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Full Length Research Paper

Detection, isolation and molecular characterisation of Shigatoxigenic O157 and non-O157 *Escherichia coli* in raw and fermented camel milk

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Prevalence and distribution of *Escherichia coli* O157 and non-O157 Shiga toxin-producing *Escherichia coli* (STEC) in samples collected along raw and fermented camel milk marketing chain was assessed. A combination of culture media and immunomagnetic separation followed by typing for associated virulence factors and serotypes was performed. Thirty six percent (36%) of the isolates haboured either single or combinations of *stx*1, *stx*2 and *eae* with 77.7% being *stx*1-positive, 18.5% *eae*-positive, 3.1% *stx*1 and *stx*2- positive and 0.8% *stx*2 and *eae*-positive. The highest percentage (56.5%) of presumptive *E. coli* isolates was isolated from EMB agar while CHROM agar and CT-SMAC enabled the detection of 23 and 20.5% of isolates, respectively. However, 100, 38.6 and 12.3% of isolates from CT-SMAC, EMB agar and CHROMagar, respectively were found to be STEC. Serotypes O157, O111 and O113 represented 94, 2 and 4% of the STEC, respectively. A higher prevalence of STEC found in camel milk in the current study compared to milk samples in other countries from other animal species indicates that the milk could be an important vehicle for transmission of STEC to humans.

Key words: Shigatoxigenic, *Escherichia coli*, virulence factors, serotype, molecular typing, camel milk, Immunomagnetic separation.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are among the most important causes of foodborne diseases (Kaufmann et al., 2006). They cause illnesses ranging from mild diarrhea to more severe conditions such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Brett et al., 2003).

STEC strains that are pathogenic to humans have been shown to belong to a broad range of O serogroups (Orden et al., 2008). Serogroup O157:H7 has however been detected in many outbreaks and sporadic cases of HUS in the United States, Japan, and Europe. However, non-O157 STEC are becoming more recognized, for their contribution to HUS and HC (Brett et al., 2003). Serotype O157:H7 together with O111 are responsible for many of the serious cases (Paton and Paton, 1998). Serogroup O113 has been recognized as a major STEC associated with cases of HUS in South Australia (Paton and Paton, 1999) and attention to this serogroup in the disease diagnosis and in epidemiological studies has increased (Dos Santos et al., 2007). However, many laboratories historically screen for only serogroup O157 and diseases associated with non-O157 STEC have probably been underreported (Kaufmann et al., 2006).

Many clinical laboratories use Sorbitol-MacConkey agar-medium (SMAC) to identify phenotype O157:H7 owing to its slow sorbitol fermentation (Novicki et al., 2000). The introduction of cefixime and tellurite (CT) into SMAC has enhanced the rate and ease of isolation of *E. coli* O157 (Bennett et al., 1995). However, this medium does not detect other, sorbitol-positive STEC serotypes and even with supplementation, the isolation of false

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positives still remains a problem (Wallace and Jones, 1996; Novicki et al., 2000). CHROMagar® O157 has been designed to address this problem (Wallace and Jones, 1996). Using CHROMagar® O157, enterohaemorrhagic *E. coli* O157 can be easily isolated and detected by typical pink colonies. However, some other STEC have also been shown to produce similar colonies, while O113 and majority of other STEC strains are blue and not distinct from strains of *E. coli* lacking Shiga-like toxins (Bettelheim, 1998). A more optimal method for isolation of STEC would be to combine both supplemented SMAC and CHROMagar® O157 (Ogden et al., 2001). A specific trypticase sheep blood agar has also been recommended because many STEC isolates elaborate enterohemolysin which this agar detect but approximately 10% do not and are left out by this technique (Novicki et al., 2000).

Immunomagnetic separation (IMS) has been extensively utilized as an efficient method to detect O157 strains in numerous clinical and epidemiological cases. However, with many and significant number of HUS being attributed to non-O157 STEC serogroups, the use of additional diagnostic methods besides IMS has been recommended (Karch et al., 1996). Pre-enrichment in tryptic soy broth (TSB) prior to plating on Eosin methylene blue agar (EMB) has been used to successfully detect and isolate both O157 and non-O157 STEC serogroups (Adwan and Adwan, 2004; Taormina et al., 1998).

Pathogenic STEC strains characteristically express Shiga toxin 1 (Stx 1) and Shiga toxin 2 (Stx 2) (Neill, 1997). Other virulence factors may or may not be present but include, intimin, the factor responsible for intimate attachment to the intestinal surface and enterohemolysin for production of attaching and effacing lesions on intestinal mucosa encoded by the genes *eaeA* and *ehxA,* respectively (Orden et al., 2008; Erickson and Doyle, 2007). Various multiplex and single PCRs have allowed the detection of STEC associated gene sequences *stx1* and *stx2* (Novicki et al., 2000). Several immunoassay techniques have also been used for reliable detection of O157 lipopolysaccharide, Stx1, and Stx2 (Novicki et al., 2000).

However, the identification of O:H types or serotypes is also important for epidemiological purposes. Serotyping, however, has limitations by consuming a lot of time, requiring skills limited to reference laboratories and cannot also identify non-motile strains (Botelho et al., 2003). These non-motile strains have been extensively found among common and non-common STEC. Restriction analysis of the flagellin encoding gene *fliC* has been recently established as efficient for the typing of both motile and non-motile and both O157:H and non O157:H STEC strains (Botelho et al., 2003; Moreno et al., 2006). Food has been a major route for transmission of both O157:H7 and non- O157:H7 STEC to humans (Erickson and Doyle, 2007). Foods of bovine origin, particularly raw beef products and milk have been linked with food poisoning outbreaks in which this organism was identified as the causal agent (Vernozy-Rozand et al., 2005; Hussein and Bollinger, 2005). Rey et al. (2006) outlined a strong association between STEC virulence profile and the animal species from which these isolates were isolated. Cattle have been considered as major natural reservoirs of STEC, but other domestic animals, particularly ruminants have been implicated as well (Kaufmann et al., 2006). Geographic factors have also been found to be very important and in some cases more than specific management practices in influencing prevalence of STEC (LeJeune et al., 2006). In many countries, except for data from domestic ruminants few studies about STEC have been undertaken in other species, and no data exist for camels.

The objectives of our study were therefore to assess the prevalence and epidemiology of *E. coli* O157 and non-O157 STEC along the raw and fermented camel milk marketing chain and to determine serotypes and associated virulence factors in the isolated strains.

MATERIALS AND METHODS

Milk sample collection

A total of 70 samples consisting of raw camel milk or spontaneously fermented camel milk (*suusac*) were collected from Isiolo, Kenya, along the marketing chain at herd level, first collection point (3-6 h after milking) and from the final market in Nairobi (>24 h after milking). The fermented milk, called *suusac,* is made by leaving milk to ferment spontaneously without prior heat treatment at ambient temperature for about 24-48 h often in unhygienic containers (Lore et al., 2005). Thirty nine samples were collected at herd level (33 from individual animals and 6 from pooled milk), 20 from first collection point (3 from *suusac* and 17 from pooled milk) and 11 from final market (5 from *suusac* and 6 from pooled milk). Both traditional free ranging and semi-intensive camel milking herds were chosen for sampling. Samples were frozen in dry ice to keep them below 4°C and transported overnight to Zürich, Switzerland for analysis.

Detection and isolation of presumptive STEC

Ten milliliters of samples were added to 90 ml of Tryptic Soy Broth, (TSB) (Becton Dickinson AG, Allschwil, Switzerland) and homogenized. The homogenate was incubated overnight at 37°C as an *E. coli* enrichment-step. A portion of the TSB broth was spread on a plate of Eosin Methylene blue agar (EMB), (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and after overnight incubation at 37°C, at least 5 *E. coli*-like colonies were selected per plate. A portion of the pre-enriched homogenate was also used to carry out the automated IMS using a Bead-Retriever instrument (Thermo Fisher Scientific Oy, Vantaa, Finland).

One disposable sample tube strip was placed into a BeadRetriever rack for each sample to be processed. The dynabeads anti-*E. coli O157* (Invitrogen AG, Basel, Switzerland) were then re-suspended by vortexing until the pellet in the bottom disappeared. Suspended dynabeads were then mixed with sample and wash buffer composed of 0.15 M NaCl and 0.01 M Sodium-Phosphate buffer, pH 7.4, with 0.05% Tween-20. The filled tubes were then inserted into the sample racks and then sterile protective tip combs were inserted into the instrument. The IMS was then performed by running the EPEC/VTEC program. One half of the bead-bacteria complexes was plated onto Sorbitol- MacConkey

Table 1. Sequences of oligonucleotide primers used and predicted lengths of PCR amplification products.

Target gene	Primer Pair*	Oligonucleotide sequence ,5-3`	Product size (bp)	Reference
stx1	stx1R stx1F	AGA GCG ATG TTA CGG TTTG TTG CCC CCA GAG TGG ATG	180	Paton and Paton, 1998
stx2	stx2R stx2F	TGG GTT TTT CTT CGG TATC GAC ATT CTG GTT GAC TCT CTT	255	Paton and Paton, 1998
eae	ep1 ep2	AGG CTT CGT CAC AGT TG CCA TCG TCA CCA GAG GA	384	Paton and Paton, 1998
rfb O157	O157 F O157 R	CGG ACA TCC ATG TGA TAT GG TTG CCT ATG TAC AGC TAA TCC	259	Paton and Paton, 1998
rfb O113	O113 F O113 R	AGC GTT TCT GAC ATA TGG AGTG GTG TTA GTA TCA AAA GAG GCT CC	593	Paton and Paton, 1998
rfb O111	O111 F O111 R	TAG AGA AAT TAT CAA GTT AGT TCC ATA GTT ATG AAC ATC TTG TTT AGC	406	Paton and Paton, 1999
fliC	F-FLIC1 R-FLIC2	ATG GCA CAA GTC ATT AAT ACC CAA C CTA ACC CTG CAG CAG AGA CA	1130 - 2600	Moreno et al., 2006

*Primers were supplied by Microsynth, Balgach, Switzerland.

Table 2. *Escherichia coli* strains used as references for PCR amplifications.

Strain	Serotype	Pathotype	Reference
SW 479	O157: H19	Non-pathogen	Kohler et al., 2008
3750/2	O111: H 21	*stx-I*$^+$ *eae*$^+$	Kohler et al., 2008
STM 1	O174: H 21	*stx-II*$^+$	Kohler et al., 2008
4115/2	ONT: H45	*eae*$^+$	Kohler et al., 2008
STM 2	O113: H4	*stx-I*$^+$	Kohler et al., 2008
DSM 8702	O127:H-	Ns	DSMZ
DSM 1103	O6	*eae*$^+$	DSMZ
DSM 8699	O119:H-	Ns	DSMZ
DSM 8698	O111: H -	Ns	DSMZ

ns = not specified; DSMZ=German collection of microorganisms and cell cultures.

(SMAC) (Sigma-Aldrich Chemie, GmbH) agar containing CT-supplement (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and the other half on CHROMagar O157 (Invitrogen AG, Basel, Switzerland). To break-up the bacteria-bead complexes, they were spread over one half of the plate with a sterile swab and then further dilution was done by streaking a loop into the previously streaked quadrant several times to ensure that the beads reached a fresh unstreaked quadrant. The plates were then incubated at 37°C for 24 h after which colourless colonies on CT-SMAC agar and pink-mauve coloured colonies on CHROMagar O157 were selected as suspect *E. coli* O157. Bacterial glycerol stocks were prepared and preserved in brain heart infusion broth (Biolife, Milan, Italy) containing 20% glycerol (Sigma-Aldrich Chemie GmbH) at -80°C for use in subsequent experiments.

Molecular typing of isolates

Multiplex PCR for stx1, stx2 and eae

The glycerol stock isolates were streaked on blood agar plates (Oxoid AG, Pratteln, Switzerland). A loopful of the colonies was suspended in 0.5 ml of sterile water and heated at 95°C for 10 min. Centrifugation was then carried out at 5,000 rpm for 5 min at 4°C. The DNA-containing supernatant was used as the source of template for further amplification. The detection of *stx*1, *stx*2 and *eae* was performed using multiplex PCR step as previously described by China et al. (1996) with modifications. Each 25 µL PCR mixture was prepared using 9 ml of double distilled water, 2 µL of template DNA, 0.25 µL of each primer (100 µM) (Table 1) and

Table 3. Virulence factor profiles of 361 *E. coli* isolates from raw and naturally fermented camel milk at different points along the marketing chain in Kenya.

Gene	Source						Total
	Herd (89)		Isiolo (193)		Nairobi (79)		
	Single	Pooled	*Suusac*	Milk	*Suusac*	Milk	
*stx*1+	12	14	8	45	10	12	101
*stx*2+	-	-	-	-	-	-	-
eae+	2	-	3	8	5	6	24
*stx*1+*stx*2	1	-	-	1	2	-	4
*stx*2+*eae*	-	-	-	1	-	-	1
Total	15	14	11	55	17	18	130

-not detected; figures in brackets indicate number of isolates; pooled = pooled milk from more than one camel; single = milk from one camel.

12.5 µL of 2x PCR mastermix (Fermentas GmbH, Le Mont-sur-Lausanne, Switzerland) composed of 0.05 U/µl *Taq* DNA polymerase, reaction buffer, 4 mM $MgCl_2$ and 0.4 mM of each dNTP (dATP, dCTP, dGTP, dTTP). DNA from characteristic control strains were included in every batch sample consisting of *E. coli* strains (Table 2). Amplified products were visualized by electrophoresis in 1.5% agarose gel (Sysmex Digitana AG, Horgen, Switzerland) stained with ethidium bromide (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland).

Multiplex PCR for rfb O157, O113 and O111

Second multiplex PCR targeting O-antigen biosynthetic genes *rfb* O157, O113 and O111 was performed on all *stx* and *eae* positive samples using the primers shown in Table 2 and the protocol by Paton and Paton (1998).

fliC gene PCR–RFLP

The whole coding sequence of *fliC* gene was amplified using primers shown in Table 2 and the protocol developed by Botelho et al. (2003) with modifications. The PCR product was purified using QIAamp DNA minikits (QIAGEN AG, Basel, Switzerland). The digestion mixture contained 0.2 µL *Rsa*I restriction enzyme (BioConcept, Allschwil, Switzerland), 2.5 µL of 10x buffer (BioConcept, Allschwil, Switzerland), 7.3 µL double distilled water and 19 µL of the purified PCR product (with approximately 50-200 ng of DNA). Digestion was done at 37°C for 180 min. Restricted DNA fragments were visualized with ethidium bromide after electrophoresis in 3.0% agarose.

The resulting fingerprints were analyzed using the GelCompar II version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium) software package. The similarity among digitized profiles was calculated using the Pearson correlation, and an average linkage unweighted pair group method with arithmetic averages (UPGMA) dendrogram was derived from the profiles.

RESULTS AND DISCUSSION

The prevalence and distribution of *E. coli* O157 and non-O157 STEC associated with camel milk was studied in 70 samples (62 raw and 8 fermented) collected along the marketing chain.

Thirty six percent of the isolates haboured either single or combinations of factors *stx1*, *stx2* and *eae*. Prevalence of isolates positive for the virulence factors increased from 25.06% at herd level to 26.3% in first market and 34.01% in the final market (Table 3). Varying prevalence of STEC in milk from different species has been reported by others. These include: 16% STEC isolated from healthy goats in Spain (Cortés et al., 2005); one *eae* positive *E. coli* O157 from raw cow's milk cheese and 0.63% from ovine pasteurised milk in a national survey in Italy (Conedera et al., 2004); 40% in fresh milk from small-holder dairy farms in Nigeria (Waziri et al., 2010); 33.5% in raw milk from Malaysia (Chye et al., 2004); 4% from cattle herds in a common European and North American study (Coia et al., 2001); 3.3% of milk samples from Brazil (Vicente et al., 2005) and 0.75- 16.2% in various dairy products (Hussein and Sakuma, 2005). In Kenya, Arimi et al. (2005) reported prevalence in *E. coli* O157:H7 of 0.8% with two positive samples one of which produced *stx 1*. However, majority of the studies reviewed reported no STEC in different milk related samples (Conedera et al., 2004; Cortés et al., 2005; Coia et al., 2001). This might be due to proper hygienic milking practices, thereby preventing milk contamination, or too low counts of bacteria for detection with the methods applied in these studies. Our results therefore indicate a higher prevalence level than in most other cases and this indicates that this milk could be an important vehicle for transmission of STEC to humans.

In the current study, 77.7% of the STEC were *stx1*-positive, 18.5% *eae*-positive, 3.1% *stx1*- and *stx2*-positive and 0.8% *stx2*- and *eae*-positive (Table 3). Development of severe disease from infection with STEC is associated with the virulence profile of the particular strain (Erickson and Doyle, 2007). The virulence profile of STEC has also been closely associated with animal species from which STEC were isolated. For example *stx*2 has been found in all sheep strains from western Spain whereas goat STEC carried both the *stx*1 and *eae* genes, beef samples from India had only *stx*1 and either *stx*2 or both *stx*1 and *stx*2 were found in seafood isolates (Erickson and Doyle, 2007). Amongst the isolates harboring more than one

Table 4. Virulence factor detection patterns using either CHROMagar, EMB agar or CT-SMAC for 361 *E. coli* isolates cultured from raw and naturally fermented camel milk.

Media	*stx*1	Gene			Total
		*stx*1+*stx*2	*stx*2+*eae*	*eae*	
CHROMagar (83)	25	4	0	3	32
EMB (204)	22	0	0	2	24
CT-SMAC (74)	54	0	1	19	74
Total	101	4	1	24	131

Figures in brackets indicate number of isolates.

Table 5. Serotypes of STEC strains isolated from raw and naturally fermented camel milk at different points along the marketing chain in Kenya.

Serotype	Source						Total
	Herd		First collection		Final market		
	Pooled[a]	Single [b]	*Suusac*	Pooled	Suusac	Pooled	
O157	nd[c]	4	9	17	6	13	49
O111	nd	nd	nd	1	nd	nd	1
O113	nd	nd	nd	2	nd	nd	2

[a] Mixed milk from more than 1 camel, [b] milk from single camel, [c] serotype not detected.

virulence factor milk from the first collection point had one isolate having *stx1 + stx2* and one isolate *stx2 + eae*. Of the four isolates with both *stx1* and *stx2*, included two isolates in *suusac* from the final market. The pathogenicity of STEC has been found to vary depending on whether they possess single or both genes (Erickson and Doyle, 2007).

The highest percentage of presumptive *E. coli* isolates, 56.51%, was isolated from EMB agar while CHROMagar and CT-SMAC enabled the detection of almost equal number (22.99 and 20.5% respectively) of presumptive isolates. However, of the isolates harboring virulence genes, 100, 38.6 and 12.3% of isolates from CT-SMAC agar, EMB agar and CHROMagar, respectively were positive (Table 4). This can be explained by the fact that IMS, which increases sensitivity in O157 detection, was not used prior to plating on EMB and also CT-SMAC agar is more specific for detecting 0157 strains. However, because a significant number of STEC belong to non-O157 serogroups, other studies have also suggested the use of such additional methods besides IMS in the detection of STEC (Karch et al., 1996).

Addition of tellurite to SMAC reduces the back-ground flora therefore making both the observation and growth of *E. coli* clearer. *E. coli* O157 is not sensitive to tellurite and therefore, CT-SMAC gives excellent results (Ogden et al., 2001; Bettelheim, 1998). Our results contrast with those by Ogden et al. (2001) who concluded that CHROMagar was equal in performance with CT-SMAC. Studies have also shown that whereas STEC can be readily isolated and noticed by typical pink colonies, some other STEC also produced pink colonies in CHROMagar and in addition to O113, many other STEC strains showed blue colour and are hard to differentiate from Shiga-like toxin-negative strains of *E. coli* (Bettelheim, 1998). The use of a combination of CT-SMAC and CHROMagar therefore enhanced detection of STEC. Ogden et al., (2001) also similarly optimised detection of STEC using combination of CT-SMAC and either Rainbow or CHROMagar. Serotype O157 predominated amongst O157, O111 and O113 with 94% (49), 2% (1) and 4% (2) respectively detected by PCR. Moreover, O111 and O113 were only detected in raw milk from the first market. Prevalence of the *E. coli* O157 was 38.2, 50.0 and 11.8% in milk from the final market, first collection point, and herd level, respectively (Table 5). In *suusac*, prevalence of the *E. coli* O157 was 60.0% and 40.0% from the first collection point and final market, respectively. PCR enabled the detection of serogroups other than O157 including O113 and O111 though O157 (94%) predominated amongst the other serogroups.

Few studies were found comparing prevalence of O157 with other serogroups. However, the prevalence of O157 compared to other serotypes in our study was much higher than that already reported. In other studies, O157:H7 serotype was isolated from 1 of 39 (0.3 %) bulk tank where 9 STEC strains (O27:H18, O45:H38, O76:H19, O91:H28, O157:H7, ONT:H7, ONT:H9 and ONT:H21) were identified in ovine and caprine milks (Rey et al., 2006). Studies on goat milk showed most prevalent serotypes as O5:H-, O76:H19, O126:H8, O146:H21, ONT:H- and ONT:H21 and no O157:H7 was detected in bulk milk samples (Cortés et al., 2005).

Analysis of RFLP profiles of the *fliC* gene using

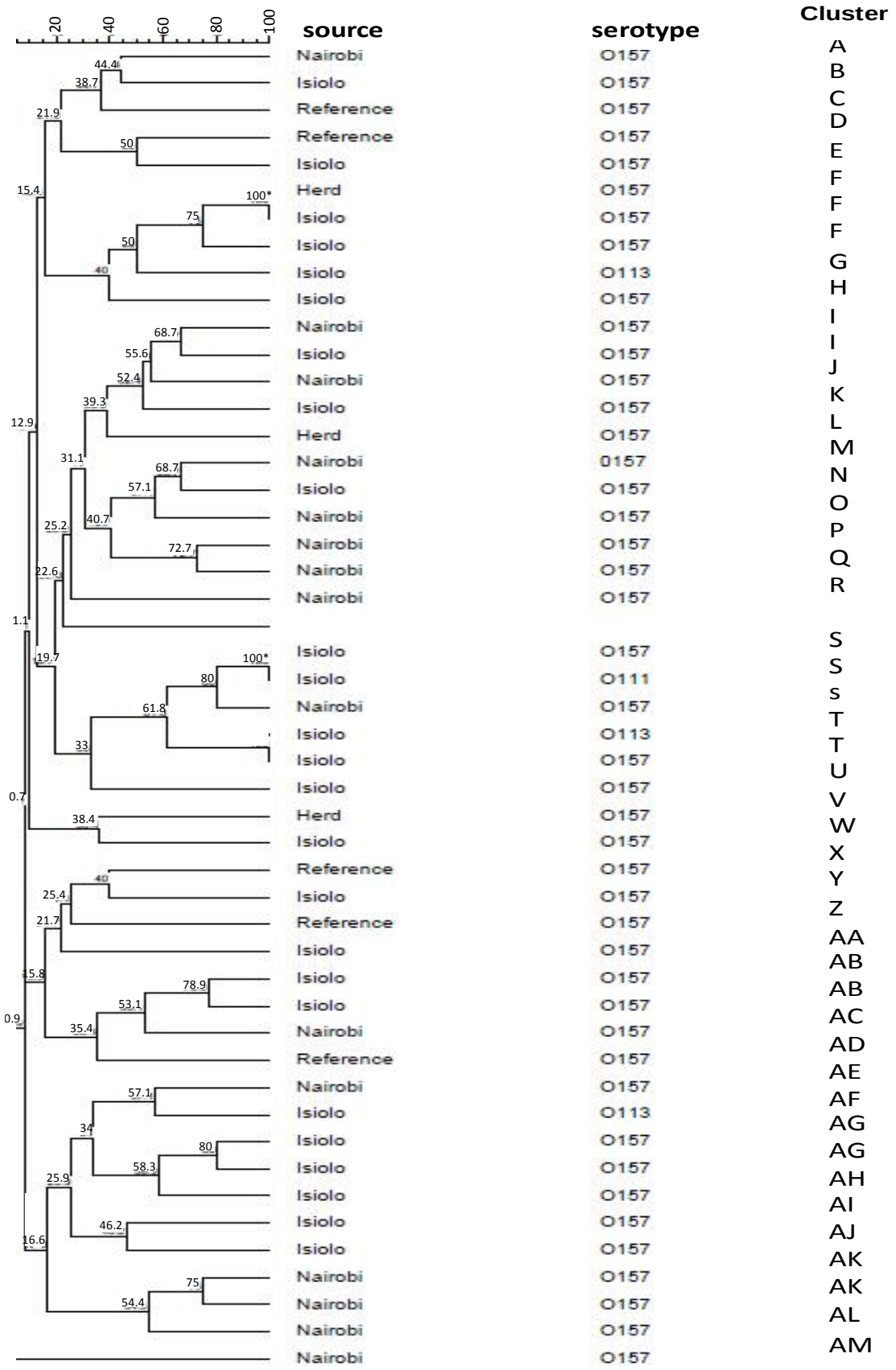

Figure 1. Restriction endonuclease digestion profiles of 43 STEC isolates from raw and fermented camel milk and 5 reference strains. Similar clusters were identified by at least 75% similarity.

GelCompar software revealed 39 different restriction endonuclease digestion profiles (REDPs) with greater than 75% similarity in banding patterns. RFLP analysis revealed 32 different REDPs amongst the serogroups O157 (Figure 1). These O157 REDPs were distributed among samples from all the sources. The highest diversity of O157 REDPs was 33 and 16.6% in milk from first collection point and *suusac* from final market, respectively. Serogroup O113 was detected only in pooled milk from the first collection point but with different REDPs. There were 7 clusters F, I, S, T, AB, AG and AK with common profiles bearing at least 75% similarity. Isolates with common profile F were collected from herd and first collection center in both milk and *suusac*, respectively. Strains with profile I were found in *suusac* from both first collection point and final market. Profile S was found both in milk and *suusac* from first collection point and final market. Profiles T and AG were found in both *suusac* and milk from first collection point. Profiles AB and AK were found in pooled milk from first collection point and final market, respectively.

Similar results have been found in different herds and farms in other studies (Zweifel et al., 2004; Schouten et al., 2005). Such profiles could also demonstrate the probability of either the persistence of strains (Schouten et al., 2005) or the contamination and spread of isolates (Conedera et al., 2001). PFGE analysis has also been used to show that a single animal might harbour O157:H7 strains that have different *Xba*I restriction endonuclease digestion profiles (Faith et al., 1996).

Some of the isolates detected as STEC in this study were from spontaneously fermented milk. Lack of a strong starter culture increases fermentation times leading to contamination with pathogenic and toxigenic bacteria and moulds, amongst other undesirable changes in the milk. Moreover, such pathogens have been found to grow faster than lactic acid bacteria and even shown to grow faster when cultured without lactic acid bacteria especially in mastitic milk (Fang et al., 1993).

Conclusions

The presence of STEC in camel milk in much higher levels than has been previously reported in Kenya calls for intervention especially because camel milk is consumed unpasteurised either raw or as *suusac* with the later being produced from camel milk by spontaneous fermentation at ambient environmental temperatures. The presence of STEC serotype O157 and non-O157 in camel milk and *suusac* emphasizes the importance of other serotypes in environmental and food sources. The distribution patterns of the serotypes as shown by RFLP patterns indicate both continued contamination and also persistence throughout the marketing chain of STEC strains. This calls for hygiene and animal health based interventions to be considered at all levels in this and similar milk marketing chains. Immuno-magnetic separation increases sensitivity in O157 detection and the use of a combination of CT-SMAC agar and CHROMagar further enhances the detection. However, a number of STEC belong to non-O157 serogroups and therefore plating on other general STEC media such as EMB will additionally enable their detection.

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