Prevalence and Molecular Determinants of Antimicrobial Resistance in Clinical Isolates of *Staphylococcus haemolyticus* from India

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Aims: Although *Staphylococcus haemolyticus* is considered as a part of normal skin flora, infections associated with them are increasing. Irrespective of the low virulence profile it poses a severe threat to patients with indwelling devices due to its multidrug-resistant nature. The aim of this study was to determine antibiotic resistance patterns and to detect the genes responsible in clinical isolates of *S. haemolyticus*.

Results: All the 356 *S. haemolyticus* isolates were susceptible to glycopeptides. 91.3% were resistant to ceftazidime, 85.4% to erythromycin, 57.3% to co-trimoxazole, 52.8% to clindamycin, whereas only 3.7% of isolates were resistant to linezolid. Tetracycline resistance was found in 16.6% of isolates with *tetK* as the major genetic determinant. Most of the ceftazidime-resistant isolates carried *mecA* gene (99.4%), whereas *dfrG* gene was found only in 57.3% of co-trimoxazole-resistant isolates. Macrolides resistance was seen in 85.4% of isolates with cMLS_B (constitutive macrolide, lincosamide, and streptogramin B) (42.5%) as the major phenotype with *ermC* and *msrAB* genes as the predominant genetic determinants. Among linezolid-resistant isolates all except one showed higher minimum inhibitory concentration (MIC) (>256 µg/mL) with chloramphenicol–florfenicol resistance (*cfr*) gene as the genetic determinant, whereas one isolate had a lower MIC (16 µg/mL) and was negative for *cfr* gene.

Conclusion: Emerging resistance to linezolid is a cause for concern. Strategies to prevent the spread of antibiotic resistance require continuous surveillance of these multidrug-resistant strains.

Keywords: *Staphylococcus haemolyticus*, multidrug resistant, linezolid-resistant *S. haemolyticus*

Introduction

STAPHYLOCOCCUS HAEMOLYTICUS, AN emerging nosocomial pathogen, is considered as the second most common species of coagulase-negative staphylococci (CoNS) and is associated with device-related infections in immunocompromised individuals.¹ Among all the CoNS studied so far, this species is known to be resistant to most antibiotics, including cephalosporins, penicillins, macrolides, tetracyclines, quinolones, and aminoglycosides.^{2,3} The presence of insertion sequences in this organism allows the addition and deletion of genes, thereby conferring a frequent genomic rearrangement that enables the organism to acquire drug resistance. This, in turn, leads to better adaptation and the ability to survive in a hospital environment. *S. haemolyticus* frequently colonizes the skin and mucous membranes of hospitalized patients and acts as a reservoir for antibiotic resistance genes.⁴ Moreover, methicillin resistance rates are much higher than in *Sta-*

phylococcus aureus, with reduced susceptibility to glycopeptides and linezolid also being reported.⁵

Very few studies have dealt with the antibiotic susceptibility pattern of *S. haemolyticus*. This study was undertaken to determine the prevalence and molecular determinants of antibiotic resistance in clinical isolates of *S. haemolyticus* obtained from two centers located in south and north India.

Materials and Methods

This study was conducted using isolates from two centers—Jawaharlal Institute of Postgraduate Medical Education & Research (JIPMER), Puducherry, India (collected from March 2016 to May 2018) and Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India (collected from May 2017 to April 2018). The study was approved by the Institute Human Ethics Committee (IEC), JIPMER, Puducherry. A total of 356 isolates (296 from JIPMER and 60 from PGIMER) were collected from

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different clinical samples such as blood, sterile body fluids, and pus. The isolates were presumptively identified as *S. haemolyticus* using conventional tests and confirmed by MALDI-TOF-MS (BRUKER Daltonics, Germany). Final confirmation was done using PCR with species-specific primers (*nuc* gene). All the isolates were tested for antibiotic sensitivity and the involved genetic mechanisms for antibiotic resistance.

Antibiotic susceptibility testing

Antibiotic susceptibility was tested by Kirby–Bauer disk diffusion method using standard procedures for the following antibiotics: cefoxitin (30 µg), penicillin (10 U), erythromycin (15 µg), clindamycin (2 µg), tetracycline (30 µg), ciprofloxacin (5 µg), trimethoprim–sulfamethoxazole (1.25/23.75 µg), teicoplanin (30 µg), linezolid (30 µg), and mupirocin (200 µg). Vancomycin screen agar (6 µg/mL; Sigma) and minimum inhibitory concentration (MIC) of vancomycin and linezolid (only for linezolid-resistant isolates) was determined using E-test strip (Biomerieux, France). The results were interpreted as per the guidelines of Clinical and Laboratory Standards Institute (CLSI).⁶ *S. aureus* ATCC 25923 (for disk diffusion) and ATCC 29213 (for E-test) were used as controls.

Determination of macrolide resistance phenotypes

The following five different types of macrolide resistance phenotypes were determined by a double-disk test using erythromycin (15 µg) and clindamycin (2 µg): (1) inducible macrolide–lincosamide–streptogramin (iMLS_B) phenotype—flattening of the zone of inhibition around clindamycin (D-test positive); (2) constitutive macrolide–lincosamide–streptogramin (cMLS_B) phenotype—erythromycin and clindamycin resistant; (3) macrolide and streptogramin B (MS) phenotype—erythromycin resistant, clindamycin sensitive, and negative for D-test; (4) lincosamide–streptogramin A/streptogramin B (LSa/b) phenotype—erythromycin sensitive and clindamycin resistant; and (5) sensitive phenotype (SP)—sensitive to both erythromycin and clindamycin.⁷

DNA extraction and PCR

Bacterial DNA was extracted using Mericon DNA Bacteria Plus kit (Qiagen, Germany) as per the manufacturer's instructions. All the isolates were screened for resistance determinants by PCR using primers previously published with slight modification of the conditions from the original reports (Table 1).^{8–13} The following genes conferring resistance to the antibiotics were tested in different reaction mixtures: *mecA* (cefoxitin), *ermA*, *ermB*, and *ermC*

TABLE 1. PRIMER SEQUENCES AND CYCLING CONDITIONS FOR ANTIBIOTIC RESISTANCE GENES

Gene	Primer sequence (5'-3')	Amplicon size (bp)	PCR conditions	References
<i>mecA</i>	F-GTAGAAATGACTGAACGTCCGATA R-CCAATTCACATTGTTTCGGTCTAA	310	94°C for 4 min; 30 cycles of 94°C for 15 sec; 58°C for 15 sec; 72°C for 30 sec; and final extension of 72°C for 10 min	8
<i>cfr</i>	F-TGAAGTATAAAGCAGGTTGGGAGTCA R-ACCATATAATTGACCACAAGCAGC	746	94°C for 5 min; 30 cycles of 94°C for 30 sec; 55°C for 30 sec; 72°C for 30 sec; and final extension of 72°C for 5 min	9
<i>tetK</i>	F-GTAGCGACAATAGGTAATAGT R-GTAGTGACAATAAACCTCCTA	360	95°C for 3 min; 30 cycles of 95°C for 30 sec; 54°C for 30 sec; 72°C for 1 min; and final extension of 72°C for 10 min	10
<i>tetM</i>	F-AGTGGAGCGATTACAGAA R-CATATGTCCTGGCGTGTCTA	158		
<i>ermA</i>	F-GTTCAAGAACAATCAATACAGAG R-GGATCAGGAAAAGGACATTTTAC	421	95°C for 3 min; 30 cycles of 94°C for 30 sec; 55°C for 30 sec; 72°C for 1 min; and final extension of 72°C for 10 min	11
<i>ermB</i>	F-CCGTTTACGAAATTGGAACAGGTAAAGGGC R-GAATCGAGACTTGAGTGTGC	359		
<i>ermC</i>	F-GCTAATATTGTTTAAATCGTCAATTCC R-GGATCAGGAAAAGGACATTTTAC	572		
<i>msrA</i>	F-GGCACAATAAGAGTGTTTAAAGG R-AAGTTATATCATGAATAGATTGTCCTGTT	940	95°C for 5 min; 30 cycles of 94°C for 1 min; 50°C for 1 min; 72°C for 90 sec; and final extension of 72°C for 10 min	12
<i>msrB</i>	F-TATGATATCCATAATAATTATCCAATC R-AAGTTATATCATGAATAGATTGTCCTGTT	595		
<i>dfrD</i>	F-CCCTGCTATTAAAGCACC R-CATGACCAGATAACTC	340	95°C for 7 min; 30 cycles of 94°C for 30 sec; 57°C for 30 sec; 72°C for 1 min; and final extension of 72°C for 10 min	13
<i>dfrK</i>	F-CAAGAGATAAGGGGTTTCAGC R-ACAGATACTTCGTTCCACTC	229		
<i>dfrG</i>	F-TGCTGCGATGGATAAGAA R-TGGGCAAATACCTCATTCC	405		
<i>dfrA</i>	F-CACTTGTAATGGCACGGAAA R-CGAATGTGTATGGTGGAAAG	270		
<i>mupA</i>	F-CCCATGGCTTACCAGTTGA R-CCATGGAGCACTATCCGAA	1.6 kb	94°C for 5 min; 30 cycles of 94°C for 15 sec; 55°C for 15 sec; 72°C for 90 sec; and final extension of 72°C for 5 min	13

(macrolide–lincosamide–streptogramin B [MLS_B]), *msrA* and *msrB* (macrolides), *tetK* and *tetM* (tetracycline), *dfrA*, *dfrD*, *dfrG*, and *dfrK* (trimethoprim–sulfamethoxazole), chloramphenicol–florfenicol resistance (*cfr*) (linezolid), and *mupA* (mupirocin). PCR was performed using Mastercycler Nexus gradient (Eppendorf, Hamburg, Germany). The PCR product was analyzed on 1.5% agarose gel stained with ethidium bromide and observed under UV (GelDoc XR; BioRad, California). For the gene targets, the PCR-amplified products were sequenced, and once confirmed, they were used as positive controls. PCR for resistance genes was performed in all the test isolates regardless of their phenotype.

Statistical analysis

Statistical analysis was carried out using IBM SPSS version 20. Chi-square or Fisher's exact test was used to calculate associations between antibiotic resistance and its determinants ($p \leq 0.05$ was considered statistically significant).

Results

Of the total 356 isolates, 100% susceptibility was observed to teicoplanin and vancomycin, whereas susceptibility to tetracycline was 83.4%, mupirocin 57.6%, clindamycin 47.2%, and trimethoprim–sulfamethoxazole was 42.7%; susceptibility to ceftioxin (8.7%) and penicillin (5.9%) was extremely low (Fig. 1). Susceptibility to most antibiotics was lower in isolates from PGIMER when compared with those from JIPMER. However, statistically significant difference was observed only for clindamycin ($p = 0.001$).

MIC of vancomycin ranged from 0.064 to 2 µg/mL, and the median MIC values differed between the two centers (MIC₅₀ in south Indian isolates was 0.75 µg/mL whereas it was 1 µg/mL among the north Indian isolates) ($p = 0.01$). However, the MIC₉₀ values were similar in both the regions (1.5 µg/mL).

Majority of the macrolide-resistant isolates belonged to cMLS_B phenotype (42.5%) followed by the MS phenotype (40.3%), iMLS_B phenotype (13.7%), and LSa/b phenotype (3.5%) (Table 2).

FIG. 1. Percentage susceptibility to different antibiotics among isolates of *Staphylococcus haemolyticus* obtained from the two study sites. CIP, ciprofloxacin; CLI, clindamycin; ERY, erythromycin; FOX, ceftioxin; MUP, mupirocin; LNZ, linezolid; PEN, penicillin; SXT, trimethoprim–sulfamethoxazole; TEC, teicoplanin; TET, tetracycline; VAN, vancomycin.

TABLE 2. DISTRIBUTION OF PHENOTYPES AND GENOTYPES AMONG MACROLIDE-RESISTANT *STAPHYLOCOCCUS HAEMOLYTICUS* ISOLATES

Phenotypes	Genes	Number positive
cMLS _B (134)	<i>ermC</i> + <i>msrA</i> & <i>B</i>	102
	<i>msrA</i> & <i>B</i>	28
	<i>ermC</i>	3
	<i>msrA</i>	1
iMLS _B (43)	<i>ermC</i>	26
	<i>ermC</i> + <i>msrA</i> & <i>B</i>	14
	<i>msrA</i> & <i>B</i>	2
	<i>ermC</i> + <i>msrB</i>	1
MS (127)	<i>msrA</i> & <i>B</i>	125
	<i>msrA</i>	1
	<i>msrB</i>	1
LSa/b (11)	<i>msrA</i> & <i>B</i>	7
SP (41)	<i>msrA</i> & <i>B</i>	8

Resistance determinants

Resistance to macrolides was mediated only by *ermC*, *msrA*, and *msrB*, whereas *ermA* and *ermB* were not detected in any of the resistant isolates. The cMLS_B phenotype was mediated predominantly by a combination of *ermC*, *msrA*, and *msrB* (Figs. 2 and 3), whereas MS-resistant phenotype was almost exclusively mediated by *msrA* and *msrB* combination (125/127; 98.4%). However, a combination of *msrA* and *msrB* was able to encode resistant phenotype among most of the isolates (155/356; 43.5%) and in 20.9% (28/134) of isolates with cMLS_B phenotype. The combination of *msrA* and *msrB* genes was also responsible for the LSa/b phenotype in this study, whereas eight macrolide-sensitive isolates were also positive for both *msrA* and *msrB* genes (Table 2).

Most of the ceftioxin-resistant isolates (99.4%) harbored *mecA* gene (Fig. 4). *mupA* gene was found in 98% of the

FIG. 2. Gel picture of multiplex PCR reaction for erythromycin-resistant genes. Lane 7 (L7): 100 bp molecular marker; L1–L4, L8: test isolates positive for *ermC* gene (572 bp); L5, L6: test isolates negative for *erm* genes; L9: PC *ermB* (359 bp); L10: PC *ermA* (421 bp); L11: PC *ermC* (572 bp); L12: NC. NC, negative control; PC, positive control.

FIG. 3. Gel picture of multiplex PCR reaction for erythromycin-resistant genes. Lane 1 (L1): 100 bp molecular marker; L2, L6, L8–L13: test isolates positive for *msrAB* genes (940 and 595 bp); L7: test isolate negative for *msr* genes; L3: PC *msrA* (940 bp); L4: PC *msrB* (595 bp); L5: NC. NC, negative control; PC, positive control.

151 phenotypically resistant isolates (Fig. 5). Among the tetracycline-resistant isolates (59), 91.5% harbored *tetK* as the major genetic determinant and 3.4% of the isolates carried the *tetM* gene (Fig. 6). However, for three (5.1%) isolates, the genetic mechanism for tetracycline resistance could not be identified. Moreover, 12 isolates positive for *tetK* gene were sensitive to tetracycline. Among the trimethoprim-sulfamethoxazole-resistant isolates (204), 89.7% harbored *dfrG* as the only genetic determinant, and in 6.9% of the isolates, it was found in combination with other genes (Fig. 7). In 10 isolates *dfrA* and *dfrD* genes were detected together, and in 7 (3.4%) of the resistant isolates the genetic mechanisms for resistance could not be determined (Table 3). None of the isolates were positive for *dfrK* genes. Among the linezolid-resistant isolates (13; 3.7%), the MIC was very high (≥ 256 $\mu\text{g/mL}$) in 12 isolates, whereas one isolate had a lower MIC at 16 $\mu\text{g/mL}$. There was a significant association between high-level linezolid resistance and *cfr* gene, with this gene being absent in the low-level-resistant isolate (Fig. 8). Three phenotypically sensitive isolates positive for *cfr* gene had an MIC of 1.5 and

FIG. 4. Gel picture of PCR reaction for cefoxitin-resistant genes. Lane 1 (L1): 100 bp molecular marker; L2–L6: test isolates positive for *mecA* gene (310 bp); L7: PC *mecA* (310 bp); L8: NC. NC, negative control; PC, positive control.

FIG. 5. Gel picture of PCR reaction for mupirocin-resistant genes. L5, L14: 1 kb molecular marker; L1, L2, L4, L6, L8, L10, L16, L18–L21: test isolates positive for *mupA* gene (1.6 kb); L3, L7, L9, L11, L15, L17: test isolates negative for *mupA* gene; L12: PC *mupA* (1.6 kb); L13: NC. NC, negative control; PC, positive control.

3 $\mu\text{g/mL}$, respectively. Phenotypic and genotypic test results for *S. haemolyticus* isolates are shown in Supplementary Data.

No difference was observed in the resistance determinants for the different antibiotics among the isolates between the two centers.

Discussion

Most of the *S. haemolyticus* isolates (310/356, 87.1%) tested in this study were found to be multidrug resistant (254/296, 85.8% from JIPMER and 56/60, 93% from PGI-MER). The multidrug resistance was defined as resistance to at least one agent in three or more different classes of antibiotics. The commonest combination of antimicrobial classes exhibiting resistance was beta lactams, quinolones, and trimethoprim-sulfamethoxazole (184/356, 51.7%), whereas a combination of linezolid, tetracycline along with mupirocin was least frequent. The greatest number of isolates were resistant to five class of antibiotics (108) followed

FIG. 6. Gel picture of PCR reaction for tetracycline-resistant genes. Lane 7 (L7): 100 bp molecular marker; L1, L2, L3, L9, L10: test isolates positive for *tetK* gene (360 bp); L11: test isolate positive for *tetM* gene (158 bp); L8: test isolate negative for *tetK* & *tetM* genes; L5: PC *tetK* (360 bp); L6: PC *tetM* (158 bp); L4–NC. NC, negative control; PC, positive control.

FIG. 7. Gel picture of PCR reaction for trimethoprim-sulfamethoxazole-resistant genes. L1, L14, L27, L40: 100 bp molecular marker; L8–L11: test isolates positive for *dfrA* (270 bp) gene; L12: PC *dfrA*; L21–L24: test isolates positive for *dfrD* (340 bp) gene; L25: PC *dfrD*; L28–L31, L33: test isolates positive for *dfrG* (405 bp) gene; L36: PC *dfrG*; L2–L7, L15–L20, L32, L34, L35, L38, L39, L41–L52: test isolates negative for *dfr* genes; L13, L26, L37, NC: negative control; PC, positive control.

by four class of antibiotics (72), six class of antibiotics (71), three class of antibiotics (46), and seven class of antibiotics (12), and least frequent was resistant to eight class of antibiotics (1). Rajkumar *et al.* noted that among the CoNS species, *S. haemolyticus* isolates displayed greater multidrug resistance than *S. epidermidis*.¹⁴

Methicillin resistance in staphylococci is because of the expression of a modified penicillin-binding protein, PBP2a, which is encoded by *mecA* or *mecC* genes. This study noted most isolates to be ceftiofur resistant (99.4%). Two ceftiofur-resistant isolates were negative for the *mecA* gene, wherein resistance might have occurred due to other mechanisms such as *mecC* or hyperproduction of penicillinase. Although this is a rather rare finding, Barros *et al.* reported five of the methicillin-resistant *S. haemolyticus* isolates to be negative for the *mecA* gene.²

The MLS resistance phenotype patterns among staphylococci could differ across geographical regions. In this study, both cMLS_B and MS phenotypes were high (42.5% and 40.3%) among the isolates, whereas iMLS_B phenotype was found in only 13.7% among all the erythromycin-resistant isolates. The rarest detected resistance phenotype was LSa/b (3.5%). In an Indian study, Zachariah *et al.* noted that among the MRCoNS, the most prevalent phenotype was the MS phenotype (34%), followed by cMLS_B (22%) and iMLS_B phenotypes (18%).⁷ However, Mišić *et al.* in 2017 reported iMLS_B (33.4%) to be the most common phenotype

among all staphylococci, with MS being detected in 17.6%, cMLS_B in only 8.9%, and LSa/b among 1% of the isolates.¹⁵

Clindamycin is generally recommended for treating skin and soft tissue infections and among the 167 isolates from such sites, 80 (47.9%) were clindamycin resistant. These high rates of macrolide with concomitant clindamycin resistance (either inducible or constitutive) preclude their use in skin and soft tissue infections.

Erythromycin resistance in *S. haemolyticus* is encoded by *ermA/B/C* and *msrA/B* genes. All *S. haemolyticus* isolates that were found resistant to erythromycin by phenotypic methods contained at least one of the macrolide resistance genes. Inducible or constitutive expression of *erm* genes results in the production of enzymes that methylate 23S rRNA thereby altering binding sites for MLS_B antibiotics. Active efflux mediated by the *msr* (macrolides-streptogramin B) gene also confers resistance to erythromycin. Drug modification is mediated by *lnu* (A/B) gene, which codes for lincosamide nucleotidyl transferases, with resistance to lincosamides.⁷

FIG. 8. Gel picture of PCR reaction for linezolid-resistant genes. Lane 7 (L7): 100 bp molecular marker; L1–L4, L6, L8–L11: test isolates positive for *cfr* gene (746 bp); L5: test isolate negative for *cfr* gene; L12: PC *cfr* gene (746 bp); L13: NC. NC, negative control; PC, positive control.

TABLE 3. DISTRIBUTION OF PHENOTYPES AND GENOTYPES AMONG TRIMETHOPRIM-SULFAMETHOXAZOLE-RESISTANT *STAPHYLOCOCCUS HAEMOLYTICUS* ISOLATES (N=204)

<i>dfr</i> genes	Number positive	Number negative
<i>dfrG</i>	183	7
<i>dfrA</i> and <i>dfrD</i>	10	
<i>dfrA</i> and <i>dfrG</i>	2	
<i>dfrA</i> , <i>dfrD</i> , and <i>dfrG</i>	2	

In this study, most of the *S. haemolyticus* isolates harbored *ermC* gene—either alone or in the combination with *msrA* and *B* genes. The *msrA* and *msrB* genes were found to be more frequent in MS resistance phenotype (98.4%); however, the cMLS_B phenotype was determined by a combination of *ermC*, *msrA*, and *msrB* in most of the isolates (76.1%). Nevertheless, a small number of isolates harbored the unusual LSa/b phenotype, with a combination of *msrA* and *msrB* genes. This finding is consistent with that of Mišić *et al.*, which determined that the MS phenotype in staphylococci was a combination of *msrA* and *msrB* (95.6%) genes and the cMLS_B phenotype was a combination of *ermC*, *msrA*, and *msrB* (13.2%) genes.¹⁵ Although *ermA* gene was reported more frequently in *S. aureus*, the *ermC* gene was reported mostly in CoNS, according to Lim *et al.*¹⁶ Furthermore, a few studies reported *ermC* as one of the most common genes in *S. aureus* isolates.¹⁷ Eight macrolide-susceptible isolates harbored macrolide resistance genes, with all being positive for both *msrA* and *msrB* genes. This result was concordant with similar studies conducted by Duran *et al.* who noted 48.7% of the staphylococcal isolates to be resistant to erythromycin, according to the phenotypic method, and 55.4% of the isolates harboring resistance genes, according to the multiplex PCR method.¹⁸

Tetracycline-resistant *S. haemolyticus* isolates acquired either one of the resistance genes, that is, *tetK* or *tetM*, with resistance mechanisms mediated by active drug efflux or ribosomal protection, respectively.¹⁹ In this study, a higher number of tetracycline-resistant isolates (91.5%) carried *tetK* gene. However, recent genome-wide analyses of *S. haemolyticus* by Cavanagh *et al.* in 2014 found that the majority of isolates (86.8% of 38 tetracycline-resistant isolates) had *tetL*, whereas *tetK/tetL* was found in only one isolate.²⁰ Duran *et al.* found 32.7% of tetra-resistant isolates to carry *tetK* gene, whereas the predominant resistance determinant was *tetM* (67.4%) among CoNS.¹⁸ These differences in distribution of tetracycline resistance genes can be due to differences in their prevalence among other staphylococci. Although performed on veterinary specimens, El-Razik *et al.* found 50% of CoNS to have the *tetK* gene.²¹ Moreover, 12 isolates that were positive for the *tetK* gene were sensitive to tetracycline. Duran *et al.* too reported 15 staphylococcal isolates to be positive for *tetK* and *tetM* genes, but these isolates were phenotypically sensitive.¹⁸

Most of the 151 mupirocin-resistant isolates carried *mupA* gene (98%). This gene is considered to be the major determinant for high-level mupirocin resistance. It encodes for altered *IleRS*, which has no affinity for mupirocin.²² Meanwhile, three resistant isolates did not demonstrate the presence of the *mupA* gene; it could be that these isolates carried some other mupirocin resistance genes (*mupB*). Moreover, Oommen *et al.* reported that 28.2% of MRCoNS were mupirocin resistant, whereas only 2.08% of methicillin-resistant *S. aureus* (MRSA) were resistant.²³ In a multicentre study, Desroches *et al.* also found that mupirocin resistance was identified in 10.3% of CoNS and 2.2% of MRSA.²² For the past 5 years, the rates of mupirocin resistance among *S. aureus* isolates from our hospital were observed to be very low (3–4%). In contrast, 42.4% of the *S. haemolyticus* isolates exhibited mupirocin resistance. The emergences of high-level mupirocin resistance in *S. haemolyticus* isolates demonstrate an expanding reservoir

of plasmids encoding mupirocin resistance. These plasmids can be transferred from *S. haemolyticus* to other *Staphylococcus* species.

Two resistance mechanisms of trimethoprim are known till date. One of them involves the mutation of the chromosomal dihydrofolate reductase (DHFR) gene and the other involves resistance genes that encode the variant DHFRs.²⁴ In this study, 89.7% of the trimethoprim-sulfamethoxazole-resistant isolates were *dfrG* positive, and the most common detected gene combinations were *dfrA* and *dfrD* (4.9%). However, among 2% of the isolates, it was found in combination with other genes (*dfrA* and *dfrD*; *dfrA* and *dfrG*). Similarly, Nurjadi *et al.* reported that 94% of *S. aureus* isolates were positive for the *dfrG* gene.²⁴ In this study, none of the isolates were positive for *dfrK* genes. Nevertheless, Argudín *et al.* noted that 39% of *dfrK* and 21% of *dfrG* genes were found in livestock-associated *S. aureus* (ST398), among the strains from Europe.¹² In addition, in seven (3.4%) of the resistant isolates, the genetic mechanisms for resistance could not be determined.

In this study, resistance to glycopeptides was not noted. The median MIC value for vancomycin was 0.75 µg/mL (ranging from 0.064 to 2 µg/mL in the strains from JIPMER) and 1 µg/mL (ranging from 0.25 to 2 µg/mL in the strains from PGIMER). Reduced susceptibility to glycopeptides among *S. haemolyticus* is now increasingly being reported. Kristóf *et al.*²⁵ reported 32% of the 184 isolates to exhibit reduced susceptibility to teicoplanin. They also tested for the presence of vancomycin-heteroresistant strains and found isolates with higher MIC (≥2 µg/mL) had heteroglycopeptide intermediate (45%) and glycopeptide intermediate (36%) phenotype.²⁵ In another study by Nakipoglu *et al.* six of the seven *S. haemolyticus* of nosocomial origin (among the patients and hospital personnel) were heteroresistant to both vancomycin (MIC 5–8 µg/mL) and teicoplanin (32–64 µg/mL). They also found that acquisition of heteroresistance is more frequent among CoNS than *S. aureus*.²⁶ Although in this study, resistance to glycopeptides was not detected, the heteroresistance may be still be present and needs to be tested for. Unpublished data from our centre demonstrated thickening of the cell wall in isolates of CoNS with reduced susceptibility to vancomycin.

A single gene, *cfr* gene, is a methyltransferase gene, conferring resistance to different classes of antibiotics such as phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramins (PhLOPS_A) by specifically methylating the drug target site (A2503 in the 23S rRNA).²⁷ Linezolid resistance is more common among CoNS species, although a few reports exist for MRSA as well. Among the *S. haemolyticus* isolates, 3.7% of linezolid resistance was encountered; one isolate had MIC at 16 µg/mL but was negative for the *cfr* gene. Here the resistance might have been due to other mechanisms such as mutation in 23S rRNA and ribosomal proteins L3 and L4. Most of the linezolid-resistant isolates displayed the unusual pattern of erythromycin sensitivity and clindamycin resistance. Chamon *et al.*²⁸ from Brazil characterized 10 linezolid-resistant CoNS isolates from blood or catheter tips and these isolates were negative for *cfr* gene, whereas mutation in 23S rRNA was the predominant resistance mechanism. These mutations were also common among linezolid-resistant enterococci.

Among the 13 linezolid-resistant isolates, only one isolate did not harbor *cfr* gene, whereas the mutations in 23S rRNA were not tested for. This was a limitation of this study. Interestingly a very few linezolid-susceptible isolates had *cfr* gene (3/343). Similar *cfr*-gene (15.6%; 20/128) presence among the linezolid sensitive LA-MRSA has been reported.²⁹ Gene expressions in these isolates may have been suppressed by as yet unknown factors. *Cfr* gene of both linezolid susceptible (3) and resistant isolates (12) were sequenced and submitted to GenBank under the following accession numbers (MN103293–MN103307). There was no difference in sequences between the two groups of isolates. However, as only a portion of the gene and not the entire gene was sequenced, it is still possible that differences exist in nonsequenced region of the gene. Mutations in genes coding for 23S rRNA were also found to be responsible for reduced susceptibility to linezolid.³⁰ Owing to these facts, the role of *cfr* gene alone to provide resistance to linezolid becomes questionable. However, measuring the expression levels of *cfr* gene along with the detection of mutations responsible for linezolid resistance can shed light on molecular mechanisms of linezolid resistance in this organism. The reduction in cost and simplified analyses makes genome sequencing and total gene expression profiling viable methods when compared with traditional gene sequencing and Quantitative Polymerase Chain Reaction (qPCR)-based gene expression studies. One limitation of our study was failure to detect mutation in 23S rRNA especially in the linezolid-resistant and *cfr* gene negative isolate.

In conclusion, a high antibiotic resistance rate in *S. haemolyticus* isolates is a cause for concern as they may act as a source of resistance determinants to the more pathogenic *S. aureus*. As the L_{Sa}/b phenotype was common among linezolid-resistant isolates, it may be used as marker to predict linezolid resistance.

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Supplementary Material

Supplementary Data

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