NOTTINGHAM TRENT UNIVERSITY

Aetiology of potentially pathogenic bacteria from neonatal feeding tubes.

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A thesis submitted in partial fulfilment of the requirement of Nottingham Trent University of the degree of Doctor of Philosophy.

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Declaration;

I hereby certify that the work presented herein is the result of my own research work, except where reference has been made to published literature. I have composed the thesis and the work has not been submitted for any other degree or professional qualification. All the work was conducted in the School of Science and Technology at the Nottingham Trent University. You may copy up to 5% of this work for the private study or personal, non-commercial research. Any information used from this thesis should be fully cited.

Mohamed Taher A. Saad

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Manuscripts in preparation

- *In situ* colonization of nasogastric feeding tubes of neonates in intensive care by *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Escherichia coli.*

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List of abbreviations;

ACT	Artemis comparison tool
ANOVA	Analysis of Variance
AU	Absorbance units
BSAC	British Society for Antimicrobial chemotherapy
BLAST	Basic local alignment search tool
bp	Base pairs
BPW	Buffered peptone water
BRIG	BLAST ring image generator
Caco-2	Human colonic carcinoma epithelial cells
CASAD diffusion	Chrome Azurol S agar
CLB	Cell lysis buffer
CSB	Cell Suspension Buffer
COSHH	Control of substances hazardous to health
CV	Crystal violet
DNA	Deoxyribonucleic acid
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extra intestinal pathogenic *E. coli*
ESBL	Extended spectrum beta-lactamase
FAO	Food and Agriculture Organization of the UN
FDA	Food and Drug Administration

GIT	Gastrointestinal tract
HBMEC	Human brain microvascular endothelial cells
HCL	Hydrochloric acid
HDTMA	Hexadecyltrimethylammonium bromide
ICU	Intensive care units
IF	Infant formula
ISA	Iso-Sensitest agar
MSHA	Mannose sensitive haemagglutination
MRHA	Mannose resistant haemagglutination
MLST	Multi-locus sequence typing
NGT	Nasogastric tube
NEC	Necrotising enterocolitis
NEFT	Neonateal enteral feeding tube
NICU	Neonatal intensive care unit
NTU	Nottingham Trent University
OD	Optical density
PBS	Phosphate buffered saline
PBP	Penicillin binding proteins
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
PIF	Powdered infant formula
SNPs	Single Nucleotide Polymorphisms
Spp.	Species

ST	Sequence type
T24	Human bladder carcinoma cell line
TAE	Tris/acetate/EDTA
TBE	Tris base/ Boric acid / EDTA
TEB	Tris EDTA buffer
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
U937	Macrophage Cell Line
UPGMA	Unweight pair group method with arithmetic mean
UTI	Urinary tract infection
UV	Ultraviolet
VREF	Vancomycin resistant *Enterococcus faecalis*
VRBGA	Violet red bile glucose agar
WHO	World Health Organization
XLD	Xylose lysine deoxycholate agar
α	Alpha
β	Beta
ɣ	Gamma

Abstract;

Recently, the incidence of neonatal infections, particularly in those born with low birth weight (< 2000g) has increased due to *Enterobacteriaceae* including *Escherichia coli*, *Klebsiella* and *Serratia* spp., and other Gram-positive bacteria such as *Enterococcus* spp. These are known to be responsible for major neonatal intensive care unit (NICU) infections. Mortality among neonates is attributed to infectious causes, preterm birth complications, intrapartum-related complications, sepsis and meningitis. Therefore, this study aimed: (a) to evaluate the potential risk to neonates posed by ingestion of *E. coli* and *Klebsiella* spp, *E. hormaechei* and *E. faecalis* either through powdered infant formula, contaminated milk, or by medical equipment, (b) to categorise isolates of these organisms into high, medium and low potential pathogenicity to neonates, and (c) to conduct a longitudinal study to determine whether the same strain colonises both the feeding tube and intestine of a premature baby in the NICU over time and to determine their virulence potential and genetic relationships.

This study used, 76 *K. pneumoniae* strains previously isolated from neonatal feeding tubes, from two Jordanian hospitals from May to Dec 2011 and 36 isolates (*E. coli* n=14 and *Klebsiella* n=22) from Queen's Medical Centre and Nottingham City Hospital (QMC & NCH, Nottingham), from neonatal enteral feeding tubes and sepsis cases between 2007 and 2015. In addition, 14 isolates (*E. faecalis* n= 8 and *E. hormaechei* n= 6) were collected from four samples (two nasogastric feeding tube and two faecal samples) from a single premature baby at age 6 and 8 weeks of life in the NICU at QMC, Nottingham.

The isolates were identified by sequence analysis of the *rpoB* gene, 16S rDNA and genotyped using pulsed-field gel electrophoresis (*XbaI, SpeI and SmaI* restriction digestion), subsequently profiled using specific PCR probes for virulence genes (K1, 2, and 5, fimbria type, invasion gene and yersiniabactin). Isolates were examined for potential virulence factors including biofilm, capsule production, serum resistance, siderophores, heat resistance, desiccation tolerance and antibiotic resistance. Potential virulence traits were predicted from whole genome sequences. In addition, *in vitro* tissue culture assays (attachment, invasion and macrophage survival) were used in a comparative study between

representative *K. pneumoniae* strains isolated from EFT Jordanian hospitals and Nottingham NEFTs isolates and sepsis isolates.

The *K. pneumoniae* Jordanian strains clustered into five pulsotypes, pulsotypes 1 and 2 were *rpoB* allele profile 4, cluster 3 and 4 were *rpoB* profile 25 and pulsotype 5 was *rpoB* profile 21. The curli fimbriae and hypermucoviscous phenotype were observed in 10.7 % and 67.9 % of isolates respectively. Capsular serotypes included K1 (17.9%) and K2 (78.6%). All isolates showed resistance to imipenem, meropenim and ceftriaxone. An extended spectrum beta-lactamase (ESBL) was identified phenotypically in strains with resistance to cefotaxime + clavulanate and cefpodoxime + clavulanate, and most isolates showed susceptibility to ciprofloxacin. All strains had ɣ-haemolytic activity on sheep blood and most showed ß-haemolytic on horse blood. Most isolates were able to form biofilms on plastic surfaces at 25 ^{o}C and 37 ^{o}C. Most of the strains were able to survive in pH 3.5 for up to 2 hours and tolerated human serum.

For the *E. coli* (QMC) strains, PFGE showed two *E. coli* strains 2113 and 2114 clustered together, whereas the other strains were all unique. Thirteen *E. coli* strains from QMC & NCH belonged to phylogenetic group B2 and strain 2255 was group D. The majority of isolates showed ɣ-haemolytic activity on both horse and sheep blood, capsular type K1 and type 1 fimbria were detected. Almost all of the strains were resistant to augmentin, whereas only 7% of the strains were resistant to ceftazidime. ESBL production was identified phenotypically in 99% of the strains with resistance to cefotaxime + clavulanate (CTX+CV) and cefpodoxime + clavulanate (CPD+CV). Furthermore, 77% of the strains were multidrug resistant. All strains were able to form biofilms at 25^{o}C and 37^{o}C.

In addition, *Klebsiella* spp. (QMC) strains revealed eight different *rpoB* alleles profiles; 1,2,4,10,13,14,15,40. Using PFGE, strains 498 and 500 were clustered together, whereas the other strains were all unique. Phenotypically, all isolates were able to lyse horse erythrocytes, showing ß-haemolytic activity, and were ɣ-haemolytic on sheep blood. Furthermore, most of the strains possessed plasmids and were able to produce cellulose, hyperviscous capsule and biofilm in TSB and infant formula milk (IF). Most strains were able to form capsular material on XLD and IF agar. Only strain 1444 expressed curli fimbriae and produced high levels of biofilm on both media. The great majority of these *K. pneumoniae*

isolates were able to adhere and invade T24, HMBEC and Caco2 cell lines. Unexpectedly, *K. pneumoniae* strains isolated from FT from Jordan were significantly better at surviving within macrophage (U937) cells than strains isolated from sepsis blood cultures from QMC, Nottingham.

The longitudinal study indicated that the *E. hormaechei* and *E. faecalis* strains isolated persisted in the baby's gut across the whole period of study without genotypic and phenotypic changes. Some isolates from feeding tubes and faeces of the baby over time were found to be the same strain based on their sequence type and genomic analysis. All of these strains were shown to have high pathogenic potential.

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Chapter 1. Introduction:

1.1. General background:

Some of the most life threatening infections acquired in the early stages of human life would include bacterial neonatal infections such as bacteraemia, septicaemia and meningitis, which can lead to high mortality and morbidity among infants (Chávez-Bueno and McCracken, 2005). Despite an advancement in healthcare, the average number of deaths among new-born babies is still around 2.6 million per year worldwide, of which, one million deaths occur during the first week of life, accounting for nearly 38.5% of the total. More than 80% of deaths were associated with premature babies, delivery, and infections such as pneumonia, bacteraemia, sepsis and meningitis (UNICEF data 2018). Furthermore, prolonged hospitalisation, preterm neonates and low birth weights increase the risk of infection to neonates (Stoll *et al*., 2005; Klinger *et al*., 2010).

The annual number of deaths among toddlers below five years has decreased by more than half in recent decades, from 12.7 million cases in 1990 to 5.9 million cases in 2015 (UNICEF, 2017; UN Sustainable Development, 2017). Despite the fact that overall neonatal death rates have declined in the last two decades, the mortality rate among neonates in the first few days after live birth has not significantly reduced (UN Sustainable Development, 2017). The infant death rate within the first week of life is estimated to be nearly 1.7 times higher in rural zones than in urban regions. Furthermore, the survival rate of new-born babies with educated mothers is three times higher than those with un-educated mothers. Therefore, the UN Sustainable Development goals (2017) states that universal efforts are required for non-developed countries where education is lacking or limited, to support awareness for expectant mothers about the risk of microbial pathogenicity during pregnancy and feeding, to protect their babies from infection.

In the UK, the death rate among neonates was 3.7 deaths/1000 births in 2015, a slight increase from 3.6/1000 births in 2014. There are several significant risk factors that are associated with increased mortality rate including low birthweight, immaturity, birth asphyxia, pneumonia, sepsis, the parents' poor socio-economic situation and young age of the mother at birth of the child (Black *et al,* 2010; Liu *et al,* 2012; Patel, 2017). The mortality

rate among neonates (< 1 month) in Europe was 148,000 cases in 2008; 18% of these were immaturity complications and infections caused 33% of the deaths (Lawn *et al*., 2005; Black *et al*., 2010). Neonatal sickness is partly due to bacterial infection, and in early life the baby is highly vulnerable due to their underdeveloped immune system.

Members of the ESKAPE pathogens group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and Enterobacter species) are responsible for most hospital- and community-acquired infections and present serious threats in nosocomial pathogenesis (Rice, 2008). Furthermore, some members of the Enterobacteriaceae are considered to be the most common opportunistic pathogens globally, particularly *Klebsiella* spp., *E. coli*, *Salmonella* spp., *Enterobacter* spp., and *Serratia* spp., which are widely associated with neonatal morbidity and mortality (Adamson *et al*., 2012; Friedland *et al*., 2003). *K. pneumoniae* is an example of an opportunistic pathogen within the Enterobacteriaceae associated with nosocomial infection outbreaks such as sepsis, bacteraemia, infantile meningitis, typically in immunocompromised patients.

1.1.1. The ESKAPE group:

The ESKAPE group represents the most important pathogens that are responsible for the most common nosocomial infections within modern hospitals, which are capable of evading the vast majority of antibiotics and biocidal actions. These pathogens are also associated with high mortality and morbidity especially among immunocompromised patients Table *1-1*. There is great concern due to their prevalence within hospitals, especially in neonatal intensive care units (NICUs) and they cause more mortality among patients than HIV and tuberculosis combined (Klevens *et al*., 2006; Boucher & Corey, 2008; Boucher *et al*., 2009; Rice *et al*., 2008; Rice *et al*., 2010).

The transmission of bacterial pathogens in hospital environments is influenced either by direct contact with contaminated surfaces or through contaminated gloves, hands or medical equipment of hospital medical staff, spillage of contaminated drops on sterile surfaces, as well as sharp and invasive tools used on patients. The presence of a potential reservoir (contaminated medical devices, medical staff and surfaces) in the hospital may

increase the potential risk for nosocomial infections (Hota, 2004; Inweregbu *et al*., 2005; Boyce, 2007; Kelsey, 2013). Nosocomial infections are infections acquired when the patient is admitted to the hospital for treatment of other illnesses and contact with medical facilities or any other hospital environments occurs (Girard *et al*., 2002).

Table 1-1: Pathogenic characteristics of ESKAPE pathogens.

Strain	Pathogenisis
Enterococcus faecium	*E. faecium* enters the human body through contact (ingestion, open wounds, etc.) with contaminated sources. Once infected, the patient suffers from septicaemia and the mortality rate can be as high as 50%.
Staphylococcus aureus	*S. aureus* can multiply and colonize a patient in a short time frame, especially in immunocompromised patients. This can result in a range of diseases including boils, skin sepsis, post-operative wound infections, enteric infections, septicaemia, endocarditis, osteomyelitis, pneumonia, impetigo, meningitis, and arthritis.
Klebsiella pneumoniae	Poor hand hygiene of staff, patients, and visitors has been identified as one of the main sources of colonization of *Klebsiella spp* . in nosocomial settings. The weakened immune systems of the immunocompromised, both young and old, or those with open wounds are highly susceptible to infection from *Klebsiella* and colonization may lead to an infection.
Acinetobacter baumannii	*Acinetobacter* infections in immunocompromised individuals can include urinary tract infection, pneumonia, meningitis, and wound infection. There are various reports of veterans from the US and UK who were injured while on tours of Iraq or Afghanistan being colonized by *A. baumannii* .
Pseudomonas aeruginosa	Principal infections include septicaemia, skin, respiratory, urinary tract, and ear and eye. Theseinfections may occur due to burns, surgery, and open wounds. Cystic fibrosis and immunocompromised patients are prone to colonization with P. aeruginosa, which may lead to serious progressive pulmonary infections.
Enterobacter species	*Enterbacter* spp. have been associated with sporadic cases or small outbreaks of sepsis, meningitis, cerebritis, and necrotizing enterocolitis. Most of the infections are seen in low-birth-weight infants (i.e. less than 2 kg) or infants born prematurely (i.e. less than 37 weeks of gestation).

World Health Organisation (WHO), (2011)

Nosocomial infection is a global health problem. Allegranzi and co-authors (2011) reported that in Argentina the prevalence of patients with nosocomial infections was 11.5% (n = 4,249) Durlach *et al*. (2012), in Germany it was 5.1% (n = 41,539) Behnke *et al*. (2013), in six Southeast Asian countries were 9.0% (n = 12, 285) Ling *et al*. (2015), in the USA was 4.0% (n = 648,000) Magill *et al*. (2014) and 10.1% from other developing countries Allegranzi *et al*. (2011). The detection rate of bacteria in hospital environments in Germany were 24.7% for vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) and 4.9% for Gram-negative bacteria with antimicrobial resistance (Lemmen *et al*., 2004; Boyce, 2007). Furthermore, in 2018, a Malaysian tertiary hospital study investigated 358 swab and fluid samples collected from medical equipment, hands of healthcare workers,

ward sinks and toilets, and frequently touched surfaces by using biochemical tests and 16S ribosomal RNA (rRNA) sequencing. Among the isolates, the most clinically important bacteria were *Enterococcus* spp., *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter* spp*., Pseudomonas aeruginosa*, and *Enterobacter* spp. The great majority of these isolates were isolated from a few key sources: medical equipment, frequently touched surfaces, and ward sinks and toilets (Phoon *et al*., 2018).

Another study investigated blood cultures collected from babies with cancer. The ESKAPE members recovered were Enterococcus spp. (9%), *Staphylococcus aureus* (MRSA)(23%), *Klebsiella pneumoniae* (37%), *Acinetobacter baumannii* in (10%), *Pseudomonas aeruginosa* (9%) and *Enterobacter* spp.,(12%)(El-Mahallawy *et al*., 2016).

1.1.2. The Enterobacteriaceae:

The Enterobacteriaceae are Gram-negative short rods, oxidase-negative, the majority are motile, some are encapsulated, and they constitute large and differed grouping contain 44 genera and more than 176 species (Hong Nhung *et al*., 2007). This family are facultative anaerobes, non-spore forming and able to ferment various carbohydrates. Some members of this family define serologically depending on the capsular lipopolysaccharide, and flagella protein. Many virulence factors such as invasins and toxins have been studied in the Enterobacteriaceae. The vast majority of this family are opportunistic pathogens, particularly *E. coli*, *Salmonella* spp., *Klebsiella* spp., *Enterobacter* spp. and *Serratia* spp., that are linked to significant morbidity and mortality (Friedland *et al*., 2003; Adamson *et al*., 2012). Several types of infections can result from these organisms for example in the brain (meningitis), wounds (sepsis), urinary tract, and lung (pneumonia) especially in ICUs. In addition, some of these family members such as *E. coli* which is the most common, *Klebsiella*, *Citrobacter*, spp., *Serratia* and *Proteus* are responsible for nosocomial infections. Most of these organisms are part of the normal intestinal flora, and could cause such infections following an empiric antibacterial regime. The most common human associated pathogens of this family are shown in Table 1-2.

Table 1-2: The most common human associated species of Enterobacteriaceae.

Genus	Clinically importance 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

This table was adapted from Liu *et al*., (2012).

1.2. Neonatal infectious diseases:

Hospitalised babies, especially premature neonates are highly susceptible to infections from several routes such as intravascular catheters, nasogastric feeding tubes, medical devices and immature mucosal and epithelial barriers. The neonatal gastrointestinal tract is sterile at birth, but shortly afterwards is rapidly colonised by microorganisms (Kaufman and Fairchild, 2004; Ramasethu, 2017). Previous studies have stated that environmental and healthcare practices in neonatal units (for example diet, antibiotic exposure) have significant effects on the incidence of intestinal sepsis in preterm neonates. Furthermore, the feeding tubes, on which some premature babies are dependent, may act as a site for potential pathogenic bacterial colonisation and a reservoir for infection. It has also been stated that different feeding regimes could influence bacterial colonisation of feeding tubes (Hurrell *et al*., 2009a and 2009b). These pathogens are considered to be resistant to such environmental stresses like neonatal stomach pH, and then proliferate in high numbers.

Several studies have stated that neonatal deaths data globally may represent countries or regions with high mortality rates where the babies are predominantly born outside hospitals (at home), such as in rural areas (Seale *et al.,* 2009; Bang *et al.,* 2001). In support of this notion, several reports have documented that microbial neonatal infections have been

conducted in low income countries in tertiary centres in urban areas, but most of the babies are born outside of the hospital at home in the rural areas (Laving *et al*., 2003; Simiyu *et al*., 2005; Bang *et al*., 2001). Talbert *et al*. (2010) reported that several studies conducted in these countries could not confirm whether the infection was nosocomial infection or following delivery in hospital or at home. Therefore, due to a lack or limitation of good hygiene and microbiology facilities in under-developed countries, the data concerning causative organisms are still not accurate globally, particularly among delivery-associated and nosocomial infections.

Bacterial neonatal infections can lead to serious diseases such as acute diarrhoea, pneumoniae, bacteraemia, sepsis and meningitis, which are the most common causes of neonatal deaths (Cortese *et al.,* 2016). Several studies reported that the death of new-born babies within the first week of life refer to vertical infection (cross infection) from the mother during delivery, whereas the deaths that occur later in the neonatal period are attributed to environmental microbial acquisition (horizontal infection), such as child handling, hospital instruments and social behaviours (Camacho-Gonzalez *et al.,* 2013; Chan *et al.,* 2013). The most predominant microbial neonatal infections therefore might vary based on the geographical conditions (Darmstadt *et al.,* 2011; Cortese *et al.,* 2016). In under-developed countries, the rate of neonatal infection ranged from 3 to 20 times higher than in developed countries. Furthermore, toddlers who were born at hospitals in these countries are more exposed to infection from environmental microbes especially *Staphylococcus aureus, Klebsiella* spp., and *Pseudomonas* spp., due to less qualified nursing teams and poor hygiene in NICUs, delivery rooms and sterilisation units (Talbert *et al.,* 2010; Darmstadt *et al.,* 2011).

1.3. Conditions and concerns in the NICU:

Frequently, premature babies born before the 37th week of pregnancy suffer from problems with feeding, breathing and/or infections. Therefore, these babies require special medical nursing, which is available only in NICUs (American Academy of Paediatrics, 2014). It is not safe for these babies to feed through their mouth due to breathing problems or immaturity of the neurological system; therefore, they are fed via either nasogastric or enteral feeding tubes. Generally, during the first days of life enteral feeding is preferred over parenteral

nutrition, which promotes endocrine adaptability and the maturation of intestinal motility (McGuire *et al*., 2004).

The World Health Organisation (WHO) guideline for preparation of PIF products recommends aseptic hygiene, hot water (>70 °C) and short storage times of prepared PIF at 4 ˚C to reduce microbial contamination (Codex Alimentarius Commission, 2008). On the other hand, babies at NICUs are frequently fed via enteral feeding tubes (EFTs). These tubes could remain in place for several days to weeks to reduce the pain for the baby by gag reactions (Holý and Forsythe, 2014). However, the great majority of Enterobacteriaceae have ability to colonise feeding tubes at 37 °C that receive fresh feeds regularly within the tube (Hurrell *et al*., 2009a). This is not associated with neonates fed via nasogastric or enteral feeding tubes only; neonates who are receiving rehydrated PIF by feeding bottles are also included. Regardless of the feeding regime, complying with the WHO/FAO instructions such as good hygiene practice and suitable temperature during reconstitution of PIF is crucial (Holý and Forsythe, 2014). The majority of countries previously did not follow FAO/WHO recommendation instructions during reconstitution of the PIF with hot water above 70 °C (e.g. the USA). In the UK, the advice during reconstitution of PIF is to boil water in the kettle, then leave it for about 30 minutes to cool, with consideration to the size and kind of kettle during the cooling curve. This is impractical for new-born babies that require a small amount of formula and feed every 3 hours. Some parents prefer to complement the nutritional value of PIF by adding breast milk fortifier; in this case, PIF is not reconstituted in water and cannot be treated with heat to kill intrinsic bacteria (Holý and Forsythe, 2014).

1.4. Bacterial outbreaks in NICUs;

Gastmeier *et al*. (2007) reported that all NICU outbreaks estimated to represent 37.9%, and neonatology outbreaks represent around 87.6% of these outbreaks. The most common causative organisms were *Klebsiella* spp. (20.3%), *Staphylococcus* spp. (15.9%), *Serratia* spp. (12%), *Enterobacter* spp. (9.4%), *Pseudomonas* spp. (5.4%), *E. coli*, *Salmonella* serovars, *Candida* spp., and *Acinetobacter* spp., were 4.7% for each individual organism. The Enterobacteriaceae group (*Klebsiella* spp., *Serratia* spp., *Enterobacter* spp., *E. coli*, *Salmonella* serovars) accounts for 52.5% of NICU outbreaks. Recently, vancomycin-resistant *enterococci* (VRE), ESBL-producing *K. pneumoniae* and methicillin-resistant *Staphylococcus*

aureus (MRSA) have increased due to the extensive use of broad-spectrum antibiotics throughout the world (Khoury *et al*., 2005; Mitra *et al*., 2011; Shamshad *et al*., 2012; Lacobelli *et al*., 2013; Ramsing *et al*., 2013; Losifidis *et al*., 2013). Moreover, bloodstream infections were the most frequent type of infection (62.7%), which may be due to prolonged extensive use of indwelling catheters and intravenous feeding tubes, followed by gastrointestinal infections (20.7%), and central nervous system infections (19.9%), a lower frequency of infections were detected in the urinary tract, lower respiratory tract, and surgical sites (Gastmeier *et al*., 2007).

In recent decades, medical microbiological research has become more concerned with hospital outbreaks and nosocomial infections, within the NICUs than other types of intensive care units, due to the increase of potential health risks among low birth weight and premature babies (Stoll *et al*, 2002; Brady 2005; Boghossian *et al*, 2013). The frequency of neonatal infections due to Enterobacteriaceae has increased in neonatal intensive care units (NICUs), particularly those born with low birth weight (< 2000g) and fed via nasogastric feeding tubes. Gastmeier *et al*. (2007) and Stoll *et al*. (2005; 2011) have stated that the most common neonatal Enterobacteriaceae pathogens reported are *Klebsiella* spp. (23.4%), *Serratia* spp. (13.8%) and *E. coli* (although occurring less frequently), are the leading causes of neonatal meningitis and sepsis and they are also the second most common cause of meningitis after Group B *Streptococcus* during the neonatal period. Supporting this notion, in 2017, Ramasethu reported that among Enterobacteriaceae members, *Klebsiella* spp., *Enterobacter* spp. and *Serratia* spp., followed by *Staphylococcus aureus* (MRSA), were responsible for most of the reported neonatal outbreaks. Additionally, some of *Salmonella* spp., are significantly connected with high mortality and morbidity in neonates (Friedland *et al.,* 2003; Adamson *et al.,* 2012). Within this family, *K. pneumoniae* and *E. coli* are the most common producers of ESBLs, and they are recognised as the main pathogens responsible for NICU outbreaks with a link to significant mortality rates (Romero *et al*., 2007; Stapleton *et al.*, 2016).

Currently, among Gram-positive bacteria the major risk factor among NICU outbreaks are the two most commonly encountered: methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). VRE colonisation has been associated with

long-term antibiotic treatment, prematurity and low birth weight babies (Sherer *et al.,* 2005). On the other side, colonisation of MRSA in NICUs is still mostly anonymous (Zervou *et al*., 2014). Actually, *S. aureus*, in particular MRSA, remain the second most common causative agent of bloodstream infections, particularly among premature babies, due to the inability of their immune system to mount a response (Mease, 1990; Koenig and Yoder, 2004; Hocevar *et al*., 2012). In 2015, at the St. Anna Hospital Wuppertal, Germany, the NICU group decided to control any kind of microorganisms within the NICU, especially MRSA, methicillin-sensitive *Staphylococcus aureus* (MSSA) and VRE, and after that period no outbreaks or colonisation infections have been recorded (Hensel *et al*., 2017).

1.5. Pathogenic bacteria existence in powdered infant formula:

In 1988, Muytjens and co-workers investigated 120 PIF samples and they found that 10% of these samples were contaminated with *Cronobacter* spp. In addition, Iversen and Forsythe (2004b), by using Druggan-Forsythe-Iversen (DFI selective agar), examined 120 and 49 samples of PIF and dry baby food, respectively. They recovered three *Cronobacter* spp. from PIF and five strains from dry baby food. A study done by Fernandez-Baca *et al*. (2001) on NICUs environments found opportunistic *Enterobacter cloacae* isolates present in these samples. This bacterium, as well as *Enterobacter agglomerans*, were isolated from PIF by Muytjens *et al*. (1988).

Potential contamination of PIF with *Salmonella* serovars or *Cronobacter* spp. is a global concern and controversial area due to contamination during PIF production, which can occur suddenly and no one can predict when or where it can occur. In addition, *Cronobacter* spp. have been isolated from many different environments, including food products (cereal, powdered milk, potato, chocolate and pasta) (Lehner *et al*., 2004). Most *Cronobacter* spp., especially *C. sakazakii* thermo tolerant strains, can survive during PIF production or after the pasteurisation process and may remain a contaminant of PIF after reconstitution procedures (Nazarowec-White and Farber, 1997; Breeuwer *et al*., 2003; Osaili and Forsythe, 2009; Kalyantanda *et al*., 2015). Recently in 2016, the CDC reported that *Cronobacter* infection outbreaks do not occur very frequently, but they are deadly in neonates, usually occurring within the first day or first week of the baby's life. Furthermore, the CDC stated that there

are around 4-6 cases of neonatal infection with *Cronobacter sakazakii* yearly, but reporting is not usually required (CDC, 2017).

In addition to *Cronobacter* and *Salmonella*, many other bacteria may contaminate PIF, and are also reported to be linked with neonatal infections such as *Bacillus cereus, Clostridium perfringens*, *S. aureus*, and some members of the Enterobacteriaceae (Forsythe, 2005). Among Enterobacteriaceae members, *E. coli, E. cloacae, E. hormaechei, K. oxytoca, K. pneumoniae* and *Citrobacter freundii* have been isolated from PIF previously (Muytjens *et al*., 1988; Townsend *et al*., 2008a). According to the FAO/WHO (2004 and 2006), these organisms were categorised as category B, which is defined as 'causality reasonable' but did not present considerable potential to cause neonatal infection through ingestion of rehydrated PIF, and there was no evidence to emphasise their responsibility for any NICU outbreaks associated with PIF Table 1-3. On the other hand, category A included *Cronobacter* spp. and *Salmonella enterica*, which present clear evidence of causality.

Table 1-3: Categorisation of microorganisms in PIF depend on their strength of illness in infants.

Organism	Category	Strength of evidence of a causal association among their presence in PIF illness in infants
Salmonella enterica	A	clear evidence of causality
Cronobacter sakazakii *		
Pantoea agglomerans	B	causality plausible but not demonstrated
Escherichia vulneris		
Hafnia alvei		
Klebsiella pneumonia		
Citrobacter freundi		
Citrobacter koseri		
Klebsiella oxytoca		
Enterobacter cloacae		
Escherichia coli		
Serratia spp		
Bacillus cereus	C	causality less plausible or not demonstrated
Clostridium difficile		
Clostridium perfrigens		
Clostridium botulinum		
Listeria monocytogenes		
Staphylococcus aureus		
coagulase-negative staphylococci		

This table was adapted from FAO/WHO, (2004), * in 2008, the name *Enterobacter sakazakii* was changed to *Cronobacter* genus

According to Iversen *et al*. (2008) and the World Health Organization/Food and Agriculture Organization (FAO/ WHO, 2004 and 2006) the microbiological safety of PIF was the main

concern due to the association of *Salmonella* and *Cronobacter* with contamination of PIF and linked with neonatal infections. It is known that PIF is not sterile, but PIF preparation is in accordance with international microbiological standards (Codex Alimentarius Commission, 2008). Nonetheless, presence of pathogens in PIF may lead to fatal diseases and also sheds light on the importance of careful preparation, handling and storing of PIF in the healthcare settings.

1.6. Bacterial infections in neonatal feeding tubes:

In NICUs, enteral feeding tubes (EFTs) are typically used alternatively, when the new baby is unable to feed naturally from their mother or reconstituted PIF. In 2009, a study carried out by Hurrell and co-workers in cooperation with a team of NICUs at two Nottingham hospitals analysed more than 127 neonatal enteral feeding tubes (NEFTs) revealed that Enterobacteriaceae were recovered extensively (>10^7 CFU/tube) from biofilm formation within the NEFTs, hence, showing a higher potential risk to the neonatal health than *Cronobacter* alone. These isolates included *E. coli, K. oxytoca, K. pneumoniae, E. hormaechei, E. cancerogenus, S. marcescens, S. liquefaciens* and *Raoutella* spp. Furthermore*, Y. enterocolitica* and *Cronobacter* spp. were also isolated from PIF. During this study, all *S. marcescens* isolates were co-amoxiclav- and amoxicillin-resistant. Of importance from this study, a quarter of *E. hormaechei* strains were 3rd generation cephalosporins cefotaxime- and Ceftazidime-resistant. In addition, extended-spectrum beta-lactamases (ESBLs) were detected in *S. marcescens* and *K. pneumoniae* isolates, and both species were able cause infection in the two NICUs. It was interesting to note that the babies analysed in this study had received different feeding regimes (mixed feeding regime, breast milk, fortified breast milk, reconstituted PIF and ready to feed formula). In fact, most PIF were prepared at room temperature not with hot water (>70 °C) so did not comply with the recommended standards of FAO/WHO (Hurrell *et al.,* 2009a; 2009b).

Contamination of PIF may happen during the manufacturing process (during spray-drying, heat or handling) and reconstitution by healthcare workers. This was reflected in several outbreaks including *Cronobacter* associated with PIF, and among these outbreaks in 1986 3 cases were in Iceland (1986), 4, 11 and 2 cases in the USA (1988, 2001 2008, respectively), 12 cases in Belgium (1998), and 3 cases in France (2004). *Salmonella enterica* outbreaks

connected with PIF have been reported in 1976 in Trinidad and Tobago (3,000 cases), in 1986, in the UK (76 cases), in 2005, in France (141 cases), and in 2008 in Spain (42 cases). All of these outbreaks are due to low water activity food products among milk powder or PIF (Podolak *et al*., 2010; Beuchat *et al*., 2013; Forsythe, 2014). In addition, a study of 250 babies from multiple outbreaks between 1985 and 2005, that found only six cases of salmonellosis associated with contaminated PIF (Toyofuku *et al*., 2006). But more recently, in January 2018, an outbreak of *Salmonella* Agona connected to the consumption of PIF had affected 39 babies (children <1 year of age); 37 infants in France, one in Greece and one in Spain, which were later confirmed by whole-genome sequencing (WGS) that this batch of PIF was distributed to 66 countries, including 12 European Union (EU) countries since December 2017 (Jourdan-da Silva *et al*., 2018; EFSA, 2018; ECDPC, 2018).

In 2017, Ogrodzki *et al*. (2017), carried out a longitudinal study (28 days) investigating the potential effect of nosocomial bacterial colonisation in NEFTs and faecal samples from premature babies in the NICU at QMC hospital, Nottingham. It was found that different kinds of Gram-positive and Gram-negative bacteria were prevalent and the predominant species in both NEFTs and faecal samples were *E. faecalis* and *E. hormaechei.* Another study by Alkeskas *et al*. (2015) investigated NEFTs from 30 neonates in NICUs at two different hospitals, where these babies were fed by different feeding regimes. They recovered indistinguishable *E. coli* K1 ST95 strains from 11 different neonates. The most important conclusion from these studies was that microbiological neonatal feeding safety should not be focussed on *Cronobacter* spp. and rehydrated PIF only. Instead it should also focus on how to prepare feeding tubes in order to decrease possible neonatal health risk exposure from other bacteria, which may carry virulence factors and multidrug resistance genes. Thus, prolonged placement NEFTs need to be considered to reduce the probability of biofilm formation on NEFTs that may lead to neonatal infection (Hurrell *et al*., 2009a; 2009b).

1.7. The neonatal intestinal flora:

New-born babies are known to have a sterile gut lumen and the intestinal tract during birth and first few weeks of life; however, after that the baby's gut will be exposed to a wide range of different commensal organisms and become a natural bacterial reservoir. (Orrhage and Nord, 1999; LaTuga *et al*., 2011; Brooks *et al*., 2014). Some of these bacteria have the ability

to colonise the baby's intestinal tract. The first colonisation depends on either type of delivery, nutrition type (PIF or breast milk), medical staff, inanimate objects or environment (Guenthner *et al*., 1987; Bezirtzoglou, 1997; Heshmati *et al*., 2011; Unger *et al*., 2015). The human gastrointestinal tract contains many different bacterial species, where each species may represent various bacterial strains. The number of bacterial cells in the human bowel is estimated to be more than 10^{11} bacterial cells. This number is 10 times more than that of the gut lumen cells (Guarner and Malagelada, 2003). Nevertheless, the number of bacterial cells among gut lumen sites differs, such as the lower tract of the stomach which ranges between 10^{11} and 10^{12} cells per gram, whereas the upper tract ranges between 10^{1} and 10^{3} cells per gram (Sekirov *et al*., 2010). The lower bacterial number in the upper part of the stomach is due to several inhibitory factors like acid, bile and salivary enzymes, which kill most ingested bacteria. Aside from this, food movement toward the end of the ileum by motor activity plays a crucial role in bacterial colonisation of the lumen (Riskin *et al*., 2011). Intestinal bacteria are categorised into three groups. These groups have been identified as pathogenic bacteria, potentially pathogenic bacteria, and beneficial bacteria. Pathogenic bacteria are responsible for feed spoilage, harmful inflammatory infections and causes serious infections such as bacteraemia, sepsis and meningitis particularly among immature babies (Dash *et al*., 2006). Potentially pathogenic bacteria may not have positive or negative effects even if they are present in high numbers, for example *Streptococcus*, *E. coli*, and *Enterobacter* spp. Despite this, some of these bacteria may become pathogenic, even if existing in low numbers, such as *Staphylococcus* spp., *E. coli* O157:H7 and *Klebsiella* (Dai and Walker, 1999). Beneficial bacteria act in a way to balance out effects in the intestine by inhibiting growth of harmful bacteria, stimulate the immune system, and convert parts of dietary fibres to some beneficial amino acids (Westerbeek *et al*., 2006).

Several studies on neonatal intestinal flora were focused on faecal samples by using traditional microbiological techniques with plating on specific growth media. There are a limited number of studies on neonatal faecal samples using culture-based and molecular techniques to monitor the changes in the neonatal intestinal flora during the first days of life. Studies by Favier *et al*. (2002) and Fanaro *et al*. (2003) have examined the neonatal microbial flora according to their mode of feed (nutrition) and birth weight. They concluded that the diversity of neonatal flora is influenced by the environment and the diet of the

neonate (Harmsen *et al*., 2000; Wold and Adlerberth, 2002; Alderberth *et al*., 2011). Supporting this notion, Gomez *et al*. (2016) investigated ready-to-use reconstituted PIF, which were collected from the external part of the neonatal entering feeding tube, as well as from faecal samples at different time points. 16S rDNA sequencing and pulsed-field gel electrophoresis were performed to confirm the existence and clonal relatedness of bacterial isolates among infant milk and faecal samples. It was stated that among 4,000 bacterial strains isolated, the dominant isolates in both neonatal faecal and milk samples were *E. coli*, *Enterococcus faecalis*, *E. faecium*, *Staphylococcus aureus*, *S. epidermidis*, *Klebsiella pneumoniae* and *Serratia marcescens* (Gomez *et al*., 2016). Another recent study investigated the colonisation and development of bacterial flora of neonatal intestines among healthy Chinese babies and compared with those from other countries. They found that the intestinal microbiota of two-month-old babies is more varied than that of new-born babies, as indicated by the relative prevalence of *Prevotella*, *Bacteroides*, *Collinsella, Veillonella* and *Lactobacillus* and lower levels of *Enterococcus* and *E. coli*, which were confirmed by 16S rDNA sequencing (Kuang *et al*., 2016).

The presence, composition, and development of neonatal intestinal microbiota depends on the type of nutrition available. Significantly, the breast-fed microbiota is very different from that of powdered infant formula, with regards to composition and bacterial diversity. Breast-fed neonates contain more heterogeneous bacterial populations and higher taxonomic diversity than that of infant formula-fed babies (Schwartz *et al*., 2012). The microbiota isolated from PIF include *E. coli*, *Citrobacter freundii*, *C. koseri*, Serratia spp., *Hafnia alvei*, *Enterobacter cloacae*, *E. agglomerans, K. pneumoniae, K. oxytoca, Salmonella, Listeria monocytogenes*, *Acinetobacter* spp., *Staphylococcus aureus*, *Clostridium botulinum*, *C. difficile* and *C. perfringens* (FAO/WHO, 2004 and 2006). In 1997, Bezirtzoglou stated that breast milk largely contains low numbers of bifidobacteria, lactobacilli, streptococci, staphylococci, micrococci and diphteroids. The colonisation of staphylococci on breast-fed neonates mostly occurs due to direct contact with the babies and their mothers during feeding.

Several studies reported that *Streptococcus*, *Lactobacillus* and *Bifidobacterium* species were the predominant intestinal bacteria during the first weeks of life among full-term infants,

who were breast-fed (Koenig *et al*., 2011; Scholtens *et al*., 2012). On the other hand, *Bifidobacterium*, *Staphylococcus*, *Enterococcus, Clostridium*, *Bacteroides* spp., *Klebsiella* spp., and *E. coli* dominated in babies fed with PIF (Rubaltelli *et al*., 1998; Harmsen *et al*., 2000).

Another study investigated the bacterial diversity through breast-feeding and weaning periods among two babies. Wang *et al*. (2004) revealed that babies were colonised by *Streptococcus*, *Staphylococcus, Enterococcus*, *Bacteroides*, *Veillonella* and members of the Enterobacteriaceae. Furthermore, the predominant types of bacteria during breast-feeding were *Bacteroides* and Enterobacteriaceae, whereas and after weaning, the prevalence of Enterobacteriaceae had decreased but clostridia had increased. Cabrera-Rubio *et al*. (2012) stated that the human milk microbiome could be influenced by many factors such as proteins and nucleotides, and these factors play a vital role in the immune system and microbial growth. A study by Rueda and co-authors (1998) stated that the growth and colonisation of bifidobacteria strains in formula-fed babies was faster and higher when the infant formula was complemented with gangliosides. However, Crawley and Westland, (2012) stated that the incidence of diarrhoeal episodes was reduced when infant formula was supplemented with nucleotides and plasma antibody response had increased. Twenty years ago, a study by Gewolb and co-workers collected stool samples from twenty-nine low birth weight (<1000 g) babies at three different time points: 10, 20 and 30 days. At day 30, the most prevalent species among Gram-positive bacteria were *Enterococcus faecalis*, *Staphylococcus haemolyticus, S. epidermidis*, whereas among Gram-negative bacteria they were *E. coli*, *Enterobacter cloacae* and *K. pneumoniae*. The *Bifidobacterium* and *Lactobacillus* genera were detected in only one baby. Furthermore, *E. coli*, *E. faecalis*, and *S. haemolyticus* were recovered from those babies (Gewolb *et al*., 1999). During this study, two babies were fed by infant formula and sadly, one baby had died.

Although most of studies which mentioned above stated that several strains such as *E. faecalis*, *E. hormaechei* and *S. aureus* revealed their ability to persistence in the neonatal intestine, however, potential pathogenicity and persistence ability of these strains were poorly understood (not covered). Besides to that, these strains were identified by either 16S rDNA or traditional methods that cannot confirm either the same clone or other strain

persistence within the neonatal gut in all time points. Therefore, in the current study, the representative strains (*E. faecalis* and *E. hormaechei*) were conducted to determine their pathogenicity and Whole genome sequencing was performed to determine strains clonality.

1.8. Bacterial mechanisms for pathogenicity:

Bacterial pathogens have several mechanisms of causing disease in the host. Among these strategies includes the ability of bacterial attachment to the host cell, and this feature could be unique for the organism or conserved between different species. Moreover, another factor is secreted enzymes, for example extended-spectrum beta lactamase enzymes to inactivate beta lactam antibiotics (Sansonetti, 2002). Furthermore, biofilm formation can occur on any suitable surface with adequate nutrients available. The attachment can occur on biotic surfaces such as human skin, epithelial cells and animal tissue, or on abiotic surfaces, for instance medical devices, plastic, glass and metal. In 2009, Annous and co-authors reported on several factors associated with biofilm formation on medical device surfaces, in particular curli fimbriae, which is known to play an important role in biofilm formation (Kim *et al*., 2012). Lehner and collaborators (2005) documented that most species of the Enterobacteriaceae have the ability to form biofilm on glass and polyvinyl chloride. The majority of these materials are involved in food manufacturing and infant feeding equipment, which likely protect bacteria from environmental stresses and antimicrobial agents. Risk of neonatal infection will therefore increase with contamination of these materials. The identification, characterisation, and differentiation all of these features among pathogens is the key factor to eradicate bacterial infections. Therefore, this study focusses on the characterisation of these strategies used by bacterial pathogens to cause infections.

1.9. Bacterial interaction with mammalian cells:

Bacterial colonisation of neonatal intestinal walls is a basic step in the interaction with epithelial cells. The neonatal gut walls are usually sterile, but are quickly colonised by bacteria through the consumption of food and interaction with the environment. *E. coli* is one of the earliest colonisers of neonatal intestinal walls (Grajek and Olejnik, 2004). However, most intestinal pathogens can be influenced by different factors, for example the

colonisation period, nutrition, antibacterial agents and environmental exposure (Lindhorst and Oscarson, 2009; Abraham and Medzhitov, 2011). The innate defence system of the gastrointestinal tract can also inhibit bacterial persistence and dissemination within the gut, by sloughing from intestinal surfaces (Kim *et al*., (2010). Nevertheless, some pathogenic bacteria have ability to survive and persist within the gut and cause infections (Townsend *et al*., 2008b; Kim *et al*., 2010).

The corneum stratum in premature babies is thin and under-developed when compared to full-term neonates, toddlers, and adults. The mature corneum is composed of between 10 and 20 layers of cells, whilst in in premature babies of less than 30 weeks old the immature corneum consists of 2 to 3 layers of cells, which may increase the opportunity for invasion by harmful pathogens (Evans and Rutter, 1986; Hoeger, 2006).

Several bacterial pathogens have the ability to attach, invade and persist intracellularly, causing tissue damage to the host (Wilson *et al*., 2002). In addition, some pathogens are able to produce toxins or different compounds, such as polysaccharides, to escape from the host immune defence (Kim *et al*., 2010). Furthermore, some bacterial pathogens use two different mechanisms to cause infections, such as *Yersinia enterocolitica*, entero-pathogenic *E. coli*, *Shigella flexneri* and *Salmonella enterica*, which initially are able to attach, invade, and colonise host epithelial cells in the gut, and secondly are able to persist within the macrophages and spread from cell to cell (Reis and Horn, 2010). In 2000, Greiffenberg and co-authors reported that there are two methods of bacterial interaction with the host cell surface. Firstly, some bacteria revealed an ability to adhere to the smooth cell surfaces without any modification action to the membrane, and secondly, some bacteria require appendages such as microvilli for attachment (Greiffenberg *et al*., 2000). Additionally, Mogensen and Otzen (2005) stated that the outer membrane in Gram-negative bacteria plays a crucial role in the attachment of cells to the external environment (Mogensen and Otzen, 2005).

1.9.1. Bacterial adhesion:

Bacterial adherence to the host tissues is a very important and essential first step that results in pathogens colonising the host tissues, which as a result, contributes to necrotizing

enterocolitis (NEC), UTIs, meningitis, and bacteraemia, and could lead to systemic morbidity and mortality (Lindhorst and Oscarson, 2009; Bjarnsholt *et al*., 2014). However, the host uses several mechanical forces to protect the body surfaces such as blood flow, mucous flow, saliva secretion, coughing, and sneezing. Although, the host defence system acts against the invading bacterial cells, the pathogen is able to express virulence factors that bind to the host cell surface. In addition, most bacterial intestinal infections occur due to interactions between the host mucosa and invading bacteria, such as adherence and invasion (Hu and Kopecko, 2008). The processes of bacterial adherence to the host tissues can be mediated by protein adhesins such as fimbriae (pili) or via polysaccharides such as capsules (Wilson *et al*., 2002; Melville and Craig, 2013). Fimberial adhesins are classified into two categories, fimbrial and afimbrial. Fimbrial adhesins are tightly packed accessory proteins, which protrude from the bacterial cell surface as a helical cylinder. This type of adhesin is most common among Gram-negative bacteria such as *E. coli*, *Neisseria* species, *Pseudomonas aeruginosa* and *Vibrio cholerae* (Hahn, 1997; Donnenberg, 2000). On the other hand, afimbrial adhesins are mediated by proteins known as adherence factors, which intimately adhere the pathogen to the host cells over a shorter domain than fimbriae, for example in mycobacterial pathogens such as *Streptococcus* spp., *Staphylococcus* spp., *Neisseria* spp., *Y. pseudotuberculosis* and entero-pathogenic *E. coli* (Joh *et al*., 1999; Bermudez and Sangari, 2000; Merz and So, 2000; Donnenberg, 2000). Following adhesion, the bacterial pathogen is able to proliferate and secrete specific toxins which contribute to causing infections (Wilson *et al*., 2002).

1.9.2. Bacterial invasion:

Following adherence, several bacterial species have other pathogenic strategies, such as establishing deeper invasion into the host tissues for continuation of the infection. These strategies are divided into two categories: extracellular and intracellular invasions (Wilson *et al*., 2002). Extracellular invasion occurs when the pathogen destroys the tissue barriers of the host in order to spread their enzymes inside the tissues whilst the pathogens remain outside of the tissues, such as *Vibrio cholerae*, which secretes hemagglutinin/protease to destroy the extracellular domain, which is a key component of the tight junctions (between cells) (Wu *et al*., 2000). Additionally, *S. aureus* is an example of a pathogen which secretes

haemolysin toxins as well as beta-haemolytic group A *Streptococcus* (Walker, 1998; Kim *et al*., 2010). There are three different types of haemolysin: alpha-haemolysin, beta-haemolysin and gamma-haemolysin. These haemolysins have capability to damage the host cells and some cells may also result in their distribution inside host tissues (Schmidt *et al*., 1995; Wilson *et al*., 2002). Intracellular invasion is the ability of pathogens to penetrate, survive and proliferate within the host tissues. Many Gram-positive and Gram-negative organisms, and *Mycobacterium tuberculosis*, have the ability to penetrate mammalian tissues (Finlay and Falkow, 1997; Cleary and Cue, 2000).

1.9.3. Intracellular lifestyles:

After invasion, several pathogens have the ability to survive, proliferate and persist inside the host tissues such as macrophages, epithelial and endothelial cells. The host will employ many defence strategies such as macrophages and neutrophils, which are the main phagocytic defence cells that able to destroy ingested pathogens by activation of destructive protease and production of reactive oxidative intermediates. Nevertheless, several bacterial pathogens have ability to escape from the host immune system (phagocytosis). For instance, adherent-invasive *E. coli* (AIEC) recovered from patients with Crohn's disease were able to survive and multiply inside macrophages in large vacuoles. The ability of bacteria to persist within the host cell and the ability to proliferate and transmit from one cell to another within the body might allow them to use macrophages as a vehicle to establish a successful infection in other tissues/organs of the neonate (Wilson *et al*., 2002; Bringer *et al*., 2006).

1.9.4. Bacterial toxins:

Bacterial cytotoxicity is classified into two groups based on their cell wall structure (Gram-negative and Gram-positive bacteria). Bacteria produce toxic proteinaceous or non-proteinaceous components in their cell wall that lead to destruction of the host cells and cause infection (Wilson *et al*., 2002; Forsythe, 2010).

Proteinaceous toxins are known as exotoxins which are mainly enzymes. These exotoxins are classified into two groups based on their secretory method to mammalian cells; they are either deliver directly into the host tissues or via injection within the cytoplasm of the host cell (Finlay and Falkow, 1997a and b). The clinical manifestations of these toxins often

include vomiting and acute watery diarrhoea. Furthermore, the vast majority of these toxins are linked to infections of the digestive system (Forsythe, 2010). Another study reported that the most virulent proteins, such as the heat-labile toxin of enterotoxigenic *E. coli* (ETEC) and cholera toxin of *Vibrio cholera* were found to be predominantly associated with damaging gut epithelial cells *vivo* and *in vitro* (Chakraborty *et al*., 2008). Several studies reported that some *Enterococcus faecalis* strains have a unique toxin known as cytolysin, which has an ability to damage a broad range of target cells, including mammalian cells and even some Gram-positive bacteria. This toxin makes a significant contribution to the pathogenicity of *E. faecalis* (Elsner *et al*., 2000; Karen Carniol, 2006; Van Tyne *et al*., 2013). Another study investigated the effect of the virulence factors of *Cronobacter* spp. *in vivo* and they found that this organism expresses enterotoxins (Pagotto *et al*., 2003). Another study by Raghav and Aggarwal (2007) reported that a 66 KD enterotoxin of *Cronobacter* spp. was active at pH 6.0 and more stable at 90 ^{o}C for up to 30 minutes. Non-proteinaceous toxins (endotoxins) are lipopolysaccharides (LPS) associated with Gram-negative bacteria. These toxins are complex lipopolysaccharides within the bacterial cell wall of pathogens such as *Shigella, Salmonella* and *E. coli* (Cetin *et al*., 2004a; Cetin *et al*., 2004b). According to Townsend and co-authors (2007), the LPS is heat-tolerant and provides bacteria with the ability to persist in PIF during preparation, as well as enhancing the translocation of enteric pathogens from the intestinal tract to the blood-brain barrier, which may increase the risk of neonatal infections (Townsend *et al*., 2007b).

1.9.5. Iron uptake:

Iron is essential for pathogenic bacteria to survive and multiply within their host. Therefore, the availability of iron in the host can enhance the pathogenic potential of bacteria, thus influencing their ability to express virulence-associated genes. The presence of iron within the host varies due to complexes formed from iron binding proteins such as lactoferrin, ferritin, haemoglobin, and transferrin. Nevertheless, bacteria are unable to utilise iron from these sources directly, therefore they require iron acquisition systems to attain iron from their host, such as siderophores (Lin *et al*., 2012). Siderophores are chelators of small organic molecules (low-molecular-weight), and are considered as a virulence mechanism in bacterial pathogenicity. Siderophores are synthesised by pathogenic bacteria which solubilise and

acquire iron from eukaryotic iron-binding proteins like transferrin and lactoferrin (Ratledge and Dover, 2001; Eijkelkamp *et al.,* 2011; Penwell *et al*., 2012). In 2003, Raymond and co-authors noted increased virulence pathogenicity of *Yersinia*, *Pasteurella*, *Klebsiella*, *Salmonella*, *Escherichia*, *Shigella*, *Neisseria*, *Listeria*, and *Vibrio* associated with an increase in iron availability in the host (Raymond *et al*., 2003).

A study was carried out by Banin *et al*. (2005), who reported that bacterial growth and biofilm formation was prevented due to iron starvation. Henderson and Payne (1994) stated that bacteria have developed many strategies to overcome iron deficiency from the host, including the production of proteases which cleave the iron-binding proteins to obtain free iron, by reduction of the ferric ion Fe^{3+} to F^{2+}, followed by the liberation of iron from the protein complex (Henderson and Payne, 1994). Thus far, bacterial iron acquisition by siderophores is possibly the most studied strategy in bacterial pathogens. Bacterial iron depletion from the host depends on high-affinity iron compounds to act as intermediary compounds for siderophores. According to Neil (1995), at least one Siderophore is produced by aerobic and facultatively anaerobic bacteria, as well as around 500 siderophores which are present in selected microorganisms (Balagurunathan and Radhakrishnan, 2007). Some of the most important siderophores are presented in Table 1-4.

Table 1-4: Most important bacterial siderophores

Strain	Siderophore
Staphylococcus aureus	Aureochelin
Klebsiella spp.	Aerobactin
Salmonella spp.	Aerobactin
Aeromonas hydrophila	Amonabsactin
Acinetobacter calcoacaticus	Acinetobactin
Aerobacter aerogenes	Aerobacin
Escherichia coli	Enterobactin
Mycobacterium tuberculosis	Mycobactin
Pseudomonas aeruginosa	pyochelin and Pyoverdin
Vibrio cholerae	Vibriobactin
Yersinia pestis	Yersniabactin

In 2000, Bach and co-workers documented that the siderophore Yersiniabactin is part of the iron-uptake system first discovered in *Yersinia* spp., which is located on the high

pathogenicity island (HPI). The *fyuA* and *irp2* genes are the main genes of this locus. The *irp1* and *irp2* genes are recognised as two iron-repressible high-molecular-weight proteins which are involved in the biosynthesis of Yersiniabactin, whereas the *fuyA* gene encodes for the Yersiniabactin receptor (Lucier *et al*., 1996; Bach *et al*., 2000 and Schubert *et al*., 2000). A study by Tu *et al*. (2016) investigated the influence of the *irp2* and *fyuA* genes in the HPI among avian pathogenic *E. coli* (APEC) by knocking out these two genes and inoculated them into DF-1 cells (immortalised cell-line of chicken embryo fibroblasts). The examined strains revealed a significantly lower capacity of attachment to DF-1 cells compared to the wild type. Another study by Paauw (2008b) investigated a nationwide outbreak in the Netherlands caused by multidrug-resistant *E. hormaechei*. These strains revealed acquisition of mechanisms to obtain iron from their host by producing Yersiniabactin.

1.10. Bacterial diversity of antibiotic resistance:

Over recent decades, the spread of multidrug resistance among nosocomial pathogens has become a serious health problem worldwide (Wood *et al*., 1996; Goldmann *et al*., 1996; Wilson *et al*., 2002). According to the European Antimicrobial Resistance Surveillance Network (EARS-Net, 2016) the percentage of antimicrobial resistance across European countries is widely varied. South-eastern and southern Europe had a higher resistance percentage when compared to northern Europe. For *E. coli*, the level of antimicrobial resistance has increased significantly in the European Union (EU)/European Economic Area (EEA) between 2013 and 2016, particularly against aminoglycosides, for both 3rd generation cephalosporins and combined resistance to 3rd generation cephalosporins and fluoroquinolones. Also, during this surveillance in 2016, *K. pneumoniae* comprised more than one-third of the strains that were resistant to three or more combined antibiotic groups, phenotypically. In addition, carbapenems are considered the drug of choice for treatment of infections caused by antimicrobial-resistant Gram-negative pathogens, such as *E. coli* and *K. pneumoniae*. In 2016, most European countries reported that carbapenem resistance among *K. pneumoniae* is very low and in *E. coli* it is rare. On the other hand, a few countries with high levels of bacterial antimicrobial resistance reported that the percentage of carbapenem resistance for *K. pneumoniae* is considerably high.

In countries with high levels of antimicrobial resistance (south-east Europe), especially for carbapenems, there are a few therapeutic options available, other than carbapenems; this includes colistin, which is last-line for the treatment of carbapenem-resistant bacteria. In these countries, if the bacteria are revealed to be resistant to carbapenems and colistin it represents a serious concern because the treatment options become limited, particularly when colistin resistance (*mcr-1*) became mobilised on a plasmid, allowing faster spread between bacteria. Moreover, between 2013 and 2016, the percentage prevalence of MRSA in the EU/EEA had significantly decreased. Despite this promising development, MRSA continues to present a global public health problem; in European countries, the reported percentage prevalence of MRSA was greater than 25%. Furthermore, vancomycin-resistant *Enterococcus faecium* (VREF) has noticeably increased among 7 out of 25 countries. An increase in VREF has been reported in countries which commonly report high percentage levels of antimicrobial resistance. (EARS-Net, 2016). In a recent report, the WHO (2017) has warned that a small injury following the post-antibiotic period may lead to more deaths if we do not overcome these antimicrobial-resistant pathogens. There are a greater number of deaths worldwide, particularly among immunocompromised patients. In the United States of America up to 63,000 deaths occur every year from bacterial nosocomial infections (Aminov and Mackie, 2007; WHO, 2017). Additionally, in Europe, it is estimated that there are around 25,000 cases resulting in death every year from multidrug-resistant bacterial infections (Freire-Moran *et al*., 2011).

Several multidrug-resistant bacterial isolates represent a cause for considerable concern among diarrhoeal pathogens, such as *E. coli* (IPEC), *E. faecium*, *Salmonella* and *Shigella*; respiratory pathogens like *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *K. pneumoniae* and *Pseudomonas aeruginosa*; urinary tract pathogens such *E. coli* (UPEC), *Enterococci* and *Mycobacterium tuberculosis* which, is still responsible for death from a single infectious disease worldwide (Wood *et al*., 1996; Mazel and Davies, 1999; Chen *et al.,* 2016; Solayide *et al*., 2017). Strains of several bacterial species such as *E. faecalis*, *S. aureus*, *E. coli*, *Klebsiella*, and *Enterobacter* are predominantly associated with community- and hospital-acquired infections. Those strains have the ability to disseminate within the hospitals easily through contaminated food, medical equipment, water and hand carriage, and are able to acquire genetic elements horizontally via plasmids or transposons, and this

may contribute to multidrug resistance (Arias *et al*., 2010; Partridge, 2011; Toleman and Walsh, 2011; Downie *et al.*, 2013).

The vast majority of pathogenic bacteria use several mechanisms to inhibit or inactivate the antibacterial agent either through permeability of the cell wall or via enzymes, particularly the beta-lactamase (β-lactamase) enzyme. These enzymes (β-lactamases) are classified into four groups, carbapenems, penicillins, cephalosporins and monobactam, based on their chemical structure (Livermore, 2003; Nordmann *et al*., 2012). Bacterial ESBL producers remain important due to reported therapeutic failures of β-lactam antibiotics, which may lead to a serious effect on infection control (Paterson and Bonomo, 2005; Zhao and Hu, 2013; Calbo and Garau, 2015).

In 2009, Hurrell and co-workers investigated the antimicrobial susceptibility for Enterobacteriaceae strains isolated from NEFTs in the NICUs of two different hospitals. They reported that among isolated strains, one-quarter of *E. hormaechei* strains were found to be resistant to 3rd generation cephalosporins, ceftazidime and cefotaxime. Also, *S. marcescens* strains were resistant to amoxillin and augmentin. Furthermore, *S. marcescens* and *K pneumoniae* were responsible for infections within the two NICUs, and all these isolates had exhibited ESBLs (Hurrell *et al.,* 2009b). Another study by Adesida *et al*. (2017) investigated antibacterial susceptibility for seventy-three *Enterococcus* spp., isolated from healthy carriers. They found all isolated strains were resistant to ceftizoxime, cefuroxime and ceftriaxone. *E. faecium* presented resistance levels of 88.9%, 77.8%, 63.9%, 44.4%, 19.4% and 16.7% for erythromycin, gentamicin, augmentin, ofloxacin, teicoplanin and vancomycin, respectively. On the other hand, *E. faecalis* revealed resistance to teicoplanin (27.7%) and was the least resistant (13.8%) to vancomycin (Adesida *et al*., 2017).

The presence of virulence traits such as antimicrobial resistance, siderophores (iron uptake), haemolysin activity, biofilm formation, serum resistance, polysaccharide capsule (mucoid) production, capsular serotypes, invasion and adherence to the mammalian cells, which are the most significant virulent traits for bacterial pathogens and likely represents a serious threat to the babies especially premature in NICUs. Therefore, in the current study I am trying take into account to determine virulent traits linked with these isolates, which may form a significantly high risk to the health of neonates in NICUs.

1.11. Genomic studies:

Bacterial identification, characterisation and subtyping are increasingly important; these can be used to investigate whether the strains are clonally associated and potentially to infer case linkage. Previously, there have been a number of genotypic and phenotypic techniques used such as the API ID32E kit, MLST, *rpoB* gene screening and PFGE, which were used to determine bacterial identification and classification (Tenover *et al*., 1995; Nazarowec-White and Farber, 1999). Nowadays, microbial molecular typing methods in public and private laboratories have become commonplace. Strain-level subtyping is a very crucial tool, including investigation into whether the isolates are clonally related and linked with virulence traits, in order to determine the importance of these strains as potential pathogens, and to determine the source of infection, potential cross-transmission events and the diversity of these isolates (Adamsson *et al*., 2000). This has been developed and expanded because of the use of whole-genome sequencing in routine clinical care (Bick and Dimmock, 2011). For instance, a comparative investigation between PFGE and WGS was done by Salipante *et al*. (2015), who reported that a total of 28.9% of bacterial strains were indistinguishable by PFGE, whereas by WGS, these isolates were easily distinguishable (non-clonal). In addition, *A. baumannii* was investigated, which is recognised as a species for high levels of horizontal gene transfer. It was found that 16.2% of strains were non-identical by PFGE, but were clonally identical by WGS. The investigators suggested a new molecular epidemiology approach, such as WGS, is needed instead of PFGE which is prone to giving false-positive or false-negative results.

In 1988, a scientific article was published on the incidence of *Cronobacter* spp. in powdered infant formula. To date, the citation of this paper still high and is considered to be the initial key study of this topic. Despite that, the isolated strains were identified by traditional techniques such as biochemical tests (Muytjens *et al*., 1988). Whereas Townsend *et al*. (2008a) from NTU re-identified these isolates by using 16S rDNA sequencing, and they found that some of these isolates had been mis-identified and were *E. hormaechei*. However, another study carried by Ogrodzki *et al*. (2017) investigated *E. faecalis* strains isolated from NEFT by using 16S rDNA and they confirmed that all presented strains were clonally related and indistinguishable. When these isolates were subjected to WGS, it was confirmed that

the *E. faecalis* isolates formed two distinct clusters, according to their sequence type. Additionally, in 2015, the CDC recommended using WGS technology, such as the Illumina high-throughput HiSeq, instead of PFGE for bacterial surveillance (CDC, 2015).

Nosocomial infections and NICU outbreaks particularly those caused by multidrug-resistant pathogens still a frequent threat to sensitive hospitalised patients globally. Therefore, we believe that WGS will open the door within microbial molecular typing methods and it may contribute to reduce the burden of healthcare-associated infections. In addition, WGS offers easier workflows, high-quality resolution and is a universally applicable technique. In this study, a combination of PFGE, MLST and whole-genome sequencing were used to determine the relatedness of the strains.

The great majority of research that conducted on the NEFTs have been focused on bacterial potential pathogenicity rather than their ability to persist and multiply in the neonatal gut and feeding tube. In addition, the influence the neonatal intestine on the phenotypic and genotypic behaviour of these organisms. These studies represents "snapshots" – single without follow-up samples, that could not determine whether the baby was re-infected by the same strains or by different strains, once or multiple times, and whether these strains persistent prolonged within the baby gut.

1.12. Study objectives:

In this study, Enterobacteriaceae recovered from NEFTs in NICUs from two different hospitals in Nottingham (chapter 3) and *Klebsiella pneumoniae* strains from NEFTs in NICUs from two different hospitals in Jordan (chapter 4) were phenotypically and genotypically characterised. In addition, *Enterococcus faecalis* and *Enterobacter hormaechei* strains were isolated from two NEFT and two faecal samples taken at different time points from the same premature baby in the NICU at QMC hospital, Nottingham to conduct a longitudinal study (chapter 5).

The project aims were: (a) to evaluate the potential risk to neonates posed by ingestion of *E. coli* and *Klebsiella* spp, *E. hormaechei* and *E. faecalis* either through powdered infant formula, contaminated milk, or by medical equipment, (b) to categorise isolates of these organisms into high, medium and low potential pathogenicity to neonates, and (c) to

conduct a longitudinal study to determine whether the same strain colonises both the feeding tube and intestine of a premature baby in the NICU over time.

Chapter 2. Materials and Methods;

2.1. Safety consideration

Health and safety code of practice for microbiology level two containment laboratories at Nottingham Trent University was considered before carrying out any experiments and protocols in this project. The procedural COSHH forms were also completed and considered. Category 2 organisms and materials were disposed of according to the recommended instructions. For tissue culture laboratories health and safety regulations, hepatitis B antibodies and vaccination were assessed before starting any tissue culture experiments.

2.2. Ethics.

Ethical approval was given by NRES Committee East Midlands. This permitted the collection of feeding tubes, faeces and metadata, but did not permit the storage of human tissue, which by definition included faeces.

2.3. Sterilisation and aseptic techniques

All buffers, solutions, media, and equipment were decontaminated by autoclave sterilisation at 121°C under 15 psi pressure for 15 minutes, 121°C for 5 minutes, filtered using 0.2 µm pore size filters (Thermo Fisher Scientific, UK) or sprayed with 70% ethanol, as appropriate.

2.4. Bacterial storage

All the studied isolates and any additional positive and negative control strains were stored at -80°C and -20°C in TSB/ glycerol (80%) (Thermo Fisher Scientific, UK) for long term storage. When required, bacteria were recovered from frozen stock and subcultured on Trypton Soya Agar (TSA) and incubated aerobically at 37°C for 18 h. For short periods of use, bacteria were streaked on TSA and stored at 4°C.

2.5. General stock reagents and buffers

2.5.1. Phosphate buffered saline (PBS)

The PBS solution was prepared by dissolving one PBS tablet (Sigma Aldrich, UK) into 100 ml of distilled water and autoclaved at 121°C for 15 minutes.

2.5.2. Saline Solution (0.85 %)

For preparing the 0.85% saline solution, one tablet of saline (Thermo Fisher Scientific, UK) was dissolved into 500 ml of distilled water and autoclaved at 121°C for 15 minutes.

2.5.3. Hydrochloric acid (HCl)

One molar hydrochloric acid (HCl) was prepared by adding 86ml of 37% HCl (Thermo Fisher Scientific, UK) to 914 ml of distilled water. The mixture was gently mixed and used to adjust the pH of solutions used in this project.

2.5.4. TRITON X-100 (1%)

In order to prepare 1% Triton X-100, 1 ml of TRITON X-100 (Thermo Fisher Scientific, UK) was added to 99ml of distilled water, then autoclaved and then stored at room temperature until use.

2.5.5. 1 M Tris-HCl (pH8)

For preparing 1 M Tris-HCL, 60.55 g of Tris base (Thermo Fisher Scientific, UK) was dissolved into 400 ml of distilled water and then the mixture was adjusted to pH 8 by adding HCl and measured by pH meter (HANNA, USA). The volume was brought up to 500 ml with distilled water and then autoclaved at 121°C for 15 minutes.

2.5.6. 0.5 M EDTA (Ethylenediamine tetra-acetic acid, sodium hydroxide) (pH8)

The preparation of 0.5M EDTA was obtained by dissolving 93 g of EDTA (Sigma Aldrich, UK) into 400 ml of distilled water and the mixture then was adjusted to pH 8 by adding sodium hydroxide pellets (NaOH) (Sigma Aldrich, UK) and the pH was measured by a pH meter. The total volume of the mixture was completed to be 500 ml and then it was autoclaved at 121°C for 15 minutes.

2.5.7. 10 X TBE Buffer (Tris base, boric acid and EDTA buffer)

The preparation of 10X TBE buffer was obtained by dissolving 108 g Tris base (Thermo Fisher Scientific, UK), 55 g boric acid (Thermo Fisher Scientific, UK) into 800 ml of distilled water. Once the chemicals were completely dissolved, 40 ml of 0.5 EDTA pH 8 was added and the

mixture volume was adjusted to 1000 ml by adding distilled water. The prepared mixture then autoclaved at 121°C for 15 minutes.

2.5.8. 1 X TAE buffer (1 X Tris-acetate-EDTA buffer)

In order to prepare 1X TAE buffer, 20ml of 50X TAE buffer (National Diagnostics, UK) was diluted with 980 ml of distilled water. The 1X TAE diluted buffer was used for preparing agarose gel and filling the gel electrophoresis tanks.

2.5.9. Glycerol (80 %)

The preparation of 80% of glycerol was completed by adding 80 ml of glycerol (Thermo Fisher Scientific, UK) to 20 ml of distilled water. The mixture was autoclaved and stored at room temperature until use.

2.5.10. Iron III Solution

This solution was prepared by dissolving 0.0027 g of $FeCl_3$-6 H_2O (Sigma Aldrich, UK) into 10 ml of 10 mM HCl.

2.5.11. Chrome Azurol Sulphate (CAS) SOLUTION

CAS solution was prepared by mixing 0.06 g CAS (Sigma Aldrich, UK) with 50ml of sterile distilled water.

2.5.12. Hexadecyltrimethylammonium bromide (HDTMA)

Exactly 0.1458 g of HDTMA (H6268, Sigma Aldrich) was dissolved in 80 ml of distilled water.

2.5.13. Sodium hydroxide solution

This solution was prepared by dissolving 10 g of NaOH into 20 ml distilled water.

2.5.14. Lysozyme Solution (for gram-positive bacteria only)

The manufacturer's instructions for preparing a stock solution of lysozyme were followed supplier. To prepare 1 ml of Lysozyme Solution (L4919), 2.115 × 106 units of lysozyme were dissolved in 1 mL of Gram-Positive Lysis Solution (L7539). For each DNA preparation to be performed, 200 µL of Lysozyme Solution was required.

2.6. General culture media

2.6.1. Tryptone Soya Agar (TSA)

According to manufacturer's instructions for TSA (Thermo Fisher Scientific, UK) the media was prepared by dissolving 40 grams of TSA agar in 1L of distilled water. The mixture was boiled to completely melt the agar and then autoclaved at 121°C for 15 minutes. The media was cooled to 50°C and then dispensed into petri dishes about 20 ml each. These plates were dried and stored at 4°C until use.

2.6.2. Trypticase soy broth (TSB)

Exactly 15 g of TSB (Thermo Fisher Scientific, UK) were dissolved into 500 ml of distilled water. This mixture was dispensed to 100 ml in separate bottles. These 100 ml bottles were autoclaved at 121°C for 15 minutes and then stored at room temperature.

2.6.3. MacConkey agar

To prepare 1 litre, 51.5 g of MacConkey agar (Thermo Fisher Scientific, UK) were added to 1 litre of distilled water. The mixture was boiled to completely dissolve the powder and then autoclaved at 121°C for 15 minutes after which it was cooled to 50°C and dispensed into petri dishes and stored at 4°C until needed.

2.6.4. Violet Red Bile Glucose Agar (VRBGA)

The medium was prepared by dissolving 38.5 g of VRBGA (Thermo Fisher Scientific, UK) into 1 litre of distilled water. The medium was boiled then cooled to 50°C to be dispensed into petri dishes.

2.6.5. Luria-Bertani agar (LBA)

The manufacturer's directions were followed to prepare Luria-Bertani Agar (Merck KgaA, Darmstad, Germany; 1102830). Thirty-seven gram were dissolved in 1 litre of water, mixed and then sterilized for 15 min at 121°C.

2.6.6. Xylose lysine deoxycholate agar (XLD)

Fifty-three grams of XLD (Thermo Fisher Scientific, UK) agar was added to 1 litre of distilled water and heated with frequent agitation until the mixture boiled. The medium was transferred immediately to a 50°C water bath and then poured into petri dish plates.

2.6.7. De Man, Rogosa, Sharpe Agar (MRS agar)

Sixty-two grams of MRS (Oxoid Thermo Fisher Scientific, UK) agar was added to 1 litre of distilled water. The mixture was boiled to completely dissolve the powder and then autoclaved at 121°C for 15 minutes after which it was cooled to 50°C and dispensed into petri dishes.

2.6.8. Brain Heart Infusion Agar (BHIA)

Brain Heart Infusion Agar (CM131B, Oxoid Thermo Fisher Scientific) was prepared according to the manufacturer's instructions. Briefly, 47 g/ liter of BHIA was suspended in 1 litre of distilled water, and sterilised at 121°C for 15 minutes. After cooling to 45-50°C it was mixed gently and dispensed into sterile Petri dishes. These were stored in the fridge at 4ºC until required.

2.6.9. Calcofluor media

Cellulose production was investigated by streaking the bacterial strains on supplemented LBA medium with Calcofluor White Stain (18909 Sigma, UK). Calcofluor media was prepared by dissolved of 5 grams of tryptone, 2.5 grams yeast extract and 7.5 grams of technical agar (LP0013 Agar No 3; Oxoid Thermo Fisher; UK) in 500 ml of distilled water. It was mixed until completely dissolved and autoclaved at 121°C for 15 minutes. Then it was cooled to 50°C. The agar was supplemented by 25ml of Calcofluor White Stain (18909 Sigma, UK), and about 15 - 20 ml of media was poured in each sterile Petri dish. The plates were then stored in a dark and cool place for up to 2 weeks at 4°C, followed inoculation & incubation for 18 h at 37°C and 48h at 30°C.

2.6.10. Tributyrin agar, base (Lipase activity).

The lipase activity assay was performed by using Tributyrin agar (pre-prepared Oxoid, UK). The pre-prepared agar was rehydrated and then poured into petri dishes. The petri dish plates were left at room temperature to dry for two to three days and after that strains were streaked on tributyrin agar and incubated for 72 h at 37^{o}C. Each strain was evaluated for lipase activity by visual observation. A positive result was defined as a clear zone around the bacterial colonies.

2.6.11. Iso-Sensitest agar (ISA)

Exactly 31.4 g were suspended in 1 litre of distilled water and next the mixture was boiled to dissolve the agar and then autoclaved at 121°C for 15 minutes. The medium was cooled to 50°C and dispensed into petri dishes.

2.7. Identification methods and molecular typing

2.7.1. Bacterial strains and their sources.

A total of 111 strains of *Enterobacteriaceae* (*E. coli*, *K. pneumoniae* and *K. oxytoca*) from different clinical sources were used in this study. Thirty-six strains had been isolated from neonatal enteral feeding tube/ sepsis from two local Nottingham hospitals (QMC and NCH), in chapter 3 and reported in a previous Nottingham NICU study by Hurrell *et al*. (2009b). Seventy-five enteral feeding tube (EFT) and flushed milk isolates of *K. pneumoniae* from Jordan were also analysed in chapter 4. Further, 14 strains of *E. hormaechei* (n=6) and *E. faecalis* (n=8) were isolated from NEFT and faecal samples of a premature baby in the NICU at QMC during a 28-day period. Isolates information such as, source, site and date of isolation are presented in Table 2-1, Table 2-2 and Table 2-3.

Table 2-1: *E. coli* and *Klebsiella* spp. isolated from neonatal enteral feeding tubes in NICUs at Nottingham Hospital, UK (chapter 3)

NTU	Strain	Source	Country	Year	*A	*B	*C
605	*E. coli K5*	NEFT	UK	N.A	✓	✓	
1980	*E. coli K1*	NEFT	UK	2012	✓	✓	✓
1981	*E. coli K5*	NEFT	UK	2012	✓	✓	
1982	*E. coli K1*	NEFT	UK	2012	✓	✓	✓
1983	*E. coli K1*	NEFT	UK	2012	✓	✓	✓
1984	*E. coli K5*	NEFT	UK	2013	✓	✓	✓
1985	*E. coli K5*	NEFT	UK	2013	✓	✓	✓
1986	*E. coli K5*	NEFT	UK	2013	✓	✓	
2113	*E. coli K1*	NEFT	UK	2014	✓	✓	✓
2114	*E. coli K1*	NEFT	UK	2014	✓	✓	✓
2255	*E. coli K5*	NEFT	UK	2014	✓	✓	
2286	*E. coli*	Sepsis	UK	2015	✓	✓	✓
2297	*E. coli*	Sepsis	UK	2015	✓	✓	✓
2300	*E. coli*	Sepsis	UK	2016	✓	✓	✓
498	*K. pneumoniae*	N.A	UK	N.A	✓	✓	✓
500	*K. pneumoniae*	N.A	UK	N.A	✓	✓	✓
1443	*K. pneumoniae*	Blood	UK	2010	✓	✓	✓
1446	*K. pneumoniae*	Blood	UK	2011	✓	✓	✓
453	*K. pneumoniae*	N.A	UK	N.A	✓	✓	✓
497	*K. pneumoniae*	N.A	UK	N.A	✓	✓	✓
502	*K. pneumoniae*	N.A	UK	N.A	✓	✓	✓
1444	*K. pneumoniae*	Blood	UK	2010	✓	✓	✓
673	*K. pneumoniae*	N.A	UK	N.A	✓	✓	✓
2291	*K. pneumoniae*	Sepsis	UK	2015	✓	✓	✓
2298	*K. pneumoniae*	Sepsis	UK	2015	✓	✓	✓
2312	*K. pneumoniae*	Sepsis	UK	2016	✓	✓	✓
599	*K. oxytoca*	N.A	UK	N.A	✓	✓	
601	*K. oxytoca*	N.A	UK	N.A	✓	✓	
672	*K. oxytoca*	N.A	UK	N.A	✓	✓	
1445	*K. oxytoca*	Blood	UK	2011	✓	✓	
1989	*K. oxytoca*	Blood	UK	2013	✓	✓	
671	*K. oxytoca*	N.A	UK	N.A	✓	✓	
674	*K. oxytoca*	N.A	UK	N.A	✓	✓	
670	*K. oytoca*	N.A	UK	N.A	✓	✓	
600	*K. oxytoca*	N.A	UK	N.A	✓	✓	
2310	*K. oxytoca*	Sepsis	UK	2016	✓	✓	✓

*A – Physiological and phenotyping study as part of this project was performed, *B – Genotypic analysis as part of this project was performed, *C– Whole genome sequencing was performed, N.A= Not available.UK= United Kingdom. In the current study, thirty-six clinical isolates were collected from NEFTs and neonates with sepsis cases on intensive care units from two local hospitals; QMC and NCH (Nottingham). Of particular note is the fact that these collections are unique and have not been studied before. Unfortunately, metadata (hospital infection) associated to some of the studied babies were not available because it has been a long time between sample collection and analysis. Furthermore, new strains were isolated from neonatal sepsis cases for which the genomes and strain history are available, and it was revealed that all these isolates carry more virulent factors and antibiotic resistance genes. I have therefore included these strains in my study as well. In order to understand more and identify new virulence traits contributing to causing disease to the babies.

Table 2-2: *K. pneumoniae* strains used in this study isolated from enteral feeding tubes on neonatal intensive care units at Jordan Hospitals (chapter 4).

NTU number	Strain	Source		Country	Date	*A	*B	*C
		FL	T					
1729	*K pneumoniae*	✓		Jordan	15\7\2011			
1681	*K pneumoniae*	✓		Jordan	23\7\2011	✓	✓	✓
1682	*K pneumoniae*	✓		Jordan				
1683	*K pneumoniae*		✓	Jordan	23\7\2011	✓	✓	
1684	*K pneumoniae*		✓	Jordan				
1685	*K pneumoniae*		✓	Jordan				
1713	*K pneumoniae*	✓		Jordan				
1714	*K pneumoniae*	✓		Jordan				
1715	*K pneumoniae*	✓		Jordan	23\7\2011			
1716	*K pneumoniae*	✓		Jordan				
1718	*K pneumoniae*		✓	Jordan	23\7\2011	✓	✓	
1719	*K pneumoniae*		✓	Jordan				
1720	*K pneumoniae*	✓		Jordan				
1721	*K pneumoniae*	✓		Jordan				
1734	*K pneumoniae*		✓	Jordan	7\12\2011	✓	✓	✓
1736	*K pneumoniae*	✓		Jordan				
1738	*K pneumoniae*	✓		Jordan	10\12\2011			
1737	*K pneumoniae*	✓		Jordan		✓	✓	
1739	*K pneumoniae*		✓	Jordan	10\12\2011			
1740	*K pneumoniae*	✓		Jordan				
1741	*K pneumoniae*	✓		Jordan	10\12\2011	✓	✓	
1742	*K pneumoniae*		✓	Jordan				
1743	*K pneumoniae*		✓	Jordan				
1752	*K pneumoniae*		✓	Jordan	12\12\2011			
1753	*K pneumoniae*		✓	Jordan		✓	✓	
1745	*K pneumoniae*	✓		Jordan	15\12\2011			
1746	*K pneumoniae*	✓		Jordan	15\12\2011	✓	✓	
1747	*K pneumoniae*	✓		Jordan				
1748	*K pneumoniae*		✓	Jordan	DETAILS MISSING	✓	✓	
1749	*K pneumoniae*		✓	Jordan				
1750	*K pneumoniae*	✓		Jordan	15\12\2011	✓	✓	
1751	*K pneumoniae*		✓	Jordan				
1754	*K pneumoniae*	✓		Jordan	20\12\2011	✓	✓	
1755	*K pneumoniae*	✓		Jordan				
1756	*K pneumoniae*	✓		Jordan	20\12\2011			

Table 2.2 continued;

NTU number	Strain	Source		Country	Date	*A	*B	*C
		FL	T					
1727	*K pneumoniae*		✓	Jordan	22\5\2011	✓	✓	
1728	*K pneumoniae*		✓	Jordan				
1699	*K pneumoniae*	✓		Jordan	31\5\2011	✓	✓	✓
1700	*K pneumoniae*	✓		Jordan				
1701	*K pneumoniae*		✓	Jordan	31\5\2011	✓	✓	✓
1702	*K pneumoniae*		✓	Jordan				
1703	*K pneumoniae*	✓		Jordan		✓	✓	
1704	*K pneumoniae*	✓		Jordan				
1705	*K pneumoniae*		✓	Jordan	31\5\2011	✓	✓	
1706	*K pneumoniae*		✓	Jordan				
1707	*K pneumoniae*	✓		Jordan				
1708	*K pneumoniae*	✓		Jordan		✓	✓	
1686	*K pneumoniae*	✓		Jordan	4\7\2011	✓	✓	
1687	*K pneumoniae*	✓		Jordan		✓	✓	
1688	*K pneumoniae*		✓	Jordan				
1689	*K pneumoniae*		✓	Jordan				
1690	*K pneumoniae*		✓	Jordan	4\7\2011	✓	✓	
1691	*K pneumoniae*		✓	Jordan				
1692	*K pneumoniae*	✓		Jordan				
1709	*K pneumoniae*			Jordan	6\7\2011	✓	✓	
1710	*K pneumoniae*		✓	Jordan				
1711	*K pneumoniae*	✓		Jordan				
1712	*K pneumoniae*	✓		Jordan				
1722	*K pneumoniae*	✓		Jordan	6\7\2011	✓	✓	
1723	*K pneumoniae*	✓		Jordan				
1724	*K pneumoniae*		✓	Jordan				
1725	*K pneumoniae*	✓		Jordan	6\7\2011	✓	✓	✓
1726	*K pneumoniae*	✓		Jordan				
1717	*K pneumoniae*		✓	Jordan	7\7\2011	✓	✓	
1730	*K pneumoniae*		✓	Jordan	9\7\2011			
1731	*K pneumoniae*		✓	Jordan		✓	✓	
1732	*K pneumoniae*	✓		Jordan				
1733	*K pneumoniae*	✓		Jordan				
1693	*K pneumoniae*		✓	Jordan	9\7\2011	✓	✓	
1694	*K pneumoniae*		✓	Jordan				
1695	*K pneumoniae*	✓		Jordan				
1696	*K pneumoniae*	✓		Jordan				
1697	*K pneumoniae*		✓	Jordan	9\7\2011	✓	✓	
1698	*K pneumoniae*		✓	Jordan				

*A – Physiological and phenotyping study as part of this project was performed, *B – Genotypic analysis as part of this project was performed, *C– Whole genome sequencing was performed, FL= flushed milk, T= tube. Based on PFGE analysis and associated neonatal metadata, representative isolates were selected for further characterisation of various virulence traits.

Table 2-3: *E. faecalis* and *E. hormaechei* strains included in this study. Strains were isolated from Neonatal Feeding Tube and faecal samples isolated from a single premature baby at QMC (chapter 5).

NTU	Strain	Source	Country	Year	*A	*B	*C
2315	*Enterobacter hormaechei*	Tube 1	UK	2015	✓	✓	✓
2318	*Enterobacter hormaechei*	Tube 2	UK	2015	✓	✓	✓
2320	*Enterobacter hormaechei*	Tube 2	UK	2015	✓	✓	✓
2316	*Enterobacter hormaechei*	Faecal 1	UK	2015	✓	✓	✓
2319	*Enterobacter hormaechei*	Faecal 1	UK	2015	✓	✓	✓
2317	*Enterobacter hormaechei*	Faecal 2	UK	2015	✓	✓	✓
2324	*Enterococcus faecalis*	Tube 1	UK	2015			✓
2326	*Enterococcus faecalis*	Tube 1	UK	2015			✓
2329	*Enterococcus faecalis*	Tube 1	UK	2015	✓	✓	✓
2325	*Enterococcus faecalis*	Tube 2	UK	2015			✓
2328	*Enterococcus faecalis*	Tube 2	UK	2015			✓
2321	*Enterococcus faecalis*	Tube 2	UK	2015	✓	✓	✓
2323	*Enterococcus faecalis*	Faecal 1	UK	2015	✓	✓	✓
2322	*Enterococcus faecalis*	Faecal 2	UK	2015	✓	✓	✓

*A – Physiological and phenotyping study as part of this project was performed, *B – Genotypic analysis as part of this project was performed, *C– Whole genome sequencing was performed.

2.7.2. Biochemical confirmation;

Suspected colonies of *E. faecalis* and *E. hormaechei* were picked for confirmation by biochemical testing. The colonies selected based on colony morphology on each media, were first subjected to Gram staining, catalase test and oxidase test. After that, the presumptive isolates were subjected to various phenotypic and genotypic analysis.

2.7.2.1. Gram stain;

Gram stain is very crucial step for identifying, differentiation and classifying bacteria as either Gram positive or Gram negative. There are four components required for Gram staining. In the Gram stain procedure single pure colonies of *E. faecalis* and *E. hormaechei* were heat fixed onto a clean and labelled microscope slide by passing it over a flame. Then the smear was flooded with primary crystal violet dye for about 45 seconds. Before applying Gram's iodine, the slide was washed off with sterile water or by tap water to remove the primary stain; the Gram's iodine was performed for about 25 seconds. The Gram's iodine was washed off with sterile water. Then ethanol 95% was used to wash the dye off. Finally safranin (red dye) was used for 45 seconds, the slide gently was washed off and then dried before it was viewed under a microscope with the oil immersion objective lens. The Gram-

staining components that were used in this study were from the Remel™ Gram Stain Kit (Thermo Scientific, UK).

2.7.2.2. Oxidase test;

Oxidase reaction was determined with filter paper saturated with N,N,N,N'-tetramethyl-p-phenylene diamine dihydrochloride (Alfa Products, Damers, MA). Isolates were streaked onto the filters with wooden applicator sticks. Purple to black discoloration within 10 seconds constituted a positive reaction

2.7.2.3. Catalase test;

A well-grown 18-24 hours old culture on TSA was picked up and spread on a clean glass slide, a drop of 3 % hydrogen peroxide (H_2O_2) (Thermo Scientific, UK) added on the smear and observed for the immediate development of bubbles which was considered as positive for catalase.

2.7.3. Phenotyping

Four different media were used to assess the diversity of the colony morphology of examined strains according to recovery on TSA, XLD, VRBGA and MRS agar.

2.7.4. Genotyping assays

2.7.4.1. Pulsed field gel electrophoresis (PFGE):

PFGE was used in order to determine whether there were indistinguishable strains collected from four different hospitals and the sites or departments. The experiment used has been standardised according to the CDC and PulseNet (2004) protocol.

PFGE analysis for these collections was performed by using the three restriction enzymes *Xbal*, *Spel* and *Smal* (Promega, UK). The CHEF-DR II system (BIO-RAD, Belgium) was used to separate the bands at 6V, 14°C for 20 h with initial and final switch of 5 and 50 sec respectively. BioNumerics software version 3.5 (Applied Maths, Belgium) was used to analysed DNA bands profiles. Less than 95 % of band similarity value was used to consider the isolates to be non-clonal (Tenover *et al*., 1995).

2.7.4.2. Tris EDTA Buffer (TEB)

TEB was prepared aseptically by diluting 10 ml of 1M Tris pH8 and 2ml of 0.5 M EDTA pH 8 with 988ml distilled water. This buffer was used for making and washing the PFGE plugs.

2.7.4.3. Cell Suspension Buffer (CSB)

The preparation of CSB was obtained aseptically by diluting 10 ml of 1 M Tris pH8 and 20 ml of 0.5 M EDTA pH8 with 70ml of distilled water.

2.7.4.4. Cell Lysis Buffer (CLB)

CLB was prepared aseptically by diluting 2.5 ml of 1 M Tris pH8 and 5 ml of 0.5 M EDTA pH8 with 42.5 ml distilled water. 1% Sarkosyl NL (N-Dodecanoyl-N-methylglycine sodium salt) (Sigma Aldrich, UK) was dissolved into the mixture before adding 20 mg/mL of proteinase K (Sigma Aldrich, UK).

2.7.4.5. DNA preparation in agarose plugs

The strains of interest and a marker strain *Salmonella* strain 732 reference standard H9812 (NTU 732) were screened in this project. About two full loops of overnight culture on TSA of the isolates and reference strain were individually suspended into cell suspension buffer and centrifuged at 7000 rpm for 4 minutes. The previous step was repeated two times and then, using the same buffer, the optical density (OD) of each bacterial suspension was adjusted to be between 1.35-1.5 at 610 nm. Exactly 400 µl of bacterial suspension was pipetted into a 1.5 ml Eppendorf tube and while they were incubated in water bath at 37°C for 10 minutes, 1% agarose gel in TEB was microwaved for melting and 0.5% sodium dodecyl sulfate (SDS) was added and the gel was left in 55°C water bath. Each Eppendorf tube was mixed with 25 µl proteinase K and then mixed with 400 µl of TEB agarose and immediately about 100 µl of this mixture was dispensed into five well plug molds. The plugs were transferred into 15 ml falcon tube contain 5 ml of CLB and incubated in a shaking water bath at 50°C for 2 hours. After the period of incubation, the plugs were washed twice with 15 ml of warmed sterile distilled water and incubated in shaking water bath at 50°C for 15 minutes. Finally, the DNA plugs were washed three times in 15 ml warmed TEB in a shaking water bath at 55°C for 15 minutes and stored in 5 ml of TEB at 4°C until needed.

2.7.4.6. Digestion and Electrophoresis;

Three restriction enzymes *Xbal*, *Spel* and *Smal* (Promega, UK) were used for digestion. About 2 millimetres of each plug was transferred into a 2 ml Eppendorf tube containing 2 µl BSA, 20 µl 10X buffer D and 178 µl sterile distilled water and incubated in a 37°C water bath for 15 minutes. After the incubation, the content of tubes was removed and digestion was performed on each plug by adding 2 µl BSA, 20 µl 10X buffer D, 5 µl *Xbal* or *Smal* enzyme and 173 µl sterile distilled water or 2 µl BSA, 20 µl 10X buffer B, 3 µl *Spel* enzyme and 175 µl sterile distilled and incubated in water bath at 37°C for 4 hours. A 1% agarose gel was prepared in 100 ml of 0.5XTBE buffer and kept in 55°C water bath. The restricted plugs were then loaded on a 15-tooth comb (BIO-RAD Laboratory; Belgium). The comb was inserted in the gel tray and the 1% agarose gel was carefully poured into the gel tray. After the gel solidified, the comb was removed, it was placed in an electrophoresis cell and covered with 2400 ml of 0.5X TBE buffer. The system used for PFGE was CHEF-DR II system (BIO-RAD, Belgium), it was performed at 14°C, 6V for 20 hours, and initial and final switch was 5 and 50 seconds respectively.

2.7.4.7. Staining and Analysis:

The gel was stained with 0.1 µg/ml ethidium bromide (Sigma Aldrich, UK) for 45 minutes, visualised under UV light and photographed using In Genius® gel documentation system (Syngene, UK). The DNA band profiles were analysed using BioNumerics software version 3.5 (Applied Maths, Belgium).

2.7.4.8. DNA extraction.

GeneEluteTM kit (NA2110-1KT, Sigma, UK) was used for DNA extraction from target strains. The instructions were followed as explained by the manufacturer. The DNA concentration was confirmed by using a NanoDrop® ND-2000 UV-Vis spectrometer (Thermo Scientific, UK), and the DNA in accepted concentration was stored at -20°C for up to 6 months.

2.7.4.9. PCR 16S rDNA Sequence Analysis;

The partial sequencing of the 16S rDNA loci (528 bp) Table 2-4 was performed using primers described by Iversen *et al*., (2006). Cycling conditions were an initial denaturation at 95°C for 10 minutes; 30 cycles of denaturation at 95°C for 30 sec, primer annealing at 62.6°C for

30 sec, extension at 72°C for 45 sec; followed by a final extension step of 72°C for 10 minutes. Regarding to *E. faecalis*, the partial sequencing of the 16S rDNA loci (520 bp, Table 2-4) was performed using primers described by Jiménez *et al*., (2008). Cycling conditions were an initial denaturation of 94°C for 5 minutes; 25 cycles of 94°C for 30 seconds, primer annealing at 48°C for 30 seconds, extension at 72°C for 45 seconds and followed by a final extension step of 72°C for 4 minutes.

2.7.4.10. Screening for *rpoB* gene for *Klebsiella* sp.

Profiling the *rpoB* gene Table 2-4 was performed as described by Mollet *et al*., (1997). PCR products were visualized on a 1% agarose gel stained with SYBR safe. The PCR product (637 bp) length was sequenced and aligned with additional sequences from *Klebsiella* locus/sequence definitions on the Pasteur MLST database.

(http://bigsdb.web.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef_public&page=batchSequenceQuery).

The *rpoB* gene sequences were analysed using the MEGA6 software to construct and analyse the phylogenetic relationship between the studied strains.

2.7.4.11. Phylogenetic grouping of *E. coli* isolates:

Phylogenetic grouping (A, B1, B2 or D) of the *E. coli* isolates was performed using a multiplex PCR method as described by Clermont *et al*., (2000). Each PCR reaction was carried out in a 25-µl reaction volume that included 5 µl of 10X PCR buffer (Promega, UK), 2 µl of 15 mM MgCl2 (Promega), 1.0 µl of dNTP solution (Promega, 10 mM each dNTP), 2.5 µl of the forward and reverse primers (10 pmol/µl for each primer), 0.25 µl of Taq DNA polymerase (Promega; 5 Units/µl), and 10.75 µl of PCR-grade water.

Table 2-4: Primers used to amplify 16S rDNA, Phylogenetic grouping and *rpoB* genes

Primer name		Prime sequences	PCR product size
ChuA	F	GACGAACCAACGGTCAGGAT	279
	R	TGCCGCCAGTACCAAAGACA	
YjaA	F	TGAAGTGTCAGGAGACGCTG	211
	R	ATGGAGAATGCGTTCCTCAAC	
TspE4C2	F	GAGTAATGTCGGGGCATTCA	152
	R	CGCGCCAACAAAGTATTACG	
16S rDNA	F	TGGAGAGTTTGATCCTGGCTCAG	528
	R	TACCGCGGCTGCTG-GCAC	
16S rDNA	F	AGAGTTTGATCCTGGCTCAG	520
	R	GGCTGCTGGCACGTAGTTAG	
rpoB	F	GGCGAAATGGCWGAGAACCA	501
	R	GAGTCTTCGAAGTTGTAACC	

2.7.4.12. PCR detection of virulence factor genes (VFG):

The presence of 30 VFGs for *E. coli* were determined using 5 multiplex PCR–based assays as described by Johnson and Stell, (2000) using primers Table 2-5. The gene classes included toxins, invasins, adhesins, siderophores, capsule and others. Reactions were heated to 95 ^{o}C for 12 min, followed by 25 cycles of denaturation 94 ^{o}C for 30 sec, annealing 63 ^{o}C for 30 sec and extension 68 ^{o}C for 3 min and a final extension 72 ^{o}C for 10 min. With respective to *Klebsiella* spp. VFGs were determined using one tube multiplex PCR for K1, K2, K5, *fyuA* and *Irp2* were performed according to (Turton *et al*., 2008). The reaction mixture was kept at 95°C for 5 min, followed by 40 temperature cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min, and 72°C for 7 min. Successful PCR products were confirmed by agarose gel electrophoresis.

2.7.4.13. Confirmation of PCR products by agarose gel electrophoresis;

Agarose gel was used to visualize the amplification and size of PCR products. The gel was prepared as follows: 1% (w/v) agarose (Fisher Scientific, UK) was dissolved in 1X Tris-acetate-EDTA (TAE) buffer (Geneflow, UK). The solution was heated in a microwave oven to dissolve the agarose. 0.1 µ/ml (v/v) of SYBR® Safe DNA gel stain (Life Technologies – Invitrogen, UK) was added and dissolved well in the agarose solution. The gel was then dispensed into the tray of gel and left for 20 to 30 minute to cool. After making wells, 10 µl of 1kb ladder

(Promega, UK) was loaded as a marker into two wells and 10 µl PCR products were loaded into each of the well. Gel electrophoresis was performed for 40 minutes at 100 V in 1X TAE buffer. The gel was then visualized under ultraviolet (UV) light. DNA bands were observed using the InGenius® gel documentation system (Syngene, UK).

2.7.4.14. Purification of PCR product.

The amplified products were purified using the MinElute PCR Purification Kits (Qiagen, UK) according to the manufacturer's protocol. The concentration and purity of the samples was checked by using the Nano drop 2000 (Thermo Scientific, UK). The purity of DNA samples with minimum 260/280 nm values of 1.8 and concentration with minimum 260/280 nm values of 15 ng were used. The products were finally eluted in 50 µl of molecular biology grade water (Fisher Scientific, UK).

2.7.4.15. PCR product sequencing.

PCR products were Sanger sequenced by Eurofins MWG Operon (London, UK) and Source Bioscience (Nottingham, UK). The nucleotide sequences were determined on each DNA strand using nested sequencing primers.

Table 2-5: Primers used for the detection of virulence factor genes

Primer name		Prime sequences	PCR product size
AlleleI	F	TCGTGCTCAGGTCCGGAATTT	461
	R	TGGCATCCCCCAACATTATCG	
AlleleI'	F	CTACTATAGTTCATGCTCAGGTC	474
	R	CTGACATCCTCCAACATTATCGA	
Allele II	F	GGGATGAGCGGGCCTTTGAT	190
	R	CGGGCCCCCAAGTAACTCG	
Allele III	F	GGCCTGCAATGGATTTACCTGG	258
	R	CCACCAAATGACCATGCCAGAC	
sfa	F	CTCCGGAGAACTGGGTGCATCTTAC	410
	R	CGGAGGAGTAATTACAAACCTGGCA	
SfaS	F	GTGGATACGACGATTACTGTG	240
	R	CCGCCAGCATTCCCTGTATTC	
FocG	F	CAGCACAGGCAGTGGATACGA	360
	R	GAATGTCGCCTGCCCATTGCT	
Afa	F	GGCAGAGGGCCGGCAACAGGC	559
	R	CCCGTAACGCGCCAGCATCTC	
bmaE	F	ATGGCGCTAACTTGCCATGCTG	507
	R	AGGGGGACATATAGCCCCCTTC	
gafD	F	TGTTGGACCGTCTCAGGGCTC	952
	R	CTCCCGGAACTCGCTGTTACT	
nfaE	F	GCTTACTGATTCTGGGATGGA	559
	R	CGGTGGCCGAGTCATATGCCA	
FimH	F	TGCAGAACGGATAAGCCGTGG	508
	R	GCAGTCACCTGCCCTCCGGTA	
hly	F	AACAAGGATAAGCACTGTTCTGGCT	1177
	R	ACCATATAAGCGGTCATTCCCGTCA	
cnf	F	AAGATGGAGTTTCCTATGCAGGAG	498
	R	CATTCAGAGTCCTGCCCTCATTATT	
cdt-a	F	AAATCACCAAGAATCATCCAGTTA	430
	R	AAATCTCCTGCAATCATCCAGTTTA	
cdt-s	F	GAAAGTAAATGGAATATAAATGTCCG	430
	R	GAAAATAAATGGAACACACATGTCCG	
FyuA	F	TGATTAACCCCGCGACGGGAA	880
	R	CGCAGTAGGCACGATGTTGTA	
AerJ	F	GGCTGGACATCATGGGAACTGG	300
	R	CGTCGGGAACGGGTAGAATCG	
kpsII	F	GCGCATTTGCTGATACTGTTG	272
	R	CATCCAGACGATAAGCATGAGCA	
KpsIII	F	TCCTCTTGCTACTATTCCCCCT	392
	R	AGGCGTATCCATCCCTCCTAAC	
K1	F**	TAGCAAACGTTCTATTGGTGC	153
K5	F**	CAGTATCAGCAATCGTTCTGTA	159
rfc	F	ATCCATCAGGAGGGGACTGGA	788
	R	AACCATACCAACCAATGCGAG	
ibe10	F	AGGCAGGTGTGCGCCGCGTAC	170
	R	TGGTGCTCCGGCAAACCATGC	
ColV-C	F	CACACACAAACGGGAGCTGTT	680
	R	CTTCCCGCAGCATAGTTCCAT	
TraT	F	GGTGTGGTGCGATGAGCACAG	290
	R	CACGGTTCAGCCATCCCTGAG	
RPAi	F	GGACATCCTGTTACAGCGCGCA	930
	R	TCGCCACCAATCACAGCCGAAC	

Followed; Primers used for the detection of virulence factor genes

Primer name		Prime sequences	PCR product size
K1	F	GGTGCTCTTTACATCATTGC	1283
	R	GCAATGGCCATTTGCGTTAG	
K2	F	GACCCGATATTCATACTTGACAGAG	641
	R	CCTGAAGTAAAATCGTAAATAGATGGC	
K5	F	TGGTAGTGATGCTCGCGA	280
	R	CCTGAACCCACCCCAATC	
FyuA	F	GCGACGGGAAGCGATGATTTA	547
	R	TAAATGCCAGGTCAGGTCACT	
Irp2	F	AAGGATTCGCTGTTACCGGAC	287
	R	TCGTCGGGCAGCGTTTCTTCT	
Irp1	F	GTCGGCACCAGACTGATTGA	429
	R	TAAAGAACGGGTATCCGGCG	
PapA	F	ATGGCAGTGGTGTCTTTTGGTG	720
	R	CGTCCCACCATACGTGCTCTTC	
PapC	F	GTGGCAGTATGAGTAATGACCGTTA	200
	R	ATATCCTTTCTGCAGGGATGCAATA	
PapEF	F	GCAACAGCAACGCTGGTTGCATCAT	336
	R	AGAGAGAGCCACTCTTATACGGACA	
pG	F	CTGTAATTACGGAAGTGATTTCTG	1170
	R	ACTATCCGGCTCCGGATAAACCAT	
pG1	R*	TCCAGAAATAGCTCATGTAACCCG	1190

pG1-R* Use with pG-F, K1 and K5-F** Use with kpsII-R.

2.7.4.16. Whole Genome Sequencing;

Bacterial DNA was extracted from 1-day old cultures using the GenElute bacterial genome kit (Sigma Aldrich, UK) using the manufacturer's protocol. Genomes were sequenced on an Illumina MiSeq by Pauline Ogrodzki. Genome comparisons were applied to find the key physiological and virulence genes of the organisms such as environmental stress, attachment, invasion, capsule, cellulose, curli fimbriae and antimicrobial resistance genes. This comparative analysis was performed using WebACT comparative tool, artimes comparative tool (ACT) for genome alignment, which has been developed by Carver *et al*. (2005). In addition, BLAST searches were performed using NCBI BLAST research facility at;

https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome

A collaborative approach was taken in this study, and credit must be given to my colleague Pauline Ogrodzki (NTU), who carried out the whole-genome sequencing, as part of a parallel PhD study.

2.7.5. Physiological assays.

Based on PFGE analysis and associated neonatal metadata, representative isolates were selected for further characterisation of various virulence traits.

2.7.5.1. Haemolysis reaction:

Isolates were plated on 5% sheep and 5% horse-blood (Oxoid Fischer Scientific, UK) agar and incubated at 37 C for 24 h. The resultant colony morphology was recorded after 24 h to determine the formation of either α-, λ or β-haemolysis. *Staphylococcus aureus* NCTC 10788 was used as a positive control for β haemolysis, *Streptococcus pneumoniae* was used as a positive control for α haemolysis and *Staphylococcus epidermidis* was used as a negative control for λ haemolysis.

2.7.5.2. Iron siderophore detection;

The siderophore detection assay was performed as described by Shin *et al*., (2001). Two solutions were used to prepare the Chrome azurolsulphate (CAS) agar. The first solution which is a dark blue liquid was prepared by using 10 ml of iron III solution (Section 2.4.11), 50 ml of CAS solution (Section 2.4.12), and 40 ml of HDTMA (Section 2.4.13) and then autoclaved at 121°C for 15 minutes, (the total volume of dark solution is 100 ml). The second solution was prepared by mixing 900 ml of DW, 15 g agar, 30.24 g PIPES (Sigma Aldrich, UK) and 12 g NaOH and then autoclaved at 121°C for 15 minutes. After autoclaving, the first solution was mixed with the second solution and then the media was dispensed into the petri dishes. Immediately before use, 5mm diameter holes were punched into the agar using a 5mm diameter gel plug cutter. The bacterial suspension was prepared by inoculating five colonies from TSA culture into 10 ml TSB broth containing 200 µM of 2,2′-dipyridyl (Sigma Aldrich, UK) and incubated at 37°C in a shaking incubator at 200 rpm for 20 h. Then the samples were centrifuged at 5000 rpm for 10 minutes and 70 µl of the supernatant was placed into a specific hole. The agar was incubated at 37^{o}C for up to 8 hours and observed for the presence of an orange zone around the hole which indicated that the strain is positive

for siderophore production. Y*ersinia enterocolitica* strain 1880 and PBS were used as positive and negative controls respectively.

2.7.5.3. Protease activity assay;

Ten percent skimmed milk powder (Thermo Fisher Scientific, UK) was used to prepare the skimmed milk solution (SMS) and autoclaved for 5 minutes at 121°C. PCA plate count agar was prepared by adding 22.5 grams to 1 litre of distilled water and autoclaved at 121°C under 15 psi pressures for 15 minutes. 20 ml of 10 % SMS was aseptically mixed with 980 ml of PCA and then dispensed into Petri dishes to be stored at room temperature for 2 days. The tested strains were streaked on the dried plates and incubated at 37°C for 72 h and monitored every 24 h. *Bacillus cereus* was used as a positive control while *E. coli* DH5α NTUCC407 was used as a negative control.

2.7.5.4. Cellulose production

LB agar without salt was prepared as mentioned in 2.6.9 and supplemented with 200 µg/ml calcofluor white stain (Fluka, UK). The medium was poured in petri dishes and left to dry for 2 days. Representative strains were streaked and incubated either at 37°C or 30°C for 24 h and then the cellulose production was visualised by using ultraviolet light at 366 nm.

2.7.5.5. Congo red morphotype

To prepare LB agar without salt, 5 g of tryptone (Thermo Fisher Scientific, UK), 2.5 g of yeast extract (Melford laboratories, UK) and 7.5 g agar (Thermo Fisher Scientific, UK) were mixed in 500 ml distilled water and then autoclaved. Congo red solution dye was prepared by dissolving 32 mg of Congo red powder (Sigma Aldrich, Uk) in 8 ml SDW and the solution was filtered with 0.20µm pore filter. After this, LB agar without salt was cooled to 55°C, 5 ml of filtered Congo red solution was added, gently mixed and dispensed into petri dishes.

2.7.5.6. Biofilm Formation:

Eighteen hour TSB cultures were used to inoculate 5 ml of TSB and adjusted to O.D = 0.3 reading at 600nm. In three 96 plates, 600 µl of each inoculated TSB was dispensed into 3 wells (200 µl into each well) of each 96 well plate. Also liquid infant formula (Cow & Gate Premium 1) was used instead of TSB to detect and compare the ability of examined isolates

to form biofilm in both media. After measuring the O.D of TSB culture (TSB was adjusted to a cell density of an O.D = 0.3) then the bacteria were resuspended in PIF. Loading plates were incubated for 24h at 25°C and 37°C. After 24 hours of incubation, the plates were emptied from the TSB or the milk and washed twice with sterile distilled water (SDW) the plates then left for 10 minutes at room temperature to dry. Two hundred microliter of 1% crystal violet (CV) was added to each well and left for 30 minutes after which all the wells were washed three times with SDW. Finally, 200 µl of absolute ethanol was added to each well and after 15 minutes the content of the plates was transferred into new plates and read using ELx800 absorbance microplate reader (BioTek, UK) at absorbance 600nm.

2.7.5.7. Capsule production assay:

For detecting the ability of the strains to produce capsule, two types of media were used; XLD and PIF agar were used in this study. For preparing milk agar 800 ml of Plate Count Agar (PCA) was made according to the manufacture of instructions. Gently 200 ml of milk (20% final concentrated) was added to PCA and mixed and the media then dispensed into petri dishes. All plates were left at room temperature for drying. The test strains were plated on the PCA with milk agar and XLD and incubated at 37°C for 24h.

2.7.5.8. Serum resistance:

A single isolated colony was inoculated into TSB and incubated at 37 ^{o}C for 24h with shaking. The bacterial suspension was then diluted 1:10 in new TSB incubated under the same conditions for 2 hrs to approximately 10^{6} cfu/ml. Fourty microliters of the bacterial culture were added to 360 µl of 50% human serum (Sigma, UK). Strains were compared with negative and positive controls which were *Escherichia coli* K-12 and *Salmonella* Eenteritidis respectively. The remaining viable bacterial cells after 24 hrs were counted using the Miles and Misra technique. All strains were tested in triplicate and in three independent experiments.

2.7.5.9. Acid tolerance:

Acid tolerance was studied following the method of Edelson-Mammel *et al*. (2006) with some modification. A pure single colony was inoculated into 5 ml of TSB and incubated overnight at 37 ^{o}C. One millilitre of the bacterial cell suspension was inoculated into acidified

rehydrated PIF at pH 3.5 in a water bath at 37 ^{o}C. Viable cells were enumerated after 0, 15, 30, 60, 90 and 120 minutes using the Miles and Misra technique. All experiments were performed in triplicate from separate overnight cultures.

2.7.5.10. Thermo-tolerance.

Thermal resistance of representative strains were investigated at 55°C. The isolates of interest were investigated by suspending 1 ml overnight culture in 20 ml of temperature equilibrated sterile liquid infant formula (Cow & Gate Premium 1) in a water bath 55°C. At timed intervals, 0.1 ml aliquots were transferred to 2 ml TSB at room temperature and the number of surviving bacterial cells determined. The number of survivors at this temperature was plotted against time. The best fit-line was extrapolated and the D values were determined (-1/slope of the regression line) (Breeuwer *et al*., 2003). Each single number is an average of three replicate experiments. The standard deviations of the D value were calculated.

2.7.5.11. Desiccation stress and determining of sublethaly injured cells;

All strains were grown on TSA plates overnight at 37°C. A single colony from a purity TSA plate was inoculated into 5 ml in sterile liquid infant formula (Cow & Gate Premium 1) and incubated overnight at 37°C. After that the bacterial cell density was approximately 10^{11} CFU/ml. Aliquots (0.2 ml) of the suspension were transferred into six-well plates and air-dried overnight in a class II cabinet at room temperature (20 to 25°C) as described by (Caubilla-Barron and Forsythe, 2007). After desiccation, the test strains were re-suspended in 0.2 ml of sterile water and viable counts were determined in triplicate on TSA, MRS agar and VRBGA. Plates were incubated overnight at 37°C. The sublethaly-injured cells were defined as the difference between the viable counts obtained on MRS and VRBGA (selective agar) and those obtained on TSA (nonselective agar).

2.7.5.12. Antimicrobial susceptibility assay:

Susceptibility to antimicrobial agents were tested by the Kirby-Bauer method, as described by British Society for Antimicrobial Chemotherapy guidelines (BSAC, 2015). The antibiotics tested were augmentin, imipenem, meropenem, amikacin, ceftazidime, ampicillin, chloramphenicol, gentamicin, tobramycin, cefotaxime, vancomycin and ciprofloxacin. ESBL

production was determined by the combination of cefotaxime + clavulanate, cefpodoxime + clavulanate discs according to the manufacturer's instructions (Mast Diagnostics, Bootle, United Kingdom). Four colonies of fresh TSA culture were suspended in 3ml of sterile normal saline and the OD adjusted to be equivalent to a 0.5 McFarland standard. Suspensions were swabbed onto ISA (OXOID, UK) and then the antibiotic disks were applied onto the surface of the ISA plates. The plates were incubated at 37 ^{o}C for 20 hours. The diameters of zones of inhibition were measured and interpreted according to the BSAC Protocol (2015). *Escherichia coli* strain 10418 and *Escherichia coli* 13353 were used as negative and positive controls respectively.

2.7.6. Determination of bacterial pathogenicity using tissue culture;

2.7.6.1. Bacterial strains

The strains shown in Table 2-6 were used in Chapter 4. *Salmonella* Eenteritidis strain number NCTC 3046 358, *Citrobacter koseri* strain number SMT319 48, and uropathogenic *E. coli* strain CFT073 (ST73) were used as positive controls for HMBEC, Caco-2, and T24 cell lines respectively and *E. coli* K12 MG1655 was the negative control for all cell lines. A single colony of each test and control strain was inoculated into 5 ml of TSB and incubated in a shaking incubator at 200 rpm at 37°C for 18 h. 120 µl of overnight culture was added to 5 ml of appropriate infection culture media as described below in Section 2.7.6.2 and incubated for a further 2 h to reach the OD of 0.3-0.5 at 600 nm using the spectrophotometer (JENWAH, UK). The bacteria in the infection medium were then diluted to obtain 4x10^{6} cfu/ml, which equated to a multiplicity of infection (MOI) 1:100 on the cells.

Table 2-6: K*. pneumoniae* strains subjected for tissues culture

NTU strain	Strain	Source of bacteria		Country	Date
		Flushed milk	Tube		
1681	*Klebsiella pneumoniae*	✓		Jordan	23/07/2011
1699	*Klebsiella pneumoniae*	✓		Jordan	31/05/2011
1701	*Klebsiella pneumoniae*		✓	Jordan	31/05/2011
1725	*Klebsiella pneumoniae*	✓		Jordan	06/07/2011
1734	*Klebsiella pneumoniae*		✓	Jordan	07/12/2011
453	*Klebsiella pneumoniae*		✓	UK	N.A
497	*Klebsiella pneumoniae*		✓	UK	N.A
1446	*Klebsiella pneumoniae*		✓	UK	13/03/2011
2291	*Klebsiella pneumoniae*	Sepsis cases		UK	01/10/2015
2298	*Klebsiella pneumoniae*	Sepsis cases		UK	01/10/2015
2312	*Klebsiella pneumoniae*	Sepsis cases		UK	07/04/2016

N.A= Not available.UK= United Kingdom

2.7.6.2. Culture Media for Caco-2, HBMEC and T24 cell lines

All media and reagents for cell culture were obtained from Sigma Aldrich, UK unless otherwise stated. The growth medium for Caco-2 cells was Minimum Essential Medium (MEM) with 10% fetal bovine serum (FBS), 1% non-essential amino acids solution and 1% Penicillin-Streptomycin. HBMEC cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% non-essential amino acid solution and 1% Penicillin-Streptomycin. T24 cells were grown in McCoy's 5A Medium with 10% FBS, 1% non-essential amino acid solution and 1% Penicillin-Streptomycin. The infection medium for all the above-mentioned cell lines was same as the growth medium but lacking the 1% Penicillin-Streptomycin.

2.7.6.3. Human cell lines

Four human eukaryotic cell lines were used to determine the bacterial adhesion and invasion ability, as shown in Table 2-7. All cell lines were stored in liquid nitrogen. When a cell line was required, a vial was taken from the liquid nitrogen and thawed quickly. The content of the cell line tube was mixed into 6 ml of suitable pre-warmed growth medium, as explained above in Section 2.7.6.2, in a 15 ml falcon tube. The tube was then centrifuged at 1200 rpm for 5 minutes to harvest the cells. After discarding the supernatant, the cell pellet was re-suspended into 6 ml pre-warmed growth medium and transferred into a 25 cm^3 tissue culture flask. Finally, the flask was incubated for 48 hours at 37°C in the presence of 5% CO_2. After the cell line achieved a confluent monolayer, the medium was decanted out and the cells were detached using 5 ml of TrypLe™ express (Life Technologies, UK). The cell suspension was mixed with 5 ml pre-warmed growth medium and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded and the cell pellet was re-suspended in pre-warmed growth medium and then transferred into a new 75 cm^3 tissue culture flask containing 20 ml of pre-warmed growth medium. The cell lines were maintained and split routinely twice a week to keep the continuity of growth.

Table 2-7: Cell lines used in this study.

Cell line	Reference code	Source
Human colonic carcinoma epithelial cells (Caco-2)	ECACC #86010202	European Collection of Cell Cultures
Human brain microvascular endothelial cells (HBMEC)	#P10354	Inooprot, Spain
Macrophage cell line (U937)	ATCC#CRL-1593.2	American Type Culture Collection
human bladder carcinoma epithelial cells (T24)	ATCC #HTB-4	American Type Culture Collection

2.7.6.4. Determination of the bactericidal concentration of gentamicin.

Before tissue culture assays could be carried out, preliminary experiments were needed in order to determine the susceptibility of test strains to gentamicin as this is used to discriminate between attached and internalized bacterial cells. Strains were tested at the gentamicin concentrations of 50 and 75 µg/ml. Due to the resistance of these strains, this concentration was increased to 125 µg/ml and strains were re-tested. The antibiotic susceptibility testing was conducted in accordance with the BSAC guidelines, (2015). The inhibitory effects of the gentamicin were determined by the micro-dilution method (BSAC, 2015). Bacterial cultures were grown in TSB for 18-20h at 37°C. Their turbidity was adjusted to McFarland Standard 0.5 (~ $1.5xl0^8$ cfu/ml) before being diluted to 1:10 and 1:100. Equal volumes of these dilutions and the gentamicin solution were added at a final concentration of 50, 75, and 125 µg/ml to 96-well plates. Blank bacterial suspensions (without gentamicin) were also included as controls for each strain. Plates were incubated for 20h at 37 ^{o}C. Aliquots (~10 µl) of these overnight suspensions were inoculated into TSA plates. The presence or absence of growth recovery was checked 24 hrs later. Duplicate results were recorded for each strain.

2.7.6.5. *K. pneumoniae* attachment to human cells

For Caco-2 and HBMEC the cells were grown as described above in Section 2.7.6.3. The adhesion assay was conducted as described by Townsend *et al*., (2008). The cells were seeded into 24-well plates (Sarstedt, Germany) at a concentration of $4x10^4$ cells/well in growth medium and incubated at 37°C under 5% CO_2 for 48 h to achieve a confluent monolayer. For the T24 cell line, the cells were seeded in 24-well plates at a concentration of $5x10^4$ cells/well and incubated as previous condition for 24h. After achieving the confluent monolayer, the *K. pneumoniae* strains of interest and control strains were grown as described in Section 2.7.6.1. Bacterial suspension was added to the wells at a concentration

of $4x10^6$ cfu /well, which equated to an MOI of 1:100 on the cells, and 24-well plates were incubated at 37°C with 5% CO_2 for 2 h. After the incubation period, the plates were washed 3 times with PBS (Sigma Aldrich, UK) to remove unbound bacteria, and attached bacterial cells were released by lysing the human cells with 1% Triton X-100 (Thermo Fisher Scientific, UK). Serial dilution and Miles Misra method on TSA were performed to determine the overall number of viable attached bacterial cells. All bacterial strains were added to cells in similar numbers and the cfu/well of the original bacterial suspensions were determined.

2.7.6.6. *K. pneumoniae* invasion of human cells;

The preparation of cell lines and bacterial infection dose were done as described in Section 2.7.6.4. However, after 3 times washing with PBS the wells were filled with 500 µl infection media supplemented with gentamicin at a concentration of 125 µg/ml and the plates were incubated in the same conditions for 1 h to kill any extracellular bacteria. The plates were washed a further 3 times with PBS (Sigma Aldrich, UK) and invasive bacterial cells were released by lysing the human cells with 1% Triton X-100 (Thermo Fisher Scientific, UK). Serial dilution and Miles Misra method on TSA were performed to determine the overall number of viable invaded bacterial cells.

2.7.6.7. Uptake and persistence of *K. pneumoniae* in macrophage cell line U937;

2.7.6.7.1. Culture media

The macrophage cell line U937 was grown in RPMI medium containing 2 mM L-glutamine, 10 mM HEPES, and 1 mM sodium pyruvate. The growth medium was supplemented with 10% FBS, 1% non-essential amino acids solution and 1% Penicillin-Streptomycin while the infection medium was supplemented just with 10% FBS and 1% non-essential amino acid solution. The condition of incubation was 37°C under 5% CO_2 for 24 h.

2.7.6.7.2. *K. pneumoniae* uptake and persistence in human macrophages

The experiment was performed as described by Townsend et al., (2007a). The macrophage cells were seeded into four 24-well plates (Sarstedt, Germany) at a concentration of $4x10^4$ cells/well in growth medium and incubated at 37°C under 5% CO_2 for 72 h to achieve a confluent monolayer. However, before seeding the cells into 24-well plates, the macrophage cell suspension was supplemented with phorbol 12-myristate 13-acetate (PMA; Sigma

Aldrich, UK) at a concentration of 0.1 µg/ml to promote cell adhesion. The seeded plates were washed 3 times with PBS before cells were infected with the determined infection dose. All tested and control strains were prepared as described previously in Section 2.6.6.1; however, the infection dose was at a concentration of $4x10^5$ cfu/ml which is equivalent to an MOI of 1:10. The plates were incubated at 37°C under 5% CO_2 for 1h and the bacterial infection medium was replaced with infection medium supplemented with gentamicin at a concentration of 125 µg/ml and incubated in the same condition for one hour further. Three plates were washed 3 times with PBS and infection medium with gentamicin at a concentration of 50 µg/ml was added into the wells and incubated for interval periods of 24 h, 48 h and 72 h. The infection medium with gentamicin was changed every day. In different time point 1h, 24 h, 48 h and 72 h, each plate was washed 3 times with PBS and treated with 1% Triton X-100 to lyse the macrophage cells. Serial dilution and Miles Misra method on TSA were performed to determine the overall number of viable intracellular bacteria.

2.8. Statistical analysis;

All the conducted experiments in this study were repeated at least twice. The statistical analysis was carried out using unpaired t-test and One-way and Two-way ANOVA (Graph Pad Prism Software Version 7.0) to determine statistical significance. A P value of < 0.05, < 0.01 and < 0.001 was considered statistically significant, highly significant and very highly significant respectively.

Chapter 3. Characterisation of *E. coli* and *Klebsiella* isolates from Neonatal Feeding Tubes and Sepsis Cases.

3.1. Introduction:

3.1.1. General background

Neonatal sepsis is a globally widespread cause of mortality and morbidity. Lawn *et al*. (2005), estimated that over 26% of neonatal deaths worldwide were due to sepsis Figure 3-1. In addition, in developing countries sepsis is estimated to be responsible for about 30% to 50% of neonatal deaths each year (Agnihotri *et al*., 2004; Al-Rabea *et al*., 1998). In 2005, the World Health Organisation (WHO) documented that globally, more than 70% of deaths in children were those under the age of five and 40% of them occurred within the first month of life (WHO, World Health Report, 2005). Artero and co-authors (2012) reported that the global epidemiology of sepsis is still poorly described due to the lack of a universally accepted definition of sepsis. Sepsis is globally problematic with an estimated 1.7 million neonatal sepsis cases worldwide in 2010, and approximately 140,000 deaths Liu *et al.* (2012), and with 600,000 cases in Sub-Sahara Africa (Seale *et al*., 2013). It has been difficult to estimate the prevalence and aetiology of sepsis in developing countries and Africa due to lack of data.

In many cases, neonatal sepsis occurs within the first 28 days of life, due to the presence of bacteria in the neonatal pulmonary or urinary tract system, with subsequent progression to the bloodstream. This, as a result, could lead to septicaemia, arthritis, osteomyelitis, and meningitis (NNPD, NNF 2002-2003). Singh *et al.* (1994) reported that neonatal sepsis is classified into two categories according to the age of the infected neonate. Early onset sepsis occurs within the first 72 hours of life, and the source of infection is usually vertical transmission through the maternal genital tract from the mother to the new baby. Infections after 72 hours of age are referred to as late onset sepsis. Generally, the source of this infection is horizontal transmission - either nosocomial or community-acquired. Prolonged admissions in neonatal intensive care units (NICUs), prematurity, low birth-weight, bottle-feeding and use of feeding tubes are all factors that may increase the risk of neonatal sepsis

(Hurrell *et al*., 2009a and b; Ballot *et al.*, 2012). Moreover there has only been limited consideration that the nasogastric enteral feeding tube (NEFT) may act as a site for bacterial colonisation and a locus for infection. Ogrodzki *et al*. (2017) studied correlation between neonatal feeding tube flora with their colonization of the baby's gut and found several indistinguishable isolates. In the UK, the national press have highlighted in several recent reports multiple cases of mortality and morbidity in neonatal intensive care units due to sepsis and nosocomial infections. Health care practice (antibiotic exposure, diet) and the care environment in NICUs play a significant role in nosocomial infections (Forsythe, 2009; Hurrell *et al*., 2009a, b; Black *et al*., 2010; Liu *et al*., 2012).

Neonatal sepsis is caused by a range of different Gram-negative and Gram-positive bacteria. These include *E. coli*, *K. pneumoniae*, *L. monocytogenes*, *H. influenza*, *Serratia* spp., *Enterobacter* spp. *Acinetobacter* spp., *Pseudomonas*, coagulase negative *Staphylococcus* spp., *S. aureus*, *Streptococcus* group B, *Candida* and anaerobes. The incidence of these pathogens, which are involved in neonatal sepsis in developed countries, vary from those isolated from developed countries. Overall, Gram-negative bacteria such as *E. coli*, *Klebsiella*, *Salmonella* and *Pseudomonas* are more common causes of neonatal sepsis than Gram-positive bacteria (Moreno *et al.,* 1993; Tallur *et al.,* 2000; Stoll *et al.,* 2011). Whereas, within Gram-positive bacteria, coagulase negative *Staphylococcus* spp., *S. aureus*, *S. pyogenes* and *S. pneumoniae* are more commonly isolated from neonatal sepsis cases than other Gram-positive genera. In developing countries, *E. coli*, *Listeria*, *Enterococcus*, *Enterobacter* and Gram-positive *Staphylococcus* are mostly linked with early onset sepsis whilst *Klebsiella*, *S. aureus* and *Acinetobacter* are associated with both early and late onset sepsis (Hyde *et al.,* 2002; Scuchat *et al.,* 2000). In Australia, a retrospective study done by Sanghvi and co-authors (1996) in NICUs over a five year period revealed that coagulase negative *Staphylococcus* and group B *Streptococcus* were the most common causes of neonatal sepsis (38.8% and 20.1%, respectively).

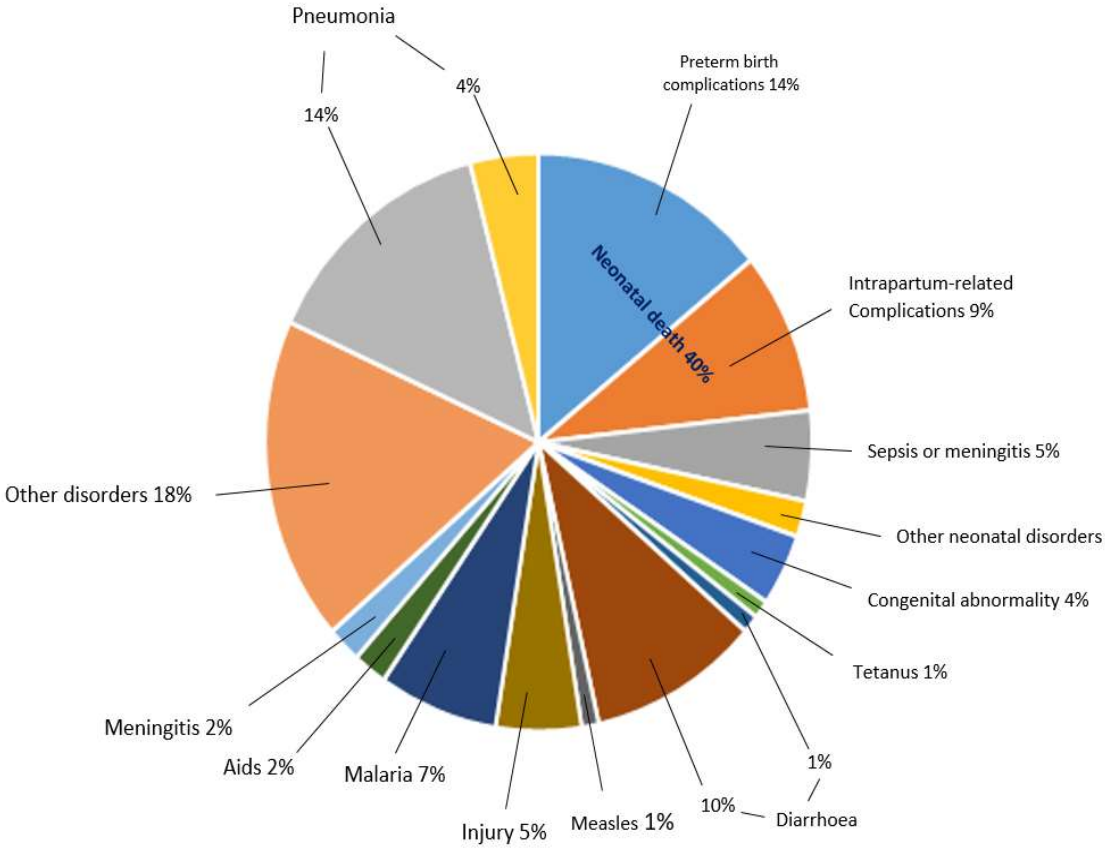


Figure 3-1: Global causes of childhood deaths in 2010 Liu *et al*., (2012)

The prevalence of neonatal infections due to *Enterobacteriaceae* has increased and these are seen as dominant causative agents in NICUs (McGuire *et al*., 2004; Kaufman *et al*., 2004; Gastmeier *et al*., 2007). *Enterobacteriaceae*, such as *Escherichia coli* and *Klebsiella* spp. are a large group of bacterial pathogens that inhabit humans, birds and other mammalian intestinal tracts. This family are Gram-negative bacilli, oxidase negative, some of which are motile and have capsules, facultatively anaerobic and catalase-positive (Hafsa *et al*., 2011). Studies by Gastmeier *et al*. (2007) and Stoll *et al*. (2005) reported that the most prevalent neonatal pathogens of the *Enterobacteriaceae* are *Klebsiella* spp. (23.4%) and *Serratia* spp. (13.8%). Another pathogenic species, which occurs less frequently, is *E. coli* group B2 and D, which are a leading cause of acute infections, such as sepsis and neonatal meningitis. Also, these two groups are recognised as a major cause of bacteraemia and are the second most common cause of meningitis during the neonatal period. Among this family, *K. pneumoniae* and *E. coli* are most commonly antimicrobial-resistant and producers of extended spectrum beta lactamase (ESBLs) (Romero *et al*., 2007). Globally, there is considerable concern due to

the high levels of resistance to antibiotics used in the treatment of infections caused by these bacteria.

In 2014, WHO declared that bacterial antimicrobial resistance has increased, becoming a globally problematic threat to health. Of particular concern, available neonatal data revealed wide spread antimicrobial resistance to gentamicin, ampicillin and third generation of cephalosporins therapy for both community and hospital-acquired neonatal sepsis (Downie *et al.*, 2013; Aiken *et al.*, 2011; Thaver *et al.*, 2009; Bates *et al.*, 2014;Zaidi *et al.*, 2005). The beta-lactam family of antibiotics are widely administered to treat bacterial infections due to the presence of a beta-lactam ring, which is responsible for the inactivation of the penicillin binding protein (PBP). Among these families, the carbapenems represent one of the most effective antibiotics, thus providing broad-spectrum activity due to the presence of a beta-lactam ring. This feature gives carbapenems high stability against most beta-lactamase producers. Therefore, carbapenem resistance constitutes a global health problem (Sanchez, 2015). In 2006-2007, National Healthcare Safety Network stated that carbapenem resistance was evident in 10.8% and up to 4.0% of *K. pneumoniae* and *E. coli* isolates, respectively, that were associated with medical device infections (Hidron *et al.,* 2008).

3.1.2. Impacts of Enterobacteriaceae

The majority of the *Enterobacteriaceae* are referred to as opportunistic pathogens, especially *Klebsiella* spp., *E. coli*, *Salmonella* spp., *Serratia* spp., and *Enterobacter* spp., and a significant majority of these strains are associated with morbidity and mortality (Adamson *et al*., 2012; Friedland *et al*., 2003). These organisms have the ability to cause several different types of infections such as urinary tract infections (UTIs), sepsis, meningitis and pneumonia in ICUs (Ibrahim *et al*., 2000; Kollef *et al*., 1999). The majority of these isolates, like *Klebsiella* spp., *E. coli*, *Citrobacter* spp., *Serratia* spp. and *Proteus* spp., are recognized as normal flora, possibly able to cause infections following an empiric antimicrobial regime (Stoll *et al*., 2005; Iversen *et al*., 2004a). Table 3-1 shows the relatedness of microbial pathogens and their risk factors with neonatal sepsis (Camacho-Gonzalez *et al*., 2013).

Table 3-1. Showing relatedness of microbial pathogens and their risk factors with neonatal sepsis.

Neonatal sepsis	Microbial pathogens	Risk factors
Early-onset	Group B *streptococcus*	Maternal Group B streptococcal colonization
	Escherichia coli	Chorioamnionitis
	Streptococcus viridans	Premature rupture of membranes
	Enterococcus	Prolonged rupture of membranes (> 18 h)
	Staphylococcus aureus	Preterm birth (< 37 weeks)
	Pseudomonas aeruginosa	Multiple gestation
	Other gram-negative bacilli	
Late-onset	Coagulase-negative *Staphylococcus*	Prematurity
	Staphylococcus aureus	Low birth weight
	Candida albicans	Prolonged indwelling catheter use
	Escherichia coli	Invasive procedures
	Klebsiella pneumoniae	Ventilator associated pneumonia
	Enterococcus	Prolonged antibiotics
	Pseudomonas aeruginosa	
	Group B *streptococcus*	

Camacho-Gonzalez *et al.,* (2013).

3.1.3. Clinical impacts of *E. coli* strains

Pathogenic *E. coli* isolates are able to cause human extra-intestinal or enteric infections. Generally, enteric infections are divided into six patho-types: Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC, including *Shigella* sp), Enteroaggregative *E. coli* (EAEC) and Diffusely Adherent *E. coli* (DAEC), based on the strain's pathogenicity characteristics involving clinical pathology, virulence trait and phylogenetic background (Kaper, Nataro & Mobley, 2004). Additionally, pathogenic *E. coli* is responsible for other infections outside the gastrointestinal tract, such as sepsis-causing *E. coli* (SEPEC), uropathogenic *E. coli* (UPEC), meningitis-associated *E. coli* (MNEC). Recently these *E. coli* K1 have been grouped as extra intestinal pathogenic (ExPEC) because they commonly cause neonatal meningitis and neonatal sepsis, UTIs and community-acquired bacteraemia. It considered a second most common causes neonatal

meningitis after *Streptococcus* group B. Additionally, they can cause other infections such as osteomyelitis, cellulitis, intra-abdominal infections and wound infections (Johnson & Russo, 2002ab; Russo & Johnson, 2000). In 2015, Alkeskas and co-workers isolated indistinguishable *E. coli* K1 (ST95) (neonatal meningitis *E. coli*) from multiple nasogastric feeding tubes.

3.1.4. Clinical impacts of *Klebsiella* species

In the genus *Klebsiella*, it is considered that *K. pneumoniae* pathogenic strains are responsible for about 75% to 86% of *Klebsiella* infections. Furthermore, the second predominant species is *K. oxytoca*, responsible for about 13% to 25% of *Klebsiella* infections (Broberg, Palacios & Miller, 2014). *Klebsiella* spp. are opportunistic pathogens responsible for both nosocomial and community-acquired infections, and are associated with several infections, including Friedlander's pneumonia, septicaemia, meningitis, intravenous, urinary tract infections (UTIs), gastrointestinal tract (GIT) infections, liver abscess, and soft tissue (Green *et al*., 2009; Dworkin *et al*., 2006a). Gastmeier *et al*. (2007) documented that NICUs bacterial colonisation such as *Klebsiella* spp., have a high potential risk of acquisition of antibiotic-resistance genes for more than three days and are linked with a prolonged stay in NICUs. Further, these bacteria are known as more tolerant to desiccation, having the ability to persist on the skin more than other members of the *Enterobacteriaceae*. Moreover, *Klebsiella* spp. can act as opportunistic pathogens by colonizing the GIT and nasopharynx, causing infection (Podschun & Ullmann, 1998).

3.1.5. Physiological and genotypic virulence tests;

The physiological studies in this thesis include determining biofilm formation by *Klebsiella* spp. and *E. coli* strains on powder infant formula (PIF), tryptone soya broth (TSB), presence of curli fimbriae, haemolysis, motility and the presence of extended spectrum beta lactamase (ESBL). Capsule production was determined by colony morphology on XLD and PIF agar. The susceptibilities of isolates to antimicrobial agents was determined by the breakpoint method on antibiotic supplemented Iso-Sensitest agar (ISA) according to the British Society for Antimicrobial Chemotherapy (BSAC Methods, 2015).

Genotyping, 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. The DNA fingerprinting via pulsed-field gel electrophoresis (PFGE), considered for many years to be a gold standard technique for molecular typing and epidemic surveillance of bacteria before the increased availability of next generation sequencing (CDC, 2004; Tenover *et al*., 1995; Bick *et al*., 2011). Additionally, among the most significant virulent factors, specific PCR probes such as capsules, especially K1 and K2, K5, kpsMT III and hypermucoviscosity are very important for virulence, increasing resistance avoidance of host serum complement factors to the host phagocytic cells (Lai *et al*., 2003; Wu *et al*., 2009). In addition, fimbrial genes (*fim* and *mrk* operons) facilitate adherence to the host tissues and surfaces, and some strains have siderophores (iron-acquisition systems), which are involved in systematic survival for these bacteria (Struve *et al*., 2009). According to the approach of Clermont (2000), it was stated that *E. coli* can be categorised into four main phylogenetic groups A, B1, B2 and D based on *chuA*, *yjaA*, and *TspE4.C2* genes. Phylogroups B2, and D are mainly associated with *E. coli* strains causing extraintestinal infections, including those which are able to cause urinary tract infections (UTIs), sepsis, and newborn meningitis, whilst phylogroups A and B1 are associated mainly with commensal strains. More recently, due to use of whole genome sequencing methods, it became clear that *E. coli* can be categorised into more than four phylogenetic groups, resulting in seven phylogenetic groups in total (A, B1, B2, C, D, E, and F) (David *et al*., 2008; Clermont *et al*., 2013). Of interest is the improved triplex PCR method which is very crucial to help discriminate between potentially pathogenic strains (B2 or D) and commensal strains (A and B1). Quadruplex PCR, however, can identify all seven of the *E. coli* phylogenetic groups (A, B1, B2, C, D, E, and F) based on *arpA*, *chuA*, *yjaA*, and *TspE4.C2* genes (David et al., 2008; Clermont et al., 2013). Alkeskas and co-authors (2015) studied thirty *E. coli* strains previously isolated from NEFTs in the NICUs of two different hospitals in Nottingham. During this study, neonatal meningitic pathovar *E. coli* K1 isolates were recovered from multiple neonatal feeding tubes.

3.1.6. Aim of the chapter

This project is a continuation of previous research and a part of the continued research at NTU, since the initial studies by Hurrell *et al*. (2009a and b), which involved the collection all the isolates from neonatal enteral feeding tubes (NEFTs) in the NICUs of two different hospitals in Nottingham. Furthermore, new strains were isolated from neonatal sepsis cases (blood culture) were conducted for phenotypic, genotypic analysis and strain history are available, and it was revealed that all these isolates carry more virulence factors and antibiotic resistance genes. I have therefore included these strains in my study as well. In order to understand more and identify new virulence traits contributing to causing disease to the babies. Therefore, the current study used thirty-six clinical isolates collected from NEFTs and from neonates with sepsis cases in intensive care units from two local hospitals; QMC and NCH (Nottingham). The rationale and significance of this research needs to be highlighted because indistinguishable *E. coli* K1 ST95 strains were isolated from multiple NEFTs collected by Hurrell *et al*., (2009). Of particular note, these are unique collections not studied before. Unfortunately, metadata (hospital infection) associated with some of the studied babies were not available because there was a long time between sample collection and analysis (all isolates were from sporadic cases at either QMC or NCH collected from 2007 and 2015).

These isolates were selected because they are important neonatal pathogens isolated from NEFTs and sepsis cases. There is a need to identify risk factors in bacterial pathogens that affect neonates. Additionally, there is also a need for better characterisation of these pathogens so that differential diagnosis is possible. It is not yet well understood how neonates acquire infections, therefore the analysis in this chapter is aimed at evaluation and characterisation of the potential risk factors of these isolates, in order to determine any unique virulence, source and relatedness of these strains by using PFGE as a DNA fingerprinting method, and MLST techniques. Further, phenotypic and genotypic traits such as capsule production, biofilm formation, acid tolerance at pH 3.5, virulence-related genes K1, K2, K5, S fimbriae, type 1 fimbriae, cell invasion and β-lactamase genes were investigated to confirm whether these isolates are more virulent than commensal bacteria. These traits may help the pathogens to induce sepsis in new babies or support them to persist in the

environment, consequently increasing the potential risk of exposure to the babies. The identification and evaluation of these virulence-linked traits could be important to combat the pathogens, thus reducing infections which are caused by pathogens, and saving precious babies' lives. Therefore, this study focused on the evaluation and characterisation of the isolates, not epidemiological outbreak determination. Thus, these findings would reveal the potential risk and the exposed level of these organisms to immune-compromised babies in NICUs. Both *E. coli* K1 and *K. pneumoniae* are important pathogens, however *K. pneumoniae* is less well studied than *E. coli* K1. So, in this chapter I characterised both pathogens and in the following chapter I will focus on a further set of *K. pneumoniae* isolates.

3.2. Materials and Methods:

The methodology of this chapter is described in detail in Chapter 2 above (Section 2). In the current study, thirty-six isolates of *E. coli* (n=14) and *Klebsiella* spp. (n=22) were used. Most of these isolates were originally obtained by Hurrell *et al.* (2009b) from NETFs and another recent isolates were recovered from neonates with sepsis cases from two local hospitals in Nottingham (NCH and QMC); Table 3-2. These strains were isolated from NEFT and neonates with sepsis in intensive care units and subjected for further analysis to investigate their potential virulence factors. In addition, these are interesting and very unique collections, as well as these are potentially high pathogenic due to possessing virulent traits that can cause a risk to the babies. Furthermore, 10 representative *E. coli* strains 1980, 1982, 1983, 1984, 1985, 2113, 2114, 2286, 2297 and 2300 were selected based on their PFGE, physiological and genotypic virulent traits and were subjected to whole genome sequencing analysis in order to investigate further molecular characterisations, such as genes associated with physiological and virulent traits. Please note, due to the complimentary teamwork of the research group, the whole genome sequencing and SNP analysis reproduced here was done by my colleague Pauline Ogrodzki (NTU), as part of a parallel Ph.D. study.

Table 3-2: Description of *E. coli* and *Klebsiella* spp. strains isolated from NEFTs and neonatal with sepsis from QMC and NCH.

NTU	Organism	Hospital	Source	Sex	DOB	D.O.I
605	*E. coli* K5	N.A	NEFT	N.A	N.A	N.A
1980	*E. coli* K1	QMC	NEFT	M	21.4. 12	16.5. 12
1981	*E. coli* K5	QMC	NEFT	M	19.7. 12	21.7. 12
1982	*E. coli* K1	QMC	NEFT	F	6.7. 12	30.7. 12
1983	*E. coli* K1	NCH	NEFT	F	29.10. 12	30.10. 12
1984	*E. coli* K5	QMC	NEFT	F	30.1. 13	5.2. 13
1985	*E. coli* K5	QMC	NEFT	F	1.4. 13	12.4. 13
1986	*E. coli* K5	QMC	NEFT	M	2.4. 13	21.5.13
2113	*E. coli* K1	NCH	NEFT	F	5.2. 14	27.3.14
2114	*E. coli* K1	NCH	NEFT	M	5.3. 14	1.4.14
2255	*E. coli* K5	QMC	NEFT	F	21.7. 14	29.7.14
2286	*E. coli*	QMC	Sepsis	N.A	N.A	30.07.15
2297	*E. coli*	QMC	Sepsis	N.A	N.A	18.11.15
2300	*E. coli*	QMC	Sepsis	N.A	N.A	06.01.16
498	*K. pneumoniae*	N.A	NEFT	N.A	N.A	N.A
500	*K. pneumoniae*	N.A	NEFT	N.A	N.A	N.A
1443	*K. pneumoniae*	NCH	NEFT	M	5.5. 10	28.7.10
1446	*K. pneumoniae*	NCH	NEFT	F	1.3. 11	13.3.11
453	*K. pneumoniae*	N.A	NEFT	N.A	N.A	N.A
497	*K. pneumoniae*	N.A	NEFT	N.A	N.A	N.A
502	*K. pneumoniae*	N.A	NEFT	N.A	N.A	N.A
1444	*K. pneumoniae*	QMC	NEFT	F	18.8. 10	28.8.10
673	*K. pneumoniae*	N.A	NEFT	N.A	N.A	N.A
2291	*K. pneumoniae*	QMC	Sepsis	N.A	N.A	01.10.15
2298	*K. pneumoniae*	QMC	Sepsis	N.A	N.A	01.10.15
2312	*K. pneumoniae*	QMC	Sepsis	N.A	N.A	07.04.16
599	*K. oxytoca*	N.A	NEFT	N.A	N.A	N.A
601	*K. oxytoca*	N.A	NEFT	N.A	N.A	N.A
672	*K. oxytoca*	N.A	NEFT	N.A	N.A	N.A
1445	*K. oxytoca*	QMC	NEFT	F	9.3. 11	20.4.11
1989	*K. oxytoca*	NCH	NEFT	M	30.12. 12	8.2.13
671	*K. oxytoca*	N.A	NEFT	N.A	N.A	N.A
674	*K. oxytoca*	N.A	NEFT	N.A	N.A	N.A
670	*K. oxytoca*	N.A	NEFT	N.A	N.A	N.A
600	*K. oxytoca*	N.A	NEFT	N.A	N.A	N.A
2310	*K. oxytoca*	QMC	Sepsis	N.A	N.A	24.03.16

QMC= Queen medical Hospital, NCH= Nottingham City Hospital, NEFT= Neonatal enteral feeding tube, D O B= Date of birth, D. O. I= Date of Isolation and N.A= Not available.

3.3. Results

3.3.1. PFGE analysis

BioNumerics software (version 3.5) was used in this study to detect a band assignment and a dendogram for the PFGE profiles from all isolates. By using *Xbal* restriction enzyme, *E. coli*

isolates gave 14 to 20 comparable DNA fragments per strain, and *Klebsiella* spp. isolates gave 10 to 17 DNA fragments; Figure 3-2 and Figure 3-3. The band similarity of the non-clonal strains was less than 95% (Tenover *et al*., 1995). In this study, the optimisation and tolerance of the bands was 1.5% for the isolates. *Salmonella* strain 732 (*Salmonella* strain H9812) was used as the reference strain (universal size standard) that covered a large range of DNA fragment sizes and gave distinct bands in the *XbaI* (Promega, UK) digested PFGE profile. In PFGE the strains are clustered in a clonal group based on the pattern of their restriction enzyme digestion and size of the fragments. The electronic image (TIFF format) was normalised by using reference strain (universal size standard) to establish reference positions (restriction fragments detectible in the pattern). The experiment used the standardised PFGE method as according to the CDC and PulseNet (2004) protocol with 2 sets of strains from QMC.

As shown in Figure 3-2 and Figure 3-3 [and the original gel images in Appendix A & B], respectively, the PFGE typing was undertaken for 14 *E. coli* strains and 22 *Klebsiella* spp. isolates from NEFTs and neonatal sepsis cases from NICU at Queen's Medical Centre (QMC) and Nottingham City Hospital (NCH) Nottingham. Figure 3-2 and Table 3-3 showed *E. coli* strains 2113 and 2114 clustered together, whereas the other strains were all unique. Isolates 2113 and 2114 had been isolated over a one-week period (27 March to 01 April 2014) from two different neonates in the same hospital. Figure 3-3 and Table 3-4 show *Klebsiella* spp. strains 498 and 500 clustered together, whereas the other strains were all unique. It should be noted that all strains were isolated from NEFTs and neonatal with sepsis. Unfortunately, no clinical history was available for these patients.

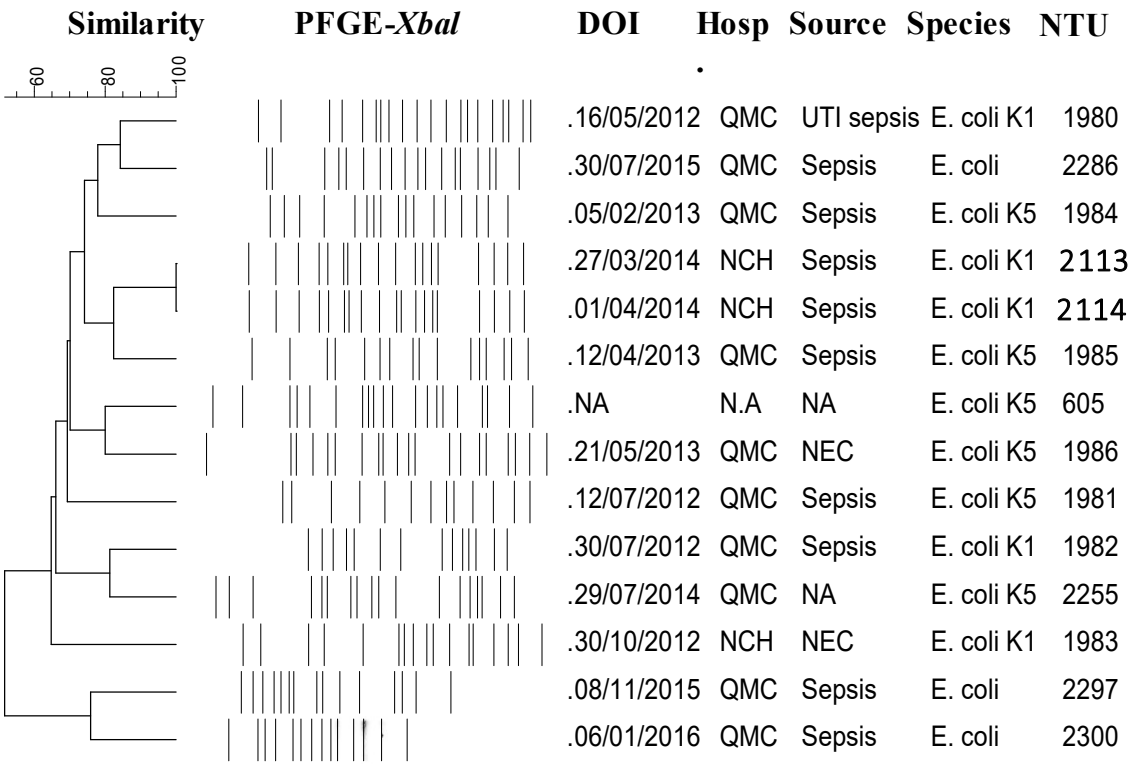


Figure 3-2: PFGE cluster analysis of *E. coli* strains isolated from NEFTs and neonates with sepsis cases from QMC and NCH Nottingham.

DOI= Date of isolation, Hosp. = Hospital (Queen's Medical Centre (QMC) or Nottingham City Hospital (NCH)), N.A= Not available. The dendrogram was generated by BioNumerics software, version 3.5, dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimisation in the band was1.50%.

Table 3-3: Summary of PFGE analysis of *E. coli* strains.

Species	Hospital	Strain number	PFGE Cluster	No. of Strains	Period of isolation	Patients	Genetic relatedness
E. coli	QMC & NCH	2113 and 2114	EcC1	2	Five days	Two	Closely related
		605,1980,1981, 1982, 1983, 1984, 1985, 1986, 2255, 2286, 2297 and 2300	U	12	Over five years	11	Unrelated

QMC= Queen's Medical Centre, NCH= Nottingham City Hospital, U= unique strains. This table showed the analysis of fourteen (NEFTs and sepsis) strains of *E. coli*, which were isolated previously from QMC and NCH [Appendix A]. All of these isolates were collected from 14 neonates; two strains (2113 and 2114) out of fourteen isolates were clustered into the same pulsetype and revealed a 100% similarity coefficient. Whereas, the remaining strains were all unique clonally non related. These strains were isolated during a period of three years (14thMay2012 and 30th July 2015).

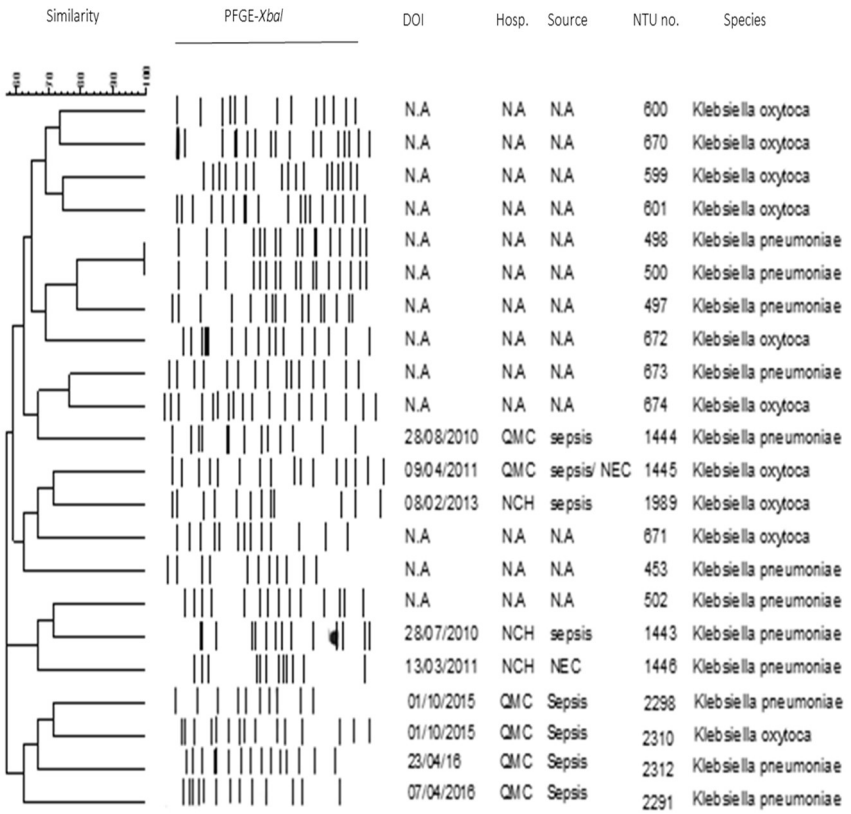


Figure 3-3 PFGE cluster analysis of *Klebsiella* spp. strains isolated from NEFTs and neonates with sepsis cases from QMC and NCH Nottingham.

DOI= Date of isolation, Hosp. = Hospital, N.A= Not available. The dendrogram was generated by BioNumerics software, version 3.5, dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimisation in the band was 1.50%.

Table 3-4: Summary of PFGE analysis of *Klebsiella* spp., strains.

Species	Hospital	Strain number	PFGE Cluster	No. of Strains	Genetic relatedness
K. pneumoniae	QMC & NCH	498 and 500	KpC1	2	Closely related
		453, 497, 502, 1444, 1443,1446, 673, 2291, 2298, 2312	U	10	Unrelated
K. oxytoca	QMC & NCH	599, 600, 601, 670, 671, 672, 674, 1445, 1989 and 2310	U	10	Unrelated

QMC= Queen's Medical Centre, NCH= Nottingham City Hospital, U= unique cluster. This table revealed the analysis of twenty-two (NEFTs and sepsis) strains of *Klebsiella* spp which were isolated previously from QMC and NCH [Appendix B]. All of these isolates were collected from 13 neonates, two strains (498 and 500) were clustered into the same pulsetype and shown a 100% similarity coefficient. Whereas, the remaining strains were all unique (clonally unrelated), were isolated during a period of three years (14thMay2012 and 30th July 2015).

3.3.2. Bacterial genomic analysis of *E. coli* strains;

Isolates subjected to whole genome sequencing differed from each other by 29–47827 SNPs; Figure 3-4 and Figure 3-5. Two *E. coli* isolates (2113 & 2114) which had been isolated from two different babies had 29 SNPs difference. It was notable that the two *E. coli* strains (2113 & 2114) with the lowest SNPs difference (29) were from two different babies within the same hospital. These two strains had been obtained 5 days apart.

3.3.3. *E. coli* phylogenetic groups

Clermont *et al*. (2000) have established a simple multiplex (triplex) PCR technique which has allowed categorisation of the phylogenetic structure of *E. coli* into four main groups: A, B1, B2 and D. Groups B2 and D are associated with extra-intestinal infections, whereas groups A and B1 are associated with commensal strains (Picard *et al*., 1999). This experiment was based on PCR detection of the *chuA* and *yjaA* genes and DNA fragment *TSPE4.C2*, *chuA* is known to be involved with heme transport while *yjaA* is involved in cellular response to hydrogen peroxide and acid stress. The function of TspE4.C2 is not discovered yet (Carlos *et al*., 2010). Thirteen *E. coli* strains from QMC & NCH belong to group B2, and strain 2255 was group D as shown in Table 3-5.

Table 3-5: PCR amplification of the *chuA* and *yjaA* genes and DNA fragment *TSPE4.C2* in *E. coli* isolates from NEFTs and neonatal sepsis and allocation of strains to phylogenetic groups.

NTU	chuA	yjaA	TSPE4.C2	Phylogenetic
605	+	+	-	group B2
1980	+	+	-	
1981	+	+	-	
1982	+	+	-	
1983	+	+	-	
1984	+	+	-	
1985	+	+	-	
1986	+	+	-	
2113	+	+	-	
2114	+	+	-	
2286	+	+	-	
2297	+	+	-	
2300	+	+	-	
2255	+	-	-	group D
939 (C+)	+	+	-	group B2
1230 (C-)	-	-	-	group B1

C+= control positive, C-= control negative

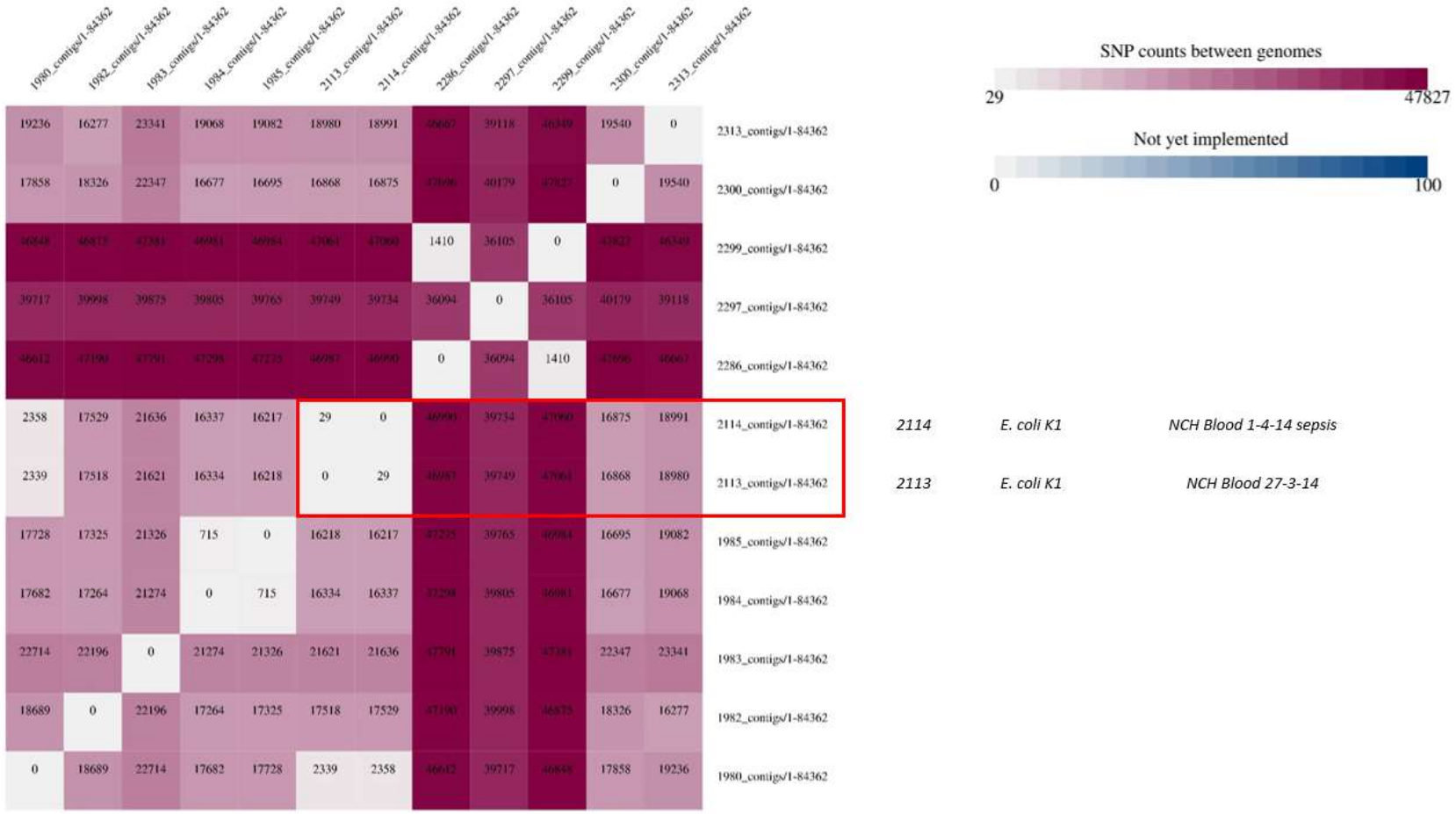
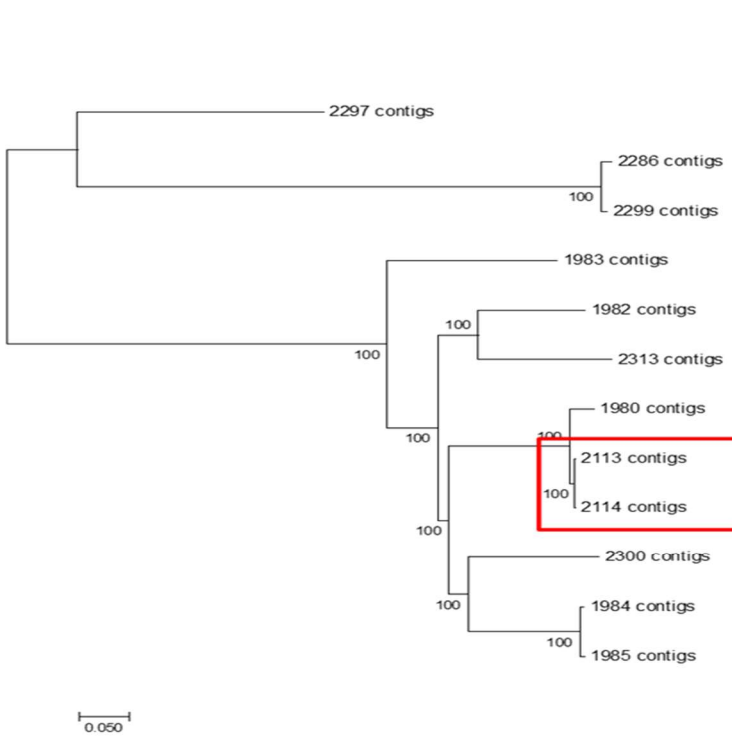


Figure 3-4: Numbers of single nucleotide polymorphisms (SNPs) detected between the selected *E. coli* strains.

The heat map shows the numbers of SNPs difference between each genome in the alignment. SNPs were called using the program CSI Phylogeny version 1.4 and the heat map was generated by Pauline Ogrodzki (NTU), as part of a parallel PhD study.



2297	*E. coli*	*QMC NNU 18-11-15 Blood*
2286	*E. coli*	*NNUN 30-7-15 Preterm/sepsis blood*
2299	*E. coli*	*Shoulder tissue 6-12-15 QMC osteomyelitis/musculoskeletal*
1983	*E. coli K1*	*NCH 30-10-12 sepsis term bloody stools rotavirus +*
1982	*E. coli K1*	*QMC 30-7-12 Blood sepsis severe hypotension deceased*
2313	*E. coli*	*NNUN Blood 4-5-16 mum-chorioamnionitis*
1980	*E. coli K1*	*QMC Blood 16-5-12 sepsis term*
2113	*E. coli K1*	*NCH Blood 27-3-14*
2114	*E. coli K1*	*NCH Blood 1-4-14 sepsis*
2300	*E. coli*	*QMC 24-12-15 Peritoneal bowel perf/poss NEC Post-op*
1984	*E. coli K5*	*QMC Blood 5-2-13 Intra-abdominal*
1985	*E. coli K5*	*QMC Blood 12-4-13 Intra-abdominal Peritonitis*

Figure 3-5: Core genome phylogenetic tree of 10 selected *E. coli* strains.

The SNP tree was generated by Pauline Ogrodzki (NTU), as part of a parallel PhD study. SNPs were called using the program CSI Phylogeny version 1.4.

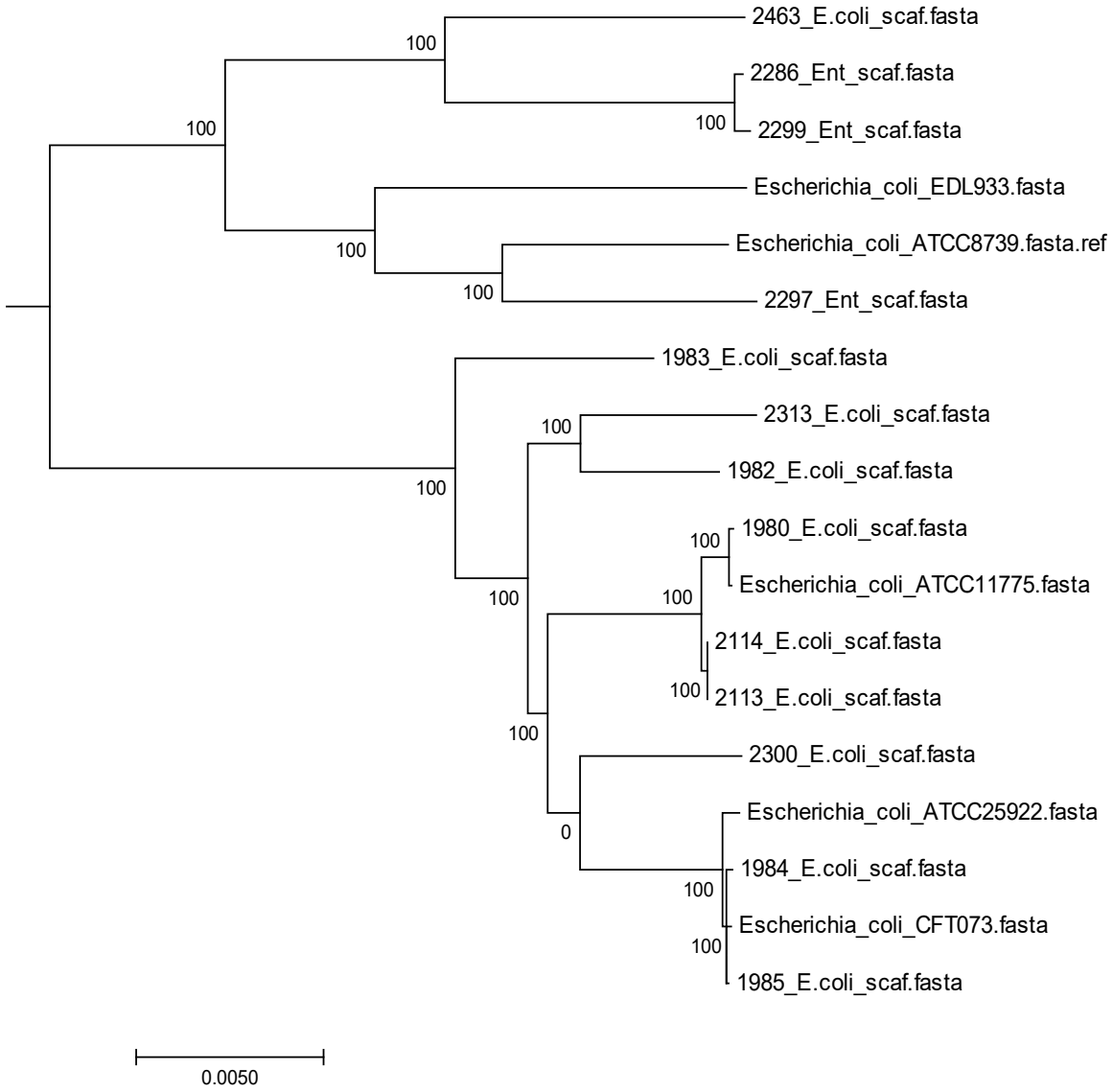


Figure 3-6: Comparative core genome alignment of 10 selected *E. coli* strains versus *E. coli* CFT073, *E. coli* ATCC8739, *E. coli* ATCC11775, *E. coli* ATCC25922 and *E. coli* EDL 933.

The tree was generated by Pauline Ogrodzki (NTU), as part of a parallel PhD study. SNPs were called using the program CSI Phylogeny version 1.4.

3.3.4. Phylogenetic analysis of *Klebsiella* spp

The *rpoB* gene has emerged as a key gene for identification of bacteria and phylogenetic analyses, particularly when analysing closely related strains. The *rpoB* has allowed scientists to refine bacterial community studying and define new bacterial species. Rowland *et al*. (1993) reported that the *rpoB* gene has the possibility to be used as a powerful molecular chronometer. Further, sequence analysis of the different *rpoB* for twenty-two *Klebsiella* (QMC and NCH) isolates are shown in Figure 3-7. *Klebsiella* spp. isolated from QMC and NCH revealed eight different *rpoB* alleles profiles; 1, 2, 4, 10, 13, 14, 15, 40. The PCR product (637 bp length) was sequenced and aligned with additional sequences from the *Klebsiella* locus/sequence definitions Pasteur MLST database.

(http://bigsdb.web.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef_public&page=batchSequenceQuery). According to PFGE, all of them were unique except two strains (498, 500), which were from the same pulse type (clonally related).

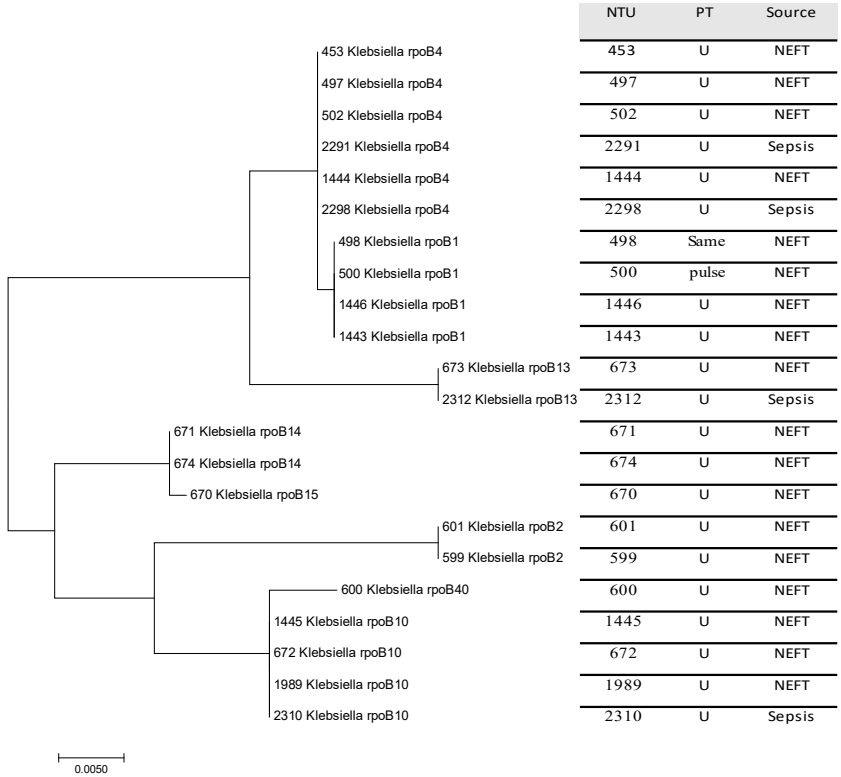


Figure 3-7 shows phylogenetic tree of rpoB gene (501 bp) of *Klebsiella* spp. isolates.

The tree was generated using MEGA7. Eight *rpo*B allele profiles were revealed *rpo*B1, *rpo*B4 and *rpo*B13 for *K. pneumoniae*, *rpo*B2, *rpo*B10, *rpo*B14, *rpo*B15 and *rpo*B40 for *K. oxytoca.* PT= pulse type.

3.3.5. Detection of virulence factor genes (VFGs) for *E. coli* strains

Table 3-6 shows the characterisation of fourteen whole genome sequenced *E. coli* strains isolated from QMC and NCH , for the presence or absence of 30 virulence genes, including genes encoding for toxins, invasions, adhesins, siderophores, capsule and others. All isolates encoded type1 fimbriae *fimH* adhesion gene, and strains 605, 1980, 1981, 1985, 1986 and 2255, encoded the *papACEF* adhesion gene, whereas *sfaS* adhesion gene was found only in strains 2113 and 2114 which were from two different babies and were the same pulsotype, and the *papG* adhesion gene was present only in strains 605 and 1986. However, *bmaE*, *Afa/draBC*, *gafD*, *nfaE* and *papG*1&2 adhesion genes were not found in all strains. The *ibeA* invasin gene was only found in the *E. coli* K1 strains, whereas *hlyA* and *cnf1* toxin genes were found in most of *E. coli* K5 isolates. *FyuA*, *iutA* and *PAI* siderophore genes were found in the majority of the isolates. Serum resistance associated gene *traT* was present in all strains except strains 605 and 1982, *cvaC* gene was found only in *E. coli K1* strains 1980, 2113 and 2114, whereas *rfc* gene was detected only in strain 1986.

Table 3-6. Distribution of potential virulence genes among of *E. coli* isolates selected from NEFTs and neonatal with sepsis cases from two local hospitals, Nottingham

E. coli strains	Pulsetype	Phylogenetic	Pili (Fimbriae)																Invasion	Toxins			Capsule				Siderophores					Others		
			Afa/ draC	bmaE	focG	fimH	gafD	papEF	papA	papC	nfaE	Sfa/ focDE	papG allele II	papG1	papG II,III	papG allele	sfaS	papG allelel	ibeA	hlyA	cnf+	cdtB	K1	K5	kpsMT II	kpsMT III	fyuA	iutA	PAI	Irp1	Irp2	rfc	cvaC	traT
1982	U	B2	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	-	+	-	+	-	+	-	-	-
1983	≠	≠	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	-	+	-	+	-	+	-	-	+
2113	EcC 1	≠	-	-	-	+	-	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-	+	-	+	-	+	+	+	-	+	-	+	+
2114	≠	≠	-	-	-	+	-	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-	+	-	+	-	+	+	+	-	+	-	+	+
1980	U	≠	-	-	-	+	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+	+	-	+	-	+	+
1986	≠	≠	-	-	-	+	-	+	+	+	-	+	-	-	-	-	-	+	-	+	+	-	-	+	+	-	+	-	+	-	+	+	-	+
605	≠	≠	-	-	+	+	-	+	+	+	-	+	+	-	+	-	-	+	-	+	-	-	-	+	+	-	+	+	+	-	+	-	-	-
1981	≠	≠	-	-	-	+	-	+	+	+	-	-	+	-	+	-	-	-	-	+	-	-	-	+	+	-	+	+	+	-	+	-	-	+
1984	≠	≠	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	+	+	-	+	-	+	-	+	-	-	+
1985	≠	≠	-	-	+	+	-	+	+	+	-	+	+	-	+	-	-	-	-	+	+	-	-	+	+	-	+	+	+	-	+	-	-	+
2300	≠	≠	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	+	-	+	-	-	+
2297	≠	≠	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	+
2255	≠	D	-	-	-	+	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-	-	+	-	-	+
2286	≠	B1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	+	-	-	+

U = unique, EcC = cluster group of Pulsed Field Gel Electrophoresis and ≠ = same. All virulence genes for presented *E. coli* were detected by using PCR primers.

Table 3-7 Genome analysis of representative *E. coli* isolates within distinguishable isolates from NEFTs and sepsis cases at two local hospitals over six years period.

Isolate number	Species	Isolation information (source, date, other)	Sequence type	Serotype	Virulence	Plasmids	Aminoglycoside	Beta-lactam	Macrolide	Sulphonamide	Tetracycline	Trimethoprim	*kps*	related *kps* genes
1980	*E. coli* K1	N. sepsis(QMC/NCH)	ST95	O45:H7	iss-gad-mchF-iroN-vat-iss-ireA	IncFII, IncB/O/K/Z, IncFIB	strA,strB	blaTEM-1C		sul2			M:T-U:D:orf:F	epsM:legI:neuA:neuC-orf:orf
1982	*E. coli* K1	N. sepsis(QMC/NCH)	Unk ST	O83:H6	gad-celb-vat	ColpVC							F:orf:D:U-T:M	epsJ:-epsM:epsL
1983	*E. coli* K1	N. sepsis(QMC/NCH)	ST538	O13:H4	gad-iss-iroN-vat-iss	IncI1, IncFIB,IncFIC							F:orf:D:U-T:M	neuC:neuA:legI:epsM1-epsM2:epsL
1984	*E. coli* K5	N. sepsis(QMC/NCH)	ST73	O22:H1	pic-gad-iss-senB-cnf1-vat	IncFIB,IncFII, Col156	aadA1	blaSHV-1		sul1,sul2			T:M-F:orf:D	epsM:epsL
1985	*E. coli* K5	N. sepsis(QMC/NCH)	ST73	O6:H1	cnf1-mchB-iha-sat-mchC-iroN-vat-pic-ireA-mchF-iss-gad-mcmA	Col, ColVC, IncFIB,IncFII	strA,strB,aadA5	blaTEM-1B	mph(A)	sul1,sul2	tet(A)	dfrA17	F:orf:D:U-T:M	epsM:epsL
2113	*E. coli* K1	N. sepsis(QMC/NCH)	ST95	O18:H7	sfaS-gad-iroN-iss-iss-mchF	IncFIA, IncFIB, IncX1							F:orf:D:U-T:M	epsM_2:legI:neuA:neuC
2114	*E. coli* K1	N. sepsis(QMC/NCH)	ST95	O18:H7	sfaS-gad-iroN-iss-iss-mchF	IncFIA, IncFIB, ColpVC, IncX1							F:orf:D:U-T:M	epsM_2:legI:
2286	*E. coli*	Shiu NNUN 30-7-15 Preterm/sepsis blood	ST69	no O:H2	eilA-gad-iss-air-gad-sat-iha-ipfA-senB	Col156, IncQ1, IncFII, IncFIB	strA,strB,aadA5	blaTEM-1B	mph(A)	sul1,sul2	dfrA17		M:orf:orf:T-U:D	epsM:epsL
2297	*E. coli*	Shiu QMC NNU 18-11-15 Blood sepsis/poss meningitis	ST120	O9:H9	iss-gad-capU-ipfA	IncQ, IncFIB,IncFII	strA,strB	blaTEM-1B	ere(A)	sul2	tet(B)	dfrA5	D	-
2300	*E. coli*	bowel perforation NEC? Post-operative	ST452	O81:H27	celb-iha-capU-ireA-iss-sigA	ColRNAI, IncB/O/K/Z							T:M-U:D:orf:F	epsM:epsL

Based on PFGE analysis, neonatal meningitic pathovar *E. coli* K1 isolates, distribution of virulent traits among these strains and associated neonatal metadata, 10 *E. coli* isolates were selected for further characterization of various physiological and genomic virulence traits. As given in the Table 3-7 above, genomic analysis of the *E. coli* isolates against the virulence factor database of bacteria (VFDB), showed that three isolates (1980, 2113 and 2114) belonged to ST95 serogroup (O45:H7, O18:H7 and O18:H7) respectively, two isolates (1984, 1985) to ST73 serogroup (O22:H1 & O6:H1) respectively, strains 1983, 2297 and 2300 to ST538 serotype O13:H4, ST120 serotype O9:H9 and ST452 serotype O81:H27 respectively, while strain 2286 to ST69 serotype O unknown:H2 and strain 1982 to ST unknown serogroup O83:H6. The representative isolates 1980, 1982, 2113 and 2114 were 045:H7:K1, 083:H7:K1 and O18:H7:K1 Table 3-7. The genomic analysis showed the majority of *E. coli* strains encoded genes for colanic acid and curli fimbriae, which are associated with biofilm formation and extracellular matrix production. In addition, *E. coli* strains 1980 (ST95), 1984 and 1985 (ST73), 2286 (ST69), and 2297 (ST120) had an additional β–lactamase gene compared with the other strains 1982, 1983, 2113, 2114 and 2300 (ST unknown, ST538, ST95, ST95, and ST452 respectively). This revealed the range of antibiotic resistance encoding genes among *E. coli* strains isolated from NEFTs and neonates with sepsis cases. Furthermore, *E. coli* strains 1985 (ST73) and 2286 groups. (ST69) had three streptomycin resistance associated genes (*strA*, *strB* and *aadA5*) in the aminoglycoside resistance class and two sulphonamide class resistance associated genes (*sul1* and *sul2*). In addition, the genomic analysis showed the strains 1985, 2286 and 2297 had encoded genes for macrolide and tetracycline resistance (*mphA* & *ereA*) and (*tetA*, *dfrA*17 and *tetB*) respectively. Beside, trimethoprim resistance genes (*dfrA*17 and *dfrA*5) were detected in strains 1985 and 2297 respectively.

3.3.6. Detection of virulence factors genes (VFGs) for *Klebsiella* spp. strains

The fimbrial adhesins, capsular serotype, lipopolysaccharide and iron acquisitions systems are very important traits involved in the virulence of *K. pneumoniae* isolates (Williams *et al.,* 1990; Fang *et al.,* 2005). The plentiful polysaccharidic capsule is very important because it protects *Klebsiella* spp. against environmental stresses, serum bactericidal action and

impairs phagocytes ability to engulf the bacteria, and may be considered as the most important virulence trait playing a pivotal role in pathogenesis. Capsular serotypes K1, K2, K4 and K5 have been described as the most virulent among the 77 capsular (K) serotyping scheme. These are associated with severe infections in animals and humans and are often used in experimental infection in mice (Ørskov 1980; Simoons-Smit *et al.,* 1984; Ofek *et al.,* 1993).

The ability of bacteria to chelate and remove iron (Fe) from the host is very crucial because they need it for their variety of metabolic processes and replications. Thus, iron is a key factor or might be a necessary feature for bacterial pathogenesis. Furthermore, *Klebsiella* similar to other *Enterobacteriaceae* secretes siderophores (enterobactin, aerobactin, yersiniabactin, and salmochelin) that acquire ferrous iron (*Fe II*) and ferric iron (*Fe III*) from host binding proteins and then re-enter the bacterial cell via specific-siderophores receptors (Garenaux *et al.,* 2011). A total of 22 *Klebsiella* spp. strains were collected from NEFTs and neonates with sepsis cases from two local hospitals. Twelve of twenty two strains (54.5%) were isolated and identified as *Klebsiella pneumoniae* phenotypically, and by their physiological and biochemical characteristics, and the results confirmed by *rpoB* gene sequencing. *K. pneumoniae* isolates were subjected to K1, K2, and K5 serotyping, only one strain 1444 (8.3%) of 22 strains among these strains was identified as K2 serotype and three 453, 497 and 502 (25%) of twenty two strains were K5, whereas K1 was not detected. Furthermore, as shown in

Table 3-8 the ability of subjected isolates to produce siderophore gene *fyuA* was detected in 19 (86.4%) of 22 strains except strains 673, 1446 and 1989, while gene *Irp2* was present in 17 (77.3%) of 22 isolates except *Klebsiella* strains 497, 673, 1443, 1444 and 1446.

It has been shown that two major adhesive fimbriae structures in *Klebsiella*, type 1 pili (T1P) and type 3 pili (T3P), are mainly responsible for adherence to the host cells. T1P, composed of *FimA* the major fimbrial subunit and *FimH* a minor fimbrial subunit, and T3P composed of *MrkA* was the major fimbrial subunit and *MrkD* the minor fimbrial subunit. Both T1P and T3P play an important colonisation factor in biofilm associated urinary tract infections due to indwelling urinary catheter.

As shown in Table 3-8, 100% of strains carried the *mrkA* and *mrkD* adhesion genes. While 100% of isolates carried *fimA*, the remaining 81% carried *fimH.* The fimbrial adhesins, capsular serotype, lipopolysaccharide and iron acquisitions systems these are very important traits involved in the virulence of *K. pneumoniae* isolates (Williams *et al.,* 1990; Fang *et al* 2005). The plentiful polysaccharidic capsule is very important because it protects *Klebsiella* spp. against environmental stresses, serum bactericidal action and impairs phagocytes ability to engulf the bacteria, and may be considered as the most important virulence trait playing a pivotal role in pathogenesis. Capsular serotypes K1, K2, K4 and K5 have been described as the most virulent among the 77 capsular (K) serotyping scheme. These are associated with severe infections in animals and humans and are often used in experimental infection in mice (Ørskov 1980; Simoons-Smit *et al.,* 1984; Ofek *et al.,* 1993).

Table 3-8: Virulence distribution of *Klebsiella* spp. and capsular serotype prevalence of *K. pneumoniae* isolated from NEFT and neonates with sepsis from two hospitals Nottingham

NTU	Species	*rpo* B allele	Pulse type	Capsule serotype			Siderophores		Fimbriae			
				K1	K2	K5	*Irp2*	*fyuA*	*mrkA*	*mrkD*	*FimA*	*FimH*
498	*K. pneumoniae*	*rpoB* 1	Same pulse	-	-	-	+	+	+	+	+	+
500	*K. pneumoniae*			-	-	-	+	+	+	+	+	+
1443	*K. pneumoniae*		U	-	-	-	-	+	+	+	+	+
1446	*K. pneumoniae*			-	-	-	-	-	+	+	+	+
453	*K. pneumoniae*	*rpo* B4		-	-	+	+	+	+	+	+	+
497	*K. pneumoniae*			-	-	+	-	+	+	+	+	+
502	*K. pneumoniae*			-	-	+	+	+	+	+	+	+
1444	*K. pneumoniae*			-	+	-	-	+	+	+	+	+
2291	*K. pneumoniae*			-	-	-	+	+	+	+	+	+
2298	*K. pneumoniae*			-	-	-	+	+	+	+	+	+
673	*K. pneumoniae*	*rpo* B13		-	-	-	-	-	+	+	+	+
2312	*K. pneumoniae*			-	-	-	+	+	+	+	+	+
599	*K. oxytoca*	*rpo* B2					+	+	+	+	+	+
601	*K. oxytoca*						+	+	+	+	+	+
672	*K. oxytoca*	*rpo* B10					+	+	+	+	+	+
1445	*K. oxytoca*						+	+	+	+	+	-
1989	*K. oxytoca*						+	-	+	+	+	-
2310	*K. oxytoca*						+	+	+	+	+	+
671	*K. oxytoca*	*rpo* B14					+	+	+	+	+	-
674	*K. oxytoca*						+	+	+	+	+	+
670	*K. oxytoca*	*rpo* B15					+	+	+	+	+	-
600	*K. oxytoca*	*rpo* B40					+	+	+	+	+	+
0	*Y. enterocolitica*						+	+	x	x	x	x

3.3.7. Physiological Virulence Traits:

3.3.7.1. Biofilm formation:

Relative biofilm formation was determined by dividing the isolate value by the equivalent control value. Then, the results were categorised as; ≤ 0.3x OD = Control, > 0.3 ≤1.3x OD = Low biofilm formation, >1.3 ≤2.3x OD= Moderate biofilm formation and >2.3x OD = High biofilm formation:

Concerning *E. coli*, this experiment was conducted to evaluate the level of biofilm formation of fourteen *E. coli* strains (11 isolates from NEFT and 3 strains from neonates with sepsis cases) on plastic surfaces according to two incubation temperatures (25°C and 37°C), in powdered infant formula (PIF) and trypton soya broth (TSB). Figure 3-8 reveals the ability of *E. coli* strains to form biofilm on plastic surfaces at both temperatures 25 ^{o}C and 37 ^{o}C compared with the control, there was clear variation between strains. On TSB, some *E. coli* strains such as 1982, 1984, 1985, 2113 and 2114 were able to form more biofilm at 25^{o}C, nevertheless, all of the *E. coli* strains formed biofilm better at 37 °C than at 25 °C. Strains 605, 1980, 1982, 1983 and 1984 were able to form high biofilm at both temperatures.

On the other hand, when the PIF was used for biofilm formation instead of TSB the amount of biofilm formation considerably increased particularly at 37 ^{o}C. Figure 3-9 showed the optimal temperature for *E. coli* strains to form high biofilm was the same when TSB was used. One-way ANOVA test was used to compare the effect of temperatures. This revealed significantly higher biofilm formation at 37°C ($p < 0.0001$) compared to 25°C when TSB was used Figure 3-10. Additionally, the biofilm formation in PIF at 37^{o}C was higher ($p < 0.0001$) in comparison with 25°C; Figure 3-10. Most *E. coli* isolates revealed their ability to form biofilm at 37°C in PIF compared with the control. All *E. coli* isolates were able to form a high biofilm at 37°C in PIF except strains 605, 2286, 2297 and 2300 which formed moderate biofilm compared with the control, and strains 1981, 1984, 2113, 2114 and 2255 had the highest biofilm formation above 3.5 absorbance units (AU) Figure 3-9. Overall, all strains showed the lowest amount of biofilm formation in TSB at both temperatures (25°C and 37°C) in comparison with PIF. Strains 1982 and 1984 showed their ability to form more

biofilm than other strains in both PIF and TSB at 37°C. All *E. coli* strains formed significantly more biofilm in PIF than in TSB media ($p < 0.05$). The maximum absorbance in PIF was up to 3.5 AU, whereas in TSB was up to 0.4 AU.

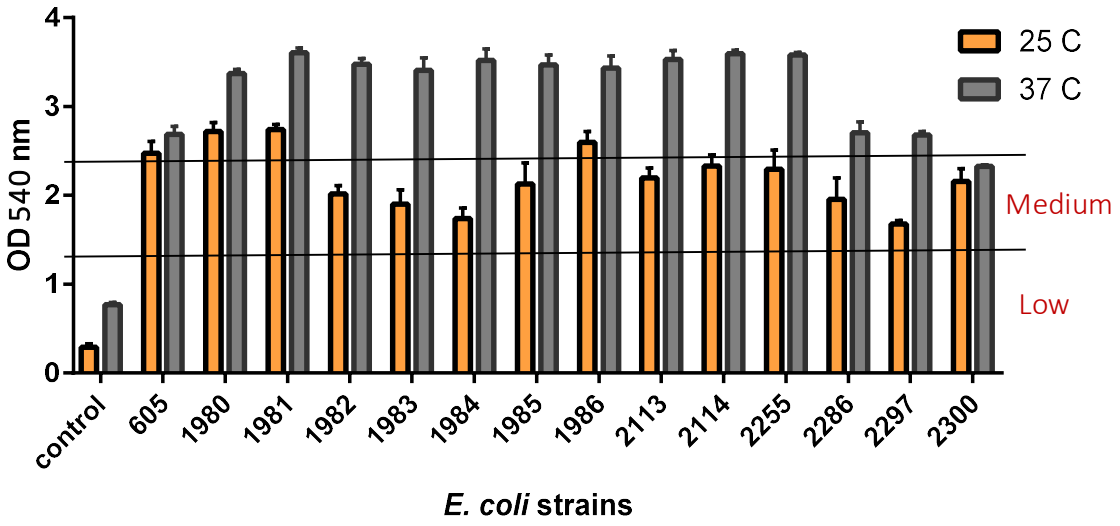


Figure 3-8 Biofilm Formation of *E. coli* isolates at 25 °C and 37 °C in TSB. Shows the preferred temperature is 37 °C

All strains produced biofilm, compared to the control. Isolates 1980, 1982, 1983, 1984, 1985, 2113 and 2114 formed more biofilm than other isolates. Biofilm formation stained with crystal violet 0.01% (w/v) and evaluated at wavelength 540nm; the control was non-inoculated media, the experiments were undertakes in three independent times. Error bars represented using standard error.

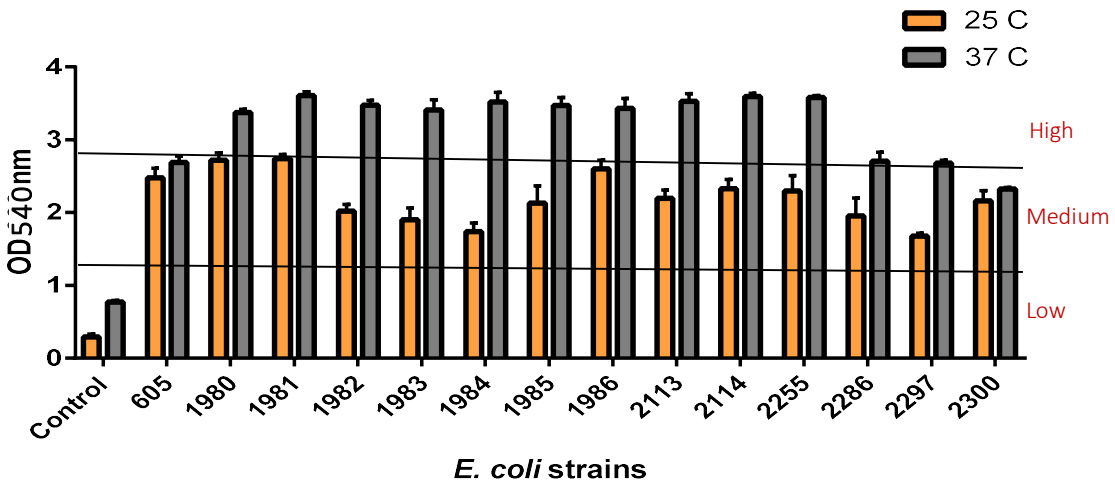


Figure 3-9: Biofilm formation of *E. coli* isolates at 25 °C and 37 °C in PIF agar plates. Shows the preferred temperature is 37 °C,

Biofilm formation stained with crystal violet 0.01% (w/v) and evaluated at wavelength 540nm; the control was non-inoculated media, the experiments were undertaken in three independent times. Error bars represented using standard error.

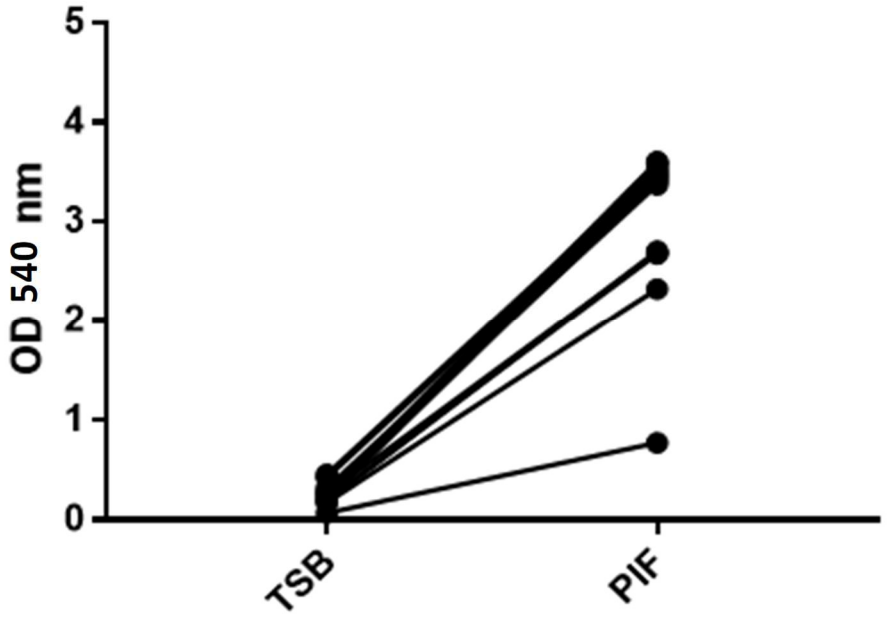


Figure 3-10: T-test revealed *E. coli* isolates form high significantly biofilm in PIF than in TSB at 37°C

Paired t-test shows *E. coli* strains form significantly more biofilm in PIF than in TSB ($P < 0.0001$).

With regards to *Klebsiella* strains, Figure 3-11 shows the ability of *Klebsiella* spp. strains to form biofilm on TSB compared with the control, and there was clear variation between

strains. In general, the highest biofilm former in TSB at both temperatures (25°C & 37°C) was strain 1444, and the lowest was strain 453. Strains 599 and 1444 were able to form a moderate amount of biofilm on TSB and PIF at both temperatures (25°C & 37°C) compared to the control, whereas the lowest amount of biofilm formation was shown by the other *Klebsiella* isolates. At 25°C, there were only four strains (453, 497, 673 and 502) which produce a weak amount of biofilm that was close to the control value. In contrast, these isolates formed more biofilm when growing in PIF Figure 3-12 .

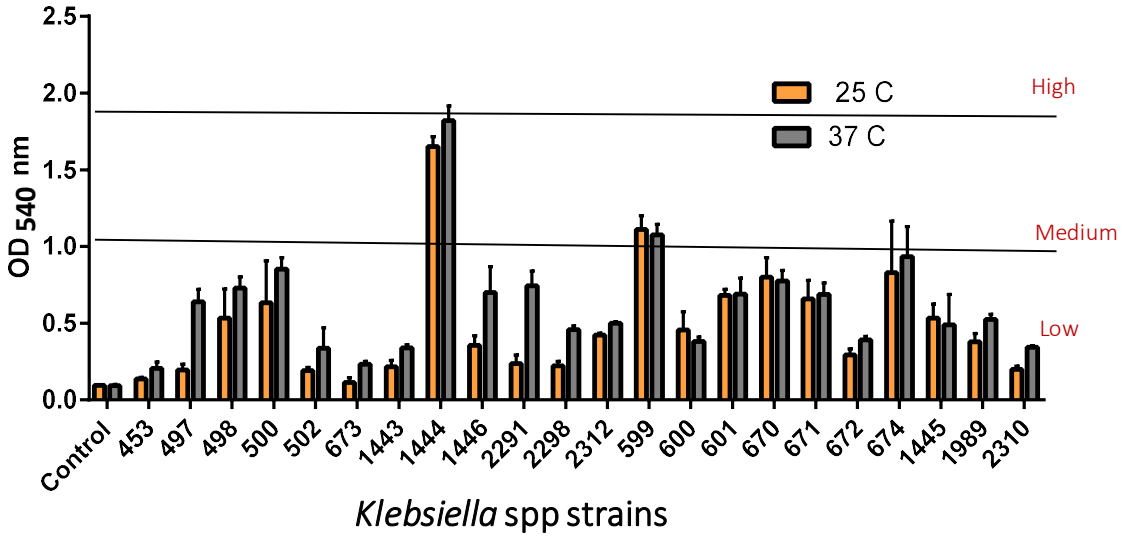


Figure 3-11: Biofilm formation of *Klebsiella* spp. isolates at 25°C and 37°C in TSB. Showing the optimal temperature is at 37°C.

K. pneumoniae strain 1444 was able to form more than other isolates. Biofilm formation was stained with crystal violet 0.01% (w/v) and evaluated at wavelength 540nm; the control was non-inoculated media, the experiments were investigated in three independent times. Error bars represented using standard error.

The majority of *Klebsiella* spp. strains formed biofilm in PIF at 37 C Figure 3-12. Generally, the highest values of biofilm formation in PIF at 25°C presented by strain 497 the absorbance unit was 1.4, and the lowest value was by strain 2298 the AU was 0.26833 compared to the control. Whereas the highest value of biofilm formation in PIF at 37°C was by strain 502, the AU was 2.5 and strain 1444 presented lowest biofilm value with above 1.5 AU compared to control. This figure showed that strains 453, 497, 599, 600, 601, 670, 673, 1445, 1446, 1989 and 2291 had the ability to form moderate biofilm in PIF at 25°C,

while the other isolates were able to form a low amount of biofilm compared to the control. In contrast to this, at 37°C strains 453, 498, 500, 502, 600, 670, 671, 673, 1443, 1445, 2291 and 2312 were able to form a high amount of biofilm in PIF compared to the control. While, the other strains were able to form a moderate amount of biofilm compared to the control. In addition, by One-way ANOVA test there was an obvious high significant effect of the PIF and the temperatures on biofilm formation by *Klebsiella* isolates compared to TSB media ($p < 0.0001$); Figure 3-13.

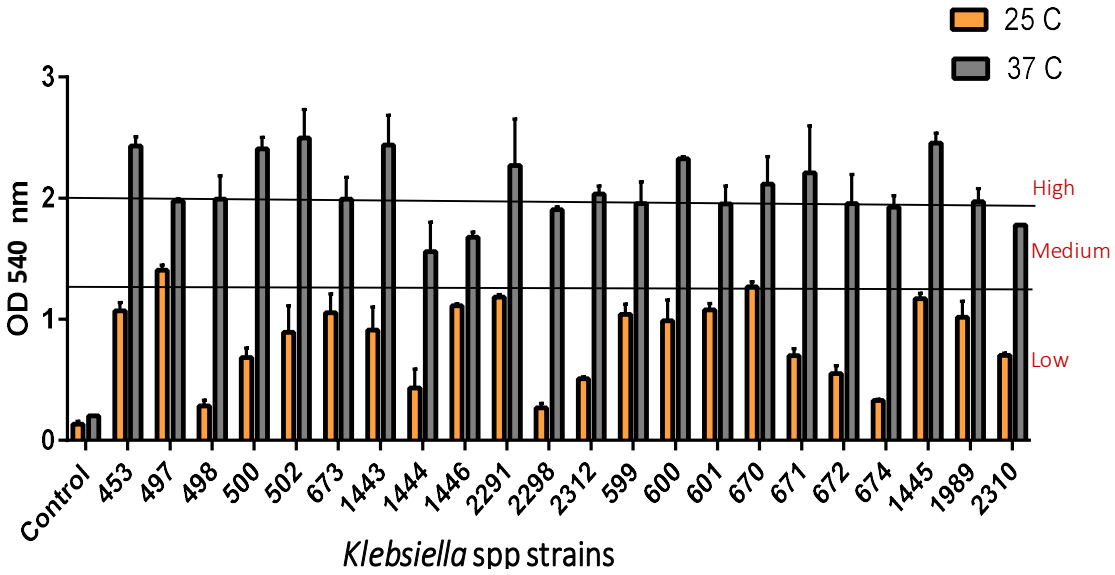


Figure 3-12: Biofilm formation of *Klebsiella* spp. isolates at 25°C and 37°C in liquid PIF.

Shows the preferred temperature is at 37°C. *K. pneumoniae* strains 502 showed their ability to form highest value of biofilm than other isolates compared to control. Biofilm formation stained with crystal violet 0.01% (w/v) and evaluated at wavelength 540nm; the control was non-inoculated media, the experiments were investigated in three independent times. Error bars represented using standard error.

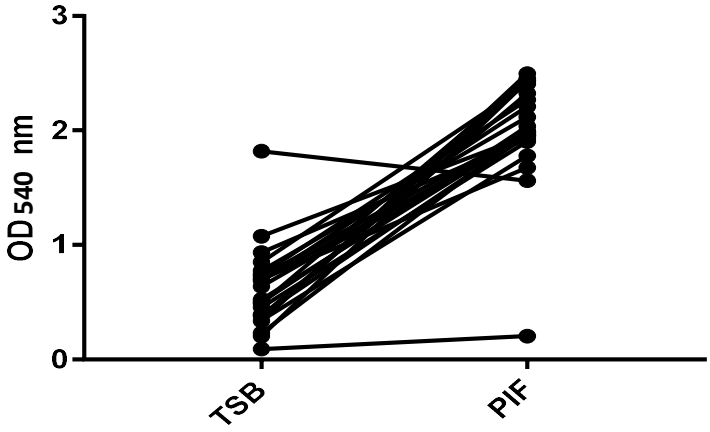


Figure 3-13: T-test revealed *Klebsiella* spp., isolates form high significantly biofilm in PIF than in TSB at 37°C

Paired t-test shows *Klebsiella* spp. strains form significantly more biofilm in rehydrated PIF than in TSB ($P < 0.0001$).

3.3.7.2. Production of curli fimbriae

In this project, fourteen isolates of *E. coli* were investigated using Congo red agar at 25 °C and 37 °C for 48 h. On Congo red agar, red morphotype colony expression indicates that the bacterium has ability to express curli fimbriae. Whereas, pink colony expression that reveals inability of bacterium to express curli fimbriae. The majority of isolates belonging to the dominant morphotypes produced pink colonies on Congo red indicating that these isolates are unable to produce curli fimbriae. However, strains 605, 1982, 1983, 2255 and 2297 were able to produce red colonies on Congo red showing the expression of curli fimbriae; Figure 3-14 and Table 3-9. With respect to *Klebsiella* spp., Figure 3-14 and Table 3-9 shows the result for 22 strains of *Klebsiella* spp. grown on Congo red agar at 25 °C and 37 °C for 48 h. Strains 601, 672, 674, 1444 and 2312 were able to produce red colonies on Congo red showing the expression of curli fimbriae, the others showed inability to produce curli fimbriae

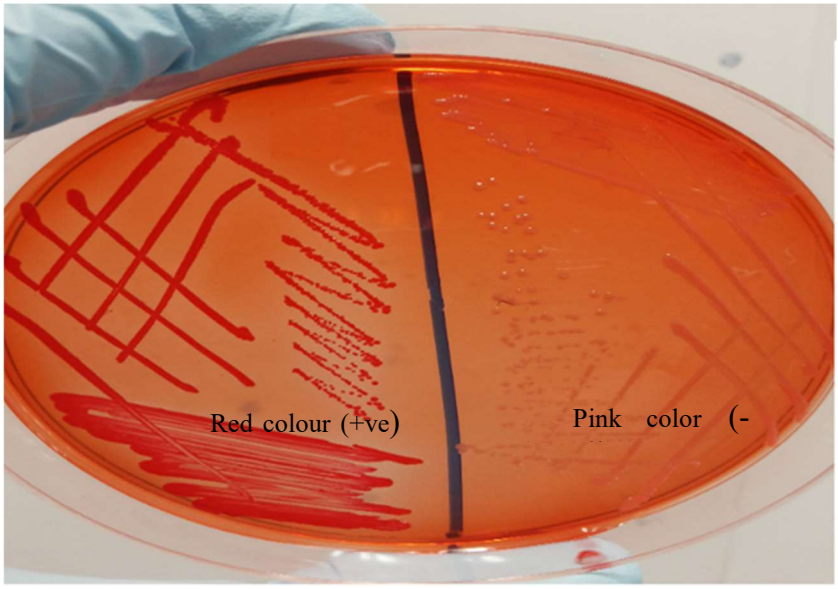


Figure 3-14: revealed colony morphology colour on Congo red agar media.

(Red mean bacteria expressed curli fimbriae and pink colour as a negative).

3.3.7.3. Cellulose Production on Calcofluor agar

The ability of *E. coli* and *Klebsiella* spp. isolates to produce cellulose on Calcofluor agar was investigated by the Calcofluor binding assay. All strains were streaked on LBA medium supplemented with Calcoflour White stain. Visually, the cellulose expression and appearance of colonies were detected by using ultra-violet (366 nm) fluorescence, then cellulose expression categorised as strong (+++), moderate (++) and weak (+) based on the strength of fluorescent signal. Regarding the *E. coli* strains, most isolates expressed cellulose, nevertheless the cellulose production was clearly variant between the isolates. Further, Figure 3-15 and Table 3-9 revealed only one strain 605 (7.69%) which exhibited a strong fluorescence signal at 366 nm, 5 (38.5%) strains; 1982, 1983, 1984, 1986 and 2300 exhibited moderate fluorescence signals and two isolates (15.4%) exhibited low fluorescence signal with the remaining isolates unable to express cellulose on Calcofluor agar.

With regards to *Klebsiella* spp., all strains indicated the ability to produce cellulose from Calcofluor agar with notable variation between strains. Table 3-9 show 8 (36.1%) isolates 500, 502, 599, 600, 601, 670, 671 and 674 gave a strong fluorescence signal at 366nm,

followed with two strains 2 (9.1%) 1446 and 1989 had exhibited a weak fluorescence signal. As well as the majority of examined strains, which were 12 (54.5%), 498, 1443, 453, 497, 1444, 673, 672, 1445, 2291, 2298, 2310, 2312, had showed moderate fluorescence signal at 366nm.

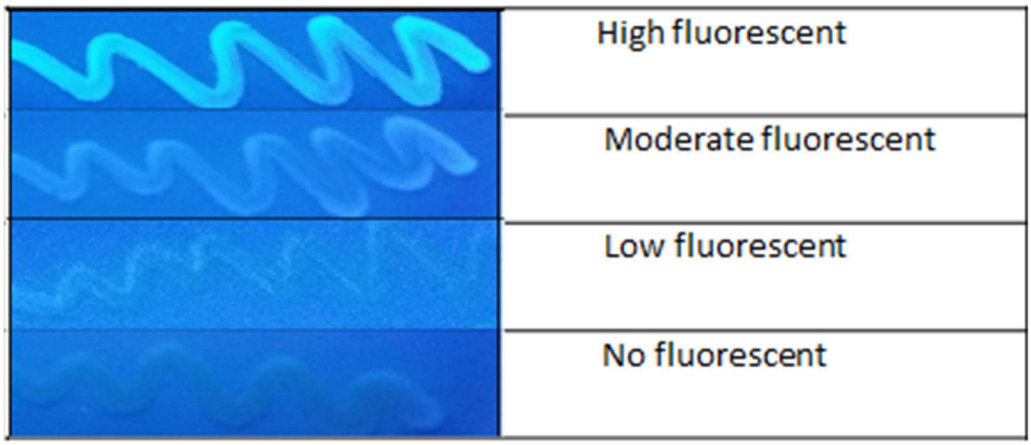


Figure 3-15: Cellulose production (expression) on Calcofluor.

A positive test is indicated by the presence of fluorescence around the isolate, as seen in the positive control (+ve). In the negative control (-ve) there is no fluorescence

3.3.7.4. Detection of iron siderophore by using chrome azurol S agar diffusion (CASAD)

Iron is a very essential micronutrient for nearly all living organisms. The ability of bacteria to obtain iron from the environment or the host is very important for their pathogenicity to cause infection. Bacteria have several known mechanisms for acquiring iron from the host, one of these are siderophores (chelating molecules) which are more widespread in pathogenic isolates than others in commensal isolates. In this experiment, all *E. coli* and *Klebsiella* spp. isolates demonstrated their ability to produce siderophores on CASAD agar by producing orange halos around the wells. Additionally, NTU strains *C. sakazakii* (520) and *Y. enterocolitica* (8081) were used as negative and positive controls respectively; Table 3-9 and Figure 3-16. These results were in agreement with the siderophore gene results, which were attained from PCR Figure 3-8.

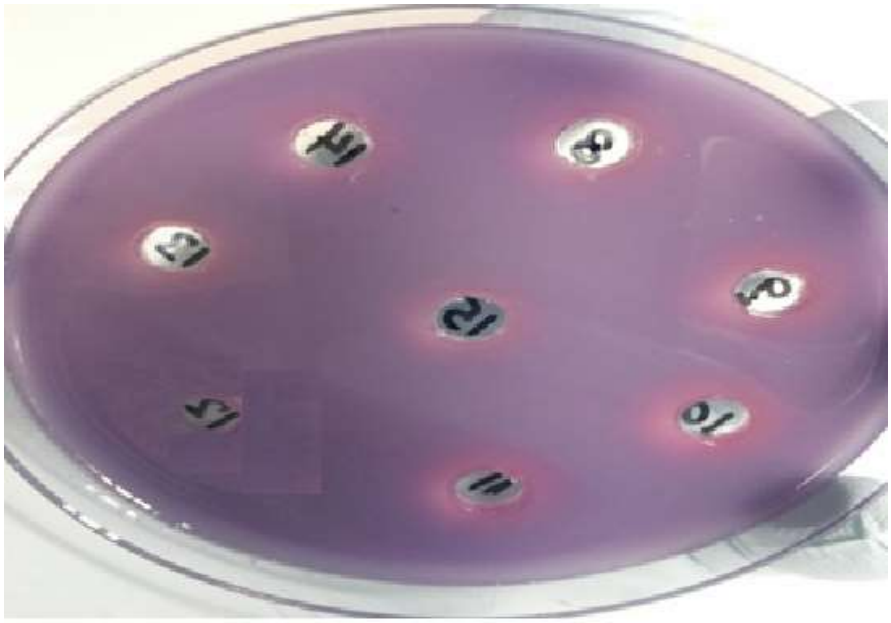


Figure 3-16: shows the ability of pathogens to produce siderophores on CASAD

This figure shows activity of siderophore using CASAD analysis, wells were filled with cell free culture supernatant of different clinical strains of *E. coli and Klebsiella* spp. All of these isolates were able to produce iron siderophores. CAS agar showing orange halo around the site of inoculation, nevertheless *Y. enterocolitica* strains (8081) was positive control and strains *C. sakazakii* (520) was negative.

Table 3-9 Congo red morphotype (curli fimbriae), cellulose production and siderophore production

Organism	Strain	Phylogenetic	Congo red phenotype	Cellulose production	Siderophores
E. coli K1	1980	B2	Pink	-	+
E. coli K1	1982	B2	Red	++	+
E. coli K1	1983	B2	Red	++	+
E. coli K1	2113	B2	Pink	-	+
E. coli K1	2114	B2	Pink	-	+
E. coli K5	605	B2	Red	+++	+
E. coli K5	1981	B2	Pink	-	+
E. coli K5	1984	B2	Pink	++	+
E. coli K5	1985	B2	Pink	+	+
E. coli K5	1986	B2	Pink	++	+
E. coli	2286	B2	Pink	+	+
E. coli	2297	B2	Red	-	+
E. coli	2300	B2	Pink	++	+
E. coli K5	2255	D	Red	-	+
K. pneumoniae	498	*rpo*B1	Pink	++	+
K. pneumoniae	500	*rpo*B1	Pink	+++	+
K. pneumoniae	1443	*rpo*B1	Pink	++	+
K. pneumoniae	1446	*rpo*B1	Pink	+	+
K. pneumoniae	453	*rpo*B4	Pink	++	+
K. pneumoniae	497	*rpo*B4	Pink	++	+
K. pneumoniae	502	*rpo*B4	Pink	+++	+
K. pneumoniae	1444	*rpo*B4	Red	++	+
K. pneumoniae	673	*rpo*B13	Pink	++	+
K. pneumoniae	2291	*rpo*B13	Pink	++	+
K. pneumoniae	2298	*rpo*B13	Pink	++	+
K. pneumoniae	2312	*rpo*B13	Pink	++	+
K. oxytoca	599	*rpo*B2	Pink	+++	+
K. oxytoca	601	*rpo*B2	Red	+++	+
K. oxytoca	2310	*rpo*B2	Pink	++	+
K. oxytoca	672	*rpo*B10	Red	++	+
K. oxytoca	1445	*rpo*B10	Pink	++	+
K. oxytoca	1989	*rpo*B10	Pink	+	+
K. oxytoca	671	*rpo*B14	Pink	+++	+
K. oxytoca	674	*rpo*B14	Red	+++	+
K. oxytoca	670	*rpo*B15	Pink	+++	+
K. oxytoca	600	*rpo*B40	Pink	+++	+

Cellulose expression categorised as strong (+++), moderate (++) and weak (+).

3.3.7.5. Capsule production;

Capsule production by 36 isolates of *E. coli* and *Klebsiella* species was investigated morphologically by appearance of the colony on two different media, including XLD and reconstituted powdered infant formula (PIF); as shown in Table 3-10 and Table 3-11; some isolates were clearly able to produce capsular materials on both PIF and XLD media. Nonetheless, notable variation was seen among strains examined for material produced on the XLD and PIF that was used. Therefore, the ability of these strains to produce capsules were categorised into four groups according to the relative scale of high, medium, low and non-mucoid that are represented by +++, ++, + and – respectively.

On PIF agar, the capsule formation in the *E. coli* strains was detected only in strain 1981, which produced low level of capsular materials, whilst other strains were unable to produce capsular materials, as shown in Table 3-10.

With respect to *Klebsiella* spp., the majority of these isolates displayed a mucoid appearance on PIF agar. As presented in Table 3-11, *Klebsiella* spp. strains, 453, 502, 599, 673, 1446, 1989 and 2298 were able to produce high levels of capsular material. The other strains 497, 498, 500, 670, 1443, 2291 and 2312 produced medium levels of capsular material, while lower levels of capsular material were noticed in isolates 600, 674, 1444, 1445 and 2310. In contrast, three strains 601, 671 and 672 were unable to form capsular material. Of particular note is strain 1446, which formed considerable capsular material on PIF agar Table 3-11. On XLD agar, all *E. coli* isolates revealed an inability to produce capsular material. On the other hand, the medium and low levels of capsular material production was formed by *Klebsiella* spp. isolates 502, 1446, 1989 & 2298 and 497, 498, 453, 500, 674, 1443, 1445, 2291 & 2312 respectively; Table 3-11. While, the strains 599, 600, 601, 670, 671, 672, 673, 1444 and 2310 were not able to produce capsular material.

3.3.7.6. Blood haemolysis:

In the current study, thirty six isolates of *Enterobacteriaceae* including, fourteen strains of *E. coli* and twenty-two strains of *Klebsiella* spp. were investigated for their capability to lyse horse and sheep erythrocytes. All *E. coli* strains showed gamma (ɣ)-type haemolytic activity (non-haemolysis) on both horse and sheep blood, except strains 2113, 2114 and 2300,

which showed β-haemolytic activity on horse blood agar. All isolates belonged to pathogenic phylogroup B2, except strain 2255, which belonged to phylogroup D Table 3-10. Twenty-two strains of *Klebsiella* spp. were investigated for their capability to lyse horse and sheep erythrocytes. Table 3-11 showed that all had gamma (ɣ) haemolytic activity on sheep blood, and the majority showed β-haemolytic activity on horse blood, while strains 600, 601, 674, 1445 and 1989 were gamma (ɣ) haemolytic on horse blood.

Table 3-10 Summary of virulence factors detected among *E. coli* strains isolated from NEFTs and neonates with sepsis

NTU	Species	Phylogenetic	Curli fimbriae and cellulose		Capsule production		Biofilm formation				Haemolyses	
			Curli	Cellulose	XLD	PIF	PIF		TSB		S	Ho.
							25°C	37°C	25°C	37°C		
2113	E. coli K1	Group B2	-	-	-	-	M	H	L	M	ɣ	β
2114	E. coli K1		-	-	-	-	M	H	L	M	ɣ	β
1980	E. coli K1		-	-	-	-	L	H	L	M	ɣ	ɣ
1982	E. coli K1		+	++	-	-	M	H	L	M	ɣ	ɣ
1983	E. coli K1		+	++	-	-	L	H	L	M	ɣ	ɣ
605	E. coli K5		+	+++	-	-	L	M	L	M	ɣ	ɣ
1981	E. coli K5		-	-	-	-	M	H	L	M	ɣ	ɣ
1984	E. coli K5		-	++	-	-	L	H	L	H	ɣ	ɣ
1985	E. coli K5		-	+	-	-	L	H	L	M	ɣ	ɣ
1986	E. coli K5		-	++	-	-	M	H	L	M	ɣ	ɣ
2286	E. coli K5		-	-	-	-	L	M	L	M	ɣ	ɣ
2297	E. coli		-	-	-	-	L	M	L	M	ɣ	ɣ
2300	E. coli		-	-	-	-	L	M	L	M	ɣ	ɣ
2255	E. coli K5	group D	+	-	-	-	M	H	L	M	ɣ	ɣ

PIF= powdered infant formula, TSB= trypton soya broth, S= sheep, Ho.= horse, M= medium, L= low and H= high.

Table 3-11 Phenotypic distribution of virulence factors among *Klebsiella* spp. strains isolated from NEFTs and neonates with sepsis

NTU	Species	*Rpo*B allels	Curli fimbriae and cellulose		Capsule production		Biofilm formation				Haemolyses	
			Curli	Cellulose	XLD	PIF	PIF		TSB		S	Ho.
							25°C	37°C	25°C	37°C		
1443	*K. pneumoniae*	Kp1	-	++	++	+++	L	M	L	L	ɣ	β
498	*K. pneumoniae*	Kp1	-	++	++	+++	M	H	M	M	ɣ	β
500	*K. pneumoniae*	Kp1	-	+++	++	+++	M	H	M	H	ɣ	β
1446	*K. pneumoniae*	Kp1	-	+	++	+++	L	H	L	M	ɣ	β
453	*K. pneumoniae*	Kp4	-	++	+	+++	M	H	L	L	ɣ	β
497	*K. pneumoniae*	Kp4	-	++	+	+++	L	H	L	M	ɣ	β
502	*K. pneumoniae*	Kp4	-	+++	+++	+++	M	M	L	L	ɣ	β
1444	*K. pneumoniae*	Kp4	+	++	-	++	M	H	H	H	ɣ	β
2291	*K. pneumoniae*	Kp4	-	++	+	+++	M	H	M	M	ɣ	β
2298	*K. pneumoniae*	Kp4	-	++	++	+++	M	H	M	H	ɣ	β
673	*K. pneumoniae*	Kp13	-	++	-	+++	L	M	L	L	ɣ	β
2312	*K. pneumoniae*	Kp13	-	++	+	++	M	H	M	M	ɣ	β
599	*K. oxytoca*	Ko2	-	+++	-	+++	L	H	H	H	ɣ	β
601	*K. oxytoca*	Ko2	+	+++	-	+	M	H	M	M	ɣ	ɣ
672	*K. oxytoca*	Ko10	+	++	-	-	L	M	L	L	ɣ	β
1445	*K. oxytoca*	Ko10	-	++	+	++	M	M	M	M	ɣ	ɣ
1989	*K. oxytoca*	Ko10	-	+	++	+++	M	H	M	M	ɣ	ɣ
2310	*K. oxytoca*	Ko10	-	++	+	++	M	H	M	M	ɣ	β
674	*K. oxytoca*	Ko14	+	+++	+	+	L	M	M	M	ɣ	ɣ
671	*K. oxytoca*	Ko14	-	+++	-	+	M	H	H	H	ɣ	β
670	*K. oxytoca*	Ko15	-	+++	-	+++	L	H	H	H	ɣ	β
600	*K. oxytoca*	Ko40	-	+++	-	+	M	M	M	M	ɣ	ɣ

PIF= powdered infant formula, TSB= trypton soya broth, S= sheep, Ho.= horse, M= medium, L= low and H= high

3.3.7.7. Acid sensitivity

In 2009, Hurrell and co-authors stated that the stomach pH of infant's fed milk formula is ranged between 2.5 and 4.3. In the present study, 36 strains of *E. coli* and *Klebsiella* spp. were investigated for their acid tolerance (pH 3.5) at five different time-points 0 min, 30 min, 60 min, 90 min and 120 min. This experiment was carried out in order to recognize which strains could persist and multiply in a pH that mimics the neonatal stomach acidity and acidified food. Strains were subjected to powdered infant formula pre-adjusted to pH 3.5 at 37^{o}C for 2 hours. Generally, the inoculum (initial viable count) of the isolates was between 8.3 to 8.74 $\log_{10}$ CFU/ml Figure 3-17 and Figure 3-18.

Figure 3-17, showed all *E. coli* strains were tolerant to pH 3.5 for 2 hours exposure and there was notable variation between the strains. Additionally, most strains started to multiply after 30 min, with the exception of strains 1982, 1985 and 2286. In general, all *E. coli* isolates tested were able to resist acid but their overall numbers did not change very much over time.

Figure 3-18, shows that most *Klebsiella* spp. isolates tolerated rehydrated PIF pH 3.5; the majority of these strains started to multiply after 30 min, and others after 60 min. Strains 1446 and 2298 revealed resistance by multiplying initially, and then after 60 min, the viable count partially decreased to log 7.2 and log 6.5, respectively. Strain 674 was only able to multiply and resist acidity for up to 30 min and then their viable count decreased from $\log_{10}$ 8.4 to $\log_{10}$ 5 indicating poorer tolerance of acid than the other strains. In general, most of the *Klebsiella* isolates tested were able to resist acid but their overall numbers did not change much over 120 minutes. The exception is strain 674.

The capability of these strains to multiply and persist in the acidic environment (pH 3.5) similar to the stomach acidity means these isolates are most likely to be more virulent and capable of invading the human intestinal and cause disease particularly in immunocompromised patients.

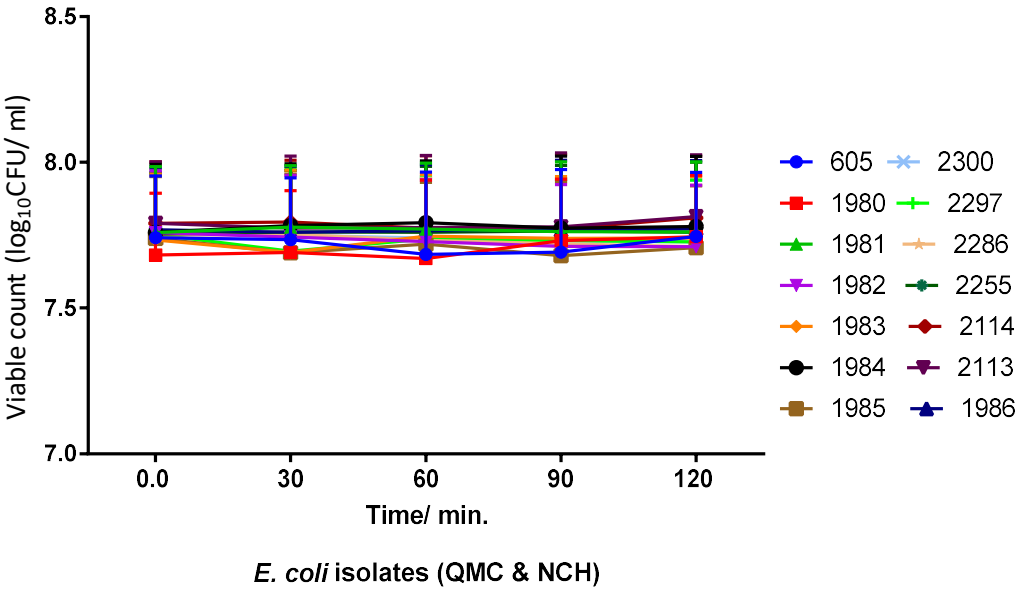


Figure 3-17: The survival of *E. coli* strains after the exposure to acidified PIF (pH 3.5) at 37°C for 120 minutes

The survival curve reveals the susceptibility of *E. coli* isolates cultivated in acidified PIF (pH 3.5) at 37oC for 120 minutes. Survivals was measured at 0.0, 30, 60, 90 and 120. The number of survival cells in this assays were performed in duplicate in two independent experiments. Error bars represented using standard error.

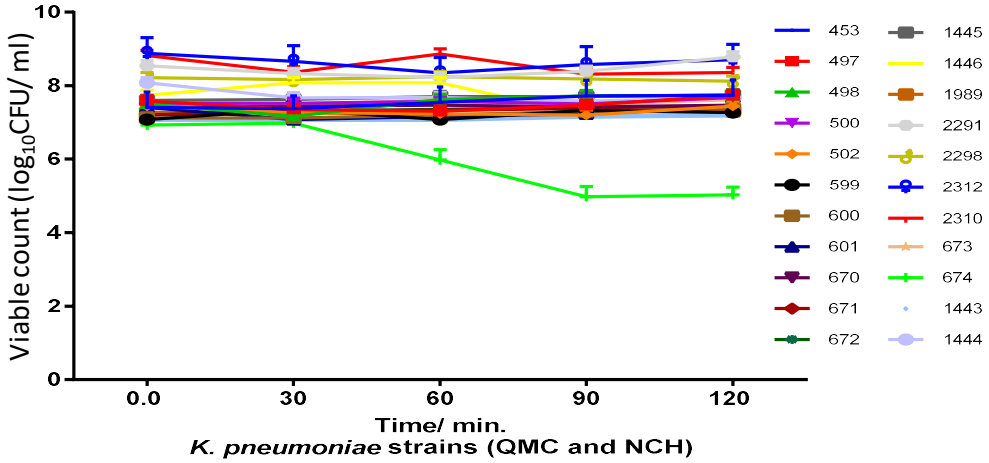


Figure 3-18: The survival of *Klebsiella* spp., strains after the exposure to acidified PIF (pH 3.5).

The survival curve reveals the susceptibility of *Klebsiella* spp., isolates cultivated in acidified PIF (pH 3.5) at 37°C for 120 minutes. Survival count was measured at 0, 30, 60, 90 and 120 minutes. The number of surviving cells in this assays were performed in duplicate in two independent experiments. Error bars represented using standard error.

3.3.7.8. Serum Resistance:

Figure *3-19* and Figure 3-20 showed that all *E. coli* and *Klebsiella* spp. strains from QMC and NCH were tolerant to serum (≥0.5 log difference) at four time points up to 3 hours. High tolerance to human serum was noted by *E. coli* isolates 1980, 1983, 2113, 2114, 1986, 2255, & 2300 and *Klebsiella* spp. strains 453, 498, 500, 1446, 2291 & 2298. However, *E. coli* strains 1982 appear to have a lower starting concentration and 2286 was the least resistant to human serum. *E. coli* K12 strain 1230 and *Salmonella* Enteritidis strain 358 were used as completely sensitive and resistant to human serum controls, respectively. These data were given as a viable count (log_{10}cfu/ml) by applying log_{10} differentiation among the bacterial viable count at initial time and after four hours exposure.

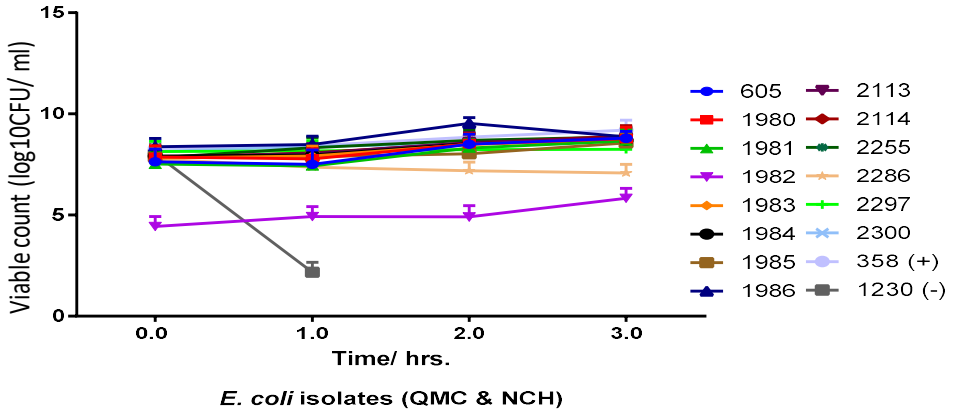


Figure 3-19: Sensitivity of *E. coli* isolates to human serum cultivated at 37°C for 3 hrs

S. Enteritidis (358) and *E. coli* K12 (1230) were used as positive and negative controls respectively. There was variation among isolates the highest tolerance was noted by *E. coli* isolates 1980, 1983, 2113, 2114, 1986, 2255, and 2300. Nevertheless, *E. coli* strains 1982 and 2286 were the least resistant to human serum. All examined strains were significantly better at surviving serum compared to the *E. coli* K12 (1230) negative control. The assays were performed in duplicate in two independent experiments. Error bars represented using standard error. Presented data was showing viable count (log_{10}cfu/ml) among the isolates.

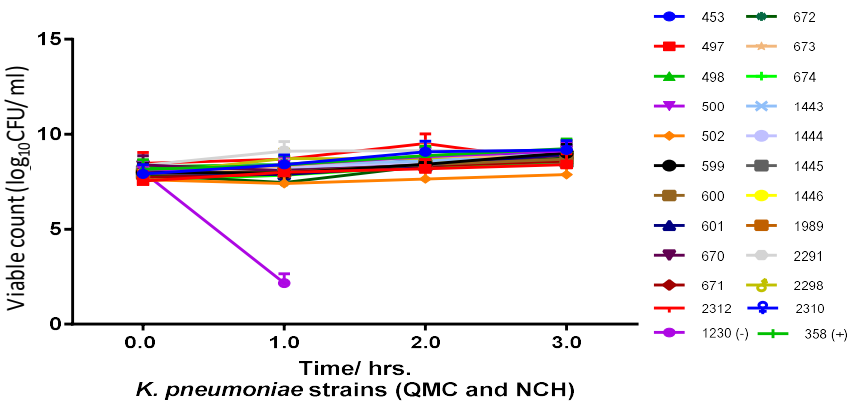


Figure 3-20: Sensitivity of *Klebsiella* spp. isolates to human serum cultivated at 37°C for 3 hrs

S. enteritidis (358) and *E. coli* K12 (1230) were used as positive and negative controls respectively. There was variation among subjected isolates the highest tolerance was noted by *K. pneumoniae* isolates 453, 498, 500, 1446, 2291 and 2298. All examined strains showed significance increases in their growing compared to the *E. coli* K12 (1230) negative control. The assays were performed in duplicate in two independent experiments. Error bars represented using standard error. Presented data was showing viable count (log10 cfu/ml) among the isolates.

3.3.7.9. Antibiograms:

Antibacterial susceptibility of the *Klebsiella* spp. and *E. coli* strains were investigated, for six antibiotic groups; miscellaneous (chloramphenicol and tetracycline) which are only agent available in their class. This meaning that they are unique in their action and not comparable to other antibiotics. Although their spectrum and side effect act maybe similar to other antibiotics, carbapenems (Meropenem and Imipenem), cephalosporins (Ceftazidime, Cefotaxime and Cefpodoxime), penicillins (Piperacillin / Tazobactam Ampicillin, and Augmentin) mode of action of these groups inhibiting cell wall biosynthesis, fluoroquinolones (Ciprofloxacin) their mode of action is by inhibition of DNA replication, and aminoglycosides (Gentamicin and Amikacin) their mode of action bind to the 30s ribosomal sub-unit inhibit protein synthesis. In addition, two antibiotics were examined to detect ESBL production; Cefotaxime /Clavulanic acid and Ceftazidime /Clavulanic Acid. *E. coli* NCTC 10418 was used as a control strain susceptible to all antibiotics examined.

As shown in Table 3-12, all *E. coli* strains were resistant to augmentin and amikacin antibiotics. In contrast, all *E. coli* isolates were susceptible to imipenem, meropenem, chloramphenicol and ciprofloxacin, with the exception of only one strain 1981 was resistent to ciprofloxacin. Beside, most isolates were susceptible to ceftozidime and

gentamycin. The majority of *E. coli* strains were resistant to ampicillin, except for strains 1982, 1983, 2113 and 2114, which were susceptible. ESBL production was detected phenotypically using the combination disc method. All of the *E. coli* isolates produced ESBLs. With respect to *Klebsiella* spp., these were only susceptible to one antibiotic (ciprofloxacin 5µg) out of the 11 antibiotics tested. Whereas, the rest of the bacteria were resistant, ESBL was produced by all isolates as well; Table 3-13. Thus, there is a high potential infectivity of these bacteria to new babies particularly premature babies that could be exposed to these organisms in NICU.

Table 3-12: Susceptibility of *E. coli* strains to antibiotics.

NTU No.	Species	Penicillin's		Cephalosporins		Aminoglycosides		EBSLs		C 30 µg	CAZ 10 µg	CIP 5 µg
		Aug 30 µg	AP 10 µg	IMI 10 µg	MEM 10 µg	AK 30 µg	GM 10 µg	CTX+CV 30 µg + 10 µg	CPD+CV 10 µg +1 µg			
1980	*E. coli* K1	R	R	S	S	R	S	R	R	S	R	S
1982	*E. coli* K1	R	S	S	S	R	R	R	R	S	S	S
1983	*E. coli* K1	R	S	S	S	R	R	R	R	S	S	S
2113	*E. coli* K1	R	S	S	S	R	S	R	R	S	S	S
2114	*E. coli* K1	R	S	S	S	R	S	R	R	S	S	S
605	*E. coli* K5	R	S	S	S	R	S	R	R	S	S	S
1981	*E. coli* K5	R	R	S	S	R	S	R	R	S	S	R
1984	*E. coli* K5	R	R	S	S	R	R	R	R	S	S	S
1985	*E. coli* K5	R	R	S	S	R	R	R	R	S	S	S
1986	*E. coli* K5	R	R	S	S	R	R	R	R	S	R	S
2255	*E. coli* K5	R	R	S	S	R	S	R	R	S	S	S
2286	*E. coli*	R	R	S	S	R	S	R	R	S	R	S
2297	*E. coli*	R	R	S	S	R	R	R	R	S	S	S
2300	*E. coli*	R	R	S	S	R	R	R	R	S	R	S

Augmentin (AUG), Imipenem (IMI), Meropenem (MEM), Cefotaxime + Clavulanate (CTX+CV), Amikacin (AK), Ceftazidime (CAZ), Cefpodoxime + Clavulanate (CPD+CV), Ampicillin (AP), Chloramphenicol (C), Gentamicine (GM) and Ciprofloxacin (CIP), S= susceptible and R= resistant.

Table 3-13: Susceptibility of *Klebsiella* spp., strains to the agents of antibiotic.

NTU No.	Species	*rpoB* allele	Carbapenem		Cephalosporin's				ESBLs		AP	GM	CIP
			IMI	MEM	CTX	CRO	CAZ	ZOX	CPD+CV	CTX+CV			
			10 µg	10 µg	30 µg	30 µg	10 µg	30 µg	10 µg +1 µg	30 µg + 10 µg	10µg	10µg	5µg
453	*K. pneumoniae*	Kp4	R	R	R	R	R	R	R	R	R	R	R
497	*K. pneumoniae*	Kp4	R	R	R	R	R	R	R	R	R	R	R
502	*K. pneumoniae*	Kp4	R	R	R	R	R	R	R	R	R	R	R
1444	*K. pneumoniae*	Kp4	R	R	R	R	R	R	R	R	R	R	S
2291	*K. pneumoniae*	Kp4	R	R	R	R	R	R	R	R	R	R	S
2298	*K. pneumoniae*	Kp4	R	R	R	R	R	R	R	R	R	R	S
1443	*K. pneumoniae*	Kp1	R	R	R	R	R	R	R	R	R	R	R
498	*K. pneumoniae*	Kp1	R	R	R	R	R	R	R	R	R	R	S
500	*K. pneumoniae*	Kp1	R	R	R	R	R	R	R	R	R	R	S
1446	*K. pneumoniae*	Kp1	R	R	R	R	R	R	R	R	R	R	S
673	*K. pneumoniae*	Kp13	R	R	R	R	R	R	R	R	R	R	S
2312	*K. pneumoniae*	Kp13	R	R	R	R	R	R	R	R	R	R	S
599	*K. oxytoca*	Ko2	R	R	R	R	R	R	R	R	R	R	S
601	*K. oxytoca*	Ko2	R	R	R	R	R	R	R	R	R	R	S
672	*K. oxytoca*	Ko10	R	R	R	R	R	R	R	R	R	R	S
1445	*K. oxytoca*	Ko10	R	R	R	R	R	R	R	R	R	R	S
1989	*K. oxytoca*	Ko10	R	R	R	R	R	R	R	R	R	R	S
2310	*K. oxytoca*	Ko10	R	R	R	R	R	R	R	R	R	R	S
674	*K. oxytoca*	Ko14	R	R	R	R	R	R	R	R	R	R	S
671	*K. oxytoca*	Ko14	R	R	R	R	R	R	R	R	R	R	S
670	*K. oxytoca*	Ko15	R	R	R	R	R	R	R	R	R	R	S
600	*K. oxytoca*	Ko40	R	R	R	R	R	R	R	R	R	R	S

Cefotaxime (CTX), Imipenem (IMI), Meropenem (MEM), Cefotaxime + Clavulanate (CTX+CV), Ceftriaxone (CRO), Ceftazidime (CAZ), Cefpodoxime + Clavulanate (CPD+CV), Ampicillin (AP), Chloramphenicol (C), Gentamicine (GM) and Ciprofloxacin (CIP), S= susceptible and R= resistant.

3.3.8. Summary of strain characterisation data;

Table 3-14 and Table 3-15 summarise the association between certain genes expressed genotypically and physiological traits, in addition to the potential pathogenicity of *E. coli* and *Klebsiella* spp. strains used in this study. The *E. coli* strains 1980, 2113 and 2114, which were isolated from NEFTs, belonging to ST95 (meningitic strains), had very high potential pathogenicity to neonates, and in particular, immunocompromised premature babies. Additionally, *E. coli* strains 2113 and 2114 were demonstrated to be clonally related by PFGE and whole genome sequencing. With regards to *K. pneumoniae* strains, all isolates analysed in this study demonstrated resistance to all antibiotics used, with the exception of ciprofloxacin. This would suggest that these strains are likely difficult to treat, which could result in a delay in neonatal recovery. *K. pneumoniae* strains 498 & 500 were clonally related as demonstrated by PFGE and whole

genome SNP analysis. No phylogenetic relationships could be detected between the other *E. coli* and *Klebsiella* spp., strains examined.

Table 3-14: Results summary and assessment of phenotypic and genotypic analysis of *E. coli* isolates (QMC and NCH).

NTU	Species	Source	ST	SG	PG	PT	Phenoypic Traits											Genotypic traits							Assessment of pathogenicity
							BF in PIF/°C		CUR	CP	CF ON		AT	SR	HAE		ESBL	CS	IA	Siderophores			TG	Fimbriae		
							25	37			XLD	PIF			H	S			*ibeA*	*Irp* 2	*fyu* A		*cnf+*	*fim* H	*sfa* S	
1980	*E.coli* K1	NEFT and sepsis cases	95	045:H7	B2	U	L	H	-	-	-	-	+	+	ɣ	ɣ	+	K1	-	+	+		-	+	-	High
2113	*E.coli* K1		95	018:H7		Same pulse	M	H	-	-	-	-	+	+	β	ɣ	+	K1	+	+	+		-	+	+	
2114	*E.coli* K1		95	018:H7			M	H	-	-	-	-	+	+	β	ɣ	+	K1	+	+	+		-	+	+	
1982	*E.coli* K1		Unk	083:H6		U	M	H	+	++	-	-	+	+	ɣ	ɣ	+	K1	+	+	+		-	+	-	Moderate
1983	*E.coli* K1		538	013:H4			L	H	+	++	-	-	+	+	ɣ	ɣ	+	K1	+	+	+		-	+	-	
605	*E.coli* K5		NA	NA			L	M	+	+++	-	-	+	+	ɣ	ɣ	+	K5	-	+	+		-	+	-	
1981	*E.coli* K5		NA	NA			M	H	-	-	-	-	+	+	ɣ	ɣ	+	K5	-	+	+		-	+	-	
1984	*E.coli* K5		73	022:H1			L	H	-	++	-	-	+	+	ɣ	ɣ	+	K5	-	+	+		+	+	-	
1985	*E.coli* K5		73	06:H1			L	H	-	+	-	-	+	+	ɣ	ɣ	+	K5	-	+	+		+	+	-	
1986	*E.coli* K5		NA	NA			M	H	-	++	-	-	+	+	ɣ	ɣ	+	K5	-	+	+		+	+	-	
2255	*E.coli* K5		NA	NA	D		M	H	+	-	-	-	+	+	ɣ	ɣ	+	K5	-	+	+		-	+	-	
2286	*E.coli*		69	*:H2	B2		L	M	-	-	-	-	+	+	ɣ	ɣ	+	non	-	+	+		-	+	-	Low
2297	*E.coli*		120	09:H9			L	M	-	-	-	-	+	+	ɣ	ɣ	+	non	-	+	+		-	+	-	
2300	*E.coli*		542	018:H27			L	M	-	-	-	-	+	+	ɣ	ɣ	+	non	-	+	+		-	+	-	

NEFT= neonatal enterla feeding tubes, H= high, M= medium, L= low, *= not detected, U= unique, NA= not applicable, Ukn=unknown, ST= sequence type, SG= serogroup, PG=phylogenetic group, PT= puleotype, BF= biofilm formation, PIF= powdered infant formula, CUR= curli fimbriae, CP= cellulose production, CF= capsule formation, AT= acid tolerant, SR= serum resistant, HAE. = haemolysis, H= horse, S= sheep, ESBL= extended spectrum beta lactamase, IA= invasin gene, CS= capsular eu/, TG= toxin gene.

Table 3-15: Results summary and assessment of phenotypic and genotypic analysis of *Klebsiella* spp. isolates (QMC and NCH).

NTU	Species	Source	*rpo*B allele	PT	Phenoypic Traits											Genotypic traits				Assessment of pathogenicity
					BF in PIF/°C		CUR	CP	Capsule on		AT	SR	Haemolysis		ESBL	Capsule serotype	Siderophores		Fimbriae	
					25	37			XLD	PIF			S	Ho.			*Irp*2	*fyu*A	*fim*H	*mrk*D	
1444	*K. pneumoniae*	NEFTs and Sepsis cases	Kp4	U	M	H	+	++	-	++	+	+	ɣ	β	+	K2	-	+	+	+	High
453	*K. pneumoniae*		Kp4		M	H	-	++	+	+++	+	+	ɣ	β	+	K5	+	+	+	+	
497	*K. pneumoniae*		Kp4		L	H	-	++	+	+++	+	+	ɣ	β	+	K5	-	+	+	+	
502	*K. pneumoniae*		Kp4		M	M	-	+++	+++	+++	+	+	ɣ	β	+	K5	+	+	+	+	
498	*K. pneumoniae*		Kp1	Same pulse	M	H	-	++	++	+++	+	+	ɣ	β	+	ND	+	+	+	+	Moderate
500	*K. pneumoniae*		Kp1		M	H	-	+++	++	+++	+	+	ɣ	β	+	ND	+	+	+	+	
2291	*K. pneumoniae*		Kp4	U	M	H	-	+++	+	+++	+	+	ɣ	β	+	ND	+	+	+	+	
2298	*K. pneumoniae*		Kp4		M	H	-	+++	++	+++	+	+	ɣ	β	+	ND	+	+	+	+	
2312	*K. pneumoniae*		Kp13		M	H	-	++	+	++	+	+	ɣ	β	+	ND	+	+	+	+	
1443	*K. pneumoniae*		Kp1		L	M	-	++	++	+++	+	+	ɣ	β	+	ND	-	+	+	+	
1446	*K. pneumoniae*		Kp1		L	H	-	+	++	+++	+	+	ɣ	β	+	ND	-	-	+	+	
673	*K. pneumoniae*		Kp13		L	M	-	+++	-	+++	+	+	ɣ	β	+	ND	-	-	+	+	
599	*K. oxytoca*		Ko2		L	H	-	+++	-	+++	+	+	ɣ	β	+	ND	+	+	+	+	
670	*K. oxytoca*		Ko15		L	H	-	+++	-	+++	+	+	ɣ	β	+	ND	+	+	-	+	
1989	*K. oxytoca*		Ko10		M	H	-	+++	++	+++	+	+	ɣ	ɣ	+	ND	+	-	-	+	
1445	*K. oxytoca*		Ko10		M	M	-	++	+	++	+	+	ɣ	ɣ	+	ND	+	+	-	+	
601	*K. oxytoca*		Ko2		M	H	+	+	-	+	+	+	ɣ	ɣ	+	ND	+	+	+	+	
672	*K. oxytoca*		Ko10		L	M	+	-	-	-	+	+	ɣ	β	+	ND	+	+	+	+	
674	*K. oxytoca*		Ko14		L	M	+	+	+	+	+	+	ɣ	ɣ	+	ND	+	+	+	+	
2310	*K. oxytoca*		Ko10		M	H	-	++	+	++	+	+	ɣ	β	+	ND	+	+	+	+	
671	*K. oxytoca*		Ko14		M	H	-	+	-	+	+	+	ɣ	β	+	ND	+	+	-	+	
600	*K. oxytoca*		Ko40		M	M	-	+	-	+	+	+	ɣ	ɣ	+	ND	+	+	+	+	

NEFT= neonatal enterla feeding tubes, PT= puleotype, U= unique, BF= biofilm formation, PIF= powdered infant formula, H= high, M= medium, L= low, CUR= curli fimbriae, CP= cellulose production, AT= acid tolerant, SR= serum resistant, Ho.= horse, S= sheep, ESBL= extended spectrum beta lactamase, ND= not detected.

3.4. Discussion:

Distinguishing bacteria at the strain level or bacterial pathogen categorising is very crucial for treatment, diagnosis and tracking source of bacterial infection. Genera of *E. coli* and *Klebsiella* are ubiquitous, belonging to the family of *Enterobacteriaceae*, therefore bacterial environmental and epidemiological investigation require typing schemes. Especially, those genera commonly cause NICUs nosocomial infection such as septicaemia, bacteraemia, necrotising enterocolitis (NEC) and meningitis especially in immunocompromised patients (Adamsson et al., 2000; Almuneef *et al*., 2001). Additionally, these bacteria are rarely isolated from PIF. Furthermore, some phenotypic procedures such as culturing bacteria on selective media lead to a quick hypothetical identification of causative agent of infection. In many laboratories, biochemical tests are still recommended and widely used for initial identification.

However, the use of biochemical tests alone is now understood to be unreliable (not be discriminatory), particularly with members of the *Enterobacteriaceae*. Thus, the use of molecular typing techniques is preferred to achieve high accuracy identification (Cetinkaya *et al*., 2013; Baldwin *et al*., 2009; Iversen *et al*., 2007). Molecular subtyping methods, such as PFGE, are an important tool to know which bacteria are transferred within the neonatal units, if these strains are from common sources and how diverse they are. Therefore, this will help in determining the potential cross-transmission events or a common source of infection, which is causing nosocomial outbreaks. Furthermore, it is very important to control and reduce outbreaks rapidly (Adamsson *et al*., 2000). PFGE is used to cluster strains before studying their physiological and virulence related traits. This project is a continuation of in-depth studies conducted by our research group since the initial studies by Hurrell *et al.* (2009a and b), in order to better understand which virulence traits contribute in causing disease. Hurrell *et al.* 2009a and b; Adamson *et al*. 2012; Almuneef *et al*. 2001; Goldmann, 1988; Tullus *et al*. 1988; Parm *et al*. 2011, established that most general nasogastric and neonatal enteral feeding tubes were colonised with members of *Enterobacteriaceae*, primarily *Klebsiella*, *E. coli*, *Serratia* and *Enterobacter*.

In the current study, thirty-six clinical isolates were collected from NEFTs and neonates with sepsis cases on intensive care units from two local hospitals; QMC and NCH (Nottingham). Of particular note is the fact that these collections are unique and have not been studied before. Unfortunately, metadata (hospital infection) associated to some of the studied babies were not available because it has been a long time between sample collection and analysis. However, the aim of evaluating and characterising these isolates is very important, therefore, this study focused on the identification and characterisation of the isolates, not epidemiological outbreak determination. In this project, DNA fingerprinting was used to analse isolates of *E. coli* and *Klebsiella* spp. via PFGE, in order to determine whether the same isolates were isolated from different neonates within the NICUs. It was also important to look at how diverse these isolates are, as well as to characterise their potential to cause serious infections.

The results showed that all of the *E. coli* isolates (n=14) were unique except two strains 2113 and 2114, which were clonally related and clustered together Figure 3-2. This pair of strains had been isolated over a one-week period (27 March to 01 April 2010, from NEFT) from two different neonates at NCH. The *Klebsiella* spp. strains 498 and 500 which are isolated from NEFTs also clustered together but unfortunately meta data for these two isolates are not available. The other *Klebsiella* strains (n=22) were all unique Figure 3-3. This indicates that a wide range of strains were isolated from the NICUs, but there was some evidence of the cross-transmission of indistinguishable strains (clone) between different neonates over a short period of time in the same NICU. This transmission within the NICU suggests a common origin source such as either carers, powdered infant formula or the environment. Previous studies by Castro *et al.* 2010; Wojkowska-Mach *et al.* 2013 reported similar findings when they investigated *E. coli* and *Klebsiella* spp isolated from six NICUs in Poland. Supporting to this notion, Alkeskas *et al.* (2015) isolated indistinguishable *E. coli* K1 (ST95) recovered from multiple neonatal feeding tubes on NICUs.

In 2013, Pires *et al*. argued that the persistence of the same clone within different sites in the hospital may reflect existence of the same isolate during the follow-up period in these sites. This may also be attributed to its capability of surviving and multiplying well under adverse conditions, which therefore becomes a potential origin of nosocomial infection

(Bradford, 2001; Kristof *et al*., 2007; Viswanathan *et al*., 2010). The isolation of clonally unrelated strains may reflect different origins of strains. Given the circulation of the isolates within the hospital environment, it is not unexpected that the neonates could have acquired these strains from different origins. For example, several studies in different countries have showed genotypic diversity between isolates. In Japan, a study on 57 strains of *Citrobacter* spp. using PFGE found 18 unique clones and 13 different clusters (Kanamori *et al.,* 2011). Other studies at NTU, UK by Alkeskas *et al.* (2013) and Hurrell *et al.* (2009) on different sets of isolates belonging to family of *Enterobacteriaceae* including neonatal meningitis *E. coli* K1 (ST95), *Klebsiella* spp., *Enterobacter* spp. and *Serratia* spp. isolated from neonatal entering feeding tubes also showed large diversity in PFGE profiles among these strains. This diversity indicates the neonatal enteric feeding tubes may become contaminated from different sources. (Singer *et al*., 2004; Gomez *et al*., 2016; Ogrodzki *et al*., 2017) reported that PFGE is considered a beneficial technique within deep-rooted limitations. For example, similarity among strains or groups as determined by PFGE may not reflect the genetic similarity of the isolates.

Representative *E. coli* strains (10) from NEFTs and neonatal with sepsis cases were subjected to whole genome sequencing. Genomic analysis assigned two strains (1984 & 1985) as ST73, three strains (1980, 2113 & 2114) as ST95 and the remaining strains 1983, 2286, 2297 and 2300 as ST538, 69, 120 and 452 respectively. In study done by Alkeskas *et al*. (2015) they recovered indistinguishable *E. coli* K1 (ST95) serotype O1:K1:NM from 11 neonates who were on different feeding regimes from NICUs. SNP analysis clustered only two strains (2113 and 2114) ST95 together. These isolates had only 0–29 SNPs difference between them, they were regarded as nearly indistinguishable strains isolated from two different babies within one week. Of interesting is that, genome sequencing results agreed with PFGE results.

There are seven known phylogenetic groups of *E. coli* (A, B1, B2, C, D, E, and F), four of which (A, B1, B2, D) are the main phylogenetic groups, and each of these groups display ecological speciality and vary in their abilities to cause disease (Jacoby *et al*., 2005; Shaikh *et al*., 2015). Therefore, knowing the phylogenetic group is very crucial to recognising the infectious diseases epidemiology. *E. coli* phylogenetic groups B2 and D are attributed to causing extra-intestinal infections. These groups are able to cause UTIs, sepsis, and neonatal meningitis,

whilst groups A and B1 are associated with commensal strains (Nawaz *et al.,* 2008; David *et al*., 2008; Picard *et al*., 1999). Several authors have argued that most strains of *E. coli* containing mulitple virulence factors cluster within phylogenetic group B2 or are shared among groups B2 and D (Boyd & Hartl, 1998; Picard *et al*., 1999). Duriez *et al.* (1984) investigated 168 commensal *E. coli* strains isolated from faecal samples from three differential geographical human populations to determine their phylogenetic groups. They found that the most common phylogenetic groups were group A and B1, followed by phylogenetic group D.

Moreno *et al.* (2008) compared *E. coli* strains isolated from stool and urine from the same patients, and showed that group B2 were at significantly higher prevalence in urine than in stool. Obata Yasuoka *et al.* (2002) studied prevalence of phylogenetic groups among 88 vaginal *E. coli* clones, and showed that the majority of isolates belonged to group B2. Besides this, Bingen-Bidois *et al.* (2002), reported that *E. coli* groups B2 and D were the most predominant phylogenetic groups isolated from urosepsis patients. Supporting to these notions, in 2015 Alkeskas and co-authors studied 30 strains of *E. coli* isolated from NEFT from two hospitals at different times. They found all studied isolates were belonging to group B2. Our data revealed that phylogenetic group B2 was the most predominant among strains from NEFTs and neonatal with sepsis cases. Thus, these data are in agreement with several previous studies, which revealed that *E. coli* strains phylogenetic group B2 were more widespread in urine than stool samples, as well as prevalent in NEFTs, vaginal and urosepsis samples (Moreno *et al*., 2008; Obata Yasuoka *et al.,* 2002; Bingen-Bidois *et al*., 2002; Alkeskas *et al*., 2015).

The treatment of infections is very important, requiring appropriate and correct diagnostics. In clinical microbiology laboratories the identification of infectious microorganisms can be very difficult and strains are often misclassified. Accurate identification of these organisms is crucial for molecular epidemiological studies and improved taxonomic classification of these groups. Some members of the *Enterobacteriaceae* family such as *E. aerogenes*, *K. oxytoca*, and *K. pneumoniae* are difficult to identify using classical phenotypic/biochemical tests.

The *rpoB* gene was used for identification of bacteria and as a locus for phylogenetic analysis. When comparing the phenotypic results of *R. platicola*, *K. pneumonia*, *K. oxytoca*, and *K. variicola* strains with their *rpoB* sequence, these species were effectively identified, with high confidence (Mollet *et al*., 1997; Monnet *et al.,* 1994). Drancourt and Raoult, in 2005 reported that there was less than 97% similarity in the *rpoB* gene sequences or 16S rRNA genes within the bacterial strains belonging to different species. For the 47 *Klebsiella* isolates analysed, the BLASTn search showed 98% similarity in *rpoB* sequence for the classification proposed by bio-typing profiles, and therefore, *rpoB* sequences could confirm the biochemical bio-types of all analysed strains (Fevre *et al*., 2005; Drancourt *et al*., 2005).

Mollet *et al*. (1997) and Ade´kambi *et al*. (2009) reported that the *rpoB* gene might be a powerful bacterial discrimination and identification tool, particularly when analysing closely related isolates. In the present study, the *rpoB* gene sequence was used to analyse the 22 *Klebsiella* (QMC, NCH) strains revealing eight different *rpoB* alleles profiles; 1, 2, 4, 10, 13, 14, 15, 40. Multiple alignments data were analysed according to the Pasteur MLST database; Figure 3-7. These results confirmed that the *rpoB* allele sequence is a powerful identification tool allowing reliable differentiation and speciation of isolates. Recently, the main focus in bacterial pathogenesis is the identification and description of the molecular virulent trait genes which allow bacteria to cause hospital infections (Wilson *et al*., 2002). Further, Gibreel *et al*. (2011) reported that the capacity of *E. coli* isolates to carry one or several virulent genes enhance its ability to cause various clinical infections. For instance, strains can carry curli fimbriae and K1 capsular genes leading to attachment and protecting them from engulfment by the immune system. A previous study suggested that group B2 *E. coli* strain carriage of the *sfa/foc* operon and *hly* operon was the cause of neonatal meningitis (Bingen *et al*., 1998). Additionally, *E. coli* serotypes O18:K1:H7, O1:K1, O7:K1, O83:K1 and recently O45:K1:H7 were primarily linked with neonatal meningitis (Bonacorsi *et al.,* 2005; Bonacorsi *et al.,* 2003; Peigne *et al.,* 2009). These are sequence type (ST) 95 and in *E. coli* extraintestinal pathogenic group B2 (Achtman scheme).

In addition, historical studies of new serotypes O18:K1:H7 and O7:K1 for meningitic *E. coli* strains have recently begun such as O45:K1:H7 in France and O83:K1 in the Netherlands (Bonacorsi *et al*., 2003; Mulder *et al*., 1984). In France and Hungary, serotype O45:K1:H7

seems to be more frequent than the O18:K1:H7 serotype (Bonacorsi *et al*., 2003; Czirok *et al*., 1993). Alkeskas *et al*. (2015) investigated 30 *E. coli* strains that were isolated from neonatal enteral feeding tubes and the results revealed that *E. coli* K1 isolate (ST95) serotype O1:K1:NM was isolated from 11 neonates tubes at the same hospital. Our results showed that out of 3 *E. coli* K1 isolates (ST95), two are indistinguishable strains of *E. coli* K1 (ST95) serotype O18:H7:K1 and had been isolated from two different babies at different time period and *E. coli* K1 (ST95) serotype O45:H7:K1. Nowadays, considerable research focuses on *E. coli* group B2 isolates of serotype O1, O2, O18, and O45 which are frequently in ST95. In the present study, the presence of 30 potential virulence factor genes (VFGs) in the isolates was assessed. These included genes for adhesion, capsule, siderophores, toxins and others (Johnson *et al.,* 2000). The VFGs *fyuA*, *PAI*, *kpsMTII*, *fimH* and *traT* were present in the majority of isolates sequenced in this study Table 3-6. In addition, it was found that strains 1980, 1982, 1983, 2113 and 2114 possessed genes for the K1 capsular antigen. K1 capsule is known as an essential meningeal virulence trait and is strongly associated with neonatal meningitis and *E. coli* pathogenicity (Saukkonen*et al*., 1988; Peigne*et al*., 2009). A notable difference in the prevalence of virulence-associated genes was observed among the isolates, depending on the capsular antigen and sequence type Table 3-7. For instance, *E. coli* strains of ST95 serotype O18:H7:K1 were β-haemolytic and possessed type 1 *fimH*, *Sfa/focDE* and *sfaS* fimbriae adhesion genes, *ibeA* invasin gene and Colicin V – the marker for *colV* plasmid gene *cvaC*. Bert *et al*. (2010) stated that among screened *E. coli* strains, the *cavC* gene was significantly linked with ST95 isolated from neonates with cases of bacteraemia. In contrast, *E. coli* strains of ST73 serotype O6:H1:K5 and *E. coli* K5 strain 605 which were not carrying K1 capsular antigen possessed the *focG*, *papEF* and *papG II, III* fimbriae adhesion genes, *hly* haemolysin A genes, and *cnf+* Cytotoxic necrotizing factor-1 gene. Several recent studies reported that most ST73 strains which possessed *cnf+* and haemolysin genes were linked with UTIs caused by UPEC (Smith *et al*., 2008). That may suggest these VFGs are commonly associated with disease.

Moreover, *hly* haemolysin A genes, and *cnf+* Cytotoxic necrotizing factor-1 gene are involved in tissue injury and host immune dysfunction (Blanco *et al.,* 1992; Hofman *et al.,* 1998). After investigation of the characteristic virulence traits of urosepsis *E. coli* strains, the authors

suggested that *cnf+* and *hly* genes are probably a virulence entity, because they form the backbone of the PAI (Bingen-Bidois *et al.,* 2002). Bingen *et al*., (1997, 1998); Bonacorsi *et al*., (2003) reported that nearly half of the isolates studied were thought to capable of penetrating the blood-CSF barrier despite lacking specific adhesins such as *ibeA* and adhesins such as *sfaS*.

In addition, the *pap* gene is a member of a family of adhesion genes, and the most important virulence trait present. Presence of *pap* genes among UTI causative isolates is associated with pyelonephritis as well as these strains having the ability to penetrate the bloodstream to cause sepsis and complicated infections (Mora *et al.,* 2009; Johnson 1991). Earlier studies stated that the occurrence of different adhesion pap genes vary among 50% to 79% of UTI *E. coli* isolates (Johnson *et al*., 2001; Leflon-Guibout *et al*., 2008). In the present study, the prevalence of pap adhesion genes found was relatively low, ranging between 0 to 42% and this might be due to a small sample size or the nature of the isolates. However, during this study some virulent genes were completely absent, for instance *Afa/draC*, *bmaE*, *gafD*, *nfaE*, *papG* and *cdtB* indicating that these genes are not crucial to the virulence of UPEC isolates.

Capsule production in *Klebsiella* is considered one of the most importance virulent factors and they play an important role in pathogenicity and protect the bacteria from harsh environments. Among the 77 capsular (K) serotype scheme, generally K1, K2, K4 and K5 capsular serotypes are considered as highly virulent traits that are associated with most severe infections in mammalians. In addition, several reports documented that most *K. pneumoniae* isolates from liver abscess patients were associated with K1 and K2 capsular serotype (Ma LC *et al.,* 2005; Simoons-Smit *et al.,* 1982; Nassif *et al.,* 1986). Furthermore, the K2 serotype is the second most predominant cause of pyogenic liver abscess after the K1 capsular serotype (Fung *et al.,* 2002). In Taiwan, over 80% of liver abscesses are caused by K1 and K2 capsular serotypes, and these serotypes are also linked with increased virulence and lethality in lab mice (Kim *et al.,* 2003; Ayinala *et al.,* 2001; Lederman *et al.,* 2005). Moreover, Lawlor *et al*. (2007) and Wu *et al*. (2009) reported that K1 and K2 capsular serotype in *Klebsiella* are associated with pyogenic liver abscess as well as being frequently reported in hospital infections and community-acquired pneumonia.

In 2007, Fang *et al*; studied capsular serotypes of *K. pneumoniae* strains isolated from an outbreak of community acquired pneumonia in Taiwan. They found that the predominant capsular serotypes were K1, K2, K5 and K20. Moreover, they stated that majority of these serotypes were associated with liver abscess patients in Taiwan. Our results from a total of 22 *Klebsiella* spp., isolated from NEFTs and neonates with sepsis cases (QMC & NCH), showed that only one strain 1444 was identified as K2 serotype and strains 453, 497 and 502 were K5, whereas serotype K1 was not detected. It was noted that the capsular serotypes (K2 and K5) were detected in the strains, which are isolated from NEFT only Table 3-8. The presence of virulence traits in *Klebsiella,* such as capsular serotypes might indicate the risk of infection to the neonates in NICUs.

With regards to iron starvation, iron acquisition from the host is very important for metabolic processes in pathogenic bacteria to cause infection. Furthermore, in the human body and blood circulation particularly in the presence of oxygen the availability of iron for bacteria is extremely limited which will inhibit growth of pathogenic bacteria. For this reason, pathogenic bacteria with a scarcity of iron secrete siderophores which have high affinity to remove (chelate) iron from the binding proteins for bacterial survival (Negre *et al*., 2004; Braun & Winkelman, 1987; Banin *et al.* 2005). According to Schubert *et al*. (2000) all members of *Enterobacteriaceae*, which were investigated carried siderophores genes such as *irp1*, *irp2* and *fyuA* in high pathogenicity island (HPI) revealed as iron uptake genes, moreover these genes are linked with pathogenicity in *Yersinia* species. In the present study, all isolates except 673, 1446 and 1989 possessed the iron uptake gene *fyuA* and the *irp2* siderophore gene was detected in all strains except 497, 673, 1443, 1444 and 1446. The gene *irp1* was absent from all investigated isolates Table 3-8**.**

Of interest, we noted that all isolates including those lacking of *irp1*, *irp2* and *fyuA* genes, were able to express siderophores on CASAD agar by producing an orange halo around the wells. This suggests that these isolates may use an alternative iron uptake system or there is another gene related to the siderophores not yet detected in these strains.

Several reports have stated that severe nosocomial infections are increasingly being caused by *K. pneumoniae*, and the ability of this species to adhere and multiply on biotic and abiotic

surfaces may lead to persistence in the hospital environment. The virulence factors related to the adherence of *K. pneumoniae* on inanimate surfaces are not very well understood (Podschun and Ullmann, 1998). According to (Martynenko *et al*., 1992; Schurtz *et al*., 1994; Livrelli *et al*., 1996; Podschun *et al*., 2001) the prevalence of type 1 and type 3 fimbriae in environmental and clinical *K. pneumoniae* strains was high. Furthermore, type 1 fimbriae in *K. pneumoniae* have no effect on the strain's ability to colonize and infect the lung; however, they are essential for the initiation urinary tract infections (Struve *et al.,* 2008). On the other hand, type 3 fimbriae have no influence on pathogenicity, particularly in pulmonary and intestinal infections, but are a major contributor in biofilm formation (Struve *et al.,* 2009). Moreover, Alcántar-Curiel and colleagues investigated the ability of 69 strains of *K. pneumoniae* to form biofilm, and demonstrated that 55/69 isolates were able to form biofilm and possessed type 1 and type 3 fimbriae and the other isolates did not have type 1 and type 3 fimbriae (Rao *et al.,* 2009; DeMartino 2003). Of particular note is that type 3 fimbriae share functional attributes with curli fimbriae and both have the ability to interact with polyester and glass surfaces, and both mediate bacterial adherence (biofilm-associate) to extracellular matrix (ECM) proteins. In this study, twenty-two *Klebsiella* strains from NEFTs and neonates with sepsis from NICUs at two local hospitals were characterised for fimbriae possession and ability to form biofilm.

As shown in Table 3-8 and Table 3-11, the majority of investigated strains produced high biofilm and 100% of isolates carryed type 3 fimbriae, while 86% of isolates possessed type 1 fimbriae. These findings are in agreement with the previous reports that prevalence of type 1 and type 3 fimbriae in *Klebsiella* are high. Furthermore, the presence of these virulent traits may contribute to adherence of this bacteria on medical devices and persistence in the hospitals particularly in long term catheterised patients and in infant feeding tubes, which could leave patients at risk of serious infection.

Biofilm formation is one of the main virulent traits for pathogenic bacteria, leading to attachment and replication on abiotic and biotic surfaces such as medical equipment, food contact surfaces and food. Consequently, biofilm allows bacteria to become more resistant to environmental stresses such as human stomach acidity, disinfectants, extreme temperature, desiccation and antibiotics (Kim *et al.*, 2006; Dancer *et al.*, 2009). In this study,

two incubation temperatures (25°C and 37°C), PIF and TSB were used to investigate the capability of the *E. coli* and *Klebsiella* spp. strains to form a biofilm. Previous reports stated that biofilm formation is affected by different variables such as O_2, temperature, osmolarity and pH (Romeo, 2008).

In this study, the temperature was clearly the key factor in biofilm formation Table 3-10 and Table 3-11. Biofilm formation was significantly higher for most isolates at 37 °C in both PIF and TSB compared with 25°C Table 3-9 and Figure 3-12. However, the majority of strains showed lower biofilm formation with TSB at both 25°C and 37°C temperatures in comparison with PIF. Biofilm formation was high in PIF at 37°C, which might be due to the milk nutrient, and these findings corroborate previous studies (Kim *et al*., 2006; Meadows, 1971). Previous studies also suggest that media components and nutrients affect bacterial attachment to different materials surfaces (Hood and Zottola, 1997). These results are in agreement with another report (Kim *et al*., 2006). In particular, *E. coli* strains 2113 and 2114 (same pulse type) produced the same quantity of biofilm as each other in all conditions tested. In agreement with the PFGE and genome sequencing data, this suggests that the same strain cross-infected two different neonates within a one week in NICU at QMC. In 2009, Hurrell *et al.* reported that the neonate's infection risk at NICUs has increased due to bacterial biofilm formation on neonatal entering feeding tubes, so biofilm might be problematic in the hospitals settings and in the food industry.

Many reports have stated that some members of the *Enterobacteriaceae* encode curli fimbriae genes, which play an important role in biofilm formation and adherence (Olsén *et al*., 1993; Römling *et al*., 1998; Zogaj *et al*., 2003; Romeo, 2008; Lee *et al*., 2011). Furthermore, after incubation on Congo red agar at 37 °C for 72 h, the colonies exhibited the colour indicating the Congo red dye binding to the curli fimbriae. In this investigation, 36 strains of *E. coli* and *Klebsiella* spp. (n=14 and n= 22) were examined on Congo red agar to detect the cell wall expression of curli fimbriae. Table 3-9 showed that *E. coli* isolates 605, 1982, 1983 & 2255 and *Klebsiella* spp. isolates 601, 672, 674 & 1444 respectively have ability to bind with Congo red dye and express red colour colonies on Congo red agar indicating that these strains carry curli fimbriae. However, with regards to *Klebsiella* 601, 672, 674 and 1444 isolates the gene searching (*csg*BAC and *csg*DEFG) did not show any homologues

(absence of this gene cluster) which means Congo red might react with other materials and give a false positive. On the other hand, the other strains were unable to bind to Congo red dye and gave pink colony colours indicating that their ability to produce cellulose on Congo red agar (Bokranz *et al.,* 2005). Biofilm formation in *E. coli* isolates has been associated with expression of cellulose and curli fimbriae (Bokranz *et al.,* 2005). In 2006, Ghigo and Da Re stated that in *Salmonella* and *E. coli* cellulose is very important and one of the main ingredients of the biofilm matrix. Our data showed no link between the presence of curli fimbriae genes (*csg*BAC and *csg*DEFG) and detection of curli fimbriae phenotypically by binding to Congo red dye in *E. coli* and *Klebsiella* spp. Besides, concerning to *E. coli* isolates there is no link between biofilm formation and cellulose production Table 3-9 and Table 3-10. In contrast, there is link between biofilm formation and cellulose in *Klebsiella* spp., strains Table 3-9 and Table 3-11.

Capsules in bacteria, are considered as one of the most important virulent factors, and play an important role in protecting them from harsh environments such as phagocytic cells, desiccation, acidity and serum activity (Guerry & Szymanski, 2008; Ogrodzki & Forsythe, 2015). In the present study, Table 3-10 shows there was no obvious correlation between biofilm formation and capsule production for *E. coli* strains. For instance, most *E. coli* isolates were not able to produce capsule on PIF agar nor XLD but they formed biofilm. Hurrell *et al*. (2009b) found that some *E. coli* strains did not form capsules on PIF agar, but produced more biofilm. However, the majority of *Klebsiella* isolates in this study were able to form capsules on XLD and PIF agars Table 3-11. This is important because it indicates that PIF allows the strains to produce capsules. Van Acker *et al.* (2001) stated that PIF composition is important for bacteria for pre-attachment to feeding tubes to survive and grow. In addition, Caubilla-Barron *et al*. (2007) showed that species growing on PIF could yield notable hyper-mucoviscosity that led to growth on the inverted lid of the agar plate by dripping. Capsule formation is considered a highly virulent trait in these isolates, which could pose a high risk to neonatal health.

Haemolysin is an important virulence factors which is found in clinical isolates, such as *Serratia* species, *Klebsiella* spp. and *E. coli* (Schmidt *et al*., 1995). There are three different haemolysis categories; partial or Alpha-haemolytic (α), complete or Beta-haemolytic (β) and

Gamma-haemolytic (γ) indicating the absence of haemolysis. In this current study, *E. coli* and *Klebsiella* species were investigated for their capability to lyse sheep and horse erythrocytes. All were ɣ-haemolytic on sheep blood; Table 3-10 and Table 3-11. Whereas, most showed ß-haemolytic activity on horse blood. Ring *et al*. (2002) reported that β-haemolysin plays a crucial role in streptococcal group B sepsis by contributing to high mortality and liver failure. Therefore, these findings indicate that the potential risk factor for the neonatal health is the ability of these isolates to produce β-haemolysis, consequently destroying the cell membrane of red blood cells.

Usually, pathogenic bacteria such as *Klebsiella* or *E. coli* when ingested with food will be affected by the stomach acidity, which hinders the survival of the ingested pathogens. Acidic tolerance was determined by exposing strains to acidified PIF after adjusting the pH to 3.5 for two hours at body temperature; Figure 3-17 and Figure 3-18. Hurrell *et al*. (2009a) reported that the acidity of human stomach ranged between 2.5 to 4.3 depending on the ingested food type. Most of the *E. coli* strains tested in the current study were able to tolerate the 2 hours exposure time at pH 3.5 with the exception of *E. coli* strains 1982 and 1985 which were only resistant for up to 1 h and then their viable count slightly decreased. Of the *Klebsiella* strains tested, all were resistant to pH 3.5 for two hours except for *Klebsiella oxytoca* strain 674, which was only resistant for up to 30 min and then their viable count gradually decreased from log 7.5 to log 5. The ability of these isolates to persist and multiply in conditions mimicking stomach acidity means these strains may be able to invade the intestinal tract and cause neonatal illness. According to figures 3.9 & 3.12 and 3.17 & 3.18 there was no correlation between the degree of biofilm formation and strain survival in low acidity. Nevertheless, strain 674 was unable to form significant biofilm and was more susceptible to acid compared to other strains. Together, these data suggest that strain 674 might be less capable causing a gastrointestinal infection from feeding tube contamination than the other strains tested. The capability of isolates to survive exposure to low pH may be due to their ability to adhere within the feeding tube and form biofilm. Bacteria in biofilms may also be intrinsically more resistant to acid than planktonic bacteria. For example, McNeill and Hamilton, (2006), reported that *Streptococcus mutans* biofilm is very highly resistant to low acidity.

Human serum contains a wide range of factors with antimicrobial activity against most of the microorganisms, in particular Gram-negative bacteria. Therefore pathogenic bacteria have the ability to escape from the bactericidal effect of serum (Seigfried *et al.,* 1994). In the present study, all *E. coli* and *Klebsiella* spp. strains were able to tolerate the human serum bactericidal activity at four time-points up to 3 hours Figure 3-19 and Figure 3-20. This is consistent with the ability of these strains, isolated from NEFTs and sepsis cases, to survive in the bloodstream. Several previous reports documented that the majority of Gram-negative bacteria, especially members of *Enterobacteriaceae* have the ability to survive in human serum. In addition, they stated that regardless of the severity in symptomatic UTI patients, serum resistance plays a crucial role in the pathogenicity (Johnson *et al.,* 1991). Furthermore, several recent studies have suggested that proteins such as protease, outer membrane protein (*ompA*), siderophores and plasmid-encoded proteins *traT* may play important roles in human serum resistance (Bogard and Oliver, 2007; Phan *et al*., 2013; Miajlovic *et al*., 2013). On the other hand, Kanukol *et al*. (1985), reported that about half (58%) of serum resistant extraintestinal *E. coli* strains carrying *traT* gene and was found frequently human serum sensitive isolates. Surprisingly, *E. coli* 605 and 1982 isolates which lack the *traT* gene, were able to tolerate human serum which suggests that there is no association between *traT* gene and serum resistance in the strains we studied. This is consistent with previous studies which have suggested that there are other factors that contribute to human serum resistance.

Sepsis is a neonatal life-threating condition, which requires immediate appropriate antibiotic therapy, so drug choice is very important (Taheri *et al.,* 2001). In recent years, antimicrobial resistance among *K. pneumoniae* and *E. coli* has emerged and become globally problematic (Rezaee *et al.,* 2011). In 2001 and 2010, Bradford and Bush reported that *E. coli* and *Klebsiella* are the most common species among Gram-negative bacteria to appear in both community and hospital settings. In addition, they were responsible for numerous hospital outbreaks in Europe and USA, and in Italy, these species were responsible for nosocomial infections (Perilli *et al*., 2002; Woodford *et al*., 2011) and in France, single-clone epidemics in neonatal intensive care unit (Bagattini *et al*., 2006). In 2006, Talbot and co-authors stated that the Infectious Diseases Society of America through the Antimicrobial

Availability Task Force listed that multidrug-resistant and ESBL-producing *Enterobacteriaceae* (*E. coli* and *Klebsiella* spp.) are considered as one of six problematic antimicrobial-resistant pathogens worldwide. Additionally, in 2017, *E. coli* and *Klebsiella* strains ranked third and fourth respectively of critical priority pathogens according to WHO classification (Tacconelli *et al*., 2017). In the present study, phenotypically, the majority of *E. coli* strains tested were susceptible to various antibiotics; imipenem, meropenem, ceftazidime and ciprofloxacin and some strains showed low sensitivity to ampicillin and gentamycin. However, most strains were resistant to augmentin and amikacin Table 3-12.

Furthermore, the majority of these isolates were able to produce ESBL. Of particular note is the ability of some of these isolates to resist the antibiotics. This was confirmed by genome sequencing of those strains which were found to carry genes encoding multidrug efflux pumps Table 3-7. In contrast, antimicrobial susceptibility testing profile of the *Klebsiella* spp. showed that most of the isolates were resistant to almost all antibiotics commonly used for the treatment sepsis except ciprofloxacin. In addition, all of the isolates were ESBL producers. These findings agree with (Talbot *et al.,* 2006), further, it suggests that neonates may be directly exposed to antibiotic-resistant isolates in NICUs, which might contribute to resistant infections becoming refractory to treatment with the available antibiotics. Thus, there is a high potential of infectivity of these strains to newborn babies that have weak immune systems.

In this chapter, I characterised strains isolated from NEFTs and neonatal sepsis cases at QMC and NCH hospitals. *K. pneumoniae* is still particularly under studied so, in the next chapter, I will focus in more detail on *K. pneumoniae* strains, broadening the study to also include strains isolated from feeding tubes (EFT). Nottingham NEFTs isolates (453, 497, 1446) and sepsis isolates (2291, 2298, 2312) from this chapter were selected according to their *rpo*B, PFGE and virulence profiles for comparison versus representative *K. pneumoniae* strains 1681, 1699, 1701, 1725 and 1734 which were isolated from EFT from Jordan hospitals in order to compare the two strain sets genomic-ally and phenotypically. The aim of the next chapter was to attain a comprehensive understanding of how *K. pneumoniae* can cause infections and persist in various environments.

Chapter 4. Characterisation of *Klebsiella pneumoniae* isolated from neonatal nasogastric feeding tubes (Jordan strains)

4.1. Introduction;

4.1.1. General background;

Several research groups have reported the ability of bacteria to form biofilms on inanimate surfaces such as plastic and glass. Moreover, many studies have found that bacteria can also form biofilms on various medical devices, including ventilator tubes, catheters, and enteral feeding tubes (Donlan and Costerton, 2002; Iversen *et al*., 2004; Juma and Forsythe, 2015). Biofilm formation on medical devices has been associated with hospital-acquired infections (HAI), which may lead to protracted hospitalisation and debilitating infections (Mitchell *et al.,* 2016).

Premature infants are highly susceptible to infection due to having underdeveloped immune systems. Impaired immunity will lead to an increase in intestinal mucosa permeability and immature microflora of the gut (Greenough, 1996; Mehall *et al*., 2002ab; Townsend & Forsythe, 2008). The nasogastric enteral feeding tube (NGT) may act as a site for bacterial colonisation, and is therefore a possible source of infectious organisms, which would influence the neonatal intestinal microbiome (Hurrell *et al*., 2009a).

Many studies found that neonatal feeding tubes are inhabited by several types of flora as well as opportunistic bacterial pathogens. Hurrell *et al*. (2009a), Alkeskas *et al*. (2015), Ogrodzki *et al*. (2017) and Gomez *et al*. (2016) reported that 76% of NEFT samples collected from neonatal intensive care units contained biofilms, with up to 10^7 bacterial colony-forming units per tube (CFU/tube). The microbial flora complex in these tubes included various bacterial species of the Enterobacteriaceae (such as *E. coli* K1, *Enterobacter hormaechei, E. cancerogenus, Cronobacter sakazakii, Serratia marcescens*, and *Klebsiella pneumoniae*), as well as *Pseudomonas fluorescens, P. luteola*, staphylococci, lactic acid bacteria, and fungal species such as *Candida albicans*.

Among these pathogenic organisms, *K. pneumoniae* is a very important opportunistic pathogen which is responsible for about 75% of nosocomial and community-acquired *Klebsiella* infections, such as septicaemia, urinary tract infections (UTIs), gastrointestinal infections and pneumonia (WHO, 2011; Kanevsky-Mullarky *et al*., 2014). These bacteria can express several virulence factors which contribute to causing host infections. These virulence factors include capsular polysaccharides, siderophores, lipopolysaccharide (O-antigen) and fimbriae (Podschun *et al*., 2001; Li *et al*., 2014); Figure 4-1 and Table 4-1. Other factors that contribute to pathogenesis include antimicrobial resistance and haemolysin.

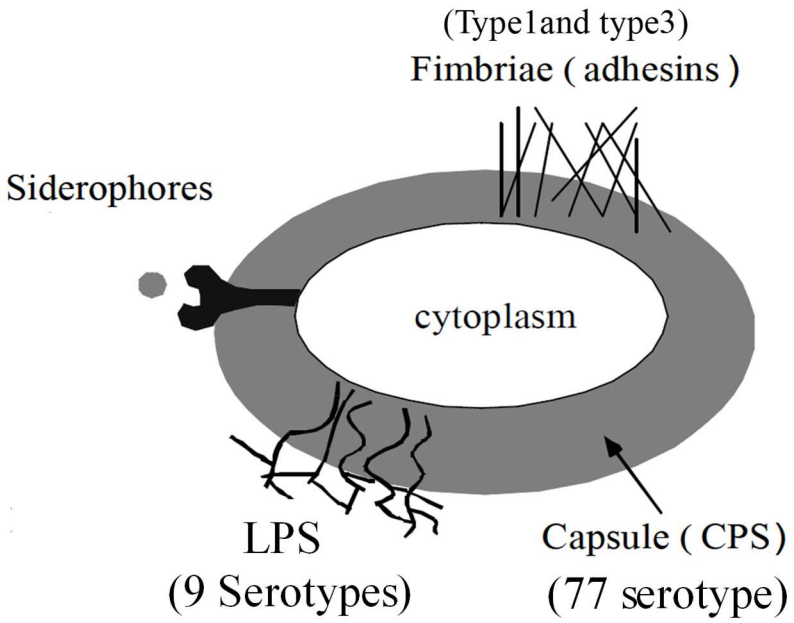


Figure 4-1 Virulence factors of *K. pneumoniae*; Podschun et al., (1998).

Table 4-1: Summary of virulence factors associated with the pathogenicity of *K. pneumoniae.*

Virulence factor	Role in pathogenisis
Capsule	Capsules in bacteria, are considered as one of the most important virulence factors, and play an important role in protecting them from harsh environments such as phagocytic cells, desiccation, acidity and serum activity
siderophores	Siderophores which have high affinity to remove (chelate) iron from the binding proteins for bacterial survival
Fimbriae (adhesins)	Important mainly responsible for biofilm formation on biotic and abiotic surfaces
LPS	O antigen provides serum resistance

In 2017, the World Health Organisation (WHO) declared that bacterial antimicrobial resistance has increased, becoming a globally problematic threat to human health. Available neonatal data revealed widespread antimicrobial resistance to gentamycin, ampicillin and third generation cephalosporins for both community and hospital-acquired neonatal sepsis (Downie *et al*, 2013; Aiken *et al*, 2011; Thaver *et al*, 2009; Bates *et al*, 2014 and Zaidi *et al*, 2005). The β-lactam family of antibiotics are widely administered to treat bacterial infections depends on species, due to the presence of a β-lactam ring, which is responsible for the inactivation of the penicillin-binding protein (PBP). Among these families, the carbapenems are the most effective antibiotics, thus providing broad-spectrum activity due to the β-lactam ring, together with a carbapenem. This feature gives carbapenems high stability against most β-lactamase producers. Therefore, carbapenem resistance constitutes a global health problem because of the drug of last resort as treatment option (Sanchez, 2015). In 2006-2007, the National Healthcare Safety Network stated that carbapenem resistance was evident in 10.8% and up to 4.0% of *K. pneumoniae* and *E. coli* isolates, respectively, which were associated with medical device infections (Hidron *et al*., 2008). Supporting to this notion, Wilson and Török, (2018) viewed that the spread of multidrug resistance pathogens such as carbapenem-resistant among Enterobacteriaceae especially *K. pneumoniae* continued increasing, becomes one of the biggest health threats globally.

4.1.2. Impacts of *K. pneumoniae*;

Today, *K. pneumoniae* is considered second to *E. coli*, which is recognised as the most common pathogenic Enterobacteriaceae species causing bacteraemia, septicaemia and UTI. *K. pneumoniae* can spread more readily than *E. coli* in hospital environments (Bouchillon *et al.*, 2013; Hennequin, C. and F. Robin., 2016). Additionally, *K. pneumoniae* is commonly associated with nosocomial and community-acquired infections, particularly in premature babies and the elderly (Chung *et al*., 2007; Hennequin, C. and F. Robin., 2016). Rice LB, (2008) stated that *K. pneumoniae* is a member of the ESKAPE pathogens group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species). It poses a high threat to public health, due to its ability to cause hospital- and community-acquired infections. The pathogenicity of *K. pneumoniae* is based on various virulence factors, such as capsules, iron acquisition system (siderophores) and adhesions (Brisse *et al*., 2009). Among these virulent traits, the capsule plays an important role in protecting the bacterium from the engulfment during infection (Domenico *et al*., 1994). Fung *et al*. (2007) reported that among 77 capsular (K) serotyping scheme, it is considered that K1, K2, K4 and K5 are linked with severe infections and very high virulence traits, particularly the K1 and K2 serotypes, which are highly associated with pyrogenic liver abscesses (PLA). With regards to the iron acquisition (siderophores) virulence trait. *K. pneumoniae* like any other pathogen, secretes siderophores (enterobactin, aerobactin, yersiniabactin, and salmochelin) that acquire ferrous and ferric iron from host binding proteins and then re-enter the bacterial cell by specific-siderophores receptors. Siderophores are therefore a key factor and very important step for their variety of metabolic processes and replications in pathogens (Ofek *et al*., 1993; Garenaux *et al*., 2011). Invasive procedures such as urinary catheters and ventilators can allow *K. pneumoniae* to invade the bloodstream of immuno-compromised hospitalised patients, contributing to serious infections with high mortality and morbidity (Borer *et al*., 2009; CDC. 2011). Several previous studies have documented that *K. pneumoniae*, which is responsible for causing liver abscesses, is also the most common causative agent of diabetes mellitus in Taiwan, with a prevalence ranging from 47–75%. Similar findings were also reported later in Singapore, Thailand and the USA (Wang *et al*., 1998; Chou *et al*., 1997; Yeoh

et al., 1997). Several recent studies have reported that *K. pneumoniae* has become significantly problematic worldwide. In Italy, for example, carbapenem-resistant *K. pneumoniae* was found to be associated with severe and fatal infections in patients with haematological malignancies (Trecarichi *et al*., 2015; Pagano *et al*., 2014; Girmenia *et al*., 2015). Furthermore, these bacteria are known to be more tolerant to desiccation and heat, demonstrated by their ability to persist on the skin more than other members of the Enterobacteriaceae. Alshara (2010) investigated 597 strains isolated from children at the Princess Rahmah Hospital, in Jordan. The most predominant pathogens found were *E. coli* (72.9%), followed by *Klebsiella* spp. (19.9%), which can develop multidrug resistance to commonly used antibiotics.

4.1.3. Physiological and genotypic virulence tests;

The physiological studies described in this chapter include determination of biofilm formation on powdered infant formula (PIF), presence of curli fimbriae, haemolysis and the presence of extended-spectrum β-lactamases (ESBLs). Capsule production was determined by colony morphology on XLD and PIF agar. The susceptibilities of isolates to antimicrobial agents was determined by the breakpoint method on antibiotic supplemented Iso-Sensitest agar (ISA) according to the British Society for Antimicrobial Chemotherapy (BSAC Methods, 2015). In order to determine the virulence potential and possible pathogenicity of isolates. Genotyping through identification and characterisation of bacterial isolates and their subtypes is becoming increasingly important, as it can be used to determine whether the strains are clonally related. Such methods would include DNA fingerprinting via pulsed-field gel electrophoresis (PFGE), according to CDC (2004), Tenover *et al*. (1995), and Bick *et al*. (2011). Among the most significant virulence factors, specific PCR probes such as genes involved in capsule formation and capsule typing, especially K1, K2, K5, and hyper-muco-viscosity are very important for virulence. For examples, these factors are responsible for increasing resistance to the host serum complement factors, and thus avoidance of the host phagocytic cells (Lai *et al*., 2003; Wu *et al*., 2009). In addition, fimbrial genes (*fim* and *mrk* operons) facilitate adherence to the host tissues and surfaces, and some strains have

siderophores (iron-acquisition systems), which are involved in systematic survival for these bacteria (Struve *et al*., 2009).

4.1.4. Aims of the Study:

The aims of this project were to apply phenotypic and genotypic methods to characterise a collection of seventy-five clinical isolates of *K. pneumoniae* collected from feeding tubes from two Jordanian hospitals: King Abdallah Hospital (KAH) and Princess Rahma Hospital (PRH). This project is a part of a larger study conducted by NTU groups since the initial studies by Hurrell in 2009, in order to further understand and identify new virulence traits that contribute to disease. Of particular note in this study is that this collection of *K. pneumoniae* strains is unique and they have not been studied before. This study includes investigations into virulence, physiological traits and genetic profiling using molecular tools including:

1- Physiological traits, including biofilm formation, cellulose and curli fimbriae, capsule production, and antibiotic sensitivity (breakpoint method).

2- Virulence traits, including serum resistance and ability of bacterial colonies to induce haemolysis when grown on blood agar.

3- Genotyping strains profiled using *rpoB* gene and PFGE schemes to determine if indistinguishable strains were isolated on different occasions within or between the two hospitals.

Specific PCR probes for virulence-associated genes K1, K2, K5, fimbriae types, invasion, detection of β-lactamase genes and siderophores were used. Following this, interesting isolates of *K. pneumoniae* strains 1681, 1699, 1701, 1725 and 1734, were selected according to their *rpo*B, PFGE and virulence profiles for comparison against representative strains, which were chosen from those in chapter 3 (Nottingham; NEFTs isolates 453, 497 and 1446, sepsis strains 2291, 2298 and 2312). Based on their *rpo*B, PFGE and virulence profiles, strains were subjected to whole genome sequencing to compare a range of virulence traits with *in vitro* tissue culture models of pathogenesis to evaluate and characterise the potential risk factors of these isolates, in order to determine any unique virulence, source and relatedness

of these strains. This included adhesion and invasion assays using different cell lines, such as survival in macrophages, and ability of these strains to adhere to and invade human epithelial and endothelial cells. *K. pneumoniae* is recognized as an important opportunistic pathogen that frequently causes urinary tract infections (UTI), septicaemia, meningitis, or pneumonia, particularly in immunocompromised individuals. Therefore, I used T24 (human bladder carcinoma cells), (Caco2) human colonic carcinoma epithelial cells, (HBMEC) human brain microvascular epithelial cells and macrophage cell line (U937) to determine the ability of these strains (*K. pneumoniae*) to attach, invade and survive within these cell lines. Additionally, haemolysis genes, genes involved in biofilm formation and any other features of interest were assessed following further genome analysis. The invasive potential of these clinical isolates in the human body is of great importance and has not been investigated extensively to date. It is therefore important to identify the infectivity potential of these bacteria in immuno-compromised neonates, especially those exposed to high levels of these organisms within the hospital environment, with the aim being to confirm the presence or absence of specific genes that are responsible for some traits.

4.2. Materials and Methods:

The methodology of this chapter is described in detail in chapter 2 Materials and Methods (Section 2). In the current study, seventy-five enteral feeding tube (biofilm material from inside tube) and flushed milk (flushed feed from neonatal nasogastric feeding tubes) isolates of *K. pneumoniae* from two Jordanian hospitals (KAH) and (PRH) were analysed for further analysis to investigate their potential virulence factors Table 4-2. Furthermore, heat, desiccation, whole genome sequencing and *in vitro* tissue culture model assays were used in a comparison study between 11 *K. pneumoniae* strains isolated from EFT Jordanian hospitals (1681, 1699, 1701, 1725 and 1734) and Nottingham neonatal enteral feeding tubes (NEFTs) isolates (453, 497, and 1446) and sepsis isolates (2291, 2298, and 2312) from previous chapter (chapter 3) Table 4-3, which were selected based on their PFGE, physiological and genotypic virulence factors. A collaborative approach has been taken in this study, and credit must be given to my colleague Pauline Ogrodzki (NTU), who carried out the whole genome sequencing, as part of a parallel PhD study.

Table 4-2: Summary table of *K. pneumoniae* strains isolated from EFT (KAH and PRH) used in this chapter.

Source of sample	D.O.I	NTU strain	Source of bacteria		A.O.N	Kind of IF
			Flushed milk	Tube		
KAH 6	22/05/2011	1727		*Klebsiella pneumoniae*	5 days old	bebelac
		1728		*Klebsiella pneumoniae*		12 meal
KAH 17	31/05/2011	1699	*Klebsiella pneumoniae*		26 days old	neosure
		1700	*Klebsiella pneumoniae*			12 meal
KAH16	31/05/2011	1701		*Klebsiella pneumoniae*	24 days old	neosure
		1702		*Klebsiella pneumoniae*		12 meal
		1703	*Klebsiella pneumoniae*			
		1704	*Klebsiella pneumoniae*			
KAH1 15	31/05/2011	1705		*Klebsiella pneumoniae*	15 days old	S26
		1706		*Klebsiella pneumoniae*		12 meal
		1707	*Klebsiella pneumoniae*			
		1708	*Klebsiella pneumoniae*			
KAH 34	07/12/2011	1734		*Klebsiella pneumoniae*	18 days old	neosure
		1735		*Klebsiella pneumoniae*		
		1736	*Klebsiella pneumoniae*			
PRH A	04/07/2011	1686	*Klebsiella* pneumoniae		21 days old	bebelac
		1687	*Klebsiella* pneumoniae			12 meal
		1688		*Klebsiella* pneumoniae		
		1689		*Klebsiella* pneumoniae		
PRH B	04/07/2011	1690		*Klebsiella pneumoniae*	12 days old	bebelac
		1691		*Klebsiella pneumoniae*		12 meal
		1692	*Klebsiella pneumoniae*			
PRH H	06/07/2011	1709		*Klebsiella pneumoniae*	16 days old	bebelac
		1710		*Klebsiella pneumoniae*		12 meal
		1711	*Klebsiella pneumoniae*			
		1712	*Klebsiella pneumoniae*			
PRH I	6\7\2011	1722	*Klebsiella pneumoniae*		39 days old	bebelac
		1723	*Klebsiella pneumoniae*			12 meal
		1724		*Klebsiella pneumoniae*		
PRH G	06/07/2011	1725	*Klebsiella pneumoniae*		15 days old	bebelac
		1726	*Klebsiella pneumoniae*			12 meal
PRH F	07/07/2011	1717		*Klebsiella pneumoniae*	35 days old	bebelac
PRH E	09/07/2011	1730		*Klebsiella pneumoniae*	19 days old	Bebelac
		1731		*Klebsiella pneumoniae*		12 meal
		1732	*Klebsiella pneumoniae*			
		1733	*Klebsiella pneumoniae*			
PRH C	09/07/2011	1693		*Klebsiella pneumoniae*	37 days old	bebelac
		1694		*Klebsiella pneumoniae*		12 meal
		1695	*Klebsiella pneumoniae*			
		1696	*Klebsiella pneumoniae*			
PRH D	09/07/2011	1697		*Klebsiella pneumoniae*	23 days old	beblac
		1698		*Klebsiella pneumoniae*		12 meal
PRH N	15/07/2011	1729	*Klebsiella pneumoniae*			12 meal
PRH P	23/07/2011	1681	*Klebsiella pneumoniae*		21 days old	bebelac
		1682	*Klebsiella pneumoniae*			12 meal
PRH J	23/07/2011	1683		*Klebsiella* pneumoniae	9 days old	bebelac
		1684		*Klebsiella* pneumoniae		12 meal
		1685		*Klebsiella* pneumoniae		
		1713	*Klebsiella pneumoniae*			
		1714	*Klebsiella pneumoniae*			
PRH L	23/07/2011	1715	*Klebsiella pneumoniae*		9 days old	bebelac
		1716	*Klebsiella pneumoniae*			12 meal
PRH O	23/07/2011	1718		*Klebsiella pneumoniae*	9 days old	bebelac
		1719		*Klebsiella pneumoniae*		12 meal
		1720	*Klebsiella pneumoniae*			
		1721	*Klebsiella pneumoniae*			
		1738	*Klebsiella pneumoniae*			
		1737		*Klebsiella pneumoniae*		12 meal
		1739	*Klebsiella pneumoniae*			
		1740	*Klebsiella pneumoniae*			
		1741	*Klebsiella pneumoniae*			
		1742		*Klebsiella pneumoniae*		
		1743		*Klebsiella pneumoniae*		
		1752		*Klebsiella pneumoniae*		
		1753		*Klebsiella pneumoniae*		
		1744		*Klebsiella pneumoniae*		12 meal
		1745	*Klebsiella pneumoniae*			
PRH AE	15/12/2011	1746	*Klebsiella pneumoniae*		7 days old	S26L
		1747	*Klebsiella pneumoniae*			12 meal
PRH AT		1748		*Klebsiella pneumoniae*		S26
		1749		*Klebsiella pneumoniae*		
		1750	*Klebsiella pneumoniae*			12 meal
		1751		*Klebsiella pneumoniae*		
PRH W	20/12/2011	1754	*Klebsiella pneumoniae*		11 days old	S26
		1755	*Klebsiella pneumoniae*			12 meal
		1756	*Klebsiella pneumoniae*			

KAH= King Abdallah Hospital, PRH= Princes Rahma Hospital, D.O.I= Date of Isolation, A.O.N= Age of Neonate, IF= Infant formula

Table 4-3: *K. pneumoniae* isolates selected for a comparison study.

Source of sample	D.I.O	NTU strain	Source of bacteria		A.O.N	Kind of PIF
			Flushed milk	Tube		
PRH P	23/07/2011	1681	*Klebsiella pneumoniae*		21 days old	bebelac
KAH 17	31/05/2011	1699	*Klebsiella pneumoniae*		26 days old	neosure
KAH16	31/05/2011	1701		*Klebsiella pneumoniae*	24 days old	neosure
PRH G	06/07/2011	1725	*Klebsiella pneumoniae*		15 days old	bebelac
KAH 34	07/12/2011	1734		*Klebsiella pneumoniae*	18 days old	neosure
QMC or NCH	N.A	453		*Klebsiella pneumoniae*	N.A	N.A
QMC or NCH	N.A	497		*Klebsiella pneumoniae*	N.A	N.A
NCH	13/03/2011	1446		*Klebsiella pneumoniae*	13 days old	N.A
QMC	01/10/2015	2291	*Klebsiella pneumoniae* Sepsis cases		N.A	N.A
QMC	01/10/2015	2298	*Klebsiella pneumoniae* Sepsis cases		N.A	N.A
QMC	07/04/2016	2312	*Klebsiella pneumoniae* Sepsis cases		N.A	N.A

KAH= King Abdallah Hospital, PRH= Princes Rahma Hospital, QMC= Queen medical Hospital, NCH= NottinghamCity Hospital, D.O.I= Date of Isolation, A.O.N= Age of Neonate, PIF= Infant formula, N.A= Not available, pale orange color for Jordanian strains, bright turquoise and turqoise colors for neonatal enteral feeding tube (NEFTs) and sepsis cases, respectively (Nottingham).

4.3. Results

4.3.1. Molecular analysis;

4.3.1.1. Pulsed Field Gel Electrophoresis

Correia, Martin, and Castro, (1994) reported that the PFGE technique is useful in defining the relatedness among different isolates within the same species, and is also a highly discriminatory technique to be used in outbreaks or local epidemiological studies. In this study, *XbaI* and *SpeI*-restriction enzymes (Promega, UK) were used to analyse *K. pneumoniae* strains isolated from flushed feed residues and inner tube biofilm collected from the NICUs in two separate Jordanian hospitals. BioNumerics software (v 3.5) was used in this study to detect a band assignment and a dendrogram for all isolates. Dice coefficient cluster analysis was used for unweighted pair group method with arithmetic mean (UPGMA), and the optimisation and tolerance of the bands was 1.5%. The band similarity of the non-clonal strains was less than 95% (Tenover *et al.*, 1995). The experiment used the standardised PFGE method in accordance with the CDC and PulseNet (2004) protocol, with 2 sets of strains from QMC and from Jordanian isolates.

PFGE for 75 *K. pneumoniae* isolates from Jordan are shown in Figure 4-2 and Table 4-4. These formed three distinguishable pulsotypes and two unique strains. Sixty-one strains were

isolated over a six-month period (04 July to 20 December 2011). These strains belonged to pulsotype Kp1. Of particular interest in this pulsotype were thirteen strains (1746, 1681, 1683, 1686, 1693, 1697, 1709, 1715, 1717, 1722, 1725, 1730, and 1754) isolated from 12 different neonates. These had been fed different types of reconstituted powdered infant formula. Seven of these neonates received bebelac, four received S26 and one received neosure powdered infant formula. Pulsetype Kp1 is composed of two sub-clusters; the difference between these sub-clusters was only one band. Two strains (1709, 1722) belonging to pulsetype Kp1 and strain 1725 belonging to pulsetype Kp2, had been isolated on the same day (06 July 2011), from three different neonates and two different hospitals. These babies were fed bebelac infant formula. Two strains (1701, 1705) belonged to Pulsetype Kp3 and strain 1699 (unique) were isolated on the same day (31 May 2011) from three different neonates fed S26 and neosure reconstituted infant formula. In addition, there were two unique strains (1699 and 1725). Three strains of *K. pneumoniae* (1734, 1735, and 1736) belonged to pulsetype Kp5, which were isolated on the same day (7 December 2011), from the same neonate, receiving neosure infant formula.

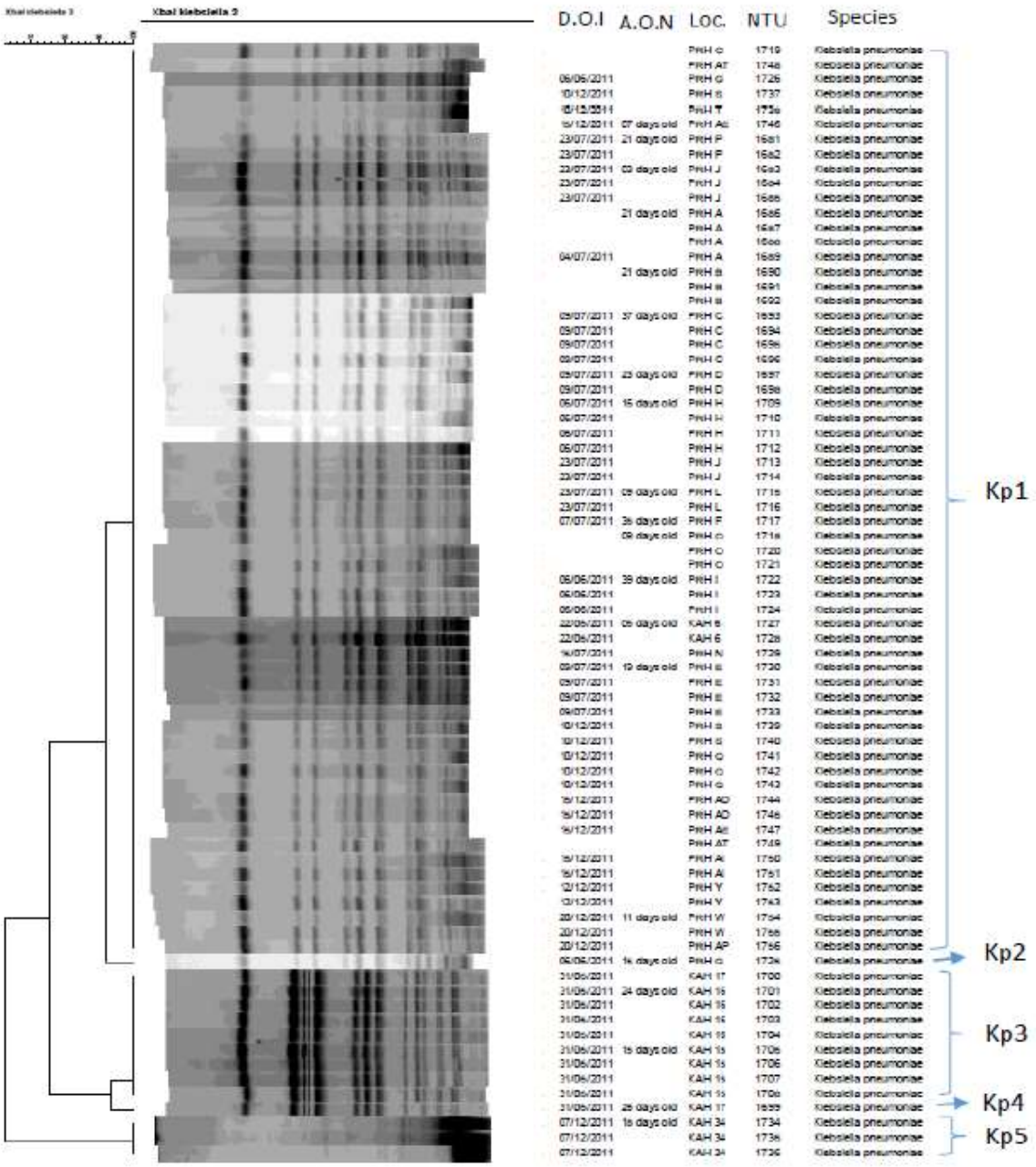


Figure 4-2: PFGE cluster analysis of *K. pneumoniae* strains isolated from EFT in two local Jordanian hospitals (KAH and PRH).

D.O.I= date of isolation, A.O.N= age of neonate, Loc. = location. XbaI digestive enzyme was used to separate DNA fragments to obtain restriction profiles. BioNumerics software, version 3.5 was used in this study to detect a band assignment and a dendogram for all isolates. CHEF-DR II (BIO-RAD, Belgium) system was used for bands separation at 6V, 14^{o}C for twenty hours with 5 seconds initial and 50 seconds final. Dice coefficient, cluster analysis was used for unweight pair group method with arithmetic mean (UPGMA).

Table 4-4: Summary of PFGE analysis of *K. pneumoniae* strains isolated from Jordan

Species	Hospital	Strain number	PFGE	N.O.I	P.O.I	Patients
K. pneumoniae	KAH	1727, 1728,	Kp1	2	One day	1
	PRH	1686, 1687, 1688, 1689, 1690, 1691, 1692, 1709, 1712, 1722, 1723, 1724, 1726, 1717, 1730, 1731, 1732, 1733, 1693, 1694, 1695, 1696, 1697, 1798, 1729, 1681, 1682, 1683, 1684, 1685, 1710, 1711, 1713, 1714, 1716, 1718, 1719, 1720, 1721, 1738, 1737, 1739, 1740, 1741, 1742, 1743, 1752, 1753, 1744, 1745, 1746, 1747, 1748, 1749, 1750, 1751, 1754, 1755, 1756		59	Over seven months	14
		1725	Kp2	1	One day	1
	KAH	1700, 1701, 1702, 1703, 1704, 1705, 1706, 1707, 1708	Kp3	9	One day	2
		1699	Kp4	1	One day	1
		1734, 1735, 1736	Kp5	3	One day	1

This table presents the PFGE analysis of Jordan strains. There were seventy five EFT isolates of K. pneumoniae from two hospitals in Jordan which were PRH (n=60) and KAH (n=15). The strains were clustered into 3 pulsotypes and there were two unique strains. KAH= King Abdallah Hospital, PRH= Princes Rahma Hospital, N.O.I= Number of Isolates and P.O.I= Period of isolation.

Representative strains were confirmed by *SpeI* restriction enzyme. The combined PFGE profile for both *XbaI* and *SpeI* restriction enzymes is shown in Figure 4-3, which demonstrates that the representative isolates are grouped into 5 pulsetypes. All these strains were isolated from flushed milk and lumen enteral feeding tubes, and the age of neonates ranged from 3 to 37 days old Table 4-4.

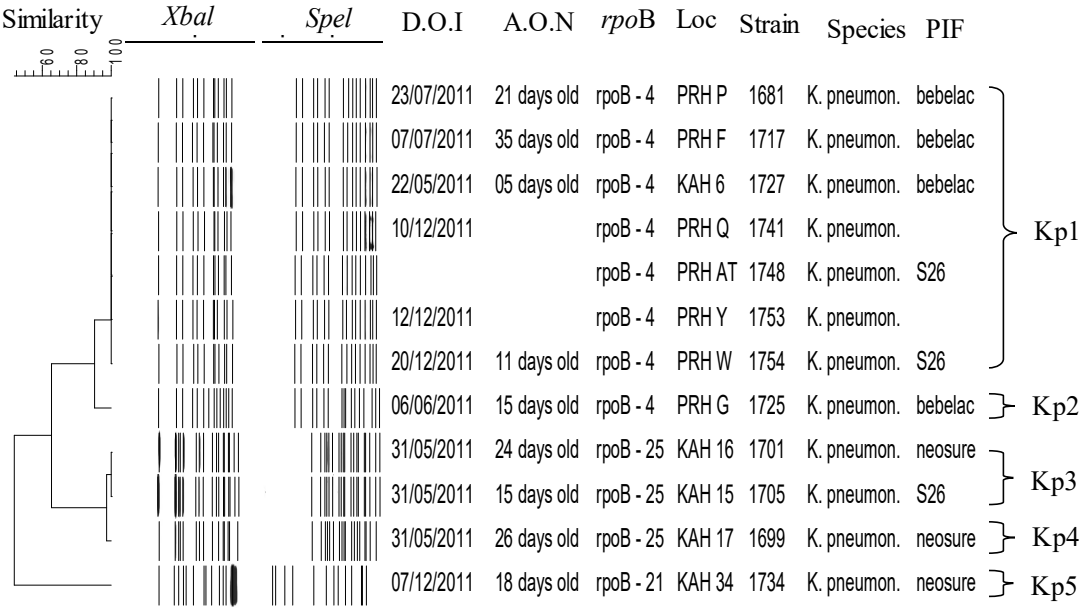


Figure 4-3: analysis using *XbaI* and *SpeI* for 12 *K. pneumoniae* strains isolated from NEFT, additional data of rpoB allele, sampling date, and source.

D.O.I= date of isolation, A.O.N= age of neonate, Loc= location, PIF= powdered infant formula, N.A= Not available. The dendrogram was generated by BioNumerics software, version 3.5, dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimisation in the band was1.50%. Three *rpoB* allele profiles were revealed *rpoB4*, *rpoB21* and *rpoB25*.

4.3.1.2. Phylogenetic analysis of *K. pneumoniae*;

In 2006, Kupfer and collaborators documented that analysis of the *rpoB* gene sequence not only identifies species within a phylogenetic framework, but also the discriminates between closely related isolates, as well as contributing useful information even at the sub-strain level. In addition, Rowland *et al.* (1993) reported that the *rpoB* gene has the potential to be used as a powerful molecular chronometer.

In this study, 75 *K. pneumoniae* strains isolated from two separate Jordanian hospitals were subjected to *rpoB* sequence analysis by PCR and comparison was made with *rpoB* sequences in the *K. pneumoniae* Pasteur Pub MLST database. There were three different *rpoB* profiles (4, 21 and 25) which correlated well with PFGE pulsetypes. *K. pneumoniae* belonging to pulsetypes 1 and 2 were all *rpoB* allele profile 4, whereas clusters 3 and 4 were *rpoB* profile 25 and pulsetype 5 was identified as *rpoB* profile 21 [Appendix C].

4.3.1.3. Multi-locus sequence typing (MLST) clonality analysis.

Nine strains of *K. pneumoniae* were selected as a representative sample for further characterisation by MLST using the sequences of seven housekeeping genes; these strains were 1681, 1690, 1693, 1715, 1725, 1699, 1701, 1705 and 1734. These were chosen based on PFGE grouping, *rpoB* analysis and associated neonatal metadata. Table 4-5 shows the sequence types (ST) obtained for each of the nine isolates, according to the Pasteur MLST database. Three different STs (ST247, ST111 and ST526) were identified across all of the PFGE groups.

Table 4-5: MLST profile of selected *K. pneumoniae* strains

NTU	Species	Hos.	PFGE	Housekeeping gene							ST
				*gap*A	*inf*B	*Mdh*	*Pgi*	*pho*E	*rpo*B	*ton*B	
1681	*K. pneumoniae*	PRH	Kp1	2	1	5	1	17	4	42	111
1690	*K. pneumoniae*	PRH	Kp1	2	1	5	1	17	4	42	111
1693	*K. pneumoniae*	PRH	Kp1	2	1	5	1	17	4	42	111
1715	*K. pneumoniae*	PRH	Kp1	2	1	5	1	17	4	42	111
1725	*K. pneumoniae*	PRH	Kp2	2	1	5	1	17	4	42	111
1699	*K. pneumoniae*	KAH	Kp4	10	7	2	2	3	25	4	247
1701	*K. pneumoniae*	KAH	Kp3	10	7	2	2	3	25	4	247
1705	*K. pneumoniae*	KAH	Kp3	10	7	2	2	3	25	4	247
1734	*K. pneumoniae*	KAH	Kp5	38	19	53	58	73	21	130	526

Hos= Hospital, KAH= King Abdallah Hospital, PRH= Princes Rahma Hospital and ST= sequence type.

4.3.1.4. Detection of virulence factors genes for *K. pneumoniae* strains (VFGs)

Based on PFGE analysis and associated neonatal metadata, 28 *K. pneumoniae* isolates were selected as representative isolates for further characterisation of various virulence traits.

In this study, a total of 28 from 75 *K. pneumoniae* isolates were selected as representative isolates for further characterisation based on PFGE analysis and associated neonatal metadata. These strains were collected from flushed lumen residues and inner tube biofilm from the NICUs in two hospitals. PCR was used to detect the presence/absence of virulence associated genes, twenty-two instances of capsular serotype K2 (78.57%) and five instances of K1 (17.85%) were identified, while K5 was not found in any of the isolates tested Table 4-6. Additionally, siderophore genes *fyuA* and *Irp2* were found in all isolates, except strains 1734, 1735 and 1736, which belonged to pulsetype Kp5.

With regards to bacterial attachment in *Klebsiella*, there are two main fimbrial types (T1P and T3P) that play an important role in bacterial cell adhesion. Both T1P and T3P fimbriae enhance *K. pneumoniae* biofilm formation on biotic and abiotic surfaces. Furthermore, T1P fimbriae plays a crucial role in colonisation, while T3P fimbriae is capable of agglutinating erythrocytes and the indwelling of urinary catheters (Murphy *et al.,* 2013; Stahlhut *et al.,* 2012). As shown in Table 4-6, all tested strains were found to harbour T1P and T3P fimbrial genes, and these findings are in agreement with phenotypic data showing that 100% of the isolates were able to form biofilms on plastic surfaces at 25 °C and 37 °C Figure 4-4.

Table 4-6: Capsular serotype and virulence distribution of *K. pneumoniae* isolated from EFT (KAH and PRH).

NTU strain	Source	Pulstype	rpoB	Genotypic								
				Capsule serotype			Adhesion (fimbriae)				Siderophores	
				K1	K2	K5	*fimH*	*fimA*	*mrkD*	*mrkA*	*Irp2*	*fyuA*
1681	PRH	Kp1	4	-	+	-	+	+	+	+	+	+
1683				-	+	-	+	+	+	+	+	+
1718				-	+	-	+	+	+	+	+	+
1686				-	+	-	+	+	+	+	+	+
1687				-	+	-	+	+	+	+	+	+
1690				-	+	-	+	+	+	+	+	+
1693				-	+	-	+	+	+	+	+	+
1697				-	+	-	+	+	+	+	+	+
1709				-	+	-	+	+	+	+	+	+
1722				-	+	-	+	+	+	+	+	+
1717				-	+	-	+	+	+	+	+	+
1731				-	+	-	+	+	+	+	+	+
1737				-	+	-	+	+	+	+	+	+
1741				-	+	-	+	+	+	+	+	+
1744				-	+	-	+	+	+	+	+	+
1746				-	+	-	+	+	+	+	+	+
1748				-	+	-	+	+	+	+	+	+
1750				-	+	-	+	+	+	+	+	+
1753				-	+	-	+	+	+	+	+	+
1727	KAH			-	+	-	+	+	+	+	+	+
1754	PRH			-	+	-	+	+	+	+	+	+
1725		Kp2	4	-	+	-	+	+	+	+	+	+
1701	KAH	Kp3	25	+	-	-	+	+	+	+	+	+
1703				+	-	-	+	+	+	+	+	+
1705				+	-	-	+	+	+	+	+	+
1708				+	-	-	+	+	+	+	+	+
1699		Kp4	25	+	-	-	+	+	+	+	+	+
1734		Kp5	21	-	-	-	+	+	+	+	+	-

KAH= King Abdallah Hospital, PRH= Princess Rahma Hospital and PT= Pulsetype, +/- = presence/ absence, 4, 21 and 24= number of rpoB allel profiles.

4.3.2. Physiological Virulence Factors;

4.3.2.1. Biofilm formation;

An investigation was conducted in order to evaluate the level of biofilm formation of 28 from 75 *K. pneumoniae* strains on plastic surfaces when grown under two different incubation temperatures (25 °C and 37 °C) and types of PIF. Figure 4-4 and Table 4-7 shows that all strains have the ability to form biofilm at both temperatures compared to the control, and there was clear variation observed between the isolates. Overall, the majority of isolates at 37 °C were able to form a high amount of biofilm, whereas strains 1683, 1690, 1693, 1703, 1705, 1718, 1753 and 1754 were able to form medium biofilm. On the other hand, at 25 °C, strains 1683, 1690, 1690, 1709, 1727, 1737 and 1754 produced only a small amount of

biofilm, which was closer to the amount of biofilm produced by the control strain (media only). The remaining strains formed moderate levels of biofilm at the same temperature. It was evident from these observations that the incubation temperature played a crucial role in the ability of these isolates to form biofilm and the investigation revealed a significantly higher amount of biofilm formation at 37 °C ($P < 0.05$; one-way ANOVA) when compared to 25 °C Figure 4-5.

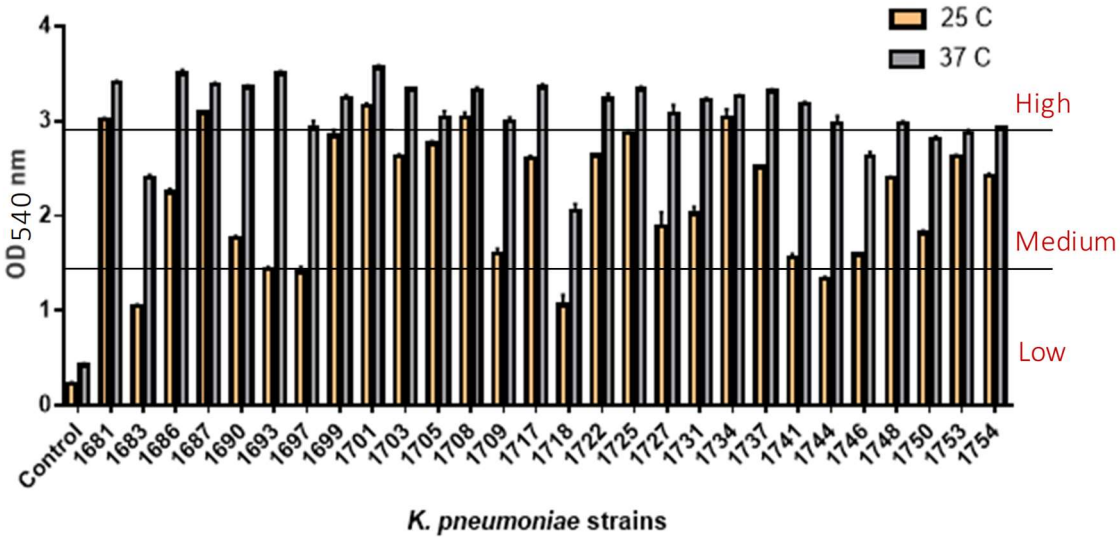


Figure 4-4: Biofilm formation of *K. pneumoniae* isolates at 25°C and 37°C in liquid PIF. Shows the preferred temperature is 37 °C

All strains produced biofilm, compared to the control, biofilm formation stained with crystal violet 0.01% (w/v) and evaluated at wavelength 540 nm; the control was non-inoculated media, the experiments were investigated in three independent times. Error bars represented using standard error.

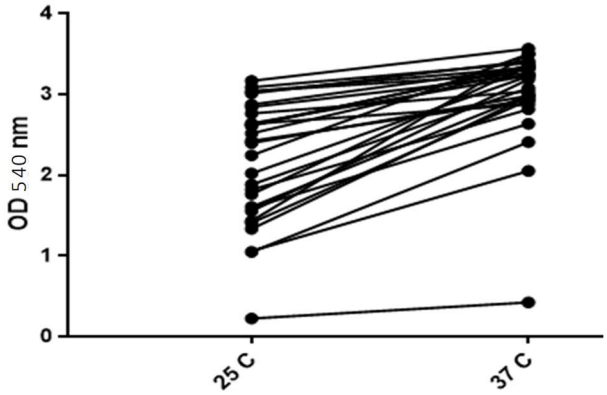


Figure 4-5: *K. pneumoniae* isolates formed significantly more biofilm in PIF at 37°C than at 25 °C (paired t test, $p < 0.0001$).

4.3.2.2. Production of curli fimbriae:

Several previous reports recommend the use of Congo red agar to provide phenotypic indication of the presence of curli fimbriae. Isolates that exhibited red-coloured colony morphology indicated the ability to produce curli fimbriae, whereas isolates demonstrating pink coloured colony morphology on Congo red agar indicated an inability to produce curli fimbriae.

Table 4-7 and Figure 4-6 shows the results for 28 strains of *K. pneumoniae* (obtained from Jordan) grown on Congo red agar at 25 °C and 37 °C for 48 h. It was revealed that only strains 1734, 1741 and 1754 showed an ability to produce red colonies on Congo red agar, and thus, an ability to express curli fimbriae. All the other isolates, on the other hand, were unable to produce curli fimbriae.

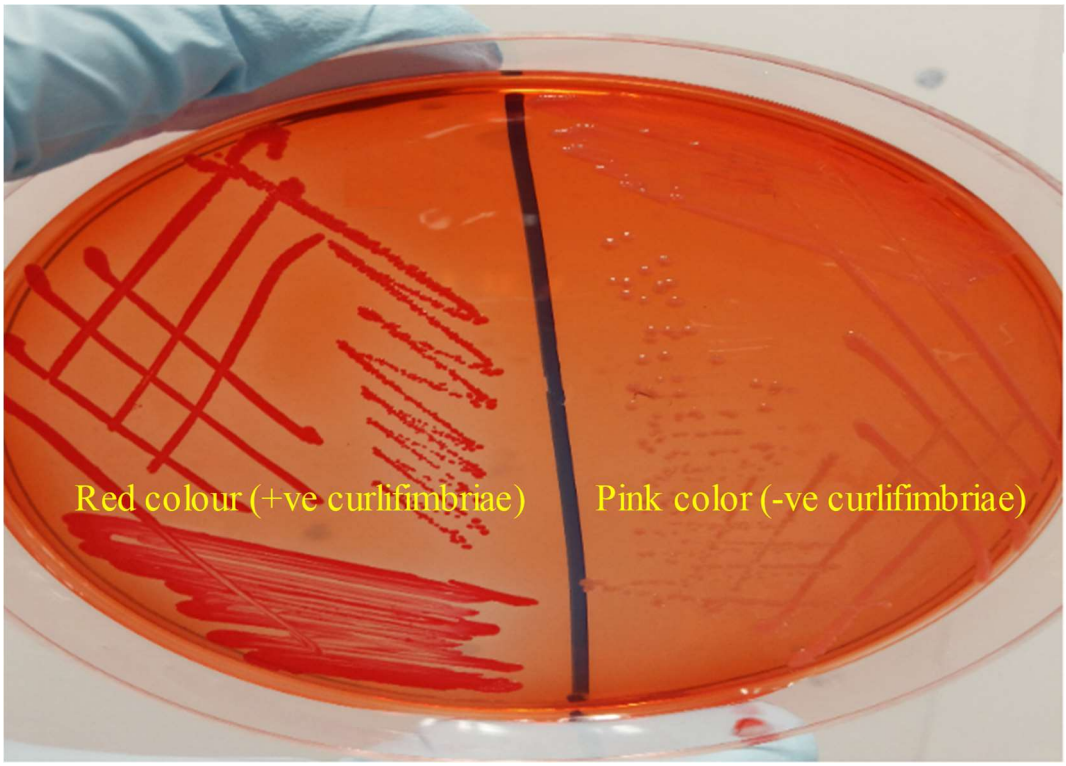


Figure 4-6: revealed colony morphology colour on Congo red agar media.

(Red mean bacteria expressed curli fimbriae and pink colour as a negative).

4.3.2.3. Cellulose production (Calcofluor);

In the Calcofluor binding assay, LBA medium supplemented with Calcofluor White stain was used to investigate the capability of 28 from 75 *K. pneumoniae* strains to express cellulose production. Macroscopically, the colony appearance and cellulose production was determined by using ultra-violet (366 nm) fluorescence. Figure 4-7 and Table 4-7 revealed that all examined isolates were able to produce cellulose, and clear variation in cellulose production was noted among isolates. Strains 1683, 1686, 1687, 1744, 1746, 1748, 1750, 1753 and 1754 grew on Calcofluor and exhibited moderate (++) fluorescence at 366 nm, whereas the remaining isolates expressed strong (+++) fluorescence indicating high level cellulose production.

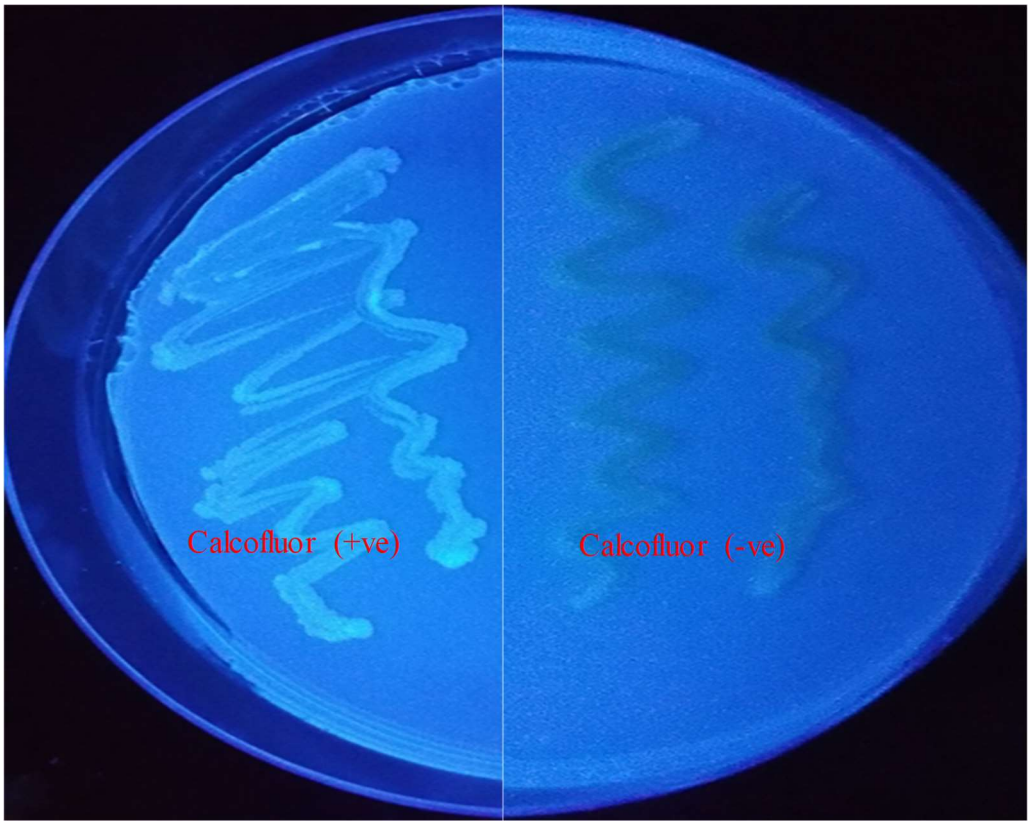


Figure 4-7: showing cellulose production (expression) on Calcofluor.

This figure reveals cellulose production (expression) on Calcofluor. A positive test is indicated by the presence of fluorescence around the isolate, as seen in the positive control (+ve), negative control (-ve) there is no fluorescence.

4.3.2.4. Detection of iron siderophores by using CASAD

Of the known virulence factors associated with pathogenicity of *Klebsiella* spp., siderophores (Iron acquisition system) are essential for many microorganisms and are considered a major virulence trait for *Klebsiella* spp.(Eijkelkamp *et al.,* 2011). In this study, a total of 28 from 75 *K. pneumoniae* isolates were selected as representative isolates for further characterisation based on PFGE analysis and associated neonatal metadata. These strains were subjected to culture on CAS agar, and revealed their ability to express siderophores on CAS agar as they demonstrated an orange halo around the inoculated wells Figure 4-8 and Table 4-7 A *Y. enterocolitica* strain (8081) was used as a positive control and NTU strain *C. sakazakii* 520 was used as a negative control.

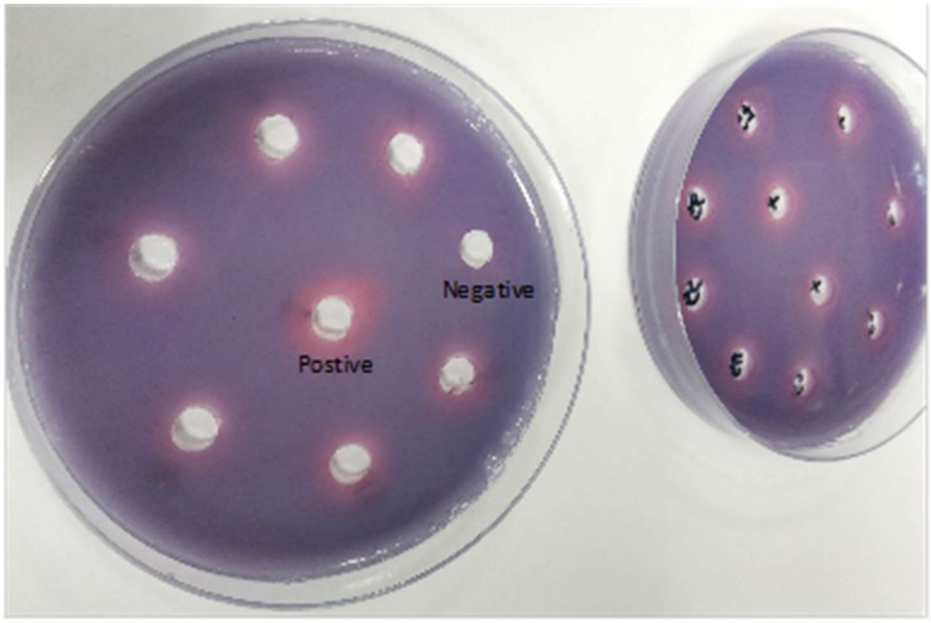


Figure 4-8: Siderophore production on CASAD

Wells were filled with cell free culture supernatant of *K. pneumoniae* isolates. All isolates were able to produce iron siderophores. CAS agar showed orange halo around the site of inoculation indicating siderophore production. *Y. enterocolitica* strain 8081 was the positive control and *C. sakazakii* strain 520 was the negative control.

4.3.2.5. Capsular production;

Capsule production of the 28 *K. pneumoniae* strains was investigated phenotypically by the appearance of the colony morphology on two different media including, XLD agar and agar

containing powdered infant formula (PIF). The ability of isolates to produce capsular material was classified into four categories according to the relative amounts of capsular material produced, and these were defined as: high (+++), medium (++), low (+) and non-mucoid (-). As presented in Table 4-7, there was notable variation in capsule production observed among the 75 strains examined, which formed colonies on XLD and PIF. Strains 1681, 1682, 1686, 1687, 1709, 1717, 1731, 1746 and 1748 demonstrated an ability to produce high hyper-mucoid capsular materials on both PIF and XLD; Figure 4-9. Furthermore, on PIF, strains 1737, 1741 and 1744 produced moderate amount of capsular materials. In addition, strains 1750, 1753 and 1754 showed an ability to produce only low levels of capsular material on PIF, as well as strain 1699, which produced a low level of capsular material on XLD. On the other hand, strains 1737, 1741, 1744, 1750, 1753, 1754 and 1734 were unable to produce capsular material on XLD. It was interesting to note that most of *K. pneumoniae* was able to produce extensive amounts of capsule surrounding the cell, as indicated by Anthony's stain (copper sulphate staining) Figure 4-9 C.

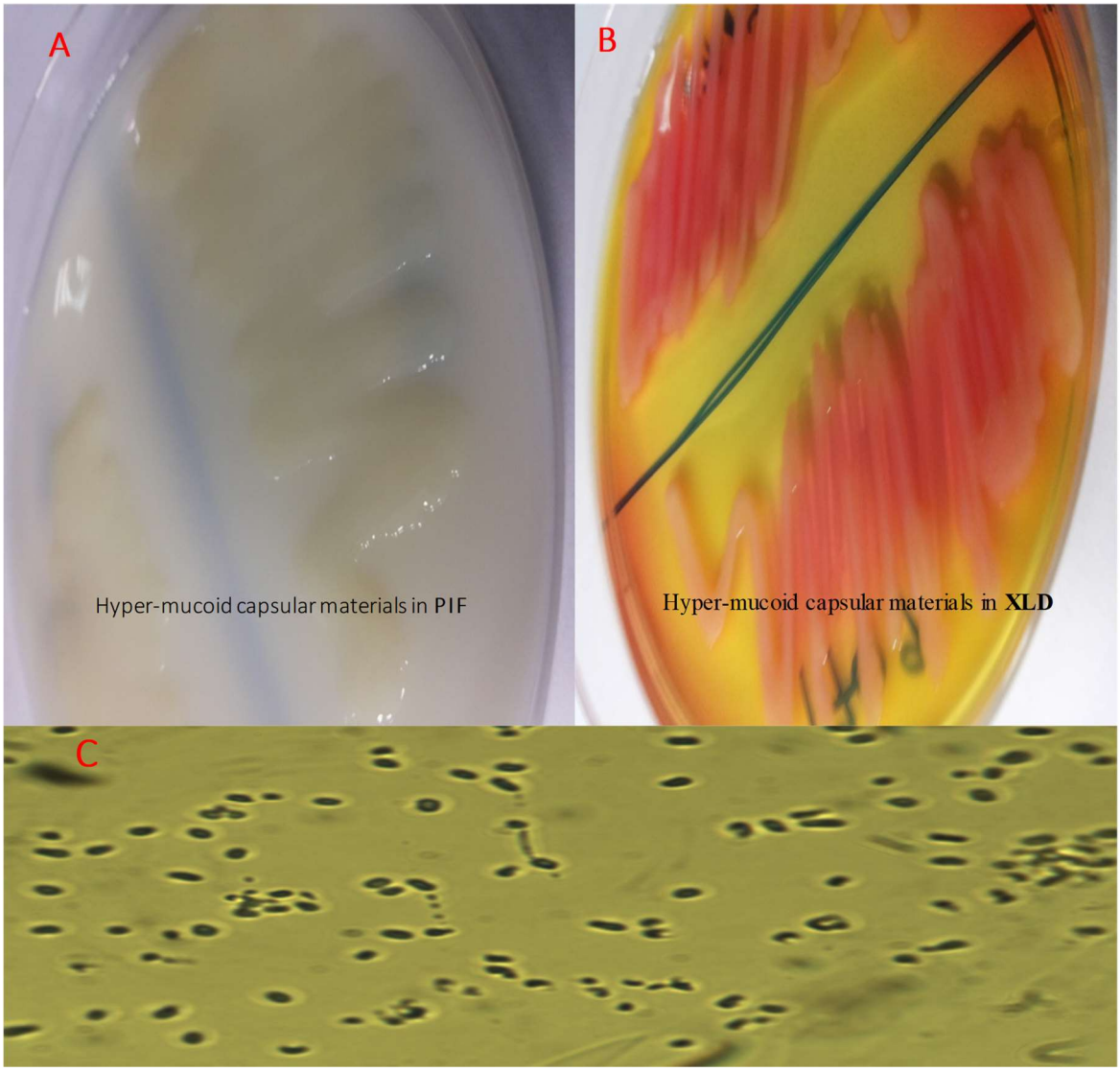


Figure 4-9: Morphology of mucoid *K. pneumoniae* isolates on PIF agar (A), XLD (B) and capsule staining.

K. pneumoniae isolates were cultured on XLD and PIF agar for 24 hrs at 37 °C and photographed. The example strain shown in these Figures exhibited hypermucoviscosity on PIF (A), was mucoid on XLD (B) and capsule was visible after staining with Anthony's stain (C).

Table 4-7: Phenotypic characterization of *K. pneumoniae* isolates from neonatal nasogastric feeding tubes

Isolate	Pulstype	Hospital	Date of isolation	Source	Congo red	Calcofluor	Capsule production		Biofilm formation		Haemolysis	
							XLD	IF	IF 37 C	IF 25 C	Sheep	Horse
1681	Kp1	1	23/07/2011	F	-	+++	+++++	+++++	M	H	ɣ	β
1683	Kp1	1	23/07/2011	BM	-	++	+++++	+++++	L	M	ɣ	β
1718	Kp1	1	23/07/2011	BM	-	+++	+++	+++++	M	M	ɣ	β
1686	Kp1	1	04/07/2011	F	-	++	+++++	+++++	M	H	ɣ	β
1687	Kp1	1	04/07/2011	F	-	++	+++++	+++++	M	H	ɣ	β
1690	Kp1	1	04/07/2011	BM	-	+++	+++	+++++	L	M	ɣ	β
1693	Kp1	1	09/07/2011	BM	-	+++	+++	+++++	L	M	ɣ	β
1697	Kp1	1	09/07/2011	BM	-	+++	+++	+++++	M	H	ɣ	β
1709	Kp1	1	06/07/2011	BM	-	+++	+++++	+++++	L	H	ɣ	β
1722	Kp1	1	06/07/2011	F	-	+++	+++	+++++	M	H	ɣ	β
1717	Kp1	1	07/07/2011	BM	-	+++	+++++	+++++	M	H	ɣ	β
1731	Kp1	1	09/07/2011	BM	-	+++	+++++	+++++	M	H	ɣ	β
1737	Kp1	1	10/12/2011	BM	-	+++	-	++	L	H	ɣ	β
1741	Kp1	1	10/12/2011	F	+	+++	-	++	M	H	ɣ	β
1744	Kp1	1	15/12/2011	BM	-	++	-	++	M	H	ɣ	β
1746	Kp1	1	15/12/2011	F	-	++	++++	+++++	M	H	ɣ	β
1748	Kp1	1	15/12/2011	BM	-	++	++++	+++++	M	H	ɣ	β
1750	Kp1	1	15/12/2011	F	-	++	-	+	M	H	ɣ	β
1753	Kp1	1	12/12/2011	BM	+	++	-	+	M	M	ɣ	β
1754	Kp1	1	20/12/2011	F	+	+++	+++	+++++	L	H	ɣ	β
1727	Kp1	2	22/05/2011	BM	-	+++	+++	+++++	M	H	ɣ	β
1725	Kp2	1	06/07/2011	F	-	++	-	+	L	M	ɣ	β
1701	Kp3	2	31/05/2011	BM	-	+++	+++	+++++	M	H	ɣ	β
1703	Kp3	2	31/05/2011	F	-	+++	+++	+++++	M	M	ɣ	β
1705	Kp3	2	31/05/2011	BM	-	+++	++	+++	M	M	ɣ	β
1708	Kp3	2	31/05/2011	F	-	+++	++	+++	M	H	ɣ	β
1699	Kp4	2	31/05/2011	F	-	+++	+	+++++	M	H	ɣ	β
1734	Kp5	2	07/12/2011	BM	+	+++	-	++++	M	H	ɣ	β

1: PRH, 2: KAH. F: Flushed feed from neonatal nasogastric feeding tubes, BM: Biofilm material from inside neonatal nasogastric feeding tubes. PRH: Princesses Rahma Hospital. KAH: King Abdulla Hospital, L= low, M= moderate, H= high, IF= infant formula.

4.3.2.6. Blood haemolysis;

In the current study, the haemolytic activity of *K. pneumoniae* isolates was investigated for their capability to lyse horse and sheep erythrocytes using TSA supplemented with 5% blood

(Horse & Sheep). Table 4-7 shows that all tested strains had ɣ-haemolytic activity on sheep blood and ß-haemolytic activity on horse blood.

4.3.2.7. Antimicrobial susceptibility;

Antibacterial susceptibility profiling and investigation into ESBL production for the *K. pneumoniae* strains was carried out. Antimicrobial susceptibility was performed for six antibiotic groups: miscellaneous (chloramphenicol and tetracycline), carbapenems (Meropenem and Imipenem), cephalosporins (Ceftazidime, Cefotaxime and Cefpodoxime), fluoroquinolones (Ciprofloxacin), penicillins (Piperacillin/Tazobactam Ampicillin, and Augmentin), and aminoglycosides (Gentamicin). Additionally, ESBL tests conducted included: Cefotaxime/Clavulanic acid and Ceftazidime/Clavulanic acid. *E. coli* NCTC 10418 was used as a control strain that is susceptible to all antibiotics tested Table 4.8.

As presented in Table 4-8, all of the screened *K. pneumoniae* strains were shown to be resistant to 10 of the 11 antibiotics tested, but were sensitive to ciprofloxacin (5 µg). Furthermore, all isolates were revealed to be ESBL-positive.

Table 4-8: Susceptibility of *K. pneumoniae* strains to antibiotics.

Strins	Pulstype	Carbabenem		Cephalosporins				ESBL family				
		IMI 10µg	MEM 10µg	CTX 10µg	CRO 30µg	CAZ 10µg	ZOX 30µg	CTX+CV 30/10µg	CPD+CV 10/1µg	GM 10 µg	AP 10µg	CIP 5µg
1681	Kp1	R	R	R	R	R	R	R	R	R	R	I
1683	Kp1	R	R	R	R	R	R	R	R	R	R	I
1686	Kp1	R	R	R	R	R	R	R	R	R	R	I
1687	Kp1	R	R	R	R	R	R	R	R	R	R	S
1690	Kp1	R	R	R	R	R	R	R	R	R	R	S
1693	Kp1	R	R	R	R	R	R	R	R	R	R	S
1697	Kp1	R	R	R	R	R	R	R	R	R	R	S
1709	Kp1	R	R	R	R	R	R	R	R	R	R	S
1717	Kp1	R	R	R	R	R	R	R	R	R	R	S
1718	Kp1	R	R	R	R	R	R	R	R	R	R	S
1722	Kp1	R	R	R	R	R	R	R	R	R	R	S
1727	Kp1	R	R	R	R	R	R	R	R	R	R	S
1731	Kp1	R	R	R	R	R	R	R	R	R	R	S
1737	Kp1	R	R	R	R	R	R	R	R	R	R	S
1741	Kp1	R	R	R	R	R	R	R	R	R	R	S
1744	Kp1	R	R	R	R	R	R	R	R	R	R	S
1746	Kp1	R	R	R	R	R	R	R	R	R	R	S
1748	Kp1	R	R	R	R	R	R	R	R	R	R	S
1750	Kp1	R	R	R	R	R	R	R	R	R	R	S
1753	Kp1	R	R	R	R	R	R	R	R	R	R	S
1754	Kp1	R	R	R	R	R	R	R	R	R	R	S
1725	Kp2	R	R	R	R	R	R	R	R	R	R	S
1701	Kp3	R	R	R	R	R	R	R	R	R	R	S
1703	Kp3	R	R	R	R	R	R	R	R	R	R	I
1705	Kp3	R	R	R	R	R	R	R	R	R	R	S
1708	Kp3	R	R	R	R	R	R	R	R	R	R	S
1699	Kp4	R	R	R	R	R	R	R	R	R	R	I
1734	Kp5	R	R	R	R	R	R	R	R	R	R	S

R= resistant, I= intermediate, S= susceptible, Imipenem (IMI), Meropenem (MEM), Cefotaxime (CTX), Ceftriaxone (CRO), Ceftazidime (CAZ), Ceftizoxime (ZOX), Cefotaxime + Clavulanate (CTX+CV), Cefpodoxime + Clavulanate (CPD+CV), Gentamicin (GM), Ampicillin (AP), and Ciprofloxacin (CIP).

4.4. Further analysis;

This part of the study was to compare and assess the potential virulence of *K. pneumoniae* isolated from flushed and neonatal enteral feeding tubes (NEFTs) from Jordanian hospitals and strains isolated from NEFTs and neonatal sepsis cases at QMC and NCH hospitals, Nottingham and assess whether they pose a potential risk in causing neonatal infections. Based on previous findings on isolates from NEFTs and neonatal sepsis cases from QMC and NCH (Chapter 3), six strains of *K. pneumoniae* (453, 497, 1446, 2291, 2298 and 2312) were selected based on their *rpoB* allele sequence, PFGE and virulence profiles for further analysis. Accordingly, five strains of *K. pneumoniae* (1681, 1699, 1701, 1725 and 1734), were also obtained from NEFTs from Jordanian hospitals. These isolates were investigated for further phenotypic and genotypic characterisation such as heat tolerance, desiccation,

tissue culture and whole genome analysis. The results obtained from these two groups were then compared for genetic relatedness.

4.4.1. Heat tolerance

Bacterial thermo-tolerance is another factor that may lead to serious neonatal infections. In this experiment, the heat tolerance of *K. pneumoniae* isolates was investigated according to previously published methodology by Iversen *et al*. (2004), in order to determine the ability of these isolates to tolerate heat at 55 °C. The bacterial survival at 55 °C was quantified and plotted against time. D-values were extrapolated at the time desired for one $\log_{10}$ reduction [(−1) / (slope of the regression line)] in the viable count. Furthermore, D-values were categorised into three groups: heat-labile strains (D_{55}= ≤ 4), moderate (5≥ D_{55} ≤9) and high heat tolerance (D_{55}= ≥10). As shown in Figure 4-10 and Table 4-9, there was a clear variation in thermo-tolerance among the representative *K. pneumoniae* strains tested. *K. pneumoniae* strain 1701 (ST247), which was isolated from EFT in Jordan, was more heat-tolerant than the remaining strains. It had a D-value of 12.9 minutes at 55 °C in the liquid infant formula. Whereas strains 1446 (ST37), 2298 (ST35) and 2291 (ST35) showed moderate heat tolerance, as they had only tolerated heat for up to 20 minutes and then their viable count slightly decreased (D_{55} values were 9.7, 5.10 and 5 minutes, respectively). Additionally, strains 2312 (ST34), 1725 (247), 1699 (ST247), 453 (ST105), 1681 (ST111), 497 (ST147) and 1734 (ST526) revealed a slight susceptibility to heat. Their D_{55} values were therefore lower than 5 minutes (D_{55}= 4.8, 4.5, 4.4, 4.0, 3.6, 2.7 and 1.2 respectively).

More recent studies stated that there is a genetic locus which could be responsible for heat tolerance, and it has been recognised in certain isolates such as *E. coli*, *C. sakazakii* and *K. pneumoniae* (Gajdosova *et al*., 2011; Mercer *et al*., 2015; Bojer *et al*., 2010). They suggested that the locus *clpK* ATPase play an important role in the survival and persistence of *K. pneumoniae* in certain harsh hospital environments, such as heat. As shown in Table 4-10, there was clear variation among presented strains in possession of *clpK* ATPase family genes. Interestingly, strain 1701, the most heat resistant strain, was the only strain with *clpC*2 gene, whereas, the strains 497 and 1734 each had 3 *Clp* genes missing and had the lowest D values

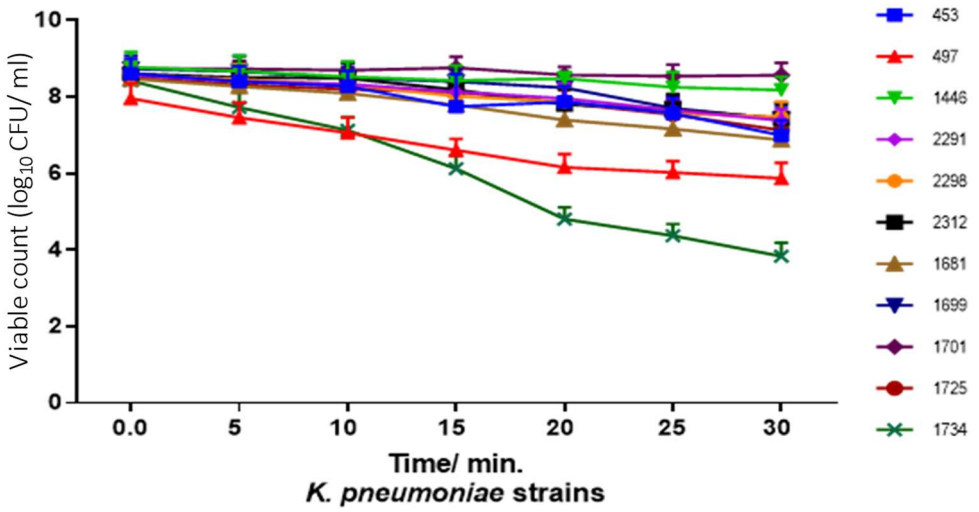


Figure 4-10: Survival of *K. pneumoniae* strains in liquid infant formula at 55°C.

Table 4-9: Survival of *K. pneumoniae* in liquid powdered infant formula at 55°C.

Strain	Species	Source	ST	O-antigen	K-type	D-value (min)	Category
1701	*K. pneumoniae*	EFT (Jordan)	247	O3	K1	12.9	R
1446	*K. pneumoniae*	NEFTs (Nottingham)	37	O2	ND	9.7	M
2291	*K. pneumoniae*	Sepsis (Nottingham)	35	ND	ND	5	M
2298	*K. pneumoniae*	Sepsis (Nottingham)	35	O8	ND	5.1	M
2312	*K. pneumoniae*	Sepsis (Nottingham)	34	O2	ND	4.8	M
453	*K. pneumoniae*	NEFTs (Nottingham)	105	O4	K5	4	S
497	*K. pneumoniae*	NEFTs (Nottingham)	147	O12	K5	2.7	S
1681	*K. pneumoniae*	EFT (Jordan)	111	O1	K2	3.6	S
1699	*K. pneumoniae*	EFT (Jordan)	247	O3	K1	4.4	S
1725	*K. pneumoniae*	EFT (Jordan)	111	O12	K2	4.5	S
1734	*K. pneumoniae*	EFT (Jordan)	526	O2	ND	1.2	S

ST= sequence type, R= resistant, M= moderate, S= sensitive, EFT= enteral feeding tube, NEFTs= neonatal enteral feeding tuibes, ND= not detected

Table 4-10: presence/ absence of the thermo-tolerant family of proteins genes in *K. pneumoniae*

Gene				Clp ATPase family									D-Value (minutes)
				clpA	*clpB1*	*clpB2*	*clpC1*	*clpC2*	*clpP1*	*clpP2*	*clpS*	*clpX*	
FUNCTION				Protease ATP-binding subunit	Chaperon protein	Chaperon protein	Protease ATP-binding subunit	Protease ATP-binding subunit	Proteolytic subunit precursor	Proteolytic subunit precursor	Protease adaptor protein	Protease ATP-binding subunit	
Strain	Species	Source	ST										
1701	*K. pneumoniae*	EFT (Jordan)	247	+	+	+	+	+	+	-	+	+	12.9
1446	*K. pneumoniae*	NEFTs (Nottingham)	37	+	+	+	+	-	+	-	+	+	9.7
2298	*K. pneumoniae*	Sepsis (Nottingham)	35	+	+	+	+	-	+	-	+	+	5.1
2291	*K. pneumoniae*	Sepsis (Nottingham)	35	+	+	+	+	-	+	-	+	+	5
2312	*K. pneumoniae*	Sepsis (Nottingham)	34	+	+	+	+	-	+	-	+	+	4.8
1725	*K. pneumoniae*	EFT (Jordan)	111	+	+	+	+	-	+	-	+	+	4.5
1699	*K. pneumoniae*	EFT (Jordan)	247	+	+	+	+	-	+	-	+	+	4.4
453	*K. pneumoniae*	NEFTs (Nottingham)	105	+	+	+	+	-	+	-	+	+	4
1681	*K. pneumoniae*	EFT (Jordan)	111	+	+	+	+	-	+	-	+	+	3.6
497	*K. pneumoniae*	NEFTs (Nottingham)	147	+	+	+	+	-	-	-	+	+	2.7
1734	*K. pneumoniae*	EFT (Jordan)	526	+	+	-	-	-	+	+	+	+	1.2

ST= sequence type, EFT= enteral feeding tube, strain 1701, the most heat resistant strain, was the only strain with *clpC2* gene. Also the strains 497 and 1734 each had 3 *Clp* genes missing and had the lowest D values.

4.4.2. Desiccation and sub-lethal injured cells

In 2004 and 2006, FAO-WHO documented their concerns about the persistence of opportunistic pathogenic bacteria in PIF, which may increase the potential risk of neonatal infection during feeding, particularly in immuno-compromised infants. In the current study, desiccation of *K. pneumoniae* strains in rehydrated PIF incubated for 3 weeks at room temperature on non-selective media (TSA) and selective media (VRBGA), the inoculum of examined isolates was around 8.5 $\log_{10}$ CFU/ml. On the other hand, the recovery of all tested isolates was greater on TSA than on VRBGA ($P < 0.05$), and these differences revealed sub-lethally injured cells throughout the desiccation period. Overall, on TSA, the recovery of cells on TSA during the three-week period ranged from 8.5 to 5.3 $\log_{10}$ CFU/ml Figure 4-11. Whilst on VRBGA, the cell recovery was slightly lower, ranging from 8.2 to 3.8 $\log_{10}$ CFU/ml Figure 4-12. In addition, by the first week of incubation on TSA, all tested strains showed the highest cell recovery value of 8.5 $\log_{10}$. By the third week of incubation on TSA, however, strains 2298, 453, 2291, 1699 and 2312 showed slightly decreased survival, ranging from log 8.0 to log 6.0, and the remaining isolates decreased to log 5.0 when compared to the inoculum (first desiccation time point). In contrast to this, by the first week of incubation on VRBGA,

all strains were able to persist and had showed the highest cell recovery value of up to 8.0 log_{10}. However, by the third week of incubation on VRBGA, the majority of the isolates had reduced in viability. It was found that Jordanian strains 1699, 1681, 1734, and QMC strain 2291 showed the highest desiccation survival values of log 5.3, log 4.8, 4.0 and 4.0 respectively.

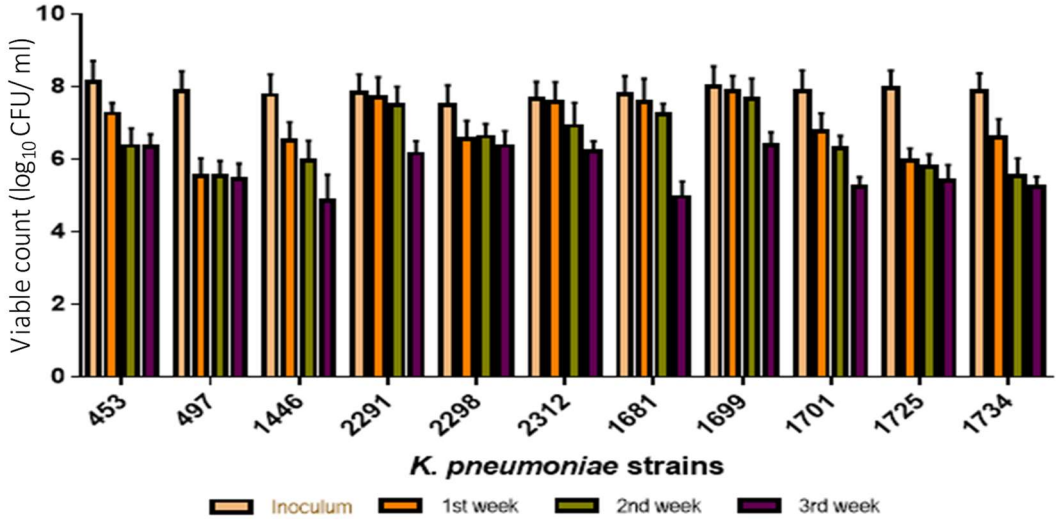


Figure 4-11: Viable cell counts for *K. pneumoniae* on TSA for three weeks after reconstituted in PIF.

K. pneumoniae survival of dessication stress. The strains were dessicated for up to three weeks in powdered infant formula then recovered in reconstituted liquid infant formula and quantified by plating on TSA. The experiments were investigated in three independent times. Error bars represent 95% confidence intervals.

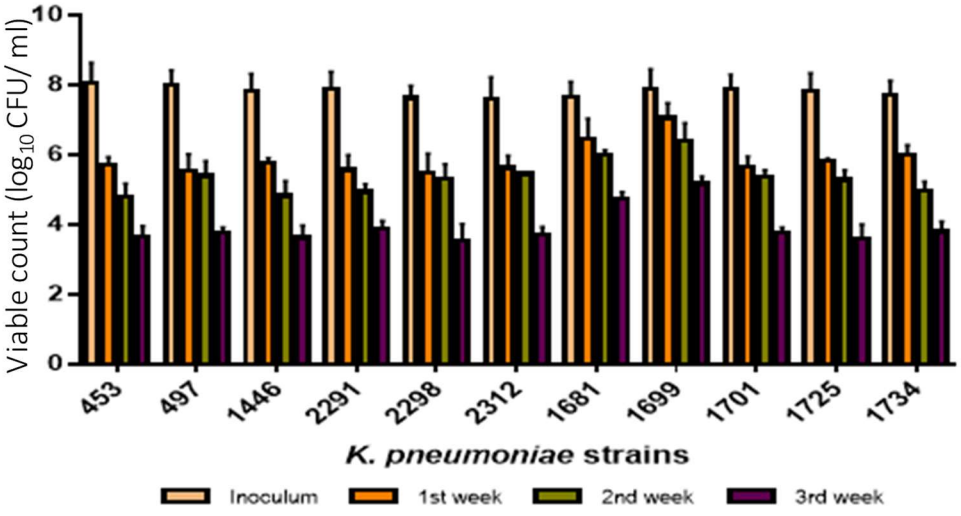


Figure 4-12: Viable cell counts for *K. pneumoniae* on VRBGA for three weeks in liquid PIF.

K. pneumoniae survival of dessication stress. The strains were dessicated for up to three weeks in powdered infant formula then recovered in reconstituted liquid infant formula and quantified by plating on VRBGA. The experiments were investigated in three independent times. Error bars represent 95% confidence intervals.

The differences between dead cells on TSA and sub-lethal injured cells on VRBGA are illustrated in Figure 4-13 and Table 4-11. The number of colonies on TSA would indicate live cells, and VRBGA is more stressful so fewer colonies seen, because the sub lethal injured cells can not grow. The highest amount of sub-lethal injured cells were detected in strains 2291 and 2312 at log 2.49 and 2.21 cfu/ml, respectively. It was found that the lowest amount of sub-lethal injured cells was identified in strain 497 at log 0.71 cfu/ml.

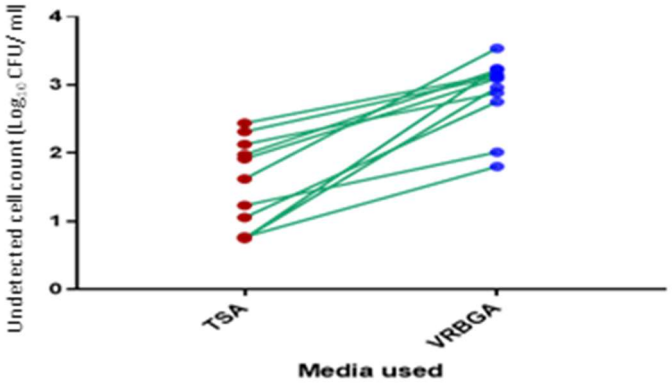


Figure 4-13: Paired t test showing strains gave significantly higher recovery on TSA than VRBGA.

Paired t-test shows strains form significantly a greater recovery on TSA than on VRBGA agar ($P < 0.0001$).

Table 4-11: Comparison of viable counts for *K. pneumoniae* on TSA and VRBGA after desiccation and then reconstitution in infant formula

NTU	ST	Source	Sublethal injured cells on TSA (log cfu/ ml)	Sublethal injured cells on VRBGA (log cfu/ ml)	Sub-lethal injured cells (log cfu/ ml)
453	105	QMC and NCH	1.62	3.53	1.91
497	147		2.44	3.15	0.71
1446	37		1.97	3.21	1.24
2291	35		0.74	3.24	2.49
2298	35		1.05	2.75	1.69
2312	34		0.75	2.96	2.21
1681	111	KAH and PRH	1.23	2.01	0.77
1699	247		0.77	1.8	1.03
1701	247		1.91	3.09	1.17
1725	111		2.31	3.11	0.8
1734	526		2.12	2.88	0.75

The numbers, which are in the table, comes from $\log_{10}$ of inoculum (TSA and VRBGA) for each strain – average of $\log_{10}$ 1st wk, 2nd wk and 3rd wk (TSA and VRBGA) for each strain.

There are several known regulatory genes which play an important role in microbial survival under challenging environments such as heat stress, desiccation, acid stress and osmotic stress conditions (Dong 2009; Stasic *et al.,* 2012). The *rpoS* gene is an alternative sigma factor in Gram-negative bacteria, particularly *E. coli*, which is implicated in resistance to desiccation, heat and other stress conditions (Stasic *et al.,* 2012; Hryckowian *et al.,* 2013). All strains examined in this study were found to possess this regulatory gene (*rpoS*). Although these strains were found to possess this gene, which is linked to desiccation tolerance, their gene sequences varied between strains and clustered by sequence type as showing in Figure 4-14.

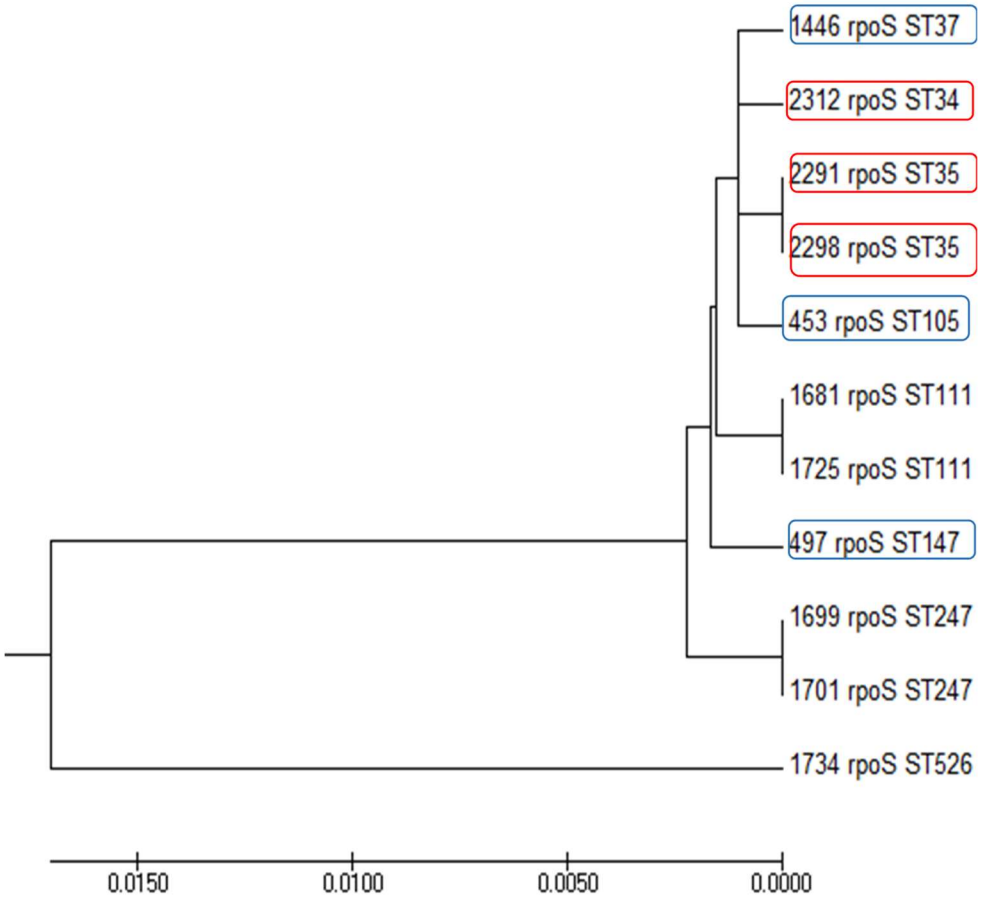


Figure 4-14: Maximum likelihood tree for rpoS gene sequences for 11 K. pneumoniae strains.

RpoS gene is a proposed stress protection gene, for *K. pneumoniae* isolates. The NTU strains IDs, name of gene and sequence type ST number are shown on the tree, blue outline box refere to NEFTs (Nottingham), red outline box refer to sepsis strains and the remaining were from EFTs (Jordan). The alignment was constructed using MEGA7.

4.5. Tissues culture investigations (Caco-2, T24 and HBMEC) of representative *K. pneumoniae;*

In the current study, three different cell lines (Caco2, HBMEC and T24) were used to evaluate the capability of selected *K. pneumoniae* isolates to attach to and invade human cells. The ability of these isolates to attach was investigated by quantifying CFU/ml after two hours of exposure to the mammalian cells and the attachment ability was expressed as % of the inoculum (initial dose). Furthermore, the gentamicin protection technique was performed to determine bacterial invasion of the endothelial and epithelial cells after three hours exposure. The number of bacteria surviving gentamicin treatment was determined and invasion ability was expressed as % of the initial inoculum. For comparison, *Salmonella* Eenteritidis NCTC strain 3046 (NTU strain 358) and *Citrobacter koseri* SMT319 (NTU strain 48) were included as positive controls in Caco2 and HBMEC cell lines, respectively. Additionally, uropathogenic *E. coli* strain CFT073 (ST73) was used as a positive control in the T24 cell line, whereas, non-pathogenic *E. coli* K12 (NTU strain 1230) was employed as a non-attaching, non-invasive negative control strain for all cell lines used in the current study (Townsend *et al*., 2008).

4.5.1. Attachment to Caco-2 (human intestinal epithelial cells)

Bacterial adherence to human tissues is a crucial feature that results in pathogens colonising the host tissue. Figure 4-15 shows the ability of representative *K. pneumoniae* isolates to adhere to Caco-2 intestinal epithelial cells. All strains tested except for the negative control *E. coli* K12 strain were able to attach to the Caco2 cell line, however, clear variation was noticeable between the examined strains. The highest attachment was exhibited by QMC strain 453 (ST105) and the lowest level of attachment was exhibited by QMC strain 2312 (ST34). Statistical analysis by means of one-way ANOVA revealed significantly higher attachment levels of all tested strains when compared to the negative control ($P < 0.05$). Giemsa staining and microscopy was used to confirm the ability of these strains to attach to the Caco2 cell line.

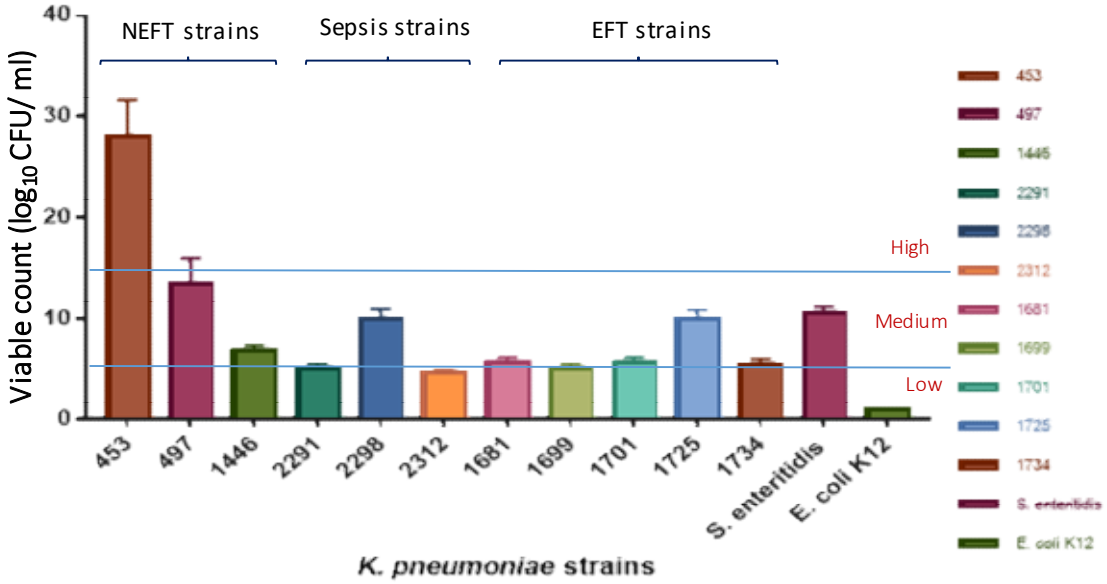


Figure 4-15: *K. pneumoniae* attachment assay using Caco-2 cells, after 2-h of incubation.

The NTU *S.* Enteritidis (358) and *E. coli* K12 (1230) strains were used as positive and negative controls, respectively. NEFTs and sepsis strains were from Nottingham, EFT strains were from Jordan. The attachment is shown as % of the initial inoculum. The mean results from three independent replicates are shown +/- standard error.

4.5.2. Invasion of Caco-2 (human intestinal epithelial cells)

The ability of selected strains to invade the Caco2 cell line was investigated by carrying out the gentamicin protection assay. Using this method, gentamicin kills extracellular bacteria and the invasive intracellular organisms escape from the bactericidal effect of gentamicin (Elsinghorst, 1994). This experiment was therefore able to determine the ability of strains to invade the Caco2 cell line. From Figure 4-16, it is clear that only one of the *K. pneumoniae* strains tested, QMC strain 453 (ST105), was able to invade epithelial cells at a significant level when compared to the negative control strain *E. coli* K12 (1230) ($P < 0.05$). It was interesting to note that this particular QMC strain (453) was also found to have the greatest level of attachment on the Caco2 cell line Figure 4-15. On the other hand, the Jordanian strain 1725 (ST11) derived from EFT showed very low invasion levels on Caco2, whilst the remaining strains tested were not able to invade the Caco2 cell line.

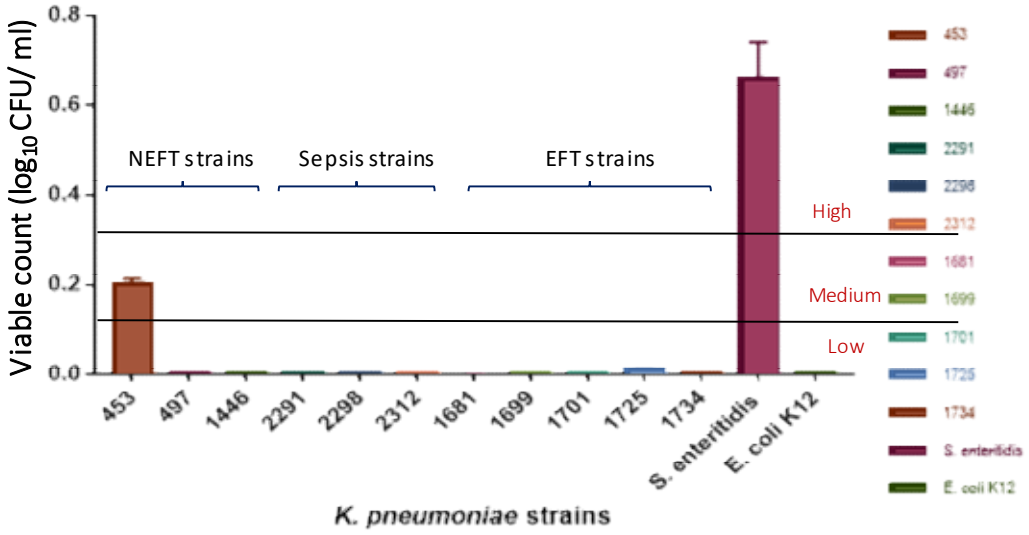


Figure 4-16: *K. pneumoniae* invasion assay using Caco-2 cell line over 3 h of incubation.

The NTU *S.* Enteritidis (358) and *E. coli* K12 (1230) strains were used as positive and negative controls, respectively. NEFTs and sepsis strains were from Nottingham, EFT strains were from Jordan. The invasion is shown as % of the initial inoculum. The mean results from three independent replicates are shown +/- standard error.

4.5.3. Attachment to T24 (Urinary Bladder Epithelial Cells)

In 1991, Johnson and co-authors reported that the ability of pathogens to adhere to the host tissue protects them from being carried away from the human body. In urinary tract infections, for instance, bacterial attachment to the epithelial cells lining the urinary tract helps them avoid being swept away by urine. In this assay, urinary bladder epithelial cells (T24) were used to investigate the capability of representative *K. pneumoniae* strains to attach to these cells. For comparison, uropathogenic *E. coli* strain CFT073 (ST73) and *E. coli* K12 (1230) were used as positive and negative controls, respectively. If more than 20% of the initial bacterial inoculum could be recovered from the T24 cells after incubation and washing, this was classified as 'high attachment'; if the cfu/ml recovered was between 10-20% of the initial inoculum, this was defined as 'moderate attachment'. Figure 4-17 shows that all of the isolates tested were able to attach to the T24 cell line, with significantly greater attachment than the negative control strain ($p \leq 0.001$, one way ANOVA). In addition, there was clear variation among strains. QMC strains 453, 497 and 1446 (ST105, 147 and 37) and

Jordanian (EFT) strains 1725 and 1701 (ST111 and 247) had high levels of attachment to the T24 cell line. In contrast, the remaining strains exhibited moderate attachment.

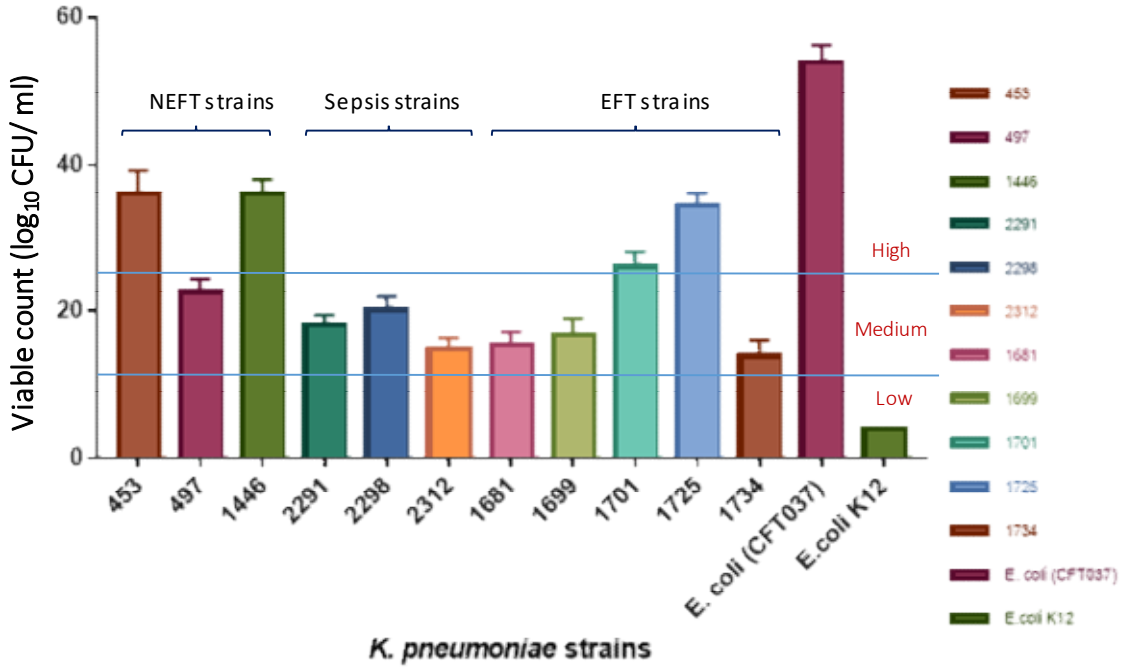


Figure 4-17: *K. pneumoniae* strains attachment assay using T24 cell line after 2-hour incubation.

E. coli CFT037 and *E. coli* K12 were used as positive and negative controls, respectively. NEFTs and sepsis strains were from Nottingham, EFT strains were from Jordan. The attachement is shown as % of the initial inoculum. The mean results from three independent replicates are shown +/- standard error.

4.5.4. Invasion of T24 (Urinary Bladder Epithelial Cells)

In this experiment, the gentamicin-based invasion assay revealed that all of the strains tested were able invade the T24 bladder epithelial cell line, with clear variation ($P<0.05$) between some of the isolates Figure 4-18. Jordanian strain 1725 (ST111) from EFT and QMC strain 1446 (ST37) from NEFT were the most invasive strains tested. The least invasive strain was Jordanian strain 1701 (ST247) from EFT, whilst the remaining isolates had moderate invasion levels. All of the *K. pneumoniae* strains tested were significantly better at invading T24 cells than the negative control non-pathogenic *E. coli* K12 ($p \leq 0.05$), but had significantly lower invasion ability than the positive control strain UPEC CFT037 ($p \leq 0.001$).

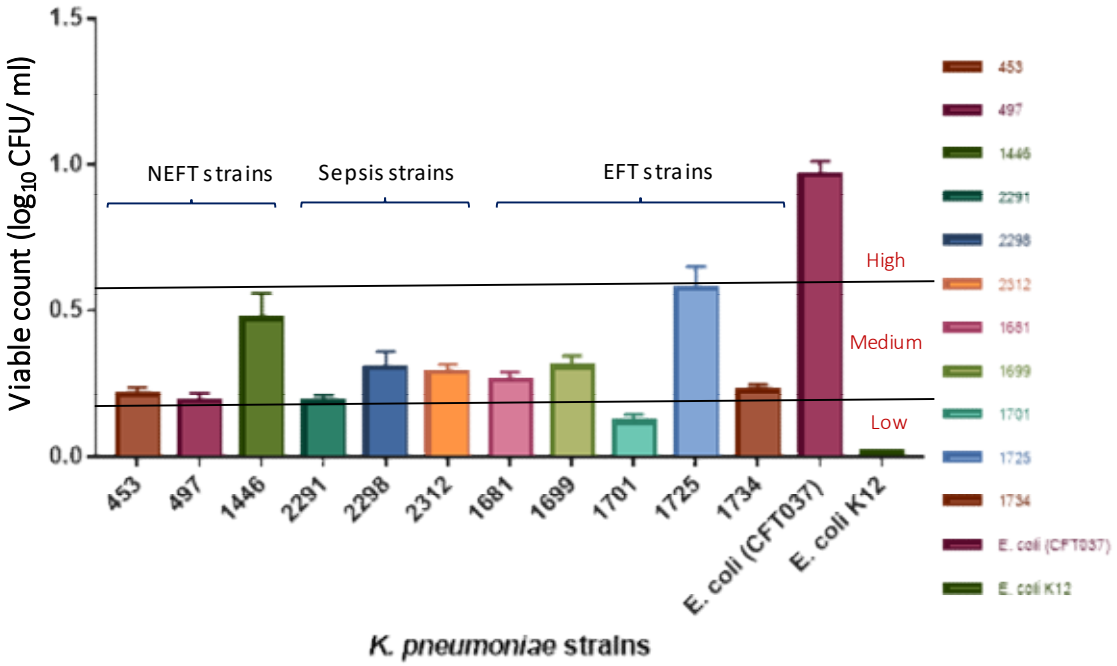


Figure 4-18: *K. pneumoniae* invasion assay using T24 cell line over 3 hours of incubation.

E. coli CFT037 and *E. coli* K12 were used as positive and negative controls, respectively. NEFTs and sepsis strains were from Nottingham, EFT strains were from Jordan. The invasion is shown as % of the initial inoculum. The mean results from three independent replicates are shown +/- standard error.

4.5.5. Attachment to HBMEC (Human brain microvascular epithelial cells)

Previously, pathogens with the potential to cause meningitis such as group B *Streptococcus*, *E. coli* K1, and *S. aureus* have been studied using the HBMEC cell line (Brouwer *et al*., 2010; Thigpen *et al*., 2011). The ability of selected *K. pneumoniae* strains to attach to the HBMEC cell line was assessed in this experiment Figure 4-19. Strains *C. koseri* (48) and *E. coli* K12 (1230) were used as positive and negative controls, respectively. All of the *K. pneumoniae* strains tested were significantly better at attaching to the HBMEC cell line than the negative control *E. coli* K12 strain ($p \leq 0.001$). Furthermore, there was clear variation observed among isolates, with the majority showing a higher level of attachment than the positive control *C. koseri* strain.

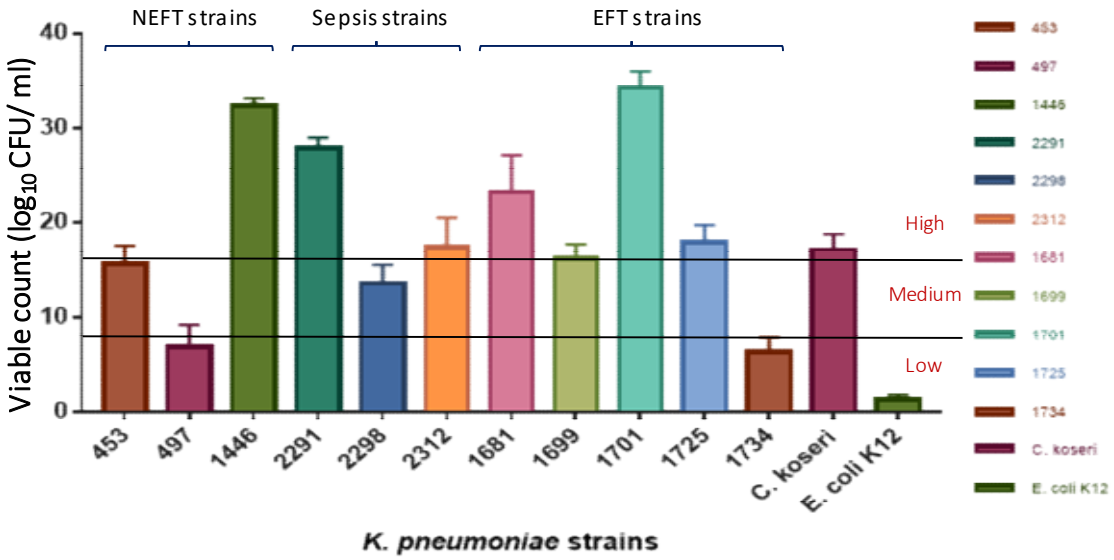


Figure 4-19: *K. pneumoniae* attachment assay using HBMEC cell, after 2 h of incubation.

The NTU *C. koseri* (48) and *E. coli* K12 (1230) strains were used as positive and negative controls respectively. NEFTs and sepsis strains were from Nottingham, EFT strains were from Jordan. The attachment is shown as % of the initial inoculum. The mean results from three independent replicates are shown +/- standard error.

4.5.6. Invasion to HBMEC (Human brain microvascular epithelial cells)

The gentamicin protection assay was used to quantify HBMEC cell invasion ability, with *C. koseri* (48) and *E. coli* K12 (1230) used as positive and negative control strains, respectively. Figure 4-20 shows that all of the *K. pneumoniae* strains tested were significantly more invasive of HBMEC cells than the negative control *E. coli* K12 ($p = 0.0294$, one way ANOVA). Additionally, notable variation in invasion ability was detected among the strains tested, with some showing higher invasion levels than the positive control *C. koseri* (48).

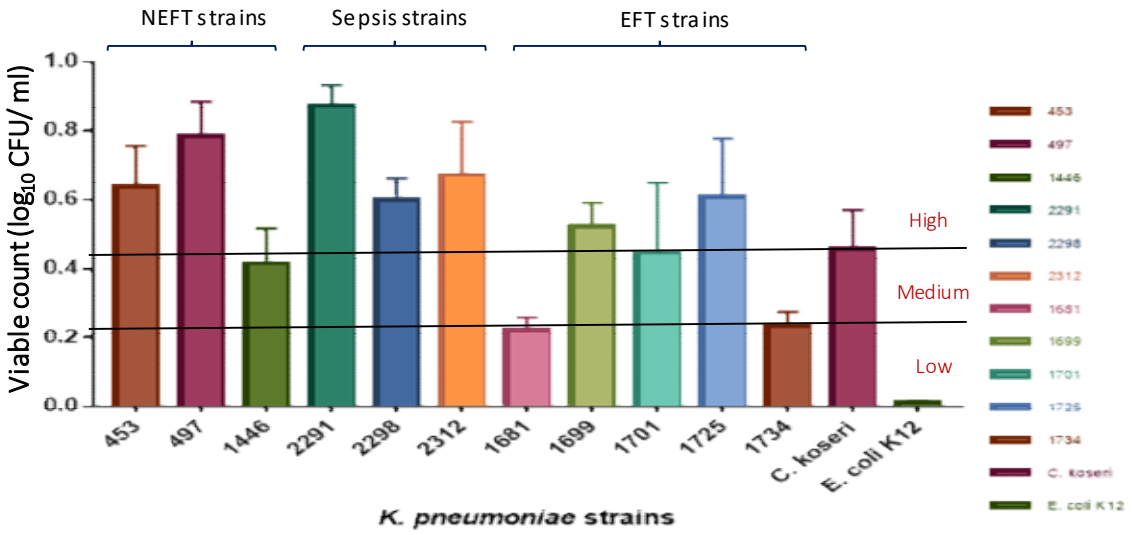


Figure 4-20: *K. pneumoniae* invasion assay using HBMEC cell line over 3 h of incubation.

The NTU *C. koseri* (48) and *E. coli* K12 (1230) strains were used as positive and negative controls respectively. NEFTs and sepsis strains were from Nottingham, EFT strains were from Jordan. The invasion is shown as % of the initial inoculum. The mean results from three independent replicates are shown +/- standard error.

4.5.7. *K. pneumoniae* adhesion associated genes;

The presence and absence of adhesion (*fimH* and *mrkD*) genes from presented isolates were detected by PCR and sequenced for phylogenetic analysis. The results are shown in Figure 4-21, the presence of adhesion-associated genes were detected in all examined isolates; there was clear variation among presented strains in possession these genes. Although, all tested strains possessed these genes which are linked with adhesion, but some of the strains had high, moderate and low attachment to the mammalian cells, it possible that some defaults that makes these genes were poorly expressed or non-functional in these isolates.

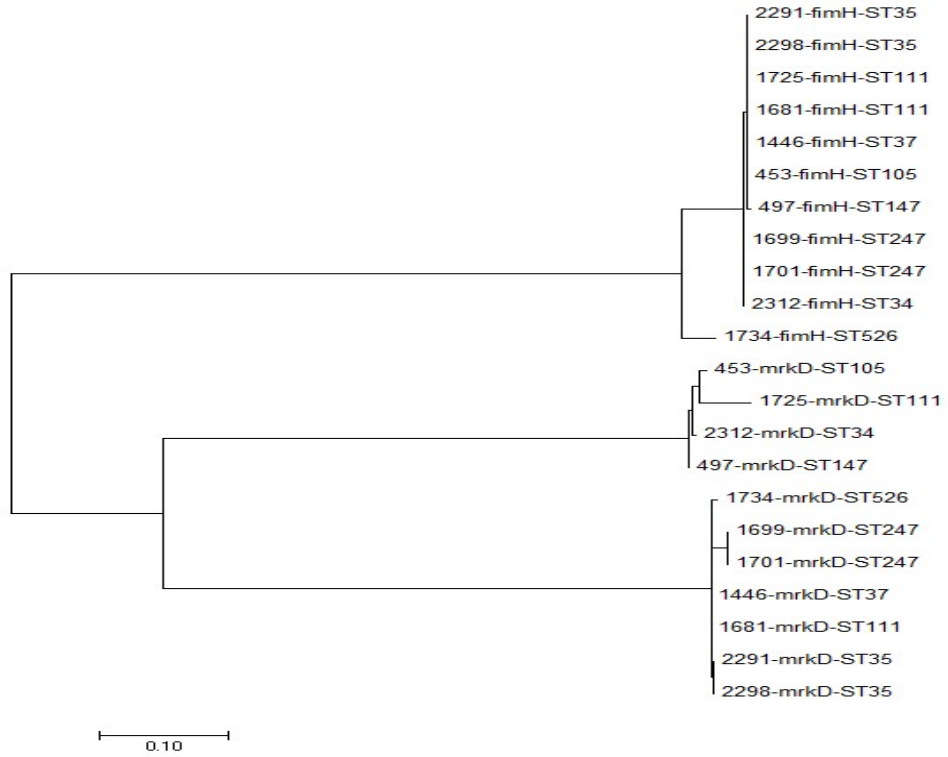
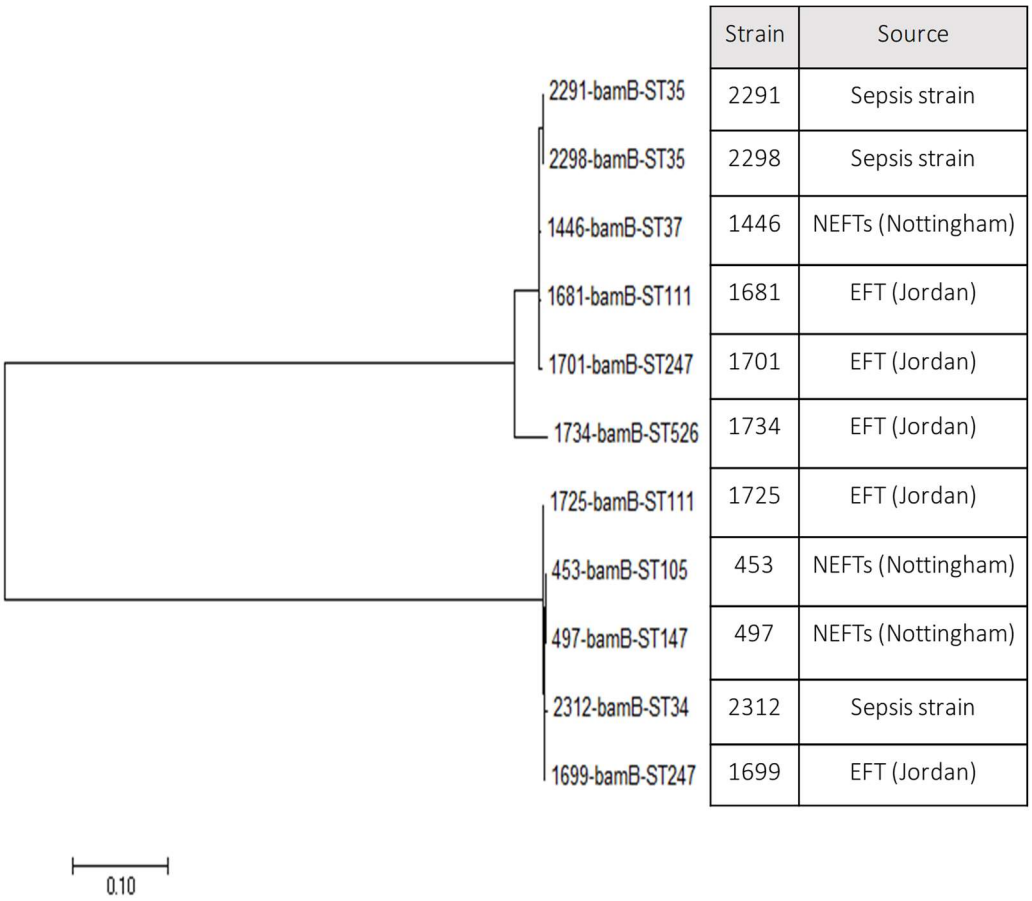


Figure 4-21: Maximum likelihood tree for fimH and mrkD fimbriae genes for *K. pneumoniae* strains.

The NTU strains IDs, name of gene and sequence type ST number are shown in the tree. The alignment was constructed using MEGA7.

4.5.8. *K. pneumoniae* Invasions associated genes;

The ability of *K. pneumoniae* to invade the epithelial cells remains undefined as there are other factors which play crucial roles in contributing to the pathogenicity of *K. pneumoniae*. Furthermore, several reports have suggested that there are various genes that associated with invasion (Rosen *et al.,* 2008; Sahly *et al.,* 2000). These genes include *yaeT, bamB* (*YfgL*), *nlpB, YfiO* and *smpA*, of which, only *bamB* was found to be essential for invasion in *K. pneumoniae*. Figure 4-22 shows the DNA sequences of *bamB*.



Strain	Source
2291	Sepsis strain
2298	Sepsis strain
1446	NEFTs (Nottingham)
1681	EFT (Jordan)
1701	EFT (Jordan)
1734	EFT (Jordan)
1725	EFT (Jordan)
453	NEFTs (Nottingham)
497	NEFTs (Nottingham)
2312	Sepsis strain
1699	EFT (Jordan)

Figure 4-22: maximum likelihood tree for bamB invasion gene for *K. pneumoniae* strains.

The NTU strains IDs, name of gene and sequence type ST number are shown in the tree. The alignment was constructed using MEGA7.

4.5.9. Uptake and Persistence into U937 (Macrophage Cell Line)

The human innate immune system, including macrophages, is considered a front-line defence mechanism against pathogens. Cell lines, such as macrophage U937, are widely used to evaluate the ability of pathogens to persist and multiply within these cells. In the current study, *C. koseri* (48) and *E. coli* K12 (1230) isolates were used as positive and negative controls, respectively, for comparative analysis with the tested isolates. After 60 minutes of incubation, *K. pneumoniae* strains 497 (ST147), 2291 and 2298 (ST35), and 2312 (ST34) revealed no uptake or were killed rapidly by macrophage (U937) cells through the initial incubation period (60 min). It was noticeable that *K. pneumoniae* strains 1681 and 1725 (ST111); 1701 and 1699 (ST247); 1446 (ST37) and 453 (ST105) were taken up by the macrophage cell line Figure 4-23. Different uptake levels were observed among the strains

tested, particularly isolate 1699 (ST247), which was the strain with poorest survival of the initial phase of engulfment. After 24 hours incubation, all of the strains that had survived 60 minutes incubation with macrophages were still viable and proliferating inside the macrophage (U937) cells. Of interest was strain 1734 (ST526), which persisted and was recovered from macrophages and showed increased levels of recovery after 24 hours. After 48 hours incubation, all previous strains exhibited an ability to persist and survive, and recovery levels had increased within macrophage (U937) cell. A noticeable amount of variation in recovery levels was observed among examined strains, with the highest amount noted in strain 1725 (ST111), whilst the lowest amount was detected in strain 453 (ST105). Additionally, a dramatic increase was noticed in strain 1734 (ST526) from 0.06% to 0.9% recovery. After 72 hours incubation, strain 1681 (ST111) demonstrated continuous survival and replication within macrophage (U937) cells. However, the remaining isolates showed a decrease in survival, with differing recovery levels. Additionally, strains 1725 (ST111), 1699 and 1701 (ST247), and 453 (ST105) showed decreased slight decrease in their intracellular survival rates. A dramatic decline in survival was noted by strain 1734 (ST526), with a decrease from 0.9% to 0.4%, as well as strain 1446 (ST37), which decreased from 0.72% to 0.4%. Interestingly, *K. pneumoniae* strains 1681 and 1725 (ST111); 1701 and 1699 (ST247) isolated from EFTs from Jordanian hospitals revealed their ability to survive and proliferate within the macrophage (U937) cells over the 72 hours ($p < 0.0001$; one-way ANOVA). Whereas, only *K. pneumoniae* strains 1446 (ST37) & 453 (ST105) which were isolated from NEFTs at QMC and NCH, Nottingham showed their ability to survive and replicate up to 48 hrs Figure 4-23.

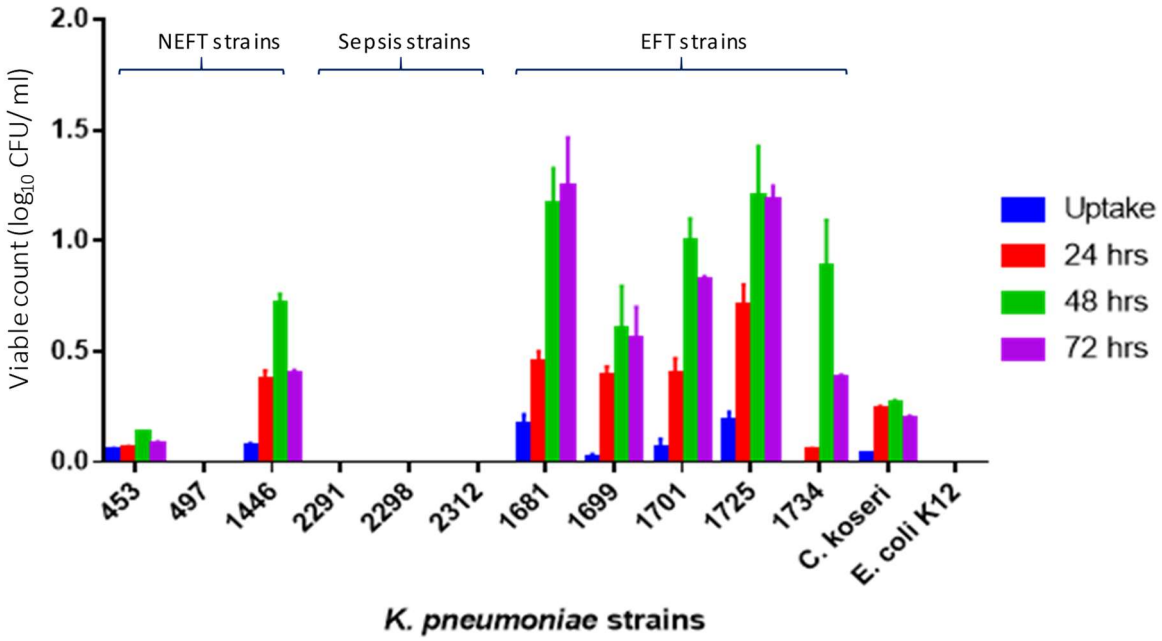


Figure 4-23: Uptake, persistence and replication of *K. pneumoniae* strains in U937 cells over 72 hours of incubation.

The NTU *C. koseri* (48) and *E. coli* K12 (1230) strains were used as positive and negative controls respectively. NEFTs and sepsis strains were from Nottingham, EFT strains were from Jordan. The survival is shown as % of the initial inoculum after lysis of U937 cells to release any phagocytosed bacteria. The mean results from three independent replicates are shown +/- standard error.

4.5.10. Level of *K. pneumoniae* cytotoxicity on Caco2 cells.

K. pneumoniae is considered to be one of the major pathogens responsible for nosocomial and community acquired infections globally. This pathogen is known to carry more than one type of virulence mechanism, such as fimbriae, multidrug resistance and stress resistance genes. The cytotoxicity of representative *K. pneumoniae* strains towards Caco2 cells was determined. The experiment was performed according to the MTT assay protocol in the ISO 10993-5 (ISO, 2009). This assay is widely used to investigate cytotoxicity by means of a colorimetric reaction, based on the correlation between cell viability (eukaryotic cells) and mitochondrial activity. In this experiment, *Salmonella* Eenteritidis strain (358) was used as a positive control, whereas the negative control comprised uninfected cells incubated in media without any bacteria. Low levels of MTT reduction were observed for strains 453, 1681, 2291, 1725, 1734 and 1446, indicating higher levels of cytotoxicity Figure 4-24. In contrast to this, strains 497, 2312, 1699 and 1701 were moderately cytotoxic when

compared to the negative control. Only strain 2298 had low cytotoxicity close to that of the negative control. All examined strains, except strain 2298, were significantly cytotoxic compared to the negative control ($p < 0.001$; one-way ANOVA).

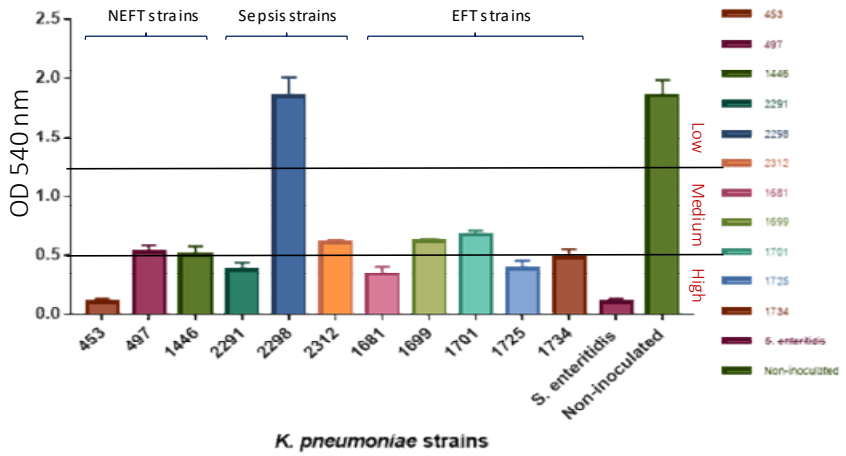


Figure 4-24: Cytotoxicity of *K. pneumoniae* strains on Caco2 cells after 3 hours of incubation.

MTT reduction was used to determine the cytotoxicity of *K. pneumoniae* isolates. Only viable Caco2 cells are able to reduce MTT to its insoluble purple form formazan, so low absorbance (low MTT reduction) indicates higher levels of cytotoxicity. NEFTs and sepsis strains were from Nottingham, EFT strains were from Jordan.The mean results from three independent replicates are shown +/- standard error.

4.5.11. Genomic comparison of virulence factor genes among representative *K. pneumoniae* strains

Further analysis was carried out, based on the distribution of virulence factor genes among representative strains that are associated with heat resistance, desiccation resistance, antimicrobial resistance, and attachment and invasion of eukaryotic cells. Virulence-associated genes (VAGs) that may be responsible for various clinical infections were identified using whole genome sequences of representative strains generated by Pauline Ogrodski as part of a related PhD project in our research group. This analysis also sought to evaluate the usefulness of whole genome sequencing in identifying VAGs in clinical isolates. Nucleotide BLAST of whole genomes against the Virulence factors database (VFRD) allowed genomic profiling analysis of these isolates Table 4-12 revealed that there was clear variation among the tested strains. With regards to strains isolated from EFT Jordanian hospitals, only two strains, 1681 and 1725 (ST111), were found to carry more than two plasmids encoding several genes associated with multidrug resistance, such as aminoglycoside resistance genes

(*aadA2*, *strA*, *aac(6')lb-cr*, *aac(3)-lld*, *armA*, *strB*). These strains also contained more β–lactamase genes than the other strains (each of strains 1681 and 1725 contained four β–lactamase genes: bla_{SHV-11}, $bla_{CTX-M-15}$, bla_{TEM-1B}, bla_{OXA-1}). Furthermore, these two strains were also found to each possess three fluoroquinolone resistance genes (*oqxA*, *oqxB*, *aac(6')lb-cr*), as well as sulphonamide class resistance associated genes (*sul1* and *sul2*). In addition, macrolide (*mphA* & *msrE*), tetracycline (*tetA*) and trimethoprim resistance genes, (*dfrA*12 and *dfrA*14) were also detected. *K. pneumoniae* strain 1734 (ST526), isolated from EFT Jordanian hospitals, possessed three plasmids (*IncFII, IncFIB, IncR*), with two resistance genes associated with aminoglycosides (*aac(6')-lb aadA*1), and had an additional four β lactamase genes ($blaO_{XA-9}$, $bla_{OKP-A-8}$, bla_{TEM-1A}, bla_{SHV-12}) as well as three fluoroquinolone resistance genes (*oqxA*, *oqxB*, *aac(6')lb-cr*). In contrast, genomic analysis of *K. pneumoniae* strains isolated from NEFTs at QMC and NCH revealed that strains 453 (ST105), 497 (ST147) and 1446 (ST37) possessed two plasmids [(IncFIB(K), IncFII), (IncFIA(HI1) and (IncY, IncFIB)], respectively. Furthermore, strains 453 (ST105) and 1446 (ST37) contained only one aminoglycoside resistance gene (*aadA2*), whereas strain 497 (ST147) exhibited three different aminoglycosides resistance genes (*aac(6')lb-cr*, *strA*, *strB*), as well as strains 453 (ST105) and 497 (ST147), which both possessed the same two β–lactamase genes (bla_{SHV-11}, bla_{OXA-1}). Strain 1446 (ST37), on the other hand, had two different β lactamase genes (bla_{SHV-11}, bla_{TEM-1B}). In addition, strain 497 (ST147) had three fluoroquinolone resistance genes (*aac(6')lb-cr*, *oqxA*, *oqxB*), whereas strains 453 (ST105) and 1446 (ST37) had the same genes associated with fluoroquinolone resistance (*oqxA*, *oqxB*) and trimethoprim resistance genes (*dfrA*14 and *dfrA*16), which were detected in strains 497 (ST147) and 1446 (ST37), respectively. Regarding *K. pneumoniae* strains 2291, 2298 (ST35) and 2312 (ST34), which were isolated from neonatal sepsis cases at QMC, these strains exhibited plasmid IncFIB, and possessed an additional β-lactamase gene bla_{SHV-33} in addition to two fluoroquinolone resistance genes *oqxA* and *oqxB*.

Table 4-12: Distribution of virulence factor genes among representative *K. pneumoniae* strains isolated from EFT, NEFT and sepsis.

Isolate number	Species	Isolation information	Sequence type	Plasmids	Aminoglycoside	Beta-lactam	Fluoroquinolone	Fosfomycin	Macrolides	Sulphonamide	Tetracycline	Trimethoprim
453	*K. pneumoniae*	NEFTs (Nottingham)	ST105	IncFIB(K), IncFII	aadA2	blaSHV-11, blaOXA-1	oqxA, oqxB	fosA		sul1		
497	*K. pneumoniae*	NEFTs (Nottingham)	ST147	IncFIA(HI1), IncR	aac(6')Ib-cr, strA, strB	blaSHV-11, blaOXA-1	aac(6')Ib-cr, oqxA, oqxB	fosA		sul2		dfrA14
1446	*K. pneumoniae*	NEFTs (Nottingham)	ST37	IncY, IncFIB	aadA2	blaSHV-11, blaTEM-1B	oqxA, oqxB	fosA		sul1		dfrA16
2291	*K. pneumoniae*	N. Sepsis QMC	ST35	IncFIB		blaSHV-33	oqxA, oqxB	fosA			tet(D)	
2298	*K. pneumoniae*	N. Sepsis QMC	ST35	IncFIB		blaSHV-33	oqxA, oqxB	fosA			tet(D)	
2312	*K. pneumoniae*	N. Sepsis QMC	ST34	IncFII,IncFIB		blaSHV-33	oqxA, oqxB	fosA				
1681	*K. pneumoniae*	EFT (Jordan)	ST111	IncFII(K), IncFIB(K), IncL/M	aadA2, strA, aac(6')Ib-cr, aac(3)-IId, armA, strB	blaSHV-11, blaCTX-M-15, blaTEM-1B, blaOXA-1	oqxA, oqxB, aac(6')Ib-cr	fosA	mph(E), msr(E)	sul1, sul2	tet(A)	dfrA12, dfrA14
1725	*K. pneumoniae*	EFT (Jordan)	ST111	IncFII(K), IncFIB(K), IncL/M	aadA2, strA, aac(6')Ib-cr, aac(3)-IId, armA, strB	blaSHV-11, blaCTX-M-15, blaTEM-1B, blaOXA-1	oqxA, oqxB, aac(6')Ib-cr	fosA	mph(E), msr(E)	sul1, sul2	tet(A)	dfrA12, dfrA14
1699	*K. pneumoniae*	EFT (Jordan)	ST247	IncFIB(Mar)		blaSHV-1	oqxA, oqxB	fosA				
1701	*K. pneumoniae*	EFT (Jordan)	ST247	IncFII, IncFIB(Mar), IncFI(K)	aadA1	blaSHV-1	oqxA, oqxB	fosA		sul1		
1734	*K. pneumoniae*	EFT (Jordan)	ST526	IncFII, IncFIB, IncR	aac(6')-Ib, aadA1	blaOXA-9, blaOKP-A-8, blaTEM-1A, blaSHV-12	oqxA, oqxB, aac(6')Ib-cr	fosA				

This table shows that some of the *K. pneumoniae* isolates studied carry two or more plasmids encoding several genes associated with multidrug resistance, such as β–lactamase, aminoglycoside resistance, fluoroquinolone and trimethoprim resistance genes, N.A= not available, NEFTs= neonatal enteral feeding tubes, EFT enteral feeding tube, N. sepsis= neonatal sepsis, yellow color indicate for Nottingham strains and blue sky color indicate for Jordan strains.

4.5.12. *K. pneumoniae* O-antigen diversity;

In the present study, whole genome sequencing was performed for representative *K. pneumoniae* strains to determine their O serotyping profile and to ascertain whether there is an association between O serotyping and K serotyping in *K. pneumoniae* infections. The genomes of strains were visualised using the WebAct comparative tool and Artemis comparative tool (Carver *et al.*, 2005). Furthermore, BLAST searches of genomes were carried out using NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

According to Fang *et al.* (2016), the results revealed that six O-antigen serogroups were detected: O1, O2, O3, O4, O8 and O12. Strain 1681 (ST111) was typed as O1; three strains (2312, 1446 and 1734) were typed as O2; serogroup O3 comprised two strains (1701 and 1699); strain 453 was typed as O4; strain 2298 was typed as O8; serogroup O12 comprised two strains (497 and 1725). It could not be determined which serogroup strain 2291 belonged to; Table 4-13

Table 4-13: Distribution of *K. pneumoniae* serogroups among EFT, NEFT and sepsis isolates

Strain	Source	ST	O-antigen	K-type
1681	EFT (Jordan)	111	O1	K2
2312	Sepsis strain	34	O2	ND
1446	NEFT (Nottingham)	37	O2	ND
1734	EFT (Jordan)	526	O2	ND
1701	EFT (Jordan)	247	O3	K1
1699	EFT (Jordan)	247	O3	K1
453	NEFT (Nottingham)	105	O4	K5
2298	Sepsis strain	35	O8	ND
497	NEFT	147	O12	K5
1725	EFT (Jordan)	111	O12	K2
2291	Sepsis strain	35	ND	ND

EFT= enteral feeding tube, NEFT= neonatal entering feeding tube, ST= sequence type, ND= not detected

4.5.13. Summary of the associations between genotypic and physiological factors, and the potential pathogenicity of the *K. pneumoniae* strains used in this study.

The key results presented in Table 4-14 summarise possible associations between certain genes and physiological factors, in addition to an assessment of the potential pathogenicity of the *K. pneumoniae* strains used in this study. All of the strains were categorised as having high pathogenic potential due to their ability to express capsular material on PIF agar, lyse horse blood (β -haemolytic), and produce biofilms. Additionally, all of the strains possessed the main adhesion, siderophore and capsular serotype K1, K2 and K5 genes. Also of interest, was a noticeable correlation between the *rpoB* allele type and capsular serotypes. There was also a clear correlation between ST247 and the O:3 and K1 capsular serotype. Strains with the *rpo*B4 allele were capsular serotype K2, whilst strains with the *rpo*B25 allele type were serotype K1. The strains were also classified as having high, moderate or low pathogenic potential based on their ability to persist and replicate within macrophages and attach to and invade epithelial and endothelial cell lines Table 4-15.

In summary, the data generated in this study indicate that *K. pneumoniae* strains, which were isolated from flushed and NEFTs, harbour several potential virulence factors than *K. pneumoniae* strains, which were isolated from neonates with sepsis cases. The virulence factors genes such as capsular serotype K1 and K2, O1 and O2 antigens, siderophores and adhesion genes. In addition, they demonstrate a propensity to adhere to and invade epithelial and endothelial cells. Furthermore, some of these strains were found to carry more than three plasmids as well as multidrug resistance-associated genes which could result in un-treatable infections, thus presenting a high potential health risk to neonates in the future.

Table 4-14: Results summary and assessment of phenotypic and genotypic analysis of *K. pneumoniae* isolates isolated from EFT.

NTU	Source	Pulstype	rpoB	Phenotypic						Genotypic									Virulence Potential
				Capsule (Mucoid)		Biofilm formation In IF		Haemolysis		Capsule serotype			Adhesion (fimbriae)				Siderophores		
				XLD	IF	25 C	37 C	S	Ho.	K1	K2	K5	*fimH*	*fimA*	*mrkD*	*mrkA*	*Irp2*	*fyuA*	
1681	PRH	Kp C1	4	+++++	+++++	M	H	ɣ	β	-	+	-	+	+	+	+	+	+	High
1683				+++++	+++++	L	M	ɣ	β	-	+	-	+	+	+	+	+	+	High
1718				+++	+++++	M	M	ɣ	β	-	+	-	+	+	+	+	+	+	High
1686				+++++	+++++	M	H	ɣ	β	-	+	-	+	+	+	+	+	+	High
1687				+++++	+++++	M	H	ɣ	β	-	+	-	+	+	+	+	+	+	High
1690				+++	+++++	L	M	ɣ	β	-	+	-	+	+	+	+	+	+	High
1693				+++	+++++	L	M	ɣ	β	-	+	-	+	+	+	+	+	+	High
1697				+++	+++++	M	H	ɣ	β	-	+	-	+	+	+	+	+	+	High
1709				+++++	+++++	L	H	ɣ	β	-	+	-	+	+	+	+	+	+	High
1722				+++	+++++	M	H	ɣ	β	-	+	-	+	+	+	+	+	+	High
1717				+++++	+++++	M	H	ɣ	β	-	+	-	+	+	+	+	+	+	High
1731				+++++	+++++	M	H	ɣ	β	-	+	-	+	+	+	+	+	+	High
1737				-	++	L	H	ɣ	β	-	+	-	+	+	+	+	+	+	High
1741				-	++	M	H	ɣ	β	-	+	-	+	+	+	+	+	+	High
1744				-	++	M	H	ɣ	β	-	+	-	+	+	+	+	+	+	High
1746				++++	+++++	M	H	ɣ	β	-	+	-	+	+	+	+	+	+	High
1748				++++	+++++	M	H	ɣ	β	-	+	-	+	+	+	+	+	+	High
1750				-	+	M	H	ɣ	β	-	+	-	+	+	+	+	+	+	High
1753				-	+	M	M	ɣ	β	-	+	-	+	+	+	+	+	+	High
1727	KAH			+++	+++++	L	H	ɣ	β	-	+	-	+	+	+	+	+	+	High
1754	PRH			-	+	L	M	ɣ	β	-	+	-	+	+	+	+	+	+	High
1725		Kp C2	4	+++	+++++	M	H	ɣ	β	-	+	-	+	+	+	+	+	+	High
1701	KAH	Kp C3	25	+++	+++++	M	H	ɣ	β	+	-	-	+	+	+	+	+	+	High
1703				+++	+++++	M	M	ɣ	β	+	-	-	+	+	+	+	+	+	High
1705				++	+++	M	M	ɣ	β	+	-	-	+	+	+	+	+	+	High
1708				++	+++	M	H	ɣ	β	+	-	-	+	+	+	+	+	+	High
1699		Kp C4	25	+	+++++	M	H	ɣ	β	+	-	-	+	+	+	+	+	+	High
1734		Kp C5	21	-	++++	M	H	ɣ	β	-	-	-	+	+	+	+	+	-	High

KAH= King Abdallah hospital, PRH= Prences Rahma hospital, H= high, M= medium, L= low, PIF= powdered infant formula, Ho.= horse, S= sheep.

Table 4-15: Results summary of attachment, invasion and phagocytosis survival of Caco-2, HBMEC, T24 and U937 cell lines, which were used in a comparative study between QMC and Jordanian strains.

Strain	Species	Source	ST	O-antigen	K type	Caco2		T24		HBMEC		Survival in macrophages	Pathogenicity assessment
						attach	invasi	attach	invasi	attach	invasi		
1681	*K. pneumoniae*	EFT*	111	O1	K2	M	Neg	M	M	H	M	Persist+ replicate	High
1699	*K. pneumoniae*	EFT*	247	O3	K1	M	Neg	M	M	M	H	Persist+ replicate	High
1701	*K. pneumoniae*	EFT*	247	O3	K1	M	Neg	H	VL	H	H	Persist+ replicate	High
1725	*K. pneumoniae*	EFT*	111	O12	K2	M	VL	H	H	M	H	Persist+ replicate	High
1446	*K. pneumoniae*	NEFT**	37	O2	ND	M	Neg	H	H	H	H	Persist+ replicate	High
1734	*K. pneumoniae*	EFT*	526	O2	ND	M	Neg	M	M	M	M	Persist+ replicate	High
453	*K. pneumoniae*	NEFT**	105	O4	K5	H	M	H	M	M	H	Low persist	Medium
497	*K. pneumoniae*	NEFT**	147	O12	K5	M	Neg	H	L	L	H	Killed	Low
2291	*K. pneumoniae*	Sepsis	35	ND	ND	M	Neg	M	L	H	H	Killed	Low
2298	*K. pneumoniae*	Sepsis	35	O8	ND	M	Neg	M	M	M	H	Killed	Low
2312	*K. pneumoniae*	Sepsis	34	O2	ND	L	Neg	M	M	M	H	Killed	Low

EFT*= enteral feeding tube Jordannian strains, NEFT**= neonatal entering feeding tubes Nottingham strains, ST= sequencing type, H= high, M= moderate, L= low, VL= very low, Neg= negative, attach= attachment, invasi= invasion

4.6. Discussion;

4.6.1. *K. pneumoniae* collected from flushed and feeding tubes from two Jordanian hospitals (KAH and PRH);

The presence of *K. pneumoniae* in PIF or in any enteral diets associated with the feeding of hospitalised babies would seem to be a possible source of infection, particularly in immunocompromised individuals, such as premature babies. Furthermore, the incidence of *K. pneumoniae* infection is likely to increase because those individuals are highly susceptible to nosocomial infections. Hospital-acquired infections are globally problematic due to their clinical impacts as well as financial and ethical influences. Of significance is the inability to reduce the impact of these infections due to the difficulty of controlling outbreaks worldwide (WHO, 2011).

The identification, differentiation and determination of the source of transmission of pathogens is very important to effectively reduce and control outbreaks, and this requires good strategies and appropriate techniques (Almuneef *et al*., 2001). Cetinkaya and co-authors (2013) reported that molecular subtyping techniques, such as PFGE and MLST, are considered more accurate than phenotypic methods. These techniques are useful in determining if strains are clonally related, defining epidemiological studies, as well as determining the source of infection and the diversity among pathogens.

In 2004 and 2006, respectively, the FAO and WHO reported that non-sterile PIF products could be attributed as the main reservoir of bacterial pathogens which could cause serious infantile diseases (*viz*. in children aged < 1 year). The majority of pathogens belonging to the Enterobacteriaceae that are readily isolated from PIF include *K. pneumonia*, *Salmonella* spp., *E. coli*, *E. hormaechei, Cronobacter* spp., and *C. freundii* (Townsend *et al.,* 2008; Caubilla-Barron *et al.*, 2007). Alkeskas and co-authors (2013) investigated *E. coli* K1 strains isolated from neonatal enteral feeding tubes from two local hospitals in the UK. They found 4 pulse types by PFGE, one of which was unique; 3 strains clustered into each of the pulse types 1, 2 and 4, whilst 19 strains had clustered into pulse type 3. Furthermore, Zhang *et al.* (2012) investigated 51 strains of *Citrobacter* spp., from different sources, by using PFGE, demonstrating a 48% similarity among isolates from the same source. Another study, by

Kanamori and colleagues (2011), was carried out on 57 isolates harbouring ESBLs in Japan, and found that 13 different clusters existed, whilst 18 isolates belonged to a unique cluster.

As part of the present study, seventy-five *K. pneumoniae* strains, isolated from the EFT of neonates in NICUs from two different hospitals (KAH and PRH) in Jordan, were analysed using PFGE in order to determine the source of transmission and genotypic relatedness between strains. This analysis indicated genetically indistinguishable strains forming five pulsotypes. These strains had been isolated from different hospitals and neonates over a six-month period (22 May to 20 December 2011). Kp2 comprised a unique strain (1725) isolated from the same tube as strain 1726, which was typed as the Kp1 pulsotype. The Kp2 *XbaI* profile differed from Kp1 by 8%, and could also be distinguished from Kp1 by using *SpeI* restriction digestion Figure 4-2 and Figure 4-3. Strains belonging to pulsetype Kp3 and strain 1699 (pulsetype not determined) formed the second largest cluster isolated on the same day (31/05/2011) from three different neonates. These neonates were fed S26 and neosure reconstituted PIF. These findings associated with these neonates illustrate the possible spreading of strains within the NICU. Of particular significance was pulsotype Kp1, the largest cluster, which was composed of 61 out of 75 indistinguishable strains from 12 different neonates; two strains were isolated from KAH, eleven strains were from RAH over six months period. Seven neonates were fed bebelac, whilst four others had received S26, and one was fed neosure powdered infant formula Table 4-2.

Of particular interest was indistinguishable *K. pneumoniae* isolated from the feeding tubes of 21 neonates from two different Jordanian hospitals. Pulsetype Kp1 was the most predominant pulsetype isolated from babies of both hospitals. In addition, these hospitals had used the same brand of PIF to feed neonates, where KAH started feeding neonates in May 2011, whilst PRH had started in July. The first sample of *K. pneumoniae* was obtained from KAH and then subsequent collection was made two months later from PRH. Interestingly, clonally related strains of pulsotype Kp1 was confirmed to be the same clone that had cross contaminated different feeding tubes and prevalence of the same clone was noted among those recovered from different hospitals and neonates over a short period. This emphasises the possibility that strains were acquired due to spreading within the NICU by carers and the environment and not due to a specific feeding source, such as

contaminated PIF. Possible sources could include the PIF itself, the hands of hospital workers, or through the preparation of PIF for infants, which can be considered as a vehicle of transmission in the NICU. This consequently may lead to contamination of fresh feed in the tube lumen, leading to subsequent bacterial multiplication.

Brady *et al.* (2005) reported that contamination by health care workers may play an important role in bacterial transmission between patients. Enterobacteriaceae are the most common type of organisms that can be transmitted from the hospital environment to patient. These findings are in agreement with Muytjens *et al*. (1988) who isolated various Enterobacteriaceae from PIF, which is also consistent with the reports of the FAO and WHO (2004; 2006) stating that non-sterile PIF products may become the main reservoir for bacterial pathogens associated with serious infantile diseases. This therefore indicates that the colonisation of neonatal enteral feeding tubes by certain pathogenic lineages within NICUs could lead to increased exposure and risk to the neonates. In addition, Hurrell *et al.* (2009a and b) investigated 129 neonatal enteral feeding tubes from two local hospitals (QMC and NCH) in Nottingham. They found a wide range of Enterobacteriaceae, included *K. pneumoniae*. *E. coli*, *E. hormaechei*, *K. oxytoca*, *Cronobacter* spp., *Serratia marcescens*, *S. liquefaciens* and *E. cancerogenus.*

The identification of *Klebsiella* spp. has proven to be quite difficult and often leads to misidentification in clinical microbiology laboratories. Nevertheless, the correct identification of *K. pneumoniae* is required because these isolates vary in their virulence as well as antibiotic sensitivity (Brisse *et al*. 2014; Podschun *et al*. 2001). Previously, *K. pneumoniae* was identified by traditional methods based on morphological and physiological traits, which would usually result in incorrect identification. However, Brisse & Verhoef (2001) and Brisse & Duijkeren (2005) reported that the beta subunit of RNA (β-RNA) polymerase has been recommended as a core gene signature, which is used as a powerful identification tool for phylogenetic analysis of *Klebsiella* and differentiation among closely related strains.

In this chapter, the *rpoB* gene sequence was used to analyse the 75 *K. pneumoniae* strains, which showed three different *rpoB* alleles profiles: 4, 21 and 25. Multiple alignment data

were analysed according to the Pasteur MLST database; Appendix C. These results indicate that the *rpoB* allele sequence is a powerful identification tool that allows for reliable classification of isolates and speciation.

The existing *K. pneumoniae* MLST scheme is based on seven housekeeping genes, *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB* and *tonB*, which have been deposited in the *K. pneumoniae* Pasteur PubMLST database:

(http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html).

The MLST tool has been used in large scale epidemiological studies to discriminate between strains. MLST allows identification, detection and typing of vastly different clones. Nine strains of *K. pneumoniae* from two different NICUs Jordanian hospitals, representing different pulse types, were chosen for MLST analysis. In terms of sequence types, the tested strains divided into 3 different STs: ST111 (n = 5), ST247 (n = 3), and ST526 (n = 1). The ST profiles and PFGE clustering agreed well among isolates. For instance, *K. pneumoniae* strains 1681, 1690, 1693 and 1715, all typed as ST111 by MLST, were all indistinguishable within PFGE cluster (Kp1) even though they were isolated from 14 different babies over a six-month period. Furthermore, *K. pneumoniae* strains 1701 and 1705 revealed indistinguishable PFGE profiles (Kp3) and both belong to ST247, whilst being isolated from two different babies on the same day. This is a cause for concern because the results indicate that some strains might persist in the NICU over time and contaminate the EFT of multiple babies. From the findings of the current study, it is suggested that weekly microbiological screening is carried out, particularly in NICUs with low birthweight (immunocompromised) babies, for detecting probable sources of pathogens in order to prevent possible transmission of pathogens within these wards. A late detection of causative agents would lead to a delayed response to these agents, and therefore might contribute to difficulties in controlling such unexpected infections.

KO and co-authors (2002) reported that *K. pneumoniae* infections cause a wide spectrum of diseases, including meningitis, septicaemia, urinary tract infection, pneumonia and purulent abscess at different sites. Several virulence traits contribute to the pathogenesis of

Klebsiella, including virulence-associated genes such as capsular serotype, iron uptake system, attachment and invasion genes.

Biofilm formation is an essential step for bacterial pathogenesis that contributes to attachment and multiplying on abiotic and biotic surfaces, such as medical equipment, food contact surfaces and food. Biofilm formation not only gives the pathogen an ability to survive within the host, but also allows further persistence in inhospitable environments such as high temperature, fluid flow, variable pH, and carbon sources (Hall-Stoodley *et al.*, 2004). Balestrino and co-authors (2005) stated that *K. pneumoniae* is a significant causative agent of nosocomial infections, which is hard to treat and has an ability to persistent in the harsh conditions of hospitals. Furthermore, type 1 and type 3 fimbriae in *K. pneumoniae* strains are involved in biofilm formation, as well as type 3 pili, which are generally considered to be a virulence factor (Hennequin and Forestier, 2007; Podschun *et al.*, 2001). In addition, Romeo, (2008) has reported that biofilm formation may be affected by different circumstances such as O_2, temperature, osmolarity and pH. In this study, all of the strains tested were able to form significantly more biofilm at 37 ^{o}C in PIF than at 25^{o}C ($p < 0.05$); Figure 4-4 and Table 4-7.

Further analysis was conducted on the role of the Congo-red binding assay. Several reports documented that curli-fimbriae play an important role in bacterial biofilm formation and adherence (Romeo, 2008; Lee *et al*., 2011). Data presented in Table 4-7 showed that *K. pneumoniae* strains 1734, 1741 and 1754 expressed curli fimbriae, whereas the other isolates did not. It was interesting to note that no homologues of *csg*BAC and *csg*DEFG could be detected by whole genome sequences whilst searching for curli-fimbriae gene clusters, suggesting that the Congo red may have reacted with other materials and gave a false positive result for these three strains. However, a similar study was conducted by Zogaj *et al.* (2003) on *Klebsiella* spp. isolated from the gastrointestinal tracts of healthy people. The authors reported that this assay is not always accurate because it sometimes gives false negative results. The current study reinforces the findings of previous studies on characteristics of Calcofluor growth in *Klebsiella*. In this study, the synthesis of cellulose by *K. pneumoniae* was indicated by the observation of blue florescence colonies under UV light. The majority of the isolates tested exhibited strong a fluorescent signal indicating high level

production of cellulose, whilst a small number of strains produced moderate amounts of cellulose.

Several genes have been documented in the literature to be linked with bacterial biofilm formation, however, in *K. pneumoniae* only two genes are recognised to be associated with biofilm formation type 1 and type 3 fimbriae (*fimH* and *mrkD*), and the genome search showed the presence of these genes in all examined strains. Furthermore, it is clear that some bacterial factors such as cellulose production, motility, capsular production and curli fimbriae are factors that influence biofilm formation (Hu *et al*., 2015). There was an observable correlation between the phenotypic detection of capsule production on PIF agar, cellulose production by Calcofluor, and the biofilm formation on rehydrated PIF. This study in agreement with Hu *et al*. (2015), who reported that capsular material and cellulose production may contribute to biofilm formation.

The ability of *K. pneumoniae* to adhere to host surfaces may lead to multiplying and persistence in the hospital environment and contributing to severe nosocomial infections (Mandlik *et al.*, 2007). Generally, adhesion in Enterobacteriaceae is mediated by various types of fimbrial genes, and most *K. pneumoniae* strains possess two different types of adhesion fimbrial genes: type 1 (T1P) and type 3 (T3P) fimbrial genes. Furthermore, T1P fimbriae in *K. pneumoniae* is closely linked to T1P fimbriae exhibited by other strains of Enterobacteriaceae. These types of fimbriae enable what is known as mannose sensitive haemagglutination (MSHA), and the association of these fimbriae (T1P) with pathogenesis in UTIs is well studied in *E. coli* and *K. pneumoniae* isolates in animal models. T1P fimbriae of *K. pneumoniae* mediate many UTIs and respiratory tract infections (RTIs) (Struve *et al.*, 2008; Struve *et al*., 2009). On the other hand, type 3 fimbriae in *K. pneumoniae*, unlike any other fimbriae in Enterobacteriaceae, enable mannose resistant haemagglutination (MRHA). T3P fimbriae were once thought to be a unique type of fimbriae synthesised by *Klebsiella* (Combe *et al.*, 1994), but later reports stated that T3P fimbriae can be found in most enteric genera. Nevertheless, T3P fimbriae are apparently not identical in all enteric genera due to the expression of considerable antigenic diversity (Combe *et al.*, 1994; Old *et al.*, 1985; Murphy *et al.*, 2013; Struve *et al.*, 2010). DiMartino and co-authors (2003) reported that T3P fimbriae share functional attributes with curli fimbriae and both have the ability to interact with

polyester and glass surfaces. Additionally, both are able to mediate bacterial adherence (biofilm-associate) to extracellular matrix (ECM) proteins. According to Burmølle *et al.* (2008) and Struve *et al.* (2009), these fimbriae can bind to different human cell lines such as uroepithelial cells, and epithelial cells of the respiratory tract. They also mediate *Klebsiella* adhesion to the tubular basement membranes in the kidneys. These types of fimbriae are strongly associated with long-term UTIs in indwelling catheterised patients but not in short-term catheterised patients (Maki *et al.*, 2001; Warren *et al.*, 2001). For these reasons, the presence of the *fimH* and *mrkD* genes in the genomes of all strains was examined in this study.

As shown in Table 4-6, all the investigated isolates were able to form high levels of biofilm and carried both types of fimbriae (T1P and T3P). These findings agree with the previous work detailed above that the prevalence of type1 and type3 fimbriae in *Klebsiella* is high. The presence of these virulence traits may contribute to the adherence this bacterium on medical devices and persistence in hospitals, particularly in long-term catheterised patients, which could cause profound effects on the patient's health. Although all tested strains possess T1P and T3P fimbriae, all strains did not reveal high levels of attachment to all cell types, which suggests that the expression levels could differ among strains, or the genes could have mutated and become non-functional in some strains. More studies are needed to shed light on the importance of T1P and T3P for adherence because all presented strains possess these genes, however their expression levels had differed among strains.

Capsule production is also associated with biofilm formation in enteral feeding tubes from premature babies in NICUs (Hurrel *et al*., 2009a). According to previous studies by Pomakova *et al.* (2012) and Fodah *et al.* (2014), the possession of extremely high viscosity and thick extracellular polysaccharide capsule by *K. pneumoniae* can contribute to biofilm formation. This capsule in *K. pneumoniae* strains is a universal feature; it is considered as a highly potent host inflammatory inducer and protects the bacteria from bactericidal human serum and engulfment by phagocytes. In 2004, Fang *et al.* reported that highly serum tolerant *K. pneumoniae* associated with hyper-muco-viscosity was more predominant among invasive isolates that were linked with metastatic meningitis in mice, and often in human and animals as well. In addition, Jung *et al.* (2013) stated that it is likely that specific capsular serotypes

in *K. pneumoniae* induce pathogenicity in mice. The degree of virulence is linked with specific capsular serotypes (see below).

In this study, 25 representative *K. pneumoniae* strains were investigated to find out more about variation on two different types of media: XLD and PIF agar. There was noticeable variation among examined isolates in capsule production, and hyper-muco-viscosity was more noticeable on PIF than XLD. These data confirm that the ingredients in the media can influence bacterial capsular production. Van Acker *et al.* (2001) reported that PIF ingredients play a crucial role in bacterial attachment in feeding tubes, as well as in growth and persistence. Furthermore, bacteria grown in PIF demonstrated an ability to produce remarkable hyper-muco-viscosity that led to their growth on the inverted lid of the petri dish. Thus, this feature is considered to be an important virulence factor associated with these isolates that may contribute to their potential risk to neonatal health (Caubilla-Barron *et al*., 2007). For this reason, capsule staining may be of benefit during diagnostic testing and environmental screening for bacterial pathogens within the NICU.

The presence of capsules in *Klebsiella* is a very important virulence trait linked with severe infections. In addition, according to Lin *et al.* (2014) and Pan *et al.* (2013), among 77 capsular serotypes, only K1, K2 and K5 capsular serotypes were associated with severe infections in humans and animals. Furthermore, Siu *et al.* (2012) and Yang *et al.* (2009) reported that hyper-muco-viscosity in *K. pneumoniae* serotypes K1 and K2 is well known in Asian countries, particular in China, Korea and Taiwan. Moreover, in the last two decades in Taiwan, *K. pneumoniae* was detected as a causative agent in community acquired infections associated with serotypes K1, K2 and hyper-muco-viscosity (Yeh *et al.*, 2007; Fang *et al.*, 2004; Ma *et al.*, 2005). Although Western countries consider *E. coli* and polymicrobial infection as the predominant causes of pyogenic liver abscesses, *K. pneumoniae* was stated found to be progressively recovered from Asian patients in the USA (Rahimian *et* al., 2004; Decre *et al*., 2011). Bacteraemia and liver abscess were later found to be associated with the Taiwanese syndrome connected with virulent *K. pneumoniae* serotype K1, the first cases associated with virulent *K. pneumoniae* hyper-muco-viscosity serotype K1 from the United States was in 2005 (Fang *et al.*, 2005; Nadasy *et al.*, 2007).

It is interesting to note that pyogenic liver abscess disease cases, which are predominantly reported in Asia, is becoming increasingly prevalent worldwide. This would indicate that these infections have become globally problematic. It is notable that the majority of incidence cases recorded outside of Asia involved Asian ethnicity patients. That suggests that genetic predisposition likely plays a role, or that there may be a specific food source commonly consumed in Asia that is associated with the causative pathogen (Frazee *et al.*, 2009; Karama *et al.*, 2008; Gomez *et al.*, 2007). More recently, cases from France, Ireland, Spain, Sweden, South Africa and Argentina have been found to demonstrate a global dissemination of these strains in both Asian and non-Asian individuals (Sobirk *et al.,* 2010; Vila *et al.*, 2011; Moore *et al.,* 2013; Decre *et al.,* 2011). In 2011, Decre *et al.* reported that *K. pneumoniae* isolates from fatal bacteremia cases were all of capsular serotype K2. Another study conducted in Brazil on enteral diets (milk-based and food supplement) as used in two local hospitals, concluded that *K. pneumoniae* was more frequently isolated from these samples. In addition, the K1 and K2 serogroups were detected in 6/15 *K. pneumoniae* strains, which were considered to be hospital (clinical) endemic strains linked with nosocomial infections (Viale *et al*., 2013). With regards to the presence of *K. pneumoniae* in the nutritional sector of hospitals, a similar study conducted by Pereira and Vanetti, (2015) demonstrated that these isolates were most frequently isolated from food processing surfaces, food handlers and utensils.

In the present study, 28 representative *K. pneumoniae* strains were collected from flushed EFT lumen residues and inner tube biofilm from the NICUs of two hospitals in Jordan. Capsular serotype K2 (78.57%) and K1 (17.85%) were identified among these strains, while K5 was not present among any of the isolates tested. These findings therefore confirm the presence of high virulence capsular serotypes in these *K. pneumoniae* isolates*,* posing a potential risk of serious bacterial infection in NICUs. There was also a correlation between the *rpoB* allele type and capsular serotype in this set of isolates. Strains with the *rpoB4* allele belonged to capsular serotype K2, whilst strains with the *rpoB25* allele were of the K1 capsular serotype. Control programs are needed to prevent infections caused by such strains, especially for the most sensitive patients (immunocompromised), because therapeutic options are very limited and carry a high cost.

Iron is an essential element for bacterial growth, multiplication, and progression to infection. The ability of *K. pneumoniae* to acquire iron from the host is one of the most important traits related to its virulence. Wilson *et al.* (2002) reported that all members of the Enterobacteriaceae were able to produce siderophores. Furthermore, Schubert *et al.* (2000) reported that the *irp*1, *irp*2 and *fyu*A genes of the iron uptake system were associated with pathogenicity in *Yersinia* strains.

In this study, the iron uptake genes *irp*2 and *fyu*A were detected in all tested isolates, except strain 1734 which belonged to pulsotype Kp5 and did not possess the *fyu*A gene. None of the isolates tested possessed the *irp*1 gene but despite lack of *irp*1, all strains phenotypically demonstrated an ability to express siderophores on CASAD agar, by producing an orange halo around the wells. This result suggests that these isolates could use an alternative pathway in the iron uptake system or there may be other genes related to siderophores not studied in this investigation.

Another virulence factor studied in this chapter was haemolysin activity. Haemolysins are essential toxins secreted by pathogenic bacteria, which lyse mammalian red blood cells to cause disease. There are three different categories of haemolysins: Alpha-haemolysin (α), Beta-haemolysin (β), and Gamma (γ) absence of haemolysin activity (Goebel *et al*., 1988). In the current study, *K. pneumoniae* strains were investigated for their capability to lyse sheep and horse erythrocytes. All examined isolates revealed an ability to lyse horse blood cells; this was detected as β -haemolytic activity, whereas no haemolytic activity was observed on the sheep blood. In 2002, Ring *et al.* carried out a study on haemolysin activity in group B *streptococcus* isolated from patients with sepsis. The authors reported that β-haemolysin plays an important role in the pathogenicity of *Streptococcus* by destroying the cell membrane of red blood cells and contributing to high mortality and liver failure. Therefore, the data presented show that all tested strains had similar haemolytic activities, that haemolysis is associated with disease so this suggests that these strains would be capable of causing disease to the neonatal health.

The advent of antibiotics in the treatment of microbial infections is arguably the greatest discovery in medical history as it allows us to take control of microbial infectious diseases

that contribute to morbidity and mortality in humans (Aminov RI, 2010). However, recently there has been a serious concern regarding the increase of antimicrobial resistance, and additionally, the widespread appearance of antimicrobial resistant infections worldwide.

In the present study, all of the *K. pneumoniae* strains tested were resistant to 10 of the 11 antibiotics typically used, being susceptible only to ciprofloxacin. According to Wang *et al.* (1998), *K. pneumoniae* isolated from patients with liver abscesses were highly sensitive to all antibiotics used in their study, except for carbenicillin and ampicillin. This study, however, is relatively dated having been conducted around two decades previously. In contrast, more recent studies have identified *K. pneumoniae* isolates resistant to the carbapenems, penicillins, and cephalosporins globally, and resistance to this extended spectrum of antibiotics has been documented in more than 34% of *K. pneumoniae* isolates in a medical centre of one major population area in New York (Landman *et al.,* 2007).

Additionally, Chen *et al.* (2016) reported that a patient who died in a Nevada hospital from septic shock owing to a multidrug-resistant (MDR) *K. pneumoniae* infection. The strain responsible for this infection was resistant to all 27 antibiotic that used, and the strain carried multiple antibiotic resistance genes including NDM. In 2017, the CDC stated that *K. pneumoniae* strains were the most common carbapenem-resistant among Enterobacteriaceae and they were classified as one of the top three antimicrobial resistant threats. This report stated that there were more than 7,900 infection cases and about 520 deaths in the US each year due to carbapenem-resistant *K. pneumoniae* strains. Moreover, these isolates typically possess genes that confer resistance to several other classes of antimicrobials, resulting in multidrug resistance (CDC, 2017). Carbapenems (imipenem and meropenem) are recommended as the drugs of choice and first line of treatment for severe infections caused by extended-spectrum β-lactamase producing Enterobacteriaceae, such as *K. pneumoniae* and *E. coli* strains, in particular. Carbapenems are often the last drug of choice in such infections and the spread of carbapenem-resistant strains would represent a significant threat due to limited treatment options (Van Duin *et al.,* 2013; Pitout *et al.*, 2008; Nordmann *et al.*, 2002).

In response to these findings, it is suggested that the colistin antibiotic could be used to treat carbapenem-resistant *K. pneumoniae*. However, colistin resistance is also increasing now, and mobilisation of the colistin resistance gene mcr-1 on plasmids has been a cause for concern. Therefore, colistin susceptibility testing is recommended for future work. All of the *K. pneumoniae* strains analysed in the current study were resistant to carbapenems. This is consistent with the findings of another independent study Shin-hee *et al*. (2005) that reported that the majority of foodborne MDR pathogens, such as *Campylobacter* spp., *Salmonella*, *E. coli* O157:H7, and other enteric bacteria isolated from farms were resistant to penicillins, cephalosporins, aminoglycosides and carbapenems. The present study revealed similar phenotypes of multidrug resistance, as noted in isolates previously (Schroeder *et al*., 2002; Schroeder *et al*., 2003; Swartz, 2002). In fact, the high level of antimicrobial resistance observed among the examined strains in this study is of huge concern, even despite the observed susceptibility to ciprofloxacin. The data generated in the current study reveal the high prevalence of specific *bla* genes, which are responsible for extended-spectrum β-lactamase production; multidrug resistance could also be due to the presence of a multidrug efflux pump gene, which was detected within the genomes of these strains.

Several recent studies stated that virulence and antimicrobial resistance genes present in *K. pneumoniae* genomes may lead to more challenges in the control and treatment of infections caused by these isolates. In the last two decades, antimicrobial resistance, particularly due to extended-spectrum β-lactamases (ESBLs), has increased dramatically within clinical members of the Enterobacteriaceae. Furthermore, during last decade, the $bla_{\text{CTX-M}}$ gene became a major ESBL type circulating worldwide, more so than the β-lactamase gene bla_{SHV} (Zhao and Hu, 2013; Calbo and Garau, 2015). Surveillance studies in Europe, Asia and South America showed dramatically increases to cephalosporin resistance among *Klebsiella* species and *E. coli* strains, mainly contingent on the dissemination of $\text{bla}_{\text{CTX-M}}$ type ESBLs (Kumarasamy *et al*., 2010; Zhao and Hu, 2013). Furthermore, Pitout (2013) reported that $bla_{\text{CTXM-15}}$ was the most prevalent in the majority of European countries, and it has also recently spread to Latin America, the USA and Canada (Denisuik *et al*., 2013; Wang G. *et al*., 2013; Kazmierczak *et al*., 2015). Similarly, in a tertiary hospital in China, ESBL-producing *K.*

pneumoniae strains were found to be responsible for about 50% of nosocomial infections, with *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-15}$ being the most predominant ESBL types identified in this study (Wang *et al*., 2013; Li *et al*., 2014; Yang *et al*., 2015). In addition, it has been suggested that there were several types of plasmids, namely IncN, IncI, IncF, IncK, IncHI2, and IncL/M groups, which were found to be associated with the *bla*$_{CTX-M}$ genes. Furthermore, the IncF plasmid group contributes to spread of the bla$_{CTX-M-15}$ gene (Zhao and Hu, 2013). With regards to quinolone resistance-associated genes, the *oqxAB* gene encoding an efflux pump was found in the genomes of 163 out of 167 *K. pneumoniae* strains, indicating that this gene is extremely conserved in *K. pneumoniae* (Kumar *et al*., 2011; Perez *et al*., 2013). Moreover, 16 aminoglycoside resistance genes were identified in the same genomes, with the *aac(6')-Ib-cr* and *ant(3")-Ia* genes being the most frequently reported genes in 6 genomes (Hansen *et al*., 2004). In support of these findings, studies were performed on *K. pneumoniae* strains BG130 and BG141 in Madagascar (2007) and Vietnam (2008), respectively. These isolates were revealed to harbour bla$_{CTX-M-15}$ and genes conferring resistance to fluoroquinolones and aminoglycosides. Additionally, a bla$_{CTX-M-15}$ producing strain was reported previously in South Korea (Hu *et al*., 2008; Shin *et al*., 2014). Furthermore, in a subsequent study on *K. pneumoniae* strains isolated from Labbafi nejad Hospital in Tehran, *bla*$_{SHV-11}$, *bla*$_{SHV-12}$ and *bla*$_{TEM-1}$ were found to be the most prevalent ESBL types (Feizabadi *et al*., 2010a; 2010b). In the current study, the *bla*$_{CTX-M-15}$ gene was detected in only two *K. pneumoniae* strains (1681 and 1699; ST11) which were recovered from EFTs. All presented strains showed possession of the IncF plasmid group, which likely plays an important role in the dissemination of bla$_{CTX-M-15.}$ In addition, *bla*$_{SHV-11}$ and *bla*$_{TEM-1}$ were the commonly detected β-lactamase genes in the majority of subjected isolates, in addition to the fluoroquinolone resistance gene (*oqxAB*), which was present in all isolates, whereas the *aac(6')Ib-cr* gene was found only in strains 497 (ST147), 1681 and 1699 (ST111), and 1734 (ST526). Of note, strains 1681 and 1699(ST111), which were isolated from EFT, had expressed more than 4 aminoglycoside resistance-associated genes. Furthermore, macrolides, sulphonamides, tetracyclines and trimethoprim resistance associated genes were also detected Table 4-12. These findings are consistent with several previous studies (Hu *et al*., 2008; Kumar *et al*., 2011; Denisuik *et al*., 2013; Wang

G. *et al*., 2013; Zhao and Hu, 2013; Perez *et al*., 2013; Shin *et al*., 2014; Kazmierczak *et al*., 2015).

The phenotypic and genotypic features identified in the current study indicate that healthcare workers, hospital environment and PIF could be a significant source of *K. pneumoniae* strains. The presence of haemolysin activity, biofilm formation, siderophores, serum resistance, polysaccharide capsule (mucoid) production and capsular serotypes K1 and K2, which are the most significant virulent traits produced by *K. pneumoniae*, indicate an extremely high potential risk to neonates. The existence of *K. pneumoniae* either in the healthcare workers, hospital environment or PIF needs to be considered as a possible contamination source for babies. Furthermore, this may increase infections due to prescribing PIF to highly susceptible neonates in the NICUs, suspected to have prolonged indwelling in NICUs, and thus exposed to possible infection. We know these babies are being fed every 2 hours, which would mean these babies ingest these bacterial pathogens every 2 hours (Hurrell *et al*., 2009b). The findings of this study therefore take into account highly virulent traits linked with these isolates, which may form a significantly high risk to the health of neonates in NICUs.

Weekly microbiological screening in the hospital sector, particularly in NICUs with low birthweight, immunocompromised babies, could be beneficial for detecting probable sources of pathogens, in order to prevent possible transmission of pathogens within and between wards. Late detection of causative agents leads to delays in response, which may contribute to the difficulties in controlling such unexpected infections. The data presented in this study contribute to the understanding of the characterisation and diversity of these organisms. In addition, this study aimed to identify possible methods of prevention, which could limit the risk of contamination in neonatal food products.

4.6.2. Comparison of selected *K. pneumoniae* (Jordan) isolates against representative *K. pneumoniae* (Nottingham) strains

In the last few decades, bacterial heat-tolerant contaminations have increased and have become a source for concern, particularly in human food safety. Despite numerous Enterobacteriaceae strains expressing different heat tolerance levels after heat shock, only

a relatively small number of strains of this family have innate thermo-tolerance, phenotypically (Mercer *et al*., 2015; Bojer *et al*., 2010). In 2009, Forsythe reported that in some neonatal care units, the PIF and fortified breast milk were reconstituted at room temperature rather than at higher temperatures (Forsythe, 2009). Rosset *et al.* (2007) stated that samples were reconstituted with water at room temperatures in some French hospitals as well. Current recommendations are PIF should be prepared following the official guidelines (using sterile bottles and boiled water allowed to cool to 55^{o}C, which is sufficient to kill most bacterial pathogens). But Iversen and co-authors (2004a) reported that some microbes are able to tolerate and survive at 55 °C which could contribute to pathogens causing neonatal infections in NICUs even when formula preparation guidelines are followed correctly. Additionally, they found that *C. sakazakii* isolates were able to grow in PIF between 5-45 °C and adhere to infant feeding equipment, which may thus become a source of neonatal infection in NICUs. Additionally, the WHO and FAO (2006) reported that the reconstitution of PIF with water between 40 - 50 ^{o}C and then keeping feeding bottles at room temperature for a long time may be associated with the increase in neonatal infections, so the bottles should be used immediately. The heat tolerance analysis carried out in this study was designed to evaluate the ability of strains to tolerate temperatures of more than 54 °C for 30 minutes in liquid infant formula. The findings showed that *K. pneumoniae* strains vary in their tolerance to 55 °C. These isolates were classified into three groups based on their ability to tolerate heat. Strain 1701 (ST247-O3-K1), which was isolated from EFT, had high thermo tolerance with D_{55} = 12.9 minutes. Strains 1446 (ST37-O2-ND), 2298 (ST35-O2-ND) and 2291 (ST35-ND-ND), which were isolated from NEFTs and neonates with sepsis, had moderate heat tolerance with D_{55} = 9.7, 5.10 and 5.0 minutes, respectively. In contrast, the remaining *K. pneumoniae* strains 2312 (ST34-O2-ND), 1725 (247-O12-K2), 1699 (ST247-O3-K1), 453 (ST105-O4-K5), 1681 (ST111-O1-K2), 497 (ST147-O12-K5) and 1734 (ST526-O2-ND) were susceptible to heat with D_{55} = 4.8, 4.5, 4.4, 4.0, 3.6, 2.7 and 1.2, respectively Figure 4-10 and Table 4-9. These data showed that there was no correlation between heat tolerance, strain source (EFT vs NEFTs and sepsis, Jordan vs Nottingham), O-antigen type, and capsule serotype. It has been previously suggested that capsular serotype and O-antigen may increase the ability of *K. pneumoniae* isolates to tolerate heat (Tomas *et*

al., 1986; Fang *et al*., 2004; Pan *et al*., 2011), but no such links were evident in the current study.

There is a genetic locus which could be responsible for heat tolerance, in species including *E. coli*, *C. sakazakii* and *K. pneumoniae* (Gajdosova *et al*., 2011; Mercer *et al*., 2015; Bojer *et al*., 2010). A study by Bojer and co-workers (2010), in Danish hospitals, found that phenotypically multidrug-resistant *K. pneumoniae* strains were able to persist in reusable contaminated endoscopes. They suggested that the adaptability of these strains might be due to their resistance to environmental stresses, such as heat. They then discovered a locus encoding small heat shock proteins clp ATPase (*clpK*). Furthermore, the *clpK* family were newly described in clinical *K. pneumoniae* strains. They contribute to the survival and persistence of *K. pneumoniae* in certain harsh hospital environments, such as heat stress on endoscopes. In addition, *clp*K is present in the genomes of the majority of Gram-negative bacteria, and has been identified in genomic regions of *C. sakazakii*, possibly explaining the high thermo-tolerance of this organism (Bojer *et al*., 2010; Gajdosova *et al*., 2011). In support of this notion, Jørgensen and co-authors (2016) reported that two-thirds of all *K. pneumoniae* strains isolated from clinical environments harboured *clpK* genes, which play a crucial role in the heat resistance phenotype in order to colonise and survive on medical equipment successfully. Lee and co-authors (2016) reported that this locus, acquired horizontally in some bacteria, is not associated with specific isolates of Enterobacteriaceae, but nevertheless is present widely in other proteobacteria. Therefore, in the present study, screening of the genomes of representative *K. pneumoniae* strains for the presence of the *clp* family of proteins was carried out. All of the strains tested were found to harbour heat shock resistance genes Table 4-10. Although, all tested strains possessed these genes which are linked with heat tolerance, but some of the strains had high, moderate and low heat resistance, it possible that some defaults that makes these genes were poorly expressed or non-functional in these isolates. This suggests that heat resistance has become a very important virulence trait in *K. pneumoniae*, and likely represents a potential threat in PIF and other food products that require heat treatment. *K. pneumoniae* thus poses a serious threat to premature, immunocompromised babies in NICUs.

PIF is the main nutritional source for most hospitalised neonates who unable to feed from the breast, especially premature babies that feed through nasogastric feeding tubes. The presence of *K. pneumoniae* in the manufacture of PIF, or any other food products, indicates a lack of hygiene in food production. Sometimes, during the PIF desiccation process, sub-lethally injured cells are difficult to detect. In the current study, 11 *K. pneumoniae* strains (6 strains, n=3 from NEFTs, n=3 from neonatal sepsis blood cultures, Nottingham and 5 strains from EFT, Jordan) were exposed to dry stress conditions in PIF and then cultured on two different media (TSA and VRBGA) in order to determine the effect of these media on bacterial cell recovery. The injured cells cannot grow on VRBGA, because the conditions in this media are harsher than TSA. As shown in Figure 4-11 and Figure 4-12, the recovery of *K. pneumoniae* cells on non-selective media (TSA) was significantly higher ($P < 0.05$) than on selective media (VRBGA) during a three-week period, when compared to the initial inoculum. On the other hand, the number of dead and sub-lethally injured (undetected) cells was higher on VRBGA than on TSA during this period Figure 4-13 and Table 4-11. Of particular interest was the poor survival of NEFTs strains 453 and 497, at 5.5 and 5.4 $\log_{10}$ CFU/ml, respectively. Although these strains were found to possess the *rpoS* gene, which is linked to desiccation tolerance, it is possible that the *rpoS* gene was poorly expressed or non-functional in these strains, or that there were other explanations for their poor desiccation tolerance. On VRBGA, Jordanian strains 1699, 1681, 1734 and only one QMC strain (2291) showed high recovery with 5.3, 4.8, 4.0 and 4.0 $\log_{10}$ CFU, respectively. These findings indicate the ability of some, but not all, of the *K. pneumoniae* isolates to preserve their viability during the process of desiccation in PIF. These data are in agreement with the FAO-WHO publication (2006), and confirm again the potential high risk of neonatal infection by *K. pneumoniae* during ingestion of contaminated food.

Bacterial recovery after desiccation can be contributed to by many factors such as capsule formation, ingredients of media and milk contents. These components could provide protection to the bacteria during the drying process and lead to live attenuate (Lian *et al*., 2002; Caubilla-Barron and Forsythe, 2007). Moreover osmotic effects during the PIF formulation process could contribute to the ability of *K. pneumoniae* to tolerate desiccation (Caubilla-Barron and Forsythe, 2007; Osaili and Forsythe, 2009). According to Wesche *et al.*

(2009), bacterial sub-lethally injured cells are able to mend their cellular damage and recover their features, including virulence traits. Therefore, sub-lethally injured cells of *K. pneumoniae* isolates in this study could repair their viability and virulence characteristics and ultimately cause neonatal infections. There is a potential problem with false negative results during investigations of *K. pneumoniae* in dried food products, particularly PIF, if samples are plated onto selective media such as VRBGA. To avoid false negative results in such cases, it is suggested to use enrichment media (TSB, BPW or BHI) which can provide a chance for damaged cells to improve their viability before culturing onto selective agar.

There are several regulatory genes that contribute to the ability of microorganisms to withstand environmental stresses. For instance, the *rpoS* regulatory gene contributes to resistance to environmental stresses such as desiccation, cold stress, oxidative stress and osmotic stress in *E. coli* and other related bacterial species (Coldewey *et al*., 2007; Hryckowian *et al*., 2013; Stasic *et al.,* 2012; Alvarez-Ordonez *et al.,* 2012). In the current study, screening for the *rpoS* gene revealed that all subjected strains possess this regulatory gene Figure 4-14. The differences seen among strains, with regards to their ability to tolerate desiccation, may be due to other genes that play a crucial role in desiccation, such as colanic acid exopolysaccharide and cellulose biosynthesis genes (Grim *et al*., 2013). Findings from this study predicts that the *rpoS* is likely to an enhancing component to keep *K. pneumoniae* persistent for long-term survival in case of environmental stress, such as low nutrients, desiccation, dry stress, and acidity. It could also provide *K. pneumoniae* with a competitive advantage when nutrition is limited. This study also found that there was no significant difference in dessication resistance between strains from NEFTs, neonatal sepsis cases and from EFTs.

Klebsiella cause host infections using several strategies including adhesion, invasion, serum resistance and other virulence traits. In this study, the potential virulence of 11 *K. pneumoniae* strains [n = 3 NEFTs, n =3 sepsis strains (Nottingham) and n = 5 EFT strains (Jordan)] was assessed in attachment/invasion, cytotoxicity and survival assays using the Caco-2 epithelial cell line (representing the intestinal tract), T24 epithelial cells (representing the urinary tract), HBMEC endothelial cell line (representing the blood brain barrier), and U937 macrophage cell line (representing the immune response). These particular cell lines

were used in order to investigate the potential ability of the *K. pneumoniae* isolates to cause NEC, UTI, meningitis, and bacteraemia, which are infections that can contribute to systemic morbidity, and even mortality.

All of the *K. pneumoniae* strains tested were able to attach to mammalian epithelial and endothelial cells. There was some variation between strains in attachment levels, but no clear correlation between strain source (EFT vs NEFTs and sepsis) and cell attachment was evident. Microbial attachment to host cells is an important first step in infection (Wilson *et al.,* 2002). After attachment, the microbes are then able to cause disease via specific virulence traits such as invasion, toxin secretion, and multiplication.

After adhesion to the host cell surface, some bacterial pathogens are able to invade epithelial and endothelial cells allowing progression towards more invasive disease (Townsend *et al*., 2008). Bacterial adhesion to host surfaces is an essential first step in the invasion of host cells. The representative strains were therefore investigated to determine their ability to invade the cell lines mentioned previously. Only one QMC strain (453; ST105) was able to invade the Caco2 cell line ($p < 0.05$), and the EFT strain 1725 (ST11) was also able to invade Caco2 cells at low levels. However, the remaining isolates were unable to invade the Caco2 cell line Figure 4-16. It was interesting to note that QMC strain 453 (ST105) also had the highest level of attachment to the Caco2 cell line. Beyond this, there was no evident link between attachment and invasion in the Caco2 model; for instance, *K. pneumoniae* strains 497, 2298 and 1725 had elevated levels of attachment, but were nevertheless unable to invade Caco2 Figure 4-15 and Figure 4-16.

With regards to invasion of the T24 cell line, all examined strains were able to invade these cells, with some variation in the invasion levels between strains Figure 4-18. There was no clear correlation between strain source (EFT versus NEFTS and sepsis) and invasion, or between attachment and invasion levels on the T24 cell line. The majority of strains tested exhibited a high level of adhesion to the T24 cell line when compared to Caco2 cells. This suggests that the strains tested are more able to invade human bladder cells than human intestinal cells. This is consistent with studies done by Martinez *et al*. (2000) and Meier *et al*. (1996) who demonstrated that *C. freundii* and *E. coli* isolates displayed very high levels of

invasion on T24 cells and very low levels of invasion on Caco2 cells. Another study by Struve and Krogfelt in 2003 reported that bacterial capsules may play a significant role in invasion of *K. pneumoniae* to the urinary tract of an animal model, but did not have the same effectiveness in an animal intestinal model. Similarly to Struve and Krogfelt (2003), the findings of this study revealed that capsule potentially contribute to *K. pneumoniae* invading urinary tract model rather than an intestinal model.

Regarding HBMEC cell invasion, all examined isolates showed high invasion levels ($p < 0.02$) with the exception of strain 497 (ST147), which exhibited moderate levels of invasion Figure 4-20. It was noticeable that the strains tested had double and triple the level of invasion in HBMEC when compared to T24 and Caco2, respectively. Similarly, this was notable in the study conducted by Alkeskas *et al.* (2015), which revealed that *E. coli* K1 isolated from neonatal nasogastric feeding tubes showed an ability to attach and invade HBMEC. Additionally, Townsend *et al*. (2008b) studied a fatal NICU outbreak in France, which was caused by *C. sakazakii*. They found that all *C. sakazakii* isolates were able to invade and attach to Caco2 and HBMEC, and they stated that these traits may contribute to the pathogen's ability to evade the host immune defences and disseminate through the body. In addition, the ability of *K. pneumoniae* to invade HBMEC cells indicates the potential ability to penetrate the blood brain barrier (BBB) and cause neonatal meningitis. Kim (2003) stated that the ability of microbes to invade the brain and bladder endothelial and epithelial cells is certainly a major concern, particularly in *Klebsiella* spp., *E. coli*, *E. cloaceae* and *Serratia* spp., as these pathogens are able to colonise enteral feeding tubes.

Bacterial adhesion to host surfaces is a successful survival strategy when pathogens cause infection, particularly parts of the hospital environment such medical devices linked infections (Francolini, 2010). It has been recently reported that type 1 (*fim*H) and type 3 (*mrk*D) fimbriae in *Klebsiella* are considered virulent traits associated with biofilm formation (Wilksch, 2011; Bandeira, 2014). These fimbriae play an important role in adhering to several types of mammalian cell lines such as urinary bladder, respiratory tissues and endothelial tissues (Schurtz, 1998); Di Martino, (1996). Cordeiro and co-authors (2016) stated that curli fimbriae in *E. coli* play a crucial role in colonisation to the host surfaces. On the other hand, T1P and T3P fimbriae in *Klebsiella* are considered to be strongly associated with adhesion to

the host cells. Several authors have suggested that type 1 (*fimH*) and type 3 (*mrkD*) fimbrial genes are associated with biofilm formation in *Klebsiella* spp. Type 1 fimbriae (*fimH*) in *K. pneumoniae* contribute to bacterial adhesion at the host cell surface and extend beyond the capsule (Rosen *et al.,* 2008). This type of fimbriae play a crucial role in establishing a urinary tract infection. Nevertheless, type 1 fimbriae do not contribute to *K. pneumoniae* infection of lung cells or adherence to intestinal cells (Struve *et al.*, 2008). Type 3 (*mrkD*) fimbriae are important in mediating biofilm formation in *K. pneumoniae*, but have no role in lung and intestinal infections (Struve *et al*., 2009; Stahlhut *et al*., 2013). Both *fimH* and *mrkD* fimbriae enhance *K. pneumoniae* colonisation on indwelling urinary catheters (Murphy *et al*., 2013). Consistent with these previous reports, all of the strains analysed in the current study possessed the genes for type 1 and 3 fimbriae and were able to invade urinary cells but unable to invade intestinal cells.

There are several genes associated with cell invasion in *K. pneumoniae*, such as *yaeT*, *bamB* (YfgL), *nlpB*, *yfiO* and *smpA* (Bolla *et al*., 1988; Wu *et al*., 2005; Malinverni *et al.,* 2006; Pei-Fang *et al.,* 2016). Among these genes, lipoprotein gene *bamB* serves as a mediator for invasion of host cells (Rolhion *et al*., 2005; Fardini *et al*., 2007). This gene, when deleted in mutant *S. enteritidis*, reduced the invasion of enterocytes in comparison with the wild type strain (Amy *et al*., 2004). A similar study found that the deletion of this gene from *E. coli* strain LF82 decreased invasion capability into intestinal epithelial cells (Rolhion *et al.,* 2005). Hsieh and co-authors (2016) reported that the *bamB* (YfgL) gene in *K. pneumoniae*, *E. coli*, and *S. Enteritidis* has an analogous function in invasion and attachment to the host cells. All of the *K. pneumoniae* isolates in the current study were confirmed positive for the *bamB* (*YfgL*) invasion gene Figure 4-22. As all of examined strains have this gene, but not all of these isolates were good at invading cells; gene knockouts (deletion) are needed for these strains to determine the invasion ability was reduced in the deletion mutants.

The majority of the *K. pneumoniae* isolates tested were able to persist, survive and replicate within macrophages for more than 72 hours of incubation. Four strains, 497, 2291, 2298 and 2312 of *K. pneumoniae*, isolated from NEFT and neonates with sepsis, were either killed or not taken up through the macrophage phagocytic process. Jordanian strains 1681, 1725, 1701 and 1699 were able to persist, survive and replicate significantly ($p < 0.01$) within

human macrophages (U937) for up to 48 hours in comparison with other isolates. These strains were isolated from neonatal enteral feeding tubes in an NICU. The ability of these isolates to persist and replicate within bactericidal macrophages, might allow them to use the macrophage as a vehicle to establish a successful infection in other tissues/organs of the neonate.

Macrophages, through their capacity for phagocytosis, play an important role in the protection of hosts from pathogens. This was observed previously through the deletion of either alveolar macrophages or neutrophils *in vivo*, which resulted in the decreased killing of *K. pneumoniae* (Broug-Holub *et al*., 1997; Cheung *et al*., 2000). It can be inferred from this that *K. pneumoniae* relies on virulence factors to counter the effects phagocytosis by macrophages and neutrophils. In support of this notion, capsulated *K. pneumoniae* was resistant to phagocytosis by macrophages and neutrophils, whereas mutant *K. pneumoniae* isolates which lacked capsule were unable to infect lung and bladder cells (Alvarez *et al*., 2000; Cortes *et al*., 2002; Regueiro *et al*., 2006; Camprubi *et al*., 1993; Lawlor *et al*., 2005). A number of studies have shown that *K. pneumoniae* can be internalised by different cell types *in vitro* showing their ability to persist and replicate in macrophages for up to 48 hours (Oelschlaeger and Tall, 1997). The presence of *Klebsiella* spp. within epithelial lung, mouse alveolar and human macrophages *in vivo* has been documented in previous studies (Cortes *et al*., 2002b; Willingham *et al*., 2009; Greco *et al*., 2012; Fevre *et al*., 2013). The previous studies reported that capsule is an important trait to protect *Klebsiella* from phagocytosis. By contrast, in the current study, all examined strains were capsulated, however, some of them were unable to survive and persist in the macrophage. That suggest the capsule may had no protection roles among these isolates or other factors may be at play.

Bacterial cytotoxicity is considered as one of the most important virulence factors for the pathogenicity of *K. pneumoniae* and their ability to infect eukaryotic cells (Schneditz *et al.*, 2014; Darby *et al.*, 2014). This may lead to the lack of cell membrane integrity, thus allowing bacterial cells to penetrate and invade eukaryotic cells and cross epithelial barriers. The capability of representative *K. pneumoniae* isolates to cause a cytotoxic effect on Caco2 cells was determined by the reduction of MTT. As shown in Figure 4-24, *K. pneumoniae* strains 453, 1681, 2291, 1725, 1734, 1446, in addition to *S. Eenteritidis*, were highly cytotoxic to

Caco2 cells ($p < 0.05$). Isolates 497, 2312, 1699 and 1701 were moderately cytotoxic and strain 2298 had a low level of cytotoxicity which was almost comparable to that of the negative control. Overall, there is no significant difference among representative isolates when compared to each other. *K. pneumoniae* is a leading cause of nosocomial infections and is responsible for more than 70% of *Klebsiella* infections, including those of soft tissue, septicaemia, bacteraemia, UTIs and pneumonia infections. *K. pneumoniae* isolates can be differentiated by their liposaccharide (O antigen) type and capsular polysaccharide (K antigen) type. The O:K serotypes in *K. pneumoniae* have a very crucial clinical and epidemiological significance (Fang *et al*., 2007; Hsieh *et al*., 2012; Hansen *et al*., 2002). For instance, *K. pneumoniae* serotype K1, which is newly identified as a hypervirulent strain, is associated with pyogenic liver abscesses as well as septic ocular and central nervous system complications. Also, serotype O1 is linked with a hypervirulent isolate that causes pyogenic liver abscess. Furthermore, both O and K serotypes are known to assist in the establishment of *K. pneumoniae* clonality within nosocomial outbreak examinations (Hansen *et al*., 2002; Brisse *et al*., 2009; Struve *et al*., 2015). Several previous studies have reported that O1 and O2 serotypes in *K. pneumoniae* are responsible for more than 65% of all *Klebsiella* human diseases, and the O1 serotype is more prevalent than the O2 serotype (Trautmann *et al*., 1997; Trautmann *et al*., 2004; Fang *et al*., 2015; Follador *et al*., 2016). Similarly, a previous study conducted by Hansen *et al*. (1999) had investigated 638 clinical strains of *K. pneumoniae* isolated from the US, Denmark and Spain. They found that more than 89% of these strains were positive for O antigen, with the O1 antigen being the most prevalent serotype. Additionally, K serotype has more discriminatory power in epidemiological studies than O serotype due to the limited number of O serotypes compared to the K serotype. At present, there are 77 K serotypes, whereas only 9 O serotypes have been identified (Podschun *et al*., 1998). Supporting this notion, epidemic *K. pneumoniae* K25 strain harbouring extended-spectrum β-lactamase which is prevalent in Belgium and France. Similarly, in the London area, multidrug-resistant *K. pneumoniae* K41 strain was isolated from 150 patients in 14 hospitals (Aucken *et al*., 1998; Yuan *et al*., 1998). In the present study, six O antigen serogroups were detected: O1, O2, O3, O4, O8 and O12. Table 4-13. The strains identified as O1 and O2 serotypes in particular could represent a serious potential

threat to premature babies in NICUs. Interestingly, these O1 and O2 serotypes were recognised among EFT, NEFTs and sepsis strains together. Comparable analysis of the various virulence traits among the EFT, NEFTs and sepsis isolates revealed that strain 1681 (O1:K2), which was isolated from EFT Jordan, could be more virulent than the sepsis strains studied.

Taken together, the data described in this chapter suggest that these *K. pneumoniae* strains possess a concerning combination of both high risk virulence factors and high-level antimicrobial resistance which may represent a serious threat to public health. The high virulence and antimicrobial resistance were not restricted to strains isolated from sepsis cases. Strains 1681 and 1699 (ST11) and 1734 (ST526), which were isolated from EFT, were contained a greater number of virulence traits than the strains isolated from neonates with sepsis.

In the previous chapters (3 and 4), several strains such as *E. coli*, *K. pneumoniae*, *K. oxytoca* were obtained from the NICUs at different hospitals (EFT from two Jordanian hospitals, NEFTs and sepsis cases from Nottingham hospitals) and were characterised. These strains were very virulent, possessing virulence traits that can cause toxicity, adherence, and invasion of mammalian cells, tolerance of environmental stress and resistance to several antibiotics. Nevertheless, it was not possible to determine whether the NEFTs colonising strains went on to infect the babies or whether the same strain could persist in NEFTs and in the baby's gut over time. Therefore, in the next chapter (5) a longitudinal study focusing on *Enterococcus faecalis* and *Enterobacter hormaechei* will be described. These strains were isolated from paired nasogastric feeding tubes and faecal samples, obtained from a single premature baby of estimated gestational age of 25 weeks in the NICU at QMC hospital Nottingham. Samples were collected from the same baby at age 6 weeks and 8 weeks after birth. The aim of this work was to determine the virulence potential of these bacteria that colonised the feeding tubes and intestines. Additionally, this study aimed to determine whether the baby was re-infected by the same strains or by different strains, once or multiple times, and whether these strains were persistent over time.

Chapter 5. Longitudinal study of *E. hormaechei* and *E. faecalis* strains colonising the feeding tube and intestine of a premature baby over time.

5.1. Introduction

5.1.1. General background

The incidence of nosocomial and neonatal infections caused by members of the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter. baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species), has increased in recent years (Rice 2008, 2010; Holt *et al*., 2015). These organisms are known to be the major causative agents in neonatal intensive care unit (NICU) infections and multidrug-resistant infections (Bialek-Davenet *et al*., 2014; Holt *et al*., 2015). At present, there are serious concerns over the microbiological safety of infant feeds, which may act as a reservoir for bacterial colonisation and thus a route of transmission leading to infection in premature babies. Premature infants are highly susceptible to infection due to having an underdeveloped immune system. Impaired immunity will lead to an increase in intestinal mucosa permeability and immature microflora of the gut (Greenough, 1996; Mehall *et al*., 2002ab; Townsend & Forsythe, 2008). Moreover, the increase in mucosal permeability in preterm neonates will allow colonisation by microorganisms and subsequent infection when exposed to opportunistic bacterial pathogens. Furthermore, the infection may disseminate systemically through translocation across the permeable intestinal mucosa (Townsend *et al*., 2008). Bacterial analysis of enteral feeding tubes is therefore of great importance with regard to identifying and characterising bacteria that neonates could be exposed to during enteral feeding.

Many studies have found that neonatal feeding tubes are inhabited by several types of flora as well as opportunistic bacteria with pathogenic potential. Hurrell *et al*. (2009a) and Alkeskas *et al*. (2015) reported that 76% of the nasogastric feeding tube (NGT) samples they collected from neonatal intensive care units contained biofilms, with up to 10^7 bacterial colony-forming units (CFU)/tube. The microbial flora complex includes various bacterial

species such as *Enterobacteriaceae* species (*E. coli* K1, *Cronobacter sakazakii, E. cancerogenus, Enterobacter hormaechei, K. pneumoniae* and *Serratia marcescens*), *Pseudomonas luteola*, *Pseudomonas fluorescens,* lactic acid bacteria, *Staphylococcus* spp., as well as fungi such as *Candida albicans*. Some of these organisms, such as *E. hormaechei*, *C. sakazakii,* and *Staphylococcus* spp., are associated with serious neonatal infections and can be found in infant feeding formula. Among these pathogenic organisms, *E. faecalis* and *E. hormaechei* are very important opportunistic pathogens, which are responsible for most hospital-acquired infections, such as septicaemia, urinary tract infections (UTIs), and gastrointestinal infections. These bacteria can express several virulence factors, which contribute to causing host infections. These virulence factors include capsular polysaccharides, siderophores, biofilm formation, antimicrobial resistance and haemolysin (Townsend *et al*., 2008a; Hurrell *et al*., 2009b; WHO, 2011; Madsen *et al*., 2017). Many studies have reported that *E. hormaechei* were found to be resistant to 3rd generation cephalosporins (cefotaxime and ceftazidine). Moreover, *S. marcescens* strains were found to be resistant to amoxicillin and augmentin (Holy & Forsythe, 2014; Forsythe *et al*., 2014; FAO-WHO 2004, 2006, 2008). Another study reported that multidrug resistance in *Enterococcus* spp. has increased, particularly among three first-choice drugs (penicillins, aminoglycoside and glycopeptides) for enterococcal infections (Werner *et al*., 2008; Arias *et al*., 2010; Nishiyama *et al*., 2017). In 2014 & 2017, the World Health Organisation (WHO) declared that bacterial antimicrobial resistance has increased, becoming a globally problematic threat to health. Of particular concern, available neonatal data revealed widespread antimicrobial resistance to aminoglycosides, glycopeptides, penicillins, third generation cephalosporins and extended-spectrum β-lactamase (ESBL) therapy for hospital-acquired neonatal infections (Downie *et al.*, 2013; Aiken *et al.*, 2011; Thaver *et al.*, 2009; Bates *et al.*, 2014; Zaidi *et al.*, 2005; Nishiyama *et al*., 2017).

5.1.1.1. Impacts of *E. faecalis*

Enterococcus faecalis are catalase-negative, Gram-positive cocci, occurring as single coccus or as chains of cocci, and are also non-spore forming facultative anaerobes. Over the past few decades, *Enterococcus* species were known as harmless and non-clinically important

strains (Moreno *et al*., 2006). Recently, it became one of the most common hospital-associated infections with a mortality rate of up to 61% (Lopes *et al*., 2005). It described as a 'triple-threat' pathogen, presenting idealistic colonisation of the skin and the intestinal tract with *S. aureus* and Enterobacteriaceae, respectively within hospital environments (Abt *et al*., 2016; Galvan *et al*., 2016). Enterococcal infections include endocarditis, urinary tract infections, neonatal sepsis, bacteraemia and surgical wound infections (Poh *et al*., 2006; Pinkston *et al*., 2014). Several studies reported that in Europe *Enterococcus* was categorised as the third-most common cause of bacteraemia and the second-most common cause of UTIs and surgical wound infections (Lopes *et al*., 2005; ECDC, 2010). According to the Health Protection Agency (2007) in 2005, there were 7,066 cases of *Enterococcus* bacteraemia in the UK, and 63% of these cases were *E. faecalis*, which have increased antibiotic resistance. Multidrug resistance among *Enterococcus* is of increasing concern, particularly with glycopeptides such as vancomycin, aminoglycosides, and penicillins (Kacmaz and Aksoy, 2005; Solayide *et al*., 2017). Supporting to these notions, in April 2017, the UK health protection (use agency home) reported that the average of enterococcal bacteraemia in 2016 in Wales, England and Northern Ireland were 15.4, 12.4 and 13.1 per 100,000 population, respectively. Furthermore, in England, more than 75% of babies enterococcal bacteraemia occurred within the first month of life.

5.1.1.2. Impacts of *Enterobacter hormaechei*

Enterobacter hormaechei is a member of family of *Enterobacteriaceae* associated with neonatal nosocomial infection (Paauw *et al*., 2008a). For example, it was recovered from neonates with septicaemia during an outbreak occurring in six NICUs among five Brazilian hospitals in 2004 (Campos *et al*., 2007). Jackson *et al*. (2015), used 16S rDNA, multilocus sequence typing, and whole-genome sequencing to re-identify an *E. hormaechei* strain, isolated from a hospital in Mexico, which had been misidentified as *C. sakazakii.* In another study carried out by Aldova *et al*. (1983), an *E. hormaechei* subspecies *steigerwaltii* strain recovered from neonatal rehydrated powdered infant formula from the Czech Republic had been identified as *C. sakazakii.* Most *E. hormaechei* isolates were resistant to fluoroquinolones, 3rd generation cephalosporins (ceftazidime and Cefotaxime) and carried

ESBLs enzymes (Townsend *et al*., 2008a). The clinical significance of these isolates is that they have proved difficult to treat and identify thus far.

5.1.1.3. Physiological and genotypic virulence tests

The physiological studies described in this chapter include isolation and identification of isolated strains, determination of biofilm formation on reconstituted powdered infant formula (PIF), and detection of haemolysis, protease activity, lipase activity and the presence of ESBLs. Capsule production was determined by colony morphology on trypticase soya agar (TSA) and PIF agar. Additionally, human serum resistance, acid tolerance in rehydrated PIF, heat tolerance in rehydrated PIF at 55 ^{o}C, and desiccation resistance for three weeks were all investigated. The susceptibilities of isolates to antimicrobial agents were determined by the breakpoint method on antibiotic supplemented Iso-Sensitest agar (ISA), according to the British Society for Antimicrobial Chemotherapy (BSAC Methods, 2015).

Genotyping through identification and characterisation of bacterial isolates and their subtypes is becoming increasingly important, as it can be used to determine whether the strains are clonally related. Such methods would include DNA fingerprinting via pulsed-field gel electrophoresis (PFGE), 16S rDNA and whole-genome sequencing analysis (CDC, 2004; Tenover *et al*., 1995) in order to further molecular characterise strains by identifying genes associated with physiological and virulence factors, which are associated with bacteria inside the feeding tubes of a preterm infant in a NICU.

5.1.1.4. Aims of this chapter

In chapters 3 and 4, detailed characterisation of isolates from neonatal enteral feeding tubes, flushed milk and neonatal sepsis cases from different hospitals in the UK and Jordan were described. These isolates represented “snapshots” – single samples for which detailed metadata (eg. patient information) and follow up samples were not available. While such studies are useful for confirming the pathogenic potential of isolates, they have a number of limitations. In particular it was impossible to determine from the information available whether the neonatal enteral feeding tubes (NEFTs) colonising strains went on to infect the babies or whether the same strain could persist in NEFTs and in the baby’s intestinal tract

over a long time. No clinical information about the health of the babies was available for the strains described in chapters 3 and 4.

For this chapter, four samples comprising two nasogastric feeding tubes (NFT) and two faeces samples (FS) were collected from a single premature baby with an estimated gestational age of 25 weeks in the neonatal intensive care unit (NICU) at QMC hospital, Nottingham. The first two samples from NFT and FS were collected on the same day at 6 weeks of age, while the second sample from NEFT was collected at age 8 weeks and the second sample from FS was collected 8 days after the first samples.

The aims of this chapter which describes a longitudinal study were to determine the pathogenic potential of the *E. faecalis* and *E. hormaechei* strains that colonised the feeding tubes and intestine of this baby, and to find out whether the baby was re-infected or persistently infected by the same strains or by different strains over time.

This study formed part of a wider collaborative work conducted by Pauline Ogrodzki, Khaled Dahmani and myself. The original intention was to continue investigation of Gram negative pathogens in this longitudinal study (following on from chapters 3 & 4 of this thesis) but the majority of strains isolated were unexpectedly Gram positive so the study was adapted to include characterisation of both Gram negative (*E. hormaechei*) and Gram positive (*E. faecalis*) organisms in this chapter. Of the bacteria isolated for this study, *Streptococcus* can cause neonatal infection, while *Enterococcus* can cause various infections to the neonate, and *Staphylococcus* can cause neonatal infection as well. The acquisition of these strains represented a unique opportunity to study colonising pathogens over time and answer some of the questions that are central to this thesis, to help inform pathogen control in NICUs in the future.

5.2. Materials and Methods;

The methodology for this chapter is described in detail in chapter 2 (Section 2). In the current study, as shown in Table 5-1, eight strains of *E. faecalis* and six strains of *E. hormaechei* were collected from four samples (two nasogastric feeding tubes and two faecal samples) which

were collected from a premature baby with an estimated age of 25 weeks in the neonatal intensive care unit (NICU). The first two samples of NFT and FS were collected on the same day at 6 weeks of age, the second NEFT sample was collected at age 8 weeks and the second FS was collected 8 days after the first samples. These isolates (n=14) were selected based on their PFGE, 16S rDNA, genomic profile, and found in both faecal and feeding tube samples whole period of study and were then subjected for identification and characterisation, and further analysis to investigate their potential virulence factors. In addition, these are unique collections of great interest, which are potentially highly pathogenic due to possessing virulence traits that can cause an increased risk to new-born babies. Furthermore, heat and desiccation resistance testing and whole-genome sequencing were performed in this study. A collaborative approach was taken in this study, and credit must be given to my colleague Pauline Ogrodzki (NTU), who carried out the whole-genome sequencing, as part of a parallel PhD study.

The original faecal material was not stored due to ethical restrictions on the analysis of human tissues. Therefore, the faecal samples and the feeding tubes were separately incubated for 24 hrs at 37^{o}C in Brain Heart Infusion (BHI) growth medium. The overnight growth was mixed with glycerol (20%) and kept frozen (-80°C) in 1.5 ml aliquots.

These samples were then plated separately on four different types of media (Violet Red Bile Glucose Agar (VRBGA) for *Enterobacteriaceae*, and Brain heart infusion agar (BHIA) for general bacteria) and incubated at 37 °C for 72 h, aerobically. Samples were also streaked on Bifidus Selective Media Agar (BSMA) for Bifidobacteria, and De Man, Rogosa, Sharpe Agar (MRSA) for Lactic acid bacteria, then incubated anaerobically at 37 °C for 72 h. Three to four unique colonies were picked up from each medium based on their morphological appearance (texture, edge, elevation and opacity) re-streaked on appropriate medium; (MRSA, BHIA, VRBGA and BSMA) in order to confirm the culture purity, then incubated at 37 °C for 24–48 h. Gram staining was performed for the appearance of the Gram reaction and pure colonies.

Table 5-1: Summary of the *E. faecalis* and *E. hormaechei* strains included in this study. Strains were isolated from Neonatal Feeding Tube and faecal samples isolated from a single premature baby at QMC.

NTU	Organism	Hospital	Source	Isolation date	Sex	Age
2315	*Enterobacter hormaechei*	QMC	Tube 1	08/09/2015	Male	25 weeks
2318			Tube 2	06/10/2015		
2320						
2316			Faecal 1	22/09/2015		
2319						
2317			Faecal 2	30/09/2015		
2324	*Enterococcus faecalis*		Tube 1	18/09/2015		
2326						
2329						
2325			Tube 2	06/10/2015		
2328						
2321						
2323			Faecal 1	22/09/2015		
2322			Faecal 2	30/09/2015		

QMC= Queens Medical Centre

5.3. Results

5.3.1. Bacterial isolation and identification

As shown in Table 5-2, with regards to VRBGA, all subjected strains recovered from both NFT and FS were revealed to be Gram-negative short rods, and also, some yeasts were recovered from FS only. Additionally, on MRSA all of the strains that were recovered from both NFT and FS were shown to be Gram-positive cocci. BHIA revealed a mix of Gram-positive and Gram-negative bacteria.

5.3.1.1. Oxidase reaction:

As shown in Table 5-2, all tested isolates were oxidase-negative

5.3.1.2. Catalase test:

All strains were subjected to 3% hydrogen peroxide (H_2O_2) in order to determine their ability to release oxygen from H_2O_2, as shown in Table 5-2, all of the Gram-negative bacteria isolated were catalase-positive, whereas all of the Gram-positive bacteria were catalase-negative.

5.3.1.3. 16 S rDNA sequencing:

In this study, all of the bacterial strains which were isolated from feeding tubes and faecal samples were identified initially by 16S rDNA sequencing Table 5-2. Some strains were not included in this study because they were found either in faecal or feeding tube samples, but not in both. This includes *Klebsiella oxytoca* isolated only from FS and *Staphyloccous* and *Bifidobacteria* which were isolated from NFT1 only.

5.3.1.4. PFGE analysis:

Although multiple bacterial species were isolated, only *E. faecalis* and *E. hormaechei* were present in both NFT and faecal samples during the entire period of study. These isolates were subjected to PFGE analysis in order to determine the clonal similarity. Regarding *E. hormaechei*, the *Xbal* digestive enzyme was used to separate their DNA fragments, whereas the *Smal* digestive enzyme was used to separate DNA fragments of *E. faecalis* to obtain restriction profiles.

As shown in Figure 5-1, Figure 5-2 and Table 5-2 [and the original gel images in [Appendix D & E], respectively, the PFGE typing was undertaken for 8 *E. faecalis* strains and 6 *E. hormaechei* isolates from two NFT samples and two FS during the longitudinal study (28 days) from a premature baby in the NICU of the QMC hospital in Nottingham.

Figure 5-1, shows that all *E. faecalis* strains were clustered together as indistinguishable isolates, and Figure 5-2 also shows that all *E. hormaechei* strains clustered together as indistinguishable isolates.

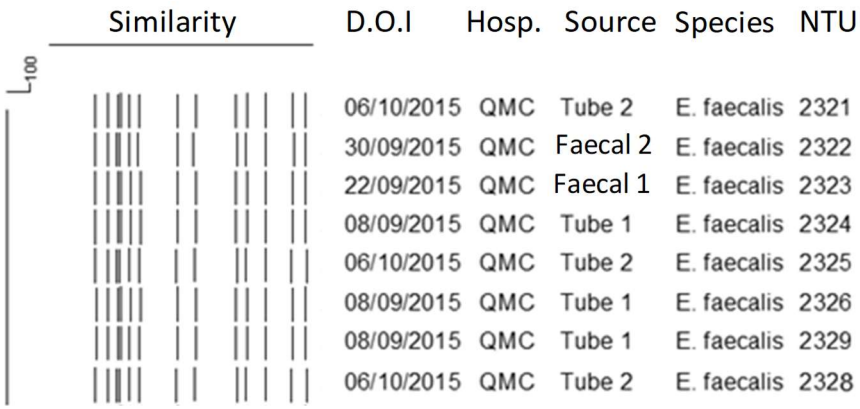


Figure 5-1: PFGE cluster analysis of *E. faecalis* strains isolated from feeding tube and faecal samples from premature baby at QMC

D.O.I= date of isolation, Hosp. = hospital, QMC= Queens Medical Centre, *Smal* digestive enzyme was used to separate DNA fragments to obtain restriction profiles. BioNumerics software, version 3.5 was used in this study to detect a band assignment and a dendogram for all isolates. CHEF-DR II (BIO-RAD, Belgium) system was used for bands separation at 6V, 14^{o}C for twenty hours with 5 seconds initial and 50 seconds final. Dice coefficient, cluster analysis was used for the unweighted pair group method with arithmetic mean (UPGMA).

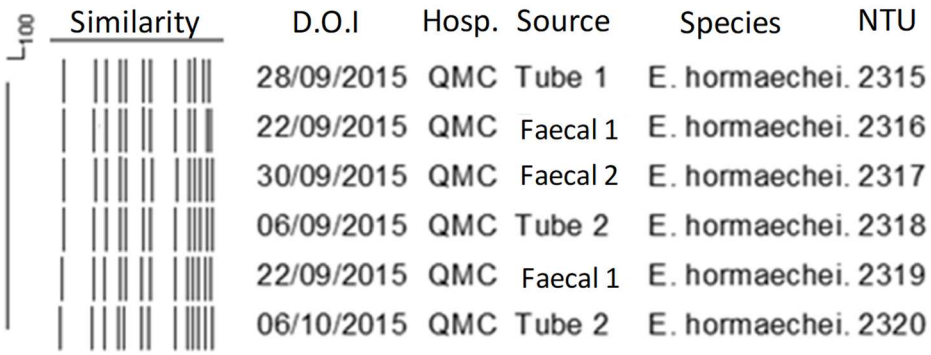


Figure 5-2: PFGE cluster analysis of *E. hormaechei* strains isolated from feeding tube and faecal samples from a premature baby at QMC.

DOI= Date of isolation, Hosp. = Hospital, QMC= Queens medical centre. *Xbal* digestive enzyme was used to separate DNA fragments to obtain restriction profiles. The dendrogram was generated by BioNumerics software, version 3.5, dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimisation in the band was 1.50%.

Table 5-2: Biochemical tests and 16S rDNA of *E. faecalis* and *E. hormaechei* strains isolated from feeding tube and faecal samples isolated from a premature baby at QMC.

NTU	Collections Date	Sample source	Pulse type	Gram stain	Oxidase	Catalase	16S rDNA	Feeding regime
2315	08/09/2015	Tube 1	Same pulse type	G -ve	-ve	+ve	*E. hormaechei*	Breast milk + Pre-Made formula
2318	06/10/2015	Tube 2		G -ve	-ve	+ve	*E. hormaechei*	
2320	06/10/2015	Tube 2		G -ve	-ve	+ve	*E. hormaechei*	
2316	22/09/2015	Faecal 1		G -ve	-ve	+ve	*E. hormaechei*	
2319	22/09/2015	Faecal 1		G -ve	-ve	+ve	*E. hormaechei*	
2317	30/09/2015	Faecal 2		G -ve	-ve	+ve	*E. hormaechei*	
2329	08/09/2015	Tube 1	Same pulse type	G +ve	-ve	-ve	*E. faecalis*	
2321	06/10/2015	Tube 2		G +ve	-ve	-ve	*E. faecalis*	
2323	22/09/2015	Faecal 1		G +ve	-ve	-ve	*E. faecalis*	
2322	30/09/2015	Faecal 2		G +ve	-ve	-ve	*E. faecalis*	

G -ve= Gram negative, G +ve= Gram positive

5.3.2. Genome analysis

Based on the 16S rDNA, PFGE, Gram stain reaction and catalase activity, the representative isolates were subjected to whole-genome sequencing in order to determine the clonal diversity among similar isolates. Additionally, whole-genome sequencing would allow identification of virulence-associated genes, and shed light on the value of whole-genome sequencing in discriminating between similar strains compared to PFGE and 16S rDNA analyses.

5.3.2.1. Bacterial genomic analysis of *E. faecalis* strains

The *E. faecalis* isolates subjected to whole-genome sequencing formed two distinct sequence types (ST191 and ST211) which were distinct from each other by 3–13259 SNPs Figure 5-3. Three *E. faecalis* isolates (2321, 2325, and 2328; ST191) which had been isolated from NFT 2 only, had 7-14 SNPs difference between isolates. Whereas, five *E. faecalis* isolates (2322, 2323, 2324, 2326, 2329; ST211) which had been isolated from NFT 1 and both FS 1 and 2, had 3-13 SNPs difference. These strains were obtained 12 days apart from each other, indicating that essentially the same *E. faecalis* strain was present in the first EFT sample and also persisted in the baby's intestine over time.

5.3.2.2. Bacterial genomic analysis of *E. hormaechei* strains

As shown in Figure 5-4, all of the representative *E. hormaechei* isolates that were subjected to whole-genome sequencing, belonged to ST106 with 28-70 SNPs difference between each strain. These strains had been isolated over a 1-month period. There were 45 SNPs difference between two *E. hormaechei* (2316 and 2319) strains that were recovered from the same FS 1, and also 59 SNPs difference were identified among two *E. hormaechei* strains (2320 and 2318), which were recovered from NFT 2. Two *E. hormaechei* strains (2317; faecal sample 2) and (2320; feeding tube 2) revealed the lowest difference of 28 SNPs. These two samples were obtained 6 days apart. This indicates persistence of essentially the same *E. hormaechei* strain over time in NFT and faecal samples of the same baby.

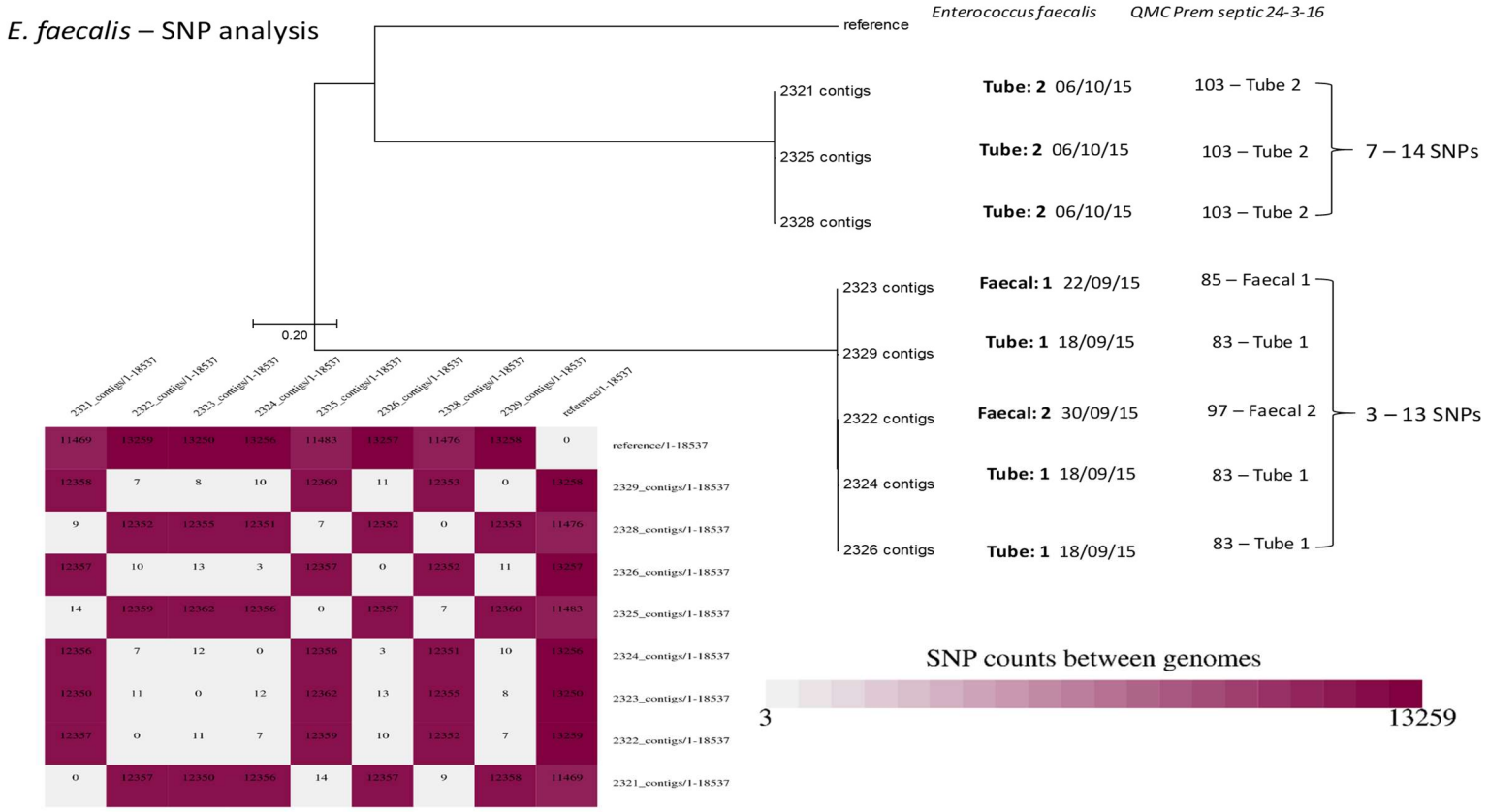


Figure 5-3: Numbers of single nucleotide polymorphisms (SNPs) detected between the selected *E. faecalis* isolates.

The heat map shows the numbers of SNPs difference between each genome in the alignment. SNPs were called using the program CSI Phylogeny version 1.4 and the heat map was generated by Pauline Ogrodzki (NTU), as part of a parallel PhD study.

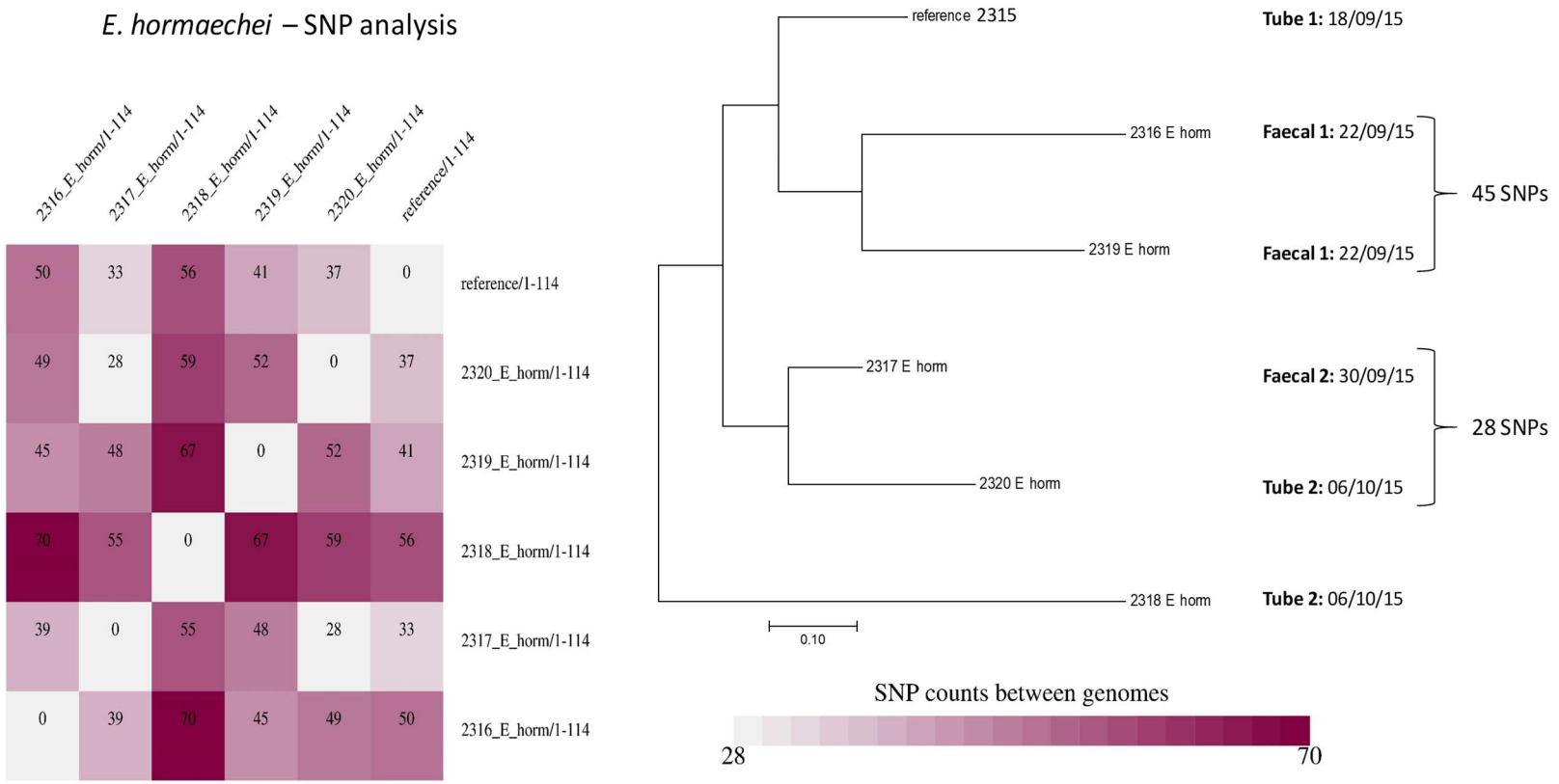


Figure 5-4: Numbers of single nucleotide polymorphisms (SNPs) detected between the selected *E. hormaechei* isolates.

The heat map shows the numbers of SNPs difference between each genome in the alignment. SNPs were called using the program CSI Phylogeny version 1.4 and the heat map was generated by Pauline Ogrodzki (NTU), as part of a parallel PhD study.

5.3.2.3. Detection of virulence factor genes for *E. faecalis* strains (VFGs)

The virulence factor genes of representative isolates were identified by using Virulence Finder 1.5 and the Artemis genome browser (Carver *et al*., 2005). Furthermore, BLAST searches of genomes were carried out using NCBI GenBank research facilities (https://www.ncbi.nlm.nih.gov/genbank/). The pathogenicity of *E. faecalis* is based on various virulence traits, such as capsules, biofilm formation, antimicrobial resistance-associated genes and adhesins. Among these virulence traits, the capsule plays an important role in protecting the bacterium from engulfment (Hancock *et al*., 2002; Thurlow *et al*., 2009).

In this study, a total of 4 representative *E. faecalis* strains were isolated from neonatal enteral feeding tube and faecal samples during a 28-day period Table 5-3 shows the presence of capsular-associated genes. There was clear variation among representative isolates of *E. faecalis* strain 2321 (ST191), isolated from feeding tube 2, which possessed *cpsA* and *cpsB* genes only. On the other hand, other *E. faecalis* strains 2322, 2323 and 2329 (ST211) expressed all cps cluster genes, except for *cpsF*, which was not detected. Additionally, biofilm formation and adherence genes *BopD*, *ebp*ABC, *Efa*A and *Scm* were detected in all examined strains and there was no variation among the isolates tested. Additionally, genes for the exoenzymes gelatinase (*gelE*) and serine protease (*sprE*) were detected in all of the strains, as well as tetracycline resistance (*tetM*) and macrolide resistance (*lsaA*) genes.

5.3.2.4. Detection of virulence factors genes for *E. hormaechei* strains (VFGs)

As shown in the Table 5-4, gene BLAST of six *E. hormaechei* strains against the virulence factor database of bacteria (VFDB), the genomic profiling analysis of these strains revealed that all of the tested strains possessed *mrkABC*, *mrkD*1 and *mrk-D*2 type 3 fimbriae, and curli fimbriae *csgABCD* genes. Furthermore fosfomycin (*fosA*) resistance, efflux pump (*acrAB*) and ESBL ($bla_{\text{ACT-15}}$) genes were detected in all of the strains. Moreover, siderophore (enterobactin and aerobactin) genes were found in all isolates. All of the examined strains also possessed haemolysin *hlyACD* and *hlyB* genes.

Table 5-3: Genome analysis of representative *E. faecalis* strains isolated from feeding tube and faecal samples from a premature baby at QMC.

Virulence factors		Related genes	*E. faecalis*			
			2329	2321	2323	2322
			Tube 1	Tube 2	Faecal 1	Faecal 2
			ST211	ST191	ST211	ST211
Adherence	Ace	*ace*	+	+	+	+
	efaAfs	-	+	+	+	+
		-	+	+	+	+
		-	+	+	+	+
	Ebp pili	*ebpA*	+	+	+	+
		ebpB	+	+	+	+
		ebpC	+	+	+	+
		srtC	+	+	+	+
	EfaA	*efaA*	+	+	+	+
	Scm	*scm*	+	+	+	+
Antiphagocytosis	Capsule	*cpsK*	+	-	+	+
		cpsJ	+	-	+	+
		cpsI	+	-	+	+
		cpsH	+	-	+	+
		cpsG	+	-	+	+
		cpsF	-	-	-	-
		cpsE	+	-	+	+
		cpsD	+	-	+	+
		cpsC	+	-	+	+
		cpsB/cdsA	+	+	+	+
		cpsA/uppS	+	+	+	+
Biofilm formation	*BopD*	*bopD*	+	+	+	+
	Fsr locus	*fsrC*	-	-	-	-
		fsrB	-	-	-	-
		fsrA	-	-	-	-
Exoenzyme	Gelatinase	*gelE*	+	+	+	+
	Hyaluronidase	-	-	-	-	-
		-	+	+	+	+
	SprE	*sprE*	+	+	+	+
Antimicrobial resistant	macrolide	*lsaA*	+	+	+	+
	tetracycline	*tetM*	+	+	+	+

Table 5-4: Genome analysis of representative *E. hormaechei* strains isolated from feeding tube and faecal samples from a premature baby at QMC.

Specis		*E. hormaechei*					
NTU		2315	2318	2320	2316	2319	2317
Collections Date		08/09/2015	06/10/2015	06/10/2015	22/09/2015	22/09/2015	30/09/2015
Sample source		Tube 1	Tube 2	Tube 2	Faecal 1	Faecal 1	Faecal 2
Sequence type		106					
Beta-lactam		bla_{ACT-15}	bla_{ACT-15}	bla_{ACT-15}	bla_{ACT-15}	bla_{ACT-15}	bla_{ACT-15}
Fosfomycin		fosA	fosA	fosA	fosA	fosA	fosA
efflux pump	*acrAB*	+	+	+	+	+	+
Siderophores	*enter*	+	+	+	+	+	+
	aero	+	+	+	+	+	+
Haemolysin	*hlyACD*	+	+	+	+	+	+
	hlyB	+	+	+	+	+	+
Type 3 fimbriae	*mrkABC*	-	-	-	-	-	-
	mrk-D1	+	+	+	+	+	+
	mrk-D2	-	-	-	-	-	-
Curli fimbriae	*csgABCD*	+	+	+	+	+	+

5.3.3. Physiological Virulence Traits

5.3.3.1. Biofilm formation

Bacterial colonisation can occur on any suitable environment where there are sufficient nutrients available. The adherence can be on biotic or abiotic surfaces. Several studies have reported that the majority of *Enterobacteriaceae* have an ability to form biofilm on plastic, glass, metal and polyvinyl chloride. These materials are associated in medical devices, manufacturing and infant feeding equipment. Thus, neonatal infection risk will increase with contamination of these materials (Kim *et al*., 2012; Lehner *et al*., 2005). Figure 5-5 shows the ability of *E. faecalis* strains to form biofilm in rehydrated infant formula (PIF) on plastic surfaces at 25 ^{o}C and 37 ^{o}C compared to the control (non-inoculated media) . The amount of biofilm formed at 37 ^{o}C was significantly higher than at 25 ^{o}C ($p < 0.001$; one way ANOVA) Figure 5-6. All *E. faecalis* strains produced biofilm, compared to the control, biofilm formation stained with crystal violet 0.01% (w/v) and evaluated at wavelength

540nm; the control was non-inoculated media, the experiments were investigated in three independent times. Error bars represented using standard error.

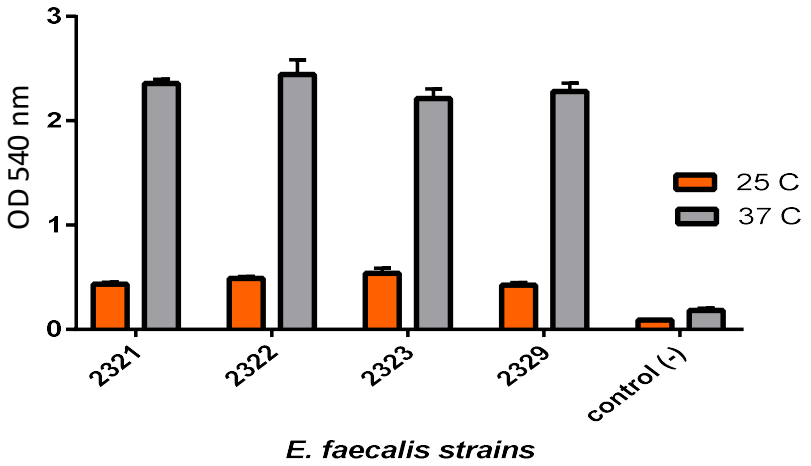


Figure 5-5: Biofilm Formation of *E. faecalis* isolates at 25 °C and 37 °C in liquid PIF.

Data shows that the preferred temperature was 37 °C, the mean of three experiments with error bars as standard deviation. The OD of blank where no bacteria added subtracted (media)

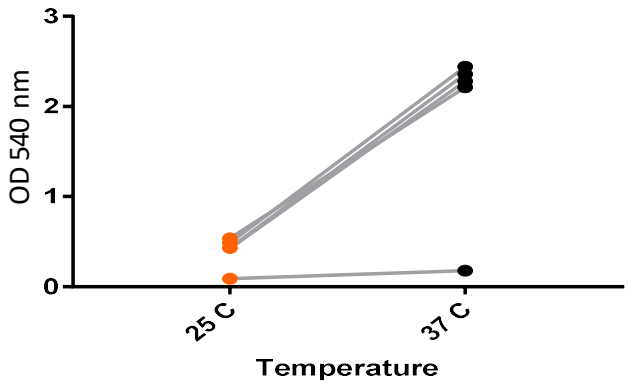


Figure 5-6: Paired t-test shows *E. faecalis* strains form significantly more biofilm in PIF at 37 °C than at 25 °C ($P < 0.001$).

For the *E. hormaechei* strains, Figure 5-7 shows that all of the strains tested could form biofilm on PIF when compared with the control, and there was no significant variation in biofilm forming ability between the strains. At 37 °C, the amount of biofilm formation was significantly higher than at 25 °C ($p < 0.0001$, paired t-test, Figure 5-8). At 25 °C there was

no biofilm formation (not significantly higher than the control – $p > 0.05$, one way ANOVA, Figure 5-7). Biofilm formation stained with crystal violet 0.01% (w/v) and evaluated at wavelength 540nm; the control was non-inoculated media, the experiments were investigated in three independent times. Error bars represented using standard error.

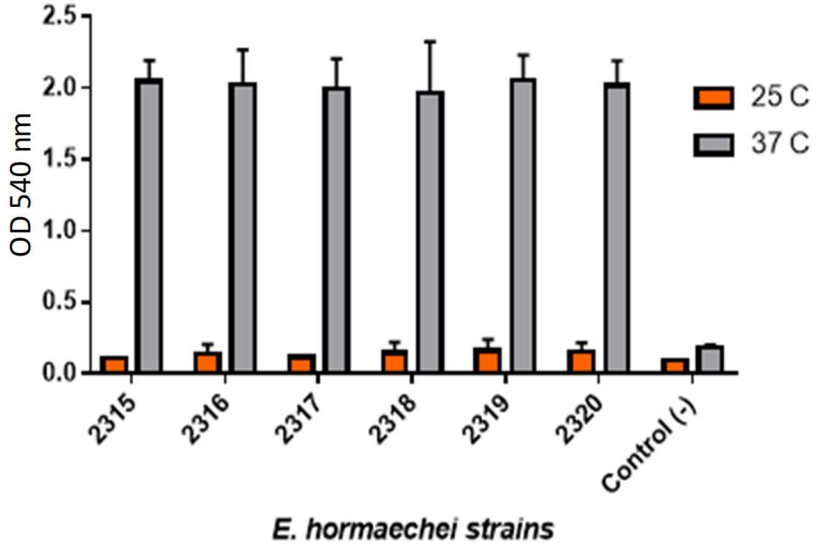


Figure 5-7: Biofilm Formation of *E. hormaechei* isolates at 25 °C and 37 °C in liquid PIF.

Data shows that the preferred temperature was 37 °C, the mean of three experiments with error bars as standard deviation. The OD of blank where no bacteria added subtracted (media).

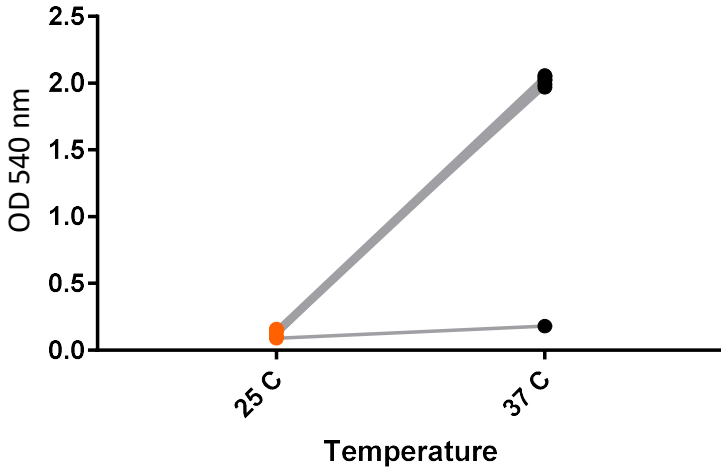


Figure 5-8: Paired t-test shows *E. hormaechei* strains form significantly more biofilm in PIF on 37°C than at 25 °C ($P < 0.0001$).

5.3.3.2. Capsule production on different media

In the present study, 4 strains of *E. faecalis* and 6 strains of *E. hormaechei* were investigated for their ability to produce capsular materials phenotypically, by the appearance of the colony morphology on two different media, including TSA agar and powdered infant formula (PIF). The ability of isolates to produce capsular material was classified into four categories according to the relative amounts of capsular material produced, and these were defined as high (+++), medium (++), low (+) and non-mucoid (-). All of the *E. faecalis* strains tested were unable to produce capsular materials on either TSA or PIF agar. In contrast, all *E. hormaechei* strains produced a medium level of capsular materials on both TSA and PIF agar Table 5-5, and there was no variation in capsule production among the examined strains.

5.3.3.3. Blood haemolysis

Table 5-5, shows that all of the *E. faecalis* strains tested had alpha (α)-type haemolytic activity (partial haemolysis) on horse blood. On the other hand, all *E. hormaechei* isolates had beta (β)-type haemolytic activity (complete haemolysis) on horse blood.

5.3.3.4. Lipase activity

The lipase activity was investigated by using tributyrin agar after 72 hours incubation at 37 ^{o}C, in order to determine the ability of subjected strains to produce lipase. As shown in Table 5-5, all tested strains of *E. faecalis* (n = 4) and *E. hormaechei* (n = 6) were able to express lipase and produce a clear inhibition zone around the colony Figure 5-9.

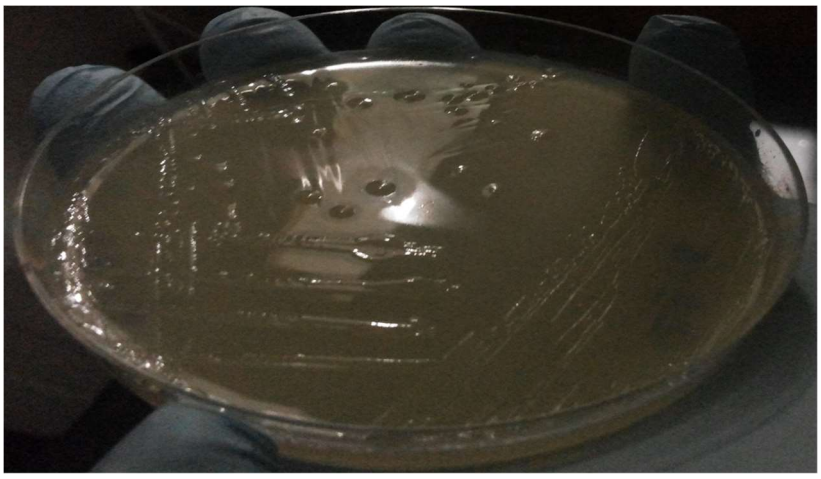


Figure 5-9: showing lipase activity on tributyrin agar.

Clear inhibition zone around colony means *E. hormaechei* and *E. faecalis* have lipase activity on tributyrin agar after 72 hours incubation at 37 ^{o}C

5.3.3.5. Protease activity

The protease activity was investigated on skimmed milk agar at 37 ^{o}C for three days, to determine the protease activity of examined strains. All of the isolates were positive for protease activity, as clear proteolytic zones were detected. Table 5-5 summarises the results of phenotypic characterisation of the *E. faecalis* and *E. hormaechei* strains.

5.3.3.6. Acid Tolerance

The 4 strains of *E. faecalis* and 6 strains of *E. hormaechei* were investigated for their acid tolerance ability (pH 3.5) at five different time-points: 0 min, 30 min, 60 min, 90 min and 120 min. This experiment was carried out in order to identify which strains could persist and multiply in a pH that mimics the neonatal stomach acidity and acidified food. Strains were inoculated into reconstituted powdered infant formula, pre-adjusted to pH 3.5 at 37 ^{o}C for 2 hours. Generally, the starting inoculum (initial viable count) at 0 minutes time point was between 8.4 to 9.01 $\log_{10}$ CFU/ml Figure 5-10 and Figure 5-11.

Figure 5-10 shows that all of the *E. faecalis* strains were tolerant to pH 3.5 for 2 hours of exposure and there was no notable variation between the strains. Additionally, all strains showed some resistance to low pH by multiplying initially, and then after 30 min, the viable

count decreased to log 8.7. In general, all *E. faecalis* isolates tested were able to resist acid, and their overall numbers did not change much over 120 minutes.

Figure 5-11 shows that all of the *E. hormaechei* isolates were also tolerant to pH 3.5; these strains also multiplied initially, and then after 120 min, the viable count partially decreased to log 7.8. In general, all *E. hormaechei* isolates tested were able to resist acid and their overall numbers did not change much over 120 minutes.

Table 5-5: Phenotypic characterization of *E. faecalis* and *E. hormaechei* strains isolated from neonatal feeding tube and faecal samples isolated from a premature baby at QMC.

NTU	Specis	Collections Date	Sample source	Pulstype	SNPs	ST	Capsule (Mucoid)		Biofilm formation by PIF		Haemolysis	Lipase	Protease	Feeding regime
							IF	TSA	25 C	37 C	Hor. B.			
2315	*E. hormaechei*	08/09/2015	Tube	Same pulse type	< 45	106	++	++	Low	High	Beta (β)	+	+	Breast milk + Pre-Made formula
2316	*E. hormaechei*	22/09/2015	Faecal				++	++	Low	High	Beta (β)	+	+	
2319	*E. hormaechei*	22/09/2015	Faecal				++	++	Low	High	Beta (β)	+	+	
2317	*E. hormaechei*	30/09/2015	Faecal		< 28		++	++	Low	High	Beta (β)	+	+	
2318	*E. hormaechei*	06/09/2015	Tube				++	++	Low	High	Beta (β)	+	+	
2320	*E. hormaechei*	06/10/2015	Tube				++	++	Low	High	Beta (β)	+	+	
2321	*E. faecalis*	06/10/2015	Tube	Same pulse type	< 14*	191	-	-	Low	High+	Alpha(α)	++	++	
2322	*E. faecalis*	30/09/2015	Faecal		< 13	211	-	-	Low	High+	Alpha(α)	++	++	
2323	*E. faecalis*	22/09/2015	Faecal				-	-	Low	High+	Alpha(α)	++	++	
2329	*E. faecalis*	08/09/2015	Tube				-	-	Low	High+	Alpha(α)	++	++	

SNPs= single nucleotide polymorphisms, ST= sequence type, PIF= liquid powdered infant formula, TSA= trypticase agar, Hor.B.= horse blood, *= the difference within the same ST

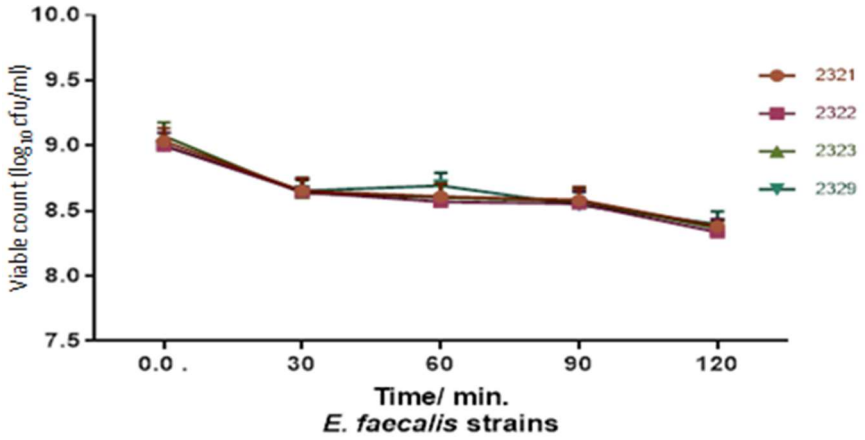


Figure 5-10: The survival of *E. faecalis* strains after the exposure to acidified PIF (pH 3.5) at 37°C for 120 minutes

The survival curve reveals the susceptibility of *E. faecalis* isolates cultivated in acidified PIF (pH 3.5) at 37oC for 120 minutes. Survival was measured at 0, 30, 60, 90 and 120 minutes. The assays were determined in duplicate in two independent experiments. Error bars represented using standard error.

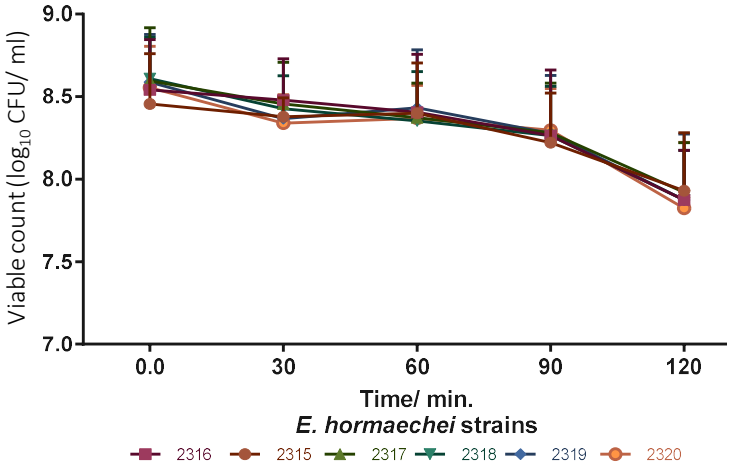


Figure 5-11: The survival of *E. hormaechei* strains after the exposure to acidified PIF (pH 3.5) at 37 °C for 120 minutes

The survival curve reveals the susceptibility of *E. hormaechei* isolates cultivated in acidified PIF (pH 3.5) at 37oC for 120 minutes. Survival was measured at 0, 30, 60, 90 and 120 minutes. The number of surviving cells in this assays were determined in duplicate in two independent experiments. Error bars represented using standard error.

5.3.3.7. Serum tolerance

Figure 5-12 and Figure 5-13 show that all *E. faecalis* and *E. hormaechei* strains isolated from both feeding tube and faecal samples were resistant to human serum, and there was no

variation among the strains tested. *E. coli* K12 strain 1230 and *Salmonella* Enteritidis strain 358 were used as controls that were completely sensitive and resistant to human serum, respectively.

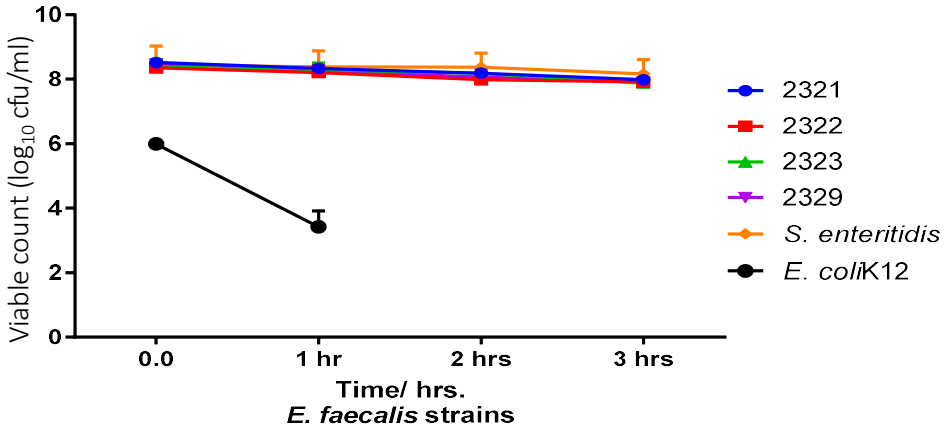


Figure 5-12: Sensitivity of *E. faecalis* isolates to human serum cultivated at 37°C for 3 hrs

S. Enteritidis (358) and *E. coli* K12 (1230) were used as positive and negative controls respectively. There was no variation among isolates. All examined strains were significantly better at surviving serum compared to the *E. coli* K12 (1230) negative control. The assays were performed in duplicate in two independent experiments. Error bars represented using standard error.

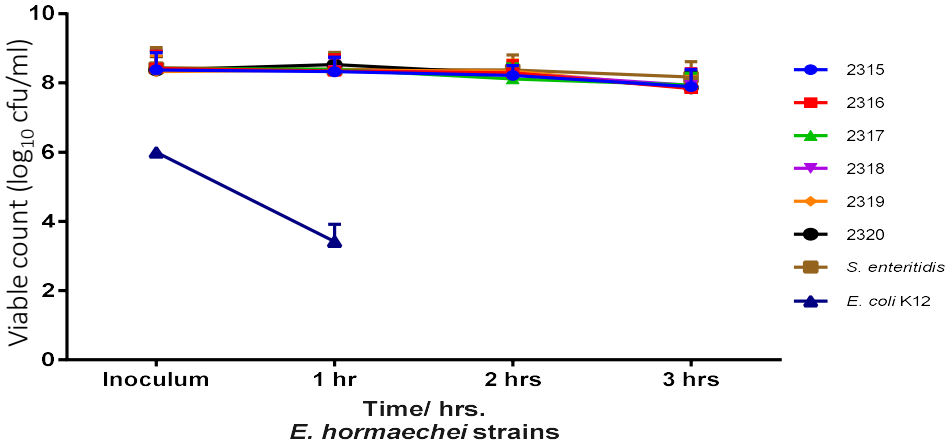


Figure 5-13: Sensitivity of *E. hormaechei* isolates to human serum cultivated at 37°C for 3 hrs

S. Enteritidis (358) and *E. coli* K12 (1230) were used as positive and negative controls respectively. There was no variation among examined isolates. All examined strains were significantly better at surviving serum compared to the *E. coli* K12 (1230) negative control. The assays were performed in duplicate in two independent experiments. Error bars represented using standard error. Presented data was showing viable count (log_{10}cfu/ml) among the isolates.

5.3.3.8. Desiccation

The capability of pathogenic bacteria to survive and persist under stressful conditions such as desiccation, osmotic pressure, and heat is well known and leads to the persistence of these pathogens during the food production process and in the food chain (Sleator *et al*., 2003). Additionally, in 2004 and 2006, FAO-WHO documented their concerns about the persistence of opportunistic pathogenic bacteria inside PIF, which may increase the potential risk of neonatal infection during feeding, particularly in immuno-compromised infants. In the current study, 4 strains of *E. faecalis* and 6 strains of *E. hormaechei* were inoculated in rehydrated PIF and incubated under dry conditions for 3 weeks at room temperature, and then plated on non-selective media (TSA) and selective media (MRS agar and VRBGA). The starting inoculum of examined isolates was around 7.8 $\log_{10}$ and 7.4 $\log_{10}$ CFU/ml, respectively, in order to determine the capability of examined strains to resist desiccation.

Concerning *E. faecalis* strains, as shown in Figure 5-14 and Table 5-6, there was a slight difference in the recovery of *E. faecalis* between TSA and MRS agar. On TSA, the recovery of all tested isolates was greater on TSA than on MRS agar ($P < 0.05$; one-way ANOVA), and these differences revealed sub-lethally injured cells throughout the desiccation period Figure 5-16. In addition, by the first week of incubation on TSA, all tested strains showed the highest cell recovery value of 6.8 $\log_{10}$. By the third week of incubation on TSA, however, all tested strains showed slightly decreased survival, ranging from 7.8 $\log_{10}$ to 4.7 $\log_{10}$, when compared to the inoculum (first desiccation time point) Figure 5-14. In contrast to this, by the first week of incubation on MRS agar, all strains were able to persist and had showed the highest cell recovery value of up to 6.2 $\log_{10}$ compared to the inoculum. However, by the third week of incubation, the majority of the isolates had reduced in viability from 7.8 $\log_{10}$ to 4.3$\log_{10}$, when compared to the inoculum Figure 5-15. Overall, there was no variation among tested isolates during the desiccation period and all strains were able to persist, with some reduction in numbers, over 3 weeks.

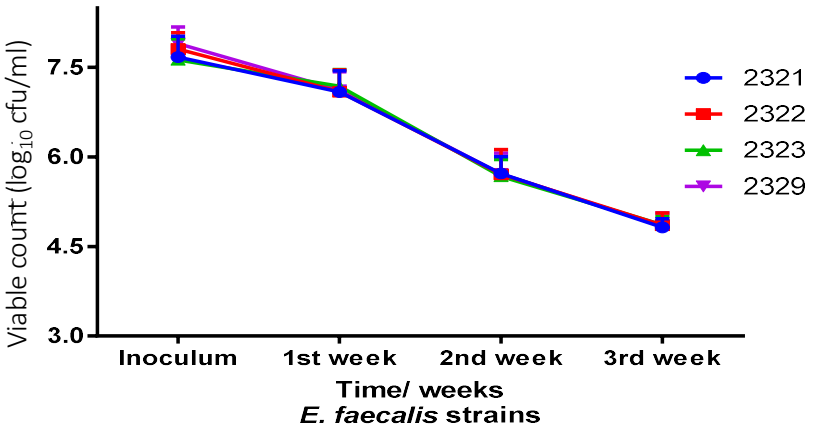


Figure 5-14: Viable cell counts for *E. faecalis* on TSA for three weeks in liquid PIF.

The figure show the viability of presented *E. faecalis* strains for desiccation stress at room temperature in dehydrsted PIF for three weeks on TSA. The inoculum of examined isolates was around 7.8 log_{10}. Error bars represented using standard error.

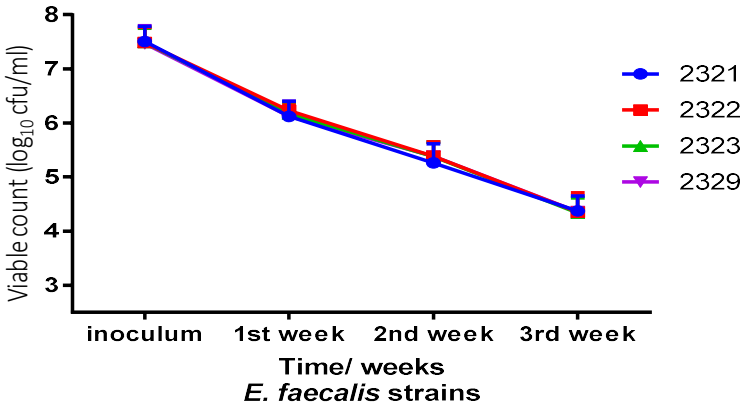


Figure 5-15: Viable cell counts for *E. faecalis* on MRS agar for three weeks in liquid PIF.

The figure show the viability of presented *E. faecalis* strains for desiccation stress at room temperature in dehydrsted PIF for three weeks on MRS agar. The inoculum of examined isolates was around 7.4 log_{10}. Error bars represented using standard error.

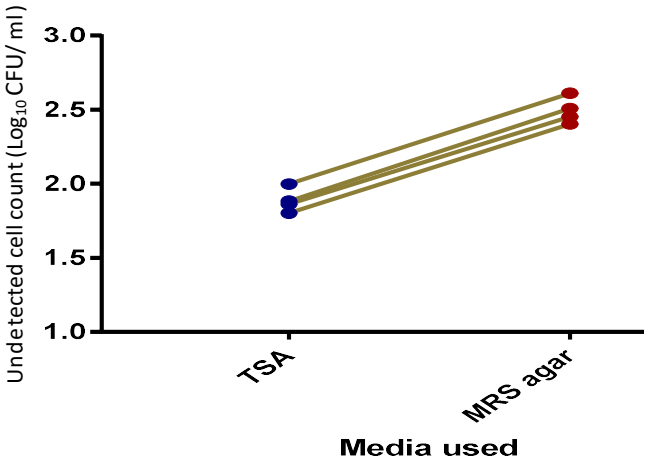


Figure 5-16: Paired t-test shows *E. faecalis* strains had significantly greater recovery on TSA than on MRS agar ($P < 0.0001$).

Table 5-6: Comparison of dead and sub-lethal injured cells for *E. faecalis* on TSA and MRS agar after desiccation and then reconstitution in powder infant formula

NTU	ST	Dead and injured cells on TSA in log_{10} CFU/ml	Dead and injured cells on MRS in log_{10} CFU/ml	Sub lethal injured cells in log_{10} CFU/ml
2321	191	2.19	2.37	0.18
2322	211	2.04	2.58	0.54
2323		2.11	2.49	0.38
2329		2.10	2.36	0.26

These data shows the number of dead cells (average of viable cells among three weeks subtracted from initial inoculum for each strain)

With regards to *E. hormaechei* strains, there was no variation among tested isolates during the desiccation period. By the first week of incubation on TSA, all subjected isolates showed the highest cell recovery value of 7.7 log_{10}. By the third week of incubation, all tested strains showed slightly decreased survival of 5.9 log_{10}, when compared to the inoculum Figure 5-17. In contrast to VRBGA, all tested strains were able to persist and presented the highest cell recovery value of up to 6.7 log_{10} during the first week of incubation. However, by the third week of incubation, all strains had reduced in their viability value from 7.8 log_{10} to 4.6 log_{10}, when compared to the inoculum Figure 5-18.

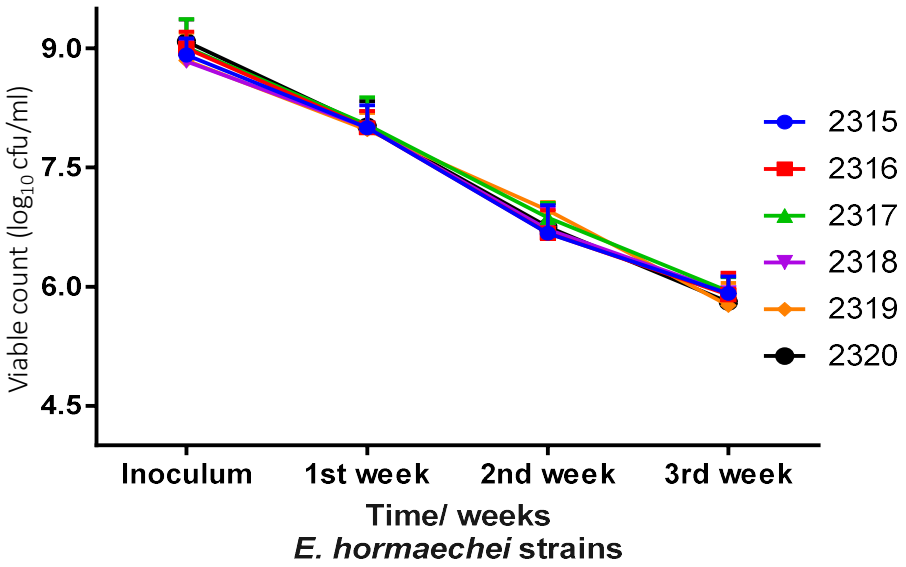


Figure 5-17: Viable cell counts for *E. hormaechei* on TSA for three weeks in liquid PIF.

The figure show the viability of presented *E. hormaechei* strains for desiccation stress at room temperature in reconstitution PIF for three weeks on TSA. The inoculum of examined isolates was around 8.8 log_{10}. Error bars represent 95% confidence intervals.

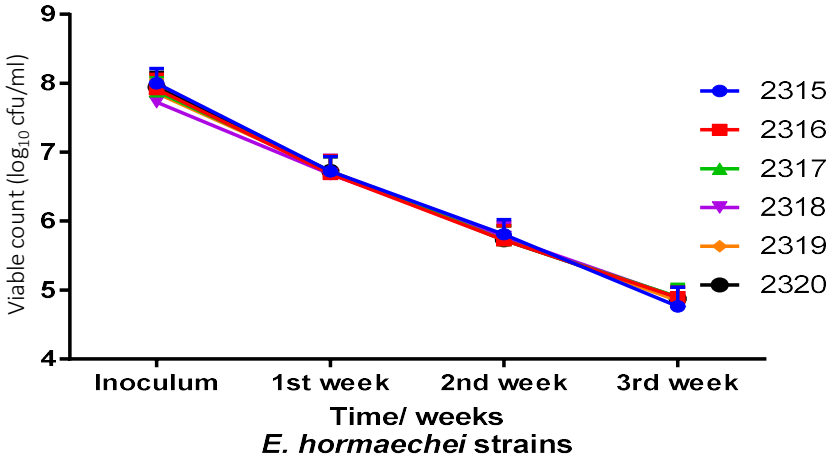


Figure 5-18: Viable cell counts for *E. hormaechei* on VRBGA for three weeks in liquid PIF.

The figure show the viability of presented *E. hormaechei* strains for desiccation stress at room temperature in reconstitution PIF for three weeks on VRBGA. The inoculum of examined isolates was around 8.0 log_{10}. Error bars represent 95% confidence intervals.

The proportion of sublethally injured cells was estimated based on the difference in the log_{10} numbers of cycles of CFU obtained after plating treated cells onto the nonselective (TSA) and selective (MRS agar and VRBGA) media. The viable counts were determined using Miles and Misra method on VRBGA or MRS agar as a selective medium and TSA as non-selective

medium to define the sublethally injured cells. The differences in recovery on TSA and VRBGA or MRS agar after desiccation reflect the number of cells that were sublethally injured during overnight desiccation. The differences between sub-lethally injured cells on VRBGA are presented in Figure 5-19 and Table 5-7. The number of undetected sub-lethally injured on TSA had varied between 1.9 $\log_{10}$ and 2.2 $\log_{10}$. Whilst the number of sub-lethally injured cells on VRBGA had ranged between 3.1 $\log_{10}$ and 3.3 $\log_{10}$. The highest amount of sub-lethally injured cells was detected in strains 2317 and 2320 at 1.18 $\log_{10}$ and 1.16 $\log_{10}$ CFU/ml, respectively. It was found that the lowest amount of sub-lethal injured cells was identified in strain 2319 at 1.10 $\log_{10}$ CFU/ml.

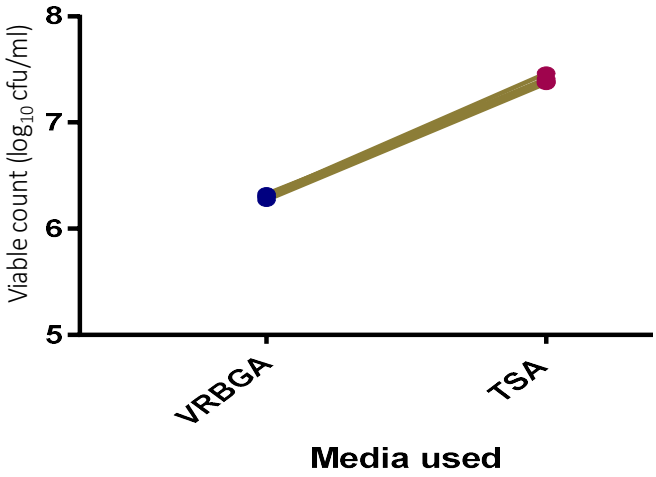


Figure 5-19: Paired t-test shows *E. hormaechei* strains form significantly a greater recovery on TSA than on VRBGA agar ($P < 0.0001$).

Table 5-7: Comparison of sub-lethal injured cells for *E. hormaechei* on TSA and VRBGA after desiccation and then reconstitution in infant formula

NTU	ST	Dead and injured cells on TSA in log CFU/ml	Dead and injured cells on VRBGA in log CFU/ml	Sub lethal injured cells in log CFU/ml
2315	106	2.00	3.13	1.12
2316		2.11	3.23	1.12
2317		2.14	3.33	1.18
2318		1.97	3.10	1.12
2319		2.05	3.16	1.10
2320		2.20	3.36	1.16

The numbers, which are in the table, comes from log10 of inoculum (TSA and VRBGA) for each strain subtracted from average of log10 1st wk, 2nd wk and 3rd wk (TSA and VRBGA) for each strain.

5.3.3.9. Heat tolerance

One of the most important bacterial virulence factors is thermo-tolerance, particularly in the food industry where bacterial survival of heat treatment may pose a high risk to the human health. In this experiment, the heat tolerance of 4 strains of *E. faecalis* and 6 strains of *E. hormaechei* were investigated according to previously published methodology by Iversen *et al*. (2004), in order to determine the ability of these isolates to tolerate heat at 55 °C. The bacterial survival at 55 °C was quantified and plotted against time. D-values were extrapolated at the time desired for one $\log_{10}$ reduction [(−1) / (slope of the regression line)] in the viable count. Furthermore, D-values were categorised into three groups: heat-labile strains (D_{55} = ≤ 10), moderate (11≥ D_{55} ≤20) and high heat-tolerant (D_{55} = ≥21).

Regarding *E. faecalis* isolates, as shown in Figure 5-20 and Table 5-8, all tested strains revealed a high level of heat resistance. There were slightly differences in thermo-tolerance among tested strains. *E. faecalis* strain 2322 (ST211), which was isolated from faecal sample 2, was more heat-tolerant than the remaining isolates. It had a D_{55} of 33.5 minutes at 55 ^{o}C in the liquid infant formula. In addition, *E. faecalis* strains 2321 (ST191) and 2329 (ST211) had the same D_{55} value of 31.6.

All of the *E. hormaechei* strains tested were also heat tolerant and there were slight differences among strains in terms of heat resistance Figure 5-21 and Table 5-8. The highest level of thermo-tolerance was noticed in strains 2316 and 2317, which were isolated from faecal samples 1 and 2, respectively. Their D_{55} values were 24.1 and 24.5 minutes at 55 ^{o}C, respectively, in the liquid infant formula. On the other hand, *E. hormaechei* strain 2320, which was isolated from NEFT sample 2, had presented the lowest D_{55} value 21.9.

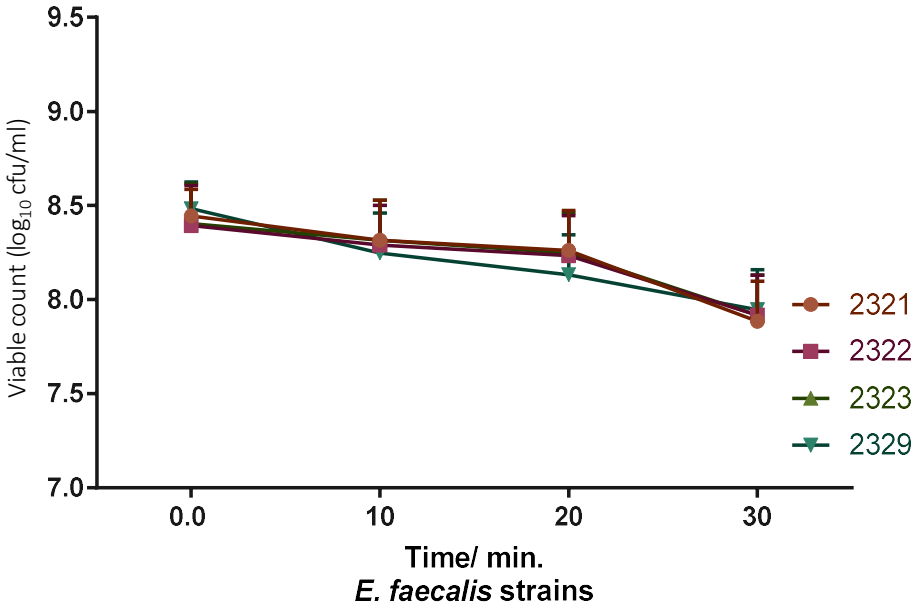


Figure 5-20: Survival of *E. faecalis* strains in liquid infant formula at 55°C.

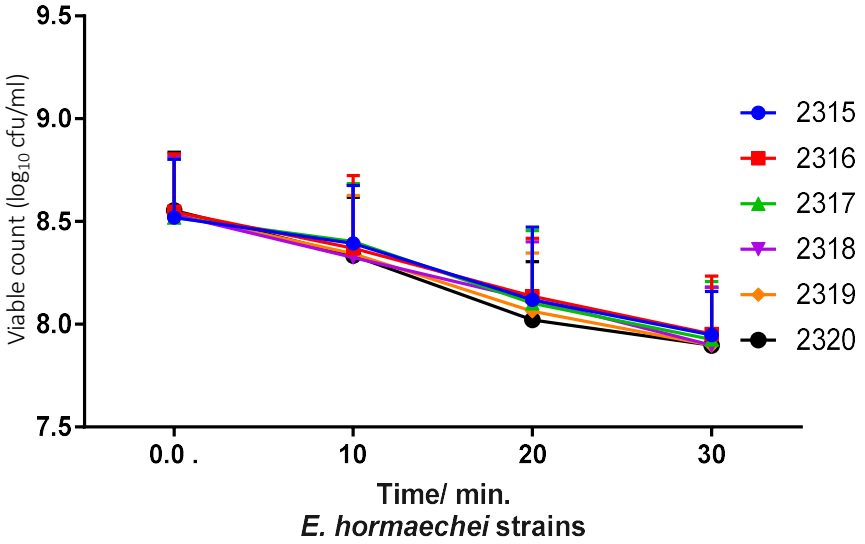


Figure 5-21: Survival of *E. hormaechei* strains in liquid infant formula at 55°C.

Table 5-8: Survival of *E. faecalis* and *E. hormaechei* in liquid powdered infant formula at 55 °C.

Strain	Species	Source	ST	D-Value (min.)	Category
2315	*E. hormaechei*	Tube 1	106	23.9	R
2318	*E. hormaechei*	Tube 2		23.0	R
2320	*E. hormaechei*	Tube 2		21.9	R
2316	*E. hormaechei*	Faecal 1		24.1	R
2319	*E. hormaechei*	Faecal 1		22.8	R
2317	*E. hormaechei*	Faecal 2		24.5	R
2329	*E. faecalis*	Tube 1	211	31.6	R
2321	*E. faecalis*	Tube 2	191	31.6	R
2323	*E. faecalis*	Faecal 1	211	32.6	R
2322	*E. faecalis*	Faecal 2		33.5	R

ST= sequence type, R= resistant, min.= minutes

5.3.3.10. Antibiotic resistance

Antibiotic susceptibility profiling and investigation into ESBL production for 4 strains of *E. faecalis* and 6 strains of *E. hormaechei* was carried out. Antimicrobial susceptibility was performed for eight different antibiotic groups: carbapenems (meropenem and imipenem), cephalosporins (ceftizoxime, ceftazidime, ceftriaxone and cefoperazone), macrolides (clarithromycin), tetracyclines (doxycycline), penicillins (ampicillin), fluoroquinolones (ciprofloxacin), glycopeptides (vancomycin) and aminoglycosides (tobramycin and gentamicin). Additionally, ESBLs testing included cefotaxime/clavulanic acid and ceftazidime/clavulanic acid. *E. coli* NCTC 13353 and *E. coli* ATCC 10418 were used as resistant and susceptible control strains, respectively.

As presented in Table 5-9, all the screened *E. hormaechei* isolates were revealed to be resistant to most antibiotics tested in this study, with the exception of cefoperazone, doxycycline, ciprofloxacin and tobramycin. Additionally, all strains had intermediate resistance to meropenem. ESBL production was detected phenotypically using the combination disc method. All the *E. hormaechei* isolates were revealed to be ESBL-producers.

The *E. faecalis* strains were only susceptible to clarithromycin, doxycycline and ampicillin. Intermediate resistance was detected for cefoperazone and vancomycin. Interestingly, clear variation was notable among the tested isolates for ciprofloxacin; strain 2321 was resistant, and strains 2322 and 2329 had intermediate resistance, whereas strain 2323 was susceptible. Furthermore, all isolates tested were shown to be ESBL-positive Table 5-9.

Table 5-9: Susceptibility of *E. hormaechei* and *E. faecalis* strains to the agents of antibiotic.

NTU	Species	Carbapenem		Cephalosporins				Macrolide	Tetra	Penicillin	Fluoroqu	Glycope	Aminoglycoside		ESBL	
		IPM	MEM	ZOX	CAZ	CRO	CFP	CLR	DO	AMP	CIP	VA	TOB	CN	CAZ+CV	CTX+CV
2315	*E. hormaechei*	R	I	R	R	R	S	R	S	R	S	R	S	R	R	R
2316	*E. hormaechei*	R	I	R	R	R	S	R	S	R	S	R	S	R	R	R
2317	*E. hormaechei*	R	I	R	R	R	S	R	S	I	S	R	S	R	R	R
2318	*E. hormaechei*	R	I	R	R	R	S	R	S	R	S	R	S	R	R	R
2319	*E. hormaechei*	R	I	R	R	R	S	R	S	R	S	R	S	R	R	R
2320	*E. hormaechei*	R	R	R	R	R	S	R	S	R	S	R	S	R	R	R
2321	*E. faecalis*	R	I	R	R	R	I	S	S	S	R	I	R	R	R	R
2322	*E. faecalis*	R	R	R	R	R	I	S	S	S	I	I	R	R	R	R
2323	*E. faecalis*	R	I	R	R	R	I	S	S	S	S	I	R	R	R	R
2329	*E. faecalis*	R	R	R	R	R	I	S	S	S	I	I	R	R	R	R

R= resistant, I= intermediate, S= susceptible, Imipenem (IMI), Meropenem (MEM), ceftizoxime (ZOX), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefoperazone (CFP), clarithromycin (CLR), doxycycline (DO), Ampicillin (AMP), Ciprofloxacin (CIP), Vancomycin (VA), Tobramycin (TOB) and Gentamicin (CN), Ceftazidime + Clavulanic acid (CAZ/CV), Cefotaxime + Clavulanic acid (CTX+CV), Fluroqu= Fluoroquinolone, Tetra= tetracycline, Glycope= glycopeptide.

5.4. Discussion

Several previous studies have reported the presence of both Gram-negative and Gram-positive bacteria inside neonatal feeding tubes (Hurrell *et al*., 2009a; Petersen *et al*., 2016; Gómez *et al*., 2016). However, the extent to which bacterial colonisation in the feeding tubes contributes to the colonisation of the infant intestines has not been studied in great detail. In order to determine whether the bacterial biofilms observed in optical coherence tomography (OCT) imaging were composed of viable organisms, the layers were sampled for microbiological analysis. Additionally, the potential influence of the feeding tube microbial flora on the neonatal intestinal flora was investigated by sampling faecal material from the same neonate, followed by identification and subsequent whole-genome analysis of isolates. The neonate was admitted to the intensive care unit due to premature birth (EGA 25 weeks). Two separate samples (faecal and enteral feeding tube) were collected at 6

weeks and 8 weeks after birth. The presence and persistence of pathogenic bacteria, especially *E. hormaechei* and *E. faecalis* in the human gut or in any enteral diets associated with the feeding of hospitalised babies, would seem to be a possible source of infection. In addition, the infection incidence of these pathogens is likely to increase because those individuals are highly susceptible to nosocomial infections. Hospital-acquired infections are globally problematic due to their clinical impacts, as well as financial and ethical influences. Of significance is the inability to reduce the impact of these infections due to the difficulty of controlling outbreaks worldwide (WHO, 2011).

The conventional phenotypic identification and differentiation methods among closely related isolates are time consuming, labour intensive and may lead to misidentification (Facklam *et al*., 2002; Domig *et al*., 2003). In 2001, Almuneef and co-authors reported that identification of, and differentiation between closely related isolates is very important, and this requires good strategies and appropriate techniques. Additionally, Cetinkaya and co-authors (2013) reported that molecular subtyping techniques, such as PCR (16S rDNA), PFGE and whole-genome sequencing, are considered more accurate than phenotypic methods. These techniques are useful in determining if strains are clonally related, for epidemiological studies, as well as determining the source of infection and the diversity among pathogens. In the present study, all tested strains were subjected to conventional methods such as Gram staining, catalase and oxidase tests for primarily identification. With regard to *E. faecalis,* all presented isolates were confirmed as Gram-positive, oxidase-negative, and catalase-negative, and all *E. hormaechei* strains were confirmed to be Gram-negative short rods, catalase-positive and oxidase-negative.

In the last few decades, the use of 16S rDNA sequencing has played an important role in accurate identification among closely related strains. 16S rDNA sequencing is broadly applicable and accurate as a clinical microbiological diagnostic technique (Cursons *et al*., 1999; Sleigh *et al* 2001; Xu *et al*., 2004). In 2008, a study was carried out by Townsend and co-authors on misidentified *Cronobacter* spp. strains recovered from an NICU outbreak in a Californian hospital. These strains were identified by conventional methods (phenotyping), but were re-identified as *E. hormaechei* by 16S rDNA sequencing. Therefore, all representative isolates in this study were confirmed to the species level using 16S DNA

sequencing and whole-genome sequencing (by core-genome alignment), which identified the strains as *E. faecalis* and *E. hormaechei* subspecies *steigerwaltii.* According to Aldova´ *et al*. (1983), the *E. hormaechei* subspecies *steigerwaltii* strain recovered from neonatal powdered infant formula from the Czech Republic had been mis-identified as *Enterobacter sakazakii* (*C. sakazakii*). In another study in The Netherlands, *E. hormaechei* subspecies *hormaechei* was reported in error as *C. sakazakii* isolated from neonatal powdered infant formula (Muytjens *et al*., 1988). A study carried out by Jackson *et al*. (2015) re-identified *E. hormaechei* isolated from a hospital in Mexico that had been misidentified phenotypically and via PCR probes as *Cronobacter sakazakii.* Further analysis, using 16S rDNA, multilocus sequence typing and whole-genome sequencing revealed that the strain was in fact *E. hormaechei.*

The genomic profiling of *E. faecalis* showed two distinct clusters according to their sequence type. Five strains (2322, 2323, 2324, 2326, 2329) belonging to ST211 had only 3–13 SNPs difference between them. These strains were isolated from feeding tube 1, and faecal samples 1 and 2 (collected 8 days apart). On the other hand, three strains (2321, 2325, 2328) belonged to ST191, with 7–14 SNPs difference between them, were isolated from feeding tube 2 only Figure 5-3.

Six strains of *E. hormaechei* subspecies *steigerwaltii* (2315, 2316, 2317, 2318, 2319, 2319 and 2320) were closely clustered belonging to ST106 with 26–70 SNPs difference between them. Two *E. hormaechei* strains (2320 & 2318) from feeding tube 2 had 59 SNPs difference, and two *E. hormaechei* isolates (2316 & 2319), which were isolated from the same faecal sample 1 had 45 SNPs difference. Of greater interest were two strains, 2320 (feeding tube 2) and 2317 (faecal sample 2), which were isolated from different sample types (collected 8 days apart) and had the lowest SNPs difference Figure 5-4. This suggested that these strains were able to colonise and persist in the feeding tube and the baby's gut over an extended period of time. During this study, it became clear that whole-genome sequencing was more sensitive for bacterial speciation than 16S rDNA. This was unsurprising, since 16s rDNA focusses only on a single region of the bacterial genome, whereas WGS considers all regions of the genome. Thus, WGS is valuable, reliable and more accurate in bacterial genome analysis than other methods.

PFGE, previously was considered an excellent, superior and highly discriminatory genotyping method to identify clonal relationships among strains (Teixeira *et al*., 2007; Dicuonzo *et al*., 2001; Domig *et al*., 2003). The *SmaI* restriction enzyme was shown to work well in differentiation and epidemiological evaluation of *Enterococcus* in nosocomial infections (Domig *et al*., 2003; Persing *et al.*, 2004). In contrast, according to Zhang *et al*. (2012) the *XbaI* restriction enzyme was reliable and was used as a method of differentiating between *Enterobacteriaceae*. Therefore, in the present study, 10 *E. hormaechei* and *E. faecalis* strains isolated from NGFT and FS of a premature baby in the NICU during a 28-day period longitudinally were analysed using PFGE, in order to determine genotypic relatedness between the strains and shed light on possible transmission routes. This analysis indicated that within each species, all strains were genotypically indistinguishable, forming only one pulsotype for each species Figure 5-1 and Figure 5-2. This suggested that strains belonging to the same clone were recovered from NEFT and FS over 28 days, indicating their capability to survive, persist and multiply under adverse conditions within the baby's gut. In addition, this suggests that the possible sources could include the PIF itself, the hands of hospital workers, or through the preparation of PIF for infants, which can be considered as a vehicle of transmission in the NICU. To identify the source of contamination, we need to sample possible sources and find a match for the strains that were present in the baby. It was not possible to do this because ethical approval did not extend to this type of study.

Moreover, in 2013, Pires *et al*. argued that the persistence of the same clone within different sites in the hospital may reflect existence of the same isolate during the follow-up period in these sites. This may also be attributed to its capability of surviving and multiplying well under adverse conditions, which therefore becomes a potential origin of nosocomial infection (Bradford, 2001; Kristof *et al*., 2007; Viswanathan *et al*., 2010). In 2015, indistinguishable *E. coli* K1 ST95 strains were recovered from 11 neonates in NICUs during a 2-week period, although these neonates were fed different regimes (Alkeskas *et al*., 2015). Furthermore, Brady *et al.* (2005) suggested that contamination by health care workers may play an important role in bacterial transmission between patients. These findings are in agreement with Alkeskas *et al*. (2015) and Muytjens *et al*. (1988) who isolated various *Enterobacteriaceae* from PIF. This is also consistent with the reports of the FAO and WHO

(2004; 2006) stating that non-sterile PIF products may become the main reservoir for bacterial pathogens associated with serious infantile diseases. This therefore indicates that the colonisation of neonatal enteral feeding tubes by certain pathogenic lineages within NICUs could lead to increased exposure and risk to the neonates.

5.4.1. Virulence factors of *E. hormaechei*:

It is known that the incidence of neonatal nosocomial infections has increased due to members of the *Enterobacteriaceae*, particularly *Enterobacter* spp., *E. coli* and *Klebsiella* spp. These are the most predominant pathogens in NICUs. Despite microbiological safety measures in place for neonatal feeds, it is possible that neonatal feeding tubes could act as a site for bacterial biofilm. Therefore, bacterial investigation of feeding tubes might be the key to identify factors associated with enteral feeding tubes (Hurrell *et al*., 2009b). However, there is currently a lack of comprehensive studies characterising *E. hormaechei* in terms of acid, heat, human serum, lipase, protease, and desiccation resistance and other virulence traits.

The persistence of pathogenic bacteria in reconstituted PIF, or any enteral diets associated with the feeding of hospitalised babies, is a possible source of infection, particularly in immunocompromised individuals, such as premature babies. According to the WHO (2011), hospital-acquired infections are globally problematic due to their clinical impacts as well as financial and ethical influences. Of significance is the inability to reduce the impact of these infections due to the difficulty of controlling outbreaks worldwide. In 2008, Romeo had reported that biofilm formation may be affected by different circumstances such as O_2, temperature, osmolarity and pH, which are considered to be very important elements with regards to biofilm formation. In 2015, Fernandez-Delgado and researchers reported that different growth conditions may play a significant role in influencing attachment and biofilm formation. Furthermore, Nyenje *et al*. (2013) reported that temperature and incubation time had a significant influence on biofilm formation in *E. cloacae.* Another study reported that curli fimbriae in *Enterobacteriaceae* has specific role in bacterial biofilm and adherence (Olsén *et al*. 1993; Römling *et al*., 1998; Zogaj *et al*., 2003; Romeo, 2008; Lee *et al*., 2011). Van Acker *et al.* (2001) stated that the PIF ingredient is important for pre-attachment of

bacteria to feeding tubes and for survival and proliferation. Hurrell *et al*. (2009b), reported that all *Enterobacteriaceae* which were recovered from neonatal enteral feeding tubes from two NICUs in Nottingham had attached inside feeding tubes and formed biofilm in both reconstituted PIF and breast milk. In this study, it was shown that all tested *E. hormaechei* strains were able to form more significant levels ($p < 0.05$) of biofilm at 37 ^{o}C in PIF than at 25^{o}C Figure 5-7, Figure 5-8 and Table 5-5. Furthermore, gene searching for curli-fimbriae gene clusters (*csg*BAC and *csg*DEFG) was carried out and homologues of these genes were detected, suggesting that the investigated isolates carry curli fimbriae Table 5-4. These findings illustrated that the effect of temperature was evident and was a key factor in biofilm formation. These data are in agreement with previous studies (Nyenje *et al*., 2013; Fernandez-Delgado *et al*., 2015).

Usually, when foodborne pathogenic bacteria are ingested with food, the bacteria would be affected by the stomach acidity, which prevent the survival of the ingested pathogens. Acidic tolerance was determined by exposing strains to acidified reconstituted PIF after adjusting the pH to 3.5 for two hours at body (37^{o}C) temperature Figure 5-11. Hurrell *et al*. (2009a) reported that the acidity of the human stomach ranged between 2.5 to 4.3 depending on the ingested food type. In the current study, all tested *E. hormaechei* strains were able to tolerate pH 3.5 for the 2-hour exposure time with a slight reduction in viable count from 8.4 $\log_{10}$ to 7.8 $\log_{10}$. The capability of these strains to multiply and persist in an acidic environment (pH 3.5), similar to that of the stomach acidity, that may led to their ability to persist and multiply within the baby intestine. Bacteria with biofilms may also be intrinsically more resistant to acid than planktonic bacteria. For example, McNeill and Hamilton, (2006) reported that the biofilm of *Streptococcus mutans* is very highly resistant to low acidity. This means that these isolates are most likely to be more virulent and capable of invading the human intestinal tract and causing disease particularly in immuno-compromised patients. Several previous studies reported that the reduction in gastric acid reinforces the persistence rate of some foodborne pathogenic bacteria, with reduction in the infectivity dose (Cash *et al.,* 1974; Peterson, 1989; Schlech *et al.,* 1993). This study in consistent with (Cash *et al.,* 1974; Peterson, 1989; Schlech *et al.,* 1993).

Bacterial capsules are considered as one of the most important virulence factors, and play a crucial role in protecting them from harsh environments such as phagocytic cells, desiccation, acidity and serum activity (Guerry & Szymanski, 2008; Ogrodzki & Forsythe, 2015). According to previous studies by Pomakova *et al.* (2012) and Fodah *et al.* (2014), the possession of extremely high extracellular polysaccharide capsule by pathogenic bacteria can be contribute to biofilm formation. Supporting this notion, the capsule material production is also associated with biofilm formation in enteral feeding tubes from premature babies in NICUs (Hurrel *et al*., 2009a). In this study, capsular material production was investigated for all representative *E. hormaechei* isolates based on colony morphology appearance on two different media: TSA and PIF agar. All of the isolates tested were able to synthesise moderate amounts of capsular materials on both media (TSA and PIF agar). There was no variation in capsule production between both media used and among examined strains. These findings revealed a strong association between production of capsular materials and formation of biofilm. More studies are required to shed more light on the types of capsule genes present and their function in pathogenicity.

In the current study, other virulence factors were predicted to be involved in the pathogenicity of *E. hormaechei.* Among these factors are haemolysin resistance, serum resistance, and protease and lipase activity.

Haemolysin activity is another virulence trait which plays an important role in bacterial pathogenicity. There are three different classes of haemolysins: alpha-haemolysin (α), beta-haemolysin (β), and gamma-haemolysin (γ) usually found in clinical strains such as *Enterobacter* spp., *Klebsiella* spp., *Serratia* spp., and *E. coli* (König *et al*., 1987; Welch, 1987; Goebel *et al*., 1988; Schmidt *et al*., 1995). In the last decade, a study was carried out by Balsalobre *et al*. (2006), and they reported that the expression of the α-haemolysin toxin was the most common virulence trait among extraintestinal *E. coli*. In 2002, Ring and co-authors carried out a study on haemolysin activity in group B *streptococcus* isolated from patients with sepsis. The authors reported that β-haemolysin plays an important role in the pathogenicity of *Streptococcus* by destroying the cell membrane of red blood cells and contributing to high mortality and liver failure. In previous decades, studies reported that the *hly* haemolysin and *cnf*+ cytotoxic necrotizing factor-1 genes are linked to tissue damage

and immune dysfunction (Blanco *et al*., 1992; Hofman *et al*., 1998). In the current study, all investigated *E. hormaechei* isolates were β-haemolytic on horse red blood cells, and possessed *hlyABC* genes Table 5-4 and Table 5-5.

The susceptibility of the *E. hormaechei* strains to human serum was investigated. In this study, all tested strains were able to survive and proliferate in human serum for up to 180 minutes. No significant differences between the examined isolates were observed. During the past two decades, several studies have reported that the most common trait among different bacterial strains causing bacteraemia is the ability of those strains to escape from the host human serum (Porto *et al*., 1989; Hol *et al*., 1995; Hoe *et al*., 1999). In addition, Townsend *et al*. (2008b), reported that *E. hormaechei* strains isolated from a neonatal nosocomial outbreak in a Californian hospital were serum-resistant. These findings indicate the ability of examined strains to survive in human serum, which would allow them to cause bacteraemia. These data are in agreement with (Townsend *et al*., 2008b).

Other virulence traits such as protease and lipase activity were also detected in all tested *E. hormaechei* isolates Table 5-5. These activities play a crucial role in bacterial infections. Previous studies have reported that protease and outer membrane proteins are involved in human serum resistance (Lambris *et al*., 2008; Schwizer *et al*., 2013). Other studies have reported that protease activity could lead to damage of the structure and function of proteins in the host tissues (Goebel *et al*., 1988; Lantz, 1997; Joseph *et al*., 2012a; Kucerova *et al*., 2010). In 2002, Wilson and co-authors reported that bacterial lipase activity leads to degradation of accumulated host oils.

Several previous studies reported that bacterial heat-tolerant infections have increased in number and have become a source for concern, particularly in human health and food safety. Despite numerous *Enterobacteriaceae* strains expressing different heat tolerance levels after heat shock, only a relatively small number of strains of this family of bacteria revealed innate thermo-tolerance phenotypically (Mercer *et al*., 2015; Bojer *et al*., 2010). The heat tolerance analysis carried out in this study was designed to phenotypically evaluate the ability of strains to tolerate temperatures exceeding 54 °C for 30 minutes in liquid infant formula. The findings elucidated that all *E. hormaechei* strains demonstrated an ability to

tolerate heat of 55 °C. The highest levels of thermo-tolerance were observed in strains 2316 and 2317, which had been isolated from faecal samples 1 and 2, respectively Figure 5-21 and Table 5-8. These data suggest that the heat resistance virulence factor may lead to serious neonatal infections, especially in immuno-compromised babies. These findings are consistent with previous studies (Forsythe, 2009; Holý and Forsythe, 2014; Rosset *et al.,* 2007).

Several previous studies reported that the ingredients of milk and media may protect the bacteria during the drying process (Lian *et al*., 2002; Caubilla-Barron and Forsythe, 2007). In 2009, Osaili and Forsythe reported that during PIF processing, osmotic effects may result in the bacteria being able to tolerate desiccation. Another study reported that in several situations, bacterial sub-lethally injured cells were able to recover their viability features, including virulence factors required to cause infections (Wesche *et al.*, 2009). Therefore, in this study, 6 representative *E. hormaechei* strains (2 strains from neonatal feeding tube and 2 strains from faecal samples) were exposed to dry stress conditions in PIF and then cultured on two different media (TSA and VRBGA) in order to determine the effect of these media on bacterial cell recovery. Our findings revealed that there was an obvious effect of the different culture media on cell recovery. As shown in Figure 5-17 and Figure 5-18, the recovery of *E. hormaechei* cells on general media (TSA) was significantly higher ($P < 0.05$) than on selective media (VRBGA) during a three-week period, when compared to the initial inoculum Figure 5-19 and Table 5-7. In general, there was no significant variation between the tested strains on TSA and VRBGA during the desiccation period. These data are consistent with studies by (Lian *et al*., 2002; Caubilla-Barron and Forsythe 2007; Osaili and Forsythe, 2009; EFSA, 2015).

In 2014, the World Health Organisation (WHO) declared that bacterial multidrug resistance has increased significantly and is becoming a worldwide problematic threat to human health. Available neonatal data revealed widespread prevalence of antimicrobial resistance to gentamycin, ampicillin, and third generation cephalosporins for both community and hospital-acquired neonatal sepsis (Downie *et al*., 2013; Aiken *et al*., 2011; Thaver *et al*., 2009; Bates *et al*., 2014; Zaidi *et al*., 2005). In 2001, several previous studies reported that the *Enterobacter* species is responsible for about 7% of nosocomial infections in the USA.

Additionally, the *E. cloacae* complex, especially *E. hormaechei*, was the most common type of strain identified among nosocomial infections (Jones, 2003; Streit *et al*., 2004; Delmas *et al*., 2006; Paauw *et al*., 2008). Another study carried by Paauw *et al*. (2006) reported that most *E. hormaechei* which were isolated from a nationwide outbreak in the Netherlands, possessed several antimicrobial resistance genes encoding resistance to fluoroquinolones, third generation cephalosporins, aminoglycosides, and extended-spectrum β-lactamase (ESBL) genes. In support of these findings, Hurrell *et al*. (2009b) stated that most *E. hormaechei* strains isolated from neonatal enteral feeding tubes from two local hospitals in Nottingham were resistant to third generation cephalosporins, and ESBLs were also detected among the examined strains. The ability of pathogenic bacteria to produce ESBLs is still a major concern for bacterial therapy, particularly among the cephalosporins (Paterson and Bonomo, 2005).

In the current study, all *E. hormaechei* strains isolated from neonatal enteral feeding tube and faecal samples demonstrated susceptibility to cefoperazone, doxycycline, ciprofloxacin, and tobramycin, whereas intermediate resistance was detected only for meropenem, with the exception of strain 2320, which was completely resistant to meropenem. In contrast, the examined strains were resistant to the remaining antibiotics such as imipenem, most of the cephalosporins, macrolides, ampicillin, vancomycin, and gentamicin. In addition, all *E. hormaechei* isolates revealed an ability to express ESBLs phenotypically Table 5-9. With regards to strain 2320, interpretation of the results suggested that this strain was closer to being resistant. Gene searching for genes associated with antibiotics resistance revealed that all examined strains possessed a specific Fosfomycin resistance (*fosA*) gene and ESBL ($bla_{\text{ACT-15}}$) gene. Multidrug resistance among these strains could also be due to the presence of a multidrug efflux pump (*acrAB*) Table 5-4. This study in agreement with (Paterson and Bonomo, 2005; Paauw *et al*., 2006; Hurrell *et al*., 2009b), and furthermore, it suggests that neonates may be directly exposed to antibiotic-resistant isolates in NICUs, which may contribute to resistant infections becoming unmanageable by treatment with the available antibiotics. Thus, there is a high potential of infectivity by these strains to new-born babies that have weak and underdeveloped immune systems.

Overall, all *E. hormaechei* strains (ST106) observed in both feeding tubes and faecal samples revealed an ability to persist over time. All isolates were closely related, and were thus deemed to be the same strain based on their sequence type and genomic analysis. There were no genotypic and phenotypic behavioural changes noticed during the time points analysed in this study, with the exception of strain 2320, which was recovered from NFT 2; this strain demonstrated resistance to meropenem phenotypically.

5.4.2. Virulence factors of *E. faecalis*

For the past few decades *Enterococcus* species were considered as harmless and medically unimportant (Moreno *et al*., 2006). Recently, this genus, and particularly *E. faecalis* and *E. faecium*, have become one of the most common nosocomial pathogens after *E. coli*, *Staph aureus* and *Pseudomonas aeruginosa*., and have a mortality rate of up to 60% (Lopes *et al*. 2005), especially in immuno-compromised patients, long-term hospitalised patients, and preceding antibiotic therapies (Giraffa 2002 and 2003; Kayser 2003; Murray, 1990). The pathogenicity of these species may be due to isolates carrying virulence factors, which may contribute to the ability of these strains to evade the immune systems of humans and animals (Borgen *et al*., 2001; Boyce *et al*., 1994; Cocconcelli *et al*., 2003; Leclerc *et al*., 1996). Furthermore, virulent isolates produce pathological changes either directly through toxin production or indirectly through inflammation (Slaminen *et al*., 2004).

Enterobacter and *Enterococcus* infections cause a wide spectrum of diseases, including meningitis, septicaemia, bacteraemia, endocarditis, intra-abdominal infections, and urinary tract infection. Several virulence factors contribute to the pathogenesis of these bacteria including virulence-associated genes such as capsular serotype, attachment and invasions genes (Guerrero-Ramos *et al*., 2016; Campos *et al*., 2007). Biofilm formation is an essential step for bacterial pathogenesis that contributes to attachment and multiplication on abiotic and biotic surfaces, such as medical equipment, food contact surfaces and food. Biofilm formation not only gives the pathogen an ability to survive within the host, but also allows further persistence in inhospitable environments such as high temperature, fluid flow, variable pH, and carbon sources (Hall-Stoodley *et al.*, 2004). The *EbpABC* gene cluster in *E. faecalis* has been described to play an important role in biofilm formation (Sillanpää *et al*.,

2013). In 2006, Nallapareddy and co-authors investigated the role of the *EbpA* gene cluster in biofilm formation, they found that mutated strains formed significantly reduced amounts of biofilm compared to the wild type, which presented a higher level of adherent cells. In addition, a more recent study demonstrated that immunisation of mice with *EbpA* prevented *E. faecalis* from binding to fibrinogen and provides a safeguard for the mice in catheter-associated bladder infections (Flores-Mireles *et al*., 2014).

Several studies have reported that biofilm formation is a crucial factor in enterococcal pathogenesis. Some authors stated that biofilm formation among *E. faecalis* is more common than other species (Creti *et al*., 2004; Mohamed *et al*., 2004; Baldassarri *et al*., 2001). In 2003, Sandoe and co-authors had investigated 108 Enterococcal strains phenotypically over a one-year period. They found that 65% were *E. faecalis* and 35% were *E. faecium* strains. They also found that all *E. faecalis* strains tested had the ability of forming biofilms.

In the present study, it was shown that all tested strains carried the *EbpABC* cluster of genes Table 5-3. Additionally, all strains were able to form high levels of biofilm, as shown in Figure 5-5 and Table 5-5. These virulence traits could contribute to the adherence of this bacterium on medical devices and persistence in hospitals, particularly in long-term catheterised patients, which could have profound effects on the patient's health. Another suggestion is that *E. faecalis* is a catalyst; it contributes significantly to the formation of polymicrobial biofilms in the feeding tube and therefore contributes to colonisation of the intestines. *E. faecalis* produces lactic acid which coagulates milk proteins leading to biofilm formation and facilitating bacterial adhesion to the tube. This study is in agreement with several previous studies (Nallapareddy *et al*., 2006; Sillanpää *et al*., 2013; Flores-Mireles *et al*., 2014; Creti *et al*., 2004; Mohamed *et al*., 2004; Baldassarri *et al*., 2001; Sandoe *et al*., 2003).

A number of studies have reported that gelatinase (*GelE*) and serine protease (*sprE*) play important roles in enterococcal pathogenesis as they are able to hydrolyse casein gelatin, haemoglobin, other bioactive peptides, as well as degrade the host tissues and provide nutrients to the bacteria (Gilmore, 2002; Franz *et al*., 2003; Koch *et al*., 2004; Mohamed & Huang, 2007). Other recent studies have found that some *Enterococcus* isolates positive for

the *gelE* gene were unable to hydrolyse gelatine phenotypically, which suggests that the presence of *gelE* is not necessarily associated with gelatinase activity (Marra *et al*., 2007). Another study stated that other genes might be correlated with *gelE* expression (Lindenstrau *et al*., 2011). Similarly, in the case of haemolysis, two strains of *E. faecalis* were found to possess a haemolysin-related gene (*cylA*) although phenotypically they lacked haemolytic activity (Eaton & Gasson, 2001; Togay *et al*., 2010; Tsikrikonis *et al*., 2012).

In 2002, Shankar and co-authors reported that *E. faecalis* strains that are haemolysin producers are more pathogenic for both animals and humans and are linked with increasing infection severity. Another study reported that *E. faecalis* strains had haemolytic activity on several animal erythrocytes except sheep and cow (Miyazaki *et al*., 1993). Other studies have found that 11% of *E. faecalis* had haemolytic activity on horse blood (Vergis *et al*., 2003). In the present study, gelatinase (*gelE*) and protease (*sprE*) genes were detected in all of the isolates tested, and all strains had strong protease activity on skimmed milk agar. Additionally, the haemolytic activity of the *E. faecalis* strains on horse blood was characterised as alpha (α)-type (partial haemolysis) although the gene search for *cylA* did not reveal any homologues (absence of this gene cluster), which suggests the involvement of other genes for haemolysis Table 5-3 and Table 5-5. Our findings are consistent with several previous studies (Gilmore 2002; Franz *et al*., 2003; Koch *et al*., 2004; Mohamed & Huang 2007; Tsikrikonis *et al*., 2012).

It is likely that the biological activity of lipase plays an important role in microbial pathogenicity and it might be considered a crucial step in bacterial infections (Jaeger *et al*., 1994; Stehr *et al*., 2003; Castro-Escarpulli *et al*., 2003). In this study, all representative *E. faecalis* strains showed lipolytic activity Table 5-5.

The capability of pathogenic bacteria to escape from the host immune defence and survive in in the blood stream is very crucial in bacterial pathogenesis. Several previous studies reported that some proteins such as protease, gelatinase and outer membrane proteins may play an important role in human serum resistance (Bogard and Oliver, 2007; Phan *et al*., 2013; Miajlovic *et al*., 2013). In the present study, all of the *E. faecalis* isolates tested were able to tolerate human serum bactericidal activity at four time-points up to 3 hours Figure

5-12. In this regard, the present study is in agreement with previous studies (Bogard and Oliver, 2007; Phan *et al*., 2013; Miajlovic *et al*., 2013).

Another major strategy used by the *Enterococcus* species is that they produce capsules to avoid killing by phagocytes, which could contribute to evasion of the host's immune system (Miyazaki *et al*., 1993; Hufnagel *et al*., 2004). In support of this notion, a study was carried out within the last two decades by Bottone (1999), where two sets of white mice were inoculated intra-peritoneally with encapsulated *E. faecalis*, and another set of mice were inoculated with non-encapsulated *E. faecalis.* Non-encapsulated *E. faecalis* was easily cleared from the peritoneum of inoculated mice. Whereas, the encapsulated *E. faecalis* isolates had persisted in the peritoneum cavity of the mice for up to 5 days, which suggests that the delay in clearance might be due to the interaction of the capsule with the phagocytic cells (Bottone, 1999). Several studies reported that the CPS capsule type in *E. faecalis* consists of nine genes *cpsC* – *cpsK*. The putative glucosyltransferase gene (*cpsF*), is possibly responsible for the sero-diversity among serotype C and D isolates and modifying the ratio of galactose and glucose among the two types of capsules (Hancock *et al*., 2002; Hufnagel *et al*., 2004; Thurlow *et al*., 2009; Thurlow *et al*., 2009b). Thurlow and co-authors (2009b) reported that the polysaccharides, which are expressed by the genes encoded in the CPS locus, are responsible for enterococcal pathogenesis by enabling resistance to phagocytosis complement mediated by bacterial serotype C and D.

In the present study, all *E. faecalis* isolates possessed capsular genes. There was clear variation among representative isolates, for example *E. faecalis* strain 2321 (ST191), which was isolated from feeding tube 2, possessed *cpsA* and *cpsB* genes only, whereas the remaining *E. faecalis* strains 2322, 2323 and 2329 (ST211) possessed all cps cluster genes, except for *cpsF*, which was not detected Table 5-3. Also of interest is that all representative *E. faecalis* isolates showed an inability to produce capsular materials phenotypically on PIF agar and TSA Table 5-5. Although most of the capsule genes were present, all present strains were unable to produce capsular materials phenotypically, which suggests that this may be due to the ingredients of media used, or otherwise the gene was silent and therefore not expressed.

Acidic tolerance was determined by exposing strains to acidified PIF at pH 3.5 for two hours at body temperature Figure 5-10. Hurrell *et al*. (2009a) reported that the acidity of the human stomach ranged between pH 2.5 to pH 4.3, depending on the ingested food type. Mishra and Prasad, (2005) reported that most lactic acid bacteria (LAB) presented higher survival rates when inoculated in pH 3.0 than at pH 2.0. Another study was carried out by Klayraung and co-authors (2008) on fermented tea leaves, pork, and garlic at pH 3.0, and they found a higher survival (> 75%) rate of LAB strains. Another study supporting this notion was performed by Khalkhali and Mojgani (2017) who observed that *E. faecalis* and *E. faecium* isolates tolerate high stress conditions, such as an acidic environment (growing in MRS broth with different set pH 2, 2,5, 3, 4, 5 and 6) and high bile salt concentration (0.1, 0.5, 0.7, 1%). These findings are probably due to the stress response of LAB to low acidity. This property contributes to the ability of LAB to persist and multiply in a high acidic environment such as the stomach. In this study, all of the *E. faecalis* strains tested showed an ability to survive in acidic conditions and their viable counts remained greater than $\log_{10}$ 8 cfu/ml during the period of exposure. This confirms the ability of these isolates to persist in acidic conditions, and may also mean that these strains are capable of surviving passage through the stomach to cause neonatal illness. This study is in agreement with several previous studies reported that LAB tolerate high acidic stress (Mishra and Prasad, 2005; Klayraung *et al*., 2008; Khalkhali and Mojgani, 2017).

Bacterial heat-tolerant contaminations of food and food products have increased and have become a source for concern, particularly in human health and food safety (Mercer *et al*., 2015; Bojer *et al*., 2010). In 2009, Forsythe reported that in neonatal care units, the PIF and fortified breast milk were sometimes reconstituted at room temperature rather than at higher temperatures (Forsythe, 2009). Iversen *et al*. (2004a) suggested that the capability of microbes to tolerate and survive heat at 55 °C may contribute to pathogens causing neonatal infections in NICUs. Additionally, they found that *C. sakazakii* isolates were able to grow in reconstituted PIF between 5-45 °C and adhere to infant feeding equipment, which could thus become a source of neonatal infection in NICUs. Additionally, the WHO and FAO (2006) reported that the reconstitution of PIF with water between 40 - 50 ^{o}C and keeping feeding bottles at room temperature for a long time may be associated with the increase in neonatal

infections, unless the bottles are used immediately, because heat-tolerant bacteria might be capable of replicating in the warm milk.

In 2015, Enache and co-authors stated that *E. faecium* was three times more heat-tolerant in talcum powder than *Salmonella*. Another study showed that *E. faecium* NRRL B-2354 was significantly more heat-tolerant (17.7 times more) than *Salmonella* in ground beef (Ma *et al*., 2007). *E. faecium* has been proven to be heat-resistant and suitable as an appropriate surrogate for *Salmonella* in food safety tests due to their stability for longer periods of time (Enache *et al*., Unpublished data; Enache *et al*., 2013). The heat tolerance analysis carried out in this study was designed to phenotypically evaluate the ability of strains to tolerate temperatures of more than 54 °C for 30 minutes in liquid infant formula, to replicate the conditions during feed preparation. The findings revealed that all tested *E. faecalis* strains demonstrated high levels of heat tolerance at 55 °C. There was a slight difference in thermo-tolerance observed among the tested strains but these differences did not reach statistical significance Figure 5-20 and Table 5-8. These data show that there was no correlation between heat tolerance and capsular serotype. This could be because I have not enough strains of each serotype to test this with sufficient statistical power. These findings suggests that bacterial heat tolerance has become very crucial virulence factor represent a potential threat in PIF and other food products. Therefore, *E. faecalis* poses a serious threat to premature, immunocompromised babies in NICUs. This study is in agreement with several previous studies (Ma *et al*., 2007; Mercer *et al*., 2015; Bojer *et al*., 2010; Enache *et al*., 2015 and 2013).

The ability of pathogenic bacteria to survive and multiply under harsh environmental stress conditions such as desiccation, heat, and osmotic pressure is well known and leads to persistence of the pathogen. Such environments would include the hospital environment and food processing environment, thus increasing the presence of pathogens on medical devices and in the food chain. There is significant concern about the ability of pathogens to tolerate the food desiccation process and persist in food products; this may affect the safety and quality of those food products. In 2015, the European Food Safety Authority (EFSA, 2015) reported that salmonellosis is steadily prevalent in Europe, as well as verotoxin-producing *E. coli* (VTEC) and listeriosis infections, which have also increased. The presence

of *E. faecalis* in the manufacture of food, or any other food products, may occur due to the lack of hygiene in food production. Sometimes, during the PIF desiccation process, sub-lethally injured cells are difficult to detect due to their exposure to dry stress conditions. These injured cells might be undetectable due to an inability to grow on selective media, but they could still survive and potentially cause disease. Thus, it is important to determine the presence of sub-lethally injured cells in PIF or any food product by culturing also on non-selective media. In the current study, 4 representative *E. faecalis* strains (2 strains from neonatal feeding tube and 2 strains from faecal samples) were exposed to dry stress conditions in dehydrated PIF and then cultured on two different media (TSA and MRS agar) in order to determine the effect of these media on bacterial cell recovery. The effect of different media on cell recovery was quite clear. As shown in Figure 5-16 and Table 5-6, the recovery of *E. faecalis* cells on non-selective media (TSA) was significantly higher ($P < 0.05$; one-way ANOVA) than on selective media (MRS agar) during a three-week period, when compared to the initial inoculum. This indicates that there were significant numbers of dead and sub-lethally injured (undetected) cells which might escape detection if only selective media were used to screen the PIF.

Overall, there was no significant variation in survival among the tested isolates during the desiccation period, it was interesting to note that the growth of *E. faecalis* isolates on TSA was higher in terms of CFU number, but with tiny colonies, whereas fewer colonies of *E. faecals* strains grew on MRS agar but each colony was larger. The results of this study show that in order to avoid false-negative results during microbiological safety testing of desiccated foods, enrichment media such as TSB, BPW, MRS or BHI should be used. These enrichment media can provide a chance for damaged cells to improve their viability before culturing on selective agar media. These data are in agreement with the EFSA (2015) and the FAO-WHO publication (2006), and reinforce the potential high risk of neonatal infection by microbial pathogens during ingestion of contaminated food.

Regarding bacterial antimicrobial resistance, there has been a serious concern recently due to the increase of antimicrobial resistance among clinically-important organisms, and additionally, the widespread spectrum of antimicrobial-resistant infections worldwide. From the present study, it was found that all tested *E. faecalis* isolates were only susceptible to

clarithromycin, doxycycline and ampicillin, and they showed intermediate resistance to cefoperazone and vancomycin, and they were resistant to the rest of the antibiotics. Interestingly, clear variation was notable among the tested isolates to ciprofloxacin; strain 2321 (ST191) which was isolated from tube 2 was resistant, and strains 2322 and 2329 (ST211), which were isolated from faecal sample 2 and tube 1, respectively, had intermediate resistance, whereas strain 2323 (ST211), which was isolated from faecal sample 1, was susceptible. That suggest the change of antibiotic development among the strains may be between early and later samples or during interpreting of the results. Furthermore, all isolates were phenotypically revealed to be ESBL-positive. For many decades enterococci presented low clinical significance, but *E. faecalis* has recently become one of the leading pathogens causing nosocomial infections (Sader *et al*., 2011; Arias and Murray, 2012).

Arias and co-authors (2010) stated that the three most important antimicrobial resistances are to ampicillin, aminoglycosides, and glycopeptides because these are considered the front-line drug choices for enterococci infections. A study in Taiwan reported that the incidence of tetracycline, erythromycin, ciprofloxacin, streptomycin and gentamicin resistance in *E. faecium* and *E. faecalis* isolated from faeces of chicken in Taiwan was very high (Lauderdale *et al*., 2007). Another study was conducted by Tay Wang and colleagues who found a high rate of aminoglycosides, macrolides and tetracycline in *E. faecalis* and *E. faecium* (Tay Wang *et al*., 2013). Another study in Germany was conducted between 2000 and 2002 and the most isolated stains were *E. faecalis* (299 isolates) followed by *E. faecium* (54 isolates), where all of these isolates were subjected to 13 antibiotics. All selected strains were susceptible to teicoplanin, vancomycin, penicillin, amoxicillin and clavulanic acid (Peters *et al*., 2003). In 2004, another study was conducted by Busani *et al*. (2004), between 1997 – 2000, on *Enterococcus* strains isolated in Italy from raw meat, and selected strains were subjected to 10 different antibiotics. These isolates showed high resistance to erythromycin and tetracycline and low resistance to chloramphenicol, and additionally these isolates showed more resistance to the β-lactams. Additional studies have reported that *E. faecalis* and *E. faecium* strains are resistant to multiple antibiotics such vancomycin,

erythromycin, ceftizoxime, ceftriaxone, cefuroxime, teicoplanin, gentamicin, ofloxacin, and amoxicillin-clavulanate (Rams *et al*., 2013; Komiyama *et al*., 2016; Solayide *et al*., 2017).

Gene searching for genes associated with antibiotic resistance in the tested isolates reveal the prevalence of specific tetracycline resistance (*tetM*) gene and macrolide resistance (*lsaA*) genes, which may be responsible for the antimicrobial resistance observed in the tested strains. But all examined isolates showed their susceptibility to tetracycline and macrolide that suggest may be due to presence of mutant gene (gene silent) or other factors may affect. In this study, we addressed many virulence factors associated with pathogenicity of *E. faecalis* such as biofilm formation, acid-tolerance, heat-tolerance, desiccation-resistance, and serum-resistance. Additionally, protease, lipase, and haemolysin activity were also investigated. In order to determine the virulence potential and possible pathogenicity of presented isolates, which may represent a serious threat to public health.

In general, two different strains of *E. faecalis* (ST191 and ST211) were isolated from this baby at different time points and different location. It was revealed that indistinguishable strains of *E. faecalis* (ST211) were isolated from tube 1, tube 2 and faecal sample 2. These isolates were considered to be the same strain according to their PFGE profiles and 16S analysis, which revealed 3-13 SNPs difference between isolates. This strain demonstrated an ability to persist for a prolonged period of time and did not develop any genetic changes during this study. In contrast, some notable phenotypic variations were observed for this strain. *E. faecalis* strain 2323 (ST211), which was isolated from faecal sample 1, was intermediate to meropenem and susceptible to ciprofloxacin. On the other hand, *E. faecalis* strains 2322 and 2329 (ST211), which were isolated from faecal sample 2 and feeding tube 1, respectively, were resistant to meropenem and showed intermediate resistance to ciprofloxacin. Recently, Gomez *et al*. (2016) investigated the potential effect of nosocomial bacterial colonisation in enteral feeding tubes and their effect on early gut colonisation of premature neonates. Among 4,000 bacterial strains recovered, it was found the predominant isolates in both milk samples and faeces were *E. coli*, *S. epidermidis*, *K. pneumoniae*, *S. aureus*, *Serratia marcescens*, *E. faecium and E. faecalis.* The present study draws striking parallelism with the study by (Gomez *et al*., 2016).

The virulence of *E. faecalis* is still not completely understood, so further studies are needed, for example using *in vitro* tissue culture to determine the ability of *E. faecalis* isolates to adhere, invade and persist in mammalian epithelial and endothelial cells. Moreover, the current study has shown that virulence and antibiotic resistance genes are present, but real-time qPCR analysis would be required to prove that these genes are expressed and at what level.

Chapter 6. Conclusion and Future Directions;

The main goals of this study were (a) to evaluate the potential risk to neonates posed by ingestion of *E. coli* and *Klebsiella* spp, *E. hormaechei* and *E. faecalis* either through powdered infant formula, contaminated milk, or by medical equipment, (b) to categorise isolates of these organisms into high, medium and low potential pathogenicity to neonates, and (c) to conduct a longitudinal study to determine whether the same strain colonises both the feeding tube and intestine of a premature baby in the NICU over time.

Enterobacteriaceae recovered from NEFTs in NICUs from two different hospitals in Nottingham (chapter 3) and *Klebsiella pneumoniae* strains from NEFTs in NICUs from two different hospitals in Jordan (chapter 4) were phenotypically and genotypically characterised. Chapter 3 was a continuation of previous research since the initial studies by Hurrell *et al*. (2009a and b), which involved the collection of isolates from neonatal enteral feeding tubes (NEFTs) in the NICUs of two different hospitals in Nottingham, UK. Subsequently, new strains (as given in Table 3.2) were isolated from neonatal sepsis cases and their genomes and strain history were available. It was found that all these isolates carry virulence factors and antibiotic resistance genes, so they were also included in this study. Chapter 4 characterised and analysed a collection of seventy-five enteral feeding tube (EFT) and flushed milk isolates of *K. pneumoniae* from two different hospitals in Jordan.

In addition, *Enterococcus faecalis* and *Enterobacter hormaechei* strains were isolated from two NEFT and two faecal samples taken at different time points from the same premature baby in the NICU at QMC hospital, Nottingham to conduct a longitudinal study using whole genome sequencing and phenotypic characterisation (chapter 5). *E. faecalis* (ST191 and ST211) and *E. hormaechei* (ST106) were recovered from both faecal samples and feeding tubes from the same baby at different time points. The key novel findings of this study are stated below;

The first phase genotypic and phenotypic assays revealed that the great majority of *E. coli* and *Klebsiella* spp., which were isolated from flushed milk, NEFTs and sepsis cases at Jordan and Nottingham hospitals, harboured several potential virulence factors such as capsular serotype

K1 and K2, O1 and O2 antigens, siderophores and adhesion genes. In addition, they were able to express capsular material on PIF agar, lyse horse blood (β -haemolytic), produce biofilms, resist antibiotics, and tolerate acid, heat and desiccation. This suggests that these strains would be able to survive in stressful environmental conditions, akin to those of the human stomach. These strains are highly virulent and would likely be capable of invading the human intestinal tract to cause disease, particularly in immunocompromised individuals.

The second phase characterised the potential pathogenicity of a representative selection of *K. pneumoniae* strains using adhesion, invasion, survival and cytotoxicity assays on a range of human cell lines. Of the strains tested only one *K. pneumoniae* strain (453) isolated from NEFT at QMC, UK and five *K. pneumoniae* strains (1681, 1699, 1701, 1725 and 1734) isolated from flushed milk and EFTs at Jordan hospitals were highly pathogenic. The remaining strains tested were low pathogenicity.

In the third phase *E. hormaechei* and *E. faecalis* strains were isolated in the longitudinal study described in Chapter 5. The findings indicated that some of these strains persisted in the baby's gut for the whole period of study without genotypic and phenotypic behavioural changes, and multiple isolates were found to be essentially the same strain based on their sequence type and genomic analysis. All of these strains were found to be highly pathogenic strains. While, these strains did not cause disease during the period of study, it is important to be aware that these strains have the *potential* to cause disease, and can persist in the baby over a prolonged period of time.

In summary, the current study proved that opportunistic pathogens such as *Klebsiella* spp., *E. coli*, *E. hormaechei* and *E. faecalis* are able to overcome several adverse environmental stress conditions, such as heat, acidity, dryness, antibacterial agents and human serum. Furthermore, *K. pneumoniae* strains isolated from NEFTs and neonatal sepsis cases were able to adhere to, invade and survive within various human cell lines. The pathogenic potential of these bacteria to cause serious infections to neonates in NICUs, particularly in babies with underdeveloped immune systems, is clear.

Completion of this study does not necessarily mean that this is the end of this research; it is only the end of the beginning. This study has offered several horizons for continuation of research of nosocomial and foodborne associated pathogens. In addition, it has allowed us to better understand how these pathogens become more aggressive and cause serious neonatal infections. Therefore, continuation of research investigations will lead to an appropriate response to control and prevent the risk of potential bacterial pathogens for hospitalised babies.

6.1. Future direction;

During this study, screening of the virulence factor genes were based on the presence/ absence of the genes only. While such studies are useful for confirming the pathogenic potential of isolates, they have a number of limitations. They could not determine the gene expression level among examined isolates. For some key virulence-associated genes in particular *bamB*, *Clp*ATPase family, *rpoS*, *fimH* and *mrkD* in *E. faecalis* and *K. pneumoniae*, all strains possess these genes, but their expression levels might vary between strains or under certain conditions such as macrophage survival, desiccation or any other environmental stress. Therefore;

- Real-time qPCR analysis would be required to determine gene expression among examined isolates especially *E. faecalis* strains;
- RNA-Seq transcriptome analysis could be used to determine gene expression that controls genotypic and phenotypic virulence factors, which would confirm gene silence within strains;
- Gene knock-out (mutagenesis) analysis could be used to confirm the role of each gene in virulence-related traits.

To date, few studies describe the importance of *E. hormaechei* in terms of its pathogenicity and multidrug resistance. Therefore, further studies are needed, using *in vitro* tissue culture to fully understand and determine the ability of *E. hormaechei* to adhere, invade and persist in epithelial and endothelial cells. In addition, proteomic analysis of protein profiles has become

a very important tool for investing the mechanisms of bacterial resistance and virulence, which contributes of understand how the bacterial pathogenicity occurs that cannot be determined by phenotypic and genotypic analysis. This also may lead to the realizing of how *E. hormaechei* induces apoptosis. Therefore;

- Proteomic analysis could be used to determine the effects of different stress conditions such as biofilm formation, dryness, pH, thermal stress and antimicrobial resistance on *E. hormaechei* and *K. pneumoniae* to see how protein changes in response to different conditions.
- More studies are required to determine relatedness between capsular K-serotype and O-serotype and their associations with pathogenicity.
- Due to limited or lack of comprehensive studies on the virulence of *E. hormaechei* more studies are needed, for instance, to determine the effects of antibiotic prophylaxis in the neonates in terms of reduced intestinal flora.

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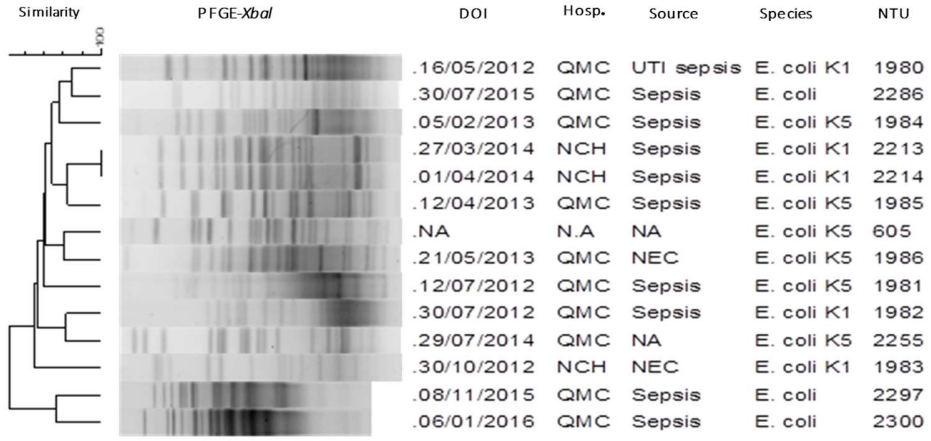
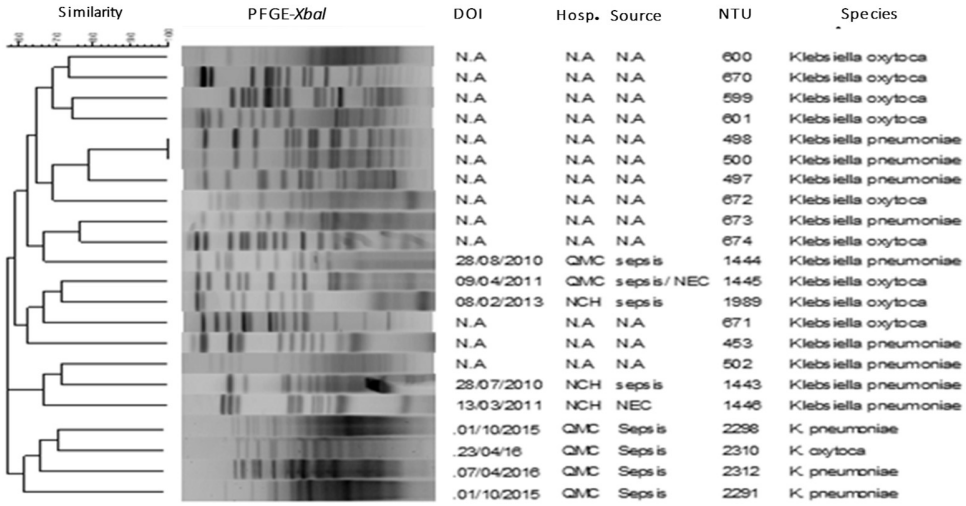
Appendices;



Appendix A**:** PFGE cluster analysis of *E. coli* strains isolated from NEFT and neonates with sepsis cases from QMC and NCH Nottingham.

DOI= Date of isolation, Hosp. = Hospital (Queen's Medical Centre (QMC) or Nottingham City Hospital (NCH)), N.A= Not available. The dendrogram was generated by BioNumerics software, version 3.5, dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimisation in the band was1.50%.

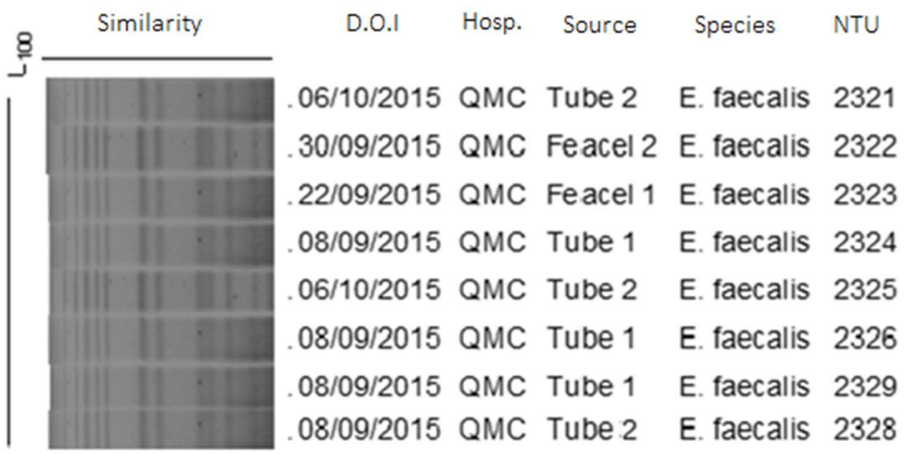


Appendix B**:** PFGE cluster analysis of *Klebsiella* spp. strains isolated from NEFT and neonates with sepsis cases from QMC and NCH Nottingham.

DOI= Date of isolation, Hosp. = Hospital, N.A= Not available. The dendrogram was generated by BioNumerics software, version 3.5, dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimisation in the band was 1.50%.

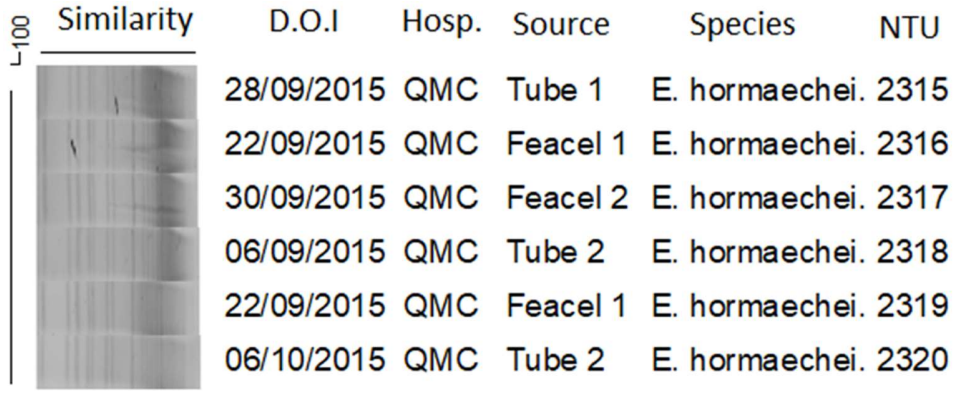
Appendix C**:** Phylogenetic tree of *rpoB* gene (501 bp) of K. pneumoniae isolates.

The tree was generated using MEGA7. Three rpoB allele profiles were revealed rpoB4, rpoB21 and rpoB25.



Appendix D: PFGE cluster analysis of *E. faecalis* strains isolated from feeding tube and faecal samples from premature baby at QMC.

D.O.I= date of isolation, Hosp. = hospital, QMC= Queens medical centre, *SmaI* digestive enzyme was used to separate DNA fragments to obtain restriction profiles. BioNumerics software, version 3.5 was used in this study to detect a band assignment and a dendogram for all isolates. CHEF-DR II (BIO-RAD, Belgium) system was used for bands separation at 6V, 14oC for twenty hours with 5 seconds initial and 50 seconds final. Dice coefficient, cluster analysis was used for the unweighted pair group method with arithmetic mean (UPGMA).



Appendix E: PFGE cluster analysis of *E. hormaechei* strains isolated from feeding tube and faecal samples from a premature baby at QMC.

DOI= Date of isolation, Hosp. = Hospital, QMC= Queens medical centre. *XbaI* digestive enzyme was used to separate DNA fragments to obtain restriction profiles. The dendrogram was generated by BioNumerics software, version 3.5, dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimisation in the band was 1.50%.