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Molecular Characterisation of Antibiotic Resistance in *Staphylococcus haemolyticus* Isolates from Chennai, South India

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Abstract

***Staphylococcus haemolyticus* is a highly resistant opportunistic pathogen having close genomic relatedness with other virulent species of staphylococci. However, compared to *Staphylococcus aureus* and *Staphylococcus epidermidis*, little is known about the resistance genes of *S. haemolyticus*. The purpose of this study was to characterise antibiotic resistance genes in *S. haemolyticus* isolates. Standard microbiological techniques were used to identify and confirm 104 *S. haemolyticus* isolates included in the study. Antibiotic susceptibility testing and D-test were performed, followed by PCR amplification of various resistance determinants *(mecA, ermA, ermC, msrA, aac(6')-Ie-aph(2"), ant(4')-Ia,aph(3')-IIIa, tetK, tetM, dfrA, fusB, fusC, fusD* and *mupA).* Methicillin resistance was observed in 93.3% of study isolates. The maximum number of isolates showed resistance to erythromycin (n=79, 76%), followed by ciprofloxacin (n=66, 63.5%) and cotrimoxazole (n=58, 55.8%). In the D-test, 8 isolates showed inducible ($iMLS_B$) and 11 showed constitutive ($cMLS_B$) resistance. Among the resistance determinants, *mecA* gene (93.3%) was the most prevalent, followed by *dfrA* (50.5%). Furthermore, *aac(6')-Ie-aph(2")* and *aph(3')-IIIa* combination was observed in 26.9% of isolates, and *aac(6')-Ie-aph(2")* alone was present in 3.8% of isolates. Among the study isolates, 17.3% exhibited *tetK* gene, whereas only 1% exhibited *tetM;* a combination of *tetK* and *tetM* was observed in one isolate. The *fusB* and *fusC* were present in 11.5% of isolates, and 12.5% of the isolates were positive for *mupA*. In conclusion, the present study underlines the concern of increasing antibiotic resistance among *S. haemolyticus* isolates. Avoiding misuse/overuse of antibiotics along with continuous surveillance programs can reduce the spread of antibiotic resistance.**

Keywords: *S. haemolyticus* Resistance, Multidrug-resistant *S. haemolyticus*, Antibiotic Drug Resistance

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INTRODUCTION

Staphylococcus haemolyticus is an opportunistic pathogen and the second most frequently isolated coagulase-negative staphylococci (CoNS), with high degree of genetic relatedness to *Staphylococcus aureus* and *Staphylococcus epidermidis*.[1] It has an average nucleotide sequence similarity of 75% with *S. aureus* and *S. epidermidis*. Thus, there is a high probability that *S. haemolyticus* could act as a reservoir of resistance genes and disseminate them, thereby posing a threat of antibiotic resistance in hospital setup.[2] Another unique feature of *S. haemolyticus* genome is that it undergoes constant rearrangement due to the presence of various insertion sequences.[3] Empirical treatment with broad-spectrum antibiotics and genomic diversity due to frequent genomic rearrangements have led to the selection of multi-resistant strains that slowly replace susceptible strains in hospitals. These findings are consistent with the fact that among CoNS, *S. haemolyticus* possesses the highest level of resistance to commonly used antibiotics.[4,5] Despite being the second most commonly isolated CoNS, there is a paucity of available data regarding its antimicrobial resistance. Hence, this study aimed to elucidate the antibiotic pattern and molecular characterisation of resistance genes in *S. haemolyticus* isolates from various clinical samples.

MATERIALS AND METHODS

Study Isolates

A total of 104 *S. haemolyticus* isolates were collected from a tertiary care centre in Chennai during March 2016–January 2017 and used for further research. Initial sampling and identification of CoNS using standard sampling methods were performed by technical experts from the Microbiology Laboratory of the tertiary care centre. Further identification and species confirmation were performed as described below. The sources of the collected isolates are given in Table 1.

Table 1. Clinical sources of the study isolates

Source of the isolate	% (No. of isolates)
Skin and soft tissues	51.9 % (n=54)
High vaginal swab	25.9 % (n= 27)
Semen	13.5 % (n=14)
Urine	5.8 % (n=6)
Ascitic fluid	1.9 % (n=2)
Sputum.	1 % (n=1)

Identification and Confirmation of *S. haemolyticus*

Staphylococcus isolates were initially identified using standard microbiological techniques such as Gram staining, catalase test, oxidation-fermentation test, coagulase test (tube and slide coagulase), DNase test, and mannitol fermentation on mannitol salt agar. Further species confirmation was performed using alkaline phosphatase, ornithine decarboxylase, urease, novobiocin and polymyxin B susceptibility, and carbohydrate (maltose, mannose, trehalose and sucrose) fermentation tests.[6]

Phenotypic Screening of Antibiotic Resistance

(i) Antibiotic Susceptibility Testing

The Kirby-Bauer disc diffusion method was used to test antibiotic sensitivity using the following antibiotic discs at the concentrations mentioned: cefoxitin (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), erythromycin (15 µg), gentamicin (10 µg), linezolid (30 µg), tetracycline (30 µg), rifampicin (5 µg), fusidic acid (10 µg), mupirocin (200 µg), and vancomycin (30 µg).[7] The zone diameter was measured and interpreted according to the Clinical Laboratory Standards Institute guidelines (CLSI, 2015).[8] *S. aureus* ATCC 25923 was used as the control strain.

(ii) Detection of Methicillin Resistance

The Kirby-Bauer disc diffusion method was performed to screen for resistance to methicillin using cefoxitin antibiotic disc (30 µg).[7] For *S. haemolyticus*, a zone diameter of ≤ 24 mm was considered methicillin resistant.

(iii) Detection of Inducible and Constitutive Clindamycin Resistance

The D-test was performed to detect inducible clindamycin resistance of the isolates, and the results were interpreted according to the CLSI guidelines, 2015.[8] Briefly, erythromycin (15

Table 2. Primer sequence, product size, PCR cycling conditions and reference of the various genes screened in the study

Gene	Sequence	Product size (bp-basepair)	Cycling condition (˚C-degree Celsius, min-minute, sec-seconds)			Ref.
			Denaturation	Cycles	Final extension	
mvaA	F:5'GGTCGCTTAGTCGGAACAAT-3' F:5'CACGAGCAATCTCATCACCT-3'	271 bp	92˚C for 3 min	30 cycles of 92˚C -1 min, 56˚C -1 min, 72 ˚C -1 min	72 ˚C for 3 min	9
mecA	F:5'TGCTATCCACCCTCAAACAGG-3' R:5'AACGTTGTAACCACCCCAAGA-3'	286 bp	94˚C for 4 min	25 cycles of 94˚C-30 sec, 54˚C-30 sec, 72 ˚C -1 min	72 ˚C for 5 min	10
aph(3')-IIIa	F:5'CGATGTGGATTGCGAAAACT-3' R:5'CACCGAAATAACTAGAACCC-3'	175 bp	94˚C for 4 min	30 cycles of 94˚C-1 min, 57˚C-2 min, 72 ˚C- 1 min	72 ˚C for 5 min	11
aac(6')-Ie-aph(2'')	F:5'CATTATACAGAGCCTTGGGA-3' R:5'AGGTTCTCGTTATTCCCGTA-3'	279 bp				
ant(4)-I	F:5'ATGGCTCTCTTGGTCGTCAG-3' R:5'TAAGCACACGTTCCTGGCTG-3'	367 bp				
ermA	F:5'AAGCGGTAAACCCCTCTGA -3' R:5'TTCGCAAATCCCTTCTCAAC-3'	190 bp	94˚C for 4 min	30 cycles of 94˚C-1 min, 54˚C-30 sec, 72 ˚C-1 min	72 ˚C for 5 min	12
ermC	F:5'AATCGTCAATTCCTGCATGT-3' R:5'TAATCGTGGAATACGGGTTTG-3'	299 bp				
tetK	F:5'GTAGCGACAATAGGTAATAGT-3' R:5'GTAGTGACAATAAACCTCCTA-3'	360 bp				
tetM	F:5'AGTGGAGCGATTACAGAA-3' R:5'CATATGTCCTGGCGTGTCTA-3'	158 bp				
msrA	F:5'GAAGCACTTGAGCGTTCT-3' R:5'CCTTGTATCGTGTGATGT-3'	287 bp	94˚C for 4 min	30 cycles of 94˚C-1 min, 50˚C-30 sec, 72 ˚C- 30 sec	72 ˚C for 5 min	13
dfrA	F:5'CTCACGATAAACAAAGAGTCA–3' R:5'CAATCATTGCTTCGTATAACG – 3'	201 bp				
mupA	F:5'TATATTATGCGATGGAAGGTTGG-3' R:5'AATAAAATCAGCTGGAAAGTGTTG-3'	456 bp	94˚C for 2 min	30 cycles of 94˚C-45 sec, 53˚C-30 sec, 72 ˚C-45 sec	72 ˚C for 2 min	14
fusB	F:5'CCGTCAAAGTTATTCAATCG 3' R:5'ACAATGAATGCTATCTCGACA 3'	496 bp	94˚C for 2 min	30 cycles of 94˚C-45 sec, 53˚C-30 sec, 72 ˚C -45 sec	72 ˚C for 2 min	15
fusC	F:5'GGACTTTATTACATCGATTGAC 3' R:5'CTGTCATAACAAATGTAATCTCC 3'	128 bp				
fusD	F:5'AATTCGGTCAACGATCCC 3' R:5'GCCATCATTGCCAGTACG 3'	525 bp				

Table 3. Antibiotic resistance data comparing resistant phenotypes and genotypes (n-104)

Antibiotic	Phenotypic Resistance			Genotypic Resistance {Respective Genes- No. (%)}
	No. of Non-Susceptible Isolates (N-104)	(R- resistant, I- intermediate susceptibility)	(%)	
Cefoxitin	97	R-97	93.3%	*mecA* - 97 (93.3%)
Erythromycin	79	R-70, I-9	76%	*ermC* - 40 (38.5%) *msrA* - 33 (31.7%) *msrA+ermC* - 5 (4.8%) *msrA+ermA* - 1 (1%)
Cotrimoxazole	58	R-50, I-8	56%	*dfrA* - 54 (50.5%)
Gentamicin	40	R-28, I-12	38.5%	*aac* - 4 (3.8%) *aac+aph* - 28 (26.9%)
Tetracycline	25	R-19, I-6	24%	*tetK* - 18 (17.3%) *tetM* - 1 (1%) *tetK+tetM* - 1 (1%)
Mupirocin	13	R-13	12.5%	*mupA* - 13 (12.5%)
Fusidic acid	24	R-24	23.1%	*fusB* - 12(11.5%) *fusC* - 12 (11.5%)

µg) disc and clindamycin (2 µg) disc were placed 15 mm apart (measured from the edge of the disc) in a previously swabbed lawn culture of the isolates with growth matching the turbidity of 0.5 McFarland standard. The zone of inhibition was observed the following day after incubation at 37°C. Blunting of the clindamycin zone near the erythromycin antibiotic disc (D-shape) showed inducible macrolide-lincosamide-streptogramin B ($iMLS_B$) resistance phenotype, whereas resistance to both erythromycin and clindamycin showed constitutive resistance ($cMLS_B$).

Genotypic Methods

DNA Extraction and Polymerase Chain Reaction

DNA extraction from all the study isolates was performed using the boiling lysis method; the extracted DNA was amplified for each of the resistance genes by polymerase chain reaction (PCR) in Mastercycler® Gradient (Eppendorf, Hamburg, Germany). The PCR products were then subjected to agarose gel electrophoresis, and the respective bands were visualised using Gel Logic 212 PRO imaging system. Analysis was carried out using the Carestream Molecular Imaging Software (Carestream Health, Incorporated, USA).

(i) Molecular Confirmation of *S. haemolyticus*

PCR amplification of the *mvaA* gene was performed for molecular confirmation of *S. haemolyticus* isolates.[9]

(ii) Genes conferring Antibiotic Resistance

The genes conferring resistance screened in the study were as follows: *mecA*- gene conferring methicillin resistance, *aac(6')-Ie-aph(2'')*, *aph(3')-IIIa* and *ant(4')*- aminoglycoside modifying enzymes, *msrA, ermA* and *ermC*- genes conferring macrolide resistance, *dfrA*- gene conferring trimethoprim resistance, *tetK* and *tetM*- genes conferring tetracycline resistance, *fusB, fusC* and *fusD*- fusidic acid resistant genes, *mupA*- mupirocin resistant gene. The primers, PCR cycling conditions, and reference for the respective resistance determinants are shown in Table 2.

Statistics

GraphPad Prism version 9 was employed to perform Fischer's exact test. The association between antibiotic resistance and its respective resistance determinants was tested ($p \leq 0.05$ was considered statistically significant).

RESULTS

All the phenotypically identified *S. haemolyticus* isolates (n=104) were confirmed by the presence of *mvaA* gene.

Phenotypic Screening of Antibiotic Resistance

(i) Antibiotic Susceptibility Testing

To vancomycin and linezolid, 100% susceptibility was shown by all tested isolates. The highest resistance (n=79, 76%) was observed for erythromycin, followed by ciprofloxacin (n=66, 63.5%) and cotrimoxazole (n=58, 55.8%). Relatively lower level of resistance was observed for gentamicin (n=40, 38.5%), followed by tetracycline (n=25, 24%), fusidic acid (n=24, 23.1%), clindamycin (n=19, 18.3%), mupirocin (n=12, 11.5%) and rifampicin (n=11, 10.6%). The overall antibiotic resistance profiles of the isolates are given in Figure 1.

(ii) Methicillin Resistance

The cefoxitin disc diffusion result revealed that majority of *S. haemolyticus* isolates were resistant to methicillin (n=97, 93.3%).

(iii) Detection of Inducible and Constitutive Clindamycin Resistance

Nineteen of the 104 isolates were non-susceptible to clindamycin, of which 14 were resistant, and the remaining five showed intermediate susceptibility. Inducible clindamycin resistance ($iMLS_B$) was observed in eight isolates, and the remaining 11 isolates showed constitutive resistance ($cMLS_B$).

Genotypic Screening of Antibiotic Resistant Genes

S. haemolyticus isolates (n=97, 93.3%) expressed the *mecA* gene, indicating resistance to methicillin. A high number of isolates were non-

Table 4. Sample wise antibiotic resistance profile

N (%)	Skin & soft tissues	High Vaginal swab	Semen	Urine	Ascitic fluid	Sputum
Cefoxitin	52 (50%)	25 (24%)	11 (10.6%)	6 (5.8%)	2 (1.9%)	1 (1%)
Ciprofloxacin	40 (38.5%)	14 (13.5%)	6 (5.8%)	4 (3.8%)	1 (1%)	1 (1%)
Erythromycin	46 (44.2%)	19 (18.3%)	9 (8.7%)	2 (1.9%)	2 (1.9%)	1 (1%)
Clindamycin	14 (13.5%)	2 (1.9%)	2 (1.9%)	1 (1%)	-	-
Cotrimoxazole	40 (38.5%)	12 (11.5%)	3 (2.9%)	1 (1%)	1 (1%)	1 (1%)
Tetracycline	10 (9.6%)	8 (7.7%)	5 (4.8%)	1 (1%)	1 (1%)	-
Gentamicin	27 (26%)	8 (7.7%)	2 (1.9%)	1 (1%)	1 (1%)	1 (1%)
Rifampicin	6 (5.8%)	2 (1.9%)	2 (1.9%)	-	1 (1%)	-
Mupirocin	10 (9.6%)	3 (2.9%)	-	-	-	-
Fusidic Acid	10 (9.6%)	7 (6.7%)	4 (3.8%)	1 (1%)	1 (1%)	1 (1%)

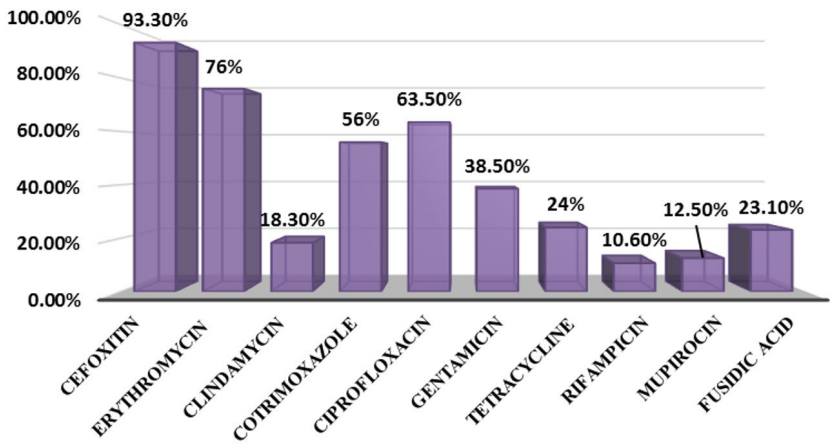

Figure 1. Antibiotic resistance profile of study isolates.

susceptible to erythromycin (n=79), of which 40 isolates (38.5%) were positive for the *ermC* gene and 31.7% (n= 33) were positive for the *msrA* gene. Five isolates (4.8%) contained a combination of the *msrA* and *ermC* genes, whereas one isolate (1%) showed a combination of the *msrA* with *ermA* genes. Non-susceptibility to cotrimoxazole was observed in 58 isolates (50: resistance, 8: intermediate resistance). The trimethoprim resistance-encoding gene *dfrA* was present in 54 isolates (52%). PCR detection of aminoglycoside modifying enzymes was performed for all gentamicin non-susceptible isolates (28: resistance, 12: intermediate resistance). A combination of the *aac(6')-Ie-aph(2'')* and *aph(3')-IIIa* genes was detected in 28 isolates (26.9%), and the *aac(6')-Ie-aph(2'')* gene alone was observed in four isolates (3.8%). Nineteen isolates were resistant and six showed intermediate resistance to tetracycline (n=25, 24%), of which both genes were present in one of the isolates (1%). The *tetK* gene alone was present in 18 isolates (17.3%), and the *tetM* gene alone was present in one isolate (1%). Fusidic acid resistance was phenotypically observed in 24 isolates, of which 12 (11.5%) were positive for the *fusB* gene, and the remaining 12 (11.5%) were positive for the *fusC* gene; however, the *fusD* gene was absent. High level of mupirocin resistance was observed in 13 isolates; all 13 isolates (12.5%) were positive for the *mupA* gene. The representative gel pictures of the resistance genes screened in the present study are given in Figure 3. The complete antibiotic resistance profiles of the study isolates with both resistant phenotypes and genotypes are listed in Table 3. The correlation between the isolate source and antibiotic resistance was also determined (Figure 2). Strains isolated from the skin and soft tissue infections exhibited a comparatively high percentage of resistance to all antibiotics, followed by isolates from genital tract samples, such as high vaginal swab and semen, which showed increased antibiotic resistance. The sample-wise distribution of antibiotic resistance and its determinants is given in Figure 2 and Table 4 and 5.

Statistical Analysis

No significant difference was observed between the antibiotic resistance and its determinants.

DISCUSSION

S. haemolyticus has been well known for its resistance to multiple antibiotics, which is also evident from the fact that it acquired methicillin

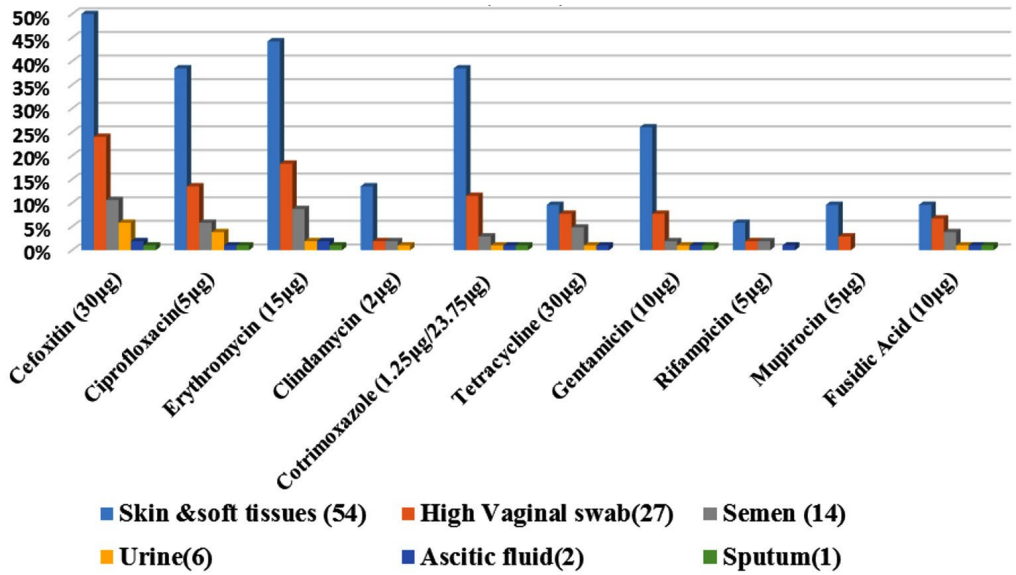

Figure 2. Correlation between source of sample and antibiotic resistance (n - 104).

resistance much earlier than other species of staphylococci.[1] In this study, 93.3% of the isolates were methicillin resistant, and all the resistant isolates exhibited the *mecA* gene; however, in the study conducted by Barros et al.,[4] among 64 methicillin-resistant *S. haemolyticus* isolates, 87% showed the *mecA* gene. In another study by Silva et al.,[16] the *mecA* gene was present in 26 of 27 methicillin-resistant isolates. This proves that the phenotypic method cefoxitin disc diffusion is economical and can be reliably performed in a limited setup for surveillance of methicillin resistance.

Identification of a high percentage (85%) of multi-resistant strains was consistent with the results of other studies. Multidrug-resistant (MDR) strains in the study were defined as those "acquired non-susceptibility to at least one agent in three or more antimicrobial categories" (Magiorakos et al.).[17] *S. haemolyticus* genome undergoes constant rearrangements, which is attributable to its multidrug resistance.

Indiscriminate and inappropriate use of broad-spectrum antibiotics has significantly increased the incidence of antibiotic resistance. This was reflected in the study results. Erythromycin non-susceptibility was observed in the maximum number of isolates (76%), followed by ciprofloxacin (63.5%) and cotrimoxazole (55.8%), the three being the most commonly prescribed broad-spectrum drugs in clinical setting. Surprisingly, last resort and the least prescribed drugs such as vancomycin and linezolid have shown 100% susceptibility. Similar results were reported by Krzyminska et al.[18]

MLS antibiotics, though chemically different, have similar resistance mechanism of ribosomal modification encoded by the erythromycin ribosome methylation (erm) gene.[19,20] MLS antibiotics are clinically significant in the treatment of Gram-positive infections. Hence, cross-resistance between them is a clinical concern.[21] In this study, among erythromycin-resistant isolates, the MS_B phenotype was

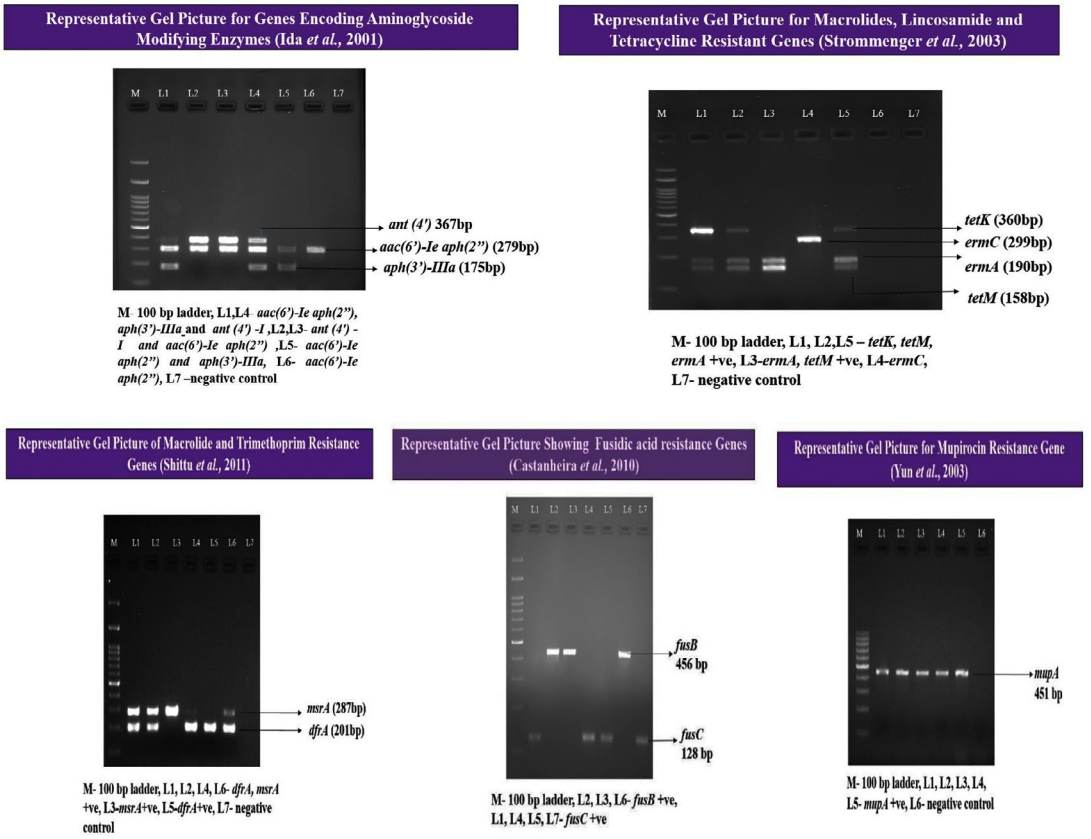

Figure 3. Representative gel pictures of the resistance genes screened in the study.

Table 5. Sample wise distribution of antibiotic resistance determinants

	CX (n-97)	GEN (n-40)		COT (n-58)	ERY (n-79)				TET (n-25)			MUP (n-13)	FUS (n-24)	
Genes	*mecA*	*aac*	*aac+ aph*	*dfrA*	*msrA*	*ermC*	*msrA+ ermC*	*msrA+ ermA*	*tetK*	*tetM*	*tetK+ tetM*	*mupA*	*fusB*	*fusC*
Source of Infection														
Skin &soft tissues (54)	52 (50%)	4 (3.8%)	15 (14.4%)	29 (27.9%)	25 (24%)	28 (26.9%)	3 (2.9%)	1(1%)	6 (5.8%)	-	1 (1%)	10 (9.6%)	4 (3.8%)	6 (5.8%)
High Vaginal swab(27)	25 (24%)	-	8 (7.7%)	12 (11.5%)	4 (3.8%)	6 (5.8%)	1 (1%)	-	7 (6.7%)	-	-	3 (2.9%)	3 (2.9%)	4 (3.8%)
Semen (14)	11 (10.6%)	-	2 (1.9%)	8 (7.7%)	2 (1.9%)	3 (2.9%)	-	-	4 (3.8%)	1 (1%)	-	-	2 (1.9%)	2 (1.9%)
Urine(6)	6 (5.8%)	-	1 (1%)	2 (1.9%)	1 (1%)	1 (1%)	-	-	1 (1%)	-	-	-	1 (1%)	-
Ascitic fluid(2)	2 (1.9%)	-	1 (1%)	2 (1.9%)	1 (1%)	1 (1%)	1 (1%)	-	-	-	-	-	1 (1%)	-
Sputum(1)	1 (1%)	-	1 (1%)	1 (1%)	-	1 (1%)	-	-	-	-	-	-	1 (1%)	-
TOTAL (n-104)	97	4	28	54	33	40	5	1	18	1	1	13	12	12

[CX-cefoxitin, GEN- gentamicin, COT-cotrimoxazole, ERY-erythromycin, TET-tetracycline, MUP-mupirocin, FUS-fusidic acid].

predominant (n=60, 57.7%), followed by $cMLS_B$ (n=11, 10.6%) and $iMLS_B$ (n=8, 7.7%). Furthermore, MLS phenotypes are considered to vary according to the geographic location. Hence, when analysing a similar Indian study by Manoharan et al.,[5] on isolates from southern India, mainly Puducherry, $cMLS_B$ and MS_B phenotypes had almost the same predominance (42.5% and 40.3%, respectively), whereas the MS_B phenotype was predominant in the present study from Chennai.

Trimethoprim resistance is either chromosomally mediated that occurs due to mutations in the *dfrG* gene encoding dihydrofolate reductase (DHFR), the enzyme involved in the folate pathway, or plasmid mediated that occurs due to variants of DHFR having low affinity for trimethoprim.[22] These DHFR variants are encoded by the *dfrA, dfrD* and *dfrK* genes, of which the *dfrA* gene is the most common. In the present study, 54/58 cotrimoxazole-resistant isolates exhibited the *dfrA* gene. The results were in concordance with the study by Aggarwal et al.,[23]; they screened three trimethoprim resistance genes from *S. aureus* isolates, of which the majority of isolates (45/74) carried the *dfrA* gene. In contrast, Manoharan et al.[5] reported that among *S. haemolyticus* study isolates, 89.7% of cotrimoxazole-resistant isolates were *dfrG*-positive, and the *dfrA* gene in combination with other genes, including *dfrD* and *dfrG*, was present only in 2% of the isolates.

Aminoglycoside resistance in staphylococci is due to target site modification, leading to inactivation of the drug caused by aminoglycoside modifying enzymes.[24] Plasmid mediated genes {*aac(6')-Ie-aph(2'')*, *aph(3')-IIIa* and *ant(4')*} encoding three commonly found aminoglycoside modifying enzymes {AAC(6')/APH(2'), APH(3')-III, and ANT (4')-I, respectively} were screened in this study. A high number of aminoglycoside-resistant isolates (26.9%) exhibited a combination of the *aac(6')-Ie-aph(2'')* and *aph(3')-IIIa* genes in the present study. These findings disagree with those of other published studies that revealed the presence of *aac(6')-Ie-aph(2'')* alone rather than in combination. Both studies showed the lowest prevalence of the *ant(4')* gene, whereas it was completely absent in the present study.[25,18]

Tetracycline resistance in staphylococci is either due to active efflux by acquiring the plasmid-mediated genes *tetK* and *tetL* or the chromosomal resistance genes *tetM* and *tetO*.[26] In this study, the most frequently observed genes, *tetK* and *tetM*, were screened. Among the 25 tetracycline non-susceptible isolates, 72% were positive for the *tetK* gene. Unlike the findings of the present study, the *tetM* gene was predominant (67%) and the *tetK* gene was present in only 33% of the isolates in the study conducted by Duran et al.,[27] However, the findings of the study by Manoharan et al.,[5] were similar to those of the present study, with 91.5% prevalence of the *tetK* gene. Apart from the resistant isolates, susceptible isolates exhibiting resistance genes were also observed in both previous studies but was absent in the present study.

The most common resistance mechanism to fusidic acid is protecting the target site by the genes encoding the fusB family of proteins, thereby preventing the translocation of elongation factor G (EF-G) from the ribosome, leading to inhibition of protein synthesis. Casanteira et al.,[18] compared the occurrence rates of fusidic acid resistance in Australia, Canada and the USA, and observed that the prevalence of fusidic acid resistance in CoNS was the highest in Canada (20%), followed by Australia (10.8%) and the USA (7.2%). In this study, the occurrence of fusidic acid resistance in Chennai, southern India, was 23% among *S. haemolyticus* study isolates. Half of the isolates exhibited prevalence of the *fusB* gene, and the remaining 50% exhibited the *fusC* gene. However, other studies have demonstrated a higher prevalence of the *fusB* gene than that of the *fusC* gene.[15,28]

Mupirocin is a bacteriostatic antibiotic that inhibits protein synthesis. Among the two phenotypes, high level of resistance is mediated by plasmid carrying the *iles2* or *mupA* gene that encodes a novel tRNA synthetase. Among the study isolates, 12.5% exhibited high-level phenotypic resistance to mupirocin, all of which carried the *mupA* gene. These findings are consistent with those of other studies.[29,30] Universal methicillin-resistant *Staphylococcus aureus* (MRSA) decolonisation protocol followed

in hospitals is an important reason for increased resistance to mupirocin. Thus, stabilising the use of mupirocin with proper surveillance and target-based decolonisation may be of great help in controlling mupirocin resistance.

The isolate source was correlated with the resistance phenotypes and genotypes. It is well known that staphylococci normally inhabit the skin and mucous membranes in humans. Hence, the predominant *S. haemolyticus* isolates having the highest resistance to various antibiotics were from the skin and soft tissues. These findings were consistent with those of Palestine and Ethiopia.[31,32] Interestingly, all resistant genotypes and their combinations were observed in isolates from the skin and soft tissues in the present study. In addition to skin and soft tissue infection samples, genital tract samples, such as high vaginal swab and semen, also exhibited high level of antibiotic resistance. Other samples (urine, ascitic, and sputum) were low in number to draw conclusions.

CONCLUSION

A high percentage of antibiotic resistance in opportunistic pathogens such as *S. haemolyticus* is a concern, as it may lead to treatment failure, prolonged hospital stay and increased mortality rate. In addition, there is a greater risk of disseminating resistance genes to other virulent species of staphylococci, making them increasingly arduous in hospital setup.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

REFERENCES

1. Czekaj T, Ciszewski M, Szewczyk EM. Staphylococcus haemolyticus – an emerging threat in the twilight of the antibiotics age. *Microbiology.* 2015;161(11):2061-2068. doi:10.1099/mic.0.000178
2. Cavanagh JP, Hjerde E, Holden MTG, Kahlke T, Klingenberg C, Flægstad T, Parkhill J, Bentley SD, Sollid JUE. Whole-genome sequencing reveals clonal expansion of multiresistant Staphylococcus haemolyticus in European hospitals. *Journal of Antimicrobial Chemotherapy.* 2014;69(11):2920-2927. doi:10.1093/jac/dku271
3. Takeuchi F, Watanabe S, Baba T, Yuzawa H, Ito T, Morimoto Y, Kuroda M, Cui L, Takahashi M, Ankai A, Baba S- ichi, Fukui S, Lee JC, Hiramatsu K. Whole-Genome Sequencing of *Staphylococcus haemolyticus* Uncovers the Extreme Plasticity of Its Genome and the Evolution of Human-Colonizing Staphylococcal Species. *Journal of Bacteriology*. 2005;187(21):7292-7308. doi:10.1128/jb.187.21.7292-7308.2005
4. Barros EM, Ceotto H, Bastos MCF, Dos Santos KRN, Giambiagi-deMarval M. Staphylococcus haemolyticus as an Important Hospital Pathogen and Carrier of Methicillin Resistance Genes. *Journal of Clinical Microbiology.* 2011;50(1):166-168. doi:10.1128/jcm.05563-11
5. Manoharan M, Sistla S, Ray P. Prevalence and Molecular Determinants of Antimicrobial Resistance in Clinical Isolates of *Staphylococcus haemolyticus* from India. *Microbial Drug Resistance*. 2021;27(4):501-508. doi:10.1089/mdr.2019.0395
6. Koneman EW. Koneman. Lippincott Williams & Wilkins; 2006.
7. Bauer AW, Kirby WM., Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. *American journal of clinical pathology*. 1966; 45(4): 493–496.
8. Performance Standards for Antimicrobial Susceptibility Testing, Twenty-Fifth Informational Supplement. CLSI document M100-S25, Wayne, PA: Clinical and Laboratory Standards Institute. CLSI 2015.
9. Pereira EM, Schuenck RP, Malvar KL, et al. Staphylococcus aureus, Staphylococcus epidermidis and Staphylococcus haemolyticus: Methicillin-

resistant isolates are detected directly in blood cultures by multiplex PCR. *Microbiological Research*. 2010;165(3):243-249. doi:10.1016/j.micres.2009.03.003

10. Abimanyu N. Use of Triplex PCR for Rapid Detection of PVL and Differentiation of MRSA from Methicillin Resistant Coagulase Negative Staphylococci. *Journal of clinical and diagnostic research*. Published online 2013. doi:10.7860/jcdr/2013/4523.2731
11. Ida T, Okamoto R, Shimauchi C, Okubo T, Kuga A, Inoue M. Identification of Aminoglycoside-Modifying Enzymes by Susceptibility Testing: Epidemiology of Methicillin-Resistant Staphylococcus aureus in Japan. *Journal of Clinical Microbiology*. 2001;39(9):3115-3121. doi:10.1128/jcm.39.9.3115-3121.2001
12. Strommenger B, Kettlitz C, Werner G, Witte W. Multiplex PCR Assay for Simultaneous Detection of Nine Clinically Relevant Antibiotic Resistance Genes in Staphylococcus aureus. *Journal of Clinical Microbiology*. 2003;41(9):4089-4094. doi:10.1128/jcm.41.9.4089-4094.2003
13. Shittu AO, Okon K, Adesida S, et al. Antibiotic resistance and molecular epidemiology of Staphylococcus aureus in Nigeria. *BMC Microbiology*. 2011;11(1):92. doi:10.1186/1471-2180-11-92
14. Yun H-J . Prevalence and mechanisms of low- and high-level mupirocin resistance in staphylococci isolated from a Korean hospital. *Journal of Antimicrobial Chemotherapy.* 2003;51(3):619-623. doi:10.1093/jac/dkg140
15. Castanheira M, Watters AA, Mendes RE, Farrell DJ, Jones RN. Occurrence and molecular characterization of fusidic acid resistance mechanisms among *Staphylococcus* spp. from European countries (2008). *Journal of Antimicrobial Chemotherapy*. 2010;65(7):1353-1358. doi:10.1093/jac/dkq094
16. Silva PV, Cruz RS, Keim LS, et al. The antimicrobial susceptibility, biofilm formation and genotypic profiles of Staphylococcus haemolyticus from bloodstream infections. *Memórias do Instituto Oswaldo Cruz*. 2013;108(6):812-813. doi:10.1590/0074-0276108062013022
17. Magiorakos AP, Srinivasan A, Carey RB, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect*. 2012;18(3):268-281. doi:10.1111/j.1469-0691.2011.03570.x
18. Krzymińska S, Szczuka E, Dudzińska K, Kaznowski A. Virulence and the presence of aminoglycoside resistance genes of Staphylococcus haemolyticus strains isolated from clinical specimens. *Antonie van Leeuwenhoek*. 2015;107(4):857-868. doi:10.1007/s10482-015-0378-6
19. Schmitz F-J, Verhoef J, Fluit AC, The Sentry Participants Group. Prevalence of resistance to MLS antibiotics in 20 European university hospitals participating in the European SENTRY surveillance programme. *Journal of Antimicrobial Chemotherapy.* 1999;43(6):783-792. doi:10.1093/jac/43.6.783
20. Lim J-A. Prevalence of resistance to macrolide, lincosamide and streptogramin antibiotics in Gram-positive cocci isolated in a Korean hospital. *Journal of Antimicrobial Chemotherapy*. 2002;49(3):489-495. doi:10.1093/jac/49.3.489
21. Szemraj M, Czekaj T, Kalisz J, Szewczyk EM. Differences in distribution of MLS antibiotics resistance genes in clinical isolates of staphylococci belonging to species: S. epidermidis, S. hominis, S. haemolyticus, S. simulans and S. warneri. *BMC Microbiology*. 2019;19(1). doi:10.1186/s12866-019-1496-5
22. Vickers AA, Potter NJ, Fishwick CWG, Chopra I, O'Neill AJ. Analysis of mutational resistance to trimethoprim in Staphylococcus aureus by genetic and structural modelling techniques. *Journal of Antimicrobial Chemotherapy*. 2009;63(6):1112-1117. doi:10.1093/jac/dkp090
23. Aggarwal S, Jena S, Panda S, et al. Antibiotic Susceptibility, Virulence Pattern, and Typing of Staphylococcus aureus Strains Isolated From Variety of Infections in India. *Frontiers in Microbiology*. 2019;10. doi:10.3389/fmicb.2019.02763
24. Doi Y, Wachino J, Arakawa Y. Aminoglycoside Resistance. *Infectious Disease Clinics of North America*. 2016;30(2):523-537. doi:10.1016/j.idc.2016.02.011
25. Perumal N, Murugesan S, Krishnan P. Distribution of genes encoding aminoglycoside-modifying enzymes among clinical isolates of methicillin-resistant staphylococci. *Indian Journal of Medical Microbiology*. 2016;34(3):350-352. doi:10.4103/0255-0857.188339
26. Grossman TH. Tetracycline Antibiotics and Resistance. Cold Spring Harbor Perspectives in Medicine. 2016;6(4):a025387. doi:10.1101/cshperspect.a025387
27. Duran N, Ozer B, Duran GG, Onlen Y, Demir C. Antibiotic resistance genes & susceptibility patterns in staphylococci. *The Indian journal of medical research*. 2012; 135(3): 389–396.
28. Hung W-C, Chen H-J, Lin Y-T, et al. Skin Commensal Staphylococci May Act as Reservoir for Fusidic Acid Resistance Genes. de Lencastre H, ed. *PLOS ONE*. 2015;10(11):e0143106. doi:10.1371/journal.pone.0143106
29. Bathoorn E, Hetem DJ, Alphenaar J, Kusters JG, Bonten MJM. Emergence of High-Level Mupirocin Resistance in Coagulase-Negative Staphylococci Associated with Increased Short-Term Mupirocin Use. *Journal of Clinical Microbiology.* 2012;50(9):2947-2950. doi:10.1128/jcm.00302-12
30. Rudresh MS, Ravi GS, Motagi A, Alex AM, Sandhya P, Navaneeth BV. Prevalence of Mupirocin Resistance Among Staphylococci, its Clinical Significance and Relationship to Clinical Use. *Journal of Laboratory Physicians*. 2015;7(02):103-107. doi:10.4103/0974-2727.163127
31. Al Laham DN, Abou Elkhair E, Bashir A, Abdelateef N. Resistance profiles and biofilm formation of coagulase negative staphylococci isolated from clinical specimens in a tertiary care hospital in Palestine. *The International Arabic Journal of Antimicrobial Agents*. 2017;7(3). doi:10.3823/814
32. Deyno S, Fekadu S, Seyfe S. Prevalence and antimicrobial resistance of coagulase negative staphylococci clinical isolates from Ethiopia: A meta-analysis. *BMC Microbiology.* 2018;18(1). doi:10.1186/s12866-018-1188-6