Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2025.260

RESEARCH ARTICLE

Molecular Characterization and Antibiogram Profiling of Methicillin- and β-Lactam-Resistant *Staphylococcus aureus* in Dogs with Respiratory Infections in Lahore, Pakistan

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ARTICLE HISTORY (25-467)

Received: May 06, 2025
Revised: August 30, 2025
Accepted: September 01, 2025
Published online: October 07, 2025

Key words:
BRSA
In silico analysis
MRSA
Respiratory tract infections
Risk factor analysis

ABSTRACT

Methicillin and β-lactam-resistant *Staphylococcus aureus* (*S. aureus*) are pathogens that prolong the respiratory infection in dogs and have major zoonosis concern for humans living in the vicinity of these infected animals. Overall, 384 nasal swab samples from dogs with respiratory tract infections were collected and processed for the confirmation of *S. aureus*, methicillin-resistant (MRSA), and β-lactam-resistant *S. aureus* (BRSA) using phenotypic and molecular methods (PCR and sequencing). The results showed 38.02% prevalence of *S. aureus,* and among those, 43.15% and 50.00% isolates were approved as MRSA and BRSA on cefoxitin and penicillin disc diffusion test. PCR results confirmed 47.26% and 51.37% isolates as MRSA and BRSA, respectively. The *mecA* and *blaZ* gene sequences were submitted to NCBI, and accession numbers were attained, followed by the phylogenetic evaluation and *in silico* analysis of respective genes using bioinformatics tools. Phylogenetic analysis revealed genetic similarities with isolates from neighboring regions, suggesting cross-boundary transmission, while *in-silico* analysis showed significant similarity of the isolated samples with reported 3D protein templates, and confirmed their conformational stability. Antimicrobial susceptibility of isolates revealed a higher resistance to commonly prescribed β-lactams (penicillin, cefoxitin, amoxicillin, cephalosporins) and aminoglycosides, leaving doxycycline and fluoroquinolones as partially effective options. Risk factor analysis revealed various animal and management based variables to be significantly associated with staphylococcal respiratory infections. The findings suggested that the emergence of antimicrobial-resistant *S. aureus* in dogs underscores a growing therapeutic challenge with potential zoonotic implications.

To Cite This Article: Nasir F, Ijaz M, Ahmed A and Ali M 2025. Molecular characterization and antibiogram profiling of methicillin- and β-Lactam-resistant *Staphylococcus aureus* in dogs with respiratory infections in Lahore, Pakistan. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2025.260

INTRODUCTION

Dog keeping is a widespread and well-accepted phenomenon in today's society due to their cognitive and friendly nature (Benz-Schwarzburg *et al*., 2020). However, these companion animals, due to their intervening lifestyle, are a potential source of transmission of pathogens to humans around them (Chomel, 2014; Yaseen *et al*., 2025). Gram-positive *Staphylococcus aureus* (*S. aureus*) bacteria have been associated with several potentially fatal infections in animals, including dogs. *S. aureus* has emerged as a pathogen of significant concern for both human and animal health because of its invasive confinement, a diversity of pathogenic aspects, and the acquisition of mechanisms to get escape from the host immune system and resist antibiotics (Sabir *et al.*, 2024) Over the previous 20 years, methicillin-resistant *S. aureus* (MRSA) has drawn worldwide attention as a human pathogen but the recent reports have shown that MRSA has emerged as a potential pathogen for pet animals including dogs (Faires *et al.*, 2010). However, the studies focusing on specific infections, associated clinical signs, and risk factors are lacking. *S. aureus* and specifically MRSA have been associated with respiratory tract infections in different animals, including pet animals, leading to chronic infections and lower cure rate (Faires *et al.*, 2010).

Antimicrobial resistance (AMR) is one of the major threats to animal and human health (Pinheiro *et al*., 2020). Bacteria may adjust to a variety of environmental challenges because of their extraordinary genetic flexibility

and ability to develop antimicrobial resistance mechanisms (Munita *et al*., 2016; Zaib *et al*., 2019)). Among various microorganisms that are resistant to antibiotics, *S. aureus* has developed various antimicrobial-resistant strains, including methicillin (MRSA) and β-lactam-resistant *S. aureus* (BRSA), which can disseminate between different species by horizontal or vertical transfer of resistance genes (Bakht *et al.*, 2024). The interspecies spread of several multidrug-resistant bacteria, distinctly methicillin-resistant *S. aureus* (MRSA), is well documented by molecular and epidemiological evidence (Morris *et al.*, 2012). *S. aureus* is regarded as a multidrug-resistant bacterium due to resistance against the antibacterial actions of penicillin and other groups of drugs like aminoglycosides, chloramphenicol, tetracycline, macrolides, and fluoroquinolones (Lee, 2003). These antimicrobial resistant bacterial strains are associated with increased rates of illness, mortality, and huge economic losses.

Although the molecular epidemiology of antimicrobial resistant *S. aureus* has been widely studied in different livestock animals of Pakistan including cattle (Muzammil *et al.*, 2022), buffalo (Ahmed *et al.*, 2022), sheep (Sabir *et al.*, 2024), goat (Javed *et al.*, 2024), camel (Aqib *et al.*, 2018), and horses (Achek *et al.*, 2020; Rasheed *et al.*, 2023), the information regarding prevalence, genetic determinants, risk factors, and phylogenetic lineages of staphylococcal AMR in dogs remains scarce. Dogs have received comparatively less attention despite their close contact with humans, which could facilitate bidirectional transmission and the emergence of novel strains. Keeping in view the paucity of molecular-level investigations in dogs, particularly in Pakistan, the goal of the current study was to look into staphylococcal AMR, underlying genetic mechanisms (e.g., *mecA*, *blaZ*), and their phylogenetic relationships.

MATERIALS AND METHODS

Study rationale: The research was conducted in Lahore district, Punjab, Pakistan (Fig. 1). A total of 384 nasal swabs were accumulated from the dogs (male: n=231; female: n=153) showing respiratory tract infections. A convenient sampling approach was adopted based on the availability of animals at clinics, shelters, and households, and sampling was done despite of age, sex, and animals breed. The samples were collected from the upper respiratory tract of dogs by nasal swabs employing the standards of Anderson and Weese, (2007). All collected samples were promptly sent to the Medicine Research Laboratory in the Department of Veterinary Medicine, University of Veterinary and Animal Sciences (UVAS) Lahore, by maintaining a cold chain.

The study was approved by the Ethical Review Committee of the University of Veterinary and Animal Sciences, Lahore (approval no. DR/489, dated 01-11-2023). A written informed consent was acquired from all owners prior to sample collection and the collection of information. Participation of owners was voluntary, and sampling was conducted using non-invasive procedures with full consideration of animal welfare.

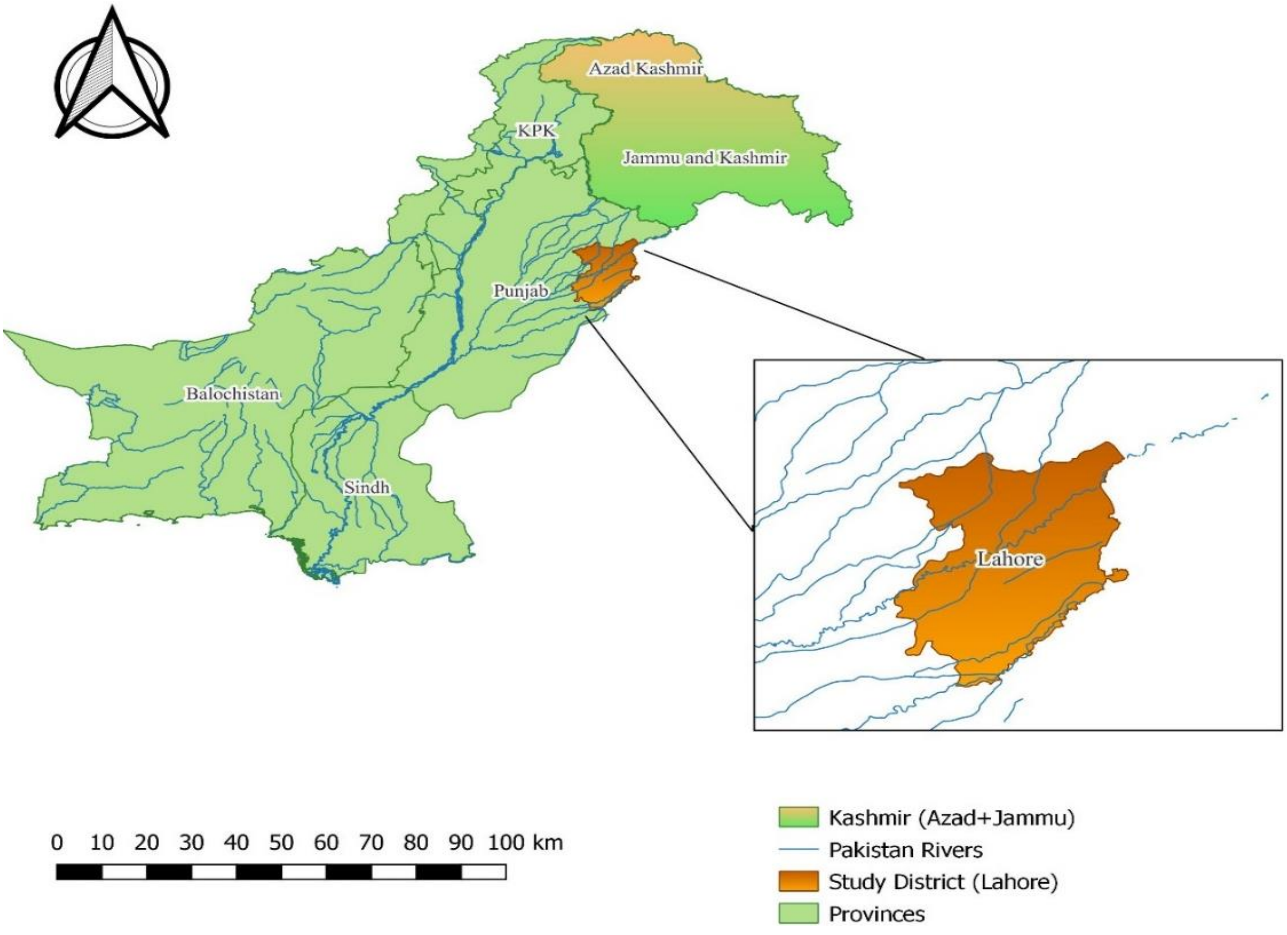


Fig. 1: The QGIS map of the study district.

Additionally, information concerning several assumed animal and management-related risk factors was obtained from the dog owners using a predesigned data capture form. The information was later analyzed to check the association of different risk factors with staphylococcal respiratory infection of dogs.

Inclusion and exclusion criteria: Dogs showing any clinical signs of respiratory tract infections including coughing, sneezing or nasal discharge (purulent or muco-purulent) were included while dogs with none of the above-mentioned clinical signs were not included in the study.

***S. aureus* isolation and identification:** Following collection, the nasal swabs were inoculated into 5 ml brain heart infusion (BHI) enrichment broth (proteose peptone 10 g/l, dextrose 2 g/l, sodium chloride 5 g/l, and disodium phosphate 2.5 g/l) then a 24-hour incubation period at 37°C as per protocol followed by Rasheed *et al*., (2023). Following enrichment, swabbing of samples on 5% sheep blood agar was done, and an overnight incubation at 37°C was provided. *S. aureus* identification was made upon the colonial morphology and pattern of hemolysis. Catalase test, coagulase test, and Gram's staining were utilized for *S. aureus* verification (Ahmed *et al*., 2022). To acquire a pure colonies of *S. aureus*, samples were underwent secondary culturing on mannitol salt agar (MSA), the selective media for *S. aureus* growth. *S. aureus* was confirmed on MSA based on mannitol fermentation and golden-yellow to brown colored raised colonies (Ahmed *et al*., 2022).

Molecular validation of *S. aureus*:: For molecular confirmation of *S. aureus*, DNA extraction of all the phenotypic *S. aureus* was conducted by employing commercial DNA extraction kit (Gene Jet Genomic DNA Purification kit, Thermoscientific) following the manufacturer's guidelines (Ahmed et al., 2023). The DNA quantity was checked using Nanodrop. All DNA samples were subjected to PCR for molecular identification of *S. aureus* by targeting thermonuclear *nuc* gene (270 bp) utilizing primers mentioned in Table 1 (Louie *et al.*, 2002) and thermocycler conditions as followed by Muzammil *et al*., (2022).

Table 1: Primer sequences used in this study

Sr. No.	Target gene	Gene size (bp)	Primer sequences	References
1.	*nuc*	270	F= GCGATTGATGGTGATACGGTT R=AGCCAAGCCTTGACGAACTAAAGC	(Louie *et al*., 2002)
2.	*mecA*	310bp	F=TGGCATTCGTGTCACAATCG R=CTGGAACTTGTTGAGCAGAG	(Galdiero *et al.*, 2003)
3.	*blaZ*	421bp	F=CAAAGATGATATAGTTGCTTATTCTCC R= TGCTTGACCACTTTTATCAGC	(Kaase *et al*., 2008)

Phenotype-based identification of antibiotic-resistant isolates: Phenotypic confirmation of methicillin and β-lactam-resistant *S. aureus* isolates was conducted using the disc-diffusion antibiotic susceptibility test. Mueller Hinton agar plates were plated and streaked with 0.5 McFarland adjusted active growth of *S. aureus.* Antibiotic discs of cefoxitin (30µg) and penicillin (10µg) were used for the identification of resistance against MRSA and BRSA, respectively. After incubation at 35°C-37°C temperature for 24 hours, the diameter of zones of growth inhibition (ZoI) encircling the discs was assessed by a vernier caliper and interpreted based on the Clinical and Laboratory Standard Institute (CLSI) (CLSI, 2019) guidelines. Isolates with ZoI ≤17 mm to cefoxitin and ≤28 mm to penicillin were declared as phenotypic MRSA and BRSA, respectively. Similarly, isolates with ZoI ≥17 mm to cefoxitin and ≥29 mm to penicillin were declared as phenotypic methicillin and β-lactam sensitive (MSSA and BSSA), respectively.

Genotypic confirmation of MRSA and BRSA isolates: For genotypic confirmation of MRSA and BRSA, Polymerase Chain Reaction (PCR) amplification of antibiotic encoding genes *nuc*, *mecA* and *blaZ* was performed. The primers mentioned in Table 1 were used to target the *nuc*, *mecA* and *blaZ* genes of *S. aureus*. PCR protocol for the *mecA* gene began with an initial denaturation at 94°C for 5 minutes, followed by 34 cycles comprising denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute, and extension at 72°C for 1 minute. The reaction was finished using final extension at 72°C for 10 minutes. For the *blaZ* gene, PCR amplification was carried out with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step was performed at 72°C for 7 minutes. After amplification, the amplicon was subjected to gel electrophoresis (1.5% agarose gel with ethidium bromide as a dye) and subsequent illumination under a UV-illuminator to visualize the PCR bands at their specific bp. Isolates that showed bands at 310 bp were reported as genotypic MRSA, whereas isolates displaying bands at 421 bp were reported as genotypic BRSA.

Sequencing and molecular characterization of *mecA* and *blaZ* genes: PCR products positive for antibiotic resistance gene carriage were subjected to purification utilizing a commercially available kit (WizPrep Gel/PCR purification kit) considering the instructions provided by the manufacturer. Purified gel bands were delivered to a renowned laboratory (1st BASE, JTC MedTech Hub, Tukang Singapore 618,305) for sequencing of the amplified samples.

On reception of the sequencing data, the similitude of research samples with the benchmark bacterial samples previously reported on NCBI was checked using the Basic Local Alignment Search Tool (BLAST). Multiple alignment of study isolates was performed using the *ClustalW* feature of Bioedit software. Sequences of the study isolates were also submitted to NCBI, and accession numbers for all *mecA* gene (Accession no: PP760507, PP760508, PP760509, PP760510, PP757496) and *blaZ* gene (Accession no: PP757490, PP757491, PP757493, PP757494, PP757495) were acquired. Finally, Mega X software with maximum likelihood method and 1000 bootstrap settings was used for the construction of respective phylogenetic trees to assess the evolutionary relationship of the research isolates with one another and with previously published sequences.

In silico* analysis of MRSA and BRSA isolates*: Nucleic acid and protein sequences were aligned using an analytical tool named Clustal Omega software. The Multiple EM for Motif Elicitation (MEME) suite was utilized to build nucleic acid and protein motifs. Moreover, Swiss model software was employed to determine the 3D assembly of protein. With the help of STRING, protein-protein interactions were analyzed, and the physical and chemical properties of the protein were evaluated by ProtParam. While Secondary structure motifs comparison was performed with the help of SOPMA (Self-Optimized Prediction Method with Alignment). Furthermore, the Ramachandran plot was devised to forecast the configurational properties.

Antibiogram profiling of MRSA and BRSA isolates: Antibiogram profiling of bacterial cultures positive for *mecA* gene (n=17), *blaZ* gene (n=17), and bacterial cultures positive for both *mecA* and *blaZ* gene (n=17) was done. Three to five colonies of respective isolates were taken and suspended in 5 mL of nutrient broth. The broth culture's turbidity was calibrated to 0.5 McFarland standard containing 1.5×10^8 CFU/ml and 0.1 ml of broth culture was incorporated onto the surface of Mueller Hinton agar plates and antibiotic discs including cefoxitin (30µg), amoxiclav (30µg), cefoperazone (75µg), ceftriaxone sodium (30µg), gentamicin (20µg), ciprofloxacin (30µg), cephalexin (30µg), cefotaxime (10µg), penicillin (10µg), streptomycin (30µg), and doxycycline (30µg) was placed using a disc dispenser. Plates after inoculation were incubated at 37°C for a period of 18–24 hours. After the incubation period, a vernier caliper was used to measure the zones of inhibition (ZoI) surrounding the antibiotic discs. These zones were compared with CLSI guidelines to categorize study isolates as sensitive, intermediate, or resistant to the respective antibiotics.

Statistical analysis: The positive percentage of *S. aureus*-associated respiratory tract issues in dogs was calculated by dividing the number of samples positive for *S. aureus* by the total number of samples tested for *S. aureus*. The information regarding risk variables was entered into a database and coded for analysis. The association of these risk variables with the staphylococcal respiratory tract infections was evaluated by the Chi-square test. Variables with $p < 0.20$ in univariable analysis were included in a multivariable logistic regression model to adjust for confounding. Results were reported as odds ratios (OR) with 95% confidence intervals (CI), and a p-value < 0.05 was considered statistically significant. Descriptive statistical tools like percentage were used to assess the antibiogram profiling of the study isolates. The Statistical Package for Social Sciences (SPSS) version 20.0 was used for all statistical analyses.

RESULTS

Prevalence of *S. aureus*: In this research, *S. aureus* was isolated from 38.02% of dogs suffering from respiratory tract infections by bacteriological, biochemical, and molecular methods. The most frequent prevalence of *S. aureus* was noted in shelter home dogs at 53.96% followed by Kennel reared dogs at 36.11% while the lowest prevalence was recorded in domestic dogs at 34.27%. (Table 2).

Table 2: Prevalence of *S. aureus*, MRSA, and BRSA

Rearing Type	Animal numbers	*S. aureus* (%)	Phenotypic resistance (%)		Genotypic resistance (%)	
			Cefoxitin	Penicillin	*mecA*	*blaZ*
Domestic dogs	213	73 (34.27)	27 (36.98)	36 (49.31)	28 (38.35)	34 (46.57)
Kennel reared dogs	108	39 (36.11)	18 (46.15)	13 (33.33)	19 (48.71)	18 (46.15)
Shelter home dogs	63	34 (53.96)	18 (52.94)	24 (70.58)	22 (64.70)	23 (67.64)
Total	384	146 (38.02)	63 (43.15)	73 (50.00)	69 (47.26)	75 (51.37)

Phenotypic and genotypic estimation of MRSA and BRSA: For phenotypic detection of MRSA, cefoxitin was used, while for the BRSA, penicillin disc was used. Phenotypically, MRSA was found in 43.15% and BRSA was found in 50.00% of the dog nasal swab samples. The highest level of phenotypic MRSA was recorded in shelter home dogs at 52.94% followed by Kennel reared dogs at 46.15% and domestic dogs at 36.98%. Similarly, the highest level of phenotypic BRSA was also recorded in shelter home dogs at 70.58% followed by domestic dogs (49.31%) and Kennel reared dogs (33.33%).

Concerning genotypic prevalence, *mecA* gene carrying *S. aureus* was found in 47.26% while *blaZ* gene carrying *S. aureus* was found in 51.37% dogs. The slightly higher frequency of *blaZ* gene indicates widespread β-lactamase–mediated resistance, reflecting common resistance against penicillin derivatives. The detection of *mecA* is of greater clinical importance because it encodes PBP2a, leading to resistance to nearly all β-lactams and significantly limiting treatment choices. The highest infection rate of MRSA was recorded in shelter home dogs (64.70%), followed by kennel-reared dogs (48.71%) and domestic dogs (38.35%). Similarly, genotypic BRSA was prevalent in shelter home dogs at (67.64%), followed by domestic dogs (46.57%) and Kennel reared dogs (46.15%) (Table 2).

Analysis of risk factors associated with staphylococcal respiratory issues in dogs: Risk factors analysis revealed that breed (p=0.009), vaccine status (p=0.049), stocking density (p=0.002), body condition score (p=0.045), house hygiene (p=0.000), respiratory distress (p=0.005), wound history (p=0.041), human contact (p=0.009), diagnostic facilities (p=0.004), and history of antibiotic therapy (p=0.002) showed significant association with incidence of *S. aureus* linked respiratory tract infections. (Table 3). Multiple logistic regression analysis revealed that Labrador breed showed a 1.965 times and mixed breed showed 2.851 times higher rate of staphylococcal respiratory infection than the German Shepherd. Moreover, vaccinated dogs had 0.659 times lesser chances, dogs with higher stocking density had 1.937 times increased risk, dogs with medium and poor BCS had 1.821 times and 1.944 times higher likelihood and dogs kept in poor hygiene practices had 2.280 times higher chances of suffering from staphylococcal respiratory infections compared to the reference groups. Similarly, the respiratory distress (OR=1.995), history of wounds (OR=1.543), frequent human contact (OR=1.735), Lack of diagnostic facilities increased (OR=1.851), and prior history of antibiotic therapy (OR=1.923) were found potential risk factors associated with staphylococcal respiratory issues (Table 4).

Table 3: Analysis of assumed risk factors in relation to *S. aureus*-associated respiratory infections in dogs

Variables	Variable levels	Respiratory infections associated *with S. aureus*		
		Total	Positive (%)	*p*-value
Breed*	German Shepherd	138	38(27.54)	0.009*
	Labrador	121	43(35.54)	
	Mixed Breed	125	65(52.00)	
Sex	Male	231	90(38.96)	0.641
	Female	153	56(36.60)	
Age	<1yr	211	83(39.34)	0.558
	>1yr	173	63(36.42)	
Vaccine status*	Yes	193	64(33.16)	0.049*
	No	191	82(42.93)	
Stocking density*	High	223	99(44.39)	0.002*
	Low	161	47(29.19)	
BCS*	Good	214	74(34.58)	0.045*
	Medium	97	35(36.08)	
	Poor	73	37(50.68)	
Season	Winter	203	77(37.93)	0.391
	Summer	106	36(33.96)	
	Autumn	75	33(44.00)	
House hygiene*	Poor	222	102(45.95)	0.000*
	Good	162	44(27.16)	
Respiratory distress*	Present	273	116(42.49)	0.005*
	Absent	111	30(27.03)	
Transport history	Yes	164	60(36.59)	0.617
	No	220	86(39.09)	
Living area	Open	194	72(37.11)	0.711
	Closed	190	74(38.95)	
Wound history*	Yes	206	88(42.72)	0.041*
	No	178	58(32.58)	
Human contact*	Yes	191	85(44.50)	0.009*
	No	193	61(31.61)	
Diagnostic facilities*	Available	164	76(46.34)	0.004*
	Not available	220	70(31.82)	
Presence of other livestock animals	Yes	210	76(36.19)	0.417
	No	174	70(40.23)	
History of antibiotic therapy*	Yes	185	85(45.95)	0.002*
	No	199	61(30.65)	

*indicates significant association.

Table 4: Analysis of potential risk factors by multiple Logistic Regression analysis

Variables	Variable level	Odds Ratio	CI 95%	S.E	*p*-value
Breed	German Shepherd	Ref			
	Labrador	1.965	1.178, 3.278	0.261	0.01
	Mixed Breed	2.851	1.708, 4.759	0.261	0.000
Vaccine status	Yes	0.659			
	No	Ref	0.436, 0.998	0.212	0.049
Stocking density	High	1.937	1.259, 2.978	0.220	0.003
	Low	Ref			
BCS	Good	Ref			
	Medium	1.821	1.135, 3.331	0.275	0.015
	Poor	1.944	0.981, 3.379	0.315	0.057
House hygiene	Poor	2.280	1.475, 3.523	0.222	0.000
	Good	Ref			
Respiratory distress	Present	1.995	1.231, 3.233	0.246	0.005
	Absent	Ref			
Wound history	Yes	1.543	1.016, 2.343	0.213	0.042
	No	Ref			
Human contact	Yes	1.735	1.144, 2.632	0.213	0.010
	No	Ref			
Diagnostic facilities	Available	Ref			
	Not available	1.851	1.218, 2.811	0.213	0.004
History of antibiotic therapy	Yes	1.923	1.266, 2.920	0.213	0.002
	No	Ref			

Molecular characterization of *mecA* gene of *S. aureus*: Molecular characterization of *mecA* harboring isolates, PP757494, PP757495, PP757490, PP757491, and PP757493, was performed, and their homology with previously published NCBI sequences was analyzed by applying BLAST and subsequent phylogenetic tree construction via MEGA XI. Current research isolates PP757494 and PP757495 were more alike to each other than the remaining study isolates, making a clade with each other. Overall, our isolates were more closely related to each other than other isolates from Pakistan or neighboring countries of China or Iraq. Comparison of our sequences with previously published *mecA* nucleotide sequences obtained from NCBI showed that they were more similar to *mecA* bacterial sample from India (CP102576) while PP757493 demonstrated stronger genetic similarity with *mecA* isolates from Pakistan (OL409021, ON643120) rather than other isolates from Iraq, USA, and China (MZ359763, CP092561, CP130551, CP130540, CP130543, and CP130528). Our research isolates revealed notable variance from the isolates of *mecA* isolated from China (CP130534, CP130518) and the least resemblance with the isolate from Iraq (MZ359763), making an out-group as shown in (Fig. 2).

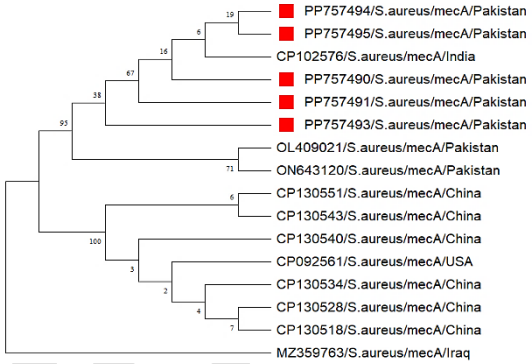


Fig. 2: Phylogenetic analysis of *mecA* gene of MRSA isolates.

Molecular characterization of *blaZ* gene of *S. aureus*: Molecular investigation of *blaZ* harboring study isolates, PP760508, PP760510, PP757496, PP760507, and PP760509, and their comparison to previously documented NCBI sequences revealed that our study isolates, PP760510, PP757496, PP760507, and PP760509, demonstrated more resemblance to one another than to the reference sequences from NCBI. Whereas a comparative study of our sequence PP760508 with that of previously documented sequences for *blaZ* on NCBI indicated that the isolate PP760508 resembles more with the isolates of *blaZ* from Italy, Australia, Ghana and China (U58139, CP127820, CP096249, and CP094853) than that of the other study isolates of *blaZ*. Significant variation was observed between our research isolates and the reference isolates of *blaZ* isolated from Taiwan, USA, and Japan (CP039449, CP117979, CP102976, and AP025684), and made an out-group (Fig. 3).

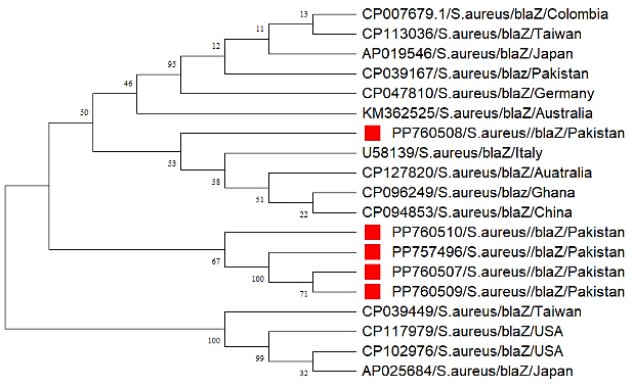


Fig. 3: Phylogenetic analysis of *blaZ* gen of BRSA isolates.

***In silico* analysis of *S. aureus* isolates**

In silico* analysis of *mecA* gene of MRSA*: Pattern of nucleic acid showed variations among reference sequences and research isolates, while the amino acid arrangement pattern showed that the benchmark sequence and research isolates were shown to be 100% identical. Comprehensive motif evaluation of all nucleic acid sequences, including (reference + local) isolates, revealed that *p* value falls between 1.34e-64 to 1.59e-64 (Fig. 4). Motif discrepancies were illustrated with various colors, and the amino acid motif analysis of the benchmark sequence and all local isolates showed *p value* is equal to 2.83e-86 (Fig. 5).

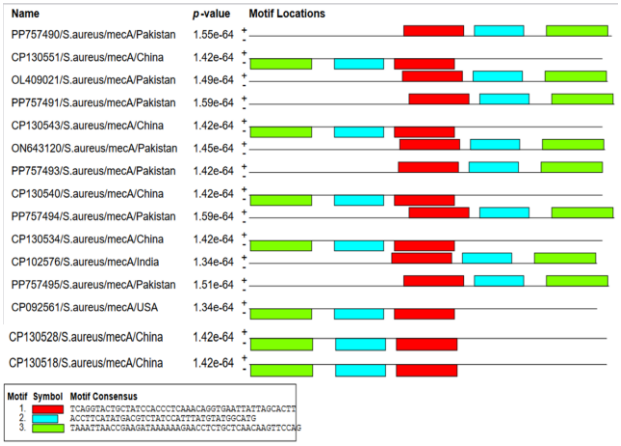


Fig. 4: Conserved motif analysis of *mecA* gene of *S. aureus.*

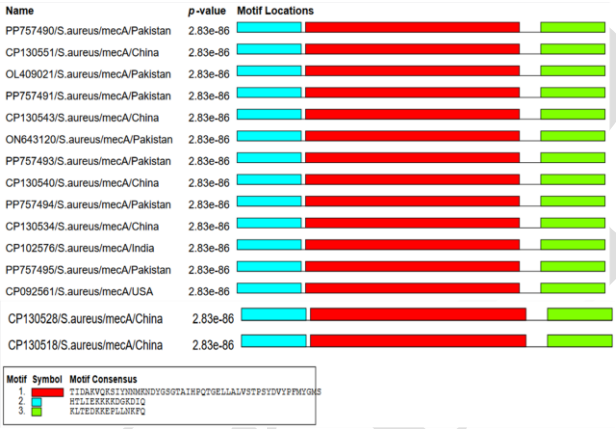


Fig. 5: Conserved motif analysis of protein PBP2a (penicillin-binding protein 2a).

With the help of the Swiss Model, the 3D analysis of protein (PBP2a) was performed. The protein-protein connection of target protein (PBP2a) with other proteins such as mecI (methicillin resistant regulatory protein), mecR (methicillin resistance protein) and blaI (penicillinase repressor) showed significant association by using STRING (Search Tool for the Retrieval of Interacting Genes/ Proteins) (Fig. 6), and connecting lines with various colors exhibited neighborhood, gene co-occurrence, fusion of genes and gene co expression of different genes with PBP2a. Physical and chemical characteristics of protein (PBP2a) of S. aureus were shown in (Table 5). Secondary level of protein structure comparison from SOPMA demonstrated a high percentage of random coil, then an alpha helix, extended strand, and β-turn (Fig. 7). Ramachandran plot depicts that most of the protein residues fall in the most allowed region, which confirms the authenticity of the protein (PBP2a). Moreover, right-handed alpha helices were greater in number than β sheets, and mostly alpha helices and β sheets fall in the most allowed region. Apart from this, 0.4% residues fall in the disallowed region, and Glycine and Proline residues are 83 and 32 in numbers, respectively (Fig. 8).

In silico* analysis of *blaZ* gene of β-lactamase producing *S. aureus: Nucleic acid sequence alignment demonstrated that the benchmark sequences and regional isolates differed significantly from each other. The *p*-value ranged from 1.22e-63 to 7.52e-63 for the nucleic acid motif analysis. (Fig. 9). Significant variations between the research and reference isolates were also shown, and the Analysis of amino acid motifs disclosed that the *p*-value falls between 1.05e-129 to 7.68e-129 (Fig. 10).

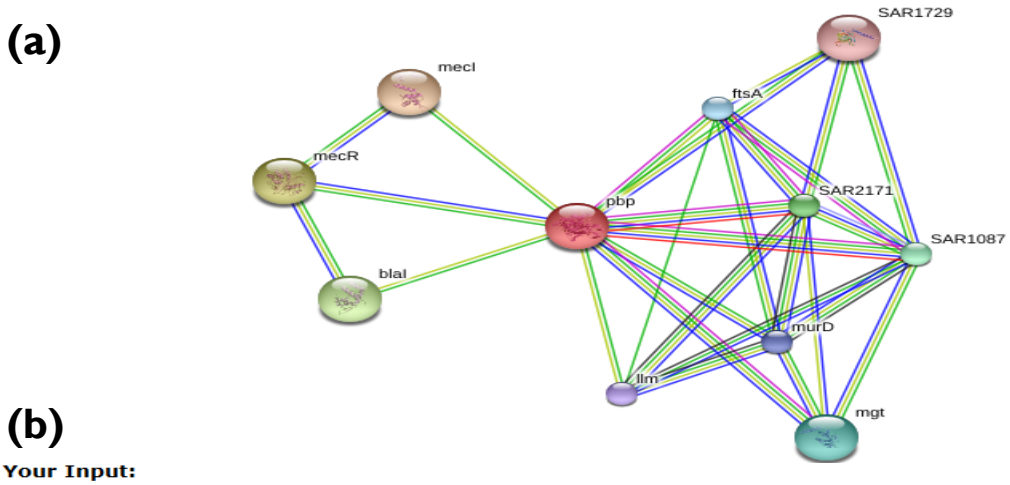
The Swiss model was used to assess the protein's (β-lactamase) three-dimensional structure. Protein association of examined protein (β-lactamase) disclosed remarkable association with other proteins like *blaI* (penicillinase repressor), *blaR1* (β-lactamase regulatory protein), and *pbp* (Penicillin-binding protein 2 prime) via the STRING tool (Fig. 11). Physical and chemical characteristics of β-lactamase protein of *S. aureus* were presented in Table 6). High numbers of alpha helices were found in the secondary structure comparison from SOPMA, succeeded by random coils, extended strands, and β-turns (Fig. 12).

Table 5: Physico-chemical properties of PBP2a protein

Accession numbers	Molecular weight	Number of Amino Acids	Theoretically Pi	TNC	TPC	Half-life (hours)	Instability index (II)	Aliphatic index (AI)	GRAVY
PP757490	10242.62	90	6.65	12	12	7.2	39.00	73.67	-0.806
PP757491	10256.65	90	6.93	12	12	1.4	37.12	74.78	-0.781
PP757493	10041.44	88	6.93	12	12	20	38.70	76.48	-0.751
PP757494	10343.72	91	6.69	12	12	1.9	36.82	73.96	-0.781
PP757495	10142.54	89	6.65	12	12	7.2	38.37	75.62	-0.751

Table 6: Physico-chemical properties of β-lactamase protein of *S. aureus* from ProtParam

Accession numbers	Molecular weight	Number of Amino Acids	Theoretically pI	TNC	TPC	Half-life (hours)	Instability index (II)	Aliphatic index (AI)	GRAVY
PP757496	14314.55	128	9.70	15	26	1.3	16.09	83.05	-0.758
PP760507	14186.38	127	9.65	15	25	2.8	16.14	83.70	-0.733
PP760508	14082.31	127	9.65	15	25	2.8	18.51	87.56	-0.662
PP760509	14082.31	127	9.65	15	25	2.8	16.14	83.70	-0.733
PP760510	14314.55	128	9.70	15	26	1.3	16.09	83.05	-0.758



(b)

Your Input:

pbp penicillin-binding protein 2 prime (668 aa)
(Staphylococcus aureus MRSA252)

Predicted Functional Partners:

		Neighborhood	Gene Fusion	Cooccurrence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
mecI	methicillin resistance regulatory protein MecI; Transcriptional repressor that constitutively b [...] (114 aa)									0.959
mecR	methicillin resistance protein MecR1; Penicillin-interactive protein and potential antirepresso [...] (585 aa)									0.892
blaI	penicillinase repressor; Transcriptional repressor that constitutively blocks expression of bet [...] (126 aa)									0.892
SAR2171	hypothetical protein (400 aa)									0.892
SAR1087	putative cell division protein (408 aa)									0.850
mgt	glycosyltransferase; Involved in the biosynthesis of cell wall peptidoglycan. Responsible for t [...] (269 aa)									0.837
ftsA	putative cell division protein; This protein may be involved in anomalous filament growth. May [...] (468 aa)									0.759
murD	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase; Cell wall formation. Catalyzes the additi [...] (449 aa)									0.758
llm	putative glycosyl transferase (351 aa)									0.752
SAR1729	rod shape-determining protein MreC (280 aa)									0.745

Fig. 6: (a) Protein-Protein interaction by STRING (Network view) and (b) Interaction of reference protein with other proteins (predicted functional partners).

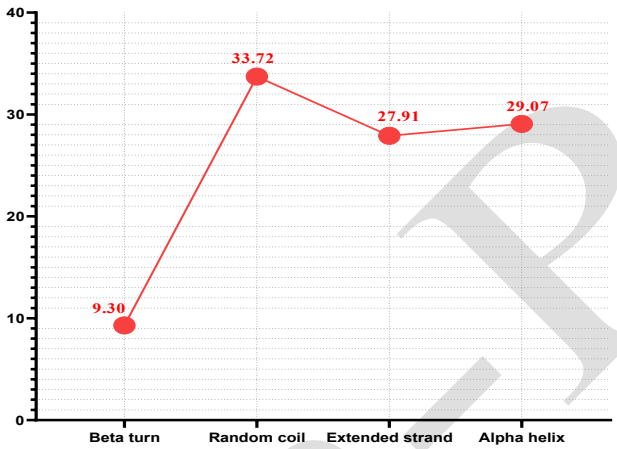


Fig. 7: Secondary structure comparison of PBP2a from SOPMA.

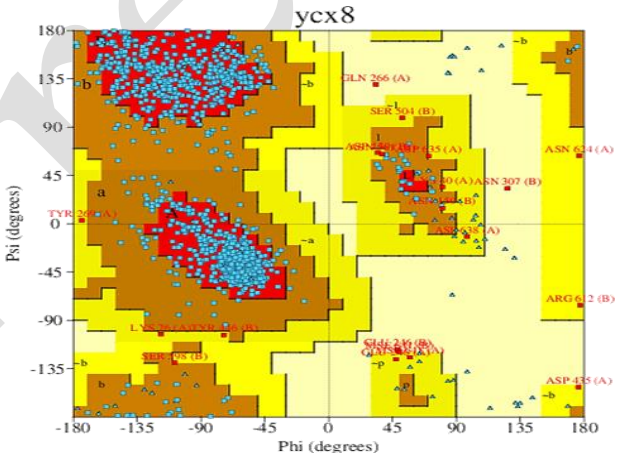


Fig. 8: Ramachandran plot of PBP2a.

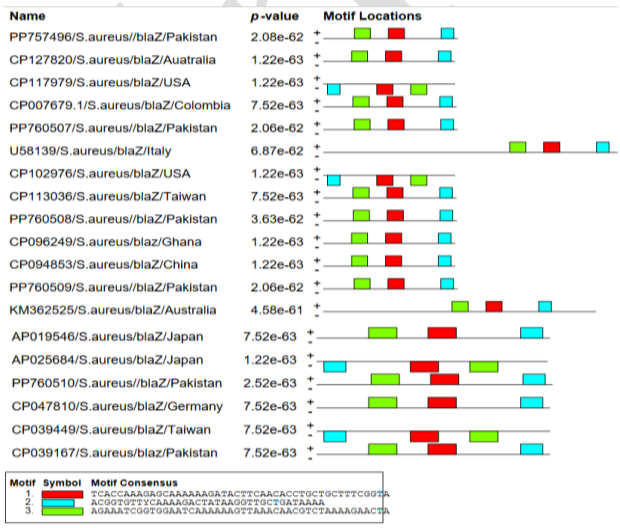


Fig. 9: Conserved motif analysis of *blaZ* gene of *S. aureus.*

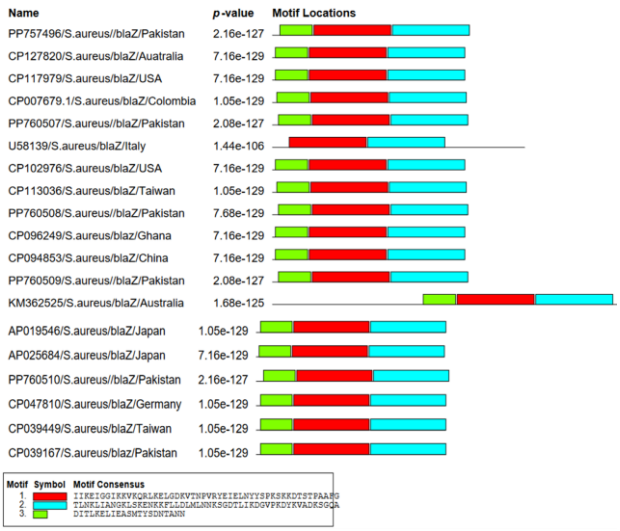


Fig. 10: Conserved motif analysis of β -lactamase protein.

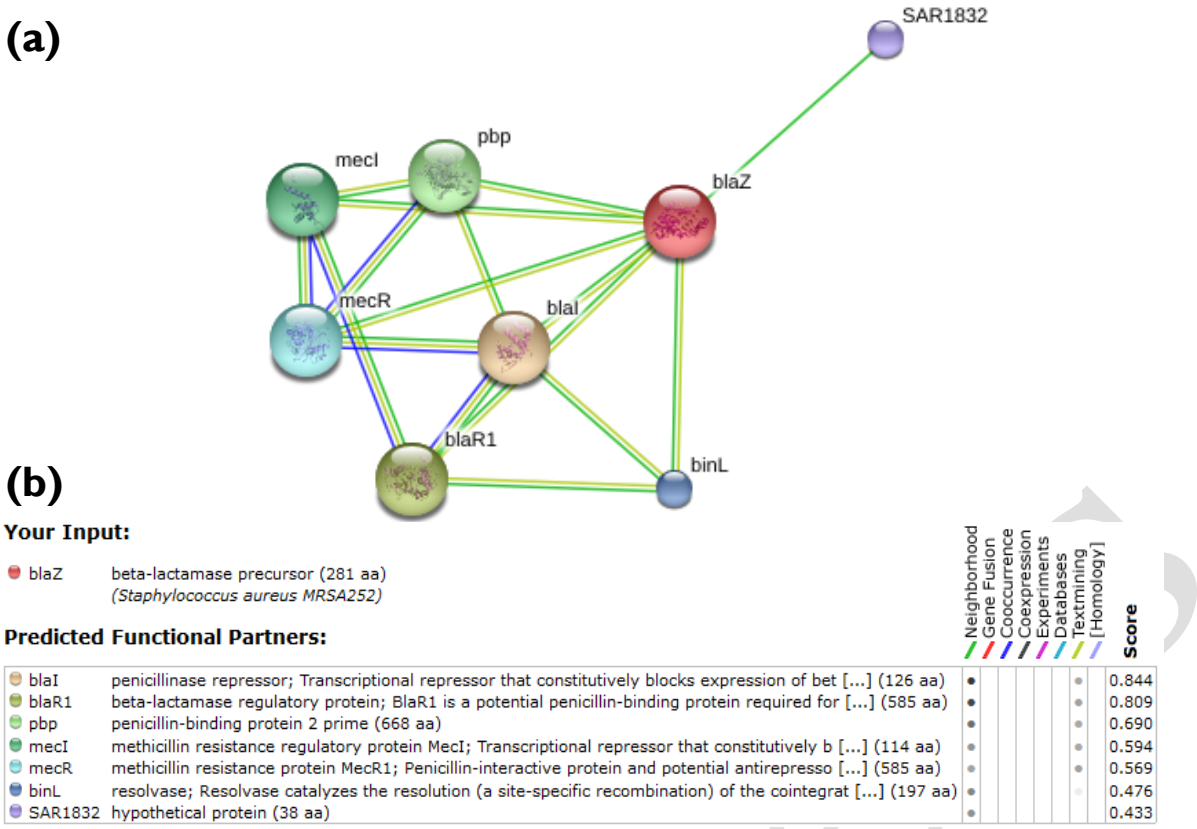


Fig. 11: (a) Protein-Protein interaction by STRING (Network view) and **(b)** Interaction of reference protein with other proteins (predicted functional partners).

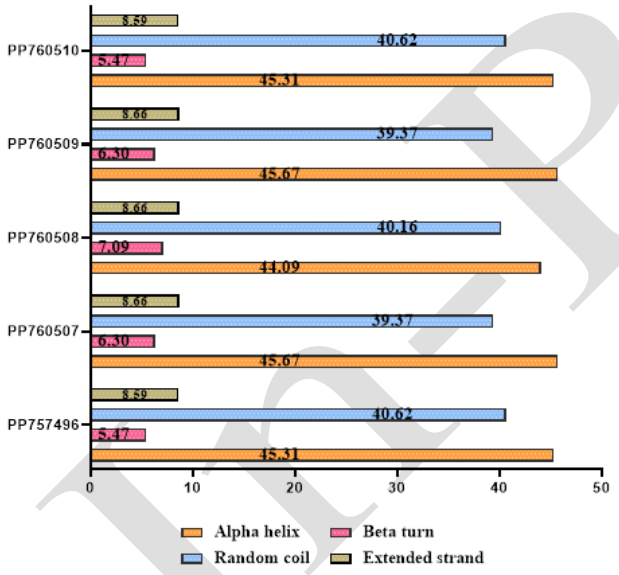


Fig. 12: Secondary structure comparison of β -lactamase from SOPMA.

Ramachandran plot of β-lactamase revealed that a high number of protein residues are in the most allowed region, which gave proof of protein authenticity. Furthermore, right-handed alpha helix was higher in number than β sheets, and mostly alpha helices and β sheets fall in most allowed regions. No protein residue found in the disallowed region, and Glycine and Proline residues are 11 and 9 in numbers respectively, as shown in (Fig. 13).

Apart from the physicochemical properties of genes, SOPMA, Swiss model, and String databases further analyzed the secondary and tertiary structure of proteins. High proportion of α-helices by SOPMA contributes to rigidity and proper positioning of binding sites, ensuring resistance activity against β-lactam antibiotics. STRING analysis confirmed the involvement of proteins in broader interaction networks regulating cell wall biosynthesis and β-lactamase expression. Moreover, the present study was designed as a preliminary *in silico* characterization of the protein.

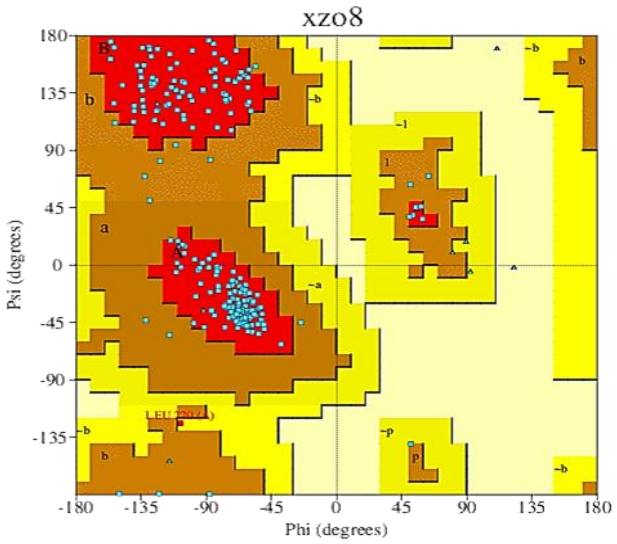


Fig. 13: Ramachandran plot of β-lactamase protein.

Antibiogram Profiling of MRSA and BRSA isolates: The results of the antibiotic susceptibility testing demonstrated that genotypic MRSA (*mecA*-positive)

isolates showed complete resistance (100%) against penicillin, followed by cefoxitin (88.23%), and the least resistance (11.76%) was observed against moxifloxacin. Overall resistance pattern for genotypic MRSA isolates was penicillin > cefoxitin > amoxicillin > ceftriaxone sodium + gentamicin > doxycycline > ciprofloxacin + cephalexin (Fig. 14a). These findings indicate that commonly used β-lactams, which are often considered first-line options in veterinary practice, are largely ineffective against MRSA strains in dogs.

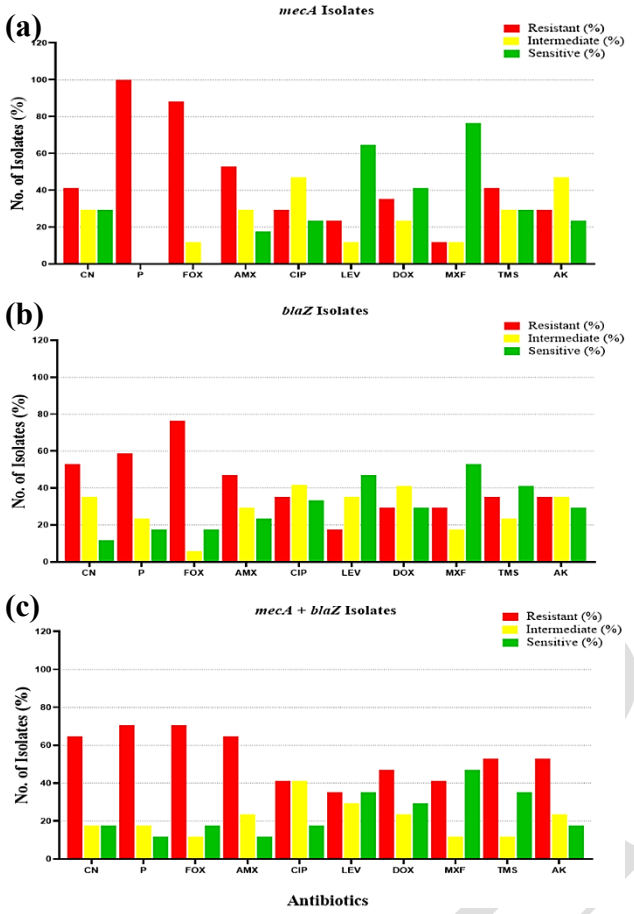


Fig. 14: Antibiogram profiling of *S. aureus* isolates positive for (a) *mecA* gene (b) *blaZ* gene (c) *mecA* + *blaZ* gene

In *blaZ*-positive isolates, the highest resistance was observed to cefoxitin (76.47%), followed by penicillin (58.82%) and gentamicin (52.94%). The overall resistance pattern exhibited by genotypic BRSA isolates was cefoxitin > penicillin > gentamicin > amoxicillin > ceftriaxone sodium + cephalexin + ciprofloxacin > doxycycline + moxifloxacin (Fig. 14b). This suggests that even β-lactamase–mediated resistant strains are showing reduced susceptibility to both aminoglycosides and cephalosporins, limiting their therapeutic value.

In case of *S. aureus* samples containing both *mecA* and *blaZ* genes, the most significant resistance was against cefoxitin and penicillin, followed by gentamicin (64.1%), while resistance (52.94%) was observed against ceftriaxone and cephalexin. The overall resistance pattern recorded was penicillin + cefoxitin > gentamicin + amoxicillin > ceftriaxone sodium + cephalexin > doxycycline > ciprofloxacin + moxifloxacin (Fig. 14c). The co-occurrence of both resistance determinants indicates that multidrug resistance is a significant challenge in canine respiratory infections, leaving only a limited number of antimicrobials, such as doxycycline and fluoroquinolones, with partial activity.

DISCUSSION

S. aureus commonly exists as a normal commensal organism in both humans and animals, frequently colonizing the skin and nasal passages of healthy individuals; nevertheless, it has the potential to cause serious infections. The prevalence of *S. aureus* in canines with respiratory tract infections and the emergence of antibiotic-resistant strains is a significant public health concern.

The study reported a 38.02% prevalence of *S. aureus* in dogs having respiratory tract infections, with notable variation across different breeds and housing conditions. Research conducted in Australia by Ma *et al.* (2020) reported *S. aureus* prevalence of 67.3% in dogs, which was found higher than the current study, although similar environmental conditions were maintained in both countries. Whereas a lower prevalence of *S. aureus* was reported by Boost *et al*.,f (2008) and Morrissey *et al.* (2016), who reported 8.8% and 2.8% prevalence of *S. aureus* in dogs. Likewise, zero prevalence of *S. aureus* in dogs was reported by Grönthal *et al.* (2015). This low prevalence can be due to stark variance in the environmental conditions and housing factors. Furthermore, the high prevalence of *S. aureus* in certain breeds such as Rottweilers and mixed breeds aligns with previous findings suggesting breed-specific susceptibilities. A very low MRSA prevalence was found in Nigeria conducted by Yakubu *et al.* (2022) who reported MRSA in 18% of pet animals as compared to stray animals that was recorded 12%. The variability across studies may be explained by differences in animal management, antibiotic use practices, and diagnostic approaches. Importantly, the relatively high frequency of MRSA in our samples underscores the growing role of companion animals as reservoirs of multidrug-resistant bacteria in Pakistan. The strong association with certain breeds and housing systems suggests that host-related susceptibility and management factors could influence colonization and infection dynamics.

The correlation of the number of risk variables, like human contact, with the incidence of *S. aureus* respiratory tract issues is supported by (Chomel, 2014; Stull *et al.*, 2013), who identified management factors like kissing, sleeping, and being licked as key determinants in the spread of zoonotic infections. Previous history of antibiotics found no significant association with *S. aureus*-associated respiratory infection according to Boost, *et al.,* (2008), and these findings were in contrast with the current study. The role of wound history and human contact, as noted in our study, validates findings by Eiff *et al.* (2001), who identified nasal passages as common sites for *S. aureus* colonization and subsequent infection. A study by Udell and Wynne (2008) highlighted the cognitive abilities of dogs and their close interaction with humans, which can contribute to the transmission of resistant strains within them. This is particularly relevant to the one health approach, where human contact is strongly linked with the

outbreak of *S. aureus*. The close bonds between pets and their owners, while beneficial for emotional support, can unknowingly facilitate the transmission of resistant pathogens. Also, interspecies transmission of multidrug-resilient bacteria and MRSA between humans and companion animals was documented by Morris *et al.* (2012).

MRSA and β-lactam-resistant *S. aureus* are a major global concern due to its multidrug resistance and are the major source of infection in domestic and farm animals. MRSA arises when methicillin-susceptible *S. aureus* (MSSA) acquires the methicillin resistance gene, *mecA*, through a mobile genetic element known as staphylococcal cassette chromosome *mec* (SCC*mec*), while β-lactam resistance is primarily regulated by BlaR13, a receptor that detects β-lactams by the acylation of its sensor domain. This process triggers transmembrane signaling and activates the metalloprotease domain on the cytoplasmic side. It is believed that SCC*mec* can be transmitted between different staphylococcal species (Alexander *et al.*, 2023; Tsubakishita *et al.*, 2010). In *S. aureus*, resistance to methicillin is primarily due to the *mecA* gene, which encodes a penicillin-binding protein 2a (PBP2a) with low avidity for β-lactams. A new protein similar to PBP2a, encoded by the *mecC* gene, has been identified in the literature. This protein has only 70% similarity to the PBP2a produced by *mecA* (Alexander *et al.*, 2023). The molecular characterization of the *mecA* and *blaZ* genes in *S. aureus* isolates provided insights into the genetic diversity and evolutionary potential of these resistant strains. The results of the phylogenetic study showed significant homology between research isolates and those reported in neighboring regions, suggesting potential regional transmission routes. This genetic similarity emphasizes the interconnected nature of AMR across borders and the importance of a One Health approach in tackling this issue. The molecular characterization of the *mecA* and *blaZ* genes in our isolates showed homology with strains from India and Pakistan, indicating spread within regions and societies. Similar findings have been reported by Anwaar *et al.* (2023). Comparative studies, such as those by Galdiero *et al.* (2003) have demonstrated the widespread distribution of these resistance genes.

Despite notable nucleic acid sequence variations, protein sequence alignments of *mecA* and *blaZ* showed no significant differences, indicating that protein structures and functions remain largely conserved (Liu *et al.*, 2013). This may suggest that nucleotide changes did not greatly affect protein functionality or that evolutionary pressures had preserved protein integrity. More investigation is required to examine the effects of these differences on protein function and to detect any subtle biochemical effects. *In silico* analysis supported that *blaZ* is frequently plasmid-associated and regulated by adjacent *blaR1/blaI* elements, while *mecA* is typically carried within the SCCmec cassette, a mobile genetic element responsible for interspecies transfer of methicillin resistance (Kayowa *et al*., 2022). The Ramachandran plot confirms the protein's authenticity, with most residues in allowed regions, indicating favorable backbone angles. PBP2a has more right-handed alpha helices than β sheets compared with *blaZ*, suggesting structural stability, though 0.4% of residues are in disallowed regions, possibly indicating local strain (Mohamed *et al.*, 2019). Overall, these results validate PBP2a's structural integrity and stability. This structural stability, *mecA* and *blaZ* interacts with cell wall synthesis proteins according to String tool linked to the high phenotypic resistance against methicillin and penicillin. Moreover, high resistance demonstrated by antibiogram against penicillin highlighted the clinical data supported the antimicrobial resistance pattern.

In canine medicine within the studied region, antibiotics are frequently prescribed empirically for skin, respiratory, gastrointestinal, and urinary tract infections. The most commonly used drugs include β-lactams (such as amoxicillin–clavulanic acid), cephalosporins, fluoroquinolones, aminoglycosides, and tetracyclines. The antibiotic resistance patterns observed in our study, with MRSA showing 100% resistance to penicillin and 88.23% to cefoxitin, reflect similar trends reported globally, such as reported by (Anwaar *et al.*, 2023; Bakht *et al.*, 2024; Sabir *et al.*, 2024) indicating a significant public health crisis due to limited treatment options. These high resistance rates to commonly used antibiotics, such as penicillin and cefoxitin, highlight the critical need for judicious use of antimicrobials in veterinary practice to mitigate the spread of resistant strains. These findings of genotypic carriage of resistance-encoding genes and expression of resistance are also coherent with the findings of Lee *et al*., (2003) and who has previously documented the extensive genetic adaptability of bacteria like *S. aureus*, leading to resistance against multiple drug classes, including penicillin, aminoglycosides, and fluoroquinolones. These classes often overlap with those in which resistance was observed in our isolates, suggesting that frequent and sometimes inappropriate use in routine practice may be driving the selection of resistant strains. In addition, over-the-counter availability of certain antibiotics and incomplete treatment courses further aggravate this problem. Such practices highlight the urgent need for antimicrobial stewardship in companion animals to preserve therapeutic efficacy and reduce the risk of resistant bacteria being transferred from dogs to humans.

The current study highlighted a significant prevalence of *S. aureus* in dogs suffering from respiratory tract infections, with a noted presence in domestic, kennel-reared, and shelter-reared dogs. However, the single geographical location and convenience sampling, and lack of human sampling to check the AMR at one health perspective are main limitations of the study. From a One Health perspective, the close association of dogs with human and environment underscores the need for integrated surveillance and judicious antimicrobial use across sectors. Incorporating household pets into antimicrobial resistance monitoring programs and promoting collaborative efforts between veterinarians, physicians, and public health professionals will be essential to limit the spread of these pathogens.

Conclusions: This study reported the high prevalence of *S. aureus* and antibiotic-resilient strains of MRSA and BRSA associated with respiratory tract infections. Several risk variables were also revealed a significant link with the *S. aureus* association. In this investigation, a specific degree of breed-associated risk was also noted. The phylogenetic assessment revealed the homology of research isolates with

other isolates from Pakistan and adjacent countries, while antibiotic resistance pattern evaluation revealed that commonly used antibiotics subjected to high levels of resistance by MRSA and BRSA isolates.

Credit authorship contribution statement: Faraz Nasir: Writing – original draft. Muhammad Ijaz: Conceptualization. Arslan Ahmed: Data curation. Muhammad Muddassir Ali: Writing – review & editing.

Declaration of competing interest: The authors declared no conflicts of interest.

Statement on animal for research: All experiments on animals in this study were undertaken in compliance with the institutional guidelines of ethical review committee University of Veterinary and Animal Sciences Lahore, Pakistan. Both male and female animals were included in this study.

Acknowledgment: Thanks to the medicine research laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan for providing instruments.

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