Virulence capabilities of endemic bacteriophages against Colistin and Extended spectrum beta-lactam resistant non-O157 *Escherichia coli* strains

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DECLARATION

I, MF Mtimunye, declare that this dissertation titled: "**Virulence capabilities of endemic bacteriophages against Colistin and Extended spectrum beta-lactam resistant non-O157 *Escherichia coli* strains**" submitted to the North-West University, Mafikeng Campus, for the degree Master of Science in Biology (Molecular Microbiology) and the work contained therein is my own work in design and execution and has not previously been submitted by me to another university for a degree. I further declare that, this is my work in design and execution and that all materials contained herein have been duly acknowledged.

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DEDICATION

This work is dedicated to my parents Ms K. Mtimunye and Mr S.A Sibanyoni, my family and friends. I thank them all for their support and input towards the fulfillment of this study.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

DEFINITION OF CONCEPTS

Bacteriophages: are bacterial viruses that only infect and replicate within their specific hosts.

Biocontrol: the practice or processes by which an undesirable organism is controlled by means of another organism.

Colistin: An antibiotic with broad-spectrum activity against Gram-negative bacteria that belongs to the Polymyxin, cationic polypeptides.

Endemic: restricted to a certain region.

Enteropathogenic: are microorganisms that cause intestinal tract diseases.

***Escherichia coli*:** is a Gram-negative, facultative anaerobic, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms.

***Escherichia coli* O157:H7:** Are an enterohaemorrhagic serotype of the bacterium *Escherichia coli* and a cause of illness, typically through consumption of contaminated food.

Host range: is the spectrum of strains of bacterial species that a given strain of phage can infect.

Haemolytic-uremic syndrome (HUS): is a severe complication of *E. coli* infection, characterized by haemolytic anemia, acute renal failure (uraemia), and a low platelet count (thrombocytopenia).

Phage lytic capability: is the ability of a viral particle to lyse bacterial cells.

Plaques: are clear zones formed in a lawn of bacterial cells due to lysis by phage.

Serotype: is a subdivision of a species distinguishable from other strains based on antigenic character.

STEC: are shiga toxin-producing *E. coli* (STEC)are members of *E. coli* that produces a toxin called shiga toxin, which is responsible for the bloody diarrhoea, HUS, and other symptoms associated with its infection.

Thrombotic thrombocytopenic purpura: is a disorder of the blood-coagulation system, causing microscopic blood to form in the small blood vessels throughout the body.

Virulence factors: are molecules produced by pathogens that contribute to the pathogenicity of the organism.

LIST OF ABBREVIATIONS

The following abbreviations have been used throughout this dissertation and follow the style recommended by the American Society for Microbiology for Journals.

EAEC **:** enteroaggregative *E. coli*

E. coli **:** *Escherichia coli*

EHEC **:** enterohaemorrhagic *E. coli*

EIEC **:** enteroinvasive *E. coli*

EPEC **:** enteropathogenic *E. coli*

ESBL **:** extended spectrum beta-lactamase

ETEC **:** enterotoxigenic *E. coli*

FCD **:** foodstuffs, cosmetics and disinfectant

FLAG **:** food legislation advisory group

HACCP **:** hazard analysis critical control point

HC **:** haemorrhagic colitis

HMS **:** hygiene management system

HUS **:** haemolytic uremic syndrome

MOI **:** multiplicity of infection

PFGE **:** pulsed field gel electrophoresis

PFU **:** plaque-forming unit

STEC **:** shiga toxin-producing *E. coli*

TEM **:** transmission Electron Microscopy

TTP **:** thrombotic thrombocytopenic purpura

USDA **:** United States Department of Agriculture

SABS **:** South African Bureau of Standards

ABSTRACT

STEC *Escherichia coli* O157 and non-O157 strains are known to cause severe food-borne infections as that may occur as either sporadic case or outbreak cases in humans even in countries with advance public health policies. The treatment of these infections caused by bacteria pathogens is generally achieved through the administration of antibiotics. However, the use of antibiotics in the treatment of STEC infections is generally discouraged since antibiotics have been reported to increase the release of shiga-toxins thus increasing the severity of disease. In addition, some previous studies have revealed that large proportions of environmental STEC strains are multi-drug resistant and this therefore indicates the need to search for other alternative control strategies. This study assesses the potential of using endemic bacteriophages as control agents against Colistin and Extended spectrum beta-lactam non-O157 Shiga-toxin producing-*E. coli* (STEC).A In the present study, non-O157 STEC strains were targeted and they were successfully isolated from cattle faeces samples. Isolates belonged to the serogroups O111, O104, O161 and O145, with O111 and O145 that are classified as non-O157 *E. coli* "big six" STEC group. The prevalence and antibiotic resistance profiles of shiga-toxin producing non- O157 *E. coli* strains isolated were determined. The strains were further characterised by molecular methods for the presence of shiga-toxin virulence genes and antibiotic resistance genes of Colistin and Extended spectrum beta lactams. Two hundred and forty-two (242) non-O157 *Escherichia coli* strains were isolated and a large proportion (104; 42.97%) of the 242 isolates possessed the stx_1 gene while 161 (66.52%) of these possessed the stx_2 gene. On testing these isolates for their resistance to Colistin and ESBLs agents, multiple antibiotic resistance (MAR) was observed in some of the isolates. A proportion of 6.19% isolates were resistant, 41.32% were intermediate resistant and 52.48% were susceptible to Colistin, while (83.06%) of them were

confirmed to also be ESBL-producing isolates phenotypically. In addition, the ESBL genes *bla*$_{OXA}$, *blaCTX*$_{-M}$, *bla*$_{SHV}$ and *bla*$_{TEM}$ were detected in 20 (66.67%), 11 (36.67%), 6 (20%) and 5 (16.67%) of the isolates respectively. Bacteriophages were successfully isolated in this study using confirmed environmental non-O157 *E. coli* STEC bacterial hosts strains obtained in this study. The isolated phages possessed visible heads, neck and tail regions based on electron microscopy data. Phage virulence assays revealed that these phages displayed lytic potentials. Three of the employed bacteriophages BNEO1575E, BNEO1574D and BNEO1574C were able to inhibit bacterial growth of more than one bacterial strain. These findings indicate that phages isolated in this study have displayed characteristics of being effective agents for biological control of environmental non-O157 STEC strains that also possess multidrug resistant determinants. These findings are of great epidemiological significance given the recent detection and clinical implications of Colistin and ESBL-producing bacteria strains particularly in hospital settings.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Escherichia coli are rod shaped Gram-negative, facultative anaerobic bacteria that live as normal flora in the gastrointestinal tract of human beings and warm blooded animals (Weintraub, 2007; Sillankorva *et al.*, 2012; Alonso *et al.*, 2016). These organisms belong to the family *Enterobacteriaceae* and have the ability to form mutualistic relationships with their hosts for very long periods (Weintraub, 2007). Despite this, a number of *E. coli* strains have recently been linked to a large proportion of water and foodborne disease outbreaks worldwide (Brooks *et al.*, 2005; Caprioli *et al.*, 2005). *E. coli* strains belonging to the serotype O157:H7 have attracted a lot of attention in many countries lately due to their pathogenic nature and therefore been classified as pathogens of huge public health concerns (Ateba *et al.*, 2008; Ateba and Bezuidenhout, 2008; Karmali, 2009; Ateba and Mbewe, 2011; Bolton, 2011; Ateba and Mbewe, 2014).

Despite the public health significance of *E. coli* O157 strains, a number of non- O157 shiga-toxin producing *E. coli* (STEC) strains particularly those belonging to serotypes O26, O91, O103, O111, O118, O145 and O166 have also been associated with a number of outbreaks and sporadic cases of human infections (Karmali, 1989; Kropinski *et al.*, 2013). Infections caused by these non- O157 STEC strains range from simple and uncomplicated diarrhoea to the more complicated haemolytic colitis (HC), haemorrhagic uraemia syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Karmali, 1989; Kropinski *et al.*, 2013). These diseases are more severe in young children, elderly and immune-compromised individuals (O'hanlon *et al.*, 2005). These non-O157 strains have also been associated with two separate multistate foodborne outbreaks in the USA and epidemiological investigations indicated that

complications in humans resulted from the spread of a rare strains consumed from Chipotle (a smoked hot chili pepper) served in a Mexican grill restaurant (CDC, 2015). The potential of these isolates to cause disease outbreaks even in countries that have advanced public health policies greatly amplifies the need to monitor their occurrence in the food chain since findings may provide opportunities for the implementation of strict control measures.

The pathogenicity of non-O157 STEC strains result from the possession of virulence gene determinants (Paton and Paton, 1998b; Martorelli *et al.*, 2017). Shiga toxins are the primary virulence factors of STECs and two major forms designated Stx_1 and Stx_2 have been identified and characterised (Gyles, 2007; García-Aljaro *et al.*, 2009; Karmali *et al.*, 2010). In addition to these, a number of Stx_1 and Stx_2 subtypes that include *Stx1a, Stx1c, Stx1d, Stx2a, Stx2b, Stx2c, Stx2dact, Stx2e, Stx2f* and *Stx2g* have also been categorized (Karmali *et al.*, 2010). Epidemiological studies suggest that stx_2 rather than stx_1 is more often associated with severe disease and also largely contributes to the development of HUS in patients (Proulx *et al.*, 2001; Matussek *et al.*, 2003). In addition to the shiga-toxins, other putative virulence determinants that include intimin and enterohaemolysin genes designated *eaeA* and *hlyA* respectively have been associated with diseases in humans (Frankel *et al.*, 1998; Paton and Paton, 1998a; Donnenberg and Whittam, 2001; Ateba and Bezuidenhout, 2008; Fernández *et al.*, 2013).

Domestic animals, especially cattle, are generally known to be the principal reservoir *E. coli* O157 and non-O157 STEC isolates (Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011; Kh *et al.*, 2011). However, previous studies conducted in the study area focused on isolates belonging to the serotype O157, and findings indicated that pigs rather than cattle are

a potential reservoir for these pathogens (Ateba *et al.*, 2008; Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011).

Despite the fact that non-O157 STEC outbreaks are rare, they have been reported to primarily occur through the consumption of contaminated food as well as person-to-person transmission (Gould *et al.*, 2013; Luna-Gierke *et al.*, 2014) . Unfortunately, non-O157 STEC infections were under-recognized and under-reported worldwide until the year 2000 due to inadequate epidemiological and laboratory surveillance as well as specific pathogen source tracking schemes (Stratakos *et al.*, 2018). In addition, identification of non-O157 STEC strains is complicated by the fact that they do ferment sorbitol and therefore requires adequate and effective screening of isolates belonging to different serotypes (Scott *et al.*, 2008).

Infections caused by non-O157 *E. coli* strains are more difficult to control especially if isolates also habour antibiotic resistance determinants (Mora *et al.*, 2005). A number of studies have revealed the presence of multiple antibiotic resistance determinants in non-O157 *E. coli* strains (Brooks *et al.*, 2005; Luna-Gierke *et al.*, 2014; Shen *et al.*, 2015; Kennedy *et al.*, 2017). Colistin is an old antibiotic that is used as a last resort drug for treating infections caused by multiple antibiotic resistant *Enterobacteriaceae* particularly those harboring carbapenemase resistance determinants. However, recent studies have revealed the presence of plasmid-mediated Colistin resistance genes designated *mcr1* and *mcr2* in *Escherichia coli* and *Klebsiella pneumoniae* as well as a variety of bacteria strains (Hasman *et al.*, 2015; Wong *et al.*, 2016). Despite the fact that antibiotic resistance is usually expected to be a slow-moving crisis, Colistin resistance determinants are spreading rapidly and this highlights the need to return to the pre-antibiotic era and search for novel control agents. Bacteriophages are viruses that infect bacteria and they have a history of safe use, can be highly host specific,

and capable of replicating within a host. These attributes gives them the potential to serve as novel options to controlling foodborne pathogens (Hudson *et al.*, 2005) particularly virulent and antibiotic resistant non-O157 *E. coli* isolates (Nagy *et al.*, 2015; Shen *et al.*, 2015) .

Despite the fact that the occurrence of *E. coli* O157 strains has been extensively studied in the area (Ateba *et al.*, 2008; Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011; Ateba and Mbewe, 2014), to the best of our knowledge there is currently no information documenting the prevalence of antibiotic resistant non-O157 STEC strains. Data generated in this study may be of great epidemiological importance and may provide the public health implications of these non-O157 strains in the South African food production chain. In addition, the characterization of phages that are effective against antibiotic and virulent non-O157 STEC strains advanced control strategies.

1.2 Problem statement

Antibiotic resistant isolates present a severe challenge to public health due to the fact that they have the potential to complicate therapy (Bonelli *et al.*, 2014). Despite this, an important achievement in drug discovery has been the ability to constantly develop antimicrobial agents that are effective against pathogens associated with life-threatening infections. Unfortunately, the emergence of multidrug-resistant (MDR) Gram-negative bacteria has drastically narrowed down the therapeutic options for treating infections they cause (Bonelli *et al.*, 2014; Nathan and Cars, 2014). Colistin is an old antibiotic that was used as a last resort drug for treating infections caused by multiple antibiotic resistant *Enterobacteriaceae* particularly those harboring carbapenemase resistance determinants. However, the administration of the antibiotic Colistin was discontinued in the early 1980s due to its high nephrotoxicity and neurotoxicity (Li *et al.*, 2006; Pintado *et al.*, 2008). Despite the fact that this antibiotic was

discontinued, recent studies have revealed the presence and increasing prevalence of Colistin resistance determinants among bacteria (Elnahriry *et al.*, 2016).

Colistin comprises a mixture of at least 30 different compounds that are administered as inactive derivatives in the treatment of infections caused by multi-drug resistant Gram-negative bacteria (Couet *et al.*, 2012) and hence may be responsible for the selective pressure that is currently available in the environment. Given that there is clinical evidence that multidrug-resistant Gram-negative bacteria pose the greatest risk to public health (Kumarasamy *et al.*, 2010), there is need to determine the prevalence of these recently emerging and fast disseminating Colistin resistance determinants in food producing animals.

Colistin resistance in Gram-negative bacteria results through structural modifications of lipopolysaccharide on the bacterial cell-wall (Falagas *et al.*, 2005). Modifications can be due to mutations in the *mgrB* gene or addition of 4-amino-4-deoxy-t-arabinose or phosphoethanol amine caused by chromosomal mutations. Recently, it has been shown that the *mcr1*-gene that encodes phosphoethanol amine transferase is easily transferred horizontally among bacteria. In addition, some strains may possess either Carbapenemase or Extended Spectrum Beta-Lactamase genes together with the *mcr1*-gene on the same plasmid, which may lead to pan-drug-resistance.

Antimicrobial agents are also known to induce phages that may be haboured by pathogenic STEC O157 as well as non-O157 strains (D'herelle, 1917; Lu and Koeris, 2011). Recently bacteriophages have been regarded as effective agents that could be used for biological control and therapeutic approaches intended to eliminate pathogenic bacteria from the environment and animals (Sillankorva *et al.*, 2012). A number of studies that focused on

shiga-toxigenic *E. coli* have isolated antibiotic resistant *E. coli* O157:H7 from animals, food products, water and humans in the North West Province (Ateba *et al.*, 2008; Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011; Ateba and Mbewe, 2013). This may have been due to a very huge attention that this pathogen received worldwide (Müller *et al.*, 2001; Douëllou *et al.*, 2017). There is no study that has been conducted to determine the occurrence, virulence potentials as well as antibiotic resistance profiles of non-O157 *E. coli* serotypes in the area. This study is therefore aimed at expanding on previous investigations by isolating, identifying and determine the virulence profiles of non-O157 *E. coli* isolates from cattle as well as determining the correlation between Colistin and ESBL resistant determinants in the isolates. A further objective will be to isolate non-O157 *E. coli* specific bacteriophages, determine their morphologies and assess their effectiveness against Colistin resistant isolates.

1.3 Aim

The aim of this study was to determine virulence capabilities of endemic bacteriophages against Colistin and Extended spectrum beta-lactam resistant non- O157 *Escherichia coli* strains from cattle.

1.4 Objectives

The objectives of the study were:

- To isolate and confirm the identities of non-O157 *E. coli* isolates from cattle faecal samples using *E. coli* PCR (16S rRNA, *E. coli* specific PCR, *E. coli* O157 PCR and non-O157 serological assays)
- To determine the virulence gene profiles of the non-O157 *E. coli* isolates

- To determine the presence of Colistin resistant determinants using phenotypic assays to confirm the presence of ESBL resistant determinants in the isolates and compare their association with Colistin resistant determinants
- To isolate and determine the morphologies of non-O157 *E. coli* bacteriophages using transmission electron microscopy
- To determine the effectiveness of bacteriophages against Colistin and ESBL resistant non-O157 *E. coli* isolates using the virulence micro-plate assays.

CHAPTER 2

LITERATURE REVIEW

2.1 Classification of *Escherichia coli*

Escherichia coli are gram-negative bacilli, facultative anaerobic bacteria that belong to the *Enterobacteriaceae* family (Ewing, 1986). They reside in the gastrointestinal tract of numerous animals and of human beings; it is part of the human and warm-blooded animal's natural microbiota (Ercoli *et al.*, 2016; Osińska *et al.*, 2017). Genotypically, it is a microorganism of a great variety as it can be a harmless strain in the laboratory, probiotic, commensal or pathogenic (Do *et al.*, 2017). It is again used as a contamination indicator in food, soil and water ecosystems (Li *et al.*, 2015; Titilawo *et al.*, 2015). *E.coli* falls under the most isolated gram-negative bacteria and this is due to its ability of being pathogenic (Xie *et al.*, 2017). It is the causative agent of various diseases in humans and in animals (Do *et al.*, 2017) .

E. coli is a type of bacteria that is able to populate various animal host species and the environments of the non-hosts (Power *et al.*, 2005; Kon *et al.*, 2007; Chandrasekaran *et al.*, 2015). Therefore a phylogenetic analysis was created so that *E. coli* strains could be grouped according to the type of host they are found in (Clermont *et al.*, 2013). These phylogroups: A, B1, B2, C, D, E, F, and E clade I have host relations attached to them, for example group A(40.5%) and B2(25.5%) are mostly identified from humans and then group B1(41%) are usually identified from animals (Tenaillon *et al.*, 2010). Furthermore, not only can this microorganism be found in feacal matter of either humans or animals, but it can also be found in wastewater treatment plants and this is due to its significant level of host-non-host

adaptation (Zhi *et al.*, 2016b; Zhi *et al.*, 2016c). *Escherichia coli* as mentioned before populate the human natural flora, specifically harmlessly in the intestines, though it may cause alarming diseases. Thus they are classified in two major pathotypes, namely, Extraintestinal *E. coli* and Diarrheogenic *E. coli* (Hussain and Hussain, 2015). In the Diarrheogenic *E. coli* there are six classes which the different strains are grouped under: Enteropathogenic *E.coli* (EPEC), Enterohaemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E.coli* (EIEC) and Diffusely adherent *E.coli* (DAEC) (Nataro and Kaper, 1998; García *et al.*, 2016). Strains in these pathotypes are placed depending on their pathogenicity, diseases they cause, the host and the virulent capabilities (Robins-Browne *et al.*, 2016).

Beside the pathotypes, *E. coli* strains can be placed in subtypes using a formula that is unique for individual strains and these subtypes include serotypes (Robins-Browne *et al.*, 2016). Serotypes have a functional formula for distinguishing pathogenic *E. coli* strains in samples and in making epidemiology understandable (Wang *et al.*, 2010). Therefore, this microorganism is serotyped using the surface antigen profiles: O (Somatic), H (Flagella) and K (Capsular); in which they occur in various combinations that create a specific serotype (Robins-Browne, 1987; Nataro and Kaper, 1998). One of the known serotype is the Shiga toxigenic *E. coli* (STEC) which consists of the notable *E. coli* O157:H7 and the “big six” non-O157 serogroups (O26, O145, O121, O45, O111 and O103), they are all known to be the causative agents of diseases in humans and some animals (Brooks *et al.*, 2005; Scallan *et al.*, 2011). Thus it is imperative not only to understand, but also to classify this versatile microorganism accordingly as this may be helpful in the laboratory, understanding its pathogenicity and virulence and in a situation of an outbreak (Hussain and Hussain, 2015).

2.2 Pathogenicity

Pathogenicity is the ability of a microorganism to damage the host and to cause a disease, and this is achieved when a pathogen possesses a certain gene that initiates the mechanism (Henderson's, 2008). The first step to pathogenicity of *E. coli* species is to populate the hosts intestine and adhesion to the intestinal epithelial cell; Though these vary from host to host and strain to strain (Malik *et al.*, 2017). Etymological agents of intestinal or Extraintestinal diseases are the two subdivisions of *E. coli*; that is by virtue of the acquisition of virulence factors excluding non-pathogenic commensal isolates (Müller *et al.*, 2016).

There are two major groups of pathogenic *E. coli*, the first one consists of pathotypes EPEC, STEC (and its subgroup EHEC), ETEC, EAEC, EIEC and DAEC, which causes characteristic symptoms of gastrointestinal diseases (Nataro and Kaper, 1998). Typical EPEC strains are pathogenic for humans and have not yet been found in animals, while the atypical EPEC and EHEC are pathogenic for humans and young animals (Malik *et al.*, 2017). The second group (termed Extraintestinal pathogenic *E. coli* (ExPEC) causes infections outside the gastrointestinal system and it consists of avian pathogenic *E. coli* (APEC), which causes respiratory tract infections, and uropathogenic *E. coli* (UPEC) (Kaper *et al.*, 2004). Predominantly, the primary reservoir for UPEC is said to be the human intestinal tract, where it is capable of spreading to the urogenital tract and later causing urinary tract infections (Pitout, 2012; Singer, 2015).

2.2.1 STEC: Shiga toxin producing *E. coli*

Pathogenic shiga toxin producing *Escherichia coli* are major microorganisms that are more involved in causing foodborne outbreaks and severe infections in both humans and animals; and the prevalent strain amongst these STECs is *E. coli* O157:H7. Despite that, many

outbreaks across the world are associated with non-O157 STECs strains (Pizarro *et al.*, 2013; Stratakos *et al.*, 2018). In the United States, non-O157 serogroups such as O121, O26, O103, O45 and O145 are involved with sporadic and epidemic infections (Schulz *et al.*, 2015). There are types of toxins produced by the STEC, two of which are the main virulent factors Stx_1 and Stx_2. These are the potent phage encoded cytotoxins named shiga-toxins, which causes cell destruction through the inhibition of protein synthesis, which leads to cell death (leading to the lining collapsing and to haemorrhage) and cause damage to the endothelial cells of the target organ. Moreover, the protein intimin (*eaeA*), which is responsible for intimate attachment of STEC to the intestinal epithelial cells causing attachment and effacing (A/E) lesions in the intestine is also expressed by STEC harboured by human and cattle (Acheson *et al.*, 1996; Matussek *et al.*, 2003; Pizarro *et al.*, 2013; Ahsan, 2016; Otero *et al.*, 2017). They lead to the disease outbreaks, causing diseases such as non-bloody and bloody diarrhoea, haemorrhagic colitis, haemolytic uremic syndrome (leading to thrombocytopenia, renal failure and haemolytic uremia) and thrombotic thrombocytopenic purpura (Acheson *et al.*, 1996; Karch *et al.*, 2005).

2.2.2 Reservoir and transmission

Two domains where *E. coli* revolves are the intestines of humans and animals, where it usually resides for a long period. The environment (water, sediment and soil) being the second domain after being expelled from the warm blooded animals, the required nutrients should however be provided by the environment for survival (Ahsan, 2016). According to (Gyles, 2007; Fernández *et al.*, 2013) the main source of the non-O157 STEC is recognized to be cattle. These microorganisms are transferred to humans through the consumption of under cooked meat, ground beef, feacal contaminated water or vegetables, unpasteurized dairy products, contact with animal carriers and person-to-person.

2.3 Epidemiology

There is an increasing incidence of ESBL-producing *E. coli* in community and hospital settings, which are known to cause serious infection in humans (Datta *et al.*, 2014). ESBL-producing bacteria such as *E. coli*- particularly, O-antigen type and *K. pneumonia*, are increasingly spreading globally creating a severe threat; their antimicrobial resistance development is multi-factorial, with antibiotic consumption being the major factor (Daoud *et al.*, 2014; Hayakawa *et al.*, 2017). Cases of infections caused by these bacteria have been reported in different countries. In Germany, February 2012 there was a large outbreak of ESBL-Producing *K. Pneumonia* in a neonatal intensive care unit; which lead to a shut down for over 2 years (Haller *et al.*, 2015). Similar bacteria were found among pigs and other livestock to a herd level of 44–85% (Fischer *et al.*, 2017). The two scenarios show that there was transmission of these particular bacteria that occurred. Hence, ESBL producing strains epidemiological factors should be documented to help generate information about the strains and to help with administering treatment on time (Datta *et al.*, 2014).

2.4 Mechanism of antimicrobial agents

Antimicrobial agents are organic compounds that are produced by microorganisms which selectively inhibits the growth of other microorganisms, these substances are used greatly as form of medication in both animals and humans to treat or prevent infections and can also be used as growth promoters (Henderson's, 2008; Kemper, 2008; Nathan and Cars, 2014). The antibiotics work through these five mechanisms of activity: interference with the cell wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis, inhibition of metabolic pathway and disorganization of the cell membrane (Shaikh *et al.*, 2015). The decrease in the productiveness of an antimicrobial agent in treating a disease or condition is called antibiotic resistance, and an organism that is multidrug resistant- can cause numerous

antimicrobial agents to be ineffective towards inhibiting its growth. Thus some bacterial strains may harbour various types of resistant mechanism (Fisher and Mobashery, 2010; Shaikh *et al.*, 2015).

2.4.1 Antibiotic inactivation

In hydrolysis process, various antibiotics contain chemical bonds such as amides and esters which are hydrolytically susceptible. Moreover, a number of enzymes are learned to disintegrate antibiotic activity by targeting and cleaving bonds and are mostly excreted. While in redox process, pathogenic bacteria are rarely exploited in oxidation or reduction of antimicrobial agent; such as the oxidation of tetracycline antibiotics by the *tet*X enzyme (Yang *et al.*, 2004). Which may lead to antibiotics that are modified are damaged while binding to a target.

Antibiotic resistance through target modification- Resistance mechanism is the modification of the antimicrobial agent target site so that the antibiotic is ineffective to bind correctly, though it is achievable for mutational adjustments to arise in the target that decrease susceptibility to inhibition while retaining cellular function (Spratt, 1994). Furthermore, there are genetics of antimicrobial resistance which can also occur through either one of the mechanisms: Antibiotic resistance through mutations. These can be mutations of the sequences of genes encoding the target of certain antibiotics such as resistance to rifampicin and fluoroquinolones is caused by mutations in the genes encoding the targets of these molecules, *Rpo*B and DNA-topoisomerases, respectively (Ruiz, 2003). On the other hand, difference in the expression of antibiotic uptake or of the efflux systems may also be modified by mutation such as the reduced expression or absence of the OprD porin of *Pseudomonas aeruginosa* reduces the permeability of the cell wall to carbapenems (Wolter *et al.*, 2004); and antibiotic resistance through horizontal gene transfer. A predominant

mechanism for the increase of antimicrobial resistance is through horizontal transfer of genetic material where antibiotic resistance genes may be transported by various mechanisms of conjugation, transformation or transduction.

The target for antimicrobial activity of Colistin is the bacterial cell membrane, where the beginning attachment of Colistin with the bacterial membrane arises through electrostatic interactions between the cationic polypeptide (Colistin) and anionic lipopolysaccharide molecules in the outer membrane of the gram-negative bacteria; this leads to derangement of the cell membrane. Colistin then displaces magnesium and calcium that usually stabilizes the lipopolysaccharide molecules, from the negatively charged lipopolysaccharide causing a local disarrangement of the outer membrane. Therefore, this process results in an increase in the permeability of the cell envelope, discharging of cell contents and eventually cell death (Newton, 1956; Davis *et al.*, 1971; Schindler and Osborn, 1979; Falagas *et al.*, 2005). The two mechanisms of resistance that can be developed by Gram-negative bacteria against Colistin may occur either through a mutation mechanism- a low level and independence of constant existence of the antimicrobial agent; or through adaptation mechanism (Moore *et al.*, 1984; Moore and Hancock, 1986; Groisman *et al.*, 1997; Falagas *et al.*, 2005).

2.5 Increase of antibiotic resistance in *Escherichia coli*

Antimicrobial agents were introduced into medicine in the year 1940 and they have been the main modern form of therapeutics (Gelband *et al.*, 2015; Nesme and Simonet, 2015). Unfortunately infections related to *Escherichia coli* cannot be prevented with either immunization or medication, though it can be managed by the practice of proper hygiene and following the standard protocols of food safety (Ahsan, 2016). For this reason, antimicrobial agents are now used as a form of therapeutic to minimize or eliminate the infection caused by

bacteria. Regrettably, bacterial strains find an opportunity to modify and form resistant genes (Zhu *et al.*, 2013) that are transferred to human beings through the food chain (Zhi *et al.*, 2016a). Antibiotic residues in human foods are found due to the overuse and abused utility of antimicrobial agents in animal cultivation and agriculture (Lam *et al.*, 2013; Liu and Wong, 2013; Liu *et al.*, 2017), this therefore may contribute to the increased resistance.

2.6 Transfer of resistant determinants

The transfer of resistant determinants in bacteria can occur either through intrinsic or acquired mechanisms; where the intrinsic mechanism includes the specific naturally occurring genes or enzymes found in bacterial chromosomes being expressed, such as the beta-lactamases that can destroy an antibiotic before it is activated. However, the acquired mechanism involves the de novo mutation or acquisition of resistance genes from other organisms through the transfer of plasmids, bacteriophages or transposons that possess these resistant determinants (Tenover, 2006; Tang *et al.*, 2014; Brown-Jaque *et al.*, 2015). Therefore, the emergence of resistant strains is caused by the use of antimicrobial agents, furthermore activating these resistant mechanisms that may occur by attaining resistant genes from bacterial hosts or through spontaneous mutations (Tenover, 2006; Da Costa *et al.*, 2013). Lastly, these resistant determinants can also occur through transformation, conjugation or transduction, where the transfer may be facilitated by transposons and an incorporation of acquired resistant genes into the plasmids or genome of the host (Tenover, 2006; Von Wintersdorff *et al.*, 2016). Hence, the detection of bacterial resistant determinants is of health and clinical importance since they have severe implications.

2.6.1 Transformation

This is a process where bacteria with released DNA complement into the environment after cell lysis, and then it has its DNA segment acquired and incorporated by other bacteria. This changes the genotype of the recipient cell and also can move resistant genes into previously susceptible strains (Tenover, 2006). Therefore exchanging antibiotic resistant genes (Von Wintersdorff *et al.*, 2016).

2.6.2 Conjugation

Conjugation is a commonly employed process that is mediated by plasmids (Von Wintersdorff *et al.*, 2016). During this process in Gram-negative bacteria, a plasmid-carrying resistant genes from one bacterium is transferred to an adjacent bacterium through a sex pilus-an elongated proteinaceous structure that joins two organisms (Tenover, 2006).

2.6.3 Transduction

It is a rare mechanism during which the transfer of resistant genes between bacteria is achieved through bacteriophages (Tenover, 2006). Moreover, bacteriophages may play a bigger role in the spread of resistant genes.(Von Wintersdorff *et al.*, 2016). In a figure shown below, it is the summary of the explained processes.

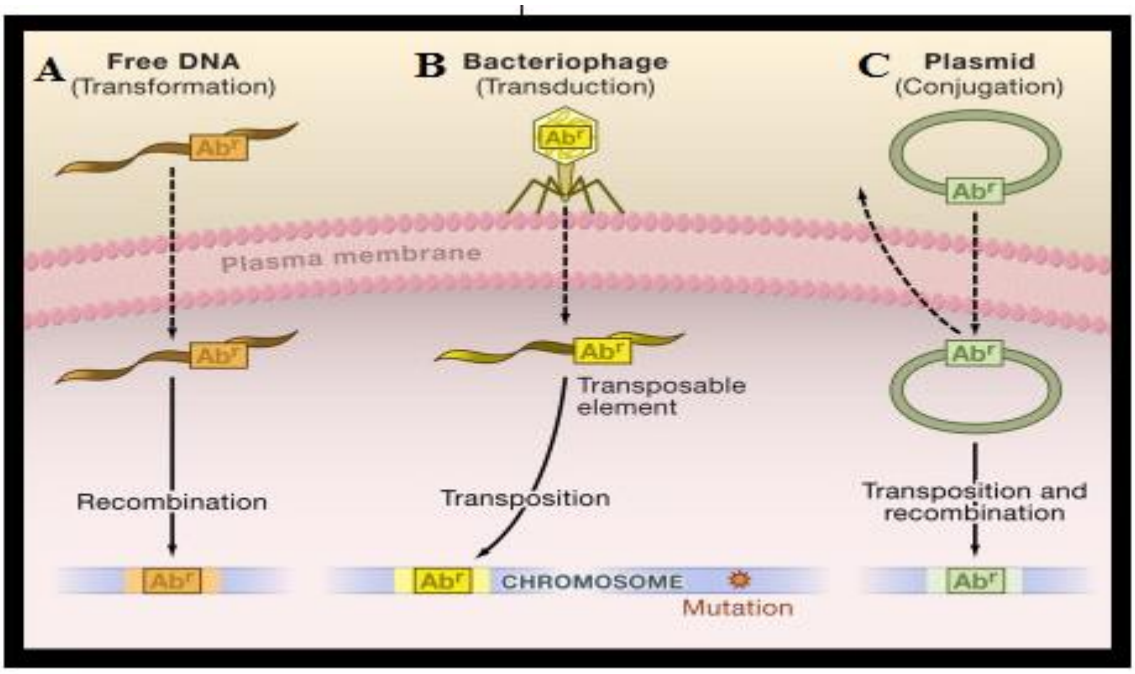


Figure 2.1: Processes of Antimicrobial Resistance Acquisition.

A= Transformation process, B= Transduction process and C= Conjugation process (Alekshun and Levy, 2007).

2.7 Extended spectrum beta-lactamase in *Escherichia coli*

2.7.1 Beta-lactamases

Beta-lactamases are classified as globular proteins that are grouped based on the similarity in the amino acid sequence or on their substrate and inhibitor profile; they share structural features that are similar and they possess alpha helices and beta- pleated sheets. They are also classified under class A- penicillinases, class B-metallo-beta—lactamases, class C-cephalosporinases and class D- oxacillinases; and their antibiotics are known to inhibit cell wall synthesis (Babic *et al.*, 2006; Bush, 2013). These enzymes are a great cause for the broad spectrum antibiotic resistance; they have capabilities that deactivate beta-lactam based antibiotics (Gazin *et al.*, 2012). Consequently studies show that these enzymes provide a worthy example of how resistance mechanisms evolve mostly in Gram-negative bacteria

(Gniadkowski, 2008); considering that beta-lactam antibiotic resistance is also experienced in *Enterobacteriaceae*, basically due to the B-lactam-mediated antibiotic hydrolysis while a small part is engaged by an altered expression of efflux pumps or porins (Gazin *et al.*, 2012). Furthermore, the resistance mechanism in Gram-negative bacteria caused by the destruction of beta-lactam antibiotics beta-lactamases produced by these bacteria is of great importance (Babic *et al.*, 2006) since it leaves minimal treatment that can be administered.

2.7.2 Extended Spectrum beta-lactamases

Extended Spectrum beta-lactamases are generated by *Enterobacteriaceae* as enzymes that hydrolyze beta-lactams, plasmids that regularly carry genes that transfer resistance to different antimicrobial classes (Paterson and Bonomo, 2005). The types of genes encoded by ESBLs are CTX-M, SHV and TEM amongst others (Shaikh *et al.*, 2015). Not only these enzymes are extensively spread between *Enterobacteriaceae* especially in *E. coli* and *Klebsiella pneumonia* species due to the misuse of antimicrobial agents, but also can cause a treatment to be complex due to their ability to promote and hydrolyze resistance to a diverse class of beta-lactam antimicrobial agents (Mosqueda-Gómez *et al.*, 2008; Peirano *et al.*, 2012; Deng *et al.*, 2017). Moreover, *E. coli* has developed resistance to recent 3rd generation cephalosporins, this makes it challenging for this generation of cephalosporins to treat ESBL producers (Gelband *et al.*, 2015). The high prevalence of beta-lactamases in some bacteria such as ampicillin hydrolyzing TEM-1 and SHV-1 beta-lactamases in *Escherichia coli* resulted in the development of Cephalosporins- antibiotics that are effective against beta-lactamase producing organisms; which were also used to minimize the transfer of beta-lactamases to new hosts (Paterson and Bonomo, 2005). Moreover, the existence of resistant *E. coli* expressing ESBL presents limited alternatives of antibiotic therapy and treatment, also

suspends time for patients who are infected to be administered with proper therapy (Mcdanel *et al.,* 2017).

2.7.3 Extended Spectrum Beta Lactamases types

2.7.3.1 CTX-M beta-lactamase type

CTX- M type beta-lactamases are enzymes under Class A of ESBLs due to a distinct lineage of molecular class A beta-lactamases that they constitute; they are a fast growing group that has more than 50 allelic variants that are grouped into sub-lineages (Rossolini *et al.,* 2008). CTX is an abbreviation for cefotaxamine, against which beta-lactamases reflect a potent hydrolytic activity (Paterson and Bonomo, 2005). These enzymes are the most widely spread and some variants such as CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-5 are most recognized in ESBL strains (Bonnet, 2004). CTX-M is dominant in most settings, especially in the ESBL producing *E. coli* and *K. pneumonia* (Rossolini *et al.,* 2008). For example, CTX-M-15-located on highly mobile IncFII plasmids and associated with mobile genetic elements, is now found in other *Enterobacteriaceae* species and at first it was only found in *Escherichia coli* (Bush and Fisher, 2011; Woodford *et al.*, 2011). Furthermore, CTX-M carrying plasmids are transferable through conjugal transfer amongst bacteria (Rossolini *et al.*, 2008).

2.7.3.2 SHV beta lactamase type

SHV beta lactamase type is also an enzyme that belongs to the class A ESBLs; they hydrolyze beta-lactam antibiotics that have the oxyimino side-chain (Paterson *et al.,* 2004; Shaikh *et al.,* 2015). The inhibition of SHV activity by p-chloromercuribenzoate was substrate related, as a result SHV refers to sulfhydryl variable (Paterson and Bonomo, 2005). SHV beta lactamase can be divided into subgroups influenced by their functional and molecular characteristics; SHV beta lactamase type is mostly found in ESBL producing

Enterobacteriaceae, especially *Escherichia coli* and *K. pneumonia*- which is also learned to be the precursor (Liakopoulos *et al.*, 2016). This enzyme also has variant alleles, the first one being SHV-1 residing in bacterial chromosome and is commonly found in *K. pneumonia* ;it was later moved into the plasmid which caused the transfer to other *Enterobacteria species* (Shaikh *et al.*, 2015; Liakopoulos *et al.*, 2016). SHV-1 therefore confers resistance to a wide range of penicillins, but not to the oxymo-substituted cephalosporins (Livermore, 1995; Shaikh *et al.*, 2015).

2.7.3.3 TEM beta lactamase type

The TEM-type beta lactamase is a derivative of TEM-1 and TEM-2. TEM is an abbreviation for Temoneira-a patient from which the first *E. coli* harbouring this gene was isolated (Datta and Kontomichalou, 1965; Paterson and Bonomo, 2005). TEM-1 is unable to attack the oxyimino cephalosporin, it is capable of hydrolyzing penicillin and first generation cephalosporins; while TEM-2 a derivative of TEM-1, has similar hydrolytic profiles except it has more active native promoter and a different isoelectric point. They are also inhibited by a clavulanic acid (Paterson and Bonomo, 2005; Shaikh *et al.*, 2015). Various other TEM derivatives were discovered, for example the TEM-3- which has increased activity against ESBLs (Shaikh *et al.*, 2015); these derivatives have reduced affinity for beta-lactamase inhibitor (Paterson and Bonomo, 2005). Therefore, this justifies the need of studying the relation of this gene with bacteria such as *Escherichia coli.*

2.7.3.4 OXA beta lactamase type

The OXA-type enzymes have the ability to hydrolyze Oxacillin, hence abbreviated OXA (Shaikh *et al.*, 2015), they are characterized by the rates of hydrolysis for cCoxacillin and benzyl penicillin (Bush *et al.*, 1995). These enzymes belong to the Class D ESBLs (Pfeifer *et al.*, 2010) and they are predominantly found in *Pseudomonas aeruginosa* (Weldhagen *et al.*, 2003). Despite that, they have been found in other Gram-negative bacteria, in *E. coli* amongst

others at 1-10%, which has the OXA-1 type (Livermore, 1995). Most of the OXA-type does not hydrolyze the extended spectrum cephalosporins, unlike the other beta-lactamase types that hydrolyze it; hence, it is not regarded as an ESBL. Despite this the OXA-type is transferrable, though with a low epidemiological data (Paterson and Bonomo, 2005).

2.7.3.5 Resistance to Beta-lactam antibiotics

Antibiotics exist in various classes that are grouped based of their mechanism of action. Unfortunately, bacteria have developed resistance mechanisms against these classes of antibiotics (Singh *et al.*, 2017). Beta-lactam antibiotics are one of the various classes that were effective in treating bacterial infection, but are now considered to be the cause of beta-lactam antibiotic resistance directly or indirectly in Gram-negative bacteria globally (Bush and Fisher, 2011; Shaikh *et al.*, 2015). This is due to the fact that beta-lactam antibiotics were highly used in treating infection, especially if the causative agent is unknown; they have a broad-spectrum of target organisms (Seiffert *et al.*, 2013). Moreover, they have less toxicity compared to other antibiotics, excellent penetration ,can be manageable and is also versatile in the clinic. Which also made it interesting to use in veterinary medicine and be implemented as feed additives to promote growth (Seiffert *et al.*, 2013). Thus beta-lactam-mediated antibiotic resistance is the most thoroughly studied mechanism compared to the other resistant mechanisms (Bush and Fisher, 2011).

There are three types of mechanisms that are employed by Gram-negative bacteria and may be the cause of resistance to beta-lactam antibiotics. These mechanisms occur either through mutations in the penicillin-binding proteins (PBPs), or reduced permeability of the cell wall synthesis (disruption of porin proteins, efflux system) and production of beta-lactamase enzymes able to hydrolyse and inactivate the beta-lactam ring- this is the most frequent mechanism in the family *Enterobacteriaceae* (Seiffert *et al*., 2013). These mechanisms are

learned to be responsible for resistant patterns in beta-lactam antibiotics, they can either be observed individually or united in bacteria (Bush and Fisher, 2011).

2.8 Treatment options for ESBLs producing *E. coli* infections

Microorganisms that are ESBL-producing were previously associated with *K. pneumoniae*, but recently they are also associated with community-acquired infections caused by *Escherichia coli* and this remains a threat in public health (Paterson and Bonomo, 2005; Blaak *et al.*, 2014). ESBL-producing *E. coli* are present in commensal of food-producing animals and healthy humans and generally do not cause disease, though the spread of ESBL-producing variants between animals and humans raises concern (Trott, 2013; Blaak *et al.*, 2014). Given the fact that ESBL-producing pathogens may degrade majority of antibiotics belonging to the beta-lactam group, other cephalosporins including 3rd and 4th generation and are resistant to other numerous antibiotic classes (Blaak *et al.*, 2014; Harris *et al.*, 2015). Infection caused by these types of bacteria therefore presents with limited treatment option, leading to last-resort antibiotics such as carbapenems being used in high rates (Cantón *et al.*, 2012).

Despite the fact that carbapenems are regarded as first choice treatment for ESBL caused infections, particularly in *Enterobacteriaceae* (Cantón *et al.*, 2012), resistance to these antibiotics by Gram negative bacteria has also been reported (Harris *et al.*, 2015). Moreover, the treatment options of ESBL-producing bacteria by carbapenems is reduced to the selection pressure for carbapenems resistance (Harris *et al.*, 2015). Carbapenem- resistance has led to Colistin or Tigecycline last line antibiotics to be employed as treatment options (Harris *et al.*, 2015; Yu *et al.*, 2016). Regardless, treatment options are narrowing due to constant emergence of resistance.

2.9 Colistin and emerging resistance

Recently, the increased emergence of antibiotic resistance is currently recognized as the greatest threat to human health world-wide (Liu *et al.*, 2016). Especially of the Colistin antibiotic-since it is active against multidrug-resistant gram-negative bacteria and it belongs to the last line of antibactericidal antimicrobial drugs (Wong *et al.*, 2016). Colistin-also known as Polymyxin E has a broad spectrum activity and it is a family member of the Polymyxins, cationic peptides (Liu *et al.*, 2016), has recently developed a plasmid-mediated Colistin resistant gene *mcr-1* in *E. coli* which may result in this antibiotic being inefficient in treating *mcr-1* carrying *Enterobacteriaceae* infections (Klevens *et al.*, 2006; Wong *et al.*, 2016).

Until recently, the Polymyxin resistance mechanism was reported to be chromosomally mediated and not known to be transferable from cell to cell, the mechanism is learned to be the modification of a "lipid A" evolving to the Polymyxin affinity being minimized (Falgenhauer *et al.*, 2016; Liu *et al.*, 2016). However, in *Escherichia coli* and *Klebsiella pneumoniae*, a transferable plasmid conferring resistance to Colistin was detected and it has been detected in animals, animal products and human bacterial species (Hasman *et al.*, 2015; Falgenhauer *et al.*, 2016; Liu *et al.*, 2016; Stoesser *et al.*, 2016). Comparing the prevalence of isolates between animals and humans (clinical isolates) harbouring the *mcr-1* gene around the world, literature indicates that animals and animal products are the prospective origin of this gene in humans; which may be caused by the careless use of Colistin in agriculture and poultry industry (Elnahriry *et al.*, 2016). This plasmid-encoded resistance can be transferred through horizontal transfer to humans from the infected farm kept animals that are being treated with Colistin (Falgenhauer *et al.*, 2016). The life span of Polymyxins as the resolution

of the prescribed medication against the multi-drug resistant gram-negative bacteria will be limited and unavoidable (Paterson and Harris, 2016). Hence, it should be emphasized that resistance is largely detected on mobile genetic elements such as plasmids, transposons, integrons, gene cassettes and bacteriophages, indirectly transporting the ability for resistance from non-pathogens to pathogenic micro-organisms and in between *E. coli* strains (Kemper, 2008; Paterson and Harris, 2016), since it may lead to current therapy alternatives being challenged (Falgenhauer *et al.,* 2016).

2.9.1 Colistin resistant genes

2.9.1.1 mcr-1

The mobile/mediated Colistin resistant-1 (*mcr-1*) gene encodes resistance to Colistin in *E. coli* isolates from animals and human, its mechanistic aspect are not yet fully known even though it is said to be a plasmid-encoded gene (Gao *et al.,* 2016; Liu *et al.,* 2016). *MCR-1* is capable of horizontal transfer between various bacterial strains and species; it is the first Polymyxin resistant gene known to be capable of this form of transfer (Haenni *et al.,* 2016; Liu *et al.,* 2016). Moreover, the mechanism of action is initiated through the modification of the Colistin target, therefore catalyzing the transfer of the phosphoethanolamine (PEA) onto the glucosamine saccharide of lipid A in the outer membrane of the bacteria (Hinchliffe *et al.,* 2017). Consequently, *mcr-1* causes a great concern since it is spreading worldwide.

2.9.1.2 *mcr-2*

MCR-2 is a recently discovered gene which is also a plasmid-mediated Colistin resistance gene that encodes resistance to Colistin through the phosphoethanolamine transferase, can modify the intensity of a lipopolysaccharide; hence it is known to share most of the amino acid activity with mcr-1 (Liassine *et al.*, 2016; Xavier *et al.*, 2016). This gene was discovered in an *E. coli* strain isolated from a pig (Xavier *et al.,* 2016). Thus far, the route of transfer,

mechanism and origin of the gene are unknown; MCR-2 present another public health threat, therefore an instant global surveillance is required (Sun *et al.*, 2017).

2.10 Correlation of Colistin and ESBL resistance

According to literature, multi-drug resistant microorganisms have restricted the treatment of infections leading to Polymyxins, especially Polymyxin B and E (Colistin) being mostly used as the last resort of antibiotics to treat these severe infections caused by multi-drug resistant Gram-negative bacteria to prevent high rates of morbidity and mortality (Gales *et al.*, 2011; Bonelli *et al.*, 2014; Bradford *et al.*, 2016). Unfortunately, shortly after the re-introduction of Colistin, not only there was an emergence of Colistin and carbapenems resistance but also an extended-spectrum beta lactamase producing *K. pneumoniae* that has a high rate of Colistin resistance has been reported in France (Caspar *et al.*, 2017). An example of this case was learned in carbapenems-resistant *K. pneumoniae* isolates obtained in various countries (Gales *et al.*, 2011). Therefore the observation of ESBL genes and Colistin *mcr-1* gene possessed by the same plasmid raises concern since it can lead to multi-drug resistance (Baron *et al.*, 2016; Schwarz and Johnson, 2016). Moreover this *mcr-1* gene has been found in bacteria such as *Escherichia, Klebsiella, Salmonella, Shigella* and *Enterobacter*, especially in *E. coli* which was reported late in 2015 and again in 1980s a different group reported Colistin resistance in *E. coli* (Baron *et al.*, 2016). Another report extended to observe that pathogens carrying different resistant genes such as bla_{NDM-5}, bla_{CTX-M} and bla_{KPC} also carried the *mcr-1* gene (Du *et al.*, 2016; Falgenhauer *et al.*, 2016; Haenni *et al.*, 2016).

Extended spectrum beta-lactamase encoding genes mostly possess resistance genes that are situated on plasmids and are highly mobile, and may also affect antibiotic classes such as aminoglycoside and trimethoprim/ sulfamethoxazole (Paterson and Bonomo, 2005). Thus it is

known that the description of horizontal gene transfer amongst bacteria and animals is rising and this type of genetic transfer has the potential to influence evolution especially in animals (Hotopp, 2011). Furthermore, other studies show that the spread of these antibiotic resistant bacteria around communities are increased by environmental contamination, poor sanitation, crowded living conditions and non-portable drinking water supply (Bonelli *et al.*, 2014).

2.11 Treatment options for Colistin resistant *E. coli*

Multi-drug resistant Gram-negative bacteria are increasing, while novel antibiotics are decreasing and leaving little to zero treatment options for infections caused by these bacteria (Ozsurekci *et al.*, 2016). Combination therapy could be the only treatment option left currently, this can be achieved by employing two or more antimicrobial agents to eliminate or reduce the pathogens. Few cases have been reported that have used this method (De Maio Carrillho *et al.*, 2017). Despite that combination therapy has succeeded in some cases, more treatment options such as bacteriophage therapy should be explored since drug-resistance is a growing public threat.

2.12 Bacteriophages

2.12.1 Classification of phages

Bacteriophages, also referred to as phages, are viruses that are naturally occurring, they only infect and replicate inside bacteria(Harper *et al.*, 2014; Sagona *et al.*, 2016). They were discovered in 1917 by Felix d'Herelle, phages do not affect the microflora when attacking the bacterial cells of the host due to their highly specific activity and most of them target only one species (D'herelle, 1917; Kutateladze and Adamia, 2010; Sagona *et al.*, 2016). Bacteriophages can co-exist with their host in two forms, which is either lysogenic, insert themselves into bacterial genome and cause modifications or either lytic to replicate inside

the host and release more new phages that will infect more bacteria thus destroying host bacteria (Harper *et al.*, 2014). Their capabilities therefore give the phages a potential role in treating infectious diseases an emphasis, hence scientists and physicians are examining the possibility of phage therapy being developed as another option of reliable treatment since the rate of antibiotic resistant pathogens is rising (Kutateladze and Adamia, 2010).

2.12.2 Phage mode of action

Bacteriophages are obligate parasites that consists of two distinct modes of action characterizing their life style depending on the phage type or genetics and interaction with their bacterial host (Ptashne, 2004; Kittler *et al.*, 2017). The two life cycles are known as lytic and lysogenic cycles (Feiner *et al.*, 2015), and the factors influencing the decision of either life cycles by the phage were studied for *E. coli* infected by the lambda phage (Erez *et al.*, 2017).

2.12.2.1 Lytic life cycle

Replication of a lytic phage starts upon infection; it replicates its genome and packages into progeny phage particles that are later released through bacterial lysis (Feiner *et al.*, 2015; Kittler *et al.*, 2017). Furthermore this cycle leads to the destruction of the bacterial cell, hence in phage therapy lysogenic phages are preferred (Jassim and Limoges, 2014).

2.12.2.2 Lysogenic life cycle

Lysogenic phages may also be known as temperate phages due to their ability to induce both the lysogenic and lytic cycles over time (Kittler *et al.*, 2017). During the lysogenic cycle the phage genome integrates into the genome of the bacterium and then replicate along with the

replication of bacterial cells, resulting in the lysogenised bacteria being immune to further infection by the same-phage (Erez *et al.*, 2017; Kittler *et al.*, 2017). Furthermore a lytic cycle is induced by stressful conditions such as DNA damage (Feiner *et al.*, 2015). The depicted figure below, show the summary of the two life cycles.

A=It is the lytic life cycle and B= It is the lysogenic life cycle (Feiner *et al.*, 2015).

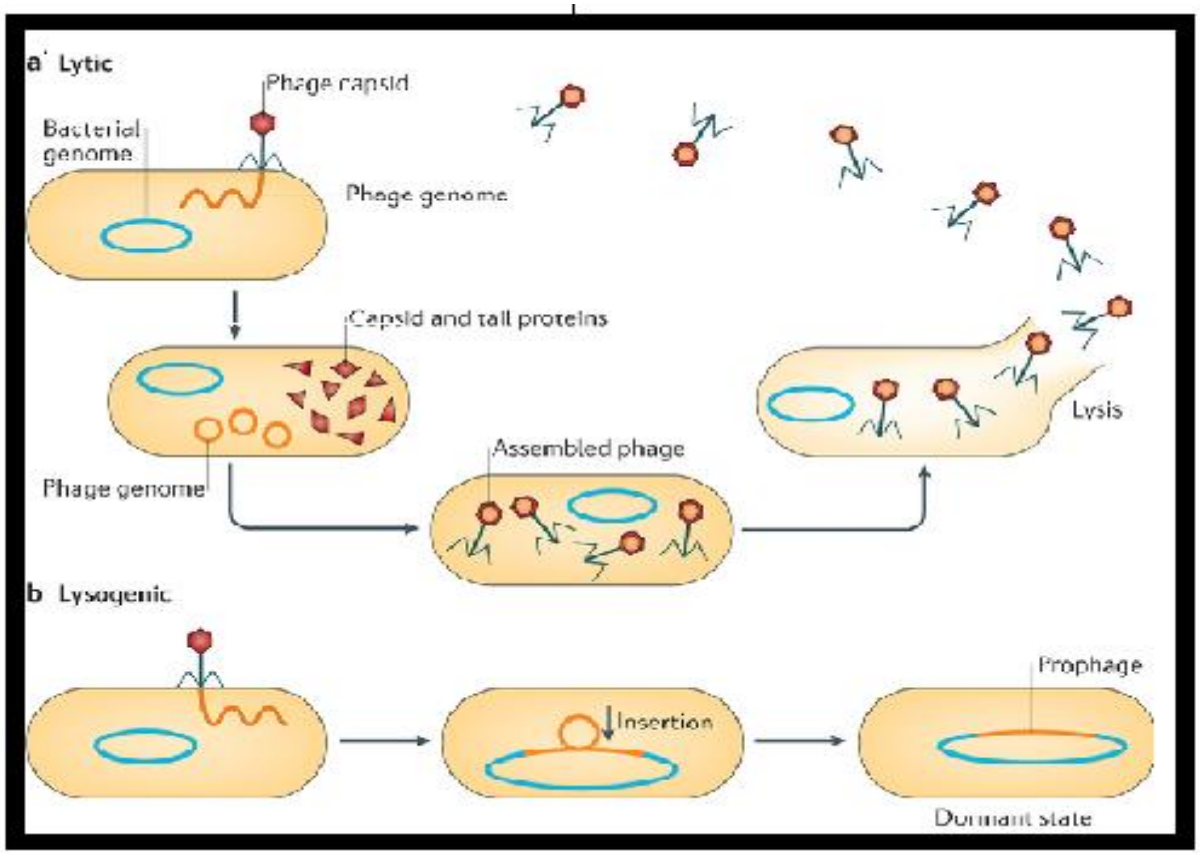


Figure 2.2: Phage replication cycles (Feiner *et al.*, 2015)

2.12.3 Phage therapy

An increasing incidence of bacterial antibiotic resistance to majority of available antibiotics is a serious challenge which is now leading to the possibility of phage therapy (Tanji *et al.*, 2004). Phage therapy is the use of bacteriophages to reduce or destroy bacteria which may express resistance to antibiotics; moreover, lysogenic phages are the preferred type for therapy (Harper *et al.*, 2014). Since the 1980s, phage therapy has been applied to control and

eliminate challenges caused by bacterial contaminants from food surfaces, gastrointestinal diseases caused by bacteria and food-borne spoilage bacteria; they have also been proposed as another option of treatment to antimicrobial agents in animal health in recent years (Garcia *et al.*, 2008). They can either be used as mono-phage preparations (where only one type of phage is involved) or as a cocktail (where a combination of various phages is used) to also reduce the challenge arising of the development of resistance of bacteria against single phages (Tanji *et al.*, 2004). For example newly added members of the bacteriophage family were isolated and characterized phages against the ESBL-producing *E. coli, K. pneumoniae* and mycobacteria which might be of importance in phage cocktail production (Ahmad *et al.*, 2015; Lu *et al.*, 2015; Teng *et al.*, 2015). In 2007, the US Department of Agriculture (USDA) had bacteriophage products targeting *Salmonella* species and *E. coli* O157:H7 approved (Aminov *et al.*, 2017). Nonetheless, not all isolated phages are acceptable to be used for therapy since complications are encountered in the interactions between the bacteriophage and its host (Wei *et al.*, 2015).

2.13 Conclusion

Literature has shown that both Colistin and ESBL- producing *Enterobacteriaceae* have become a huge health and public concern in veterinary health and in public since they present with an increased prevalence of antibiotic resistance. These ESBL-producing strains have been associated with a number of outbreaks worldwide especially those that are isolated from *E. coli* and *K. pneumoniae* species, which are known to cause severe infections in humans. In addition, the plasmid-mediated genes conferring resistance to the last line of antibiotics present with limited options, which may increase the mortality and morbidity rates globally. Despite the emergence of antibiotic resistance, phage therapy may be the rescue.

Antibiotics being used on food-producing animals are the cause of increased antibiotic resistance and these animals are the sole reservoir of ESBL-producing strains, therefore bacteria with resistant determinants may be transferred to food products which at a later stage could be transferred to humans. Proper hygiene should be implemented at all times, for example in animal slaughtering and food preparation. Thus in this study investigating the effectiveness of bacteriophages against the emerging of Colistin resistance and ESBLs is important in order to help the epidemiology and to provide the public health implications of these non-O157 strains in the South African food chain.

CHAPTER 3

MATERIALS AND METHODS

3.1 Ethical clearance

Ethical clearance for the study was obtained from the Mafikeng Animal Research Ethics Committee and an ethics number (NWU-00066-15-S9) was assigned.

3.2 Control strains

Bacteria strains *E. coli* (ATCC 25922) and *Salmonella enterica* subsp diarizonae (ATCC 12325) were used as positive and negative controls respectively.

3.3 Sample size

This study was conducted at the North West University, Mafikeng Campus North West Province, South Africa. Cattle faeces samples were randomly collected from both communal and commercial farms. The number of samples that was collected during the current study was determined using the formula 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%)

For estimation of the prevalence of ESBL-producing *Enterobacteriaceae,* the sample size for this study was determined by using the prevalence of 8 % according to (Reist *et al.*, 2013) at

the 95% confidence level and absolute error of 5% using the formula described by (Charan and Biswas, 2013). Accordingly, the minimum sample size required for the study was 114 samples.

3.4 Collection of cattle faeces samples

One hundred and eighteen (118) faeces samples were collected from both communal and commercial cattle in randomly selected farms around the North West Province. The number of samples collected from the different sampling points is shown in Table 3.1. Animals were put in crush pens and minimally restrained. Feacal samples were collected directly from the rectum of the animals using sterile arm-length gloves by trained Animal Health technicians. The samples were placed in sterile sample collection bottles, labeled properly and immediately transported on ice to the Laboratory for analysis. Upon arrival in the laboratory, the samples were analysed for the presence of STEC strains. However, when it was not possible to analyze samples immediately, the samples were stored at 4°C and analysed within 24 hours.

Table 3.1: Stations from which samples were collected in the study

Nature of the farm	**Sample station**	**No. of samples collected**
Communal	Lokaleng	23
	Zeerust	15
Commercial	Koster	30
	Rooigrond	50
Total		**118**

3.5 Isolation of *E. coli* strains from feacal samples

Approximately 1gram of faeces samples was dissolved in 10 mL 2% (w/v) peptone water and homogenized by vortexing for 1 minute. The samples were serially diluted ten-folds using

sterile peptone water. Aliquots of 100 µL from each dilution were spread-plated on Sorbitol-MacConkey agar (SMAC). Plates were incubated aerobically at 37 ^{o}C for 24 hours. Non-sorbitol fermenting colonies were purified by sub-culturing on SMAC and plates were incubated aerobically at 37^{o}C for 24 hours. Pure colonies were then subjected to biochemical identification tests specific for *E. coli*.

3.6 Bacteria Identification tests

3.6.1 Genomic DNA extraction from presumptive isolates

Bacterial DNA was isolated from presumptive non-O157 *E. coli* isolates using the Zymo Research Fungal/Bacterial DNA MiniPrep^{TM} kit (Lot No: ZRC178482) obtained from the Epigenetics Company, USA according to the manufacturers' instruction. Overnight cultures were prepared by inoculating single pure colonies into 5 mL of sterile nutrient broth. Cultures were incubated at 37°C for 24 hours. Aliquots of 1.0 µL from each overnight culture were transferred into 1.5 µL sterile Eppendorf tubes and centrifuged at 10 000 rpm for 10 minutes. The supernatant was discarded and the pellet was re-suspended in 100 µL of sterile distilled water after which 95 µL of 2X digestion buffer as well as 5 µL of proteinase K were also added. The contents in the tubes were mixed by inverting and tubes were incubated at 55°C for 20 minutes on a pre-heated Bio-Rad heating block (Digital dry heat Bio-Rad). Following incubation, 700 µL of genomic lysis buffer was added to the tubes and to facilitate the lysing cells the tubes were thoroughly vortexed. The contents in the tube were later transferred into a Zymo-spin™ IIC Columns that were placed in collection tubes, and the samples were centrifuged at 10 000 rpm for 1 minute. The flow-through in the collection tubes was discarded and the columns were placed in new collection tubes. Aliquots of 200 µL of DNA pre-wash buffer were added to each spin column and centrifuged at 10 000 rpm for 1 minute. After centrifugation, 400 µL of DNA wash buffer was added to the spin columns and

centrifuged at 13 500 rpm for 1 minute. The flow-through in the tubes was discarded and the Zymo-spin™ IIC Columns were transferred into sterile 1.5 µL Eppendorf tubes. Aliquots of 70 µL of DNA elution buffer were added into the spin Columns. The tubes were incubated at room temperature for 3 minutes and centrifuged at 14 800 rpm for 30 seconds to elute the DNA. Presence of DNA was determined through separation on a 1% (w/v) agarose gel electrophoresis using 1X TAE buffer. A ChemiDoc imaging system (Bio-Rad ChemiDoc™ MP Imaging System, UK) was used to capture images using Gene Snap (version 6.00.22) software. The purity and concentration of the DNA extracted was determined using the Nano drop Lite spectrophotometer obtained from Thermo Fisher Scientific, USA. DNA samples were stored at -4°C for future use.

3.6.2 Amplification of 16S rRNA gene fragments

Bacterial 16rRNA gene fragments were amplified using DNA extracted from the isolates. DNA from 242 presumptive isolates was used to amplify the 16S rRNA universal gene fragments. The PCR reactions were performed using the oligonucleotide primer sequences in Table 3.3. Amplification was performed using a DNA thermal cycler (model- Bio-Rad C1000 Touch ^{TM} Thermal Cycler). Reactions were prepared in 25 µL standard volumes that constituted 12.5 µL of 2X DreamTag Green Master Mix, 0.5 µL both primers and 1µL of template DNA and 11 µL of nuclease free distilled water. Cycling conditions comprised an initial strand separation step of 94^{o}C for 3 minutes; 25 cycles of 94^{o}C for 1 minute, 55^{o}C for 1 minute, 72^{o}C for 2 minutes and a final extension step of 72^{o}C for 10 minutes (Korzeniewska and Harnisz, 2013). The PCR reagents were obtained from the Inqaba Biotechnical Industry Ltd, Sunnyside, Pretoria, South Africa.

3.6.3 Identification of *E. coli* by PCR analysis

The identities of a total 242 presumptive *E. coli* isolates were determined through amplification of the *uidA* gene fragments (Anastasi *et al*., 2010; Anbazhagan *et al*., 2011). control reaction tubes containing DNA from *E. coli* (ATCC 25922) and *Salmonella enterica* subsp diarizonae (ATCC 12325) were included as both positive and negative controls, respectively, in the experiments. Amplifications were performed using DNA thermal cycler (model- Bio-Rad C1000 Touch TM Thermal Cycler). The PCR reactions were performed using the oligonucleotide primer sequences in Table 3.3. The reactions were prepared in 25 µL volumes that constituted 12.5 µL of 2X DreamTag Green Master Mix, 11 µL RNase free distilled water, 0.5 µL mixture of both the forward and reverse primers and 1 µL of template DNA. The PCR reagents were obtained from the Inqaba Biotechnical Industry Ltd, Sunnyside, Pretoria, South Africa.

3.6.4 PCR for identities of *E. coli* O157 isolates

In order to determine the proportion of non-O157 *E. coli* isolates present among the collection, *E. coli* O157:H7 specific PCR was used to screen the identities of 242 confirmed *E. coli* isolates. This was used as the main exclusion criteria for possible *E. coli* O157:H7 isolates. Bacteria isolates were screened for characters of *E. coli* O157:H7 through amplification of the rfb_{O157} gene fragments (Morin *et al.*, 2004). PCR to amplify the rfb_{O157} gene fragments were performed using oligonucleotide primer sequences that appear in Table 3.4. The reactions were prepared in 25 µL standard volumes that constituted 12.5 µL of 2X DreamTag Green Master Mix, 11 µL RNase free distilled water, 0.5 µL of both the forward and reverse primers and 1 µL of template DNA. PCR cycling conditions comprised an initial denaturation of 94^{o}C for 3 minutes, 30 cycles of template denaturation at 94^{o}C for 40 seconds, 52^{o}C for 60 seconds and 72^{o}C for 20 seconds and a final elongation step of 72^{o}C for

10 minutes. The PCR reagents were obtained from the Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. The amplicons were stored at 4^{o}C until they were separated by electrophoresis.

3.6.5 Serological identification of non-O157 *E. coli* isolates using specific latex slide agglutination test

All the 242 *E. coli* isolates were negative for the O157 specific PCR and therefore were subjected to non-*E. coli* O157 serotyping by the slide agglutination assay using specific monovalent antisera targeting serotypes O161, O111, O104 and O145 respectively. All the antisera were obtained from Mast Diagnostics, UK. The test was performed according to the manufacturers' instruction and results were recorded.

3.7 Determining the haemolytic patterns of non-O157 *E. coli* isolates

Haemolytic activity of the 242 confirmed non-O157 *E. coli* isolates was determined by culturing on blood agar (Davis Diagnostics, South Africa), supplemented with 5% (v/v) sheep blood for phenotypic detection of EHEC hemolysin. The plates were incubated at 37°C for 24 hours and later assessed for the presence of haemolytic patterns.

3.8 PCR for the detection of STEC virulence genes in non- O157 *E. coli* isolates

All the 242 non-O157 *E. coli* isolates were screened for STEC virulent genes *stx*$_{1,}$ *stx*$_{2,}$ (encoding the shiga toxins) (Pass *et al.,* 2000), *eaeA* (attaching and effacing) and *hlyA* (encoding hemolysin). The oligonucleotide primer sequences that appear in Table 3.4 were used in PCR analysis. The reactions were prepared in 25 µL volumes that constituted 12.5 µL of 2X DreamTag Green Master Mix, 11 µL RNase free distilled water, 0.5 µL mixture of the

forward and reverse primers and 1 µL of template DNA. PCR cycling conditions comprised of an initial denaturation step at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 62.5°C for 30 seconds and elongation at 72°C for 30 seconds followed by a final elongation step at 72°C for 5 minutes. The PCR reagents were obtained from the Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. Amplicons were separated on a 2% (w/v) electrophoresis agarose gel. A ChemiDoc imaging system (Bio-Rad ChemiDoc™ MP Imaging System, UK) was then used to capture the image using Gene Snap (version 6.00.22) and determine relevant amplicon sizes.

3.9 Detection of Colistin resistance among non-O157 *E. coli* isolates

3.9.1 Antibiotic resistance assay for detection of Colistin resistance phenotypes

All the 242 isolates were subjected to the agar disc diffusion test in order to determine their susceptibility to Colistin based on standard protocols (CLSI, 2007). In order to perform the test, isolates were reviewed by culturing on Nutrient agar and plates were incubated at 37°C for 24 hours. Bacteria suspensions of the isolates were prepared and aliquots of 100 µL were spread-plated on Muller Hinton agar obtained from Merck Diagnostics, South Africa. Commercially obtained antibiotic discs impregnated with Colistin (25 µg) was placed on the inoculated plates and plates were incubated at 37°C for 24 hours. After incubation, the antibiotic growth inhibition zone diameter data were measured and results interpreted based on standard reference values (Table 3.2)

Table 3.2: Details of antibiotics used during the study. The concentration used as well as inhibition zone diameter data in (mm) were used to classify isolates as resistant (R), intermediate resistant (I) and susceptible (S) to a particular antimicrobial agent (Galani *et al*., 2008) .

Group	Antibiotic	Abbreviation	Disc conc.	R	I	S
Polymyxins	Colistin	C	25 µg	≤11	12-13	≥14

3.9.2 Molecular detection of Colistin resistant determinants

All non-O157 *E. coli* isolates were examined for the presence of Colistin resistant genes (*mcr-1* and *mcr-2*) by specific PCR analysis (Lui *et al.,* 2015). Oligonucleotide primer sequences used are shown in Table 3.5. PCR conditions comprised of an initial denaturation step at 94°C for 15 minutes, 25 cycles of 94°C for 30 seconds, annealing at 58°C for 90 seconds and elongation at 72°C for 60 seconds followed by a final elongation step of 72°C for 10 minutes. PCR products were stored at 4°C and resolved by electrophoresis. All isolates that possess Colistin resistant determinants were subjected to molecular typing.

3.10 Detection of ESBL resistant genes in Colistin resistant isolates

This test was done to determine the correlation between the prevalence of Colistin and ESBL resistant genes in non-O157 isolates, only those isolates that were positive for Colistin resistance determinants were subjected to ESBL specific PCR designed to amplify the bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ genes using specific PCR and primer sequences that appear in Table 3.5.

3.11 Pulsed Field Gel Electrophoresis of Escherichia coli non-O157

Representative non-O157 isolates (30) were subjected to genotypic characterization using the Pulse Field Gel Electrophoresis in order to determine their molecular subtypes by comparing the fingerprints of isolates that were collected from the different sampling points. In order to perform this, overnight cultures were prepared for each isolate using nutrient broth. Aliquots of 200 µL from each overnight culture were transferred into a sterile 1.5 mL eppendorf tube and 200 µL of molten agarose tube. The contents of the tube were mixed by pipetting, immediately transferred into the wells of the plug mold. Plugs were kept at room temperature to solidify and soaked in 5 mL of cell lysis buffer (50 mM Tris; 50 mM EDTA (pH = 8.0); 1% Sarcosyl containing proteinase K (0.1 mg/mL) for 15 minutes. The tubes containing the plugs were incubated at 55^{o}C using a Labex shaking incubator [(Labcom Laboratory Equipment PTY, LTD; USA] at 175 rpm for 2 hours. Plugs were washed in 10 mL of pre-heated sterile ultrapure water and sterile TE buffer, respectively. Chromosomal DNA in the agarose plugs were restricted using *Xba*I 200 µL of enzyme master mix (2.5 µL of *Xba*I, 175.5 µL sterile ultrapure water, 2.0 µL of Bovine serum albumin (BSA) and 20 µL of restriction buffer) and by incubating the plugs at 37°C for 2 hours. Slices of the restricted plugs were loaded on the comb, sealed with 1% (w/v) Seakem Gold agarose in TBE [Tris (0.1 M); Boric acid (0.1 M) and EDTA (0.002 M)] and allowed solidify. The comb was placed into the gel-casting tray and a 1% (w/v) agarose gel was prepared and allowed to solidify. The comb was removed and the wells were sealed using 1% (w/v) Seakem Gold agarose. Gels were placed in the electrophoresis chamber and electrophoresis was conducted using a CHEF DR-II (Bio-Rad, USA). Electrophoresis conditions included an initial switch time of 6.76 seconds, a final switch time of 35.38 seconds, voltage of 6V, included angle 120° and a run time of 19 hours. Gels were stained with ethidium bromide (0.01 µg/mL) for 30 minutes and later distained with deionized water for 30 minutes. Images were captured

using a Bio-Rad imaging system (Model Bio-RAD ChemiDoc^{TM} MP Imaging System, UK) using Gene Snap (version 6.00.22) software. Bacteria fingerprints were analysed using the Applied Maths Bio-numerics software (version 7.6.2).

3.12 Agarose gel electrophoresis

PCR products were resolved by electrophoresis on 1% (w/v) agarose gels (Sambrook *et al.*, 1989). Gels were stained with ethidium bromide (0.01µg/mL) and electrophoresis was conducted using a horizontal Pharmacia biotech equipment system containing 1X TAE buffer for an hour at 70V. A Quick-Load® 1 kb or 100 bp DNA molecular weight Ladder (#N0467S) obtained from New England Biolabs Ltd (UK) was included in each gel in order to confirm the sizes of the amplicons. Gels were viewed under the UV light Trans illuminator (Sambrook *et al.*, 1989), and images were captured using a Bio-Rad imaging system (Model Bio-RAD ChemiDoc^{TM} MP Imaging System, UK) using Gene Snap software (version 6.00.22)

3.13 Sequence analysis of PCR amplicons

The PCR amplicons were sequenced at Inqaba Biotec Pretoria and the identities of the isolates were confirmed using a Blast Search with the NCBI Search Tool: (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.14 Isolation of non-O157 *E. coli* specific bacteriophages from cattle faeces

Non-O157 *E. coli* environmental strains were used to specifically isolate bacteriophages from cattle faeces. To achieve this, 2 g of faeces was suspended in 10 mL lambda diluent [10 mM Tris Cl (pH 7.5), 8 mM $MgSO_4.7H_2O$], vortexed thoroughly and kept at room temperature for 60 minutes. Samples were later centrifuged at 5250 *rpm* for 10 minutes using a Hermle Ultra

Centrifuge (model Z300) to sediment the faeces. Aliquots of 1.8 mL from each supernatant was extracted, centrifuged at 11,000 *rpm* for 10 minutes and filtered through a 0.22 µm pore syringe filter (GVS Filter Technology, USA). Bacteriophages in the filtrates were enriched using Tryptic Soy broth (TSB) for 60 minutes at 37 ^{o}C . The enriched samples were later used for detection of phages based on the double layer agar assay (Niu *et al.*, 2012). In order to isolate bacteriophages, ten-fold serial dilutions of the phage lysates were prepared using Lambda diluent. Overnight cultures of competent log phase host bacteria cells (O.D of 0.4 at 600 nm) were also prepared using TSB containing 10 Mmol/L $MgSO_4$. Aliquots (100 µL) of each diluted phage lysate was added to 100 µL of the log phase bacteria culture and kept at room temperature for 10 minutes to facilitate attachment. An aliquot of 3 mL of 0.6 % (w/v) agarose was added into each tube containing the diluted phage lysate and the bacteria host culture. The contents were mixed by swirling and poured on solidified modified Nutrient agar plates. The plates were kept at room temperature to solidify and incubated aerobically at 37°C for 24 hours. Plates were observed for the presence of plaques.

3.15 Purification and enumeration of phages in stocks

Phages were purified three times by single-plaque isolation and stock filtrates were prepared using suitable host strains as described previously (Niu *et al.*, 2012). Titres of isolated phages in stock filtrates were also determined using the soft layer overlay technique (Sambrook and Russell, 2001).

3.16 Phage propagation

Plaques were picked from plates using sterile pipette tips and inoculated in 1.5 mL of Difco phage broth (DPB) and incubated at 4°C for phage to diffuse. Broth was centrifuged at 10000 g for 2 minutes. One milliliter of filtrate and 1 ml of overnight broth culture of each *E. coli*

strains were added to a Falcon tube containing 5 ml of DPB and incubated at 37°C in a shaking water bath for 6-7 hrs. The phage lysates were centrifuged at 9 000 g for 15minutes to remove bacterial debris, and the supernatant was filtered through 0.2 µm pore-size super acrodisc syringe filter.

3.17 Characterization of bacteriophages

3.17.1 Electron microscopy

Phages were purified by PEG (polyethylene glycol) using a standard procedure (Sambrook and Russell, 2001). Phage suspensions were centrifuged at 25000 g for 60 minutes and washed twice in ammonium acetate (0.1 mol l^{-1}, pH 7.0). Sediments were subjected to transmission electron microscopy in order to determine the morphology of the phages.

3.17.2 Bacteriophage virulent assay

The bacteriophage virulent assay was performed using the microtiter plate technique. Overnight cultures of isolates were prepared in TSB along with phage lysates. An amount of 180 µL of the overnight cultures was mixed with 20 µL of the filtered phage lysates and incubated at 37°C for 5 hours. TSB was used as a blank while the overnight cultures were used as the negative control. A spectrophotometer was used to read the results. In addition, plates were assessed visually for lysis of bacteria and thus bacteria growth inhibition.

Table 3.3: Oligonucleotide primers used for amplification of 16S rRNA and uidA *E. coli* specific gene fragments

Primers	**Sequence (5′-3′)**	**Targeted gene**	**Amplicon size (bp)**	**PCR conditions and cycles**	**Reference**
27F	AGAGTTTGATCATGGCTCAG	16S rRNA	1420	1 cycle of 3 minutes at 94°C, 25 cycles of 1 minute at 94°C, 1 minute at 55°C, 2 minutes at 72°C; 1 cycle of 10 minutes at 72°C	Korzeniewska and Harnez (2013)
1492R	GGTACCTTGTTACGACTT				
*uidA*F	CTGGTATCAGCGCGAAGTCT	*uidA*	556	1 cycle of 10 minutes at 95°C, 35 cycles of 45 seconds at 95°C, 30 seconds at 59°C, 1 minute 30 seconds at 72°C; 1 cycle of 10 minutes at 72°C	Anbazhagan *et al.* (2011)
*uidA*R	AGCGGGTAGATATCACACTC				

Table 3.4: Oligonucleotide primers used for molecular identification of *E. coli* O157:H7 isolated during the study, detection of shiga-toxins and the different cycling conditions utilized

Primer Name	**Oligonucleotide primer sequence (5'-3')**	**Target gene**	**Product size (bp)**
stx1F	ACGTTACAGCGTGTTGCRGGGATC	*stx*$_1$	180
stx1R	TTGCCACAGACTGCGTCAGTRAGG		
Stx2F	TGTGGCTGGGTTCGTTTATACGGC	*stx*$_2$	255
Stx2R	TCCGTTGTCATGGAAACCGTTGTC		
eaeAF	TGAGCGGCTGGCATGATGCATAC	*eaeA*	241
eaeAR	TCGATCCCCATCGTCACCAGAGG		
hlyAF	GCATCATCAAGCGTACGTTCC	*hlyA*	534
hlyAR	AATGAGCCAAGCTGGTTAAGCT		
rfbO157F	CGGACATCCATGTGATATGG	*rfb*$_{O157}$	259
rfbO157R	TTGCCTATGTACAGCTAATCC		

Table 3.5: Oligonucleotide primer sequences used to detect ESBL genes in non-O157 *E. coli* isolates

Primer Name	**Primer sequence (5' – 3')**	**Target gene**	**Amplicon size (bp)**
CLR F	CGGTCAGTCCGTTTGTTC	mcr-1	
CLR R	CTTGGTCGGTCTGTAGGG		
MCR2-IF	TGTTGCTTGTGCCGATTGGA	mcr-2	
MCR2-I R	AGATGGTATTGTTGGTTGCTG		
blaTEMF	AAACGCTGGTGAAAGTA	bla_{TEM}	822
blaTEMR	AGCGATCTGTCTAT		
blaSHVF	ATGCGTTATATTCGCCTGTG	bla_{SHV}	753
blaSHVR	TGCTTTGTTATTCGGGCCAA		
blaCTX-MF	CGCTTTGCGATGTGCAG	bla_{CTX-M}	550
blaCTX-MR	ACCGCGATATCGTTGGT		
blaOXAF	ATATCTCTACTGTTGCATCTCC	bla_{OXA}	619
blaOXAR	AAACCCTTCAAACCATCC		

CHAPTER 4

RESULTS

4.1 Detection of non-*E. coli* O157 isolates from cattle faecal samples

One hundred and eighteen (118) samples were collected and analysed for characteristics of non-O157 *E. coli* using CT-SMAC and 242 non-replicative isolates that presented macroscopic colonial morphologies were selected and subjected to bacterial identification tests that appear in Sections 3.6 and 3.7. The 242 isolates were then presumed to be non-O157 *E. coli* isolated since they tested negative to the rfb_{O157} primer.

4.2 Extraction of DNA from presumptive *E. coli* isolates.

DNA was extracted from all presumptive isolates (242) and control strain (*E. coli* (ATCC 25922) and *Salmonella enterica* subsp diarizonae (ATCC 12325) as described in Section 3.2. The snap shows the presence of DNA in the isolates, which was confirmed by electrophoresis on a 1% (w/v) agarose gel and DNA was of high quality with little or no fragmentation. Figure 4.1 indicates an agarose gel image of genomic DNA extracted from the isolates in the study.

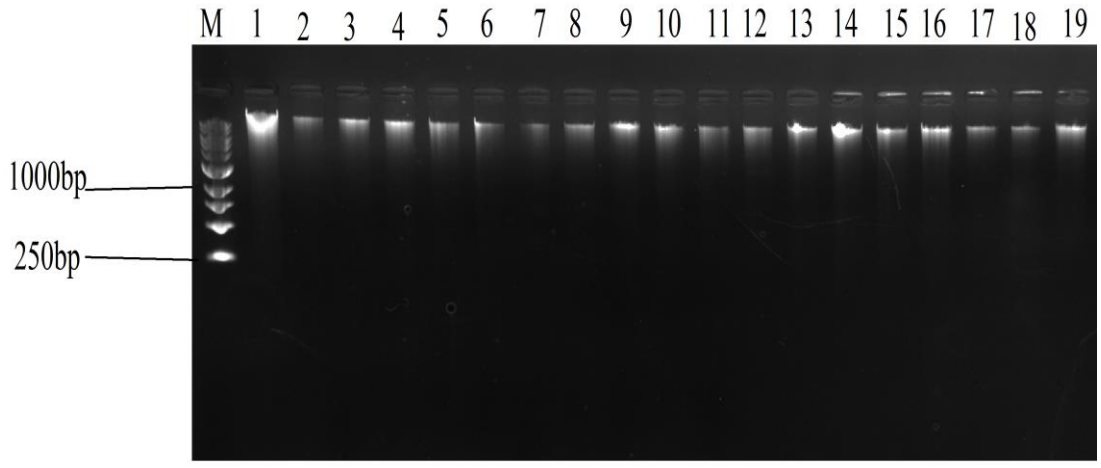


Figure 4.1: Agarose gel image showing DNA extracted from non-*E. coli* O157 isolates.

Lane M= DNA marker (O'GeneRuler 1 kilo base pairs DNA ladder), Lane 1-19 = Genomic DNA of isolates from cattle faeces samples.

4.3 The 16SrRNA gene analysis

Bacterial 16S rRNA gene fragments were amplified from all the 242 presumptive isolates. All (242; 100%) the isolates were positive for the universal 16S rRNA gene PCR analysis and detailed results are shown in Table 4.2. Figure 4.2 indicates a 1% (w/v) agarose gel image of bacterial 16S rRNA gene fragments amplified during the study. Amplicons possessed the expected size (1420 bp) and all these isolates were subjected to an *E. coli* species-specific PCR assay.

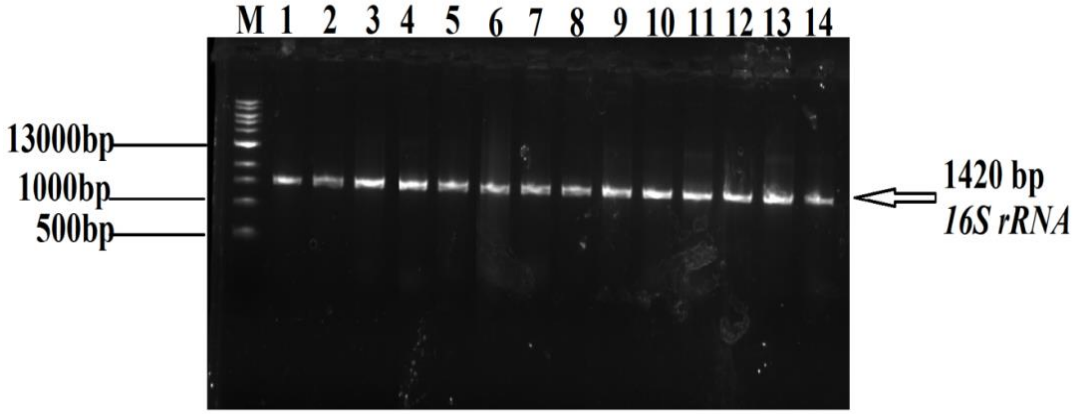


Figure 4.2: Agarose gel (1% w/v) image of 16S rRNA gene fragments.

Amplified from DNA extracted from non-O157 *E. coli* isolates and control strains. Lane M= 1 kb DNA ladder; Lane 1= 16S rRNA gene fragment amplified from DNA extracted from *E. coli* (ATCC 25922) positive control strain, Lanes 2-14= 16S rRNA gene fragments amplified from DNA extracted from non-O157 *E. coli* isolates obtained from cattle faeces.

4.4. Proportion of isolates confirmed as *E. coli* through PCR amplification of *uidA* gene

Escherichia coli species specific PCR was performed on all isolates that were positive for 16S rRNA gene by amplifying the *uidA E. coli* housekeeping gene. All isolates (242 = 100%) possessed the *uidA* gene fragment and were therefore confirmed as *E. coli* isolates. Detailed results are shown in Table 4.2. The expected amplicon size of 556 bp was obtained. Figure 4.3 indicates a 1% (w/v) agarose gel image of *uidA* gene fragments amplified from *E. coli* isolates and the *E. coli* (ATCC 25922) positive control strain.

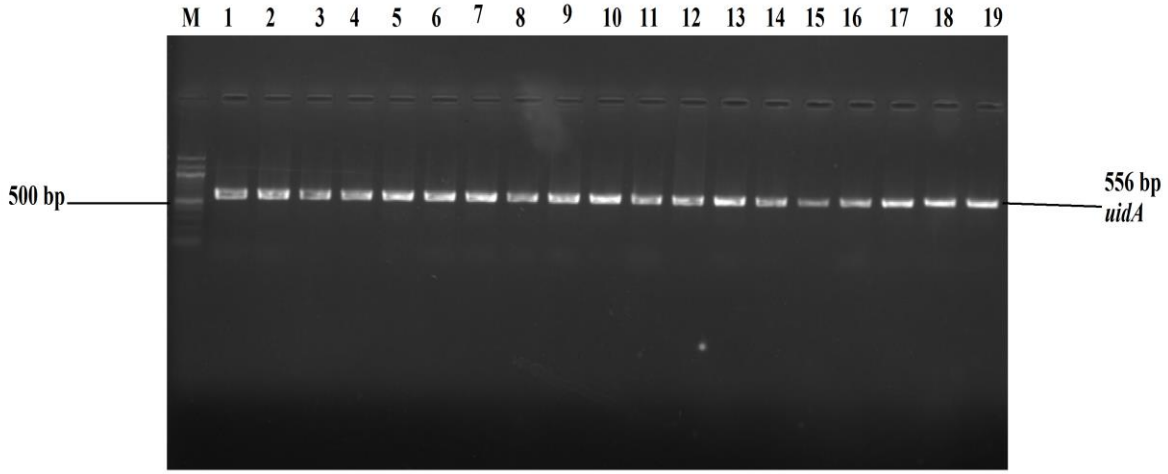


Figure 4.3: Agarose gel (1% w/v) image of *uidA* gene fragments.

Amplified from all *E. coli* isolates and *E. coli* (ATCC 25922) positive control strain. Lane M = 100 bp DNA molecular weight marker, Lane 1= uidA gene fragments amplified from *E. coli* (ATCC 25922) positive control strain, Lane 2 - 19= uidA gene fragments amplified from *E. coli* isolates.

4.5 Proportion of isolates confirmed as non-O157 *E. coli* using *E. coli* rfb_{O157} PCR analysis.

The identities of non-O157 *E. coli* isolates were confirmed for all isolates that were positive for *uidA E. coli* gene and this was achieved by performing an rfb_{O157} gene specific PCR.

Isolates were considered non-O157 strains if they were negative for the *rfb*$_{O157}$ gene sequence. In this study, all (242; 100%) the isolates did not possess the *rfb*$_{O157}$ gene fragment and were confirmed as non-O157 *E. coli* strains. The expected amplicon size of 259 bp was not obtained. Detailed results are shown in Table 4.1. The non-O157 isolates were subjected to serological assays in order to determine the serotypes that they belong to.

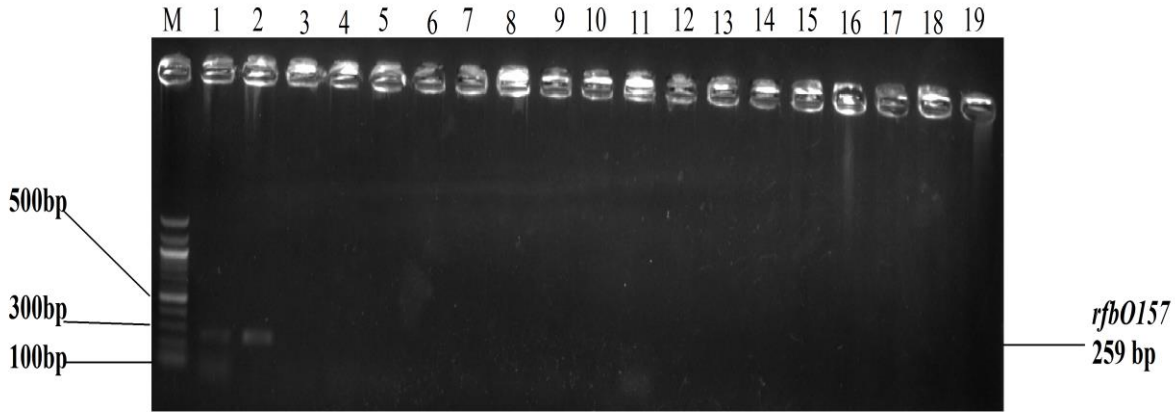


Figure 4.4: Agarose gel (1% w/v) image of *rfb*$_{O157}$ gene.

PCR performed on *E. coli* isolates and *E. coli* (ATCC 25922) positive control strain. Lane M = 100 bp DNA molecular weight marker, Lanes 1-2 = rfbO157 gene fragments amplified from an *E. coli* O157:H7 environmental positive control strain previously identified in our laboratory, Lane 3 – 19 = isolates that were negative for the rfbO157 specific PCR.

Table 4.1: Proportion of isolates that were positive for the different genes targeted during the study.

Sample site	**No. tested**	***16S rRNA***	**Genus specific gene**	**O157 specific gene**
			uidA	*rfb*$_{O157}$
Lokaleng	NT	48	48	48
	NP	48	48	0
Zeerust	NT	24	24	24
	NP	24	24	0
Koster	NT	88	88	88
	NP	88	88	0
Rooigront	NT	82	82	82
	NP	82	82	0
Total	**NT**	**242**	**242**	**242**
	NP	**242**	**242**	**0**

NT = Number tested; NP = Number positive

4.6 Proportion of non-O157 *E. coli* isolates Identified based on serological assays

Two hundred and forty-two (242) isolates were successfully isolated from cattle feacal samples and identified as non-O157 based on their potential to produce agglutination using four specific antisera (O161, O111, O104 and O145) that are designed to target isolates belonging to the different serogroups. Results obtained indicated that 55 (24.93 %) isolates were positively identified as members belonging to the targeted serogroups. Detailed results indicating the number of isolates belonging to each serogroup are shown in Table 4.2. As shown in Table 4.2, the proportion of isolates that were positive for the *E. coli* O161 serogroup was larger (22; 8.26%) than *E. coli* O111 (12; 4.96%), *E. coli* O104 (12; 4.96%) and *E. coli* O145 (11; 4.55%). *E. coli* O145 and *E. coli* O111 that belong to the non- *E. coli* O157 "big-six" serogroups were isolated in the current study. *E. coli* O104 has been associated with a recent foodborne outbreak that occurred in some European countries that include France, Germany, Belgium and Switzerland in 2011.

Table 4.2: Proportion of isolates that were positive for the different non-O157 *E. coli* serogroups targeted during the study. Superscripts "*" indicate that the number of isolates tested reduced since the number of isolates that tested positive for the previous serotype was deducted.

Serogroup	Positive isolates	Percentage of positive isolates
E. coli O161	NT = 242	8.3%
	NP = 20	
E. coli O111	NT = 222*	6.0%
	NP = 12	
E. coli O104	NT = 210*	5.0%
	NP = 12	
E. coli O145	NT = 198*	5.0%
	NP = 11	
Total	**Total NT = 242**	**24.3%**
	Total NP for the 4 antisera = 55	
Other non-O157 *E. coli* serotypes	**NP = 187**	**77.3%**

NT = Number tested; NP = Number positive

4.7 Proportion of non-O157 *E. coli* isolates positive for the different virulence genes

Two hundred and forty-two (242) isolates derived from cattle faeces were further screened for the presence of STEC virulent genes using specific PCR assays. The results obtained indicated that large proportions of the isolates possessed the stx_1 (104; 42.97%), stx_2 (161; 66.52 %), *eaeA* (178; 73.6%) and the *hlyA* (212; 87.6 %). Detailed results are shown in Table 4.3. As indicated in Table 4.3, a large proportion of the isolates possessed all the four virulence genes that were targeted and these isolates may be of great public health and clinical significance.

Figures 4.5, 4.6, 4.7 and 4.8 show gel images of the stx_1 (180 bp), stx_2 (255 bp), *eaeA* (384 bp) and *hlyA* (534 bp) gene fragments respectively amplified from the non-O157 *E. coli* isolates in the study.

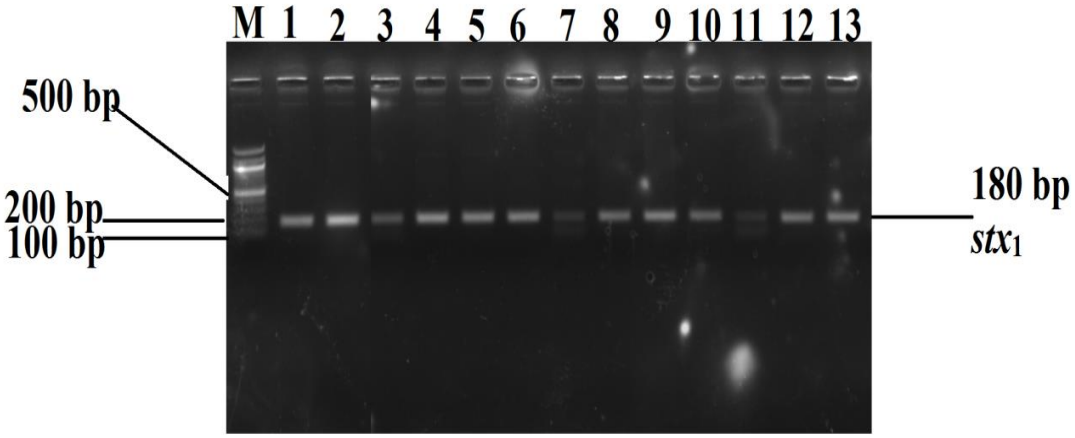


Figure: 4.5: Agarose (2% w/v) gel image of *stx*$_1$ gene fragments.

Amplified from all non-O157 *E. coli* isolates and the control strains. Lane M= DNA marker (100 base pairs DNA ladder), Lane 1=, Lane 2-13= stx1 gene fragments amplified from *E. coli* isolates.

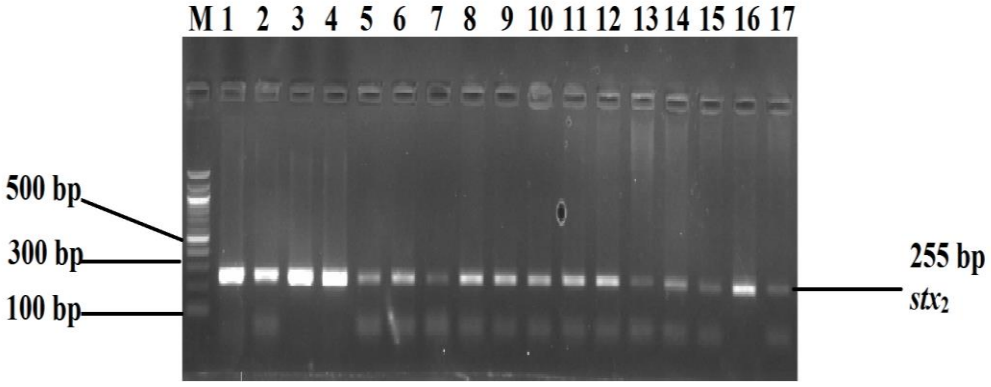


Figure: 4.6: Agarose (2% w/v) gel image of *stx2* gene fragments.

Amplified from all non-O157 *E. coli* isolates and the control strains. Lane M= DNA marker (100 base pairs DNA ladder), Lane 1=, Lane 2-17= stx2 gene fragments amplified from *E. coli* isolates.

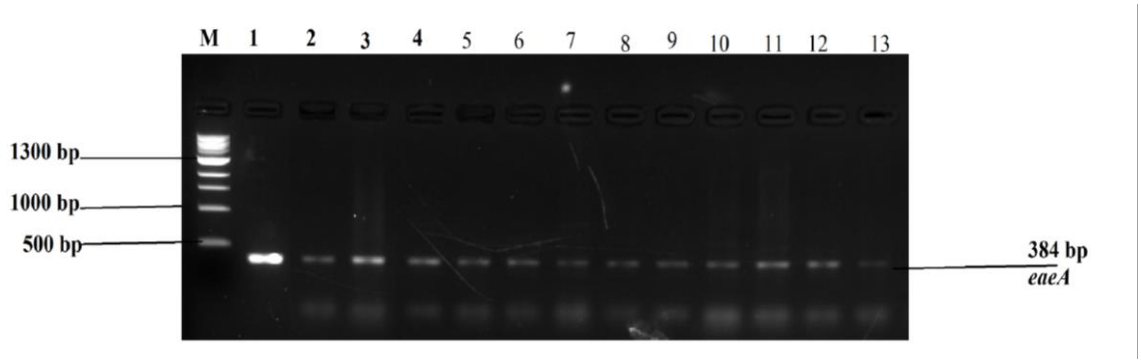


Figure: 4.7: Agarose (2% w/v) gel image of *eaeA* gene fragments.

Amplified from all non-O157 *E. coli* isolates and the control strains. Lane M= DNA marker (100 base pairs DNA ladder), Lane 1=, Lane 2=, Lane 3-13= eaeA gene fragments amplified from *E. coli* isolates.

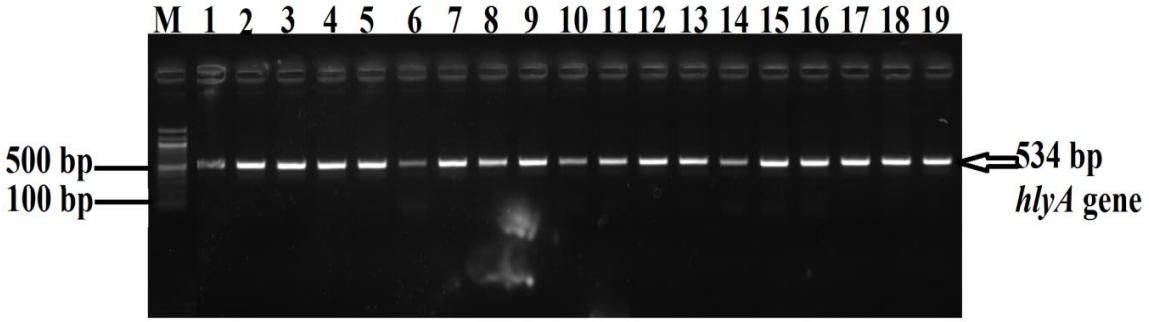


Figure: 4.8: Agarose (2% w/v) gel image of *hlyA* gene fragments.

Amplified from all non-O157 *E. coli* isolates and the control strains. Lane M= DNA marker (100 base pairs DNA ladder), Lane 1=, Lane 2-19= hlyA gene fragments amplified from *E. coli* isolates.

4.8 Haemolytic patterns of non-O157 *E. coli* isolates

All the 242 non-O157 *E. coli* isolates were screened phenotypically to assess their potential to produce haemolytic patterns on blood agar. Results obtained indicated that a large proportion (145; 59.9%) of these isolates were beta (β) haemolytic (Table 4.3).

Table 4.3: Proportion of isolates that were positive for the different STEC virulent genes targeted in the study.

Sample station	**No. tested**	**Virulence genes**				**Number positive for haemolysin production**
		***stx*$_1$**	***stx*$_2$**	***eaeA***	***hlyA***	
Lokaleng	NT	48	48	48	48	48
	NP	33	38	40	36	30
Zeerust	NT	24	24	24	24	24
	NP	8	14	15	21	15
Koster	NT	88	88	88	88	88
	NP	12	49	64	76	38
Rooigront	NT	82	82	82	82	82
	NP	51	37	59	79	62
Total	**NT**	**242**	**242**	**242**	**242**	**242**
	NP	**104**	**161**	**178**	**212**	**145**

NT = Number tested; NP = Number positive

4.9 Percentage resistance of non-O157 *E. coli* isolates to Colistin

All the 242 non-O157 *E. coli* isolates were screened phenotypically to assess their antibiotic resistance profiles to Colistin. Despite the fact that a large proportion (127; 52.5%) of these isolates were susceptible to colistin (Figure 4.9), a significantly higher proportion (100; 41.3%) displayed intermediate resistance to this antimicrobial agent. In addition, 15 (6.2%) of the isolates were resistant to Colistin and this was a cause for concern (Figure 4.9). The x-axis represent the number of isolates.

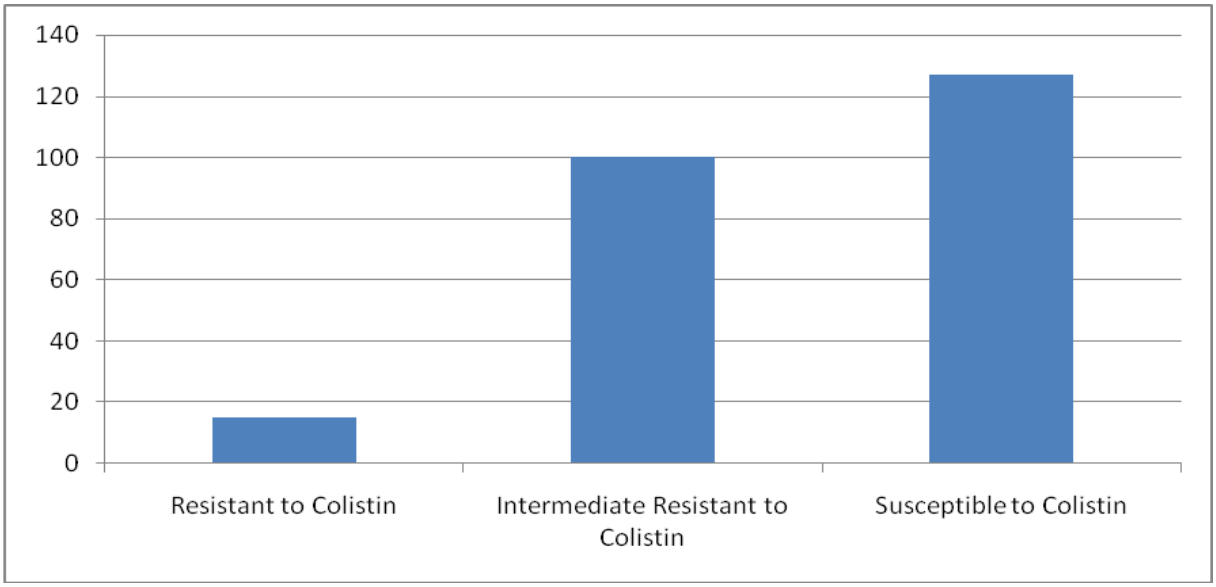


Figure: 4.9: Percentage antibiotic resistance of non-O157 *E. coli* isolates that were resistant to Colistin.

4.10 ESBLs profiles of non-O157 *E. coli* isolates

4.10.1 ESBL producing profiles on chromogenic agar

All the 242 non-O157 *E. coli* isolates were screened phenotypically to assess their potential to produce Extended Spectrum Beta-Lactamases (ESBLs) using the chromogenic Brilliance ESBL agar (Oxoid, UK). Results indicated that a large proportion (201; 83.1%) of the isolates produced blue colonies and were identified as *E. coli* isolates that produced ESBLs (Oxoid, UK).

4.10.2 Proportion of non-O157 *E. coli* isolates positive for the ESBL genes

Thirty (30) isolates that were resistant to Colistin and also displayed the potential to produce ESBLs on the chromogenic Brilliance ESBL agar (Oxoid, UK) were screened for the presence of ESBL genes using specific PCR assays. The results obtained indicated that large proportions of the isolates possessed the bla_{OXA} (20; 66.67%), $bla_{CTX\text{-}M}$ (11; 36.67 %), bla_{SHV} (6; 20%) and the bla_{TEM} (5; 16.67 %) genes. Detailed results are shown in Table 4.4. As indicated in Table 4.4, a large proportion of the isolates possessed bla_{OXA}, and a small

proportion of the isolates possessed the other three ESBL genes that were targeted and these isolates may be of great public health and clinical significance.

Table 4.4: Proportion of isolates that were positive for the different ESBL genes targeted in the study.

No. tested	**ESBL gene**			
	***bla*$_{OXA}$**	***bla*$_{CTX-M}$**	***bla*$_{SHV}$**	***bla*$_{TEM}$**
NT	30	30	30	30
NP	20	11	6	5
Percentage (NP/NT)	**66.67%**	**36.67 %**	**20%**	**16.67 %**

NT = Number tested; NP = Number positive

4.11 Sequence analysis of PCR amplicons

Bacterial 16S rRNA gene sequence data indicated that *E. coli* isolates possessed great (99%) similarities to *E. coli* strain Lc 16S ribosomal RNA gene partial sequence (Accession No: MF 104544.1), *E. coli* strain Lb 16S ribosomal RNA gene partial sequence (Accession No: MF 104543.1) and *E. coli* strain HCD38-2 16S ribosomal RNA gene partial sequence (Accession No: MH111663.1), *E. coli* strain HCD22-4 16S ribosomal RNA gene partial sequence (Accession No: MH111571.1), *E. coli* strain HCD41-3 16S ribosomal RNA gene partial sequence (Accession No: MH111682.1), *E. coli* strain HCD 39-1 16S ribosomal RNA gene partial sequence (Accession No: MH111669.1), *E. coli* strain HCD17-4 16S ribosomal RNA gene partial sequence (Accession No: MH111539.1) and *E. coli* strain HCD16-5 16S ribosomal RNA gene partial sequence (Accession No: MH111532.1).

4.12 Characterization of non-O157 *E. coli* specific bacteriophages

4.12.1 Transmission electron microscopy

Bacteriophages isolated from cattle faeces using environmental non-O157 *E. coli* host strains were subjected to morphological characterization using Transmission electron microscope. Transmission electron micrographs of the phages are shown in Figure 4.10 and phages were

most often identified to be intact with head, neck and tail regions. Morphological classification of the phages was achieved using previous guidelines (Ackermann, 2007; Ackermann, 2011; Lopes *et al.*, 2014), and the phages may belong to the family *Siphoviridae.*

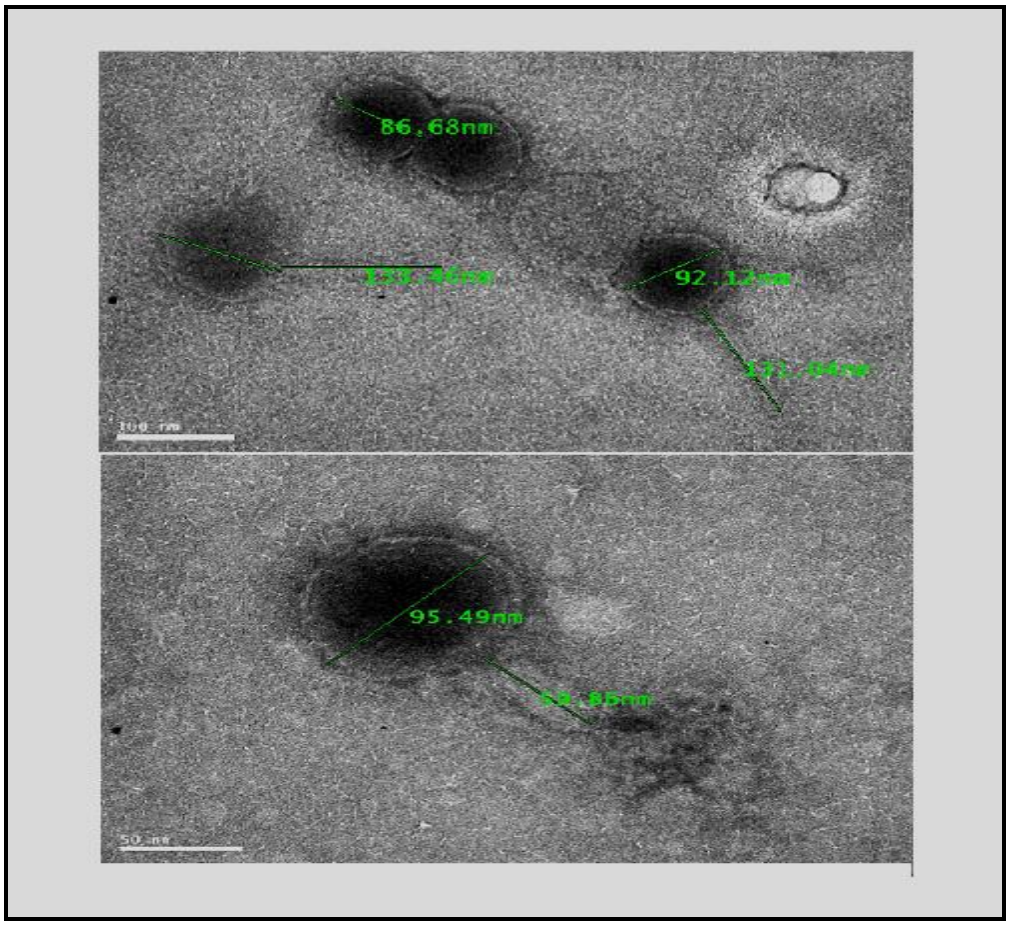


Figure 4.10: Transmission electron micrographs of non-O157 specific bacteriophages isolated from cattle faeces.

4.12.2 Bacteriophage virulent assay

Three bacteriophages designated BNEO1575E, BNEO1574D and BNEO1574C were used to assess their potentials to biologically control twenty environmental non-O157 *E. coli* isolates based on the virulence assay technique. Results were interpreted based on a previous report and presented as positive (+) when there was inhibition of bacteria growth or negative (-) when bacteria growth was not inhibited. The bacteriophage BNEO1575E was active against bacteria strains NEO1571C, NEO1572C and NEO1576C. However, the bacteriophages

BNEO1574D and BNEO1574C were active against bacteria strains NEO15710C, NEO1571E, NEO1578E, NEO15733E, NEO15752D, NEO15753D and NEO15778D and bacteria strains NEO15753D, NEO15778D, NEO1571E, NEO1578E, NEO15733E, NEO15752D, NEO1571C, NEO1572C, NEO1576C and NEO15710C respectively. Interestingly, more than one bacteriophage was active against the same set of bacterial strains. Results indicated that bacteriophages BNEO1575E and BNEO1574C were active against NEO1571C, NEO1572C and NEO1576C while bacteriophages BNEO1574D and BNEO1574C were active against NEO15710C, NEO1571E, NEO1578E, NEO15733E, NEO15752D, NEO15753D and NEO15778D. A summary of the results is shown in Table 4.5. Detailed results of the microplate assays are shown in the Appendix B tables of virulent assays.

Table 4.5: Virulence potentials of bacteriophages against potential environmental resistant bacteria non-O157 *E. coli* isolates.
The acronym BNEO157 indicates a bacteriophage isolated using a non-O157 *E. coli* isolate as the host bacteria strain. Isolates with the superscript "**" were susceptible host strains for phages BNEO1574D and BNEO1574C while those with the superscript "*+" were susceptible to phages BNEO1575E and BNEO1574C.

	Bacteriophage ID and plaque morphology* / virulence potentials		
Bacteria strains	**BNEO1575E (Small*)**	**BNEO1574D (Small*)**	**BNEO1574C (Small*)**
NEO1571C*+	+	-	-
NEO1572C*+	+	-	-
NEO1576C*+	+	-	-
NEO15710C**	-	+	-
NEO1571E**	-	+	-
NEO1578E**	-	+	-
NEO15733E**	-	++	-
NEO15752D**	-	+	-
NEO15753D**	-	++	-
NEO15778D**	-	++	-
NEO15753D**	-	-	+
NEO15778D**	-	-	+
NEO1571E**	-	-	+
NEO1578E**	-	-	+
NEO15733E**	-	-	++
NEO15752D**	-	-	+
NEO1571C*+	-	-	+
NEO1572C*+	-	-	++
NEO1576C*+	-	-	+
NEO15710C**	-	-	+

BNEO157 = Bacteriophage Non-*E. coli* O157; + = bacteria growth inhibited; - = bacteria growth not inhibited.

4.13 Pulsed-Field Gel Electrophoresis *Xba*l of non-O157 *E. coli* isolates

A total of 30 multidrug resistant non-O157 representative isolates that possessed both Colistin and ESBL resistant traits were subjected to genotypic characterization using the Pulse Field Gel Electrophoresis in order to determine their molecular subtypes. Figure 4.11 indicates a 2% (w/v) agarose gel of the Pulse-Field gel electrophoresis patterns of chromosomal DNA profiles using an *Xba*l enzyme for the non-O157 *E. coli* isolates. The

PFGE of *Xba*l-digested chromosomal DNA patterns from all the 30 isolates produced stable and reproducible fingerprints. Isolates were clustered into two main groups designated (Clusters I and II) as shown in Figure 4.12. The two clusters were further analysed for associations of isolates from the different farms (Table 4.6). The largest cluster (cluster I) with 21 non-O157 STEC Colistin and ESBL-producing isolates comprised isolates from all the two Communal farms in Lokaleng and Zeerust as well as both Commercial farms in Koster and Rooigrond respectively. On the contrary, the smaller cluster (Cluster II) contained 9 isolates from three farms. The make-up of isolates in this cluster comprised 5 and 3 isolates from the commercial farms in Koster and Rooigrond as well as one isolate from the communal farm in Zeerust. Generally, both clusters were considered to be mixed since they contained isolates from more than one farm (Figure 4.12). The findings of this study also indicate a high genetic similarity between isolates from the same as well as different farms thus suggesting high levels of cross-contamination in the area.

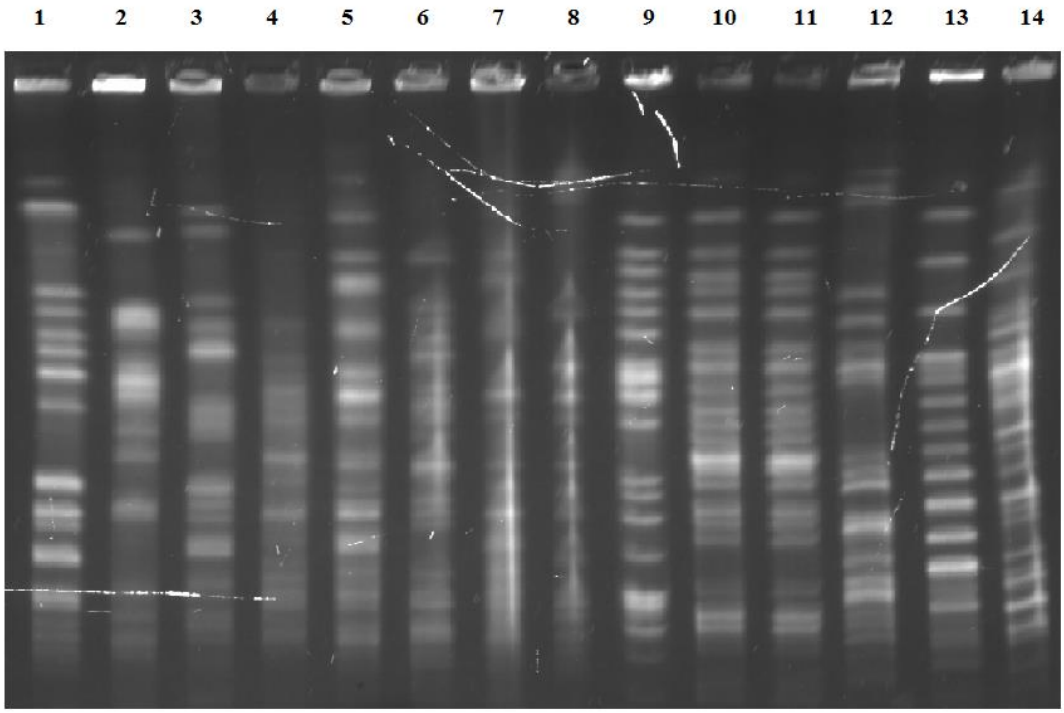


Figure 4.11: Pulse-Field gel electrophoresis image of non-O157 STEC that are Colistin and ESBL-producing, using the *Xba*l enzyme

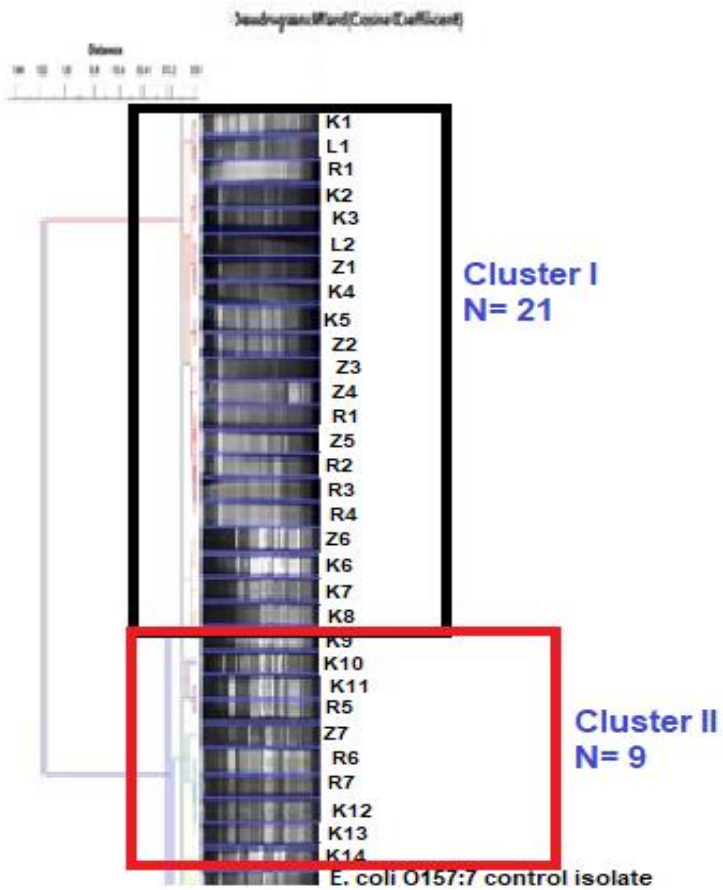


Figure 4.12: Dendrogram of PFGE patterns of non-O157 STEC Colistin and ESBL-producing isolates based on *Xba*I PFGE analysis.

Table 4.6: PFGE clusters of *E. coli* O157 isolates from different farms

PFGE clusters		No. of isolate per cluster	No. of isolates from farms per cluster	**Farms**
I	Communal farms	21	2	Lokaleng
			6	Zeerust
	Commercial farms		9	Koster
			4	Rooigrond
II	Commercial farms	9	5	Koster
			3	Rooigrond
	Communal farm		1	Zeerust

CHAPTER 5

GENERAL DISCUSSION

The main objective of this study was to determine virulence capabilities of endemic bacteriophages against non-O157 *E. coli* isolates from cattle that possessed Colistin and extended spectrum beta-lactam resistance determinants. This was motivated by the fact that a number of studies conducted in the area have revealed the presence of multiple antibiotic resistant *E. coli* O157 STEC strains from water intended for human consumption, animals and humans (Ateba *et al.*, 2008; Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011). In addition, all these studies focused on the serotype O157 that received significant attention worldwide mainly due to its very high pathogenic nature. However, recent studies have indicated that some non-O157 isolates, in particular those belonging to six serotypes O26, O45, O103, O111, O121 and O145 have been linked to a growing number of foodborne illnesses (Brooks *et al.*, 2005; Bosilevac and Koohmaraie, 2011; Monaghan *et al.*, 2011; Schulz *et al.*, 2015). These serotypes have been termed the "big six" (Brooks *et al.*, 2005). Due to their pathogenic nature, food-regulating authorities in the USA such as the United States Department of Agriculture (USDA) have included them in routine testing particularly in meat and raw ground beef (Alocilja and Radke, 2003).

Access to safe and affordable food is a basic human right because contaminated food may pose severe complications to consumers. In order to prevent the occurrence of foodborne infections in humans, regulatory procedures must be implemented during the production and processing of food products. In South Africa, the Department of Agriculture, Department of Health and the Department of Trade and industry are mandated to improve food safety nets

and food emergency management systems. The Agricultural Product Standards Act (1990) (Agriculture, 1990) is enforced by the Department of Health to ensure that it regulates food safety and quality of agriculture and animal products. However, the Department of Health is expected to ensure that all foodstuffs are safe for human consumption in terms of the Foodstuffs, Cosmetics and Disinfectant (FCD) Act of 1972 (Foodstuffs and Act).

The South African Bureau of Standards (SABS) operates under the umbrella of the Department of Trade and Industry and is mandated to ensure canned meat, frozen and canned fishery products are safe for consumption based on public health standards (Standards Act, 1993). In order to understand the risks associated with contaminated food as well as to conveniently develop regulations under the FCD Act, there is a need to have expert knowledge in a number of areas such as microbiology, mycotoxins, additives, animal husbandry and irradiation of food. Therefore, the government of South Africa has established the Food Legislation Advisory Group (FLAG) that is made up of academics, scientists, consumers and industry representatives. The FCD Act is delegated to provincial and local health authorities.

Global reports on the number of foodborne related illnesses and deaths as well as food safety scares recorded worldwide have amplified the need to implement strict meat safety control strategies (Newell *et al.*, 2010; Forsythe, 2011). To address this problem, most countries including South Africa have put in place preventative and systematic control systems particularly in abattoirs based on the Hazard Analysis Critical Control Point (HACCP) to ensure meat safety. In addition, South Africa has a recently regulated Hygiene Management System (HMS) derived from the HACCP principles to be utilized in abattoirs. Despite the availability of these food safety control systems, farm management techniques may greatly

affect the quality of meat products especially if processes in the HACCP and HMS systems are not fully implemented. It is against this background that the present study was designed to assess the occurrence of non-O157 *E. coli* isolates in beef cattle and further determine the potential of using endemic bacteriophages as bio-control agents against resistant strains. Data obtained from this study may be of great epidemiological importance and may provide only one strategy, control using bacteriophages, investigated for controlling non-O157 strains in the South African food chain.

The primary objective of this study was to isolate and confirm the identities of non-*E. coli* O157 isolates from cattle using sequence specific *E. coli* O157 PCR analysis and specific serogroup serological assays. In the present study, non-O157 *E. coli* STEC strains were successfully isolated and isolates belonged to the serogroups O111, O104, O161 and O145. However, isolates belonging to the serogroups O111 and O145 belonging to the non-O157 *E. coli* "big six" group and these have also been implicated in disease amongst consumers (Schulz *et al.*, 2015; Stratakos *et al.*, 2018).

Ruminants, and cattle in particular, have been identified as reservoirs for non-O157 *E. coli* isolates (Ateba and Bezuidenhout, 2008; Kh *et al.*, 2011). Given that non-O157 *E. coli* (STEC) strains particularly those belonging to serotypes O26, O45, O103, O111, O121 and O145 were linked to numerous foodborne outbreaks and sporadic cases of human infections ranging from uncomplicated diarrhoea to complicated HC (Brooks *et al.*, 2005; Bosilevac and Koohmaraie, 2011; Monaghan *et al.*, 2011; Schulz *et al.*, 2015), there is need for constant monitoring of these strains. In addition, results obtained from previous studies designed to report on the prevalence of *E. coli* O157 strains indicated that the proportion of isolates in pigs were higher than those in cattle despite the fact that cattle are considered the principal

reservoir (Ateba *et al.*, 2008; Ateba and Mbewe, 2011). It was suggested that the principal reservoir of *E. coli* O157 isolates in animal species in a given area could not be assumed which raises the need to monitor their occurrence in the North-West Province.

Another objective of the study was to determine the virulence gene (*stx*$_{1,}$ *stx*$_{2,}$ *eae*A and *hly*A) profiles of the non-O157 *E. coli* strains. A previous study conducted indicated significant diversity and variability of the virulence genes among *E. coli* O157 strains and not of non-O157 *E. coli* strains (Kim *et al.*, 1999; Ateba *et al.*, 2008). Since such a study had not been previously conducted in this area, it motivated the need for it to be done in this study considering that the prevalence of STEC virulence genes being unknown in this area.

Infections caused by STEC are exposed to the community, especially children, and this is spread by the practices of human-cattle contact. In the socio-economic and cultural activities, especially in rural and developing communities cattle play a central role (Germinario *et al.*, 2016). Moreover, literature stipulates that numerous factors such as geographical location and cattle management may influence STEC prevalence and the shiga-toxin content in cattle (Majalija, 2009). In this study 104 (42.97 %) of 242 isolates possessed the *stx*$_1$ gene while 161 (66.52%) possessed the *stx*$_2$ gene. Therefore, in this study the prevalence of *stx*$_2$ was higher than that of *stx*$_1$. The prevalence of *hly*A 212/ 242 (87.60 %) of the isolates was higher than that of *eae*A 178 (73.55 %) of 242 isolates. Of all the STEC genes, *hlyA* was the most frequently detected gene followed by *eae*A, *stx*2 and *stx*1 respectively.

STEC carrying *stx*1 strains are associated with mild diseases such as watery diarrhea while strains carrying *stx*2 are associated with bloody diarrhea and HUS (Majalija, 2009). Consequently, *hly*A is a plasmid- encoded virulence marker that encodes for

enterohaemolysin; this gene is less prevalent in *E. coli* isolated from cattle compared to pigs. While *eae*A is a constituent gene of the locus for enterocyte effacement, it encodes for intimin and is responsible for attaching and effacing lesions. *eae*A is the significant gene that is the accessory factor in correlation with severe diseases, thus the absence of this gene does not conclude that there will not be a disease development (Paton and Paton, 1998a; Paton and Paton, 1998b; Ateba *et al.*, 2008).

An additional objective of this study was to determine the antibiotic resistance profiles of non-O157 *E. coli* isolates using phenotypic assays. Antibiotic resistance against beta-lactams, carbapenems, ESBLs and Polymyxin has increased, especially against *E. coli* species (Shaikh *et al.*, 2015). These antimicrobial agents are used as treatment, growth promoters and prophylaxis in animal dairy – farming (Economou and Gousia, 2015). Not only does their use result in the development of resistant determinants in pathogenic, environmental and commensal bacterial strains; but also can be a factor in the increasing spread of antibiotic resistance genes by contaminating food products (Newell *et al.*, 2010) . These determinants may either be resistance genes or multidrug resistance determinants, which are possible to be horizontally transferred amongst bacterial strains through mobile genetic elements like the transposons and a plasmids (Paterson and Bonomo, 2005). Isolates that are resistant to Colistin have been reported to pose a serious threat to the health facilities, food production industries and consumers. Therefore, this type of resistance poses a challenge to the medical industry, as infections caused by these bacterial strains may be impossible to treat (Li *et al.*, 2006; Chen *et al.*, 2011; De Maio Carrillho *et al.*, 2017). Thus, it is of great importance to determine the rates at which food-producing animals are resistant to Colistin and other significant antimicrobial agents.

Two hundred and forty-two (242) isolates from this study were subjected to a phenotypic antibiotic profile assay using Colistin (Polymyxin E) disc. A proportion of 6.19 % isolates were resistant, 41.32 % were intermediate resistant and 52.48 % were susceptible to the antibiotic. Therefore, this indicates the emerging rate of resistance to Colistin. Considering that samples were obtained from different sites, these findings may suggest that transmission of antibiotic resistance determinants may have occurred across the farms. Results obtained from previous studies that were conducted in this study area revealed that *E. coli* strains which possessed multiple drug resistant profiles were present in humans, pigs and cattle stool samples (Ateba *et al.*, 2008; Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011). Hence in the present study cattle was considered as the sample species. *Escherichia coli* non-O157 was therefore successfully isolated from cattle located around North-West province for this study.

Another objective of this study was to confirm the presence of ESBL specific gene determinants in the isolates, which were resistant to Colistin. General results obtained from this study revealed that 242 (100%) were confirmed to be non-O157 *E. coli* strains, of which 201(83.06%) of them were confirmed to also be ESBL-Producing phenotypically on the ESBL Brilliance agar. Moreover, a small proportion of ESBL genotypic traits were observed and the observed results indicated that bla_{OXA} (20; 66.67%) was the dominating detected gene, followed by (11; 36.67 %), bla_{SHV} (6; 20%) and the bla_{TEM} (5; 16.67 %) respectively. Results obtained in this study are different from those that were found in previous studies were $bla_{\text{CTX-M}}$ was the most common gene (Moussé *et al.*). Despite the different results obtained from this study and previous studies, which may have been influenced by the different sites of sampling, geographical locations, source of specimen and protocol used, the presence of these genes in the isolates remains a concern in the public health. Therefore,

these results show that shiga toxin and ESBL-producing *E. coli* are likely to be isolated from the same set of samples, which is a cause for concern.

Studies also indicated that ESBL-producing *E. coli* pose serious challenges to the food production industry since they constantly contaminate meat, meat products, during slaughtering of animals and during food preparation if hygiene measures are not properly practiced (Blaak *et al.*, 2014; Economou and Gousia, 2015). These bacteria have also been implicated in severe and untreatable human infections (Seiffert *et al.*, 2013; Cantas *et al.*, 2016). Despite that they are mostly isolated from human clinical samples and hospital settings; ESBL-producing *E.coli* can also be isolated from ruminants (Alonso *et al.*, 2016; Ibrahim *et al.*, 2016).

A further objective of the study was to isolate and determine the morphologies of non-O157 *E. coli* bacteriophages using electron microscopy. Bacteriophages are found in habitats occupied by bacteria, anywhere in the biosphere; they reside in places such as top-soils, plants, animals or water (Lopes *et al.*, 2014). The goal was to isolate bacteriophages from cattle feacal samples, phages that are to be effective against ESBL-producing non-O157 *E. coli* strains with emphasis on the strains that are resistant to Colistin. The increase in the number of the available bacteriophages makes it difficult to understand their genomic and structural diversity. More than 96% of phages isolated are tailed and constitute the Caudovirales order, which is an example of showing that bacteriophages were grouped based on the nature of their encapsulated nucleic acid and their virion morphology (Ackermann, 2011; Lopes *et al.*, 2014). The nature of the tail is used to classify bacteriophages of the order Caudovirales under one of the three families, namely, Siphoviridae, Myoviridae and Rodoviridae. Other families are illustrated in Figure 5.1. The nature of the tail can be long

and non-contractile, long and contractile or short (Lopes *et al.*, 2014). Little is known about the phages in "Black Africa" (Ackermann, 2011). This is a motivation to classify the bacteriophages isolated from this study and to grow the bacteriophage world.

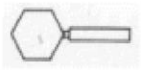
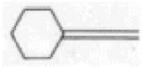
Shape	Order or family	Nucleic acid, particulars, size	Member	Number
	Caudovirales	dsDNA (L), no envelope		
	*Myoviridae*	Tail contractile	T4	1312
	*Siphoviridae*	Tail long, noncontractile	λ	3262
	*Podoviridae*	Tail short	T7	771
	*Microviridae*	ssDNA (C), 27 nm, 12 knoblike capsomers	ϕX174	38
	*Corticoviridae*	dsDNA (C), complex capsid, lipids, 63 nm	PM2	3?
	*Tectiviridae*	dsDNA (L), inner lipid vesicle, pseudo-tail, 60 nm	PRD1	19
	*Leviviridae*	ssRNA (L), 23 nm, like poliovirus	MS2	38
	*Cystoviridae*	dsRNA (L), segmented, lipidic envelope, 70–80 nm	ϕ6	3
	*Inoviridae*	ssDNA (C), filaments or rods, 85–1950 x 7 nm	fd	66
	*Plasmaviridae*	dsDNA (C), lipidic envelope, no capsid, 80 nm	MVL2	5

Figure 5.1: Classification and overview of bacteriophage.

(Picture obtained from (Ackermann, 2007)

Furthermore, bacteriophages were successfully isolated from this study using non-O157 *E. coli* isolates also confirmed from this study as bacterial host. Isolated phages consisted of a head, neck and tail. Classification of these phages based on their morphologies comparing

them with the morphologies described by (Ackermann, 2007; Ackermann, 2011; Lopes *et al.*, 2014), the phages can be classified to belong under the family *Siphoviridae.*

The last objective of the study was to determine the effectiveness of the bacteriophages against ESBL-producing non-O157 *E. coli* isolates resistant to Colistin using the virulent micro-plates assay. Unfortunately, not all isolated phages are acceptable to be used for therapy since complications are encountered in the interactions between the bacteriophage and its host (Wei *et al.*, 2015). Despite that, bacteriophages extracted from this study showed the potential of being lytic phages. They have showed characteristics of being effective against environmental non-O157 *E. coli* bacterial host strains that were used in this study. Three of the employed bacteriophages BNEO1575E, BNEO1574D and BNEO1574C were able to inhibit bacterial growth of more than one strain. Therefore, from the results observed in this study it is considered that phage therapy can be applied to control and eliminate challenges caused by bacterial contaminants from food surfaces since they have been proposed as another option of treatment to antimicrobial agents in animal health in recent years (Garcia *et al.*, 2008).

Epidemiological investigations provide data that may assist in the detection and surveillance as well as control of bacteria strains that are responsible for both sporadic and outbreaks of disease in a given geographical location. Comparison of genetic fingerprints for isolates from difference areas and/or species may provide an understanding of whether causative strains are resident or transient bacteria populations. In this study, PFGE fingerprinting was employed to compare the genetic relatedness of non-O157 STEC Colistin resistant and ESBL isolates from different farms of the North-West Province, South Africa. The great genetic similarities between isolates from these farms indicate the need to improve hygiene practices or farm

management techniques. This will greatly reduce the risk of farm-to-fork cross-contamination with these pathogens and thus also reduce the occurrence of foodborne infections in humans.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

The present study examined the virulence capabilities of the endemic bacteriophages against Colistin and Extended-spectrum beta-lactam resistant non-O157 *Escherichia coli* strains from cattle feacal samples. To the best of our knowledge, this is the first study to be conducted in the North-West Province of South Africa. Isolates obtained were confirmed through PCR to be non-O157 *E. coli*, which were ESBL-producing and a small proportion of them possessed ESBL genes while a large proportion possessed shiga toxins. Moreover, an evaluation of antibiotic resistant patterns of isolates revealed that a significant proportion of these isolates were also resistant to the antimicrobial agent Colistin and thus may present severe health complication on humans if standard operating procedures in the farms are not strictly implemented.

The findings of the study also revealed the presence of endemic bacteriophages from cattle faecal samples with wide host ranges against a number of non-O157 *E. coli* environmental strains. Electron micrographs revealed that the bacteriophages were classified and belonged to the family of *Siphoviridae* based on their morphologies. The virulent capabilities of the phages were also confirmed through the micro-plate assay, thus it can safely be concluded that the phages have lytic capabilities thus making them effective bio-control agents against Colistin resistant and ESBL-producing non-O157 STEC strains.

Therefore, based on the findings of this study, it is recommended that a large scale study be conducted to compare the occurrence of these Colistin resistant and ESBL-producing non-

O157 STEC strains in food-producing animals, humans and food production industries. In addition, both the Colistin resistant and ESBL-producing non-O157 STEC strains as well as the bacteriophages should be subjected to Whole Genome Sequence (WGS) analysis rather than just the conventional bacterial 16S rRNA gene sequence analysis that was used to confirm the identities of bacteria in this study. WGS data will provide gene hotspots as well as all the possible targets that will facilitate the generation of valuable epidemiological data that may be of great clinical significance. Knowledge of the entire bacteriophage gene profiles from WGS data may also provide information on the safety analysis of using these agents as potential bio-control options for these multidrug resistant bacteria, thus increasing chances of expanding treatment options and presenting new approaches on how to improve food safety.

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APPENDICES

APPENDIX A: Materials used during the study

Culture media

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

20g of these materials were added in 1L of distilled water and dissolved. It was autoclaved at 121°C for 15minutes, then after cooling off it was poured into sterile falcon tubes. Buffered peptone water (Biolab, Merck Diagnostic, South Africa) was used as a pre-enrichment medium for isolation of *Escherichia coli*.

Nutrient Broth	**g/L**
Meat extracts	1.0
Yeast extract	2.0
Peptone	5.0
Sodium Chloride	8.0

16g of these materials were added in 1L of distilled water and dissolved. It was autoclaved at 121°C for 15minutes, then after cooling off it was poured into sterile falcon tubes. Nutrient Broth (Biolab, Merck Diagnostic, South Africa) was used as a pre-enrichment medium for DNA extraction overnight cultures.

Sorbitol-MacConkey Agar	**g/L**
(Supplemented with Cefixime and potassium tellurite)	
Peptone	20.0
Sorbitol	10.0
Bile salts	1.5
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.001
Agar	15.0
Cefixime	1m/L
Potassium tellurite	2m/L

50g of these materials were added in 1L of distilled water and dissolved. The media was autoclaved at 121°C for 15minutes, then after cooling off, the media was supplemented with cefixime and potassium tellurite then it was poured into sterile petri-dishes. Sorbitol-MacConkey Agar (Biolab, Merck Diagnostic, South Africa) was used as differential medium for isolation of *Escherichia coli*.

Muller-Hinton Agar	**g/L**
Meat Infusion	5.0
Casein Hydrolysate	17.5
Soluble starch	1.5
Agar	14.0

38g of these materials were added in 1L of distilled water and dissolved. The media was autoclaved at 121°C for 15minutes, then after cooling off it was poured into sterile petri-dishes. The medium was used to determine the antimicrobial susceptibility profiles of isolates.

Modified Nutrient Agar (MNA)	**g/L**
Nutrient Broth	20.0
NaCl	8.5
Agar	10.0
$CaCl_2$	8.3mg
$FeCl_3$	1.1mg
$MgSO_4$	0.5
30% Glucose (added after autoclaving)	10.0ml

These materials were added in 1L of distilled water and dissolved. The media was autoclaved at 121°C for 15minutes. Later after cooling off glucose was added to the media then it was poured into sterile petri-dishes. The medium was used for plaque development during phage work.

Top Agar	**g/L**
UltraPure Agarose	6.0
$MgSO_4.\ 7H_2O$	2.46

These materials were added in 1L of distilled water and dissolved. The top agar was autoclaved at 121°C for 15minutes. Later after cooling off, it was stored at 4°C and melted every time it was to be used for plaque development during phage work.

Tris-Cl (1M)	**g/L**
Tris Base	121.1

Tris-base was dissolved in 800ml of distilled water then adjusted pH to desired value by adding HCl (For a pH of 7.4, **7.5**, 8.0 amount of HCl 70ml,**60ml**, 42ml was added respectively). The volume was then adjusted to 1L by adding distilled water and autoclaving the solution at 121°C for 15minutes. The solution was used during phage work.

Lambda Diluent	**g/L**
1M Tris-Cl (pH 7.5)	10.0ml
$MgSO_4$. $7H_2O$	2.0

These materials were added in 1L of distilled water and dissolved. The diluent was autoclaved at 121°C for 15minutes. Later after cooling off, it was stored at room temperature and it was used for plaque development during phage work.

Tryptic Soy Broth (TSB)	**g/L**
TSB powder	30.0

The powder was added to 1L of distilled water and dissolved. Then it was autoclaved at 121°C for 15minutes, then after cooling off it was stored at 4°C. The broth was used as a pre-enrichment broth during phage work.

CHEMICALS

Buffers (50X TAE)

Thermo scientific 50 X TAE-Electrophoresis-buffer (40mM Tris, 20mM Acetic acid and 1mM EDTA) stock solutions was supplied to by Thermo Scientific, Johannesburg, South Africa. Resolving DNA or Amplified PCR products, a 1X TAE buffer was prepared for gel electrophoresis.

Sodium Hypochlorite

10% (v/v) sodium hypochlorite working solution was prepared by aliquoting 10ml of stock solution sodium hypochlorite into 1L Duran bottle with 900ml of distilled water. The solution was kept at room temperature and used as a disinfectant of the working station.

70% Ethanol

Absolute ethanol (90% v/v) which was supplied for by Merck, Diagnostics, South Africa was used to prepare a 70% (v/v) working solution by; aliquoting 750ml of absolute ethanol into

1L Duran bottle with 300ml of distilled water. The solution was kept at room temperature and used as a disinfectant of the working station.

Ethidium Bromide

In a 5ml Duran bottle a stock solution of 10mg/ml was prepared by dissolving the powder into distilled water and a masking tape was used to protect the solution from light, and then stored at room temperature. A final concentration of 1µl was used for staining the gel for electrophoresis for a clear visual of DNA or PCR products amplicons.

DNA Loading Dye (6X)

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol FF

30% (w/v) glycerol

The above contents were mixed into a 50ml Duran bottle. The solution was filter sterilized using 0.45µm filter and stored at room temperature. The solution was used for agarose gel electrophoresis of extracted DNA.

DNA Ladder/Marker

The standard DNA ladders GeneRuler 100 base pairs and O'GeneRuler 1 Kilo base pairs were used to determine the relative amplicon sizes after the gel electrophoresis. The ladders were supplied for by Thermo Scientific, Johannesburg, South Africa.

Oligonucleotide Primers

The primer (forward and reverse) sets used in this study to amplify the *E. coli* housekeeping genes, colistin and ESBLs genes were synthesized and supplied for by the Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside, Pretoria, South Africa. All primers were supplied in a lyophilized state and provided a synthesis report on how to reconstitute a 100µM stock solution. A 1.5µl sterile eppindorf tube was used to create and prepare a

working solution by aliquoting the required volume of both the forward and reverse primers sets. The primer sets were stored in their separate tubes at -4°C for future use.

ENZYMES

Master Mix (2X DreamTag Green)

2X Dream tag master mix (0.4mM dATP, 0.4mM dCTP, 0.4mM dGTP and 0.4mM dTTP, 4mM $MgCl_2$ and loading buffer) was used for PCR amplification of target genes and stored at -20 °C for future use. The enzyme was produced by Fermentas, USA and supplied for by Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside, Pretoria, South Africa.

Protein K

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

APPENDIX B: Virulent assay detailed results

Plate No: Plate 1	cut off: 0	Test Mode: Plate test
WL1: 630	LP range: 0	Test Doctor:
WL2: none	determine symbol: >	Test Date: 2017-12-05

	1	2	3	4	5	6	7	8	9	10	11	12
A	0002 0.6336 +	0003 0.6561 +	0004 0.6445 +	0005 0.6467 +	0006 0.5833 +	0007 0.6378 +	0008 0.6843 +	0009 0.6836 +	NC 0.7581	NC 0.7534	BLK 0.2259	BLK 0.2312
B	0010 0.6838 +	0011 0.6866 +	0012 0.6615 +	0013 0.6568 +	0014 0.6603 +	0015 0.6533 +	0016 0.7086 +	0017 0.7201 +	NC 0.7747	NC 0.7931	BLK 0.2299	BLK 0.2298
C	0018 0.7288 +	0019 0.7161 +	0020 0.6523 +	0021 0.6715 +	0022 0.6587 +	0023 0.6432 +	0024 0.7528 +	0025 0.7181 +	NC 0.7635	NC 0.7403	BLK 0.2280	BLK 0.2288
D	0026 0.7002 +	0027 0.7075 +	0028 0.6818 +	0029 0.6644 +	0030 0.6594 +	0031 0.6526 +	0032 0.7439 +	0033 0.7167 +	NC 0.6327	NC 0.3844	BLK 0.2329	BLK 0.2320
E	0034 0.6986 +	0035 0.6605 +	0036 0.6916 +	0037 0.6679 +	0038 0.6426 +	0039 0.6420 +	0040 0.7185 +	0041 0.7069 +	NC 0.4716	NC 0.2890	BLK 0.2224	BLK 0.2229
F	0042 0.6872 +	0043 0.7210 +	0044 0.6887 +	0045 0.6650 +	0046 0.6442 +	0047 0.6445 +	0048 0.7102 +	0049 0.7143 +	NC 0.2588	NC 0.1085	BLK 0.2239	BLK 0.2222
G	0050 0.7044 +	0051 0.7282 +	0052 0.6998 +	0053 0.6826 +	0054 0.6605 +	0055 0.6445 +	0056 0.7067 +	0057 0.7250 +	NC 0.1993	NC 0.0760	BLK 0.2336	BLK 0.2411
H	0058 0.6782 +	0059 0.6869 +	0060 0.6875 +	0061 0.6719 +	0062 0.6271 +	0063 0.6473 +	0064 0.6750 +	0065 0.6782 +	NC 0.2188	NC 0.0455	BLK 0.2370	BLK 0.2386

Plate No: Plate 2	cut off: 0	Test Mode: Plate test
WL1: 630	LP range: 0	Test Doctor:
WL2: none	determine symbol: >	Test Date: 2017-12-05

	1	2	3	4	5	6	7	8	9	10	11	12
A	0002 0.6509 +	0003 0.6839 +	0004 0.5761 +	0005 0.6342 +	0006 0.5168 +	0007 0.5899 +	0008 0.6930 +	0009 0.7153 +	NC 0.7667	NC 0.7648	BLK 0.2240	BLK 0.2294
B	0010 0.6579 +	0011 0.6769 +	0012 0.6765 +	0013 0.6673 +	0014 0.6240 +	0015 0.6078 +	0016 0.7354 +	0017 0.7395 +	NC 0.8723	NC 0.8402	BLK 0.2189	BLK 0.2287
C	0018 0.6535 +	0019 0.6711 +	0020 0.6708 +	0021 0.6658 +	0022 0.6280 +	0023 0.6332 +	0024 0.7492 +	0025 0.7485 +	NC 0.8671	NC 0.9043	BLK 0.2193	BLK 0.2226
D	0026 0.6558 +	0027 0.6810 +	0028 0.6810 +	0029 0.6902 +	0030 0.6190 +	0031 0.6207 +	0032 0.7252 +	0033 0.7207 +	NC 0.7771	NC 0.7620	BLK 0.2194	BLK 0.2241
E	0034 0.6917 +	0035 0.6771 +	0036 0.6719 +	0037 0.6779 +	0038 0.6343 +	0039 0.6376 +	0040 0.7392 +	0041 0.7452 +	NC 0.3792	NC 0.2707	BLK 0.2170	BLK 0.2218
F	0042 0.6726 +	0043 0.6157 +	0044 0.6753 +	0045 0.6673 +	0046 0.5957 +	0047 0.6376 +	0048 0.7348 +	0049 0.7234 +	NC 0.1839	NC 0.3453	BLK 0.2220	BLK 0.2202
G	0050 0.6993 +	0051 0.6748 +	0052 0.6854 +	0053 0.6729 +	0054 0.6081 +	0055 0.6096 +	0056 0.7363 +	0057 0.7218 +	NC 0.0743	NC 0. 1535	BLK 0. 2247	BLK 0.2360
H	0058 0.7213 +	0059 0.7093 +	0060 0.6558 +	0061 0.6566 +	0062 0.6722 +	0063 0.6948 +	0064 0.7272 +	0065 0.7258 +	NC 0. 0415	NC 0.1530	BLK 0.2337	BLK 0.2402

Plate No: Plate 3 cut off: 0 Test Mode: Plate test

WL1: 630 LP range: 0 Test Doctor:

WL2: none determine symbol: > Test Date: 2017-12-05

	1	2	3	4	5	6	7	8	9	10	11	12
A	0002 0.6841 +	0003 0.7057 +	0004 0.7447 +	0005 0.7708 +	0006 0.6285 +	0007 0.6545 +	0008 0.6713 +	0009 0.6748 +	NC 0.8648	NC 0.8503	BLK 0.2220	BLK 0.2255
B	0010 0.7424 +	0011 0.7270 +	0012 0.7918 +	0013 0.7603 +	0014 0.6690 +	0015 0.6504 +	0016 0.6833 +	0017 0.6788 +	NC 0.8532	NC 0.8751	BLK 0.2170	BLK 0.2218
C	0018 0.7084 +	0019 0.7321 +	0020 0.7365 +	0021 0.7301 +	0022 0.6914 +	0023 0.7079 +	0024 0.7271 +	0025 0.7327 +	NC 0.8286	NC 0.8650	BLK 0.2093	BLK 0.2204
D	0026 0.7128 +	0027 0.7253 +	0028 0.7631 +	0029 0.7330 +	0030 0.6693 +	0031 0.6461 +	0032 0.7324 +	0033 0.7301 +	NC 0.8268	NC 0.8385	BLK 0.2169	BLK 0.2271
E	0034 0.6967 +	0035 0.7108 +	0036 0.7224 +	0037 0.7496 +	0038 0.6979 +	0039 0.7211 +	0040 0.7216 +	0041 0.7511 +	NC 0.2435	NC 0.6158	BLK 0.2031	BLK 0.2132
F	0042 0.6789 +	0043 0.6701 +	0044 0.7146 +	0045 0.7255 +	0046 0.7597 +	0047 0.8072 +	0048 0.7070 +	0049 0.7100 +	NC 0.1918	NC 0.2246	BLK 0.2134	BLK 0.2208
G	0050 0.6978 +	0051 0.7060 +	0052 0.7242 +	0053 0.7244 +	0054 0.1867 +	0055 0.1875 +	0056 0.7211 +	0057 0.7267 +	NC 0.0707	NC 0. 2089	BLK 0. 2137	BLK 0.2213
H	0058 0.8302 +	0059 0.6814 +	0060 0.7163 +	0061 0.7194 +	0062 0.1867 +	0063 0.1869 +	0064 0.6982 +	0065 0.6422 +	NC 0.1281	NC 0.1611	BLK 0.2342	BLK 0.2374

Plate No: Plate 4 cut off: 0 Test Mode: Plate test

WL1: 630 LP range: 0 Test Doctor:

WL2: none determine symbol: > Test Date: 2017-12-05

	1	2	3	4	5	6	7	8	9	10	11	12
A	0002 0.6836 +	0003 0.6367 +	0004 0.6792 +	0005 0.1991 +	0006 0.6749 +	0007 0.6695 +	0008 0.7031 +	0009 0.6371 +	NC 0.7450	NC 0.7719	BLK 0.2274	BLK 0.2321
B	0010 0.7173 +	0011 0.6406 +	0012 0.6809 +	0013 0.1964 +	0014 0.6498 +	0015 0.5829 +	0016 0.7219 +	0017 0.6917 +	NC 0.8166	NC 0.8200	BLK 0.2239	BLK 0.2323
C	0018 0.7391 +	0019 0.6227 +	0020 0.6378 +	0021 0.1950 +	0022 0.6189 +	0023 0.6367 +	0024 0.6896 +	0025 0.7191 +	NC 0.8576	NC 0.8069	BLK 0.2178	BLK 0.2189
D	0026 0.6736 +	0027 0.6485 +	0028 0.6260 +	0029 0.1963 +	0030 0.6255 +	0031 0.6491 +	0032 0.6833 +	0033 0.7721 +	NC 0.7573	NC 0.4485	BLK 0.2177	BLK 0.2281
E	0034 0.6633 +	0035 0.6624 +	0036 0.6488 +	0037 0.1960 +	0038 0.6124 +	0039 0.6230 +	0040 0.6854 +	0041 0.1951 +	NC 0.5860	NC 0.2304	BLK 0.2139	BLK 0.2220
F	0042 0.6632 +	0043 0.6042 +	0044 0.6371 +	0045 0.1936 +	0046 0.5916 +	0047 0.6192 +	0048 0.6583 +	0049 0.1919 +	NC 0.3138	NC 0.2170	BLK 0.2127	BLK 0.2186
G	0050 0.6599 +	0051 0.1614 +	0052 0.6410 +	0053 0.1917 +	0054 0.6132 +	0055 0.6196 +	0056 0.6425 +	0057 0.1932 +	NC 0.2247	NC 0.0322	BLK 0.2311	BLK 0.2344
H	0058 0.6142 +	0059 0.1932 +	0060 0.6558 +	0061 0.1919 +	0062 0.6069 +	0063 0.6425 +	0064 0.6272 +	0065 0.1910 +	NC 0.1126	NC 0.0365	BLK 0.2415	BLK 0.2585

Plate No: Plate 5 cut off: 0 Test Mode: Plate test

WL1: 630 LP range: 0 Test Doctor:

WL2: none determine symbol: > Test Date: 2017-12-05

	1	2	3	4	5	6	7	8	9	10	11	12
A	0002 0.7754 +	0003 0.7876 +	0004 0.7018 +	0005 0.6691 +	0006 0.6257 +	0007 0.6436 +	0008 0.7557 +	0009 0.7850 +	NC 0.8778	NC 0.8416	BLK 0.2227	BLK 0.2261
B	0010 0.7439 +	0011 0.7733 +	0012 0.6677 +	0013 0.8382 +	0014 0.6264 +	0015 0.6287 +	0016 0.7557 +	0017 0.7834 +	NC 0.8959	NC 0.9131	BLK 0.2221	BLK 0.2272
C	0018 0.7113 +	0019 0.7077 +	0020 0.6268 +	0021 0.6135 +	0022 0.6205 +	0023 0.7029 +	0024 0.8481 +	0025 0.7511 +	NC 0.8424	NC 0.8727	BLK 0.2187	BLK 0.2258
D	0026 0.7315 +	0027 0.7504 +	0028 0.6350 +	0029 0.1947 +	0030 0.6091 +	0031 0.8000 +	0032 0.7558 +	0033 0.7342 +	NC 0.3630	NC 0.5059	BLK 0.2234	BLK 0.2317
E	0034 0.7179 +	0035 0.6964 +	0036 0.6008 +	0037 0.1954 +	0038 0.7809 +	0039 0.1942 +	0040 0.7560 +	0041 0.8062 +	NC 0.1836	NC 0.2181	BLK 0.2190	BLK 0.2219
F	0042 0.7066 +	0043 0.6935 +	0044 0.5838 +	0045 0.1955 +	0046 0.6889 +	0047 0.1947 +	0048 0.7475 +	0049 0.8360 +	NC 0.0261	NC 0.1134	BLK 0.2149	BLK 0.2260
G	0050 0.7000 +	0051 0.7095 +	0052 0.6498 +	0053 0.1936 +	0054 0.7078 +	0055 0.1946 +	0056 0.7620 +	0057 0.5705 +	NC 0.0132	NC 0.0219	BLK 0.2301	BLK 0.2393
H	0058 0.7053 +	0059 0.7405 +	0060 0.6279 +	0061 0.1940 +	0062 0.6350 +	0063 0.1912 +	0064 0.6968 +	0065 0.1937 +	NC 0.0154	NC 0.0147	BLK 0.2369	BLK 0.2401