VIRULENCE POTENTIAL OF *ENTEROBACTERIACEAE* ISOLATED FROM NEONATAL ENTERAL FEEDING TUBES

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STATEMENT

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ABSTRACT

In recent years, there has been a rise in the incidence of neonatal infections due to *Enterobacteriaceae* including *Escherichia coli*, *Enterobacter*, *Klebsiella* and *Serratia* spp. These are major causative agents in neonatal intensive care unit (NICU) infections. Neonates, especially those born with low birth weight (< 2000g), are fed via a nasogastric tube. Despite recent concerns over the microbiological safety of infant feeds, there has been no consideration that the nasogastric enteral feeding tube may act as a site for bacterial colonisation and act as a locus for infection. Therefore bacterial analysis of used feeding tubes is of importance with regard to identifying risk factors during neonatal enteral feeding.

The aims of this study were to determine whether neonatal nasogastric enteral feeding tubes are colonised by opportunistic pathogens in the *Enterobacteriaceae*, and whether their presence was influenced by the feeding regime.

In this research a collection 224 *Enterobacteriaceae* strains previously isolated from the enteral feeding tubes of neonates on intensive care units have been analysed. This study describes the use of DNA finger printing, via pulsed-field gel electrophoresis (PFGE), to determine if the same strains were isolated on different occasions from the NICUs. Therefore indicating whether certain strains have colonised the NICUs leading to increased exposure and risk to the neonates.

The second project aim compared the virulence potential of *Enterobacteriaceae*, many of which were associated with feeding tubes. Twenty strains were chosen that represented species from the major genera isolated; *E. coli*, *Serratia*, *Klebsiella* and *Enterobacter* spp. Virulence to mammalian cells has been assessed using attachment and invasion studies of human colonic carcinoma epithelial cells (Caco-2), rat blood brain barrier cells (rBECE4) and human brain microvascular endothelial cells (HBMEC) tissue culture cells. Macrophage survival was studied using the (U937) cell line of human monocyte cells.

The analysis has covered attachment and invasion of human intestinal cells, survival in macrophages, and invasion of rat and human brain cells. The results show that certain strains of *E. coli* k1 isolated from neonatal feeding tubes are able to persist in macrophages and hence be dispersed and potentially cause systemic infections.

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DEDICATION

THIS THESIS IS DEDICATED TO, MY MUM AND DAD

MY WIFE, DAUGHTERS AND SON

ALL LOVED ONES

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LIST OF ABBREVIATIONS

AA	Aggregative adherence
bp	Base pairs
Caco-2	Human colonic carcinoma epithelial cells
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CLB	Cell lysis buffer
CNS	Central nervous system
CSB	Cell Suspension Buffer
CV	Crystal violet
DA	Diffuse Adherence
DAEC	Diffuse Adherence E. coli
DFI	Druggan Forsythe Iversen medium
dH2O	Distilled and sterilised water
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
dNTPs	Deoxyribonucleotide Triphosphate
EAEC	Enteroaggregative *E. coli*
EDTA	Ethylenediamine tetra-acetic acid
EPEC	Enteropathogenic *E. coli*
ESBL	Extended spectrum β-lactamases
ExPEC	Extraintestinal pathogenic *E. coli*
HBMEC	Human brain microvascular endothelial cells
HCL	Hydrochloric acid
HIV	Human Immunodeficiency Virus
ICU	Intensive care units
IF	Infant formula
kbp	Kilo base pair
LA	Localized Adherence
LB	Luria-Bertani
LBA	Luria-Bertani Agar

LPS	Lipopolysaccharide
MDGs	Millennium Development Goals
MHA	Mueller-Hinton Agar
MLST	Multi-locus sequence typing
MOI	Multiplicity of infection
NICU	Neonatal intensive care unit
NNIS	National nosocomial infections surveillance
OD	Optical density
OMP	Outer membrane protein
OMPs	Outer membrane proteins
P.S	Product size
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
PIF	Powdered infant formula
PIF	Powdered infant formula
PMA	Phorbol 12-myristate 13-acetate
R	Resistance
rBCEC4	Rat Blood Brain Barrier cells
rpm	Revolutions per minute
S	Sensitive
SC	Similarity coefficient
SDS	Sodium dodecyl sulphate
Spp.	Species
STs	Sequence types
TAE	Tris/acetate/EDTA
TBE	Tris base/ Boric acid / EDTA
TEB	Tris EDTA buffer
TIFF	Tagged Image File Format
Tm	Melting temperature
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth

U	Unique
U937	Macrophage Cell Line
UN	United Nations
UPGMA	Unweight pair group method with arithmetic mean
UV	Ultraviolet
v/v	Volume per volume
VF	Virulence factor
w/v	Weight per volume
α	Alpha
β	Beta

Chapter 1 **Introduction**

1.1 **Introduction**

Despite improvements in health care, still an average of 4 million babies out of 130 million born every year die in the first 4 weeks of life (Lawn *et al*., 2005). The United Nations (UN) Millennium Development Goals (MDGs) represent the widest commitment in history to addressing global poverty and ill health. The fourth UN goal (MDG-4) commits the international community to reducing mortality in children aged younger than 5 years by two-thirds between 1990 and 2015 (Haines and Cassels, 2004). Although between 1960 and 1990, the risk of dying in the first 5 years of life was halved, achieving MDG-4 will depend on mortality reductions even greater in percentage terms than those already achieved (Lawn *et al*., 2005). A decade before the target date of 2015, there are predictions that the goal will not be met. A major challenge, which is less frequently identified in policy analysis, is the slow progress in reducing global neonatal mortality. Between 1980 and 2000, child mortality after the first month of life between 2 months and 5 years fell by a third, whereas the neonatal mortality rate was reduced by only about a quarter (Travis *et al*., 2004). Hence, an increasing proportion of child deaths are now in the neonatal period. In 2000, approximately 38% of all deaths in children younger than age of 5 years happened in the first month of life. Deaths in the first week of life have shown the least progress in reduction. In 1980, only 23% of deaths occurred in the first week of life and by the year 2000 this had risen to an estimated 28% (3 million deaths). To meet MDG-4, a substantial reduction in neonatal mortality rate in high-mortality countries is needed, and reduction deaths in the first week of life will be essential to achieve this target. In year 2000 in Europe, the

average neonatal mortality rate per 1000 live births was 11 (range 2 - 38 across countries), the number of deaths being 116 /1000. Three-quarters of neonatal deaths arose in the first week of life. The major direct causes were infections 36%, preterm birth 28% and birth asphyxia 23%. 60 – 80% of neonatal deaths were in low birth weight babies (Lawn *et al*., 2005). In 2008, globally, there were 8.8 million deaths in children less than 5 years in age, and 41% (3.6 million) of these deaths occurred in neonates. The most significant risk factors identified were pre-term complications, birth asphyxia, sepsis and pneumonia. In Europe, in 2008 there were 148,000 neonatal deaths, 18% of these were due to preterm complications and various infections comprised 33% of the deaths (Lawn *et al*., 2005; Black *et al*., 2010).

Premature neonates are vulnerable to infections from several routes. Major clinical risk factors include the presence of medical devices e.g. nasogastric feeding tubes, intravascular catheters, immaturity of their immune system, and immaturity of their epithelial and mucosal barriers. The gastrointestinal tract of the neonate is sterile at birth, yet it is rapidly colonised by bacteria afterwards (Kaufman and Fairchild, 2004).

Recent reported events in the UK, highlighted by the national press, indicated that nosocomial infections and sepsis continue to be significant causes of mortality and morbidity in preterm infants on neonatal intensive care units (Black *et al*., 2010; Liu *et al*., 2012). Furthermore, the care environment can be a major source of potential pathogens. Earlier studies have shown that healthcare practice in neonatal units (antibiotic exposure, diet) has significant effect on the incidence of intestinal sepsis in premature neonate. Others have also demonstrated that the care

environment also plays a significant role in these nosocomial infections. Previous work at NTU has already demonstrated that feeding tubes, which most premature neonates are dependent upon, can be rapidly colonised with high levels potentially pathogenic organisms and act as a reservoir that may perturb the normal colonising process. They also showed that different feeding regimes can modify this colonisation pattern (Hurrell *et al*., 2009a and 2009b).

A portion of neonatal illnesses are due to bacterial infection, to which the neonate is highly susceptible due to their immature immune status. Therefore rates of early exposure to bacteria must be carefully considered to evidence infections by opportunistic pathogens, which include members of the *Enterobacteriaceae*.

1.2 *Enterobacteriaceae*

Enterobacteriaceae are a family of Gram-negative bacteria that contains 44 genera and more than 176 species (Hong Nhung *et al*., 2007). The first identified member of the family was *Serratia marcescens*, which was described by Bizio in 1823, who found it had grown on Italian barley dish. More than half a century later, in 1880s, *Klebsiella* and *Proteus* were described. Initially biochemical methods were used to define the *Enterobacteriaceae* family. Currently the family has been reclassified according to phenotyping and genotyping methods (Janda and Abbott, 2006).

The *Enterobacteriaceae* are a large and varied grouping of Gram negative rods. Members are relatively small, non-spore forming bacilli typically about 0.5 µm in width and 1 to 5 µm in length. Most are motile. Some have capsules. They grow under both aerobic and anaerobic conditions, and ferment various carbohydrates.

They are oxidase negative; some species are further defined serologically based on flagella protein, polysaccharide capsule and lipopolysaccharide. Several virulence factors including, invasion and toxins are the most studied pathogenicity factors of the *Enterobacteriaceae.*

Most *Enterobacteriaceae* are opportunistic pathogens, especially *Escherichia coli, Klebsiella* spp., *Serratia* spp., *Enterobacter* spp. and *Salmonella*, and are associated with significant morbidity and mortality (Friedland *et al*., 2003; Adamson *et al*., 2012). These organisms can cause many different kinds of infections in wounds (sepsis), brain (meningitis), pneumonia, and urinary tract infections especially in intensive care units (ICU) (Kollef *et al*., 1999; Ibrahim *et al*., 2000). Nosocomial infections are caused by *Enterobacteriaceae* such as *E. coli* (most common), *Klebsiella*, *Proteus, Serratia* and *Citrobacter* spp. Some of these organisms are part of the normal flora, such as *E. coli* and *Enterobacter cloacae,* and may cause such infections via selection following an empiric antimicrobial regime (Iversen and Forsythe, 2004a; Stoll *et al*., 2005). The most commonly encountered human pathogens of the family *Enterobacteriaceae* causing infections are showed in Table 1.1 (Liu *et al*., 2012; Podschun and Ullmann, 1998; Pazhani *et al*., 2005; Fraser and Arnett, 2006; Chaudhry *et al*., 2007; Hammerum and Heuer, 2009; Bisi-Johnson *et al*., 2011).

Table 1.1 The most commonly clinical important species of *Enterobacteriaceae* causing infections

Genus	Clinically important species	Clinical Presentation
Citrobacter	*freundii*	Pneumonia, meningitis, septicaemia, wound and urinary tract infections
Enterobacter	*aerogenes, cloacae*	Pneumonia, septicaemia, wound and urinary tract infections
Escherichia	*coli*	Diarrhoea, meningitis, septicaemia and urinary tract infections
Klebsiella	*pneumoniae, oxytoca*	Pneumonia, septicaemia and urinary tract infections
Morganella	*morganii*	Septicaemia and urinary tract infections
Plesiomonas	*shigelloides*	Diarrhoea and septicaemia
Providencia	*rettgeri, stuartii*	Urinary tract infections
Salmonella	*enteritica*	Diarrhoea, typhoid fever, septicaemia, osteomyelitis and urinary tract infections
Serratia	*marcescens, liquefaciens*	Pneumonia, septicaemia, wound and urinary tract infections
Shigella	*sonnei, flexneri*	Diarrhoea
Yersinia	*pestis, enterocolitica*	Diarrhoea, septicaemia plague and enteritis

1.3 **Bacteria, breast milk and formula-fed**

Human breast milk can be replaced with infant formula (IF) when the infant is unable to obtain mothers' milk or the amount is insufficient for normal development. In some cases mothers are unable to produce the milk or too little to feed her baby. Infant formula can also be required as a consequence of separating the child from their mother for health reasons, being at work, or the death of the mother (FAO/WHO, 2004 and 2006). Following delivery, the intestinal bacterial flora is usually sparse during the first few days of life, independent of feeding habits and environmental factors may be more important than breastfeeding (Mackie *et al*., 1999; Dominguez-Bello *et al*., 2010). The colonisation of the neonate intestinal tract is initially dependent on the neonates' environment (Rosberg-Cody *et al*., 2004). The majority studies of infants intestinal gut flora were on faecal samples using the classical plating techniques with culturing on specific media. There have been

a limited number of studies, using cultivation and molecular techniques to follow the changes in the neonatal gut flora in the first weeks of life. Some of these studies have distinguished neonates according to birth weight, and mode of nutrition (ie. breast or formula-fed) (Favier *et al*., 2002; Fanaro *et al*., 2003). The environment and hospital staff are a major source of the colonising bacteria (Bezirtzoglou, 1997). After this initial colonization, the diversity of the subsequent bacterial flora community is influenced by the diet (Harmsen *et al*., 2000; Wold and Adlerberth, 2002; Alderberth *et al*., 2011).

Diet is an important factor in composition and development of microbiota in early of life. The microbiota of breast-fed and formula-fed infants was found to differ significantly in both composition and diversity. Breast-fed babies contain the microbiota that is more heterogeneous than that of formula-fed babies and contain a higher taxonomic diversity (Schwartz *et al*., 2012). The bacteria isolated from infant formula include *Enterobacter agglomerans*, *Hafnia alvei*, *K. pneumoniae*, *Citrobacter koseri*, *Citrobacter freundii*, *K. oxytoca*, *E. cloacae*, *E. coli*, *Serratia* spp., *Acinetobacter* spp., *Bacillus cereus*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium botulinum*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella* (FAO/WHO, 2004 and 2006). *S. aureus*, *B. cereus*, and *Salmonella* serovars could be linked to neonate infections (Cahill *et al*., 2008; Forsythe, 2005). A published study (Bezirtzoglou, 1997) shows that breast milk often has low numbers of streptococci, micrococci, lactobacilli, staphylococci, diphteroids, and bifidobacteria. Whereas colonisation by staphylococci is more common in breast-fed infants, and may be occur due to contact with the mother's skin during feeding.

A study of the predominant intestinal microbes in full term infants who were breastfed, found bifidobacteria as most dominated and become stable during the first few days of life, lactobacilli and streptococci (Koenig *et al*., 2011; Scholtens *et al*., 2012). Whereas *Enterobacteriaceae* (*E. coli* and *Klebsiella* spp.), *Staphylococcus*, *Clostridium*, *Bifidobacterium*, *Enterococcus* and *Bacteroides* spp. were the predominant flora in infant's formula-fed, (Harmsen *et al*., 2000; Rubaltelli *et al*., 1998). Another study of the microbial variety during breast-feeding and weaning periods in two infant by Wang *et al*. (2004) shows that infants were colonized by *Enterobacteriaceae*, *Bacteroides*, *Enterococcus*, *Streptococcus*, *Staphylococcus* and *Veillonella*. In addition the *Enterobacteriaceae* and *Bacteroides* were more predominant during breast-feeding. After weaning the clostridia were increased, while the *Enterobacteriaceae* were reduced.

Heavey and Rowland (1999) compared the intestinal microbial flora in breastfed and formula-fed, and reported that the bifidobacteria were more dominant on breast-fed infants neonates. This may be attributable to environment and improvements in formula feeds (Balmer *et al*., 1994; Rueda *et al*., 1998). Also human milk composition such as protein, nucleotides and gangliosides play a role in both the immune systems and growth microbes. Cabrera-Rubio *et al*. (2012) indicates that the microbiomes in milk are influence by several factors such as composition of milk. The study by Rueda *et al*. (1998) found that the counts of bifidobacteria was higher in formula-fed of infant, and also indicate that the colonization by bifidobacterial was faster when milk formula supplemented with gangliosides. While Helen and Susan (2012) reported that feeding infant's formula supplementary nucleotides are

attributable to a lower incidence of episodes of diarrhoea and also rose to plasma antibody response to some immunisations.

A comparative study of microbial diversity in 40 infants during the first 2 years from birth using real-time PCR from month 7 to month 24, showed that the numbers of *Bacteroides* and *Desulfovibrio* spp. increased, while *E. faecalis* decreased. In addition microbial diversity increased which number of bifidobacteria in breast-fed babies, and the higher levels were desulfovibrio in bottle-fed children (Hopkins *et al*., 2005; Penders *et al*., 2005). A comparative study of predominant *Bifidobacterium* spp., *E. coli* and *Clostridium difficile* in faecal samples of 50 breast-fed and 50 formula-fed infants, showed that *C. difficile* were highest in the formula-fed group (10^7 CFU/g) than in breast–fed infants (10^3 CFU/g). The predominance of *E. coli* in the breast-fed and formula-fed was 80% and 94% respectively at ca. 10^9 CFU/g, while the predominance of *Bifidobacterium* spp. was similar in both groups (Penders *et al*., 2005).

Twenty-nine infants with low birth weight (<1000 g) had stool samples collected on days 10, 20 and 30. In day 30 the major of species were *Enterococcus faecalis*, *E. coli*, *S. epidermidis*, *E. cloacae*, *K. pneumoniae*, and *Staphylococcus haemolyticus*. Members of the *Lactobacillus* and *Bifidobacterium* genera were identified in only one infant. The total counts and numbers of species identified increased in breast milk fed infants over time. During the study, two babies were fed by infant's formula, but one of those babies died. In addition those babies were colonized by *E. coli*, *E. faecalis*, and *S. haemolyticus* (Gewolb *et al*., 1999).

1.4 *Enterobacteriaceae* infections in neonatal feeding tubes and bottles

The most important problem for new-born infants and premature infants especially those born with low birth weight (< 2000g) are bacterial infections. These neonates are fed via a nasogastric tube. Despite recent concerns over the microbiological safety of infant feeds, there has been little consideration that the nasogastric enteral feeding tube may act as a site for bacterial colonisation due to biofilm formation, and act as a locus for infection (Wiener *et al*., 1999). The nasogastric tube is usually between ambient (outer portion) and body temperature (inner portion), with regular (every 2 h) additions of nutrients from the infant feed and in-place over sufficient time periods for bacterial multiplication. Previously, nasogastric feeding tubes of adults in a nursing home have been shown to be a reservoir for *E. coli* and *Klebsiella* with extended spectrum β–lactamase (ESBL) (Wiener *et al*., 1999).

In recent years, there has been a rise in incidence of neonatal infections due to *Enterobacteriaceae*, and they have become the predominant causative agents in neonatal intensive care unit (NICU) outbreaks (Kaufman and Fairchild, 2004; McGuire *et al*., 2004; Gastmeier *et al*., 2007). *Klebsiella* spp. infections outnumber staphylococci infections, and *Serratia* spp., are the third most common causative pathogen (Gastmeier *et al*., 2007). Pathogenic strains of *E. coli* are one of the leading causes of neonatal meningitis and sepsis (Stoll *et al*., 2005). Neonates may be particularly prone to Gram negative infections (i.e. *Enterobacteriaceae*) as their innate immune cells have low responses to lipopolysaccharide (part of the Gram negative cell wall structure) and macrophage response (Levy *et al*., 2004).

A recent study at NTU analysed the enteral feeding tubes from >125 neonates in two local hospital neonatal intensive care units (NICU) (Hurrell *et al*., 2009a and 2009b).

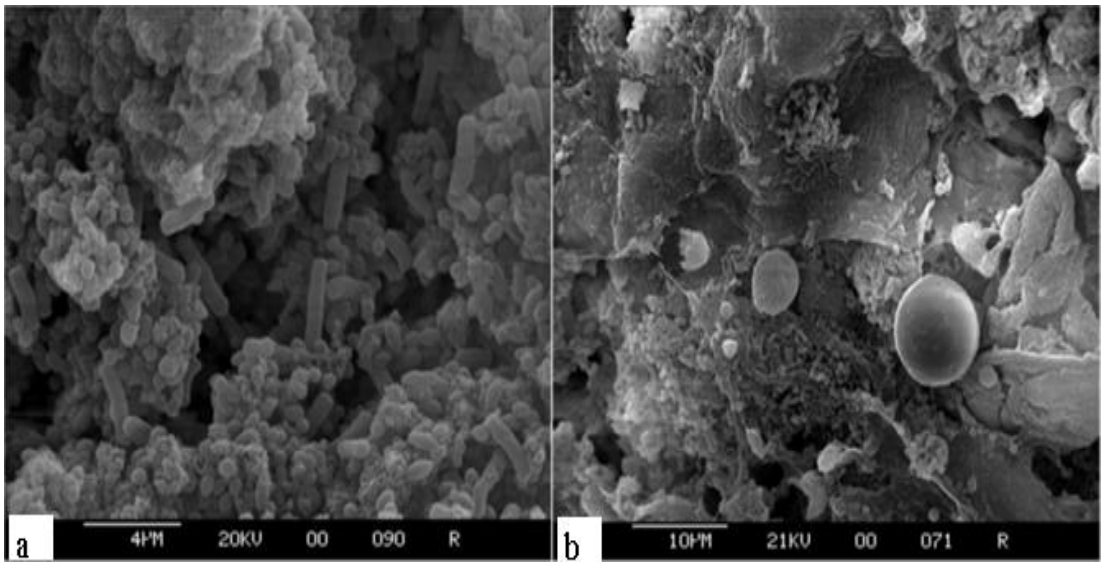

Figure 1.1 Electron microscopy of enteral feeding tube inner wall from:

(a) Neonate fed breast milk and ready to feed formula. Bar indicates 4 µm size marker. (b) Neonate fed breast milk and reconstituted PIF with added thickener. Bar indicates 4 µm size marker. (Hurrell *et al*., 2009a)

Enterobacteriaceae were isolated in high numbers (up to 10^7 CFU/tube) from biofilms growing inside the feeding tubes, and identified see Figure 1.1. Of particular interest was the frequent isolation of the opportunistic pathogens *E. coli, K. oxytoca, K. pneumoniae, S. marcescens, Enterobacter cancerogenus*, and *E. hormaechei*. In addition, *Cronobacter sakazakii* and *Yersinia enterocolitica* were also isolated Table 1.2. The presence of the *Enterobacteriaceae* was irrespective of the feeding regime. During the period of this study there were 15 infections cases in the NICUs due to *E. coli, K. pneumoniae, K. oxytoca, S. marcescens*, and *E. cloacae.*

Table 1.2 Isolation of *Enterobacteriaceae* from biofilms on nasogastric enteral feeding tubes of neonates

Feeding regime (n,%)	Number of samples positive for *Enterobacteriaceae*	*Enterobacteriaceae* species							
		E. coli	*E. cancerogenus*	*E. hormaechei*	*K. pneumoniae*	*R. planticola*	*R. terrigena*	*S. liquifaciens*	*S. marcescens*
Breast milk (21,16)	11(52)	3(4)	8(38)	**12**(57)	2(10)			2(10)	7(33)
Fortified breast milk (37,29)	29 (78)	10(27)	19(51)	10(27)	21(58)	1(3)	1(3)	5(14)	5(14)
Ready to feed formula (26,20)	21(81)	7(27)	8(31)	4(15)	7(27)		3(14)	4(15)	18(69)
Reconstituted PIF (8,6)	7(88)	5(6)	3(4)				6(75)	2(25)	3(38)
Mixed (27,21)	21(78)	1244()	13(48)	14(52)	1(4)	3(11)	3(11)	2(7)	5(19)
Nil by mouth (10,8)	8(80)		2(20)	4(40)	1(10)				8(80)
Total 129 (%)	97 (75)	37 (29)	53 (41)	44 (34)	32 (25)	4 (3)	13 (10)	15 (12)	46 (36)

Identification of *Enterobacteriaceae* isolated from biofilms inside neonatal enteral feeding tubes collated according to the feeding regime of the neonate (Hurrell *et al*., 2009a)

Levy *et al*. (1989) reported on 309 formula bottles over a 1 year period and found 83 (27%) were contaminated. Unfortunately, the source of contamination was unknown; though plasmid profiles of *E. cloacae* isolated from feeding liquid remained identical for several months, which may indicate a common source. Plasmid profiling however is not as discriminatory as PFGE, which would be applied to similar studies these days. The authors reported that *E. cloacae* nosocomial sepsis isolates over a 7-year period (1979 - 1985) had plasmid profiles that matched them to contaminated enteral nutrition solutions. Two out of seven *K. pneumoniae* bacteraemia over a six-month period could also be related to contaminated enteral feeds.

1.5 *Enterobacteriaceae* found in powdered infant formula

In a study by Muytjens *et al*. (1988), 10% of powdered infant formula (PIF total 160 samples), were shown to be contaminated with *Cronobacter* spp. Another study by Iversen and Forsythe (2004b) using Druggan-Forsythe-Iversen (DFI selective agar), examined other dry baby food for the presence of *Cronobacter*. They isolated three *Cronobacter* spp. from 102 PIF samples and 5 isolates from 49 samples of baby food. *Enterobacter cloacae*, an opportunistic pathogen, were isolated from a neonatal intensive care unit (Fernandez-Baca *et al*., 2001). This bacterium and *Enterobacter agglomerans* were isolated by Muytjens *et al*. (1988) from powdered infant formula.

During production powdered infant formula is heated and spray dried which is expected to kill most bacteria. However, contamination may occur after the heat

treatment process is over. Moreover, it is likely that contamination may happen during reconstitution (Smeets *et al*., 1998). Contamination can occur via carer professionals (Farmer *et al*., 1980). Previously Waters *et al*. (2004) indicated that cross-transmission with certain Gram-negative such as *S. marcescens* by the hands of the health care team has a high probability. Likewise, Weir (2002) asserted that preparing PIF must be done for neonatal intensive care units (NICUs) following the manufacturer's instructions in a designated area by trained personnel under clean and aseptic conditions. Finally, according to the FAO/WHO (2004) the refrigerated reconstituted formula should not be used after 24 hours. Continuous enteral feeding administration, on the other hand, should not exceed more than four hours.

In addition FAO/WHO (2004 and 2006) collated information reported that *Enterobacteriaceae* sometimes found in powdered infant formula, which was also reported to be associated with neonatal infections. These were divided into groups A and B. *Salmonella enterica* and *Cronobacter* spp. were placed in Group A 'Clear evidence of causality', while *E. cloacae, C. koseri, Citrobacter freundii, Hafnia alvei, K. pneumoniae, K. oxytoca* and *Pantoea agglomerans* were considered as Group B 'Causality plausible, but not demonstrated'(Table 1.3).

Table 1.3 The *Enterobacteriaceae* in FAO/WHO (2004 and 2006) Category B

Organism	Infection	Reference
Citrobacter freundii	Urinary tract infections respiratory and meningitis	Drelichman and Band, 1985
Citrobacter koseri	Neonatal sepsis and meningitis	Graham and Band, 1981
Enterobacter cloacae	Nosocomial, including bacteraemia, lower respiratory tract infections, skin and soft tissue infections	Fernández-Baca *et al*., 2001
Escherichia coli	Neonatal meningitis	Harvey *et al*., 1999
Escherichia vulneris	Wounds, osteomyelitis, urosepsis, bacteraemia, meningitis	Brenner *et al*., 1982
Hafnia alvei	Pneumonia, meningitis, abscesses, and septicaemia.	Albert *et al*., 1992
Klebsiella oxytoca	Neonatal bacteraemia and meningitis	Tullus *et al*., 1992
Klebsiella pneumoniae	Pneumonia, neonatal meningitis	Harvey *et al*., 1999
Pantoea agglomerans	Rare systemic infections	Rostenberghe *et al*., 2006
Serratia spp.	Urinary tract infections, wound infections, and pneumonia	Anahory *et al*., 1998

1.6 **Neonates' intestinal flora**

In utero and before delivery the body surfaces, the gut lumen and the intestinal tract must be sterile, but during birth and after birth the baby is exposed to a wide-range of different bacteria (Orrhage and Nord, 1999). However not all of bacteria are able to colonise the baby. The initial colonisation is related to the type of delivery, the type of nutrition (breast milk or formula feeding), environment, hospital staff, human contacts and inanimate objects (Guenthner *et al*., 1987; Bezirtzoglou, 1997; Heshmati *et al*., 2011).

The gastrointestinal tracts of humans contain more than 500 different species of bacteria. Moreover each species of bacteria comprise different bacterial strains. The estimated number of in the gut lumen contains as many as 10^{11} bacterial cells. These numbers of bacteria are comparable to those growing under conditions of

a laboratory. Those numbers are 10 times greater than the number of the gut lumen cells (Guarner and Malagelada, 2003). However, the number of bacteria varies between gut lumen sites, such as the stomach $10^1 – 10^3$ cells per gram and 10^{11}-10^{12} cells per gram in the lower tract (Sekirov *et al*., 2010). The lower bacterial number in the upper lumen is because of factors such as acid, enzymes in saliva and bile, which kills most ingested bacteria. In addition, the function of motor activity, which moves the food towards the end of ileum, also plays a role in the bacterial colonisation of the lumen (Riskin *et al*., 2011).

Bacterial species inside the intestinal lumen of human can be classified into three groups. These groups have been recognised as pathogenic bacteria, beneficial bacteria and potentially pathogenic bacteria. Pathogenic bacteria can effect feeding intolerance, produce harmful materials inflammation, infections and make serious problems such as meningitis and necrotising enterocolitis especially with a premature baby (Dash *et al*., 2006). Beneficial bacteria are able to inhibit the growth of pathogenic bacteria such as outnumber pathogenic bacteria, activate the immune system, digestion of component parts and fermentation of dietary fibre (Westerbeek *et al*., 2006). Potentially pathogenic bacteria have neither a positive or negative impact if present in high numbers for example *E. coli*, *Enterobacter* spp. and streptococci. Although some of potentially pathogenic possibly will become pathogenic, even if present in low numbers for instance *Klebsiella*, *E coli* O157:H7 and staphylococci (Dai and Walker, 1999). In the review by Westerbeek *et al*. (2006) five studies were considered that the beneficial bacteria and the potentially pathogenic bacteria colonised the intestine of premature baby. In brief, the studies

show that the intestine of premature babies is colonised with beneficial bacteria during the late period. While potentially pathogenic bacteria such as *Enterobacter* spp. and *E. coli* were found in the faeces of premature baby high numbers during the whole study period.

1.7 **Neonatal infections**

New-born babies and particularly those with very low birth-weight, have a weak immune system (Levy *et al*., 2004). This allows to the opportunistic pathogen of bacteria and other microbes to cause morbidity and mortality. The estimated numbers of newborns die in developing countries; about one million newborns die from infections (Ganatra and Zaidi, 2010). Thus, the study of neonatal infections is very important for describing and recognising the epidemiology of infections (Ganatra and Zaidi, 2010). In addition, the rates of risk and infection to neonates increased with decreased in birth weight, early age, additionally prolonged hospitalization (Stoll *et al*., 2005; Klinger *et al*., 2010).

A study by Liu *et al*. (2012) shows that the estimated mortality in children younger than 5 years in 2010 were seven million and six hundred-thousand from different countries of the world. According to the causes of mortality, this number was distributed into two groups. First group occurred in neonates younger in 28 days, which was attributed to infectious causes, preterm birth complications, intrapartum-related complications, and sepsis or meningitis and other older which were attributed to pneumonia, diarrhoea and malaria. This is summarised in

Table 1.4 below which shows the rates of mortality of two groups, with the high rate in both groups of leading killers, are pneumonia and preterm birth.

Table 1.4 Estimated number of mortalities in children younger than 5 years in 2010

Causes	Estimated death per millions	Estimated deaths (%)
Neonates aged 0 – 27 days		
Preterm birth complications	1·078 (0·916 – 1·325)	14
Intrapartum-related complications	0·717 (0·610 – 0·876)	9
Sepsis or meningitis	0·393 (0·252 – 0·552)	5
Pneumonia	0·325 (0·209 – 0·470)	4
Congenital abnormalities	0·270 (0·207 – 0·366)	4
Other neonatal disorders	0·181 (0·115 – 0·284)	2
Tetanus	0·058 (0·020 – 0·276)	1
Diarrhoea	0·050 (0·017 – 0·151)	1
Children aged 1 – 59 months		
Other disorders	1·356 (1·112 – 1·581)	18
Pneumonia	1·071 (0·977 – 1·176)	14
Diarrhoea	0·751 (0·538 – 1·031)	10
Malaria	0·564 (0·432 – 0·709)	7
Injury	0·354 (0·274 – 0·429)	5
Meningitis	0·180 (0·136 – 0·237)	2
AIDS	0·159 (0·131 – 0·185)	2
Measles	0·114 (0·092 – 0·176)	1

Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000 (Liu *et al.*, 2012)

In Table 1.5 summaries of information obtained from national nosocomial infections surveillance (NNIS) from 1986 to 1989 by Emori and Gaynes (1993), 1989 to 1990 by Jarvis and Martone (1992) and 1981 and 1999 by Hsueh *et al*. (2002) about pathogens causing nosocomial infections in hospitals. Their study shows that *E. coli* was the most common isolate.

Table 1.5 Pathogens isolated from nosocomial infections reported to the national nosocomial infections surveillance (NNIS)

Pathogens isolated	% of isolates		
	1986 and 1989	1986 and 1990.	1981 and 1999
E. coli	16	13·7	18.6
Enterococci	12	10.7	-
Pseudomonas aeruginosa	11	10.1	11
Staphylococcus aureus	10	11.2	-
Coagulase-negative staphylococci	9	9·7	-

Lee *et al*. (2004) studied the rate of bacteramia in a neonatal intensive care unit (NICU). Fifty-eight out of 623 (9.31%) patients had bacteremia, with 12 deaths. Among the bacterial pathogens isolated in sort as follows, were coagulase-negative Staphylococci 29%, *S. aureus* 22%, and *E. cloacae* 17%. In the Chittagong area of Bangladesh, study was performed to determine the incidence of bacterial neonatal sepsis. The study shows that 104 isolated were collected from 1400 blood samples of neonatal sepsis infection. 102 out of 104 isolated species of bacteria were belonged to Gram-negative and 2 belonged to Gram-positive. The main Gram-negative bacterial isolated were *K. pneumoniae* 79 (75.96%), *S. marcescens* 19 (18.27%) and *Pseudomonas aeruginosa* 4 (3.85%). The two (1.92%) *S. aureus* were isolated. Most of the Gram-negative bacteria showed resistance to ampicillin, ceftriaxone and gentamicin (Hafsa *et al*., 2011). Gram-negative bacteria isolated from hospital-acquired infections and bloodstream infections in NICU are leading causes of high rates of morbidity and mortality. This study showed that *E. coli* was the most common organism isolated (Morfin-Otero *et al*., 2012).

1.8 **Infant meningitis**

Bacterial meningitis is the inflammation of the lining around the brain and spinal cord, which are a significant cause of mortality and morbidity worldwide (Chávez-Bueno and McCracken, 2005; Shmaefsky *et al*., 2010). Consequently, bacterial meningitis is one of the most serious infection diseases and causes serious problems in children (Mashouf *et al*., 2006). Infection usually occurs between the fourth and eight day after birth and it can be fatal within hours to days following the first diagnosis (Muytjens *et al*., 1983). The estimates of infant sepsis and meningitis are approximately 1 to 5 infants per 1000 live births (Unhanand *et al*., 1993). The main risk factor for meningitis could be the lack of immunity to opportunistic pathogens that infect premature and infant babies, other risk factors infected by pathogenic bacteria, close interaction with individual having invasive disease (Feikin and Klugman, 2002; Al-Rawazq, 2010).

Bacterial causes of meningitis are divided by age into two groups; first group premature and new-borns babies up to three months. Common causes meningitis of these group are group B streptococci and those that normally inhabit the gut lumen and the intestinal tract such as *E. coli* (Kim *et al*., 2005; Zhu *et al*., 2010). Older children are affected by *Neisseria meningitidis*, *Streptococcus pneumoniae* and third group under five *Haemophilus influenzae* (Sáez-Llorens and McCracken, 2003; Tunkel *et al*., 2004).

The most common Gram-negative organism causing neonatal meningitis is *E. coli* K1 (Janda and Abbott, 2006; Kim *et al*., 2005; Zhu *et al*., 2010; Logue *et al*.,

2012; Ribes, 2013) and also can occur due to a variety of other bacterial pathogens, such as *E. cloacae, C. koseri, Neisseria meningitidis, Serratia* spp., *Enterobacter* spp. and, *Cronobacter* spp.; and are also responsible for considerable morbidity and mortality (Friedland *et al*., 2003; Iversen and Forsythe, 2003; Adamson *et al*., 2012). Studies of Gram- negative enteric bacillary meningitis in neonates and infants from 1969-1989 have been reported by Unhanand *et al*. (1993) with ninety-eight neonates and infant patient identified from 1 day to 2 years. The isolated organisms were *E. coli* 53%, *Enterobacter* spp. 16%, *Citrobacter koseri* 9%, *Salmonella* spp. (9%), *Proteus mirabilis* 4%, *Serratia marcescens* 3%, *Bacteroides fragilis* 3%, and *Aeromonas* spp. 2%. A number of these organisms have been isolated from nasogastric enteral feeding tubes, and their presence was not necessarily limited to the ingestion of reconstituted infant formula.

1.9 **The bacterial interaction with mammalian cells**

The diversity and high mass of microbes in the gastrointestinal tract leads to an interaction between them and host cells. These interactions can be commonly beneficial or can have detrimental effects. Human epithelial lining are colonized by diverse range of microbes and different bacterial species use different means of interaction with the host epithelium. The communities of microbes on the host epithelium can be affected by different factors such as nutrition, antibiotic treatment, environmental exposure to microorganisms, and microbial colonization in the neonatal period (Lindhorst and Oscarson, 2009; Abraham and Medzhitov, 2011). Additionally, the innate defence mechanisms of the gastrointestinal tract acts to wash microbes from surfaces include epithelial integrity, the fast renewal of old epithelial

cell, rapid propulsion of infected cells, auto-phagocytosis, and innate immune responses (Kim *et al*., 2010).

Infants, particularly premature infants are more to prone diseases due to the vulnerability of natural epidermal, epithelial barriers and secreted products which are part of the innate immune system. For instance, the outer epidermal layer in a full-term infant is composed of 16 layers, whereas with premature infants at first few weeks this is only 3 layers, which could be more prone to invasive processes (Evans and Rutter, 1986). Many bacterial pathogens are able to avoid these defences of immune systems for example bacterial pathogens secrete various components and toxins (Kim *et al*., 2010). Also pathogenic bacteria are able to create an antiphagocytic surface layer usually containing of polysaccharide or by expressing their adhesions on polymeric structures that extend out from the cell surface (Kline *et al*., 2009).

Infections by enteropathogenic *E. coli*, *S. enterica*, *Shigella flexneri*, and *Yersinia enterocolitica* are determined by the ability of microbes to attach, invade, colonize the intestinal epithelium, intracellular persistence and transmission from cell to cell (Reis and Horn, 2010). These bacteria use different infection strategies; for instance bacteria survive attack by resident macrophages by the injection, through a plasmid encoded type III secretion systems. Another strategy by the bacteria is the ability to invade the epithelial barrier by inducing changes in the intracellular compartment in which they reside, such bacteria survive and grow inside macrophages (Reis and Horn, 2010; Sansonetti, 2002). Bacteria can interact with the host cell surface in two ways. Some bacteria show attachment to the smooth surface

of the cells without any specific membrane action and other bacteria attach to the host cells by surface microvilli (Greiffenberg *et al*., 2000). In addition, another factor is the outer membrane which could play a role in interaction between Gram-negative bacteria and their external environment (Mogensen and Otzen, 2005).

1.9.1 **Adhesion**

Following contact of the epithelial layer with bacteria, adhesion to host cells is the initial step in interactions between bacteria and host cells (Reis and Horn, 2010). The host uses several strategies to protect the body and wash microbes from surfaces. Such as the epithelial layer being composed of ciliated and non-ciliated cells. The ciliated cells create an unstable environment for adhesion by their cleaning mechanisms. However microbial pathogens can express factors that bind to host tissue cells (Wilson *et al*., 2002). Adhesion of bacteria to host cells is an important step in the pathogenesis of infection (An and Friedman, 2000; Lindhorst and Oscarson, 2009). The processes of microbial adherence to host cells can be via fimbria or polysaccharides such as capsule. Gram negative bacterial pathogens such as *E. coli* in particular use fimbriae to attach to host cells (Wilson *et al*., 2002).

Bacterial diseases of the gut commonly arise from interactions between bacterial and the mucosa host such as adherence and invasion (Hu and Kopecko, 2008). In 1908, there was the first report of the adhesion of bacteria to host cells. *E. coli* was reported to agglutinate animal cells by appendages, which was later determined to be multimeric pili (Kline *et al*., 2009). Curli fimbriae play a role in the adhesion of *E. coli* to its contact hosts (Pawar *et al*., 2005). Many of bacterial pathogens

including *Y. enterocolitica*, *Salmonella*, *Shigella* spp. and *C. jejuni* develop specific interactions as adhesion, invasion (Hu and Kopecko, 2008). Following adhesion the microbial pathogens are able to start specific biochemical processes that will led to causes infection such as toxin secretion and host cell invasion (Wilson *et al*., 2002).

1.9.2 **Invasion**

The interaction between bacterial cells and host cells largely depends upon invasion ability. The ability of bacterial pathogens to enter deeper within the host tissues can result in the infection disease. Invasion by bacterial pathogens into host tissues could be divided into two groups: extracellular and intracellular (Wilson *et al*., 2002). Extracellular invasion happens when bacterial pathogens damage the barriers of host tissue, but the microbes remain outside of the host tissue. This is a strategy used by bacteria, which produce toxins such as haemolysin; there are three different of haemolysin, α-haemolysin, β-haemolysin and γ-haemolysin. Secreted α-haemolysin is formed in clinical isolates, such as *E. coli* and *Serratia* spp. (König *et al*., 1987; Welch, 1987; Schmidt *et al*., 1995). In addition, haemolysins expressed by these species did not only damage cells, but could also be contributing to distributing the microbes within host tissues (Wilson *et al*., 2002). While intracellular invasion happens when a microbe enters and survives within the cells of a host tissue. A number of Gram-negative pathogens have demonstrated their ability to penetrate host cells. Bacteria such as *E. coli* and *Salmonella* serovars have been found to invade intestinal cells. Moreover, some of these bacteria are able to interact with macrophages, while others can cross the blood brain barrier and invade brain cells. Some experiments have shown that *Cronobacter* spp. can invade Caco-2 cells

in vivo. A study by Kim and Loessner (2008) investigated the likelihood of invasion by *Cronobacter* spp. of human intestinal epithelial cells. The study concluded that this is receptor mediated, as well as actin filament and microtubule dependent. *Cronobacter* spp. strains are able to persist in human macrophage cells. Townsend *et al*. (2007a) concluded that *Cronobacter* strains differ in their persistence and replication in a human macrophage cell line. The authors also found that the *Enterobacteriaceae* were able to translocate through the blood brain barrier and thus lead to brain damage for the infected rat pups (Townsend *et al*., 2007b).

1.9.3 **Intracellular lifestyles**

Numerous bacterial pathogens have been developed to survive and replicate within epithelial, endothelial or macrophages cells after attachment and invasion. The ability to survive and replicate within phagocytic cells is becoming well characterised. Bacterial pathogens use strategies to avoid killing mechanisms such as the production of reactive oxidative and survive at low of acidic (Wilson *et al*., 2002). A phagocytic cell such as macrophages is produces pro-inflammatory cytokines, oxygen radicals and nitric oxide to protect the body and killing pathogenic microbes (Burmester and Pezzutto, 2003; Urban *et al*., 2006). In addition intracellular infections can persist for years and require extensive antibiotic treatment (Wilson *et al*., 2002). A previous study by Bringer *et al*. (2006) of adherent invasive *E. coli* (AIEC) isolated from Crohn's disease patients showed the organism's ability to replicate within macrophages in large vacuoles.

1.9.4 **Bacterial toxins**

Based on their cell wall structure, bacteria can be divided into two groups: Gram-positive and Gram-negative bacteria. The cell wall of bacteria can contain toxic components which are proteinaceous or non-proteinaceous. These are produced by bacteria to damage the host cell and cause disease (Wilson *et al*., 2002; Forsythe, 2010). Non-proteinaceous toxins for Gram-negative bacteria are lipopolysaccharides (LPS) which are also known as endotoxins. Endotoxins are complex lipopolysaccharides in the cell wall of Gram-negative bacteria such as *E. coli*, *Salmonella* and *Shigella* (Cetin *et al*., 2004a; Cetin *et al*., 2004b). LPS is a heat stable endotoxin that can persists during the preparation of powdered infant formula milk (Townsend *et al*., 2007b). The author concluded that endotoxin can enhance the translocation of *Enterobacteriaceae* across the intestinal tract and blood brain barrier, which could increase the risk of neonatal infections. Cetin *et al*. (2004b) found that endotoxin increases Ras homolog A (RhoA) activation which leads to impairment enterocyte migration consequently, this could be increase in focal adhesions and enhanced cell adhesiveness and also inhibit tissue repair following bacterial infection.

Proteinaceous toxins (exotoxins) are generally enzymes. These toxins are divided into two groups according to methods of delivery to eukaryotic cells; (1) secretion into the host cell (2) injection into the host cell cytoplasm (Finlay and Falkow, 1997a and 1997b). The result of bacteria producing exotoxins is extremely watery diarrhoea. In addition, exotoxins are specific for intestinal cells associated with digestive system diseases such as vomiting and diarrhoea (Forsythe, 2010). A study

by Chakraborty *et al*. (2008) of the major virulence proteins of *Vibrio cholerae* and enterotoxigenic *E. coli* (ETEC) are cholera toxin (CT) and labile toxin (LT) respectively, which were found that these virulence proteins predominantly responsible of effects and damaging intestinal epithelial cells in both *in vitro* and *in vivo*. A report by Pagotto *et al*. (2003) described the virulence factors of *Cronobacter* spp. They investigated the effect of *Cronobacter* spp. *in vivo*, and found the organism produces an enterotoxin. Another study of *Cronobacter* spp. concluded that a 66 KD enterotoxin was more active at pH 6 and was stable for 30 min at 90ºC (Raghav and Aggarwal, 2007).

1.9.5 **Curli fimbriae**

Numerous Gram-negative bacteria have filamentous appendices, which extend from the cell surface called fimbriae or pili. These extensions consist of structural proteins named pilins. Fimbriae or pili are distinguished into two groups: (A) Tight contact, which enable the pathogenic bacteria to adherence to host cells, and (B) sex contact, which are responsible for the attachment of donor and receiver cells in bacterial conjugation (Brooks *et al*., 2007). In Gram negative bacteria there are various pilus structures, such as type 1 pili, P-pili, type IV pili and S-pili (Kline *et al*., 2009). The first recognized curli fimbriae were found in 1980s on *E. coli* strains that caused bovine mastitis (Olsén *et al*., 1989; Barnhart and Chapman, 2006). Similar fibrous structures also called thin aggregative fimbriae, which were found in other *Enterobacteriaceae* such as *Shigella*, *Citrobacter* and *Salmonella enteritidis* (Olsén *et al*., 1998; Romeo, 2008). Curli fimbriae enable bacteria to attach proteins in the extracellular matrix for instance fibronectin, laminin, and plasminogen

(Olsén *et al*., 1989). Furthermore, curli can be adhesive fibers and also play a role in biofilm formation and cell to cell interactions (Romeo, 2008).

Curli fimbriae expression is dependent upon environmental conditions such as low temperature, low nitrogen, phosphate, and iron; to slow growth (Barnhart and Chapman, 2006; Olsén *et al*., 1993; Maurer *et al*., 1998; Maurer *et al*., 1998). Römling *et al*. (1998) and Gerstel and Römling (2008) reported that curli fimbriae expression correlated with different environment factors of salt, nitrogen, phosphate, and iron. Also Barnhart and Chapman (2006) reported that most strains in the laboratory produced curli fimbriae under low incubation temperatures such as less than 30ºC. Olsén *et al*. (1989) showed that in *E. coli* the production of curli fimbriae was at growth temperatures less than 37ºC. While Bian *et al*. (2000) grew pathogenic *E. coli* strains at 37ºC and found that 24 out of 46 were able to produce curli fimbriae. Most commensal isolates of *E. coli* and *Salmonella* only express curli at room temperature. Though a number of clinical isolates of *E. coli* expressed curli at 37ºC (Kline *et al*., 2009). Other studies indicated that *E. coli* curli fimbriae can be expressed in a biofilm and at 37ºC (Romeo, 2008). Bian *et al*. (2000) investigated to detect curli fimbriae production. Forty-six strains of *E. coli* collected from Karolinska hospital were investigated using Congo red stain at 28ºC for 48 hours and 37ºC for 24 hours. Results showed that 31 strains were able to produce curli fimbriae at 37ºC and 24 strains at 28ºC. On the other hand two strains were produced curli fimbriae at 37ºC but not 28ºC. It was concluded that a human *E. coli* sepsis isolates were capable of producing curli fimbriae at 37ºC.

Congo red indicator agar is a suitable method to recognize genes important expression of curli fimbriae production (Barnhart and Chapman, 2006; Hammar *et al*., 1995). Where Barnhart and Chapman (2006) reported that different morphotypes could be appear when bacteria growth on media supplemented with Congo red indicator. Four defined morphotypes are rdar (red, dry, and rough) indicates curli fimbriae and cellulose production, pdar (pink, dry, and rough) indicates cellulose only, bdar (brown, dry, and rough) indicates only curli fimbriae are produced, Saw (smooth and white) colonies indicates neither curli nor cellulose (White *et al*., 2006).

The genes of curli biogenesis in *E. coli* are called *csg* (Römling *et al*., 1998) and are grouped in two divergent operons: the *csg*BA and the *csg*DEFG. They encode, respectively, the structural component of curli and encoding the transcriptional regulator *csg*D and the curli export machinery *csg*E-G (Romeo, 2008). The *csg*A operon encodes the major subunit protein of the fibre outside of the cell (Hammar *et al*., 1995) whereas *csg*B nucleates the structural subunit into a fibre (Barnhart and Chapman, 2006). Nenninger *et al*., (2011) shows the major and minor curli fimbriae subunits are encoded by the genes *csg*A and *csg*B, whereas the extracellular localization and assembly of curli subunits into fibres are genes *csg*E, *csg*F and *csg*G. In addition, they found that the outer membrane lipoprotein *csg*G aids the secretion and stability of *csg*A and *csg*B. Biofilm formation, invasion and virulence factors of bacteria were linked with the activity of the *csgD* gene (Uhlich *et al*., 2002). The over expression of *csgD* can overcome temperature dependent control of curli encoding *csgBA* operon (Gualdi *et al*., 2008).

The expression of curli fibers and *csgA* were reported to induce cytokines production in response to *E. coli* in human sepsis (Bian *et al*., 2000). In *Salmonella* spp. a homologous gene has been identified and called *agf* (thin aggregative fibres) and is grouped in two operons encoding *agf*BA and *agf*DEFG (Römling *et al*., 1998; Barnhart and Chapman, 2006).

1.10 **Study objectives**

The project aims (a) to consider possible reasons why certain *Enterobacteriaceae* are more frequently associated with neonatal enteral feeding tubes (b) determine the potential virulence of such *Enterobacteriaceae* organisms and (c) assign isolates from neonatal enteral feeding tubes into 'high risk' and 'low risk' categories.

1.10.1 **Strain selection and DNA profiling**

Initial studies will review our culture collection of enteral feeding tube isolates, and additional clinical isolates provided by Dr Shiu (Queen's Medical Centre, Nottingham). The range of genera will include *E. coli*, *Klebsiella* spp., *Serratia* spp. and *Enterobacter* spp. It is plausible that during the previous study, the same strains were either isolated from the same neonate on more than one occasion, or even from different neonates. Therefore DNA fingerprinting via pulsed field gel electrophoresis (PFGE) will be used to identify if the same strains were isolated on different occasions from the NICUs. In addition, multi-locus sequence typing (MLST) of *E. coli* will be used as a highly discriminatory choice for typing and detecting epidemiologically important strains.

1.10.2 **Physiological and virulence tests**

Representative strains from the four genera listed above will be selected according to PFGE groups, will be subject to a range of physiological and virulence related tests. The physiological studies will include determining biofilm formation on enteral feeding tubes, curli fimbriae, haemolysis, acid resistance, motility, the presence of extended spectrum β-lactamases (ESBL). Capsule production will be determined by colony morphology on milk formula agar plates. The susceptibilities of *Enterobacteriaceae* isolates to antimicrobial agents will be determined by the breakpoint method on antibiotic supplemented Iso-Sensitest agar according to the British Society for Antimicrobial Chemotherapy protocol.

1.10.3 **Virulence related traits.**

A range of virulence traits will be determined which are relevant to an enteroinvasive bacterial pathogen. These will include attachment and invasion of human intestinal cell line Caco-2, and brain cells humans and rats, as well as survival in macrophages, and detection of various virulence factor (VF) genes.

Chapter 2 **Materials and methods**

2.1 **Safety considerations**

Experiments described in this study were started after consideration of health and safety codes practice for microbiology level two containment laboratories of Nottingham Trent University, under the supervision of Professor Stephen Forsythe. All procedures and preparation of materials were assessed and completed on the appropriate COSHH forms. Category 2 organisms and all materials were disposed of according to recommended procedures.

2.2 **Bacterial strains**

Two hundred and twenty four strains of *Enterobacteriaceae* were used for the initial part of this study; Table 2.1 - Table 2.4. These were *E. coli* (n = 30), *Serratia* spp. (n = 60), *Enterobacter* spp. (n = 50) and *Klebsiella* spp. (n = 84). All of these strains had been isolated from enteral feeding tubes on neonatal intensive care units at Nottingham City Hospital (n = 174) and Queen's Medical Centre (n = 50). These strains were studied at the beginning of the project with PFGE to determine if they were clonal variants, and distinguishable. For the second part of the study, twenty three strains were chosen for virulence factors determination.

It should be noted that later towards the end of the research period an additional collection of thirty-eight *E. coli* strains from neonatal enteral feeding tubes from Jordan were obtained for preliminary profiling; Table 2.5.

Table 2.1 *E. coli* strains used in this study

Species	Strain number	Source	Date of isolation	Neonate
E. coli	780	NCH	16/01/2007	4
	796		30/01/2007	17
	786		17/02/2007	37
	904		24/04/2007	55
	905			55
	910		03/05/2007	64
	912			64
	913			65
	917			65
	923		08/05/2007	67
	924			67
	926		01/05/2007	61
	927			61
	929			62
	933			62
	934			63
	937		08/05/2007	68
	939			69
	943			70
	944			70
	947			71
	949			71
	1008		06/06/2007	99
	1009		12/06/2007	101
	1010			102
	1015			104
	1016			104
	1047	QMC	22/10/2007	116
	1050			116
	1051			116

E. coli isolated from enteral feeding tubes on neonatal intensive care units at Nottingham City Hospital and Queen's Medical Centre. NCH: Nottingham City Hospital (n = 27), QMC: Queen's Medical Centre (n = 3)

Table 2.2 *Serratia* spp. strains used in this study

Species	Strain number	Source	Date of isolation	Neonate
Serratia marcescens	791	NCH	23/01/2007	13
	793			14
	804		30/01/2007	19
	805		06/02/2007	23
	809		07/02/2007	25
	811		06/02/2007	26
	812			26
	813			26
	821		17/02/2007	37
	822			37
	823			31
	837		28/02/2007	39
	838			39
	840			39
	844		02/04/2007	45
	846			45

Serratia marcescens	849	NCH	10/04/2007	48
	852			48
	853		17/04/2007	49
	854			49
	898		15/05/2007	73
	899			76
	903		24/04/2007	58
	911		03/05/2007	64
	918		08/05/2007	66
	919			66
	920			66
	921			66
	925		01/05/2007	61
	935			63
	936		08/05/2007	68
	940			69
	941			69
	942			69
	945			71
	946			71
	951		09/05/2007	72
	952		22/05/2007	79
	953			79
	954			80
	955			80
	958			82
	959			82
	961			83
	962			85
	963			85
	964			85
	965			86
	967			86
	970			87
	973			88
	974			88
	975			88
Serratia liquifaciens	1030	QMC	16/10/2007	108
	1036		22/10/2007	111
	1041			113
	1045		16/10/2007	115
	1055		22/10/2007	118
	1076		16/10/2007	119
	1080		12/11/2007	126

Serratia spp. isolated from enteral feeding tubes on neonatal intensive care units at Nottingham City Hospital and Queen's Medical Centre, UK. NCH: Nottingham City Hospital (n = 53), QMC: Queen's Medical Centre (n = 7).

Table 2.3 *Enterobacter* spp. strains used in this study

Species	Strain number	Source	Date of isolation	Neonate
Enterobacter horm. steigerwaltii	790	NCH	16/01/2007	4
Enterobacter hormaechei	795		30/01/2007	17
	797			17
	798			18
	799			18
	800			19
	801			20
	802			21
	803			22
	806		06/02/2007	24
	856		17/04/2007	50
	859			51
	860			52
	861			52
	862			52
	863			52
Enterobacter horm. steigerwaltii	960		23/05/2007	83
Enterobacter cloacae	979		16/02/2007	36
	980			36
	981			36
	983			36
	986			36
	987			36
	993			36
	997			36
	1027	QMC	16/10/2007	108
	1028			108
	1032			110
	1033			110
	1034			110
	1035			110
	1037			112
	1038		22/10/2007	113
	1039			113
	1040			113
	1044		16/10/2007	115
	1052			117
	1053			117
	1066		12/11/2007	126
	1067			126
	1068		19/11/2007	128
	1069			128
	1074		16/10/2007	117
	1075			117
Enterobacter cancerogenus	1077		31/10/2007	120
Enterobacter cloacae	1081		12/11/2007	126
	1084			126
	1088		19/11/2007	128
	1089			128
	1094			128

Enterobacter spp. isolated from enteral feeding tubes on neonatal intensive care units at Nottingham City Hospital and Queen's Medical Centre, UK. NCH: Nottingham City Hospital (n = 25), QMC: Queen's Medical Centre (n = 25).

Table 2.4 *Klebsiella* spp. strains used in this study

Species	Strain number	Source	Date of isolation	Neonate
Klebsiella pneumoniae	778	NCH	16/01/2007	1
	779			2
Klebsiella oxytoca	781		17/01/2007	6
	782		16/01/2007	7
	783		16/01/2007	8
	784		23/01/2007	9
Klebsiella pneumoniae	785			9
	787			10
	788			11
Klebsiella oxytoca	789			12
Klebsiella pneumoniae	792			13
	794			14
Klebsiella oxytoca	807		06/02/2007	24
	808			24
	810			26
	814		11/02/2007	27
	815			27
	816			27
	817			27
	818		13/02/2007	28
	824		16/02/2007	36
	825		17/02/2007	37
	826			37
	827		20/02/2007	33
	828			33
	829			33
	830			33
	831			33
	832		16/02/2007	36
	833			36
	834		20/02/2007	35
Klebsiella pneumoniae	839		28/02/2007	39
	841			39
Klebsiella oxytoca	842		20/03/2007	42
	845		02/04/2007	45
	847			45
	848		10/04/2007	48
	850			48
	851			48
	907		24/04/2007	58
	908			58
	909			58
	957		22/05/2007	81
	966			86
	969			87
	972			87
Klebsiella pneumoniae	976		30/05/2007	89
	977			89
Klebsiella oxytoca	982		16/02/2007	36
	985			36
	988			36
	989			36
	990		17/02/2007	37
	991			37
	992		16/02/2007	36
	994			36
	996			36

Klebsiella oxytoca	998	NCH	16/02/2007	36
Klebsiella pneumoniae	999		05/06/2007	94
	1000			95
	1001			96
	1002			95
	1003			96
	1004			94
	1005			95
Klebsiella pneumoniae	1006			94
	1012		12/06/2007	103
	1013			103
	1014			103
Klebsiella oxytoca	1042	QMC	22/10/2007	114
Klebsiella pneumoniae	1046			116
	1048			116
	1049			116
Klebsiella oxytoca	1054			118
	1056			118
	1058		16/10/2007	119
	1059			119
	1071			108
	1078		31/10/2007	120
	1079			121
Klebsiella pneumoniae	1090		19/11/2007	129
	1091			129
	1092			129
	1093			129

Klebsiella spp. isolated from enteral feeding tubes on neonatal intensive care units at Nottingham City Hospital and Queen's Medical Centre, UK. NCH: Nottingham City Hospital (n = 69), QMC: Queen's Medical Centre (n = 15).

Table 2.5 *E. coli* strains used in this study isolated from enteral feeding tubes on neonatal intensive care units at Jordan Hospital

Species	Strain number	Date of isolate	Neonate	Comment
E. coli	83	11/12/2011	22	Free liquid
	84		22	
	87	10/12/2011	36	Biofilm
	104	12/12/2011	19	Free liquid
	105		19	
	106		19	Biofilm
	107	15/12/2011	13	Free liquid
	110		13	
	115		13	
	116		13	
	117		13	Biofilm
	118		13	
	119		13	Free liquid
	123		24	
	126		24	Biofilm
	127		10	Free liquid
	131		15	
	132		15	
	133		15	Biofilm
	134		15	
	139		7	
	140		7	
	141		7	Free liquid

E. coli	157	20/12/2011	5	Biofilm
	158		5	
	160		5	Free liquid
	162		12	Biofilm
	164		12	Free liquid
	165		18	Biofilm
	166		18	
	167		18	Free liquid
	168		18	
	171		11	Biofilm
	172		11	
	173		27	Free liquid
	174		27	
	175		27	Biofilm
	176		27	

JH: Jordan Hospital (n=38)

2.3 **Bacterial storage and culture**

All strains of *Enterobacteriaceae* were stored for a long term at -80°C and -20°C in 20% glycerol broth. When required, cells were recovered from frozen stock and subcultured on Tryptone Soya Agar (TSA) and incubated under aerobic conditions at 37°C for 24 h. All culture strains were stored for short periods at 10°C as appropriate.

2.4 **Primers used for amplification and sequencing**

All the primers used in this study were purchased from Sigma-Aldrich (UK) and shown in Table 2.6 and Table 2.8 below.

Table 2.6 Primers used for the seven selected house keeping genes used in *E. coli* MLST profiling

Oligo	Tm (ºC)	Sequence 5' to 3'	P.S (bp)	Comment
adk f adk r	54	ATTCTGCTTGGCGCTCCGGG CCGTCAACTTTCGCGTATTT	583	Adenylate kinase
fumC f fumC r	54	TCACAGGTCGCCAGCGCTTC GTACGCAGCGAAAAAGATTC	806	Fumarate hydratase
gyrB f gyrB r	60	TCGGCGACACGGATGACGGC ATCAGGCCTTCACGCGCATC	911	DNA gyrase
icd f icd r	54	ATGGAAAGTAAAGTAGTTGTTCCGGCACA GGACGCAGCAGGATCTGTT	878	Isocitrate/isopropylmalate dehydrogenase

mdh f mdh r	60	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT	932	Malate dehydrogenase
purA f purA r	54	CGCGCTGATGAAAGAGATGA CATACGGTAAGCCACGCAGA	816	Adenylosuccinate dehydrogenase
recA f recA r	58	CGCATTCGCTTTACCCTGACC TCGTCGAAATCTACGGACCGGA	780	ATP/GTP binding motif

P.S (bp) = Product size (base pairs). Tm = the melting temperature of the oligonucleotide (Wirth *et al*., 2006)

Table 2.7 Primers used for screen for different β-lactamase genes

Oligo	Tm (ºC)	Sequence 5' to 3'	P.S (bp)	β-lactamase
shv f shv r	57	CTTTATCGGCCCTCACTCAA AGGTGCTCATCATGGGAAAG	237	SHV
tem f tem r	63	CGCCGCATACACTATTCTCAGAATGA ACGCTCACCGGCTCCAGATTTAT	445	TEM
ctx-m f ctx-m r	68	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYCAGCGG	593	CTX-m
oxa f oxa r	54	ACACAATACATATCAACTTCGC AGTGTGTTTAGAATGGTGATC	813	OXA

P.S (bp) = Product size (base pairs). Tm = the melting temperature of the oligonucleotide (Fang *et al*., 2008)

Table 2.8 Primer used for the detection of virulence factor genes

ADHESINS				
Oligo	Tm (ºC)	Sequence 5' to 3'	P.S (bp)	Comment
PapA f PapA r	68	ATGGCAGTGGTGTCTTTTGGTG CGTCCCACCATACGTGCTCTTC	720	Pyelonephritis associated pili.
PapC f PapC r	64	GTGGCAGTATGAGTAATGACCGTTA ATATCCTTTCTGCAGGGATGCAATA	200	
PapEF f PapEF r	65	GCAACAGCAACGCTGGTTGCATCAT AGAGAGAGCCACTCTTATACGGACA	336	
pG f pG r	60	CTGTAATTACGGAAGTGATTTCTG ACTATCCGGCTCCGGATAAACCAT	1070	Flanking regions
pG1"r*	66	TCCAGAAATAGCTCATGTAACCCG	1190	
AlleleI-f AlleleI-r	69	TCGTGCTCAGGTCCGGAATTT TGGCATCCCCCAACATTATCG	461	Internal region
AlleleI'-f AlleleI'-r	64	CTACTATAGTTCATGCTCAGGTC CTGACATCCTCCAACATTATCGA	474	Internal region specific to canine isolate
AlleleII-f AlleleII-r	70	GGGATGAGCGGGCCTTTGAT CGGGCCCCCAAGTAACTCG	190	Internal regions
AlleleIII-f AlleleIII-r	70	GGCCTGCAATGGATTTACCTGG CCACCAAATGACCATGCCAGAC	258	
sfa1 sfa2	69	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	410	sfa/foc operon
SfaS f SfaS r	59	GTGGATACGACGATTACTGTG CCGCCAGCATTCCCTGTATTC	240	S fimbriae
FocG f FocG r	68	CAGCACAGGCAGTGGATACGA GAATGTCGCCTGCCCATTGCT	360	F1C fimbriae
Afa f Afa r	74	GGCAGAGGGCCGGCAACAGGC CCCGTAACGCGCCAGCATCTC	559	Dr family
bmaE-f bmaE-r	68	ATGGCGCTAACTTGCCATGCTG AGGGGGACATATAGCCCCCTTC	507	M blood group antigen specific M fimbriae
gafD-f gafD-r	66	TGTTGGACCGTCTCAGGGCTC CTCCCGGAACTCGCTGTTACT	952	Glucosaminyl-specific G fimbriae

nfaE-f nfaE-r	62	GCTTACTGATTCTGGGATGGA CGGTGGCCGAGTCATATGCCA	559	Non fimbrial adhesin-1
FimH f FimH r	71	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA	508	Mannose-specific adhesin subunit of type 1 fimbriae
TOXINS				
hly f hly r	66	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	1177	Haemolysin A
cnf1 cnf2	65	AAGATGGAGTTTCCTATGCAGGAG CATTCAGAGTCCTGCCCTCATTATT	498	Cytotoxic necrotizing factor-1
cdt-a1 cdt-a2	62	AAATCACCAAGAATCATCCAGTTA AAATCTCCTGCAATCATCCAGTTTA	430	Cytolethal distending toxin
cdt-s1 cdt-s2	61	GAAAGTAAATGGAATATAAATGTCCG GAAAATAAATGGAACACACATGTCCG		
SIDEROPHORES				
FyuA f FyuA r	66	TGATTAACCCCGCGACGGGAA CGCAGTAGGCACGATGTTGTA	880	Yersinia bactin
AerJ f AerJ r	71	GGCTGGACATCATGGGAACTGG CGTCGGGAACGGGTAGAATCG	300	Aerobactin
POLYSACCHARIDE COATINGS				
kpsII f kpsII r	65	GCGCATTTGCTGATACTGTTG CATCCAGACGATAAGCATGAGCA	272	All group II capsules
KpsIII f KpsIII r	63	TCCTCTTGCTACTATTCCCCCT AGGCGTATCCATCCCTCCTAAC	392	All group III capsules
K1-f	61	TAGCAAACGTTCTATTGGTGC	153	Only K1 capsule region
K5-f	59	CAGTATCAGCAATCGTTCTGTA	159	Only K5 capsule region
MISCELLANEOUS				
rfc-f rfc-r	65	ATCCATCAGGAGGGGACTGGA AACCATACCAACCAATGCGAG	788	Participates in O4 LPS biosynthesis. O4 serotype marker.
ibe10 f ibe10 r	76	AGGCAGGTGTGCGCCGCGTAC TGGTGCTCCGGCAAACCATGC	170	Invasion of brain endothelium gene.
ColV-C f ColV-C r	67	CACACACAAACGGGAGCTGTT CTTCCCGCAGCATAGTTCCAT	680	Colicin V. Marker for colV plasmids
TraT f TraT r	71	GGTGTGGTGCGATGAGCACAG CACGGTTCAGCCATCCCTGAG	290	Serum survival gene
RPAi f RPAi r	73	GGACATCCTGTTACAGCGCGCA TCGCCACCAATCACAGCCGAAC	930	Generic marker for uropathogenic PAIs

P.S (bp) = Product size (base pairs). Tm = the melting temperature of the oligonucleotide (Johnson and Stell, 2000)

2.5 **Sterilisation**

All equipment, media, buffers and solutions were sterilised by autoclaving at 121°C for 15 min, washed with 70% alcohol or filtration using 0.2 pore size µm filters.

2.6 **Stock reagents and buffers**

The chemicals in the study were obtained from either Sigma Aldrich or Fisher UK. All buffers were prepared in double distilled and sterilised water (dH_2O) as follows:

2.6.1 **Phosphate buffered saline**

Phosphate buffered saline (PBS) containing sodium and magnesium chloride solution was obtained from Sigma Aldrich, UK (D8662). The phosphate buffered saline was used during the work with tissue culture.

2.6.2 **Saline solution**

A 0.85% saline used as a suspension medium in bacteriological studies was prepared by one tablet of Oxoid Fisher Scientific, UK (BR0053G) dissolved into 500 ml of distilled water (dH_2O). This was distributed into glass bottles in 50 ml amounts.

2.6.3 **Hydrochloric acid**

A 1 M of hydrochloric acid (HCl) was used to control the pH of solution used in study. This was prepared using 1 M HCl 86 ml slowly added to 914 ml of distilled water and mixed quietly. This was transferred into a plastic container.

2.6.4 **Triton X-100**

Triton X-100 was used during the tissue culture studies, which was obtained from Fisher Scientific Fluka, Canada (BPE151-100). This was prepared by adding 100 µl of Triton X-100 to 10 ml of sterile distilled water (dH_2O).

2.6.5 **1 M Tris-HCl**

Tris base was obtained from Fisher Scientific, UK (BP152-500) to use in the PFGE analysis. 121.1 g of Tris base was placed in 1 litre flask and dissolved by adding 700 ml of (dH_2O) and then placed on hot plate magnetic stirrer at 56°C. The pH was adjusted to pH 8.0 with concentrated HCl. The volume was brought to 1 L with ultra-pure water then transferred to 1 L bottle. The solution was autoclaved and stored at room temperature.

2.6.6 **0.5 M EDTA (Ethylenediamine tetra-acetic acid, sodium hydroxide)**

The 0.5 M EDTA was prepared during work with pulsed field gel electrophoresis. This was made by adding 186.1 g of EDTA (E5134) to 700 ml of distilled water. Sodium hydroxide (NaOH) from Sigma Aldrich, UK (S8045) was added to adjust the pH to 8.0 with approximately 20 g of NaOH and mixed well to dissolve. The volume was made up to 1 L with de-ionised water and the solution was sterilized by autoclaving and store at room temperature.

2.6.7 **10 X TBE Buffer (Tris base, boric acid and EDTA buffer)**

The 10 X TBE Buffer was prepared by dissolved 108 g Tris base, 55 g boric acid Fisher Scientific; UK (10043-35-3) in 700 ml of (dH_2O) then 80 ml 0.5 M EDTA pH 8.0 was added to the mixed and adjusted to 1 L with distilled water. The solution was autoclaved and stored at room temperature.

2.6.8 **50 X TAE buffer (50 X Tris-acetate-EDTA buffer)**

The 50 X TAE buffer was obtained from National Diagnostics, UK (EC-872). The compositions of TAE buffer contain (2.0 M Tris-acetate and 100 mM sodium EDTA). 1 X of TAE was used in the preparation of agarose gels; Bio-Rad, UK (162 - 0137) and in the gel electrophoresis tanks. This was prepared by diluting 20 ml of the concentrated buffer in 980 ml distilled water.

2.7 **Culture media**

All culture media were obtained from Oxoid Thermo-Fisher and Sigma Aldrich.

2.7.1 **Tryptone Soya Agar (TSA)**

Tryptone Soya Agar Oxoid Thermo-Fisher, UK (CM0131) was prepared in accordance with the manufacturer's instructions. Forty grams of TSA agar was dissolved onto 1 L of distilled water. It was boiled until the agar was completely melted. The mixture was autoclaved at 121ºC for 15 min. After it had cooled to 50ºC it was poured into Petri dishes 20 ml each. These were dried and then stored in the refrigerator at 4ºC until required.

2.7.2 **Tryptone Soya Broth (TSB)**

Tryptone Soya Broth Oxoid Thermo-Fisher, UK (CM0876) was prepared in accordance with the manufacturer's instructions. Thirty grams of TSB was dissolved into 1 L of distilled water. The mixture was dispersed in 50 ml volumes poured in

and to 100 ml bottles and autoclaved at 121ºC for 15 min. The media were stored at room temperature.

2.7.3 **Luria-Bertani Agar (LBA)**

Luria-Bertani Agar Merck KgaA, Darmstad, Germany (1102830) was prepared according to the manufacturer's instructions. Thirty seven grams of LBA was mixed in 1 L of distilled water. It was mixed until dissolved and then autoclaved at 121ºC for 15 min.

2.7.4 **Luria-Bertani Broth (LB)**

Luria-Bertani Sigma Aldrich, UK (L3022) was prepared in accordance with the manufacturer's instructions. Twenty five grams LB was dissolved into 1 litre of distilled water. The mixture was dispersed in 50 ml volumes poured in and to 100 ml bottles and autoclaved at 121ºC for 15 min.

2.7.5 **Mueller-Hinton Agar (MHA)**

Twenty one grams of Mueller-Hinton agar Sigma, Aldrich, UK (70191) were added to 1 L of distilled water and mixed to dissolve. Autoclaved at 121ºC for 15 min. After it had cooled to 50ºC it was poured into Petri dishes 20 ml each and then stored in the refrigerator at 4ºC until required.

2.7.6 **Motility medium**

Four grams of bacteriological agar LabM agar, UK (No 2) and thirty grams of TSB Oxoid UK (CM0876) were dissolved into 1 L of distilled water. The mixture was autoclaved at 121ºC for 15 min. It was then cooled for three hours at 55ºC before further cooling with mixing at about 45ºC. Finally, the medium was poured into Petri dishes allowed to set at room temperature, and stored at 4ºC for maximum of 8 weeks.

2.7.7 **Milk agar**

To mimic the growth conditions in infant formula, a milk agar had been designed by Caubilla-Barron *et al*. (2007). Three grams of agar Oxoid Thermo-Fisher, Ltd, Basingstoke, UK (LP0011) with 0.4 g of ammonium sulphate were dissolved into 40 ml of distilled water. The medium was autoclaved at 121ºC for 15 min. Then it was mixed with 200 ml of warmed 55ºC sterile liquid formula (whey based). Finally, the mixture was poured into Petri dishes, and stored in a cooled place at 4ºC.

2.7.8 **LBA - Congo red medium**

LBA-Congo red medium was used to investigate curli fimbriae phenotype. LBA was prepared as given in section 2.7.3. Final concentration 40 µg/ml Congo red dye Sigma Aldrich, UK (C6277) was added to the media after it had cooled to ~ 50ºC. Then the mixture was dispensed into Petri dishes and stored in a cooled place at 4ºC.

2.7.9 **TSA - blood agar**

TSA- blood agar was used to detect haemolysis. TSA was prepared as described in section 2.7.1. sheep blood final concentration 5% (v/v) from Oxoid Thermo-Fisher; UK (CM0854) was added to the TSA media after it had melted, sterilized and cooled to ~ 45ºC. Then the mixture was dispensed into Petri dishes and stored in a cooled place at 4ºC.

2.8 **Pulsed Field Gel Electrophoresis**

Pulsed field gel electrophoresis (PFGE) was performed to determine whether the strains were clonal or distinguishable. PFGE analyses of *Enterobacteriaceae* was as described by the PulseNet (USA) protocol for molecular subtyping of *E. coli* O157, non-typhodial *Salmonella* serotypes, and *Shigella sonnei* (CDC, 2004; Ribot *et al*., 2006). The strains used are listed in; Table 2.1 -Table 2.5.

2.8.1 **Tris EDTA buffer (TEB)**

TE buffer was used to make the plug agarose and to wash lysed PFGE plugs. It was prepared as follows:

10 mM Tris, pH 8.0

1 mM EDTA, pH 8.0

Dilute with sterile ultrapure water.

Volume of TEB buffer = 15 ml (dH_2O)*t*n

Where t = time of wash, n = number of samples

2.8.2 **Cell Suspension Buffer (CSB)**

The CSB was prepared as follows (100 mM Tris; 100 mM EDTA pH 8):

V total = 3 ml* n

V added of Tris pH 8.0 = 0.3 ml* n

V added of EDTA pH 8.0 = 0.6 ml* n

$VdH_2O = 3\ ml^* n - (V_{add\ Tris} + V_{add\ EDTA})$

Where n = number of samples, V = Volume

2.8.3 **Cell lysis buffer (CLB)**

The CLB was prepared as follows (50 mM Tris; 50 mM EDTA, pH 8.0) (1% Sarkosyl NL (N-Dodecanoyl-N-methylglycine sodium salt) Sigma Aldrich, UK (L-5125) + 20 mg /mL proteinase K Sigma Aldrich, UK (P2308)):

V total = 5 ml * n

V added of Tris, pH 8.0 = 0.25 ml * n

V added of EDTA, pH 8.0 = 0.5 ml * n

Sarkosyl NL = 0.05 g *n

V proteinase k = 25 µl * n

$VdH_2O = 5\ mL * n - (V_{add\ Tris} + V_{add\ EDTA} + V_{add\ proteinase\ k})$

Where n= number of samples, V=Volume

2.8.4 **DNA preparation in agarose plugs**

Two hundred and twenty six strains of *Enterobacteriaceae* and *Salmonella enterica* serovar Typhimurium reference standard H9812 (732 NTU) were analysed in the study. The isolates and marker strains were grown overnight on TSA at 37ºC. Approximately a full swab of bacteria was suspended in buffer. The cell density was adjusted to an optical density (OD) of between 1.3 - 1.4 at 610 nm in the CSB. Three-hundred microliters of bacterial suspension was distributed into a 1.5 ml eppendorf tube. The TEB + 1% agarose + 1% SDS were boiled in a microwave oven until became melted and then 300 µl of the mixture were dispensed into a 1.5 ml eppendorf tube and placed in a thermo block at 55ºC.

2.8.5 **Casting and washing**

Casting plugs were made by mixing carefully 300 µl of bacterial suspension, 300 µl of TEB agarose and 15 µl of proteinase K (20 mg/ml). One hundred microliters of the mixture were dispensed into five well plug molds. After that the plugs were transferred into polypropylene tubes with 5 ml of cell lysis buffer. The mixture was placed in a shaking water bath at 54ºC for 1.5 - 2 h at 150 - 175 rpm. The DNA plugs were washed by removing the CLB and adding 15 ml of ultra-pure water, followed by shaking in a shaking water bath at 50ºC for 15 min. In the next step the plugs

were washed 4 times in 15 ml TEB in a shaking water bath at 50ºC. After washing it was stored in 5 ml of TEB in a fridge at 4ºC until analysis.

2.8.6 **Digestion and electrophoresis**

Two millimetres of each plug was cut and transferred to 135 µl water + 15 µl buffer, for 30 min at room temperature. The plugs were then digested with a restriction enzyme mixture 131.25 µl distilled water, 15 µl buffer and 3.75 µl *Xba*I enzyme, Promega, UK (R6181) at 37°C for 2 h [Appendix 7.1]. The fragments in the plugs were resolved by adding 2 litres of 0.5 X TBE buffer into the running tank. At the same time, 100 ml of 0.5 X TBE buffer 1% agarose was added into the plugs in the comb. The running parameters were as follows: the gel was run at 14°C with pulse times of 2.2 sec to 63.8 sec for 20 h at 6 v.

2.8.7 **Staining and analysis**

Gels were stained in ethidium bromide (1 µg /ml) for 30 to 60 min. Gels were viewed under Ultraviolet (UV) and photographed. Digital images were stored as Tagged Image File Format (TIF) and analysed by using BioNumerics software, version 3.5.

2.9 **Bacterial strains for physiological experiments**

Following PFGE analysis, representative strains of the pulse-types were chosen for further analysis. These were eight strains of *E. coli*, eight strains of *Serratia* spp. and four strains of *E. cloacae* were selected for physiological experiments see Table 2.9.

Table 2.9 Bacterial strains for physiological experiments

Organism	Strain	Pulsetype
E. coli	780	EcC4
E. coli	904	EcC3
E. coli	923	≠
E. coli	939	≠
E. coli	1008	U
E. coli	1009	EcC2
E. coli	1047	CcE1
E. coli	1050	≠
S. marcescens	791	SC2
S. marcescens	805	≠
S. marcescens	936	SC1
S. marcescens	942	≠
S. marcescens	946	≠
S. marcescens	952	≠
S. liquefaciens	1030	SC3
S. liquefaciens	1036	SC4
E. cloacae	979	EbC5
E. cloacae	1027	EbC1
E. cloacae	1028	U
E. cloacae	1074	EbC3

≠ = same

It should be noted that to overcome the ambiguity of phenotypic identification of *Enterobacter* and *Klebsiella*, all strains were subject to PFGE. After PFGE cluster analysis some strains were no longer studied as they had been initially misidentified. Instead they were given to H. Abdalla (NTU PhD student) for her studies of *Klebsiella* and *Enterobacter hormaechei*.

2.10 **Motility Test**

The motility assay of the bacterial strains was performed by first inoculating a single colony from each strain into 3 ml of TSB at 37°C with shaking incubator at 200 rpm. The culture was diluted to 10^4 CFU/ml and 3 µl from the prepared suspension was

stabbed in TSB supplemented with 0.4% agar as described above in section 2.7.6. After 10 min the inoculated plates were incubated overnight at 37°C. Strains were analysed twice, each time in triplicate. Data were analysed using EXCEL^{Tm} software.

2.11 **Capsule formation test**

The capsule formation of the bacterial strains was performed as follows. As described above in section 2.7.7, milk agar was prepared to investigate the effect of formula on capsule formation and determine the effect of formula components on capsule formation. Strains were streaked on milk agar and incubated overnight at 37ºC and room temperature. The colony morphology was used as the main parameter to compare capsule production between different strains and the effect of formula type on capsule production.

2.12 **Biofilm formation**

The biofilm formation was performed according to the crystal violet (CV) method as described by Zhang *et al*., (2004) and Stepanović *et al*., (2004). A single colony of each twenty strains were grown in TSB with shaking at 200 rpm at 37ºC for 18 h after harvesting from TSA plates for 24 h at 37ºC under aerobic conditions. Two types of sterile infant formula (whey and casein based) from local shops were examined in this study. These formulas were purchased as they contain different protein and were sterile.

After incubation for 18 h, the growth of bacterial was diluted by 10^{-4} in the formula. A 0.6 ml of cell suspension was dispensed in triplicate into plastic surfaces 24-well

ELISA tray for different temperatures incubation at 25ºC and 37ºC for 18 h incubation. After 18 h, the suspension was removed gently, and then the wells were washed three times by adding 1 ml of normal saline in each well, with shaking for 10 min at 200 rpm. 0.6 ml of 0.01% (w/v) of CV was then added to stain the cells for 15 min at room temperature and then the wells were rinsed again with normal saline to remove the unbound dye. One millilitre of absolute ethanol was added to each well, and then incubated at room temperature for 10 min. A half millilitre from each well was then transferred to new ELISA plates. Finally, the absorbance was measured at 540 nm with a spectrophotometer. Data were analysed using EXCEL^{Tm} software.

2.13 **Acid sensitivity**

The effect of acid on *Enterobacteriaceae* viability was performed to determine the survival of bacterial strains in low level of pH according to investigation by Edelson-Mammel *et al*. (2006). A single colony from a purity TSA plate was used to inoculate into 5 ml of TSB and incubated overnight at 37ºC. The pH of an infant whey-based formula was adjusted to pH value of 3.5 units with hydrochloric acid. This was used to mimic the stomach. Three millilitres of overnight culture were inoculated into 12 ml of the acidified infant formula, and which then were distributed into three sterile tubes and incubated in water bath to 37ºC. Viable cells were enumerated after 0, 15, 30, 60, 90 and 120 min. At known time intervals of incubation 200 µl was transferred from each tube, diluted in normal saline and plated in triplicate on TSA plates using the Miles Misra technique. TSA plates were

incubated at 37ºC for 24 h before enumeration of cells. All experiments were performed in triplicate from separate overnight cultures.

2.14 **Congo red morphotype (curli fimbriae or cellulose)**

Curli fimbriae or cellulose phenotype was examined by streaking strains on LBA-Congo red plates as described previously in section 2.7.8 and incubated at 37ºC for 48 h. The colony morphology was recorded at 48 h to determine the formation of their Congo red phenotype. Morphology was defined according to the following pigmentation: red which indicate curli fimbria and pink which indicate express cellulose.

2.15 **Haemolysis on blood agar**

Haemolysis was examined by streaking a single colony from each strain into blood agar plates which prepared as described before in section 2.7.9, and then incubated for 24 h for at 37ºC. A response of colony morphology was recorded at 24 h to determine the formation of their α-haemolysis with a green-brown zone surrounding the colony and β-haemolysis with clear zone surrounding the colony.

2.16 **Antimicrobial Susceptibility**

Susceptibility to β-lactam antibiotics and other antimicrobials were tested by the disc diffusion method, as described by the National Committee for Clinical Laboratory Standards (NCCLS). In brief, eight strains of *E. coli* were streaked onto TSA and incubated at 37ºC overnight to obtain isolated colonies. After incubation, using a swab ~5 colonies were transferred into a tube containing 5 ml of a sterile saline and

mixed thoroughly via vortex. The suspension was then adjusted to optical equivalent the 0.5 McFarland standards. After adjusting the turbidity a sterile cotton swab was dipped into the suspension and gently the swab was used to streak Mueller Hinton agar which prepared as explained above in section 2.7.5. The plates were allowed to dry for about 3 to 5 min. β-lactamase and other antibiotic disc given in Table 2.10 were obtained from MAST Group; UK. Single discs from each antibiotic was placed and pressed down to ensure complete contact with the surface of the MHA plates by using flame sterilized forceps. After addition of the antibiotic discs the plates were inverted and incubate at 37ºC for 24 h. For each antibiotic the diameter of the zone was measured and then compared with standard measurements in Table 2.10 to determine if the strains were resistant or sensitive to the antibiotic (Andrews, 2009; EUCAST, 2011). Control strains included were *E. coli* NCTC 13351 (+), *E. coli* NCTC 13352(+), *E. coli* NCTC 13353 (+) and *E. coli* NCTC 10418 (–).

Table 2.10 Zone size interpretative standards for *E. coli* for selected antimicrobial disks

Antibiotic Groups	Antibiotic	Abbreviation	Disc content (µg)	Zone diameter breakpoint (mm)	
				S ≥	R <
Cephalosporins	Cefpodoxime /Clavulanic Acid	CPD / CLAV	30/10	Z(CPD / CLAV)-Z(CPD)≥5mm ESBL +ve	
	Cefotaxime /Clavulanic Acid	CTX / CLAV	30/10	Z(CTX /CLAV)-Z(CTX)≥5mm ESBL +ve	
	Ceftazidime /Clavulanic Acid	CAZ / CLAV	30/10	Z(CAZ /CLAV)-Z(CAZ)≥5mm ESBL +ve	
	Cefpodoxime	CPD 30	30	20	20
	Cefotaxime	CTX 30	30	20	17
	Ceftazidime	CAZ 30	30	22	19
Penicillins	Ampicillin	AP 10C	10	14	14
	Augmentin	AUG 10C	10	17	17
	Piperacillin / Tazobactam	PTZ 36C	30/ 6	20	17
Miscellaneous agents	Chloramphenicol	C 30C	30	17	17
Fluoroquinolones	Ciprofloxacin	CIP 5C	5	22	19
Aminoglycosides	Gentamicin	GM 10C	10	17	14
Carbapenems	Imipenem	IMI 10C	10	22	17
	Meropenem	MEM 10C	10	22	16

µg = Microgram, mm = Millimeter, S = Sensitive, R = Resistance (Andrews, 2009; EUCAST, Testing 2011)

2.17 **Tissue culture investigations**

2.17.1 **Attachment and invasion assays**

Attachment and invasion assays were completed to examine the capability of selected bacterial strains to attach and invade mammalian cells (Caco2, rBCEC4 and HBMEC) as described by Townsend *et al*. (2007a). Attachment and invasion assays were performed at the same time using the same cell line passage with same inoculum of mammalian cell line and bacterial suspension respectively.

2.17.1.1 **Mammalian cell lines**

Bacterial attachment and invasion of eukaryotic epithelial cells was determined *in vitro* by performing adhesion and invasion assays using Caco-2 cells (Human colonic carcinoma epithelial cells, ECACC# 86010202), obtained from the European Collection of Cell Cultures, rBCEC4 cells (Rat Blood Brain Barrier cells), obtained from I. E. Blasig (Forschungsinstitut für Molekulare Pharmakologie, Berlin, Germany) and HBMEC cells (Human brain microvascular endothelial cells), supplied by Dr M. Rittig (University of Nottingham) and originally used by Lum *et al*., (2003). All cell lines were stored in the liquid nitrogen.

2.17.1.2 **Culture Media**

All media were obtained from Sigma Aldrich; UK. Caco-2 cells are grown in Minimum Essential Medium, Media Sigma Aldrich, UK (M4655) supplemented with 10% fetal bovine serum Sigma Aldrich, UK (F7524), 1% non-essential amino acid

Sigma Aldrich, UK (M7145) solution and 1% Penicillin-Streptomycin Sigma Aldrich, UK (p4333). The infection media was minimum essential medium with 10% fetal bovine serum and 1% non-essential amino acid solution. Growth medium for rBCEC4 and HBECE was 4.5 g / l, 4 mM glutamine, 110 g/l sodium pyruvate (Sigma Aldrich, UK (M4655) with 10% fetal bovine serum 1% penicillin and streptomycin and 1% non-essential amino acid solution. The infection medium for rBCEC4 and HBECE was 4.5 g /l, 4 mM glutamine and 110 g /l sodium pyruvate Sigma Aldrich, UK (M4655) with 10% fetal bovine serum and 1% non-essential amino acid solution.

2.17.1.3 **Bacterial strains**

Following PFGE analysis, representative strains of the pulse-types were chosen for further analysis Table 2.10. These were eight strains of *E. coli*, eight strains of *Serratia* spp. and four strains of *E. cloacae* and *Salmonella Entertidis* strain number NCTC 3046 (358 NTU) as a positive control for Caco-2 cell line, *Citrobacter koseri* strain number SMT319 (NTU 48) as positive control for rBCEC4 and HMBEC cell lines and *E. coli* K12 (1230) was the negative control for all experiments of tissue culture. A single colony was growth in TSB after harvesting from TSA plates for 18 h at 37ºC under aerobic conditions. Overnight cultures (120 µl) were inoculated into 5 ml of infection media and incubated with shaking 200 rpm at 37ºC for ~ 2 h to obtain the OD of 0.3 - 0.5 at 600 nm, using a spectrophotometer (JENWAH, UK). The suspension was then diluted to obtain the number of 2 x 10^8 cells per well to obtain Multiplicity of infection (MOI) of 1 - 100.

2.17.1.4 **Mammalian cells culture**

All cell lines (Caco-2, rBCEC4 and HBMEC) were stored in liquid nitrogen until required. In this investigation a volume of frozen vials for each cell line were thawed quickly until only a small amount of ice remained in the vial. All the contain was transferred to a 10 ml tube containing 6 ml pre-warmed growth medium as mentioned above in section 2.17.1.2. The cells were harvested by centrifugation. After that, in a 10 ml tube the pellet was suspended gently in 6 ml of growth medium adding with 100 µl of gentamicin just in the first passage. The suspension then was transferred to 25 cm^3 tissue culture flasks and incubated at 37ºC under 5% CO_2 for 48 h to achieve confluent monolayer cells. The cells were then harvested by detachment using trypsin-EDTA solution and centrifugation of the released suspended cells at 1300 rpm for 5 min. All cells were split routinely twice a week under growth continued. For attachment and invasion assays these were done to obtain the number of 2 x 10^4 cells (0.5 ml/well) in 24 tissue culture plates.

2.17.1.5 **Adhesion Assay**

Mammalian cells (Caco-2, rBECE4 and HBECE) were grown as described in section 2.17.1.4. Cells were seeded at 2 x 10^4 /well. The organism was then added at 1 x 10^8 CFU /well. This was then incubated for 2 h at 37ºC under 5% CO_2. In order to determine the average of adhesion, cells were washed three times with PBS before harvesting with Triton X-100. This assay showed the total number of bacteria that were attached to the surface and those that were intracellular. The overall number of

viable bacteria was determined using the serial dilution and Miles Misra method on TSA plates.

2.17.1.6 **Gentamicin assay (Invasion Assays)**

For invasion assays as per attachment studies mammalian cells (Caco-2, rBCEC4 and HBMCE) were seeded at 2 x 10^4 /well. The organism was then added at 1 x 10^8 cfu per well and this was then incubated for 3 h at 37ºC under 5% CO_2. Then the cells were washed three times with PBS before adding gentamicin 0.1 mg/ml to each well followed by further incubation for 1 h to kill attached bacteria. The cells were washed three times before harvesting with Triton X-100. The total number of viable bacteria was determined using serial dilution and Miles Misra method on TSA plates. All strains were analysed in triplicate.

All strains of bacteria were assayed in three independent assays.

2.17.2 **Uptake and persistence into macrophage cell line U937**

Macrophages were obtained from the American Type Culture Collection (ATCC) (CRL-1593.2) U937. Mammalian cell lines U937 used in this study were stored in the liquid nitrogen.

2.17.2.1 **Culture media**

Macrophage cells were cultivated in RPMI medium Sigma Aldrich, UK (1640) with 2 mM L-glutamine Sigma Aldrich, UK (R8758), and modified to contain 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g /l glucose, 1.5 g /l sodium bicarbonate, and

supplemented with 10% fetal bovine serum. At least 24 h prior to infection, cells were treated with 0.1 µg/ml of PMA (phorbol 12-myristate 13-acetate) Sigma Aldrich, UK (P8139), placed into 75 cm^2 tissue culture flasks at 37ºC, under 5% CO_2 to become activated.

2.17.2.2 **Macrophage Assays**

The selected bacterial strains were investigated for their uptake and persistence in macrophages as described by Townsend *et al*. (2003). In summary, one ampoule of macrophage cells (U937) was collected from the liquid nitrogen and thawed quickly, then seeded in 10 ml of RPMI supplemented with nutrients to meet the growth conditions as previously described into 75 cm^2 tissue culture flasks. Then it was incubated under 5% CO_2 at 37ºC for cell cultivation. After activation and cultivation the cells were centrifuged at 1500 g for 5 min. Cells in the pellet were then counted low and re-suspended into maturation medium as described previously. The cells were seeded 500 µl into 24 well plates. At 1 x 10^5 cells in each well and incubated 48 h at 37ºC, 5% CO_2 incubator to allow cell maturation and adherence. One day prior to cell infection, one bacterial colony was isolated from TSA and inoculated into 3 ml TSB which was then incubated aerobically overnight at 37ºC with vigorous shaking. Three millilitres of infection medium were inoculated using 60 µl of the inoculated TSB and incubated 2 hours at 37°C with vigorous shaking to obtain the OD between 0.3 and 0.5 at 600 nm. The bacteria were then diluted into 6 ml infection medium to get obtain the multiplicity of infection 1: 100. For the tissue culture well plates the medium was discarded from the wells and the cells were then washed twice with 500 µl phosphate buffer saline to remove the residual PMA. The

tissue culture plates were divided into 4 groups to determine macrophage uptake and bacterial persistence into the cell line for 72 h. Then 500 µl was dispensed into each well and incubated for 1 h at 37ºC in a 5% CO_2 incubator to allow the matured macrophage cells to take-up the bacterial cells. The infection medium was then discarded, and the plates washed gently twice using phosphate buffer saline. Five hundred microliters of the concentration of gentamicin 100 µg/ ml were dispensed into each well and incubated for 1 h at 37ºC into CO_2 incubator to eliminate untaken bacteria. Plates were then washed gently twice with 500 µl phosphate buffer saline. One group was then treated with 100 µl of 1% Triton X-100 with 10 times pipette flush to determine the macrophage uptake serially diluted and the number of viable bacterial cells was enumerated using the Miles Misra technique. For the other three groups, 500 µl of growth medium supplemented with 10 µg/ml gentamicin were added to each well. One group was taken for each 24 h washed twice and treated with 100 µl Triton X 100, the viable bacteria was enumerated as described above. The persisted bacteria was calculated by calculating the number of cells that was survived against the number of bacteria after 45 min or of the persistence after 24, 48 and 72 h survived per well was expressed as a percentage of the bacterial number added to the well at the start of the experiment.

All strains of bacteria were assayed in 3 independent assays.

2.17.3 **Giemsa staining**

The Giemsa stain was performed to determine the adherence pattern of selected strains. The assays for Caco-2 and Hep-2 cells were undertaken as described above

in section 2.17.1.5 except small modifications that cells monolayers were grown over tissue culture coverslips in six-well tissue culture plated (Rüttler *et al*., 2006). Briefly, Chamber slides were seeded with 2 x 10^4 Caco-2 and same for Hep-2 cells line and then incubated at 37ºC with 5% CO_2 for 48 h. After incubation period the coverslip monolayers were infected with 10^8 per well overnight growth of bacteria as described in 2.17.1.2 and were incubated at 37ºC under 5% CO_2 for 2 h. After the incubation period the coverslip was washed three times with sterile PBS. The coverslip slides were fixed with absolute methanol for 5 min and allowed to air dry. The cells were stained with 5% of Giemsa stain (Life Technologies™; UK 10092 - 013) for 15 min, washed with sterile PBS and allowed to air dry. The slide was examined using light microscopy (X100) with oil immersion.

2.18 **Oxidative and nitrosative stress**

The investigation of oxidative and nitrosative stress of *Enterobacteriaceae* were determined by exposing strains to four using the with a disk diffusion assay approach on agar plates as described by Baillon *et al*., (1999). *S*-nitrosoglutathione (N4148), Methyl viologen (856177), cumene hydroperoxide (247502) and hydrogen peroxide (88597) were obtained from Sigma Aldrich; UK. From frozen stock strains were plated on TSA, incubated for 18 h at 37ºC. After incubation, one colony was plated out again in 3 ml of TSB and incubated with shaking 180 rpm for 18 h at 37ºC. Overnight cultures (60 µl) were inoculated into 3 ml of TSB and incubated with shaking 175 rpm at 37ºC for ~ 2 h to obtain the optical density of ~ 0.4 at 600 nm, using a spectrophotometer (JENWAH, UK). After adjusting OD, the 200 µl of culture was inoculated into 4 ml of soft TSA agar and poured onto TSA plates. After

solidification of the soft TSA agar, four sterile 6mm disks were placed on each agar plate. The concentrations of chemicals were prepared at 100 mM of *S*-nitrosoglutathione; 3% (w/v) of methyl viologen; 3% (w/v) of cumene hydrogenperoxide and 3% (w/v) hydrogen peroxide. 3 µl for each chemical were inculcated onto each disk. After dispensing the chemicals the agar plates were incubated 18 h at 37ºC. Finally for each chemical the diameter of the zone was measured to determine if the strains are resistant or sensitive to the chemicals.

2.19 **Molecular biology investigations**

2.19.1 **Bacterial strains**

Eight strains of *E. coli* were chosen in this part after show interesting results in physiological and tissue culture studies Table 2.11.

Table 2.11 Bacterial strains of molecular biology investigations

Organism	Strain	pulsetype
E. coli	780	EcC4
E. coli	904	EcC3
E. coli	923	≠
E. coli	939	≠
E. coli	1008	U
E. coli	1009	EcC2
E. coli	1047	CcE1
E. coli	1050	≠

≠ = same

2.19.2 **DNA extraction**

DNA extraction from of *E. coli* and references strains was performed using the GeneElute^{TM} kit Sigma Aldrich, UK (NA2110-1KT) according to the manufacturer's instructions. Briefly, a single colony from overnight culture TSA plate was inoculated into 3 ml of TSB and incubated for 24 h at 37ºC. Afterwards, 1.5 ml of overnight culture was centrifuged at 13,000 ×g, at 4ºC for 2 min. The pellet was suspended in 180 µl of lysis solution T and incubated for ~2 min at room temperature. A 20 µl of the proteinase K solution 20 mg/ml then were added to the mixture and incubate at 55ºC for 30 min. after incubation 200 µl lysis solution C was added to the mixture, mixed and incubated for 10 min at 55ºC. In parallel, during incubation time a column was prepared by adding 500 µl of column preparation solution and then it was centrifuged at 13,000 ×g, at 4ºC for 1 min. Subsequently, the mixture was transferred into the binding column after lysed cells by adding with 200 µl of absolute ethanol and then spin for 1 min at ≥ 6500 ×g. washing the column, 500 µL of wash Solution 1 and 2 were added 1 and 3 times and centrifuged at 6500 ×g, 13,000 ×g, at 4ºC for 1 min respectively with change the column each time. After washing DNA samples were eluted 200 µl of elution solution, centrifuged at 6500 ×g at 4ºC for 1 min, and DNA concentration confirmed by using a NanoDrop® ND-2000 UV-Vis spectrometer (Thermo Scientific; USA), and stored at -20ºC.

2.19.3 **Multilocus sequence typing (MLST)**

Multilocus sequence typing (MLST) of *E. coli* strains was performed as described by Wirth *et al*., (2006) (http://mlst.ucc.ie/mlst/dbs/Ecoli). Seven housekeeping genes of *E.coli* were amplified by PCR using the primers, *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase), and *recA* (ATP/GTP binding motif). See Table 2.6 for details.

2.19.4 **Polymerase chain reaction amplification**

The total volume of PCR reaction was 30 µl, which contained 6 µl (5X PCR buffer), 1 µl (5 µM primer pair working solution) of each primer, 0.6 µl (10 mM dNTPs) 1 µl (50 mM $MgCl_2$), 0.2 µl (5 U/µl Taq polymerase), 2 µl (50 ng template DNA) and 19 µl of ultrapure water. The PCR reactions was achieved with the PCR machine as follows: 1 cycle at 95ºC for 1 min followed by 95ºC for 30 sec, 54 - 60ºC (according to the variable for primer pairs temperature) for 30 sec, and at 72ºC for 30 sec and a final elongation step at 72ºC for 10 min. Successful PCR product size was confirmed by agarose gel electrophoresis.

2.19.5 **Confirmation of PCR products by agarose gel electrophoresis**

The PCR amplification products were visualised by gel electrophoresis on agarose gels (Life Science ® Company, UK, BIO-41025). Electrophoresis was performed by mixing a 1% (w/v) agarose gel in 1 X TAE buffer which prepared as described in above in section 2.6.8. The mixture was microwaved until melted and then the

molten gel cooled to ~50ºC. 0.001% of SYBR® Safe DNA Gel Stain Life Technologies, Invitrogen™, UK (S33102) was added into molten gel and mixed before pouring into a gel tray. The gel was left to set at room temperature, and then placed into a Mini-Sub® Cell GT tank (Bio-Rad Laboratories Ltd., Hertford, UK) containing 1 X TAE buffer. Loading gel was done with 5 µl of sample and a 100 bp DNA ladder (Promega, Southampton, UK) to indicate product size [Appendix 7.2]. The run condition of the gel was 90 V for 30 min and DNA bands were visualised and photographed using UV InGenius® gel documentation system (Syngene, UK).

2.19.6 **Purification of PCR products**

PCR amplification products were purified using QiaQuick PCR purification kit (Qiagen, UK, 28106) to remove other components from the PCR reactions to use for DNA sequencing. Purification was completed as described in the manufacturer's protocol. Briefly, 125 µl of buffer PB was added to 25 µl of the PCR sample and mixed. The mixture was placed into a QIAquick spin column in a provided 2 ml collection tube and then centrifuged at 13,000 rpm for 1 min to binding DNA. Prior to washing flow-through was discarded. 750 µl Buffer PE was added to the QIAquick column and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and the QIAquick column was placed back in the same tube and centrifuged at 13,000 rpm for an additional 1 min. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube. Elute DNA was performed by adding 50 µl elution buffer to the centre of the QIAquick membrane and centrifuged at 13,000 rpm for 1 min. The purified PCR product was stored at -20ºC until required.

2.19.7 **MLST DNA Sequencing**

The reaction products and primers volumes and concentration for MLST sequencing were adjusted as follow: sample about 8 - 10 ng/µl and primer about 3.5 pmol/µl. About 10 µl for each reaction, forward and reverse of the reaction products were then sequenced by Source Bioscience Sequencing http://www.lifesciences.sourcebioscience.com.

2.19.8 **Phylogenetic of DNA sequence**

After sequencing, the reaction products were imported as the FASTA files and then the sequences from both strands of a given locus of the same isolate were aligned using the software CLC Sequence Viewer 6.6 http://www.clcbio.com. To analyse, trim and assign the sequences were determined via MLST *E. coli* database website http://mlst.ucc.ie/mlst/dbs/Ecoli. The trimmed sequences were compared against the MLST database and the sequence types (STs) were determined by submitting the allele number to the MLST *E .coli* database website http://mlst.ucc.ie/mlst/dbs/Ecoli.

2.20 **Detection of virulence factor (VF) genes**

The presence of 29 VFGs for *E. coli* was determined using previously published 5 multiplex PCR–based assays (Johnson and Stell, 2000) using primers Table 2.8. VFGs gene classes were included adhesins (*papAH, papC, papEF, papG, sfa/focDE, sfaS, focG, afa/draBC, bmaE, gafD, nfaE* and *fimH*), toxins (*hlyA, cnf1, cdtB),* siderophores (*fyuA, iutA*), polysaccharide coatings (*kpsMT* II *kpsMT* III, *kpsMT* K1, *kpsMT* K5), invasins (*ibeA)* and others (*rfc, cvaC, traT,* PAI). Multiplex polymerase

chain reaction amplification was performed in a 25 µl reaction mixture containing 5 µl (5X PCR buffer), 0.5 µl (30 µM) of each primer (except for those marked "*", which were used 0.25 µl at a concentration of 30 µM), 1 µl (20 µM dNTPs) 4 µl (25 mM $MgCl_2$), 0.5 µl (5 U/µl Taq polymerase), 2 µl (50 ng template DNA) and the volume was then made up to 25 µl with ultrapure water according to primer pools. The 5 primer pools Appendix 7.3, with the 29 primer pairs were listed as follows: pool 1: PAI (930), *papA* (720), *fimH* (508), *kpsMT* III (392), *papEF* (336), and *ibeA* (171); pool 2, *fyuA* (880), *bmaE* (507), *sfa/focDE* (410), *iutA* (302), *papG* allele III (internal; 258), and K1 (153); pool 3: *hlyA* (1177), *rfc* (788), **nfaE* (498), **papG* allele I (internal; 491), **kpsMT* II (272), and **papC* (200); pool 4: *gafD* (952), *cvaC* (680), *cdtB* (430), *focG* (360), *traT* (290), and *papG* allele II (internal; 190); and pool 5: *papG* allele I (flanking; 1190), *papG* alleles II and III (flanking; 1070), **afa/draBC* (559), **cnf1* (498), **sfaS* (240), and K5 (159). Reactions were heated to 95ºC in an automated thermal cycler (PTC-100-96; MJ Research, Watertown, MA) for 12 min to activate the AmpliTaq Gold. This was followed by 25 cycles of denaturation (94ºC, 30 sec), annealing (63ºC, 30 sec), and extension (68ºC, 3 min) and a final extension (72ºC, 10 min). Successful PCR products were confirmed by agarose gel electrophoresis.

2.21 **Detection of β-lactamase genes**

The presence of 4 β-lactamase genes for *E. coli* were determined by screen for the resistance genes *SHV*, *TEM*, *CTX-M*, and *OXA* (Table 2.7) by a multiplex PCR assay (Fang *et al*., 2008). PCR amplification reactions were performed in a volume of 25 µl containing 5 µl (5 X PCR buffer), 2 µl (2.5 µM each primer), 0.25 µl (25 µM

dNTPs) 2.5 µl (50 mM $MgCl_2$), 0.25 µl (5 U/µl Taq polymerase), 2 µl (50 ng template DNA) and 11 µl of ultrapure water. The cycling parameters were as follows: 1 cycle at 94ºC for 1min followed by 94ºC for 30 sec, 54ºC for 30 sec, and at 72ºC for 30 sec and a final elongation step at 72ºC for 10 min. The amplified PCR products were subjected to electrophoresis at a 2% agarose gel in 1 X TAE buffer. Strains with known β-lactamase types were included as references. These were NCTC 13351 *E. coli TEM*-3, NCTC 13353 *E. coli CTX-M*-15 (*TEM*, *CTX-M*, *OXA*), NCTC 13368 *K. pneumoniae SHV*-18 (*TEM*).

2.22 **Statistical analysis**

All experiments were repeated at least twice in triplicate. One way ANOVA was used to obtain the consistence of the independent experiments, the significance was set at $p < 0.05$.

Chapter 3 **Genotyping profiles of bacterial isolates from neonatal enteral feeding tube**

3.1 **Introduction**

The identification and characterization of bacterial isolates and their subtyping is increasingly important, and can be used to determine whether the strains are clonally related. There are a number of phenotypic and genotypic techniques which previously have been employed for microbial identification and classification (Tenover *et al*., 1995; Nazarowec-White and Farber, 1999). These days molecular typing methods of microbes have become commonplace in public and private laboratories. Subtyping of isolates to the strain level is an important tool, including studying if the bacteria are clonally related as this would help identify a common source in nosocomial outbreaks or potential cross-transmission events and sources of infection (Adamsson *et al*., 2000). This has been even been extended to the use of whole genome sequencing, which becoming central to research of human disease and also start to be used in routine clinical care (Bick and Dimmock, 2011).

Numerous genotypic methods are available for the identification and characterisation of micro-organisms. These methods include pulsed-field gel electrophoresis (PFGE) for the whole genome (CDC, 2004; Tenover *et al*., 1995; Bick and Dimmock, 2011), random amplification of polymorphic DNA (RAPD) (Olive and Bean, 1999) amplified fragment length polymorphism (AFLP) (Goulding *et al*., 2000), restriction fragment length polymorphisms (RFLP) (Foley *et al*., 2009), multi-locus enzyme electrophoresis (MLEE) (Caugant *et al*., 1986), and multi-locus sequence typing (MLST) (Wirth *et al*., 2006; Maiden *et al*., 1998). Molecular typing techniques can be additional classified into two subgroups; DNA-sequence-based and DNA pattern-based methods.

PFGE has been referred to as the gold standard of molecular typing methods. It is has a high discriminatory power with high resolution and reproducibility for many species, compared to other typing methods (Olive and Bean, 1999). The Centres for Disease Control and Prevention (CDC) and PulseNet have standard protocols for the comparison of PFGE patterns for major foodborne pathogens *Salmonella*, *E. coli* O157:H7, *Shigella*, *Campylobacter jejuni*, *Listeria monocytogenes* and *Yersinia pestis* (CDC, 2004; Cooper *et al*., 2006).

PFGE involves the digestion of whole bacterial DNA by restriction enzymes. There are many different restriction enzymes used such as *Xba*I, *Spe*I, *Apa*I, *Sma*I, and *Not*I. These are often used to improve characterized and discrimination among bacteria (Tenover *et al*., 1995). The restriction enzymes cut the genomic DNA into a few fragments (8 – 25) which are separated using agarose gel electrophoresis. Banding patterns can be stored electronically and analysed using commercially available software such as BioNumerics (CDC, 2004).

A criterion proposed by Tenover *et al*., (1995) for the analysis of restriction patterns is often used to interpret the PFGE patterns in epidemiological investigations. PFGE patterns showing no differences in banding pattern can be considered the same strains and if between 1 - 3 different bands they can be considered closely related to each other (i.e. clonal). Isolates showing between 4 - 6 different bands can be considered possibly related to each other. Strains showing 7 different bands or more are considered to be unrelated to each other (Ribot *et al*., 2006). In addition the comparison of patterns is possible via the internet or software such as BioNumerics , which were used in this study (Hunter *et al*., 2005). The similarity values are based

on the Dice coefficient, unweight pair group method with arithmetic mean band profile of reference standard (Vauterin and Vauterin, 2006).

This chapter investigates further the genotypic similarities and differences between collections of enteral feeding tube isolates by PFGE. The bacterial genera will include *Klebsiella*, *Serratia*, *Enterobacter* and *Escherichia*. It is plausible that during the previous study by Hurrell *et al*. (2009a); Table 1.2 that the same strains were either isolated from the same neonate on more than one occasion, or even from different neonates. Therefore DNA fingerprinting via PFGE will be used to identify if the same strains were isolated on different occasions from the neonatal intensive care unit (NICUs). This may also reveal if certain strains were indigenous to the NICU, and if there are different populations between the two NICUs. The PFGE profiles will be compared with clinical isolates to determine if any are directly linked to previous neonatal infections. These organisms commonly carry extra-chromosomal material (encoding antibiotic resistance factors) for each strain will be determined.

MLST is a highly discriminatory method for typing and detecting epidemiologically important strains (Urwin and Maiden, 2003). Wirth *et al*., (2006) constructed a comprehensive MLST scheme for *E. coli* based on the partial sequence analysis of 7 housekeeping genes *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase) and *recA* (ATP/GTP binding motif). Therefore, we investigated whether the frequency of isolation by *E. coli* is associated with particular genotype by compiling patient

details and isolation site, for clinical *E. coli* isolates and comparing these with the isolates ST profile.

3.2 **Materials and methods**

The key methods, culture media and culturing conditions for this part were described previously in chapter 2 of materials and methods.

3.3 **Results**

3.3.1 **Pulsed Field Gel Electrophoresis**

A dendrogram and band assignment of all strains was achieved using BioNumerics software, version 3.5. Dice coefficient, unweight pair group method with arithmetic mean (UPGMA) were used for cluster analysis. Less than 95% of band similarity value was used to consider the isolates to be non-clonal (Tenover *et al*., 1995). The tolerance and optimization of the bands was 1.5%. Two-hundred and sixty-two strains of *Enterobacteriaceae* which had been isolated from neonatal enteral feeding tubes, different patients and date were analysed. The PFGE analysis of these isolated were based on the analysis criteria that strains differing in their restriction fragment patterns belonged to different clonal clusters. PFGE patterns were compared of between 9 - 18 bands, with an average of 13 bands per gel. *Salmonella enterica* serovar Typhimurium reference standard H9812 (NTU strain 732) was used as the reference strain and gave distinct bands in the *XbaI*-digested PFGE profile; [Appendix 7.1].

3.3.2 **PFGE profile analysis *Enterobacteriaceae* strains from enteral feeding tubes**

As shown in Figure 3.1, the typing of thirty of *E. coli* isolates from neonatal enteral feeding tubes from City Hospital (NCH) and Queen's Medical Centre (QMC), Nottingham, showed the strains clustered into four pulsetypes, and one unique strain. Figure 3.2 shows PFGE typing of sixty PFGE banding patterns for *Serratia* isolated from neonatal enteral feeding tubes from two local hospitals were formed four distinguishable pulsetypes. Fifty PFGE patterns for *Enterobacter* which were isolated from neonatal enteral feeding tubes from neonatal enteral feeding tubes also from Nottingham are shown in Figure 3.3 which was formed eight distinguishable pulsetypes and six unique strains. Figure 3.4 shows forty eight PFGE patterns of *Klebsiella* isolates from neonatal enteral feeding tubes from QMC and NCH. These strains were formed 13 distinguishable pulsetypes. Figure 3.5 shows the PFGE typing of thirty eight of *E. coli* isolated from Jordan were formed three distinguishable pulsetypes and one unique strain. It should be noted that all strains were isolated from neonatal enteral feeding-tubes. Unfortunately no clinical data were provided for the patients.

To overcome the ambiguity of phenotypic identification of *Enterobacter* and *Klebsiella*, all strains were subject to PFGE. After PFGE cluster analysis, a number of *E. coli* strains were selected for MLST profiling. It should be noted that Figure 3.1 - Figure 3.4 are the bacterial species identification as according to phenotyping, however some strains were later renamed following PFGE analysis and MLST

sequence analysis. Some strains were re-identified as different species, and were given to H. Abdalla for her studies of *Klebsiella* and *Enterobacter hormaechei*.

3.3.2.1 *E. coli* strains from QMC and NCH

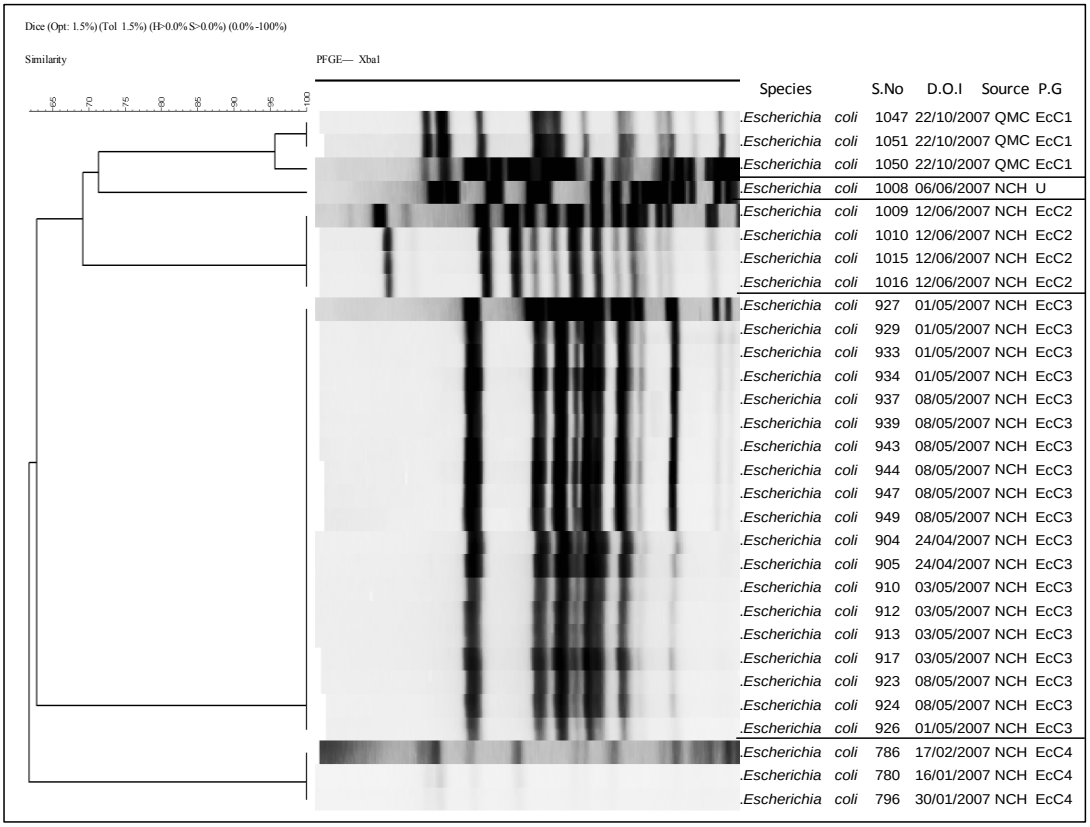

Figure 3.1 PFGE profiles of thirty *E. coli* strains isolated from neonatal enteral feeding tubes

Dendrogram obtained from cluster analysis by Bionumerics software, version 3.5, Dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimization in the band was 1.5%. The first column from right to left, PFGE cluster group, U = unique, the hospital identifier, NCH = Nottingham City Hospital (n = 27), QMC = Queen's Medical Centre (n = 3), DOI = Date of isolation, S.No = Strain number.

Table 3.1 Cluster analysis of *E. coli* isolates

Species	Hospital	Strain Number	PFGE cluster	Number of strains	Period of isolation	Patients	Feeding source
E. coli	NCH	1009, 1010, 1015, 1016	EcC2	4	Same day	3	BMF, BM
		904, 905, 910, 912, 913, 917, 923, 924, 926, 927, 929, 933, 934, 937, 939, 943, 944, 947, 949	EcC3	19	Two weeks	11	BMF, BM, PIF, IF, Th, RTF
		780, 786, 796	EcC4	3	One month	2	BM, BMF, PIF, Th
		1008	U	1	One day	1	BMF, PIF, Th
	QMC	1047, 1050, 0151	EcC1	3	One day	1	RTF

NCH = Nottingham City Hospital. QMC = Queen's Medical Centre. U = Unique. EcC = E. coli cluster BM = Breast milk, BMF = Breast milk fortified, PIF = Reconstituted powdered infant formula, IF = Infant formula, RTF = Ready to feed formula, Th = Thickener added to feed

Table 3.1 summarises the results of Figure 3.1. The thirty isolates of *E. coli* were from two local hospital NCH (n = 27, 90%) and the remaining 3 were from QMC. These strains were isolated from enteral feeding tubes as a biofilm. The strains were clustered into four pulsetypes; EcC1, EcC2, EcC3 and EcC4 and are described briefly below.

Table 3.1 shows that a number of neonates were described as receiving a 'mixed feeding regime'. For example, pulsetype EcC3 contain 19 strains were isolated from 11 neonates; those neonates received breast milk and fortified breast milk, whereas others received breast milk and reconstituted PIF. A thickener was added to feeds to reduce reflux for neonates receiving fortified breast milk, ready to feed formula, reconstituted PIF, and mixed feed [Appendix 7.8].

- Three strains belonged to pulsetype EcC1, which were isolated on the same day (22 October 2007), and from the same patient.

- Four strains formed pulsetype EcC2 which were isolated on same day (12 June 2007) from 3 different patients.
- A total of nineteen strains were isolated over a two week period (24 April, 3 May, and 8 May 2007). These strains were belonged to pulsetype EcC3, and were isolated from 11 different patients.
- Three strains belonged to pulsetype EcC4 and were isolated over a one month period (16 January, 30 January and 16 February 2007). They were isolated from 2 different patients.
- In addition there was one unique strain.

3.3.2.2 ***Serratia* spp.**

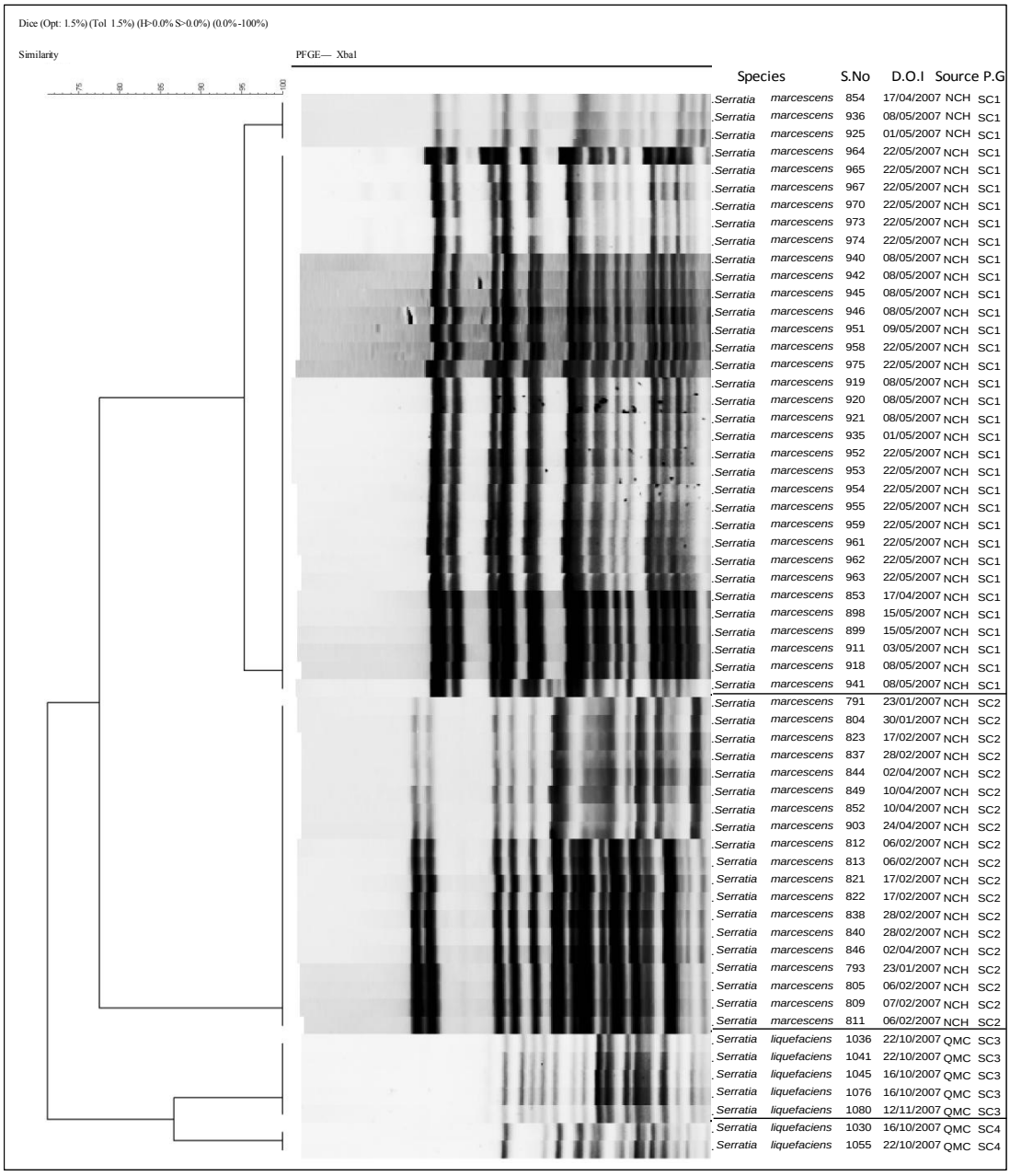

Figure 3.2 PFGE profiles of sixty *Serratia* spp. isolated from neonatal enteral feeding tubes

Dendrogram obtained from cluster analysis by Bionumerics software, version 3.5, dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimization in the band was 1.5%. The first column from right to left, PFGE cluster group, the hospital identifier NCH: Nottingham City Hospital (n = 53), QMC: Queen's Medical Centre (n = 7), DOI = Date of isolation, S.No = Strain number.

Table 3.2 Cluster analysis of *Serratia* spp.

Species	Hospital	Strain Number	PFGE cluster	Number of Strains	Period of isolation	Patients
S. marcescens	NCH	853, 854, 898, 899, 911, 918, 919, 920, 921, 925, 935, 936, 940, 941, 942, 945, 946, 951, 952, 953, 954, 955, 958, 959, 961, 962, 963, 964, 965, 967, 970, 973, 974, 975	SC1	34	Over one month	20
		791, 793, 804, 805, 809, 811, 812, 813, 821, 822, 823, 837, 838, 840, 844, 846, 849, 852, 903	SC2	19	Over three months	12
S. liquifaciens	QMC	1036, 1041, 1076, 1045, 1080	SC3	5	About one month	5
		1030, 1055	SC4	2	One week	2

NCH = Nottingham City Hospital. QMC = Queen's Medical Centre**.** SC = *Serratia* cluster

Figure 3.2 shows the dendrogram of sixty isolates of *Serratia* species from NCH (n = 53, 88.3%) and QMC (n = 7, 11.7%). Table 3.2 summarises these results. These isolates were grouped into four distinguishable pulsetypes SC1, SC2, SC3 and SC4. Strains of each pulsetypes SC2, SC3 and SC4 showed 100% of similarity coefficient (SC). Whereas pulsetype SC1 is composed of two sub-clusters: 3 and 31. These were composed of identical strains (SC 100%) and the similarity coefficient of 854, 936 and 925 with others was SC 95%. Pulsetypes SC1 and SC2 were *S. marcescens*. These were isolated from Nottingham, City Hospital, while pulsetypes SC3 and SC4 were *S. liquifaciens* which were isolated from Queen's Medical Centre. Overall, the *Serratia* pulsetypes were isolated from a large number of patients over a wide time period.

- A total of 34 *S. marcescens* strains belonged to pulsetype SC1. These were isolated over a prolonged period of over one month (17 April, 1, 3, 8, 9,

15 May and 22 May 2007), and furthermore were isolated from 20 different patients.

- Nineteen strains belonged to pulsetype SC2. This pulsetype was isolated over a prolonged period of four months (23, 30 January, 6, 7, 17, 28 February, 2, 10 April and 24 April 2007), and were from 12 different patients.
- Five strains belonged to cluster SC3. These were isolated over extended periods of about one month (16, 22 October and 12 November 2007).They were also isolated from 5 different patients.
- There were two strains in pulsetype SC4, which were isolated at one week period (16 and 22 October 2007), and were from different patients.

3.3.2.3 ***Enterobacter* spp.**

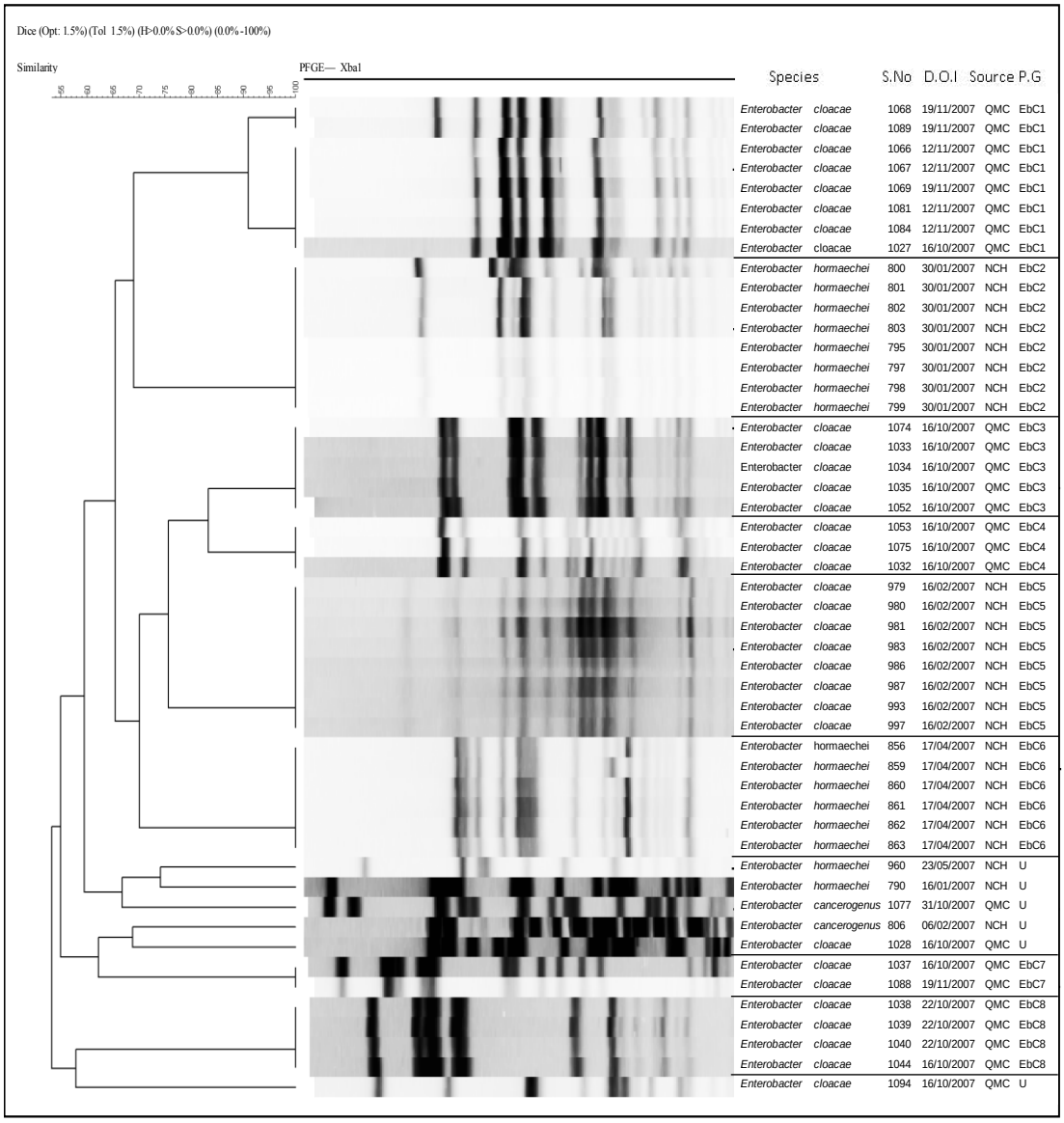

Figure 3.3 PFGE profiles of fifty *Enterobacter* spp. isolated from neonatal enteral feeding tubes

Dendrogram obtained from cluster analysis by Bionumerics software, version 3.5, Dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimization in the band were 1.5%. The first column from right to left: PFGE cluster group, U = unique, the hospital identifier NCH = Nottingham City Hospital (n = 25), QMC = Queen's Medical Centre (n = 25), DOI = Date of isolation, S.No= Strain number.

Table 3.3 Summary of cluster analysis for *Enterobacter* spp.

Species	Hospital	Strain Number	PFGE cluster	Number of Strains	Period of isolation	Patients
E. hormaechei	NCH	795, 797, 798, 799, 800, 801, 802, 803	EbC2	8	Same day	6
E. cloacae		979, 980, 981, 983, 986, 987, 993, 997	EbC5	8	Same day	1
E. hormaechei		856, 859, 860, 861,862, 863	EbC6	6	Same day	3
E. cloacae	QMC	1027,1066, 1067, 1068, 1069, 1081, 1084, 1089	EbC1	8	One month	3
		1033, 1034, 1035, 1052, 1074	EbC3	5	Same day	2
		1032, 1053, 1075	EbC4	3	Same day	2
		1037 ,1088	EbC7	2	Three days	2
		1039, 1044, 1038, 1040	EbC8	4	One week	2
		1028	U	1	One day	1
		1094		1	One day	1
E. cancerogenus		1077		1	One day	1
E. cancerogenus	NCH	806		1	One day	1
E. hormaechei		960		1	One day	1
		790		1	One day	1

Strains, study neonate, date of isolation and hospital, NCH = Nottingham City Hospital. QMC = Queen's Medical Centre. U = Unique. EbC = *Enterobacter* cluster

The result of Figure 3.3 are summarised in Table 3.3. Fifty isolates of *Enterobacter species* from NCH (n=25, 50%) and QMC (n=25, 50%). These isolates were clustered into eight distinguishable pulsetypes (EbC1 – EbC8) and six unique strains as shown in the dendrogram. All strains of each pulsetypes showed 100% of similarity coefficient (SC). The strains were isolated from neonatal enteral feeding-tubes but no further clinical data were provided. The *Enterobacter* spp. pulsetypes were isolated from a number of patients, and over a considerable time period:

- Over more than one month (16 October, 12 November and 19 November 2007) eight strains of *E. cloacae* were isolated which belonged to pulsetype EhC1. These strains were isolated from 3 different patients.

- Eight strains of *E. hormaechei* in pulsetype EhC2, were isolated on the same day (30 January 2007), nonetheless were isolated from 6 different patients.
- Five strains of *E. cloacae* formed pulsetype EhC3 were isolated on the same day (16 October 2007), and isolated from 2 different patients.
- Three strains of *E. cloacae* were formed pulsetype EhC4, which were isolated on the same day (16 October 2007). There were isolated from 2 different patients.
- Eight strains of *E. cloacae* formed pulsetype EhC5 were isolated on the same day (16 February 2007), and were isolated from same patient.
- Six strains of *E. hormaechei* formed pulsetype EhC6 were isolated on the same day (17 April 2007), from 3 different patients.
- Two strains of *E. cloacae* belonged to pulsetype EhC7, which were isolated over a one month (16 October and 19 November 2007). There were isolated from 3 different patients.
- Four strains of *E. cloacae* were belonged to pulsetype Eh8, which were isolated at one week period (16 and 22 October 2007), also were isolated from 2 different patients.

3.3.2.4 ***Klebsiella* spp.**

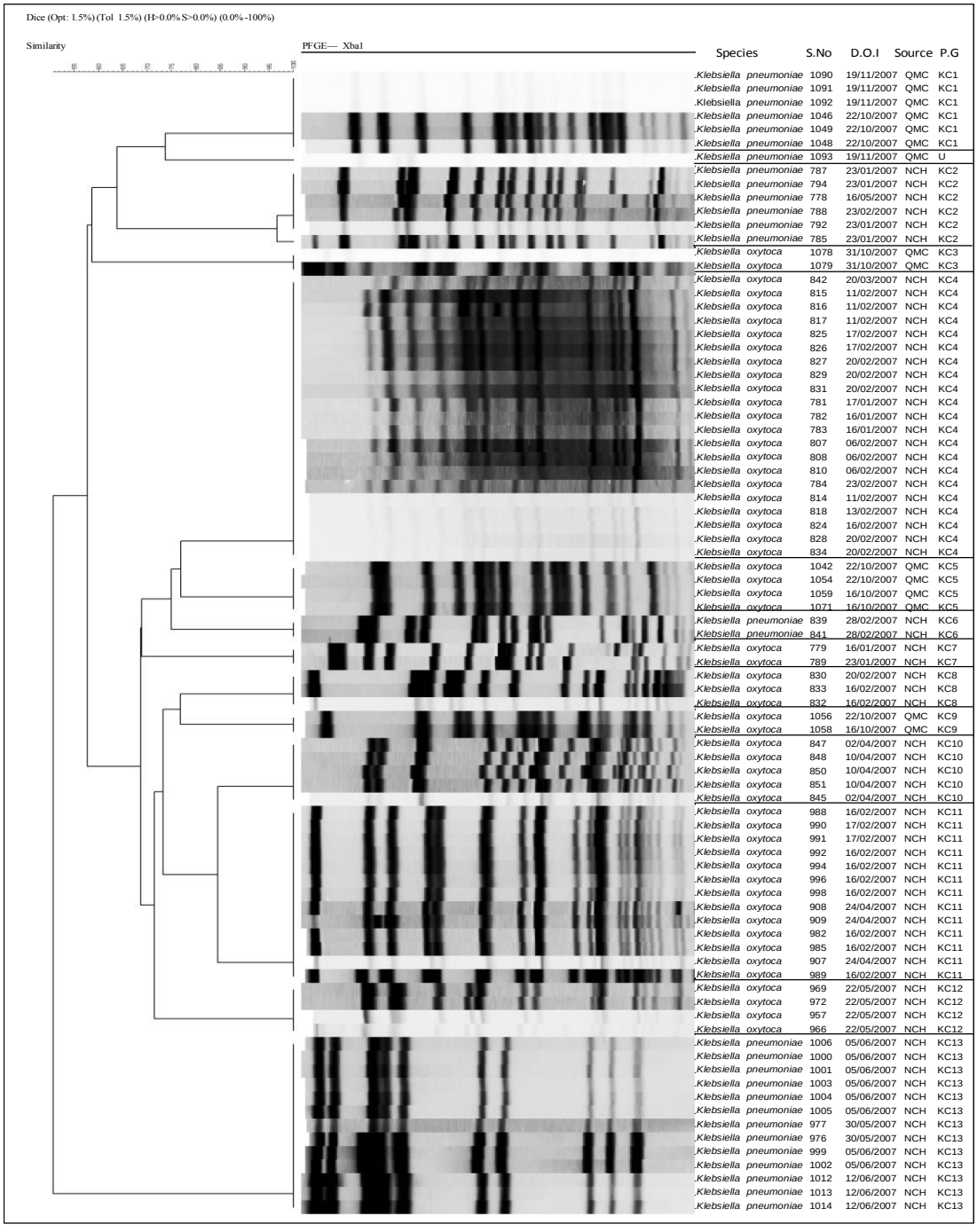

Figure 3.4 PFGE profiles of forty-eight *Klebsiella* spp. isolated from neonatal enteral feeding tubes

Dendrogram obtained from cluster analysis by Bionumerics software, version 3.5, Dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimization in the band were 1.5%. The first column from right to left, PFGE cluster group, U = unique, the hospital identifier NCH = Nottingham City Hospital (n = 69) QMC = Queen's Medical Centre (n = 15), DOI = Date of isolation, S.No = Strain number.

Table 3.4 Cluster analysis of *Klebsiella* spp.

Species	Hospital	Strain Number	PFGE cluster	Number of Strains	Period of isolation	Patients
K. pneumoniae	NCH	778, 785, 787, 788, 792, 794	KC2	6	Over five months	6
K. oxytoca	NCH	781,782,783,784, 807, 808, 810, 814, 815, 816, 817, 818, 824, 825, 826, 827, 828, 829, 831, 834, 842	KC4	21	Over two months	12
K. oxytoca	NCH	839, 841	KC6	2	One day	1
K. oxytoca	NCH	779, 789	KC7	2	One day	1
K. oxytoca	NCH	830,832,833	KC8	3	One week	2
K. oxytoca	NCH	845, 847, 848, 850, 851	KC10	5	One week	2
K. oxytoca	NCH	907, 908, 909, 982, 985, 988, 989, 990, 991, 992, 994, 996, 998	KC11	13	Over two months	3
K. oxytoca	NCH	957, 966, 969, 972	KC12	4	Same day	3
K. pneumoniae	NCH	976, 977, 999, 1000, 1001, 1002, 1003, 1004, 1005, 1006, 1012, 1013, 1014	KC13	13	Over two weeks	5
K. oxytoca	QMC	1046,1048, 1049, 1090, 1091, 1092	KC1	6	One month	2
K. oxytoca	QMC	1078, 1079	KC3	2	One day	1
K. oxytoca	QMC	1042, 1054, 1059, 1071	KC5	4	One week	3
K. oxytoca	QMC	1056, 1058	KC9	2	One week	1
K. pneumoniae	QMC	1093	U	1	One day	1

NCH = Nottingham City Hospital. QMC = Queen's Medical Centre. U = Unique. KC = *Klebsiella* cluster

Table 3.4 above is a summary of the results in Figure 3.4. The forty-eight isolates of *Klebsiella* spp. were from NCH (n = 69, 82.3%) and QMC (n = 15, 17.7%). These were clustered together into 13 pulsetypes KC1 – KC13 and one unique strain. In addition, all strains in each pulsetypes showed 100% of similarity coefficient (SC). All strains were isolated from neonatal enteral feeding-tubes and no clinical data was provided for the patients. These pulsetypes were clustered of strains isolated over a wide time period.

- Six strains of *K. pneumoniae* were belonged into pulsetype KC1. These strains were isolated from 2 different patients during one month (22 October and 19 November 2007).

- Pulsetype KC2 was composed of 4 strains of *K. pneumoniae*. These strains were isolated over a prolonged period of more than five months (23 January, 23 February and 16 May 2007), and were isolated from 3 different patients.
- Two strains of *K. oxytoca* belonged to pulsetype KC3, which were isolated on the same day (31 October 2007) and from same patient.
- Twenty-one strains of *K. oxytoca* were formed pulsetype KC4, which were isolated over more than two months (16, 17 January, 6, 11, 13, 16, 17, 20, 23 February and 20 March 2007). These were from 12 different patients.
- At one week period (16 and 22 October 2007) four strains of *K. oxytoca* were isolated which belonged to pulsetype KC5. These were isolated from 2 different patients.
- Pulsetype KC6 was composed of two strains of *K. pneumoniae*. These strains were isolated on the same day (28 February 2007), and similarly were isolated from 1 patient.
- Pulsetype KC7 was composed of two strains of *K. oxytoca* were isolated during one week (16 and 23 January 2007), and were isolated from 2 patients.
- Three strains of *K. oxytoca* belonged to pulsetype KC8, which were isolated on one week (16 and 20 February 2007), and isolated from 2 different patients.
- Two strains of *K. oxytoca* were belonged to pulsetype KC9, which were isolated over a one week period (16 and 22 October 2007). These strains were isolated from 1 patient.
- Five strains of *K. oxytoca* formed pulsetype KC10, which were isolated over a one week period (2 and10 April 2007), and were from 2 different patients.

- From 3 different patients 13 isolates of *K. oxytoca* were recovered which belonged to pulsetype KC11. They were isolated over more than two months (16, 17 February and 26 April 2007).
- Four strains of *K. oxytoca* belonged in pulsetype KC12, which were isolated same day (22 May 2007), and were from 3 different patients.
- A total of 13 strains of *K. pneumoniae* belonged to pulsetype KC13, which were isolated over a two week period (30 May, 5 and 12 June 2007), and from 5 different patients.

3.3.2.5 ***E. coli* isolated from neonatal enteral feeding tubes in Jordan**

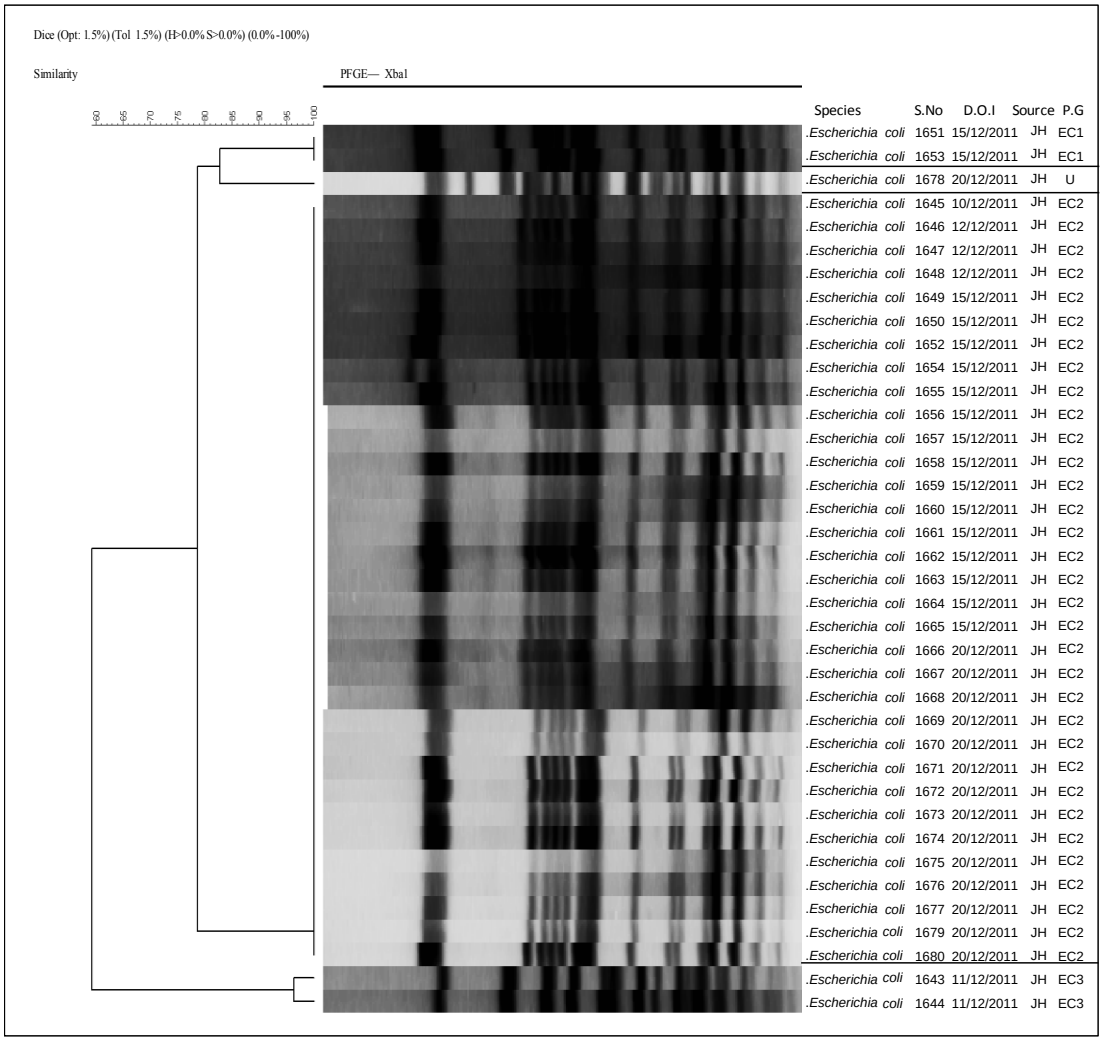

Figure 3.5 PFGE profiles of thirty eight strains of *E. coli*

Dendrogram obtained from cluster analysis by Bionumerics software, version 3.5, Dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimization in the band were 1.5%. The first column from right to left, PFGE cluster group, U = unique, the hospital identifier, DOI = Date of isolation, S. No. = Strain number.

Table 3.5 Cluster analysis of *E. coli* from neonatal enteral feeding tubes in Jordan

Species	Strain Number	PFGE cluster	Number of Strains	Period of isolation	Patients
E. coli	1651, 1653	EC1	2	Same day	1
	1645, 1646,1647,1648, 1649, 1650, 1652, 1654, 1655, 1656, 1657, 1658, 1659, 1660, 1661, 1662, 1663, 1664, 1665, 1666, 1667, 1668, 1669, 1670, 1671, 1672, 1673, 1674, 1675, 1676, 1677, 1679, 1680	EC2	33	Ten days	12
	1643, 1644	EC3	2	Same day	1
	1678	U	1		1

U = Unique. EC = *E. coli* cluster

Table 3.5 above summarises the cluster analysis shown in Figure 3.5. Thirty-eight strains of *E. coli* isolated from neonatal enteral feeding tubes from a hospital in Jordan were grouped into three pulsetypes; EC1, EC2 and EC3 and one unique strain.

- Two strains belonged to pulsetype (EC1), which were isolated on same day (16 and 22 October 2007), and were from the same patient.
- Total of 33 strains were formed pulsetype (EC2) which isolated over one week period (16 and 22 October 2007). These were isolated from 12 different patients.
- Two strains belonged to pulsetype (EC3) were isolated on ten days; in addition they were isolated from the same patients.

3.3.3 **Multi-locus sequence typing (MLST) of *E. coli* isolates from Nottingham neonatal intensive care units**

Eight strains of *E. coli* were selected for MLST*;* 780, 904, 923, 939, 1008, 1009, 1047 and 1050. These were selected according to pulsotype grouping and represented thirty strains of *E. coli* isolated from neonatal enteral feeding tubes used in Nottingham City Hospital and Queen's Medical Centre, UK.

The sequence types (ST) obtained for eight strains are given in Table 3.6 . Five STs were identified across the pulsotype groups, and were internally consistent within the clonal group. EcC1, EcC2, EcC3, EcC4, and unique isolates corresponded with sequence types ST2076, ST73, ST95, ST127 and ST394, respectively. According to the MLST database at the ERI, University College Cork (http://mlst.ucc.ie/mlst/dbs/Ecoli/GetTableInfo_html) all strains were belonged to the *E. coli* pathogenic group B2.

Table 3.6 MLST profile of selected *E.* coli strains

***E .coli* strain**	**PFGE pulsotype**	**Housekeeping gene**								
		adk	***fumC***	***gyrB***	***icd***	***mdh***	***purA***	***recA***	**ST**	**PG**
780	EcC4	21	35	61	52	5	5	4	394	B2
904	EcC3	37	38	19	37	17	11	26	95	〃
923	〃	37	38	19	37	17	11	26	95	〃
939	〃	37	38	19	37	17	11	26	95	〃
1008	U	13	14	19	36	23	11	10	127	〃
1009	EcC2	36	24	9	13	17	11	25	73	〃
1047	EcC1	21	35	61	52	5	77	4	2076	〃
1050	〃	21	35	61	52	5	77	4	2076	〃

EcC = *E. coli* cluster, ST sequence type, PG = pathogenic group, 〃 = same

3.4 **DISCUSSION**

To control and reduce outbreaks, it is very important to rapidly and accurately discriminate and identify sources and routes of transmission of bacterial pathogens. Molecular subtyping methods is an important tool in epidemiological studies and if the strains are clonally related then this will assist in determining a common source of infection causing nosocomial outbreaks or potential cross-transmission events and sources of infection (Adamsson *et al*., 2000).

In this study a collection two hundred fifty six *Enterobacteriaceae* strains isolated from the enteral feeding tubes of neonates on intensive care units from two local hospitals in Nottingham UK were analysed as the primary research focus. However later an additional collection of thirty-eight *E. coli* strains from Jordan were obtained for preliminary profiling. Unfortunately, because of the long time between the collection and this study, clinical details (ie. infection) were not available to us. Therefore, this study is not an epidemiological outbreak investigation. Instead, it has focused on the discrimination and identification of the isolates. Nevertheless, neonatal information regarding, strain, study neonate, date of isolation are shown in the tables above. This project describes the use of DNA fingerprinting of these *Enterobacteriaceae* strains via PFGE, to determine if the same strains were isolated on different occasions from the NICUs. Therefore indicating whether certain strains have colonised the neonatal enteral feeding tubes could be led to colonise the NICUs leading to increased exposure and risk to the neonates.

This study shows that over the period from 17th January to 19th November 2007, neonatal enteral feeding tubes were colonised by four pulsetypes of *E. coli*, four pulsetypes of *Serratia* spp., eight pulsetypes of *Enterobacter* spp. and thirteen pulsetypes of *Klebsiella* spp. for which no definitive sources were identified. The pulsetypes of *E. coli* and *Serratia* spp. were less diverse than the other species. This indicates that a narrower range of strains was colonising the enteral feeding tubes.

The PFGE profiles of strains indicated the existence of clones and were designated as genetically indistinguishable; for example *E. coli* isolates exhibited three small pulsetype groups EcC1, EcC2 and EcC4 which containing 3, 4 and 3 strains respectively and a large group (EcC3) which comprising 19 strains. The EcC3 pulsetype was isolated during two week period from 26 April to 9 May. It was isolated from eleven patients. *Serratia* spp. isolates encompassed two species *S. marcescens* and *S. liquifaciens*. *S. marcescens* formed two large pulsetype groups SC1 and SC2, which included 34 and 19 strains respectively. These pulsetypes were isolated over a prolonged period from 19 April to 23 May and from 24 January to 26 April respectively. In addition, SC1 and SC2 were isolated from twenty and twelve patients respectively. This indicates the existence of a clone for each group, which were showed 100% of similarity coefficient (SC).

Interestingly the clonally related strains of *E. coli* EcC3 could be confirmed that the same clone were cross contamination to different feeding tube and prevalence of the same clone among those recovered from different patients for short term. In addition this could be confirm that, with rustle of collection of *E. coli* from Jordan, these collection shown large pulsetype group EC2 comprised 33 strains out of 38 strains

E. coli Table 3.5. While SC1 and SC2 of *S. marcescens* were demonstrated analysis indicates that the *S. marcescens* more prevalence indistinguishable clone were contaminated different feeding tube. This in addition provided cross-transmission indistinguishable clone were recovered from different patients for long period. Our findings also indicate that the neonates were fed by shows that a number of Patients were described as receiving a 'mixed feeding regime'. For example, pulsetype EcC3 contain 19 strains were isolated from 11 neonates; those neonates received breast milk and fortified breast milk, whereas others received breast milk and reconstituted PIF. A thickener was added to feeds to reduce reflux for neonates receiving fortified breast milk, ready to feed formula, reconstituted PIF, and mixed feed that may possibly originate from the same sources, especially for substances such as environment or carer see Table 3.1 and for more information see [Appendix 7.8].

In contrast with *E. coli* and *Serratia* spp., the *Enterobacter* species and *Klebsiella* species pulsotypes showed a large diversity (Figure 3.3 and Figure 3.4), showing that wider ranges of strains were isolated from the enteral feeding tubes. *Enterobacter* species and *Klebsiella* spp. exhibited multiple pulsetypes KC 8 and KC 13 respectively. This diversity in PFGE profiles indicates the contamination of feeding tube could be from different sources. Nonetheless pulsetype KC4 of *K. oxytoca* were recovered from twelve patients and also were isolated over more than two months.

Eight strains of *E. coli*, representing the various pulsetypes were selected for MLST analysis of *E. coli*. These were from two neonatal intensive care units. As shown in Table 3.6, the concatenated MLST sequences showed clear discrimination between

the 8 strains of *E. coli* ST2076, ST73, ST95, ST394 and ST127. All the STs comprised a varying number of isolates. Interestingly, included in the current study, remarkable genetic similarity among the PFGE profiles observed among isolates of STs, for example *E. coli* strains 904, 923 and 939 exhibited indistinguishable PFGE profiles and were the same ST. *E. coli* ST95 was isolated over a two week period from three neonates, and therefore of concern due to the continued exposure.

Reviewing the MLST database at the ERI, University College Cork (http://mlst.ucc.ie/mlst/dbs/Ecoli/GetTableInfo_html) revealed that the prevalent ST95 in this study has been associated with various infections, including meningitis, diarrhoea, septicaemia and invasion. According to the database three strains out of one-hundred sixty-four strains of ST95 had caused meningitis. These strains are reference named as ISUNMEC48, ISUNMEC36 and ISUNMEC70; two from Europe and one from North America respectively.

Chapter 4 **Physiological and virulence related traits of *Enterobacteriaceae* isolated from neonatal enteral feeding tubes.**

4.1 **Introduction**

Newborns baby are at increased risk of bacterial infection and currently our understanding of the fundamental mechanisms of the immune system in neonates is incomplete. In addition, bacterial infections of newborn babies are associated with higher morbidity and mortality than older children (Levy *et al*., 2004). Many neonates are not feed breast milk when they are born, but reconstituted powdered infant formula. Despite the common mis-conception that such products are sterile, the Codex Alimentarius Commission reported that powdered infant formula does contain a variety of bacteria, including opportunistic pathogens (CAC, 2007). The FAO/WHO, (2004 and 2006) reviewed the microbiological status of powdered infant formula and reported that *Enterobacteriaceae* present were 'causality plausible, but not yet demonstrated' with respect to neonatal infections and their occurrence in powdered infant formula and follow-on formula. Previous studies by Gastmeier *et al*. (2007) and Kaufman and Fairchild (2004) showed the rate of neonatal infections by *Enterobacteriaceae* has increased and are one of the most predominant pathogen in intensive care units outbreaks. Gastmeier *et al*. (2007) reported that the most common bacterial pathogen causative infections are *Klebsiella*, staphylococci and *Serratia* spp. The study by Stoll *et al*. (2005) proposed that pathogenic *E. coli* are one of the causative neonatal infections such as meningitis and sepsis. Hurrell *et al*. (2009b) found that the neonatal enteral feeding tube acts as loci for in the attachment and colonisation by *Enterobacteriaceae*. Consequently, enteral feeding tubes are an important risk factor with respective to neonatal infections.

Hurrell *et al*., (2009b) investigated *Enterobacteriaceae* isolated from the enteral feeding tubes which had been collected from two NICUs in Nottingham. The organisms had attached inside the feeding tubes and formed biofilms Figure 1.1 and Table 1.2. In addition, they found that the *Enterobacteriaceae* biofilms formed in tube which had been used to feed babies in both infant formula and breast milk. Another study by Hurrell *et al*. (2009a) used laboratory-based experiments to investigate the effect of acidity on bacteria in reconstituted powdered infant formula and breast milk. They found that bacteria can survive at acid pH values in the range 2.5 to 4.3.

The bacterial capsule is a layer on the outside of bacterial cells. It is composed of a polymeric material consisting of polysaccharides, polypeptides or polynucleotides (Forsythe, 2010). The capsule formation as a stress protection factor by bacteria and is considered a virulence factor which can protect the organisms from macrophages (Daffe and Etienne, 1999). Capsule formation by *Enterobacteriaceae* is linked to pathogenicity (Pluschke *et al*., 1983). In addition capsulated bacteria are more resistant to stress conditions (Caubilla-Barron *et al*., 2007; Beuchat *et al*., 2009). Investigations by Iversen *et al*., (2004) found that the capsulated strains of *Cronobacter sakazakii* were able to form more biofilms than non-capsulated strains.

Curli fimbriae also have a role in biofilm formation (Barnhart and Chapman, 2006). *E. coli*, *Salmonella* serovars and other *Enterobacteriaceae* produce curli fimbriae and/or cellulose (Zogaj *et al*., 2003). Austin *et al*., (1998) found *Salmonella* Enteritidis expressed curli fimbriae that were able to form biofilm on abiotic surfaces. Most pathogenic strains of *E. coli are* able to produce curli fimbriae

(Bian *et al*., 2000). Type 1 fimbriae are a feature of *E. coli* strains and have a role in adherence and invasion of intestinal epithelial cells (Boudeau *et al*., 2001).

In the immune system, the phagocytic cells such as macrophages produce reactive oxygen species (ROS) and nitric oxide species (NOS) to kill invading microbes (Burmester and Pezzutto, 2003; Urban *et al*., 2006; Kehrer, 2000). ROS comprise two major groups: free radicals, O_2 or hydroxyl moiety (OH) and non-radical of O_2 like hydrogen peroxide (H_2O_2) or peroxynitrite ($ONOO^-$) (Paravicini and Touyz, 2008) the effect of oxidative stress on cells leads to oxidation, DNA damage, methylation of DNA, deamination and depurination (Ames *et al*., 1993). Nitric oxide synthase (NOS) produces NO. Three different NOS are produced by endothelial: NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Kone *et al*., 2003). Bogdan *et al*., (2000) reported that the killing of microbes was dependent upon iNOS-derived NO.

In this chapter the experimental work was focussed on comparing enteral feeding tube isolates of *E. coli*, *Serratia* spp. and *Enterobacter cloacae*. These strains were subjected to a range of physiological and virulence related tests. The physiological studies included determining biofilm formation, as neonatal exposure to the bacteria may in part be due to the extent of biofilm formation on the enteral feeding tube. This will be determined by biofilm formation on polymeric substances. Capsule production will be determined by colony morphology on milk formula agar plates. To mimic ingestion, bacteria will be exposed to the acidity of the neonate stomach. Hence survival curves for the strains will be determined at 37°C in infant formula

adjusted with HCl to pH3, as representative of the neonate stomach (Hurrell *et al*., 2009b).

Virulence traits determined were those considered relevant to an enteroinvasive bacterial pathogen. These included motility, curli fimbriae, haemolysis on blood agar and oxidative stress. Many of these have been previously used at NTU to study the related *Enterobacteriaceae C. sakazakii* and *E. hormaechei*. The susceptibilities of *E. coli* isolates to antimicrobial agents will be determined by the breakpoint method on antibiotic supplemented Iso-Sensitest agar according to the British Society for Antimicrobial Chemotherapy protocol. Extended spectrum β-lactamases (ESBL) production will be detected using the combination disc method and presence of ESBL for *E. coli*. These experiments will assess the potential susceptibility of neonates to infections and efficacy of first choice antibiotics in subsequent treatment.

4.2 **Materials and methods**

The methods section, culture media and culturing conditions for this part were as previously described in Chapter 2 Materials and Methods.

4.2.1 **Result**

4.2.2 **Biofilm formation**

Biofilm formation was studied in twenty strains of *Enterobacteriaceae*. These were eight strains of *E. coli* (780, 904, 923, 939, 1008, 1009, 1047 and 1050), eight strains of *Serratia* species (792, 805, 936, 942, 946, 952, 1030 and 1036) and four strains of

E. cloacae (979, 1027, 1028 and 1074) Table 2.9. Strains were selected as representatives of the PFGE pulsotypes. In this study pulsotypes group were used to represent groups of *Enterobacteriaceae* strains, which were isolated from enteral feeding tubes.

The ability of *Enterobacteriaceae* strains, isolated from enteral feeding tubes at two local hospitals in Nottingham during 2007, to form biofilms on plastic surfaces is shown in Figure 4.1 and Figure 4.2. Two factors influenced biofilm formation: incubation temperature (25°C and 37°C) and type of formula (casein and whey).

Figure 4.1 shows the biofilm formation of eight strains of *E. coli*, six strains of *S. marcescens*, two strains of *S. liquifaciens* and four strains of *E. cloacae*. All strains showed the ability of form biofilm compared with the control, and there was variation between strains. The highest values were by *S. liquifaciens* in both formula types. The average of the absorbance at 540 nm was between 1.5 to 1.75 absorbance units (AU). All *E. coli* strains showed the lowest amount of biofilm formation with whey formula, the average of the absorbance at 540 nm was between 0.75 to 1 AU, and were higher when grown in casein formula. *S. marcescens* and *E. cloacae* showed the same values in both formula types. Their average absorbance values at 540nm was between 1 - 1.5 AU.

Figure 4.1 and Figure 4.2 compared the production of biofilm according to the incubation temperature and type of formula. The effect of temperature was apparent. All selected strains of *Enterobacteriaceae* were able to form biofilm at 37ºC compared with the control sample; Figure 4.1. While at 25ºC all strains only produced a small amount of biofilm which was close to the control value; Figure 4.2.

One way ANOVA test was performed to compare the effect of temperatures. This showed significantly higher biofilm formation at 37ºC ($p < 0.05$) compared to 25ºC.

There was no influence due to the type of formula on biofilm formation at 25ºC, with all strains showing same rate of biofilm formation, whereas the effect of the formula type was apparent at 37ºC for some strains. All *E. coli* strains, two strains of *S. marcescens* (791 and 805) and *S. liquifaciens* (1036) in casein based formula was significantly higher compared to biofilm formation in whey based formula ($p < 0.05$). While the others strains did not shown any variation attributable to formula types.

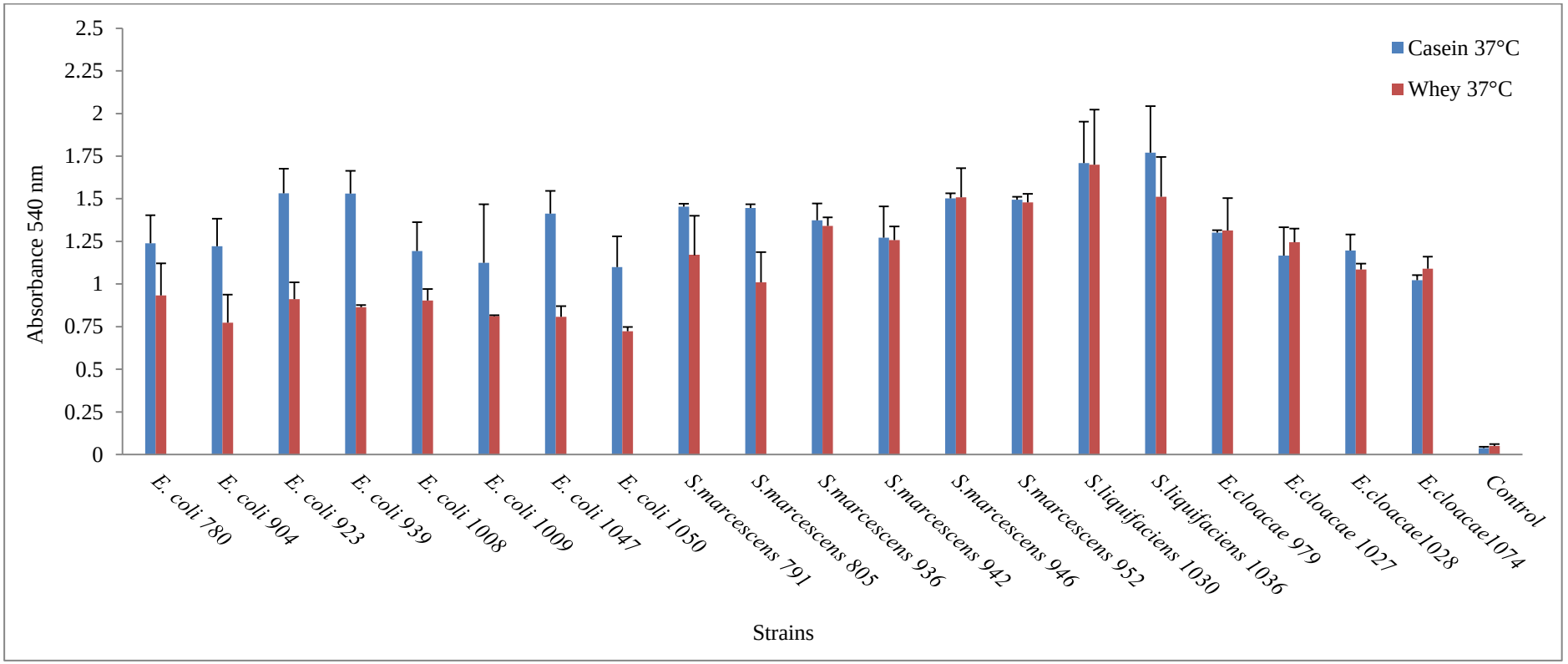

Figure 4.1 Biofilm formation at 37ºC temperatures in two different formula types (whey and casein)

Biofilms were stained with 0.01% (w/v) crystal violet, which was analysed at 540 nm; control was inoculated formula. Experiments were assayed in three independent assays. Error bars represented using standard error.

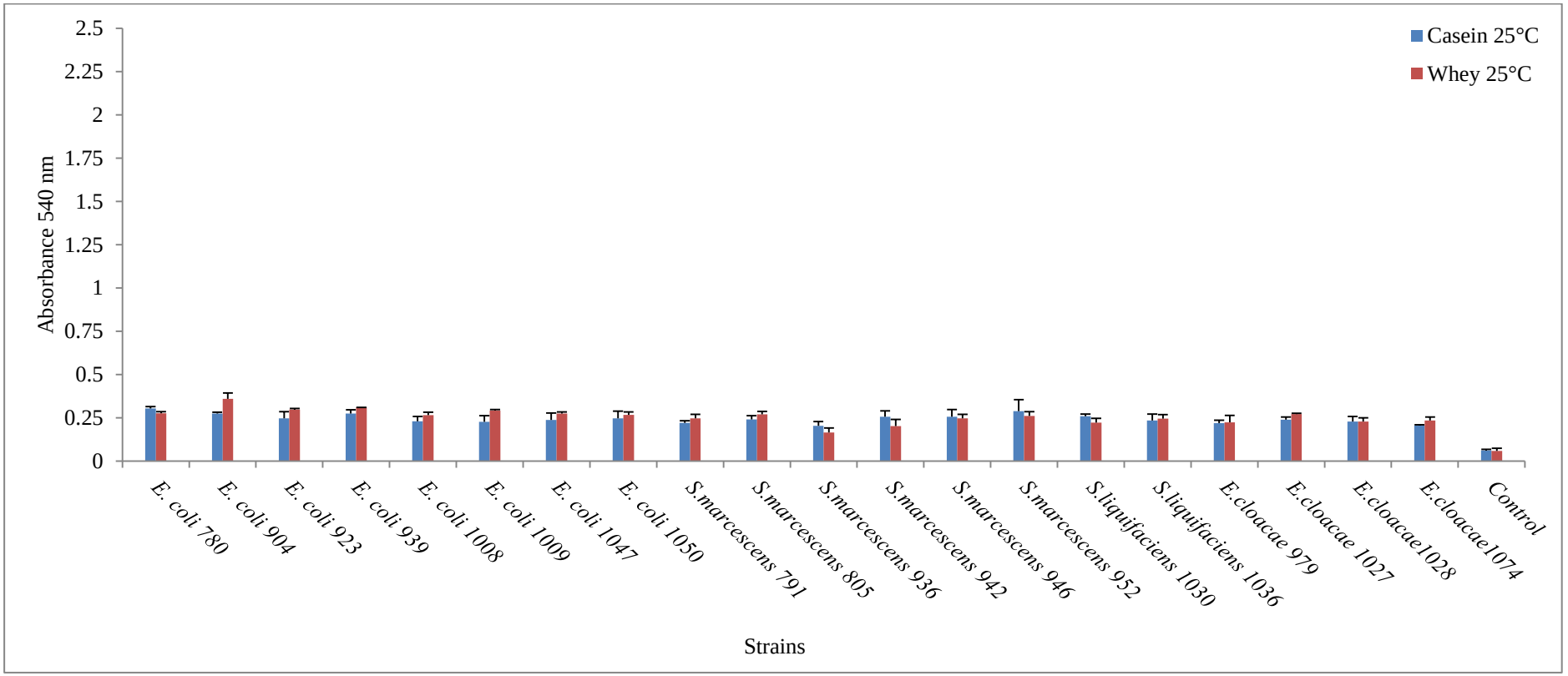

Figure 4.2 Biofilm formation at 25ºC temperatures in two different formula types

Data presented in this figure are the same of the presented data in figure one. Biofilms were stained with 0.01% (w/v) crystal violet which was analysed at 540 nm; control was taken as formula without inoculation. Experiments were assayed in three independent assays. Error bars represented using standard error.

4.2.3 **Motility**

The motility of the *Enterobacteriaceae*, *E. coli*, *Serratia* species and *E. cloacae* strains were demonstrated by growth on TSB supplemented with 0.4% agar by measuring the diameter of growth after 24 h the results and are shown in Figure 4.3. Strains showed a wide-range of motility zone diameter. *S. liquifaciens* strain 1030 showed highest amount of motility, approximately 40mm diameter. On the other hand, five *E. coli* strains 780, 904, 923, 939 and 1009, *S. marcesens* strain 936, *S. liquifaciens* 1036 and *E. cloacae* 979 were non-motile. While the remaining strains were showed different swarm zone diameters between 15 to 35 mm. For example the motility zone for *E. cloacae* strain 1028, *S. marcesens* strain 946 and *E. coli* strain 1047 were 20 mm, 30 mm and 35 mm respectively.

There was a variation range in motility between strains in the same pulsetype; Figure 4.3. Two strains of *E. coli* 1047 and 1050 from same pulsetype EcC3 showed different swarm zone diameters. These were 8 mm and 18 mm, respectively. Three strains 942, 946 and 952 of *S. marcescens*, which belong to pulsetype SC1, demonstrated high motility. The diameter of their swarm zone was nearly 30 mm, whereas a fourth SC1 strain; 939 was non-motile. The motility diameters for strains 791 and 805 both pulsetype SC2 were 25.5 and 16.5 mm, respectively. In general, for comparison data of all strains, *E. coli* was less motile than *Serratia* spp. and *E. cloacae* strains.

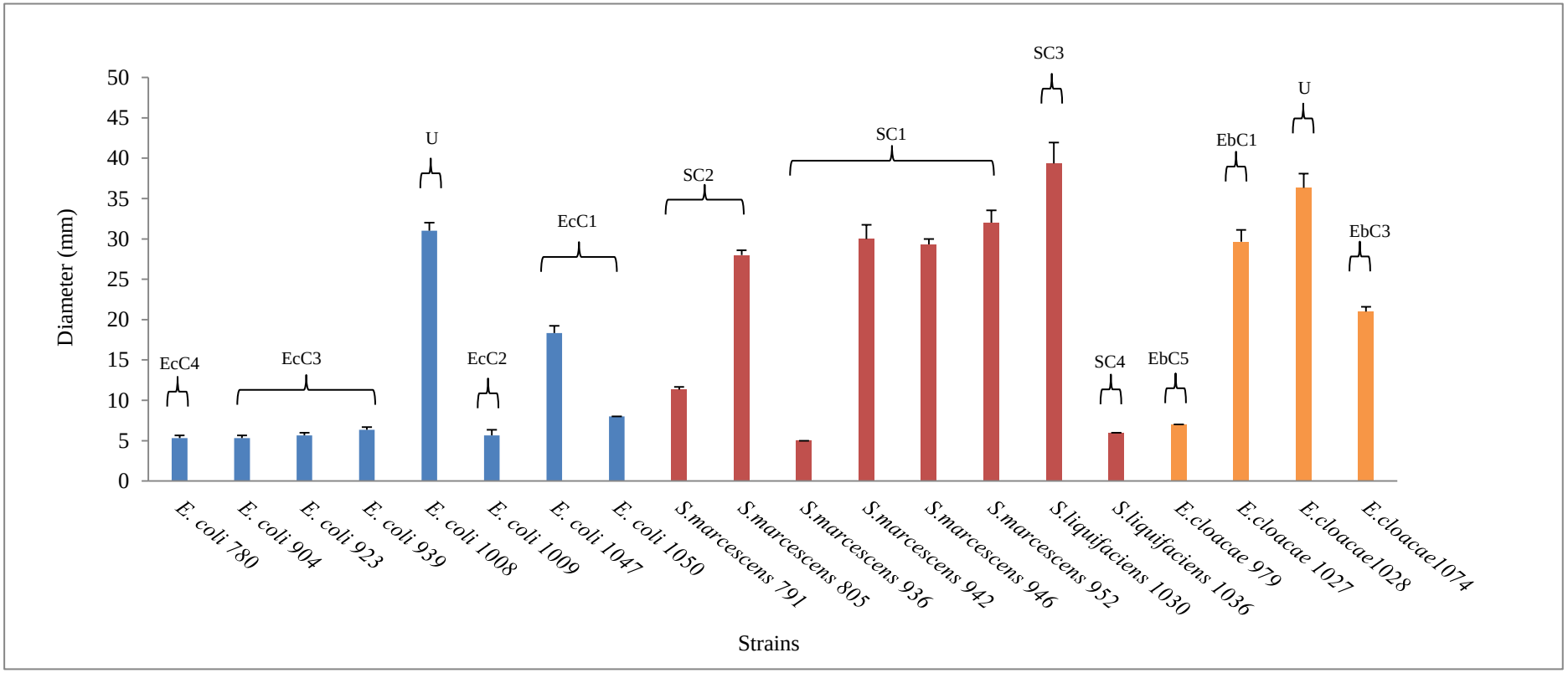

Figure 4.3 Motility of *E. coli*, *Serratia* spp. and *E. cloacae* strains at 37ºC for incubated 18 h

The diameter for the motility zone was measured in millimetres. Standard deviation represents the error bars, Experiments were assayed in three independent assays. U= unique. EcC, SC and EbC= cluster group of Pulsed Field Gel Electrophoresis.

4.2.4 **Acid sensitivity**

Twenty strains of *Enterobacteriaceae*, including eight strains of *E. coli*, *Serratia* spp. and *E. cloacae* were investigated for their tolerance to pH 3. This was used to mimic the neonatal stomach. They were exposed to pre-adjusted infant formula at pH 3 for two hours at body temperature. Figure 4.4, Figure 4.5 and Figure 4.6 show the results for the different species.

In general, the initial viable count for all strains was between 5.25 $\log_{10}$ CFU/ml to 5.75 $\log_{10}$ CFU/ml. The duration of the experiment was two hours, and all strains *Serratia* spp. and *Enterobacter cloacae* were resistant to pH 3; Figure 4.5 and Figure 4.6.

E. coli strains showed a variation in resistant to pH 3 over the two hour period. Three different groups are shown in Figure 4.4. The viability of Group 1 of E. *coli* representative of pulsetypes; EcC4 strain (780), EcC3 strains (904, 923 and 939) and unique strains (1008) remained stable. Group 2 containing *E. coli* strain 1009 representative of pulsetype EcC2 was able resistant to pH 3 and started to multiply after 90 min. Group 3 pulsetype EcC1 included *E. coli* strains 1047 and 1050, both of which belonged to EcC1, showed resistant initially, and then the viable count decreased from 5.30 $\log_{10}$ CFU/ ml after 1 hour to 4.80 $\log_{10}$ CFU/ ml after 2 hours.

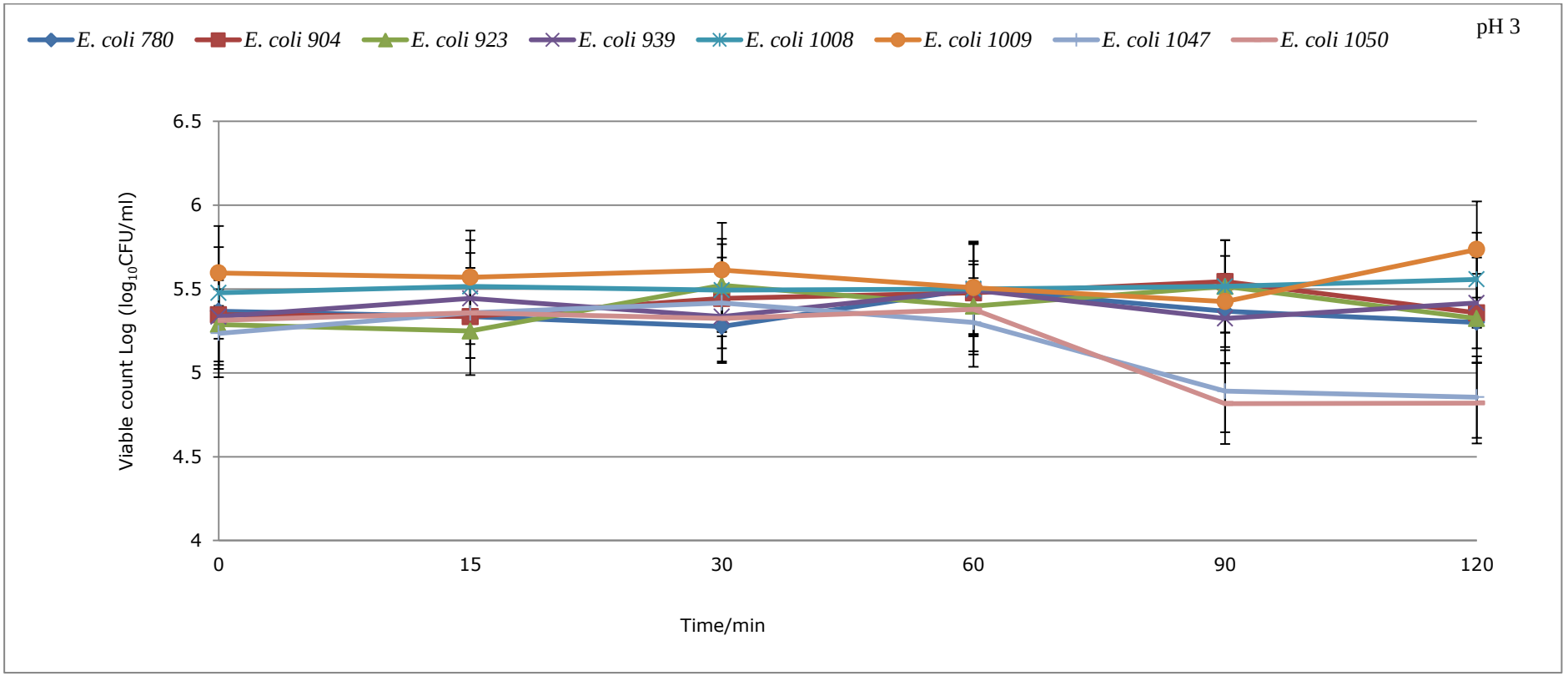

Figure 4.4 Survival of *E. coli* after the exposure to pH 3

The survival was measured for up to 2 h at 0, 15, 30, 60, 90 and 120 minutes. The number of recovered cells was obtained from two independent experiments in triplicate.

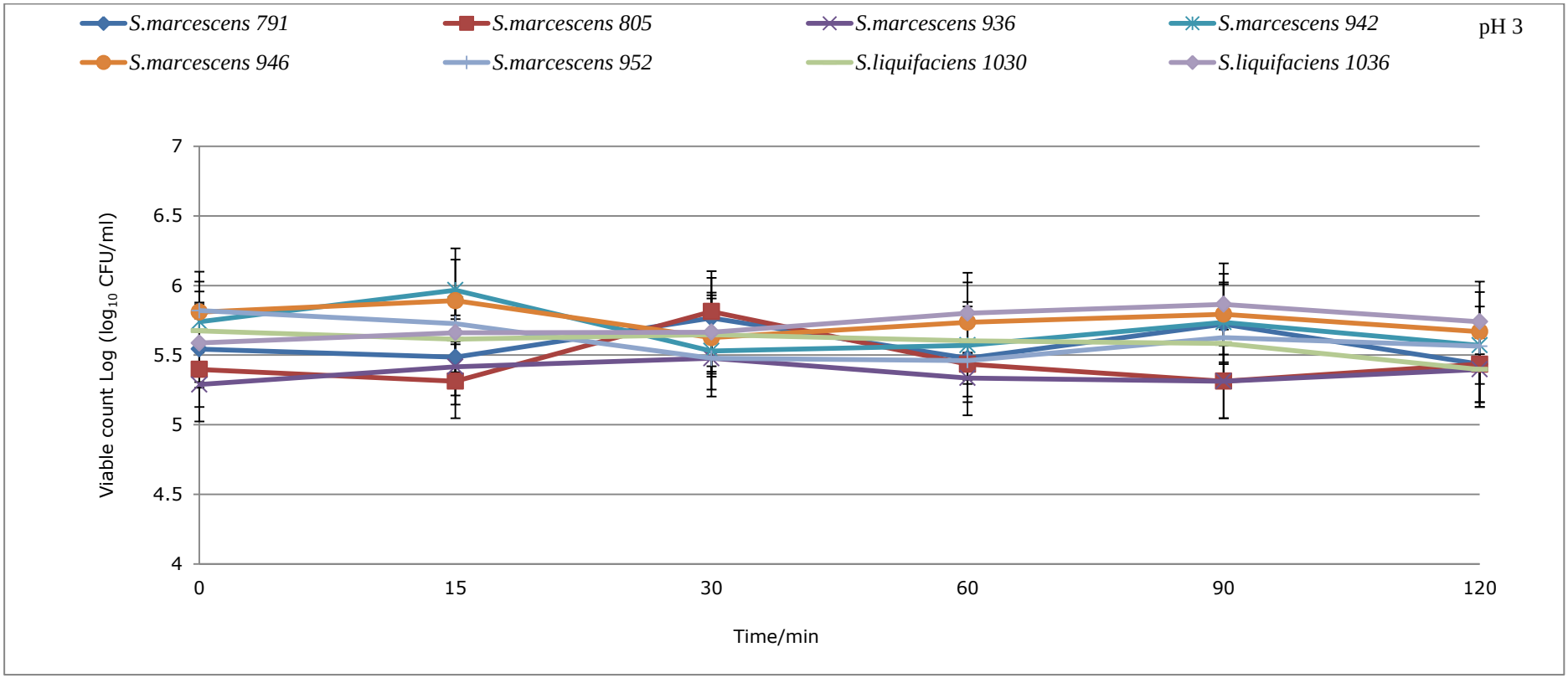

Figure 4.5 Survival of *Serratia* spp. after the exposure to pH 3

The survival was measured for 2 h at 0, 15, 30, 60, 90 and 120 minutes. The number of recovered cells was obtained from two independent experiments in triplicate.

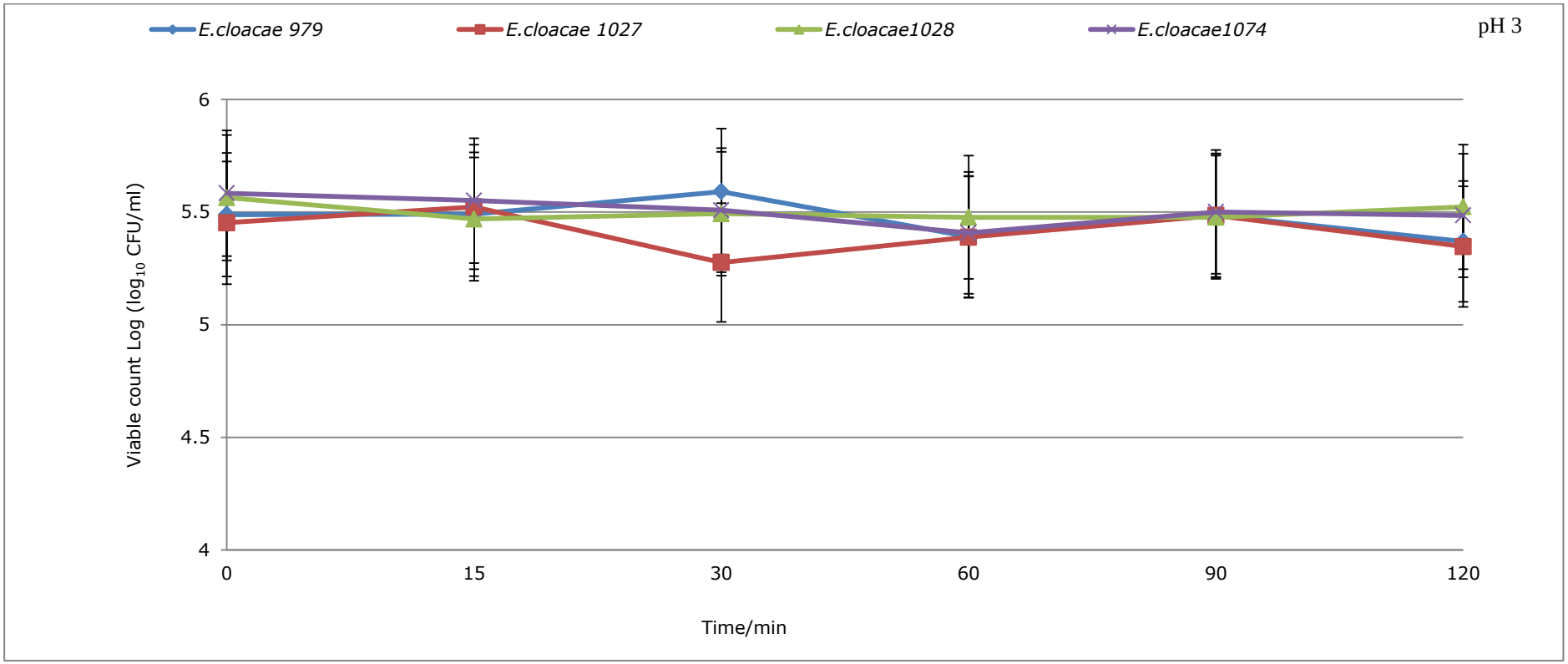

Figure 4.6 Survival of *E. cloacae* after the exposure to pH 3

The survival was measured for 2 hours at 0, 15, 30, 60, 90 and 120 minutes. The number of recovered cells was obtained from two independent experiments in triplicate.

4.2.5 **Capsule production**

Table 4.1 below shows the results of an investigation into capsule production on whey-based formula milk agars for twenty strains of *Enterobacteriaceae* including *E. coli*, *Serratia* spp. and *E. cloacae* strains. All strains formed creamy white colonies on the agar. There was a notable variation between strains in their ability to produce capsules and Table 4.1 shows the relative results. There were three groups according to the relative scale of high, medium and low capsule production are represented by +++, ++ and + respectively. Strains of *E. cloacae* 1047 and *S. marcescens* 791 & 805 were able to form high levels of capsular materials. *E. coli* strains 780, 1008 & 1009 and 904, 923, 939, 1047 & 1050 produced medium and lower levels of capsule production, respectively. In contrast, all other strains of *Serratia* spp. and *E. cloacae* strains formed medium levels of capsule, except *E. cloacae* 979, which was only able to form a small amount of capsular material.

Table 4.1 Capsule formation on milk agar

Organism	Strain	Pulsetype	Capsule formation
E. coli	780	EcC4	++
E. coli	904	EcC3	+
E. coli	923	〃	+
E. coli	939	〃	+
E. coli	1008	U	++
E. coli	1009	EcC2	++
E. coli	1047	CcE1	+
E. coli	1050	〃	+
S. marcescens	791	SC2	+++
S. marcescens	805	〃	+++
S. marcescens	936	SC1	++
S. marcescens	942	〃	++
S. marcescens	946	〃	++
S. marcescens	952	〃	++
S. liquifaciens	1030	SC3	++
S. liquifaciens	1036	SC4	++
E. cloacae	979	EbC5	+
E. cloacae	1027	EbC1	++
E. cloacae	1028	U	++
E. cloacae	1074	EbC3	+++

Capsule production; +++ high, ++ medium, and + low. U= unique. EcC, SC and EbC= cluster group according to PFGE, 〃 = same

4.2.6 **Congo red morphotype (curli fimbriae or cellulose)**

Table 4.2 shows the pigment production on Congo red agar for each strain of *Enterobacteriaceae* including *E. coli*, *Serratia* spp. and *E. cloacae* strains. Morphology was defined according to the following pigmentation: red, pink or white. The expression red morphotype on Congo red agar indicates the ability of the bacterium to express curli fimbria. Whereas the expression pink morphotype on Congo red agar; that means it is able to express cellulose. The morphology of *E. coli* strains and *Serratia* species colonies were pink. All but one strain of *E. cloacae* were pigmented pink, except 1028 which was pigmented red.

Table 4.2 Congo red morphotype (curli fimbriae or cellulose)

Organism	Strain	Pulsetype	Congo red phenotype
E. coli	780	EC4	Pink
E. coli	904	EC3	Pink
E. coli	923	≠	Pink
E. coli	939	≠	Pink
E. coli	1008	U	Pink
E. coli	1009	EC2	Pink
E. coli	1047	CE1	Pink
E. coli	1050	≠	Pink
S. marcescens	791	SC2	Pink
S. marcescens	805	≠	Pink
S. marcescens	936	SC1	Pink
S. marcescens	942	≠	Pink
S. marcescens	946	≠	Pink
S. marcescens	952	≠	Pink
S. liquifaciens	1030	SC3	Pink
S. liquifaciens	1036	SC4	Pink
E. cloacae	979	EbC5	Pink
E. cloacae	1027	EbC1	Pink
E. cloacae	1028	U	Red
E. cloacae	1074	EbC3	Pink

U= unique. EcC, SC and EbC= cluster group of PFGE, ≠ = same

4.2.7 **Haemolysis on blood agar**

Haemolysis on blood agar for twenty strains of *Enterobacteriaceae* including eight strains of *E. coli*, eight strains of *Serratia* spp. and four strains of *E.* cloacae are shown in Table 4.3. All strains, with the exception of *E. coli* strain 1008 were produced α-haemolysis, while strain 1008 was produced β-haemolysis. All strains of *Serratia* spp. and *E.* cloacae were produced α-haemolysis.

Table 4.3 Bacterial Haemolysis on blood agar

Organism	Strain	Pulsetype	Types of haemolysis
E. coli	780	EcC4	α
E. coli	904	EcC3	α
E. coli	923	〃	α
E. coli	939	〃	α
E. coli	1008	U	β
E. coli	1009	EcC2	α
E. coli	1047	CcE1	α
E. coli	1050	〃	α
S. marcescens	791	SC2	α
S. marcescens	805		α
S. marcescens	936	SC1	α
S. marcescens	942	〃	α
S. marcescens	946	〃	α
S. marcescens	952	〃	α
S. liquifaciens	1030	SC3	α
S. liquifaciens	1036	SC4	α
E. cloacae	979	EbC5	α
E. cloacae	1027	EbC1	α
E. cloacae	1028	U	α
E. cloacae	1074	EbC3	α

α= α-haemolysis, β= β-haemolysis. U= unique. EcC, SC and EbC= cluster group based on PFGE, 〃 = same.

4.2.8 ***Enterobacteriaceae*** **oxidative and nitrosative stress response**

The results of Table 4.4 show the sensitivity of *E. coli, Serratia* species and *E. cloacae* to oxidative and nitrosative stress. There was no significant variation between *E. coli, Serratia* spp. and *E. cloacae* ($p > 0.05$). While there was significant variation between *E. coli, Serratia* species and *E. cloacae* compared to the control strains *E. coli* K12 and *C. koseri* ($p < 0.001$) in their sensitivity to oxidative and nitrosative agents.

The sensitivity to methyl viologen was produced zones of inhibition with diameter in the range between 9 mm and 12 mm for *E. coli* strain 939 and *S. marcescens* strain 791. The zone of *E. coli* K12 and *C. koseri* were 18 and 23 mm respectively. For sensitivity to cumene hydroperoxide *E. coli* were inhibition zone was between 28 mm and 31 mm, whereas *Serratia* species and *E. cloacae* had zones between 20 mm and 23 mm and 18 mm and 19 mm respectively.

Hydrogen peroxide sensitivity was investigated. The inhibition zone was 10 mm and 13 mm for *E. coli*. There was no influence of hydrogen peroxide on *S. marcescens.* Two strains of *S. liquifaciens* and *E. cloacae* showed no significant variation ($p < 0.01$) and their inhibition zones were between 12 mm - 14 mm. The sensitivity of *E. coli, Serratia* species and *E. cloacae* strains to S-nitrosoglutathione was in the same range, as *E. coli* K12 and *C. koseri*.

Table 4.4 Sensitivity for *E. coli*, *Serratia* spp. and *E. cloacae* to agents of oxidative and nitrosative stress

Organism	Strain	Pulsetype	Methyl viologen (mm)	Cumenehydroperoxide (mm)	Hydrogen peroxide (mm)	S-Nitrosoglutathione (mm)
E. coli	780	EcC4	10.33 ± 2.52	29.33 ± 1.15	10.67 ± 1.15	5.00 ± 0.00
E. coli	904	≠	10.00 ± 1.00	28.67 ± 1.15	11.33 ± 0.58	5.00 ± 0.00
E. coli	923	≠	9.67 ± 0.58	28.67 ± 1.15	11.00 ± 0.00	5.00 ± 0.00
E. coli	939	≠	10.33 ± 1.15	30.67 ± 0.58	10.33 ± 0.58	5.00 ± 0.00
E. coli	1008	U	11.67 ± 1.53	31.33 ± 1.53	13.00 ± 1.00	5.00 ± 0.00
E. coli	1009	EcC2	10.33 ± 0.58	30.00 ± 2.65	11.00 ± 0.00	6.00 ± 0.00
E. coli	1047	CcE1	10.67 ± 0.58	28.33 ± 0.58	11.33 ± 0.58	5.00 ± 0.00
E. coli	1050	≠	11.33 ± 2.08	28.67 ± 2.08	12.00 ± 1.00	5.00 ± 0.00
S. marcescens	791	SC2	12.33 ± 0.58	20.67 ± 1.53	5.00 ± 0.00	6.33 ± 0.58
S. marcescens	805	≠	10.33 ± 0.58	20.33 ± 1.15	5.00 ± 0.00	5.00 ± 0.00
S. marcescens	936	SC1	11.67 ± 2.89	22.00 ± 1.00	6.00 ± 0.00	5.00 ± 0.00
S. marcescens	942	≠	10.33 ± 1.15	20.67 ± 1.53	6.00 ± 0.00	6.33 ± 0.58
S. marcescens	946	≠	12.00 ± 1.00	20.00 ± 1.73	5.00 ± 0.00	5.00± 0.00
S. marcescens	952	≠	8.67 ± 0.58	20.00 ± 1.00	5.00 ± 0.00	5.00 ± 0.00
S. liquifaciens	1030	SC3	10.00 ± 0.00	23.00 ± 1.00	12.67 ± 0.58	5.00 ± 0.00
S. liquifaciens	1036	SC4	10.67 ± 1.15	22.33 ± 0.58	13.33 ± 0.58	5.00 ± 0.00
E. cloacae	979	EbC5	11.00 ± 1.00	18.33 ± 0.58	12.33 ± 0.58	5.00 ± 0.00
E. cloacae	1027	EbC1	10.00 ± 1.00	18.33 ± 0.58	13.00 ± 1.00	5.00 ± 0.00
E. cloacae	1028	U	9.00 ± 1.00	19.67 ± 0.58	14.67 ± 0.58	7.67 ± 0.58
E. cloacae	1074	EbC3	11.33 ± 2.08	19.67 ± 0.58	13.67 ± 0.58	5.00 ± 0.00
C. koseri	48	-	18.33 ± 0.58	35.33 ± 1.73	12.33± 0.58	5.00 ± 0.00
E. coli K12	1230	-	23.33 ± 0.58	34.00 ± 1.53	13.33 ± 0.58	5.00 ± 0.00

Results represent the diameter of the zone of inhibition. Experiments were assayed in three independent assays. Standard deviation (±) represents the error between diameters of the zones of inhibition. U= unique. EcC, SC and EbC= cluster group of PFGE, ≠ = same.

4.2.9 **Antimicrobial Susceptibility of *E. coli***

Antimicrobial susceptibility of the *E. coli* strains was examined. This included screening sensitivity to six antibiotic groups; cephalosporins (Cefpodoxime, Cefotaxime and Ceftazidime), penicillins (Ampicillin, Augmentin and Piperacillin / Tazobactam), miscellaneous (Chloramphenicol), fluoroquinolones (Ciprofloxacin), aminoglycosides (Gentamicin) and carbapenems (Imipenem and Meropenem). In addition, three antibiotics were tested to determine ESBL phenotype; Cephalosporins (Cefpodoxime /Clavulanic Acid, Cefotaxime /Clavulanic Acid and Ceftazidime /Clavulanic Acid), Table 4.5. The analysis of the *E. coli* strains in antibiotic susceptible and antibiotic resistant strains used representative strains based on MLST and pulsetypes groups.

As shown in Table 4.5, strains 1008 and 1009 which belonged to STs 127 and 73 and pulsotypes U and EcC2 respectively were susceptible to all antibiotics. The sequence type ST2076 including *E.coli* strains 1047 and 1050, which belonged to EcC1, showed resistance to the penicillin's antibiotic group and susceptible to all others. The sequence type ST95, including three *E. coli* strains 904, 923 and 939 from pulsotype EcC3, displayed susceptibility to all the antibiotics with the exceptional resistance towards ampicillin. *E. coli* strain 780 ST394 belonged to EcC4 and displayed resistance towards three antibiotics (Ampicillin and Augmentin) and Meropenem which not expected. While strain 780 was susceptible to the other tested antibiotics. In addition analyses for phenotypic of an ESBL detection of ESBL-producing strains was performed. As presented in Table 4.6 , all strains were shown to be ESBL negative.

Table 4.5 Sensitivity for *E. coli* to agents of Antibiotic

Antibiotic	Antibiotic	Zone diameter (mm)		D.C (µg)	EcC4 ST 394	EcC3 ST 95	EcC3 ST 95	EcC3 ST 95	U ST127	EcC2 ST73	EcC1 ST2076	EcC1 ST2076
		S ≥	R <		780	904	923	939	1008	1009	1047	1050
Cephalosporins	Cefpodoxime /Clavulanic Acid	Z (CPD/CLAV) - Z (CPD) ≥ 5mm ESBL +ve		30/10	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	Cefotaxime /Clavulanic Acid	Z (CTX/CLAV) - Z (CTX) ≥ 5mm ESBL +ve		30/10	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	Ceftazidime /Clavulanic Acid	Z (CAZ/CLAV) - Z (CAZ) ≥ 5mm ESBL +ve		30/10	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	Cefpodoxime	20	20	30	S	S	S	S	S	S	S	S
	Cefotaxime	20	17	30	S	S	S	S	S	S	S	S
	Ceftazidime	22	19	30	S	S	S	S	S	S	S	S
Penicillins	Ampicillin	14	14	10	R	R	R	R	S	S	R	R
	Augmentin	17	17	10	R	S	S	S	S	S	R	R
	Piperacillin / Tazobactam	20	17	30/ 6	S	S	S	S	S	S	R	R
Miscellaneous	Chloramphenicol	17	17	30	S	S	S	S	S	S	S	S
Fluoroquinolones	Ciprofloxacin	22	19	5	S	S	S	S	S	S	S	S
Aminoglycosides	Gentamicin	17	14	10	S	S	S	S	S	S	S	S
Carbapenems	Imipenem	22	17	10	S	S	S	S	S	S	S	S
	Meropenem	22	16	10	R	S	S	S	S	S	S	S

U= unique, EcC = *E. coli* cluster group of Pulsed Field Gel Electrophoresis and ST = sequence typing.

4.2.10 **Detection of β-lactamase genes of *E. coli***

The presence of the β-lactamase genes in *E. coli* strains was also screened for in the study; Table 4.6. The bla$_{tem}$ gene was found in *E. coli* ST95, ST394 and ST2076 of isolates and none harboured bla$_{SHV}$, bla$_{CTX-M}$ and bla$_{OXA}$. ST127 and ST73 did not carry any of these genes.

Table 4.6 Genotypic traits for ESBL *E. coli* isolates

			ESBL genotype				Phenotypic
E. coli strain	Pulsetype	ST	*bla$_{shv}$*	*bla$_{tem}$*	*bla$_{ctxm}$*	*bla$_{oxa}$*	ESBL-producing
780	EcC4	394	-	+	-	-	-
904	EcC3	95	-	+	-	-	-
923	≠	≠	-	+	-	-	-
939	≠	≠	-	+	-	-	-
1008	U	127	-	-	-	-	-
1009	EcC2	73	-	-	-	-	-
1047	EcC1	2076	-	+	-	-	-
1050	≠	≠	-	+	-	-	-

U= unique, EcC= cluster group of Pulsed Field Gel Electrophoresis, STs= sequence typing, ≠ = same

4.1 **DISCUSSION**

In recent years there has been a rise in incidence of neonatal infections due to *Enterobacteriaceae*, especially *E. coli*, *Serratia* spp. and *Enterobacter* spp. These are major causative agents in neonatal intensive care unit (NICU) infections. Despite recent concerns over the microbiological safety of infant feeds, there has been no consideration that the nasogastric enteral feeding tube may act as a site for bacterial colonisation as a biofilm. Consequently microbial analysis of feeding tubes could add further information on factors related to enteral feeding (Hurrell *et al*., 2009b).

This chapter investigated whether neonatal nasogastric enteral feeding tubes were colonised by opportunistic pathogenic strains of *E. coli, S. marcescens, S. liquifaciens* and *E. cloacae*, and whether their presence was influenced by the feeding regime.

In this research a collection of *Enterobacteriaceae* strains were used, which had been isolated from the enteral feeding tubes of neonates on intensive care units. PFGE was used to cluster strains before studying their physiological and virulence related traits. Selected strains were chosen to represented pulsotype group. These strains included eight strains of *E. coli*, 6 strains of *S. marcescens*, two strains of *S. liquifaciens* and four strains of *E. cloacae*.

Bacterial pathogens can form biofilms on food and food contact surfaces, consequently enhancing the capability of these bacteria to survive under harsh environments and resist antimicrobial treatments (Annous *et al*., 2009). Numerous opportunistic pathogens within the *Enterobacteriaceae* family are known to be able

to attachment, multiplication and colonize nasogastric enteral feeding tubes (Hurrell *et al*., 2009a). The comparison of *E. coli, S. marcescens, S. liquifaciens* and *E. cloacae* in Figure 4.1 and Figure 4.2 generally reflects common knowledge of their ability to form biofilms. In this investigation only 2 variables (incubation temperature and formula composition) were used.

These investigations showed the ability of strains to form biofilms; Figure 4.1 and Figure 4.2. The effect of temperature on biofilm formation was evident. Biofilm formation was significantly higher ($p < 0.05$) at 37ºC for all strains compared 25ºC. Biofilm formation may be affected by the formula composition. Two different types of formulas were used for biofilm investigations. The results did not show any correlation of formula type on biofilm production by *S. marcescens, S. liquifaciens* or *E. cloacae;* Figure 4.1 and Figure 4.2. However, formula composition affected *E. coli* biofilm production. For example strains of *E. coli* 939 and 1047 grown in whey-based formula showed absorbance units approximately 0.57 AU while biofilm formation in casein-based formula was about 1.5 AU; Figure 4.1.

Previous studies have shown that conditions, which affect biofilm formation, are temperature, pH, O_2, hydrodynamics, and osmolarity. These conditions affect enzymatic and structural elements which are important for biofilm formation (Romeo, 2008). Proliferation of bacteria is a significant effect only in some cases during the first steps of form biofilm (Chavant *et al*., 2002). In study by Else *et al*., (2003) the lowest and highest of humidity values led to the best biofilm development. Temperature may also affect he production of extracellular polymeric substances, which is known to increase the biofilm formation. Another study

indicated that curli fimbriae in members of *Enterobacteriaceae* play a role in adherence and biofilm formation (Romeo, 2008; Olsén *et al*., 1993; Römling *et al*., 1998; Zogaj *et al*., 2003; Lee *et al*., 2011). *E. coli* could be affected by the formula composition to form biofilm; Figure 4.1. The study by Kucerova *et al*., (2011) found that the whey and casein based formulae, commonly used in infants feeding, and affected the growth rate and death of *Enterobacteriaceae*. Finally, the study by Hurrell *et al*., (2009b) showed that biofilm formation by bacteria on neonatal feeding tubes increases the risk of baby infection at NICUs.

In this study, the motility test of the selected strains was investigated to determine any correlation of motility with biofilm. There was a different range in motility shown by strains of *E. coli*, *S. marcescens, S. liquifaciens* and *E. cloacae*; Figure 4.3. In general, most strains of *E. coli* were less motile compared to *Serratia* species and *Enterobacter cloacae* strains. Investigation showed no correlation between motility and biofilm formation. In addition, the study showed a variation range in motility between strains belonged to same pulsetypes. For example, strain 939 of *S. marcescens,* which belong to pulsetype SC1 was non-motile, whereas the other strains of the same pulsetype SC1 group were motile, with the same swarm diameter of ~ 30 mm. However, further analyses is necessary in order to confirm the isolates are related or not by used another restriction enzyme then those strains could be confirmed as the same. There is biofilm data showed can be relationship between motility Figure 4.3 and biofilm formation Figure 4.1. Previous studies of *E. coli* and *Salmonella* reported that motility was not required for initial attachment and biofilm formation (Pratt and Kolter, 1998; Prigent-Combaret *et al*., 2000). This contrasts

with a study of *E. coli* K-12 which showed correlation between motility and biofilm formation (Wood *et al*., 2006).

The ingestion of microbes during feeding exposed them to acidity stress in the stomach. The infants' stomach pH of infant's fed milk formula is between 2.5 and 4.3 (Hurrell *et al*., 2009a) in this study the role of biofilm in the resistance of cells to pH 3 was investigated. The same selected strains for biofilm studies were screened for pH 3 tolerance. *Serratia* species and *E. cloacae* strains showed persistence at pH3 for up to 2 hours; Figure 4.5 and Figure 4.6. As well as in Figure 4.4, *E. coli* strains shown to resist pH 3 for 2 hours with the exception of strains 1047 and 1050 which were only resistant for up to 1 h and then their viable count slightly decreased. The survival of these strains at low pH may increase the risk of exposure to bacterial pathogens by neonates. In addition, the persistence of strains in low pH may be because of their ability to form a biofilm inside the feeding tube. Previous study of acid tolerance by biofilm cells of *Streptococcus mutans* (McNeill and Hamilton, 2006) showed the cells in the biofilm are highly resistant to low pH.

Capsule morphology was also assessed. *E. coli, S. marcescens, S. liquifaciens* and *E. cloacae* showed different capsule morphology; Table 4.1. Study observed there was no correlation between capsule formation and biofilm on milk agar for 20 strains studied as shown in Table 4.1 and Figure 4.1. For instance, *S. marcescens* 791 and 805 produce more capsules, nevertheless those strains were produced less biofilm. *E. coli* pulsotype EcC3 produced more biofilm than the most capsulated *S. marcescens* strains 791 and 805. Hurrell *et al*., (2009b) found that strains, which did not produce any capsule on milk agar plates, produced more biofilm. Capsules may have played

roles in resistance to desiccation, serum activity and contribute to the survival of the organism after uptake by phagocytic cells (Guerry and Szymanski, 2008).

In addition to capsule production, there is another production by cell wall, which is the expression of cellulose and / or curli fimbriae. The role of curli fimbriae in adherence and invasion has been described in members of *Enterobacteriaceae* (Olsén *et al*., 1993; Zogaj *et al*.; 2003; Boudeau *et al*., 2001; Lee *et al*., 2011; Römling *et al*., 2003). In this study, 20 *Enterobacteriaceae* isolates were characterised on media characterising the cell wall expression of cellulose and/or curli fimbriae. The morphotypes are shown in Table 4.2. All isolates belonging to the dominant morphotypes produced pink colonies on Congo red indicating that these isolates are able to produce cellulose and not curli fimbriae. However, one strain *E. cloacae* 1028 produced red colonies on Congo red showing the expression of curli fimbriae. Pink morphotype on Congo red agar indicate that the cells are producing cellulose (Bokranz *et al*., 2005). The same authors also reported the capability of *E. coli strains* to form biofilms was correlated with expression of fimbriae and cellulose. In *E. coli* and *Salmonella*, cellulose is one of the major components of the biofilm matrix (Da Re and Ghigo, 2006).

A number of supposed virulence factors play a role in the pathogenesis of bacteria. Toxins production by bacteria are major important virulence factors in bacterial disease. Haemolysin is one of the toxins producted by bacteria. There are three different of haemolysins; α-haemolysin, β-haemolysin and γ-haemolysin. Secreted haemolysin are found in clinical isolates, such as *E. coli* and *Serratia* species (König *et al*., 1987; Welch, 1987; Schmidt *et al*., 1995).

In this current study, *E. coli*, *S. marcescens*, *S. liquifaciens* and *E. cloacae* were investigated for their capability to lyse sheep erythrocytes. All showed α-haemolytic activity on sheep blood, except *E. coli* 1008 which was ß-haemolytic; Table 4.3. The α-haemolysin is an important virulence factor generally expressed by extraintestinal pathogenic *E. coli* (Balsalobre *et al*., 2006). All *E. coli* in this study belonged to the *E. coli* pathogenic group B2, extraintestinal pathogenic *E. coli*. Gianotti, (1999) found that most clinical isolates of *S. marcescens*, *S. liquifaciens* and other *Serratia* species were β-haemolytic.

During infection, pathogenic bacteria are engulfed by phagocytic cells, such as macrophages. The engulfed bacteria are then exposed to high concentrations of ROS and NOS, which often leads to killing (Burmester and Pezzutto, 2003; Urban *et al*., 2006; Kehrer, 2000). However, some of pathogenic bacteria are able to adapt to oxidative and nitrosative stress (Soares *et al*., 2010). NrfA enzymes and cytoplasmic Fdp proteins, which are produced by *Wolinella succinogenes*, *E. coli*, *C. jejuni* and other bacteria contribute to the conversion of nitric oxide to ammonium and then to nitrous oxide (Kern *et al*., 2011). The sensitivity of *E. coli*, *S. marcescens*, *S. liquifaciens* and *E. cloacae* to a range of oxidative and nitrosative were studied. As shown in Table 4.4, strains of *E. coli*, *S. marcescens*, *S. liquifaciens* and *E. cloacae* did slightly vary in their response to methyl viologen, cumene hydroperoxide, and hydrogen peroxide between strains. Nonetheless, strains were less sensite to these compounds compared to control strains, for example *S. marcescens* which was not affected by hydrogen peroxide, whereas other strains and were effaced Table 4.4. Strains were not affected by S-nitrosoglutathione Table 4.4. These traits could be links between oxidative stress resistance and tissue culture results.

Antimicrobial resistance and ESBL were determined for eight strains of the *E. coli* isolated; Table 4.5 and Table 4.6. The *E. coli* strains were highly sensitive to miscellaneous, cephalosporins, fluoroquinolones, and aminoglycosides. However *E. coli* strains showed low sensitivity to penicillin's, for example ST2076 of *E. coli* strains 1047 and 1050 were resistances to Penicillin's. Although strains were not demonstrated phenotypic ESBL activity, nonetheless all strains note according to Table 4.6 of *E. coli* carried bla_{tem} gene of ESBL. Strains of *E. coli* not detected of phenotypic ESBL activity, whereas ST394, ST95 and ST2076 of *E. coli* strains were harboured bla_{tem} gene of β-lactamase Table 4.6. Strains of *E. coli* did not expression of phenotypic ESBL that could be β-lactamase genes are locked or may be it need change the concentration of the antimicrobial agent.

Chapter 5 **Virulence determination of *Enterobacteriaceae* isolated from neonatal enteral feeding tubes.**

5.1 **Introduction**

Numerous enteric pathogens require surface molecules such as adhesins for their adherence to host cells, leading to colonization and infection. Recently, important evidence has shown that markedly diverse microbial pathogens use common strategies to cause infection and disease. For example, several pathogenic bacteria share a common strategy to adhere, invade, and subvert host cells and tissues, as well as to survive within host cells and causes infection (Wilson *et al*., 2002; Miller, 1995).

Enterobacteriaceae infections start after the bacteria adhere to the host cell surface (Shoaf-Sweeney and Hutkins, 2008). Many bacterial pathogens including *E. coli, Salmonella*, *Shigella* and *Yersinia* have evolved specific pathogen: host interactions e.g. actively enter eukaryotic cells (Finlay and Falkow, 1988). Additionally, microbes such as *Salmonella* spp., *Streptococcus pneumoniae, E. coli*, *Shigella* are able to invade and survive within host cells, causing a number of infectious diseases (Horwitz, 1983; Formal *et al*., 1983; Isberg and Falkow, 1985; Ernst *et al*., 1999).

Caco-2 as a model of small intestine is the main epithelial cell line used *in vitro* to study the mechanisms of bacterial adhesion and invasion of human pathogens (Grajek and Olejnik, 2004; Cencič and Langerholc, 2010). The Caco-2 cell line was proposed by Szymanski *et al*. (1995) as a model to investigate the invasion of *Campylobacter jejuni* and *Campylobacter coli* of intestinal epithelia. The ability of *E. coli* isolated from children to invade Caco-2 cells was shown to be significantly more than controls ($p \leq 0.001$) (Geyid *et al*., 1998). The cell line has previously used

at NTU to study that *E. hormaechei* strains vary in their ability to invade intestinal and brain cells, and persistence in macrophages (Townsend *et al*., 2008).

In contrast, HEp-2 or Hela epithelial cell lines are the most common cell line for differentiating between enteropathogenic *E. coli* (EPEC), Diffuse Adherence *E. coli* (DAEC) and enteroaggregative *E. coli* (EAEC). In earlier studies adherence tests had been used to detect distinct the adherence patterns; localized adherence (LA), diffuse adherence (DA) and aggregative adherence (AA) (Janda and Abbott, 2006; Rüttler *et al*., 2006).

Phagocytic cells are the first defence in the body against microbes' infection. However many pathogenic microbes are able to survive and even replicate within phagocytic cells (Lindgren *et al*., 1996). Fields *et al*. (1986) investigated *Salmonella* Typhimurium *in vitro* for its ability to survive and replicate within macrophages. An investigation by Townsend *et al*. (2003) used the human macrophages U937 to determine the virulence of *C. koseri*. The study detected the ability of *C. koseri* to survive and replicate within macrophages which could be contributing to chronic central nervous system infection including brain abscesses. Previous studies showed that *Citrobacter* causes meningitis and brain abscess formation in babies (Kline *et al*., 1988b). Lucchini *et al*. (2005) found that *S. flexneri* are able to persist within macrophages (U937) cell line.

In spite of the advances of increased health care, there remains a significant amount of mortality and morbidity associated with bacterial meningitis (Lucchini *et al*., 2005). Studies of bacteria translocation in brain endothelial cells to the central nervous system (CNS) can be studied *in vitro* because of the availability of human

brain microvascular endothelial cells HBMEC (Huang *et al*., 1995; Stins *et al*., 2001). Kim *et al*. (2003) investigated the ability of *E. coli* K1 to invade human brain microvascular endothelial cells HBMEC, although it remains unclear how *E. coli* K1 crosses HBMEC. Khan *et al*. (2007) demonstrated that type 1 fimbriae, specifically *Fim*H attached to brain endothelial cells and triggers signalling events that promote *E. coli* K1 to invade in HBMEC. Townsend *et al*. (2008) investigated the virulence of *E. hormaechei* using the invasion of rat brain capillary endothelial cell line rBCEC4 cell line as a model to investigate the bacterial invasion of brain cells.

A number of virulence factors have been identified in *E. coli,* although many others are probably not yet totally elucidated (Sussman, 1997; Manges and Johnson, 2012). *E. coli* are responsible for extraintestinal infections such as neonatal meningitis (Manges and Johnson, 2012). The virulence potential of extraintestinal pathogenic *E. coli* (ExPEC) is determined largely by the presence of particular virulence factors (VFs), such as adhesins, invasins, capsule, toxins and siderophores (Johnson and Stell, 2000).

In this chapter a range of virulence determinants in *E. coli*, *Serratia* species and *E. cloacae* strains have been investigated, which are relevant to an enteroinvasive bacterial pathogen. These will include attachment and invasion of Caco-2, HBMEC, rBCEC4, and macrophage cell line U937. A selection of virulence factor genes for *E. coli* was considered and any observed differences would be compared to the pulsetypes group; Table 2.8.

5.2 **Materials and methods**

The methods section, culture media and culturing conditions for this part were previously described in Chapter 2 Materials and Methods.

5.3 **RESULTS**

5.3.1 **Attachment and invasion assays (Caco-2, rBECE4 and HBECE)**

The virulence determination of *Enterobacteriaceae* strains has been studied *in vitro* by investigating their attachment and invasion of three different cell lines, Caco-2, rBCEC4 and HBMEC. The bacterial attachments to epithelial and endothelial cells were determined after two hours of the exposure of the mammalian cells and compared to the initial inoculum size. The invasion experiment was performed by using a gentamicin protection method after three hours exposure epithelial and endothelial cells with strains compared to the viable count of the initial inoculum [Appendix 7.5 -Appendix 7.7]. *Salmonella* Enteritidis strain number NCTC 3046 (NTU strain 358) was used as positive control for the Caco-2 cell line. While *C. koseri* strain number SMT319 (NTU strain 48) was used as positive control for rBCEC4 and HBMEC. Both were used in this investigated as standard enteric pathogens capable of attachment and invasion. While *E. coli* K12 (NTU 1230) was used as a negative control for all cell lines used in this study, which was non-pathogenic and incapable of attachment to Caco-2 cell line (Townsend *et al*., 2008).

5.3.1.1 **Caco-2 attachment**

Figure 5.1 below shows the attachment to the mammalian epithelial cell line Caco-2 by *E. coli*, *Serratia* species and *E. cloacae*. In addition, *E.coli* K12 and *Salmonella* Enteritidis 358 were also used as reference strains for comparative data. As shown in Figure 5.1, all strains attached to Caco-2 cells when inoculated (T = 0) and later both the initial bacterial count and the count after 3 hours (T = 3). Statistically one-way ANOVA all strains showed significantly more attachment than *E. coli* K12 ($p < 0.001$).

Most strains were able to attach to the Caco-2 cell line readily and the number of recovered cells was in the range 5.5 $\log_{10}$ - 6 $\log_{10}$ CFU/ml. Two strains of *E. coli* type (strain 1008 and 1009), strain 1030 of *S. liquefaciens* and *E. cloacae* showed a lower attachment rate than the other bacterial strains.

5.3.1.2 **Caco-2 invasion**

The gentamicin protection assay was performed to investigate the ability of *Escherichia coli*, *Serratia* species and *Enterobacter cloacae* to invade the Caco-2 cell line. *Salmonella* Enteritidis 358 and *E. coli* K12 were used as positive and negative controls, respectively for comparative data. All strains invaded Caco-2 cells at a significantly ($p \leq 0.001$) greater value than *E. coli* K12.

Figure 5.2 below shows a range of invasion abilities of *E. coli*, *Serratia* spp. and *E. cloacae* to Caco-2. The number of recovered cells was in the range between 1.5 $\log_{10}$ to 4 $\log_{10}$ CFU/ml.

The *E. coli* strain 1050 showed the highest level of invasion. As given above, this strain also had the highest attachment value. *E. coli* and *E. cloacae* showed the higher levels of invasion than *Serratia* spp. One strain of *E. coli* 1008, five strains of *Serratia* spp. (type strain 791, 805, 942, 1030, and 1036) and one strain of *E. cloacae* (1047) were shown to be unable to invade Caco-2 cells. Three *Serratia* strains (type strain 936, 946 and 952), which previously had the highest attachment rates, showed low invasion rates of about 1 $\log_{10}$ CFU/ml. In addition, *E. coli* 1009 showed low invasion value of about one $\log_{10}$ CFU/ml and had low attachment rates.

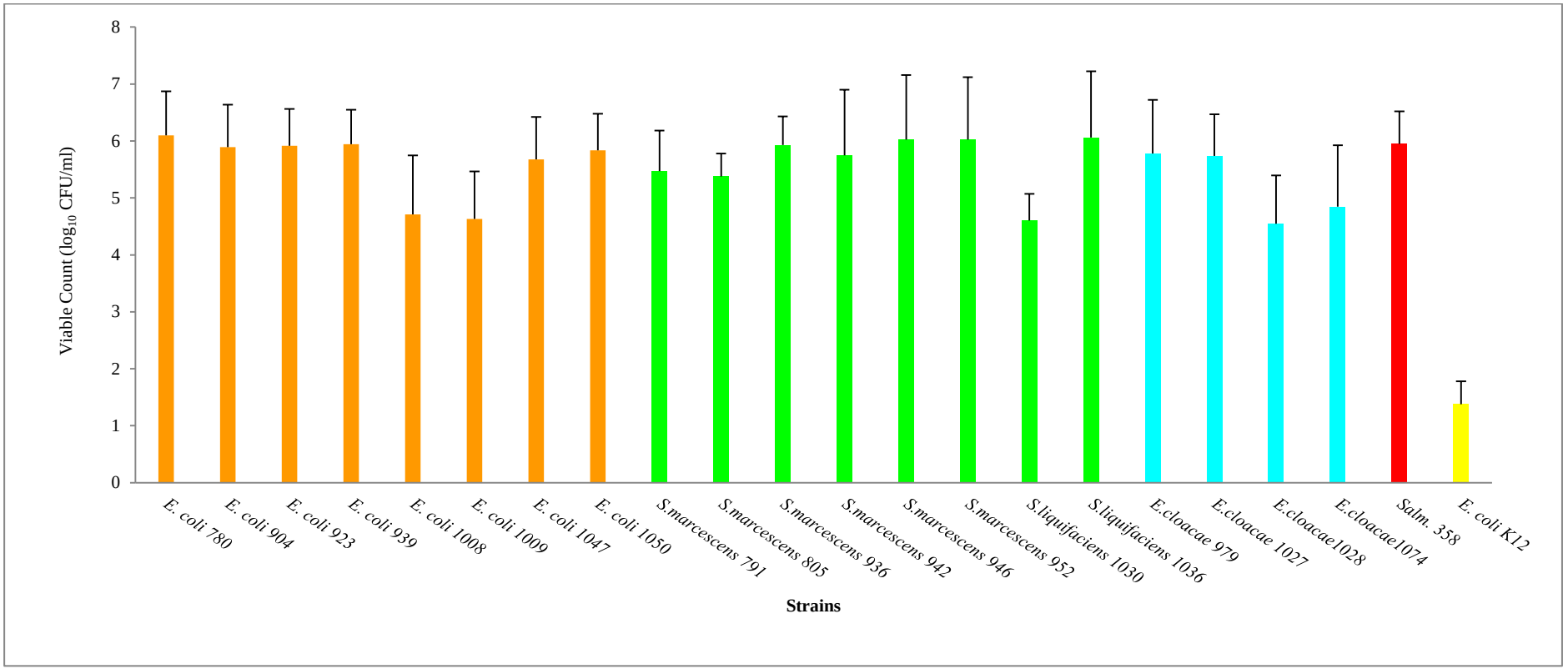

Figure 5.1 Attachment of CaCo-2 cells by *E. coli*, *Serratia* spp. and *E. cloacae*

S. Enteritidis 358 and *E. coli* K12 were used as positive and negative controls respectively

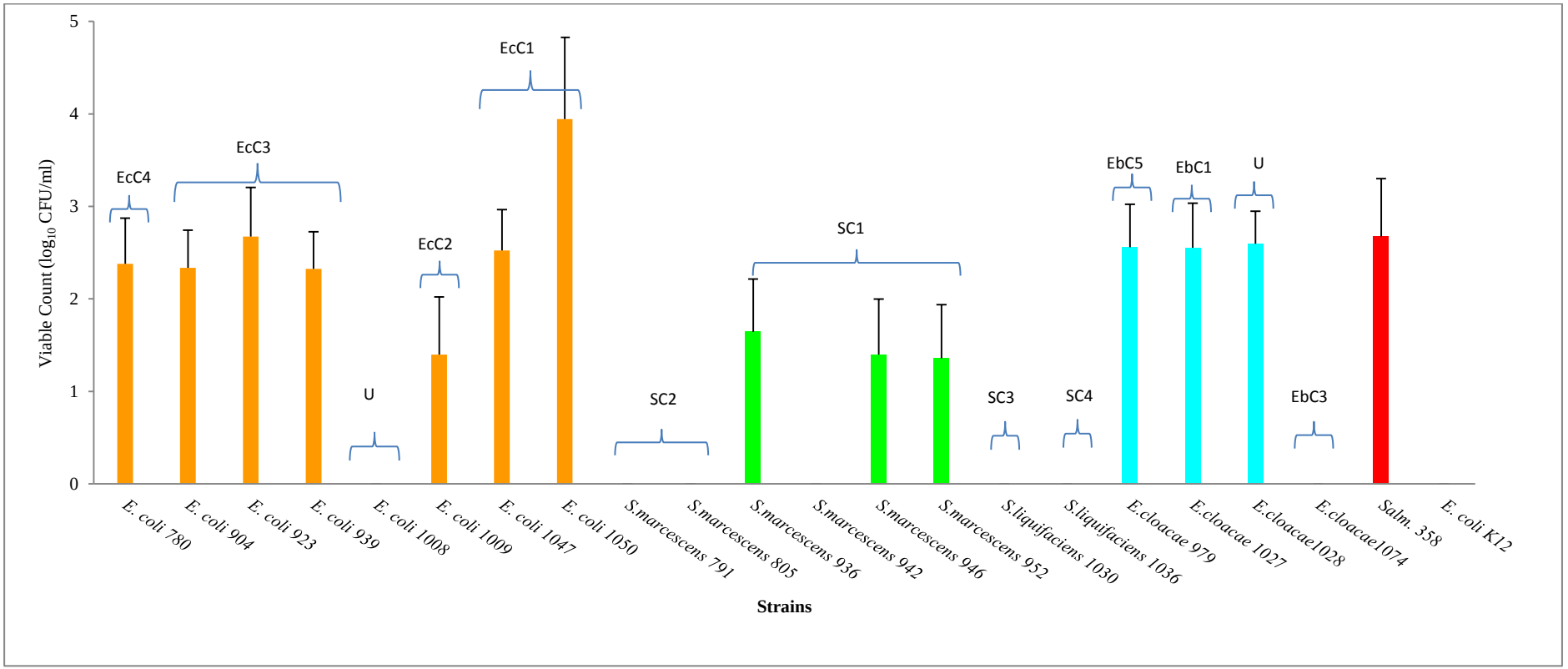

Figure 5.2 Invasion of Caco-2 cells by *E. coli, Serratia* spp. and *E. cloacae*

S. Enteritidis 358 and *E. coli* K12 were used as positive and negative controls, respectively. U= unique. EcC, SC and EbC= cluster group of Pulsed Field Gel Electrophoresis.

5.3.1.3 **rBCEC4 Attachment**

Figure 5.3 below displays the level of the attachment of the selected *Enterobacteriaceae* strains to the mammalian endothelial cell line rBCEC4 by the same strains as used previously with Caco-2 cells. *C. koseri* 48 and *E. coli* K12 were used as positive and negative controls, respectively for comparative data.

The selected strains were varied in their attachment to rBCEC4cells, that there was a three-log variation in invasion values between strains. The number of recovered cells was in the range between 2.5 $\log_{10}$ – 6 $\log_{10}$ CFU/ml. All strains showed significantly more attachment than *E. coli* K12 ($p \leq 0.001$).

Strains of *E. coli* with the exception of 1009 showed the attachment to rBCEC4 cells at higher values approximately 6 $\log_{10}$ CFU/ml. Three *Serratia* strains (805, 857 and 936) had an attachment rate higher than *C. koseri* 48, which were recovered in the range approximately 5.5 $\log_{10}$ CFU/ml. These were significantly greater than *C. koseri* 48 ($p \leq 0.001$). All strains of *E. cloacae* and six strains of *Serratia* species (type strain 791, 942, 952, 968, 1030, and 1036) had similar values and the lowest attachment, which was lower than *C. koseri* 48, although significantly greater than *E. coli* K12 ($p \leq 0.001$)

5.3.1.4 **rBCEC4 Invasion**

The ability of *E. coli, Serratia* species and *E. cloacae* strains to invade into the rBCEC4 cells are shown in Figure 5.4 below. The rBCEC4 cell line was used to investigate the ability and interaction of strains to invade brain capillary endothelial

cells *in vitro*. The gentamicin protection assay used *C. koseri* 48 as the positive control as it very able to invade this cell line and *E. coli* K12 as a negative control as it is unable to invade this cell line whereas the number of recovered cells was in the range between 2.5 $\log_{10}$ to 3.5 $\log_{10}$ CFU/ml. Four strains of *E. coli* (780, 904, 923 and 939) and one strain of *Serratia* spp. 936 showed the ability to invade rBCEC4, which had an attachment rate higher, while all other strains were unable to invade endothelial cell line rBCEC4.

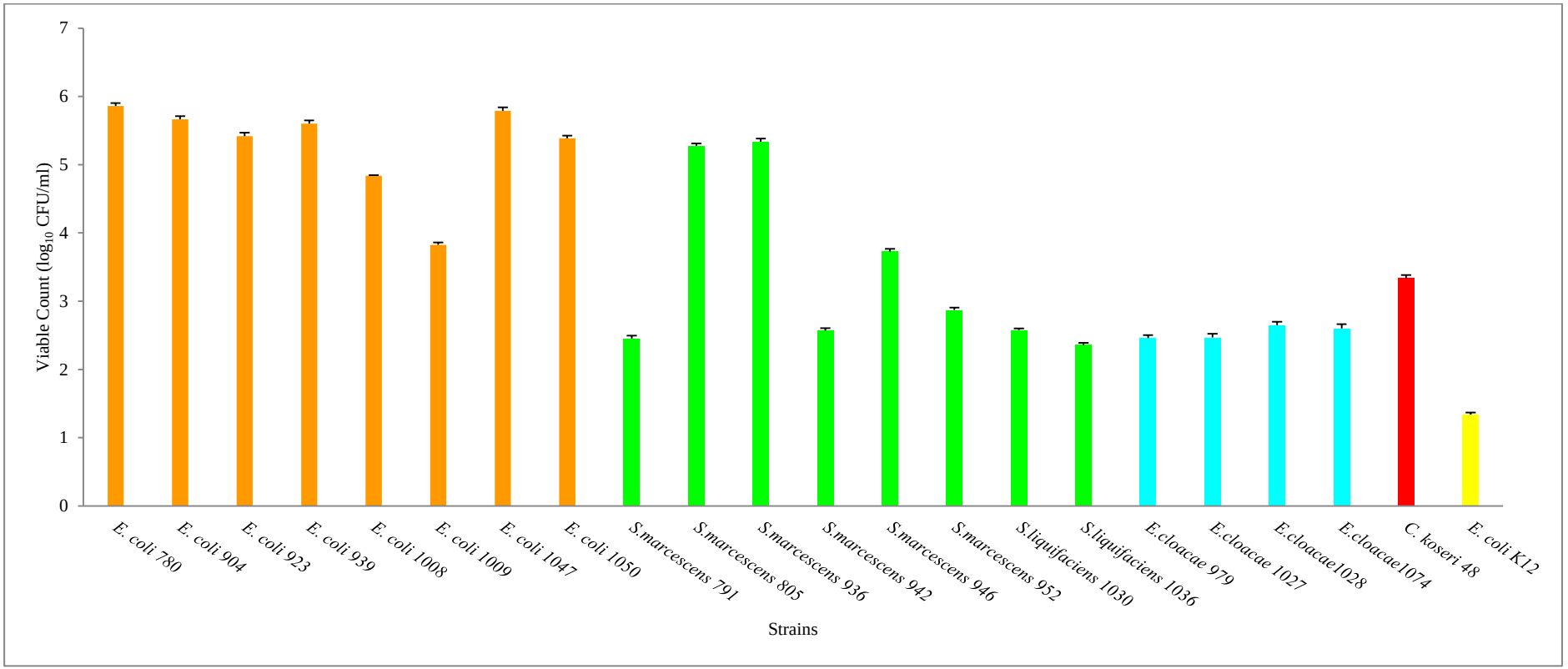

Figure 5.3 Attachment of rBCEC4 cells by *E. coli*, *Serratia* spp. and *E. cloacae*

C. koseri 48 and *E. coli* K12 were used as positive and negative controls, respectively.

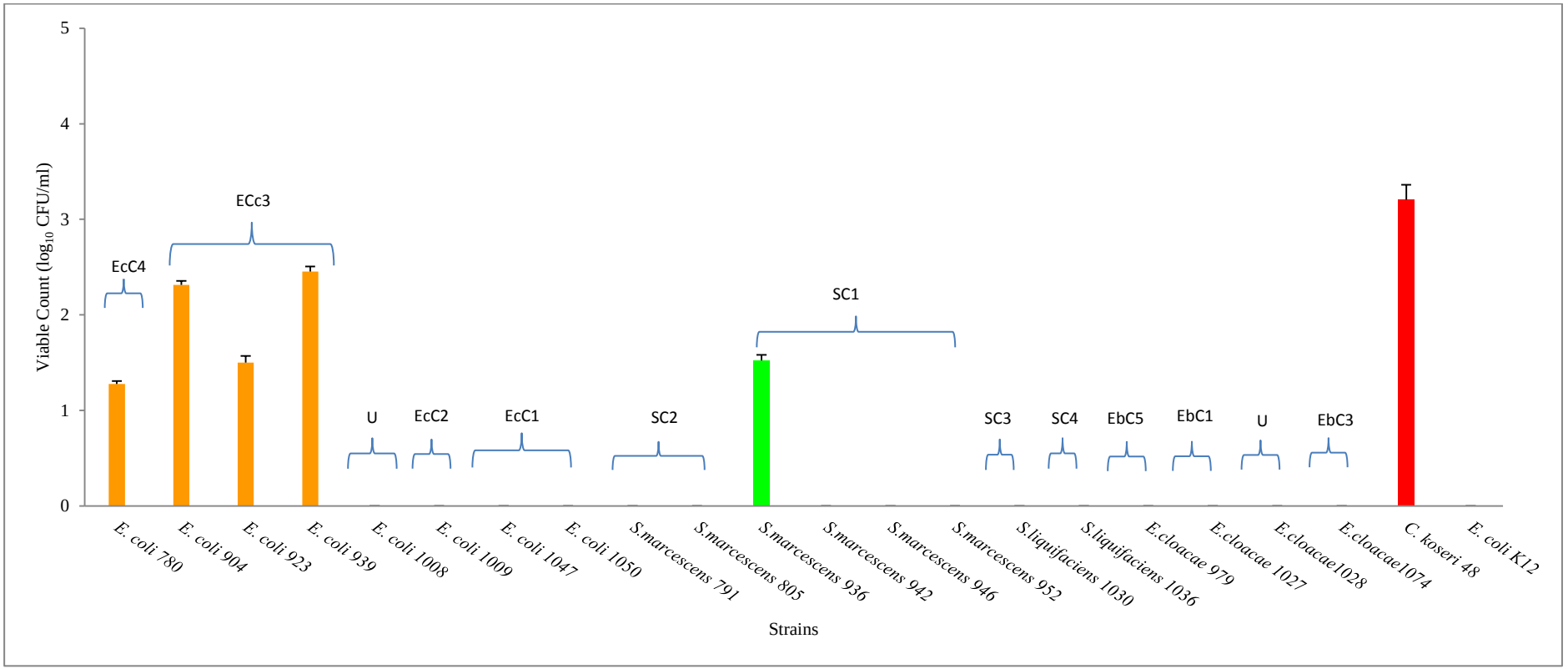

Figure 5.4 Invasion of rBCEC4 cells by *E. coli*, *Serratia* species and *E. cloacae*

C. koseri 48 and *E. coli* K12 were used as positive and negative controls, respectively. U= unique. EcC, SC and EbC= cluster group of pulsed field gel electrophoresis

5.3.1.5 **HBMCE Attachment**

Figure 5.5 summarizes the attachment to HBMEC by *E. coli*, *Serratia* species and *E. cloacae*. *C. koseri* 48 and *E. coli* K12 were included for comparison data as positive and negative controls.

All strains were able to attach to the HBMCE cell line and the number of recovered cells was in the range between 4 $\log_{10}$ to 6 $\log_{10}$ CFU/ml. In addition all strains showed significantly more attachment than *E. coli* K12 ($p \leq 0.001$).

Based on data presented in Figure 5.5, the four strains of *E. coli* (780, 904, 923, 939) and three strains of *Serratia marcescens* showed the greatest attachment and there was significantly more attachment than *E. coli* K12 ($p \leq 0.001$) and higher than *C. koseri* 48. Two strains of *E. coli* (type strain 1008 and 1009) showed low attachment values, about 4.5 $\log_{10}$ CFU/ml. These strains also showed low values for attachment and invasion of Caco-2 cells. Three strains of *E. cloacae* showed the lowest rate of attachment of ~ 3.5 $\log_{10}$ CFU/ml

5.3.1.6 **HBMCE Invasion**

Figure 5.6 below displays the abilities of *E. coli*, *Serratia* spp. and *E. cloacae* strains to invade the human blood brain barrier endothelial cell line HBMCE. The gentamicin protection assay used *C. koseri* 48 as a positive control and *E. coli* K12 as a negative control that was unable to invade this cell line and the number of recovered cells was in the range between approximately 2.5 $\log_{10}$ to 3 $\log_{10}$ CFU/ml.

All *E. coli* strains except strain number 1008, *Serratia* spp. (strains 857, 936, 946 and 952) and *E. cloacae* strains 979 and 1028 were significantly higher invaders compared with *E. coli* K12 ($p \leq 0.001$). These strains were not different from *C. koseri* 48, which were recovered in the range 2.5 $\log_{10}$ to 3 $\log_{10}$ CFU/ml. However all other strains were unable to invade this cell line. *E. coli* strains were more invasive than *Serratia* spp. and *E. cloacae*.

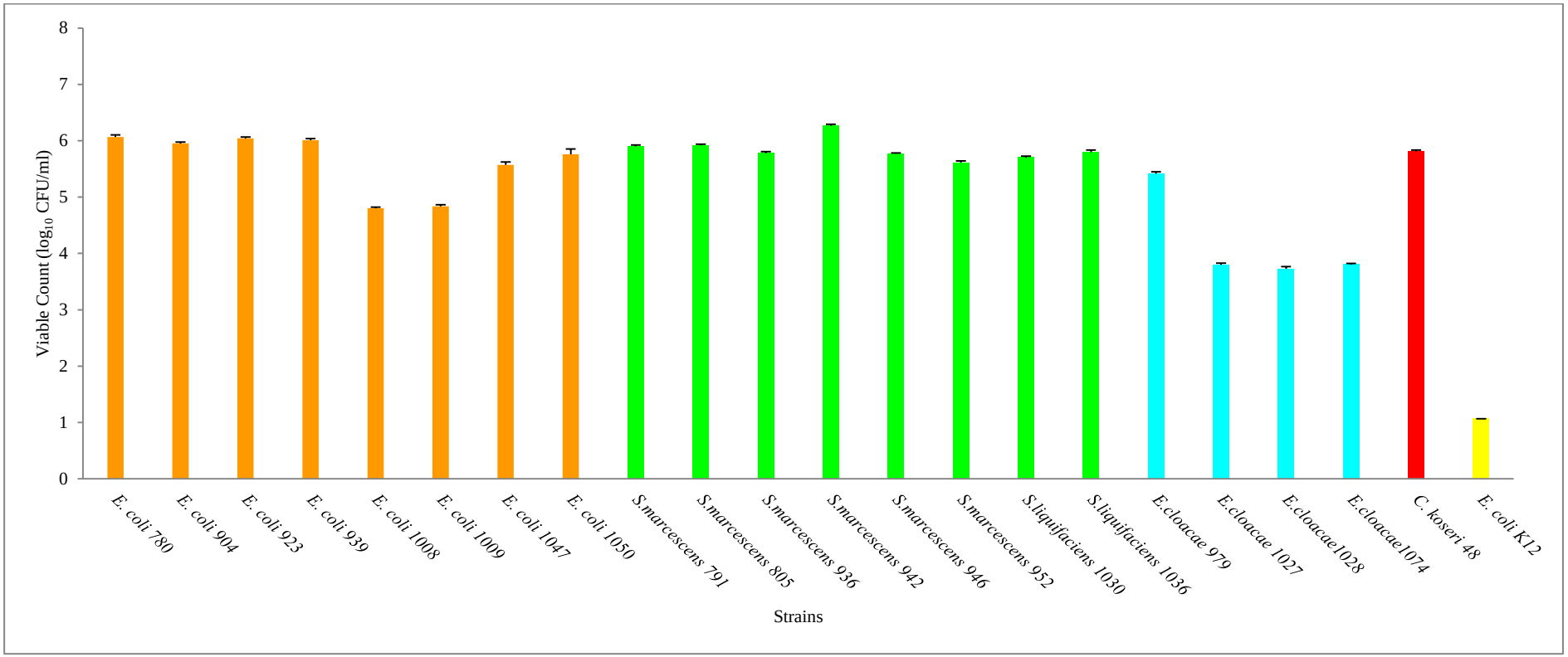

Figure 5.5 Attachment to HBMCE cells by *E. coli*, *Serratia* spp. and *E. cloacae*

C. koseri 48 and *E. coli* K12 were used as positive and negative controls, respectively

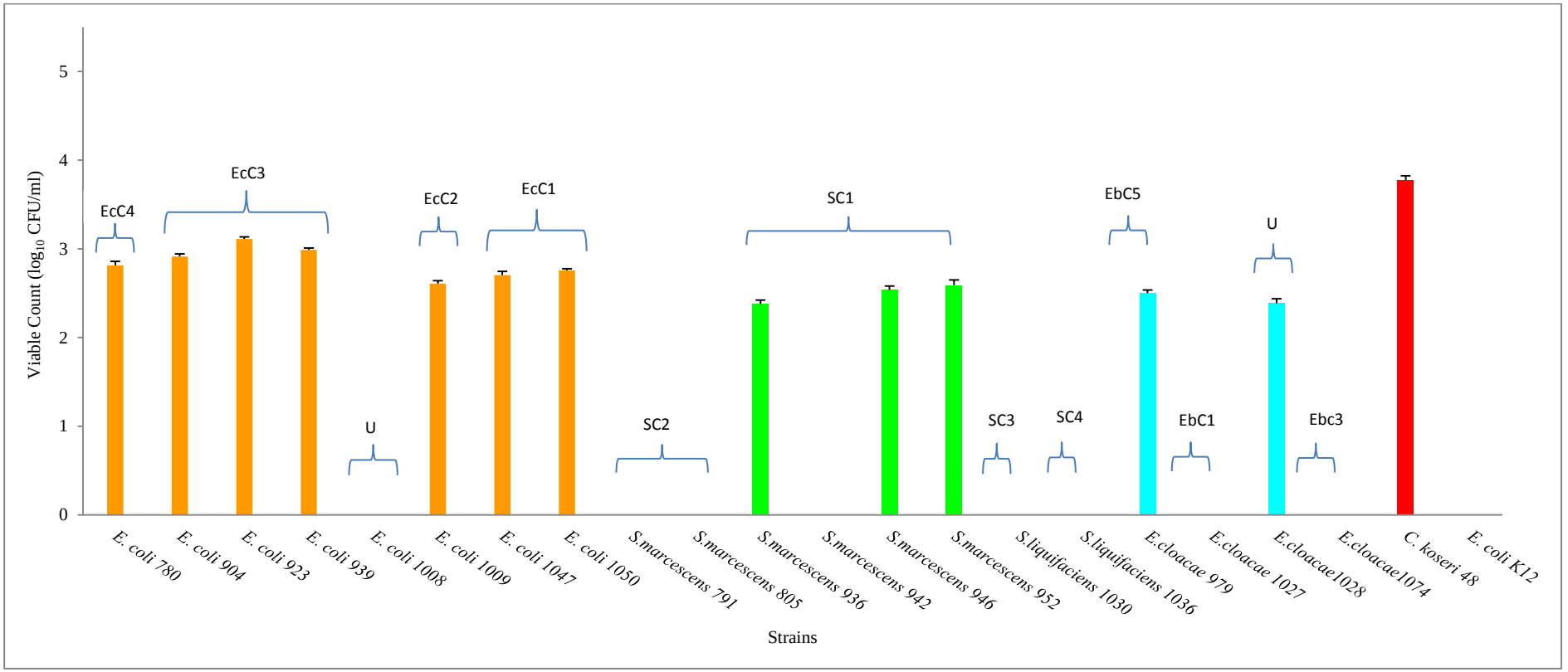

Figure 5.6 Invasion to HBMCE cells by *E. coli*, *Serratia* spp. and *E. cloacae*

C. koseri 48 and *E. coli* K12 were used as positive and negative controls, respectively. U= unique. EcC, SC and EbC= cluster group based on PFGE.

5.3.2 **Uptake and Persistence into Macrophage Cell Line (U937)**

Macrophages are immune cells, which engulf invading microbes and kill them to protect the host body. This is known as an innate immune process that plays a role in human antimicrobial defence mechanisms. The capabilities of some bacteria to persist or even grow within these long-lived immune cells provide them with protection and evasion from the immune response.

Figure 5.7 shows the results for twenty strains of *Enterobacteriaceae* which were investigated for their ability to persist in human macrophages after phagocytosis. This was performed using the U937 macrophage cell line. The same strains of *E. coli*, *Serratia* spp. and *E. cloacae* strains as used with previous cell lines were used to infect the U937 macrophage cell line. Strains of *C. koseri* SMT319 (NTU 48) and *E. coli* K12 (NTU strain 1230) were used as positive and negative controls respectively. The same strains of *Enterobacteriaceae* tested were able to persist in macrophages for up to 72 h.

All bacterial strains were shown to be taken up by the macrophages following the initial 45 min incubation. However, different levels were taken up by the macrophage after inoculation in the initial 45 min period, and there were between 2.5 $\log_{10}$ to 4.5 $\log_{10}$ CFU/ml.

Despite all bacterial strains were taken up by the macrophages following the initial 45 min incubation, only *E. coli* strains 780, 904, 923, 939, 1009, 1047, and 1050 were shown to persist and replicate inside macrophages; Figure 5.7. In contrast, *E. cloacae* strains showed decrease levels of persistence after the initial 45 min period.

E. coli strain 1008 and *Serratia*, strains 952, 1030, and 1036 showed the least resistance to macrophage engulfment, and were killed during the initial time (45 min). Whereas *Serratia* strains 971, 805, 942, and 1036 persisted and were recovered from macrophages at detectable levels after 48 h. Strains 939 and 946 of *Serratia* and *E. cloacae* 1074 did not show any persistence in the macrophages after 24 h.

5.3.3 **Summary of virulence factors for *Enterobacteriaceae***

When assessing opportunistic pathogen virulence potential, macrophage survival and invasion of CaCo-2, rBCEC4 and HBMCE cells are an important consideration. Using the data summarised in Table 5.1, twenty strains of *Enterobacteriaceae* used in study can be compared with respect to virulence potential. Their comparison showed that strains could be grouped as follow; high, moderate and low in their virulence potential. Group 1 high in their potential risk, which includes EcC3 and EcC4 of *E. coli* persisted or replicated in macrophages, showed high attachment and invasion of CaCo-2, rBCEC4 and HBMCE cells. These were followed by group 2 with moderate potential hazards. Group 2 involved *E.coli* strains 1009, 1047 and 1050; *S. marcescens* strains 936 and *E. cloacae* strains 979 and 1028. Third group of strains low in their potential risk such as *E. coli* 1008, *Serratia* strains 791, 805, 946, 952 and 1052 and *E. cloacae* 1074 and 1027. These strains were differed from group 1, with respect to lower CaCo-2, rBCEC4 and HBMCE invasion and killed by macrophage cells.

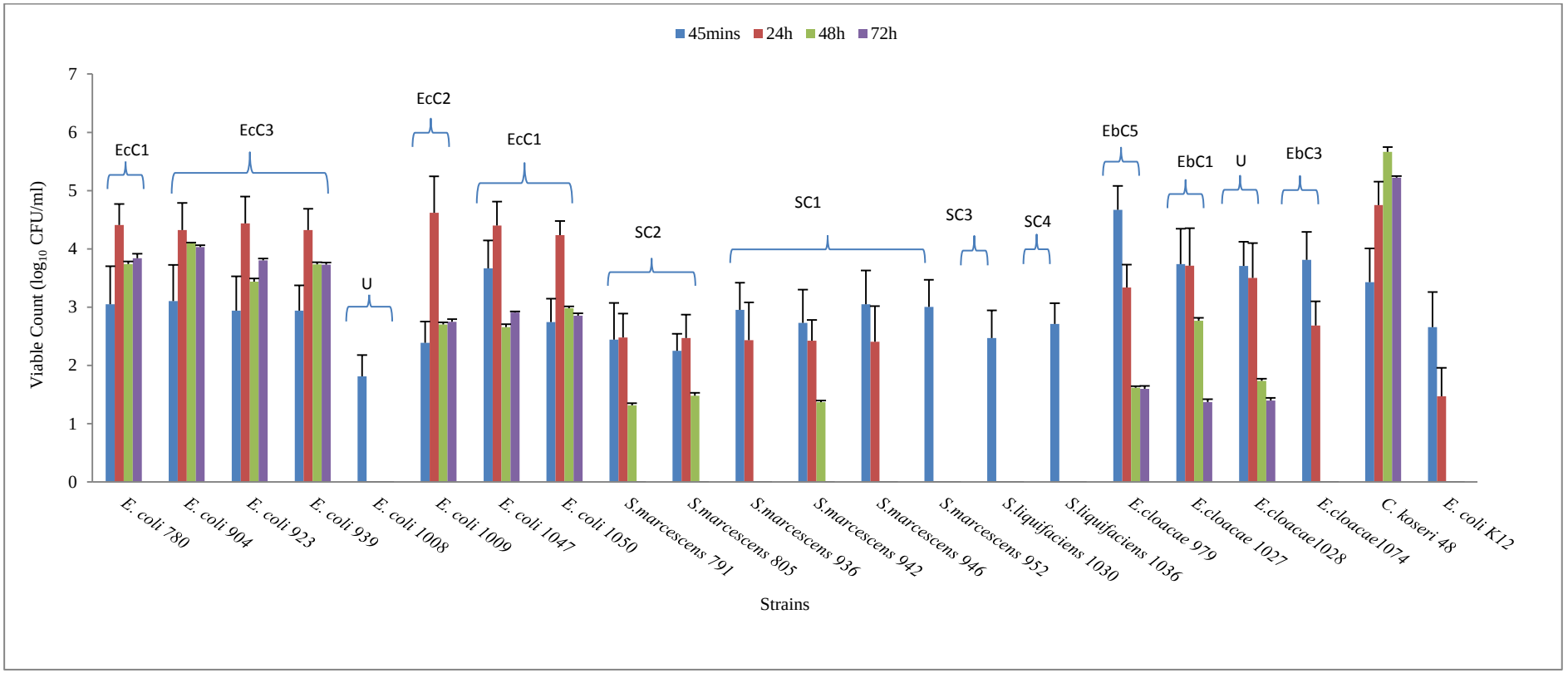

Figure 5.7 *E. coli*, *Serratia* spp. and *E. cloacae* survival in U937 macrophage cells after 45 min, 24h, 48h and 72h incubation

C. koseri 48 and *E. coli* K12 were used as positive and negative controls, respectively. U= unique. EcC, SC and EbC= cluster group of PFGE.

Table 5.1 Summary of virulence factors for *E. coli*, *Serratia* spp. and *E. cloacae*

Species	Strain	Pulsetype	Caco-2		Macrophage response	rBCEC4		HBMCE		Potential risk
			Attachment	Invasion		Attachment	Invasion	Attachment	Invasion	
E. coli	780	EcC4	Very High	High	Replicates- Persist	Very High	Moderate	Very High	High	High
E. coli	904	EcC3	Very High	High	Replicates- Persist	Very High	High	Very High	High	High
E. coli	923	≠	Very High	High	Replicates- Persist	Very High	Moderate	Very High	High	High
E. coli	939	≠	Very High	High	Replicates- Persist	Very High	High	Very High	High	High
E. coli	1008	U	Very High	Not able	Killed	Very High	Not able	High	Not able	Low
E. coli	1009	EcC2	Very High	Moderate	Replicates- Persist	Very High	Not able	High	High	Moderate
E. coli	1047	EcE1	Very High	High	Replicates- Persist	Very High	Not able	Very High	High	Moderate
E. coli	1050	≠	Very High	Very High	Replicates- Persist	Very High	Not able	Very High	High	Moderate
S. marcescens	791	SC2	Very High	Not able	Persist -Killed	High	Not able	Very High	Not able	Low
S. marcescens	805	≠	Very High	Not able	Persist -Killed	Very High	Not able	Very High	Not able	Low
S. marcescens	936	SC1	Very High	Moderate	Persist -Killed	Very High	High	Very High	High	Moderate
S. marcescens	942	≠	Very High	Not able	Persist -Killed	High	Not able	Very High	Not able	Low
S. marcescens	946	≠	Very High	Moderate	Persist -Killed	Very High	Not able	Very High	High	Low
S. marcescens	952	≠	Very High	Moderate	Killed	High	Not able	Very High	High	Low
S. liquefaciens	1030	SC3	Very High	Not able	Killed	High	Not able	Very High	High	Low
S. liquefaciens	1036	SC4	Very High	Not able	Killed	High	Not able	Very High	Not able	Low
E. cloacae	979	EbC5	Very High	High	Persist	High	Not able	Very High	High	Moderate
E. cloacae	1027	EbC1	Very High	High	Persist	High	Not able	High	Not able	Low
E. cloacae	1028	U	Very High	High	Persist	High	Not able	High	High	Moderate
E. cloacae	1074	EbC3	Very High	Not able	Persist -Killed	High	Not able	High	Not able	Low

U = unique, EcC, SC and EbC= cluster group of PFGE, ≠ = same

5.3.4 **Giemsa staining**

Based on the adhesion to Caco-2 and HEp-2 cells, *E. coli*, *Serratia* spp. and *E. cloacae* strains were investigated by staining with Giemsa stain to visualise their patterns of adherence. *Enterobacteriaceae* were distinctly in two patterns: Diffuse Adherence (DA), in which attach pattern of strains was dispersed to the whole cell surface and aggregative adherence (AA), in which attach pattern were appearance strains attach to the cell aggregated and stacked.

Table 5.2 show the attachment pattern of *E. coli* Hep-2 cell line and Table 5.3 shows adherence patterns of *E. coli*, *Serratia* spp. and *E. cloacae* to Caco-2 cell line. All strains of *E. coli* with the exception of strain 1008 showed aggregative attachment (AA), while strain 1008 showed diffuse attachment. These attachment patterns were the same with Caco-2 and Hep-2. Two strains of *Serratia* 952 and 1030 showed aggregative attachment (AA), whereas the others showed diffuse attachment (DA). An *E. cloacae* 979 was diffusely attachment (DA). Whereas the other *E. cloacae* strains aggregatively attached (AA) [Appendix 7.4].

Table 5.2 Adherence patterns of *E. coli* to Hep-2 cell line

Organism	Strain	Pulsetype	Adherence pattern
E. coli	780	EcC4	AA
E. coli	904	EcC3	AA
E. coli	923	EcC3	AA
E. coli	939	EcC3	AA
E. coli	1008	U	DA
E. coli	1009	EcC2	AA
E. coli	1047	CcE1	AA
E. coli	1050	CcE1	AA

AA = aggregative adherence, DA = diffuse adherence, U = unique.

Table 5.3 Adherence patterns of *E. coli, Serratia* spp. and *E. cloacae* to Caco-2 cell line

Organism	Strain	Pulsetype	Adherence pattern
E. coli	780	EcC4	AA
E. coli	904	EcC3	AA
E. coli	923	≠	AA
E. coli	939	≠	AA
E. coli	1008	U	DA
E. coli	1009	EcC2	AA
E. coli	1047	CcE1	AA
E. coli	1050	≠	AA
S. marcescens	791	SC2	DA
S. marcescens	805	≠	DA
S. marcescens	936	SC1	DA
S. marcescens	942	≠	DA
S. marcescens	946	≠	DA
S. marcescens	952	≠	AA
S. liquefaciens	1030	SC3	AA
S. liquefaciens	1036	SC4	DA
E. cloacae	979	EbC5	DA
E. cloacae	1027	EbC1	AA
E. cloacae	1028	U	AA
E. cloacae	1074	EbC3	AA

AA = aggregative adherence, AD = diffuse adherence, U = unique, ≠ = same

5.3.5 **Detection of virulence factor genes (VFGs)**

Eight strains of *E. coli* isolates were selected from the 30 *E. coli* feeding tube isolates for virulence factor genes based on their pulsetypes groups and virulence related traits. Virulence genotyping of *E. coli* strains were from the two local hospitals; QMC and NCH. The 30 virulence genes investigated, included a range of traits including genes encoding for adhesins, invasins, capsule, toxins, siderophores and others.

The virulence factor genes were detected between 0 to 75% of the strains and their incidence differed depending on the strain pulsotype; Figure 5.8. Adhesin genes *afa/draBC, bmaE, focG, fimH, gafD, papEF, papA, papC, nfaE, sfa/focDE, papG* allele II, *papG* I, *papG* II III, *papG* allele, *sfaS* and *papG* allele I. Figure 5.8 and Table 5.4 were present in 37.5%, 50%, 50% and 6.25% of EcC3, U, EcC2 and EcC1 strains, respectively, while adhesion genes were not found in EcC4. No strains carried the invasion gene *ibeA* therefore either false negative or other mechanisms involved in invasion. For capsular genes, *kpsMT III, kpsMT II, k1* and *k5* were found in 75.5%, 50%, 75.5%, 50% and 75% of EcC4, EcC3, unique strain 1008, EcC2 and EcC1 strains, respectively. The unique strain (1008) and EcC2 encoded the genes for toxins *hlyA, cnf*1 and *cdtB* and were present in 66.7% of these pulsotypes whereas no toxins genes were detected in other pulsotypes. Additionally, siderophore genes *fyuA, iutA* and pathogenic isolated gene *PAI* were found in 6.25%, 12.5%, 12.5% and 18.75% of EcC4, EcC3, unique strain of *E. coli* 1008 and EcC2 respectively and not found in EcC1. The other genes *rfc, cvaC* and *traT* occurred in only 6.25% of EcC4, EcC3, U and EcC1, while not detected in EcC2.

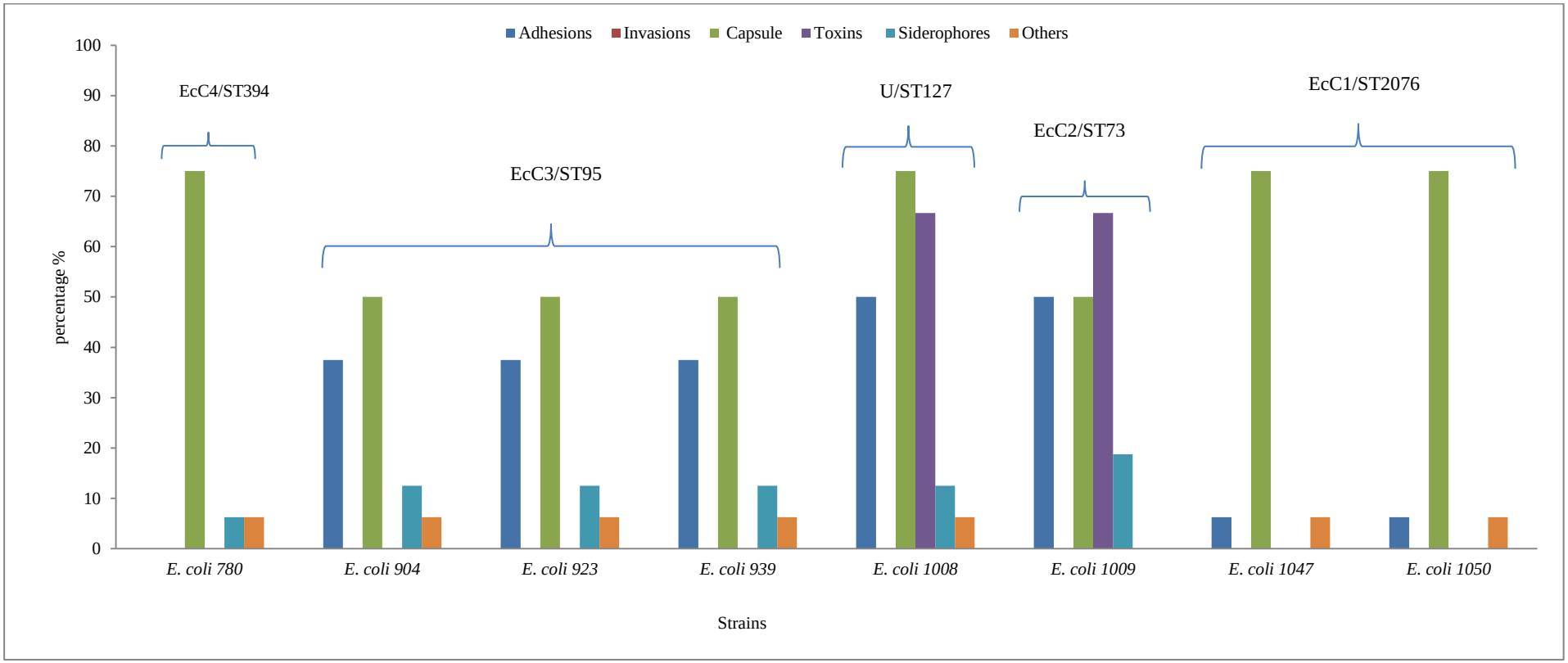

Figure 5.8 Virulence factor genes prevalence in *E. coli* isolates from feeding tube of two local hospitals in Nottingham

U = unique. EcC, SC and EbC = cluster group of Pulsed Field Gel Electrophoresis and STs = sequence typing.

Table 5.4 Distribution virulence factors among of *E. coli* isolates selected based on pulsotype

			Adhesions																Invasion	Capsule				Toxins			Siderophores			Others		
Strains of *E. coli*	Pulsetype	ST	*afa/draBC*	*bmaE*	*focG*	*fimH*	*gafD*	*papEF*	*papA*	*papC*	*nfaE*	*sfa/focDE*	*papG allele II*	*papG I*	*papG II,III*	*papG allele*	*sfaS*	*papG allele I*	*ibeA*	*kpsMT III*	*kpsMT II*	*K1*	*k5*	*hlyA*	*cnf+*	*cdtB*	*fyuA*	*iutA*	*PAI*	*rfc*	*cvaC*	*traT*
780	EcC4	394	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	+	-	-	-	-	+
904	EcC3	95	-	-	-	+	-	+	+	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-	-	+	-	+	-	-	+
923	=	=	-	-	-	+	-	+	+	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-	-	+	-	+	-	-	+
939	=	=	-	-	-	+	-	+	+	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-	-	+	-	+	-	-	+
1008	U	127	-	-	-	+	-	+	+	+	-	+	-	+	-	+	+	-	-	+	+	-	+	+	+	-	+	-	+	-	-	+
1009	EcC2	73	-	-	+	+	-	+	+	+	-	+	-	+	-	+	-	-	-	-	+	-	+	+	+	-	+	+	+	-	-	-
1047	EcC1	2076	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	+
1050	=	=	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	+

U = unique, EcC, SC and EbC = cluster group of Pulsed Field Gel Electrophoresis and STs = sequence typing, = = same

5.4 **Discussion**

The ability of different strains of *Enterobacteriaceae* to cause different clinical diseases are attributable to one or more virulence factors such as, attachment, invasion and survival with mammalian cells (Wilson *et al*., 2002; Miller, 1995). The genera of *E. coli* (most common), *Enterobacter*, *Serratia*, and other *Enterobacteriaceae* are opportunistic pathogens responsible for a wide range of infections. Many of these genera are members of the normal intestinal flora (Baron and Guentzel, 1996; Logue *et al*., 2012).

This part of the study assessed the potential virulence of strains from neonatal enteral feeding tubes and whether they pose an infection risk to cause neonates was investigated. Twenty *Enterobacteriaceae* composed of *E. coli*, *Serratia* spp. and *E. cloacae*, isolated from feeding tubes were chosen that represented species from the major genera isolated. The virulence potential of these 20 strains to mammalian cells was assessed using attachment and invasion studies of Caco-2, rBCEC4 and HBMEC tissue culture cells. Macrophage survival was studied using the U937 cell line of human monocyte cells.

The ability of *E. coli*, *Serratia* spp. and *E. cloacae* to attachment and invade Caco2, HBMCE and rBCEC4 mammalian cell lines are shown in Figure 5.1 - Figure 5.6. All strains were able to attach to epithelial and endothelial cell lines. The viable counts showed that *Enterobacteriaceae* strains were able to attach to Caco2, HBMCE and rBCEC4 cells. The viable counts showed that *Enterobacteriaceae* strains were able to attach to Caco2 cells, whereas *E. cloacae* strains were at relatively low numbers

compared to *E. coli*, *Serratia* Figure 5.1, Figure 5.3 and Figure 5.5. However all strains were able to attach to epithelia and endothelial cell line. Attachment to host cells may be a key of causes infection, where this step of interaction of pathogen to host surfaces, then microbes able by specific biochemical developments to result in disease including outbreak, toxin secretion, host cell invasion (Wilson *et al*., 2002).

Following attachment to the host surface, some bacterial pathogens are able to invade mammalian cells. It is believed that bacterial infection is the ability of microbes to invade host epithelial and endothelial cells (Townsend *et al*., 2008). Certainly, the capability for invasion of epithelial cells and brain endothelial cells is a concern (Kim, 2002), especially if *E. coli*, *Serratia* spp. and *E. cloacae* are found to colonise enteral feeding tubes. Consequently, their virulence potential was investigated to better understand the pathogenic potential of *E. coli*, *Serratia* and *E. cloacae*.

Comparable invasion values had obtained using Caco-2, HBMCE and rBCEC4 culture cells. The gentamicin protection assay demonstrated differing capacity of *E. coli*, *Serratia* spp. and *E. cloacae* to invade the intestinal epithelium (Caco-2). In detail, *E. coli*, and *E. cloacae* were more able to invade the intestinal epithelium than *S. marcescens*. Additionally, *S. liquifaciens* was not able to invade the intestinal epithelium Caco-2 and brain endothelial HBMCE and rBCEC4; Figure 5.2, Figure 5.4 and Figure 5.6. Further invasion studies investigated the potential of *E. coli*, *Serratia* and *E. cloacae* to invade the HBMCE and rBCEC4. This investigation suggests that *E. coli* showed more ability to invade HBMCE than *S. marcescens* and *E. cloacae*. As for rBCEC4, just four strains of *E. coli* pulsetypes EcC4, EcC3, and one of *S. marcescens* 936 showed the ability to invade rBCEC4 while others were

not able to invade; Figure 5.4 and Figure 5.6. This tendency was also observed in macrophage persistence experiments, which showed that the *E. coli* strains, except strain 1008, were capable of persistence and replicate within human macrophages, whereas *E. cloacae* strains only persisted at lower levels within the macrophages after 24 h. *S. marcescens* persisted at lower levels within the macrophages up to 48 then were killed. Whereas *E. coli* 1008 and *S. liquefaciens* were killed by macrophages, furthermore they were not able to invade the epithelial cells and brain endothelial cells.

Each assay determined the presence of virulence factors of selected strains, and demonstrated which virulence traits might be used by individual strains during infection. In addition when assessing opportunistic pathogen virulence potential of strains, to survive and replicate within epithelial, endothelial or macrophages cells are an important consideration. So according to summary of Table 5.1 *E. coli* 1008, *S. marcescens*, *S. liquefaciens* and *E. cloacae* 1074 isolated from an enteral feeding tube are not recognized as pathogens, this may be due in part to an inability to invade cells and showing less level to persist in human macrophage cells. Extra-intestinal pathogenic *E. coli* are commonly implicated in neonatal meningitis, urinary tract infections and septicemia in humans. Therefore, the ability of *E. coli* to invade the epithelial cells, persist and replicate in human macrophage cells and brain endothelial cells, and it is isolated from neonatal enteral feeding tubes at NICU may be a cause for concern.

Selected strains of *E. coli*, *Serratia* spp. and *E. cloacae* exhibited two characteristic adherence patterns diffuse adherence (DA) and aggregative adherence (AA), which

could be distinguished. Furthermore, in a number of strains a mixture of those two patterns could be observed. In several studies, strains of opportunistic pathogens such as entero-pathogenic *E. coli* has been categorised based on their adherence pattern to tissue culture cells: aggregative patterns, diffuse adhesion and localized (Janda and Abbott, 2006; Rüttler *et al*., 2006).

The results of the study showed that *E. coli* strains exhibited aggregative adhesion in both of cell line Caco-2 and Hep-2; except *E. coli* strain 1008 which showed was diffuse adhesion Table 5.2 and Table 5.3. In addition, *E. cloacae* strains exhibited aggregative adhesion except *E. cloacae* strain 979, which showed a diffuse pattern. While most strains of *Serratia* spp. showed diffuse adhesion, two strains had aggregative adhesion. These results indicate there could be a relationship between the adhesion patterns and invasion the epithelial cells, persist and replicate in human macrophage cells and brain endothelial cells, for example strains of *E. coli* that showed aggregative adhesion, invaded endothelial cells and persist and replicated in macrophages, in contrast to *E. coli* strain 1008. The aggregative, diffuse and localized pattern of adhesion were previously recognized in *Klebsiella, Enterobacter,* and *Serratia* strains associated with neonatal colitis and was found to be most prevalent phenotype in nosocomial strains (Livrelli *et al*., 1996). Nevertheless, for other bacteria such as *E. coli*, the association of adhesion patterns with different clinical cases is still contentious (Levine *et al*., 1988; Gomes *et al*., 1989). Although *E. coli* strains with localized adherence have been recognized enteric pathogens in several parts of the world, while the role of diffuse and aggregative adherence patterns are still unclear (Gomes *et al*., 1989).

A major focus in bacterial pathogenesis in recent years is the identification and characterization of the molecular virulence factors of genes that allow pathogens to cause infection in the hospital (Wilson *et al*., 2002). The capability of *E. coli* to cause diverse clinical infection is attributable to the cumulative impact of one or several virulence factors traits or virulence factor genes carried by these strains (Gibreel, 2011). For example, the carriage of genes for the K1 capsule contributes avoidance of neonatal host immunity (Johnson, 1991). Previous study reported that carriage of *hly* operon and the *sfa / foc* operon were often harboured by group B2 of *E. coli* meningitis strains (Bingen *et al*., 1998).

In this study, the prevalence of virulence factors was investigated. With regard to VFGs possession, it was found that *fim*H, *pap*EF, *pap*A, *pap*C, *pap*G1, *fyu*A, *kps*MT II, *tra*T and *PAI* were present in high levels across the most strains. Figure 5.8 and Table 5.4. For individual VFGs profile, ST95 strains harboured the K1 capsular antigen, *papG allele II* adhesions gene and type 1 fimbriae *Fim*H adhesions gene. However, the K1 capsule is compatible an essential meningeal virulence determinant and strongly linked it to neonatal meningitis *E. coli* strains and pathogenicity (Saukkonen *et al*., 1988; Peigne *et al*., 2009). This study shows that ST73 and ST127 of *E. coli* were not possessed the K1 capsular antigen, *papG allele II* adhesions gene and type 1 fimbriae *Fim*H adhesions gene. Those STs were show Low levels of invasion of epithelial cells, persist and replicate in human macrophage cells and brain endothelial cells. This could suggest that the key to ST95 elevated virulence potential cannot be attributed to specific VFGs set or increased carriage of VFGs in general.

Chapter 6 **General discussions**

6.1 **General discussion**

Neonates are at high risk of nosocomial infection in neonatal intensive care units (NICUs). Nosocomial infections are a most important problem in neonatal intensive care units (NICUs), and the estimated rates of infection at NICUs can be 10 – 20%; these are higher than in most paediatric and many adult wards. In addition, many outbreaks at NICUs have been reported (McGuire *et al*., 2004; Vergnano *et al*., 2005; Brady, 2005; Phillips *et al*., 2008). Nosocomial infection via *Enterobacteriaceae* is commonly caused by commensal bacterial strains that already colonise the infants (Al muneef *et al*., 2001). *Klebsiella* spp., *E. coli, Serratia* spp., and *Enterobacter* spp. are opportunistic pathogens that colonize the gut and sometimes causes invasive, meningitis, pneumonia and are responsible for considerable morbidity and mortality (Friedland *et al*., 2003; Adamson *et al*., 2012). Hurrell *et al*. (2009a) found that the neonatal enteral feeding tubes act as loci for in the attachment and colonisation by numerous opportunistic pathogens within the *Enterobacteriaceae* family. Subsequently, these organisms will enter the stomach as a bolus with each feed. So, enteral feeding tubes are an important risk factor with respective of neonatal infections.

Molecular subtyping methods are an important tool in determining a common source of infection causing nosocomial outbreaks or potential cross vertical transmission or horizontal transmission events and sources of infection (Adamsson *et al*., 2000; Almuneef *et al*., 2001). Molecular typing techniques such PFGE are commonly used in outbreak investigations. In this study, PFGE was used to track the spread of *Enterobacteriaceae* in two NICUs with the absence of an outbreak.

For improve health care and avoid outbreaks and nosocomial infections there are debates how to investigate avoided infection by microbes. Where a previous study suggested that directed investigation of outbreaks and nosocomial infections rather than study colonization and environments microbes (White *et al*., 1981). While others, considering that studying the colonisation by microbes is a more important for avoided infection (Goldmann, 1988). In this study a collection of two-hundred and twenty-four *Enterobacteriaceae* strains isolated from the lumen and the inner wall as biofilm of neonatal enteral feeding tubes of 2 NICUs in Nottingham UK were investigated. Tubes received from infants that were 'nil by mouth'. In addition, the neonates were fed by diversity of feeds including breast milk and sterile feed formula Table 1.2 and for more information see Appendix 7.8. These are described as receiving a 'mixed feeding regime'. The enteral feeding tubes were in place between < 6 h to > 48 h and the neonates was received fresh fed of milk at every 2 - 3 h. The ages of the neonates were from 1 week, to 4 weeks.

Later additional collections of thirty-eight strains of *E. coli* from Jordan were obtained for preliminary profiling. Unfortunately, because of the long time between the collection and this study, less information is currently available on the clinical details. This could be useful for detecting reservoirs of potentially pathogenic microbes. Consequently that can be leading to increased exposure and risk to the neonates at NICUs.

This study shows that over the period from January to November 2007, neonatal enteral feeding tubes were contaminated by twenty-nine pulsetypes of *Enterobacteriaceae*. Which was distribute as follow four, four, eight and thirteen

pulsetypes of *E. coli*, *Serratia* spp., *Enterobacter* spp. and *Klebsiella* spp. for which no sources were identified. These has been established by others and is considered to be the most infants were colonized with *Enterobacteriaceae*, primarily *E. coli, Klebsiella, Enterobacter, Serratia* and *Citrobacter* spp. (Adamson *et al*., 2012; Almuneef *et al*., 2001; Goldmann, 1988; Tullus *et al*., 1988; Parm *et al*., 2011).

Although multiple *Enterobacteriaceae* pulsetypes were isolated, this study demonstrated that the presence of the prevalent strains. The indistinguishable; pulsetypes of *E. coli* and *Serratia* spp. were less diverse than the other species. This indicates a narrower range of strains were colonising the enteral feeding tubes. For example, pulsetype group EcC3 of *E. coli* comprised 19 strains, isolated during the two-week period from 26 April to 9 May and isolated from eleven patients. Similarly, for two large pulsetype groups SC1 and SC2 of *S. marcescens*, each group had including 34 and 19 strains and was isolated over a prolonged period for one month and four months, respectively. In addition may be provided cross-transmission clone was recovered from different patients for long periods. Our findings in this study indicate that indistinguishable clone of EcC3 strain of E. coli was colonising feeding tube from 11 different patients. Where those patients were fed by different kind of milk Table 3.1 and for more information see [Appendix 7.8]. So that indicates the contamination not from the milk, may possibly originated from the same sources, especially for substances such as environment or carer. In addition this study indicated that *E. coli* could be more of a prevalent and cross-transmission clone, whereas the later collection of *E. coli* strains from Jordan were shown large pulsetype group EC2 comprised 33 strains out of 38 strains; Figure 3.5. In contrast, the *Enterobacter* spp. and *Klebsiella* spp. showed a large diversity of PFGE profiles

(Figure 3.3 and Figure 3.4), reflecting a wider range of strains was isolated from the enteral feeding tubes. This diversity in PFGE profiles indicates the contamination of feeding tubes could be from different sources.

An important finding in this investigation was the same pulsetypes spread among enteral feeding tubes of infants in the same NICUs. The result of specific strains belonged to same pulsetypes EcC3 and EcC2 of *E. coli* and SC1 and SC2 of *S. marcescens* associated with enteral feeding tubes or NICUs could be there is no doubt that there was a common source of contamination, such as the feed or carers (i.e. nurse). *Enterobacteriaceae* was isolated from PIF include *E. cloacae*, *K. pneumoniae, K. oxytoca, E. hormaechei,* and *E. coli* (Muytjens *et al*., 1988). The FAO/WHO, (2004 and 2006) considered these organisms as *'causality plausible, but not yet demonstrated'* with respect to their potential source to cause neonatal infection via ingestion of reconstituted PIF.

On the other hand, it was clear that certain strains of pulsetypes EcC3 and EcC2 of *E. coli* and SC1 and SC2 of *S. marcescens* were isolated from different patients and isolated over a prolonged period in which medical care could be provided by a single medical team. Transmission of *Enterobacteriaceae* is commonly from patient to patient by way of the hands of the health care team (Brady, 2005). Previous study indicated that a potentially higher probability of cross-transmission with certain Gram-negative such as *S. marcescens* by the hands of the health care team (Waters *et al*., 2004).

Eight strains of *E. coli*, from two NICUs in Nottingham, UK, representing the various pulsetypes were selected for MLST analysis. As shown in clear

discrimination between the eight strains of *E. coli* ST2076, ST73, ST95, ST394 and ST127. According to UCC database, these STs belonged to the *E. coli* pathogenic group B2, extraintestinal pathogenic *E. coli* (ExPEC). Based on the type of infection ExPEC were subtypes into: uropathogenic *E. coli* (UPEC); neonatal meningitis associated *E. coli* (NMEC) and sepsis-associated *E. coli* (Köhler and Dobrindt, 2011). In this study MLST profile were confirmed the result of PFGE, where STs group strains of *E. coli* isolated showed as well as similar PFGE patterns, which suggest that these isolates could be related. For example *E. coli* strains 904, 923 and 939 were indistinguishable by their PFGE profiles as well as being the same ST. *E. coli* ST95 was isolated over a two week period from three neonates, and therefore of concern due to the continued exposure. The study by Manges *et al*. (2008) indicated that *E. coli* members isolated from both study locations showed similar PFGE patterns and MLST patterns, which indicated that, they are likely related.

Reviewing the MLST database at the ERI, University College Cork (http://mlst.ucc.ie/mlst/dbs/Ecoli/GetTableInfo_html) revealed that the prevalence ST95 in this study has been associated with various infections, including meningitis, diarrhoea, septicaemia and invasion. According to Achtman database three strains out of 146 strains of *E. coli* ST95 had caused meningitis. These strains were reference numbers ISUNMEC48, ISUNMEC36 and ISUNMEC; two were from Europe and one North America respectively. The study by Gibreel *et al*., (2012) found that uropathogenic *E. coli* strains UTI89, NU14 and RS218 were causes of neonatal meningitis. These strains were also ST95. Additionally more information from database shows that there are 34 strains out of 146 also ST95 which caused septicaemia.

Following subgroup *Enterobacteriaceae* collection, data generated in this study using 20 isolates were chosen for the represented pulsetype group. These strains included eight strains of *E. coli*, six strains of *S. marcescens*, two strains of *S. liquefaciens* and four strains of *E. cloacae*.

The work included in this thesis, focuses on studying biofilm formation and composition, which related factors with relevance for enteral feeding tubes. *E. coli*, *S. marcescens*, *S. liquifaciens* and *E. cloacae*, were abundant bacteria in the enteral feeding tubes biofilm. It was previously known that opportunistic pathogens within the *Enterobacteriaceae* family are known to be able to attach, multiply and colonize nasogastric enteral feeding tubes (Hurrell *et al*., 2009a). In NICUs, low birth weight infants are fed via enteral feeding tubes, and these may be left in-place over prolonged periods (> 24 h). Consequently, this will enhance bacterial attachment and multiplication as biofilm (Hurrell *et al*., 2009b). As shown in this study *Enterobacteriaceae*, such as *E. coli*, *S. marcescens*, *S. liquifaciens* and *E. cloacae* are able to form biofilm. The biofilm detected by crystal violet staining allows quantifying the production of biofilm and comparing different strains or different conditions (Stepanović *et al*., 2004). Average OD of producing biofilm by 20 *Enterobacteriaceae* strains was 1.35 AU; Figure 4.1 and Figure 4.2.

In a NICU the tubes will be between ambient body temperature, and receive fresh nutrients from the infant feed at every 2 – 3 h, which may have an additional affected formation as well as biofilm is temperature. In this study the effect of temperature was evident an in biofilm formation. This was significantly higher ($p < 0.05$) at 37ºC for all strains compared 25ºC. According to Else *et al*. (2003) temperature may also

affect the production of extracellular polymeric substances, which is known to increase the biofilm formation of bacteria. Another factor which may have an effect on biofilm formation is the type of formulas. The results show that formula composition may effect of biofilm production, where all pulsetypes group of *E. coli* and SC2 of *S. marcescens* were shown form value of biofilm with casein-based more than whey-based formula, for example strains 939 and 1047 grown in whey-based formula showed absorbance units approximately 0.57 (AU) while biofilm formation in casein-based formula was about 1.5 (AU); Figure 4.1.

In addition the study has shown a variation range in motility between strains belonged to same pulsetypes. For example strains 939 and 942 of *S. marcescens,* belong to pulsetype SC1 were one non-motile and the other swarm diameter of ~ 30 mm respectively; Figure 4.3. So, further analyses are necessary in order to confirm the isolates are related or not by used another restriction enzyme then those strains could be confirmed as the same or not. According to the results shown in Figure 4.1 and Figure 4.3 there was no relationship between motility and biofilm formation. Previous studies of *E. coli* and *Salmonella* reported that motility was not required for initial attachment and biofilm formation (Pratt and Kolter, 1998; Prigent-Combaret *et al*., 2000).

As the biofilm ages the *Enterobacteriaceae* become detached and break off as clumps. These detached cells with fresh feed, will subsequently enter the neonate stomach. The ingestion of microbes during feeding will expose them to acidity stress in the stomach. Although the pH of stomach is ~ 2.5, which kills the common of swallowed bacteria, however this is not true for the neonate. While the infants'

stomach pH of the infant fed milk formula is remaining ~ 3.5 to 4.3 during the feeding regimes (Hurrell *et al*., 2009a). In this study the selected strains were screened for pH 3 tolerance to mimic infant's stomach pH of the infant fed milk formula. *E. coli* strains, *Serratia* spp. and *E. cloacae* strains showed persistence at pH 3 for up to 2 h; Figure 4.4 to Figure 4.6. The protection of these strains from the low pH acidity could be because of the detached cells being in clumps. In addition, the persistence of strains in low pH may be because of their ability to form a biofilm inside the feeding tube. A previous study of acid tolerance by biofilm cells of *Streptococcus* (McNeill and Hamilton, 2006) showed the cells in the biofilm are highly resistant to low pH. The survival of these strains at low acid may increase the risk of exposure bacterial pathogens in neonates. Furthermore, capsule formation may have role in protecting strains from the low pH.

In this study the production of capsule was investigated for *E. coli*, *S. marcescens*, *S. liquifaciens* and *E. cloacae*. All strains were showed different capsule morphology; Table 4.1. There was no correlation between capsule formation and biofilm on milk agar for 20 strains studied. For example, *S. marcescens* 791 and 805 produce more capsules, nevertheless those strains produced less biofilm. *E. coli* pulsotype EcC3 produced more biofilm than the most capsulated *S. marcescens* strains 791 and 805. Capsules may have a role in resistance to desiccation, serum activity and contribute survival of the organism after uptake by phagocyte cells (Guerry and Szymanski, 2008). The reason for the difference is incretion between microbes and cell surface structure. Also it may be linked to attachment factors such as curli fimbriae and cellulose production. The role of curli fimbriae in adherence and invasion has been described in members of *Enterobacteriaceae* (Olsén *et al*.,

1993; Römling *et al*., 1998; Zogaj *et al*., 2003; Boudeau *et al*., 2001; Lee *et al*., 2011).

Through this study the morphotypes are shown in Table 4.2. All isolates belonging to the dominant morphotypes produced pink colony on Congo red agar formation, so that indicates that strains are producing cellulose, with exception strain 1028 of *E. cloacae* which expressed red colony which mean showing the expression of curli fimbriae Bokranz *et al*. (2005). The author also reported the capability of *E. coli* which formed biofilms and correlated with expression fimbriae and cellulose. In *E. coli* and *Salmonella*, cellulose is one of the major components of the biofilm matrix (Da Re and Ghigo, 2006).

This study demonstrates that 20 strains of *E. coli*, *Serratia* spp. and *E. cloacae* are able to form biofilms. In addition the strains surviving in low acidity produce capsules and express cellulose. These factors is associated with these organisms could be constitute a risk factor to neonatal health.

Survival strains of *E. coli*, *S. marcescens*, *S. liquifaciens* and *E. cloacae* up to 2h in the neonate's stomach due to protection from the low acidity. This will enhance cells to pass through from neonate's stomach to the neonate's intestine. These could result to increased risk subsequently greater potential to colonise and infect the neonate via toxins productions by bacteria such as α-haemolysis, which may damage the host cell membrane or invasion cell line. Alpha-haemolytic activity was detected in *E. coli*, *S. marcescens*, *S. liquifaciens* and *E. cloacae*, except *E. coli* 1008 which was ß-haemolytic; Table 4.3. The α- haemolysin is an important virulence factor generally expressed by extraintestinal pathogenic *E. coli* (Balsalobre *et al*., 2006).

This part of the study assessed the potential virulence of 20 strains composed of *E. coli*, *Serratia* spp. and *E. cloacae* strains from neonatal enteral feeding tubes and whether they pose an infection risk to cause neonates. The virulence potential was assessed using standard using attachment and invasion studies of Caco-2, rBCEC4 and HBMEC tissue culture cells. Macrophage survival was studied using the U937 cell line of human monocyte cells. The ability of organisms to attachment, invasion and survival within mammalian cells, that would be cause different clinical diseases and in some cases, may be responsible for considerable morbidity and mortality at NICU (Wilson *et al*., 2002; Miller, 1995).

Comparable attachment and invasion values were obtained using Caco2, HBMCE and rBCEC4 mammalian cell lines and are shown in Figure 5.1- Figure 5.6. All strains were able to attach to epithelial and endothelial cell lines. This demonstrated that CaCo-2, HBMCE and rBCEC4 cells did not lack attachment factors required for *E. coli*, *Serratia* species and *E. cloacae* strains that may support the robustness of the epithelial cell invasion assay. The viable counts showed that *Enterobacteriaceae* strains were able to attach to Caco2, HBMCE and rBCEC4 cells. The viable counts showed that *Enterobacteriaceae* strains were able to attach to Caco2 cells, whereas *E. cloacae* strains were at relatively low numbers compared to *E. coli*, and *Serratia* spp.*;* Figure 5.1, Figure 5.3 and Figure 5.5. However all strains were able to attach to epithelia and endothelial cell line. Attachment to host cells may be a key of causes infection, where this step of interaction of pathogen to host surfaces, then microbes able by specific biochemical developments to result in disease including toxin secretion and host cell invasion (Wilson *et al*., 2002).

Following attachment to the host surface, some bacterial pathogens are able to invade mammalian cells. The gentamicin protection assay demonstrated differing capacity of *E. coli, Serratia* spp. and *E. cloacae* to invade the intestinal epithelium Caco-2. It was noted that *E. coli* and *E. cloacae* showed higher attachment and invasion values of Caco-2 than *S. marcescens*; Figure 5.2. This investigation detected that *E. coli* pulsetypes EcC4, EcC3 showed more ability to invade HBMCE than *S. marcescens* and *E. cloacae*. As for rBCEC4, just four strains of *E. coli* pulsetypes EcC4, EcC3, and one of *S. marcescens* 936 showed the ability to invade rBCEC4, while others were not able to invade; Figure 5.4 and Figure 5.6.

When assessing bacterial strains for virulence potential, macrophage survival is an important consideration in Figure 5.7. For example all pulsetypes groups of *E. coli*, EcC1, EcC2, EcC3 and EcC4 (except strain 1008), persisted and replicated within human macrophages. *E. cloacae* strains only persisted at lower levels within the macrophages after 24 h. *S. marcescens* were persisted at lower levels within the macrophages up to 48 than were killed. Whereas *E. coli* 1008 and *S. liquefaciens* were killed by macrophages, furthermore they were not able to invade the epithelial cells and brain endothelial cells. In this work there was not relationship between sensitivity of *E. coli, Serratia species* and *E. cloacae strains* to oxygen radicals and persistence of these strains within macrophages.

Using the details summarised in Table 5.1 the five pulsetypes groups of *E. coli* EC4, EC3, EC2, CE1 and U; four pulsetypes *Serratia species* SC2, SC1, SC3, SC4 and four pulsetypes *E. cloacae* EbC5, EbC1, U, EbC3 can be compared with respect to

virulence potential. Such comparison confirms that the four pulsetypes groups of *Enterobacteriaceae* vary in their virulence potential, which was distributed as follow:

- Cluster 1 organisms considered high potential risk EcC3 and EcC4 of *E. coli* showed high attachment and invasion of Caco-2, HBMCE and rBCEC4 cells persisted and replicated in macrophages.
- Cluster 2 organisms considered moderate potential risk EcC1and EcC2 of *E. coli,* EbC5 and strains 936 and 1028 of *S. marcescens* with respect ability Caco-2 and HBMCE attachment and invasion and persist up to 48 then killed in macrophages.
- Cluster 3 organisms considered low potential risk all other pulsetypes were killed by macrophage cells and not able to invade Caco-2, HBMCE and rBCEC4.

Therefore, the ability of *E. coli* to invade the epithelial cells, persist and replicate in human macrophage cells and brain endothelial cells, and it is isolation from neonatal enteral feeding tubes at NICU may be a cause for concern. It is believed to be that bacterial infection would be capable of invasion of host epithelial and endothelial cells (Townsend *et al*., 2008). Certainly, the capability for invasion of epithelial cells and brain endothelial cells is a concern (Kim, 2002).

The ability of *E. coli* strains to invade to mammalian cell lines could be linked with adherence patterns. Strains showing aggregative adhesion pattern showed high invasion rates. For example, results in this study showed that *E. coli* collations, EcC3 and EcC4 strains exhibited with Caco-2 and Hep-2 aggregative adhesion Table 5.2 and Table 5.3. Those pulsetypes showed high invasion. *E. coli* strain 1008 of *E. coli*

showed diffuse adhesion and was not able to invade to Caco-2, HBMCE or rBCEC4. In addition, *E. cloacae* strains exhibited aggregative adhesion except *E. cloacae* strain 979, showed a diffuse pattern and were more invasive. Whereas most of strains *Serratia* spp., showed diffuse adhesion and shows lees invasive. *E. coli* strains with localized adherence have been recognized enteric pathogens in several parts of the world (Gomes *et al*., 1989).

This study was aimed at investigating the prevalence of virulence factor genes of *E. coli*. The ability of different strains of *E. coli* to cause nosocomial infections in NICUs could be attributed to virulence factors harboured by these strains. For example K1 capsule is compatible an essential meningeal virulence determinant and strongly linked it to neonatal meningitis *E. coli* strains and pathogenicity (Saukkonen et al., 1988; Peigne et al., 2009). A carriage gene of K1 capsule allows evasion of neonatal host humoral immunity (Johnson, 1991). Kim et al. (2003) investigated the ability of *E. coli* K1 to invade human brain microvascular endothelial cells HBMEC, although it remains unclear how *E. coli* K1 crosses HBMEC. Khan et al. (2007) demonstrated that type 1 fimbriae, specifically FimH attached to brain endothelial cells and also triggers signalling events that promote *E. coli* K1 to invade in HBMEC. Type 1 fimbriae, specifically *Fim*H adhesion, which was harboured by our strains of Table 5.4; is not only an adhesive organelle that provides bacteria with a foothold on brain endothelial cells but also triggers signalling events that promote *E. coli* K1 invasion in HBMEC (Khan et al., 2007) . Furthermore, *E. coli* K1 binding to and invasion of HBMEC. Taken together, these findings indicate that *Fim*H induces host cell signalling cascades that are involved in *E. coli* K1 invasion of HBMEC and CD48 is a putative HBMEC receptor for *Fim*H. With regard to VFGs

possession, it was found that K1 capsular, *papG allele II* adhesions gene and type 1 fimbriae *Fim*H adhesions gene were harboured by ST95 isolates Table 4.6. This group shows more invasions to Caco-2, HBMCE and rBCEC4 and in addition were persist and replicate in human macrophage cells Table 5.1. ST95 belonged to pulsetype group EcC3 of *E. coli* which comprised 19 strains, and shows more the prevalence of period and between 11 patients.

Chapter 7 **Conclusions**

7.1 **Conclusion**

Finally, the first part of the this study considered possible reasons why certain *Enterobacteriaceae* are more frequently associated with enteral feeding tubes and possible links with neonatal infections at NICUs and determining a common source of infection, based on DNA fingerprinting via PFGE. The conclusions of this part can be summarised as follows:

- The findings some strains were genotypecally related or differing by restriction fragments belonged to different PFGE groups.
- Genotyping using PFGE would be an appropriate strategy for studying relationship between isolates e.g. for enteral feeding tubes pathogens.
- An important finding in this investigation was the same pulsetypes were spread across enteral feeding tubes of different infants in the same NICUs. A consistent profile of *E. coli* EcC3 and *S. marcescens* SC1, SC2 repeatedly appeared in patients were over an extended time period.
- All pulsetype EcC3 strains were ST95.
- *E. coli* strains studied (n= 30) belonged to the pathogenic group B2, extraintestinal pathogenic *E. coli* (ExPEC).

The second part of the study focused on comparative physiological and virulence related tests of 20 strains *E. coli*, *Serratia* spp. and *E. cloacae*.

- Incubation temperature affected biofilm formation on plastic materials by *E. coli, Serratia* spp. and *E. cloacae.*
- Type of formula has an influence on biofilm formation by *E. coli.*

- Strains belonging to the same pulsetype, differed in their motility one non motile and the other motile. It would be of use to use another restriction enzyme to confirm these as indistinguishable strains or otherwise.
- Strains in this study were able to survive at low acidity (pH 3) for up to 2h and may increase the risk of exposure to bacterial pathogens by neonates.
- The detected of haemolytic activity (α and β) by most of strains in study could indicate an increased risk to neonates.

The third part of the study concentrated on virulence traits which are relevant to an enteroinvasive bacterial pathogen.

- All strains used in study were able to attach to Caco-2, rBCEC4 and HBMCE cell line.
- Many stains were able to invade human intestinal epithelial cells Caco-2 and brain endothelial cells HBMCE and rBCEC4. These can be used to find mechanisms of invasion and spreading in the infected host.
- The average invasion rate of the *E. coli* strains was greater than *Serratia* spp. and *E. cloacae*.
- Most of the *E. coli* strains show macrophage survival values whereas strains of *Serratia* spp. and *E. cloacae* not persist after 24 hours.
- This study revealed that strains showing the aggregative adhesion pattern also showed more invasions.
- In this study found K1 capsular gene was only harboured by *E. coli* ST95 strains, which considered high potential risk, also showing high

attachment and invasion of Caco-2, HBMCE and rBCEC4 cells. In addition were show persisted and replicated within macrophages.

7.2 **Future work**

- Visualisation of bacterial adherence patterns and engulfment by Caco-2 intestinal cells using GFP labelled bacterial cells.
- Rearrangement of host cell cytoskeleton on exposure to bacterial cells. Any re-arrangement will be visualised using fluorescent microscopy using such anti-actin antibody labelled with dyes.
- Measuring pro-inflammatory and anti-inflammatory cytokines, and NF-KB production by host cells on bacterial exposure. Caco-2 colonic cells invasion, inflammation of the human and rat brain endothelial cells will be determined to demonstrate the cause of meningitis.

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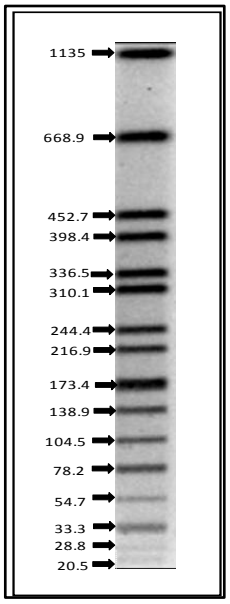
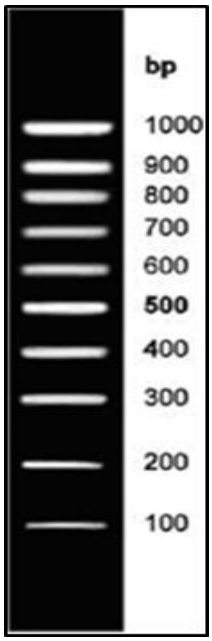
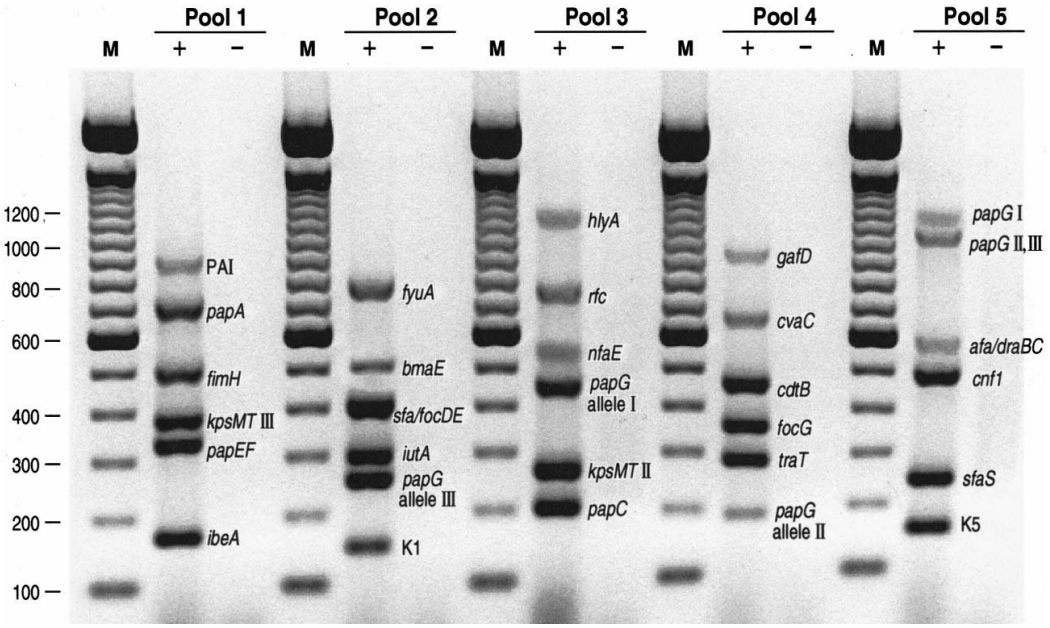
Appendix



Appendix 7.1 approximate band sizes in Kbp of the *Salmonella*, restricted with *XbaI* and run under the PulseNet standardized electrophoresis conditions.



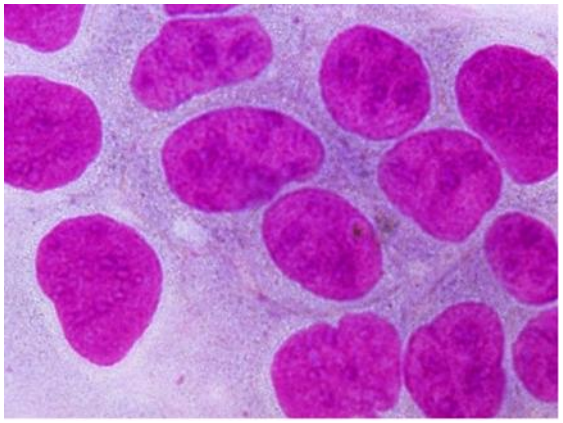
Appendix 7.2 100 bp PCR sizer ladder run at 90v.

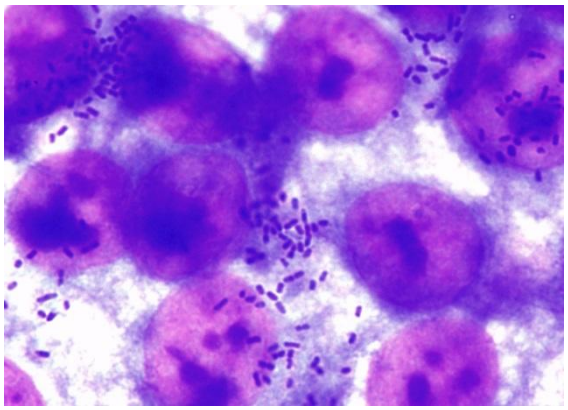


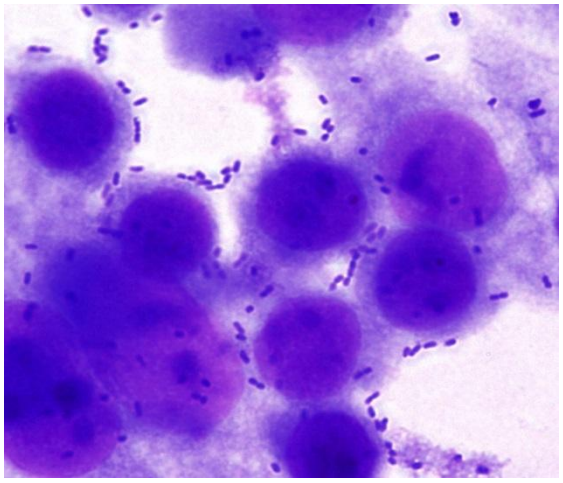
Appendix 7.3 Agarose gel showing polymerase chain reaction (PCR) products from multiplex virulence factor

primer pools 1–5. M, 100 bp PCR sizer ladder; + = positive control; - = negative control.

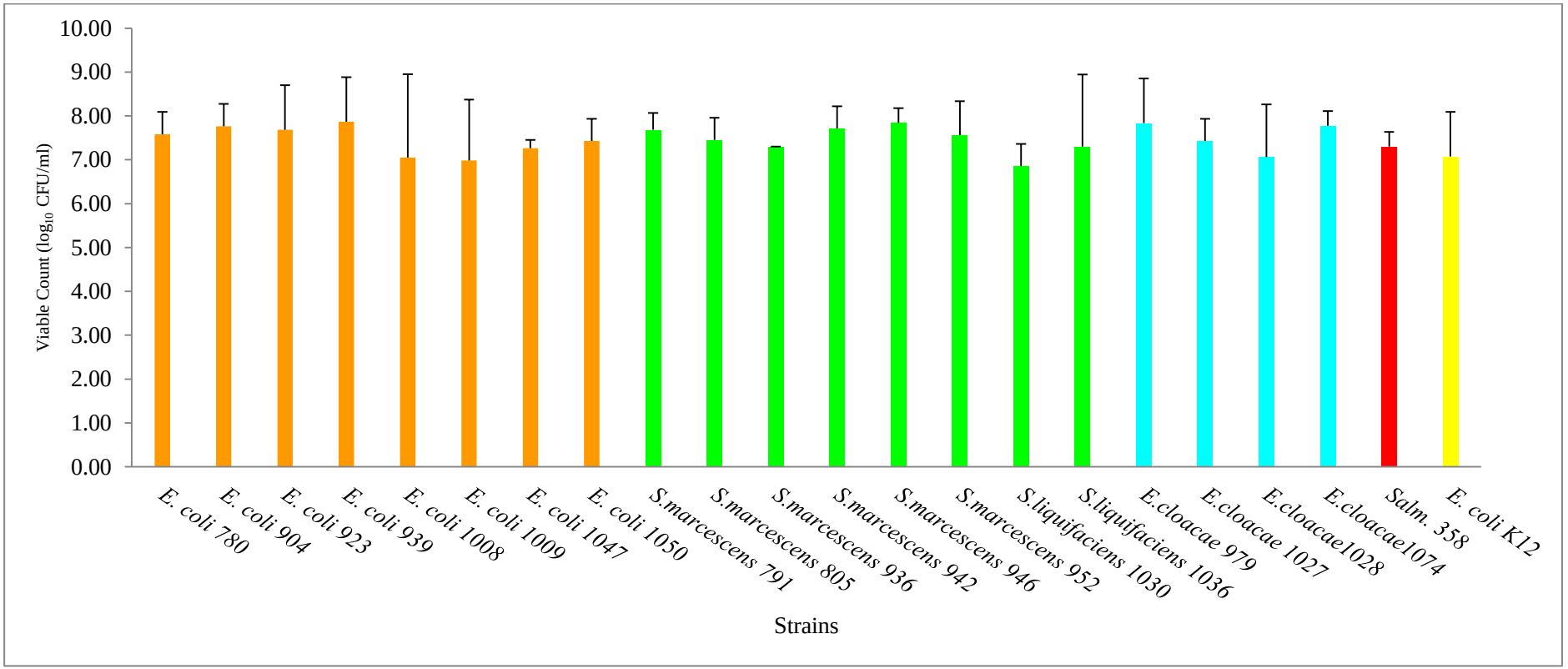
Extended virulence genotypes of Escherichia coli strains from patients with urosepsis in relation to phylogeny and host compromise (JOHNSON, 2000).

A 

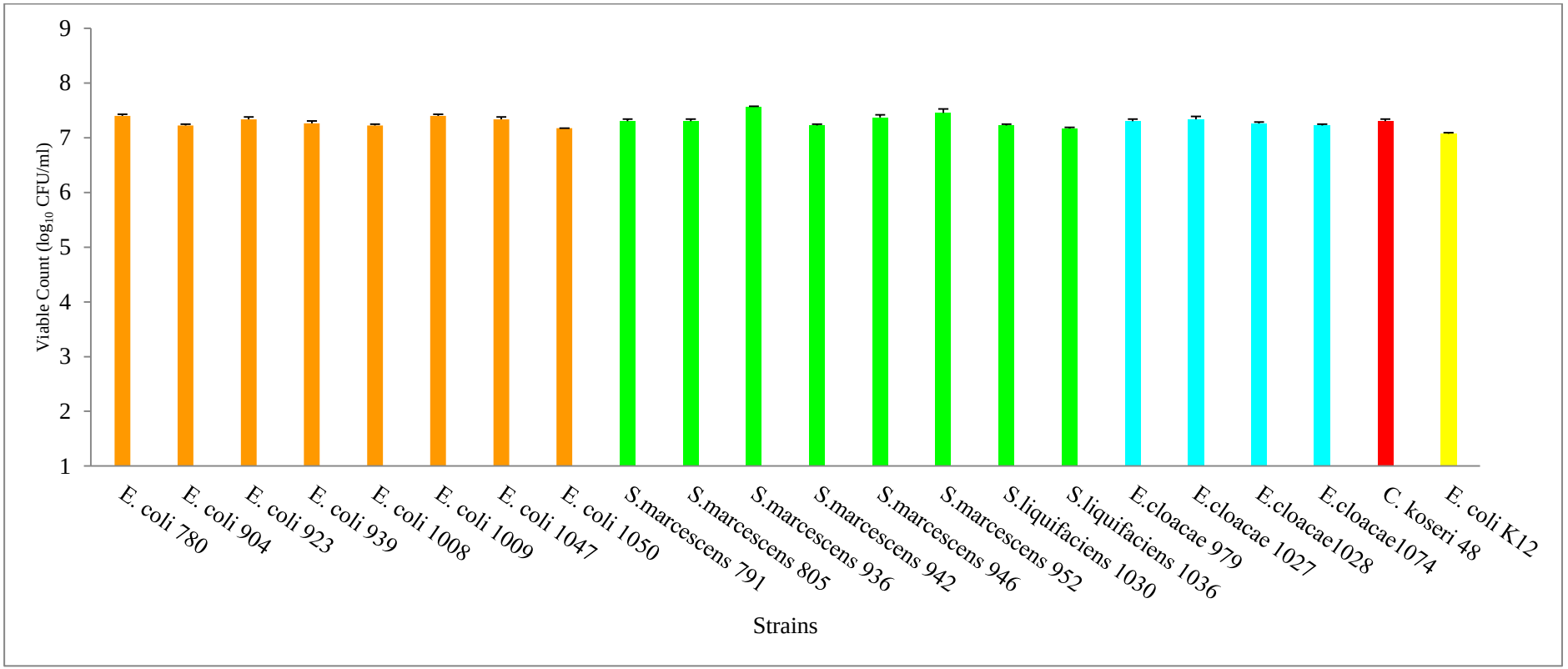
B 

C 

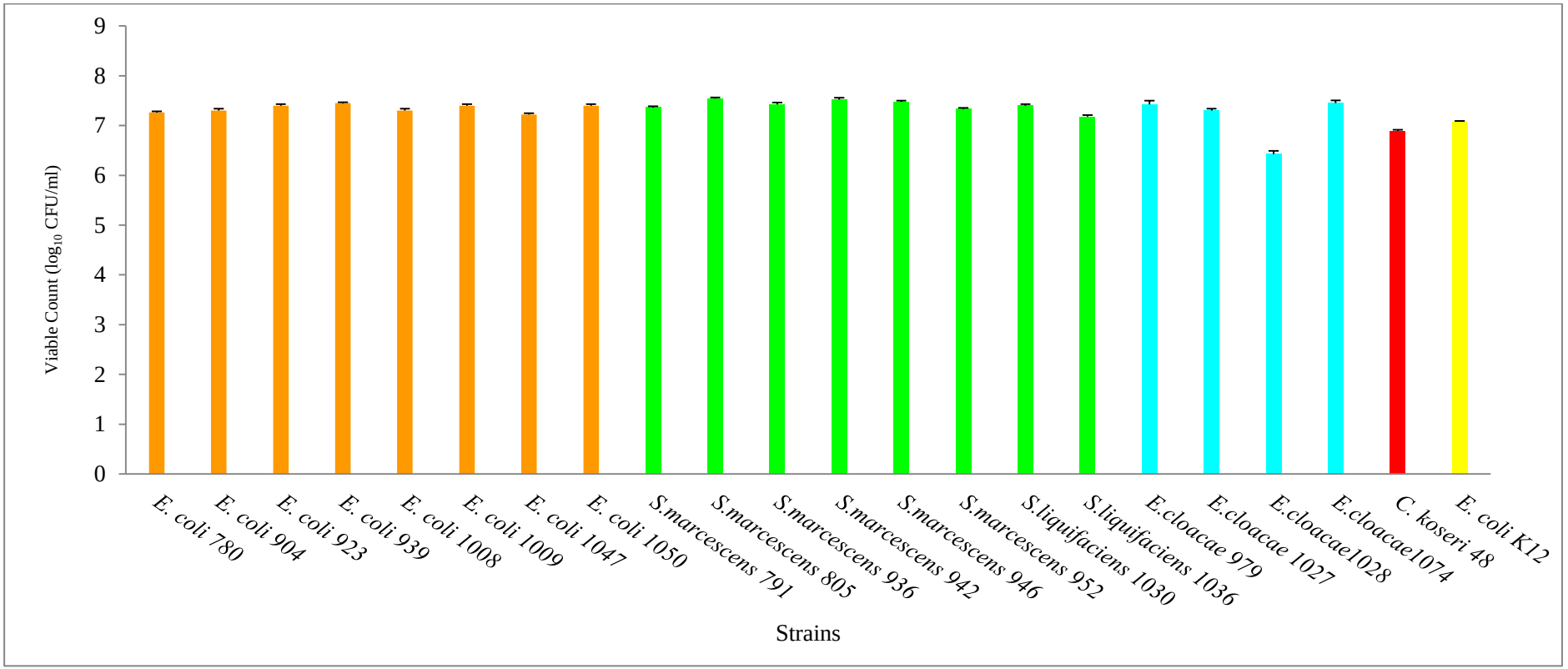
Appendix 7.4 Adherence patterns (A) control, (b) Aggregative Adherence (AA) and (c) Diffuse adherence (DA)



Appendix 7.5 Initial inoculum of *E. coli*, *Serratia* spp., *E. cloacae*, positive control and negative control to CaCo-2 cells



Appendix 7.6 Initial inoculum of *E. coli*, *Serratia* spp., *E. cloacae*, positive control and negative control to rBCEC4 cells



Appendix 7.7 Initial inoculum of *E. coli*, *Serratia* spp., *E. cloacae*, positive control and negative control to HBMCE cells

Species	Pulsetype cluster	Strain number	Location recovered from NGT	Source	Date of isolation	Neonate	Feeding source	Duration (h)	Age (wk)	Frequency of feeding
E. coli	EcC4	780	Biofilm	NCH	16/01/2007	4	BM	18-24	>4	Every 2h
		796			30/01/2007	17	BM, PIF	6-12	>4	Every 2h
		786			17/02/2007	37	BMF,PIF, Th	18-24	>4	Every 3h
	EcC3 K1	904		NCH	24/04/2007	55	BM,IF	24-48	3-4	Every 3h
		905	Lumen							
		926	Biofilm		01/05/2007	61	BMF, RTF	6-12	>4	Cont.
		927	Lumen							
		929	Biofilm			62	PIF	>48	>4	Cont.
		933	Lumen							
		934	Biofilm			63	RTF	18-24	3-4	Every 3h
		910			03/05/2007	64	BMF, RTF	>48	1-2	Every 2h
		912	Lumen							
		913	Biofilm			65	RTF	24-28	>4	Cont
		917	Lumen							
		923	Biofilm		08/05/2007	67	BMF	12-18	1-2	Every 2h
		924	Lumen							
		937				68	BMF, RTF	18-24	3-4	Every 2h
		939	Biofilm			69	BMF	<6	1-2	Every 2h
		943				70	RTF, Th	24-48	>4	Every 2h
		944	Lumen							
		947				71	PIF, Th	<6h	>4	Every 4h
		949	Biofilm							

E. coli	U	1008	Lumen	NCH	06/06/2007	99	BMF, RTF, Th	>48	2-3	Every 3h
	EcC2	1009	Biofilm	NCH	05/06/2007	101	BMF	>48	>4	Every 2h
		1010			12/06/2007	102		12-18	>4	Every 2h
		1015				104	BM	24-48	3-4	Every 2h
		1016	Lumen							
	EcC1	1047	Biofilm	QMC	22/10/2007	116	RTF	12-18	2-3	Every 2h
		1050	Lumen							
		1051								

Appendix 7.8 Summary of neonates' age and feeding regimes

BM = Breast milk, BMF = Breast milk fortified, PIF = Reconstituted powdered infant formula, IF = Infant formula, no further description given, RTF = Ready to feed formula, Cont = Continuous feed, Th = Thickener added to feed, NCH = Nottingham City Hospital and QMC = Queens medical Centre