Phenotypic and Genotypic Characterization of Beta-lactams Resistant \textit{Staphylococcus aureus} Isolates from Bovine Mastitis and its Zoonotic Implications

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\textbf{A B S T R A C T}

Rise of antibiotic resistant \textit{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 \textit{S. aureus} from mastitic Nilli Ravi buffaloes and nasal carriage of milkers. Phenotypic profile of \textit{S. aureus} isolates was conducted against penicillin, ampicillin, and cefoxitin. PCR analysis revealed presence of \textit{bla\textsuperscript{Z}} gene and \textit{mec\textsuperscript{A}} gene from \textit{S. aureus} isolated from milk and milkers samples. Sequence and phylogenetic analysis depicts the divergence of \textit{mec\textsuperscript{A}} gene originated from bovine and human but for the \textit{bla\textsuperscript{Z}} gene, no divergence was detected. The high degree of genetic relatedness among \textit{bla\textsuperscript{Z}} and \textit{mec\textsuperscript{A}} genes in bovine and human \textit{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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\textbf{INTRODUCTION}

\textit{Staphylococcus aureus} (\textit{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 \textit{S. aureus} is a predominant causative agent of mastitis, resulting in tremendous economic losses. Methicillin-resistant \textit{S. aureus} (MRSA) has been reported in different localities of Pakistan (Ullah et al., 2016). In dairy buffaloes, \textit{\beta}-lactam antibiotics (penicillin, methicillin, cephalosporin, and ampicillin) are used frequently for the treatment of mastitis. Genetic determinants such as \textit{mec\textsuperscript{A}} and \textit{bla\textsuperscript{Z}} are responsible for resistance to \textit{\beta}-lactam antibiotic in \textit{S. aureus}. \textit{\beta}-lactamase are encoded by \textit{bla\textsuperscript{Z}} gene \textit{mec\textsuperscript{A}} gene encodes a penicillin-binding protein, called PBP2A. PBP2A shows resistance to \textit{\beta}-lactam antibiotics by not binding with \textit{\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 \textit{mec\textsuperscript{A}} and \textit{bla\textsuperscript{Z}} gene harboring \textit{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.

\textbf{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 (\textit{nuc}) gene which is specific for \textit{S. aureus} was used to identify \textit{S. aureus}. 

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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 blaZ and mecA resistant genes, plasmid extracted by alkaline lysis was used for blaZ gene amplification and genomic DNA was used for mecA gene amplification. Two sets of overlapping, flanking and gene-specific primers were designed, based on the blaZ gene sequence with accession number KJ756353. While four primer pairs were designed to amplify overlapping segments of the mecA gene for sequencing purpose and primers were based on the nucleotide sequence of S. aureus (AB236888). For mecA gene identification, one pair of mecA2 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 mecA gene, whereas whole gene sequencing was performed for blaZ 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 (Altuf 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 blaZ and mecA 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 blaZ and mecA 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     | AGTTGATGTTGTCCGCGTTTGG | 59.02 | 497 |           |
|       | mecA1-R     | CATTCCTTTAGGAGATGCCCA | 60.08 |             |           |
| 2     | mecA2-F     | TCCAGGAATGCGAAAGACCC | 60.20 | 669 |           |
|       | mecA2-R     | TCACCTGTGGAGGTTGATGAT | 60.36 |             |           |
| 3     | mecA3-F     | GGTATCGTGGTCAATCTGGTT | 60.01 | 548 |           |
|       | mecA3-R     | TCTTACTGCTGCTAATTCGAGTCG | 59.93 |             |           |
| 4     | mecA4-F     | ATCTTGGGGGTGTTACACACG | 59.71 | 725 |           |
|       | mecA4-R     | GTTACCGATTGCTTCACTG | 59.34 |             |           |
| 5     | blaZ1-F     | GCCATTCACACCATCCTCTTCA | 61.36 | 668 |           |
|       | blaZ1-R     | AAAGTCTTACCGGAAAGCAGCA | 59.17 |             |           |
| 6     | blaZ2-F     | GAAATCGTGGTAAATCTAAA | 57.52 |             |           |
|       | blaZ2-R     | CTGTGCTTTTATGCTTATG | 59.30 |             |           |
| 7     | nuc1-F      | ATATGATGCGAATCTGGTTTCAAT | 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) |
| No. (%) | Sensitive | Resistant | No. (%) | Sensitive | Positive No. (%) | Negative No. (%) | Positive No. (%) | Negative No. (%) |
| 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%) | 8 (53%) | 8 (47%) | 11 (64.7%) | 6 (35.3%) |
Analysis with the NCBI BLAST program resulted in 99% homology of sequenced \textit{blaZ} gene to the reference sequence KJ756353 while sequenced \textit{mecA} gene showed 98% homology to the reference sequence AB236888.

Using the pairwise method 0.2-0.4% nucleotide sequence distance were found between the \textit{blaZ} gene sequence of \textit{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 \textit{S. aureus} and \textit{S. hemolyticus} isolates (Fig. 1). Phylogenetic analysis revealed clustering of \textit{S. aureus} \textit{blaZ} 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 \textit{mecA} gene sequence of \textit{S. aureus} isolates of bovine (buffaloes) and human (milkers) origin of this study and already reported bovine isolates. 72% nucleotide differences were found between \textit{mecA} gene of \textit{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 \textit{mecA} gene identified two main clusters in which human origin \textit{Staphylococcal} species were separated from bovine origin \textit{Staphylococcal} species (Fig. 2).

All isolates which were penicillin resistant were also carrying \textit{blaZ} 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 mecA 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 mecA gene in MRSA (Aqib et al., 2018).

Sequencing and phylogenetic analysis revealed that sequences of blaZ gene of S. aureus from bovine and human origin were indistinguishable. This raise the likelihood of conserved nature of blaZ gene. But further studies are required in this context. However, cladal separation of mecA 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 blaZ and mecA 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.

Conclusions: the genetic relatedness between mecA and blaZ 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 designed 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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