Pakistan J. Zool., vol. 51(1), pp 307-315, 2019. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2019.51.1.307.315

Drug Susceptibility Profile of *Staphylococcus aureus* Isolated from Mastitic Milk of Goats and Risk Factors Associated with Goat Mastitis in Pakistan

Amjad Islam Aqib[1,*], Shagufta Nighat[2], Rais Ahmed[3], Saba Sana[3], Muhammad Ameen Jamal[4], Muhammad Fakhar-e-Alam Kulyar[2], Naimat Ullah Khan[5], Mian Saeed Sarwar[5], Muhammad Asif Hussain[5], Asadullah[5], Attaur Rahman[5] and Sadeeq ur Rahman[5,*]

[1]*Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan*

[2]*Department of Zoology, Government Postgraduate College for Women, Sub Campus, University of Gujrat, Rawalpindi, Pakistan*

[3]*Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan*

[4]*Yunnan Agricultural University, Kunming, China*

[5]*College of Veterinary Sciences and Animal Husbandry, Abdul Wali Khan University, Mardan, Pakistan*

Article Information
Received 07 April 2018
Revised 24 May 2018
Accepted 13 June 2018
Available online 21 December 2018

Authors' Contribution
AIA, RA, MFAK and SS conceived the idea and did research work. AIA, SR and MAJ analyzed the data. MAH, AIA, AR, NUK and Asadullah wrote the article.

Key words
Goat milk, Subclinical caprine mastitis, *Staphylococci*, Coagulase positive, Coagulase negative.

ABSTRACT

The current study investigates the incidence of subclinical mastitis (SCM), associated risk factors, involvement and antimicrobial susceptibility of *Staphylococcus aureus* in the development of SCM in District Faisalabad of Pakistan. For this purpose, a total of 384 goat milk samples were screened for SCM through surf field mastitis test (SFMT) and mastitis-positives cases were further investigated for isolation of *S. aureus* using standard procedures. Coagulase gene was PCR amplified from the clinical isolates to categorize them into Coagulase positive *Staphylococci* (CPS) and coagulase negative *Staphylococci* (CNS). A questionnaire was used to record risk factors associated with occurrence of SCM and results were analyzed using non-probability statistical analysis. Results indicated that an overall 63.28% (243/384) of goats were found positive for SCM, of which 58.85% (143/243) revealed *Staphylococcal* growth among them 69.93% (100/143) were CPS, while 30.07% (43/143) were found as CNS. Drug susceptibility against penicillins, cephalosporins, macrolides and other drugs showed that 50% of CPS and 30% of CNS were found multi-drug resistant-exhibiting resistance against more than two or more than two classes of drugs. None of the CPS while 20 % from CNS isolates were 100% susceptible to all kinds of drugs tested. Finally, age, grazing system, use of beta-lactam antibiotics, parity, and poor hygiene were potential risk factors. Altogether, the study concluded high incidence of SCM and isolation rate of *staphylococci* that were found resistant against most of the commonly used antibiotics.

INTRODUCTION

Mastitis is an inflammatory condition of udder(s) and its surrounding tissues characterized by changes in physical and chemical characteristics of udder and milk (Khan and Khan, 2006). Three major types of mastitis, clinical mastitis (CM), sub-clinical mastitis (SCM) and chronic mastitis (ChM) are recognized in animals including goats. Changes in milk characteristics are readily observed in CM condition along with redness and pain in the udder, while, generally no obvious changes (except in somatic cell counts and presence of pathogenic organisms) in milk and clinical signs in the udder are observed in SCM. SCM adversely affects the milk production ability, deteriorates the quality of milk causing severe economic losses to farmers (Halasa *et al*., 2007). SCM in goats has been randomly reported from Pakistan (Ali *et al*., 2010; Islam *et al*., 2012; Najeeb *et al*., 2013) as well from other countries of the world (Ameh and Tari, 1999; McDougall *et al*., 2002), and is considered a challenging disease all around the world. In fact, SCM remains one of the most

* Corresponding authors: sdeeeq@awkum.edu.pk; amjad.aqib@uaf.edu.pk
0030-9923/2019/0001-0307 $ 9.00/0

important infectious diseases of small ruminants and as such it is crucial to identify the cause of SCM in order to prevent its occurrence.

Goat farming is an important source of income of Pakistani farmers. The country witnesses an estimated population of more than 63 million goats producing 0.799 million tons of goat milk and 0.629 million tons of mutton (Rehman *et al*., 2017; Wasti, 2015). An overall country-wide comprehensive data regarding the prevalence of SCM in goats in Pakistan is not available, and published reports are random studies from different regions of the country (Ali *et al*., 2010; Najeeb *et al*., 2013). A study from Punjab indicated an incidence rate of 45% of subclinical mastitis in goats (Najeeb *et al*., 2013), while it has been reported as 53.3% in Kohat region of Khyber Pakhtunkhwa province of Pakistan (Ali *et al*., 2010). Furthermore, information regarding associated risk factors of occurrence of SCM are also crucial for designing control strategies of SCM (Megersa *et al*., 2010). In Pakistan, SCM in goats is usually treated by broad spectrum antibiotics in combination with anti-inflammatory drugs (Khan and Khan, 2006). Due to recurrent mastitic-infection, long term usage of antibiotics remains the only option resulting emergence of antimicrobial resistance in mastitis causing pathogens (Ali *et al*., 2016, 2017; Rahman *et al*., 2018b). Knowledge regarding mechanism of pathogenicity and development of drug resistance in mastitis causing pathogens in goats is vital to understand transmission frequency, improving management strategies and designing effective therapeutic interventions (Merz *et al*., 2016).

Staphylococcus aureus has been commonly isolated from raw milk and in addition to its key involvement in the development of SCM in dairy animals is also found associated with food poisoning (Najeeb *et al*., 2013; Shamila-Syuhada *et al*., 2016). Since, goat herds in Pakistan are either smaller or its farming has not yet been mechanised, therefore, are commonly milked by hands. In such scenario, risk of mastitis due to *S. aureus* may be increased mainly due to poor hygiene practices of milk handlers and herd management (Popov *et al*., 2014). *Staphylococci* are divided into two categories based on coagulase production *i.e.* coagulase positive *Staphylococci* (CPS) and coagulase negative *Staphylococci* (CNS). Coagulase production has remained an important phenotypic determinant in *S. aureus* and has been often associated with virulence (Moreillon *et al*., 1995). Furthermore, coagulase gene of *S. aureus* has also been frequently implicated in typing of clinical isolates based on its polymorphism (da Silva and da Silva, 2005). *Staphylococcus* spp. are the most commonly diagnosed causative micro-organisms (Contreras *et al*., 2003; Marogna *et al*., 2012). The aim of this study was to investigate the incidence of SCM in goats and involvement of *S. aureus* and associated risk factors in the development of SCM in District Faisalabad of Pakistan.

MATERIALS AND METHODS

Ethical approval

This study was approved from the ethical committee of University of Agriculture, Faisalabad, Pakistan and all procedures of animals handling and sample collection were performed following local and national guidelines of animal ethics.

Milk sample collection

The goat milk samples (n=348) from goats with history of milk drop were obtained from herds located in sub-districts Faisalabad, Samundary and Jaranwala of Punjab province. Subclinical mastitis was determined by surf field mastitis test (SFMT) as reported earlier (Schalm *et al*., 1971). A questionnaire was used to assess risk factors associated with incidence of subclinical mastitis.

Isolation of staphylococci and phenotypic identification of coagulase positive and negative isolates

Milk samples were initially streaked onto blood agar base (Columbia agar base supplement with 5% sheep blood) and incubated aerobically at 37 °C for 24-48 h. Tentative *Staphylococcus* isolates were identified based on colonial morphology and gram staining, and presumptive colonies were further streaked on Staph 110 medium to confirm Staphylococcus as described earlier (Aqib *et al*., 2017; Memon *et al*., 2012). The purified colonies were then confirmed again on colonial morphology, gram staining followed by catalase test and, and were further subjected to slide and tube coagulase test in order to identify coagulase positive and negative *Staphylococcus* isolates (Khan *et al*., 2013).

PCR based identification of coagulase positive isolates

Beside phenotypic identification of coagulase positive and negative *Staphylococcus* isolates were also confirmed on molecular level by targeting amplification of coagulase gene. For this purpose the chromosomal DNA were extracted using commercial kit (VivantisTechn., Sdn, Bhd, Malaysia) according to manufacturer's instruction. Quality of extracted DNA was evaluated through gel electrophoresis as well as by Nano-Drop (Thermo-Scientifics, USA). PCR was used to amplify coagulase gene with primers For: 5`CGA GAC CAA GAT TCA ACA AG 3`, and Rev: 5`AAA GAA AAC CAC TCA CAT CA3` (Annemüller *et al*., 1999; Goh *et al*., 1992). PCR reaction was performed in a total of 25 µL reaction mixture with 12.5 was master (Accuprim TM super mix11), 1.5 µL of

10 picomole of each primer, and 1.5 µL extracted DNA as template and 8 µL distilled water. PCR conditions were set as initial denaturation at 94ºC for 3 min. followed by 30 cycles of denaturation at 94ºC, followed by annealing at 54ºC for 30 sec and elongation at 72ºC for 1 min in thermocycler (Eppendorf-Mastercycler ®5330, Germany) with a final extension time of 5 min. at the end of all cycles. A PCR amplicon of 970 bp resolved on 1% agarose gel was considered as coagulase positive isolate (Annemüller *et al*., 1999; Goh *et al*., 1992).

Antibiotic susceptibility profile

All isolates including those confirmed for *coag* gene through PCR were put to antibiotic susceptibility by Kirby-Bauer disc method on Muller Hinton agar and interpreted as described by Clinical and Laboratory Standard Institute (CLSI, 2015). Antibiotic disks used were amoxicillin/calvalanic acid, ampicillin, amoxicillin, oxacillin, cefotaxime, cefoxitin, trimethoprim+sulphamethoxzole, gentamicine, amikacin, fluoroquinolone, ciprofloxacillin, enrofloxacin, vancomycin, chloramphenicol and linezolid. The zones of inhibition (mm) formed around disks were measured after incubation of 24 h at 37°C. The zones of each antibiotic disk were compared against the standards as mentioned by CLSI in order to find out resistant, sensitive or intermediate susceptibility. *Staphylococcus aureus* ATCC 25923 (American Type Culture Collection, Rockville, Maryland, USA) was used as reference quality control strain for the above experiment.

Table I.- Prevalence of subclinical mastitis; and coagulase positive and coagulase negative *Staphylococci* from different sub-districts.

Site		Subclinical mastitis*	Coagulase positive *Staphylococci***	Coagulase negative *Staphylococci***
Samundary sub-district	No. observed	90/128	35/55	20/55
	Prevalence	70.31	63.64	36.36
	CI (95%)	61.9-77.54	50.43-75.07	24.93-49.57
Jaranwala sub-district	No. observed	75/128	40/53	13/53
	Prevalence	58.59	84.90	24.53
	CI (95%)	49.93-66.75	62.43-85.07	32.65-58.54
Faisalabad sub-district	No. observed	78/128	25/35	10/35
	Prevalence	60.94	71.43	28.57
	CI (95%)	52.29-68.96	54.95-83.67	16.33-0.45.05
Overall	No. observed	243/384	100/143	43/143
	Prevalence	63.28	69.93	30.07
	CI (95%)	58.22-68.07	61.97-76.84	23.16-38.03

*Among different sub-districts subclinical prevalence, p=0.120; **Coagulase positive and coagulase negative *Staphylococci* among different sub-districts p=0.397; p<0.05, significant difference.

Table II.- Prevalence of coagulase positive and coagulase negative *Staphylococci* (PCR based) with hemolytic characteristics.

Hemolysis toxins		Coagulase positive *Staphylococci***	Coagulase negative *Staphylococci***	Total
Alpha hemolysis	No.	25	6	31
	Prevalence	25 (25/100)	13.59 (6/43)	21.68 (31/143)
	CI (95%)	0.1712-0.3484	0.058-0.2862	0.1541-0.295
Beta hemolysis	No.	30	8	38
	Prevalence	30 (30/100)	18.60 (8/43)	26.57 (38/143)
	CI (95%)	0.2145-0.4011	0.0892-0.3391	0.197-0.3472
Alpha-beta hemolysis	No.	35	4	39
	Prevalence	35 (35/100)	9.3 (4/43)	27.27 (39/143)
	CI (95%)	0.2591-0.4526	0.0302-0.2305	0.2032-0.3546
No hemolysis	No.	10	25	35
	Prevalence	10 (10/100)	58.14 (25/43)	24.48 (35/143)
	CI (95%)	0.0516-0.1804	0.4221-0.7263	0.1785-0.3251
Total		100	43	143

Statistical analysis

Descriptive statistics was used for quantification of antibiotic susceptibility results. The prevalence was calculated as per formula described by Thrusfield (2007) while association of disease determinants with mastitis was estimated by chi-square analysis at 5% probability using SPSS version 22.

RESULTS

Incidence of caprine mastitis, isolation and characterization of Staphylococcus *isolates*

In the current study, a total of 384 milk samples were analyzed by surf filed mastitis test and results indicated that 243 were found positive for subclinical mastitis suggesting a high frequency (63.28%) with non-significant difference among the sub-districts. The incidence rate of sub-district Samundary was found to be the highest (70.31%) followed by sub-district Faisalabad (60.94%) and sub-district Jaranwala (58.59%). A total of 143 *Staphylococcus* isolates were recovered from 384 milk samples with a prevalence rate of 37.24%. Phenotypic identification and subsequent molecular confirmation by amplifying coagulase gene through PCR indicated that 100 (69.93%) isolates were coagulase positive, while 43 (30.07%) were found coagulase negative (Table I). Coagulase positive isolates (84.90%) were recovered in higher number from Jaranwala sub-district as compared to the rest of the two sub-districts. Overall, the coagulase positive *Staphylococcus* (CPS) isolates recovered from these cases of mastitis were found 2.33 times higher than the Coagulase negative *Staphylococcus* (CNS) suggesting possible involvement of CPS in goat mastitis in the under study population. Furthermore, alpha-beta hemolysis on blood agar was found higher compared to other types of hemolysis. Interestingly, hemolysis intensity was noted two times higher in CPS as compared to CNS. Moreover, we found that 10% of CPS while 25% from CNS isolates showed no hemolysis on blood agar. Beta hemolysis was found 3.13 times lower than non-hemolytic isolates, while it was 1.37 and 2 times higher than the isolates displaying alpha and alpha-beta hemolysis, respectively (Table II).

Table III.- Antibiotic susceptibility profile of clinical isolates.

Antibiotics	Coagulase positive *Staphylococci* (%)			Coagulase negative *Staphylococci* (%)		
	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
Penicillin						
Amoxicillin Calvalanic acid	60	0	40	90	0	10
Ampicillin	40	0	60	60	0	40
Amoxicillin	50	0	50	70	0	30
Oxacillin	40	0	60	70	10	20
Average	47.5	0	52.5	72.5	2.5	25
Cephalosporin						
Cefotaxime	50	10	40	70	10	20
Cefoxitin	40	20	40	60	30	10
Average	45	15	40	65	20	15
Sulphonamide						
Trimethoprim+Sulphamethoxzole	80	0	20	90	0	10
Gentamicine	50	20	30	60	20	20
Amikacin	40	20	40	60	30	10
Average	56.67	13.33	30	70	16.67	13.33
Fluoroquinolone						
Ciprofloxacillin	70	20	10	80	10	10
Enrofloxacin	60	20	20	100	0	0
Average	65	20	15	90	5	5
Miscellaneous						
Vancomycin	70	0	30	90	0	10
Chloramphenicol	70	10	20	100	0	0
Linezolid	80	10	10	90	10	0
Average	73.33	6.67	20	93.33	3.33	3.33

Antibiotic susceptibility profile

Results of the antibiotic susceptibility profile are displayed in Table III. Different classes of antimicrobials were used. Among the penicillin group, amoxicillin was found to be the most effective with 50% of isolates were found susceptible. Overall, penicillin group of antibiotics were found less effective as compared to other groups of antibiotics, and CPS were found resistant against most of the antibiotics applied in comparison with CNS. Resistance against cefotaxime and cefoxitin was 40% in CPS, while it was 20% and 10% in CNS, respectively. Interestingly, 56.67% of CPS were found susceptible and 30% were found resistant against sulfonamides, while, 70% and 13.3% CNS were found susceptible and resistant, respectively. Of note, all CNS isolates were sensitive to enrofloxacin and chloramphenicol, whereas 80% of CPS were found susceptible against linezolid and Trimethoprim+Sulphamethoxzole, respectively.

Table IV.- Risk factor association with different disease determinants.

Parameters	Levels	Examined	No. positive	Prevalence (%)	CI (95%)	p-value
Grazing Type	Mixed with bovine	205	101	49.27	0.4226-0.563	0.000
	Alone	179	42	23.46	0.176-0.3048	
Milk yield	Low	206	78	37.86	0.3129-0.4489	0.000
	High	178	165	92.70	0.8757-0.959	
Parity	1-2	90	40	44.44	0.3409-0.5527	0.224
	3-4	164	60	36.59	0.2932-0.445	
	>5	130	43	33.08	0.2523-0.4195	
Feeding system	Grazing alone	185	135	72.97	0.6587-0.791	0.000
	Only stall fed	154	65	42.21	0.3664-0.533	
	Grazing plus stall fed	45	43	95.56	0.8364-0.9923	
Body condition	Thin	95	35	36.84	0.2735-0.4741	0.000
	Fat	103	76	73.79	0.6404-0.8174	
	Normal	186	32	17.20	0.1222-0.2357	
Milker's hygiene	Poor	201	185	92.04	0.8717-0.9523	0.000
	Good	183	58	31.69	0.3816-0.5632	
Housing	Congested	225	165	73.33	0.6696-0.7888	0.000
	Spacious	159	78	49.06	0.411-0.5707	
Milking hygiene	Practiced	120	40	33.33	0.2515-0.4259	0.000
	Nor practiced	264	203	76.89	0.7124-0.8174	
Farm hygiene awareness	Nominal to Basic	220	138	62.73	0.5594-0.6907	0.145
	Medium	134	95	70.90	0.6232-0.7826	
	Advanced	30	10	33.33	0.1794-0.5286	
Use of germicidal teat dips	Regular	60	10	16.67	0.087-0.2898	0.000
	Occasional	121	45	37.19	0.2872-0.4649	
	Never	203	188	92.61	0.8787-0.9566	
Antibiotics use in mammary gland ailments	Penicillin group	264	185	70.08	0.641-0.7546	0.000
	Other	120	58	48.33	0.3918-0.5759	
Peri-parturion hygiene	Observed	104	33	31.73	0.2314-0.4169	0.000
	Not observed	280	210	75.00	0.6942-0.7987	
Disease management assistance	Self	185	166	89.73	0.842-0.9354	0.000
	Veterinary assistant	125	65	52.00	0.4292-0.6095	
	Qualified veterinarian	74	12	16.22	0.0902-0.2701	
Kid milk feeding	Direct from doe	301	185	61.46	0.5568-0.6694	0.159
	Bottle feeding	83	58	69.88	0.2513-0.3903	

Risk factors of occurrence of caprine mastitis

Data regarding risk factors association with occurrence of subclinical goat mastitis is categorized in Table IV. Non-probability statistical tests proposed grazing type, milk yield, feeding system, body condition, milker's hand hygiene, milking hygiene, germicidal teat dip practice, antibiotics' use, peri-parturition hygiene, and disease management assistance were significantly associated with risk of onset of subclinical mastitis in the under study population of goats. Moreover, goats with high milk yield (92.7%), grazing plus stall fed (95.5%), poor hygiene of milker (92.0%) and self-management of disease (89%) were found highly associated with occurrence of subclinical mastitis in under study population of goats. Finally, factors like grazing alone (23.4%), parity (>5), normal body condition (17%), use of germicides before and after milking (16.6%) and asking help of a qualified veterinary professional during disease (16.2%) were found linked to healthy udder and low incidence of subclinical mastitis in the under study population of goats (Table IV).

DISCUSSION

Sub clinical caprine mastitis remains un-highlighted in Pakistan despite its high incidence rate in different regions of the country and its associated economic losses. Knowledge regarding the etiological agent(s) and risk factors associated with caprine mastitis is crucial in order to design control and effective therapeutic-strategy against mastitis. A significant population of farmers is associated with goat farming in Faisalabad, and is solely dependent on the goat-milk and meat production. Here, we highlight the incidence of goat SCM among random goat population of District Faisalabad, and further report on the drug susceptibility profile and risk factors associated with *Staphylococcus*-causing goat SCM.

Our study identifies 63.28% (243/384) incidence of SCM in goat population under study with more than 58% cases contaminated with *Staphylococcus* suggesting its involvement in the disease progression. Our study identified higher incidence rate as compared to other reports from other parts of Pakistan, such as Ali and colleagues reported 53% and Najeeb and colleagues recorded 47% incidence rate of SCM in goats (Ali *et al*., 2010; Najeeb *et al*., 2013). Our higher rate of SCM may be due to the factor that we choose randomly those apparently looking healthy goats with a history of drop of milk production over the last few weeks. We assume that the SCM incidence would be a bit lower in the region in normal population of milking goats. *S. aureus* has been previously reported as one of most important pathogens causing mastitis in goats (Ali *et al*., 2010; Goh *et al*., 1992; Islam *et al*., 2012; Najeeb *et al*., 2013). In our study, CPS were found to be the most prevalent causing SCM in goat population under study in agreement with other studies (Aqib *et al*., 2017; Goh *et al*., 1992); however, other reports suggested increased involvement of CNS in the development of SCM (Mishra *et al*., 2018). Furthermore, few of the CPS were non hemolytic possibly due to lack of expression of hemolytic toxins or absence of such encoding elements. Besides as a molecular marker for typing *S. aureus*, coagulase gene has been considered as an important phenotypic and virulent determinant among clinical isolates (da Silva and da Silva, 2005).

Mastitis is known for its reversion and irresponsiveness to treatment mainly due to development of antimicrobial resistance by mastitis-causing pathogens (Ali *et al*., 2016; Rahman *et al*., 2018b), and these resistant organisms can be shed in the environment (Adnan *et al*., 2017) or in milk (Ali *et al*., 2018; Aqib *et al*., 2017; Shamila-Syuhada *et al*., 2016; Rahman *et al*., 2018a, b) and thus could become a serious food poisoning issue as well. Hence it is important to imply antibiotics judiciously to treat mastitis cases and to reduce the chance of emergence of antibiotic resistance. Antibiotic susceptibility testing should preferably be performed prior to prescription of antimicrobials to avoid excessive and unnecessary usage of antibiotics. In the current study, we tested most of the commonly used antimicrobials against the clinical isolates of *S. aureus* in order to understand the level of drug resistance in the isolated pathogens. Our studies indicated that more than half of the isolates were found resistant against the commonly used antimicrobials including penicillin, cephalosporin, macrolides and other miscellaneous drugs (Table III). However, CNS isolates were more sensitive to most of the antibiotics as compared to CPS (Table III). *S. aureus* has been notorious for its drug resistance and has successfully evolved a number of drug resistance mechanisms such methicillin resistant *S. aureus* (Enright *et al*., 2002; Shamila-Syuhada *et al*., 2016). Isolation of multidrug resistant *S. aureus* from mastitic milk has been reported earlier in agreement with our findings (Ali *et al*., 2010; Islam *et al*., 2012; Memon *et al*., 2012; Najeeb *et al*., 2013).

It is important to understand risk factors associated with increased risk of SCM in goats for the improvement of udder health (Koop *et al*., 2013). Poor treatment protocol can cause the reoccurrence of infection or the risk of infection (Koop *et al*., 2016). Major factors involve in mastitis prevalence are low body score, late lactation, long teats, season, milk fever and hygiene prophylactic management (Koop *et al*., 2009; Megersa *et al*., 2010). Animals with low body score have five times more tendency of occurrence of SCM than those with a better body score. Similarly, animals near to parturate and having long teats are at more risk for udder infections as compared to others (Megersa *et al*., 2010). In the current

study, we found that Age, grazing system, use of beta-lactam antibiotics, parity, and poor hygiene were potential risk factors for occurrence of SCM in the study population of goats. Age is one of the most important factors in determining SCM in goats such as with increased age the chances are increased mainly due increased somatic cell counts (Ali *et al*., 2010; Clark and García, 2017). In agreement with our findings, increased parity was found associated high prevalence of mastitis in ewes and goats (Boscos *et al*., 1996). Finally, high incidence of mastitis has also been reported previously at drying-off and at parturition in relation with environmental contamination mainly due to poor hygienic practices during milking and herd management (Ali *et al*., 2010; Bergonier *et al*., 2003). Altogether, our study concludes a high incidence of SCM in the studied population of goats in District Faisalabad with concomitant higher isolation rate of *S. aureus*, which was resistant to most of the commonly used antibiotics. Furthermore, we identified age, grazing system, use of beta-lactam antibiotics, parity, and poor hygiene as potential risk factors for occurrence of SCM in goats.

CONCLUSION

We report on high incidence rate of SCM in goats in District Faisalabad with increased isolation rate of *S. aureus* that were mostly found resistant against commonly used antimicrobials. Factors, such as age, parity number, poor hygiene, and grazing system were found positively associated with spread of SCM. Increased hygienic practices, proper diagnosis of diseases, and judicious use of antibiotics may be included in designing prevention strategies against occurrence of SCM.

ACKNOWLEDGEMENTS

No funding was available for the current study. The Authors are highly thankful for the kind support of the department of clinical medicine of the University of Agriculture, Faisalabad, and the Livestock and dairy development department of district Faisalabad for facilitating collection of milk samples from goat herds.

Statement of conflict of interest

Authors have declared no conflict of interest.

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