Detection of high levels of resistance to linezolid and vancomycin in *Staphylococcus aureus*

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**Abstract**

Both methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) are rapidly overcoming the current array of drugs. One hundred and fifty isolates from a hospital were studied for resistance towards linezolid and vancomycin. Fifty-four (36.0 %) isolates were MRSA. Both MRSA and MSSA showed high resistance towards linezolid when using the disc diffusion method, with the figures being 48.1 and 29.2 %, respectively. The figures for the E-test were 46.3 and 27.0 %, respectively. The vancomycin resistance was remarkable in MRSA (14.8 %), but relatively low in MSSA (3.1 %). The E-test results were 13.0 and 4.16 %, respectively. The *cfr* gene was detected in 78 % of linezolid-resistant isolates and the *vanA* operon was detected in 74 % of vancomycin-resistant isolates. This level of resistance against linezolid and vancomycin is unprecedented. These results are alarming and highlight the threat of non-treatable *S. aureus* strains.

The rapid emergence of methicillin resistance in *Staphylococcus aureus* has greatly enhanced the threat posed by these organisms [1]. Multidrug resistance is also emerging rapidly in methicillin-sensitive *S. aureus* (MSSA) [2]. Resistance is even being detected against specific drugs, such as vancomycin and linezolid [3].

In this study, 150 pyogenic methicillin-resistant *S. aureus* (MRSA) and MSSA isolates from a local hospital were studied for resistance towards linezolid and vancomycin, the two drugs of choice.

Linezolid is an oxazolidinone agent that inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit, blocking the formation of the initiation complex [4]. It inhibits protein synthesis by binding to the peptidyl transferase centre on the ribosome [5].

In a comparison of the agreement level between various methods, including VITEK 2, E-test, Phoenix, disk diffusion and VITEK, Tenover *et al.* [4] found that automated methods had no advantage over conventional methods in the case of linezolid. They also found the values to be very close for the disc diffusion method and the E-test, with this being 88.0 and 90.0 %, respectively. The overall categorical agreement levels for VITEK 2, E-test, Phoenix, disk diffusion and VITEK were 93.0, 90.0, 89.6, 88.0 and 85.9 %, respectively.

One of the common mechanisms of linezolid resistance is the presence of a transmissible *cfr* ribosomal methyltransferase. This is the leading mechanism in *S. aureus* [2]. Among coagulase-negative staphylococci, the mutation G2576T in 23S rRNA is the more common reason [6].

Vancomycin is the drug of choice for the treatment of infections due to methicillin-resistant *S. aureus* (MRSA), but the increase in vancomycin use has led to the emergence of two types of glycopeptide-resistant *S. aureus*. The first one, designated vancomycin intermediate-resistant *S. aureus* (VISA) is associated with a thickened and poorly cross-linked cell wall, resulting in the accumulation of acyl-d-alanyl-d-alanine (X-D-Ala-D-Ala) targets in the periphery that sequester glycopeptides. The second type, vancomycin-resistant *S. aureus* (VRSA), is due to acquisition of the *vanA* operon, carried by transposon Tn1546, from *Enterococcus* spp. resulting in high-level resistance [7].

We used the disc diffusion test and the E-test to gauge resistance towards linezolid and vancomycin in both MRSA and MSSA. The phenotypic results were supported by the amplification of *cfr* and *vanA* genes.

Pus samples from wounds, ear and skin were collected on sterile swabs from indoor patients at Madinah Teaching Hospital (600 beds), University of Faisalabad, Faisalabad.
Pakistan during March–July 2016. They were transferred to the laboratory within 1 h and processed immediately.

After inoculation on blood agar and nutrient agar plates overnight at 37 °C, observations were made for the presence of Staphylococcal colonies. The suspected colonies were subcultured on the same media to obtain pure cultures and record the colonial morphology. Probable Staphylococcal colonies were found in 255 cases. They were processed for biochemical and molecular identification.

Smears from suspected isolates were observed after Gram-staining. The coagulase test was performed by the tube method [8] and 150 isolates were labelled as S. aureus.

The cefoxitin disk diffusion test is the most accurate phenotypic test to check for the presence of the mecA gene (a major cause of methicillin resistance) in S. aureus [9]. The test isolates were inoculated heavily on Mueller–Hinton agar (Merck, UK) under standardized conditions and a cefoxitin disk (30 µg) was applied. A zone size of <22 mm indicated S. aureus methicillin resistance [9].

Antimicrobial susceptibility testing was performed by the disc diffusion method and interpreted according to the CLSI guidelines [10]. Disc concentrations of 30 µg were used for both linezolid and vancomycin.

We also used the E-test to determine the MICs for linezolid and vancomycin by using strips provided by bioMérieux Vittek, Marcy-l’Etoile, France, and interpreted the results according to the manufacturer’s guidelines. Isolates with linezolid MICs of >4 µg ml⁻¹ were considered to be resistant [6]. In the case of vancomycin, isolates with MICs ≥16 µg ml⁻¹ were classified as VRSA.

For DNA extraction, we followed a reported procedure [11]. The DNA pellet was dissolved in 80 µl of 10 mM Tris buffer (pH 8) and resolved on 0.8 % agarose gel electrophoresis to check integrity.

The 16S rRNA and nuc genes were targeted to confirm the staphylococcus genus and Staphylococcus aureus, respectively [12]. Three primers were used to detect normal and mutated mecA genes (Table 1).

For 16S rRNA amplification, 100 µl of reaction mixture was made up of 7 µl dNTPs, 5 µl of 10× PCR buffer, 3 µl MgCl₂, 2 µl of each primer, 1 µl Taq polymerase (Fermentas, USA), 10 µl template DNA and 10 mM Tris buffer (pH 8.3) to make the volume. The thermal cycler conditions for first step were 1 cycle at 94 °C for 10 min, followed by 35 cycles each of heating at 94 °C for 60 s, at 45 °C for 60 s, and at 72 °C for 75 s, and a final step at 72 °C for 10 min. The amplification products were analysed by electrophoresis at 100V using 2.5 % agarose gel.

The conditions for the other PCR reactions were similar, except for the annealing temperature, which was 50 °C for nuc, 48 °C for mecA, 45 °C for cfr and 55 °C for vanA gene amplification.

The 16S rRNA and nuc genes that confirm that S. aureus was detected in all isolates. Fifty-four (36.0 %) isolates were methicillin-resistant phenotypically. Forty-eight of them (88.9 %) were confirmed genotypically.

Remarkably, both MRSA and MSSA showed relatively high resistance towards linezolid, with the figures being 48.1 and 29.2 %, respectively, as determined by disc diffusion test. The respective figures for the E-test were 46.3 and 27.0 %, respectively. Vancomycin resistance was also significant in MRSA (14.8 %), although it was very low in MSSA (3.1 %), as determined by the disc diffusion test. The respective figures for the E-test were 13.0 and 4.16 %, respectively.

The presence of the cfr gene indicates resistance to linezolid. It was detected in 78 % of linezolid-resistant isolates. Similarly, the vanA operon, which is the cause of complete vancomycin resistance, was detected in 74 % of resistant isolates.

We used primers [13] that can detect not only the mecA gene, but also its mutated forms. The reason for some negative genotypic results may be that some other genes, such as mecR1 and mecl, may also be responsible for methicillin resistance.

Our results for MRSA are comparable to those of Gade and Qazi (42.8 %) [14] and Tiwari et al. (38.4 %) [15], but lower than those in a report from a previous study from Pakistan (51 %) [16].

Linezolid is a leading antimicrobial for use against S. aureus. Resistance to linezolid has been reported to be growing steadily since the first report [3]. In USA, the reported resistance level is less than 2 % [2]. In Spain, only 256 linezolid-resistant S. aureus (2.8 %) were isolated between 2005 and 2009 [6], whereas in India the occurrence has been reported to be between 2–20 % [17]. Our study shows alarmingly high resistance to linezolid, which is unprecedented. The figures were 48.1 % for MRSA and 29.2 % for MSSA, as determined by the disc diffusion test. The results of the E-test were similar.

Because of the importance of these results, we decided to obtain genotypic support for our findings. The most common mechanisms for linezolid resistance are the presence of a transmissible cfr ribosomal methyltransferase or a mutation (G2576T) to the 23S rRNA [2].

Quiles-Melaro et al. [6] studied seven linezolid-resistant S. aureus isolates. They belonged to sequence type ST125 and all carried the cfr gene. Their linezolid MICs ranged from 8 to 16 µg ml⁻¹. Similarly, Molares et al. [18] detected the cfr gene in all 12 linezolid-resistant S. aureus isolates that they studied. However, among coagulase-negative staphylococci, the mutation G2576T was dominant and was detected in 67 out of 79 linezolid resistant isolates.

We targeted the cfr gene for PCR amplification using the method described by Morales et al. [18]. Although the above mentioned authors found all linezolid-resistant
isolates to be positive for the cfr gene, we only detected this gene in 78% of isolates. This may be because our sample size was much larger (55 as compared to 7 and 12).

We decided to only detect VRSA because there is much confusion regarding the definition of VISA [19], and also because the results of VRSA can be supported more easily by molecular methods.

According to the available reports, although there are sporadic reports of resistant isolates, vancomycin remains a very effective drug. In India, Tiwari et al. [15] reported 0.33% resistance. In USA only 13 cases of VRSA were reported up to 2013 [20]. However, in this study, unprecedentedly high resistance to vancomycin (7.3%: 14.8% for MRSA and 3.1% for MSSA) was observed via the disc diffusion test. The E-test results were similar.

Intermediate and complete resistance to vancomycin involves two different mechanisms. VISA arises due to cell wall changes and VRSA due to the acquisition of vanA operon [7]. As we were only interested in finding complete resistance, we targeted vanA operon.

We detected the vanA gene in 72.7% of isolates using the methodology described by Okolie et al. [21]. Although vanA is the most important vancomycin resistance-related gene, it is one of four possible loci [22], which may explain the negative results.

This study presents an unprecedentedly high level of resistance towards linezolid and vancomycin, the frontline drugs against both MRSA and MSSA, and highlights the need to find newer drugs or ways to improve the present options to avoid the scenario of untreatable *S. aureus*.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


