Short Communication

Methicillin-Susceptible Teicoplanin-Resistant *Staphylococcus haemolyticus* Isolate from a Bloodstream Infection with Novel Mutations in the *tcaRAB* Teicoplanin Resistance Operon

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SUMMARY: *Staphylococcus haemolyticus* is a coagulase-negative staphylococcus that is frequently isolated from blood cultures. Here, we report a case of methicillin-susceptible *S. haemolyticus* that is resistant to teicoplanin (TEC) and heteroresistant to vancomycin (VAN). The isolate was susceptible to cefoxitin and resistant to TEC by Etest. Population analysis profile-area under the curve analysis confirmed the presence of a VAN heteroresistant subpopulation. Next-generation sequencing analysis of the genome revealed the presence of *blaZ* and *msr(A)*, which encode cross-resistance to macrolide, lincosamide, and streptogramin B, and the quinolone resistance-conferring gene *norA*. In addition, several amino acid substitutions were observed in the TEC resistance operon *tcaRAB*, including I3N, I390N, and L450I in *tcaA* and L44V, G52V, and S87P in *tcaR*, as well as in the transpeptidase encoding gene *walk* (D336Y, R375L, and V404A) and L315 and P316 in *graS*. We hypothesized that this combination of mutations could confer TEC resistance and reduced VAN susceptibility.

Staphylococcus haemolyticus is the second most frequently isolated coagulase-negative *Staphylococcus* (CoNS) species from blood cultures. The combination of the increased use of intravascular indwelling devices, such as catheters and mechanical heart valves, and hospitalization of immunocompromised patients has established CoNS as significant nosocomial pathogen (1). This opportunistic pathogen is known to exhibit multidrug resistance including heteroresistance to glycopeptides (2).

Glycopeptides such as vancomycin (VAN) and teicoplanin (TEC) are being increasingly used to treat multidrug-resistant CoNS, and this increased use has led to the emergence of strains with reduced susceptibility to glycopeptides. Among the CoNS, resistance to glycopeptides is most commonly seen in *Staphylococcus epidermidis* and *S. haemolyticus*, which are 2 strains of significant clinical concern (3). In CoNS species, TEC resistance has now become more common than VAN resistance (4–6). Heteroresistance to VAN in methicillin-resistant CoNS is increasingly being reported in hospital settings (7). However, homogenous resistance to glycopeptides is very rarely seen. Here, we report the first case of a methicillin-susceptible *S. haemolyticus* bloodstream infection in India, showing homogenous intermediate-resistance to TEC and heterogeneous resistance to VAN.

A TEC-resistant *S. haemolyticus* isolate was recovered

from a patient at Christian Medical College, Vellore, a 2,600-bed tertiary level hospital in South India. The patient, a 46-year old woman, who had type II diabetes mellitus for 15 years and a cerebrovascular accident with left hemiplegia, was diagnosed with colonic pseudo-obstruction and was admitted for further clinical management. On the 5th day of hospital admission, she developed sepsis, and a blood sample was collected for culture. She was started on empirical therapy with VAN and meropenem. The blood culture grew *S. haemolyticus* (VB31546). Based on the blood culture result, the initial therapy was replaced with nafcillin and ceftriaxone.

S. haemolyticus was identified using both standard microbiological methods and the Vitek 2 compact system (BioMerieux, Etoile, France). The isolate (VB31546) was susceptible to cefoxitin, gentamicin, netilmicin, clindamycin, rifampin, tetracycline, trimethoprim-sulfamethoxazole, and linezolid but resistant to penicillin and erythromycin. The VB31546 strain was susceptible to VAN (MIC, 1.5 µg/ml) and homogeneously non-susceptible to TEC (MIC, 12 µg/ml). The isolate was screened for VAN heteroresistance with the glycopeptide resistance detection (GRD) Etest, and the results were confirmed by the population analysis profile-area under curve (PAP-AUC) method. The isolate was positive in the GRD Etest. Therefore, this isolate was heteroresistant to VAN with a PAP-AUC ratio of 0.92.

Next generation sequencing (NGS) was used to evaluate the molecular mechanisms of antibiotic resistance, virulence, and biofilm formation in this *S. haemolyticus* strain. Sequencing was performed on the Ion Torrent Personal Genome Machine platform (Life Technologies, Carlsbad, CA, USA) with the 400-bp kit according to the manufacturer's instructions. The sequencing reads were *de novo* assembled using

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AssemblerSPAdes (ver. 5.0.0.0) in Torrent Suite Server (ver. 5.0.3). *De novo* assembled sequences were annotated by submitting the sequences to PATRIC, the bacterial bioinformatics database and analysis resource <<http://www.patricbrc.org>> (8); Rapid Annotation using Subsystem Technology (RAST) pipeline <<http://rast.nmpdr.org/>> (9); and the National Center for Biotechnology Information (NCBI) Prokaryotic Genome Automatic Annotation Pipeline (PGAP). This whole genome shotgun sequence has been deposited in DDBJ/ENA/GenBank under accession no. MAUC00000000.1

The assembled sequence contained 143 contigs, with an average coverage of $56\times$, representing the genome of *S. haemolyticus* (2,526,650 bp). The resistance gene profile was determined with ResFinder 2.1 from the CGE server. The genome of this *S. haemolyticus* strain showed the presence of 3 antibiotic resistant genes, *blaZ*, for beta-lactamase production; *msr(A)* for cross-resistance to macrolides, lincosamide, and streptogramin B; and *norA* for quinolone resistance. Interestingly, this isolate had an empty staphylococcal cassette chromosome *mec* (SCC*mec*) type II without a *mecA* gene (*mecA* dropout), which confers methicillin resistance. The genomic sequence revealed the presence of the IS256 and IS1272 insertion sequence.

Analysis of the genome for the presence of virulence factors showed the absence of the *ica* operon, which is

crucial for biofilm production in staphylococcal species. However, the genes encoding several *ica*-independent biofilm-associated virulence factors such as fibrinogen binding protein (*fbe*), capsular polysaccharide synthesis enzyme (*cap8D*), virulence-associated cell wall anchored protein (*sasH*), staphylococcal accessory regulator (*sarA*), the two-component regulatory system (*arlRS*), and the major autolysin (*atl*) were detected in the *S. haemolyticus* sequence.

The whole genome sequence of *S. haemolyticus* JCSC1435 (GenBank ID: NC_007168) was used as the reference strain for mutational analysis of the glycopeptide resistance determinant locus. The TEC resistance operon (*tcaRAB*) plays a vital role in determining glycopeptide susceptibility. Deletion or mutation of the *tcaRAB* locus has been reported to increase TEC resistance. Three amino acid substitutions were identified in *tcaA* (I3N, I390N, and L450I), a key gene in the *tcaRAB* locus that determines the level of glycopeptide resistance. Mutations were also observed in the transcriptional regulator *tcaR*. Mutation of candidate genes involved in the development of heteroresistance to VAN were detected in the genomic sequence (Table 1). However, no mutation was observed in the *vraSR* two-component regulatory system (TCR). Amino acid substitutions were observed in the WalK/WalR TCR; D336Y, R375L, and V404A were observed in the

Table 1. Genomic characteristics of methicillin-susceptible, vancomycin-heteroresistant and teicoplanin non-susceptible *S. haemolyticus* from bloodstream infection

Functional role	Candidate gene	Amino acid substitution
Teicoplanin resistant gene	<i>tcaA</i>	I3N I390N L450I
	<i>tcaB</i>	—
	<i>tcaR</i>	L44V G52V S87P
Vancomycin resistance-associated gene	<i>vraS</i>	—
	<i>vraR</i>	—
	<i>vraT/yvqF</i>	—
Glycopeptide resistance-associated gene	<i>graS</i>	V256A Insertion of L at 315 Insertion of P at 316
	<i>graR</i>	—
Transpeptidase encoding gene	<i>walK</i>	D336Y R375L V404A
	<i>walR</i>	—
RNA polymerase β -subunit	<i>rpoB</i>	—
Autolysin encoding gene	<i>lytS</i>	Q75H L160I A319V S395A G436E
Clp protease proteolytic subunit	<i>clpP</i>	D194E
Secretory protein	<i>saeS</i>	—
Phosphorylase	<i>phoR</i>	—
N-acetylmuramyl-L-alanine amidase	<i>atl</i>	A63T C1182R
Penicillin binding protein	PBP4	A88V D149N
	PBP2	S704N

—, no mutation.

histidine sensor kinase WalK, but none were detected in WalR. Interestingly, insertion of 6 nucleotides tta at 943–945 and cct at 946–948 led to the addition of a leucine at position 315 and a proline at position 316 in the glycopeptide resistance-associated histidine kinase GraS.

Glycopeptide heteroresistance is common in methicillin-resistant *S. haemolyticus* but rare in methicillin-susceptible *S. haemolyticus*. Moreover, the interpretative breakpoints for TEC resistance recommended by Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) are > 32 µg/ml and > 4 µg/ml, respectively. This wide variation in the interpretative breakpoint further complicates the categorization of staphylococcal strains according to antibiotic susceptibility. Here we report the first case of a methicillin-susceptible, TEC-resistant *S. haemolyticus* strain that is heteroresistant to VAN. Reduced TEC susceptibility can occur without a demonstrated reduction in VAN susceptibility, as was previously reported in *Staphylococcus aureus* (10).

The *tcaRAB* locus determines TEC susceptibility. Inactivation of *tcaA* or deletion of the *tcaRAB* operon increases TEC resistance (11). Similarly, strain VB31546 showed a combination of inactivation of TcaA and amino acids insertions L315 and P316 in GraS, which might increase TEC resistance. These are novel mutations in TEC-resistant *S. haemolyticus* that are reported here. Irrespective of oxacillin resistance, reduced susceptibility to glycopeptides is associated with multidrug-resistant *S. haemolyticus*, and the levels of resistance to glycopeptides and β-lactams are often inversely correlated (12).

In addition to *tcaRAB*-mediated TEC resistance, overproduction of PBP2 or reduction in PBP4 expression can also cause TEC resistance (13,14). Remarkably, the isolated strain was fully susceptible to oxacillin but showed reduced glycopeptide susceptibility. The presence of the 2 insertion sequences, IS256 and IS1272, has been attributed to the adaptation, evolution, and pathogenicity of *S. haemolyticus* (15).

This is the first report of a methicillin-susceptible *S. haemolyticus* strain that is resistant to TEC with reduced VAN susceptibility isolated in India. The propensity of *S. haemolyticus* toward multi-drug resistance and its pathogenic potential in immunocompromised patients emphasize the importance of species-level identification of CoNS and routine testing for both VAN and TEC susceptibility. These results demonstrate the need for surveillance of nosocomial CoNS isolates for

glycopeptide resistance.

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Conflict of interest None to declare.

REFERENCES

1. Natoli S, Fontana C, Favaro M, et al. Characterization of coagulase-negative staphylococcal isolates from blood with reduced susceptibility to glycopeptides and therapeutic options. *BMC Infect Dis.* 2009;9:83.
2. Tacconelli E, Tumbarello M, Donati KG, et al. Glycopeptide resistance among coagulase-negative staphylococci that cause bacteremia: epidemiological and clinical findings from a case-control study. *Clin Infect Dis.* 2001;33:1628-35.
3. Biavasco F, Vignaroli C, Varaldo PE. Glycopeptide resistance in coagulase-negative staphylococci. *Eur J Clin Microbiol Infect Dis.* 2000;19:403-17.
4. Sloos JH, van de Klundert JA, Dijkshoorn L, et al. Changing susceptibilities of coagulase-negative staphylococci to teicoplanin in a teaching hospital. *J Antimicrob Chemother.* 1998;42:787-91.
5. Tsakris A, Papadimitriou E, Douboyas J, et al. Emergence of teicoplanin-resistant *Staphylococcus haemolyticus* clinical isolates in Greece. *J Antimicrob Chemother.* 2000;46:1040-1.
6. Blans M, Troelstra A. Glycopeptide resistance in *Staphylococcus haemolyticus* during treatment with teicoplanin. *Infect Control Hosp Epidemiol.* 2001;22:263-4.
7. Ma XX, Wang EH, Liu Y, et al. Antibiotic susceptibility of coagulase-negative staphylococci (CoNS): emergence of teicoplanin-non-susceptible CoNS strains with inducible resistance to vancomycin. *J Med Microbiol.* 2011;60:1661-8.
8. Wattam AR, Abraham D, Dalay O, et al. PATRIC, the bacterial bioinformatics database and analysis resource. *Nucl Acids Res.* 2014;42:D581-91.
9. Overbeek R, Olson R, Pusch GD, et al. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res.* 2014;42:D206-14.
10. Biavasco F, Vignaroli C, Lazzarini R, et al. Glycopeptide susceptibility profiles of *Staphylococcus haemolyticus* bloodstream isolates. *Antimicrob Agents Chemother.* 2000;44:3122-6.
11. Brandenberger M, Tschierske M, Giachino P, et al. Inactivation of a novel three-cistronic operon *tcaR-tcaA-tcaB* increases teicoplanin resistance in *Staphylococcus aureus*. *Biochim Biophys Acta.* 2000;1523:135-9.
12. Domaracki BE, Evans A, Preston KE, et al. Increased oxacillin activity associated with glycopeptides in coagulase-negative staphylococci. *Eur J Clin Microbiol Infect Dis.* 1998;17:143-50.
13. Shlaes DM, Shlaes JH, Vincent S, et al. Teicoplanin-resistant *Staphylococcus aureus* expresses a novel membrane protein and increases expression of penicillin-binding protein 2 complex. *Antimicrob Agents Chemother.* 1993;37:2432-7.
14. Wootton M, Bennett PM, MacGowan AP, et al. Strain-specific expression levels of *pbp4* exist in isolates of glycopeptide-intermediate *Staphylococcus aureus* (GISA) and heterogeneous GISA. *Antimicrob Agents Chemother.* 2005;49:3598-9.
15. Bouchami O, de Lencastre H, Miragaia M. Impact of insertion sequences and recombination on the population structure of *Staphylococcus haemolyticus*. *PLoS One.* 2016;11:e0156653.