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Biodiversity and Enterotoxigenic Potential of Staphylococci Isolated from Raw and Spontaneously Fermented Camel Milk

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Authors' contributions

This work was carried out in collaboration between all authors. 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

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ABSTRACT

Aims: To characterise the diversity, genotypic and phenotypic properties of coagulase negative and coagulase positive staphylococci from camel milk.
Place and Duration of Study: Laboratory of Food Biotechnology, Department of Health Sciences and Technology (D-HEST), Swiss Federal Institute of Technology, Zurich, Switzerland, between July 2009 and June 2011.
Methodology: Staphylococci isolated from 59 raw and spontaneously fermented camel milk (suusac) samples from Kenya and Somalia were identified, pheno- and genotypically characterized. Preliminary screening of colonies was done by catalase test, Gram staining reactions, clumping factor/protein A and microscopy. Further identification was done by 23S rDNA species PCR, thermostable nuclease gene (*nuc*) PCR and rep-PCR followed by staphylococcal genus ID32 Staph system and coagulase negative species specific PCR. PCR amplification of the genes encoding capsular polysaccharides cap5 and cap8, and staphylococcal enterotoxins SEA to SEE and SEG to SEJ was also carried out.

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Results: From a total of 235 BP medium isolates, staphylococci were 146 (62 %) of which, 66 (45 %) were *Staphylococcus aureus*. *S. epidermidis* accounted for 43 % of the coagulase negative staphylococci (CNS). The rest of the CNS were 25 % *S. simulans*, 16.3 % *S. saprophyticus*, 2.5 % *S. haemolyticus*, 2.5% *S. hyicus*, 2.5 % *S. xylosus*, 2.5 % *S. lentus*, 1.3 % *S. carnosus* and 1.3 % *S. microti*. Capsular polysaccharide gene *cap5* was present in 15 % and *cap8* in 23 % of the *S. aureus* isolates. Enterotoxin genes were detected in 47 % of the staphylococci with *sej* in 33 %, *seb* in 6 %, *sed* in 5 % and *seg* in 3 % of the isolates. Within the species enterotoxin genes were detected in 100 %, 64.7 %, 38.5 % and 22.7 % of the *S. simulans*, *S. epidermidis*, *S. sapropyticus* and *S. aureus* respectively.
Conclusion: The diversity of CNS is remarkable and the prevalence of enterotoxin genes amongst CNS and CPS further informs generalizations for other milk and hygienic situations in similar production environment.

Keywords: Staphylococcus; biodiversity; enterotoxigenic potential; molecular typing; camel milk.

1. INTRODUCTION

S. aureus belonging to CPS is implicated in the aetiology of a series of infections in both man and animals whereas CNS have been assumed to be rarely pathogenic [1]. *S. aureus* produces several virulence factors such as exoproteins and various cell surface proteins that contribute to the pathogenicity of this organism most of which have been well characterized (2). *S. aureus* are named according to whether or not they have been proved to cause emesis, 11 staphylococcal enterotoxins (SEs) (SEA-E, SEG-I, SER-T), and 10 enterotoxin-like (SEI) (SEIJ-Q, ESIU and SEIV) *S. aureus* are known (3). The detection of SEs facilitates monitoring of outbreaks and enterotoxigenicity of strains. This is now most often based on molecular biological techniques (4) rather than immunological tools [1].

Little attention has been paid to the toxigenic potential of CNS though some investigators emphasize that they could produce toxic shock syndrome toxin alone or in combination with staphylococcal enterotoxins [5,6,7]. The important role of CNS as pathogens has been recognized only recently, and specific factors involved in pathogenesis are now under exploration [8,5,9]. Modern species identification is another prerequisite for such attempts in CNS and it relies mostly on the 16S rRNA gene, *rpoB*, *groEL*, *hsp60*, *femA*, *tuf*, *gap*, *sodA*, *rpoB*, *dnaJ,* and *kat* genes [10,11,12].

Reports are scarce and limited information is available on the diversity, genotypic and phenotypic characteristics of both CNS and CPS in milk and milk products. Camel milk plays an important role in the diet of nomads in the arid and semiarid lands of East Africa where 10.5 million of the world's estimated 24.2 million camel population is found [13] . *S. aureus*, a CPS, has been ranked as either the most or second most frequent bacterium isolated from udder infections in camels but CNS are also incriminated as a major bacterium causing mastitis in the camel [14,15]. Staphylococci are therefore of great clinical and epidemiological importance not only in camel milk but also in other environmental and food matrices through cross-contamination and potential transfer of virulence determinants. Taking this into account, CNS and CPS from raw and fermented camel milk were identified and further characterized for their pathogenic potential.

2. MATERIALS AND METHODS

2.1 Camel Milk Sample Collection

Total of 59 samples of raw, fermenting or fully fermented camel milk were collected from Kenya (in Isiolo, Nanyuki, Mandera and Garissa) and Somalia (in Burco and Garowe) in July and August year 2007 and May of year 2008. Raw camel milk was sampled at points along the market chain in Nanyuki at herd level as individual camel and pooled milk, first collection point and from the final market in Nairobi.

The time taken between milking and the first collection point varied from 4 to 7 hours while it took at least 27 hours between milking and final market. The pastoralists hold the milk at ambient temperatures during transit due to lack of refrigeration facilities.

In both locations, the traditional free ranging and intensive camel dairy herds were chosen for sampling. Samples were also collected from milk collection containers. Samples were frozen in dry ice and transported to the laboratory within 8 hours of collection.

2.2 Isolation and Enumeration of Staphylococci

Appropriate dilutions of samples were surface plated on Baird-Parker agar (Biolife, Milan, Italy) supplemented with 5 % egg yolk tellurite emulsion. After incubation at 37 ºC for 48 h, enumeration was done and representative colonies selected based on colony morphology and presence or absence of halos on agar and colour. Three colonies per morphology were selected and purified by 3 times repetitive streaking. A total of 235 isolates were isolated and transported frozen to Zurich. After characterization by catalase test (3 % H_2O_2, VWR International), Gram 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). *Staphylococcus* isolates were then preserved in brain heart infusion broth (Biolife) containing 20 % glycerol (Sigma-Aldrich Chemie GmbH) at -80ºC for use in subsequent experimentation.

2.3 Staphylococcus Reference Strains

The isolates were analysed and genomic data profiles compared with the following reference strains: *Staphylococcus aureus* DSM 1104, *Staphylococcus epidermidis* DSM 20044T, *Staphylococcus xylosus* DSM 6179, *Staphylococcus simulans* DSM 20322T and *Staphylococcus saprophyticus* DSM 20229 were used as PCR controls for species identification. *S. aureus* strains 463 (*seb*, *seg*, *sei*), 117 (*sea*, *seg*, *sej* and *sei*), 129 (*sea*, *seg*, *sei*, *sej*), 266 (*seb*, *seg*, *sei*), 216 (*sec*, *seg*, *sei*), 235 (*sec*, *seg*, *sei*), 243 (*sed*, *seg*, *sei*, *sej*) and 238 (*sed*, *seg*, *sei*, *sej*) previously isolated and characterized in the study by Stephan et al. (16) were used as control strains for enterotoxin gene typing.

2.4 Phenotyping and Genotyping of Staphylococci

DNA was extracted from single colonies as described by Goldenberger et al. [17]. The DNA was first evaluated by *Staphylococcus* genus-specific PCR targeting the *tuf* gene, which encodes the elongation factor Tu [18], then for a *S. aureus*-specific section of the 23S rDNA intergenic spacer region [19] and also for the thermostable nuclease (*nuc* gene) [20]. For

further differentiation and grouping both *S. aureus* and other *Staphylococcus* isolates were subjected to rep-PCR fingerprinting with the single oligonucleotide primer $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) [21] which enabled differentiation and grouping into clusters. This enabled selection of fewer isolates from similar clusters for further work. The resulting fingerprints were analyzed using the GelCompar II version 5.10 (Applied Maths, Saint-Martens-Latem, Belgium) software package. The similarity among digitized profiles was calculated using the Pearson correlation, and an average linkage (UPGMA or unweighted pair group method with arithmetic averages) dendrogram was derived from the profiles. Staphylococci other than *S. aureus* were selected based on similarity amongst the clusters and were further characterized using the ID32 Staph system according to the recommendations of the manufacturer (bioMérieux, Genéve, Switzerland). Species identified within CNS clusters identified as *S. saprophyticus*, *S. epidermidis* and *S. carnosus* were confirmed by multiplex PCR using method by Morot-Bizot et al., [11] slightly modified by use of 1 µl template DNA and annealing temperature and time of 55ºC and 45 seconds respectively. *Staphylococcus xylosus* was confirmed as described by Aymerich et al. [22]. The strains were additionally investigated by PCR amplification of the capsular polysaccharide encoding genes *cap5* and *cap8* [23] and the enterotoxin encoding genes. Enterotoxin genes typed and the corresponding references for primers and protocols were *sea* [24], *seb* [25], *sec* and *see* [26], *sed* and *sej* [27] and *seg*, *seh* and *sei* [28]. All PCR assays were performed in Biometra®TGradient Cycler (Biolab Châtel-St. Denis, Switzerland).

3. RESULTS AND DISCUSSION

3.1 Identification of Staphylococci

According to genus specific PCR and ID32 Staph system, 146 of the 235 BP-medium isolates (62 % of colonies picked from BP-agar) were identified as members of the genus *Staphylococcus, Macrococcus*, *Aerococcus* and *Dermacoccus*. Of these, 66 (45 %) were identified as *S. aureus* (Table 1). Highest occurrence of *S. aureus* was found in *suusac* (29 %), pooled morning milk (24 %) and milk during fermentation (18 %). The presence of staphylococci in samples from different points in the marketing chain indicated significant contamination and/or microbial build up under handling at ambient temperatures. Such observation has also been made in cow milk from Mali [29]. This shows that raw milk quality is a prerequisite for subsequent microbiological quality of products. CPS might originate from mastitis. They are frequently isolated from camel milk and are considered to be a cause of subclinical mastitis in camels [14].

S. epidermidis, *S. simulans* and *S. saprophyticus* were the most frequently isolated *species*, accounting for 43 % (n = 34 isolates), 25 % (n = 20) and 16.3 % (n = 13) of the 80 CNS respectively (Table1). Ruaro et al. [30] also reported *Staphylococcus lentus*, *Staphylococcus simulans* and Staphylococcus xylosus as predominant CNS isolated from raw milk and cheese of North Italy. Additionally, *Aerococcus viridans* (1 isolate), *Macrococcus caseolyticus* (1 isolate) and *Dermacoccus nishinomiyaensis* (1 isolate) also isolated from BP-agar as presumptive staphylococci were identified (Table 1). CNS which have long been regarded as nonpathogenic and dismissed as cultivation contaminants were also studied. Their important role as pathogens has been recognized only recently, and specific factors involved in pathogenesis are currently explored [31].

Table 1. *Staphylococcus* and related species identified in camel milk and containers from different sources in the marketing chain

Species	*Suusac* n = 26	Pooled morning n = 8	Raw[a] n= 21	Fermentation[b] n = 33	1st collector n = 5	Final market n = 4	Container n = 8	Total
S. aureus	19	16	4	12	7	6	2	66
S. epidermidis	8	7	6	8	5	-	-	34
S. carnosus	1	-	-	-	-	-	-	1
S. haemolyticus	-	-	-	2	-	-	-	2
S. hyicus	-	-	-	-	2	-	-	2
S. lentus	-	-	2	-	-	-	-	2
S microti	-	-		-	1	-	-	1
S. saprophyticus	2	1	2	4	1	3	-	13
S. simulans	4	6	5	1	4	-	-	20
S. xylosus	-	-	-	1	-	-	1	2
Aerococcus viridans	1	-	-	-	-	-	-	1
Dermacoccus nishinomiyaensis	-	-	1	-	-	-	-	1
Macrococcus caseolyticus	-	-	-	-	1	-	-	1
Total	35	30	20	28	21	9	3	146

[a]Milk directly expressed from the udder
[b]Partially fermented suusac
- Not detected (< $10^{2.5}$ cfu/ml)
n = number of samples

Rep-PCR revealed 42 and 25 clusters of *S. aureus* and CNS with greater than 95 % similarity respectively. Thirty nine of 42 strains typed using ID32 Staph were confirmed by rep-PCR fingerprinting. However *S. simulans* (1 isolate), *S. microti* (n = 1) and *Macrococcus caseolyticus* (n = 1) confirmed by molecular techniques had been previously identified by ID32 as *Micrococcus varians*, *S. simulans* and *S. hominis* respectively. *S. simulans* (5 isolates), *S. epidermidis* (n = 5), *S. lentus* (n = 1) and *S. saprophyticus* (n = 2) showed a coagulase positive reaction which is typical of *S. aureus*. Biochemical tests and use of biochemical-based commercial kits as a final confirmation of CNS isolates may result in a number of misidentified strains. Different types of phenotypic tests have been shown in past clinical studies to achieve varying levels of accuracy for different phenotypic kits [32,33]. For example, commercial identification systems have been shown to commonly misidentify *S. hominis*, *S. schleiferi* and *S. warneri* with error rates as high as 33, 50 and 17.9 %, respectively [32,34]. Giammarinaro et al. [35] reported failure by VITEK 2 (bioMe´rieux) to identify 21% of clinical isolates and 84% of food and food plant *Staphylococcus* isolates. Phenotypic characterization shows limitations particularly because expression of characters varies and the observation of results is often ambiguous [22]. This shows that although conventional commercial identification kits give rapid results, they may result in misidentification of some species.

3.2 Toxigenic Potential of the Isolated Staphylococci

The virulence factors detected were distributed among the various sample types (Table 2). The gene *cap5* was observed for 15% (10 isolates), and *cap8* for 23 % (n = 15) of the *S. aureus* isolates. These frequencies of *cap5* and *cap8* in *S. aureus* differ from the 96 % and 4 % *S. aureus* harboring *cap5* and *cap8,* respectively in camel milk from Sudan [36]. The occurrence of either *cap5* or *cap8* may depend amongst other factors on geographical origin of isolates [37]. *S. aureus* strains harboring either of capsular polysaccharides encoding genes *cap5* or *cap8* have an increased protection against phagocytosis by polymorphonuclear leucocytes [37]. Enterotoxin genes were observed in 47 % (68 isolates) of the staphylococci with *sej* in 32.8 % (49 isolates), *seb* in 6 % (9 isolates), *sed* in 5 % (7 isolates), and *seg* in 3 % (4 isolates) of the *Staphylococcus* isolates (Table 2). Highest numbers of enterotoxin gene positive isolates were detected in milk at the herd level (n = 23), *suusac* and *garoor* (n = 20) and milk during fermentation (n = 13). None of the strains harbored the genes encoding SEA, SEC, SEE, SEH and SEI.

Amongst the predominant Staphylococcus species, 100 % of the *S. simulans*, 64.7 % of *S. epidermidis*, 38.5 % of *S. saprophyticus* and 22.7 % of *S. aureus* harboured enterotoxin genes. *S. epidermidis*, *S. simulans*, *S. saprophyticus*, *S. hyicus* and *S. lentus* have been reported elsewhere to contain enterotoxigenic genes [1,7,6].

The incidence of enterotoxin genes in CNS is higher than that reported in milk from other animals. For example occurrence of enterotoxigenic isolates amongst CNS was 5 % in cow's milk [38], 5.3 % in goat's milk and cheese [39], 39.4 % in ewe's milk [6], and 13.6 % in goat's milk [7]. In contrast, Even et al., [9], reported a low occurrence of toxin genes amongst CNS. However they similarly found genes encoding staphylococcal toxin in *S. epidermidis* consisting of 76 % of the 129 strains of food and clinical origin. We also found enterotoxin genes in the recently described CNS species *S. microti* [40] and the non *Staphylococcus* isolate *Dermacoccus nishinomiyaensis* also isolated from BP-agar both of which have not been previously described as SE producers (Table 3).

Table 2. Distribution of enterotoxin, nuclease and capsular polysaccharide encoding genes in 146 staphylococci from 59 camel milk associated samples

[a]Property	*Suusac*	*Garoor*[b]	Pooled morning	Raw[c]	Fermentation[d]	First collector[e]	Container	Final market	Total
sea	_	_	_	_	_	_	_	_	0
seb	3	_	_	_	2	2	2	_	9
sec	_	_	_	_	_	_	_	_	0
sed	_	_	1	_	6	_	_	_	7
see	_	_	_	_	_	_	_	_	0
seg	1	2	_	_	_	1	_	_	4
seh	_	_	_	_	_	_	_	_	0
sei	_	_	_	_	_	_	_	_	0
sej	10	4	9	13	5	7	_	_	48
nuc	10	8	14	1	12	12	2	2	61
*cap*5	3	1	2	_	4	_	_	_	10
*cap*8	4	2	4	_	1	2	1	1	15
Total (SE_S)	14	6	10	13	13	10	2	0	

[a]sea, seb, sec, sed, see, seg, seh, sei and sej represent Staphylococcal enterotoxins A, B, C, D, E, G, H, I, and J respectively; [b]Fermented longer than suusac; [c]Milk directly expressed from the udder; [d]Partially fermented suusac; [e]Collection point at first market; - Not detected by PCR or gene not present; SEs staphylococcal enterotoxins; cap5 and cap8 capsular polysaccharide 5 and 8 respectively

CNS in camel milk could therefore harbor enterotoxigenic genes and therefore this group of staphylococci could be as important as *S. aureus* as potential safety hazard. Such findings are of concern given that camel milk is consumed either as raw or fermented raw milk. Furthermore, CPS have been found to persist in pasteurised contaminated milk where enterotoxin genes were found in 9 of 10 isolated strains [41]. However, molecular techniques, especially DNA amplification allows the detection of potentially enterotoxigenic isolates based on the gene sequences whether these strains will further express the toxin genes or not.

Table 3. Presence of enterotoxin, nuclease and capsular polysaccharide genes in 146 staphylococci and related species isolated from 59 camel milk associated samples

[a]Species	seb	sed	seg	Sej	Nuc	cap5	cap8	Total
Aerococcus viridans	_	_	_	_	_	_	_	_
S. aureus	6	1	4	4	61	10	15	101
S. carnosus	_	_	_	_	_	_	_	_
S. epidermidis	1	4	_	17	_	_	_	22
S. haemolyticus	_	_	_	_	_	_	_	_
S. hyicus	_	_	_	1	_	_	_	1
S. lentus	2	_	_	_	_	_	_	2
S. microti	_	_	_	1	_	_	_	1
S. saprophyticus	_	2	_	3	_	_	_	5
S. simulans	_	_	_	21	_	_	_	21
S. xylosus	_	_	_	_	_	_	_	_
D. nishinomiyaensis	_	_	_	1	_	_	_	1
Macrococcus caseolyticus	_	_	_	_	_	_	_	_

[a]*S- Staphylococcus, D- Dermacoccus; - Not detected by PCR or gene not present*

4. CONCLUSION

The presence of staphylococci in samples from different points in the marketing chain indicated significant contamination and/or microbial build up due to handling of milk at ambient temperatures. There was a remarkable diversity of CNS which emphasises their potential role in milk safety. The incidence of enterotoxin genes in CNS was higher than that reported in milk from other animals and CNS in camel milk could be as important as *S. aureus* as potential safety hazard especially because this milk is consumed either as raw or fermented raw milk.

Phenotypic characterization showed that although conventional commercial identification kits gave rapid results they may result in misidentification of some species.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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