Prevalence and antibiogram of some swine associated Shiga toxin producing *Escherichia coli* Serogroups and *Salmonella* species in Nkonkobe Municipality, Eastern Cape Province, South Africa

Masters Dissertation

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Declaration

I hereby declare that the dissertation I have submitted to the University of Fort Hare for the degree of Master of Science in Microbiology is my original work, and has not been previously submitted for any degree at this institution or any other university.

Signature/date _____

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Prof A.I. Okoh (Supervisor)

Dedication

This work is dedicated to our Almighty and all sufficient God, the ancient of days, my great provider, for his wisdom, strength, protection and provision.

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Summary

Gastrointestinal illnesses have continually become a global public health issue. Exposure to zoonotic food borne pathogens such as *Salmonella* and diarrhoegenic *E. coli* either by direct or indirect contact through the consumption of food producing animals is likely an important mode of infection to humans. More so, the use of antibiotics in farm animals similar to those used in humans can select for resistance in bacteria frequently harboured by them. These resistant strains can be passed on to humans through contaminated meat products and water leading to resistant infections with consequences such as prolonged illnesses, treatment failures, and increased morbidity and mortality. In animals, these can lead to reduced productivity. Monitoring the level of resistance among bacteria from animal isolates will help in generating data that could be used to create awareness of their presence in the environment and aid in preventing a potential epidemic in the community.

In this study, we investigated the prevalence and antimicrobial resistance profile of *Escherichia coli* serogroups and *Salmonella* species in faecal samples collected from pigs in Nkonkobe Municipality in the Eastern Cape Province, South Africa between April – July, 2014. A total of 310 presumptive Shiga toxin producing *Escherichia coli* (STEC) were confirmed as *E. coli* spp using polymerase chain reaction (PCR) technique by amplification of the *uidA* gene, out of which 179 (58%) were confirmed positive. Approximately, serogrougs O157:H7, O145 and O26 made up 24% (n=43), 8% (n=14) and 20% (n=35) of the *E. coli* population respectively. Only *E. coli* O26 was positive for *stx2* gene in 31% of the isolates harbouring the gene, while the other serogroups were non-pathogenic. Susceptibility of the isolates to 18 antibiotics was carried out in vitro by the standardized agar disc-diffusion method. All the isolates were susceptible to imipenem. Similarly, a relatively high susceptibility was observed in norfloxacin (83-100%), ciprofloxacin (63-100%), gentamycin

(77-100%), and chloramphenicol (77-100%). However, all the isolates were resistant to tetracycline and its long acting counterpart oxytetracycline. Resistances observed against other antimicrobials are as follows: ampicillin (84-91%), streptomycin (14-100%), erythromycin (91-100%), ceftazidime (35%). Multiple antimicrobial resistance patterns and indices ranged from 3 to 12 and 0.2 to 0.7 to respectively. Genes encoding resistances to ampicillin (*ampC*), streptomycin (*strA*) and tetracycline (*tetA*) were frequently detected in 50-100%, 22-29% and 40-86% of the resistant isolates respectively.

In the other arm of the dissertation, two hundred and fifty eight presumptive isolates of *Salmonella* were recovered from the faecal samples of pigs. Specific primers targeting serogroups A, B, C1, C2, and D were used to delineate the isolates into different serogroups using PCR. Only serogroup A (n=48) was detected. These isolates were examined for antimicrobial susceptibility by disc diffusion method using 18 antibiotics. The results showed that a large proportion of the isolates were resistant to tetracycline (100%), oxytetracycline (100%), ampicillin (75%), sulphamethoxazole/trimethoprim (75%) and streptomycin (75%). Majority of the isolates exhibited multidrug resistances with the predominant multiple antibiotic resistance (MAR) phenotype being against eleven antibiotics. A high multiple antibiotic resistance (MAR) index in a range of 0.3- 0.6 was observed. The incidence of genes encoding resistance against tetracycline (*tetA*), streptomycin (*stra*), and ampicillin (*ampC*) were 54%, 44% and 61% respectively.

These findings reveal that pigs within the Nkonkobe Municipality in the Eastern Cape Province could harbour Shiga toxins and multidrug resistant serogroups of *E. coli* as well as resistant *Salmonella* which could be transmitted to humans through the food chain. To ensure public health safety, continuous monitoring and sufficient sanitation in swine industries must be ensured.

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List of abbreviations

DNA	Deoxyribonucleic acid
AEMREG	Applied Environmental Microbiology Research Group
AGPs	Antibiotic Growth Promoters
CLSI	Clinical Laboratory Standard Institute
DAEC	Diffusely-adherent *E. coli*
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
EAEC	Enteroaggregative *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
LEE	Locus of enterocyte effacement
MAR	Multiple antibiotic resistance
MARI	Multiple antibiotic resistance index/indices
MDR	Multidrug resistance
NTS	Non-typhoidal *Salmonella*
PAIs	Pathogenicity islands
SMAC	Sorbitol MacConkey Agar
STEC	Shiga toxin - producing *Escherichia coli*
Stx	Shiga toxin
T3SS	Type III secretion system
VTEC	Verotoxigenic *E. coli*

CHAPTER 1

General introduction

1.1 Background of study

Pig is known to be one of the best meat-producing animals in the world (Rajkhowa & Sarma, 2014) with South Africa contributing to about 0.2% of the global pork production, and about 1.6 million pigs raised commercially (Mohlatlole *et al*., 2013). In 2010/2011, pork , the third most produced meat in the country contributed to 16.3% of the gross value of agricultural production (Mohlatlole *et al*., 2013). Animals such as pigs frequently harbour bacteria pathogenic to man in their intestinal tracts. These bacteria which are known as zoonotic agents include *Escherichia coli, Salmonella, Campylobacter* etc. In modern intensive production systems, animals receive antibiotics for therapy, control and prevention of diseases and as growth promoters (Rosengren *et al*., 2009; Vo *et al*., 2006). The use of antimicrobials for prophylaxis in food producing animals has been a great concern, because it is believed to be an important factor in the emergence of strains with resistance to certain antimicrobials (Threlfall *et al*., 2000).

The most commonly used antimicrobials in pigs include, tylosin, sulphonamides, and tetracycline , even though other drugs are also used (McEwen & Fedorka-cray, 2002). Many studies have report a direct relationship between antimicrobials use as antibiotic growth promoters (AGPs) in farms and the emergence and spread of multi resistant strains even against drugs that were never used in the farms, one of which is the transmission of resistance genes to humans through the food chain (Marshall & Levy, 2011). Development of resistances in these bacteria constitutes a public health risk, primarily through increased rates of treatment failures and disease burden. Also, antimicrobial-resistant pathogens also pose severe and costly animal health problems, as they prolong illness and lessen efficiency due to increased morbidity and death rates (Yang *et al*., 2004).

Escherichia coli are facultative anaerobes and component of the usual intestinal flora in humans and animals (Schroeder *et al*., 2002). The bacterium belongs to the Enterobacteriac*eae* family and is ubiquitously found in faeces of healthy humans, swine, and other domestic, wild mammals and birds. Human infections caused by this organism include urinary tract infections, neonatal meningitis, septicaemia, and post-operative infections (Schroeder *et al*., 2002). Among pathogenic strains identified, the commonest is the Shiga toxin-producing *E. coli* (STEC), others being enterotoxigenic *E. coli* (ETEC), enteropathogenc *E. coli* (ETEC), Enteroaggregative *E. coli* (*EAE*C), Enteroinvasive *E. coli* (EIEC) and Diffusely adherent *E. coli* (DAEC) (Bolton, 2011).

Shiga toxin-producing *E. coli* is a pathogen of zoonotic origin and a major cause of diarrhoea worldwide. STEC can cause haemorrhagic colitis (HC) in humans, which can possibly progress to and cause severe extra-intestinal complications, such as haemolytic-uremic syndrome (HUS) (Paton & Paton, 1998). *E. coli* O157:H7 are the main strains of STEC that responsible for infections in humans in various parts of the world as well South Africa (Dunn *et al*., 2004). Although domestic ruminants, particularly cattle, are the main reservoirs of Shiga toxin-producing *E. coli* (Prendergast *et al*., 2011) pigs have also been found to be important carriers of this pathogen (Meng *et al*., 2014).

The major clinical signs of illness caused by STEC arise as a result of the production of Shiga toxin1 (*stx1*), or Shiga toxin 2 (*stx2*) or combinations of these toxins (Wang *et al*., 2002). Intimin, another virulent factor is an outer membrane protein encoded by the *eae* gene which enables the intimate attachment of STEC to intestinal epithelial cells (Kaspar *et al*., 2010; Ju, 2013). STEC strains may also be haemolytic due to the presence of either α-hemolysin or enterohemolysin or both. The α-hemolysin gene *hly*A is situated on the chromosome (Marcade *et al*., 2009) while the enterohemolysin (*ehx*A) is harboured by a

plasmid (Cookson *et al*., 2007). Studies on Shiga toxin–producing *E. coli* (STEC) in food production systems have focused primarily on serotype O157:H7 with less attention given to other non- O157 serogroups. However, human cases of non-O157 STEC have been reported to exceed O157 STEC cases in the US and Europe (Brooks *et al*., 2005). The Center for Disease Control and Prevention(CDC) has reported that six non-O157 STEC groups (O26, O45, O103, O111, O121, and O145) are responsible for over 70% of illnesses caused by non-O157 STEC in the US (Brooks *et al*., 2005; Lee *et al*., 2009; Molla *et al*., 2010; Ojo *et al*., 2010; Scallan *et al*., 2011; Varma *et al*., 2012). Even though products such as fruits, nuts and leafy vegetables have been implicated in non-O157 outbreaks of human illness (Painter *et al*., 2013), ruminants, including cattle, and other warm blooded animals like pigs and birds are considered a major reservoir of non-O157 STEC (Bettelheim, 2000).

Salmonella enterica causes salmonellosis, a global food borne ailment of humans and livestock, characterised by diarrhoea, abdominal cramps and fever and a major cause of food related hospitalizations worldwide (Scallan *et al*., 2011; Varma *et al*., 2012). Farm animals, especially poultry and pigs, are considered to be the most important ecological reservoirs of *Salmonella* (Vo *et al*., 2006). Most of the cases of *Salmonella* infections in humans are linked with the consumption of food products such as meat, poultry, eggs, seafood, and dairy products that are contaminated (Threlfall *et al*., 2000). Contamination occurs when organisms come in contact with areas and replicate in foodstuff, due to poor storage temperatures, insufficient food preparation, or cross-contamination of ready-to-eat food (Skyberg *et al*., 2006).

Several virulence factors that aid pathogenicity of *Salmonella* have been identified and characterized. Some of these genes are invasion protein (*inv*A), cytolethal distending toxin B (c*dt*B), Iron N (*iro*N), flagelin-encoded gene (*fli*C), Mg2+ transport protein (*mgt*C), plasmid-

encoded fimbriae *(pef*A), pathogenicity island I effector protein (*sip*B), plasmid virulence protein (*spv*C) etc (Skyberg *et al*., 2006). Molecular assays such as the polymerase chain reaction (PCR)–based methods can be used for detecting the characteristics that induce pathogenicity and resistance in bacteria (Huehn *et al*., 2010).

All types of *E. coli* acquire antimicrobial resistance frequently linked with diverse animal and environmental sources. This explains why they are normally included in studies involving antimicrobial resistance surveillance (Erb *et al*., 2007). Resistance to sulfonamide aminoglycosides, cephalosporins, and fluoroquinolones is common in *E. coli* isolated from animals and humans and may affect treatment outcomes considering that these drugs classes are normally used to treat infections caused by Gram-negative bacteria (Hammerum & Heuer, 2009). Specifically, occurrence of resistance to clinically important 3rd and 4th generation cephalosporins mediated by extended-spectrum β-lactamases (ESBLs) has serious implications for human and veterinary medicine (Pfeifer *et al*., 2010). Resistance in *E. coli* is often facilitated by plasmids that encode antimicrobial resistance genes (Carattoli, 2009).

Antimicrobial-resistant *Salmonella* may possibly result from usage of antimicrobials in food producing animals, and these antimicrobial resistant *Salmonella* are consequently spread to humans, usually through food. The antimicrobial resistance patterns of isolates from humans with *Salmonella* infections have been shown to demonstrate more resistance to antibiotics used in husbandry than to those used for the treatment of *Salmonella* infections in humans (Angulo *et al*., 2000). Multidrug resistance (MDR) in *Salmonella* is a concern worthy of note as treatment options may be very narrow, thereby making management of these infections difficult. *Salmonella typhimurium* is one of the most prevalent MDR *Salmonella* serovars isolated from humans and animals in the United States (Brunelle *et al*., 2013). One of the major multi drug resistant strains are usually *Salmonella typhimurium* definitive phage type

104 (DT104), which is frequently observed to be resistant to five antibiotics namely ampicillin, chloramphenicol, streptomycin, sulphamethoxazole, and tetracycline (ACSSuT) (Glenn *et al*., 2011). Due to detected increases in morbidity and mortality in antimicrobial resistant infections, it has been suggested that resistant *Salmonella* are more virulent than sensitive strains. However, research into this has been lacking, and some studies have revealed that resistance to some antimicrobials such as fluoroquinolones actually reduces virulence in *Salmonella* (Frye & Jackson, 2013).

1.2 Statement of Research Problem

In order to improve livestock production, antibiotics are often used. The use of antibiotics in food animals similar to those used in humans may be responsible for the increasing resistance to antimicrobials used in human populations which constitute a public health risk. The use of antibiotics in food animals acts as a selective pressure for the development of bacteria resistant to antimicrobial agents used in humans, and these might be transmitted through the food chain and water and cause infections in humans (Phillips *et al*., 2004). Furthermore, the use of antimicrobial agents selects for resistance genes in non-pathogenic bacterial species which can be transferred to pathogenic ones (Aarestrup, 2004). As a result of vertical and horizontal gene transfers among bacterial species, it may not be unusual to observe same antibiotic resistance profile of bacteria isolates from humans and farm animals that are regularly treated with antibiotics used for controlling human diseases. This indiscriminate use of antibiotics in animal husbandry has aggravated the current problems being experienced in management of bacterial infections in humans. It is unlikely that new drugs will become available soon as most pharmaceutical companies are not investing on discovery of new antibiotics.

Animals are known to spread bacteria to farm workers and those strains with resistance show up in meat products and in the environment like water. As it is not completely possible to ban antibiotic usage in veterinary medicine, regular sentinel studies like this are necessary to monitor the level of resistance among bacterial isolates from animals. Besides, surveys and reports on antibiotic resistance in isolates from farm animals in South Africa are few and most of them are done in areas close to Johannesburg and Pretoria (Henton *et al*., 2011). Also, with the high diversity of *E. coli* strains that are capable of producing Shiga toxins, little is known about the prevalence of STEC O157 and non-O157 along with the health implications of food infection by *Salmonella enterica* and the antibiotic resistance profiles of these organisms commonly found in gut of farm animals but posing health challenges to humans in the Eastern Cape Province. Taking into consideration the human health implications of these organisms and the danger posed by bacterial ever increasing resistance to antibiotics which are the available weapon to counter them, it is therefore imperative to probe into their presence in our environment as these organisms are zoonotic thus posing a health problem to humans. As it is known, knowledge of the prevalence of a potential pathogen is very important in its epidemiological control. With paucity of data on the prevalence of these zoonotic pathogens in Eastern Cape Province, it was very crucial that a study of this type be carried out in order to generate data which could create awareness of their presence in the environment and aid in preventing a potential epidemic in the community.

1.3 Hypothesis

This research is premised on the hypothesis that pigs within the Nkonkobe Municipality are potential reservoirs for pathogenic and antimicrobial resistant strains of STEC and *Salmonella* spp.

1.4 Aim and objectives

1.4.1 Aim

The aim of this research was to determine the prevalence and antimicrobial resistance profile of *E. coli* and *Salmonella* spp. and the Shiga toxin producing capability of *E. coli* from swine

1.4.2 Specific Objectives

i. To collect faecal samples of swine in the Nkonkobe Municipality.
ii. To isolate *E. coli* O157:H7, non O157 *E. coli* and *Salmonella* spp from the faecal samples.
iii. To carry out antimicrobial susceptibility profiling of the confirmed isolates
iv. To determine the genetic presence of relevant antimicrobial resistance genes in the isolates

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CHAPTER 2

LITERATURE REVIEW

2.1 Enterobacteriaceae

Members of this family are found primarily in the guts of humans and warm blooded animals. They are Gram-negative, rod -shaped, and non-sporulating facultative anaerobes. They also ferment different carbohydrates using them as the carbon source. They may grow as mucoid colonies when grown on agar plates but only *Klebsiella* spp are truly encapsulated. The most infections caused by the Enterobacteriaceae are the urinary tract infections. Others include wound infections, pneumonia, septicaemia and infections involving the nervous system. Clinically important members of the family include *Escherichia, Salmonella, Citrobacter, Enterobacter, klebsiella, Proteus, Providencia, Serratia, Shigella, Yersinia, Enterobacte*r, etc. Some genera in this family are known to cause intestinal infections such as enteritis and diarrhoea.(Tärnberg, 2012)

2.2 *Escherichia coli*

Escherichia coli (*E. coli*) are facultative anaerobes and are part of the normal intestinal flora in both humans and animals (Kaper *et al*., 2004). The main habitats of *E. coli* are the intestinal tract of humans and warm-blooded animals, where they appear virtually ubiquitously. *E. coli* are mostly commensal bacteria and do not cause any disease except to immunocompromised people. However, some strains are highly virulent carrying added genes in plasmids and/or the chromosomes (Chandra *et al*., 2013). About six pathotypes of *E. coli* are associated with gastrointestinal ilnesses: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffuse-adherent *E. coli* (DAEC). Each of these pathotypes possesses different virulence characteristics that enable them to cause

diseases by different mechanisms which result in variable clinical symptoms (Nataro & Kaper, 1998; Chandra *et al*., 2013).

Pathogenic *E. coli* also cause of extra-intestinal infections. These strains of *E. coli* known as (ExPEC), possess virulence traits that enable it to attack, inhabit, and cause disease in body locations outside the gastrointestinal tract. The uropathogenic *E. coli* (UPEC) is one of the main causes of community-acquired urinary tract infections (UTI), responsible for both lower infections of the urinary tract (cystitis) and upper infections (pyelonephritis). The meningitis/sepsis-associated *E. coli* (MNEC) is another extra-intestinal pathotype (Lavigne & Blanc Potard, 2008). The STEC are the most overwhelming and a major public health concern for its association with large foodborne outbreaks and life-threatening haemolytic uremic syndrome (HUS) (Bolton, 2011).

2.2.1 Shiga toxin producing *Escherichia coli* (STEC)

These are strains of *E. coli* that produce one or more types of cytotoxins known as Shiga toxin (Stx) or Verocytotoxin (VT). STEC was discovered in North America in 1982 in stool isolates of *Escherichia coli* from sporadic cases of haemolytic uraemic syndrome. Since then they have been implicated in major foodborne illnesses reported in both developed countries and developing countries (Karmali *et al*., 1983; Gyles, 2007). Shiga toxin producing *E. coli* can also be referred to as Verocytotoxin-producing *E. coli* (VTEC) for its toxigenic effect to Vero cells (Xia *et al*., 2010). Largely, STEC is frequently used in America, whereas VTEC is mostly used in Europe (Bolton, 2011). Various diseases caused by STEC include watery diarrhoea, bloody diarrhoea, haemorrhagic colitis (HC), and haemolytic uraemic syndrome (HUS). Hemolytic uremic syndrome is a rare disorder characterized by microangiopathic hemolytic anaemia, microthrombi, and multiorgan injury. it is one of the major causes of acute kidney failure in children globally (Tarr *et al*., 2005). Occurrences of illnesses caused

by STEC have been epidemiologically associated with contact with animals and consumption of meat and fresh products (Kaspar *et al*., 2010). The STEC causing HC and HUS are also called enterohemorrhagic *E. coli* (EHEC) (Gyles, 2007).

Effective procedures are available for detection of O157, but the same methods cannot be applied to the non-O157 serogroups due to the complexity and diversity of these pathogens, which has prohibited the development of a standardized isolation and culturing method (Conrad *et al*., 2014).

2.2.2 Pathogenicity of STEC

Virulence in pathogenic bacteria is accomplished by acquisition of mobile genetic components such as prophages, transposons, plasmids, and genomic islands. Pathogenicity islands (PAI) is a class of genomic island that comprises virulence genes responsible for host invasion and infection. They constitute a flexible gene pool that contributes to the development and acquisition of virulence determinants and can serve as genetic markers for novel and evolving pathogens. The locus of enterocyte effacement (LEE) is a chromosomally borne PAI which encodes a type III secretion system (T3SS) essential for the attaching and effacing lesion in STEC, characteristic of disease associated with this strain. Outstandingly, the T3SS is a crucial genetic determinant of both colonization and tenacity in animal reservoirs and for virulence in humans after zoonotic transmission (Coombes *et al*., 2011). The pathogenic strains of *E. coli* O157 result from the production of virulence genes of which *stx1*, *stx2* and its variants, *eae* and *hly*A are the most commonly recognized (Ateba & Bezuidenhout, 2008). Shiga toxins (Stx) are the primary virulence factors in STEC and are extremely related to the toxin produced by *Shigella dysentriae* serotype I (O'Brien *et al*., 1984). Shiga toxin can be divided into two groups: Stx1 and Stx2 along with their variants (Jaeger & Acheson, 2000). They are mostly categorised by their hexameric conformation and

are composed of two units of A and B subunits respectively. The B subunit is further made up of five B subunits, which permits toxin attachment to their host cell receptor known as the enterocyte receptor; Gb3, which is also present in endothelial cells of glomerular capillaries (Hoey *et al*., 2003). On the other hand, the "A" subunit, is catalytically active and functions by blocking translation of mRNA to protein, thus interfering with protein synthesis, and inducing inflammatory responses hence leading to cell death (Fraser *et al*., 2004; Kaspar *et al*., 2010).

The possession of Shiga toxins correlates strongly with bloody diarrhoea and haemolytic uremic syndrome (Scheiring *et al*., 2008). Even though several *E. coli* strains are capable of producing one or both Shiga toxins (Stx1 and Stx2), not all of them are important human pathogens (Gyles, 2007). According to Bolton, (2011), many STEC strains that produce Stx do not cause HUS, which implies that additional virulence factors may be required to cause illness in humans. Numerous variants of these proteins have been well-defined and some are more frequently identified in certain serotypes or certain host animals**.** The Stx2 has been shown to be the more potent toxin, and strains producing this toxin are generally associated with more acute illness(AMézquita-López *et al*., 2014). Various pathogenic STEC strains also produce intimin, an outer membrane protein encoded by the *eae* gene which enables the intimate attachment of STEC to intestinal epithelial cells (Kaspar *et al*., 2010). Besides the Shiga toxins and intimin, human pathogenic STEC strains also possess a heat stable enterotoxin EAST1 encoded by the *ast*A gene (Savarino *et al*., 1993). This toxin initiates diarrhoea by activating the cyclic guanosine monophosphate (GMP) pathway that phosphorylates the chloride channels on the apical membranes of intestinal epithelial cells (IECs). This phosphorylation stimulates chloride secretion, and the prevention of sodium chloride absorption by the villus tips of cells. This leads to an increase in luminal ion content

and causing water to pull through the paracellular pathway, resulting in osmotic diarrhoea (AMézquita-López *et al*., 2014; Nataro & Kaper, 1998)**.** The detection of these virulence genes in STEC strains would provide vital evidence for the identification of risk factors that may potentially contribute to the development of human disease (AMézquita-López *et al*., 2014).

2.2.3 Epidemiology of STEC

Shiga toxin producing *E. coli* O157 is has been shown to cause approximately 73,500 cases in the United States annually**.** Occurrences of illness due to STEC have been epidemiologically associated with interaction with animals and eating of meat and fresh products (Kaspar *et al*., 2010). Severe infections in humans have been associated with more than 100 serotypes of STEC. Although it is well known that O157 causes most of the HUS infections and foodborne outbreaks worldwide, findings show that non-O157 STEC are as important as O157 and illnesses caused by non-O157 are rising worldwide (Nataro & Kaper, 1998; Gyles, 2007). More so, epidemiological studies have indicated that six non-O157 serogroups, O26, O45, O103, O111, O121, and O145, have been linked with severe disease symptoms in North America (AMézquita-López *et al*., 2014)**.** Furthermore, the CDC has reported that non-O157 STEC are responsible for about 37,000 cases of illness annually, even though with relatively fewer cases of HUS compared to O157:H7. Though many strains of non-O157 STEC appear to be less pathogenic than *E. coli* O157:H7, a 2008 outbreak of STEC strain O111 in Oklahoma caused illness in at least 314 people, HUS in 17 cases, and one death (Kaspar *et al*., 2010).

Although not in outbreak proportions, *E. coli O157* illness has also been reported in Nigeria (Chigor *et al*., 2010). The first incidence of *E. coli* O157:H7 in South Africa was reported in 1990, where the bacterium was isolated from a man in Johannesburg (Abong & Momba,

2008). Another study done in 2008 by Ateba & Bezuidenhout (2008) reported the presence of *E. coli* O157 in stools of suspected and confirmed HIV/AIDS patients with diarrhoea, as well as water samples and meat products. In the Eastern Cape province, *E. coli* O157:H7 has equally been found in milk obtained from dairy cows (Caine *et al*., 2014).

2.2.4 Treatment of STEC

Treatment of STEC infections with antibiotics may induce the production of Shiga-toxins and increase the chances of the disease progressing to HUS, hence it is discouraged. Nonetheless, studies have reported that *E. coli* O157 isolates are resistant to antibiotics. Detecting the antibiotic resistant capacity of these pathogens may be helpful in revealing the distribution of antibiotic resistant genes within the population and this might be helpful in the control of antibiotic resistance (Ateba & Bezuidenhout, 2008).

2.3 *Salmonella*

Salmonella enterica is a Gram-negative, facultative intracellular human and animal pathogen causing major global public health concern (Galanis *et al*., 2006). They are flagellated rod-shaped bacteria about 2-3 x 0.4-0.6 µm in size, and can proliferate under various environmental conditions outside the living hosts. They can grow in the presence of 0.4 to 4% sodium chloride. Most *Salmonella* species can thrive at temperatures ranging between

5 to 47°C with optimum temperature of about 35 to 37°C but some can grow at low temperature such as 2 to 4°C or as high as 54°C. They are sensitive to heat and often killed at temperature of 70°C or above. Their optimum pH for survival is between 6.5 and 7.5. They also have the ability to survive in dried foods, with water activity (a_w) as low as < 0.2. Complete inhibition of growth occurs at temperatures <7°C, pH <3.8 or water activity <0.94. The current system of nomenclature classifies *Salmonella* into two major species, *Salmonella enterica* and *Salmonella bongori* (formerly subspecies V). *Salmonella enterica* is further

classified into six subspecies namely, (I) *S. enterica* subsp. *enterica*; (II) *S. enterica* subsp. *salamae*; (IIIa) *S. enterica* subsp. *arizonae*; (IIIb) *S. enterica* subsp. *diarizonae*; (IV) *S. enterica* subsp. *houtenae*; and (VI), *S. enterica* subsp. *Indica*. *Salmonella enterica* subsp. *enterica* is responsible for almost all infections in mammals while *Salmonella bongori* are rarely isolated and mostly found in cold-blooded animals as well as the environment. About 2,500 serovars of *Salmonella* have been identified (Hidayah, 2011; Tindall *et al.*, 2005). These serovars can be divided into two clinically relevant groups according to the disease they cause. Those that cause typhoid or enteric fever in humans such as *S. typhi* and *S. paratyphi* which cause an invasive, life- threatening systemic disease (Parry *et al*., 2002), as well as the non-typhoidal serovars (NTS) normally which elicit self-limiting gastroenteritis, associated with intestinal inflammation and diarrhoea (Zhang *et al*., 2003).

Salmonella is therefore one of the major causes of foodborne illness worldwide (Glenn *et al*., 2011) with pork, poultry and egg products being the common sources of infections (Verraes *et al*., 2013)

2.3.1 Pathogenecity of *Salmonella*

The disease usually begins when bacteria cells multiply in the lumen of the small intestine following ingestion and this could further spread to the liver, spleen, lymph nodes and blood (McCormick *et al*., 1996). In humans , infection with *Salmonella* can be divided into four patterns of illness namely enteric fever, gastroenteritis, bacteraemia and as well as other complications of nontyphoidal salmonellosis (Pui *et al*., 2011). *Salmonella typhi* causes typhoid fever whereas Paratyphi A, B and C cause paratyphoid fever with milder symptoms and lower mortality rate. Both serovars are exclusively human pathogens and infection usually occurs due to consumption of food or water contaminated with human waste. Nontyphoidal salmonellosis or enterocolitis is caused by at least 150 *Salmonella* serotypes

with *Salmonella typhimurium* and *Salmonella enteritidis* being the most common serotypes in the U.S. Infection always occurs via ingestion of water or food contaminated with animal wastes (Pui *et al*., 2011). This disease pattern is characterized by gastroenteritis, alongside intestinal inflammation and diarrhoea. The infection is usually self-limited to the intestine, but the bacteria can also spread beyond the intestine and cause bacteraemia and systemic infections (Lavigne & Blanc Potard, 2008). If salmonellosis is left untreated, about 8% of result in situation in which bacteria will enter the bloodstream through the intestinal barrier. Such conditions have been linked to extremely invasive serotypes like Cholearaesuis or Dublin. It is advised that patients with bacteraemia and other complications be treated with antibiotics (Pui *et al*., 2011).

Bacterial cells have the ability to withstand extremes of temperature, oxygen shortage, digestive enzymes bile salts as well as other flora that might compete with them (McCormick *et al*., 1996). *Salmonella* has the ability to tolerate the hostile environment within the gastro intestinal tract and this enhances the transcription of genes required for invasion and attachment to the host, which could trigger inflammatory response which manifests as diarrhoea in the host (McCormick *et al*., 1996).The virulence factors in *Salmonella* are encoded on the five pathogenicity islands (SPI-1 to SPI-5), which are located on the chromosome. These might have been acquired through horizontal gene transfer from other bacteria species (Lavigne & Blanc Potard, 2008). Invasion of non-phagocytic epithelial cells is the main mechanism of virulence. This involves the typeIII secretion systems (T3SS) which are encoded by the SPI1, containing about 25 genes. The SPI-2 encodes additional type III secretion systems which enables survival of bacteria in epithelial cells and macrophages. This is responsible for the induction of systemic illnesses. The SPI-3 contains genes which enable the survival of *Salmonella* in environments lacking Magnesium ions

(Mg^{2+}). Furthermore, putative virulence factors have been identified as detailed studies have not been carried out for SPI-4. These genes include those that may encode a type I secretion system and a gene that codes for survival within the macrophage. Finally, the SPI-5 encodes two effector proteins namely SopB and PipB (Herold *et al*., 2004).

Several virulence factors that aid pathogenicity of *Salmonella* have been identified and characterized. Some of the genes include effector protein (*avr*A), cytolethal distending toxin B (*cdt*B), type 1 fimbrial protein (*fim*A), flagelin-encoded gene (*fli*C), invasion protein (*inv*A), Iron N (*iro*N), outer membrane receptor (*fep*A), long polar fimbrial outer membrane usher protein (*ipf*C), Mg2+ transport protein (*mgt*C), plasmid-encoded fimbriae (*pef*A), outer membrane usher protein (*sef*C), secreted effector protein(*sif*A), pathogenicity island I effector protein (*sipB*), pathogenicity island I effector protein(*sipC*), iron transporter (*sitC*), transcriptional regulator (*sly*A), cell invasion protein (*sop*B), *Salmonella* plasmid virulence outer membrane protein (*sop*E*1*), tyrosine phosphatase (*spt*P), and plasmid virulence protein (*spv*C) (Skyberg *et al*., 2006).

2.3.2 Epidemiology of *Salmonella*

Annually, there are approximately 400 confirmed *Salmonella infections* in Ireland (7.8 cases per 100,000), which is less than the European average (21.5 per 100,000). Pork is an important source of human infection and it has been estimated that between 15 and 23% of all cases of human salmonellosis are related to its consumption (Bolton *et al*., 2013).

Typhoid cases are stable with low numbers in developed countries, whereas in the developing world; it is responsible for 5 - 30% deaths. The World Health Organization (WHO) estimates that 16 to 17 million cases occur annually, resulting in about 600,000 deaths (Pui *et al*., 2011).On the other hand, Non-typhoidal salmonellosis (NTS) in industrialised countries is one of the most common food-borne bacterial diseases in humans (Schlundt *et al*., 2004).

Also, about 5% of NTS cases could be invasive, with extra-intestinal disease progressing to bacteraemia and systemic infections. In Sub-Saharan Africa, non- typhoidal *Salmonella* which are invasive, have developed as a major cause of bacteraemia in children and adults, with about 175–388 cases per 100,000 children and 2000–7500 cases per 100,000 HIV-infected adults occurring yearly (Feasey *et al*., 2012). Records of salmonellosis are rare in many countries of Asia, Africa and South and Central America where only 1 to 10% of cases are reported (Pui *et al*., 2011)

2.3.3 Treatment of *Salmonella* infection in humans

Most infections in humans are self-limiting, and antimicrobial agents might not be necessary for treatment. However, severe forms of the disease such as invasive infections may occur, and will require treatment. Fluoroquinolones and third-generation cephalosporins are the drugs-of-choice for invasive *Salmonella* infections in humans; folic acid pathway inhibitors can also be used alternative antimicrobial choices even though their use is limited by increasing antimicrobial resistance, limited efficacy, and less desirable effects of the drugs (Angulo *et al*., 2000). The β-lactams are mostly used in treating children and pregnant women due to fluoroquinolone's interference with cartilage formation; this therefore contributes significantly to resistance to β-lactams by *Salmonella.* In the treatment of multidrug resistant strains of *Salmonella* infections, the last treatment options are usually the aminoglycoside, amikacin or the carbapenems, imipenem or meropenem (Frye & Jackson, 2013).

2.4 Use of antimicrobials in farm animals

Demands for products of animal origin are increasing worldwide due to the significant growth in the human population and changes in health, wealth and life expectancy of people (Webb & Erasmus, 2013). There is a similar increase in demand for good quality protein

from animal products in developing countries (Webb, 2013) where large numbers of people suffer from hunger and starvation, while problems associated with malnutrition, obesity and the metabolic syndrome are rising. In fact, global livestock production is estimated to double by 2050 in order to satisfy the rising demands for animal products (Ilea, 2009). Most of this growth is likely to occur in developing countries, which are often poverty ridden and faced with civil wars or poor governance (Webb & Erasmus, 2013).

Increasing demands for agricultural products became a reality during the Industrial Revolution from about 1760 to 1820, which involved the adoption of new manufacturing practices. This can be seen from a shift from the use of wood and other biofuels to coal, steam power, machines and then modern tools (Delgado *et al*., 2001), leading to an increase in numbers of livestock reared especially in developing countries (Steinfeld, 2004)As time went on, there was a need to improve productivity. This probably led to the intensification of agricultural production systems. Significant progress has been made in terms of breeding, feeding, and management of livestock in different production systems in order to increase the level and efficiency of animal production (Webb & Casey, 2010).

Swine and poultry farms are usually operated in intensive systems, which are systems where "the movement of animals is confined and they are raised in high density, usually with stimulated feeding, and weight gain optimized so as to decrease time to mature weight." (Silbergeld *et al*., 2008). As demand for food of animal origin increases, the confined livestock production followed suit since it allows for cost advantages. Raising large numbers of animals in a confinement usually require prophylactic, metaphylactic, and growth promotional antimicrobial use in order to prevent diseases and deaths, to ensure animal well-being, and for increased productivity and profit. In swine industry for example,

antimicrobials are used majorly for the treatment or prophylaxis of respiratory and enteric diseases (Rosengren *et al*., 2009).

There are three key reasons for the use of antimicrobials use in farm animals: treatment of ill animals, prophylaxis and disease control, and enhanced efficiency. Prevention and control can be divided into metaphylactic or prophylactic measures. Metaphylactic application involves the treatment of a collection of animals to check the spread of infection when only a few have been identified as diseased. Prophylaxis involves the preventive treatment of an animal or group of animals at a time when it may likely be susceptible to infection (McEwen & Fedorka-cray, 2002), depending on the animal species, the production system, and the ailment (Rosengren *et al*., 2009). Another reason for antimicrobial use in livestock is for promotion of growth. The mode of action of antimicrobials used as growth promoters is unclear. However, they are believed to cause a reduction in growth of bacteria in the intestinal tract and thereby leading to less breakdown of beneficial nutrients by microbial flora, and the prevention of infections with pathogenic bacteria (Aarestrup, 2000).

Generally, antimicrobials used for the purpose of growth promotion are incorporated at doses lower than those permitted for treatment purposes and are given to the animals for a longer period than antimicrobials used for prevention and control (Rosengren *et al*., 2009). Therefore, continuous exposure to antimicrobials can increase the tendency for development and persistence of antimicrobial resistance (Prescott, 2008). Although the use of antimicrobials as prophylactics is criticized for its role on the selection of resistance among pathogenic bacteria, treatment at these key periods for disease incidence seems like an unavoidable measure in the current pork and beef producing systems This probably explains why it is a controversial issue globally (Chen *et al*., 2005)

These antimicrobials were referred to as 'antibiotic growth promoters' or AGPs, and European countries raised concern about the use of these substances soon after their adoption (Cogliani, *et al*., 2011). For example, Swedish farmers requested a ban on AGPs in 1986 because of a drop in consumer confidence in animal products owing to the excessive use of these substances for growth promotion (Webb & Erasmus, 2013). This might be due to increased awareness by the consumers.

2.5 Development of antimicrobial resistance

Perhaps, one of the greatest significant findings in medicine was the discovery and development of antimicrobials. The introduction of antibiotics came with great optimism with success in treating bacterial infections both in human and veterinary medicine, hence a decrease in patient morbidity and mortality. It is however unfortunate as the "pendulum has swung in the opposite direction" (Rosengren *et al*., 2009). Some authors are of the opinion that "the end of the antibiotic era may be near and are wondering if continuous occurrence of multidrug-resistant pathogens signifies an unwinnable war" (Perez *et al*., 2007).

Understanding the molecular basis for the development of resistance is vital as it allows the development of new methods for managing infections caused by these bacteria and also to create new strategies for the development of new treatments against these bacteria (Alanis, 2005). For antibiotic resistance to develop, two essential processes occur; the presence of an antimicrobial agent capable of inhibiting most of the bacteria present in a colony and a diverse colony of bacteria where at least one bacterium carries the genetic determinant capable of expressing resistance to the antibiotic. Hence, the susceptible strains in the colony will die leaving the resistant ones. These persisting bacteria therefore harbour the genes that code for the type and level of resistance to be expressed by the bacterial cell. Selection of these bacteria results in the selection of these genes that can now spread and disseminate to

other bacteria (Marshall & Levy, 2011). In addition**,** susceptible bacteria can acquire resistance to antimicrobial agents by either genetic mutation or by acquiring antimicrobial resistance genes from other bacteria. These genes are usually situated in specific fragments of DNA known as transposons (sections of DNA containing "sticky endings"), allowing the resistance genes to move easily from one plasmid to another (Djordjevic *et al*., 2013; Li *et al*., 2007; Pezzella *et al*., 2004; Roberts, 2005; Velge *et al*., 2005; Walsh, 2010). Furthermore, some transposons may possess an exceptional and complex DNA fragment called "integron", a location capable of mixing different antibiotic resistance genes enabling them to confer multiple antibiotic resistances to bacteria. Integrons have been identified in both Gram-negative and Gram-positive bacteria, and they seem to confer high-level multiple drug resistance to the bacteria that transmit and express them (Marshall & Levy, 2011).

Genetic mutation could occur causing a change in the bacterial DNA, and the transfer of genetic material among bacteria through several means. Antimicrobial resistance can be intrinsic (natural) or acquired, and can also be transmitted horizontally or vertically. The natural method of antibiotic resistance usually occurs by a spontaneous gene mutation without any selective pressure by antibiotics, and is less commonly observed than the acquired one (Alanis, 2005).

2.5.1 Molecular mechanisms of transfer of resistance determinants among bacteria

Conjugation, transformation and transduction are the most common means of genetic transfer among bacteria (Alanis, 2005).The most common and most important mechanism of transmission of antimicrobial resistance in bacteria is by conjugation. This mechanism is usually facilitated by circular fragments of DNA known as plasmids which have the ability to replicate independently of the chromosome. Transmission of plasmids among bacteria occur through the formation of a hollow tubular structure (pilus) between bacteria that are next to

each other, therefore linking them momentarily and allowing the passage of these DNA fragments (Alanis, 2005). Transformation occurs when free DNA (naked DNA) passes from one cell to another. This free DNA usually comes from dead bacteria that are fragmented close to the receiving bacteria. The receiving bacteria thus incorporate the free DNA into their own DNA (Alanis, 2005). The mechanism of transfer due to transduction occurs through the means of a vector, most commonly, viruses that have the ability to infect bacteria, known as bacteriophages. The viruses carry the bacterial gene which codes for antibiotic resistance and infects the new bacterial cell thereby introducing the genetic material into it. Also, the bacteriophage introduces its own viral DNA to the receiving bacteria, causing the cell to produce more copies of the infecting virus until the bacterial cell dies and releases new bacteriophages, which go on to infect other cells (Alanis, 2005).

2.5.2 Discovery of antimicrobials

Antimicrobials are chemical compounds of natural (from fungi or bacteria), synthetic or semi-synthetic origin that kill or inhibit growth of microorganisms with little or no harm to the host. Antibiotics were described by the Nobel Laureate Selman Waksman as natural compounds of microbial origin. It is used interchangeably with antimicrobials. Also, antimicrobials that target bacteria in both people and animals are generally referred to as antibacterial agents (WHO, 2011). The era of antibiotics began in 1927, when the antibiotic penicillin was discovered and since then, different classes of penicillins have been developed. In 1928, Alexander Fleming discovered penicillin when he noticed that a mould *Penicillin notanum*, produced a substance that inhibited the growth of *Staphylococcus.* Florey and Chain further purified it for use. This was followed by discovery of newer classes of antimicrobials between 1950s and 1970s, a period known as the golden era (Aminov, 2010).

Since then several antimicrobials have been developed and grouped according to different classes.

2.5.3 Mechanisms of action and resistance of different classes of antimicrobials in *Salmonella* and *E. coli*

The antibiotics are grouped into classes based on chemistry, mode of action, and other properties as follows;

2.5.3.1 β -lactams and Cephalosporins

One of the earliest β-lactams to be developed for clinical use in humans was the penicillin, and it was also one of the first antibiotics to which bacteria developed resistant to. β -lactam is a broad name for all antibiotics that contain a β-lactam ring, a heteroatomic ring structure, comprising of three carbon atoms and one nitrogen atom (Wilke *et al*., 2005). They act by inactivating enzymes known as penicillin binding proteins (PBPs), located in the bacterial cell wall which are also involved in the third step of cell wall synthesis (Poole, 2004).

β-lactams and cephalosporins are well-tolerated even in farm animals, thus making them useful for treatment of disease (Girlich *et al*., 2007; Li *et al*., 2007). Resistance to β-lactams are due to the production of β-lactamases by bacteria. The β-lactamases are enzymes produced by bacteria that cleave the β-lactam ring and prevent it from bonding to and inactivating cell wall enzymes (Girlich *et al*., 2007; Li *et al*., 2007). New β-lactams were therefore synthesized through structural modification of the chemical groups around the β-lactam ring to yield products that are resistant to the β-lactamases. Further modifications were also done to enhance their activity on specific bacteria or increase access to some sites of infection. Products of these modifications include the development of modified penicillins such as methicillin and oxacillin; cephalosporins like cephalothin, cefoxitin, ceftriaxone, and cefipime, which are 1st, 2nd , 3rd and 4th generation cephalosporins respectively; and the

carbapenems such as imipenem and meropenem (Girlich *et al*., 2007; Li *et al*., 2007). The carbapenems possess the broadest spectrum of activity (Nordmann *et al*., 2012). They are active against many Gram-positive, Gram-negative as well as anaerobic bacteria. Furthermore, they are stable to most β-lactamases including *amp*C β-lactamases and this is why they are used among bacteria resistant to β-lactamases especially Enterobacteriaceae (Zhanel *et al*., 2007). However, occurrence of mutations in β-lactamase genes has led to the production of enzymes that can destroy these new generation β-lactams, some of which include the extended spectrum β-lactamases (ESBLs), cephalosporinases (Labia *et al*., 2001; Mammeri *et al*., 2008) and carbapenemases (Miriagou *et al*., 2010). Nonetheless, some β-lactamases inhibitors have been synthesized to inactivate β-lactamases, by binding irreversibly to particular β-lactamases, therefore allowing the β-lactam to exert it antibacterial action. Example of such is clavulanic acid which is used in combination with amoxicillin (ampicillin/clavulanicacid) (Girlich *et al*., 2007; Li *et al*., 2007).

The use of these antimicrobial agents in farm animals has been contentious. This is because they are very beneficial in treatment of infections in the animals and are also a part of the most important antimicrobials used in humans. Resistance in *E. coli* isolated from farm animals to β -lactam and cephems has been reported (Li *et al*., 2007). The genes that confer resistance to β-lactams are similar in both *E. coli* and *Salmonella* isolated from animals (Li *et al*., 2007). The *amp*C gene is chromosomally mediated, and it has been shown to confer resistance mainly to ampicillin and cephalosporins. Other genes that have been detected include bla_{TEM-1}, which encodes resistance to ampicillin, bla_{CMY-2} and bla_{PSE-1} encoding resistance to ampicillin, 1st, 2nd, and 3rd generation cephalosporins, and the β-lactamase inhibitor, clavulanic acid (Glenn *et al*., 2011; Frye & Jackson, 2013). Also, the emergence of

metallo - β- lactamse which confers resistance to carbapenems often used as the last option in treatment of Gram negative infections is a cause for concern (Walsh, 2010).

2.5.3.2 Aminoglycosides

Aminoglycoside antimicrobials are protein synthesis inhibitors, which act by binding irreversibly to the 30S subunit of the bacterial ribosomes. Protein synthesis inhibitors take advantage of the difference between the structure of ribosomes in bacteria and those of eukaryotic cells, (Tenover, 2006). This structural difference confers selective toxicity, whereby the growth of the bacteria will be inhibited while the host is unharmed. Their use in farm animals is restricted due to their toxicity and presence as drug residue in tissue of animals. The use of drugs in this class such as gentamycin, streptomycin, and neomycin in animals such as swine have been reported (Schwarz *et al*., 2001).

Resistance to the aminoglycosides is mainly due to active efflux or enzymatic modification of the 16S rRNA subunit preventing the drug from binding to the bacterial ribosomes. Enzymes that are responsible for modification of the compound are acetyltransferases, nucleotidyltransferases, and phosphotransferases (Ramirez & Tolmasky, 2011). The gene which codes for acetyltransferases is the *aac* which confers resistance to gentamycin, tobramycin and kanamycin. Whereas, the *aad* and *ant* group of genes code for nucleotidyltransferases and are responsible for resistance to gentamycin, streptomycin and tobramycin. The phosphotransferases are encoded by the *aph* gene which confers resistance tobramycin and neomycin and the *stra* and *strB* genes which confer resistance to streptomycin (Frye & Fedorka-Cray, 2007; Glenn *et al*., 2011; Yang *et al*., 2004). These genes are plasmid borne and can be transferred between *E. coli* and *Salmonella* (Deschamps *et al*., 2009; Johnson *et al*., 2006).

2.5.3.3 Quinolones/ Flouroquinolones

This class of antimicrobials act by inhibiting topoisomerases, enzymes required for DNA replication in bacteria. Fluoroquinolones are useful for the treatment of *Salmonella* , *E. coli*, and other bacterial infections in humans (Nelson *et al*., 2007). A number of fluoroquinolones have been used in food animals such as danofloxacin, enrofloxacin, sarafloxacin, orbifloxacin, etc (Hopkins *et al*., 2005; Rodríguez *et al*., 2012). Since these drugs exhibit similar mode of action as those used in humans, the possibility of occurrence of cross resistance to fluoroquinolones used in animals and humans is high. This probably explains why flouroquinolones were banned from being used in animals after the introduction of enrofloxacin in animal husbandry and the discovery ciprofloxacin-resistant *Campylobacter jejuni*, in poultry (Nelson *et al*., 2007).

Bacteria demonstrate resistance to fluoroquinolones by reducing their permeability through the cell, formation of efflux pumps, or mutations in the genes that encode the DNA gyrase or topoisomerase enzymes such as *gyr*A, *gyr*B, *par*C, and *par*E genes (Hopkins *et al*., 2005; Schwarz & Chaslus-dancla, 2001; Yang *et al*., 2004). These mutations usually occur in a conserved site of the enzymes targeted by these antimicrobials , known as the quinolone resistance determining region (QRDR), targeted by these antimicrobials (Abatcha *et al*., 2014; Chen *et al*., 2005; Hopkins *et al*., 2005; Velge *et al*., 2005; Yang *et al*., 2004). The progression of resistance from to the quinolone, nalidixic acid and then to fluoroquinolones has been described as a gradual process of mutations in the QRDR region which eventually leads to the production of an enzyme with a target region that quinolones cannot bind to (Chen *et al*., 2005). Other resistance mechanisms have also been identified such as the *qnr* efflux system, and an aminoglycoside acetyltransferase, *aac*(6')-1b including , which can modify and inactivate ciprofloxacin (Aarestrup, 2004). Also, some studies have reported

plasmids harbouring quinolone resistance genes (*qnr*A, *qnr*B, and *qnr*S) (Frye & Jackson, 2013) Resistance to nalidixic acid and fluoroquinolones like ciprofloxacin has been reported worldwide in human isolates of bacteria. However, according to Frye & Fedorka-Cray (2007), animal isolates of *Salmonella* in the U.S. have very low levels of resistance while *E. coli* has higher levels of resistance.

2.5.3.4 Folic acid pathway inhibitors

These compounds act at two different stages in the folic acid pathway by interfering with the synthesis of dihydrofolic acid in bacteria. They include the sulphonamides which inhibits the dihydropteroate synthase (DHPS) and trimethoprim which inhibits dihydrofolate reductase (DHFR) enzymes (Skold, 2001). Sulfonamides and trimethoprim are usually combined together to treat *Salmonella* infections that are resistant to other antimicrobials (Lauderdale *et al*., 2006; Zewdu & Cornelius, 2009). Also, they have also been used widely used as growth promoters in swine and for treatment of diseases such as colibacillosis in swine and coccidiosis in poultry (Girlich *et al*., 2007; Li *et al*., 2007). Sulfonamides inhibit bacterial growth (bacteriostatic) when used alone or becomes bactericidal when used in combination with trimethoprim (Walsh, 2010).

Resistance to sulfonamides is widespread. It occurs when these bacteria acquire genes encoding enzymes that do not bind these compounds. The *sul* genes; *sul*I, *sul*II, and *sul*II, have been shown to encode DHPS enzyme and are found in *Salmonella* worldwide. In the United States, majority of the resistance is due to either *sul*I or *sul*II. Resistance to trimethoprim is caused by DHFR encoding genes, either *dhfr* or *dfr*, both of which have been found in *Salmonella* isolates from animals in the U.S. (Girlich *et al*., 2007; Glenn *et al*., 2011; Li *et al*., 2007).

2.5.3.5 Tetracyclines

This class of compounds are wide-spectrum antibiotics that inhibit synthesis of protein in bacteria, by preventing the binding of aminoacyl-tRNA to the bacterial ribosome. The tetracyclines such as oxytetracycline and chlortetracycline have been used as growth promoters in swine ,cattle, and poultry (Bryan *et al*., 2004). The *tet* genes have been found to be responsible for conferring resistance to tetracyclines. They code for any of the three mechanisms of resistance: efflux pumps, ribosomal protection, or direct enzymatic inactivation of the drug. The production of efflux pumps are the commonest mechanism among Gram-negative bacteria, while ribosomal protection are more common among Gram-positive organisms. In *E. coli* the common *tet* genes reported are *tet(*A), *tet* (B), *tet* (C), *tet* (D), *tet* (E), *tet* (G), *tet*(J), *tet* (L), *tet* (Y). Although *tet* (M) have been reported in some cases (Frye & Jackson, 2013). However, in *Salmonella* , the most commonly observed active efflux systems include *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(G), and *tet*(H) (Bryan *et al*., 2004; Feasey *et al*., 2010; Roberts, 2005). Largely, the rapid spread of tetracycline resistance among bacteria is due to the localization of *tet* genes on plasmids, transposons, and integrons (Glenn *et al*., 2011).

2.5.3.6 Phenicols

This class includes Chloramphenicol, and florphenicol. They act by inhibiting protein synthesis by binding to 50S subunit of bacterial ribosome (Tenover, 2006). The use of chloramphenicol in both human and veterinary medicine has been banned in the United States and other countries due to its toxicity in humans. It is mainly used in the invasive salmonellosis caused by bacteria that are resistant to other drugs of choice (Frye & Jackson, 2013). This probably explains the reason why low resistance to chloramphenicol is reported. Resistance to these compounds is due the production of inactivating enzymes such as

chloramphenicol acetyltransferase, *cat1* and efflux pumps such as *flo*R and *cml*A .These genes are plasmid mediated (Girlich *et al*., 2007; Li *et al*., 2007).

2.6 Consequences of antimicrobial resistance of food borne bacteria to humans

About 50 years ago, scientists became aware of the link between antibiotic resistance and the use of antimicrobials in humans and food-producing animals (Mathew *et al*., 2007). In fact, numerous studies have been reported to support this relationship (Verraes *et al*., 2013). The debate about the misuse of antimicrobials and antimicrobial resistance continued for almost 40 years before the sub-therapeutic use of antimicrobials in food-producing animals was banned by the European Union (EU) in January 2006 (Moyane *et al*., 2013). In the USA, an incomplete ban on the use of certain tetracycline and penicillin drugs in animals was declared in 2012 to prevent these drugs from losing their effectiveness in the management of illnesses in humans.

When food pathogens develop resistance, more so multiple resistances, it leads to physicians having to alter their treatment as the infection will not respond to any commonly used antimicrobial substances (Threlfall, 2002). This results in prolonged illness with potentially more severe symptoms. The worst situation is a bacterial infection that is refractory to all available treatments (Travers & Barza, 2002). This could lead to death if not noticed on time (Salisbury *et al*., 2002).

2.7 Addressing antimicrobial resistance

2.7.1 Antimicrobial resistance surveillance

Monitoring of antimicrobial resistance is a necessity in evaluating the degree of the menace. Usually, bacterial isolates from either healthy or diseased animals are used. These isolates are categorized into three namely; animal pathogens, zoonotic bacteria and indicator bacteria. The use of animal pathogens is useful in observing antimicrobial resistance trends among

pathogens However, indicator bacteria have the advantage of being easily isolated from healthy animals, hence giving a true picture of the résistance in the entire animal population unlike pathogenic ones. For example, in the Danish surveillance programme *Escherichia coli* were chosen to represent Gram negative bacteria and *Enterococcus faecalis*/*faecium* to represent Gram-positive ones. The zoonotic bacteria can develop resistance in their animal reservoirs which when transmitted to humans, makes treatment of infections caused by them difficult to treat. The most commonly included zoonotic bacteria are *Salmonella* , *Campylobacter coli, Campylobacter jejuni* and *Yersinia enterocolitica* (Aarestrup, 2004; Rosengren *et al*., 2009).

2.7.2 Prudent use of antibiotics

Prudent or cautious use of antimicrobials entails decreasing misuse and choosing the ideal drug, dose, and duration when use is necessary, so as to eventually reduce the development of antimicrobial resistance. Another measure is that antimicrobial use should be based on susceptibility testing. More so, narrow spectrum antimicrobials should be used for the shortest time possible whenever antimicrobials are required (Rosengren *et al*., 2009). It is rather unfortunate in situations where uses of drugs especially in farm animals are not restricted to the veterinarians alone; untrained farm workers tend to misuse these drugs.

South Africa is part of the four countries (including India, Vietnam and Kenya) forming the Global Antibiotic Resistance Partnership (GARP**)** (Moyane *et al*., 2013)**.** According to GARP, (2011), South Africa has the most active antibiotic surveillance of any country in Africa. The Group for Enteric Respiratory and Meningeal disease Surveillance in South Africa (GERMS-SA) and the National Antibiotic Surveillance Forum (NASF)/South African Society for Clinical Microbiology (SASCM) are the two main groups that have been active for over 10years. The sexually transmitted infections (STI) center in collaboration with the

National Department of Health (NDOH), also conducts surveillance. The Enteric Diseases Reference Unit (EDRU) gathers data on patients presenting with both invasive and non-invasive diarrhoea-causing bacteria in the whole of South Africa. This unit collates patient and isolate information under a single record, compiled from 2003 onward. EDRU attempts to represent the entire country by offering free serogrouping, serotyping and antibiotic susceptibility testing to all diagnostic laboratories throughout the country. Despite these surveillance systems in South Africa, there seems to be no evidence of active surveillance going on in the country, since the available data has not been translated into policy (Moyane *et al*., 2013).

2.7.3 Use of Vaccines

Vaccination reduces the demand for antibiotic treatment of certain vaccine-preventable bacterial infections and significantly reduces morbidity and mortality in populations at risks. Furthermore, some viral diseases, e.g. rotavirus diarrhoea, are vaccine preventable, and inappropriate use of antibiotics for such clinical conditions again results in decreased appropriate use of antibiotics (GARP, 2011)

2.7.4 Bacteriophages

Bacteriophages (phages) are a diverse group of viruses that infect bacteria. They are employed for use in biotechnology, research and therapeutics. The use of phages in therapy is an important substitute to antimicrobials, in this current dispensation of drug resistant pathogens and have been used for approximately five decades now (Golkar *et al*., 2014; Jassim & Limoges, 2014). Several advantages of phages over antibiotics have been highlighted; they do not cause secondary infections or super infections due to host specificity, i.e. they do not that target both pathogens and commensals. Secondly, they only focus at the site of infection where they are expected to lyse the pathogens (Sulakvelidze & Alavidze,

2001). Also, no side effects have been reported. Furthermore, developing the phages is more cost effective and takes shorter duration than developing new antimicrobials (Golkar *et al*., 2014). Although, bacteria can become resistant to phages, these viruses can transform to counter the bacteria resistant to them (Ho, 2001). Phages have various applications including use in the treatment of cystic fibrosis, chronic otitis caused by *Pseudomonas auruginosa* (Jassim & Limoges, 2014), biocontrol agent in the prevention of food borne infections (Biswas *et al*., 2002) as well as in the treatment of bacterial infections in animals such as the treatment of respiratory infections caused by *E. coli* in chickens (Huff *et al*., 2003).

However several concerns have been raised as regards the use of bacteriophages. These include safety and efficacy, immune response, optimization of their growth, amongst others. Nevertheless, with the speedy growth in the area of biotechnology, there is still hope that phages will eventually be of help in combating antimicrobial resistance (Golkar *et al*., 2014).

2.7.5 Crude plant extracts as a promising alternative

According to Sibanda & Okoh, (2007), plants have provided a source of optimism for the development of new compounds owing to their vast composition and chemical diversity. Several of such compounds have been discovered with myriad modes of action, including resistance modifying activities.

These breakthroughs seem promising, however, with the ever increasing emergence of strains that have intrinsic antimicrobial resistance mechanisms and the fact that measures needed to combat resistance have not been fully achieved globally, it becomes tempting to question if new compounds will make any significant impact. In the same vein, it usually takes a long time in addition to high cost, for a novel compound to go through the different stages of drug development; from clinical trials up to its approval. It is imperative for the already existing

ones to be safe guarded. This therefore means that certain policies regarding the use of antimicrobials should be strictly implemented.

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CHAPTER 3

Prevalence and antibiogram of *Escherichia coli* O157 and non-O157 isolated from swine in Nkonkobe Municipality, Eastern Cape Province, South Africa

Abstract

We investigated the prevalence and antibiogram of *Escherichia coli* serogroups in swine faecal samples in Nkonkobe Municipality. A total of 310 presumptive STEC were subjected to identity confirmation using polymerase chain reaction technique for confirmation of *uid*A gene, out of which 179 isolates were confirmed positive. Serogroups O157: H7, O145 and O26 occurred in (24%), 0145 (8%) and (20%) of isolates, respectively. Only *stx2* gene was detected in 22 confirmed isolates, 7 of them belonged to *E. coli* O26 serogroup while the remaining 15 belonged to un targeted serogroups Antibiotic susceptibility test revealed that all the isolates were susceptible to imipenem. Similarly, a relatively high susceptibility was observed in norfloxacin (83-100%), ciprofloxacin (63-100%), gentamycin (77-100%), and chloramphenicol (77-100%). All the isolates were resistant to tetracycline, its long acting counterpart oxytetracycline. Varied resistances were observed against ampicillin (84-91%), streptomycin (14-100%), erythromycin (91-100%), ceftazidime (35%). Multiple antimicrobial resistance patterns and indices ranged from 3 to 12 and 0.2 to 0.7 to respectively. Genes encoding resistances to ampicillin (*amp*C), streptomycin (*str*A) and tetracycline (*tet*A) were detected in 50-100%, 22-29% and 40-86% respectively. These findings reveal that pigs within the Eastern Cape could harbour Shiga toxins and multidrug resistant *E. coli* O26, and O145 and O157:H7. Hence, adequate sanitation coupled with monitoring and surveillance of antimicrobial usage in swine industries is advocated in order to safeguard public health.

Key words: STEC, antimicrobial resistance, pigs, susceptibility, *E. coli*

3.1 Introduction

Escherichia coli are mostly commensal bacteria whose habitats are the guts of humans and warm blooded animals (Kaper *et al.,* 2004) and do not cause disease except in immunocompromised persons especially children and aged ones. They are used as foremost indicator organisms for faecal contamination and breaches in hygiene. However, some of them carry virulence factors that have enabled them adapt to new environments and cause serious infections. Among these are pathotypes responsible for causing gastrointestinal infections namely, enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffuse-adherent *E. coli* (DAEC). Each of these pathotypes possesses different virulence properties that enable them to cause diseases by different mechanisms which result in variable clinical symptoms (Nataro & Kaper, 1998; Chandra *et al*., 2013).

Among the defined pathotypes, is the STEC which have emerged as important zoonotic food-borne pathogens and it is the most overwhelming diarrhoegenic pathotype. Due to their association with numerous food-borne disease outbreaks, they have become a major public health concern (Bolton, 2011; Ghanbarpour & Kiani, 2013). Shiga toxin-producing *Escherichia coli* (STEC), also recognized as verotoxin- producing *E. coli* (VTEC) are food borne pathogens encompassing a serologically different group of pathogens that cause disease in humans and animals (Barman *et al*., 2008; Conrad *et al*., 2014). Symptoms of infections caused by STEC include diarrhoea, haemolytic uraemic syndrome (HUS), haemorraghic colitis, thromobocytopenia and acute renal failure (Momtaz *et al*., 2013).

The most common serotype of STEC associated with human disease and considered to be a major public health concern is *E. coli* O157:H7. However, there has been increasing

awareness overtime that over 100 non-O157 STEC serogroups which have emerged as important pathogens causing outbreaks of food-borne diseases. Conrad *et al.* (2014) reported that about 20-50% (equivalent to 37,000) illnesses are associated with STEC annually in the US. Amongst these, six serogroups have been associated with major illnesses caused by the non - STEC namely, *E. coli* O26, O103, O111, O121, O45, and O145 in order of occurrence in the United States (Farrokh *et al*., 2013; Smith, Fratamico, & Gunther, 2014). In fact, they are being referred to as the "top six" non O157 serogroups and classified as adulterants in beef trim by the U.S Department of Agriculture, Food, Safety and Inspection Service (USDA-FSIS), and requiring routine monitoring (Conrad *et al*., 2014) . Furthermore, the severity of illnesses due to STEC O157 and non O157 has been shown to be equivalent (Johnson *et al*., 2006). Although cattle are the major reservoirs of this group of organisms, they have also been detected in pigs in so many countries such as the US, Germany, France, Switzerland, etc (Ferens & Hovde, 2011). In South Africa, a prevalence of 9.5% of *E. coli* O157 has been reported in North West Province (Ateba *et al*., 2008).

The pathogenicity of STEC is connected to numerous virulence factors. The major ones are the Shiga toxins (Stx) 1 and 2 encoded by *stx1* and *stx2* genes respectively which act by inhibiting protein synthesis in mammalian cells. Others include the intimin encoded by *eae* gene, responsible for adherence; α-haemolysin encoded by *hly*A and enterohaemolysin encoded by *ehx*A which are responsible for haemolysis, amongst others (Meng *et al*., 2014).

Treatment of STEC using antibiotics has not yielded any favourable results after HUS development. Although the actual mechanism is not known but it is assumed that bacterial lysis could increase the amount of Shiga toxins released into the systemic circulation (Buvens *et al*., 2010). However, the development and spread of antimicrobial resistance in both commensal and pathogenic strains of bacteria has been recognized to be a major public health

problem globally. This is because they serve as reservoirs for resistance and even multiple antibiotic resistances (MAR) determinants that could be spread to foodborne and other zoonotic pathogens (Zhang *et al*., 2009; Joris *et al*., 2011).

Several factors have been attributed to the occurrence of antimicrobial resistance. These include the use of antibiotics in humans and animals for treatment and prevention of diseases, and also the use at sub therapeutic levels for growth promotion in animals. The consequence of this is the promotion of resistance in bacteria that is commensally present in the intestinal tracts of animals. This reduces the efficacy of antimicrobials used in both human and veterinary medicine, thus posing a serious public health threat (Schroeder *et al*., 2002; Buvens *et al*., 2010).

To the best of our knowledge, the prevalence of virulence and antimicrobial resistance determinants of STEC from pigs in the Eastern Cape Province of South Africa have been scantily investigated, considering their potential health threats posed to their consumers. In the light of this, this study was designed to investigate the aforementioned matter to increase our understanding of both pathogenic and antimicrobial resistance mechanisms present among Shiga toxin producing *E. coli* in food producing animals and proffer some possible ways out to reduce their incidence in the swine industry for safeguarding the health of the public.

3.2 Materials and Methods

3.2.1 Description of study area

The Nkonkobe Municipality is situated along the Southern slopes of the Winterberg Mountain range and escapement, and is within the greater Amathole District Municipality in the Province of the Eastern Cape. The Municipal area being the second largest covers approximately 3725 Km2 and constitutes 16% of the surface area of the Amatole District

Municipality. It is a rural Municipality largely driven by the agricultural sector. (Nkonkobe Municipality IDP, 2013/2014; 2014/15).

3.2.2 Sample collection and isolation of presumptive organisms

Faecal samples were collected from healthy pigs over a period of four months (April – July 2014) using sterile swab sticks. The samples were transported immediately on ice packs to the Applied Environmental Microbiology Research Group (AEMREG) laboratory of the University of Fort Hare, South Africa where microbiological analysis was conducted. Samples were pre-enriched by inoculating into Tryptic soy broth (TSB) (Merck, South Africa) and incubated for 24 hours at 37°C. Tubes with growth were then streaked onto Sorbitol MacConkey agar (Mast Group Ltd, Mayerside, UK) supplemented with 1mg/L potassium tellurite and incubated for 18-24 hours at 37°C. Non O157 were isolated by picking characteristic pink (sorbitol fermenting) colonies while pale colonies (sorbitol non-fermenting) were picked as presumptive *E. coli* O157 and incubated for 24 hours at 37°C. The pure colonies were inoculated into TSB and incubated for 24hours at 37°C. They were then stocked into 30% glycerol and refrigerated stored at -80°C till further analyses.

3.2.3 DNA Extraction

Prior to molecular identification of isolates, DNA was extracted from presumptive isolates. The isolates were first resuscitated by inoculating unto Tryptic soy broth (Merck, SA) and incubated at 37 °C for 24hours. They were further streaked unto nutrient agar (Merck, South Africa) and incubated at 37°C for 24hours. The extraction was done using boiling method as described by Maugeri *et al*. (2004). Briefly, about 3-5 colonies were picked using a sterile wire loop into sterile DNAase/ RNAase free Eppendorf tubes (Biologix, USA) containing 200µl sterile nuclease free water (Thermoscientific, USA). The suspension was vortexed using the Vortex mixer (Digisystem Laboratory, Taiwan) and the cells were lysed by heating

in a heating block (Lasec, UK) at 100^{o}C for 15 min. The cell debris was removed by centrifugation using a centrifuge (Lasec, UK) at 13,500 rpm for 15min. The supernatant containing the genomic DNA was carefully transferred into a sterile Eppendorf tube and stored at -20^{o}C to be used for further assays.

3.2.4 Molecular identification of *E.coli* isolates/ serogrouping

The presumptive isolates were confirmed as *E. coli* using primer pair targeting the *uid*A gene. *E. coli* ATCC 25922 was used as a positive control strain Table 3.1.Serogroups including O26, O45, O103, O111, O121, O145, and O157 were screened using polymerase chain reaction assays. Primer sequences and the expected amplicon sizes for the serogroups and virulence genes are listed in Table 3.1 and 3.2 respectively. The primers were synthesized by Inqaba Biotechnical industries (Pty) Ltd, South Africa. Each reaction mixture consisted of 12.5µl 2x Master Mix (Thermo scientific, USA), 1µl each of the forward and reverse primers, 5.5µl of nuclease free water and DNA template in a final volume of 25µl. The PCR cycling conditions for *uid*A amplification were as described by Tsai *et al*.(1993), with little modification as follows; initial denaturation at 94^{o}C for 5min., 35 cycles of 30 seconds denaturation at 95^{o}C, primer annealing at 58^{o}C for 1min., extension at 72^{o}C for 1min. and final extension at 72^{o}C for 8 min. whereas the same conditions was used for the serogroups and virulence genes following the description of Franck *et al*. (1998) and Perelle *et al*. (2004) with little modification as follows; initial denaturation at 94^{o}C for 2min., 35 cycles of 1min. denaturation at 94^{o}C, annealing at 55^{o}C for 45 seconds, extension at 72^{o}C for 1min. and final extension at 72^{o}C for 5min.. All reactions were performed in a MyCyclerTM Thermal Cycler PCR system (BioRad, USA). *E. coli* O157:H7 (ATCC 35150) was used as the positive control for STEC O157. Negative controls were used in all reactions, comprising of the reaction mixture except DNA template which was replaced by nuclease free water. The PCR

products (5 µl) were subjected to 1.5% agarose gel electrophoresis (Seperations, South Africa) stained with ethidium bromide (0.001µg/ml) using 0.5X Tris-borate EDTA (TBE) buffer at 100V for 60min. The gel was visualized under the UV transilluminator (Alliance 4.7). A 50-bp DNA ladder (Promega, USA) was used as molecular size standard for band sizes below 100bp on each gel, and 100bp ladder (Promega, USA) for expected band sizes of 100bp and above. Tables 3.1 and 3.2 show the primer sequences, amplicon sizes of serogrous and virulence genes expected targeted respectively.

Table 3.1: Oligonucleotide sequences and predicted sizes for PCR amplification of serogroups of Shiga toxin- producing *E. coli*

Gene/primer sequence(5'-3')	**Amplicon size (bp)**	**Reference**
wzy (O121) F -GCA ATG AGG ACC GGT ATA TCT C R -CAC GCC CGT GTT AAT ATT CC	318	(Perelle *et al*., 2004)
*rfb*E(O157) F -TTT CAC ACT TAT TGG ATG GTC TCA A R -CGA TGA GTT TAT CTG CAA GGT GAT	88	(Perelle *et al*., 2004)
wbdI (O111) F -CGA GGC AAC ACA TTA TAT AGT GCT TT R -TTT TTG AAT AGT TAT GAA CAT CTT GTT TAG C	146	(Perelle *et al*., 2004)
wzx (O26) F -CGC GAC GGC AGA GAA AAT T R- AGC AGG CTT TTA TAT TCT CCA ACT TT	135	(Perelle *et al*., 2004)
ihpl (O145) F -CGA TAA TAT TTA CCC CAC CAG TAC AG R -GCC GCC GCA ATG CTT	142	(Perelle *et al*., 2004)
$flic_{H7}$ (O157:H7) F- GCG CTG TCG AGT TCT ATC GAG R- GTCGGCAACGTTAGTGATACC	230	(Wang *et al*., 2002)
*uid*A F-AAAACG GCA AGA AAA AGC AG 147 R- ACG CGTGGTTAA CAG TCT TGC G	147	(Tsai *et al*., 1993)

Table 3.2: Oligonucleotide sequences and predicted sizes for PCR amplification of virulence genes of Shiga toxin- producing *E. coli*

Gene/primer sequence(5'-3')	**Amplicon size (bp)**	**Reference**
Stx1 F-TTC GCT CTG CAA TAG GTA R -TTC CCC AGT TCA ATG TAA GAT	555	(Franck *et al*., 1998)
*stx*2 F -GTG CCT GTT ACT GGG TTT TTC TTC R- AGG GGT CGA TAT CTC TGT CC	118	(Franck *et al*., 1998)
eae F-ATA TCC GTT TTA ATG GCT ATC T R -AAT CTT CTG CGT ACT GTG TTC A	425	(Perelle *et al*., 2004)
ehxA F -GGT GCA GCA GAA AAA GTT GTA G R-TCT CGC CTG ATA GTG TTT GGT A	1551	(Perelle *et al*., 2004)

3.2.5 Antimicrobial susceptibility testing

Phenotypic antimicrobial susceptibility testing was performed on all identified *E. coli* serogroups using disk diffusion assay following Clinical Laboratory Standard Institute guideline (CLSI, 2012). Briefly, isolates were grown on nutrient agar (Merck, SA) at 37^{o}C for 18- 24hour. A sterile inoculating loop was used to pick about 4- 5 colonies growing on the agar (Conda – pronadisa, USA) and suspended in 0.85% solution of sodium chloride (NaCl). The cell density was adjusted to a 0.5 McFarland turbidity standard (equivalent to 1.5 x 10^{8}) using normal saline. A sterile cotton swab was dipped into the standardized bacterial test suspension and used to evenly spread the entire surface of Muller Hinton agar plate. After the agar surface was dried for 5min., the appropriate antibiotic disks (Mast Diagnostics, UK) were placed on the plates using the using the antibiotic disc dispenser (Mast Diagnostics, UK).

The choice of these drugs was based on the drugs used to commonly treat infections caused by *E. coli* and those used in the in the farms where samples were collected as shown in Table 3.3. The antimicrobials tested include tetracycline (T-30 µg), oxyteracycline (OT - 30µg), ampicillin (AMP -10 µg), sulphamethoxazole/trimethoprim (TS- 25), streptomycin, gentamycin (GM-10µg), amikacin (AK-30µg), ceftazidime (CAZ-30µg), cephalothin (KF-30µg), cefotaxime (CTX-30µg), chloramphenicol (C-10µg), norfloxacin (NOR-10µg), ciprofloxacin (CIP-5µg), nalidixic acid, cefuroxime, imipenem and polymycin B belonging to ten different classes of antimicrobials The plates were incubated at 37^{o}C. After 24hours of incubation, determination of resistance or susceptibility of isolates was performed by measuring to the nearest millimetres, the zones (diameter) of inhibition and interpreted according to the Clinical and Laboratory Standards institute (CLSI, 2012) guideline. Multiple antibiotic resistance (MAR) phenotypes, patterns and indexing were generated for the

resistant isolates (Ateba *et al*., 2008). The MAR index of individual isolates of the each serogroup identified was calculated using the formula described by Krumperman (1983). MAR index of isolate = No. of antibiotics to which isolate was resistant / Total no. of antibiotics to which isolate was exposed. A MAR index of ≥ 0.2 indicate high risk environment where antibiotics are often used (Osundiya *et al*., 2013).

3.2.6 Prevalence of antimicrobial resistance genes

Based on the phenotypic results, the *amp*C, *tet*A and s*tr*A genes encoding for resistance to ampicillin, tetracyclines, and streptomycin respectively, were genetically investigated using specific primers in a monoplex PCR. The primer sequences, PCR conditions and amplicon size of the target genes are shown in Table 3.4.

3.2.7 Data Analysis

Data were captured into Microsoft excel sheet 2010 and simple descriptive statistics of Statistical Package for Social Sciences (SPSS) version 22 was used in analysing the data

Table 3.3 list of antibiotics (classes) commonly used in the farms studied

Farm A	**Farm B**
Procaine benzyl penicillin (penicillins)	Procaine penicillin (penicillins)
Sulphadizine + trimethoprim (Folate pathway inhibitor)	Tylosin (Macrolides)
	Amoxicillin (beta lactams; ampicillin)
	Sulphadiazine trimethoprim (Folate pathway inhibitor)
Ampicillin (β-lactams; ampicillins)	
	Danofloxacin (fluoroquinolones)
Oxytetracycline (tetracyclines)	

Source: From the staff of the farms

Table 3.4 The Primer sequences, PCR conditions and amplicon size of antibiotic resistance genes targeted among all the *E. coli* isolates

Antimicrobial (gene)	**Primer sequence(5' – 3')**	**PCR condition**	**Product size**	**Reference**
Ampicillin (*bla ampC)*	F-AATGGGTTTTC- CGGTCTG R-GGGCAGCAAATGTG-GAGCAA-	5 min initial denature at 94°C followed by 35 cycles of 94°C for 1min, 55°C for 1min and 72°C for 1•5 min final incubation at 72°C for 5 min	191bp	(Forward *et al*., 2001)
Tetracycline (*tetA*)	F – GGCCTCAATTTCCTGACG R-AAGCAGGATGTAGCCTGTGC	5 min initial denature at 94°C followed by 35 cycles of 94°C for 1min, 55°C for 1min and 72°C for 1•5 min final incubation at 72°C for 5 min	372bp	(Guillaume *et al*., 2000)
Streptomycin (*StrA*)	F- CCAATCGCAGATAGAAGGC R-CTTGGTGATAACGGCAATTC	5 min initial denature at 94°C followed by 35 cycles of 94°C for 1min, 55°C for 1min and 72°C for 1•5 min final incubation at 72°C for 5 min	548bp	(Thong, 2010)

3.3 Results

3.3.1 *E. coli* isolation

Five hundred faecal samples were collected from two commercial farms (Farm A and Farm B) within the Nkonkobe Municipality in the Eastern Cape Province, South Africa. After preliminary screening using selective culture medium, a total of 310 presumptive isolates were obtained as shown in Table 3.5.

3.3.2 Molecular confirmation and molecular serotyping of *E.coli* isolates

The presumptive isolates were further subjected to molecular confirmation for *E. coli* spp and a total of 179 (57.7%) of the presumptive STEC tested positive for the *uid*A gene. Molecular serogrouping was carried out on these confirmed isolates and only serogroups O26, O145, and O157:H7 were detected as shown in Table to 3.5. Figures 3.1 to 3.5 below show the gel electrophoresis of all the amplified products.

Molecular serogrouping of the confirmed *E. coli* isolates was conducted using primers specific for each serogroup. About 19.6% (n=35) belonged to the O26 serogroup while 7.8% (n=14) and 24% (n=43) belonged to the O145 and O157 serogroups respectively, as shown in Table 3.3. *E. coli* serogroups O45, O103, O111, and O121 were not detected. Figures 3.2, 3.3 and 3.4 show the electrophoresis gel pictures of amplified products of serogroup O26, O145 and O157:H7 respectively.

3.3.3 PCR detection of virulence genes

Shiga toxin gene (*stx2*) was detected in 13.1% (n=22) of the confirmed isolates. Thirty one percent (n=7) of the *stx* positive isolates belonged to the O26 serogroup, while about 68% (n=15) of the *stx* positive isolates belonged to serogroups that were not among those screened for. None of the remaining serogroups possessed either the *stx1* genes or the other virulence

genes as seen in Table 3.4. Figure 3.5 shows the electrophoresis products of amplified *stx2* gene harboured by *E. coli* O26.

Table 3.5: Results of isolation and identification of *E. coli* serogroups

Location	**No of faecal samples collected**	**No of presumptive STEC isolates**	**Serogroups**							
			No of Confirmed isolates (%)	**026**	**045**	**0103**	**0111**	**0121**	**0145**	**0157**
Farm A	50	43	23 (53.4)	0	0	0	0	0	0	7
Farm B	450	267	156 (58.4)	35	0	0	0	0	14	36
Total (%)	**500**	**310**	**179 (57.7)**	**35(19.6)**	**0 (0)**	**0 (0)**	**0 (0)**	**0 (0)**	**14(7.8)**	**43(24)**

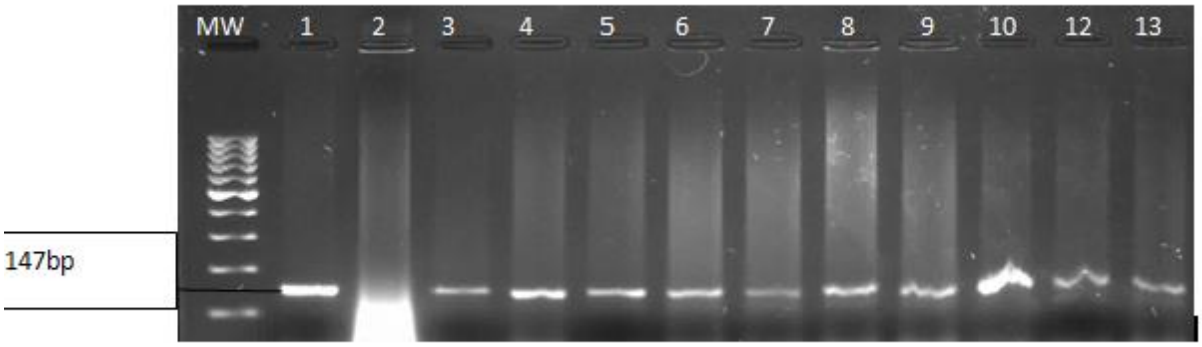

Fig 3.1: Agarose gel electrophoresis of PCR products of *uid*A gene amplification for identification of *E. coli spp***.** Lane MW: 100 bp ladder; Lane 1: positive control (*E. coli* ATCC 25922); Lane 2: negative control; Lanes 3-13: positive isolates.

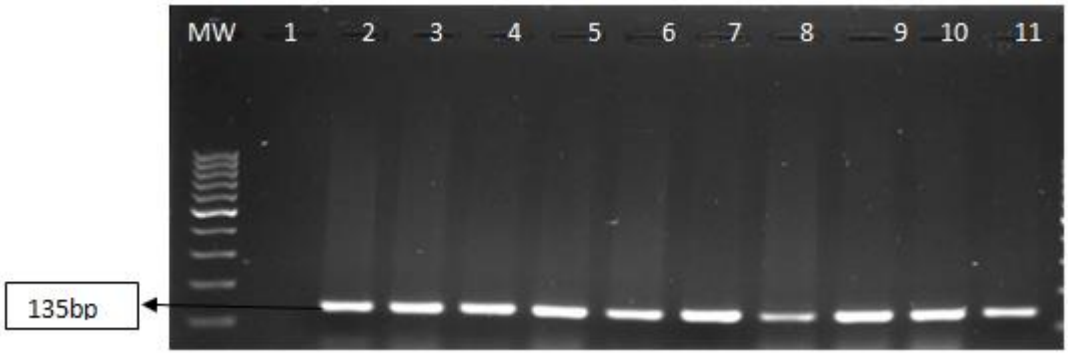

Fig 3.2: Agarose gel electrophoresis of PCR products of *wzx* gene amplification for identification *E. coli* O26. Lane MW: 100 bp ladder; Lane 1: negative control; Lanes 2-11: positive isolates.

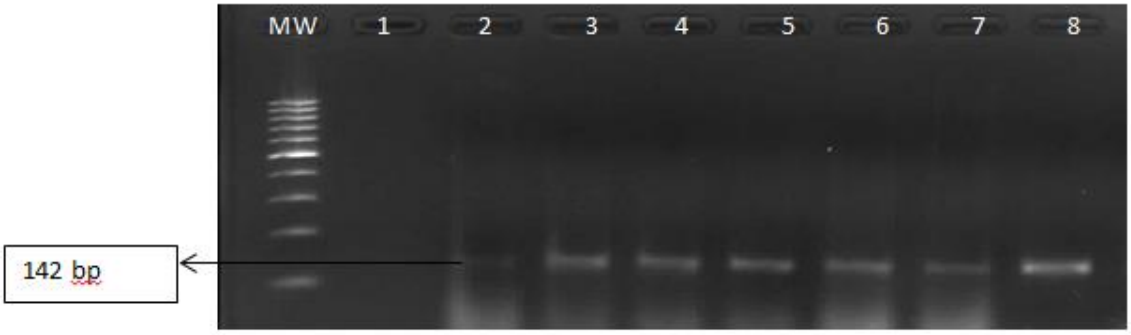

Fig 3.3: Agarose gel electrophoresis of PCR products of *ihpl* gene amplification for identification of *E. coli* O145. Lane MW: 100 bp ladder; Lane 1: negative control:

Lanes 2-8: positive isolates.

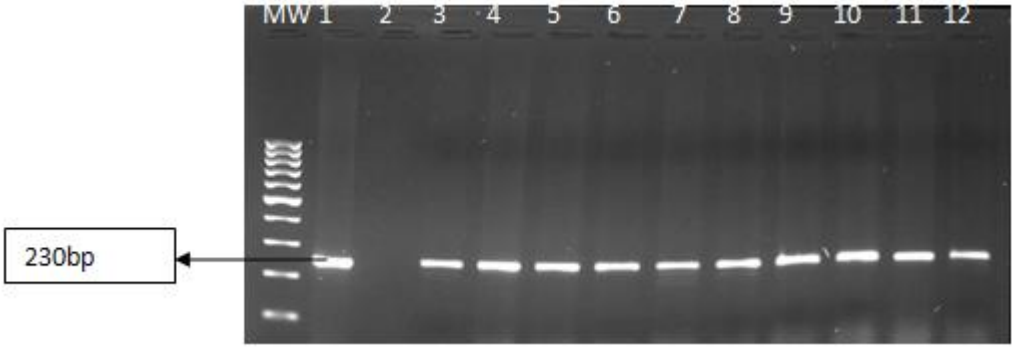

Fig 3.4: Agarose gel electrophoresis of PCR products of *flic*H7 gene amplification for identification of *E. coli* O157:H7. Lane MW: 100 bp ladder; Lane 1 positive control (*E. coli* ATCC 35150); Lane 2: negative control; Lanes 3-12: positive isolates.

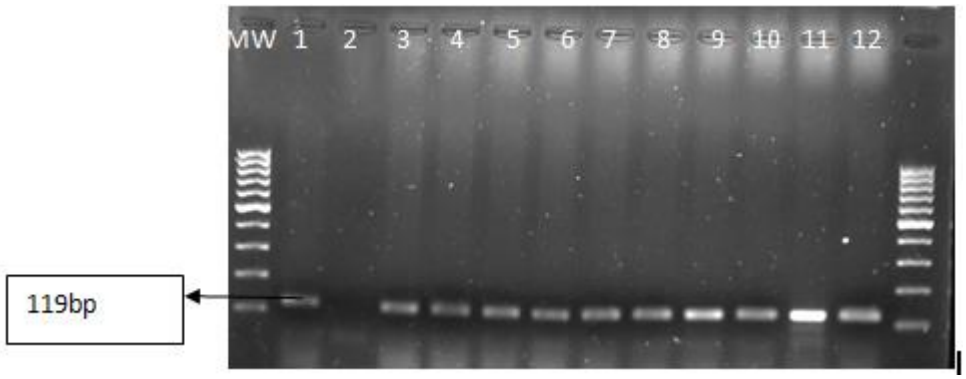

Fig 3.5: Agarose gel electrophoresis of PCR products of *stx2* gene amplification. Lane MW: 100 bp ladder; Lane 1 positive control (*E. coli* O157:H7 ATCC *35150*); Lane 2: negative control; Lanes 3-12: some positive isolates**.**

Table 3.6 Distribution of virulence genes among *E. coli* serogroups

Serogroups	**Number of STEC isolates having virulence isolates**			
	***stx*1**	***stx*2**	***eae***	***ehxA***
026	0	7	0	0
045	0	0	0	0
0103	0	0	0	0
0111	0	0	0	0
0121	0	0	0	0
0145	0	0	0	0
0157	0	0	0	0
NTS	0	15	0	0

NTS: Non targeted serogroup

3.3.4 Antibiogram Profile

Hundred percent of *E. coli* O26 strains were resistant to tetracycline, oxytetracycline, (Table 3.7). Similarly, 91.4% of them exhibited resistance to erythromycin. Resistance was also observed to the first generation cephalosporin, cephalothin (25.7%). Similarly, serogroups O145 and O157:H7 exhibited high resistance against tetracycline, oxytetracycline and amplicillin. Unlike O26 and O157:H7, serogroup O145 demonstrated 100% resistance to streptomycin. Serogroup O157:H7 showed a relatively high resistance to cephalothin (42%), cefuroxime (56%) and ceftazidime (35%) which are first, second and third generation cephalosporins respectively. All the isolates were resistant to at least 3 antibiotics, hence they are categorised as multidrug resistant strains. High sensitivity to the fluoroquinolones was observed. Tables 3.7, 3.8 and 3.9 show the antibiogram profile of *E. coli* O26, O145 and O157:H7 respectively.

Table 3.7 Antibiotic susceptibility pattern of *E. coli* O26 (n=35)

Antimicrobial agent	**Disc Code**	**Potency**	**R**	**I**	**S**
				n (%)	
Tetracycline	T	30	35(100)	0(0)	0(0)
Oxytetracycline	OT	30	35(100)	0(0)	0(0)
Ampicillin	AMP	10	32 (91.4)	3(8.6)	0(0)
Cephalothin	KF	30	9(25.7)	17(48.6)	9(25.7)
Cefuroxime	CXM	30	1(2.9)	0(0)	34(97.1)
Ceftazidime	CAZ	30	4(11.4)	1(2.9)	30(85.7)
Cefotaxime	CTX	30	0(0)	0(0)	35(100)
Erythromycin	E	15	32(91.4)	3(8.6)	0(0)
Sulphamethoxazole/Trimethoprim	TS	25	13(37.1)	0(0)	22(62.9)
Chloramphenicol	C	10	8(22.9)	0(0)	27(77.1)
Nalidixic acid	NA	30	2(5.7)	6(17.1)	27(77.1)
Ciprofloxacin	CIP	5	0(0)	2(5.7)	33(94.3)
Norfloxacin	NOR	10	1(2.9)	0(0)	34(97.1)
Gentamycin	GM	10	8(22.9)	0(0)	27(77.1)
Amikacin	AK	30	0(0)	2(5.7)	33(94.3)
Streptomycin	S	10	5(14.2)	20(57.14)	10(28.6)
Imipenem	IMI	10	0(0)	0(0)	35(100)
Polymycin B	PB	300	15(42.9)	0(0)	20(57.1)

R: Resistant, I: Intermediate, S: Susceptible, T: Tetracycline, OT: Oxytetracycline, AMP: Ampicillin, KF: Cephalothin, CXM: Cefuroxime, CAZ: Ceftazidime, CTX: Cefotaxime, E: Erythromycin, TS: Sulphamethoxazole/Trimethoprim, C: Chloramphenicol, NA: Nalidixic acid, CIP: Ciprofloxacin, NOR: Norfloxacin, GM: Gentamycin, AK: Amikacin, S: Streptomycin, IMI: Imipenem, PB: Polymycin B

Table 3.8 Antibiotic susceptibility pattern of *E. coli* O145 (n=14)

Antimicrobial class	Antimicrobial agent	Code	Potency	R	I	S
					n (%)	
Tetracycline	Tetracycline	T	30	14(100)	0(0)	0(0)
	Oxytetracycline	OT	30	14(100)	0(0)	0(0)
Ampicillins	Ampicillin	AMP	10	12(85.7)	2(14.3)	0(0)
Cephalosporins	Cephalothin	KF	30	2(14.3)	2(14.3)	10(71.4)
	Cefuroxime	CXM	30	2(14.3)	0(0)	12(85.7)
	Ceftazidime	CAZ	30	0(0)	0(0)	14(100)
	Cefotaxime	CTX	30	4(28.6)	8(57.1)	2(14.3)
Macrolides	Erythromycin	E	15	14(100)	0(0)	0(0)
Folate pathway inhibitor	Sulphamethoxazole/ Trimethoprim	TS	25	0(0)	0(0)	14(100)
Phenicols	Chloramphenicol	C	10	0(0)	0(0)	14(100)
Quinolone	Nalidixic acid	NA	30	2(14.3)	10(71.4)	2(14.3)
Floroquinolone	Ciprofloxacin	CIP	5	0(0)	0(0)	14(100)
	Norfloxacin	NOR	10	0(0)	0(0)	14(100)
Aminiglycosides	Gentamycin	GM	10	0(0)	2(14.3)	12(85.7)
	Amikacin	AK	30	8(57.1)	4(28.6)	2(14.3)
	Streptomycin	S	10	14(100)	0(0)	0(0)
Carbapenems	Imipenem	IMI	10	0(0)	0(0)	14(100)
	Polymycin B	PB	300	8(57.1)	0(0)	6(42.9)

R: Resistant, I: Intermediate, S: Susceptible, T: Tetracycline, OT: Oxytetracycline, AMP: Ampicillin, KF: Cephalothin, CXM: Cefuroxime, CAZ: Ceftazidime, CTX: Cefotaxime, E: Erythromycin, TS: Sulphamethoxazole/Trimethoprim, C: Chloramphenicol, NA: Nalidixic acid, CIP: Ciprofloxacin, NOR: Norfloxacin, GM: Gentamycin, AK: Amikacin, S: Streptomycin, IMI: Imipenem, PB: Polymycin B

Table 3.9 Antibiotic susceptibility pattern of *E. coli* O157:H7 (n=43).

Antimicrobial agent	**Code**	**Potency**	**R**	**I**	**S**
				n (%)	
Tetracycline	T	30	34(79)	4(9)	5(12)
Oxytetracycline	OT	30	40(93)	1(2)	2(5)
Ampicillin	AMP	10	36(84)	7(16)	0(0)
Cephalothin	KF	30	18(42)	21(49)	4(9)
Cefuroxime	CXM	30	24(56)	11(25)	8(19)
Ceftazidime	CTX	30	15(35)	1(2)	27(63)
Cefotaxime	CAZ	30	0(0)	9(21)	34(79)
Erythromycin	E	15	42(98)	0(0)	1(2)
Sulphamethoxazole/ Trimethoprim	TS	25	22(51)	3(7)	18(42)
Chloramphenicol	C	10	9(21)	2(5)	32(74)
Nalidixic acid	NA	30	11(26)	28(65)	4(9)
Ciprofloxacin	CIP	5	13(30)	3(7)	27(63)
Norfloxacin	NOR	10	0(0)	7(16)	36(84)
Gentamycin	GM	10	4(9)	5(12)	34(79)
Amikacin	AK	30	3(7)	18(42)	22(51)
Streptomycin	S	10	36(84)	5(12)	2(5)
Imipenem	IMI	10	0(0)	0(0)	43 (100)
Polymycin B	PB	300	43(100)	0(0)	0(0)

R: Resistant, I: Intermediate, S: Susceptible, T: Tetracycline, OT: Oxytetracycline, AMP: Ampicillin, KF: Cephalothin, CXM: Cefuroxime, CAZ: Ceftazidime, CTX: Cefotaxime, E: Erythromycin, TS: Sulphamethoxazole/Trimethoprim, C: Chloramphenicol, NA: Nalidixic acid, CIP: Ciprofloxacin, NOR: Norfloxacin, GM: Gentamycin, AK: Amikacin, S: Streptomycin, IMI: Imipenem, PB: Polymycin B

3.3.5 Multiple antibiotic resistance (MAR) phenotypes and MAR indices (MARI)

E. coli O26, O145 and O157:H7 were characterized for their MAR phenotypes and MAR indices (MARI) as shown in Tables 3.10., 3.11, and 3.12 respectively. All the isolates were resistant to at least two antibiotics. Multiple antibiotic resistances were observed against 4 -11 antibiotics. The predominant MAR phenotype was T-AMP-S-OT-E, which occurred in 14%, 85%, and 53% in *E. coli* O26, O145, and O157:H7 respectively. On the other hand, the MARI for all the serogroups ranged between 0.2- 0.7 with the average being 0.4.

Table 3.10 Multiple antibiotic resistance (MAR) pattern and MAR indices (MARI) of *E. coli* O26 isolates

MAR pattern	No. of antibiotics	MARI
T-GM-OT-AMP-T/S-NA-E	6	0.3
T-GM-OT-KF-AMP-C-NOR-E-CXM-PB	10	0.6
T-OT-T/S-E	4	0.2
T-OT-KF-AMP-C-E-PB	7	0.4
T-GM-OT-KF-AMP-C-E-PB	8	0.4
T-OT-AMP-T/S-E	5	0.3
T-GM-OT-AMP-T/S-NA-E	6	0.3
T-OT-AMP-T/S-E-PB	6	0.3
T-GM-OT-KF-AMP-C-NOR-E-CXM-PB	10	0.6
T-OT-AMP-T/S-E	5	0.3
OT-AMP-T/S-E	4	0.2
T-OT-KF-AMP--C-E-PB	7	0.4
T-GM-OT-AMP-T/S-NA-E	7	0.4
T-CAZ-S-OT-AMP-T/S-C-E	8	0.4
T-GM-OT-KF-AMP-C-NOR-E-CXM-PB	10	0.6
T-OT-AMP	3	0.2
T-OT-AMP-T/S-E	5	0.3
T-OT-AMP-T/S-CXM	5	0.3
T-GM-OT-KF-AMP-C-E	7	0.4
T-CAZ-S-OT-AMP-T/S-C-E	8	0.4
T-OT-KF-AMP-C-E-PB	7	0.4
T-GM-OT-AMP-T/S-NA-E	7	0.4
T-CAZ-S-OT-AMP-T/S-C-E	8	0.4
T-GM-OT-KF-AMP-C-E-CXM-PB	9	0.5
T-GM-OT-KF-AMP-C-E-PB	8	0.4
T-OT-AMP-T/S-E-PB	6	0.3
T-GM-OT-KF-AMP-C-E-PB	8	0.4
T-OT-AMP-C-E	4	0.2

Table 3.11 Antibiotic resistance pattern and MARI of *E. coli* O145 isolates

MAR pattern	No. of antibiotics	MARI
T-S-E-OT-AMP-AK-CTX-PB	8	0.4
T-S-E-OT-AMP	5	0.3
T-S-E-OT-AMP-AK-PB	7	0.4
T-S-E-OT-AMP	5	0.3
T-S-E-OT-AMP	5	0.3
T-S-E-OT-KF-AMP-CTX	6	0.3
T-S-E-OT-AMP	5	0.3
T-S-E-OT—NA	5	0.3
T-S-E-OT-AMP-AK-PB	7	0.4
T-S-E-OT-AMP	5	0.3
T-S-E-OT-AMP-AK-PB	7	0.4
T-S-E-OT-AMP-NA	6	0.3
T-S-E-OT-AMP-AK-CTX-PB	8	0.4
T-S-E-OT	4	0.2

Table 3.12 Antibiotic resistance pattern and MARI of *E. coli* O157:H7 isolates

Resistance pattern	No. of resistance	MARI	Resistance pattern	No. of antibiotics	MARI
CIP-E-OT-KF-PG-T/S-NA	7	0.4	S-E-OT-KF-PG-T/S-NA	7	0.4
CAZ-E-OT-PG-AK-CXM	6	0.3	T-S-E-OT-PG-T/S-C-CTX-CXM	9	0.5
CAZ-S-0T-KF-PG-T/S-NA-C	8	0.4	T-CAZ-S-E-OT-KF-PG-T/S-NA	9	0.5
T-S-E-OT-KF-PG-T/S-NA	8	0.4	T-CAZ-S-E	4	0.2
T-KF-PG-T/S-C-CTX-CXM-PB	8	0.4	T-OT-KF-PG-T/S-NA-C-AK-CTX-CXM	10	0.6
T-CAZ-S-E-OT-KF-PG-T/S-NA-IMI	10	0.6	T-CAZ-S-E-OT-KF-PG-T/S-NA-C-CTX-CXM	12	0.7
T-S-E-OT-PG-T/S-CTX-CXM	8	0.4	T-S-E-OT-PG-C-AK-CTX-CXM-PB	10	0.6
T-CAZ-S-E-OT-KF-PG-T/S-NA-C	10	0.6	T-E-OT-PG-T/S-CTX-CXM-PB	8	0.4
T-S-E-KF-PG-T/S-CTX-CXM	8	0.4	E-KF-PG-R-CTX-CXM-PB	7	0.4
T-S-GM-E-OT-KF-PG-T/S	8	0.4	S-E-CTX-CXM	4	0.2
S-E-OT-PG-T/S-C-CTX-CXM	8	0.4	CAZ-E-OT-KF-PG-T/S-NA-C-CTX-CAM-PM	11	0.6
T-CAZ-S-E-OT-PG-T/S-	7	0.4	S-E-OT-KF-PG-CTX-CXM	7	0.4
S-E-OT-PG-CTX-CXM	6	0.3	T-S-E-OT-KF-PG-T/S-CTX-CXM	8	0.4
CAZ-S-PG-T/S-CTX-CXM	6	0.3	T-S-GM-E-OT-PG-T/S-C-AK-CTX-CXM	11	0.6
T-CAZ-S-CIP-E-OT-PG-C	8	0.4	T-CAZ-S-E-OT-PG-T/S-C-CTX-CXM	10	0.6
T-S-GM-OT-PG-T/S-NA	7	0.4	T-S-E-OT-KF-PG-T/S-NA-CTX	9	0.5
T-CAZ-S-E-OT-PG-T/S-NA-CTX-CXM	10	0.6	T-S-GM-E-OT-PG-T/S-CTX	8	0.4
T-S-E-OT-KF-PG-T/S-CTX-CXM	9	0.5	T-CAZ-S-E-OT-KF-PG-T/S-NA-CTX-CXM	11	0.6
CAZ-S-E-OT-KF-PG-T/S-C-CTX-CXM	10	0.6	T-S-E-OT-PG	5	0.3
T-E-OT-KF-PG-T/S-NA	7	0.4	T-S-E-OT-PG	5	0.3
T-E-OT-KF-PG	5	0.3	T-S-E-OT-PG	5	0.3
			T-S-E-OT-PG	5	0.3

3.3.6 Distribution of antimicrobial resistance genes

Resistance genes *tet*A, *str*A, and *amp*C were assessed for their possible involvement in tetracycline, streptomycin and ampicillin resistance among the isolates. The frequencies of these resistance determinants in the different serogroups identified are shown in Table 3.13 where *E. coli* O145 had the highest frequency (86%) of *tet*A genes followed by O26 (43%) and O157:H7 (40%). Furthermore, *E. coli* O145 and O157:H7 resistant to streptomycin were shown to harbour the *str*A gene (29%) and 21 % respectively, unlike *E. coli* O26 serogroup which was negative for the gene. *E. coli* O145 had the highest frequency of the *amp*C with all the ampicillin resistant isolates possessing the gene. Fig 3.6, 3.7 and 3.8 show the electrophoresis of the amplification of *tet*A, *amp*C and *str*A genes among the resistant isolates respectively.

Table 3.13 Distribution of the resistant determinants of the *E. coli* isolates from both farms

Antimicrobial agent (resistance gene)	**Serogroup**	**No. of resistant isolates**	**No. Of isolates harbouring resistance gene (%)**
Tetracycline (*tet*A)	026	35	15 (42.9)
	0145	14	12 (85.7)
	0157:H7	40	16 (40)
Streptomycin (*Str*A)	026	15	0 (0)
	0145	14	4 (28.5)
	0157:H7	36	8 (22)
Ampicillin (*amp*C)	026	32	18 (50)
	0145	12	12 (100)
	0157:H7	36	32 (88.9)

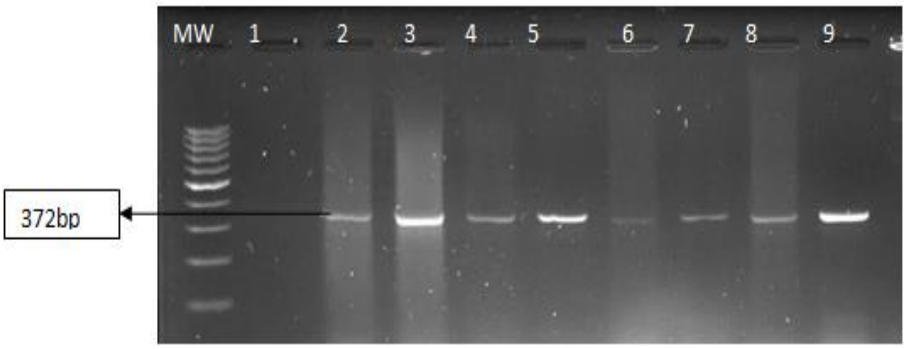

FIG 3.6: Electrophoresis of PCR products of *tet*A gene amplification among *E. coli* isolates

(Lane MW: 100 bp ladder; Lane 1: negative control; Lanes 2-9: positive isolates).

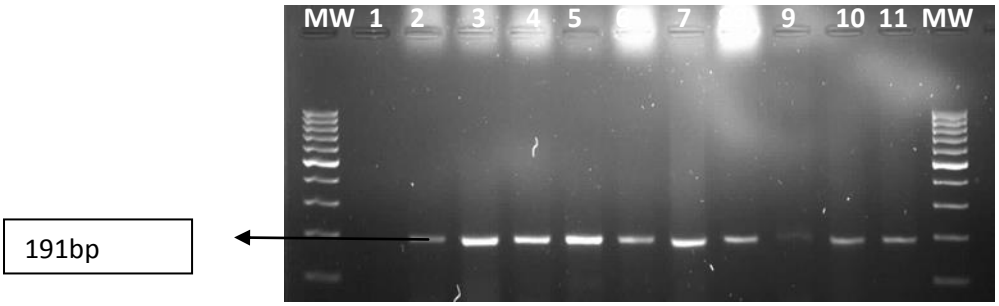

FIG 3.7: Electrophoresis of PCR products of *amp*C gene amplification among *E. coli* isolates

(Lane MW: 100 bp ladder; Lane 1: negative control; Lanes 2-11 positive isolates).

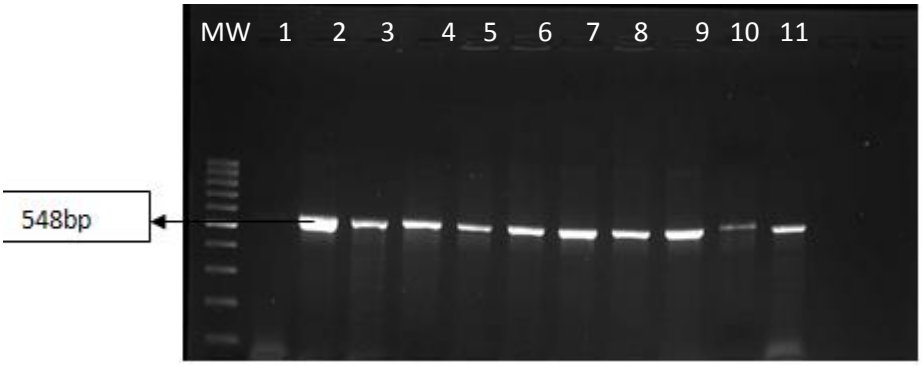

FIG 3.8: Electrophoresis of PCR products of *str*A gene amplification among *E. coli* isolates

(MW: 100 bp ladder; Lane 1-11: some positive isolates).

3.4 Discussion

Many studies aimed at detecting STEC serogroups have been done in cattle (Jenkins *et al*., 2008; Joris *et al*., 2011) and few in pigs, probably because cattle are the major reservoirs of STEC. Although *E. coli* O157 and non O157 are predominantly found in cattle, this study was motivated by the need to determine their occurrences in pigs being an important economic resource in South Africa and the consequent potential health risk that they could pose as reservoirs of the pathogens.

During isolation, both sorbitol and non-sorbitol fermenting colonies were picked, and the Sorbitol MacConkey agar was not supplemented with cefixime to increase the chances of detecting the non O157 *E. coli* whose growth might be inhibited by cefixime since there is no standard method for isolating non O157 STEC (Farrokh *et al*., 2013). It was observed that the culture medium was not absolutely selective for STEC isolates, as colonies which grew on it turned out to be negative for the target serogroup.

With respect to distribution of the serogroups, *E. coli* O157:H7 was more frequently detected than the O26 and O145 in decreasing order. This is unlike the results from Hofer *et al*. (2012) who reported more non *E. coli* O157 (O111, O26, O103 and O145) than O157 in faeces of cattle. The source of outbreaks or sporadic cases of STEC O26 is rarely found in literature but they have been isolated from animals including pigs. This finding also supports the fact that route of transmission of this organism can be linked to animals and their faeces (Chase-Topping *et al*., 2012).

It has also been shown that STEC O26 is the commonest non O157 STEC isolated from animals (Khandaghi *et al*., 2011). Same has been reported in human infections in Switzerland where 28.9% of O26 was isolated followed by 10.3% of O145 (Hofer *et al*., 2012). Reports on prevalence of O26 in the faeces of pigs are very rare. The prevalence of STEC O26

obtained from this study is 6.7% which is similar to that obtained from bovine faeces in East-Azarbaijan Iran where they reported a prevalence of 8.01%.

Even though the overall prevalence of STEC (O26 and untypable serogroup) observed in this study was about 13.1%, it still calls for concern since it could be underestimated considering that there is no standard method for isolating non O157 STEC (Jenkins *et al*., 2008). *E. coli* O145 was the least detected in this study. However in another study in Nigeria aimed at detecting the serogroups in faeces of finished pigs, it was not detected at all in the faeces of finished pigs (Ojo *et al*., 2010). This probably implies that serogroup O145 is not commonly found in pigs. Also, in some studies in involving cattle, this serogroup was less prevalent (Jenkins *et al*., 2003; Joris *et al*., 2011) . From this study, isolates belonging to this serogroup didn't possess any virulence gene. Also, this study showed that isolates belonging to this serogroup did not possess any virulence gene. This is in collaboration with the report by Joris *et al*. (2011) in slaughter cattle where O145 isolates were negative for Shiga toxin genes. Their study also showed that O26 was more prevalent than O145.

Both the *rfb*E and *flic*H7 genes in order to detect *E. coli* O157:H7. No sample was positive for *rfb*E gene but some were positive for the flicH7 gene only. According to Jeshveen *et al*. (2012), primer sequences which are unique for the target species determines the specificity in detecting a microorganism. This probably explains the uniqueness of the *fliC* H7 gene which is specific for *E. coli* O157:H7. From this study, the *fli*C H7gene was detected in 24% (n=43) of the confirmed *E. coli* isolates. This finding is similar to that of Kumar *et al* (2013) who reported that the primer pair for fliCH7 was highly specific for the detection of *E. coli* O157:H7 in milk samples. They used *uid*R primers based on the β-glucoronidase gene found in all *E. coli* strains in combination with the *fliC*H7. They also evaluated the specificity of these primer sets by testing them on non-*E. coli* strains such as *Salmonella* , *Shigella*

dysenteriea, etc, and found out that none of the organisms produced the right amplification. A similar study by Sarimehmetoglu *et al.* (2009) referred to the flagella H7 as one of the virulence factors for this pathogen where they used the primers to evaluate the presence of O157:H7 in fresh ground beef samples. It was on these bases that we found the use of the fliC: H7 primers reliable for targeting the *fliC* gene in O157:H7. The overall prevalence of *E. coli* O157:H7 in this study was 24.4% (Table 3.1). Although Farm A has lesser number of animals, the prevalence was higher than Farm B. This could be attributed to better hygienic practices observed in Farm B. Also, farm A is in close proximity to a cattle farm, where these cattle are left to graze around the area, hence a possible risk of cross contamination especially from the cattle which are known to be the major reservoirs of this pathogen. The prevalence obtained in this study was lower than what was reported in a study in the North West Province of South Africa by Ateba *et al*. (2008) where the prevalence in swine was 44-50%, and the prevalence in commercial farms higher than what was obtained from the communal farms. The same study reported a higher prevalence of *E. coli* O157 in pigs than cattle. In the Western part of Nigeria, Ojo *et al*. (2010) reported a prevalence of 4.9% which was lower than what was obtained in our study. Also, in another study from our group carried out in the Eastern Cape Province of South Africa, we reported that 54% of raw milk samples from cattle were positive for *E. coli* O157:H7, which was higher than reported in this current study, and further supports that cattle are the major reservoirs of this pathogen (Caine *et al*., 2014). Even though the reference strain possessed all the virulence genes tested in this study, none of the *E. coli* O157:H7 detected possessed any of the Shiga toxins and other virulence genes. This serogroup may be referred to as a non-toxigenic strain with low potential to cause disease in humans. Although, most studies, reported that *E. coli* O157:H7 harbour Shiga toxins, a study in the US, reported a prevalence of 1.2% of O157 that did not possess Shiga toxin genes, in accordance to our finding in this study (Keen *et al*., 2006) as observed in this

study, suggesting that not all *E. coli* O157:H7 are Shiga toxin producers. However, rare cases of human disease have been reported including haemolytic uraemic syndrome caused by non-toxigenic *E. coli* O157:H7 in Germany (Schmidt *et al*., 1999).

Findings from antimicrobial resistance patterns of the isolates showed that despite the use of danofloxacin a flouroquinolone, in the farms, most strains were highly sensitive to the fluoroquinolones with sensitivity of 63-100% and 83-100% for ciprofloxacin and norfloxacin respectively. This might be attributed to infrequent use in the farms. For the aminoglycosides the isolates were more resistant to earlier generation streptomycin than the newer ones like amikacin. Even though relatively low level of resistance was observed to the 3 classes of cephalosporins, a relatively (35%) resistance was observed against cefotaxime by *E. coli* O157:H7 strain. This could be attributed to the production of extended spectrum β-lactamases and/or plasmid-mediated *amp*C beta-lactamases, which can hydrolyse β -lactam antibiotics such as ampicillin and extended spectrum β - lactams. Similar high level of resistance to tetracycline, oxytetracycline was observed among all the isolates. This is in accordance with the study by Simon & Carlos (2009) who reported a high level of resistance of *E. coli* isolated from pigs to tetracycline in the North West Province of South Africa. They attributed this to both commercial and communal farmers relying on tetracycline due to its broad spectrum activity, cost effectiveness and availability. A high resistance rate was also reported in US, where 81% of *E. coli* strains recovered from swine exhibited resistance to tetracycline (Schroeder *et al*., 2002). Bryan *et al*., (2004) reported a prevalence of 78% resistance to tetracycline form *E. coli* from pigs., while another study reported a prevalence of 83% (Kozak *et al*., 2009).

In this study, a small proportion of the isolates showed resistance to chloramphenicol (21%). This could be attributed to lack of its use in farm animals as also reported by Tadesse *et al*.

(2012). Resistance to streptomycin (40-100%) was higher than other members of the aminoglycosides tested i.e. gentamycin and amikacin. This supports similar reports elsewhere (Schroeder *et al*., 2002; Tadesse *et al*., 2012).

A relatively moderate resistance (31-51%) to sulphamethoxazole /trimethoprim was observed in this study, which is quite similar to that (31%) reported by (Schroeder *et al*., 2002) among swine in the US. Susceptibility of some isolates to this drug could be as a result of synergistic activity arising from the combination sulphonamide, sulphamethoxazole and trimethoprim. Almost all the isolates displayed resistance to ampicillin in corroboration of the report by. This is similar to the report by Rajkhowa & Sarma, (2014). The high resistance might be attributed to the extensive use or misuse of penicillins used for treatment of infections in animals as well as supplements in feeds which is a common practice in the farms used in this study.

A large proportion of *E. coli* isolates from this study, showed multiple antimicrobial resistances, with the most dominant MAR pattern being T-AMP-S-OT-E which occurred more in O145 (85%) than O157:H7 (53%) and O26(14%). This is higher than previously reported finding by Simon & Carlos (2009) in pig farms in North West Province , South Africa, where E-OT-SMX was the most prevalent phenotype. The similar MAR phenotypes among the isolates in this study, suggests that they have a common origin and similar history of antibiotic exposure (Hayes *et al*., 2004; Simon & Carlos, 2009) . Hence, high MAR phenotypes could pose a health risk for humans. The MAR index observed in this study (0.2-0.7) is also similar to that reported by Simon & Carlos, (2009) who obtained MAR indices of 0.4369 and 0.3864 from two locations, suggesting an extensive use of antibiotics in the farms; hence a high risk source of multidrug resistant organisms to humans.

From this study, a high occurrence (50-100 %.) of *ampC* gene was observed among the isolates. This has a public health importance as these organisms may serve as a reservoir for

β - lactamase producing bacteria in humans. This finding is similar to the prevalence among swine bacterial isolates (*Salmonella* spp, *Escherichia coli* and *Pasteurella multocida)* where a prevalence of 45% was reported (Chander *et al*., 2011).

A rapid spread of resistance to tetracycline among bacteria is due to the localization of *tet* gene on plasmids, transposons and integrons and can be transferred from one bacteria to another (Bryan *et al*., 2004; Ibekwe *et al*., 2011). The Prevalence of 50-100% of *tet*A gene was observed among *E. coli* isolates. This is higher than the findings reported by Bryan *et al*. (2004), where 35% of isolates from pigs harboured the *tet*A genes and 28% reported among swine (Kozak *et al*., 2009). The tetracycline resistant isolates that didn't harbour the *tet*A genes, their resistance could be as a result of other *tet* genes not assessed in this study. The percentage of isolates resistant to streptomycin that were found to harbour the *strA* gene was between 21-29% and similar to previous finding by Kozak *et al*. (2009) who reported a prevalence of 28%.

3.5 Conclusion

Isolates from swine showed the presence of *E. coli* serogroups O26, O145 and O157:H7, with only serogroup O26 harbouring the *stx2* gene. Both the phenotypic and genotypic resistance profile of all the isolates depicts high level of multidrug antibiotic resistance. In the same vein, the MARI in all the serogroups exceeded the threshold limit, indicating excessive use of antibiotics in the swine farms. If the products from this study must be safe and healthy, proper hygiene, and sanitation must be put into consideration. Furthermore, the indiscriminate use of antibiotics as well as their use as growth promoters should be

discouraged. More studies on the prevalence of STEC in swine is advocated to add to the body of knowledge as their remains a lot to be done in this area world wide

3.6 References

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CHAPTER 4

Prevalence and antimicrobial susceptibility of *Salmonella* species isolated from commercially raised swine in the Nkonkobe Municipality, Eastern Cape Province South Africa.

Abstract

We evaluated the prevalence and antibiogram of *Salmonella* species in pig herds from two commercial farms in Nkonkobe Municipality in the Eastern Cape Province of South Africa. Two hundred and fifty eight presumptive *Salmonella* isolates were recovered from the faecal samples of 500 pigs. Specific primers targeting *Salmonella* serogroups A, B, C1, C2, and D were used to ascertain the prevalence of different serogroups. Only serogroup A (n=48) was detected, none was positive for other serogroups. These isolates were examined for antimicrobial susceptibility by disc diffusion method against a panel of 18 antibiotics. All the 48 isolates were resistant to tetracycline and oxytetracycline while 75% were resistant to ampicillin, sulphamethoxazole/trimethoprim and streptomycin. Majority of the isolates exhibited multidrug resistance with the predominant multiple antibiotic resistances (MAR) phenotype being against eleven antibiotics. A high multiple antibiotic resistance (MAR) index in a range of 0.3- 0.6 was observed. The incidence of genes encoding resistance against tetracycline (*tetA*), streptomycin (*str*A), and ampicillin (*amp*C) were 54%, 44% and 61% respectively. These findings imply that pigs are potential vehicles of multidrug resistant *Salmonella* that could be transmitted to humans through the food chain, hence, posing significant public health consequences.

Keywords: *Salmonella*, pigs, antimicrobial resistance, food chain, resistance genes

4.1 Introduction

Salmonella species are Gram negative flagellated facultatively anaerobic bacilli and intestinal pathogens that can penetrate into the intestinal barrier (Suez *et al*., 2013; Wang *et al*., 2013). Infections caused by *Salmonella* are amongst the most common food borne bacterial infections worldwide, with about 1.4 million cases and about 600 deaths occurring annually in the United States (Lan *et al*., 2009). Consumption of contaminated foods including pork, predispose people to infection due to *Salmonella* (Abatcha *et al*., 2014). The Kauffman-White serotyping Scheme is used in most laboratories in characterizing *Salmonella* isolates. A serotype is determined on the basis of somatic (O) and flagella (H) antigens present in the cell walls of *Salmonella* organisms. The O factors determine the grouping while the H factors define the serotype identity of a *Salmonella* strain (Herrera-León *et al*., 2007). Serotyping is the most commonly used method for phenotypic characterization for identification of *Salmonella* serogroups, namely A, B, C1, C2, D and E. For example, *Salmonella paratyphi* A, B, C, and *Salmonella typhi* belong to the serogroups A, B, C1 and D respectively. It is required to determine the relationship between disease and source of *Salmonella.* The traditional method has been shown to be time consuming, expensive, laborious, and lacks standardised methods of determining antisera to be used for its detection. It is therefore imperative to integrate molecular methods to overcome these short comings (Liu *et al*., 2011).

The emergence of antimicrobial resistance as well as multidrug resistance patterns of *Salmonella* and other enteric pathogens have raised concerns all over the world (Wang et al., 2013). The acquisition of resistance factors by *Salmonella* has led to difficulty in managing infection hence reducing the treatment options available (Glenn *et al*., 2011). Also, the use of antimicrobials for treatment, prevention of infections as well as growth promotion in farm

animals, can potentially lead to widespread transmission of antimicrobial resistant bacteria through the food chain (Abatcha *et al.*, 2014). In this paper, we report on the prevalence and antibiogram of *Salmonella* in faeces of healthy pigs in Nkonkobe Municipality as part of our larger study on the reservoirs of antibiotic resistance determinants in the environment.

4.2 Materials and methods

4.2.1 Sampling site and sample collection

Swine faecal samples were collected from two commercial farms located within the Nkonkobe Municipality, which are situated along the Southern slopes of the Winterberg Mountain range and escapement, within the greater Amathole District Municipality in the Province of the Eastern Cape Province, South Africa. The Municipal area being the second largest covers approximately 3725 Km^2 and constitutes 16% of the surface area of the Amatole District Municipality. It is a rural Municipality largely driven by the agricultural sector. (Nkonkobe Municipality IDP, 2013/2014; 2014/15).

Faecal samples were collected from five hundred pigs in the two farms located within the Nkonkobe Municipality of the Eastern Cape Province. This was done over a period of four months (April – July, 2014) using sterile swab sticks. The samples were transported immediately in ice to the Applied Environmental Microbiology Research Group (AEMREG) laboratory of the University of Fort Hare, South Africa for analyses.

4.2.2 Isolation of *Salmonella* species

Salmonella species were isolated following the method described by (Karou *et al*., 2013), with slight modification. Briefly, samples were pre- enriched by inoculating unto tryptic soy broth (TSB) and incubated at 37^oC for 18 -24hours. Enrichment was done by adding 1 ml of pre-enrichment to 9 ml of Muller-Kauffman tetrathionate broth, and incubated at 37^oC for 48

hours. Tubes showing growth were selectively plated onto xylose lysine deoxycholate agar (XLD) (Merck, South Africa) and incubated aerobically at 37^{o}C for 22-24 hours. Red colonies with unique black centers characteristic of the desired isolate were presumed positive for *Salmonella.* They were purified on Nutrient agar (Merck, South Africa) picked, grown in TSB and made into 30% glycerol stocks and stored at -80^{o}C for further use.

4.2.3 DNA Extraction

Prior to molecular identification of the isolates, DNA was extracted from the presumptive isolates. The isolates were first resuscitated by inoculating unto tryptic soy broth and incubated at 37^{o}C for 24hours.They were further streaked unto Nutrient agar (Merck, SA) and incubated at 37^{o}C for 24hours. The extraction was done using boiling method as described by (Maugeri *et al*., 2004). Briefly, about 3-5 colonies were picked using a sterile wire loop into sterile DNAase/RNAase free Eppendorf tubes (Biologix, USA) containing 200µl sterile nuclease free water (Thermoscientific, USA). The suspension was vortexed using the Vortex mixer (Digisystem Laboratory, Taiwan) and the cells were lysed by heating in a heating block (brand) at 100°C for 15 minutes. The cell debris was removed by centrifugation using a centrifuge (Lasec, UK) at 13,500rpm for 10minutes. The supernatant containing the genomic DNA template was carefully transferred into another Eppendorf tube and stored at -20^{o}C for further assays.

4.2.4 Identification of *Salmonella* Serogroups by PCR

The presumptive isolates were screened for the different *Salmonella* serogroups namely A, B, C1, C2 and D using polymerase chain reaction technique. Primer sequences and the expected amplicon sizes are listed in Table 4.1. The primers were synthesized by Inqaba Biotechnical industries (Pty) Ltd, South Africa. Each reaction mixture consisted of 12.5µl of 2x Dream Taq Master mix (Thermo scientific, USA), 1µl each of the forward and reverse primers, 5.5µl

of nuclease free water (Thermo scientific, USA) and DNA template in a final volume of 25µl. PCR amplification was carried in out in MyCycler^{TM} Thermal Cycler PCR system (BioRad, USA) with an initial denaturation of 94 °C for 3 minutes, 35 cycles of 94 °C for 50 seconds, 60 °C for 50 seconds and 72 °C for 50 seconds, and then with a final extension at 72 °C for 10 min (Liu *et al*., 2011). The same conditions were used in identifying all the serogroups. Negative controls were used in all reactions that comprised of the reaction mixture except DNA template which was replaced by nuclease free water since positive controls were not available as at the period of study. Five microlitre aliquots of the amplicons were resolved in 1.5% agarose gel (Seperations, South Africa) at 100V for 60min. The gel was stained with ethidium bromide and visualized under the UV transilluminator (Alliance 4.7). A 100bp DNA ladder (Promega, USA) was used on each gel as molecular size standard.

Table 4.1 Primers used for the specific detection of *Salmonella* serogroups

Primer set name	Primer name	Sequences (5′–3′)	Product size (bp)
SA	SA-L	F-AACAGATCCTGCACCATATC	350
	SA-R	R-CAGTTTCATGATGGCAGAG	
SB	SB-L	F-CGATGAGGGTTTCTAATCTC	177
	SB-R	R-TCTTGCTTCAGTATCCCTTG	
SC1	SC1-L	F-CAGTCACAACCTGGAAGA	623
	SC1-R	R-ATACAAGCCGCTGAGTGA	
SC2	SC2-L	F-CAGTAGAGACGACGGAGTTC	540
	SC2-R	R-TACATGCTTGGCTGAGACTA	
SD	SD -L	F-GCCAATAAACTCCACAACAT	466
	SD -R	R-GGATCATGCGTTAAATGTCT	

(Liu *et al*., 2011)

4.2.5 Antimicrobial susceptibility testing of Salmonella serogroup A isolates

Antimicrobial susceptibility testing (phenotypes) was performed on all confirmed *Salmonella* serogroup A isolates using disk diffusion assay following Clinical Laboratory Standard Institute guideline (CLSI, 2012) on Mueller – Hinton agar (Conda – pronadisa, USA). Briefly, Positive isolates were grown overnight in nutrient agar (Merck, South Africa) at 37^{o}C. Cells were harvested from the surface of the growth medium and suspended in 0.85% sterile normal saline and the cell density adjusted to a 0.5 McFarland turbidity standard. A sterile cotton swab was dipped into the standardized bacterial test suspension and used to evenly spread the entire surface of the Muller Hinton agar plate. After the agar surface was dried for 5min, the appropriate antibiotic disks (Mast Diagnostics, UK) were placed on it using the using the disc dispenser (Mast Diagnostics, UK). The plates were immediately placed in an incubator at 37^{o}C. After 24hr incubation, the zone sizes (diameter) were measured to the nearest millimetres and interpreted according to the CLSI standard (CLSI, 2012). The panel of antibiotics include, tetracycline (T-30 µg), oxytetracycline (OT - 30µg), ampicillin G (AMP- 10 µg), Sulphamethoxazole/trimethoprim (TS- 25), streptomycin, gentamycin (GM-10µg), amikacin (AK-30µg), ceftazidime (CAZ-30µg), cephalothin (KF-30µg), cefotaxime (CTX-30µg), chloramphenicol (C-10µg), norfloxacin (NOR-10µg), ciprofloxacin (CIP-5µg), nalidixic acid, cefuroxime, imipenem and polymycin B belonging to ten different classes of antimicrobials.

The choice of these drugs was based on the drugs used to commonly treat infections caused by *Salmonella* and those used in the in farms where samples were collected as shown in Table 4.3. Multiple antibiotic resistance (MAR) phenotypes were generated for isolates that exhibited resistance to 3 or more antibiotics (Ateba *et al*., 2008).

Table 4.2: List of antibiotics commonly used in the Farms studied

FARM A	FARM B
Procaine benzyl ampicillin (ampicillins)	Procaine ampicillin (ampicillins)
Sulphadizine + trimethoprim (Folate pathway inhibitor)	Tylosin (Macrolide)
	Amoxicillin (beta lactams; ampicillin)
	Sulphadiazine trimethoprim (Folate pathway inhibitor)
Ampicillin (β-lactams;ampicillins)	
	Danofloxacin (fluoroquinolones)
Oxytetracycline (tetracyclines)	

4.2.6 Multiple antibiotic resistances (MAR) Indexing

The Multiple Antibiotic Resistance (MAR) index of the serogroup A isolateswas calculated and interpreted according to (Krumperman, 1983) using the formula: a/b, where 'a' represents the number of antibiotics to which a particular isolate was resistant and 'b' the total number of antibiotics tested. Isolates classified as intermediate on the basis of inhibition zone were considered as sensitive for the MAR index. A MAR index of ≥ 0.2 indicate high risk environment where antibiotics are often used (Osundiya *et al*., 2013).

4.2.7 Detection of antibiotic resistance genes

The *bla ampC, tetA* and *StrA* genes encoding for resistance to ampicillin, tetracyclines, and streptomycin respectively, were investigated using PCR. The primers used were synthesized by Inqaba Biotechnical Industries (Pty) Ltd, South Africa. The Primer sequences, PCR conditions and amplicon size of the target genes are shown in Table 4.3

4.2.8 Data Analysis

Data were captured into Microsoft excel sheet 2010 and simple descriptive statistics of Statistical Package for Social Sciences (SPSS) version 22 was used in analysing the data.

Table 4.3 Primer sequences, PCR conditions and amplicon size of antibiotic resistance genes targeted among *Salmonella* group A isolates

Antimicrobial (gene)	**Primer sequence(5' – 3')**	**PCR condition**	**Product size**	**Reference**
Ampicillin*(ampC)*	F-AATGGGTTTTC- CGGTCTG R-GGGCAGCAAATGTG-GAGCAA-	5 min initial denature at 94°C followed by 35 cycles of 94°C for 1min, 55°C for 1min and 72°C for 1•5 min final incubation at 72°C for 5 min	191bp	(Forward *et al*., 2001)
Tetracycline (*tetA*)	F – GGCCTCAATTTCCTGACG R-AAGCAGGATGTAGCCTGTGC	5 min initial denature at 94°C followed by 35 cycles of 94°C for 1min, 55°C for 1min and 72°C for 1•5 min final incubation at 72°C for 5 min	372bp	(Guillaume *et al*., 2000)
Streptomycin (*strA*)	F-CCAATCGCAGATAGAAGGC R-CTTGGTGATAACGGCAATTC	5 min initial denature at 94°C followed by 35 cycles of 94°C for 1min, 55°C for 1min and 72°C for 1•5 min final incubation at 72°C for 5 min	548bp	(Thong, 2010)

4.3 Results

4.3.1 Isolation and molecular serotyping

Two hundred and fifty eight (258) presumptive isolates were recovered from 500 pig faecal samples. They were screened for the five serogroups and only serogroup A was detected in 48 samples 18.6% (n=48) all from farm A only. None of the other serogroups were detected (Table 4.4). Figure 4.1 shows the gel electrophoresis of the amplified products.

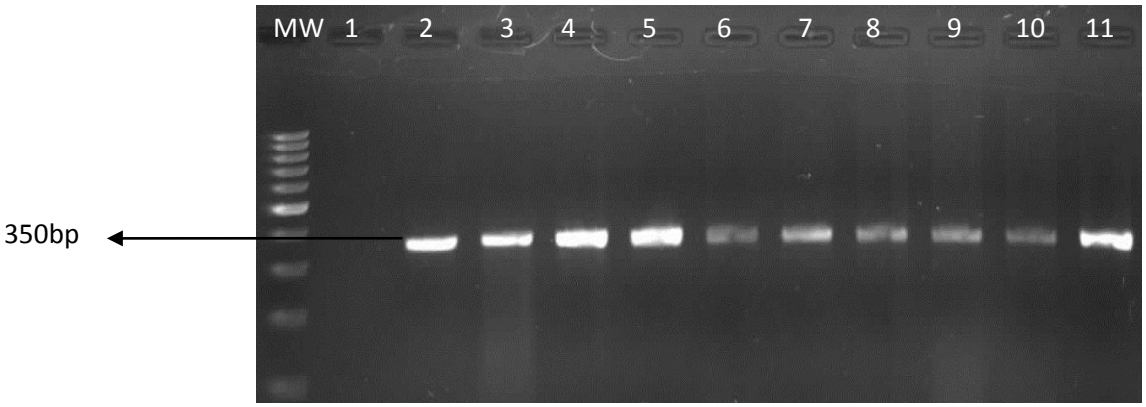

Fig 4.1 Gel electrophoresis of the amplicons of some of the positive *Salmonella* serogroup A isolates (MW: 100bp DNA ladder; lane 1: negative control; lanes 2 – 11 positive isolates).

Table 4.4: Results of molecular delineation of the presumptive *Salmonella* isolates into their Serogroups

Serogroups	**Number of isolates identified** (%)
A	48 (18.6%)
B	0 (0%)
C1	0 (0%)
C2	0 (0%)
D	0 (0%)

4.3.2. Antimicrobial susceptibility testing of the *Salmonella* group A isolates

The distribution of antimicrobial resistance in *Salmonella* group A isolates obtained in this study is summarized in Table 4.5. All the isolates were sensitive to imipenem, whereas 91.7%, 83.3%, and 66.7% were sensitive to norfloxacin, gentamycin, and ciprofloxacin respectively.

All the isolates were resistant to erythromycin, tetracycline and oxytetracycline. A large proportion was also resistant to ampicillin (75%), sulphamethoxazole/trimethoprim (75%), nalidixic acid (75%), and streptomycin (75%). Furthermore, close to half of the isolates (42%) were resistant to cefotaxime, a third generation cephalosporin while 25% of the isolates showed resistance to chloramphenicol.

Table 4.5 Antimicrobial susceptibility pattern of the *Salmonella* serogroup A isolates

Antimicrobial agent	**Code**	**Potency(µg)**	**R**	**I**	**S**
			n (%)	**n (%)**	**n (%)**
Tetracycline	T	30	48(100)	0(0)	0(0)
Oxytetracycline	OT	30	36(75)	0(0)	0(0)
Ampicillin	AMP	10	36(75)	12(25)	0(0)
Cephalothrin	KF	30	4(8)	4(8)	40(83)
Cefuroxime	CXM	30	8(17)	8(17)	32(67)
Ceftazidime	CTX	30	12(25)	24(50)	12(25)
Cefotaxime	CAZ	30	20(42)	16(33)	12(25)
Erythromycin	E	15	48(100)	0(0)	0(0)
Sulphamethoxazle/trimethoprim	TS	25	36(75)	0(0)	12(25)
Chloramphenicol	C	10	12(25)	32(67)	4(8)
Nalidixic acid	NA	30	36(75)	12(25)	0(0)
Ciprofloxacin	CIP	5	0(0)	16(33)	32(67)
Norfloxacin	NOR	10	4(8)	0(0)	44(92)
Gentamycin	GM	10	4(8)	4(8)	40(83)
Amikacin	AK	30	8(17)	20(42)	20(42)
Streptomycin	S	10	36(75)	0(0)	12(25)
Imipenem	IMI	10	0(0)	0(0)	48(100)
Polymycin B	PB	300	44(92)	4(8)	0(0)

4.3.3 MAR phenotypes and MAR indexing

The resistance pattern of each *Salmonella* serogroup A isolate is as shown in Table 4.6. All the isolates were resistant to at least 3 antibiotics. The highest frequency of MAR phenotype was against 11 antibiotics and was demonstrated by 16.7% of the isolates (n=8), whereas 4.2% (n=2) of the isolates showed the lowest frequency against 6 antibiotics. The MAR indices ranged between 0.3 – 0.6.

4.3.4 Prevalence of resistance genes in the *Salmonella* serogroup A isolates

Approximately 61% , 44% and 54% of the resistant serogroup A isolates were positive for *tet*A, *str*A and *amp*C resistance genes respectively. The gel electrophoresis pictures showing the PCR amplification of the genes are shown in Figures 4.3, 4.4 and 4.5 respectively.

Table 4.6. Antimicrobial resistance pattern of the *Salmonella* serogroup A isolates

Resistance pattern of *Salmonella* (n = 48)	No. of isolates	MARI	Resistance pattern of *Salmonella* (n = 48)	No. of isolates	MARI
T-CAZ-S-E-OT-AMP-NA-AK-PB	9	0.5	T-CAZ-S-GM-E-OT-AMP-T/S-NA-CTX-PB	11	0.6
T-S-E-OT-T/S-NA-PB	7	0.4	T-S-E-OT-T/S-NA-PB	7	0.4
T-S-E-OT-AMP-T/S-NA-PB	7	0.4	T-CAZ-S-E-OT-AMP-NA-AK-PB	7	0.4
T-S-E-OT-AMP-NA-AK-CTX-NOR-PB	10	0.6	T-S-E-OT-AMP-T/S-NA-PB	7	0.4
T-CAZ-S-E-OT-AMP-T/S-NA-CTX-CXM-PB	11	0.6	T-S-E-OT-T/S-PB	6	0.3
T-CAZ-S-E-OT-AMP-T/S-NA-C-CTX-CXM	11	0.6	T-S-E-OT-AMP-NA-AK-CTX-NOR-PB	10	0.6
T-S-E-OT-KF-AMP-T/S-NA-PB	9	0.5	T-CAZ-S-GM-E-OT-AMP-T/S-NA-CTX-PB	11	0.6
T-E-OT-AMP-C-CTX-PB	7	0.4	T-CAZ-S-E-OT-AMP-T/S-NA-C-CTX-CXM	11	0.6
T-S-E-OT-T/S-PB	6	0.3	T-S-E-OT-AMP-T/S-NA-PB	7	0.4
T-CAZ-S-E-OT-AMP-T/S-NA-AK-PB	10	0.6	T-CAZ-S-E-OT-AMP-T/S-NA-AK-PB	10	0.6
T-CAZ-S-GM-E-OT-AMP-T/S-NA-CTX-PB	11	0.6	T-S-C-E-OT-T/S-NA-PB	8	0.4
T-S-C-E-OT-T/S-NA-PB	8	0.4	T-S-E-OT-AMP-NA-AK-CTX-NOR-PB	10	0.6
T-S-E-OT-AMP-NA-AK-CTX-NOR-PB	11	0.6	T-CAZ-S-E-OT-AMP-T/S-NA-AK-PB	10	0.6
T-CAZ-S-E-OT-AMP-NA-AK-PB	9	0.5	T-CAZ-S-E-OT-AMP-T/S-NA-CTX-CXM-PB	11	0.6
T-S-E-OT-AMP-NA-AK-CTX-NOR-PB	10	0.6	T-S-E-OT-T/S-PB	6	0.3
T-S-E-OT-KF-AMP-T/S-NA-PB	9	0.5	T-CAZ-S-E-OT-AMP-T/S-NA-C-CTX-CXM	11	0.6
T-S-E-OT-KF-AMP-T/S-NA-PB	7	0.4	T-CAZ-S-GM-E-OT-AMP-T/S-NA-CTX-PB	11	0.6
T-S-E-OT-T/S-NA-PB	7	0.4	T-S-C-E-OT-T/S-NA-PB	8	0.4
T-E-OT-AMP-C-CTX-PB	7	0.4	T-S-E-OT-AMP-T/S-NA-PB	7	0.4
T-S-E-OT-KF-AMP-T/S-NA-PB	9	0.5	T-E-OT-AMP-C-CTX-PB	7	0.4
T-CAZ-S-E-OT-AMP-T/S-NA-AK-PB	10	0.6	T-CAZ-S-E-OT-AMP-T/S-NA-C-CTX-CXM	11	0.6
T-CAZ-S-E-OT-AMP-NA-AK-PB	9	0.5	T-S-C-E-OT-T/S-NA-PB	8	0.4
T-S-E-OT-T/S-PB	6	0.3	T-S-E-OT-T/S-NA-PB	7	0.4
T-CAZ-S-E-OT-AMP-T/S-NA-AK-PB	9	0.5	T-CAZ-S-E-OT-AMP-T/S-NA-CTX-CXM-PB	11	0.6

Table 4.7 Prevalence of antimicrobial resistance genes in the resistant *Salmonella* serogroup A isolates

Antimicrobial agent (resistant gene)	No. of resistant isolates	Prevalence of resistance gene (%)
Tetracycline (*tet*A)	48	26 (54.17)
Streptomycin (*str*A)	36	16 (44.4)
Ampicillin (*amp*C)	36	22 (61.1)

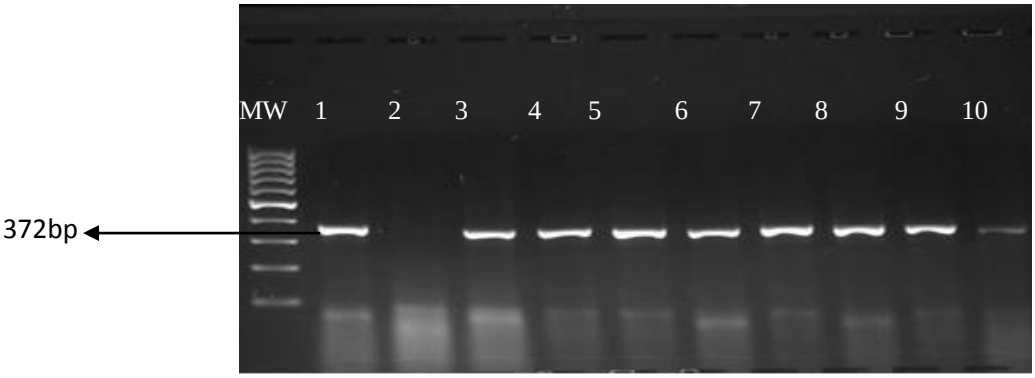

Fig 4.2 Electrophoresis of *tet*A gene amplification among *Salmonella* serogroup A isolates

(Lane MW :100 bp ladder; Lane 1: positive isolate; Lanes 2: negative control; Lanes 3-10: positive isolates).

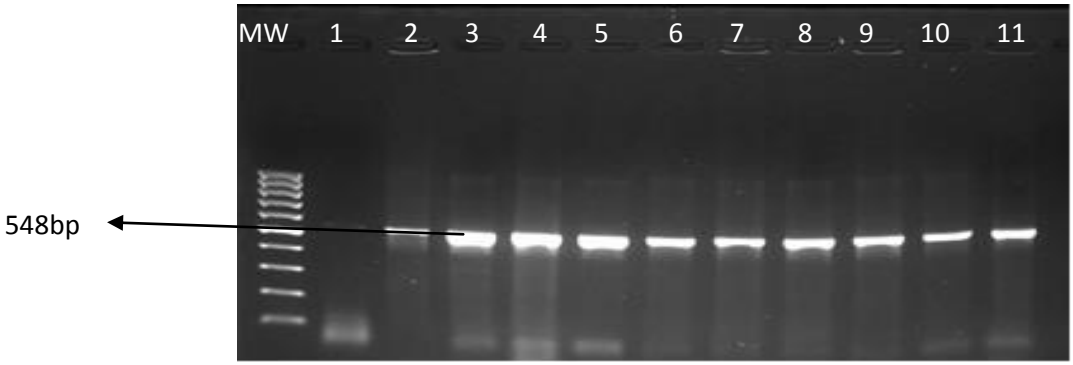

Fig 4.3 Electrophoresis of *str*A gene amplification among *Salmonella* serogroup A isolates (Lane MW: 100 bp ladder; Lanes 1-11: positive isolates).

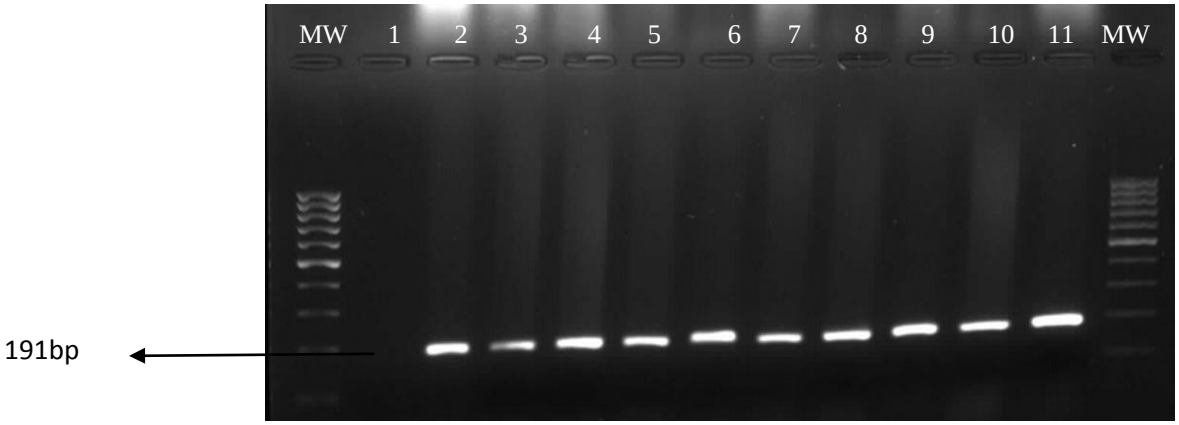

Fig 4.4 Electrophoresis of *amp*C amplification among *Salmonella* serogroup A isolates

(Lane MW: 100 bp ladder ; Lane 1: negative control; lanes 2-11: positive isolates).

4.4 Discussion

Serotyping of *Salmonella* is important as it provides information for the determination and tracing of *Salmonella* during outbreaks as well as for strain identification. Molecular methods have been employed to reduce the limitations associated with the traditional methods and several studies have been used to validate the workability of this method (Herrera-León *et al*., 2007; Yoshida *et al*., 2007; Cardona-Castro *et al*., 2009). In this study, molecular serogrouping of *Salmonella* isolates obtained from pig faeacal materials was carried out using specific primers targeting serogroups A, B, C1, C2 and D. The only serogroup identified in this study was the serogroup A in about 19% (48/258) of the presumptive isolates. *Salmonella paratyhphi A*, and *Salmonella typhimurium* have been shown to be the commonest serovars belonging to this serogroup (Nori & Thong, 2010; Liu *et al*., 2011). Generally, *S. typhimurium* has been the predorminantly isolated serovar from pigs (de Jong *et al*., 2014). Also, in South Africa, an 11 year (1996-2006) retrospective study of the incidence of *Salmonella* in pigs showed that majority was due to *S. typhymurium* (Kidanemariam *et al*., 2010). The results in this study, showed the prevalence of *Salmonella* spp to be approximately 19%. It is therefore evident that pigs can be reservoirs of *Salmonella spp.* A similar prevalence of 17.2% was reported by Molla *et al*. (2010) from certain swine production units in the US. A relatively similar finding was also observed by Kishima *et al.* (2008) who reported a 15.1% (26/172) prevalence of *S.typhimurium* from the faeces of pigs in a study conducted to determine the nationwide prevalence and distribution of *Salmonella* in Japan between 2003 and 2005. However, a lower prevalence was reported by Kikuvi *et al.* (2010) from a slaughter house in Kenya, where the prevalence of *Salmonella* in faecal samples of pigs was 8.6% (5/58). From the same study however, a higher prevalence of 19% (11/58) was observed from carcass samples, suggesting that environmental contamination during slaughtering can increase the prevalence of *Salmonella* in pork. In canada, a higher

prevalence of 31.5% (113/359) of *Salmonella* was reported by Farzan *et al*. (2010), and majority of them were *S.typhimurium*.

Results of antimicrobial susceptibility assay showed that no isolate was susceptible to all the antimicrobials tested. The highest frequency of resistance observed was against tetracyclines (100%), followed by ampicillin (75%), sulphamethoxazole/trimethoprim (75%) and streptomycin (75%). Similar results have been reported in other studies from Spain (Agustín *et al*., 2005; García-Feliz *et al*., 2008) and other parts of the world; New Zealand (Gebreyes *et al*., 2000). Resistance to these antimicrobials is generally attributed to the frequent use of these compounds in the treatment of infections and as growth promoters, since they are affordable and readily available (Kikuvi *et al*., 2010). Conversely, Wolf *et al*. (1999) reported in a study in Netherlands a high susceptibility (93.9%) of *Salmonella* isolates to ampicllin. This could be attributed to infrequent use of this drug or its structural analog in the farms. Our study showed a relatively high resistance rate of 42% to cefotaxime, used in human medicine, whereas another study reported high rate (63%) against ceftiofur , a drug in the same class as cefotaxime but approved for veterinary use (Chander, Oliveira, & Goyal, 2011). The high sensitivity of *Salmonella* isolates to gentamycin in this study is consistent with the findings of some authors such as García-Feliz *et al*. (2008) in Spain, and Sisak *et al*., (2006) in Czech republic. This could be attributed to its infrequent use in the farms visited. Although, danofloxacin which is a flouroquinolone was used in the farm studied, a low resistance rate to ciprofloxacin, the drug of choice in the treatment of salmonellosis in humans was observed. This is also in accordance with several studies in developed and developing countrieses (García-Feliz *et al*., 2008; DANMAP, 2012; de Jong *et al*., 2014). The resistance of isolates to chloramphenicol was realtively low, probably because it is not used in the farms, moreover, its use has not been allowed in veterinary medicine in South Africa

over two decades now (Henton *et al*., 2011). All the serogroup A isolates were resistant to more than 4 antibiotics. The most predominant resistance phenotype was observed against eleven antibiotics, similar to the report by Agustín *et al*. (2005), although the highest resistance phenotype recorded by them was against 13 antibiotics. Furthermore, 4 of the resistant isolates demonstrated the penta resistance pattern AMP- C- S- T/S- T, typical of the, *Salmonella typhimurium* DT104 which has gene encoded resistance pattern ACSSuT (ampicillin, chloramphenicol, streptomycin, sulphonamides, tetracyclines) (Threlfall, 2002; Gebreyes & Thakur, 2005). This serovar has been isolated from pigs and pork products in many countries (Kishima *et al*., 2008) and has become a potential threat for animal husbandry and human medicine (Esaki *et al*., 2004). In addition MAR index ranged between 0.3 – 0.6, indicating high level of antibiotic use in the swine farm studied.

From the distribution of the resistance genes in this study, the isolates which showed penotypic resistance seemed to express the corresponding resistance genes. From our study, the prevalence of the *amp*C gene was 61.1% of the resistant isolates. This was lower though than the finding by Chander *et al.* (2011) who obsereved 11 out of 12 resistant *Salmonella* harbouring the *amp*C gene**.** In addition**,** this study showed that *tetA* and *strA* genes were present in 54% and 44% of resistant isolates respectively, in contrast with the findings of Pezzella *et al*. (2004) carried out in Italy on *Salmonella* spp isolated from food animals where they found the prevalence of *tet*A and *Str*A genes to be 84% and 68% respectively. Reason for this high prevalence could be attributed to the fact that the isolates were gotten from a collection of various serovars isolated from animals and foods of animal origin.

4.5 Conclusion

The present study revealed the importance of pigs as source of *Salmonella* species with multiple resistances to commonly used antimicrobials including ampicillin, chloramphenicol, streptomycin and tetracycline. To reduce the incidence of *Salmonella* carriage in pigs, it is important to identify contamination sources at the farm and develop useful practices aimed at limiting the transmission of MDR *Salmonella* serotypes through the food chain. The indiscriminate use of antibiotics by unqualified personnel should be discouraged, while their use as growth promoters should be reviewed.

4.6 References

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CHAPTER 5

General Discussion, recommendations and conclusions

5.1 General discussion

Bacterial food-borne zoonotic diseases are among the most serious health problems affecting public health and development worldwide. *Salmonella* and STEC are among the most common food borne pathogens (Galanis *et al.*, 2006). The most common serotype of STEC associated with human disease and considered to be a major public health concern is *E. coli* O157:H7. However, there is increasing awareness that several non O157 serogroups are emerging and have been shown to cause similar infections as O157 (Smith *et al*., 2014). Six major serogroups belonging to the non O157, and requiring routine monitoring are O26, O103, O111, O121, O45, and O145 (Cernicchiaro *et al.*, 2013).

The most important animal reservoirs in terms of human infections are cattle and pigs, with cattle being the major carriers (Rivera *et al.*, 2012). Their pathogenicity is majorly due to the production of Shiga toxins 1 and 2 encoded by *stx1* and *stx2* genes respectively. Other virulence factors include intimin encoded by *eae* gene, which is responsible for adherence; α-haemolysin encoded by *hly*A and enterohaemolysin encoded by *ehx*A which are responsible for haemolysis, amongst others (Etcheverría & Padola, 2013; Meng *et al*., 2014). From this study, STEC belonging to the non O157 (*E. coli* O26) was isolated from healthy pigs. Other serogroups identified which didn't harbour any of the virulence factors were O145 and O157:H7 serogroups. Studies reporting the prevalence of STEC O26 in pigs are scanty (Leomil *et al*., 2005), many have been done in cattle and humans (Jenkins *et al*., 2008; Khandaghi *et al*., 2011). Furthermore, among the variants of *stx2* gene, the stx2e has been shown to be associated with oedema in pigs (Nataro & Kaper, 1998; Kim *et al*., 2010).

Therefore the isolation of STEC remains a public health concern. Serogroups O145 and O157:H7 were also isolated in this study, but they didn't harbour any of the virulence genes. This is in accordance with some studies (Keen *et al*., 2006; Ateba & Bezuidenhout, 2008; Ojo *et al*., 2010). However, most studies on occurrence of O157; H7 have reported the presence of Shiga toxins and or other virulence genes (Wang *et al*., 2002; Rivera *et al*., 2012). Nevertheless, some rare cases of HUS have associated with strains without Shiga toxins. This could be attributed to either absence or loss of these genes during culturing (Joris *et al.*, 2011)

Salmonella strains isolated from this study belongs to the serogroup A. this further confirms the possibility of using molecular serotyping succesfully. However other studies have identified other serogroups like B, C1, and C2 and D (Lim & Thong, 2009; Helmy *et al*., 2012). This could be attributed to the fairly good hygienic practices in the farms used in this study. The strains can be said to belong to non-typhoidal *Salmonella*. These strains have been implicated in infections in humans and animals alike. *Salmonella typhimurium*, the commonest serovar isolated in pigs have been reported in many studies (Davies *et al*., 2004). None of the isolates harboured the genes which are probably plasmid borne.

E. coli and *Salmonella* strains isolated from this study had similar antimicrobial susceptibility profile. They were all sensitive to imipenem while most of them exhibited high sensitivity to the flouroquinolones; ciprofloxacin and norfloxacin as well as chloramphenicol and varied sensitivities to the cephalosporins. Most of the isolates were resistant to tetracycline, oxytetracycline, ampicillin, and streptomycin. Close to 50% of the isolates were resistant to the third generation cephalosporin, ceftazidime, probably due to the production of extended spectrum beta lactamases. This is a cause for concern as it might limit the treatment option for its structural analog, ceftriaxone which is normally used in humans to treat invasive salmonellosis, especially in children whom the flouroquinolones are contraindicated.

Unlike the *E. coli* isolates, *Salmonella* spp demonstrated high resistance to nalidixic acid. In the same vein, *Salmonella* exhibited a higher resistance rate to sulphamethoxazole and trimethoprim than the *E. coli*. This could be attributed to intrinsic bacteria species differences. Both *Salmonella* and *E. coli* exhibited multiple resistance patterns of over 10 antibiotics, and their MAR indices exceeded limits. Similarity in the resistance pattern of these isolates is possibly because they were all isolated from the same source and would have undergone similar exposure to antimicrobials in the farms. Also, It has been shown that the greatest amount of antibiotics used in farm animals in South Africa are in the commercial piggery and poultry farms (Henton *et al*., 2011). This intensive exposure of bacteria to antimicrobials probably explains the high multiple antibiotic resistance and MAR indices observed among these isolates. It is also possible that prolonged use of a single antibiotic selects for resistance to numerous antibiotics that are structurally unrelated, through transfer of genes on mobile genetic elements such as plasmids and transposons (Marshall & Levy, 2011). For example, oxytetracycline in feed supplement led to multidrug resistance pattern of *E. coli* strains to streptomycin, sulphonamide and tetracycline (Khachatryan *et al*., 2008).

The detection of the resistance genes *tet*A, *str*A, and *amp*C in relatively high amounts shows that the phenotypic resistance observed is due to the presence of the genes that confer resistance to tetracycline, streptomycin, and ampicillin respectively. Resistance was encoded by genes that are widespread in other enterobacteriaceae and that are known to be commonly located on transposons, mobile genetic elements that play an important role in the transmission and dissemination of antimicrobial resistance determinants.

5.2 Conclusions

E. coli O157:H7, O26 and O145 were isolated from pigs. *E. coli* O26 is said to be STEC as it was found to harbour Shiga toxin gene, while other serogroups do not. *Salmonella* strains belonging to serogroup A were the only group detected in this study. Furthermore, both *E. coli* and *Salmonella* had similar resistance pattern with most of them being resistant to tetracyclines, streptomycin, ampicillin and erythromycin. Varied resistances were also observed against sulphamethoxazole/trimethoprim and ceftazidime (third generation cephalosporin). However, they were mostly sensitive to imipenem, ciprofloxacin, norfloxacin and gentamycin. Both organisms exhibited high multidrug resistance pattern and high multiple antibiotic resistance indices. They also harboured *tet*A, *str*A and *amp*C genes that confer resistance to tetracyclines, streptomycin and ampicillin respectively.

This study identified pigs as potential reservoirs of STEC and *Salmonella*, which exhibited high multidrug resistance. This therefore calls for urgent attention

5.3 Recommendations

- The misuse of antibiotics by untrained personnel should be discouraged. Administration of newer classes of drugs such as the flouroquinolones and cephalosporins, should be restricted to veterinarians, to help reduce further transfer of resistance genes.

- The use of antibiotics as growth promoters should be cautiously applied.

- Continuous monitoring of occurrence and antimicrobial resistance among food borne pathogens in swine and other food producing animals is important in order to detect new and emerging resistance trends.

- Good farming practice should focus on reducing the risk of the dissemination of these pathogens in the farming environment and from the animals to the carcass during slaughter.

Further research should be done to identify the variants of the *stx2*, especially the *stx2e* gene responsible for oedema in pigs. This will raise awareness to the farmers of this public health threat. Effective measures to reduce antibiotic use in swine production should be investigated and results published to further enlighten relevant stakeholders .In addition, there is a need to further evaluate antimicrobial usage in both humans and swine by obtaining actual drug usage records instead of relying on rough estimates.

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