Phenotypic and Genotypic Antibiotic Resistance Patterns of *Staphylococcus aureus* From Raw and Spontaneously Fermented Camel Milk

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Authors' contributions

Author CJ participated in milk sample collection, isolation and enumeration of staphylococci. Authors JW, CL and LM conceived the study, its design and coordination and helped to draft the manuscript. Author PMKN took part in all experiments of the study and drafted the manuscript. All authors read and approved the final manuscript.

| Research Article |

ABSTRACT

Aims: To pheno- and genotypically characterise *Staphylococcus aureus* isolated from raw and fermented camel milk from Kenya and Somali for their antibiotic resistance.
Methodology: Microdilution assays to determine minimal inhibitory concentrations (MICs) were done using to 20 different antibiotics. Further tests with selected antibiotics were done using disk diffusion test. Genotypic antibiotic resistance was tested using by microarray hybridization with selected isolates and consequent screening of antibiotic resistance genes by PCR.
Results: Prevalence of antibiotic resistance among the 47 *S. aureus* tested were ampicillin 26% (12), gentamicin 26% (12), streptomycin 11% (5), tetracycline 13% (6), trimethoprim 6% (3) and fusidic acid 2% (1). Multi-resistance was detected with three isolates resistant to two antibiotics, six to three antibiotics and six to four or more

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antibiotics. Three multi-resistant *S. aureus* isolates were positive for the β-lactamase resistant genes (*bla*Z), the tetracycline resistance gene *tet*38 and the Panton-Valentine leukocidin gene *pvl* according to microarray hybridization assays. Two of the three isolates harbored additionally streptomycin resistance gene *ant*(6)-*Ia*. The tetracycline resistance gene *tet*(K) was also detected by microarray in four isolates. PCR detected *tet*(K) and *bla*Z in 2 and 7 additional isolates respectively.

Conclusion: Controlled antibiotic therapy in camels should be introduced to prevent the increase of AB resistant bacteria for this and similar milk and hygienic situations in similar production environment. Detection of the Panton-Valentine leukocidin gene *pvl* by microarray hybridization calls for further research on possibility of community-acquired methicillin-resistant *S. aureus* (CA-MRSA) in the milk as CA-MRSA with high virulence potential has been associated with the gene *lukF*-PV (*pvl*).

1. INTRODUCTION

As antibiotic use became commonplace in human medicine and food animal production, selective pressure has led to maintenance of resistance genes in many groups of bacteria. Bacterial evolution has included mechanisms to retain, accumulate, and disperse resistance genes among bacterial populations [1].

Staphylococci are among the most significant pathogens causing a wide range of diseases in both humans and animals. In humans, nosocomial and community-acquired staphylococcal infections have been most commonly reported [2]. Coagulase-positive (CPS) and coagulase-negative staphylococci (CNS) are important mastitis pathogens in animals [3,4].

Resistance of staphylococci to various ABs has been previously reported [5]. Since the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) in 1961, options for treatment of *S. aureus* infection have been significantly limited. Antibiotic resistant pathogens have been noted at significant levels in dairy cattle and some isolates also showed resistance to two or more antibiotics [6].

S. aureus has been ranked as the most frequent or second most frequent microorganism isolated from udder infections in camels [7]. Resistance to tetracycline by six and ampicillin by one *S. aureus* isolate has been previously reported [8] indicating that there could be presence of various antibiotic resistance determinants amongst the *S. aureus* in camel milk. However, reports on antibiotic resistance in milk *S. aureus* are scarce. Furthermore there is no conclusive study using molecular tools to investigate the extent of antibiotic resistance in bacterial isolates from camel milk.

Camel milk plays an important role in the nutrition of people in Arid and Semi Arid Lands of Kenya and Somalia where more than 60% of the one humped camel population is found [9]. Fermentation is the only means of preserving milk under the warm conditions where refrigeration in technically and economically not feasible. The spontaneously fermented milk, known as *suusac*, is made by leaving the unheated milk in smoke treated calabash or recently plastic containers at ambient temperatures until it becomes sour [10].

This study investigated the antibiotic resistance patterns of *S. aureus* isolates associated with raw and spontaneously fermented camel milk using a combination of phenotypic and genotypic tools with aim to assess consumer safety hazards arising from uncontrolled use of anibiotics.

2. MATERIALS AND METHODS

2.1 Sample Collection

A total of 59 raw milk, fermenting or fully fermented camel milk samples were collected from Kenya (in Isiolo, Nanyuki, Mandera and Garissa regions) and Somalia (in Burco and Garowe regions). Raw camel milk was sampled at points along the market chain in Nanyuki and Isiolo at herd level as individual and pooled camel milk, first collection point and from the final market in Nairobi.

2.2 Isolation and Identification of *S. aureus*

Appropriate dilutions of samples were surface plated on Baird-Parker agar (Biolife, Milan, Italy) supplemented with 5% egg york tellurite emulsion. Representative colonies were selected after incubation at 37 °C for 24 h based on colony morphology and presence or absence of halos on agar and colour. Three colonies per visual morphology-type derived from plates containing > 30 colonies were selected and purified by three times repetitive streaking. A total of 235 isolates were isolated. After characterization by catalase test (3% H_2O_2, VWR International), Gram-staining reactions (3% KOH, Sigma-Aldrich) and microscopic examination, isolates were further phenotyped by detection of the clumping factor/protein A by latex agglutination with the Staphytect-Plus test system (Oxoid AG, Pratteln, Switzerland).

S. aureus DSM 1104, *S. epidermidis* DSM 20044[T], *S. xylosus* DSM 6179, *S. simulans* DSM 20322[T] and *S. saprophyticus* DSM 20229 were used as PCR controls. DNA was extracted from single colonies as described by Goldenberger et al. [11]. Such DNA was first evaluated by *Staphylococcus* genus-specific PCR targeting the *tuf* gene, which encodes the elongation factor Tu [12], then for a *S. aureus*-specific section of the 23S rRNA intergenic spacer region [13].

2.3 Antibiotic Resistance Determination

2.3.1 Phenotypic antibiotic resistance profile tests

2.3.1.1 Antibiotic susceptibility test by microdilution assay

S. aureus subsp. *aureus* ATCC 25923/DSM 1104 and *S. aureus* subsp. *aureus* ATCC 29213/DSM 2569 were used as reference strains for antibiotic susceptibility testing. Microdilution assays to determine minimal inhibitory concentrations (MICs) were done according to the method by Klare et al. [14] but using Mueller-Hinton (MH) broth in place of LSM broth.

MICs were tested using 20 different ABs (Fig. 1). Subsequently, MICs were calculated as the lowest concentration of an antimicrobial agent at which growth was inhibited. Results were compared with target MICs from Clinical and Laboratory Standards Institute (CLSI),

Swedish Reference Group for Antibiotics (SRGA), European Committee on Antimicrobial Susceptibility Testing (EUCAST) and British Society for Antimicrobial Chemotherapy (BSAC) (Fig. 1).

Antibiotic	Concentration [µg ml⁻¹]																	
	≤	0.02	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	512	≥
Amoxicillin[†]	1		1	15	16	2		2	1	2	5	2						
Ampicillin[*]	2			6	21	6		1		1	6	2	2					
Chloramphenicol[*]	2						1	18	18	8								
Cloxacillin[*]	5			20	16	5	1											
Erythromycin[*]	2		4	19	17	4	1											
Gentamicin[*]	4						6	25	10	2								
Mupirocin[*]	45			y	2													
Nalidixic acid[†]	1									3	16	16	8		2	1		
Novobiocin[**]	46												1					
Penicillin G[‡]	35			1			x	3	1		5	1	1					
Rifampicin[*]	47																	
Streptomycin[*]	2							4	16	16	3	1	1			2	2	
Tetracycline[*]	42								3	1	1							
Trimethoprim[§]	12						22	7	2	1	2	1						
Vancomycin[*]	1					5	36	5										
Fusidic acid[*]	43				1	1	1	1										
Oxacillin[*]	44		3															
Methicillin[‡]	2			4	28	12	1											
Clindamycin[*]	20	1	16	7	3													
Kanamycin[*]	1				1	3	7	28	6		1							

Fig. 1. Dilution ranges tested, number of isolates observed at each MIC value and the resistance breakpoints for antimicrobials tested

Region between grey (■) shades demarcates tested dilution ranges; region shaded (▨) demarcates resistance region; [*]EUCAST, [†]BSAC, [‡]CLSI, [§]SRGA, [**]No MIC breakpoint found

2.3.1.2 Antibiotic susceptibility test by disk diffusion technique

Disk diffusion was used to confirm the susceptibilities shown by antibiotic susceptibility testing with microdilution assay. For this, oxacillin, rifampicin, tetracycline and vancomycin whose resistance is sometimes not detectable by applying the broth microdilution method were used [15,16,17].

A single colony of the test organism was picked up with the help of a disposable loop, emulsified in 5 ml saline solution (0.85% NaCl; Prolabo) in a test tube to match the turbidity with 0.5 McFarland's Standard (bioMérieux, Geneva, Switzerland).

The suspension of the test organism was then spread on MH agar (Biolife, Italiana S.r.l) surface with the use of a cotton swab soaked in the suspension tube. Disks containing tetracycline, vancomycin or rifampicin (each 30 µg) or oxacillin (1 µg) were placed in

duplicate MH agar plates for each isolate and then incubated at 37°C for 20 hours. The inhibition zone around the disk was then measured and compared with CLSI standards for disk diffusion [18].

2.3.2 Genotypic antibiotic resistance profiling

2.3.2.1 Microarray hybridization

Microarray hybridization allowing a fast detection of 90 antibiotic resistance genes was applied to six isolates showing phenotypic multi-resistance as a guide on the choice of target genes for subsequent PCR typing. Isolation and labeling of the bacterial DNA prior to hybridization was done as previously by [19]. The hybridized probes were stained [19] followed by online detection of the staining in an array tube reader (Clondiag GmbH) for 15 minutes at 25°C. The data was optically analyzed and location of the positive spots compared with the microarray's key.

2.3.2.2 Screening of antibiotic resistance genes by PCR

Microarray hybridization was complemented with PCR testing the genes *tet*(K) and *blaZ* detected using microarray hybridization. This enabled screening of the *S. aureus* isolates not tested using microarray hybridisation for the presence of these genes.

DNA was extracted according to Goldenberger et al. [11] and amplifications done using a modified method and primers previously described [19]. Briefly, the 25-µl PCR mixture contained 2 µl of the extracted DNA (approximately 10-50 ng), 0.25 µl of each of the 100 µmol l^{-1} primer pair (Microsynth), 12.5 µl of 2X MasterMix (GE Healthcare) and 10 µl of autoclaved double distilled water. Initial denaturation was done at 94ºC for 5 min followed by 35 times of denaturation at 94ºC for 30 sec, annealing at 54ºC for 30 sec and extension at 72ºC for 1.5 min. A final extension was done at 72ºC for 7 min.

As reference strains for the PCR *S. aureus* DSM 1104 (*mecA*-negative, β-Lactamase-negative) and DSM 2569 (*mecA*-negative, β-Lactamase-positive and oxacillin susceptible) were selected as recommended by CLSI [18]. Furthermore, three isolates numbers 20, 34 and 51, tested with Microarray were used, either as positive or negative control, depending on the target gene. PCR products were subjected to gel electrophoresis using 2% agarose gel in 1 x TAE (pH 8), stained in an ethidium bromide and visualized under UV light (302 nm, Alphalmager^{TM}).

3. RESULTS

3.1 Identification of *S. aureus*

Coagulase positive *Staphylococcus* isolates as detected using latex agglutination test were 100. The genus specific PCR revealed that 146 of the 235 isolates (62% of colonies picked from Baird Parker agar) belonged to genus *Staphylococcus*. Total of 47 (32%) of the *Staphylococcus* were identified as *S. aureus* by 23S rRNA PCR.

3.2 Phenotypic Antibiotic Resistance

3.2.1 Antibiotic susceptibility by microdilution assay

Eleven isolates numbered as 20, 21, 25, 26, 31, 34, 37, 38, 44, 48 and 51 were resistant to ampicillin in ranges of the MIC between 4 and 32 mg l^{-1} (Table 1). These isolates together with number 45 were also resistant to amoxicillin in the MIC range between 1 and 16 mg l^{-1}. Isolates 20, 21, 25, 34, 37, 38, 48 and 51 were resistant to penicillin in the MIC range between 2 and 32 mg l^{-1}. MIC values for fusidic acid in the tested range were found in only four of the 47 tested strains with one fully resistant (1 mg l^{-1}). The twelve isolates 1, 7, 10, 13, 24, 25, 31, 32, 33, 34, 45, and 51 were resistant to gentamicin in MIC ranges between 2 and 4 mg l^{-1}. Isolates 25, 26, 33, 34 and 37 showed resistances to streptomycin with MICs from 32 to ≥256 mg l^{-1}. Five isolates 21, 34, 46, 47 and 51 were resistant to tetracycline with MICs in in the range 2-8 mg l^{-1}. Isolates 32, 33 and 46 displayed resistance MIC values for trimethoprim from 8 to 16 mg l^{-1}. Isolates 29, 32 and 33 were resistant to nalidixic acid at between 128 and 256 mg l^{-1} (Table 1). Multiresistant isolates included 15 isolates resistant to between two and six antibiotics (Table 1). There were 3 isolates resistant to 2 antibiotics, 6 to 3 antibiotics and 6 to 4 or more antibiotics.

The six isolate numbers 20, 21, 25, 26, 34 and 51 (Table 1) were selected for microarray hybridization based on the multi-resistance.

3.2.2 Antibiotic susceptibility assayed by disk diffusion

Isolates 1, 18, 36, 38, 43, and 49 were resistant to oxacillin with inhibition halos ≤ 10 mm. None of these resistances to oxacillin were detected by microdilution assay. All of the isolates showed susceptibility for rifampicin in ranges 24 to 62 mm. Three isolates 13, 21, 25 presented an intermediate susceptibility to tetracycline and six isolates 26, 34, 37, 46, 47, and 51 were resistance with inhibition halos lower or equal to 14. Four isolates 16, 18, 36 and 38 were resistant to vancomycin.

3.3 Genotypic Antibiotic Resistance

Genotypic antibiotic resistance was typed first by microarray hybridization for isolates showing significant phenotypic resistances and for the positive resistance genes, confirmation by PCR for the rest of the *S. aureus* isolates.

3.3.1 Antibiotic resistance genes by microarray hybridization

Isolates 20, 34 and 51 all harbored the β-lactamase resistant genes (*bla*Z), tetracycline resistance with gene *tet*38 and the Panton-Valentine leukocidin gene *pvl*. Additionally, isolate numbers 34 and 51 harbored streptomycin resistance gene *ant*(6)-*Ia*. Tetracycline resistance through the gene *tet*(K) was found in isolates 20, 21, 26, 34 and 51.

Table 1. Phenotypic and genotypic resistance profiles of *S. aureus* isolates harboring antibiotic resistant determinants

Isolate no.	Method			
	Phenotypic		Genotypic	
	*MIC	†DD	Microarray	‡PCR
1	Gen	Oxa	Nd	-
4	-	-	Nd	*bla*Z
7	Gen	-	Nd	-
10	Gen	-	Nd	-
13	Gen	-	Nd	-
16	-	Van	Nd	-
18	Fus	Oxa, Van	Nd	-
20	Amo, Amp, Gen	-	*bla*Z, *tet*(38), *pvl*	*bla*Z
21	Amo, Amp, Pen, Tet	-	*tet*(K)	*tet*(K), *bla*Z
24	Gen	-	Nd	-
25	Amo, Amp, Gen, Pen, Str	-	Nd	*bla*Z
26	Amo, Amp, Str	Tet	*tet*(K)	*tet*(K), *bla*Z
29	Nal	-	Nd	-
31	Amo, Amp, Gen	-	Nd	*bla*Z
32	Gen, Nal, Tri	-	Nd	-
33	Gen, Nal, Str, Tri	-	Nd	-
34	Amo, Amp, Gen, Pen, Str, Tet	Tet	*bla*Z, *tet*(38), *pvl*, *tet*(K), *ant*(6)-*Ia*	*tet*(K)
36	-	Oxa, Van	Nd	-
37	Amo, Amp, Pen, Str	Tet	Nd	*bla*Z
38	Amo, Amp, Pen	Oxa, Van	Nd	*bla*Z
43	-	Oxa	Nd	-
44	Amo, Amp	-	Nd	-
45	Amo, Gen	-	Nd	*bla*Z
46	Tet, Tri	Tet	Nd	*bla*Z, *tet*(K)
47	Tet	Tet	Nd	*tet*(K)
48	Amo, Amp, Pen	-	Nd	-
49	-	Oxa	Nd	-
50	Tri	-	Nd	-
51	Amo, Amp, Gen, Pen, Tet	Tet	*bla*Z, *tet*(38), *pvl*, *tet*(K), *ant*(6)-*Ia*	*tet*(K)

Minimum inhibitory concentration method; † disc diffusion method (tested for oxacillin, rifampicin, tetracycline and vancomycin); ‡ tested for blaZ and tet(K); -, not detected; nd, not determined; Amp, ampicillin; Tet, tetracycline; Gen, gentamycin; Str, streptomycin; Oxa, oxacillin; Van, vancomycin; Amo, amoxicillin; Fus, fusidic acid; Pen, penicillin G; Tri, trimethoprim; Amo, amoxicillin

3.3.2 Antibiotic resistance genes by PCR

The isolates numbers 21, 26, 34, 46, 47 and 51 were positive for *tet*(K) (Fig. 2). The isolate numbers 4, 20, 21, 25, 26, 31, 37, 38, 45 and 46 were positive for *blaZ* (Fig. 3). Isolates numbers 46 and 21 harbored both *tet*(K) and *blaZ* genes (Table 1).
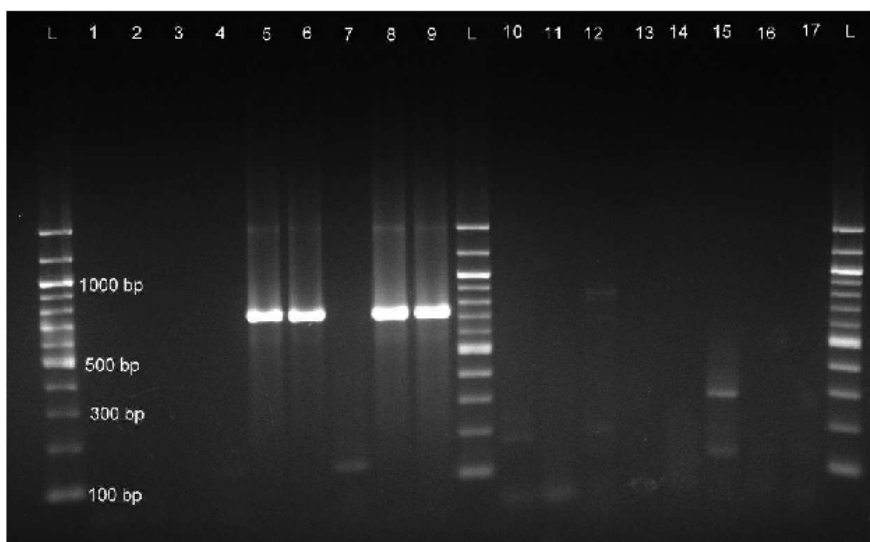
Fig. 2. Illustrative *tet*(K) simplex PCR amplification gel electrophoresis picture with isolates numbers 34, 46, 47 and 51 amongst the *tet*(K) positive isolates
Lanes indicated at the top contained the PCR amplicons from indicated isolates or references: [Lane, isolates or references]; L, 100-bp DNA ladder (New England Biolabs); 1, no DNA in amplification assay; 2, S. aureus DSM 1104; 3, S. aureus DSM 2569; 4, sample no. 20; 5, no. 34; 6, no. 51; 7, no. 45; 8, no. 46; 9, no.47; 10, no.48; 11, no.49; 12, no.50; 13, no. 12; 14, no. 24; 15, no.25; 16, no.27; 17, no.28
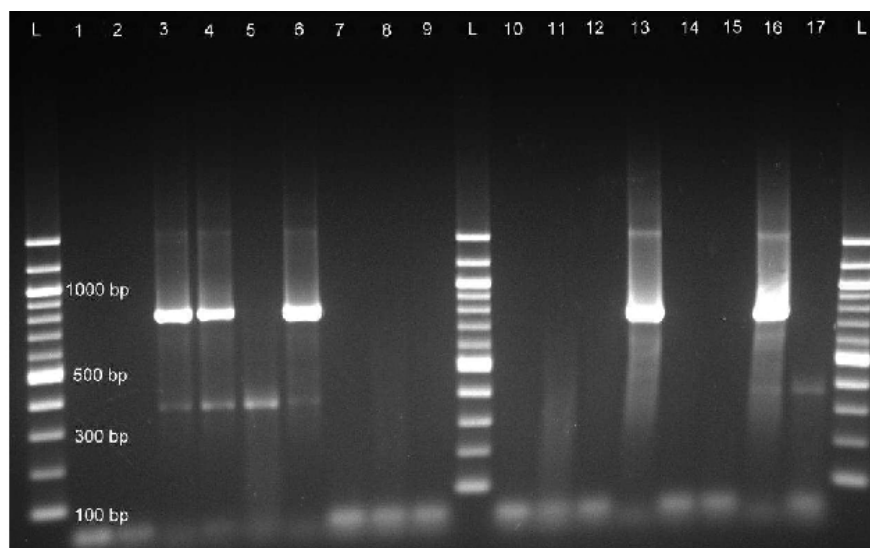
Fig. 3. Illustrative *blaZ* simplex PCR amplification gel electrophoresis picture of *S. aureus* isolates and references showing positive samples 20, 21 and 25
Lanes indicated at the top contained the PCR amplicons from indicated isolates or references: [Lane, isolates or references]; L, 100-bp DNA ladder (New England Biolabs); 1, no DNA in amplification assay; 2, S. aureus DSM 1104; 3, S. aureus DSM 2569; 4, sample no. 20; 5, no. 34; 6, no. 51; 7, no. 14; 8, no. 15; 9, no.16; 10, no.17; 11, no.18; 12, no.19; 13, no. 21; 14, no. 22; 15, no.24; 16, no.25; 17, no.26

2. DISCUSSION

Up to now many studies have focused on characteristics of bovine *S. aureus* and there is limited information on *S. aureus* associated with other species and their antibiotic susceptibilities.

Resistance to single or multiple antimicrobials was detected in the *S. aureus* isolates from camel milk. Resistance to β-lactam ABs including penicillin, ampicillin, amoxicillin and oxacillin was detected. It was noted that all isolates resistant to a β-lactam antibiotic except for oxacillin showed resistance to additional ABs especially from the β-lactam class. The β-lactamase structural gene *blaZ* is present on elements commonly carried by a diverse group of plasmids which invariably exhibit resistance to one or more other antimicrobial agents [20].

This was evident also from genotyping using microarray and PCR where 11 isolates were *blaZ* positive (Table 1). Different species of staphylococci when present in the same environment, like the skin of dairy cows, have been found to exchange *blaZ* genes when the appropriate bacterial conditions are met [21].

Six isolates were resistant to oxacillin none of which was found by microdilution assay. Each susceptibility test has inherent advantages and limitations [17]. Even though the microdilution method has been shown to be both rapid and accurate, detection of resistance in isolated colonies has been shown to fail in some species using this method as compared to agar-based methods ([15,16,17]. This could be reason for the differences in detection in the present study. On the other hand, lack of proper attachment of the disks to the agar surface could give false positives and longer drying periods of the agar plates after the inoculation, gives smaller zones of inhibition [22].

Five isolates were resistant by MIC method to tetracycline all except one of which were colloborated by the disk diffusion tests (Table 1). All the five isolates were also positive by microarray or PCR for *tet*(K) and two of those tested by microarray additionally harboured *tet*(38). TET(K) efflux protein encoded by *tet*(K) confers resistance to tetracycline in majority of the cases [20]. Gene *tet*(38) is a novel chromosomal tetracycline transporter associated with TetK. The Tet38 efflux pump imparts tetracycline resistance and shares 46 % similarity with the tetracycline resistance TetK protein in *S. aureus* [23]. High expression of the *tet*(38) gene could lead to 32 times increase in resistance to tetracycline [23]. Tetracycline resistance has become particularly important in some countries because of low cost of the antibiotic and its prescription to treat a variety of infections including the ones caused by staphylococci [24].

In the isolate numbers 21, 26, 34, 46 and 51 multiresistance was detected with both *blaZ* and *tet*(K) present and in all except number 46, phenotypic resistance was detected for both β-lactam antibiotics and tetracycline. Resch et al. [25] reported a relationship between phenotypic resistances to β-lactam antibiotics, lincomycin and tetracycline and the occurrence of the genes *bla*Z, *lnu*A and *tet*K, respectively.

Twelve isolates were resistant to gentamicin. Gentamicin resistance in *S. aureus* has been reported since 1975 and it has now spread worldwide [26]. However, all isolates were susceptible to kanamycin the other aminoglycoside tested. For fusidic acid, only one of the 47 tested was resistant. Fusidic acid, a narrow spectrum AB, has been used in the treatment

of *S. aureus* infections for the last 35 years [27]. Susceptibility by the all *S. aureus* was however found to 10 of the 20 antibiotics.

Microarray hybridization enabled detection of the Panton-Valentine leukocidin gene *pvl*. Community-acquired methicillin-resistant *S. aureus* (CA-MRSA) with high virulence potential has been associated with the genes *lukS*-PV and *lukF*-PV (*pvl*) that encodes the subunits of the Panthon-Valentine leukocidin [28]. Phenotypic resistance to methicillin was however not detected in this study. Microarray hybridization allows detection of many resistance genes in only one experiment with 90 genes in this case being tested [19]. On the other hand it is very expensive, laborious and requires considerable skills especially when labeling the DNA which is a critical step in this technique.

These results also reflect the antimicrobial use pattern in the region. Estimates show that tetracycline, sulfonamides, β-lactams and aminoglycosides are the most used ABs for food animals. Tetracycline contribute to approximately 55% of the consumption [29]. A number of these ABs including tetracyclines, macrolides, penicillins and aminoglycosides have been listed as critically important for both human health and animal health by WHO and OIE [30].

3. CONCLUSIONS

Antibiotic therapy in the camels remains largely uncontrolled and there is poor milking hygiene practice [7]. The detection of such resistance profiles therefore implies the need for controlled antibiotic therapy in camels and the introduction of measures aimed at reducing milk contamination. Instruction is necessary on the use of appropriate and timely mastitis detection tests because mastitis is also reported a source of *S. aureus* contamination in camel milk [7]. Detection of the Panton-Valentine leukocidin gene *pvl* by microarray hybridization calls for further investigation on possibility of community-acquired methicillin-resistant *S. aureus* (CA-MRSA) in the milk as CA-MRSA with high virulence potential has been associated with the gene *lukF*-PV (*pvl*).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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