Molecular characterization of virulence determinants in *Escherichia coli* pathotypes isolated from abattoirs

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DECLARATION

I declare that, the dissertation entitled "Molecular characterization of virulence determinants in Escherichia coli pathotypes isolated from abattoirs", hereby submitted for the Degree of Master of Science in Biology (Molecular Microbiology) at the North-West University (Mafikeng Campus), has not been submitted by me for a degree at this or any other university. This is my own work in design and execution and that all material contained herein has been duly acknowledged.

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DEDICATION

The work is dedicated to my parents Mr Emmanuel Tchatchouang and Mrs Tchatchouang Born Djouonang Emilienne.

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ABSTRACT

Escherichia coli are Gram negative organisms that live as normal flora in the gastrointestinal tract of humans and warm blooded animals. Despite the fact that these organisms were previously considered to be non-pathogenic a number of species are currently known to cause diseases to their hosts worldwide. Infections caused by *E. coli* are usually associated with the consumption of contaminated food products especially those that are of animal origin. Meat products particularly beef have been implicated as causative agents of foodborne disease outbreaks as well as sporadic cases of infections such as diarrhoea caused by *E. coli* strains in many countries worldwide. Diarrhoeagenic *E. coli* pathotypes (DEP) are classified into different pathological groups depending on their virulence characteristics and comprise enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and enteroinvasive *E. coli* (EIEC). These *E. coli* strains can easily be transmitted to meat if standard operating procedures as well as proper hygiene are not implemented in abattoirs. The main aim of the study was to characterize *E. coli* pathotypes isolated from beef carcasses in selected abattoirs in North West Province, South Africa. In the present study, a total of 196 swab samples were collected from beef carcasses and analysed using Eosin Methylene Blue agar (EMBA). A total of 291 presumptive *E. coli* isolates that were metallic sheen based on macroscopic morphologies were obtained from 152 samples. The cellular morphology of presumptive *E. coli* isolates was determined by Gram-staining and Gram negative rods were subjected to both preliminary (oxidase, carbohydrate metabolising and citrate utilization) and confirmatory (*uidA* specific PCR analysis) tests. Bacterial 16S rRNA genes were also amplified through PCR as an internal control. Confirmed *E. coli* isolates were further subjected to PCR analysis designed to amplify the *stx1*, *stx2*, *eaeA* and *hlyA* virulence gene determinants. The *E. coli* isolates were further analysed for combinations of the various virulence genes detected per isolate and a summary of data indicating the different genotypes obtained for isolates

from the different sampling sites was generated. Generally, a large proportion 152 (77.6%) of the samples were positive for *E. coli* based on macroscopic morphologies. In addition, large proportions (80.3% to 80.7%) of the samples from Kareespruit and Potchefstroom were potentially contaminated with *E. coli* strains. All the isolates from Kareespruit and Potchefstroom were Gram negative rod- shaped bacteria that were also oxidase negative. In addition, these isolates also fermented sugars in the TSI medium but did not produce hydrogen sulphide gas. Results for the citrate utilization test indicated that 86%, 57.3% and 40% of the isolates from Kareespruit, Potchefstroom and Zeerust respectively were citrate negative. Bacterial 16S rRNA gene fragments were successfully amplified for all the 291 (100%) isolates. Out of the 291 isolates, a large proportion 256 (88 %) possessed the *uidA* gene which is specific for *E. coli*. The proportion of isolates that possessed the *uidA* gene was higher among the isolates from Kareespruit 95 (95%) and Potchefstroom 88 (91.7%) than those from Zeerust 73 (76.8%). Sixteen major genotypes designated G1 to G16 were identified in the study and large proportions (70.7% to 77.7%) of the isolates screened possessed the stx_1, stx_2 and *eaeA* genes respectively. Despite this the stx_1 was the most frequently identified virulence determinant among isolates from Kareespruit 79 (83.2%) and Zeerust 61 (83.6%) while the stx_2 gene was commonly detected 60 (82.2%) among isolates from Zeerust. A large proportion 49 (67.1%) of the *E. coli* isolates from Zeerust also possessed the *hlyA* gene. On the contrary, *eaeA* gene that codes for intimin was frequently detected 72 (75.8%) among isolates from Kareespruit. There was no major difference in the number of isolates obtained in this study that possessed the stx_1, stx_2 and *eaeA* genes and only 3 (1.2%) harboured both stx_1, and stx_2 shiga toxin genes as shown in genotype (G6). In addition, only 1 (0.4%) isolate that is classified as genotype (G12) harboured all these three genes. Despite this, the number of isolates that possessed the stx_1 gene, i.e. 199 (77.7%), was higher than those with the stx_2 gene in this study, 6 (2.3%) of the *E. coli* isolates screened harboured all the four virulence gene determinants investigated. Only 16 (6.3%) of the *E. coli* isolates possessed the stx_1 and stx_2 genes respectively in combination with the two accessory virulence gene determinants (*eaeA* and *hlyA*) as indicated in

genotypes G14, G15 and G16. ERIC-PCR analysis of the 256 *E. coli* isolates revealed between 1 and 17 polymorphic bands per isolate. Isolates produced band sizes that ranged from 100 bp to 1 kb. A large band of approximately 2 kb as well as a smaller band of 400 bp were very common in most of the isolates typed.

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ACRONYMS AND ABBREVIATIONS

ATCC	: American Type Culture Collection
API	: Analytical Profile Index
DEC	: Diarrhoeagenic *E. coli*
DEP	: Diarrhoeagenic *E. coli* pathotypes
E. coli	: *Escherichia coli*
EPEC	: Enteropathogenic *Escherichia coli*
EHEC	: Enterohaemorrhagic *Escherichia coli*
ETEC	: Enterotoxigenic *Escherichia coli*
STEC	: Shiga toxin *Escherichia coli*
EAEC	: Enteroaggregative *Escherichia coli*
EIEC	: Enteroinvasive *Escherichia coli*
NCBI	: National Center for Biotechnology Information
NCTC	: National Collection Type Culture
ERIC-PCR	: Enterobacterial Repetitive Intergenic Consensus

DEFINITION OF CONCEPTS

Abattoirs: A place in which animals are killed and slaughtered with the intention of being processed as food.

Enteroaggregative *Escherichia coli*: Considered as emerging pathogens, the EAEC are the second cause of travelers' diarrhea after ETEC in developed countries and emerging development.

Enterohaemorrhagic *Escherichia coli*: are zoonotic pathogens and bacteria which are found in water and sometimes in foods.

Enteroinvasive *Escherichia coli*: are responsible for dysentery characterized by high fever, abdominal cramps and nausea, watery diarrhea accompanied by rapidly progressing to dysentery (diarrhea with blood and mucus).

Enteropathogenic *Escherichia coli*: responsible for severe diarrhea in children in developing countries.

Enterotoxigenic *Escherichia coli*: are causing watery diarrhea episodes, moderate to severe low fever, associated with nausea and abdominal cramps.

***Escherichia coli*:** A bacterium commonly found in the intestines of human and other animals, some strains of which can cause severe food poisoning.

Fingerprint: specific banding pattern displayed by isolates on application of one or more typing method.

Pathogenicity: it is ability to cause disease that is determined by its virulence factors.

Pathotypes: Any of a group of organisms which are of the same species that have the same pathogenicity in a specified host.

Shiga toxin *Escherichia coli*: Shiga-toxin (*Stx*) also known as verocytotoxin (*Vtx*), which is a characteristic of *E. coli* of the group of "Shiga toxin Producing *E. coli*" (STEC) which EHEC O157:H7 is the main virulence factor EHEC.

Species: collection of bacterial cells which share an overall similar pattern of traits in contrast to other bacteria whose pattern differ significantly.

Typing: A phenotypic and/or genetic analysis of bacterial isolates below the species level that is employed to generate strain specific fingerprints that could be used in investigating cross contaminations, transmission patterns and/or sources of infections in humans or consumers of a particular food product.

Virulence: The degree of pathogenicity within a group or species of parasites as indicated by case fatality rates and/or the ability of the organism to invade the tissues of the host.

CHAPTER ONE

INTRODUCTION AND PROBLEM STATEMENT

CHAPTER ONE

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1. INTRODUCTION

1.1 GENERAL INTRODUCTION

Escherichia coli are Gram negative rod-shaped bacteria that live as normal flora in the gastrointestinal tract of humans and warm blooded animals (; Harakeh *et al.*, 2005; Nettleman, 2011). Despite the fact that these organisms were previously considered to be non-pathogenic a number of species are currently known to cause disease to their hosts worldwide (Karmali, 1989; Nataro and Kaper, 1998; Wieler, *et al.,* 2001). Diseases caused by *E. coli* are usually associated with the consumption of contaminated food and water (Riley *et al.*, 1983; Armstrong *et al.*, 1996; Mühldofer *et al.*, 1996; Müller *et al.*, 2001, Dunn *et al.*, 2004). Food products of animal origin, especially meat have been implicated as causative agents of disease outbreaks as well as sporadic cases of infections caused by *E. coli* strains in many countries worldwide (FAO, 2005; Rhoades *et al.*, 2009; Karmali *et al.,* 2010).

E. coli species that cause disease especially diarrhoea in humans are termed diarrhoeagenic *E. coli* pathotypes (DEP). These strains are classified into different pathological groups depending on their virulence characteristics and the clinical symptoms in their hosts (Levine, 1987; Nataro and Kaper, 1998). The pathotypes include enterohemorrhagic *E. coli* (EHEC) subgroup of STEC, which is responsible for hemorrhagic colitis and hemolytic-uremic syndrome, Shiga toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC) which causes diarrhoea in children and animals, Enterotoxigenic *E. coli* (ETEC) which causes traveler's diarrhoea and porcine and bovine diarrhoea, enteroinvasive *E. coli* (EIEC) which causes watery diarrhoea and dysentery, which causes urinary tract infections in humans and animals, enteroaggregative *E. coli* (EAEC) which causes persistent diarrhoea in humans and diffusely

adherent *E. coli* (DAEC), a subclass of enteroaggregative *E. coli* which causes diarrhoea in children (Levine, 1987; Nataro and Kaper, 1998). Despite the fact that EIEC and EAEC strains have been reported to be found only in humans and not in animals, coupled with the fact that both humans and animals are potential hosts for *E. coli* strains this presents a serious challenge when developing methods for identifying the different *E. coli* pathotypes. This is further complicated by the fact that bacterial genomes are not only very dynamic but the ability of the organisms to acquire genetic elements and virulence determinants from one another in the environment is a potentially aggravating situation (Ochman *et al.,* 2000).

Cattle are usually considered the main reservoir of *E. coli* strains that belong to all the currently known pathotypes (Riley *et al.*, 1983; Mühldorfer *et al.*, 1996; Müller *et al.*, 2001; Dunn *et al.,* 2004). However, results obtained from studies have also revealed that shiga-toxigenic *E. coli* strains were more prevalent in pigs than in cattle (Ateba *et al.,* 2008) and therefore it is suggested that the presence of these pathogens in animals in a given area largely depends on host pathogen interactions. Pathogenic *E. coli* strains are usually transmitted to raw food products such as meat when proper hygiene practices as well as standard operating procedures are compromised either on the farms where the animals are housed or in the facilities where they are slaughtered (Bell, 1997; Barkocy-Gallagher *et al.,* 2001). This usually results when faeces get in contact with the carcasses and pathogens contaminate the food product (Bell, 1997; Barkocy-Gallagher *et al.*, 2001). In addition to meat, outbreaks of infection caused by DEP have also resulted from the consumption of contaminated vegetables and sprouts even in a number of European countries that usually have advanced public health policies (Buchholz *et al.,* 2011; Gault *et al.,* 2011). Epidemiological investigations have revealed that the genetic profiles of pathogenic *E. coli* strains isolated from food products and water are similar to those from animal species (Ateba and Mbewe, 2013; 2014). Against this background it is important to constantly monitor the occurrence of these pathogens in animals since meat products such as lamb (Werber *et al.*, 2007), deer meat (Keene *et al.*, 1997; Rabatsky-Her *et al.*,

2002;Rangel *et al.*, 2005), pork (Stevenson and Hanson, 1996; Belongia *et al.*, 1991); salami (Tilden *et al.*, 1996; Williams *et al.*, 2000), and fermented sausages (Paton *et al.*, 1996; Martinez *et al.*, 2001; Werber *et al.*, 2008) are known to be potential sources of pathogenic *E. coli* strains. In addition, pathogenic *E. coli* strains have also been isolated from dairy products such as yogurt, milk, cheese and other food products such as raw vegetables and salads (Wachtel *et al.*, 2004; Food Safety Network, 2006). Moreover radish, sprouts, alfalfa sprouts, spinach, cider, unpasteurized apple juice produced from contaminated fruits have also been implicated in *E. coli* associated infections (Besser *et al.*, 1993; Tamblyn *et al.*, 1999). The findings from these studies have increased the awareness of the involvement of pathogenic *E. coli* as agents of food-borne infections in humans (Estrada-García *et al.,* 2005, 2009; Paniagua *et al.,* 2007; Ateba and Mbewe, 2011; Paredes-Paredes *et al.,* 2011) as well as the need to implement strategies to prevent cross contamination with these organisms. Given that most hospitals in the study area do not perform routine screening for DEP in patients, coupled with the fact that most individuals do not report diarrhoeal cases to health care facilities, the incidence of complications caused by these pathogens are usually under reported (WHO, 2007a). This therefore explains the need to generate more extensive data that provides an overview of the prevalence as well as the virulence profiles of pathogenic bacteria such as DEP isolated from food products since this may not only indicate the need for improved pathogen surveillance strategies but also highlight the clinical significance of these organisms in the area.

E. coli strains are able to cause disease in their hosts due to their ability to produce various potent toxins and a number of accessory virulence determinants such as specific invasion plasmids, colonization factors, fimbriae and adhesins (Croxen and Finlay, 2009; WHO, 2016). Differences in virulence determinants or pathogenic markers are responsible for variations in the pathogenesis of strains belonging to different pathotypes as well as clinical signs and symptoms in patients (Nataro and Kaper, 1998). EHEC strains produce shiga-toxins and two major toxins designated shiga-toxin 1 and shiga-toxin 2 have been isolated

and identified (Kaper *et al.*, 2004; Müthing *et al.*, 2009). In addition, there are other variants of shiga-toxin 2 that are designated *stx2a*, *stx2b*, *stx2c* (Scheutz *et al.*, 2012) as well as some putative virulence gene determinants such as *hlyA* and *eaeA* (Mingle *et al.*, 2012) that have also been associated with EHEC. More over *stx2*e is associated with oedema disease in pork (Laura Ercoli *et al.*, 2016). ETEC are usually associated with the production of enterotoxins including heat-labile (LT) and/or heat-stable (ST) enterotoxins (Nataro and Kaper, 1998) as well as colonization factors (CFs). While the virulence potentials of EAEC strains usually result from the possession of a plasmid that encodes aggregative adherence fimbriae, designated *AAFI* (Nataro *et al.*, 1992), a number of toxins including *Pic* and *Shigella* enterotoxin 1 (*ShET1*) have also been detected in these organisms. In addition, a variety of virulence factors that are regulated by a single transcriptional activator, *AggR,* are expressed by EAEC strains (Kaper *et al.*, 2004).

The pathogenic traits of EIEC strains are very similar to those displayed by *Shigella* species and therefore possess a plasmid-borne type III secretion system that codes for a number of proteins including the *IpaA*, *IpaB*, *IpaC* and *IpgD* (Sansonetti *et al.*, 2000). In addition to this EIEC strains also possess an *IpaH* gene that encodes for an invasive plasmid antigen "H" that is located on both the chromosome and the invasion plasmid (Dutta *et al.*, 2001). The pathogenesis of DAEC is associated with the possession of a LEE island (Beinke *et al.*, 1998) and adhesins designated *draA-E* and *draP*) (Nowicki *et al.*, 2001; Berger *et al.*, 2004). Contamination of meat and its associated products with pathogenic organisms is known to have significant health implications on consumers and may also result in significant financial loss to the meat production industry (Bell, 1997). This therefore justifies the need to investigate the epidemiological distribution of the different *E. coli* pathotypes as well as the critical points during meat production that may facilitate their transmission of these pathogens from animals to humans through the consumption of undercooked contaminated meat.

1.2 PROBLEM STATEMENT

In South Africa, the consumption of poultry, beef and pork is approximately 2.9 million tons per annum and a large proportion (60%) of the meat consumed comprises of poultry (South Africa Meat Market, 2015). Despite this, a significantly large proportion of individuals rely on meat particularly, beef and pork, as a source of protein and these meat products are frequently used during socialization activities such as outdoor braaisc. In addition, steady economic growth coupled with an increase in the average household income has resulted in the rapid increase in meat consumption (South Africa Meat Market, 2015). This has the potential to expose consumers to pathogenic organisms such *E. coli* strains if these food products were contaminated and consumed undercooked (Ateba and Mbewe, 2013).

Food of animal origin can be contaminated either at the abattoir during processing or at the butchery and the sale point in supermarkets depending on the hygiene practices and the manner in which they are handled (Ghosh *et al.*, 2007). Despite the fact that the Department of Agriculture has put in place the Meat Safety Act which outlines standard operating procedures to be followed in abattoirs (Meat Safety Act 40 of 2000), it is reported that in most developing countries, such as South Africa, hygiene practices are not strictly followed during meat processing and handling (Harakeh *et al.*, 2005; Magwira *et al.*, 2005). This therefore provides opportunities for microbial contaminants present in the equipment used in cutting meat as well as the environment in which the carcasses are stored to get in contact with the raw meat (Elder *et al.*, 2000; Conedera *et al.*, 2004; Magwira *et al.*, 2005). This therefore deserves the constant monitoring of the conditions under which these food products are either produced or handled since microbiological quality of the raw products may provide an indication of the possible health impacts that may be posed on consumers.

In South Africa, and the North West Province in particular, a number of studies have been conducted to assess the prevalence of *E. coli* strains and results reveal the presence of these pathogens in animals, meat products and water samples (Müller *et al.*, 2001; Ateba *et al.*, 2008; Ateba and Mbewe, 2011; Ateba and Mbewe, 2013). Nevertheless, these studies focused on the detection and characterization of shiga-toxigenic *E. coli* O157 strains mainly due to their highly pathogenic nature as well as the recent attention that this pathogen has received worldwide (Elder *et al.*, 2000; Conedera *et al.*, 2004; Magwira *et al.*, 2005; Ateba *et al.*, 2008; Ateba and Mbewe, 2011; Ateba and Mbewe; 2013). The current study was designed to expand previous investigations by isolating and determining the virulence profiles of different *E. coli* pathotypes in cattle carcasses in some randomly selected beef abattoirs in the North West Province, South Africa. It is envisaged that data obtained may serve as a valid tool for assessing the effectiveness of the implementation of hygiene practices in these production facilities and may also be of epidemiological significance.

1.3. AIM AND OBJECTIVES

1.3.1. Aim of the study

The main aim of the study was to characterize *E. coli* pathotypes isolated from beef carcasses from some selected abattoirs in the North West Province, South Africa.

1.3.2 Specific objectives

The specific objectives of the study were to:

i) determine the level of potential *E. coli* contamination in abattoir samples using morphological and preliminary biochemical identification tests.

ii) determine the occurrence of *E. coli* isolates using specific PCR analysis.

iii) amplify and determine the presence of virulence gene determinants in the *E. coli* isolates in order to establish the pathotypes.

iv) determine the genetic relationships of isolates using ERIC PCR analysis.

CHAPTER TWO

LITERATURE REVIEW

CHAPTER TWO

LITERATURE REVIEW

2.1. HISTORICAL BACKGROUND

Escherichia coli was first described by Theodor E. Escherich, a Bavarian pediatrician in 1885. He worked with the gut flora of children and later revealed that an organism which he called *Bacterium coli* was present as normal microbial inhabitant in healthy individuals (Shulman *et al.*, 2007). In 1919, the organism *Bacterium coli* was renamed to *Escherichia coli* in honour of Theodor Escherich. *Escherichia coli* is a ubiquitous bacterium that generally resides as normal flora in the gastrointestinal tract of warm-blooded animals and humans (Serna and Boedeker, 2008). Despite the fact that these organisms comprise a small proportion of the total faecal flora, they are the main facultative anaerobes that generally reside in the colon (Wei *et al.*, 2006).

E. coli strains have been reported to possess pathogenic traits and are known to cause a wide variety of disease in humans (Battisti *et al.*, 2006). In recent years *E. coli* strains have been most often identified as the causative agent of a large proportion of diarrhoeal diseases resulting in an increase in the isolation and characterization of new as well as emerging pathogenic strains (Svensson *et al.*, 2011). Emerging *E. coli* strains have been found to possess a variety of genetic determinants that serve as virulence factors and are responsible for pathological conditions associated with the high morbidity and mortality caused by these organisms (Johnsen *et al.*, 2001). Continuous isolation and characterization of virulent strains is essential for the implementation of control strategies.

2.2 TAXONOMY

Escherichia coli is Gram negative, facultative anaerobic and non-spore forming rod shaped bacterium that belongs to the family *Enterobacteriaceae* (Law, 2000). This bacterium is classified in the Phylum *Proteobacteria*, order *Enterobacteriales* and family *Enterobacteriaceae*. Organisms within this family belonging to the genera *Escherichia, Shigella, Salmonella, Klebsiella, Enterobacter, Serratia marcescens, Proteus, Morganella, Citrobacter* and *Yersinia* are the prominent pathogens that are most often associated with disease and these organisms ferment glucose (De Ryck *et al.,* 1994). Cells of *E. coli* strains have peritrichous flagella and produce fimbriae also known as pili as well as capsules that enhance their virulence (Quinn *et al.,* 2002). In addition, *E. coli* are able to produce three types of antigens namely the somatic or "O" antigen, the flagellar or "H" antigen as well as the capsular or "K" antigen (Quinn *et al.,* 2002).

Over 186 "O", 56 "H" and 103 "K" antigens have been identified among *E. coli* strains and these antigens play a significant role in the classification of these strains and hence facilitates identification especially during outbreaks of infections (Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011). Based on genetic differences in the virulence determinants that are harboured by a particular diarrhoeagenic *E. coli* strain these organisms are classified into Enterotoxigenic (ETEC), Enteropathogenic (EPEC), Enteroinvasive (EIEC), Enteroaggregative (EaggEC) *E. coli,* Verotoxigenic (VTEC)/ Shiga-toxin (STEC) and Enterohemorrhagic (EHEC) which is a subgroup of Verotoxigenic (VTEC)/ Shiga-toxin (STEC) (Gamian *et al.*, 1992, Frenzen and Drake, 2005). Despite the fact that it is difficult to clearly associate a particular virulence determinant to a given set of *E. coli* strains, this classification is important since it assists in the determination of genotypes during disease outbreaks. In addition, classification based on virulence

determinants may also provide information necessary for the development of treatment protocols since infections caused by some pathotypes may not be treated with antibiotics (Wong *et al.,* 2000).

2.3 ISOLATION AND IDENTIFICATION OF E. COLI

E. coli is a facultative anaerobic bacterium that is easily recovered from animal faeces (Ateba *et al.,* 2008; Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011), water (WoseKinge *et al.,* 2010; Phokela *et al.,* 2011, Dikobe *et al.,* 2011) and clinical specimens (Vogt and Dippold, 2005) using general-purpose or selective media by incubating samples at 37°C under aerobic conditions (Edwards and Ewing, 1972). MacConkey agar and Eosin methylene-blue agar have been used in most laboratory studies to isolate *E. coli* species (WoseKinge *et al.,* 2010). This is based on the fact that cultures of these organisms produce characteristic lactose fermenting pink and metallic sheen colonies on MacConkey agar and Eosin methylene-blue agar respectively and therefore easy to differentiate them with other members of the *Enterobacteriaceae* on the basis of these macroscopic morphologies (Balows *et al.,* 1991). However, the identification of *E. coli* based on these differential morphologies must be supplemented with other more reliable tests since lactose fermenting colonies on MacConkey agar may not necessarily be true *E. coli* isolates (Nicoletti *et al.,* 1988). In addition, some diarrhoeagenic *E. coli* strains are typically lactose negative and may not produce the characteristic pink colour on MacConkey agar (Nicoletti *et al.,* 1988). The indole test has also been used in the presumptive identification of *E. coli* strains and generally the *Enterobacteriaceae* are differentiated from other bacteria since these organisms are able to split the amino acid tryptophan to form the compound indole (Nataro and Kaper, 1998).

E. coli strains are known to produce acid from the fermentation of glucose, lactose, mannitol and arabinose and may produce gas as a by-product (Sojka, 1965). In addition, *E. coli* strains can be identified using other

biochemical characteristics that include catalase positive, oxidase negative, methyl red positive, and inability to grow on Simmon's citrate medium (Edwards and Ewing, 1972). However, atypical *E. coli* strains that are citrate positive have also been identified (Ishiguro and Sato, 1979; Lee and Choi, 1983; Kim and Tak, 1984; Dubey and Sharda, 2001, Choudhary, 2012). It has also been reported that some *E. coli* isolates have the potential to produce H_2S which is a typical characteristic of *Salmonella* strains (Sutariya, 1993; Mishra *et al.*, 2002).

Given the challenges resulting from the identification of different types of Diarrheagenic Escherichia coli based on macroscopic morphologies on agar plates as well as the lack of consistency within phenotypic biochemical results it is suggested that relevant biochemical tests, serotyping and molecular detection methods be included in each identification protocol (Stenutz *et al.*, 2006). This will reduce the possibilities of false positive as well as false negative results.

2.3.1 Serotyping of *E. coli*

E. coli isolates like many other bacterial species can be identified through serotyping, a technique that occupies a central position in the history of these organisms. Serotyping involves the differentiation of organisms within a genus in which case a group of closely related microorganisms are placed together based on similarities of the set of antigens they possess. Before the development of molecular techniques, diarrhoeagenic *E. coli* strains were identified and differentiated based on differences in their antigenic compositions. According to the scheme developed by Kauffman *E. coli* strains are classified into different serogroups based on their associated somatic, flagellar and capsular antigen combinations that are present on the cell surface (Edwards and Ewing, 1972; Lior, 1996). Whitham *et al.*, (1993) also revealed that a "serotype" of an isolate is defined by a specific combination of O and H antigens while the K antigen codes

for capsules. With 186 O antigens, 103 K antigens, and 56 H antigens among *E. coli* species, different O, K, and H antigens combinations are found in cells. This therefore means there are more than 100000 *E. coli* serotypes in nature (Orskov and Orskov, 1992). Against this background, serotyping is central in the diagnosis of infections caused by pathogenic *E. coli* (Durso *et al.*, 2005). Therefore, genetic determinants of serotypes and serogroups serve as readily identifiable chromosomal markers that correlate with specific virulent strains.

2.3.2 Antigenic structure

E. coli species are a group of genetically and phenotypically diverse organisms whose antigenic structure is vital for identification. Given that about 186 O antigens, 103 K antigens and 56 H antigens have been documented (Gamian *et al.*, 1992, Frenzen and Drake, 2005), the antigenic pattern of a strain is assigned by the number of the specific antigens it possesses. K antigens are subdivided into the thermolabile, thermostable A and B antigens found on enteropathogenic strains associated with infantile diarrhoea. Later it was shown that the B antigen was not a separate entity. In addition, K antigens are therefore currently classified into two groups, I and II, generally corresponding to the former "A" and "L" antigens.

Furthermore, the "O" antigen consists of various replicate oligosaccharide units that are incorporated in the lipopolysaccharide of the outer membrane of Gram-negative bacteria. The biosynthetic process involves the donation of a sugar phosphate that is sequential followed by sugars from particular nucleotides to the carrier lipid, undecaprenyl phosphate (UndP). Polymerization of the O units takes place by conversion of UndP into polysaccharide chains, subsequently turning it to free synthesized core lipid A to form LPS (Reeves, 1994). *E. coli* O antigens are grouped into the *galF* and *gnd* genes based on gene specific classification (Reeves *et al.*, 1996). The inconsistency on the cell surface is an enormous contributing factor

towards the antigenic potential of isolates. When pathogenic *E. coli* cells are present in the host, the immune response depends on the nature and type of the O antigen that is present on the cell wall of the pathogen.

2.4 PCR BASED CHARACTERIZATION OF *E. COLI*

The microbiological safety status of meat products is an important public health concern. Numerous epidemiological reports have identified pathogenic *E. coli* as the main cause of disease outbreaks associated with contaminated meat (Olsvik *et al.,* 1991; Meng and Doyle, 1998). Strains of pathogenic *E. coli*, that have acquired virulence factors, are able to cause diarrhoeal disease in humans (Lee *et al.,* 2009). Different molecular methods, such as DNA hybridization and PCR, have been developed for identification of different categories of DEC, and these methods are based on the detection of genes that are related to the pathogenicity of organisms belonging to each category (Nataro and Kaper, 1998).

Multiplex PCR has been widely used as a diagnostic tool for the simultaneous amplification of multiple target genes that are generally associated with different strains but in a single PCR reaction (Nataro and Kaper, 1998; Toma *et al.,* 2003; Vidal *et al.,* 2004; Aranda *et al.,* 2007; Vilchez *et al.,* 2009). The application of nucleic acid amplifications requires selecting appropriate oligonucleotide primers and optimizing conditions to maximize sensitivity and specificity and a number of multiplex PCR detection assays have been used to determine the presence of diarrhoeagenic *E. coli* strains in food (Lopez-Saucedo *et al.,* 2003, Dhanashree and Mallya, 2008; Farooq *et al.,* 2009; Xia *et al.,* 2010). Multiplex PCR has also been used in the identification of virulence genes in enteroaggregative (EAEC), enteroinvasive (EIEC), enteropathogenic (EPEC), enterotoxigenic (ETEC) and shiga toxin-producing *E. coli* (STEC) (Chomvarin *et al.,* 2005; Kimata *et al.,* 2005; Rajendran *et al.,* 2010; Ali *et al.,* 2012; Kagambega *et al.,* 2012b; Mohammed *et al.,* 2012;

Chen *et al.*, 2013; Hassan and Alireza, 2013; Souza *et al.*, 2013) from food products of animal origin particularly poultry.

2.5 TYPING TECHNIQUES

Phenotypic methods and molecular-based techniques are currently used to determine the source of faecal contamination of pathogens in environmental samples (Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2013; 2014). In addition, molecular-based typing techniques, such as Pulsed-Field Gel Electrophoresis (PFGE), Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Automatic Ribo-typing, rep-PCR, Multi-Locus Sequence Typing (MLST) have been reported to be more reliable than phenotypic typing tools when comparing the relatedness and resolve differences among pathogens obtained from different sources (Garaizar, *et al.*, 2000; Tsen and Lin 2001). This is based on the fact that these typing systems are designed to generate genetic fingerprints based on the DNA nucleotide composition of the isolates. Molecular typing is therefore very useful for bacteria source tracking especially during outbreaks of infections.

2.5.1. Pulsed-field gel electrophoresis (PFGE)

PFGE is a valuable tool for assessing the relatedness of pathogens and its application is based on comparing bacterial DNA restriction patterns of isolates under investigation (Schwartz and Cantor, 1984). The PFGE technique has made tremendous improvements in the epidemiological studies since large DNA molecules of sizes 40 to 50 kb are difficult to migrate efficiently in agarose gels due to their size-independent co-migration, known as reptation (Singh *et al.*, 2006; Slater, 2009). Therefore the success made through PFGE is based on the fact that the direction of the electrical field in which large DNA

molecules are suspended is changed periodically which allows for the separation of DNA molecules over 1000 kb in size. It is evident that PFGE typing coupled with epidemiological data can enhance the source-tracing of bacterial contaminants and provide an indication of the occurrence of food-borne diseases in a given area (Zhao *et al.*, 2007).

2.5.2. Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from the digestion of genomic DNA or an amplified gene. To achieve this genomic DNA from test organism is digested with restriction enzymes and fingerprinting patterns comprising of fragments that differ in lengths are generated. The genetic relationships of test organisms are based on similarities in the patterns generated. Although complex RFLP patterns can be difficult to interpret, RFLP remains a reproducible and highly discriminatory typing system for molecular epidemiology studies (Suthienkul *et al.*, 1996).

2.5.3. Random Amplification of Polymorphic DNA (RADP)

Random Amplification of Polymorphic DNA (RAPD) is a molecular typing technique that involves the random amplification of segments of DNA isolated from test organisms and it is commonly used as a molecular marker in genetic diversity studies. RAPD protocols involve the use of non-specific primers that are usually 10 bases in length, to generate genetic fingerprints that are specific to different organisms. Variations in the genetic profiles of RAPD assays facilitate efficient differentiation of isolates as well as generation of reliable genetic diversity clusters resulting from the fact that this protocol is able to generate random markers from the entire bacteria genome. Previous studies on *E. coli* strains in the North West

Province have utilized ERIC, REP and BOX-AIR PCR in determining the genetic relatedness of the strains obtained from animals, food products and human stool samples (Ateba and Bezuidenhout, 2013; 2014). In addition, Cetinkaya *et al.* (2000) reported that RAPD analysis was reliable in providing the strong similarity between MRSA isolates responsible for an outbreak and those isolated from contaminated surgical dressing containers as well as those from nasal cavity of staff in a given health care facility. This therefore outlines the importance of these typing systems during source tracking surveys.

2.5.4. Multi-Locus Sequence Typing (MLST)

Multi-Locus Sequence Typing (MLST) is a DNA sequence-based method developed by Maiden *et al.* (1997) and Chan *et al.* (2001) and it is known to provide highly discriminatory approaches for clustering of bacterial isolates based on the generation of 450 bp internal fragments of housekeeping genes (Hunter and Gaston 1988). Usually different sequences are assigned as distinct alleles, and each isolate is defined by the alleles present in the housekeeping loci and this is termed the allelic profile or sequence type (ST). Therefore isolates with similar sequence types are considered to have originated from a single clone (Thompson *et al.,* 1998). An advantage of MLST typing is that sequence data for isolates are highly reproducible and it provides unambiguous DNA sequence data that can be easily exchanged and compared between laboratories through worldwide web databases (Enright and Spratt, 1999).

2.5.5. Repetitive Eubacterial palindromic sequences (REP) and Enterobacterial repetitive intergenic consensus (ERIC) PCR

Bacterial source tracking is important in determining the origin of microorganisms, particularly pathogens, in any given area (Duan *et al.,* 2009). However, it is very difficult to achieve this using culture-based techniques and the situation is even further complicated by the fact that traditional bacterial taxonomy is

problematic in differentiating strains that share a close genetic relationship (Duan *et al.,* 2009). Against this background, genotypic techniques such as Enterobacterial repetitive intergenic consensus (ERIC) and repetitive extragenic palindromic (REP)-PCR have been found to be useful in bacterial source tracking studies (Ahmed *et al.,* 2005; Griffith *et al.,* 2003; Indest *et al.,* 2005; Ateba and Mbewe, 2013; 2014). These genetic typing methods are able to distinguish between closely related bacteria by producing DNA fingerprints that are specific for individual strains.

Enterobacterial repetitive intergenic consensus (ERIC) and repetitive extragenic palindromic (REP)-PCR methods respectively utilize primers complementary to specific sequences in the bacterial genome. ERIC sequences are 126 bp long and appear to be restricted to transcribed regions of the genome; whereas REP sequences consist of highly conserved 33 bp inverted repeat sequences (Versalovic *et al.*, 1991). The discriminative abilities of these PCR methods are usually similar to those obtained from PFGE and ribotyping techniques. REP-PCR typing is highly effective in determining the relatedness of isolates when compared to ERIC-PCR due to the fact that fingerprints generated are highly reproducible (Wong and Lin, 2001).

2.6. E. COLI PATHOTYPES

Despite the fact that *Escherichia coli* is found in intestinal micro-flora of a variety of animal species including humans, some strains have been found to cause disease in their hosts (Ochman et al., 2000). Pathogenic *E. coli* strains can be divided into intestinal pathogens that are able to cause diarrhoea in their hosts and extra-intestinal *E. coli* (ExPEC) that are known to cause a variety of infections in both humans and animals including urinary tract infections (UTI), meningitis and septicaemia (Kaper *et al.,* 2004). ExPEC are the primary Gram-negative bacterial strains that are most often associated with neonatal meningitis and

are second only to group B Streptococci in terms of the diseases they cause (Furyk *et al.,* 2011, Bonacorsi and Bingen, 2005). Given that the present study is designed to isolate and characterize diarrhoeagenic *E. coli* from abattoirs as the main focus, ExPEC will not be further investigated or discussed.

The diarrhoeagenic *E. coli* (DEC) are bacterial strains that are able to alter the movement of ions and water in the intestine thus changing the osmotic balance of fluids resulting in excessive secretion that leads to diarrhoeal diseases (Hodges and Ravinder, 2010). DEC strains are among the most common etiological agents of diarrhoea and based on their specific virulence factors as well as their phenotypic traits they are divided into different pathotypes that include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), vero toxin-producing or shiga toxin-producing *E. coli* (VTEC/STEC) which include its well-known subgroup enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). Despite the pathotype to which a strain may be classified, all isolates belonging to these groups have been reported to present severe health challenges to humans and are generally of considerable clinical importance. This therefore explains why it is very important to constantly monitor their occurrence in a given area. Results obtained from such surveillance studies may not only be of great epidemiological importance but may provide an indication of the health risks associated with the consumption of contaminated food products and water.

2.6.1 Classification of E. coli pathotypes

Each *E. coli* pathotype is defined by a characteristic set of virulence associated factors that in turn determine the clinical, pathological and epidemiological features of the disease they cause. On the basis of these virulence associated determinants, diarrhoeagenic *E. coli* have been characterised into five principal pathotypes (Robins-Browne and Hartland, 2002) that are outlined in the following sub-sections.

2.6.1.1 Enterotoxigenic E. coli (ETEC)

ETEC is one of the main etiological agents of infantile and traveller's diarrhoea that is more prevalent in less developed countries (Robins-Browne and Hartland, 2002). The pathogenicity of organisms belonging to this group of diarrhoeagenic *E. coli* is based on the fact that they are able to secrete at least one of two enterotoxins namely a heat-stable enterotoxin designated *ST* and a heat-labile enterotoxin designated *LT* (Robins-Browne and Hartland, 2002). These toxins are responsible for the pathological conditions that are associated with this group of diarrhoeal disease pathogens. Clinical symptoms include loose stools, nausea, vomiting and abdominal cramps (Clarke, 2001). Given that the natural hosts of ETEC are humans and animals and the fact that these organisms are acquired mainly through the ingestion of contaminated food and water; coupled with the fact that they are capable of colonising the proximal small intestine (Levine, 1987) there is a need to implement strategies to limit cross-contamination.

2.6.1.1.1 Heat-stable enterotoxin (ST)

The *ST* is a low molecular weight peptide comprising 19 amino acids and shares significant homology with the intestinal paracrine hormone guanylin (Robins-Browne and Hartland, 2002). The presence of multiple cysteine residues and their disulfide bonds accounts for the stability of this monomeric enterotoxin when exposed to heat (Nataro and Kaper, 1998). The *ST* has two classes namely *STa* and *STb*, both of which are unrelated and are plasmid mediated (Clarke, 2001). The main receptor for *STa* is a membrane-spanning enzyme called guanylate cyclase C (GC-C), located in the apical membrane of intestinal epithelial cells (Nataro and Kaper, 1998) while the receptor for *STb* is still unknown. The *ST* has also been in other Gram negative bacteria, including *Yersinia enterocolitica* and *Vibrio cholerae* (Nataro and Kaper, 1998). These toxins may not be denatured during cooking and therefore ETEC strains that harbour this genetic

determinant are able to express this protein and produce the clinical signs and symptoms associated with them.

2.6.1.1.2 Heat-labile enterotoxin (LT)

LT is an oligomeric toxin that shares an 80% protein sequence homology with the cholera enterotoxin designated "CT" expressed by *V. cholerae* (Nataro and Kaper, 1998). Generally ETEC strains are able to produce two types of *LT* designated *LT-I* and *LT-II*. Both these toxins are composed of a single A subunit (28 kDa), and five larger identical B subunits (11.5 kDa), the latter of which can bind ganglioside GM1, a receptor that is distributed widely in all tissues of the body (Nataro and Kaper, 1998). It is the A subunit however that is solely responsible for the enzymatic activity of the toxin by activating adenylate cyclase (Clarke, 2001). This results in increased intracellular levels of cyclic adenosine monophosphate (cAMP), which in turn leads to diarrhoea due to an alteration in electrolyte balance, a net result of decreased sodium absorbance by villous epithelial cells and stimulated chloride secretion by crypt cells (Clarke,2001).

2.6.1.2 Enteroinvasive *E. coli* (EIEC)

EIEC are an important cause of diarrhoea and are a significant cause of morbidity and mortality in young children in under-developed countries especially when the level of hygiene is low (Robins-Browne and Hartland, 2002). EIEC strains were first reported to be causative agents of diarrhoea (DuPont *et al.*, 1971) and these strains are closely related to *Shigella* species based on biochemical, genetic and pathogenicity potentials (Nataro and Kaper, 1998). However, EIEC strains are significantly different from ETEC because the latter remains within the intestinal lumen throughout the course of infection. EIEC are able to proliferate within the epithelial cells of the gastrointestinal tract (Robins-Browne and Hartland, 2002) and this results in inflammation and ulceration of the mucosa and ultimately leads to cell death (Clarke, 2001). Infections

caused by EIEC are usually associated with fever; severe abdominal cramps, malaise, and watery diarrhoea followed by scanty dysenteric stools containing blood and mucus (Levine, 1987).

2.6.1.3 Enteropathogenic *E. coli* (EPEC)

EPEC are an important cause of infantile diarrhoea in most developing countries worldwide (Nataro and Kaper, 1998). Despite the fact that EPEC strains do not produce any specific classical toxins, a number of serotypes have been associated with disease that is caused by the presence of a variety of other virulence determinants (Clarke, 2001). In contrast to EIEC, EPEC do not penetrate the intestinal mucosa after infection cause histopathological changes through the establishment of attaching and effacing lesions (A/E lesions) (Nataro and Kaper, 1998; Robins-Browne and Hartland, 2002). This histopathological phenotype is known to be the hallmark of EPEC pathogenesis. The establishment of A/E lesions is responsible for intimate adherence of cells of the pathogen to the gut epithelial cells of the host causingto significant changes in the host cell cytoskeleton as well as the formation of actin-rich pedestals at the site of bacteria cell attachment (Nataro and Kaper, 1998).

The genes required for the formation of A/E lesions are located within a pathogenicity island (PAI) on the bacterial chromosome termed the locus of enterocyte effacement (*LEE*) (Elliott *et al.,* 1998; McDaniel *et al.,* 1995; Schmidt, 2010) which is made up of 40 genes. *LEE* encodes a type III secretion system (T3SS), multiple secreted proteins and a bacterial adhesin called intimin and these facilitate the establishment of A/E lesions in EPEC strains (Jarvis *et al.,* 1995; Elliott *et al.,* 1998; Nataro and Kaper, 1998; Iguchi *et al.,* 2009; Schmidt, 2010). In addition, EPEC strains also possess a large virulence plasmid that comprises genes encoding for bundle forming pili (*bfp*) (Clarke, 2001) which also ensures initial contact of EPEC with the intestine (Robins-Browne and Hartland, 2002). Clinically, EPEC illness is characterised by fever,

malaise, vomiting, and diarrhoea with large amounts of mucus in the stool. In infants, EPEC infection tends to be clinically more severe than other diarrhoeal infections, and diarrhoeal episodes can persist for as long as two weeks (Levine, 1987). The exact mechanism(s) of diarrhoea during EPEC infections still remain(s) to be fully elucidated, although several mechanisms have been proposed. These include mal-absorption due to the dramatic loss of microvilli resulting from the formation of A/E lesions, alteration of ion transport in epithelial cells by EPEC, and diarrhoea due to inflammation as well as increased intestinal permeability (Nataro and Kaper, 1998).

2.6.1.4 Enteroaggregative *E. coli* (EAEC)

EAEC, like the other pathotypes are considered a significant cause of diarrhoea in developing countries and are associated with acute and persistent diarrhoea, particularly in young children (Clarke, 2001). These organisms derive their name from their ability to produce distinctive adherence patterns on epithelia cells despite the fact that they do not induce A/E lesions like EPEC strains (Clarke, 2001). In addition, these isolates are able to form a characteristic bacterial layer that possesses a stacked-brick configuration. Infections caused by EAEC strains are characterised by increased mucus secretion from the mucosal surface of epithelial cells (Nataro and Kaper, 1998).

Currently the pathogenesis of EAEC infections is generally not well understood when compared to the other diarrhoeagenic *E. coli* strains. Virulence factors of EAEC include and enteroaggregative heat-stable enterotoxin designated EAST1 that is functionally similar to the heat-stable enterotoxin (ST) (Clarke, 2001; Nataro and Kaper, 1998). In addition to these toxins, two fimbriae have been characterised from EAEC, termed aggregative adherence fimbriae I and II (*AAFI* and *AAFII*) (Clarke, 2001). These plasmids encode for fimbriae that are considered to be responsible for the Aggregative Adherence (AA) phenotype (Nataro

and Kaper, 1998). Although the site of EAEC infection in the human intestine has yet to be demonstrated and the clinical features of EAEC diarrhoea are not well defined, evidence suggests that either watery or mucoid diarrhoea, with little or no vomiting, largely represents the symptoms of EAEC infection (Nataro and Kaper, 1998).

2.6.1.5 Enterohaemorrhagic *E. coli* (EHEC)

EHEC constitute a subset of serotypes of Shiga toxin producing *E. coli* (STEC) that have been associated with haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) that is common in industrialised countries (Caprioli *et al.,* 2005; Chase-Topping *et al.,* 2008). These organisms are regarded as emerging pathogens (Riley *et al.,* 1983; Karmali *et al.,* 1985) based on the fact they have recently received significant attention resulting from their ability to cause diseases in humans worldwide. EHEC strains are also known as verotoxigenic *E. coli* designated VTEC based on the fact that they are cytotoxic to vero cells (Konowalchuck *et al.,* 1977; Nataro and Kaper, 1998). STEC strains also produce one or more shiga toxin and two main types designated stx1 and stx2 have been fully characterised. There are variants of stx2 together with other accessory virulence genes that have also been associated with disease.

2.7 PREVALENCE OF *E. COLI*

Foodborne illness resulting from the consumption of undercooked contaminated food products is a serious public health threat to humans in both developing and developed countries worldwide (Lynch *et al.,* 2009; Potter *et al.,* 2012). The health burden associated with foodborne complications has made food safety an important global issue requiring serious attention (Di Pinto *et al.,* 2006). The presence of pathogenic microorganisms in food products has resulted in the recall of products especially those that are of animal origin as such operational standards play a significant role in determining the quality of finished products

(Potter *et al.,* 2012). Given that the natural hosts of *E. coli* are animals especially cattle, there is a need to empower employees, food producers and suppliers with the skills and abilities that are required in a retail as well as food production facility to ensure food safety (Potter *et al.,* 2012).

A number of studies designed to trace and determine the source of pathogens in food products and compare their genetic and phenotypic profiles with those isolated from humans suffering from infections have revealed strong similarities between the isolates (Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2013; 2014). The implication is that these foodborne pathogens that normally reside in the gastrointestinal tract of animals also have the potential to contaminate food processing facilities and be transmitted to food products such as meat. Given that bacterial isolates that contaminate food products have been reported to possess a variety of virulence determinants their potential to cause diseases in their hosts cannot be under estimated (Gillespie *et al.,* 2000; Suresh *et al.,* 2000; Zhao *et al.,* 2001; Kobayashi *et al.,* 2002; Johnson *et al.,* 2005; Adesiyun *et al.,* 2006; Singh, 2012; Hasan and Alireza, 2013). Unfortunately, most individuals who suffer from diarrhoeal diseases resulting from the consumption of contaminated food products usually do not report cases to health care facilities and therefore official estimates of the prevalence of foodborne pathogens and diseases are highly unrepresentative of the true figures (O'Brien, 2014). In addition, a lack of routine screening for diarrhoeal pathogens within health care facilities especially in developing countries makes it difficult to assess the actual incidence of diseases caused by foodborne contaminants. To overcome any possible bias and effectively provide timely reports on the exact prevalence of these pathogens and *E. coli* strains in particular in a given area, routine surveillance programmes must be implemented.

The prevalence of pathogenic *E. coli* isolates has been determined for a number of poultry, poultry products, cattle, beef, pigs, pork and humans (Jiménez *et al.,* 2003; Musgrove *et al.,* 2004; Ateba *et al.,*

2008; Ateba and Bezuidenhout, 2008; Hossain *et al.*, 2008; Akond *et al.*, 2009; Keeratipibul *et al.*, 2010; Nzouankeu *et al.*, 2010; Saikia and Joshi, 2010; Ateba and Mbewe, 2011; Ghasemian *et al.*, 2011; Kagambega *et al.*, 2012a). Despite the fact that *E. coli* strains are generally considered to be highly prevalent in cattle, other animal species including pigs have been reported to habour these pathogens more than cattle (Ateba and Mbewe, 2011). This therefore amplifies the need for actual monitoring studies that will provide reliable information of *E. coli* prevalence rates in a given area or food production facilities as well as humans.

2.8 CROSS CONTAMINATION

An abattoir is a labour-intensive working environment, and the employees who handle meat must be well trained to ensure they adhere to both personal and general hygiene to ensure the health and safety of the consumer. Staffs in abattoirs are required to ensure that pathogenic organisms that normally reside in the gastrointestinal tract of animals do not enter the food production chain and this is only possible if the recommended standard operating procedures are implemented during the processing, storage and preparation of meat. Mishandling and disregard for hygiene measures in abattoirs have resulted in foodborne outbreaks caused by a number of bacteria including *E. coli* (Abd-Elaleem *et al.*, 2014).

E. coli resides in the gastrointestinal tract of animals and this explains why it is used as a preferred indicator of faecal contamination in both food and water that is intended for human consumption (Bucci *et al.*, 2011). Given that the composition of beef meat is approximately 70-73% of water, 20-22% of protein and 4.8% of lipids (Alan *et al.*, 1995). These chemical constituents favour the growth and survival of microorganisms and therefore once meat is exposed to contaminants there is the potential for the raw food product to spoil if it is not stored under appropriate conditions to limit the growth of mesophilic organisms

(Hudson *et al.,* 1996, Abd-Elaleem *et al.,* 2014). Given that healthy animals have been reported to be carriers' of most pathogenic bacteria including a number of *E. coli* strains without showing any signs of disease, it is important for the hides as well as the intestinal contents to be considered at all times to possess pathogens and therefore be removed properly in order to reduce any chances of cross contamination. This will go a long way to reducing human sufferings associated with foodborne infections. In addition, foodborne complications are known to be more severe in infants, elderly and immuno-compromised individuals and therefore the importance of surveillance studies in countries with significantly higher incidences of HIV/AIDS cannot be over-emphasized.

CHAPTER THREE

MATERIALS AND METHODS

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3.1 STUDY AREA AND SAMPLE SIZE DETERMINATION

The present study was conducted at North West University-Mafikeng Campus, South Africa. The number of samples collected during the study was determined using the formula outlined below:

$$\text{Sample (n)} = \frac{Z_{1-\alpha/2}^2 P (1-P)}{d^2}$$

$Z_{1-\alpha/2}$ = standard normal variable (at 5% type I error (P< 0.05) it is 0.05)

P = expected prevalence in population based on previous studies

d = absolute error or precision (which is 5%)

$$\text{Sample size (n)} = \frac{(1.96)^2(0.86)(1-0.86)}{(0.05)^2} = 185.0011$$

For estimation of the prevalence of *E. coli* pathotypes, the sample size for this study was determined using a prevalence of 86.2% obtained in the study of Kalchayanand *et al.,* (2007) in South Africa to be the expected prevalence with the 95% confidence level and desired precision of 5% using the formula described by Charan and Biswas (2013). Accordingly, the minimum sample size required for the study was 185 swabs from beef carcasses. During the collection of samples a total of 196 samples were obtained from abattoirs and analyzed microbiologically.

3.2 ETHICAL CLEARANCE

Ethical clearance for the study was obtained from the Mafikeng Animal Research Ethics Committee (MAREC) at North West University, South Africa with an ethics clearance number (NWU-00532-16-S9) assigned to the project.

3.3 SAMPLE COLLECTION

A total of 196 swabs were collected from the selected abattoirs in the North West Province (Table 3.1). The selection of the abattoirs was based on the handling and slaughtering capacity as defined by the Meat Inspectors Manual for the Veterinary Public Health Programme of the National Department of Agriculture, South Africa. Sterile swabs were used to collect samples from the surfaces of meat carcasses in the selected abattoirs. The swabs were immersed into transport medium (sodium glycerophosphate, sodium thioglycollate, cysteine hydrochloride, calcium chloride, methylene blue, and agar, pH 7.4 ± 0.2), (Oxoid, UK), labeled properly and immediately transported on ice to the laboratory for selective isolation of *E. coli*.

Table 3.1: Different abattoirs of North West Province from where samples were collected

Location	**No of Samples collected**
Kareespruit^{L}	61
Potchefstroom^{H}	83
Zeerust^{L}	52
Total number of samples	**196**

Capacity of abattoir: H = High; L = Low

3.4 SELECTIVE ISOLATION OF *E. COLI*

The swabs were washed in 5 mL of 2% (w/v) peptone water and aliquots of 100 µL were spread-plated on the Eosin Methylene Blue agar (EMBA) plates. All plates were incubated aerobically at 37°C for 24 hours. After incubation each plate was observed for the presence of characteristic metallic sheen colonies and these were purified by sub-culturing on EMB plates. The plates were incubated aerobically at 37°C for 24 hours. Pure metallic sheen colonies were preserved in 1 mL of 20% (v/v) glycerol stock solutions and stored at -80°C. The isolates that were used for further identification tests.

3.5 BACTERIAL STRAINS

A total of 291 presumptive isolates were obtained from 152 samples that were positive for *E. coli* based on preliminary findings. These isolates were picked from EMBA plates based on differences in their colonial appearances.

3.6 CONTROL STRAINS

In the present study *E. coli* (ATCC 25922) obtained from the American Type Culture Collections and supplied by BioMérieux, South Africa was used as a positive control strain in all experiments while *Klebsiella pneumoniae* (ATCC 13883) was used as a negative control.

3.7 PRELIMINARY IDENTIFICATION OF *E. COLI*

Presumptive isolates were identified using the following criteria:

3.7.1 Cellular morphology

The cellular morphology of presumptive *E. coli* isolates was determined by Gram-staining using a standard protocol (Cruikshank *et al.*, 1975). *E. coli* species are Gram negative rods and isolates that satisfied this

criterion were retained and subjected to further biochemical identification tests specific for *Enterobacteriaceae*.

3.7.2 Oxidase test

Cytochrome is an iron containing protein which is present in all aerobic organisms and thus these organisms are capable of exhibiting oxidase activity (Winn and Koneman, 2006). Presumptive isolates were screened using the Oxidase™ reagent (PL.390) obtained from Mast Diagnostics (Neston, Wirral, U.K.) in accordance with the manufacturer's protocol. Using a sterile tooth pick the test isolate was spotted on the oxidase strip and the strip was observed for colour change within 10 seconds. Strips were observed for the formation of a deep blue or violet colour which was recorded as a positive reaction for the test and vice versa. For quality control purposes, *E. coli* (ATCC 25922) strain was used as an internal control. All isolates that were oxidase negative were analyzed using the triple sugar iron(TSI) test in order to determine their ability to utilize three different carbohydrates at varying concentrations.

3.7.3 Triple Sugar Iron (TSI) test

The TSI test is a carbohydrate utilization biochemical test which differentiates Gram negative enteric bacteria based on their ability to breakdown the sugars glucose, lactose and sucrose present at concentrations of 0.1%, 1% and 10% respectively in the medium. All the composition and preparation of the media used in this study was tabled in Appendix 1. The medium was prepared in a 1 L Duran Schott bottle and approximately 5 mL of TSI medium was dispensed into McCartney bottles and autoclaved. After autoclaving, the bottles were placed in slanting positions to ensure butts and slants were formed when the medium solidified. The TSI medium in McCartney bottles was stab-inoculated with the test isolate in the butt while the slant was later streaked with the same isolate in a *zig-zag* motion (Prescott, 2002). The

inoculated medium was incubated aerobically at 37°C for 24 hours. After incubation the TSI medium was observed for colour change from pink to yellow as well as gas and hydrogen sulphide production (Forbes and Weissfeld, 1998).

3.7.4 Simons Citrate test

Some enteric bacteria are able to use citrate as a sole source of carbon in the absence of fermentable sugars. To screen isolates for this ability Simmons citrate agar is used to determine the ability of isolates to utilize citrate as the sole source of carbon (MacFaddin, 2000). The media was prepared in a 1 L Duran Schott bottle and about 5 mL dispensed into McCartney bottles and autoclaved. After autoclaving, the bottles were placed in slanting positions to ensure butts and slants were formed when the media solidified. The Citrate medium in McCartney bottles was stab-inoculated with the test isolate in the butt while the slant was later streaked with the same isolate in a *zig-zag* motion. The medium was incubated aerobically at 37°C for 24 hours. After incubation the agar was observed for color change from green to blue and the production of gas. Colour change from green to blue indicated a positive result for citrate utilization and vice versa.

3.8 BACTERIAL STORAGE

Presumptive isolates that satisfied the preliminary identification tests were stored for future use and in order to achieve this 1 mL of 20 % (v/v) glycerol was aliquoted into 1.5 µL sterile Eppendorf tubes. A single pure colony for each isolate was transferred into the tubes using sterile disposable plastic loops. The contents of the tubes were homogenised by vortexing and stored at -80°C.

3.9 MOLECULAR CHARACTERIZATION

3.9.1. DNA extraction

In order to extract chromosomal DNA, presumptive isolates were revived by sub-culturing homogenized cultures that were stored at -80°C on Nutrient agar plates. The plates were incubated aerobically at 37°C for 24 hours. Genomic DNA was extracted from all presumptive isolates using the Zymo Research Genomic DNA™ Tissue MiniPrep Kit supplied by Inqaba Biotec Industries (Pty) Ltd Pretoria, South Africa based on the manufacturer's instructions. The DNA samples were stored at -20°C for further molecular identification and characterization.

3.9.2. Determination of DNA quality and quantity

The quality and quantity of DNA extracted was determined using a UV visible spectrophotometer (model S-22, Boeco, Germany) at the wavelengths of 260 nm and 280 nm. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of the DNA extracted (Sambrook *et al.*, 1989).

3.9.3. PCR amplification of 16S rRNA gene fragment

The 16S rRNA gene fragments are sections of prokaryotic DNA found in all bacteria and archaea that possess unique sequences in each bacterium (Song, 2005). These sequences have high discriminatory powers making them very useful for the identification of different bacteria species. Bacterial 16S rRNA gene fragments were thus amplified as an internal control for all presumptive isolates. DNA samples from all the 196 presumptive isolates were used for amplification of 16S rRNA gene fragments in the isolates using the protocol of Korzeniewska and Harnisz (2013). The PCR reactions were performed using a pair of universal 16S rRNA oligonucleotide primers that are shown in Table 3.2. Amplifications were performed using a DNA thermal cycler (model- Bio-Rad C1000 Touch™ Thermal Cycler) obtained from Life Science Group, USA).

The reactions were prepared in 25 µL volumes made up of 12.5 µL of 2X DreamTaq Green Master Mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4mM $MgCl_2$ and loading buffer), 0.25 µL of each primer, 1 µL of template DNA and nuclease free sterile water. All the PCR reagents used in this study were obtained from Thermo Fisher Scientific Inc., USA and supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. The following PCR conditions were used during the amplification of 16S rRNA genes: an initial denaturation at 94°C for 3 minutes, followed by 25 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes. A final extension step was performed at 72 °C for 10 minutes. DNA from *E. coli* (ATCC 25922) was included in this experiment as a positive control. The 16S rRNA amplicons were resolved by electrophoresis on a 1% (w/v) agarose gel. A 100 bp molecular weight marker was included in each gel and used to confirm the relative sizes of amplicons. A ChemiDoc Imaging System (Bio-RAD ChemiDoc™ MP Imaging System, UK) was used to capture the images.

3.10. GENUS SPECIFIC PCR FOR E. COLI

3.10.1 PCR for confirming the identities of *E. coli* isolates through amplification of *uidA* gene

The identities of all the 291 potential *E. coli* isolates were confirmed by PCR amplification of *uidA* house-keeping gene that is specific for *E. coli* (Anbazhagan *et al.*, 2011). The PCR reactions were prepared using oligonucleotide primer sequences shown in Table 3.2. A positive control reaction tube containing DNA from *E. coli* (ATCC 25922) was included in all the experiments. Amplifications were performed using DNA thermal cycler (model- Bio-Rad C1000 Touch™ Thermal Cycler). Reactions were carried out in 25 µL volumes that constituted 11 µL of 2X Dream*Taq* Green Master Mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4 mM $MgCl_2$ and loading buffer), 0.25µL of each primer, 1 µL of template DNA and 12.5 µL nuclease free sterile water. All PCR reagents were obtained from Thermo Fisher Scientific Inc.,

USA products supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. The cycling conditions were as follows: 95°C for 10 minutes, 35 cycles 95°C for 45 seconds, 59°C for 30 seconds and 72°C for 90 seconds and a final elongation at 72°C for 10 minutes. The amplicons were separated by electrophoresis on a 2% (w/v) agarose gel. A 100 bp molecular weight marker was included in each gel and used to confirm the relative sizes of amplicons. A ChemiDoc Imaging System (Bio-RAD ChemiDoc™ MP Imaging System, UK) was used to capture the image.

3.11 PCR AMPLIFICATION OF VIRULENCE GENES

Isolates that were positively confirmed as *E. coli* by species specific PCR were further subjected to a PCR protocol designed to amplify virulence genes that are harboured by *E. coli* strains. The *stx1*, *stx2*, *eaeA* and *hlyA* genes were amplified using primer sequences shown in Table 3.3. PCR amplification consisted of an initial denaturation of 95°C for 15 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, primer annealing at 56°C for 1 minute and extension at 72°C for 1 minute. A final extension was performed at 72°C for 5 minutes (Mohini Joshi and Deshpande, 2011). The amplicons were resolved by electrophoresis on a 1% (w/v) agarose gel on a horizontal Pharmacia biotech equipment system (model Hoefer HE 99X). A 100 bp molecular weight marker was included in each gel and used to confirm the relative sizes of the amplicons. The gels were stained with ethidium bromide (0.001 µg/mL) for 15 minutes and observed under UV light at a wavelength of 420 nm (Sambrook *et al.*, 1989). A ChemiDoc Imaging System (Bio-RAD ChemiDoc™ MP Imaging System, UK) was used to capture images.

3.12 GENOTYPIC TYPING OF *E. COLI* ISOLATES USING ERIC-PCR

The genetic relatedness of all confirmed *E. coli* isolates was determined using ERIC-PCR analysis based on a previously described protocol (Ateba and Mbewe, 2014). The PCR reactions were performed using an

oligonucleotide primer ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3'). Amplifications were performed using a DNA thermal cycler (model Bio-Rad C1000 Touch™ Thermal Cycler). The reactions were prepared in 25 µL standard volumes that constituted 12.5 µL of 2X DreamTaq Green Master Mix (0.4 mM dATP, 0.4 Mm dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4mM $MgCl_2$ and loading buffer), 0.25 µL of each primer, 1 µL of template DNA and nuclease free distilled water,. All the PCR reagents were obtained from Thermo Fisher Scientific Inc., USA products supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. PCR cycling conditions comprised an initial denaturation at 95°C for 2 minutes, 30 cycles of 94°C for 3 seconds, 50°C for 1 minute, 65°C for 8 minutes and a final elongation at 65°C for 8 minutes. The amplicons were resolved by electrophoresis on a 2% (w/v) agarose gel using a horizontal Pharmacia biotech equipment system (model Hoefer HE 99X). A mixture of 1 kb and 100 bp DNA gene rulers were included in each gel and used to confirm the relative sizes of the amplicons. The gels were stained with ethidium bromide (0.001 µg/mL) for 15 minutes and observed under UV light at a wavelength of 420 nm (Sambrook *et al.*, 1989). A ChemiDoc Imaging System (Bio-RAD ChemiDoc™ MP Imaging System, UK) was used to capture images

Table 3.2: Oligonucleotide primers used for amplification of 16S rRNA universal *uidA* gene fragments

Primers	**Sequence (5'-3')**	**Targeted gene**	**Amplicon size (bp)**	**References**
27F	AGAGTTTGATCATGGCTCAG	*16S rRNA*	1420	Lane *et al.*, (1991)
1492R	GGTACCTTGTTACGACTT			
uidAF	CTGGTATCAGCGCGAAGTCT	*uidA*	556	Anbazhagan *et al.*, (2011)
uidAR	AGCGGGTAGATATCACACTC			
uspAR	ACGCAGACCGTAGGCCAGAT			

Table 3.3: Oligonucleotide primers used for amplification of the different virulence genes

Primers	Sequence (5'-3')	Targeted gene	Amplicon size (bp)	References
Stx1F	ATAAATCGCCATTCGTTGACTAC	*Stx1*	180	Paton and Paton, 1998
Stx1R	AGAACGCCCACTGAGATCATC			
Stx2F	GGCACTGTCTGAAACTGCTCC	*Stx2*	255	
Stx2R	TCGCCAGTTATCTGACATTCTG			
eaeAF	GACCCGGCACAAGCATAAGC	*eaeA*	384	Paton and Paton, 1998
eaeAR	CCACCTGCAGCAACAAGAGG			
hlyAF	GCATCATCAAGCGTACGTTCC	*hlyA*	534	Paton and Paton, 1998
hlyAR	AATGAGCCAAGCTGGTTAAGCT			

3.13 STATISTICAL ANALYSIS

The genetic fingerprints of the isolates were compared and analyzed with the TotalLab Phoretix 1D Pro software (TotalLab Ltd., Newcastle, UK). The unweighted pair group method with arithmetic mean (UPGMA) and complete linkage algorithms were used to analyze the percentage similarity and matrix data. Relationships between the various profiles and/or lanes were expressed as dendrograms. Data from groups of related lanes were compiled and reported on cluster tables.

CHAPTER FOUR

RESULTS

CHAPTER FOUR

RESULTS

4. RESULTS

4.1 DETECTION OF *E. COLI* ISOLATES FROM BEEF CARCASS SAMPLES FROM DIFFERENT ABATTOIRS BASED ON MACROSCOPIC MORPHOLOGIES

A total of 196 swab samples were collected from beef carcasses in selected abattoirs in the North West Province during the months of November 2015 to May 2016 and analyzed for macroscopic characters of *E. coli* using Eosin Methylene Blue agar (EMB). The number of samples collected from the different abattoirs and analyzed as well as the proportion that was positive for *E. coli* are shown in Table 4.1. Generally, a large proportion 152 (77.6%) of the samples were positive for *E. coli* based on macroscopic morphologies. In addition, a large proportion (80.3% to 80.7%) of the samples from Kareespruit and Potchefstroom were potentially contaminated with *E. coli* strains (Table 4.1). From these samples, 291 isolates that presented with different macroscopic colonial morphologies were selected and subject to both preliminary and confirmatory biochemical tests for specific identification of *E. coli*.

Table 4.1: Number of samples collected from the different abattoirs and the proportion that was positive for presumptive *E. coli*.

Sampling station	**Number of samples collected and analyzed for presumptive *E. coli***	**Number of samples positive for presumptive *E. coli***
Kareespruit	61	49 (80.3%)
Potchefstroom	83	67 (80.7%)
Zeerust	52	36 (69.2%)
Total	**196**	**152 (77.6%)**

4.2 IDENTIFICATION OF ISOLATES BASED ON PRELIMINARY BIOCHEMICAL TESTS

A total of 291 isolates that were successfully isolated from the samples based on macroscopic colonial morphologies were analysed through Gram staining and preliminary biochemical (oxidase, TSI and citrate utilization) tests. A summary of the number of isolates that was positive for these tests is shown in Table 4.2. All the isolates were Gram negative rod- shaped bacteria that were also oxidase negative and possessed the cytochrome oxidase. In addition, all the isolates fermented sugars in both the butt and slant TSI medium respectively but did not produce hydrogen sulphide gas hence were presumptive *E. coli* species. Results for the citrate utilization test indicated that 86%, 57.3% and 40% of the isolates from Kareespruit, Potchefstroom and Zeerust respectively did not grow on the citrate medium and therefore could not produce the enzyme citrate permease that converts citrate to pyruvate. Due to the absence of the enzyme the pH of the medium was not altered and hence there was no change in colour from green to blue. Isolates possessed this characteristic satisfied the preliminary identification criterion for *E. coli* based on the citrate utilization test.

Table 4.2: Proportion of isolates identified using various biochemical tests

Sampling station	Isolates with Metallic sheen colonies	Gram staining (-ve rod)	Oxidase (-ve)	TSI					Citrate (+/-)
				Glu (+ve)	Suc (+ve)	Lac (+ve)	Gas (+ve)	H_2s (-ve)	
Kareespruit	100	100	100	100	100	100	100	100	14^{+} **86^{-} (86%)***
Potchefstroom	96	96	96	96	96	96	96	96	41^{+} **55^{-} (57.3%)***
Zeerust	95	95	95	95	95	95	95	95	57^{+} **38^{-} (40%)***
Total	**291**	**291**	**291**	**291**	**291**	**291**	**291**	**291**	**112^{+} 179^{-} (61.5%)***

For the citrate utilization test, $^{+}$ = able to grow in the medium and produce a colour change from green to blue; $^{-}$ = unable to grow in the medium and hence did not produce any colour change since medium remained green after incubation. Therefore * = proportion of potential *E. coli* isolates from the different sampling stations.

4.3 BACTERIAL STRAINS AND DNA EXTRACTED

To avoid any bias, chromosomal DNA was extracted from all presumptive isolates (291) and control strains (*E. coli* - ATCC 25922) as described in Section 3.9.1. The presence of DNA was confirmed by electrophoresis on a 1% (w/v) agarose gel and Figure 4.1 indicates genomic DNA from the isolates. The DNA was of good quality without any fragmentation.

Figure 4.1: Agarose gel (1% w/v) image depicting DNA extracted from *E coli* isolates and control strains. Lane M= DNA marker (O'GeneRuler 1kilo base pairs DNA Ladder), Lane 1= DNA extracted from *E. coli* (ATCC 25922) and lane 2-14= DNA extracted from *E. coli* isolates.

4.4 BACTERIAL 16S rRNA GENE PCR ANALYSIS

Bacterial 16S rRNA gene fragments were amplified from all the 291 presumptive isolates. The 16S rRNA gene fragments were successfully amplified all the 291 (100%) isolates. Figure 4.2 indicates a 1% (w/v)

agarose gel image of the 16S rRNA gene fragments amplified in the study. Amplicons possessed the expected sizes of 1420 bp.

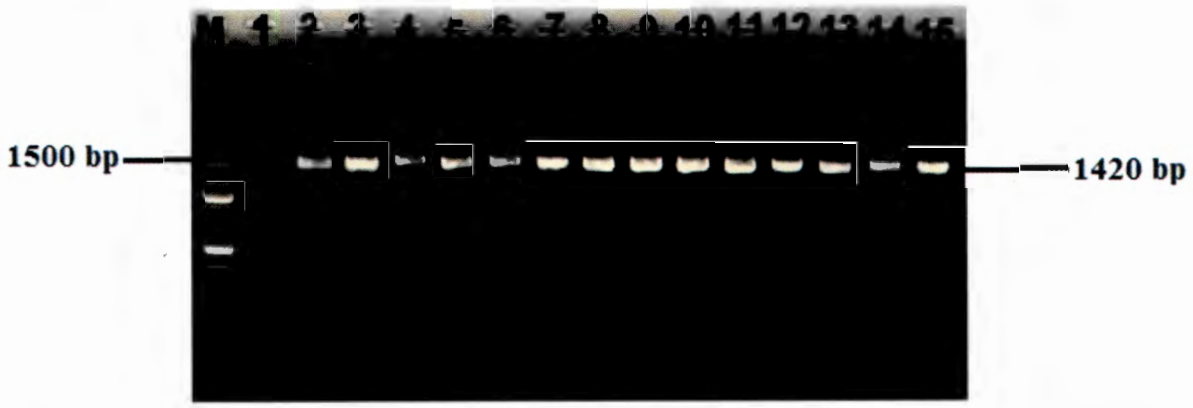

Figure 4.2: Agarose [1% w/v] gel image of 16S rRNA gene fragments amplified from all *E. coli* isolates and *K. pneumoniae* (ATCC 13883), *E. coli* (ATCC 25922) control strains. Lane M= DNA marker (O'GeneRuler 100 bp base pairs DNA Ladder), Lane 1= *K. pneumoniae* (ATCC 13883) negative control, Lane 2= *E. coli* (ATCC 25922) positive control, Lane 3-15= 16S rRNA gene fragments of isolates obtained from swab samples.

4.5 PROPORTION OF *E. COLI* ISOLATES OBTAINED THROUGH PCR AMPLIFICATION OF *uidA* GENE

All the isolates were subjected to *E. coli* specific PCR designed to amplify the *uidA E. coli* housekeeping gene. Out of the 291 isolates, a large proportion 256 (88 %) were positive for the *uidA* gene based on PCR and were confirmed as *E. coli* species. Detailed results of the isolates that possessed this gene from the different areas are shown in Table 4.3 while a 1% agarose gel of representative isolates that were positive for the *uidA* gene are shown in Figure 4.3. *UidA* gene fragments possessed the expected 600 bp nucleotide sequences and this was also verified using the *In Silico* PCR amplification tool (http://www.insilico.ehu.es/PCR/) which provides opportunities for PCR assays to be simulated against up-

to-date sequenced prokaryotic genomes. As indicated in Table 4.3, a total of 256 (88%) of the isolates were positive for the *uidA* gene. The proportion of isolates that possessed the *uidA* gene was higher among the isolates from Kareespruit (n=95) (95%) and Potchefstroom (n=88) (91.7%) than isolates from Zeerust (n=73) (76.8%). Despite this the fact that *E. coli* was frequently detected in beef carcasses in these abattoirs was a cause for concern since these isolates may pose severe public health challenges in consumers if they harbour virulence and antibiotic resistance gene determinants.

Table 4.3: Proportion of isolates that were positive for the *E. coli* specific *uidA* gene fragments

Sampling stations	**Number of isolates tested**	**Positive isolates positive for the *uidA* gene**
Kareespruit	100	95 (95%)
Potchefstroom	96	88 (91.7%)
Zeerust	95	73 (76.8%)
Total	291	256 (88%)

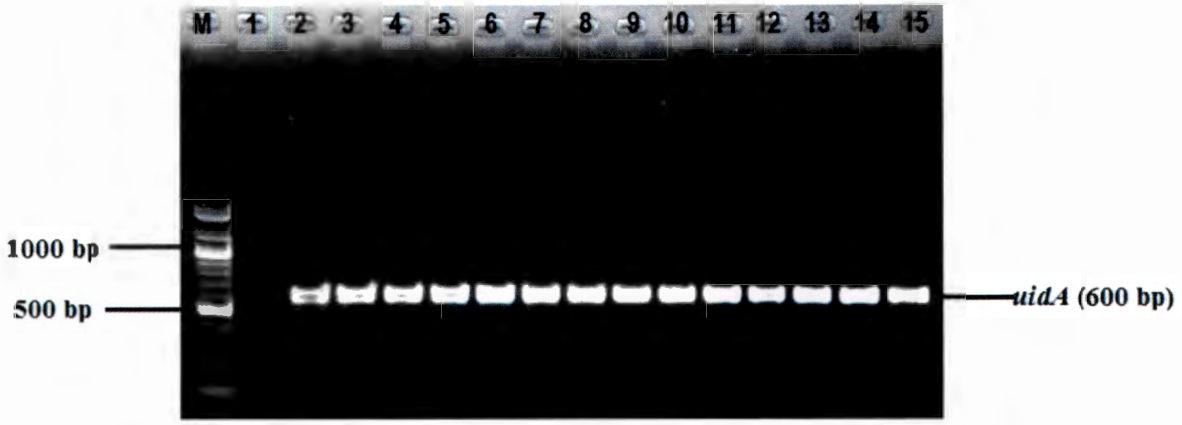

Figure 4.3: Agarose [2% w/v] gel image of *uidA* gene fragments amplified from all *E. coli* isolates and *K. pneumoniae* (ATCC 13883), *E. coli* (ATCC 25922) control strains. Lane M= DNA marker (100 base pairs DNA Ladder), Lane 1= *uidA* gene fragments amplified from *K. pneumoniae* (ATCC 13883) negative control, Lane 2= *uidA* gene fragments amplified from *E. coli* (ATCC 25922) positive control and Lanes 3-15= *uidA* gene fragments amplified from *E. coli* isolates.

4.6 PCR FOR THE DETECTION OF VIRULENCE GENE DETERMINANTS IN THE *E. COLI* ISOLATES THROUGH AMPLIFICATION OF *stx1*, *stx2*, *eaeA* and *hlyA* GENE FRAGMENTS

A total of 256 positively identified *E. coli* isolates were screened by specific PCR for the presence of virulence gene determinants that comprised the *stx1*, *stx2*, *eaeA* and *hlyA*. The proportions of the various genes detected in *E. coli* isolates from the different sampling stations are shown in Table 4.4. The *E. coli* isolates were further analyzed for combinations of the various virulence genes detected per isolate and a summary of data indicating the different genotypes obtained for isolates from the different sampling sites is also shown in Table 4.5. Sixteen major genotypes designated G1 to G16 were identified in the study (Table 4.5). However, as shown in Table 4.5, large proportions (70.7% to 77.7%) of the isolates screened possessed the stx_1, stx_2 and *eaeA* genes respectively. Despite this the stx_1 was the most frequently identified determinant among isolates from Kareespruit 79 (83.2%) and Zeerust 61 (83.6%). In addition, the

stx$_2$ gene was commonly detected 60 (82.2%) among isolates from Zeerust. Similarly, a large proportion 49 (67.1%) of the *E. coli* isolates from Zeerust also possessed the *hlyA* gene. On the contrary, e*aeA* gene that codes for intimin was frequently detected in 72 (75.8%) of the isolates from Kareespruit (Table 4.4). The data in Table 4.4 indicates that there was no major difference in the number of isolates obtained in this study that possessed the *stx*$_1$, *stx*$_2$ and e*aeA* genes respectively, only 3 (1.2%) harboured both shiga toxin genes as shown in genotype (G6). In addition, only 1 (0.4%) isolates that is classified as genotype (G12) harbour all these three genes. Despite this, the number of isolates that possessed the *stx*$_1$ gene, i.e. 199 (77.7%), was higher than those with the *stx*$_2$ gene (Table 4.4). In this study, 6 (2.3%) of the *E. coli* isolates screened harboured all the four virulence gene determinants investigated. Only 16 (6.3%) of the *E. coli* isolates possessed the *stx*$_1$ and *stx*$_2$ genes in combination with the two accessory virulence gene determinants (e*aeA* and *hlyA*) as indicated in genotypes G14, G15 and G16.

Table 4.4: PCR for the detection of virulence gene determinants in the *E. coli* isolates using PCR amplification of *stx1*, *stx2*, *eaeA* and *hly* genes from the different abattoirs.

Virulence gene(s)	*E. coli O157* (ATCC 43895)	*Klebsiella pneumoniae* (ATCC 13883)	Number of isolates with indicated of virulence gene(s)			
			Kareespruit (NT = 95)	Potchefstroom (NT = 88)	Zeerust (NT = 73)	Total (%) (NT = 256)
Stx1	+	-	NP = 79 % = 83.2%	NP = 59 % = 67.0%	NP = 61 % = 83.6%	NP = 199 % = 77.7%
Stx2	+	-	NP = 62 % = 65.3%	NP =51 % = 58%	NP = 60 % = 82.2%	NP = 173 % = 67.6%
eaeA	+	-	NP = 72 % = 75.8%	NP = 65 % = 73.9%	NP = 44 % = 60.3%	NP = 181 % = 70.7%
hlyA	+	-	NP= 46 % = 48.4%	NP = 31 % = 35.2%	NP = 49 % = 67.1%	NP = 126 % = 49.2%

+ = Gene was present in the control strains; - = Gene was absent in the control strains

Table 4.5: Proportion of sixteen different gene combinations (genotypes) identified in *E. coli* isolated. The notations G1 to G16 indicate gene combinations of these ten genotypes.

	Gene combinations for *E. coli* isolated					**Proportion of *E. coli* isolates with the different gene combinations per sample station**			
Genotype	***uidA***	***stx*$_1$**	***stx*$_2$**	***eaeA***	***hlyA***	**Kareespruit**	**Potchefstroom**	**Zeerust**	Total
G1	+	-	-	-	-	19	10	18	
G2	+	+	-	-	-	2	5	1	
G3	+	-	+	-	-	9	8	9	
G4	+	-	-	+	-	7	3	16	
G5	+	-	-	-	+	15	19	10	
G6	+	+	+	-	-	1	1	1	
G7	+	+	-	+	-	3	3	2	
G8	+	+	-	-	+	6	1	3	
G9	+	-	+	+	-	3	1	1	
G10	+	-	+	-	+	16	8	1	
G11	+	-	-	+	+	6	7	5	
G12	+	+	+	+	-	1	0	0	
G13	+	+	+	-	+	1	12	0	
G14	+	+	-	+	+	2	1	4	
G15	+	-	+	+	+	1	2	0	
G16	+	+	+	+	+	1	4	1	

G = genotype

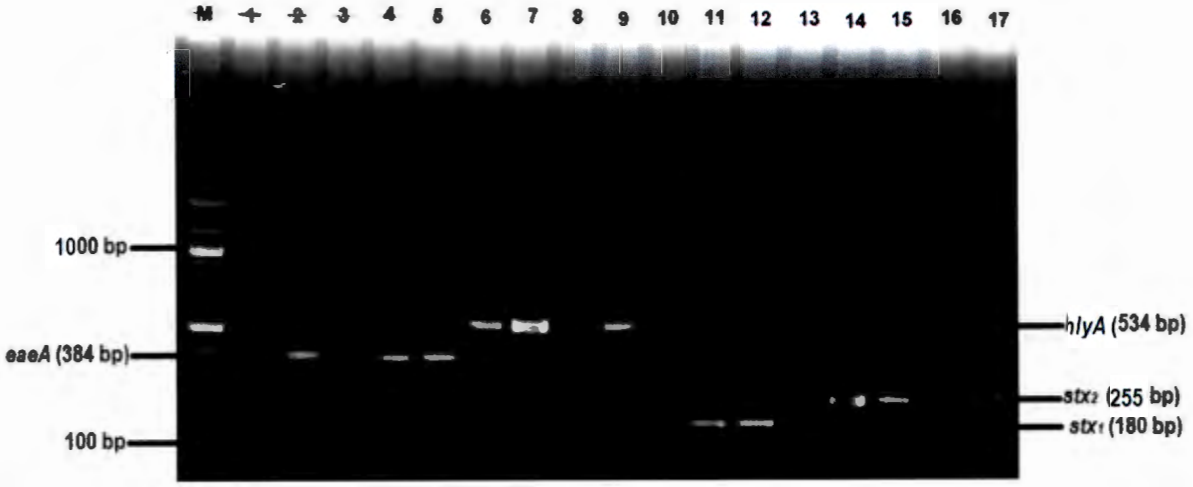

Figure 4.4: A 2% (w/v) agarose gel image showing the *stx*$_1$, *stx*$_2$, *eaeA* and *hlyA* gene fragments amplified from all *E. coli* isolates and *E. coli* control strain. Lane M= DNA marker (100 base pairs DNA ladder), Lane 1= negative control strain *K. pneumoniae* (ATCC 13883), Lanes 2-5= *eaeA* gene fragments amplified from *E. coli* isolates, Lanes 6-9= *hlyA* gene fragments amplified from *E. coli* isolates, Lanes 10-13= *stx*$_1$ gene fragments amplified from *E. coli* isolates, Lanes 14-17=*stx*$_2$ gene fragments amplified from *E. coli* isolates.

4.7 ERIC PCR ANALYSIS

A total of 256 *E. coli* isolates obtained in the study were analysed using ERIC-PCR order to determine their genetic profiles. A comparison of the ERIC-PCR banding patterns generated using chromosomal DNA from the *E. coli* isolates revealed between 1 and 17 polymorphic bands per isolate. Isolates produced band sizes that ranged from 100 bp to 1 kb. A large band of approximately 2 kb as well as small bands of 400 bp were very common in most of the isolates typed (Figures 4.5 – 4.7).

The genetic fingerprints of 40 randomly selected *E. coli* isolates generated through ERIC PCR analysis were subjected to cluster analysis and a comparison of the clustering patterns revealed 2 major cluster (Clusters 1 and 2). Cluster 1 was subdivided into two sub cluster (Clusters 1A and 1B) while cluster 2 was

composed of four sub-clusters (sub-clusters 2A – 2D). The clusters were analysed for association of isolates from the different sampling sites based on similarities in the genetic profiles. The largest sub-cluster (sub-cluster 2A) had 12 isolates and large proportions (9/12) were from Zeerust. In addition, a large proportion of the isolates in sub-cluster 1A were from Kareespruit but these isolates cluster together with 2 isolates from Zeerust (Figure 4.8). Isolates from the abattoir in Potchefstroom also clustered together in sub-cluster 2B. These data revealed that there is a need to reduce the level of contamination in abattoirs to avoid cross contamination of meat products.

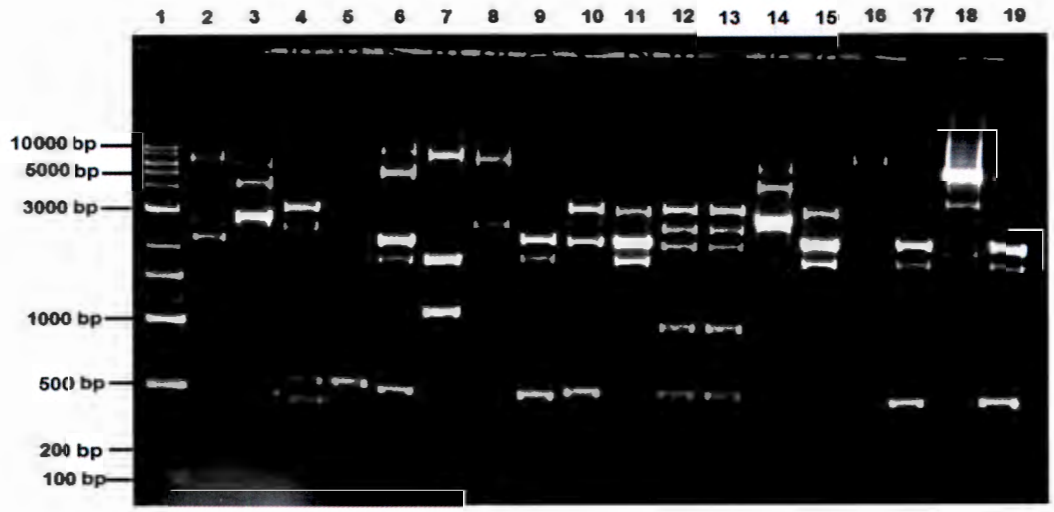

Figure 4.5: Agarose [2% w/v] gel image of ERIC fingerprints of representative *E. coli* isolates obtained from the abattoir in Kareespruit.

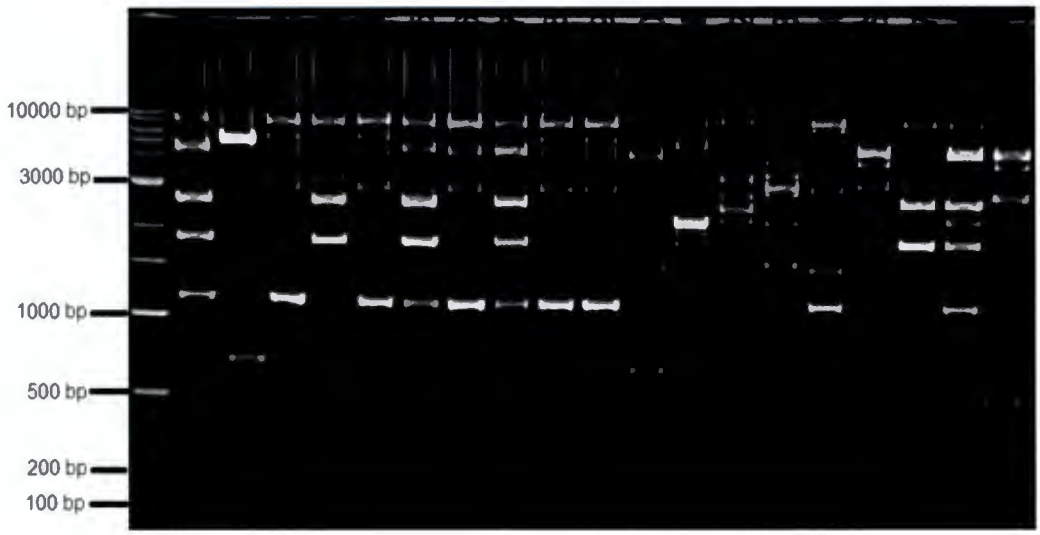

Figure 4.6: Agarose [2% w/v] gel image of ERIC fingerprints of representative *E. coli* isolates obtained from the abattoir in Potchefstroom.

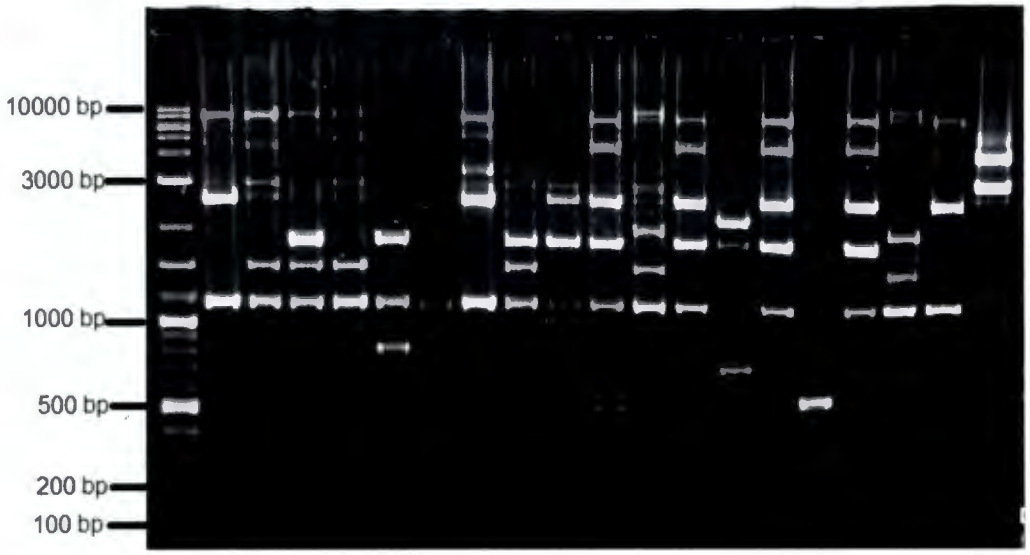

Figure 4.7: Agarose [2% w/v] gel image of ERIC fingerprints of representative *E. coli* isolates obtained from the abattoir in Zeerust.

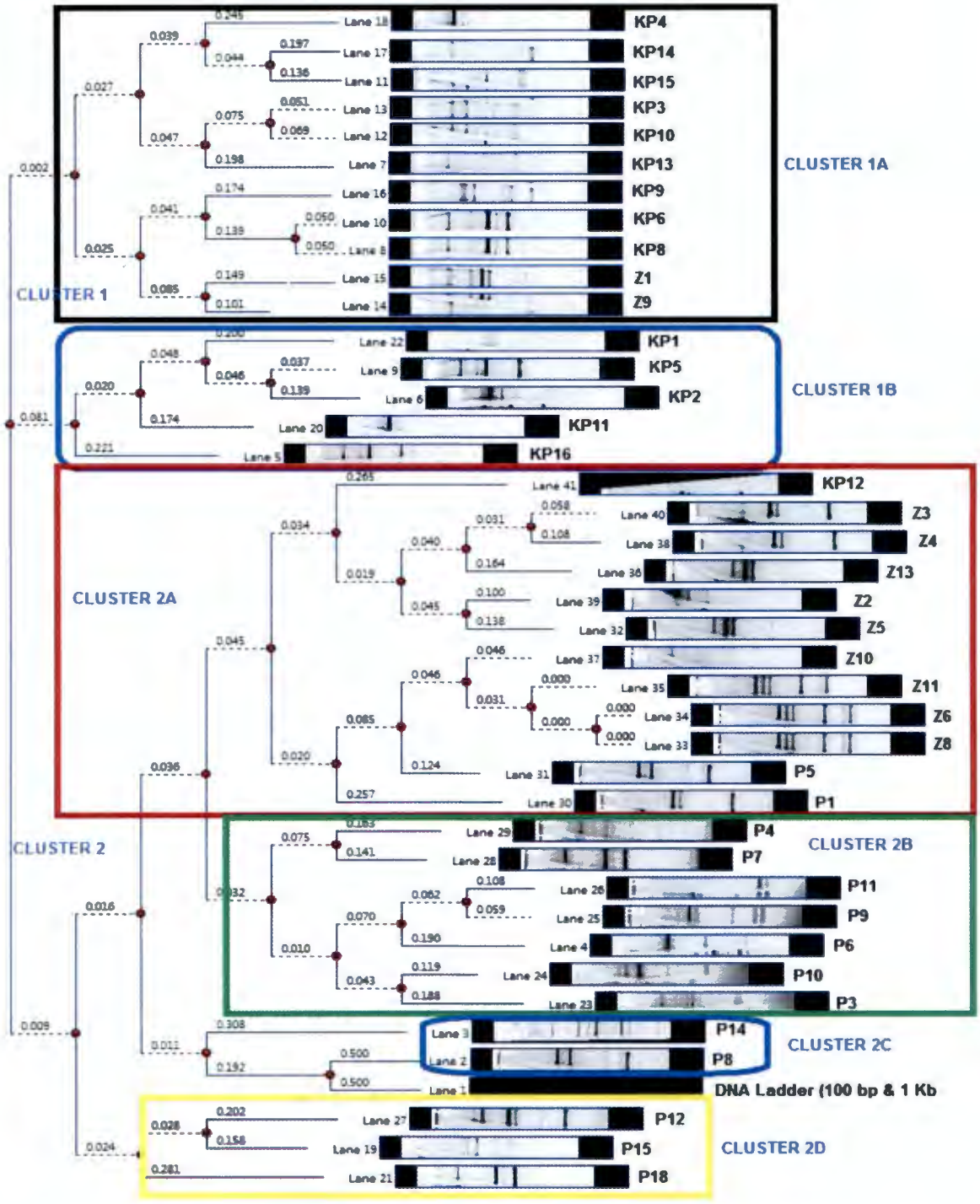

Figure 4.8: Dendrogram showing the genetic relationship of *E. coli* isolates obtained from the different sample stations using ERIC-PCR analysis

CHAPTER FIVE

DISCUSSION

CHAPTER FIVE

DISCUSSION

5. DISCUSSION

5.1 GENERAL DISCUSSION

The primary objective of this study was to isolate and confirm the identities of *E. coli* isolates obtained from beef carcasses in selected abattoirs in the North West Province of South Africa. This was motivated by the fact that food-producing animals especially cattle are known to serve as reservoirs for enteric bacteria species particularly those belonging to the family *Enterobacteriaceae* (Gundogan and Avci, 2013; Haenni *et al.*, 2014; Valentin *et al.*, 2014; Iweriebor *et al.*, 2015). In addition to this, microbes that reside as normal flora in the gastro-intestinal tract of animals have been found to contaminate both the environment in which animals are slaughtered and the equipment used for cutting meat in an abattoir. It is also evident that microbial contaminants that are present in the abattoir environment as well as equipment used for cutting can easily be transferred to meat if proper hygiene is not implemented (Bouvet *et al.*, 2001; Tutenel *et al.*, 2003; Yilmaz *et al.*, 2006, Ateba and Mbewe, 2011).

A number of studies conducted in the area have revealed the presence of *E. coli* strains in cattle and pigs including their corresponding meat products such as beef and pork (Ateba and Bezuidenhout, 2008; Ateba *et al.*, 2008, Bezuidenhout and Moneoang, 2009; Ateba and Mbewe, 2011). In addition, isolates obtained in some of these studies were typed using phenotypic and genotypic techniques and data generated revealed very close similarities between isolates from animals, meat samples as well as humans (Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2013; Ateba and Mbewe, 2014; Makhubalo *et al.*, 2016). Given the high demand for meat particularly beef in South Africa coupled with the fact that meat is rich in nutrients

that are needed to support the growth of microorganisms (Magnus, 1981, Rawlings *et al.,* 2013), there is need to implement strategies that will greatly reduce cross-contamination with pathogenic bacteria such as toxigenic, enteroinvasive and enterohaemorrhagic *E. coli* strains. Extensive studies have been designed to isolate and characterise pathogenic *E. coli* strains from animals and meat samples (Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2013; Ateba and Mbewe, 2014; Makhubalo *et al.,* 2016) in the study area as well as South Africa in general (Peirano, *et al.,* 2011; Gqunta and Govender, 2015; Iweriebor *et al.,* 2015). However, to the best of our knowledge, this is the first study that has been conducted in the study area designed to assess the level of contamination with pathogenic *E. coli* at the level of the abattoir. It is evident that these baseline data may be of great epidemiological importance since it has been reported that the fast growth of the cattle and meat industries may introduce significant pressures on production systems thus providing opportunities for increased health risks associated with the consumption of pathogenic *E. coli* in contaminated food products (Vidovic and Korber, 2006).

The *uidA* gene, which encodes for βglucuronidase, has been used for detecting *E. coli* in previous studies (Bej *et al.*, 1991; Martins *et al.*, 1993; Tsai *et al.*, 1993; McDaniels *et al.*, 1996; Iqbal *et al.*, 1997; Lasalde *et al.*, 2003; Abib and AL-zuwainy, 2014). Bacterial βglucuronidase produced by the aerobic Enterobacteriaceae such as *E. coli* is an essential enzyme which deconjugates bilirubin diglucuronide, resulting in the release of free bilirubin and glucuronic acid (Swidsinski and Lee, 2001). It is assumed that the enzyme is one of the factors playing a role in the pathogenesis of *E. coli* (Lee *et al.,* 1999; Abib and AL-zuwainy, 2014).

In the present study, an overall prevalence of 88% was detected for *E. coli* of isolated from beef carcasses in the abattoirs sampled. In addition, the proportion of *E. coli* isolates was higher in samples obtained from Kareespruit 95 (95%) and Potchefstroom 88 (91.7%) than in Zeerust 73 (76.8%). Despite the fact that the

abattoir in Potchefstroom is a high capacity facility while those in Kareespruit and Zeerust are low capacity abattoirs, there was no difference in the level of *E. coli* contamination detected. Similarly high prevalence rates (78.6% to 86.2%) for non-*E. coli* O157 STEC strains have been reported for pelts, pre-evisceration carcass, and post-intervention carcass samples in United the States of America (Kalchayanand *et al.*, 2007). These findings are higher than those previously reported for *E. coli* isolates obtained from raw beef in which 10.8% (Ateba and Mbewe, 2011) and 15.89 % of the samples were contaminated. Vorster *et al.* (1994) also reported a prevalence of 74.5% of *E. coli* O157:H7 contamination in meat and meat products in South Africa. In another study 30% of minced meat and polony samples were contaminated with *E. coli* O157 strains (Abong'o *et al.*, 2008). Despite the fact that results obtained from these microbial quality assessment studies differ significantly, it is generally known that these data largely depend on the hygiene conditions that are implemented on the farms were animals are kept, that of the equipment used in abattoirs, the surrounding environment and the personnel in the facility. In addition, the assessment of slaughter processes and facilities based on determination of microbial contamination levels especially with respect to *E. coli* that indicates the presence of faecal contamination is very important for evaluating the level of adherence to standard operational procedures in an abattoir (Milios *et al.*, 2014; Nyamakwere *et al.*, 2016). Against this background, the implementation of strict control measures at the farms, in the abattoirs and handling of meat at sale point may reduce foodborne infections in humans.

In South Africa environmental management is governed by the National Environmental Management Act (NEMA), 1998 (Act 107 of 1998) and the management of abattoirs as well as other wastes from animals are controlled by NEMA. NEMA also requires an environmental impact assessments be conducted in abattoirs to determine the effect of current procedures relating to the disposal and treatment of wastes in these facilities since they are known to have a direct implication on the microbial quality of the raw meat products. Due to the fact that the management of abattoirs can be very complex and may depend on a

wide range of issues an Abattoir Waste Management Facilities (AWMF's) guideline has been developed to ensure that all facilities adhere to common and standard procedures. Despite all this the World Health Organization also recommends that water used for washing of carcasses in an abattoir must be free from *E. coli* to avoid cross-contamination (WHO, 2004). The quality of water in the North West province that is supplied by municipal treatment plants is an issue of great public health concern, it is believed that water may also increase the level of contamination of carcasses during the production process.

The safety of meat products is also regulated through the Meat Safety Act (MSA), 2000 (Act 40 of 2000) which is designed to promote the safety of meat and animal products and therefore to establish and maintain essential national standards. Despite the fact that cattle have been found to be contaminated with *E. coli* during transportation from the farm to the abattoir through contact with faeces of other animals (Hui, 2012), procedures such as skinning, evisceration and splitting may contribute to carcass contamination during slaughter. In addition, poor personal hygiene in abattoirs and butcheries can increase the level of microbial contamination of carcasses (Nel *et al.,* 2004; Haileselassie *et al.,* 2013). This explains why hot water, sanitizers and detergents are recommended as important agents that greatly reduce microbial contamination on slaughter equipment and the hands of employees (Griffith, 2000; Redmond and Griffith, 2003; Nel *et al.,* 2004; O'Brien *et al.,* 2005; Abd-Elalee *et al.,* 2014; Nyamakwere *et al.,* 2016) and are very useful during the slaughter process, especially hide removal, which can provide opportunities for *E. coli* to be transferred into the food chain.

Another objective of the study was to determine the virulence gene determinants of *E. coli* isolates using specific PCR analysis. A total of 256 *E. coli* isolates and control strains were analysed. Despite the fact that there was no major difference in the number of isolates that possessed the stx_1 and stx_2 genes respectively, only 3 (1.2%) isolates harboured both of these shiga toxin genes. Similar observations have

been reported in which only a small proportion of isolates from meat and faecal samples of animals possessed both the shiga toxin genes (Ateba and Mbewe, 2011). However, this finding is contrary to a previous report in which *E. coli* O157 pathotypes did not possess any of the shiga-toxin genes (Ateba and Bezuidenhout, 2008). Although the *stx* genes are considered the primary virulence factors of shiga-toxigenic *E. coli* but the stx_2 gene is usually more prevalent among these pathogens (Paton and Paton, 1998, Johnsen *et al.,* 2001 and Villani *et al.,* 2005). Despite this stx_1 gene was more prevalent in the isolates when compared to stx_2. However, since the stx_2 gene rather than stx_1 has been most frequently isolated STEC genotypes that are associated with disease in humans (Bidet *et al.,* 2005). On the contrary, a number of *E. coli* serotypes with varied isolation frequencies have been isolated from patients suffering from HUS and HC (Goldwater and Bettwlheim, 1996). Despite the fact that the number of isolates positive for these genes may be greatly affected by methodological bias (Karmali, 1989; Griffin, 1995; Gyles *et al.,* 1998) studies designed to correlate the presence of specific primary or putative virulence factors with either the occurrence or severity of disease indicate that no single factor is responsible for the virulence of *E. coli* strains. This therefore explains the need for screening patients before the administration of treatment especially due to the fact that antibiotics are generally not recommended as options for shiga-toxigenic *E. coli* pathotypes.

The prevalence of two putative virulence determinants *hlyA* and the *eaeA* was determined. Contrary to a previous finding in which the *hlyA* was most frequently detected among shiga-toxigenic *E. coli* (Ateba and Mbewe, 2011), the *eaeA* gene was dominant among a large proportion 181 (70.7%) of the isolates analyzed in the current study. Despite this the *eaeA* gene was detected in 126 (49.2%) of the isolates. The plasmid-encoded *hlyA* gene gives any bacterium that harbours the gene the ability to display haemolytic activity on 5% sheep blood agar (Beutin *et al.,* 1998; Schmidt *et al.,* 1999) while the *eaeA* gene is associated with the production of intimin that facilitates intimate attachment of host cells to the epithelia

cells of the human host resulting from the production of attaching and effacing intestinal lesions (Knutton *et al.*, 1989).

Generally, ERIC PCR was able to distinguish between isolates from the different sampling sites since isolates from a particular abattoir produced similar genetic fingerprints based on ERIC PCR analysis and clustered together. In a previous study it was reported that isolates with huge sequence similarities usually reflects that they are descents from a common ancestor, and this accounts for the reason why they cluster together in a typing assay (Panangala *et al.*, 2005). Moreover, *E. coli* isolates from a particular geographic location with similar genetic and antibiotic resistant profiles had been reported to be related genetically (Nielsen and Scheutz, 2002). The findings of the study revealed that ERIC-PCR was very sensitive in detecting slight differences between isolates from different species and therefore the major implication is that the ERIC PCR analysis could serve as a more effective tool in the routine surveillance of *E. coli* especially in abattoirs in the area.

CHAPTER SIX

CONCLUSION

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6. CONCLUSION

6.1 GENERAL CONCLUSION AND RECOMMENDATIONS

In both developing and developed countries meat safety currently remains a very important issue of public health concern particularly in a world where there is great focus on the one health programme. In recent years, a number of foodborne diseases as well as outbreaks of infections associated with pathogenic *E. coli* particularly those belonging to the serotype O157 have been reported even in countries such as the United States of America in which meat producers adhere to the standard operating procedures. The occurrence of these diseases that are associated with the consumption of contaminated meat products have brought about meat safety issues to the forefront of societal concerns (Sofos, 2008).

During the processing of beef carcass in abattoirs, *E. coli* associated with the carcass of cattle can increase or decrease during processing depending on factors such as the levels of contamination of live animals, efficiency of evisceration and hygienic practices that are implemented in the abattoir (Rigobelo *et al.,* 2006). Against this background, abattoirs are generally required to screen carcasses for the presence of *E. coli* strains since this may provide an indicator of the adequacy of the plant's ability to control faecal contamination (Anonymous, 1994). In addition, the level of *E. coli* contamination may also provide an indication of the health risks associated with the consumption of undercooked contaminated meat products in a given area.

In the present study *E. coli* strains were successfully isolated from beef carcasses in the selected abattoirs in the North West Province of South Africa. *E. coli* strains belonging to different pathological types that also harboured various virulence genes were detected and these bacterial contaminants can easily be transmitted to humans who consume the products. In addition, the detection of these organisms serves as a valid assessment of the hygiene practices that are implemented in these abattoirs but may also raise issues relating to farm management techniques practiced where animals are kept. The higher rate of *E. coli* contamination in the present study may be attributed to the lack of standard operating procedures and poor hygienic practices such as flaying, evisceration and splitting of carcass as well as the poor quality of water used for washing carcasses in the abattoirs. It is also recommended that contact between the hides and intestinal contents of animals and the carcass be prevented to reduce transmission of these pathogens to meat and hence limit human infections.

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APPENDICES

APPENDIX A

Appendix 1A: Details of all the materials; culture media, chemicals enzymes and reagents used in this study.

1.1 CULTURE MEDIA

1.1.1 Buffered Peptone Water	**g/L**
Peptone	10.0
Sodium Chloride	5.0
Di-sodium Hydrogen Phosphate	3.5
Potassium Dihydrogen Phosphate	1.5

Twenty grams (20 g) of the above components were dissolved in 1L of distilled water. The media was distributed into McCartney bottles and autoclaved at 121°C for 15 minutes. Buffered peptone water (Biolab, Merck Diagnostic, South Africa) was used as a pre-enrichment medium for isolation of *Enterobacteriaceae*.

1.1.2 Nutrient broth	**g/L**
Meat extract	1.0
Yeast extract	2.0
Peptone	5.0
Sodium Chloride	8.0

Sixteen grams (16 g) of the above components were dissolved in 1L of distilled water. The media was distributed into McCartney bottles and autoclaved at 121°C for 15 minutes. Nutrient broth (Biolab, Merck

Diagnostic, South Africa) was used as pre-enrichment medium for overnight cultures during DNA extraction.

1.1.3 MacConkey Agar (With Crystal Violet and Salt)	**g/L**
Peptone	20.0
Lactose	10.0
Bile Salts No 3	1.5
Sodium Chloride	5.0
Neutral Red	0.03
Crystal Violet	1.001
Agar	13.5

Fifty grams (50 g) of the above components were dissolved in 1L of distilled water. The media was autoclaved at 121°C for 15 minutes. MacConkey agar (Biolab, Merck Diagnostic, South Africa) was used as a differential medium for isolation of *Enterobacteriaceae*.

1.1.4 Triple Sugar Iron (TSI)	**g/L**
Meat extract	3.0
Yeast extract	3.0
Tryptone	18.0
Meat peptone	2.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferric Ammonium Sulphate	0.2

Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Phenol Red	0.024
Agar	10.5

Sixty three grams (63 g) of the above components were dissolved in 1L of distilled water. The media was distributed into McCartney bottles and autoclaved at 121°C for 15 minutes. TSI agar (Biolab, Merck Diagnostic, South Africa) was used as a medium for preliminary identification of *E. coli* based on its biochemical profile.

1.1.5 Simmons Citrate Agar	**g/L**
Ammonium Dihydrogen Phosphate	1
Dipotassium Phosphate	1
Sodium chloride	5
Sodium citrate	2
Magnesium Sulphate	0.2
Bromothymol Blue	0.08
Agar	15

Twenty two grams (22 g) of the above components were dissolved in 1 L of distilled water. The medium was distributed into McCartney bottles and autoclaved at 121 °C for 15 minutes. Simons Citrate agar (Biolab, Merck Diagnostic, South Africa) was used as a medium for preliminary identification of *E. coli* based on its biochemical profile.

1.1.6 Eosin Methylene Blue Agar	**g/L**
Peptic digest of animal tissue	10.0

Dipotassium phosphate	2.0
Lactose	5.0
Sucrose	5.0
Eosin – Y	0.40
Methylene blue	0.065
Agar	13.5
Final pH (at 25°C)	7.2±0.2

Suspend thirty-five point nine six (35.96) grams in 1 L distilled water. Mix until suspension is uniform. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

2.1 CHEMICALS

2.1.1 Buffers (50X TAE)

Thermo Scientific (#B49) 50X TAE Electrophoresis Buffer (40mM Tris, 20mM Acetic Acid and 1mM EDTA) stock solution was supplied by Thermo Scientific, Johannesburg, South Africa. A 1X TAE buffer working solution was prepared and used for resolving either DNA or amplified PCR products by agarose gel electrophoresis.

2.1.2 Sodium Hypochlorite

A 10% (v/v) sodium hypochlorite (working solution) was prepared by aliquoting 10ml of sodium hypochlorite (stock solution) into 1L Duran bottle and the volume adjusted to 1L by adding 900ml of distilled water. The solution was stored at room temperature and used for disinfecting the working area.

2.1.3 Ethanol (70%)

Absolute ethanol (96% v/v) was supplied by Merck, Diagnostics, South Africa. A 70% (v/v) working solution was prepared by aliquoting 729 ml of absolute ethanol into 1L Duran bottle and the volume adjusted to 1L by adding 271 ml of distilled water. The solution was stored at room temperature and used for sterilising the working area.

2.1.4 DNA loading dye (6X)

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol FF

30% (w/v) glycerol

A working solution was prepared by mixing 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol into 50 ml Duran bottle. The solution was filter sterilised using 0.45 µm filter and stored at room temperature. The solution was used for agarose gel electrophoresis of extracted DNA or amplified PRC products.

2.1.5 Ethidium Bromide

A stock solution of 10mg/ml was prepared in 5 ml Duran bottle by dissolving the powder in distilled water and the solution protected from light by wrapping the bottle with a masking tape and stored at 4°C. A final concentration of 0.1 µl was used for visualising DNA and PCR products in electrophoresis gel.

3.1 ENZYMES

3.1.1 Protein K

Proteinase K stock solution (20 g/ml) for DNA extraction was prepared by adding 260 µl of Protein K Storage Buffer to the tube containing Proteinase K powder. The stock solution was stored at -20°C for future use.

3.1.2 PCR Master Mix (2X DreamTaq Green)

Thermo Scientific (Catalog #K1081 200 rxns) 2X DreamTaq Green Master Mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4mM $MgCl_2$ and loading buffer) was used for PCR amplification of target genes. This enzyme was produced by Fermentas, USA and supplied by Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside, Pretoria, South Africa. The Master Mix was stored at -20°C when not in use.

3.2 DNA LADDER OR DNA MARKER

The standard DNA markers, O'GeneRuler 1 Kilo base pairs and GeneRuler 100 base pairs ranging from 250-10000 bp to 100-1000 bp fragments were supplied by Thermo Scientific Company and used to determine the relative sizes of all amplicons after agarose gel electrophoresis.