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EVALUATION OF PHENOTYPIC METHODS OF BETA-LACTAMASE PRODUCING METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS (MRSA) IN A TERTIARY CARE HOSPITAL

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ABSTRACT

Background: Methicillin-Resistant Staphylococcus aureus (MRSA) is one of the most common causes of nosocomial and community infections, and the majority of clinical isolates are β-lactam and multidrug-resistant.

Resistance to β-lactam antibiotics is frequently caused by the development of penicillin-binding proteins (PBPs) and β-lactamases. The identification of β-lactamases is critical for selecting the most effective antibiotic treatment.

Objective: To compare various phenotypic methods for the detection of β-lactamase producing Methicillin-Resistant Staphylococcus aureus (MRSA) in a Tertiary Care Hospital.

Material and Methods: A Prospective Hospital study conducted from January 2023 to December 2023 from various clinical specimens that were sent for processing in the Department of Microbiology, United Institute of Medical Sciences, Prayagraj, from patients attending various OPDs and admitted in IPDs.

A total of 1877 patients' sample, received from various departments were processed. All MRSA isolates were tested for β-lactamase production by using various phenotypic methods like cloverleaf test, Masuda double-disc test, Double disc method and Penicillin disc diffusion method.

Result: Characterization of β-lactamases is important for choosing appropriate antibiotic therapy; therefore 97*S. aureus* were isolated with 49 MRSA and 48 MSSA. The findings of this study revealed that the Cloverleaf test and Masuda test had high accuracy (100%) compared with other methods tested for the detection of MRSA β-lactamase production.

Conclusion: The study shows that the Cloverleaf and Masuda double-disc were more accurate and superior methods in the detection of β-lactamase producing *S. aureus* and suggests that these methods can be done as routine tests in the Microbiology Laboratory.

KEYWORDS: β-lactamase, *S. aureus*, MRSA, Cloverleaf test, Masuda double disc test.

INTRODUCTION

According to the European Center for Disease Control (ECDC), antimicrobial resistance is currently the single biggest threat facing the world in infectious diseases. With the progression of the discovery of antibiotics and their prophylactic use, drug resistance to one or more drugs has emerged. Antibiotic resistance is a natural selection process where microorganisms are treated with different antibiotics, and microorganisms tend to escape this selection pressure with a greater ability to survive and thus show antibiotic resistance (Majumder et al.2020) (Ahmad et al.2021). In contrast, bacteria with a sensitive nature are killed by exposed antibiotics. Emerging resistance to β-Lactamase Antibiotics is a serious health problem that causes a major obstacle in the treatment of bacterial infections (Vrancianu et al.2020).

The state of drug resistance is primarily developed by the increasing and indiscriminate use of antibiotics in clinical diseases, unregulated sales of antibiotics, long treatment times and poor public health infrastructure. According to a hospital survey, more than 80% of clinical samples of *S. aureus* were found to be resistant to leading antibiotics, including methicillin (Gurung et al., 2020). It has been reported that 70% of nosocomial bacterial pathogens have developed resistance to more than one antibiotic during the treatment of chronic infections. In contrast, an alarming increase in community-acquired bacterial resistance has also been observed, with significantly high rates in both acute and chronic bacterial infections. The occurrence of drug-resistant strains of gram-positive (*Staphylococcus, Enterococcus, Streptococcus spp.*) and gram-negative (*Pseudomonas, Klebsiella, Enterobacter, Acinetobacter, Salmonella spp.*) bacteria is more serious in the current therapeutic scenario (Mancuso et al., 2021) (Motbainor et al., 2020).

Staphylococcus aureus represents one of the most challenging pathogenic bacteria. Resistance in *S. aureus* strains is increasing constantly; this has increased the ability of these pathogens to spread in hospital and community settings. *S. aureus* has remarkably developed resistance to antimicrobial drugs in several ways. When treated with antibiotics, bacteria tend to overcome the selection pressure of the drug through morphological and genetic changes or inactivation of the drug. Changes in membrane integrity and transfer of resistance genes from one strain to another are common examples of β-Lactamase Antibiotic-mediated resistance in *S. aureus*. β-Lactamase Antibiotics, penicillin was initially successful in treating *S. aureus* infections, but widespread and long-term use of penicillin was no longer effective and resistance emerged soon after in the 1950s (Guo et al.2020).

Antibiotic resistance can be a characteristic of the bacterial species (intrinsic resistance) or acquired by an individual organism that is naturally susceptible (acquired resistance). Acquired resistance is the result of chromosomal mutations or the acquisition of resistance genes through horizontal gene transfer (Arnold et al., 2022) (Rodríguez-Beltrán et al.2021). Resistance to multiple β-LAs can be acquired by individual strains, leading to multi-resistant phenotypes. The high prevalence of drug resistance is mainly due to the unregulated sale of antibiotics without a medical prescription, long treatment, indiscriminate use of drugs and poor health infrastructure. The mobility and mortality of drug resistance in public health is difficult to assess (Guliy et al., 2021) (Caro et al.2021).

According to a report by WHO, MRSA remains among the high-priority multidrug-resistant organisms that need new efforts in the field of research and development of new antibiotics and innovative prevention approaches. Both MRSA and methicillin-susceptible *S. aureus* (MSSA) can produce β-lactamases (Sulis et al.2022). Even in PBP2-null strains, these enzymes alone may be responsible for the borderline methicillin and oxacillin-resistance phenotype. In addition to PBP2, most MRSA strains produce β-lactamase. One of the main mechanisms of resistance to β-lactam antibiotics is production of β-lactamase by *Staphylococci*. Approximately the majority of clinical isolates are resistant to β-lactams due to enzyme production (Bush & Bradford, 2020) (Panchal et al.2020) (Alfei & Schito, 2022).

This enzyme is secreted into the environment where β-lactam hydrolysis occurs before the drug binds to PBP in the cell membrane. Transcription of mecA and blaZ resistance genes can be controlled by

a homologous two-component system consisting of the sensor inducers BlaR1 and MecR1 and the repressors BlaI and MecI. Interestingly, despite cross-resistance to virtually all β-lactams provided by mecA, the majority (95%) or more of MRSA today are still positive for the β-lactamase locus. Moreover, the blaZ regulators BlaR1 and BlaI can induce mecA transcription efficiently and faster than the natural mecA regulators MecR1 and MecI (Schwendener and Perreten2022) (Boonsiri et al.2020). Furthermore, many MRSA strains lack a functional mecI-mecR1 gene due to polymorphisms in the mecA regulatory region, so mecA transcription is probably only under the control of the blaI-blaR1 gene. mecA acquisition and stabilization has been shown to be promoted by the presence of blaZ locus (Mlynarczyk-Bonikowska et al.2022) (Deekshit & Srikumar, 2022).

MATERIALS AND METHODS

Bacterial isolates: Ninety-seven isolates of *S. aureus* were isolated from a total of 1877 specimens collected from different clinical samples such as urine, burns, wounds, and blood. These specimens were received at the Department of Microbiology, UIMS, Prayagraj for Culture and Sensitivity testing between January 2023 to December 2023. The identification of isolates was based on their morphological features in the culture medium and biochemical tests following the standard operating procedure.

Ethical Clearance: The study was performed after obtaining the ethical clearance from the institution UIMS, Prayagraj.

β-Lactamase Production Assay

1. *Clover Leaf Assay:* Muller-Hinton agar plates (Himedia, India) were used to culture *Escherichia coli* ATCC 25922. A penicillin disc (10U) (Himedia, India) was then positioned at the centre of each plate, and four test isolates were streaked outward from the disc to create 0.25 cm wide outgrowths. The plates were then incubated at 37° C for 24 hours to observe the production of β-lactamase by the isolates, which would result in the formation of cloverleaf patterns (Gupta et al.2020).
2. *Masuda Double Disc Test*: The experiment involved streaking *Escherichia coli* ATCC 25922 onto a plate and placing a 10 U penicillin disc in the middle. Around the central disc, a filter paper disc with the test isolate was positioned 10 mm away. If an inhibition zone around the central disc was caused by the disc with the test isolate, it indicated positive β-lactamase production (Gupta et al.2020).
3. *Double Disc Synergy Test* (DDST): β-lactamase enzyme production was detected by DDST using a combination of piperacillin (PI) 100/10 μg and piperacillin/tazobactam (PI/PIT) 100/10 μg discs. (Himedia, India). A 0.5 McFarland bacterial suspension was prepared in saline for each isolate and plated on MHA plates. The PI and PI/PIT discs were manually placed 15 mm apart edge-to-edge. Plates were read after 24 hours of incubation at 35°C. β-Lactamase production was noted by the development of a synergistic reaction (Dirar et al., 2020).
4. *Penicillin Disc Diffusion Test*: This experiment is founded on the technique introduced by Bauer et al. (1966). It is considered the most detailed D.D. method in the MHA medium, with established standards for interpretation and CLSI data support. The isolates to be tested were applied to MHA plates. A 10 U penicillin disc was then placed on the plate's surface. After incubating the plates at 37°C for 24 hours, sharp edges around the plates indicated whether the isolates produced beta-lactamase (Bauer AW et al. 1966).

RESULTS AND DISCUSSION

Several different approaches were meticulously and comprehensively employed to detect the production of β-lactamase in Gram-positive bacteria with great precision and thoroughness. The primary goal of this study was to carefully evaluate and analyze four specific phenotypic methods to accurately detect the presence of β-lactamase in a total of 49 Methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from the prestigious UIMS, Prayagraj.

Tale 1: Sample isolates distribution

Total Sample	1877
Culture Positive Isolates **1. Gram-negative Bacilli** **2. Gram-positive cocci**	745 558 187
Staphylococcus aureus **1. Methicillin Sensitive *S. aureus* (MSSA)** **2. Methicillin Resistance *S. aureus* (MRSA)**	97 48 49

The first table provides an overview of bacterial isolates from a total of 1877 samples. Of these, 745 samples (39.7%) were culture-positive, indicating significant bacterial growth. The isolates were categorized into two main groups: Gram-negative bacilli, which accounted for 558 (74.9%) of the positive cultures, and Gram-positive cocci, representing 187 (25.1%) of the isolates. This distribution suggests a higher prevalence of Gram-negative bacteria in the sampled population.

The table also focuses on *Staphylococcus aureus*, a clinically significant pathogen. Out of 97 *S. aureus* isolates, which constitute 13% of all culture positives, there was an almost even split between Methicillin Sensitive S. aureus (MSSA) and Methicillin-resistant S. aureus (MRSA), with 48 and 49 isolates respectively. This near 1:1 ratio of MSSA to MRSA indicates a high prevalence of antibiotic-resistant *S. aureus* strains, which is a significant concern for healthcare settings.

Table 2: Comparison of synergetic Test

Total Samples	MRSA	Cloverleaf	Masuda Double-Disc	Double Disc Synergy (PI/PIT)	Penicillin disc zone edge
1877	49	49 (100%)	49 (100%)	24 (44.4%)	4 (7.4%)

The second table compares different testing methods for detecting Methicillin Resistant *Staphylococcus aureus* (MRSA) among the 1877 total samples. It identified 49 MRSA isolates, representing 2.6% of all samples. Four different testing methods were evaluated for their efficacy in detecting MRSA.

The Cloverleaf test and the Masuda Double-Disc test both demonstrated 100% sensitivity, successfully identifying all 49 MRSA isolates. In contrast, the Double Disc Synergy (PI/PIT) test showed lower sensitivity, detecting only 24 out of 49 MRSA isolates (44.4%). The Penicillin disc zone edge test performed poorly, identifying just 4 out of 49 MRSA isolates (7.4%). These results highlight significant variations in the effectiveness of different MRSA detection methods. The perfect sensitivity of the Cloverleaf and Masuda Double-Disc tests suggests they may be preferable for accurate MRSA identification, while the other two methods, particularly the Penicillin disc zone edge test, appear less reliable for this purpose.

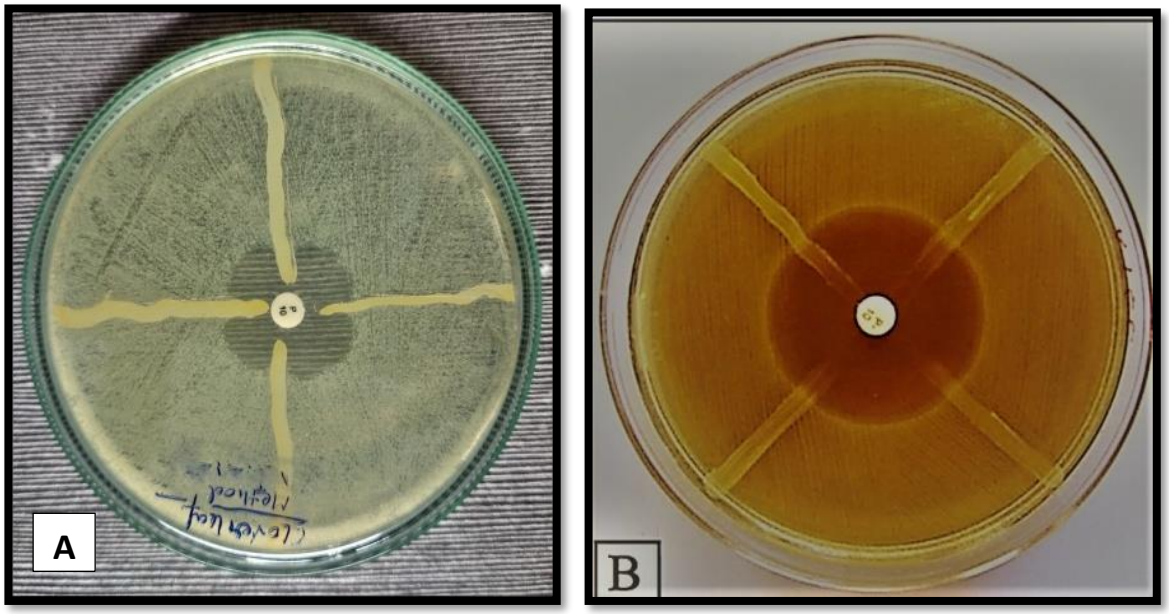

Figure 1 *shows the detection of β-lactamases production using the* ***Cloverleaf method****. In the positive result (A), a distinct and visually appealing cloverleaf pattern is observed around the penicillin (P10U) disc by the tested isolates. On the other hand, in the negative result (B), no cloverleaf pattern is formed as expected, observed in ATCC 25922, which serves as a negative control. This demonstrates the efficacy of the Cloverleaf method in identifying β-lactamases production.*

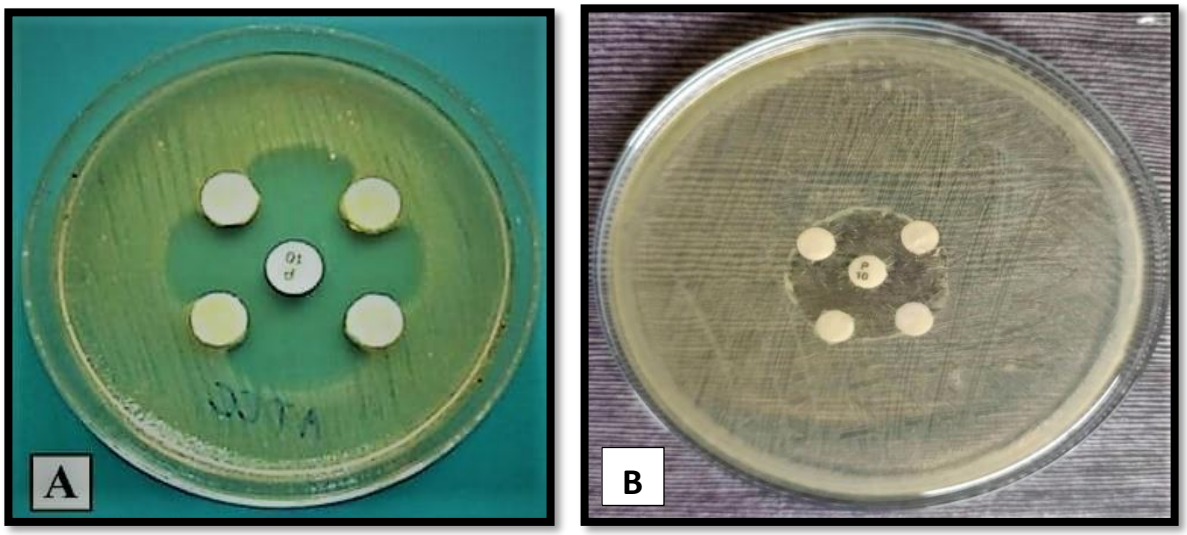

Figure 2*shows the detection of β-lactamase production using the* ***Masuda method*** *(A) Demonstrates isolates exhibiting a positive result for β-lactamase production, leading to a disruption of inhibition around the penicillin disc at the center (10 U); (B) Where the absence of an irregular inhibition zone is viewed as a positive result, with ATCC 25922 serving as the negative control.*

Image 3 *shows the detection of β-lactamases production using the* ***penicillin disc diffusion zone edge method****. (A) it is observed that there is no zone of inhibition around the penicillin disc, indicating constitutive resistance by 49 isolates in the present study. (B) shows a sharply demarcated zone edge with fully developed individual colonies within the inhibition zone, seen in 2 isolates. This appearance indicates that the isolate tested is a β-lactamase producer. (C) displays a zone edge with gradual tapering of growth, observed in 3 isolates. This suggests that the MSSA isolate tested is not a β-lactamase producer.*

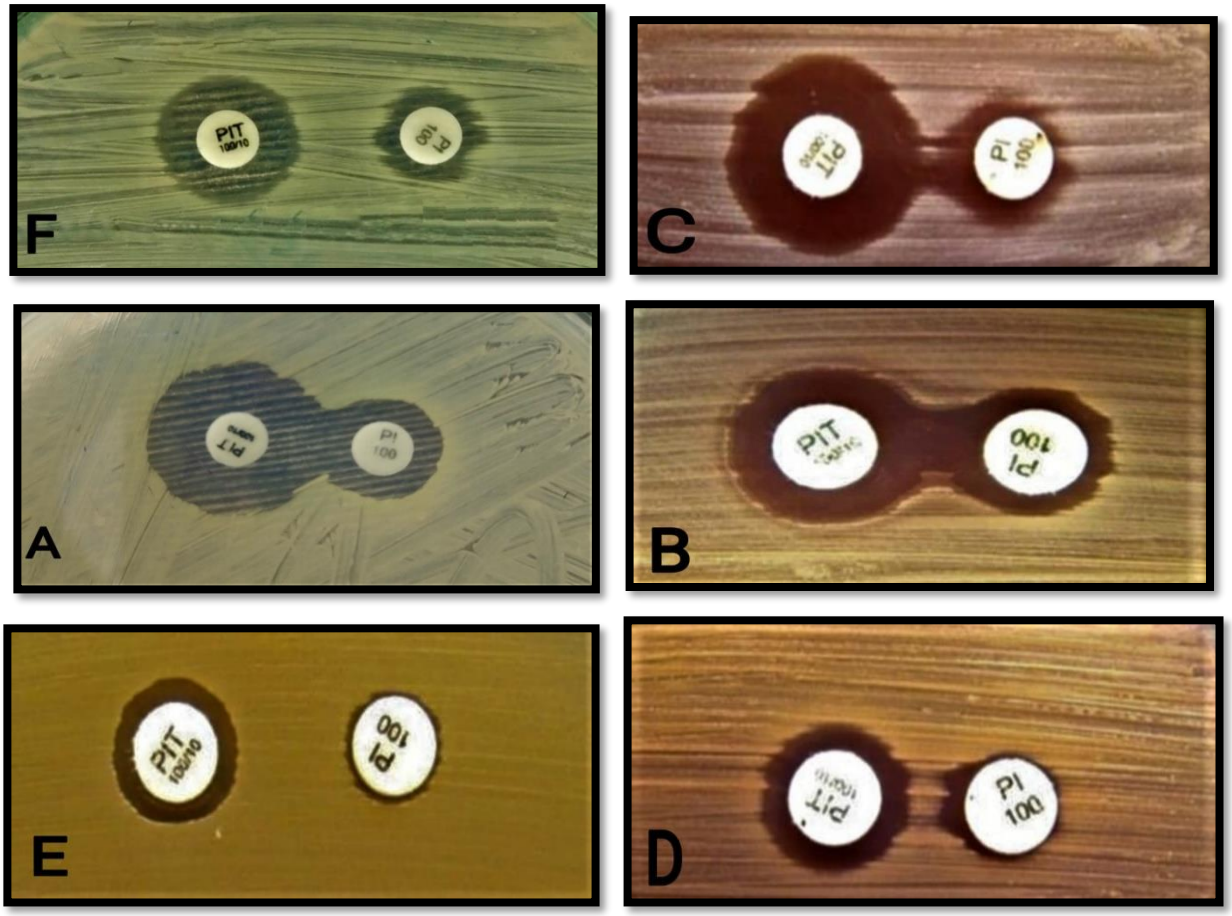

Image 4 *shows the identification of β-lactamases through a* ***double-disc synergy test using a combination of piperacillin*** *(PI 10µg) and* ***piperacillin-tazobactam*** *(PIT 100/10 µg) discs. Positive results for β-lactamase production were observed in MRSA isolates A, B, C, and D, while isolate E exhibited constitutive resistance to both PI and PIT. Isolate F, which served as a negative control, showed a negative result. All results were confirmed following the guidelines set by the CLSI.*

DISCUSSION

The present study aimed to evaluate various phenotypic methods for detecting β-lactamase production in Methicillin-Resistant Staphylococcus aureus (MRSA) isolates. The findings provide valuable insights into the efficacy of different testing methods and contribute to our understanding of antimicrobial resistance in *S. aureus*. This discussion will analyze the results in the context of existing literature, highlighting similarities, differences, and implications for clinical practice.

Our study identified 49 MRSA isolates out of 97 *S. aureus* strains, representing a prevalence of 50.5%. This high prevalence of MRSA is consistent with several recent studies. For instance, Gurung et al. (2020) reported MRSA rates ranging from 30% to 70% in various clinical settings. The near-equal distribution between MSSA and MRSA in our study (48 vs. 49 isolates) underscores the significant presence of antibiotic-resistant strains in the clinical environment.

The high prevalence of β-lactamase production among MRSA isolates, as indicated by the 100% positive results in both the Cloverleaf and Masuda double-disc tests, aligns with previous research. Bush and Bradford (2020) noted that the majority of clinical *S. aureus* isolates produce β-lactamases, which is a primary mechanism of resistance to β-lactam antibiotics. This finding emphasizes the critical role of β-lactamase in antibiotic resistance and the importance of accurate detection methods.

Our study found that both the Cloverleaf and Masuda double-disc tests demonstrated 100% sensitivity in detecting β-lactamase production among MRSA isolates. This high accuracy is particularly noteworthy and suggests these methods could be valuable tools in clinical microbiology laboratories (Figure 1). The effectiveness of the Cloverleaf method aligns with findings by Gupta et al. (2020), who reported high sensitivity and specificity of this test in detecting β-lactamase production in Enterobacteriaceae. While their study focused on different bacterial species, the principle of the test remains similar, indicating its broad applicability across various β-lactamase-producing organisms.

The Masuda double-disc test's high sensitivity in our study corroborates its efficacy as reported in previous research. Although less commonly discussed in recent literature, our findings suggest that this method deserves renewed attention as a reliable technique for β-lactamase detection in MRSA (Figure 2).

The DDST using piperacillin and piperacillin/tazobactam showed lower sensitivity (44.4%) compared to the Cloverleaf and Masuda tests. This result is somewhat surprising, as DDST is often considered a reliable method for detecting extended-spectrum β-lactamases (ESBLs). Dirar et al. (2020) reported higher sensitivity for DDST in Enterobacteriaceae, suggesting that its efficacy might vary depending on the bacterial species and the specific β-lactamases involved (Figure 3). The lower sensitivity of DDST in our study could be attributed to the unique characteristics of MRSA β-lactamases or potential technical variations in the test procedure. This finding highlights the need for caution when interpreting DDST results for MRSA and suggests that it may not be the most suitable method for this particular pathogen.

The penicillin disc diffusion test showed the lowest sensitivity (7.4%) among all methods tested. This poor performance is noteworthy, especially considering that the penicillin disc test is often used as a routine method in many laboratories. Our findings suggest that this method may significantly underestimate β-lactamase production in MRSA (Figure 4). This result contrasts with some earlier studies that found the penicillin disc test to be reasonably reliable. However, it aligns with more recent research indicating the limitations of this method, especially for detecting low-level β-lactamase production or in the presence of alternative resistance mechanisms (Panchal et al., 2020).

The high prevalence of β-lactamase production in MRSA isolates, as demonstrated by our study, underscores the critical need for accurate and reliable detection methods. The superior performance of the Cloverleaf and Masuda tests suggests that these methods should be considered for routine use in clinical laboratories, particularly when dealing with suspected MRSA infections. Our findings also highlight the potential limitations of commonly used methods like the penicillin disc test. The low sensitivity of this test in detecting β-lactamase production in MRSA raises concerns about its reliability as a standalone method. Laboratories relying solely on this test might underestimate the

prevalence of β-lactamase-producing MRSA, potentially leading to inappropriate antibiotic choices and treatment failures.

The varying sensitivities of different methods observed in our study emphasize the importance of using multiple testing approaches for a comprehensive assessment of antibiotic resistance. This aligns with recommendations by Mlynarczyk-Bonikowska et al. (2022), who advocate for a combination of phenotypic and genotypic methods to accurately characterize resistance mechanisms in S. aureus.

While our study focused on phenotypic detection methods, it's important to consider the underlying mechanisms of β-lactamase production in MRSA. Boonsiri et al. (2020) described the complex interplay between mecA and blaZ genes in conferring β-lactam resistance. The high prevalence of β-lactamase production observed in our MRSA isolates suggests the widespread presence of these resistance genes. Schwendener and Perreten (2022) provided an in-depth analysis of the bla and mec families of β-lactam resistance genes in *Staphylococcus* and related genera. Their work highlights the diversity of β-lactamases in these organisms and the potential for horizontal gene transfer, which could explain the high prevalence of β-lactamase production observed in our study.

The concept of 'silent' antimicrobial resistance genes, as discussed by Deekshit and Srikumar (2022), is also relevant to our findings. The discrepancy between different phenotypic tests in our study could potentially be explained by the presence of unexpressed or partially expressed resistance genes, which might be detected by some methods but not others. The high prevalence of β-lactamase-producing MRSA observed in our study has significant implications for clinical practice and public health. As noted by Majumder et al. (2020), antimicrobial resistance is a global threat requiring coordinated efforts in antimicrobial stewardship. Our findings underscore the importance of accurate detection methods as a crucial component of these efforts.

The superior performance of the Cloverleaf and Masuda tests suggests that incorporating these methods into routine clinical practice could improve the detection of β-lactamase-producing MRSA. This, in turn, could lead to more appropriate antibiotic choices and better patient outcomes. However, the implementation of these methods may require additional resources and training in clinical laboratories.

The limitations of the penicillin disc test observed in our study highlight the need for caution in interpreting routine antimicrobial susceptibility tests. Clinicians should be aware of the potential for false-negative results and consider using more sensitive methods when MRSA is suspected. Our findings also have implications for surveillance and epidemiological studies of antibiotic resistance. The choice of detection method can significantly impact reported prevalence rates of β-lactamase-producing MRSA. Standardization of testing methods across different laboratories and regions would be crucial for accurate comparison and tracking of resistance trends.

Limitations of the Study

While our study provides valuable insights, it's important to acknowledge its limitations. The sample size, although substantial, was from a single institution, which may limit the generalizability of the findings. Additionally, we focused solely on phenotypic methods and did not include genotypic confirmation of β-lactamase production. Future studies incorporating molecular techniques could provide a more comprehensive understanding of the resistance mechanisms.

CONCLUSION

In conclusion, our study demonstrates the high prevalence of β-lactamase production among MRSA isolates and highlights the varying efficacy of different phenotypic detection methods. The Cloverleaf and Masuda double-disc tests showed superior sensitivity compared to the DDST and penicillin disc diffusion test. These findings have important implications for clinical microbiology practices and emphasize the need for accurate and reliable detection methods in the ongoing battle against antimicrobial resistance. The results align with much of the existing literature on the prevalence of β-lactamase production in MRSA but provide new insights into the comparative efficacy of different detection methods. Our findings contribute to the growing body of knowledge on antimicrobial

resistance detection and highlight areas for future research and improvement in clinical practice. As antibiotic resistance continues to pose a significant threat to global public health, studies like ours play a crucial role in refining our approach to detection and management of resistant pathogens. By improving our ability to accurately identify β-lactamase-producing MRSA, we can enhance antibiotic stewardship efforts and work towards more effective treatment strategies for these challenging infections.

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