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Resistance to linezolid observed in clinical isolates of Staphylococcus aureus obtained from a tertiary care facility in Hyderabad, India

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Abstract
Background: *Staphylococcus aureus* has become increasingly prevalent in recent decades, serving as a primary source of infections acquired in both hospital and community settings. Severe MRSA (Methicillin resistant *Staphylococcus aureus*) infections pose a challenge in terms of therapeutic options, as they are limited. Linezolid, due to its antimicrobial spectrum, favorable short-term safety profile, and effectiveness, is extensively utilized in critical care settings.
Methods: The study was conducted within the microbiology department of a tertiary care hospital located in Hyderabad from December 2021 to December 2023.The identification of MRSA was carried out using the cefoxitin disk diffusion technique. The detection of LRSA (Linezolid resistant *Staphylococcus aureus***)** was carried out using the Kirby Bauer disc diffusion method, while the detection of MIC (Minimum Inhibitory Concentration) was conducted through the E-strip method and broth dilution methods for all the MRSA samples.
Results: Among the 221 *S.aureus*isolates examined, the cefoxitin method revealed that 100 samples were determined to be MRSA, whereas the remaining 121 samples were categorized as MSSA. Six out of the 100 MRSA isolates were determined to be LRSA using the Kirby Bauer disc diffusion method. Three isolates were termed as LRSA using the E-strip method, whereas the broth dilution method identified four isolates as LRSA.
Conclusion: This study show's both the E-strip method and broth dilution method exhibited a sensitivity rate of 96% and 97% respectively. The determination of MIC can be accomplished using either of these methods. In order to halt the dissemination of resistant strains, healthcare facilities should enhance the Standard Operating Procedures (SOPs) of Hospital Infection Control Committees (HICC) to mitigate the impact of antibiotic resistance and nosocomial infections.

Keywords: *Staphylococcus aureus*, Methicillin resistant *Staphylococcus aureus*, MIC, LRSA

Introduction
The most clinically significant species of Staphylococci, *Staphylococcus aureus*, has been linked to human disease for over a century. It is a problem of reinfection and dissemination. It is one of the top three major potential pathogens that cause infections in hospitals and the community. These infections can range from relatively minor skin and soft tissue infections to potentially fatal systemic infections that can be toxin- or non-toxin-mediated and have a high global morbidity and mortality rate. Multiple antibiotic resistances have made *Staphylococcus aureus* a growing threat in the medical community [1].
MRSA strains pose a serious risk to public health because they can result in costly and difficult-to-treat hospital-acquired illnesses [2]. Vancomycin is a common antibiotic used to treat people infected with MRSA strains because many of these strains are only responsive to it. Numerous publications have reported on the rise of VISA (*Vancomycin-intermediate S. aureus*) and VRSA (Vancomycin Resistant Staphylococcus aureus), however. Additionally, MIC of vancomycin slightly below cutoff value is reported to be the reason for treatment failure for MRSA infections. Elevated minimum inhibitory concentration (MIC) of vancomycin for MRSA, which is susceptible to the antibiotic, could suggest antibiotic resistance [3]. MRSA has the ability to withstand all β-lactam antibiotics, such as cephalosporin's and carbapenems, and is more likely to develop resistance to macrolides, quinolones, and aminoglycosides [4].

For VRSA and VISA (Vancomycin-intermediate S. aureus) infections, linezolid is a treatment and life-saving option. First made available in 2000, linezolid is an antibiotic belonging to the oxazolidinone category. For resistant Staphylococcus infections [5], it is the sole antibiotic that is accessible in an oral formulation. Nosocomial pneumonias, such as VAP (Ventilator-associated pneumonia), infective endocarditis, and MRSA meningitis, can all be effectively treated with it [6]. Additionally, MRSA colonization in the throat and nose can be completely eliminated with it. Linezolid inhibits bacterial protein synthesis via attaching to the 50S ribosomal subunit's peptidyl transferase center (PTC).Mutations in PTC's ribosomal proteins L3, L4, and L22 are also associated with resistance to linezolid [7].

Linezolid, an oxazolidinone derivative, is used for the treatment of infections caused by methicillin-resistant Staphylococcus aureus (MRSA). MRSA is a pathogenic bacterium that can lead to a diverse range of infections, varying from uncomplicated wound infections to severe and life-threatening illnesses. Consequently, it is crucial to conduct antimicrobial susceptibility testing and administer appropriate treatment for MRSA. The initial instance of linezolid resistance was identified in July 2004 [8].

It is preferred for outpatient treatment because it is the only antibiotic with strong activity against MRSA that is accessible in an oral formulation. It can be used on adults, children, and babies with safety and effectiveness. This medication is safe to use for short periods of time in patients of all ages, including those with liver disease or impaired kidney function [9].

Our research aimed to determine the minimum inhibitory concentration of linezolid on MRSA by the use of the E strip method and micro broth dilution.

Materials and Methods

This study was carried out in the microbiology department at the tertiary care hospital in Hyderabad between December 2021 and December 2023.

Various clinical samples were used in the investigation, including blood, pus, sputum, urine, and other bodily fluids. Blood agar, MacConkey agar, and Nutrient agar were used to culture all specimens, with the exception of urine. Cysteine lactose electrolyte deficient agar (CLED Agar) was utilized for urine analysis. [9].

Staphylococcus aureus was isolated and identified using standard microbiological techniques, including colony morphology, Gram stain, the catalase test, coagulase, the mannitol fermentation test, and DNase production [10]. Following a lawn culture of 0.5 McFarland suspension of isolates on Muller Hinton Agar plate, all isolates were evaluated using the Cefoxitin disc diffusion test using 30 µg disc. 18 to 24 hours were spent reading plates at 37 °C. Methicillin-resistant isolates were those that displayed a zone of inhibition with cefoxitin of < 21 mm [10].

By using the Kirby Bauer disc diffusion method, all Staphylococcus aureus samples were tested for antibiotic susceptibility in accordance with CLSI [10]. Clinical & Laboratory Standards Institute) recommendations. To evaluate the antimicrobial sensitivity pattern, 30 µg of Linezolid from Himedia was utilized. Eight of the 100 MRSA isolates showed resistance to linezolid when tested using the disc diffusion method.

The E Strip and Micro Broth Dilution Method were used to determine the Minimum Inhibitory Concentration of Linezolid resistant.

E –test method

Using E-test strips (Hi-media laboratories Pvt. Ltd., Mumbai), the lowest inhibitory concentration of linezolid was determined for each MRSA isolate.

Mueller-Hinton agar plates were used for the lawn culture after the isolate's 0.5 McFarland standard suspension was made. After ensuring that the agar surface was completely dry, using the applicator, E-test strip was held and its bottom edge was placed against the inoculated agar surface. After ensuring that the agar surface was completely dry, using the applicator, E-test strip was held and its bottom edge was placed against the inoculated agar surface. For 24 hours, plates were incubated at 37 °C [10].

Micro broth dilution method

Pickup 3-4 colonies from the pure culture plate, suspend them in 3-4 milliliters of peptone water, and incubate for one to two hours at 37 °C. Adjust to McFarland 0.5, to prevent further growth, the suspension should be utilized for inoculation within 15 minutes. Sterile normal saline was used to dilute antibiotic solutions at various concentrations, starting from 0.5, 1, 2, 4, 8, 16, 32µg/ml.

With the aid of a pipette, 50 µl of the inoculum suspension was added to each well of the microtitre trays. Each well holds roughly 2.5 x 10^4 cells. 50 microliters of an antibiotic solution were added, each at a different dilution. For 18 to 22 hours, plates were sealed and incubated at 37 ºC. To get accurate endpoints, the incubation period is crucial. Test strains are performed concurrently with quality control strains. While *S. aureus* ATCC 29212 was used without an antibiotic as the positive control, nutritional broth was used as the negative control [11].

Results

This study includes a total of 221 clinical isolates of S. aureus**.**

Table 1: The Kirby Bauer method is used to identify MRSA and MSSA using cefoxitin (30 ug) disk

Cefoxitin (30ug)	**Sensitive (%)**	**Resistant (%)**
MRSA	-	100 (45.24%)
MSSA	121 (54.75%)	-
Total (n)	221 (100)	

Methicillin-sensitive S. aureus (MSSA) was found to be 54.75% in this study, whereas MRSA was reported to be 45.24%.

The pattern of MRSA's antibiotic sensitivity to different antibiotics is displayed in the table below. Linezolid resistance was present in 7 isolates. Cefotaxime showed 86% resistance and cephalexin 100% resistance. The resistance pattern for Teicoplanin was 14%, that of amikacin and gentamicin was 35% and 32%. Four isolates were reported to be nitrofurantoin resistant out of 14 urine samples.

Table 2: Pattern of antibiotic susceptibility of different drugs to MRSA (n=100)

Antibiotic class	Antibiotics	Sensitive no (%)	Resistant no (%)
Aminoglycosides	Amikacin	65	35
	Gentamicin	68	32
Quinolones	Ofloxacin	51	49
	Levofloxacin	53	47
Cephalosporin's	Cefotaxime	14	86
	Cephalexin	0	100
Macrolides	Clarithromycin	45	55
	Erythromycin	37	63
	Azithromycin	28	72
	Clindamycin	51	49
Sulfonamides	Co-trimoxazole	63	37
Glycopeptides	Teicoplanin	86	14
	Vancomycin	98	2
Oxazolidinones	Linezolid	94	6
Nitrofuron	Nitrofurantoin	10	4

*Urine sample only tested for nitrofurantoin (14)

Table 3: Kirby Bauer disc diffusion method: antibiotic susceptibility pattern of six LRSA isolates

S. No	Antibiotics	LRSA 1	LRSA 2	LRSA 3	LRSA 4	LRSA 5	LRSA 6
01	Amikacin	S	R	R	R	R	R
02	Gentamicin	S	R	R	R	S	R
03	Ofloxacin	R	R	R	R	S	R
04	Levofloxacin	R	R	S	R	R	R
05	Cefotaxime	R	R	R	R	R	R
06	Cephalexin	R	R	R	R	R	R
07	Clarithromycin	R	R	R	R	R	R
08	Erythromycin	R	S	R	S	S	R
09	Azithromycin	R	R	R	R	R	R
10	Clindamycin	R	R	S	R	S	S
11	Co-trimoxazole	R	R	R	R	S	S
12	Teicoplanin	S	R	S	R	R	S
13	Vancomycin	S	S	S	S	S	S

Table 4: Key attributes of six LRSA (Linezolid Resistant *Staphylococcus aureus*) samples

S.no	Sex	Age	Ward	Type of sample	Medical Diagnosis
01	Female	45	Ortho	Wound swab	Osteomyelitis
02	Male	47	CCU	ET Secretion	Pneumonia
03	Male	34	Ortho	PUS	Septic arthritis
04	Female	38	CCU	Blood	Sepsis
05	Male	8	PICU	Blood	Pyrexia
06	Male	27	GMW	PUS	Gangrene of 5th finger

Ortho: Orthopedic; CCU: Critical Care Unit; PICU: Pediatric Intensive Care Unit; GMW: General Medical Ward; ET: Endotracheal Secretion

Vancomycin exhibited susceptibility against all the 6 isolates. LRSA 1 shows susceptibility to amikacin, gentamycin, and teicoplanin. Erythromycin exhibited susceptibility solely against LRSA 2 and LRSA 4 isolates. Levofloxacin, clindamycin, and teicoplanin were all effective against LRSA 3.Co-trimoxazole, erythromycin, gentamycin, ofloxacin, and Clindamycin are sensitive to the LRSA 5 isolate. Teicoplanin, Clindamycin, and Co-trimoxazole are all susceptible to the LRSA 6isolate.

Out of the six isolates, three exhibited sensitivity to erythromycin, while the remaining three shown sensitivity to Teicoplanin.

The characterization of six LRSA isolates is displayed in Table 4. In the pediatric age category, there was only one isolate (08 years).Conversely, the remaining isolates were obtained from adult patients. Two isolates were obtained from the orthopedic ward, while two isolates were collected from the critical care unit (CCU) wardand one each from GMW and PICU. Two of the six LRSA isolates were obtained from pus specimens. One from an ET sample and one from a wound swab. From blood samples, two isolates were found.

Table 5: Linezolid minimum inhibitory concentration among MRSA isolates as determined by the E test (n = 100)

S. No	E test	MRSA (%)
01	<0.5µg/ml	7%
02	<1µg/ml	41%
03	<2 µg/ml	32%
04	<4 µg/ml	17%
05	<8 µg/ml	3%

The results indicated that 41% of the samples showed a minimum inhibitory concentration (MIC) of less than 1µg/ml, whereas 32% had an MIC of less than 2µg/ml. additionally, 17% displayed an MIC of less than 4µg/ml, and 7% exhibited an MIC of less than 0.5µg/ml. Only 3% of the samples showed resistance to Linezolid.

Table 6: The Micro Broth Dilution Method was utilized to ascertain the MRSA isolates' minimum inhibitory concentration of Linezolid.

S. No	Micro broth dilution	MRSA (%)
01	<0.5µg/ml	8
02	<1µg/ml	53
03	<2 µg/ml	26
04	<4 µg/ml	9
05	<8 µg/ml	4

The findings shown that over half of the isolates (53%) showed a MIC below 1µg/ml, with 26% having a MIC below 2µg/ml. Moreover, 9% of the isolates exhibited a MIC below 4µg/ml, while 8% had a MIC below 0.5µg/ml. Merely 4% of the isolates indicated a MIC below 8µg/ml.

Discussion

S. aureus remains a prevalent source of nosocomial infections, notably contributing to pneumonia, surgical site infections, and bloodstream infections. Furthermore, it persists as a significant factor in the occurrence of community-acquired infections [12].

In this study, a total of 221 Staphylococcus aureus strains were screened for MRSA. Among the 221 isolates, 100 (45.24%) were found to be MRSA, while 121 (54.75%) were identified as MSSA.

Among the 100 MRSA isolates, it is observed that males exhibit a higher prevalence rate of 62%, while females demonstrate a prevalence rate of 38%.In contrast to our research findings, the investigation carried out by Sharlee *et al*. [7]. Revealed that a majority of MRSA cases were detected in male patients at a rate of 67.7%, while female patients accounted for 32.3% of the cases.

The study conducted by Nadia Aslam *et al*. [13]. Also yielded comparable results, with the majority of MRSA cases being identified in male patients (65.2%) and the remaining cases in female patients (34.8%).

Blood showed a higher prevalence compared to pus, urine, ET, and sputum, with percentages of 42%, 37%, 14%, 6%, and 1% respectively. Muneeba Wali *et al*. [14]. Reported comparable results, where 42% of MRSA isolates were identified from blood samples.

In this study, a total of 100 isolates were tested. The results indicated that the majority (41%) had a minimum inhibitory concentration (MIC) of less than 1µg/ml, followed by 32% with an MIC of less than 2µg/ml, 17% with an MIC of less than 4µg/ml, and 7% with an MIC of less than 0.5µg/ml. Additionally, 3% of the isolates demonstrated resistance to Linezolid when tested using the E strip method. In contrast to our investigation, similar MIC values were observed in the research carried out by Stefan Riedel *et al*. Their study revealed that 65.1% of the samples had MIC values below 1µg/ml, 27.9% had MIC values below 2µg/ml, 4.7% had MIC values below 0.5µg/ml, and 2.3% had MIC values below 4µg/ml.

In our investigation, the majority of isolates (53%) exhibited a MIC of less than 1µg/ml, while 26% had a MIC of less than 2µg/ml. Additionally, 9% of isolates displayed a MIC of less than 4µg/ml, and 8% had a MIC of less than 0.5µg/ml. Only 4% of isolates demonstrated a MIC of less than 8µg/ml. Research carried out by R. Sharlee and colleagues revealed that the majority, specifically 63.1% of the isolates, exhibited a minimum inhibitory concentration (MIC) of less than 1µg/ml. This was followed by 26.1% of isolates with an MIC of less than 2µg/ml, 6.1% with an MIC of less than 4µg/ml, and 4.7% with an MIC of less than 0.5µg/ml. The broth dilution method revealed that all isolates are susceptible to linezolid, as demonstrated by their findings. However, our study presents a contrasting result, indicating a 4% resistance to linezolid.

In this study, a comprehensive analysis was conducted on a total of 100 MRSA isolates, it was observed that 6 isolates exhibited resistance to linezolid when tested using the Kirby Bauer method. To further investigate, all 100 isolates were subjected to the E strip and micro broth dilution methods. The results revealed that 3% of the isolates were resistant when tested using the E strip method, while 4% showed resistance when tested using the broth dilution method.

Conclusion

Both the E-strip method and broth dilution method exhibited a sensitivity rate of 96% and 97% respectively. The determination of minimum inhibitory concentration (MIC) can be accomplished using either of these methods. Performing a minimum inhibitory concentration (MIC) test before reporting resistance to high-end antibiotics solely based on the Kirby Bauer disk method is considered a best practice. By obtaining knowledge of the MIC, physicians can gain valuable information to aid in making appropriate prescriptions.

The rise of resistance to over-the-counter medications such as linezolid poses a significant obstacle.

In all tertiary care hospitals, it is imperative to establish a well-defined antibiotic policy through collaboration between clinicians and microbiologists. Furthermore, clinicians should diligently adhere to a stringent antibiotic regimen.

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