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

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Abstract

Cattle are a major reservoir of Shiga toxin-producing *Escherichia coli*. This study investigated the occurrence of seven major STEC serogroups including O157, O145, O103, O121, O111, O45 and O26 among 578 STEC isolates previously recovered from 559 cattle. The isolates were characterized for 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 distinct serotypes. 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 five farms was variable rang-ing 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); *ehx*A, 92.5% (223/241); and both *stx1* and *stx2*, 62.2% (150/241) of isolates. These findings are evidence that cattle in South Africa carry STEC that belong to 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 *ehx*A but lacked *eaeA*, a major STEC virulence factor in human disease.

KEYWORDS
cattle, cow–calf operations, major seven STEC, non-O157, serotypes, virulence

1 | INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are zoonotic foodborne pathogens that cause infections in humans characterized by mild to severe diarrhoea, haemorrhagic colitis (HC) and complications such as the haemolytic uraemic syndrome (HUS), a major cause of kidney failure in young children and the elderly (Nataro & Kaper, 1998). Humans acquire STEC after consumption of contaminated foods of animal origin (Hussein & Bollinger, 2005; Hussein & Sakuma, 2005), contaminated water and vegetables (Feng, 2014; Swerdlow et al., 1992). Furthermore, contact with infected animals or a contaminated environment, and persons to person has been documented as routes of transmission (Belongia et al., 1993; 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 *Escherichia coli* O:H serotypes exist and ~400 STEC serotypes have been associated with human disease worldwide (Bettelheim, 2007; Beutin & Fach, 2014; European Food Safety Authority, 2013; Hussein & Bollinger, 2005; Hussein & Sakuma, 2005). STEC O157:H7 is the predominant serotype in human infections (Johnson, Thorpe, & Sears, 2006). However, 70%–80% of human infections have been attributed to isolates that belong to non-O157 STEC serogroups (Gould et al., 2013; Johnson et al., 2006; Luna-Gierke et al., 2014). Several studies have documented that STEC O157, O26, O45, O103, O121, O111 and O145 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" (Beutin & Fach, 2014; Fratamico, Bagi, & Abdul-Wakeel, 2017).

Shiga toxins (Stx1 and Stx2) are the major virulence factors of STEC. Typical STEC may either harbour *stx1* or *stx2* or both genes (O'Brien et al., 1984; Strockbine et al., 1986). Another important STEC virulence factor is intimin (*eaeA*). The *eaeA* gene is located on a pathogenicity island termed the locus of enterocyte effacement (LEE) (McDaniel, Jarvis, Donnenberg, & Kaper, 1995). Intimin facilitates bacterial cell adhesion and colonization of human enterocytes and produces classical attaching and effacing lesions that were first observed "in vivo" when *eaeA*-positive STEC adhered to piglet intesti-nal epithelial cells (Knutton, Tzipori et al., 1986). Furthermore, STEC strains also possess the plasmid-encoded enterohemolysin (*ehx*A) which is responsible for lysing human erythrocytes and has been associated with haemorrhagic diarrhoea in human STEC infections (Schmidt, Beutin, & Karch, 1995).

When the first human outbreak of STEC occurred in 1982, ground beef was incriminated as the source of STEC (Riley et al., 1983). Since then, cattle have been recognized as a major STEC reservoir and cattle-derived food products (ground beef, unpasteurized milk and cheese) have been associated with a number of STEC outbreaks worldwide (Hussein & Bollinger, 2005; Hussein & Sakuma, 2005). Although human STEC outbreaks have occurred in South Africa (Effler et al., 2001; Smith, Tau, Sooka, & Keddy, 2011), epidemiological data on STEC in South Africa remain 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 (a) investigate the occurrence of STEC O157 and top six STEC serogroups including STEC O26, O45, O103, O121, O111 and O145 in adult healthy cattle on cow–calf operations in South Africa and (b) characterize STEC isolates by serotype and major virulence genes.

Impacts

- Shiga toxin-producing *Escherichia coli* have been incriminated in human disease worldwide and cattle are considered the major STEC reservoir.
- However, data on the role played by cattle as a reservoir of STEC remain scarce in developing countries including South Africa.
- This study showed cattle on cow–calf operations in South Africa are a major reservoir of STEC serogroups O26, O45, O103, O121, O145 and O157 and provides much needed data for STEC monitoring and surveillance in South Africa.

2 | MATERIALS AND METHODS

2.1 | Cattle study population

A cross-sectional study of five cow–calf operations was conducted from July 2015 and April 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. Only cow–calf operations in which cattle were maintained on grazing pasture or rangeland all year were considered for the study. Samples were collected during routine pregnancy diagnosis checks. Fresh faecal samples were collected from the rectum of adult cows and heifers using a new pair of disposable nitrile gloves for each animal. Faecal 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 faecal samples were collected from 559 cattle (one sample/cow) in this study.

2.2 | Detection of STEC

Each sample (5 g) 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 microlitre aliquots of the enrichment were spread on Drigalski Lactose agar (CM0531; Oxoid, Basingstoke, UK) and CHROMagar STEC (https://www.chromagar.com) and incubated at 37°C for 18–24 hr. All Drigalski Lactose agar and CHROMagar STEC plates showing growth after 18–24 hr of incubation were screened for STEC by PCR (Paton & Paton, 1998). In brief, a sterile inoculating loop was used to harvest colony sweeps from Drigalski Lactose agar and CHROMagar plates. The bacterial culture was suspended in a sterile 1.5 ml Eppendorf tube containing 1 ml of FA buffer (Becton Dickinson and Company Sparks, USA). Bacterial suspensions were mixed and washed by vortexing, followed by centrifugation for 5 min. 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, Beisaw, & Feng, 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 hr at 37°C. Three suspect single colonies were taken from each positive agar plate and subcultured on Luria Bertani for multiplication and purification. Once again, DNA was extracted (Monday et al., 2007), from pure colonies and the multiplex PCR (Paton & Paton, 1998) was used to verify and confirm the STEC status of the pure colonies. Confirmed STEC 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. A total of 578 confirmed STEC isolates were recovered from 559 adult healthy cattle and used in this study.

2.3 | *Confirmation of E. coli status*

PCR was performed to confirm the *E. coli* status of isolates using primers and PCR cycling conditions as previ-ously described (Table 1) (Doumith, Day, Hope, Wain, & Woodford, 2012). Before carrying out PCR, the frozen mixture containing DNA was thawed at room temperature, centrifuged at 12,000 rpm for 5 min and stored on ice. In brief, each PCR reaction (25 μl) con-tained 2.5 μl of 10X Thermopol reaction buffer, 2.0 μl of 2.5 mM dNTPs (deoxynucleotide triphosphates), 0.25 μl of 100 mM $MgCl_2$, 1.6 μl of each primer (0.64 μM final concentration), 1U of Taq DNA Polymerase (New England BioLabs® *Inc*.) and 5 μl of DNA lysate tem-plate. 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).

2.4 | 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). In brief, each PCR reaction (25 μl) contained 2.5 μl of 10X Thermopol reaction buffer, 2.0 μl of 2.5 mM dNTPs, 0.25 μl of 100 mM $MgCl_2$, 0.5 μl of each primer (0.2 μM final concentration), 0.25 μl of Taq DNA Polymerase (New England BioLabs, USA) and 5 μl 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 (0.05 mg/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 PCR serotyping: STEC-C210-03 (O157), STEC-ED476 (STEC O111), STEC-C1178-04 (STEC O145), STEC-C125-06 (STEC 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).

2.5 | Virulence gene profiling

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

2.6 | 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). Associations between farms, serogroups and serotypes were assessed using Fisher's exact test. Variables with p values of <0.05 were considered statistically significant.

3 | 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 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% CI: 13.48–19.80) (Table 2) which were positive. 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 fol-lowing 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). Occurrence rates for individual serogroups among the 559 cattle were as follows: STEC O26, 10.2% (57 cattle); STEC O45, 2.9% (16 cattle); STEC O145, 2.5% (14 cattle); STEC O157, 1.4% (8 cattle); STEC O121, 1.1% (6 cattle); and STEC O103; 0.4% (2 cat-tle) (Figure 1).

3.1 | STEC Serotypes

Further H typing revealed that the 241 STEC isolates belonged to 14 H types (Table 2). However, 14.9% (36/241) of the isolates were classified as H-non-typeable (HNT). Overall, 85.1% (205/241) of the isolates were fully serotypeable and assigned to 33 O:H serotypes.

TABLE 1 Nucleotide sequences used as primers in the PCR reaction to identify O-antigen and virulence genes

O or H antigen	Target gene	Sequence	Amplicon size (bp)	References
Escherichia 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, Fratamico, Roberts, Davis, and Liu (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)
	*ehx*A	F: GCATCATCAAGCGTACGTTCC R: AATGAGCCAAGCTGGTTAAGCT	584	Paton and Paton (1998)
H2	*fliC2*	F: TAGCGGGTACCCAGCCTCAAG R: CATCGCTTCCACCCAGTTCAA	305	Singh et al. (2015)
H4	*fliC4*	F: AGCGCGGCGAAACTGAC R: ACTTCCGCTGCACCAACA	215	Singh et al. (2015)
H7	*fliC7*	F: CTGGCGCGAAGTTAAACACCA R:ACCCGCGGTAAACCCAATAGTT	670	Singh et al. (2015)
H8	*fliC8*	F: CGGCGCGGTTAAGAATGATG R: GCTCTGCGCCAGTGTTGTTAGTAA	196	Singh et al. (2015)
H10	*fliC10*	F: GTGTGCGTGAGCTGACTGTT R: TGGCTTTAAGTGCAGAAGCA	555	Singh et al. (2015)
H11	*fliC11*	F: AGCGGCTAAGAATAAAGCACAGA R: GTGGCAGCCTTGTTATCAACTTTG	237	Singh et al. (2015)
H16	*fliC16*	F: TCCTTACCACCCACCTGAAC R: CCATTGAGATTGCCCTTGAT	403	Singh et al. (2015)
H19	*fliC19*	F: CCGCGACTGCAAGCAATGTA R: AGCCGCGTCTTTTAACACCTGA	493	Singh et al. (2015)
H21	*fliC21*	F: CGTGCTTCCTGTTTTCTTGG R: TGAATTCACTATTTCGGGGAGT	172	Singh et al. (2015)
H25	*fliC25*	F: TGGCGATAAACCTGTTGATGT R: GGCCATTAGCCTTGTAACAG	759	Singh et al. (2015)

(Continues)

TABLE 1 (Continued)

O or H antigen	Target gene	Sequence	Amplicon size (bp)	References
H28	*fliC28*	F: ACTGGCATACAACAGGCACACC R: TTACCATCCGCTGAAACATAGACTG	387	Singh et al. (2015)
H34	*fliC34*	F: CGGTTCGATGAAAATTCAGG R: AATGCCCGTAAATGCAGATG	727	Singh et al. (2015)
H38	*fliC38*	F: GCCGCCTTGAAGAATAACAC R: GCAGAGTCAGTGGATCGTTG	172	Singh et al. (2015)
H45	*fliC45*	F: CAAAGGCACTATTGCGAACA R: CAGCCGCTGGTTTCAGAGT	272	Singh et al. (2015)

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 2). 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 4 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 serotype.

Particular serotypes colonized cattle on specific farms ($p < 0.000$) (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.

3.2 | 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 *ehx*A, 92.5% (223/241). Both *stx1* and *stx2* occurred in 62.2% (150/241) of isolates (Table 3). The majority of STEC isolates carried *stx1 stx2 ehx*A (61%, 147/241), and *stx2 ehxA*, (22.8%, 55/241) as the major gene combination pathotype. Minor gene combinations were also observed: *stx2 eaeA ehx*A, 5.8% (14/241); *stx1 stx2*, 3.7% (9/241); *stx1 ehx*A, 1.7% (4/241) and *stx1 eaeA ehx*A, 1.2% (3/241) (Table 3). The *eaeA* gene was found in 7.1% (17/241) of isolates. Isolates that carried *eaeA* 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 3).

4 | DISCUSSION

Although a few studies have investigated the occurrence of STEC in cattle in South Africa (Ateba, Mbewe, & Bezuidenhout, 2008; Iweriebor, Iwu, Obi, Nwodo, & Okoh, 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 (Cernicchiaro et al., 2013; Joris, Pierard, & Zutter, 2011; Paddock, Shi, Bai, & Nagaraja, 2012; Pearce et al., 2006). However, a number of studies in different countries have also recorded STEC O157, STEC O103, STEC O45 or STEC O145 as the most frequent serogroups in cattle (Barlow & Mellor, 2010; Ekiri et al., 2014; Lynch, Fox, O'Connor, Jordan, & Murphy, 2012; 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 6 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; Stanford, 2016). Furthermore, studies that were carried out in Australia and

TABLE 2 Distribution of STEC serotypes on the five 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

(Continues)

France registered considerably lower prevalences of 7.7% and 1.8%, respectively, in cattle faeces (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 6 non-O157 STEC (O145, O103, O121, O111, O45 and O26) that were observed in feedlot cattle (Cernicchiaro et al., 2013; Stanford, Johnson, Alexander, McAllister, & Reuter, 2016; Stromberg et al., 2015). 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 (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 the cow–calf operations system (Gunn et al., 2007). Concentrate feeding creates a conducive environment in the gut for the proliferation of STEC strains resulting in excretion of STEC in large numbers in the farm environment (Diez-Gonzalez et al., 1998).

TABLE 2 (Continued)

Farm	Farm prevalence	Serogroup (No. of cattle +) *n* = 92	Cattle ID	STEC Serotype	Isolate (*N* = 241)
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

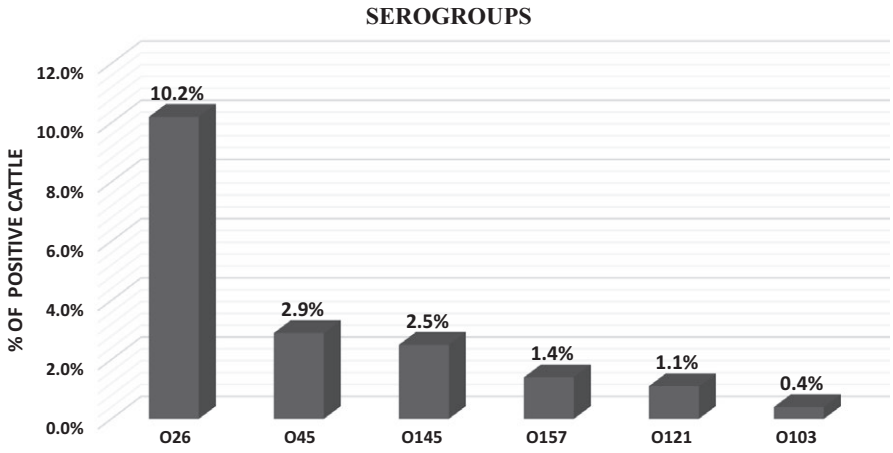

FIGURE 1 The frequency of seven major STEC serogroups in cattle

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, 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, Brod, Rodrigues, Carvalhal, & Aleixo, 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,

TABLE 3 STEC major virulence factors and gene combination

Serotype	No.	*stx1*	*stx2*	*eaeA*	*ehxA*	Gene combination
O26:H2	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[BD]	1	+	+	−	−	*stx1*, *stx2*
O26:H8	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	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*

(Continues)

TABLE 3 (Continued)

Serotype	No.	*stx1*	*stx2*	*eaeA*	*ehxA*	Gene combination
O45:HNT	1	–	+	–	–	*stx2*
O45:HNT	1	–	+	–	+	*stx2*, *ehxA*
O157:H7 [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	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	7	+	+	–	+	*stx1*, *stx2*, *ehxA*
O121:H8	2	–	+	–	+	*stx2*, *ehxA*
O121:HNT	1	+	+	–	+	*stx1*, *stx2*, *ehxA*
O103:H2 [BD,	1	+	–	+	+	*stx1*, *eaeA*, *ehxA*
O103:H21	1	–	+	–	–	*stx2*
Total	241	167	232	17	223	
%Positive		69.3	96.3	7.1	92.5	

Notes. BD: blood diarrhoea; D: diarrhoea; HUS: haemolytic uraemic syndrome (based on European Food Safety Authority, 2013).

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 (Bibbal et al., 2015; Blanco et al., 1996; Menrath et al., 2010). Of particular interest, one cow carried more than five serotypes including STEC O26:H8, O26:H11, O26:H28, O26:H38 and O121:H21.

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 diarrhoea, bloody diarrhoea and HUS worldwide (Bettelheim, 2007; Beutin & Fach, 2014). STEC O157:H7 is the serotype predominantly associated with diarrhoea, bloody diarrhoea (BD) and HUS outbreaks globally (Majowicz et al., 2014; Nataro & Kaper, 1998). 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 (Gould et al., 2013; Johnson et al., 2006; Luna-Gierke et al., 2014). STEC O26:H11 has been previously associated with outbreaks of severe human illness in Japan (Hiruta, Murase, & Okamura, 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). However, 14.9% of the isolates were H-non-typeable (HNT) 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). 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 sero-types 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% (Cull et al., 2017; Stanford et al., 2016), 73% to 98.6% for *stx2* (Bibbal et al., 2015; Paddock et al., 2012) and 52.5% to 53% of STEC isolates carried both *stx1* and *stx2* genes (Cernicchiaro et al., 2013; Cull et al.,

2017). STEC isolates harbouring *stx2* have been frequently associated with severe disease including HUS in humans in comparison to STEC strains which carry *stx1* only or both *stx1* and *stx2* (Friedrich et al., 2002; Ostroff et al., 1989). Almost all STEC isolates carried *ehx*A (92.5%). This was in agreement with similar studies in the USA which reported the presence of the *ehx*A gene in STEC isolates ranging from 74.5% to 99.7% (Cernicchiaro et al., 2013; Stanford et al., 2016). The *ehx*A 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).

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). 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 *ehx*A 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 (Friedrich et al., 2002; Ostroff et al., 1989). 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, 2013).

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 are harboured by 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; Cernicchiaro et al., 2013; Cull et al., 2017; Lee, Kusumoto, Iwata, Iyoda, & Akiba, 2017; Mellor et al., 2016; Paddock et al., 2012; Stanford et al., 2016). 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 foodborne human disease outbreaks 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 STEC isolates from nonhuman sources belonging to one of the "top seven" STEC serogroups be typed beyond O-grouping in routine surveillance studies before they are considered clinically relevant.

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 *ehx*A (enterohemolysin) genes but lacked *eaeA*. 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.

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CONFLICT OF INTEREST

The authors declare that the study was conducted in the absence of any commercial or financial relationship that could be interpreted as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

MK and AK conceptualized and designed the study; TT collected samples; AM, BT, MM and MK designed laboratory assays and generated data; AM and MK analysed data; AM and MK drafted the manuscript.

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