Antimicrobial Resistance of Foodborne Pathogens in Retail Pork

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Dedicated to my best friend and great companion of nearly 14 years, my beloved dog ‘Rocky’ who passed away shortly after the beginning of this project. 🐾

ABSTRACT

The risk of potential transmission of antimicrobial resistance in bacteria from animals to humans via the food chain needs to be extensively evaluated. Retail pork products, including organic, non-organic and free-range, were purchased from four different sources and tested, along with pig carcass swabs taken from a small local non-commercial abattoir, for the presence of multidrug-resistant (MDR) *E. coli*, *Salmonella*, *Staphylococcus aureus* and *Yersinia enterocolitica*. Isolation methods were based on the ISO standards for each organism but some method variations were tested to improve isolation frequency. Eight *Escherichia* isolates were detected, six of which were identified as *E. coli*, twelve *Yersinia* isolates with six being identified as *Yersinia enterocolitica,* five as *Yersinia frederiksenii* and one as *Yersinia* sp. A total of four Gram positive coccoid isolates were detected with two identified as *Staphylococcus aureus.* No *Salmonella* isolates were obtained. All isolates were tested for phenotypic resistance using disc diffusion assays and antibiotic discs from fifteen antibiotic classes. Five of the eight *Escherichia* isolates, 12/12 *Yersinia* isolates and 1/3 *Staphylococcus* isolates were resistant to three or more antibiotics from different classes and so could be defined as MDR. Resistances observed among *E. coli* isolates include ampicillin, ampicillin/clavulanic acid, sulphonamides, streptomycin, tetracycline and chloramphenicol, while in *Yersinia* isolates resistances observed were to ampicillin, ampicillin/clavulanic acid, cefoxitin, cefpodoxime, sulphonamides and nitrofurantoin. PCRs were performed and all *Escherichia* and *Yersinia* isolates were positive for the possession of chromosomal AmpC. *Staphylococcus* isolates were tested for the presence of the *mecA* gene conferring resistance to methicillin but none showed possession of the gene. PCR

was also performed for *Staphylococcus* isolates to detect if they were CC398 strains but none were. Gram-negative isolates were also tested for metal resistance using PCR for the detection of *merA*, *merC* and *intl1* genes associated with mercury resistance, *pcoE* gene for copper resistance and *silE* and *silP* genes for silver resistance. Only two *E. coli* isolates were shown to possess genes associated with metal resistance. *Escherichia* isolates were tested for the presence of the pathogenic *eae*, *astA*, heat-labile (LT) and heat-stable (ST) toxin genes. Five of the eight *Escherichia* isolates tested positive for the possession of enteroaggregative *astA* gene and none tested positive for the rest of the genes. *Yersinia* isolates were tested for the chromosomal *ail* and *inv* pathogenic genes and for the virulence plasmid genes *yadA* and *virF*. All *Y. frederiksenii* tested positive for the possession of *yadA* gene and 2/5 *Y. frederiksenii* were positive for the *ail* gene, findings that are quite unusual since both genes are associated with pathogenic *Yersinia*. None of the *Y. enterocolitica* isolates showed possession of any pathogenic genes. This study demonstrated the possible transmission of MDR foodborne pathogens to humans via the food chain.

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ABBREVIATIONS

ACDP	Advisory Committee on Dangerous Pathogens
AGPs	Antimicrobial Growth Promoters
AMR	Antimicrobial Resistance
CA-MRSA	Community-Associated MRSA
CDC	Centre for Disease Control and Prevention
CE	Common Era
CLSI	Clinical and Laboratory Standards Institute
DAEC	Diffusely-adherent *E. coli*
EAEC OR EAggEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ESBLs	Extended-spectrum β-lactamases
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa* and *Enterobacter* species
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
ETEC	Enterotoxigenic *E. coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing

FDA	Food & Drug Administration
HA-MRSA	Healthcare-Associated MRSA
ISO	International Organisation of Standardization
LA-MRSA	Livestock-Associated MRSA
MDR	Multi-Drug Resistance
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistant Staphylococcus aureus
MSSA	Methicillin Sensitive Staphylococcus aureus
MVPs	Veterinary Medicinal Products
PCR	Polymerase Chain Reaction
PCU	Population Correction Unit
STEC	Shiga-toxin producing *E. coli*
UTI	Urinary Tract Infection
VARSS	Veterinary Antimicrobial Resistance and Sales Surveillance
VMD	Veterinary Medicine Directorate
VTEC	Verocytotoxin producing *E. coli*
WHO	World Health Organisation

1. Introduction:

1.1 Herbalism: The History of Antimicrobials

Herbalism, which is defined by the Oxford dictionary as the study and practice of medicinal use of plants, was an honourable profession that aided the development of modern medicine and pharmacy. Homeopathy was a system developed in the early 1800s by Samuel Hahnemann which relied on the theory that a disease can be treated by remedies which are given in very low doses despite the fact that if the same drugs are given in higher doses they can cause the person symptoms similar to the disease itself. Around 60% of homeopathic remedies are naturally botanical (Hoffmann, 2003). In history, herbs, acupuncture and prayer were the most commonly used means for the treatment of diseases. In fact, there is evidence unearthed in 1960 from a burial site that showed that around 60,000 years ago plants such as hollyhocks were used most likely as part of a burial ritual, for purposes that still remain unclear, in the Shanidar caves in Northern Iraq, by people who were thought to be the Neanderthals. In total eight plant species were detected with seven of them still being used today in medicine (Saad *et al.*, 2005; Ibrahim, 2016). Plant extracts found popularity in the US around the late 1990's as the sales of botanical medicines had risen by 37% from 1995 to 1996. By 1999 about one-quarter to one-half of the pharmaceuticals sold in the United States were made with higher-plant ingredients. However, these were not intended for antimicrobial use since the discovery of bacterial and fungal derived compounds have taken over from plant-derived products as medical treatments of infectious diseases. In the European world, herbs have replaced treatments that involved in toxic practices back in 1800's such as syphilis treatment with the use of mercury

baths and hallucinogens as an antidote for tuberculosis (Cowan, 1999). The four systems of Herbalism as described by Saad *et al.* (2005) are the Traditional Chinese Herbalism, Ayurvedic Herbalism, Western Herbalism, which originated from Greece, and Arab Traditional medicine. In Chinese medicine for more than 2000 years they used to use the herb ephedra for the treatment of asthma and respiratory conditions. Even nowadays the commercial medicines to treat such conditions consist of a high percentage of plant substances including ephedrine, an active component of ephedra. On the other hand, the Egyptians were focused on a diet that would provide them with essential vitamins and minerals in order to keep them healthy. To be more specific, Egyptian workers were fed a diet rich in radish, garlic and onion, which nowadays are known to contain the natural antimicrobials raphanin, allicin and allistatin, in order to stay healthy and be able to keep up with the large workforce they were facing during the construction of the pyramids (Shuttleworth, 2010; SIRC, 2014). Other natural antioxidants include phenols and phenolic acids, such as caffeic acid, found in the herbs tarragon and thyme are known to be effective for viruses and bacteria (Cowan, 1999). Furthermore, quinones (which produce the brown reaction of cut fruit and vegetables) interact irreversibly with nucleophilic amino acids in proteins and cause inactivation which subsequently leads to loss of protein function, hence they have great antimicrobial potential. Additionally, plant lectin molecules are other natural sources that proved to be powerful against viral adsorption (Cowan, 1999). Plant lectins are even studied for the treatment of human immunodeficiency virus (HIV). It was estimated that around 36.7 million people were living with HIV by the end of 2015 alone and around 1.1 million have died from it (WHO, 2017). Particularly mannose

and *N*-acetylglucosamine-specific lectins are qualified inhibitors of the virus cell fusion process. In fact, these plant lectins block syncytium formation between the healthy and HIV infected cells without preventing the attachment of the virus to the cells. Moreover, they can restrict its replication. Apart from HIV, lectins are qualified molecules to work with against other viruses as well such as Influenza A virus (De Clercq, 1995).

Essential oils from herbs are also naturally occurring preservatives against microorganisms. In a study performed using Indian medicinal plants it was observed that as little as 1 mM of plant extracts could confer antibacterial activity against Gram-negative organisms. Essential oils carvacrol and thymol found in thyme were shown to decrease the intracellular ATP content of *E. coli* cells when at the same time the extracellular ATP was increased. Similarly, oils extracted from cumin, caraway, basil and coriander were found to have inhibitory properties on microorganisms such as *Staphylococcus aureus* (Brul & Coote, 1999).

1.2 History and Evolution of Antibiotic Resistance

The word 'antibiotic' has been reported to first be used as a noun by Selman Waksman in the early 1940s to describe small molecules produced by microbes, such as fungi and soil bacteria, and antagonise the growth of other microbes (Clardy *et al.,* 2009). However, some antibiotics have been developed synthetically and semi-synthetically; manufactured by fully chemical routes or by chemical synthesis using a natural product as a starting material, respectively. An example of chemically synthesised antibiotics are sulphonamides (Wright *et al.,* 2014). Antibiotics are known to inhibit or kill bacteria by several different ways of which interrupting either their cell wall synthesis or protein synthesis are the main ones.

Some are known to disrupt both of those processes (Dipalma, 1969). Since their discovery, these drugs have managed to significantly reduce illness and death from numerous infectious diseases (CDC, 2017). However, most bacteria are now able to interfere with and disrupt the action of antibiotics resulting in the ineffectiveness of drugs against them and thus treatments become unsuccessful and illnesses persist longer (WHO, 2017). This ability to stop antimicrobial actions is now defined as 'antimicrobial resistance'.

Bacteria and their ability to rapidly acquire resistances against antibiotics has become a major health threat across the globe. Moreover, this increasing problem has led to the need to discover newer, more powerful drugs that could fight against diseases caused by these resistant organisms. However, the real challenge is to understand how these pathogens acquire their resistance and whether there are any means of preventing that from happening. Table 1.1 summarizes the discovery of some main antibiotic classes, a few of which are discussed further.

Table 1.1: History of discovery of some main antibiotic classes (Modified from Aminov, 2017).

Arsphenamines	1910
β-lactams	1929
Sulphonamides	1935
Polypeptides	1939
Aminoglycosides	1943
Tetracyclines	1945
Amphenicols	1947
Lipopeptides	1947
Macrolides	1950
Oxazolidinones	1952
Glycopeptides	1953
Streptogramins	1953
Ansamycins	1957
Quinolones	1962
Lincosamides	1963

It may appear to some that antibiotic resistance in pathogenic bacteria is quite a recent phenomenon due to the great attention surrounding it in the past few years, nevertheless evidence from metagenomic analysis on DNA from 30,000-year-old Beringian permafrost sediments recorded genes encoding resistance to antibiotics such as β-lactams, tetracycline and glycopeptides. A gene called *vanA* is known to confer resistance to vancomycin, a glycopeptide antibiotic. Analysis of this resistance element from the ancient DNA revealed similarities to recent variants suggesting resistance existed long before it was thought to exist. Similarly, natural product antibiotics are estimated to have been derived 2 billion to 40 million years ago indicating resistance could be as old too (D'Costa *et al.*, 2011). Penicillin, a β-lactam antibiotic, was one of the first drugs to be discovered after arsphenamines (a phenolic amine that was used to treat syphilis). Penicillin was discovered fortuitously by Alexander Fleming, a Scottish scientist, back in 1928. Its mass production and distribution was not until 1945 after it proved to be a valuable antibiotic for treating ill and wounded soldiers during World War II and saving around 15% of lives by decreasing infections. However, the first ever group of antibiotics to undergo a large-scale production was that of sulphonamides, also known as sulpha drugs, which took place around the late 1920s and early 1930s. The first compound synthesized by German chemists in Bayer AG laboratories and which acted as a precursor for the actual active drug was sulphonamidochrysoidine. Its antimicrobial activity was tested in animal disease models and it was shown to be predominantly active against streptococcal infections. In the period between 1937 and 1943 sulpha drugs managed to increase human life expectancy by approximately 0.7 years (Aminov, 2017). Resistance to sulphonamides was first

reported in the late 1930s, only a few years after their introduction, with the same resistance mechanisms found some 70 years later (Davies & Davies, 2010).

In 1943 and after the success penicillin encountered, Selman Abraham Waksman led a team into the research for new antibiotics in the University of Rutgers. Later in that year one of the students isolated two *Streptomyces* strains (Aminov, 2017), from which streptomycin was the purified antimicrobial compound obtained. Streptomycin falls into the antibiotic class of aminoglycosides and is an inhibitor of protein synthesis. Furthermore, it can act against penicillin- resistant Gram negative bacteria. At the time it was also particularly important as it was highly effective against the tubercle bacilli such as *Mycobacterium tuberculosis* (Lewis, 2013). Despite that aminoglycosides were broadly used in antimicrobial chemotherapy, around the 1980s they were replaced with other newer and more efficient drugs that had less side effects such as carbapenems (Aminov, 2017).

Carbapenems are related to β-lactam antibiotics, such as penicillins, with olivanic acid and thienamycin being the first two discovered simultaneously around the mid-1970s. *Streptomyces olivaceus* was the organism which produced the olivanic acids which subsequently led to the discovery of clavulanic acid (Moellering *et al.,* 1989). Both olivanic acid and thienamycin were shown to be chemically unstable and therefore they were not clinically utilised. A more stable derivative of thienamycin was imipenem, a broad spectrum drug which also can result in resistance to various β-lactamases, the enzymes that provide resistance to β-lactam antibiotics. However, resistance to carbapenems was developed in infections caused by *Pseudomonas aeruginosa* that were treated purely with imipenem. One of imipenem's most important properties is the ability to penetrate well via the outer

cell membrane of Gram negative bacteria. This resistance possibly came as a result of the cell permeability changes such pathogens undergo. Meropenem is a newer more promising carbapenem that comprises ultra-broad-spectrum activity as well as resistance to β-lactamases and an improved intrinsic activity against Gram negatives (Moellering *et al.*, 1989).

Chlortetracycline and oxytetracycline, produced by *Streptomyces aureofaciens* and *Streptomyces rimosus* respectively, were the first two tetracyclines to be discovered and described within this group during the 1940s. Aureomycin was the trade name of the purified antibiotic obtained from chlortetracycline which gained FDA approval for clinical use in December 1, 1948, and soon after experienced great success by saving patients from a wide spectrum of infections. Tetracycline was synthesized as a derivative from the catalytic hydrogenation of Aureomycin and was approved for clinical use by FDA in 1954 (Nelson & Levy, 2011).

Tetracyclines became an important class of drugs and soon experienced great interest as they were the first group that exhibited the growth promoting properties of antibiotics in animals in 1949 when it was accidentally noticed that low levels of tetracycline administration were beneficial to the growth rates and feed utilisation of young chickens (Chopra & Roberts, 2001). However, it is thought that the rapid acquisition of resistance to this antibiotic was mainly due to its use by the food animal sector. This resistance expanded quickly throughout the 1980s (Nelson & Levy, 2011). To overcome this growing problem second-generation tetracyclines were developed from 1965 to 1972. In 2005 the first third-generation tetracycline to represent this class (known as tigecycline) gained FDA approval for hospital use in 2006. It is administered intravenously to act against infections caused by multi-

drug resistant (MDR) pathogens. Despite antibiotics and resistance to them being associated with the modern era of medicine, there is evidence of tetracycline being present in human bone remains from the ancient Sudanese Nubia region dating back to 350-550 CE, which indicated some sort of exposure to tetracycline-most likely through diet (Aminov, 2010). Similarly, *tetM*-related resistance genes were detected in permafrost sediments as noted above, in the study of D'costa *et al.* (2011).

Polypeptide antibiotics were independently discovered from *Brevibacillus brevis* around 1939-beginning of 1940s when this soil organism was shown to produce inhibitory substances against some pathogenic microbes and fungi (Aminov, 2017). René Dubos was an American-French microbiologist who first discovered tyrothricin, a cyclic polypeptide antibiotic mixture, in 1939. These antibiotics act as membrane channels that tend to damage the ion gradient between the cell cytoplasm and the extracellular environment when increasing the permeability of the cell's membrane. A multicomponent polypeptide drug is polymyxin E, also known as colistin. This antibiotic consists primarily of colistin A and B and found its way into clinical use around 1949. Nearly two decades later, around the 1970s, its use was suspended for its nephrotoxicity and colistin was replaced by drugs that were considered less toxic. Despite being a relatively old antibiotic, as it was discovered more than 60 years ago, colistin has never undergone any drug development process in order to comply with up to date regulatory requirements (Li *et al.*, 2006). However, it was re-introduced into clinical practices after nearly 20 years as a last resort antibiotic for the treatment of life-threatening infections caused primarily by MDR- Gram negative bacteria, such as those caused by *Pseudomonas aeruginosa,* especially in

patients suffering from cystic fibrosis (Michalopoulos *et al.*, 2005). Colistin, which is a last resort antibiotic, did not escape the development of resistance either. It has been suggested that most of the resistance mechanisms in Gram-negative pathogens are related to changes in the outer membrane. According to Li *et al.* (2006), MDR- *Pseudomonas aeruginosa* did not show resistance towards colistin in Denmark and the UK. Nonetheless, mutational resistance to colistin has been recorded in a number of ESKAPE pathogens at high frequencies. For instance, heteroresistance, which has been described by Falagas *et al.* (2008) as the resistance to specific antibiotics expressed by a microbial subpopulation that is generally considered susceptible to those drugs according to in-vitro testing, to colistin is more common in MDR- *Acinetobacter baumanii* than MDR- *Pseudomonas aeruginosa* (Li *et al.*, 2006). ESKAPE pathogens are of great importance nowadays due to the resistance they confer against multiple antibiotics. The ESKAPE group is predominantly important in hospitals around the world (Rice, 2008), and consists of six pathogens; *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species, altogether they cause the majority of nosocomial infections and hence any resistance acquired by these pathogens is of great importance to hospital and care unit environments (Lewis, 2013). It was also reported that healthcare-associated infections with significant mortality rates caused by this group is usually related with intensive care unit patients and previous antibiotic use. For example, a study on episodes of bacteraemia in solid-organ transplant recipients revealed that approximately 20% of the time it was due to resistant ESKAPE strains (Bodro *et al.*, 2013). The most common resistances observed from these pathogens are MRSA,

vancomycin-resistant *Enterococcus faecium,* carbapenem and quinolone-resistant *Pseudomonas aeruginosa*, ESBL-producing *Klebsiella pneumoniae,* carbapenem-resistant *Acitenobacter baumannii* and de-repressed chromosomal β-lactamases producing *Enterobacter* species (Boucher *et al.*, 2009; Bodro *et al.*, 2013).

Macrolides are considered the second most prescribed antibiotic class after the β-lactams and are the first to be used in patients suffering from penicillin allergies. In this class, pikromycin was the first to be isolated from the bacterium *Streptomyces venezuelae* by Brockmann and Henkel in 1950 (Aminov, 2017). For many years, macrolides were used as an alternative for penicillins and cephalosporins for treating infections caused by Gram-positive bacteria such as β-haemolytic streptococci. In the 1990s and after macrolides resistance has evolved, semisynthetic derivatives were produced with the hope of overcoming resistance to erythromycin, the first successful commercial macrolide. However, despite their improved pharmacokinetics and acceptability they were still unable to overcome erythromycin resistance (Leclercq, 2002).

The discovery of cephalosporins, another class of β-lactam antibiotics, began back in 1945 when a professor of hygiene named Guiseppi Brotzu isolated the seawater fungus *Cephalosporium acremonium* and discovered its ability to produce substances that act against Gram-positive and Gram-negative bacteria (Abraham, 1979). The first extractable material was named cephalosporin P due to its antimicrobial activity only against Gram-positive organisms. After experimental work, cephalosporin N was detected and showed antimicrobial activity against primarily Gram-negative but also Gram-positive bacteria. However, further work on this substance proved it to be analogous to penicillin and therefore it was

considered as a new type of penicillin (Penicillin N) which entered clinical trials and was recommended as an alternative to chloramphenicol for the treatment of typhoid fever (Abraham, 1979). Cephalosporin C was discovered in 1953 and was the third substance obtained from *C. acremonium*, and was in fact concentrated through penicillin N purification. Cephalosporins along with other semisynthetic antibiotics account for around 64% of all antimicrobial new chemical entities classified between 1981 and 2005. Every new generation of antibiotic is developed mainly as a result of acquired resistance to the previous generation of antibiotic (Fischbach & Walsh, 2010). Similarly, second and third generation cephalosporins, such as cefuroxime and cefotaxime, are designed to be more resistant to β-lactamase enzymes as well as being more effective in penetrating the outer membrane of Gram-negatives (Fischbach & Walsh, 2010).

Another major class of antibiotics are quinolones. Their discovery took place in the 1960s and they are chemically synthetic compounds, although structurally related compounds can be found in the environment as well. For this reason, quinolones attracted much attention after their discovery. Some of their good characteristics include a broad spectrum activity, bioavailability, both oral and intravenous formulations and low incidence of side-effects (Andersson & MacGowan, 2003). The first representative compound of the group was nalidixic acid which was introduced into clinical practice for the treatment of urinary tract infection (UTI) in 1962. Fluoroquinolones were derived from the addition of a fluorine molecule, which enhanced the potency of the drug by providing a 10-fold increase in gyrase inhibition. The main antimicrobial activity of these compounds is known to be their formation of a DNA gyrase-quinolone-DNA complex that is capable of blocking the

chromosome replication in bacteria and eventually resulting in cell death (Amivon, 2017). In comparison with the original quinolones, fluoroquinolones are rapidly and nearly fully absorbed in the gastrointestinal tract (King *et al.,* 2000). Furthermore, it is worth mentioning that their activity is dependent on concentration, which means that the more antibiotic concentration increases, the more the bacterial killing increases. Table 1.2 demonstrates the proposed classification of quinolones in chronological order.

Table 1.2: Proposed classification of Quinolones (Based on Andersson & MacGowan, 2003).

Agents	**Activity**	**Clinical application**
Urinary agents (1960-1985)		
nalidixic acid	Activity against common Enterobactericeae	Main use in UTIs
cinoxacin		
enoxacin		
norfloxacin		
Gram-negative systemic agents (1985-1995)		
ciprofloxacin	Wide activity against Gram-negatives, marginal activity against Gram-positives	Used against tissue-based infections and UTIs
ofloxacin		
levofloxacin		
Broad spectrum systemic agents (1990-2000)		
temafloxacin	Wide activity against Gram-negatives and Gram-positives, some activity against anaerobes	Main use in broad-range of tissue-based infections
clinafloxacin		
trovafloxacin		
Respiratory agents (1995 onwards)		
levofloxacin	Wide activity against Enterobacteriaceae, active against Gram-positives and atypical bacteria	Main use in respiratory tract infections
sparfloxacin		
grepafloxacin		
moxifloxacin		
gatifloxacin		
gemifloxacin		
garenoxacin		

1.3 The Use of Antibiotics in Livestock

It has been emphasized repeatedly that one of the major factors causing AMR to arise, and possibly the most important one, is the overuse or misuse of antibiotics in livestock. Antimicrobials used in swine production account for around $1.7 billion

dollars, the equivalent of 34% of the global animal health antimicrobial market (Burch *et al.,* 2008). In order to keep record of the use, the European Commission launched a request back in 2009 for the development of a synchronised approach to collecting and reporting the data of antimicrobials used in animals within the European Union and European Economic Area member states. This request has been implemented by the European Medicines Agency which started the ESVAC project and now publishes a yearly report with all the data collected from the EU/EEA countries. However, not all countries contributed immediately in providing information on their antibiotic sales. It is important to mention that in the UK, veterinary antimicrobials can only be given under prescription and distributed by a veterinarian or veterinarian pharmacist. Moreover, the supplier has to be authorised by the Veterinary Medicines Directorate in the UK (European Medicines Agency, 2011). An annual tonnage of sales of antibiotics does not solely allow reliable trend analysis due to the variation in animal populations taking place over time. Therefore, the 'population correction unit' (PCU) has been implemented to take into account relative usage. According to the Veterinary Antibiotic Resistance and Sales Surveillance (VARSS) report (2014), PCU "represents the estimated weight at treatment for each species and the estimated numbers of each species which will have been eligible for treatment over a 12-month period. This enables year-to-year comparison to see whether sales for livestock are changing in real terms". According to the first VARSS report, which provide details of UK veterinary antibiotic resistance and sales surveillance, published for the periods of 2005 to 2009 the most widely sold antibiotic classes in mg of active ingredient per PCU in the UK were tetracyclines, sulphonamides and penicillins which accounted for

approximately 76%-81% of total sales in each year. Similarly, other European countries showed such results with tetracyclines being the predominant class in the Czech Republic, France and the Netherlands whilst penicillins were the predominant ones in Finland, Sweden and Norway. Denmark showed almost equal use of both the above drug classes. Switzerland on the other hand was predominantly prescribing sulphonamides (European Medicines Agency, 2011). The following year (2010) statistics remained the same with tetracyclines, penicillins and sulphonamides being the highest-selling veterinary antibiotics amongst the 19 different EU countries that provided data. For 3rd and 4th generation cephalosporins, overall for all 19 countries, around 59% of sales (in tonnes) were in injectable forms while for fluoroquinolones only 20.8% were sold by injections since the highest sales of pharmaceutical form for this class was the oral solutions with 78.5%. The most notable variations amongst all 19 countries were observed for macrolides, fluoroquinolones and 3rd and 4th generation cephalosporins. However, variations are believed to have occurred due to different animal production systems, availability of antimicrobials, animal species and prices (European Medicines Agency, 2012). In addition, apart from Norway, Sweden, Iceland and Finland, it seems that administration of antimicrobial veterinary medicinal products (VMPs) was mainly due to herd treatment. In the United Kingdom in 2011 there was a 25% decrease in sales of veterinary antibiotics particularly the tetracyclines and penicillins, which were considered the highest-selling ones for the previous years. This decrease was not implemented for long as sales of tetracyclines have increased again in 2012 from 51 to 66 mg/PCU (European Medicines Agency, 2013). In 2013 the vast majority of the antimicrobials sold between the 26

countries that participated in the report were in the form of premixes and oral powders. Spain was the number one country with the highest amount of active antimicrobial ingredients sold in tonnes for food producing animals with 2,201.9 tonnes sold compared to only 422.0 tonnes in the UK. Moreover, just as in the previous few years, tetracyclines, penicillins and sulphonamides were the most popular selling drugs and accounted for around 70.8% of total sales (European Medicines Agency, 2015). In the UK there was a slight decrease in the sales in 2013 compared to 2012 especially for tetracyclines and sulphonamides. Additionally, cephalosporins and polymyxins were the antimicrobials sold least for the period of 2010 to 2013. In 2014 an increase was observed particularly in the sales of sulphonamides and macrolides compared to 2013 (European Medicines Agency, 2016). However, between 2014 and 2015 a slight decrease was generally noticed with -9% in total sales in tonnes, -11% in sales of 3rd and 4th generation cephalosporins (mg/PCU) and -3% for fluoroquinolones (mg/PCU). Furthermore, colistin, which is an antibiotic of last resort, was also included in the report with sales in the UK for 2015 being at 0.12 mg/PCU, below the recommended guideline of 1 mg/PCU (Veterinary Medicines Directorate, 2015).

There is a variation amongst the different countries and their use of antimicrobials in food-producing animals. Therefore, in order to tackle AMR it is essential for all countries to minimise the use of antimicrobials and to only administer them to animals when absolutely needed. Figure 1.1 illustrates the tonnes of active ingredient sold in the UK during the past decade from 2005 to 2015.

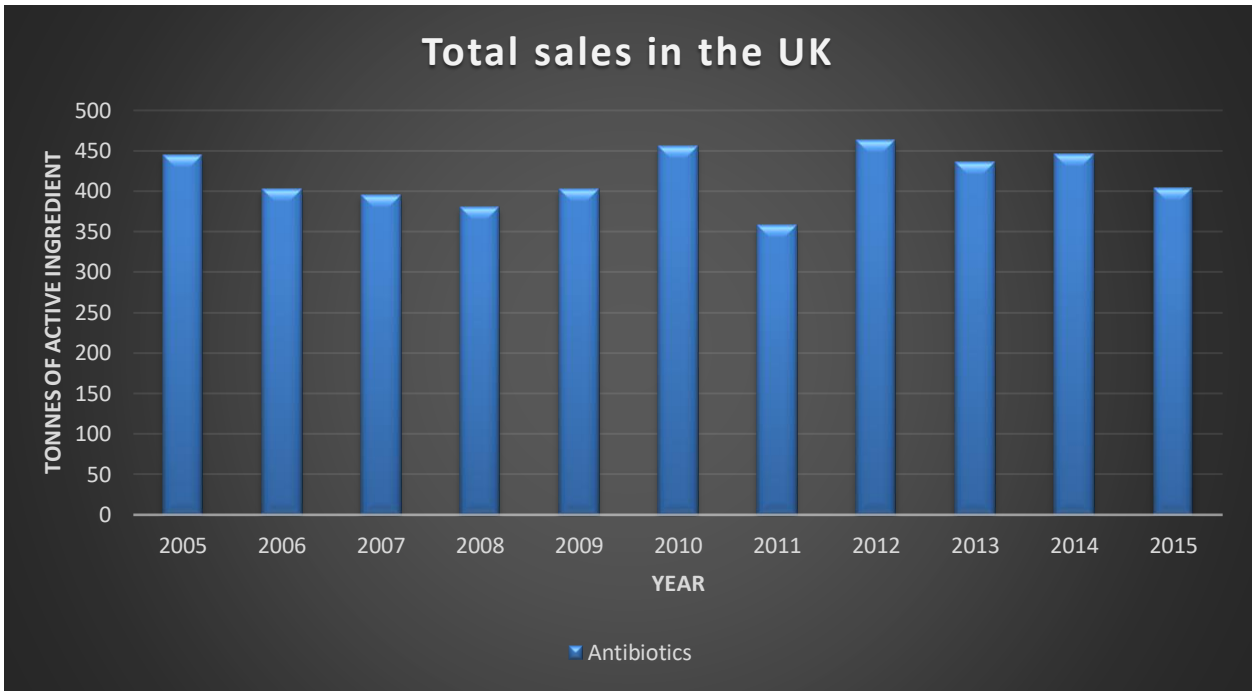


Figure 1.1: Sales in tonnes of active ingredient of veterinary antimicrobial agents for food-producing animals including horses (data to create chart obtained from ESVAC reports).

1.3.1 Overuse and Misuse of Antibiotics in Livestock

Farmers play the most important role in the administration of antibiotics to their animals. Generally in food-producing animals, prophylactic use of medicines is considered important in order to maintain lower mortality rates within the herd by decreasing disease and thus improve their production. In Belgium during 2010 it was observed that such prophylactic treatments were carried out shortly after birth in fattening pig herds (Callens *et al.*, 2012). Additionally, monitoring of antibiotic use revealed that injectable medicines were overdosed while orally administered products were underdosed (Callens *et al.*, 2012). In order to control the emergence of resistance it is very important to appropriately assess the selection pressure applied from the use of antimicrobial products. Inappropriate use such as wrong dosage often lead to irreversible consequences. In Belgium the fact that approximately 43% of the income of a pig veterinarian is earned from selling

medicines could possibly have acted as a contributing factor in the misuse of drugs since selling more could translate to earning higher sums. Countries such as Denmark, Sweden and Netherlands have forbidden the use of antimicrobials for prophylaxis (Cogliani *et al.*, 2011). German guidelines suggest prescriptions only for therapeutic or metaphylactic reasons and only after the causal pathogen has been tested and antimicrobial susceptibility profile has been revealed (Callens *et al.,* 2012). For prophylactic and/or metaphylactic preventive use, drugs were also added in animal feed. Generally, in Europe guidelines state that premixes for medicated feeding stuff should only be given under controlled prescriptions for both prevention and treatment purposes. Medicated feed was intended to be used only when there was a threat of a disease outbreak and that this preventative method should only last for a short period of 5-10 days (Barton, 2014). However, it is very common for antimicrobials to be given to pigs via soluble forms in their drinking water as they pass through the animals' stomaches more quickly than solid/semi-solid feed and therefore they can be rapidly absorbed (Burch *et al.,* 2008). Additionally, unwell pigs have reduced appetite and therefore time-consuming preparation of feed can be wasted sometimes as their feed intake varies when being sick, hence soluble formulations are advantageous over medicated feed. Apart from the use of antibiotics in food animals for the purpose of disease treatment, prophylaxis and/or metaphylaxis for more than 50 years, antimicrobials were also added to animal feeds to promote growth. Their growth promoting properties were discovered in the 1940s when animals fed mycelia of *Streptomyces aureofaciens* containing chlortetracycline showed an improved growth rate (Castanon, 2007). This growth-promoting effect is said to be associated with

microbial interactions within the gut population. Antibiotics such as tetracyclines and penicillins were the first to be included in poultry feeds and were authorised without veterinary prescription for poultry up to 10 weeks old. This decision was approved by the United States Food and Drug Administration in 1951 and in the years to follow each European country permitted its own national regulations (Castanon, 2007). Resistance was soon discovered after this implementation, with reports published as early as 1951, and reporting the connection of tetracycline resistance when growth promoting levels of the drug were fed to poultry (Dibner & Richards, 2005). Similar findings were observed in England with glycopeptide resistant enterococci being isolated from food animals in 1993 when in fact this group of antimicrobials was not used for treatment but only fed as a growth promoter, for example avoparcin (Dibner & Richards, 2005). Although in the United States recommendations for the reduction and elimination of antimicrobials in animal feed were made in the 1980s, in Europe Sweden was the first country to ban antimicrobial growth promoters (AGPs) on farm use in 1986. In 1995 Denmark followed by prohibiting the use of avoparcin as it can cause cross-resistance to vancomycin and thus select for vancomycin-resistant enterococci (VRE) (Nilsson, 2012). Two years later, in 1997, the Commission of the European Union implemented the ban in all EU member states (Cogliani *et al.,* 2011). In addition, in 1999 the Danish swine industry stopped all remaining antimicrobial growth promoters for pigs under 35kg and in 2000 the country restricted the antibiotic use for treatment to prescription only. Despite the ban, the Danish swine market has seen an increase in their pig production in the period from 1992 to 2008 with 90%

of the pork produced being exported. All remaining AGPs, including classes being used in human medicine, were phased out in the EU by 2006.

Research is now focusing on developing and/or finding alternative products that can act similarly in pigs as antibiotics and their growth promoting effects. Some of the already existent alternatives are enzymes, probiotics, acidifiers and nutraceuticals such as copper and zinc. Peptides with antimicrobial activities, such as bacteriocins, are known to be active against a wide range of pathogens including fungi and some viruses. In weaned pigs beneficial effects are seen in the use of clay minerals added into their diet that contribute to reducing toxicity by binding toxic materials in the gastrointestinal tract. Despite the fact that their use has shown a reduction in diarrhoea in young pigs, it appeared that there was no great improvement regarding their growth performance and thus they were not considered viable alternatives to antibiotic growth promoters (Thacker, 2013). Prebiotics and probiotics have also shown benefits to the animal's health. Prebiotics have the advantage of stimulating the growth of commensal bacteria that are normally present in the gut of that particular animal and therefore are already adapted to that environment, while probiotics and especially *Lactobacillus* species used in feed supplementation illustrated positive results on resistance to *Clostridium* and *Campylobacter* infections in poultry (Yang *et al.,* 2009). Finally, another tested alternative was the egg-yolk antibody referred to as IgY. It has been tested against swine pathogens such as rotavirus*, Salmonella* and *E. coli* and successfully demonstrated a pig performance equal to that of antibiotic use. However, there is always the possibility of failing to perform due to the sensitivity to low pH which makes it a challenge to survive the passage through the gut (Thacker, 2013).

1.4 Metal Resistance

Trace minerals, such as copper and zinc, are considered important for many physiological processes and vital for a number of cellular enzymes. Hence, they are required in small amounts in order to meet nutrient requirements for those processes. For example, zinc acts as a co-factor for numerous enzymes and contributes to animal growth, immune development, gene regulation and more. Similarly, copper enhances processes performed by zinc due to particular enzymes needed for such functions being copper-dependent (Richards *et al.*, 2010). Normally, 5 to 10 ppm (parts-per-million) of copper is said to be enough for covering these dietary needs (Jacela *et al.*, 2010). However in pig production, copper and zinc are supplied in much higher quantities. For instance, copper is supplied at high concentrations, 100 to 250 ppm, as it was found to positively contribute towards the growth rate of pigs in a mechanism that still remains unclear (Jacela *et al.*, 2010). Unlike Europe and Russia, countries like China have no implemented directives in the use of copper as a dietary supplement and hence it can be easily oversupplied (Xiong *et al.*, 2010). One of the main concerns, which has been reported in many studies, is the impact on bacterial resistance towards the different metals and trace minerals used in agriculture and food-producing animals. Considering that China is the world's largest pork producer, supplying more than 40% of pork meat globally, lack of such regulations can lead to uncontainable dissemination of such resistances (Xiong *et al.*, 2010). There are studies that also reported the presence of heavy metals, such as mercury, within liquid pig manure (Hölzel *et al.*, 2012). Mercury was used in agriculture since the early 1900s for the control of plant diseases. Inorganic forms were soon replaced by aryl, alkoxy and alkyl organomercurials. Despite the known toxicity, mercurials

were not banned until the 1970s and even though are no longer used, mercury and other heavy metals can still be found widely in the environment as they are still used in industrial processes, alloys and electrical apparatus (Huisingh, 1974), although the largest current use of mercury is through the use of dental amalgams in humans which consist of 50% Hg(0) and 35% Ag(0) (Hobman & Crossman, 2015). In fact, it has been reported that the slowly released mercury from amalgams selects for mercury-resistant gut bacteria (Gupta *et al.*, 2001). Ag(0) on the other hand has not been studied well as to whether it selects for resistance. Silver ions, nevertheless, are known to be highly toxic to bacteria but, despite the fact, Ag^{+}-resistant bacteria were previously reported (Gupta *et al.*, 2001). Additionally, silver compounds, particularly salts, are widely used as biocides in agriculture and livestock farming but also as clinical antimicrobial agents in antiseptics, disinfectants and antiinfectives in healthcare (Pal *et al.*, 2017).
The majority of metal ion resistance systems are encoded on bacterial plasmids, despite the fact in some microorganisms related systems were found to be determined by chromosomal genes (Silver, 1996). AMR is somehow linked to metal resistance in the sense of co-selection as both systems share similar structural and functional characteristics. Since metals are not subjected to degradation, they could possibly contribute towards antimicrobial resistance and act as a long-term selection pressure. Since both antibiotic and metal resistance genes can be found on transposable elements, many studies have already shown evidence of co-selection of resistance (Baker-Austin *et al.*, 2006).

1.5 Pathogens & Resistance

1.5.1 *Escherichia coli*

The genus *Escherichia* was named after the German bacteriologist Theodor Escherich who first discovered the type species of the genus in the human colon in 1885 (Clark, 2005). *E. coli* are Gram-negative bacteria of the Enterobacteriaceae family and they normally live in the intestines of humans and warm-blooded animals. Despite the fact that *E. coli* bacteria play an important part in the healthy human intestinal tract, there are strains that can be extremely pathogenic and thus cause some serious health illnesses (Rasko *et al.*, 2008). The pathogenic strains are divided into six types, also known as pathotypes: Enteropathogenic *E. coli* (EPEC), Enterotoxigenic (ETEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC) and Shiga-toxin producing *E. coli* (STEC) (Estrada-Garcia *et al.,* 2009), which is also known as verocytotoxin (VTEC) or enterohaemorrhagic (EHEC). According to the Centre for Disease Control and Prevention (CDC, 2015), altogether these are also referred to as diarrhoeagenic *E. coli*. One of the most commonly associated diseases caused by *E. coli*, and particularly extra-intestinal pathogenic *E. coli* (ExPEC), is urinary tract infection (UTI) when the pathogen can be transferred to the bladder via the urethra or via the blood. By circulating in blood, the bacteria can attach to different tissues and cause infection. The most notorious strain in relation to public health is STEC O157:H7 which causes bloody diarrhoea and in some instances, due to circulation of toxins in the body, infections lead to a severe condition called haemolytic-ureamic syndrome (HUS) which subsequently causes kidney failure and even death (Fratamico & Smith, 2017). Ruminant animals such as sheep, goats and deer act as important reservoirs for this pathogen, although the primary one seems to be

cattle. Infections caused by ExPEC have also been reported for domestic mammals, for example dogs and cats, which have been suggested as potential reservoirs for the transmission of the pathogen to humans (Bélanger *et al.*, 2011). The main route of transmission of *E. coli* to humans is via the consumption of contaminated food. Undercooked or raw meat, raw milk, food cross-contaminated during preparation and/or water contaminated with the faeces of an infected animal can all lead to the transmission of the pathogen (WHO, 2016).

E. coli is usually used as the sentinel organism for AMR since it has proved to acquire resistance easily. Additionally, this pathogen is present in a wide range of hosts making it advantageous for the transmission of resistance genes from one host to the other as well as transmitting to closely-related bacteria such as *Salmonella* (Tadesse *et al.*, 2012). A recent study illustrated how fast *E. coli* isolated from their natural environment were able to adapt and domesticate into a new habitat through genetic modification. It was demonstrated that genetic changes in the isolates during handling for short periods with mutants being identified within as little as three days (Liu *et al.*, 2017).

One of most interesting debates over MDR bacteria (bacteria that are resistant to three or more antibiotic classes), is whether there is a linkage between animals and humans. Recently, a number of studies have shown that there is a link and supporting evidence in defence of this statement. *E. coli* strains isolated from patients with UTI, community-dwelling humans (not in assisted living), broiler and imported meat were investigated for AMR and phylogrouping. The study took place in Denmark and cluster analysis of antibiotic resistance data showed that isolates from patients with UTI were grouped with those isolated from meat and animals.

Similarities were also found in the AMR phenotypes with MDR being more frequent from imported meat sample isolates; a result that reflects differences in the veterinary antimicrobial practise among different countries (Jakobsen *et al.*, 2010). One of the main mechanisms by which bacteria develop drug resistances has been shown to be the possession of efflux pumps. Although mostly chromosomally-encoded they can also be plasmid-encoded and hence resistance genes can be transferred between DNA molecules (Li & Nikaido, 2009). *E. coli* genome analysis has revealed a minimum of thirty-seven single and/or MDR efflux transporters. These transporters are divided into five different families with the resistance-nodulation-cell division (RND) family accommodating one of the predominant efflux pump systems for commonly used antimicrobial substances (Li & Nikaido, 2004). Therefore, pumps belonging to this family play a vital role in clinically related resistance, especially in Gram-negative pathogens such as *E. coli*. The main difference between Gram-negative and Gram-positive efflux pumps is that the majority of efflux pumps in Gram-negative bacteria are RND transporters which function as proton/drug antiporters. The efflux system also comprises membrane fusion proteins and an outer membrane channel protein. On the other hand, in Gram-positive efflux pumps are non-RND but often characterised by singleton protein pumps (Li & Nikaido, 2009). Figure 1.2 illustrates a typical drug efflux mechanism for both Gram-negative and Gram-positive bacteria.

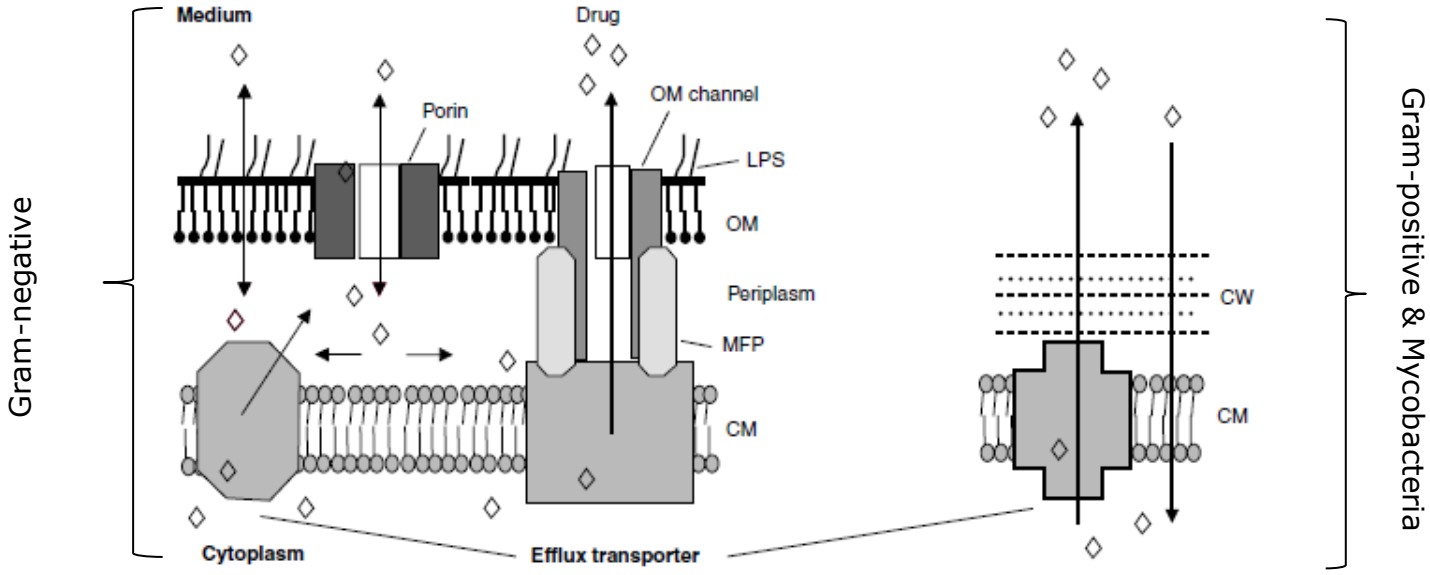


Figure 1.2: Scheme of a typical drug efflux pump where in a) Gram-negative drugs are travelling across the outer membrane (OM) via porin channels or lipopolysaccharides (LPS)/phospholipid bilayer and once into the cytoplasm are expelled in the periplasm by transporters (illustrated as an octagon). They are then ejected into the medium by a tripartite transport complex. b) In Gram-positives, drugs are pumped directly into the medium by simple transporters since cell wall (CM) does not offer resistance to diffusion of small molecules. CM=cytoplasmic membrane, MFP=membrane fusion proteins (Modified from Li & Nikaido, 2004).

1.5.1.1 β-Lactamases & ESBL

β-lactamase enzymes are primarily responsible for resistance to the β-lactam antibiotics. The AmpC β-lactamase was the first ever enzyme reported to destroy penicillin and was found in *E. coli* (Jacoby, 2009). Although the genes conferring β-lactam resistance seem to have originated from Gram-negative bacilli (Chapman, 2015), they quickly spread via plasmids into other pathogens resulting in the dissemination of resistance such as the destruction of benzylpenicillin activity against *Staphylococcus* strains (Livermore, 1995). Their mode of action is the catalytic inactivation of the amide bond of the β-lactam ring resulting in the inactivation of the antibiotic (Livermore, 1995). The classification of these enzymes

was firstly proposed and then expanded in the 1970s. However, it was later revised and re-proposed based on substrate preference, susceptibility to inhibitors and whether the β-lactamases are chromosomal or plasmid-mediated (Livermore, 1995). Classification of these enzymes falls within four molecular classes (Classes A, B, C and D) that are based on protein sequence. Classes A, C and D act, via a serine ester mechanism, while enzymes of class B are known to hydrolyse the β-lactam ring by utilising zinc ions and hence are described as metallo- β-lactamases (Bush & Jacoby, 2010). Class C enzymes are cephalosporinases, which encoded on the chromosome of many Enterobacteriaceae, while class D are known as oxacillinases and found in plasmids and integrons and can hydrolyse cloxacillin, oxacillin and in some instances carbapenems (Poirel *et al.,* 2010). Table 1.3 briefly summarises the main classes and subgroups of β-lactamases with some of the major enzyme families involved.

Chromosomal AmpC β-lactamases are expressed in the majority of Enterobacteriaceae except for *Salmonella.* Nevertheless, differentiating AmpC β-lactamases from penicillin binding proteins (PBPs) is quite difficult by sequencing alone due to PBPs which retain identical structure and conserved nucleotide sequences near the serine active site. For instance, *E. coli* is capable of producing a PBP, known as AmpH, which is structurally associated with AmpC but lacks the β-lactamase activity (Jacoby, 2009). In addition, AmpC in *E. coli* is regulated by a promoter and attenuation mechanisms because it lacks the *ampR* regulator gene. Therefore, expression of AmpC is non-inducible. Due to possession of low levels of non-inducible enzymes, *E. coli* is intrinsically sensitive to ampicillin and narrow-spectrum cephalosporins (Livermore, 1995). Nevertheless, resistance due to

mutations in the PBP gene resulting in the upregulation of chromosomal cephalosporinase AmpC in *E. coli* has been reported (Moya *et al.*, 2009). Resistance was also reported in other bacteria that do not possess an inducible AmpC enzyme and was soon found to be due to transferable AmpC genes most widely known as plasmid-mediated AmpC β-lactamases. These are derived from chromosomal AmpC genes from several Enterobacteriaceae such as *Enterobacter cloacae* and *Citrobacter freundii* (Pérez-Pérez & Hanson, 2002). Plasmid-mediated AmpC families were named after the resistance they produce and/or site of discovery such as CMY for cephamycins resistance; FOX for cefoxitin and DHA for Dhahran hospital in Saudi Arabia. Other plasmid-mediated AmpC families include OXA, TEM, SHV and CTX.

Table 1.3: Classification scheme of β-lactamases with some of the major plasmid-mediated families (Modified from Bush & Jacoby, 2010).

Groups	Class	Subgroups	Enzyme families	Function/Characteristics
Group 1: cephalosporinases	C	1	CMY, ACT, FOX, DHA, MIR	Better action against cephalosporins and active on cephamycins (e.g cefoxitin)
		1e	CMY, GC1	Greater activity against ceftazidime and other oxyimino-β-lactams
Group 2: serine β-lactamases	A	2b	TEM, SHV	Readily hydrolyse penicillins and cephalosporins
		2be	TEM, SHV, CTX-M, PER, VEB	Extended-spectrum cephalosporins, monobactams. Hydrolyse one or more oxyimino-β-lactams
		2br	TEM, SHV	Broad-spectrum β-lactamases, resistant to clavulanic acid, sulbactam, tazobactam
		2ber	TEM	Extended-spectrum cephalosporins, monobactams. Relatively resistant to clavulanic acid inhibition, sulbactam, tazobactam
	D	2d	OXA	Hydrolyse cloxacillin or oxacillin
		2de	OXA	Extended-spectrum. Hydrolyses cloxacillin, oxacillin and oxyimino-β-lactams (exc. carbapenems)
		2df	OXA	As 2de but with carbapenem hydrolysing activity
	A	2f	GES, KPC, SME	Greater hydrolysis of carbapenems, oxyimino-β-lactams and cephamycins
Group 3:Metallo-β-lactamases	B	3a	IMP, VIM, IND	Broad-spectrum carbapenem hydrolysis, inhibited by metal ion chelators

The genesis of these plasmid-mediated β-lactamases eventually led to the term 'extended-spectrum β-lactamases' also referred to with the abbreviation ESBL. It was demonstrated in 1976 that mutations could improve the catalytic action of TEM-1 which resulted in better hydrolysis of cephalosporin C. It was then noted that mutations could enhance the β-lactamase spectrum of activity. ESBLs have arisen

by mutations in two types of enzymes: TEM and SHV-type. TEM-1 is also the most common plasmid-mediated enzyme in ampicillin-resistant Gram-negatives (Philippon *et al.,* 1989). In all extended-spectrum β-lactamases the active serine site remains at position 68 for the TEM-type and position 66 for SHV-type enzymes. According to British Society for Antimicrobial Chemotherapy, testing for resistance against certain third-generation cephalosporins can indicate the type of ESBLs. Ceftazidime is best for indicating TEM and SHV-derived ESBLs while cefotaxime indicates CTX-M types. However, Harwalkar *et al.* (2013) showed that among the third-generation cephalosporins used in phenotypic screening tests for detection of ESBLs, cefpodoxime is generally considered a good indicator for all types with better resistance results (increased inhibition zone).

ESBLs are considered a major health threat. ESBL-producing *E. coli* have been isolated from poultry, pig and even rabbit farms. Therefore, commensal *E. coli* strains of food-producing animals are said to act as a reservoir (Blanc *et al.*, 2006). Additionally, ESBL-producing *E. coli* has also been reported in imported raw chicken (Warren *et al.*, 2008), illustrating the potential of ESBL getting transferred to humans via food.

1.5.2 *Salmonella*

Salmonella is one of the major foodborne pathogens with around 3 million deaths worldwide every year. It is another Gram-negative rod shape bacterium, and predominantly motile (Pui *et al.*, 2011). It belongs to the Enterobactericeae family and is one of the four key causes of diarrhoeal/enteric diseases worldwide. Nomenclature of this bacterium is so complex due to the fact it is divided into species, subspecies, subgenera, groups, subgroups and finally to the different

serotypes/serovars (Brenner *et al.*, 2000). According to Public Health England (2015) there are two validly published species; *Salmonella bongori* and *Salmonella enterica* which consists of six subspecies; *Salmonella enterica* subspecies *arizonae*, *Salmonella enterica* subspecies *diarizonae*, *Salmonella enterica* subspecies *enterica*, *Salmonella enterica* subspecies *houtenae*, *Salmonella enterica* subspecies *indica* and *Salmonella enterica* subspecies *salamae. Salmonella* species are categorised into serotypes in a concept that was first proposed by Kauffmann (the Kauffman-White scheme) and is maintained by the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France. These serotypes are categorised according to the O-somatic antigen and then subdivided according to the H-flagellar antigens and there are currently more than 2,500 known *Salmonella* serovars around the globe (Agbaje *et al.*, 2011). The majority of them fall within *Salmonella enterica* subspecies *enterica,* with the groups A, B, C1, C2, D and E being the most regular O-antigen serogroups that account for nearly 99% of infections in warm-blooded animals including humans (Brenner *et al.,* 2000). Despite being prevalent in warm-blooded animals, *Salmonella* can also be found in cold-blooded animals indicating a wide range of host adaptability. Foodborne salmonellosis, sometimes referred to as non-typhoidal salmonellosis, is a result of the ingestion of the cells of more than 150 serotypes with *Salmonella* Typhimurium and *Salmonella* Enteritidis being the most prevalent (Pui *et al.*, 2011). Contaminated food and/or water are the major mode of transmission to acquire foodborne salmonellosis. Having an incubation time from 12 to 72 hours, gastroenteritis is associated with diarrhoea, abdominal cramps and fever (Dhama *et al.,* 2013) but is normally self-limited to approximately a week. In

systemic disease, caused by the serovar Typhi bacteria which are limited to humans, the pathogen survives the low pH of the stomach and gains access into the host's circulation through the invasion of phagocytic epithelial cells on Peyer's patches which helps them move across the intestinal epithelium. Surviving the phagocytic killing mechanism has been found to play a vital role in virulence (Coburn *et al.,* 2007). *Salmonella* virulence arises from a combination of genes that are not only found on plasmids but also are encoded within the chromosome in the form of large cassettes that are also known as pathogenicity islands. These are responsible for particular interactions of the pathogen with the host. The most well characterised ones are SPI1 and SPI2 of which both possess type III secretion systems responsible for delivering virulence factors into the host (Marcus *et al.,* 2000). SPI1 can be horizontally acquired and aids the bacterial penetration in the intestinal epithelium while studies illustrated that SPI2 is more important for the bacterial growth in host tissues as its genes have been shown to be rapidly induced straight after penetration into the host cells and to being repeatedly expressed throughout the infection (Marcus *et al.,* 2000).

Pigs are generally considered carriers of the pathogen and therefore they can act as a reservoir that poses a threat to both animal and human health. Although vertical transmission (from mother to baby) in pigs has not been validly established yet, other routes of transmission such as pig-to-pig contact, the faecal-oral route, rodents, insects and infected humans have been reported (Gilbert *et al.*, 2010). In addition, *Salmonella* are capable of surviving long periods in dried meals hence contaminated feed can also act as a route of transmission for the pathogen (Gilbert *et al.*, 2010). In the United States from the period of 2000-2008, non-typhoidal

salmonellosis was responsible for an estimated of 1,000,000 illnesses and 380 deaths per year (CDC, 2012). Increasing AMR in *Salmonella* serotypes has already been reported since the 1990s. However, some serotypes are prone to acquire more resistances than others; e.g *Salmonella* Typhimurium shows greater resistance in comparison to *S.* Enteritidis (Boyen *et al.*, 2008). *Salmonella* are the only Enterobacteriaceae that do not possess chromosomal β-lactamases (Livermore, 1995); hence resistances are primarily acquired through conjugative transfer of plasmids (Chen *et al.*, 2004). Serotypes that are swine adapted, but can also cause disease in other species, are *Salmonella* Typhisuis and Choleraesuis. The latter is known to be able to generate hybrid plasmids that carry both virulence and resistance genes and therefore it serves as a threat for both animals and humans (Boyen *et al.,* 2008). There are numerous studies on AMR found in *Salmonella* isolates from retail meat. A recent study investigated the resistance in foodborne outbreaks caused by the pathogen in the United States from 2003-2012 (Brown *et al.*, 2017). Findings showed that MDR outbreaks involved a minimum of one isolate conferring resistance to three or more antibiotic classes. From all the resistant outbreaks reported from 2003 to 2012, 78% were MDR and mostly associated with foods derived from land animals in contrast to those derived from plants or marine animals. In addition, it was also noticed that hospitalisation and bloodstream infections were significantly higher in patients infected with resistant *Salmonella* than those with strains susceptible to all antimicrobial drug classes (Brown *et al.,* 2017). Particularly important is *Salmonella* Typhimurium phage type DT104 associated with pigs and pork meat as it was found to be MDR with integrons incorporated into its chromosome making it a stable clone able to disseminate its

resistances even when antimicrobial selection is absent (Chen *et al.,* 2004). Antibiotics used to treat human infections caused by the pathogen include fluoroquinolones and extended-spectrum cephalosporins. Often enough, isolates recovered from raw retail pork were found to be resistant to these antibiotics (Chen *et al.*, 2004) with ceftriaxone (a third-generation cephalosporin) resistance being the most important as it is crucial for treating salmonellosis (Brown *et al.*, 2017). This resistance is attributed to presence of the plasmid-mediated AmpC β-lactamase bla_{CMY-2} gene and on cross-resistance with another third-generation cephalosporin, ceftiofur, which is used in food producing animals (Chen *et al.,* 2004).

1.5.3 *Staphylococcus aureus*

Staphylococcus aureus is a Gram positive, non-motile coccus that belongs to the family of Staphylococcaceae. In the genus *Staphylococcus* there are 47 validly recognised species with *S. aureus, S. epidermidis* and *S. saprophyticus* being the most commonly associated species with regards to human infections (Public Health England, 2014). Generally, Gram positive cocci are transmitted via food of animal origin with milk and milk products being the most commonly known sources for *S. aureus.* The production of enterotoxins in the food is the main mechanism of foodborne illness caused by the pathogen that is usually characterised by nausea and vomiting (Dhama *et al.,* 2013). This pathogen is part of the normal skin flora and mucus membranes of birds and mammals and therefore it can be easily spread throughout the environment where it can survive for long periods. Its main routes of transmission into food are via an infected human while handling or processing food and in dairy animals due to mastitis disease (Hennekinne *et al.,* 2012).

Staphylococcal food poisoning is quite common and is caused by the ingestion of an adequate amount (suggested around 10-20 μg for an adult) of one or more preformed enterotoxins. Enterotoxins produced by *S. aureus* are strong gastrointestinal exotoxins that can endure extreme conditions such as heat treatment, freezing, drying and low pH, making them capable of surviving the digestive tract and proteolytic enzymes without losing their activity. Moreover, these enterotoxins are neurotoxins acting by stimulating receptors on the abdominal viscera which subsequently transmit signals via the vagus nerve to the vomiting centre located in the brain (Argudín *et al.,* 2010).This organism is of great importance to hospitalised patients as it is one of the most common pathogens causing infections in hospitals. Apart from foodborne gastroenteritis: toxic shock syndrome, pneumonia, septic shock, acute endocarditis and surgical wound infection are some of the infections the pathogen is known to prominently cause (Archer, 1998). In animals, cows in particular, *S. aureus* causes mastitis which is a potentially serious infection. This infection causes major losses in the farm and dairy industry as the animal can be asymptomatically harbouring the organism on the surface of the udder and/or in the teat canal and thus contaminating the milk (Swift, 2010). Where visible contamination is observed, the milk is discarded.
In terms of AMR, the most widely known resistant phenotype is MRSA. This phenotype has evolved when MSSA acquired the *mecA* gene responsible for encoding methicillin resistance. This resistance was due to a mutant gene product (Penicillin-binding protein 2a) which has low affinity for penicillin and subsequently to other β-lactam drugs except from ceftaroline (Pottinger, 2013). It has been reported that more than 50% of MRSA strains are also resistant to macrolides,

lincosamides, fluoroquinolones and aminoglycosides. Additionally, resistance has also been reported for vancomycin which was one of the effective antimicrobials against MRSA as it was observed that *S. aureus* can successfully gain the *vanA* gene from enterococci (Sieradzki *et al.,* 1999).

There were two main MRSA types: healthcare-associated (HA-MRSA) which involved mostly immunocompromised people and individuals that have spent time in hospitals, and community-associated (CA-MRSA) which infects healthy people in the wider community. However, in the past decade or so livestock-associated (LA-MRSA) has become an important health concern as it is mainly found in food production animals and was found to act as a reservoir for infections in humans (Frana *et al.*, 2013). Numerous studies tried to demonstrate the transmission from animals to humans with surprisingly positive results. LA-MRSA first appeared as a concern when it was reported in 2005 in France and the Netherlands where a particular multilocus sequence type (398) was detected in high counts in swine workers and hence it was initially referred to as swine-associated. In Germany, the prevalence of MRSA in pig farms was studied and it was observed that MRSA was significantly more prevalent in fattening than breeding farms. Additionally, environmental samples from the air in the farms showed that the number of positive pigs was correlated with that of the positive air samples within the barn, meaning that the more positive MRSA pigs were present the higher the amounts of MRSA in the air and vice versa (Friese *et al.*, 2012). Similarly, the study conducted by Frana *et al.* (2013) demonstrated the association between people and positive-MRSA pigs by examining veterinary students following their visit to the farms. It was observed that 22% of the students tested positive after exposure to a MRSA-

positive pig farm for at least a day. However, carriage quickly decreased when exposure was removed. This finding shows the comparison between long-term exposure (eg swine-workers) and short-term in terms of acquisition and endurance of the carriage.

In recent years, the clonal complex 398 (CC398) has also been reported in animals within the UK. Profiling studies using whole genome sequencing illustrated that European isolates showed separate lineage to non-European ones. Furthermore, non-EU lineages showed greater diversity of AMR genes in comparison with EU and UK isolates with genes encoding resistance to at least three drug classes including tetracyclines, β-lactams and macrolides (Sharma *et al.*, 2016). Infection analysis of this clonal complex revealed high cytotoxin potential compared to CA-MRSA. LA-MRSA CC398 isolates showed a strong upregulation of the RNAIII transcription which is the vital effector molecule for the *agr* quorum sensing system that influences the production of *S. aureus* exotoxin (Ballhausen *et al.*, 2014). Therefore, this strain is posing a great threat to human health.

1.5.4 *Yersinia enterocolitica*

Yersiniae are Gram negative, facultative anaerobes and coccobacilli that belong to the Enterobacteriaceae family. They are motile at temperatures below 30°C with the exception of *Yersinia pestis.* This genus consists of 17 validly recognised species but only three are known to be pathogenic and associated with human and animal illnesses (Public Health England, 2015c); *Yersinia pestis* which is a zoonotic pathogen that causes the life-threatening systemic disease known as plague and *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* responsible for yersiniosis, a gastrointestinal illness (Francis, 2013). The isolation and identification process of

Yersinia enterocolitica strains is very challenging and time-consuming when carried out via different phenotypic tests which provide results that are not always reliable (Fredriksson-Ahomaa & Korkeala, 2003). *Y. enterocolitica* is considered an important foodborne pathogen as, unlike other members of the Enterobacteriaceae family, *Y. enterocolitica* strains are psychrotrophic; i.e. capable of growing at 4^{o}C, making the pathogen able to survive in refrigerated food products (Wannet *et al.*, 2001). Yersiniosis caused by this bacterium is mainly characterised by mild fever, diarrhoea, abdominal cramps and sometimes skin rashes (Dhama *et al.,* 2013). The infection is usually self-limiting. In humans, *Y. enterocolitica* is generally transmitted via ingestion of contaminated food or water but mainly through consumption of contaminated pork meat. Animals and pigs in particular harbour the pathogen and they are usually considered asymptomatic carriers (Thoerner *et al.*, 2003). In fact, strains isolated from both humans and pigs proved to have indistinguishable genotypes when restriction endonuclease analysis of their chromosomal or plasmid DNA was performed (Fredriksson-Ahomaa *et al.*, 2006). Most of the time infections are sporadic and foodborne outbreaks are normally rare from this pathogen. Nonetheless, there were small outbreaks reported between 2006 to 2011 that were mainly due to consumption of pork products, salads and milk (Fredriksson-Ahomaa, 2017). In the United States, an estimated 98,000 illnesses caused by *Y. enterocolitica* were reported annually from 2000 to 2008 with 29 of them resulting in death every year; a record significantly higher than that of diarrhoeagenic *E. coli* (non-ETEC and STEC) with only 12,000 reported incidents annually for that period (CDC, 2012).

Classification of *Y. enterocolitica* strains is based on biochemical and genetic

features which divide them into six different biotypes/biovars; biovar 1A which consists of non-pathogenic strains, biovars 2-5 with low-virulence strains and biovar 1B with highly virulent strains (Garzetti *et al.*, 2014). Biotypes, however, are further subdivided into different serotypes that are mainly determined from the expression of the O-antigen on the cell surface lipopolysaccharides (Public Health England, 2015c). According to Wannet *et al.* (2001) there are over 60 serotypes but only 11 are usually associated with human infections. The most common strains associated with cases of human yersiniosis in the UK were those of biotype 3 serotypes O:9 and O:5,27, biotype 2 serotype O:9 and biotype 4 serotype O:3 (McNally *et al.*, 2004). Nevertheless, different geographical areas have different predominant serotypes. For instance, bioserotype 2/O:9 is more commonly isolated from pigs in Europe, 2/O:5,27 from pigs in the United States, 3/O:3 mainly from pigs in Asia while 4/O:3 is frequently isolated from pigs worldwide (Fredriksson-Ahomaa, 2017). Apart from being a faecal commensal bacterium, the pathogen is commonly isolated from the oral cavities of the pigs with tonsils being the most predominant source (Nesbakken *et al.*, 1991). Therefore, in the event of slaughter it can be spread and contaminate the surface of the carcass which is one of the reasons *Y. enterocolitica* is encountered as a surface contaminant and mostly found on fresh retail pork products.

Pathogenesis in *Yersinia* is dependent on the presence of the 70-kb pYV virulence plasmid along with chromosomally encoded proteins (Mikula *et al.*, 2013). This plasmid is highly variable among different species and strains within them, having variable genetic architecture. It encodes the Ysc type III secretion system (T3SS) responsible for injecting *Yersinia* outer proteins (Yops proteins) into the host via

direct contact which results in the apoptotic death of the macrophages. The plasmid with the genetic locus encoding the Ysc T3SS is carried by all pathogenic *Yersinia* species including *Y. enterocolitica* (McNally *et al.*, 2016). However, depending on the culture conditions, the plasmid can be easily lost and thus the pathogenicity of a strain should not rely solely on the presence of the plasmid but in fact on the possession of other virulence genes. Very important is the possession of a chromosomally encoded locus responsible for attachment and invasion processes. Responsible for this is the *ail* gene which is only found in pathogenic strains. In addition, *ystA* is another chromosomally encoded gene that is needed for the production of a heat-stable enterotoxin (YST) (Thoerner *et al.,* 2003). This heat-stable enterotoxin is produced at temperatures below 30°C (Singh & Virdi, 2004). Invasin on the other hand is a protein present on the outer membrane that is expressed during stationary phase and plays a vital role in the first stages of infection as it triggers internalisation of the bacteria. Similarly, plasmid-borne virulence genes can also contribute significantly in pathogenesis. *yadA* is a plasmid-borne adhesion that protects against phagocytosis and also facilitates adhesion to collagen, important in reactive arthritis infections (Mikula *et al.,* 2013). All virulence factors that are encoded on the plasmid are only expressed at mammalian body temperatures (37°C) while chromosomally encoded *ail* and *inv* genes are expressed at temperatures below that but never at body temperature (McNally *et al.,* 2016). However, the exception is serotype O:3 where *inv* is highly activated even at body temperature (Schaake *et al.*, 2014), hence it is thought this is why these are human pathogens.

Studies have also illustrated that strains isolated from slaughtered pigs and pork products carry the pathogenic genes, which proposes the link between porcine isolates and human infection. An example of that is the study performed by Gürtler *et al.* (2005) in Germany when samples obtained from fattening pigs throughout the rearing period and after the slaughter process showed that all isolates obtained harboured the *ail* gene. Interestingly enough, all isolates belonged to the bioserotype 4/O:3. In fact this bioserotype was reported as the most common cause of sporadic human yersiniosis in Germany and Finland (Fredriksson-Ahomaa *et al.,* 2006). In Northern Italy, pig tonsil samples were also tested for the prevalence of 4/O:3 and it was observed to be the most frequently isolated bioserotype. Furthermore, all 4/O:3 isolates were found to be resistant to the antibiotics ampicillin and cephalothin while high resistance levels were also observed for sulphonamides, streptomycin and chloramphenicol. Among all the isolates around 91% were shown to be MDR (Bonardi *et al.*, 2014). *Yersinia enterocolitica* can possesses chromosomal β-lactamases which confer resistance to ampicillin, cephalothin and carbenicillin. In addition, infections are usually treated with antibiotics such as trimethoprim-sulfamethoxazole, aminoglycosides, third-generation cephalosporins and fluoroquinolones; with resistance being mostly developed as a result of a gene mutation and/or efflux mechanisms (Dekker & Frank, 2015). Antibiotic susceptibility testing was also compared among the different biovars. The majority of them illustrated minor differences between them with the non-pathogenic biovar 1A also possessing chromosomally encoded β-lactamases demonstrating the diverse and complex expression of these enzymes among different biovars (Stock & Wiedemann, 1999). Such findings led to the

suggestion and possible hypothesis that minority strains can act as reservoirs of resistance genes that can be easily transmitted from one organism to the other. It is usually assumed that where possession of virulence factors is absent, disease cannot be caused. Non-pathogenic species are ignored most of the time and research is primarily focused on pathogens. However, findings do not always support the above statement. In regards to *Yersinia,* species other than the three pathogenic ones are being neglected. The remaining eight species comprising the *Yersinia* genus are referred to as *enterocolitica*-like. Several studies have managed to illustrate that despite the absence of the virulent pYV plasmid, the strains were still able to cause disease in model animals, i.e. mice, and also showed successful invasion of epithelial cells even if the chromosomally encoded *ail* gene was absent too (Sulakvelidze, 2000). It is beyond any doubt that there is a need for more research in order to fully understand virulence mechanisms and possible reservoirs.

1.6 The Project

From this literature review, it can be seen that AMR bacteria are frequently reported in food-producing animals and meat products, posing concerns over human health. This research project aims to study the antimicrobial resistance of foodborne pathogens isolated from retail pork meat and aims to contribute to the already existing data on the prevalence of MDR bacteria from retail meat products. The project is primarily focused on the isolation and identification of *E. coli*, *Yersinia enterocolitica*, *Salmonella* and *Staphylococcus aureus.* Antimicrobial resistance profiles, metal resistance and pathogenicity of any isolated pathogen will be studied. The project aimed at illustrating the effect of antibiotic use in food-

producing animals and the likelihood of antibiotic-resistant bacteria being transferred from animals to humans through the food-chain.

2 Materials & Methods

2.1 Media & Solutions

All media used were prepared according to manufacturer's instructions, dispensed into glass containers, and autoclaved at 121°C for 15 minutes unless otherwise stated. The media were then cooled to 55°C in a water bath before approximately 25 ml were poured in each petri dish.

2.1.1 Maximum Recovery Diluent (MRD)

4.75 g of MRD powder (Oxoid, CM0733) was dissolved in 500 ml of Reverse Osmosis (RO) water. The medium was then autoclaved and stored at ambient temperature.

2.1.2 Buffered Peptone Water (BPW)

10 g of BPW powder (Oxoid, CM0509) was dissolved in 500 ml of Millipore water (ultrapure water), autoclaved and stored at ambient temperature.

2.1.3 Xylose Lysine Deoxycholate Agar (XLD)

26.5 g of XLD powder (Merck, 105287) was dissolved in 500 ml of RO water. The medium was then heated with frequent agitation until boiling. It was then cooled and poured into petri dishes. The prepared plates were then stored at 2-8°C.

2.1.4 Brilliant Green Agar (BGA)

25 g of BGA powder (Oxoid, CM0263) was dissolved in 500 ml of RO water. The medium was then autoclaved and prepared plates were stored at 2-8°C.

2.1.5 Nutrient Agar

14 g of Nutrient agar powder (Oxoid, CM0003) was dissolved in 500 ml of RO water. The medium was then autoclaved and prepared plates were stored at 2-8°C.

2.1.6 MacConkey Agar

26 g of MacConkey powder (Oxoid, CM0007) was dissolved in 500 ml of RO water. The medium was then autoclaved and prepared plates were stored at 2-8°C.

2.1.7 Minerals Modified Agar (MMA)

Double strength: 11.35 g of Minerals Modified Medium base powder (Oxoid, CM0607), 6.35 g of sodium glutamate (Oxoid, LP0124), 2.5 g ammonium chloride (Fisher Chemicals) and 7.5 g of Bacteriological Agar (Oxoid, LP0011) were dissolved in 500 ml of RO water. The medium was then autoclaved at 115°C for 10 minutes. The prepared plates were stored at 2-8°C.

2.1.8 Peptone Sorbitol Bile (PSB) Broth

31 g of PSB broth powder (Sigma-Aldrich, 17192) was dissolved in 1 L of RO water. The medium was then dispensed into 100 ml Duran bottles and autoclaved. The prepared broth was stored at 2-8°C.

2.1.9 Rappaport-Vassiliadis (RVS) Broth

6 g of RV broth powder (Oxoid, CM0669) was dissolved in 200 ml of RO water. 10 ml aliquots were then dispensed into Universal bottles and autoclaved at 115°C for 10 min. The prepared broths were stored at 2-8°C.

2.1.10 Baird Parker Agar (BP)

31.5 g of BP powder (Oxoid, CM0275) was dissolved in 500 ml of RO water. The medium was then autoclaved. Once cooled, 25 ml of egg yolk tellurite emulsion (Oxoid, SR0054) were added to the medium. The prepared plates were stored at 2-8°C for no longer than a week.

2.1.11 Irgasan-Ticarcillin Chlorate Broth (ITC)

76 g of ITC broth powder (Sigma-Aldrich, 17156) was dissolved in 970 ml of RO water. The medium was then autoclaved. One vial of Ticarcillin supplement (Sigma-Aldrich, 17778) was added to the medium along with 30 ml of sterile potassium chlorate (ThermoFisher Scientific, UK) solution (see Section 7.2). The prepared broth was stored at 2-8°C.

2.1.12 SSDC Agar (Salmonella Shigella Agar with Deoxycholate and Calcium Chloride)

The medium was prepared using 30 g of Salmonella-Shigella (SS) agar (Merck, 107667) supplemented with 2.5 g of yeast extract (Oxoid, LP0021), 5 g of sodium deoxycholate (Sigma-Aldrich, D6750) and 0.5 g of calcium chloride (ThermoFisher Scientific, UK) in 500 ml of purified water. The medium was then heated until boiling. The prepared plates were stored at 2-8°C.

2.1.13 Tryptone Bile X-Glucuronide Medium (TBX)

18.3 g of TBX powder (Oxoid, CM0945) was dissolved in 500 ml of RO water and autoclaved. The prepared plates were stored at 2-8°C.

2.1.14 Yersinia Selective Agar (CIN)

29 g of CIN agar powder (CM0653) was dissolved in 500 ml of RO water and autoclaved. Once cooled, one vial of Yersinia Selective supplement (Oxoid, SR0109 –see Section 7.2) was added to the medium. The prepared plates were stored at 2-8°C for up to 24 hours.

2.1.15 Muller-Kauffman Tetrathionate-Novobiocin (MKTTn) Broth

44.75 g of MKTTn broth powder (Oxoid, CM1048) was dissolved in 500 ml of RO water and heated until boiling. The medium was then left to cool. Once cooled, 10 ml of iodine – iodide solution was prepared (see Section 7.2) and added to the

broth along with two vials of novobiocin supplement (Oxoid, SR0181 – see Section 7.2).

2.1.16 Tryptone Broth

3.75 g of Tryptone powder (Oxoid, LP0042) was dissolved in 250 ml of RO water and autoclaved. The prepared broth was stored at ambient temperature.

2.1.17 Brain Heart Infusion (BHI) Broth

9.25 g of BHI broth powder (Oxoid, CM1135) was dissolved in 250 ml of RO water and autoclaved. The prepared medium was stored at ambient temperature.

2.1.18 Blood Agar (with 5% Defibrinated Horse blood)

40 g of Blood agar Base No.2 powder (Oxoid, CM0271) was dissolved in 1 L of RO water and autoclaved. Once cooled, 5% of sterile defibrinated horse blood (Oxoid, SR0051) was added to the medium. The prepared plates were stored at 2-8^{o}C.

2.1.19 Urea Agar (Christensen's agar)

12 g of Urea agar base (Oxoid, CM0053) powder was dissolved in 475 ml of RO water and autoclaved. Once cooled, 25 ml of 40% urea (Sigma-Aldrich, U0631) solution was filter-sterilised and added to the medium. The prepared plates were stored at 2-8^{o}C.

2.1.20 0.5% Potassium Hydroxide (KOH)

0.58 g of KOH (ThermoFisher Scientific, UK, P/5560/60) flakes were dissolved in 100 ml of sterile RO water. The prepared solution (oxidizing agent) was kept in a plastic bottle at ambient temperature.

2.1.21 CHROMagar Orientation Agar

8.25 g of CHROMagar powder (CHROMagar, France. Product code ES372) was dissolved in 250 ml of purified water. To enhance growth, 0.125 g of Tween 80 (Sigma-Aldrich, P1754) was added to the medium (following manufacturer's

instructions). The medium was then autoclaved and prepared plates were stored at 2-8°C for up to a week. This chromogenic medium differentiates urinary tract pathogens according to their colony appearance and was particularly used as an additional step in confirming presumptive *E. coli* and *Staph. aureus*. *E. coli* appears as dark pink to reddish, *Staph. aureus* as small golden opaque colonies and *Enterococcus* as turquoise blue.

2.1.22 Mueller-Hinton Broth & Mueller-Hinton Agar

11.5 g of MH broth powder (Sigma-Aldrich, 70192) was dissolved in 500 ml of RO water and autoclaved. The prepared medium was stored at ambient temperature. For MH agar, 7 g of Bacteriological Agar (Oxoid, LP0011) was added to the MH broth (per 500 ml solution) before autoclaving. The prepared plates were used within 24 h.

2.1.23 Luria-Bertani (LB) Broth & Agar

10 g of LB broth powder (L3022, Sigma-Aldrich) were dissolved in 500 ml of Milli-Q water and autoclaved. For the agar 7.5 g of Bacteriological Agar (Oxoid, LP0011) was added to the above composition before autoclaving.

2.1.24 TE Buffer (pH 8)

0.0372 g of disodium EDTA (ethylenediamine tetra-acetic acid) and 0.12 g of Tris Base were dissolved into 80 ml of RO water in order to prepare 10 mM Tris-HCl and 1 mM EDTA buffer. The pH of the solution was then adjusted to pH 8 using concentrated stock of HCl by slowly adding the HCl into the solution while measuring with a pH meter. The solution was then made up to 100 ml of final volume and autoclaved at 121°C for 15 min. The prepared solution was stored at room temperature.

2.1.25 10% SDS Lysis Buffer

10 g of electrophoresis-grade sodium dodecyl sulfate (SDS) was dissolved into 80 ml of RO water. The solution was then diluted to a final volume of 100 ml and stored at room temperature.

2.1.26 Agarose Gels

To make 1% agarose gel, 1 g of agarose powder (A9539, Sigma-Aldrich) was dissolved in 100 ml of TAE buffer (prepared by dissolving 242 g of Tris base in water, adding 57.1 ml of glacial acetic acid and 100 ml of 500 mM EDTA pH 8) by microwaving for about 3 minutes at 600 watts. Once cooled, 2 µl of ethidium bromide (10 mg/ml stock) were carefully added and the gel was poured into a gel tray and left to set.

2.2 Pork Meat Samples

Samples were purchased from four different locations at different time periods and therefore experiments were divided according to each time. The first three samples (for Sampling 1) were purchased from the Victoria Meat Market at the Victoria Centre in Nottingham (Source A). The samples purchased were pork mince, pork feet and pork belly with skin. For Sampling 2, two samples were purchased from a high street retail chain (Source B) in Victoria Centre, Nottingham city. The samples purchased were pork mince and pork belly with skin. For Sampling 3 one sample of free-range pork loin steaks was purchased from another high street retail chain (Source C) at Nottingham city centre. For Sampling 4 one sample of organic pork belly with skin was purchased from the farmer's market at Sutton Bonington campus at The University of Nottingham (Source D).

All samples were purchased fresh and kept at refrigerated temperatures in their original packing. When analysis was not achievable on the same day as purchase,

samples were kept up to 24 h in refrigerated conditions. All samples were tested within 2 h after being taken out of refrigerated conditions in order to minimise the time of temperature abuse.

2.2.1 Pork Carcass Sampling Using Sponge Swabs

Two pork carcasses from a small local non-commercial abattoir used for teaching were also tested by taking samples using sponge swabs. Feet were swabbed for both pig carcasses and the belly was only swabbed from pig carcass 1. All swabs were transported to the lab for testing immediately after they were taken.

2.3 Isolation Process

Isolation and detection methods for each pathogen were carried out in the same way for all samples tested from all four experiments unless otherwise stated. All samples were plated on duplicate plates.

2.3.1 *Salmonella*

The method used was in compliance with the ISO Standards (ThermoScientific Microbiology, 2013) (Section 7.1, Fig. A). 10 g of each sample were placed in a stomacher blender bag, diluted 10-fold with BPW (pre-enrichment stage) and placed in a stomacher (Stomacher® 400 circulator, Seward UK) at medium speed for 30 sec to homogenise. The samples were then incubated at 37°C for 18-24 hours. After incubation, 0.1 ml of each sample was transferred into 10 ml of RV broth and incubated at 42°C for 24 h. 1 ml of each sample was also transferred into 10 ml of MKTTn broth and incubated at 37°C for 24 h (selective enrichment step). Duplicate XLD and BGA selective plates were then streaked, from each selective broth, for single colonies and incubated at 37°C for 24 h. Typical colonies, red with black centre on XLD and red colonies on red medium on BGA agar were further

streaked onto NA to obtain pure cultures in order to proceed with biochemical and/or serological confirmation.

2.3.2 *Escherichia coli*

Two methods were performed for the isolation of *E. coli* both of which are illustrated in Section 7.1 (Fig. C) as described by the ISO Standard (ThermoScientific Microbiology, 2013).

2.3.2.1 Detection Method Using Cellulose Ester Membranes

10 g of food sample was diluted 1:10 with BPW and placed in a stomacher (Stomacher® 400 circulator, Seward UK) at medium speed for 30 sec to homogenise sample. 1 ml of the homogenised sample was then plated onto an MMA agar with a cellulose ester membrane (85 mm diameter and 0.45-1.2 µm pore size; Millipore, UK) and let to absorb for 5 min. Plates were then incubated facing upwards at 37°C for 4 h. After 4 h the membranes were aseptically transferred onto TBX agar and incubated at 44°C for 24 h. Plates were incubated on stacks of four. Typical colonies appear blue/green in colour. Where typical colonies were observed, selected colonies were streaked onto NA plates and incubated at 37°C for 24 h to obtain pure cultures.

2.3.2.2 Detection Method Using Tryptone Bile X-Glucuronide Medium (TBX) only

10 g of food sample were diluted 1:10 with BPW and placed in the stomacher at medium speed at 30 sec to homogenise. 0.1 ml was then spread onto TBX plates (instead of 1 ml instructed by the ISO guidelines) and left to dry for about 5 minutes. The plates were then incubated inverted at 44°C for 24 h. Typical colonies appear blue/green in colour. Where typical colonies were observed, selected colonies were streaked onto NA plates and incubated at 37°C for 24 h to obtain

pure cultures. Any typical blue/green colonies observed on TBX agar were also streaked on CHROMagar Orientation plates and further tested for catalase, oxidase and indole tests (Section 2.4).

2.3.3 *Staphylococcus aureus*

The isolation method for staphylococci was based on the ISO standards method using Baird-Parker selective agar for the isolation of coagulase-positive staphylococci (ThermoScientific Microbiology, 2013) (Section 7.1, Fig. B). 10 g of the food sample was diluted 1:10 with BPW and placed in the stomacher (Stomacher® 400 circulator, Seward UK) at medium speed for 30 sec to homogenise. 0.1 ml of the homogenised sample was then spread onto Baird-Parker plates and left to dry for about 5 min. The plates were then incubated at 37°C for 24 h. After 24 h the plates were examined for presumptive colonies and re-incubated for another 24 h at 37°C. Typical black/grey colonies surrounded by a zone of clearing were picked for biochemical testing and also streaked on CHROMagar Orientation plates.

Black/grey colonies with a halo were picked and streaked onto NA plates to obtain pure cultures. The plates were incubated at 37°C for 24 h. Purified colonies from NA plates were subjected to the coagulase test (Section 2.4).

2.3.4 *Yersinia enterocolitica*

The isolation method for *Yersinia enterocolitica* was taken from Van Damme *et al.,* (2010) as described in Section 7.1 (Fig. D). In this study, additional combinations of the selective broth dilutions were carried out.

2.3.4.1 Procedure & Materials

Samples were treated in three different dilution methods as described in the sections below.

2.3.4.1.1 Dilution Method No.1 (Pre-enrichment)

Four different trials were made. 10 g of food sample were diluted 10-fold with PSB broth and placed in a stomacher (Stomacher® 400 circulator, Seward UK) at medium speed for 30 sec to homogenise. From the first trial 100 µl were directly plated onto a Yersinia Selective agar (CIN) and SSDC agar and incubated at 30°C for 24 h. The homogenate of the second trial was incubated at 25°C for 2 days and the homogenate of the third trial at 25°C for 5 days. Homogenate of fourth trial was incubated at 4°C for 2 weeks. After incubation 100 µl of samples were spread onto CIN agars and incubated at 30°C for 24 h. Homogenates from second, third and fourth trials were also subjected to alkaline treatment by adding 0.5 ml of the enriched homogenised sample into 4.5 ml of 0.5% KOH solution (Section 2.1.20), mixed for 20 sec, then 100 µl were spread onto fresh CIN plate. Plates were incubated at 30°C for 24 h.

2.3.4.1.2 Dilution Method No.2 (Direct Selective Enrichment in ITC Broth)

10 g of food sample were diluted 10-fold with ITC broth (ITC broth) and placed in a stomacher (Stomacher® 400 circulator, Seward UK) at medium speed for 30 sec to homogenise. The homogenate was then incubated at 25°C for 2 days and then 100 µl were spread onto SSDC and incubated 30°C for 24 h.

2.3.4.1.3 Dilution Method No.3 (Selective Enrichment)

10 ml were taken from the homogeneous food sample from dilution method No.1 and aseptically transferred into a sterile stomacher bag. 90 ml of ITC broth were

then added to the sample and incubated at 25°C for 2 days. After incubation, 100 µl were spread onto SSDC and incubated 30°C for 24 h.

2.4 Biochemical Testing

A series of biochemical tests was carried out for each presumptive isolated pathogen in order to further and correctly identify the species.

2.4.1 Catalase Test

The test is used to detect whether an organism possesses the catalase enzyme which converts hydrogen peroxide to oxygen and water. The test was performed by placing a loopful of the organism taken from a pure culture onto a glass microscope slide. Then one drop of hydrogen peroxide (3%) was added onto the bacterial culture. If the mixture produced bubbles then it was an indication of the production of oxygen. The organism was then recorded as catalase-positive. If no bubbles were observed, then the organism was catalase-negative.

2.4.2 Oxidase Test

The test is used to detect bacteria that possess cytochrome c oxidase or indophenol oxidase, which are enzymes of the bacterial electron transport chain. Possession of the enzymes means the bacteria can catalyse the transport of electrons from donor compounds to electron acceptors (Public Health England, 2015b). The test was performed using a strip (40560, Sigma-Aldrich) impregnated with a redox indicator that turns purple when oxidised. The strip was smeared on a pure bacterial culture. Results were read within 10-20 sec. Oxidase positive organisms turned the strip purple while oxidase negative left it colourless.

2.4.3 Lactose Fermentation

This test was performed using MacConkey agar (Section2.1.6) which is a selective and differential agar to isolate and discriminate between Gram-negative enteric bacteria based on their lactose fermentation. Fresh MacConkey agars plates were streaked from a fresh pure bacterial culture and incubated at 37^{o}C for 24 h. Lactose fermenters utilise lactose in the medium and appear as red/pink colonies due to the pH change whilst non-lactose fermenters appear as colourless colonies. This medium was also used for the differentiation of *Yersinia enterocolitica* from *Pasteurella* species as *Yersinia enterocolitica* shows growth after 24 h incubation.

2.4.4 Indole Test

The test is used to determine the ability of an organism to convert tryptophan into indole. The test was performed by inoculating 10 ml of tryptone broth (Section 2.1.16) with a loopful of a fresh bacterial colony. The broth was then incubated at 37^{o}C for 24 h. After 24 h 0.5 ml of Kovacs' reagent (ThermoFisher Scientific, UK, Pcode: R21227) was added to the broth. A positive result is illustrated by a bright pink colour that appears as a top layer on the broth. A colourless or slightly yellow/green layer indicates a negative result.

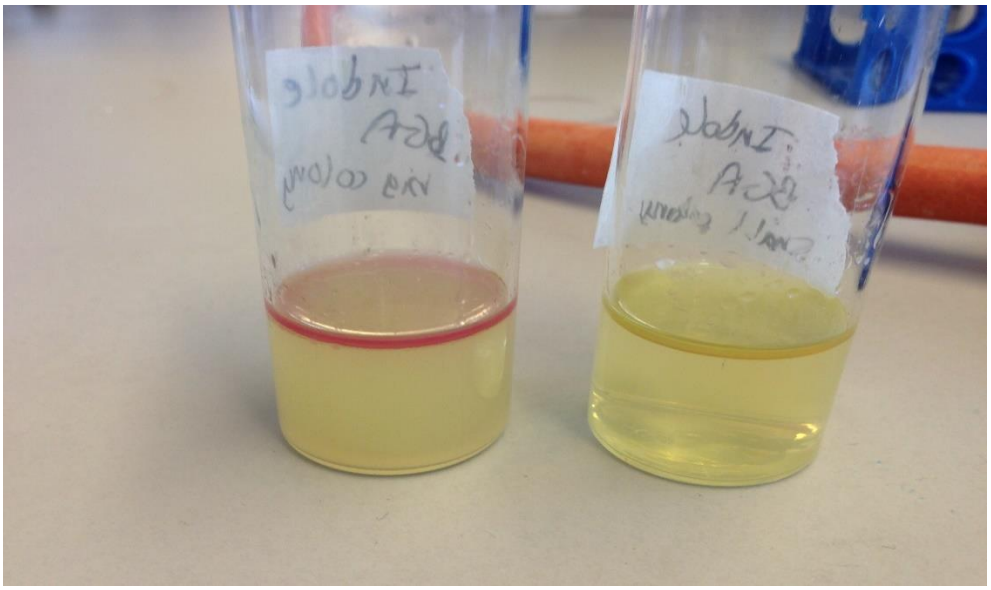


Figure 2.1: Picture illustrates a positive (left) and a negative (right) indole test result.

2.4.5 Urease Test

The test is used to determine the ability of an organism to hydrolyse urea through the production of urease enzyme. The test was performed using Christensen's urea agar (Section 2.1.19) which consists of 2% urea and phenol red that acts as a pH indicator. When pH increases due to the production of ammonia, the medium changes colour from light orange to bright pink indicating a positive result (Public Health England, 2015d). The test was performed by streaking a fresh pure bacterial colony onto fresh urea agar plates. The plates were incubated at the optimum temperature of the particular isolate (e.g at 30°C for *Yersinia enterocolitica*). Plates were incubated between 24-48 h and observed for a colour change as indicated.

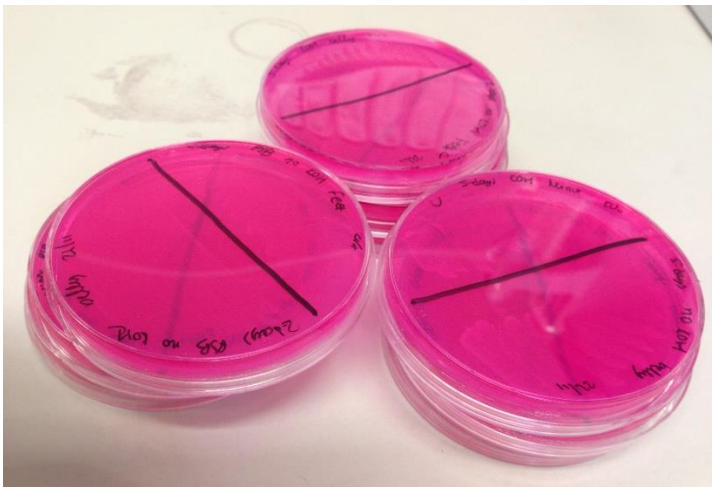


Figure 2.2: Inoculated urease agar showing a strong positive urease result due to production of ammonia which resulted in colour change of the medium from orange to bright pink.

2.4.6 Streptococcal Lancefield Grouping

The Streptococcal Grouping latex agglutination test (DR0585, Oxoid) was used in order to identify the Streptococcal Lancefield group of the unknown isolate. The unknown isolate was tested for Lancefield groups A, B, C, D, F and G following the manufacturer's instructions. A fresh culture was made by growing the isolate on ablood agar overnight at 37°C. An extraction enzyme bottle (provided in the kit) was reconstituted as instructed and 2-3 colonies were emulsified in the enzyme solution. The enzyme-culture solution was then incubated at 37°C for 10 min. Meanwhile the latex reagents were brought to room temperature. The reaction card provided consisted of six circular rings, one for each reagent. One drop of each reagent was dispensed into the circular rings on the reaction card. Using a pasteur pipette one drop from the enzyme-culture mixture was added in each ring and using a separate mixing stick (provided) for each reagent the mixture was spread onto the entire circular area of the ring. The card was then gently rocked for a few seconds. The ring with a visible agglutination indicated a positive result and the organism falls in the group of the equivalent reagent used in that reaction.

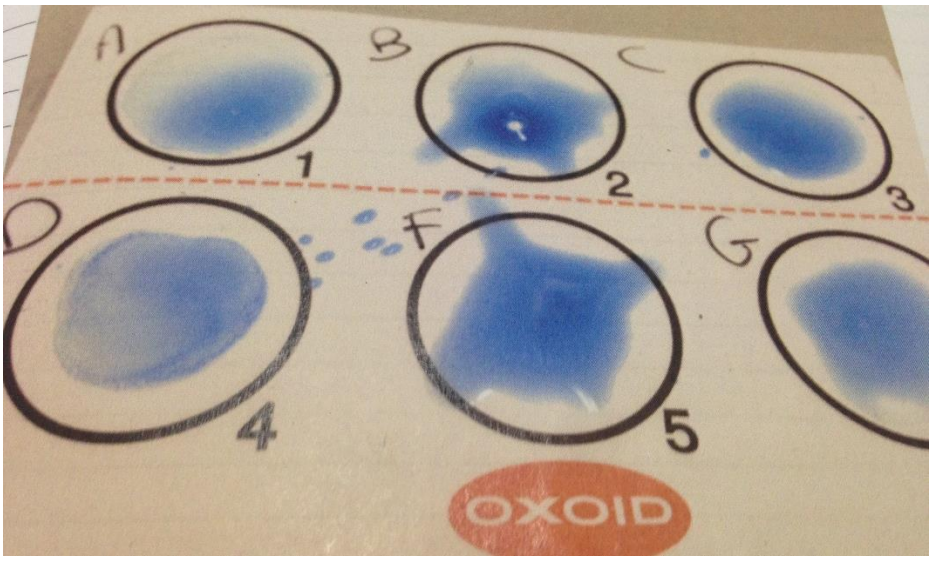


Figure 2.3: Picture illustrates agglutination results of Streptococcal group D (circle 4).

2.4.7 Coagulase Test

A coagulase tube test was performed by adding 0.5 ml of a fresh pure BHI broth culture (grown aerobically overnight at 37°C of a 10 ml broth) into a sterile test tube. 0.5 ml of a re-hydrated rabbit plasma (ThermoFisher Scientific, UK, Pcode: R21050, see Section 7.2) was then added to the tube. The mixture was then mixed thoroughly to emulsify. The tube was then incubated, slightly angled, at 37°C. The tube was observed for clotting every 30 min by slightly and gently slanting it. If no clot was visible after 4 h, the tube was left in the incubator for 24 h. After overnight incubation if there was no clot observed then the organism was recorded as coagulase negative.

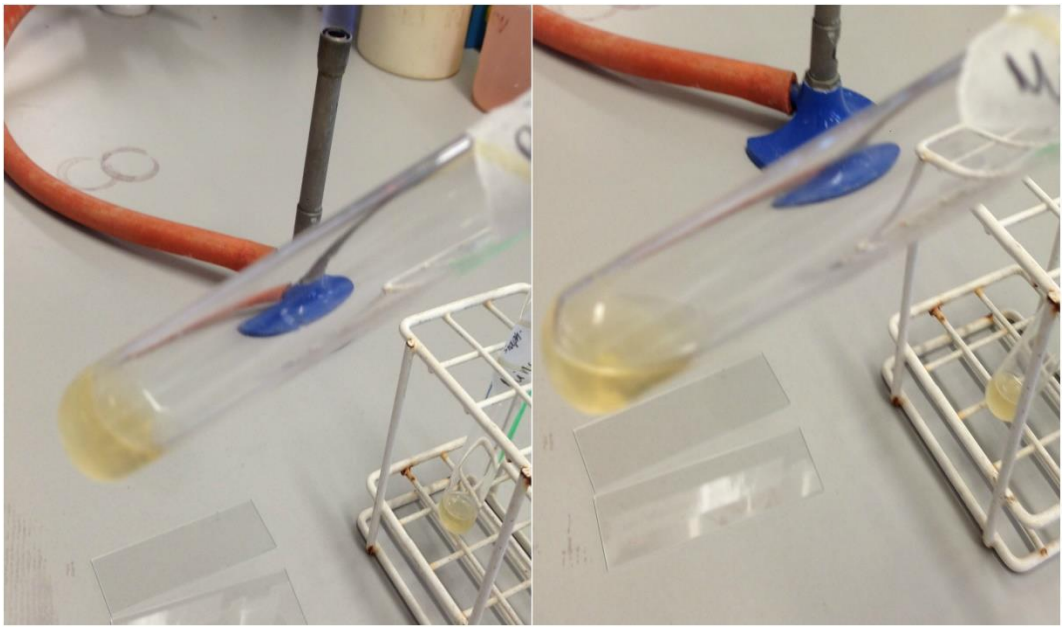


Figure 2.4: Pictures illustrate a positive (left) and negative (right) coagulase tube test result.

2.4.8 Gram Staining

A loop of sterile RO water was placed on a microscope slide. One colony was then picked of a fresh overnight NA plate culture using a sterile loop. It was then mixed with the RO water on the glass slide. The sample on the slide was heat fixed by passing the slide over a Bunsen flame until the sample has dried on the slide. The prepared slides were then placed in a rack and placed into a crystal violet solution for 60 sec. This solution acts as a primary stain for the cells. The slides were then washed in water to remove excess crystal violet and placed in iodine solution for 30 sec. Iodine is considered a mordant that binds crystal violet and is not able to exit Gram-positive cells. The slides were washed again and then placed in absolute ethanol for 60 sec in order to decolourise and remove crystal violet from Gram-negative cells. The slides were washed and placed into a final solution of safranin for 30 sec, which acts as a counter stain. Finally, the slides are dried by passing them over a Bunsen flame and then observed under a 100x oil immersion lens.

Gram-positive cells are stained purple (from crystal violet) while Gram-negative are pink (from safranin).

2.5 Identification Using API Biochemical Tests

The Analytical Profile Index (API) provide a fast identification of bacteria based on a series of biochemical reactions. There are a number of test kits based on the provisional classification of the bacteria. All kits were used following the manufacturer's instructions (bioMérieux, France).

2.5.1 API 20E (BioMérieux)

This test system is used for the identification of Enterobacteriaceae and other non-fastidious Gram-negative rods. Each sample contains an incubation box and a strip that contains a combination of 20 tests.

A fresh bacterial culture was prepared by streaking the unknown isolate from a selective medium onto nutrient agar and incubating overnight at 37°C. The incubation box was prepared by dispensing distilled water into the honey-combed wells of the tray in order to create humid conditions. The bacterial inoculum was prepared by emulsifying a bacterial colony from the NA plate into a tube containing 5 ml of sterile distilled water and adjusting to a turbidity of 0.5 McFarland standards. The turbidity was checked visually using a Wickerham card. Using a pipette the bacterial suspension was then dispensed into each individual microtube on the strip carefully in order to avoid the formation of any bubbles. For the tests CIT, VP and GEL both the tube and cupule were filled with the bacterial suspension. The tests ADH, LDC, ODC, H_2S and URE were overlaid with sterile mineral oil in the cupule to create anaerobic conditions. The strip was then placed in the tray and closed with the lid. The box was then incubated at 37°C for 18-24 h. After

incubation a drop of TDA reagent was added to the TDA tube, a drop of JAMES reagent was added to the IND test and one drop each of VP1 and VP2 were added to the VP test. A numerical profile was then obtained according to interpretation of the results from the reading table provided by the manufacturer. The numerical profile was then uploaded onto the APIweb software to identify the unknown isolate.

(https://apiweb.biomerieux.com/servlet/Authenticate?action=prepareLogin).

2.5.2 API Staph (BioMérieux)

The test is used for the identification of the genera *Staphylococcus, Micrococcus* and *Kocuria.* Each sample contains an incubation box and a test strip. Distilled water was distributed into the honey-combed wells of the incubation tray in order to create a humid atmosphere. A fresh bacterial culture was prepared by incubating the presumptive *Staphylococcus* isolates on a blood agar overnight at 37°C. The inoculum was prepared by emulsifying a distinct bacterial colony into a sterile tube containing 5 ml of sterile distilled water and adjusted to a turbidity of 0.5 McFarland standard. The turbidity was checked visually using a Wickerham card. Using a pipette the inoculum was then carefully distributed into the microtubes on the test strip without forming any bubbles. Only the tubes were filled with the inoculum and not the cupules. The tests ADH and URE were overlaid with mineral oil on the cupule in order to create anaerobic conditions. The tray was closed with the lid and the incubation box was incubated at 37°C for 18-24 h. After incubation one drop each of VP1 and VP2 reagents were added in the VP microtube. One drop each of NIT 1 and NIT 2 were added in the NIT test microtube and finally one drop each of ZYM A and ZYM B were added in the PAL test microtube. The reagents were left for

5-10 min to react and a numerical profile was then obtained according to interpretation of the results from the reading table provided by the manufacturer. The numerical profile was then uploaded onto the APIweb software to identify the presumptive isolate.

2.6 Antimicrobial Susceptibility Testing

Testing was carried out according to the Clinical and Laboratory Standards Institute method (CLSI, 2012).

2.6.1 Inoculum Preparation

A fresh bacterial culture was prepared by streaking on a nutrient agar (NA) and incubating overnight at 37°C (except *Yersinia enterocolitica* which was incubated at 30°C). The inoculum was prepared by adding 10 ml of Mueller-Hinton broth (Section 2.1.22) into a sterile tube and emulsifying 1-2 distinct colonies from the fresh bacterial culture. The suspension was adjusted to 0.5 McFarland standard to achieve equivalent turbidity (approximately 1 to 2 x 10^8 colony-forming units per ml). The turbidity was checked visually using a Wickerham card.

2.6.2 Inoculation of Test Plates

Within 15 min of the inoculum preparation, a sterile cotton swab was used to inoculate the test plates. The swab was dipped into the adjusted suspension and the excess fluid was removed by firmly pressing on the side of the tube. Then the dried surface of a fresh Mueller-Hinton agar plate (Section 2.1.22) was inoculated by streaking the swab over the entire surface of the agar. To ensure even distribution of the inoculum, the plate was rotated approximately 60° and each time the swab was dipped in the adjusted inoculum. Lastly, the rim of the agar was

swabbed. The inoculated agar was left to rest for 5 min in order to allow for any excess moisture to be absorbed.

2.6.3 Application of Drug-Impregnated Discs

The antibiotic discs (Table 2.1) were either dispensed aseptically onto the inoculated agar surface using sterile tweezers or via the use of an Antibiotic Dispenser for 120 mm square plates (Pro-Lab Diagnostics, REF: PL.800256). Each disc was pressed down to ensure complete contact with the agar. The discs were applied evenly over the surface with no less than 24 mm distance from centre to centre. Discs which produce small diameter inhibition zones, e.g vancomycin, were placed next to discs which give larger inhibition zones, such as the cephalosporins, to avoid overlapping. Once all discs were dispensed, the plates were inverted and incubated at 37°C (30° for *Yersinia*) for 24 h.

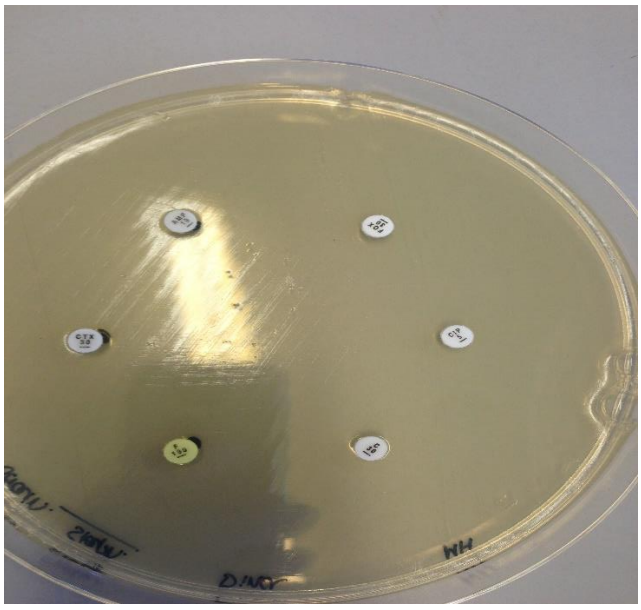


Figure 2.5: Antibiotic discs on MH agar prior to incubation.

Table 2.1: Antibiotic discs (n=26) used in this study.

Antibiotics Discs	
Abbreviation	**Concentrations**
AMP	Ampicillin 10 µg
AMC	Amoxicillin/Clavulanic acid 2:1 30 µg
ATM	Aztreonam 30 µg
CTX	Cefotaxime 30 µg
CAZ	Ceftazidime 30 µg
CPD	Cefpodoxime 10 µg
FOX	Cefoxitin 30 µg
C	Chloramphenicol 30 µg
CIP	Ciprofloxacin 5 µg
ETP	Ertapenem 10 µg
IPM	Imipenem 10 µg
MEM	Meropenem 10 µg
NA	Nalidixic acid 30 µg
F	Nitrofurantoin 100 µg
OT	Oxytetracycline 30 µg
SXT	Sulphamethoxazole/trimethoprim 25 µg
S	Streptomycin 10 µg
CFQUI	Cefquinome 30 µg
VA	Vancomycin 30 µg
E	Erythromycin 15 µg
DA	Clindamycin 2 µg
S3	Compound Sulphonamides 300 µg
TE	Tetracycline 30 µg
CT	Colistin 10 µg
EFT	Ceftiofur 30 µg
ENR	Enrofloxacin 5 µg

2.6.4 Interpretation of the Results

The diameter of each zone of clearing around each disc was measured and referred to as resistant, intermediate or susceptible according to the referencing standards for each pathogen from CLSI and EUCAST breakpoints (CLSI, 2014; EUCAST, 2017) (see Section 7.5).

2.6.5 Clindamycin-Inducible Resistance Test

This test (also known as the D-test) was performed only for *Staphylococcus* and *Enterococcus* isolates as macrolide-resistant isolates are known to induce clindamycin resistance. The D-zone test was performed by placing a 2 µg

clindamycin disc 15 mm away (12 mm away for *Enterococcus* isolates) from a 15 µg erythromycin disc on a Mueller-Hinton plate inoculated with the isolates as described previously. Inducible clindamycin resistance is illustrated with a flattening zone (D-shape zone) of the clindamycin disc adjacent to the erythromycin (Figure 2.6).

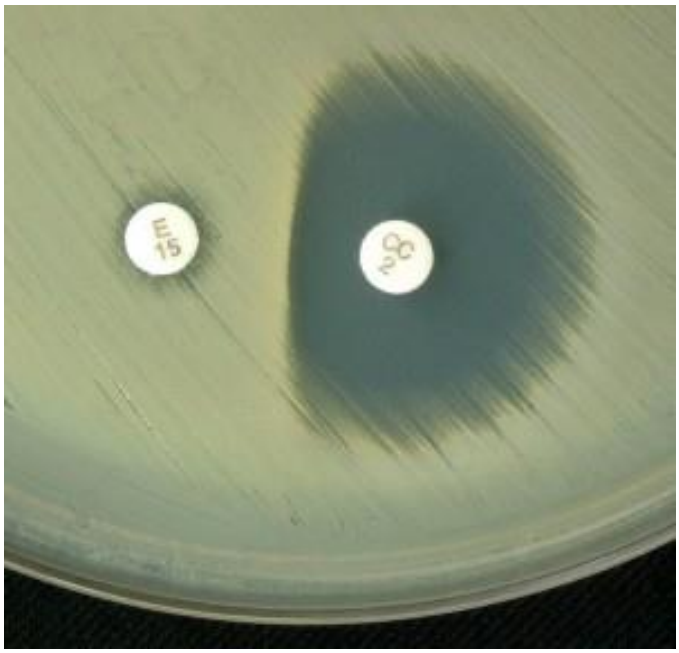


Figure 2.6: Picture illustrates a positive D-test result.

2.7 Molecular Methods

2.7.1 Crude DNA Extraction by Boiling Method

100 µl of sterile RO water was added into a sterile 1.5 ml micro centrifuge tube. Two to three distinct colonies from a fresh pure culture were picked and resuspended in sterile RO water. The tube was then heated in a dry heating block (QBT2, Grant Instruments, UK) at 100°C for 30 min. The tube was then transferred into a centrifuge and spun at 15,493 *g* for 15 min. The supernatant was then transferred into a fresh sterile 1.5 ml micro centrifuge tube and stored at -20°C.

The NanoDrop®ND-1000 Spectrophotometer (ThermoFisher Scientific, UK) was used to measure the DNA concentration and purity (in ng/µl). The spectrophotometer was cleaned using lens tissue and 2 µl of RO water was then loaded onto the machine to initiate BLANK measurement. Then 2 µl of extracted DNA was loaded and measurement initiated. Samples with a DNA yield below 30 ng/µl were repeated.

2.7.2 DNA Extraction Methods Used for the Gram-Positive Isolates

2.7.2.1 Boiling Procedure Using TE Buffer (pH 8)

100 µl of TE buffer (pH 8, Section 2.1.24) and 5 µl of Proteinase K (ThermoFisher Scientific, 20 mg/ml) were added into a sterile 1.5 ml micro centrifuge tube. Two distinct colonies from a fresh pure culture were then emulsified into the tube. The tube was heated in a dry heating block for 1 hour 30 minutes at 65°C and then at 100°C for 10 min. The tube was then centrifuged at 15,493 *g* for 15 min. The supernatant was then transferred in a fresh sterile tube. The DNA concentration was measured as described in Section 2.7.1.

2.7.2.2 Boiling Procedure Using a Lysis Buffer

100 µl of TE buffer (pH 8, Section 2.1.24), 10 µl of 10% SDS buffer (Section 2.1.25) and 5 µl of Proteinase K (ThermoFisher Scientific, 20 mg/ml) were dispensed into a sterile 1.5 ml micro centrifuge tube along with two colonies from a fresh pure culture. The tube was then heated in a dry heating block at 65°C for 10 min and then at 100°C for another 10 min. The tube was then centrifuged using a refrigerated microcentrifuge at 4°C at 15,493 *g* for 5 min and the supernatant removed to a fresh tube followed by an ethanol precipitation step.

2.7.2.2.1 Ethanol Precipitation

2.5 volumes of 100% (v/v) ethanol were added to the supernatant tube (e.g for 100 µl sample – 250 µl of absolute ethanol). The tube was then kept at -80°C for 30 min. It was then centrifuged using a refrigerated microcentrifuge at 4°C for 10 min at 15,493 *g*. The supernatant was discarded and the pellet was resuspended with 70% (v/v) ethanol. The pellet was then air-dried using a Vacuum Concentrator (RC 1022 ThermoElectron Corporation) at heat level 2 for around 30 min. Once fully dried, the pellet was resuspended with 100 µl of TE buffer (pH 8). The DNA suspension was stored at -20°C.

2.7.3 16S rDNA Sequencing

For presumptive *E. coli* and *Yersinia enterocolitica* DNA was extracted using the method described in Section 2.7.1. For presumptive *Staphylococcus* DNA was extracted using the methods in Section 2.7.2.1 and for presumptive *Enterococcus* the method in Section 2.7.2.2. PCR was performed in 25 µl reactions. Preparation of the PCR mixture was performed using 12.5 µl of DreamTagGreen PCR Master Mix (2X) (ThermoFisher Scientific), 8.5 µl of nuclease-free water (ThermoFisher Scientific), 1 µl of 10 mM stock of forward primer 16SV1-4:8F, 1 µl of 10 mM stock of reverse primer 16SV1-4:805R (Table 2.2) and 2 µl of DNA template (~100-150 ng). Where the volume of DNA template was increased in the mixture, nuclease-free water was decreased proportionally. The PCR was run with an initial denaturation step at 95°C for 5 min followed by 30 cycles of 94°C for 1 min, 52°C for 1 min and extension at 72°C for 2 min. Final elongation of the cycle was at 72°C for 10 min. 5 µl of each PCR sample were electrophoresed on a 1% TAE agarose gel (Section 2.1.26) for 1 h at 85 V. The gel was then visualised under UV light using

the Gel-Doc XR system (Bio-rad, UK). The remaining PCR mixture was purified as described in Section 2.7.3.1 and sent for sequencing by Source Bioscience, UK.

2.7.3.1 DNA Purification

The PCR mixtures were purified using the Monarch PCR & DNA Cleanup Kit (New England BioLabs, UK). Five volumes of DNA cleanup binding buffer were added to one volume of PCR sample. The mixture was then transferred onto a column provided and the column was inserted in a collection tube provided. The sample was spun for 1 min at 15,493 *g*. The flow-through was discarded and 200 µl of DNA wash buffer were added to the column and spun for another minute at the same speed. This step was repeated. The flow-through was discarded and the column was transferred into a sterile 1.5 ml micro centrifuge tube. 10 µl of DNA Elution Buffer were then added to the column and left to sit for 1 min. The microfuge was then spun for 1 minute at 13,000 rpm to elute DNA. The purified DNA concentration was measured as described in Section 2.7.1. Purified DNA samples were stored at -20°C.

2.7.4 *Yersinia enterocolitica* Patho-Serotyping

DNA was extracted as described in Section 2.7.1. Method was slightly modified from Garzetti *et al*. (2014). PCR was performed using 12.5 µl of DreamTagGreen PCR Master Mix (2X), 8.5 µl of nuclease-free water, 1 µl of 10 mM stock of forward primer (*per, wzt, wbbU*), 1 µl of 10 mM stock of reverse primer (*per, wzt, wbbU*) (Table 2.2) and 2 µl of DNA. Three different primer sets were used to detect particular genes present in certain serotypes. The *per* gene was used to detect serotype O:9 strains, *wbbU* for O:3 and *wzt* for O:5,27. The PCR cycle was as follows: 95°C for 5 minutes, then 30 cycles of 95°C for 40 seconds, 58°C for 40

seconds, 72°C for 30 seconds and final extension at 72°C for 8 minutes. 5 µl of each PCR product were electrophoresed on a 1.5% agarose gel at 85 V for 1 h.

2.7.5 *Yersinia enterocolitica* Pathogenicity

PCRs were performed in order to detect the chromosomal *ail* and *inv* and plasmid-mediated *virF* and *yadA* pathogenic genes. Method was slightly altered from Thoerner *et al*. (2003).

2.7.5.1 *virF* and *yadA* Plasmid Virulent Genes

DNA was extracted as described in Section 2.7.1. PCR was performed using 12.5 µl of DreamTagGreen PCR Master Mix (2X), 7.5 µl of nuclease-free water, 1 µl of 10 mM stock of forward primer (*virF, yadA*), 1 µl of 10 mM stock of reverse primer (*virF, yadA*) (see table 2.2) and 3 µl of DNA. The PCR cycle consisted from 95°C for 10 min followed by 30 cycles of 95°C for 30 sec, 63°C (for *virF*) / 60°C (for *yadA*) for 30 sec and 72°C for 20 sec. Final extension was at 72°C for 7 min. 7 µl from each PCR product were electrophoresed at a 1.5% (w/v) TAE agarose gel at 85 V for 1 h.

2.7.5.2 Chromosomal *ail* and *inv* Genes

DNA and PCR were extracted and performed, respectively, as described in Section 2.7.5.1. The primers used (*ail, inv*) are listed in Table 2.2. The PCR cycle for the *ail* gene was as follows: 95°C for 5 min, the 30 cycles of 95°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec and final extension at 72°C for 7 min. The PCR cycle for the *inv* gene consisted of 95°C for 10 min, then 30 cycles of 95°C for 30 sec, 61°C for 30 sec, 72°C for 20 sec and final extension at 72°C for 7 min. 7 µl of each PCR product were electrophoresed on a 1.5% (w/v) TAE agarose gel at 85 V for 1 h.

2.7.6 *E. coli* Pathogenic Gene Detection

Control strains used are summarised in Table 2.3. Method was modified from Wei (2013).

2.7.6.1 EPEC/EHEC *eae* Gene Detection

DNA was extracted as described in Section 2.7.1. PCR was performed using 12.5 µl of DreamTagGreen PCR Master Mix (2X), 8.5 µl of nuclease-free water, 1 µl of 10 mM stock of forward primer *eae*, 1 µl of 10 mM stock of reverse primer *eae* (Table 2.2) and 2 µl of DNA. The PCR cycle was run at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 64°C for 1 min and 72°C for 40 sec. Final extension was at 72°C for 5 min. 7 µl of each PCR product were electrophoresed on a 1.5% (w/v) TAE agarose gel at 85 V for 1 h.

2.7.6.2 Enteroaggregative *astA* Gene Detection

DNA and PCR mixture were prepared as described in the previous Section (2.7.6.1). The primers used (*astA*) are listed in Table 2.2.The PCR cycle was run as follows: 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. Final extension was at 72°C for 10 min. 5 µl of each PCR product were electrophoresed on a 2% (w/v) TAE agarose gel at 85 V for 1 h.

2.7.6.3 ETEC Heat-Stable (ST) and Heat-Labile (LT) Gene Detection

DNA extraction and PCR mixture were prepared as described previously in Section 2.7.6.1. The primer used (LT, STA) are listed in Table 2.2. The PCR cycle was run at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min. Final extension was carried at 72°C for 1 min. 5 µl from each PCR product were electrophoresed on a 2% (w/v) TAE agarose gel at 85 V for 1 h.

2.7.7 Chromosomal and Plasmid-Mediated AmpC Detection

2.7.7.1 Chromosomal AmpC

Method was modified from Ibrahim (2016). All *E.coli* isolates were tested for the presence of chromosomally encoded β-lactamases. DNA was extracted as described in Section 2.7.1. PCR was performed using 12.5 µl of DreamTagGreen PCR Master Mix (2X), 8 µl of nuclease-free water, 1.25 µl of 10 mM stock of forward primer chromosomal AmpC, 1.25 µl of 10 mM stock of reverse primer chromosomal AmpC (Table 2.2) and 2 µl of DNA. The PCR cycle was run at 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 20 sec. Final extension was carried at 72°C for 7 min. 7 µl from each PCR product were electrophoresed on a 1.5% (w/v) TAE agarose gel at 85 V for 1 h.

2.7.7.2 Plasmid-Mediated AmpC for the FOX Family

Isolates that exhibited phenotypic resistance to cefoxitin were further tested for plasmid-mediated AmpC of the FOX family, which confers resistance to cefoxitin. DNA extraction and PCR mixture were as described in the previous Section (2.7.7.1). The primers used (MultiFOX) are listed in Table 2.2. The PCR cycle was carried at 94°C for 10 min followed by 30 cycles of 94°C for 40 sec, 60°C for 40 sec and 72°C for 20 sec. Final extension was carried at 72°C for 7 min. 7 µl from each PCR product were electrophoresed on a 1.5% (w/v) TAE agarose gel at 85 V for 1 h.

2.7.7.3 *Yersinia enterocolitica* Chromosomally Encoded β-Lactamases

Yersinia isolates were tested for chromosomally encoded β-lactamases using specific primers for the detection of *blaA* and *blaB* genes obtained from Stock *et al.* (1999). DNA extraction was as described in Section 2.7.1. The PCR mixture consisted of 12.5 µl DreamTagGreen PCR Master Mix (2X), 7.5 µl of nuclease-free

water, 1 µl forward primer (*blaA, blaB*), 1 µl reverse primer (*blaA, blaB*) (Table 2.2) and 3 µl DNA template. PCR cycle was performed at 95°C for 5 min followed by 25 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for 1 min. Final extension was at 72°C for 5 min. 7 µl of PCR product was electrophoresed on a 1.5% (w/v) TAE agarose gel at 85 V for 1 h.

2.7.8 *Staphylococcus aureus*

2.7.8.1 *mecA* Gene Detection

All *Staphylococcus* isolates were tested for the detection of the *mecA* gene which confers resistance to methicillin. DNA was extracted as described in Section 2.7.2.1. PCR was performed using 12.5 µl of DreamTagGreen PCR Master Mix (2X), 8.5 µl of nuclease-free water, 1 µl of 10 mM stock of forward primer *mecA*, 1 µl of 10 mM stock of reverse primer *mecA* (Table 2.2) and 2 µl of DNA. PCR cycle was run at 94°C for 3 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. Final extension was at 72°C for 4 min. 7 µl from each PCR product were electrophoresed on a 2% (w/v) TAE agarose gel at 85 V for 1 h.

2.7.8.2 Identification of CC398 Strains

All *Staphylococcus* isolates were tested to detect if they were CC398 strains using PCR detection of the clonal complex 398 by targeting the restriction-modification system carrying *saul-hsdS1* in *Staph. aureus* following a published method by Stegger *et al.* (2011). DNA preparation and PCR procedures were as described in Section 2.7.8.1. The primers used (AF, CC398r1) are listed in Table 2.2. PCR cycle was run at 95°C for 12 min followed by 35 cycles of 95°C for 30 sec, 61°C for 30 sec and 72°C for 30 sec. Final extension was at 72°C for 10 min. 7 µl from each PCR product were electrophoresed on a 2% (w/v) TAE agarose gel at 85 V for 1 h.

2.7.9 Metal Resistance

All *E. coli* and *Yersinia* isolates were subjected to phenotypic and genotypic testing for mercury, copper and silver resistance. Control strains used are summarised in Table 2.3.

2.7.9.1 Mercury Resistance

2.7.9.1.1 Phenotypic Resistance

LB agar plates were prepared (Section 2.1.23) and mercury solution was added to them. Plates with two different mercury concentrations were prepared. Isolates were tested with 25 µg/ml and 50 µg/ml of $HgCl_2$. 25 µg/ml plates were prepared by adding 12.5 µl of 50 mg/ml $HgCl_2$ stock solution into 25 ml of LB agar (to make 1 agar plate). 50 µg/ml plates were prepared by adding 25 µl of 50 mg/ml $HgCl_2$ stock solution into 25 ml of agar. Fresh bacterial cultures were then streaked onto the plates and incubated for 24 h at 37°C for *E. coli* and 30°C for *Yersinia*. The plates were then observed for growth.

2.7.9.1.2 Genotypic Resistance

Multiplex-PCR was performed for the detection of *merA, merC* and *intl1* genes. DNA was extracted as described in Section 2.7.1. The PCR mixture was prepared by adding 12.5 µl of DreamTag Green (2X), 0.5 µl of 10 µM stock of each primer *merA*, *merC*, *intl1* (Table 2.2), 9.5 µl of nuclease-free water and 2 µl of DNA. For *Yersinia* isolates 3 µl of DNA were added and 8.5 µl of nuclease-free water. The PCR cycle was performed at 95°C for 5 min followed by 30 cycles of 94°C for 1 min, 64°C for 1 min and 72°C for 2 min. Final extension step was performed at 72°C for 10 min. 7 µl of PCR product were electrophoresed on 1.5% (w/v) TAE agarose gel at 80 V for 80 min.

2.7.9.2 Copper Resistance

2.7.9.2.1 Phenotypic Resistance

LB agar plates (Section 2.1.23) with added $CuSO_4$ (Sigma Aldrich, C1297) were prepared in the following concentrations: 2, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18 and 20 mM. For example, for 2 mM stock 0.064 g of $CuSO_4$ were added into 200 ml of LB agar. Fresh bacterial cultures were then streaked and the plates were incubated and examined as described in Section 2.7.9.1.1.

2.7.9.2.2 Genotypic Resistance

DNA was extracted as described in Section 2.7.1. PCR was performed as for detection of the *pcoE* gene. The PCR mixture was prepared by adding 12.5 µl of DreamTag Green (2X), 1 µl of 10 µM stock of each *pcoE* primer (Table 2.2), 7.5 µl of nuclease-free water and 2 µl of DNA. The cycle was performed at 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 48°C for 30 sec and 72°C for 1 min. Final extension was at 72°C for 7 min. 7 µl of PCR product were electrophoresed on 1.5% (w/v) TAE agarose gel at 80 V for 90 min.

2.7.9.3 Silver Resistance

2.7.9.3.1 Phenotypic Resistance

This method was based on the study by Gupta *et al.* (1998). LB agar plates were prepared without adding NaCl. For 200 ml of agar, 3 g of agar, 2 g of tryptone and 1 g of yeast extract were added. Highly purified water (Milli-Q, Merck) was used to prepare the media, and the agar was then autoclaved. Plates were prepared containing the following $AgNO_3$ (Sigma Aldrich, 209139) concentrations: 100 µM, 200 µM, 400 µM and 600 µM. Fresh bacterial cultures were then streaked and the plates were incubated and examined as described in Section 2.7.9.1.1.

2.7.9.3.2 Genotypic Resistance

PCR was performed for the detection of *silE* and *silP* genes (Table 2.2). DNA extraction and PCR mixture were prepared as described in Section 2.8.9.2.2. The PCR cycle for *silP* was carried at 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 50°C for 30sec and 72°C for 2 min. Final extension was carried at 72°C for 7 min. *silE* was performed using the same steps except for the annealing temperature and elongation which were performed at 55°C for 30 sec and 72°C for 1 min, respectively. 7 µl of PCR product were electrophoresed on 1.5% (w/v) TAE agarose gel at 80 V for 90 min.

Table 2.2: Oligonucleotides.

Primer	Sequence	Amplicon(bp)	Test	References
16S rRNA				
16SV1-4:8F	AGAGTTTGATCCTGGCTCAG	800	16S rRNA gene	Universal primers (taken from Litten, 2016, unpublished work)
16SV1-4:805R	GACTACCAGGGTATCTAATC			
Chromosomal & Plasmid-mediated AmpC				
MultiFOX-F-	CTACAGTGCGGGTGGTTT	162	Plasmid AmpC for the FOX family	(Dallenne *et al.*, 2010)
MultiFOX-R-	CTATTTGCGGCCAGGTGA			
Chromosomal AmpC-F-	AAT GGG TTT TCT ACG GTC TG	191	Chromosomal AmpC	(Dierikx *et al.*, 2012)
Chromosomal AmpC-R-	GGGCAGCAAATGTGGAGCAA			
***Y. enterocolitica* serotyping & pathogenicity**				
per (O:9)-F-	TCCTTCTCCAAATATATAGGTGCCA	837	Patho-serotyping for *Yersinia enterocolitica*	(Garzetti *et al.*, 2014)
per (O:9)-R-	ATGCGGCATTAGATGAGATGGA			
wbbU(O:3)-F-	ACCTCGTATTTTTGAAGATGATCGC	463		
wbbU(O:3)-R-	GTACTCAATAACTTGCTGTTCGGA			
wzt (O:5,27)-F-	GTTAGTTCCTGCATCTGATCGCC	662		
wzt (O:5,27)-R-	ATCCAGCATCCATGGCTCC			
ail-F-	TAATGTGTACGCTGCGAG	351	*Yersinia enterocolitica* chromosomal genes	(Thoerner *et al.*, 2003)
ail-R-	GACGTCTTACTTGCACTG			
inv-F-	CGGTACGGCTCAAGTTAATCTG	183		
inv-R-	CCGTTCTCCAATGTACGTATCC			
virF-F-	GGCAGAACAGCAGTCAGACATA	561	*Yersinia enterocolitica* virulence plasmid genes	
virF-R-	GGTGAGCATAGAGAATACGTCG			
yadA-F-	CTTCAGATACTGGTGTCGCTGT	849		
yadA-R-	ATGCCTGACTAGAGCGATATCC			
Staph. aureus				
AF-F-	AGGGTTTGAAGGCGAATGGG	296	LA-MRSA CC398 strain	(Stegger *et al.*, 2016; Stegger *et al.*, 2011)
CC398r1-R-	CAGTATAAAGAGGTGACATGACCCCT			
mecA-F-	AAAATCGATGGTAAAGGTTGGC	532	Methicillin-resistant *Staph. aureus*	(Strommenger *et al.*, 2003)
mecA-R-	AGTTCTGCAGTACCGGATTTGC			
***E. coli* pathogenicity**				
eae-F-	TCAATGCAGTTCCGTTATCAGTT	482	EPEC/EHEC	(Vidal *et al.*, 2004)
eae-R-	GTAAAGTCCGTTACCCCAACCTG			
astA-F-	CCATCAACACAGTATATCCGA	111	EAggEC	(Jenkins *et al.*, 2006)
astA-R-	GGTCGCGAGTGACGGCTTTGT			
LT-F-	GCACACGGAGCTCCTCAGTC	218	ETEC	(Stacy-Phipps

LT-R-	TCCTTCATCCTTTCAATGGCTTT			*et al.,* 1995)
STA1 (F)	TCTTTCCCCTCTTTAGTCAG	166		
STA2 (R)	ACAGGCCGGATTACAACAAAG			
Primer	**Sequence**	**Amplicon(bp)**	**Test**	**References**
Mercury Resistance				
merA-F-	ACCATCGGCACCTGCGT	1237	mercuric reductase	(Liebert *et al.*, 1997)
merA-R-	ACCATCGTCAGGTAGGGGAACAA			
merC-F-	CATCGGGCTGGGCTGGGCTTCTTGAG	364	Mercury transport protein	
merC-R-	CATCGTTCCTTATTCGTGTGG			
intI1-F-	CCTCCCGCACGATGATC	280	Mercury resistance operon of transposon Tn*21*	(Goldstein *et al.*, 2001)
intI1-R-	TCCACGCATCGTCAGGC			
***Y. enterocolitica* chromosomal AmpC**				
blaA-F-	AAATGCGCTACCGGCTTCAG	439	*Yersinia enterocolitica* chromosomally encoded β-lactamases	(Stock *et al.*, 1999)
*bla*A-R-	AGTGGTGGTATCACGTGGGT			
*bla*B-F-	CCCACTTTATACCTTGGCACAAA	781		
*bla*B-R-	GAACATATCTCCTGCCTGGAAAT			
Copper Resistance				
PcoE-F-	ATGAATATATTAATCACGAC	450	Periplasmic copper binding protein	(Asiani, 2016)
PcoE-R-	TTACCTGGTCGAATACAGCC			
Silver Resistance				
SilE-F-	ATGAAAAATATCGTATTAGC	432	Periplasmic chaperon of Ag^+	(Asiani, 2016)
SilE-R-	TCAGCCTGCACTGAGCATGC			
SilP-F-	ATGCTTCAGATATGCATAAG	2,475	ATPase that exports Ag^+	
SilP-R-	TTATTTCCCGAGCCTGACA			

Table 2.3: Control strains.

Strain	Characteristics	Use	Original Reference
E. coli ATCC® 25922	Quality control strain susceptible to all antibiotics tested. Possesses chromosomal AmpC but no reference for other β-lactamases.	Used as a negative control for the following tests: antibiotic disk assays, MultiFOX PCR, chromosomal *ail* & *inv* genes, plasmid *virF* & *yadA* genes Used as positive control for chromosomal AmpC PCR	(CLSI, 2013)
E. coli H10407	Enterotoxigenic (ETEC) strain possessing a heat stable, heat labile and the enteroaggregative heat stable toxin *ast*A	ETEC positive control strain used for the detection of *astA*, LT and ST genes	(Crossman *et al.*, 2010)
0157/SAKI-STX (EDL933)	*E. coli* 0157 without Shiga-toxin	Used as a positive control for the detection of *eae* gene	(Pacheco-Gómez *et al.*, 2012)
Salmonella Typhimurium LT2	Principal strain for cellular and molecular biology in *Salmonella*	Used as a negative control for the chromosomal AmpC PCR	University of Nottingham culture collection
Yersinia enterocolitica NCTC 10460	Known *Y. enterocolitica* strain	Used as positive control for the detection of *ail* gene	University of Nottingham culture collection
Enterobacter cloacae NCTC 13406	AmpC β-lactamase de-repressed	Used as negative control for the detection of EAEC *astA* gene	University of Nottingham culture collection
J53 (pMG101)	K-12 derivative strain carrying the pMG101 plasmid which confers resistance to silver, copper and mercury and multiple antibiotics	Used as a positive control for PCR assays for the detection of *silE*, *silP*, *merA*, *merC* and *intI1*	(Gupta *et al.,* 1998)
J53	K-12 derivative strain non-plasmid carrying	Used as a negative control for silver PCR gene detection	(Clowes & Rowley, 1954)
E. coli MG1655	K-12 derivative strain	Used as negative control for mercury PCR gene detection	(Blattner *et al.*, 1997)

Strain	Characteristics	Use	Original Reference
E. coli ED8739 pRJ1004	K-12 derivative strain carrying the pRJ1004 plasmid encoding copper resistance	Used as positive control for PCR for the detection of *pcoE* gene	(Brown *et al.*, 1995)
E. coli ED8739	K-12 derivative strain non-plasmid carrying strain	Used as negative control for PCR for the detection of *pcoE* gene	

*Note: All control strains were obtained from The University of Nottingham Culture Collection stock.

Table 2.4: Known strains used for phylogenetic analysis.

Strain	GenBank Number	Reference paper
E. coli O104:H4 str. C227-11	CP011331	(Beaulaurier *et al.*, 2015)
E. coli strain 042	FN554766.1	(Chaudhuri *et al.*, 2010)
E. coli O157 strain 644-PT8	CP015831	(Cowley *et al.*, 2016)
Yersinia enterocolitica subsp. *enterocolitica* 8081	AM286415.1	(Hao *et al.*, 2016)
Yersinia kristensenii ATCC 33639	CP008955.1	
Yersinia ferderiksenii ATCC33641	KN150731.1	
Staphylococcus aureus subsp. *aureus* ATCC25923	CP009361.1	(Treangen *et al.*, 2014)
Staphylococcus vitulinus ATCC 51145	NR_024670.1	(Takahashi *et al.*, 1999)

2.8 Storage of the Isolates

All isolates were stored on Microbank beads containing specially formulated broth (PL.170/M, Pro-Lab Diagnostics, UK) and kept in -80°C. A loopful from pure NA cultures was swirled into the beads and left to set for about 2-3 min. Using a pipette, the broth was removed from the tubes and discarded. The microbanks were kept at -80°C.

2.9 Bioinformatics for Phylogenetic Analysis

Sequences were aligned and phylogenetic trees were constructed based on the 16S rRNA gene copies of the isolates obtained from sequencing (Section 2.7.3) using Kimura's 2-parameter distance and the neighbour-joining method (MEGA 7.0). 16S rRNA gene copies from the whole genome of known strains (Table 2.4) were used

in order to validate the 16S results (Tables 3.4, 3.5, 3.6) and further identify any isolates identified only to genus level.

3 Results

3.1 Isolation Process & Confirmatory Testing

The primary aim of the project was to study the antimicrobial resistance in foodborne pathogens isolated from retail pork products. Different retail sources were chosen randomly to examine different origins of the products (i.e farms). Different pork samples were also purchased in order to isolate from a wider selection of products. Products with skin included pork belly and pig feet. Product without the skin was pork loins and the processed product was pork mince. Isolation and detection from a variety of products could also help study other factors affecting the contamination of the retail meat such as that of cross-contamination and hygiene practices inside slaughterhouses. Additionally, three different production categories were tested, i.e. products from conventional farms, free-range pork and organic pork. ISO standard methods were chosen as the isolation methods primarily used in this study. This was to mirror those used in industry during food quality testing; however, in this project the focus was not on viable counting of any foodborne pathogen but rather for determining presence or absence from retail products. In this project, no presumptive *Salmonella* isolates were detected.

3.1.1 *E. coli*

Two different isolation methods were used (Section 2.3.2), the membrane isolation method, which includes a recovery step prior to selection on TBX, and direct plating on TBX agar. Table 3.1 summarises the samples tested and from which methods

the presumptive isolates were obtained. Swab samples were directly streaked onto TBX with no pre-enrichment step being performed.

Table 3.1: Sample type and detection of presumptive *E. coli* by the isolation methods.

Sample type	*E. coli* detected by:	
	Membrane method	TBX
Pork carcass 1- feet swab	n/a[1]	N[2]
Pork carcass 1- belly swab	n/a	Y[3]
Pork carcass 2- feet swab	n/a	Y
Pork belly- Source A	Y	N
Pork mince- Source A	N	N
Pork feet- Source A	Y	N
Pork belly- Source B	N	N
Pork mince- Source B	N	N
Pork loins- Source C	N	N
Pork belly- Source D	Y	Y

1: not applicable, 2: no, 3: yes.

Presumptive colonies (blue/green colonies on TBX) were streaked onto CHROMagar Orientation plates and typical dark pink/reddish colonies were then tested for Gram stain catalase, oxidase and indole production (Section 2.4). Isolates that appeared as Gram negative non-spore forming rods, were catalase positive, oxidase negative and positive for indole production were considered isolates of *E. coli* and confirmed using 16S rDNA sequencing (Section 3.2). A total of eight presumptive isolates were sent for sequencing: two from pork carcass swabs, one from the mince sample of sampling 1, one from the pork belly sample of sampling 1 and four from organic pork belly (sampling 4) (Table 3.7).

3.1.2 *Staphylococcus aureus*

The isolation method on Baird Parker agar (Section 2.3.3) was slightly altered for sampling 2 in order to allow better cell recovery and aid detection since direct

plating of sampling 1 resulted in no presumptive colonies (Table 3.2). The initial homogenised sample was incubated at 37°C for 24 h instead of directly being plated onto selective media. 0.1 ml of the homogenate was then spread onto Baird-Parker plates and incubated at 37°C for 24 h. The plates were then examined and re-incubated at 37°C for another 24 h. Presumptive grey/black colonies surrounded by a halo were then subjected to biochemical testing including catalase, oxidase and coagulase tests. Table 3.2 summarises the results.

Table 3.2: Sample types, isolation methods and biochemical reactions of Gram-positive isolates.

Sample	Isolation method		Biochemical reactions		
	Direct plating	**24 h pre-enrichment**	**catalase**	**oxidase**	**coagulase**
Pork mince (Source A)					
Pork belly (Source A)					
Pork feet (Source A)					
Pork mince (Source B)		Presumptive	+	-	+
Pork belly (Source B)		Presumptive	+	-	+
Organic belly (Source D)		Presumptive	+	-	-
Free-range loins (Source C)		Presumptive	-	-	n/a

A Gram stain was also performed and isolates were confirmed as Gram positive cocci before testing further. A total of four isolates were tested. Two isolates, one from pork mince and one from pork belly (sampling 2), were catalase positive, oxidase negative and coagulase positive and therefore presumptive *Staph. aureus* and these were further tested using an API Staph (Section 2.5.2). Results showed for both isolates as 97.7% S (similarity) with *Staphylococcus aureus*. One isolate,

from organic pork belly, was catalase positive, oxidase negative and coagulase negative and in view of the latter reaction, therefore it is not likely to be *Staph. aureus.* Another isolate from free-range pork was shown to be both catalase and oxidase negative illustrating that this isolate does not belong to the *Staphylococcus* genus. The latter isolate was then subjected to a streptococcal agglutination test (Section 2.4.6) for the identification of streptococcal groups. Agglutination was observed for group D (*Enterococcus* genus). In addition, CHROMagar plates (Section 2.1.21) showed growth of turquoise blue colonies, typical for *Enterococcus* while the rest of the isolates showed growth of golden/cream coloured colonies which are typical for *Staphylococcus.* All four isolates were then sent to Source Bioscience for 16S sequencing (Section 3.2).

3.1.3 *Yersinia enterocolitica*

Isolation of this pathogen proved to be time-consuming and very challenging. According to results obtained after each round of sampling, the method was altered in order to improve recovery rates for every newly purchased sample. Presumptive colonies appear as a transparent red bull's-eye on CIN agar and 1 mm round colourless colonies on SSDC agar. For sampling 1, diluting the meat samples in ITC broth solely (Figure 3.1) (Section 2.3.4.1.2) recovered very few presumptive colonies and no confirmed *Yersinia* according to 16S sequencing results (Section 3.2) and therefore this isolation method was not repeated for samplings 2, 3 and 4. Direct plating onto the selective media was also excluded after sampling 1 mainly because of the abundance of other background flora which made it difficult to select for presumptive colonies. Cold incubation at 4^{o}C for 14 days was then incorporated into the isolation method using the pre-enrichment and selective enrichment

isolation methods (Section 2.3.4.1). Using the pre-enrichment isolation method (Figure 3.1), samples were only spread onto CIN agars after 2 and 5-days incubation at 25°C and at 4°C for 2 weeks (all with and without alkaline treatment). Using the selective enrichment isolation method (Figure 3.1) the samples' incubations periods were kept the same as detailed in the pre-enrichment method but plating was altered. SSDC plates were spread from samples from all three incubation conditions and/or periods, but unlike in Van Damme *et al.* (2010), CIN agars were also spread. Alkaline treatment was only performed for plating onto CIN agar as SSDC has previously been proved an unsuitable medium for plating after alkaline treatment according to Wauters *et al.* (1988).

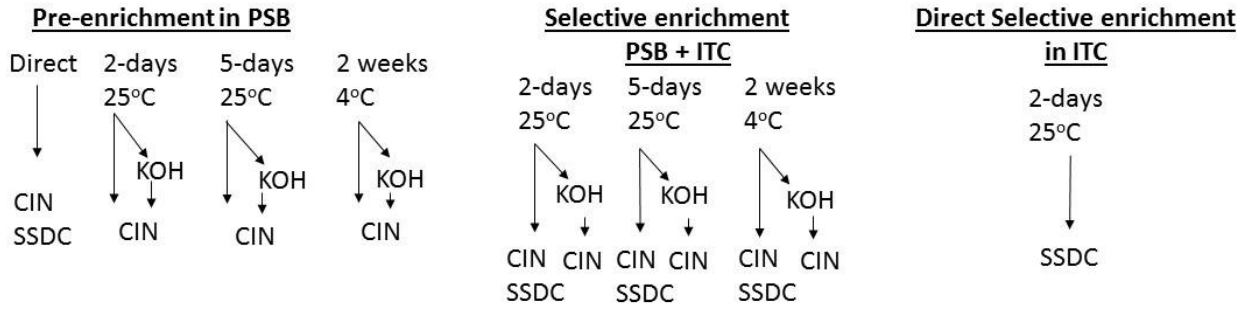


Figure 3.1: Diagram of the steps involved for the isolation of *Yersinia enterocolitica*.

Despite the fact that presumptive colourless colonies were observed on SSDC plates, other background flora also grew (Figure 3.2). Higher dilutions were required for plating on SSDC in order to reduce colonies counts. CIN, on the other hand, when inoculated with samples after alkaline treatment proved to be much better for the recovery of *Yersinia enterocolitica* as it was observed that growth of

background flora was inhibited and colony count was also reduced making it easier to select for presumptive colonies.

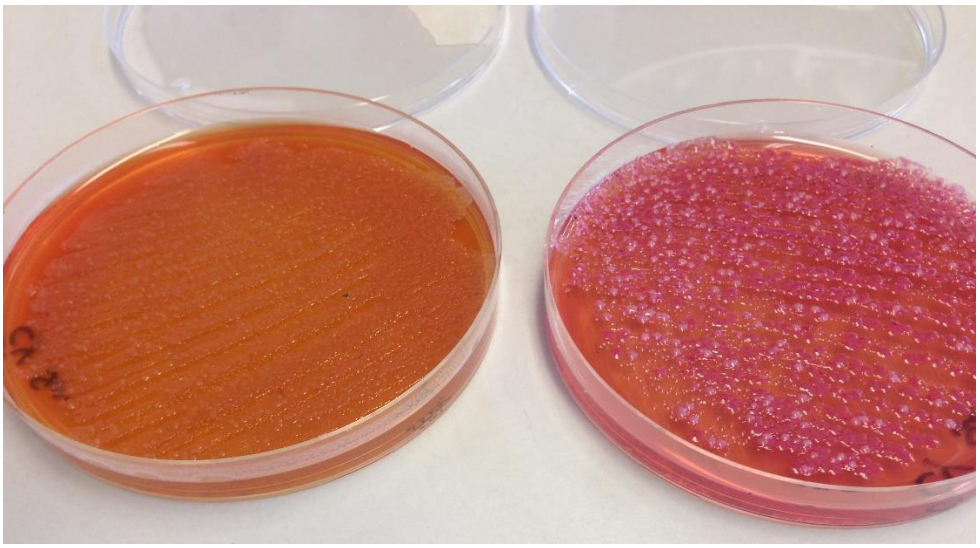


Figure 3.2: Comparison of sample growth mixed colonies on *Yersinia* selective SSDC (left) and CIN (right) agars.

Presumptive *Yersinia* colonies from SSDC and CIN agars were re-streaked onto NA plates (Section 2.1.5) to obtain pure cultures. The colonies were then streaked onto MacConkey agar (Section 2.1.6). *Yersinia* appears as colourless colonies on MacConkey agar (typical for non-lactose fermenters). MacConkey agar differentiates the three pathogenic *Yersinia* species from *Pasteurella* species when incubated at 35°C. *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* grow at 35°C unlike *Pasteurella* species (Oxoid, 2017). However, results were variable as MacConkey plates were initially incubated at 37°C and growth was not observed for the majority of the plates at that temperature. The feet sample from Sampling 1 showed growth at 37°C and were further tested using API 20E (Section 2.5.1). API showed 80.8% S to *Raoultella ornithinolytica* and 12.6% S to *Serratia odorifera*. Therefore, lactose fermentation was then omitted from the detection method and isolates, which were Gram-negative non-spore forming coccobacilli, catalase

positive, oxidase negative and urease positive were considered *Yersinia* isolates. Table 3.3 summarises all selected isolates and their biochemical testing results to the three main tests performed: catalase, oxidase and urease. *Yersinia enterocolitica* is catalase positive, oxidase negative and urease positive. However, all isolates were 16S sequenced (Section 3.2) for identification despite the biochemical testing results. This allowed observation of false-positive organisms that grow as presumptive on the selective agar but also showed other organisms that can survive the *Yersinia* isolation methodology.

Table 3.3: Biochemical testing of unknown isolates obtained from *Yersinia* isolation methods.[1]

Unknown isolates	Catalase	Oxidase	Urease
Sampling 1: Source A			
Direct on SSDC feet	+	+	-
2 days PSB KOH feet	+	-	+
2 days PSB no KOH feet	+	-	+
Cold incubation feet	+	+	-
2 days ITC mince	+	+	+/-
2 days ITC on SSDC belly	+	-	+
2 days PSB no KOH mince	+	-	+/-
5 days PSB KOH belly	+	-	+
5 days PSB no KOH belly	+	-	+/-
2 days ITC feet	+	+	-
2 days PSB no KOH belly	+	-	+
5 days PSB no KOH feet	+	-	+/-
5 days PSB KOH mince	+	-	+/-
5 days PSB no KOH mince	+	-	+/-
Cold incubation belly	+	-	-
Cold incubation Mince	+	-	-
Sampling 2: Source B			
5 days KOH mince	+	-	+

[1] Isolates presumed as *Yersinia* are highlighted.

Unknown isolates	Catalase	Oxidase	Urease
5 days no KOH mince	+	-	+/-
2 days PSB+ITC belly	+	-	-
2 days PSB KOH mince	+	-	+
2 days PSB no KOH mince	+	-	+
2 days PSB no KOH belly	+	-	+/-
2 days PSB+ITC mince	+	-	-
Cold incubation PSB no KOH mince	+	-	-
Cold incubation PSB+ITC mince	+	-	-
Cold incubation PSB no KOH belly	+	-	+
2 days PSB KOH belly	+	-	-
5 days PSB no KOH belly	+	-	+
Sampling 3: Source C			
2 days PSB KOH	+	-	+
2 days PSB no KOH	+	-	+
5 days PSB KOH	+	-	+
5 days PSB no KOH	+	-	+
Cold incubation PSB+ITC	+	+	+/-
Cold incubation PSB no KOH	+	-	+
Cold incubation PSB KOH	+	-	+
Sampling 4: Source D			
2 days PSB KOH	+	-	+
2 days PSB no KOH	+	-	-
5 days PSB KOH	+	-	+
5 days PSB no KOH	+	-	+
Cold incubation KOH	+	-	+
Cold incubation no KOH	+	-	+

3.2 16S rDNA Sequencing

For all isolates which were provisionally identified as *E.coli*, *Yersinia* or *Staphylococcus*/*Enterococcus,* amplification of the V1-4 of 16S rDNA was undertaken as described in Section 2.7.3. From the sequence results (Table 3.4), the eight isolates tested were confirmed as belonging to the *Escherichia* genus, with

six of them identified as *Escherichia coli.* Two of the isolates came from swabs taken from pork carcasses from a small local non-commercial abattoir used for teaching; two were from pork meat purchased from Source A in Nottingham city. The rest of the isolates derived from organic pork belly (Source D), including the two isolates that were only identified to genus level. The majority of the confirmed isolates were obtained using the membrane method (TBX + MEM) (Section 2.3.2.1). The two *Staphylococcus aureus* isolates identified from API Staph were also confirmed using 16S rDNA sequencing. The one coagulase-negative isolate was identified as *Staphylococcus* sp*.* and so sequencing did not improve its identification. The one *Enterococcus* sp. isolate was identified as *Enterococcus faecalis.* Results are shown in Table 3.5. The *Yersinia enterocolitica* isolation method resulted in many false-positives. However, 12 of the total of 43 putative isolates that were tested were identified as *Yersinia* spp (Table 3.6). Half of those isolates were confirmed to be *Yersinia enterocolitica* strains, one was only identified to genus level and the rest were identified as *Yersinia frederiksenii*.

Table 3.4: Sequencing results for presumptive *E. coli*.

Unknown samples	**Expected**	**16S sequencing results**	**% ID**	**Strain accession number**
Sampling 1: Source A				
Belly on TBX + MEM	*Escherichia coli*	*Escherichia coli*	99	KX349997.1
Feet on TBX + MEM	*Escherichia coli*	*Escherichia coli*	100	CP022912.1
Sampling 4: Source D				
Belly on TBX + MEM	*Escherichia coli*	*Escherichia* sp.	99	KR148972.1
Belly on TBX direct	*Escherichia coli*	*Escherichia* sp.	99	KR148973.1
Belly on TBX + MEM	*Escherichia coli*	*Escherichia coli*	99	KU156690.1
Belly on TBX + MEM	*Escherichia coli*	*Escherichia coli*	99	MF428963.1
Abattoir swabs				
Pig carcass 1- feet on TBX	*Escherichia coli*	*Escherichia coli*	99	MF682399.1
Pig carcass 2- feet on TBX	*Escherichia coli*	*Escherichia coli*	99	MF682399.1

Table 3.5: Sequencing results for Gram-positive isolates.

Unknown samples	**Expected**	**16S sequencing results**	**% ID**	**Strain accession number**
Exp. 2: Source B				
Belly	*Staphylococcus aureus*	*Staphylococcus aureus*	99	MF157603.1
Mince	*Staphylococcus aureus*	*Staphylococcus aureus*	100	AP017922.1
Exp. 3: Source C				
Pork loins	*Enterococcus* spp.	*Enterococcus faecalis*	99	MF369842.1
Exp. 4: Source D				
Belly	*Staphylococcus aureus*	*Staphylococcus* sp.	100	JN853151.1

Table 3.6: Sequencing results of unknown isolates obtained from *Yersinia* isolation method.[2]

Unknown samples	**Expected**	**16S sequencing results**	**% ID**	**Strain accession number**
Sampling 1: Source A				
Direct on SSDC feet	*Yersinia enterocolitica*	*Aeromonas hydrophila*	99	MF716692.1
2 days PSB KOH feet	*Yersinia enterocolitica*	*Serratia* sp.	99	KR189431.1
2 days PSB no KOH feet	*Yersinia enterocolitica*	*Citrobacter braaki*	99	KP739920.1
Cold incubation feet	*Yersinia enterocolitica*	*Shewanella* sp.	100	FJ002584.1
2 days ITC mince	*Yersinia enterocolitica*	*Pseudomonas qessardii*	100	KT382252.1
2 days ITC on SSDC belly	*Yersinia enterocolitica*	Enterobactericeae bacterium – not defined	99	KX817233.1
2 days PSB no KOH mince	*Yersinia enterocolitica*	*Citrobacter freundii*	99	MF716708.1
5 days PSB	*Yersinia*	*Yersinia*	99	CP009456.1

[2] Highlighted isolates are those presumed as *Yersinia* after biochemical testing results in Table 3.3.

KOH belly	*enterocolitica*	*enterocolitica*		
Unknown samples	**Expected**	**16S sequencing results**	**% ID**	**Strain accession number**
5 days PSB no KOH belly	*Yersinia enterocolitica*	*Citrobacter* sp.	99	KY938131.1
2 days ITC feet	*Yersinia enterocolitica*	*Pseudomonas plecoglossicida*	99	AY972231.1
2 days PSB no KOH belly	*Yersinia enterocolitica*	*Citrobacter braaki*	99	KY404158.1
5 days PSB no KOH feet	*Yersinia enterocolitica*	*Citrobacter* sp.	99	MF457854.1
5 days PSB KOH mince	*Yersinia enterocolitica*	*Yersinia enterocolitica*	99	CP009456.1
5 days PSB no KOH mince	*Yersinia enterocolitica*	*Citrobacter freundii*	98	MF716708.1
Cold incubation belly	*Yersinia enterocolitica*	*Hafnia* sp.	99	KX378947.1
Cold incubation Mince	*Yersinia enterocolitica*	*Rahnella* sp.	99	JF494812.1
Sampling 2: Source B				
5 days KOH mince	*Yersinia enterocolitica*	*Yersinia* sp.	100	KF811209.1
5 days no KOH mince	*Yersinia enterocolitica*	*Rahnella* sp.	99	KR189413.1
2 days PSB+ITC belly	*Yersinia enterocolitica*	*Hafnia* sp.	99	KJ191378.1
2 days PSB KOH mince	*Yersinia enterocolitica*	*Serratia liquefaciens*	99	KY048397.1
2 days PSB no KOH mince	*Yersinia enterocolitica*	*Serratia* sp.	98	KF737363.1
2 days PSB no KOH belly	*Yersinia enterocolitica*	*Citrobacter freundii*	98	KY378909.1
2 days PSB+ITC mince	*Yersinia enterocolitica*	*Hafnia alvei*	99	KY940341.1
Cold incubation PSB no KOH mince	*Yersinia enterocolitica*	Enterobactericeae bacterium-not defined	99	KY681873.1
Cold incubation PSB+ITC mince	*Yersinia enterocolitica*	*Rahnella aquatilis*	99	DQ440548.1
Cold	*Yersinia*	*Yersinia*	99	CP011286.1

incubation PSB no KOH belly	*enterocolitica*	*enterocolitica*		
Unknown samples	**Expected**	**16S sequencing results**	**% ID**	**Strain accession number**
2 days PSB KOH belly	*Yersinia enterocolitica*	*Serratia liquefaciens*	99	KY643700.1
5 days PSB no KOH belly	*Yersinia enterocolitica*	*Yersinia enterocolitica*	99	CP009456.1
Sampling 3: Source C				
2 days PSB KOH	*Yersinia enterocolitica*	*Yersinia enterocolitica*	99	CP009456.1
2 days PSB no KOH	*Yersinia enterocolitica*	*Yersinia enterocolitica*	99	CP009456.1
5 days PSB KOH	*Yersinia enterocolitica*	*Yersinia frederiksenii*	98	KC776773.1
5 days PSB no KOH	*Yersinia enterocolitica*	*Yersinia frederiksenii*	98	KC776773.1
Cold incubation PSB+ITC	*Yersinia enterocolitica*	*Pseudomonas psychrophila*	99	KJ626249.1
Cold incubation PSB KOH	*Yersinia enterocolitica*	*Yersinia frederiksenii*	99	KC776773.1
Cold incubation PSB no KOH	*Yersinia enterocolitica*	*Yersinia frederiksenii*	99	KC776773.1
Sampling 4: Source D				
2 days PSB KOH	*Yersinia enterocolitica*	*Serratia* sp.	97	HM055762.1
2 days PSB no KOH	*Yersinia enterocolitica*	*Rahnella aquatilis*	100	KF817729.1
5 days PSB KOH	*Yersinia enterocolitica*	*Serratia* sp.	97	HM055762.1
5 days PSB no KOH	*Yersinia enterocolitica*	*Yersinia frederiksenii*	99	KC776773.1
Cold incubation KOH	*Yersinia enterocolitica*	*Serratia* sp.	100	JQ322839.1
Cold incubation no KOH	*Yersinia enterocolitica*	*Serratia* sp.	99	KP318035.1

All of the confirmed *Yersinia* isolates were isolated on CIN agars inoculated with samples that were incubated in PSB broth solely. Interestingly, the ratio between confirmed isolates that were subjected to alkaline treatment to those that were not, was 50-50. 16.7% of the isolates were from samples incubated for 2 days at 25°C, 58.3% were from samples incubated for 5 days at 25°C and 25% were from samples incubated at 4°C for 14 days. The other 72% of the isolates were false-positives that survived the isolation process and managed to grow on the selective media used.

Table 3.7 summarises the confirmed isolate identifications, their given name codes and the meat products they were isolated from.

Table 3.7: Confirmation of isolates and their sources.

Isolates	Codes		Experiment
Staphylococcus aureus	2StB[3]	*Staph*. from belly	Sampling 2: Source B pork belly
	2StM	*Staph*. from mince	Sampling 2: Source B pork mince
***Staphylococcus* sp.**	4St	Sampling 4, *Staph*.	Sampling 4: Source D organic pork belly
Escherichia coli	Sw1F	Swab carcass No.1, feet	Abattoir swabs
	Sw2F	Swab carcass No.2, feet	Abattoir swabs
	1B	Belly	Sampling 1: Source A pork belly
	1F	Feet	Sampling 1: Source A pork feet
	4EcRB	*Escherichia coli* from mem+tbx	Sampling 4: Source D organic pork belly
	4EcRX		Sampling 4: Source D organic pork belly
***Escherichia* spp.**	4Etbx	*Escherichia* from direct tbx	Sampling 4: Source D organic pork belly
	4Emem	*Escherichia* from membrane	Sampling 4: Source D organic pork belly
Yersinia enterocolitica	1PK5B	5 days PSB KOH belly	Sampling 1: Source A pork belly
	1PK5M	5 days PSB KOH mince	Sampling 1: Source A pork mince
	2PNK5B	5 days PSB no KOH belly	Sampling 2: Source B pork belly
	2CPNKB	Cold incubation PSB no KOH belly	Sampling 2: Source B pork belly
	3PK2	2 days PSB KOH	Sampling 3: Source C pork loin
	3PNK2	2 days PSB no KOH	Sampling 3: Source C pork loin
***Yersinia* sp.**	2PK5M	5 days PSB KOH mince	Sampling 2: Source B pork mince
Yersinia frederiksenii	3PK5	5 days PSB KOH	Sampling 3: Source C pork loin
	3PNK5	5 days PSB no KOH	Sampling 3: Source C pork loin
	3CPNK	Cold incubation PSB no KOH	Sampling 3: Source C pork loin
	3CPK	Cold incubation PSB KOH	Sampling 3: Source C pork loin
	4PNK5	5 days PSB no KOH	Sampling 4: Source D organic pork belly
Enterococcus faecalis	3E	Sampling 3, *enterococcus*	Sampling 3: Source C pork loin

[3] The first number of every code indicates the sampling number.

3.3 Phylogenetic Analysis of 16S rDNA Sequences

Phylogenetic analysis was conducted using the 16S rRNA of all isolates from the sequencing results (Section 2.7.3) along with the 16S rRNA sequences of some strains from the data base (Section 2.9) in order to confirm identified isolates and to try to further categorise the unidentified isolates. Ribosomal RNAs are considered valuable molecular chronometers. They occur in all organisms, are functionally constant and different positions within their sequences change at very diverse rates, thus allowing phylogenetic relationships to be measured from the most distant to the most recent (Woese, 1987).

3.3.1 *E. coli*

Eight *Escherichia* isolates were obtained from this study, with six of them identified as *E. coli* (Table 3.7). The phylogenetic analysis (Figure 3.3) clustered the isolates into groups according to their relatedness. Isolate 1F and isolate 4Emem are sister taxa, thus they are each other's closest relatives. Therefore, since 1F is an *E. coli* and 4Emem did not cluster as an outgroup, it is reasonable to conclude that this isolate is an *E.coli* species. The second unidentified isolate, 4Etbx, clustered with an *E. coli* O104:H4 strain suggesting, as well, the possibility of falling within the same species. Similarly clustered were isolates Sw1F, Sw2F and 1B which appeared very

closely related with a Shiga-toxin producing *E. coli* O157 strain.

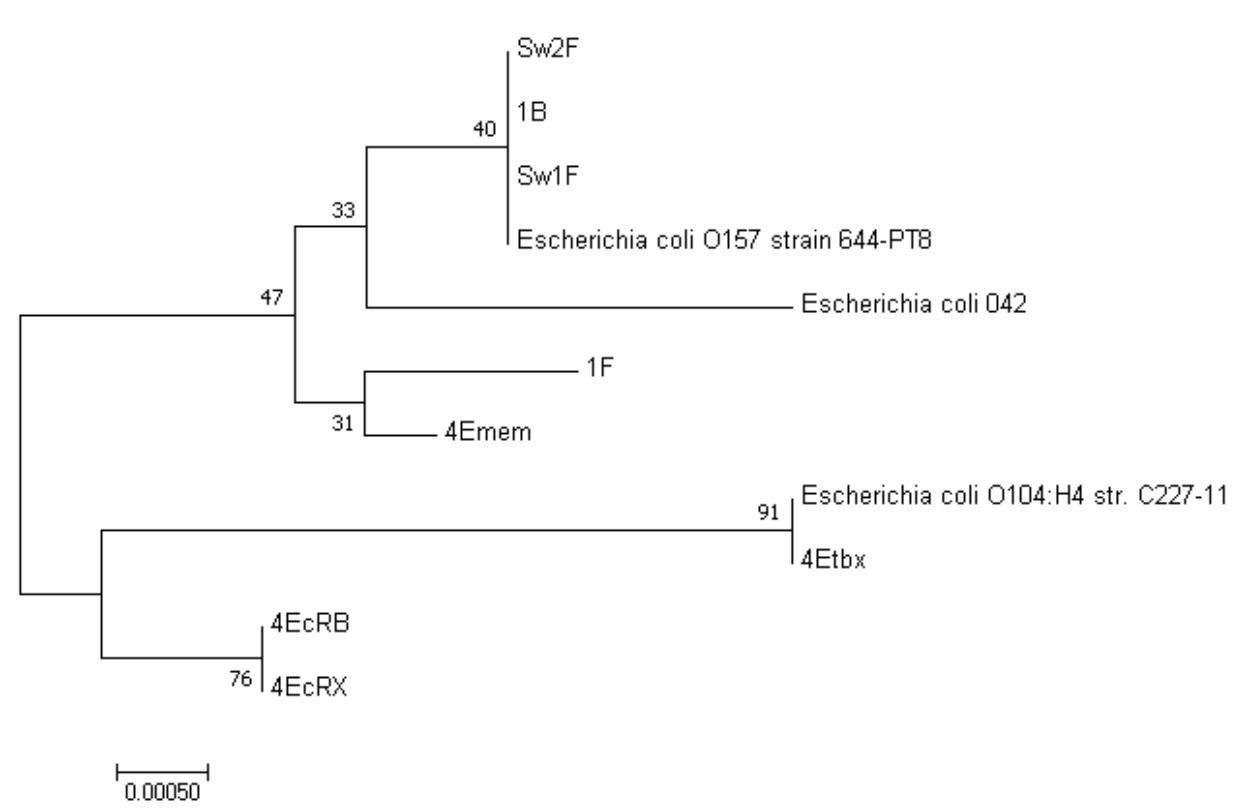


Figure 3.3: Evolutionary relationship of *E. coli* isolates. The phylogenetic tree was constructed using Kimura's 2-parameter distance and the neighbour-joining method. The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are in the units of the number of base substitutions per site. The analysis involved 11 16S rDNA V1-V4 region nucleotide sequences and positions containing gaps and missing data were eliminated. There were a total of 649 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7 software.

3.3.2 *Yersinia*

In Figure 3.4, it is clearly indicated that the isolates identified by 16S sequencing are correctly closely related with the known strains of the same species. For example, the isolates grouped at the top branch were all identified as *Yersinia frederiksenii* and correctly clustered with the known strain ATCC 33641. The unknown *Yersinia* isolate 2PK5M was clustered with *Yersinia kristensenii* ATCC 33639 and are also considered as sister taxa. The six *Yersinia enterocolitica* isolates

clustered at the bottom branches of the phylogenetic tree are shown to be more closely related to a known *Yersinia enterocolitica* strain. Therefore, the clusters produced using the phylogenetic analysis support the identifications produced using a similarity comparison of the data and hence it is reasonable to suggest that isolate 2PK5M is *Yersinia kristensenii.*

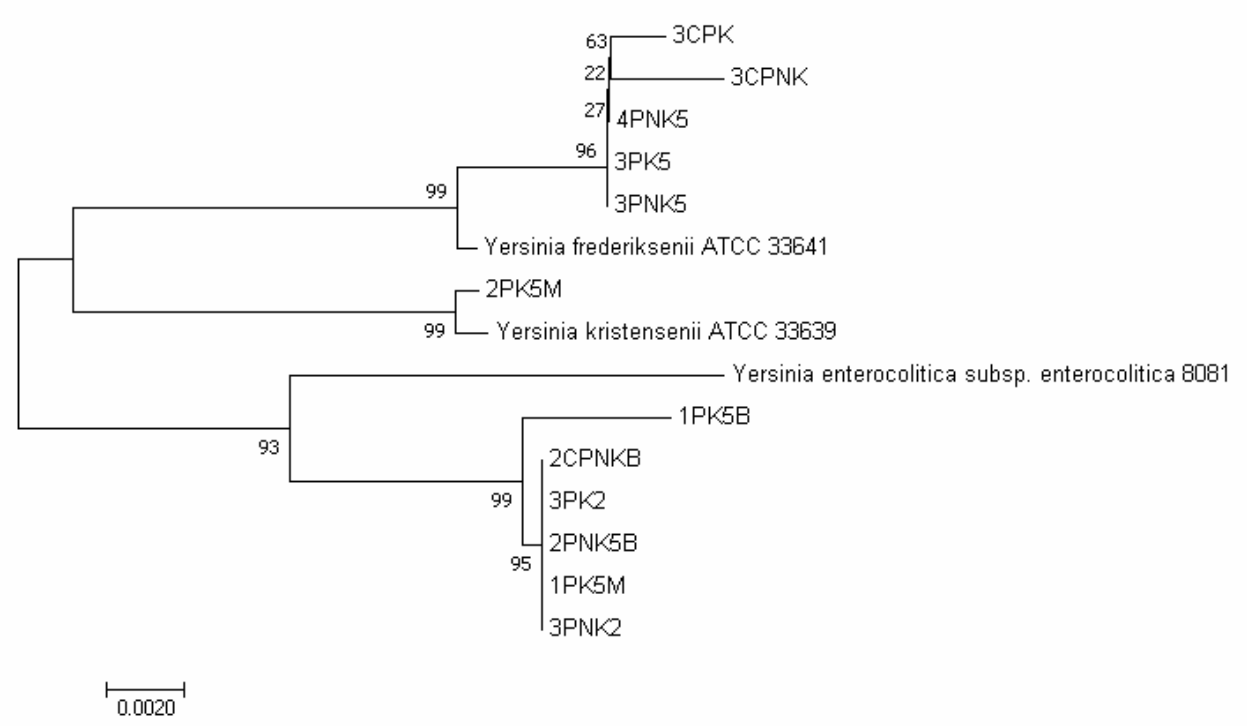


Figure 3.4: Evolutionary relationship of *Yersinia* isolates. The phylogenetic tree was constructed using Kimura's 2-parameter distance and the neighbour-joining method. The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are in the units of the number of base substitutions per site. The analysis involved 15 16S rDNA V1-V4 region nucleotide sequences and positions containing gaps and missing data were eliminated. There were a total of 692 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7 software.

3.3.3 *Staphylococcus aureus*

In Figure 3.5, both *Staph. aureus* isolates 2StB and 2StM fully correlated to the known ATCC 25923 strain whilst the unknown 4St isolate clustered with a known

Staphylococcus vitulinus strain, suggesting it is a *S. vitulinus* species. Once more, the phylogenetic analysis was consistent with the identifications produced using a direct similarity comparison of the 16S results and helped to further identify the unknown isolates, where this latter analysis did not.

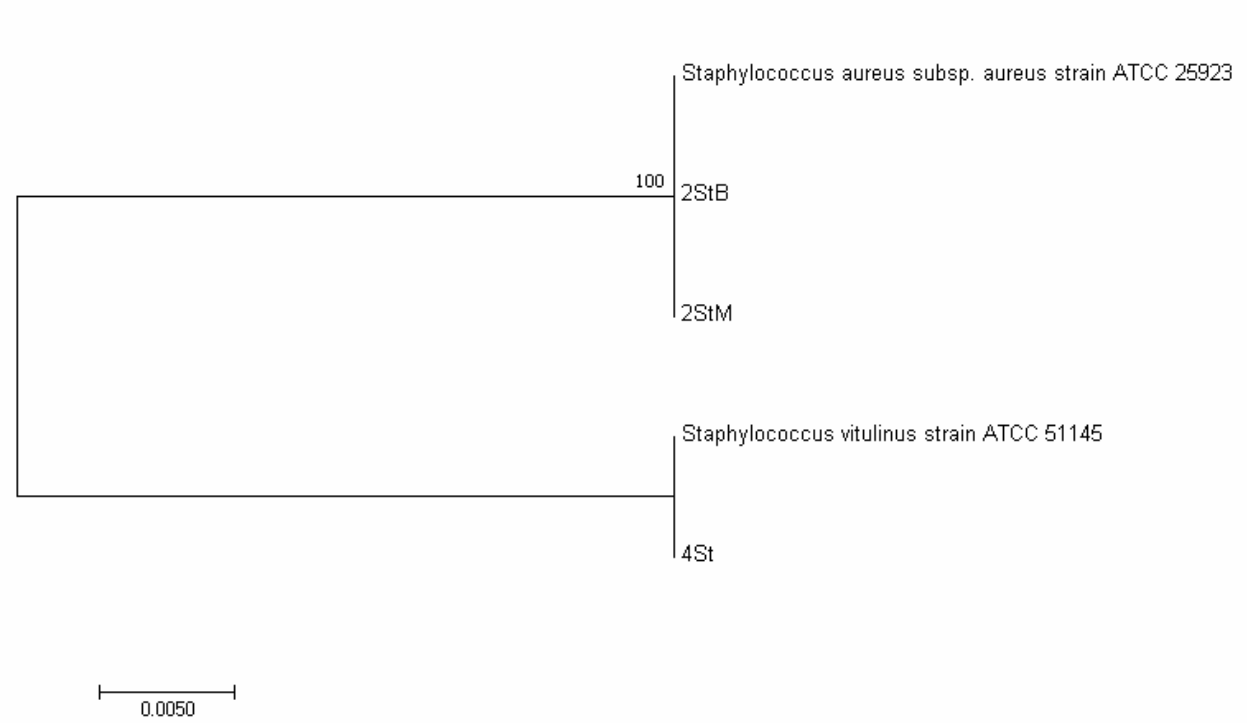


Figure 3.5: Evolutionary relationship of *Staphylococcus* isolates. The phylogenetic tree was constructed using Kimura's 2-parameter distance and the neighbour-joining method.The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are in the units of the number of base substitutions per site. The analysis involved 5 16S rDNA V1-V4 region nucleotide sequences and positions containing gaps and missing data were eliminated. There were a total of 730 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7 software.

3.4 Antimicrobial Disc Diffusion Assays

E. coli strain ATCC 25922 was used as an antibiotic sensitive negative control in the antibiotic disc diffusion assays for the Enterobactericeae tested (Table 2.3).

3.4.1 *E. coli*

All isolates were tested using fifteen different antibiotic classes according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2012). Results are shown in Table 3.8. Resistance was found in the *E. coli* isolates for eight of those drug classes. Six isolates showed AMR while two isolates were sensitive to all antibiotics tested. Four isolates showed resistance to ampicillin and ampicillin with clavulanic acid. Four showed resistance to sulphonamides and no isolates were resistant to cephalosporins. Isolate 1B showed resistance to tetracyclines, sulphonamides and chloramphenicol while isolates Sw1F, Sw2F and 4Emem exhibited colistin resistance. Therefore, 62.5% of the isolates (5 isolates) can be considered as multidrug resistant (MDR; as defined by Ibrahim *et al.* 2016) as they exhibited resistance to three or more antibiotic classes; with isolate 1B exhibiting the highest number of resistances in six drug classes. MDR isolates Sw1F, Sw2F, 1B and 1F were derived from two different sources. Sw1F and Sw2F were obtained from pig carcasses while 1B and 1F were from pork products purchased from Source A, although not from the same sample (Table 3.7). The first two demonstrated the same resistances (and were from the same source) while 1B and 1F showed differences with the latter being sensitive to ampicillin, ampicillin with clavulanic acid and sulfamethoxazole / trimethoprim. Isolate 4Etbx showed resistance to three antibiotic classes while 4Emem was resistant only to one. In contrast, isolates 4EcRB and 4EcRX were sensitive to all antibiotics despite being isolated from the same pork sample (organic pork) as 4Etbx and 4Emem.

Table 3.8: Antimicrobial susceptibility testing results for *E. coli* isolates.

	Antibiotic Class																					
	Penicillins	B-Lactam/ase inhibitor	Second Gen Ceph	Third Gen Cephalosporin				Fourth Gen Ceph	Monobactam	Carbapenem			Amino-glycosides	Tetracyclines		Fluoro-quinolones		Quinolones	Folate Pathway Inhibitors		Phenicols	Nitrofurans	Polymyxin
Isolates	AMP	AMC	FOX	CAZ	CTX	CPD	EFT	CFQ	ATM	MEM	ETP	IPM	S10	TE	OT	ENR	CIP	NA	S300	SXT	C	F	CT
Sw1F	15	15	25	27	31	22	26	31	30	26	27	28	17	24	21	34	37	23	0	21	26	18	11
Sw2F	13	16	25	25	31	22	28	31	30	26	28	28	17	25	22	35	37	23	0	22	23	19	12
1B	0	10	27	33	35	28	33	31	37	26	32	30	13	0	0	42	41	25	0	0	9	20	15
1F	17	20	28	33	37	30	29	32	40	28	32	31	13	0	0	38	40	27	0	25	15	21	15
4Emem	20	21	27	32	36	28	28	33	40	28	35	31	18	28	24	34	40	25	23	20	30	18	9
4Etbx	10	9	25	31	36	23	28	31	35	21	37	37	21	27	25	36	40	25	22	22	27	22	15
4EcRB	20	19	26	28	35	27	28	36	30	26	35	33	20	28	26	35	36	24	22	29	25	22	14
4EcRX	20	21	26	30	36	25	27	37	32	29	38	33	19	28	27	34	40	26	22	27	28	21	15
ATCC 25922	22	20	28	29	33	28	28	31	33	38	35	38	23	33	30	33	42	29	20	26	32	26	14

[4]

[4] Numbers refer to the diameter of zones of inhibition observed around the discs. Green colour indicates isolate is susceptible to the antibiotic, yellow indicates intermediate resistance and red indicates isolate is resistant. *E. coli* ATCC 25922 was used as a negative control. Interpretation of results was according to CLSI (2014)and Veterinary practice (CLSI, 2010)for cefquinome, ceftiofur and enrofloxacin. For the full name of the antibiotics used, refer to Table 2.1 in Section 2.6.3.

3.4.2 *Yersinia enterocolitica*

All isolates were tested using the same antibiotic groups as *E. coli,* Table 3.9. All showed resistance towards ampicillin, ampicillin and clavulanic acid and cefoxitin (a 2nd generation cephalosporin). The majority of the isolates (83.3%) also exhibited intermediate resistance to cefpodoxime, a 3rd generation cephalosporin. Additionally, sulphonamide resistance was common among the isolates with five out of six being resistant. One of the isolates also showed resistance towards nitrofurantoin. All isolates are considered MDR since they have been shown to be resistant to three or more antibiotic classes, with isolate 3PNK2 showing resistance to six different antibiotic classes. Similarities in resistances were observed among isolates from the same products. Isolates 2CPNKB and 2PNK5B were both obtained from pork belly purchased at Source B and were resistant to the same drug classes. Isolates 3PK2 and 3PNK2 were isolated from the same product obtained from the same source, but the latter isolate exhibited an additional resistance (to nitrofurantoin) that was not seen for 3PK2. Isolates 1PK5B and 1PK5M were obtained from different products of the same source (A). Resistances observed were quite similar; however, isolate 1PK5M was sensitive to 3rd generation cephalosporins while 1PK5B was sensitive to sulphonamides instead.

3.4.3 *Yersinia frederiksenii*

All of the non-*Y. enterocolitica* isolates only showed resistances to four out of the fifteen drug classes tested (Table 3.10); half of those seen with *E. coli.* They all exhibited resistance to ampicillin and full to intermediate resistance for ampicillin and clavulanic acid combo. Resistance was also observed for sulphonamide compounds in five out of six isolates and two if the isolates also showed intermediate resistance to trimethoprim/sulfamethoxazole. Interestingly enough, all

isolates were resistant to nitrofurantoin. All isolates are considered MDR since they demonstrated resistance towards three or more antibiotic classes. 66.6% of the isolates were obtained from the product purchased at Source C and all exhibited resistance to nitrofurantoin. The *Y. enterocolitica* isolate that exhibited resistance to nitrofurantoin was also isolated from the same product. Only one isolated from that product appeared sensitive towards the antibiotic. Resistance to sulphonamides was also common among isolates from the same product origin, with all isolates, both *Y. enterocolitica* and *Y. frederiksenii*, from Source C being resistant. Interestingly, isolate 4PNK5 from organic pork belly purchased at Source D was resistant to ampicillin, ampicillin/clavulanic acid, sulphonamides and nitrofurantoin (same resistances as other *Y. frederiksenii* isolates), unlike *E. coli* isolates from the same pork sample.

Table 3.9: Antimicrobial susceptibility testing results for *Yersinia enterocolitica* isolates.[5]

	Antibiotic Class																					
	Penicillins	B-Lactam/ase Inhibiot comb	Second Gen Ceph	Third Gen Cephalosporin				Fourth Gen Ceph	Monobactam	Carbapenem			Amino-glycosides	Tetracyclines		Fluoro-quinolones		Quinolones	Folate Pathway Inhibitors		Phenicols	Nitrofurans	Polymyxin
Isolate	AMP	AMC	FOX	CAZ	CTX	CPD	EFT	CFQ	ATM	MEM	ETP	IPM	S10	TE	OT	ENR	CIP	NA	S300	SXT	C	F	CT
1PK5B	0	7	9	30	35	20	29	40	33	32	41	32	21	33	35	40	45	35	23	19	26	18	16
2CPNKB	0	8	9	29	34	19	30	39	35	30	40	31	20	31	30	42	45	36	15	31	30	18	16
2PNK5B	0	10	11	28	32	20	32	38	33	30	38	31	19	32	32	39	40	35	0	30	30	17	16
1PK5M	0	7	17	31	35	25	30	38	30	30	39	34	20	31	33	42	47	33	16	30	27	17	16
3PK2	0	0	11	28	33	18	29	38	32	29	39	32	22	31	33	37	42	31	12	28	30	17	14
3PNK2	0	0	11	31	35	20	0	38	33	28	36	31	20	29	31	41	43	34	0	27	28	14	15

[5]Results were interpreted as per Table 3.8.

Table 3.10: Antimicrobial susceptibility testing results for *Yersinia* sp.* and *Yersinia frederiksenii* isolates. [6]

	Antibiotic Class																					
	Penicillins	B-Lactam/ase Inhibiot comb	Second Gen Ceph	Third Gen Cephalosporin				Fourth Gen Ceph	Monobactam	Carbapenem			Amino-glycosides	Tetracyclines		Fluoro-quinolones		Quinolones	Folate Pathway Inhibitors		Phenicols	Nitrofurans	Polymyxin
Isolate	**AMP**	**AMC**	**FOX**	**CAZ**	**CTX**	**CPD**	**EFT**	**CFQ**	**ATM**	**MEM**	**ETP**	**IPM**	**S10**	**TE**	**OT**	**ENR**	**CIP**	**NA**	**S300**	**SXT**	**C**	**F**	**CT**
2PK5M*	0	9	28	33	43	31	32	43	35	33	43	37	25	34	36	42	48	40	28	23	29	13	18
3PK5	0	16	29	32	37	24	35	41	32	31	44	38	22	29	31	41	42	35	9	15	33	8	16
3PNK5	0	16	27	33	39	25	30	37	33	28	37	33	24	31	28	40	43	34	0	15	31	8	17
3CPK	0	12	27	32	34	23	31	36	30	28	42	36	21	33	34	40	45	33	0	20	37	15	16
3CPNK	0	12	26	32	36	23	31	40	30	29	42	36	20	31	32	41	40	34	9	25	32	15	16
4PNK5	0	16	27	33	37	25	31	40	32	32	42	39	20	30	30	41	41	32	10	16	30	12	17

[6]Results were interpreted as per Table 3.8.

3.4.4 *Staphylococcus aureus*

Isolates were tested for sensitivity to thirteen antibiotic classes and results are shown in Table 3.11. *Staphylococcus aureus* isolate 2StM is a MDR isolate that exhibited full resistance towards oxytetracycline, clindamycin and erythromycin. All isolates showed intermediate resistance to enrofloxacin. None of the isolates demonstrated clindamycin-inducible resistance from the D-zone tests performed (Section 2.6.5). Additionally, according to the phenotypic assay, none were MRSA strains since resistance to cefoxitin was not observed.

3.4.5 *Enterococcus faecalis*

This isolate was tested for sensitivity to ten antibiotics, primarily to search for vancomycin-resistant enterococci (VRE), as it is a major clinical concern since glycopeptides, such as vancomycin, are first line treatment drugs against infections cause by enterococci (Dutka-Malen *et al.,* 1995). Results are shown in Table 3.12. However, no resistances were observed apart from trimethoprim-sulfamethoxazole, which is uncertain against enterococci, and therefore a wild type population is generally categorised as intermediate for resistance (EUCAST, 2017). No further tests were carried out on this particular isolate.

Table 3.11: Antimicrobial susceptibility testing results for *Staphylococcus aureus* and *Staphylococcus* sp.* isolates. [7]

		Antibiotic Class																	
		Penicillins	B-Lactamyase Inhibiot combo	Second Gen Ceph	Third Gen Cephalosporin			Carbapenem			Tetracyclines	Fluoro-quinolones		Folate Pathway Inhibitors	Phenicols	Nitrofurans	Glycopeptides	Lincosamides	Macrolides
coagulase	**Isolate**	**AMP**	**AMC**	**FOX**	**CTX**	**CPD**	**EFT**	**MEM**	**ETP**	**IPM**	**OT**	**ENR**	**CIP**	**SXT**	**C**	**F**	**VA**	**DA**	**E**
positive	2StM			30			28				0	25	30	28	23	17	17	0	0
positive	2StB			30			28				28	25	26	28	26	17	17	30	27
negative	4St*			33			28				31	25	32	23	27	27	18	31	31

Note: According to CLSI (2014), cefoxitin-susceptible staphylococci are also susceptible to: amoxicillin/clavulanate, cefpodoxime, cephalosporins I, II, III and IV, ertapenem, imipenem, meropenem. Cefoxitin results can be applied to methicillin. Additionally, the vancomycin disk does not differentiate susceptible from intermediate isolates of *S. aureus* nor susceptible, intermediate or resistant coagulase-negative staphylococci (CLSI, 2014).

[7]Results were interpreted according to CLSI Standards (2014). Ciprofloxacin results of coagulase-negative isolates were according to EUCAST breakpoints (EUCAST, 2017). Vancomycin, enrofloxacin and ceftiofur breakpoints were according to veterinary practice (CLSI, 2010). Green indicates susceptible, yellow intermediate resistance and red resistant.

Table 3.12: Antimicrobial susceptibility testing results for *Enterococcus faecalis*. [8]

		Antibiotic Class									
		Penicillins	B-Lactam/ase Inhibiot combo	Carbapenem	Tetracyclines	Fluoro-quinolones	Folate Pathway Inhibitors	Phenicols	Nitrofurans	Glycopeptides	Macrolides
Isolate	**Isolate**	**AMP**	**AMC**	**IPM**	**TE**	**CIP**	**SXT**	**C**	**F**	**VA**	**E**
Enterococcus faecalis	3E	34	33	35	28	28	29	28	24	21	28

[8]Breakpoints were according to CLSI (2014) except for ampicillin, imipenem and trimethoprim-sulfamethoxazole, which were taken from EUCAST breakpoints (2017).

3.5 Antimicrobial Resistance Gene Detection

Since phenotypic resistances were observed in some of the isolates, possession of resistance genes was also tested. As already discussed in Section 1.4.1.1, chromosomal AmpC is usually expressed by the majority of Enterobactericeae; therefore both *E. coli* and *Yersinia* isolates were tested. This enzyme is capable of destroying penicillins and therefore conferring resistance to them (Jacoby, 2009). However, AmpC enzymes can be either chromosomal or plasmid-mediated. Plasmid-mediated AmpC are divided into different enzyme families and apart from being able to be transferred into different bacteria, they confer resistances to a broad-spectrum of antibiotics and particularly β-lactams, with cephalosporins being the predominant group (see Table 1.3). PCR methods for *E. coli* chromosomal AmpC detection and plasmid-mediated AmpC were modified from those published by Ibrahim (2016) and Ibrahim *et al*. (2016), while PCR detection of chromosomal AmpC in *Yersinia* isolates was modified from that published by Stock *et al.* (1999). In all gel images shown in this section, lanes are numbered from left to right.

3.5.1 *E. coli* Chromosomal AmpC Detection

Isolates were tested for the possession of chromosomal AmpC as described in Section 2.7.7.1. All *E. coli* isolates appeared positive for the possession of chromosomal AmpC by producing a distinct amplicon on the gel at the correct position indicative of a product of around 191-base pairs (Table 2.2). Strain ATCC 25922 was the positive control resulting in a positive band while *Salmonella* Typhimurium LT2 was the negative control. However, phenotypic resistances to cephalosporin antibiotics were not seen in any of the isolates and therefore further testing for the presence of plasmid-encoded AmpC enzymes was not performed.

3.5.2 Detection of Plasmid-Mediated AmpC of the FOX-Family

Isolates exhibiting resistance to cephalosporins were further tested for the possession of plasmid-mediated AmpC. All *Yersinia enterocolitica* isolates exhibited phenotypic resistance to cefoxitin, a 2nd generation cephalosporin. There are different plasmid-encoded AmpC families (Table 1.3) according to the resistance they produce and the FOX-family produces resistance to cefoxitin (Philippon *et al.,* 2002). Therefore, isolates with phenotypic resistance to cefoxitin were further tested as described in Section 2.7.7.2. However, PCR using particular primers targeting the plasmid-mediated β-lactamase FOX gene (162 bp product, Table 2.2) obtained from Dallenne *et al.* (2010) resulted only in some apparently non-specific binding and amplification (Figure 3.6) and therefore isolates were assumed as negative for the possession of the plasmid-encoded FOX gene.

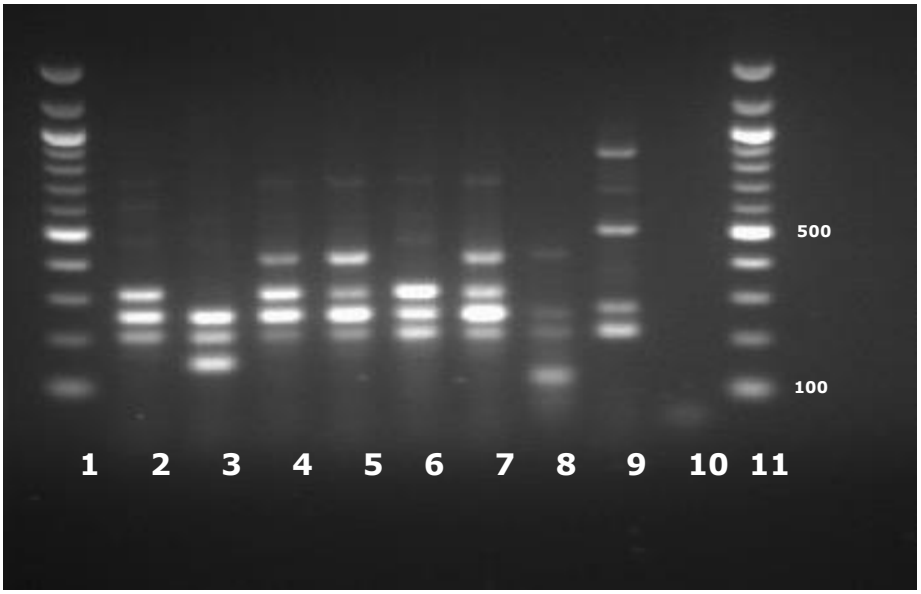


Figure 3.6: PCR amplification of the plasmid-mediated AmpC gene for the FOX family (1.5% TAE agarose gel, 85 V for 1 h). Expected product size 162 bp. Lanes 1 & 11: 100 bp DNA marker, Lanes 2-9: Sw1F, Sw2F, 1B, 1F, 4EcRB, 4EcRx, 4Emem, 4Etbx, Lane 10: H_2O.

3.5.3 Detection of Chromosomal β-Lactamase Genes *blaA* and *blaB* in *Yersinia*

Cefoxitin resistance can be both plasmid-borne and chromosomal since AmpC β-lactamases are cephalosporinases conferring resistance, among other β-lactams, to cephalosporins (see Table 1.3). Hence, specific primers were used to carry out PCR to target the chromosomally encoded β-lactamase genes *blaA* and *blaB* in *Yersinia enterocolitica.* BlaA is a constitutive broad-spectrum penicillinase while BlaB is an inducible class C cephalosporinase (Sharma *et al.*, 2006). All *Yersinia* isolates were tested including non-*Y. enterocolitica* isolates as described in Section 2.7.7.3. Amplification of the *blaA* gene, which according to Stock *et al.* (1999) expresses a class A enzyme related to those of *Klebsiella oxytoca* and *Proteus vulgaris,* resulted in multiple bands (top section in Figure 3.7), but none of the requisite size predicted apart from the positive control. Similar findings have also been observed in previous studies that suggested such results are possibly due to genetic variability of the *Y. enterocolitica blaA* genes (Sharma *et al.*, 2006). On the other hand, all isolates were shown to be positive for *blaB* (bottom section in Figure 3.7); an inducible class C enzyme associated with AmpC β-lactamases of other Enterobactericeae (Stock *et al.,* 1999).

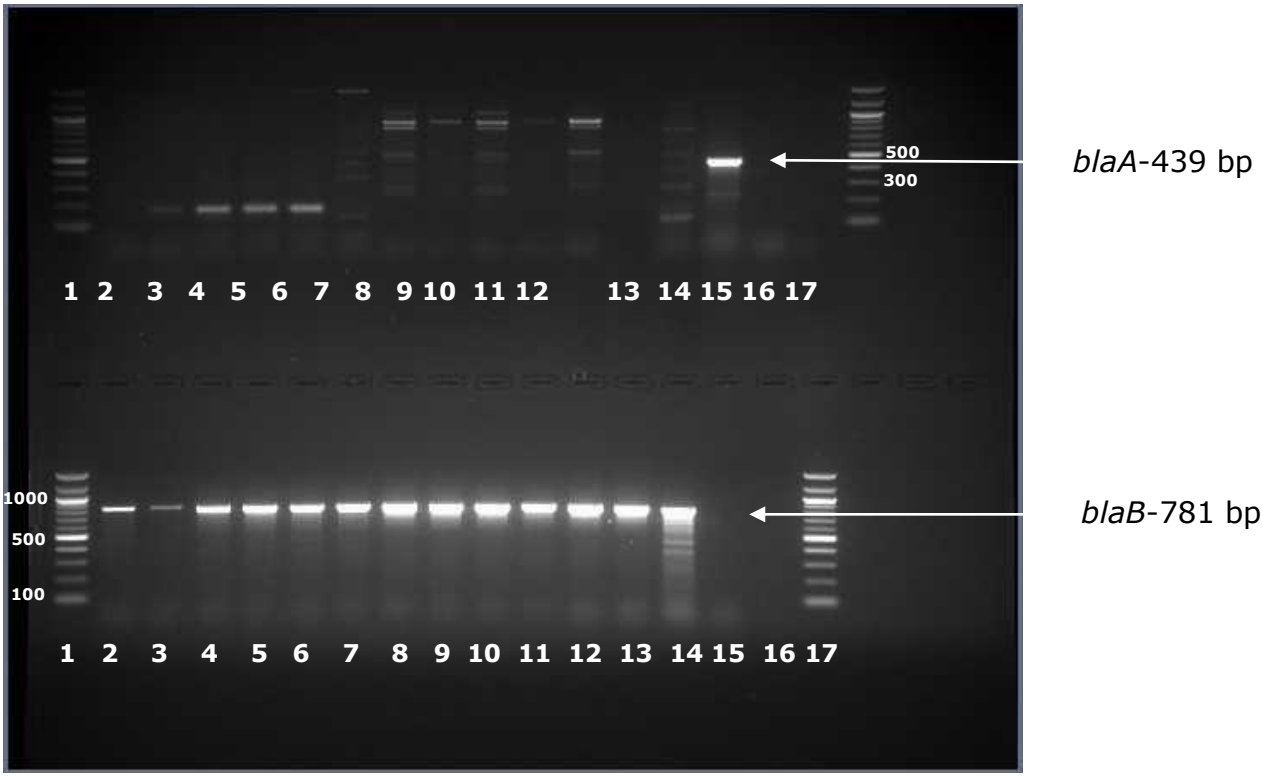


Figure 3.7: PCR detection of *blaA* and *blaB* genes (1.5% TAE agarose gel, 85 V for 1 h). Top of gel *blaA* amplification; bottom of gel *blaB* amplification. Lanes 1 & 17: 100 bp DNA marker, Lanes 2-13: 3PK2, 3PNK2, 1PK5M, 1PK5B, 2CPNKB, 2PNK5B, 3PK5, 3PNK5, 3CPK, 3CPNK, 4PNK5, 2PK5M, Lane 14: *Y. enterocolitica* strain NCTC 10460 (positive control), Lane 15: *Salmonella* Typhimurium LT2 (negative control) and Lane 16: H_2O.

3.6 *Yersinia enterocolitica* patho-serotyping

There are particular biotypes of *Y. enterocolitica* that are most common in causing human yersiniosis. Those include serotypes O:3, O:9 and O:5,27 (Section 1.4.4). PCR amplification using specific primers (Table 2.2) designed to target particular O-antigen encoding genes that are serotype-specific was described by Garzetti *et al*. (2014). Amplification of the O-antigen encoding genes was carried out for the common serotypes O:9, O:3 and O:5,27. The genes amplified, *per*, *wbbU* and *wzt,* were from specific regions of the O-antigen genetic clusters of each serotype mentioned, respectively. Only isolate 1PK5B tested positive for the gene *wzt* indicative of serotype O:5,27 (Figure 3.8) and responsible for encoding proteins for

the transport of O-polysaccharide into the periplasm. Therefore, the result suggests that the rest of the isolates do not fall within these three serotypes.



wzt-662 bp

Figure 3.8: PCR amplification of *wzt* gene of serotype O:5,27 (1.5% TAE agarose gel, 85 V for 1 h). Lanes 1 & 12: 100 bp DNA marker, Lanes 2-7: 3PK2, 3PNK2, 1PK5M, 1PK5B, 2CPNKB, 2PNK5B, Lane 8: H_2O (negative control).[9]

3.7 *Yersinia* Pathogenicity

Pathogenicity of the *Yersinia* genus depends on the possession of a virulence plasmid that encodes certain genes essential to cause illness. However, it has been reported by McNally *et al.* (2016) that particular chromosomally-encoded genes are equally important for the pathogenesis of the *Y. enterocolitica* species (Section 1.4.4). PCRs were performed for the detection of plasmid-borne genes *virF* and *yadA* located on the virulence plasmid pYV and chromosomal genes *ail* and *inv* as described in Section 2.7.5. The chromosomal genes *inv* and *ail* are invasion loci essential for the entry process into mammalian cells (Pepe & Miller, 1993). The plasmid-borne *virF* gene is a transcriptional activator for *yadA;* another plasmid-borne adhesin which is involved in numerous processes including autoagglutination, serum resistance and adhesion (Thoerner *et al.*, 2003). None of the isolates

[9]Lanes 9-11 are empty.

possessed *virF* and *inv*. No *Y. enterocolitica* isolates tested positive for possession of the *yadA* gene, but *Y. frederiksenii* isolates 4PNK5, 3CPK, 3CPNK, 3PNK5 and 3PK5 were positive for *yadA* (Figure 3.9), essential for virulence in *Yersinia enterocolitica* as it promotes initial attachment and invasion of the pathogen into the cells (Mikula *et al.*, 2013).

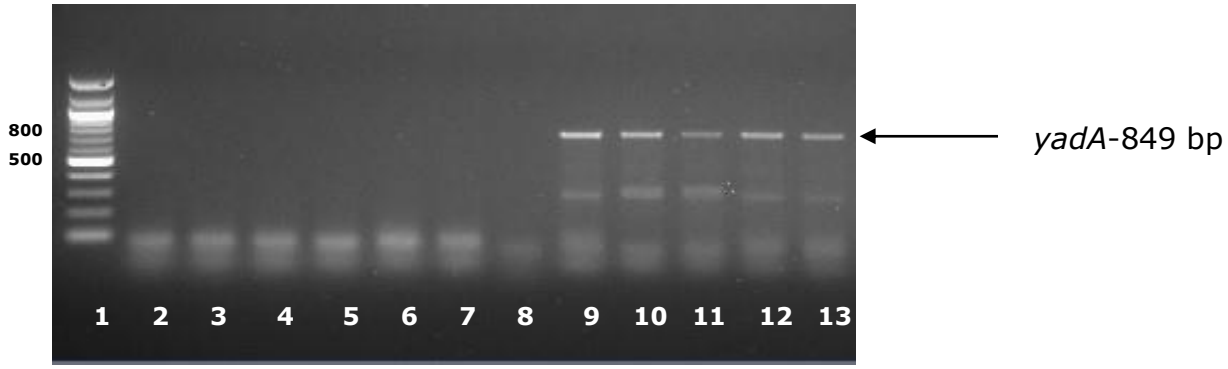


Figure 3.9: PCR amplification of *yadA* gene (1.5% TAE agarose gel, 85 V for 1 h). Lane 1: 100 bp DNA marker, Lanes 2-7: 3PNK2, 3PNK2, 1PK5M, 1PK5B, 2CPNKB, 2PNK5B, respectively. Lane 8: H_2O, Lanes 9-13: 3PK5, 3PNK5, 3CPK, 3CPNK, 4PNK5 respectively.

The method performed for the detection of the *ail* gene (Section 2.7.5.2) resulted in non-specific DNA amplification. Therefore, in each PCR tube containing the PCR mixture in a total volume of 25 µl, 5 µl of Q-solution (see Section 7.2) was added. Q-solution is a betaine (neutral chemical compound) that alters the melting temperature of nucleic acids and helps improve the PCR reaction that initially did not perform well using standard conditions (Qiagen, 2008) and consequently reduces non-specific amplification. The PCR was performed twice, in duplicate reactions each time in parallel with and without Q-solution. Results were inconsistent without Q-solution and consistent with Q-solution. Isolates 3CPK and 3CPNK tested positive for the possession of the gene (Figure 3.10) for both PCRs performed using Q-solution but they only tested positive once in the two PCRs performed without Q-solution. Figure 3.10 illustrates the positive results both with

(bottom section) and without (top section) Q-solution. A known *Y. enterocolitica* strain (NCTC 10460) was used as a control and was positive for the *ail* gene. However, NCTC did not provide enough information about the strain as to whether it carries the *ail* gene, but according to the results, this strain appeared to be a good positive control for this assay and could be further validated by sequencing the *ail* band and determining if it has the correct sequence.

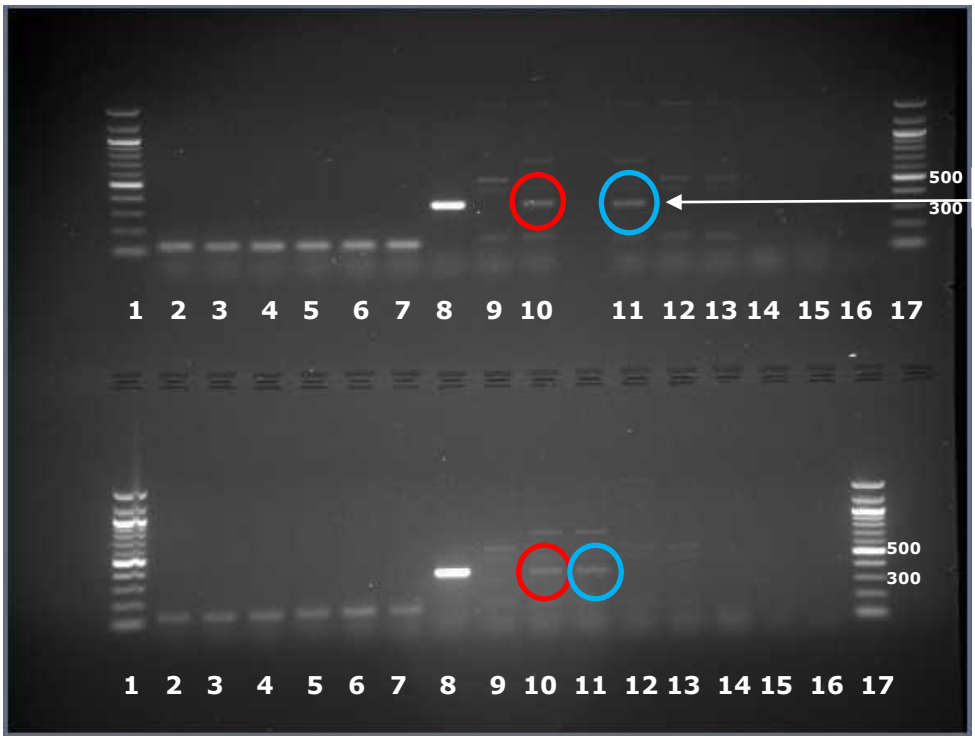


Figure 3.10: PCR amplification of the *ail* gene in the presence (bottom part) and absence (top part) of Q-solution in the PCR reaction mix (1.5% TAE agarose gel, 85 V for 1 h). Lanes 1 & 17: 100 bp DNA marker, Lanes 2-7: 3PK2, 3PNK2, 1PK5M, 1PK5B, 2CPNKB, 2PNK5B, respectively. Lane 8: NCTC 10460 (positive control), Lane 9: 2PNK5M, Lane 10: 3CPK (red circle), Lane 11: 3CPNK (blue cirle), Lanes 12-14: 3PK5, 3PNK5, 4PNK5, Lane 15: *Sal.* Typhimurium LT2 (negative control), Lane 16: H_2O.

3.8 *E. coli* Pathogenicity

There are different pathotypes of *E. coli* and each of them is related to a particular type of illness (see Section 1.4.1). The isolates were tested as described in Section 2.7.6 for the possession of particular genes specifically found in the EPEC/EHEC, EAggEC and ETEC pathotypes: the EPEC/EHEC *eae* gene, which is responsible for

intimate attachment and microvillus effacement; the EAggEC *astA* gene responsible for producing the heat-stable enterotoxin 1; and finally the ETEC heat-stable toxin and heat-labile toxin (STA and LT) genes responsible for causing severe diarrhoea. None of the isolates possessed the heat-stable toxin, heat-labile toxin and *eae* genes. However, five out of 8 isolates (62.5%) tested positive for possession of enteroaggregative *astA* gene (Figure 3.11). Positive isolates were *E. coli* 1F, 4EcRB, 4EcRx and *Escherichia* spp. 4Emem and 4Etbx.

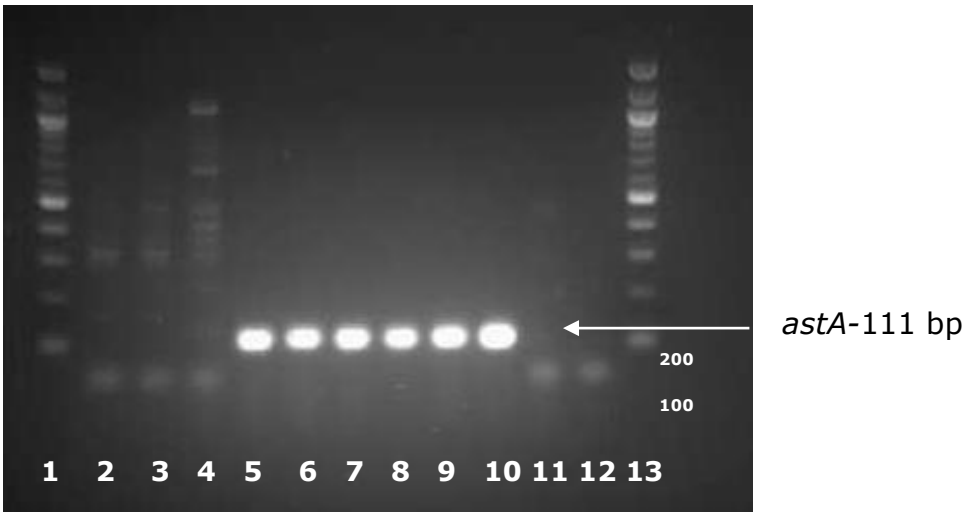


Figure 3.11: PCR amplification of *astA* gene (2% TAE agarose gel, 80 V for 1 h). Lanes 1 & 13: 100 bp DNA marker, Lanes 2-9: Sw1F, Sw2F, 1B, 1F, 4EcRB, 4EcRX, 4Emem, 4Etbx, respectively. Lane 10: positive control *E. coli* H10407, Lane 11: negative control NCTC 13406, Lane 12: H_2O.

3.9 *Staphylococcus aureus*

MRSA is one of the most well-known pathogens of clinical importance causing severe infections. In recent years an LA-MRSA strain type (CC398) has become widespread among pigs and has also been detected in farmers and individuals that are in close-proximity with animals that are carriers, hence posing a threat to human health (Section 1.4.3). CC398 was also found on retail pork (Hadjirin *et al.*, 2016). The isolates were tested as described in Section 2.7.8 for the possession of the *mecA* gene, responsible for conferring resistance to methicillin, and for the

identification of CC398 strain, by using the primers of by Stegger *et al.* (2016). All three isolates were shown to be negative for the possession of *mecA* gene. The findings match the antimicrobial susceptibility testing results where isolates phenotypically sensitive to methicillin (Table 3.11). Additionally, none of the isolates was of the clonal complex 398.

3.10 Metal Resistance

Just like antibiotic use for growth-promoting effects in animals has been said to contribute to antibiotic resistance, the use of heavy metals in animal farming could also play a significant role in developing resistance against them. It has also been reported that antimicrobial agents other than drugs have the ability to co-select for antibiotic resistance (Seile *et al.*, 2012), therefore in this project the possession of particular genes that confer resistance to the metals mercury, silver and copper was also examined.

3.10.1 Mercury Resistance

The mercury phenotypic resistance was examined by culturing on plates containing 25 µg/ml and 50 µg/ml $HgCl_2$ (Section 2.7.9.1.1). Resistant isolates should be able to show growth on the plates at 25 µg/ml of mercury but not at 50 µg/ml, including the positive control strain, as the higher concentration becomes toxic to the bacteria. None of the *Yersinia* isolates exhibited mercury resistance (phenotypic or genotypic). *Escherichia* isolates were tested and only *E. coli* isolate 1F demonstrated phenotypic resistance to 25 µg/ml $HgCl_2$. PCR was performed for the detection of *merA, merC* and *intl1* (Section 2.7.9.1.2). *merA* is the most important gene related with resistance as it encodes for the mercury reductase enzyme that converts Hg^{2+} to the less toxic Hg^0 which can easily diffuse out of the cell (Seiler *et*

al., 2012). *merC* is an inner membrane protein that transports ionic mercury into the cytosol (Pal *et al.*, 2017); whilst *intl1* is an integrase gene, an essential component of an integron where any resistance genes can be incorporated and become functional (Carattoli, 2001). This can be found on the mercury resistance transposon Tn*21*. Isolate 1F was shown to possess all three (Figure 3.12) supporting the phenotypic results. However, *E. coli* isolate 1B also appeared to possess *merC* and *intl1* (Figure 3.12) but not *merA* and therefore phenotypic resistance could not be fully expressed.

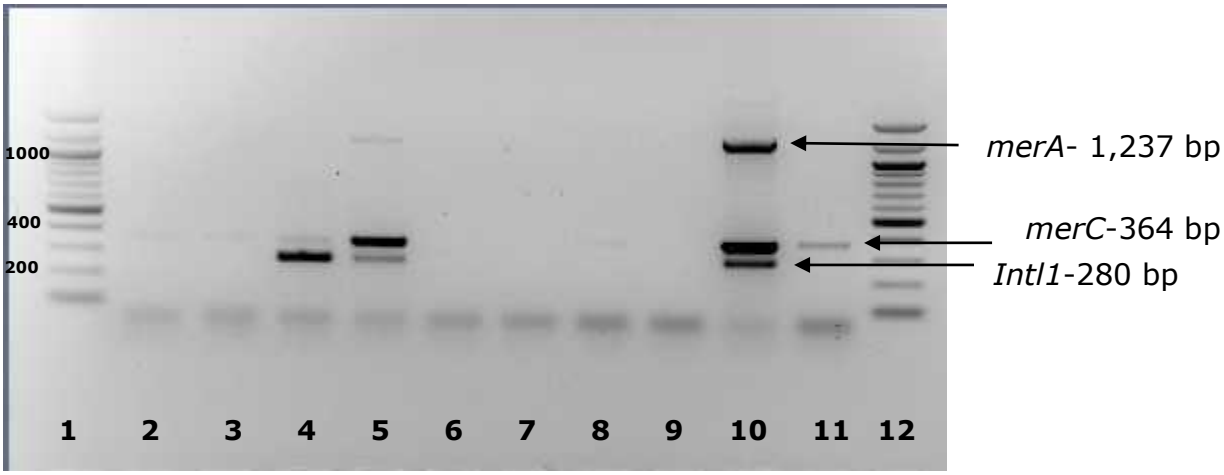


Figure 3.12: PCR amplification of *merA*, *merC* and *intl1* for the detection of mercury resistance (1.5% TAE agarose gel, 80 V for 80 min). Lanes 1 & 12: 100 bp DNA marker, Lanes 2-9: Sw1F, Sw2F, 1B, 1F, 4EcRB, 4EcRX, 4Emem, 4Etbx, respectively. Lane 10: positive control pMG101, Lane 11: negative control MG1655.

3.10.2 Copper Resistance

Phenotypic copper resistance was examined by culturing isolates on LB agar plates containing 2, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18 and 20 mM $CuSO_4$ (Section 2.7.9.2.1). Strains possessing the plasmid carrying the copper resistance (*pco*) system can tolerate up to 20 mM $CuSO_4$. In phenotypic assays, *Yersinia enterocolitica* isolates 1PK5B, 2PNK5B, 1PK5M and 3PNK2 showed growth up to 4 mM $CuSO_4$. However, *Yersinia frederiksenii* isolates 3CPK and 3CPNK exhibited

growth up to 8 mM while the rest of the isolates grew up to 6 mM (5 mM for 4PNK5). *E. coli* isolates 1B, 4EcRB, 4EcRX, 4Etbx and 4Emem showed growth up to 6 mM, isolate 1F up to 5 mM whilst isolates Sw1F and Sw2F were very sensitive and did not survive even at the minimum concentration. PCR was also performed for the detection of the *pcoE* gene (Section 2.7.9.2.2) as an indicator of resistance. None of the *Yersinia* isolates possessed *pcoE*. However, *E. coli* isolates 1B and 1F appeared positive (Figure 3.13) for the product amplification.

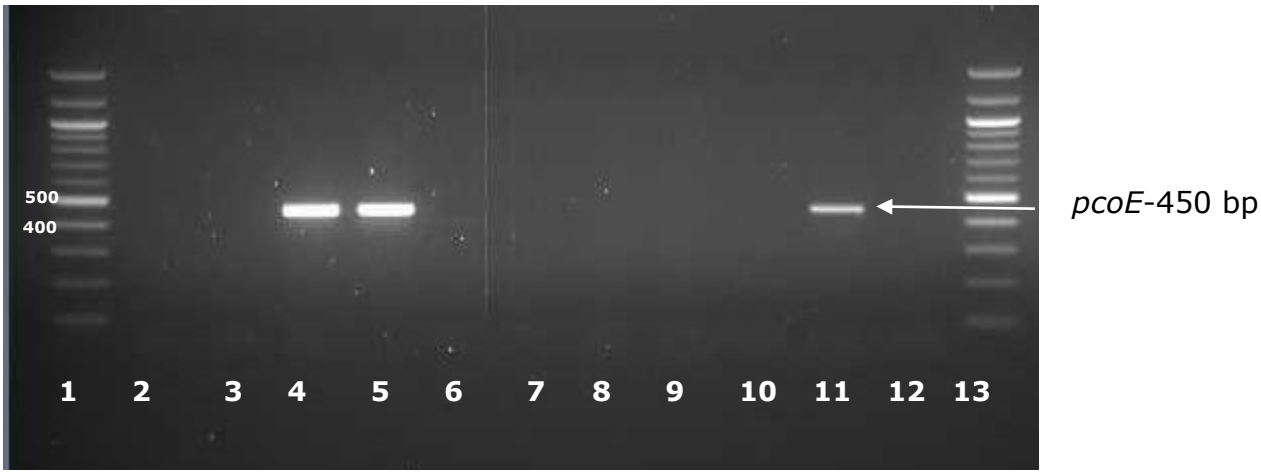
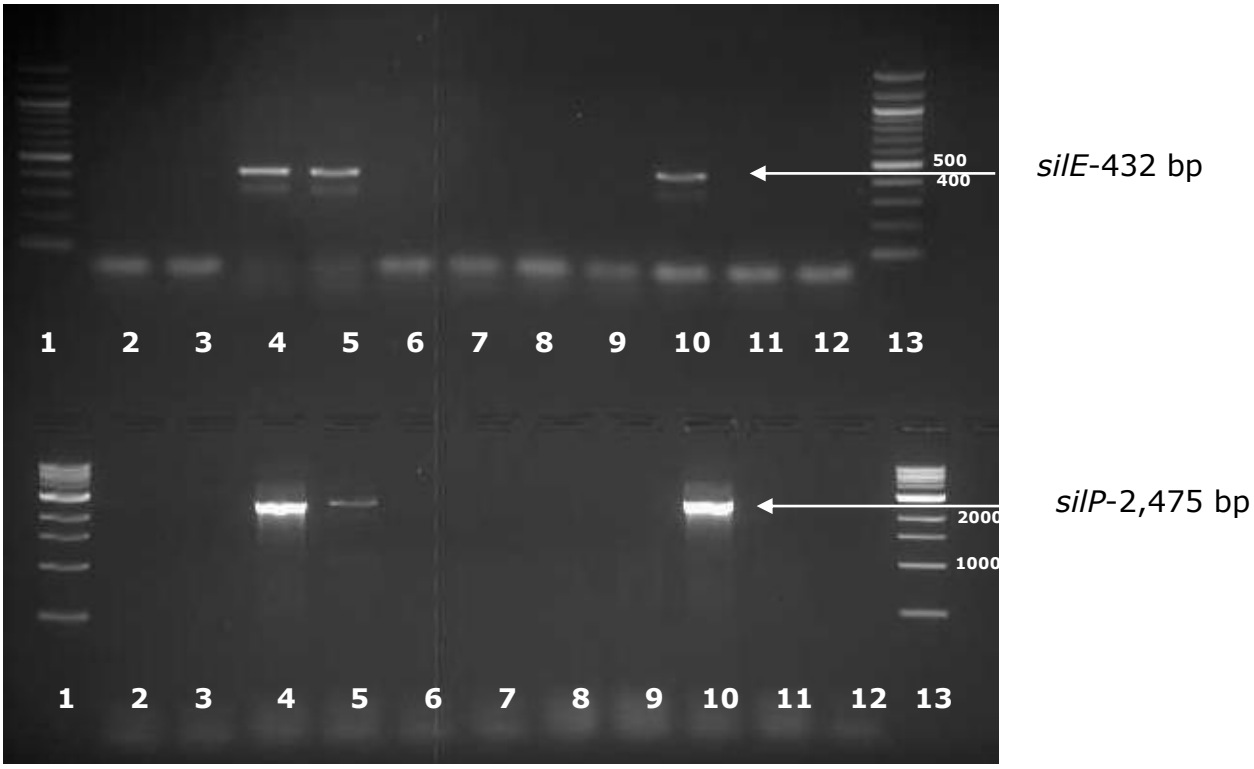


Figure 3.13: PCR amplification of *pcoE* (1.5% TAE agarose gel, 80 V for 90 min). Lanes 1 & 13: 100 bp DNA marker, Lanes 2-9: Sw1F, Sw2F, 1B, 1F, 4EcRB, 4EcRX, 4Emem, 4Etbx, respectively. Lane 10: negative control ED8739, Lane 11: Positive control pRJ1004, Lane 12: H_2O.

3.10.3 Silver Resistance

Phenotypic silver resistance was tested by culturing isolates on LB (no salt) agar plates containing 100, 200, 400 and 600 µM $AgNO_3$ (Section 2.7.9.3.1). The positive control strain showed phenotypic resistance at 600 µM $AgNO_3$. All *Yersinia enterocolitica* isolates and *Yersinia frederiksenii* 3PNK5 showed growth up to 200 µM $AgNO_3$. The rest of the strains exhibited no growth even at the minimum concentration $AgNO_3$ used. Similarly, *Escherichia* sp. isolate 4Emem showed growth at 200 µM whilst the rest of the *E. coli* isolates appeared sensitive. PCR was also

performed for the detection of *silE* and *silP* genes (Section 2.7.9.3.2) as an indication of resistance. Interestingly, none of the *Yersinia* isolates carried the genes. *E. coli* isolates 1B and 1F (Figure 3.14), carried both genes and also the *pcoE* gene, but neither of these strains showed phenotypic resistance to silver.



silE-432 bp

silP-2,475 bp

Figure 3.14: PCR amplification of *silE* and *silP* (1.5% TAE agarose gel, 80 V for 90 min). Lane 1 & 13 (top half): 100 bp DNA marker, Lane 1& 13 (bottom half): 1 kb DNA marker, Lanes 2-9: Sw1F, Sw2F, 1B, 1F, 4EcRB, 4EcRX, 4Emem, 4Etbx, respectively. Lane 10: positive control pMG101, Lane 11: negative control J53, Lane 12: H_2O.

4 Discussion

4.1 Isolation Methods

4.1.1 *E. coli*

One of the most commonly used methods for isolation of *E. coli* from foodstuffs is direct plating on TBX medium. This method (ISO 16649-2:2001) is also approved by ISO as a standard for isolation, and was the method used in this project. However, in this study it was observed that direct isolation on selective TBX medium proved less successful in isolating *E. coli* than the membrane method, which incorporates a resuscitation step on non-selective agar before selective plating, which yielded better results with the majority of the isolates being obtained using the membrane method. Similarly, this isolation method had also proved to be more successful in a previous study on 'Antimicrobial resistance in *E. coli* from pork products in the food chain' (Borisova, 2016). At low temperature microbial growth is suppressed but that does not mean that any microorganisms present will be killed. Refrigerated conditions, which the meat samples were exposed to, can cause sublethal injury to mesophilic microbial cells, which may be difficult later on to detect if directly exposed to selective conditions which cause additional stress. For this reason, it has been suggested by Kalchayanand *et al*. (2013) that this can be overcome by self-repair which occurs when using a resuscitation step where samples are incubated in non-selective media that do not contain selective agents that can further reduce cell count. Hence, the use of membranes on MMA agar led to better cell recovery results. Over the years, numerous studies have evaluated a number of methods but still, according to Rivas *et al.* (2015), there is no single method used to enrich, isolate and select for the multiple pathotypes of this

particular pathogen. The membrane method can help in cell detection as colonies appear larger and thus could be readily detected. The advantage of the membrane method over the direct selective plating is that it also allows for viable counting. Roberts & Greenwood (2008) described at least nine different isolation methods for *E. coli* specific for different purposes; eg a specific method for the detection of O157 strain. However, chromogenic media, such as TBX and CHROMagar, tend to be the most popular as they provide faster results by eliminating the need for subculturing and further biochemical testing (Manafi, 2000). However, elimination of subculture and biochemical testing is not quite efficient since confirmation is always required for validating the results. In addition, biochemical testing was performed in this study despite the use of chromogenic media since false-positives can occasionally occur even on chromogenic media; eg non-*E. coli* β-D-glucuronidase positive organisms on TBX show the same colony as *E. coli*.

4.1.2 *Staphylococcus aureus*

In this project the ISO 6888-1:1999 + A1:2003 method using Baird-Parker medium proved insufficient for the detection of *Staph. aureus* from the pork samples. This appeared to be due to the absence of a resuscitation step, since when samples were pre-enriched for 24 h prior to selective plating, detection was significantly improved. This was possibly because pre-enrichment increased cell numbers to a detectable level. In addition, Baird Parker agar, although it is the most widely used medium, and is still the first choice for direct plating and enumeration of the pathogen both in Europe and the United States (Baird & Lee, 1995), has limitations. Similar colonies of other species, or even genera, can still show growth, and in particular lecithinase-negative strains of *Staph. aureus* could be ignored (Capita *et*

al., 2001). For instance, in the current study isolate 3E which was identified as *Enterococcus faecalis*, was isolated with the same method, despite that the haloes around the colonies were nearly indistinguishable on the medium. The need for detection of *Staph. aureus* in foods is primarily due to the enterotoxic strains responsible for causing food poisoning. The main characteristic that can distinguish the enterotoxin-producing strains is the coagulase test; although it has been previously reported that some coagulase negative organisms are capable of forming enterotoxins (Baird-Parker, 1962). In a study performed by Viçosa *et al.* (2010) Baird-Parker agar gave lower frequencies of confirmed coagulase-positive colonies, in comparison with rabbit plasma fibrinogen agar (RPFA) and the Petrifilm™ Staph Express count system (STX), with 59% of the tested colonies (gray/black shiny colonies encircled by 2 halos; opaque from lipolysis and translucent from proteolysis) isolated on BP being coagulase-negative. On the other hand, higher frequencies of coagualase-positive colonies were observed using RPFA and STX agar.

Just like with *E. coli*, the use of newer media such as chromogenic media is gaining popularity against older ones. CHROMagar *Staph. aureus* agar has been shown to have higher specificity for the detection of S. aureus, providing rapid and more accurate results for the identification of the pathogen (Kluytmans *et al.*, 2002). Furthermore, when compared with oxacillin resistance screening agar for the detection of MRSA using a well-defined collection of staphylococci strains, it proved to be a better medium with significantly higher specificity (Kluytmans *et al.*, 2002). However, isolating a particular species from food samples could provide different results mainly because of the background flora present. In addition, strains of a

target species from different samples may also exhibit different characteristics. Despite that, a general chromogenic medium for the isolation of urinary tract pathogens was used in this particular study and it sufficiently provided more reliable results, with the isolates appearing the right shape (small, round, opaque) and colour (golden) confirmed as coagulase-positive *Staph. aureus.* Additionally, coagulase-negative staphylococci in this study appeared as a different colour shade (creamy colour) which was clear enough to be distinguished from typical *Staph. aureus*. Overall, the use of chromogenic media may provide faster and more accurate results as long as a resuscitation step in pre-enrichment is allowed in order to recover stressed cells and/or increase the cell number to a detectable level; particularly helpful when testing for absence/presence of a target organism.

4.1.3 *Yersinia enterocolitica*

The isolation of this pathogen proved to be the most challenging compared to the rest of the pathogens. Isolates presumed as *Yersinia* (highlighted in Table 3.3) were not all confirmed as *Yersinia* through 16S rDNA sequencing (highlighted in Table 3.6), illustrating that the biochemical tests performed (Table 3.3) could not differentiate the isolates. Therefore, it can be suggested that the method is not sensitive enough. Another reason could be the fact that those false-positive organisms can survive the same conditions as *Yersinia* cells; such as pH, temperature and salt concentration. Competition is therefore important, especially if other flora were more dominant on the pork products than *Yersinia* cells, and therefore managed to outgrow them.

The isolation pathway involved multiple steps in different enrichment broths prior to plating, as well as an alkaline treatment. In this project a modified version of the

method used by Van Damme *et al.* (2010) was carried out. In the Van Damme *et al.* (2010) study different isolation methods were performed and compared, including the one complying with ISO standard 10273. The ISO 10273 procedure for isolation of *Yersinia enterocolitica* from foods involves the suspension of the samples in an initial enrichment broth, peptone-sorbitol-bile broth, and subsequently in a selective enrichment medium: irgasan, ticarcillin and potassium chlorate broth. The selective agar plates used are primarily cefsulodin, irgasan and novobiocin (CIN) agar and SSDC agar (BSI, ISO 10273, 2015). SSDC was first described by Wauters (1973). It is a *Salmonella-Shigella* agar that is supplemented with deoxycholate, yeast extract and calcium chloride and intended particularly for the detection of biovar 4 serotype O:3. However, the ISO method involves an alkaline treatment in 0.5% KOH, which according to Wauters *et al.* (1988) is not suitable for use with SSDC medium and hence treated samples were only then plated onto CIN agar. CIN has been described as the most commonly and widely used medium for the isolation of *Yersinia enterocolitica* (de Boer, 1992). In this project, both agar media proved quite inefficient as they allowed growth of many false-positive organisms, making the whole isolation process laborious. SSDC allowed the growth of *Morganella*, *Proteus*, *Serratia* and *Aeromonas* and differentiation of *Yersinia* from these pathogens was quite challenging as noted by de Boer (1992). In fact, in this study none of the *Yersinia* isolates was isolated from SSDC (Table 3.6). Other microorganisms isolated from CIN and identified by 16S sequencing included *Citrobacter* species, *Serratia* species and *Pseudomonas* species*;* all known to give a similar colony morphology on CIN to *Yersinia enterocolitica* (Oxoid, 2017) despite that when it was first formulated, CIN was said

to be inhibitory to *Pseudomonas* (de Boer, 1992). Van Damme *et al.* (2013) demonstrated that, in the absence of a background flora, *Yersinia enterocolitica* appears as typical red bull's eye colonies on CIN agar while among an abundance of growth it can appear as non-typical small white colonies. In this study, only typical colonies were selected and therefore it is likely that many isolates were being ignored. Thus, recovery rates of the pathogen from the samples might be underestimated. Additionally, another limitation of the two selective media was their inability to discriminate potentially pathogenic *Y. enterocolitica* from non-pathogenic and other *Yersinia* (Denis *et al.*, 2011).

The alkaline treatment has been reported to facilitate the isolation of *Y. enterocolitica* by reducing or inhibiting non-*Yersinia* microflora and thus improving its detection (Aulisio *et al.,* 1980). However, it does not entirely remove non-*Yersinia* flora (Hudson *et al.*, 2008). Counts on CIN were significantly lower in this study in samples that had undergone KOH treatment, which made it much easier to select for typical colonies. Nevertheless, there was no obvious difference in terms of the confirmed isolates as the isolation rate between those isolated after KOH treatment from those without treatment was 50-50. Van Damme *et al.* (2010) commented on a better recovery rate using KOH treatment, however their study came to the conclusion that higher isolation rates were obtained within shorter incubation periods; when reducing incubation from 5 to 2-days. Similarly, Bonardi *et al.* (2014) stated that direct plating was a significantly better method followed by a 2-day enrichment in ITC broth as the second most efficient method and 2-days enrichment in PSB plus KOH treatment as the third. In the current project, more successful isolation of *Y. enterocolitica* was achieved with longer incubation periods:

50% of the *Yersinia enterocolitica* isolates were obtained after 5-days enrichment in PSB, 33.3% from 2-days enrichment in PSB and 16.6% from cold enrichment (2-weeks incubation) in PSB. None of the isolates were obtained by direct plating which would suggest, as described previously, that cell numbers on the samples tested were below the detectable level and therefore pre-enrichment aided their detection. In the studies mentioned above, the samples tested were pig tonsils, which were sampled straight out of slaughterhouses. Since *Yersinia enterocolitica* is known to inhabit the oral cavities of healthy pigs (Fredriksson-Ahomaa *et al.*, 2000) it is then reasonable that it is present in high numbers that could easily be detected. Moreover, the samples were likely unprocessed if taken from the slaughterhouse, thus cells were possibly not subjected to stress which may have subsequently contributed to an easier recovery of the organism. In contrast, the samples tested in this project were retail products that had already been subjected to some form of process such as handling, packaging and refrigerating which could have resulted in the injury of the cells present, hence a longer time in pre-enrichment resulted in better recovery rates. ITC broth is predominantly effective for the recovery of the O:3 pathogenic serotype whilst cold enrichment yields better results for non-pathogenic strains (de Boer, 1992); their findings are in agreement with those observed in this study. Interestingly, 33.3% of the non-*Y.enterocolitica* isolates of this study were obtained from cold enrichment in PSB; double that of the pathogenic isolates where only 16.6% of isolates were recovered from cold enrichment. In addition, poor recovery rates from ITC broth could reflect the fact that the isolates belong to different serotypes and not one in particular.

An alternative to SSDC agar has recently been suggested in ISO 10273 (2015)

method by the use of a chromogenic medium. YECA is a new *Y. enterocolitica* selective chromogenic medium with tryptophan which allows differentiation of pathogenic from non-pathogenic *Y. enterocolitica* strains. Colonies appear red fuschia due to the presence of a colour indicator revealed by sugar fermentation (Denis *et al.*, 2011). The use of this medium in conjunction with ITC broth can generate positive results for the detection of pathogenic strains within 72 hours making it less time-consuming than the standard ISO technique. Furthermore, the medium can also be used with human faecal samples for the detection of yersiniosis (Denis *et al.*, 2011). Chromogenic media are, nevertheless, more expensive than the conventional counterfeits, hence their use for routine testing is not as cost effective as other media. However, their use can reduce the overall testing needed when using media that are less specific and sensitive, thus resulting in a lower total cost. When testing a much bigger number of samples, chromogenic media seem a more cost and time-effective choice.

4.2 Antimicrobial Resistance

Antimicrobial resistance has increasingly become a major health concern over recent years. In the beginning, infections related with resistance were mainly associated with hospital and health-care units, until recently there has been seen a spread to the wider community. Survival of resistant bacteria to the exposure of drugs allows them to grow and freely spread due to the lack of competition from other strains (O'Neill, 2016). The misuse and overuse of antibiotics has helped in rapidly developing resistance. In some parts of the world antibiotics are being prescribed more freely than in others, but apart from that, lack of education on the subject has revealed many times that patients cannot differentiate between basic

knowledge such as antibiotics not being efficient against viral infections that cause cough, cold and fever (WHO, 2015). Interviews conducted in nine different countries revealed that patients would exaggerate their symptoms in order to get a prescription while others admitted not to completing their treatment fully after feeling better and thus keeping leftover medication for future use (Pechère, 2001), illustrating another example of misuse. However, the use of antibiotics in livestock as growth promoters and/or used for prophylaxis against infections has contributed to the levels of environmental antibiotic contamination (Berglund, 2015). Now antibiotic residues are making their way to the environment through multiple routes posing potential complications in tackling the problem. For example, Chee-Sanford *et al.* (2001) demonstrated the presence of all eight tetracycline-resistance genes in waste lagoons underlying swine farms and noticed that those genes were seeping into the groundwater downstream of the lagoons. This could have been as a result of the antibiotics being excreted from animal waste into the farm environment and subsequently disseminated into groundwater. Additionally, it was observed that groundwater isolates harboured an identical gene to the lagoon isolates that is not predominant in environmental samples. Therefore, this finding suggests that soil microbiota can act as vectors and help in the dissemination of the genes.

4.2.1 Phenotypic Resistance

Extraintestinal *E. coli* are the most common Gram-negative pathogens in humans and one of the most frequent cause of UTIs, which are a leading cause of adult bacteremia (Poolman & Wacker, 2016). Additionally, *E. coli* is a significant foodborne pathogen. Resistant strains can therefore impose serious threat to human health as they can be transmitted via the food chain (WHO, 2014). There

are numerous studies discussing multi-drug resistant *E. coli* isolated from pigs or pork products (Nijsten *et al.*, 1996; Enne *et al.*, 2008; Gousia *et al.*, 2011). Organic animal producers are prohibited by law from using antibiotics on their farms and in order for their animal products to be sold as organic, the animals should only consume 100% organic feed. However, where treatment is absolutely necessary, injectable antibiotics can be administered, but meat products of those particular animals that received antibiotic treatments cannot be sold as organic (Winter & Davis, 2006). Since antibiotics are prohibited in organic farms, it is likely to expect commensals, such as *E. coli* isolates, to be sensitive. However, resistances were also observed which illustrates that resistance genes are widespread in the environment. On the other hand, cross-contamination from other pig carcasses from conventional farms during the slaughter process could also be a reason for the resistances observed.

Isolates from organic pork in this study showed fewer resistances overall in comparisson with isolates from non-organic pork samples. Isolate 4Etbx from organic pork belly showed resistance to ampicillin, ampicillin with clavulanic acid and intermediate resistance to meropenem, whilst isolate 4Emem from the same meat product showed resistance to colistin. The rest of the isolates from organic pork were susceptible to all antibiotics tested. These findings are in agreement with those of Ŏsterberg *et al.* (2016) who investigated the resistances found in *E. coli* isolates from pigs in both organic and conventional farms in four different European countries and found that although overall resistance was lower in isolates from organic pigs, resistant isolates could still be present suggesting that despite regulations being applied the same way throughout the EU, there are country-

specific factors that affect the emergence, spread and persistence of resistance. Colistin resistance was also observed for isolates Sw1F and Sw2F. Colistin was withdrawn from human treatment use in the 1980s until recent years when increasing numbers of infections caused by MDR bacteria, such as *E. coli*, and lack of other effective drugs has caused it to re-emerge as a last resort treatment in humans (Catry *et al.*, 2015). However, colistin is widely used in veterinary medicine and according to the guidelines for antimicrobial use in swine production, it is the first choice drug for treating piglet scours (Burch *et al.,* 2008). It has been proposed that resistance to colistin might be due to several mechanisms, including gene mutations, but mainly from modifications in lipopolysaccharide structure of cell membranes (Prim *et al.*, 2015). Phenotypic resistance using the disc diffusion assay is not an accepted method for reporting colistin resistance as CLSI-EUCAST recommendations also require the MIC of the isolates (EUCAST, 2016). Therefore, the resistance reported in these isolates could be inaccurate. Galani *et al*. (2008), showed that the disc diffusion assay is inadequate and unreliable for evaluating resistance or susceptibility to colistin due to its poor diffusion characteristics in solid media. Many of the isolates tested in their study fell into the intermediate category and therefore additional confirmation was required and performed using the MIC measurement by Etest or broth dilution method.

All non-organic pork isolates exhibited resistance to sulphonamides and one also showed resistance to the trimethoprim/sulphamethoxazole combination. The latter is still the first line of treatment in pigs with neonatal scours and mastitis, metritis and agalactia (MMA) syndrome caused by *E. coli* (Burch *et al.,* 2008). Sulphonamides are structural analogues of ρ-amino-benzoic acid that bind the

catalytic enzyme dihydropteroate synthase (DHPS) in a reaction that inhibits the formation of dihydrofolic acid (Byrne-Bailey *et al.*, 2009). Sulphonamide resistance is either due to the acquisition of either of the two *sulI* and *sulII* genes, encoding forms of DHPS which are not inhibited by the antibiotic, previously found on different mobile elements (Enne *et al.*, 2001), or from mutations occurring on the chromosomal DHPS *folP* gene (Enne *et al.*, 2001). This resistance continues to persist despite restrictions in the UK taking place in 1995 for the use of trimethoprim/sulfamethoxazole, as studies have demonstrated trimethoprim alone is as effective for treating UTIs and hence combination should only be used when there is a good reason (Enne *et al.*, 2001); such as failure of treatment when trimethoprim is used alone.

According to the veterinary antibiotic resistance and sales surveillance (VARSS) report in 2015 (Veterinary Medicines Directorate, 2015) the most common resistances observed from *E. coli* isolates were streptomycin, tetracycline and ampicillin. Two of the isolates, 1B and 1F, found in this study showed phenotypic resistance to both tetracycline and oxytetracycline. Because of its use in animal agriculture, particularly for prophylactic, therapeutic or metaphylactic purposes, resistance may occur in the animals' gut flora which is therefore a substantial reservoir. Nevertheless, tetracyclines are also not fully absorbed from the animals' gut. It was reported that around 25% of the oral tetracycline dose administered to pigs is excreted in faeces and another 50% approximately in urine (Mackie *et al.*, 2006); thus contributing to the dissemination of the antibiotic and its metabolites into the farm environment.

The same isolates also showed resistance to chloramphenicol. Chloramphenicol was extensively used in veterinary practice but was banned for use in food animals by the U.S Food and Drug Administration in the 1980s when concerns about its toxicity emerged (Bischoff *et al.*, 2002). Its use in European animal farms was banned in 1994 (EURACTIV, 2010). Despite the fact that it has not been used in pig production and generally in food-producing animals in the EU for many years, resistant isolates are still being detected. It was proposed that resistance is likely occurring due to co-selection via the use of other antibiotics (Osterberg *et al.*, 2016). Similar findings were also observed in Oklahoma, US, where chloramphenicol-resistant beta-haemolytic *E. coli* were found to be associated with diarrhoea in neonatal swine. It was revealed that at the time of its use it was the most preferred antibiotic by veterinarians for the treatment of neonatal colibacillosis which, perhaps, resulted in selected resistant-strains and thus persistent rates of resistance could still be seen years after its withdrawal (Bischoff *et al.*, 2002). Drugs of the phenicol family, such as chloramphenicol, are strong inhibitors of protein synthesis. Several resistance mechanisms have been reported in the past including enzymatic inactivation, reduction in influx of the drug through permeability barriers and ribosomal resistance to the action of the antibiotic (Burns *et al.*, 1985). Kehrenberg *et al.* (2005) showed that the presence of the plasmid-encoded *cfr* gene from *Staphylococcus sciuri* confers resistance to chloramphenicol in both *Staphylococcus sciuri* and *E. coli* by demonstrating decreased binding of the antibiotic to ribosomes. Apart from the presence of particular genes, the study also pointed out that mutations in the RNA at the binding site are also responsible for resistance. However, in Gram-negatives the most commonly reported mechanism is

the plasmid-mediated production of acetyltransferase which catalyses the reaction (diacetylation) of chloramphenicol with acetyl coenzyme A that results in a product that no longer inhibits protein synthesis (Burns *et al.*, 1985) and thus bacteria can still survive the presence of the drug. The group of genes responsible for encoding chloramphenicol acetyltransferases in Gram-negatives are known as *catB* genes and are largely found in mobile gene cassettes. Apart from *catB* genes, another cassette-associated gene, *cmlA*, encodes an inner membrane protein that also confers resistance to the drug through an active efflux mechanism (Hall & Collis, 1998).

Resistance to 3rd generation cephalosporins was not observed from randomly selected pigs in the UK during monitoring in 2015. The sales of 3rd and 4th generation cephalosporins in 2015 was 0.17 mg/PCU compared to 0.19 mg/PCU in 2014 (Veterinary Medicines Directorate, 2015). Despite the drop in the sales of these critically important drugs, ESBL-producing *E. coli* were detected in 24.7% of the randomly tested pigs. In this study, none of the *E. coli* isolates exhibited resistance to 3rd or 4th generation cephalosporins. In contrast, a recent study by Ibrahim *et al.* (2016) showed that 57.9% of *E. coli* isolates from a single dairy farm were MDR including resistance to 3rd and 4th generation cephalosporins. Their findings however reflect the use of these antibiotics in that particular farm where ESBL drugs were used to treat mastitis in the cattle. Hence, the antibiotic treatment of farm animals can influence which resistances are observed in organisms isolated from the animals.

Regarding *Yersinia enterocolitica*, there are numerous studies demonstrating resistance levels of the pathogen (section 1.5.4). However, common resistance

mechanisms of the Enterobacteriaceae family could also occur in this genus (Advisory Committee on the Microbiological Safety of Food, 1999). All isolates of this study showed resistance to ampicillin and ampicillin with clavulanic acid, a combination treatment used to inhibit resistance by β-lactamase-producing bacteria which inactivate penicillins (Harold & Fu, 1978). 83.3% of the isolates also showed resistance to sulphonamides. These resistances were also shared by the non-*Y. enterocolitica* isolates. Numerous studies have reported resistant *Yersinia enterocolitica* isolates from around the world predominantly isolated from pigs and pig products. For example, in Italy MDR-isolates of *Y. enterocolitica* from pig tonsils at slaughter were tested for antimicrobial resistance and it was found that 100% of the isolates were resistant to ampicillin and 91% to sulphonamides (Bonardi *et al.*, 2014). In Spain Fàbrega *et al.* (2015) reported strains belonging to bioserotypes 1A, 2, 3 and serotype O:5,27 had a higher prevalence of amoxicillin/clavulanic acid resistance than pathogenic 4/O:3. On the other hand, 4/O:3 isolates from slaughter pigs in Latvia showed resistance towards ampicillin, cephalothin, erythromycin, streptomycin, sulfamethoxazole, and tetracycline but proved to be sensitive to combination treatments such as amoxicillin/clavulanic acid and trimethoprim/sulfamethoxazole (Terentjeva & Bērziņs, 2010). Occurrence of pathogenic serotypes in pigs were also reported in Czech Republic by Simonova *et al.* (2008) which demonstrated resistance of the pig isolates to tetracycline and erythromycin. Erythromycin is administered to pigs for bacterial gastroenteritis (Simonova *et al.,* 2008) and tetracycline is among the drugs used to treat human yersiniosis (Fàbrega *et al.,* 2015). In regards to more critically important antibiotics, all *Yersinia enterocolitica* isolates showed resistance to cefoxitin (2nd

generation cephalosporin) and 83.3% showed intermediate resistance to cefpodoxime (3rd generation cephalosporin). Third-generation cephalosporins are administered to baby piglets for prophylactic use of wound infections from processes like tail docking and castration (Burch, 2013). Therefore, it is no surprise resistance to this drug class is also reported among *Yersinia* strains. In fact, resistance to cephalosporins has been reported in *Yersinia enterocolitica* isolates from both animals and humans. Intermediate resistance to third-generation cephalosporins was also reported in isolates from retail pork in India (Anju *et al.*, 2014). These studies have also demonstrated that resistance is shared by *Yersinia intermedia* strains which are not consider pathogenic. Intermediate resistance was observed for ceftriaxone in those isolates, as was found in this study where *Yersinia frederiksenii* isolates showed the same resistance patterns as *Y. enterocolitica* isolates to ampicillin, ampicillin/clavulanic acid and sulphonamides.

In Greece *Y. enterocolitica* isolates from pork products were shown to carry resistance to a wide-spectrum of antibiotics including cephalosporins. However, 3rd and 4th generation cephalosporins such as ceftiofur and cefquinome are approved by the Greek National Drug Organisation for veterinary use in pig production (Gousia *et al.*, 2011). It would have been quite reasonable that isolates derived from the same sample would be expected to share the same set of resistances if those resistances arose from antibiotic treatment of that particular animal. However, among the isolates from this study, differences were observed. For example, *Y. enterocolitica* isolate 1PK5B and *E. coli* isolate 1B were isolated from the same pork belly sample purchased at Source A. Despite the fact that both isolates are considered MDR, isolate 1B was resistant to six different drug classes

whilst 1PK5B was resistant to three, and the resistances they shared were those of ampicillin and ampicillin/clavulanic acid. Similarly, differences in resistance were also observed between *Y. enterocolitica* and *Y. frederiksenii* isolates derived from the same sample. *Y. enterocolitica* isolates 3PK2 and 3PNK2 and *Y. frederiksenii* isolates 3PK5, 3PNK5, 3CPK and 3CPNK were all derived from the same sample purchased at Source C. They shared the same resistances to ampicillin, ampicillin/clavulanic acid and sulphonamides. However, the *Y. frederiksenii* isolates were not resistant to 2nd and 3rd generation cephalosporins unlike the *Y. enterocolitica* isolates. Moreover, the *Y. frederiksenii* isolates were all resistant to nitrofurantoin whilst only *Y. enterocolitica* isolate 3PNK2 was resistant to that drug. Therefore, isolates obtained from the same sample may not necessarily carry the same set of resistances because of animal treatment. It could be the case some of these strains could be present on the products as a result of cross-contamination within the slaughterhouse environment. High numbers of enterobacteria on pig carcasses after evisceration have already been reported (Warriner *et al.*, 2002). Hence, since antibiotic resistance genes originate within a variety of bacterial sources, the genomes of all bacteria are then considered as a single global gene pool from where genes can randomly be moved from one bacterial cell to another (without being related to the gene donor) usually by horizontal gene transfer that involves the help of a bacterial plasmid or through a different transfer system such as a transposon (Bennett, 2009). This can therefore partly explain why isolates obtained from the same samples showed a different set of resistances.

Two of the MDR *Y. enterocolitica* isolates (2CPNKB and 2PNK5B) of this study were isolated from a product that, according to the retailer, was imported from the EU.

Therefore, resistance could reflect the antimicrobial usage and population of the particular country of origin.

An interesting observation, however, is seen with the non-pathogenic isolates that all showed some level of resistance to nitrofurantoin. There are two types of nitroreductase; type I which is insensitive to oxygen and type II which is inhibited by oxygen (Sandegren *et al.*, 2008). However, resistance has mainly been reported to be due to mutation in the *nfsA* and *nfsB* genes which encode oxygen-insensitive nitroreductases (Giske, 2015). The EU banned its use in food animals in 1993 after toxicology studies demonstrated genotoxic and carcinogenic properties of the nitrofurans (Antunes *et al.,*2006). In human medicine, patients take this drug orally for lower UTIs as it rapidly excreted in urine. Resistance levels to this antibiotic are generally quite low in EU countries (Giske, 2015) although resistant *Y. enterocolitica* isolates have occasionally been reported in some European countries (Gousia *et al.*, 2011), suggesting that despite the ban in animal use, resistance genes can still persist. In this study, only one of the *Y. enterocolitica* isolates showed resistance to this antibiotic unlike the non-pathogenic strains. Sandegren *et al*. (2008) showed that nitrofurantoin resistance affects bacterial fitness and that generally antibiotic resistance is accompanied by a decrease in fitness, whether this is a reduction in growth rate or reduction in virulence; which could perhaps partly explain why this resistance was mostly observed in non-virulent strains. Reduction in growth rate and virulence would cause a bigger impact in pathogens than in non-virulent strains.

Another critically important pathogen is methicillin-resistant *Staph. aureus.* Its increasing resistance towards multiple antibiotics has made its elimination difficult

to achieve. Methicillin was introduced as a treatment against penicillin-resistant staphylococcal infections in humans. However, about two years after its introduction the first reports of resistance emerged (Enright *et al.*, 2002). A more recent incident is that of direct pig to farmer transmission of LA-MRSA that was observed particularly in France and the Netherlands (Frana *et al.*, 2013). This particular LA-MRSA type, known as CC398, has recently been detected in retail pork products raising concerns over public health. LA-MRSA CC398 is endemic in pig farms around Europe and specifically in Denmark, which is Europe's biggest pork exporter and the predominant exporter to the UK (Harvey *et al.*, 2015). Surprisingly, given this background, none of the isolates from this study showed phenotypic resistance towards any antibiotics of the β-lactam family. All isolates showed intermediate resistance to enrofloxacin. Enrofloxacin is an antibiotic of the fluoroquinolone-family that is globally approved for use in veterinary, and also human, medicine to treat a number of bacterial infections including those caused by staphylococci (Ihrke *et al.,*1999). Resistance to these drugs quickly developed among *S. aureus* strains and more particularly within MRSA isolates. As a result, their use for treating staphylococcal infections was significantly reduced (Lowy, 2003). Fluoroquinolones act by inhibiting DNA synthesis by interacting with DNA complexes and either of the two DNA gyrase and topoisomerase IV target enzymes (Hooper, 2001). DNA gyrase, a drug target in Gram-negative bacteria, consists of two subunits GyrA and GyrB which are respectively homologues to subunits ParC and ParE of topoisomerase IV, a drug target in Gram-positive bacteria. Therefore, resistance can be developed through chromosomal mutations in the genes encoding the subunits of the drugs' target enzymes (Hooper, 2001). Apart from target site

mutations, fluoroquinolone resistance can also occur either via transmissible mechanisms such as genes encoding resistance mechanisms on mobile genetic elements (i.e plasmids) that decrease sensitivity to quinolones, or through efflux pumps and alterations in membrane permeability which affect the penetration of the drug (Redgrave *et al.*, 2014).

In this particular study only one isolate, 2StM, showed resistance to other antibiotics including erythromycin, clindamycin and oxytetracycline. Macrolides (erythromycin) and lincosamides (clindamycin) are widely used for treating staphylococcal infections and hence resistance is quite frequent. Resistance is thought to occur via three ways: target-site modification or mutation, mediated by one or more *erm* genes, preventing binding to the ribosome, efflux pump (encoded by *msrA* or *msrB*) and drug inactivation (such as enzymes EreA and EreB) (Leclercq, 2002). Co-resistance to macrolides, lincosamides and streptogramins B (a drug class used for vancomycin-resistant isolates) is due to *erm* genes which cause alterations on the ribosomal target site. Genes *ermA* and *ermC* are found in staphylococcal isolates (Leclercq, 2002). In addition, *linA'* is a gene detected in *S. aureus* and confers resistance to lincosamides only; however, it has been reported that this was never detected alone but rather in combination with *ermC* (Lina *et al.*, 1999). This is further supported by a study carried out in the Netherlands, where 23% of MRSA isolates from pigs and pig farmers that were resistant to erythromycin were also cross-resistant to clindamycin (de Neeling *et al.*, 2007). In conclusion, targeting particular genes that are known to confer resistance could explain any phenotypic resistances observed among isolates. Nevertheless, isolates were only tested for the *mecA* gene, responsible for methicillin resistance and for

the identification of CC398 since it is a raising concern and has already been detected in retail pork product within the UK. Phenotypic resistance to lincosamides and macrolides was only observed for one *Staph. aureus* isolate and if time was not a limiting factor in this project, it would have been interesting to search for target genes, such as *erm* and *linA'*, in order to further define the phenotypic resistances seen.

4.2.2 Genotypic Detection of Resistances

4.2.2.1 Chromosomal and Plasmid-Mediated AmpC

Particular interest in studying AmpC β-lactamases started back in the 1970s. In an *E. coli* K-12 strain it was first noticed that the chromosomal *ampC* gene belonged to a different class of β-lactamases than those which already existed (A and B); the *ampC* β-lactamases were known as class C, which exhibit substrate specificity for cephalosporins (Bergstrom *et al.,* 1982). These chromosomally-encoded β-lactamases can be inducible or non-inducible. In *E. coli* these chromosomal cephalosporinases are constitutively produced, hence antibiotics and external effectors do not influence expression. Moreover, they are generally produced at such low levels that resistance to β-lactams is not conferred. It has been established that poor expression is a result of an inefficient promoter for *ampC* (Sanders, 1987). Such findings could perhaps explain why chromosomal *ampC* positive strains can still be susceptible to antibiotics such as ampicillin, as seen with 50% of the isolates in this study that were shown to be phenotypically sensitive to ampicillin. In contrast, increased production of the enzyme leads to high levels of resistance to different β-lactams including 3rd generation cephalosporins. This over-expression has been linked to different mutations in the attenuator and promoter

(Corvec *et al.*, 2007). Other Enterobactericeae have been shown to also express chromosomally-encoded β-lactamases, with particular studies demonstrating extensive sequencing and regulation similarities between an *E. coli* K-12 strain and *Shigella sonnei* (Bergstrom *et al.,* 1982). In addition, DNA sequence analysis of *E. coli* ampicillin-resistant isolates that overproduced chromosomal *ampC* were found to be almost identical (except for one base pair difference) to that of low-producing *Shigella sonnei*. However, when one-step hyper-producing *Shigella* mutants were characterised, it was revealed that mutation had taken place in the single base which differentiates low-producing *Shigella* from *E. coli* isolates, suggesting the hyper-producing ability of those *E. coli* isolates were the results of a natural gene exchange between the two genera (Olsson *et al.*, 1983). Similarly, *Citrobacter freundii ampC* shares an identical 77% amino acid sequence with the *E. coli* K-12 chromosomal *ampC,* proposing that genes encoding for these chromosomal β-lactamases in enterobacteria could be a result of divergent evolution from a common source (Lindberg & Normark, 1986).

As was mentioned previously, these enzymes can also be inducible and hence β-lactams can affect their expression. In the late 1980s these inducible chromosomal genes were discovered on plasmids in certain Enterobactericeae (*Enterobacter cloacae*, *Morganella morganii*, *Citrobacter freundii* and *Hafnia alvei*) (Pérez-Pérez & Hanson, 2002) and subsequently transported to other microorganisms that do not normally express them, such as *Salmonella* spp (Hanson, 2003). There are six groups (Table 1.3). of which CMY-2 is said to be the most common one (Liebana *et al.*, 2013). However, on many occasions resistance caused by AmpC proteins is misidentified as ESBL (Hanson, 2003). Plasmid-mediated *ampC* genes do not

normally confer resistance to 4th generation cephalosporins and carbapenems but they do confer resistance for β-lactam/inhibitor combinations (eg ampicillin/clavulanic acid) and cephamycins, whilst ESBLs tend to confer resistance for 4th generation cephalosporins and monobactams but not usually to carbapenems and cephamycins (Liebana *et al.*, 2013).

Food animals and humans have been found to carry pathogens harbouring these plasmid *ampC* β-lactamases and in many cases these are present in their commensal gut flora such as commensal *E. coli,* which can act as a reservoir of the resistance genes. In the case of chromosomal resistance genes, transfer to other bacteria can occur via bacterial conjugation, a mechanism of horizontal gene transfer including genetic transformation (Spratt *et al.*, 1992) or transduction. Blanc *et al.* (2006) detected plasmid-mediated *ampC* and ESBLs in poultry, pig and rabbit farms with CMY-2 producing *E.coli* isolates being discovered for the first time in rabbits. This transfer of plasmid-mediated *ampC* β-lactamases was also shown by Winokur *et al.* (2001) who demonstrated that the plasmids would be transferred between food animal and human isolates. In their study, *Salmonella* isolates from food animals and humans were shown to express an identical CMY-2 β-lactamase. Additionally, *E. coli* isolates with the same resistances were isolated from animals and humans within the same geographic region and it was revealed via molecular studies that they were carrying an identical CMY-2 β-lactamase. Conserved regions between CMY-2 plasmids from *Salmonella* and *E. coli* of both animal and human origin were also demonstrated, suggesting the possibility of transmission not only between these genera but also from food animals to humans. In the current study, none of the *E. coli* isolates exhibited resistance to cephalosporins and therefore

testing for the possession of plasmid-mediated *ampC* was not undertaken. Interestingly, all *Yersinia enterocolitica* isolates exhibited phenotypic resistance to cefoxitin, a 2nd generation cephalosporin, and 83.3% also exhibited resistance to cefpodoxime, a 3rd generation cephalosporin. Initially, these phenotypic resistances were thought to have derived from plasmid-borne β-lactamases and particularly the FOX family which confers resistance to cefoxitin. However, when specific primers were used to detect for the presence of plasmid-borne *ampC* of the FOX family in those isolates, no amplified products could be detected. Hence, a further search was carried out in the literature about *ampC* in *Yersinia*. *Yersinia enterocolitica* is among a group of enterobacteria that is naturally resistant towards aminopenicillins and early cephalosporins by a resistance phenotype that is mediated by chromosomal β-lactamases that belong to class C of these enzymes, as those described previously for *E. coli* did (Poirel *et al.*, 1999). The majority of *Y. enterocolitica* strains produce two different ones; the first being a class A penicillinase (*blaA*), related to that of *Klebsiella* and the second an inducible *ampC* cephalosporinase (*blaB*) that matched the sequence of other known group I β-lactamases (Seoane *et al.,* 1992). BlaA, apart from being related to the plasmid-borne TEM-type enzyme (Seoane & Lobo, 1991), was previously found to be completely inhibited by clavulanic acid but not by aztreonam while BlaB was inhibited by aztreonam and cloxacillin but not by clavulanic acid (Stock *et al.,* 1999). Consequently, PCR was performed on all isolates using primers that allow the detection of *blaA* and *blaB* and amplification showed that all isolates, including non-*Y. enterocolitica* isolates possessed *blaB* but none had *blaA*. Seoane & Lobo (1991) carried out cloning experiments on these genes and concluded that when

both are simultaneously present in the chromosome, they evolve in a particular way such that only one of them is active. Counter to these findings, a different study on molecular characterisation of *blaA* demonstrated that PCR amplification resulted in multiple faint bands, as observed in this study, and was hypothesised to be due to genetic variability of the *Y. enterocolitica bla* genes isolated in various parts of the world (Sharma *et al.*, 2006). This latest explanation could perhaps be a reasonable explanation for our *blaA* results. However, despite possessing *blaB*, *Y. frederiksenii* isolates did not exhibit phenotypic resistance towards cephalosporins. The reason for this finding is not clear yet, and antimicrobial resistance studies related to non-pathogenic *Yersinia* strains are quite limited as the focus is, largely on pathogenic strains.

Nonetheless, Mittal *et al.* (2007) were the first to report characteristics of β-lactamases in *Y. frederiksenii* and *Y. intermedia* isolates from an Asian region. Their study showed that sequence identity of the *blaB* gene of these two species was 85% with that of *Y. enterocolitica* biovars 1A, 1B and 2. Expression studies of both BlaA and BlaB in biovars 2, 4 and 5 of *Y. enterocolitica* showed that uninduced biovar 4 strains mainly expressed *blaA* but, when induced, *blaB* predominated instead; in biovars 2 and 5 either of the two can be predominant and after induction *blaB* is exclusively expressed (Stock *et al.,* 1999).

Another study carried out on retail foods in China between 2011 to 2014, showed that all *Y. enterocolitica* isolates obtained belonged to biotype 1A and *blaA* and *blaB* β-lactamases were detected in 97% and 100% of the isolates, respectively (Ye *et al.*, 2016). Despite these findings poorly explaining the lack of resistance conferred in these isolates, it shows that levels of expression and increase of activity differs

between different strains and biovars and hence it sheds some light on a topic that definitely needs more research in order to fully understand the expressed resistance patterns.

4.2.2.2 Staphylococcal *mecA* Gene and Clonal Complex 398 Detection

MRSA have arisen from MSSA strains by acquiring the *mecA* gene which confers resistance to methicillin. The gene is part of a mobile staphylococcal chromosome cassette and, although origins of MRSA clones are not clearly understood yet, it has been suggested that MRSA was either descended from a single ancestor that acquired the gene and then spread around the world, or that the gene has been transferred between *S. aureus* lineages, mostly based on studies showing the divergence of MRSA strains (Enright *et al.*, 2002). The resistance to increasing numbers of different antibiotics seen by this pathogen are a rising concern (section 1.5.3). The evidence that the *mecA* gene resides on a mobile element that encodes recombination enzymes for its excision from, and integration in the pathogen's chromosome, could perhaps suggest the acquisition of other genetic elements, such as transposons, which could confer resistances to other antibiotic classes, hence explaining the MDR-profile of emerging isolates (Wielders *et al.*, 2002). Isolation of LA-MRSA from pigs and pork products has also been reported in different studies. Friese *et al.* (2012) showed evidence that MRSA could possibly be transmitted through air, which could contribute to the contamination of barns and subsequently be transferred to pig herds. Possession of the *mecA* gene was also detected in all MRSA isolates from packaged hamburgers in a study conducted by Shahraz *et al.* (2012) who also showed that all isolates exhibited phenotypic resistance to methicillin prior to gene detection. In the present study, methicillin resistance was

recorded according to the cefoxitin disc results which is used as a surrogate for oxacillin. Oxacillin resistance is mediated by *mecA* and therefore its results could be applied to other penicillinase-stable penicillins (CLSI, 2014). All isolates, however, were negative for phenotypic resistance to methicillin. Nevertheless, Wielders *et al.* (2002) showed in their study that 10% of their European MRSA isolates that were phenotypically susceptible to methicillin contained the *mecA* gene and 5% did not possess the gene but appeared phenotypically resistant. On this basis a PCR was performed for the *mecA* gene on all isolates, however, it showed that none possessed the *mecA* gene and hence were considered as non-MRSA.

MRSA isolates have been identified in both pigs and pig farmers, rising concerns for their role in human infection. In Ontario, Canada, samples were collected from 20 pig farms and their workers to reveal 45% MRSA colonisation in farms (in 9/20 farms), including 24.9% prevalence of MRSA in pigs and 20% carriage among workers. In addition, no workers tested positive on any farm where MRSA was not detected in pigs, sparking debate whether there is a transmission between the pigs and the workers. Analysis demonstrated that strains present in pigs were indistinguishable from those in humans; with 59.2% of isolates being the CC398 strain (Khanna *et al.*, 2008). LA-MRSA is said to belong predominantly to the clonal complex 398 (CC398) in which most strains belong to the sequence type 398 (ST398), known to cause severe infections (Graveland *et al.*, 2011). After a published report on the detection of LA-MRSA CC398 in retail meat from across the UK that was said to be supplied by a UK farm (Hadjirin *et al.*, 2016), isolates from this study were also tested using the specifically designed primers that target the restriction-modification system carrying the *sau1-hsdS1* gene (Stegger *et al.,* 2016)

that helps identify CC398 strains. Despite its reported occurrence in UK retail meat products, none of our isolates were indentified as being CC398 strains. However, findings that support correlation of the colonisation of this strain between animals and humans (such as those mentioned above) is a reminder of the need of more surveillance systems and control actions even if CC398 is said to be a poor persistent coloniser in humans as opposed to animals (Graveland *et al.*, 2011).

4.3 Pathogenicity

4.3.1 *E. coli*

Despite most strains of *E. coli* being commensal organisms that naturally inhabit the intestines of humans and animals, certain pathotypes of *E. coli* are food-borne pathogens that are associated with infections characterised with symptoms from diarrhoea to a life-threatening haemolytic uremic syndrome (HUS). In this study, the pathogenicity of the isolates was examined for the presence of the heat-stable (ST) and heat-labile (LT) toxin genes, the enteroaggregative *astA* gene and finally the *eae* intimin protein gene. Unfortunately, due to the work taking place in an ACDP 2 laboratory, testing for the presence of Shiga-toxin genes (*stx*) was not allowed to be performed. Therefore, only β-glucuronidase-positive *E.coli* were selected, which excludes most O157 strains. In a previous study on *E. coli* isolates from UK pigs at slaughter (Wei, 2013), there was a high prevalence of potentially pathogenic strains present. The study illustrated that among 1065 isolates, 71.9% possessed at least one pathogenicity-related gene with 13 isolates testing positive for the EHEC *stx* gene, particularly stx_{2e}, and 19 for the *eae* (Wei, 2013). A study in China demonstrated the prevalence of Shiga-toxin producing isolates in retail raw meat, including pork samples, with 166 samples testing *stx*-positive out of 853,

with subtype stx_{2e} being the predominant one. Meanwhile, 6.3% of the STEC isolates carried the *eae* gene and 11.1% *astA* (Bai *et al.*, 2015). *stx* genes are divided into two major types, 1 and 2, which are then further divided into different genetic variants. Pigs are said to be natural reservoirs of stx_{2e} which is mainly connected in the animal with uncomplicated diarrhoea or it can be asymptomatic. However, stx_{2e} is not associated with human disease (Bai *et al.*, 2015). The link between pigs and this *stx*-type is a possible indication of pork meat being cross-contaminated with faecal material during the slaughter process (Beutin *et al.*, 2007). The *eae* gene was not detected in any of the isolates in this study. This gene encodes the intimin protein responsible for creating attaching and effacing lesions in human intestinal epithelial cells and its presence in combination with *stx* genes has been associated with increased virulence (Bai *et al.*, 2015); especially the risk of bloody diarrhoea. Most EHEC strains retain both these genes. Despite person-to-person contact and consumption of contaminated meat being said to be the primary routes of transmission for such infections, there are also links with ingestion of contaminated vegetables and water or direct contact with animals and soil (Loukiadis *et al.*, 2006). A recent example was the outbreak in Germany in 2011 which resulted from sprouted seeds contaminated with an enteroaggregative strain that acquired the Shiga toxin (Frank *et al.,* 2011). However, this EHEC strain does not possess the *eae* gene, unlike most EHEC strains. *stx*-genes are carried by lysogenic bacteriophage and *eae*-positive strains can acquire them by horizontal transfer thus enhancing their virulence. In fact, a possible dissemination into the environment of both these genes was demonstrated when Loukiadis *et al.* (2006) detected *stx* and *eae* positive *E. coli* isolates from treated effluent and sludge from

wasterwater of slaughterhouses in France. That study suggested the dissemination of virulence factors into the environment can play an important role in the already existing bacterial population and act as an additional pressure for newly emerging pathogenic strains. In the current study, 62.5% of the isolates obtained exhibited possession of the *astA* gene illustrating the likelihood of *E. coli* strains on retail pork products carrying at least one pathogenic factor and thus potentially could cause illness if ingested. Additionally, among the isolates which tested *astA*-positive there were the two unidentified isolates: 4Emem and 4Etbx. More interestingly, in the phylogenetic analysis isolate 4Etbx clustered with an *E. coli* O104 enteroaggregative strain, which also possesses *astA.* Therefore, it is likely that acquiring new virulence factors can develop new pathogenic strains potentially harmful to humans, just as seen with EHEC O104:H4. EAggEC is the most recently recognised category of *E.coli* pathotype and strains in this category are capable of producing a heat-stable toxin 1 (EAST1) which is a product of the *astA* gene which can be located either on the chromosome or on plasmids in one or multiple copies (Veilleux & Dubreuil, 2006). EAST1-positive strains have mainly been associated with swine and cattle. Positive strains were detected in pigs with post-weaning diarrhoea and from pigs with edema disease (Choi *et al.,* 2001; Osek, 2003). Both those studies illustrated a significant relationship between *astA* gene and a fimbrial marker (F4) which contribute to the adherence of the pathogen to porcine intestinal epithelial cells as it seems that the majority of the times, isolates positive for *astA* are also positive for F4. Furthermore, EAST1 is not solely present in EAggEC strains but also in other human and animal diarrhoeagenic strains, and sequence of the toxin from a pig isolate demonstrated it is identical to the human pathogenic strain

O42 (Veilleux & Dubreuil, 2006), thus illustrating that EAST1 from pigs may play a critical role in pathogenicity associated with diarrhoea.

4.3.2 *Yersinia*

Yersiniosis was the 4th most frequently zoonosis in the EU in 2011 with *Y. enterocolitica* bio-serotypes 4/O:3 and 2/O:9 said to be the most prevalent around Europe (Bonardi *et al.*, 2014). In the UK human cases of yersiniosis were mainly associated with 3/O:9, 3/O:5,27, 2/O:9 and 4/O:3 while strains from biotype 1b and 4/O:3 were found to be largely isolated in the United States (McNally *et al.*, 2004). Since healthy pigs are known to be carriers of the human pathogenic 4/O:3 bio-serotype (Bonardi *et al.,* 2014), there is no doubt why this is the most reported pathogenic strain. Laukkanen *et al.* (2009) showed that carcasses were contaminated at slaughterhouses with pathogenic strains which belonged to bioserotype 4/O:3, a similar genotype to that of strains isolated from the animals at the farm, hence demonstrating that pork product contamination is likely derived from cross-contamination during the slaughter process. Cross-contamination events during the slaughter process act as a major factor for contaminating the carcass and therefore the meat, especially in the pig slaughter process where scalding and singeing cannot actually control cross-contamination since evisceration is at a later stage in the process (Warriner *et al*., 2002). Similarly, a study on characterisation of pathogenic strains from different slaughterhouses revealed 4/O:3 as the only bio-type isolated (Fredriksson-Ahomaa *et al.*, 2000).

Patho-serotyping of the isolates in the current study was performed for the detection of O:3, O:9 and O:5,17 serotypes which are the most commonly detected as mentioned previously. A multiplex PCR was performed (Garzetti *et al.* 2014), for

identification of the O-antigen gene clusters of each particular serotype. Only one *Y. enterocolitica* isolate gave a positive result for any of the serotypes described above. Isolate 1PK5B was shown to belong to serotype O:5,27 while the rest of the isolates remained unidentified. This serotype has previously been isolated from caecal and faecal contents of pigs at slaughter (Kotula *et al.*, 1993), suggesting pigs are also asymptomatic carriers of this pathogenic serotype just as is seen with 4/O:3. Similar findings were observed during an investigation of the different biotypes isolated from animals at slaughter and patients with yersiniosis in Great Britain when O:5,27 was the predominant pathogenic type isolated from animals, and particularly pigs and sheep (McNally *et al.,* 2004).

The pathogenicity of each isolate depends on the possession of particular virulence factors. In this study the isolates were tested for two chromosomally encoded genes, *ail* and *inv,* and two plasmid-encoded genes, *virF* and *yadA,* which all play an important role in the pathogenicity of *Y. enterocolitica* strains (Thoerner *et al.*, 2003). The invasion of the tissue is believed to be the initial step of pathogenesis. Pathogenesis in *Yersinia* depends on a virulence plasmid as described in Section 1.4.4 of this report. However, genes responsible for invasion are thought to be located on the chromosome rather than the plasmid, as strains lacking the plasmid could still be invasive (Miller & Falkow, 1988). As noted above, in *Y. enterocolitica* there are two chromosomal loci that confer an invasive phenotype, *inv* and *ail*. Invasin (*inv*) facilitates the entry by directly attaching onto mammalian cell receptors and is essential for effective penetration of the intestinal barrier. Moreover, it is more highly expressed at ambient temperatures (~ 25^{o}C), unlike the *ail* gene which is expressed more at 37^{o}C, possibly to prime the system for the

initiation of the infection after contact with a host (Pepe & Miller, 1993). Furthermore, *inv* expression is regulated in response to temperature, growth rate and pH, where at 26°C and pH 8 it is maximally expressed, whereas expression at body temperature requires pH 5.5. The thermoregulation of *inv* depends on the levels of RovA; a MarR-type regulator that triggers *inv* expression both *in vitro* and *in vivo* (Fàbrega & Vila, 2012). Several studies have reported that an additional promoter provided by an IS*1667* element inserted into the *invA* promoter region can increase expression of the *inv* gene (Schaake *et al.*, 2014; McNally *et al.*, 2016). Additionally, this nearly constitutive expression of *inv* is specifically occurring in O:3 strains which because of this are also able to invade human cells at 37°C (Schaake *et al.*, 2014). Garzetti *et al.* (2014) have illustrated in their study that strains with serotype O:5,27 were *inv*-positive, unlike isolate 1PK5B from this study which appeared negative for the possession of the gene. However, strains lacking invasin would require an increased number of cells in order to initiate an alternative and less productive invasion route to infection (Pepe & Miller, 1993). The second invasion locus, *ail*, has mainly been found associated with pathogenic strains and hence it is considered the most important invasion locus. However, in this study results proved otherwise as two *Y. frederiksenii* isolates, 3CPK and 3CPNK, showed possession of the gene. There is no information about possession of this gene from *Y. frederiksenii* strains in the literature but nevertheless, some *Y. enterocolitica* biotype 1A strains isolated from pigs and pork products, which are considered non-pathogenic, showed possession of this gene (Garzetti *et al.* 2014), thus possession of this gene alone may not denote strain pathogenic potential. Despite *Y. frederiksenii* strains being considered non-pathogenic, mainly due to the

absence of the virulence plasmid, it has been reported that strains of this species were associated with acute diarrhoeal disease (Sulakvelidze, 2000).

The plasmid-encoded *virF* gene encodes for a transcriptional activator of *yop* and *yadA* genes, both vital virulence determinants of the *Yersinia* genus. In *Y. enterocolitica* strains that are pYV-positive the expression of *yadA* is said to be strictly dependent upon *virF* (de Rouvroit *et al.,* 1992), which seems possible since both genes are located on a plasmid essential to cause infection. Neither *virF* nor *yadA* were detected among *Y. enterocolitica* isolates of this study, which would suggest that the isolates are probably pYV-negative, including the isolate identified as patho-serotype O:5, 27. Pathogenic strains lacking the virulence plasmid were also reported by Fukushima *et al.* (1993) when they differentiated successfully strains O:5,27 into biotypes 2 and 3, both of which can carry the virulence plasmid, and they showed that out of the 138 isolates tested, fifteen of them lacked the virulence plasmid.

Paradoxically, the *yadA* gene was detected in all *Y. frederiksenii* isolates. *yadA* is essential to *Y. enterocolitica* as it protects from the bactericidal activity of human serum and helps it persist in the Peyer's patches (El Tahir & Skurnik, 2001). Plasmid-borne genes *virF* and *yadA* have previously been reported in a *Y. intermedia* strain but have not been reported in other non-pathogenic species including *Y. frederiksenii* (Schaake *et al.*, 2014). Possession of the *yadA* gene suggests that the isolates possess the virulence plasmid pYV, which also suggests that the *virF* gene should be present too. However, the *virF* gene was not detected in any of the isolates. One reasonable explanation could be that the primers used for the detection of *virF* may have not been very specific and therefore it would be

interesting to repeat the PCR using a different set of primers if there had been more time. Using a positive control strain positive for *virF* would also be useful to check whether the PCR was working with the conditions used. Similarly, the results observed for the *yadA* gene would need to be validated by sequencing the amplicons to check the nucleic acid sequence needed to is correct. A positive strain carrying the virulence plasmid and therefore *yadA* would also be a useful positive control for the PCR. Nevertheless, Sulakvelidze (2000) states that some of the non-pathogenic *Yersinia* species carry large plasmids discrete from pYV, and despite little being known about genes encoded on these plasmids, it has been reported that non-pathogenic *Y. enterocolitica*-like strains containing such a large plasmid were strongly related with human diarrhoea. Therefore, *yadA* could also be carried on such a plasmid and perhaps due to the absence of other virulence genes normally present on pYV, the pathogenicity of the strains carrying it has not fully been established. Consequently, it is essential to further study non-pathogenic isolates in order to understand a pathogen's evolution. As McNally *et al.* (2016) stated: "the role of the accessory gene pool in pathogenic bacteria and pathogenesis may be better understood when studied in the wider context of non-pathogenic members of the genus". Additionally, the acquisition of virulence factors by non-pathogenic organisms could perhaps be important for their survival against competitors within their own environment and it does not necessarily have to be associated with mammalian pathogenesis.

4.4 Metal Resistance

Unlike other elements, mercury has no positive role in cellular processes and it has, in fact, been in use for many years both in medicine and agriculture as an

antimicrobial (Hobman & Crossman, 2015). Bacteria have evolved different resistance mechanisms to deal with the many forms of mercury, with plasmid-encoded resistance being as common as antibiotic resistance and hence can be easily found in many bacterial genera (Summers, 1986). All Gram-negative isolates were tested both phenotypically and genotypically for mercury resistance (section 2.7.9.1). Phenotypic assays showed only *E. coli* isolate 1F was resistant to mercury. Therefore, PCR was performed to detect three specific genes: *merA* and *merC* which are involved in resistance and *intl1* which is associated with an integron carried within Tn*21*-family mercury resistance transposons, which can carry antibiotic resistances. Isolate 1F carried all three genes, thus confirming the results of the phenotypic assays, but also suggesting the possession of an integron carrying mercury resistance (due to *intl1* gene). Isolate 1B also carried *merC* and *intl1* but not *merA.* The resistance mechanism strictly requires *merA*, which encodes for the enzyme mercuric reductase, to reduce Hg^{2+} to the less reactive volatile form of Hg^{0} in the bacterial cytoplasm; since 1B did not possess the gene it appeared phenotypically sensitive. *merC* is an operon-encoded transmembrane protein, particularly found in Tn*21*-family transposons, which pumps ionic mercury into the cytosol (Pal *et al.*, 2017). *intl* is a gene encoding for an integrase which is a necessary component of an integron and many class 1 integrons (*intl1*) are found on Tn*21*-like transposons (Mazel *et al.*, 2000). The data suggest that since 1F possesses *merC* and *intl1*, a Tn*21*-like transposon is present. The *mer* transposon is particularly important as it acts as a vector for co-resistance because it is known to accumulate antibiotic resistance genes as well as conferring resistance to mercury (Pal *et al.*, 2017). For example, mercury resistant *E. coli* were detected significantly

more regularly in inhabitants with high carriage rates of antibiotic-resistant *E. coli.* Moreover, humans that are exposed to mercury through their living environment have been found to carry significantly more resistant *E. coli* than people living in areas where exposure to mercury is insignificant, illustrating the environmental impact on gene selection (Skurnik *et al.*, 2010). The interaction between antibiotic resistance and metals could be due to the fact that some multidrug efflux pumps are induced by heavy metals and also due to metal cations that complex with antimicrobial elements such as tetracycline in order to prevent their absorption (Hölzel *et al.*, 2012). Co-transferability on plasmids has previously been described for streptomycin, tetracycline and chloramphenicol resistance and tolerance to mercury (Nakahara *et al.*, 1977). Interestingly, both isolates 1F and 1B, which showed possession of the mercury genes, also were the only two to confer resistance to tetracycline, chloramphenicol and streptomycin (Table 3.8).

Unlike mercury, copper is involved in many cellular processes and therefore is an essential cofactor for most organisms (Zimmermann *et al.*, 2012). Copper is commonly used in pig feed and usually is supplied in concentrations higher than those recommended in order to counterweigh for dietary factors that could cause reduction in its absorption. As a consequence, isolation of organisms with Cu resistance could be expected. PCR was performed on all Gram-negative isolates for the detection of the *pcoE* gene as an indicator for carriage of the plasmid-borne copper resistance system. As was seen with mercury, isolates 1B and 1F were the only ones positive for possessing *pcoE*. It has been suggested that resistance to copper arises as a result of copper being used in agriculture and particularly in pig production, and resistant *E. coli* were isolated from weaned pigs that were fed with

copper as a growth promoter (Brown *et al.*, 1995). Resistance is conferred after a succession of events from a combination of both chromosomal and plasmid-encoded genes (Agga *et al.*, 2014). In *E. coli* the primary mechanism for copper homeostasis is the chromosomal *cue* system, which is an efflux system responsible for maintaining normal cellular copper concentrations in both aerobic and anaerobic conditions (Pal *et al.*, 2017). However, in more extreme copper concentrations or under anaerobic conditions another efflux system is used: the *cus* system. Both systems are chromosomally-encoded (Hobman & Crossman, 2015). Plasmid-encoded resistance is conferred by the *pco* system which was first found on a plasmid isolated from pigs that were given copper feed supplements (Rouch *et al.*, 1985). This cluster contains seven genes, including *pcoE* which is transcribed from a separate promoter (Brown *et al.*, 1995). This gene is a periplasmic copper-binding protein strongly induced by the presence of cupric salts under the control of the chromosomal regulator *cus*RS in the *cus* system. Its expression reduces recovery time from copper stress in *E. coli* cells but overexpression of this gene alone showed very little effect on resistance (Zimmermann *et al.*, 2012). The relationship between copper resistance and antibiotic resistance has also been described with particular relation to an increased resistance against β-lactams (Hölzel *et al.*, 2012). However, such a correlation was not observed in this study with copper resistant isolates 1B and 1F (Figure 3.13) since only isolate 1B was phenotypically resistant to ampicillin and ampicillin/clavulanic acid while 1F did not exhibit those resistances.

Another example of co-resistance is that of copper and silver. The *pco* and *sil* operons have regularly been found together in an identical arrangement in plasmids

but also on the chromosomes of Gram-negative organisms (Asiani, 2016). Both systems share similarities, with the silver resistance mechanism being predicted through homology studies to the copper mechanism. In addition, it was noted that the *cus* system for copper resistance is capable of conferring resistance to low levels of silver ions (Asiani, 2016). For the presence of silver resistance, the isolates were tested for possession of two genes; *silP* and *silE*. The SilE protein is homologous to PcoE with 48% sequence identity (Zimmermann *et al.*, 2012). It is, as well, a periplasmic binding protein whose expression is controlled by its own promoter and is highly expressed when silver ions are present. SilP, on the other hand, is an efflux pump of the ATPase-type that transports the ions from the cytoplasm into the periplasm (Asiani, 2016); similar to the function of PcoD and PcoC. Therefore, since copper and silver are an example of co-resistance, it was to be expected that the only isolates possessing the genes tested would be those expressing copper resistance. Silver is known to be toxic to bacteria but just like copper, its use through various products and processes may have contributed towards development of resistance. Silver was approved as an antimicrobial in the early 20th century and was particularly used to treat ulcers. However, when antibiotics were discovered its usage reduced but was re-introduced again around the 1960s in a solution form of silver-nitrate for the treatment of burn patients in combination with sulphonamides (Chopra, 2007). As a result, it could be assumed that silver resistance could possibly co-select for resistance to sulphonamides if strains started acquiring those genes. Such results were actually observed in this study with both isolates, 1B and 1F, possessing *silE* and *silP* also conferring phenotypic resistance to sulphonamides. Co-resistance was also documented for

plasmid pMG101, on which the silver resistance locus was first discovered, which is known as a MDR-plasmid containing multiple genes encoding for antibiotic resistances (Pal *et al.*, 2017), with chloramphenicol, sulphonamides, streptomycin, ampicillin and tetracycline being a few of them (Hobman & Crossman, 2015). Both isolates exhibited a combination of the above antibiotic resistances, including resistance to chloramphenicol which was not as common in this study. Silver is still widely used in medicine, from medical devices made with silver polymers to dental amalgams but also in the non-medical sector as a biocide. Additionally, is also involved in water treatments (Silver, 2003); all of this can contribute to the slow release of silver and add to the resistance gene pool in both environmental and biological settings.

Unlike the mercury resistance results, the phenotypic results for copper and silver resistance did not match the genotypic ones. The silver resistance phenotype was not expressed for isolates 1F and 1B despite the presence of the resistance genes in both isolates. According to Gupta *et al.* (1998) sensitive strains can grow in up to 100 μM of $AgNO_3$, while strains with plasmid pMG101 continued to grow in up to 600 μM. Gupta *et al.* (2001) showed that an *E. coli* strain carrying the pMG101 plasmid had high and constitutive level of transcription of *sil* genes, which could explain why the control strain showed perfect growth for up to 600 μM, while isolates 1F and 1B did not exhibit phenotypic resistance.

However, *Y. enterocolitica* isolates, *Y. frederiksenii* isolate 3PNK5 and *E. coli* isolate 4Emem showed growth up to 200 μM but none showed possession of either *silP* or *silE*. Similar results were observed for copper phenotypic assays with isolates 1F and 1B showing possession of the *pcoE* gene but appearing phenotypically sensitive

to copper as growth on plates was only observed up to 5 and 6 mM $CuSO_4$, respectively. The *pco* system responsible for plasmid-mediated copper resistance is encoded by nine clustered genes that are arranged as two operons and one separate gene (*pcoE*) (Pal *et al.*, 2017). Additionally, expression of *pcoE* is regulated by a different promoter than *pcoABCD* (Zimmermann *et al.*, 2012). Isolates 1B and 1F which were shown to possess *pcoE,* might not have fully expressed the rest of the genes involved in the copper resistance system. PcoE has been said to act as a first line of defence copper 'sponge' protein that binds copper while the rest of the proteins are being expressed. Nevertheless, full copper resistance requires proteins PcoC and PcoD (Hobman & Crossman, 2015). Therefore, this could explain why isolates 1B and 1F were shown to be phenotypically copper sensitive but *pcoE* positive. Strains expressing full resistance can tolerate up to 20 mM $CuSO_4$ while plasmid-free strains usually do not endure more than 4 mM (Rouch *et al.*, 1985). However, if pigs were exposed to conditions where the metals were in abundance and thus their gut flora became tolerant, then it could explain why the isolates withstand slightly higher concentrations than expected. *Y. frederiksenii* isolates 3CPK and 3CPNK in particular, tolerated a higher copper concentration at 8 mM compared to the rest of the isolates. As was mentioned previously, both those isolates showed possession of two pathogenic genes, *yadA* and *ail.* It was described by McNally *et al.* (2016) that possession of pathogenic genes might be important for the survival of the strains in their environment and not necessarily correlated with mammal infection. Likewise, the ability to tolerate higher copper concentrations could perhaps have resulted from competition for survival. Finally, although plasmid-encoded copper resistance is

said to be inducible, the level of resistance in induced and uninduced cells varies accordingly with the growth phase of the culture (Rouch *et al.*, 1985). To further investigate copper resistance amongst the isolates, it would have been interesting (if there had been more time) to test for the presence of other *pco* genes, particularly in isolates 1B and 1F which appeared phenotypically sensitive despite possessing *pcoE*. The same can be said for *Y. frederiksenii* isolates 3CPK and 3CPNK which tested negative for *pcoE,* but managed to grow on plates with up to 8 mM $CuSO_4$, a concentration slightly higher than that tolerated by a copper-sensitive strain.

4.5 Phylogenetic Analysis

Interpretation of the phylogenetic trees was conducted as described by Gregory (2008). On a phylogenetic tree, moving from the roots to the tips is a move forward in time (from ancestor to modern species). Sister taxa are species that share a common ancestor that is not shared by other species. For example, 1F and 4Emem are sister taxa (Figure 3.3). The branch length indicates some measure of divergence. For instance, the lineage that led to 4Emem has undergone less change than that of 1F. Isolates 1B, Sw1F and Sw2F are clustered with the known strain O157 str. 644-PT8. Similarly, the unidentified *Escherichia* isolate 4Etbx clustered with *E. coli* O104:H4 str. C227-11. However, although 16S rRNA gene is useful for assigning different bacterial strains to species, it should not be assumed that the isolates of this study are identical to the known strains they showed compatibility with because the analysis was based only on the 16S rRNA gene and not the whole genome sequence. According to Hao *et al.* (2016), the 16S rRNA gene solely is sometimes insufficient for distinction to species level due to the high sequence

similarities among particular species and heterogeneity within copies at the intragenomic level.

According to Chaudhuri & Henderson (2012), groups A, B1, E, D1 and D2, B2 and C-I are the major groups in the evolution of *E. coli* phylogeny. The majority of the strains causing human infections belong to groups B2 and D with those groups possessing the most virulence factors, whilst the group with the lowest prevalence of virulence factors is group A (Corvec *et al.*, 2007). Figure 4.1 illustrates a modified version of the phylogenetic tree published by Chaudhuri & Henderson (2012).

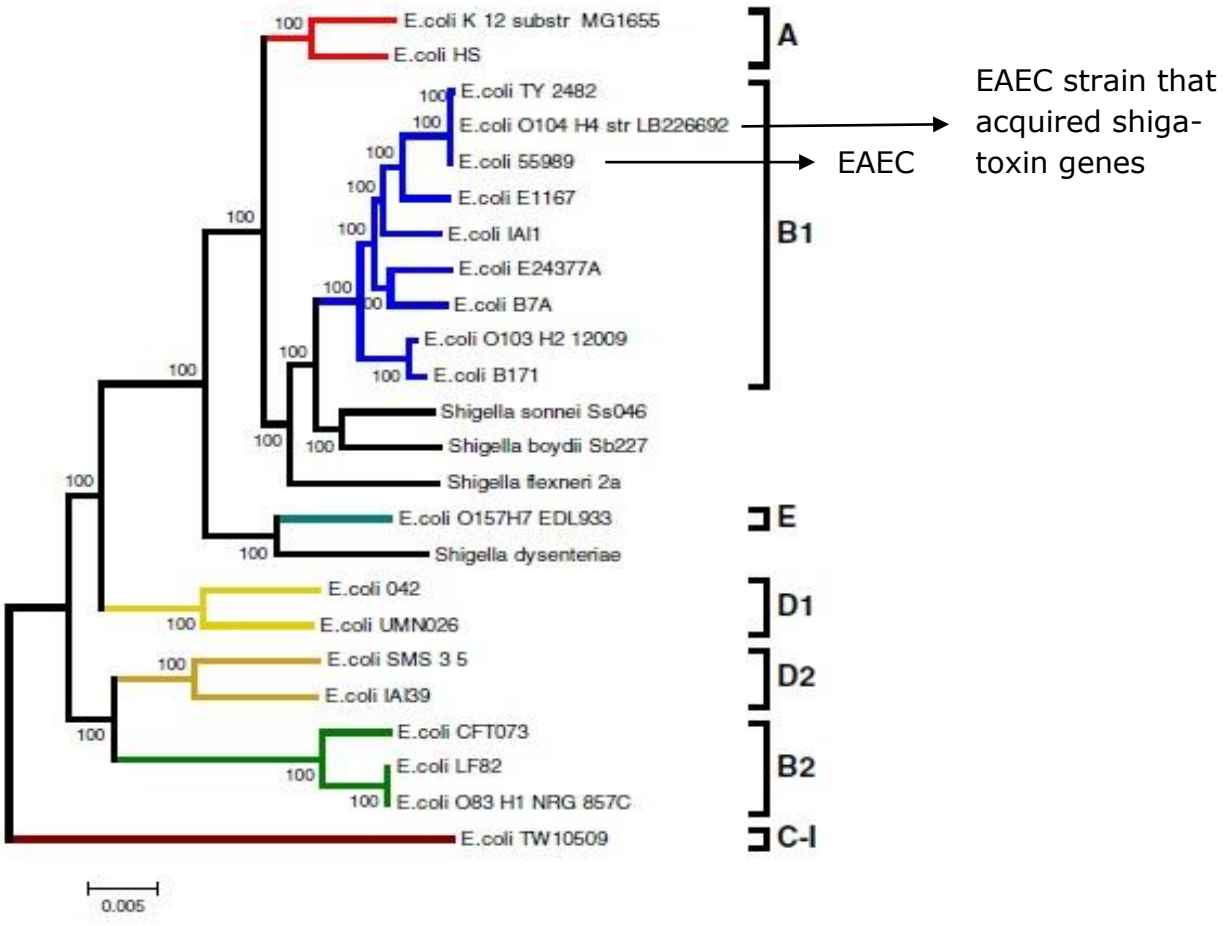


Figure 4.1: Major groups of the *E. coli* phylogeny determined by phylogenetic analysis of complete and draft genome sequences available in GenBank.

Constructing a phylogenetic tree using a partial 16S rDNA phylogenetic analysis allowed a similar cluster pattern to part of Figure 4.1, which further allowed the identification of unknown species (4Emem and 4Etbx) as *E. coli*. Therefore, taking into consideration the phylogenetic tree constructed for this study (Figure 3.3) and comparing results with the above phylogenetic tree it could be that isolate 4Etbx is categorised into group B1 and isolates Sw1F, Sw2F and 1B into group E. However, such a suggestion would only be supported if the isolates were positive for the possession of a number of virulence factors that make every group strain distinct from one another; although virulence genes in *E. coli* are usually located on plasmids, such as the pAA plasmid, or phage and therefore can be easily transmitted between quite unrelated isolates (Fratamico & Smith, 2017). For example, the strain associated with the outbreak in Germany in 2011 was a serotype not typically related to EHEC and it showed possession of a number of genes normally found in EAEC. For fact, it can be observed from the above phylogenetic tree that there is a close relationship between strain O104:H4 and *E. coli* 55989 which is an EAEC isolated from an immunocompromised individual with persistent diarrhoea (Chaudhuri & Henderson, 2012). *E. coli* O157:H7 strain 644-PT8 is a STEC strain that caused a foodborne outbreak at a restaurant but was genetically dissimilar with STEC O157:H7 disseminating among the local population. It was later clustered with strains from the whole genome sequence database to reveal it was closely related with strains linked with foreign travel to Egypt and Israel (Cowley *et al.*, 2016). None of the isolates of this study showed possession of *eae* gene which most EHEC possess. However, isolate 4Etbx did show possession of the EAEC *astA* gene but the presence of additional virulence factors is not known

and therefore it cannot be proposed that there is a close relationship with strain O104:H4. Correspondingly, the same can be said for Sw1F, Sw2F, 1B and O157:H7. Nevetheless, the phylogenetic analysis indicates the possible association of the isolates with specific linages from which known pathogens arise.

Regarding antimicrobial resistance among the different groups, Corvec *et al.* (2007) demonstrated that isolates which were AmpC overproducers belonged more regularly to the non-pathogenic group (A) and less frequently to the more virulent groups D and B2. Such findings suggest that commensal bacteria are perhaps more regularly exposed to antibiotics and thus developing resistance faster than pathogens. On the other hand, a different study illustrated that ESBL-producers belonged more frequently to groups D and B2 and to a lesser extent to groups B1 and A (Pitout *et al.*, 2005). Consequently, such findings show that AMR is not exclusively found within particular strains but instead is widespread and can be expressed from both pathogenic and non-pathogenic strains.

Yersinia sp*.* isolate 2PK5M was shown to be closely related to a known *Yersinia kristensenii* strain. *Y. kristensenii*, just like *Y. frederiksenii*, is also referred to as a *Y. enterocolitica*-like species. It has been isolated from multiple sources including fresh water, soil, foods, animals as well as humans. This species has a different H antigen pattern than *Y. enterocolitca*, but they share some of the O antigens. Additionally, genetic studies of *Y. enterocolitica* and *Y. enterocolitica*-like species demonstrated that *Y. kristensenii* strains isolated from individuals with diarrhoea are more genetically related to one another than environmental isolates of the same species (Sulakvelidze, 2000). Although *Y. kristensenii* is normally a non-pathogenic species that lacks the virulence plasmid, it has been previously

associated with disease. It has also been reported to cause fatal infection in iron-loaded mice, perhaps due to the fact that non-pathogenic species like *Y. kristensenii* and *Y. frederiksenii* carry chromosomal DNA homologous to the *inv* pathogenic gene (Robins-Browne *et al.*, 1991). Antimicrobial susceptibility testing performed on isolates from various foods including pork and sausages taken from pork butchers have showed that *Y. kristensenii* isolates were the most susceptible to ampicillin and carbenicillin. Similarly, they were also sensitive to non-lactam antibiotics including tetracycline and chloramphenicol (Ahmedy *et al.*, 1985). However, antimicrobial genes can be selected at any time and be taken from non-pathogenic strains which can then act as reservoir for the genes for potential pathogens, such as the results observed with the non-pathogenic isolates of this study.

According to the staphylococcal phylogeny, isolate 4St which is a coagulase-negative *Staphylococcus* sp., seemed to be related to a *Staph. vitulinus* strain. This particular species is a member of the *Staphylococcus sciuri* group that are predominantly associated with farm animals, pets or wild animals but have also been isolated from different foods of animal origin (Stepanovic *et al.*, 2005). In addition, this species is considered novobiocin-resistant. Antimicrobial susceptibility testing of isolates obtained from humans, slaughterhouse and animals showed oxacillin resistance using the MIC method and around 30% of the isolates were also tetracycline resistant. However, when tested for the presence of the *mecA* gene, all isolates were shown to be negative (Ćirković *et al.*, 2008). Sinlapasorn *et al.* (2015) isolated a *Staph. vitulinus* strain from a pig which showed resistance against oxacillin, cefoxitin, erythromycin and tetracycline. Despite being mainly found in

animals, members of the *S. sciuri* group have been associated in the past with severe infections including peritonitis, wound infection and UTI (Stepanovic *et al.*, 2005).

5 Conclusion

This project aimed to study the AMR of potential foodborne pathogens isolated from retail pork meat products. The study showed the prevalence of pathogenic and non-pathogenic organisms on raw pork products highlighting the importance of hygiene practices in slaughterhouses.

Apart from the presence of foodborne pathogens in pork products, this study also illustrated that most of the obtained isolates were MDR, including *E. coli* isolate 1F which tested positive for chromosomal AmpC, possession of the enteroaggregative *astA* gene and showed resistance to mercury, copper and silver. Table 7.1 (Section 7.3) summarises the gene possession of all Gram-negative isolates obtained in this study. Some resistances were due to genes located on the chromosome while others were possibly acquired via plasmids or other genetic mobile elements. It was also noted that the resistances observed could have been selected from the animals' gut flora during treatment period with certain drugs. Resistance genes seemed to persist and therefore co-selection can take place and spread among organisms. For example, chloramphenicol resistance was observed for two *E. coli* isolates. This antibiotic has been banned from the EU for use in farm animals for many years (Bywater *et al.*, 2004) but resistance towards it is still being observed, showing that the genes are persisting. The same can be said for nitrofurantoin. In Canada it has been withdrawn from use in pigs for over two decades, but resistance among pig *E. coli* isolates still persists (Akwar *et al.*, 2008). In this study nitrofurantoin resistance was shared among all non-pathogenic *Yersinia* isolates while in pathogenic isolates only one *Y. enterocolitica* isolate was shown to be resistant. This result highlights another important finding of the study; that non-pathogenic species should not be ignored and excluded from studies. In one way or

another, they can still contribute towards the resistance gene pool as they were shown to be potential reservoirs of resistance genes. Furthermore, studying the non-pathogenic species will provide a better understanding on the virulence of the pathogens and any new emerging pathogenic strains.

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7 Appendix

7.1 Schematic Diagrams of the Isolation Methods

Figure A: Scheme for the ISO 6579:2002 + A1:2007 of the horizontal method for the detection of *Salmonella* species

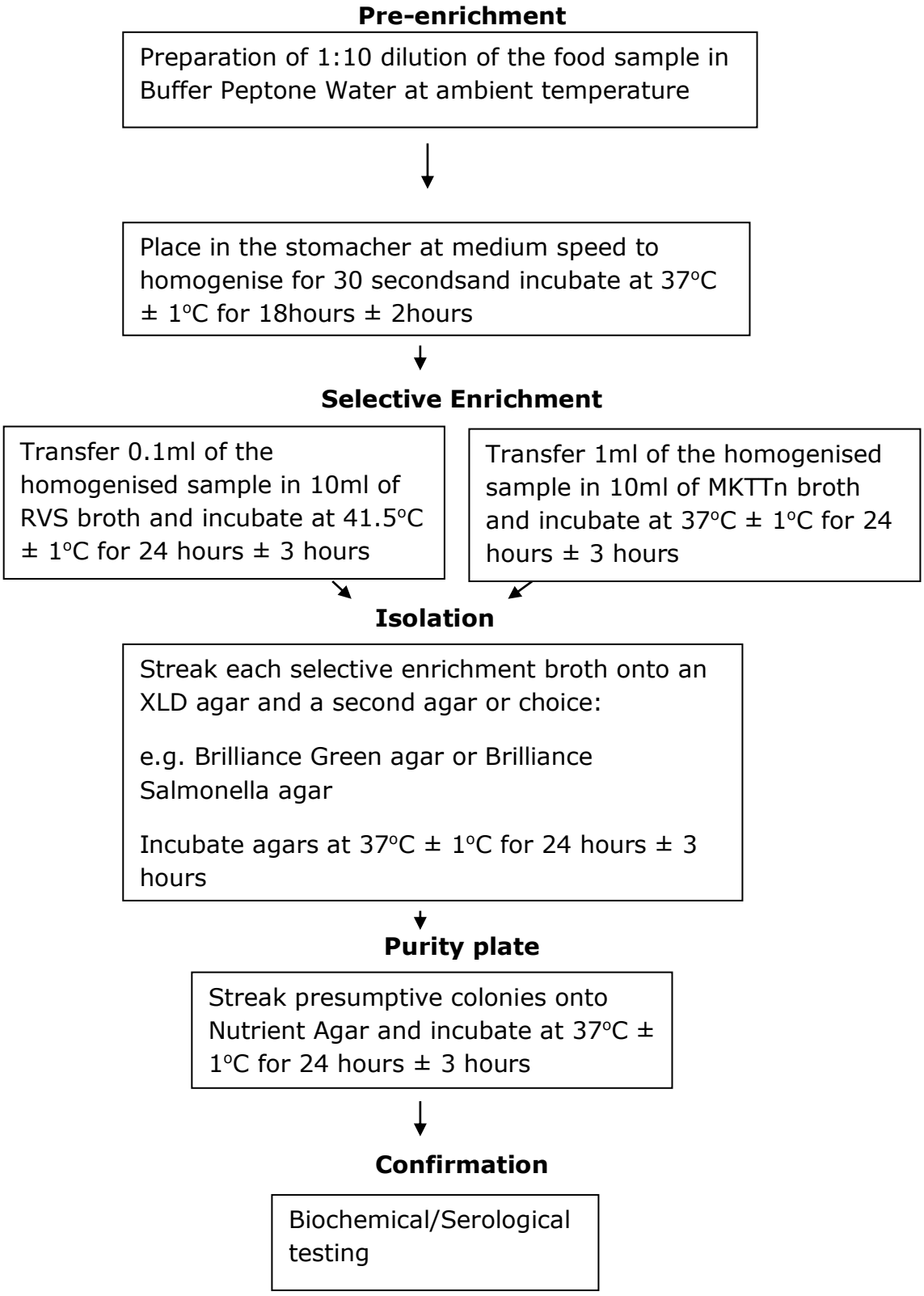


Figure B: Scheme for the isolation of Coagulase-positive Staphylococci according to ISO 6888-1:1999 + A1:2003 using Baird-Parker medium

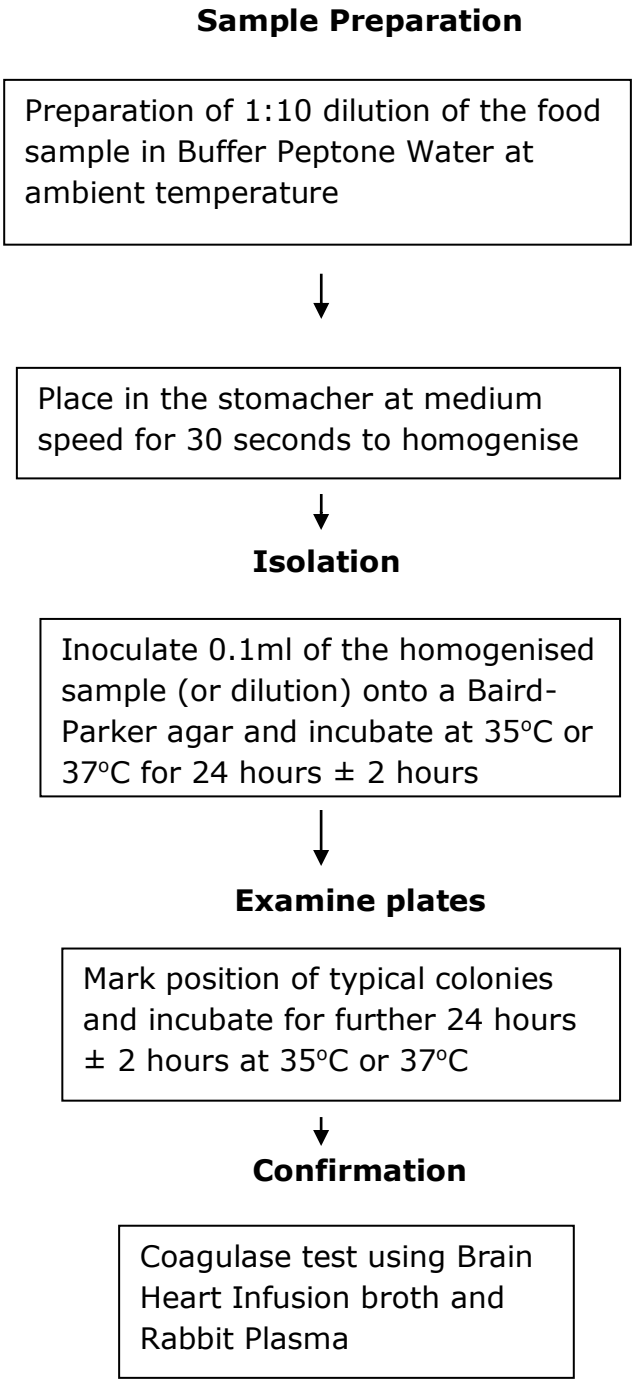


Figure C: Scheme for the detection of β-glucuronidase-positive *Escherichia coli* according to ISO 16649-2:2001 using 5-bromo-4-chloro-3-indolyl- b-D-glucuronide with and without membranes

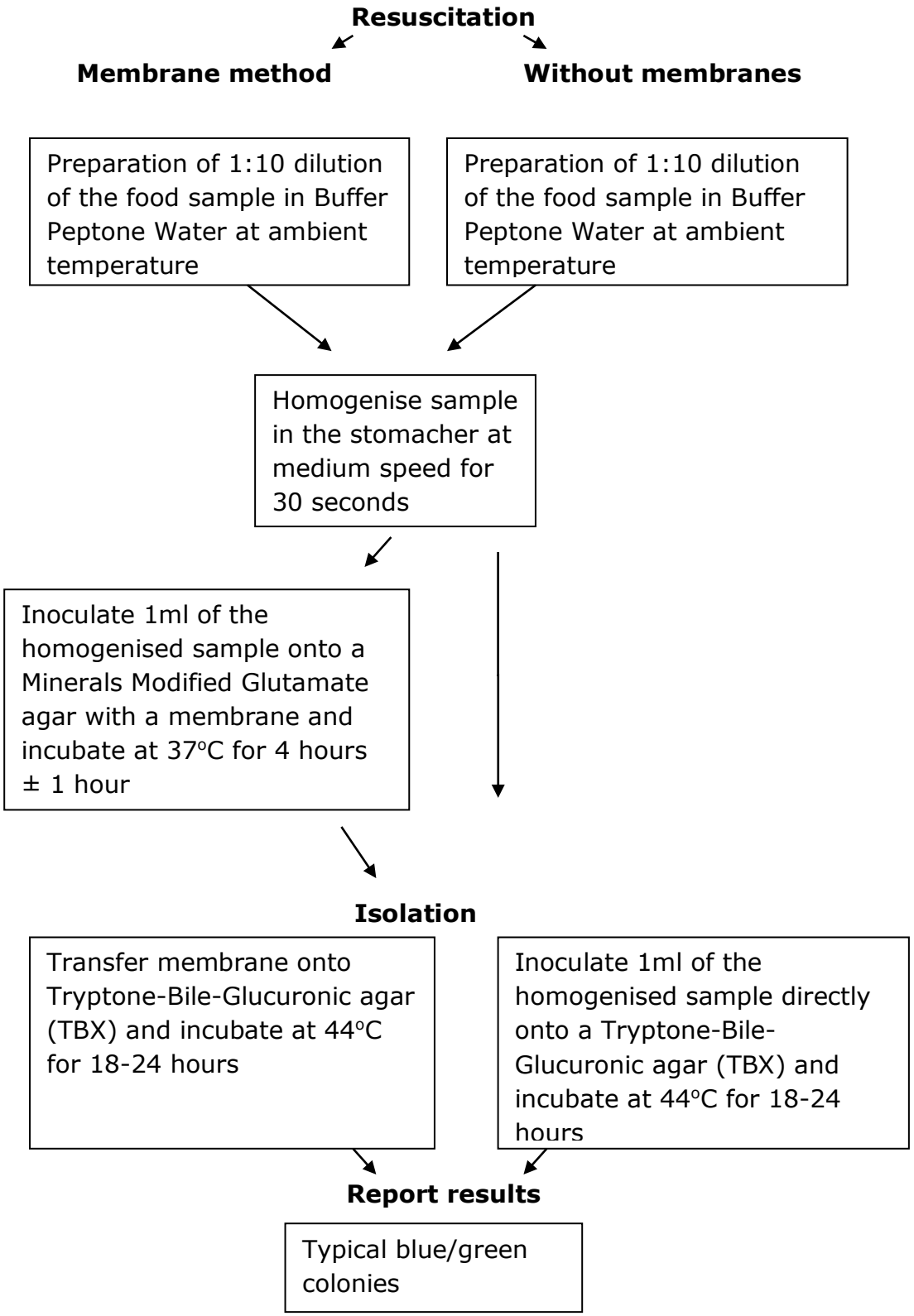
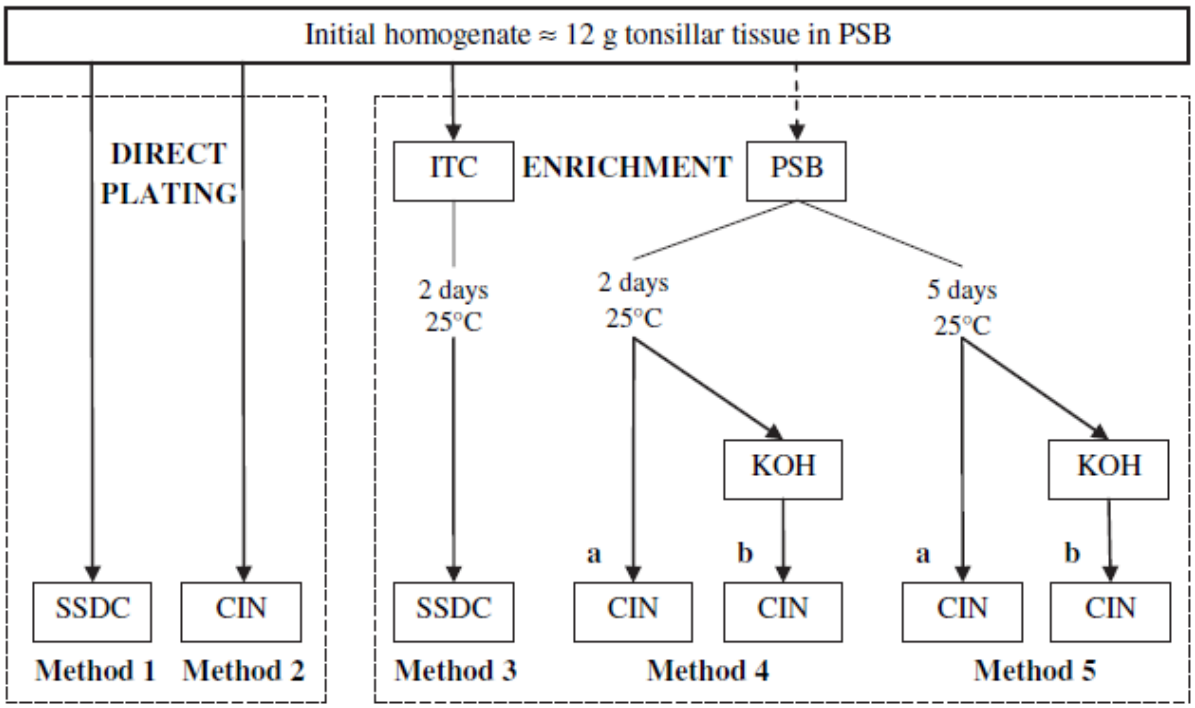


Figure D: Enumeration & detection method for *Yersinia enterocolitica* in slaughter pig tonsils as used by Van Dammee*t al.* (2010)



7.2 Supplements & Solutions

- **Ticarcillin Supplement**: Each vial was rehydrated by adding 2 ml of sterile RO water.
- **Potassium Chlorate (for ITC broth)**: 1 g of Potassium Chlorate was dissolved in 30 ml of sterile RO water.
- **Yersinia Selective Supplement**: Each vial was rehydrated by adding 1:1 ethanol and sterile RO water.
- **Novobiocin Supplement**: Each vial was rehydrated by adding 2 ml of sterile RO water.
- **Iodine-Iodide Solution**: to prepare 20 ml of the solution, 25 g of Potassium iodide (ThermoFisher Scientific, UK, Pcode: p/5880) were dissolved in 10 ml of sterile RO water. 20 g of Iodine (ThermoFisher Scientific, UK, Pcode: I/0450) were then added to it and the solution was diluted to 100 ml with sterile RO water.
- **Rabbit Plasma**: for the use of coagulase test with BHI broth (according to manufacturers' instructions).
- **Kovacs' Reagent**: Used for the purpose of the ability of organisms to split indole from tryptophan.

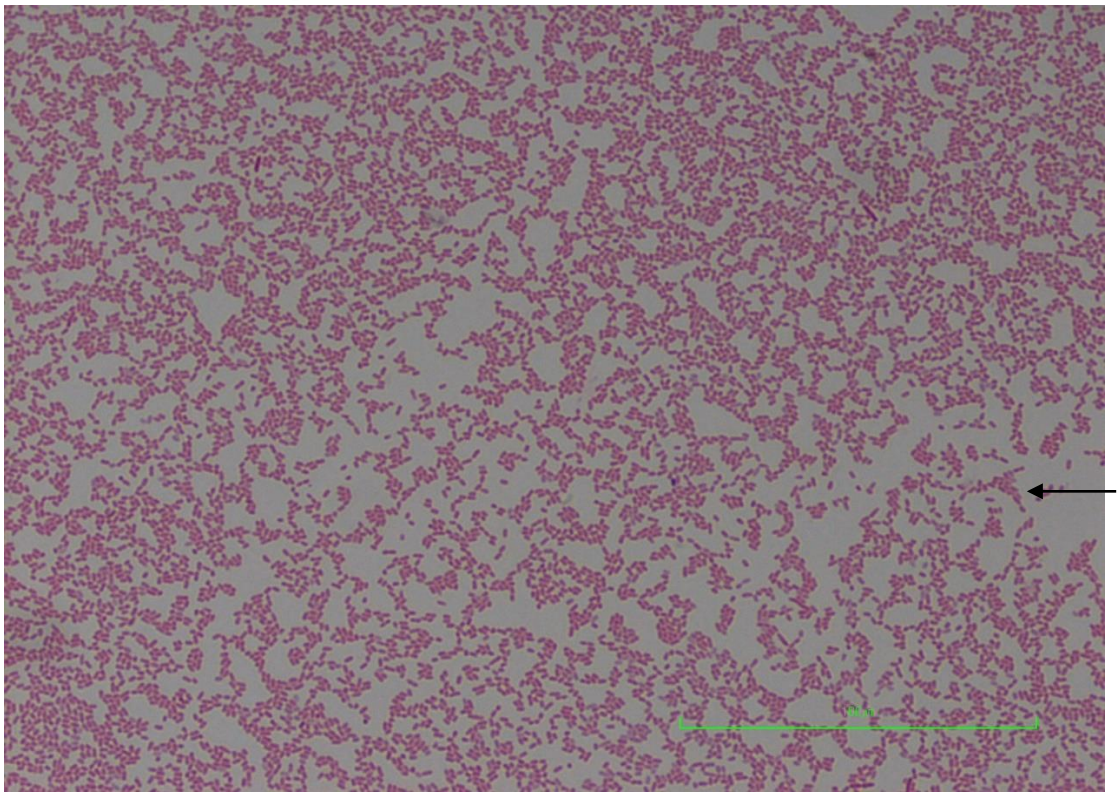
- **Q-solution**: Betaine solution that helps improving PCR conditions that previously failed by changing the melting behaviour of amino acids (Available at:https://www.qiagen.com/us/shop/pcr/end-point-pcr-enzymes-and-kits/regular-pcr/qiagen-multiplex-pcr-kit/#productdetails).
- **Ethidium Bromide Solution**: It is used to visualise DNA in agarose gels. It is classified as a toxic solution and a mutagen (Bio-Rad, 2017).

7.3 Gram-Negative Isolates

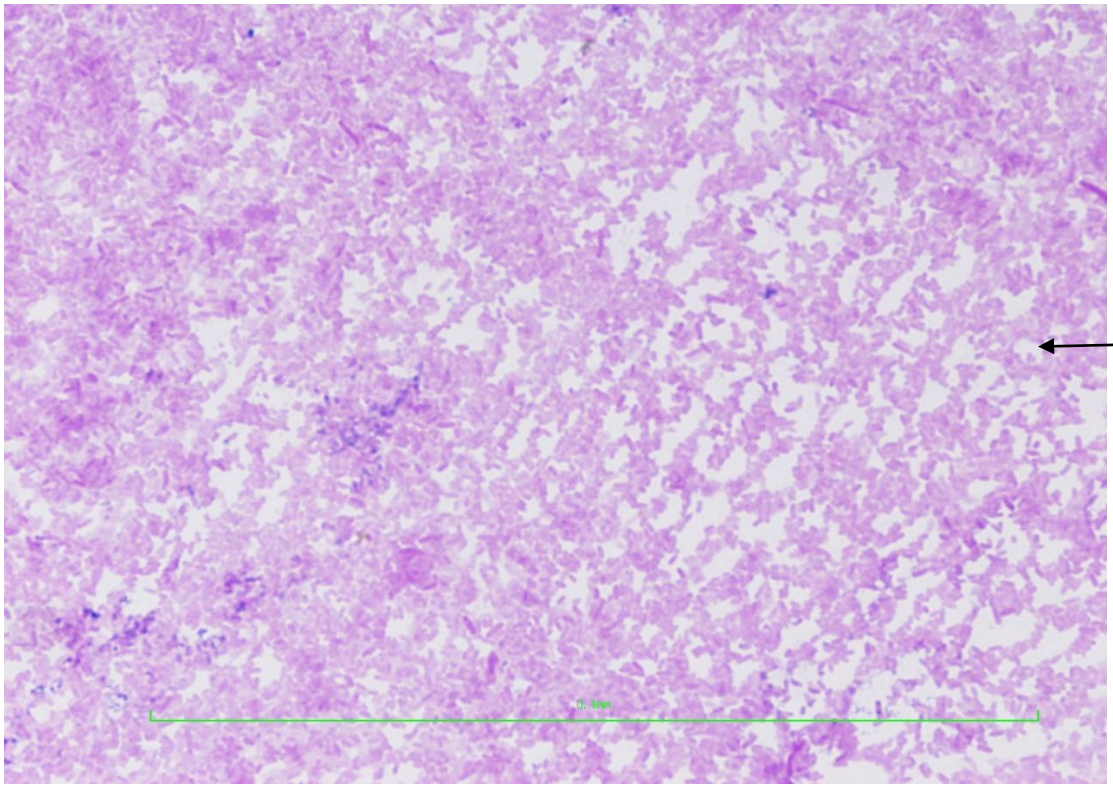
Table 7.1: Summary of resistance and virulence gene of the gram-negative isolates obtained in this study.

	Isolates	**Chromosomal AmpC**	**Pathogenic genes**	***merA***	***merC***	***IntI1***	***SilP***	***SilE***	***PcoE***
E. coli	Sw1F	√							
	Sw2F	√							
	1F	√	*astA*	√	√	√	√	√	√
	1B	√			√	√	√	√	√
	4EcRB	√	*astA*						
	4EcRX	√	*astA*						
	4Emem	√	*astA*						
	4Etbx	√	*astA*						
Y. enterocolitica	1PK5B	√							
	2CPNKB	√							
	2PNK5B	√							
	1PK5M	√							
	3PK2	√							
	3PNK2	√							
Y. sp	2PK5M	√							
Y. frederiksenii	3PK5	√	*yadA*						
	3PNK5	√	*yadA*						
	3CPK	√	*yadA & ail*						
	3CPNK	√	*yadA & ail*						
	4PNK5	√	*yadA*						

7.4 Morphology of *Yersinia enterocolitica* & *Yersinia frederiksenii*



Yersinia enterocolitica



Yersinia frederiksenii

7.5 Reference Tables for Zones of Inhibition

Differentiation of susceptible, intermediate and resistant isolates according to the diameter (mm) of each zone of clearing around each antibiotic disc. Diameter of each disc is according to EUCAST (2017) and CLSI (2014) breakpoints.

Table 7.2: Breakpoints of Gram-negative isolates.

	Zones of Inhibition (mm)		
	Sus	Int	Res
Amoxicillin-clavulanic acid (AMC)	>18	14-17	<13
Ampicillin (AMP)	>17	14-16	<13
Aztreonam (ATM)	>21	18-20	<17
Chloramphenicol (C)	>18	13-17	<12
Ceftazidime (CAZ)	>21	18-20	<17
Cefquinome (CFQ)	>23	22-20	<19
Ciprofloxacin (CIP)	>21	16-20	<15
Colistin (CT)	>14	12-13	<11
Cefotaxime (CTX)	>26	23-25	<22
Cefpodoxime (CPD)	>21	18-20	<17
Ertapenem (ETP)	>22	19-21	<18
Nitrofurantoin (F)	>17	15-16	<14
Cefoxitin (FOX)	>18	15-17	<14
Imipenem (IPM)	>23	20-22	<19
Nalidixic Acid (NA)	>19	14-18	<13
Oxytetracycline (OT)	>15	12-14	<11
Sulfonomide (S300)	>17	13-16	<12
Streptomycin (S10)	>15	12-14	<11
Trimethoprim-sulfamethoxazole (SXT)	>16	11-15	<10
Meropenem (MEM)	>23	20-22	<19
Tetracycline (TE)	>19	15-18	<14
Enrofloxacin (ENR)	>26	25-19	<18
Ceftiofur (EFT)	>23	22-20	<19

Table 7.3: Breakpoints of *Staphylococcus* isolates.

	Zones of Inhibition (mm)		
	Sus	Int	Res
Erythromycin	>23	14-22	<13
Chloramphenicol (C)	>18	13-17	<12
Nitrofurantoine (F)	>17	15-16	<14
Tetracycline (TE)	>19	15-18	<14
Ciprofloxacin (CIP)	>21	16-20	<15

Ciprofloxacin (CIP) - CoNS	>24		<24
Trimethoprim-sulfamethoxazole (SXT)	>16	11-15	<10
Clindamycin (DA)	>21	15-20	<14
Vancomycin (VA)	>16	15-14	<13
Cefoxitin (FOX)	>22		<21
Cefoxitin (FOX) - CoNS	>25		<24
Enrofloxacin (ENR)	>26	25-19	<18
Ceftiofur (EFT)	>23	22-20	<19

Table 7.4: Breakpoints of *Enterococcus faecalis*.

	Zones of Inhibition (mm)		
	Sus	Int	Res
Amoxicillin-clavulanic acid (AMC)	>17		<16
Ampicillin (AMP)	>17		<16
Chloramphenicol (C)	>18	13-17	<12
Ciprofloxacin (CIP)	>21	16-20	<15
Nitrofurantoin (F)	>17	15-16	<14
Imipenem (IPM)	>21	19-20	<18
Trimethoprim-sulfamethoxazole (SXT)	>50		<21
Vancomycin (VA)	>17	15-16	<14
Erythromycin (E)	>23	14-22	<13
Tetracycline (TE)	>19	15-18	<14