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RESEARCH ARTICLE

Molecular Characterization of Methicillin Resistant *Staphylococcus aureus* (MRSA) and Associated Risk Factors with the Occurrence of Goat Mastitis

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A B S T R A C T

The current study was designed to determine the prevalence of *Staphylococcus aureus* (*S. aureus*) related Subclinical Mastitis (SCM) and detection of Methicillin Resistant *Staphylococcus aureus* (MRSA) in dairy goats in Pakistan. A total of 385 milk samples were collected from Mianwali (n=192) and Narowal (n=193) districts and initially screened using Surf Field Mastitis Test (SFMT). SFMT based positive samples were processed for the isolation and identification of *S. aureus* by using mannitol salt agar. The positive samples were subjected to disc diffusion test using oxacillin discs and further confirmed through amplification of mecA gene to detect resistance against Methicillin in *S. aureus* isolates. Hypothesized risk factors for the occurrence of SCM were recorded and were analyzed through logistic regression model. The study revealed 39.2% (151/385) prevalence of SCM by SFMT which was mainly 80.8% (122/151) caused by *S. aureus*. MRSA prevalence through disc diffusion test was 18.8% (23/122) while PCR based prevalence was 6.5% (8/122). All the study isolates showed 99% homology with MRSA isolates of India, Turkey and Japan with accession numbers MH798869, EU790488 and NG047938, respectively, available in NCBI database. Milker's care and hygienic measures during milking, milk yield, use of teat dips, presence of ticks, mixed type of grazing and services by professionals were proved to be the key risk factors associated with the occurrence of SCM in goats. This is the first report regarding the molecular characterization of MRSA isolated from dairy goats in Pakistan and the study will be helpful to provide information for developing control strategies against mastitis in goats.

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INTRODUCTION

Mastitis (inflammation of udder) is a disease of economic importance in dairy animals; it affects both quality and quantity of milk (Najeeb *et al*., 2013). Mastitis in goats results in decreasing the income of poor rural families in terms of profit loss from goat farming, decreased milk production, depriving kids from milk and reducing their growth rates (Koop *et al*., 2016). Goats are termed as the cow of the poor people. The goat milk is suitable and recommended for those who are allergic to cow milk, it may also be used in human infants those are deprived or cannot be fed with their mother milk.

Goat mastitis is mainly caused by contagious pathogens such as *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae* and some environmental pathogens like *Escherichia coli*, coagulase negative staphylococci, *Pseudomonas aeruginosa* and *Streptococcus uberis*. Among these pathogens, *S. aureus* is the most common pathogen causing mastitis (Ribeiro *et al*., 2007) The prevalence of *S. aureus* subclinical mastitis in goats usually varies from 5.6 to 37% (Aras *et al*., 2012). When *S. aureus* is present in subclinical mastitis, they are transferred to milk without causing any apparent change to the milk. Through infected milk and milk by products they may be transferred to humans (Caruso *et al*., 2016).

S. aureus may develop resistance against antibiotics. Resistance against methicillin shows resistance to all beta-lactams therefore these isolates may also be called as multidrug resistant (MDR) isolates. Methicillin resistant *S. aureus* (MRSA) develop resistance by acquiring mecA gene, which encodes a protein PBP2a that help in

bacterial cell wall synthesis (Luini *et al*., 2015). Due to this resistance, not only treatment costs are increased but also MRSA may be transferred to humans through consuming animal products or by handling of contaminated animals and it has been found that almost 60% emerging pathogens in humans come from animal sources (Feingold *et al.,* 2012).

MRSA associated with livestock (Livestock associated -LA-MRSA) in these days became prominent in many countries worldwide (Caruso *et al*., 2016). When MRSA infected milk is consumed by humans particularly by infants, it may lead to emerging issue of livestock associated -LA-MRSA in humans. The current study aims to determine the prevalence of subclinical mastitis and molecular characterization of MRSA isolated from dairy goats of Pakistan.

MATERIALS AND METHODS

Sampling and Screening for sub-clinical mastitis: Assuming 50% prevalence at 95% confidence interval (CI), sample size was calculated that resulted in 385 milk sample (Thrusfield, 2005). The milk samples were collected from 385 dairy goats from two districts of Punjab (192 from Mianwali and 193 from Narowal) by convenient sampling technique and screened for SCM using SFMT as described by Muhammad *et al*. (1995). SFMT based positive milk samples were collected aseptically into sterile screw capped tubes. These samples were maintained in cold chain at 4°C in ice packs and immediately shifted to the laboratory in Department of Clinical Medicine and Surgery, University of Veterinary and Animal Sciences Lahore and stored at -20°C till further processing.

Risk factors analysis: During sample collection, data regarding various risk factors were recorded, i.e. parity, physiological status, milking frequency, milker's care during milking, hygiene during milking, use of teat dips, milk yield, presence of ticks, body health, grazing type, feeding systems and veterinary services were recorded assuming that these risk factors may be the determinants of the disease.

Culturing and Biochemical Confirmation of *S. aureus* and identification of MRSA by Disc Diffusion Method: SCM based positive milk samples (3 ml each) were processed for the culturing and isolation of *S. aureus* by using selective media mannitol salt agar (TM Media, Titan Biotech Ltd, India) as described by Ali *et al*. (2018). For identification of MRSA, Oxacillin discs (1µg, Bioanalyse Turkey) were used. These Oxacillin discs were placed aseptically using disc dispenser on activated growth of *S. aureus* (0.5 McFarland) on Muller Hinton agar plates (TM Media, Titan Biotech Ltd, India). The plates were incubated at 37°C for 24 hours. The zones of inhibition around disc were measured by vernier calipers and compared with standard zone of inhibition provided by Clinical and Laboratory Standard Institute (CLSI, 2015).

Molecular identification of mecA gene of MRSA: For molecular confirmation of mecA gene in MRSA responsible for the development of resistance in *S. aureus*, the DNA was extracted from MRSA colonies using bacterial DNA extraction kit (WizPrepTM gDNA cell/tissue kit, Korea). Quantification of extracted DNA was performed by Nano-drop (Thermoscientific™-NanoDrop2000). Polymerase chain reaction (PCR) was carried out for all the extracted DNA samples for mecA gene of *S. aureus* using primers P1:5' TGGCATTCGT GTCACAATCG-3' and P2: 5'- CTGGAACTTGTTGAG CAGAG-3' (Galdiero *et al*., 2003) with amplicon of 310bp. The reaction mixture of PCR was made by mixing 3 µl DNA. Reaction was adjusted by 35 cycles after initial denaturation for 5 minutes at 95°C, denaturation at 95°C, annealing at 58°C, extension at 72°C, every step was run for 30 seconds with final elongation at 72°C for 10 minutes was carried out. The amplified PCR products were observed as positive bands (310bp) by running on 1.5% agarose gel using 100bp ladder under UV light illuminators.

Sequencing: Amplified fragments showing bands at 310bp were then sliced using cutter on UV illuminator and were purified using Gene All® gelpurification kit (Cat# 102-102; Lot: 10216B12009) following the manufacturer's instructions. Samples were sent for sequencing to 1st Base biological technology, Singapore. Previously published mecA gene sequences of MRSA (MH798869, KC243784, KM505044 and KR936060) and of current study (310-bp) were analyzed in Bioedit by CLUSTAL W alignment method. Phylogenetic tree was then constructed using the neighbor-joining (NJ) methods on Mega 7.0 Software.

Statistical analysis: To find out the assumed risk factors association with the occurrence of SCM in goats, all variables were initially tested by univariable analysis (Bursac *et al*., 2008), those variables which produced $P<0.2$ were analyzed in multivariable logistic regression model. The statistical analysis was conducted on R statistical software (version 3.2.1., http://www.r-project.org).

RESULTS

Epidemiology of Subclinical Mastitis: The current study revealed an overall 39.22% (151/385) prevalence of SCM by SFMT from dairy goats in study area (Fig. 1). The prevalence was recorded higher in Narowal 44.55% (86/193) district as compared to Mianwali 33.85% (65/192) district, however statistically significant relationship could not be found between the study districts. The samples which were found positive for SCM were further processed for the isolation of *S. aureus*, by initially swabbing milk samples aseptically on 5% sheep blood ager and incubating at 37°C for 24 hours. The colonies were further streaked to mannitol salt agar (Fig. 2). The *S. aureus* colonies were identified on basis of typical morphological characteristics, Gram's staining and subsequently by biochemical tests like catalase test and coagulase test. 122/151 isolates were found positive for the presence of *S. aureus*. Out of these *S. aureus* isolates, 57.37% were from Narowal and 42.63% were from Mianwali district. For further isolation of MRSA, the samples that were found positive for *S. aureus* were subjected to disc diffusion test using oxacillin discs and

further confirmed through PCR for mecA gene, being responsible for the development of resistance. The prevalence of MRSA through disc diffusion test was 18.85% (23/122) with 19.23% (10/52) in Mianwali and 18.57% (13/70) in Narowal. PCR based confirmation of MRSA revealed 6.55% (8/122) prevalence of MRSA having mecA gene with 5.7% (3/52) in Mianwali and 7.14% (5/70) in Narowal.

The relation of assumed risk factors like parity of animal, physiological status, frequency of milking, milker's care during milking, hygiene during milking, milk yield, use of teat dip, presence of ticks, body condition, feed and water, feeding system, grazing type and veterinary services were analyzed statistically to find out association with disease occurrence (Table 1). The association of risk factors with the occurrence of SCM was analyzed by multivariable logistic regression model. Initially twelve variables that produced P<0.2 in univariable analysis (Table 1) were included in the multivariable logistic regression model. The final model contained seven statistically significant variables (Table 2).

Poor milker's care during milking was found to be significantly (P<0.05) associated risk factor for the occurrence of subclinical mastitis. The animals having poor care during milking were affected more compared to the animals having good care during milking and the prevalence for SCM was 58.2 and 12.5%, respectively. The odds of having subclinical mastitis in goats on farms with poor milker's care was 5.18 times as high as on farms where milker's care was good. Similar type of findings was found for hygiene during milking, the animals not having hygienic conditions were at 6.6 odds of having SCM. High milk producing goats were found at 3.2 times more risk of having SCM as compared to those having low milk yield. It was found that the risk of SCM was 8.3 times more in those animals in which teat dips were not practiced as compared to those in which teat dips were practiced; similarly, 15.9 times more chances of SCM in those animals that were grazed with other animals as compared to those that were grazed separately. Presence of ticks and disease management by veterinary services were also found to be associated significantly (P<0.05) with the occurrence of subclinical mastitis in goats. Parity and physiological status of the animal and provision of feed and water were statistically associated (P<0.05) with disease dynamics by univariable analysis while they became non-significant when analyzed with multivariable analysis.

MRSA mecA gene analysis: This is the first study regarding the molecular characterization of mecA gene of MRSA isolated from dairy goats of Pakistan. A partial fragment of mecA gene (310 bp) was amplified by PCR from 08/122 milk samples (Fig. 3). PCR based positive samples were purified by using gel extraction kit and sequencing was conducted for mecA gene of MRSA using the same set of primers that were used for PCR. All of the sequences were then blasted through Basic Local Alignment Search Tool (BLAST), all of the isolated sequences showed 99% homology with mecA gene of MRSA with accession numbers MH798869, KC243784, KM505044 and KR936060 available in NCBI database.

Table 1: Survey of sub-clinical mastitis in goats: Summary of risk factors included in the questionnaire

Variable	Variable levels	Positive (%)	Negative	P value
District	Mianwali	71 (37.0)	121	0.369
	Narowal	80 (41.5)	113	
Parity	1st	42 (55.5)	31	<0.001
	2nd	29 (28.2)	74	
	3rd	32 (28.8)	79	
	>3rd	48 (49.0)	50	
Physiological Status	Lactating	133 (37.7)	220	0.042
	Dry	18 (56.2)	14	
No. of milking	Once	132 (38.9)	207	0.758
	Twice	19 (41.3)	27	
Milker's care during milking	Poor	131 (58.2)	94	<0.001
	Good	20 (12.5)	140	
Hygiene during milking	Yes	20 (13.5)	128	<0.001
	No	131 (55.3)	106	
Milk yield	Low	121 (36.4)	211	0.006
	High	30 (56.6)	23	
Use of Teat dips	Yes	02 (7.1)	26	<0.001
	No	149 (41.7)	208	
Presence of ticks	Yes	30 (75.0)	10	<0.001
	No	121 (35.1)	224	
Body health	Normal	135 (38.6)	215	0.186
	Thin	15 (51.7)	14	
	Emaciated	01 (16.7)	5	
Feed and water	Well-fed	140 (37.8)	230	0.006
	Underfed	11 (73.3)	4	
Feeding system	Stall Feeding	61 (39.4)	94	0.452
	Grazing	25 (33.3)	50	
	Grazing + Stall Feeding	65 (41.9)	90	
Grazing Type	Mixed	84 (75.0)	28	<0.001
	Separate	67 (24.5)	206	
Veterinary services	Veterinary Officer	42 (25.9)	120	<0.001
	Veterinary Assistant	84 (44.7)	104	
	Self	25 (71.4)	10	

Table 2: Summary of key risk factors associated with the occurrence of subclinical mastitis in dairy goats: variables included in final logistic regression model

Study variable	Response categories	OR*	95% **CI	*Std. Error*	P value
Milker's care during milking	Poor	5.18	2.67-10.51	0.348	<0.001
	Good	1			
Hygiene during Milking	No	6.62	3.31-14.02	0.366	<0.001
	Yes	1			
Milk yield	High	3.25	1.36-8.02	0.450	0.009
	Low	1			
Use of teat dip	No	8.32	1.88-63.76	0.867	0.015
	Yes	1			
Presence of ticks	Yes	3.71	1.38-10.82	0.521	0.012
	No	1			
Grazing type	Mixed	15.94	7.81-35.03	0.381	<0.001
	Separate	1			
	Self	3.88	1.34-11.9	0.553	0.014
Veterinary services	Vet. assistant	3.33	1.73-6.64	0.342	<0.001
	Vet. Officer	1			

*OR = Odds Ratio; **CI = Confidence Interval.

The comparison of the study isolates with the isolates available in GeneBank database (MH798869, KC243784, KM505044 and KR936060) revealed substitution of T with A at position 265 in MRSA Pakistan 1 and 5 samples (Fig. 4).

Phylogenetic analysis: All the study sequences and previously submitted sequences obtained from NCBI database were then aligned and subjected to phylogenetic analysis. Tree was then constructed using neighbor joining bootstrapping method at 1000 replications (Fig. 5). Two of the study isolates MRSA Pakistan 1 and 5 clustered with each other separately while MRSA Pakistan 3 clustered with MH798869, KC243784, KM505044 and KR93606 isolates previously submitted in NCBI database.

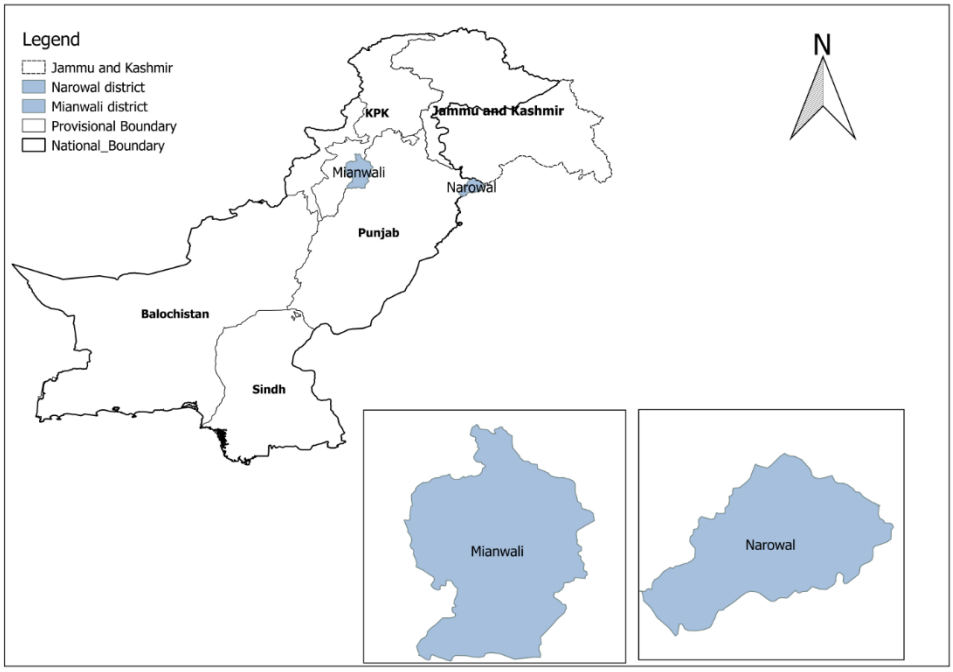

Fig. 1: Map showing the study districts in Pakistan.

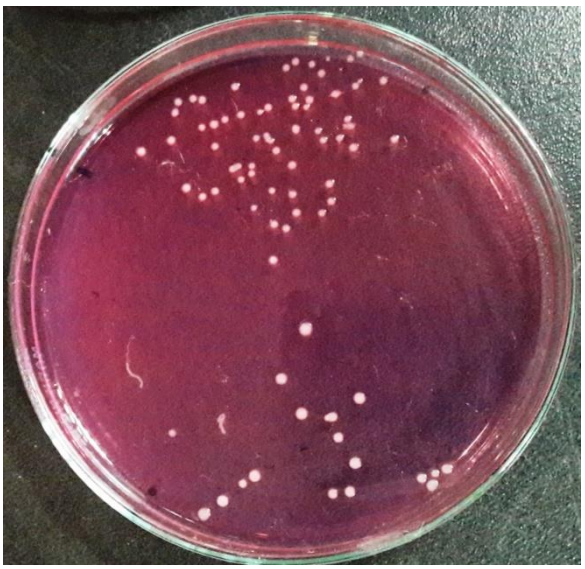

Fig. 2: Staphylococcus culture plate on Mannitol salt agar.

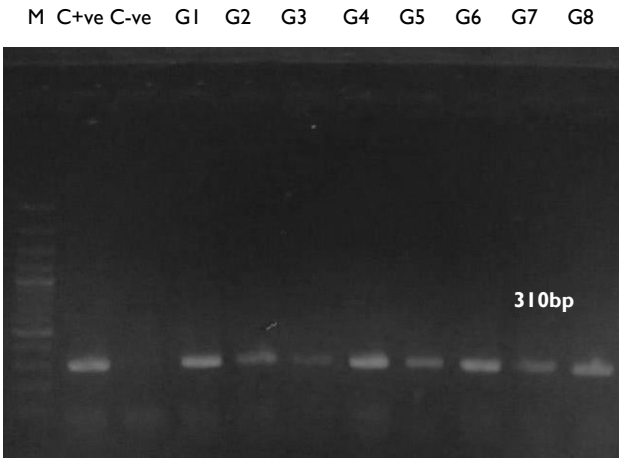

Fig. 3: Gel picture for the amplification of MRSA mecA gene (310bp). Lane M indicates 100bp molecular weight marker, Lane C+ve indicates control positive, Lane C-ve indicates control negative, Lane G1-G8 indicates MRSA positive samples isolate from goats.

DISCUSSION

SCM in goats causes huge economic losses by increased treatments costs, decreased milk production and there is transfer of contagious pathogens to humans which usually remains unnoticed (Caruso *et al*., 2016; Koop *et al*., 2016). Information about causative agent(s), their characterization and associated risk factors are important for effective treatment and control strategies. This study highlights the prevalence of SCM, *S. aureus* and MRSA isolated from milk samples of dairy goats along with the molecular characterization of MRSA.

Epidemiology: The current study revealed 39.22% prevalence of SCM from dairy goats and these findings are in agreement with the findings of Pirzada *et al*. (2016) and Moroni *et al*. (2005) who have reported 38% and 40.2% prevalence of SCM from goats, respectively. Ali *et al*. (2010) and Najeeb *et al*. (2013) have reported 47% and 53% prevalence, which is higher than the current study findings. The difference might be due to variation in sampling techniques as samples were collected from apparently healthy goats with no history of mastitis in this study or due to some management or therapeutic practices.

The study presented 80.79% prevalence of *S. aureus* among SCM positive cases. Being important pathogen of mastitis, *S. aureus* has previously been reported in caprine SCM (Ali *et al*., 2010; Aras *et al*., 2012; Islam *et al*., 2012; Najeeb *et al*., 2013). The high percentage of *S. aureus* in SCM might be due to its ability to produce exopolysaccharides (Slime) that provides protection and resists to both immune system of body and the chemotherapy (Contreras *et al*., 2003).

Proper prevention of SCM with improved udder health needs understanding of disease determinants or risk factors (Koop *et al*., 2013). Improper treatment or without decreasing the effects of risk factors, reoccurrence of the infection may be evident (Koop *et al*., 2016). Prevalence of mastitis depends on many factors like body condition,

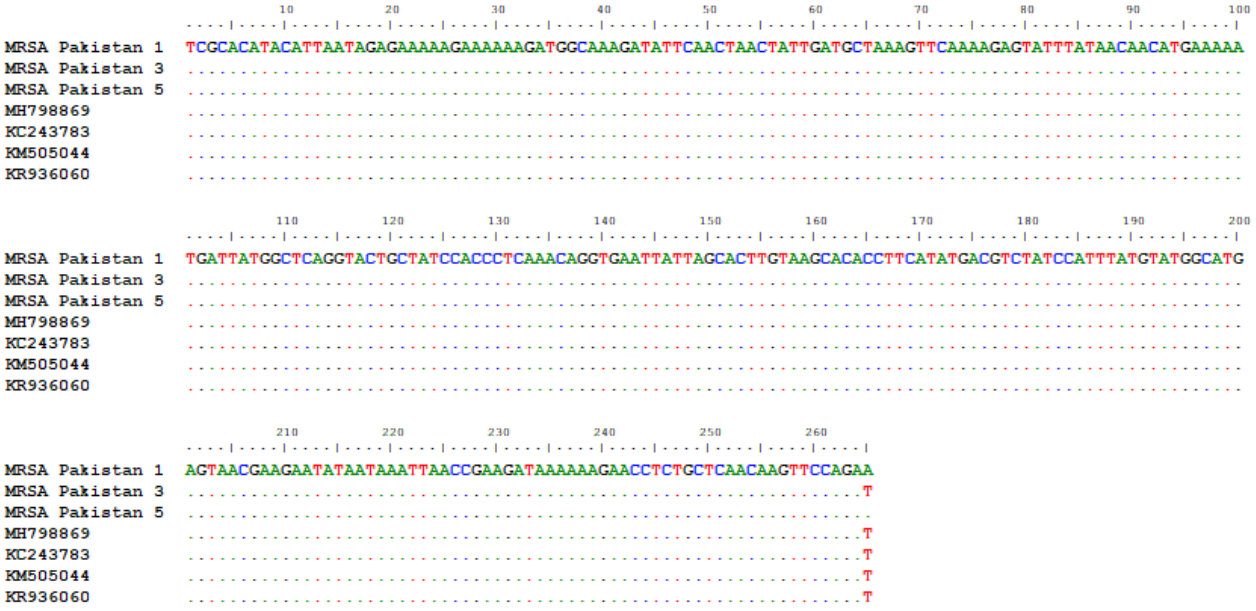

Fig. 2: Blast alignment of current study isolates with NCBI reported isolates of MRSA mecA gene.

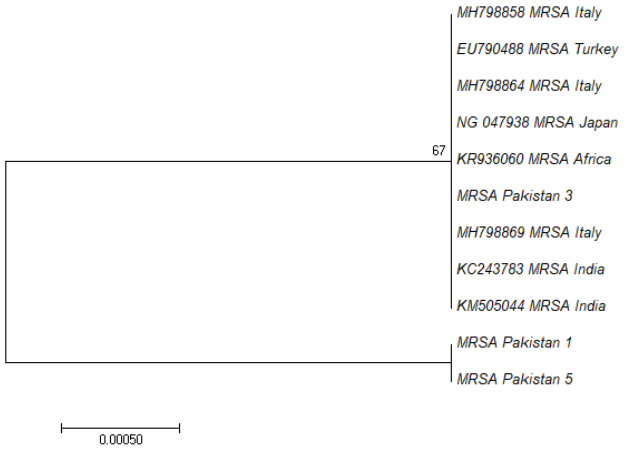

Fig. 5: Phylogenetic tree of MRSA mecA gene sequence.

hygienic measures during milking, teat problems and late lactation (Koop *et al*., 2009; Megersa *et al*., 2010). Animals having poor conditions are five times more prone to subclinical mastitis as compared to healthy ones. Similarly, before the time of parturition, when the development of udder and teats begins, animals are also more prone to udder infections (Megersa *et al.*, 2010).

This study showed that milker's care and hygienic measures during milking, milk yield, lack of teat dipping, presence of ticks and mixed type of grazing were important determinants and directly related with the occurrence of subclinical mastitis in goats. These findings were supported by various research conducted on risk factors for mastitis (Moroni *et al*., 2005; Megersa *et al*., 2010; Koop *et al*., 2013).

MRSA and its Molecular characterization: Presence of MRSA in caprine SCM not only affects quality and quantity of milk but it may also be transferred to humans leading to the development of antibacterial resistance problems in humans posing to a great zoonotic threat. It has been found that MRSA is prominent and common hospital acquired pathogenic organism being responsible for endemic and epidemic infections in healthcare centers throughout the world (Nikbakht *et al*., 2008). Many scientists have isolated and reported increasing prevalence of MRSA carrying mecA gene over last two decades exhibiting resistance to various classes of antibiotics (Al-Ashmawy *et al.*, 2016; Obaidat *et al*., 2018). In these days LA-MRSA has become important and cause huge economic losses. LA-MRSA has been isolated and reported from different animal species (Turutoglu *et al.*, 2009; Stastkova *et al*., 2009; Caruso *et al*., 2016; Aqib *et al.*, 2017). Bovine milk samples with mecA MRSA have been reported in many countries (Holmes and Zadoks, 2011; Aqib *et al.*, 2017; Obaidat *et al.*, 2018). Small ruminants especially goats having significant contribution as a source of milk in many countries throughout the World but there is scarcity of data regarding MRSA prevalence in goats.

In current study MRSA is isolated from SCM cases of goats. In Pakistan, previously MRSA has been reported in bovine mastitis (Aqib *et al.*, 2017) but there is no report of MRSA from goat mastitis. In goats MRSA has been isolated from raw milk and their handlers in Czech Republic (Stastkova *et al*., 2009). The current study revealed 6.5% MRSA from subclinical mastitis of goats, on the base of PCR by targeting mecA gene. This prevalence is slightly higher to that reported in Turkey in clinical mastitis of goats by Aras *et al.* (2012) they have isolated 4.8% MRSA from clinical cases of goat mastitis. A higher prevalence (14.3%) of MRSA in SCM of goat has been reported in Indonesia (Suwito *et al*., 2014). MRSA has also been isolated from bulk tank milk of goats with its prevalence 0-2% reported in Italy (Cortimiglia *et al*., 2015; Caruso *et al*., 2016) while its higher prevalence was reported in Jordan (Obaidat *et al*., 2018).

MRSA strains isolated from bovines were similar to those MRSA strains of humans on the base of mecA gene sequence analysis (Turutoglu *et al*., 2009). Stastkova *et al.* (2009) reported MRSA transmission from goat to their handler and from handler to goat. All the study isolates

showed 99% homology with previously submitted human MRSA isolates in NCBI database. Similar MRSA genotype from handlers and goat milk suggesting its zoonotic importance has been reported (Curso *et al*., 2016; Ramadhan *et al*., 2017; Obaidat *et al*., 2018). These results show that MRSA strains can pose great threat to veterinarian and public health through consuming unpasteurized milk of goat.

Conclusions: This study provides the first insight of the genetic characterization of MRSA isolated from dairy goats in Pakistan. Risk factors like milker's care and hygienic measures during milking, milk yield, use of teat dips, presence of ticks, mixed grazing and disease management by veterinary services were proved to be important risk factors affecting the occurrence of MRSA related subclinical mastitis in dairy goats. Isolation of MRSA indicates huge economic losses and a great threat to veterinarians and public health due to development of resistance against all beta-lactam group of antibiotics in Pakistan. This study will be helpful to provide information for developing strategies to control mastitis in goats.

Authors contribution: MI, MKI and MA designed the project. The sampling, data collection, processing and interpretation of results were made by MA, AG. The data analysis was made by RMA, MKI and AR. The manuscript was written by MA, MI, AG and AR. All the authors read the manuscript and approved the contents.

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