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SHORT COMMUNICATION

Phenotypic and Genotypic Characterization of Beta-lactams Resistant *Staphylococcus aureus* Isolates from Bovine Mastitis and its Zoonotic Implications

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ABSTRACT

Rise of antibiotic resistant *Staphylococcus aureus* is a higher risk and great concern to global health. Zoonotic transfer of such strains is well documented. Present study evaluated the presence of resistant *S. aureus* from mastitic Nilli Ravi buffaloes and nasal carriage of milkers. Phenotypic profile of *S. aureus* isolates was conducted against penicillin, ampicillin, and cefoxitin. PCR analysis revealed presence of *bla*Z gene and *mec*A gene from *S. aureus* isolated from milk and milkers samples. Sequence and phylogenetic analysis depicts the divergence of *mec*A gene originated from bovine and human but for the *bla*Z gene, no divergence was detected. The high degree of genetic relatedness among *bla*Z and *mec*A genes in bovine and human *S. aureus* isolates from same farm suggests the potential transfer of antimicrobial resistance genes between buffaloes and milkers, highlighting the importance of one-health approach to promote global health.

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is highly pathogenic gram-positive bacteria causing diseases in both humans and animals. It is the most common cause of bacteremia, skin and soft tissue infections in humans. In dairy animals *S. aureus* is a predominant causative agent of mastitis, resulting in tremendous economic losses. Methicillin-resistant *S.aureus* (MRSA) has been reported in different localities of Pakistan (Ullah *et al*., 2016). In dairy buffaloes, β-lactam antibiotics (penicillin, methicillin, cephalosporin, and ampicillin) are used frequently for the treatment of mastitis. Genetic determinants such as *mec*A and *bla*Z are responsible for resistance to β-lactam antibiotic in *S. aureus*. β-lactamase are encoded by *bla*Z gene *mec*A gene encodes a penicillin-binding protein, called PBP2A. PB2A shows resistance to beta-lactam antibiotics by not binding with beta lactam (Zapun *et al*., 2008).

Considering the wide spread occurrence of antibiotic resistance strains, this study was designed to study the potential zoonotic transfer of *mec*A and *bla*Z gene harboring *S.aureus* isolates from Nilli Ravi buffaloes and humans in dairy farms in Lahore, Pakistan. Furthermore, association between phenotypic and genotypic resistance was also analyzed.

MATERIALS AND METHODS

Sampling was carried out from different household farms of Lahore. A total of 22 fresh milk samples from Nili-Ravi buffaloes with clinical and sub-clinical mastitis and 23 nasal swabs were collected from milkers in direct contact with the mastitic buffaloes. Clinical mastitis was screened by visible signs and sub-clinical mastitis was diagnosed by Surf Field Mastitis Test (SFMT). Milk and nasal samples were transported to the immediately to the laboratory, and stored at 4°C. Then for isolation and identification all samples were cultured on selective Staph 110 agar medium (HIMEDIA, India) and then appeared colonies were streaked on mannitol salt agar (Oxoid, UK). Gram staining and catalase test were also performed. Thermonuclease (*nuc*) gene which is specific for *S.aureus* was used to identify *S.aureus*.

S. aureus specific *nuc* gene PCR was also performed. Genomic DNA was extracted and already published sequence of primers were used (Table 1) (Gao *et al.*, 2011). PCR was performed in a final volume of 15 µl consisting of 7.5 µl Master Mix, 2 µl of template DNA sample and 10 pmol of each primer. The cycling conditions were initial denaturation of 95°C for 5 min; followed by 35 cycles of denaturation 94°C for 30 sec, annealing 55°C for 45 sec and extension 72°C for 60 sec followed by a final extension step at 72°C for 10 min. PCR product was observed on 2% agarose gel. The bands observed at 395 bp level were considered positive.

Confirmed milk and nasal carriage *S. aureus* isolates were subjected to antibiotic susceptibility test. The strains were tested against three different β-lactam antibiotics (penicillin, cefoxitin, and ampicillin) by disc diffusion method on Mueller-Hinton medium (Oxoid, UK) and 0.5 McFarland turbidity standards (1.5 X 108 CFU/ml). After following standard procedure of susceptibility testing, zones of inhibition were measured in millimeters.

For the molecular identification of *bla*Z and *mec*A resistant genes, plasmid extracted by alkaline lysis was used for *bla*Z gene amplification and genomic DNA was used for *mec*A gene amplification. Two sets of overlapping, flanking and gene-specific primers were designed, based on the *bla*Z gene sequence with accession number KJ756353. While four primer pairs were designed to amplify overlapping segments of the *mec*A gene for sequencing purpose and primers were based on the nucleotide sequence of *Staphylococcus aureus* (AB236888). For *mec*A gene identification, one pair of *mec*A2 primer was used. The PCR products observed for positive bands on 2% Agarose gel. All the primers used in this study along with their amplification conditions are available upon request.

Two representative isolates from buffaloes and two from milkers were selected and partial sequencing was performed for *mec*A gene, whereas whole gene sequencing was performed for *bla*Z gene.

The phylogenetic tree was constructed by neighbor-joining method using p-distance model with 1000 bootstrap values in MEGA6. The bootstrap test inferred from 1000 replicates was used. Association between phenotypic and genotypic resistance was determined by chi-square test using SPSS 16 program and p value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

β-lactam antibiotic resistant *S. aureus* is a contagious pathogen causing bovine mastitis. Keeping this fact in view many studies have been conducting to report the prevalence and antibiotic susceptibility pattern of *S. aureus* in cattle from Pakistan. Out of 22 bacterial isolates recovered from buffalo's milk and 23 isolates from the nasal carriages, 21 (95.4%) and 22 (95.6%) showed growth on staph 110 agar, respectively. Presence of mixed colonies was observed. Further microbiological identification tests confirmed 17/21 (81%) milk isolates and 18/22 (82%) human isolates as *S. aureus*. Furthermore, by *nuc* gene PCR 15/17 (88.2%) isolates from milk and 17/18 (94%) isolates from milker's were molecular confirmed as *S. aureus* positive. *S. aureus* prevalence in dairy settings of Lahore was evaluated as 68.18% and 73.91% in milk and nasal carriage, respectively. These findings are in accordance with our finding which shows 68.18% bovine mastitis caused by *S. aureus* (Altaf *et al*., 2019). But it is higher than reported *S. aureus* in buffaloes of Kasur district (Maalik *et al*., 2019). This may be due to management differences in urban and rural areas. Higher prevalence of *S. aureus* in human nasal carriages was observed than previously reported from Algerian farmers (Akkou *et al*., 2016).

15 Isolates recovered from milk manifested phenotypic resistance against penicillin, ampicillin and cefoxitin as 6/15 (40.0%), 8/15(53.3%) and 2/15 (13.3%), respectively. While Genotypic characterization of these isolates resulted as 6/15 (40.0%) and 3/15 (20%) isolates positive for *bla*Z and *mec*A gene, respectively (Table 2). In Milkers nasal *S. aureus* isolates, Penicillin, ampicillin and cefoxitin resistance was observed in 11 (64.7%), 12 (70.5%) and 7 (41.2%) isolates, respectively. While *bla*Z and *mec*A genes were observed in 11 (64.7%) and 8 (47.0%) nasal carriage isolates, respectively (Table 2). Significant association was observed between genotypic and phenotypic penicillin resistance (Table 3).

Table 1: Primer sequences used to amplify *nuc* gene, blaZ gene and mecA gene of *Staphylococcus aureus.*

Sr. No	Primer name	5'-3' Sequence	Tm	Product size (bp)	Reference
1	mecA1-F	AGTTGTAGTTGTCGGGTTTGG	59.02	497	
	mecA1-R	CATTCTTTGGAACGATGCCTA	60.08		
2	mecA2-F	TCCAGGAATGCAGAAAGACC	60.20	669	
	mecA2-R	TCACCTGTTTGAGGGTGGAT	60.36		
3	mecA3-F	GGCTATCGTGTCACAATCGTT	60.01	548	
	mecA3-R	TTCTTACTGCCTAATTCGAGTGC	59.93		
4	mecA4-F	ATCTTGGGGTGGTTACAACG	59.71	725	
	mecA4-R	CGTTACGGATTGCTTCACTG	59.34		
5	blaZ1-F	GCCATTTCAACACCTTCTTTCA	61.36	653	
	blaZ1-R	AAAGTCTTACCGAAAGCAGCA	59.17		
6	blaZ2-F	GAAATCGGTGGAATCAAAAA	57.52	668	
	blaZ2-R	CGTTGCTTTTTCGATTGATG	59.30		
7	nuc1-F	ATATGTATGGCAATCGTTTCAAT		395	(Gao *et al*., 2011)

Table 2: Frequencies and averages of phenotypic and genotypic beta-lactam antibiotics resistance in *S. aureus* isolated in this study

	Phenotyping results				Genotyping results							
	Strains from milk (n=15)		Strains from nostrils (n=17)		Strains from milk (n=15)				Strains from nostrils (n=17)			
	Resistant	Sensitive	Resistant	Sensitive	Positive No. (%)		Negative No. (%)		Positive No. (%)		Negative No. (%)	
	No. (%)	No. (%)	No. (%)	No. (%)	*bla*Z	*mec*A	*bla*Z	*mec*A	*bla*Z	*mec*A	*bla*Z	*mec*A
Penicillin	6 (40%)	9 (60%)	11 (64.7%)	6 (35.3%)	6 (40%)		9 (60%)		11 (64.7%)		6 (35.3%)	
Cefoxitin	2 (13.3%)	13 (86.6%)	7 (41.2%)	10 (58.8%)		3 (20%)		12 (80%)		8 (47%)		8 (53%)
Ampicillin	8 (53.3%)	7 (46.6%)	12 (70.5%)	5 (29.4%)								

a

Genetic distance of blaZ gene	1	2	3	4	5	6	7	8	9
1. S.aureus (F4)human									
2. S.aureus (F4) bovine	0.00								
3. S.aureus (F11) bovine	0.02	0.02							
4. S.aureus (F11) human	0.02	0.02	0.00						
5. S.aureus strain SA101 (bovine)	0.04	0.04	0.02	0.02					
6. S.haemolyticus (bovine)	0.56	0.56	0.56	0.56	0.57				
7. S.aureus strain JKD6008 (human)	0.00	0.00	0.02	0.02	0.04	0.56			
8. S.aureus strain SM39 (human)	0.00	0.00	0.02	0.02	0.04	0.56	0.00		
9. S.aureus isolate SA304 (bovine)	0.02	0.02	0.03	0.03	0.04	0.55	0.02	0.02	
10. S.epidermidis strain SE90 (human)	0.00	0.00	0.02	0.02	0.04	0.56	0.00	0.00	0.02

b

Genetic distance of mecA gene	1	2	3	4	5	6	7	8	9
1. S.aureus (F4)human									
2. S.aureus (F4) bovine	0.00								
3. S.aureus (F11) bovine	0.02	0.02							
4. S.aureus (F11) human	0.02	0.02	0.00						
5. S.aureus strain SA101 (bovine)	0.04	0.04	0.02	0.02					
6. S.haemolyticus (bovine)	0.56	0.56	0.56	0.56	0.57				
7. S.aureus strain JKD6008 (human)	0.00	0.00	0.02	0.02	0.04	0.56			
8. S.aureus strain SM39 (human)	0.00	0.00	0.02	0.02	0.04	0.56	0.00		
9. S.aureus isolate SA304 (bovine)	0.02	0.02	0.03	0.03	0.04	0.55	0.02	0.02	
10. S.epidermidis strain SE90 (human)	0.00	0.00	0.02	0.02	0.04	0.56	0.00	0.00	0.02

Fig. 1: Genetic distance matrix depicting *bla*Z (a) and *mec*A (b) genes from this study and already submitted sequence of human and bovine origin.

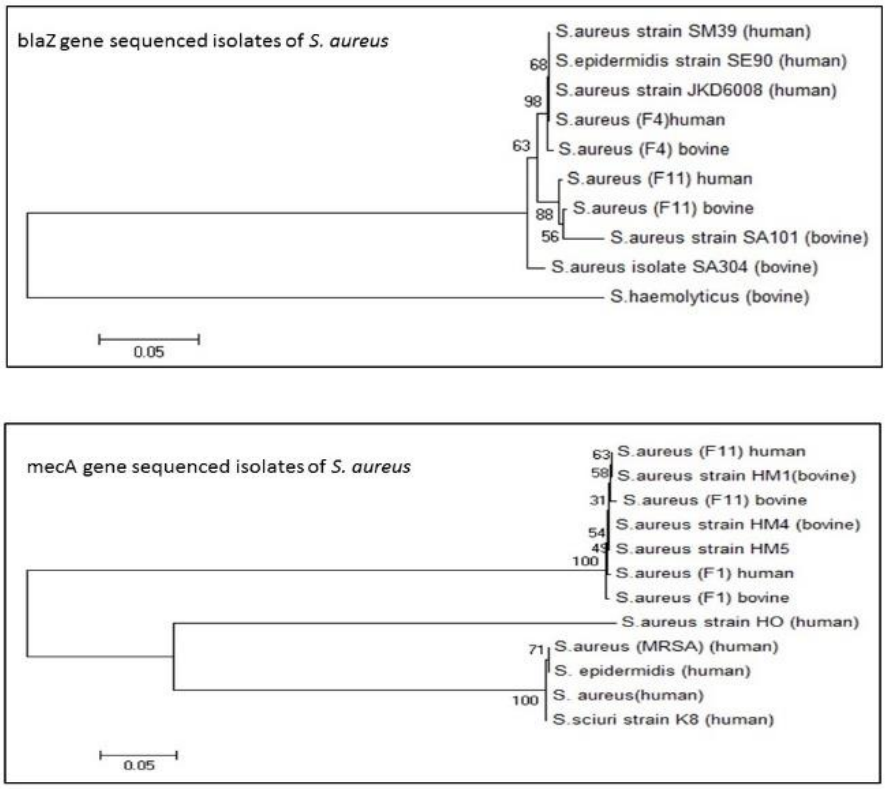

Fig. 2: Phylogeny of *bla*Z (a) gene detected in this study (F4 bovine, F4 human, F11 bovine, F11 human) and *mec*A (b) gene detected in this study (F1 bovine, F1 human, F11 bovine, F11 human).

Table 3: Chi square test results for phenotypic and genotypic penicillin resistance

	Variables	Value	df	p-value
Pearson Chi square	Penicillin resistance	35.000	1	0.000
Pearson Chi square	methicillin resistance	12.778	1	0.000

P≤0.05 was considered as significant;

Analysis with the NCBI BLAST program resulted in 99% homology of sequenced *bla*Z gene to the reference sequence KJ756353 while sequenced *mec*A gene showed 98% homology to the reference sequence AB236888.

Using the pairwise method 0.2-0.4% nucleotide sequence distance were found between the *bla*Z gene sequence of *S. aureus*, isolates of buffaloes and milkers origin of this study and the already studied sequences of human and bovine originated isolates. 56% nucleotide differences were observed between *S. aureus* and *S. hemolyticus* isolates (Fig. 1). Phylogenetic analysis revealed clustering of *S. aureus bla*Z gene from bovine and human origin. The sequences of buffalo isolate and its milkers from same farm (i.e., human and bovine (F4)) were more closely related to each other than isolates from other (F11) farm (Fig 1). 0-0.1% nucleotide sequence distance was found between the *mec*A gene sequence of *S. aureus* isolates of bovine (buffaloes) and human (milkers) origin of this study and already reported bovine isolates. 72% nucleotide differences were found between *mec*A gene of *Staphylococcal* species of human origin and the sequences of this study. This difference (72%) was also observed between already studied bovine and human originated isolates (Fig. 1). Phylogenetic tree for *mec*A gene identified two main clusters in which human origin *Staphylococal* species were separated from bovine origin *Staphylococal* species (Fig. 2).

All isolates which were penicillin resistant were also carrying *bla*Z gene. This strong association has been elucidated by the fact that acquisition of phenotypic

resistance is due to changes on genetic level i-e mutation, horizontal gene transmission of resistance genes and induction mechanisms (Corona and Martinez, 2013). Absence of *mec*A gene in methicillin resistance *S. aureus* (MRSA) has previously been reported. Our findings also support this phenomenon to some extent as one milk and one nasal isolate were mecA negative but phenotypically positive. Beta lactamase overproduction and different amino acids alteration in penicillin binding protein 1, 2 and 3 elaborate the absence of *mec*A gene in MRSA (Aqib *et al.,* 2018).

Sequencing and phylogenetic analysis revealed that sequences of *bla*Z gene of *S. aureus* from bovine and human origin were indistinguishable. This raise the likelihood of conserved nature of *bla*Z gene. But further studies are required in this context. However, cladal separation of *mec*A gene sequences of human and bovine origin is in agreement with the study of Melo and colleagues (Melo *et al*., 2014).

With the increasing population intensity and misuse of antibiotics in animals, potential zoonotic transfer of resistant *S. aureus* is a major concern in today's world. MRSA transfer to humans from dogs, equine and cows have been reported (Melo *et al*., 2014). The possible transmission of *S. aureus* isolates from buffaloes to human is the key finding of our study. Both *bla*Z and *mec*A gene sequence relatedness and phylogenetic analysis depict the transmission of *S. aureus* between buffaloe and its respective milkers. Juhász-Kaszanyitzky and coworkers has also reported MRSA transmission between cows and human by using spa typing (Juhász-Kaszanyitzky *et al*., 2007). These observations indicate the possibility of buffaloes as a reservoir for the spread of pathogenic strains. However, to well establish this phenomenon, adaptation of more genetic methods for further investigations are required.

In conclusion, the genetic relatedness between *mec*A and *bla*Z genes in *S. aureus* originating from buffalo milk and human in the same farm highlight the urgent need for improvement of hygiene and proper animal care as well as the consumer awareness.

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Authors contribution: AK, SF, IK and ARA design and executed the study. SF, MT and AZ conceived, helped in molecular assays and interpreted the data. MT provided microbiology lab facilities. MZS helped in microbiological tests. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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