Extended-spectrum β-lactamase, shigatoxin and haemolysis capacity of O157 and non-O157 *E. coli* serotypes from producer-distributor bulk milk.

Victor Ntuli[a], Patrick M.K Njage[a], Elna M. Buys[a]*

[a]Department of Food Science, University of Pretoria, Private Bag X20, Hatfield, Pretoria 0028, South Africa

*Corresponding Author: Elna. M. Buys,
Email: elna.buys@up.ac.za
Tel: +27 12 4203209
Fax: +27 12 4202839

Abstract

We investigated for virulence genes (*stx1*, *stx2* and *hlyA*), serotypes and extended-spectrum β-lactamases (ESBLs) producing capacity in O157 and non-O157 *Escherichia coli* isolated from producer-distributor bulk milk (PDBM). Fifteen different *E. coli* O-serogroups were observed from the isolates (n=121). The prevalence of *stx1* and *stx2* genes among the *E. coli* isolates was 8.3% and 11.6% (n=121), respectively, while 5.8% harboured both *stx1* and *stx2*. Four *E. coli* isolates (3.3%) had ESBLs producing capacity, resisted multiple cephalosporins and aztreonam, and carried *stx* genes. Cluster analysis using GTG_5 finger printing revealed a diversity of *E. coli* seropathotypes in PDBM which are known to be associated with human diarrhoeal diseases. These results highlight a potential risk posed on human health by the consumption of PDBM contaminated with pathogenic *E. coli*. A further quantitative risk assessment of the impact of pathogenic *E. coli* contamination in PDBM on human health is therefore recommended.

1. Introduction

In the last few decades numerous studies have targeted *E. coli* O157:H7 serotype in food borne illness outbreaks due to its association with life-threatening diseases (Khan et al., 2002). However, in recent years, the prevalence of emerging non-O157 *E. coli* pathotypes, in particular non-O157 shigatoxin producing *E. coli* (STEC), infections and foodborne diarrhoeal disease has increased (Constantiniu, 2002). The STEC group is very diverse, and has emerged as an important food borne pathogen owing to its insidious strains belonging to a broad range of O & H serotypes that have been implicated to human and animal diseases (Paton & Paton, 1998). Gillespie, O'Brien, Adak, Cheasty, and Willshaw (2005) reported that the estimated risk of death in STEC outbreaks in European countries was at least five times more than outbreaks attributed to other intestinal pathogens. The frequency of outbreaks and sporadic cases of infection caused by pathogenic *E. coli* in food is often high also including in developed countries despite the implementation of proper public health policies and control measures (CDC, 2005; EFSA, 2015). Continuing epidemiological and clinical reports around the globe on O157 and emerging non-O157 *E. coli* serotypes has emphasised the need for research on these pathogens in foods in order to monitor their incidence and spread in an attempt to protect public health (Constantiniu, 2002). The public health impact of pathogenic and commensal *E. coli* has also increased over the years due to acquisition of antibiotic resistance genes and subsequent evolution of strains which are resisting commonly used antibiotics (da Silva & Mendonça, 2012). The spread of resistance in *E. coli* has threatened treatment of infections by this pathogen in both humans and animals and is also of increasing public health concern (Allocati, Masulli, Alexeyev, & Di Ilio, 2013). Recently, reports in the US have highlighted unprecedented foodborne outbreaks associated with antibiotic resistant pathogens (CDC, 2013). *E. coli* is reported among the top six drug resistant microbes

requiring urgent development of novel therapies (Shah, Hasan, Ahmed, & Hameed, 2004). Reports of *E. coli* strains being capable of producing Extended Spectrum β-lactamases (ESBLs) and hydrolysing the new generation cephalosporins and aztreonam further necessitates new therapies (Rawat & Nair, 2010). To our knowledge, very little information has been published about Enterobacteriaceae producing ESBLs in milk.

In addition to consequences on human health, the distribution of resistance in *E. coli* pathotypes is associated with genetic mobile elements, such as plasmids, that may also carry virulence determinants (da Silva & Mendonça, 2012). STEC O157 and non-O157 serotypes are characterised by plasmid carrying *stx1*, *stx2* and *eae* virulence factors which may cause severe hemorrhagic colitis (HC) and Haemolytic Uremic Syndrome (HUS) (da Silva & Mendonça, 2012). However, association of virulence and antibiotic resistance in non-O157 pathotypes in particular is underreported (Orden, Ruiz-Santa-Quiteria, Garcia, & Cid, 2000).

Food, especially of bovine origin, is one of the major transmitters of *E. coli* and its pathotypes to humans (CDC, 2005; De Buyser, Dufour, Maire, & Lafarge, 2001; EFSA, 2015). One of the most incriminated foods of bovine origin which is linked to disease outbreaks around the word is milk and its products (Oliver, Jayarao, & Almeida, 2005). In South Africa (SA) a significant population, especially the poor and vulnerable, is supplied with either raw or pasteurised milk directly from producer known as producer-distributor bulk milk (PDBM). This category of milk in SA, though a significant source of income to the farmers, is reported to pose a risk to the public due to pathogens, including *E. coli* (Caine, Nwodo, Okoh, Ndip, & Green, 2014; Lues, de Beer, Jacoby, Jansen, & Shale, 2010; O'Ferrall-Berndt, 2003). Very few studies have been reported about characterisation of pathogenic *E. coli* in PDBM. However, a recent study on bulk milk from a province in SA reported diarrheagenic *E. coli* belonging to enteroaggregative *E. coli*

(EAEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and uropathogenic *E. coli* (UPEC) from the milk (Caine et al., 2014). We previously identified *E. coli* in PDBM from different geographical regions sold directly from producer to the public in SA (Ntuli, Njage, & Buys, 2016). This study was carried out to characterise 121 STEC O157 and non-O157 strains from PDBM for prevalence and distribution of virulence genes encoding for shigatoxin and haemolysis genes, serogroups and ESBLs producing capacity. Genotypic and phenotypic relationship of the *E. coli* isolates was also determined. This was done with the aim of enhancing often limited data, which is important in assessing the risk on human health posed by pathogenic *E. coli* in milk, particularly in developing countries. Information obtained from this study will be used to carry out a quantitative risk assessment of the impact of STEC on public health in PDBM.

2. Materials and Methods

2.1 Escherichia coli strains

E. coli isolates (n=121) used in the current study had previously been isolated from raw and pasteurised PDBM from different provinces in South Africa and identified using MALDI-TOF (Ntuli et al., 2016).

2.2 Screening for β-lactamase producing Escherichia coli

ESBLs producing *E. coli* isolates were first screened using a disc diffusion test with extended-spectrum cephalosporins (Ceftriaxone (CRO 30 µg), Cefpodoxime (CPD 10 µg), Amoxiclav (AMC 30 µg), Ceftazidime (CAZ 30 µg), Cefotaxime (CTX 30 µg)) and aztreonam (ATM 30 µg) (Bio-Rad Laboratories, Hercules, CA, USA) on Mueller-Hinton agar II (Njage & Buys,

2015; Smet et al., 2008). *E. coli* isolates which showed resistance (CLSI, 2011) to any one of the six antibiotics were further screened for phenotypic production of ESBLs. Confirmation of ESBLs producing *E. coli* isolates was done using the modified double disc diffusion method on Mueller-Hinton agar II (Pitout, Reisbig, Venter, Church, & Hanson, 2003). Cefotaxime + clavulanic acid (30 µg + 10 µg), ceftazidime + clavulanic acid (30 µg + 10 µg), Amoxicillin + clavulanic acid (30 µg + 10 µg) discs (Bio-Rad Laboratories) were used. Extended-spectrum β-lactamase was positive when the zone diameters given by the discs with clavulanate were ≥ 5 mm larger than those without the inhibitor for at least one of the combinations. *E. coli* ATCC 25922 (ESBL negative control), *E. coli* ATCC 35218 (ESBL positive control) were used in the study (Njage & Buys, 2015). Data for antibacterial agent susceptibility tests performed during screening for ESBLs were recorded as zone diameters (mm) and a hierarchical cluster analysis was performed using Euclidean distance matrix with un-weighted pair group method and arithmetic means using XLSTAT version 2014.08.09 (www.**xlstat**.com).

2.3 Extraction of genomic DNA

Extraction of DNA for all the PCR analyses was done as follows: Bacterial strains were grown in 2 mL tryptone soy broth and incubated at 37 ºC for 24 h. The cultures were harvested by centrifugation at 9184 rcf (g) for 10 min. Each bacterial pellet was resuspended in 200µL of lysis buffer (10mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 % Triton X-100 (SIGMA, St. Louis, MO, USA)) (Goldenberger, Perschil, Ritzler, & Altwegg, 1995). The mixture was incubated at 95 ºC for 30 min with agitation. This was then cooled to 4 ºC for 10 min and centrifuged at 13000 rpm for 10 min. The DNA containing supernatant was purified using phenol chloroform method as described by Wilson (2001). DNA concentration was measured using a NanoDrop (Jeneway

Genova Nano, Staffordshire, UK) and concentration was standardized to 100 ng/μL. DNA extracts were stored at 4 ºC before use. *E. coli* isolates were confirmed using PCR detection of the *uidA* gene (Bej, DiCesare, Haff, & Atlas, 1991). All the *E. coli* isolates were characterised using primers and PCR thermal cycle conditions depicted in Table 1.

2.4 Haemolysis on blood agar

Columbia agar supplemented with 5% (v/v) sheep blood (Bio-Rad, Marnes-la-Coquette, France) were used to identify haemolysis activity in the *E. coli* isolates. Indication of alpha-haemolysin and all haemolysin types (beta and gamma) were examined after 6 h and 24 h respectively (Schmidt & Karch, 1996). To test for the presence of *hlyA* gene, PCR was then performed on the isolates which had given positive results for alpha-haemolysis activity (Lorenz et al., 2013). The 20 μL reaction mixture consisted of 1 μL (100ng) DNA template, 2 μL (10 μM) of each *hlyA* primers (Table 1), 10 μL of 2 times KAPA Taq ReadyMix PCR Kit and PCR grade water.

2.5 **E. coli** ***O157:H7 and virulence gene determination***

A multiplex PCR assay using three sets of primers targeting *stx1*, *stx2* and *uidA* genes was used to identify *E. coli* O157:H7 and shigatoxin virulence genes (Cebula, Payne, & Feng, 1995). For the *uidA* gene, mismatch amplification mutation assay (MAMA) primers, specific for a unique base substitution in *uidA* of *E. coli* O157:H7, were used (Table 1). *E. coli* O157:H7 (ATCC 35150) was used as a positive control. The 20 μL reaction mixture consisted of 1 μL (100ng) DNA template, 0.5 μL (10 μM) of each *stx1*, *stx2* and *uid*A primers, 10 μL of 2 times KAPA Taq ReadyMix PCR Kit and PCR grade water.

2.6 GTG_5 Repetitive extragenic palindromic (REP)-PCR fingerprinting of the **E. coli** *isolates.*

Fingerprints were generated for the 46 *E. coli* isolates including the O157:H7 reference strain. The 46 fingerprinted isolates had previously given a positive presumptive test on STEC media (Ntuli et al., 2016). The 20 µL reaction mixture consisted of 2 µL (100 ng) DNA template, 0.3 µL (0.2 µM) of the single oligonucleotide $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) primer (Versalovic, Schneider, De Bruijn, & Lupski, 1994), 10 µL of 2 times KAPA Taq ReadyMix PCR Kit, 0.4 µL (1%) dimethylsulphoxide and PCR grade water. The PCR thermal cycling was performed as follows: Initial denaturation at 95 ºC for 10 min; 35 cycles at 95 ºC for 30 s; 40 ºC for 60 s and 65 ºC for 3 min and a final elongation step at 65 ºC for 8 min. The PCR amplified DNA was separated on 2% agarose gel (55 V for 4 h). The GTG_5 fingerprints of the *E. coli* isolates were analysed using GelCompar II version 6.5 (Applied Maths NV, Sint-Martens-Latem, Belgium). The similarity of digitized bands patterns was calculated using Pearson's correlation coefficient, and unweighted pair group method with arithmetic means. Complete linkage algorithms were used to construct an average linkage dendrogram to show relationship of isolates. Isolates were considered to be within a clonal cluster if relatedness was 70% and above (Stackebrandt et al., 2002).

2.7 O- Serotyping

A method by Coimbra et al. (2000) was modified and used for serotyping the *E. coli* isolates. The O-antigen gene cluster (*rfb*) was amplified prior to restriction digestion using restriction enzyme *Mbo*II. JUMPstart and *gnd* primers (Wang & Reeves, 1998) were used (Table 1) to amplify the O-gene cluster in *E. coli.* A touchdown PCR cycle was done as follows: denaturation at 94 °C for 10 s, annealing at 64 °C for 30 s, and extension at 68 °C for 15 min, repeated ten

Table 1: Oligonucleotide primers used in the study for detection of targeted genes of *E. coli* isolated from producer-distributor bulk milk and the different thermal cyclic conditions applied.

Target Gene	Primer name	Sequence (5' - 3')	Fragment size bp	Source	PCR cycling conditions
Alpha-haemolysin	*hlyA F*	GTCTGCAAAGCAATCCGCTGCAAATAAA	561	(Kerényi et al., 2005)	**35 X[a]** 95 ºC for 60s, 54 ºC for 60s, 72 ºC for 60s
	hlyA R	CTGTGTCCACGAGTTGGTTGATTAG			
*uid*A	*uidA F*	GGTCACTCATTACGGCAAAG	379	(Bej, DiCesare, Haff, & Atlas, 1991)	**30 X[b]** 94 ºC for 60s, 59 ºC for 60s, 72ºC for 60s
	uidA R	CAGTTCAACGCTGACATCAC			
Shigatoxin 1	*SLT-1LP 30*	CAGTTAATGTGGTGGCGAAGG	348	(Cebula, Payne, & Feng, 1995)	**35 X[c]** 94 ºC for 30s, 58 ºC for 45s, 72 ºC for 90s
	SLT-1LP 31	CACCAGACAATGTAACCGCTG			
Shigatoxin 2	*SLT-2LP 43*	ATCCTATTCCCGGGAGTTTACG	584	(Cebula et al., 1995)	**35 X[c]** 94 ºC for 30s, 58 ºC for 45s, 72 ºC for 90s
	SLT-2LP 44	GCGTCATCGTATACACAGGAGC			
Mutated *uid*A for *E. coli* O157:H7	*uidA PT-2*	GCGAAAACTGTGGAATTGGG	252	(Cebula et al., 1995)	**35 X[c]** 94 ºC for 30s, 58 ºC for 45s, 72 ºC for 90s
	uidA PT-3	TGATGCTCCATAACTTCCTG			
rfb gene cluster	*JUMPstart*	CACTGCCATACCGACGACGCCGATCTGTTGCTTGG	1500 -1700	(Coimbra et al., 2000)	As explained in the method
	gnd	ATTGGTAGCTGTAAGCCAAGGGCGGTAGCG			

The 2X ReadyMix contains KAPA Taq DNA Polymerase (1 U per 50 µl reaction), KAPA Taq Buffer, dNTPs (0.2 mM of each dNTP at 1X), $MgCl_2$ (1.5 mM at 1X) and stabilizers (Boston, MA, USA). PCR grade water (KAPA bio-systems, Salt River, CPT, SA). Unless stated, all PCR products were electrophoresed (120 V for 45 min) in 1.5 % agarose gels, stained with GelRed (Biotium, CA, USA) and visualised using Gel documentation system (Gel Doc EZ Imager, BioRad, Hercules, CA, USA). A GeneRuler 1 kb DNA ladder (Thermo Scientific, Waltham, MA, USA) was used as a size marker. All PCR reactions were done using the thermocycler (C1000 Touch ThermalCycler CFX96TM Real Time System (Bio-Rad). [a]Initial denaturation step of (95 ºC for 15 min) and final strand extension steps of (72 ºC for 7 min). [b,c]Initial denaturation step of (95 ºC for 3 min) and final strand extension step of (72 ºC for 8 min).

times. For the next 20 cycles, extension time was increased by 20 s each time. Initial denaturing step (94 °C for 2 min) and final elongation (72 °C for 7 min) steps were included. Amplicons were electrophoresed in 1.5% agarose gels and their sizes were estimated using a GeneRuler 1 kb plus DNA ladder (Thermo Scientific, Waltham, MA, USA). In cases where multiple bands were obtained, the band with the highest molecular weight within size range 0.5 to 1.7 kbp was isolated and purified using an E-Gel® Electrophoresis System (E-Gel® Safe Imager Real-time Transilluminator (Invitrogen, Carlsbad, CA, USA)) with E-Gel® Precast Agarose (2%) (Invitrogen) prior to restriction digestion.

Restriction analysis was as follows: 10 µL of the PCR product was mixed with 1 µL of *Mbo*II (FastDigest enzyme), 17 µL of nuclease free water and 2 µL of 10 X FastDigest buffer (Thermo Scientific, Waltham, MA, USA). After incubation at 37 ºC for 5 min the enzyme was denatured at 65 ºC for 5 min. The restriction product was then electrophoresed in 2% agarose at 55 V for 5 h. Gel images were taken under UV illumination. BioNumerics software version 6.0 (Applied Maths NV, Sint-Martens-Latem, Belgium) was used to interpret the Restriction Fragment Length Polymorphism (RFLP) patterns by construction of a dendrogram based on the patterns. A dendrogram was constructed on the basis of a binary table in which the presence or absence (1/0) of restriction fragments was recorded. The "Ward" algorithm of the R-routine "hclust" was used (BioNumerics, Applied Maths, Sint-Martens-Latem, Belgium). A 1.5% band position tolerance was applied for comparison of RFLP fingerprinting patterns (Karger et al., 2011). Isolates were considered to be within a clonal cluster if relatedness was 70% and above (Stackebrandt et al., 2002). Representative isolates within a cluster were sent for serotyping using agglutination assays at an ISO 17025 certified national reference laboratory (Agricultural Research Council-Onderstepoort Veterinary Institute, Pretoria, SA).

3. Results

3.1 Virulence genes and ESBLs in E. coli

Resistance to cephalosporins (Ceftriaxone (CRO), Cefpodoxime (CPD), Amoxiclav (AMC), Ceftazidime (CAZ), Cefotaxime (CTX)) and aztreonam (ATM) used during screening of ESBLs was detected in all the *E. coli* isolates tested. While a total of 97.5% of the isolates were susceptible to cefotaxime, cefpodoxime was the most frequently resisted antibiotic (66.1%) (Fig 1a). Resistance patterns to cephalosporins and aztreonam revealed six different antibiotic resistance phenotypes (resistant to either a single or a combination of cephalosporins and aztreonam) and resistance to cefpodoxime gave the highest frequency (24.8%) of phenotypes (Fig 1b). Resistance to cefpodoxime was observed to be common in all the phenotypes resistant to multiple cephalosporins and aztreonam, and ATM-CPD was the phenotype combination with the highest frequency (14.0%, n=121) (Fig 1b). Four (3.3%) *E. coli* isolates belonging to serogroups O2, O9 and O68 had ESBLs producing capacity, resisted multiple cephalosporins and aztreonam, and also carried either *stx1* or *stx2*. Out of the 121 *E. coli* isolates, 31 (25.6%) harboured shigatoxin virulence genes. A total of 8.3% (n=121) carried *stx1* genes and 11.6% were positive for *stx2*, while 5.8% exhibited both (Table 2). Five *E. coli* isolates from the milk were positive for alpha-haemolysis (two O9, one O83 and two O112) on sheep blood agar and only 3 (one O9 and two O112) carried the *hlyA* gene (Table 2). The isolates which carried the *hlyA* gene also exhibited either *stx1* or *stx2* genes. A total of 25 (20.6%, n=121) *E. coli* isolates were capable of producing ESBLs. Eight of the serotypes (one O2, twoO9, one O20, one O68 and three O157) which produced ESBLs also carried either one or a combination of *stx1* and *stx2* genes (Table 2).

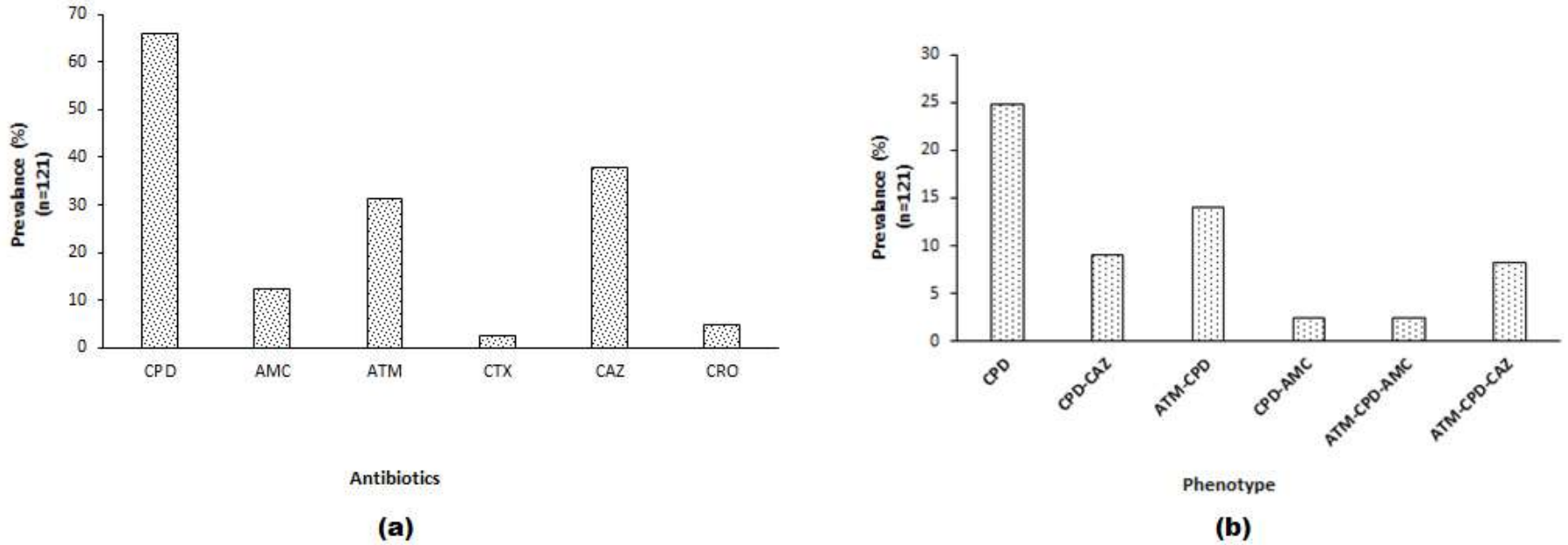

Fig 1: Prevalence of: (a) *E. coli* isolates resistant to different cephalosporins and aztreonam; (b) resistant *E. coli* phenotypes isolated from producer-distributor bulk milk. The cephalosporins and aztreonam were used to screen for ESBLs producing *E. coli*.

(CRO – Ceftriaxone, ATM – Aztreonam, CPD – Cefpodoxime, AMC – Amoxiclav, CAZ – Ceftazidime, CTX – Cefotaxime).

Table 2: Number of *E. coli* strains with virulence genes and/or ESBLs producing capacity for O157 and non-O157 STEC isolates from producer-distributor bulk milk.

Virulence gene(s) and/or ESBLs producing capacity	Serotype																Total (%)
	O2	**O4**	**O9***	**O20**	**O43**	**O64**	**O68**	**O83***	**O85**	**O109**	**O112***	**O119**	**O147**	**O155**	**O157**	**Rough**	
*hly*A (only)	-	-	1	-	-	-	-	-	-	-	2	-	-	-	-	-	**3** (2.45%)
Stx1 (only)	1	-	1	1	1	1	-	-	-	-	1	-	-	-	4	-	**10** (8.3%)
Stx2 (only)	-	-	3	-	-	1	1	-	-	-	-	-	-	1	8	-	**14** (11.6%)
Stx1 and *Stx2* (only)	-	-	-	-	-	-	-	1	-	-	1	-	-	-	5	-	**7** (5.8%)
ESBLs (only)	-	-	7	1	-	1	1	-	-	-	-	-	2	1	-	4	**17** (14.0%)
ESBLs and *Stx1/Stx2*	1	-	2	1	-	-	1	-	-	-	-	-	-	-	3	-	**8** (6.6%)

(-) not detected, ESBLs – Extended Spectrum β-lactamases, *Stx* – shigatoxin gene, *hly*A – haemolysis gene. Serotypes with asterisk (*) were positive for alpha haemolysis on sheep blood agar (two O9, one O83and two O112).

3.2 Serotyping (O-gene cluster and restriction analysis)

Amplification of the O-gene cluster generated one amplified fragment which ranged in size from 0.2 – 2kbp. After *MboII* digestion, clear bands were obtained for most of the serogroups. The number of bands per pattern varied from 4 to 10 (Fig 2). Cluster analysis of RFLP patterns revealed 16 clusters at 75% similarity level (Fig 3). A total of 32 representative isolates; 2 from each sub- clusters were sent for serum agglutination assay. The assumption drawn was that clonal strains clustered at ≥75% similarity. The dendrogram revealed that out of the 121 *E. coli* strains from the milk, 90 (74.2%) strains were associated with 15 different O-serogroups and 31 (25.6%) were considered rough strains (Fig 3). Rough *E. coli* strains were untypeable isolates with serum agglutination due to auto-agglutination with all the O-antisera used. The dominant and most frequently identified O-serotype were O9 (19.8%), followed by O157 (14.0%) serogroup, while O4 (0.8%), O43 (0.8%) and O119 (1.7%) had lower prevalence (Fig 4). A total of 26.6% of the serotypes (O2, O9, O64, O157 and Rough) were associated with both pasteurised and raw PDBM while the rest of the serotypes were identified in raw milk only.

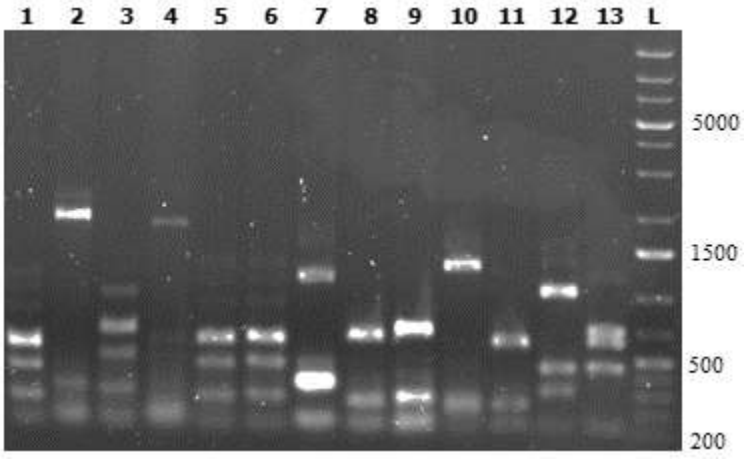

Fig 2: Illustrative agarose gel electrophoresis image of DNA fragments after *MboII* digestion.
Lanes 1= O9, 2= O43, 3= O9, 4= O4, 5= O9, 6= O9, 7= O112, 8= O64, 9= O64, 10= O155, 11= O64, 12= O147, 13= O64, L= molecular weight maker.

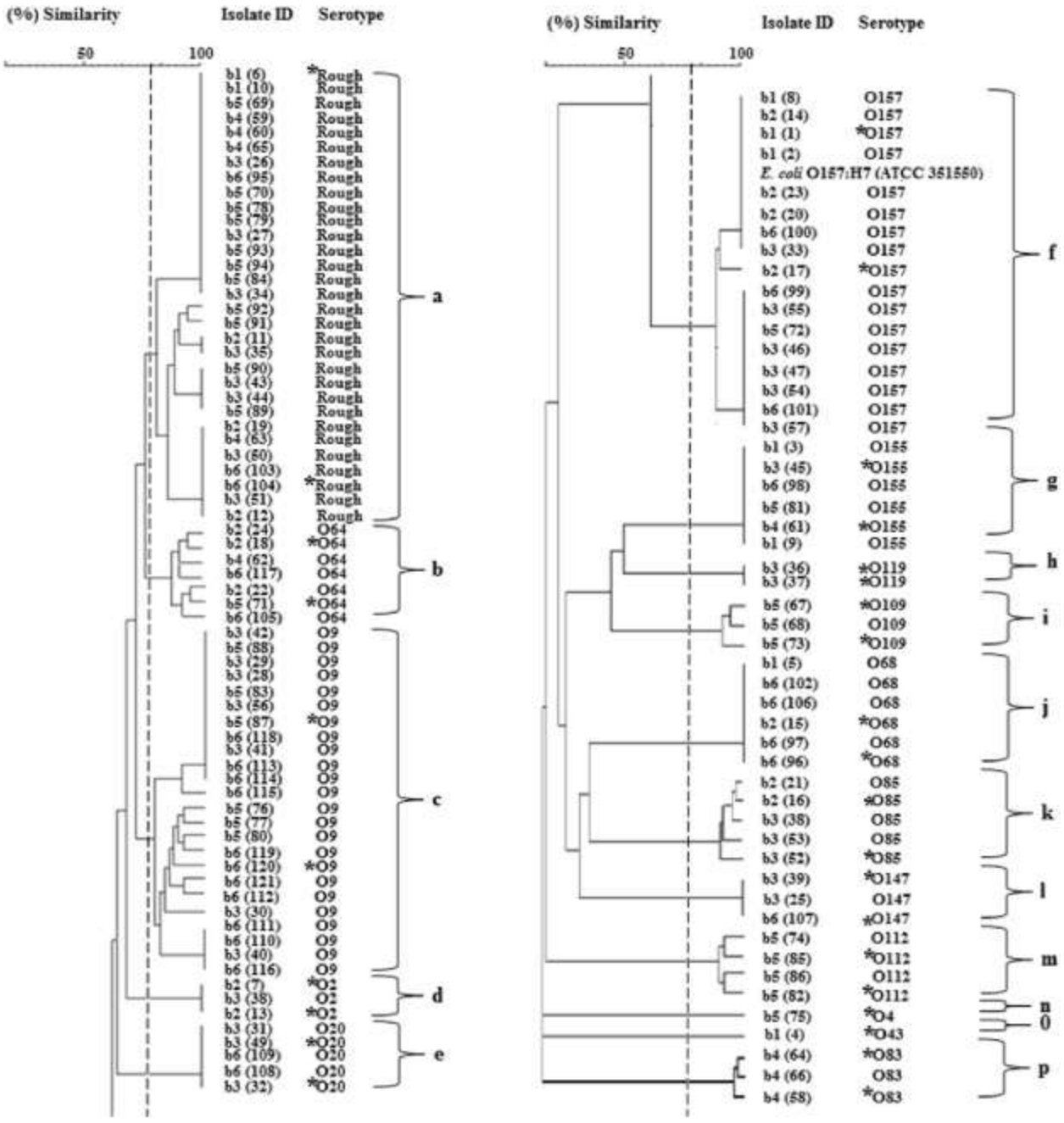

Fig 3: Dendrogram for RFLP fingerprints of *E. coli* isolates obtained from producer-distributor bulk milk. Clusters defined at ≥75% similarity. Isolates within a cluster in asterisks (*) were sent for serotyping using serum agglutination assays at an ISO 17025 certified national reference laboratory (Agricultural Research Council-Onderstepoort Veterinary Institute, Pretoria, SA).

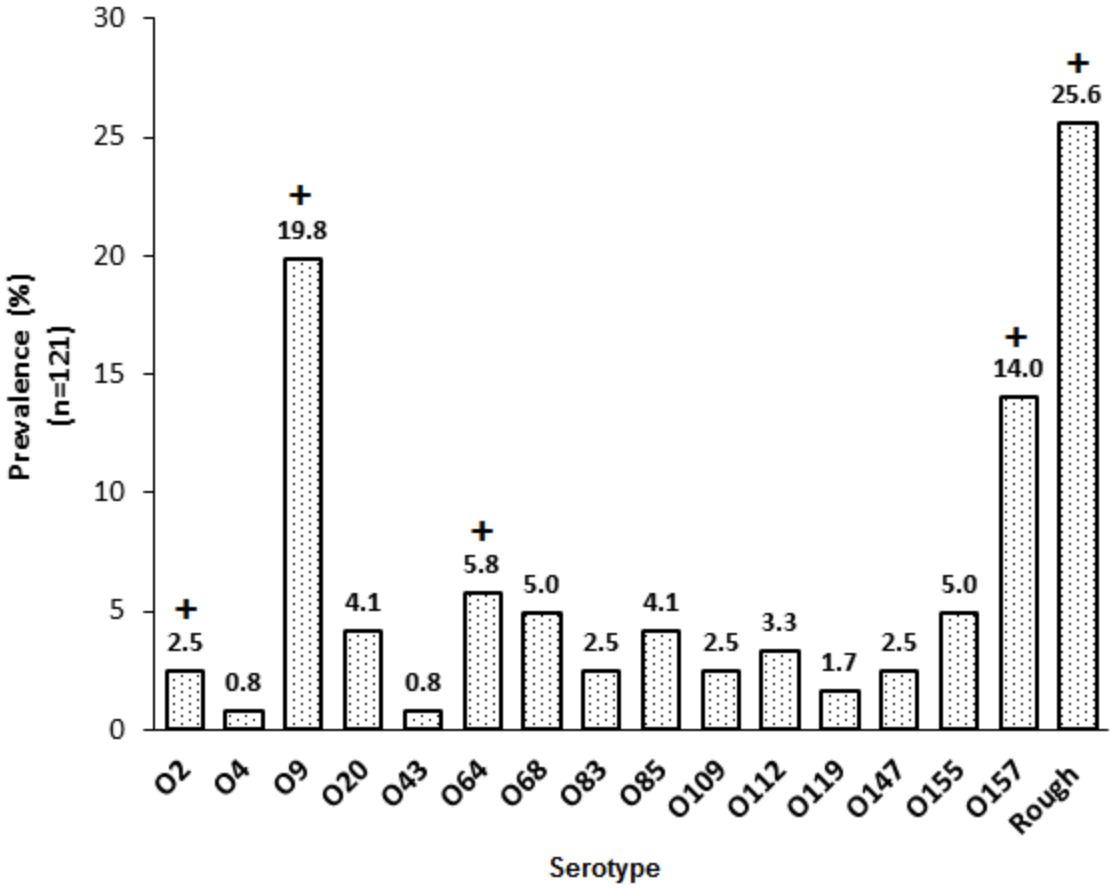

Fig 4: Prevalence of *E. coli* serotypes isolated from producer-distributor bulk milk (n=121). *E. coli* serotypes with plus sign (+) at the top were isolated from both raw and pasteurised retail bulk milk samples whereas the rest were isolated from raw milk only. Rough *E. coli* strains were untypeable isolates with serum agglutination due to auto-agglutination with all the O-antisera used

3.3 Cluster analysis of E. coli *serotypes in terms of phenotypic and genotypic relationship*

In order to establish the phenotypic and genotypic relationship of *E. coli* isolates according to serogroup, we observed the clustering trends on dendrograms generated from antibiotic susceptibility data (used during screening for ESBLs) and GTG_5 finger prints. Cluster analysis was applied to 25 *E. coli* isolates which produced ESBLs. This was done to determine trends in common phenotypes and relationship of isolates according to serogroup. Figure 5 presents six major clusters (A – F), at 75% similarity, that were further analysed for patterns of association of

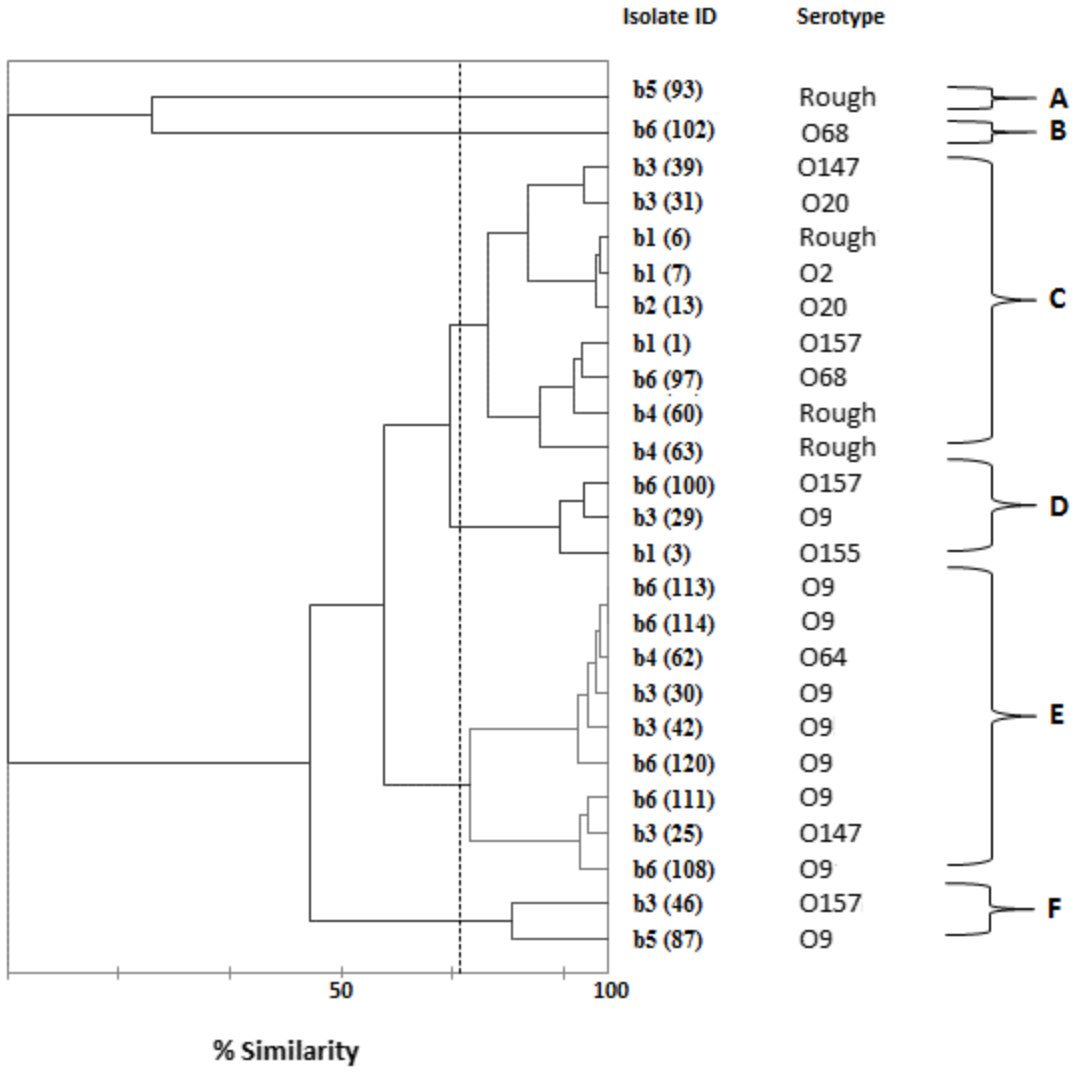

Fig 5: Two-dimensional hierarchical cluster analysis dendrogram showing relationship amongst 25 extended beta lactamase spectrum (ESBLs) producing *E. coli* isolated from producer-distributor bulk milk. Clusters defined at ≥75% similarity. The dendrogram was based on inhibition zone diameter data for antibiotics used for screening ESBLs producing *E. coli*.

E. coli isolates. There was no trend of clustering which was observed amongst the ESBLs *E. coli* isolates, though serotype O9 was distributed in most of the clusters. Cluster group E was dominated by serotype O9 (66.6%). The common phenotypes in clusters C, E and F were resistant to CPD-CAZ, ATM-CPD and ATM-CPD-CAZ respectively. In general clustering of

isolates on basis of antibiotic resistance phenotypic characteristics revealed no relationship of the *E. coli* isolates based on serogroup. However, a genetic relatedness of 31 STEC and 15 non STEC isolates using rep- PCR fingerprints revealed clustering which was based on serogroups (Fig 6).

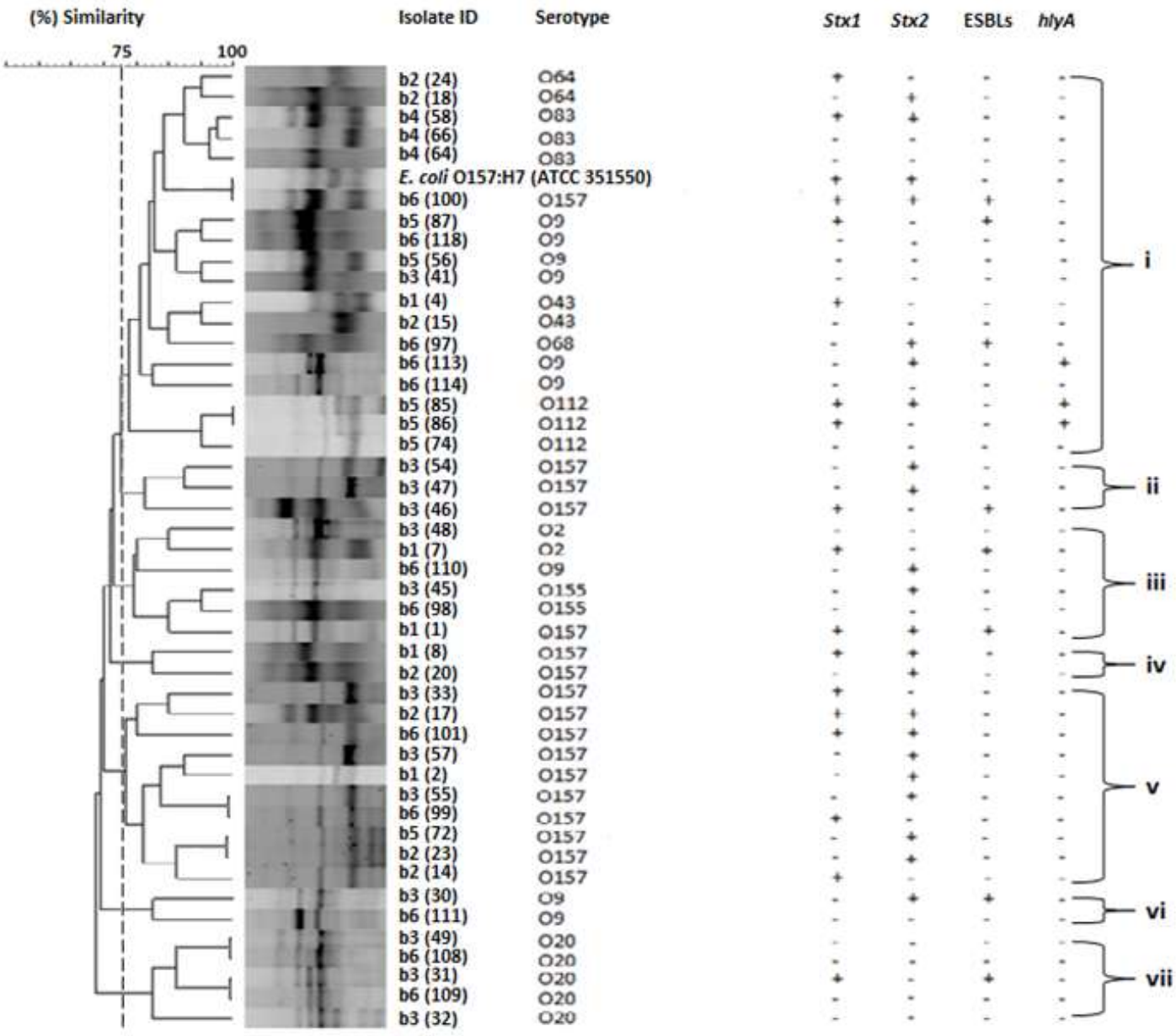

Fig 6: Dendrogram for rep-PCR fingerprints showing the relationships based on serotype, and virulence profiles amongst 46 isolates from retail producer-distributor bulk milk. Clusters defined at ≥75% similarity.

Figure 6 presents seven clusters which were generated from the dendrogram. The largest cluster (i) was dominated by serogroup O9 followed by O83 and O112. Further analysis of the cluster revealed that isolates were also grouping based on *stx* and *hlyA* genes. There was no common antibiotic resistance patterns observed in this cluster. The second largest cluster (v) comprised of serotype O157. Relatedness of these isolates was based on genotypic and phenotypic characteristics. The isolates that grouped in this cluster had been found to have common antibiotic phenotypic patterns (resistant to CPD and CPD – CAZ). Seventy percent of serotypes in cluster (v) carried *stx 2* genes. Cluster groups (ii), (iv), (vi) and (vii) were unique for serotype O157, O157, O9 and O20 respectively. In addition, common resistance to CPD had also been recoded for isolates that grouped in cluster (vii).

4. Discussion

We detected multiple resistant *E. coli* to cephalosporins and aztreonam during screening for ESBLs producing *E. coli*, with 20.6% of the isolates to be phenotypically positive for ESBLs production (Fig. 1; Table 2). Reports on acquisition of antimicrobial resistance genes in *E. coli*, and emergence of new strains capable of hydrolysing new generation cephalosporins, have escalated over the years around the globe (da Silva & Mendonça, 2012). However, acquisition of antimicrobial resistance genes by bacteria in environments such as food has not been studied extensively (Straley et al., 2006). This probes for more research work in this area of study. Again, very few studies are available on the presence of ESBLs Enterobacteriaceae in milk (Geser, Stephan, & Hächler, 2012; Hammad, Ahmed, Ishida, & Shimamoto, 2008). However, studies carried out in different countries around the world have isolated ESBLs Enterobacteriaceae in dairy cattle, livestock and vegetables (Girlich et al., 2007; Njage & Buys,

2015; Watson et al., 2012). ESBLs genes are plasmid- mediated and molecular detection of the genes is recommended as plasmids have been implicated in the spread of resistance. Rough *E. coli* strains in this study were noted to produce ESBLs. Even though rough strains are mostly non-pathogenic, this is a cause of concern since commensal *E. coli* are reported as reservoirs of antibiotic resistance genes and can transfer these genes, through lateral gene transfer, to the pathogenic strains (Straley et al., 2006). In the current study, 66.1% of *E. coli* were found to be resistant to cefpodoxime. Resistant to cefpodoxime has been reported to be mediated by a mutation in the attenuator or the weak promoter of the chromosomal *ampC β-lactamase* gene and this lead to enhanced production of AmpC, resulting in resistance to extended-spectrum cephalosporins (Caroff, Espaze, Berard, Richet, & Reynaud, 1999).

STEC seropathotypes with different combination of virulence genes and ability to cause haemolysis were identified (Table 2). Similar observations to our present study, on prevalence of STEC with different combinations of virulence genes, have been reported for STEC in milk (Altalhi & Hassan, 2009; Rey et al., 2006). Thirty one *E. coli* isolates in our study harboured either single or a combination of *stx1* and *stx2* genes and this was prominent in O157 serogroup. A total of 74.4% *E. coli* isolates in this study did not harbour the *stx* gene. However, other virulence genes, which were not tested for in the current study but known to cause diarrheagenic diseases, could be present in these isolates. A survey by Caine et al. (2014) on raw milk from two commercial dairy farms in SA did not detect *stx* (n=100) from *E. coli* isolates. However the authors detected *fliC*H7 gene (43%, n=100) used as an identification of *E. coli* O157:H7 serotype and also other virulence factors in *E. coli* associated with EPEC, EAEC, ETEC and UPEC. Therefore, to fully understand the risk posed to the public by consumption of PDBM, a comprehensive study on the virulence factors of diarrheagenic *E.coli* in recommended. Similar

distribution patterns of *stx* genes (*stx1* (19%) and *stx2* (20.8%) have been reported in studies on *E. coli* O157:H7 in milk and dairy cows (Hoffmann et al., 2014). STEC non-O157 serogroups (O2, O4, O9, O20, O43, O64, O68, O83, O85, O109, O112, O119, O147, O155) we reported were different in comparison to commonly isolated STEC non-O157 (O111, O26, O103, and O145) serogroups from foods such as milk in continental Europe (Eklund, Scheutz, & Siitonen, 2001). The dominant *stx* virulence gene in *E. coli* O157:H7 is *stx2* as reported by Khan et al. (2002) and this was also evident in our study. This high proportion of *stx2* in *E. coli* from PDBM poses an important risk for consumers since the gene is reported to produce the most virulent Shiga toxin for humans compared to *stx1* (Karch, Tarr, & Bielaszewska, 2005).

In the present study, 4.1% of the *E. coli* isolates were phenotypically haemolytic and the *hlyA* gene was only present in 2.4% of the isolates (Table 2). *E. coli*, in the current study, which exhibited haemolytic expression on blood agar could have carried other hemolysin genes such as *sheA*, *ehxA*, and *e-hlyA* (Kerényi et al., 2005*)*. We detected genotypic and phenotypic enterohaemolysin activity in non-O157 *E. coli* in our study. The presence of alpha-hemolysin gene is often a characteristic of *E. coli* belonging to serogroup O157 and is less frequently associated with STEC of other serogroups particularly noted in the present study.

A total of 3.3% *E. coli* isolates in the current study had a combination of ESBLs, multiple resistance to cephalosporins and aztreonam and *stx* virulence factors.

A review study by da Silva and Mendonça (2012) revealed an association between antibiotic resistance and virulence factors in *E. coli*. Rasko et al. (2011) reported that acquisition of genes in *E. coli* through lateral gene transfer can result in an accretion of synergistic antibiotic resistance and virulence factors. Bacteria subjected to antibiotic selection pressure can reduce genetic barriers between distantly related bacteria resulting in a rapid emergence of antibiotic

resistance and the import of virulence genes by lateral gene transfer (Wirth et al., 2006). Presence of pathogenic *E. coli* in this study, which was antibiotic resistant, is of considerable importance from the standpoint of public health since it reduces the effectiveness of medicines. Outbreaks caused by pathogenic *E. coli* in food/milk have been reported (CDC, 2005; EFSA, 2015).

In this study we adopted and modified a method by Coimbra et al. (2000) which integrated genotypic and phenotypic data for serotyping our *E. coli* isolates. The method demonstrated cost effectiveness and was less time intensive and improves the possibility of detecting the often minimal amount of variation between strains. Sizes of amplified *rfb* gene region fragments for the isolates which ranged from 0.2 – 2 kbp (Fig 2) were far lower than what was reported by Coimbra et al. (2000). This could be as a result of the plasticity of O-antigen genes in *E. coli* and more so, the diversity of the genetic pool in different geographical regions (Aydanian, Tang, Morris, Johnson, & Stine, 2011). Restriction digestion using *MboII* generated O-patterns with less than 30 band fragments. This is ideal even for visual inspection in case where computer software are not available (Collins & Ross, 1984). Han et al. (1991) reported that large numbers of DNA fragments generated by restriction endonucleases are difficult to interpret, especially when the bands are densely distributed and need complicated computer software. In our study, similarities and differences in O-patterns among different strains were clear and easily determined by a computer software (BioNumerics software version 6.0).

Cluster analysis of *E. coli* isolates, based on RFLP data (Fig 3), grouped some genetically different but phenotypically similar *E. coli* strains into unique clusters. *E. coli* can lose or gain genetic mobile elements through lateral gene transfer (da Silva & Mendonça, 2012). Some of the isolates in the unique clusters had been found to carry *stx* virulence genes. *E. coli* serotypes with

either *stx1* and/or *stx2* genes and different antibiotic profiles produced identical O-antigen RFLP patterns and cluster together. This is important for epidemiological and clinical purposes as variations between *E. coli* pathotypes can be detected (Khan et al., 2002). Conventional serotyping using serum agglutination assays poses problems of cross-reaction (i.e. reaction of one O-antigen with O-antisera developed against another O-antigen) between O-serogroups of *E. coli* (Harrigan & McCance, 2014) and the method we used is more reliable since the indistinctness is resolved. Unlike conventional serotyping, the method used in the current study, also uses genotypic in addition to the phenotypic characteristics. In our study, 25.6% (Fig 4) of *E. coli* isolates were untypeable using serum agglutination due to auto-agglutination with all the O-antisera used and were considered rough strains. Rough *E. coli* strains do not produce the O-antigen as a result of mutation within the *rfb* gene clusters (Coimbra et al., 2000). We did not detect *stx* or haemolysis activity from the rough strains. However, Menrath et al. (2010), Eklund et al. (2001) reported rough *E. coli* strains harbouring *stx 2*, EHEC-*hly A* and *eae* virulence factors and the strains were isolated from diseased humans with HUS and thrombotic-thrombocytopenic purpura (TTP).

Previous studies on *E. coli* in milk focused more on the eminent O157 serogroup (Bielaszewska et al., 1997; Keene et al., 1997; Upton & Coia, 1994). However, in the current study, attention was on both O157 and non-O157 serogroup in both raw and pasteurized PDBM. Non-O157 STEC are increasingly recognized as an important human pathogen, responsible for several human outbreaks (Jacob et al., 2013) and both the European Food Safety Authority (EFSA) and the U.S. Department of Agriculture (USDA) have issued recommendations for laboratory testing for these pathogens (Eblen, 2007; EFSA, 2007). Serogroups O9 and O157 were dominant among the 121 *E. coli* isolates in our study (Fig 4). We identified 15 different O-serotypes and these

results were similar to Zweifel et al. (2010) who observed 13 different O-serotypes of *E. coli* in raw milk. Similar serotypes found in the present study have previously been isolated from cattle and humans; of which 64.0% of the serotypes in the present study were commonly isolated from humans; which suggest cross contamination during the milk chain (Constantiniu, 2002).

In this study we clustered the *E. coli* isolates using phenotypic and genotypic data in a bid to understand relatedness of the isolates. Phenotypic clustering (based on antibiotic susceptibility data) showed no trend of the *E. coli* isolates clustering according to serogroup. We observed *E. coli* isolates within the clusters which had common antibiotic resistance patterns (Fig 5). This particular clustering can be as a result of localized selection pressure from the characteristic antimicrobial agents commonly used as per particular geographical province. (Parveen, Portier, Robinson, Edmiston, & Tamplin, 1999). Owing to the above explained phenomenon, antibiotic resistance profiles have been used in bacterial source tracking in different studies (Meays, Broersma, Nordin, & Mazumder, 2004; Scott, Rose, Jenkins, Farrah, & Lukasik, 2002). GTG_5-clustering (Fig 6) in our study showed a different trend among the *E. coli* isolates compared to the clustering we identified using phenotypic data. REP-cluster analysis of the *E. coli* isolates showed a particular trend with respect to serogroups. *E. coli* belonging to the same serogroups have the same DNA regions that are flanked by conserved sequence which generate similar genetic fingerprints when GTG_5 technique is employed (Nürnberg et al., 1989). Studies have shown that cluster analysis using rep- PCR fingerprints (GTG_5) groups *E. coli* isolates according to common ancestry (Aijuka, Charimba, Hugo, & Buys, 2014; Njage & Buys, 2015). *E. coli* isolates in the present study were genetically diverse and such degree of genetic diversity has been associated with versatility and genomic plasticity in *E. coli* from either food or the

environment (Van Elsas, Semenov, Costa, & Trevors, 2011). Cluster analysis of *E. coli* isolates using GTG_5 fingerprints revealed 7 groupings which were based on serogroups in some of the clusters (Fig 6) and this supported the earlier articulated clustering results from RFLP fingerprinting (Fig 3) which was used in serotyping in this study. Thus, REP-PCR finger prints can also be used in serotyping following the procedure outlined in this study. However, given that grouping of the same serotypes into different clusters and different serotypes into unique serogroups can also occur, use of GTG_5 fingerprinting requires caution.

5. Conclusion

The results obtained in the current study revealed a diversity of *E. coli* seropathotypes (with different shigatoxin virulence factors and ESBLs producing capacity) known to be associated with human diarrhoeal diseases in PDBM. Isolation of *E. coli* serotypes carrying virulence genes that are known to cause human diseases and are also resistant to antibiotics can present a significant public health risk, especially to the vulnerable members of the community. We detected serotypes in the present study that are commonly isolated from humans and this suggest cross contamination during the milk chain. This study highlights a potential risk posed on human health by consuming PDBM contaminated by pathogenic *E. coli*. We recommend further quantitative risk assessment of the impact on human health by pathogenic *E. coli* contamination in PDBM in order to formulate appropriate food safety objectives and adequate levels of protection that can manage the risk and meet public health goals.

Acknowledgements

The authors would like to thanks Milk South Africa for funding the project (Pretoria, Republic of South Africa).

Reference

Aijuka, M., Charimba, G., Hugo, C. J., & Buys, E. M. (2014). Characterization of bacterial pathogens in rural and urban irrigation water. *Journal of Water and Health, 13*, 103-117.

Allocati, N., Masulli, M., Alexeyev, M. F., & Di Ilio, C. (2013). Escherichia coli in Europe: An overview. *International Journal of Environmental Research and Public Health, 10*, 6235-6254.

Altalhi, A. D., & Hassan, S. A. (2009). Bacterial quality of raw milk investigated by Escherichia coli and isolates analysis for specific virulence-gene markers. *Food control, 20*, 913-917.

Aydanian, A., Tang, L., Morris, J. G., Johnson, J. A., & Stine, O. C. (2011). Genetic diversity of O-antigen biosynthesis regions in Vibrio cholerae. *Applied and Environmental Microbiology, 77*, 2247-2253.

Bej, A. K., DiCesare, J. L., Haff, L., & Atlas, R. M. (1991). Detection of Escherichia coli and Shigella spp. in water by using the polymerase chain reaction and gene probes for uid. *Applied and Environmental Microbiology, 57*, 1013-1017.

Bielaszewska, M., Janda, J., Blahova, K., Minarikova, H., Jikova, E., Karmali, M., Laubova, J., Šikulová, J., Preston, M., & Khakhria, R. (1997). Human Escherichia coli O157 [ratio] H7 infection associated with the consumption of unpasteurized goat's milk. *Epidemiology and Infection, 119*, 299-305.

Caine, L.-A., Nwodo, U. U., Okoh, A. I., Ndip, R. N., & Green, E. (2014). Occurrence of Virulence Genes Associated with Diarrheagenic Escherichia coli Isolated from Raw Cow's Milk from Two Commercial Dairy Farms in the Eastern Cape Province, South Africa. *International Journal of Environmental Research and Public Health, 11*, 11950-11963.

Caroff, N., Espaze, E., Berard, I., Richet, H., & Reynaud, A. (1999). Mutations in the ampC promoter of Escherichia coli isolates resistant to oxyiminocephalosporins without extended spectrum β-lactamase production. *FEMS Microbiology Letters, 173*, 459-465.

CDC. (2005). Escherichia coli O157:H7 Infection Associated with Drinking Raw Milk --- Washington and Oregon, November--December 2005.

CDC. (2013). Antibiotic Resistance Threats in the United States, 2013. http://www.cdc.gov/drugresistance/threat-report-2013/ (Accessed 13/11/2015)

Cebula, T. A., Payne, W. L., & Feng, P. (1995). Simultaneous identification of strains of Escherichia coli serotype O157: H7 and their Shiga-like toxin type by mismatch amplification mutation assay-multiplex PCR. *Journal of Clinical Microbiology*, *33*, 248-250.

CLSI. (2011). Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing, 21st informational supplement. CLSI document M100-S21. Clinical and Laboratory Standards Institute, Wayne, PA.

Coimbra, R. S., Grimont, F., Lenormand, P., Burguière, P., Beutin, L., & Grimont, P. A. (2000). Identification of Escherichia coli O-serogroups by restrictionof the amplified O-antigen gene cluster (rfb-RFLP). *Research in Microbiology, 151*, 639-654.

Collins, D., & Ross, D. (1984). Restriction endonuclease analysis of Campylobacter strains with particular reference to Campylobacter fetus ss. fetus. *Journal of Medical Microbiology, 18*, 117-124.

Constantiniu, S. (2002). Escherichia coli enterohemoragic–an emerged pathogen of human infections Part II. Non-o157 Escherichia coli enterohemorrhagic. *J. Prev. Med, 10*, 57-73.

da Silva, G. J., & Mendonça, N. (2012). Association between antimicrobial resistance and virulence in Escherichia coli. *Virulence, 3*, 18-28.

De Buyser, M.-L., Dufour, B., Maire, M., & Lafarge, V. (2001). Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *International Journal of Food Microbiology, 67*, 1-17.

Eblen, D. (2007). Public health importance of non-O157 Shiga toxin-producing Escherichia coli (non-O157 STEC) in the US food supply. *US Department of Agriculture, Food Safety and Inspection Service. Available at: http://www. fsis. usda. gov/PDF/STEC_, 101207*.

EFSA. (2007). Scientific opinion of the panel on biological hazards on a request from EFSA on monitoring of verotoxigenic Escherichia coli (STEC) and identification of human pathogenic types. *EFSA Journal, 579*, 1–61.

EFSA. (2015). European Food Safety Authority (EFSA). 2015. Scientific Opinion on the public health risks related to the consumption of raw drinking milk. *EFSA Journal 13, 3940*.

Eklund, M., Scheutz, F., & Siitonen, A. (2001). Clinical isolates of non-O157 shiga toxin-producingEscherichia coli: serotypes, virulence characteristics, and molecular profiles of strains of the same serotype. *Journal of Clinical Microbiology, 39*, 2829-2834.

Geser, N., Stephan, R., & Hächler, H. (2012). Occurrence and characteristics of extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae in food producing animals, minced meat and raw milk. *BMC veterinary research, 8*, 21.

Gillespie, I., O'Brien, S., Adak, G., Cheasty, T., & Willshaw, G. (2005). Foodborne general outbreaks of Shiga toxin-producing Escherichia coli O157 in England and Wales 1992-2002: where are the risks? *Epidemiology and Infection, 133*, 803-808.

Girlich, D., Poirel, L., Carattoli, A., Kempf, I., Lartigue, M.-F., Bertini, A., & Nordmann, P. (2007). Extended-spectrum β-lactamase CTX-M-1 in Escherichia coli isolates from healthy poultry in France. *Applied and Environmental Microbiology, 73*, 4681-4685.

Goldenberger, D., Perschil, I., Ritzler, M., & Altwegg, M. (1995). A simple" universal" DNA extraction procedure using SDS and proteinase K is compatible with direct PCR amplification. *Genome Research, 4*, 368-370.

Hammad, A. M., Ahmed, A. M., Ishida, Y., & Shimamoto, T. (2008). First characterization and emergence of SHV-60 in raw milk of a healthy cow in Japan. *Journal of Veterinary Medical Science, 70*, 1269-1272.

Han, N., Hoover, C., Winkler, J., Ng, C., & Armitage, G. (1991). Identification of genomic clonal types of Actinobacillus actinomycetemcomitans by restriction endonuclease analysis. *Journal of Clinical Microbiology, 29*, 1574-1578.

Harrigan, W. F., & McCance, M. E. (2014). *Laboratory methods in microbiology*: Academic Press.

Hoffmann, S. A., Pieretti, G. G., Fiorini, A., Patussi, E. V., Cardoso, R. F., & Mikcha, J. M. G. (2014). Shiga-Toxin Genes and Genetic Diversity of Escherichia coli Isolated from Pasteurized Cow Milk in Brazil. *Journal of Food Science, 79*, M1175-M1180.

Jacob, M., Foster, D., Rogers, A., Balcomb, C., Shi, X., & Nagaraja, T. (2013). Evidence of Non-O157 Shiga Toxin–Producing Escherichia coli in the Feces of Meat Goats at a US Slaughter Plant. *Journal of Food Protection®, 76*, 1626-1629.

Karch, H., Tarr, P. I., & Bielaszewska, M. (2005). Enterohaemorrhagic Escherichia coli in human medicine. *International Journal of Medical Microbiology, 295*, 405-418.

Karger, A., Ziller, M., Bettin, B., Mintel, B., Schares, S., & Geue, L. (2011). Determination of serotypes of Shiga toxin-producing Escherichia coli isolates by intact cell matrix-assisted

laser desorption ionization-time of flight mass spectrometry. *Applied and Environmental Microbiology*, *77*, 896-905.

Keene, W. E., Hedberg, K., Herriott, D. E., Hancock, D. D., McKay, R. W., Barrett, T. J., & Fleming, D. W. (1997). A prolonged outbreak of Escherichia coli O157: H7 infections caused by commercially distributed raw milk. *Journal of Infectious Diseases, 176*, 815-818.

Kerényi, M., Allison, H. E., Bátai, I., Sonnevend, A., Emödy, L., Plaveczky, N., & Pál, T. (2005). Occurrence of hlyA and sheA genes in extraintestinal Escherichia coli strains. *Journal of Clinical Microbiology, 43*, 2965-2968.

Khan, A., Das, S., Ramamurthy, T., Sikdar, A., Khanam, J., Yamasaki, S., Takeda, Y., & Nair, G. B. (2002). Antibiotic resistance, virulence gene, and molecular profiles of Shiga toxin-producing Escherichia coli isolates from diverse sources in Calcutta, India. *Journal of Clinical Microbiology, 40*, 2009-2015.

Lorenz, S. C., Son, I., Maounounen-Laasri, A., Lin, A., Fischer, M., & Kase, J. A. (2013). Prevalence of hemolysin genes and comparison of ehxA subtype patterns in Shiga toxin-producing Escherichia coli (STEC) and non-STEC strains from clinical, food, and animal sources. *Applied and Environmental Microbiology, 79*, 6301-6311.

Lues, J. F. R., de Beer, H., Jacoby, A., Jansen, K. E., & Shale, K. (2010). Microbial quality of milk, produced by small scale farmers in a peri-urban area in South Africa. *African Journal of Microbiology Research, 4*, 1823-1830.

Meays, C. L., Broersma, K., Nordin, R., & Mazumder, A. (2004). Source tracking fecal bacteria in water: a critical review of current methods. *Journal of Environmental Management, 73*, 71-79.

Menrath, A., Wieler, L. H., Heidemanns, K., Semmler, T., Fruth, A., & Kemper, N. (2010). Shiga toxin producing Escherichia coli: identification of non-O157: H7-Super-Shedding cows and related risk factors. *Gut pathogens, 2*, 1.

Njage, P. M., & Buys, E. M. (2015). Pathogenic and commensal Escherichia coli from irrigation water show potential in transmission of extended spectrum and AmpC β-lactamases determinants to isolates from lettuce. *Microbial biotechnology*.

Ntuli, V., Njage, P. M. K., & Buys, E. M. (2016). Characterization of Escherichia coli and other Enterobacteriaceae in producer-distributor bulk milk. *Journal of Dairy Science, 99*, 9534-9549.

Nürnberg, P., Roewer, L., Neitzel, H., Sperling, K., Pöpperl, A., Hundrieser, J., Pöche, H., Epplen, C., Zischler, H., & Epplen, J. (1989). DNA fingerprinting with the oligonucleotide probe (CAC) 5/(GTG) 5: somatic stability and germline mutations. *Human Genetics, 84*, 75-78.

O'Ferrall-Berndt, M. M. (2003). A comparison of selected public health criteria in milk from milk-shops and from a national distributor. *Journal of the South African Veterinary Association, 74*, 35-40.

Oliver, S. P., Jayarao, B. M., & Almeida, R. A. (2005). Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. *Foodbourne Pathogens & Disease, 2*, 115-129.

Orden, J., Ruiz-Santa-Quiteria, J., Garcia, S., & Cid, D. (2000). In vitro susceptibility of Escherichia coli strains isolated from diarrhoeic dairy calves to 15 antimicrobial agents. *Journal of Veterinary Medicine, Series B, 47*, 329-335.

Parveen, S., Portier, K. M., Robinson, K., Edmiston, L., & Tamplin, M. L. (1999). Discriminant analysis of ribotype profiles of Escherichia coli for differentiating human and nonhuman sources of fecal pollution. *Applied and Environmental Microbiology, 65*, 3142-3147.

Pitout, J., Reisbig, M., Venter, E., Church, D., & Hanson, N. (2003). Modification of the double-disk test for detection of Enterobacteriaceae producing extended-spectrum and AmpC β-lactamases. *Journal of Clinical Microbiology, 41*, 3933-3935.

Rawat, D., & Nair, D. (2010). Extended-spectrum ß-lactamases in gram negative bacteria. *Journal of global infectious diseases, 2*, 263.

Rey, J., Sánchez, S., Blanco, J., de Mendoza, J. H., de Mendoza, M. H., García, A., Gil, C., Tejero, N., Rubio, R., & Alonso, J. (2006). Prevalence, serotypes and virulence genes of Shiga toxin-producing Escherichia coli isolated from ovine and caprine milk and other dairy products in Spain. *International Journal of Food Microbiology, 107*, 212-217.

Schmidt, H., & Karch, H. (1996). Enterohemolytic phenotypes and genotypes of shiga toxin-producing Escherichia coli O111 strains from patients with diarrhea and hemolytic-uremic syndrome. *Journal of Clinical Microbiology, 34*, 2364-2367.

Scott, T. M., Rose, J. B., Jenkins, T. M., Farrah, S. R., & Lukasik, J. (2002). Microbial source tracking: current methodology and future directions. *Applied and Environmental Microbiology, 68*, 5796-5803.

Shah, A., Hasan, F., Ahmed, S., & Hameed, A. (2004). Extended-spectrum β-lactamases (ESBLs): characterization, epidemiology and detection. *Critical Reviews in Microbiology, 30*, 25-32.

Smet, A., Martel, A., Persoons, D., Dewulf, J., Heyndrickx, M., Catry, B., Herman, L., Haesebrouck, F., & Butaye, P. (2008). Diversity of extended-spectrum β-lactamases and class C β-lactamases among cloacal Escherichia coli isolates in Belgian broiler farms. *Antimicrobial agents and chemotherapy, 52*, 1238-1243.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A., Kämpfer, P., Maiden, M. C., Nesme, X., Rosselló-Mora, R., Swings, J., & Trüper, H. G. (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology, 52*, 1043-1047.

Straley, B., Donaldson, S., Hedge, N., Sawant, A., Srinivasan, V., Oliver, S., & Jayarao, B. (2006). Public health significance of antimicrobial-resistant gram-negative bacteria in raw bulk tank milk. *Foodbourne Pathogens & Disease, 3*, 222-233.

Upton, P., & Coia, J. (1994). Outbreak of Escherichia coli 0157 infection associated with pasteurised milk supply. *The Lancet, 344*, 1015.

Van Elsas, J. D., Semenov, A. V., Costa, R., & Trevors, J. T. (2011). Survival of Escherichia coli in the environment: fundamental and public health aspects. *The ISME. J, 5*, 173-183.

Versalovic, J., Schneider, M., De Bruijn, F. J., & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology, 5*, 25-40.

Wang, L., & Reeves, P. R. (1998). Organization of Escherichia coli O157 O antigen gene cluster and identification of its specific genes. *Infection and Immunity, 66*, 3545-3551.

Watson, E., Jeckel, S., Snow, L., Stubbs, R., Teale, C., Wearing, H., Horton, R., Toszeghy, M., Tearne, O., & Ellis-Iversen, J. (2012). Epidemiology of extended spectrum beta-lactamase E. coli (CTX-M-15) on a commercial dairy farm. *Veterinary Microbiology, 154*, 339-346.

Wilson, K. (2001). Preparation of genomic DNA from bacteria. *Current protocols in molecular biology*, 2.4. 1-2.4. 5.

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L. H., Karch, H., Reeves, P. R., Maiden, M. C., & Ochman, H. (2006). Sex and virulence in Escherichia coli: an evolutionary perspective. *Molecular Microbiology*, *60*, 1136-1151.

Zweifel, C., Giezendanner, N., Corti, S., Krause, G., Beutin, L., Danuser, J., & Stephan, R. (2010). Characteristics of Shiga Toxin–Producing Escherichia coli Isolated from Swiss Raw Milk Cheese within a 3-Year Monitoring Program. *Journal of Food Protection*, *73*, 88-91.