PREVALENCE AND ANTIBIOTIC SENSITIVITY OF ENTEROTOXIGENIC *ESCHERICHIA COLI* ISOLATES IN SOUTH AFRICAN PIG POPULATION

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ABSTRACT

Escherichia coli (E. coli) are among the leading bacterial causes of diarrhoea and edema in newborn and weaned pigs. Pathogenic strains of *E. coli* are classified into enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroaggreagative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC) and diffusely adherent *E. coli* (DAEC) based on virulence factors. Infection with *E. coli* is achieved by adherence of fimbrial and/or non-fimbrial adhesins to the intestines and the release of toxins thereafter. The increasing rates of antimicrobial resistance are posing a threat in the treatment of porcine *E. coli* infections. The aim of this study was to determine the prevalence of pathogenic *Escherichia coli* virulence genes and antibiotic sensitivity of enterotoxigenic *Escherichia coli* isolates from neonatal and post-weaning pigs in Limpopo and Eastern Cape provinces of South Africa. For this purpose, 325 rectal swabs were collected from pigs from the Eastern Cape and Limpopo provinces of South Africa to investigate the prevalence of ETEC relative to other *E. coli* strains. Classical microbiological tests were conducted for confirmation of *E. coli* and PCR was used for the detection of fimbrial, non-fimbrial adhesins and toxin genes. In addition, antimicrobial susceptibility of ETEC positive isolates was determined by the Kirby-Bauer disk diffusion method. Of the 325 swabs collected, 303 isolates were identified as *E. coli* with 67% (205/303) harboring at least one of the tested virulence genes (*LT*, *STa*, *STb*, *EAST-1*, *Stx1*, *Stx2*, *Stx2e*, *VT1*, *VT2*, *hlyA*, *F4*, *F5*, *F6*, *F18*, *F41*, *AIDA-1*, *EAE* and *PAA*) and categorized into 48 pathotypes. A total of 36 (11.9%) isolates was classified as ETEC, having heat-labile (*LT*) enterotoxin as the most prevalent. Only a single isolate (2.8%) carried fimbriae (*F4/F5*). Instead, non-fimbrial adhesins *PAA, AIDA-1* and *EAE* were detected. The ETEC positive isolates displayed 47.2%, 38.9% and 36.1% resistance to oxytetracycline, ampicillin and trimetroprim respectively. Most of the ETEC isolates were sensitive to florphenicol (100%), cefotaxime (97.2%) and enrofloxacin (77.8%). Multi-drug resistance was detected in 50% of the isolates. The study demonstrated that there are various *E. coli* pathotypes in South Africa. The detection of non-fimbrial adhesins reinforces existing knowledge that fimbriae are not the only colonization factors associated with ETEC. Based on the antimicrobial

susceptibility patterns observed, florphenicol, cefotaxime and enrofloxacin could be used for the treatment of ETEC infections in South African pigs.

DECLARATION

I, **Zizile Emelda Lilly Sikhosana**, declare that:

i. The research reported in this thesis is my original work except where acknowledged.

ii. This thesis has not been submitted for any degree or examination at any other university but the University of KwaZulu-Natal.

iii. Information and pictures obtained from other sources have been acknowledged.

iv. Assistance received while conducting the research and writing this thesis has been acknowledged.

v. This work was conducted under the supervision of Mr E.F. Dzomba, Dr E. Madoroba and Dr F.C. Muchadeyi.

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AIDA-1	Adhesin involved in adherence factor 1
A/E	Attaching and effacing
AMR	Antimicrobial resistance
astA	Enteroaggregative heat-stable enterotoxin type 1 encoding gene
Bp	Base pair
cAMP	Cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CFs	Colonization factors
CTX	Cefotaxime
DAFF	Department of Agriculture, Forestry and Fisheries
DNA	Deoxyribonucleic acid
EAE/*eae*	Attaching and effacing factor
EAEC	Enteroaggregative *Escherichia coli*
EAST-1	Enteroaggregative heat-stable enterotoxin 1
E. coli	*Escherichia coli*
e.g.	*Exempli gratia*, for example
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
ELISA	Enzyme linked immunosorbent assay
ENR	Enrofloxacin
et al	(*et alia*) and others
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
ESBLs	Extended spectrum β-lactamases
FFC	Florphenicol

HC	Haemorrhagic colitis
HUS	Haemolytic Uraemic Syndrome
hlyA	Haemolysin gene
K	Kanamycin
LEE	Locus of enterocyte effacement
LS	Linconmycin
LT	Heat-labile enterotoxin
MDR	Multi-drug resistant
mm	millimeter
mPCR	Multiplex polymerase chain reaction
ºC	Degrees Celcius
OT	Oxytetracycline
PAA	Porcine attaching and effacing factor
PCR	Polymerase chain reaction
PI	Pathogenicity island
PB	Polymyxin B
PWD	Post-weaning diarrhoea
SAS	Statistical Analysis System
ST	Heat-stable enterotoxin
STa	Heat-stable enterotoxin variant 1
STb	Heat-stable enterotoxin variant 2
STEC	Shiga toxin-producing *Escherichia coli*
Stx	Shiga toxin
Stx1	Shiga toxin variant 1 encoding gene
Stx2	Shiga toxin variant 2 encoding gene
Stx2e	Oedema associated shiga toxin
Taq	*Thermus aquaticus*
Tir	Translocated intimin receptor

VT	Verotoxins
W	Trimethoprim
-	Negative
+	Positive
α	Alpha
β	Beta
γ	Gamma
µg	Microgram
µl	Microlitre
%	Percentage

CHAPTER 1

General Introduction

1.1 Introduction

Escherichia coli is one of the natural inhabitants of the intestinal tract of humans and other warm-blooded animals although it forms a minor component of the gut microflora (Martins *et al*., 2011). Unless *E. coli* acquire genetic elements that code for virulence genes, they remain harmless commensals. When *E. coli* acquires virulence genetic elements, the organism causes a plethora of infections. *E. coli* is one of the agents of enteritis and extraintestinal diseases (Martins *et al*., 2011). *Escherichia coli*, an important enteric pathogen causes diarrhoea in neonatal and post-weaning pigs (Frydendahl, 2002; Blanco *et al*., 2006). Significant economic losses result mostly from *E. coli* induced diarrhoea (Khac *et al*., 2006).

Six diarrheagenic *E. coli* strains have been identified based on virulence genetic elements (Croxen and Finlay, 2010). These include enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggreagative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC). However, a strain of shiga toxin-producing *E. coli* (STEC), *E. coli* O157 known to cause infections in humans has been reported to be more prevalent in pigs and cattle than in humans (Ateba and Bezuidenhout, 2008). In addition, ETEC, STEC and EAEC have been reported in South Africa where ETEC was more predominant, occurring in 46.2 % of the isolates that carried the tested virulence genes (Mohlatlole *et al*., 2013). In this latest study, enteroaggregative *E. coli* (EAEC) and STEC were detected in 43.4 % and 0.9 % of the sampled isolates, respectively (Mohlatlole *et al*., 2013).

Of the six pathogenic *E. coli* groups identified, ETEC is the main aetiological agent that is associated with diarrhoea in pigs (Blanco *et al*., 2006). Its pathogenesis involves colonization of the epithelial surface of the porcine small intestine by means of specific fimbrial adhesion factors and the

production of one or more enterotoxins (Vidotto *et al*., 2009). Adhesion allows the bacteria to resist flushing by gut peristalsis (Madoroba *et al*., 2009). Fimbriae associated with ETEC infection in neonatal piglets and weaned pigs include *F4* (*K88*), *F5* (*K99*), *F6* (*987P*), *F18* and *F41* (Vidotto *et al*., 2009). Upon colonization, ETEC produces heat-labile (*LT*) and/or heat-stable (*STa* and *STb*) enterotoxins (Kobayashi *et al*., 2003) that cause hypersecretion of fluids and electrolytes (Amezcua *et al*., 2002; Liu *et al*., 2014). The imbalance between water and electrolytes in the small intestine results in diarrhoea and dehydration (Frydendahl, 2002; Liu *et al*., 2014).

ETEC-induced diarrhoea accounts for a high proportion of losses in pig production. Effective control measures that are required to manage and minimize losses are usually expensive (Smith *et al*., 2010). The ETEC virulence factors and pathogenesis need to be understood to develop alternative and effective control measures. This includes understanding the associated fimbrial and non-fimbrial adhesins and enterotoxins produced (Madoroba *et al*., 2009).

Use of antibiotics to treat infected neonatal and post-weaning pigs is practised in most pig production systems. However, the industry has been faced with challenges of most bacterial and fungal pathogens developing resistance to antibiotics in use (Amezcua *et al*., 2002; Wang *et al*., 2006). Antimicrobial resistance (AMR) is a global problem in veterinary medicine (Boerlin *et al*., 2005). Resistance to drugs has been identified in pigs with diarrhoea (Smith *et al*., 2010). This response has generally been allied with recurrent exposure of the pathogen to antimicrobial agents in pigs and other livestock systems (Boerlin *et al*., 2005). The increase of multi-drug resistant (MDR) phenotypes and the possible emergence of resistant clones with virulence factors exhibiting disease severity are of great concern to the pig industry (Smith *et al*., 2010). Therefore, research is necessary to determine effective antimicrobial agents.

1.2 Justification

Pigs are a major contribution in agricultural production in South Africa with Limpopo Province being the largest pork producer (DAFF, 2012). Moreover, Limpopo and Eastern Cape provinces have large numbers of Indigenous pigs kept in households. Despite enterotoxigenic *E. coli* being the most predominant cause of diarrhoea in pigs (Mohlatlole *et al*., 2013), the significance of ETEC has received limited attention. Therefore, there is a need to understand the virulence genes associated with ETEC and their prevalence in South Africa (Blanco *et al*., 2006). Mohlatlole *et al*. (2013) gave an insight on the possible causes of colibacillosis in pigs in South Africa. However, the study only sampled from two farms (the experimental populations at the Agricultural Research Council in Irene and the Middledrift Pig Farm in the Eastern Cape which was used as a reference population) which did not represent the diversity of pig production systems in South Africa. Sampling from many farms under varying management practices allows for the unraveling of trends of various *E. coli* types and their virulence profiles across the country. *E. coli* isolates demonstrate drug-resistance to a wide range of antimicrobials (Costa *et al*., 2010). In South Africa there is a dearth of information on ETEC virulence genes and antimicrobial resistance putting the pig industry at risk of severe losses due to ineffective management programs. Studies aimed at understanding prevalent ETEC virulence genes and antimicrobial resistance profiles are therefore necessary.

Knowledge of the most prevalent virulence factors in the South African pig population will aid in the generation of specific and effective control measures against multi-drug resistance. Information on antimicrobial resistance profiles will inform farmers, veterinary extension officers and other stakeholders on drugs that can still be used and those that should be avoided. Therefore, antimicrobial resistance profiling is a prerequisite in the South African pig industry.

1.3 Objectives

This study was primarily conducted to determine the prevalence of pathogenic *E. coli* and antibiotic sensitivity of ETEC in South Africa.

The specific objectives of the study were to:

a) isolate and identify *E. coli* from rectal swabs obtained from neonatal and post-weaning pigs from Limpopo and Eastern Cape provinces of South Africa,

b) determine the prevalence of ETEC in relation to other *E. coli* types (DAEC, EAEC, EHEC and EPEC) in neonatal and post-weaning pigs in South Africa,

c) establish predominant ETEC fimbriae and enterotoxins from South African *E. coli* isolates, and

d) determine antimicrobial susceptibility of ETEC positive isolates.

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CHAPTER 2

Literature Review

2.1 *Escherichia coli*

Escherichia coli were identified in 1885 and have become one of the most studied bacteria (Clements *et al*., 2012). This is due to *E. coli* strains being easy to grow and the ease with which the organism can be manipulated in the laboratory (Clements *et al*., 2012). *Escherichia coli* is a Gram-negative facultative anaerobic bacterium belonging to *Enterobacteriaceae* that forms part of the gut microbiota (Dubreuil, 2012; Bardiau *et al*., 2010). Microbiota are responsible for helping the host fight against exogenous pathogenic bacteria. This can be achieved by the production of vitamins and other substances (Dubreuil, 2012).

Escherichia coli were previously characterized as non-pathogenic bacteria that form part of the normal flora (Dubreuil, 2012). However, recent studies have reported of pathogenic *E. coli* (enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC) or shiga toxin-producing *E. coli* (STEC), enteroaggreagative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC) and diffusely adherent *E. coli* (DAEC) responsible for diarrhoea in humans and livestock (Nagy and Fekete, 2005; Olaniran *et al*., 2011). *Escherichia coli* pathogenicity is achieved through the production of toxic proteins referred to as enterotoxins (Olaniran *et al*., 2011). The mechanism of pathogenesis, however, varies from strain to strain.

2.2 Diarrheagenic *E. coli*

Enteric *E. coli* are primarily classified according to their virulence factors and the associated clinical disease (Clements *et al*., 2012). The virulence factors that determine *E. coli* pathogenicity are adhesins, invasins, haemolysins, toxins, effacement factors, cytotoxic necrotic factors and capsules (Olaniran *et al*., 2011). Six major groups of diarrheagenic *E. coli* are enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggreagative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC),

diffusely adherent *E. coli* (DAEC) and enterotoxigenic *E. coli* (ETEC) (Bardiau *et al*., 2010). Of the six mentioned pathogenic *E. coli* types, ETEC remains the leading cause of severe diarrhoea in newborn calves and weaned pigs (Nagy and Fekete, 2005).

2.2.1 ETEC

Enterotoxigenic *E. coli* (ETEC) is defined by its fimbriae and enterotoxins (Garabal *et al*., 1997; Zhang *et al*., 2007). To manifest pathogenesis, ETEC isolates attach to the epithelial cells by means of fimbriae initiating colonization (Frydendahl, 2002). Thereafter, heat-labile (*LT*) and/or heat-stable (*ST*) enterotoxins are produced (Kagambega *et al*., 2012) which then cause diarrhoea. ETEC accounts for traveler's diarrhoea in humans, neonatal diarrhoea in calves and also post-weaning diarrhoea (PWD) in pigs (Johnson and Nolan, 2009). This results in loss of productivity and health care cost. In a study conducted in South Africa aimed at determining the prevalence of ETEC, STEC and EAEC in pigs, ETEC was the most dominant (18.6 %) (Mohlatlole *et al*., 2013). Also, ETEC was found in combination with STEC and also with EAEC (Mohlatlole *et al*., 2013).

2.2.2 EPEC

Enteropathogenic *E. coli* (EPEC) produces a specific lesion referred to as the attaching and effacing (A/E) lesion on the host's intestinal cells (Kagambega *et al*., 2012). The mechanism is characterized by attachment of bacteria to the intestinal epithelial cells and effacement of enterocyte microvilli (Bardiau *et al*., 2010; Jafari *et al*., 2012). Bacterial attachment to the outer cell membranes is mediated by a protein called intimin encoded by the *eae* gene located on a 35-kb pathogenicity island (PAI) the locus of enterocyte effacement (LEE) (Kaper *et al*., 2004; Jafari *et al*., 2012). There are three stages associated with binding to the enterocytes and these are: non-intimate association carried out by pili, signal transduction, and intimate contact (Adams and Moss, 2008). A bacterial type III secretion (Adams and Moss, 2008) inserts Tir (translocated intimin receptor) into the enterocyte and becomes part of the cell's membrane where it functions as a receptor for the intimin outer-membrane protein

(Kaper *et al*., 2004). Such mechanism illustrates the ability of a pathogen to provide its own receptor for binding. This stage is associated with increased levels of intracellular calcium ions, inositol phosphate release and activation of tyrosine kinase (Adams and Moss, 2008). The enterocytes develop pedestal-like surface structures on which bacteria perch (Kaper *et al*., 2004). Consequently, deformation and loss of some microvilli occurs and this is thought to cause diarrhoea through disrupting the balance between absorption and secretion in the small intestine (Adams and Moss, 2008). EPEC strains have been found in humans, bovine, dogs, cats, rabbits, pigs, goats and sheep (Bardiau *et al*., 2010). In the 1940s and 1950s, EPEC strains were the cause of frequent diarrhoea outbreaks in infants (Bardiau *et al*., 2010). There is, however, a need to determine the prevalence of EPEC in South African pigs.

2.2.3 EHEC

The main virulence property of enterohemorrhagic *E. coli* (EHEC) is the production of Shiga toxins encoded by *stx1* and *stx2* or their variants. Elaboration of Shiga toxins results in bloody diarrhoea, haemorrhagic colitis (HC) and the haemolytic uraemic syndrome (HUS) (Bugarel *et al*., 2011). Osek (1999) states that *E. coli* strains producing a variant of *stx2* termed *stx2e* have been isolated in pigs with oedema disease. This variant has targets cells such as endothelial cells of small blood vessels leading to edema at specific locations (Choi *et al.*, 2001). However; some reports have revealed the presence of this toxin in diarrheic isolates. Therefore, *stx2e* has a role in post-weaning diarrhoea (Osek, 1999). Mohlatlole *et al*. (2013) detected Shiga toxin in 0.4 % of the pigs from South Africa. Also a combination between ETEC and STEC was detected in 0.8 % of this population. In addition to the *stx* gene, EHEC strains carry the *eae* gene that encodes the adherence factor intimin (Kagambega *et al*., 2012). Intimin is involved in the attachment of the bacteria to the surface of the epithelial cells in the gastrointestinal tract (Kim *et al*., 2010). Adherence of the bacteria to the host then results in watery diarrhoea (Kobayashi *et al*., 2003). Furthermore, there are additional virulence factors associated with EHEC. One of the known additional factors is enterohaemolysin encoded by the *hlyA* gene (Botteldoorn *et al*., 2003; Kagambega *et al*., 2012). These two virulence factors, *eae* and *hlyA*

are thought to enhance pathogenicity (Arthur *et al*., 2002). Although Shiga toxins were detected in a relatively low proportion in South Africa, their presence cannot be ignored. Therefore, further investigations are required.

2.2.4 EAEC

Enteroaggregative *E. coli* (EAEC) initiates pathogenesis by employing an aggregative adherence pattern carried out by aggregative-adhesive fimbriae (Kagambega *et al*., 2012). Its mechanism involves three steps: adherence of the bacteria to the intestinal mucosa by aggregative-adherent fimbriae, followed by the production of mucus-mediated biofilm on the enterocyte surface. Lastly, toxins affecting inflammatory response, mucosal toxicity as well as intestinal secretion are released by the bacteria (Johnson and Nolan, 2009). Though EAEC has a pathogenicity that is not yet fully understood, it produces an enterotoxin called enteroaggreagative heat-stable enterotoxin 1 (EAST-1) (Choi *et al*., 2001). This gene is not only confined in EAEC but also carried in human, porcine and bovine ETEC strains (Choi *et al*., 2001). Also, the *astA* gene encoding *EAST-1* has been detected in EPEC and STEC from animals and humans (Vu-Khac *et al*., 2007; Kim *et al*., 2010). This pathogen is recognized as a diarrhoeal agent especially in developing countries. Mohlatlole *et al*. (2013) reported that in South African pigs, EAEC was the second most dominant pathogen after ETEC where it was detected in 17.5 % of the 40.3 % of the isolates containing the tested virulence genes. In addition, a combination of ETEC and DAEC was detected in 3 % of the isolates (Mohlatlole *et al*., 2013). Though EAEC pathogenesis is not clearly understood, its prevalence suggests that it is a potential pathogen either inducing colibacillosis or increases virulence where colibacillosis has already occurred.

2.2.5 EIEC

Enteroinvasive *E. coli* (EIEC) is defined as almost indistinguishable from *Shigella* spp. because of the similarity in virulence factors as well as disease symptoms (Clements *et al*., 2012; Hill, 2013).

Infection with EIEC is associated with diarrhoea and dysentery (Kagambega *et al*., 2012; Mainil, 2013). These bacteria cause inflammation and ulceration through invasion and multiplication within the epithelial cells of the colon. Invasion is mediated by genes such as those encoding *Ipa* proteins and their transcription regulator *invE* (Kagambega *et al*., 2012).

2.2.6 DAEC

The diffusely adherent *E. coli* (DAEC) consist of a heterogenous group, which creates a diffuse adherence pattern on HeLa and HEp2 cells (Jafari *et al*., 2012). Pathogenesis is achieved by diffuse adherence to the surface of the epithelial cells (Hill, 2013). Although DAEC produce no toxins, it has been associated with diarrhoea (Kaper *et al*., 2004). The DAEC has been divided into *AIDA-1*-dependent group and the adhesins encoded by a family of related operons called fimbrial and afimbrial adhesins (Jafari *et* al., 2012; Clements *et al*., 2012; Mainil, 2013). In addition to DAEC, *AIDA-1* gene can be carried by porcine ETEC strains (Ha *et al*., 2003). In a study by Mohlatlole *et al*. (2013) where the presence of *F4*, *F5*, *F6*, *F18* and *F41* was investigated in South African pigs, none of these were detected. However, the non-fimbrial adhesin *AIDA-1* was detected in 14.2 % of the isolates, which agrees with other studies where *AIDA-1* was found to be prevalent (Zhang *et al*. 2007; Lee *et al*. 2008).

2.3 Enterotoxigenic *E. coli*

Enterotoxigenic *E. coli* (ETEC) belongs to a heterogenous family of lactose-fermenting *E. coli* (Qadri *et al*., 2005). This pathogen is associated with a wide variety of O antigenic types (Qadri *et al*., 2005). Enterotoxigenic *E. coli* causes diarrhoea by means of two virulence factors: adhesins (fimbriae) and enterotoxins (Garabal *et al*., 1997; Zhang *et al*., 2007). The hair-like proteinaceous appendages (fimbriae) mediate attachment to the epithelial cells initiating bacterial colonization (Frydendahl, 2002). Fimbriae types associated with ETEC are *F4* (*K88*), *F5* (*K99*), *F6* (*987P*), *F18* and *F41* (Toledo *et al*., 2012). *F4* (*K88*) contains three antigenic variants *K88ac*, *K88ab* and *K88ad* (Garabal *et*

al., 1997). Enterotoxigenic *E. coli* isolates can contain one or more of the mentioned fimbriae (Duan *et al*., 2012). Upon colonization, heat-labile (*LT*) and/or heat-stable (*STa* and *STb*) enterotoxins are produced (Casey and Bosworth, 2009). The most frequently occurring enterotoxin in piglets is *STb* (Casey and Bosworth, 2009). The released enterotoxins disrupt intestinal fluid homeostasis and fluid hypersecretion leading to diarrhoea (Zhang *et al*., 2007). In addition, some strains do not always carry the known colonization factors. Other non-fimbrial adhesions, therefore, play a role in the adhesion of ETEC to the host. Recently, enteroaggreagative *E. coli* heat-stable enterotoxin (*EAST-1*) initially identified in EAEC has been associated with ETEC strains (Dubreuil, 2012).

2.4 Pathogenesis

To cause disease, two defining traits are associated with ETEC. These include host colonization and enterotoxin production. Host colonization is mediated by pili/fimbriae (Johnson and Nolan, 2009). The colonization factors (CFs), pili/fimbriae adhere to the intestinal epithelium, thereafter heat-labile (*LT*) and/or heat-stable (*ST*) enterotoxins are produced, resulting in diarrhoea (Qadri *et* al., 2005; Johnson and Nolan, 2009). The colonization factors are encoded by plasmids and are age-specific (Johnson and Nolan, 2009). The ETEC strains, therefore, contain different CF types since CF types are host-specific.

2.4.1 Colonization factors

2.4.1.1 F5

In neonatal piglets, diarrhoea is primarily a result of infection with ETEC strains containing *F5*. The *F5* antigen is encoded by a ~78-kb conjugative plasmid (Johnson and Nolan, 2009). These fimbriae mediate binding to the small intestinal glycolipid ganglioside of newborn piglets. *F5* expression is

temperature-dependent, activated at body temperature while inhibited at lower temperatures. There are, however, no completed *F5* plasmid sequences to date (Johnson and Nolan, 2009).

2.4.1.2 F6

Like *F5*, *F6* fimbriae are in porcine ETEC strains causing diarrhoea in neonatal pigs (Johnson and Nolan, 2009). *F6* are responsible for adhesion to the intestinal cells. Johnson and Nolan (2009) reported that the operon encoding *F6* can be positioned in the plasmids or bacterial chromosomes. Similar to *F5*, no *F6*-encoding plasmids have been sequenced (Johnson and Nolan, 2009).

2.4.1.3 F4

In addition to neonatal diarrhoea in pigs, ETEC is also responsible for post-weaning diarrhoea. This disease is characterized by decreased weight gain and death, which normally occur a week after weaning. The ETEC fimbriae known to induce post-weaning diarrhoea are *F4*. The *F4* antigen described in 1961 was the first porcine ETEC CF to be discovered (Johnson and Nolan, 2009). The *F4* antigen is encoded by transmissible plasmids. Studies reveal that *F4* antigens account for enteritis and oedema disease in pigs. These antigens, which are classified into three genetic variants all adhere to porcine epithelial mucosa. The three variants are *K88ab*, *K88ac* and *K88ad* (Kwon *et al*., 2002). The most common variant is *K88ac* (Johnson and Nolan, 2009). The mentioned variants target erythrocytes, intestinal mucosa and carbohydrates or glycoconjugants found in the intestinal epithelial cells. There have been no *F4*-encoding plasmids sequenced (Johnson and Nolan, 2009). However, draft sequencing on plasmid preparations of $K88ab^{+}$ and $K88ac^{+}$ isolates has been conducted. Operons from these plasmids are similar to one another and located on plasmids with a similar arrangement and core backbone (Johnson and Nolan, 2009).

2.4.1.4 F18

The virulence factor *F18* is implicated in ETEC-induced post-weaning diarrhoea. The plasmid encoded *F18* fimbrial adhesins are observed as a zig-zag pattern when subjected to an electron microscope and occur as two antigenic variants, *F18ab* and *F18ac*. *F18* adhesins are not only identified in porcine ETEC strains, they are also found in veterotoxin-producing individuals associated with oedema diseases. Draft sequencing done on $F18^{+}$ isolate revealed seven plasmids ranging from 1 to over 120kbp. Currently, no completed F18-encoding plasmid sequences are available (Johnson and Nolan, 2009).

2.4.1.5 F41

Unlike most ETEC adhesins which are plasmid-encoded, *F41* is chromosomally encoded (Anderson and Moseley, 1988). Genetic determinants of *F41* have shown nucleotide sequence homology with the plasmid-encoded *F4* genes (Anderson and Moseley, 1988). The antigen *F41* occurs less frequently in *E. coli* isolated from post-weaning diarrhoea in pigs. It has, however, been found in neonatal pigs with diarrhoea (Osek, 1999).

2.4.2 Receptors

Fimbria-receptor interaction is required for ETEC to adhere and colonize the small intestines. The susceptibility of pigs to ETEC is, therefore, determined by the presence of adhesion receptor genes (Nagy and Fekete, 2005). The *F5* fimbriae referred to as newborn specific bind to small intestinal glycolipid ganglioside (NeuGc-GM3) (Nagy and Fekete, 2005). The *F6* fimbriae recognize glycoproteins and sulphatides (Nagy and Fekete, 2005). The *F4* and *F18* are highly associated with post-weaning diarrhoea and require small intestinal glycoproteins on the microvilli and/or in the mucus (Nagy and Fekete, 2005). The existence of several receptor variants for *F4* (*F4ab*, *ac* and *ad*) poses difficulty to the determination of *F4* receptor gene. Nagy and Fekete (2005) reported that alleles

control the presence of *F4* receptors where adherence dominates over non-adherence. Similarly, *F18* receptors are controlled by a dominant allele and the absence of a recessive allele (resistant phenotype).

The presence or absence of receptor genes results in host-specificity of ETEC to different animal species (Nagy and Fekete, 2005). The expression of receptors influences age-related resistance/susceptibility to ETEC adhesion. With increased age, *F5* glycolipid receptors are decreasingly expressed. In contrast, *F6* receptors are over-expressed with age resulting in free receptors accumulating in the intestinal lumen and covering *F6* fimbriae, thus blocking adherence. Both these responses induce decreased adhesion of *F5* and *F6* with age (Nagy and Fekete, 2005). However, *F18* receptors are not expressed in neonatal pigs but increasingly expressed up to four weeks. The pigs are, therefore, susceptible during the weaning period. Nevertheless, a number of receptors and their genetic backgrounds remain unknown (Nagy and Fekete, 2005).

2.4.3 Enterotoxins

Enterotoxins are defined as plasmid-regulated secreted proteins or peptides of ETEC bacteria that act on the intestinal epithelium (Nagy and Fekete, 2005). These include large-molecular-weight heat-labile enterotoxin (*LT*) and small-molecular-weight heat-stable enterotoxin (*ST*) (Nagy and Fekete, 2005). Enterotoxins are responsible the stimulation of secretion of electrolytes and fluids by intestinal epithelial cells, causing diarrhoea and dehydration (Choi *et al*., 2001).

2.4.3.1 Heat-labile enterotoxins

ETEC strains are characterized by the production of large molecular weight heat-labile enterotoxins (Olaniran *et al*., 2011). These enterotoxins are structurally and functionally similar to cholera enterotoxin (CT) expressed by *Vibrio cholerae* (Dubreuil, 2012). Two major serogroups of *LT* described are *LT-I* and *LT-II* (Qadri *et al*., 2005). Enterotoxin *LT-I* is expressed in both animals and

humans. However *LT-II* is found primarily in animals, rarely in humans and is not associated with clinical disease symptoms (Olaniran *et al*., 2011).

Heat-labile enterotoxin is characterized by a single A domain and five B subunits containing 240 and 103 amino acids, respectively (Nagy and Fekete, 2005). Present on the cell surface is the monosialganglioside GM1 ganglioside receptor where the B subunits bind into. After fixation of the enterotoxin to the surface, a fragment of A domain (A1) translocates through the cell into the endoplasmic reticulum and activates the adenylatecyclase system thus increasing cyclic adenosine monophosphate (cAMP) level (Nagy and Fekete, 2005). This results in increased fluid and electrolyte secretion as well as decreased absorption. These *LT* effects are irreversible (Nagy and Fekete, 2005).

2.4.3.2 Heat-stable enterotoxins

Heat-stable enterotoxins are small monomeric toxins containing a number of cysteine residues whose disulphide bonds define heat stability of these toxins (Olaniran *et al*., 2011). The first discovered *ST* was the 2 kDa peptide composed of 11 to 18 amino acids called *STa* (Nagy and Fekete, 2005). Heat-stable enterotoxin *STa* induces fluid secretion only in newborn pigs (Nagy and Feteke, 2005). In contrast, *STb* causes diarrhoea in both newborn and weaned pigs (Nagy and Fekete, 2005). The *STa* receptors are heterogenous: with glycoproteins on the cell surface and a particulate transmembrane form of guanylatecyclase-c (pGC-c) (Nagy and Fekete, 2005). Pathogenesis of *STa* is initiated by reversible binding of *STa* to a glycoprotein receptor coupled to guanylatecyclase (Olaniran *et al*., 2011). Activation of the guanylatecyclase results in accumulation of cyclic guanosine monophosphate (cGMP) leading to reduced absorption of water and electrolytes on villus tips and simultaneous increased secretion of Cl^- and water in crypt cells (Nagy and Fekete, 2005; Olaniran *et al*., 2011). The effect of this mechanism is diarrhoea. The *STa* toxin genes are situated on plasmids and a part of the Tn1681 transposon flanked by inverted repeats of IS1 (Nagy and Fekete, 2005).

Another *ST* produced in porcine ETEC is *STb*, a 48-amino-acid peptide. *STb* binds to the sulphatide of the intestinal epithelium (Nagy and Fekete, 2005). Binding results in the stimulation of non-chloride anion secretion by intestinal epithelial cells. Nagy and Fekete (2005) reported that *STb* opens the G protein-linked calcium channel. Accumulated Ca^{2+} can activate prostaglandin endoperoxidase synthetase, thus causing development of prostaglandins. The known receptor associated with STb is sulphatide of the intestinal epithelium (Nagy and Fekete, 2005). *STb* has been recognized as the potent enterotoxin in weaned pigs but not in newborn (Nagy and Fekete, 2005). Like *STa*, *STb* is regulated in the plasmid and has been described as part of a Tn4521 transposon (Nagy and Fekete, 2005).

Another kind of *ST*, called *EAST-1*, has been detected. It has been acquired by some ETEC, EPEC and EHEC strains (Nagy and Fekete, 2005). This toxin interacts with *STa* receptor GC-c thereafter stimulating cyclic GMP (Nagy and Fekete, 2005). There is, however, no homology between the two structures. The toxin alone is, however, not sufficient to cause diarrhoea (Nagy and Fekete, 2005). In addition, *EAST-1* in combination with *LT* produces more severe diarrhoea than *LT* on its own (Nagy and Fekete, 2005).

Figure 2.1 illustrates the pathogenic mechanism of ETEC. ETEC is attached to the host by colonization factors (CFs) (Croxen and Finlay, 2010). The *LT* particularly targets adenylatecyclase. The B subunit binds to the ganglioside GM1. The A subunit then transfers an ADP-ribosyl moiety from NAD to the alpha subunit of the GTP-binding protein G_s responsible for stimulation of adenylatecyclase (Nataro and Kaper, 1998). Adenylatecyclase is permanently activated by ADP-ribosylation of the $G_{s\alpha}$ subunit. Intracellular cyclic AMP is then increased. Thereafter, cAMP-dependent protein kinase A (PKA) is activated resulting in activation of the chloride channel CFTR (Nataro and Kaper, 1998). The *STa* however binds reversibly to guanylatecyclase receptors. This results in the increase of intracellular levels of cyclic GMP (Croxen and Finlay, 2010). Similar to *LT*, the chloride channel is activated thus resulting in Cl^- secretion and inhibition of NaCl absorption.

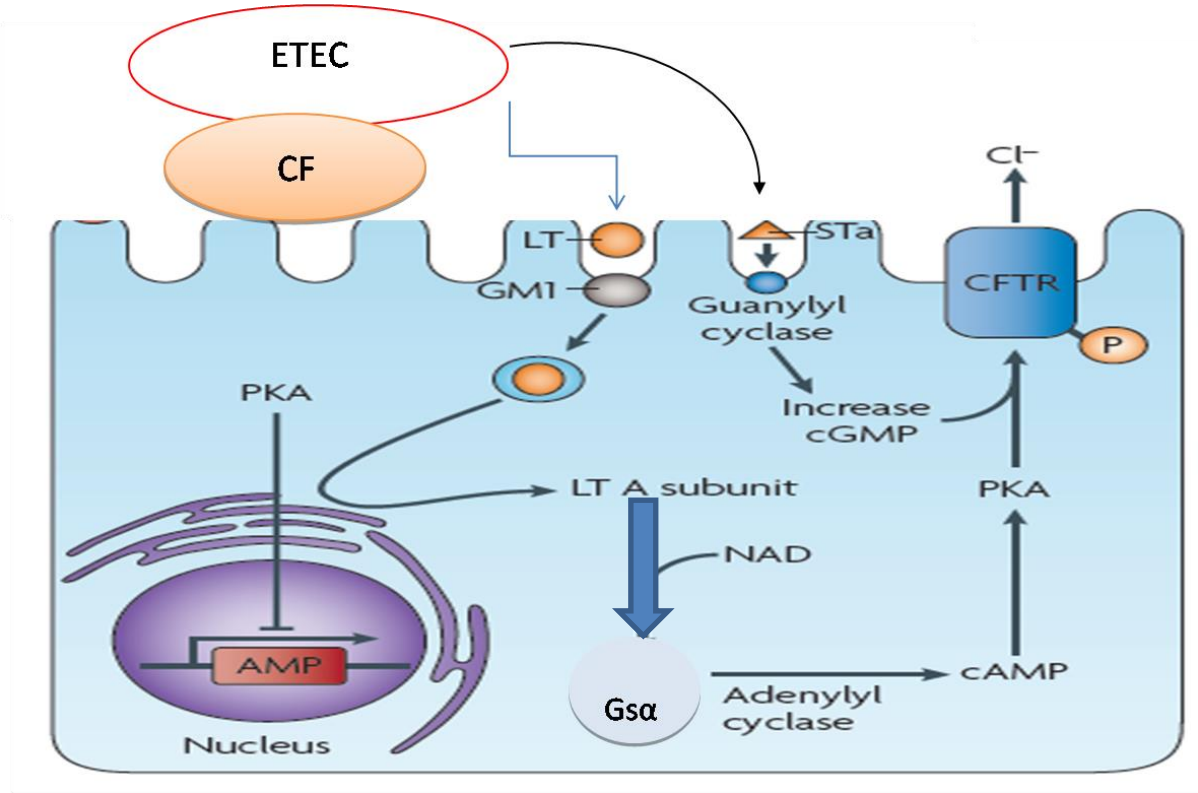

Figure 2.1: Pathogenic mechanism of enterotoxigenic *E. coli* (Croxen and Finlay, 2010)

Figure 2.2 illustrates the enterotoxins produced (*LT*, *STa* and *STb*) and the mechanism employed. As *LT* and *STa* appear on Figure 2.1, only *STb* is highlighted in Figure 2.2.

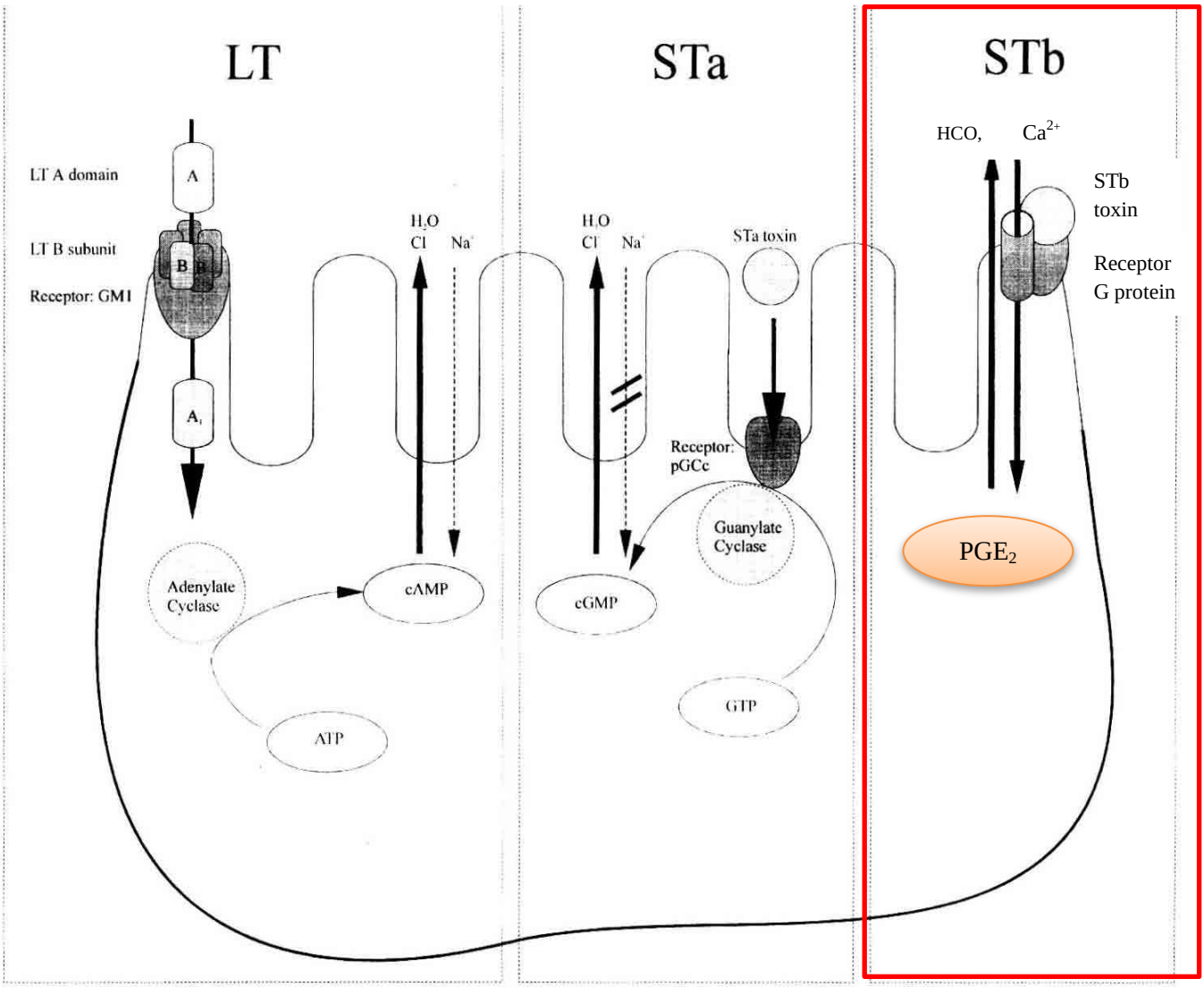

Figure 2.2: Mechanisms of action of enterotoxigenic *E. coli* enterotoxins *LT*, *STa* and *STb* (Nagy and Fekete, 1999)

The *STb* mechanism of action is less known (Nagy and Fekete, 1999). This enterotoxin stimulates a non-chloride anion secretion by intestinal epithelial cell. No intracellular cyclic nucleotide elevation is associated with *STb*; however Nagy and Fekete (1999) suggest that *STb* opens a G protein-linked calcium channel. The increased Ca^{2+} can activate prostaglandin endoperoxidase synthatase, which may result in the formation of prostaglandins (Nagy and Fekete, 1999; Nagy and Fekete, 2005).

2.5 Isolation and identification of *E. coli*

Escherichia coli strains are easy to grow in laboratories, hence are one of the most studied bacteria (Clements *et al*., 2012). Isolation and identification of pure *E. coli* involves classical microbiological methods and molecular diagnosis.

2.5.1 Microbiological methods

Escherichia coli from the *Enterobacteriaceae* family are classified as Gram-negative (Nataro and Kaper, 1998). This species can be recovered easily from clinical specimen by using a general or selective media at 37°C under aerobic conditions. MacConkey agar is commonly used as it selectively grows *Enterobacteriaceae* members (Frydendahl, 2002). In addition, it allows for differentiation of these members based on colony morphology and lactose fermentation (Nataro and Kaper, 1998). MacConkey agar is composed of bile salts and crystal violet dye that inhibit growth of most Gram-positive bacteria, thus selecting for Gram-negative bacteria. In addition, the agar contains lactose which is fermented by some bacteria. Peptone which serves as a source of proteins and neutral red indicator that stains microbes with the ability to ferment lactose are also components of MacConkey agar. Upon streaking onto MacConkey agar, bacteria such as *E. coli* will utilize lactose and produce acid which then lowers the pH of the medium resulting in red or pink colonies. Bile salts precipitate around the colonies causing the surrounding medium to become hazy in appearance. Non-lactose fermenting bacteria will use peptone instead resulting in the formation of ammonia that raises the pH of the agar. This mechanism yields white or colourless colonies. To test haemolysis, the differential and enriched blood agar is used. Some bacteria have the ability to cause lyse red blood cells by release of hemoglobin. Bacteria that produce incomplete hemolysis characterized by greening of the medium are called alpha-hemolytic. Individuals with the ability to completely destruct red cells in the agar zones surrounding colonies are beta-hemolytic. Beta-hemolysis is characterized by clear zones around the growth colonies. Bacteria without the ability to lyse cells are described as non-hemolytic or gamma-hemolytic.

In addition, biochemical tests are employed to identify *Enterobacteriaceae.* Lee *et al*. (2008) carried out indole, methyl-red, Voges-Proskauer and citrate tests which are employed often in other studies. Based on these biochemical tests, indole production, glucose fermentation, lactose fermentation as well as citrate utilization are determined (Vidotto *et al*., 2009).

i) Indole Test

Tryptophan is converted into indole by an enzyme called trypophanase. Bacteria are grown at 37°C in peptone water overnight. Few drops of Kovac's reagent are then added to detect the presence of indole. Indole reacts with the aldehyde in the reagent. A red or pink-coloured ring at the top of the tube denotes a positive.

ii) Methyl-red and Voges-Proskauer tests

These tests are used to test if bacteria use glucose. The end products are, however not the same. Methyl-red test is used to test the ability of bacteria to ferment glucose to produce and maintain stable acid end products. The Voges-Proskauer test tests the ability of organisms to use glucose and produce acetoin. Bacteria are inoculated in a glucose phosphate medium and incubated at 37°C for 48 hours. Thereafter the inoculated broth is separated into two tubes to test for end products. For the methyl-red test, drops of methyl-red (pH indicator) are added. A red-coloured solution symbolizes a positive test and yellow denotes a negative test. For the Voges-Proskauer test, alpha-naphthol 40% KOH are added to the broth then shaken. The tube is then allowed to stand for at least 20 minutes. A pink colour at the top of the tube indicates a positive test.

iii) Citrate test

This test is performed to test the ability of an organism to use citrate as the sole carbon source. Bacteria are inoculated in a medium composed of sodium citrate, inorganic ammonium salts and bromothymol blue (pH indicator). Bacterial colonies are steaked onto Simmon's agar slant and incubated overnight at 37°C. Change in colour from green to royal blue means citrate was used as the carbon source (positive test).

These methods should, however, be used with caution as not all *E. coli* strains are lactose positive, only about 90 % of the bacteria ferment lactose (Nataro and Kaper, 1998). Enteroinvasive *E. coli* strains are lactose negative (Nataro and Kaper, 1998). The indole test is the single best test for differentiation of other family members (Nataro and Kaper, 1998).

2.5.2 Molecular diagnosis

Advances in research have led to the development of molecular detection methods. Employed are DNA-based molecular detection methods (Nagy and Fekete, 2005). Among the first strains to be used in the development of molecular diagnosis, diarrheagenic *E. coli* strains were involved (Nataro and Kaper, 1998). Methods developed include nucleic-acid probes and polymerase chain reaction (PCR) methods (Nataro and Kaper, 1998). Among other methods, real-time PCR is another method used for rapid detection of enteric pathogens such as ETEC (Nagy and Fekete, 2005). However, classical methods such as culture assays, enzyme linked immunosorbent assay (ELISA) or immunofluorescence tests are required when testing gene expression and to make sure that a chance of determining new virulence attributes is not limited (Nagy and Kaper, 2005).

2.5.2.1 Nucleic acid probes

The development and use of DNA probes to detect heat-labile and heat stable toxins in ETEC laid a foundation in the study of these organisms (Nataro and Kaper, 1998). This led to the replacement of costly and complex animal models.

There are polynucleotide and oligonucleotide probes. Polynucleotide probes may originate from genes encoding a particular phenotype or are empirical probes selected from extensive testing revealing a link between the probe and phenotype (Nataro and Kaper, 1998). Probes representing the virulence genes are generally superior to empirical probes. From a DNA sequence of the gene of interest, oligonucleotides are derived. There is the need to be precise with factors such as annealing temperatures, other hybridization conditions and also washing with oligonucleotide probes (Nataro and Kaper, 1998). In addition, false-negative results may be obtained due to slight strain-strain differences within the virulence genes. However, oligonucleotide probes are faster and often yield cleaner results compared to those achieved from the use of polynucleotide probes (Nataro and Kaper, 1998).

2.5.2.2 PCR

A number of diagnostic methods have been developed for the detection of pathogenic microorganisms (Lee *et al*., 2008). Polymerase chain reaction is identified as the major advance and has been used to detect ETEC. Polymerase chain reaction assays that have been used to determine the prevalence of virulence genes in *E. coli* isolates involve single or multiplex reactions for the detection of multiple virulence genes (Casey and Bosworth, 2009). In a multiplex assay, several primers are used in a single reaction tube for the detection of known virulence factors (Nagy and Fekete, 2005). Reference strains serve as controls (Wang *et al*., 2006). In several diagnostic laboratories, multiplex PCR tests are preferred. A great advantage of PCR is the high specificity and sensitivity of the primers. In addition,

real-time PCR is another sensitive and rapidly used method for detection of enteric pathogens such as ETEC (Nagy and Fekete, 2005).

2.6 Treatment and prevention of *E. coli*-induced diarrhoea in pigs

Treatment and prevention of diarrhoea are critical for successful pig production. Vaccination, medication, optimization of nutrition and improved facility sanitation are commonly used in the control of diarrhoea in pigs (Amass *et al*., 2003). Farm management plays a huge role in the spread of the pathogen. Transmission from the infected pigs to susceptible pigs can even occur through caretakers moving from one pig to the other if the necessary biosecurity procedures are not followed (Amass *et al*., 2003). The acquired knowledge on ETEC virulence factors has led to the development of vaccines (Nagy and Fekete, 2005). Although it is possible to breed resistant pigs, vaccination is more realistic (Nagy and Fekete, 1999). Vaccines for blocking adherence of *E. coli* to the intestines have been developed (van Beers-Schreurs *et al*., 1992). Prevention measures aimed at inhibiting colonization of the small intestine by blocking *E. coli* adherence to the villi are of great interest (van Beers-Schreurs *et al*., 1992).

According to Nagy and Fekete (1999) control measures that do not involve antibiotics should be considered. These include reduction of management factors likely to contribute to disease manifestation such as the weaning age, weight, diet, overstocking and environment contamination from previous stocks.

Successes have been observed with vaccination against neonatal diarrhoea since adhesins (*K88*, *K99* and *987P*) and toxins (*LT*) became standard vaccine components (Nagy and Fekete, 1999). Nowadays, infections in suckling pigs can be prevented through maternal immunization and early supply of colostrum (Nagy and Fekete, 2005). Maternal vaccines, especially for parental application in pregnant sows are available on the market (van Beers-Schreurs *et al*., 1992; Nagy and Fekete 2005). The vaccines are either carrying activated bacteria with protective antigens or purified antigens

applied in late pregnancy. The passively acquired antibodies through colostrum provide protection given that the offspring ingest immune colostrum just after birth (within the first 12 hours of life) thus inhibiting virulence factors and proliferation of ETEC in the intestines (Nagy and Fekete, 1999; Nagy and Fekete, 2005). The supply of colostrum antibodies metaphylactically can be used, albeit with less success.

Control measures for post-weaning diarrhoea are quite different. Promising results are achieved with live oral vaccination applied before weaning (Nagy and Fekete, 1999). Another approach to reduce post-weaning diarrhoea (PWD) is the combination of live oral and killed parenteral administration (Nagy and Fekete, 1999). Protection against diarrhoea, has, however been a problem for farmers and veterinarians since *E. coli* has the ability to acquire new virulence factors or mask these genes, thus those posing disadvantages for survival leading to resistance (Nagy and Fekete, 1999). Therefore, further work is required on effective control and prevention measures.

2.7 Antimicrobial resistance

Since the last decade, the emergence of antimicrobial resistance has been observed worldwide (Da Silva and Mendonça, 2012). Bacteria acquire resistance to therapeutic antibiotics (Hill, 2013). *Escherichia coli*, in particular, is associated with antibiotic resistance (Hill, 2013). Antibiotics are not only used for therapeutic purposes in animals and humans, but also used as prophylactic agents and as growth promoters in animals (Da Silva and Mendonça, 2012). Antimicrobial resistance is associated with intensive use and misuse of drugs (Da Silva and Mendonça, 2012). Resistant bacteria are, therefore, experienced in both farm animals and humans (Da Silva and Mendonça, 2012). Transmission of these resistant bacteria can occur directly or indirectly from animals to humans or vice versa via the food chain (Da Silva and Mendonça, 2012).

Antibiotics employ different modes of action depending on their structure and target sites on the bacteria. The different modes of action are:

i) Inhibition of cell wall synthesis

Antibiotics targeting the cell wall can either kill (bactericidal) or inhibit (bacteriostatic) bacterial organisms. For example β-lactams and vancomycin are responsible for the inhibition of peptidoglycan synthesis and disruption of peptidoglycan cross-linkage, respectively.

ii) Inhibition of protein synthesis

The synthesis of protein is necessary for multiplication and survival of bacterial cells. Several antibiotics target protein synthesis by binding to either the 30S or 50S subunits of intracellular ribosomes resulting in the disruption on cellular metabolism. This could either result in the death of the organism or growth inhibition. Examples include aminoglycosides and macrolides which irreversibly bind 30S ribosomal proteins (bactericidal) and reversibly bind 50S ribosome blocking peptide elongation (bacteriostatic), respectively.

iii) Alteration of cell membrane

Disruption or damage of cell membranes, which are responsible for regulating the flow of intra- and extracellular substances, could result in the leakage of important solutes required by bacteria for survival. Bacitracin is an example and is responsible for disrupting cytoplasmic membranes.

iv) Inhibition of nucleic acid synthesis

Certain antibiotics bind to components involved in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis, thus interfering with normal cellular processes. For example, quinolones

inhibit DNA gyrases required for DNA supercoiling and bacitracin inhibits RNA transcription. This mechanism affects bacterial survival and multiplication.

v) Antimetabolite activity

Some bacteria inhibit certain metabolic pathways required for bacterial survival. Trimethophrim for example, inhibit dihydrofolate reductase, an enzyme required for folic acid production.

Responses from bacteria to antibiotics have however posed concerns. These include antibiotic degradation by enzymes such as β-lactamases (Hill, 2013). Also, the antibiotic can be removed by efflux pumps before it even acts in the cell. In addition, down-regulation of porin genes may occur resulting in prevention of antibiotic entry. The occurrence of modifications as well as spontaneous mutations resulting in the alteration of the cell binding site or target site also causes resistance to the antibiotic.

The mentioned resistance mechanisms may be acquired either vertically or horizontally. Between bacteria, resistance genes can be transferred through conjugation, transformation and/or transduction (Hill, 2013). The transfer of these resistant genes between different bacterial strains or species occur where the resistance traits are located in genetic elements such as plasmids, transposons and integrons (Hill, 2013; Da Silva and Mendonça, 2012). They are therefore highly mobile (Hill, 2013). Nowadays, there are *E. coli* strains that are resistant to all conventional therapeutic antibiotics. Also, a number of *E. coli* strains have displayed resistance to modern antibiotics due to the advent of extended spectrum β-lactamases (ESBLs) (Pitout and Laupland, 2008). In a study conducted in Ontario where surface water and faecal samples were tested, samples revealed resistance to a wide range of antibiotics (Hill, 2013). Interestingly, samples collected from combined sewage outfalls

displayed multiple drug resistance to ampicillin, ciprofloxacin, gentamicin, and tetracycline (Hill, 2013).

2.8 Summary

Enterotoxigenic *E. coli* is the major cause of diarrhoea in both neonatal and post-weaning pigs. The advent of diagnostic methods such as PCR has allowed for the detection of virulence genes. The major concern worldwide is the development of antimicrobial resistance. Resistance poses difficulties in treating or preventing ETEC-induced diarrhoea in pigs. There is a need to determine the prevalence of ETEC as well as the antimicrobial resistance patterns prevalent in South Africa. Such information will be used to improve *E. coli* control measures, thus, minimizing economic losses. The study was therefore conducted with the intention of determining the prevalence of pathogenic *E. coli* and antibiotic sensitivity of ETEC isolates in South African pigs.

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CHAPTER 3

Detection of enterotoxigenic *Escherichia coli* (ETEC) virulence profiles relative to enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) by multiplex PRC in South African pigs

Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of colibacillosis in piglets worldwide. The objective of the current study was to investigate prevalent *E. coli* strains and determine the prevalence of ETEC relative to EAEC, DAEC, EHEC and EPEC in pigs from Limpopo and Eastern Cape provinces. A total number of 303 isolates classified as *E. coli* by classical microbiological methods were tested for the presence of enterotoxins (*LT*, *STa* and *STb*), enteroaggreagative heat-stable enterotoxin (*EAST-1*), Shiga toxins (*Stx1*, *Stx2* and *Stx2e*), verotoxins (*VT1* and *VT2*), enterohaemolysin (*hlyA*), fimbriae (*F4*, *F5*, *F6*, *F18* and *F41*), adhesin involved in adherence (*AIDA-1*), attaching and effacing factor (*EAE*) and porcine attaching and effacing factor (*PAA*) using multiplex polymerase chain reaction (mPCR). The most prevalent strain was EAEC observed in 33.3 % of the tested population. A significant 11.9% of the isolates belonged to ETEC. The most dominant enterotoxin was *LT* (33.3%) followed by *STb* (25 %). *STa* was found in 22.2 % of the isolates. Verotoxins, *hlyA* and *Stx1* were detected in 2.97, 9.57 and 1.65 % of the isolates, respectively. The adhesin *EAE* was found in 5.25% of the isolates where it was only positive in the presence of other genes. The adhesins *AIDA-1* and *PAA* occurred as individual genes accounting for 0.99 and 1.65 % of the isolates. From ETEC positive isolates 61.1 % contained *AIDA-1* (30.6 %), *PAA* (8.3 %), *EAE* (2.8 %), *AIDA-I/PAA/EAE* (2.8 %) and lastly *PAA/AIDA-1* and *EAE/AIDA-1* with an equal proportion of 8.3 %. Overall, virulence genes were detected in 67.7 % of the isolates and were represented in 48 pathotypes. The most prevalent pathotypes were *EAST-1* (33.3 %), *EAST-1/PAA* (4.95 %), *HLY* (2.65 %) and *HLY/EAST-1* (1.94 %). Only one isolate harboured *F4/F5* fimbriae. The study gives an insight into the role of ETEC and the prevalent *E. coli* pathotypes likely

to cause colibacillosis in South African pigs. This information is important when designing effective control measures for prevention and treatment of colibacillosis.

Keywords: ETEC, colibacillosis, fimbriae, enterotoxins, non-fimbrial adhesins, EAEC, DAEC, EHEC, EPEC, pigs, virulence genes, pathotypes

3.1 Introduction

The pork industry is relatively large in South Africa with leading producers in Limpopo and North West provinces accounting for 44% of total production (DAFF, 2012). In the year 2010/2011, more than 2 million tons of pork were produced (DAFF, 2012). Both commercial and indigenous breeds are raised in South Africa particularly in Limpopo and Eastern Cape provinces. *Escherichia coli* infections are a major concern in the pig industry in South Africa and worldwide (Henton and Engelbrecht, 1997). Enteric colibacillosis occurs most frequently in pigs where it affects neonates and those in the immediate post weaning period (Henton and Engelbrecht, 1997). During farrowing, the percentage of pathogenic *E. coli* strains increases noticeably resulting in transmission from sow to the piglets at an early stage (Henton and Engelbrecht, 1997). A contributing factor in weaned piglets is stress which could be due to loss of maternal contact, change of environment (new pens) and fluctuations in environmental temperature.

Diarrhoeal diseases are commonly due to infection with one or the other strain of *Escherichia coli* (Choi *et al*., 2001a). Based on virulence factors and clinical diseases, six well-known major groups identified to date are enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC). Enterotoxigenic *E. coli* is the most predominant cause of *E coli*-induced diarrhoea in pigs (Liu *et al*., 2014). Enterotoxigenic *E. coli* adhere to the small intestinal epithelial cells without causing any significant changes in morphology (Nagy and Fekete, 2005). Upon adhesion, enterotoxins are released which then elevates water secretion by enterocytes. The absorption of water and electrolytes from the gut is also decreased, resulting in osmotic diarrhoea (Nataro and Kaper, 1998; Nagy and Fekete, 2005). Adhesion factors (*F4*, *F5*, *F6*, *F18* and *F41*) and enterotoxins (*LT*, *STa* and *STb*) are the main ETEC virulence factors (Osek, 1999).

Identification of ETEC fimbriae and enterotoxins is a very necessary tool for understanding ETEC prevalence and for the development of effective control measures (Qi *et al*., 2012). To aid in detecting

enterotoxigenic *E. coli*, several diagnostic methods have been generated (Lee *et al*., 2008). The polymerase chain reaction (PCR) is one of the popular methods used for determining ETEC virulence genes (Lee *et al*., 2008). There are various PCR assays employed to establish the most prevalent virulence genes in porcine *E. coli* isolates (Casey and Brosworth, 2009). These include single reactions or multiplex PCR (mPCR) assays. Multiplex assays are performed with the purpose of determining multiple virulence genes in a single reaction (Casey and Brosworth, 2009). The use of either real-time or gel electrophoresis for product detection has been explored (Casey and Brosworth, 2009).

In South Africa, limited research on porcine enteropathogens has been conducted. To name a few, Ateba and Bezuidenhout (2008) characterized *E. coli* O157 strains from pigs, cattle and humans in the North-West province of South Africa. Mohlatlole *et al*. (2013) determined the prevalence of ETEC, EHEC and EAEC in South African pigs. With only limited research carried out to date, there is scarce information on the epidemiology of diarrheagenic *E. coli* strains present in South Africa. We tested the hypothesis that infection with pathogenic *E. coli* is prevalent in pigs, with ETEC classified as the most predominant strain. There is, therefore, the need to determine the prevalence of the mentioned pathogenic *E. coli* strains relative to enterotoxigenic *E. coli* characterized as the main pathogen responsible for diarrhoea and causing losses in both neonatal and weaning pigs. The objective of the current study was, therefore, to isolate and identify *E. coli* and determine the prevalence of ETEC in comparison to other types of *E. coli* from rectal swabs of pigs.

3.2 Materials and Methods

3.2.1 Sampling

Samples used in the study were collected from the pigs' rectum using COPAN^{R} swabs containing Amies transport media. A total of 325 pigs were sampled from the villages of Thohoyandou in Limpopo and Alice, King Williams Town, Umtata and Port St Jones regions of the Eastern Cape

Province during the months of June and September 2012. Selected pigs ranged from as young as 4 days to 16 weeks old. These pigs belonged to various breeds including indigenous, Large white, Duroc and exotic. The feed supply varied between these pigs. Neonatal pigs depended on the sows for milk. Weaning pigs especially those raised commercially were fed pig grower while free-ranging pigs scavenged for food or were given any food remains from humans. The collected rectal swabs were stored in ice filled bags during sampling according to Vidotto *et al*. (2009) and were transported to the research laboratory at the Bacteriology section of the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI) where further analysis were conducted.

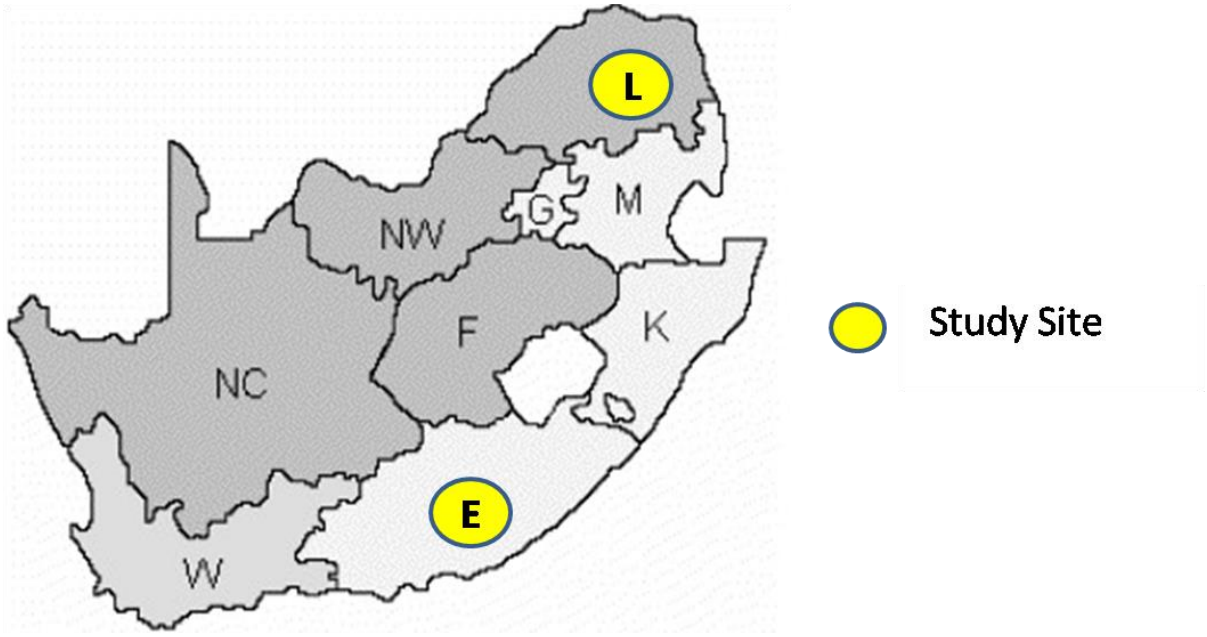

Figure 3.1: Map illustrating the two provinces of South Africa; Eastern Cape (E) and Limpopo (L) sampled in this study (Fielding and Shields, 2006)

3.2.2 Classical microbiological methods

The rectal swabs were cultured directly onto MacConkey plates and incubated at 37ºC overnight (Lee *et al*., 2008; Vu-Khac *et al*., 2007). Suspect colonies (pink rods achieved as a result of utilization of lactose leading to a decrease in the pH of the agar) were further streaked onto sheep blood agar for overnight growth at 37ºC to determine the presence of α-, β- and γ-haemolysis according to Kim *et al*.

(2010). Therefore, *E. coli* colonies were identified based on colony morphology and haemolysis (Costa *et al*., 2010). In addition, presumptive *E. coli* were selected and confirmed as *E. coli* by standard biochemical procedures that included indole, methyl-red and citrate tests (Lee *et al*., 2008; Vu-Khac *et al*., 2007). Indole, methyl-red and citrate tests were used to (i) test the production of indole from tryptophan using tryptophanase, (ii) test for the ability of an organism to produce and maintain stable acid products from fermenting glucose and (iii) to test for the ability of an isolate to utilise citrate as the sole carbon and energy source, respectively. Further culturing on nutrient agar was done and the colonies obtained thereof were used in DNA preparation.

3.2.3 DNA extraction

Crude bacterial DNA used for PCR was prepared by overnight incubation of *E. coli* cultures on nutrient agar plates at 37ºC. The obtained colonies were suspended into 1000 µl of sterile distilled water in eppendorf tubes. Thorough shaking was done so to mix the bacteria with water. Samples were then boiled at 99ºC for 20 minutes using a heating block (Casey and Brosworth, 2009). To prevent pressure build-up during boiling, eppendorf lids were pierced with sterile needles prior to boiling. After cooling at room temperature, suspensions were then centrifuged at 13 000 x *g* for 5 minutes to separate the supernatant to be used as DNA template for PCR from the cell debris (Lee *et al*., 2008).

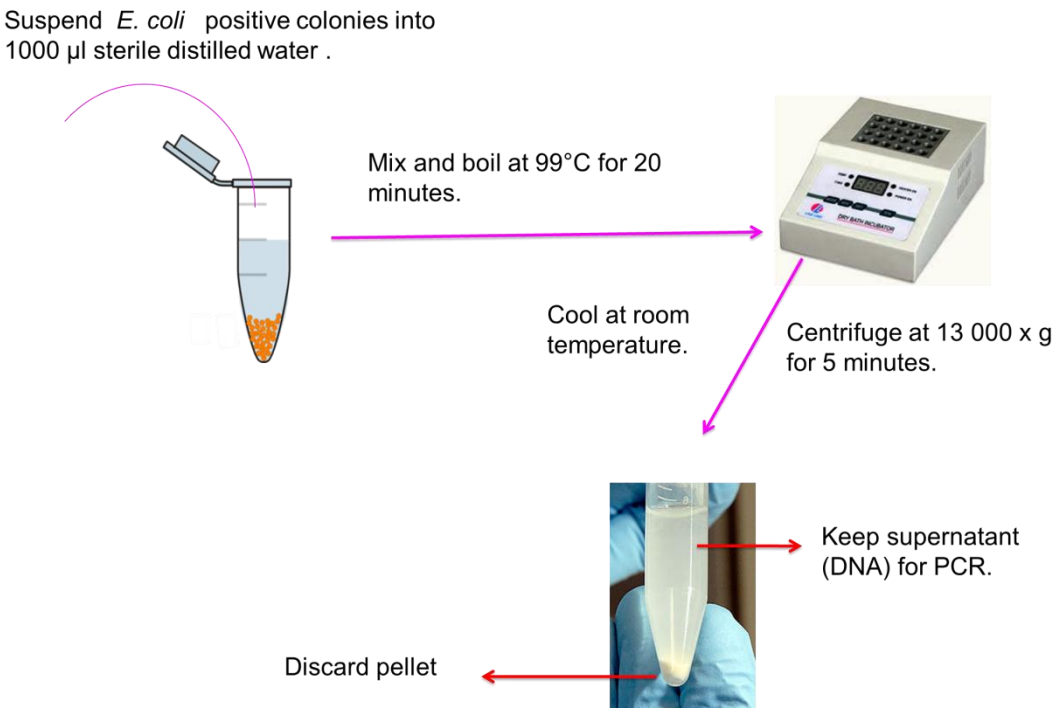

Figure 3.2: Steps involved in the extraction of crude DNA

3.2.4 Multiplex PCR

To determine virulence factors (represented in Table 3.1) present in the porcine DNA samples, multiplex PCR assays with various combinations of primer sets were conducted. Reactions involved primers targeting toxins, fimbriae and non-fimbrial adhesins. Targeted virulence genes were *LT*, *STa*, *STb*, *Stx1*, *Stx2*, *Stx2e*, *hlyA*, *VT1*, *VT2* and *EAST-1*. These isolates were further tested for the presence of *F4*, *F5*, *F6*, *F18* and *F41* fimbriae and non-fimbrial adhesins *AIDA-1*, *EAE* and *PAA*. This was done to determine the common virulence genes present in the South African pig population. In addition, the prevalence of ETEC in the given pig population was determined. Primers used for amplification are tabulated in Table 3.2. Reference samples were obtained from ARC-OVI Bacteriology culture collection from previous studies. Serving as negative controls were *E. coli* ATCC 25922 and a no template reaction. The 25µl PCR reactions contained 12.5 µl of Dream *Taq* Green PCR Master Mix, 0.5 µl of each primer, 5 µl of crude DNA and nuclease free water. Deoxyribonucleic acid amplification was achieved by initial denaturation at 95ºC for 5 minutes,

followed by 35 cycles of denaturation at 95ºC for 30 seconds, annealing at 56ºC for 30 seconds, extension at 72ºC for 1 minute and final extension at 72ºC for 7 minutes.

Table 3.1: Virulence genes associated with *Escherichia coli* categories targeted in this study

***Escherichia coli* type**	**Virulence mechanism**	**Virulence gene**
ETEC	Enterotoxins	*LT, STa, STb*
	Fimbriae	*F4, F5, F6, F18, F41*
EHEC	Shiga-toxins/Vero-toxins	*Stx1, Stx2, VT1, VT2*
	Variant	*Stx2e*
	Intimin	*EAE*
	Enterohaemolyin	*hlyA*
EPEC	Intimin	*EAE*
	Adhesin	*PAA*
EAEC	Enterotoxin	*EAST-1*
DAEC	Adhesin	*AIDA-I*

3.2.5 Gel electrophoresis

Ten microliters of the amplified DNA products were run on 2% agarose gel to separate the PCR products. To identify amplified products and validate findings, reference positive strains and negative controls along with a 100 bp ladder were used (Toledo e*t al*., 2012) and visualised using a gel documentation system (Bio-Rad Trans-UV, Japan) .

3.2.6 Statistical analysis

The *E. coli* types were grouped into different categories based on the toxins, fimbriae and adhesins. ETEC strains were identified as those carrying toxins *LT, STa* and *STb* and fimbriae *F4, F5, F6, F18* and *F41*. Individuals carrying *Stx1, Stx2, Stx2e, VT1, VT2* and *hlyA* were characterized as EHEC. Enteroaggregative *E. coli* was represented by *EAST-1*. The adhesins *PAA, AIDA-1* and *EAE* known to occur in various *E. coli* strains including ETEC were also determined. The frequencies of the different *E coli* (ETEC, EAEC, EHEC, EPEC and DAEC) were determined by direct counting of the respective types relative to the total number of *E. coli* positive isolates (N = 303).

3.2.7 Pathotype grouping

Pathotype (the presence of one or more virulence factor) determination was also conducted. Grouping was based on the presence of genes occurring in the same isolate whether from the same or different *E. coli* group. As a result, *E. coli* isolates carrying the same gene combinations were then classified under the same pathotype.

Table 3.2: Polymerase chain reaction primers used for amplification of virulence genes of *E. coli* isolates

Virulence Factors	Product size (bp)	Reference
LT	282	Ngeleka *et al*., 2003;Mohlatlole *et al*., 2013
STa	183	Cai *et al*., 2003; Ngeleka *et al*., 2003
STb	360	Cai *et al*., 2003; Ngeleka *et al*., 2003
F4	841	Cai *et al*., 2003; Qi *et al*., 2012
F5	543	Cai *et al*., 2003; Qi *et al*., 2012
F6	463	Cai *et al*., 2003; Qi *et al*., 2012
F18	513	Ngeleka *et al*., 2003; Mohlatlole *et al*., 2013
F41	682	Cai *et al*., 2003; Qi *et al*., 2012
Stx1	664	Cai *et al*., 2003; Mohlatlole *et al*., 2013
Stx2	281	Cai *et al*., 2003; Mohlatlole *et al*., 2013
Stx2e	454	Cai *et al*., 2003; Mohlatlole *et al*., 2013
VT1	256	ARC-OVI Bacteriology collection
VT2	185	ARC-OVI Bacteriology collection
hlyA	561	ARC-OVI Bacteriology collection
EAST-1	125	Cheng *et al.*, 2006
PAA	360	Cheng *et al.*, 2006
AIDA-1	585	Cheng *et al.*, 2006
EAE	790	Cheng *et al.*, 2006

3.3 Results

3.3.1 Classical microbiological methods

Pink rods were observed on MacConkey plates (Figure 3.3a). *Escherichia coli* is known to ferment lactose, however not all *E. coli* strains are lactose positive, only about 90% (Nataro and Kaper, 1998). Haemolysis was observed on sheep blood agar plates (Figure 3.3b). Colony morphology was not conclusive enough to confirm the presence of *E. coli.* Identification and confirmation of *E. coli* was achieved by standard biochemical tests (indole, methyl-red and citrate tests). These tests were aimed at determining indole production, glucose fermentation as well as citrate utilization (Vidotto *et al*., 2009).

a **b**

Figure 3.3: Colony morphology observed on a) MacConkey and b) Blood plates after overnight incubation at 37°C to culture *E. coli*

Most *E. coli* isolates tested positive for indole and methyl-red but negative for citrate as expected (Figure 3.4). The indole test is, however, characterized as the best test for identification and differentiation of *Enterobacteriaceae* (Nataro and Kaper *et al*., 1998). Based on the biochemical tests obtained, a total of 303 (93.2 %) isolates out of 325 rectal swabs collected were identified as *E. coli* positive. The 303 isolates were then used for DNA extraction and mPCR.

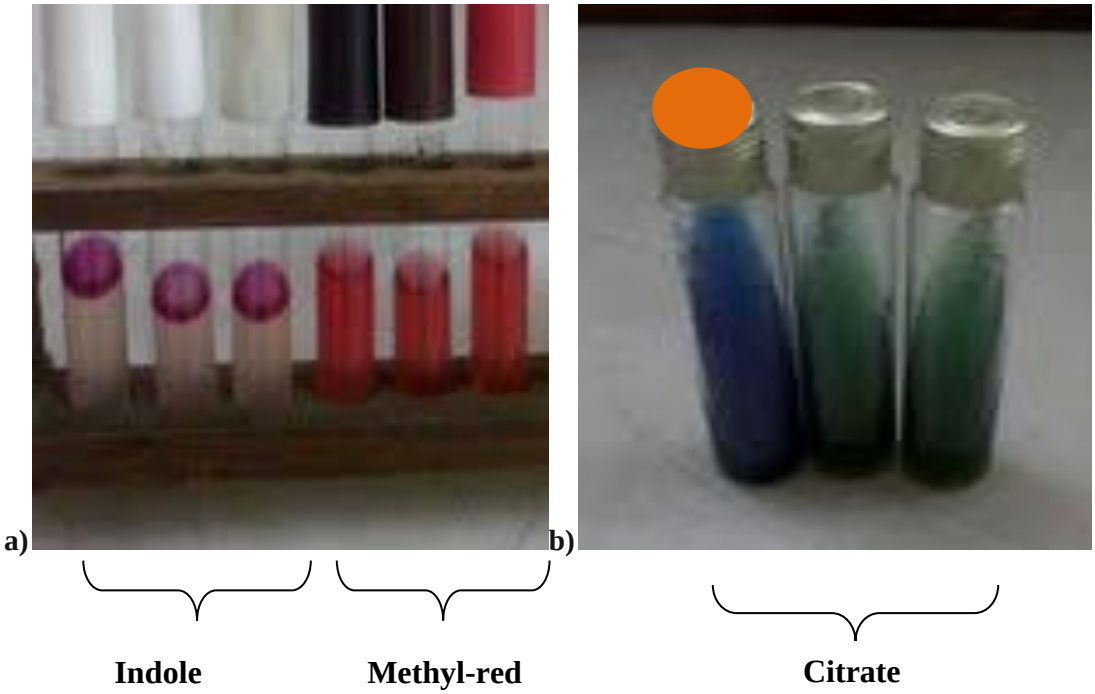

Figure 3.4: *Escherichia coli* isolates after overnight incubation at 37°C on indole and methyl-red (a) and citrate (b) where **C** denotes negative control (*Klebsiella pneumonia*)

3.3.2 Genotyping

(i) Prevalence of different types of *E. coli*

Based on the multiple PCR reactions involving primers specific to *E. coli* virulence genes (Table 3.1), grouping of the positive isolates into different pathogenic *E. coli* was done. Of the 303 tested isolates, 205 harboured the tested virulence genes and could be categorised into different *E. coli* types (Table 3.3). The isolates carried one or more virulence genes of the same strain and/or different strains. The isolates were, therefore, identified as EAEC, ETEC, EHEC, DAEC and EPEC or a combination of the *E. coli* types. Majority of the isolates (101, 33.3%) were identified as EAEC. Enteroaggregative *E. coli* was detected in combination with other types (EPEC, EHEC, ETEC and DAEC) in 21, 11, 7 and 4 isolates, respectively. Other combinations involved EPEC with DAEC (2 isolates) and EHEC (2 isolates). Isolates harbouring virulence genes belonging to three *E. coli* types were also observed. In total, these isolates belonged to nine groups, five combinations of which were associated with ETEC. A total of six isolates had a combination of four *E. coli* types. Of these six isolates, four were classified as EAEC/EPEC/DAEC/ETEC and two were classified as EHEC/EAEC/DAEC/ETEC.

Table 3.3: Distribution of *E. coli* strains among 205 isolates harbouring virulence genes

***E. coli* type**	**Frequency (n=303)**	**Prevalence (%)**
EAEC	101	33.3
ETEC[a]	8	2.64
EHEC	16	5.3
DAEC	3	0.99
EPEC	5	1.65
EAEC+ETEC[a]	7	2.31
EAEC+EHEC	11	3.63
EAEC+DAEC	4	1.32
EAEC+EPEC	21	6.93
EPEC+DAEC	2	0.66
EHEC+EPEC	2	0.66
EAEC+DAEC+ETEC[a]	9	2.97
EAEC+EPEC+ETEC[a]	2	0.66
EHEC+EPEC+ETEC[a]	1	0.33
EHEC+EAEC+ETEC[a]	1	0.33
EPEC+DAEC+ETEC[a]	2	0.66
EHEC+EPEC+DAEC	1	0.33
EHEC+EAEC+DAEC	1	0.33
EAEC+EPEC+DAEC	1	0.33
EHEC+EAEC+EPEC	1	0.33
EHEC+EAEC+DAEC+ETEC[a]	2	0.66
EAEC+EPEC+DAEC+ETEC[a]	4	1.32

[a] ETEC detected

(ii) Molecular detection of toxins

The most frequently encountered toxin gene was *EAST-1* which was observed in 163 (53.8%) isolates. Of the three shiga-toxin genes investigated in this study, *Stx1* was the only one present and observed in five (1.65%) isolates. In addition to shiga-toxins, verotoxins *VT1* and *VT2* were observed in 0.99% and 1.65% of the isolates, respectively. One isolate carried both *VT1* and *VT2*. Toxin *hlyA* was detected in 9.57%. ETEC virulence genes were observed either purely alone and/or in combination with other virulence genes belonging to various *E. coli* types. Based on Table 3.3, ETEC occurred alone and in combination with other types in 11.9% (36/303) of the *E. coli* positive isolates. Classification was based on the presence of enterotoxins *STa*, *STb* and/or *LT*. Figure 3.5 illustrates some of the ETEC positive isolates. The most prevalent enterotoxin was *LT* (33.3%). Enterotoxins *STb* and *STa* were found in 25 % and 22.2 % of the positive isolates, respectively. The ETEC isolates were also observed to carry more than one of the ETEC enterotoxins in the proportions 11.1 %, 5.6 % and 2.8 % for *LT/STb*, *LT/STa* and *STa/STb* respectively (Table 3.4). None of the isolates were however positive for all three ETEC enterotoxins.

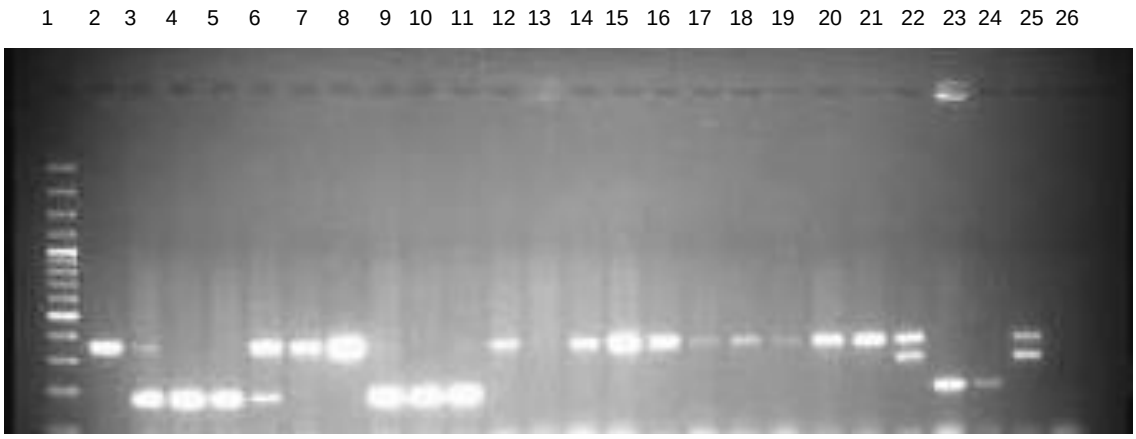

Figure 3.5: A 2% agarose gel illustrating amplification of enterotoxigenic *E. coli* obtained using primers *STa* (183 bp), *STb* (360 bp) and *LT* (282 bp) for amplification of ETEC enterotoxins where the Lanes were loaded as follows, Lane 1: 100 bp DNA ladder, Lanes 2-12 and Lanes 14-21: ETEC positive isolates, Lanes 22-26: *K88* (*F4:F5:LT:STb*); *K99* (*F5:STa*); 1883-2 (*F41:F5:STa*), 1883-5 (*LT:STb*) and *E. coli* ATCC 25922 (negative control)

Some ETEC positive isolates did not only possess ETEC enterotoxins (*LT*, *STa* and *STb*) but also carried the virulence factor, heat stable toxin-1 (*EAST-1*) of enteroaggregative *E. coli.* This toxin was present in 69.4 % (25) of the 36 ETEC positive isolates.

Table 3.4: Proportions of enterotoxins and *EAST-1* in the ETEC positive population (n=36)

Virulence genes	Number of isolates (n=36)	Proportion (%)
LT	12	33.3
STa	8	22.2
STb	9	25
LT/STa	2	5.6
LT/STb	4	11.1
STa/STb	1	2.8
EAST-1	25	69.4

(iii) ETEC colonization factors

The 36 ETEC positive isolates were tested for the ETEC associated fimbriae *F4*, *F5*, *F6*, *F18* and *F41*. Of these, only one isolate (2.8 %) carried a combination of *F4* and *F5* fimbrial genes. The other 35 isolates had none of the tested ETEC fimbriae (*F4*, *F5*, *F6*, *F18* and *F41*). The 36 isolates were further tested for the presence of non-fimbrial adhesins belonging to other *E. coli* strains that included *AIDA-1*, *PAA*, and *EAE*. Of these 36 isolates, 38.9 % were negative for the three non-fimbrial adhesion genes tested. The most common adhesin was *AIDA-1* which was found in 30.6 % of the population (Table 3.5). The *PAA* and *EAE* adhesin genes were found in 8.3 and 2.8 % of the population, respectively. Some isolates carried more than one adhesion gene. Isolates carrying combinations of *PAA* and *AIDA-1* were observed in 8.3 % of the isolates. Another 8.3 % of the isolates had both *EAE* and *AIDA-1*. Only one isolate (2.8 %) carried all the three adhesin factors.

Table 3.5: Distribution of adhesin factors *AIDA-1*, *PAA* and *EAE* in ETEC isolates

Adhesin factors	Number of isolates (n=36)	Proportion (%)
AIDA-1	11	30.6
PAA	3	8.3
EAE	1	2.8
PAA/AIDA-1	3	8.3
EAE/AIDA-1	3	8.3
AIDA-1/PAA/EAE	1	2.8

(iv) Prevalence of *E. coli* pathotypes

Of the 303 *E. coli* positive isolates, 205 tested positive for the tested *E. coli* strains. Tables 3.6a and 3.6b show the occurrence of different *E. coli* pathotypes in the 205 isolates. A total of 48 pathotypes were observed.

Table 3.6a: Distribution of the different *E. coli* pathotypes within the 205 isolates containing the tested virulence genes

Pathotype	Frequency (n=303)	Percentage (%)
AIDA-1	3	0.99
AIDA-1/LT[a]	1	0.33
EAE/AIDA-1	2	0.66
EAE/PAA/AIDA-1/STb[a]	1	0.33
EAST-1	101	33.3
EAST-1/AIDA-1	4	1.32
EAST-1/EAE	5	1.65
EAST-1/LT[a]	1	0.33
EAST-1/PAA	15	4.95
EAST-1/STa[a]	3	0.99
EAST-1/STb[a]	3	0.99
EAST-1/VT1	1	0.33
HLY	8	2.64
HLY/EAE	1	0.33
HLY/EAE/PAA/AIDA-1	1	0.33
HLY/EAST-1	6	1.98
HLY/PAA	3	0.99
HLY/PAA/STa[a]	1	0.33
HLY/VT1	1	0.33
LT[a]	3	0.99
LT/STa[a]	1	0.33
LT/STb[a]	1	0.33
LT/STb/F4/F5[a]	1	0.33
PAA	5	1.65

[a] denotes the presence of ETEC virulence genes either alone or in combination with other genes

Table 3.6b: Distribution of the different *E. coli* pathotypes within the 205 isolates containing the tested virulence genes

Pathotype	Frequency (n=303)	Percentage (%)
STa[a]	1	0.33
Stx1/VT2	1	0.33
Stx1/EAST-1	1	0.33
VT1	1	0.33
VT2	3	0.99
HLY/EAST-1/EAE/STa[a]	1	0.33
EAST-1/AIDA-1/STa[a]	2	0.66
EAST-1/PAA/AIDA-1/LT/STa[a]	1	0.33
EAST-1/PAA/AIDA-1/LT[a]	1	0.33
EAST-1/PAA/AIDA-1/STa/STb[a]	1	0.33
EAST-1/PAA/STb[a]	2	0.66
EAST-1/AIDA-1/STb[a]	1	0.33
HLY/EAST-1/PAA	1	0.33
EAST-1/PAA/AIDA-1	1	0.33
Stx1/EAST-1/VT1/VT2	1	0.33
Stx1/HLY/VT2	1	0.33
EAST-1/AIDA-1/LT[a]	5	1.65
Stx1/HLY/EAST-1/AIDA-1/STb[a]	1	0.33
EAE/AIDA-1/STb[a]	1	0.33
EAST-1/EAE/AIDA-1/LT/STb[a]	1	0.33
HLY/EAST-1/AIDA-1	1	0.33
EAST-1/AIDA-1/LT/STb[a]	1	0.33
HLY/EAST-1/EAE	2	0.66
HLY/EAST-1/EAE/AIDA-1/LT[a]	1	0.33

[a] denotes the presence of ETEC virulence genes either alone or in combination with other genes.

The most prevalent pathotypes identified in this study were *EAST-1* (101 isolates), *EAST-1/PAA* (15 isolates), *HLY* (8 isolates) and *HLY/EAST-1* (6 isolates). Interestingly, only one isolate displayed fimbriae (*LT/STb/F4/F5*).

3.4 Discussion

Diarrhoea is a major cause of morbidity and mortality in both neonatal and recently weaned pigs worldwide (Ngeleka *et al*., 2003). Even though *E. coli* is the major part of the commensal intestinal microflora of pigs, some *E. coli* strains can cause diseases such as diarrhoea and edema. Colibacillosis is responsible for significant losses in the economy as a result of mortality. Determination of the specific virulence and colonization factors (Cheng *et al*., 2006) is an important step in the management and control of colibacillosis in any livestock production system. PCR is routinely used in molecular diagnostics of *E. coli* because of its sensitivity and specificity (Cheng *et al*., 2006). The study was aimed at investigating the potential role of ETEC in colibacillosis in South African pig populations. From a total of 325 rectal swabs collected, 303 isolates were identified and confirmed as *E. coli* based on their ability to produce indole from tryptophan, producing and maintaining stable acid products and their inability to utilise citrate as the sole carbon and energy source. These positive isolates were then subjected to multiplex PCR assays to screen for toxins, fimbriae and adhesin genes and determine the relative prevalence of ETEC relative to DAEC, EAEC, EHEC and EPEC.

Multiplex PCR was used for the detection of isolates harbouring virulence genes with the aim to classify strains as EAEC, ETEC, EHEC, DAEC and EPEC. A significant proportion of 67.7 % (205/303) carried one or more virulence genes and classified as EAEC, ETEC, EHEC, DAEC and EPEC. The prevalence of each *E. coli* type was based on detection of the strain in singleton or in combination with other strains. The most predominant group identified in this study was EAEC detected singularly in 33.3 %. The groups EHEC, ETEC and EPEC were also detected, in proportions of 5.3 % (16/303), 2.64 % (8/303) and 1.65 % (5/303), respectively. The least dominant group was DAEC detected in 0.99 % of the isolates. However, there were isolates carrying virulence genes associated with two *E. coli* types which included EAEC detected in combination with EPEC, EHEC, ETEC and DAEC in 21, 11, 7 and 4 individuals, respectively. Some isolates had a combination of EPEC with DAEC (2 isolates) and EHEC (2 isolates). Combinations involving three groups of *E. coli* revealed that 5/9 combinations had ETEC as the common group. Based on these results, the most

dominant group observed in the tested population was EAEC. Also, a significant number of combinations had ETEC as the primary group. Using these classification criteria, a total of 36 isolates were therefore classified as ETEC. Lastly, a total of 6 isolates revealed the presence of four *E. coli* types in which four isolates were classified as EAEC/EPEC/DAEC/ETEC and 2 classified as EHEC/EAEC/DAEC/ETEC.

Enteroaggreagative *E. coli* (EAEC) is one of the most recently recognised category of diarrheagenic *E. coli* and has a pathogenesis that is not yet fully understood (Choi *et al*., 2001b). This strain produces 38 amino acid enterotoxins called enteroaggreagative heat-stable enterotoxins 1 (*EAST-1*) (Choi *et al*., 2001b; Ngeleka *et al*., 2003). The most prevalent toxin was *EAST-1* which was observed as a single gene (33.3 %) and also co-expressed with other virulence genes (20.5 %). These findings are in line with results from previous studies where *EAST-1* was prevalent. In a study by Toledo *et al*. (2012) where enterotoxin genes were detected in 424 isolates out of 953, *EAST-1* (153, 36.1 %) was the second most common enterotoxins after *STa* (159, 37.5 %). Prevalence of the *estA* gene encoding for *EAST-1* was reported in Slovakia in diarrhoeic (144/220; 65 %) and healthy (8/30; 27 %) piglets respectively (Vu-Khac *et* al., 2007). The presence of *EAST-1* in healthy pigs suggests that the expression of *EAST-1* alone is insufficient to cause diarrhoea in piglets thus implying that *EAST-1*-producing *E. coli* can only cause infection in the presence of other virulence factors (Vu-Khac *et al*., 2007; Toledo *et al*., 2012). Among other strains, isolates carrying ETEC (36/303) which accounted for 11.9 % of the population had *EAST-1* (25/36, 69.4 %). This is in accordance with literature (Choi *et al*., 2001b) stating that the *EAST-1* gene is carried in human, porcine and bovine ETEC strains. The gene has also been reported in other pathogenic strains (EPEC and STEC) from humans and animals (Vu-Khac *et al*., 2007; Toledo *et al*., 2012). This is evident in this study as individuals having EAEC with EPEC, ETEC, EHEC and DAEC were detected. The role of *EAST-1* toxin in swine colibacillosis has not yet been clearly determined. This toxin however has been commonly detected in *E. coli* strains associated with diarrhoea in suckling and weaning piglets (Toledo *et al*., 2012). There is, however, the need for further studies on whether *EAST-1* alone or in association with other toxins is sufficient to induce diarrhoea.

Verotoxin-producing *E. coli* (VTEC) causes diseases in humans and animals (Kim *et al*., 2010). Two major cytotoxins associated with VTEC are *VT1* and *VT2* (Parma *et al*., 2000). Since veterocytotoxin is homologous to the Shigella toxins of Shigella dysenteriae they are also referred to as Shiga toxin-producing *E. coli* (Parma *et al* 2010). To cause disease, production of one or more types of Shiga toxins is required (Martins *et al*., 2011). These toxin include Shiga toxin 1 (*Stx1*) and Shiga toxin 2 (*Stx2*) and their variants (*Stx1c*, *Stx1d*, *Stx2c*, *Stx2d*, *Stx2e*, *Stx2f* and *Stx2g*) (Osek, 1999; Kim *et al*., 2010; Toledo *et al*., 2012). The *Stx2e* variant has been associated with edema disease in swine (Osek, 1999, Vu-Khac *et al*., 2007; Kim *et al*., 2010; Toledo *et al*., 2012). Studies by Osek (1999) and Vu-Khac *et al*. (2007) only found the *Stx2e* gene in 9.1 % and 4 % of the pig isolates respectively. No isolates studied contained *Stx1* and *Stx2* were detected. Contrary to these findings, *Stx1* was the only Shiga toxin gene detected in this study, though in a small proportion (5/303, 1.65 %). Kim *et al*., (2010) found *Stx1* and *Stx2* in 4 % and 25 % of the isolates respectively. Also, in a study by Toledo *et al*. (2012) *Stx1* and *Stx2* were found in 0.4 % and 1 % of the isolates. The presence of Shiga toxins in this study is of interest as it could suggest the possibility of piglets being reservoirs of STEC pathogenic for humans (Kim *et al*., 2010).

Enterohemorrhagic *E. coli*, a subgroup of VTEC (Botteldoorn *et al*., 2003) is characterized by the production of verotoxins and an attaching and effacing (A/E) lesions encoded by *eaeA* gene (Bardiau *et al*., 2010). The A/E lesions are characterized by way of attachment to the epithelial cell membrane and destruction of microvilli where adherence occurs (Martins *et al*., 2000; Botteldoorn *et al*., 2003). In addition to *VT1* and *VT2* and attaching and effacing lesions EHEC contains another virulence factor, a plasmid-encoded enterohaemolysin encoded by *hlyA* gene (Botteldoorn *et al*., 2003). Verotoxins were observed in 2.97 % (9/303). Within this proportion *VT1* and *VT2* occurred alone in 0.33% and 0.99% of the isolates respectively. In addition to single carrying isolates, *VT1* and *VT2* also occurred in combinations with other genes (5/303, 1.65 %). Ho *et al*. (2013) also detected a very low prevalence of 2 %. Virulence gene *EAE* was also observed but only together with other genes (16/303, 5.28 %). The prevalence of *hlyA* was detected in 9.57 % of the population where it was expressed alone (8/303, 2.64 %) and co-expressed with other genes (21/303, 6.93 %). Contrary to Botteldoorn

and colleagues (2003) who found that none of their tested isolates carried a combination of virulence genes, in this study isolates harbouring EHEC virulence genes carried additional virulence genes. The isolates carrying combinations of virulence genes are likely to be more virulent even to humans. The South African pigs are therefore more likely to be reservoirs of EHEC.

Enterotoxigenic *E. coli* (ETEC) has been incriminated as the major cause of diarrhoea in pigs (Ngeleka *et al*., 2003). Infection with ETEC involves adherence of the bacteria to porcine intestinal epithelial cells by fimbriae (*F4, F5, F6, F18* and *F41*) after which disease-causing toxins heat-labile enterotoxins (*LT*) and heat-stable enterotoxins (*STa* and *STb*) are produced (Chen *et al*., 2004; Cheng *et al*., 2006; Ngeleka *et al*., 2003). Multiplex PCR analysis showed that ETEC was present in the tested porcine isolates (Figure 3.5). Thirty six isolates (11.9 %) carried ETEC enterotoxins (*LT, STa* and/or *STb*). These isolates harboured genes encoding one or more toxins (Table 3.4). In this present study, *LT* was the most prevalent gene observed in 33.3 % of the isolates. Heat-stable enterotoxins (*STb* and *STa*) were less prevalent as they were manifested in 25 % and 22.2 % of the ETEC isolates respectively. Similar results were observed in Brazil where the most prevalent toxin was *LT* (71%) followed by *STb* (47 %) and *STa* (40 %) (Vidotto *et al*., 2009). Blanco *et al*. (2006) found that *STb* was the most prevalent toxin accounting for 69.5 % of Cuban piglets and also Vu-Khac *et al*. (2007) found *LT* and *STb* to be more prevalent than *STa* in Slovakia. Kim *et al*. (2010) also obtained a high prevalence for *LT* compared to other toxins (23 %). However, Chen *et al* (2004) found that *STa* was the most commonly found gene (130/215, 60.5 %) while *LT* was only found in one out of 215 isolates. Mohlatlole *et al*. (2013) also detected that *LT* was the least common toxin accounting for only 0.4 % of the tested isolates from South African pigs. Such differences suggest that the prevalence of virulence genes varies with the geographical area (Toledo *et al*., 2012; Mohlatlole *et al*., 2013). In addition to these enterotoxins occurring singularly, some isolates carried more than one of these enterotoxins. The observed combinations were *LT/STb*, *LT/STa* and *STa/STb* distributed in 11.1 %, 5.6 % and 2.8 % of the ETEC population respectively. Qi *et al*. (2012) found *LT/STa* and *STa/STb* combinations in 10 % and 3 % of the isolates respectively. On the other hand Kim *et al*. (2010)

reported 0.5 % for *LT/STb* and approximately 5 % for *LT/STa* and *STa/STb*. These results suggest the diversity and complexity of these toxin combinations (Kim *et al*., 2010).

Among the 36 isolates observed to carry ETEC enterotoxins, only 1 isolate (2.8 %) co-expressed two fimbriae (*F4/F5*). None of the 36 isolates contained *F6*, *F18* and *F41*. The presence of *F4/F5*, though in a small proportion should be taken into consideration when formulating vaccines against colibacillosis in South Africa. Do *et al*. (2006) determined that *F4* and *F5* were the most common fimbriae observed in 53.1 % and 16.7 % isolates from Vietnam. Also in Do *et al*. (2006), *F6*, *F18* and *F41* were not detected. The observed absence of the usually reported colonization factors in porcine strains is of interest. Osek (1999) states that the absence of fimbriae in isolates carrying toxins may be explained by the loss of plasmids encoding these colonization factors during storage of the strains or, even though less likely, fimbriae were not expressed under in vitro conditions used. Ngeleka and colleagues (2003) state that recent studies have reported an increase of *E. coli* isolates from piglets possessing ETEC-related toxins yet lacking classical fimbrial adhesins. These findings have therefore led to speculations suggesting that there exists an emerging group of diarrheagenic *E. coli* in piglets (Ngeleka *et al*., 2003). Further research ought to be done to confirm these suggestions. Pathogenic *E. coli* do not only use fimbriae to adhere to the intestinal walls of piglets, but also utilize other modes of attachment involving adhesins (Mohlatlole *et al*., 2013). These include adhesins involved in diffuse adherence (*AIDA-1*), porcine attaching and effacing-associated factor (*PAA*) and attaching and effacing factor (*EAE*) (Mohlatlole *et al*., 2013). The plasmid-encoded outer membrane protein (*AIDA-1*) of 100 kDa (Ha *et al*., 2003; Fang *et al*., 2005) is involved in the diffuse adherence phenotype of DAEC (Ha *et al*., 2003). However, the *AIDA-1* gene is not only restricted to DAEC but has also been observed in porcine ETEC strains (Ha *et al*., 2003). The *eae*A gene responsible for the production of a 94 kDa-protein intimin which mediates the effacement and disruption of microvilli of the intestinal mucosa (Martins *et al*., 2000; Botteldoorn *et al*., 2003) has previously been reported to be associated with porcine post-weaning diarrhoea (Ngeleka *et al*., 2003). Porcine attaching and effacing-associated factor (*PAA*) has also been associated with porcine diarrhoea (Zhang *et al*., 2007). The *PAA* gene was reported necessary for the development of attaching and effacing (AE) lesion by human EPEC strains

(Ngeleka *et al*., 2003; Zhang *et al*., 2007; Duan *et al*., 2012). Similar to *AIDA-1*, *EAE* and *PAA* have been considered as adherent molecules of porcine ETEC (Lee *et al*., 2008; Duan *et al*., 2012).

In the study, *PAA* (1.65 %) and *AIDA-1* (0.99 %) occurred as single genes and also together with other virulence genes. The third adhesin, *EAE*, was only found in combination with other virulence genes not expressed alone (5.2 %). Zhang *et al*. (2007) found that *AIDA-1* and *PAA* were the most frequently occurring than *EAE* as they were detected in 26 % and 43.8 % of all isolates respectively while *EAE* was present in 2.3 % of the detected strains. Similarly, Mohlatlole and colleagues (2013) found that *AIDA-1* (14.2 %) and *PAA* (17.9 %) were more prevalent than *EAE* (2.8 %) in South Africa. As mentioned above, all three non-fimbrial adhesins were present with other virulence genes. Lee *et al*. (2008) mentioned that ETEC strains with none of the recognised fimbriae are becoming more common. This challenge has been reported in South Africa whereby none of the tested isolates had fimbriae (Mohlatlole *et al*., 2013). Also in this particular study, only a single isolate out of the 36 ETEC positive isolates contained the tested fimbriae (*F4/F5*). Such findings were very striking and induce curiosity as to which other adhesins could be present in the ETEC positive strains. Table 3.5 illustrates the adhesins obtained from the ETEC positive isolates (n=36). From the 36 isolates that tested positive for ETEC, 22 (61.1 %) carried the adhesins *AIDA-1*, *PAA* and *EAE* either alone or combinations. The most prevalent adhesin was *AIDA-1* (11/36, 30.6 %) followed *PAA* (3/36, 8.3 %) and the least common adhesin was *EAE* with only a proportion of 2.8 % (1/36). In addition to these genes occurring alone, *AIDA-1* was found together with *PAA* (*PAA/ AIDA-1*) and *EAE* (*EAE/AIDA-1*) separately and with equal proportions (3/36, 8.3 % for each pathotype). In addition, the three adhesins were all observed in one isolate (1/36, 2.8 %). These findings are similar to Lee *et al*. (2008) who detected *AIDA-1* and *PAA* to be contributing to ETEC diarrhoea. Although frequently present in ETEC strains, the significance of *AIDA-1* in porcine diarrhoea still remains unknown (Zhang *et al*., 2007). Whether the adhesin alone can initiate sufficient colonization for diarrhoea is questionable as it has been found in non-enteropathogenic *E. coli* strains (Ngeleka *et al*., 2003). Similarly, *PAA* which was also detected in diarrheic and non-diarrheic piglets raises questions on the virulence and role of the adhesins in causing diarrhoea (Ngeleka *et al*., 2003). None of these three potential virulence

factors have been well characterised on the basis of their significance in porcine diarrhoea (Duan *et al*., 2012). Therefore, more studies aimed at determining the roles of *AIDA-1*, *PAA* and *EAE* in porcine diarrhoea are needed.

A total of 28 pathotypes were recovered in this present study. The most frequent associations between enterotoxins and fimbrial and non-fimbrial adhesins were *EAST-1*, *EAST-1/PAA*, *HLY* and *HLY/EAST-1*. Only a single isolate was positive for *F4* and *F5* fimbriae. This result together with findings by Mohlatlole *et al*. (2013) suggests that fimbriae were not frequently associated with pathogenic *E. coli* in South African pig populations. Lack of these colonization factors may mean decreased disease severity (Mohlatlole *et al*., 2013). Similar results were observed in a study by Mohlatlole *et al*. (2013) where *EAST-1* was the most dominant and none of the isolates displayed any of the tested fimbriae. Increased severity is generally associated with the high number of virulence genes present in *E. coli* pathotypes (Mohlatlole *et al*., 2013). In this study *EAST-1/PAA/AIDA/LT/ST*a, *EAST-1/PAA/AIDA/STa/STb*, *EAST-1/EAE/AIDA/LT/STb*, *Stx1/HLY/EAST-1/AIDA/STb* and *HYL/EAST-1/EAE/AIDA/LT* were therefore expected to have enhanced pathogenicity. Though the prevalence of these pathotypes is unknown, the results however provide information on the diversity of the virulence factors present in pigs of South Africa. These findings could be used for the development of rational preventive measures for colibacillosis in pigs.

3.5 Conclusion

The two most common strains of *E. coli* were EAEC and ETEC. From the ETEC positive isolates, *LT* was the most dominant ETEC enterotoxin. The low prevalence of fimbriae was unexpected as fimbrial adhesins were initially identified in *E. coli* strains isolated from pigs in previous studies. The mechanism of colonization for ETEC could probably be through *AIDA-1*, *EAE* and *PAA*. These findings, therefore, provide an idea on prevalent pathotypes in South African pigs, thus creating a platform for improved vaccine development.

3.6 References

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CHAPTER 4

Antimicrobial susceptibility of enterotoxigenic *Escherichia coli* isolates from South African pigs

Abstract

The Kirby-Bauer disk diffusion method was employed to investigate the sensitivity of 36 enterotoxigenic *Escherichia coli* (ETEC) isolated from rectal swabs of neonatal and post-weaning pigs of Limpopo (n = 16) and Eastern Cape (n = 20) provinces of South Africa to nine antimicrobials. Differences in the sensitivity patterns observed between the two provinces reflected differences in antibiotic use amongst these regions. Overall, significant rates of resistance to oxytetracycline, ampicillin and trimetroprim (47.2, 38.9 and 36.1 % of isolates, respectively) were observed. Resistance to two or more antimicrobials was observed in 50% of the ETEC positive isolates. However, highest sensitivity to florphenicol, cefotaxime and enrofloxacin was observed in 100, 97.2 and 77.8 % of the population, respectively. These three effective antimicrobials could, therefore, be recommended as drugs of choice for the treatment of ETEC infections in the South African pig industry.

Keywords: Kirby-Bauer disk diffusion, sensitivity, Enterotoxigenic *Escherichia coli*, antimicrobials, resistance

4.1 Introduction

Colibacillosis is one of the major diseases in pig production in South Africa (Henton, 2010). Infection with *E. coli* is commonly represented by watery diarrhoea, dehydration, loss of weight and mortality of infected pigs. Losses are incurred through expenses associated with antibiotics (Chen *et al*., 2004; Viott *et al*., 2013) and reduced feed efficiency. Diarrhoea remains the major cause of morbidity and mortality in suckling and post-weaning piglets across the globe (Ngeleka *et al*., 2003) and enterotoxigenic *E. coli* (ETEC) is the dominant bacterial agent responsible (Osek, 2003). In certain geographical regions, diarrhoea is responsible for approximately 10.8% of preweaning mortality (Ngeleka *et al*., 2003).

Antimicrobials are used widely for therapy of animals infected with colibacillosis (Sukuruman *et al*., 2012). Antimicrobials are also used for growth promotion in most food producing animals including pigs (Sukuruman *et al*., 2012). As a result, antimicrobial agents can be found present in environments such as sewage effluents surrounding areas where the drugs are used extensively e.g. farms where animal feed contains these drugs (Sukuruman *et al*., 2012). Antimicrobials have been used to control and prevent *E. coli* infections in pigs (Costa *et al*., 2010). The high usage of antibiotics has a negative impact on food-producing animals, human health and the environment (Camerlink *et al*., 2010). The continuous feeding of antibiotics to enhance pig growth of these animals are not only associated with increased resistance in faecal flora but also with resistance in bacteria (Docic and Balkei, 2003; van den Bogaard *et al*., 2000; Wang *et al*., 2011). These are pathogens resistant to various antimicrobial agents and ETEC is one of them (Lim *et al*., 2007; Abraham, 2011). Resistance observed in bacterial populations can spread from one ecosystem to another and is, therefore, an increasing problem (Sukuruman *et al*., 2012). Antimicrobial resistant pathogens can enter the food chain through consumption of contaminated pork, thus resulting in the transfer of resistance to commensal or pathogenic *E. coli* causing severe health concerns in humans in the event of food borne illnesses (Docic and Belkei, 2003; Wang *et al*., 2011; Abraham, 2011). There is, therefore, a need for rational

and appropriate use of antibiotics to reduce drug failure and increased antibiotic resistance (Costa *et al*., 2010).

Studies in the recent years have revealed the occurrence of multi-drug resistant *E. coli* (Costa *et al*., 2010). Most common resistant patterns in *E. coli* have been observed towards tetracyclines, sulphonamides and streptomycin or spectinomycin (Boerlin *et al*., 2005). The emergence of resistance in *E. coli* poses difficulty in treatment regimes as new drugs have to be sought.

Colibacillosis in pigs causes tremendous economic losses, weight loss, increased use of antibiotics and mortality (Mohlatlole *et al*., 2013). Ateba and Bezuidenhout (2008) reported higher resistance to tetracycline, sulphamethoxazole and erythromycin in *E. coli* O157 isolated from pigs compared to those from cattle and humans. There is, however, no information in South Africa on the antibiotic resistance of ETEC strains which are the most common cause of *E. coli* induced diarrhoea in pigs worldwide.

There is a need to determine the antibiotic resistance profiles of ETEC strains in the South African pig population. This information will reveal patterns of resistance towards certain antibiotics and inform on the effective antibiotics that should be considered when treating ETEC-induced infections. Such knowledge is significant for controlling antibiotic resistance and development of improved therapy measures aimed at reducing infection with ETEC. The study was, therefore, designed to determine antimicrobial resistance and/or susceptibility of enterotoxigenic *E. coli* isolates from South African pigs.

4.2 Materials and Methods

4.2.1 E. coli isolates

A total of 325 rectal swabs were collected from neonatal and post-weaning pigs from Limpopo and Eastern Cape Provinces during the months of June and September 2012. The collected swabs were transported to the laboratory at the Bacteriology section of the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI) for microbial and molecular analysis. Rectal swabs were streaked onto MacConkey and blood agar plates and incubated at 37ºC overnight (Lee *et al*., 2008; Kim *et al*., 2010). Suspect colonies were subjected to biochemical tests (indole, methyl red and citrate) for *E. coli* confirmation followed by the extraction of DNA from *E. coli* positive isolates. A total of 303 isolates were identified as *E. coli* positive and DNA from these samples were used for PCR. Several multiplex PCRs containing various target genes were conducted. The multiplex reactions contained ETEC enterotoxins *LT*, *STa* and *STb*; ETEC fimbriae *F4*, *F5*, *F6*, *F18* and *F41*; non-fimbrial adhesins *EAE*, *PAA* and *AIDA-1*. In addition, reactions involving *EAST-1*, *hlyA*, Shiga toxins (*Stx1*, *Stx2* and *Stx2e*) and verotoxins (*VT*1 and *VT2*) were performed. In total, 205 isolates contained the tested genes, 36 of which were confirmed to be ETEC. These 36 ETEC positive isolates were then screened for antimicrobial susceptibility.

4.2.2 Antimicrobial susceptibility testing

Susceptibility of confirmed enterotoxigenic *E. coli* isolates of porcine origin was determined by the Kirby-Bauer disk diffusion method (Lim *et al*., 2007). To grow the pathogenic organism, Mueller-Hinton agar was used (Lim *et al*., 2007). Most organisms plated on this medium grow easily without any inhibition as this is a non-selective and non-differential medium. Also, the medium contains starch which is good for the absorption of bacterial toxins to avoid their interference with the

antibiotics. The medium is not composed of thick or dense agar, thus allowing better diffusion of antibiotics. This allows for determination of true zones of inhibition.

Single colonies of each ETEC bacterium were suspended in 4 ml of nutrient broth. The suspensions were adjusted to the turbidity of a 0.5 McFarland standard prior to streaking onto Muller-Hinton agar plates (Lim *et al*., 2007). Sterile swabs were used for streaking of the inoculum over the entire Mueller-Hinton agar plates to ensure even distribution. A multi-disk dispenser with antibiotics of known concentrations was then used to dispense the antibiotic disks onto inoculated plates. Plates were then incubated at 37ºC overnight. Isolates were tested against ampicillin (10µg), cefotaxime (30µg), florphenicol (30µg), kanamycic (30µg), oxytetracycline (30µg), trimethoprim (5µg), enrofloxacin (5µg), polymycin B (300 units) and lincomycin (109 units). Susceptibility and/or resistance of the isolate to each compound tested against were determined by the size of the zones formed around the disks. Susceptibility and/or resistance of *E. coli* isolates were measured in accordance with the Clinical Laboratory Standards Institute (previously called the NCCLS) guidelines illustrated in Table 4.1 (Boerlin *et al*., 2005; Kalantar *et al*., 2013).

Table 4.1: Zone sizes (mm) and interpretation of the 9 tested antibiotics

Antibiotics	**Resistant** (≤)	**Intermediate**	**Susceptible** (≥)
Ampicillin (AMP-10)	13	14-16	17
Cefotaxime (CTX-30)	14	15-22	23
Florphenicol (FFC-30)	14	15-18	19
Kanamycin (K-30)	13	14-17	18
Oxytetracycline (OT-30)	14	15-18	19
Trimethoprim (W-5)	10	11-15	16
Enrofloxacin (ENR-5)	16	17-22	23
Polymyxin B (PB-300)	8	9-11	12
Linconmycin (LS-109)	14	15-20	21

4.2.3 Statistical analysis

The proportion of isolates that were susceptible and resistant to the different antibiotics was analysed using the frequency procedure of the statistical analysis system (SAS, 2012). The effects of province of origin on the susceptibility or resistance of isolate to antibiotics was analysed using a Chi-square test also in SAS where a *p*-value less than 0.05 was considered as significant (Docic and Bilkei, 2003; Wang *et al*., 2011).

Multi-drug resistance was defined as an isolate being resistant to two or more of the tested drugs. The proportion of isolates presenting multi-drug resistance was analysed using the frequency procedure of the Statistical Analysis System (SAS, 2012). Effect of province of origin on multi-drug resistance was also analyzed using the Chi-square test of SAS (2012).

4.3 Results

4.3.1 Sample structure

A total of 303 *E. coli* positive isolates obtained from rectal swabs of neonatal and post-weaning pigs of indigenous and Large White breeds were used in the study. Of these, 205 contained the tested virulent strains and 36 (11.9 %) of these isolates were characterized as ETEC positive and were further analysed for antimicrobial resistance. Of the 36 ETEC positive isolates, 16 were from Limpopo and 20 from the Eastern Cape Province. Twenty-six of these pigs were from the Large White breed. These 36 ETEC positive isolates were investigated for resistance to 9 antimicrobial drugs indicated in Table 4.1.

4.3.2 Frequency of susceptible and resistant isolates

The distribution of isolates resistant and susceptible to the different antimicrobial drugs is shown in Table 4.2 and illustrated in Figure 4.1. The results showed that 100, 97.2, 77.8, 63.9, 61.1, 55.6, 55.6, 47.2 and 33.3 % of the isolates were susceptible to florphenicol, cefotaxime, enrofloxacin, kanamycin, polymycin B, trimethoprim, ampicillin, oxytetracycline and lincomycin, respectively. Antibiotics florphenicol, cefotaxime and enrofloxacin were the most effective in the total population displaying effectiveness on 36, 35 and 28 isolates, respectively. None of the 36 isolates were resistant to florphenicol. Over 30 % of the isolates were resistant to oxytetracycline (47.2 %), ampicillin (38.9 %) and trimethoprim (36.1 %).

Table 4.2: Frequency of antimicrobial response of enterotoxigenic *E. coli* positive isolates from neonatal and post-weaning piglets

Drug	**Number of Isolates**		
	Susceptible (%) n=36	**Intermediate (%) n=36**	**Resistant (%) n=36**
Ampicillin	20 (55.6)	2 (5.6)	14 (38.9)
Cefotaxime	35 (97.2)	1 (2.8)	0 (0)
Florphenicol	36 (100)	0 (0)	0 (0)
Kanamycin	23 (63.9)	6 (16.7)	7 (19.4)
Oxytetracycline	17 (47.2)	2 (5.6)	17 (47.2)
Trimethoprim	20 (55.6)	3 (8.3)	13 (36.1)
Enrofloxacin	28 (77.8)	3 (8.3)	5 (13.9)
Polymycin B	22 (61.1)	9 (25)	5 (13.9)
Lincomycin	12 (33.3)	14 (38.9)	10 (27.8)

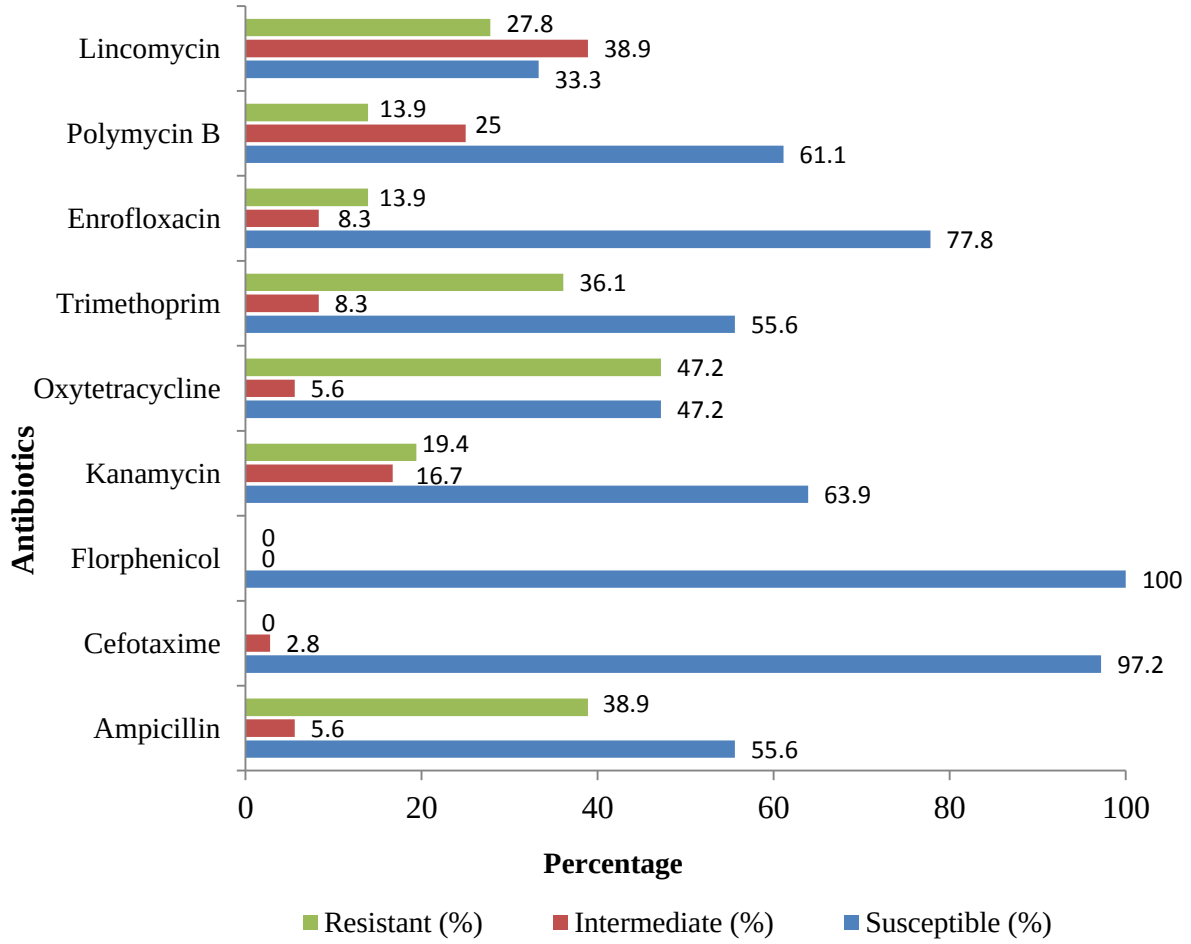

Figure 4.1: Frequency of susceptible, intermediate and resistant ETEC isolates to antimicrobial agents

4.3.3 Effects of province of origin on isolate's resistance/susceptibility to antimicrobial drugs

Antimicrobial response of the 36 tested individuals varied between provinces. In the Eastern Cape, CTX, ENR as well as FFC were effective on the whole population (n=20). In addition, AMP and W were effective on a significant number of isolates (16). Similarly in Limpopo FFC and CTX were the most effective as 16 and 15 individuals displayed susceptibility towards these two antibiotics respectively (n=16). In addition, 12 individuals were susceptible to PB in the Limpopo population (Table 4.3).

Table 4.3: Susceptibility patterns in the whole population and between the two provinces, Eastern Cape and Limpopo

	Susceptibility			
Antibiotic	**Eastern Cape (n=20)**	**Limpopo (n=16)**	**P value**	**Total (n=36)**
AMP	16	4	<0.05	20
CTX	20	15	NS	35
ENR	20	8	<0.05	28
FFC	20	16	<0.05	36
K	14	9	NS	23
LS	7	5	NS	12
OT	12	5	NS	17
PB	10	12	NS	22
W	16	4	<0.05	20

p-value >0.05: not significant *p*-value <0.05: significant

A proportion of individuals were resistant to certain antibiotics used in this study. Resistance was observed towards OT, AMP and W where these three antibiotics were not effective against 17, 14 and 13 of the ETEC isolates (Table 4.4).

In both provinces, resistance was observed towards OT and W. In the Eastern Cape (n = 20), 6 and 4 isolates were resistant to OT and W respectively. For Limpopo (n=16), 11 and 9 isolates displayed resistance to OT and W respectively. Six isolates from Eastern Cape were resistant to LS. In addition, 12 isolates from Limpopo were resistance to AMP (Table 4.4).

Table 4.4: Resistance patterns in the whole population and between the two provinces Eastern Cape and Limpopo

	Resistance			
Antibiotic	**Eastern Cape (n=20)**	**Limpopo (n=16)**	**P value**	**Total (n=36)**
AMP	2	12	<0.05	14
CTX	0	0	NS	0
ENR	0	5	<0.05	5
FFC	0	0	-	0
K	2	4	NS	6
LS	6	4	NS	10
OT	6	11	NS	17
PB	3	2	NS	5
W	4	9	<0.05	13

p-value >0.05: not significant *p*-value <0.05: significant

4.3.4 Multiple antimicrobial resistance patterns

Resistance to two or more antimicrobials was observed in 18/36 (50%) of the isolates. Such response resulted in the grouping of the resistance patterns into multi-drug resistance profiles as shown in Table 4.5. Four isolates displayed multi-drug resistance to ampicillin, oxytetracycline, trimethoprim and enrofloxacin.

Table 4.5: Multi-drug resistance patterns of enterotoxigenic *E. coli* from rectal swabs of pigs (n=36)

Antimicrobial resistance patterns	**Frequency (n-36)**
OT/PB	1
OT/LS	1
W/LS	1
OT/W/LS	1
K/OT/LS	1
AMP/OT/LS	1
AMP/OT/W	1
AMP/K/W	1
AMP/OT/W/PB	1
AMP/K/OT/PB	1
AMP/OT/W/ENR	4
AMP/K/OT/W/PB	1
AMP/K/OT/W/LS	2
AMP/K/OT/W/ENR/LS	1
Total	18

4.4 Discussion

For decades, antimicrobial agents have been used for disease prevention and growth support in farm animals (Kalantar *et al*., 2013). The recurring use and misuse of antimicrobial agents may lead to resistance in various bacteria thus causing difficulty in treating bacterial infections (Sukuruman *et al*., 2012). Resistance phenotypes can therefore manifest a selective advantage to bacteria (Kalantar *et al*., 2013). Antimicrobials are used to control and prevent *E. coli* infections in swine (Costa *et al*., 2010) and misuse of these agents results in the development of antibiotic resistance bacteria (Wang *et al*., 2011). In small-holder farms such as those sampled in the study, misuse and/or frequent use of antibiotics due to limited resources and absence of veterinarians or health technicians might be common thus resulting in antibiotic resistance. A study conducted in the North-West Province of South Africa reported antibiotic resistance to tetracycline, sulphamethoxazole and erythromycin in *E.*

coli O157 strains isolated from pigs (Ateba and Bezuidenhout, 2008). There is no information on the antibiotic resistance of ETEC in South Africa. This is regardless of ETEC being one of the major causes of diarrhoea in pigs and other livestock species (Osek, 2003). The current study was therefore conducted with the intention to develop antibiotic resistance profiles of enterotoxigenic *E. coli* strains from pig populations from Limpopo and the Eastern Cape provinces of South Africa.

In the study, the modified Kirby-Bauer method was used to determine the antimicrobial response of ETEC positive isolates. A total of 36 ETEC positive isolates from neonatal and post-weaning pigs of Eastern Cape and Limpopo provinces were tested against nine antibiotics. Prevalence of antibiotic resistance was defined by growth of the isolates on the plates containing the antimicrobial agents (Nijsten *et al*., 1996). Of the nine antibiotics tested, the highest rate of resistance was recorded for oxytetracycline (47.2 %). These results are in line with findings by Nijsten *et al*. (1996) and Habrun *et al*. (2010) where in both cases *E. coli* isolates exhibited highest resistance to oxytetracycline (100 % and 98 % respectively). Nijsten *et al*. (1996) states that oxytetracycline is mainly given orally to pigs for mass medication rather than individual treatment. Treatment of the entire herd with an antibiotic is therefore likely to result in development and spread of resistance. In addition, excessive use of oxytetracycline antibiotics and faecal-oral contact between pigs are possible causes of the observed high prevalence of resistant isolates. Even though no history is available on the use of oxytetracycline in South African pigs, the detected resistance suggests that this drug is no longer effective and should not be used for treatment of *E. coli* in this pig population.

Significantly high sensitivity rates ranging from 77 to 100 % were observed for enrofloxacin, cefotaxime and florphenicol. The broad-spectrum florphenicol which is effective on many Gram-negative and Gram-positive bacteria has been approved for veterinary use in food animals since 1999 (Wang *et al*., 2011). Wang *et al*. (2011) mentioned that florphenicol resistance in *E. coli* isolates of poultry and cattle origin has been reported. The observed effectiveness of florphenicol (100 %) is therefore consistent with results from literature. These results suggest that florphenicol might be the potential antibiotic that will be effective for ETEC-induced infections in South Africa.

A significantly high degree of susceptibility to cefotaxime (97.2 %) was also detected. These findings are in agreement with findings by Habrun and colleagues (2010) where the highest sensitivity rate to cefotaxime (86 %) was also observed. In addition to florphenicol and cefotaxime having the highest susceptibility rates, enrofloxacin also displayed a significantly high proportion (77.8 %). In contrast, Wang *et al*. (2011) observed high resistance rates to cefotaxime (78 %). Similar to florphenicol, cefotaxime and enrofloxacin are therefore the potential antibiotics for control of *E. coli* infections in South African pigs.

Good management is important to prevent the development and spread of infections. These include best nutrition supply, good environment, cleanliness and disinfection of pens or cubicles, low-stock densities within each space and veterinary services. Inconsistency observed in susceptibility patterns between Limpopo and the Eastern Cape Province could possibly be due to differences in the geographical location in terms of environmental conditions, population and management practices (Kibret and Abera, 2011). Sampling of the two provinces was carried out during different seasons. Limpopo pigs were sampled in June 2012 while Eastern Cape pigs were sampled in September 2012. Therefore this difference in time is significant as it is reflects temperature changes. Also, the two populations are different in terms of feed and water availability and general animal husbandry. Management practices vary from farm to farm especially between communal and commercial farms (Hove *et al*., 2005). Commercially reared pigs are kept under good hygienic conditions such as clean pens with sufficient food and water while in communal pig farming housing and hygiene are generally poor (Hove *et al*., 2005). In addition, backyard pigs scavenge for food and sometimes fed crop residues and household waste and therefore lack good nutrition (Hove *et al*., 2005). Pigs kept outdoors may not be easily monitored like indoor pigs. These pigs are possibly with no shelter and exposure to unfriendly environmental conditions. In an indoor system, temperature, ventilation and lighting and accumulation of excessive waste such as faeces and urine can be monitored. Unlike commercial farms, there are limited veterinary services in communal farms and use of antibiotics is limited. These factors therefore affect the prevalence of diseases in pigs and also their response to

antibiotics. In this study, both commercial and communal pigs were sampled which could explain the varied response to antibiotics between populations.

A variation in antimicrobial susceptibility pattern was observed between Eastern Cape and Limpopo isolates. Eastern Cape individuals displayed significant susceptibility to ampicillin and trimethoprim as 16/20 individuals displayed susceptibility to these two antibiotics. However, Limpopo isolates were resistant to ampicillin (12/16) and trimethoprim (9/16). Differences in feed supplies and environmental (particularly temperature) between Limpopo and the Eastern Cape could explain the observed differences between the provinces. Differences in antimicrobial response that were observed between the two provinces could be due to geographical variation in factors that affects antimicrobial susceptibility (Kibret and Abera, 2011). Smallholder pigs in general, are not given the proper feed and in most cases are fed on household leftover food. Therefore, whatever form of feed that may be abundant in Limpopo could be limited in Eastern Cape thus resulting in feed differences administered between the two provinces. In addition, there are observable temperature differences as Limpopo has higher temperatures than the Eastern Cape which is along the coast. Results from these two provinces suggest differences in the antibiotics used between the two farming regions with some indication of overuse of ampicillin in the Limpopo province. There is however dearth of information on previously used antibiotics in the two provinces due to absence of farm records on management practices and minimum veterinary interventions.

Habrun *et al*. (2010) reported the occurrence of resistance to ampicillin (85 %) and also trimethoprim (87 %) in their study on large pig breeding farms of Croatia. Resistance to ampicillin (99 %) dates back to the 1990s (Nijsten *et al*., 1996). Emerging resistance observed in *Enterobacteriaceae* poses difficulty in handling infections associated with them (Paterson, 2006). The most contributing factor to resistance in Gram-negative pathogens is the production of β-lactamases (Pitout and Laupland, 2008). Bacterial enzymes β-lactamases inactivate β-lactam antibiotics by hydrolysis resulting in ineffective compounds (Pitout and Laupland, 2008). Along with *Klebsiella pneumonia*, *Escherichia coli* is the major extended-spectrum β-lactamase-producing organism worldwide (Pitout and

Laupland, 2008). Extended-spectrum β-lactamases (ESBLs) is one group of β-lactamases that hydrolyses and causes resistance to various newer β-lactam antibiotics including third-generation cephalosporins, monobactams and penicillins (Pitout *et al*., 1997; Pitout and Laupland, 2008). The mentioned facts therefore justify the observed resistance to ampicillin.

Multiple drug resistance to two or more antibiotics was also evident (50 %) in the studied population. In total, 14 multiple-drug resistance profiles were observed. The AMP/OT/W/ENR profile was more prevalent and was observed in 11% of the isolates. Thirteen of the 14 multidrug-resistance profiles included resistance to oxytetracycline. This could suggest that *E. coli* strains showing resistance to oxytetracycline possibly have enhanced ability to develop resistance to other antimicrobials. A similar conclusion was made by Lim *et al*. (2007), who observed tetraxycline resistance in all of the multi-drug resistance combinations.

This study showed resistance patterns to individual antibiotics and also resistance to two or more antibiotics (multiple resistance patterns). These results support the notion that ETEC strains of porcine origin have developed antibiotic resistance. This is therefore a benchmark for future use of antibiotics for controlling colibacillosis in pigs of South African origin.

4.5 Conclusion

Resistance to individual antimicrobials as well as multi-drug resistance was detected in the South African pig populations raising concerns on the effectiveness of current antibiotic treatment programs. Oxytetracycline was not effective on the tested isolates as significant resistance rates were detected for this antibiotic. Nonetheless, significantly high susceptibility to enrofloxacin, cefotaxime and florphenicol implies that these three antibiotics can be considered as effective antibiotics for *E. coli* control in the South African pig populations.

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CHAPTER 5

5.1 General Discussion

In South Africa, pork is the third most consumed meat accounting for 0.2% of the world's pig population (South African Pork Producers' Organisation, 2011). Pigs are raised in commercial farms and also in the marginal regions dominated by indigenous breeds. Approximately 1.6 million pigs are raised for pork production in South Africa (South Africa Pork Producers Organisation, 2011). Colibacillosis is regarded as one of the major porcine diseases in South Africa and affects production (Henton, 2010).

Colibacillosis remains a serious cause of diarrhoea and oedema diseases and accounts for considerable economic losses in the pig industry worldwide (Zhao *et al*., 2009; Zajacova *et al*., 2012; Viott *et al*., 2013). Enterotoxigenic *E. coli* (ETEC) which is characterized by fimbrial adhesins *F4*, *F5*, *F6*, *F18* and *F41* and heat-labile (*LT*) and heat-stable (*STa* and *STb*) enterotoxins, has been reported to be a major cause of colibacillosis infections in pigs and is responsible for post-weaning (PWD) and neonatal diarrhoea (ND) (Zajacova *et al*., 2012). There is a need to determine the prevalence of ETEC in South African pigs. Treatment of enteric infections in pigs usually involves the use of antibiotics (Habrun *et al*., 2010). There has, however, been an emergence of antimicrobial resistance in the past few years particularly among gram negative bacteria, such as *Enterobacteriaceae* (Abraham, 2011). Antibiotic resistance makes treatment of neonatal and post-weaning diarrhoea difficult (Habrun *et al*., 2010). When exposed to lethal antibiotics, bacteria develop antibiotic resistance as a defensive mechanism. Therefore, there is a need to accurately detect and identify porcine pathogens so as to develop fitting treatment regimes of affected pigs and prevention programs for pig populations.

In this current study, we sought to isolate and identify *E. coli* from rectal swabs obtained from neonatal and post-weaning pigs from the different agroecological regions (Limpopo and Eastern Cape provinces) of South Africa and determine the prevalence of ETEC. A further objective was to investigate the prevalent fimbriae (*F4*, *F5*, *F6*, *F18* and *F41*) and enterotoxins (*LT*, *STa* and *STb*) from S.A. *E. coli* isolates and to determine antimicrobial susceptibility of the ETEC positive isolates.

In Chapter 3, 325 pig rectal swabs were collected from suckling and post-weaning piglets of Eastern Cape and Limpopo province, South Africa. The swabs were streaked onto MacConkey and sheep blood agar plates and incubated at 37°C overnight with the aim of culturing *E. coli*. Presumptive *E. coli*, isolates producing pink colonies were then subjected to indole, methyl red, and citrate tests for confirmation of *E. coli*. Based on these biological tests, a total of 303 isolates were identified as *E. coli*. To determine the prevalence of ETEC in South African pigs, the presence of other *E. coli* strains was determined. These 303 *E. coli* positive isolates were used in multiplex PCR reactions performed to determine the various virulence genes associated with ETEC, DAEC, EAEC, EHEC and EPEC.

Overall, 205 isolates contained one or more virulence genes either of the same or different *E. coli* strains. The most prevalent *E. coli* strain observed in 33.3 % isolates was EAEC. The groups EHEC, ETEC, EPEC and DAEC were detected in proportions 5.3 %, 2.64 % and 1.65 % and 0.99 % respectively when considered on their own. In addition, some isolates belonged to more than one *E. coli* group. The detection of EAEC in a significantly high proportion was expected as this strain was found to be the second most dominant in a study conducted in South African pigs where it was found in 43.4 % of the tested isolates (Mohlatlole *et al*., 2013). Though the pathogenesis and contribution of EAEC towards colibacillosis still remains unknown, this strain should be included in future control measures. When considering the occurrence of ETEC alone and in combination other strains, this strain was observed in 11.9 % of the population. Previously, Henton and Engelbrecht (1997) and Mohlatlole *et al*. (2013) found ETEC in significantly high proportions than other strains. Therefore, the presence of ETEC in this current study cannot go unnoticed and should be considered when developing programs aimed at promoting pig production by controlling the disease.

Of all the investigated toxins in this study, *EAST-1* was the most dominant. These findings confirm those by Mohlatlole *et al*. (2013). However, the role of *EAST-1* in colibacillosis is unknown. Furthermore, ETEC enterotoxins were detected in 36 isolates either occurring alone or combined with other pathogenicity traits, with *LT* being the most prevalent (33.3 %). Only one isolate contained a combination of *F4* and *F5* fimbriae, while the other 35 had none of the tested fimbriae. This significantly low identification of fimbriae raised questions which led to the development of PCR assays targeting non-fimbrial adhesins *AIDA-1*, *PAA* and *EAE*. From these adhesins, *AIDA-1* was the most frequently occurring.

Based on these results, it is clear that ETEC is endemic in South African pigs. There was however minimal fimbriae detected. Instead, ETEC isolates contained non-fimbrial adhesins. These findings agree with the findings of Mohlatlole *et al.* (2013) that none of the tested isolates from South African pigs contained the tested fimbriae with the majority of isolates containing *PAA* and *AIDA-1*. Therefore, strains with none of the recognized fimbriae are probably becoming more common in South Africa. Whether or not these adhesins alone are sufficient enough to cause disease is still unclear (Ngeleka *et al*., 2003). Further studies are however necessary in order to understand ETEC pathotypes commonly occurring in South Africa. Once these are detected, successful therapy programs aimed at preventing and treating ETEC induced diarrhoea can be devised. A total of 48 pathotypes were recovered in this study thus suggesting that there are various virulence gene combinations present in South African pigs. Of these, *EAST-1* (33.3 %) and *EAST-1/PAA* (4.95 %) were the most dominant.

In Chapter 4, antimicrobial susceptibility testing of 36 ETEC positive isolates against nine selected antibiotics was performed using the Kirby-Bauer disk diffusion method. The highest rate of sensitivity was recorded for florphenicol (100 %), cefotaxime (97.2 %) and enrofloxacin (77.8 %). In addition, the isolates exhibited significant rates of resistance to oxytetracycline (47.2 %), ampicillin (38.9 %) and trimetroprim (36.1 %). Also, multidrug resistance was observed with 50 % of the ETEC isolates

resistant to two or more antibiotics. These finding indicated that florphrnicol, cefoxamine and enrofloxacin were effective as a treatment regime for the South African pigs and therefore can still be used in ETEC-induced neonatal and post-weaning diarrhoea. However, the patterns of resistance detected require further elaboration. Previously, a study conducted in the North-West province of South Africa on *E. coli* O157 isolates from pigs, humans and cattle revealed high resistance frequencies to tetracycline, sulphamethoxazole and erythromycin (Ateba and Bezuidenhout, 2008). The origins of these resistance patterns observed in this study are not fully understood because there are no reports on studies of resistance profiles on previously used antibiotics to combat ETEC in South African pigs. The current pattern could suggest the possible misuse or persistent use of these antibiotics. The detection of antibiotic resistance in ETEC could be reflecting potential therapy failure in animals (Abraham, 2011). In addition, the tested ETEC isolates exhibited geographically different responses towards the involved antibiotics.

Some of the challenges associated with antibiotic therapy include limited availability of new antibiotics with novel mechanisms of action, high cost and benefits risk ratio and the rapid development of bacterial resistance when antibiotics are used excessively (Carlet *et al*., 2012). There might be a need to resort to alternative therapy measures such as *E. coli* based probiotics with prophylactic and also therapeutic potential (Abraham, 2011).

5.2 Conclusion

The South African pig industry is faced with infection with *E. coli*. Virulence genes such as *EAST-1* and enterotoxins associated with ETEC were detected and hence play a role in the pig industry. Heat-labile (*LT*) was the most prevalent ETEC enterotoxin. The results show that in South Africa, infection of piglets by *E. coli* is not mediated by fimbriae since they are not the major colonization factor. Instead, non-fimbrial adhesins *AIDA-1*, *PAA* and *EAE* were detected with *AIDA-1* particularly, being the most observed. The antibiotics, florphenicol, cefotaxime and enrofloxacin appear to be the most effective for ETEC treatment. Resistance to single drugs (oxytetracycline, ampicillin and

trimetroprim) and also to multiple drugs was also observed. However, resistance patterns observed cannot be ignored. These findings therefore give an insight on prevalent pathotypes, possible effective drugs and antibiotic resistance patterns in South African pigs. This information could be necessary in the design of effective control measures for colibacillosis in pigs.

5.3 Recommendations

i. There is a need for routine screening of pigs for virulence genes so to have a profile on frequently and newly developed pathotypes potentially responsible for colibacillosis. This will aid veterinarians in developing effective control strategies.

ii. Fimbriae are not the primary colonization factors in South Africa. It is, therefore, advisable to consider adhesins *AIDA-1*, *PAA* and *EAE* rather than fimbriae when developing vaccines for preventing colibacillosis in pigs.

iii. The significance of *EAST-1*, *PAA*, *AIDA-1* and *EAE* in porcine diarrhoea is not yet known; therefore future research could be aimed at determining the role of these virulence factors.

iv. The detected pathotypes should be investigated to determine their role in colibacillosis infections in pigs.

v. The effective antibiotics florphenicol, cefotaxime and enrofloxacin are the possible solution to ETEC infection in South African pigs. However, there is a need for surveillance of antibiotic resistance and recording of antibiotic profiles in order to understand antibiotic resistance and possible development of resistance in future.

vi. There is a need to improve farming practices such as: feeding procedures and routine sow immunoprophylaxis for prevention of neonatal colibacillosis.

vii. The effect of differences in regions or provinces, farms and management practices on the isolate's response to antibiotics should be investigated.

viii. Genotyping of the ETEC positive isolates can be done to determine the genes responsible for resistance in this population. Through this procedure, genetic associations and the evolution of the genes can be determined.

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