Antibiotic resistance patterns and plasmid profiles of methicillin resistant *Staphylococcus aureus* isolates from human samples

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ABSTRACT

Antibiograms and plasmid profile are commonly used to characterize MRSA epidemiologic studies. However, antibiograms are frequently inadequate to accomplish the differentiation. Plasmid profile being more informative has been reported to be useful in tracing the epidemiology of antibiotic. Antibiotic resistance patterns and plasmid profiles of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from human specimens were investigated to determine the discriminatory power of plasmid profile analysis in conjunction with antibiotic susceptibility pattern. Specimens were analyzed using disc diffusion assay and restriction enzymes analysis of plasmid DNA procedure. The 51 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were grouped into 18 groups using their resistogram. Twenty four (47.1%) strains out the 51 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates harbored plasmids. Single plasmid isolates were 14(27.5%), double plasmid isolates were 6(11.8%) while tripple plasmid isolates were 4(7.8%). The 24 isolates containing plasmids were categorized into 14 groups based on their resistogram. Plasmid profile showed greater similarity between isolates (10 profiles) than antibiotic resistance pattern which showed a higher disparity (14 patterns). However, resistance to various antimicrobial agents was not consistent with the presence of plasmids. No particular molecular size plasmid could be associated with any particular antimicrobial resistance patterns. Resistance was observed in isolates with various molecular size plasmids as well as in those that had no plasmids. Nonetheless, 2 pairs of isolates with the same plasmid profile also have similar (almost the same) resistance pattern. Plasmid profile analysis in conjunction with the antibiotic resistance typing is valuable in the epidemiological investigation of methicillin-resistant *Staphylococcus aureus* (MRSA).

Keywords: MRSA, antibiogram, plasmid profiling, human isolates.

1. INTRODUCTION

Staphylococcus aureus (*S. aureus*), a spherical aerobic Gram-positive, catalase positive, non-motile, non-spore forming coccus [1,12,26], is an opportunistic pathogen in human [1,9] and animal [12,24]. The pathogen is responsible for broad spectrum of human and animal diseases ranging from skin infections [9] to such severe disease as pneumonia [11,25], endocarditis [10], osteomyelitis [12,20], septicaemia and enterocolytis [3]. Such that

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infections involving antibiotic resistance strain may impact on human health [21]. Methicillin-resistant *S. aureus* (MRSA) is responsible for hospital-acquired (HA) infections [13, 16] and presently community acquired (CA) infections [15,17,18,29]. Hospital associated (nosocomial) staphylococcal infections have been reported to be resistant to as many as 20 antimicrobial compounds, including antiseptics and disinfectants. Resistance to penicillin among *S. aureus* strains appeared a few years after the introduction of penicillin therapy. Introduction of other antibiotics such as streptomycin, tetracycline and chloramphenicol, and the macrolides was similarly followed by emergence of resistant organisms. Resistant organisms that had acquired resistance to these antibiotics were reported to be usually resistant to penicillin through the production of penicillinase. This resulted in the evolution of organisms with a wide spectrum of resistance and a marked ability to survive and spread in the hospital environment. Such multiply resistant *S. aureus* strains were of global significance as early as 1950s [19].

The genetic exchange of plasmids containing antibiotics resistant determinants between bacteria is believed to play a critical role in the evolution of antibiotic resistant bacteria and this has been shown in *S. aureus* [2]. Plasmids may contain resistance genes for single or multiple antimicrobial agents and they have been reported to transfer these resistance genes from one bacteria to another. Indeed, resistance within the staphylococci to several therapeutically useful antibiotics, including streptomycin, rifampicin, fusidic acid and novobiocin is thought to be derived from chromosomal mutation.

Molecular techniques such as plasmid profiling [4, 10], restriction endonuclease analysis of plasmid DNA [2,27], and analysis of chromosomal DNA after enzyme restriction [23,28] are currently being used in differentiating strains of bacteria isolates and opinions vary as to the discriminating power of the various types of these techniques [2], while others have opined that no single technique was clearly superior to the other for typing *S. aureus* and that a combination of different techniques is sometimes necessary [2]. Plasmid profiles have been useful in the epidemiological surveillance of disease outbreaks and in tracing antibiotic resistance [6].

Individual plasmid mediated resistance in MRSA isolates [6] and plasmid carried by resistance isolates [10,29], have been studied. At present, MRSA has become an endemic pathogen worldwide [22] and has also become multidrug resistant [14]. This study was therefore conducted to analyze the antibiotic resistance and resistance plasmids of MRSA isolates from healthy humans and consequently determine the discriminatory power of plasmid profile analysis in conjunction with antibiotic susceptibility pattern (antibiogram) in differentiating different strains of methicillin-resistant *S. aureus* (MRSA).

2. MATERIALS AND METHODS

2.1 Sample Collection

A total of 84 pathological specimens (nose swab or ear swab or urine) were obtained from 84 otherwise healthy individuals in Uturu community, Abia State Nigeria with the use of sterile swab sticks and universal bottles. The specimens were labeled accordingly with the first initial of the site of specimen collection (N for nose, E for ear and U for urine). Each specimen was streaked on mannitol salt agar (MSA), nutrient agar (NA) and incubated at 37°C for 24 hours at the Department of Plant Science and Biotechnology Laboratory, Abia State University, Nigeria.

2.2 Bacterial Identification

Colonies that appeared white or yellow or cream-coloured on MSA plates with yellow surrounding (indicative of fermentation) were considered as *S. aureus* and stock cultures were immediately prepared . Isolates were further identified as *S. aureus* by testing their

ability for DNAse production on agar plates. Gram stain was performed and isolates were identified by their ability to produce catalase, coagulase and oxidase enzymes then identification was finally confirmed by Bergey's manual [31], as described by Al-Jumaily *et al*. [5].

2.3 Oxacillin Susceptibility Testing

The oxacillin disc used was procured from Oxoid, Germany. The antimicrobial susceptibility profile of the isolates was determined using the disc diffusion technique assay as described by Orji *et al*. [20]. All other discs used were prepared by MAYO diagnostic laboratory, Nigeria.

With a sterile wire loop, few colonies of each of the isolates were emulsified in 5ml of sterile peptone water to a turbidity corresponding to 0.5 McFarland standard (corresponding to approximately 10^8 cfu/ml). Then 0.5 ml of each inoculum was dispensed unto the surface of dried Mueller Hinton agar plate using sterile Pasteur pipette. These were spread evenly on the agar surface with sterile swab stick (one for each inoculum). The excess inocula were discarded into a disinfectant jar. The inoculated plates were kept on the bench for 3 minutes to dry. The oxacillin disc (1 µg) was then placed centrally on the inoculated plates aseptically using a sterile forceps.

The preparations were incubated aerobically for 24 hours at 35°C. The diameter of zone of inhibition produced by each of the disc was measured, recorded and the isolates were classified as resistant (≤ 10 mm) or sensitive (≥ 13 mm) based on the standard interpretative chart as described by the Clinical and Laboratory Standard Institute [7,8].

2.4 Susceptibility Testing of MRSA Isolates to other Conventional Antibiotics

The following antimicrobial sensitivity discs were used; ampicillin (10µg), ciprofloxacin (5µg), ofloxacin (5µg), erythromycin (15µg), gentamicin (10µg), vancomycin (30µg), amoxicillin (10µg), and ceftriaxone (30µg), to determine the susceptibility profile of all the methicillin-resistant strains using the disc diffusion technique assay as described by Orji *et al*. [21].

With a sterile wire loop, few colonies of each of the isolates were emulsified in 5ml of sterile peptone water to a turbidity corresponding to 0.5 McFarland standard. Then 0.5 ml of each of the inoculum was dispensed unto the surface of dried Mueller Hinton agar plates using sterile Pasteur pipettes. These were then spread evenly on the agar surface with sterile swab sticks. The excess inocula were discarded into a disinfectant jar. The plates were kept on the bench for 3 minutes to dry. The various discs were placed on the inoculated plates at 25 mm away from one another and 15 mm away from the edge of the plates aseptically using a sterile forceps.

The preparations were incubated aerobically for 24 hours at 35°C. The diameter of the zone of inhibition produced by each of the discs was measured, and recorded and interpreted based on the standard interpretative chart as described by the Clinical Laboratory Standard Institute [7].

2.5 Plasmid Profile Analysis

Plasmid DNA was isolated as described by Birnboim and Doly modified by use of lysostaphin for lysing the cell wall as described by Ombui *et al*. [20]. The analysis was carried out at the International Institute of Tropical Agriculture (IITA), Ibadan. Each MRSA strain was inoculated into 3 ml tryptic soy broth and incubated overnight on a roller drum at 37°C. About 1.5 ml of each overnight broth culture was transferred into eppendorf tubes and centrifuged for one minute at 15000 rpm at room temperature. The supernatant was discarded and 2 µl of lysotaphin solution (1.0 µg/ml in distilled water) added to the pellet. Tubes were capped, vortexed and placed in ice for 30 minutes. Two hundred microlitres of

alkaline detergent solution (0.2 N NaOH; 1% SDS) was added and tubes inverted several times and then kept in the water bath for five minutes. One hundred and fifty microlitres of 3 M Sodium acetate (pH 4.8) was added and tubes inverted several times to mix and then kept in ice for at least 10 minutes. The tubes were centrifuged at room temperature at 15000 rpm for five minutes and the supernatant transferred into new eppendorf tubes. One millilitre of 95% ice cold ethanol was added to the tubes, which were then kept at -20°C for five minutes. After five minutes, they were centrifuged at 15000 rpm for three minutes, supernatant discarded and the sediment re-suspended in 40 µl of sterile distilled water and 10 µl of Tris/ETDA (10 mM Tris HCl and 1 mM ETDA. pH 8.0) was added as well. This plasmid DNA was subjected to electrophoresis according to the method mentioned below for molecular weight estimation or be stored at -20°C.

2.6 Restriction Endonuclease Analysis of Plasmid DNA

One hundred µl of Tris/ETDA (10 mM Tris HCl and 1 mM ETDA. pH 8.0) buffer were added to about 100 µl of the plasmid DNA solution that remained after the molecular weight determination, and this solution was mixed. One millilitre of 95% ice cold ethanol was added to the tube, which was then kept at -20°C for five minutes. After five minutes, it was centrifuged at 15000 rpm for three minutes, supernatant discarded. The pellet was dried by evaporation under a vacuum and dissolved in 40 µl of autoclaved water. For restriction enzyme analysis, 10 µl of 5 x –concentrated restriction endonuclease assay buffer and 5 U of endonuclease solution (EcoRI) was added to the material and allowed to react for 3 hours. The assay buffer used and the reaction temperature were according to the instructions of the manufacturer. After digestion, the sample was subjected to electrophoresis as described below.

2.7 Agarose Gel Electrophoresis

Electrophoresis was carried out in a horizontal agarose gel electrophorectic unit. The agarose gel (0.8%) was prepared in Tris-Boric acid-EDTA (TBE) buffer, allowed to cool and 3 drops of ethidium bromide was added.
Using a micropipette, 20 µl of each sample plasmid preparation, with added 0.2 µl bromophenol blue dye (tracking dye) was applied into the horizontal well containing 50 ml of agarose gel. Into the first well was added 20 µl of DNA molecular Weight Marker (1kb DNA ladder) to which 0.2 µl of bromophenol blue had also been added as the standard. Electrophoresis was performed at 120v at room temperature for 2 hours. The electrophoretic bands were visualized with the aid of UV light transilluminator and the photograph was taken using a digital camera.

2.8 Molecular Weight Estimation

The size of each plasmid was estimated by directly comparing with the molecular weight marker bands (ladder 1kb) which has 13 distinctive observed bands with molecular weight ranging from 300bp-1kb. Each strain of MRSA was characterized by the number of EcoRI and size of plamid DNA fragment it contained. Isolates with the same number and size of EcoRI plasmid DNA fragment (same plasmid profile) were considered the same strain.

3.0 RESULT AND DISCUSSION

Pathological specimens from 84 healthy individuals were examined and amongst them, 69(82.1%) were positive for *S. aureus* and 51(60.7%) were methicillin (oxacilin)-resistant. All the 51 MRSA isolates were grouped into 18 groups following their antibiotic resistant

patterns. Eighteen isolates were resistant to ampicillin and amoxicillin while only 9 isolates were resistant to ampicillin alone (table 1). Resistance to ampicillin cut across all groups.

Table 1: The resistogram of MRSA isolated from healthy individuals
n = 51

Isolates	Resistance pattern
18	Ap, Am
9	Ap
3	Ap, Va, Am
2	Ap, Am, Ce
1	Ap, Ge
4	Ap, Va
2	Ap, Ci, Of, Er, Ge, Va, Am, Ce
1	Ap, Ci, Er, Ge, Va, Am, Ce
1	Ap, Of, Er, Va, Am, Ce
1	Ap, Ci, Er, Va, Am, Ce
1	Ap, Er, Ge, Va, Am
1	Ap, Ci, Of, Er, Ge, Va, Am
1	Ap, Er, Va, Am
1	Ap, Va, Am, Ce
2	Ap, Ge, Am
1	Ap, Er, Va
1	Ap, Ci
1	Ap, Gen, Ce

Ap = ampicillin, Ci = ciprofloxacin, Of = ofloxacin, Er = erythromycin, Ge = gentamycin, Va = vancomycin, Am = amoxicillin, Ce = ceftriaxone

A total of 24 isolates representing 47.1% of the total MRSA isolates were found to possess plasmid. Single plasmid isolates were 14(27.5%), double plasmid isolates were 6(11.8%) while tripple plasmid isolates were 4(7.8%). Twenty seven isolates were found to have no plasmid (table 2). The 24 isolates containing plasmids were categorized into 14 groups based on their resistogram. Plasmid profile showed greater similarity between isolates (10 profiles) than antibiotic resistance pattern which showed a higher disparity (14 patterns).

Table 2: Plasmid profile of MRSA isolated from healthy individuals
n = 51

Plasmid profile group	Plasmid profile	No of isolates (%)
0	-	27(52.9)
1	3000	2(3.9)
2	500, 1000	1(2.0)
3	4000	7(13.7)
4	500	2(3.9)
5	300	3(5.9)
6	500, 4000	3(5.9)
7	300, 4000	1(2.0)
8	300, 500, 700	4(7.8)
9	1000, 1500	1(2.0)

In characterizing MRSA isolates epidemiologically, antibiotic profile is poorly discriminatory, type specific antisera for serologic tests are expensive, phage typing are often non-reproducible and some strains are non-typeable.
Restriction endonuclease analysis of plasmid DNA resolved all the isolates in this study into 10 EcoRI profiles (figure 1and table 2).

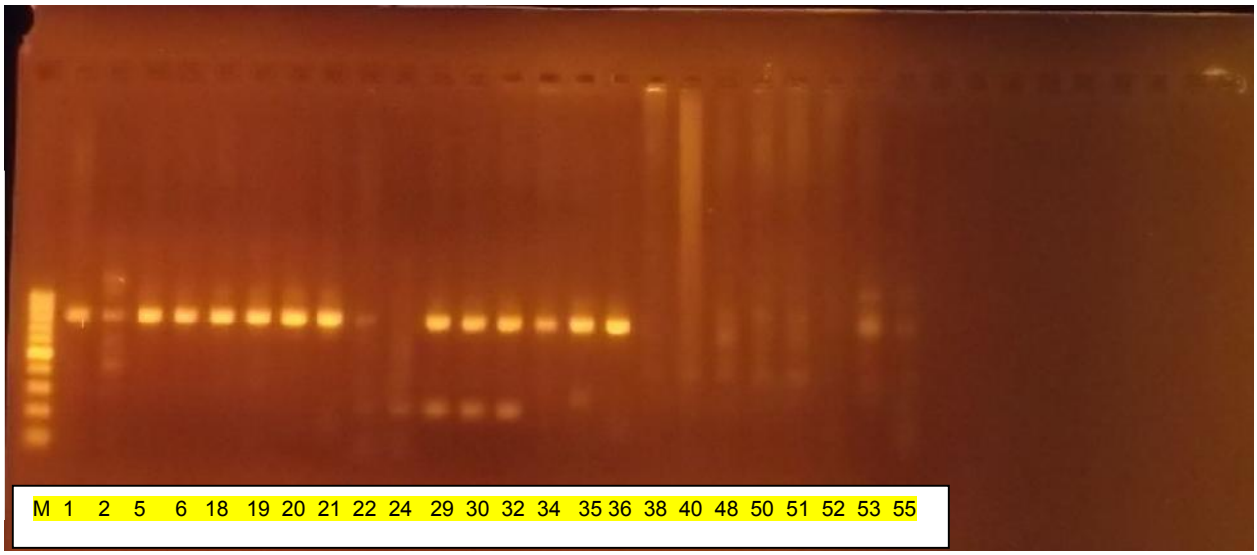

Figure 1: Plasmid profile of MRSA after the EcoRI restriction digestion reaction set up with the selected 24 plasmid DNA.

Even though studies have demonstrated the stability and diversity of restriction enzyme profile of plasmid DNA by finding very few strains of MRSA obtained from various sources and locations to lack plasmid entirely [27], plasmid profile appeared to be of low discriminatory power in the investigation of MRSA epidemiology because of the non-detection of plasmid in majority of the isolates of MRSA in this study. This could be due to the fact that most isolates have low copy number plasmids which were not detected.
Plasmid profile has been reported to be useful in tracing the epidemiology of antibiotic resistance [19]. However, in this study, resistance to various antimicrobial agents was not consistent with the presence of plasmids. This was because no particular molecular size plasmid could be associated with any particular antimicrobial resistance patterns. Resistance was observed in isolates with various molecular size plasmids as well as in those that had no plasmids. The reason for this could not be explained but could be due to various attitudes of the participants towards use of antibiotics. Nonetheless, while there existed in this study isolates with same resistance pattern but with different plasmid profile, 2 pairs of isolates with the same plasmid profile also have similar (almost the same) resistance pattern (table 3).

Table 3: Plasmid profile and resistogram of plasmid DNA containing isolates
n = 24

Serial number	Lab number	Resistance pattern	Plasmid profile (bp)
1	1=E_1	Ap, Ci, Er, Ge, Va, Am, Ce	3000
2	2= E_3	Ap, Of, Er, Va, Am, Ce	500, 1000
3	5= E_6	Ap, Ge, Am	4000

4	6= E_7	Ap, Am	4000
5	18= E_{12}b	Ap, Er, Va	4000
6	19= E_{17}a	Ap, Am	4000
7	20= E_{17}b	Ap	4000
8	21= E_{18}	Ap	4000
9	22= E_{19}	Ap, Ci	500
10	24=N_1	Ap, Va, Am	300
11	29= N_8	Ap, Am	500, 4000
12	30= N_9	Ap, Am	500, 4000
13	32= N_{11}	Ap, Ci, Of, Er, Ge, Va, Am, Ce	500, 4000
14	34= N_{12}	Ap, Am, Ce	3000
15	35= N_{13}	Ap, Am	300, 4000
16	36= N_{15}	Ap, Ge	4000
17	38= N_{16}b	Ap, Am	300
18	40= N_{18}	Ap, Am	500
19	48=U_{14}	Ap	300, 500, 700
20	50= U_{16}a	Ap, Am	300, 500, 700
21	51= U_{16}b	Ap	300, 500, 700
22	52 U_{10}a	Ap, Va, Am	300
23	53= U_{10}b	Ap, Va	1000, 1500
24	55= U_9a	Ap, Ge, Ce	300, 500, 700

Ap = ampicillin, Ci = ciprofloxacin, Of = ofloxacin, Er = erythromycin, Ge = gentamycin, Va = vancomycin, Am = amoxicillin, Ce = ceftriaxone.

The resistance of MRSA isolates to various antimicrobials may be located either on chromosomes, plasmids or transposons. Methicillin resistance gene (mec4916) has a chromosomal locus, and is probably maintained on mobile genetic element while tetracycline resistance observed in *S. aureus* strain DU4916 was reported to be encoded by a 4.3 kb plasmid, and in some strains resistance was found to be encoded by a chromosomal gene [19].

4. CONCLUSION

In conclusion, this study showed that the genetic basis for antibiotic resistance in the study area is not entirely plasmid mediated. Plasmid profile analysis in conjunction with the antibiogram is valuable in differentiating various strains of community acquired MRSA.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS CONTRIBUTIONS

This work was carried out in collaboration between all authors. Author CI designed the study, searched out the literature and carried the experiments. Authors CI did the analysis and wrote the first draft of the manuscript. Author RAO supervised the entire work. Author SCU contributed to analysis of the results and writing the manuscript. All authors read and approved the final manuscript.

CONSENT

All authors declare that verbal informed consent was obtained from the participants for publication of this result.

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