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RESEARCH ARTICLE

Identification of Coagulase Gene in *Staphylococcus aureus* Isolates Recovered from Subclinical Mastitis in Camels

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ABSTRACT

The current study was conducted to estimate coagulase positive *Staphylococcus aureus* (*S. aureus*) being a nuisance pathogen among mastitis etiology from subclinical cases of camel mastitis and to investigate the association of assumed risk factors. A total of 235 subclinical positive milk samples were collected from camels kept at different localities of Cholistan desert, Punjab Pakistan by using Surf Field Mastitis Test (SFMT). Data regarding some possible risk factors associated with subclinical mastitis were also collected and analysed by chi-square techniques at 95% confidence interval. The study revealed 50.54% (235/465) prevalence of subclinical mastitis in camels. *Staphylococci* were cultured in 74.04% (174/235) of milk samples from subclinical camel mastitis. Results showed 88.5% (154/174) prevalence of coagulase positive *S. aureus* using slide and tube agglutination techniques, while 54.02% (94/174) prevalence of coagulase positive *S. aureus* was confirmed by PCR technique.The quarter based prevalence of subclinical mastitis was examined in 30.93% (520/1681) quarters, whereas 9.6% (179/1860) quarters were blocked in camels. Chi-square and Odd's ratio analysis showed significant ($P<0.05$) association of mastitis with age, parity and udder pathology while non significant association with unhygienic condition of udder, lack of teat dipping practice, lactation status and feeding management. It was concluded that coagulase positive *S. aureus* was the major cause of mastitis in camel and there was positive association of some risk factors with occurring of mastitis.

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INTRODUCTION

Camel population in Punjab is estimated to be one third of the total camel population in Pakistan. Camel serves as the major animals for milk purpose, transportation and food for people living in desert areas (Hussain *et al.,* 2016). Camel milk is enriched with nutrients that make it acceptable as staple food with additional characteristics of higher minerals and other valuable contents. The lower sugar levels, higher vitamin C and low level of cholesterol as compared to cattle milk make it healthy food for people living in semi-arid and arid areas (Mullaicharam, 2014; Ali *et al.,* 2016). The milk yield in camels is ensured even in stress conditions like drought period with peak production on pasture (Khan and Iqbal, 2001). The quality and quantity of milk is deteriorated with mastitis. Mastitis in camels has been over looked in Pakistan. Camel mastitis has been reported in all camel rearing countries including Pakistan (Ahmad *et al*., 2012; Ali *et al.,* 2016). Mastitis, the inflammatory disease of mammary glands not only decreases the milk production but also makes human and suckling calves vulnerable to poor health. This transfer continues to other animals and hence to herd resulting in public challenge (da Silva *et al*., 2004). In camels, epidemiological studies on mastitis are already very few which need to be given more attention regarding S*taphylococcus aureus* (*S. aureus).* Mastitis caused by *S. aureus* has been

documented to be one of the most important udder infections in dairy animals (Qayyum *et al.,* 2016a; Qayyum *et al.,* 2016b). *S. aureus* has ability to survive even at high environmental temperature and higher salt concentration. It has been reported that *S. aureus* is frequently isolated from clinical, subclinical and chronic udder infections (Khan *et al.,* 2013). *S. aureus* is known to associate with decreased milk production for longer period starting from initial reaction in mammary glands (Hussain *et al.,* 2012). Recently comprehensive plans have decreased mastitis incidence but *S. aureus* still remains eminent organism in dairy animals affiliated with both subclinical and clinical mastitis all over the world (Momtaz *et al*., 2011; Qayyum *et al.,* 2016b). The mastitis caused by *S. aureus* results in culling of animals because of its resistance to antibiotics.

Coagulase positive *S. aureus* has ability to yield toxins such exfoliative, toxic shock syndrome toxins and panton-valentine leucodin (Unal *et al*., 2012).The traditionally infectious agents are identified on the basis of different tests like bacterial culturing, gram staining and biochemical procedures, which are being considered as gold standard bacterial identification protocols. However, drawbacks like unable to identify non-cultivable organisms and ambiguity in characterizing at strain level is crucial for exact diagnosis. Polymerase chain reaction serves the purpose as specific gold standard test for bacterial identification (Khan *et al.,* 2013; Qayyum *et al.,* 2016b). Polymerase chain reaction has been effective and simple technique to diagnose coagulase positive *S. aureus* from humans and animals (Khan *et al.,* 2013; Qayyum *et al.,* 2016b). Early and accurate identification of pathogenic organism is needed not only for the sake of treatment but also to monitor farm level infection (Aqib *et al.*,2017).Studies are scanty to estimate the prevalence of mastitis with reference to coagulase positive *S. aureus* in camel. The mammary gland infection in camel is equally important and might be given much attention as is in case of bovines for effective control plan. Therefore, keeping in view the importance of the issue this study was designed to estimate the prevalence of coagulase positive *S. aureus* in camel mastitis and to find some risk factor associated with mastitis.

MATERIALS AND METHODS

Study area and sampling methodology: Camel herds kept at various desert localities including tehsils Bahawalpur, Ahmadpur East, Liaqatpur of district Bahawalpur and district Raheem Yar Khan were included to estimate subclinical infection. All the lactating camels were screened to determine the subclinical mastitis using Surf Field Mastitis Test (SFMT). Milk samples (10mL) were collected per standard protocol from a total of 465 animals (Schalm *et al.,* 1971). All the milk samples were tested by SFMT. The severity of subclinical infection (0 to 3) was determined on the basis of SFMT reaction as (0=no or trace, 1=weak positive, 2=moderate positive and 3=strong positive. The positive milk samples were transferred, to laboratory of Microbiology, Faculty of Veterinary Science, University of Veterinary and Animal Sciences, Lahore for further microbiological examination. The information regarding age, parity, involvement of quarters, udder hygiene, lactation status, feeding status, udder pathology, milking frequency, body condition and application of teat dips were also recorded.

Microbiological examination: All the positive milk samples were cultured on blood agar plates (Columbia agar base supplemented with 5% defibrinated sheep blood) for 24 and 48 hours at 37ºC. Sub-culturing was done by streaking colonies on mannitol salt agar as selective medium. The purified colonies were subjected to Gram's staining and catalase test. The *Staphylococci* (n=174) identified on the basis of biochemical tests were subjected to slide and tube coagulase method to determine the coagulase positive *S. aureus* (Khan *et al*., 2013).

Extraction of DNA and quantification: The biochemically identified *Staphylococci* were further analyzed to determine the presence of coag gene (coagulase gene) by polymerase chain reaction technique (PCR). For this purpose bacterial DNA was extracted by using DNA extraction kit (GF-1 Nucleic Acid extraction kit, Vivantis Technologies Sdn. Bhd, Malaysia) and stored at -20ºC. The previously designed primers forward Coag 2 (CGA GAC CAA GAT TCA ACA AG), reversed Coag 3 (AAA GAA AAC CAC TCA CAT CA) approximately 970bp product size were used for bacterial confirmation (Momtaz *et al*., 2011). A total of 25 µl PCR reaction mixture containing 12.5 µl of master mix, 1.5µl each forward and reverse primer, 8µl distilled water and 1.5µl DNA template was processed. The PCR conditions included initial denaturation at 94ºC for 10 min for single time, denaturation at 94ºC for 45 seconds followed by annealing for 1min at 54ºC, extension at 72ºC for 2 min and final extension at 72ºC for 10 minutes. Amplified PCR products were stained with ethidium bromide stain (Qayyum *et al*., 2016b) and photographed under ultraviolet illumination.

Statistical analysis: Risk factors associated were analyzed by MH Chi square (Hussain *et al.,* 2013). The Odd's ratio was used to estimate the association with an exposure and outcome. Results showing $P<0.05$ were considered significant.

RESULTS

Prevalence of subclinical mastitis and association of risk factors: An overall 50.54% (235/465) prevalence of subclinical mastitis was recorded in camel present at Cholistan desert of Pakistan. The comparison of different localities revealed non-significant difference ($P>0.05$) of prevalence of subclinical mastitis in camels. However, higher prevalence of subclinical camel mastitis 55.56% (80/144) was recorded at Ahmadpur East tehsil followed by tehsil Liaqatpur 48.44% (109/225) and tehsil Bahawalpur 47.91% (46/96). *Staphylococci* appeared at higher percentage (74.04%) in sub-clinically infected camels. Ahmadpur East tehsil was found harboring slightly higher percent of *Staphylococci* than tehsil Bahawalpur and tehsil Liaqatpur with 75.00, 73.39, and 73.91%, respectively. The quarter based prevalence of subclinical mastitis revealed 30.93% (520/1681) infected quarters. The 37.80% right rear (RR) quarters were infected

Table 1: Bivariate frequency analysis of different parameters in mastitic and healthy camels from district Bahawalpur and Raheem Yar Khan

Parameters	Positive		Negative	95% C.I.	MH Chi-sq P value/ Odd Ratio
	N	%			
Age groups (Years)					
1-4	45	27.27	120	20.89- 34.45	0.001
5-7	113	62.43	68	55.20- 69.26	
8-11	77	59.69	52	51.05- 67.90	
Lactation status					
Dry	152	64.96	82	58.68- 70.87	3.31/0.30
Lactating	83	35.93	148	29.94- 42.28	
Parity					
1	11	13.25	72	7.17-21.86	0.041
2-3	37	22.70	126	16.76-29.60	
>4	68	31.05	151	25.19-37.41	
Teat/udder Pathology					
Normal	40	20.0	160	14.89- 25.97	P = 0.001
Udder ticks	45	78.95	12	66.95- 88.06	
Inflammation/swelling	38	61.29	24	48.78-72.77	
Teat skin abrasions	54	77.14	16	66.24- 85.84	
Necrosis at teat/udder	58	85.29	10	75.35-92.28	
Body condition					
Normal	94	44.98	115	38.32-51.77	
Thin	141	55.08	115	48.94- 61.10	0.67 / 1.50
Feeding system					
Well fed	84	35.90	150	29.94- 42.20	
Underfed	151	65.37	80	59.06-71.30	0.30 / 3.37
Teat dips					
No	178	68.46	82	62.62- 73.89	
Yes	57	27.80	148	22.00 -34.24	5.64 / 0.18
Milking Frequency					
Once	111	49.78	112	43.24- 56.32	
More than once	124	51.24	118	44.95- 57.50	0.94 / 1.06

Table 2: Prevalence of coag gene from *staphylococci* in camels in district Bahawalpur and Raheem Yar Khan

Herd area	Total examined	Positive	Prevalence (%)	P-value	95% CI
Bahwalpur	34	16	47.06		30.87- 63.73
Ahmadpur East	60	36	60.00		47.19- 67.10
Liaqatpur	80	42	52.50	0.733	41.56- 63.26
Overall	174	94	54.02		46.58- 61.33

with subclinical mastitis followed by front right (FR), front left (FL) and rear left (RL) with 32.53, 28.17 and 25.58%, respectively. Results showed 9.6% (179/1860) of blocked quarters in camel. The study found right side of udder was blocked more than that of left side. However, there was no significant difference (P>0.05) among frequency of teat blockage. Results revealed that 11.83% right rear teats were blocked while 7.53, 8.39 and 10.75% prevalence of block quarters in camels in rear left (RL), front left (FL) and front right (FR) quarters was recorded respectively. The results showed significant association of different risk factors with subclinical mastitis (Table 1). Significantly increased prevalence of mastitis was recorded in adult animals (5-7 year of age) than young animals (1-4 year) and older ones (>8 year). Results showed higher prevalence of subclinical mammary gland infection in dry animals than lactating animals. The odd's ratio revealed that animals in dry status were 3.37 times more prone to mastitis. The occurrence of mastitis was significantly increased with increase in parity number. Results revealed significantly higher prevalence of mastitis in animals of second and third parity groups. Udder pathologies were strongly (P<0.001) associated with occurrence of mastitis in camels. Among various determinants of udder pathology, animals with teat lesions, skin abrasions, necrosis at udder and swelling showed significantly high prevalence of mastitis. Thin body condition was shown to be 1.5 times more prone to mastitis occurrence. The chances of occurrence of mastitis were increased in camels in which the teat dipping was not carried out. The frequency of infection was non significantly increased with increase milking frequency.

Prevalence of coagulase positive *Staphylococcus aureus*

A total of 88.05% (154/174) of coagulase positive *S. aureus* isolates were identified by slide/coagulase test from biochemically coagulase negative *Staphylococci*. No significant results were obtained in prevalence of *S. aureus* on the basis of different localities (P>0.05). However, Tehsil Bahawalpur revealed higher prevalence coagulase positive *S. aureus* followed by Liaqatpur and tehsil Ahmadpur East with 91.18, 90 and 85% respectively. Coagulase positive *S. aureus* (Fig. 1) was identified from *Staphylococci* (n=174) by PCR techniques. Results revealed 54.02% (94/174) prevalence of coag gene (Table 2). Conventional technique presented 38.96% higher coagulase positive *S. aureus* compared to molecular technique. Localities did not display significant results in prevalence of coagulase positive *S. aureus* (P>0.05), however Ahmadpur East had higher percent of coagulase gene followed by Liaqatpur and Bahawalpur with 60, 52.5, and 47.06% prevalence respectively.

DISCUSSION

Prevalence of subclinical mastitis in camel 50.54% (235/465) in present study is closely related with results of previous studies (Ahmad *et al*., 2012; Aqib *et al.*, 2017) who reported 41, 57 and 46% of prevalence of subclinical mastitis in camels. However contrary to our results different studies reported lower prevalence 24.5, 24, 29, 24 and 22.5% of subclinical mastitis in camels (Abera *et al.*, 2009; Saleh and Fave, 2011; Al-Juboori *et al.*, 2013). In present study coagulase positive *S. aureus* isolates were also identified on the basis of tube and slide methods. Previously different studies reported 18% and 22% prevalence of coagulase positive *S. aureus* isolates recovered from infected milk samples in Fieri region of Albanaia (Sulaj *et al.*, 2013). The higher prevalence of coagulase positive *S. aureus* in current study might be due to poor immune status of animal and virulence of infectious agent (Ibrahim *et al.*, 2011). The higher frequency of *S. aureus* in mammary gland infections in dairy animals could be due to ability of *S. aureus* to stay and survive in keratin layers of teat canal of animals (Ibrahim *et al.*, 2011; Khan *et al.*, 2013; Qayyyum *et al.*, 2016b). In present study coagulase positive *S. aureus* isolates were confirmed by amplification of coagulase gene. It has been reported that molecular based investigations are effective tools to identify the circulating infectious strains for eradication and control measures. Previously in camels no reports is available about the frequency of coagulase positive *S. aureus* isolates and less data is available regarding subclinical mastitis (Ali *et al.*, 2016). In our study PCR products about 970bp were amplified for coagulase genes suggestive of *S. aureus* infections. Previously, in contrast to these results different amplicons of coagulase gene (390bp, 500bp, and 600 bp) showing coagulase gene polymorphisms in Cholistani cattle have also been reported (Qayyum *et al.*, 2016a).

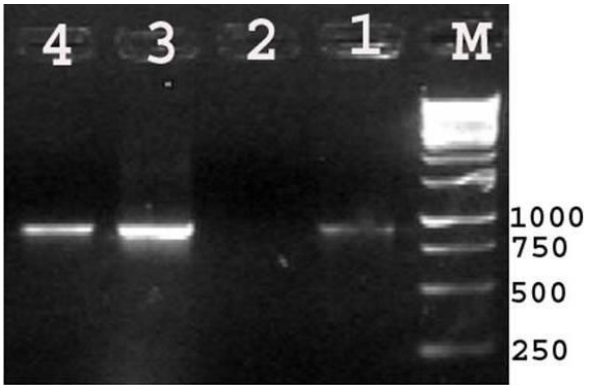

Fig. 1: PCR amplification and gel electrophoresis of coagulase gene approximately 970bp (Lane: 1, 3, 4) stained with ethidium bromide. M: 1kb DNA marker.

Quarter based prevalence (30.9%) of subclinical mastitis in present study is less than (39.72% and 45.3%) to previous studies (Tuteja *et al.,* 2003; Ahmad *et al.,* 2012). Results showed that the prevalence of mastitis was more in right rear quarters. However, different earlier studies in camels have reported higher prevalence of mastitis in left rear (Shittu *et al.,* 2012) and right front quarters (Tuteja *et al.,* 2003). Higher prevalence in right quarters in present study might be due to relaxed teat sphincters in association with high milk yield in these quarters (Hussain *et al.,* 2013; Zenebe *et al.,* 2014; Qayyum *et al.,* 2016). Significantly higher prevalence of mastitis in rear quarters has also been reported in cattle (Hussain *et al.,* 2012b; Tripura *et al.,* 2014; Qayyum *et al.,* 2016b). In present study 9.62% prevalence of blind quarters was recorded which is higher than previous reports in Pakistan (Ahmad *et al.,* 2012**).** The increased prevalence of blocked quarters in present study might be due to development of fibrosis, teat cistern and teat canal lesions. The prevalence of mammary gland infection in current study was significantly increased with increase in age of camel which is in line with findings of previous studies in camels (Zeleke and Bekele, 2000; Ahmad *et al.,* 2012). These results might be due to more prone to disease susceptibility as the animals in later age have poor immunity and repeated exposure of infections. Higher susceptibility of mastitis occurrence at middle age of animals is contrary to the findings of earlier report (Ahmad *et al.,* 2012). Higher prevalence of mastitis during 3rd and 4th parity was associated with higher milk production (Raziq *et al.,* 2008). Previously similar reports are also available about the prevalence of mastitis in dry animals (Tuteja and Dixit, 2007). Results showed that different udder pathological lesions were strongly associated with occurrence of mastitis in camels. Animals having different teat and udder lesions such as skin lesions, necrosis at teat and inflammatory conditions revealed increase rate of infection. The increased prevalence of mastitis in camels having teat and udder lesions might be due to higher susceptibility of infectious agents to disseminate in udder (Hussain *et al.,* 2013). Increased prevalence of mastitis in animals having teat and other pathological lesions has also been reported (Abdurahman, 2006; Hussain *et al.,* 2013). Animals with tick infestation on their udder and animals having thin body condition showed higher prevalence of mastitis which might be due to poor health and immune status. Previously it is reported that the infestation of ticks results in bacterial entry into teat canal (Woubit *et al.,* 2001). Furthermore higher prevalence of mastitis in camels with thin body condition might be due to poor efficiency of immune response. The results of this study suggest that age, parity, lactation stage and different pathological lesions are useful predictor of udder infection and presence of different coagulase positive *S. aureus* isolates in camels.

Authors contribution: Milk sampling, data collection and interpreted the results was made by AIA, MI, AR, SS and SHF. Data analysis was made by RH and MI. The manuscript was written by AIA, MI, AZD and RH. All the authors read the manuscript and approved the contents.

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