Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
32 (3): 343-353, 2026
DOI: 10.9775/kvfd.2026.36030

RESEARCH ARTICLE

Molecular Evidence, Phylogenetic Evaluation, and Antibiogram Profiling of Methicillin-Resistant *Staphylococcus aureus* Isolated from Respiratory Tract Infections of Cats in Pakistan

Ali Abdullah JABIR [1], Muhammad IJAZ [1 (*)], Arslan AHMED [1], Muhammad Umar JAVED [1], Hamza RASHEED [1], Maria BATOOL [1], Ashraf ALI [1], Kehkshan SHAHID [1], Gulbaz ANWAR [1], Mahpara SAFDAR [1]

[1] Department of Veterinary Medicine, University of Veterinary and Animal Sciences, 54000, Lahore - PAKISTAN

(*) **Corresponding author:**
Muhammad Ijaz
Phone: +92 3336255049
E-mail: mijaz@uvas.edu.pk

How to cite this article?

Jabir AA, Ijaz M, Ahmed A, Javed MU, Rasheed H, Batool M, Ali A, Shahid K, Anwar G, Safdar M: Molecular Evidence, Phylogenetic Evaluation, and Antibiogram Profiling of Methicillin-Resistant *Staphylococcus aureus* Isolated from Respiratory Tract Infections of Cats in Pakistan. *Kafkas Univ Vet Fak Derg*, 32 (3): 343-353, 2026.
DOI: 10.9775/kvfd.2026.36030

Article ID: KVFD-2026-36030
Received: 01.01.2026
Accepted: 23.04.2026
Published Online: 29.04.2026

Abstract

Antimicrobial-resistant *Staphylococcus aureus* particularly methicillin-resistant *Staphylococcus aureus* (MRSA) is a worldwide challenge for both veterinary and human health. This study investigated MRSA in respiratory tract infections of cats, associated risk factors, antibiogram profiling and synergy testing for resistance modulation. A total of 103 nasal swabs collected from cats were processed to identify and confirm *S. aureus* and MRSA using microbiological, biochemical, and molecular tests. Furthermore, antibiogram profiling and in-vitro trials for resistance modulation against MRSA were also conducted. The molecular prevalence of *S. aureus* in respiratory tract infections in cats was 37.86%. Among infected cats, phenotypic and genotypic prevalence of MRSA was 64.10% and 38.46%, respectively. Antibiotic susceptibility trials against MRSA revealed highest resistance against Ceftriaxone, Cefixime, and Penicillin G (100%). Resistance modulation trials revealed synergism between Ceftriaxone with Ketoprofen against MRSA. Risk factors analysis revealed significant association including respiratory illness, housing hygiene, human contact, and diagnostic resources. Phylogenetic analysis of *mecA* sequences revealed four study isolates (PP768062, PP852380, PP848319, PP848320) showing significant similarity with each other and with mecA sequences already reported from Myanmar and Pakistan, while one isolate (PP848321) showed significant similarity with *mecA* from Nigeria, India, Pakistan, and Iraq. The study provided useful insights into resistance profiling and resistance modulation to tackle challenge of antimicrobial resistance in *S. aureus*.

Keywords: Antimicrobial resistance, Molecular characterization, MRSA, Phylogenetic analysis, *Staphylococcus aureus*

INTRODUCTION

Antimicrobial resistance (AMR) is among the top ten global health risks to humans and animals [1]. Antimicrobial-resistant organisms can easily transmit between the environment, humans, and animals, especially when inadequate infection control practices are coupled with a sharp rise in emerging bacterial resistance [2]. Due to genetic flexibility, bacteria can adapt themselves to different environmental extremes, such as antibiotics that can endanger their survival, ultimately leading to the emerging and prevailing issue of AMR [3]. Companion animals like cats and dogs, which remain in close vicinity of human beings, may act as a potential source of transmission of these antimicrobial-resistant pathogens to human beings, leading to severe health consequences [4-6].

Among antibiotic-resistant bacteria, *Staphylococcus aureus* is a notorious pathogen due to its multidrug-resistant clones[7] and its resistance to the majority of currently prescribed antibiotics [8]. *S. aureus* is a gram-positive bacterium that has been incriminated in several potentially fatal illnesses in humans and animals, including pets [9]. Community and hospital-acquired *S. aureus* infections put a significant burden in the form of healthcare costs, morbidity, and mortality [10]. *S. aureus* is a common nasal microflora present in both immune-compromised and healthy pet animals, including cats and dogs [11]. The habitat of *S. aureus* in pets is the anterior nares [5], and being an opportunistic pathogen, it can worsen the respiratory tract infections of cats. Moreover, *S. aureus* has the potential to spread from companion animals to humans, therefore spreading antimicrobial resistance to other species [12].

S. aureus sheds from mucous membranes and skin of colonized animals, contaminating the environment and eventually serving as a cause of many diseases [13].

S. aureus can develop multidrug resistance (MDR), which helps bacteria evade antimicrobial effects along with the host defense system [6,14,15]. Among antimicrobial-resistant strains of *S. aureus*, methicillin-resistant *S. aureus* (MRSA) is of high significance among veterinary and human medicine. The *mecA* gene is considered responsible for methicillin resistance, which encodes the production of the PBP2a protein. PBP2a protein has a lower affinity for beta-lactam group of antibiotics (penicillin, cephalosporins, carbapenems), which makes *S. aureus* resistant to these commonly used antimicrobials [16].

Due to potential household interspecies transmission of *S. aureus* and MRSA, it is pertinent to study the nasal carriage of these pathogens in cats. The MRSA has been documented in cattle [8], equines [13,17], buffalo [6,18], goats [4], and sheep [19] in Pakistan, but comprehensive research on MRSA in respiratory tract infections of cats to assess the likelihood of interspecies transmission is lacking in Pakistan. This study provides molecular evidence of MRSA from respiratory tract infections of cats, especially focusing on sequencing and phylogenetic analysis. The main purpose of this study was to investigate the prevalence of *S. aureus* and specifically MRSA prevalence in client-owned and cattery cats, along with the analysis of possible associated risk factors. Moreover, antibiotic susceptibility profiling of study isolates, along with possible resistance modulation strategies, were also evaluated in this study.

Material and Methods

Ethical Approval

The study was approved by the Advanced Studies and Research Board (ASRB), University of Veterinary and Animal Sciences, Lahore-Pakistan vide letter no. DAS/498; Dated 15.04.2024.

Study Design and Population

The sampling was done from client-owned cats brought to the veterinary clinics and cats reared in catteries based in the district Lahore, Punjab, Pakistan *(Fig. 1)*. A total of 103 samples (n=103) consisting of client-owned cats (n=36) and cattery cats (n=67) were collected using convenient sampling technique from January 2023 to December 2023 [4]. Animals with fever, anorexia, and respiratory signs such as coughing, nasal discharge, and dyspnea were considered for sampling.

Sample Collection and Risk Factors Analysis

Nasal swabs were collected from client-owned cats and catteries following the guidelines of Shoaib et al.[20]. A sterile swab dipped in Phosphate Buffered Saline (PBS) was used for sampling. A swab was inserted into the cat's nostrils and withdrawn after proper contact with the mucosa. Maintaining sterility, nasal swabs dipped in normal saline were transferred to the Medicine Research Laboratory, Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore, Pakistan. A predesigned questionnaire was also filled out while sampling, which included information regarding possible risk factors that can be connected with *S. aureus* and MRSA-associated respiratory tract infection of cats. Major risk factors include species, sex, age, housing type, respiratory signs, general physical condition, presence of other animal species, stocking density, antibiotic usage, outdoor access, housing hygiene, human contact, and season.

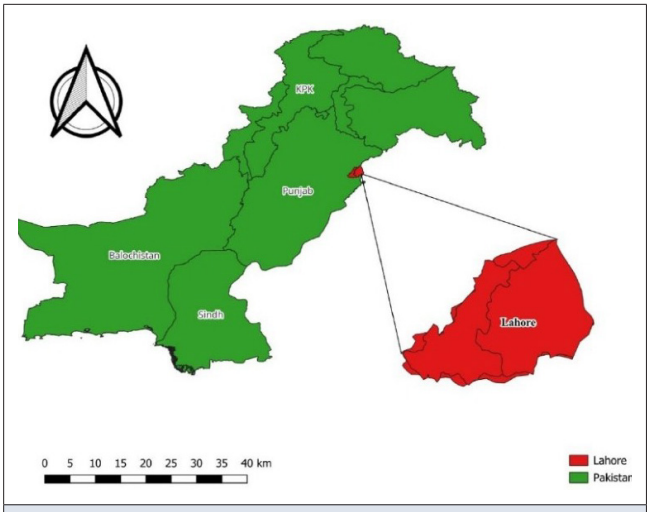

Fig 1. QGIS Map highlighting study area in red color

Isolation and Confirmation of *S. aureus*

First of all, the nasal swab samples were processed for enrichment by placing them in Brain Heart Infusion broth containing dextrose 2 g/L, disodium phosphate 2.5 g/L, proteose peptone 10 g/L, and NaCl 5 g/L, accompanied by an incubation at 37°C for 24 h. After enrichment, swabbing was performed on blood agar for hemolysis pattern and then culturing on selective media, MSA for *S. aureus* [13]. *S. aureus* isolates were identified based on characteristic features of colonies, fermentation of mannitol sugar present in MSA, purple color on gram staining, and positive results on coagulase and catalase tests [6,21]. For genotypic detection of *S. aureus*, DNA was isolated from phenotypically positive *S. aureus* isolates using the DNA extraction kit (Thermoscientific Gene JET Genomic DNA purification kit K0721) method [6]. The DNA samples were then stored at -20°C for subsequent analysis [18]. DNA quantification was performed by the nano-drop assay. After quantification, isolates were genotypically confirmed by PCR targeting the presence or absence of the *nuc* gene. The 270 base pair *nuc* gene

fragment was amplified using the amplification conditions and primers (**P1:** GCGATTGATGGTGATACGGTT, **P2:** AGCCAAGCCTTGACGAACTAAAGC) described by Louie et al.[22] *(Fig. 2)*.

Phenotypic Identification of Methicillin-Resistant *S. aureus*

Methicillin-resistant *S. aureus* was phenotypically identified by the *Kirby-Bauer disc diffusion method using Cefoxitin discs.* A cefoxitin disc was placed on *S. aureus* growth on MHA plates. Agar plates were then incubated at 37°C for 24 h. After incubation, isolates were classified as methicillin sensitive or methicillin resistant based on zones of inhibition measurements as per the guidelines of [23]. Isolates that showed zones of ≥22 mm were considered as methicillin-sensitive isolates, while isolates with inhibition zones of ≤21 mm were considered as methicillin-resistant isolates.

Genotypic Confirmation of Methicillin-Resistant *S. aureus*

MRSA was genotypically identified by PCR targeting the *mecA* gene. Primers used for PCR were documented by [24]. The primers were P1: 5′-TGGCATTCGTGTCACAATCG-3′, P2: 5′- CTGGAACTTGTTGAGCAGAG-3′ with a product size of 310 bp. The conditions of PCR are listed as: initial DNA denaturation at 94°C for 5 min, accompanied by a second step in which 34 cycles of final denaturation at 94°C for 1 min, annealing at 54°C for 1 min, initial extension at 72°C for 1 min, and then final extension at 72°C for 10 min [21]. The amplified PCR product was analyzed using ethidium bromide dye on a 1.5% agarose gel and observed via ultraviolet light [25,26] *(Fig. 3)*.

Sequencing and Phylogenetic Analysis of the Staphylococcal *mecA* Gene

The product obtained after gel electrophoresis was purified using a gel extraction kit (Thermoscientific GeneJET). Purified gel was forwarded for sequencing to the renowned laboratory (Address: 1st BASE, JTC MedTech Hub, Tukang Singapore 618,305).

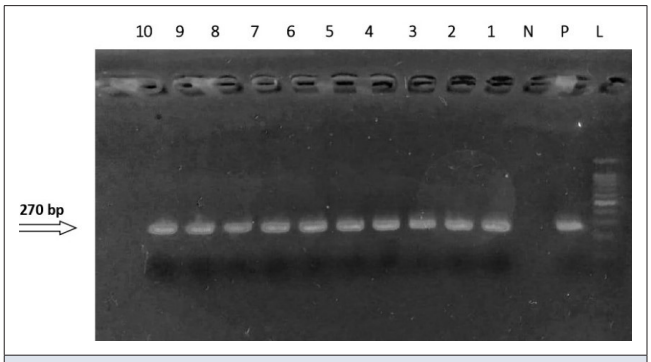

Fig 2. Amplified PCR product of nuc gene illuminated under UV-illuminator. L = Ladder, P = Positive control, N = Negative control, 1-10 = Sample 1 to 10

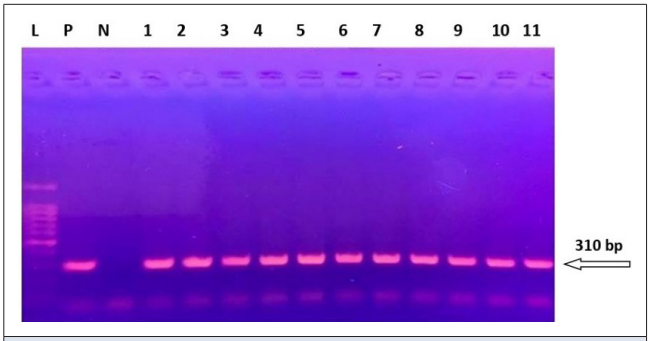

Fig 3. Amplified PCR product of mecA gene illuminated under UV-illuminator. L = Ladder, P = Positive control, N = Negative control, 1-11 = Sample 1 to 11

For phylogenetic analysis of sequenced isolates, sequences of the *mecA* gene reported by different countries were obtained from NCBI. Using specialized tools, including BLAST and the ClustalW method of MEGA-X (version 11.0.13), nucleotide sequences were compared and aligned. After sequence alignment, MEGA-X (version 11.0.13) was used to construct a phylogenetic tree with Maximum Likelihood method at 1000 bootstrap values [13].

In vitro Antibiotic Susceptibility Trials

First of all, the susceptibility of phenotypically and molecularly confirmed *mecA*-positive isolates was checked against commonly used antibiotics using the disc diffusion test and well diffusion assay. After this, susceptibility against NSAIDs (Ketoprofen and Meloxicam) alone was checked using the well diffusion assay. Finally, susceptibility against antibiotics that were highly resistant in combination with NSAIDS was assessed via the well diffusion assay to look for resistance modulation.

Susceptibility of MRSA Isolates Against Antibiotics

Antibiotic discs were purchased in order to carry out disc diffusion trials from an industry named Bioanalyse. The antibiotics include Penicillin G (10 µg), Ampicillin (10 µg), Amoxicillin (25 µg), Cephalexin (30 µg), Ceftriaxone (30 µg), Cefixime (5 µg), Vancomycin (30 µg), Cefoxitin (30 µg), Tylosin (30 µg), Linezolid (30 µg), Levofloxacin (5 µg), Oxytetracycline (30 µg), Doxycycline (30 µg), as well as Ciprofloxacin (5 µg). Muller Hinton agar was swabbed with bacterial growth (1×10^8 CFU/mL), and then antibiotic discs were placed aseptically on it. Plates were incubated at 37°C for 24 h. After 24 h, the inhibition zones around the discs were measured. These zones were compared with standard zones according to Clinical and Laboratory Standards Institute [23] to classify *S. aureus* isolates as resistant, intermediate, and sensitive to various antibiotics [27-29].

Well Diffusion Assay for Non-Antibiotics and Resistant Antibiotics

The well diffusion assay was used for the evaluation of the effectiveness of highly resistant antibiotics and NSAIDs

alone as well as in combinations against MRSA isolates. For this purpose, bacterial culture (1× 10^8 CFU/mL of activated staphylococcal growth) was evenly spread across the surface of pre-seeded MHA plates, accompanied by the well formation of 6- 8 mm with the help of a sterile cork-borer [30]. Antibiotics (Sigma Aldrich®) and NSAIDs (Nexgen®) powders were made in dilutions of specific concentrations according to guidelines of [31] and poured alone and in combination, into wells according to desired volumes, accompanied by incubation at 37°C for 24 h following the guidelines of Ahmed et al.[6]. The well having no dilution of drug was considered a negative control. Antimicrobial drugs diffuse in agar, thus inhibiting the growth of the bacterial strain. The zones of growth inhibition of antimicrobials were assessed according to the guidelines of Aqib et al.[21]. After this, modulation factors were calculated using the formula [31]. The increase in ZOI in combination, in comparison with zones of drugs alone, was deemed as effective [32].

Data Analysis

The Thrushfield formula was used to assess the prevalence of *S. aureus* and MRSA [33]. To analyze the association of different risk factors with staphylococcal respiratory infections, the Chi-square test and Logistic regression analysis were used at a 5% probability using SPSS version 22 [34]. The combinations of resistant antibiotics and NSAIDs were evaluated based on the modulation factor, assessed as ZOI of antibiotics divided by ZOI of the antibiotics and NSAIDs combination, as calculated by Ahmed et al.[6]. The modulation factor <0.5 indicates synergistic interaction of resistant antibiotics and NSAIDs in inhibiting the bacterial growth.

Results

Prevalence of *S. aureus* and MRSA

As per the current study, the molecular prevalence of *S. aureus* was 37.86% in cats with a history of respiratory disease. The findings indicated that *S. aureus* prevalence was higher (44.78%) in samples taken from catteries than from client-owned cats (25%). The Cefoxitin disk diffusion test confirmed a 24.27% (25/103) prevalence of MRSA phenotypically. In contrast to that, genotypic confirmation by the PCR targeting the *mecA* gene revealed that MRSA is 14.5% (15/103) prevalent *(Table 1)*.

Analysis of Risk Factors Associated with *S. aureus* İnfection in Cats

The analysis of possible risk factors using the Chi-square test revealed that respiratory illness, age, season, housing hygiene, housing type, human contact, availability of diagnostic facilities, and presence of other livestock species show a P-value <0.05 and were regarded to have a significant association with *S. aureus* infection in cats. The comparative risk factors analysis for *S. aureus* and MRSA shows that breed is insignificantly associated with *S. aureus* as well as MRSA, even though some of the risk factors have an insignificant association with *S. aureus* but are significantly associated with MRSA and vice versa. For instance, age, season, housing type, and presence of other livestock species were significantly associated with *S. aureus* infection in cats, having an insignificant association with MRSA. In contrast to that, antibiotic usage and outdoor access were significant risk factors for MRSA infection, while these were found to have an insignificant association for *S. aureus*. However, most of the risk factors were significantly associated with both *S. aureus* and MRSA, such as housing hygiene, respiratory illness, human contact, and availability of diagnostic facilities. The significant factors were considered for in-depth analysis via the Logistic Regression model *(Table 2)*.

Regression Analysis of Significant Risk Factors

Regression analysis of significant risk factors with S. aureus: Significant risk factors for *S. aureus* and MRSA were analyzed in comparison. The analysis revealed that respiratory illness in cats contributes 2.72 times more towards *S. aureus* infection in the respiratory tract of cats in comparison to healthy cats. Likewise, cats reared in catteries were 2.43 times more prone to contract *S. aureus* in comparison to client-owned cats. Poor housing hygiene contributed 3.01 times more towards *S. aureus* infection in cats in comparison to good hygienic management. Cats with human contact turned out to be 2.55 times higher likelihood of *S. aureus* infection as a secondary pathogen during respiratory tract infection than cats without human contact. Similarly, there was 2.17 times more risk of *S. aureus* infection in cats that were reared along with other animal species, such as dogs, in comparison to cats that were reared alone. Cats in the winter season had 2.33 times more chances of

Table 1. *Phenotypic and genotypic prevalence of S. aureus and MRSA in respiratory tract infections of cats*

Source of Sample	No. of Samples	*S. aureus* Prevalence (%)		MRSA Prevalence (%)	
		Phenotypic	Genotypic	Phenotypic	Genotypic
Cattery cats	67	41	30 (44.78)	17 (25.37)	9 (13.43)
Client-owned cats	36	15	9 (25)	8 (22.22)	6 (16.66)
Total	**103**	**56 (54.37)**	**39 (37.86)**	**25 (24.27)**	**15 (14.5)**

Table 2. *Analysis of risk factors associated with MRSA infection in cats*

Variable	Variable Name	*S. aureus*			MRSA		
		Total	Positive (%)	P-value	Total	Positive	P-value
Breed	Domestic short hair	51	19 (37.25)	0.454	51	7 (13.72)	0.744
	Persian	24	10 (41.66)		24	4 (16.66)	
	Mix breed	20	9 (45.00)		20	4 (20.00)	
	Siamese	5	0 (0.00)		5	0 (0.00)	
	Himalayan	3	1 (33.33)		3	0 (0.00)	
Sex	Male	61	24 (39.34)	0.709	61	8 (13.11)	0.616
	Female	42	15 (35.71)		42	7 (16.66)	
Age	<1 year	40	23 (57.5)	**0.001***	40	9 (22.5)	0.069
	>1 year	63	16(25.39)		63	6 (9.52)	
Stocking density	Low	46	15 (32.60)	0.323	46	5 (10.87)	0.340
	High	57	24 (42.10)		57	10 (17.54)	
Season	Winter	50	24 (48.00)	**0.039***	50	9 (18.00)	0.337
	Summer	53	15 (28.30)		53	6 (11.32)	
Housing Hygiene	Poor	67	31 (46.26)	**0.016***	53	12 (22.64)	**0.017***
	Good	36	8 (22.22)		50	3 (06.00)	
Respiratory illness	Yes	62	29 (46.77)	**0.022***	62	13 (20.96)	**0.023***
	No	41	10 (24.39)		41	2 (4.87)	
Outdoor access	Yes	46	20 (43.47)	0.291	43	10 (23.25)	**0.034***
	No	57	19 (33.33)		60	5 (8.33)	
Housing type	Cattery	67	30 (44.78)	**0.048***	67	13 (19.40)	0.057
	Client owned	36	9 (25.00)		36	2 (5.55)	
Human contact	Yes	57	27 (47.36)	**0.027***	41	10 (24.39)	**0.021***
	No	46	12 (26.08)		62	5 (8.06)	
Diagnostic facilities	Yes	50	25 (50.00)	**0.014***	35	9 (25.71)	**0.021***
	No	53	14 (26.41)		68	6 (8.82)	
Other animal spp. present	Absent	54	14 (25.93)	**0.009***	43	5 (11.62)	0.475
	Present	49	25 (51.02)		60	10 (16.66)	
Antibiotic usage	Yes	54	21 (38.88)	0.822	32	8 (25.00)	**0.044***
	No	49	18 (36.73)		71	7 (9.85)	

** Statistically significant risk factor*

S. aureus infection than during the summer season. Also, occurrence of *S. aureus* was 2.78 times more in areas where advanced diagnostic facilities were available as compared to the areas where diagnostic facilities were lacking. There was 3.97 times more risk of *S. aureus* infection in younger cats (<1 year) as compared to older (>1 year) ones *(Table 3)*.

Regression analysis of significant risk factors with MRSA: The results revealed that poor housing hygiene in cats accounted for 4.58 times more risk for the MRSA-associated respiratory infections than proper hygiene management. Likewise, cats with respiratory illness were 5.173 times more at risk of MRSA infection than healthy cats. Outdoor access contributed 3.01 times more towards MRSA infection in cats in comparison to indoor cats. There was 3.67 times more risk of MRSA-associated respiratory infections in cats with frequent human contact than without human contact. Also, the occurrence of *S. aureus* was 3.57 times more in areas where advanced diagnostic facilities were available as compared to the areas where diagnostic facilities were lacking. Antibiotic usage in the past accounted for a 3.04 times higher risk for MRSA infection in cats *(Table 4)*.

Table 3. *Risk factors included in the final logistic regression model for S. aureus infection in cats*

Variable	Response	OR	95% C.I.	S.E.	P-value
Age	<1 year	3.974	1.706, 9.256	0.4321	0.001
	>1 year	Ref			
Season	Winter	2.338	1.035, 5.285	0.416	0.041
	Summer	Ref			
Housing Hygiene	Poor	3.014	1.200, 7.569	0.470	0.019
	Good	Ref			
Respiratory illness	Yes	2.724	1.141, 6.503	0.444	0.024
	No	Ref			
Housing type	Cattery	2.432	0.994, 5.953	0.457	0.052
	Client owned	Ref			
Human contact	Yes	2.550	1.102, 5.899	0.428	0.029
	No	Ref			
Diagnostic facilities	Available	2.786	1.221, 6.355	0.421	0.015
	Not available	Ref			
Other animal spp. presence	Present	2.976	1.301, 6.806	0.422	0.010
	Absent	Ref			

OR = Odds Ration, C.I.= Confidence Interval, S.E.= Standard Error, Ref = Reference

Phylogenetic Analysis of Sequenced Study Isolates

For phylogenetic analysis of study isolates, five representative sequences (Accession number: PP768062, PP852380, PP848319, PP848320, and PP848321) were selected from sequenced isolates based on highest similarity index and query cover when they were run on nucleotide BLAST of NCBI. These selected study sequences were matched against already documented sequences of the *Staphylococcal mecA* gene from different countries on GenBank of NCBI. Four out of five study isolates Accession number: PP768062, PP852380, PP848319, PP848320 depicted highest similarity with each other as well as with already documented sequences of *mecA* from Myanmar (Accession number: LC727174) and Pakistan (Accession number: ON643114), moderate similarity with *mecA* sequences reported from Kenya, South Korea, Germany, Egypt, and India (accession numbers: CP141508, CP085690, CP127021, OP651956 and MH221030) while least similarity with sequences reported from Nigeria, India, Saudia Arabia, Pakistan, and Iraq (Accession

Table 4. *Risk factors included in the final logistic regression model for MRSA infection*

Variable	Response	OR	95% C.I.	S.E.	P-value
Housing Hygiene	Poor	4.585	1.209,17.385	0.680	0.025
	Good	Ref			
Respiratory illness	Yes	5.173	1.101,24.301	0.789	0.037
	No	Ref			
Outdoor access	Yes	3.333	1.048,10.601	0.590	0.041
	No	Ref			
Human contact	Yes	3.677	1.154,11.721	0.591	0.028
	No	Ref			
Diagnostic facilities	Available	3.577	1.156,11.072	0.577	0.027
	Unavailable	Ref			
Antibiotic use age	Yes	3.048	0.997,9.318	0.570	0.051
	No	Ref			

P-value <0.05=significant results
OR = Odds Ration, C.I.= Confidence Interval, S.E.= Standard Error

number: OK040766, MK125082, KY467026, MW558050, KX870024, and MZ359763). However, the remaining one study isolate (Accession number: PP848321) has shown significant variation from remaining study isolates but it has shown highest similarity with mecA sequences from Nigeria, India, Saudia Arabia, Pakistan, and Iraq (Accession number: OK040766, MK125082, KY467026, MW558050, KX870024, and MZ359763) *(Fig. 4)*.

Antibiogram Profiling of *S. aureus* Isolates

Antibiotic susceptibility testing revealed that (100%) study isolates showed resistance against three antibiotics, including Penicillin, Cefixime, and Ceftriaxone while 75% of study isolates showed sensitivity against oxytetracycline and doxycycline. Study isolates showed the highest sensitivity (91.6%) against Trimethoprim/ Sulphamethoxazole and gentamicin. Isolates showed considerable resistance against other antibiotics such as Ampicillin (75%), Vancomycin (75%), Cephalexin (66.7%), Amoxicillin (41.6%), Tylosin (33.3%), and Ciprofloxacin (25%) *(Table 5)*.

Well Diffusion Assay for Non-Antibiotics and Resistant Antibiotics

Combination of resistant antibiotics (Ceftriaxone, Cefixime, and Penicillin) with minimum inhibitory concentrations (MIC) of NSAIDs showed a considerable increase in the zones of inhibition, as previously reported by Mansoor et al.[35]. In case of Ceftriaxone, Ketoprofen exhibited synergistic activity with Ceftriaxone, having modulation factor of 0.48. Ketoprofen also showed synergism with Cefixime as well (modulation factor = 0.44). Meloxicam also resulted in a considerable increase in ZOI when combined with Penicillin G, with a modulation factor of 0.48 (synergistic effect). The combination of the remaining two antibiotics (Ceftriaxone and Cefixime) with meloxicam and the combination of Penicillin G with Ketoprofen did not show synergism *(Table 6)*.

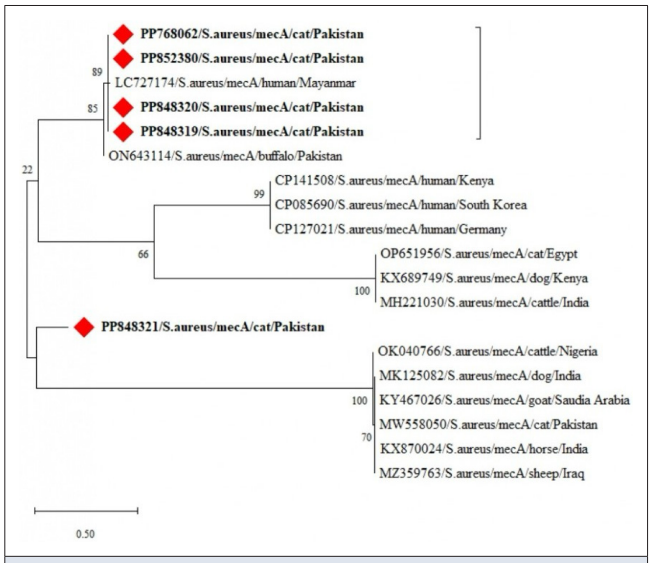

Fig 4. Phylogenetic analysis of staphlococcal mecA gene isolated from cats. The isolates with red bullets indicate study isolates

Table 5. *Antimicrobial susceptibility profiling of MRSA isolates*

Antibiotics	Abbreviations	Breakpoint Zone Diameter (mm)			*mecA* Positive Isolates (n=12)		
		R	I	S	R (%)	I (%)	S (%)
Penicillin G	P	≤28	-	≥29	12 (100)	0 (0)	0 (0)
Amoxicillin	AMX	≤16	-	≥23	5 (41.6)	5 (41.6)	2 (16.66)
Ampicillin	AM	≤28	-	≥29	9 (75)	3 (25)	0 (0)
Cephalexin	CL	≤14	15-17	≥18	8 (66.7)	2 (16.66)	2 (16.66)
Ceftriaxone	CRO	≤13	14-20	≥21	12 (100)	0 (0)	0 (0)
Cefixime	CFM	≤15	16-18	≥19	12 (100)	0 (0)	0 (0)
Gentamicin	CN	≤12	13-14	≥15	1 (8.33)	0 (0)	11 (91.6)
Tylosin	TY	≤14	15-18	≥19	4 (33.33)	3 (25)	5 (41.6)
Oxytetracycline	T	≤14	15-18	≥19	1 (8.33)	2 (16.66)	9 (75)
Doxycycline	DOX	≤12	13-15	≥16	2 (16.66)	1 (8.33)	9 (75)
Ciprofloxacin	CIP	≤15	16-20	≥21	3 (25)	6 (50)	3 (25)
Levofloxacin	LEV	≤15	16-18	≥19	1 (08.33)	2 (16.66)	9 (75)
Trimethoprim + Sulphamethoxzole	TMS	≤10	11-15	≥16	1 (08.33)	0 (0)	11 (91.6)
Vancomycin	VA	≤17	18-20	≥21	9 (75.00)	3 (25)	0 (0)

R: Resistant; I: Intermediate; S: Sensitive

Table 6. *Results of antibiotic resistance modulation by NSAIDs using the well diffusion assay*

Resistant Antibiotics	NSAIDs	ZOI for Antibiotics Alone (mm)	ZOI for NSAIDs Alone (mm)	Zone of Inhibition in Combination (mm)	Modulation Factor
Ceftriaxone	Ketoprofen	12	17	25	**12/25=0.48**
	Meloxicam	13	10	17	13/17=0.76
Cefixime	Ketoprofen	8	14	18	**8/18=0.44**
	Meloxicam	10	9	12	10/12=0.83
Penicillin	Ketoprofen	22	18	29	22/29=0.76
	Meloxicam	15	20	31	**15/31=0.48**

**ZOI = Zones of inhibition; Modulation Factor <0.5=Synergism*

Discussion

S. aureus is a commensal bacterium in animals as well as humans since it usually inhabits the respiratory tract, especially nares, in healthy animals; however, it also causes diseases that may be even fatal [36]. Cats live near human beings and can transmit pathogens of zoonotic importance [37]. This study was done to assess the prevalence of methicillin-resistant *S. aureus* both phenotypically (Disc diffusion method) and genotypically (PCR targeting the *mecA* gene) in the upper respiratory tract of cats. This study is conducted for molecular characterization, especially focusing on sequencing and phylogenetic analysis of MRSA residing within the respiratory tract of cats in Pakistan.

In this study, *S. aureus* was found to be 37.86% prevalent on the molecular basis in cats. It is about double that reported by Bierowiec et al.[11]. This higher prevalence may be due to the adoption of poor housing hygiene practices, especially in cats reared in catteries. *S. aureus* was found to be more prevalent in catteries than client-owned cats. This is possibly due to higher stocking density, poor ventilation, and hygiene conditions in catteries. Previously a research conducted by Habibullah et al.[38] in Dhaka reported a prevalence of 40.86% in pets, including dogs and cats. The prevalence reported by Lilenbaum et al.[39] in cats of Brazil is about one-fourth (9.4%) of that of the current study. A research done by Bierowiec et al.[40] to evaluate comparative prevalence of different *Staphylococcal* species from different anatomical sites in cats revealed a prevalence of 7.37% for *S. aureus* in cat nares. In a comparative study by Moon et al.[41] to evaluate the burden of different pathogens in respiratory samples of cats, *S. aureus* was not detected as a significantly prevalent species.

In the present study, methicillin-resistant *Staphylococcus aureus* (MRSA) was phenotypically detected in 24.27% of isolates using the Cefoxitin disc diffusion method. Previously, phenotypic prevalence of MRSA was reported to be 26.6% in Philadelphia [42], which is comparable to the results of the current study. MRSA prevalence carriage was reported to be 0.46% and 2.16% in apparently healthy and diseased cats, respectively, in Greater London [43], which is quite less than the current study. The possible reason behind this difference may be poor housing hygiene and irrational use of antimicrobial agents in Pakistan. The MRSA prevalence reported by Habibullah et al.[38] in Dhaka aligns with the current findings, which may be attributed to the shared geographical region. Previously, Shoaib et al.[20] reported a 30.43% phenotypic prevalence of MRSA in cats in Pakistan using the Oxacillin disc diffusion method, which is marginally higher than the prevalence observed in the present study.

Genotypically MRSA was 14.5% prevalent in this study. Previously, genotypic prevalence in Pakistan was reported at 30.34% in cats based on PCR targeting the *mecA* gene [20], which is about double that of the current results. Moreover, the phenotypic and genotypic prevalence of MRSA were similar in that study, in contrast to our study results, where phenotypic (24.27%) and genotypic (14.5%) prevalence had a significant difference. This difference may be due to a discrepancy between phenotypic and genotypic methods to detect MRSA. This discrepancy indicates that Cefoxitin is not an effective alternative for identifying the *mecA* gene [13]. In a study conducted in Libya, the PCR-based confirmation for *mecA* revealed the prevalence of 13% in cats [44] , which is very close to our study results. In another study conducted in Dhaka, MRSA was confirmed by PCR in 5.88% of cats [38],which is about one-third of our study results. This difference may be due to poor hygienic measures, environmental changes, excessive human contact, and irrational antimicrobial use age in Pakistan.

Antibiogram profile of current study isolates showed that isolates were highly resistant against Penicillin G, Ceftriaxone, and Cefixime. This resistance may be due to the excessive antibiotics in some countries [45] and different genetic mutations that the microorganisms have acquired over time [46]. This study showed that the susceptibility results slightly contradict the findings of Shoaib et al.[20]. In the current study, 25% of isolates were resistant to ciprofloxacin, in contrast to the Shoaib et al.[20] where all

the isolates (100%) were sensitive to ciprofloxacin. Resistance against vancomycin was also higher (75%) in comparison to the results of Shoaib et al.[20]. Susceptibility findings of the current study isolates were similar to results reported by Anwaar et al.[17] in case of Tylosin, while sensitivity against oxytetracycline was higher (75%) in the current study. When the study results were compared to those reported by Rasheed et al.[14], it revealed that results vary significantly in case of Trimethoprim/ Sulphamethoxazole while results are almost similar in case of Amoxicillin.

Phylogenetic analysis of the *mecA* revealed substantial similarity within study isolates and the isolates from various other countries (Myanmar, India, Saudi Arabia, Iraq, Nigeria, Egypt, Germany, South Korea, and Kenya). The analogy between local isolates and isolates from different countries indicated that there is dissemination of MRSA between countries, possibly due to trade of animals, especially import of pet animals, and transboundary mobility of human beings, leading to worldwide presence of these notorious pathogens [17] .

Resistance modulation trials using different combinations of NSAIDs and antibiotics showed synergistic effects. In a study, Bakht et al.[47] found that NSAIDs increased the zones of inhibition of resistant antibiotics when antibiotics were administered in combination with various NSAIDs to combat MRSA. As some NSAIDs also have antimicrobial activities along with their antipyretic, analgesic, and anti-inflammatory activities [48]. So, this approach can be used to resensitize MRSA against commonly used antibiotics. Synergistic activity of antibiotics in combination with NSAIDs against MRSA has also been well established by Aqib et al.[31] Altaf et al.[49], and El-Deeb et al.[50], and which can be because of bacteria targeting various sites [49].

Furthermore, this study revealed a significant association of different risk factors that is season, age, respiratory illness, housing hygiene, housing type, human contact, availability of diagnostic facilities, and presence of other animal species, with *S. aureus* infection in the respiratory tract of cats. The findings of various risk factor associations turned out to be similar to the findings as per Bierowiec et al.[11] and Shoaib et al.[20]. For instance, the presence of other livestock species, human contact, and availability of diagnostic facilities were significant risk factors in both of these studies and the current study as well. Some of the risk factors were very peculiar as they were reported novel in this study, such as housing hygiene and respiratory illness. The association of housing hygiene with *S. aureus* infection in cats may be due to poor hygienic measures [51] and likewise association of respiratory illness with *S. aureus* infection may be due to immunosuppression due to viral infections that leads to *S. aureus* infection as a secondary pathogen [52]. Housing type was also found to be significantly associated with *S. aureus*. This is because of close contact of cats with each other, especially in catteries [17]. Comparative risk factors analysis for *S. aureus* and MRSA exhibited that frequent use of antibiotics is significantly associated with MRSA, but it is an insignificant risk factor for *S. aureus* infection in cats. This significant association of antibiotic usage may be due to resistance acquired by the bacterium, which helps it to evade the action of antibiotics and helps it to colonize the respiratory tract. In contrast to that, season turned out to be a significant risk factor associated with *S. aureus,* while it is insignificant for MRSA infection. This is possibly due to better conditions for *S. aureus* to colonize during the winter season [53].

In conclusion, this study revealed that MRSA is a significantly prevalent pathogen in cats with respiratory tract infections. However, these *S. aureus* infections can be controlled in cats by controlling various risk factors, which turned out to be associated significantly with occurrence of *S. aureus* infections according to the study, such as better management practices like appropriate hygiene, good housing, and proper treatment of respiratory illness can control MRSA infection in cats. This study demands an in-depth investigation into the transmission of MRSA from companion animals to humans and the environment from the perspective of one health. Furthermore, well diffusion assay of combinations (Resistant antibiotics + NSAIDs) revealed significant resistance modulation in MRSA. This combinational therapy (Antibiotics + NSAIDs) can be implemented as an alternative therapeutic option against antimicrobial-resistant strains of *S. aureus* in the future.

This study is subject to certain limitations. The sample collection was restricted to a limited number of veterinary clinics and geographic locations, which may not adequately represent the overall feline population in Pakistan, thereby potentially limiting the phylogenetic and antibiogram analysis. Furthermore, the molecular detection strategy relied on PCR assays targeting established resistance determinants, such as the *mecA* gene, which may preclude the identification of novel or less-characterized resistance genes and genetic variants, leading to an underestimation of the true diversity of methicillin resistance. In addition, antimicrobial susceptibility testing performed using disk diffusion may have limited sensitivity in detecting certain phenotypic expressions of resistance. It is also important to note that in vitro susceptibility profiles may not consistently translate into in vivo therapeutic efficacy, as host-related and environmental factors can significantly influence antimicrobial performance, thereby constraining the direct clinical application of the observed resistance patterns.

Declarations

Availability of Data and Material: Data and materials for this research are available upon request.

Acknowledgments: The Researchers would like to thank Medicine Research Laboratory in the Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore.

Funding Support: No funding support was available

Ethical Approval: The study was approved by the Advanced Studies and Research Board (ASRB), University of Veterinary and Animal Sciences, Lahore-Pakistan vide letter no. DAS/498; Dated 15.04.2024.

Competing Interest: The authors declare that there is no conflict of interest

Generative Artificial Intelligence: No Generative Artificial Intelligence was used in this research.

Author Contribution: MI contributed to the conceptualization, literature search, AAJ contributed to data collection, original draft preparation, AA critical revision of the manuscript. MUJ was responsible for manuscript structuring, HR contributed to data interpretation, MB contributed in figure and table preparation, AA and KS editing, GA and MS were responsible for proofreading.

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