resistant isolates, and the isolate sent to the reference laboratory for confirmation (figure1). A dilution series (to 10^{-8} from a 0.5 MacFarland suspension) following the method from Wilkinson *et al.* 2012 was performed using 20 known positive isolates with 5 different mechanisms and each dilution was plated onto the 4 selective media.

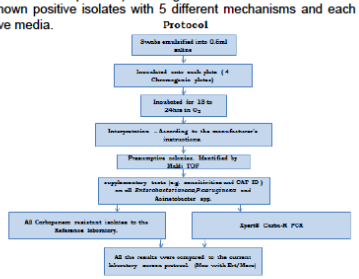

Figure 1: flowchart showing the method

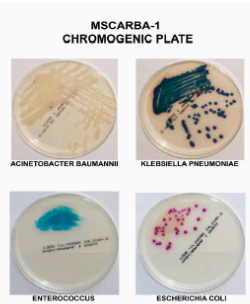

Figure 2:

	Mac with Mero/Ert	BCRE	Chrom ID Carba	CXmSCARBA-1	CXmSCARBA-2
NEG	213	204	204	206	206
POS (CPO)	23	32	32	30	30
Non-CPO	19	28	19	18	17

Table 1: Number of positive and negative CPE's isolated using different chromagenic media

Media	NDM	OXA48	KPC	VIM	IMP
BCRE	10^{-6}	10^{-6}	10^{-7}	10^{-6}	10^{-6}
Chrom ID CARBA	10^{-6}	0.5 Mac	10^{-5}	10^{-6}	10^{-6}
CXmSCARBA-1	10^{-6}	10^{-6}	10^{-7}	10^{-7}	10^{-6}
CXmSCARBA-2	10^{-6}	10^{-5}	10^{-5}	10^{-5}	10^{-5}

Table 2: Limit of detection of the chromagenic media

3. Results

49 different isolates were identified and sent to the reference lab for confirmation, yielding 33 confirmed CPEs (32 NDM, 1 OXA-48), and 19 false positive Enterobacteriacae (a combination of ESBL and AmpC hyperproducing strains with porin loss). None of the media detected all of the strains - Brilliance CRE and ChromID carba each missed 1 NDM, and CXmSCARBA-1 and CXmSCARBA-2 each missed 3 NDM isolates; MacConkey agar missed 10. The false positive detection rate was as follows: Brilliance CRE 28, ChromID Carba 19, CXmSCARBA-1 18, CXmSCARBA-2 17, MacConkey 19 (Table 1).

Out of the validation of four plates, CXmSCARBA-1 was selected as our screening method as it showed 91% specificity and sensitivity for the detection of carbapenames. It also excelled in displaying the colony morphology of all carbapenamse producing organisms. The study also concluded the limit of detection of CXmSCARBA-1(10^{-6}) was lower than the Brilliance CRE, Chrome ID CARBA and CXmSCARBA-2. Genotypic assay of Xpert® Carba-R PCR showed 100% specificity and sensitivity for the detection and differentiation of the *bla*KPC,*bla*NDM,*bla*VIM,*bla*OXA-48 and *bla* IMP-1 genes associated with carbapenem resistance. The MAST CAT-ID discs showed 100% sensitivity and 85% specificity and proved to be a good indicator for the presence of carbapemase enzyme in *Enterobacteriaceae* but its low specificity was due to the detection of false positives (i.e. isolates with porin loss).

In the dilution experiments, CXmSCARBA-1 was most sensitive (10^{-6} to 10^{-7} for all isolates). ChromID Carba was unable to detect the OXA48 isolates even from an undiluted 0.5 MacFarland suspension (Table 2).

CXmSCARBA-1 was the agar selected for the routine screening program and over the first 6 months 19,506 rectal screening swabs were received. 790 (4%) grew Gram negative colonies which required further work, and 283 were sent to the reference laboratory. 59 isolates were confirmed carbapenemase producers (giving a prevalence of 0.3%), including 51 confirmed as CPEs (predominantly NDM and OXA48). Xpert Carb-R-PCR gave 100% concordance with the reference lab PCR for the genes contained in the PCR, though some isolates were identified which were not included in the PCR (subsequently found to be GES-5 positive).

4. Conclusions

The sensitivity of all chromogenic agar tested was higher than using a reference method (MacConkey) agar. The Xpert PCR performed well for in-house confirmation, allowing earlier and more accurate information to be passed to infection control and clinicians, but was restricted to 5 genes. Laboratory detection and confirmation remains challenging as there is phenotypic overlap with ESBL/AMPC hyper production with permeability which led to a delay in the recognition of the GES-5 producing isolates. The protocol chosen was able to support a widespread screening program.

*PS and JD contributed equally to this work.

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Xpert® Carba-R 1 301-2437, Rev. B June 2014.

Disclosures: Media provided free of charge from the following companies; BioMerieux, Oxoid, E&O
Subsequently received part funding from ChromAgar to attend ECCMID.

Imperial College Healthcare NHS Trust

APPENDIX 3

Successful IBMS funding application.

IBMS Research Grant Application Form

PLEASE COMPLETE IN BLOCK CAPITALS

SECTION ONE – PERSONAL DETAILS

First Names:	Preetha		Surname:	Shibu
Title:	Mrs		Suffix:	
Gender:	Male ☐	Female ☒	Date of Birth:	03/08/1966

IBMS Membership Number	00378030	Membership Grade	Fellow

Is this for an Overseas Research Grant only?	☐ Yes	☒ No

Please indicate which address should be used for correspondence:			
Home Address	☒	Employment Address	☐

Home Address		Current Employment/Educational Establishment Address	
Address Line 1	10, Hitherhook Hill	Job Title	Senior Biomedical Scientist
Address Line 2		Company/Hospital	Imperial College NHS Trust Healthcare
Address Line 3		Address Line 1	Fulham Palace Road
Town	Binfield	Town	Hammersmith
County	Berkshire	County	London«Firmcounty_descr»
Postcode	RG42 4QQ	Postcode	W6 8RF
Country	United Kingdom	Country	United Kingdom
Telephone Number	07588 615 309	Telephone Number	020 3311 17814
Fax		Fax	
Email	preethashibu@hotmail.com	Email	Preetha.Shibu@imperial.nhs.uk

Previous Employment and Professional History		
Dates (to-from)	Company/Organisation	Post Held (Grade & Title)
July 2010 – Present	Imperial College Healthcare NHS Trust, Charing Cross Hospital	Senior Biomedical Scientist in Microbiology, Band 7
2001 – July 2010	Hammersmith Hospital NHS Trust	Senior Biomedical Scientist in Microbiology, Band 7 (Promoted to Band 7 in 2005)
1990 – 2001	Chelsea and Westminster Hospital NHS Trust	Biomedical Scientist in Microbiology, Band 6 (Started as a trainee band 5 – (promoted to band 6 in 1992)
1988 – 1990	Royal Postgraduate Medical School, Hammersmith Hospital	Trainee Laboratory Technician in Biochemistry / Endoscopy Unit

Previous Qualifications		
Dates	Organisation	Qualification
Sept 2014 – Present	University of Westminster	Professional Doctorate in Health Sciences (2nd year)
Oct 2006 – Dec 2008	University of Ulster	Biomedical Sciences (MSc with distinction and offer for PhD)
Sept 1990 – July 1992	University of Westminster	BTEC Higher National Certificate in Medical Sciences (Merit)

Publications:
Recent and/or Relevant Publications only

Preetha Shibu*, Jyothsna Dronavalli*, Monica Rebec, Sweenie Goonesekera, Nigel Bartholomew, Siddharth Mookerjee, Jon Otter, Hugo Donaldson, Frances Davies.Evaluation of different media for introduction of a CPE screening program at a UK hospital.European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) ,Amsterdam, Netherlands 9-12 April 2016.

SECTION TWO – PROJECT DETAILS

Project Summary			
Project Title	Isolation of lytic bacteriophages active against antibiotic-resistant *Klebsiella pneumoniae*		
Please attach separate sheet(s) clearly detailing the information required below. For items 2-4 inclusive do not exceed 1000 words in length. (Please note that not all members of the Selection Committee can be fully conversant with your subject area, and abbreviations/jargon should be avoided)			
	1. Title of project (or subject of study). 2. Purpose and background of proposed study. It is likely that this will describe the existing problem and how the project seeks to contribute to its solution. This may take the part of an original hypothesis. 3. Outline of methodology, subject and materials. 4. The major benefits of a successful outcome to the project in terms of the advancement in the practice of laboratory science and/or an improvement in patient care and outcome. 5. Key references.		
Ethical Approval	Is Ethical Approval Required? *	Yes ☒	No ☐
	Has Ethical Approval been obtained?	*Yes* ☒	*No* ☐
	***Please note successful bids for grants will only be awarded once ethical approval has been confirmed.**		
Proposed Start Date	1 September 2016	Proposed Duration	12 months«Firmcounty_descr»
Location of Project, if different from employment address			

Name and address of joint applicant or, in the case of a student applicant, the project supervisor:				
First Names:	Lesley		Surname:	Hoyles
Title:	Dr		Suffix:	CBiol
Gender:	Male ☐	Female ☒	Date of Birth:	15/02/1974
Address Line 1	Computational and Systems Medicine, Department of Surgery and Cancer			

Address Line 2	Imperial College London, Sir Alexander Fleming Building, Exhibition Road
Town	London
County	-
Postcode	SW7 2AZ
Country	United Kingdom
Telephone Number	07905 527 467
Email	lesley.hoyles11@imperial.ac.uk

Project Support		
Please specify precisely what the grant is required for. Sums of money indicated as miscellaneous are not admissible. If support is needed to cover attendance at meetings, specific details of these meetings must be given, together with explanation of why attendance is essential to the project. Costs should be shown in pounds sterling and should include VAT (or other local taxes) where appropriate. Append a separate sheet if necessary.		
		Totals (£)
Consumables	Syringe filter Minisart sterile PES 28mm 0.45µm pore size (box of 50), x2 Syringe 5mL disposable plastic x 2mL (box of 50), x2 Eppendorf tubes (bags of 1000), x1 Filter tips (10 ul, 10 boxes of 96 tips), x1 Filter tips (200 ul, 10 boxes of 96 tips), x3 Filter tips (1000 ul, 10 boxes of 96 tips), x3 Sodium chloride (1 kg), x1 Bijou bottles, plastic (box of 700), x2 Universal bottles, plastic (box of 500), x2 Nutrient agar, 500 g, x1 Nutrient broth, 500 g, x1 GeneJET Viral DNA/RNA Purification Kit, x1 Deoxyribonuclease I from bovine pancreas, x1 Ribonuclease A from bovine pancreas, salt-free lyophilized powder, x1 Polyethylene glycol 8000 (PEG 8000), x1 DNA gel loading buffer, 10x, 6×500 ml, x1 1 kb Ready Ladder™, pre-mixed with loading dye, sufficient for 100 lanes, x2 100 bp Ready Ladder™, pre-mixed with loading dye, sufficient for 50 lanes, x1 Petri dishes with 3 vents, x5 Sequencing of phage and bacterial genomes (via microbesng, academic rate), x25	200 413 15 70 210 210 24 120 100 70 70 161 19 42 44 37 200 125 138 2125
Equipment	No equipment is requested.	
Other expenses	No other expenses are requested.	
Grand Total		4023
Other Support *(Please indicate if you have access to alternative funding arrangements (for example employer or HEI if you are postgraduate student).*		

I am a self-funded student studying for a Professional Doctorate. I do not have access to alternative funding arrangements, and wish to use any monies awarded from this application to contribute towards my postgraduate studies. Bioinformatics and computing support for my study are being provided by my supervisor, Dr Lesley Hoyles, at no cost.	

SECTION THREE – PAYMENT DETAILS

Research Grants to successful applicants will be paid directly into a nominated bank account by BACS payment method. Please provide bank details below (personal bank accounts are not accepted):	
Name of Bank	
Bank Address	
Sort Code	
Account Number	
Account Name	
BIC	
IBAN	
Special Instructions	If the project is funded, Manfred Almedia (Laboratory Manager, Charing Cross Hospital E-mail address : Manfred. Almedia@imperial.nhs.uk) should be contacted and he will provide the bank details.

REGULATIONS AND CONDITIONS	
RESEARCH GRANTS IBMS Research Grants are awarded annually to members of the Institute to support original investigations and other suitable research work. The grants awarded are usually between £500 and £4500 **OVERSEAS RESEARCH GRANTS** A single grant of up to £2500 to support original investigations may be awarded annually to a member of the Institute employed outside the United Kingdom or the Republic of Ireland. (Note; Such members may apply for either category of grant, but must indicate only one category on page 1 of the application form.)	**CONDITIONS** 1. Applicants must be fully paid up members of the Institute in the correct class of membership for their qualifications and experience. 2. Each application shall be made on the official form and shall be submitted to the Institute by 30 April in the year of application 3. The award of a grant in one year does not preclude a subsequent application provided criteria for each award are met. 4. Grants may be awarded to assist members with a practical project integral to a course of study leading to a post-graduate education award. 5. The results of the project will be written up and formally submitted in the first instance to the British Journal of Biomedical Science. Guidance will be available from the Editor. Should the Editor decline to publish, the researcher may submit elsewhere, but the Institute must be acknowledged as the funding source, and a copy of the publication lodged with the Institute. 6. Within 12 months from receipt of the grant a statement of expenditure, a brief report on the research project and a statement about any publications arising from the research must be submitted to the Institute.

DECLARATION

I declare that that I have read the Regulations and Conditions and that the information given is complete and correct.

I shall be actively engaged as a principal in this project.

I agree to this application being referred to the regional member of Council (for UK and Republic of Ireland applicants) for informal enquiry should the Selection Committee deem it necessary.

I declare that within a year of the date of the award I shall submit a schedule of the items on which the grant was spent. I shall also submit a short report on the research project and a statement saying whether or not any publications have of will be made as a result of the research.

Sign: *Preetha Shibu*

Print Name:Preetha Shibu Date:17/04/2016

RETURN ADDRESS

Postal: Institute of Biomedical Science, 12 Coldbath Square, London, EC1R 5HL

Email: education@ibms.org

1. Isolation of lytic bacteriophages active against antibiotic-resistant *Klebsiella pneumoniae*

2. Purpose and background of proposed study

The global spread of carbapenemase-producing *Enterobacteriaceae* – especially *Klebsiella pneumoniae* – has reached a concerning level and is a critical medical and public health issue [1]. The number of confirmed carbapenemase-producing organism (CPO) isolates referred from UK laboratories has increased from 5 per year (2003–2007) to as many as 1,000 (2013) [2]. CPOs are extremely multidrug-resistant (carbapenems, penicillins and cephalosporins) and often carry genes that confer resistance to other antimicrobials (aminoglycosides and quinolones) leading to limited therapeutic options [3]. *K. pneumoniae*-associated infections (urinary tract, blood, pneumonia, burn-wound) occur mostly in adults in high-dependency units. Capsular type K1 carbapenem-resistant *K. pneumoniae* strains causing community-acquired pyogenic liver abscess are on the increase globally [4]. Information on capsular types associated with other infections is lacking; Public Health England's screening programme can only identify five of the 78 known capsular types of *K. pneumoniae* [5]. However, capsular type and other strain characteristics can be determined using whole-genome sequence data (http://bigsdb.web.pasteur.fr).

Controlling the spread of *K. pneumoniae* is difficult because of the diversity of carbapenem-hydrolysing enzymes and because resistance genes spread among different bacteria [2]. *K. pneumoniae* inhabits the gastrointestinal tract and decolonisation therapy only reduces the number of bacteria without elimination [2]. Most people colonised with *K. pneumoniae* have no further complications but, in immunocompromised patients, colonisation can lead to endogenous infection, which can be a cause of cross-infection [2]. Patients with invasive CPO infections are twice as likely to die as those infected with antibiotic-sensitive strains [6].

Bacteriophage therapy has been suggested as an alternative or adjunct treatment option in CPO-associated infections [7]. Bacteriophages are ubiquitous viruses of bacteria, found in water sources, sewage and the intestines of animals [8]. Lytic bacteriophages are recommended for phage therapy because they kill bacteria, infecting only the target bacterium and not affecting the microbiota or human cells. In bacteriophage therapy, lytic bacteriophages multiply in their host bacterium at the site of the infection until the bacteria are killed, then the phages are excreted from the body in urine and faeces [9]. Lytic bacteriophages active against *K. pneumoniae* have shown good therapeutic potential in mice [10–13]. No lytic bacteriophages active against carbapenemase-resistant *K. pneumoniae* have been isolated and characterised. Strains

included in bacteriophage studies are not well characterized, making it impossible to know which bacteriophages should be used to treat *K. pneumoniae* infections in a clinical setting. In addition, the genomes of bacteriophages included in these studies are not well-characterised, limiting the clinical applications of bacteriophage gene products (such as holins and lysins) as adjuncts or alternatives to antibiotic therapy.

It is hypothesised that bacteriophages and their gene products can be used to treat *K. pneumoniae*-associated infections *in vitro*, and ultimately used *in vivo* and in decolonization therapy to reduce transmission of *K. pneumoniae* from the gut of patients upon admission to hospital and hands of hospital personnel. This project aims to (i) isolate, from sewage samples, lytic bacteriophages active against a collection of 13 well-characterized antibiotic-resistant *K. pneumoniae* strains isolated in west London hospitals since 2010; fully characterise (genomically and phenotypically) the bacteriophages and their host bacteria; screen the bacteriophages against a panel of 200 randomly selected *K. pneumoniae* clinical isolates to determine their host ranges; and use the bacteriophages singly and in phage cocktails to determine their *in vitro* effectiveness at killing *K. pneumoniae*.

3. Methods

Permission has been granted to collect sewage samples from the Mogden and Ascot Sewage Treatment Works (Thames Water). These samples will be centrifuged and filter-sterilized to remove debris and bacteria, leaving only bacteriophages [8]. The following applies to each of the 13 antibiotic-resistant isolates to be used in the study. To 1 ml of sterile 10x concentrated nutrient broth, 9 ml of filtered sewage will be added. The sample will be inoculated with 200 microlitres of an overnight culture of *K. pneumoniae*, and incubated overnight at 37 °C. Cultures will then be centrifuged and the supernatants used in spot assays using the double-agar method [8] to determine whether bacteriophages have been isolated. Bacteriophage-positive samples will be processed [10] so that bacteriophages can be propagated to purity; their DNA will then be extracted [10]. Molecular fingerprints will be generated for all bacteriophages to identify those that are unique [14]. Unique bacteriophages will be characterized phenotypically [14], and their whole-genome sequences (and those of their host bacteria) determined (microbesNG; http://microbesng.uk). Genomes will be annotated [10], and bacteriophage gene products with potential clinical applications will be identified for future work. Lytic activity of bacteriophages will be tested against all the antibiotic-resistant isolates and 200 clinical isolates. *In vitro* killing activity of bacteriophages will be tested against strains singly and in bacteriophages cocktails [15] to identify bacteriophages suitable for future *in vivo* studies.

4. Major benefits of a successful outcome to the project

One of the major problems facing the Imperial College Healthcare Trust (ICHT) is the rapid emergence of antibiotic-resistant *K. pneumoniae* (CPO) in high-dependency units. Presence of antibiotic-resistant *K. pneumoniae* in patients is due to previous antibiotic therapy and transmission of organisms from the guts of patients and hands of hospital personnel. Currently no decolonisation therapy is available for gut carriage of CPOs: antibiotic therapy leads to eradication of the normal gut microbiota, which can result in the emergence of opportunistic pathogens and development of potentially life-threatening conditions such as *Clostridium difficile*-associated diarrhoea. Bacteriophages isolated in this study will ultimately be used in a topical form to suppress or eradicate gut carriage of CPOs once patients have screened positive, thereby preventing infection with these organisms which in turn will help to control CPO-associated infections in hospitals and provide better patient care and management. This study will result in at least one publication. Outputs of studies will be offered for oral/poster presentations at national and international conferences. The work will be presented internally to staff at Charing Cross Hospital, to disseminate the findings of the work to clinicians, technicians and researchers working in the field.

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