Antimicrobial Resistance, Plasmid Profiles and Sequence Typing of Enterotoxigenic *Escherichia coli* isolates causing Colibacillosis in Neonatal and Weaning Piglets of South Africa

by

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Genetics, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research was financially supported by the National Research Foundation (NRF) and the Agricultural Research Council.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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I, Mary Ranketse, declare that:

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(iii) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

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DECLARATION 2: PUBLICATIONS

My role in each paper and presentation is indicated. The * indicates corresponding author.

1. Ranketse, M.*, Madoroba, E. Muchadeyi, F. C. and Dzomba, E. F. 2015. Analysis of antibiotic resistant genes, plasmid profiles and multilocus sequence typing of *Escherichia coli* isolates causing colibacillosis in South African piglets. In Proceedings of the University of KwaZulu-Natal, College of Agriculture, Engineering and Science, 2015 Postgraduate Research Day, 22 September 2015, Pietermaritzburg, South Africa. (Abstract). Work was presented at the CAES Research Day conference held on 22nd of September 2015. Presented by M. Ranketse. (Awarded College prize for best presenter)
2. Ranketse, M.*, Madoroba, E. Muchadeyi, F. C. and Dzomba, E. F. 2017. Whole Genome Sequence Analysis Reveals Antimicrobial Resistance Genes, Plasmids and Potentially Unique Sequence Types of Enterotoxigenic *Escherichia coli* isolates causing Colibacillosis in Neonatal and Weaning Piglets of South Africa. In Proceedings of the Plant and Animal Genome XXV 2017, 14-18 January 2017, San Diego, California, United Stated of America. (Abstract). Work was presented at the Swine workshop held on 14th of January. Presented by M. Ranketse.

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) are a pathogenic strain of *E. coli* that cause colibacillosis in animals and humans. Colibacillosis causes diarrhoea, dehydration and eventual death if left untreated. In pigs, colibacillosis mostly affects neonatal and post-weaning piglets, and is a problem as the death of animals and expensive control methods results in major profit losses for farmers. The misuse of antimicrobials in the livestock sector has lead to elevated levels of antimicrobial resistance. Plasmids are extra-chromosomal genetic elements which are implicated in the dissemination and persistence of antimicrobial resistance between and amongst different bacterial communities. There are limited studies relating to ETEC affecting piglets in South Africa. The aim of this study was to determine the sequence types of, assess the antimicrobial resistance profiles and genes confering such resistance and to screen for plasmids in ETEC recovered from piglets from the Limpopo and Eastern Cape provinces of South Africa. A total of 20 samples were analysed in this study. Multilocus sequence typing (MLST) using whole-genome sequencing data was used to determine the sequence types present. Plasmid profiling was performed and compiled with antimicrobial susceptibility profiles using the Kirby-Baur method of twenty samples, and twelve of these samples were used for further analysis. Whole-genome sequencing (WGS) was further used to determine the antimicrobial resistance genes and plasmids present in the samples. Multilocus sequence typing determined six different sequence types (ST10, ST56, ST101, ST4253, ST4704, ST1830) using the www.cge.cbs.dtu.dk//services/MLST and http://mlst.warwick.ac.uk/mlst/dbs/Ecoli databases. Plasmid profiling identified 10 plasmids of various sizes ranging from 1.3kb to >10kb with six samples having multiple plasmids. Antibiogram analysis showed that samples were resistant to ampicillin, oxytetracycline, trimethoprim, polymyxin B and lincomycin, with enrofloxacin and kanamycin resistance shown in only 2 samples. Whole-genome sequencing identified seven different plasmids originating from *Klebsiella pneumonia*, *Salmonella enterica*, *E. coli* K-12, β-lactamase CTX-M-15 plasmids, as well as Incompatibility groups FII and I2. The plasmids identified belong to different species of bacteria indicating the ability of plasmids to be transferred across species most probably through horizontal gene transfer. In addition, samples carried antimicrobial resistance genes for tetracycline A (*tetA*), tetracycline B (*tetB*), gentamycin (*aac(6')-Ii*) and the MLS class of antimicrobials (*msr(C)*). The antibiogram showed samples

exhibiting multiple antimicrobial resistance, and resistance was shown towards commonly used antimicrobials used in South Africa. This is a major problem with regards to controlling colibacillosis in South African pigs. This study expands our understanding of ETEC affecting pigs in South Africa which would aid in controlling colibacillosis.

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CHAPTER 1

INTRODUCTION

1.1. Rationale for the research

Colibacillosis is a major problem that affects neonatal and post-weaning piglets (Akol 1999; Francis 1999; Nagy & Fekete 2005; Coelho *et al.* 2009). The disease is caused by pathogenic *Escherichia coli* (*E. coli*) which include enterotoxigenic *E. coli* (ETEC) (Weiner *et al.* 2004; Olasz *et al.* 2005), shiga toxin *E. coli* (STEC) (Römer *et al.* 2012) and enteroaggregative *E. coli* (EAEC) (Weiner *et al.* 2004). The symptoms of colibacillosis include excessive diarrhoea, severe dehydration and if left untreated it may lead to death (Wray & Morris 1985; Coelho *et al.* 2009; Liu *et al.* 2014). Colibacillosis results in huge loses for both commercial and small-holder farmers because affected piglets fail to gain the desired weight, and are smaller compared to thier contemporary age group (Liu *et al.* 2014). Strains of *E. coli* carrying a combination of either two or more virulence genes associated with the three pathogenic groups have been identified (Francis 1999). Compared to all the pathogenic strains, ETEC has been frequently implicated in piglet diarrhoea (Do *et al.* 2005; Lee *et al.* 2009). In South African piglets, 25 pathotypes were identified from *E. coli* strains belonging to ETEC, STEC and EAEC (Mohlatlole *et al.* 2013).

Enterotoxigenic *E. coli* strains are classified on the basis that they produce heat-stable toxins (STa, STb) and/ or heat-labile toxin (LT) (Francis 1999; Olasz *et al.* 2005) and/ or enteroaggregative *E. coli* heat-stable enterotoxin-1 (EAST-1). In addition to toxins, ETEC also carry colonisation factors known as adhesins (Weiner *et al.* 2004). Adhesins enable the bacterium to attach to and colonise the small intestine and belong to two types, fimbrial and non-fimbrial. Documented fimbrial adhesins include F4, F5, F6, F7 and F18 (Francis 1999; Zhang *et al.* 2007). Non-fimbrial adhesins include: adhesin involved in diffuse adherence 1 (AIDA-1), attaching and effacing factor (EAE), porcine attaching- and effacing-associated factor (PAA) (Liu *et al.* 2014). Adhesion is a principal step to successful infection (Francis, 2002, Nagy and Fekete, 2005, Nataro and Kaper, 1998). Colonisation facilitates ETEC-induced infection, which causes symptoms such as excessive diarrhoea, resulting in fluid and

electrolyte imbalance and severe dehydration which eventually lead to death (Wray & Morris 1985; Coelho *et al.* 2009; Žutić *et al.* 2010).

A good undestanding of the epidemiology and virulence of prevailing *E. coli* pathotypes is crucial in disease management and control. Multilocus sequence typing (MLST) is a technique that is often used to study the epidemiology and track relationship between disease causative agents. This method makes use of housekeeping genes of pathogenic micro-organisms to track disease outbreak and progression (Esimone *et al.* 2010). Multilocus sequence analysis was used to study *Leishmania braziliensis* that caused an outbreak in the coastal region of Santa Catarina, Brazil. The results showed that the clonal complexes of the isolates that caused the outbreak formed a single cluster and were closely related (Marlow *et al.* 2014). Kusumoto *et al.* (2014) used MLST to determine the spread of insertion sequence excision enhancer (*iee*) gene amongst ETEC belonging to different serotypes from domestic animals in Japan. Fifty samples including enterohemorrhagic *E. coli* samples were included in the analysis. The results show that instead of being limited to just specific lineages, the *iee* gene had spread to different lineages of ETEC and EHEC through horizontal gene transfer. Wu *et al.* (2012) determined the relatedness of 39 extraintestinal pathogenic *E. coli* (ExPEC) isolated from domestic animals in Wales and England during the years 1999 and 2008. The study identified 29 sequence types, and found ST23, ST10, ST155 and ST117 to be the most prevalent clonal complex and further found that isolates belonging to ST10, ST23 and ST155 clonal complex harbour multiple *E. coli* pathotypes. The study found no association between sequence type, antimicrobial resistance and virulence genes, also that these samples had a similar clonal origin to human and other animal originating ExPEC isolates (Wu *et al.* 2012).

Despite its importance in both commercial and smallholder pig production systems, there has been no investigation of MLST on ETEC in South African pigs. Multilocus sequence typing of South African *E. coli* might provide useful information on the epidemiology and virulent sequence types of the prevalent pathotypes, which would be of value to the development of disease control strategies.

Due to the major losses occurring because of ETEC colibacillosis, several control strategies have been employed in pigs which include feed additives (Montagne *et al.* 2004), immunotherapy, vaccines (Nagy & Fekete 2005) and antimicrobials (Johnson & Nolan 2009;

Costa *et al.* 2010; Henton *et al.* 2011). Although vaccines and feed additives have proven to be efficient, antimicrobials are most preferred by pig breeders as they are affordable and easily administered (Habrun *et al.* 2011; Henton *et al.* 2011). In South Africa, microlides; pleuromutilins; tetracyclines; sulphonamides and penicillins are the most extensively used, whereas chloramphenicol and nitrofuras are not used in livestock (Henton *et al.* 2011). The mode of action of antimicrobials is to inhibit the growth of microbe(s) that cause diseases (Davison *et al.* 2000), either by destroying the bacterial cell or by hindering multiplication (Byarugaba 2010). Antimicrobials admisitered subtherapeutically are also used to enhance the growth of livestock (Emborg *et al.* 2001; Teuber 2001). It has been shown that antimicrobials administered on a daily basis at low levels have a positive impact on growth in livestock (Emborg *et al.* 2001) and to prevent disease (Teuber 2001). Antimicrobials are therefore administered to livestock for preventing the onset of disease, treating diseases and as growth promoters (Aarestrup 1999; WHO 2014).

Antimicrobials are important for livestock growth enhancement and disease control. Microbes however have been found to develop resistance against most antimicrobials over time, and this resistance is easily spread between species including humans (Tadesse *et al.* 2012). Factors contributing to resistance include overuse of antimicrobials (Wegener 2003; WHO 2014), and the genetic evolution of pathogens which results in increased virulence (Do *et al.* 2005). In addition, the overuse of antimicrobials has a negative effect on the microbial flora of the animal as the antimicrobials may select for bacteria that are naturally antimicrobial-resistant. This suppresses the growth of normal flora, a phenomenon known as the "competitive effect" (McEwen & Fedorka-Cray 2002). Alternatively the "selective effect" could occur when the antimicrobial is administered during exposure to a resistant pathogen, in which case the antimicrobial aids infection (McEwen & Fedorka-Cray 2002). It has been found that plasmids present in pathogenic bacterial strains harbour genes that code for antimicrobial resistance (Carattoli 2009; Svara & Rankin 2011; Adzitey *et al.* 2013). Further investigation into plasmids and the spread of resistance revealed that plasmids, transposons, integrons and gene cassettes, which are mobile genetic elements are actually responsible for the spread of resistance (Hall & Collis 1998; White *et al.* 2001; Domingues *et al.* 2012; Deng *et al.* 2015).

Plasmid profiling is used to characterise plasmids found in bacteria based on size. Plasmid profiling can be used to study epidemiology of diseases and disease pathogens by determining the plasmids shared between isolates (Mayer 1988). The dissemination of antimicrobial resistance, and multidrug resistance within a sample population can also be indirectly determined using plasmid profiling (Motayo *et al.* 2013). Plasmid profiling and antibiogram analysis can help in determining the correlation between plasmid incidence and antimicrobial resistance (Alam *et al.* 2013; Subba *et al.* 2013). Plasmids of various sizes have been isolated from ETEC affecting pigs. Olasz *et al.* (2005) isolated plasmids from wild ETEC strains Ec2173, Ec2172, Ec2152, Ec2188 and Ec2085 that ranged between 13.8 kb and 200 kb. Mainil *et al.* (1998) isolated ETEC plasmids ranging between 65 Mda and 200 Mda from piglets, and Pachaury *et al.* (2013) isolated ETEC plasmids from kids, calves and lambs that sizes ranged between 10 kb and 15 kb in size.

Plasmid profiling has been successfully used in many epidemiology surveillance cases to identify the cause of foodborne disease outbreaks. The American Centre for Disease Control first used plasmid profiling in 1981 in order to determine the strain of *Salmonella* Muenchen had caused a gastroenteritis epidemic which was associated with smoking marijuana (Wachsmuth 1985). Plasmid profiling analysis in two concurrent outbreaks of *S.* Newport in the states of New Jersey and Pennsylvania identified the source of the foodborne contamination to be from precooked roast beef that was available in these two states (Riley *et al.* 1983). Wells *et al.* (1983) used plasmid profiling to successfully identify a rare *E. coli* O157:H7 that caused two outbreaks of hemorrhagic colitis in Oregon and Michigan. No plasmid profiling studies of ETEC has been performed in South Africa. It is hypothesised that plasmid profiling will shed some light on the types of plasmids present linked to virulence traits and the spread of antimicrobial resistance genes in South African ETEC present in piglets.

Whole-genome sequencing using next-generation sequencing allows for massively parallel sequencing which enables millions of fragments from a single sample to be sequenced at once, enabling assembly of a complete genome within short intervals (Grada & Weinbrecht 2013). Next-generation sequencing is increasingly becoming exceptionally fast, and low cost (Loman *et al.* 2012). Next-generation sequencing is used to analyze the genomes of all living organisms from model organisms to plants, animals and humans (Lui *et al.* 2012). Sequencing

of bacterial genomes such as ETEC allows for comparative and phylogenetic analysis and also enables the identification of virulence genes, antimicrobial resistance genes, plasmids, transposons and other interesting genes these bacteria may harbour (Sahl *et al.* 2011; Wyrsch *et al.* 2015). Shepard *et al.* (2011) conducted whole-genome sequencing at 30X depth using Roche 454 GS-FLX pyrosequencing on ETEC isolated from piglets in Minnesota and Iowa. The authors compared the two ETEC strains they sequenced, UMNF18 and UMNK88, with the human ETEC strain EC24377A and *E. coli* lab reference strain K-12 MG1655. The study revealed that the pig ETEC genomes (UMNF18 - 5.3 MB, UMNK88 – 5.1 MB) are larger than those of the human ETEC (4.98 MB) and the K12 (4.6 MB) strain and the pig ETEC strains each harboured unique plasmids encoding various pathogenic traits.

1.2. Problem statement and justification

Enterotoxigenic *E. coli* infections are a challenge in pig production in South Africa. They increase the cost of production and reduce profit gains directly with purchase of medication and indirectly as a result of poor production performance and mortality (Henton & Engelbrecht 1997). This is a challenge as methods used in controlling the disease are increasingly becoming ineffective. *Escherichia coli* isolated from communal and commercial pigs in North West province of South Africa were found to be resistant to a number of commonly used antimicrobials (Moneoang & Bezuidenhout 2009). In order to improve return on investment in the pig industry, general disease infections including ETEC that cause major setbacks in performance needs to be controlled. A recent study showed that virulence profiles found in ETEC affecting South African piglets (Mohlatlole *et al.* 2013), was different from those identified two decades back (Henton & Engelbrecht 1997). The evolution of ETEC strains imply that there is need to adapt a different strategy of controlling the pathogen if efficient control is to be achieved.

Currently there are limited studies investigating the pathogenesis and efficiency of control methods of ETEC in South Africa. One study conducted in South Africa characterised *E. coli* O157 strain isolated from humans, cattle and pigs based on antimicrobial sensitivity and virulence genes (Ateba & Bezuidenhout 2008). Another study investigated antimicrobial sensitivity and vancomycin-resistant genes in *E. coli* from pigs (Moneoang & Bezuidenhout 2009). A more recent study determined the antimicrobial sensitivity profile of ETEC from pigs (Sikhosana 2015). Further characterisation of South African ETEC isolates from piglets

is required in order to bridge the information gap and make strides in the efficient control of colibacillosis. The use of MLST will allow for the identification of ETEC sequence types found in South Africa, and the phylogenetic comparison of South African isolates to those found internationally. Next-generation sequencing will allow for the screening of the complete genome allowing for the determination of antimicrobial resistance genes as well as identification of plasmids which harbour virulence genes.

The ongoing antimicrobial resistance found in *E. coli* strains will continue to be a great challenge to pig producers if the same antimicrobials are used. Considering antimicrobials are the most affordable and popular measure of controlling ETEC-induced infection in piglets, there is a need for a study on the factors causing resistance in these strains. It has already been shown that antimicrobial resistance is increasingly becoming common in South Africa. Therefore a study on the characteristics of factors contributing towards resistance, as well as their antimicrobial resistance profile will be relevant if an effective antimicrobial control method is to be implemented.

This study forms part of a larger study on *E. coli* colibacillosis affecting post-weaning and neonatal piglets in South Africa. Mohlatlole *et al.* (2013) studied the prevalence of, and virulence factors of ETEC; STEC and EAST-1 including toxins; fimbrial and non-fimbrial adhesin factors of *E. coli* strains associated with colibacillosis in South African pigs. Chaora (2013) analysed the relationship between pig breeds and ETEC susceptibility using adhesion analysis. Sikhosana (2015) analysed the prevalence of, and antimicrobial susceptibility profiles of ETEC samples. There are however, still some gaps regarding South African ETEC strains, such as the molecuar typing and genetic profiles associated with antimicrobial resistance.

1.3. Aims and objectives

The aim of the study was to characterise *E. coli* causing colibacillosis in neonatal and post-weaning piglets of South Africa by determining plasmid profiles present within this population, and to use next-generation sequencing to analyse plasmids and antimicrobial resistance genes as well as to determine the sequence types of these enterotoxigenic *E. coli* isolates.

The specific objectives were to:

i. Determine the sequence types and perform phylogenetic analysis of enterotoxigenic *E. coli* strains isolated from South African neonatal and post-weaning pigs using Multilocus sequence typing by Polymerase chain reaction and Next-generation sequencing.

ii. Perform plasmid profiling and determine the association between the plasmid profile and antibiogram of enterotoxigenic *E. coli* isolated from South African neonatal and post-weaning pigs.

iii. Determine antimicrobial resistance genes and plasmids present in enterotoxigenic *E. coli* strains from South African neonatal and post-weaning piglets using Next-generation sequencing data.

1.4 Dissertation layout

This dissertation analyses enterotoxigenic *E. coli* isolated from pigs at a molecular level. Chapter one is a general introduction of ETEC which includes virulence, plasmids and antimicrobial resistance. Chapter two gives an overview of ETEC literature, including phenotype and genotypes, further discussion of sequence typing, plasmids, antimicrobial resistance and the methods used to research these topics such as MLST, plasmid profiling, antibiogram and whole-genome sequencing using next-generation sequencing tools. Chapter three looks specifically at MLST and phylogenetics. Chapter four compares the plasmid profiles and antimicrobial resistance profiles and Chapter five determines the plasmids and antimicrobial resistance genes present using Next-generation sequencing. Chapter six is the concluding chapter which gives an overview of the results and recommendations for future studies.

CHAPTER 2

CHARACTERISATION OF ENTEROTOXIGENIC *ESCHERICHIA COLI* AFFECTING PIGLETS IN SOUTH AFRICA USING EPIDEMIOLOGY AND GENOMIC TOOLS

Abstract

Pork is an essential part of a healthy, well-balanced diet as it is packed with many vitamins and minerals and is low in intramuscular fat. Pigs are produced in all the regions of South Africa, in both the commercial and communal sectors. An important disease affecting piglets is colibacillosis which is a diarrhoeal disease caused by the enterotoxigenic *Escherichia coli* (ETEC) strain. This strain is characterised by virulence factors such as adhesin factors which enable the bacteria to attach to the microvillus of the small intestine thereby causing colonisation, and heat-labile and heat-stable toxins from the bacteria are released into the lumen which causes the cells to excrete water and ions. It is important to study epidemiology of bacteria in order to understand their pathogenicity. This review provided an overview of enterotoxigenic *Escherichia coli* as a pathogen causing colibacillosis, its epidemiology, and the use of antimicrobials to control and manage colibacillosis. The review further looked at methods to investigate diversity and epidemiology of *E. coli*. Multilocus sequence typing is a highly efficient method used for epidemiology which involves sequencing of housekeeping genes to determine sequence types and trace phylogeny of bacteria. Multilocus sequencing typing (MLST) utilizes genomic data, therefore it is highly accurate, as compared to previous epidemiological methods such as serotyping and Multilocus-enzyme electrophoresis. A review on plasmids and their role in antimicrobial resistance is provided followed by methods to screen for plasmids and determine the genetic elements conferring antimicrobial resistance. Colibacillosis is traditionally controlled using antimicrobials. In South Africa, the pig and poultry sector has the highest use of antimicrobials as compared to other livestock. Antimicrobial resistance has grown and spread rapidly with most bacteria being resistant to majority of the antimicrobials being used to control infections. Resistance is either genetically (intrinsic or acquired) or non-genetically mediated. Intrinsic resistance is due to mechanisms found within the chromosome such as mutations, low cell permeability and efflux amongst others. Acquired resistance is due to extrachromosomal elements such as plasmids,

transposons and gene cassettes, some of which are mobile or mobilizable. Due to plasmids being mostly implicated in the transfer of antimicrobial resistance and virulence factors, within and between bacterial species, it is essential to determine what plasmids are harboured by ETEC isolates. Plasmid profiling is a technique that determines the total plasmid content of isolates, therefore can also be used as an epidemiological tool. Further analysis of plasmids can be performed using Next-generation sequencing (NGS) tools. With sequencing getting cheaper, it is possible to sequence whole bacterial genomes and extract more information. Next-generation sequencing allows for the complete analysis of bacteria, such as virulence genes, antimicrobial resistance, extrachromosomal genetic elements, epidemiology and much more.

Key words: *pig; ETEC; MLST; antimicrobial resistance; plasmids; whole-genome sequencing*

2.1 Introduction

Pork is an economically important source of lean meat (SAPPO 2008; Murphy *et al.* 2012), and the preferred choice of meat over beef for many health conscious consumers (SAPPO 2008). The intramascular fat content of pork is lower than that of beef and lamb, and the total fat found in pork has been reduced with time through breeding and nutrition (Kanis *et al.* 2005). South African produced pork has been recognized to be lower in fat than most other countries internationally and the South African pork industry is affiliated with the Heart and Stroke Foundation (SAPPO 2008). Pork has high quality protein that is rich with essential amino acids and contains many essential vitamins and minerals such as vitamin B1, iron, zinc, calcium, potassium, phosphorus and sodium (SAPPO 2008). In the commercial sector, pork is produced under intensive production systems using grain as a basic energy source (Ramsay *et al.* 2006).

Escherichia coli is a predominantly commensal bacteria which resides in the gut of animals and humans (Krogfelt 1991; Nataro & Kaper 1998). Pathogenic strains of the bacteria are known to cause infections such as gastroenteritis, meningitis, peritonitis, urinary tract infections and septicaemia (Johnson & Nolan 2009). In pigs, virulent strains such as enterotoxigenic *E. coli* (ETEC), shiga toxin *E. coli* (STEC) and enteroaggregative *E. coli* (EAEC) are responsible for colibacillosis in piglets (Francis 1999; Nagy & Fekete 2005; Mohlatlole *et al.* 2013). These strains are characterized by toxins, fimbrial and non-fimbrial adhesin factors which are responsible for their pathogenesis (Nataro & Kaper 1998). Virulent genes for these pathotypes have been identified and are known to be transferrable between isolates (Nagy & Fekete 2005).

Treatment of colibacillosis is through the use of antimicrobials (Tadesse *et al.* 2012). However, it has been observed that resistant and multiple-drug resistant *E. coli* strains are present in the South African population (Ateba & Bezuidenhout 2008; Sikhosana 2015). Understanding the antimicrobial resistant profiles and genetic mechanisms that pathogens use to resist or tolerate antimicrobials is crucial in the development of effective control programs. Resistance to antimicrobials is either genetically (Zhang 2007; Davies & Davies 2010) or non-genetically (Zhang 2007; El-Halfway & Valvano 2012; Corona & Martinez 2013) controlled. Non-genetic resistance can be characterized as changes in bacterial metabolism (leading to an increase or decrease of antimicrobial resistance), due to the presence of small

molecules which are metabolic byproducts such as indole; polyamines and volatile compounds (El-Halfway & Valvano 2012). Non-genetic resistance is also found in populations consisting of persistent cells and biofilms (Corona & Martinez 2013). Genetic resistance can be further characterised as either extrachromosomal or chromosomal (Levy & Marshall 2004). Extrachromosomal resistance is due to the acquisition of mobile genetic elements such as plasmids and transposons that harbour genes for antimicrobial resistance (Levy & Marshall 2004; Carattoli 2009; Johnson & Nolan 2009). This resistance is also deemed as a form of "acquired" resistance mechanism (Ammor *et al.* 2008; Hollenbeck & Rice 2012). Chromosomal resistance occurs in the absence of plasmids or transposons whereby the bacteria creates mutations in the chromosome, leading to resistance (Levy & Marshall 2004). This type of resistance is also deemed as "intrinsic" and is naturally occurring within the bacterial genome (Ammor *et al.* 2008; Hollenbeck & Rice 2012).

This chapter will discuss pork production in South Africa, colibacillosis and its effects, as well as the role of ETEC in colibacillosis. The review will look at the use of antimicrobials in the control and management of colibacillosis and discuss cases of antimicrobial resistance. The role of plasmids and the genes conferring antimicrobial resistance are reviewed followed by a discussion on the use of molecular technologies including MLST and next-generation sequencing in understanding the epidemiology of *E. coli*, plasmid profiling and screening of antimicrobial resistance genes. The chapter concludes with a review of previous research performed on *E. coli* in South Africa and the gaps that that still exist.

2.2 Pork production in South Africa

South Africa has approximately 4000 commercial producers, 19 stud breeders and 100 smallholder farmers (DAFF 2012). The commercial sector owns approximately 100 000 sows, and the smallholder farmers approximately 25 000 sows (DAFF 2012). Limpopo and North West provinces produce almost 50% of the total pig production in South Africa, with Northern Cape and Eastern Cape producing the lowest at 2% and 6% respectively (DAFF 2012).

According to the 2002 agricultural statistics on South African farm animals, pigs represented the smallest number of farmed livestock compared to cattle, sheep, goats, poultry and ostrich. In the Eastern Cape, majority of pigs are reared in the communal areas, with very

few being farmed commercially (DAFF 2012). In Limpopo, like the Eastern Cape, most pigs are farmed in the communal areas, with a few being farmed in the northern and southern commercial areas. In KwaZulu-Natal, pigs are mostly farmed in the Midlands and the northern areas. Pigs are farmed in the eastern parts of the North West, and in the Free State pigs are farmed in all areas except the southern and western areas (DAFF 2012). Production indices for pigs were higher than cattle and sheep before the year 2005, thereafter the production index was below that of the other two livestock species (DSaEA 2013). Approximately 2.4 million pigs were slaughtered which yielded more than 2 million tons of pork in 2011 (DAFF 2012). South Africa produces a much higher volume of pork than that consumed, with with consumption only being approximately 250 tons. Surprisingly, more pork is imported compared to that exported, with a difference of approximately 30 million tons reported in 2011. This large difference is due to a higher demand of processed pork food products (DAFF 2012).

The advantages of pig farming include quicker turnaround time compared to the other red meats, farms do not require a large space for production, pig feed products cost lower than other livestock and demand for pork has increased over recent years (DAFF 2012). The quicker turnaround time for pigs is related to their advantage to turn feed into meat, and their ability to efficiently convert poor feed (kitchen swill, brewer's grain, maize grains and vegetables) into meat as seen in the communal areas (Gcumisa 2013). Pigs are also reported to be more prolific as compared to other livestock, reaching maturity earlier and are able to reproduce twice a year (Gcumisa 2013). The disadvantages of pig farming are that pigs are highly susceptibility to diseases resulting in health, safety and phyto-sanitary issues. Water shortages directly affects cleaning and safety and pig farming is more labour intensive than other livestock farming (DAFF 2012).

2.3 Colibacillosis in pig production

Colibacillosis is a condition that affects neonatal and post-weaning piglets (Francis 1999; Alexa *et al.* 2005; Coelho *et al.* 2009). Pathogenic strains of *E. coli* cause diseases such as enteric colibacillosis and colibacillary toxaemia in piglets (Wray & Morris 1985; Quinn *et al.* 2011). Neonatal colibacillary diarrhoea is the most common type of disease affecting piglets 1-3 days after birth (Žutić *et al.* 2010; Fekete *et al.* 2012). The disease is caused by pathogenic strains of *E. coli* colonising the small intestine by attaching fimbrial and non-

fimbrial adhesins to the microvilli of the small intestines (Krogfelt 1991). When an infection is established, enterotoxins are released into the lumen, which affects the enterocytes and causes them to release water and electrolytes into the intestine causing diarrhoea and downstream symptoms (Zhang *et al.* 2007; Janjatović *et al.* 2009; Taxt *et al.* 2010; Haycocks *et al.* 2015). Post-weaning colibacillosis is caused by an increase in enteric *E. coli* and this may be due to environmental factors such as changes in the environment and the diet that these pigs are fed, as well as the decrease of maternal immunity (antibodies present in maternal milk) due to termination of suckling (Alexa *et al.* 2005; Lin *et al.* 2013).

2.3.1 Bacteria causing colibacillosis

The pathology of colibacillosis is characterized by various pathotypes of *E. coli* such as enterotoxigenic *E. coli* (ETEC), shiga toxin *E. coli* (Choi *et al.* 2001; Römer *et al.* 2012) and enteroaggregative *E. coli* (EAEC) (Choi *et al.* 2001). In order to cause infection, the bacteria first needs to adhere to and colonize the intestine, thereafter toxins are produced and released into the lumen (Nataro & Kaper 1998; Zhang *et al.* 2007). Pathogenic *E. coli* virulence factors are mainly fimbrial adhesin factors: F4 (K88), F5 (K99), F6 (987P), F18ab/ac (F107, 2134P) and F41 (Francis 1999; Zhang *et al.* 2007; Gyles *et al.* 2010); non-fimbrial adhesin factors: adhesin involved in diffuse adhesin (AIADA-1), porcine attaching- and effacing-associated factor (PAA) and attaching and effacing factor (EAE) (Zhang *et al.* 2007; Gyles *et al.* 2010), and enterotoxins: heat-labile toxins (LT), heat-stable toxins (STa/b), shiga toxins (Stx1/2/2e) and enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) (Gyles *et al.* 2010; Mohlatlole *et al.* 2013).

2.4 Antimicrobials used to control colibacillosis

Current methods utilized for the control of colibacillosis include immunisation of sows and piglets (Alexa *et al.* 2005; Lin *et al.* 2013), the use of antimicrobials (Costa *et al.* 2010), and chemicals such as cleaning products (WHO *et al.* 2003), or the culling of affected livestock (FAO/INRA 2009).

In 1928 Alexandra Flemming discovered that penicillin, produced by mould, had antibacterial properties and that marked the age of antimicrobials as we know it (Sengupta *et al.* 2013; Sharma *et al.* 2013). In later years antimicrobials were chemically modified (semi-synthetic) to enhance their properties for use in controlling bacterial infections (WHO *et al.*

2003). There are also a number of completely synthetic antimicrobials (van Hoek *et al.* 2011), such as fluoroquinolones, sulphonamides and trimethoprim (Davies & Davies 2010). The last batch of new classes of antimicrobials were discovered in the 1980's and no new antimicrobials have been discovered since (Cole 2014; WHO 2014). Since the introduction of antimicrobials, used against microbes, have played an immense role in preventing and eradicating diseases in animals, humans (Tadesse *et al.* 2012), horticulture and aquaculture (Hall & Collis 1998).

Antimicrobials are used in the livestock sector for three different reasons of (i) treating disease outbreaks (therapeutic), (ii) prevention of disease when they are administered before the onset of disease (prophylactic) and (iii) as growth enhancers or promoters (sub-therapeutic) (Schwarz *et al.* 2001; Wegener 2003). Antimicrobials are often given to the entire herd in feed or water resulting in both sick and healthy individuals receiving the same amount of antimicrobials. In this instance, sick animals consume a lesser amount of the antimicrobial due to their loss of appetite (Wegener 2003). Metaphylaxis is a form of administering antimicrobials to the whole herd at therapeutic levels for short periods of time, upon possible onset of an epidemic (Schwarz *et al.* 2001; McEwen & Fedorka-Cray 2002). The antimicrobials are put into food or water and sick animals receive antimicrobials as treatment while healthy animals receive antimicrobials as control or preventative infection measures (McEwen & Fedorka-Cray 2002). In swine production antimicrobials are usually administered to the entire herd through feed for growth promotion or disease prophylaxis. These antimicrobials are administered for the duration of the growth up until the finishing stages when the antimicrobials are withdrawn to prevent residues from staying in meat (McEwen & Fedorka-Cray 2002).

Antimicrobials are classified based on their effect on the three different stages of bacterial cell replication, i.e. cell wall biosynthesis, protein synthesis and DNA synthesis (Peach *et al.* 2013). Antimicrobials are further characterized into classes based on their mode of action against bacterial cells. Certain antimicrobials inhibit bacterial wall synthesis by activating enzymes that disrupt the cell wall or cause an increase in cell membrane permeability causing leakage of cellular components (Kohanski *et al.* 2010; van Hoek *et al.* 2011). Other antimicrobials cause lethal inhibition of protein synthesis and others inhibit synthesis of nucleic acids (Kohanski *et al.* 2010; van Hoek *et al.* 2011). Generally, the effects of

antimicrobials are either bactericidal meaning that the mode of action leads to the death of the cell, or bacteriostatic meaning that they inhibit the growth and proliferation of cells (Rosengren *et al.* 2009). Resistance against antimicrobials and the mechanisms conferring resistance varies in line with the mode of action of the different antimicrobials (Byarugaba 2010; Munro 2015).

2.4.1 Antimicrobial use in livestock in South Africa

In South Africa the classes of antimicrobials most extensivley used include macrolides, pleuromutilins, tetracyclines, sulphonamides and penicillins. Tylosin, tetracyclines, sulphonamides and penicillins are the most extensively sold. Chloramphenicol and nitrofurans are not used in the livestock sector (Henton *et al.* 2011). Tetracycline, tylosin, sulphamethazine and other sulpha's are used most frequently in swine production (McEwen & Fedorka-Cray 2002) including trimethoprim (Mazurek *et al.* 2015; van Rennings *et al.* 2015). Antimicrobials that are used as growth promoters include ionophores, macrolides, quinoxalines, polypeptides, streptogramins, glycolipids, oligosaccharides, phosphonic acids and polymeric compounds; all of which are banned from use in the European Union (Henton *et al.* 2011).

In a study conducted by Eagar *et al.* (2012) the authors collated data from a survey taken between 2002 to 2004 to analyze the antimicrobials available for use in food animals and the volumes used for metaphylaxis, prophylaxis and sub-therapeutically. Two hundred and thirty four (234) antimicrobials are registered under two separate acts: Act 36 of 1936 and Act 101 of 1965 for use in food animals. Five in-feed premixes were registered as veterinary medicine. Sixty four (64) in-feed antimicrobials used sub-therapeutically were registered as stock remedies and are available over-the-counter. Of these, a total of 11 antimicrobial classes are included with majority belonging to tetracyclines, ionophores, macrolides, lincosamides, pleuromutilins, glycolipids and polypeptides. In terms of volumes (kg %) of antimicrobial consumption, the four classes most consumed in descending order are microlides and pleuromutilins class (42.4 %), tetracyclines (16.7 %), sulphonamides (12.4 %) and penicillins (10.7 %). Of the volume in kg %, of antimicrobials administered in parenteral form, penicillins were the largest class at 60 %, and tetracylcines were the second largest at 32 %. In terms of modes of administration, in-feed constituted the most at 69 %, followed by parenteral at 17.5 %; water soluble at 12 %, with intramammaries and other dosage forms (such as

tropical ointments, tablets, etc.) the lowest at 4 % and 1.5 % respectively. Unfortunately, the authors were unable to conclude the use of the antimicrobials whether for metaphylaxis, prophylaxis or sub-therapeutically, as that data were not available. This information was compiled as a means of initiating surveillance systems for veterinary antimicrobial use, which will be utilized in part for assessing and monitoring veterinary antimicrobial resistance.

Previous studies have reported the presence of antimicrobial residues in meat, and sometimes chemical residues still remain in meat, even after cooking (Kaneene & Miller 1997; Heshmati 2015). This poses a serious health problem as these residues, when consumed by people can result in allergic reactions, liver damage, yellowing of teeth and imbalance of intestinal bacteria due to antimicrobial selection pressure (Fritz & Zuo 2007; Gajda & Posyniak 2009). Long term effects of humans consuming low levels of these residual antimicrobials include alterations of normal microflora and possible resistance to antimicrobials thus making antimicrobial treatment of diseases redundant (Moyane *et al.* 2013).

It was found that the pig and poultry sector has the highest use of antimicrobials (Krnjaić *et al.* 2005; Henton *et al.* 2011). In 2007 the South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs (SANVAD) surveillance programme observed that *E. coli* had a high resistance of 67 % to the most commonly used antimicrobials, mainly tetracyclines, sulphonamides and fluoroquinolones, with pigs and poultry having the highest resistance (Henton *et al.* 2011). The greater use of antimicrobials in pigs and poultry may be due to the intensive farming practise used to rear these animals as animals are kept indoors in large numbers during their entire growth period thus allowing rapid disease transmission (Krnjaić *et al.* 2005; Habrun *et al.* 2011; Henton *et al.* 2011). An alarming problem due to the extensive use of antimicrobials especially in an intensive farming system is the emergence of resistant pathogens as antimicrobials are mainly used to control diseases, and are administered to the entire group (Krnjaić *et al.* 2005; Rahman *et al.* 2008; Henton *et al.* 2011).

2.4.2 Antimicrobial resistance

The overuse of antimicrobials in the livestock industry, as growth promoters and to treat infections has resulted in pathogenic bacterial strains such as ETEC, STEC and EAST-1

developing resistance to a wide range of antimicrobials. Antimicrobials are produced as a bi-product (secondary metabolites) of metabolism (morphological development) by various microorganisms (Sengupta *et al.* 2013), and as a result of this, these bacteria have natural resistance towards those antimicrobials as a form of self-preservation (Chu *et al.* 2012). Resistance mechanisms towards antimicrobials have co-evolved with naturally synthesized antimicrobials (Bernal *et al.* 2013; Munro 2015). This was described before the first antimicrobial penicillin was made available for use with the discovery of β-lactamase in *E. coli* (Bradford 2001; van Hoek *et al.* 2011) and penicillin resistant *Staphylococcus aureus* in the 1940s (Chambers 2001; Lowy 2003; Fernández & Hancock 2012). It is hypothesized that the antimicrobial-producing bacterial strains transferred their resistance determinants to pathogenic strains via horizontal gene transfer. Another theory for the uncontrolled emergence and spread of resistant bacteria is the increased use or misuse of antimicrobials which creates selective advantage for antimicrobial resistance (Keen & Montforts 2012).

Multiple antimicrobial resistance (MAR) or multidrug resistance (MDR) was identified in Japan in the 1950's with *Shigella* species (Hall & Collis 1998), and enterobacteria (Fernández & Hancock 2012). Multi-drug resistance is a phenotype whereby an isolate exhibits resistance to more than three antimicrobial classes. This poses a serious problem as firstly, controlling diseases with a suitable antimicrobial is no longer effective and secondly, commensal bacterial strains act as reservoirs for MDR traits. There now exists a large number of microorganisms that exhibit MDR to most currently used antimicrobial drugs and these pathogens are commonly known as "superbugs" (Fernández & Hancock 2012). Most Gram-negative species are resistant to majority of the present antimicrobial classes (Moyane *et al.* 2013). The possiblity of new antimicrobials to control MDR strains being made available in the near future is slim (Moyane *et al.* 2013). Mobile genetic elements such as plasmids and transposons are most commonly implicated in MDR (Byarugaba 2010). Multidrug resistance can also be influenced by chromosomal genes, which code for regulatory proteins (such as MarA and SoxS) that control the expression of other chromosomal genes and are involved in intrinsic resistance mechanisms (Levy & Marshall 2004).

Bacteria are able to resist the action of antimicrobials through either producing enzymes that destroy or inhibit the action of the antimicrobials. This is achieved by positively removing antimicrobials that enter the cell through efflux pumps, or by genetically altering

the cellular target of an antimicrobial so that it can no longer bind and cause effect. Bacteria are also able to reduce the amount of antimicrobial that enters the cell through modification of the cell wall (Fernández & Hancock 2012). Permeability reduction is another resistance mechanism that determines if the antimicrobial can enter the cell and reach its target (Kumar & Varela 2013). Mutations allow bacterial populations to adapt to pressures created in a changing environment depending on such changes. Mutational selection for antimicrobial resistance is an example of bacterial survival mechanism caused by the selection pressure from antimicrobials (Laxminarayan *et al.* 2013).

Bacteria exhibit three types of resistance mechanisms which include intrinsic, adaptive and acquired (Fernández & Hancock 2012; Blair *et al.* 2015). Intrinsic resistance is inherently present within microbial cells and is species-specific. Alternatively acquired resistance is the result of a microbe acquiring new genetic material such as plasmids encoding resistant genes or via mutations. Adaptive resistance is a phenotype which is due to alterations in gene and/or protein expression and is a result of changes in environmental conditions such as stress, nutrient conditions, growth state or exposure to sub-inhibitory levels of antimicrobial drugs (Keen & Montforts 2012; Bernal *et al.* 2013). Intrinsic and acquired resistance are stable and can be transferred to subsequent generations. Adaptive resistance however is reversible upon the re-equilibration of environmental conditions (Keen & Montforts 2012). Therefore intrinsic resistance can be recognised as a number of chromosomal genes that are responsible for resistance, and is not based on exposure to antimicrobials. Acquired resistance however is attributed to genetic mutations based on exposure to antimicrobial/s or acquisition of mobile genetic elements conferring resistance (El-Halfway & Valvano 2012).

Efflux-mediated antimicrobial resistance is an important mechanism used by both acquired and intrinsic resistance mechanisms. Efflux pumps are responsible for the removal of toxic substrates from the inside of the cell to the environment outside. These pumps and are coded for by both chromosomal and plasmid mediated genes (Webber & Piddock 2003) for either antimicrobial-specific, antimicrobial class-specific or multidrug resistance (Poole 2005). Efflux pumps are incriminated mostly in acquired clinical resistance (mutations to intrinsic gene). The over-expression of efflux pumps due to mutations enable the bacterium to exhibit resistance to a number of antimicrobials from different classes in addition to some dyes, detergents and disinfectants, and may even result in cross-resistance (Webber & Piddock

2003). Unregulated over-expression however can result in decreased fitness and reduction of virulence potential (Webber & Piddock 2003; Fernández & Hancock 2012). These mutations are useful in the presence of antimicrobials therefore bacteria need to regulate the expression of these genes, and expression is regulated by global and local regulatory genes (Webber & Piddock 2003; Fernández & Hancock 2012).

The prevalence of antimicrobial resistance depends on a number of factors such as type of antimicrobial agent, species and strain of micro-organism, dose, time and mode of administration of antimicrobial agent (through feed or intravenous) (Aarestrup 1999).

2.4.2.1 Non-genetic mechanisms of antimicrobial resistance

Non-genetic resistance can be described as a state whereby bacteria are resistant to antimicrobials in the absence of genetic change, which could be transient resistance, as seen with biofilms, persistents and drug indifference (Levin & Rozen 2006). Other mechanisms of non-genetic resistance are evidenced by phenotypic changes depending on metabolic state of the bacteria (Corona & Martinez 2013). Studies have shown that a large number of genes are involved in phenotypic resistance, and some of these genes are also involved in bacterial metabolism. It can therefore be said that as the phenotype for resistance is under metabolic control, changes in bacterial metabolism due to external and internal factors can alter susceptibility to antimicrobials (Corona & Martinez 2013).

The state of bacterial drug indifference to antimicrobials is usually seen in the stationary phase of bacterial growth. Bacteria in stationary phase are resistant to some antimicrobials, and are moderately susceptible to others, as such, antimicrobials have a reduced level of activity against bacteria as compared to when cells are actively multiplying (Levin & Rozen 2006; Corona & Martinez 2013). Persistent cells are a subpopulation of bacterial cells that are dormant in a culture, and therefore not affected by antimicrobial treatment when the rest of the population dies out (Shah *et al.* 2006; Vázquez-Laslop *et al.* 2006; Corona & Martinez 2013). Persisters can resume growth during the absence of the antimicrobial, but become susceptible and die if the antimicrobials is reintroduced (Lewis 2005; Corona & Martinez 2013). The mechanism of resistance implicated in persistent cells is due to toxin-antitoxin module (Karen *et al.* 2004; Lewis 2005; Hansen *et al.* 2008).

Biofilms are consitant of bacteria growing slowly in an encapsulated matrix attached to a surface (Costerton *et al.* 1999). Under these conditions, a gradient is formed due to the various depths in the biofilm, nutrients and oxygen alter the metabolic state of the bacteria at different depths indirectly making the bacteria resistant to a number of antimicrobials (Corona & Martinez 2013). The diffusion of certain antimicrobials through the biofilm is impaired due to compounds in the matrix (Lewis 2005), resulting in reduced free antimicrobial concentration (Corona & Martinez 2013). Another mechanism known as quorum sensing can alter the susceptibility of antimicrobials within the depths of biofilms (Corona & Martinez 2013). Persistent cells are implicated in maintenance and survival of biofilms (Lewis 2005; Hansen *et al.* 2008).

The presence of inducers, reactive oxygen species, changes in temperature, and metabolic conditions can all alter susceptibility to antimicrobials by causing bacteria to modify their permeability. Bacteria are able to alter thier lipopolysaccharide layer leading to a cascade of processes. These processes include reducing the binding of the antimicrobial, increasing surface area to minimise the effect of the antimicrobial per cell, altering the number or type of porins to prevent antimicrobials from entering the cell and the expression of efflux pumps to remove the antimicrobials if it enteres the cytoplasm of the cell (Corona & Martinez 2013).

El-Halfway and Valvano (2012) analysed the role of chemical signals of metabolic by-products in antimicrobial resistance. In *E. coli* indole is produced in higher quantities under stress and is used as a cell-signalling infochemical that induces protection of cells against certain antimicrobials in a population (El-Halfway & Valvano 2012). Polyamines are produced by bacterial cells, including *E. coli*, and this compound is also found to induce antimicrobial resistance in different bacterial species under various concentrations (El-Halfway & Valvano 2012). Ammonia is a volatile compound that is found to increase resistance to certain antimicrobials in *E. coli* (El-Halfway & Valvano 2012).

2.4.2.2 Genetic elements causing antimicrobial resistance

2.4.2.2.1 Chromosomal mechanism of antimicrobial resistance

Chromosomal resistance, also known as intrinsic resistance, refers to the naturally present resistance genes within the bacterial genome that are not due to previous exposure to an antimicrobial or acquisition of new genes which generate a resistance phenotype (Fajardo *et*

al. 2008; El-Halfway & Valvano 2012). An example of this resistance is antimicrobial producing bacteria, such as actinomycetes, which have genes coding for resistance to the antimicrobials they produce. In some cases, the resistance mechanism involves enzymatic modification of the antimicrobial. In nature, these bacteria are found to exhibit MDR to up to eight antimicrobials, this collection of resistance genes is known as the resistome (Davies & Davies 2010). The resistome is largely non-specific and genes in the resistome play a role in other mechanisms involved in basic physiological functions of the microbe such as signalling, growth inhibition and virulence activation (Fajardo *et al.* 2008; Sengupta *et al.* 2013). At some point during bacterial population growth dynamics, the cells experience a reduction in nutrients thus slowing down their growth rate and readjusting their metabolism which alters cellular structures and compositions (Greenway & England 1999). These changes as well as stressful conditions (e.g. introduction of antimicrobials and biocides) lead to activation and expression of the intrinsic resistance determinants ensuring long-term survival of the bacterial population (Greenway & England 1999). Some chromosomally encoded resistance mechanisms include increasing or mutating antimicrobial target genes, low permeability of the antimicrobial, efflux pumps and digestion of the antimicrobial (Davies & Davies 2010; Sengupta *et al.* 2013).

Evidence for antimicrobial resistance in bacterial communities that have never been exposed to antimicrobials and the discovery of ancient antimicrobial resistant bacteria dating as far back as 4 million years further highlights that resistance is not only dependent on exposure, but also chromosomally-mediated (Sengupta *et al.* 2013). Resistance can therefore also be a result of evolution (Sengupta *et al.* 2013).

2.4.2.2.2 Extrachromosomal genetic elements causing antimicrobial resistance

2.4.2.2.2.1 Plasmids

Plasmids are extrachromosomal, transmissible, self-replicating, circular genetic elements that contain genes not necessary for cellular functions (Carattoli 2009). Transmissible plasmids require various genetic traits such as conjugal donor ability, autonomous replication and incompatibility determinants (Santos *et al.* 1975; Norman *et al.* 2009). Plasmids are responsible for the evolution of microbial populations and provide a competitive advantage to bacteria (Mølbak *et al.* 2003; Ojala *et al.* 2013). Contained within plasmids are genes that code for transfer and replication, antimicrobial resistance, virulence and the metabolism of

rare substances such as mercury and copper (Johnson & Nolan 2009). Plasmids also contain genes that attribute to a wider variety of function such as resistance to metal ions and sequesteration of ions (Dale & Park 2010; Masood & Malik 2013), fermentation of sugars, urea hydrolysis, hydrogen sulphide production, degradation of toxic compounds (Dale & Park 2010), elaboration or production of colicins (Riley & Wertz 2002; Cascales *et al.* 2007), proteases (Rao *et al.* 1998) and bacterial appendages (Dionisio *et al.* 2002). It has been found that *E. coli* harbouring plasmids have a higher frequency of antimicrobial resistance (Uma *et al.* 2009), and that a higher incidence of plasmids reflect resistance to a wider variety of antimicrobials (Nsofor & Iroegbu 2013).

Plasmids are classified into incompatibility groups. Incompatibility grouping implys that not all plasmids can coexist in the same bacterial host due to their replication mechanisms. This means that plasmids that share the same replication mechanism or origin of replication cannot reside within the same host (Johnson & Nolan 2009). The plasmid replicon type determines the incompatibility group that it belongs to, with majority of *E. coli* plasmids belonging to incompatibility group F (Johnson & Nolan 2009). However Velappan *et al.* (2007) found that plasmid incompatibility is more complex and not solely based on replication mechanism, but rather plasmids are more compatible than previously thought. Plasmid compatibility depends on other factors such as plasmid size, copy number, toxicity and virulence genes harboured (Velappan *et al.* 2007).

Gyles *et al.* (1977) first discovered a plasmid (pCG86) that contained heat-labile (LT) and heat-stable (ST) enterotoxin genes as well as genes encoding resistance to tetracycline, streptomycin, sulphonamide and mercury in ETEC from piglets with diarrhoea. The authors conducted transconjugation experiments to determine the genetic component of this combined resistance and found that the transconjugants harboured three plasmids. Further analysis concluded that the three plasmids actually form a single plasmid, which dissociated into three separate plasmids after being transfered into the tranconjugate cells.

Mazaitis *et al.* (1981) analyzed the physical structure of the pCG86 plasmid isolated from ETEC in pigs in order to determine its origin. It was known that there were plasmids harbouring solely enterotoxin genes (Ent plasmids) and plasmids encoding resistance to many different antimicrobials (R-plasmids). It was therefore hypothesized that this plasmid pCG86

had arisen from the recombination of Ent plasmids and R-plasmids. Further analysis proposed that the recombined plasmid pCG86 may have been a product of recombination between EntP307 (an LT-ST plasmid) and an IncFII R-plasmid (resistance plasmid) or of an insertion of a tetracycline transposon and R-determinant into an EntP307 plasmid.

Enterotoxigenic *E. coli* plasmid pTC isolated from pigs was the first plasmid of animal origin to have been completely sequenced by Fekete *et al.* (2012). The plasmid is 91,019 bp in size and comprises of genes encoding STa and STb enterotoxins (toxin-specific locus – TSL), tetracycline resistance (Tn10 transposon), plasmid transfer (tra) responsible for self conjugation, ColE1-like origin of replication and plasmid maintenance/stability. The TSL region is a large 16,839 bp fragment containing heat-stable toxin *STa* and *STb* genes, 18 transposases, 4 hypothetical proteins and IS- elements. The Tn10 transposon region is 9,146 bp and encodes resistance genes *tetR*, *tetA* and *tetC* flanked by IS10 which is responsible for the independent mobility of this region. The plasmid transfer (tra) region is the largest, spanning a total of 33,729 bp. It was hypothesized that the pTC plasmid may have originated from the family of *Shigella* plasmids. Plasmid pTC has a selective advantage in that it is self-transmissible, enabling it to spread pathogenicity and antimicrobial factors. Most importantly, it was discovered that pTC from porcine ETEC differs from the pEntH10407 plasmid isolated from human ETEC, which indicates that there is low or no zoonotic potential of pTC (Fekete *et al.* 2012).

2.4.2.2.2.2 Transposons, integrons and cassette genes

Transposons are transposable elements that contain insertion sequences which are inverted- or direct-repeat sequences at the end. The inverted- or direct-repeat sequences enable transposons to insert and remove itself from anywhere in the genome. Transposons usually carry genes encoding antimicrobial resistance and virulence factors. Antimicrobial resistance genes in Gram-negative bacteria were also found to be associated with smaller mobile genetic elements called gene cassettes (Rechia & Hall 1995; Hall & Collis 1998), and integrons (Domingues *et al.* 2012). Integrons and gene cassettes were discovered along with resistance plasmids in the mid-1960's (Bennett 1999). These elements consist of the resistant gene and a recombination site, named 59-base element or 59-be, that allows for mobility (Hall & Collis 1998; Bennett 1999; White *et al.* 2001). Gene cassettes are small, ranging between 500 bp and 1000 bp (Bennett 1999). The structure of a gene cassette is made up of a single open reading

frame (ORF), a recombination site (attC), a short non-coding region which may contain a ribosome binding site, a start codon for the internal ORF and a stop codon consisting of a few inverted repeats which are the integrase binding domains (Gillings 2014). Some gene cassettes may also have more than one ORF, and others may not contain an ORF at all (Gillings 2014). Gene cassettes usually harbour antimicrobial resistance genes and some even contain genes for metabolic functions of bacteria (Domingues *et al.* 2012), plasmid maintenance, virulence, surface properties, DNA modification, phage functions, polysaccharide and amino acid biosynthesis, transporters and efflux systems (Gillings 2014). Cassettes are coupled with mobile companion elements which are integrons (found in transposons or defective transposon derivatives) (Domingues *et al.* 2012).

Integrons contain genes which encode cassette mobility, site-specific recombination and a promoter which enable the expression of the genes contained within the cassette (Domingues *et al.* 2012). Multiple gene cassettes, each coding for different antimicrobial resistance, combine with an integron to give rise to multiple antimicrobial resistance or multidrug resistance profiles (Hall & Collis 1998). Expression of the genes contained on gene cassettes are dependant on the promoter gene of an integrons, the level of expression of genes contained on a cassette are proportional to the distance from the promoter (Gillings 2014). In most cases, the genes found further away from the promoter may not be expressed. However, it has been found that some gene cassettes contain internal promoters that aid in the expression of genes that are further away from the integron promoter (Gillings 2014). Gene cassettes, integrons and transposons are able to shuffle between other transposons or plasmids, even across different species of bacteria (Domingues *et al.* 2012). Some cassettes located on integrons have lost their transposition genes and are unable to move independently, same with some transposons (Hall & Collis 1998). These mobilizable elements can however transfer via linkage to conjugative elements or transduction using bacteriophages (Domingues *et al.* 2012).

Intergrons play an important role in the transmission of antimicrobial resistance genes in animals (Mazel 2004; Gillings 2014). The components of integrons include a site-specific recombinase (intergrase *intI* gene), a cassette insertion site (attI1site - now IS) and a promoter (Ps) (Hall & Collis 1998; Bennett 1999; Mazel 2004; Gillings 2014). The conserved upstream intI and attI region is referred to as the 5'-conserved segment (5'-CS) (Hall & Collis 1998),

and the 3'-conserved segment (3'-CS) is located downstream (Bennett 1999). The 5'-CS is approximately 1.4 kb and the 3'-CS is approximately 2 kb (Bennett 1999). There are three classes of integrons, which are differentiated by the variation in the integrase genes (Mazel 2004).

Class 1 integrons have similar or identical sequences in the *intI* gene and attI1 site whereas this region of Class 2 and Class 3 integrons have very distinctive sequences. Class 1 integrons are widely spread and are mostly implicated in most integron-mediated antimicrobial resistance, and are commonly associated with the Tn402 transposon (Xu *et al.* 2007; Gillings 2014). The integrase gene of Class 2 integrons are non-functional in clinical samples as they are inactivated by an internal stop codon. They also have a limited gene cassette array and are usually associated with the Tn7 transposons (Xu *et al.* 2007; Gillings 2014). Class 3 integrons are mostly limited to Japan where they were discovered (Gillings 2014) and Portugal (Xu *et al.* 2007). They are comparable to Class 1 integrons in that they share similar but limited gene cassettes and evolved from the same transposon Tn402, but are arranged in opposite orientations (Collis *et al.* 2002). The integrase gene of Class 3 integrons is the least active compared to the other integron classes (Gillings 2014). Another class of integrons, called chromosomal superintegrons, have two structural variants relative to simple integrons. They are able to possess a larger number of cassettes and there is a high level of homology between the attC site of the cassettes unlike simple integrons (Mazel 2004). It is believed that superintegrons are ancestral to integrons (Mazel 2004; Xu *et al.* 2007; Gillings 2014).

Horizontal gene transfer (HGT) is the most common way for bacteria to transmit their pathogenic traits which reside on plasmids, transposons and integrons amongst each other (Hall & Collis 1998; Domingues *et al.* 2012). Horizontal gene transfer occurs via conjugation, transformation and transduction events (Bennett 2008; Huddleston 2014). Transference is possible both intra- and interspecies. The most common genetic element involved in HGT is plasmids as they contain transfer (tra) genes which enables them to conjugate or allow them to be self-transferrable (Norman *et al.* 2009; Venturini 2011). Conjugative plasmids are also able to enable the transfer of plasmids which are mobilisable (Bennett 2008; Norman *et al.* 2009).

2.4.3 Plasmid profiling

Pathogenic *E. coli* has been found to contain multiple plasmids of various sizes (Nsofor & Iroegbu 2013). The total plasmid content of a bacterial strain is known as the plasmid profile (Samadpour 2001), which is based on the size and number of plasmids contained within the cell (Myaing *et al.* 2005). Due to their traits, plasmids may be used to study epidemiology (Bradford 2001; Myaing *et al.* 2005) as well as understand evolution (Domingues *et al.* 2012) and antimicrobial resistance mechanisms (Samadpour 2001; Alam *et al.* 2013). Plasmid profiling was first used in 1981 by the Centre for Disease Control in order to determine which strain of *Salmonella* Muenchen had caused a gastroenteritis epidemic in a commuity smoking marijuana (Wachsmuth 1985). They were able to positively identify the strain of *S.* Muenchen that had caused the epidemic, due to the presence of two plasmids exclusively present in that particular strain isolated from the gastroenteritis cases, as opposed to the other *S.* Muenchen strains from unrelated outbreaks (Wachsmuth 1985). Plasmid profiling however has shortfalls for epidemiological applications due to the instability of plasmid DNA, high probability of genetic rearrangements, lack of plasmids in some samples and the possibility of the same plasmid content in other outbreak-unrelated samples (Serre 2006). Genomic tools for plasmid analysis have been created, and are highly informative as genomics allows for the analysis of the sequence information contained on plasmids. Also genomic techniques are reproducible, and the data can easily be shared between different labs.

Polymerase chain reaction (PCR) can be performed in order to determine the presence of plasmids in an isolate (Piece 2002). Specific primers have been designed for gene regions specific for each plasmid. Plasmid analysis using PCR has been utilized in various studies (Palmer *et al.* 2000; Rayamajhi *et al.* 2011; Rump *et al.* 2012). Palmer *et al.* (2000) developed a multiplex PCR to differentiate β-lactamase resistant plasmids found in *Neisseria gonorrhoeae* from different geographic regions. Rump *et al.* (2012) used PCR to determine the presence of plasmids in their analysis of enterohemorrhagic *E. coli* strains. Rayamajhi *et al.* (2011) performed PCR for plasmid replicon types to profile plasmids from *Escherichia fergusonii* and other Enterobacteriaceae isolated from farm animals. Another study performed by Mahony *et al.* (1993) compared different plasmid and chromosome-based PCR assays to determine the efficiency of detecting *Chlamydia trachomatis*. Polymerase chain reaction for plasmid analysis is a highly useful technique as it is simpler, requires lesss preparation-time and little amounts of sample material as compared to the other typical plasmid extraction and

analysis techniques (Palmer *et al.* 2000). The limitation of PCR however, is that the sequence of the starting material (plasmids) needs to be known in order to design specific primers for increased sensitivity (Palmer *et al.* 2000; Garibyan & Avashia 2013).

2.5 Epidemiological studies to characterise *E. coli*

2.5.1 Current epidemiological methods

It is important to identify, characterise and understand epidemiological disease-causing agents as the information discovered can assist in identifying the cause and source of disease, and the genetic diversity of the implicated isolates can assist in tracing the route of transmission (Grad *et al.* 2012). Two approaches were originally developed to track disease outbreaks. The first uses the identification of fast evolving, highly variable genetic loci using methods such as ribotyping, PCR with repetitive elementary primers and pulsed-field gel electrophoresis (PFGE) (Maiden *et al.* 1998). The second uses the identification of slowly evolving loci which are under neutral selection using multilocus enzyme electrophoresis (MLEE) (Urwin & Maiden 2003). Methods that make use of fast evolving loci are useful for short-term epidemiology such as for localised disease outbreaks, and to determine if the disease outbreak is caused by a single strain or different strains (Maiden *et al.* 1998). The method that makes use of slowly evolving loci is more useful for long-term epidemiology, looking at global disease outbreaks with the goal to determine the relatedness of strains isolated from different geographic areas (Maiden *et al.* 1998). Multilocus enzyme electrophoresis is also useful in identifying disease-causing lineages, and has been used to successfully identify clusters of closely-related strains, termed clones or clonal complexes, that are likely to cause diseases (Ashton 1990; Whittam *et al.* 1993). Multilocus enzyme electrophoresis does however have a major shortfall in that the data obtained is not reproducible and as a result, is not transferable between labs (Rajkhowa *et al.* 2010). A more refined and improved method that has been sought to overcome the limitations of MLEE is Multilocus sequence typing (MLST). Multilocus sequence typing makes use of actual nucleotide sequences in order to identify variation between strains (Maiden *et al.* 1998). The inception of high throughput next-generation sequencing has led to great improvements in epidemiological studies (Grad *et al.* 2012).

2.5.2 Multilocus sequence typing (MLST)

Multilocus sequence typing is an improved method from MLEE that is used for molecular typing of bacteria, more specifically pathogens, which involves the sequencing of bacterium housekeeping genes (Pagatto *et al.* 2005). This method was developed in 1998 for for improved epidemiological analysis of *Neisseria meningitidis* (Larsen *et al.* 2012). Multilocus sequence typing is applied in epidemiology and bacterial phylogenetics. Housekeeping genes are used for MLST analysis as they are under stabilizing, selective pressure therefore are slowly evolving (Urwin & Maiden 2003) and experience neutral genetic variation (Lemee *et al.* 2004; Woodford & Johnson 2004). They are ideal for global and long-term surveillance (Esimone *et al.* 2010) as they are conserved within a species (Pagatto *et al.* 2005). The allelic combination of all loci is defined as the sequence type (ST), which is then used to draw a phylogentic tree showing relatedness amongst strains. Mutational and recombination events that introduce diversity occur regularly and a number of genes are therefore used for typing in order to obtain better resolution to correctly group related strains. For this reason most studies use seven genes for MLST analysis (Maiden *et al.* 1998; Turrientes *et al.* 2014). The amplicon size of the housekeeping gene fragments ranges between 450-500 bp, which is ideal as it can be sequenced accurately on both strands and provides sufficient variation to identify a number of unique alleles within a population (Enright & Spratt 1999). Short-term epidemiology however requires greater variation between isolates, therefore genome sequence data is best used for this purpose as MLST may not provide enough discriminatory power (Pagatto *et al.* 2005).

Identified sequences for *E. coli* houskeeping genes are collected and stored on a large database managed by the University of Warwick, and is one of the schemes of PubMLST (http://mlst.warwick.ac.uk/mlst/mlst/dbs/Ecoli) (Wirth *et al.* 2006). Seven housekeeping genes are used for MLST analysis of *E. coli*, they are *icd* (isocitrate dehydrogenase); *mdh* (malate deydrogenase); *adk* (adenylate kinase); *fumC* (fumarate hydratase); *gyrB* (DNA gyrase); *recA* (ATP/GTP binding motif) and *purA* (adenylosuccinate dehydrogenase) (Wirth *et al.* 2006). The website has extensive information on housekeeping genes, primer sequences, PCR protocols and the allelic profile of *E. coli* MG1655 strain (Wirth *et al.* 2006). The data derived from sequencing can be entered into the website for each locus in order to determine if a new allele has been discovered or if a new strain contains a known allele (Aanensen & Spratt 2005).

Unfortunately no known literature for the MLST profile of South African ETEC is available. It is therefore important to characterise South African strains, so that we may be able to determine their relationship, and compare them with international strains.

2.6 Genomic tools

Whole-genome sequencing (WGS) by next-generation sequencing (NGS) tools allows for the sequencing of all genomic material present in the bacterial cell (Kisand & Lettieri 2013; Kwong *et al.* 2015). The first bacterial genome to be completely sequenced using random shotgun sequencing was *Haemophilus influenzae,* which was completed in 1995 (Fleischmann *et al.* 1995). Since then bacterial genome sequencing has become a standard method, as the cost of sequencing has been drastically reduced with the inception of next-generation sequencing tools (Land *et al.* 2015). Sequencing allows for the classification of bacteria and the information derived can be applied to various fields such as genome-scale metabolic modelling, biosurveillance, bioforensics and epidemiology (Land *et al.* 2015). Whole-genome sequencing of bacterial genomes has been used for numerous analyses such as evolution (Mwangi *et al.* 2007; Sahl *et al.* 2011; Wyrsch *et al.* 2015), epidemiology (Lewis *et al.* 2010; Aarestrup *et al.* 2012; Francis *et al.* 2013) plasmid and virulence profiling (Iguchi *et al.* 2009; Sahl *et al.* 2011; Wyrsch *et al.* 2015), gene-environment interactions (Pagatto *et al.* 2005) and the identification of metabolic pathways (Edwards & Palsson 2000; Thiele & Palsson 2010). Whole-genome seqquencing is highly advantageous as both chromosomal and extrachromosomal regions are sequenced. Sequencing of bacterial plasmids has also provided useful information, such as the evolution of plasmids (Kennedy *et al.* 2010; Rump *et al.* 2012).

A study conducted by Wyrsch *et al.* (2015) analysed two epidemiologically-unrelated ETEC O157 isolates from pigs, and determined their phylogenetic relationship with EHEC O157. The study identified virulence genes, antimicrobial and heavy metal resistance genes, mobile genetic elements (including plasmids carrying enterotoxin genes and scaffolds from plasmids originating from other bacterial species) and sequence types of the ETEC O157 isolates. Three strains of human ETEC H10407 (Crossman *et al.* 2010), E24377A (Sahl & Rasko 2012) and B2C (Vipin Madhavan *et al.* 2014); and animal ETEC W25K (Ren *et al.* 2014), UMNK88 and UMNF18 (Shepard *et al.* 2011) have been sequenced.

PlasmidFinder and ResFinder are web-based tools used for the *in silico* identification of plasmids and antimicrobial resistance genes from bacterial whole-genome sequence data. These tools are available from the Centre for Genomic Epidemiology website (www.geneticepidemiology.org). The consensus database used by the Centre for Genomic Epidemiology was constructed from the NCBI nucleotide database (Zankari *et al.* 2012; Carattoli *et al.* 2014). The ResFinder interface has been available for use since 2012 (Zankari *et al.* 2012) and PlasmidFinder was made available in 2014 (Carattoli *et al.* 2014). PlasmidFinder 1.2 identifies plasmid sequences from bacteria belonging to the Enterobacteriaceae and Gram-positive groups. The consensus database for PlasmidFinder contains 116 replicon sequences and 559 plasmid sequences from Enterobacteriaceae (Carattoli *et al.* 2014). ResFinder identifies horizontally acquired antimicrobial resistance gene sequences from 12 different antimicrobial classes which totals to more than 1862 resistance genes (Zankari *et al.* 2012). These tools are highly useful for the identification of plasmids and antimicrobial resistance genes present in bacterial isolates.

2.7 Summary and concluding remarks

The South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs (SANVAD) is a programme set up under the *Office International des Épizooties* guidelines to manage antimicrobial resistance in animals in South Africa (Henton *et al.* 2011). The Global Antibiotic Resistance Partnership (GARP) is another initiative formed between South Africa, India, Vietnam and Kenya. This partnership is responsible for assessing and addressing antimicrobial resistance in developing countries (Suleman & Meyer 2012; Moyane *et al.* 2013). The aim of these organisations is to address antimicrobial resistance and make strides in understanding and reducing antimicrobial resistance burden within the dynamics of each of these countries, with plans to extend it to other countries (Suleman & Meyer 2012; Moyane *et al.* 2013). The objectives of these initiative are to manage the use of antimicrobials in human health and livestock production better. Another objective is to reduce the demand for antimicrobials by reducing the incidence of infection in the hospital, community and on the farm (Winters & Gelband 2011).

In South Africa, not much research has been conducted regarding *E. coli* causing colibacillosis. Ateba and Bezuidenhout (2008) characterised *E. coli* O157:H7, isolated from pigs and other sources, based on antimicrobial sensitivity and also investigated the prevalence

of virulence gene. Ateba and Mbewe (2011) conducted a study to determine the presence of shiga toxin virulence gene in *E. coli* O157:H7 strains isolates from various sources (water, pig and cattle meat and faeces and human stools). Virulence genes were identified in *E. coli* O157:H7 from all sources tested, but pigs and pork products contained significantly higher levels of *E. coli* O157:H7 when compared to other sources. Ateba and Mbewe (2013) analysed the genetic relationship of *E. coli* O157:H7 isolated from different sources using various molecular methods such as ISR-, BOXAIR- and REP-PCR. The study reported that *E. coli* O157:H7 from cattle, pigs and pork, water and human stools were genetically related indicating human contamination may have occurred through consuming contaminated meat or water. These results indicated the inefficient hygiene management systems on the farm and downstream processes in the food sector as some isolates shared 80-100 % similarity (Ateba & Mbewe 2013).

A few studies have characterized and determined the relationship between *E. coli* isolated from pigs in South Africa. Moneoang and Bezuidenhout (2009) characterised enterococci and *E. coli* from communal and commercial pig farms using antimicrobial sensitivity testing. A dendogram was constructed using the antimicrobial sensitivity results. The study by Moneoang and Bezuidenhout (2009) reported that the *E. coli* isolates were resistant to all antimicrobials tested with the exception of vancomycin which was not used on the farms sampled. The clustering analysis indicated that the strains isolated from the two different farms were either closely related or have been exposed to similar antimicrobial treatment (Moneoang & Bezuidenhout 2009).

A study was performed by Mohlatlole *et al.* (2013) focusing on the prevalence of, and virulence factors of ETEC; STEC and EAST-1, the various toxins, fimbrial and non-fimbrial adhesin factors of *E. coli* strains isolated from South African pigs. The study reported the presence of ETEC, STEC and EAEC toxin genes as well as non-fimbrial adhesin factors (Mohlatlole *et al.* 2013). Fasina *et al.* (2015) performed a study on *E. coli* isolated from weaning piglets in South Africa suffering from colisepticaemia, the researchers found multidrug resistant enteroaggregative *E. coli* and the *EAST-1* enterotoxin gene present in these samples.

Chaora (2013) analysed the susceptibility of South African piglets to ETEC and EAEC infections. The study correlated *E. coli* adhesin phenotypes for F4, PAA and EAST-1 to polymorphisms at selected candidate genes for Mucin (*Muc4, Muc13* and *Muc20*) and the Transferrin receptor (TFRC). Lower levels of polymorphism at the Muc13 and TFRC loci, and moderate polymorphism at the Muc4 and Muc20 loci were reported. An association between pig breeds (Large White, indigenous and crossbred) and adherence intensity (adhesive, weakly adhesive and non-adhesive) of F4 and PAA was also reported. No association was found between F4 adhesion and candidate genotypes, and between the adhesin phenotypes and Muc4 (g.8227G>C; Muc20 g.191C>T) mutations.

Previous research has suggested the presence of MAR in *E. coli* strains in the South African livestock populations (Ateba & Bezuidenhout 2008; Moneoang & Bezuidenhout 2009; Malokosta *et al.* 2014; Sikhosana 2015) Ateba and Bezuidenhout (2008); (Moneoang & Bezuidenhout 2009; Malokosta *et al.* 2014; Sikhosana 2015). However, information gaps exist regarding the resistance mechanisms and antimicrobial resistant genes prevalent in the South African pig population. It has also been identified that pathogenic phenotypes such as non-fimbrial adhesin factors and toxin genes are present (Chaora 2013; Mohlatlole *et al.* 2013). Although it is generally known that plasmids are responsible for carrying and spreading genes encoding pathogenic traits, there is a need for the identification and characterisation of those plasmids prevalent in South Africa. Such information will be important in the use of effective antimicrobials and other disease control strategies.

CHAPTER 3

ISOLATION AND MULTILOCUS SEQUENCE TYPING (MLST) OF ENTEROTOXIGENIC *E. COLI* ISOLATES FROM THE SOUTH AFRICAN PIG POPULATION

Abstract

Enterotoxigenic *Escherichia coli* causes colibacillosis in young pigs during the neonatal, weaning and post-weaning stages. The symptoms of colibacillosis include excessive diarrheoa, weightloss, severe dehydration and eventual death. Epidemiological studies of disease causing agents are important to help understand the pathogenicity and spread of disease and facilitate proper control measures. Multilocus sequence typing is an epidermiological tool that uses sequence data in order to determine the sequence types and clonal complexes of bacterial isolates. The sequence types can be used to determine the phylogenetic relationship and evolution of the isolates. The aim of this study was to determine the sequence types and phylogeny of ETEC affecting young pigs in South Africa, and to infer the relationship between the South African and international isolates. Multilocus sequence typing was performed using whole-genome sequencing at 30X coverage on the Illumina HiSeq 2500 sequencing platform. The MLST analysis showed diversity within the South African *E. coli* isolates that belonged to seven different sequence types. The sequence types observed include ST1830 which was the most prevalent in 24% (n=3) of the population ST10 and ST101 which were both prevalent at 18% (n=2) each, and ST4704; ST5766 and ST56 which were each prevalent in 9% (n=1) of the sample population. The sequence types observed were associated with pathogenic strains of *E. coli* such as ST10 and ST101, which harbour extended spectrum β-lactamases. ST1830 is is part if the clonal complex 10 which includes ST10. Phylogenetic analysis revealed that ST10 was the earliest evolved ST and ST5766 was the most recent evolved ST. Some of the South African samples clustered together whilst three STs were dispersed throughout the phylogeny. The South African samples clustered with STs from Japan, Hungary, Brazil and the UK. The results informed on the diversity and pathogenicity levels of the South African isolates and assited with understanding the epidemiology of the South African ETEC isolates, which is important for developing better control strategies for colibasillosis.

Keywords: *pig; enterotoxigenic E. coli; Multilocus sequence typing; Next-generation sequencing; sequence types*

3.1 Introduction

Multilocus sequence typing (MLST) involves analysing allelic sequences of housekeeping genes in order to track long-term/global epidemiology of microbes (Cooper & Feil 2004; Aanensen & Spratt 2005). Multilocus sequence typing is used in many fields, such as epidemiology to track global disease outbreaks, evolutionary biology of microbial genomes and phylogenetic analysis to determine relationships between isolates (Sabat *et al.* 2013). Actual sequence information is used to identify variation at the nucleotide level (Feil & Enright 2004). The unique sequence of each allele is assigned a random integer number and the combination of alleles at each locus is termed an "allelic profile" and gives the sequence type (ST) (Enright & Spratt 1999; Larsen *et al.* 2012). A total of 7 housekeeping genes (Feil *et al.* 2000; Aanensen & Spratt 2005) are used to determine the MLST profile of *E. coli* (Wirth *et al.* 2006), with increased accuracy and sufficient discrimination of isolates (Larsen *et al.* 2012). Housekeeping genes evolve slowly and are under stabilizing, selective pressure and therefore ideal for MLST. In MLST analysis, loci that conform to housekeeping standards are used to identify clonal linkages whilst loci that are hypervariable can be used to analyze specific clones and to look for evidence of microevolution (Cooper & Feil 2004).

Sequence typing of ETEC is important as it will inform on what strains are present in the population and their epidemiology. Numerous typing methods exist such as pulsed-field gel electrophoresis, serotyping, randomly amplified polymorphic DNA, repetitive-element PCR, multilocus-enzyme electrophoresis, multilocus sequence typing and whole-genome sequencing to name a few (Sabat *et al.* 2013). Although serotyping is highly useful, its major shortfall is that is requires specific anti-sera, which are not always readily available and are expensive (Girardini *et al.* 2012). Molecular methods such as PCR and whole-genome sequencing are better in that the results are reproducible, quickly generated and highly efficient (Tang *et al.* 1997; Sabat *et al.* 2013).

Numerous MLST databases have been created for fungal and bacterial strains which can all be accessed from the centralized PubMLST server which is hosted by the University of Oxford (http://pubmlst.org) (Larsen *et al.* 2012). The MLST database for *E. coli* is hosted by

the University of Warwick found at (http://mlst.warwick.ac.uk/mlst/mlst/dbs/Ecoli/) (Wirth *et al.* 2006). This database contains information on seven housekeeping gene as well as protocols for the amplification of these alleles using PCR. An allelic profile is the combination of the seven alleles and is known as the isolate's sequence type (ST). The ST can be obtained by amplifying an approximate 500 bp product of each housekeeping locus, sequencing, and seaching the seven query sequences against what is present on the database (Wirth *et al.* 2006). A dendogram showing the phylogenetic relationship can be constructed from the sequences using methods such as unweighted pair group method with arithmetic mean (UPGMA) and Neighbour-Joining (Feil & Enright 2004).

The seven housekepping genes used for *E. coli* MLST include *icd* (isocitrate dehydrogenase); *mdh* (malate deydrogenase); *adk* (adenylate kinase); *fumC* (fumarate hydratase); *gyrB* (DNA gyrase); *recA* (ATP/GTP binding motif) and *purA* (adenylosuccinate dehydrogenase) (Wirth *et al.* 2006). Traditionally MLST is performed by doing PCR to amplify the internal sequences of each loci followed by sequencing of the PCR products, this process is time consuming and costly (Larsen *et al.* 2012). Next-generation sequencing has drastically reduced the cost of sequencing whole bacterial genomes, which makes analysis more rapid and efficient. Software for analysis of WGS data is often freely available (Larsen *et al.* 2012). The Centre for Genomic Epidemiology database is a centralised server that allows for MLST analysis of a large number of microorganisms, found at (www.cbs.dtu.dk/services/MLST). The database uses whole-genome sequence data or short sequence reads in order to determine allele identity and sequence types. The database uses the BLAST algorithm to search the bacterial genome for alleles at each of the 7 MLST loci. The best matching allele is selected and the sequence type of the sample determined as the combination of matched alleles (Larsen *et al.* 2012). The database was curated in 2011 and is available as freeware for the analysis of whole-genome sequence data (Larsen *et al.* 2012). The resulting output includes the nucleotide sequence of matching alleles, which can be used to construct a phylogenetic tree (Larsen *et al.* 2012).

Another software that is freely available for the analysis of MLST data is Sequence Type Analysis Recombinational Tests (START) which is available from PubMLST (updated to START2 (http://pubmlst.org/software/analysis/start2/) (Jolley *et al.* 2001). This software is able to perform data summary, lineage assignment, tests for recombination, test for selection

and to contruct phylogenetic trees (Jolley *et al.* 2001). The software can be downloaded to you computer and is extremely useful and user friendly.

Bacterial populations are thought to be highly clonal, meaning that bacterial isolates form a cluster of very closely-related genotypes, often termed clonal complexes (CCs). These CCs are slowly evolving and are dispersed over wide geographical regions (Feil & Enright 2004). The founding genotype or clone of each CC is considered to be the genotype that differs from the highest number of other genotypes in that complex at one out of the seven loci (Feil & Enright 2004). A previous study observed that vancomycin-resistant Enterococci isolated from pigs in Denmark and Switzerland were of the same clonal complex (Boerlin *et al.* 2001) indicating that all of the sequence types evolved from a single clone (Cooper & Feil 2004) regardless of geographical separation.

The few studies on *E. coli* in animals in South Africa reported that ETEC is prevalent in livestock including pigs. Enterotoxigenic *E. coli* was isolated from beef and pig at a frequency of 3.8 % in a study looking at the detection of *E. coli, Staphylococcus aureus* and *Salmonella* from pig and beef abattoirs (Tanih *et al.* 2015). Henton and Engelbrecht (1997) serotyped *E. coli* causing colibacillosis collected from pig samples collected over a 20 year period. Mohlatlole *et al.* (2013) analysed virulent *E. coli* strains from neonatal and weaning piglets and reported ETEC to be prevalent in 55.1 % of the samples tested. Chaora (2013) determined the breed susceptibility of South African pig populations (i.e. Large White, Landrace, Duroc, Indigenous breeds and crossbreeds) to ETEC infections, and Sikhosana (2015) determined the prevalence of and the antimicrobial resistance of the isolates. There is not much information available regarding ETEC sequence types and phylogeny and none that analysed the relationship of South African sequence types to international isolates. This study performed multilocus sequence typing using next-generation whole-genome sequencing tools to determine the relation within South African *E. coli* isolates and between South African and international *E. coli*. The aim of this study was to determine the sequence type, resulting clonal complexes and phylogenetic relationship of enterotoxigenic *E. coli* strains isolates from neonatal and weaning piglets sampled from two provinces of South Africa.

3.2 Materials and methods

3.2.1 Background of samples

This study was part of a larger study (Chaora 2013; Sikhosana 2015) for which *E. coli* isolates were screened and characterised. For this study, only samples positive for enterotoxigenic *E. coli* were analysed (Chaora 2013; Sikhosana 2015). These isolates were collected from two provinces of South Africa from the Thohoyandou village in Limpopo province and King Williams Town, Umtata, Alice and Port St Johns in the Eastern Cape province. Pigs ranging between 4 days and 16 weeks old belonging to the indigenous, Large White, Duroc and exotic breeds were included in the study. The isolates were from neonatal piglets that were feed only milk and were healthy and did not show signs of diarrhoea.

In the study conducted by Sikhosana (2015), thirty six isolates tested positive for ETEC based on the presence of enterotoxin genes (*LT, STa* and *STb*) and fimbrial genes (*F4, F5, F6, F18* and *F4*1). Of these 36 isolates, 11 were used in this study.

3.2.2 Culturing of bacterial isolates

The 11 isolates positive for ETEC were inoculated from freeze-dried samples onto nutrient broth and grown overnight at 37°C. A loopful of culture was then streaked onto MacConkey agar plates and incubated overnight at 37°C. Pure single colonies were thereafter streaked onto nutrient agar plates and grown overnight at 37°C.

3.2.3 DNA extraction for whole-genome sequencing

DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufactures protocol for DNA extraction from Gram-negative bacteria. One ml of overnight culture was pippetted into 1.5 ml microcentrifuge tube and centrigufed at 500 rpm for 10 minutes using the Eppendorf MiniSpin centrifuge (Eppendorf, Germany). The supernatant was discarded and the cell pellet kept for DNA extraction. Thereafter the DNA was extracted following the manufactures protocol.

The cell pellet was resuspended into 180 µl Buffer ATL and 20 µl proteinase K added, and thereafter vortexed thoroughly and incubated at 56°C for two hours on a shaker. The suspension was then vortexed thoroughly, 200 µl Buffer AL and 200 µl ethanol (96-100%)

was added and further vortexed. The suspension was then pippetted into the DNeasy mini spin column placed in a two ml collection tube and centrifuged at 10 000 rpm for one minute. The flow-through and collection tube were discarded and the spin column placed into a clean two ml collection tube. Buffer AW1 (500 µl) was added to the spin column and centrifuged at 10 000 rpm for one minute, the flow through and collection tube were discarded and the spin column placed into a clean two ml collection tube. Buffer AW2 (500 µl) was added to the spin column and centrifuged for three minutes a 13 000 rpm, the flow through and collection tube was discarded and the spin column placed into a clean 1.5 ml microcentrifuge tube. The DNA was then eluted from the spin column by pippetting 50 µl Buffer AE directly onto the spin column, which was then incubated at room temperature for five minutes and thereafter centrifuged at 13 000 rpm for one minutes to increase DNA yield. The DNA was stored at -20°C until use.

3.2.4 Whole-genome sequencing and assembly

Extracted DNA from ETEC positive isolates was sent to the Agricultural Research Council Biotechnology Sequencing Platform for sequencing. The genomes were sequenced at 30X coverage, using 125 x 125 bp paired-end sequencing at the Illumina HiSeq 2500 sequencer and 0.5 GB sequence data was generated.

CLC-Bio v6 was used to analyse sequence data for Quality Control (QC) and *De novo* assembly of contigs. Quality Control was performed directly on sequenced reads. Reads were trimmed to remove adapters and screened for quality and ambiguity using default CLC-Bio parameters. Nextera v2 transposase 2 adapter sequences, regions of ambiguity and low quality reads were trimmed. The trim settings were as follows: 0.05 limit of low quality reads, limit of two for ambugious nucleotides and a PHRED score of 30, reads below 15 bp and above 1000 base pairs were discarded.

3.2.5 Multilocus Sequence Typing

The Multilocus Sequence Typing interface on Centre for Genomic Epidemiology (CGE-MLST) database was used to analyse MLST sequence types using whole-genome data (https://cge.cbs.dtu.dk/services/MLST-1.8/) (Larsen *et al.* 2012). Sequence files following QC were uploaded onto the server and analysed using the *E. coli* scheme1 (genes: *adk, icd, fumC, mdh, gyrB, purA* and *recA*) (Wirth *et al.* 2006). The MLST *E. coli* database at The University

of Warwick (UoWMLST) (http://mlst.warwick.ac.uk/mlst/mlst/dbs/Ecoli/) (Wirth *et al.* 2006) was used to confrm the sequence types.

3.2.6 Phylogenetic analysis

Phylogenetic analysis was performed in order to determine the population structure of the sample population using MLST sequence data. Phylogenetic analysis was also performed for South African samples and international ETEC samples isolated from pigs to determine the relationship between local samples and international samples. Sequence information for the international samples were taken from the MLST *E. coli* database on http://mlst.warwick.ac.uk/mlst/mlst/dbs/Ecoli/) (Wirth *et al.* 2006). The data was analysed using Sequence Type Analysis Recombinational Tests (START) software using unweighted pair group method with arithmetic averages (UPGMA) (Jolley *et al.* 2001).

3.3 Results

3.3.1 Whole-genome sequencing and assembly

The trimming results from CLC-Bio are summerized in Table 3.1. The number of reads before trimming ranged from 2.2 million to 7.9 million and the average length for all the samples was 125.0 bp. After trimming, the number of reads ranged from 2.1 million to 7.8 million with the average length ranging from 103.0 bp to 113.5 bp. The percentage of reads after trimming was above of 98 %. The number of contigs from *De novo* assembly ranged between 90 bp and 3021 bp. The contig lengths ranged between 182 for the smallest contig, and the largest contig was 421744 bp.

Table 3.1 Summary of trimming results

Sample	Before	After quality control			*De novo*	
	Number of	Number of	% reads	Average	Number of	Contigs
9	3,319 194	3, 269 406	98. 5	108.1	128	182 - 321486
11	7,578 760	7,481 572	98.72	104.7	630	200 - 202375
13	2,197 312	2,166 593	98.6	113.5	397	156 - 363480
19	6,715 488	6,644 246	98.94	104.3	3021	199 - 166345
35	6,610 176	6,531 886	98.82	103.2	1842	199 - 338956
40	2,982 314	2,938 037	98.52	108.3	198	201 - 295894
41	4,728 798	4,677 373	98.91	105.0	134	204 - 320650
46	6,731 096	6,645 091	98.72	105.3	1272	200 - 105262
47	7,983 606	7,886 453	98.78	103.0	98	204 - 397173
97	5,441 214	5,373 155	98.75	105.2	90	180 - 421744
121	3,045 714	3,007 335	98.74	105.5	86	210 - 397180

3.3.2 MLST using whole-genome sequencing

The 11 samples analysed for MLST had 100 % identity to each loci, with the exception of sample number 46 at the mdh locus, which had a 99.78 % identity due to a point mutation of G→A (Table 3.2). The number of shared alleles per gene amongst the samples gives an indication of the population gene diversity. From the 11 samples, *fumC, icd* and *adk* only had four different alleles. Genes *gyrB, mdh* and *adk* only had three different alleles (amongst the 11 samples, and *purA* had a total of 5 different alleles shared by the 11 samples.

	ETEC MLST alleles							
Sample	***fumC***	***icd***	***purA***	***adk***	***gyrB***	***mdh***	***recA***	**Sequence type**
9	4	18	5	6	4	24	14	56
11	41	18	7	43	15	11	6	101
13	4	1	18	6	1	8	2	5766
19	11	8	8	10	4	8	2	10
35	4	1	18	6	1	8	2	5766
40	11	8	302	10	4	8	2	4253
41	41	18	7	43	15	11	6	101
46	11	10	8	10	4	8[a]	2	4704
47	290	8	8	10	4	8	2	1830
97	290	8	8	10	4	8	2	1830
121	290	8	8	10	4	8	2	1830

a – 99.78 identity (G→A point mutation)

3.3.3 Frequencies of sequence types

Seven different sequence types were observed from the 11 samples in the frequencies of nine percent for ST56, ST10, ST4704 and ST4253, 18 % for ST101 and ST5766, and 27 % for ST1830. All of the sequence types were identifiable on CGE-MLST and UoWMLST, except for ST5766, which was only identified on the UoWMLST database. Only three sequence types clustered into clonal complexes with ST56 in CC155, ST101 in CC101 and ST10 in CC10. The other STs did not cluster into any CCs (Table 3.3).

Table 3.3 The clonal complex and frequency of MLST sequence types

Clonal complex	**CC10**	**CC10**	**CC101**	**CC155**	**none**	**none**	**none**
Sequence type	ST10	ST1830	ST101	ST56	ST4253	ST4704	ST5766
Frequency (%)	9 (1)	27 (3)	18 (2)	9 (1)	9 (1)	9 (1)	18 (2)

Number in brackets denotes absolute number

3.3.4 Phylogenetic analysis

The Sequence Type Analysis Recombinational Tests (START) software (Jolley *et al.* 2001) was used to determine the population structure of the South Africa ETEC samples, and also to determine the phylogenetic relationship with international ETEC samples derived from pigs. Four major clades were identified (Figure 3.1). The first clade consists of two samples belonging to ST5766 and the second clade only has one sample which belongs to ST56. The third major clade collapsed into four subclades that included ST4253, ST4704 and ST1830. The fourth major clade consisted of ST101. The population structure shows that ST101 is that oldest clade, and ST5766 is the youngest clade and these two STs do not share any similar alleles, nor does ST101 share any alleles with any of the other STs.

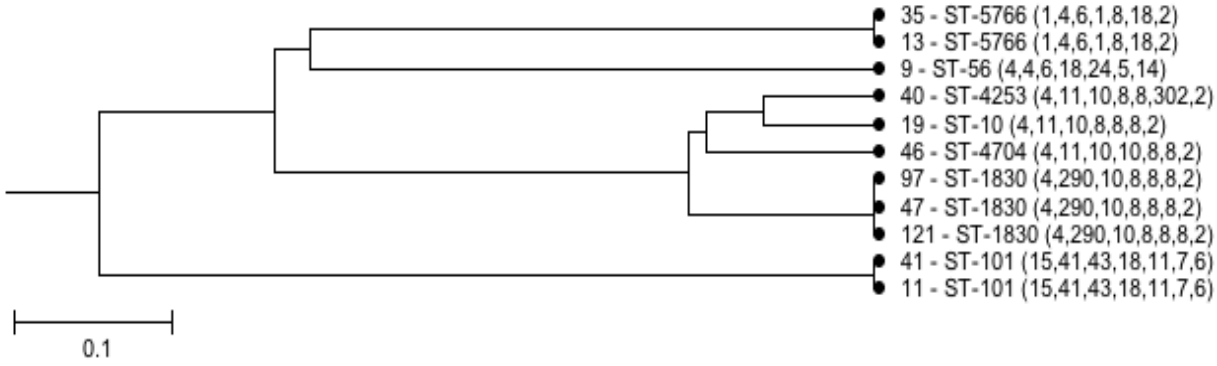

Figure 3.1 Phylogenetic relationship between South African ETEC samples isolated from pigs using UPGMA. The scale bar indicates linkage distance.

The relationship between the South African and international ETEC samples still show the same evolutionary pattern seen in Figure 3.1, whereby ST101 was an earlier evolved clade and ST5766 is one of the younger evolved sequence types. The South African samples however were interspersed within the international samples as the phylogeny is based on allele similarity and shows the evolution of the samples. A total of 30 STs all originating from 42 samples of pig ETEC samples in eight countries were included in the phylogeny (Table 3.4). The international samples originated from Australia, Brazil, Canada, Hungary, Japan, United Kingdom and USA respresenting all the continents. The South African STs formed four separate clades, one clade clustered with Brazilian STs and the other clade clustered with the Hungarian STs. The third clade which only has one sample ST56 did not cluster with any other samples, whilst the fourth clade which included two samples, ST101 also did not cluster with any other samples. Samples from other countries are interspersed on the tree.

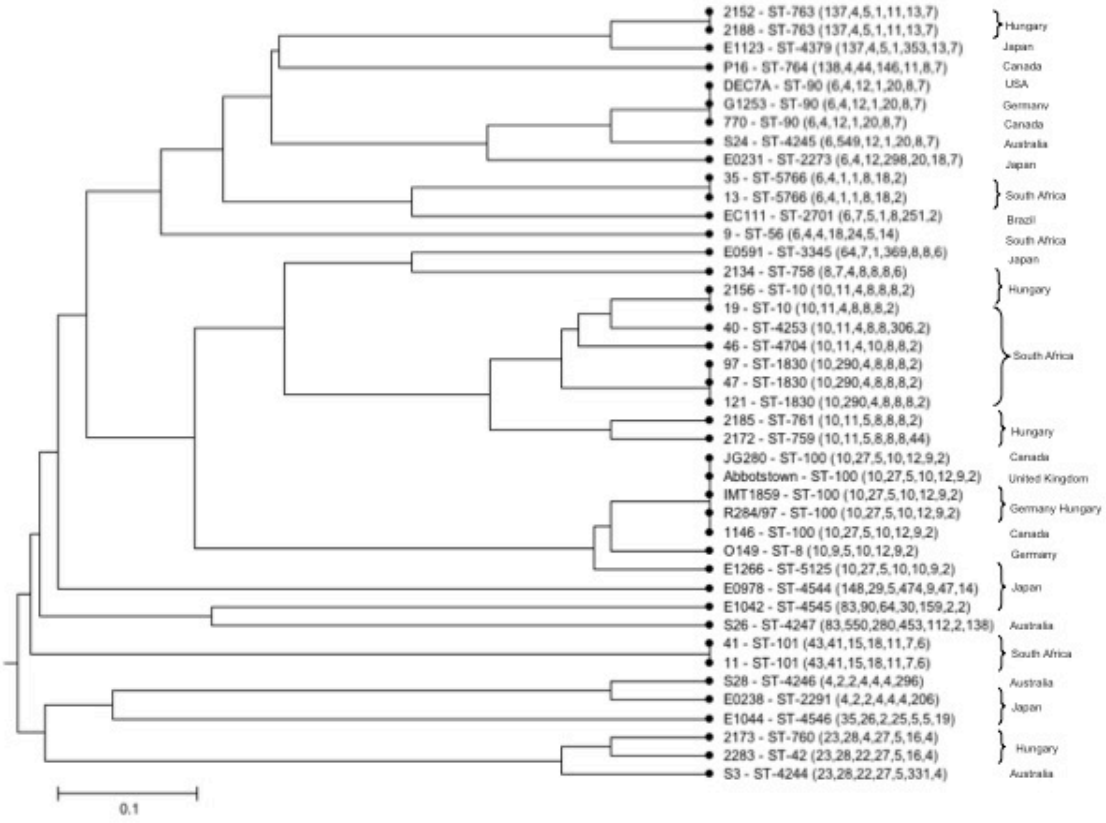

Figure 3.2 Phylogenetic relationship between South African and international ETEC samples isolated from pigs using UPGMA. The sequence types are shown next to the sample number and the alleles are given in brackets. The sample country origin is on the right panel. The scale bar indicates linkage distance.

Table 3.4 Summary of phylogenetic tree of international enterotoxigenic *E. coli* samples

Clade	Number of isolates	Sequence Types	Geographic locations
1	3	ST763 (2), ST4379 (1)	Hungary (2) Japan (1)
2	1	ST764	Canada
3	5	ST90 (3), ST4245 (1), ST 2273 (1)	USA, Germany, Canada, Australia, Japan
4	3	ST5766 (2), ST2701 (1)	South Africa (2), Brazil (1)
5	1	ST56	South Africa
6	11	ST3345, ST758, ST10 (2), ST4253, ST4704, ST1830 (3), ST761, ST759	Japan (1), Hungary (4), South Africa (6)
7	7	ST100 (5), ST8, ST5125	Canada (2), United Kingdom, Germany (3), Japan
8	1	ST4544	Japan
9	2	ST4545, ST4247	Japan, Australia
10	2	ST101	South Africa
11	2	ST4246, ST2291	Australia, Japan
12	1	ST4546	Japan
13	3	ST760, ST42, ST4244	Hungary (2), Australia
Total	42	30	8

3.4 Discussion

The population dynamics of ETEC isolated from pigs in South Africa are not known. Multilocus sequence typing is a technology that allows for the analysis of molecular epidemiology and population structures of bacteria, as it makes use of the core genome which comprises housekeeping genes and therefore reflects microevolution (Ewers *et al.* 2012). An array of MLST typing schemes have been developed for *E. coli*, ranging from the inclusion of 5 – 10 housekeeping genes, but the most widely used scheme includes a combination of seven genes namely; *adk, icd, fumC, mdh, gyrB, purA* and *recA* which are used to determine the sequence type of the bacteria (Larsen *et al.* 2012). The aim of the study was to determine the

sequence types of enterotoxigenic *E. coli* present in the South African pig population and to determine their phylogenetic relationship.

The samples used in this study were taken from pigs from both the commercial and communal (village) sectors from six towns encompassing two provinces, Eastern Cape and Limpopo. The management practices of these two sectors are quite different. Neonatal pigs were allowed to suckle from the sow, and the diet of weaning piglets differed between the commercial and village farming. Commercial piglets were fed a diet of pig grower and the village piglets were left to scavenge and/or fed household kitchen leftover. Commercial pigs are kept in houses whereas the village pigs are left to scavenge and roam freely. Commercial pigs were given antimicrobials on a regular basis whereas village pigs had little or no antimicrobial influence. As a result of the differences between the farming management systems, it was expected that the ETEC isolates would be largely diverse.

Chaora (2013) performed a study on a larger number of this pig population to determine their susceptibility to *E. coli* infections particularly with regard to association between *E. coli* F4ab/ac fimbraie and candidate genes, mucin (*MUC4, MUC13, MUC20*) and transferring receptor (TFRC), encoding receptor cells within the pig. It was found that *MUC13* and *TFRC* loci were not polymorphic in the populations analysed and the susceptible alleles C at these two loci were close to fixation at over 90 % of the three breeds (local, imported and crossbreed) analysed. The other two candidate genes *MUC4* and *MUC20* were polymorphic and the resistant allele G for *MUC4* and C for *MUC20* were present mostly in the imported breeds (Landrace, Large White and Duroc). Chaora (2013) also analysed the adhesion of ETEC strains F4, PAA and EAST-1 for intestinal brush borders in these three pig breeds. The study found that the indigenous pigs had the highest frequency of adhesive, weakly adhesive and non-adhesive intestines, followed by the crossbred and then the Large-White populations. Adhesion was higher in suckling than weaning piglets from the indigenous and crossbred populations, indicating that neonatal piglets are more susceptible to ETEC infections. The indigenous population may have had a higher susceptibility to ETEC infection due to the farming practice used to rear them, whereas the Large White had improved management with the use of antimicrobials and vaccination of sows thus reducing their susceptibility to ETEC. The results of the Chaora (2013) study highlight variation in this population with regards to their virulence genes and response to ETEC infections.

Whole-genome sequence data was used to analyse MLST loci of the samples in order to determine their sequence type and phylogenetic relationship. The MLST *E. coli* scheme 1 server on the Centre for Genomic Epidemiology database was used for the analysis. The seven MLST loci *adk*, *icd*, *fumC*, *mdh*, g*yrB*, *purA* and *recA* had alleles that ranged between 450 and 550 bp. The MLST genes from the samples had 100 % identity match to already known alleles, with the exception of the *mdh* allele for sample 46 which had 99.87 % match. The sequence type was determined from the combination of the six alleles, and the sequences types are further grouped into clonal complexes which is a cluster of closely-related genotypes.

Whole-genome sequencing is extremly efficient in that it allows for the screening of all seven MLST loci concurrently and there are MLST databases which allow for immediate screening (Larsen *et al.* 2012). Sanger sequencing however, requires the amplification of each individual gene using PCR, followed by separate sequencing of each gene, thereafter each gene sequence data must be entered onto the MLST database to screen for the different alleles (Köser *et al.* 2012). Therefore WGS is better than Sanger sequencing in that it reduces hands on time, and overall cost (Köser *et al.* 2012; Larsen *et al.* 2012). Ashton *et al.* (2016) typed *Salmonella* species using WGS performed on the Illumina HiSeq 2500, which is the same sequencing platform used in this study and used Short Read Sequence Typing to assign sequence types. Winstanley *et al.* (2015) characterised *Campylobacter* species, also using the Illumina HiSeq sequencing platform and used an in-house bioinformatics pipeline to extract and analyse MLST data. Jolley *et al.* (2012) analysed a meningococcal outbreak caused by *Neisseria meningitidis* using the Illumina sequencing platform and used BIGSdb Genome Comparator from PubMLST to analyse MLST data.

Multilocus sequence typing identified seven different STs originating from 11 samples in this study. This result is similar to that found in other studies whereby an increased number of STs were identified in their samples. A study performed in Denmark and Norway on sulphonamide resistant *E. coli* collected from clinical, animal and meat samples identified 45 different STs from 68 samples (Trobos *et al.* 2009). Thirty-three ETEC samples isolated from Bolivian children clustered into 18 different STs (Rodas *et al.* 2011). Kusumoto *et al.* (2014) identified 33 different STs from 50 ETEC samples isolated from pigs in Japan. This abundance of various STs is possible as ETEC is polyphyletic meaning that it does not

originate from a specific clonal lineage and therefore is not restricted to any particular phylogenetic group (Turner *et al.* 2006).

The different STs identified include ST10, ST56, ST101, ST1830, ST4253, ST4704 and ST5766. Three of the STs belonged to clonal complexes (CC), ST10 and ST1830 (CC10), ST56 (CC155) and ST101 (CC101). The other three STs did not group into any known CC and are known as singletons. Clonal complexes are formed amongst bacterial populations that experience homologous recombination, which are bacterial STs that differ from each other by not more than two alleles (Wirth *et al.* 2006). Clonal complexes differ from each other by three or more loci (Wirth *et al.* 2006).

The most prevalent sequence type was ST1830 observed in 27 % of the population. Izdebski *et al.* (2013) isolated ST1830 from their study on *E. coli* harbouring extended-spectrum β-lactamases and AmpC-type cephalosprinase isolated from humans. Accoding to Izdebski *et al.* (2013) ST1830 belongs to CC10. This CC also includes ST10, which was identified in 1 % of this study population. Sequence type 10 was also isolated in the studies by Izdebski *et al.* (2013) from *E. coli*, and Nyholm *et al.* (2015) from ETEC and STEC at low frequencies. Bergeron *et al.* (2012) isolated *E. coli* ST10 from chicken, human and pork samples in their study performed in Canada. Trobos *et al.* (2009) found *E. coli* samples isolated from pig, poultry and humans belonging to CC10, and Okeke *et al.* (2011) isolated enteroagregative *E. coli* belonging to CC10 from children in Nigeria. Clonal complex 10 is the largest clonal complex of *E. coli* and spans across various species and geographic locations (Trobos *et al.* 2009). This CC is part of the *E. coli* clones that are associated with extended-spectrum β-lactamases and AmpC-type cephalosprinases and is widely distributed in the world (Ewers *et al.* 2012; Izdebski *et al.* 2013).

Sequence type 101 was found in two percent of the ETEC population. *E. coli* samples belonging to ST101 is associated with the New-Delhi metallo-β-lactamase (NDM-1) which is a class B carbapenemase first isolated from a Swedish patient in New Delhi, India (Mushtaq *et al.* 2011; Peirano *et al.* 2011). Hammerum *et al.* (2012) also isolated ST101 in their study of ETEC from pigs and they had detected *blaCTX-M*- resistance genes. Wu *et al.* (2012) isolated this ST from extra-intestinal *E. coli* samples from cows and sheep and Tartof *et al.* (2005) isolated this ST from pigs and cows. Trobos *et al.* (2009) isolated ST101 in their study

on *E. coli* samples isolated from pig, poultry and humans. Bacteria carrying this ST are Gram-negative and mainly include *E. coli* and *Klebsiella pneumoniae* and have been found to be spread over 50 countries (Liu *et al.* 2014).

Hauser *et al.* (2013) observed ST10, ST101 and ST56 in their study comparing food-borne shiga-toxin *E. coli* with haemolytic-uremic syndrome *E. coli*. ST56 belongs to CC155, and this ST was also found in the study by Trobos *et al.* (2009). Izdebski *et al.* (2013) isolated STs belonging to ST155 in their study. Unfortunalty no literature was found regarding ST4704, ST4253 and ST5766 identified in this study, these STs have been identified by other researchers but the data is not yet published.

Enterotoxigenic *E. coli* affects both humans and animals, and host specifity is determined by colonization factors, which are encoded on mobile genetic elements. The same STs have been found in both animals and human ETEC samples (eg. ST10 and ST29) which indicate that different lineages of ETEC are not absolutely host specific and a particular genetic backbone is not necessary for species specificity (Turner *et al.* 2006). This result was also seen in the study by Trobos *et al.* (2009) which found *E. coli* of the same STs and clonal complexes from sources of poultry, pig and human and in different geographic locations.

Phylogenetic analysis showed that genetic structure of the South African ETEC isolates is diverse and clusters into four major clades. It was observed that ST101 is older than the other sequence types in terms of evolution and this is reflected in the international phylogenetic tree aswell. Sequence type 101 is in a stand-alone clade and does not share alleles with the other STs identified. Poirel *et al.* (2010) states that the NDM-1 ST101 is distantly related to the other metallo-β-lactamase, this observation is reflected in the present study. The STs in clade 3 share two alleles, and the samples in clades 1 and 2 also share two alleles. The clades are grouped solely based on their alleles, and does not reflect any geographic relation as the isolates from Limpopo and the Eastern Cape are completely dispersed within the tree. Other studies also observed similar patterns whereby STs do not group according to geographic location (Turner *et al.* 2006; Deng *et al.* 2015). The lack of geographic relationship in the phylogenetic trees is also reflected in Figure 3.2 whereby ST90 and ST100 were isolated in different geographic locations, even across different continents and Table 3.4 shows that STs found within the same clade are distributed across various locations. Turner *et al.* (2006)

found that ETEC STs are not geographically exclusive, the same STs occur in different geographic locations around the globe as seen in this study and the studies.

Figure 3.2 is a phylogenetic tree that includes the South African isolates with ETEC isolated from pigs from international locations. The tree has 30 different STs which group into 13 clades resulting from 42 samples originating from eight countries. The STs from different countries are dispersed throughout the tree, this result is typical of ETEC STs which has a polyphyletic origin (Turner *et al.* 2006). The South African clade 6 (Figure 3.2) with the most samples clustered closely with the Hungarian samples 2134 and 2156, 2185 and 2172 and the Japanese E0591. Strains 2156, 2185 and 2172 all belong to the O149 serogroup and have the same electrophoretic type (ET-17) from Multilocus enzyme electerophoresis analysis (Nagy & Fekete 1999) therefore their clustering is expected. The Hungarian 2134 strain which belongs to the O157 serogroup has a different ET clusters together with the Japanese E0591. These Hungarian isolates are implicated in piglet diarrhoea (Olasz *et al.* 2005). Two South African isolates, 13 and 35, clustered together with the Brazilain isolate Ec111 (Clade 4), which is a commensal *E. coli* animal isolate associated with biofilm production (Rendueles *et al.* 2011) isolated from feacal samples from Brazilian pigs (Wirth *et al.* 2006). South African isolates 41 and 11 belonging to ST101 (Clade 10) form an isolated cluster whilst isolate 9 belonging to ST56 (Clade 5) did not cluster with any of the international isolates.

3.5 Conclusion

Multilocus sequence typing using WGS was sucessful in identifying sequence types present in the South African sample pig popultion, and determing the relationship amongst the South African isolates and also between the South African and international isolates. The sequence types isolated in this study belonged to pathogenic *E. coli* clonal complexes. Some of the sequence types identified are reported in global populations outside South Africa. The South African isolates were phylogenetically related to international ETEC isolates and is suggestive that strains that cluster together based on their sequence type share similar pathogencities and virulence potential. It was concluded from the analysis that the South African isolates are largely diverse. Future analysis of a larger number of isolates from more regions will give a better understanding of the nature and dynamics of the ETEC from South African pig populations.

CHAPTER 4

PLASMID AND ANTIMICROBIAL PROFILE OF ENTEROTOXIGENIC *E. COLI* ISOLATES FROM SOUTH AFRICAN PIGS

Abstract

Enterotoxigenic *Escherichia coli* (ETEC) causes colibacillosis in young livestock such as piglets, calves and chickens. The disease symptoms include excessive diarrhoea, weight loss and eventual death. One of the methods for treatment and control of colibacillosis is with the use of antimicrobials. In the livestock sector, antimicrobials are used for disease treatment, control and as growth promoters. Due to the excessive and improper use of antimicrobials, bacteria such ETEC have grown resistant to some of the antimicrobials currently being used. This is a serious problem as antimicrobials are one of the main mode of control for infectious agents. Plasmids are largely implicated in the spread of antimicrobial resistance within and between various bacterial species due to their ability to be conjugative or mobilizable. This situation is worsened due to plasmids being able to acquire multiple resistance genes therefore allowing bacteria to be resistant to a multitude of antimicrobials. The aim of this study was to determine the plasmid profile of ETEC isolated from piglets in two provinces of South Africa and to compare the plasmid profile against the antimicrobial resistance profile to determine if there was any association between antimicrobial resistance and the type and number of plasmids present in an isolate. Plasmid extraction was performed using the Qiagen® Spin Miniprep Kit according to the manufactures protocol. Gel electrophoresis was done on a one percent ethidium bromide stained gel. Chi-squared tests of association (significance: $P = 0.05$) was used to determine if there was any statistically significant association between antimicrobial resistance and plasmid presence. The South African ETEC isolates used in this study were resistant to most antimicrobials used in the livestock sector such as oxytetracycline, which had the highest rate of resistance (39 %), ampicillin (35 %) and trimethoprim (22 %). Seventeen percent of the isolates were resistant towards both polymyxin B and lincomycin and the lowest resistance (2 %) was observed for kanamycin and enrofloxacin. Some isolates were resistance to multiple antimicrobials. Plasmid profiling found plasmids ranging from 1.3 kb up to >10 kb, with some samples having multiple plasmids. Chi-square anaylsis found a significant ($P = 0.036$) association between

antimicrobial resistance and plasmid presence, however no direct correlation was observed between resistance to a specific antimicrobial and plasmid size. This study confirmed an association between plasmids and antimicrobial resistance. Screening for plasmids can give an indication of pathogenic traits that may be present within a bacterial population, as genes for virulence and antimicrobial resistance may be carried on such plasmids.

Keywords: *ETEC; pigs; colibacillosis; antimicrobial resistance; plasmids*

4.1 Introduction

Antimicrobials are extremely important for their antimicrobial properties, however they are becoming less effective with time due to high levels of resistance (Levy & Marshall 2004; Cole 2014). Antimicrobial resistance emerged shortly after the discovery and use of antimicrobials (Davies & Davies 2010; CDC 2013). For example, penicillin was introduced for use in 1943 but antimicrobial resistance towards penicillin had already been discovered in 1940 (Fernández & Hancock 2012). According to CDC (2013), tetracycline was available in the 1950s whilst resistance for this drug was observed during the same time. Methicillin was released in 1960 and by 1962 resistance to the drug was evident (CDC 2013). Over time, antimicrobials resistance has had a snowball effect whereby resistance has spread for all classes of antimicrobials to most bacterial species (WHO 2014). This is of major concern as antimicrobials are the most common method of control for all bacterial, viral and parasitic infections (Levy & Marshall 2004; Sosa *et al.* 2013).

The high usage of antimicrobials in the intensive pig farming sector (Krnjaić *et al.* 2005; Henton *et al.* 2011), presents a high risk of transference of antimicrobial resistant enteric and zoonotic bacteria to humans via the food chain (Delsol *et al.* 2010; Moyane *et al.* 2013). There have been reports of foodborne disease outbreaks linked to the presence of viable zoonotic bacteria present in poorly cooked (Rosengren *et al.* 2009) and contaminated meat and meat products (Bester & Essack 2010; Marshall & Levy 2011). Such a scenario is alarming because the bacteria which are likely harbouring antimicrobial resistance genes colonise human systems and become part of/or compete with normal flora (Bester & Essack 2010), and transfer their antimicrobial resistance genes to the human flora (Rahman *et al.* 2008; Bester & Essack 2010). Studies have reported humans to be harbouring antimicrobial resistant bacteria that may have been transmitted to humans via the food chain (Marshall &

Levy 2011; Moyane *et al.* 2013). Antimicrobial residues have been found in meat, and unfortunately cooking only reduces the levels of antimicrobial residues (Moyane *et al.* 2013).

Antimicrobial resistance can be classified as either non-genetic (Corona & Martinez 2013) or genetic (Davies & Davies 2010). Non-genetic resistance mechanisms are usually exhibited by bacteria in biofilms and persistent cells, or due to metabolic changes resulting from environmental changes (Corona & Martinez 2013). Genetic resistance can either be intrinsic (chromosomal-mediated), adaptive (temporary alteration of gene and/or protein expression) or acquired (extrachromosomal-mediated) (Davies & Davies 2010; Bootsma *et al.* 2012). Acquired antimicrobial resistance is of great interest as this mechanism is mediated by extra-chromosomal elements such as plasmids (Carattoli 2009), transposons, integrons and gene cassettes (Domingues *et al.* 2012). Acquired resistance can easily spread in either homogenous or heterogenous bacterial communities (Domingues *et al.* 2012). Genes encoding antimicrobial resistance have been identified and are used as diagnostic tools for screening bacteria that show resistance to antimicrobials (Smith *et al.* 2010). Antimicrobial resistance genes have been observed in major classes of antimicrobials such as tetracyclines, sulphonamides, streptomycins, ampicillins, ceftiofur, chloramphenicol and trimethoprim amongst others (Smith *et al.* 2010). Co-selection or cross-resistance of related and unrelated groups of antimicrobials have also been observed. For example, the use of ceftiofur antimicrobial can result in resistance to tetracycline (Rosengren *et al.* 2009). Antimicrobial resistance genes are present both on the bacterial chromosome and also on mobile genetic elements such as plasmids, transposons and gene cassettes. Plasmids however are mostly implicated in the dissemination of antimicrobial resistance between bacteria (Rosengren *et al.* 2009; Adzitey *et al.* 2013).

Antimicrobial resistant bacteria can pass on their resistance genes to other bacteria and more especially commensal bacteria using plasmids via horizontal gene transfer mechanisms (Rahman *et al.* 2008). Plasmids are able to carry multiple antimicrobial resistance (MAR) genes and thus transfer MAR to other bacteria, both inter- and intra-species, which poses an even bigger problem for diseases control efforts (Rosengren *et al.* 2009; Marshall & Levy 2011).

Plasmids are extra-chromosomal, self-replicating, mobilizable/conjugative genetic elements that have the ability to transfer between bacteria (Bennett 2008; Yamashita *et al.* 2014). Plasmids are known to carry a wide variety of genes that encode antimicrobial resistance, virulence factors, metabolism of rare metals and degradation of organic compounds amongst other traits (Bennett 2008; Ibrahim *et al.* 2014). These traits add to their ability to provide a survival advantage to those bacterial strains that harbour them within a diverse ecosystem (Martínez & Baquero 2002). Pathogenic bacterial strains often contain more than one plasmid, and the total plasmid content in an isolate is known as the plasmid profile. Plasmid profiles are used in epidemiology to track local and global disease outbreaks and determine the phylogenetic relationship between isolates (Rosengren *et al.* 2009). They can also be used to infer upon the antimicrobial status of the bacteria (Turner *et al.* 2014). The information derived from antibiograms and plasmid profile can be used in epidemiological studies to investigate disease outbreaks (Rahman *et al.* 2008; Rosengren *et al.* 2009). Knowing the plasmid and antimicrobial resistance profiles and bacteria can assist in understanding the degree of resistance of bacterial communities so that proper control methods can be developed (Bennett 2008; Marshall & Levy 2011).

No studies have been performed in South Africa regarding plasmids harboured by the prevalent ETEC. Previous studies performed on South African pigs showed that ETEC isolates are resistant to most antimicrobials (Sikhosana 2015). However no data is available regarding the mechanisms of these antimicrobial resistance. Determining the presence of plasmids, which are known to be responsible for resistance integration and dissemination, will aid in finding improved ways to control ETEC diseases. The aim of this study was therefore to perform plasmid profiling of ETEC isolates sampled from neonatal and weaning piglets from two provinces of South Africa. In addition, the study determined the association between antimicrobial resistance and the plasmids profile of the ETEC isolates.

4.2 Materials and methods

4.2.1 Sample background

Samples were collected from two provinces of South Africa, the Thohoyandou village in Limpopo, and King Williams Town, Umtata, Alice and Port St Johns in the Eastern Cape as part of larger project (Chaora 2013; Sikhosana 2015). Pigs ranging between 4 days and 16

weeks old, belonging to the indigenous, Large White, Duroc and exotic breeds were included in the study. Neonatal piglets were only fed milk from the sows, and commercial weaning piglets were fed pig grower whereas the free-ranging piglets scavenged for feed or were given household kitchen leftovers. Samples were collected from the pig's rectum using the COPANR fecal swabs containing Amies transport media. The swab samples were kept on ice during sampling and transportation to the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI), Bacteriology section's Feed and Food Analysis laboratory for further analysis. Following the end of the project by Chaora (2013) the identified ETEC samples were freeze-dried and twenty of these samples were used in the present study. Sikhosana (2015) investigated the antimicrobial resistance profiles of these ETEC samples using the Kirby-Bauer disk diffusion method, findings of which are presented in Table 4.1

Table 4.1 Antibiogram of enterotoxigenic *E. coli* samples

	Antimicrobials						
Sample	**Amp**	**K**	**OT**	**W**	**ENR**	**PB**	**LS**
2	S	S	S	S	S	S	I
8	S	S	I	S	S	I	R
9	S	S	S	S	S	S	S
11	R	S	R	R	S	R	S
13	R	R	R	S	I	R	I
16	S	S	S	S	S	I	S
19	S	S	S	S	S	R	S
20	S	S	S	S	S	I	R
24	R	S	R	R	R	S	S
35	R	S	R	R	R	S	I
40	S	I	S	S	S	S	S
41	R	S	S	I	S	S	S
46	R	S	R	R	S	I	S
47	S	S	S	S	S	S	S
52	S	S	S	S	S	S	I
56	S	S	R	S	S	I	R
83	S	I	S	I	S	S	I
97	S	I	I	S	S	I	S
109	R	R	S	R	S	S	I
121	R	S	R	I	S	S	R
Total	8 (40 %)	2 (10 %)	9 (45 %)	5 (25 %)	2 (10 %)	4 (20 %)	4 (20 %)

4.2.2 Culturing of bacterial isolates

Twenty samples positive for ETEC, and tested for antimicrobial resistance by Sikhosana (2015) (Table 4.1) were inoculated from freeze-dried samples onto nutrient broth and grown overnight at 37°C. A loopful of culture was then streaked onto MacConkey agar plates and incubated overnight at 37°C. Pure single colonies were thereafter inoculated into one ml Luria Bertani broth and grown overnight at 37°C on a shaker. The cultures were used for plasmid extraction.

4.2.3 Plasmid extraction

Plasmids were extracted using the Qiagen® Spin Miniprep Kit. Briefly, an overnight culture was centrifuged at 13 000 rpm (revolutions per minute) on the Eppendorf MiniSpin (Eppendorf, Germany) for three minutes. The supernatant was discarded and the cells were resuspended in 250 µl Buffer P1 after which 250 µl buffer P2 was added to the tube and the suspension inverted several times. 350 µl Buffer P3 was added and mixed immediately to prevent clumping of debris. The mixture was centrifuged at 13 000 rpm for ten minutes after which the pellet containing cell debris was discarded and the supernatant was transferred into a QIAprep spin-column and centrifuged at 13 000 rpm for one minute. The flow-through from the spin-column was discarded and 500 µl Buffer PB was added to the spin-column and centrifuged at 13 000 rpm for one minute. The flow-through was discarded, and 750 µl Buffer PE was added to the spin column and centrifuged at 13 000 rpm for one minute, the flow through was discarded and the spin column was centrifuged for a further one minute at 13 000 rpm to remove residual buffer. The column was placed into a clean 1.5 ml microcentrifuge tube and 50 µl elution Buffer EB was added directly to the spin column membrane, allowed to stand for five minutes at room temperature and thereafter centrifuged at 13 000 rpm for two minutes. The spin column was discarded and the elution containing plasmid DNA was stored at -20°C.

4.2.4 Plasmid profiling

Ten µl plasmid DNA was run on a 1 % agarose gel (Seakem LE Agarose, Lonza, Rockland ME, USA) stained with ethidium-bromide (Merck, Germany) in 1X TAE buffer (Bio-Rad, Munich) at 90 V for 150 minutes. The gel was visualised using gel documentation system (Bio-Rad Trans-UV, Japan). Plasmid DNA sizes were analysed against a GeneRuler™ 1 kb DNA ladder, ready-to-use (Fermentas, Thermo Scientific) (Table 4.2 and Figure 4.1).

Table 4.2 1 kb molecular weight marker showing band sizes, log values and distance travelled (mm)

Log table		
Size (bp)	**log bp**	**Distance (mm)**
250	2.398	50
1000	3	39
1500	3.176	34
2000	3.301	30
2500	3.398	27
3000	3.477	26
4000	3.602	23
5000	3.699	21
6000	3.778	19
8000	3.903	17
10000	4	15

The log values of the size in base pairs was calculated (Table 4.2). The log was then plotted against the distance in mm (Figure 4.1) in order to extrapolate the size of the plasmids from the gel (Figure 4.2) (Lee *et al.* 2012).

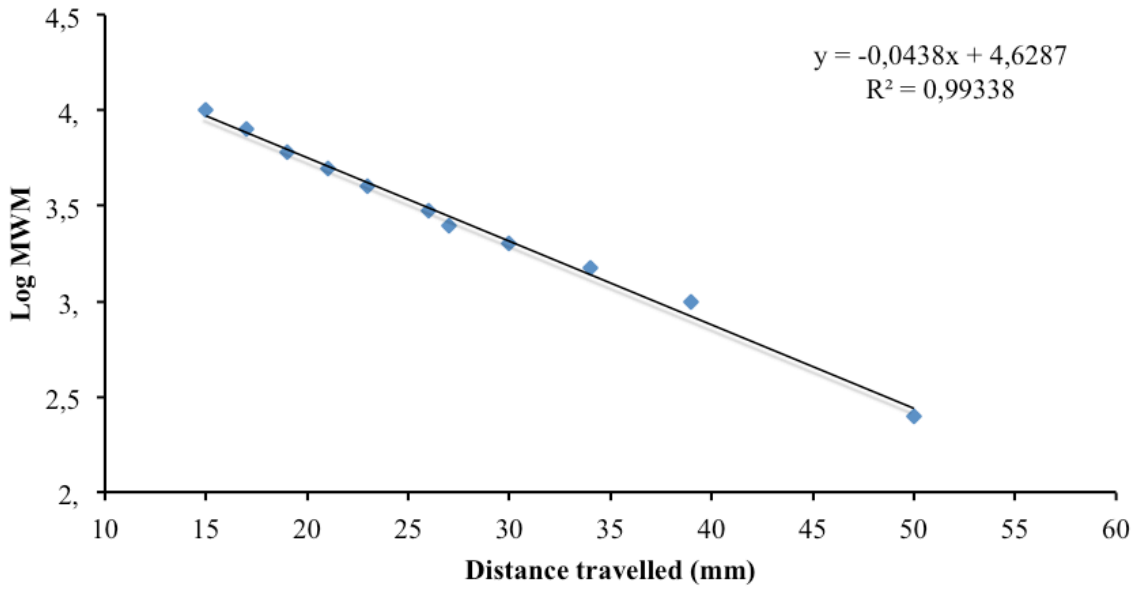

Figure 4.1 Graph of log 1 kb molecular weight marker vs. distance travelled in mm

4.2.5 Association between plasmid and antimicrobial resistance profiles

In order to determine if there was any association between the plasmids and antimicrobial resistance, statistical analysis was performed using Chi-square test at a significance level of $P< 0.05$ in SAS.

4.3 Results

4.3.1 Plasmid profile

Ten plasmids of various sizes were found in the 20 ETEC isolates examined. The distribution of plasmids for the chosen samples are shown in Figure 4.2. Plasmids ranged from 1.3 kb up to >10 kb. Some samples harboured more than one plasmid, with one isolate having up to six plasmids of various sizes. There were no plasmids recovered from samples 16, 20, 24, 35, 52, 56, 83 and 109.

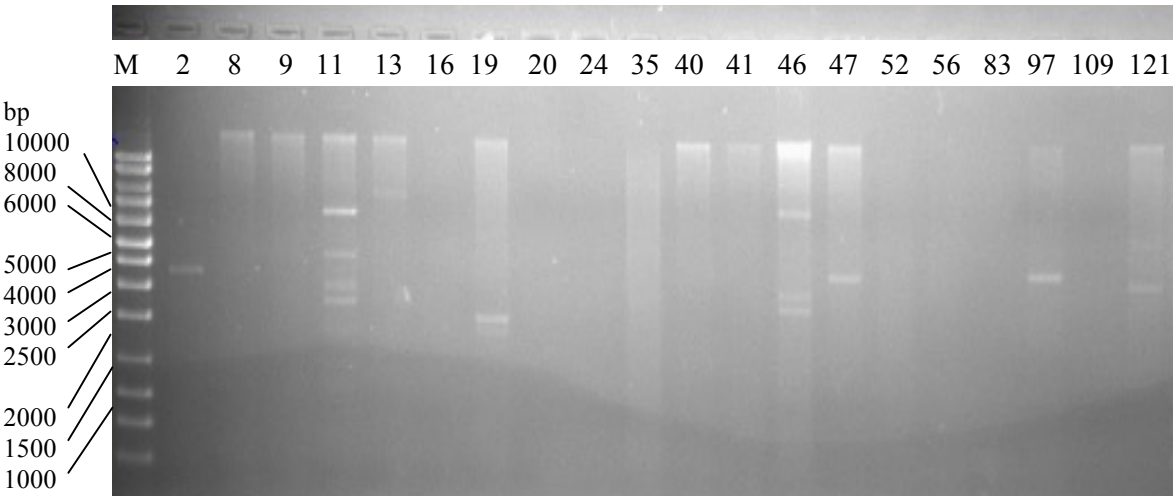

Figure 4.2 Plasmid profile of enterotoxigenic *E. coli* isolates on 1 % agarose gel. Lanes 1: 1 kb Molecular Weight Marker. Lanes 2-21: ETEC samples

Table 4.3 shows the presence/absence of plasmids in ETEC samples. The most common plasmid was >10 kb, which was observed in 11 isolates. This was followed by the 2.2 kb plasmid, which was present in three isolates. The 6 kb, 3 kb, 2.5 kb and 1.3 kb plasmids were each observed in only one sample, and the 5 kb, 2 kb, 1.6 kb and 1.5 kb plasmids were each observed in two samples. Sample 11 had the highest number of plasmids (six), followed by sample 46 which had four plasmids. Sample 109 had three plasmids whilst samples 19, 47 and 97 had two plasmids each. The rest of the samples had one plasmid each.

Table 4.3 The number and size of plasmids harboured by the enterotoxigenic *E. coli* isolates from South African piglets

Samples	Plasmid sizes										Total
	>10 kb	6 kb	5 kb	3 kb	2.5 kb	2.2 kb	2 kb	1.6 kb	1.5 kb	1.3 kb	
2	0	0	0	0	0	1	0	0	0	0	1
8	1	0	0	0	0	0	0	0	0	0	1
9	1	0	0	0	0	0	0	0	0	0	1
11	1	0	1	0	1	0	1	1	0	1	6
13	1	1	0	0	0	0	0	0	0	0	2
19	1	0	0	0	0	0	0	0	1	0	2
40	1	0	0	0	0	0	0	0	0	0	1
41	1	0	0	0	0	0	0	0	0	0	1
46	1	0	1	0	0	0	0	1	1	0	4
47	1	0	0	0	0	1	0	0	0	0	2
97	1	0	0	0	0	1	0	0	0	0	2
121	1	0	0	1	0	0	1	0	0	0	3
TOTAL	12	1	2	1	1	3	2	2	2	1	

0 – denotes no plasmid present. 1- denotes presence of plasmid. Samples that did not have any plasmids are not shown

4.3.2 Antimcrobial resistance and plasmid profile

The plasmid profiles were compared against the antibiogram to determine if any association could be established between plasmids and antimicrobial resistance phenotype. Five samples did not show resistance to any of the antimicrobial tested, but they contained up to two

plasmids. Three samples showed resistance to only one antimicrobial and these samples also contained up to two plamsids. Two samples showed resistance towards three antimicrobials and these samples also carried three and four plasmids each. Two samples were resistant towards four antimicrobials, one of the samples contained two plasmids, and the other sample had up to six plasmids. With regards to samples that did not harbour any plasmids, three of these samples did not exhibit resistance towards any antimirobials, one sample was resistant towards one, two and three antimicrobials respectivley and two samples where resistant towards four antimicrobials. Those samples that exhibited resistance towards more than two antimicrobials are considered.

Statistical analysis using Chi-square found an overall significant (P = 0.039) association between antimicrobial resistance and plasmid presence, however no significant association was found between individual plasmid size and resistance to a particular antimicrobial.

4.4 Discussion

Enterotoxigenic *E. coli* is a pathogenic strain of *E. coli* that causes colibacillosis in young animals such as pigs (Do *et al.* 2005; Li *et al.* 2012), chickens (Akbar *et al.* 2009) and cows (Kolenda *et al.* 2015). Antimicrobials are extensively used for disease treatment, metaphylaxis, prophylaxis and growth promotion (Eagar *et al.* 2012). Colibacillosis is commonly treated using antimicrobials (Nagy & Fekete 1999). The pig and poultry farming industry has the highest use of antimicrobials due to intensive farming system of these animals (Henton *et al.* 2011; Moyane *et al.* 2013). The high use of antimicrobials in farming has resulted in bacteria such as ETEC developing resistance against antimicrobials. Most of the genes encoding antimicrobial resistance are haboured on plasmids, along with other pathogenic traits. Plasmids are extra-chromosomal, self-replicating, conjugal genetic elements that are implicated in the extensive spread of antimicrobial resistance in bacterial communities in agriculture, the natural environment as well as the clinical environment (Bennett 2008; Carattoli 2009). An investigation of the plasmids carried by ETEC in the South African pig population and the antimicrobials that these bacteria are resistant to is important for the establishment of effective control methods of *E. coli*. The aim of this study was therefore to determine the plasmid profiles of South African ETEC samples from pigs, and to determine if there was any association between the plasmid profiles and antibiograms of the samples.

Based on the the study by Sikhosana (2015), 70 % of the 20 samples studied showed resistance to one or more of the tested antimicrobials. Nagy and Fekete (1999) reported that colibacillosis was treated with antimicrobials such as neomycin, colistin, aminosidine, kanamycin and polymyxin as these antimicrobials are absorbed slowly or not at all from the intestine making them ideal to cure intestinal infection. Polymyxin, quionlones and flouroquinolone antimicrobials were used to treat ETEC diarrhoeal infections (Nagy & Fekete 1999) and ampicillin is used to treat colibacillosis in pigs (Nagy & Fekete 1999; Marshall & Levy 2011). Oxytetracycline is often used as a growth promoter and a broad-spectrum antimicrobial to treat a number of diseases in pig farming (Chopra & Roberts 2001; Marshall & Levy 2011). Sulponamide antimicrobials form the same class of antimicrobials trimethoprim, they are sometimes used in conjunction to treat Gram-negative bacterial infections and exhibit cross-resistance to each other (Huovinen *et al.* 1995) and Gram-positive infections (Mazurek *et al.* 2015). A study by Sikhosana (2015) found that, oxytetracycline had the highest rate of resistance folowed by ampicillin and then trimethoprim. These results reflect the 2007 SANVAD statistics performed in South Africa which found that tetracylines, penicillins and trimethoprim are amongst the most sold antimicrobial classes for use in food animals (Henton *et al.* 2011). The extensive use of these antimicrobials in pig production could account for the high prevalence of resistance.

Polymyxin B and lincomycin were both resistant towards 17 % of the isolates. Polymyxin B does not have an official breakpoint in the CLS guidelines, therefore the manufactures instructions were used for the intepretation of results. Lincosamides are used in the pig and poultry industry for both metaphylaxis and prophylaxis (Moyane *et al.* 2013) to treat swine dysentery (Marshall & Levy 2011). The lincomycin group of antimicrobials which include microlides; lincosamides and pleuromutilins (MLEs), were the most sold group of antimicrobials in a study conducted between 2002 and 2004, with more than 200 000 kg's sold in each of those three years in South Africa (Moyane *et al.* 2013). Polymyxin B is of the polymyxin group of antimicrobials along with colistin (polymyxin E), and they both exhibit cross resistance to the polymyxin group of antimicrobials (Velkov *et al.* 2013; Catry *et al.* 2015). The lowest resistance (2 %) was observed for kanamycin and enrofloxacin. Sikhosana (2015) concluded that enrofloxacin can be used to effectively control colibacillosis as samples showed high susceptibilty to this antimicrobial.

Multiantimicrobial resistance to two or more antimicrobials was observed in the study by Sikhosana (2015). Constant treatment of bacteria with antimicrobials has allowed for a selective pressure towards resistance (Levy 2002; Davies & Davies 2010), which instead of eliminating pathogenic bacteria such as ETEC it promotes their growth and increases pathogenicity (DeBoy *et al.* 1980; Boerlin *et al.* 2005).

An alarming observation made by the SANVAD surveillance was that pigs and poultry had the highest levels of resistance, with commensal *E. coli* strains showing greater resistance than pathogenic strains (Henton *et al.* 2011), and this may be due to prophylactic and metaphylactic use of antimicrobials. Antimicrobial resistance determinants are typically spread between bacteria, animals, humans and the environment by plasmids via horizontal gene transfer. As such, antimicrobial resistance can be related to the plasmid content of the bacteria (Mayer 1988). Therefore it is important to assess the plasmid status of these ETEC samples so that strategies can be made to control and curb the spread of these pathogenic bacteria.

Plasmid profiling has been used in epidermiology due to their ability to self-replicate, self-conjugate and their harbouring of various pathogenic gene determinants, and specifically their infectious nature of easy dissemination between bacteria in different ecosystems (Mayer 1988). Plasmids are known to contain genes for various pathogenic traits such as virulence factors, antimicrobial resistance and metabolism of rare metals (Mayer 1988; Bennett 2008). Enterotoxigenic *E. coli* contains toxin as well fimbrial genes which are coded for on plasmids. The heat-labile (LT) and heat-stable (ST) genes are located on Ent plasmids (Trachman & Maas 1998; Ochi *et al.* 2009) and genes for fimbrial and non-fimbrial colonisation factors are also plasmid encoded (Jordi *et al.* 1992; Nagy & Fekete 2005).

The plasmid profiling analysis found the most common plasmid to be >10 kb. Plasmids sizes in this study ranged from as small as 1.3 kb, going up to 1.5 kb, 1.6 kb, 2 kb, 2.2 kb, 3 kb, 5 kb, 6 kb and <10 kb. The results of this study corroborate with the results found in other studies. Woodard *et al.* (1990) isolated plasmids from *E. coli* sourced from diarrhoegenic pig and observed plasmid sizes ranging from 4 kb – 8 kb. Mazurek *et al.* (2015) analysed the plasmid profile of *E. coli* isolated from piglets during metaphylaxis and sows post-treatment with trimethoprim and sulphonamide and found that samples harbourbed between one and

five plasmids ranging between 1.5 -100 kb. In the study by Mazurek *et al.* (2015) the larger plasmids (13 kb, 60 kb and 100 kb) were found in piglets during metaphylactic antimicrobial treatment, whereas the smaller plasmids (3.8 kb, 4.5 kb, 6 kb and 8 kb) were isolated from sows post-treatment. The most common plasmids isolated in the study by Mazurek *et al.* (2015) were 56 kb, 7 kb and 1.5 kb respectively and were isolated from both treatment groups. The results of the current study are similar to other studies perfomed on *E. coli* which found plasmids smaller than 10 kb. Plasmid sizes can range from as small as <2 – 3 kb and these carry only two or three genes, to very large sizes that equate to approximately 10 % of the chromosomal genome, which can accomadate up to 400 or more genes (Bennett 2008). This study only measured plasmids up to 10 kb, so it is possible that larger plamids could have been missed. Future studies would have to measure plasmids of up to 200 kb.

Pachaury *et al.* (2013) isolated a single sized plasmid of approximately 10-15 kb in their study on ETEC from lambs, kids and calves. Nsofor and Iroegbu (2013) determined the plasmid profile of *E. coli* from different domestic animal species, including cattle, sheep, goats and pigs. They isolated plasmids of sizes 1.5 kb, 2 kb, 2.5 kb, 3 kb, 5 kb, 95 kb and 120 kb across all the samples and an additional 1.4 kb plasmid isolated strictly from pigs. A study conducted in South Africa on *E. coli* isolated from poultry found plasmids that ranged between 1.7 kb and 89 kb, and strains harbouring multiple plasmids (two - four) were found in equal proportation (20 %) (Georgnaras *et al.* 2001). In the study by Kalantar *et al.* (2010), the authors isolated plasmids from various *E. coli* strains found in children with diarrhoea, the isolated plasmids ranged between 1.7 kb and 4.5 kb. Studies have shown that bacteria can easily transfer their plasmids between agricultural, environmental and clinical settings (Davies & Davies 2010). It was therefore not unusual that the plasmids observed in this study would be of similar sizes to those reported from different sources.

In this study, isolates had between one to six plasmids, with one sample having six plasmids. The most common number of plasmids per sample was two, as observed in four samples. These results are in agreement with those by Mazurek *et al.* (2015) who isolated between one and five plasmids from *E. coli* in piglets. The *E. coli* E2173 strain that causes post-weaning diarrhea in pigs analysed by Olasz *et al.* (2005) harboured a total of six plasmids, which also corroborate with the results of this study, although the plasmid sizes were different.

Multiple antimicrobial resistance is a result of either more than one resistance gene determinant being present within the bacteria. Samples that exhibited MAR were found in this study, these are samples that were resistant to two or more antimicrobials. Multiple antimicrobial resistant samples also harboured multiple (two to six) plasmids. Costa *et al.* (2010) detected plasmids in 85.9 % of diarrhoegenic pig samples, 85.7 % of non-diarrheic pig samples and 100 % of environmental samples. Costa *et al.* (2010) further observed that diarrhoegenic samples resistant to between five and seven antimicrobials had a higher prevalence of plasmids. It has been observed however that bacteria that contain a higher number of plasmids are more likely to exhibit MAR as most antimicrobial resistance genes are plasmid-mediated (Bennett 2008; Nikaido 2009). Antimicrobial resistance genes are chromosome-mediated whilst others are plasmid-mediated. Genes encoding resistance are mostly contained on transposable elements which are mobile genetic elements that can be transferred between the plasmid and bacterial chromosomes such as plasmids, transposons, integrons and gene cassettes (Bennett 2008). *Escherichia coli* is known to aquire resistance towards antimicrobials very rapidly (da Silva & Mendonça 2012; Eze *et al.* 2015).

Alternatively, this study found samples that did not contain plasmids, but exhibited MAR to three and four antimicrobials. These results are reflected in the study by Costa *et al.* (2010) who observed MAR in many isolates that did not contain plasmids, these resistance markers may be chromosomally located, such as genes for enrofloxacin and β-lactams. Chromosomal resistance may be mediated by the incorporation of transposons, integrons and genes cassettes into the chromosome (Domingues *et al.* 2012), or the presence of the resistome (Davies & Davies 2010; Landecker 2015). It has been observed that the administration of a single antimicrobial could lead to MAR though cross-resistance (Levy 2002; Mazurek *et al.* 2015). The studies by Levy *et al.* (1976); Levy (1978) showed that the administration of oxytetraclycine to newly-hatched chicks illicited resistance in 70 % of *E. coli* samples during the study. Oxytetracycline resistance was found to cause cross-resistance to tetracycline, ampicillin, sulphonamides and streptomycin; and resistance determinants were found on transferable plasmids which had spread the resistance to farm personnel (DeBoy *et al.* 1980).

The Chi-square test showed that there was an overall significant ($P < 0.05$) association between plasmid presence and antimicrobial resistance ($P = 0.039$). This association is corraborated by other studies have shown that there is a direct correlation between

antimicrobial resistance and plasmid transmission (Turner *et al.* 2014; Yamashita *et al.* 2014). In this study, plasmids of a particular size were not found to convey resistance to any particular antimicrobial. There were also samples that exhibited resistance, but did not contain plasmids. These results are in agreement to those reported in other studies performed by Amaechi *et al.* (2015) who found similar results when they analysed *E. coli* from pigs and poultry. Adzitey *et al.* (2013) also observed no direct association between individual plasmid size and antimicrobial resistance in *E. coli* isolated from ducks. Similarly, Wonglumsom *et al.* (2011) observed no direct relationship between particular plasmid profiles and antimicrobial resistance in their study on *E. coli* from various sources including chickens, pets, pet owners, water and vegetables.

4.5 Conclusion

This study found an overall significance (P = 0.039) between plasmid profiles and animicrobial resistance profile, however no significant association was found between individual plasmids and particular antimicrobials. It can therefore be concluded that plasmid profiling only indicates the presence of plasmids, and does not give a direct representation of antimicrobial resistance (Garner 2002; Costa *et al.* 2010). The shortfall of this study is that although it is known that plasmid presence indicates antimicrobial resistance, the actual dynamics of the plasmids found is still unknown. Also, it is unknown if the antimicrobial resistance observed is due to the presence of resistance genes only, or if there are other factors such as co-selection also involved. It is also unknown whether the resistance observed is plasmid- or chromosomal-mediated. Further analysis of these isolates using next-generation sequencing will assist in answering some of these points raised above. This will give a better understanding of the antimicrobial status and the plasmids that are harboured within the ETEC populations isolated from pigs in South Africa.

CHAPTER 5

PLASMID PROFILING AND ANTIMICROBIAL RESISTANCE GENE ANALYSIS USING WHOLE-GENOME SEQUENCE DATA

Abstract

Enterotoxigenic *E. coli* causes colicabillosis in piglets and antimicrobials are used to treat the disease. Unfortunately ETEC has grown resistant to the antimicrobials commonly used to treat the disease. Plasmids are mobile genetic elements that are mostly responsible for the dissemination of antimicrobial resistance within and between bacterial species. Conventionally, antimicrobial resistance is determined using the disc diffusion method, which does not necessarily indicate what resistance genes are present in the bacterium. Plasmid profiling is traditionally done by extraction and gel electrophoresis, a method that only indicates the size of the plasmids without additional information of the type of plasmids and their characteristics. Whole-genome sequencing (WGS) using next-generation sequencing technologies has revolutionised the world of microbiology. Whole-genome sequencing enables the analysis of complex bacterial genomes including extra-chromosomal features and pathogenic traits that are of most importance in controlling diseases. The aim of this study was to investigate antimicrobial resistance genes and plasmid profiles of enterotoxigenic *E. coli* isolated from pigs from two provinces of South Africa. Eleven samples were chosen for WGS based on the presence of plasmids and antimicrobial resistance phenotype. DNA was extracted using the Qiagen Blood and Tissue Kit following the manufactures protocol. For each isolate 0.5 Gb of data was sequenced at 30X coverage using paired-end, 125 x 125 bp sequencing on the HiSeq 2500 platform. CLC-Bio v6 Genomics Workbench was used to determine the quality of the data, which was thereafter trimmed and mapped to the *E. coli* K12 MG1655 reference genome. Unmapped reads were collected and *De novo* assembled for further analysis. The Center for Genomic Epidemiology database web-servers Plasmidfinder and ResFinder were used to identify antimicrobial resistance genes and plasmids present in the samples, National Center for Biotechnology Information (NCBI) BLASTn server was also used to identify plasmids. ResFinder identified four different antimicrobial resistance genes *tet(A)*, *tet(B)*, *msr(C)*, *aac(6)-Ii* in 55 % of the samples. Plasmidfinder identified seven plasmids that originated from *Klebsiella pneumonia*, *Salmonella enterica* serovar Typhi, *E.*

coli K12, plasmid FII and incompatibility group IncI2. NCBI BLAST also identified plasmids belonging to *Shigella* spp. and other bacterial strains. Plasmid analysis found that plasmids from bacteria other than *E. coli* where being harboured by ETEC, highlighting the ability of plasmids to disseminate within and between bacterial species. The study emphasised the importance of analysing the antimicrobial resistance and plasmid profiles of South African ETEC population for better understanding of disease epidemiology and development of effective control measures.

Keywords: *ETEC; antimicrobial resistance gene; plasmids; pigs; whole-genome sequencing*

5.1 Introduction

Enterotoxigenic *Escherichia coli* (ETEC) are the cause of colibacillosis in neonatal and post-weaning piglets. The disease causes excessive diarrhoea, severe dehydration and eventual death if there is no treatment. The control strategy for colibacillosis includes the use of antimicrobials. However, it has been observed that most bacteria including ETEC are resistant to most antimicrobials on the market due to the over-use of antimicrobials as growth promoters in the livestock sector (Henton *et al.* 2011; WHO 2014). Many bacterial strains have been observed to exhibit multiple antimicrobial resistance (MAR) (CDC 2013; WHO 2014). Genes encoding resistance to most antimicrobials have been identified and most of the genes are co-selected. Plamids have mostly been implicated in the dissemination of antimicrobial resistance (Rosengren *et al.* 2009).

Plasmids are self-replicating, mobile, circular genetic elements that are transferrable between different bacteria in an ecosystem. Plasmids contain genes for processes such as maintenance, self-replication and transfer. Most importantly, plasmids contain genes that confer pathogenic properties such as virulence traits and antimicrobial resistance (Bennett 2008). Plasmids and other traits can be analysed using whole-genome sequencing (Kennedy *et al.* 2010; Rump *et al.* 2012).

Whole-genome sequencing of bacterial genomes using next-generation sequencing (NGS) tools has allowed for advanced developments in antimicrobial resistance identification, antimicrobial development, epidemiologic surviellance of diseases (including the emergence and spread of diseases), elucidation of host-pathogen interaction (especially in the case of

zoonotic diseases), predicting the spread of diseases and drug resistance, diagnoses of diseases and disease control (Köser *et al.* 2012). Further advances of NGS technologies results in much faster, high throughput and cheaper protocols that make it feasible to use NGS in routine diagnostics and surveillance (Ståhl & Lunderberg 2012).

The first bacterial genome, *Haemophilus influenzae Rd* was sequenced in 1995 using Sanger sequencing and it was expensive and time-consuming project (Loman *et al.* 2012). *Escherichia coli* K12 M1655 was one of the first organisms to be completely sequenced and the project took six years to complete (Blattner *et al.* 1997). Since then three ETEC strains affecting humans have been completely sequenced H10407 (Crossman *et al.* 2010), E24377A (Sahl & Rasko 2012), B2C (Vipin Madhavan *et al.* 2014) and ETEC strains UMNK88 and UMNF18 isolated from pigs have also been sequenced using Roche 454 pyrosequencing (Shepard *et al.* 2011). Ren *et al.* (2014) were the first to completely sequence an ETEC strain (W25K) that was isolated from diarrhoeal pigs, using the Illumina MiSeq sequencing platform.

Bacteria are known to carry pathogenic traits on mobilizable plasmids, which are involved in the acquisition and dissemination of disease traits. Plasmid pTC isolated from pigs was the first animal-derived ETEC plasmid to be sequenced in 2012 (Fekete *et al.* 2012). Plasmid pTC is a self-conjugative plasmid of 90 kb in size. The plasmid contains genes encoding STa and STb enterotoxins on the toxin-specific locus, tetracycline resistance on the Tn10 transposon, plasmid transfer which is responsible for self-conjugation, a colE1-like origin of replication and genes for plasmid maintenance and stability (Fekete *et al.* 2012).

Next-generation sequencing improves upon plasmid profiling and the antimicrobial sensitivity testing in that it gives the actual plasmids and antimicrobial resistance genes present. The aim of this chapter was to perform WGS using the HiSeq 2500 next-generation sequencing platform in order to investigate plasmid and antimicrobial resistance genes present in ETEC isolates from the South African pig population.

5.2 Materials and methods

5.2.1 DNA extraction and quantification

Following plasmid profiling, only samples that contained plasmids were chosen for whole-genome sequencing, with the exception of sample 35 which did not have any plasmids, however it was resistant to four antimicrobials and was therefore included (refer to CHAPTER 4).

DNA was extracted from one ml overnight cell culture using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufactures protocol for DNA extraction from Gram-negative bacteria. The extracted DNA concentration was measured using the Qubit® Floruometer 3.0 (Life Technologies, CA) using the dsDNA Assay following the manufactures protocol.

5.2.2 Sequencing and assembly

DNA was extracted from 11 samples and sequenced at the Agricultural Research Council Biotechnology Sequencing Platform. The genomes were sequenced using paired-end sequencing (125 x 125 bp) on the Illumina HiSeq 2500 platform at 30X coverage. Approximately 0.5 GB of data was generated per sample.

Read quality control and data analysis was performed using CLC-Bio Genomics Workbench v6 Software (Germanton, MD). Reads were trimmed to remove Nextera v2 transposase 2 adapter sequences, regions of ambiguity and low quality reads. The trim settings were as follows: 0.05 limit of low quality reads. The trimmed reads were mapped against the reference genome *Escherichia coli* K-12, strain MG1655, Genbank accession number U00096.3. Sequences that did not align to reference genome (unmapped reads) were considered to be putative plasmids or strain specific transposable elements and were collected for further analysis.

5.2.3 Analysis of sequence data

De novo assembly was performed on the unmapped reads in order to arrange them into contigs. The data was analysed using National Center for Biotechnology Information (NCBI) BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Centre for Genomic Epidemiology

(CGE) (http://www.genomicepidemiology.org/) to search for plasmids and antimicrobial resistance gene sequences. ResFinder (Zankari *et al.* 2012) and PlasmidFinder (Carattoli *et al.* 2014) on CGE were used to determine which resistance genes and plasmids were present in the samples respectively.

5.3 Results

The raw sequence files were exported into CLC Bio Genomics Workbench. Quality control was performed on the reads, at a PHRED score of 30 to maintain good quality reads. The number of reads before trimming ranged between 2.2 million and 8 million, with the average length of 125 bp (Table 5.1). After trimming, the number of reads ranged between 2.17 million and 7.9 million, with the average length ranging between 103 bp and 113.5 bp. The percentage of trimmed reads was approximately 98.7 %. Trimmed reads where thereafter mapped to the *E. coli* K12 MG1655 reference genome.

Table 5.1 Summary statistics of pre- and post-trimmed sequence data for eleven enterotoxigenc *E. coli* isolates

Sample	Number of	Average length	Number of reads	% after	Average length
9	3,319 194	125.0	3, 269 406	98. 5	108.1
11	7,578 760	125.0	7,481 572	98.72	104.7
13	2,197 312	125.0	2,166 593	98.6	113.5
19	6,715 488	125.0	6,644 246	98.94	104.3
35	6,610 176	125.0	6,531 886	98.82	103.2
40	2,982 314	125.0	2,938 037	98.52	108.3
41	4,728 798	125.0	4,677 373	98.91	105.0
46	6,731 096	125.0	6,645 091	98.72	105.3
47	7,983 606	125.0	7,886 453	98.78	103.0
97	5,441 214	125.0	5,373 155	98.75	105.2
121	3,045 714	125.0	3,007 335	98.74	105.5

A high percentage of the reads mapped to the reference genome. Samples 47, 97 and 121 had the highest mapping percentages of 92 %, and sample 46 had the lowest percentage of 74 %. An average of 85 % of the reads mapped to the reference genome, and the number of reads ranged between 1.8 million and 7.2 million. The percentage of reads that did not map to the reference ranged between 7.6 % and 19 %. Paired reads accounted for approximately 75 % of the total reads (Table 5.2).

Table 5.2 Summary of mapping statistics of the eleven enterotoxigenic *E. coli* isolates against the *E. coli* K12 MG1655 reference genome

Sample	Mapped reads			Un-mapped reads			Reads in pairs		
	Count	%	Average	Count	% reads	Average	Count	%	Average
9	2,822 641	87.67	109.39	397 073	12.33	98.65	2,545 184	79.05	201.31
11	5,985 810	81.06	105.74	1,398 696	18.94	100.05	5,386 510	72.94	180.22
13	1.834 889	85.91	114.84	301 001	14.09	105.44	1,583 656	74.15	215.69
19	5,492 870	83.57	104.94	1,080 160	16.43	100.78	4,769 226	72.56	153.38
35	5,676 465	87.96	103.92	777 159	12.04	97.48	4,902 398	75.96	154.66
40	2,506 366	86.61	109.26	387 494	13.39	102.11	2,330 042	80.52	211.75
41	3,977 865	85.99	105.85	648 105	14.01	99.79	3,521 762	76.13	156.69
46	4,875 141	74.32	105.88	1,684 099	25.68	103.25	4,503 654	68.66	192.82
47	7,200 161	92.44	103.79	589 193	7.56	92.91	6,207 950	79.70	154.22
97	4,884 634	92.07	105.96	420 514	7.93	95.52	4,141 352	78.06	166.24
121	2,736 050	92.15	106.16	232 954	7.85	97.04	2,373 536	79.94	169.91

Reads that did not map to the *E. coli* K12 reference genome are summarized in Table 5.3. The read count of the isolates ranged between 200 000 and 1.6 million reads with an average length of 102 bp. For the assembly, the average number of matching reads ranged between 198 000 and 1.59 million, with an average length of 105 bp. Between 5000 and 54 000 reads did not match and these had an average length of 65 bp. The number of contigs created from *De novo* assembly ranged from as little as 77 with an average length of 2586 bp for sample 121. Sample 19 had the highest number of contigs (2397) and these had an average length of 1354 bp. Sample 46 had contigs of the longest contig length (2919 bp) and had a low number of contigs (250). The read count in pairs ranged from 131 828 to 1.4 million, and an average length ranging between 116.7 bp to 167.34 bp.

Table 5.3 Summary of *De novo* assembly of unmapped reads from enterotoxigenic *E. coli* samples

Sample	Reads		Matched		Not matched		Contigs		Reads in pairs	
	Count	Average	Count	Average	Count	Average	Count	Average	Count	Average
9	351 962	104.27	318 433	108.35	33529	65.51	210	2 230	244 740	152.11
11	1,314208	102.78	1,263931	103.87	50277	75.87	675	1 096	1,071400	143.66
13	275 810	110.6	222 004	114.03	53806	96.44	340	1 568	174 434	185.53
19	1,029680	103.32	992 439	104.19	37241	80.08	2 397	1 354	830 796	139.99
35	713 052	101.68	662 088	103.25	50964	81.19	1 902	594	554 638	140.72
40	354 940	107.28	342 143	108.6	12797	72.03	236	2 405	276 984	164.82
41	602 354	103.07	579 463	104.29	22891	72.33	212	2 701	480 386	133.6
46	1,622602	105.08	1,591216	105.91	31386	62.29	250	2 919	1,390894	167.34
47	514 496	100.2	498 354	101.45	16142	61.65	87	2 357	401 832	120.13
97	368 280	103.08	354 267	104.31	14013	74.41	84	2 241	260 000	119.46
121	203 166	104.96	197 845	106.16	5 321	60.23	77	2 586	131 828	116.7

The assembled contigs from the unmapped reads were used for identification of plasmids and antimicrobial resistance genes. PlasmidFinder on the CGE website was used for the identification of plasmids (Table 5.4). Six different plasmids were found, each having an identity percentage greater than 90 % to the unmapped contigs mentioned (Table 5.3). A few of the plasmids harboured by these the samples originated from various bacterial species, such as *Klebsiella pneumoniae* and *Salmonella enterica* serovar Typhi. One plasmid originated from plasmid F of *E. coli* K12, and one from incompatibility group IncI2. Plasmid CTX-M-15 is a plasmid known to harbour extended-spectrum-β-lactamases, and was found in a few of the samples.

Table 5.4: Summary of PlasmidFinder results for enterotoxigenic *E. coli*

Sample	Plasmid	% identity	Contig	Accession	Group identified
9	none				
11	FII (pCoo)	95.82	1	CR942285	Enterotoxigenic
	ColRNAI (pIGMS32)	95.12	2	DQ298019	*Klebsiella pneumoniae*
13	FII	95.04	1	AY458016	CTX-M-15 (pC15-1a)
19	FIB (pHCM2)	91.89	1	AL513384	*Salmonella enterica*
35	FII	95.04	1	AY458016	CTX-M-15 (pC15-1a)
40	FII	95.04	1	AY458016	CTX-M-15 (pC15-1a)
	FIB (AP001918)	98.68	2	AP001918	*Escherichia coli* K12
41	FII (pCoo)	97.33	1	CR942285	Enterotoxigenic
	I2	97.83	2	AP002527	Incompatibilty group
46	FIB (pHCM2)	91.43	1	AL513384	*Salmonella enterica*
	ColRNAI (pIGMS32)	92.23	2	DQ298019	*Klebsiella pneumoniae*
47	ColRNAI (pIGMS32)	93.01	1	DQ298019	*Klebsiella pneumoniae*
97	ColRNAI (pIGMS32)	93.41	1	DQ298019	*Klebsiella pneumoniae*
121	ColRNAI (pIGMS32)	93.41		DQ298019	*Klebsiella pneumoniae*

The CGE ResFinder resistance gene database was used to identify resistance genes (Table 5.5). Only three antimicrobial resistance genes were observed on the unmapped contigs, each with greater than 98 % identity. The genes identified include tetracycline resistance *tet(A)* and *tet(B)*, and the aminoglycoside acetyltranferase *(aac(6')-Ii)*. There was no antimicrobial resistance genes identified in five of the samples (Samples 9, 11, 40, 41 and 46). A fourth

resistance gene (*msr(C)*) encoding erythromycin resistance which forms part of the microlides, lincosamides and sulphonamide (MLS) group of antimicrobials was found on the chromosomal reads (mapped reads) for sample 19.

Table 5.5 Summary of ResFinder results for enterotoxigenic *E. coli*

Sample	Antimicrobial	% identity	Contig	Antimicrobial class
9	none			
11	none			
13	*tet(B)*	100	1	Tetracycline
19	*aac(6')-Ii*	98.52	1	Aminoglycoside
	tet(A)	100	2	Tetracycline
	*msr(C)**	99.42	772	MLS
35	*tet(B)*	100	1	Tetracycline
40	none			
41	none			
46	none			
47	*tet(A)*	100	1	Tetracycline
97	*tet(A)*	100	1	Tetracycline
121	*tet(A)*	100	1	Tetracycline

The msr(C) gene was found on mapped (chromosomal) reads. MLS – Microlide, Lincosamide and Sulphonamide

The unmapped contigs were also analysed on the NCBI BLASTn database to determine other sequences the South African ETEC isolates matched against (Table 5.6). Majority of the contigs matched to plasmid sequences from *E. coli* spp., *Shigella* spp., *Klebsiella* spp. and *Salmonella* spp.

Table 5.6 Summary of NCBI Blast results for enterotoxigenic *E. coli*

Sample	Description
9	*E. coli*
11	*E. coli, Shigella, Salmonella, Klebsiella, Stx2a* toxin gene
13	*E. coli, Shigella, Salmonella, Klebsiella*
19	*E. coli, Shigella, Salmonella, Klebsiella*
35	*E. coli* ST540
40	*E. coli*
41	*E. coli, Shigella, Salmonella enterica*
46	*E. coli*
47	*E. coli, Shigella, Salmonella, Klebsiella,*
97	*E. coli, Shigella, Salmonella, Klebsiella, Stx2a* toxin gene
121	*E. coli, Shigella, Salmonella, Klebsiella*

5.4 Discussion

Disease epidemics caused by pathogenic bacteria have always been a problem throughout history, and one way of controlling such bacteria is through the use of antimicrobials (WHO *et al.* 2003). Unfortunately, most bacterial strains have acquired the ability to show resistance to most current antimicrobials on the market (Fernández & Hancock 2012). There is therefore a need to characterise the bacteria to better understand what is contained in their genome and the mechanisms employed that continually make them stronger and resistant to modern medicine. Whole-genome sequencing is a technology that has been a benchmark in the

science world (Stoesser *et al.* 2013). Next-generation sequencing allows for the rapid sequencing of bacterial genomes due to the high-throughput technology (Grada & Weinbrecht 2013). Sequencing of bacteria allows for the analysis of virulence, antimicrobial resistance and epidemiology. Not much is understood about enterotoxigenic *E. coli* that cause colibacillosis in pigs in South Africa, especially with regards to antimicrobial resistance and the plasmids contained within this ETEC population. Therefore the aim of this study was to determine the antimicrobial resistance genes and plasmids present in ETEC isolated from pigs in South Africa using next-generation sequencing.

CLC-Bio was used for initial analysis of data, which involved the trimming of reads, mapping against the reference genome and collection of unmapped reads for further analysis. NCBI BLAST and the Centre for Genomic Epidemiology were used to screen for plasmids and antimicrobial resistance. Rump *et al.* (2012) used CLC-Bio to analyse plasmid sequences from enterohemorrhagic *E. coli*, and Ahmed *et al.* (2012) used CLC-Bio to analyse enteroaggregative *E. coli*.

The sequence reads were trimmed and then mapped to the *E. coli* K12 strain MG1655 genome (accession number: U00096.3). *Escherichia coli* K12 was the first organism to be sequenced due its importance in biochemical genetics, molecular biology and biotechnology as a model organism for research. This *E. coli* strain is 4,639,221 bp, and the genome consists of 87.8 % of protein coding genes, 0.8 % of stable RNAs genes, 0.7 % is noncoding repeats and approximately 11 % codes for regulatory and other functions (Blattner *et al.* 1997).

Mapping percentage ranged between 74.32 % and 92.44 % (samples 46 and 47 respectively), and between 25.68 % and 7.56 % of reads did not map. The average count for the unmapped reads ranged between 200 000 reads and 1.6 million (samples 121 and 46 respectively). Since majority of the reads mapped to the reference genome, it can be proposed that the function of the mapped reads are known. The unmapped reads were then assembled into contigs using *De novo* assembly and subjected to antimicrobial resistance and plasmid identification. The Centre for Genomic Epidemiology database was used for the analysis of antimicrobial resistance genes (ResFinder) and plasmids (PlasmidFinder). The PlasmidFinder web-based tool was designed by (Carattoli *et al.* 2014) and was succefully used in other studies for the identification of plasimds and antimicrobial resistance genes. Nyholm *et al.*

(2015) used PlasmidFinder to identify plasmids harboured by STEC and ETEC and Johnson *et al.* (2015) also identified plasmids in samples from extraintestinal pathogenic *E. coli* strains. The ResFinder web-based tool was designed by Zankari *et al.* (2012). Studies that have used ResFinder include Kleinheinz *et al.* (2014) to identify antimicrobial resistance genes in bacteriophages and prophages, and Gonzalez-Escalona *et al.* (2013) were able to identify antimicrobial resistance genes in *E. coli* implicated in hemorrhagic colitis.

Six different plasmids were identified in 10 samples: FII (pCoo) originating from ETEC, FIB (AP001918) originating from the *E. coli* K12 plasmid F family, ColRNAI (pIGMS32) originating from *Klebsiella pneumoniae*, FIB (pHCM2) originating from *Salmonella enterica* serovar Typhi, FII originating from the CTX-M-15 (pC15-1a) family of plasmids, and I2 originating from the Incompatibilty group IncI2 family of plasmids. Most of the plasmids were identified in contigs 1 and 2 from the *De novo* assembly. Sample nine did not contain any plasmid, samples 13, 19,35, 47, 97, 121 only harboured one plasmid, and samples 11, 40, 41 and 46 each harboured 2 plasmids.

The FII (pCoo) plasmid of ETEC origin was found in two samples, 11 and 41 with 95.3 % and 97.44 % identity respectively. The pCoo plasmid was the first human ETEC CF-encoding plasmid to be sequenced by Froehlich *et al.* (2005). It is 98,369 bp and a combination of two replicons (R64 – IncI1 plasmid and R100 – IncFII plasmid) separated by an insertion sequence 100 (IS100). The R64 and R100 replicons both contribute to the improved functionality of the pCoo plasmid, and the R64 replicon contributes virulence while the R100 replicon contributes pathogenicity (Froehlich *et al.* 2005). The plasmid was found to be homologous to Rep1I plasmid R64 originating from *Salmonella enterica* serovar Typhimurium, and and RepFIIA plasmid R100 from *Shigella* spp. (Johnson & Nolan 2009). The pCoo plasmid is not self-conjugative and contains four virulence genes (cooA, -B, -C, -D) which code for CS1 pili and colonization factor antigen 1 (CFA/1) which is essential for colonization by attaching the bacteria to cells in the small intestine (Froehlich *et al.* 2005). In some CS1-producing strains which have the pCoo plasmid, the plasmid also encodes heat-labile and heat-stable genes and CS3 colonization factor. Pevious studies have shown that the pCoo plasmid can coexist with the R64 and R100 replicons in a cell (Froehlich *et al.* 2005). Carattoli *et al.* (2014) analysed the pCoo plasmid along with other plasmids using the PlasmidFinder web-based tool. Crossman *et al.* (2010) compared plasmid pCoo with the

plasmids found in the commensal *E. coli* strain H10407 and Rasko *et al.* (2008) compared the pCoo plasmid with those plasmids originating in from other *E. coli* strains.

Three samples, 13, 35 and 40 harboured the CTX-M-15 plasmid pC15-1a, all with 95.04 % similarity. This plasmid contains genes which exhibit extended-spectrum beta-lactamase CTX-M-, and is mostly associated with the Inc FII replicon group and vary vastly in size (50-200 kb) (Carattoli 2009). CTX-M-15 was initially isolated from *E. coli*, *Klebsiella pneumoniae* and *Enterobacter aerogene* (Boyd *et al.* 2004). Sequencing revealed that the pC15-1a plasmid was 92,353 bp resulting from a combination of three regions, a 64 kb fragment from plasmid R100, a 24.1 kb region composed of unique combinations of six to eight multidrug resistant genes and finally a 9 bp region repeated in reverse order (Boyd *et al.* 2004). Cavaco *et al.* (2008) performed a study on the CTX-M- plasmids harboured by *E. coli*, persisting in the the gut of pigs being treated with amoxicillin, ceftiofur or cefquinome. The researchers found that the pigs already harboured *E. coli* that contained CTX-M- plasmids, and that the use of cephalosporins select for resistant bacteria, which continue to pesist even after the antimicrobial withdrawal period (Cavaco *et al.* 2008). In a study identifying antimicrobial resistance phenotype and genotypes, the researchers observed that majority of the isolates conferring β-lactam resistance contained the CTX-M-15 resistance plasmid (Stoesser *et al.* 2013).

The pHCM2 plasmid sequence was found in two samples, 19 and 46 with 91.89 % and 91.43 % identity respectively. This plasmid belong to the IncFIB incompatibility replicon group (Carattoli *et al.* 2014). The pHCM2 plasmid is a rare plasmid of 106.516 bp from *Salmonella enterica* serovar Typhi origin (Parkhill *et al.* 2001). The putative genes present on the plasmid are mostly responsible for DNA biosynthesis, replication and modification (Kidgell *et al.* 2002).

The pIGMS32 plasmid sequence was found in six samples, 11, 46, 47, 97 and 121 with above 92.23 % identity. The pIGMS32 plasmid is 9492 bp and originates from *Klesbiella pnuemoniae*. The replication mechanism of the plasmid is controlled by antisense RNA – ColRNAI. Putative genes found on pIGMS2 code for conjugation (MOBpIGMS32 - making it conjugative), a toxin-antitoxin system encoding ParE family toxin and a phenotypic module coding for cloacin production (Smorawinska *et al.* 2012).

Plasmid IncI2 was present in sample 41 with 97.83 % identity. Plasmid IncI2 is of incompatibility group I which is distinct from incompatibility group F in a number of ways. Firstly, IncI2 it is larger than IncF, IncI2 plasmids produce two different types of sex pili, a thin flexible pilus and a thick rigid one, IncI2 plasmids carry a multiple inversion system called shufflon, and IncI2 plasmids carries the *sog* gene which codes for the suppression of *E. coli* dnaG mutations, which are transferred into the recipient cell during conjugation along with other donor proteins. An example of an IncI2 plasmid that has been sequenced is the R64 plasmid isolated from *Salmonella enterica* serovar Typhimurium (Sampei *et al.* 2010). The plasmid R64 has putative genes that code for replication, drug resistance (arsenic, tetracycline and streptomycin), stability, transfer leading and transfer regions (Sampei *et al.* 2010).

ResFinder is an online web server on the CGE database that analyses whole-genome sequence data in order to identify acquired antimicrobial resistance genes in bacteria (Zankari *et al.* 2012). Resistance genes were only identified in 54.5 % (six) of the samples, and only four resistance genes were identified: *tet(A)*, *tet(B)*, *aac(6')-Ii* and *msr(C)*. Five of the six samples harboured only one resistance gene, and the sixth sample harboured three different resistance genes. Genes encoding tetracycline resistance (*tet(A)* and *tet(B)*) were predominant. Resistance gene *tet(A)* was found in samples 19, 47, 97, 121 (66.7 %) and *tet(B)* was found in samples 13 and 35 (33 %). Olasz *et al.* (2005) performed a study on ETEC strains from pigs, and reported a higher frequency (86 %) of the isolates harboured the *tet(B)* resistance gene, as apposed to *tet(A)* and *tet(C)* (<10 %) which are in contrast to the results of the current study. Findings from this study also in contrast with observations by Szmolka *et al.* (2013) who found *tet(B)* at a higher frequency (38 %) as apposed to *tet(A)* whoch was foud in 26 % of ETEC isolates from pigs. Tetracyclines are broad-sprectrum antibotics used to treat a large range of Gram-positive and Gram-negative bacterial infections, as well as for growth promotion in livestock (Chopra & Roberts 2001).

The resistance gene *aac(6')-Ii* confers resistance to gentamicin (Davis *et al.* 2010) and is of the aminoglycoside family, was found only in sample 19. Mathew *et al.* (2001) found that a higher percentage of piglets were resistant to gentamicin, however they found that the resistance was not necasarily due to the use of antimicrobials during the growth phase, but rather the wide spread presence of antimicrobial resistance determinants in enteric bacteria.

The resistance gene *msr(C)*, which confers erythromycin resistance was observed in sample 19. The *msr(C)* gene is involved in efflux mechanism of resistance, as is the *tet(A)* and *tet(B)* resistance genes (Roberts 2011). This antimicrobial resistance gene is commonly found in *Enterococcus*, and has been associated with tetracycline and animoglycoside resistance genes in broiler chickens (Werner *et al.* 2013). No antimicrobial resistance genes were identified in 45 % of the samples (samples 11, 40, 41 and 46) despite these samples having shown resistance against antimicrobials such as ampicillin, oxytetracycline, trimethoprim and polymyxin B (CHAPTER 4). These samples also did have plasmids, based on WGS analysis.

NCBI BLASTn was used to identify other plasmid and antimicrobial resistance sequences in the unmapped contigs. Most of the samples had hits for *E. coli* spp., *Shigella* spp., *Klebsiella* spp. and *Salmonella* spp. which was also not surprising as there is constant sharing and recombination of mobile genetic elements such as plasmids and gene cassettes in bacterial communities (Johnson & Nolan 2009). Ahmed *et al.* (2012) also found matches to plasmid sequences originating from *Salmonella* and *Shigella* in their analysis of *E. coli* O104:H4 that had acquired an *stx2* gene. Dionisio *et al.* (2002) observed a high rate of plasmid transfer between heterogenous bacteria. Other studies have found high level of congruence for plasmids between species, for example between *Salmonella* and *E. coli* in the natural environment share similar plasmids (Stoesser *et al.* 2013). Studies by Mirza *et al.* (2000) and Hunter *et al.* (1992) showed that *Salmonella* and *E. coli* are able to transfer multidrug resistant plamids between each other. It is therefore not unusual to see plasmids from different bacterial strains, as with plasmids originating from *Salmonella* and *Klebsiella* in ETEC observed in this study.

5.5 Conclusions

This study highlights the need to perform whole-generation seuencing studies that give an indication of the genes and plasmids present in a population, allowing for the creation of better control measures. PlasmidFinder, Resfinder and NCBI BLASTn analysis gave very interesting results in terms of the resistance genes and plasmids identified in these ETEC samples. The presence of plasmids from *Klebsiella* and *Salmonella* origin in the ETEC samples confirms the ability of plasmids to be shared and exchanged interspecies. The results of this study show that WGS can identify antimicrobial resistance genes present within the genome. Whole-genome sequence data is also better at identifying plasmids, as traditional

plamid profiling only indicates the size of the plasimds present in the sample, whereas WGS provides detailed information on the exact plasmid identified, enabling a better understanding of what is harboured by these bacteria.

CHAPTER 6

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE WORK

6.1 General discussion

Colibacillosis is a disease that effects neonatal and post-weaning piglets and is caused by enterotoxigenic *E. coli* (ETEC), shiga-toxin *E. coli* and enteroaggregative *E. coli* (EAEC) (Francis 2002; Mohlatlole *et al.* 2013). The symptoms caused by colibacillosis include excessive diarrhoea, severe dehydration, weight loss, and eventual death if left untreated (Coelho *et al.* 2009). Colibacillosis is an important disease affecting the South African pig industry including the commercial and small-holder farmers, causing huge losses in terms of pig production and capital (Henton & Engelbrecht 1997).

Not much is understood about the population dynamics of South African ETEC affecting piglets, therefore the aim of this study was to characterise *E. coli* causing colibacillosis in neonatal and weaning piglets of South Africa using MLST and next-generation sequencing together with plasmid profiling. Multilocus sequence typing (MLST) is technology that is used to track epidemiology using sequence data of hypervariable housekeeping genes (Urwin & Maiden 2003). Chapter 3 determined the sequence types of *E. coli* present in the population using whole-genome sequence data from ETEC samples. Seven housekeeping loci: *adk, gyrB, mdh, icd, recA, purA* and *fumC* were used to determine the sequence types present (Wirth *et al.* 2006). Seven different sequence types were identified, ST10, ST56, ST101, ST4253, ST4704, ST1830 and ST5766. Eighteen percent of the samples belong to ST101 and ST5766 each, and 27 % of the samples belonged to ST1830. The rest of the STs were observed in one sample each. Not much literature is available pertaining to the sequence types present in this study, and three of the STs identified were not reported in literature. The STs reported in literature were associated with pathogenic bacterial strains such as shiga-toxin *E. coli* strains – ST56, ST10 and ST101 (Hauser *et al.* 2013). Sequence type 101 has been found to harbour extended-spectrum β-lactamase (Coelho *et al.* 2011) and metallo-β-lactamases (Mushtaq *et al.* 2011; Peirano *et al.* 2011). Sequence type 1830 was found to harbour extended-spectrum β-lactamases (Izdebski *et al.* 2013). Phylogenetic analysis of these strains using MLST showed that ST101 is an ealier evolved ST compared to the other STs, as observed in both the

national and international phylogenies. Two STs, ST101 and ST56 did not cluster with any other STs, but formed independent clades on the phylogeny. Enterotoxigenic *E. coli* ST5766 formed a clade with a Brazilian isolate; and ST1830, ST4704, ST4253 and ST10 formed a clade with Hungarian isolates. The phylogenetic analysis shows that the South African ETEC isolates are largely diverse and that they belong to STs present globally. More samples are needed to get a more informed overview of the diversity of the South African isolates.

Plasmid profiles of the samples was analysed and compared to antimicrobial resistance profiles that were reported by Sikhosana (2015) using the conventional Kirby-Bauer disk diffusion method, in Chapter 4. A total of ten plasmids of various sizes were present in the population, ranging from 1.3 kb up to >10 kb. The number of plasmids per sample ranged from zero up to six, with most samples having two plasmids. Plasmids harbour various pathogenic genes (Carattoli 2009). Multiple antimicrobial resistance to three or more antimicrobials was identified in seven samples. Most samples were resistant to ampicillin, oxytetracycline, trimethoprim, polymyxin B and lincosamides, with enrofloxacin and kanamycin resistance observed in only two samples. The tetracyclines, penicillins, microlides and polypeptides were the most used antimicrobial classes in the South African livestock sector and it is therefore not surprising that most samples were resistant to these classes of antimicrobials.

Overall there was a statistically significant association between plasmid presence and antimicrobial resistance ($P = 0.039$), however no statistical significance could be made between individual plasmids and antimicrobial resistance. This is because some isolates did not contain plasmids but exhibited antimicrobial resistance. Similar results were identified in other studies (DeBoy *et al.* 1980; Costa *et al.* 2010). This could be due to the resistance mechanism being inferred upon by resistance genes present on the bacterial chromosome (Domingues *et al.* 2012), or the bacterial resistome (Landecker 2015). Antimicrobial resistance genes are also present on transposons which are mobile genetic elements transferrable between the chromosome and plasmid, although most resistance genes are plasmid mediated (Levy & Marshall 2004; Domingues *et al.* 2012).

Whole-genome sequencing (Chapter 5) was further used to analyse plasmid and antimicrobial resistance genes present. More than 80 % of reads matched to the *E. coli* K-12

MG1655 genome, with the exception of one sample which matched at 74 %. The unmapped read lengths ranged from 351 962 bp up to 1 622 602 bp. The unmapped reads were assumed to contain plasmids, and pathogenic sequences. Seven different plasmids were identified using PlasmidFinder and these originated from ETEC; *Klebsiella pneumoniae*; *Salmonella enterica*; *E. coli* K-12; β-lactamase CTX-M-15 plasmids and Incompatibility groups FII and I2 plasmids. From the seven plasmids identified, only two plasmids originate from *E. coli*, FII (pCoo) originating from ETEC and FIB (AP001918) originating from *E. coli* K12. The presence of plasmids from other bacterial species, remaining five plasmids, indicates the ability of plasmids to be transferred across bacterial species (Hunter *et al.* 1992; Dionisio *et al.* 2002).

Antimicrobial resistance gene sequences were identified in 45 % of the sample. The resistance genes identified include *tet(A)* and *tet(B)* which were predominant and confer resistance to tetracycline, *aac(6')-Ii* gene which confers resistance to gentamycin (Davis *et al.* 2010) and the *msr(C)* gene which conferes ressitance to erythromycin (Roberts 2011). Tetracycline resistance genes also confer resistance to oxytetracycline (Chopra & Roberts 2001). In this study, majority of the samples exhibited resistance to oxytetracycline, and tetracycline resistance genes were observed in most of the samples. One samples harboured three antimicrobial resistance genes *tet(A)*, *aac(6')-Ii* and *msr(C)* this showed that ETEC in this sample population can contain multiple resistance genes. Furthermore three of the genes where found in the unmapped reads and one was found on the mapped reads, highlighting that resistance is chromosomally (Sengupta *et al.* 2013) and extra-chromosomally mediated (Bennett 2008). The other resistance genes were observed on the unmapped sequences further indicating that these genes are found on extra-chromosomal elements such as plasmids and transposons (Martínez & Baquero 2002).

Bacterial genomes are constantly evolving, primarily due to the transfer of mobile genetic elements such as plasmids, transposons, gene cassettes that harbour antimicrobial resistance and virulence traits (Bennett 1999; Davies & Davies 2010). Within the cell itself, translocations occur with recombinations between different plasmids, and between plasmids and the bacterial chromosome. Traditional analysis of plasmids and antimicrobial resistance found that there is an overall statistical significance between plasmid presence and antimicrobial resistance. However, this relationship breaks down when comparing individual

plasmids against the antimicrobial resistance profile of the sample. This may be due to the complicated mechanism of antimicrobial resistance which are either genetically or non-genetically medited, with non-genetic resitance being due to metabolic activity and genetic resistance either being intrinsic or acquired (Davies & Davies 2010; Sengupta *et al.* 2013). Comparing WGS results (Chapter 5) to those of the antibiogram (Chapter 4), some ETEC exhibited multiple resistance in the antibiogram, however, only one sample contained more than one antimicrobial resistance gene. This anomaly may be due to antimicrobial cross-resistance or other resistance mechanisms such as efflux pumps (Webber & Piddock 2003). Plasmid identification using WGS and analysis on PlasmidFinder and NCBI BLAST identified plasmids originating from different species such as *Klebsiella* spp., *Salmonella* spp. and *Shigella* spp. These observations further highlight the ability of plasmids to easily transfer within and across different species using mechanisms such as horizontal gene transfer (Levy & Marshall 2004; Norman *et al.* 2009).

6.2 Conclusion

Multilocus sequence typing shows that the sequence types observed in the South African ETEC samples from the pig population are also found internationally. In this sample population, ST1830 was the most predominant sequence type and is representative of *E. coli* containing extended-spectrum β-lactamases and AmpC-type cephalosprinases. Plasmid profiling revealed various plasmids ranging from 1.3 kb up to >10 kb with samples containing up to six plasmids. A few samples showed multiple antimicrobial resistance against up to four antimicrobials. Oxytetracycline had the highest resistance, followed by ampicillin and trimethoprim. Analysis of unmapped reads from WGS confirmed the presence of plasmids and antimicrobial resistance genes on extra-chromosomal elements. Plasmids from various sources such as *Klebsiella* and *Salmonella* were found in the ETEC samples, highlighting the ability of plasmids to transfer across species. Antimicrobial resistance genes for tetracycline, *tet(A)* and *tet(B)*, and gentamycin (*aac(6')-Ii*) were found in most of the samples tested. The only chromosomal-mediated resistance gene found was *msr(C)* which is an efflux gene was found in one sample. The results show alarming antimicrobial resistance to a number of commonly used antimicrobials, as well as the presence of plasmids which originate from different bacterial species other than ETEC. The results of this research shed light on antimicrobial resistance of ETEC and the plasmids they contain, this will aid in finding better methods to control colibacillosis.

6.3 Recommendations and further research

Future research regarding ETEC isolates from the South African pig population need to focus on increasing the population size and landscape to improve upon the epidemiological relationship. The extent of antimicrobial resistance, especially for antimicrobials that are used the the pig farming industry need to be further investigated. Analysing the antimicrobial resistance genes and plasmids present will also aid in understanding the population dynamics of ETEC. Increased knowledge in this regard will aid in our ability to better manage ETEC infections in South Africa.

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