Pakistan J. Zool., vol. 55(2), pp 831-842, 2023 DOI: https://dx.doi.org/10.17582/journal.pjz/20220316080346

Evaluation of Methicillin Resistance in Field Isolates of *Staphylococcus aureus*: An Emerging Issue of Indigenous Bovine Breeds

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Article Information
Received 16 March 2022
Revised 25 April 2022
Accepted 18 May 2022
Available online 21 June 2022 (early access)
Published 02 January 2023

Authors' Contribution
NZG and MI conceptualization, methodology, data curation, investigation, resources, project administration, funding acquisition, writing review, and editing. MUJ and AA methodology, data curation, investigation, software, visualization, validation. IM writing original draft. AR writing review, and editing.

Key words
MRSA, Indigenous bovine, phylogenetic analysis, comparative risk factor analysis, SDS-PAGE

ABSTRACT

The emergence of resistant strains of *Staphylococcus aureus* particularly methicillin-resistant *S. aureus* (MRSA) confers overwhelming economic losses to the global dairy industry. The current study was planned to investigate the *S. aureus* and MRSA associated subclinical mastitis in 345 milk samples (Cattle n=173, Buffalo n=172) collected from indigenous bovines of district Rawalpindi, Pakistan. The milk samples were screened for *S. aureus* and the confirmed isolates were subjected to disc diffusion test, PCR, and SDS-PAGE analysis for the confirmation of methicillin resistance. The farm-associated and individual animal-associated risk factors were analyzed to check the association with *S. aureus* and MRSA-associated subclinical mastitis. The results revealed an overall molecular prevalence of 28.70% for *S. aureus* among which MRSA-associated mastitis was found 47.62% prevalent. The SDS-PAGE analysis depicted the presence of a 78KDa protein band specific for *PBP2a* protein in MRSA. The comparative risk factor analysis showed significant variation among risk factors associated with *S. aureus* and MRSA-induced mastitis. The phylogenetic analysis of MRSA *mec*A gene showed a high resemblance of the study isolates with MRSA isolates of the USA, Turkey, India, Africa, and Brazil. This is the first study regarding molecular characterization and phylogenetic analysis of MRSA isolates from the study area.

INTRODUCTION

Worldwide milk production is mainly dominated by dairy cows but water buffaloes also account for 13.2% of non-cattle milk according to Food and Agriculture Organization (Giovanni *et al*., 2020). In Pakistan, the major indigenous dairy breeds include Sahiwal cattle and Nili-Ravi buffalo (Afzal *et al*., 2007) among which the Nili-Ravi breed of buffalo is producing about 75% of all the milk produced in the country (Sharif *et al*., 2009). Among the major economically devastating health hazard of dairy animals, mastitis comes on top of the list (Ji *et al*., 2020). *Staphylococcus aureus*, due to its low cure rate, transmission between animals during milking, ability to reside in the mammary gland leading to subclinical infection, and the emergence of antibiotics resistant strains, is considered a major pathogen deteriorating both milk quality and production (El-Ashker *et al*., 2020; Monistero *et al*., 2018). The pathogenicity of *S. aureus* comprises a combination of its invasive property, pathogenicity-mediated genes, immune evasion, and antibiotic resistance which negates the therapeutic approaches (Chua *et al*., 2014).

The diverse genetic abilities of *S. aureus* have led to the emergence of strains that resist the β-lactam antibiotics, of which methicillin-resistant *S. aureus* (MRSA) is of paramount importance (Abdeen *et al*., 2021; Zaatout and Hezil, 2021). The methicillin resistance in *S. aureus* is primarily governed by the acquisition of the *mecA* gene which encodes penicillin-binding protein 2a (*PBP2a*) having a lower affinity for methicillin and all other β-lactam antibiotics (Zhao *et al*., 2021; Javed *et al*., 2021). MRSA is an emerging zoonotic bacteria of veterinary and public health importance (Algammal *et al*., 2020b) and has been listed by the World Health Organization as a pathogen of utmost priority for further research and treatment (Shrestha *et al*., 2021). The prevalence of MRSA in livestock is higher in Asia as compared to other continents (Zaatout and Hezil, 2021) which might be due to inadequate accomplishment of standard farm and milking hygiene practices with indiscriminate and undue usage of

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0030-9923/2023/0002-831 $ 9.00/0

antibiotics prescribed without veterinary consultation in underdeveloped and developing countries (Kayitsinga *et al*., 2017).

The rapid determination of *S. aureus* isolates, either to be methicillin-resistant or not, is critically important for both treatment and control measures. For the identification of bacteria various phenotypic and genotypic methods have been used (Elhaig and Selim, 2015). Phenotypic methods like E-tests, micro-dilution, and oxacillin disc diffusion tests have been used for MRSA detection but some of these techniques are not specific and may overestimate the MRSA prevalence leading to false-positive results (Olowe *et al*., 2013). In addition, these methods are also not reliable to distinguish between MRSA and methicillin-sensitive *S. aureus* (MSSA). However, the differentiation of MRSA from MSSA can be done based on the *mecA* gene, an important marker for the indication of methicillin-resistance in *S. aureus*, which is present in resistant isolates and absent in susceptible ones (Baddour *et al*., 2007). The *mecA* gene confirmation can be done using molecular methods like PCR which is considered reliable and the 'Gold Standard' test for MRSA detection. Moreover, to evaluate the protein expression of MRSA and MSSA, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis can be performed. The SDS PAGE of whole-cell MRSA revealed the separation of mutant *PBP2a* protein at 78kDa, which is exclusively present in MRSA isolates (Doan *et al*., 2013). *PBP2a* protein is encoded by the *mecA* gene in the chromosomal cassette of the foreign DNA region that is integrated into the MRSA chromosome (Senna *et al*., 2003).

The current study was designed to estimate the phenotypic and molecular prevalence of *S. aureus* and MRSA associated subclinical mastitis along with a comparative analysis of associated risk factors in indigenous breeds of Pakistan. In addition, molecular characterization of local MRSA isolates was performed and their genetic relatedness with the reported MRSA isolates was also checked. Moreover, protein expression of MRSA and MSSA was also evaluated using SDS-PAGE.

MATERIALS AND METHODS

Sampling design

The study was set to investigate the *S. aureus* and MRSA-associated subclinical mastitis in indigenous dairy cattle and buffalo located in three tehsils (Rawalpindi, Gujar Khan, and Taxila) of district Rawalpindi, Pakistan (Fig. 1), between the period of December 2020 to July 2021. A total of 345 bovine milk samples (n=173 cattle; n=172 buffalo from each tehsil) were collected aseptically as per guidelines of the National Mastitis Council, USA (Reyher and Dohoo, 2011). The milk samples were examined for subclinical mastitis by California mastitis test (CMT) and positive samples were immediately dispatched to Medicine Research Laboratory, University of Veterinary and Animal Sciences, Lahore, maintaining the cold chain at 4°C.

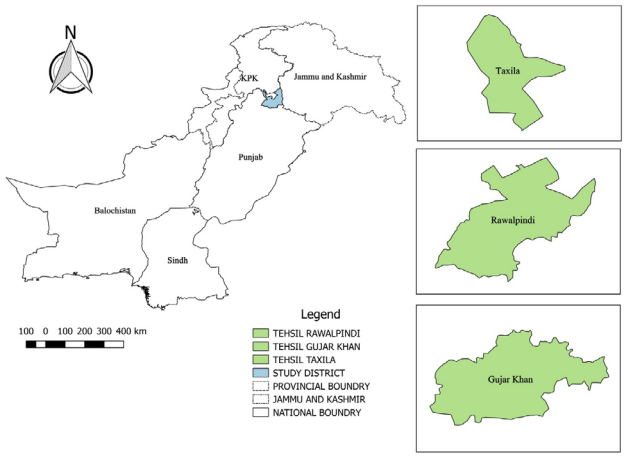

Fig. 1. QGIS map showing the study area.

The information regarding animal-based and herd-based management practices was collected on a predesigned data capture form for the comparative analysis of assumed risk factors associated with *S. aureus* and MRSA-associated subclinical mastitis in indigenous dairy animals. The purpose was to pinpoint the potential risk factors more significantly associated with MRSA-associated mastitis compared to *S. aureus*-associated mastitis.

Isolation and identification of S. aureus

The bacterial growth was isolated from milk samples by primarily streaking on 5% sheep blood agar followed by an incubation of 24-48 hours at 37°C. The bacterial colonies were subjected to Gram staining and identified by standard biochemical tests (catalase and coagulase) as per recommendations of Bergey's Manual of Determinative Bacteriology (Holt, 1977). Presumptive *S. aureus* colonies were further streaked on mannitol salt agar (MSA) for the confirmation of *S. aureus* (Altaf *et al*., 2019). Molecular confirmation of *S. aureus* was done by amplification of *nuc* gene using primers as (F= GCG ATT GAT GGT GAT ACG GTT, R= AGC CAA GCC TTG ACG AAC TAA AGC) and conditions as initial denaturation 94°C for 5 min, followed by 35 cycles and final denaturation at 94°C for 30s, annealing 55°C for 30s, with initial extension at 72ºC for 1 min and final extension for 10-min at 72°C as reported by Louie *et al*. (2002). The phenotypic and genotypic confirmed *S. aureus* isolates were further analyzed for the

evaluation of methicillin resistance.

Phenotypic identification of MRSA and MSSA isolates

Methicillin susceptibility of confirmed *S. aureus* isolates was assessed by performing a disc diffusion assay according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2019). The (0.5 McFarland) adjusted bacterial suspension of *S. aureus* was streaked on Muller Hinton agar (MHA). The oxacillin (1µg) and cefoxitin (30 µg) antibiotics discs (Bioanalyse ® Turkey) were aseptically applied using a sterile disc dispenser and subjected to incubation at 37°C for 20-24 h. The zones of inhibition were compared with standards, provided by CLSI (2019). The isolates exhibiting inhibition zone of size ≤17mm and ≤21 mm respectively for each disc were considered MRSA while isolates having greater zones were considered as MSSA.

Molecular confirmation of mecA *gene*

To evaluate the molecular confirmation of methicillin resistance, the genomic DNA was extracted using a bacterial DNA extraction kit (Vivantis Technologies Sdn. Bhd, Malaysia) following the manufacturer's instructions. All phenotypically confirmed MRSA isolates were subjected to conventional PCR (Scilogex TC1000-G) for the amplification of 310bp fragment of *mecA* gene using primers (P1: 5′-TGGCATTCGTGTCACAATCG-3′ and P2: 5′- CTGGAACTTGTTGAGCAGAG-3′) reported by Galdiero *et al*. (2003) using initial denaturation at 95ºC for 5 min followed by 35 cycle with final denaturation of 95ºC for 30s with annealing at 58ºC for 30s and extension at 72ºC for 30s and 72ºC for 10 min, respectively. The PCR products obtained after amplification were subjected to gel electrophoresis at 120 volts using 200 mAmp and run electrophoresis for 30 min using a gel electrophoresis apparatus (Cleaver Scientific Ltd). The resultant bands were visualized on a UV trans-illuminator along with standard 100bp DNA ladder (Bioshop® Canada Inc.) as a molecular weight marker. The isolates showing bands on 310bp were considered MRSA (*mecA* positive) and those found negative for bands were assumed MSSA (*mecA* negative) isolates.

Sequencing and phylogenetic analysis

The *mecA* positive bands, after the gel purification, were shipped to a renowned sequencing lab for sequencing. The resulted obtained sequences of study isolates were evaluated using the basic local alignment search tool (BLAST) of NCBI (National Centre for Biotechnology Information) to check the similarity of the study isolate's nucleotide sequence with other *S. aureus* isolates showing methicillin-resistance reported in various countries of the world. The multiple sequence alignment was done using the Clustal W method by using BioEdit software (Version 7.2.5). A phylogenetic tree was then constructed based on sequence distance using neighborhood joining methods on MEGA-X software.

Protein expression by SDS PAGE

The whole-cell protein profile of *mecA* positive and *mecA* negative isolates was analyzed for general protein expression by SDS PAGE analysis using (omni PAGE mini vertical Protein electerophoresis by Cleaver Scientific Ltd) apparatus. The difference between proteins expression of both isolates was noted specifically for the expression of *PBP2a* protein at 78kDa present in MRSA and absent in MSSA isolates (Hartman and Tomasz, 1986). The SDS PAGE analysis was performed according to procedures followed by (Berber *et al*., 2003). The protein bands were visualized with fluorescent light and relative flow (Rf) values of each sample were measured. Molecular weights of all isolates were determined through a standard curve made between Rf values and logarithmic molecular weights of known protein marker bands (Fig. 4). The presence of protein bands was assessed and compared with each other for differentiation between MRSA and MSSA isolates.

Data analysis

The phenotypic and genotypic prevalence of *S. aureus*, MRSA, and MSSA isolated from bovine milk samples was estimated using pre-described formula reported by (Thrushfield, 2013). The risk factors related to *S. aureus* and MRSA-associated mastitis were initially analyzed and compared using the chi-square method and multivariable logistic regression technique at 5% probability. All the statistical analysis was performed using SPSS statistical computer program.

RESULTS

Prevalence of S. aureus *and MRSA associated subclinical mastitis*

The current study revealed that an overall prevalence of subclinical mastitis (SCM) in district Rawalpindi was 52.75% based on CMT. While the *S. aureus*-associated SCM were found 28.70% prevalent. The phenotypic evaluation of methicillin resistance by oxacillin/cefoxitin disc diffusion test revealed that 42.42% *S. aureus* isolates were found resistant (MRSA) while 57.58% isolates were sensitive (MSSA) to antibiotic discs. Molecular analysis of phenotypically positive MRSA revealed that 47.62% of isolates were found positive for the *mecA* gene (MRSA) while the rest of the isolates were found negative for the *mecA* gene (MSSA) as shown in (Table I). The genotypic results of the current study showed that among indigenous

bovines, the cattle were more prone to *S. aureus* and MRSA-associated mastitis compared to buffalo (Table I). The prevalence of MRSA associated SCM was found more in tehsil Gujar khan (58.82%) followed by tehsil Rawalpindi (42.86%) and tehsil Taxila (36.36%) as shown in (Table II). The results depicted that phenotypic methods like bacterial culture and disc diffusion test can overestimate the infection load of *S. aureus* and MRSA in bovine mastitis and molecular methods like PCR and sequencing are more specific and reliable methods for methicillin resistance screening.

Comparative risk factor analysis

The relationship of various animal-based and herd-based assumed risk factors with the occurrence of *S. aureus* and MRSA-associated mastitis was analyzed and compared using statistical tools. A significant variation between an association of risk factors with *S. aureus* and MRSA-induced mastitis was observed.

The results revealed that among animal-based risk factors, physiological status of animals, mastitis history, and teat lesion were found significantly ($p<0.05$) associated with both *S. aureus* and MRSA induced SCM (Table III). The parity ($p=0.01$) was found significantly associated with *S. aureus* but not with MRSA-associated mastitis (Table III). It was also noted that animals having teat lesions were 2.215 times more prone to *S. aureus*-associated mastitis (Table VI). The results further showed that usage of antibiotics ($p=0.02$) was significantly associated with MRSA-associated mastitis but was found non-significant risk factor for *S. aureus*-based mastitis. Animals, in which beta-lactam antibiotics usage was common, were 3.054 times more prone to MRSA-associated mastitis compared to *S. aureus*-associated mastitis (Table V).

The data analysis of herd-base risk factors (Table IV) showed that herd size, hygiene during milking, milking person hygiene, and milking methods were found to be significantly ($p<0.05$) associated with both *S. aureus* and MRSA associated mastitis while the presence of vectors and other livestock species were found to be non-significant ($p>0.05$) risk factors for *S. aureus*-associated mastitis and MRSA associated mastitis as shown in (Table IV). The final logistic regression model showed that animals with hand milking method and poor milking person hygiene were 3.134 and 2.825 times more prone to MRSA infection (Table V).

Table I. Prevalence of subclinical mastitis (SCM), methicillin resistant (MRSA) and methicillin sensitive (MSSA) *Staphylococcus aureus* from indigenous breeds of cattle and buffalo.

Animal	No. of animals	SCM (%)	*S. aureus*	Phenotypic (%)		Genotypic (%)	
				MRSA	MSSA	MRSA	MSSA
Cattle	173	97/173 (56.07)	57/173 (32.95)	23/57 (40.35)	34/57 (59.65)	12/23 (52.17)	11/23 (47.83)
Buffalo	172	85/172 (49.42)	42/172 (24.42)	19/42 (45.24)	23/42 (54.76)	8/19 (42.11)	11/19 (57.89)
Total	345	182/345 (52.75)	99/345 (28.70)	42/99 (42.42)	57/99 (57.58)	20/42 (47.62)	22/42 (52.38)

MRSA, methicillin resistant *Staphylococcus aureus*; MSSA, Methicillin Sensitive *Staphylococcus aureus*; SCM, Sub clinical mastitis.

Table II. Prevalence of SCM, MRSA and MSSA in indigenous cattle and buffalo breeds of tehsil Rawalpindi, Gujar khan and Taxila.

Area	Animal Spp.	No. of animals	SCM (%)	*S. aureus*	Phenotypic (%)		Genotypic (%)	
					MRSA	MSSA	MRSA	MSSA
Rawalpindi	Cattle	58	32(55.17)	19(32.76)	08(42.11)	11(57.89)	04(50.00)	04(50.00)
	Buffalo	57	28(49.12)	12(21.05)	06(50.00)	06(50.00)	02(33.33)	04(66.67)
	Total	115	60(52.17)	31(26.96)	14(45.16)	17(54.84)	06(42.86)	08(57.14)
Gujar Khan	Cattle	58	36(62.07)	20(34.48)	09(45.00)	11(55.00)	06(66.67)	03(33.33)
	Buffalo	57	33(57.89)	14(24.56)	08(57.14)	06(42.86)	04(50.00)	04(50.00)
	Total	115	69(60.00)	34(29.57)	17(50.00)	17(50.00)	10(58.82)	07(41.18)
Taxila	Cattle	57	29(50.88)	18(31.58)	06(33.33)	12(66.66)	02(33.33)	04(66.66)
	Buffalo	58	24(43.38)	16(27.59)	05(31.25)	11(68.75)	02(40.00)	03(60.00)
	Total	115	53 (46.09)	34(29. 57)	11(32.35)	23(67.65)	04(36.36)	07(63.64)
Total animals		345	182(52.75)	99(28.70)	42(42.42)	57(57.58)	20(47.62)	22(52.38)

For abbreviation see Table I.

Table III. Comparative analysis of animal-based risk factors associated with *Staph* and MRSA-associated subclinical mastitis.

Variable	Variable level	*S. aureus* associated mastitis			MRSA associated mastitis		
		Total (n=345)	Positive (%)	p value	Total (n=345)	Positive (%)	p value
Body condition	Healthy	166	43 (25.90)	0.27	122	05 (04.10)	0.20
	Thin	88	24 (27.27)		127	06 (04.72)	
	Emaciated	91	32 (35.16)		96	09 (09.37)	
Parity	First	30	09 (30.00)	0.01*	32	02 (06.25)	0.77
	Second	101	19 (18.81)		102	06 (05.88)	
	Third	123	39 (31.71)		128	09 (07.03)	
	> Third	84	32 (38.10)		83	03 (03.61)	
Physiological status	Lactating	280	94 (33.57)	0.00*	184	15 (08.15)	0.04*
	Dry	65	05 (07.69)		161	05 (03.10)	
Mastitis history	Present	211	72 (34.12)	0.00*	198	16 (08.08)	0.03*
	Absent	134	27 (20.15)		147	04 (02.72)	
Use of β-lactam antibiotics	Frequent use	203	60 (29.56)	0.67	177	15 (08.47)	0.02*
	Seldom use	142	39 (27.46)		168	05 (02.98)	
Treatment person	Veterinarian	141	34 (24.11)	0.11	199	09 (04.52)	0.23
	Self	204	65 (31.86)		146	11 (07.53)	
Teat lesion	Present	227	77 (33.92)	0.00*	176	15 (08.52)	0.02*
	Absent	118	22 (18.64)		169	05 (02.96)	

*Indicate significantly associated risk factors.

Table IV. Comparative analysis of herd-based risk factors associated with *Staph* and MRSA-associated subclinical mastitis.

Variable	Variable level	*S. aureus* associated mastitis			MRSA associated mastitis		
		Total (n=345)	Positive (%)	p value	Total (n=345)	Positive (%)	p value
Milking person hygiene	Yes	159	36 (22.64)	0.02*	164	05 (03.05)	0.03*
	No	186	63 (33.87)		181	15 (8.29)	
Presence of vectors	Present	135	43 (31.85)	0.29	207	13 (6.28)	0.63
	Absent	210	56 (26.67)		138	07 (5.07)	
Hygiene during milking	Good	142	26 (18.31)	0.00*	146	04 (2.74)	0.03*
	Poor	203	73 (35.96)		199	16 (8.04)	
Herd size	Small	115	47 (40.87)	0.00*	94	11 (11.70)	0.006*
	medium	129	33 (25.58)		120	07 (05.83)	
	large	101	19 (18.81)		131	02 (01.53)	
Milking methods	Hand milking	169	58 (34.32)	0.02*	148	13 (8.78)	0.04*
	Machine method	176	41 (23.29)		197	07 (3.55)	
Other livestock species	Present	199	60 (30.15)	0.48	180	11 (06.11)	0.79
	Absent	146	39 (26.71)		165	09 (05.45)	

*indicate significantly associated risk factors.

Characterization of MRSA mecA *gene*

After sequencing, one representative isolate was selected each for cattle and buffalo population of all three tehsils, and the BLAST and CLUSTAL W alignment

tool was used to align and compare these local isolates nucleotide sequences with already reported *mecA* gene sequences. The local isolates revealed up to 99% homology with MRSA isolates reported from the USA, Turkey, India, and Brazil. After alignment, the similarity and significant variations among already reported sequences and current study sequences were explained by constructing a phylogenetic tree.

Table V. Risk factors included in final logistic regression model for MRSA-associated mastitis.

Variable	Response	OR	95% C.I	S.E	p value
Mastitis history	Present	2.92	1.056-8.035	0.518	0.039
	Absent	1			
Use of β-lactam antibiotics	Frequently	3.054	1.015-9.184	0.562	0.047
	Seldom	1			
Milking person hygiene	Poor	2.825	1.132-7.054	0.467	0.026
	Good	1			
Milking method	Hand	3.134	1.042-9.425	0.562	0.042
	Machine	1			

Table VI. Risk factors included in final logistic regression model for *S. aureus*-associated mastitis.

Variable	Response	OR	95% C.I	S.E	p value
Mastitis history	Present	1.952	1.191 - 3.198	0.252	0.008
	Absent	1			
Teat lesion	Present	2.215	1.312 - 3.739	0.267	0.003
	Absent	1			

The phylogenetic tree, constructed by using the neighbor-joining method, showed that the study isolates clustered together exhibiting high similarity among themselves. All isolates showed similarity and making in group with all other countries isolates except Brazil (Accession no: KF058902) which is out grouped and showing significant variations in nucleotide pattern to local study isolates (Fig. 2). Furthermore, the phylogenetic tree also reported current study isolates showed more evolutionary relationships among themselves as compared to other reported *mecA* gene sequences from NCBI.

SDS PAGE analysis

The protein profile of both *mec*A positive and *mec*A negative isolates was compared by SDS PAGE using 12.5% polyacrylamide resolving gel. The molecular weight of protein bands produced by MRSA isolates ranges from 26.90KDa to 122.38KDa along with a characteristic band of 78KDa indicative of *PBP2a* which is present in MRSA and not seen in MSSA. Moreover, some other proteins bands were found in MRSA and absent in MSSA isolates. The comparative protein profile reveals a significant difference between MRSA and MSSA isolates as shown in Figure 3.

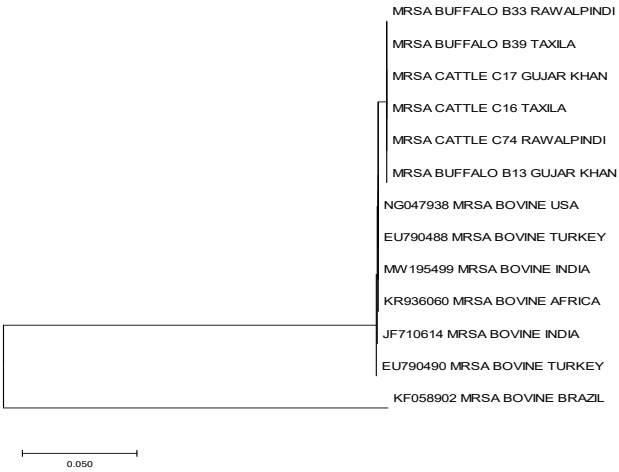

Fig. 2. Phylogenetic Tree showing comparison of the study isolates of MRSA with reported isolates.

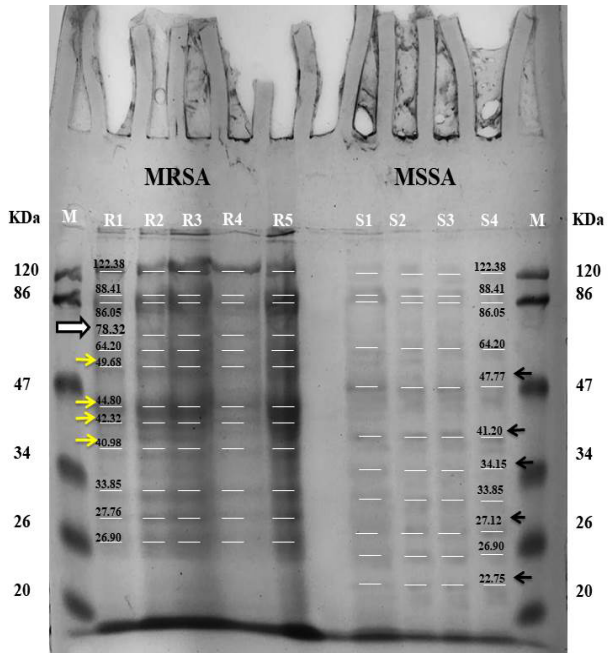

Fig. 3. Manually labeled SDS-PAGE comparison results of MRSA and MSSA with a determined molecular weight of each band. (R1 to R5) shows MRSA isolates, while, (S1 to S4) shows the MSSA. The white arrow shows the presence of 78kDa band of *PBP2a* protein of MRSA and absent in MSSA. (Yellow arrows) Shows the difference in protein bands present in MRSA, (Black arrows) shows the difference in protein bands present in MSSA.

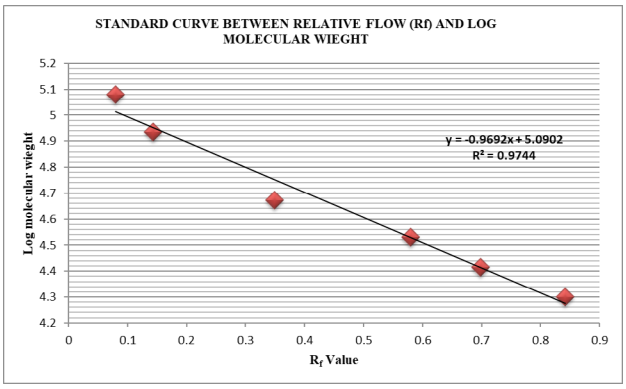

Fig. 4. A standard Curve obtained by plotting a scatter graph between Rf values and log of known molecular weight of protein marker. The X-axis shows the Rf values ranging from 0 to 0.9. Y-axis shows the log of molecular weight ranging from 4.2 to 5.2. The regression formula (y = -0.9692x + 5.0902) obtained was used to calculate the molecular weight of all the unknown proteins separated on the gel. Where Y= Rf value of that specific protein band and X= Molecular weight of unknown protein band.

DISCUSSION

Bovine mastitis caused by *S. aureus* is a major cause of milk production losses in the Asian buffalo and cattle populations (Badua *et al*., 2020). *S. aureus* is a leading pathogen in the causation of clinical, sub-clinical, and chronic mastitis in bovines (Aqib *et al*., 2017). The current study revealed that *S. aureus* associated subclinical mastitis was prevalent in 32.95% of cattle and 24.42% of buffaloes with an overall prevalence of 28.70%. The pathogen is involved in one-third of subclinical and clinical mastitis cases in the cattle population (Li *et al*., 2017). The current results were supported by the findings of various other researchers who reported an overall prevalence of 28%-33% in various countries (Abo-Shama, 2014; Pamuk *et al*., 2012; Saka *et al*., 2018; Sharma *et al*., 2011). However, contrary to the findings of the current study, some reports depicting the higher prevalence rates of *S. aureus* have also been documented (Abera *et al*., 2013; Ganai *et al*., 2016). The wide-ranging dissemination of this pathogen on the udder and teats surface along with survival inside the mammary gland is responsible for the higher prevalence of *S. aureus* induced subclinical mastitis in bovines (Ganai *et al*., 2016).

In the last few decades, MRSA has become a major threat having zoonotic potential, and is of central importance for public health (Vandenesch *et al*., 2003). Various reports have been published on the confirmation of MRSA in dairy animals and related products in various countries. The MRSA prevalence of 47.62% found in the current study was close to the findings reported in China (47.6%), Ethiopia (42.9%), and Egypt (35.7%), However, a relatively closer prevalence has also been reported in Pakistan (34%) (Algammal *et al*., 2020a; Aqib *et al*., 2017; Girmay *et al*., 2020; Yang *et al*., 2020). Lack of hygienic measures and standard pre and post-milking practices, environmental contamination, undue usage of antibiotics without consulting the veterinarian, and use of hand milking method instead of machine milking in indigenous cattle and buffalo are the prime reasons which may be responsible for the higher prevalence of MRSA in the current study. While the lower prevalence from the Philippines (23.08%), Iran (20%), China (19%), Germany (16.7%), and Turkey (15.89%) has also been reported (Badua *et al*., 2020; Buyukcangaz *et al*., 2014; Havaei *et al*., 2015; Spohr *et al*., 2011; Vandenesch *et al*., 2003; Zhang *et al*., 2016). Contrary to the findings of the current study, a very low prevalence of 0.46% in China (Li *et al*., 2017), 9% and 13.1% in India (Kumar *et al*., 2010; Venugopal *et al*., 2019), 11.57% in Iran (Khazaie and Ahmadi, 2021), and 6.9% in Nepal (Shrestha *et al*., 2021) has also been documented from bovine mastitis. The higher prevalence (42.10%) of MRSA in indigenous dairy buffaloes might be due to hand milking practices and the lack of hygienic measures adopted in backyard buffalo farming (Badua *et al*., 2020). The discrepancies in overall MRSA prevalence in various reports might be due to variations in sampling strategies, sample size, geographical location of the study area, seasons, and farming system (backyard farming or commercial farming) (Girmay *et al*., 2020; Klibi *et al*., 2018).

The current study revealed that *S. aureus*-associated mastitis prevalence was significantly linked to the parity of the cow. Present study findings were in line with studies reported by Abrahmsén *et al*. (2014) and Tesfaye *et al*. (2019) and in contrast to the findings of Muzammil *et al*. (2021) that the risk of *S. aureus* associated mastitis significantly increased with the parity. The increased incidence of *S. aureus*-associated mastitis with parity could be explained in part by the probability of infection increases with age and parity. Furthermore, the greater the parity, or the number of times the cow has calved, the greater the chances of the pendulous udder and a mastitis history (Katsande *et al*., 2013). It has also been reported that cows with the most pendulous quarters tend to be the most susceptible to mammary infections, since the pendulous udder expose the teat and udder to damage, allowing bacteria to readily attach to the teat and get access to gland tissue (Abebe *et al*., 2016). In the current study, the mastitis history of the animal was highly significant towards the spread of *S. aureus* and MRSA. Similar results were also observed by Kemal *et al*. (2017) who concluded that previous mastitis record was a significant factor for

having an antimicrobial-resistant *S. aureus* infection in cows. The use of antibiotics was also a significant factor in the spread of MRSA in animals. MRSA is resistant to β-lactam antibiotics, which are commonly used to treat mastitis. Therefore, treatment of mastitis caused by β-lactam resistant *S. aureus*, such as MRSA usually leads to a lower cure rate (Taponen *et al*., 2003). In addition, MRSA intra-mammary infection can be chronic as *S. aureus* can form microabscesses and invade host phagocytes in the mammary glands. As a result, multiple infections can be seen in animals with mastitis caused by MRSA (Cortimiglia *et al*., 2016).

The current study also found that large herds were at a higher risk of MRSA infection than smaller herds. This finding was correlated with the observations made by Mekonnen *et al*. (2017) that *S. aureus* was isolated more frequently from large dairy herds. Similarly, Tenhagen *et al*. (2018) and Cortimiglia *et al*. (2016) also reported that a higher MRSA prevalence was observed in the large herds of cows examined. The high risk of MRSA infection in large dairy herds may be due to a high number of mastitis cases, which can lead to an increased frequency of antibiotic treatments. In addition, larger herds tend to be more likely to import new animals on the farm, which increases the risk of introducing MRSA-infected animals into the herd (Tenhagen *et al*., 2018).

Protein profiles of MRSA and MSSA were compared using SDS-PAGE along with known protein markers to be used for the determination of polypeptides separated by resolving gel. The difference of protein profile among MRSA and MSSA isolates reported in the current study is supported by the findings of Tesařová *et al*. (2016) who reported significant differences among the band's pattern of both isolates. Similar findings were also documented by Sacilik *et al*. (2000). This could be attributed due to the difference in protein profiles among both sensitive and resistant isolates. Moreover, SDS PAGE of MRSA isolates revealed the protein band of 78.2KDa which is designated for *PBP2a* altered protein particular for MRSA and absent in MSSA, and it is supported by Doan *et al*. (2013) and Hartman and Tomasz (1986). The presence of the *PBP2a* band explains the resistance mechanism of MRSA as this protein is encoded by the *mec*A gene which is exclusively related to the occurrence of resistance (Arsic *et al*., 2012; Wendlandt *et al*., 2013). However, the SDS-PAGE technique has proved to be a conventional technique to differentiate between both isolates based on their protein expression (Tesařová *et al*., 2016).

CONCLUSION

The study concluded a significant prevalence of MRSA from indigenous bovine breeds from district Rawalpindi. Molecular characterization revealed considerable variation among local field isolates of MRSA and also with already reported isolates from other countries. The comparative risk factor analysis showed that milking person hygiene, milking method, and usage of antibiotics were significantly associated with MRSA. However, mastitis history, physiological status, and teat lesion were found potential risk factors for both *S. aureus* and MRSA-associated mastitis. The current study proved the first molecular characterization report addressing resistance patterns in field isolates of MRSA which can be effective in devising control strategies.

ACKNOWLEDGMENT

The current study was conducted at Medicine Research Laboratory, Department of Veterinary Medicine, University of Veterinary and Animal Sciences (UVAS) and part of the research project number ALP AS-042 entitled as "Mechanistic study of MRSA from bovine and occupational workers, and its application in field conditions" funded by the Pakistan Agriculture Research Council (PARC). The authors would like to thank the funding agency.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Abdeen, E.E., Mousa, W.S., Abdel-Tawab, A.A., El-Faramawy, R. and Abo-Shama, U.H., 2021. Phenotypic, genotypic and antibiogram among *Staphylococcus aureus* isolated from bovine subclinical mastitis. *Pak. Vet. J.,* **41**: 289-293.

Abebe, R., Hatiya, H., Abera, M., Megersa, B. and Asmare, K., 2016. Bovine mastitis: Prevalence, risk factors and isolation of *Staphylococcus aureus* in dairy herds at Hawassa milk shed, South Ethiopia. *BMC Vet. Res.,* **12**: 270. https://doi.org/10.1186/s12917-016-0905-3

Abera, M., Demie, B., Aragaw, K., Regassa, F. and Regassa, A., 2013. Isolation and identification of *Staphylococcus aureus* from bovine mastitis milk and their drug resistance patterns in Silchar town dairy farms, N.E India. *Online Int. Interdiscip. Res. J.,* **4**: 256–260.

Abo-Shama, U., 2014. Prevalence and antimicrobial susceptibility of *Staphylococcus aureus* isolated from cattle, buffalo, sheep and goats. *Assiut Vet. Med. J.*, **60**: 63–72. https://doi.org/10.21608/

avmj.2014.170753

Abrahmsén, M., Persson, Y., Kanyima, B.M. and Bage, R., 2014. Prevalence of subclinical mastitis in dairy farms in urban and peri-urban areas of Kampala, Uganda. *Trop. Anim. Hlth. Prod.,* **46**: 99–105. https://doi.org/10.1007/s11250-013-0455-7

Afzal, M., Anwar, M. and Mirza, M.A., 2007. Some factors affecting milk yield and lactation. *Pak. Vet. J*., **27**: 113–117.

Algammal, A.M., Enany, M.E., El-Tarabili, R.M., Ghobashy, M.O.I. and Helmy, Y.A., 2020a. Prevalence, antimicrobial resistance profiles, virulence and enterotoxin-determinant genes of MRSA isolated from subclinical bovine mastitis samples in Egypt. *Pathogens*, **9**: 1–11. https://doi.org/10.3390/pathogens9050362

Algammal, A.M., Hetta, H.F., Elkelish, A., Alkhalifah, D.H.H., Hozzein, W.N., Batiha, G.E.S., Nahhas, N. El. and Mabrok, M.A., 2020b. Methicillin-resistant *Staphylococcus aureus* (MRSA): One health perspective approach to the bacterium epidemiology, virulence factors, antibiotic-resistance, and zoonotic impact. *Infect. Drug Resist*., **13**: 3255–3265. https://doi.org/10.2147/IDR.S272733

Altaf, M., Ijaz, M., Ghaffar, A., Rehman, A. and Avais, M., 2019. Antibiotic susceptibility profile and synergistic effect of non-steroidal anti-inflammatory drugs on antibacterial activity of resistant antibiotics (Oxytetracycline and Gentamicin) against methicillin resistant *Staphylococcus aureus* (MRSA). *Microb. Pathog.,* **137**: https://doi.org/10.1016/j.micpath.2019.103755

Aqib, A.I., Ijaz, M., Anjum, A.A., Malik, M.A.R., Mehmood, K., Farooqi, S.H. and Hussain, K., 2017. Antibiotic susceptibilities and prevalence of Methicillin resistant *Staphylococcus aureus* (MRSA) isolated from bovine milk in Pakistan. *Acta Trop.*, **176**: 168–172. https://doi.org/10.1016/j.actatropica.2017.08.008

Arsic, B., Zhu, Y., Heinrichs, D.E. and McGavin, M.J., 2012. Induction of the staphylococcal proteolytic cascade by antimicrobial fatty acids in community acquired methicillin resistant *Staphylococcus aureus*. *PLoS One,* **7**: https://doi.org/10.1371/journal.pone.0045952

Baddour, M.M., Abuelkheir, M.M. and Fatani, A.J., 2007. Comparison of mecA polymerase chain reaction with phenotypic methods for the detection of methicillin-resistant *Staphylococcus aureus*. *Curr. Microbiol.,* **55**: 473–479. https://doi.org/10.1007/s00284-007-9015-6

Badua, A.T., Boonyayatra, S., Awaiwanont, N., Gaban, P.B.V., Mingala, C.N., 2020. Antibiotic resistance and genotyping of mecA-positive methicillin-resistant *Staphylococcus aureus* (MRSA) from milk and nasal carriage of dairy water buffaloes (*Bubalus bubalis*) in the Philippines. *J. Adv. Vet. Anim. Res.,* **7**: 397–406. https://doi.org/10.5455/javar.2020.g434

Berber, I., Cokmus, C. and Atalan, E., 2003. Characterization of *Staphylococcus* species by SDS-PAGE of whole-cell and extracellular proteins. *Microbiology,* **72**: 42–47. https://doi.org/10.1023/A:1022221905449

Buyukcangaz, E., Kahya, S., Sen, A., Intas, K.S., Eyigor, A. and Temelli, S., 2014. MecA gene prevalence in *Staphylococcus aureus* isolates from dairy cows in turkey. *J. Biol. Environ. Sci.,* **7**: 183–190.

Chua, K.Y.L., Howden, B.P., Jiang, J.H., Stinear, T. and Peleg, A.Y., 2014. Population genetics and the evolution of virulence in *Staphylococcus aureus*. *Infect. Genet. Evol.,* **21**: 554–562. https://doi.org/10.1016/j.meegid.2013.04.026

CLSI., 2019. *CLSI M100-ED29: 2019 performance standards for antimicrobial susceptibility testing*. 29th Edition, ClSI.

Cortimiglia, C., Luini, M., Bianchini, V., Marzagalli, L., Vezzoli, F., Avisani, D., Bertoletti, M., Ianzano, A., Franco, A. and Battisti, A., 2016. Short report prevalence of *Staphylococcus aureus* and of methicillin-resistant *S. aureus* clonal complexes in bulk tank milk from dairy cattle herds in lombardy region (Northern Italy). *Epidemiol. Infect.,* **144**: 3046–3051. https://doi.org/10.1017/S0950268816001576

Doan, G., Acar, G., Gursoy, Y. and Mercan, N., 2013. Antibiotic susceptibilities and SDS-PAGE protein profiles of methicillin-resistant *Staphylococcus aureus* (MRSA) strains obtained from Denizli hospital. *Curr. Prog. biol. Res.,* **345**. https://doi.org/10.5772/55457

El-Ashker, M., Gwida, M., Monecke, S., El-Gohary, F., Ehricht, R., Elsayed, M., Akinduti, P., El-Fateh, M., and Maurischat, S., 2020. Antimicrobial resistance pattern and virulence profile of *S. aureus* isolated from household cattle and buffalo with mastitis in Egypt. *Vet. Microbiol.,* **240**: 108535. https://doi.org/10.1016/j.vetmic.2019.108535

Elhaig, M.M. and Selim, A., 2015. Molecular and bacteriological investigation of subclinical mastitis caused by *Staphylococcus aureus* and *Streptococcus agalactiae* in domestic bovids from Ismailia, Egypt. *Trop. Anim. Hlth. Prod.,* **47**: 271–

276. https://doi.org/10.1007/s11250-014-0715-1

Galdiero, E., Liguori, G., D'Isanto, M., Damiano, N. and Sommese, L., 2003. Distribution of mecA among methicillin-resistant clinical staphylococcal strains isolated at hospitals in Naples, Italy. *Eur. J. Epidemiol.,* **18**: 139–145. https://doi.org/10.1023/A:1023067930211

Ganai, A.W., Kotwal, S.K., Wani, N., Malik, M.A., Jeelani, R., Kour, S. and Zargar, R., 2016. Detection of mecA gene of methicillin resistant S*taphylococcus aureus* by PCR assay from raw milk. *Indian J. Anim. Sci.,* **86**: 508–511.

Giovanni, N., Marta, C., Elisa, S., Rosa, F., Loredana, C., Alessandra, B. and Antonio, P., 2020. Occurrence and characteristics of methicillin-resistant *Staphylococcus aureus* (MRSA) in buffalo bulk tank milk and the farm workers in Italy. *Fd. Microbiol.,* **91**: 103509. https://doi.org/10.1016/j.fm.2020.103509

Girmay, W., Gugsa, G., Taddele, H., Tsegaye, Y., Awol, N., Ahmed, M. and Feleke, A., 2020. Isolation and identification of methicillin-resistant *Staphylococcus aureus* (MRSA) from milk in shire dairy farms, Tigray, Ethiopia. *Vet. Med. Int.,* **2020**. https://doi.org/10.1155/2020/8833973

Hartman, B.J. and Tomasz, A., 1986. Expression of methicillin resistance in heterogeneous strains of *Staphylococcus aureus*. *Antimicrob. Agents Chemother*., **29**: 85–92. https://doi.org/10.1128/AAC.29.1.85

Havaei, S.A., Assadbeigi, B., Esfahani, B.N., Hoseini, N.S., Rezaei, N. and Havaei, S.R., 2015. Detection of mecA and enterotoxin genes in *Staphylococcus aureus* isolates associated with bovine mastitis and characterization of *Staphylococcal cassette* chromosome mec (SCCmec) in MRSA strains. *Iran. J. Microbiol.*, **7**: 161–167.

Holt, J., 1977. *The shorter Bergey's manual of determinative bacteriology*. cabdirect.org.

Javed, M.U., Ijaz, M., Fatima, Z., Anjum, A.A., Aqib, A.I., Ali, M.M., Rehman, A., Ahmed, A. and Ghaffar, A., 2021. Frequency and antimicrobial susceptibility of methicillin and vancomycin-resistant *Staphylococcus aureus* from bovine milk. *Pak. Vet. J.,* **41**: 463-468. https://doi.org/10.29261/pakvetj/2021.060

Ji, Y., Xiao, F., Zhu, W., Liu, S., Feng, X., Sun, C., Lei, L., Dong, J., Khan, A., Han, W. and Gu, J., 2020. LysGH15 effectively control murine mastitis caused by *Staphylococcus aureus*. *Pak. Vet. J.*, **40**: 519–522. https://doi.org/10.29261/pakvetj/2020.056

Katsande, S., Matope, G., Ndengu, M. and Pfukenyi, D.M., 2013. Prevalence of mastitis in dairy cows from smallholder farms in Zimbabwe. *Onderstepoort J. Vet. Res.*, **80**: 1–7. https://doi.org/10.4102/ojvr.v80i1.523

Kayitsinga, J., Schewe, R.L., Contreras, G.A. and Erskine, R.J., 2017. Antimicrobial treatment of clinical mastitis in the eastern United States: The influence of dairy farmers' mastitis management and treatment behavior and attitudes. *J. Dairy Sci.,* **100**: 1388–1407. https://doi.org/10.3168/jds.2016-11708

Kemal, K.E., Tesfaye, S., Ashanafi, S. and Muhammad, Hussien. A.F., 2017. Prevalence, risk factors and multidrug resistance profile of *Staphylococcus aureus* isolated from bovine mastitis in selected dairy farms in and around Asella town, Arsi Zone, South Eastern Ethiopia. *Afr. J. Microbiol. Res.*, **11**: 1632–1642. https://doi.org/10.5897/AJMR2017.8529

Khazaie, F. and Ahmadi, E., 2021. Bovine subclinical mastitis associated methicillin resistant *Staphylococcus aureus*, selective genotyping and antimicrobial susceptibility profile of the isolates in Kurdistan province of Iran. *Iran. J. Microbiol*., **13**: 65–73. https://doi.org/10.18502/ijm.v13i1.5494

Klibi, A., Jouini, A., Gómez, P., Slimene, K., Ceballos, S., Torres, C. and Maaroufi, A., 2018. Molecular characterization and clonal diversity of methicillin-resistant and-susceptible *Staphylococcus aureus* isolates of milk of cows with clinical mastitis in Tunisia. *Microb. Drug Resist.,* **24**: 1210–1216. https://doi.org/10.1089/mdr.2017.0278

Kumar, R., Yadav, B.R. and Singh, R.S., 2010. Genetic determinants of antibiotic resistance in *Staphylococcus aureus* isolates from milk of mastitic crossbred cattle. *Curr. Microbiol.,* **60**: 379–386. https://doi.org/10.1007/s00284-009-9553-1

Li, T., Lu, H., Wang, X., Gao, Q., Dai, Y., Shang, J. and Li, M., 2017. Molecular characteristics of *Staphylococcus aureus* causing bovine mastitis between 2014 and 2015. *Front. Cell. Infect. Microbiol.,* **7**: 1–10. https://doi.org/10.3389/fcimb.2017.00127

Louie, L., Goodfellow, J., Mathieu, P., Glatt, A., Louie, M. and Simor, A.E., 2002. Rapid detection of methicillin-resistant staphylococci from blood culture bottles by using a multiplex PCR assay. *J. Clin. Microbiol.*, **40**: 2786-2790. https://doi.org/10.1128/JCM.40.8.2786-2790.2002

Mekonnen, S.A., Koop, G., Melkie, S.T., Getahun, C.D., Hogeveen, H. and Lam, T.J.G.M., 2017. Prevalence of subclinical mastitis and associated

risk factors at cow and herd level in dairy farms in North-West Ethiopia. *Prev. Vet. Med.,* **145**: 23–31. https://doi.org/10.1016/j.prevetmed.2017.06.009

Monistero, V., Graber, H.U., Pollera, C., Cremonesi, P., Castiglioni, B., Bottini, E., Ceballos-Marquez, A., Lasso-Rojas, L., Kroemker, V., Wente, N., Petzer, I.M., Santisteban, C., Runyan, J., dos Santos, M.V., Alves, B.G., Piccinini, R., Bronzo, V., Abbassi, M.S., Said, M. and Ben, Moroni, P., 2018. *Staphylococcus aureus* isolates from bovine mastitis in eight countries: Genotypes, detection of genes encoding different toxins and other virulence genes. *Toxins* (*Basel*), **10**. https://doi.org/10.3390/toxins10060247

Muzammil, I., Saleem, M.I., Aqib, A.I., Ashar, A., Mahfooz, S.A., Shoaib, M., Naseer, M.A., Sohrani, I.K., Ahmad, J., Saqi, R., Lodhi, F.L. and Tanveer, Q., 2021. Emergence of pathogenic strains of *Staphylococcus aureus* in goat milk and their comparative response to antibiotics. *Pakistan J. Zool.,* **53**: 1–9. https://doi.org/10.17582/journal.pjz/20191024181008

Olowe, O.A., Kukoyi, O.O., Taiwo, S.S., Ojurongbe, O., Opaleye, O.O., Bolaji, O.S., Adegoke, A.A., Makanjuola, O.B., Ogbolu, D.O. and Alli, O.T., 2013. Phenotypic and molecular characteristics of methicillin-resistant *Staphylococcus aureus* isolates from Ekiti State, Nigeria. *Infect. Drug Resist.,* **6**: 87–92. https://doi.org/10.2147/IDR.S48809

Pamuk, S., Yildirim, Y., Seker, E., Gurler, Z. and Kara, R., 2012. A survey of the occurrence and properties of methicillin-resistant *Staphylococcus aureus* and methicillin-resistant *Staphylococcus intermedius* in water buffalo milk and dairy products in Turkey. *Int. J. Dairy Technol.,* **65**: 416–422. https://doi.org/10.1111/j.1471-0307.2012.00832.x

Reyher, K.K. and Dohoo, I.R., 2011. Diagnosing intramammary infections: Evaluation of composite milk samples to detect intramammary infections. *J. Dairy Sci.,* **94**: 3387–3396. https://doi.org/10.3168/jds.2010-3907

Sacilik, S.C., Osmanagaoglu, O., Palabiyikoglu, I., Bengisun, J.S. and Cokmus, C., 2000. Analysis of methicillin resistant *Staphylococcus aureus* isolates by polyacrylamide gel electrophoresis in an intensive care unit of Ibni-Sina Hospital. *Turk. J. Med. Sci.,* **30**: 367–371.

Saka, E. and Terzi, Gulel, G., 2018. Detection of enterotoxin genes and methicillin-resistance in *Staphylococcus aureus* isolated from water buffalo milk and dairy products. *J. Fd. Sci.,* **83**: 1716–1722. https://doi.org/10.1111/1750-3841.14172

Senna, J.P.M., Roth, D.M., Oliveira, J.S., Machado, D.C. and Santos, D.S., 2003. Protective immune response against methicillin resistant *Staphylococcus aureus* in a murine model using a DNA vaccine approach. *Vaccine*, **21**: 2661–2666. https://doi.org/10.1016/S0264-410X(02)00738-7

Sharif, A., Ahmad, T., Umer, M., Bilal, M.Q., Muhammad, G. and Sharif, M.A., 2009. Quarter based determination of milk lactose contents and milk. *Pakistan J. Zool.,* **9**: 313–321.

Sharma, D., Malik, A. and Sharma, K., 2011. Prevalence and antimicrobial susceptibility of drug resistant staphylococcus aureus in raw milk of dairy cattle biomolecules production view project antibiosis view project prevalence and antimicrobial susceptibility of drug resistant S*taphylococcus aureu*. *Int. Res. J. Microbiol.,* **2**: 466–470.

Shrestha, A., Bhattarai, R.K., Luitel, H., Karki, S., and Basnet, H.B., 2021. *Prevalence of methicillin-resistant* Staphylococcus aureus *and pattern of antimicrobial resistance in mastitis milk of cattle in Chitwan, Nepal*. pp. 1–7. https://doi.org/10.1186/s12917-021-02942-6

Spohr, M., Rau, J., Friedrich, A., Klittich, G., Fetsch, A., Guerra, B., Hammerl, J.A. and Tenhagen, B.A., 2011. Methicillin-resistant *Staphylococcus aureus* (MRSA) in three dairy herds in southwest Germany. *Zoonoses Publ. Hlth*., **58**: 252–261. https://doi.org/10.1111/j.1863-2378.2010.01344.x

Taponen, S., Jantunen, A., Pyörälä, E. and Pyörälä, S., 2003. Efficacy of targeted 5-day combined parenteral and intramammary treatment of clinical mastitis caused by penicillin-susceptible or penicillin-resistant *Staphylococcus aureus*. *Acta Vet. Scand.,* **44**: 53–62. https://doi.org/10.1186/1751-0147-44-53

Tenhagen, B.A., Alt, K., Pfefferkorn, B., Wiehle, L., Käsbohrer, A. and Fetsch, A., 2018. Short communication: Methicillin-resistant *Staphylococcus aureus* in conventional and organic dairy herds in Germany. *J. Dairy Sci.,* **101**: 3380–3386. https://doi.org/10.3168/jds.2017-12939

Tesařová, M., Horká, M., Moravcová, D., Svojanovská, L., Mlynarikova, K. and Růžička, F., 2016. SDS-PAGE and Gel IEF: Tool for differentiation of methicillin-resistant and methicillin sensitive strains of *Staphylococcus aureus*. *Curr. Microbiol.,***72**: 315-320. https://doi.org/10.1007/s00284-015-0939-y

Tesfaye, B., Matios, L., Getachew, T., Tafesse, K., Abebe, O., Letebrihan, Y., Mekdes, T. and Tilaye, D., 2019. Study on bovine mastitis with isolation

of bacterial and fungal causal agents and assessing antimicrobial resistance patterns of isolated *Staphylococcus* species in and around Sebeta town, Ethiopia. *Afr. J. Microbiol. Res.,* **13**: 23–32. https://doi.org/10.5897/AJMR2018.8909

Thrushfield, M., 2013. *Veterinary epidemiology,* Blackwell. https://doi.org/10.1016/j.ijmm.2013.02.006

Vandenesch, F., Naimi, T., Enright, M.C., Lina, G., Nimmo, G.R., Heffernan, H., Liassine, N., Bes, M., Greenland, T., Reverdy, M.E. and Etienne, J., 2003. Community acquired methicillin resistant *Staphylococcus aureus* carrying panton-valentine leukocidin genes: Worldwide emergence. *Emerg. Infect. Dis.,* **9**: 978–984. https://doi.org/10.3201/eid0908.030089

Venugopal, N., Mitra, S., Tewari, R., Ganaie, F., Shome, R., Rahman, H. and Shome, B.R., 2019. Molecular detection and typing of methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative staphylococci isolated from cattle, animal handlers, and their environment from Karnataka, Southern Province of India. *Vet. World.,* **12**: 1760–1768. https://doi.org/10.14202/vetworld.2019.1760-1768

Wendlandt, S., Feßler, A.T., Monecke, S., Ehricht, R., Schwarz, S. and Kadlec, K., 2013. The diversity of antimicrobial resistance genes among staphylococci of animal origin. *Int. J. med. Microbiol.,* **303**: 338–349. https://doi.org/10.1016/j.ijmm.2013.02.006

Yang, F., Zhang, S., Shang, X., Li, H., Zhang, H., Cui, D., Wang, X., Wang, L., Yan, Z. and Sun, Y., 2020. Short communication: Detection and molecular characterization of methicillin-resistant *Staphylococcus aureus* isolated from subclinical bovine mastitis cases in China. *J. Dairy Sci.,* **103**: 840–845. https://doi.org/10.3168/jds.2019-16317

Zaatout, N. and Hezil, D., 2021. A meta-analysis of the global prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from clinical and subclinical bovine mastitis. *J. appl. Microbiol.,* 1–15. https://doi.org/10.1111/jam.15192

Zhang, L., Li, Y., Bao, H., Wei, R., Zhou, Y., Zhang, H. and Wang, R., 2016. Population structure and antimicrobial profile of *Staphylococcus aureus* strains associated with bovine mastitis in China. *Microb. Pathog.,* **97**: 103–109. https://doi.org/10.1016/j.micpath.2016.06.005

Zhao, X., Yuan, Xiaomeng, Hu, M., Zhang, Y., Li, L., Zhang, Q., Yuan, Xuexia, Wang, W. and Liu, Y., 2021. Prevalence and characterization of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* isolated from bulk tank milk in Shandong dairy farms. *Fd. Contr.,* **125**: 107836. https://doi.org/10.1016/j.foodcont.2020.107836