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The antimicrobial resistance pattern of methicillin resistant *Staphylococcus aureus* from domestic livestock and their handlers in Punjab, India

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ABSTRACT

Methicillin Resistant *Staphylococcus aureus* (MRSA) strains cause a wide spectrum of infections in domesticated livestock owing to the acquisition of multiple antibiotic resistance and virulence genes, which increase their pathogenicity. The present study was aimed at determining the prevalence, antibiotic and virulence gene profile, as well as molecular characterization of MRSA isolates in Punjab, India. A total of 650 samples, including nasal swabs from sheep, goats and pigs, caprine milk samples and human hand swab samples, were collected from different districts of Punjab and processed for isolation of *S. aureus*, followed by confirmation by MALDI-TOF. The confirmed *S. aureus* isolates were subjected to PCR for detection of methicillin resistance genes and all the MRSA isolates obtained were studied for their culture sensitivity, antibiotic resistance and virulence gene profile. A total of 45 *S. aureus* isolates were obtained, out of which 15 carried the *mec*A gene and were thus confirmed as MRSA isolates. All 15 MRSA isolates exhibited 100% resistance to cefoxitin and penicillin. In all, 53.33% MRSA isolates were found to be multidrug resistant with the majority exhibiting a MAR index > 0.2, indicating significant antimicrobial usage in farms across Punjab. The *tet*K gene, which confers tetracycline resistance, was the most prevalent, followed by the *aac-aph*D gene, while the *eno* gene was found to be the predominant virulence gene. A total of five SCC*mec* types were observed, with SCC*mec* Type IVd being the most predominantly detected SCC*mec* type. The detection of MRSA in livestock highlights a zoonotic risk with serious veterinary and public health implications

Key words: AMR; MALDI-TOF; MRSA; SCC*mec* typing; virulence genes

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Introduction

Bacteria use a myriad of ways to evade the action of antibiotics, and with the emergence of multidrug resistant bacteria, our armamentarium of drugs effective against these pathogens is rapidly shrinking, which is a cause of grave concern. *Staphylococcus aureus* is one such pathogen that cause a wide array of infections in humans and animals. This bacterium exhibits remarkable adaptation to diverse environmental conditions, and can acquire resistance against multiple antibiotic classes which complicates treatment. This is exemplified by the emergence of Methicillin Resistant *S. aureus* (MRSA) which was first detected in the UK in 1960 (JEVONS, 1961), only one year after the introduction of Methicillin in human medicine.

MRSA was initially perceived as only being associated with health care settings such as hospitals, and people associated with such environments. However, the bacterium has recently emerged as a major cause of community-associated infections since their first description in the 1980s. Moreover, MRSA has also been detected in animals (DEVRIESE et al., 1972) with infections being reported in domesticated livestock, companion animals and even in wild terrestrial and aquatic species (CUNY et al., 2010; VAN DUIJKEREN et al., 2010; WEESE, 2010; GRAVELAND et al., 2011; DORADO-GARCIA et al., 2013). The first reported case of Livestock Associated-Methicillin Resistant *S. aureus* (LA-MRSA) in animals was documented in cows suffering from bovine mastitis in Belgium in 1972, and the MRSA isolate obtained was found to originate from humans (DEVRIESE and HOMMEZ, 1975). Since then, LA-MRSA strains have been detected in a variety of animals, such as cattle, buffalo, sheep, goats, and pigs, and are important cause of infections in economically important livestock species (LAKHUNDI and ZHANG, 2018). The molecular analysis of MRSA has led to the discovery of 11 staphylococcal chromosomal cassettes called the SCC*mec* types (Types I–XI) that carry *mec*A or *mec*A gene homologues. They encode for a modified penicillin-binding protein, PBP2a, which may be associated with MRSA isolates obtained from both human and animal origin (PETINAKI and SPILIOPOULOU, 2012). MRSA from animals have been shown to harbour a gamut of other antibiotic resistance genes and various virulence genes which increase the pathogenicity of these strains. Virulence genes, such as haemolysin-encoding genes, enterotoxin genes, immune evasion cluster genes and exfoliative toxin genes, have been detected in these isolates, which causes serious illness in animals (SUN et al., 2019, CHAI et al., 2021).

Considering the importance of MRSA in the etiology of human and animal diseases, the pathogen has been listed as a high priority pathogen in the Indian Pathogen Priority (IPPL) list (WHO, 2021). Moreover, the presence of virulence factors and multidrug resistance in MRSA further complicates the scenario and underscores the urgent need to develop newer antibiotics. With the serious threat of acquiring multidrug resistance, and because of zoonotic transmission of these pathogens, the timely detection of multidrug-resistant MRSA in animals is of paramount importance. Since the research on MRSA of animal origin, especially small ruminants and pigs, is still in its infancy, the present study was undertaken to determine the antibiotic resistance and virulence gene profile of the MRSA isolates from small ruminants and pigs. Moreover, the development of resistance to Vancomycin, the last resort antibiotic for multidrug resistant *S. aureus*, was also tested. Furthermore, since there is very limited information available on the prevalent SCC*mec* types in Punjab, a region in Northern India, the molecular typing of MRSA isolates was also performed.

Materials and methods

Sample collection. In the present study, a total of 650 samples, including 150 nasal swabs each from sheep, goats and pigs, 100 caprine milk samples, and 100 human hand swab samples from animal handlers on the farms, were collected from 15 different herds/flocks in 13 villages belonging to six districts of Punjab, India.

The nasal swab samples were collected from apparently healthy and diseased animals that exhibited the signs of respiratory diseases, such as nasal discharge and sneezing. A total of 397 nasal

swab samples were taken from healthy livestock, which included 120 samples from goats, 131 from sheep and 146 from pigs. Additionally, a total of 53 nasal swab samples were taken from diseased animals viz., 30 from goats, 19 from sheep and four from pigs. The nasal and hand swab samples were collected aseptically using sterile cotton swabs, and placed in a sterile test tube containing sterile 0.9% normal saline solution. In addition, milk samples were also collected from mastitic (n=51) and apparently healthy goats (n=49). The Sodium Lauryl Sulphate (SLS) test was performed on the milk samples for detection of mastitis.

In addition to sample collection, data on host-level factors (species, age, breed, and health status), and farm-level variables (management and hygiene practices) were recorded through direct observation during farm visits. These factors were documented using standardized data sheets and later analysed to assess their association with the isolation of *S. aureus* and MRSA.

Isolation of S. aureus and species confirmation by MALDI-TOF. All the samples were inoculated on an enriched medium such as Blood agar and Brain Heart Infusion agar for primary isolation, followed by incubation at 37°C for 24 hours. The colonies exhibiting double haemolysis on Blood agar were further purified on BHI agar, mannitol salt agar (MSA) and Baird parker (BP) agar, followed by standard biochemical tests including catalase, oxidase and coagulase. All the isolates presumptively identified as *S. aureus* were subjected to MALDI-TOF for confirmation using the Bruker version 4.1.1.0.0 compass MBT. The isolates were automatically identified by the software, by comparing them with the library.

Determination of Methicillin resistance in S. aureus isolates. All the *S. aureus* isolates obtained were screened for resistance to cefoxitin. The *S. aureus* isolates which exhibited phenotypic resistance to cefoxitin were presumptively categorized as MRSA.

PCR based detection of mecA, mecB and mecC genes. Bacterial DNA was extracted from all cefoxitin resistant *S. aureus* isolates by the hot cold lysis method, followed by endpoint PCR using *mec*A (DURAN et al., 2012), *mec*B (BECKER et al., 2018) and *mec* C (CUNY et al., 2011) gene specific primers.

Each reaction was prepared in a final volume of 25µl and consisted of 12.5µl of 2X GoTaq® green mastermix (Promega, USA), 1µl each of 20µM forward and reverse primers and 5µl of template DNA. The cycling conditions consisted of an initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, followed by final extension at 72°C for 7 minutes. The PCR amplicons were resolved on 1.5% agarose gels containing 0.5 ug/ml Ethidium bromide, followed by visualization using a UV transilluminator. Since the detection of the *mec*A/*mec* gene homologues by PCR is considered as the "gold standard" for the detection of methicillin-resistant strains, all isolates that tested positive for *mec*A/other *mec* genes were confirmed as MRSA isolates.

Determination of the resistance profile of MRSA isolates to other commonly used antibiotics. The resistance profile of all the MRSA isolates was determined by the disc diffusion method using a panel of 21 different antibiotics belonging to eight different classes viz.: beta-lactams (amoxicillin, amoxicillin/clavulanic acid, ampicillin, cefazolin, cefotaxime, cefoxitin, ceftriaxone, penicillin G, imipenem), aminoglycosides (amikacin, gentamicin, streptomycin), phenicols (chloramphenicol), fluoroquinolones (ciprofloxacin, ofloxacin), tetracyclines (doxycycline hydrochloride, tetracycline), sulphonamides (co-trimoxazole), macrolides (azithromycin, erythromycin) and glycopeptide (vancomycin by MIC) antibiotics.

Determination of multidrug resistance and MAR index. All the isolates exhibiting resistance to at least one antimicrobial drug in three or more antimicrobial classes were defined as multidrug resistant, i.e. MDRs. The Multiple Antibiotic Resistance (MAR) index was calculated as the ratio between the number of drugs an isolate is resistant to and the total number of antibiotics the organism is exposed to, as described by KRUMPERMAN (1983). A MAR index greater than 0.2 indicates a high risk source of contamination, where antibiotics are frequently used (DAVIS and BROWN, 2016).

Molecular detection of antibiotic resistance genes in MRSA isolates. The MRSA isolates were tested for the presence of various antibiotic resistance genes by PCR as described above using specific primers and cycling conditions as listed in Table 1.

Molecular detection of virulence genes in MRSA isolates. All the MRSA isolates were screened for the presence of various virulence genes, including *luk*-PV, *tsst*-1, *eta*, *etb*, *coa*, *hlg*, and *eno* which encode Panton-Valentine leucocidin, toxic shock syndrome toxin, epidermolytic toxins A and B, coagulase, gamma-hemolysin and enolase enzyme, respectively. The cycling conditions for detection of virulence genes consisted of an ini- tial denaturation of 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, except *coa* and *hlg* genes where an extension time of 1 min was used. The oligo- nucleotide sequence of the primers for detection of virulence genes are given in Table 2.

Molecular typing of MRSA isolates. All the MRSA isolates were subjected to Staphylococcal Cassette Chromosome Methicillin (SCC*mec*) typing using the set of primers and the standard cycling conditions, as described previously (ZHANG et al., 2005).

Statistical analysis. In the current study, data were collected on several host and farm level factors, including animal age, breed, health status, species, and management practices followed on the farms. These variables were analysed to assess their association with the isolation of *S. aureus* and MRSA. The chi-square (χ^2) test was employed to evalu- ate the statistical associations between categorical variables.

To investigate antimicrobial resistance in MRSA isolates further, both phenotypic resistance patterns determined through antimicrobial susceptibility testing, and genotypic resistance profiles identified by detecting specific resistance genes, were analysed. The Spearman correlation test was used to assess the relationship between phenotypic resistance and the presence of corresponding resistance genes, providing insight into the concordance between the observed resistance and genetic determinants.

Table 1. Details of the primers and PCR conditions used for detection of other resistance genes in MRSA isolates

GENE	OLIGONUCLEOTIDE SEQUENCE (5'-3')	PCR CYCLING CONDITIONS			AMPLICON SIZE (BP)	REFERENCE
		Denaturation	Annealing	Extension		
TETK	F: GTA GCG ACA ATA GGT AAT AGT R: GTA GTG ACA ATA AAC CTC CTA	94°C, 30 sec	55°C, 30 sec	72 °C, 30 sec	360	STROMMENGER et al., 2003
TETM	F: AGT GGA GCG ATT ACA GAA R: CAT ATG TCC TGG CGT GTC TA	94°C, 30 sec	55°C, 30 sec	72 °C, 15 sec	158	
TETL	F: CAT ATG TCC TGG GGT GTC TA R: GTC GTT GCG CGC GCT ATA TTC C	94°C, 30 sec	55°C, 30 sec	70 °C, 30 sec	696	HUYS et al., 2005
BLAZ	F: ACT TCA ACA CCT GCT GCT TTC R: TGA CCA CTT TTA TCA GCA ACC	94°C for 30 sec	55°C, 30 sec	70 °C, 15 sec	173	MARTINEAU et al., 2000
ERMA	F: TCT AAA AAG CAT GTA AAA GAA R: CTT CGA TAG TTT ATT AAT ATT AG	94°C for 30 sec	52°C, 30 sec	72°C, 1 min	610	SUTCLIFFE et al., 1996
ERMB	F: CTA TCT GAT TGT TGA AGA AGG ATT R: GTT TAC TCT TGG TTT AGG ATG AAA	94°C for 30 sec	52°C, 30 sec	72°C, 30 sec	142	MARTINEAU et al., 2001
ERMC	F: AAT CGT CAA TTC CTG CAT GT R: TAA TCG TGG AAT ACG GGT TTG	94°C for 30 sec	55°C, 30 sec	72 °C, 30 sec	299	STROMMENGER et al., 2003
AACA-APHD	F: TAA TCC AAG AGC AAT AAG GGC R: GCC ACA ATC AAA TCA TCC TC	94°C for 30 sec	55°C, 30 sec	72 °C, 30 sec	227	
VANA	F: TCA CCC CTT TAA CGC TAA TA R: CCG ACA ATC AAA TCA TCC TC	94°C for 30 sec	50°C, 30 sec	72°C, 1 min	1032	SAHA et al., 2008
VANB	F: AAG CTA TGC AAG AAG CCA TG R: CCG ACA ATC AAA TCA TCC TC	94°C for 30 sec	50°C, 30 sec	72°C, 45 sec	536	ELSAYED et al., 2001

Table 2. Details of the primers used for detection of virulence genes in MRSA isolates

GENE	OLIGONUCLEOTIDE SEQUENCE (5'-3')	AMPLICON SIZE (BP)	REFERENCE
LUK- PV	F: TTC ACT ATT TGT AAA AGT GTC AGA CCC ACT R: TAC TAA TGA ATT TTT TTA TCG TAA GCC CTT	180 bp	GOUDARZI et al., 2016
TSST-1	F: TTA TCG TAA GCC CTT TGT TG R: TAA AGG TAG TTC TAT TGG AGT AGG	398 bp	
ETA	F: GCA GGT GTT GAT TTA GCA TT R: AGA TGT CCC TAT TTT TGC TG	93 bp	MEHROTRA et al., 2000
ETB	F: ACA AGC AAA AGA ATA CAG CG R: GTT TTT GGC TGC TTC TCT TG	226 bp	
COA	F: ATA GAG ATG CTG GTA CAG G R: GCT TCC GAT TGT TCG ATG C	547 (559-875)	HOOKEY et al., 1998
HLG	F: GCC AAT CCG TTA TTA GAA AAT GC R: CCA TAG ACG TAG CAA CGG AT	937	KUMAR et al., 2009
ENO	F: ACG TGC AGC AGC TGA CT R: CAA CAG CAT TCT TCA GTA CCT TC	301	KOT et al., 2018

Results

Isolation and identification of MRSA isola- tes. Out of the total of 650 samples processed, 45 isolates exhibiting round, smooth, glistening, golden yellow colonies on Brain Heart Infusion agar (Fig. 1a); haemolytic colonies on Blood agar (Fig. 1b), yellow colonies on mannitol salt agar (Fig. 1c) and black coloured colonies with halo on Baird-Parker medium (Fig. 1d) were identified as *S. aureus*. These isolates appeared as Gram positive cocci in the characteristic bunch of grapes appearance, and were catalase positive, while the oxidase test was negative for all the 45 isolates.

All the 45 isolates presumptively identified as *S. aureus* on the basis of their colony characteristics, and the biochemical tests were also confirmed by MALDI-TOF. Out of 45 *S. aureus* isolates, 23 (51.11%) were obtained from pigs, 17 (37.78%) were obtained from goats, 4 (8.89%) were obtained from sheep, and only one (2.22%) isolate was obtained from an animal handler. Out of the 17 *S. aureus* isolates obtained from goats, 11 were obtained from milk samples and six were obtained from nasal swab samples.

Determination of Methicillin resistance in S. aureus isolates. Out of the 45 *S. aureus* isolates obtained, 25 (55.56%) were found to be resistant to cefoxitin. The overall prevalence of MRSA based on phenotypic resistance to cefoxitin was found to be 3.84% (25 of 650 isolates; 95% CI: 2.48%-5.59%).

Detection of mecA, mecB and mecC genes. Fifteen (15) of the 25 cefoxitin resistant *S. aureus* isolates exhibited an amplicon of 331bp for the *mec*A gene. The overall prevalence of *mec*A positive MRSA was estimated as 2.3% (15 of 650 isolates; 95% CI: 1.4% to 3.8%). None of the isolates harboured *mec*B or *mec*C genes.

Out of the 15 MRSA isolates obtained, eight were isolated from pigs, five were isolated from goats and two were obtained from sheep, i.e., the highest number of isolates were obtained from pigs, followed by goats and sheep. So, the species-wise prevalence of MRSA was estimated as 5.3% in pigs, 2% in goats and 1.3% in sheep. Among the five MRSA isolates obtained from goats, four were obtained from milk samples, and one was isolated

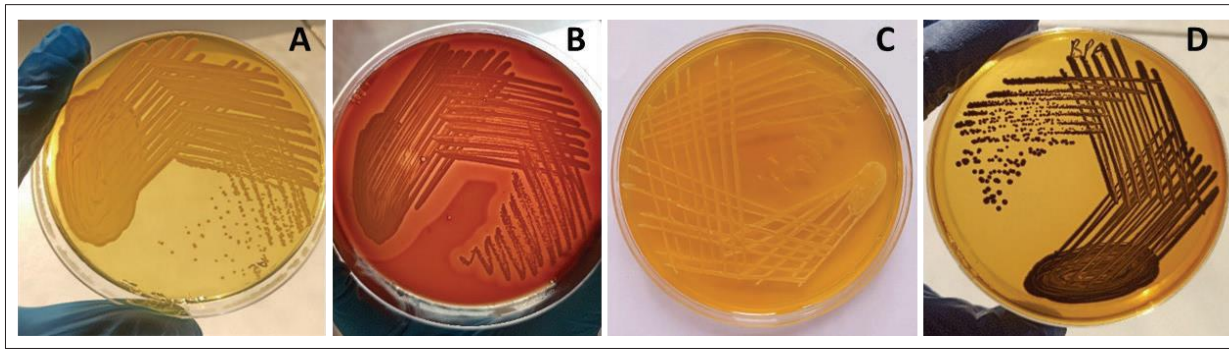

Fig. 1. Colony characteristics of *S. aureus* on different media *S. aureus* colonies exhibiting smooth, round, golden yellow colonies on Brain Heart Infusion agar (A) Double hemolysis on Blood agar (B) Yellow colonies on Mannitol salt agar (C) and Black colonies with halo on Baird-Parker medium (D)

from a nasal swab sample. So, the MRSA prevalence in the nasal cavity of goats was estimated as 0.67%, and 4% from milk.

Determination of the antibiotic resistance profile of MRSA to other commonly used antibiotics. All 15 *mec*A positive MRSA isolates exhibited 100% resistance to cefoxitin and penicillin, followed by resistance to ampicillin (93.33%). All the isolates were sensitive to cotrimoxazole, cefazolin, imipenam and ceftriaxone. Only one out of the 15 *mec*A positive MRSA isolates exhibited an MIC ≥ 16mcg/ml, and was thus considered resistant to vancomycin. Another single isolate was found to be

Table 3. The antibiotic resistance profile of MRSA isolates

S. NO.	ANTIBIOTIC DISC	CONCENTRATION	MRSA ISOLATES (N=15)			
			Resistant isolates		Sensitive isolates	
			No. of isolates	Percentage of isolates (%)	No. of isolates	Percentage of isolates (%)
1.	Amikacin (AK)	30mcg	1	6.67	14	93.33
2.	Amoxicillin (AMX)	30mcg	3	20	11	73.33
3.	Amoxicillin/Clavulanic acid (AMC)	30mcg	1	6.67	11	73.33
4.	Ampicillin (AMP)	25mcg	14	93.33	0	0
5.	Azithromycin (AZM)	15mcg	4	26.67	6	40
6.	Cefazolin (CZ)	30mcg	0	0	15	100
7.	Cefotaxime (CTX)	10mcg	2	13.33	11	73.33
8.	Cefoxitin (CX)	30mcg	15	100	0	0
9.	Ceftriaxone (CTR)	30mcg	0	0	8	53.33
10.	Chloramphenicol (C)	30mcg	1	6.67	12	80
11.	Ciprofloxacin (CIP)	5mcg	5	33.33	7	46.67
12.	Co-trimoxazole (COT)	25mcg	0	0	15	100
13.	Doxycycline hydrochloride (DO)	30mcg	1	6.67	14	93.33
14.	Erythromycin (E)	15mcg	6	40	4	26.67
15.	Gentamicin (GEN)	10mcg	7	46.67	7	46.67
16.	Imipenem (IPM)	10mcg	0	0	13	86.67
17.	Ofloxacin (OF)	5mcg	1	6.67	14	93.33
18.	Penicillin G (P)	10U	15	100	0	0
19.	Streptomycin (S)	10mcg	1	6.67	14	93.33
20.	Tetracycline (TE)	30mcg	4	26.67	8	53.33
21.	Vancomycin (VAN)	By MIC	1	6.67	13	86.66

intermediate (MIC 4-8 mcg/ml) while 13 isolates were found to be sensitive to vancomycin (MIC ≤ 2 mcg/ml). The number of sensitive and resis- tant isolates and their percentages are presented in Table 3.

Multidrug resistance and MAR index. The multi-drug resistance profile for MRSA isolates re- vealed that eight (53.33%) out of the 15 MRSA isolates were multidrug resistant, and of these four (26.66%) isolates were resistant to 3 antimicrobial classes and two (13.34%) isolates each were resis- tant to four and five antibiotic classes. Also, out of these eight MDR isolates, five (33.33%) were obtained from pigs, three (20%) were obtained from goats, but none of the ovine MRSA isolate was found to be MDR.

The MAR index of MRSA isolates revealed that the majority of the isolates exhibited a MAR index greater than 0.2, which indicates high antimicrobial usage in small ruminants and pigs in Punjab. About 40% of the isolates (n=6) had a MAR index of less than 0.20, 53.33% isolates (n=8) had a MAR index of 0.20-0.40, and 6.67% (n=1) isolates has a MAR index of 0.40-0.60, with the highest MAR value ob- served as 0.60. The MDR isolates and MAR index for MRSA isolates is presented in Table 4.

*erm*A, *erm*B, *erm*C, *van*A, *van*B, *tet*K, *tet*L, *tet*M, and *bla*Z, revealed that 11 (73.33%) isolates har- boured the *tet*K gene, followed by 10 (66.67%) isolates that had the *aac-aph*D gene. Nine (60%) isolates exhibited the presence of the *erm*C gene, seven (46.67%) had the *erm*B gene, seven iso- lates (46.67%) demonstrated the presence of the *bla*Z gene, six (40%) had the *tet*L gene, while five (33.33%) and two (16.66%) isolates carried the *tet*M and the *erm*A gene, respectively. None of the MRSA isolates carried the *mec*B, *mec*C, *van*A or *van*B genes.

Molecular detection of virulence genes in MRSA isolates. Out of the various virulence genes tested, the *eno* was found to be the predominant virulence gene present in ten of the 15 isolates (66.67%). The *luk*-PV gene was detected in nine (60%) isolates, followed by the *coa* and *tsst*-1 genes, which were detected in 13.33% isolates each. The *eta*, *etb* and *hlg* genes remained undetected in the present study

Molecular typing of MRSA isolates. All of the 15 MRSA isolates obtained were typed by SCC*mec* typing using the specific primers for types I, II, III, IV (IVa, IVb, IVc and IVd) and V. A total of five SCC*mec* types were observed. SCC*mec* type IVa was detected in eight isolates, followed by SCC*mec* type III observed in three isolates, SCC*mec* Type I was observed in two isolates, and the SCC*mec*

Table 4. Multidrug resistance pattern and Multiple Antibiotic Resistance (MAR) index for MRSA isolates

MDR PATTERN					MAR INDEX					
NO. OF ANTIMICROBIAL CLASSES	Percentage of Isolates (%)	No. of Resistant Isolates	No. of resistant isolates obtained from different species		MAR index value	Percentage of Isolates (%)	No. of Resistant Isolates	No. of resistant isolates obtained from different species		
			Pig	Goat				Pig	Goat	Sheep
THREE	26.66	4	2	2	0-0.20	40	6	2	2	2
FOUR	13.34	2	1	1	0.20-0.40	53.33	8	5	3	-
FIVE	13.34	2	2	-	0.40-0.60	6.67	1	1	-	-

Molecular detection of antibiotic resistance genes. MRSA isolates tested for the presence of 10 antimicrobial resistance genes, viz. *aac-aph*D,

Types IVc and V were observed in one isolate each.

Statistical analysis. A comprehensive analysis was conducted to assess the influence of various farm-level and animal-related parameters on the

isolation rates of *S. aureus* and MRSA. The results demonstrated that the isolation of *S. aureus* was significantly associated with the health status of the animals (P<0.01), the management practices implemented on the farms ((P<0.05), and the animal species sampled (P<0.01). These findings suggest that poor health conditions, suboptimal biosecurity measures, and specific animal reservoirs may contribute to increased colonization or infection with *S. aureus*. In contrast, the isolation of MRSA only showed a statistically significant association with the health status of the animals (P<0.05), indicating that compromised health may be a key risk factor for MRSA carriage, regardless of species or management conditions.

To explore the resistance dynamics in MRSA isolates further, a Spearman correlation analysis was performed to determine the relationship between phenotypic antimicrobial resistance patterns and the expression of corresponding resistance genes. The analysis revealed a strong and statistically significant positive correlation, with a Spearman's correlation coefficient (*r*) of 0.72 and a P-value of 0.002. This indicates a high level of concordance between the presence of resistance genes and the observed resistance to antimicrobial agents, underscoring the reliability of molecular detection methods for predicting phenotypic resistance in MRSA strains. These results highlight the importance of integrated surveillance that combines both phenotypic and genotypic approaches, for accurate detection and monitoring of antimicrobial resistance.

Discussion

India is one of the largest antimicrobial consumers in the world as over-the-counter antimicrobials are readily available, which has led to indiscriminate use and misuse. In 2010, India was ranked as the fifth largest consumer of antimicrobials in food animals (poultry, pigs, and cattle), and, with the rising demand for animal protein, antimicrobial use is projected to grow by 312% in India by 2030 (WHO, 2021). Antibiotic resistance is the most dangerous global health threat and has a major impact on food safety, public health and the economy. In the present study, the prevalence and antimicrobial resistance pattern of MRSA were ascertained in small ruminants and pigs in Punjab, a state towards the north of India. Moreover, the virulence gene profiles of the isolates were determined, along with their molecular typing.

In the present study, a total of 45 *S. aureus* isolates were confirmed by MALDI-TOF. Similarly, MALDI-TOF was used by OZBEY et al. (2022) to identify 16.7% *S. aureus* isolates from bovine milk samples. A total of 25 isolates exhibited resistance to cefoxitin, while the *mec*A gene could be determined in only 15 of these isolates, and they were thus confirmed as MRSA. None of the isolates exhibited the presence of the *mec*B or *mec*C genes. Similar results have been reported previously by MAHESH et al. (2022), who observed cefoxitin resistance in 93 *S. aureus* isolates, but the *mec*A gene was detectable in only 84 isolates. These findings are also in accordance with the study conducted by GEORGOPAPADAKOU (1993) who stated that the presence of some *mec*A-negative isolates phenotypically resistant to β-lactam antimicrobials could be due to a less common type of resistance, which may be due either to overproduction of β-lactamase or the presence of altered penicillin Binding Protein (PBP), not related to 2a or 2'.

In the present study, MRSA prevalence was found to be 5.33% in pigs. Similar results were obtained by RAJKHOWA et al. (2016), OTALU et al. (2018), KALUPAHANA et al. (2019) and BACK et al. (2020), who also reported a low prevalence of MRSA in pigs. Conversely, a higher MRSA prevalence has also been reported by various researchers, such as MROCZKOWSKA et al. (2017), PIROLO et al. (2019) and ABREU et al. (2019). Also, in this study, six out of 15 MRSA isolates were obtained from healthy pigs. This is alarming as healthy animals can continue spreading the MRSA isolates to other healthy animals, and may act as a source of infection. Also, in our study, the majority of the MRSA isolates were obtained from pigs. This may be attributed to higher antibiotic usage on commercial pig farms. The higher prevalence of MRSA in pigs may also be attributed to the fact that pigs are generally reared in intensive systems, where they are housed in crowded pens that enable close

contact between pigs, as well as due to the lack of cleanliness in these pens.

In the present study, MRSA prevalence in nasal cavities of goats and sheep was found to be low, i.e. 0.67% and 1.33%, respectively, in accordance with the results obtained by ABDEL-MOEIN and ZAHER (2019) and MECHESSO et al. (2021). No MRSA isolate was obtained from the human hand swab samples. Although human hands are the major means of transmission of diseases, the absence of bacterial isolation does not necessarily mean that the sampled surface is free of the pathogen as the sample collected only represents a snapshot of the surface from where the sample is collected. Also, the lower isolation may be attributable to increased knowledge of hand hygiene in the post Covid time. However, MRSA has been frequently isolated from animal handlers and farmers by var- ious researchers, such as MROCZKOWSKA et al. (2017), KALUPAHANA et al. (2019) and KITTL et al. (2020) where they reported the prevalence of MRSA as 13.2%, 2.2% and 5.1%, respectively.

UDOH et al. (2019) estimated the prevalence of MRSA in caprine milk as 7.5%, which is much higher than what was obtained in our study, i.e., 4%. TEGEGNE et al. (2019) estimated a prevalence of 6.12% of MRSA in caprine milk. A much higher MRSA prevalence was reported in caprine milk samples by RANA et al. (2020) and OMOSHA- BA et al. (2020). The higher MRSA prevalence in caprine milk may be due to the fact that *S. aureus* is a major cause of caprine mastitis which requires antibiotic treatment, and this may lead to the development of resistance and hence the emergence of MRSA.

All the MRSA isolates were found to be 100% resistant to cefoxitin and penicillin, followed by ampicillin (93.33%) by disc diffusion. Least resistance was observed against cotrimoxazole, cefazolin, imipenam and ceftriaxone (0% each). Similar results were observed by ALZOHAIRY (2011), EFAH et al. (2018), OMOSHABA et al. (2020) and MECHESSO et al. (2021). The sensitivity of MRSA isolates to cefazolin may be due to a type of bacterial toxin antitoxin system, called the *mazEF*, where it has been observed that the MRSA strains lacking *mazEF* expression are highly sensitive to cefazolin (MANDELL et al., 2024). All the MRSA isolates obtained in the present study were tested for vancomycin resistance, and only one isolate was found to be resistant to vancomy- cin (MIC ≥ 16mcg/ml). Similarly, OZTURK et al. (2019) and TAN et al. (2021) also reported high susceptibility to vancomycin. This is especially important as vancomycin is the traditional last-resort antibiotic for serious or suspected infections caused by MRSA. Any resistance to vancomycin is alarming as it narrows the treatment options available for therapy. About 53% of the MRSA isolates were multidrug resistant with the majority of the isolates exhibiting a MAR index of greater than 0.2, which indicates widespread antimicrobial usage in small ruminants and pigs in Punjab.

The MRSA isolates obtained in the present study were tested for the presence of 10 antimicrobial resistance genes, out of which the *tet*K gene was found to be the most prevalent. Other resistance genes, such as *aac-aph*D, *erm*A *erm*B, *erm*C, *bla*Z, *tet*L and *tet*M were also reported. Var- ious researchers, such as FELTRIN et al. (2016), HEIKINHEIMO et al. (2016) and OMWENGA et al. (2021), reported similar results. Among the virulence genes, the *eno* gene was found to be the predominant gene, followed by the *luk*-PV gene, the *coa* gene and the *tsst*-1 gene. Our results are in agreement with MOTALLEBI et al. (2016) and FIROOZEH et al. (2020) who also reported similar results. Other genes, such as the *coa*, *tsst*-1 and *luk*-PV genes, were also observed, as reported by ABDULGHANY and KHAIRY (2014) and EL-DEEB et al. (2022).

In this study, phenotypic tetracycline resistance was observed in four MRSA isolates (26.67%), while tetracycline resistance genes (*tetK*, *tetL*, *tetM*) were also detected in isolates that showed intermediate sensitivity. All three genes were present in four isolates (26.67%), *tetK* and *tetM* in one isolate (6.67%), *tetK* and *tetL* in two isolates (13.33%), and *tetK* alone in four isolates (26.67%). These results are consistent with previous findings by AGGARWAL et al. (2019), who reported resistance genes even in phenotypically sensitive isolates. For gentamicin, 46.67% of the isolates showed phenotypic

resistance, while 66.67% carried resistance genes. Similar results were observed with erythromycin resistance, where resistance was seen in 46.67% of isolates, with resistance genes detected individually or in combinations; all three (*ermA*, *ermB*, *ermC*) in two isolates (13.33%), *ermB* and *ermC* in three (20%), *ermB* alone in two (13.33%), and *ermC* alone in four (26.67%).

Molecular typing of all the MRSA isolates obtained in this study revealed five SCC*mec* types, with type IVd the most common, in accordance with the results obtained by SASAKI et al. (2022). It has been well documented that all the SCC*mec* types are frequently interchanged between animals, humans and the environment (LAKHUNDI and ZHANG, 2018) as well as between the methicillin resistant non-*S. aureus* Staphylococci (MRNAS) which might be an important SCC*mec* reservoir for MRSA, especially in pigs (VANDERHAEGHEN et al., 2012). In the present study, MRSA isolates belonging to SCC*mec* types I, III, type IVc and V were also obtained. Preliminary studies based on SCC*mec* typing revealed that five MRSA isolates could be HA-MRSA, as these belonged to SCC*mec* types I and III. Also, out of ten MRSA isolates that belonged to types IV and V, the *pvl* gene was present in six isolates, which is suggestive of these isolates being CA-MRSA. The remaining four MRSA isolates could be LA-MRSA as they belonged to types IV and V, and no *pvl* gene was detectable.

Despite the strengths of this study, including the large sample size, wide geographic coverage, and the inclusion of multiple animal species (pigs, goats, and sheep), along with human hand swab samples, several limitations should be acknowledged. First, the absence of whole-genome sequencing limited our ability to identify and characterize mobile genetic elements, such as plasmids and transposons, that may contribute to the spread of antimicrobial resistance. Additionally, hand swab sampling was conducted as a single-time point, which may not fully capture the dynamics of MRSA colonization or transmission over time. These limitations highlight the need for longitudinal studies and genomic approaches to better understand the epidemiology and evolution of MRSA in livestock and human populations.

Conclusions

The prevalence of MRSA in this study was highest in pigs, followed by goats and sheep, with all isolates showing 100% resistance to both cefoxitin and penicillin. The presence of multiple virulence genes and the detection of diverse SCC*mec* types, spanning community-associated (CA-MRSA), livestock-associated (LA-MRSA), and hospital -associated (HA-MRSA) lineages, highlights the complex epidemiology of MRSA in livestock populations. These findings underscore the urgent need to strengthen AMR surveillance programs and integrate MRSAmonitoringintoveterinaryantimicrobial stewardship efforts. Regular screening of both animals and humans in contact with livestock is critical for early detection, containment of resistant strains, and the development of evidence-based policies to reduce the spread of MRSA across sectors.

Ethics statement

Ethical approval was sought from the Institutional Animal Ethical Committee of Guru Angad Dev Veterinary and Animal Science University, vide reference no. GADVASU/2021/IAEC/61/22 dated 13/10/2021, and from the Institutional Biosafety Committee (IBSC), Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, vide reference no. IBSC/2021/1763-64 dated 13/10/2021. For collecting samples from animal handlers on the farms, permission was sought from the Institutional Ethics Committee of the Dayanand Medical College, Ludhiana, vide reference no. DMCH/R&D/2021/105.

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GAZAL, S., P. KAUR, J. BEDI, J. SINGH, A. K. ARORA, T. S. RAI: Antimikrobna rezistencija meticilin-rezistentne bakterije *Staphylococcus aureus* izolirane iz domaćih životinja i njihovih držatelja u saveznoj državi Punjab, Indija. Vet. arhiv 96, 226-241, 2026.

SAŽETAK

Sojevi meticilin-rezistentne bakterije *Staphylococcus aureus* (MRSA) uzrokuju širok spektar infekcija u domaćih životinja. Razlozi za navedeno leže u prisutnosti više gena rezistencije na antibiotike i čimbenike virulencije, koji povećavaju patogenost sojeva. Cilj istraživanja bio je utvrditi prevalenciju, profil gena rezistencije i virulencije te provesti molekularnu karakterizaciju MRSA izolata u saveznoj državi Punjab u Indiji. Ukupno je prikupljeno 650 uzoraka, uključujući brisove nosa ovaca, koza i svinja, uzorke kozjeg mlijeka te brisove ruku držatelja životinja, iz različitih okruga u Punjabu. Uzorci su obrađeni radi izolacije bakterije *S. aureus*, a prisutnost izolata potvrđena je metodom MALDI-TOF. Potvrđeni izolati *S. aureus* analizirani su PCR-om radi detekcije gena za meticilinsku rezistenciju. Također, svi dobiveni izolati MRSA-e ispitani na osjetljivost u staničnoj kulturi, antimikrobnu rezistenciju i prisutnost gena virulencije. Dobiveno je ukupno 45 izolata *S. aureus*, od kojih je 15 sadržavalo gen *mecA* te su stoga potvrđeni kao MRSA izolati. Svi MRSA izolati (100%) pokazali su otpornost na cefoksitin i penicilin. Ukupno je 53,33% MRSA izolata bilo višestruko rezistentno na antibiotike, a većina je imala indeks višestruke rezistencije (MAR) veći od 0,2, što upućuje na intenzivnu upotrebu antimikrobnih sredstava na farmama u Punjabu. Gen *tetK*, koji uvjetujerezistenciju na tetracikline, bio je najzastupljeniji, a nakon njega gen *aac-aphD*, dok je gen *eno* bio najčešći gen virulencije. Utvrđeno je ukupno pet tipova gena *SCCmec*, pri čemu je tip *SCCmec IVd* bio najučestaliji. Detekcija bakterije MRSA u domaćih životinja upućuje na zoonotski rizik s ozbiljnim veterinarskim i javnozdravstvenim implikacijama.

Ključne riječi: AMR; MALDI-TOF; MRSA; SCC*mec* tipizacija; geni virulencije