Original Research Article

Evaluation of Cefoxitin Disc Diffusion and Chromogenic agar in the detection of Methicillin Resistant *Staphylococcus aureus*.

ABSTRACT

Methicillin resistant *Staphylococcus aureus* (MRSA) has a worldwide distribution and is an important cause of clinical and epidemiological problems. The aim of this study was to evaluate the usefulness of some phenotypic methods for the detection of methicillin resistant *S. aureus* in clinical laboratories. The study is cross sectional. A total of 93 *S. aureus* isolates were tested using cefoxitin disk diffusion (CDD) and oxacillin resistance screening agar base (ORSAB) with reference to *mecA* gene PCR. Of the 93 isolates, CDD test showed 34 were MRSA, while ORSAB recorded 42. MecA gene was detected by PCR in 34 of the isolates. The CDD showed 97.1% sensitivity and 98.3% specificity and therefore superior to ORSAB with sensitivity 97.1% and specificity 84.7%. The cefoxitin disk test required no special test conditions and can improve the reliability of routine tests for the detection of MRSA. CDD test can thus be used as a cheap and reliable alternative to PCR for the detection of *MRSA* in resources limited settings.

Keywords: Cefoxitin disk, Chromogenic agar, mecA, MRSA, *Staphylococcus aureus*

INTRODUCTION

"Staphylococcus aureus is one of the important pathogens in many countries causing infection in hospitals and the community. It causes a variety of diseases, ranging in severity from boils and furuncles to more serious diseases such as septicaemia, pneumonia and endocarditis" (Lowy, 1998). "Virulent strains of these bacteria are responsible for the majority of Hospital Acquired Infections (HAIs) and can cause severe disease that can be fatal" (Deleo and Otto 2009). "Since the emergence of *S. aureus* strains with resistance to methicillin in 1961 (Jevons, 1961), it has become a well-known aetiological agent for a wide variety of infections. Methicillin-resistant *S. aureus* (MRSA) infections have become a common problem in hospital and community-acquired infections" (Kopp *et al*., 2004; Lodise and McKinnon, 2005; Nixon *et al*., 2006). "Rapid and accurate identification of MRSA is important in guiding clinicians choose appropriate antibiotics to treat and prevent spread of these strains. Many methods exist for detection of MRSA, and they include disk diffusion method which is the method used by most laboratories, use of chromogenic agars, E-test, the Minimal Inhibitory Concentration (MIC) by broth dilution or E-test, use of automated machines for identification and more recently molecular methods for detection of *mecA* gene" (Kaur *et al*., 2013). "The *mecA* gene is highly conserved among the *Staphylococci* species, and so the importance of PCR in assay of the *mecA* gene as "gold standard" for the detection of methicillin resistance in *Staphylococci* is well established" (Ekrami *et al*. 2010; Mathews *et al*., 2010; Shariati *et al*.,2010). "However, the use of PCR is not practical for routine diagnostic use in clinical laboratories due to cost and need for technical and experienced staff" (Kaur *et al*., 2013). In view of these, there is need for a reliable and simple test to detect MRSA easily in routine clinical laboratories.

MATERIALS AND METHODS

Bacterial isolates used in this study were obtained from Usmanu Danfodiyo University Teaching Hospital, Specialist Hospital and Maryam Abacha Women and Children Hospital all in Sokoto, North Western Nigeria. All isolates were identified by standard microbiological methods including colony morphology, Gram staining, catalase test, coagulase test using both slide and tube methods and deoxyribonuclease

test (Garcia and Isenberg, 2010; UKSMI, 2014). All isolates were examined for methicillin resistance by cefoxitin disk (30µg) diffusion test (CDD), Oxacillin resistance screen agar base (ORSAB) and Polymerase Chain Reaction (PCR).

Inoculum for Antibiotic Susceptibility Testing was prepared by the direct colony suspension method recommended by the Clinical Laboratory Standards Institute for testing *Staphylococci* for potential methicillin resistance (CLSI, 2012).

Cefoxitin disk diffusion test:

Cefoxitin disk 30µg (Oxoid, UK) was used on Mueller Hinton agar (Oxoid, UK). The inoculum **was** standardized by comparing with 0.5 McFarland turbidity standard and for the 0.5 McFarland standard, the absorbance at a wavelength of 625 nm was 0.09, the agar plates inoculated, inverted and incubated at 35°C for 24h. After the incubation, the zone of inhibition was measured using a metre rule (against transmitted light) and the results (zones ≤21mm indicate resistance) interpreted using the CLSI 2014 guidelines.

Oxacillin Resistance Screening Agar Base (ORSAB) Test

Chromogenic media was prepared according to manufacturer's instruction. A 0.05ml aliquot of broth was plated onto ORSA plates and incubated at 35 ^{o}C in ambient air. Growth on plates was evaluated after 18 hours of incubation as recommended by the manufacturer. Deep blue colonies on ORSAB were classified as MRSA. Coagulase Negative Staphylococci produce white colonies on ORSA (Oxoid, England). Any bacterial growths after 24 h, resulting in intense blue colonies were indicative of resistance to methicillin as described by Simor *et al*. (2001). Results obtained were compared to those obtained by cefoxitin disk diffusion test.

Molecular Analysis of the *S. aureus* Isolates.

DNA extraction for amplification was done using the method of Stephenson (2003) but with slight modification. Genomic DNA was prepared for all polymerase chain reaction (PCR) methods. Two to three colonies of 18 hours cultured *S. aureus* was added to 100 µL of a nuclease and ligase inhibitor-free water for extraction of PCR-ready template DNA in an eppendorf tube. This was vortexed for 15 seconds and incubated at 95 ^{0}C for 30 min and then centrifuged at 13,000 rpm for 5 min to allow purification of the DNA as the heavier molecules were settled at the bottom and the DNA was suspended in the supernatant. The supernatant (70 µL) was transferred into a new tube and stored at -20 ^{0}C as described by Stephenson (2003).

Duplex PCR reaction mix of 16S rRNA and *mec*A gene from the extracted DNA

RNA (16S rRNA) region with the following primer sequence was used as the quality control for the detection of extracted DNA. 27F **(**5'-**AGAGTTTGATCMTGGCTCAG-**3')907R (5'**-CCGTCAATTCMTTTRAGTTT**-3') as described by Jiang *et al*., (2006).The 25µL volume of PCR reaction mixture used, contained 3 µL of genomic DNA, 12.5 µL of Mastermix (BioLabs New England) containing; 25 units/ml Taq DNA polymerase, 32 mM (NH4)2SO4, 10mM Tris HCl, 0.05% Tween 20, 1.5 mM MgCl2, 50 Mm KCL 5% Glycerol and dNTPs: dATP, dCTP, dGTP, dTTP, then 5.5 µL nuclease free H_2O, 1 µL each of 16S rRNA primers (27F and 907R) and 1µL each of *mecA* primers (forward and reverse). *mecA* gene was amplified with the following primers: *mecA*-f: (5'-**AAAATCGATGGTAAAGGTTGGC**-3'); *mecA*-r: (5'-**AGTTCTGCAGTACCGGATTTGC**-3') with **533bp**. Negative control contains all material except

template DNA, distilled water was added to the negative control. DNA amplification was carried out for 40 cycles according to the following protocol: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min with a final extension at 72 °C for 5 min. The PCR products were then analyzed in 2% (w/v) agarose gels stained with ethidium bromide and visualized under UV light as described by Murakami *et al.* (1991).

Gel Electrophoresis:

The electrophoresis chamber was filled with the running buffer (1x TBE) and casting tray was then placed in the chamber. The gel was immersed in the buffer. 2µL of loading dye was added to each of the PCR products. The amplicon (10µL) was loaded into each well. A 1kb plus molecular weight marker- *Hind* III Lambda DNA ladder was then loaded into the first and the last well as standard for estimating the size of the resulting DNA fragment. The electrophoresis chamber was then connected to the power source and the DNA run at 160V for 45 min as described by Murakami *et al.* (1991). The DNA bands (976 bp for 16s and 533bp for *mec*A) were then viewed by illumination with UV light and images recorded by photography (Biorad imager).

RESULTS

Table 1 shows comparism between two methods; Disc diffusion and ORSAB chromogenic method for the detection of Methicillin resistance among the isolates. Of the 93 isolates, 34 with a zone of inhibition ≤21mm (36.6%) showed Methicillin resistance using the disc diffusion method and 42(45.2%) showed Methicillin resistance using the ORSAB chromogenic medium. All except one of the isolates that were cefoxitin resistant demonstrated resistance using the ORSAB Medium. Table 2 shows the comparism between PCR and Cefoxitin disc diffusion. Using the Chi square test, the variation between the two tests is statistically significant with a P value of 0.000.

Table 3 shows the comparism between the PCR and ORSAB methods and the variation between the two tests is also statistically significant with a P value of 0.000.

The statistical inferences in which PCR was used to compare the methods using the two by two contingency table are shown in Table 4, in which the Cefoxitin DD method was found to be more sensitive and specific with 97.1% and 98.3% than the ORSAB Method with 97.1% and 84.7% respectively. The Cefoxitin DD test was also found to have a higher positive predictive value (97.1%).

Table 1: Comparism between Cefoxitin, Disc Diffusion and Oxacillin Resistance Screening Agar for the detection of Methicillin Resistant *Staphylococcus aureus* (MRSA) in the study centers

Method		ORSAB		Total	*P* value
		Sensitive	Resistant		
Cefoxitin DD Test	Sensitive	33	1	34	0.000
	Resistant	9	50	59	
Total		42	51	93	

$\chi^2 = 58.284$

Table 2: Comparison of mecA gene detection by PCR and Disk Diffusion using cefoxitin in the detection of MRSA isolates

Method		Polymerase Chain Reaction		Total	*P* value
		Sensitive	Resistant		
Cefoxitin DD Test	Sensitive	58	1	59	0.000
	Resistant	1	33	34	
Total		59	34	93	

$\chi^2 = 84.577$

Table 3: Comparison of mecA gene detection by PCR and ORSAB in the detection of MRSA isolates

Method		Polymerase Chain Reaction		Total	*P* value
		Sensitive	Resistant		
ORSAB	Sensitive	50	1	51	0.000
	Resistant	9	33	42	
Total		59	34	93	

$\chi^2 = 58.284$

Table 4: Evaluation of Cefoxitin DD and ORSAB using PCR as gold standard for the detection of Methicillin resistance in the *Staphylococcus aureus* isolates

Statistics	Type of method evaluated	
	Cefoxitin DD Test	ORSAB

Sensitivity	97.1	97.1
Specificity	98.3	84.7
Positive Predictive Value	97.1	78.6
Negative Predictive Value	98.3	98.0

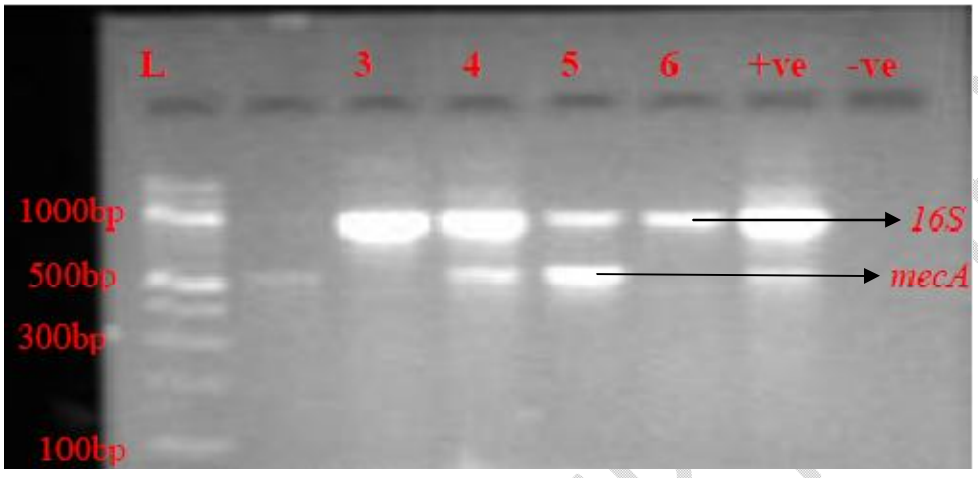

Figure 1: Results of 16s (976 bp product) and *mecA* gene Polymerase Chain Reaction (533 bp product)
Key:
L: 1kb plus molecular ladder
-ve: negative control (PCR premix only)
+ve: positive control (*S. aureus* ATCC 25923)
3: 35s
4: 2s
5:15u
6: 17s

DISCUSSION

This study showed that 34(36.6%) of the isolates obtained were methicillin resistant by the cefoxitin disc diffusion method. While oxacillin resistance screening agar base, a chromogenic agar, on the other hand, detected 42(45.2%) MRSA. Eight more MRSA than those detected by cefoxitin DD test were captured by ORSAB and is corroborated by other findings (Uzun *et al*., 2013; Rakesh *et al*., 2016; and Boutiba *et al*., 2004). This is probably because the media detect key microbial enzymes as diagnostic markers for pathogens through the use of chromogenic substrates incorporated into the agar base allowing direct colony color base identification of MRSA, as against the disc diffusion which relies on antibiotic inhibition of the bacteria. This finding is however in contrast with findings of Anand *et al*., (2009) in India, who reported that the cefoxitin DD captured more isolates with methicillin resistance than the ORSAB method. This study compared the performance of ORSAB and the Cefoxitin 30 μg disk diffusion, and it was observed that there is a statistical significant difference in the two methods ($P<0.05$). In the past decades the performance of proprietary Chromogenic media for the early identification of MRSA has improved dramatically. The ORSAB can be used by direct inoculation or from a primary culture grown on other types of media. MRSA colonies appear bluish; this makes them very easy to see against the light coloured opaque background of the media. Results can be obtained within 18 hours, this gives a rapid

turnaround time when compared with standard culture that will require on the minimum of 48 hours. This enables for rapid patient care and reduces the risk of nosocomial spread of infections(Veenemans *et al.,* 2013). However, to determine the most effective method of detecting methicillin resistance between the two methods, molecular detection of the genes responsible for methicillin resistance in *S. aureus* was done.

Polymerase chain reaction has been considered as the gold standard for determining resistance genes as these genes are highly conserved among Staphylococcal species and was therefore used in this study. Of the 93 isolates analysed to detect the presence or otherwise of *mec*A gene, 34(36.6%) were found to harbour the gene. This in agreement with findings of Angela *et al*., (2015); Adetayo *et al.,* (2014); Murakami *et al.,* (1991) and Hawraa and May (2014) who all detected *mec*A gene in *S. aureus* isolates. "PCR based assays are considered as the gold standard for the detection of MRSA due to the heteregenous resistance by various phenotypic detection methods displayed by many clinical isolates. Genotypic methods are more accurate in detecting MRSA as compared to conventional susceptibility methods" (Hawraa and May, 2014).

The sensitivity and specificity for detection of methicillin resistance using PCR as the gold standard in this study was 97.1% and 98.3% for cefoxitin DD and 97.1% and 84.7% for ORSAB. This suggests that cefoxitin DD test both sensitive and more specific in accurate detection of methicillin resistance than ORSAB. This is in agreement with findings of Rakesh *et al*., (2016) who reported sensitivity and specificity of 96.7% and 100% for cefoxitin as against 95.1% and 100% for ORSAB in India, Uzun *et al*., (2013) with 98.3% and 100% for cefoxitin DD against 96.7% and 81.6% for ORSAB in Turkey and Boutiba *et al*., (2004) with 100% and 96.5% for cefoxitin DD against 99.1% and 90.4% for ORSAB in Tunisia. All of these findings indicate that cefoxitin DD method can be used as a reliable conventional, simple and cheap alternative to PCR for detection of MRSA especially in resource constraint settings.

CONCLUSION

The cefoxitin disk test required no special test conditions and can improve the reliability of routine tests for the detection of MRSA. CDD test can thus be used as a cheap and reliable alternative to PCR for the detection of *MRSA* in resources limited settings.

CONSENT

Informed consent was sought from all the enrollees before collecting their samples.

ETHICAL APPROVAL

Ethical approval was obtained from the institutional ethical review committees of the hospitals before the commencement of the study.

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