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**Original Article**

# Prevalence and antibiogram profiling of enterotoxigenic methicillin-resistant *Staphylococcus aureus* at bovine-human interface

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## Abstract

**Background:** *Staphylococcus aureus*, a ubiquitous pathogen due to its key involvement in dairy animal mastitis, is the leading cause of food-borne diseases in humans by producing various enterotoxins. **Aims:** The present study reported the prevalence of significantly increased enterotoxigenic MRSA pathogens among bovines and dairy occupational workers, along with antibiotic-resistant patterns, using the *in-vitro* technique. **Methods:** A total of 384 bovine (n=192 cattle, n=192 buffalo) milk samples and 100 human nasal or skin swab samples were collected to find out the prevalence of *S. aureus*, MRSA, *spa*, and enterotoxin genes (*seb*, *sec*) by PCR. Also, a phylogenetic analysis was conducted to compare and analyze the prevalent enterotoxin *seb* gene sequences from bovines, workers, and other sources. **Results:** The present study revealed that out of 484 total samples, 49.79% of isolates were positive for *S. aureus* while 29.46% and 66.80% of *S. aureus* isolates were positive for MRSA, and *spa* genes among bovine and human samples collectively. The prevalence of enterotoxigenic *S. aureus* was found to be 16.18% in bovine and human staphylococcal isolates. Additionally, the enterotoxigenic strains exhibited resistance to commonly used antibiotics. **Conclusion:** The present study shows that enterotoxigenic MRSA is prevalent in bovines and dairy occupational workers of study districts, Pakistan, and study isolates revealed a varying level of resistance to different antibiotics. The various virulence factors along with the antibiotic resistance makes MRSA a potential threat at animal-human interface, highlighting the need for further research.

**Key words:** Antibiotic resistance, Bovine mastitis, Dairy workers, Enterotoxigenic MRSA, Phylogenetic analysis

## Introduction

*Staphylococcus aureus*, a predominant cause of mastitis, confers food-borne diseases in humans around the globe (Silva *et al*., 2000; Aragon-Alegro *et al*., 2007). *S. aureus* is a contagious pathogen that can be transferred from animal to animal as well as dairy occupational workers, posing a serious public health threat (Javed *et al*., 2023, 2024). The bacterial properties of *S. aureus* lower cure rate, its transmission between animal-human interface, its ability to reside in the mammary gland, and the emergence of antibiotic-resistant strains (Almuzaini, 2024; Hannan *et al*., 2024) are major deteriorating factors in disease causation ability of pathogen (Monistero *et al*., 2018; El-Ashker *et al*., 2020). The genetic variability and undue antibiotic usage have led to the emergence of methicillin-resistant *S. aureus* (MRSA) which proved a pathogen of utmost importance regarding veterinary and public health concerns (Durrani et al., 2024; Algammal *et al*., 2020; Abdeen *et al*., 2021; Li *et al*., 2023; Qureshi *et al*., 2024). MRSA has been reported in different livestock species like caprines (Javed *et al*., 2023; Javed *et al*., 2024), ovines (Sabir *et al*., 2024), equines (Rasheed *et al*., 2023), bovines (Muzammil *et al*., 2022) in Pakistan as well as bovine population in Iran 14.7% by (Dallal *et al*., 2024), Turkey (16.6%) by Turutoglu *et al*. (2009), and China (5.56%) by (Zhu *et al*., 2024). Moreover, the phagocytic avoidance of bacteria by the occurrence of cell surface-associated proteins like staphylococcal protein A (*spa*) enhances the potential virulence ability of *S. aureus* (Dunman *et al*., 2001). *Spa*, similar to many of the surface proteins of *S. aureus* is produced during the exponential phase of development and

transcriptionally down-regulated during the post-exponential phase of growth (Gao and Stewart, 2004). Moreover, this protein helps the bacteria to capture IgG molecules in an inverted position, thereby preventing the bacterial cell from phagocytosis of the host immune system (Votintseva *et al*., 2014).

The probability of infection caused by bacteria and its toxins being shed into milk creates the zoonotic potential of clinical and sub-clinical mastitis (Guimarães *et al*., 2017). However, *S. aureus*-associated food poisoning is governed by the toxic effect of Staphylococcal enterotoxins (SEs) which act on gastrointestinal emetic receptors in humans (Dinges et al., 2000; Hui *et al*., 2022). The clinical acute onset of nausea, vomiting, abdominal cramps, and diarrhea are characterized by an occurrence of Staphylococcal-induced food poisoning (Loncarevic *et al*., 2005). There are large variety of enterotoxins produced by *S. aureus*, and based on their antigenicities, it is divided into five serological types (SEA to SEE) (Benkerroum, 2018; Javed *et al*., 2023). To elude the gastrointestinal proteases and the property of thermo-resistance makes SEs more pathogenic (Nazari *et al*., 2014). However, the milk from infected animals is considered a main source of enterotoxigenic *S. aureus* of animal origin (Bryan, 1983; Gilmour and Harvey, 1990).

Several molecular and epidemiological studies have been conducted on *S. aureus*, associated antimicrobial-resistant strains, and a list of virulence factors including enterotoxins from bovines, food, and humans (Boerema *et al*., 2006). However, the studies on increasing the incidence of staphylococcal infections including food poisoning at the animal-human interface, associated spillover of MRSA to dairy workers, and any possible effect of enterotoxins on antibiogram profiling of *S. aureus* are scarce. Therefore, this study was conducted to find out the prevalence of *S. aureus* and MRSA in bovines, and associated dairy workers in Pakistan. Moreover, the prevalence of two major staphylococcal enterotoxins (SEB and SEC), molecular characterization of relevant genes, and the influence of any of these enterotoxins on antibiogram profiling of staphylococcal isolates was also investigated. The present study proved to be the first molecular report on enterotoxigenic MRSA at the animal-human interface in Pakistan.

## Materials and Methods

### Study design

This study was conducted in Muzaffargarh and Faisalabad districts, Pakistan (Fig. 1). An overall 384 bovine milk samples (n=192 cattle, n=192 buffalo) were collected using the convenient sampling technique. The collection of milk samples was done aseptically as per the National Mastitis Council guidelines. However, the teat ends of the bovine teats were cleaned with alcohol swabs and allowed to dry before sampling. After discarding the first few streams, 5 ml of milk sample was collected in sterile tubes. The collected milk samples were screened for subclinical mastitis by surf field mastitis test (SFMT) according to the guidelines of (Muhammad *et al*., 2010). Furthermore, skin and nasal swabs of occupational workers (n=100) just after milking were also collected due to the public health significance of enterotoxigenic *S. aureus* among humans. The consent was taken from dairy workers before taking the nasal, throat, and hand swabs. Then, the samples positive for subclinical mastitis along with human swab samples were promptly chilled and delivered to the Medicine Research Laboratory, Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore maintaining a cold chain at 4°C.

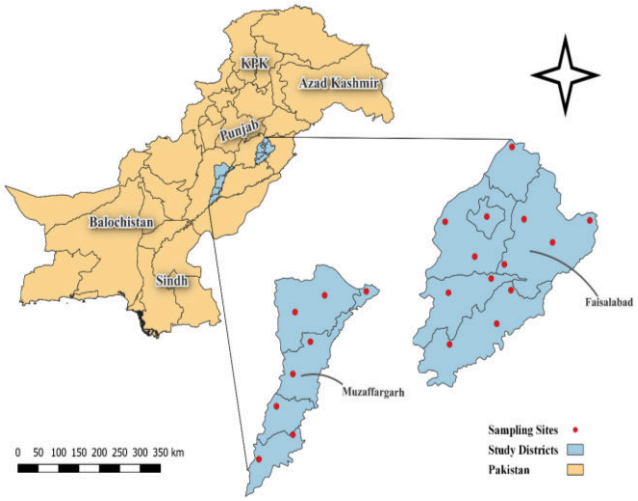


**Fig. 1:** QGIS map of the study area and sample sites

### Bacterial isolation and identification

The positive milk samples for subclinical mastitis and human samples were cultured on 5% blood agar by giving incubation of 24 h for 37°C. The culture media showing the beta-hemolytic pattern growth was assumed as *S. aureus* growth, and further streaked on Mannitol salt agar which is differential media for the selective growth of *S. aureus* for the confirmation of *S. aureus* (Ahmed *et al*., 2022; Liu *et al*., 2023; Javed *et al*., 2024). The colonies of *S. aureus* were phenotypically confirmed by mannitol fermentation, gram staining, and biochemical tests like catalase and coagulase. The genotypic confirmation of *S. aureus* was done by the amplification of a gene necessary for thermo nuclease production e.g. *nuc* gene. DNA extraction of overnight growth of *S. aureus* in BHI broth was done using a DNA extraction kit (WizPrep™ gDNA Mini kit Cell/Tissue) according to the guidelines of the manufacturer. The extracted DNA after purity concentration measurements by NanoDrop technology was further processed for molecular identification of *S. aureus* by PCR targeting the *nuc* gene using primers in (Table 1) as reported by (Louie *et al*., 2000). The conditions for the thermocycler amplification include initial denaturation at 94°C for 5 min followed by 25 cycles of final denaturation at 94°C for 30 s, annealing at 55°C for 55 s, and extension for 5 min at 72°C. The confirmed *S. aureus* isolates were further confirmed for virulence genes by PCR and sequencing.

**Table 1:** List of main primers used in the study

| Targeted gene | Primers | Base pair | References |
|---|---|---|---|
| *nuc* | F: 5´-GCGATTGATGGTGATACGGTT-3´<br>R: 3´-AGCCAAGCCTTGACGAACTAAAGC-5´ | 270 | Louie *et al*. (2000) |
| *mec*A | F: 5´-TGGCATTCGTGTCACAATCG-3´<br>R: 3´-CTGGAACTTGTTGAGCAGAG-5´ | 310 | Altaf *et al*. (2020) |
| *spa* | F: 5´-CAAGCACCAAAAGAGGAA-3´<br>R: 3´-CACCAGGTTTAACGACAT-5´ | 110-320 | Yunita *et al*. (2020) |
| *seb* | F: 5´-CCTTAAACCAGATGAGTTGCACA-3´<br>R: 3´-ACCATCTTCAAATACCCGAACA-5´ | 405 | Srinivasan *et al*. (2006) |
| *sec* | F: 5´-AGATGAAGTAGTTGATGTGTATGG-3´<br>R: 3´-CACACTTTTAGAATCAACCG-5´ | 451 | Peles *et al*. (2007) |

**Table 2:** The conditions used for PCR optimization of different genes

| Targeted gene | Initial denaturation (°C) | Final denaturation (°C) | Annealing temperature (°C) | Initial extension (°C) | Final extension (°C) | References |
|---|---|---|---|---|---|---|
| *mecA* | 95 for 5 min | 95 for 30 s | 58 for 30 s | 72 for 30 s | 72 for 10 min | Altaf *et al*. (2020) |
| *spa* | 94 for 30 s | 94 for 1 min | 60 for 1 min | 72 for 1 min | 72 for 30 s | Yunita *et al*. (2020) |
| *seb* | 94 for 5 min | 94 for 30 s | 55 for 30 s | - | 72 for 10 min | Srinivasan *et al*. (2006) |
| *sec* | 94 for 5 min | 95 for 1 min | 55 for 1 min | 72 for 1 min | 72 for 10 min | Peles *et al*. (2007) |

## Phenotypic and molecular detection of methicillin-resistant *S. aureus*

The phenotypic identification of methicillin-resistant and sensitive *S. aureus* was performed by Kirby Baurs disc diffusion assay. For phenotypic detection, the actively grown culture of *S. aureus* was swabbed on Mueller Hinton Agar followed by the placement of a cefoxitin disc (30 µg), providing the incubation at 37°C for 24 h. The zones of inhibition were measured and compared with the standards of CLSI (2019) to declare the isolate as methicillin-sensitive or methicillin-resistant *S. aureus* (Javed *et al*., 2023). Isolates that showed a zone of ≤21 mm around the cefoxitin disc were considered MRSA and isolates revealing a zone of ≥22 mm were considered as MSSA as per CLSI standards. The DNA extraction of *S. aureus* isolates from bovines and occupational workers was done using the DNA extraction kit method (Aqib *et al*., 2017) and was subjected to PCR for the molecular identification of the *mec*A gene using already reported primers and conditions (Javed *et al*., 2023). For positive control, the MRSA reported by Javed *et al*. (2024) was used as a reference strain. The isolates that showed a band on 310 bp were considered *mec*A-positive; thus, while the isolates did not carry *mecA* gene, MRSA were named methicillin-sensitive *S. aureus*.

## Molecular identification of virulence genes in *S. aureus*

The DNA samples of *S. aureus* isolates from bovine and human samples were further subjected to conventional PCR for the molecular detection of genes of three virulence factors including staphylococcal enterotoxins B and C (*seb*, and *sec*) and staphylococcal protein A (*spa*). The primers and conditions that were used are indicated in Tables 1 and 2. The samples that showed positive bands for *seb* or *sec* were termed enterotoxigenic *S. aureus* based on PCR results. The prevalence and occurrence of each virulence factor among *S. aureus* isolates were also determined from bovine and human samples, respectively.

## Sequencing and phylogenetic analysis

The PCR bands of more prevalent enterotoxin genes (*seb*) from bovine and human samples were viewed and aseptically sliced on a UV illuminator and then sent to a renowned lab for sequencing. A total of five representative *seb* bovine bands and one human *seb* were sent for sequencing. The obtained sequences were first confirmed by basic local alignment search tool (BLAST) alignment. The sequences were further subjected to *Clustal W* multiple alignment and a comparison of bovine and human gene sequences was done to check out the possible similarity among the genes. All the gene sequences from bovines and humans were also compared with retrieved sequences of *Seb* from different sources (food, feaces, liver, infection, blood, nasal swab, wound, and abiotic surfaces) to check out the significant similarities among the sequences due to public health importance of the toxin. The phylogenetic tree by maximum likelihood method was also constructed to explore the genetic relationship among the sequences from different sources. The multiple alignment and construction of the tree were executed using BioEdit and MegaX software, respectively (Ishaq *et al.*, 2022; Ghauri *et al.*, 2021).

## *In-vitro* antibiogram profiling of enterotoxigenic *S. aureus*

The *in-vitro* susceptibility trials of antibiotics against *S. aureus* isolates harboring *seb* and *sec* genes were done using the Kirby-Bauer disc diffusion method. To do so,

**Table 3:** Resistance profile of enterotoxin-producing (*seb* and *sec*) *S. aureus* isolates against antibiotics

| Antibiotics | Disc potency (µg) | CLSI standards zones of inhibition (mm) | | | Resistance pattern of enterotoxin-producing *S. aureus* isolates (n=39) | | | Multiple antibiotic resistance index (MARI) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Resistant | Intermediate | Sensitive | *seb*=24 (%) | *sec*=15 (%) | Total (%) | No. of antibiotics resistant | No. of isolates | | MARI |
| | | | | | | | | | n | % | |
| Linezolid | 30 | ≤20 | - | ≥21 | 06 (25.00) | 04 (26.67) | 10 (25.64) | 0 | 03 | 7.69 | 0 |
| Amoxicillin | 25 | ≤16 | - | ≥21 | 21 (87.50) | 13 (86.67) | 34 (87.18) | 1 | 02 | 5.13 | 0.1 |
| Gentamicin | 10 | ≤12 | 13-14 | ≥15 | 17 (70.83) | 11 (73.33) | 28 (71.80) | 2 | 11 | 28.21 | 0.2 |
| Tylosin | 30 | ≤16 | 17-19 | ≥20 | 13 (54.17) | 10 (66.67) | 23 (58.97) | 3 | 08 | 20.51 | 0.3 |
| Ciprofloxacin | 5 | ≤15 | 16-20 | ≥21 | 12 (50.00) | 06 (40.00) | 18 (46.15) | 4 | 00 | 00 | 0.4 |
| Fusidic Acid | 10 | ≤14 | 15-22 | ≥23 | 08 (33.33) | 03 (20.00) | 11 (28.21) | 5 | 04 | 10.26 | 0.5 |
| Oxytetracycline | 30 | ≤14 | 15-18 | ≥19 | 19 (79.17) | 12 (80.00) | 31 (79.45) | 6 | 06 | 15.38 | 0.6 |
| TMP+SUL | 1.25/23.75 | ≤10 | 11-15 | ≥16 | 17 (70.83) | 10 (66.67) | 27 (69.23) | 7 | 03 | 7.69 | 0.7 |
| Moxifloxacin | 5 | ≤20 | 21-23 | ≥24 | 03 (12.50) | 03 (20.00) | 06 (15.38) | 8 | 02 | 5.13 | 0.8 |
| Levofloxacin | 5 | ≤15 | 16-18 | ≥19 | 02 (08.33) | 01 (06.67) | 03 (07.70) | 9 | 00 | 00 | 0.9 |

**Table 4:** Genotypic prevalence of *S. aureus* and MRSA among bovine and human samples

| Pathogen | Prevalence (%) | | | |
|---|---|---|---|---|
| | Cattle (n=192) | Buffalo (n=192) | Human (n=100) | Total (n=484) |
| *S. aureus* | 101 (52.60%) | 93 (48.44%) | 47 (47.00%) | 241 (49.79%) |
| MRSA | 39 (38.61%) | 27 (29.03%) | 05 (10.64%) | 71 (29.46%) |

**Table 5:** Prevalence of virulence factors in *S. aureus* isolates from bovines and humans

| Virulence factors | Prevalence (%) | | | |
|---|---|---|---|---|
| | Cattle (n=101) | Buffalo (n=93) | Human (n=47) | Total (n=241) |
| *seb* | 14 (13.86%) | 06 (06.45%) | 04 (08.51%) | 24 (09.96%) |
| *sec* | 09 (08.91%) | 04 (04.30%) | 02 (04.26%) | 15 (06.22%) |
| *spa* | 71 (70.30%) | 69 (74.20%) | 21 (44.69%) | 161 (66.80%) |

the growth of enterotoxigenic *S. aureus* was adjusted to 0.5 McFarland standards and swabbed on Mueller Hinton agar. The antibiotic discs of various groups were checked against these isolates. The antibiotic discs were aseptically placed on activated growth of enterotoxigenic *S. aureus* of either toxin type (*seb* and *sec*) followed by incubation of 37°C for 24 h. The resultant zones of inhibitions were measured and compared with standard zones provided by the CLSI manual (2019) as mentioned in Table 3. The status of either resistant or sensitive antibiotics against *seb*-producing *S. aureus* and *sec*-producing *S. aureus* was noted. The cumulative resistance pattern was also declared which indicates the cumulative resistance percentage of enterotoxigenic *S. aureus* against any specific antibiotic. Moreover, the multiple antibiotic resistance index was also calculated as per the formula provided by Javed *et al*. (2023).

### Statistical analysis

The prevalence of *S. aureus* and enterotoxigenic MRSA was calculated using the formula provided by (Thrushfield, 2013) while the results of antibiogram profiling were calculated using percentage (%) of descriptive statistics.

## Results

### Prevalence of *S. aureus* and MRSA

The results of the present study showed that out of 384 milk samples from bovine, a total of 194 milk samples (comprising cattle = 101, buffalo = 93) were found positive for *S. aureus* based on thermonuclease gene (*nuc*) by PCR. PCR declared only 47 swab samples to be positive for *S. aureus* from dairy farm workers. However, the overall cumulative prevalence of *S. aureus* among bovine and human samples was found to be 49.79% on a PCR basis. Moreover, MRSA was found 38.61% and 29.03% prevalent among the *S. aureus* samples of cattle and buffalo based on *mecA* gene presence, respectively. While among the human *S. aureus* samples, only 10.64% of isolates were MRSA positive based on *mec*A gene. The overall prevalence of MRSA among all the bovine and human samples was found to be 29.46% (Table 4).

### Prevalence of enterotoxins and *spa* gene in *S. aureus* isolates

The presence of virulence activity was determined molecularly by targeting the *spa* gene. The findings showed that 70.30%, 74.20%, and 44.69% of isolates of cattle, buffalo, and humans had *spa* genes in their genome which indicated the virulence activity of the *S. aureus* isolates (Table 5). The confirmed strains of *S. aureus* also proceeded further to find out the presence of staphylococcal enterotoxins (SEs). Among all tested isolates of *S. aureus* from both bovine and humans, about 39 (16.18%) isolates were involved in the production of staphylococcal enterotoxins (SEs) (Table 5). The present study declared *S. aureus* to be enterotoxigenic based on *seb* and *sec* gene confirmation among human and bovine isolates by PCR. With an overall occurrence of 9.96% *seb* in human and bovine samples of the region, the *seb* gene was found to be 13.86%, 6.45%, and 8.51% prevalent among cattle, buffalo, and human *S. aureus* isolates, respectively. The *sec* was found to be 8.91%, 4.30%, and 4.26% prevalent among cattle, buffalo, and human samples, respectively. Comparatively, the *seb* gene was found more prevalent as compared with the *sec* gene among the enterotoxigenic *S. aureus*. Furthermore, the cumulative occurrence of both *seb* and *sec* genes among the enterotoxigenic *S. aureus* of cattle, buffalo, and human samples was also listed in (Table 5).

### Phylogenetic evaluation of enterotoxigenic *S. aureus*

The prevalent enterotoxin gene (*seb*) in this study was compared with already reported gene sequences from different sources. Moreover, the current gene sequences of dairy workers and bovines were also compared to determine the significant similarity among the sequences at the molecular level. All the sequences were aligned by multiple alignment software. The alignment revealed that the present study isolates showed substitution, deletion, and addition at different places in comparison with each other or with other isolates from different countries. The graphic representation of *Clustal W* multiple alignments among sequences in the present study revealed common substitution at (2nd, 5th) deletion at (13th, 22nd, 286th, 313th, 359th, 373rd) and addition at (29th, 373th) positions, respectively. However, the isolate R16 showed additional substitution at 1st, 2nd, and 3rd positions in comparison with isolate R41. Meanwhile, the comparison of *seb* gene sequence from dairy workers with bovine milk-associated *seb* gene showed a difference at only 3 positions which could explain the possible spread of enterotoxigenic *S. aureus* between bovines and dairy workers at the molecular level (Fig. 2).

The already reported *seb* gene sequences from different sources revealed significant differences and similarities with local study sequences. The reported sequences showed common substitution at (1st, 2nd, 4th, and 29th) and deletion at (13th, 88th, 286th, 313th, 359th, 373rd) positions. All the reported sequences showed the common addition of nucleotide (A) at position 22nd in alignment. The gene sequences with accession No.: KX168632 (feces) and AB630021 (abiotic surfaces) also exhibited additional substitution at 5 different places. Similarly, other isolates (accession No.: CP053183 (liver), KX168631 (infection), CP045468 (blood), KX168629 (nasal swab), KC428707 (wound) also showed substitutions at different places in comparison with local study *seb* isolates. Moreover, the gene sequence from India (accession No.: AM158256) showed less difference and was found most similar to the present study sequences of the *seb* gene as compared with others (Fig. 2). The significant difference among the current sequences of bovine and human *seb* with already reported gene sequences from different sources showed toxigenic *S. aureus* to be similar among bovine, human, and other sources and its occurrence is escalating among the dairy worker population increases the chances of food poisoning and foodborne maladies in them.

The phylogenetic analysis by the construction of a tree via the Maximum likelihood method at 1,000 replication bootstrapping technique revealed that all the isolates in the present study showed more similarity with

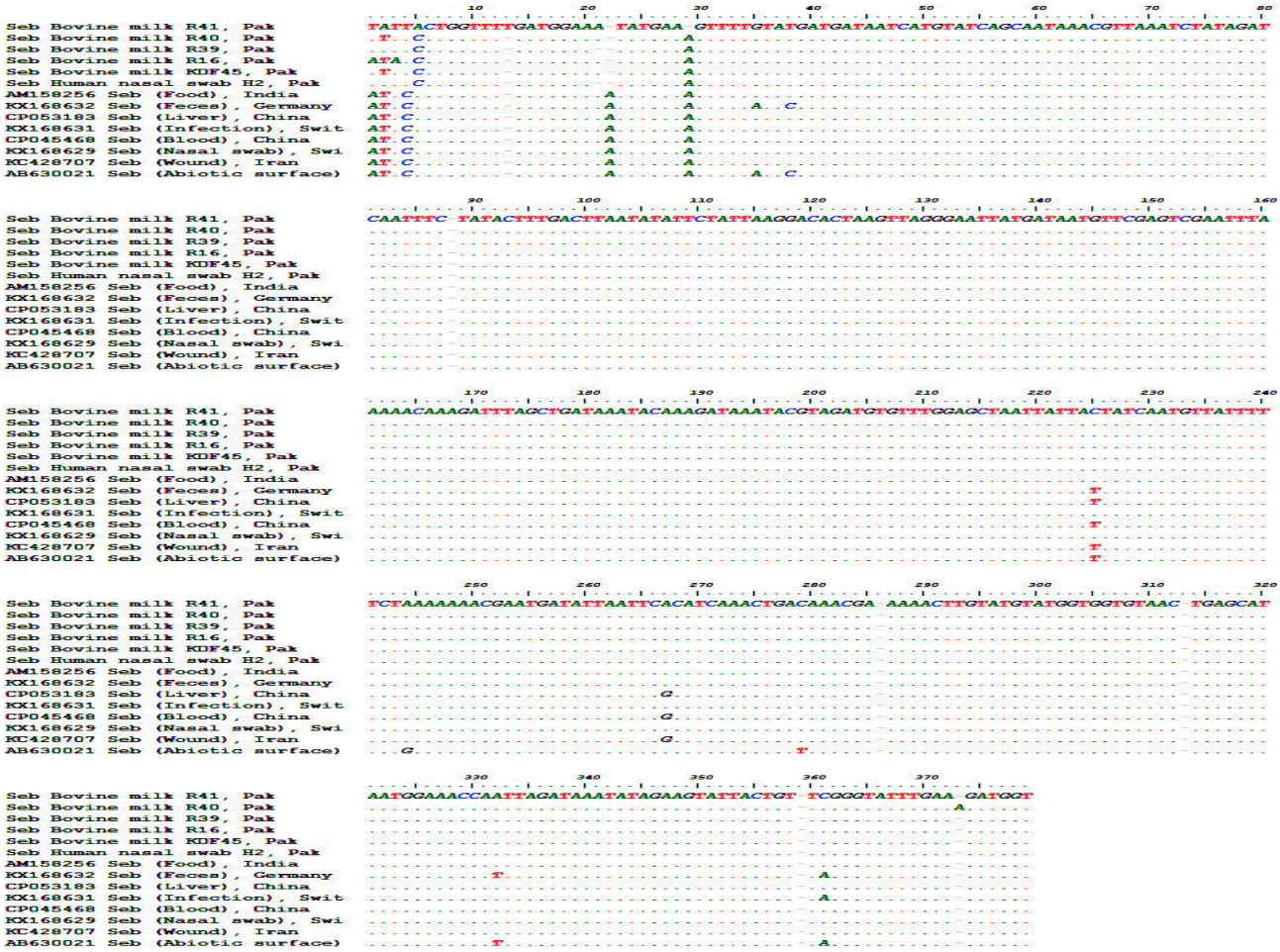


**Fig. 2:** Clustal W multiple alignment of local study sequences of Enterotoxin B (*seb*) gene with already reported sequences from different sources

each other as compared with sequences in other countries. Also, the study isolates R40 and KDF45 showed more resemblance with each other as compared with other study isolates and formed a separate clad. The study isolates showed less similarity with other isolates reported from different countries including the sequence from Japan (abiotic surfaces; accession No.: AB630021), Germany (feces; accession No.: KX168632), Switzerland (infection; accession no.: KX168631), India (food; accession No.: AM158256) and they made separate clad to study isolates (Fig. 3).

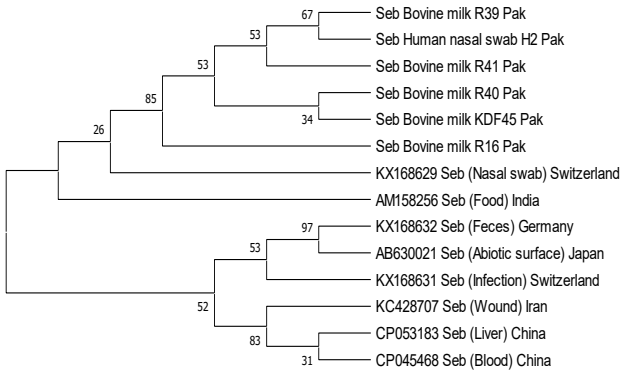


**Fig. 3:** Phylogenetic analysis of Enterotoxin B (*seb*) gene of the study isolates with NCBI Genbank reported sequences from different sources using the maximum likelihood method and 1000 bootstrap technique

### Antibiotic resistance of enterotoxigenic *S. aureus* isolates

The antibiogram of *S. aureus* producing enterotoxins (n=39) was evaluated against different antibiotics using the Kirby-Bauer technique. The findings of the antibiogram profile depicted that maximum isolates of *S. aureus* producing enterotoxins (B, C) were found resistant against amoxicillin (87.18%), tetracycline (79.45%), and trimethoprim+sulfamethoxazole (69.23%). Moreover, more antibiotics were found resistant to the *seb*-producing *S. aureus* isolates as compared with the isolates which produce *Sec* enterotoxin. Among the fluoroquinolones, levofloxacin (7.70%) and moxifloxacin (15.38%) were found sensitive against enterotoxin-producing *S. aureus*. Moreover, linezolid was also found sensitive against the study isolates. Enterotoxin-producing *S. aureus* (n=39) was also evaluated using the multiple drug resistance index (MARI). 23/39 (58.97%) of the 39 examined isolates had a MARI score larger than 0.2 and declared as multiple drug-resistant because they have resistance to more than three antimicrobial classes. Of the 39 tested isolates, 41.03% (16/39) have a MARI value below and equal to 0.2. There are no isolates with a MARI index of 1.0.

## Discussion

*S. aureus* accounts for almost 30% of intra-mammary infections around the globe (Barkema *et al*., 2006). The pathogenic characteristics and toxin-producing ability of *S. aureus* make it the most prevalent mastitogen of subclinical mastitis in various countries of the world (Bramley *et al*., 1996). The acquisition of antibiotic-resistance genes and the production of enterotoxin in *S. aureus* has led to the evolution of most emerging problems regarding public health concerns (Algammal *et al*., 2020).

In the present study, the genotypic prevalence of *S. aureus* was found to be 52.60%, 48.44%, and 47% among cattle, buffalo, and human samples, respectively. The study results were in line with the findings of several other authors who reported an overall prevalence in the range of 28% to 33% in bovines of various countries (Sharma *et al*., 2011; Pamuk *et al*., 2012; UH *et al*., 2014; Saka and Terzi Gulel, 2018). However, MRSA was found prevalent at 38.61%, 29.03%, and 10.64% in cattle, buffalo, and human samples, respectively. These results were concomitant with the overall prevalence of 34% of bovines in the area of Pakistan (Aqib *et al*., 2017). While, other studies reported higher MRSA prevalence in China (47.6%), Ethiopia (42.9%), and Egypt (35.7%) (Algammal *et al*., 2020; Girmay *et al*., 2020; Yang *et al*., 2020). The present study reports the prevalence of MRSA as 10.63% which is in agreement with the results of (Weese *et al*., 2005) who reported a 13% prevalence of MRSA from animal occupational workers. The reason for the prevalent MRSA pathogen in the present study area could be due to poor hygienic conditions at farm level, environmental contamination, unsatisfactory milking practices, and overuse of antibiotics without consulting a veterinarian.

In the present study, the overall activity of virulence factors was noted as 16.18% in *S. aureus* isolates from bovine and human samples. While, the prevalence of enterotoxin genes (*seb* and *sec*) in cattle (13.86%, 8.91%) and buffalo (6.45%, 4.30%) were found in agreement with results reported by (Nazari *et al*., 2014) who also observed a 16.6% occurrence of *seb* gene in cow mastitis milk while all the isolates were found negative regarding *sec* gene. Similarly, some other studies have also reported a lesser 2.17% prevalence of *sec* in cow milk (Ewida and Al-Hosary, 2020). However, the prevalence of 11.8% enterotoxin genes was also found in buffalo milk (Saka and Terzi Gulel, 2018). In the present study, the higher prevalence of the enterotoxin gene (SEC) shows its key role in *S. aureus* persistency in intra-mammary infections, and it also helps to evade the bovine intra-mammary immune response as explained by (Ferens *et al*., 1998). Moreover, in our study, the 12.76% prevalence of enterotoxigenic *S. aureus* in occupational workers indicates the spillover potential of the pathogen at the human-animal interface. Enterotoxigenic *S. aureus* not only confers the persistent intra-mammary infection but also plays a significant role in food poisoning maladies in humans (Weese, 2012). The bioinformatics analysis revealed that the *seb* gene sequence from humans resembles the gene sequence of *seb* from the bovine origin, which indicates the possible transfer of pathogen between dairy workers and animals as *S.*

*aureus* is a contagious organism and can transfer horizontally. The escalating issue of enterotoxigenic *S. aureus* among dairy workers is posing a serious threat and can be a cause of increasing food/milk-borne maladies among humans. However, good milk hygiene practices and implementing stringent mastitis control programs can reduce the chances of pathogen occurrence and transfer.

The findings of antibiogram profiling of enterotoxigenic *S. aureus* showed that the highest percentage of resistance was seen towards amoxicillin (87.18%) followed by tetracycline (79.45%), and TMP+Sul (69.23%) while levofloxacin (92.3%) and moxifloxacin (15.38%) were found to be most sensitive antibiotics against the tested *S. aureus* isolates. These findings are in line with previous studies conducted in different parts of the world like Iran by Alian *et al*. (2012), Eygpt by Ali *et al*. (2017) Jordan by Obaidat *et al*. (2018), and China by Wu *et al*. (2019). Similar findings have been reported in Pakistan in different species as well (Javed *et al*., 2023; Sabir *et al*., 2024). The MAR index is considered a health risk assessment indicator to ascertain if the isolates originate from areas with high or low antibiotic usage. This study reported that 58.98% of isolates have MARI above 2 while 41.02% of isolates have MARI below 0.2 which is contrary to the findings of (Kiš *et al*., 2021; Javed *et al*., 2023). The increase in trends of resistant strains of MRSA is associated with the imprudent and irrational use of antibiotics in both veterinary and human medicine (Javed *et al*., 2023).

The antibiotic resistance against the *S. aureus* pathogen being an emerging issue could be related to toxin production as in our study the enterotoxigenic *S. aureus* shows more resistance towards Amoxicillin, Gentamicin, and Tetracycline antibiotics which supports the findings of Vintov *et al*. (2003) who explained the antibiotic resistance against more commonly used antibiotics, especially penicillin related group. Food has a significant role in the spread of antibiotic resistance. This type of transfer can happen through the ingestion of resistant strains of the original food microflora, the transfer of resistant food-borne pathogens, potent toxins, antibiotic residues in food, or resistance transfer to pathogenic bacteria (Ortega *et al*., 2010).

A multidisciplinary strategy involving veterinary medicine, human health, and environmental interfaces is needed to address this enormous problem. Public awareness campaigns stressing the safe handling and consumption of animal products, monitoring programs to track antibiotic resistance patterns, and the cautious use of antibiotics in livestock are all examples of strategies. In addition, strategies and regulations that encourage the prudent use of antibiotics in dairying are necessary to reduce the growing threat posed by MRSA that comes from animals. In light of this new issue, a coordinated effort is required to guarantee the sustainability of the environment, agriculture, and public health (Roy *et al*., 2024).

The present study reveals the higher prevalence of enterotoxigenic *S. aureus* in both dairy animals and occupational workers. This study also highlights the zoonotic importance of the pathogen as it can be transferred from dairy animals to occupational workers. The antibiotic susceptibility trial against the enterotoxigenic *S. aureus* showed that the pathogen revealed higher resistance against commonly used antibiotics in field conditions and was found susceptible to the ofloxacin group of antibiotics.

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## Conflict of interest

The corresponding author and co-authors declared no conflict of interest.

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