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STAPHYLOCOCCAL SUBCLINICAL MASTITIS IN DROMEDARY DAIRY CAMEL

A. Niasari-Naslaji[1], A.B. Atakpour[1], B. Khoramian[2], M. Emaneini[3], S. Ghaffari[1], H. Parsakiya[1], A. Barin[4], B.R. Samiei[5], M. Amjadi[6], V. Akbarinejad and A.A. Moosavi-Movahedi[7]

[1]Department of Theriogenology, [4]Department of Clinical Pathology, Faculty of Veterinary Medicine,
[7]Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran
[2]Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
[3]Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[5]Pegah Milk Company, Golestan, Iran
[6]International Dairy Federation, Tehran, Iran

ABSTRACT

Camel milk samples were randomly and aseptically collected after discarding first milk streak from 243 quarters of milking camels without clinical sign of mastitis or bacteriological investigation. There was no bacterial isolation in 61.73% samples. The bacteria isolated in remaining samples were *Staphylococci* (n=78; coagulase negative Staphylococci: 71 and *Staph. aureus:* 7) and *Streptococci* (n=26). Five new species of coagulase negative Staphylococci including *Staph. saprophyticus, Staph. delphini, Staph. capitis, Staph. chromogenes* and *Staph. caseolyticus* were identified. PFGE analysis carried out on 7 isolates of *Staph. aureus* and produced 2 distinct pulsotypes designated as pulsotypes A and B. All *Staph. aureus* isolates were found to be included into 2 spa types: t527 and t1532. Coagulase negative staphylococci were the main bacteria isolates (55.04%) and the frequency of *Staph. aureus* with 2 spa types was considered as 5.43% of total bacterial isolation.

Key words: Dairy dromedary camel, *S. aureus*, subclinical mastitis

Prevalence of clinical mastitis in camel was assumed to be low (Manefield and Tinson, 1996; Wernery and Kaaden, 2002). However, by looking at the microflora involved in teat canals and udder cisterns in non-lactating dromedary camels (Johnson *et al,* 2015a), it is expected that machine milking may predispose lactating camels to subclinical mastitis, similar to other milk-producing animals.

Various species of bacteria have been found as the main cause of mastitis in camels including *Staph. aureus*, *Strep. agalactiae,* Coagulase Negative Staphylococci (CNS), *Strep. bovis*, *Escherichia coli*, *Micrococcus* spp., *Corynebacterium* spp. and *Aerobacter* spp. (Barbour *et al,* 1985; Abdurahman, 1995; Obeid *et al,* 1996; Wernery *et al,* 2008; Johnson *et al,* 2015b). Some *Staph. aureus* isolates are drug resistant and are conserved as public health threat (Fitzgerlad, 2012; Ansari *et al,* 2014). As a result, livestock-originated *Staph. aureus* is of significance in terms of human health and are required to be further characterised (Fitzgerlad, 2012). Saleh and Faye (2011) found *S. aureus* and other species of staphylococci as main causative agent for subclinical mastitis in dromedaries.

The objective of this study was to investigate the frequency and type of bacteria causing subclinical mastitis and to find the possible genotypes of principle isolate from milk samples of dromedary camels.

Materials and Methods

Experimental design

Camel milk samples were collected from 243 quarters of milking camels (7-11 years of age, 2-4 months after calving), without any observable disease and clinical signs of mastitis. Prior to sampling, the teat was washed and the camel calf was released to stimulate milk let down from the dam. Immediately after teat engorgement, the suckling was interrupted and teats were disinfected with cotton moistened with 70% alcohol. After discarding the first few squirts of milk, about 50 ml of milk were collected into sterile bottle and kept cool these were transported to the laboratory for bacteriological investigations.

Bacteriological investigation

Bacteriological isolation was conducted according to the standard procedure (MacFaddin,

SEND REPRINT REQUEST TO A. NIASARI-NASLAJI email: niasari@ut.ac.ir

2000). In brief, milk sample (30-50 µL) was streaked on 5% sheep blood agar (Merck, Germany) and MacConkey agar (Merck, Germany). Plates were incubated at 37ºC for 5 days. Presumptive identification of bacteria on primary culture was conducted according to the morphology of colony, haemolytic characteristics, Gram stain and catalase test. *Staphylococci* were identified based on coagulase test. CNS were identified using classic chemical procedure (MacFaddin, 2000). *Streptococci* isolates were evaluated based on CAMP reaction, aesculin hydrolysis test and growth on 6.5% sodium chloride (MacFaddin, 2000). Gram-negative isolates were further tested using triple sugar iron (TSI), urease, indole, MRVP, citrate and lysine decarboxylase tests.

Isolation of Staphylococcvs aureus

The species of isolated *S. aureus* was identified using standard biochemical methods including gram staining, catalase, DNase, fermentation of mannitol and production of coagulase. To confirm the identity of the species, the nuc gene was amplified by a PCR-based method, using the specific primers (nucA-F 5′-CTGGCATATGTATGGCAATTGTT-3′ and nucA-R 5′-ATTGACCTGAATCAGCGTTGTCT-3′).

PFGE analysis

The entire genomic DNA was prepared as described previously (Fatholahzadeh *et al,* 2009). After digestion with *SmaI* endonuclease, the DNAs were separated by CHEF electrophoresis system (AP-Zoha Ltd, Tehran, Iran) for 20 h at 14ºC with an electric field of 6 V/cm in 0.5× TBE buffer. The pulse time increased from 1 to 30 s (10 h) and 1 to 3 s (10 h). The gels were stained with ethidium bromide (1µg/ml) and visualised by UV illumination. A reference strain of *S. aureus* (NCTC8325) was prepared in the same way and run as the molecular size standard. The assessment and interpretation of PFGE patterns were performed visually according to the defined criteria (Tenover *et al,* 1995).

SPA typing

The spa typing was performed according to the method described previously (Emaneini *et al,* 2011) followed by amplification and sequencing of the spa gene X region. PCR reactions were performed in a 50 µL volume consisting of 1X PCR buffer, 3 mM $MgCl_2$, and 0.4 µg/ml of each primer, 1.5 U Taq DNA polymerase, 0.2 mM dNTP Mix and 5 µl of DNA template. The PCR conditions consisted of a pre-denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 40 s, 55 °C for 40 s and 72 °C for 60 s. A final extension step was performed at 72 °C for 10 min. Sequences of both strands of the amplicons were determined at Macrogen (Seoul, South Korea). Isolates were assigned to particular spa types according to the guidelines described by the spa typing website (http://www.spaserver.ridom.de).

Results and Discussion

In 150 quarter milk samples (61.7%), no bacteria were isolated. Accordingly, quarter infection rate was 38.3% (93/243). Out of 129 bacteria isolated from 93 quarter milk samples, *Staphyloccoci* was isolated from 78 cases (60.46%), out of which 7 cases (8.97%) were *S. aureus* and the rest (71; 91.03%) were CNS (Table 1). CNS bacteria had a varied prevalence, i.e. *S. saprophyticus* (47.44%), *S. schleiferi* (16.67%) and *S. epidermidis* (14.10%). *Streptoccoci* were isolated from 26 cases (20.16%), out of which *Strep. uberis* (7.75%) and *Strep. mutans* (5.43%) had greater incidence (Table 1). The rest of bacteriological isolation was dedicated to coliforms (7.75%), *Pseudomonas* (5.43%),

Table 1. Mixed and pure bacteria isolation from quarter milk samples of dromedary camel.

Bacteria	Total	No. in Genus	Pure isolation	Mixed isolation	% in Genus	% in Total
Isolated bacteria	129					
Staphylococci	78					60.46
Staph. aureus		7			8.97	5.43
Coagulase Negative Staphylococci		71			91.03	55.04
Staph. saprophyticus		37	19	18	47.44	28.68
Staph. schleiferi		13	9	4	16.67	10.08
Staph. epidermidis		11	4	7	14.10	08.53
Staph. delphini		4	2	2	5.13	03.10
Staph. capitis		3	1	2	3.85	02.32
Staph. chromogenes		2	1	1	2.56	01.55
Staph. caseolyticus		1	1	0	1.28	0.77
Streptococci	26					20.16
Strep.uberis		10	2	8	38.46	07.75
Strep. mutans		7	1	6	26.92	5.43
Strep. agalactiae		5	2	3	19.23	3.87
Strep. alactolyticus		3	2	1	11.54	2.32
Strep. dysgalactiae		1	1	0	3.85	0.77
Coliform	10		1	9		7.75
Pseudomonas	7		1	6		5.43
Corynebacterium	6		3	3		4.65
Proteus	2		2	0		1.55

Corynebacterium (4.65%) and *Proteus* (1.55%; Table 1). There were no *E. coli* or pathogenic corynebateria isolated from the milk samples.

PFGE analysis of the 7 *S. aureus* isolates produced 2 distinct pulsotypes designated as pulsotypes A and B. All *S. aureus* isolates were found to be included into 2 spa types: t527 and t1532 (Table 2).

In the present study, no bacteria were isolated from 61.7% of milk samples (150/243), similar to the result reported from Israel (60.6%; 83/137; Chaffer *et al,* 2000) and UAE (65.87%; 195/297; Johnson *et al,* 2015b). Similar results were reported by in non-lactating dromedary camel after collecting swabs from teat canal (76%; 184/242) and cistern (79%; 189/242). This condition occurs in dairy cows with the prevalence of 30% (Philpot and Stephen, 2000). The low number of bacteria isolated from camel milk might be unique for this species. It could be explained by numerous antimicrobial agents in camel milk such as lysozyme, lactoferrin, lactoperoxydase and immunoglobulins that could limit microbial growth to higher degree than in milk from other domestic animals (Korhonen and Pihlanto, 2006; El-Hatmi *et al,* 2007; Salami *et al,* 2010).

Table 2. PFGE analysis and spa typing of 7 *S. aureus* isolates.

Isolation	PFGE	SPA Typing
17	A	t527
28	A	t527
39	A	t527
41	A	t527
167	B	t1532
174	B	t1532
220	B	t1532

Based on isolation of bacteria, quarter infection rate was 38.3% (93/243) in the present study. Several studies with great variation were reported to illustrate the prevalence of subclinical mastitis in dromedary camel, i.e. 36.87% (59/160 camels; Suheir *et al,* 2005), 15% (9/60 quarters; Alamin *et al,* 2013) in Sudan, 15.8% (80/505 quarters; Abera *et al,* 2010) and 20.7% (30/145 camels; Abera *et al,* 2010), 22% (43/195 camels, Almaw and Molla, 2000), 67.4% (433/642 quarters; Seifu and Tafesse, 2010), 39.4 % (137/348 camels, Regassa *et al,* 2013) in Ethiopia, 11.67% (21 /180 camels; Ibrahim *et al,* 2011) in Saudi Arabia, 38% (57/150 camels; Sibtain *et al,* 2012) in Pakistan and 41% (41/100 quarters) and 72% (18/25 camels) in India (Bhatt *et al,* 2004). Accordingly, it may be concluded that the prevalence of subclinical mastitis in camel could be within the range of 11-72% on specieswise and 15-67% on quarter basis, providing an indication that subclinical mastitis may be considered as existing problem in dairy dromedary camel.

The majority of isolates in the present study were of *Staphylococci* spp (60.46%) and *Streptococci* spp (20.16%). It was in consonance with previous investigations in Kenya (Wanjohi *et al,* 2013), Ethiopia (Woubit *et al,* 2001; Abera *et al,* 2010), Sudan (Alamin *et al,* 2013), Pakistan (Sibtain *et al,* 2012), India (Bhatt *et al,* 2004) and Israel (Chaffer *et al,* 2000). Among *Staphylococci* in the present study, CNS were isolated in the majority of cases (55.04%). CNS prevalence in camel was 46% in Iraq (Almaw and Molla, 2000), 20.4% in Israel (Chaffer *et al,* 2000) and 7.5% in Ethiopia (Abera *et al,* 2010). In the present study new species of CNS bacteria were identified including *S. saprophyticus*, *S. schleiferi*, *S. delphini*, *S. capitis*, *S. chromogenes* and *S. caseolyticus*. It was only *S. epidermis* that has been previously reported (Obeid *et al,* 1996; Suheir *et al,* 2005). *Staphylococci* are common inhabitants of the skin and the mucosal surfaces of humans and various animals. CNS constitutes part of the physiological flora (Werckenthin *et al,* 2001; Adegoke and Okoh, 2014). Most *staphylococcal* species are considered as facultative pathogen. The pathogenic capacity of CNS mainly depends on whether they possess virulence genes (Oogai *et al,* 2011). On the host side, the intact skin or mucosal surface represents the first mechanical barrier against these infectious agents. In addition, a functionally active host immune system as well as the tissue-specific commensal flora play important role as biological barriers against pathogenic bacteria, including *staphylococci* (Singh and Morris, 2012). Open injuries, burns, scratch and bite wounds and primary viral or parasitic infections, which could destroy this mechanical barrier, enable *Staphylococci* as inhabitants of the skin or the mucosa to reach deeper tissues and cause an either localised or generalised infection (Werckenthin *et al,* 2001). The latter happens mainly when the *staphylococci* disseminate *via* blood and can evade or suppress the host's immune system. Certain virulence factors of *Staph. aureus*, in particular the Panton-Valentine leukocidin, target and destroy components of the host immune system (Holzinger *et al,* 2012). Intramammary infections caused by CNS are common in dairy cows as well (Thorberg *et al,* 2009). In comparison with *S. aureus*, CNS has lower pathogenicity, but they could have an important role in subclinical mastitis and elevation of SCC in ruminants (Contreras *et al,* 2007; Thorberg *et al,* 2009). The prevalence of *S. aureus* was relatively low (5.43%) in the present study, similar to the report from Ethiopia (2.9 %, 39/1362; Regassa *et*

al, 2013), Israel (8.8%; Chaffer *et al,* 2000) and Sudan (5.4%; Abdurahman *et al,* 1995). In other studies, *S. aureus* was considered as the main cause of subclinical mastitis in camel in Saudi Arabia (27.91%; Ibrahim *et al,* 2011), Ethiopia (26.3%; Abera *et al,* 2010), and Sudan (20.2%; Suheir *et al,* 2005).

In this study the molecular characteristics of *S. aureus* was elucidated. There is few data about the genetic analysis of *S. aureus* isolated from camel milk (Shuiep *et al,* 2009; Monecke *et al,* 2011; Jaradat *et al,* 2014). Further studies from various geographical regions are needed to investigate the molecular characteristics of *S. aureus* by various typing methods and to determine the genetic nature of this bacterium. A variety of genotyping techniques are available for classifying *S. aureus* strains for epidemiological investigation, including band-based (e.g., PFGE) and sequence-based methods (e.g., MLST, spa typing). PFGE is the gold standard for typing of *S. aureus* strains and is known to be a highly discriminatory and valuable technique for outbreak investigation (Tenover *et al,* 1995; Eslampour *et al,* 2009). Nevertheless, spa typing contains significant advantages over PFGE such as ease of use, reproducibility, transportability and comparability of the results (Emaneini *et al,* 2011). To the best of our knowledge, this is the first study reporting the molecular characteristics of *S. aureus* isolated from camel milk by PFGE and Spa A typing methods. Results of the present study showed that all *S. aureus* isolates were of 2 genotypes. The presence of 2 genotypes of *S. aureus* might be the result of its increased resistance to the host immune response. Spa type t527 collected from different locations in Croatia, and in MRSA isolates of human and bovine samples in Iran. Spa type t1532 was found in 5 isolates from different locations in France and Portugal (http://www.spaserver.ridom.de). Concordance between PFGE results and spa typing technique in our study was 100%. Koreen *et al* (2004) showed that cross-classification concordance results between PFGE and spa typing was 98%.

The isolated *Streptococci* spp. in the present study were *Strep. uberis, Strep. mutans, Strep. alactolyticus, Strep. agalactiae* and *Strep. dysgalactiae.* Mastitis caused by *Strep. agalactiae* is prevalent in camel and has been reported in UAE (Quandil, 1984), Egypt (Karamy, 1990), India (Younan *et al,* 2001), Ethiopia (Almaw and Molla, 2000), Kenya (Younan *et al,* 2001) and Sudan (Abdurahman *et al,* 1995; Obied *et al,* 1996). Subclinical mastitis caused by *Strep. agalactiae* consisted of up to 74.4% and 50% of all cases of subclinical mastitis in India and Kenya, respectively (Younan *et al,* 2001). In addition, clinical mastitis due to *Strep. agalactiae* has been reported in Ethiopia (Almaw and Molla, 2000).

Taken together, the prevalence of subclinical mastitis was relatively low in the present study. It is well known that susceptibility to mastitis is determined by a combination of factors including bacterial virulence, environmental conditions (housing, management, feeding and milking technique) and animal-related factors (milk yield, genetics). These factors are interdependent to each other and their impact depends on the type of pathogen (Burvenich *et al*, 2003). The streak canal is relatively thin in camel which could play a role in low prevalence of mastitis in this species (Manefield and Tinson, 1996). Moreover, the cover used to prevent the calf from suckling has been suggested as a reason for low rate of mastitis in camel (Manefield and Tinson, 1996; Wernery and Kaaden, 2002). It is believed that the cover protect the animal from mechanical traumas. Yet it should be considered that the cover could be moistened with milk and become contaminated with bedding, and consequently predispose the animal to intra-mammary infections. Nevertheless, no research has been conducted in this regard, any hypothesis requires to be tested by a well-designed controlled study. In addition, machine milking is uncommon in camel, which might have contributed as additional reason for low prevalence of mastitis in this species. The other suggested factors for low prevalence of mastitis in camel are the type of resting, few contact of mammary glands with the bedding, low density of animals in the pasture and the dryness of bedding. Finally, one of the main potential factors in this context is the antimicrobial components of camel milk (Korhonen and Pihlanto, 2006; El-Hatmi *et al,* 2007; Salami *et al,* 2010). Further studies are warranted to investigate the underlying mechanisms for low prevalence of mastitis in camel.

In conclusion, *Staphylococci*, particularly coagulase negative staphylococci were the most frequent bacteria isolated from camel milk. Five new species of CNS were identified and *S. aureus* was characterised into 2 genotypes in camel milk samples.

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