

1 **Antimicrobial Resistance and Virulence Factors**
2 **of *Staphylococcus aureus* in Dairy Farms:**
3 **Insights from the Animal-Human-Environment**
4 **Interface**
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7 **ABSTRACT**

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In India, dairy farming is run by medium, small, and landless farmers and has a stable biotope at the animal-human-environment interface. Hence a cross-sectional study was conducted for the detection of antimicrobial resistance in targeting indicator bacteria *Staphylococcus aureus* at the animal-human-environment interface. A total of 280 samples were collected from dairy farms and their environment. The highest prevalence was noticed in human sources (50%), followed by animal sources (28.50%), dairy equipment (27.50%), and the lowest in farm environments (20.83%), and the overall prevalence noted was 27.85%. The prevalence of species-specific *sa* gene for *S. aureus* was 80.76%. Virulence-associated genes viz. *sea* and *seb* were detected in 7.93% and 9.52% isolates of *S. aureus*. The thermostable nuclease *nuc* gene was found in isolates from animal and human sources with an overall prevalence of 41.26%. Overall antimicrobial resistance pattern of *S. aureus* isolated from dairy farm and its environment was in the descending order i.e., highest for cefoxitin, erythromycin, amikacin, and clindamycin; moderate resistance was seen in vancomycin and streptomycin, linezolid and tetracycline and the lowest resistance was observed in chloramphenicol, gentamicin, and amoxicillin-clavulanic acid irrespective of the source. In the present study, the prevalence of *tetM* was 20% (2/10) in farm environments, 1.75% (1/57) dairy animals, and 33.33% (1/3) in dairy equipment with an overall prevalence of 5.19%.

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10 **Keywords:** *Staphylococcus aureus*, Virulence, Antimicrobial Resistance, Dairy Farms, Animal-
11 Human-Environment Interface

1. INTRODUCTION

The dairy industry in India provides self-sustainability to millions of rural households contributing about 4.11% to India's GDP and 25.6% to agricultural GDP, in which the dairy sector claims a major share by contributing 67% to total livestock output. Sustainable and profitable dairy farming demands proper management practices involving healthy animals well well-managed sheds, feeding, cleaning and sanitation, worker's hygiene, biosecurity, manure disposal, and so on. Among these, disease management is mainly focused on regular vaccination, deworming, health check-ups, and the use of antimicrobial preparations for treatment, control, and prevention of diseases of dairy animals. Unfortunately, in recent years upsurge in the use of antimicrobial preparations has been noticed mainly due to self-medication by the farmers, unwanted overuse by the paravets or sometimes by veterinarians, and lack of observance of proper withdrawal period. This irrational and unscrupulous use promotes the microbiota to develop resistance to survive against antimicrobial preparations.

Due to the rise of antibiotic-resistant pathogens in human health, animal health as well as food production, WHO has declared that AMR is one of the top 10 global public health threats facing humanity (WHO, 2022). Transmission potential of antimicrobial resistance is through indirect consumption of food, water, and produce contaminated with antimicrobial-resistant pathogens and environment via direct contact with animals and animal waste. This is a critical concern at the animal-human environment interface, where animal-origin food can be contaminated and spread further. (Ruegg et al. 2015, Kalayu et al. 2020).

Dairy farming includes regular contact with animals during milking and handling as well as exposure to manure, dust, and liquid splashes, all of which contribute transmission potential of bacteria to people, dairy animals, equipment, and the surrounding farm environment indicating multiple drivers are responsible for dissemination of AMR across the farm. To strengthen knowledge about drivers of antibiotic-resistant bacteria, the present study has targeted indicator bacteria viz. *Staphylococcus* spp. as this bacterium shares microbial biotopes in both humans and animals (Holmes et al. 2016). *Staphylococcus* spp. being commensal bacteria, colonizes on soft tissues, and skin, in the udder or milk. Among all 63 species, *Staphylococcus aureus* is one of the major etiological agents of clinical and subclinical mastitis and has negative public health implications through food-borne intoxication. Enterotoxin production has been linked to sepsis-related infections, food poisoning, pneumonia, and toxic shock syndrome (Lowy 2003). The present cross-sectional study of dairy farms and their environment analyzes the magnitude to which dairy animals may contribute to the AMR of indicator bacteria at the animal-human-environment interface in the population associated with dairy farming.

2. MATERIAL AND METHODS

Eight dairy farms from ten villages in the Satara district of Maharashtra were identified. The farms having at least 10 milking animals at the time of milking (morning or evening) were considered for sampling. A total of 35 samples were collected from each farm from different sources such as animals, human, farm environment, and dairy equipment were collected and mentioned in Table -1.

Table no 1. Details of samples collected

Sr.No.	Sample source	Samples	Total samples
1	Animals	1. Faeces	10
		2. Milk	5
		3. Udder swab	10
2	Human	4. Hand swab	2
3	Farm Environment	5. Drinking water	2
		6. Drainage water	1
		7. Feed	1
		8. Farm air	1
		9. Floor swabs	1
4	Dairy equipments	10. Can swabs	2
Total samples from each farm			35
Total samples – 35 X 10 farms			350

Isolation of *Staphylococcus aureus* was carried out by the procedure as per the Bacteriological Analytical Manual (FDA 2019). Presumptive isolates were further subjected to Gram staining and biochemical tests viz. catalase test, DNAase test (Cheesbrough 2004), Voges-Proskauer

58 test, methyl red test and oxidase test (Cruckshank et al. 1975).Antibiogram sensitivity test of isolates
 59 was conducted using Kirby Bauer disc diffusion method (Bauer 1966) for evaluating resistance
 60 pattern. Antibiotics used in this study were Erythromycin, Amikacin, Ciprofloxacin, Gentamicin,
 61 Tetracycline, Vancomycin, Clindamycin, Chloramphenicol, Cefoxitin, Streptomycin, Amoxicillin-
 62 Clavulanic acid and Linezolid. Inhibition zones were recorded and interpreted according to the
 63 manufacturer's instructions and CLSI guidelines (CLSI 2020).

64 Biochemically confirmed isolates were subjected to molecular confirmation for the detection of
 65 16s-rDNA gene and species-specific *sau* gene using multiplex PCR as per protocol described by
 66 Strommenger et al. 2003 with slight modification. DNA was extracted by boiling and snap chilling
 67 method. Multiplex PCR assay was performed according to Mehrotra et al. 2000 for the genes
 68 encoding for staphylococcal classical enterotoxins A, B, C, and D. Multiplex PCR was carried for
 69 genes *vanA* and *vanB* encoding for vancomycin according to Hizlisoy et al. 2018. A multiplex PCR
 70 assay was performed for detection of *tetK* and *tetM* genes responsible for tetracycline resistance
 71 according to Kumar et al. 2010. All primer sequences used in this study are mentioned in Table No. 2.
 72 Amplified PCR products were confirmed on 1.5% of agarose gel stained with ethidium bromide.

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Table no. 2 Primer sequences used in this study.

Gene	Primer	Oligonucleotide Sequence (3'-5')	Amplicon Size
16S-rDNA	16S-F	CAG CTC GTG TCG TGA GAT GT	420
	16S-R	AAT CAT TTG TCC CAC CTT CG	
<i>S. aureus</i> specific	<i>sau</i> -F	AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG	107
	<i>sau</i> -R	CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA	
<i>Sea</i>	<i>sea</i> -F	GGT TAT CAA TGT GCG GGT GG	102
	<i>sea</i> -R	CGG CAC TTT TTT CTC TTC GG	
<i>Seb</i>	<i>seb</i> -F	GTA TGG TGG TGT AAC TGA GC	164
	<i>seb</i> -R	CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA	
<i>Sec</i>	<i>sec</i> -F	AGA TGA AGT AGT TGA TGT GTA TGG	451
	<i>sec</i> -R	CAC ACT TTT AGA ATC AAC CG	
<i>Sed</i>	<i>sed</i> -F	CCA ATA ATA GGA GAA AAT AAA AG	278
	<i>sed</i> -R	ATT GGT ATT TTT TTT CGT TC	
<i>Nuc</i>	<i>nuc</i> -F	AGT TCA GCA AAT GCA TCA CA	400
	<i>nuc</i> -R	TAG CCA AGC CTT GAC GAA CT	
<i>vanA</i>	<i>vanA</i> -F	GTA GGC TGC GAT ATT CAA AGC	231
	<i>vanA</i> -R	CGA TTC AAT TGC GTA GTC CAA	
<i>vanB</i>	<i>vanB</i> -F	GTA GGC TGC GAT ATT CAA AGC	330
	<i>vanB</i> -R	GCC GAC AAT CAA ATC ATC CTC	
<i>tetK</i>	<i>tetK</i> -F	GTA GCG ACA ATA GGT AAT AGT	360
	<i>tetK</i> -R	GTA GTG ACA ATA AAC CTC CTA	
<i>tetM</i>	<i>tetM</i>	AGTGGAGCGATTACAGAA	158
	<i>tetM</i>	CATATGTCTGGCGTGTCTA	

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76 3. RESULTS AND DISCUSSION

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78 Isolation of *S. aureus* isolates was conducted according to the Bacterial Analytical Manual
 79 (FDA 2019) and streaking on selective media Baird-Parker media. The prevalence of *S. aureus* in 8
 80 different dairy farms was in the order with an overall prevalence of 27.85%. Among the 280 samples
 81 collected from 4 different sources, the highest prevalence was found among the dairy workers
 82 (50.00%), followed by the dairy animals (28.50%), followed by farm environment (20.83%), and lowest
 83 in dairy equipment (18.70%). Within the animal source, the highest prevalence was seen in milk
 84 (52.50%), followed by udder (31.25%), and the lowest seen in fecal samples (13.70%). Within the
 85 farm environment, the prevalence of *S. aureus* was in the decreasing order of drainage water
 86 (37.50%), floor swabs (25%), and equal prevalence in drinking water, feed, and farm air (12.50%).

87 The presence of bacteria in dairy farms indicates potential cross-transmission from dairy
 88 animals and dairy farm workers and its environment recirculation through contaminated feed, air
 89 currents, drinking water, and drainage. In the study, milk and dairy farm workers followed by udder
 90 swabs were found to be potential reservoirs of bacterium. contamination of the environment indicated
 91 its transmission and survival potential. Earlier studies related to the prevalence of *S. aureus* have
 mentioned wider variation. Gwida et al. (2021) found a high prevalence of 59.30% in dairy animals,

100% in milk, 50.00% in teat swabs, and 43.50% in fecal samples. Prevalence in fecal samples was slightly lower than the prevalence noted by Badaway et al. (2022). Multiples of authors like Lee et al. (2012), Liu et al. (2018), Regasa et al. (2019), Thakur et al. (2020), Tibebu et al. (2021), Banu & Geberemedhin (2022) and Liu et al. (2022) noted prevalence in milk in the range 15.00% to 58.33%. The highest prevalence (82.50%) in dairy farm workers was reported by Gwida et al. (2021), while lowest (3.33%) was recorded by Lee et al. (2012). Whereas other studies showed prevalence of 25.00%, 22.90%, 19.23%, 16.51% and 7.00% by Regasa et al. (2019), Liu et al. (2018), Tibebu et al. (2021), Banu & Geberemedhin (2022). A prevalence of 35.42% reported by Liu et al. (2022) in the sewage samples from dairy goat farms, 27.78% in the soil of the floor and 7.50% in feed nearly matches with present cross-sectional study. Deddefo et al. (2023) noted a 10% prevalence in water used for cleaning udder and milkers' hands which matches with the present study, while Ganai et al. (2015) reported a 46.66% prevalence of the bacterium in floor swabs which was a higher as compared to this study. Many authors reported variable presence of *S. aureus* in dairy equipment as Banu & Geberemedhin (2022) found a 12.73% prevalence and Deddefo et al. (2023). Tibebu et al. (2021) noted the prevalence of *S. aureus* at different farms in the range of 15.79% to 58.33%.

1.1 Molecular confirmation of *S. aureus*

For molecular confirmation of *S. aureus*, biochemically confirmed isolates were subjected to detect genus-specific 16S-rRNA and species-specific *sau* gene using multiplex PCR phenotypically positive isolates were confirmed genotypically using 16S-rDNA, however, 63 isolates carried *sau* gene showing overall 80.76% molecular prevalence of *S. aureus*. 47 out of 57 (82.45%) isolates including 7 out of 11 fecal (63.63%), 15 out of 21 milk (71.42) and 25 out of 25 (100%) isolates from udder swabs from the animal source were confirmed for the presence of *sau* gene. 8 isolates (100%) from dairy farm workers were positive for *S. aureus* at molecular level. From farm environment, 6/10 isolates harboured *sau* gene including one each from drinking water, drainage water, feed and farm air, and two from floor swabs. Dairy equipment swab isolates showed 2 out of 3 bacteria possessing *sau* gene. Presence of *sau* gene is considered gold standard for identification of *S. aureus* (Hanon 2017). In the present study, genotypic confirmation of the isolates was carried out using 16S-rRNA and *sau* gene, which confers coagulase gene detection.

1.2 Characterisation of virulent determinant genes of the isolates.

In the present study, most potent enterotoxin expressing genes viz. *sea*, *seb*, *sec* and *sed* were screened by the protocol given by Mehrotra et al. (2000). Overall prevalence of virulence gene *sea* and *seb* was 7.93% (5/63) and 9.52% (6/63), respectively. The prevalence of virulent gene *sea* was 7.01% (4/57) and 12.8% (1/8) from dairy animal and dairy farm workers, respectively and within the animal source, *sea* producing isolates were from milk (2) and udder-swabs (2), while the prevalence of virulent gene *seb* was 7.01% (4/57) and 25.00% (2/8) from dairy animal and dairy farm workers, respectively, within the animal source, *sea* producing isolates were from milk (1) and udder swabs (3). Even et al. 2009 stated expression of SEs gene is linked to the Agr-related quorum sensing system to host cell interaction. In the present study, SEs genes were expressed by the isolates from milk, udder swabs, and dairy farm workers. As enterotoxin production is type dependent and host-pathogen interaction and *L. lactis* competes with *S. aureus* for expression of *sec* and *sed* (Even et al. 2009), *it could be correlated with* expression of only *sea* and *seb* is seen in the current study. Present enterotoxin prevalence of *sea* was slightly lower than Liu et al. (2018). Similar pattern was observed in the case of *seb* also. The prevalence of *seb* observed in hand swabs was nearly equal to the prevalence noted by Zeinhom et al. (2015). In the present study, *sea* and *seb* were found in 5 isolates from udder swabs.

1.3 Antibiogram study of *S. aureus* isolates

All biochemically confirmed isolates were subjected to an antibiogram study using Kirby Bauer disc diffusion method (Bauer, 1966) and interpreted based on CLSI guidelines (CLSI 2020). Resistance to Cefoxitin was found in samples from every source except in dairy equipment, indicating dairy farm workers and dairy animals acting as main drivers' further spreads to the environment and equipment. Its cross-resistance is due to penicillin and cephalosporin mainly through the *mecA* gene. The resistance pattern for erythromycin, being the highest resistance in dairy animals and dairy equipment followed by equal resistance in dairy farm workers and the environment, highlighted transmission from dairy animals and dairy equipment to dairy farm workers and the environment. Antibiotics of class macrolide used at the field level were found to be azithromycin not erythromycin and Gagliotti et al. (2006) highlighted cross-resistance to erythromycin due to azithromycin. The aminoglycosides group *i.e.*, amikacin and streptomycin showed the highest resistance in dairy farm workers, but another antibiotic gentamicin was found to be the least resistance in each of the sources. In the present study, it was noticed that transmission of resistance for aminoglycosides had equally contributed by dairy animals and dairy farm workers and its persistence in its environment reflected

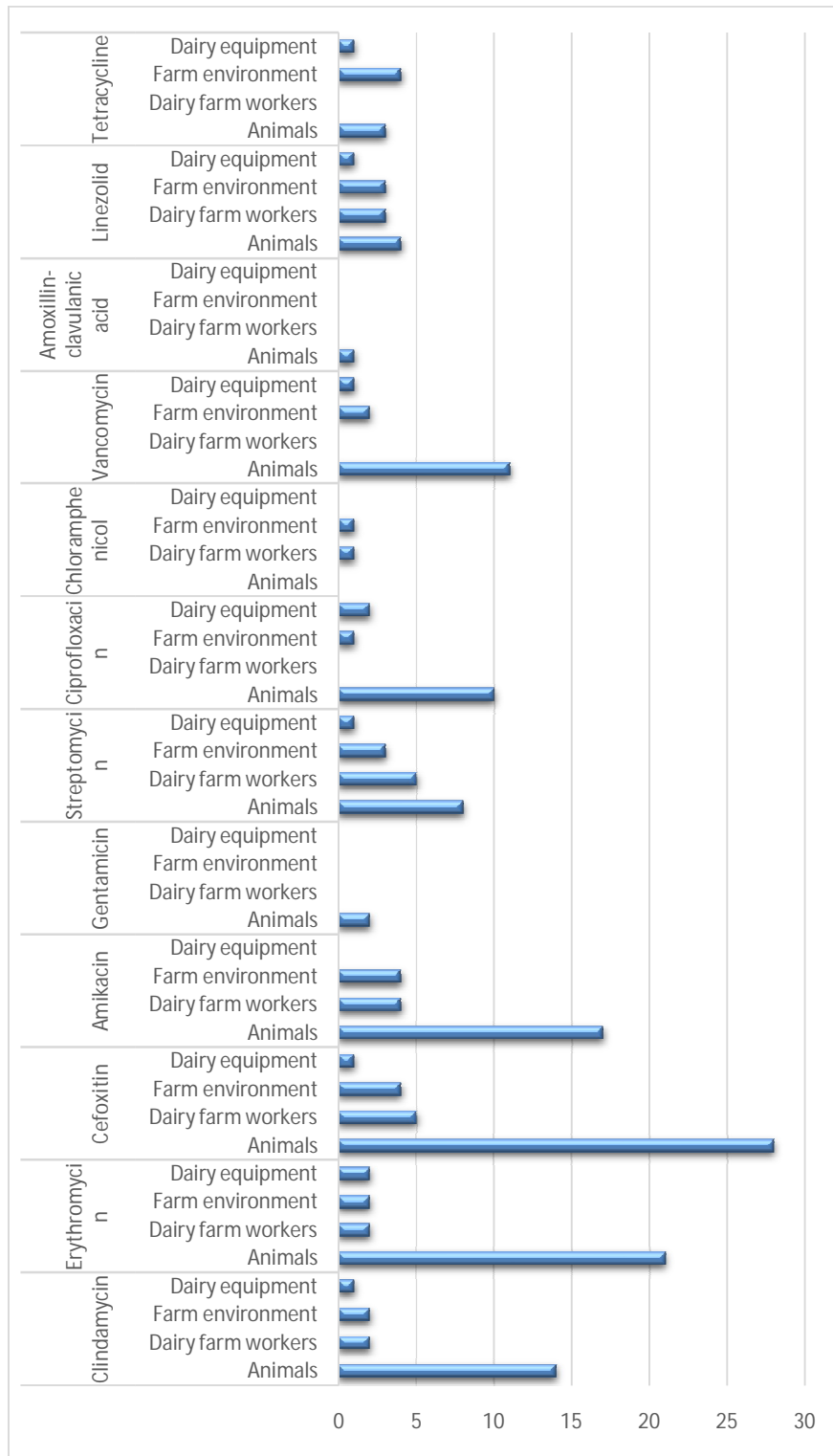
152 hygienic condition of dairy animals and dairy farm workers. AntibioGram profile for clindamycin,
153 highest resistance was seen in isolates from dairy animals indicating its source spread to dairy
154 equipment, dairy farm workers and dairy environment. Resistance to vancomycin is of utmost
155 importance. Moderate resistance was found in dairy animals' environment and dairy equipment. But
156 among these, major resistance was observed in dairy animals indicating its source even though its
157 use in veterinary practice is still not found. More interestingly, its use in human medicine was noticed
158 but zero resistance was seen. This contrasting result gained immense importance practical point of
159 view. With future aspect, its surveillance should be conducted time to time. 40% isolates from dairy
160 environment mainly drainage water, farm air and floor swabs were found to be tetracycline resistant
161 which may predict that dairy animals from previous production may be harboured and shed
162 tetracycline resistant bacteria in dairy environment. Thus, dairy environment was acting as main
163 driver. AntibioGram studies conducted by Dweba et al. (2019) revealed highest resistance to penicillin
164 G followed by ceftiofur and erythromycin and lowest resistance to ciprofloxacin and gentamicin which
165 shows similarities with current studies. Liu et al. (2018) also found moreover similar resistance
166 patterns irrespective of the source. Ganai et al. (2015) resistance to penicillin G (68.75%), ampicillin
167 (65.625%), and streptomycin (59.375%) in the dairy environment. Akindolire et al. (2015) and Liu et
168 al. (2018) noted similar resistance patterns for erythromycin but Mbindyo et al. (2021) noted quite
169 lower than the present study. Overall, the studies consistently show significant levels of resistance to
170 penicillin, ampicillin, tetracycline, erythromycin, and other commonly used antibiotics. Antibiotic
171 resistance noted by Juwita et al. (2022) from human source isolates in dairy farms was found to be in
172 contrast to the current study, due to variation in the selection of antimicrobial preparations. The
173 antibioGram study conducted by Ganai et al. (2015) is quite similar concerning amikacin, but higher
174 than the current study for streptomycin and gentamicin.

175 **3.4 Detection of antimicrobial resistant genes**

176 In the present study, tetracycline-resistant genes (*tetK* and *tetM*), vancomycin-resistant gene
177 (*vanA* and *vanB*) were screened. In this study, neither *vanA* nor *vanB* were expressed in the isolates.
178 In the present study, the prevalence of *tetM* was 20% (2/10) in farm environments, 1.75% (1/57) in
179 dairy animals, and 33.33% (1/3) in dairy equipment with an overall prevalence of 5.19%. The *tetK*
180 gene was not harbored by any one of the isolates. In the present study, the *vanA* and *vanB* genes
181 responsible for vancomycin resistance were expressed in any of the isolates and these results match
182 with Bhattacharya et al. (2016) who also noticed the absence of these genes. However, Qu et al.
183 (2019) found the *vanA* gene in 4% of staphylococcal isolates from bovine clinical mastitis but were
184 unable to detect the *vanB* gene. In contrast, Bissong and Ateba (2020) detected the *vanB* gene in 5
185 isolates but *vanA* was not detected. So also, Hizlisoy et al. (2018) showed an 11.00% prevalence of
186 the *vanB* gene but did not find the *vanA* gene in *S. aureus* isolates. Similarly, Hizlisoy et al. (2018)
187 and Liu et al. (2018) found a more predominant *tetM* gene than *tetK* which agrees with the findings of
188 the present study. AntibioGram studies have shown intermediate to high resistance and this difference
189 in phenotypic and genotypic resistance profile might be attributed to the presence of other plasmid-
190 mediated tetracycline resistant determinants viz. *tetL*, *tetN*, *tetQ*, *tetW*, *tetA*, *tetB* and *tetC* possibly
191 transferred to collected isolates (Jahantigh et al. 2020; Leroy et al. 2019).

192 **4. CONCLUSION**

193 Presence of indicator organisms viz. *S. aureus* in dairy farms along with the development of
194 antimicrobial resistance, exhibited phenotypically as well as genotypically, raises significant public
195 health concern with a possibility of cross-contamination from animal to human and vice-versa and
196 from environment too. The presence of classical enterotoxins from animal and human source samples
197 indicates public threat directly as milk is consumed by each age group of the community. The
198 possibility of transmission potential of antimicrobial-resistant genes cannot be rejected although they
199 were not exposed directly. Genotypic profiling of antimicrobial-resistant genes should be investigated
200 thoroughly including multiple resistant determinants.



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Fig. 1. Overall antimicrobial resistance pattern of *S. aureus*

205 **COMPETING INTERESTS**

206 The authors have declared that no competing interests exist.

207

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211 **AUTHORS' CONTRIBUTIONS**

212 This work was carried out in collaboration among all authors. All authors read and approved the final
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214

215 Disclaimer (Artificial intelligence)

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225 Details of the AI usage are given below:

226 1.

227 2.

228 3.

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