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Research Article

EXPLORING PREVALENCE, ANTIMICROBIAL SUSCEPTIBILITY, VIRULENCE PROFILES, AND MULTIDRUG RESISTANCE IN *STAPHYLOCOCCUS AUREUS* ISOLATES FROM RESPIRATORY DISEASE-AFFECTED SHEEP AND GOATS REARED BY THE MIGRATORY COMMUNITIES OF LOWER HIMALAYAS

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ABSTRACT: *Staphylococcus aureus* is a significant bacterium that causes substantial economic losses in the livestock sector and poses life-threatening risks to both humans and animals. This study aimed to explore the prevalence, susceptibility to antimicrobials, and profiles of virulence and antimicrobial resistance (AMR) genes in *S. aureus* isolated from nasal swabs and lung tissues of sheep and goats exhibiting symptoms of respiratory disease. A total of 194 samples were examined, resulting in the isolation and confirmation of *S. aureus* in 65 samples, indicating an overall prevalence of 33.5%. These isolates were further subjected to an AMR assay. Among the representative isolates (37), sensitivity was observed to chloramphenicol and ceftriaxone. Conversely, penicillin showed the lowest efficacy, with 83.8% of isolates demonstrating resistance, followed closely by amoxiclav, which exhibited resistance in 75.7% of isolates. Close to three-quarters of isolates carried at least one AMR gene. Virulence genes were identified in 67.6% of *S. aureus* isolates, with coa and lukpv detected in 37.8% and arcA in 32.4% of isolates. Additionally, mecA and vatC were present in 54.05%, vatB in 43.25% and aphD in 18.91% of *S. aureus* isolates. A substantial 91.9% of isolates exhibited multidrug resistance, and MAR indices exceeding 0.2 were recorded in 86.5% of *S. aureus* isolates, indicating a high public health risk. These findings underscore the importance of prioritizing infections caused by *S. aureus*, necessitating heightened attention from both veterinarians and healthcare workers in migratory communities. Furthermore, the regulated and judicious use of antimicrobials is crucial to mitigate the risks associated with antimicrobial resistance in these settings.

Keywords: *Staphylococcus aureus*, Sheep, Goat, Himalayas, MDR; MAR index, AMR genes, Virulence genes, Antibiotic susceptibility.

INTRODUCTION

In Himachal Pradesh, a north-western Himalayan state of India, close to 90% of the rural population relies significantly on sheep and goat husbandry for their livelihood. The prevailing practice of sheep and goat rearing involves migratory husbandry, subjecting these animals to challenging climatic conditions, rugged terrains, and nutritional stresses. This exposes them to various microbial infections, particularly those affecting the respiratory system. *Staphylococcus aureus*, a major opportunistic pathogen, plays a crucial role in causing challenging respiratory infections in small ruminants. Although it normally resides as a commensal in the respiratory tract mucous membranes, it can induce pathology as a secondary bacterial pathogen. The severity of the disease caused by *S. aureus* hinges on its genotype and its ability to express virulence-associated factors, given its possession of multiple virulence factors that significantly contribute to the initiation and establishment of infections [1, 2]. The emergence of multiple drug resistance in *S. aureus* poses a considerable and unavoidable challenge in therapeutic management [3], resulting in substantial economic losses in livestock production. Furthermore, it presents a potential threat to shepherds, and public health, as animals may serve as both a source of

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infection and a reservoir for methicillin-resistant *S. aureus* (MRSA) [4, 5]. Reports have indicated the existence of multidrug-resistant (MDR) strains resistant to commonly used antimicrobials, including methicillin, lincosamides, macrolides, aminoglycosides, fluoroquinolones, or combinations of these antibiotics [6,7]. Additionally, genes associated with resistant phenotypes have been identified in *S. aureus* [8, 9].

This maiden study focused on screening migratory sheep and goats in the north-western Himalayas to assess the prevalence of drug-resistant *S. aureus* in respiratory tract infections, along with an examination of their virulent and drug-resistance profiles.

MATERIALS AND METHODS

Samples, bacterial isolation, and identification

194 samples were collected, comprising 118 nasal swabs from 29 live sheep and 89 goats, along with 76 lung tissues from slaughtered 20 sheep and 56 goats. These samples were sourced from sheep and goats aged over 3 years, originating from diverse flocks owned by shepherds in Himachal Pradesh and spanning various locations in the Kangra and Shimla districts. The animals exhibited typical symptoms of respiratory diseases, including nasal discharge, dyspnea, and lung pathology. All samples were collected from flocks with a history of multiple antibiotic administrations for various ailments, either by the shepherds or veterinary staff. The samples were collected in NSS and promptly transported on ice to the Department of Veterinary Microbiology at Himachal Pradesh Agriculture University in Palampur, HP, India. Upon arrival, they were inoculated on 5% sheep blood agar and incubated at 37°C for 24-48 hours within 12 hours of collection. Bacterial colonies suspected to be *Staphylococcus aureus* were further purified on Brain Heart Infusion (BHI) agar and Mannitol Salt Agar (MSA). Standard microbiological procedures were used to identify colonies suspected of *Staphylococcus aureus* [10]. Molecular confirmation of *S. aureus* was accomplished through *in vitro* amplification and detection of the nuc gene as mentioned in Table 1 [11].

Antibiotic sensitivity profile of *S. aureus*

Antimicrobial susceptibility testing of *S. aureus* was performed [12] against the following antibiotics; Chloramphenicol (C) 30 µg, Amoxyclav (AMC) 30 µg, Tetracycline (TE) 30 µg, Ciprofloxacin (CIP) 5 µg, Rifampicin (RIF) 5 µg, Penicillin (P) 10 units, Ceftriaxone (CTR) 30 µg, Erythromycin (E) 15 µg, Streptomycin (S) 10 µg, Ofloxacin (OF) 5 µg, Co-trimoxazole (COT) 25 µg, Azithromycin (AZM) 15 µg, Gentamicin (GEN) 10 µg. The disks were purchased from HiMedia, Mumbai, India. Pure cultures of *S. aureus* were grown overnight in BHI broth at 37°C and the concentration was adjusted using sterile BHI broth until a 0.5 McFarland turbidity was attained. One hundred microliters of the culture were then swabbed onto Mueller Hinton agar (HiMedia, Mumbai). Six antimicrobial disks were placed equidistant on agar surface and plates were incubated at 37°C for 16 hours. The results were interpreted according to Clinical and Laboratory Standards Institute guidelines 2006.

Multiple antibiotic resistance (MAR) index

MAR index was determined [13] for each isolate by using the formula; MAR = a/b, where 'a' represents the number of antibiotics to which the test isolates depicted resistance and 'b' represents the total number of antibiotics to which the test isolate has been evaluated for susceptibility.

Detection of virulence and AMR genes

Multiplex PCR (Polymerase Chain Reaction) in a total volume of 25 µl was used for the detection of seh, lukpv, arcA, etd, and coa virulence genes in *S. aureus* [14, 15, 16] and for the presence of nine different AMR genes [16, 17] as shown in Table 1.

The PCR cycling conditions were: Initial denaturation (94 °C- 3min) followed by 30 cycles of (45 sec each for Denaturation at 94 °C, Annealing at 55 °C, Extension at 72 °C) and final extension at 72 °C for 5 min in a GeneAmp PCR system 9700 (Applied Biosystems, USA).

RESULTS AND DISCUSSION

Isolation and identification of *S. aureus*

Cultural, morphological, and biochemical characterization and detection of nuc (Fig. 1a) identified and confirmed that 65 bacterial isolates belonged to *S. aureus*. The overall percent recovery of *S. aureus* was 33.5% from the processed morbid samples. A greater proportion of morbid nasal swab samples (58.6%) of sheep harbored *S. aureus* followed by goat nasal swabs. The details of the isolation of *S. aureus* are presented in Table 2.

The prevalence and isolation of *S. aureus* in the nasal cavities of sheep and goats have been the subject of extensive research due to their implications for both animal health and public health. In this present investigation, *S. aureus* was successfully isolated from 33.5% of the processed samples, a significantly higher

Table 1. Details of primers used for detection of virulence and AMR genes.

Primers	Sequence	Product size (bp)	Gene	Reference
Bsetd F	CCCGTTGATTAGTCATGCAG	607	Exfoliative toxin	[15]
Bsetd R	TCCAGAATTTCCCGACTCAG			
WWarcA F	TTGCTCAAACTTTGAGAGATGAA	215	Arginine deiminase	
WWarcA R3	TTACGTACGCCAGCCATGAT			
seh F	CAACTGCTGATTTAGCTCAG	358	Enterotoxin H	
seh R	GTCGAATGAGTAATCTCTAGG			
lukpv F	ATCATTAGGTAAAATGTCTGGACATGATCCA	432	Panton-Valentine leucocidin	
lukpv R	GCATCAAGTGTATTGGATAGCAAAAGC			
coa F	ATAGAGATGCGTGTACAGG	Variable	Coagulase	[16]
coa R	CTTCCGATTGTTCGATGC			
mecA1 F	GTAGAAATGACTGAACGTCCGATAA	315	*mec*A	[14]
mecA2 R	CCAATTCCACATTGTTTCGGTCTAA			
aacA-aphD 1	TAATCCAAGAGCAATAAGGGC	277	*aacA-aphD*	
aacA-aphD 2	GCCACACTATCATAACCACTA			
ermA 1	AAGCGGTAAACCCCTCTGA	190	*ermA*	
ermA 2	TTCGCAAATCCCTTCTCAA			
ermC 1	AATCGTCAATTCCTGCATGT	299	*ErmC*	
ermC 2	TAATCGTGGAATACGGGTTTG			
tetK 1	GTAGCGACAATAGGTAATAGT	360	*tetK*	[17]
tetK 2	GTAGTGACAATAAACCTCCTA			
tetM 1	AGTGGAGCGATTACAGAA	158	*tetM*	
tetM 2	CATATGTCCTGGCGTGTCTA			
vatA 1	TGGTCCCGGAACAACATTTAT	268	*vatA*	
vatA 2	TCCACCGACAATAGAATAGGG			
vatB 1	GCTGCGAATTCAGTTGTTACA	136	vatB	
vatB 2	CTGACCAATCCCACCATTTTA			
vatC1	AAGGCCCCAATCCAGAAGAA	407	vatC	
vatC2	TCAACGTTCTTTGTCACAACC			
nuc 1	GCGATTGATGGTGATACGGTT	447	nuc	[11]
nuc 2	AGCCAAGCCTTGACGAACTAAAGC			

Table 2. Species and sample-wise details of recovery of *S. aureus*.

Species (Number)	Number and type of samples processed		Samples yielded *S. aureus* (% recovery)		
	Nasal swabs	Lung tissues	Nasal swabs	Lung tissues	Total
Goats (145)	89	56	35 (39.3)	11 (19.6)	46 (31.7)
Sheep (49)	29	20	17 (58.6)	02 (10)	19 (38.8)
Total samples (194)	118	76	52 (44.6)	13 (17.1)	65 (33.5)

rate compared to the 12.9% [18] and slightly lower than the 43.7% [19]. The nasal cavities of sheep and goats have been identified as the primary reservoir for *S. aureus* [20, 21] and the colonization of *S. aureus* organisms in the nasal carriage of small ruminants is considered a probable source of staphylococcal infections [22, 21]. Approximately 55.3% of nasal swabs yielded *S. aureus*, while only 17.1% of morbid lungs tested positive for the culture of this organism, indicating lung involvement typically occurs in advanced stages of infection. *S. aureus*, often implicated in severe infections, exhibits a high prevalence of bacterial virulence factors.

Table 3. Antibiotic susceptibility pattern of *S. aureus*.

Antimicrobial/concentration-mcg/disc	No. of isolates showing a specific pattern			Sensitive (%)
	*S	MS	R	
Chloramphenicol (C)/30	37	0	0	100
Ceftriaxone (CTR)/30	10	27	0	100
Tetracycline (TE)/30	36	0	1	97.2
Streptomycin (S)/10	35	0	2	94.5
Ofloxacin (OF)/5	32	3	2	94.5
Azithromycin (AZM)/15	31	3	3	91.8
Erythromycin (E)/15	23	9	5	86.4
Ciprofloxacin (CIP)/5	2	25	10	72.9
Gentamicin (GEN)/10	14	9	14	62.1
Rifampicin (RIF)/5	0	13	24	35.1
Co-trimoxazole (COT)/25	9	3	25	32.4
Amoxyclav (AMC)/30	9	0	28	24.3
Penicillin (P)/10 units	0	6	31	16.2

*S= Sensitive, MS=Mildly sensitive, R= Resistant.

Table 4. MAR indices of MDR *S. aureus*.

Number of antibiotics ineffective	Name of antibiotic	MDR pattern	Isolate ID	Total isolates resistant	MAR index	AMR gene (Isolate id)*
0	Sensitive to all antibiotics*	NA	Sa24, Sa25	0	0	*vatB, vatC* (Sa24)
1	RIF	NA	Sa8	1	0.08	*ermC*, *tetK*, *vatC*
2	RIF, GEN	1	Sa9, Sa10	2	0.15	*vatC* (Sa9)
3	AMC, CIP, COT	2	Sa6	8	0.23	-
	AMC, P, GEN	3	Sa19			*vatB, vatC*
	CIP, RIF, GEN	4	Sa20			*vatC*
	RIF, P, GEN	5	Sa21			*vatB*
	AMC, P, COT	6	Sa12, Sa15, Sa16, Sa23			*vatC*, *aphD*(Sa12); *ermC*, *tetK*, *vatC* (Sa15); *vatC*, *aphD* (Sa23)
4	AMC, P, COT, GEN	7	Sa14	11	0.31	*vatB, vatC*
	AMC, RIF, P, E	8	Sa28			*vatB*
	AMC, RIF, P, OF	9	Sa29			*vatC*, *aphD*
	AMC, CIP, P, COT	10	Sa13, Sa17			*vatB, vatC* (Sa13, Sa17)
	AMC, RIF, P, COT	11	Sa1, Sa18, Sa22, Sa26, Sa30, Sa31			*vatB, vatC*, *aphD* (Sa26); *ermC*, *tetK*, *vatC*, *aphD* (Sa30), *vatC* (Sa31)
5	AMC, CIP, P, COT, GEN	12	Sa5	10	0.38	*vatC*
	AMC, P, E, COT, GEN	13	Sa7			-
	AMC, CIP, RIF, P, GEN	14	Sa11			*vatB, vatC*
	AMC, RIF, P, S, COT	15	Sa27			*vatC*
	RIF, P, E, COT, GEN	16	Sa32			-
	AMC, RIF, P, COT, GEN	17	Sa33			*vatB*
	RIF, P, S, COT, AZM	18	Sa35			*vatB*
	AMC, RIF, P, E, COT	19	Sa2, Sa4, Sa36			*vatC*, *aphD* (Sa2)
6	AMC, RIF, P, COT, AZM, GEN	20	Sa3	2	0.46	*vatB, vatC*, *aphD*
	AMC, CIP, RIF, P, COT, GEN	21	Sa37			*vatB*
9	AMC, TE, CIP, RIF, P, E, OF, AZM, GEN	22	Sa34	1	0.70	*vatB*

*Resistant genes are present only in those isolates whose id is mentioned in bracket next to the resistant gene(s). If it is not mentioned, then it is present in all isolates mentioned in column under Isoate Id.

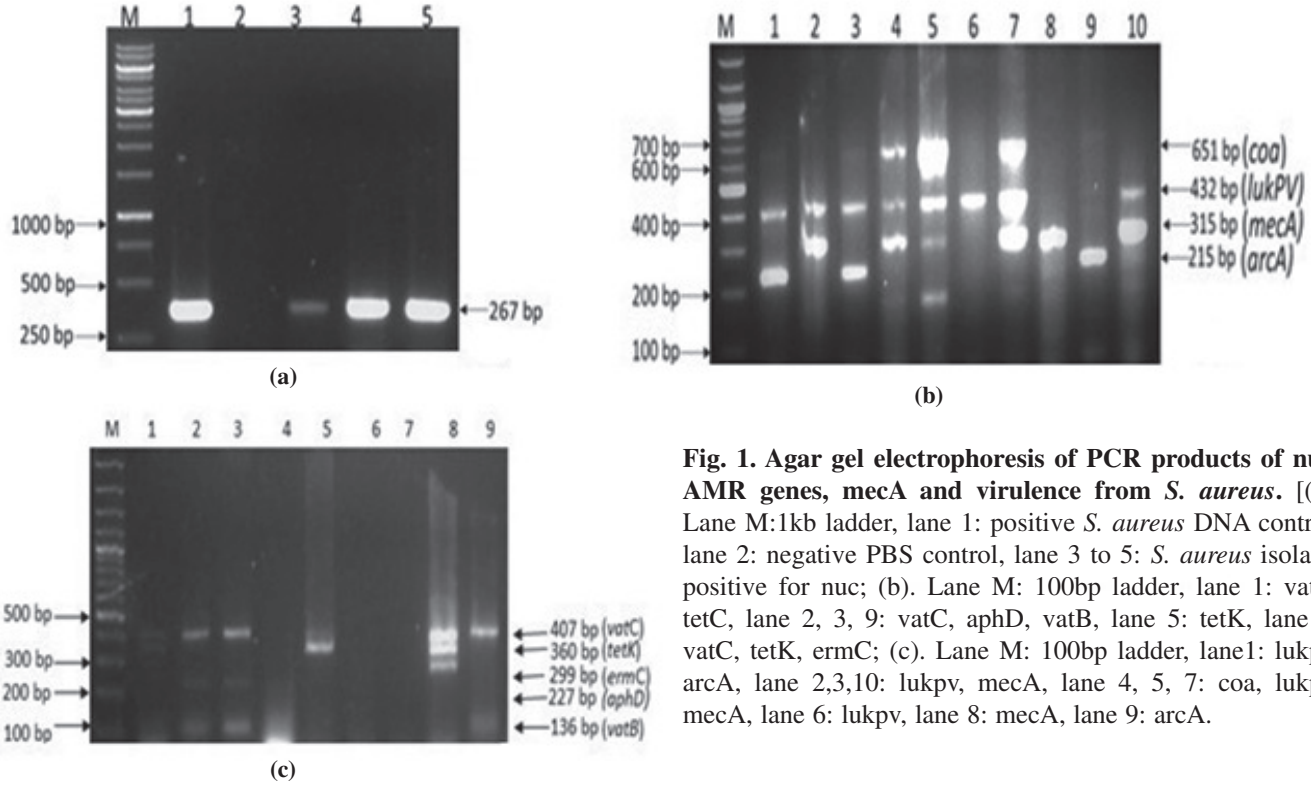

Fig. 1. Agar gel electrophoresis of PCR products of nuc, AMR genes, mecA and virulence from *S. aureus*. [(a). Lane M:1kb ladder, lane 1: positive *S. aureus* DNA control, lane 2: negative PBS control, lane 3 to 5: *S. aureus* isolates positive for nuc; (b). Lane M: 100bp ladder, lane 1: vatC, tetC, lane 2, 3, 9: vatC, aphD, vatB, lane 5: tetK, lane 8: vatC, tetK, ermC; (c). Lane M: 100bp ladder, lane1: lukpv, arcA, lane 2,3,10: lukpv, mecA, lane 4, 5, 7: coa, lukpv, mecA, lane 6: lukpv, lane 8: mecA, lane 9: arcA.

Antimicrobial susceptibility profile and distribution of antibiotic-resistant genes

Of the 65 *S. aureus*, only 37 isolates were subjected to antibiotic sensitivity and virulence profiling. The result of antibiotic susceptibility testing was reported as sensitive, mildly sensitive, and resistant and are depicted in Table 3.

All isolates were 100% susceptible to chloramphenicol and ceftriaxone followed by tetracycline (97.2%), streptomycin and ofloxacin (94.5%), azithromycin (91.8%), gentamicin (62.1%) and rifampicin (35.1%). Amoxyclav (24.3%) and penicillin (16.23%) were least effective against *S. aureus*. Overall, 75.6 % of isolates were resistant to AMC, 83.7% to P, 67.6% to COT, and 64.8% to RIF (Table 3). Twenty-two distinct types of MDR patterns were exhibited by 34 out of 37 *S. aureus* which were resistant to two or more antibiotics (Table 4). Spatial and temporal variability in antibiograms of *S. aureus*, in terms of the class of antibiotics, was evident in this study and previous reports. Factors such as the genetic makeup of the organism, environmental and management practices, regulations governing antibiotic use, antibiotic availability, strategic marketing, cost, and social awareness contribute to observed phenotypes. Indiscriminate and prolonged antibiotic use is known to enhance the development of multidrug resistance, as reported by several studies.

A total of 37 isolates were screened for the presence of nine different AMR genes (mecA, ermA, ermC, tetK, tetM, vatA, vatB, vatC, aphD). The representative amplicons have been shown in Fig. 1b. Out of 37, 26 (74.29%) isolates carried at least one AMR gene. AMR genes vatC and mecA were detected in 20 (54.05%), vatB in 16 (43.2%), aphD in 7 (18.91%), tetK and ermC in 3(8.10%) isolates of *S. aureus*. None of the isolates were positive for tetM, vatA, and ermA. The gene mecA, encoding the additional penicillin-binding protein 2a, was detected in more than half the isolates, contrasting with the 43.5% as reported earlier [14]. The association of mecA with a mobile genetic element contributes to its high occurrence. Close to 3/4th of isolates carried at least one AMR gene. The vatC gene, encoding acetyltransferase, was found in 54.05% of isolates, differing significantly from the 3.03% reported earlier [23]. Notably, vatC, mecA, vatB, aphD, tetK, and ermC were carried by *S. aureus* strains recovered from various locations, indicating their widespread distribution. In the tetracycline class of antibiotics, which is the first-line treatment for many livestock infections, the tetK gene was present in only three sheep isolates. Tetracycline-positive isolates harbor tetK over tetM, suggesting a resistance mechanism mediated by tetracycline efflux

pumps [24]. The ermC gene was present in 8.1% of *S. aureus*, and aphD was found in 18.9% of isolates, aligning closely with the earlier findings [23]. The gene aphD accounts for aminoglycoside resistance, conferring cross-resistance to clinically used aminoglycosides.

Extreme drug resistance was shown by Sa34 isolate against a group of nine antibiotics. Similarly, Sa3, Sa37 revealed resistance to six antibiotics. RIF, P, COT, and GEN were common in these two isolates for which the resistance was exhibited. The analysis of the MAR index (Table 4) revealed that 83.7% of isolates had MAR indices ranging from 0.2 to 0.4, with 86.5 % showing a high MAR index (>0.2).

This suggests that a considerable proportion may naturally carry resistant genes and/or come from flocks/ animals with imprudent antibiotic use, categorizing them as high-risk sources requiring ongoing surveillance. The MAR index is a crucial tool for monitoring antibiotic resistance trends, providing insights into population dynamics, and guiding interventions to prevent the spread of resistant bacteria. Concerns arise about the potential emergence and dissemination of antibiotic-resistant strains in sheep and goats, indicating the possible ineffectiveness of available antibiotics. This calls for the exploration of alternative antibiotic options and emphasizes the need for genetic research to understand resistance mechanisms in *S. aureus* strains from these animals. This study, consistent with previous research, did not establish a clear correlation between the detection of AMR genes and the corresponding phenotypic resistance. The resistance phenotype of a microbe depends on numerous factors, and studies like this targeting specific genes based on existing knowledge may not detect all genes associated with the resistance phenotype. Until clinically relevant genomic assays are available, *in vitro* antibiotic sensitivity results should guide decisions on antibiotic usage. The study found that the majority (91.9%) of isolates harboring antibiotic resistance were resistant to two or more antibiotics, consistent with the multiple resistances already reported [25].

Distribution of virulence-related genes

In all, 25 of 37 *S. aureus* carried at least one virulence gene, reflecting an overall carrier percentage of 67.6%. Amplicons for virulence genes from some representative isolates have been shown in Fig. 1c. The coa and lukpv genes were detected in 14 (37.8%) and arcA in 12 (32.4%) *S. aureus* isolates. None of the isolates carried Bsetd and seh virulent genes.

The lukpv gene, encoding a cytolytic toxin associated with community-associated methicillin-resistant *S. aureus* (caMRSA), was detected in 11 goats and 3 sheep *S. aureus* isolates. The lukpv serves as a useful marker for the detection of caMRSA, given its putative role in invasive infections like necrotizing pneumonia. The presence of lukpv in 37.8% of *S. aureus* recovered from morbid animals is significant, given its potential to induce serious lung pathology.

An equal percentage (37.8%) of isolates carried coa, another major virulent factor responsible for plasma clotting. The study did not note any differences in the distribution and prevalence of virulence genes between sheep and goats or between nasal and lung swabs. The elevated levels of resistance against commonly used antibiotics, along with the presence of mecA and various genes encoding virulent factors in *S. aureus*, highlight the need for attention from veterinary health professionals and livestock keepers. Considering the impact of antibacterial agents on virulence factors and the host's immune response, anti-staphylococcal regimens should account for these factors [26]. Prudent antibiotic use is crucial to decelerate the emergence of multidrug-resistant pathogens. Besides adopting rational preventive and therapeutic regimens for infectious diseases in migratory sheep and goat flocks, epidemiological evidence must be gathered regarding their occurrence in shepherds and slaughterhouse workers to gauge the full extent of the spread of antibiotic resistance.

CONCLUSION

In conclusion, 33.5% of subjects in migratory flocks comprising sheep and goats harboured *S. aureus* in their respiratory tract and lungs. Many of these bacteria possessed one or more virulent genes and carried a combination of antibiotic-resistant genes. Many isolates had the mecA gene responsible for methicillin resistance, with only 16.2% of isolates sensitive to penicillin, and 91.9% revealing resistance to two or more antibiotics. High MAR indices of the isolates imply inherent resistance mechanisms and/or widespread use of antimicrobials. The prevalence and isolation of *S. aureus* in the nasal cavities of sheep and goats are dynamic, and influenced by many factors such as geographical location, management practices, and environmental conditions. The presence of antibiotic-resistance genes and virulent genes in these isolates emphasizes the need for ongoing surveillance including screening shepherds and slaughterhouse workers for *S. aureus* carrier status and caution for regulated use of

antimicrobials in such settings. Such studies further strengthen our understanding of the evolving landscape of antimicrobial resistance and virulence, contributing to the development of effective control strategies and safeguarding both animal and human health.

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