Infection and Drug Resistance

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ORIGINAL RESEARCH

Phenotypic and molecular characteristics of methicillin-resistant *Staphylococcus aureus* isolates from Ekiti State, Nigeria

This article was published in the following Dove Press journal:
Infection and Drug Resistance
19 August 2013
Number of times this article has been viewed

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Introduction: The characteristics and antimicrobial resistance profiles of *Staphylococcus aureus* differs according to geographical regions and in relation to antibiotic usage. The aim of this study was to determine the biochemical characteristics of the prevalent *S. aureus* from Ekiti State, Nigeria, and to evaluate three commonly used disk diffusion methods (cefoxitin, oxacillin, and methicillin) for the detection of methicillin resistance in comparison with *mecA* gene detection by polymerase chain reaction.

Materials and methods: A total of 208 isolates of *S. aureus* recovered from clinical specimens were included in this study. Standard microbiological procedures were employed in isolating the strains. Susceptibility of each isolate to methicillin (5 µg), oxacillin (1 µg), and cefoxitin (30 µg) was carried out using the modified Kirby–Bauer/Clinical and Laboratory Standard Institute disk diffusion technique. They were also tested against panels of antibiotics including vancomycin. The conventional polymerase chain reaction method was used to detect the presence of the *mecA* gene.

Results: Phenotypic resistance to methicillin, oxacillin, and cefoxitin were 32.7%, 40.3%, and 46.5%, respectively. The *mecA* gene was detected in 40 isolates, giving a methicillin-resistant *S. aureus* (MRSA) prevalence of 19.2%. The *S. aureus* isolates were resistant to penicillin (82.7%) and tetracycline (65.4%), but largely susceptible to erythromycin (78.8% sensitive), pefloxacin (82.7%), and gentamicin (88.5%). When compared to the *mecA* gene as the gold standard for MRSA detection, methicillin, oxacillin, and cefoxitin gave sensitivity rates of 70%, 80%, and 100%, and specificity rates of 76.2%, 69.1%, and 78.5% respectively.

Conclusion: When compared with previous studies employing *mecA* polymerase chain reaction for MRSA detection, the prevalence of 19.2% reported in Ekiti State, Nigeria in this study is an indication of gradual rise in the prevalence of MRSA in Nigeria. A cefoxitin (30 µg) disk diffusion test is recommended above methicillin and oxacillin for the phenotypic detection of MRSA in clinical laboratories.

Keywords: evaluation, disk diffusion, *mecA* gene, MRSA, Nigeria

Introduction

Staphylococcus aureus is an important pathogen in human infections and is implicated in a wide variety of infections, from mild skin infections to more serious and invasive infections, including sepsis, pneumonia, endocarditis, deep-seated abscesses, and toxinoses including food poisoning and toxic shock syndrome.[1]

Staphylococci infections are treated with antibiotics. Over the decades, some strains of staphylococci such as methicillin-resistant *S. aureus* (MRSA) have become resistant to antibiotics that once destroyed it. MRSA strains, though first reported in 1961, did not become a major problem until the late 1970s and early 1980s when outbreaks

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were reported from many parts of the world.[2] MRSAs are resistant to methicillin, amoxicillin, penicillin, oxacillin, and many other antibiotics. They are also resistant clinically to all beta-lactam antibiotics, despite apparent in vitro efficacy. It is also important to note that MRSA are often multidrug-resistant and are resistant to antibiotics such as the macrolides and aminoglycosides.[1]

Until recently, MRSA was predominantly a nosocomial pathogen that caused hospital-acquired infections, but MRSA strains are now being increasingly isolated from community-acquired infections as well. Vancomycin has been the antibiotic of choice to treat MRSA infections, and the emergence of vancomycin nonsusceptible *S. aureus* reported in recent years is a cause of great public health concern and has made therapy of MRSA infections even more challenging for clinicians.[3] Numerous studies have indicated that *S. aureus* is among the most frequently encountered microorganisms in medical microbiology laboratories in Nigeria.[4–7]

Resistance in MRSA is mediated by the presence of penicillin-binding protein (PBP)-2a, encoded by the chromosomal *mecA* gene.[8] PBP-2a is a cell wall enzyme that has low affinity for β-lactam antibiotics, and allows cell wall formation in concentrations of a drug that render the other PBPs inactive.[9] The gene responsible for methicillin resistance in *S. aureus* is *mecA*,[10] which is part of a 21-kb to 60-kb staphylococcal chromosome cassette *mec*, a mobile genetic element that may also contain genetic structures such as Tn554, pUB110, and pT181, which encode resistance to non-β-lactam antibiotics.[11] There are four classes of the *mec* gene complex, of which only classes A (the original clone) and B have been identified in *S. aureus*, while classes C and D have been identified in coagulase negative staphylococci. These classes of staphylococcal chromosome cassette *mec* differ from each other.[12]

In line with the worldwide spread of MRSA, there are increasing reports of MRSA strains involved in clinical infections in Nigeria.[1,12–15] Most of these strains are detected by a phenotypic method using oxacillin and methicillin disks, which may not reflect the true prevalence because of many factors that affect the performance and reliability of the disk diffusion test. The cefoxitin disk was introduced a few years ago to test *S. aureus* for expression of methicillin resistance, and it was found to be reliable and even better than the oxacillin disk.[16] Although detection of the *mecA* gene by polymerase chain reaction (PCR) is the gold standard test for MRSA confirmation, only a few studies in Nigeria have employed this gold standard for MRSA detection.[14] This test is also not available for use in most routine diagnostic laboratories in Nigeria. Therefore, the onus lies on the laboratory to find the most reliable disk diffusion method for MRSA screening.

The objectives of this study are to determine the prevalence rate of methicillin resistance and antibiotic susceptibility patterns of *S. aureus* clinical isolates in Ekiti State, Nigeria, and to compare the performance of methicillin, oxacillin, and cefoxitin disk diffusion tests in detecting methicillin resistance with the gold standard *mecA* gene PCR.

Materials and methods

Specimens from various clinical infections were received at the Medical Microbiology Laboratory of University Teaching Hospital, Ado-Ekiti, between June 2010 and December 2010. The specimens were processed according to recommended guidelines by first culturing on Mannitol salt agar (Oxoid Ltd, Cambridge, UK) and incubating at 37°C for 24 hours.[17] *S. aureus* isolates were identified by colony morphology and fermentation reaction on Mannitol salt agar, Gram stain reaction, catalase, tube coagulase, and deoxyribonuclease tests.[17]

Methicillin resistance screening

This was done according to Clinical and Laboratory Standard Institute guidelines.[18] Pure cultures of each *S. aureus* isolate and control strain were used. Five colonies of each isolate and control strain were transferred to 5 mL of nutrient broth and were cultured overnight at 35°C. The overnight cultures were then diluted with sterile saline (0.85% NaCl) in Bijou bottles, and their turbidity was compared to 0.5 McFarland standards. The inocula were spread with a sterile cotton wool swab on Mueller–Hinton agar supplemented with 2% NaCl (Oxoid Ltd). Oxacillin (1 μg), methicillin (5 μg), and cefoxitin (30 μg) disks (Oxoid Ltd) were applied with sterile forceps, and the agar plates were incubated for a full 24 hours at 35°C aerobically. The inhibition zone diameter (ZD) for each isolate was measured and compared with the ZD interpretative standard.[18] MRSA National Center for Type Cultures 119040 was used as a control strain.

Antimicrobial susceptibility testing

The susceptibility of all *S. aureus* isolates to penicillin (ten units), gentamicin (10 μg), erythromycin (15 μg), pefloxacin (5 μg), tetracycline (30 μg), and vancomycin (30 μg) was determined using the modified Kirby–Bauer disk diffusion method.[19] The isolates were considered sensitive, intermediately resistant, and resistant based on the Clinical and Laboratory Standard Institute guidelines.[18]

Molecular detection of methicillin resistance (*mecA* gene)

DNA extraction

Deoxyribonucleic acid (DNA) extraction from each *S. aureus* isolate was carried out by modification of the simple crude extraction methods previously described for *Salmonella enterica*[20] and *Streptococcus pneumonia*.[21] Twenty-four-hour-old pure colonies of *S. aureus* were emulsified in 200 µL of deoxygenated water in Eppendorf tubes (Eppendorf North America, Hauppauge, NY, USA) and sealed with cellophane. The mixture was boiled at 100°C for 10 minutes in a water bath, cooled on ice, and then centrifuged at 15,000 × g for 30 seconds. The supernatant containing the DNA was stored at 4°C before use. Aliquots of 5 µL of template DNA were used for PCR.

PCR for *mecA* gene

The *mecA* DNA was amplified with the primers *mecA* F1 –AAA ATC GAT GGT AAA GGT TGG C, and *mecA* B1 –AGT TCT GCA GTA CCG GAT TTG C. PCR was carried out in a GeneAmp® PCR System 9700 (Life Technologies, Carlsbad, CA, USA) as previously described,[22] as follows: pre-denaturation at 95°C for 5 minutes, 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes. The products were detected by gel electrophoresis using 1.5% w/v agarose gel in 1 × Tris-buffered ethylenediaminetetraacetic acid for 2 hours at 80 V. The agarose gel was stained with 1 µg/mL of ethidium bromide (Sigma-Aldrich, St Louis, MO, USA) solution and visualized under ultraviolet illumination and then photographed (Syn-Gene Bioimaging System; Syngene UK, Cambridge, UK). The molecular weight marker band was used to estimate the molecular size of the amplified *mecA* gene, which was 533 base pairs. MRSA National Center for Type Cultures 119040 served as the positive control in the PCR reaction.

Data analysis

Data were entered into a Windows 7 (Microsoft Corporation, Redmond, WA, USA) laptop computer with the Statistical Package for the Social Sciences version 17.0 software package (SPSS; IBM Corporation, Armonk, NY, USA). Frequency tables were generated and analysis was performed using the appropriate statistical tools.

The sensitivity, specificity, positive predictive value and negative predictive value of the cefoxitin, oxacillin, and methicillin disk diffusion test in detecting phenotypic methicillin resistance in the *S. aureus* isolates using the presence of the *mecA* gene as "gold standard" were calculated with the formulas:

$$\text{Sensitivity} = TP/TP + FN \times 100 \quad (1)$$

$$\text{Specificity} = TN/TN + FP \times 100 \quad (2)$$

$$\text{Positive predictive value} = TP/TP + FP \times 100 \quad (3)$$

$$\text{Negative predictive value} = TN/TN + FN \times 100, \quad (4)$$

where TP (true positive) means that the disk showed the isolate to be resistant when the *mecA* gene is present, TN (true negative) means that the disk showed the isolate to be susceptible when the *mecA* gene is absent, FP (false positive) means that the disk showed the isolate to be resistant when the *mecA* gene is absent, and FN (false negative) means that the disk showed the isolate to be susceptible when the *mecA* gene is present.

Results

A total of 208 *S. aureus* isolates recovered from various clinical specimens (Table 1) were studied. Clinical data were not included. The antibiotic susceptibility pattern of the 208 isolates showing varying degrees of resistance is shown in Table 2. The highest degree of resistance was to penicillin G (82.7%; 172/208). All the isolates were susceptible to vancomycin (100%; 208/208), and were also highly sensitive to gentamicin (84.6%; 176/208) and sparfloxacin (73.1%; 152/208).

Forty of the 208 *S. aureus* isolates were *mecA* gene-positive on PCR analysis, giving a MRSA prevalence rate of 19.2% while 168 were *mecA* gene-negative (Table 1). Of the 40 *mecA* gene-positive isolates, the cefoxitin disk diffusion

Table 1 *Staphylococcus aureus* isolates from clinical specimens in Ekiti State, Nigeria

Clinical specimens	Number of isolates	*mecA* gene-positive (%) (MRSA)	*mecA* gene-negative (%) (MSSA)
Blood culture	23	0 (0)	23 (100)
Urine	53	8 (15.1)	45 (84.9)
Sputum	18	0 (0)	18 (100)
Anterior nares	20	0 (0)	20 (100)
Pleural fluid	5	0 (0)	5 (100)
Endocervical swab	11	0 (0)	11 (100)
High vaginal swab	10	4 (40)	6 (60)
Semen	8	0 (0)	8 (100)
Wound swab	46	24 (52.2)	22 (47.8)
Ear swab	14	4 (28.6)	10 (71.4)
Total	208	40 (19.2)	168 (80.8)

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*.

Table 2 Antibiotic susceptibility of *Staphylococcus aureus* isolates from clinical specimens in Ekiti State, Nigeria

Antibiotic	Susceptible number (%)	Intermediate number (%)	Resistant number (%)
Penicillin G	36 (17.3)	–	172 (82.7)
Cefoxitin	112 (53.8)	–	96 (46.2)
Methicillin	140 (67.3)	–	68 (32.7)
Oxacillin	124 (59.6)	–	84 (40.3)
Erythromycin	100 (48.1)	64 (30.7)	44 (21.2)
Gentamicin	176 (84.6)	8 (3.8)	24 (11.5)
Pefloxacin	124 (59.6)	48 (23.1)	36 (17.3)
Sparfloxacin	152 (73.1)	16 (7.7)	40 (19.2)
Tetracycline	64 (30.8)	8 (3.8)	136 (65.4)
Vancomycin	208 (100)	–	0 (0)

test detected phenotypic resistance (ZD ≤ 21 mm) in 40 of 40 (100%), the oxacillin disk diffusion test detected resistance (ZD ≤ 10 mm) in 32 of 40 (80%), and the methicillin disk diffusion test detected resistance (ZD ≤ 9 mm) in 28 of 40 (70%) *S. aureus* isolates. In the 168 *mecA*-negative *S. aureus* isolates, the cefoxitin disk detected resistance (false) in 36 (21.4%) isolates, the oxacillin disk detected resistance (false) in 52 (30.9%) isolates, and the methicillin disk detected resistance (false) in 40 (23.8%) isolates (Tables 3–5). Using the *mecA* gene as the "gold standard" test, the sensitivity of cefoxitin, oxacillin, and methicillin disks for MRSA detection were 100%, 80%, and 70% while the specificity rates were 78.5%, 69.1%, and 76.2%, respectively.

Figure 1 shows the agarose gel electrophoresis of PCR products of representative isolates with the *mecA*-positive isolate showing band at approximately 533 base pairs.

Discussion

Antibiotic resistance has been described as one of the paramount microbial threats of the 21st century.[23] *S. aureus* has always been a stumbling block for antimicrobial chemotherapy, and the introduction of new classes of antimicrobial agents is usually followed by the emergence of resistant forms of this pathogen.[24] Therefore, surveillance of the antimicrobial susceptibility patterns of *S. aureus* is of utmost importance in understanding new and emerging resistance trends, and in the management of both hospital- and community-acquired infections.

In this study, *S. aureus* was isolated from various clinical specimens obtained from different infective foci, with the highest being from urine. *S. aureus* is ubiquitous in its distribution, and although it is a normal flora of the anterior nares, skin, and genital tract of humans, it can cause infections in virtually any organ or system of the body; infections of wounds, skin, soft tissue, blood, and the lower urinary tract are particularly common. In a similar study carried out by Shittu and Lin,[25] more than 80% of the total number of isolates recovered were from infected wounds.

The antimicrobial susceptibility pattern of *S. aureus* isolates varies with place and time. The highest degree of antibiotic resistance in this study was to penicillin G (82.7%) and tetracycline (65.4%). These two drugs are available over the counter and are widely used in Nigeria without medical authorization.[25] Resistance to other antibiotics such as erythromycin, gentamicin, and fluoroquinolones appear to be relatively low, while all the isolates were susceptible to vancomycin, which agrees with previous reports.[23,25–27] This second category of antibiotics is not readily available for

Table 3 Comparison of cefoxitin disk diffusion test and *mecA* gene detection

	ZD ≤ 21 mm (R)	ZD ≥ 22 mm (S)	Total
Cefoxitin ZD (mm)			
+	40	0	40
mecA gene			
–	36	132	168
Total	76	132	208

Notes: Sensitivity = 100%; specificity = 78.6%; PPV = 52.6%; NPV = 100%.
Abbreviations: ZD, zone diameter; R, resistant; S, sensitive; PPV, positive predictive value; NPV, negative predictive value.

Table 4 Comparison of oxacillin disk diffusion test and *mecA* gene detection

	ZD ≤ 10 mm (R)	ZD ≥ 13 mm (S)	Total
Oxacillin ZD (mm)			
+	32	8	40
mecA gene			
–	52	116	168
Total	84	124	208

Notes: Sensitivity = 80%; specificity = 69.1%; PPV = 38.1%; NPV = 93.5%.
Abbreviations: ZD, zone diameter; R, resistant; S, sensitive; PPV, positive predictive value; NPV, negative predictive value.

Table 5 Comparison of methicillin disk diffusion test and *mecA* gene detection

	ZD ≤ 9 mm (R)	ZD ≥ 14 mm (S)	Total
Methicillin ZD (mm)			
+	28	12	40
mecA gene			
–	40	128	168
Total	76	132	208

Notes: Sensitivity = 70%; specificity = 76.2%; PPV = 36.8%; NPV = 96.9%.
Abbreviations: ZD, zone diameter; R, resistant; S, sensitive; PPV, positive predictive value; NPV, negative predictive value.

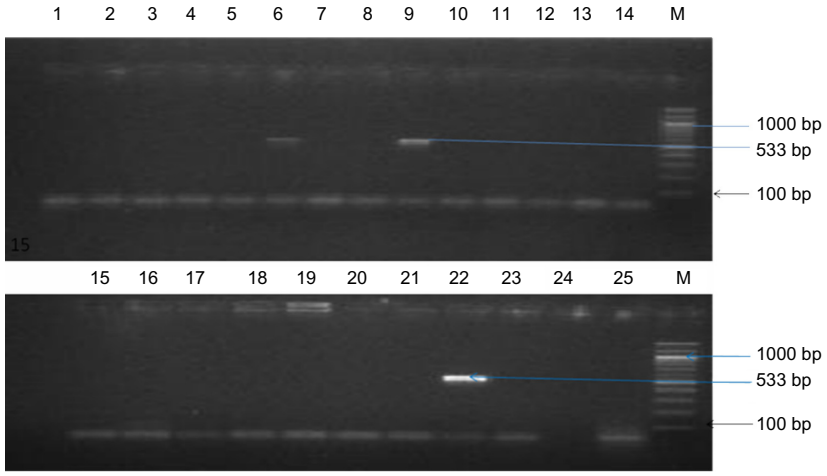

Figure 1 Agarose gel electrophoresis of PCR products of representative isolates.
Notes: The *mecA* gene is indicated by the amplification of 533 base pair products. Lanes 6 and 9 are positive for *mecA*, as indicated by the 533 base pair PCR product. Lanes 1–5, 7, 8, 10–21, 24, and 25 were negative for *mecA*. Lane 22 was the MRSA-positive control (NCTC 119040), lane 23 was the negative control (water), and lane M was the molecular weight size marker.
Abbreviations: PCR, polymerase chain reaction; MRSA, methicillin-resistant *Staphylococcus aureus*; NCTC, National Center for Type Cultures; M, molecular weight.

consumption to a majority of the populace because of its high cost. Vancomycin in particular is not available for routine clinical use in Nigeria; hence, most *S. aureus* isolates, including MRSA, are still susceptible to the drug,[1,12–15] although there are few reports of the emergence of vancomycin-intermediate and vancomycin-resistant *S. aureus* clinical isolates in some centers in Nigeria.[28,29]

Phenotypic resistance to methicillin, oxacillin, and cefoxitin were 32.7%, 40.3%, and 46.5%, respectively. These three antibiotics are commonly used in the disk diffusion susceptibility test by the clinical diagnostic laboratory in our environment to screen staphylococci isolates for resistance to methicillin. All three antibiotic disks overestimated the prevalence of MRSA, as the *mecA* gene that encodes resistance to methicillin was detected by PCR in 19.2% of the *S. aureus* isolates. The implication is that previous reports on MRSA in our environment, which were based on these disk diffusion tests, overestimate MRSA prevalence.[1,12,30] The MRSA prevalence rate of 19.2% in this study compared favorably with the rate of 22.2% reported in a recent *mecA* PCR study of MRSA isolates obtained from three major tertiary health care institutions in southwestern Nigeria.[31] When these results are compared with those of previous studies that reported 1.4%[32] and 1.5%[25] prevalence rates of MRSA using *mecA* gene PCR, we can conclude that there is a gradual increase in the prevalence of MRSA strains in Nigeria.

When compared to the gold standard *mecA* gene test for MRSA detection, the cefoxitin disk (30 µg) gave the highest sensitivity (100%) and specificity (78.5%), performing better than oxacillin and methicillin. This finding is in agreement with previous reports.[16,33] From the results of our study and those of other studies,[16,33] we recommend that the cefoxitin disk should replace the oxacillin disk or methicillin disk as a screening tool for MRSA. One advantage of the cefoxitin disk test is that incubation can take place within 18 hours at 36°C or 37°C, the usual temperature of an aerobic incubator in most microbiology diagnostic laboratories, whereas the optimal incubation temperature for oxacillin or methicillin is 35°C for a full 24 hours.[16]

Acknowledgments

The molecular biology works were all carried out in the Molecular Biology Laboratory, Department of Biomedical Sciences, Ladoke Akintola University of Technology, Mercy Land Campus, Osogbo, Nigeria. The assistance of the Management of Ladoke Akintola University of Technology is appreciated. We also wish to thank Mr Oyenike for his technical assistance.

Disclosure

The authors report no conflicts of interest in this work.

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