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Original Research Article

Vancomycin and linezolid creeping minimum inhibitory concentrations in clinical isolates of MRSA: Prelude to overt resistance

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Abstract

Background: Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) present a considerable problem in hospitals due to a lack of effective treatment options. The primary antimicrobial agents used to treat MRSA infections are vancomycin and linezolid; however, a rising trend of minimum inhibitory concentrations (MICs) for these antibiotics indicates a gradual decrease in susceptibility, which may result in treatment failure. This study aimed to determine the MIC values of vancomycin and linezolid in MRSA clinical isolates to detect any creep toward resistance.

Materials and Methods: This study included 190 MRSA isolates to determine MICs for oxacillin, vancomycin, and linezolid using the Epsilometer test, adhering to the Clinical and Laboratory Standards Institute (CLSI) 2022 guidelines. The correlation between oxacillin, vancomycin, and linezolid MICs was estimated using Spearman's rho correlation coefficient.

Results: In the present study, MRSA prevalence was 33.8% (n=562/190). Males were outnumbered, and most patients (n=83; 43.7%) belonged to the 31-50 age group. The oxacillin MIC values ranged from 0.75 to ≥256 µg/mL, vancomycin MICs from 0.38 to 2µg/mL, and linezolid MICs from 0.38 to 4µg/mL among MRSA clinical isolates. A gradual increase in vancomycin and linezolid MICs was documented. The oxacillin and vancomycin MICs showed a moderate correlation (0.666), while the oxacillin and linezolid MIC values showed no correlation with the vancomycin and linezolid MIC values.

Conclusion: The gradual increase in MICs for vancomycin and linezolid in MRSA isolates suggests a trend that could lead to total resistance. Ongoing surveillance of MIC values is crucial for enabling prompt adjustments in therapy and preventing treatment failures.

Keywords: Minimum inhibitory concentration, Epsilometer test, Vancomycin, Linezolid, Cefoxitin disk diffusion.

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1. Introduction

In recent years, Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a significant contributor to infections associated with healthcare settings as well as those acquired within the community. Outbreaks have been recorded in most major healthcare settings with increasing frequency.[1] The high prevalence of MRSA infections and colonization is largely due to the growing use of antibiotics, coupled with inadequate infection control practices in healthcare institutions.[2] Prolonged life expectancy among individuals in the community, with underlying morbidities, has contributed to the increasing number of community-acquired MRSA infections.[3]

Globally, a spike in infections with MRSA has been observed in the recent past, resulting in increased morbidity and mortality. This has limited treatment options to existing drugs like vancomycin, tedizolid, linezolid, and fifth-generation cephalosporins.[4] The increase in MRSA cases globally has resulted in excessive use of glycopeptide antibiotics, thereby reducing the efficacy of vancomycin and linezolid against MRSA, leading to therapeutic failures.[5] According to the scoping report of the ICMR Antimicrobial Resistance (AMR) surveillance network, 1.7% of MRSA

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were vancomycin-resistant out of 42.6% of (MRSA) isolates, and 0.6% were linezolid-resistant.[6]

There is a paucity of data on the correlation between the MICs of vancomycin and linezolid MICs among clinically isolated MRSA. Some studies have suggested that an elevated MIC of vancomycin is associated with a concomitant increase in MIC of several unrelated antibiotics to clinically isolated MRSA, thereby influencing their pharmacodynamics and leading to aggressive treatment strategies.[5,7,8] With this background, MIC values of oxacillin, vancomycin, and linezolid were estimated to detect any creep toward resistance.

2. Materials and Methods

This prospective study was carried out in the Microbiology Department of a tertiary care hospital over 30 months (January 2020 to June 2022). A waiver of consent was requested and granted by the Institute Ethics Committee (IEC No. RC/19/121). Clinically significant isolates of *Staphylococcus aureus* from wound, infected tissue, aspirated fluid, and blood, numbering 190 isolates, were included. Repeat isolates were excluded. The sample size 190 was determined based on an assumed correlation coefficient of 0.3, a power of 90%, and an alpha error of 5. Samples were processed, and isolates were identified as *Staphylococcus aureus* using standard microbiological procedures.[9]

2.1. Procedure for screening of Staphylococcus aureus isolates for methicillin resistance

Relevant *Staphylococcus aureus* isolates were screened for the presence of methicillin resistance by cefoxitin (30 µg) disk diffusion test on Mueller-Hinton agar (MHA) according to the Kirby-Bauer disk diffusion method and by testing on oxacillin screen agar (MHA supplemented with 6µg/mL of oxacillin and 4% NaCl). Isolates with a zone of inhibition <22mm to cefoxitin and/ or the presence of a visible growth on the oxacillin screen agar were recorded as MRSA as per the Clinical and Laboratory Standards Institute (CLSI) 2022 guidelines.[10]

2.2. Procedure for oxacillin, vancomycin, and linezolid MIC testing by Epsilometer test (E-test) method

The MHA plates were lawn cultured with the test isolate using a standardized inoculum of 0.5 McFarland for linezolid and vancomycin MIC testing. For oxacillin MIC testing, MHA plates with 2% NaCl were used. Oxacillin, vancomycin, and linezolid E-strips of MIC range 0.016 µg/mL to 256 µmL from Hi Media Pvt. Ltd. were applied on the inoculated MHA plates according to the manufacturer's instructions.

Inoculated plates were kept at 35°C ± 2°C for 18 hours for the MIC testing of linezolid and vancomycin, while oxacillin MIC testing was conducted at 35°C for 24 hours. The MIC values were recorded from the scale in µg/mL, where the symmetrical inhibition ellipse edge intersected the strip.[11] The following MIC breakpoints were used for interpretation (**Table 1**).

Additionally, MIC_{50} and MIC_{90} of oxacillin, vancomycin, and linezolid were determined for MRSA isolates. MIC_{50} was recorded as MIC, at which 50% of the isolates were inhibited, and at $MIC_{90,}$ 90% of the strains were inhibited.[12,13] All recorded data, including the patient's demographic information, were documented in a Microsoft Excel spreadsheet. Each testing batch included two standard *Staphylococcus aureus* strains, ATCC *S. aureus* (MSSA) 25923 and ATCC MRSA 43300.

2.3. Statistical analysis

The results were recorded as frequencies and percentages. The Statistical Package for the Social Sciences (SPSS, version 29.0) was used for statistical analysis. The Bar diagrams were plotted to show the MIC distribution of vancomycin and linezolid for MRSA isolates. The correlation between oxacillin MIC, vancomycin MIC, and linezolid MIC was estimated using Spearman's rho *correlation coefficient, rs.*

3. Results

Out of 562 *Staphylococcus aureus* isolates, 190 were detected as MRSA based on the cefoxitin disk diffusion method and/or by oxacillin screen agar methods, according to the CLSI 2022 standard guideline, accounting for an MRSA prevalence of 33.8% during the study period. Of these, 112 isolates were from male patients (59%), and 78 (41%) from females. The age range of the patients was from less than 1 year to 85 years, with the largest portion, 83 patients (43.7%), being in the 31-50 age group (mean and standard deviation of age: 44.1 ± 18.4 years). The clinical characteristics of patients, along with the sample distribution of 190 MRSA isolates, are presented in **Table 2**.

Table 1: MIC breakpoints for *Staphylococcus aureus* (as per CLSI M 100 document)[10]

Antimicrobial agent tested	Susceptible (µg/mL)	Intermediate (µg/mL)	Resistant (µg/mL)
Oxacillin	≤2	-	≥4
Vancomycin	≤2	4-8	≥16
Linezolid	≤4	-	≥8

Table 2: Patients' clinical characteristics and the sample distribution of 190 MRSA isolates.

S.No.	Clinical diagnosis	Clinical sample cultured	No. of patients	Percentage (%)
	Skin and soft tissue infection	Wound swab	65	34.2
		Tissue	30	15.8
		Aspirated fluid	7	3.7
	Diabetic foot ulcer	Wound swab	15	7.9
		Tissue	23	12.1
	Bone and joint infection	Tissue	19	10.0
	CSOM*	Ear swab	16	8.4
	Sepsis	Blood	15	7.9

*CSOM; chronic suppurative otitis media

Table 3: Distribution of oxacillin MICs among MRSA isolates (n=190) by E test.

Oxacillin MIC (µg/mL)	0.75	1	1.5	2	3	4	6	8	12	16	24	32	64	256
No. of isolates	1	1	2	2	7	58	38	35	6	7	5	7	8	13
Percentage (%)	0.5	0.5	1.1	1.1	3.7	30.5	20	18.4	3.2	3.7	2.6	3.7	4.2	6.8

Table 4: The MIC_{50}, $MIC_{90,}$ and mean MICs of oxacillin, vancomycin, and linezolid among MRSA isolates (n=190)

Antimicrobial agent	MIC_{50}*	MIC_{90}**	Mean MIC
Oxacillin (µg/mL)	6	64	23.92
Vancomycin (µg/mL)	1	2	1.20
Linezolid (µg/mL)	2	4	2.13

*****MIC_{50}- MIC at which 50% of the MRSA isolates were inhibited; ******MIC_{90}- MIC at which 90% of the MRSA isolates were inhibited.

Table 5: Spearman rho correlation coefficient (rs) with significance (*p* value) and their interpretation of tested antimicrobial agents.

S.No.	Antimicrobial agents	Spearman rho correlation coefficient (rs)	Significance; 2-tailed (p value)	Interpretation
	Oxacillin MIC and Cefoxitin (mm)	-0.481	p<0.001*	Highly significant and moderate negative correlation
	Oxacillin MIC and Vancomycin MIC	0.666	p<0.001*	Highly significant and moderate correlation.
	Oxacillin MIC and Linezolid MIC	-0.001	p=0.990	No significance and no correlation
	Vancomycin MIC and Linezolid MIC	-0.134	p=0.065	No significance and no correlation

* Correlation is significant at the 0.01 level (2-tailed).

All 190 MRSA isolates were tested by the E-strip method to detect oxacillin, vancomycin, and linezolid MIC.

3.1. The oxacillin MIC distribution amongst MRSA isolates (***Table 3***).

The oxacillin MIC values ranged between 0.75µg/mL to ≥256 µg/mL among these MRSA isolates.

A total of six isolates (3.2%) exhibited an oxacillin MIC of ≤ 2µg/mL, while seven isolates (3.7%) had an MIC of 3 µg/mL. These thirteen isolates (6+7=13) were identified as MRSA exclusively through the cefoxitin disk diffusion method, not the oxacillin screen agar method. Meanwhile, two MRSA isolates (1%) were resistant to oxacillin but susceptible to cefoxitin.

*3.2. The vancomycin MIC distribution amongst MRSA isolates (n=190) (**Figure 3**).*

The MIC values for vancomycin determined by the E-test method varied between 0.38µg/mL and 2µg/mL across 190 MRSA isolates. All 190 MRSA isolates demonstrated uniform susceptibility to vancomycin. The majority (n=91; 47.9%) of the isolates exhibited a vancomycin MIC of 1µg/mL, while 47 isolates (24.7%) exhibited an MIC of 1.5 µg/mL, and 24 isolates (12.6%) showed an MIC of 2µg/mL.

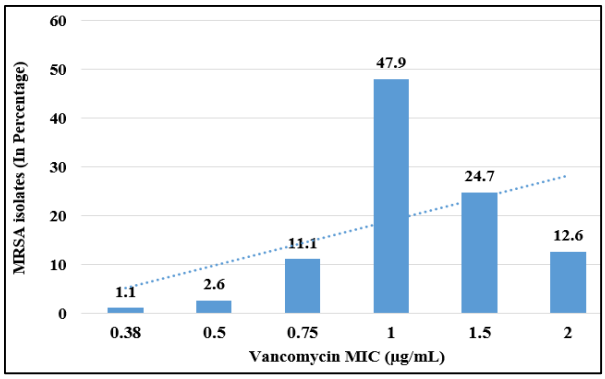

Figure 1: Distribution of vancomycin MICs for MRSA isolates (n=190).

*3.3. The linezolid MIC distribution among MRSA isolates (n=190) (**Figure 2**).*

The MIC values for linezolid varied between 0.38 µg/mL and 4 µg/mL among 190 MRSA isolates. All MRSA isolates demonstrated consistent susceptibility to linezolid. Of the MRSA isolates, 60 (31.6%) exhibited a linezolid MIC of 2 µg/mL, 34 (17.9%) had an MIC of 3 µg/mL, and 26 (13.7%) presented with an MIC of 4 µg/mL.

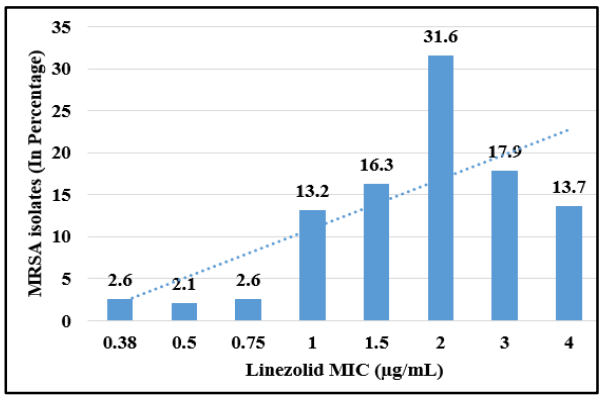

Figure 2: Distribution of linezolid MICs for MRSA isolates (n=190).

The testing of vancomycin and linezolid MICs for an MRSA isolate from a wound swab is shown in **Figure 3**.

Figure 3: Testing vancomycin and linezolid MICs for an MRSA isolate obtained from a wound swab, showing a vancomycin MIC of 1 µg/mL and a linezolid MIC of 1.5 µg/mL.

The MIC_{50}, MIC_{90}, and mean MICs of oxacillin, vancomycin, and linezolid among these MRSA isolates are shown in **Table 4**.

Spearman rho correlation coefficient values (rs) along with significance (p-value) and their interpretation between (i) oxacillin (MIC) and cefoxitin (mm), (ii) oxacillin (MIC) and vancomycin (MIC), (iii) oxacillin (MIC) and linezolid (MIC), (iv) vancomycin (MIC) and linezolid (MIC) have been given in **Table 5**.

4. Discussion

Severe infections due to MRSA isolates have become a worrisome problem in healthcare settings, especially in intensive care units. As the treatment options for these infections are limited, judicious use of available anti-MRSA antimicrobial agents, aided by microbiological culture and antimicrobial susceptibility testing, is vital in preventing an untreatable situation.[2,4,14] Most MRSA isolates express the altered penicillin-binding protein (PBP2a), mediated by the *mec A* gene, which has low binding affinities to the majority of the beta-lactam antibiotics, the most crucial class of antibiotics for treating staphylococcal infections.[10,15,16] This leaves few antibiotics for treating MRSA infections, such as vancomycin, teicoplanin, linezolid, daptomycin, and fifth-generation cephalosporins.

A slow and steady shift of MIC values towards the higher side over time is known as “MIC creep”. Recognition of this MIC creep phenomenon for anti-MRSA agents is crucial for the early detection of resistance emergence, which can lead to therapeutic failure with poor outcomes. This emphasizes the importance of routine MIC testing and monitoring for anti-MRSA drugs, which would help clinicians choose empirical antibiotics in their healthcare settings.[17]

The current study reported a prevalence of MRSA isolates at 33.8%, similar to those observed in previous studies in India and other nations.[1,2,13,15] In the present study, screening of methicillin resistance among *Staphylococcus aureus* isolates was performed using the cefoxitin disc diffusion and oxacillin screen agar method. Cefoxitin disk diffusion testing identified methicillin resistance in 188 of the 190 MRSA isolates. However, oxacillin screen agar testing detected methicillin resistance only in 95 isolates, and the remaining isolates (n=95) were recorded as susceptible; most likely, they had oxacillin MIC of <6 µg/mL. This finding suggests that oxacillin MIC testing is preferable to oxacillin screen agar for MRSA testing. Two MRSA isolates were oxacillin-resistant but cefoxitin-susceptible. These are probably borderline oxacillin-resistant *S. aureus* (BORSA) isolates due to the hyperproduction of beta-lactamases. These MRSA isolates will have high oxacillin MIC values without *mec*-gene-mediated resistance.[16]

Among MRSA isolates tested, oxacillin MICs ranged from 0.75 µg/mL to ≥256 µg/mL. Of which, 46 (24.2%)

isolates had a higher MIC of ≥12 µg/mL. About thirteen (6.9%) isolates were susceptible to oxacillin with MIC of <4µg/mL but were cefoxitin resistant. Various mechanisms responsible for methicillin resistance among *S. aureus* isolates are mediated by either *mec A* or *mec C* genes or by hyperproduction of beta-lactamases (BORSA).[16,18] The most definitive test for detecting *mecA*-mediated methicillin resistance in *S. aureus* is *mec A* PCR or PBP2a by latex agglutination. However, cefoxitin susceptibility testing can reliably predict *mec A*-mediated oxacillin resistance. This test can be a cost-effective tool in resource-poor settings for MRSA screening.[10,18,19]

The discrepancy between the interpretation of MRSA by cefoxitin and oxacillin must be considered in clinical laboratories. In the present study, *S. aureus* isolates, cefoxitin resistant and oxacillin susceptible, probably had a *mecC*-mediated mechanism for methicillin resistance. The Spearman rho correlation coefficient, *rs*, between oxacillin MIC and cefoxitin (mm) was -0.481 ($p<0.001$), indicating a moderate negative correlation. This finding highlights the need to perform a combination of cefoxitin disk diffusion and oxacillin MIC testing to detect all MRSA isolates mediated by various mechanisms.

In the current study, all MRSA isolates were uniformly susceptible to vancomycin, with MIC values ranging from 0.38µg/mL to 2µg/mL. While most (47.9%) of the isolates had an MIC of 1µg/mL, some had an MIC of 1.5 µg/mL and 2µg/mL. The MIC_{50} and MIC_{90} were 1µg/mL and 2µg/mL respectively. Over two years, these findings showed a gradual increase in the MICs. Although within the susceptible range, the gradual creep of the MIC of vancomycin towards a higher concentration may decrease therapeutic efficacy; it needs to be monitored closely (**Figure 1**). Similar observations have been reported from other parts of India.[5,18-21] In a study by Diaz R et al., MRSA isolates with higher MICs, approaching 2 µg/mL, have been associated with increased rates of treatment failure and adverse clinical outcomes.[23]

Another interesting finding was the moderate Spearman rho correlation (0.666) between oxacillin and vancomycin MICs, which was highly statistically significant ($p<0.001$). This suggests that MRSA isolates with high MIC of oxacillin also had an increased MIC of vancomycin. This observation can be extrapolated in a clinical laboratory to predict a rising MIC of vancomycin, requiring a close watch.[19,23,24]

Sixty (31.6%) MRSA isolates had linezolid MIC of 2µg/mL, 34 (17.9%) had MIC of 3µg/mL, and 26 (13.7%) isolates had 4 µg/mL. MIC values of linezolid ranged from 0.38 µg/mL to 4µg/mL within the susceptible range. A similar MIC creep was observed in linezolid MIC of 2µg/mL for 31.6%, 3µg/mL for 17.9%, and 4 µg/mL for 13.7% of the MRSA isolates. MIC_{50} andMIC_{90} were 2µg/mL and 4µg/mL respectively. This reflected the observations of Jian Y et al. in Shanghai, who documented an increase from 0.5 mg/L to 12 mg/L over ten years from 2008 to 2018, providing clear evidence of MIC creep over the years.[24] Similar observations were also reported by Iguchi et al. from Japan in 2016.[25] Linezolid resistance is rare, but it has been documented following prolonged treatment for over two weeks.[13,20,26,27]

Upon analyzing the correlation between the MIC values of vancomycin and linezolid using the Spearman correlation coefficient, the result was determined to be 0.134 ($p=0.065$). In contrast, the correlation between oxacillin and linezolid was found to be 0.001 ($p=0.990$).

This suggests no correlation between oxacillin and linezolid MIC values and vancomycin and linezolid MIC values. This finding was similarly reported by Arumugam A et al., who found a weak correlation between the MICs of oxacillin and linezolid ($r=0.41$).[13] This suggests that while elevated oxacillin MIC levels may not predict increased linezolid MIC levels, it is crucial to determine linezolid MICs for the timely identification of MIC creep; if overlooked, this may result in treatment failure. A weak correlation can be observed in MICs of various anti-MRSA agents. This may reflect similar pressure on resistance mechanisms or selection pressures, leading to higher MICs across different classes of anti-MRSA antibiotics.[13,20,28]

To detect minimum variations in MIC values, MIC_{50} and MIC_{90} were analyzed. MIC_{50} revealed the median susceptibility to the anti-MRSA antibiotics, while the range of bacterial resistance was ascertained by detecting MIC_{90} levels. These values help detect the emergence of an increased number of resistant bacteria among the isolates.[12,13] Clinical response is not optimal due to the minimum increase in MICs. The present study's findings indicate that it is essential to continuously monitor MICs of clinical isolates of MRSA, including other MDR organisms, to guide clinical judgment and improve antibiotic stewardship. This may also help in alternate treatment modalities for MRSA infections. Erythromycin or clindamycin, which have good tissue penetration, can be considered suitable options for treating less severe infections by MRSA if found susceptible.[13] This step can further prevent the development of decreased susceptibility or resistance to vancomycin or linezolid and follow the WHO-advocated AWaRe strategy.[29,30]

We found the Epsilometer test strip method (E-test) to be a better technique for detecting even intermediate MIC dilutions, which helped identify emerging MIC creep more effectively than the broth dilution method. This study had a few limitations; the confirmation of MRSA isolates by PCR for detection of *mecA/mecC* genes was not performed, and there was a lack of information regarding the treatment and its effects on the study group. The influence of MIC creep can be evaluated by monitoring patients throughout their treatment process.

5. Conclusion

Although we did not detect any resistance to vancomycin and linezolid in the MRSA isolates, it is essential to closely monitor the gradual increase in MIC to facilitate the early identification of resistance development. This study emphasizes the judicious use of linezolid and vancomycin only for MRSA isolates, rather than for methicillin-susceptible *Staphylococcus aureus* isolates.

As challenges increase in combating MRSA infections, clinical laboratories need to adopt methods to monitor and detect emerging resistance, and clinicians and management of healthcare facilities need to implement stringent antimicrobial stewardship programs. Further research is needed to identify the risk factors associated with increasing MIC and to explore alternative treatment options for managing MRSA infections.

6. Source of Funding

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7. Conflict of Interest

The authors declare that there is no conflict of interest.

8. Ethical Approval Statement

The study involved testing laboratory archived bacterial isolates, and there was no direct patient involvement. Hence, a waiver of consent was granted by the PIMS Institute Ethics Committee (IEC: RC/19/121; dated 23.12.2019) to conduct the study.

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