Prevalence and virulence gene profiling of shigatoxigenic *Escherichia coli* 0157:H7 strains isolated from cattle

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DECLARATION

I, Gosiame Pearl Koolebogile declare that this dissertation submitted to the North-West University, Mafikeng Campus, for the degree Masters in Biology and the work contained therein is my own work in design and execution and has not previously been submitted to another university for a degree, and that all the materials contained therein have been duly acknowledged.

Signed at Mmabatho on this 19th Day of May 2020

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DEDICATION

This work is dedicated to my family who kept on supporting and believing in me especially my late mom, my friends who kept on motivating and inspiring me to have passion for my research and my Laboratory family of the Antimicrobial Resistance and Phage Bio-control Research Group who kept on assisting me during the study.

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ABSTRACT

Escherichia coli and isolates belonging to serotypes O157 are Gram negative, rod shaped bacteria that have received a lot of attention recently due to their potential to cause life threatening sporadic incidents and outbreaks of infections in humans worldwide. The aim of this study was to determine the prevalence and virulence of gene profiles of Shiga-toxigenic *Escherichia coli* O157:H7 strains isolated from cattle. A total of 500 faecal samples were collected from cattle in both communal and commercial farms in the North West province, South Africa. Isolation of *E. coli* was done by sub-culturing a loop full of the bacterial colony onto Sorbitol Mac-Conkey agar (Bio lab, South Africa) supplemented with cefixime and potassium tellurite (SMAC-CT). Identification of *E. coli* isolates were performed using preliminary (serological assay) and confirmatory (PCR assay) tests. Antibiotic susceptibility test was performed using the Kirby-Bauer disc diffusion method to determine the resistance profile of the isolates against a panel of seven different antimicrobial agents (Ciprofloxacin, Gemifloxacin, Levofloxacin, Moxifloxacin, Tetracycline, Penicillin and Norfloxacin).

From 326 isolates, 119 were positive for serological assay and 62 isolates were found positive *uidA* gene fragment. Of those 62 isolates, 5 (83.3%) isolates were from Rooigront farm, 3 (37.5%) from Molelwane farm, 16 (80%) from Lokaleng farm, 16 (100%) from Rooigrond prison farm, 6 (66.6%) from Klippan, 15 (37.5%) from Ottosdal farm and 1 (5%) was from Zeerust. Fifty isolates were found positive for rfb_{O157} gene fragment. Of these 50 isolates, 3 (6%) isolates from farm, 3 (37.5%) from Molelwane farm, 16 (80%) from Lokaleng farm, 8 (50%) from Rooigrond prison farm, 5 (55.5%) from Klippan, 15 (37.5%) from Ottosdal farm and 0 (0%) from Zeerust. Fifty-five (55) of confirmed *E. coli* O157:H7 isolates were positive for shiga-toxins genes of which 30 (60%) isolates carried stx_1 virulene gene while 25 (50%) carried stx_2 gene. The persistence- of other virulence genes. *Iha*, *toxB*,

katP, *espP*, *tir* and *terD* genes were confirmed in 7 (14%), 9 (18%), 5 (10%), 13 (26%), 11 (22%) and 50 (100%) isolates respectively. In the present study most of the isolates were found to be resistant to tested antimicrobial agents. Most isolates were resistant towards Ciprofloxacin (40%), Amplicillin (100%) and Moxifloxacin (28%), while all the isolates were found susceptible to Levofloxacin, Tetracycline, Gemifloxacin and Norfloxacin. Multiple antibiotic resistant (MAR) phenotypes AP-C-M were dominant in all the tested isolates. Therefore, hypothetically it can be concluded that the bacterial isolates did have the STEC-virulence strains within their host which were then able to be expressed in the presences of antimicrobial agents especially those that are frequently utilized in veterinary and human medicine.

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DEFINITION OF CONCEPTS

Antibiotics: agents that inhibit the growth of or destroy microorganisms.

Bacteriophages: viruses which have the ability to kill bacteria and parasitize them while they do not affect cell lines from other organisms.

***Escherichia coli*:** bacteria found in the environment and food products and reside as normal flora in humans and warm-blooded animals.

***E. coli* O157 STEC strains:** *E. coli* strains that belong to the serotype O157.

Fluoroquinolones: a group of antibiotics that inhibit the activity of both the DNA gyrase and the topoisomerase IV enzymes.

Haemorrhagic colitis (HC): an acute disease caused by *E. coli* O157:H7 that is characterized by severe cramping (abdominal pain) and diarrhoea which is initially watery but becomes grossly bloody.

Haemolytic Uremic Syndrome (HUS): a disease characterized by haemolyticanaemia (anaemia caused by destruction of red blood cells), acute kidney failure (uremic), and a low platelet count (thrombocytopenia).

Penicillin: are a group of broad-spectrum antibiotic drugs, synthetic or semisynthetic, that are derived from penicillin and usually used to treat infections caused by gram-positive bacteria.

Plasmid: circular DNA molecules that can replicate independently from the chromosome and promote lateral transfer among different species of bacteria through the conjugation process.

Shiga-toxins: virulence factors produced by the bacteria *Shigella dysenteriae* and certain strains of *Escherichia coli*.

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.

LIST OF ABBREVIATIONS

E. coli	**:** *Escherichia coli*
EHEC	**:** Enterohaemorrhagic *E. coli*
HC	**:** Haemorrhagic colitis
HUS	**:** Haemolytic Uremic Syndrome
K antigen	**:** Capsular Antigens
MAR	**:** Multiple Antibiotic Resistant
O antigen	**:** Somatic Lipopolysaccharide Antigens
PFGE	**:** Pulsed Field Gel Electrophoresis
RFLP	**:** Restriction Fragment Length Polymorphism
rPCR	**:** Real-time transcriptase-polymerase chain reaction
STEC	**:** Shiga Toxin Producing *E. coli*
Stx	**:** Shiga Toxins

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CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1 Introduction

Escherichia coli species are Gram negative, rod shaped bacteria that live as normal flora in the gastrointestinal tract of humans and warm blooded animals (Serna and Boedeker, 2008). Despite this, some *E. coli* strains, particularly those belonging to the serotypes O26, O45, O111, O103, O145 and O157, have been classified as the “top six” due to their constant association with disease in humans (Brooks *et al*., 2005; Paddock *et al*., 2012). Amongst these, O157 strains have received a lot of attention due to their very low infectious dose which has resulted in their being associated with a variety of sporadic cases and outbreaks of infections in humans (Belongia *et al*., 1991; Willshaw *et al*., 1997; Vogt and Dippold, 2005; Newell *et al.*, 2010; Ateba and Mbewe, 2011; CDC, 2011).

E. coli O157 strains have thus been identified as causative agents of a variety of water and foodborne infections reported in humans worldwide (Brooks *et al*., 2005; Stromberg *et al.*, 2018). Due to their very high pathogenicity, these organisms have been reported to cause life threatening complications in humans even in countries such as USA, France, Canada, Japan, Scotland, Wales and the United Kingdom with more advanced public health and health care facilities (Belongia *et al*., 1991; Willshaw *et al*., 1997; Vogt and Dippold, 2005). Therefore,due to the reported cases of the pathogen infections ,the pathogenhave resulted in being classified as a potential threat to humans (CDC, 2011; Scallan *et al*., 2011).

Diseases caused by *E. coli* O157 strains range from non-bloody to severely bloody diarrhoea, haemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS) and thrombotic

thrombocytopenic purpura (TTP) (Tozzi *et al.*, 2003; Bidet *et al*.,2005). The pathogenesis of strains belonging to the “big six” *E. coli* groups and particularly serotype O157 is associated with the production of potent shiga-toxins that have been reported to produce severe cytopathic effects on gut epithelial cells (Karmali, 1985; Obrig *et al*., 1993). Shiga-toxins are thus known to be the major virulence factors in these *E. coli* serogoups and two main types designated stx_1 and stx_2 have been identified and are now fully characterised (Piérard *et al*., 1997). In addition to these principal pathogenic factors, *E. coli* O157 may also express some putative or accessory virulence gene determinants such as the plasmid encoded intimin gene (*eaeA*) and the enterohaemolysin (*hlyA*) (Pierard *et al*., 1997; Ateba *et al.,* 2008). Despite the fact that the exact contribution of these accessory virulence determinants is sometimes an issue of debate (Ateba and Bezuidenhout, 2008; Stromberg *et al*., 2018), it is important to mention that these genes have been associated with bacterial virulence in *E. coli* O157 isolates that did not possess these *stx* genes (Pierard *et al*., 1997; Ateba *et al.,* 2008).

The natural hosts of *E. coli* O157 are ruminants, and cattle are considered the main reservoir for this pathogen (Cobbold and Desmarchelier, 2000). In addition, the pathogen has also been isolated from a number of animal species such as deer and pigs as well as food products such as ground beef, ready to eat turkey and poultry (O’Flynn *et al*.,2004; Abuladze *et al*., 2008; Ateba *et al.,* 2008; Anany *et al*., 2011; Ateba and Mbewe, 2011). However, a previous study conducted in the North West Province of South Africa revealed that pigs rather than cattle harbor these pathogens (Ateba and Mbewe, 2011). It has therefore been suggested that the occurrence of these pathogens in animals depends on a

number of factors that include prevalence in the area, host-pathogen interactions as well as susceptibility of host amongst others (Ateba and Mbewe, 2011).

Epidemiological investigation using genetic typing techniques such as Enterobacterial Repetitive Intergenic Consensus sequences (ERIC)-PCR, IS-PCR, BOX-PCR revealed great similarities between isolates from animals such as pigs and cattle with their corresponding raw meat products, suggesting some level of cross-contamination at the abattoirs and retail shops, but does not exclude contamination that might have occurred in the farms (Ateba and Mbewe, 2011; 2013; 2014). It has been reported that the presence of pathogenic *E. coli* O157 strains in food products, particularly meat, usually results from cross contamination during the production process, and these pathogens are easily transmitted to consumers and hence there is a need for the implementation of appropriate control strategies. Unfortunately, the implementation of standard operating procedures in both farms and food production facilities may be questioned, based on data obtained from some baseline studies (Ateba and Mbewe, 2011; 2013; 2014).

The treatment of infections caused by pathogenic micro-organisms is usually achieved through the administration of antibiotics. At present, there is no specific treatment for infections caused by *E. coli* O157 strains especially HUS, and use of antibiotics is contraindicated and thus remains highly controversial (Wong *et al*., 2000; Bidet *et al.,* 2005; Tarr *et al*., 2005). Antibiotics have been reported to serve as inducers for bacteriophages in *E. coli* O157:H7 cells and the phages harbor the shiga-toxin genes. Therefore, in the presence of antibiotics, the shiga-toxin genes are expressed, thus increasing the chances for the disease to progress to the more severe clinical forms (Wong *et al*., 2000). On the contrary, it has also been demonstrated that infections such as HUS could be prevented in

infected individuals when antibiotics are administered during the early stages of *E. coli* O157:H7 infections (Slutsker *et al*., 1997). In addition, a previous baseline study that assessed the ability of two antimicrobial agents (tetracycline and ampicillin) in inducing shiga-toxin proteins in broth cultures of some antibiotic resistant *E. coli* O157:H7 isolates revealed that shiga toxins were detected in broth cultures, but this was dependent on the length of time in which cultures were incubated. It is therefore of great importance to constantly determining the antibiotic resistance profiles of shiga-toxigenic *E. coli* O157:H7 isolates against clinically relevant drugs in the South African food chain.

1.2 Problem statement

Escherichia coli O157:H7 is known to cause serious health complications in its hosts particularly humans and sporadic as well as foodborne outbreaks of *E. coli* O157:H7 infections are highly prevalent even in well developed countries that have advanced public health policies and health care facilities (Belongia *et al*.,1991; Vogt and Dippold, 2005). The natural hosts of *E. coli* O157 are ruminants, and cattle are generally considered the main reservoir for this pathogen (Cobbold and Desmarchelier, 2000). In addition, the pathogen has also been isolated from a number of animal species such as deer and pigs (O'Flynn *et al*., 2004; Abuladze *et al*., 2008; Ateba *et al*., 2008; Anany *et al*., 2011; Ateba and Mbewe, 2011). During meat production processes the pathogen may be transmitted from the faeces or hides of animals to meat, if proper hygienic practices are not followed, the pathogen is then isolated from a variety of food products such as ground beef, ready to eat turkey and poultry (O'Flynn *et al*., 2004; Abuladze *et al*., 2008; Ateba *et al*., 2008; Anany *et al.,* 2011; Ateba and Mbewe, 2011). The potential of *E. coli* O157:H7 isolates to cause disease is usually associated with the ability to process virulence genes. Despite the fact that shiga-

toxin genes designated *stx*$_1$ and *stx*$_2$ have been identified and characterized, other accessory virulence genes have been associated with diseases in humans. This study is therefore designed to expand on previous investigations that have documented the virulence gene profiles of STEC isolates from animals in the North West Province.

1.3 Aim and objectives

1.3.1 Aim

The aim of this study is to determine the effect of selected clinically relevant antibiotics on the expression of shiga-toxins and to screen the presence of the selected virulence gene determinants in *E. coli* O157:H7 using both genomic and proteomic techniques.

1.3.2 Objectives

The objectives of the study were to:

- isolate *E. coli* O157:H7 from faeces samples
- confirm the identities of the isolates using specific PCR analysis
- determine the antibiotic resistance profiles of the confirmed *E. coli* O157:H7 isolates against a panel of antibiotics.
- screen isolates for the presence of selected virulence genes determinants (*stx*$_1$, *stx*$_2$, *iha, katP, toxB, tir, terD, espP*) that are associated with STEC.

CHAPTER 2

LITERATURE REVIEW

2.1 General introduction of *Escherichia coli* O157:H7

Escherichia coli is a Gram negative, rod-shaped and non-spore forming enteric bacterial specie that live as normal flora in the gastrointestinal tract of humans and warm-blooded animals (Ateba and Mbewe, 2011). Although *E. coli* is considered to be a normal flora, the pathogenic species of the bacterium have been identified and those belonging to the sero groups O26, O45, O111, O103, O145 and O157 have been classified as the “top six” due to their constant association with disease in humans (Brooks *et al*., 2005; Paddock *et al*., 2012). Amongst them, *E. coli* O157:H7 has received significant attention recently due to its involvement in diseases that range from uncomplicated to complicated diarrhea, to the more severe clinical hemorrhagic colitis (HC), haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura in humans (Nataro and Kaper, 1998; Tarr *et al*., 2005; Ateba and Mbewe., 2011).

E. coli strains are usually motile, aerobic and oxidase negative while most of them are able to reduce nitrate to nitrite as well as ferment glucose (Maki *et al*., 1976). According to Kulasekara and Blomfield (1999*), E. coli* cells are made-up of two layered cell wall and the outer membrane has two types of lipids, namely lipopolysaccharides and phospholipids, as well as a set of proteins. The lipopolysaccharides are composed of three regions which includes the lipids A, an oligosaccharide core and polysaccharide O antigen (Maldonado *et al*., 2016). The antigen O is the important region that functions as the serotype determiner of the organism. Cytoplasmic membrane separates the cytoplasm; the periplasm occupies

the space within the outer and cytoplasmic membrane and has a layer of murein or peptidoglycan (Maldonado *et al*., 2016). *E. coli* cells often have fimbriae on their bacterial cell surface and those fimbriae facilitate attachment to intestinal epithelial cells of the host as well as contributing to their pathogenesis (Ateba and Mbewe, 2011). *E. coli* are usually isolated through culture on selective and differential media such as Mac-Conkey agar and isolates are presumptively identified based on marcroscopic morphological characteristics of pure colonies (Liu and Breukink, 2016). Figure 2.1 shows a magnified electron microscopic image of an *E. coli* cell with visible organelles that facilitate motility.

Based on their pathogenic potentials and differences in virulence factors that facilitate the establishment of disease, diarrhoeagenic *E. coli* strains can be divided into enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohaemorrhagic (EHEC), enteroinvasive (EIEC) and enteroaggregative (EAEC) (Lara *et al*., 2017). In addition, there is also diffusely adherent *E. coli* (DAEC) (Nataro and Kaper., 1998; Donnenberg and Whittam., 2001). *E. coli* has many pathogenic serotypes of which O157 is one of them, and it is categorized as a shiga toxin-producing *E. coli* (STEC). STEC is a strain of *Escherichia coli* O157:H7 that produces one or more cytotoxins, which are referred to as shiga-toxins or shiga-like toxins. Shiga-toxins are either shiga-toxin 1 (stx_1) or shiga-toxin 2 (stx_2) and these virulence factors are produced by the bacteria *Shigella dysenteriae* and certain strains of *E. coli*. There are currently no available treatments for diseases caused by toxin-producing bacteria, due to antibiotics acting as inducers for those toxins or inducers for the bacteriophages in *E. coli* O157:H7 cells (Aersten *et al*., 2005).

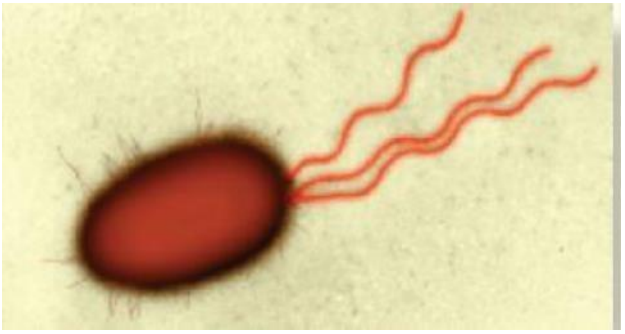

Figure 2.1: A magnified electron microscopic image of *E. coli*

2.2 Shiga-toxin producing *E. coli* (STEC)

Shiga-toxin producing *E. coli* strains, especially those belonging to the serogroup O157 cause diseases in humans through the production of toxins termed shiga-toxins (Gyles, 2007; Karama *et al*., 2019). Shiga-toxins are so called due to the fact that they structurally and biologically resemble toxins produced by *Shigella dysenteriae* (O'Brien *et al*., 1983; Beutin., 2006). Two major groups of these toxins that are designated stx_1 and stx_2 have been identified and fully characterized (Gannon *et al*., 1990). The pathogenicity of *E. coli* strains is further complicated by the fact that other variants of the stx_2 have also been identified and directly linked to disease cases in some strains (Plunkett *et al*., 1999; Barbau-Piednoir *et al*., 2018).

The shiga-toxin genes are generally known to reside in lambdoid prophage genomes (Friedman and Court, 2001) that are harbored by *E. coli* O157 strains. Expression of these genes is largely dependent on exposure of host cells to inducing agents such as UV and antibiotics (Plunkett *et al*., 1999; Wagner and Waldor, 2002). The mechanism of action of shiga toxins involves inhibiting protein synthesis within the target cell and this is very similar to that of ricin toxins which are produced by *Ricinus communis* (Sandvig and van Deurs,

2000). Thus upon infection with a shiga-toxin producing *E. coli*, the cell produces a toxin which functions as an N-glycosidase by cleaving a specific adenine nucleobase from the 28S RNA of the 60S sub-unit of the ribosome, thus stoping the process of protein synthesis (Sandvig *et al*., 2010). This therefore results in cell death and hence pathological conditions in the host.

2.2.1 Shiga-toxin producing *E. coli* and diseases in humans

Shiga-toxin producing *E. coli* O157 strains are enteric bacteria that have been identified as the major cause of most food and water-borne infections in humans worldwide (Belongia *et al*., 1993). Infections caused by *E. coli* O157:H7 often range from non-bloody diarrhea, abdominal cramps, bladder and kidney infections, pneumonia, neonatal meningitis to the more complicated hemolytic uremic syndrome, hemorrhagic colitis and thrombotic thrombocytopenic purpura (Tarr *et al*., 2005). Despite its high pathogenicity, the complexity of urgently detecting *E. coli* O157 infections especially in health care facilities that do not have advanced and highly technological genetic equipment or experts is aggravated by the fact that some infected individuals may not present symptoms immediately. Moreover, some of the symptoms that are associated with this pathogen are also common to other water and foodborne pathogens (Fremaux *et al*., 2008). Given that *E. coli* infections and in particular those caused by strains belonging to the sero groups O157 are very common in developing countries (Sandvig and van Deurs., 2000), due to factors such as poor farm management techniques, poor hygiene, lack of adherence to public health policies during food production, and poor reporting of cases, it is important to constantly assess the occurrence of these pathogens in the food chain. In addition, the generation of epidemiological data may also present detailed information on the major routes of transmission of this pathogen to healthy individuals in the given area.

2.2.2 Host range

Most pathogenic strains of *E. coli* O157:H7 infections are associated with consumption of contaminated ground beef, raw milk and other bovine products, and thus cattle are considered to be the primary reservoir for the bacteria (Ateba and Mbewe, 2011). However, the occurrence of *E. coli* O157:H7 in animals may be influenced by a number of different factors, such as the setting of the farm, the hygiene level that is used in the farm and also the animal feeds. Moreover, recent outbreaks of food borne diseases associated with consumption of fresh products have intensified concerns that those foods contaminated with STEC may be an increasing source of diseases. In the past consumption of leaf lettuce, potatoes, radish sprouts and raw vegetables were considered to being the source of infection. However, fruits are also found to be possible reservoirs of *E. coli* O157, for example, due to the consumption of fresh-pressed apple juice (Parish, 2009; Berger *et al*., 2010; CDC, 2012).

2.2.3 Epidemiology of *E. coli* O157:H7 infections

The first recognized outbreaks of illness caused by *Escherichia coli* O157:H7 happened in 1982. Undercooked hamburger meat was acknowledged as the source of transmission of the bacterial infection (Riley *et al*., 1983). Moreover, bacterial infection outbreaks appear to be increasing world-wide and these infections are reported in countries such as the USA, France, Canada, Japan, Scotland, Wales and UK that have more advanced public health and health care facilities (Vogt and Dippold, 2005).

The mortality and morbidity associated with several large outbreaks of gastro-intestinal diseases caused by *E. coli* O157:H7 strains have highlighted the threat these organisms pose to human health (Doorduyn *et al*., 2006). A large proportion of outbreaks of *E. coli* O157:H7 infections have been reported to be caused by the consumption of contaminated food (Botteldoorn *et al.,* 2003). *E. coli* attachment and effacement gene, *eae* and toxin producing genes have been studied for detection of EHEC. Studies have been conducted to determine the spread of virulent genes in *E. coli* from water samples (Bopp *et al*., 2003; Ateba and Mbewe, 2011) as well as food samples, and humans, animals and domestic animals from rural farms (Ateba and Mbewe, 2011; Amezquita-Lopez *et al*., 2014).

In South Africa, outbreaks of infections caused by *E. coli* O157:H7 have not been reported to date. However, *E. coli* O157:H7 have been isolated and studied from animals and humans (Ateba *et al*., 2008; Ateba *et al.,* 2011) and that increases the possibility of contaminated surface or groundwater and food products through improper deposition of faeces. Given the fact that the specific monitoring of pathogens such as *E. coli* O157:H7 in water and food products may suggest possible health implications for consumers (Schets *et al*., 2005) especially in developing countries, the importance of this study cannot be underestimated.

2.3 Virulence properties of *E. coli*

2.3.1 Enterotoxigenic *E. coli* (ETEC)

ETEC strains are non-invasive, but do cause diarrhoea, which usually occurs without fever, and does not leave the intestinal lumen. According to Northey *et al.*, (2007),

Enterotoxigenic *E. coli* is the main cause of bacterial diarrhea in children in developing countries. Each year it is observed that ETEC causes more than 200 million cases of diarrhea and 380000 deaths (Wenneras and Erling, 2004).

2.3.2 Enteroinvasive *E. coli* (EIEC)

EIEC are strains which are found to be intestinal pathogens and are found only in humans. Infections cause syndromes similar to shigellosis which causes epithelial invasion of the bowel that can lead to inflammation and ulceration of the mucosa, profuse diarrhea and high fever (Rendon *et al*., 2007). Enteroinvasive *E. coli* is a strain that is highly invasive and it uses adhering protein so as to bind and to enter the intestinal cells. However, most of the EIEC strains express somatic antigens which are closely related to the *Shigella* antigens. In addition, the EIEC infection cases are associated with travelers, and outbreaks of infection are due to consumption of contaminated water, food products and direct contact.

2.3.3 Enterohemorrhagic *E. coli* (EHEC)

EHEC is a strain that is found in humans, domestic and wild animals (FAO/WHO, 2008). Enterohemorrhagic *E. coli* uses bacterial fimbriae for attachment (Rendon *et al*., 2001), also it is moderately invasive, and possesses a phage –encoded shiga-toxin that can elicit an intense inflammatory response. *Escherichia coli* O157:H7 is the most famous serotype of this strain, which causes bloody diarrhea that is not characterized by fever. According to Caprioli *et al*., (1997) and Gerber *et al.,* (2002), EHEC causes hemolytic uremic syndrome (HUS) known to be the major cause of kidney failure.

2.3.4 Enteroaggregative *E. coli* (EAEC)

Enteroaggregative *E. coli* is a quite diverse group of enteric bacteria that is associated with acute or persistent diarrhoea in children and adults worldwide. Over the past decade EAEC has been reported as a cause of watery diarrhoea which was considered to be persistent. EAEC are *E. coli* strains which are diarrheal microorganisms and they are a biofilm formation on the intestinal mucosa with an adherence phenotype (stacked brick), that is related to the presence of a 60 MDa plasmid (Nataro and Kaper, 1998).

2.4 Virulence factors

There are major virulence factors which enhance the ability of *E. coli* O157:H7 to cause severe diseases in warm blooded animals and humans. These include the shiga-toxins (stx1 and stx2) or verocytotoxins (VT1 and VT2) and other variants of those toxins secreted by the *E. coli* (Leotta *et al*., 2008). *Escherichia coli* shiga-toxins (STEC) resemble the toxins produced by *Shigella dysenteriae* (O'Brien *et al*., 1983; Beutin, 2006). The stx_1 and stx_2 have been identified and characterized and they are taken to be part of the lambdoid prophages genome (Friedman and Court, 2001). Shiga-toxins prevent protein synthesis inside the target cells using a mechanism similar to that of ricin toxin which is produced by *Ricinus communis* (Sandvig and van Deurs, 2000). The protein functions as an N-glycosidase after the toxin have entered the cell and it cleaves a specific adenine nucleobase from the 28S RNA of the 60S sub-unit, thus halting the process of protein synthesis (Sandvig *et al*., 2010).

Another STEC virulence factor associated with severe diseases is the locus of enterocyte effacement (LEE) that is a 34 chromosomal pathogenicity island with an intimin, a 94 kDa

outer membrane protein that is encoded by the *eae* gene. There are bacterial mechanisms which take place, such as the intimate adherence of *E. coli* to epithelial cells, and the initiation of host signal transduction pathways and the formation of attaching and effacing intestinal lesions (Leotta *et al*., 2008). However, there is another factor that affects the virulence of *E. coli* O157:H7 termed enterohemolysis (*ehly*), referred to as the enterohemorrhagic *E. coli* haemolytic (EHEC-HlyA), which is encoded by *hlyA* gene (Leotta *et al*., 2008), whereby a *hlyA* gene will act as a potent and a cytolysis during bacterial infection.

2.5 Transmission

The primary routes of transmission of the *E. coli* are associated with ruminants which are the main reservoir of the shiga toxin-producing *E. coli.* However, transmission can also be through faecal–oral route or be spread between animals by direct contact or via water channels, shared feed, contaminated pastures or other environmental sources. The person to person route has been reported to contribute about 29% of *E. coli* infection outbreaks (Kaspar and Doyle, 2009). However, the faecal contamination route is the most common way for transmission of this pathogen to human beings, which occurs during the slaughter of animals (Kudva *et al*., 1999; Chase-Topping *et al*., 2008). In addition, it can be transmitted to humans through contaminated food and water by animal faeces or manure.

2.6 Treatment

The administration of antibiotics to patients suffering from *E. coli* O157:H7 infections are currently a controversial issue (Wong, 2000). Some studies indicate that antimicrobial agents do mend *E. coli* O157:H7 illnesses, but they do increase the risk of developing

diseases such as HUS. On the contrary, there are studies whose findings indicated that the administration of antibiotics (Aersten *et al*., 2005) and anti-diarriahoael agents like Imodium might result in the aggravation of diarriahoael diseases to more severe forms known as HC, HUS (Bidet *et al*.,2005), and TTP that may end up as renal failure in patients. However, it has been reported that antibiotics serve as inducers for bacteriophages in *E. coli* O157:H7 cells and those phages harbors the shiga-toxin genes (Aersten *et al*., 2005). Therefore, it is suggested that supportive therapy that includes hydration must be utilized to overcome infection in patients (Tarr and Philip, 1995).

2.7 General information of clinical antibiotics

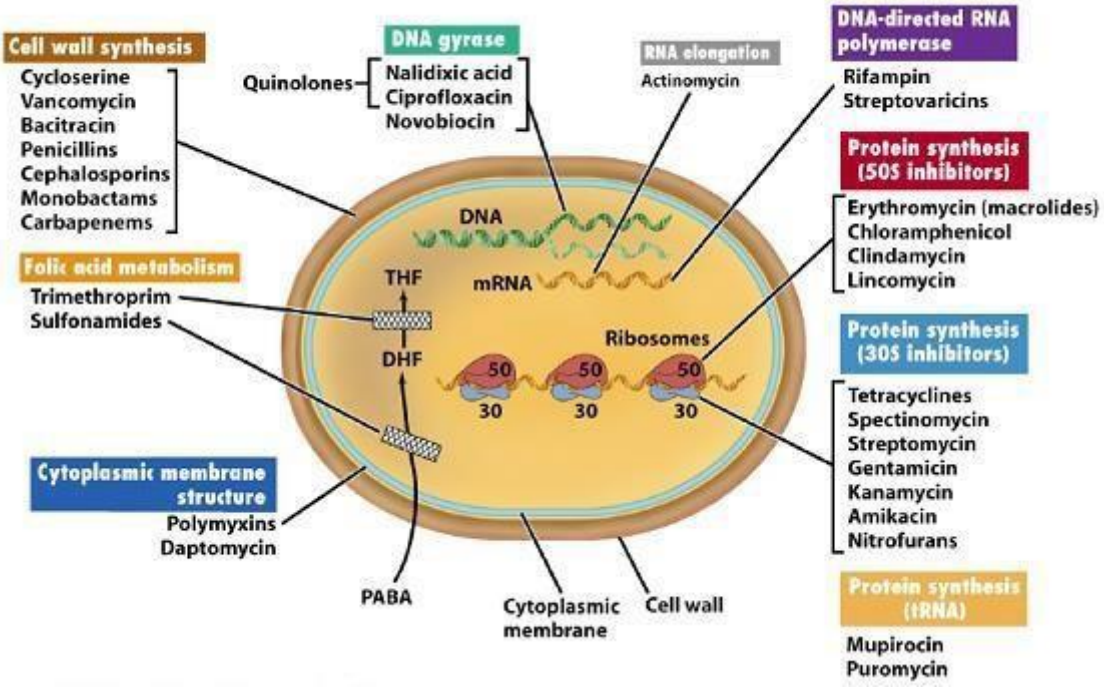

Figure 2.2: Antibiotics and their mode of action in a bacterial cell (Bbosa *et al*., 2014).

Antibiotics are antimicrobial agents which may kill or inhibit the growth of bacteria during treatment and prevention of bacterial infections. It was observed by a physician Sir William Roberts in 1874 that cultured molds of *Penicillium glaucum* used for production of blue

cheese did not show any bacterial contamination, again in 1876 a physician John Tyndall was involved (Collard and Collard., 1976). Furthermore, Louis Pasteur observed that *Bacillus anthracis* does not grow in the presence of *Penicillium notatum*. In 1897 Ernest Duchesne observed that *E. coli* was inhibited by the fungus *Penicillium glaucum* when they were both grown in the same culture, and also observed that when he inoculated laboratory animals with lethal doses of typhoid bacilli together with *Penicillium glaucum*, the animals were not infected with typhoid (Tan and Tatsumura, 2015).Antibiotics which are in use even now were first discovered before the mid-1980's in the ''Golden Age'' of antibiotics (Fernandes, 2006). According to John and Sons (2012) antibiotics cannot work against other microorganisms such as viruses, instead they may cause occurrence of resistant organisms. However, drugs which are used to inhibit viruses are called antivirals. Medical folklore was practiced before the early 20th century when it comes to treatment of bacterial infections, where ancient Egyptians and ancient Greeks used selected molds, plant materials and extracts as treatment for bacterial infections.

Recent studies have discovered natural antibacterial products made from microorganisms that might give great hope for therapeutics, as observed by Louis Pasteur when he intervened in the dislike observation of some bacteria (Kingston, 2008). In addition, the effectiveness and easy access of antibiotics has led to overuse, especially in the agricultural and medicinal industry (Lam *et al.,* 2013; Liu and Wong, 2013; Liu *et al*., 2017) which leads to the occurrence of new bacterial strains which are resistant to antibiotics. This is classified as a serious health threat by the World Health Organization (WHO).

The overuse of antibiotics in humans and animals has been reported worldwide, as the main reason for the occurrence of antibiotic resistant bacteria (Ateba and Bezuidenhout, 2008; Wose King *et al*., 2010; Phokela *et al*., 2011). In addition, about 70 antibiotics have been identified in various animal food products, including chicken, pork, beef and fish products (He *et al*., 2012; Chen *et al*., 2015; Yamaguchi *et al*., 2015; Liu *et al*., 2017). However, humans can suffer from antibiotic exposure by dietary intake of these contaminated food products. Furthermore, antibiotics enter into human systems through drug abuse (Li *et al*., 2012), drinking water (Leung *et al*., 2013; Wong *et al*., 2016b) and through inhalation of contaminated dust (Hamscher *et al.,* 2003). Antibiotic resistant *E. coli* may transfer resistant genes to other bacterial species through a horizontal process as *E. coli* strains have multidrug resistant plasmids which are ready to be transferred to other susceptible species when subjected to stress (Salyers *et al*., 2004; Shakibaie *et al*., 2009).

However, the resistance of different strains of *E. coli* to antibiotics varies (Ateba and Bezuidenhout, 2008; Wose King *et al*., 2010; Phokela *et al*., 2011). Studies have proposed that antibiotics may serve as inducers for bacteriophages that harbour the shiga-toxins genes and that may increase the disease to being severe or complicated (Craig *et al*., 2000; Zhang *et al*., 2000; Dundas *et al*., 2001). According to Slutsker *et al.,* (1997), it has also been reported that administration of antibiotics during the early stages of *E. coli* O157:H7 infections may prevent the development of haemolytic uremic syndrome (HUS). However, WHO (2014) argues that, there will be about one million deaths annually due to antibiotic resistant bacteria by 2050. Therefore, the issue of antibiotics becomes a major risk towards human health and it is important to evaluate the ability of other antimicrobial agents in inducing shiga-toxins from *E. coli* cells.

2.8 Chemical composition of clinical antibiotics

2.8.1 Ciprofloxacin

Ciprofloxacin is an antibacterial agent, one of the fluoroquinolone class. The antibiotic Ciprofloxacin has a colour that ranges between faintly yellowish to light yellow crystalline. Its chemical components are made up of 1-cyclopropyl-6fluoro-1,4-dihydro-oxo-7-(1piperazinyl)-3-quinolinecarboxylic acid. It has a molecular weight of 331.4 g/mol with empirical formula of $C_{17}H_{18}FN_3O_3$ (Masaad *et al*., 2016). It is used to treat both Gramnegative and Gram-positive bacterial infections, such as bone and joint infections, infectious diarrhoea, respiratory tract infections, skin and urinary infections as well as typhoid fever and intra-abdominal infections. It can be taken either orally or intravenously. Ciprofloxacin separates bacterial DNA by inhibiting the DNA gyrase also a type II topoisomerase and topoisomerase IV, so as to stop the cell division (Drlica and Zhao, 1997; Pommier *et al.,* 2010).

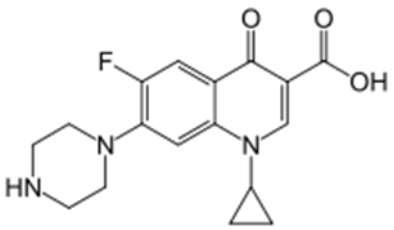

Figure 2.3: Structural composition of Ciprofloxacin

2.8.2 Gemifloxacin

Gemifloxacin is an antibacterial agent that falls under the fluoroquinolone group and it is an oral broad-spectrum. The antibiotic is active for both Gram-negative and Gram-positive bacteria. It has chemical phenotype of off-white and amorphous solid which is from the

chloroform-ethanol (O'Neil, 2006). It is used to treat acute bacterial exacerbation of chronic bronchitis and mild to moderate pneumonia. It treats the bacterial infections by stopping bacterial growth, through the process of inhibiting or binding to the DNA gyrase and topoisomerase IV, since the antibiotic has about 100 times forces which makes it bind more easily to the bacterial DNA than mammalian DNA.

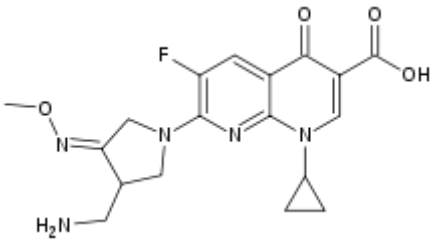

Figure 2.4: Structural composition of Gemifloxacin

2.8.3 Levofloxacin

Levofloxacin, an antibacterial agent that falls under the quinolones group (Affe *et al*., 2011), is used for treating bacterial infections such as acute bacterial sinusitis, pneumonia, urinary tract infections and chronic prostatitis which are caused by gram negative and Gram-positive bacteria. It can either be taken orally or intravenously and it is also available in eye drop form (American Society of Health-System Pharmacists., 2016). This antibiotic treats the bacterial infections by inhibiting the DNA gyrase and topoisomerase IV (Drlica and Zhao., 1997) and this abrupt's the bacterial replication and ultimately kills the bacterium (Mutschler *et al*., 2001).

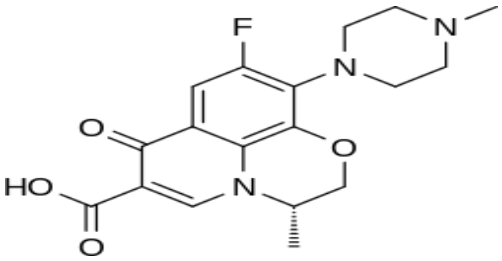

Figure 2.5: Structural composition of Levofloxacin

2.8.4 Moxifloxacin

Moxifloxacin is an antibacterial agent that falls under the fluoroquinolone class and has a better mode of action compared to levofloxacin and ciprofloxacin against the gram positive and Gram-negative bacteria. It is referred to as respiratory quinolone due to its strong mode of action against common respiratory pathogens such as *Streptococcus pneumoniae*. The moxifloxacin has a chemical phenotype of slightly yellow to yellow crystalline substance (Olaitan *et al*., 2015). The antibiotic moxifloxacin treats bacterial infections by inhibiting the bacterial DNA gyrase, type II topoisomerase and topoisomerase IV, which are enzymes that are necessary for splitting the bacterial DNA, and that stops the bacterial replication (Drlica and Zhao, 1997).

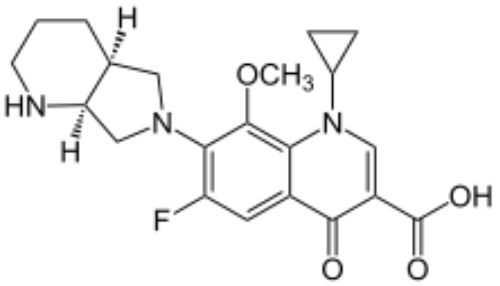

Figure 2.6: Structural composition of Moxifloxacin

2.8.5 Norfloxacin

Norfloxacin is an antibiotic that falls under the quinolone class. It is used to treat both Gram-negative and Gram-positive bacterial infections such as urinary tract infections, gynaecological infections, prostate gland inflammation, gonorrhoea and bladder infections (Rafalsky *et al*., 2006; Merck and Dohme, 2008). Unlike other antibiotics norfloxacin does

not bind to the bacterial enzyme DNA gyrase while it does bind to the substrate DNA. It inhibits the DNA gyrase, type II topoisomerase and the topoisomerase

IV so as to stop cell division.

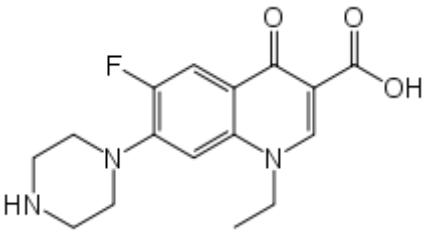

Figure 2.7: Structural composition of Norfloxacin

2.8.6 Mitomycin

Mitomycin is an antibiotic agent that falls under the aziridine family. It is an antibacterial agent which has antitumor activity (Crooke and Bradner., 1976) and it has been used for treating bacterial infections for about decades. It is one of the bi or tri functional alkylating agents which cause the cross-linking of DNA thereby inhibiting DNA replication. Mitomycin is an antineoplastic antibiotic that is obtained from *Streptomyces caespitosus* (Rahbar *et al*., 2000). This antibiotic inhibits the DNA replication of the bacteria and that causes the bacteria to be lysed (killed) and that is when the bacterial infection is treated. If the antibiotic is used at higher concentrations it does inhibit the RNA and protein synthesis of the bacterial cell.

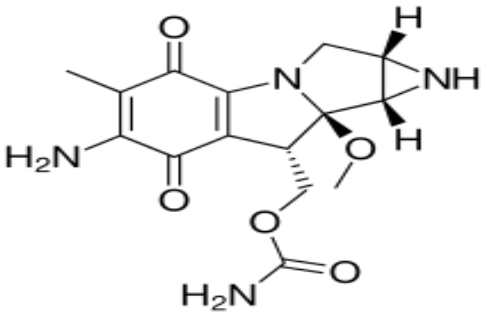

Figure 2.8: Structural composition of Mitomycin

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample size

This study was conducted in the Antibiotic Resistance and Phage Bio-control Research Laboratory, NWU in North West Province, South Africa. Faecal samples were collected from cattle in some randomly selected communal and commercial farms. The number of samples that were collected was determined using the formula outlined below:

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

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

= Expected prevalence in population based on a previous study d = Absolute error or precision (which is 5%)

$$\text{Sample (N)} = \frac{(1.96)^2 (0.259)\ (1 - 0.259)}{(0.05)^2}$$

$$= 101.6$$

For estimation of the prevalence of *E. coli* O157:H7 strains, the sample size for this study was determined by using the prevalence of 25.9 % obtained in the study by Ateba *et al*., 2008), with a 95% confidence level and desired precision of 5%, using a standard formula (Charan and Biswas, 2013). Accordingly, the minimum sample size required was 102 faecal samples.

3.2 Ethical clearance

Ethical clearance for the study was obtained from the Faculty of Natural and Agricultural Sciences Ethics Committee, North West University, and an ethics approval number (NWU-01224-19-S9) was assigned to this study.

3.3 Sample collection

Five hundred cattle faeces samples were collected from communal and commercial farms around North West Province, South Africa. Faecal samples were collected directly from the rectum using sterile arm-length gloves by trained Animal Health Technicians. They were placed in 50 mL green cap sterile bottles, and then properly labelled based on the area and the date of collection. Thereafter, the samples were transported on ice to the laboratory for microbial analysis and were processed immediately upon arrival at the laboratory. Table 3.1 indicates the number of samples collected per sampling station.

Table 3.1: Areas where samples of cattle faeces were collected

Sampling station	**Number of samples**
Lokaleng farm	42
Rooigrond prison farm	65
Ottosdal farm	140
Zeerust farm	95
Rooigrond farm	55
Molelwane farm	58
Klippan	45
Total	**500**

3.4 Isolation of *E. coli*

Approximately two grams (2 g) of each sample was dissolved in 5 mL of 2% (w/v) buffered peptone water (Biolab, South Africa). Ten-fold serial dilutions of pre-enriched samples were prepared using 2% (w/v) buffered peptone water as the diluent. Aliquots of 100µL from appropriate dilution were spread-plated onto Sorbitol MacConkey agar supplemented with cefixime and potassium tellurite (SMAC-CT) (Meichtri *et al*., 2004). The plates were incubated aerobically at 37°C for 24 hours. One non-pink colony from each SMAC-CT plate was selected and sub-cultured on Sorbitol MacConkey agar supplemented with cefixime and potassium tellurite (SMAC-CT) and the plates were incubated at 37°C for 24 hours. Pure isolates were retained and subjected to further identification tests for *E. coli* O157:H7.

3.5 Preliminary identification of *E. coli* O157:H7 isolates using a serological assay

The identities of the isolates were determined from 500 samples, using O157 and H7 latex agglutination monovalent antisera by following the manufacturer's instructions (Remel Europe Ltd., Kent, UK).

3.6 Extraction of bacterial genomic DNA

Bacterial DNA was extracted from presumptive *E. coli* O157:H7 isolates using the ZR Fungal /Bacterial DNA MiniPrep^{TM} kit (Lot No: ZRC178482) obtained from the Epigenetics Company, USA, according to the manufacture's instruction.

3.7 Determination of DNA concentration

The concentration and purity of DNA extracted was determined using the Nanodrop Lite spectrophotometer obtained from Thermo Fisher Scientific, USA.

3.8 Bacterial 16S rRNA gene PCR analysis

Bacterial 16S rRNA gene fragments were amplified for each isolate using universal primer sequences (Table 3.2). Standard 25 µl PCR reactions that constituted of 1µl of the template DNA, 0.25 µl of each oligonucleotide primer set, 12.5 µl of 1 X PCR master mix and 11 µl of RNase free distilled water were performed. All the PCR reagents used were Fermentas, USA products and supplied by Inqaba Biotec Ltd, South Africa. PCR cycling conditions comprised an initial denaturation at 94 ˚C for 3 minutes, 25 cycles of 94 ˚C for 1 minute, 55 ˚C for 1 minute, 72˚C for 2 minutes and a final elongation at 72˚C for 10 minutes. Representative *E. coli* O157:H7 isolates were sent for sequence analysis of bacterial 16S rRNA gene fragments, whose partial sequence have been previously deposited in Gene bank. All PCR products were stored at 4˚C until electrophoresis.

3.9 PCR for identification of *E. coli* isolates

The identities of *E. coli* isolates were confirmed using specific PCR analysis designed to amplify *uidA* housekeeping gene sequence in chromosomal DNA using primer sequences that appear in Table 3.2 Standard 25 µl PCR reactions that constituted of 1µl of the template DNA, 0.25 µl of each oligonucleotide primer set, 12.5 µl of 1 X PCR master mix and 11 µl of RNase free distilled water were performed. All the PCR reagents used were Fermentas, USA products and supplied by Inqaba Biotec Ltd, South Africa. PCR cycling conditions comprised an initial denaturation at 94 ˚C for 3 minutes, 25 cycles of 94 ˚C for 1

minute, 59 ˚C for 1 minute, 72˚C for 2 minutes and a final elongation at 72˚C for 10 minutes. All PCR products were stored at 4˚C until electrophoresis.

3.10 PCR for identification of *E. coli* O157:H7 isolates

All the isolates confirmed as *E. coli* strains through genus specific PCR analysis were subjected to *E. coli* O157:H7 specific PCR analysis targeting rfb_{O157} and $fliC_{H7}$ gene fragments using oligonucleotide primer sequences that appear in Table 3.2 Standard 25 µL PCR reactions that constituted of 1µl of the template DNA, 0.25 µL of each oligonucleotide primer set, 12.5 µL of 1 X PCR master mix and 11 µL of RNase free distilled water were performed. All the PCR reagents used were Fermentas, USA products and supplied by Inqaba Biotec Ltd, South Africa. PCR cycling conditions comprised an initial denaturation at 94 ˚C for 3 minutes, 25 cycles of 94 ˚C for 1 minute, 55˚C for 1 minute, 72˚C for 2 minutes and a final elongation at 72˚C for 10 minutes. All PCR products were stored at 4˚C until electrophoresis.

Table 3.2: Oligonucleotide primers used for molecular identification of bacteria isolates during the study.

Primer Name	Oligonucleotide primer sequence (5'-3')	Target gene	Amplicon size (bp)	Reference
27 F	AGAGTTTGATCATGGCTCAG	*16S rRNA*	1420	Korzeniewska & Harnisz, 2013
1492 R	GGTACCTTGTTACGACTT			
uidA F	CTGGTATCAGCGCGAAGTCT	*uidA*	600	Anbazhagan *et al*., 2011
uidA R	AGCGGGTAGATATCACACTC			
Rfb F	CGGACATCCATGTGATATGG	*rfb*$_{O157}$	259	Morin *et al*., 2004
Rfb R	TTGCCTATGTACAGCTAATCC			
H7 F	GCGCTGTCGAGTTCTATCGAG	*fliC*$_{H7}$	625	
H7 R	CAACGGTGACTTTATCGCCATTCC			

3.11 Detection of virulence genes in *E. coli* O157:H7 isolates

Confirmed *E. coli* O157:H7 isolates were subjected to specific PCR analysis for detection of virulence genes. The virulence genes *stx*$_1$, *stx*$_2$, *espP, terD, Iha, toxB, tir and katP* were screened using oligonucleotide primer sequences that appear in Table 3.3. Standard 25 µL PCR reactions that constituted of 1µL of the template DNA, 0.25 µL of each oligonucleotide primer set, 12.5 µL of 1 X PCR master mix and 11 µL of RNase free distilled water were performed. All the PCR reagents used were fermentas, USA products and supplied by Inqaba Biotec Ltd, South Africa. PCR cycling conditions for the different genes are also shown in Table 3.3. All PCR products were stored at 4˚C until electrophoresis.

Table 3.3: Oligonucleotide primers sequences that were used for detection of in the different virulence genes in *E. coli* O157:H7 isolates.

Primer name	Oligonucleotide primer sequence (5'-3')	Target gene	Product size (bp)	Reference	PCR conditions
Stx1F	ATAAATCGCCATTCGTTGACTAC	*stx1*	180	Paton and Paton (1998)	95 ˚C for 5 minutes,35 cycles at 95 ˚C for 30 seconds,62˚C,72˚C for 60 seconds,72˚C for 5 minutes
Stx1R	AGAACGCCCACTGAGATCATC				
Stx2F	GGCACTGTCTGAAACTGCTCC	*stx*$_2$	255	Paton and Paton (1998)	
Stx2R	TCGCCAGTTATCTGACATTCTG				
espP F	TGTTCGTCTATGATAACCA	*espP*	638	Rump *et al*., 2014	3 minutes at 90˚C,30 cycles at 95˚C for 15 seconds,60 ˚C for 1 minute, 72˚C for 1.5 minute, 72 ˚C for 1 minute.
espP R	AGTTCCGCCATTTTTCGC				
terD F	AGTAAAGCAGCTCCGTCAAT	*terD*	434	Rump *et al*., 2014	
terD R	CCGAACAGCATGGCAGTCT				
Iha F	CAGTTCAGTTTCGCATTCACC	*Iha*	1305	Rump *et al*., 2014	
Iha R	GTATGGCTCTGATGCGATG				
ToxB F	ATACCTACCTGCTCTGGATTGA	*toxB*	602	Rump *et al*., 2014	3 minutes at 90˚C,30 cycles at 95˚C for 15 seconds,60 ˚C for 1 minute, 72˚C for 1.5 minute, 72 ˚C for 1 minute.
toxB R	TTCTTACCTGATCTGATGCAGC				
Tir F	TCTGTTCAGAATATGGGGAATA	*tir*	407	Rump *et al*., 2014	
Tir R	TAAAAGTTCAGATCTTGATGACAT				
Kat F	CTTCCTGTTCTGATTCTTCTGG	*katP*	2126	Rump *et al*., 2014	
Kat R	AACTTATTTCTCGCATCATCC				

3.12 Electrophoresis of PCR products

The PCR products were resolved by electrophoresis in 2% (w/v) agarose gel. A horizontal Pharma biotec equipment system was used to carry out electrophoresis and gels were stained with ethidium bromide (0.1 µg/mL). The gels were run for 1 hour 20 minutes at 70 V using 1X TAE buffer (40mMTris, 1mM EDTA and 20mM glacial acetic acid, pH 8.0). Each gel contained a DNA molecular weight marker (Fermentas, USA) of 100 bp or 1 kb based on the targeted gene size. The amplicons were visualized under UV light (Sambrook *et al.,* 1989), in a gel doc machine and the image was captured in Gene snap software (version 6.00.22). The images were analysed in order to determine the targeted gene sizes of the amplicons.

3.13 Antibiotic susceptibility tests using Kirby Bauer disc diffusion assay

Antibiotic susceptibility test was performed on *E. coli* O157:H7 isolates to determine their antibiotic resistance profiles using the Kirby-Bauer disk diffusion method (Bauer *et al.*, 1966). Bacterial suspensions of pure isolates were grown in nutrient broth for 24 hours and incubated at 37°C. Aliquots of 100 µl were spread-plated on Muller-Hinton agar (Biolab, Merck, South Africa). The antibiotic discs were gently pressed onto the inoculated Muller-Hinton agar to ensure intimate contact with surface and the plates were incubated aerobically for 24 hours at 37°C (CLSI, 1999; Abo-Amer *et al*., 2018). The antibiotic used were Ciprofloxacin (5µg), Gemifloxacin (5µg), Levofloxacin (5µg), Moxifloxacin (5µg), Tetracycline (30µg), Penicillin (10µg) and Norfloxacin (10µl). These are the antibiotics which are frequently utilized in both veterinary and human medicine in the area (Ateba and Bezuidenhout, 2008; Moneoang and Bezuidenhout, 2009). The antibiotic

inhibition zone diameters were measured and the results obtained were used to classify organisms as being resistant, intermediate and susceptible to a particular antibiotic based on standard reference values (NCCLS, 2003).

CHAPTER FOUR

RESULTS

4.1 Isolation and confirmation of *E. coli* strains from cattle faeces samples

A total of 326 presumptive *E. coli* isolates were obtained from 500 faecal samples using selective media. All the 326 isolates were subjected to preliminary (serological) and confirmatory molecular (bacterial 16S rRNA, *E. coli*-specific *uidA* housekeeping gene amplification, rfb_{O157} and $fliC_{H7}$) identification tests. Out of 326 isolates, only 119 isolates were confirmed as presumptive *E. coli* isolates based on serological test. Table 4.1 shows the number of samples, number of confirmed *E. coli* isolates, serology assay and the sampling sites.

Table 4.1: Results for preliminary biochemical and confirmatory molecular tests

Sampling site	No. of isolates	Serology	16S rRNA	*uidA*	rfb_{O157}	$fliC_{H7}$
Lokaleng farm	NT	22	20	20	20	20
	NP	20	20	16	16	15
Rooigrond prison farm	NT	41	16	16	16	16
	NP	16	16	16	8	0
Klippan	NT	30	9	9	9	9
	NP	9	6	6	5	0
Molelwane farm	NT	23	8	8	8	8
	NP	8	3	3	3	0
Ottosdal	NT	117	40	40	40	40
	NP	40	20	15	15	0
Rooigrond farm (Rs)	NT	30	6	6	6	6
	NP	6	5	5	3	2
Zeerust	NT	65	20	20	20	20
	NP	20	8	1	0	0
TOTAL	NT	326	119	78	62	62
	NP	119	78	62	50	17

NT= Number tested, NP= Number positive

4.2 Molecular characterisation of *E. coli* isolates from cattle faeces

4.2.1 Isolation of Genomic DNA

The DNA was extracted from all the 119 presumptive isolates that were positively identified as *E. coli* based on serology test. Figure 4.1. shows a representative image of a 2% (w/v) agarose gel of genomic DNA extracted from the isolates. As shown in the image, DNA was of good quality with no fragmentation.

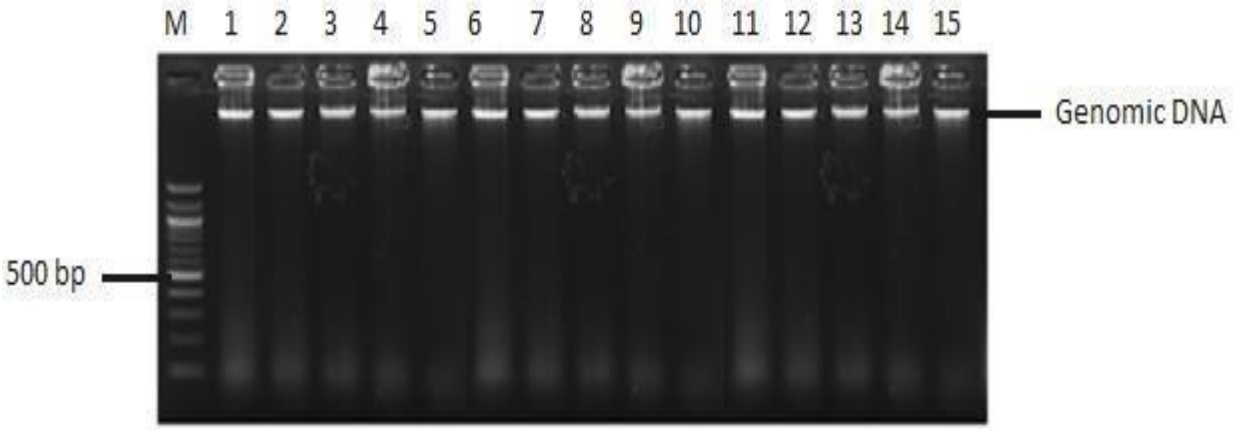

Figure 4.1: A 2% (w/v) agarose gel of genomic DNA extracted from the presumptive *E. coli* isolates. Lane M = 100 bp DNA marker; Lanes 1-15=Genomic DNA from presumptive *E. coli* isolates.

4.2.2 PCR identification of *E. coli* O157:H7 isolates using *uidA*, 16S rRNA, *rfb*$_{O157}$ and *fliC*$_{H7}$ primers

About 119 presumptive *E. coli* isolates were subjected to PCR amplification of bacterial 16S rRNA gene sequences. All the isolates were positive for the universal 16S rRNA gene sequence. *E. coli*-specific PCR identification assay targeting the *uidA* housekeeping gene confirmed 62 isolates as *E. coli* from the

family Enterobacteriaceae. As shown in Figure 4.2 make-up of the *E. coli* isolates comprised 16 from Lokaleng farm, 16 from Rooigrond prison farm, 6 from Klippan farm, 3 from Molelwane farm, 15 from Ottosdal farm, 5 from Rooigrond farm and 1 from Zeerust farm .Confirmatory PCR identification of *E. coli* O157:H7 was achieved through amplification of the rfb_{O157} and $fliC_{H7}$ gene fragments. Of the 62 E. coli isolates, a large proportion (50; 80.6%) positively identified as E. coli O157. Detailed results revealed that large proportions (16 and 15) of the isolates were from communal farms in Lokaleng and Ottosdal respectively. In addition, 8, 5, 3, 15 and 3 isolates were from the Rooigrond prison farm, a farm in Klippan, Molelwane commercial farm and a communal farm in Rooigrond. Figures 4.4, 4.5, 4.6 and 4.7 indicate 2% (w/v) agarose gels depicting the uidA, 16S rRNA, rfbO157 and fliCH7 gene fragments respectively that were amplified by PCR in the study. Amplicons of the target genes were of the expected sizes.

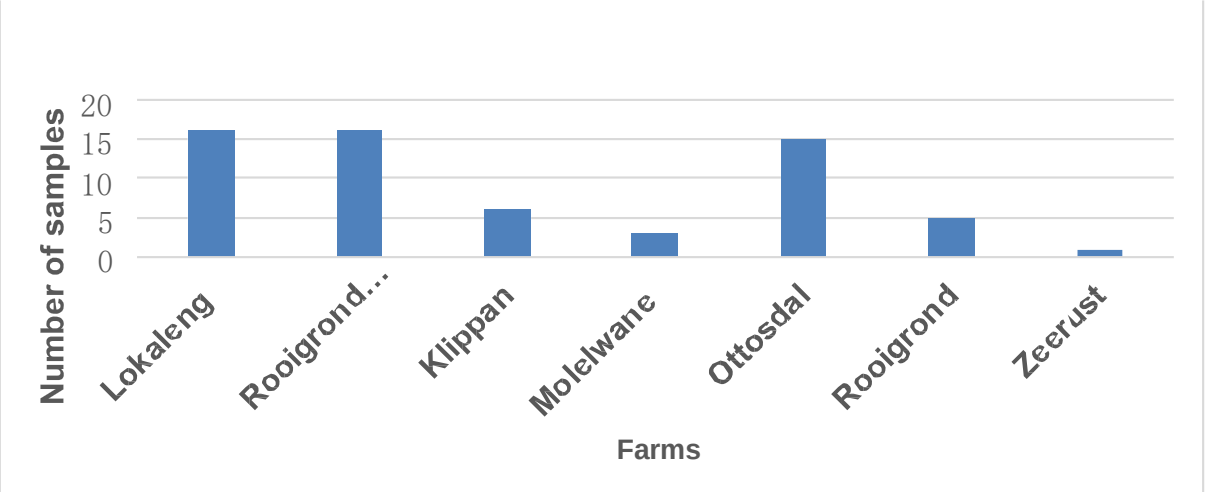

Figure 4.2: Identities of *E. coli* isolates based on analysis of the *uidA* gene fragments

4.2.3 Sequence analysis

Sequence analysis of bacterial 16S rRNA gene fragments of eight representative *E. coli* O157:H7 isolates revealed high (99% to 100%) similarities to *E. coli* O157:H7 16S ribosomal RNA gene, whose partial sequence that has been

previously deposited in Genebank. Data in Table 4.2 indicates the isolate identity, percentage similarities and accession numbers assigned. Figure 4.2 shows sequence analysis of bacterial 16S rRNA gene fragments.

Table 4.2: Identities of *E. coli* isolates based on analysis of bacterial 16S rRNA sequence data.

Isolate ID	***E. coli* strain**	**Percentage similarity**	**Accession No**
CFRC1	*E. coli* O157:H7 partial 16S rRNA gene, strain WAB1892	99 %	AM184233.1
CFOC3	*E. coli* O157:H7 16S ribosomal RNA gene, partial sequence	99%	AY513502.1
CFMC4	*E. coli* O157:H7 strain 33 16S ribosomal RNA gene, partial sequence	99%	HM007589.1
CFKC9	*E. coli* O157:H7 16S ribosomal RNA gene, partial sequence	99%	HQ658163.1
CFLC13	*E. coli* O157:H7 strain B7 HNGU 12 16S ribosomal RNA gene, partial sequence	99%	KX984112.1
CFRC20	*E. coli* O157:H7 strain CPO17446.1 16S ribosomal RNA gene, partial sequence	99%	MG663511.1
CFOC19	*E. coli* O157:H7 16S ribosomal RNA gene, partial sequence	99%	KF225555.1
CFLC16	*E. coli* O157:H7 strain Ras 4 16S ribosomal RNA gene, partial sequence	100%	KY120324.1

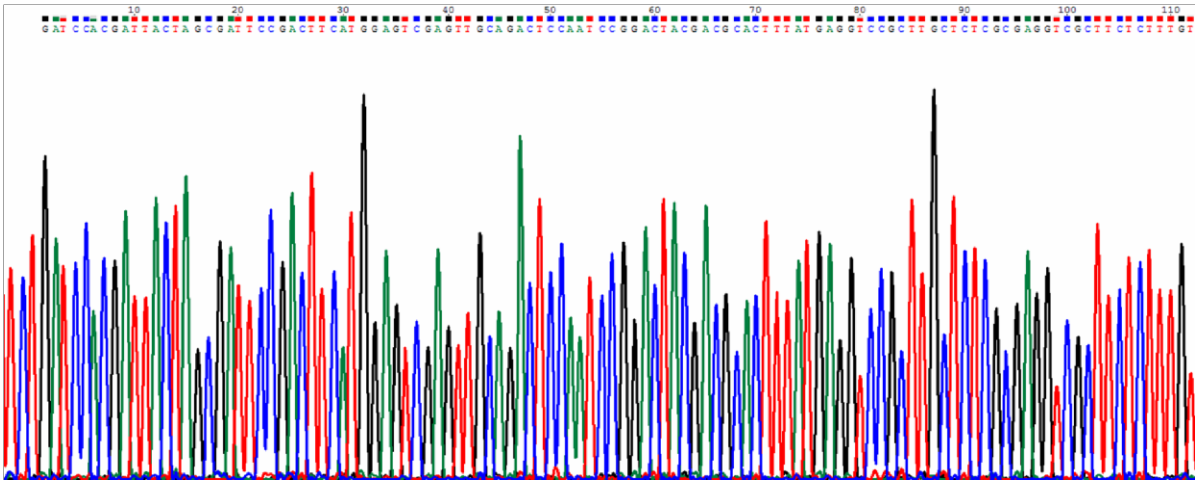

Figure 4.3: Sequence analysis of bacterial 16S rRNA gene fragments.

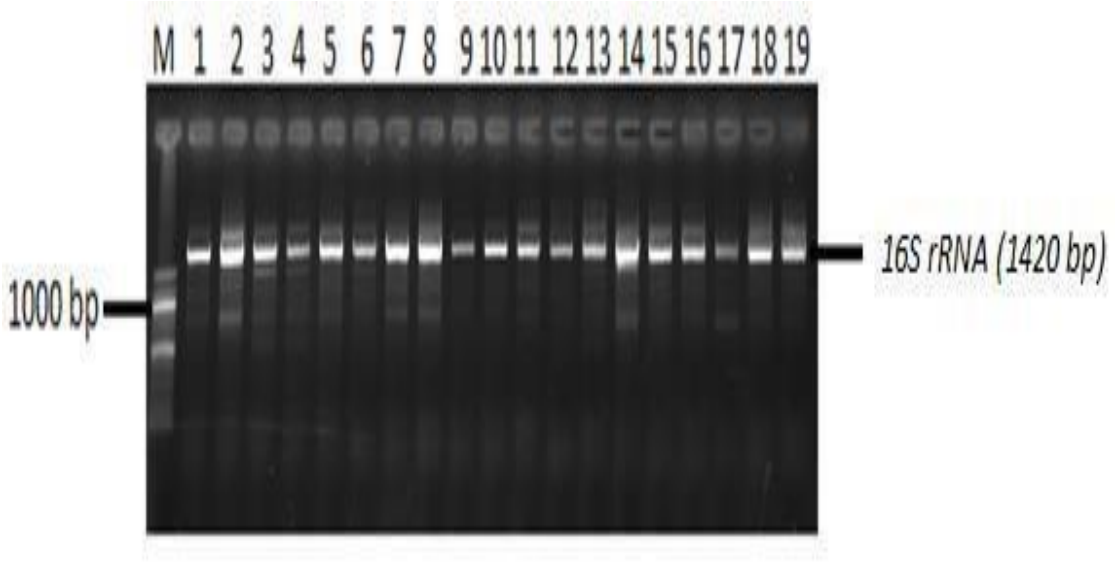

Figure 4.4: A 2% (w/v) agarose gel image of bacterial 16S rRNA gene fragments amplified from *E. coli* isolates. Lane M=1kb DNA marker; Lanes 1-19=16S rRNA gene fragments.

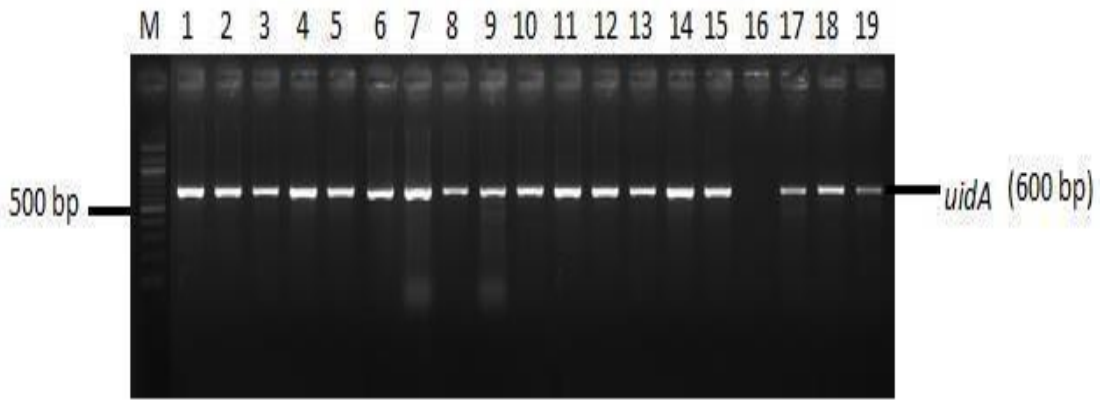

Figure 4.5: A 2% (w/v) agarose gel image of uidA gene fragments amplified from *E. coli* isolates**.** Lane M=100 bp DNA marker; Lanes 1-19=*uidA* gene fragments.

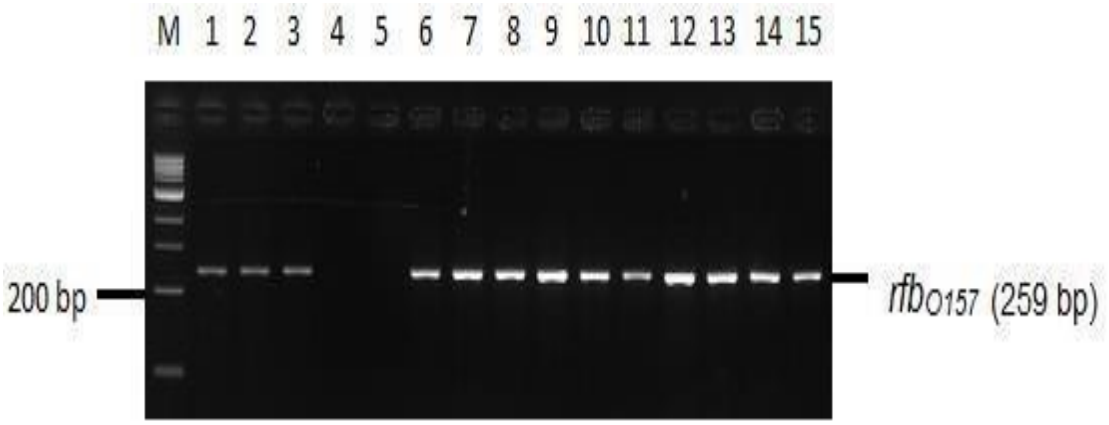

Figure 4.6: A 2% (w/v) agarose gel image of rfbO157 gene fragments amplified from *E. coli* O157:H7 isolates. Lane M=100 bp DNA marker; Lanes 1-15 = *rfb*$_{O157}$ gene fragments.

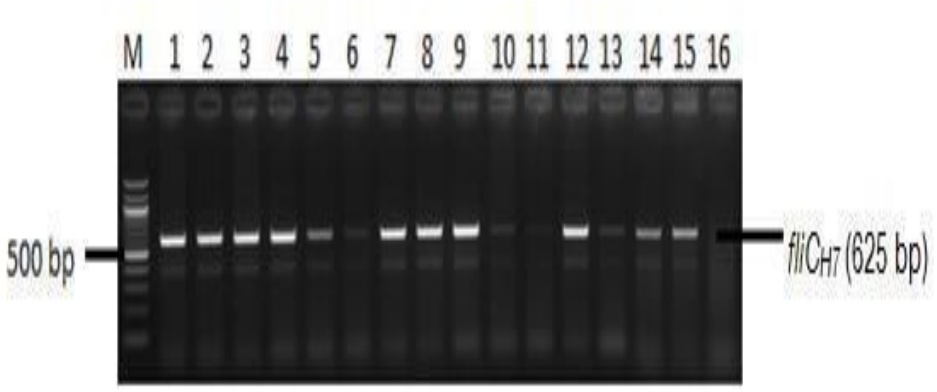

Figure 4.7: A 2% (w/v) agarose gel image of fliC$_{H7}$ gene fragments amplified from the *E. coli* O157 isolates. Lane M=100 bp DNA marker; Lanes 1-16 = *fliC$_{H7}$* gene fragments.

4.2.4 Detection of virulence genes in *E. coli* O157:H7 isolates

A total of 50 Polymerase reaction chain positively identified *E. coli* O157:H7 isolates were subjected to specific PCR analysis for the identification of various virulence genes (*stx$_1$, stx$_2$, terD, tir*, *toxB, iha, katP* and *espP*) that have been associated with pathogenicity in this strain. The proportion of isolates that were positive for the *stx$_1$* (60%) was higher than the *stx$_2$* (50%). In addition, the *stx$_1$* gene was more frequently (22%) detected in isolates from Lokaleng than in Rooigrond prison farm (16%), Ottosdal (14%), and Klippan (10%).

All the isolates from Rooigrond farm possessed this principal virulence determinant form. Similar results were observed with the *stx$_2$* in which the proportion of isolates from Ottosdal (23 %) was higher than those from Lokaleng (22%), Rooigrond prison farm (16%) and Klippan (10%). None of the

isolates from the commercial farm in Molelwane possessed the *stx*$_2$ gene. While the accessory virulence gene *terD* was detected in all the *E. coli* O157:H7 isolates, much lower proportions (26%, 22%, 18%, 14% and 10%) of these isolates harbored the *espP*, *tir*, *toxB*, *iha* and the *katP* genes, respectively (Table 4.3). In addition, there was generally very little correlation between the possession of the principal virulence factors (*stx*$_1$ and *stx*$_2$) and accessory virulence genes (*terD, tir*, *toxB, iha, katP* and *espP*). Figures 4.8, 4.9, 4.10, 4.11, 4.12, 4.13, 4.14 and 4.15 show 2% (w/v) agarose gel images of the *stx*$_1$, *stx*$_2$, *iha, toxB, tir*, *terD, katP* and *espP* gene fragments respectively that were amplified from the isolates.

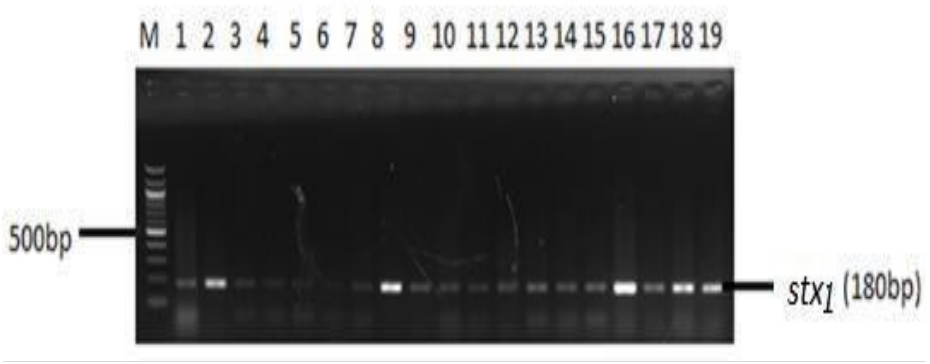

Figure 4.8: A 2% (w/v) agarose gel image of the *stx*$_1$ gene amplified from confirmed *E. coli* O157:H7 isolates. Lane M =100 bp DNA marker, Lane 1-19 = -10 *stx* $_1$ gene fragments.

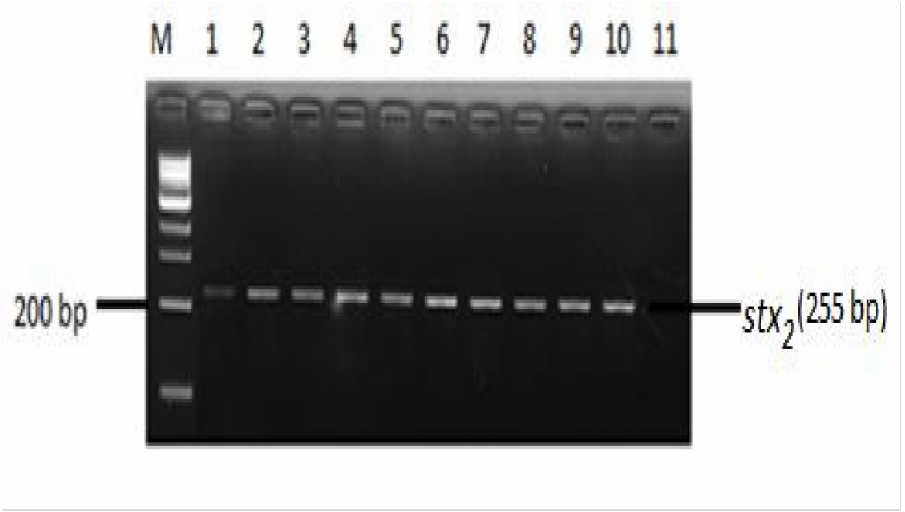

Figure 4.9: A 2% (w/v) agarose gel image of the *stx*$_2$ gene fragments from the confirmed *E. coli* O157:H7 isolates. Lane M =100 bp DNA marker, Lane 1-10 *stx*$_2$ gene fragments from the *E. coli* O157:H7 isolates.

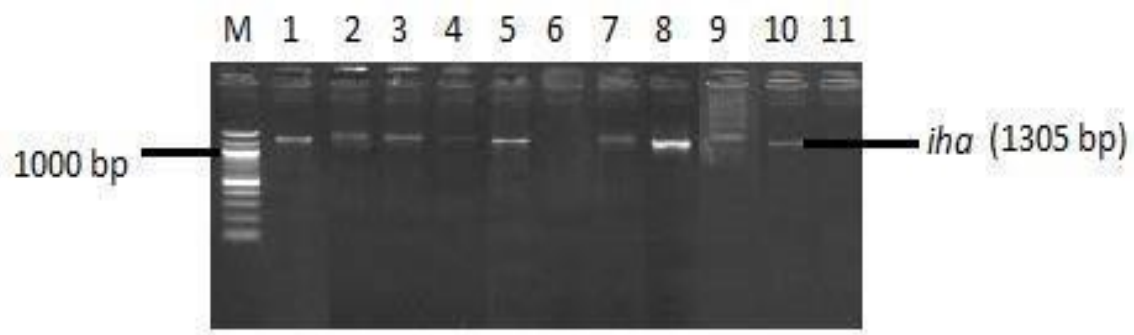

Figure 4.10: A 2% (w/v) agarose gel image of the iha gene fragments from the confirmed *E. coli* O157:H7 isolates. Lane M = 1 kb DNA marker, Lane 1-10 = *iha* gene fragments.

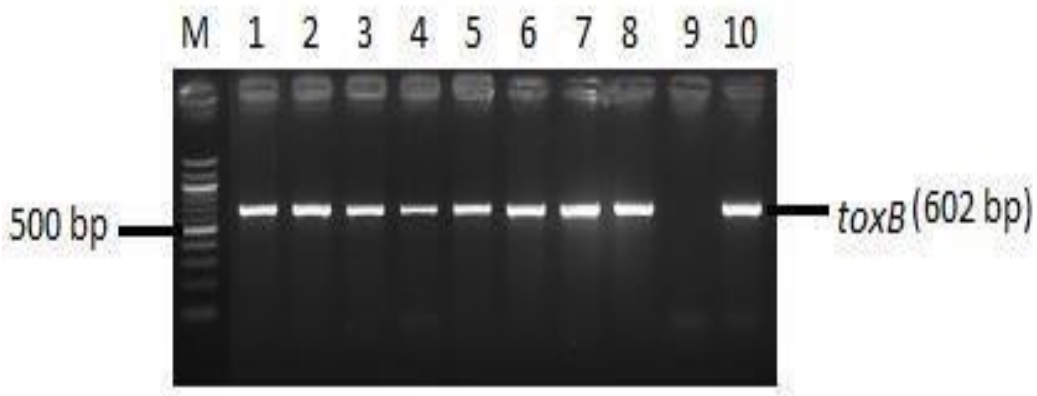

Figure 4.11: A 2% (w/v) agarose gel image of the *toxB* gene fragments from the confirmed *E. coli* O157:H7 isolates. Lane M =100 bp DNA marker, Lane 1-10 = *toxB* gene fragments from the *E. coli* O157:H7 isolated from cattle faeces.

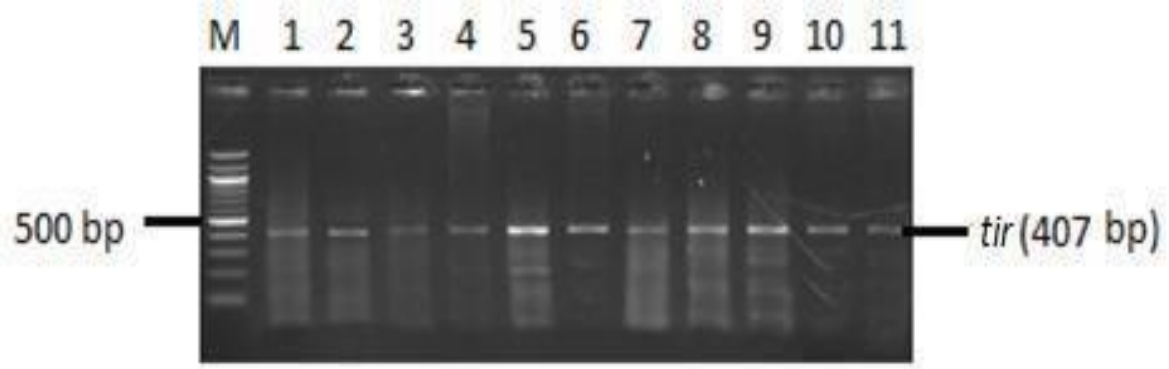

Figure 4.12: A 2% (w/v) agarose gel image of the tir gene fragments from the confirmed *E. coli* O157:H7 isolates. Lane M =100 bp DNA marker, Lane 1-11 = *tir* gene fragments from the *E. coli* O157:H7 isolates.

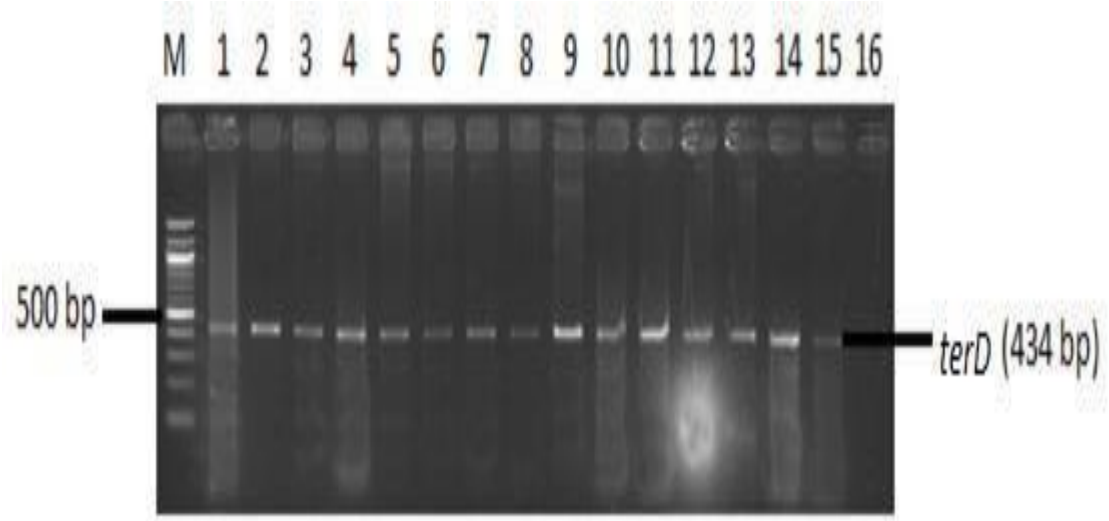

Figure 4.13: A 2% (w/v) agarose gel image of the *terD* gene fragments from the confirmed *E. coli* O157:H7 isolates. Lane M =100 bp DNA marker, Lane 1-15 =*terD* gene fragments from the *E .coli* O157:H7 isolates.

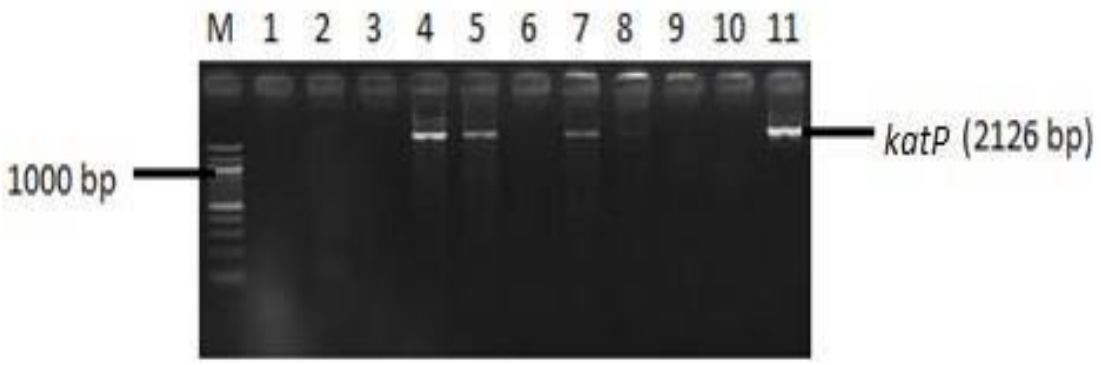

Figure 4.14: A 2% (w/v) agarose gel image of the *katP* gene fragments from the confirmed *E. coli* O157:H7 isolates. Lane M =1 kb DNA marker, Lane 1-11 = *katP* gene fragments from *E. coli* O157:H7 isolates.

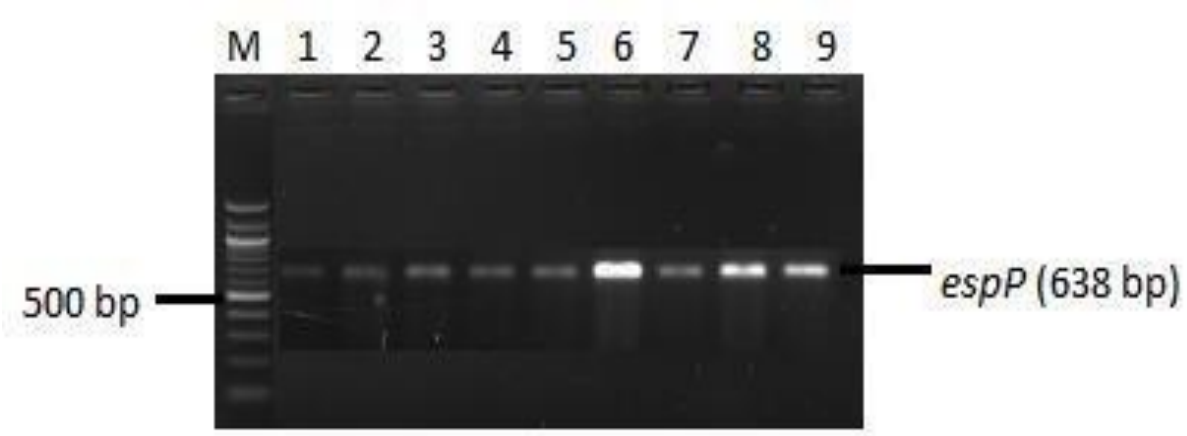

Figure 4.15: A 2% (w/v) agarose gel image of the *espP* gene fragments from the confirmed *E. coli* O157:H7 isolates. Lane M = 100 bp DNA marker, Lanes 1= *espP* gene fragments from the *E. coli* O157:H7 isolates.

4.3 Antibiotic susceptibility test for *E. coli* O157:H7 isolates

All the 50 *E. coli* O157:H7 isolates obtained from different sampling sites were screened to evaluate their susceptibilities to seven antibiotics. The susceptibility test results showed that all the 50 isolates (100%) were sensitive to Gemifloxacin, Levofloxacin, Tetracycline and Norfloxacin while they showed resistance to Ampicillin. About 40% of isolates that was constituted by 7 isolates from Ottosdal farm, 3 from Klippan farm, 2 from Molelwane farm, 3 from Rooigrond prison farm, 4 from Lokaleng farm and 1 from Rooigrond farm were twenty eight percent of isolates that was constituted by 4 from Ottosdal farm, 1 from Klippan farm, 2 from Rooigrond prison farm, 6 from Lokaleng farm and 1 from Rooigrond farm were resistant to Moxifloxacin Table 4.3 shows the results/patterns for the antibiotic susceptibility test and the names of antibiotics which were used.

Table 4.3: Antibiotic susceptibility pattern of *E. coli* O157:H7 isolates

Samples site	No. of isolates	Ciprofloxacin			Gemifloxacin			Levofloxacin			Tetracycline			Ampicillin			Norfloxacin			Moxifloxacin		
		R	IR	S	R	IR	S	R	IR	S	R	IR	S	R	IR	S	R	IR	S	R	IR	S
OTTOSDAL	NT	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15
	NP	7	8	0	0	0	15	0	0	15	0	0	15	15	0	0	0	0	15	4	10	3
KLIPPAN	NT	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	NP	3	4	0	0	0	5	0	0	5	0	0	5	5	0	0	0	0	5	1	3	0
MOLELWANE FARM	NT	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	NP	2	1	0	0	0	3	0	0	3	0	0	3	3	0	0	0	0	3	0	1	0
ROOIGROND FARM	NT	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	NP	1	2	0	0	0	3	0	0	3	0	0	3	3	0	0	0	0	3	1	3	0
LOKALENG	NT	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16
	NP	4	10	1	0	0	16	0	0	16	0	0	16	16	0	0	0	0	16	6	10	1
ROOIGROND PRISON FARM	NT	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
	NP	3	4	0	0	0	8	0	0	8	0	0	8	8	0	0	0	0	8	2	5	0
Total	NT %	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	NP %	40	58	2	0	0	100	0	0	100	0	0	100	100	0	0	0	0	100	28	64	8

R = Resistant, IR = Intermediate resistant, S = Susceptible, NT=Number of isolates tested, NP=Number of isolates positive

4.4 Multiple antibiotic resistant (MAR) phenotypes of *E. coli* O157:H7 isolates

MAR phenotypes were generated for isolates which were resistant to three or more antibiotics belonging to different classes. The antimicrobial agents used are frequently utilized in both veterinary and human medicine in the studied area. The isolates which were resistant to more than 3 antibiotics were used to create multiple antibiotic resistant (MAR) phenotypes. MAR phenotypes were generated using the abbreviations that appeared on the antibiotic discs. It was found that the AP-M-C was the only MAR phenotype.

Table 4.4: Predominant multiple antibiotic resistant (MAR) phenotypes for *Escherichia coli* O157:H7 isolates

SAMPLING SITES	**PHENOTYPES**
Lokaleng Farm	AP-M-C
Rooigrond Prison Farm	AP-M-C
Molelwane Farm	AP-M-C
Ottosdalfarm	AP-M-C
Klippan Farm	AP-M-C
Rooigrond Farm	AP-M-C

C = Ciprofloxacin; G = Gemifloxacin; L = Levofloxacin; M = Moxifloxacin; T = Tetracycline; AP = Ampicillin

CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

5.1 GENERAL DISCUSSION

The primary objective of this study was to isolate and identify *E. coli* O157:H7 from cattle faeces samples collected from selected communal and commercial farms in the North-West Province of South Africa. The main motivation is the fact that previous studies in the area have focused most often on the detection of shiga-toxin (*stx*$_1$, *stx*$_2$ and other *stx*$_2$ variants) (Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011; Akindolire and Ateba, 2018; Bumunang *et al.,* 2019; Montso *et al.,* 2019). To the best of our knowledge, this is the first study accessory virulence genes (*iha, katP, toxB, tir, terD* and *espP*).

In this study, *E. coli* O157:H7 that is a Gram-negative, rod-shaped and non-spore forming enteric bacterial species was successfully isolated. Despite the fact that *E. coli* occur as normal flora in the gastrointestinal tract of humans and warm-blooded animals (Percival *et al*., 2004), isolates belonging to the serogroup O157 and nonO157 serogroups O26, O45, O91, O103, O104, O111, O113, O121, O118, O128, O145, O148 and O174 have most frequently been associated with diseases in humans (Brooks *et al.,* 2005; Marejková *et al.,* 2013; Luna-Gierke *et al.,* 2014). Infections caused by these bacteria strains usually include diarrhoea and haemorrhagic colitis (HC), haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (Tarr *et al.,* 2005) that are known to result in severe complications, especially to young children, elderly and immunocompromised individuals (Percival *et al.,* 2004). Contamination with these pathogens, especially *E. coli* O157:H7, has been associated with the consumption of contaminated food and water with, beef reported to be the most common mode of transmission (Mora *et al*., 2007; Mohammad and

Maryam, 2015; Karmali, 2016). This explains the need to constantly determine the presence of the pathogens in the food chain and assess their pathogenicity especially to less frequently targeted virulence determinants. Moreover, these pathogens that reside in the gastrointestinal tract of warm blooded animals such as cattle can contaminate the slaughter area and the machines used for food processing if proper hygiene is not practised (Bouvet *et al.,* 2001; Ateba and Mbewe, 2011).

A variety of biochemical tests have been used for preliminary identification of *E. coli* and *E. coli* O157:H7 isolates, but the serological assay was found to be most reliable in determining the identities of the isolates. A large portion of 119 isolates were positively identified as *E. coli* species through the use of the H and O antigens. Results obtained using the serological assay were in accordance with those of a previous report (Roy *et al*., 2002). In addition, the occurance of these pathogens in animals may indirectly provide an indication of possible exposure of humans in the area (Ateba and Mbewe, 2011).

Despite the fact that serological assays have been used to determine the identities of bacteria species, they are known for frequently producing false-negative and false-positive results (Abouzeed *et al*., 2000). Against this background, PCR remains a gold standard technique for confirming the identities of bacterial isolates due to its high sensitivity and specificity (Malkawi, 2003; Zahran *et al*., 2014). The findings of this study revealed that all (119; 100%) the isolates harbored the bacterial *16S* rRNA gene while large proportions (62; 52.1% and 50; 80.6%) of these isolates were positively identified as *E. coli* and *E. coli* O157 isolates respectively, based on successful amplification of the *uidA* and rfb_{O157} gene fragments respectively.

Although this and other studies in the area as well as worldwide have documented that the principal host of this pathogen is cattle (Bouvet *et al.,* 2001; Ateba *et al.,* 2008; Ateba and Bezuidenhout, 2008; Ateba and Akindolire, 2018; Bumunang *et al.,* 2019; Montso *et al.,* 2019), previous reports have revealed that the prevalence of *E. coli* O157:H7 in pigs (44.2% to 50%) was higher than in cattle (5.4% to 20%) (Jo *et al*., 2004; Ateba and Mbewe, 2011). It is therefore suggested that the prevalence of this pathogen in animals largely depends on a number of factors.

A further objective was to determine the antibiotic and multi-drug resistance profiles of the *E. coli* O157:H7 isolates obtained in the study. This is motivated by the fact that there has been a dramatic increase in the emergence of multi-drug resistant bacteria worldwide, negatively affecting the health and well-being of humans (Li and Webster, 2018). Moreover, there is some controversy regarding the use of antibiotics in the treatment of infections caused by *E. coli* O157:H7 strains, given that antibiotics have been reported to serve as bacteriophage inducers, thus triggering the SOS response (Zhong *et al*., 2000; Dundas *et al*., 2001). Several reports have indicated that the administration of antibiotics to *E. coli* O157:H7 infected patients results in the production of shiga-toxins, thus enhancing a progression of uncomplicated cases to more severe forms of disease that include hemorrhagic colitis and haemolytic-uremic syndrome (HUS) (Bidet *et al*.,(2005). However, a previous study in the area revealed the presence of multi-drug resistant *E. coli* O157:H7 isolates in pigs, cattle and humans suffer from diarrhea (Ateba and Bezuidenhout, 2008; Magwira *et al*., 2005; Shandukani *et al*., 2016).

In the present study, large proportions of the isolates were resistant to the antimicrobial agents Ciprofloxacin, Gemifloxacin, Levofloxacin, Moxifloxacin, Tetracycline, Ampicillin and

Norfloxacin. These antimicrobial agents are frequently utilized in both veterinary and human medicine in the area and that can contribute to the bacterial antibiotic resistance. These isolates also displayed Multiple Antibiotic Resistance (MAR) phenotypes. The percentage resistance profiles of the isolates in this study were higher than those in previous studies (Ateba and Bezuidenhout, 2008; Wose King *et al*., 2010). The MAR phenotype AP-C-M was dominant among the *E. coli* O157:H7 isolates. These findings are like those previously reported by Mohammad and Maryam (2015) in which *E. coli* O157:H7 strains were most frequently resistant to ampicillin and tetracycline this might have resulted from the frequently use of the antibiotics in the veterinary and human health medicine.

The findings of this study, coupled with data on detailing the development and persistence of antibiotic resistance determinants among bacteria strains in a given area, suggest that factors such as antibiotic selective pressure and misuse of drugs in both humans and animals (Schroedev *et al*., 2002; Shandukani *et al*., 2016) may account for the patterns observed. This explains the need to constantly determine the antibiotic resistance profiles of bacteria strains including *E. coli* O157:H7. The administration of antibiotics to patients with *E. coli* O157:H7 infections without proper knowledge of the molecular basis in which the strain expresses shiga-toxin genes is highly discouraged.

The last objective of this study was to determine the pathogenicity of the *E. coli* O157:H7 isolates through amplification of the principal virulence shiga-toxin genes (stx_1 and stx_2) and most especially other STEC accessory pathogenic determinants (*terD, tir*, *toxB, iha, katP* and *espP*). A large proportion (50 to 60%) of the isolates harboured the $stx_1$and stx_2 genes and the proportion of the isolates with the stx_1 was higher than the stx_2. Similar observations have been reported earlier (Ateba and Bezuidenhout, 2008; Ateba and

Mbewe, 2011). In addition, *E. coli* O157:H7 isolates harboured other accessory virulence determinants (*terD, tir*, *toxB, iha, katP* and *espP*). However, Rump *et al*., (2014) reported that shiga-toxins are carried by phages therefore their presence in isolates may be gained or lost depending on the ecological niche. In addition, it has been reported that strains which produce shiga-toxin type 2 (stx_2) are more frequently associated with serious complications such as HUS (Kawano *et al*., 2008) in humans than to those that harbour stx_1. However, the pathogenicity of bacteria pathogens including STEC strains in their hosts depends not only on the presence of virulence determinants but also on a number of factors such as host-pathogen interactions and the immune status of the individual, just to mention a few.

5.2 CONCLUSIONS

In the present study, *E. coli* O157:H7 was successfully isolated and identified from cattle faeces samples using PCR analysis. In addition, the isolates harbored multidrug resistance determinants as well as a variety of virulence gene factors that have been reported to account for its pathogenicity. The identification of this highly pathogenic bacteria strain in the food chain is of great importance since data generated facilitates in the improvement and implementation of surveillance, preventative and control strategies. This is based on the notion that the presence of the pathogen in cattle provides opportunities for easy cross-contamination of meat during the food production process if standard operating procedures are compromised. This therefore implies the implementation of proper farm management techniques, proper hygiene and standard operational procedures in abattoirs, coupled with the effective monitoring of the presence of the pathogen in environmental sources, are critical in maintaining public health. In addition, the proper usage of antibiotics needs to be monitored especially in the veterinary and human health medicine because if a lot of

antibiotic are being used then that might cause more STEC strains to develop and become resistant to antibiotics and also that would results in more bacterial strains with more virulence genes .The less usage of antibiotics and exposer of bacteria to stress, the lesser the number of STEC strains infections in the world.

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