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Occurrence and characterisation of the seven major Shiga toxin-producing *Escherichia coli* serotypes from healthy beef cattle in South Africa.

By

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A dissertation submitted in partial fulfilment of the requirements for the degree of Master of Science (Veterinary Science).

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DEDICATION

To

My beloved wife Lydia and our children Gregory, Mercy and Merry with love and gratitude, for the encouragement and support. You had to endure my absence in order for me to realize my dreams.

God bless you.

DECLARATION:

This dissertation is my original work and has not been presented for any award or degree in another University.

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LIST OF ABBREVIATIONS

AMR Antimicrobial resistance

CLSI Clinical and Laboratory Standards Institute

AZD: Animal and Zoonotic Diseases

CLSI: Clinical and Laboratory Standards Institute

ECDC: European Center for Disease Prevention and Control

EFSA: European Food Safety Authority

GDARD-Gauteng Department of Agriculture and Rural Development

GDD: Global Disease Detection

Gb_3: Globotriaosylceramide

HC: Hemorrhagic colitis

HNT: H-non typeable

HUS: Hemolytic uremic syndrome

IASR Infectious Agents Survellance Reports

IDT: Integrated DNA Technologies

IRT: Institutional Research Theme

LB: Luria Bertani

LEE: Locus of enterocyte effacement

MDR: Multidrug resistance

MHA: Mueller Hinton agar

NM: Non-motile

NRF: National Research Foundation

OI: O-islands

PCR: Polymerase chain reaction

SPSS: Statistical package for social sciences

STEC: Shiga toxin-producing *Escherichia coli*

USDA-FSIS: United States Department of Agriculture Food Safety and Inspection Service

VRBL: Violet Red Bile Lactose

VTEC: Verocytotoxin-producing *Escherichia coli*

WHO: World Health Organization

IASR Infectious Agents Survellance Reports

THESIS SUMMARY

Shiga toxin-producing *E. coli* (STEC) is a food pathogen causing infections characterised by mild watery to severe bloody diarrhea and complications such as the hemolytic uremic syndrome (HUS). Humans acquire STEC through consumption of contaminated foods of animal origin, vegetables and water. Cattle are the main reservoir of STEC. The severity of STEC infections in humans depends on a number of virulence factors encoded in the bacterium's genome. The seven major STEC serogroups most frequently incriminated in severe human disease outbreaks and HUS worldwide include O157, O45, O103, O111, O121, O145 and, O26, commonly referred to as the "top/big seven". Although STEC has been incriminated in human disease in South Africa, data on the role of played by cattle in human disease and virulence characteristics of cattle STEC are lacking. Therefore, the objectives of this study were to (i) investigate the presence of the seven major STEC serotypes in healthy beef cattle (cow-calf operations) and (ii) characterise isolates by serotype, virulence genes and markers, and antimicrobial resistance profiles.

Polymerase chain reaction (PCR) was carried out to identify STEC serotypes (O and H antigens) and characterize the isolates by virulence factors and markers. The disk diffusion technique (Kirby Bauer test) was used to determine the antimicrobial resistance profiles of STEC isolates against a panel of 15 antimicrobials. Five hundred and seventy-eight STEC isolates (N=578), which had been previously recovered from 559 cattle from five beef farms were screened for STEC O26, O45, O103, O111, O121, O145 and O157. Confirmed STEC belonging to serogroups O26, O45, O103, O111, O121, O145 and O157 to isolates were characterised for major virulence genes including *stx1*, *stx2*, *eaeA* and *ehxA*. Furthermore, 140 isolates were characterised for

Shiga toxins (*stx*) subtypes, plasmid and pathogenicity island-encoded genes, and antimicrobials resistance profiles.

PCR serotyping revealed that 241 out of 578 STEC isolates belonged to serogroups O26, O45, O103, O121, O145 and O157. STEC O111 was not detected. The occurrence rate of each serogroup among the 559 cattle was as follows: O26, 10.2% (57/559); O45, 2.9% (16/559); O145, 2.5% (14/559); O157, 1.4% (8/559); O121, 1.1% (6/559) and O103, 0.4% (2/559). This corresponded to an overall prevalence of 16.5% (92/559) of cattle that were positive for STEC O26, O45, O103, O121, O145 and O157. STEC O111 was not detected. The 241 isolates belonged to 33 serotypes. *stx1* and *stx*2 were detected in 69.3% (167/241) and 96.3% (232/241) of isolates respectively. Both *stx1* and *stx2* were concurrently present in 62.2% (150/241) of isolates. The *eaeA* and *ehxA* genes were detected in 7.1% (17/241) and 92.5% (223/241) of isolates respectively.

Further characterisation of 140 isolates revealed that *stx1* was present in 65.7% (92/140). Among *stx1* positive isolates, *stx1c* was present in 20.7% (19/140); *stx1d* in 15.2% (14/92); and *stx1c*+*stx1d* in 6.5% (6/92) of isolates. The *stx2* gene was observed in 95.7% (134/140). Among *stx2* positive isolates, *stx2c* was the most frequent 97.8%, (131/134) followed by *stx2d* which was present in 56% (75/134). None of the isolates were positive for *stx2e*, *stx2f* and *stx2g*. The *eaeA* gene was present in 12.1% (17/140) of isolates.

The distribution of plasmid-encoded genes was as follows: *ehxA*, 90.7% (127/140); *saa*, 82.1% (115/140); *espP*, 79.3% (111/140); *sub*A, 37.9% (53/140); *kat*P, 10% (14/140) and *etp*D, 7.9% (11/140). The following proportions were observed for OI-122 markers:

pagC, 53.6% (75/140); *sen* 36.4% (51/140); *Z4333*, 28.6% (40/140) and *Z4332,* 10.7% (15/140). OI-43/48 markers were distributed as follows: *iha*, 93.6% (131/140); *terC*, 80% (112/140) and *ure*C, 55.7% (78/140).

The frequencies of non-LEE effector genes were as follows: *nleB*, 32.9% (46/140); *nleE*, 28.6% (40/140); *nleG6-2*, 33.6% (47/140); *nleG5-2*, 19.3% (27/140); *nleH1-1*, 27.9% (39/140), *nleH1-2*, 27.1% (38/140); *ent/espl2*, 34.3 (48/140); *nleG2-3*, 30.7% (43/140); *nleD*, 20.7% (29/140); *nleG9*, 16.4% (23/140); *nleB2*, 15.7% (22/140); *nleG2-1*, 14.3 (20/140); *nleC*, 12.1% (17/140); *nleA*, 12.1% (17/140); *nleF*, 5% (7/140) and *nleG*, 2.1% (3/140).

Antimicrobial resistance profiling revealed that 97.9% (137/140) of isolates were susceptible to all the 15 antimicrobials. Only 2.1% (3/140) of isolates were resistant: tetracycline, 1.4% (2/140); ampicillin, 1.4% (2/140); amoxicillin-clavulanic acid, 0.7% (2/140) and cephalothin, 0.7% (2/140).

This study showed that beef cattle (cow-calf operations) in South Africa carry STEC that belong to the six major STEC serogroups including O26, O45, O103, O121, O145 and O157. None of the isolates were identified as STEC O111. The STEC isolates represented 33 serotypes, of which 13 were serotypes that have been previously implicated in human disease. STEC O26, O45, O103, O121, O145 and O157 were recovered from 16.5% of cattle. STEC O26 serotypes were the most prevalent among the cattle surveyed. Furthermore, most STEC isolates carried both *stx1* and *stx2*, and *stx2c* and *stx2d* were most frequent *stx2* subtypes. The majority of the isolates lacked *eaeA*. The *eaeA* gene was observed in STEC O157, O145 and O103 serotypes only. Plasmid-encoded genes *ehxA*, *saa* and *esp*P genes were widespread among STEC.

However, most isolates lacked *kat*P and *etp*D. The *saa* and *subA* genes were present in *eaeA*-negative isolates only while *kat*P and *etp*D were mainly present in *eaeA* positive STEC that belonged to O157, O145 and O103 serotypes. OI-122 markers were more prevalent in *eaeA*-positive isolates while the the full complement OI-122 encoded genes was observed in STEC O157:H7 and O103:H2 isolates only.

Most of the isolates carried OI-43/48 markers *terC*, *iha* and *ureC*. *terC* and *ureC* genes were significantly associated with *eaeA*-positive STEC.

Most non-LEE effectors were detected mainly in *eaeA*-positive isolates. STEC O157:H7 and STEC O103:H2 carried the highest number of *nle* genes (13 to 15 *nle*-genes). Furthermore, half of STEC isolates lacked *nle* genes. Almost all STEC isolates were susceptible to 15 antimicrobials tested, except for three isolates that were antimicrobial resistant. The three isolates included STEC O26:H11 which was resistant to tetracycline while STEC O26:H4 and STEC O45:H21 were resitant to more than one (multiresistant) including amoxicillin-clavulanic acid, tetracycline, ampicillin and cephalothin.

In conclusion, beef cattle in South Africa are an important reservoir of the six major STEC serogroups including STEC O26, O45, O103, O121, O145 and O157. Beef cattle carried STEC isolates that belonged to various serotypes of which only a subset has been previously incriminated in severe human STEC outbreaks and HUS. Most virulence genes were detected in *eaeA*-positive STEC O157:H7, O103:H2, O145:H28 and O26:H2 strains. STEC O157:H7, O103:H2, O145:H28 and O26:H2 strains are clinically relevant or most frequently associated with human disease. Most of the beef STEC isolates under study were mainly positive fo shiga toxin encoding genes (*stx1* and *stx2*) and subtypes (stx2c and stx2d) but lacked *eaeA* and the majority of *nle* genes

which are commonly associated with highly virulent STEC strains in human disease. The absence of *eaeA,* a key STEC adhesin, and a number of major non-LEE effectors among cattle STEC isolates may explain why human STEC infections are infrequently implicated in human disease in South Africa.

1.0 CHAPTER I: GENERAL INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) also termed verocytotoxin-producing *E. coli* (VTEC) is a foodborne pathogen which causes human infections characterized by mild to severe diarrhea, hemorrhagic colitis (HC) and and complications including hemolytic uremic syndrome (HUS) as a complication. HUS is the main cause of kidney failure especially in young children (Gyles, 2007; Karmali, 2004; Karmali, 1989; Nataro and Kaper, 1998).

Ruminants, particularly cattle, are the major reservoir of STEC (Chapman et al., 1989; Chapman et al., 1993; Hancock et al., 2001; Wells et al., 1991) and are a potential source of STEC which can be transmitted to humans through fecal contamination of food and water. Humans acquire STEC through consumption of contaminated foods of animal origin including meat, milk and water (Swerdlow et al., 1992; Belongia et al., 1993; Hussein and Sakuma, 2005; Hussein and Bollinger, 2005; Ombarak et al., 2016).

Over 600 STEC serotypes have been identified from cattle, foods, humans and environment sources around the world (Bettelheim, 2007; Beutin and Fach, 2014; Hussein and Sakuma, 2005; Hussein and Bollinger, 2005; EFSA, 2013; http://idsc.nih.go.jp/iasr/virus/graph/vtec0510y.pdf). However, the majority of STEC infections in humans are due to STEC O157:H7 (Kaper and Nataro, 1998; Majowicz et al., 2014; Heiman et al., 2015). Furthermore, six major non-O157 STEC serogroups and associated serotypes including O26: (H11, H^-), O45:H2, O103: (H2, H^-), O121:H19, O111: (H2, H8, H^-) and O145:H^-, are the most implicated in mild to severe STEC disease outbreaks in humans. Furthermore, it has been suggested that these serogroups may account for 60-70% of all human STEC infections (Bettelheim, 2007; Brooks et al., 2005; Johnson et al., 2006; Majowicz et al., 2014). STEC O121, O111, O145, O26, O103, O45, and O157 are referred to as “top seven” or “Big seven” and are food adulterants

(especially in raw ground beef) by the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) because of their frequency in severe STEC disease outbreaks and HUS complication in humans (USDA FSIS, 2012).

STEC virulence factors are mainly encoded on mobile genetic elements (MGEs) including lambdoid lysogenic bacteriophages, plasmids and pathogenicity islands (Bolton, 2011; Coombes et al., 2008; Deng et al., 2004; Gal-Mor and Finlay, 2006). The main virulence factors of STEC are bacteriophages-encoded Shiga toxins (*stx1* and *stx2*) (O'Brien et al., 1984; Strockbine et al., 1986). Intimin, (*eaeA*), a key STEC adhesin which is encoded on the locus of enterocyte effacement (LEE) Pathogenicity Island is also considered a major virulence factor (McDaniel et al., 1995; Tzipori et al., 1995). Intimin is responsible for attachment of STEC to mucosal epithelial cells resulting in attaching and effacing (A/E) lesions (McDaniel et al., 1995; Tzipori et al., 1995).

A number of genes carried on plasmids are also considered important virulence markers including enterohemolysin (*ehxA*) (Schmidt et al., 1995), a catalase-peroxidase (*katP*) (Brunder et al., 1996), the extracellular serine protease (*esp*P) (Brunder et al., 1997), a type II secretion system (*etp*D) (Schmidt et al., 1997), STEC autoagglutinating adhesin (*saa*) (Paton et al., 2001) and subtilase cytotoxin (*sub*A) (Paton et al., 2004). The latter two genes are characteristic of LEE-negative STEC.

STEC are traditionally serotyped using antisera against *E. coli* surface antigens including O (somatic antigen), and H (flagellar) antigens (Guinée et al., 1981; Orskov et al., 1977; Ewing 1986). O:H serotyping is considered the “gold standard” method for STEC serotype classification (Guinée et al., 1981; Ørskov and Ørskov, 1984). However, O:H serotyping is time consuming, laborious and expensive, and can only be performed by specific reference laboratories. Therefore, rapid and accurate molecular methods have been developed to circumvent the challenges of conventional serotyping (DebRoy et al., 2011b;

Iguchi et al., 2015a; Fratamico et al., 2016; Iguchi et al., 2016). Molecular serotyping methods include the use of PCR, oligonucleotide probes targeting O-antigen biosynthesis gene clusters (O-AGC), DNA microarray assay platforms and *in silico* methods that utilize whole genome sequences (WGS) to detect and determine the O-antigen polysaccharide and the flagellar genes clusters (Iguchi et al., 2015b; Singh et al., 2015; Machado, et al., 2000; Joensen et al., 2015; Laing et al., 2009; Lacher et al., 2016).

In the recent past, there has been an increase in antimicrobial resistance (AMR) against the most frequently used antimicrobials in animal and human therapy (Walsh and Fanning, 2008; Cheney et al., 2015; Giedraitienė et al., 2011). The use of antimicrobials as growth promoters in animal production systems has further escalated the problem (Dibner and Richards, 2005; Ronquillo and Hernandez, 2017; Schwarz et al., 2001). In addition, unregulated use, overuse, misuse and abuse of antibiotics has led to emergence of resistance (MDR) among STEC isolates (Day et al., 2016; Srinivasan et al., 2007; Wang et al., 2016). STEC O157:H7 and non-O157 serogroups have developed multidrug resistance against different classes of antimicrobials and are a growing public health concern (Karama et al., 2008; Iweriebor et al., 2015; Amézquita-López et al., 2016; Beier et al., 2016). STEC as normal microflora in the gastrointestinal tract of cattle is considered an indicator of antimicrobial resistance and has been used for monitoring antimicrobial resistance in various animals and the environments. Monitoring of antimicrobial resistance in STEC provides information on abuse of antimicrobials and the potential risk of resistance spreading to humans through the food chain (Smith, et al., 2002).

Although a few reports have documented STEC O157 in cattle and in foods of cattle origin in South Africa (Ateba et al., 2008a; Iweriebor et al., 2015), data on STEC occurrence in South Africa remains scarce (Smith et al., 2011). Furthermore, data on the role of cattle as a reservoir of STEC serogroups that commonly affect humans remains scanty. In

addition, investigations on virulence characteristics and antimicrobial resistance profiles of STEC serotypes from cattle in South Africa are non-existent.

Aim and Objectives

The overall aim of this study was to investigate the occurrence and characterize STEC serogroups O26, O145, O103, O45, 111, O121 and O157 from beef cattle (cow-calf operations) in South Africa.

Specific objectives were to:

i. Serotype (O:H) STEC isolates belonging to serogroups O26, O45, O103, O111, O121, O145 and O157.
ii. Characterize the serotypes for genes encoding virulence genes and markers, and
iii. Determine the antimicrobial resistance profiles of STEC isolates.

The ultimate aim of the study was to assess the virulence potential of cattle STEC and contribute to STEC monitoring and surveillance in South Africa.

2.0 CHAPTER II: LITERATURE REVIEW

2.1 Background

Shiga toxin-producing *E. coli* (STEC) also termed Verocytotoxin-producing *E. coli* (VTEC) have emerged as important enteric foodborne zoonotic pathogens worldwide. STEC disease in humans is characterized by mild to severe diarrhea which may be complicated with hemolytic uremic syndrome (HUS) (Neill et al., 1987; Karmali, 1989; Nataro and Kaper, 1998).

STEC O157:H7 was the first serotype to be recognized and associated with an outbreak of hemorrhagic colitis (HC) in the United States, in humans who had consumed contaminated hamburgers in Oregon and Michigan states (Riley et al., 1983). STEC O157:H7 is the most frequent serotype in foodborne disease and the most significant clinically worldwide (Gould et al., 2009; Majowicz et al., 2014)**.** However, non-O157 STEC serogroups have become common in STEC outbreaks worldwide (Tozzi et al., 2003; Brooks et al., 2005; Bettelheim, 2007; Scallan et al., 2011; Majowicz et al., 2014). So far, more than 600 STEC serotypes have been reported (Bettelheim, 2007; Beutin and Fach, 2014; Hussein and Sakuma, 2005; Hussein and Bollinger, 2005; EFSA BIOHAZ 2013; http://idsc.nih.go.jp/iasr/virus /graph /vtec0510y.pdf). Based on statistics from developed countries, it is estimated that STEC account for 2,801,000 cases of acute illnesses worldwide (Majowicz et al., 2014). STEC O157:H7 causes 35.9% of the total STEC disease cases while 64.1% of cases are attributed to non-O157 STEC serogroups in the United States (Scallan et al., 2011).

STEC serogroups that are commonly incriminated in STEC foodborne illnesses in humans include O26, O103, O111, O121, O145, O45 and O157 (Brooks et al., 2005; Johnson et al., 2006; Bettelheim, 2007; Gould et al., 2013). These serogroups are significantly associated with severe human disease outbreaks and are commonly termed

the “top/big seven” (USDA FSIS, 2012; Beutin and Fach, 2014). In addition to top seven serogroups which are frequent in North America, Europe and Japan, there are other STEC serogroups of public health concern, including O91, O113, and O128 that are more common in other countries (Bettelheim, 2007; Gould et al., 2013). A number of non-O157 STEC are rarely implicated in human disease while there are also numerous STEC serotypes which have never been implicated in human disease (Hussein and Sakuma, 2005; Hussein and Bollinger, 2005).

2.2 Cattle STEC Reservoirs

Cattle are considered the major reservoir of STEC (Chapman et al., 1989; Chapman et al., 1993; Hancock et al., 2001; Wells et al., 1991). STEC is mainly transmitted to humans through the fecal-oral route after consumption of contaminated food of animal origin or direct contact with infected animals or a contaminated environment (Nataro and Kaper, 1998; Caprioli et al., 2005; Luna-Gierke et al., 2014). Cattle products that have been incriminated in human STEC infections include contaminated ground beef, milk and cheese (Bosilevac and Koohmaraie, 2011; Hussein and Bollinger, 2005; Ombarak et al., 2016; Peng et al., 2013). Furthermore, untreated water in proximity of cattle farms or feedlots and vegetables grown with cattle manure as a fertilizer have also been implicated in various STEC outbreaks (Ingham et al., 2004; Feng, 2014). Cattle are usually infected by STEC through exposure to contaminated feed and water or grazing on manure contaminated pastures (Besser et al., 2001).

STEC are found in large numbers in the gastrointestinal tract of cattle (Grauke et al., 2002; Naylor et al., 2003; Moxley, 2004). However, the terminal ileum and especially the recto-anal junction has been found to be the most contaminated part of the gastrointestinal tract in cattle (Naylor et al., 2003; Cobbold et al., 2007). Although healthy adult cattle are known to be transient carriers of STEC serotypes, no active disease has been associated with

adult STEC in cattle. Shedding of STEC occurs mostly in calves which are more susceptible to STEC colonization compared to adult cattle (Harmon et al., 1999). STEC infections have been observed in one to eight week old calves and are particularly frequent between 4-5 weeks but newborn and older calves can also be affected (Bertshinger et al., 1994). The shedding period of STEC in cattle varies extensively between animals and some animals are "super-shedders" of STEC (Widiasih et al., 2004; Matthews et al., 2006; Chase-Topping et al., 2008). Super-shedders are cattle that shed more than 10^4 CFU/g (colony forming units) of STEC in feces (Chase-Topping et al., 2008; Arthur et al., 2009). Furthermore, even when the number of super-shedders within a herd is very small, super-shedder animals are usually responsible for increasing the level of STEC contamination levels on a farm (Omisakin et al., 2003; Chase-Topping et al., 2008; Arthur et al., 2009; Menrath et al., 2010).

STEC that are shed in the environment, particularly feed and water, multiply and circulate re-exposing the cattle herd to STEC (Hancock et al., 1998; Hancock et al., 2001). STEC serotypes can survive and propagate in feed and water for long periods and can become sources of STEC contamination on farms (Hancock et al., 1998; Hancock et al., 2001; LeJeune et al., 2001). Moreover, farm soil that is contaminated with cattle feces can be a source of STEC for many months (Berry and Miller, 2005; Fremaux et al., 2008; Hancock et al., 1998). Hancock *et al.,* (1998) carried out a study to determine the prevalence of STEC serotypes in different farm environments including feeders, barn surfaces and milk filters and reported STEC O157:H7 prevalence ranging from 11 to 19% in various sites (Hancock et al., 1998).

2.3 Major STEC Serogroups

The prevalence of STEC in cattle and particularly serogroups O103, O45, O145, O121, O157, O111 and O26 may be influenced by geographic location (Ogden et al., 2004; Alam

and Zurek, 2006; Arthur et al., 2009; Cernicchiaro et al., 2013; Islam et al., 2014; Bibbal et al., 2015; Mellor et al., 2016; Lee et al., 2017). Furthermore, different serotypes associated with STEC O103, O45, O145, O121, O157, O111 and O26 have been incriminated in human disease outbreaks with variations from one region to another (Browning et al., 1990; Effler et al., 2001; Brooks et al., 2005; Johnson et al., 2006; Smith et al., 2011; Majowicz et al., 2014). Islam *et al.,* (2014) have reported an estimated cattle STEC O157:H7 global prevalence of 5.7% with differences between studies ranging from 0.13 to 62%. The world regional random effects pooled prevalence estimates for STEC O157:H7 was 31% in Africa, 7.4% in Northern America, 6.9% in Australia, 5.2% in Europe, 4.7% in Asia and 1.7% in South America (Islam et al., 2014). Furthermore, Islam *et al*., (2014) observed that STEC O157:H7 prevalence estimate levels in feedlot (19.6%) was much higher in relation to dairy cattle production systems (1.8%).

The non-O157 STEC O145, O121, O111, O103, O45 and O26 global pooled prevalence e estimate in cattle was 4.3% (Dewsbury, 2015). However, there are variations regarding non-O157 STEC prevalence in cattle in different regions of the world including Australia, Europe, Asia, South America and North America with prevalences ranging from 1.3% to 17% (Cernicchiaro et al., 2013; Jeon et al., 2006; Mellor et al., 2016; Padola et al., 2004; Shaw et al., 2004, Dewsbury, 2015). North American countries including Canada, USA and Mexico have the the highest prevalence for STEC O45, O157 O26, O121, O103, and O145 (Islam et al., 2014; Dewsbury, 2015). In most beef and dairy cattle farms, STEC and particularly STEC O157:H7 are widespread with a prevalence of up to 90% in the United States (Hancock et al., 1998; Cerqueira et al., 1999; Hancock et al., 2001; Chatterjee et al., 2012; Cernicchiaro et al., 2013; Laegreid et al., 1999).

The high numbers of STEC O157:H7 in cattle has been attributed to cattle diet (Diez-Gonzalez et al., 1998; McGee et al., 2001; Rasmussen et al., 1999). For example, feeding

cattle on grain creates an acidic environment in the gut of the animal, which selects for STEC, leading to shedding of STEC into the environment in large numbers (Diez-Gonzalez et al., 1998). A study on cattle diet, has shown that feeding hay to beef cattle can reduce the amount of STEC serotypes within the intestinal tract before slaughter (Diez-Gonzalez et al., 1998). Moreover, the type of bedding used on the floor of cattle houses and animal movement within farms also contribute to a high prevalence of STEC on farms (Cernicchiaro et al., 2009; Lejeune and Kauffman, 2005). In addition, Lejeune and Kauffman, (2005) observed that the use of sand on the floor where animals are housed reduces STEC incidence as opposed to use of sawdust.

2.4 STEC Virulence

The pathogenicity of STEC is usually a result of the expression of multiple virulence factors. A number of STEC virulence genes have been associated with mild or severe human STEC illnesses and fatal complications such as HUS observed in human disease (Coombes et al., 2008; Gyles et al., 1992). STEC virulence genes are encoded on bacteriophages, plasmids and on genomic islands termed pathogenicity islands (PAI) and can be laterally transferred from one microorganism to another or lost from the bacterial genome (Kaper et al., 1999; Lawrence, 2005; Nataro and Kaper, 1998). Pathogenicity islands (PAIs) are genetic elements on the chromosome that have a different guanine and cytosine (G-C) composition as compared to the bacterium core genome. Pathogenicity islands (PAIs) encode a collection of virulence genes and can measure from 10-200 kb (Schmidt and Hensel, 2004; Gal-Mor and Finlay, 2006). PAI DNA sequences are flanked with unstable direct repeats (DR) that are associated with tRNA and insertion sequences (Gal-Mor and Finlay, 2006; Hacker et al., 1997; Hacker and Kaper, 2000). PAIs are characterised by a different content and arrangement of guanine and cytosine (G-C % content) (Schmidt and Hensel, 2004; Gal-Mor and Finlay, 2006). PAIs are used to differentiate non-pathogenic from pathogenic bacteria as the latter carry pathogenicity

islands (PAIs) which harbor virulence genes (Hayashi et al., 2001; Perna et al., 2001; Wick et al., 2005). Plasmids are extrachromosomal DNA which carry genetic information that can be transferred from one bacteria to another through conjugation whereas bacteriophages are viruses that infect bacterial cells and inject foreign DNA (DNA from previously infected organisms), into other bacteria which can be subsequently integrated into bacteria genome (Valla, 1998; Jain and Srivastava, 2013).

The main STEC virulence factors are bacteriophage-encoded Shiga toxins (Stx) (O'Brien et al., 1984; Newland et al., 1985; Huang et al., 1986; Strockbine et al., 1986). Additional major virulence factors include *eaeA* (Beebakhee et al., 1992; Yu and Kaper, 1992), located on a PAI, and various plasmid-encoded factors which are considered virulence markers as they have been shown to enhance STEC pathogenicity in some studies (Brunder et al., 1996; Brunder et al., 1997; Schmidt et al., 1997; Schmidt et al., 1995). Furthermore, a number of genes which are encoded on various pathogenicity islands have been used in the “molecular risk assessment” as markers of highly virulent STEC (Coombes et al., 2008; Karmali et al., 2003).

2.4.1 Shiga toxin (*Stx*) subtypes

Shiga toxins are a group of AB_5 toxin compounds of about 70 kDa, consisting of a catalytic A active subunit (A subunit) which inhibits protein synthesis by cleavage of the 28S rRNA and a pentameric B subunit which binds to host enterocyte receptors (Ling et al., 1998; Donohue-Rolfe et al., 1989). Two main Shiga toxins have been identified: Stx1 and Stx2 which are antigenically different but structurally similar sharing a common operon structure, enzymatic activity, polypeptide subunit structure and specific binding to glycolipids (Strockbine et al., 1986; Scheutz et al., 2012). Shiga toxins bind to cells via a glycosphingolipid Gb3 and move into the cell through cell-mediated endocytosis (Lingwood, 1993; Obrig et al., 1993; Khine et al., 1994).

Shiga toxins (Stx1 and Stx2*)* differ at nucleotide level and immunologically (Nataro and Kaper, 1998). Both Stx1 and Stx2 also differ in terms of their epidemiological association with STEC disease. For example, HUS in humans is mostly associated with STEC strains carrying *stx2* which are considered more virulent than *stx1* positive strains or strains possessing both *stx1* and *stx2* (Ostroff et al., 1989; Nataro and Kaper, 1998; Werber et al., 2003). STEC that produce Stx1 (*Stx1)* are considered less virulent as they are rarely associated with severe human disease than Stx2 producing STEC strains (Eklund et al., 2002; Friedrich et al., 2002; Werber et al., 2003). A number of Shiga toxin subtypes have been described for both *stx1* and *stx2* (Scheutz et al., 2012).

Stx subtypes have been associated with varying degrees of clinical manifestations ranging from mild gastrointestinal illnesses to severe disease and HUS in humans (Friedrich et al., 2002; Eklund et al., 2002; Werber et al., 2003; Fuller et al., 2011). According to current nomenclature, *stx1* gene subtypes in their alphabetical designation include *stx1a, stx1b, stx1c* and *stx1d* whereas *stx2* subtypes are *stx2a, stx2b, stx2c, stx2d, stx2e, stx2f* and *stx2g* (Scheutz et al., 2012).

Posession of *stx2* is frequently associated with HC and HUS in humans (Friedrich et al., 2002), as *stx2a, stx2c, stx2d* and *stx2e* are involved in human STEC disease severity variably (Friedrich et al., 2002; Sonntag et al., 2005; Bielaszewska et al., 2006). The *stx2a* (prototypic *stx2*) is the most important and the most implicated *stx* variant in severe STEC human disease including HUS (Boerlin et al., 1999; Orth et al., 2007; Matussek et al., 2017). The *stx2c* and *stx2d* subtypes are closely related to *stx2a* (Scheutz et al., 2012), and have also been associated with high frequency of HUS in patients (Eklund et al., 2002; Friedrich et al., 2002; Fuller et al., 2011). In STEC serotypes carrying the activatable *stx2d* variant (*stx*2_{dact}), Shiga toxin production is amplified by contact with intestinal mucus and elastase causing severe human disease that may be complicated with HUS

(Bielaszewska et al., 2006). The *stx2d* genotype, especially in *eaeA*-negative STEC, may be a contributing factor to the development of HUS in patients infected with STEC strains that rarely cause HUS (Friedrich et al., 2003; Bielaszewska et al., 2006). The *stx2e* gene is mainly observed in pig STEC strains and has been associated with edema disease of piglets (Weinstein et al., 1988; Sonntag et al., 2005). The *stx2f* variant is commonly found in STEC isolates from pigeons (Schmidt et al., 2000), but it has also been recovered from STEC isolates of human origin (Etoh et al., 2009; Friesema et al., 2014; Gannon et al., 1990). Although pigeons are the natural reservoir of STEC that carry *stx2f*, there are no reports of active disease in pigeons (Morabito et al., 2001). The *stx2g* variant has been isolated from STEC serotypes (O2:H25, O2:H45 and Ont:H^-) (Leung et al., 2003), but the role *stx2g* plays in human disease is unclear.

2.4.2 Intimin (*eaeA*)

Another major STEC virulence factor is intimin (*eaeA*) and is located on the Locus of enterocyte effacement (LEE) pathogenicity island (McDaniel et al., 1995). The *eaeA* gene is responsible for colonization of human enterocytes in various LEE positive STEC serotypes (McDaniel et al., 1995; Tzipori et al., 1995). The LEE pathogenicity island (LEE-PAI) consists of several genes encoding proteins that are essential for bacterial adhesion attaching and effacement (eae) lesions and pedestal formation when *eaeA* positive STEC attach to host enterocytes (Tzipori et al., 1995; Kaper et al., 1998; McDaniel and Kaper, 1997; McDaniel et al., 1995). In addition to *eaeA*, the LEE carries a cluster of genes that encode a type III secretion system (T3SS) and a translocated intimin receptor (*tir*) (McDaniel and Kaper, 1997; Nataro and Kaper, 1998). The T3SS gene cluster acts as a molecular “syringe or needle” through which effector proteins are secreted into target host cells (Hacker and Kaper, 2000; Lory, 1998). Effector proteins are responsible for a number of functions that modulate and interfere with host cellular processes including hemolysis,

inhibition of host lymphocyte phagocytosis, interference with iron transport, cytotoxicity and destruction of the host intestinal mucosa (Nataro and Kaper, 1998; Dean, 2011).

Currently, at least 31 intimin variants have been identified and described by various authors (Mellies et al., 1999; Zhang et al., 2002; Blanco et al., 2004; Blanco et al., 2006; Garrido et al., 2006; Yamamoto et al., 2009; Williams et al., 2010; Xu et al., 2016). Intimins vary in terms of genetic sequence of their C-terminal variant which determines tissue specificity of an intimin variant in the animal host (Blanco et al., 2004). Particular STEC serotypes that carry specific intimin variants may have a high tropism for a particular host (bovine, human sheep, etc.) or tissue (Blanco et al., 2004). For instance, bovine STEC O26:H11 and O157:H7 serotypes carry β1 and *Y*1 (beta 1 and gamma 1) intimin variants that have been described in serotypes causing human disease (Blanco et al., 2004).

2.4.3 Plasmid-encoded Virulence Markers

STEC carry plasmids of varying sizes that encode accessory genes that are considered to play a role in virulence (Perna et al., 2001; Hayashi et al., 2001). Plasmid-encoded determinants include an enterohemolysin (*ehxA*), a type II secretion pathway (*etp*D), a catalase-peroxidase (*kat*P), an extra-serine protease autotransporter protein (*espP*), a subtilase cytotoxin (*sub*A) and the STEC autoagglutinating adhesin *(saa*) (Schmidt et al., 1995; Brunder et al., 1996; Brunder et al., 1997; Schmidt et al., 1997;Paton et al., 2001; Paton et al., 2004). The *ehxA* encodes a haemolysin protein which is thought to lyse human erythrocytes and subsequent release of iron from heme (Beutin et al., 1989; Schmidt et al., 1995; Schmidt and Karch, 1996). The availability of iron in the host enhances *in vivo* multiplication and survival of STEC (Schmidt et al., 1995; Schmidt and Karch, 1996). The extracellular serine protease (*esp*P), cleaves coagulation factor V which enhances gastrointestinal tract bleeding in STEC disease (Brunder et al., 1997). The catalase-peroxidase (*katP*) defends the bacteria against reactive oxygen molecules and

prevents oxidative damage by host phagocytes (Brunder et al., 1996). STEC autoagglutinating adhesin (*saa*) facilitate adherence of *eaeA*-negative STEC to host epithelial cells (Paton et al., 2001). The *etpD* gene is a marker for type II secretion pathway (Schmidt et al., 1997), which delivers exoproteins (type II proteins) from pathogenic bacteria into target host cells (Lory, 1998). The subtilase cytotoxin (*subA*) is a plasmid encoded toxin and also an AB_5 protein (Paton et al., 2004). The subtilase cytotoxin contributes to human disease pathogenesis through its lethal subtilase cytotoxin activity by suppressing host immunity and tissue damage (Morinaga et al., 2007; Paton et al., 2004; Wang et al., 2007). The *saa* and *subA* genes have been observed in *eaeA*-negative isolates exclusively (Karama et al., 2008a; Khaitan et al., 2007).

2.4.4 Virulence Genes and Markers encoded on PAI other than the LEE

Whole genome sequencing (WGS) of STEC O157:H7 EDL933 (a strain that was recovered from the 1982 STEC outbreak) and STEC O157:H7 (recovered from 1996 STEC outbreak in Sakai city, Japan) revealed that the genome of STEC O157:H7 DNA is organized into Genomic 'O' Islands. Some O-Islands are pathogenicity islands (PAIs) since they carry virulence genes (Hayashi et al., 2001; Perna et al., 2001). Sequencing of STEC O157:H7 EDL933 strain has revealed 177 O-Islands, 18 and 24 of which are multigenic phages or prophages elements in STEC O157:H7 EDL933 and Sakai strains respectively (Hayashi et al., 2001; Perna et al., 2001).

STEC virulence genes that are carried on PAIs play a vital role in STEC pathogenicity (Karmali et al., 2003; Coombes et al., 2008; Bugarel et al., 2010a). Some PAIs including OI-122, OI-71, OI-57, OI-36 and OI-43/48 are known to carry genes that may contribute to STEC virulence (Perna et al., 2001; Taylor et al., 2002; Karmali et al., 2003; Shen et al., 2004; Coombes et al., 2008). Several PAI harbor non-LEE effector (*nle*) genes. The *nle* genes encode essential effector proteins that are secreted via the T3SS but are not carried

on the LEE. *Nle* genes are considered virulence markers especially in STEC strains that are frequently incriminated in human disease outbreaks and HUS (Karmali et al., 2003; Deng et al., 2004; Coombes et al., 2008; Bugarel et al., 2010a).

The main virulence genes markers that are carried on PAI OI-122 include Z4321 (*pag*C), Z4326/*espL2* (*sen*), Z4332 (*efa1*) and Z4333 (*efa1*) (Karmali et al., 2003). Furthermore, OI-122 also encodes Z4328 (*nleB)* and Z4329 (*nleE)* (Karmali et al., 2003). The Z4321 (*pag*C) gene is homologous to "*pho*P-activated gene (*pag*C) in *Salmonella enterica serovar* Typhimurium" whereas Z4326 (*sen*) encodes a protein similar to enterotoxin 2 in *Shigella flexneri* (Miller et al., 1989). Both *pagC* and *sen* are essential for regulation of bacterial virulence and promotion of bacterial survival within the host macrophages (Gunn et al., 1995; Ju et al., 2013). The *efa1* (EHEC factor for adherence) gene encodes a protein which facilitates bacterial adherence and enhances bovine bowel colonization by suppressing proliferation of host lymphocytes (Nicholls et al., 2000). Z4328 (*nleB)*, Z4329 (*nleE)*, encode proteins that modulate host cell functions, enhance STEC colonization and virulence by possibly suppressing the host immune system and subsequently lowering STEC infection dose (Kelly et al., 2006; Wickham et al., 2007). PAI OI-57 encodes also a number of non-LEE effector proteins including Z2149 (*nleG2-3*)*,* Z2150 (*nleG6-2*) *and* Z2151 (*nleG5-2*) genes that are responsible for suppressing the host immune system (Coombes et al., 2008; Imamovic et al., 2010; Wu et al., 2010). The OI-36 PAI carries Z0985 (*nleB*2), Z0986 (*nle*C), Z0989 (*nleH1-1*) and Z0990 (*nle*D) which also play a role in STEC virulence by enhancing colonization of host intestinal mucosa by STEC and suppressing the host immune system (Dziva et al., 2004; Garcia-Angulo et al., 2008; Pearson et al., 2011).

Genomic island 71 encodes Z6010 (*nle*G), Z6020 (*nle*F), Z6021 (*nleH1-2*), Z6024 (*nle*A), Z6025 (*nleG2-1*), and Z2560 (*nleG9*) which enhance the virulence of pathogenic STEC by

facilitating efficient colonization and also inhibition of host immune proteins (Gruenheid et al., 2004; Mundy et al., 2004; Tobe et al., 2006; Coombes et al., 2008). PAI OI-43/48 encodes gene markers *iha*, *ter*C and *ure*C. The *iha* gene confers adherence of STEC to epithelial cells while *ter* genes encode tellurite resistance in STEC (Tarr et al., 2000; Taylor et al., 2002), which promotes STEC adherence (Yin et al., 2009). The *ureC* gene converts urea to ammonia and facilitate STEC survival within the host acidic gut environment (Nakano et al., 2001; Taylor et al., 2002; Orth et al., 2006; Steyert et al., 2011). Screening for PAI associated gene markers has been used in "molecular risk assessment" to determine the virulence potential and capacity of STEC to cause human disease (Karmali et al., 2003; Coombes et al., 2008; Bugarel et al., 2010a).

Table 1: Virulence Genes, their Location and Associated Functions

Gene	Name	Location	Function	Reference
ehxA	Enterohaemolysin	pO157	Lyse erythrocytes releasing haemoglobin (enterohaemolytic phenotype).	(Schmidt et al., 1995; Schmidt and Karch, 1996)
*kat*P	Catalase-peroxidase	pO157	Defends the bacterial cell against oxidative damage by host macrophages	(Brunder et al., 1996)
etpD	Complex type II secretion system	pO157	Responsible for protein transport across the outer membrane.	(Schmidt et al., 1997)
espP	Serine protease autotransporter	pO157	Cleaves coagulation factor V, enhances haemorrhage in HC	(Brunder et al., 1997)
saa	STEC agglutinating adhesin	pO113	Attachment to host epithelial cells	(Paton et al., 2001)
eaeA	intimin	LEE	STEC key adhesin, in attaching and effacing	(McDaniel et al., 1995)
subA	Subtilase cytotoxic	pO113	Suppress host immunity, host tissue structure destruction and inhibition of protein synthesis	(Paton et al., 2004)
pagC	Phop activated genes C	OI-122	Regulate STEC virulence genes and promote bacteria survival within macrophages and resistance to low pH	(Gunn et al., 1995; Ju et al., 2013)
sen	*Shigella flexneri* enterotoxin 2	OI-122	Regulation STEC virulence and promotion of bacteria survival within the host immune system	(Gunn et al., 1995; Ju et al., 2013)
efa1	EHEC factor for adherence	OI-122	Facilitates EHEC adherence	(Nicholls et al, 2000)
terC	Tellurite resistance	OI-43/48	Promotes bacteria in general stress response within the host environment	(Taylor et al., 2002)
ureC	urease	OI-43/48	Promotes STEC survival within the host immune system	(Nakano et al., 2001; Taylor et al., 2002)
iha	Iron regulated gene A homologue adhesin	OI-43/48	Confers adherence to host epithelial cells	(Tarr et al., 2000)

Table adapted from Karmali *et al.*, (2003), Yin *et al*, (2009) and Bolton, (2011)

2.5 STEC Seropathotypes

STEC strains have been grouped into five seropathotypes (A to E) based on frequency in human disease, association with severe disease, and frequency in STEC outbreaks (Karmali et al., 2003; EFSA, 2007). Seropathotypes A strains include STEC serotypes which are commonly associated with disease outbreaks and HUS in humans (Karmali et al., 2003; EFSA, 2007). Seropathotype B are STEC serotypes that are less commonly involved in outbreaks and HUS compared to seropathotype A (Karmali et al., 2003; EFSA, 2007). Seropathotype C are STEC serotypes that have been incriminated sporadically in HUS but have not been incriminated in outbreaks. Seropathotype D includes STEC serotypes that cause mild diarrhea in humans but have never been involved in disease outbreaks or HUS. Seropathotype E includes all STEC serotypes that have never been implicated in human disease (Karmali et al., 2003; EFSA, 2007). Furthermore, EFSA recently has recommended improvement of Karmali *et al.*, classification of seropathotypes based on patient clinical outcome in STEC disease (EFSA, 2007). According to the EFSA seropathotype classification, seropathotype A, B and C strains have been merged together and classified as HUS-associated serotypes (HAS) (EFSA, 2007). In addition, the EFSA has recommended the use of molecular methods targeting specific virulence genes in assigning STEC serotypes into seropathotypes (EFSA, 2007).

2.6 Molecular Serotyping of STEC

Conventional serotyping (O:H grouping) of *E. coli* including STEC, involves identification of *E. coli* O and H antigens using specific antisera. The technique is based on agglutination reaction and has been widely used to serotype *E. coli* strains (Guinee et al., 1972; Guinée et al., 1981; Orskov et al., 1977). The O-antigen is constituted of repeat units (10-25) of *E. coli* outer membrane lipopolysaccharide (LPS), and oligosaccharides (O unit) sugar residues that vary structurally in arrangement and linkage within and between the

molecules. The O unit forms the basis for serogrouping of *E. coli* (Guinee et al., 1972; Ørskov and Ørskov 1984; Ewing, 1986; Guinée et al., 1981; MacLean et al., 2010). Currently, the *E. coli* serotyping scheme comprises of 188 O-groups (serogroups) designated O1 to O188 with O-groups O31, O47, O67, O72, O94 and O122 missing from the system (Orskov et al., 1977; Scheutz et al., 2004).

Furthermore, STEC serotyping involves also flagellar antigen (H-antigen) typing. There are 53 recognized H antigens that have been designated 1-56 (with numbers 13, 22, and 50 missing) (Ørskov & Ørskov, 1992; http://www.ssi.dk). Identification of H-antigen involves subculturing *E. coli* isolates in a semi-solid medium and selecting motile *E. coli* which are reacted with specific H-antisera (Guinée et al. 1981, Ørskov, Ørskov 1984, Ewing, 1986). Non-motile (NM) flagellated bacteria that are positive for H antigen are described as NM and *E. coli* strains for which the H antigen cannot be identified are termed H-non typeable (HNT). (Guinée et al. 1981, Ørskov, Ørskov 1984, Ewing, 1986)

Although O:H serotyping is considered the “gold standard”, it is time consuming, expensive, laborious and can only be performed by a limited number of reference laboratories. In addition, difficulties in serotyping rough strains which lack an outer membrane, cross reactivity of antisera with other O groups and variation of antisera specificity and avidity in different sera batches due to production or storage of antisera can lead to false positive results (DebRoy et al., 2011a; Lacher et al., 2014).

To circumvent the challenges of conventional O:H serotyping, various molecular serotyping protocols using specific primers targeting genes encoding O and H antigens have been designed (DebRoy et al., 2011b; Iguchi et al., 2016; Iguchi et al., 2015a; Machado et al., 2000; Singh et al., 2015). Molecular protocols use PCR primers that target O-antigen biosynthesis genes and exploit genetic heterogeneity to differentiate *E. coli* into O serogroups (DebRoy et al., 2011a; Iguch et al., 2015b).

Three major groups of O-antigen synthesis genes (O-AGC) have been targeted including O unit genes (flippase and polymerase), sugar transferase genes, and nucleotide sugar synthesis genes which are involved in synthesis and translocation of the O antigen (DebRoy et al., 2011a; Iguchi et al., 2015b; Reeves et al., 1996). Currently, oligonucleotides that target the *wzx* gene which encodes the O-antigen flippase and the *wzy* gene which encodes the O-antigen polymerase sequences have been designed and used to identify specific *E. coli* serogroups (Bai et al., 2012; DebRoy et al., 2011a; Iguchi et al., 2016; Iguchi et al., 2015a; Paddock et al., 2012). In addition, oligonucleotides targeting the *wbq*F and *wbq*E markers encoding putative acetyltransferase and glycosyltransferase enzymes respectively have also been designed to classify STEC into different O serogroups (Fratamico et al., 2003; Fratamico et al., 2005; Bai et al., 2012). However, some O-AGC sequences are genetically identical and may cross react on PCR with closely related sequences encoding O antigens (Iguchi et al., 2015b; Wang et al., 2009). For example, Wang and colleagues showed that serogroup O117 and O107 primers cross react (Wang et al., 2009). For example, serogroup O129 and O135 as well as O118 and O151 primers have also been shown to cross-react (Liu et al., 2008). In addition, Iguchi *et al.*, (2015b) reported that STEC O13, O17, O44, O73, O77 and O129 are 100% genetically similar but serologically distinct. There can be also immunological cross reactivity between the *E. coli* O antigen with different bacteria genera and species (Iguchi et al., 2015b).

A number of multiplex PCR protocols have also been developed for molecular serogrouping of the top seven STEC serogroups including O157, O145, O26, O103, O121, O111 and O45 (Bai et al., 2012; Conrad et al., 2014; DebRoy et al., 2011b; Durso et al., 2005; Fratamico et al., 2005; Fratamico et al., 2011; Fratamico et al., 2003; Iguchi et al., 2015a; Paddock et al., 2012; Perelle et al., 2004).

By exploiting genetic sequence variation in the *rfb* gene cluster region of the O-polysaccharide among *E. coli* strains, Coimbra *et al*., (2000) developed an *E. coli* O-antigen genotyping method using both polymerase chain reaction technique with restriction fragment length polymorphism (PCR-RFLP). In the PCR-RFLP protocol, the *rfb gene* cluster region which carries genes encoding enzymes responsible for O-antigen synthesis is amplified, and the amplicons obtained are digested using the *Mbo*II restriction enzyme resulting in distinct patterns that are used to identify *E. coli* serogroups by gel electrophoresis (Coimbra et al., 2000). This PCR-RFLP method was able to type 100% of *E. coli* strains including rough and non-agglutinating isolates (Coimbra et al., 2000).

Recently, Iguchi et al., (2015a) using in-house designed primers and numerous primers designed by other researchers developed 20 multiplex PCR protocols for molecular serogrouping of 162 *E. coli* serogroups. The primers were designed based on O-AGCs. Further work by Iguchi *et al*., (2016) has shown that it was possible to serogroup previously untypable and/or serologically indistinguishable *E. coli* and assign them to specific serogroups. At least six novel O genotypes have been described based on the use of O-AGCs sequences encoding polymerase and glycosyltransferase genes (Iguchi et al., 2016).

Molecular serotyping methods based on flagellar antigens (H antigen) have been described for H-type identification. Molecular flagellar typing differentiates *E. coli* and assigns strains to various H-types based on polymorphisms *in E. coli* flagella gene (*fliC*) sequences (Gannon et al., 1997; Coimbra et al., 2000; Machado et al., 2000; Bai et al., 2010; Singh et al., 2015). Based on *fliC* genes encoding H-antigens, Singh *et al*., (2015) have described three multiplex PCR (mPCR) protocols for detecting 14 common *E. coli* H-antigens (Singh et al., 2015).

In addition to PCR-based protocols utilizing O-AGC sequences, DNA microarray assay platforms for identification and characterization of STEC have also been developed. DNA microarray assays are increasingly being used in molecular serotyping of STEC to simultaneously detect and characterize *E. coli* by O serogroups, H-types and a number of virulence genes (Liu and Fratamico, 2006; Bugarel et al., 2010b; Matussek et al., 2017). In microarray assays, target DNA fragments are immobilized onto a chip surface and exposed to DNA sequences labeled with a fluorescent dye within a sample of interest. Corresponding DNA fragments attach to target DNA spots on the chip. A fluorescent scanner is used to determine the intensity of fluorescent dye after washing off the excess and unattached DNA fragments (Liu and Fratamico, 2006; Lacher et al., 2014; Matussek et al., 2017). For example, the Food and Drug Administration has developed an *Escherichia coli* identification microarray assay (FDA-ECID) which has been used to serotype and characterize STEC isolates from various foods for O:H grouping/typing and virulence associated genes respectively to assess their health risk for humans (Lacher et al., 2016). The microarray assay was able to identify 68 STEC serotypes (O:H types) (Lacher et al., 2016).

With increasing availability of whole genome sequences (WGS), wide genome sequencing technologies are also being exploited to serotype *E. coli using in silico* serotyping methods In protocols based on WGS, the SerotypeFinder software is used to predict *E. coli* O-groups and H-types by searching for the sequences of genes encoding O antigens "(*wzx*, *wzy*, *wzm*, and *wzt*)" and flagellin antigens "(*fliC, flkA, fllA, flmA* and *flnA*)" in *E. coli* genome sequences (Joensen et al., 2015; Laing et al., 2009). Recently, WGS and the *in silico* serotyping method was used to predict the serotype of a large number of cattle and human *E. coli* isolates (Mainda et al., 2016). Using *in silico* serotyping, it was possible to group 550/559 and 483/559 *E. coli* isolates into H and O types respectively (Mainda et al.,

2016). Failure to serotype the remaining *E. coli* isolates into specific H and O types was ascribed to “assembly issues with short read sequences”.

2.7 STEC Occurrence in Humans

In 1977, *E. coli* strains were shown to produce a toxin that was causing cytopathic effect (CPE) on Vero cells, this led to speculation that a toxin was being secreted by particular strains of *E. coli* (Konowalchuk et al., 1977). The first human STEC outbreak occurred in 1982 when STEC O157:H7 was found in 47 human stool samples who had developed hemorrhagic colitis (HC) after consumption of undercooked ground beef in Oregon and Michigan states (Riley et al., 1983; Wells et al., 1983). A number of studies later on, showed that STEC were transmissible through consumption of contaminated animal food products, particularly ground beef (Karmali et al., 1983; Griffin and Tauxe, 1991; Armstrong et al., 1996). Subsequent studies confirmed cattle were the main reservoir of STEC serotypes that are important in human disease (Chapman et al., 1989; Wells et al., 1991; Chapman et al., 1993; Hancock et al., 2001).

World Health Organization (WHO) global estimates of foodborne disease burden in the year 2010 was 600 million illnesses of which 0.7-2.5 million acute foodborne illness cases were attributable to STEC with an annual average mortality rate of 0.06% (128 deaths) from diarrheal diseases (Havelaar et al., 2015). Furthermore, Majowicz *et al*., (2014) estimated that STEC was the fourth leading cause of foodborne illnesses worldwide accounting for 2.8 million cases of acute foodborne illnesses.

In the United States, numerous reports have shown that the common STEC serogroups causing disease in humans include O103, O121, O145 O157, O26, O45 and O111 (Brooks et al., 2005; Johnson et al., 2006; Mathusa et al., 2010; Gould et al., 2013). These serogroups are frequently incriminated in severe human disease outbreaks and have been termed the “big seven” or “top seven” STEC serogroups (USDA FSIS 2012; Conrad et al.,

2014; Smith et al., 2014). In the period from 1982 to 2002, 350 STEC outbreaks that were due to STEC O157:H7 were reported in the 48 statesof the US and ground beef was incriminated as the main source for 61% foodborne cases (Rangel et al., 2005). Ground beef is the most frequently incriminated food in STEC outbreaks followed by vegetables and dairy foods in the US (Gould et al., 2013). Gould *et al*., (2013) reported an increased occurrence rates in non-O157 STEC (O26, O103, O45, O145, O121, and O111) from 0.12 to 0.95 per 100,000 persons while STEC O157 decreased from 2.5 to 0.95 per 100,000 for the period 2000 to 2010 in the United States.

In the United Kingdom, approximately 900 STEC cases are reported annually with STEC O157 and O26 serotypes being the most prevalent causative agent of severe human disease especially in young children including HUS (Byrne et al., 2014). However, a rare serotype STEC O104:H4 was incriminated in a widespread STEC outbreak in Germany (Frank et al., 2011). In this outbreak 3816 people were affected including 22% (845) HUS cases and 54 fatalities (Frank et al., 2011).

In Australia, for the year 2000 through 2010, STEC infection rates in humans increased progressively with STEC O157 accounting for 58% of cases, while 42% of human infections were mainly ascribed to mainly non-O157 STEC O26 and O111 (Vally et al., 2012). Furthermore, the total annual incidence of all STEC infections corresponded to 0.4 per 100,000 persons for the 11 years period. There were 11 outbreaks which were ascribed mainly to STEC O157 (50%), STEC O111 (13.7%), STEC O26 (11.1%) that affected 117 people with no deaths (Vally et al., 2012). The common sources of STEC outbreaks were contaminated foods of animal origin, water and person to person (Vally et al., 2012).

In 1996, a STEC outbreak affected 12,680 children in Sakai city, Japan, of which 121 developed HUS and three died (Izumiya et al., 1997; Fukushima et al., 1999). In the Sakai

city outbreak, STEC O157:H7 was incriminated (Fukushima et al., 1999). The STEC O157:H7 outbreak was linked to consumption of radish sprouts which had been supplied to children in elementary schools (Izumiya et al., 1997). Furthermore, the Japan Infectious Agents Survellance Reports (IASR), revealed that serotypes associated with STEC O157, O111, O121, O103 and O26 were recovered from human cases in various public health facilities and health centres for the period between 2005 through 2010, (http://idsc.nih.go.jp/iasr/virus/graph/vtec0510y. pdf). Kanayama *et al*., (2015) documented an increase in STEC outbreaks in daycare centres in Japan for the period 2010 through 2013. The outbreaks were attributed to STEC O103, O121, O145, O26, O157 and O111 (Kanayama et al., 2015). A total of 68 STEC outbreaks which affected 1035 children with four cases developing HUS were also reported (Kanayama et al., 2015).

Although the burden of STEC in humans and animals is global, data on STEC occurrence in humans and animals is mainly available in developed countries. However, comprehensive reports on STEC are practically nonexistent in developing countries including South Africa (WHO, 1997; Smith et al., 2011). Although there is a limited number of published reports on STEC from South Africa, in 1965 already HUS had been described in children (including 5 deaths) in Southern Africa (Barnard, 1965). Three years later, Kibel and Bernard hypothesized that the HUS could have been caused by a mutant *E. coli* that had been lysogenized by a bacteriophage (Kibel and Barnard, 1968). In 1992, a large outbreak which was ascribed to O157:H7 occurred in Swaziland and South Africa (Effler et al., 2001). This outbreak affected 40,912 persons after consuming water, which had been contaminated with STEC O157:H7 from dead cattle carcasses after a long drought (Effler et al., 2001). Smith and colleagues have also reported the isolation of diverse STEC serogroups from human specimens in South Africa between 2006 through 2009 (). In the study by Smith et al., (2011), serogroups O26, O111 and O157 accounted for 64.3% of the total STEC serogroups, which were isolated from humans between 2006 and 2009 (Smith

et al., 2011). STEC O26 was the most predominant serogroup (55.6%), followed by STEC O111 (33.3%) and STEC O157 (11.1%) (Smith et al., 2011).

A study by Ateba et al., (2008a), showed that the prevalence of STEC O157:H7 serotype was 44%-50%, 5.4%-20% and 7.5% in pig, cattle and human stool samples respectively in South Africa. Moreover, the prevalence of STEC O157:H7 was 29.7% in commercial dairy cattle fecal samples (Iweriebor et al., 2015). This is of great concern because STEC O157:H7 is the predominant serotype known to cause severe human disease, including HUS globally (Scallan et al., 2011; Majowicz et al., 2014). However, data on the presence of STEC O103, O121, O45, O145, O26, O157 and O111 in cattle and their virulence gene characterization in South Africa remains scarce. Therefore, there is a need to investigate the occurrence of cattle STEC O103, O121, O45, O145, O26, O157 and O111 serogroups that are commonly associated with human disease.

2.8 Antimicrobial Resistance

Increase of antimicrobial resistant bacteria due to overuse (or misuse) of antimicrobial agents in both agriculture and animal production systems has become a public health concern worldwide (Schwarz et al., 2001; Dibner and Richards, 2005; Ronquillo and Hernandez, 2017). The extensive use of antimicrobial agents in the treatment and/or prophylaxis of animal diseases as well as prolonged therapy may lead to both normal intestinal flora and pathogenic bacteria acquiring resistance against antimicrobials that are commonly used in human and veterinary medicine (Khachatourians, 1998; Li and Cosgrove, 2017). Antimicrobial resistance has been associated with the frequent use of low doses of antimicrobials for treatment or prophylaxis to keep animals healthy and for growth promotion (antimicrobial growth promotants) (Khachatourians, 1998; Smith et al., 2002; Cheney et al., 2015). Global estimates show that by the year 2030, antimicrobial consumption in animal food production systems will increase by 67% (Marshall and Levy,

2011; Van Boeckel et al., 2015). This is double the percentage increase of antimicrobial consumption observed in the years 2000 through 2010 (Van Boeckel et al., 2015).

Antimicrobial resistance has also been ascribed to innate resistance in some bacteria which are naturally resistant to specific antimicrobial drugs due to substances they secrete (Van Boeckel et al., 2015). Bacteria may also acquire resistance genes by changes in DNA sequences as a result of spontaneous mutations or/and transfer of resistance genes through mobile genetic elements including plasmids, transposons, and integrons to adapt and survive in new environments (Tenover, 2006). Acquired resistance gene(s) may be transferred from resistant to susceptible bacteria through mobile genetic elements.

When gene(s) encoding antibiotic resistance are transfereed onto plasmids, as bacterial numbers multiply, plasmid replicates are transferred to members of the same or other bacteria species, genus or family (Lawrence, 2005; Jain et al., 2002; Cambray et al., 2010). Transposons also carry antibiotic resistance genes (Thomas, 2000). Antimicrobial resitance genes can also be transferred on transposons. Transposons are genetic elements that are approximately 23 kilo base pairs (kb) with a single insertion sequence at both ends of the molecule and normally carry at most two resistance genes (Hedges and Jacob, 1974). Antimicrobial resistance genes are located within the insertion sequences in the transposon, which are points of insertion into new target DNA molecules (Kleckner, 1981).

Other than plasmids and transposons, integrons integrate resistance genes into bacterial genomes. Integrons are mobile elements that encode one or more antibiotic resistance gene cassettes with the ability to integrate and remove these genes (Kleckner, 1981). Integrons contain integrase genes (*intI)* that encode site specific recombinase (responsible for insertion and excision of genes), a specific recombination site (*attI*) which is recognized by the *intI* gene and a resident promoter where transcription originates (Bennett, 2008;

Hawkey, 2008), to allow expression of acquired resistance genes into functional genes (Bennett, 2008; Hawkey, 2008). Integrons are associated with transposons, insertion sequences and plasmids that mediate their transfer within and between bacteria species (Cambray et al., 2010).

Multidrug resistance (MDR) in cattle STEC isolates has been reported for different classes of antimicrobials in different countries (Amézquita-López et al., 2016; Colobatiu et al., 2014; Eurosurveillance editorial team, 2014; Karama et al., 2008a; Srinivasan et al., 2007; Iweriebor et al., 2015). A joint report by the European Center for Disease Prevention and Control (ECDC) and the European Food Safety Authority (EFSA) showed that cattle *E. coli* strains were resistant to ampicillin, sulfonamides, tetracyclines and streptomycin at levels ranging from 24% to 31%, while the resistance to ciprofloxacin and nalidixic acid were very low (EFSA and ECDC, 2015). Furthermore, high resistance to ampicillin (up to 95%), chloramphenicol (up to 90%), cephalothin (up to 95%), tetracyclin (up to 96%), oxytetracyclin (up to 95%), streptomycin (up to 84%), amoxicillin-clavulanate (up to 84%), trimethoprim- sulfamethazole (up to 84%), cefuroxime (up to 82%), has been documented by various authors among cattle STEC isolates from different regions (EFSA and ECDC, 2015).

In South Africa, a few studies have reported on antimicrobial resistance in *E. coli* and STEC. MDR against several antibiotics commonly used in veterinary and human therapy has been reported in generic *E. coli* and STEC isolates from the environment (Olaniran et al., 2009; Abong'o and Momba, 2009), different animal species (Ateba et al., 2008b; Iweriebor et al., 2015) and humans (Galane and Le Roux, 2001; Habte et al., 2009; Brink et al., 2012). For instance, Habte et al., (2009) using laboratory hospital record data reported that uropathogenic *E. coli* were resistant to various antimicrobials including ciprofloxacin (up to 33%) gentamicin (up to 50%) amoxicillin (43% to 100%) and co-

trimoxazole (29 to 90%). In addition, Galane and Le Roux, (2001) have shown that *E. coli* isolates that were recovered from diarrheal children in Gauteng were resistant to various antimicrobials. In this study, the the highest levels of resistance were recorded for ampicillin (Galane and Le Roux, 2001).

Although STEC is a commensal bacterium in cattle, it is considered good indicator of antimicrobial resistance and is commonly used to to monitor antimicrobial resistance in humans, animals and environmental isolates (Amézquita-López et al., 2016; Karama et al., 2008; Maidhof et al., 2002; Srinivasan et al., 2007). Assessment of antimicrobial resistance levels in STEC and generic *E. coli* isolates provides information on the misuse or abuse of antimicrobial agents in animals production systems, circulation of resistance genes between animals and their environments that can be passed to humans through the food chain (Threlfall et al., 2000; Cheney et al., 2015).

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3.0 CHAPTER III: Occurrence and characterization of seven major Shiga toxin-producing *Escherichia coli* serotypes from healthy cattle on cow-calf operations in South Africa.

3.1 Abstract

Cattle are the main reservoir of Shiga toxin-producing *Escherichia coli.* This study investigated occurrence of STEC O157, O145, O103, O121, O111, O45 and O26 among 578 STEC isolates previously recovered from 559 cattle. The isolates were characterized by serotype and major virulence genes. PCR revealed that 41.7% (241/578) of isolates belonged to STEC O157, O145, O103, O121, O45 and O26 and 33 associated serotypes that b. The 241 isolates corresponded to 16.5% (92/559) of cattle that were STEC positive. The prevalence of cattle that tested positive for at least one of the six serogroups across the 5 farms was variable ranging from 2.9% to 43.4%. Occurrence rates for individual serogroups were as follows: STEC O26 was found in 10.2% (57/559); O45 in 2.9% (16/559); O145 in 2.5% (14/559); O157 in 1.4% (8/559); O121 in 1.1% (6/559) and O103 in 0.4%, (2/559). The following proportions of virulence genes were observed: *stx1*, 69.3% (167/241); *stx2*, 96.3% (232/241); *eaeA*, 7.1% (17/241); *ehxA*, 92.5% (223/241) and both *stx1* and *stx2,* 62.2% (150/241) of isolates. These findings highlight that cattle in South Africa carry STEC that belong to the six major STEC serogroups commonly incriminated in human disease. However, only a subset of serotypes associated with these serogroups were clinically relevant in human disease. Most STEC isolates carried *stx1*, *stx2*, and *ehxA* but lacked *eaeA*, a major STEC virulence factor in human disease. The absence of *eaeA* may explain why STEC outbreaks in humans remain less prevalent and sporadic in South Africa.

3.2 Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are zoonotic foodborne pathogens that cause infections in humans characterized by mild to severe diarrhea, hemorrhagic colitis (HC) and complications such as the hemolytic uremic syndrome (HUS) especially in children (Karmali, 1989; Nataro and Kaper, 1998; Neill et al., 1987). Humans acquire STEC after consumption of contaminated foods of animal origin (Hussein and Sakuma, 2005; Hussein and Bollinger, 2005; Ombarak et al., 2016), and contaminated water and vegetables (Swerdlow et al., 1992; Cieslak et al., 1993; Keene et al., 1994; Feng, 2014). Furthermore, contact with infected animals or a contaminated environment, and persons to person have also been documented as routes of transmission (Belongia et al., 1993; Locking et al., 2001; O'Brien et al., 2001; Hale et al., 2012). Majowicz *et al*., (2014) estimated that STEC accounts for 2,801,000 cases of acute human disease worldwide annually.

More than 600 *E. coli* O:H serotypes exist and approximately 400 serotypes have been implicated in STEC disease in humans worldwide (Hussein and Bollinger, 2005; Bettelheim, 2007; Blanco et al., 2004; Beutin and Fach, 2014; EFSA, 2013; http://idsc.nih.go.jp/iasr/virus /graph /vtec0510y.pdf). The most predominant serotype in human infections is STEC O157:H7 (Armstrong et al., 1996; Fukushima et al., 1999; Greenland et al., 2009; Slayton et al., 2013). However, 70% to 80% of human infections have been attributed to isolates that belong to non-O157 STEC serogroups (Johnson et al., 2006; Gould et al., 2013; Luna-Gierke et al., 2014). A number of studies have documented that STEC O157, O111, O145, O26, O45, O103, and O121 are the seven major STEC serogroups mostly incriminated in severe disease and outbreaks in humans (Gould et al., 2013; Johnson et al., 2006; Luna-Gierke et al., 2014). These serogroups are sometimes termed the “big seven group” or the “top seven STEC” (USDA FSIS, 2012; Beutin and Fach, 2014; Fratamico et al., 2017).

Shiga toxins (Stx1 and Stx2) are the major virulence factors of STEC and typical STEC may either harbor *stx1* or *stx2* or both genes (O'Brien et al., 1984; Strockbine et al., 1986). Another important STEC virulence factor is intimin (*eaeA*) (Beebakhee et al., 1992; Yu and Kaper, 1992). The *eaeA* gene is located on a pathogenicity island termed the locus of enterocyte effacement (LEE) (McDaniel et al., 1995). Intimin facilitates bacterial cell adhesion and colonization of human enterocytes and produces classical attaching and effacing lesions that are observed in the host when *eaeA* positive STEC adhere to host enterocytes (McDaniel et al., 1995; Tzipori et al., 1995). Furthermore, STEC strains also possess the plasmid-encoded enterohemolysin (*ehxA*) which is responsible for lysing human erythrocytes and has been associated with hemorrhagic colitis in human STEC infections (Schmidt et al., 1995).

When the first human outbreak of STEC occurred in 1982, ground beef was incriminated as the source of STEC (Riley et al., 1983). Cattle have been recognized as a major STEC reservoir and cattle-derived food products (raw meat and dairy products) have been associated with a number of STEC outbreaks worldwide (Hussein and Sakuma, 2005; Ferens and Hovde, 2011; Farrokh et al., 2013). Although human STEC outbreaks have occurred in South Africa (Effler et al., 2001; Smith et al., 2011), epidemiological data on STEC in South Africa remains scanty. Furthermore, data on the role played by cattle as a potential source of STEC disease for humans in South Africa are lacking. In addition, studies describing the virulence characteristics of STEC isolates from South Africa remain unavailable. Therefore, the objectives of this study were to (i) investigate the occurrence of STEC O157, O26, O45, O103, O121, O111 and O145 in beef cattle (cow-calf operations) in South Africa and (ii) characterize STEC isolates by serotypes and major virulence genes.

3.3 Materials and Methods

3.3.1 Bacterial Strains used in this study.

A total of 578 confirmed STEC isolates (Dr. Karama's STEC collection-Veterinary Public Health section, Faculty of Veterinary Science, University of Pretoria) that had previously been recovered from 559 adult healthy cattle on five cow-calf operations (Farms A, B, C, D, and E) in Gauteng and Northwest provinces in South Africa were used in this study.

3.3.2 Cattle Study population.

A cross-sectional study of five cow-calf operations was conducted from June 2015 to March 2016 in Gauteng and North-West provinces, South Africa. The cow-calf operations produce calves for sale to feedlots. Operations serviced by the Onderstepoort Veterinary Animal Hospital (OVAH) consisting of more than 20 cows/heifers were selected for sample collection. The cow-calf operations were maintained on grazing pasture all year. Samples were collected during routine pregnancy diagnosis checks. Fresh fecal samples were collected from the rectum of adult cows and heifers using a new pair of disposable nitrile gloves for each animal. Fecal samples were placed in sterile specimen bottles, transported on ice to the laboratory and stored at 4˚C until further processing. Each herd was visited only once. A total of 559 fecal and 559 animals were sampled throughout the study.

3.3.3 Detection of STEC.

Each sample (5g) was placed in 45 ml of EC Broth (CM0990; Oxoid, Basingstoke, United Kingdom) containing 20 mg/L of Novobiocin (N1628, Sigma-Aldrich, St. Louis, MO) and incubated overnight at 37˚C. One hundred microliter (100 µl) aliquots of the enrichment broth were spread on Drigalski Lactose agar (CM0531; Oxoid, Basingstoke, United Kingdom) and CHROMagar STEC (http://www.chromagar.com) and incubated at 37˚C for 18-24 hours. All Drigalski Lactose agar and CHROMagar STEC plates showing growth after 18-24 hours of incubation were screened for STEC by PCR (Paton and Paton, 1998).

Briefly, a sterile inoculating loop was used to harvest colony sweeps from Drigalski Lactose agar and CHROMagar plates. The loopful of bacterial culture was suspended in a sterile 1.5 ml Eppendorf tube containing 1ml of FA buffer (Becton Dickinson and Company Sparks, USA). The bacterial suspension was mixed and washed by vortexing, followed by centrifugation for 5 minutes. After centrifugation, the supernatant was discarded and the cell pellet was re-suspended in FA buffer. After two washes and two centrifugation cycles, the pellet was suspended in 500 µl of sterile water, vortexed and the homogeneous cell suspension was boiled at 100˚C for 15 min, and then stored at -20˚C for further processing (Monday et al., 2007). A multiplex PCR that targeted *stx1*, *stx2*, *eaeA* and *ehxA* was used to detect STEC (Paton & Paton, 1998). Drigalski Lactose agar and CHROMagar plates which were positive for *stx1* and/or *stx2* on multiplex PCR were streaked onto Drigalski Lactose agar and CHROMagar to obtain single colonies and incubated for 18-24 hours at 37˚C. Three suspect single colonies were taken from each positive agar plate and sub-cultured on Luria Bertani for multiplication and purification. Once again, DNA was extracted (Monday et al., 2007), from pure colonies and a multiplex PCR (Paton and Paton, 1998) was used to verify and confirm the STEC status of the pure colonies. Confirmed STEC pure single colonies were stored at -80˚C in sterile cryovials containing a freezing mixture comprising Brain Heart Infusion broth (70%) and glycerol (30%) for further processing.

3.3.4 Bacteria DNA extraction.

Frozen pure STEC cultures were resuscitated by overnight growth at 37°C on Violet Red Bile Lactose agar (VRBL) (Oxoid, UK). Characteristic discrete single *E. coli* colonies were multiplied by subculture onto Luria Bertani (LB) agar (Difco^{TM} Becton and Dickson &Company) and incubated at 37°C for 18-24 hours. Genomic DNA was extracted from STEC single colonies by the boiling method as previously described (Monday et al., 2007) with slight modifications. Briefly, a loopful of overnight colony sweeps was suspended in

1000 µL of sterile FA buffer (Bacto^{TM} FA Buffer, Becton and Dickson &Company) in a 1.5 mL eppendorf tube, vortexed and centrifuged at 12,000 rpm for 5 minutes. The supernatant was discarded and the bacterial pellet was re-suspended in 1000 µL of sterile FA buffer and centrifuged. This process was repeated twice. After the last centrifugation cycle, the supernatant was discarded completely. The pellet was re-suspended in 500 µL of sterile distilled water, boiled for 20 minutes on a heating block and cooled on ice for 10 minutes and frozen at -20°C for further processing.

3.3.5 Reconfirmation of *E. coli* status.

Polymerase chain reaction (PCR) was performed to confirm the *E. coli* status of isolates using primers and PCR cycling conditions as previously described (Doumith et al., 2012). Before carrying out PCR, the frozen mixture containing DNA was thawed at room temperature, centrifuged at 12,000 rpm for 5 minutes and stored on ice. Briefly, each PCR reaction (25µL) contained 2.5µL of 10X Thermopol reaction buffer, 2.0µl of 2.5mM dNTPs (deoxynucleotide triphosphates), 0.25µl of 100mM $MgCl_2$, 1.6µl of each primer (0.64µM final concentration), 1U of Taq DNA Polymerase (New England BioLabs® *Inc.*) and 5µl of DNA lysate template. DNA from *E. coli* strain ATCC 25922 and sterile water without DNA were used as positive and negative controls, respectively. All PCR reagents were supplied by New England BioLabs (NEB, USA) except for the primers which were supplied by Inqaba Biotec (South Africa) or Integrated DNA Technologies (IDT) (San Diego, USA).

3.3.6 Molecular Serotyping.

PCR was used to detect *E. coli* serogroups (O) using previously described primers and cycling conditions (**Table 1**). Flagellar (H) antigens were determined by three previously described multiplex PCR protocols, primers and cycling conditions (Singh et al., 2015). Briefly, each PCR reaction (25µL) contained 2.5µL of 10X Thermopol reaction buffer, 2.0µl of 2.5mM dNTPs, 0.25µl of 100mM $MgCl_2$, 0.5µl of each primer (0.2µM final

concentration), 1U of Taq DNA Polymerase (New England BioLabs, USA) and 5µl of the DNA lysate template. For all PCR reactions, a Veriti™ (Applied Biosystems®, USA) or a C1000 Touch™ (Bio-Rad, USA) thermal cycler was used. PCR products were electrophoresed on 2% (w/v) agarose gels in TAE (Tris–acetate–ethylenediamine tetra acetic acid) buffer, stained with ethidium bromide (0.05mg/µl) and visualized under ultraviolet (UV) light with a Gel Doc system (Bio-Rad, USA). The following *E. coli* reference strains were used as positive controls for molecular serotyping: STEC-C210-03 (O157), STEC-ED476 (O111), STEC- C1178-04 (O145), STEC-C125-06 (O103) and STEC-ED745 (O26). STEC reference positive control strains were kindly provided by Alfredo Caprioli and Rosangela Tozzoli (European Union Reference Laboratory for *Escherichia coli,* Istituto Superiore di Sanità, Rome Italy).

Table 1: Nucleotide sequences of primers used in PCR reaction

O Antigen	Target Gene	Sequence	Amplicon size (bp)	Reference
E. coli	*gad*A	**F:** GATGAAATGGCGTTGGCGCAAG **R:** GGCGGAAGTCCCAGACGATATCC	373	(Doumith et al., 2012)
O26	*wzx*	**F:** GGGGGTGGGTACTATATTG **R:** AGCGCCTATTTCAGCAAAGA	241	(Paddock et al., 2012)
O45	*wzx*	**F:** TATGACAGGCACATGGATCTGTGG **R:** TTGAGACGAGCCTGGCTTTGATAC	255	(DebRoy et al., 2005)
O103	*wzx*	**F:** TATCCTTCATAGTAGCCTGTTGTT **R:** AATAGTAATAAGCCAGACACCTG	320	(Monday et al., 2007)
O111	*wzx*	**F:** CAAGAGTGCTCTGGGCTTCT **R:** AACGCAAGACAAGGCAAAAC	451	(Paddock et al., 2012)
O121	*wzy*	**F:** CAAATGGGCGTTAATACAGCC **R:** TTCCACCCATCCAACCTCTAA	193	(Iguchi et al., 2015)
O145	*wzy*	**F:** ATGGGCAGTATCTCTGGTATTGAA **R:** TTGAAAGCCCGGATATTAGGAA	334	(Paddock et al., 2012)

O157	*wzx*	**F:** GCTGCTTATGCAGATGCTC **R:** CGACTTCACTACCGAACACTA	133	(Monday et al., 2007)
Shiga toxin	*stx1*	**F:** ATAAATCGCCATTCGTTGACTAC **R:** AGAACGCCCACTGAGATCATC	180	(Paton and Paton, 1998)
Shiga toxin	*stx2*	**F:** GGCACTGTCTGAAACTGCTCC **R:** TCGCCAGTTATCTGACATTCTG	255	(Paton and Paton, 1998)
	eaeA	**F:** GACCCGGCACAAGCATAAGC **R:** CCACCTGCAGCAACAAGAGG	384	(Paton and Paton, 1998)
	ehxA	**F:** GCATCATCAAGCGTACGTTCC **R:** AATGAGCCAAGCTGGTTAAGCT	584	(Paton and Paton, 1998)

3.3.7 Virulence Gene Profiling.

PCR was performed to detect *stx1, stx2, eaeA* and *ehxA* genes using previously described primers and PCR cycling conditions (Paton and Paton, 1998). Briefly, the final volume of each PCR reaction was 25µL including 2.5µl of 10X Thermopol reaction buffer, 2.0µl of 2.5mM dNTPs, 0.25µL of 100mM $MgCl_2$, 0.75µL of each primer (0.3µM final concentration), 1U of Taq DNA Polymerase (New England BioLabs® *Inc.*) and 5µl of DNA lysate template. STEC O157:H7 strain EDL933 and sterile water without DNA were used as positive and negative controls respectively.

3.3.8 Statistical Analysis.

Descriptive statistical analyses were performed using the statistical package for social sciences (SPSS) software version 21 (SPSS® IBM® Statistics 21, New York, NY, USA). Differences between proportions of genes were analyzed using Chi-Square test and associations between farms, serotypes and virulence genes, were assessed using Fisher's exact test. Variables with P values of <0.05 were considered statistically significant.

3.4 Results

A total of 578 STEC isolates which were previously recovered from 559 cattle on five cow-calf operations (Farms A, B, C, D, and E) were screened for O157 and top 6 non-O157 STEC serogroups including O145, O103, O121, O111, O45 and O26. Molecular serotyping revealed that 41.7% (241/578) of isolates recovered from 92 cattle belonged to STEC serogroups O157, O145, O103, O121, O45 and O26. The 241 (41.7%) STEC isolates that belonged to the six serogroups accounted for 16.5% (92/559) of cattle (95 percent CI 13.48 to 19.80) (**Table 2**).

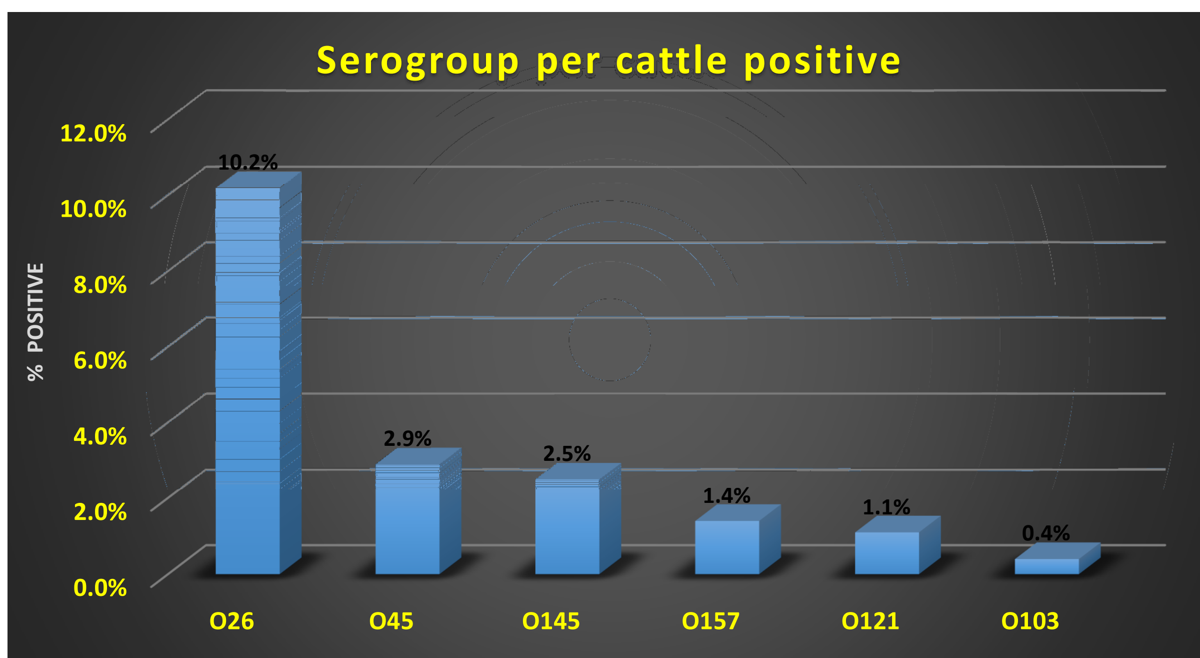
STEC O111 was not detected. The proportion of cattle that were positive for at least one of the six STEC serogroups across the 5 farms ranged from 2.9% to 43.4%. Within individual farms, the following prevalence rates were found: Farm A, 43.3% (33/76); Farm B, 14.4% (29/202); Farm C, 12.5% (19/152); Farm D, 2.9% (3/102); Farm E, 14.8% (4/27) (**Table 2**). Occurrence rates for individual serogroups among the 559 cattle were as follows: STEC O26, 10.2% (57 isolates); STEC O45, 2.9% (16 isolates); STEC O145, 2.5% (14 isolates); STEC O157, 1.4% (8 isolates); STEC O121, 1.1% (6 isolates); and STEC O103; 0.4% (2 isolates) (**Fig. 1**).

Table 2: Distribution of STEC serotypes on farms (A, B, C, D and E)

Farm	Farm Prevalence	Serogroup (No. of cattle +) n=92	Cattle ID	STEC Serotype	Isolate (N=241)
A	**43.4%** **(33/76)**	O145, 1.1% (1/92)	A67	O145:H28	3
		O157, 3.3% (3/92)	A73	O157:H2	1
			A12	O157:H7	2
			A76	O157:H28	1
		O26, 33.7% (31/92)	A (1,2, 3,11,21, 25, 32, 53, 55, 59, 69, 70, 73)	O26:H2	39
			A (24, 26,36,46,57)	O26:H8	17
			A (56, 71)	O26:H11	3
			A (15, 17, 18, 27, 63, 69)	O26:H19	13
			A (21, 28, 43, 68)	O26:H21	10
			A (4, 14,67)	O26:HNT	13
B	**14.4%** **(29/202)**	O121, 6.5% (6/92)	B (19, 25, 26, 27, 41)	O121:H8	8
			B 18	O121:H21	1
			B 26	O121:HNT	1
		O45, 17.4% (16/92)	B 8	O45:H2	1
			B (22, 28)*	O45:H8	3
			B (6, 13, 18)	O45:H11	8
			B 32	O45:H16	3
			B (24, 25, 35)	O45:H19	3
			B (3, 6)	O45:H21	2
			B 36	O45:H28	1
			B (18, 21, 39)	O45:H38	5
			B (17, 20,21, 22, 24, 28, 36, 39)	O45:HNT	12
		O26, 22.8% (21/92)	B 8	O26:H2	1
			B 50	O26:H7	2
			B (18, 19, 22, 26, 28)	O26:H8	11
			B (5, 18, 22, 28, 35)	O26:H11	7
			B (32, 50)	O26:H16	2
			B (20, 40)	O26:H19	5

			B (16, 33, 47)	O26:H21	5
			B 18	O26:H28	1
			B (18, 25, 39, 45, 47)	O26:H38	7
			B 35	O26:H45	1
			B (18, 36,45, 51)	O26:HNT	4
C	**12.5% (19/152)**	O145, 14.1% (13/92)	C 4	O145:H2	1
			C 17	O145:H7	1
			C 11	O145:H8	1
			C 3	O145:H11	1
			C (11, 13, 18, 24, 28, 34, 35)	O145:H19	13
			C (1, 2, 49)	O145:HNT	3
		O157, 3.3% (3/92)	C (27, 29)	O157:H7	2
			C 28	O157:H19	1
		O26, 9.8% (9/92)	C 7	O26:H2	1
			C 11	O26:H4	2
			C (13, 17)	O26:H7	2
			C (3, 10, 33)	O26:H8	3
			C 4	O26:H28	1
			C (4, 6)	O26:HNT	2
D	**2.9% (3/102)**	O26, 3.3% (3/92)	D 7	O26:H2	1
			D 2	O26:H21	2
			D 3	O26:HNT	1
E	**14.8% (4/27)**	O157, 2.2% (2/92)	E (2, 65)	O157:H7	5
		O103, 2.2% (2/92)	E 69	O103:H2	1
			E 41	O103:H21	1

Figure 1: The frequency of STEC Serogroups in 559 cattle



3.4.1 STEC Serotypes.

Further H typing revealed that the 241 STEC isolates belonged to 14 H types (**Table 3**). However, 14.9% (36/241) of the isolates were classified as H-non typeable (HNT) partly because we were limited to 14 pairs of primers which could only identify 14 H types that are commonly found in cattle (Singh et al., 2015). Overall, 85.1% (205/241) of the isolates were fully serotypeable and assigned to 33 O:H serotypes. The 33 STEC serotypes included O26:H2, O26:H4, O26:H7, O26:H8, O26:H11, O26:H16, O26:H19, O26:H21, O26:H28, O26:H38, and O26:H45; O45:H2, O45:H8, O45:H11, O45:H16, O45:H19, O45:H21, O45:H28, and O45:H38; O103:H2 and O103:H21; O121:H8 and O121:H21; O145:H2, O145:H7, O145:H8, O145:H11, O145H19, and O145:H28; O157:H2, O157:H7,O157:H19 and O157:H28 (**Table 3**). The 36 HNT isolates included: STEC O26: HNT (20 isolates), STEC O45:HNT (12 isolates), STEC O145: HNT (3 isolates) and STEC O121:HNT (1 isolate). STEC O26 serotypes accounted for 65.1% (157/241) of all STEC isolates identified in this study. The four most frequent O26 serotypes included STEC

O26:H2, 17.4% (42/241); O26:H8, 12.9% (31/241); O26:H19, 7.5% (18/241); and O26:H21, 7.1% (17/241). The most widespread serotypes (found on 3 farms or more) included STEC O26:H2 (4 farms), O26:H8 (3 farms), O26:H21 (3 farms) and O157:H7 (3 farms). Furthermore, 78.3% (72/92) of animals carried a single serotype while the remaining 21.7% (20/92) carried more than one serotypes.

Particular serotypes colonized cattle on specific farms (**Table 2**). STEC O45 (O45:H2, O45:H7, O45:H11, O45:H16 O45:H19, O45:H21, O45:H28, O45:H38 and O45:HNT), STEC O121 (O121:H8, O121:H21 and O121:HNT), and STEC O26 (O26:H38 and O26:H45) were found on Farm B only. STEC O103:H2 and O103:H21 serotypes were isolated on farm E only. STEC O145 (O145:H2 O145:H7, O145:H8, O145:H11 and O145:H28) occurred on Farms A and C only while STEC O157 (H2 and H28) and O157:H19 serotypes were exclusively isolated on farm A and C respectively.

Table 3: Association of H-type with O-antigen group

O-antigen Group	Associated H- type
O26	**H2**[d] (42)[a], H4 (2), H7 (4), **H8**[d] (31), **H11**[d] (10), H16 (2), **H19**[d](18), **H21**[d](17), H28 (2), H38[c](7), H45[c] (1), NT[b] (20)
O45	**H2**[d] (1)**,** H8 (3), H11 (8), H16 (3), H19 (3), H21 (2), H28 (1), H38 (5), NT (12)
O145	H2 (1), **H7** [d] (1), **H8**[d] (1), H11 (1), H19 (13), **H28**[d](3), NT(3)
O121	**H8**[d] (8), H21 (1), NT(1)
O157	H2 (1), **H7**[d] (9), H19 (1), H28[c] (1)
O1O3	**H2**[d] (1), **H21**[d] (1)

[a] The numbers in parentheses are the numbers of isolates; [b] Not typeable; [c] New serotype not found in bovine STEC in previous studies; [d]Serotypes associated with STEC human disease in previous studies.

3.4.2 Virulence Genes (*stx1, stx2*, *eaeA* and *ehxA*).

The distribution of major STEC virulence genes was as follows: *stx1*, 69.3% (167/241); *stx2*, 96.3% (232/241); *eaeA*, 7.1% (17/241) and *ehxA*, 92.5% (223/241). Both *stx1* and *stx2* occurred in 62.2% (150/241) of isolates (**Table 4**). The majority of STEC isolates

carried *stx1 stx2 ehxA* (61%, 147/241), and *stx2 ehxA*, (22.8%, 55/241) as the major gene combination pathotype. Minor gene combinations were also observed: *stx2 eaeA ehxA*, 5.8% (14/241); *stx1 stx2,* 3.7% (9/241); *stx1 ehxA*, 1.7% (4/241) and *stx1 eaeA ehxA*, 1.2% (3/241) (**Table 4**).

The *eaeA* gene was found in 7.1% (17/241) of isolates. Isolates that carried *eaeA* positive were recovered from 1.9% (10/559) of animals. The majority, 58.8% (10/17) of *eaeA*-positive STEC were STEC O157 isolates including O157:H7 (9 isolates) and O157:H28 (1 isolate). The additional seven *eaeA*-positive isolates included STEC O145:H28 (3 isolates), O145: HNT (1 isolate), O26:H2 (2 isolates), and O103:H2 (1 isolate). Most, 82.4% (14/17) of the *eaeA* positive isolates carried also *stx2* as the only Shiga toxin-encoding gene. The remaining *eaeA* positive isolates, 17.6% (3/17) belonging to STEC O26:H2 (2 isolates) and STEC O103:H2 (1 isolate) carried *stx1* only (**Table 5**).

Table 4: STEC Major Virulence Factors and Gene combinations

Serotype	No.	*stx1*	*stx2*	*eaeA*	*ehxA*	Gene combination
O26:H2^{D}	37	+	+	-	+	*stx1, stx2, ehxA*
O26:H2	1	+	+	-	-	*stx1, stx2*
O26:H2	2	+	-	+	+	*stx1, eaeA, ehxA*
O26:H2	1	+	-	-	-	*stx1*
O26:H2	1	+	+	-	-	*stx1, stx2*
O26:H4	2	+	+	-	-	*stx1, stx2*
O26:H7^{BD}	3	+	+	-	+	*stx1, stx2, ehxA*
O26:H7	1	+	+	-	-	*stx1, stx2*
O26:H8^{D}	1	+	+	-	+	*stx1, stx2, ehxA*
O26:H8	9	-	+	-	+	*stx2, ehxA*
O26:H8	20	+	+	-	+	*stx1, stx2, ehxA*
O26:H8	1	+	-	-	+	*stx1, ehxA*
O26:H11$^{HUS, BD, D}$	1	+	+	-	-	*stx1, stx2*
O26:H11	9	+	+	-	+	*stx1, stx2, ehxA*
O26:H16	1	-	+	-	-	*stx2*
O26:H16	1	-	+	-	+	*stx2, ehxA*
O26:H19	1	+	+	-	+	*stx1, stx2, ehxA*
O26:H19	4	-	+	-	+	*stx2, ehxA*
O26:H19	12	+	+	-	+	*stx1, stx2, ehxA*
O26:H19	1	+	+	-	+	*stx2, ehxA*

O26:H21[D]	12	-	+	-	+	*stx2, ehxA*
O26:H21	3	-	+	-	-	*stx2*
O26:H21	1	+	+	-	-	*stx1, stx2*
O26:H21	1	+	+	-	+	*stx1, stx2, ehxA*
O26:H28	1	+	+	-	+	*stx1, stx2, ehxA*
O26:H28	1	+	+	-	-	*stx1, stx2*
O26:H38	7	-	+	-	+	*stx2, ehxA*
O26:H45	1	+	+	-	+	*stx1, stx2, ehxA*
O26:HNT	7	-	+	-	+	*stx2, ehxA*
O26:HNT	11	+	+	-	+	*stx1, stx2, ehxA*
O26:HNT	1	+	+	-	-	*stx1, stx2*
O26:HNT	1	+	-	-	+	*stx1, ehxA*
O45:H2[D]	1	+	+	-	+	*stx1, Stx2, ehxA*
O45:H8	1	+	+	-	+	*stx1, Stx2, ehxA*
O45:H8	2	+	-	-	+	*stx1, ehxA*
O45:H11	8	+	+	-	+	*stx1, Stx2, ehxA*
O45:H16	3	-	+	-	+	*stx2, ehxA*
O45:H19	2	+	+	-	+	*stx1, Stx2, ehxA*
O45:H19	1	-	+	-	+	*stx2, ehxA*
O45:H21	1	-	+	-	-	*stx2*
O45:H21	1	-	+	-	+	*stx2, ehxA*
O45:H28	1	+	-	-	-	*stx1*
O45:H38	5	-	+	-	+	*stx2, ehxA*
O45:HNT	10	+	+	-	+	*stx1, Stx2, ehxA*
O45:HNT	1	-	+	-	-	*stx2*
O45:HNT	1	-	+	-	+	*stx2, ehxA*
O157:H7 [B, BD, HUS]	9	-	+	+	+	*stx2, eaeA, ehxA*
O157:H2	1	+	+	-	+	*stx1, stx2, ehxA*
O157:H19	1	-	+	-	+	*stx2, ehxA*
O157:H28	1	-	+	+	+	*stx2, eaeA, ehxA*
O145:H2	1	+	+	-	+	*stx1, stx2, ehxA*
O145:H7 [BD, D]	1	+	+	-	+	*stx1, stx2, ehxA*
O145:H8 [D]	1	+	+	-	+	*stx1, stx2, ehxA*
O145:H11	1	+	+	-	+	*stx1, stx2, ehxA*
O145:H19	13	+	+	-	+	*stx1, stx2, ehxA*
O145:H28 [HUS, D]	3	-	+	+	+	*stx2, eaeA, ehxA*
O145:HNT	1	-	+	+	+	*stx2, eaeA, ehxA*
O145:HNT	2	+	+	-	+	*stx1, stx2, ehxA*
O121:H8 [D]	7	+	+	-	+	*stx1, stx2, ehxA*
O121:H8	2	-	+	-	+	*stx2, ehxA*
O121:HNT	1	+	+	-	+	*stx1, stx2, ehxA*
O103:H2 [BD,]	1	+	-	+	+	*stx1, eaeA, ehxA*
O103:H21[D]	1	-	+	-	-	*stx2*
Total	**241**	**167**	**232**	**17**	**223**	
%Positive		**69.3**	**96.3**	**7.1**	**92.5**	

a STEC Serotypes in bold have been previously incriminated in human disease; **D**: Diarrhea; **BD**: Blood diarrhea; **HUS**: Hemolytic uremic syndrome.

3.5 Discussion

Although a few studies have investigated the occurrence of STEC in cattle in South Africa (Ateba et al., 2008; Iweriebor et al., 2015), data on the prevalence of STEC O157 and top six non-O157 STEC is nonexistent. This study investigated the prevalence of the seven major STEC serogroups including O157, O26, O45, O103, O111, O121 and O145 in adult healthy cattle from cow-calf operations in two provinces of South Africa. Furthermore, the isolates were characterized by serotype and virulence factors including *stx1, stx2, eaeA* and *ehxA*. The findings of this study indicate that STEC belonging to serogroups O26, O45, O103, O121, O145, and O157 colonize cattle on cow-calf operations in South Africa. The majority of cattle carried STEC O26. However, STEC O111 was not detected. Our findings are in agreement with similar studies which have observed the predominance of STEC O26 in cattle (Jenkins et al., 2003; Pearce et al., 2006; Joris et al., 2011; Paddock et al., 2012; Cernicchiaro et al., 2013; Mellor et al., 2016). However, similar studies in different countries have also recorded STEC O157, STEC O103, STEC O45 or STEC O145 as the most frequent serogroups in cattle (Barlow and Mellor, 2010; Lynch et al., 2012; Dargatz et al., 2013; Ekiri et al., 2014; Dewsbury et al., 2015; Bonardi et al., 2015; Stromberg et al., 2015). In addition, the lack of STEC O111 was not surprising and this was consistent with studies in which STEC O111 was not recovered at all or was infrequent in cattle (Pearce et al., 2006; Thomas et al., 2013).

The cumulative prevalence (16.5%) of STEC O157 and top six non-O157 STEC (O145, O103, O121, O111, O45 and O26) in the cattle surveyed was lower than previously recorded in a number of studies in the United States of America (USA) which reported prevalence rates ranging from 44.2% to 97.7% (Paddock et al., 2012; Dargatz et al., 2013; Stanford et al., 2016). Furthermore, studies that were carried out in Australia and France registered considerably lower prevalences of 7.7% and 1.8%, respectively, in cattle feces

(Bibbal et al., 2015; Mellor et al., 2016). However, the difference between the rate obtained in this study and previous studies in which far higher prevalence rates of the seven major STEC serogroups were observed may be mainly ascribed to differences in cattle populations surveyed. Furthermore, studies that are cited above reported rates of STEC O157 and top six non-O157 STEC (O145, O103, O121, O111, O45 and O26) that were observed in feedlot cattle (Cernicchiaro et al., 2013; Dargatz et al., 2013; Stromberg et al., 2015;Stanford et al., 2016). Feedlot cattle have been shown to have a higher STEC prevalence in comparison to other cattle production systems because of high numbers and mixing of cattle in a feedlot, that are mainly fed concentrate in a highly contaminated/unhygienic environment (Huntington, 1997; D. Smith et al., 2001). Although the cow-calf operations which were surveyed also kept a large number of animals, cattle in this farming system are mainly fed on pastures as opposed to a feedlot production system in which concentrates are used to feed animals, which may have accounted for the low STEC prevalence observed in cow-calf operations system (Gunn et al., 2007). Concentrate feeding creates an environment in the gut facilitating the proliferation of STEC strains resulting in excretion of STEC in large numbers on farm environment (Huntington, 1997; Stephens et al., 2009).

The prevalence of infected cattle per farm ranged from 2.9% to 43.3% consistent with reports from Japan and the United Kingdom which reported similar ranges (Ellis-Iversen et al., 2007; Gunn et al., 2007; Lee et al., 2017). However, the farms prevalence rates obtained in this study were relatively lower in comparison to studies in Brazil, South Korea and USA cattle farms which reported up to 100% for cattle that tested positive for STEC (Cull et al., 2017; Dong et al., 2017; Pereira et al., 2003).

We also observed that particular serotypes were confined to specific farms. For example, all STEC O45 and STEC O103 serotypes were isolated on farm B and farm E respectively.

In contrast, STEC O145:H28 was recovered on farm A whereas STEC O145:H2, O145:H7, O145:H8, O145:H11 and O145:H19 serotypes on farm C. Although STEC O26 serotypes were found on farms A, B, C, and D, STEC O26:H11 serotype was found on farms A and B only. The management practices, confinement of cattle on cow-calf operations within the farm environment, lack of close proximity of farms sampled, lack of cattle movement between farms and infrequency in the introduction of new stock into the farm or mixing, may have influence on the particularity of STEC serotypes on farms.

In the present study, single serogroups and/or serotypes were recovered from individual animals in most instances. However, 21.7% cattle carried multiple serotypes consistent with previous studies in Spain, Germany and France, which reported up to 24% STEC positive cattle that carried more than one serogroup (Blanco et al., 1996; Menrath et al., 2010; Bibbal et al., 2015). Of particular interest one cow was colonized by five serotypes including STEC O26:H8, O26:H11, O26:H28, O26:H38 and O121:H21 in this study. Individual cattle that shed numerous STEC serotypes have been referred to as "super-spreaders" as they can infect other animals within the herd and have an impact on farm prevalence of individual STEC serotypes (Matthews et al., 2006; Chase-Topping et al., 2008). Super-spreaders are a risk and can expose and infect herd mates directly or indirectly through environmental contamination (Matthews et al., 2006; Arthur et al., 2009).

Thirty-three different serotypes were detected in this study. The most prevalent serogroup was O26 consistent with a number of studies that have reported a high prevalence of STEC O26 in cattle (Cernicchiaro et al., 2013; Mellor et al., 2016; Paddock et al., 2012). Furthermore, STEC O26 had the highest number of serotypes circulating in the cattle populations under study. Among the serotypes identified in this study, 59.3% included STEC O26:H2, O26:H8, O26:H11, O26:H19, O26:H21, O45:H2, O103:H2, O103:H21, O121:H8, O145:H2, O145:H8, O145:H28, and O157:H7. These are clinically relevant

serotypes that have been recognized as human pathogens and previously associated with a spectrum of human illness including diarrhea, bloody diarrhea and HUS worldwide (Bettelheim, 2007; Beutin and Fach, 2014; Panel, 2013). STEC O157:H7 is the serotype predominantly associated with diarrhea, HC and HUS outbreaks globally (Armstrong et al., 1996; Slayton et al., 2013; Majowicz et al., 2014). In addition to STEC O157:H7 which is widely recognized as a human STEC involved in severe disease and complications, STEC serotypes such as O26:H11, O26:H21, O45:H2, O103:H2, O103:H21 and O145:H28 are of particular interest as they are also increasingly being reported in severe human disease and outbreaks worldwide (Johnson et al., 2006; Gould et al., 2013; Luna-Gierke et al., 2014). STEC serotype such as O26:H11 has been previously associated with outbreaks of severe human illness in Japan (Hiruta et al., 2001), and multistate outbreaks in Germany (Werber et al., 2002), United States of America (Luna-Gierke et al., 2014), and Ireland (McMaster et al., 2001). In South Africa, STEC O26:H11 was the most prevalent serotype associated with human disease between 2006 and 2013 (Musafiri Karama, personal communication). In addition, we report new STEC serotypes including O157:H28, O26:H38, and O26:H45 which to the best of our knowledge have not been recovered from cattle in previous studies. Furthermore, these new serotypes have not been implicated in human disease in South Africa and elsewhere.

Virulence characterization revealed that the majority of STEC isolates carried mainly *stx1* (69%), *stx2* (96%), and 62% of STEC isolates carried both *stx1* and *stx2* simultaneously. This was in agreement with similar studies in the USA that have reported proportions of *stx1* ranging from 65.5% to 79.4% (Dargatz et al., 2013; Stanford et al., 2016; Cull et al., 2017), 73% to 98.6% for *stx2* (Paddock et al., 2012; Dargatz et al., 2013; Bibbal et al., 2015;), and 52.5% to 53% of STEC isolates carried both *stx1* and *stx2* genes (Cernicchiaro et al., 2013; Cull et al., 2017). STEC isolates harboring *stx2* have been commonly implicated in severe disease including HUS in humans in comparison to STEC

strains which carry *stx1* only or both *stx1* and *stx2* (Boerlin et al., 1999; Friedrich et al., 2002; Fraser et al., 2004). Almost all STEC isolates carried *ehxA* (92.5%). This was in agreement with similar studies in the USA that reported the presence of the *ehxA* gene in STEC isolates ranging from 74.5% to 99.7% (Cernicchiaro et al., 2013; Dargatz et al., 2013; Stanford et al., 2016). The *ehxA* gene encodes a pore-forming enterohemolysin, which has been associated with the destruction of erythrocytes and possibly bleeding disorders that occur in human STEC disease (Schmidt et al., 1995; Schmidt and Karch, 1996).

A small portion of STEC isolates carried the *eaeA* gene (7.1%), which was substantially lower compared to other studies that have reported much higher proportions of *eaeA* in cattle STEC ranging from 18% to 77.8% (Monaghan et al., 2011; Paddock et al., 2012; Gonzalez et al., 2016). The *eaeA* gene was detected in isolates belonging to STEC O26:H2**,** O103:H2**,** O145:H28, O157:H7 and O157:H28. All the isolates that carried *eaeA* gene were also *stx2* positive and *ehxA* positive except for STEC O26:H2 and O103:H2 that carried *stx1* only. STEC serotypes that are *eaeA* and *stx2* positive at the same time are clinically significant human STEC that are usually associated with life-threatening HUS (Ostroff et al., 1989; Friedrich et al., 2002). However, in this study, a number of isolates which were identified as STEC O103:H21, O121:H8, O26:H8, O26:H11, O26:H19, O26:H21, O145:H2, O145:H8, and O145:HNT, and are considered clinically important strains in human disease were *eaeA* negative. The absence of *eaeA* may be an indication that these strains are less virulent and therefore less likely to cause disease outbreak and/or HUS in humans (Donnenberg et al., 1993; Boerlin et al., 1999).

To our knowledge, this is the first study reporting on the prevalence of the seven major STEC serogroups including O157, O26, O45, O145, O121 and O103 that colonize cattle on cow-calf operations in South Africa. It is important to note that most of the studies that have previously reported on the presence of these serogroups in cattle populations

worldwide have mainly limited their search to STEC serogrouping (O-typing) without further H-serotyping (H-typing) (Paddock et al., 2012; Cernicchiaro et al., 2013; Dargatz et al., 2013; Mellor et al., 2016; Stanford et al., 2016; Cull et al., 2017; Lee et al., 2017). The current study is among the few which characterized STEC for H-types. We demonstrated that most of the STEC isolates found in this study belonged to serotypes that have not been previously associated with human disease and only 13 were considered clinically relevant having been previously implicated in human disease globally including South Africa. This study revealed that only a subset of STEC serotypes that are associated with the so-called "top seven" STEC serogroups are responsible for human disease contrary to the widely held notion that any STEC isolate that is associated with the "top seven" major serogroups might be a clinically significant STEC. Therefore, we propose that it is necessary to investigate and serotype STEC isolates beyond the O-grouping before an STEC isolate is considered a clinically important serotype in routine surveillance studies involving STEC from non-human sources.

In conclusion, this study demonstrated that cattle on cow-calf operations in South Africa are an important reservoir of six of the seven major STEC including STEC O157, STEC O26, STEC O45, STEC O103, STEC O121 and STEC O145. A total of 33 distinct serotypes were identified in this study. Virulence characterization revealed that the majority of STEC isolates possessed *stx1, stx2* and *ehxA* (enterohemolysin) genes but lacked *eaeA*. The absence of *eaeA*, a gene encoding intimin, a key STEC adhesin, in most of the isolates under study may possibly explain why STEC disease in humans remains sporadic in South Africa. Furthermore, only a small portion of STEC serotypes which were associated with the top seven serogroups were serotypes that are clinically relevant in human disease. This study provides much needed STEC surveillance data and ascertains that cattle in South Africa are a potential source of clinically significant STEC for humans. Given that specific serotypes have been associated with foodborne disease outbreaks and

severe disease in humans, the isolates recovered in this study will need to be further characterized for more virulence factors and markers to assess fully their virulence potential for humans. Moreover, further characterisation STEC isolates for additional virulence factors and molecular is needed.

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4.0 CHAPTER IV: Virulence characterization and antimicrobial resistance among Shiga toxin-producing *Escherichia coli* O26, O45, O103, O121, O145 and O157 isolates on cow-calf operations in South Africa.

4.1 Abstract

Cattle are a major reservoir of STEC. A number of STEC strains have been isolated from cattle but only a subset of STEC strains including STEC O157, O26, O45, O103, O111, O121 and O145 are considered a major public health concern. However, little is known about the virulence characteristics of STEC O157, O26, O45, O103, O111, O121 and O145 from cattle in South Africa. In this study, 140 cattle STEC isolates that belong to serogroups O157, O26, O145, O121, O103 and O45 were characterized for virulence genes and markers by PCR. Furthermore, susceptibility profiles against 15 antimicrobials were also determined using the disk diffusion method. The distribution of virulence genes was as follows: 61.4% carried both *stx1* and *stx2*, 34.3% carried *stx*2 only, 4.3% carried *stx1* only, 20.7% carried *stx1c*; 15.2% carried *stx1d*; 95.7% carried *stx2*, 97% carried *stx2c*, 56% carried *stx2d* and 12.1% carried *eaeA*. None of the isolates carried *stx2e*, *stx2f* and *stx2g*. Plasmid-encoded markers were distributed as follows: *ehxA*, 90.7%; *subA*, 85%; *saa,* 82.1%; *espP*, 79.3%; *katP*, 10% and *etpD* 7.9%. The following proportions were observed for OI-122 encoded genes: *pagC*, 53.6%; *sen*, 34.3%; *Z4332*, 10.7% and *Z4333*, 28.6%. OI-43/48 encoded genes were distributed as follows: *iha*, 93.6%; *terC,* 80% and *ureC*, 55.7%. The following frequencies were observed for non-LEE-encoded effector genes: *ent/espL2*, 34% *nleG6-2*, 33.6%; *nleB*, 32.9%; *nleG2-3*, 30.7% and *nleE*, 28.6%. Most isolates carried both *stx1* and *stx2*, and *stx2c* and *stx2d* were the most common *stx* subtypes among STEC. *ehxA* and *espP* were the most frequent plasmid-encoded genes. However, the majority of isolates lacked *eaeA*. Possession of *eaeA* was significantly

associated with the presence of *nle* genes*, katP*, *etpD*, *ureC and terC* but *saa* and *subA* were only detected in *eaeA* negative STEC isolates. A complete OI-122 was detected in 7.1% of isolates that were classified as STEC O157:H7 and O103:H2. The *eaeA* gene was significantly frequent among STEC serotypes that have been previously implicated in STEC outbreaks and severe disease in humans including STEC O157:H7, STEC O145:H28 and O103:H2. In conclusion, only a small number of STEC serotypes harbored essential STEC virulence genes and markers that have been associated with STEC strains that have a high potential of causing severe human disease.

4.2 Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is a zoonotic foodborne pathogen characterized by mild to severe diarrhea, hemorrhagic colitis (HC) and in some cases can lead to hemolytic uremic syndrome (HUS) in humans (Karmali et al., 2010; Karmali, 1989). Cattle are the major reservoir of STEC (Hussein and Sakuma, 2005; Hussein and Bollinger, 2005). Human STEC infections are mainly acquired through ingestion of contaminated food of animal origin and water (Hussein and Sakuma, 2005; Hussein and Bollinger, 2005; Rangel et al., 2005). Direct contact with infected animals or their feces and person-to-person transmission are also considered routes of infection (Belongia et al., 1993; Hale et al., 2012).

The ability of STEC to cause disease in humans depends on a number of virulence factors. Bacteriophage-encoded Shiga toxins (Stx1 and Stx2), are the main STEC virulence factors (Scotland et al., 1983; Strockbine et al., 1986). Furthermore, a number of *stx1* and *stx2* Shiga toxin subtypes have been described (Scheutz et al., 2012). According to Scheutz *et al*., (2012), *stx1* subtypes include *stx1a*, *stx1c* and *stx1d*. At least seven s*tx2* variants have been identified including *stx2a, stx2b, stx2c*, *stx2d*, *stx2e*, *stx2f* and *stx2g* (Scheutz et al., 2012). The *stx1a* and *stx2a* genes variants represent prototypic *stx1* and *stx2* encoding genes (Scheutz et al., 2012). *stx1* variants are homogenous and conserved

with low toxicity potency while *stx2* variants are heterogeneous, differ in toxicity potency, and are commonly associated with severe disease in humans (Ostroff et al., 1989; Friedrich et al., 2002; Werber et al., 2003; Fuller et al., 2011).

The intimin protein (*eaeA*) is also considered an important STEC virulence factor (Beebakhee et al., 1992; Yu and Kaper, 1992). Intimin is encoded on the Locus of enterocyte effacement (LEE) pathogenicity island (PAI) (McDaniel et al., 1995). Intimin mediates intimate attachment of STEC to the host intestinal mucosa, and is responsible for the formation of typical attaching and effacing (A/E) lesions that are observed in STEC disease (McDaniel et al., 1995; Tzipori et al., 1995).

STEC strains harbor plasmids-encoded virulence factors including enterohemolysin (*ehxA*) (Schmidt, Beutin, and Karch, 1995), a catalase-peroxidase (*katP*) (Brunder et al., 1996), the extracellular serine protease (*esp*P) (Brunder et al., 1997) and a type II secretion system (*etpD*) (Schmidt et al., 1997). These virulence markers contribute to the survival and are known to enhance STEC pathogenicity in humans (Bolton, 2011; Dobrindt et al., 2010). The precise role of enterohemolysin in the pathogenesis of STEC infections is unknown. However enterohemolysin has been shown to lyse erythrocytes with subsequent release of heme which provides iron for multiplication and survival of STEC (Schmidt and Karch, 1996). Catalase-peroxidase is responsible for protecting STEC against oxidative damage from the host macrophages (Bortolussi et al., 1987; Welch, 1987) and extracellular serine protease cleaves human coagulase factor V increasing intestinal hemorrhage in STEC disease (Brunder et al., 1997). Other plasmid-encoded genes include the STEC autoagglutinating adhesin (*saa*) (Paton et al., 2001) which is believed to confer adherence to host enterocytes in *eaeA*-negative STEC (Paton et al., 1999; Paton et al., 2001) and subtilase cytotoxin (*sub*A) which suppresses the host immune system and facilitates STEC adherence (Paton et al., 2004; Morinaga et al., 2007; Wang et al., 2007).

A number of genes that are encoded on several O-islands in STEC are considered important STEC virulence markers (Schmidt and Hensel, 2004). O-islands that carry virulence genes are termed pathogenicity islands (PAI) (Perna et al., 2001; Karmali et al., 2003; Coombes et al., 2008; Ju et al., 2013). A number of PAIs have been identified in STEC: OI-122, OI-57, OI-71 OI-36 and OI-43/48. PAIs have been used to evaluate the virulence potential of STEC (Karmali et al., 2003; Coombes et al., 2008). OI-122 carries *pagC* (Gunn et al., 1995; Miller and Mekalanos, 1990), *sen* (*Z4326*) (Nataro et al., 1995), *efa1* (EHEC factor for adherence) (*Z4332)*, and *efa1* (*Z4333)* (Nicholls et al., 2000) which encode proteins that are essential for survival and efficient attachment of STEC to the host intestinal mucosa (Nicholls et al., 2000).

In addition, several genes located on OI-43/48 encode adherence factors (Perna et al., 2001; Tarr et al., 2000; Yin et al., 2009). The IrgA homologue adhesin (*iha*) functions as an adhesin in STEC strains (Tarr et al., 2000; Yin et al., 2009). Additional genes located in OI-43/48 include the tellurite (*ter*) resistance gene and urease gene clusters (Taylor, 1999; Taylor et al., 2002). Tellurite aids bacteria in general stress response within the host environment (Taylor, 1999; Taylor et al., 2002), while urease facilitates infection by lowering STEC infective dose and increasing bacteria survival in the host (Nakano et al., 2001; Friedrich et al., 2005).

STEC also possess a number of effector proteins that have been termed "non-LEE effector proteins" (*nles*) genes because they are carried on PAIs other than the LEE (Tobe et al., 2006; Dean and Kenny, 2009). A number of non-LEE effectors (*nles*) are located on several pathogenicity islands including OI-122 encodes (*nleB*, *nleE* and *ent/espL2*), OI-57 (*nleG2-3, nleG6-2* and *nleG5-2)*, OI-71 (*nleA, nleF, nleG*, *nleH1-2, nleG2-1* and *nleG9*) and OI-36 (*nleC*, *nleD, nleB2* and *nle*H1-1) (Deng et al., 2004; Gruenheid et al., 2004; Wickham et al., 2006; Coombes et al., 2008; Garcia-Angulo et al., 2008). Non-LEE

effectors play different roles in STEC pathogenicity including suppression of the host immune system through inactivation of specific proteins, invasion and enhancement of bacterial adherence and colonization to host intestinal epithelial cells (Karmali et al., 2003; Deng et al., 2004; Coombes et al., 2008).

Shiga toxin-producing *E. coli* (STEC) serogroups O26, O45, O103, O111, O121, O145, and O157 are the most frequent STEC in outbreaks of human foodborne illness globally. These serogroups are colloquially termed “top/big seven” because they are commonly associated with severe illness in humans and HUS (Caprioli et al., 1997; Brooks et al., 2005; Johnson et al., 2006; Bavaro, 2012; Vally et al., 2012).

A number of studies have documented that cattle may carry antimicrobial resistant *E. coli* and STEC (Schroeder et al., 2002; Pickering, 2004; Mora et al., 2005; Karama et al., 2008a; Amézquita-López et al., 2016; Mukherjee et al., 2017). Resistant STEC isolates from cattle may be transferred to humans through the food chain, occupational exposure, or manure runoff from cattle farms and this has become a public health concern (Schwarz et al., 2000).

Currently, information on virulence characteristics and antimicrobial resistance profiles of cattle STEC isolates from South Africa is scanty. The main objective of this study was to characterize STEC isolates belonging to serogroups O26, O45, O103, O121, O145 and O157 for a number of virulence genes and markers. In addition, antimicrobial resistance profiles of STEC isolates were determined. The overall aim of the study was to characterize STEC serotypes of cattle origin from South Africa, assess their public-health significance and contribute to STEC surveillance in South Africa.

4.3 Material and Methods

4.3.1 Culture of Bacteria Strains

One hundred and forty (N=140) STEC isolates representing 33 O:H STEC serotypes were used in this study. The isolates had been previously recovered from cattle on five cow-calf operations in Gauteng and Northwest provinces of South Africa. The STEC isolates included **STEC O26 serotypes:** O26:H2 (20), O26:H4 (1), O26:H7 (3), O26:H8 (8), O26:H11 (3), O26:H16 (2), O26:H19 (2), O26:H21 (7), O26:H28 (2), O26:H38 (2), O26:H45 (1) and O26:HNT (4); **STEC O45 serotypes** O45:H2 (1), O45:H8 (3), O45:H11 (8), O45:H16 (3), O45:H19 (3), O45:H21 (2), O45:H28 (1), O45:H38 (5) and O45:HNT (3); **STEC O103 serotypes** O103:H2 (1) and O103:H21; **STEC O121 serotypes** O121:H8 (8), O121:H21 (1) and O121:HNT (1); **STEC O145 serotype**s O145:H2 (1), O145:H7 (1), O145:H8 (1), O145:H11 (1), O145H19 (13), O145:H28 (3) and O145:HNT (3); and **STEC serotype O157:H2** (1), O157:H7 (9), O157:H19 (1) and O157:H28 (1).

Frozen STEC cultures (-80ºC) were propagated aerobically overnight at 37ºC on Luria Bertani (LB) agar (Difco™ Becton and Dickson &Company). Bacterial DNA was extracted using the boiling method as described previously with slight modifications (Monday et al., 2007). Briefly, a loopful of bacterial cells was suspended in 1000 µL of sterile FA buffer (Bacto™ FA Buffer, Becton and Dickson Company) in a 1.5 mL Eppendorf tube, mixed by vortexing and centrifuged at 12,000 rpm for 5 minutes. The supernatant was discarded and the bacterial cells were re-suspended in 1000 µL of sterile of FA buffer and centrifuged. This process was repeated thrice. After the last centrifugation cycle, the supernatant was discarded completely. The pellet was re-suspended in 500 µL of sterile distilled water, boiled for 20 minutes on a heating block and cooled on ice for 10 minutes. After centrifugation at 12,000 rpm for 5 minutes, the DNA was stored at -20°C for subsequent analysis.

A multiplex polymerase chain reaction (mPCR) was performed to detect *stx1, stx2, eaeA* and *ehxA* genes using previously described primers and cycling conditions (Paton and Paton, 1998). Briefly, each PCR reaction (25µL) contained 2.5µL of 10X Thermopol reaction buffer, 2.0µl of 2.5mM dNTPs, 0.25µl of 100mM $MgCl_2$, 0.3µM of each primer, 1U of Taq DNA Polymerase (New England BioLabs® *Inc.*) and 5µl of DNA lysate template. Sterile water was used to top up the reaction volume to 25µL. STEC O157:H7 EDL933 (Perna et al., 2001) and sterile water were used as positive and negative controls respectively. All PCR reagents were supplied by New England BioLabs (NEB, USA) except for the primers which were supplied by Inqaba Biotec (South Africa) or Integrated DNA Technologies (IDT) (San Diego, USA). PCR reactions were carried out in a C100 Touch™ (Bio-Rad, USA) or a Veriti™ (Applied Biosystems®, USA) thermal cycler. Amplicons were electrophoresed in 2% (w/v) agarose gels in TAE (Tris–acetate–ethylenediamine tetraacetic acid) buffer, stained with ethidium bromide (0.05mg/µl) and visualized under ultraviolet (UV) light with a Gel Doc system (Bio-Rad, USA).

4.3.2 Shiga toxin (*stx*) Subtypes

To detect *stx1a, stx1c, stx1d*, *stx2a*, stx*2c*, stx*2d*, *stx2e, stx2f and stx2g,* individual PCR assays were performed using primers and cycling conditions described elsewhere (Scheutz et al., 2012) (**Table 1**). Briefly, each PCR reaction (25µL) contained 2.5µL of 10X Thermopol reaction buffer, 2.0µl of 2.5mM dNTPs, 0.25µl of 100mM $MgCl_2$, 0.3µM final of each primer concentration, 1U of Taq DNA Polymerase (New England BioLabs® *Inc.*) and 5µl of DNA lysate template.

4.3.3 Detection of Genes encoding Virulence Factors and Markers.

Primers and cycling conditions described in previous studies were used to amplify virulence genes and markers located on plasmids and pathogenicity islands (Table 1). Amplification reactions for plasmid encoded genes *ehxA*, *saa*, *sub*A (Paton and Paton,

1998; Paton et al., 2001; Paton et al., 2004), *kat*P (Brunder et al., 1999), *esp*P (Brunder et al., 1997), and *etpD* (Schmidt et al., 1997) were conducted individually. PCR assays were also performed to screen for the presence of OI-122-encoded gene markers including *pag*C (*Z4321*), *sen* (*Z4326*), *efa1* (*Z4332* and *Z4333*) as previously described (Karmali et al., 2003). PCR amplification of non-LEE-encoded effector (*nle*) genes including *nleA*, *nleB*, *nleB$_2$*, *nleC*, *nleD*, *nleE*, *nleF*, *nleG*, *nleG2-1*, *nleG2-3*, *nleG5-2*, *nleG6-2*, *nleG9*, *nleH1*, *nleH2*, and *ent/espL2* were conducted individually as previously described (Coombes et al., 2008). PCR reactions for OI-43/48 island markers, *iha*, *ter*-island and *ure*C, were also performed according to previous studies (Janka et al., 2005; Nakano et al., 2001; Taylor et al., 2002). STEC O157:H7 EDL933 (Perna et al., 2001) and sterile water were used as positive and negative controls respectively. Primer sequences and amplicon sizes for all the PCR reactions are listed in **Table1**.

Table 1: DNA oligonucleotides used in Analysis of STEC by PCR.

Gene Location	Target Gene	Sequence (5' to 3')	Amplicon Size (bp)	References
Bacteriophage-encoded genes	*stx1*	**F:** ATAAATCGCCATTCGTTGACTAC **R:** AGAACGCCCACTGAGATCATC	180	(Paton and Paton, 1998)
	stx2	**F:** GGCACTGTCTGAAACTGCTCC **R:** TCGCCAGTTATCTGACATTCTG	255	(Paton and Paton, 1998)
	stx1c	**F1:** CCTTTCCTGGTACAACTGCGGTT **R1:** CAAGTGTTGTACGAAATCCCCTCTGA	252	(Scheutz et al., 2012)
	stx1d	**F1:** CAGTTAATGCGATTGCTAAGGAGTTTACC **R2:** CTCTTCCTCTGGTTCTAACCCCATGATA	203	(Scheutz et al., 2012)
	stx2a	**F2:** GCGATACTG**R**G**B**ACTGTGGCC **R3:** CCG**K**CAACCTTCACTGTAAATGTG	349	
	stx2c	**F1:** GAAAGTCACAGTTTTTATATACAACGGGTA **R2:** CCGGCCACYTTTACTGTGAATGTA	177	(Scheutz et al., 2012)
	stx2d	**F1:** AAARTCACAGTCTTTATATACAACGGGTG		(Scheutz et al., 2012)
		R1: TTYCCGGCCACTTTTACTGTG	179	
		O55-R: TCAACCGAGCACTTTGCAGTAG	235	

	stx2e	**F1:** CGGAGTATCGGGGAGAGGC **R2:** CTTCCTGACACCTTCACAGTAAAGGT	411	(Scheutz et al., 2012)
	stx2f	**F1:** TGGGCGTCATTCACTGGTTG **R1:** TAATGGCCGCCCTGTCTCC	424	(Scheutz et al., 2012)
	stx2g	**F1:** CACCGGGTAGTTATATTTCTGTGGATATC **R1:** GATGGCAATTCAGAATAACCGCT	573	(Scheutz et al., 2012)
	eaeA	**F:** GACCCGGCACAAGCATAAGC **R:** CCACCTGCAGCAACAAGAGG	384	(Paton and Paton, 1998)
Plasmid-encoded genes	*ehxA*	**F:** GCATCATCAAGCGTACGTTCC **R:** AATGAGCCAAGCTGGTTAAGCT	584	(Paton and Paton, 1998)
	katP	**wkat-F:** AACTTATTTCTCGCATCATCC **wkat-B:** CTTCCTGTTCTGATTCTTCTGG	2125	(Brunder et al., 1996)
	espP	**F:** AAACAGCAGGCACTTGAACG **R:** GGAGTCGTCAGTCAGTAGAT	1830	(Brunder et al., 1997)
	etpD	**D1-** CGTCAGGAGGATGTTCAG **D13R-** CGACTGCACCTGTTCCTGATTA	1062	(Schmidt et al., 1997)
	saa	**F:** CGTGATGAACAGGCTATTGC **R:** ATGGACATGCCTGTGGCAAC	119	(Paton and Paton, 2002)
	subA	**SubHCDF**: TATGGCTTCCCTCATTGC C **SubSCDR:** TATAGCTGTTGCTTCTGACG	556	(Paton and Paton, 2005)
Pathogenicity Islands-encoded genes				
OI-71	*nleA (Z6024)*	**F:** ATGAACATTCAACCGACCATAC **R:** GACTCTTGTTTCTTGGATTATATCAAA	1296	(Coombes et al., 2008)
OI-122	*nleB (Z4328)*	**F:** GGAAGTTTGTTTACAGAGACG **R:** AAAATGCCGCTTGATACC	297	(Coombes et al., 2008)
OI-36	*nleB2 (Z0985)*	**F:** GTTAATACTAAGCAGCATCC **R:** CCATATCAAGATAGATACACC	475	(Coombes et al., 2008)
OI-36	*nleC (Z0986)*	**F:** ACAGTCCAACTTCAACTTTTCC **R:** ATCGTACCCAGCCTTTCG	777	(Coombes et al., 2008)
OI-36	*nleD (Z0990)*	**F:** GGTATTACATCAGTCATCAAGG **R:** TTGTGGAAAACATGGAGC 426	426	(Coombes et al., 2008)
OI-122	*nleE (Z4329)*	**F:** GTATAACCAGAGGAGTAGC **R:** GATCTTACAACAAATGTCC	260	(Coombes et al., 2008)

OI-71	*nleF (Z6020)*	**F:** ATGTTACCAACAAGTGGTTCTTC **R:** ATCCACATTGTAAAGATCCTTTGTT	567	(Coombes et al., 2008)
OI-71	*nleG (Z6010)*	**F:** ATGTTATCGCCCTCTTCTATAAAT **R:** ACTTAATACTACACTAATAAGATCCA	902	(Coombes et al., 2008)
OI-71	*nleG2-1 (Z6025)*	**F:** ACCAGAAACCTGACTTCG **R:** CAGCATCTTCATATACTACAGC	406	(Coombes et al., 2008)
OI-57	*nleG2-3*	**F:** GGATGGAACCATACCTGG **R:** CGCAATCAATTGCTAATGC	551	(Coombes et al., 2008)
OI-57	*nleG5-2*	**F:** TGGAGGCTTTACGTCATGTCG **R:** CCGGAACAAAGGGTTCACG	504	(Coombes et al., 2008)
OI-57	*nleG6-2*	**F:** CGGGTCAGTGGATGATATGAGC **R:** AAGTAGCATCTAGCGGTCGAGG	424	(Coombes et al., 2008)
OI-71	*nleG9 (Z2560)*	**F:** GTTCGTGCCCGAATTGTAGC **R:** CACCAACCAAACGAGAAAATG	409	(Coombes et al., 2008)
OI-71	*nleH1-2 (Z6021)*	**F:** AACGCCTTATATTTTACC **R:** AGCACAATTATCTCTTCC	589	(Coombes et al., 2008)
OI-36	*nleH1-1 (Z0989)*	**F:** GTTACCACCTTAAGTATCC **R:** GTTTCTCATGAACACTCC	456	(Coombes et al., 2008)
OI-122	*ent/espL2*	**F:** GAATAACAATCACTCCTCACC **R:** TTACAGTGCCCGATTACG	433	(Coombes et al., 2008)
OI-122	*Efa1 (Z4332)*	**Z4321-a:** ATGAGTGGTTCAAGACTGG **Z4321-b:** CCAACTCCAACAGTAAATCC	521	(Karmali et al., 2003)
OI-122	*Efa1 (Z4332)*	**Z4326-a:** GGATGGAACCATACCTGG **Z4326-b:** CGCAATCAATTGCTAATGC	551	(Karmali et al., 2003)
OI-122	*sen (Z4326)*	**Z4332-a:** CTCCCAGAGATAATTTTGAGG **Z4332-b:** CAACTGTATGCGAATAGTACTC	504	(Karmali et al., 2003)
OI-122	*pagC*	**Z4333-a:** CTGTCAGACGATGACATTGG **Z4333-b:** GAAGGATGGGCATTGTGTC	547	(Karmali et al., 2003)
OI-43/48	*ure*C	**F:** TCT AAC GCC ACA ACC TGT AC **R:** GAG GAA GGC AGA ATA TTG GG	397	(Nakano et al., 2001)
OI-43/48	*Ter-island*	**F:** GAC AAA CTC TCC GGG ATA ACT CA **R:** TGC GGG TGC TGG TGT GGG ATA A	356	(Taylor et al., 2002)
OI-43/48	*iha*	**Iha-I:** CAG TTC AGT TTC GCA TTC ACC **Iha-II:** GTA TGG CTC TGA TGC GAT G	1305	(Janka et al., 2005)

4.3.4 Antimicrobial Susceptibility test.

All the 140 STEC isolates were tested against a panel of 15 antimicrobials by the disk diffusion method to determine phenotypic patterns of resistance (Bauer et al., 1966). Antimicrobial resistance testing was performed on Mueller Hinton agar (MHA) (Oxoid, UK) as described by the Clinical and Laboratory Standards Institute (CLSI, 2014). Briefly, bacterial suspensions (0.5 McFarland) of individual STEC pure colonies were prepared in 0.85% physiological saline. A sterile cotton swab was used to inoculate MHA plates to achieve a confluent growth. The antimicrobial discs were placed on the inoculated MHA plates by means of a BBL Sensi-disk dispenser or Oxoid disk dispenser and incubated aerobically at 37℃ ±2℃ for 18 hours. The panel of 15 antimicrobials consisted of amoxicillin-clavulanic acid (20 µg and 10 µg, respectively), amikacin (30 µg), ampicillin (10µg), ceftazidime (30µg), cephalothin (30µg), cefoperazone (75µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), ceftriaxone (30 µg), gentamicin (10 µg) kanamycin (30 µg), nalidixic acid (30 µg), trimethoprim-sulfamethoxazole (1.25 µg and 23.75 µg, respectively) and tetracycline (30 µg). Antimicrobial disks (BBL Sensi Disk and Oxoid Disk) were obtained from Becton Dickinson & Company and Oxoid Company respectively. *Escherichia coli ATCC* 25922 was used as the control strain. Results were classified as susceptible, resistant, or intermediate to each antimicrobial agent according to CLSI criteria and the intermediate readings were assigned to the resistant category

4.3.5 Statistical Analysis.

Descriptive statistical analyses were performed using statistical package for social sciences (SPSS) software version 21 (IBM® SPSS® Statistics 21). Fisher's exact test was used to determine if there were statistically significant differences and associations between the proportions of genes which were investigated in this study. P values of < 0.05 were considered statistically significant.

4.4 Results

4.4.1 Virulence factors and markers

Among the 140 STEC isolates, PCR revealed that 61.4% (86/140) carried *stx1+stx2*; 34.3% (48/140) carried *stx2* only and 4.3% (6/140) carried *stx1* only. Genes encoding *stx1* subtypes were distributed as follows: *stx1a,* 64.1% (59/92); *stx1c,* 20.7% (19/92); *stx1d*, 15.2% (14/92); and *stx1c+stx1d,* 6.5% (6/92) **(Table 2)**. The *stx1c* and *stx1d* subtypes were significantly detected in STEC O26 and STEC O45 serogroups. The following distribution for *stx2* subtypes was observed: *stx2a*, 95.7% (134/140); *stx2c*, 97.8% (131/134) and *stx2d*, 56% (75/134)**.** The most common toxin gene combinations among the STEC isolates were *stx2+stx2c+stx2d* in 37.1% (52/140); *stx2c+stx2d* in 35% (49/140); *stx2c+stx2d* in 5.7% (8/140); *stx1c+stx2+stx2c+stx2d* in 5% (7/140) **(Table 2)**. All isolates that carried *stx2* were negative for *stx2e, stx2f* and *stx2g* subtypes.

The *eaeA* gene was detected in 12.1% (17/140) of isolates. Among the 17 STEC isolates that carried *eaeA*, 52.9% (9/17) and 29.4% (5/17) had *stx2+stx2c+stx2d* and *stx2+stx2c* gene combinations respectively (**Table 5**). The *eaeA* gene was detected among the following STEC serotypes: O26:H2 (2 isolates), O103:H2 (1 isolate), O145:H28 (3 isolates), O145:HNT (1 isolate), O57:H7 (9 isolates) and O157:H28 (1 isolate) (**Table 5**).

Table 2: Serotypes and *stx* Genotypes

SEROTYPE	No. of Isolate Tested	*stx1c* n=92	*stx1d* n=92	*stx2* n=134	*stx2c* n=134	*stx2d* n=134	*stx* Genotype
O26:H2	1	+	-	+	+	-	*stx1c, stx2, stx2c*
O26:H2	4	+	-	+	+	+	*stx1c, stx2, stx2c, stx2d*
O26:H2	2	-	+	-	-	-	*stx1d*
O26:H2	2	-	+	+	+	+	*stx1d, stx2, stx2c, stx2d*
O26:H2	2	-	-	+	+	-	*stx2, stx2c*
O26:H2	9	-	-	+	+	+	*stx2, stx2c, stx2d*
O26:H4	1	-	+	+	-	-	*stx1d, stx2*
O26:H7	1	-	+	-	-	-	*stx1d*
O26:H7	1	-	-	+	+	+	*stx2, stx2c, stx2d*
O26:H7	1	-	-	+	+	+	*stx2, stx2c, stx2d*

O26:H8	1	-	+	+	+	-	*stx1d, stx2, stx2c*
O26:H8	3	-	-	+	+	-	*stx2, stx2c*
O26:H8	4	-	-	+	+	+	*stx2, stx2c, stx2d*
O26:H11	2	-	-	+	+	-	*stx2, stx2c*
O26:H11	1	-	-	+	+	+	*stx2c, stx2d*
O26:H16	1	-	-	+	+	-	*stx2, stx2c*
O26:H16	1	-	-	+	+	+	*stx2, stx2c, stx2d*
O26:H19	1	-	-	+	+	+	*stx2, stx2c, stx2d*
O26:H19	1	-	-	+	+	+	*Stx2, stx2c, stx2d*
O26:H21	3	-	-	+	+	-	*stx2, stx2c*
O26:H21	4	-	-	+	+	+	*stx2, stx2c, stx2d*
O26:H28	1	+	-	+	+	-	*stx1c, stx2, stx2c*
O26:H28	1	-	+	-	-	-	*stx1d*
O26:H38	1	-	-	+	+	-	*stx2, stx2c*
O26:H38	1	-	-	+	+	+	*stx2, stx2c, stx2d*
O26:H45	1	-	-	+	+	-	*stx2, stx2c*
O26:HNT	1	-	-	+	+	-	*stx2, stx2c*
O26:HNT	3	-	-	+	+	+	*stx2, stx2c, stx2d*
O45:H2	1	+	+	+	+	+	*stx1c, stx1d, stx2a, stx2c, stx2d*
O45:H8	1	+	-	-	-	-	*stx1c*
O45:H8	1	+	+	-	-	-	*stx1c, stx1d*
O45:H8	1	-	-	+	+	-	*stx2, stx2c*
O45:H11	3	+	+	+	+	+	*stx1c, stx1d, stx2, stx2c, stx2d*
O45:H11	2	-	+	+	+	+	*stx1d, stx2, stx2c, stx2d*
O45:H11	3	-	-	+	+	+	*stx2, stx2c, stx2d*
O45:H16	2	-	-	+	+	-	*stx2, stx2c*
O45:H16	1	-	-	+	+	+	*stx2, stx2c, stx2d*
O45:H19	1	+	-	+	+	+	*stx1c, stx2, stx2c, stx2d*
O45:H19	2	-	-	+	+	+	*stx2, stx2c, stx2d*
O45:H21	2	-	-	+	+	-	*stx2, stx2c*
O45:H28	1	+	+	-	-	-	*stx1c, stx1d*
O45:H38	5	-	-	+	+	+	*stx2, stx2c, stx2d*
O45:HNT	2	+	-	+	+	-	stx1c, *stx2, stx2c*
O45:HNT	2	+	-	+	+	+	*stx1c, stx2, stx2c, stx2d*
O45:HNT	5	-	-	+	+	-	*stx2, stx2c*
O45:HNT	3	-	-	+	+	+	*stx2, stx2c, stx2d*
O103:H2	1	-	+	-	-	-	*stx1d*
O103:H21	1	-	-	+	+	-	*stx2, stx2c*
O121:H8	4	-	-	+	+	-	*stx2, stx2c*
O121:H8	2	-	-	+	+	+	*stx2, stx2c, stx2d*
O121:H8	1	-	-	+	+	-	*stx2c*
O121:H8	1	-	-	+	+	+	*stx2, stx2c, stx2d*
O121:H21	1	-	-	+	+	+	*stx2, stx2c, stx2d*
O121:HNT	1	-	-	+	+	+	*stx2, stx2c, stx2d*
O145:H2	1	-	-	+	+	-	*stx2, stx2c*
O145:H7	1	-	-	+	+	+	*stx2,stx2c, stx2d*

O145:H8	1	-	-	+	+	-	*stx2, stx2c*
O145:H11	1	-	-	+	+	+	*stx2, stx2c, stx2d*
O145:H19	1	+	-	+	+	-	*stx1c, stx2, stx2c*
O145:H19	12	-	-	+	+	-	*stx2, stx2c*
O145:H28	2	-	-	+	+	-	*stx2, stx2c*
O145:H28	1	-	-	+	+	+	*stx2, stx2c, stx2d*
O145:HNT	2	-	-	+	+	-	*stx2, stx2c*
O145:HNT	1	-	-	+	+	+	*stx2, stx2c, stx2d*
O157:H2	1	-	-	+	+	+	*stx2, stx2c, stx2d*
O157:H7	7	-	-	+	+	+	*stx2, stx2c, stx2d*
O157:H7	2	-	-	+	+	+	*stx2c, stx2d*
O157:H19	1	-	-	+	+	-	*stx2, stx2c*
O157:H28	1	-	-	+	+	-	*stx2, stx2c*
TOTAL	**140**	**19**	**17**	**123**	**131**	**75**	
% Positive		**20,7**	**18,5**	**91,8**	**97,8**	**56**	

[a] Serotypes in bold have been identified previously as human pathogens causing diarrhoea, bloody diarrhoea and HUS elsewhere.

The following distribution was observed for plasmid-encoded virulence markers: *ehxA*, 90.7% (127/140), *saa,* 82.1% (115/140), *espP,* 79.3% (111/140); *subA,* 37.9% (53/140); *katP*, 10% (14/140); and *etpD*, 7.9% (11/140) (**Table 3 and fig.1; see also table 7**). The *katP* and *etpD* genes were significantly associated with *eaeA* positive isolates (*P*<0.000. All the 14 *katP* positive isolates were also *eaeA* positive and belonged to serotypes O145:H28 (3), O145:HNT (1), O157:H7 (9) and O157:H28 (1). Similarly, 10/11 *etpD* positive isolates possessed *eaeA* and belonged to serotypes O103:H2 (1), O103:H21 (1) and O157:H7 (9) (**Table 5**).

The *saa* gene was exclusively observed in *eaeA*-negative isolates. STEC O157:H7 that were *eaeA*-positive possessed the full complement of plasmid markers including *ehxA*, *sub*A*, katP, espP* and *etpD*. However, *eaeA* positive STEC O145:H28, O145: HNT and O157:H28 carried *ehxA*, *sub*A, *katP* and *espP* but lacked *etpD.*

The following distribution was observed for OI-122 encoded genes: *pagC*, 53.6% (75/140); *sen* (Z4326), 36.4% (51/140); *efa1* (*Z4333*), 28.6% (40/140) and *efa1* (*Z4332*)*,* 10.7% (15/140) (**Table 4 and fig. 2; see also Table 7**). All OI-122 markers (complete OI-22) were observed in 7.1% (10/140) of isolates. Isolates that had a complete OI-122 belonged

to two serotypes: STEC O157:H7 (9 isolates) and STEC O103:H2 (1 isolate). An incomplete OI-122 was observed in 60% (84/140) of isolates and 32.9% (46/140) of isolates carried none of OI-122 gene markers (**Table 7**).

The distribution of OI-43/48-encoded genes was: *iha*, 93.6% (131/140); *terC*, 80% (112/140) and *ureC*, 55.7% (78/140) (**Table 4 and fig. 2; see also Table 7**). Both *terC* (P=0.032) and *ureC* (P<0.000) were significantly prevalent among *eaeA*-positive STEC. All OI-43/48 markers were detected in 52.9% (74/140) of isolates. However, 2.1% (3/140) were negative for all OI-43/48 markers (**Table 7**).

The following proportions were observed for non-LEE effector (*nle*) genes: ent/espL2*,* 34.3% (48/140); *nleB*, 32.9% (46/140); *nleE*, 28.6% (40/140); *nleG2-3*, 30.7% (43/140); *nleG6-2*, 33.6% (47/140); *nleG5*-2, 19.3% (27/140); *nleH1*-2, 27.1% (38/140); *nleG9,* 16.4% (23/140): *nleG2-1,*14.3% (20/140); *nleA,* 12.1% (17/140); *nleF,* 5.0% (7/140); *nleG,* 2.1% (3/140); *nleH1-1*, 27.9% (39/140); *nleD,* 20.7% (29/140); *nleB2,*15.7% (22/140) and *nleC,* 12.1% (17/140) (**Table 4 and fig. 2; see also Table 7**). More than ten *nle* genes were observed in 12.9% (18/140) of isolates, eight to nine *nle* genes were present in 6.4% (9/140) and one to seven *nle* genes were detected in 30.7% (43/140) of isolates. The remaining 50% (70/140) isolates did not carry a non-LEE effector gene (**Table 7**).

The following OI markers and *nle*-encoding genes were significantly associated with *eaeA*: *sen* (Z4326), *efa1* (Z4332), and *efa1* (Z4333) and non-LEE effectors *nleB*, *nleD*, *nleE*, *nleF*, *nleG2-1*, *nleA*, *nleB2*, *nleG9*, *nleH1-1*, nleC, *nleG5-2*, *nleG6-2*, *nleH1-2* and *ent/espL2* (**Table 5**).

4.4.2 Antimicrobial Resistance

The disk diffusion assay was performed to determine antimicrobial resistance profiles of the 140 STEC isolates against a panel of 15 antimicrobials. Of the 140 STEC isolates, 97.9% (137/140) were susceptible to the 15 antimicrobials tested. Only 2.1% (3/140) of

STEC isolates were resistant: STEC O26:H11 was resistant to tetracycline; STEC O26:H4 was resistant to ampicillin and tetracycline; STEC O45:H21 was resistant ampicillin, tetracycline and cephalothin (**Tables 6**).

Table 3: Distribution of STEC Plasmid-encoded Genes and Markers

	LEE	**Plasmid-encoded Genes**					
N=140	*eaeA*	*ehxA*	*saa*	*subA*	*esp*P	*katP*	*etpD*
Total	17	127	115	53	111	14	11
% Positive	**12.1**	**90,7**	**82,1**	**37,9**	**79,3**	**10,0**	**7,9**
P value		0.159			0.107	0.000	0.000

Figure 1: Distribution of Plasmid-encoded Virulence Markers

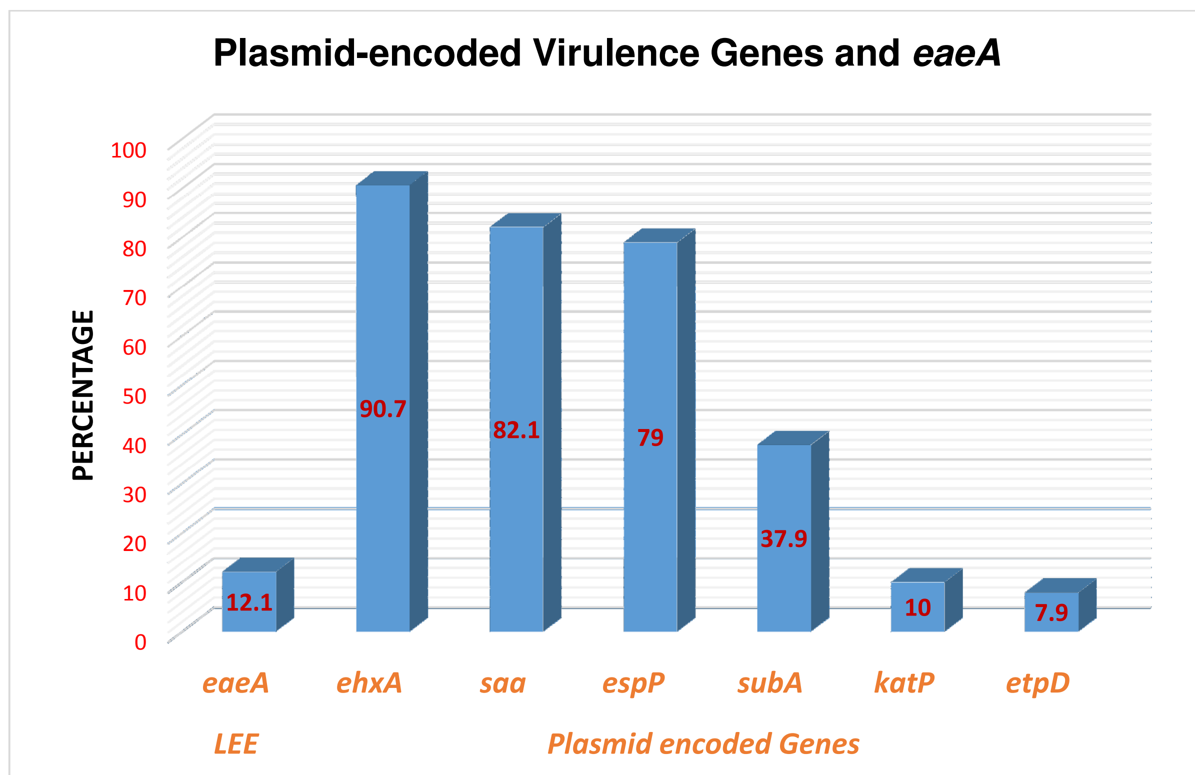
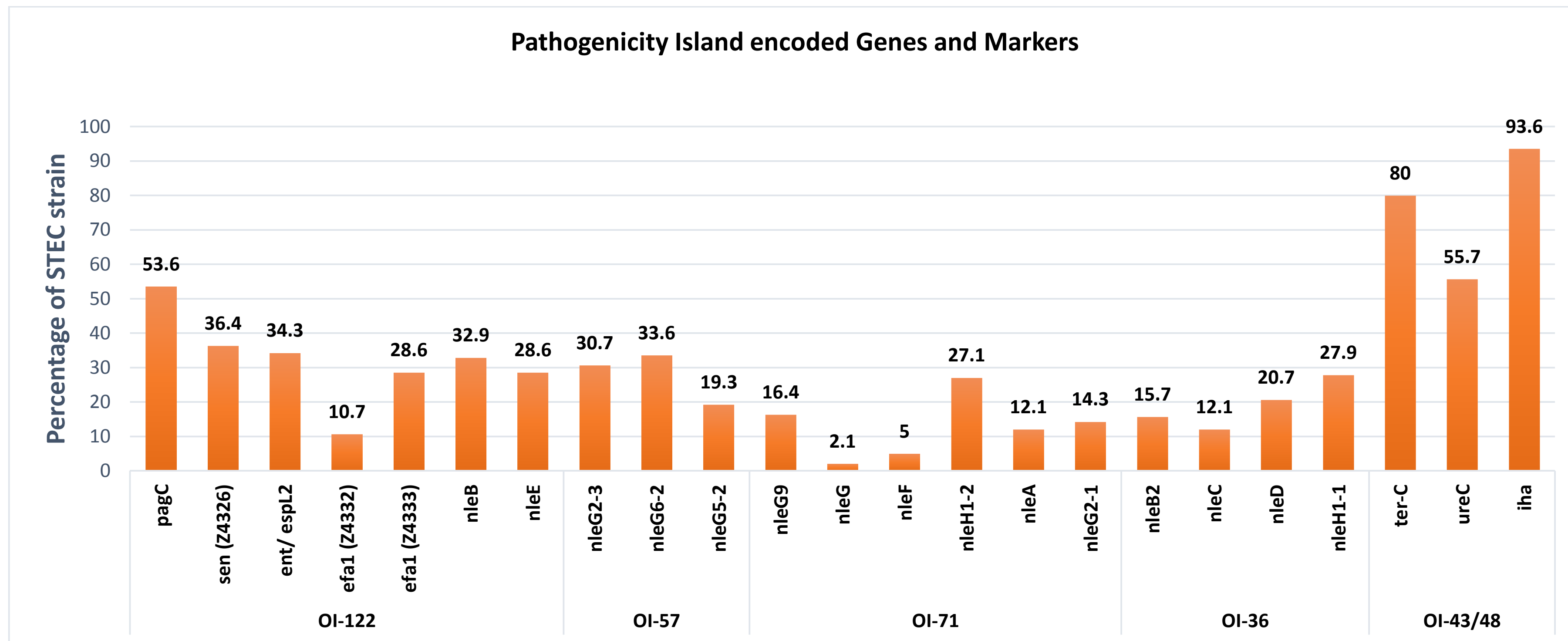


Table 4: Distribution of Pathogenicity Island encoded Genes

	OI-122							OI-57			OI-71						OI-36				OI-43/48		
N=140	*pagC*	*sen (Z4326)*	*ent/ espL2*	*efa1 (Z4332)*	*efa1 (Z4333)*	*nleB*	*nleE*	*nleG2-3*	*nleG6-2*	*nleG5-2*	*nleG9*	*nleG*	*nleF*	*nleH1-2*	*nleA*	*nleG2-1*	*nleB2*	*nleC*	*nleD*	*nleH1-1*	*ter-Island*	*ureC*	*iha*
Total	75	51	48	15	40	46	40	43	47	27	23	3	7	38	17	20	22	17	29	39	127	78	131
% Positive	53,6	36,4	34,3	10.7	28,6	32,9	28,6	30,7	33,6	19,3	16,4	2,1	5	27,1	12,1	14,3	15,7	12,1	20,7	27,9	90,7	55,7	93,6

Figure 2: Distribution of Pathogenicity Island-encoded Genes and Markers



4.5 Discussion

The emergence of STEC in human disease has become a global public health concern (EFSA, 2013). Although cattle are considered an important reservoir of STEC serogroups O26, O157, O145, O103, O45 and O121 (Paddock et al., 2012; Mellor et al., 2016; Stanford et al., 2016; Dong et al., 2017), only a few studies have investigated the virulence characteristics of various serotypes that are associated with these serogroups (Bosilevac and Koohmaraie, 2011; Amézquita-López et al., 2016). In this study, 140 cattle STEC isolates representing 33 serotypes that are associated with serogroups O157, O45, O103, O121, O26 and O145 were characterized for 38 genes encoding virulence factors and markers and for antimicrobial resistance profiles.

The majority of isolates carried both *stx1* and *stx2* with almost all isolates (95.7%) harboring *stx2*. This was consistent with previous studies which have reported high rates of *stx2* among STEC isolates from cattle (Monaghan et al., 2011; Bosilevac and Koohmaraie, 2011; Amézquita-López et al., 2014; Akiyama et al., 2017; Dong et al., 2017). A number of studies have shown that STEC that carry *stx2* are more virulent than STEC that possess *stx1* or both *stx1* and *stx2* (Ostroff et al., 1989; Boerlin et al., 1999; Werber et al., 2003). Furthermore, the *stx2* toxin has been shown to be more potent than *stx1* and frequently implicated in severe disease including HUS (Bielaszewska et al., 2006; Tesh et al., 1993). In addition, a recent study demonstrated that *stx2* was heat stable and was not inactivated at currently approved pasteurization temperatures making *stx2* carrying isolates more likely to be incriminated in human STEC disease outbreaks involving pasteurized food such as dairy products (Rasooly and Do, 2010).

The majority of *stx2* positive isolates also carried *stx2c* and/or *stx2d* consistent with a number of studies, which have shown that both genes are frequent among cattle STEC (Zweifel et al., 2005; Bosilevac and Koohmaraie, 2011; Gonzalez et al., 2016). The *stx2d* subtype identified in this study was the *stx2d*-activatable variant (Bielaszewska et al., 2006; Scheutz et al., 2012). The high occurrence of *stx2*, *stx2c* and *stx2d*-activatable is a cause of concern as STEC isolates that possess these toxin types are highly virulent and commonly implicated in severe human disease including hemorrhagic colitis and hemolytic uremic syndrome (Friedrich et al., 2002; Ethelberg et al., 2004; Bielaszewska et al., 2006; Persson et al., 2007; Fuller et al., 2011; Melton-Celsa, 2015). Both *stx2c* and *stx2d* occurred concurrently in STEC serotypes which have been previously implicated in severe human disease (STEC O157:H7, O145:H28, O145:H7, O26:H7, O121:H8, O121: HNT, O45:H2, O26:H8, O26:H16, O26:H11, O26:H2, O121:H21 and O26:H21) (Beutin and Fach, 2014; EFSA, 2013).

Among the STEC isolates that carried *stx1*, 35.1% carried *stx1c* and/or *stx1d* with *stx1c* as the most frequent in agreement with previous studies which have shown that *stx1c* is more frequent than *stx1d* in isolates of cattle origin (Bosilevac and Koohmaraie, 2011; Dong et al., 2017). STEC that possess *stx1c* have been mostly recovered from asymptomatic humans or cases of mild diarrhea (Eklund et al., 2002; Friedrich et al., 2002; Friedrich et al., 2003). Interestingly, while *stx2c* and *stx2d* were widely distributed among the STEC isolates under study, *stx1c* and *stx1d* were confined to STEC O26 and O45 strains and were detected concurrently with *stx2* and/or its subtypes. Previous reports have shown that in cases whereby *stx1c* positive STEC were implicated in bloody diarrhea in humans, they also harbored *stx2* and/or one or more *stx2* subtypes (Zhang et al., 2002; Friedrich et al., 2003;

Matussek et al., 2017). In addition, two isolates belonging to STEC O45:H2 and STEC O45:H11 carried *stx1c, stx1d, stx2, stx2c,* and *stx2d* concurrently.

As has been previously documented for cattle STEC isolates (Blanco et al., 2004; Zweifel et al., 2005; Monaghan et al., 2011; Gonzalez et al., 2016; Akiyama et al., 2017), the majority of STEC isolates lacked *eaeA* and perhaps less likely to be implicated in human STEC outbreaks or HUS (Friedrich et al., 2002; Boerlin et al., 1999; Werber et al., 2003). The *eaeA* gene was confined to seropathotypes A and B strains (STEC O157:H7, STEC O103:H2, STEC O26:H2, STEC O145:H28) that were also *stx2c* and/or *stx2d* positive, indicative of STEC isolates with a potential to cause HC or HUS (Friedrich et al., 2002; Werber et al., 2003; Ethelberg et al., 2004; Bielaszewska et al., 2006). Seropathotypes A and B strains are considered highly pathogenic for humans and more likely to be incriminated in outbreaks and HUS (Karmali et al., 2003; EFSA, 2013). However, some *eaeA*-positive STEC serotypes have never been associated with disease, and some *eaeA*-negative STEC can cause mild to severe disease outbreaks in humans including HUS, suggesting that there are other factors which contribute to STEC virulence (Karmali et al., 2003; Coombes et al., 2008).

Plasmid virulence markers *ehxA*, *espP,* and *saa* were widespread in the majority of isolates whereas *subA*, *etpD* and *katP* were detected in low numbers. Similar reports have documented high rates for *ehxA, espP* and *saa* and very low rates for *katP* and *etpD* (Wu et al., 2010; Bosilevac and Koohmaraie, 2011; Gonzalez et al., 2016; Dong et al., 2017). However, rates for these genes among cattle STEC may vary from one country to another (Zweifel et al., 2005; Lucchesi et al., 2006; Wu et al., 2010; Monaghan et al., 2011; Cadona et al., 2016; Gonzalez et al., 2016).

Our results showed that almost all isolates carried *ehxA* (90.7%), in agreement with a number of reports on cattle and human STEC isolates (Wu et al., 2010; Bibbal et al., 2015; Dong et al., 2017). In some STEC strains, the presence of *ehxA* has been associated with severe clinical disease in humans (Schmidt et al., 1995). Although the role of *ehxA* in human disease remains unclear, the EHEC enterohemolysin (EHEC-Hly) is an RTX toxin with pore-forming capacity on erythrocytes. Release of heme from lysed erythrocytes provides much needed iron for STEC multiplication and survival in the host (Schmidt et al., 1995, Schmidt, and Karch, 1996), and may be associated with bleeding disorders that are observed in STEC complications such as hemorrhagic colitis, as result of erythrocyte lysis (Beutin et al., 1989).

The lower rates of *katP* (10%) and *etpD* (7.9%) observed in this study were in agreement with a number of studies that have also detected low levels or absence of these genes in STEC of cattle origin (Khan et al., 2002; Karama et al., 2008a; Monaghan et al., 2011). Furthermore, *katP* and *etpD* were exclusively detected in *eaeA*-positive STEC (O157:H7, O103:H2 and O145:H28) that have been associated with severe disease in humans including HC and HUS, except for one isolate which was *etpD* positive but *eaeA*-negative (STEC O103:H21). This is congruent with a number of reports which have documented similar findings among human and cattle STEC (Pradel et al., 2008; Bugarel et al., 2010; Bugarel et al., 2011; Kobayashi et al., 2013). Catalase-peroxidase (*katP*) is thought to defend bacteria against oxidative damage by reactive oxygen molecules produced by host phagocytes (Brunder et al., 1996). Furthermore, *etpD* encode a type II secretion pathway responsible for delivery of exoproteins (type II proteins) from pathogenic bacteria to target host cells (Schmidt et al., 1997; Lory, 1998).

The majority of isolates carried an incomplete plasmid except STEC O157:H7 isolates which possessed all the four plasmid markers (*katP, etpD, espP* and *ehxA*) concurrently. Previous studies have also shown that STEC O157:H7 strains can have the full complement of plasmid markers *ehxA*, *etpD*, *katP* and *espP* which are usually all encoded on the large pO157 plasmid (Makino 1998; Fratamico 2011). Possession of the full complement of the four plasmid encoded genes is usually considered a marker of enhanced virulence in STEC (Karch et al., 1998; Bielaszewska et al., 2013).

The *saa* and *subA* genes were only detected among *eaeA*-negative STEC consistent with other studies which have reported that *saa* and *subA* only occur in *eaeA*-negative STEC of cattle origin (Zweifel et al., 2005; Monaghan et al., 2011; Bosilevac and Koohmaraie, 2011; Gonzalez et al., 2016; Dong et al., 2017; Akiyama et al., 2017). Both *saa* and *sub*A were detected in *eaeA*-negative STEC serotypes that have been incriminated in uncomplicated diarrhea (O26:H2, O26:H8, O26:H21, O121:H8, O45:H2 and O145:H8), hemorrhagic colitis (O26:H7 and O145:H7) and hemolytic uremic syndrome (O26:H11) in humans (Paton et al., 2004; Tozzoli et al., 2010; Buvens et al., 2010; Buvens et al., 2012; Galli et al., 2010; Michelacci et al., 2013).

Although *saa* and *subA* genes are considered important virulence markers in *eaeA*-negative STEC, their role in the pathogenesis in human disease has not been fully elucidated. The STEC autoagglutinating adhesin (*saa*) is responsible for intimate adherence to the host intestinal mucosa in intimin negative STEC (Paton et al., 1999; Paton et al., 2001). The subtilase cytotoxin is a highly potent toxin which is more cytotoxic to Vero cells than Shiga toxin and is believed to suppress the host immune system and facilitates STEC adherence (Paton et al., 2004; Morinaga et al.,

2007; Wang et al., 2007). The presence of *saa* and *subA* in clinically relevant pathogens among *eaeA*-negative STEC strains may be providing an alternative mechanisms to “cross the virulence threshold” to cause disease in humans (Paton et al., 2001; Wickham et al., 2006).

A complete OI-122 was observed in only 7.1% of isolates with most of the isolates lacking more than one OI-122 marker. Karmali et al., (2003) suggested that possession of *pag*C*, sen*, *efa1* (*Z4332*), *efa1* (*Z4333*) is indicative of a complete OI-122. A complete OI-122 was detected in STEC O157:H7 and STEC O103:H2. All isolates which had a complete OI-122 were *eaeA*-positive and belonged to serotypes which are commonly implicated in STEC disease outbreaks including HUS in humans. This is in agreement with a number of studies which have documented a complete OI-122 among clinically relevant STEC (Karmali et al., 2003; Morabito et al., 2003; Wickham et al., 2006; Buvens et al., 2012). The presence of a complete OI-122 and high prevalence of *stx2* among clinically relevant STEC serotypes, in this study, is a predictor of potentially virulent strains among STEC.

The majority (60%) of isolates had an incomplete OI-122 while OI-122 markers were absent in 32.9%. Isolates that had an incomplete OI-122 included seropathotype B and C STEC strains mainly that have been incriminated in mild or uncomplicated diarrhea (STEC O26:H2, O26:H8, O26:H21, O103:H21, O45:H2), one serotype that has been implicated in bloody diarrhea (STEC O145:H7) and two serotypes that have been associated with HUS (O26:H11 and O145:H28) in humans (Karmali et al., 2003; EFSA, 2013). However, isolates that were negative for all OI-122 markers were mainly seropathotypes D or E strains that have never been incriminated or are very rare in human disease (Karmali et al., 2003). An incomplete OI-122 observed among the isolates was indicative of the instability of OI-122 genes that are usually

encoded on mobile genetic elements that can be acquired and lost laterally (Perna et al., 2001; Karmali et al., 2003).

OI-43/48 genes including *iha* (93.6%) and the *terC* (80%) were widespread among the STEC isolates. The high frequency of *iha* was consistent with several studies that have reported predominance of this gene among cattle STEC (Wu et al., 2010; Galli et al., 2010; Bosilevac and Koohmaraie, 2011; Gonzalez et al., 2016). However, variations in *iha* occurrence rates have been observed among cattle STEC in different regions (Amézquita-López et al., 2014; Karama et al., 2009; Monaghan et al., 2011; Akiyama et al., 2017). The *iha* gene product is considered an additional adhesin in STEC strains (Tarr et al., 2000; Yin et al., 2009).

The *terC gene* was detected in the vast majority of isolates consistent with previous studies which have reported high rates of *terC* among STEC (Orth et al., 2007; Ju et al., 2013). The *terC* gene was significantly detected in *eaeA*-positive isolates congruent with other studies that have shown a correlation between *terC* and *eaeA* in both cattle and human STEC isolates (Orth et al., 2007; Ju et al., 2013). So far, the role of tellurite resistance genes in STEC virulence remains unclear but it has been hypothesized that tellurite resistance genes may promote adherence among STEC, thereby enhancing survival of bacteria in the host (Yin et al., 2009). Tellurite resistance has also been associated with inhibition and/or resistance to bacteriophage (T5) infection and pore-forming colicins (Taylor et al., 1999).

Although *ureC* gene was observed in the majority of isolates, it was less frequent compared to *terC* and *iha.* The *ureC* gene was significantly detected in *eaeA*-positive STEC isolates (17/17) in comparison to *eaeA*-negative STEC (61/123) (Friedrich et al., 2005; Friedrich et al., 2006; Orth et al., 2006; Ju et al., 2013). Furthermore,

among the isolates that carried *ureC* were serotypes that have been incriminated in mild to severe STEC illness in humans including STEC O26:H2, O26:H7, O26:H8, O26:H21, O45:H2, O103:H2, O145:H7, O145:H28 and O157:H7. The *ureC* gene has been proposed as a suitable marker for detecting pathogenic Enterohemorrhagic *Escherichia coli* (EHEC) strains among other diarrheagenic *E. coli* commonly incriminated in severe human disease (Nakano et al., 2001; Friedrich et al., 2006). The *ureC* gene also facilitates adherence and increase survival of bacteria within the acidic environment of the host (Yin et al., 2009; Steyert and Kaper, 2012).

Isolates were screened for 16 non-LEE effector encoding genes that are scattered across four O-islands (OI-36, OI-57, OI-71 and OI-122). At least ten out of sixteen *nle*-encoding genes were detected in clinically relevant serotypes that were also *eaeA*-positive including STEC O103:H2, STEC O145:H28 and STEC O157:H7. These isolates accounted for 12.9% of total isolates. The high frequency of *nle*-encoding genes in clinically relevant strains (STEC O103:H2, STEC O145:H28 and STEC O157:H7) was significantly associated with the presence of *eaeA* in these strains in agreement with studies which have reported similar findings elsewhere (Karmali 2003; Coombes et al., 2008, Bugarel et al., 2010; Bugarel et al., 2011). Furthermore, STEC O157:H7, O145:H28 and O103:H2 commonly implicated with human disease outbreaks carried the *nle* ‘virulence gene signature’ which includes the concurrent presence of *nleB*, *nleE*, *ent/espL2*, *nleG2-3, nleG5-2*, *nleG9*, *nleG2-1* and *nleB2.* It has been shown that non-LEE effectors are highly conserved among *eaeA* positive strains (Iguchi et al., 2009), suggestive of yet-undefined virulence mechanisms and evolutionary pathways among STEC carrying *nle* genes and *eaeA* concurrently.

Furthermore, we report a number of *eaeA*-negative serotypes that possessed 9 to 11 *nle*-encoding genes. These isolates belonged to STEC serotypes O26:H2, O26:H21, O157:H19, O45:H11, O45:H16 and O45: HNT. Serotypes O26:H21 and O26:H2 have been previously implicated in mild diarrhea in humans (EFSA, 2013; Beutin and Fach 2014), but STEC O157:H28 and O45:H11 have not been involved in human disease so far. The presence of the majority of *nle*-encoding genes in STEC O157:H19, O45:H11, O45:H16 and O45:HNT that have never been implicated in human disease may be an indication of emerging virulent cattle STEC strains that have a potential to cause disease in humans and should be closely monitored.

Fifty percent (50%) of isolates did not carry any *nle*-encoding gene while one to seven *nle*-encoding genes were found in 30.7% of isolates and 6.4% of isolates were positive for eight to nine *nle*-encoding genes. Isolates that did not carry any *nle*-encoding gene included a number of serotypes that have been incriminated in mild uncomplicated diarrhea (STEC O26:H2, STEC O26:H8, STEC O26:H21, STEC O121:H8) hemorrhagic colitis (STEC O26:H7, STEC O145:H7) and HUS (STEC O26:H11) in humans (Beutin and Fach, 2014; EFSA, 2013), and serotypes that have never been associated with human illness (Blanco et al., 2004; Hussein and Sakuma, 2005; Hussein and Bollinger, 2005). The lack of *nle*-encoding genes in STEC serotypes that have been previously implicated in mild to severe complicated disease in humans, suggests that the ability of STEC strains lacking these *nle* genes to cause disease in humans may not be dependent upon currently known non-LEE effectors. Furthermore, the absence of currently known *nle* in STEC isolates that have never been implicated in human disease may explain why these isolates are not pathogenic to humans.

Antimicrobial resistance profiling showed that almost all (97.9%) STEC isolates were susceptible to 15 antimicrobials tested except for three STEC isolates that showed antimicrobial resistance. These isolates belonged to STEC O26:H11, STEC O26:H4 and STEC O45:H21. STEC O26:H11 was resistant to tetracycline whereas STEC O26:H4 was resistant to tetracycline, ampicillin and STEC O45:H21 to amoxicillin-clavulanic acid and cephalothin. Similar findings were made by Dong and colleagues who reported susceptibility to 14 antimicrobials and resistance to tetracycline, ampicillin, and cefotaxime among cattle STEC (Dong et al., 2017). However, higher antimicrobial resistance levels to tetracycline, ampicillin, cephalothin and amoxicillin-clavulanic acid have been previously reported by a number of studies in STEC (Karama et al., 2008a; Iweriebor et al., 2015; Amézquita-López et al., 2016). The very low antimicrobial resistance rates observed in this study suggest that there is no selection pressure exerted on cattle farms from which the STEC isolates were recovered. Cattle on cow-calf operations in South Africa are mainly reared on pastureland and are not supplemented with feed containing antimicrobial promoters that usually exert selective pressure which may favors the proliferation and maintenance of resistant STEC.

In conclusion, the majority of STEC isolates carried *stx2, stx2c* and *stx2d* subtypes that are commonly implicated in severe human disease. Our results showed that the serotypes under study were homogenous in possession of *ehxA* and *espP* but lacked *katP* and *etpD.* In isolates which had *katP* and *etpD*, these genes were significantly observed in *eaeA*-positive STEC. In addition, a full complement of plasmid-encoded *ehxA, etpD, katP* and *esp*P was observed in STEC O157:H7. Based on the possession of O islands and *nle* genes, the STEC isolates under study were of a diverse genetic background. However, STEC O157:H7, STEC O145:H28

and STEC O103:H2 that are frequently implicated in severe STEC disease in humans carried concurrently most *nle* genes including *nleB*, *nleE*, *ent/espL2*, *nleG2-3, nleG5-2*, *nleG9*, *nleG2-1* and *nleB2* that are a “signature” of STEC strains that are highly virulent to humans. This study demonstrated that most STEC O157, O145, O121, O111, O103, O45 and O26 associated serotypes lack essential virulence genes that are necessary for causing disease in humans.

4.6 References

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4.7 Appendix 1: Supplementary Material

Table 5: Distribution of Plasmid encoded and PAI Genes and Markers in *eaeA*-positive serotypes

			Plasmid encoded Genes				OI-122					OI-57			OI-71						OI-36				OI-43/48		
STEC Serotype	**stx Variants**	*eaeA*	*ehxA*	*katP*	*espP*	*etpD*	*pagC*	*sen-(Z4326)*	*ent/espL2*	*nleB & E*	*efa1 (Z4332/Z4333)*	*nleG 2-3*	*nleG 6-2*	*nleG 5-2*	*nleG9*	*nleG*	*nleF*	*nleH 1-2*	*nleA*	*nleG 2-1*	*nleB2*	*nleC*	*nleD*	*nleH 1-1*	***terC***	***ureC***	***iha***
O26:H2 (2)	***stx1d***	**2**	**2**	0	2	0	2	2	1	2	0	2	0	1	0	0	0	0	2	2	2	1	1	0	2	2	2
O103:H2 (1)	***stx1***	**1**	**1**	0	0	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	1	1	1	1	0
O145:H28 (2)	***stx2, 2c***	**2**	**2**	2	2	0	0	2	2	2	2	2	0	2	2	0	2	0	2	2	2	0	0	0	2	2	2
O145:H28 (1)	***stx2, 2c, 2d***	**1**	**1**	1	1	0	0	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	0	1	1	1
O145:HNT (1)	***stx2, 2c, 2d***	**1**	**1**	1	1	0	0	1	1	1	0	1	0	1	1	0	1	1	1	1	0	0	1	1	1	1	1
O157:H7 (7)	***stx2, 2c, 2d***	**7**	**7**	7	7	7	7	7	7	7	7	7	7	7	7	1	0	7	7	7	7	7	7	7	7	7	7
O157:H7 (2)	***stx2, 2c, 2d***	**2**	**2**	2	2	2	2	2	2	2	2	2	2	2	2	0	0	2	2	2	2	2	2	2	2	2	2
O157:H28 (1)	***stx2, 2c***	**1**	**1**	1	1	0	0	1	1	1	1	1	0	1	1	0	1	0	1	1	1	1	0	0	1	1	1
	Total	17	17	14	16	10	12	17	16	17	14	17	10	16	15	2	6	11	16	17	16	11	14	11	17	17	16
	% Positive	**100**	**100**	**82,4**	**94,1**	**58,8**	**70,6**	**100**	**94,1**	**100**	**82,4**	**100**	**64,7**	**94,1**	**88,2**	**11,8**	**35,3**	**64,7**	**94,1**	**100**	**94,1**	**64,7**	**82,4**	**64,7**	**100**	**100**	**94,1**

Table 6: STEC Antimicrobial Susceptibility

SEROTYPE	ISOLATE	CIP	AMP	GEN	NAL	CHL	CEF	KAN	STX	CT	TET	AMC	CAZ	CRO	AMK	CFP
O26:H2	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
O26:H4	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1
O26:H7	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
O26:H8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
O26:H11	3	3	3	3	3	3	3	3	3	3	2	3	3	3	3	3
O26:H16	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
O26:H19	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
O26:H21	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
O26:H28	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
O26:H38	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
O26:H45	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O26:HNT	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
O45:H2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O45:H8	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
O45:H11	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
O45:H16	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
O45:H19	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
O45:H21	2	2	1	2	2	2	1	2	2	2	2	1	2	2	2	2
O45:H28	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O45:H38	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
O45:HNT	12	12	12	12	12	11	12	12	12	12	12	12	12	12	12	12
O103:H2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O103:H21	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O121:H8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
O121:H21	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O121:HNT	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O145:H2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O145:H7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O145:H8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O145:H11	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O145:H19	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13
O145:H28	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
O145:HNT	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
O157:H2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O157:H7	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
O157:H19	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O157:H28	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
TOTAL		140	138	140	140	139	139	140	140	140	138	139	140	140	140	140
ISOLATES SUSCEPTIBLE (%)		**100**	**98,6**	**100**	**100**	**99,3**	**99,3**	**100**	**100**	**100**	**98,6**	**99,3**	**100**	**100**	**100**	**100**
RESISTANT (%)		**0**	**1,4**	**0**	**0**	**0,7**	**0,7**	**0**	**0**	**0**	**1,4**	**0,7**	**0**	**0**	**0**	**0**

AMC (Amoxicillin – clavulanic acid); **AMK** (Amikacin); **AMP** (Ampicillin); **CAZ** (Ceftazidime); **CEF** (Cephalothin); **CFP** (Cefoperazone); **CHL** (Chloramphenicol); **CIP** (Ciprofloxacin); **CRO** (Ceftriaxone); **CT** (Colistin) **GEN** (Gentamicin); **KAN** (Kanamycin); **NAL** (Nalidixic acid); **SXT** (Trimethoprim-sulfamethoxazole); **TET** (Tetracycline).

Table 7: Distribution of Pathogenicity and Plasmid encoded Genes and Markers

			Plasmid encoded Genes						OI-122							OI-57			OI-71						OI-36				OI-43/48		
SEROTYPE	No.of Isolate	*eaeA*	*hlyA*	*saa*	*subA*	*katP*	*espP*	*etpD*	*pagC*	*sen (Z4326)*	*ent/ espL2*	*efa1 (Z4332)*	*efa1 (Z4333)*	*nleB*	*nleE*	*nleG2-3*	*nleG6-2*	*nleG5-2*	*nleG9*	*nleG*	*nleF*	*nleH1-2*	*nleA*	*nleG2-1*	*nleB2*	*nleC*	*nleD*	*nleH1-1*	*ter-C*	*ureC*	*iha*
O26:H2	7	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O26:H2	1	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
O26:H2	1	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O26:H2	1	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O26:H2	1	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
O26:H2	6	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O26:H2	1	+	+	-	-	-	+	-	+	+	-	-	-	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-	+	+	+
O26:H2	1	+	+	-	-	-	+	-	+	+	+	-	-	+	+	+	-	+	-	-	-	-	+	+	+	-	+	-	+	+	+
O26:H2	1	-	+	+	-	-	+	-	+	+	+	-	+	-	+	+	+	+	+	-	-	+	-	+	+	-	+	+	+	+	+
O26:H4	1	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-
O26:H7	1	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O26:H7	1	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O26:H7	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-
O26:H8	1	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
O26:H8	1	-	+	+	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O26:H8	1	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
O26:H8	1	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
O26:H8	1	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
O26:H8	3	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
O26:H11	1	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O26:H11	1	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
O26:H11	1	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
O26:H16	1	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
O26:H16	1	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	+	-	-	+
O26:H19	1	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
O26:H19	1	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

			Plasmid encoded Genes						OI-122							OI-57			OI-71						OI-36				OI-43/48		
SEROTYPE	No.of Isolate	*eaeA*	*hlyA*	*saa*	*subA*	*katP*	*espP*	*etpD*	*pagC*	*sen (Z4326)*	*ent/ espL2*	*efa1 (Z4332)*	*efa1 (Z4333)*	*nleB*	*nleE*	*nleG2-3*	*nleG6-2*	*nleG5-2*	*nleG9*	*nleG*	*nleF*	*nleH1-2*	*nleA*	*nleG2-1*	*nleB2*	*nleC*	*nleD*	*nleH1-1*	*ter-C*	*ureC*	*iha*
O26:H21	1	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
O26:H21	1	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
O26:H21	1	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	-	+	+	-	+	-	+	-	-	-	-	+	+
O26:H21	2	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O26:H21	1	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
O26:H21	1	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O26:H28	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
O26:H28	1	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
O26:H38	2	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
O26:H45	1	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O26:HNT	1	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
O26:HNT	1	-	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
O26:HNT	2	-	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+
O157:H7	1	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
O157:H7	1	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
O157:H2	1	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O157:H7	6	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
O157:H7	1	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
O157:H19	1	-	+	+	-	-	+	-	-	+	+	-	-	+	-	-	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+
O157:H28	1	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	-	+	+	-	+	-	+	+	+	+	-	-	+	+	+
O145:H2	1	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O145:H7	1	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O145:H8	1	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O145:H11	1	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O145:H19	2	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O145:H19	1	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O145:H19	4	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+
O145:H19	1	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O145:H19	1	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+

SEROTYPE	No.of Isolate		Plasmid encoded Genes						OI-122							OI-57			OI-71						OI-36				OI-43/48		
		eaeA	*hlyA*	*saa*	*subA*	*katP*	*espP*	*etpD*	*pagC*	*sen (Z4326)*	*ent/ espL2*	*efa1 (Z4332)*	*efa1 (Z4333)*	*nleB*	*nleE*	*nleG2-3*	*nleG6-2*	*nleG5-2*	*nleG9*	*nleG*	*nleF*	*nleH1-2*	*nleA*	*nleG2-1*	*nleB2*	*nleC*	*nleD*	*nleH1-1*	*ter-C*	*ureC*	*iha*
O145:H19	2	-	+	+	-	-	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	-	-	-	+	+	+	+	+	+
O145:H19	1	-	+	+	-	-	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	-	-	-	-	+	+	+	+	+
O145:H19	1	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O145:H28	2	+	+	-	-		+	-	-	+	+	+	+	+	+	+	-	+	+	-	+	-	+	+	+	-	-	-	+	+	+
O145:H28	1	+	+	-	-		+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+
O145:HNT	1	+	+	-	-	+	+	-	-	+	+	-	-	+	+	+	-	+	+	-	+	+	+	+	-	-	+	+	+	+	+
O145:HNT	1	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O145:HNT	1	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O121:H8	3	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O121:H8	1	-	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
O121:H8	1	-	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O121:H8	1	-	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O121:H8	1	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O121:H8	1	-	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O121:H21	1	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
O121:HNT	1	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O103:H2	1	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+	-
O103:H21	1	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
O45:H2	1	-	+	+	-	-	+	-	+	+	+	-	+	-	+	+	+	-	-	-	-	+	-	-	-	-	-	+	-	+	+
O45:H8	1	-	+	+	-	-	+	-	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O45:H8	1	-	+	+	+	-	+	-	-	+	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O45:H8	1	-	+	+	+	-	+	-	-	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O45:H11	1	-	+	+	+	-	+	-	+	+	+	-	+	+	+	+	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+
O45:H11	3	-	+	+	+	-	+	-	+	+	+	-	+	+	+	+	+	-	-	-	-	+	-	-	-	-	+	+	+	+	+
O45:H11	2	-	+	+	+	-	+	-	+	+	+	-	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	+	+	+	+
O45:H11	2	-	+	+	+	-	+	-	+	+	+	-	+	+	+	+	+	-	+	-	-	+	-	-	-	-	+	+	+	+	+
O45:H16	1	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+
O45:H16	1	-	+	+	+	-	+	-	-	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O45:H16	1	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+

			Plasmid encoded Genes						OI-122							OI-57			OI-71						OI-36				OI-43/48		
SEROTYPE	**No.of Isolate**	*eaeA*	*hlyA*	*saa*	*subA*	*katP*	*espP*	*etpD*	*pagC*	*sen (Z4326)*	*ent/ espL2*	*efa1 (Z4332)*	*efa1 (Z4333)*	*nleB*	*nleE*	*nleG2-3*	*nleG6-2*	*nleG5-2*	*nleG9*	*nleG*	*nleF*	*nleH1-2*	*nleA*	*nleG2-1*	*nleB2*	*nleC*	*nleD*	*nleH1-1*	*ter-C*	*ureC*	*iha*
O45:H19	1	-	+	+	+	-	+	-	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+
O45:H19	1	-	+	+	+	-	+	-	+	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O45:H19	1	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+
O45:H21	1	-	-	-	+	-	-	-	-	+	+	-	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+
O45:H21	1	-	+	+	+	-	+	-	+	+	+	-	+	+	-	+	+	-	-	-	-	+	-	-	-	-	-	+	+	+	+
O45:H28	1	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+
O45:H38	1	-	+	+	+	-	+	-	-	+	+	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O45:H38	1	-	+	+	+	-	+	-	-	+	+	-	+	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	+	+	+
O45:H38	1	-	+	+	+	-	+	-	-	+	+	-	+	-	+	+	+	-	-	-	-	+	-	-	-	-	-	+	+	+	+
O45:H38	1	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	+	+	+
O45:H38	1	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O45:HNT	1	-	+	+	+	-	+	-	-	+	+	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O45:HNT	2	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O45:HNT	1	-	+	+	+	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+
O45:HNT	1	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-	+	+
O45:HNT	1	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+
O45:HNT	1	-	+	+	-	-	+	-	-	+	+	-	+	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	+	+	+
O45:HNT	1	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	+	+	+	+
O45:HNT	1	-	+	+	+	-	+	-	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+
O45:HNT	1	-	+	+	+	-	+	-	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O45:HNT	1	-	+	+	+	-	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
O45:HNT	1	-	-	+	-	-	+	-	+	+	+	-	+	+	+	+	+	-	-	-	-	+	-	-	-	-	+	-	+	+	+
TOTAL	140	17	127	115	53	14	111	11	75	51	48	15	40	46	40	43	47	27	23	3	7	38	17	20	22	17	29	39	112	78	131
% Positive		12,1	90,7	82,1	37.9	10,0	79,3	7,9	53,6	36,4	34,3	10.7	28,6	32,9	28,6	30,7	33,6	19,3	16,4	2,1	5,0	27,1	12,1	14,3	15,7	12,1	20,7	27,9	80,0	55,7	93,6

NB: [a]Serotypes in bold have been identified previously as human pathogens causing diarrhoea, bloody diarrhoea and HUS

5.0 CHAPTER V: General Conclusion

Shiga toxin-producing *Escherichia coli* is an important zoonotic foodborne pathogen that has been incriminated in mild to severe diarrhoea including complications such as HC and HUS (Karmali et al, 2010). Cattle play an important role in the dissemination of STEC to humans through the food chain (Hussein and Sakuma, 2005; Hussein and Bollinger, 2005; Farrokh et al., 2013), thus a public health concern. STEC serogroups O157, O145, O103, O121, O111, O45 and O26 and associated serotypes have been widely implicated in severe human disease worldwide (Brooks et al., 2005; Johnson et al., 2006; Majowicz et al., 2014).

The importance of STEC O157, O145, O103, O121, O111, O45 and O26 serogroups is under-recognized in South Africa. The few studies that have been carried on STEC in South Africa have mainly been limited to STEC O157:H7 (Ateba et al., 2008; Iweriebor et al., 2015). This study investigated the occurrence of STEC O157, O145, O103, O121, O111, O45 and O26 and characterized isolates that had been recovered from five cattle beef farms (cow-calf operations) in South Africa by serotypes, virulence genes and markers, and antimicrobial resistance profiling.

Our findings showed that STEC O157, STEC O145, STEC O121, STEC 103, STEC O45 and STEC O26 were the most common serogroups among cattle, as 41% (241/578) of STEC isolates from cattle belonged to these seven O serogroups. STEC O111 was not detected in this study. The STEC isolates represented 33 serotypes. Most of the isolates were classified as STEC O26. The majority of serotypes that were recovered in this study had never been associated with human disease. However, 13 out of the 33 serotypes were serotypes that have been previously associated with mild to severe human STEC disease.

Virulence characterization showed that STEC isolates from cattle possessed *stx1, stx2* and *ehxA* but most of them lacked *eaeA*, a key STEC adhesin. Further subtyping of shiga toxin encoding genes on 140 isolates revealed that *stx2c* and *stx2d* were the most frequently observed shiga toxin subtypes in comparison to *stx1c* and *stx1d* which were less frequent. The majority of isolates were positive for plasmid-encoded markers *saa, subA,* and *espP* but lacked *katP* and *etp*D.

The *saa* and *subA* genes were only present in *eaeA*-negative STEC whereas *katP* and *etpD* were significantly frequent in *eaeA*-positive isolates. Furthermore, *saa* and *subA* were detected mainly among clinically relevant *eaeA*-negative isolates including STEC O26:H2, O26:H7, O26:H8, O26:H11, O26:H21 and O121:H8. The presence of *saa* and *subA* in clinically relevant pathogens that were *eaeA*-negative may provide an alternative mechanisms to “cross the virulence threshold” and cause STEC disease in humans (Paton et al., 2001; Wickham et al., 2006). Furthermore, the presence of *subA* in *eaeA*-negative STEC strains that also carried *saa*, *stx2,* and *ehxA* may be indicative of STEC strains that are highly pathogenic and pose a public health risk (Velandia et al., 2011).

This study revealed that the majority of STEC isolates carried an incomplete OI-122 markers (60%) while only a small number (7.1%) of isolates that were *eaeA* positive and belonged to STEC O157:H7 and O103:H2 strains carried a complete OI-122. Morabito *et al*., (2003) have associated the presence of *eaeA*-positive with OI-122 full complement in Enterohaemorrhagic *E. coli* (EHEC) strains. In addition, Karmali *et al*., (2003) observed that there was a significant correlation between a complete OI-122 and seropathotype A and B strains that are frequently incriminated in human disease outbreaks and HUS.

The majority of STEC isolates possessed OI-43/48 markers *terC, Iha and ureC*. The *terC* and *ureC* genes were significantly frequent among *eaeA* positive STEC. Most *nle* genes were detected in clinically relevant serotypes including STEC O103:H2, STEC O145:H28 and STEC O157:H7 which also carried *eaeA* and are commonly implicated in severe human disease (Karmali et al., 2003; Coombes et al., 2008).

Altogether, most cattle isolates belonging to STEC serogroups O157, O145, O121, O103, O45 and O26 lacked essential virulence genes and markers. However, of interest was that the *eaeA* gene was mainly observed in a small number of clinically relevant STEC serotypes that have been previously implicated in outbreaks and severe human disease including O103:H2, STEC O145:H28 and O157:H7. These serotypes also carried most of the virulence genes and markers that are commonly observed in highly virulent STEC isolates including *stx2, stx2c* and/or *stx2d*, *eaeA*, *ehxA*, *espP*, *katP* and *etpD*, a complete or almost complete OI-122 (one gene absent), all OI-43/43 markers and the majority of *nle* genes. STEC O26:H11, O103:H2, STEC O145:H28 and STEC O157:H7 represent a group of emerging human STEC in South Africa in agreement with a previous study which showed that STEC O157 and O26 were the most frequently implicated in sporadic outbreaks of human STEC disease (Smith et al., 2011; Karama, personal communication). Only a small number of STEC isolates were resistant to the antimicrobials tested including tetracycline, cephalothin, ampicillin and amoxicillin-clavulanic acid.

The limitation of this study was that a number of STEC isolates were not fully serotypable for H-types because we were limited to only 14 pairs of primers that targeted H-types which are commonly observed in cattle STEC (Singh et al., 2015). As a result, 36 STEC isolates were not assignable to a specific H-type and were classified as H-non typeable (HNT).

In conclusion, this study confirmed that cattle on cow-calf operations in South Africa are an important reservoir of STEC O157, STEC O145, STEC O121, STEC 103, STEC O45 and STEC O26. Our results support the notion that only a subset of STEC serotypes that are associated with STEC O157, STEC O145, STEC O121, STEC 103, STEC O45 and STEC O26 are clinically relevant. Furthermore, highly virulent isolates including STEC O157:H7, O145:H28, O103:H2 and O26:H11 were identified in this study. In addition, the vast majority of STEC isolates were susceptible to all antimicrobials. The information gathered in this study provides insight into the significance of cattle as a reservoir of STEC in South Africa. Comprehensive and broader investigations will be needed to establish the importance of cattle as a major reservoir of STEC in other cattle production systems including feedlots and dairy operations. It is also essential to conduct STEC surveillance programs along the food chain to determine the level of STEC contamination in foods of cattle origin. These studies are needed for creating awareness about STEC, developing mitigation strategies and initiating adequate measures for effectively controlling STEC along the food chain.

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