Original Article

Evidence-based identification and characterization of methicillin-resistant *Staphylococcus aureus* isolated from subclinical mastitis in dairy buffaloes of Pakistan

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Abstract

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA), affecting livestock and human beings, has become a global public health hazard with economic consequences. **Aims:** The current study was designed to investigate the prevailing MRSA-associated subclinical mastitis and associated risk factors in dairy buffaloes. The study also highlighted the genetic variations and in silico-based proteomic differences among MRSA isolates. **Methods:** Out of 516 milk samples, 45.93% (237/516) were found positive for subclinical mastitis, while the prevalence of *S. aureus* was recorded 56.12%. The methicillin resistance in *S. aureus* isolates was evaluated by oxacillin disc diffusion test and molecular identification of the *mecA* gene. **Results:** The results revealed a phenotypic and molecular prevalence of MRSA at 45.11% and 18.79%, respectively. The risk factor analysis revealed that among various assumed risk factors, parity, milking hygiene, milker care during milking, milk yield, housing system, and floor type were significantly associated with subclinical mastitis in buffaloes. The sequencing and phylogenetic analysis showed no significant genetic variations among study isolates and depicted a high similarity with isolates from Africa, USA, India, Italy, Turkey, and Iran. The in-silico protein analysis showed that all sequences had the same protein motifs resembling penicillin protein 2a except Buff-13, whose protein structure resembles alpha-catenin-like protein hmp-1. **Conclusion:** The current study was the first report of the genotypic characterization and in silico protein analysis of MRSA from dairy buffaloes in Pakistan. The result highlighted the importance of antimicrobial resistance (AMR) and development of control strategies against MRSA infections.

Key words: Antimicrobial resistance, Buffaloes, Mastitis, Methicillin-resistant *Staphylococcus aureus*, Phylogenetic analysis

Introduction

Buffaloes are considered dairy animals in the 21st century because of their higher adaptability and productivity in changing climatic conditions. Nili-Ravi buffalo breed in South Asia is well known for its high milk production, supporting small farmers and entrepreneurs (Siddiky and Faruque, 2018). Buffalo milk is valued for its better nutritional content as compared to cattle (Fagiolo and Lai, 2007) and use in value-added products (Locatelli *et al*., 2013). Although buffaloes are considered resistant to numerous tropical diseases prevailing in South Asia, there is a need to improve the production of high-quality buffalo milk through better management and control of diseases affecting its production such as mastitis (Guimarães *et al*., 2017).

Staphylococcus aureus is the main causative agent of mastitis in buffaloes in Asia (Wang *et al*., 2015). Antibiotics are used to treat mastitis in dairy herds (Haran *et al*., 2012). Antibiotic resistance in *S. aureus* has become a serious zoonotic concern throughout the globe. Lack of effective medicine against this pathogen and the excessive use of β-lactam antibiotics lead to rising resistance in *S. aureus* strains (Gao *et al*., 2012). The methicillin resistance in *S. aureus* is developed by the acquisition of the staphylococcal cassette chromosome (SCC) *mec*A gene that encodes penicillin-binding protein 2a (PBP2a), resulting in loss of the affinity for all β-lactam antibiotics (Cuny *et al*., 2015). Methicillin-resistant *Staphylococcus aureus* (MRSA) has a zoonotic potential and can be transmitted from infected bovine milk and environment to the animal handlers and veterinarians (Juhász-Kaszanyitzky *et al*., 2007).

The indiscriminate use of antibiotics in animal production is believed the reason of MRSA increase (Tenhagen *et al*., 2018). Overcrowding on farms and

intensive animal trade can promote the rapid spread of MRSA among farm animals (Guo *et al*., 2018). Recent studies have also shown that LA-MRSA can colonize multiple animals and associated workers (Rinsky *et al*., 2013). There are also major concerns about the treatment of mastitis caused by MRSA because MRSA is not only resistant to β-lactams, but also against other antibiotics as well (Muzammil *et al*., 2022).

As a developing country, antibiotic resistance has become an emerging issue not only in Pakistan but also for the entire human-animal population throughout the world (Ali *et al*., 2018). In Pakistan, a 38% prevalence of MRSA has been reported in buffaloes (Aqib *et al*., 2017). Weak monitoring of infections and improper and indiscriminate use of antibiotics for humans and animals have contributed to MRSA development (Lakhundi and Zhang, 2018). Moreover, the sequencing and phylogenetic analysis of MRSA reveals genetic variation in the nucleic acid pattern, elucidating the transmission chains and pathogen reservoirs (Harris *et al*., 2010). In silico subtractive genome analysis is also a reliable method used to identify specific genes, which provide information for a set of proteins essential for the pathogen to develop a unique phenotype character responsible for resistance in *S. aureus* (Hasan *et al*., 2016). Therefore, the current study was planned to investigate the phenotypic and genotypic prevalence as well as the phylogenetic and protein analysis of MRSA associated-subclinical mastitis in buffaloes of Pakistan.

Materials and Methods

Collection of milk samples

A cross-sectional study was designed to collect 516 milk samples from buffaloes in different tehsils of 3 districts (Multan, Rahim Yar Khan, and Bahawalpur) of Pakistan (Fig. 1) using previous guideline (Thrusfield, 2007). During the collection of milk samples, a questionnaire regarding various risk factors (using teat dipping, feeding system, housing system, parity, physiological status, milk yield, milk sample, hygiene during milking, and floor type) was filled out to find out the association of various risk factors with the occurrence of subclinical mastitis. The sampling was done according to the standard protocols of the National Mastitis Council. The milk samples were screened for subclinical mastitis by surf field mastitis test (Javed *et al*., 2021). All the milk samples were kept at 4°C in ice packs and immediately transferred to the laboratory for further processing.

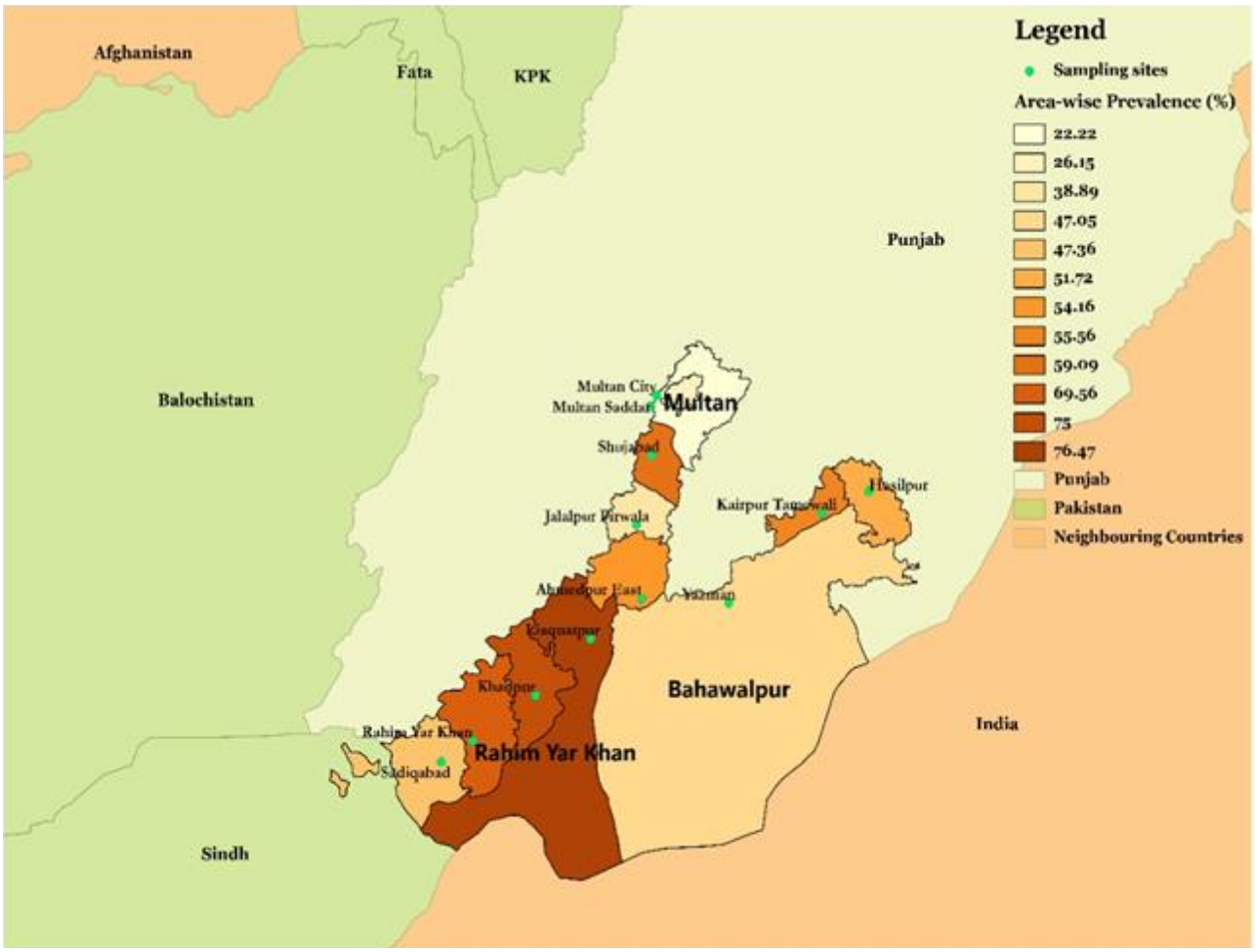

Fig. 1: GIS map of the study districts

Culturing of milk samples for *S. aureus*

SCM-positive milk samples were initially swabbed on 5% sheep blood agar. The colonies were further streaked on Mannitol salt agar for specific growth of *S. aureus* (Ghumman *et al*., 2022; Javed *et al*., 2023; Rasheed *et al*., 2023). The characteristic colonies of *S. aureus* were confirmed through Gram-staining for typical morphological characteristics of *S. aureus* followed by various biochemical tests such as catalase and coagulase tests (Aqib *et al*., 2018; Ahmed *et al*., 2022).

Phenotypic identification of MRSA

MRSA identification was carried out by placing Oxacillin discs (1 µg) on activated growth (0.5 McFarland) of *S. aureus* on Mueller Hinton agar and incubated at 37°C for 24 h (Muzammil *et al*., 2022). The zone of inhibition around Oxacillin discs was measured and compared with the standard provided by (CLSI, 2019).

Genotypic identification of MRSA by targeting *mecA* gene

Bacterial DNA was extracted from colonies on Muller Hinton agar using a DNA extraction kit, Thermoscientific (Sabir *et al*., 2023). Polymerase chain reaction (PCR) was then carried out by targeting the *mecA* gene of MRSA using previously reported primers (Forward:5´-TGG CAT TCG TGT CAC AAT CG-3´, and Reverse: 5´-CTG GAA CTT GTT GAG CAG AG-3´) (Galdiero *et al*., 2003). Initial denaturation was done (5 min at 95°C), followed by 35 cycles of denaturation (95°C for 30 s), annealing (58°C for 30 s), and extension (72°C for 30 s). The final extension was done for 8 min at 72°C. The amplified PCR products were run on 1.5% agarose gel using a 100 bp ladder under UV light illuminators. Amplicons with 310 bp size were considered positive.

Sequencing and phylogenetic analysis

Positive PCR products were excided from the agarose gel and purified using a gel purification kit (Gene All® Cat# 102-102, Lot: 10216B12009) following the manufacturer's instructions. The purified PCR products were then sent to 1st base biological technology, Singapore, for sequencing. The nucleotide sequences were initially analyzed using the BLAST search tool, aligned by Clustal W method using BioEdit software, and compared with reported sequences of *mecA* gene from GenBank database. Moreover, the similarities of current isolates were further explored by constructing a phylogenetic tree using neighborhood-joining methods on MEGA-X software.

Protein and motif analysis for PBP2a

Nucleic acid and protein alignment were done using Clustal Omega software. MEME Suit was used for the construction of nucleic acid and protein motifs. Gene structure was determined using a gene structure display server. Protein-conserved domains were predicted by the conserved domains architecture retrieval tool, the 3D structure of the protein was constructed by Swiss model software. Protein-protein interaction was found using STRING and the physical properties of proteins were calculated by ProtParam tool.

Statistical analysis

The relationship between various assumed risk factors with the occurrence of subclinical mastitis in buffaloes was analyzed statistically by univariate analysis. The variables were first tested by Chi-square test at significance level of $P<0.05$, at 95% confidence interval and 5% probability. The variables showing $P<0.2$ were further analyzed by logistic regression model to check potentially associated risk factors (Javed *et al*., 2021). Variables initially producing $P<0.2$ were further checked by multivariate analysis using the Logistic regression model on statistical software R.

Results

Prevalence of subclinical mastitis and *S. aureus*

The study showed 45.93% (237/516) prevalence of subclinical mastitis. Subclinical mastitis was higher in district Bahawalpur (51.16%) compared to 50.58% and 36.04% in district Rahim Yar Khan and Multan, respectively. The overall prevalence of subclinical mastitis caused by *S. aureus* was 56.12% (133/237) in buffaloes. A higher rate of occurrence of *S. aureus* was observed in district Rahim Yar Khan (67.81%) followed by 52.27% in district Bahawalpur and 45.16% in district Multan. The prevalence of *S. aureus* in district Rahim Yar Khan was also significantly ($P<0.0001$) associated with occurrence of sub-clinical mastitis infection in buffaloes compared to district Bahawalpur ($P=0.766$), and Multan ($P=0.062$) (Table 1).

Phenotypic and genotypic prevalence of MRSA

A total of 60 isolates of *S. aureus* from buffaloes phenotypically presented resistance to Oxacillin and exhibited a phenotypic prevalence of 45.11% (60/133) in buffaloes. MRSA phenotypic prevalence was higher in district Bahawalpur (47.82%) compared to 46.42% and 42.37% in districts Multan and Rahim Yar Khan, respectively. The PCR has detected the *mecA* gene in 25 isolates of *S. aureus*, showing the genotypic prevalence of MRSA at 18.79% (25/133). A higher genotypic prevalence of MRSA was observed in district Bahawalpur (19.56%) followed by district Rahim Yar Khan (18.64%) and district Multan (17.85%). The prevalence of MRSA infection in buffaloes was significantly associated with subclinical infection in all study districts like Rahim Yar Khan ($P=0.003$), Multan ($P=0.001$), and Bahawalpur ($P=0.003$) (Table 1).

Risk factors analysis

In univariate analysis, the parity of animals, milker care during milking, hygiene during milking, milk yield, milk sample, use of teat dips, floor type, and housing type were significantly associated ($P<0.05$) with the

occurrence of subclinical mastitis in buffaloes (Table 2).

Table 1: Prevalence of *S. aureus* and MRSA in dairy buffaloes

Name of district	Tehsils	No. of samples	SFMT N (%)	*S. aureus*		MRSA		
				N (%)	P-value	Phenotypic	Genotypic	P-value
Multan	Multan city	43	13/43 (30.23)	06/13 (46.15)	0.062	02/06 (33.33)	01/06 (16.67)	0.003*
	Multan Saddar	43	09/43 (20.93)	02/09 (22.22)		00/02 (00.00)	00/02 (00.00)	
	Shujabad	43	22/43 (51.16)	13/22 (59.09)		08/13 (61.53)	03/13 (23.07)	
	Jalalpur Pirwala	43	18/43 (41.86)	07/18 (38.89)		03/07 (42.86)	01/07 (14.29)	
Total	-	172	62/172 (36.04)	28/62 (45.16)		13/28 (46.42)	05/28 (17.85)	
RYK	Khanpur	43	28/43 (65.11)	21/28 (75.00)	<0.001*	11/21 (52.38)	05/21 (23.80)	0.001*
	Liaqatpur	43	17/43 (39.53)	13/17 (76.47)		05/13 (38.46)	03/13 (23.07)	
	Sadiqabad	43	19/43 (44.18)	09/19 (47.36)		02/09 (22.23)	01/09 (11.12)	
	RYK	43	23/43 (53.48)	16/23 (69.56)		07/16 (43.75)	02/16 (12.50)	
Total	-	172	87/172 (50.58)	59/87 (67.81)		25/59 (42.37)	11/59 (18.64)	
BWP	Ahmed Pur	43	24/43 (55.81)	13/24 (54.16)	0.766	09/13 (69.23)	04/13 (30.76)	0.003*
	Hasilpur	43	29/43 (67.44)	15/29 (51.72)		07/15 (46.67)	03/15 (20.00)	
	Khairpur	43	18/43 (41.86)	10/18 (55.56)		04/10 (40.00)	01/10 (10.00)	
	Yazman	43	17/43 (39.53)	08/17 (47.05)		02/08 (25.00)	01/08 (12.50)	
Total	-	172	88/172 (51.16)	46/88 (52.27)		22/46 (47.82)	09/46 (19.56)	
Overall	-	516	237/516 (45.93)	133/237 (56.12)		60/133 (45.11)	25/133 (18.79)	

P-value<0.05 shows significant effect

Table 2: Variables included in the questionnaire for subclinical mastitis in buffaloes

Variable	Variable levels	Total Samples	Positive (%)	Negative (%)	P-value
Parity	1st	81	31 (38.3)	50 (61.7)	0.001*
	2nd	154	61 (39.6)	93 (60.4)	
	3rd	162	71 (43.8)	91 (56.2)	
	>3rd	119	74 (62.2)	45 (37.8)	
Physiological status	Lactating	422	198 (46.9)	224 (53.1)	0.33
	Dry	94	39 (41.5)	55 (58.5)	
No. of milking	Twice	426	198 (46.5)	228 (53.5)	0.58
	Thrice	90	39 (43.3)	51 (56.7)	
Milker care during milking	Good	218	69 (31.7)	149 (68.3)	0.000*
	Poor	298	168 (56.4)	130 (43.6)	
Hygiene during milking	Yes	192	62 (32.3)	130 (67.7)	0.000*
	No	324	175 (54.0)	149 (45.9)	
Milk yield	Low	113	38 (33.6)	75 (66.4)	0.002*
	High	403	199 (49.4)	204 (50.6)	
Use of teat dips	Yes	87	27 (31.0)	60 (68.9)	0.002*
	No	429	210 (49.0)	219 (51.0)	
Milk sample	Raw milk	103	63 (61.2)	40 (38.8)	0.001*
	Bulk tank milk	413	174 (42.1)	239 (57.9)	
Body condition	Normal	447	207 (46.3)	240 (53.7)	0.89
	Thin	50	22 (44.0)	28 (56.0)	
	Emaciated	19	8 (42.1)	11 (57.9)	
Feed and water	Well-fed	480	222 (46.2)	258 (53.8)	0.59
	Underfed	36	15 (41.7)	21 (58.3)	
Feeding system	Stall feeding	216	104 (48.1)	112 (51.9)	0.46
	Grazing	103	42 (40.8)	61 (59.2)	
	Grazing + stall feeding	197	91 (46.2)	106 (53.8)	
Housing system	Conventional	301	154 (51.2)	147 (48.8)	0.005*
	Commercial	215	83 (38.6)	132 (61.4)	
Floor-type	Concrete	209	79 (37.8)	130 (62.2)	0.000*
	Earthen	263	125 (47.5)	138 (52.5)	
	Mud	44	33 (75.0)	11 (25.0)	

P-value<0.05 shows significant effect

Eight variables initially produced P<0.2 in univariate analysis (Table 2) were included in the multivariable regression model (Table 3). The final model comprised six statistically significant variables with an odds ratio greater than 1 (Fig. 2). The odds of subclinical mastitis in animals with 1st parity were 1.33 times more than in the 2nd, 3rd, and >3rd parity animals. Similarly, milker's hygiene during milking was significantly (P<0.05) associated with the occurrence of subclinical mastitis, as the animals with good milker care had 1.81 times lower chances of suffering from subclinical mastitis than those with poor milker care. Floor-type is also considered a significant risk factor for disease occurrence as the animals living in earthen and mud places have less chance 0.64 and 0.31 times, respectively of acquiring disease compared to animals living on concrete surfaces. Hygiene during milking was significantly associated with animals; hygiene during milking had 0.59 times less risk of subclinical mastitis than those with no hygiene maintained during milking. The odds of mastitis in animals with teat dipping practice before milking had 0.49 times fewer chances to disease compared to animals without teat dipping before milking. Milk sampled from bulk milk tank had 1.81 times more chance of the disease occurrence than the raw milk samples. Similarly, the housing system was also significantly associated with the risk of subclinical mastitis; conventional housing had 1.33 times more risk of getting mastitis than animals kept in the commercial housing systems (Table 3).

Molecular characterization of MRSA

The PCR products of locally identified isolates were sequenced for the partial fragment (310 bp) of the *mecA* gene of MRSA. Current study MRSA isolates sequences are available online on NCBI (National Centre for Biotechnology Information) having the following accession No. MZ814969, MZ814970, MZ814971, and MZ814972. The BLAST search of local study isolates revealed high similarity with already reported *mecA* sequences from the NCBI database. The Clustal W multiple alignments showed high similarity of the

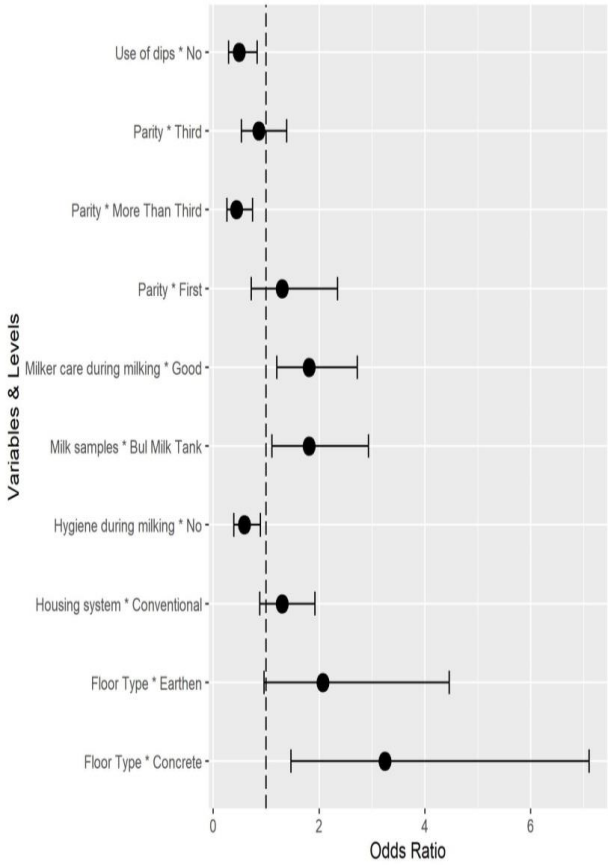

Fig. 2: Risk factors associated with the occurrence of mastitis in dairy buffaloes

Table 3: Key risk factors associated with the occurrence of subclinical mastitis in buffaloes

Variables	Variable levels	Odd ratio	95% C.I.	S.E.	P-value
			Lower-Upper		
Parity	>3rd	0.44	0.26-0.74	0.297	0.002
	3rd	0.86	0.53-1.39	0.251	0.541
	1st	1.33	0.72-2.35	0.247	0.379
	2nd	1			
Floor type	Earthen	0.64	0.43-0.96	0.376	0.029
	Mud	0.31	0.14-0.68	0.369	0.003
	Concrete	1			
Milker care during milking	Good	1.81	1.2-2.72	0.187	0.005
	Poor	1			
Hygiene during milking	No	0.59	0.39-0.89	0.190	0.012
	Yes	1			
Use of teat dips	No	0.49	0.29-0.83	0.251	0.008
	Yes	1			
Milk sample	Bulk milk tank	1.81	1.11-2.93	0.204	0.017
	Raw milk	1			
Housing system	Conventional	1.33	0.88-1.92	0.181	0.184
	Commercial	1			

P-value<0.05 and OR>1 show significant effects

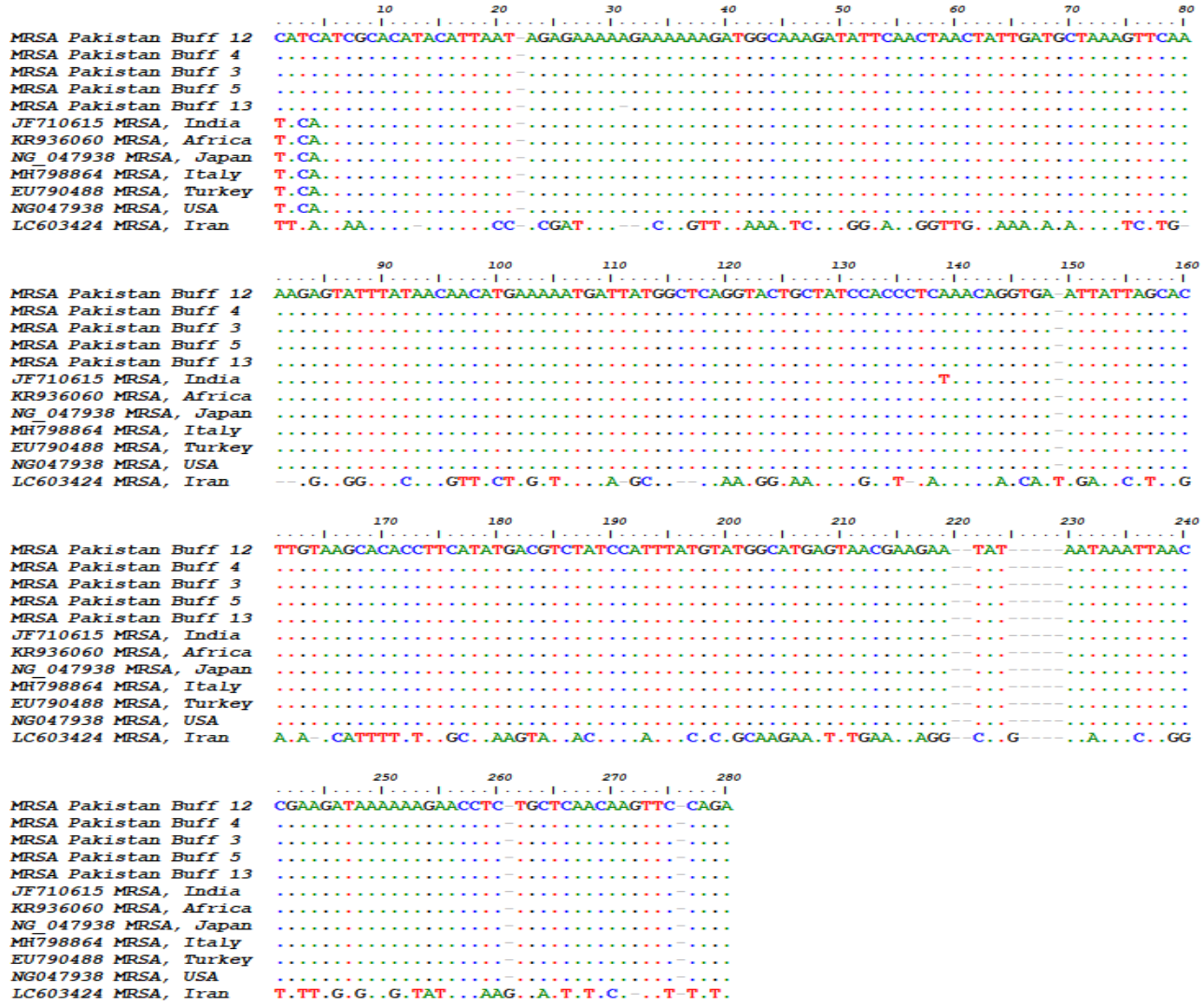

Fig. 3: Multiple clustal W alignment of the local isolates with reported isolates

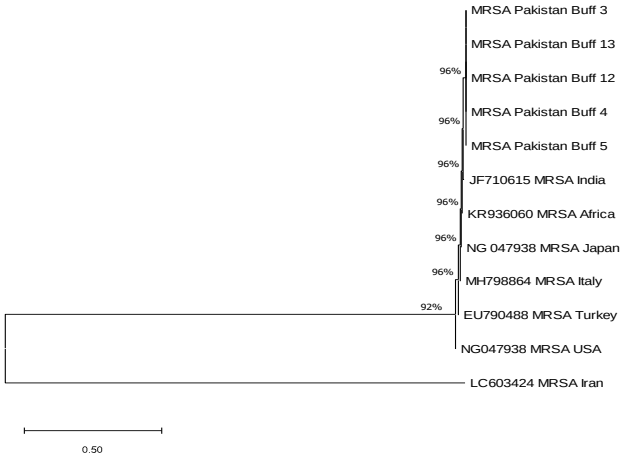

Fig. 4: Phylogenetic tree showing the relationship of local isolates of MRSA with previous reported isolates

present study isolates with reported sequences from Africa, USA, India, Italy, Turkey, and Iran having accession numbers as KR936060, NG047938, JF710615, MH798864, EU790488, and LC603424, respectively (Fig. 3). The study isolates showed no substitution or deletion at any position in comparison with each other (Fig. 4). However, the isolates from other countries showed substitution at three different positions 1, 3, and 4, compared to the study isolates, except that of Iran (LC603424). The one substitution at position 139 was also observed in sequence from India (JF710615). However, the comparison of the local study isolates with sequence from an isolate of Iran (LC603424) showed substitution and deletion at 120 and 11 positions, respectively.

The phylogenetic tree constructed by the neighborhood-joining bootstrapping method showed that all study isolates from buffaloes (MRSA Pakistan 3, 13, 12, 4, and 5) were clustered together and revealed high similarity. While other sequences with accession numbers KR936060, NG047938, JF710615, EU 790488, and MH798864 from Africa, USA, India, Turkey, and Italy showed 96% similarity with present study isolates. However, Iran isolate LC603424 formed a separate cluster from other isolates as well as current study isolates (MRSA Buff 3, 13, 12, 4, 5) which indicates a significant difference in the nucleotide sequence of the *mecA* gene reported from other countries.

Nucleic acid alignment and protein analysis

Alignment of the nucleic acid sequence revealed that Buff-12, Buff-4, Buff-3, and Buff-5 were 100% identical sequences. Polymorphism at position c.1T>C (transition), c.3C>T (transition), and c.4A>C (transversion) were observed in all sequences when compared to the reference sequence. Deletion at position c.23delA in Buff-13 sequence was also observed in Fig. 5. The nucleic acid motif of samples Buff-12, Buff-4, Buff-3, and Buff-5 had the same P-value (2.35e-113), while the reference sequence and Buff-12 had a P-value of 3.10e-112 and 2.30e-113, respectively. Nucleotide sequences of motifs were discriminated by different colours (Fig. 6). The total sequence size involved constructing nucleotide motifs was 1613 bp. Adenine and thymine frequency was 0.335 in the nucleotide sequence, while cytosine and guanine frequency was 0.165 in the nucleotide sequence. The coding region (exonic region) was involved in the nucleotide structure (Fig. 7). In the protein sequence, threonine was replaced by isoleucine (p.T1I). Other amino acids were the same in reference protein, Buff-12, Buff-4, Buff-3, and Buff-5 protein except the Buff-13 sequence (Fig. 8). All sequences have the same protein motifs, except Buff-13 (Fig. 9). The physical properties of all proteins are given in Table 4. A conserved domain of penicillin-binding protein 2a was observed in reference protein, Buff-12, Buff-4, Buff-3, and Buff-5 proteins (Table 5). The protein structure of the reference protein, Buff-12, Buff-4, Buff-3, and Buff-5 proteins resembles the penicillin protein 2a primer structures. Buff-13 protein structure resembles alpha-catenin-like protein hmp-1 (Fig. 10). Protein-protein interaction was found in reference protein, Buff-12, Buff-4, Buff-3, and Buff-5 protein. Buff-3 protein was not similar to any of the proteins in *S. aureus* (Figs. 11 and 12).

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Buff-13       CATCATCGCACATACATTAATAG-AGAAAAGAAAAAAGATGGCAAAGATATTCAACTAAC 59
Reference     TACAATCGCACATACATTAATAGAGAAAAAGAAAAAAGATGGCAAAGATATTCAACTAAC 60
Buff-12       CATCATCGCACATACATTAATAGAGAAAAAGAAAAAAGATGGCAAAGATATTCAACTAAC 60
Buff-4        CATCATCGCACATACATTAATAGAGAAAAAGAAAAAAGATGGCAAAGATATTCAACTAAC 60
Buff-3        CATCATCGCACATACATTAATAGAGAAAAAGAAAAAAGATGGCAAAGATATTCAACTAAC 60
Buff-5        CATCATCGCACATACATTAATAGAGAAAAAGAAAAAAGATGGCAAAGATATTCAACTAAC 60
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Buff-13       TATTGATGCTAAAGTTCAAAAGAGTATTTATAACAACATGAAAAATGATTATGGCTCAGG 119
Reference     TATTGATGCTAAAGTTCAAAAGAGTATTTATAACAACATGAAAAATGATTATGGCTCAGG 120
Buff-12       TATTGATGCTAAAGTTCAAAAGAGTATTTATAACAACATGAAAAATGATTATGGCTCAGG 120
Buff-4        TATTGATGCTAAAGTTCAAAAGAGTATTTATAACAACATGAAAAATGATTATGGCTCAGG 120
Buff-3        TATTGATGCTAAAGTTCAAAAGAGTATTTATAACAACATGAAAAATGATTATGGCTCAGG 120
Buff-5        TATTGATGCTAAAGTTCAAAAGAGTATTTATAACAACATGAAAAATGATTATGGCTCAGG 120
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Buff-13       TACTGCTATCCACCCTCAAACAGGTGAATTATTAGCACTTGTAAGCACACCTTCATATGA 179
Reference     TACTGCTATCCACCCTCAAACAGGTGAATTATTAGCACTTGTAAGCACACCTTCATATGA 180
Buff-12       TACTGCTATCCACCCTCAAACAGGTGAATTATTAGCACTTGTAAGCACACCTTCATATGA 180
Buff-4        TACTGCTATCCACCCTCAAACAGGTGAATTATTAGCACTTGTAAGCACACCTTCATATGA 180
Buff-3        TACTGCTATCCACCCTCAAACAGGTGAATTATTAGCACTTGTAAGCACACCTTCATATGA 180
Buff-5        TACTGCTATCCACCCTCAAACAGGTGAATTATTAGCACTTGTAAGCACACCTTCATATGA 180
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Buff-13       CGTCTATCCATTTATGTATGGCATGAGTAACGAAGAATATAATAAATTAACCGAAGATAA 239
Reference     CGTCTATCCATTTATGTATGGCATGAGTAACGAAGAATATAATAAATTAACCGAAGATAA 240
Buff-12       CGTCTATCCATTTATGTATGGCATGAGTAACGAAGAATATAATAAATTAACCGAAGATAA 240
Buff-4        CGTCTATCCATTTATGTATGGCATGAGTAACGAAGAATATAATAAATTAACCGAAGATAA 240
Buff-3        CGTCTATCCATTTATGTATGGCATGAGTAACGAAGAATATAATAAATTAACCGAAGATAA 240
Buff-5        CGTCTATCCATTTATGTATGGCATGAGTAACGAAGAATATAATAAATTAACCGAAGATAA 240
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Buff-13       AAAAGAACCTCTGCTCAACAAGTTCCAGA       268
Reference     AAAAGAACCTCTGCTCAACAAGTTCCAGA       269
Buff-12       AAAAGAACCTCTGCTCAACAAGTTCCAGA       269
Buff-4        AAAAGAACCTCTGCTCAACAAGTTCCAGA       269
Buff-3        AAAAGAACCTCTGCTCAACAAGTTCCAGA       269
Buff-5        AAAAGAACCTCTGCTCAACAAGTTCCAGA       269
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Fig. 5: Nucleic acid alignment of penicillin-binding protein 2a gene

Fig. 6: Conserved motifs of penicillin-binding protein 2a gene

Fig. 7: Structure of penicillin-binding protein 2a gene

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Buff-13       IIAHTLIEKRKKMAKIFNL-LMLKFKRVFIT-TKMIMAQVLLSTLKQVNYH--LAHLHMT    56
Reference     TIAHTLIEKKKKDGKDIQLTIDAKVQKSIYNNMKNDYGSGTAIHPQTGELLALVSTPSYD    60
Buff-12       IIAHTLIEKKKKDGKDIQLTIDAKVQKSIYNNMKNDYGSGTAIHPQTGELLALVSTPSYD    60
Buff-4        IIAHTLIEKKKKDGKDIQLTIDAKVQKSIYNNMKNDYGSGTAIHPQTGELLALVSTPSYD    60
Buff-3        IIAHTLIEKKKKDGKDIQLTIDAKVQKSIYNNMKNDYGSGTAIHPQTGELLALVSTPSYD    60
Buff-5        IIAHTLIEKKKKDGKDIQLTIDAKVQKSIYNNMKNDYGSGTAIHPQTGELLALVSTPSYD    60
               ********.** .* ::* :  *.:: : .  *   ..      : :    ::

Buff-13       SIHLCMAVTKNII--NPKIKKNLCSTSSR    83
Reference     VYPFMYGMSNEEYNKLTEDKKEPLLNKFQ    89
Buff-12       VYPFMYGMSNEEYNKLTEDKKEPLLNKFQ    89
Buff-4        VYPFMYGMSNEEYNKLTEDKKEPLLNKFQ    89
Buff-3        VYPFMYGMSNEEYNKLTEDKKEPLLNKFQ    89
Buff-5        VYPFMYGMSNEEYNKLTEDKKEPLLNKFQ    89
                 :  .::::     : **:   .. :
```

Fig. 8: Protein sequence alignment of penicillin-binding protein 2a

Fig. 9: Conserved motifs of protein sequence (penicillin-binding protein 2a sequences)

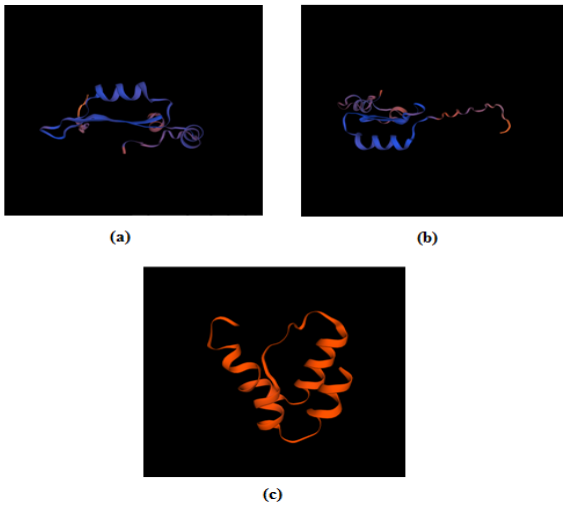


Fig. 10: (**a**) Protein structure of reference protein, (**b**) Protein structure of Buff-12, Buff-4, Buff-3, and Buff-5, and (**c**) Protein structure of Buff-13

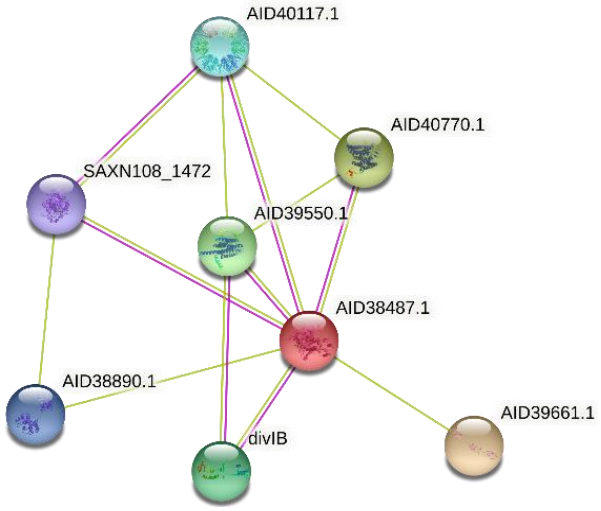


Fig. 11: Protein-protein interaction of the reference protein, Buff-12, Buff-4, Buff-3, and Buff-5 proteins

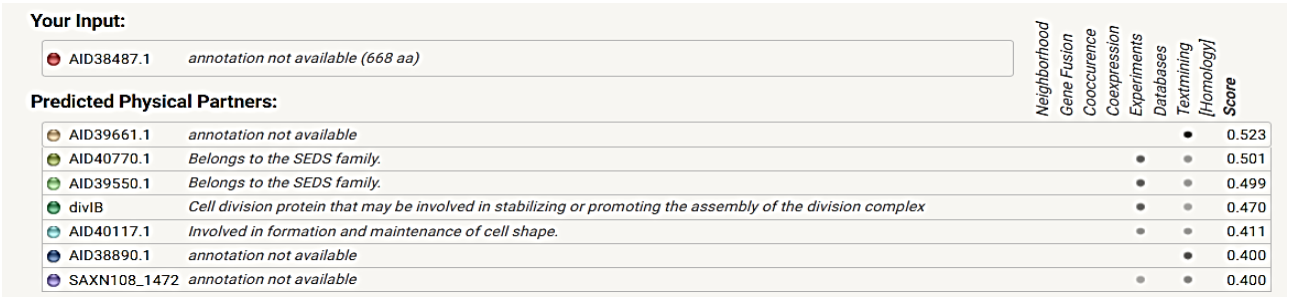
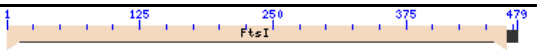
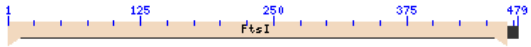
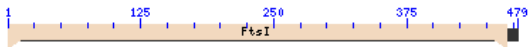
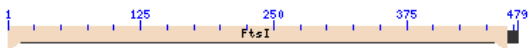
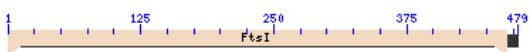


Fig. 12: Other protein interactions with the reference protein, Buff-12, Buff-4, Buff-3, and Buff-5 proteins

Table 4: Physical properties of proteins

Sample ID	Reference protein	Buff-12, Buff-4, Buff-3, and Buff-5	Buff-13
Number of amino acids	89	89	83
MW (molecular weight)	10142.54	10154.60	9632.92
pI	6.65	6.93	10.71
Number of negatively charged residues	12	12	1
Number of positively charged residues	12	12	15
Formula	$C_{453}H_{717}N_{115}O_{142}S_3$	$C_{455}H_{721}N_{115}O_{141}S_3$	$C_{436}H_{743}N_{121}O_{106}S_8$
Total number of atoms	1430	1435	1414
II	38.37	38.37	41.62
Aliphatic index	75.62	80.0	118.67
GRAVY	-0.751	-0.692	0.257

Table 5: Conserved domain structure of penicillin-binding protein 2a

Sample ID	Identified domain	Similarity score	Conserved domain structure
Reference protein	Penicillin-binding protein 2a	1	
Buff-12	Penicillin-binding protein 2a	1	
Buff-4	Penicillin-binding protein 2a	1	
Buff-3	Penicillin-binding protein 2a	1	
Buff-5	Penicillin-binding protein 2a	1	
Buff-13	-	-	This query has no valid domain hit for architecture search

Discussion

Mastitis in dairy buffaloes has evolved an emerging issue despite their lower susceptibility because the teat sphincters in buffaloes has smooth muscular support as compared to cattle which prevents the entry of microorganisms (Fagiolo and Lai, 2007). Moreover, microorganisms can multiply quickly in buffalo milk for its high nutritional content, pendulous udder, and longer teats (Fagiolo and Lai, 2007).

The study results of subclinical mastitis (45.93%) are almost in line with (Abdul *et al*., 2017), who documented a 54% prevalence of mastitis in the bovine of Pakistan. However, low mastitis prevalence has also been reported in Pakistan, including (44%) by (Ali *et al*., 2011), 39.32% by (Tassew, 2017), and 34.4% in Kenya (Gitau *et al*., 2014). The variation in prevalence might be due to different management types, environmental conditions, sampling strategies, and breeds. Risk factors like parity, milk yield, hygiene during milking, milker care during milking, milk yield of animals, teat dipping, floor type, and housing system were associated with subclinical mastitis in buffaloes. The study findings are supported by Abdul *et al*. (2017), Altaf *et al*. (2020), and Muzammil *et al*. (2021). Improper milking hygiene is a significant risk factor for mastitis due to lack of pre- and post-milking teat dipping, using the same udder cloth for more than one animal, and inappropriate use of gloves during milking (Guimarães *et al*., 2017). The current study reported parity and high milk yield as significant risk factors for mastitis. These results are supported by the findings of Oltenacu and Broom (2010), Nyman *et al*. (2014), and Taponen *et al*. (2017). Reasons for increased risk of mastitis with parity may include impaired leukocyte function due to aged animals, and changes in the teat conformation with increasing age (Rainard and Riollet, 2006). The current study reported that teat dipping was significantly associated with mastitis prevention; these findings are supported by Nururrozi *et al*. (2020) but contrary to Gleeson *et al*. (2018) who reported that teat dipping does not have a significant effect on mastitis. However, teat dipping are not only helpful to control mastitis but are also effective in reducing the transmission of bacteria through milk.

S. aureus is a major cause of mastitis in bovine (Abdul *et al*., 2017). The current study has documented a 56.12% prevalence of *S. aureus* from buffalo mastitis samples, which is comparable with the studies reported by Shah *et al*. (2019) who reported a 53.33% prevalence of *S. aureus* in India, and Awad *et al*. (2017), who described 42% prevalence of *S. aureus* in bovine mastitis. The occurrence of bovine mastitis caused by *S. aureus* in the current study is much higher compared to the 8.32% prevalence reported by Ali *et al*. (2011), and 22.14% found by Tassew (2017). The discrepancies in the *S. aureus* mastitis prevalence might be due to variations in the pathogen survival in the teat canal (Ji *et al*., 2020), biofilm formation, different bovine breeds and species, geographic locations, and management practices. Manure and bedding are sources of various contagious pathogens such as *S. aureus*. These microorganisms may also be present in soil or air as environmental microorganisms. Milker's hands, towels, tissues, and flies can spread the pathogens to healthy and clean udders during milking, hence responsible for mastitis (Aqib *et al*., 2017; Abdeen *et al*., 2021).

The current study findings for phenotypic MRSA comply with the prevalence of 47% in China (Pu *et al*., 2014) and 34% in Pakistan (Aqib *et al*., 2017). A few studies have reported low prevalence in Korea (6.3%) (Lim *et al*., 2013), Germany (16.7%) (Spohr *et al*., 2011), and the USA (4%, 1.8%, and 0.6%) (Haran *et al*., 2012). The current study has reported 18.79% MRSA by targeting the *mec*A gene. These results are coherent with the findings of Aklilu and Ying (2020) who reported 17.89% genotypic MRSA prevalence, and somewhat high genotypic prevalence of 25%, and 23.3% reported by Shah *et al*. (2019), and Guimarães *et al*. (2017), respectively. The variation might be due to overproduction of beta-lactamase or poor expression of genes, particularly *mecA* (Turutoglu *et al*., 2009). The current study has documented a much higher phenotypic prevalence of MRSA. Variations in phenotypic and genotypic prevalence might be due to other genes besides *mecA* responsible for methicillin resistance (Haran *et al*., 2012). The phenotypic detection of MRSA less reliable as compared to genotypic confirmation due to probable false-negative result following the appearance of novel resistant genes (Aqib *et al*., 2018). Resistance can occur due to genes like *mecC*, reported as an oxacillin-induced gene found in the regulatory system of *S. aureus* (Ballhausen *et al*., 2014).

The resistance development in animals due to the higher use of antibiotics has been strongly evidenced. The zoonotic transfer of resistant bacteria to people in direct contact with animals like milking, grooming, feeding, and treatment purposes is highly suspected (Cuny *et al*., 2015). The transfer of livestock-associated MRSA (LA-MRSA) is of serious concern for public health (Köck *et al*., 2012). Direct contact strongly relates to LA-MRSA colonization in patients. Biosafety measures, proper disinfection of contaminated environments, and isolation of infected animals should be applied for zoonosis prevention (Catry *et al*., 2010).

The study concludes that MRSA is a highly prevalent pathogen associated with subclinical mastitis in buffaloes. Identifying associated risk factors can help reduce the infection in buffalo herds. The confirmation of MRSA through phenotypic, genotypic, and protein analysis help in diagnosis and devise the control strategies for MRSA-associated subclinical mastitis. There are chances of misdiagnosis of MRSA by phenotypic identification, which can lead to developing novel resistant genes and serious zoonotic concerns *via* the food chain to consumers. Therefore, genotypic analysis of MRSA can confirm resistant genes, which can be helpful for the identification of protein responsible for developing resistance in *S. aureus* against commonly used antibiotics. In addition, the data from this research can also be used to inform the public about

the potential threat of MRSA from buffaloes and their dairy products.

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Conflict of interest

The authors declare no conflict of interest in the submission/publication of this data.

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