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Full Length Research Paper

Ampicillin resistance and extended spectrum β-lactamases in *Enterobacteriaceae* isolated from raw and spontaneously fermented camel milk

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The prevalence of ampicillin resistance and extended-spectrum β-lactamases (ESBL) in the dominant *Enterobacteriaceae* from raw and spontaneously fermented camel milk (*suusac*) in Kenya and Somalia was characterized both phenotypically and genotypically. Globally important SHV and CTX-M-type extended-spectrum β–lactamases (ESBLs) were tested. The *Enterobacteriaceae* belonged to 15 species from 10 genera. Dominant isolates were *Escherichia coli* (50), *Klebsiella pneumonia* subsp. *pneumoniae* (35) and *Enterobacter sakazakii* (20). *Salmonella arizonae*, *Serratia odorifera* and *E. coli* occurred at viable counts greater than 8 log cfu/ml. ESBL was studied for 96 *E. coli*, *K. pneumonia* subsp. *pneumoniae* and *E. sakazakii*. Total of 61 (63%) isolates consisting of 46 (48%) of *E. coli*, 45 (46%) *K. pneumonia* subsp. *Pneumonia* and 16 (7%) *E. sakazakii* were resistant to ampicillin. bla_{SHV}, $bla_{CTX\text{-}M\text{-}3}$-like and $bla_{CTX\text{-}M\text{-}14}$-like genes were detected in 37 (60%), 25 (40%) and 11 (18%) of the *Enterobacteriaceae* isolates respectively. *K. pneumonia* subsp. *pneumoniae* harbored majority of these *bla* genes (74%) with 1 strain possessing all 3 genes and 13 harbouring both bla_{SHV} and $bla_{CTX\text{-}M\text{-}3}$-like genes. The diversity of *Enterobacteriaceae* in camel milk calls for improved handling of camel milk. The ESBL genes in the isolates from the remote semi-arid regions emphasises the global antimicrobial resistance problem among *Enterobacteriaceae*.

Key words: Extended spectrum β-lactamase, *Enterobacteriaceae*, *Enterobacter sakazakii*, *Escherichia coli*, *Klebsiella pneumonia* subsp. *pneumonia*, camel milk.

INTRODUCTION

In Gram-negative pathogens, β-lactamase production is the most important factor complicating the treatment of nosocomial infections. β lactamases are bacterial enzymes that inactivates β-lactam antibiotics (ABs) by hydrolysis, resulting in ineffective compounds even for drugs of choice in clinical AB therapy (Shah et al., 2004; Moubareck et al., 2007). Extended-spectrum or third-generation cephalosporins have been designed towards overcoming this problem. However, some *Enterobacteriaceae* are able to produce mutant forms of the "older" β -lactamases referred to as extended-spectrum β–lactamases (ESBLs) which are capable of hydrolyzing the new-generation cephalosporins and aztreonam (Wiegand et al., 2007). The transferability of ESBLs encoding genes between various species of *Enterobacteriaceae* has contributed to a critical AB resistance situation in clinical treatments regarding global infection control issues (Shah et al., 2004).

In *Enterobacteriaceae*, a significant portion of antimicrobial resistance genes present on plasmids and transposons can also occur in integrons which play a

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Table 1. Susceptibility profiles of *Enterobacteriaceae* reference strains used in this study to ampicillin and extended-spectrum cephalosporins.

Strain	Antibiotic resistance profile[a]			
	Ampicillin	**Cefotaxime**	**Ceftriaxone**	**Ceftazidime**
K. pneumoniae ATCC 700603	Resistant	Sensitive	Intermediate resistance	Resistant
E.coli 713901	Resistant	Resistant	Resistant	Intermediate Resistant
E. coli ATCC 25922	Intermediate susceptible	Sensitive	Sensitive	Sensitive

[a]Clinical and Laboratory Standards Institute (2008).

particularly important role in the spread of multidrug resistance (Wang et al., 2008). Resistance to sulfamethoxazole, cotrimoxazole, gentamicin, tobramycin, ampicillin, piperacillin, and cefuroxime has been shown to predict the presence of such integrons (Leverstein-van Hall et al., 2003). Globally, TEM-type, SHV-type ESBLs and CTX-M-type ESBLs are the most prevalent ESBLs in *Enterobacteriaceae* (Dallenne et al., 2010).

Klebsiella pneumoniae, *Escherichia coli* and *Enterobacter sakazakii* remains the major ESBL-producing organisms isolated worldwide (Malik et al., 2005; Shah et al., 2004). ESBL-producing *Klebsiella* spp. and *E. coli* are now listed among the six drug-resistant microbes to which new therapies are urgently needed (Shah et al., 2004). Approximately 20% of *K. pneumoniae* infections and 31% of *Enterobacter* spp. infections in intensive care units in the United States are now caused by strains not susceptible to third-generation cephalosporins (Paterson, 2006).

Despite the fact that ESBL-producing *K. pneumoniae*, *E. sakazakii* and *E. coli* are now common in healthcare settings, ESBL-producing *Enterobacteriaceae* have now emerged in the community as well. Spreading of AB resistant bacteria among different environments, even in the absence of selective AB pressure, contributes to the importance of studying AB resistance dissemination among *Enterobacteriaceae* (Malik et al., 2005).

As the use of antibiotic became common in human medicine and animal food production, selective pressure has led to the maintenance of resistance genes in many groups of bacteria, and bacterial evolution has included mechanisms to retain, accumulate, and disperse resistance genes among bacterial populations (Mathew et al., 2007). Food, especially raw animal products like meat and milk are an important factor contributing to the spread of pathogens and AB resistant bacteria (Teuber et al., 1999). Camel milk, a non-industrial product consumed either fermented or fresh, has played an important role in the nutrition of the population in arid and remote zones of East African countries. The spontaneously fermented camel milk is known as *suusac*. However, reports on ESBL producing *Enterobacteriaceae* originating from foods are scarce and not available from camel milk. The aim of this study was therefore to determine the prevalence of ESBL in *Enterobacteriaceae* isolated from fresh and fermented camel milk. Additionally, ampicillin resistance was tested as a predictor of the presence of integrons mediating the spread of multi-drug resistance (Wang et al., 2008).

MATERIALS AND METHODS

Camel milk and milk product samples

A total of 105 samples were collected and analysed. Raw camel milk was sampled along distributing chains in Nanyuki and Isiolo, Kenya, at herd level as individual camel milk and pooled milk, first collection point and from the final market in Nairobi. Fully or partially fermented *suusac* was collected from Isiolo, Nanyuki, Mandera and Garissa in Kenya and Burco and Garowe in Somalia. Samples were frozen in dry ice to keep them below 4°C and transported to the laboratory within 8 h after collection.

Isolation and enumeration of *Enterobacteriaceae*

Appropriate dilutions of samples were spread in duplicate on Violet Red Bile Dextrose (VRBD) agar (VWR International AG, Dietikon, Switzerland) and incubated for 24 h at 37°C. Viable counts were enumerated and three colonies per morpho-type were selected and purified by repetitive streaking on fresh agar plates. A total of 160 presumptive *Enterobacteriaceae* were isolated and transported frozen to ETH Zurich. Initial characterization was done by catalase test (3% H_2O_2, VWR International), Gram-staining reactions (3% KOH, Sigma-Aldrich) and verification of purity was carried out by microscopic examination. Isolates were then preserved in Brain Heart Infusion (BHI) broth (Biolife, Italiana S.r.l) containing 30% glycerol (Sigma-Aldrich Chemie GmbH) at -80°C for use in subsequent experimentation.

Enterobacteriaceae reference strains

Isolates were analysed and data profiles compared with reference strains recommended by Standard Unit, Evaluations and Standards Laboratory (2008). ESBL-producing strains *Klebsiella pneumoniae* subsp. *Pneumonia* ATCC 700603 and *Escherichia coli* GSBL 713901 and ESBL-negative strain *Escherichia coli* ATCC 25922 (DSM 1103) were used. Table 1 shows their susceptibility patterns to various ABs used in this study.

Identification of isolates

Initial identification of isolates from VRBD-medium was performed

after inoculation in API 20E test-kits (bioMerieux, Geneva, Switzerland) followed by comparison of the generated numerical profile with the API 20E Analytical Profile Index.

Phenotypic antibiotic resistance tests

Total of 96 *Enterobacteriaceae* composed of 16 *E. sakazakii*, 46 *E. coli* and 34 *K. pneumonia* subsp. *pneumoniae* isolates were further tested for AB susceptibility by disc diffusion technique, ampicillin susceptibility test, extended-spectrum cephalosporins susceptibility test and double disc diffusion test.

Disc diffusion technique

A single colony of the test organism was picked up with a disposable loop and emulsified in 5 ml of saline solution (0.85% NaCl) to match the turbidity of 0.5 McFarland's Standard (bioMerieux, Geneva, Switzerland). This suspension was then spread on the surface of duplicate Mueller-Hinton (MH) agar medium using a cotton swab and the appropriate test disc placed on the agar surface.

Ampicillin and extended-spectrum cephalosporins susceptibility test

Screening for ESBL production was done as proposed by Thomson et al. (1996). This is a preliminary screening so that all *Enterobacteriaceae* presenting a decreased inhibition zone in disc diffusion test of ≤ 30 mm for at least one extended-spectrum cephalosporin (ceftazidime, cefotaxime, or ceftriaxone) or aztreonam can be selected for further ESBL production testing (Pitout et al., 1997).

As a pre-requisite, ampicillin susceptibility was tested by placing a 10 µg ampicillin disc (bioMerieux, Geneva, Switzerland) on MH agar plate for each of the tested *Enterobacteriaceae*. Discs with 30 µg ceftriaxone (OXOID), 30 µg cefotaxime (BioMerieux) and 30 µg ceftazidime (BioMerieux), were placed on MH agar plates, inoculated as described in the disc diffusion technique and incubated at 37°C for 20 h. The inhibition zone around the disc was then measured and compared to interpretative diameters according to Clinical and Laboratory Standards Institute (2008).

Double disc diffusion test

The isolates positive in the extended-spectrum cephalosporin (Ampicillin and extended-spectrum cephalosporins susceptibility test*)* test were subjected to further testing using double disc diffusion test. This ESBL detection test shows through the hydrolyzation of one or more 3rd generation cephalosporin and aztreonam, the presence of a clavulanate sensitive enzyme (Thomson et al., 1996). This test was performed as proposed by Jarlier et al. (1988) with cell density standardisation as described in the Disc diffusion technique.

Simplex polymerase chain reaction (PCR) for the detection of SHV- and CTX-M β-lactamases

Three simplex PCRs were used to detect the bla_{SHV}, $bla_{CTX\text{-}M\text{-}3}$-like and $bla_{CTX\text{-}M14}$-like genes using primers described by Chia et al. (2005). DNA of *Enterobacteriaceae* was isolated as described by Goldenberger et al. (1995). The 25 µl PCR reaction mixture contained 12.5 µl of 2X Master Mix, 0.15 µl of each primer pair of 100 µM, 11.2 µl of double distilled water and 1 µl of DNA template (10-50 ng). Initial denaturation was done at 94°C for 2 min. This was followed by 35 times denaturation at 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 1 min. Final extension was also done at 72°C for 10 min. PCR products were separated using gel electrophoresis in 3% agarose with subsequent ethidium-bromide staining prior to documentation of the gel.

RESULTS AND DISCUSSION

With recent findings of ESBL-producing *Enterobacteriaceae* in foods like meat, fish, and raw milk, assessment of animal foods as reservoirs and sources of such strains into the food production chain has been recommended (Geser et al., 2011). Fresh and fermented camel milk supply chains were therefore analysed for occurrence of ESBL in *Enterobacteriaceae*.

This bacterial family was below the detection limit of 10 colony forming units (cfu) at milking and first collection points, but was present at 10^4- 10^6 cfu/ml in final market in raw camel milk and at 10^3-10^7cfu/ml in *suusac*. These findings concur with those of Kongo et al. (2008) who reported between 5.9 and 7.0 log cfu/ml *Enterobacteriaceae* in cow milk used for manufacture of a traditional Portuguese raw milk cheese. *Enterobacteriaceae* counts ranging from 2.9-6.57 log cfu/ml were determined in cow milk from various points between milking and sale in Mali (Bonfoh et al., 2003). Udder infection, water quality, hygiene behaviour in relation to hand washing, cleaning and disinfection of containers are key areas that contribute to such contaminations in non-industrialised milk production (Bonfoh et al., 2006).

Total of 123 isolates were classified into 15 species belonging to 10 genera with a predominance of *E. coli* (Table 2). Similarly, a high species variety among *Enterobacteriaceae* such as *K. oxytoca, K. sakazakii, K. pneumonia* subsp. *pneumoniae, E. coli*, *K. ornithinolytica,* and *S. odorifera* were also reported in raw milk used for the manufacture of a traditional Portuguese raw cow milk cheese with *E. sakazakii* and *K. pneumonia* subsp. *pneumoniae* among the dominant species (Kongo et al., 2008). It was of concern that the most prevalent *Enterobacteriaceae* in our study namely *K. pneumoniae*, *E. coli* and *E. sakazakii* have been implicated as the major ESBL-producing *Enterobacteriaceae* worldwide (Malik et al., 2005).

The *Enterobacteriaceae* isolates were first screened for resistance to ampicillin as starting point for multi-drug resistance tests in order to predict the presence of integrons. Amongst the 96 isolates tested, 61 (63.5%) were resistant to ampicillin, 12 (12.5%) intermediate susceptible and 23 (24%) susceptible. Amongst the resistant isolates, 46 (47.5%) were *E. coli*, 45 (45.9%) *K. pneumonia* subsp*. Pneumonia* and 16 (6.6%) *E. sakazakii*. Twelve isolates including 6 *K. pneumonia* subsp. *pneumoniae* (50%), 5 *E. sakazakii* (41.7%) and 1 *E. coli* (8.3%) were intermediate susceptible to ampicillin. Only 23 (24%) of the isolates were susceptible to

Table 2. Identity, prevalence and viable counts of *Enterobacteriaceae* (n = 160) isolated from raw camel milk and *suusac.*

Species	No. of Isolates	Log cfu/ml[a]	
		Mean	Range
Escherichia coli	50	8.0	2.5 - 8.7
Klebsiella pneum. pneumomiae	35	6.5	3.7 - 7.0
Enterobacter sakazakii	20	7.5	2.4 - 7.9
Acinetobacter spp.	3	4.8	4.5 - 5.0
Kluyvera spp	2	5.2	Nc
Leklercia adecarboxylica	2	2.5	Nc
Serratia ficaria	2	4.8	4.5 - 4.9
Serratia odorifera	2	8.4	4.6 - 8.7
Enterobacter aerogenes	1	4.9	Nc
Enterobacter agglomerans	1	4.7	Nc
Klebsiella ornitholytica	1	6.5	Nc
Klebsiella oxytoca	1	5.4	Nc
Pseudomonas flourescence/ putida	1	5.4	Nc
Salmonella arizonae	1	8.6	Nc
Achromobacter spp.	1	4.9	Nc

[a]Nc = not calculated.

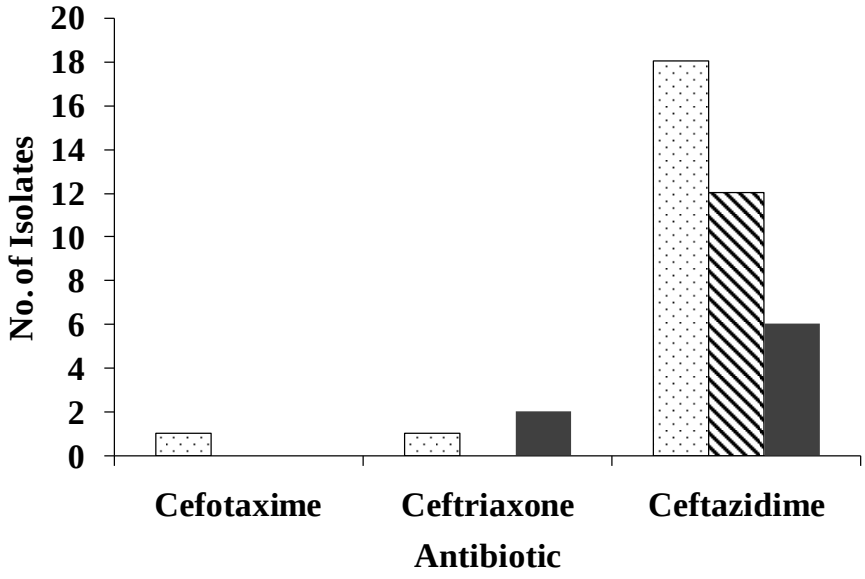

Figure 1. Presumptive ESBL resistance patterns for *E. coli*, *K. pneumoniae* and *E. sakazakii* with <30 mm diameter zone size for extended-spectrum cephalosporins.

ampicillin with 16 (70%) of them *E. coli* and none of the *K. pneumoniae* subsp. *pneumoniae*.

A total of 40 *Enterobacteriaceae* isolates among 96 tested were also positive for the cephalosporin susceptibility test, 90% of the positives were resistant to ceftazidime. The isolates including 20 (50%) *E. coli*, 12 (30%) *K. pneumonia* subsp. *pneumoniae* and 8 (20%) *E. sakazakii* displayed different susceptibilities to cefotaxime, ceftriaxone and ceftazidime (Figure 1). Two *E. sakazakii* isolates were resistant to more than one extended-cephalosporin with one isolate resistant to both cefotaxime and ceftazidime and one to ceftriaxone and ceftazidime. Although, this inhibition zone in disc diffusion test of ≤ 30 mm for extended-spectrum cephalosporins

Table 3. ESBL genes *bla*$_{SHV}$, *bla*$_{CTX-M-3}$-like and *bla*$_{CTX-M14}$-like genes in *Enterobacter sakazakii*, *Escherichia coli* and *Klebsiella pneumoniae* subsp. *pneumoniae* isolates from raw and fermented camel milk.

	Gene		
Strains	***bla*$_{SHV}$**	***bla*$_{CTX-M-3}$-like**	***bla*$_{CTX-M14}$-like**
E. coli	2	-	3
E. sakazakii	1	12	1
K. pneumoniae	34	13	7
Total	37	25	11

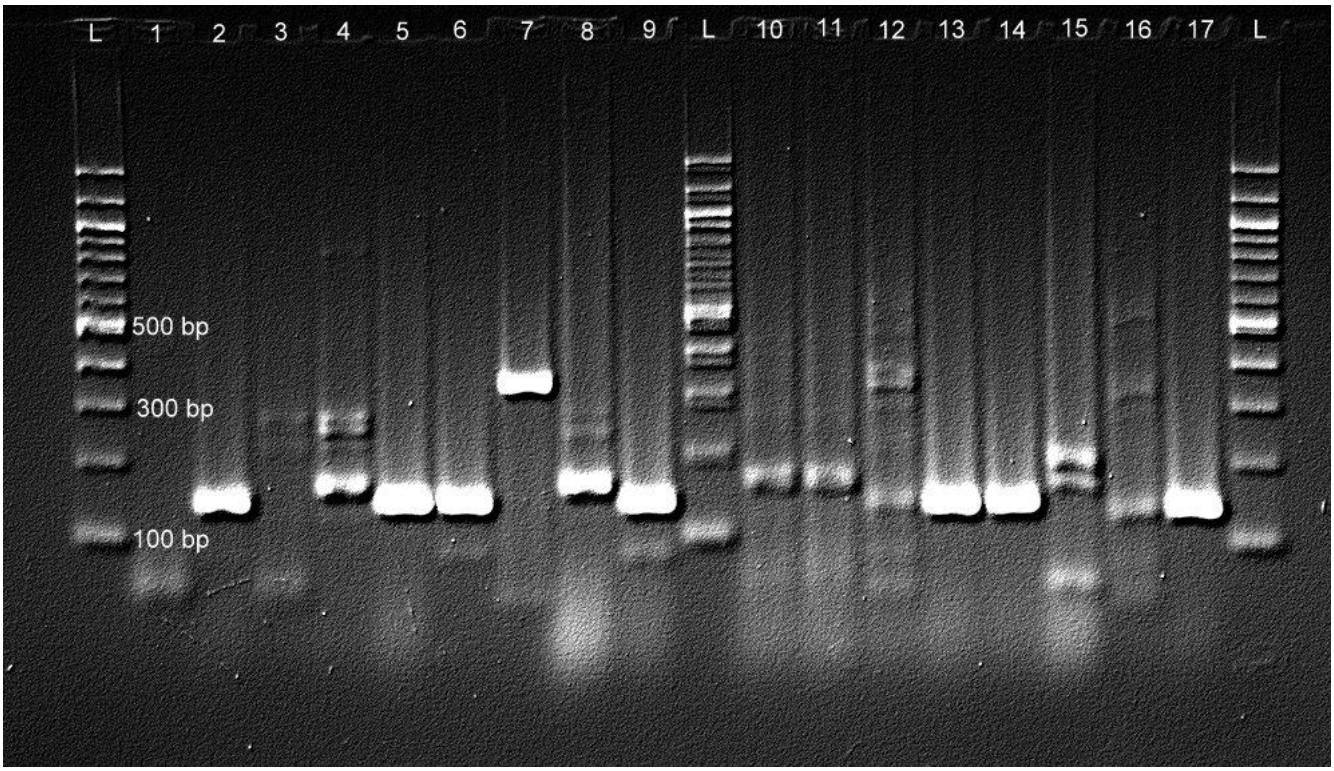

Figure 2. Gel electrophoresis filtered picture of *bla*$_{SHV}$ simplex PCR amplification of *Enterobacteriaceae* isolates. Note: Lanes 1-17 contained the PCR amplicons from indicated isolates or references: [Lane, isolate no. or references]; L, 100-bp DNA ladder (New England Biolabs); 1, no DNA in amplification assay; 2, *Klebsiella pneumoniae* ATCC 700603; 3, *E. coli* GSBL 713901; 4, *Escherichia coli* ATCC 25922; 5, no. 15 *Klebsiella pneumoniae* subsp. *pneumoniae*; 6, no. 27 *Klebsiella pneumoniae* subsp. *pneumoniae*; 7, no. 18 *Enterobacter sakazakii*; 8, no. 14 *Escherichia coli*; 9, no. 19b *Klebsiella pneumoniae* subsp. *pneumoniae*; 10, no. 10 *Escherichia coli*; 11, no. 87 *Escherichia coli*; 12, no. 9 *Escherichia coli*; 13, no. 88 *Klebsiella pneumoniae* subsp. *pneumoniae*; 14, no. 17 *Klebsiella pneumoniae* subsp. *pneumoniae*; 15, no. 90 *Escherichia coli*; 16, no. 80 *Escherichia coli*; 17, no. 44 *Klebsiella pneumoniae* subsp. *pneumoniae*.

implies ESBL production (Thomson et al., 1996; Pitout et al., 1997), the strains were all ESBL negative as tested by the double disc diffusion test at both test distances (30 and 25 mm). ESBL-producers have the ability to hydrolyze and cause resistance to third-generation β-lactam antibiotics (Pitout and Laupland, 2008).

Prevalence of *bla*$_{SHV}$, *bla*$_{CTX-M-3}$-like and *bla*$_{CTX-M-14}$-like genes, determined as PCR-positive targets, was 37 (60%), 25 (40%) and 11 (18%), respectively (Table 3). *K. pneumonia* subsp. *pneumoniae* isolates possessed 54 (74%) of the SHV (Figure 2) and CTX-M β-lactamase encoding genes detected were 14 (19%) and 5 (7%) for *E. sakazakii* and *E. coli* positive, respectively. SHV-1 β-lactamase is commonly associated with *K. pneumonia* subsp. *pneumoniae* and is accountable for up to 20% of plasmid mediated ampicillin resistance (Bradford, 2001). CTX-M, first reported in Germany in 1989, has rapidly spread worldwide in a range of bacteria but in particular

among *Enterobacteriaceae* (Bonnet, 2004). CTX-M can better hydrolyze cefotaxime than ceftazidime, both of which are third-generation cephalosporins developed to prevent spread of genes encoding these enzymes (Paterson, 2006). Additionally, plasmids with such ESBL encoding genes also encode resistances to other ABs like aminogycosides, antifolates, tetracycline and fluoroquinolones (Wiegand et al., 2007). The finding in the present study shows 13 *K. pneumonia* subsp. *pneumoniae* isolates harbouring both bla_{SHV} and $bla_{CTX\text{-}M\text{-}3}$-like genes and 1 possessing all 3 genes, further emphasising the potential health risk posed by these bacterial pathogens in camel milk.

The results show that double disc diffusion test and PCR tests differed in their detection of ESBL producing strains. *In vitro* susceptibilities of *Enterobacteriaceae* that are ESBL-producers have been found to be misleading and isolates showing susceptibility to given cephalosporins are not always effectively controlled by the same in clinical practice (Fluit et al., 2001). Furthermore, the disc diffusion breakpoints for the cephalosporins by the CLSI, were created in the 80s, before attention was paid to the role of ESBLs (Paterson, 2006). However, upon comparing the PCR results with ampicillin resistance test, we found out that twenty-eight isolates positive to SHV were also ampicillin resistant especially 27 of *K. pneumonia* subsp. *pneumoniae* isolates. Molecular methods therefore remain the most reliable methods of choice for identification of ESBL-producing isolates.

Conclusions

A high diversity of *Enterobacteriaceae* in camel milk calls for measures to improve handling of camel milk with regard to udder health water quality and hygiene practices. The finding of ESBL-positive isolates with some *K. pneumonia* subsp. *pneumoniae* harbouring more than one gene in the remote semi-arid regions shows the global antimicrobial challenge posed by these the *Enterobacteriaceae*. Molecular methods were noted to be most reliable methods of choice for identification of ESBL-producing isolates.

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