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# Vancomycin-Resistant *Staphylococcus aureus* Isolates from HIV Positive Patients in Imo State, Nigeria

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**Abstract:** Vancomycin continues to be an important antimicrobial agent for treating infections caused by *Staphylococcus* aureus strains that are resistant to oxacillin (MRSA) and other antimicrobial agents. Vancomycin-resistant *Staphylococcus aureus* (VRSA) isolates were obtained from HIV-positive patients already on HAART treatment but were not admitted in the hospital. Species identification was confirmed by standard biochemical tests and PCR amplification of the 16S rRNA gene. Vancomycin resistance was determined using the Kirby-Bauer diffusion method and confirmed by Brain Heart Infusion (BHI) vancomycin screen agar plate containing 6µg/ml vancomycin. A total of 8 VRSA were identified from the 59 isolates obtained from the patients. Five out of the eight VRSA isolates were resistant to all the antibiotics tested. However, one unusual strain which was resistant to all the antimicrobial agents tested contained no plasmid, *Mec A* gene and PVL toxin gene. One VRSA isolate contained a large plasmid (~21.2 kb) and four small plasmids of ~5, 2.5, 1.2 and 0.8 kb respectively. The minimum inhibitory concentration (MIC) for vancomycin susceptibility was >15 µg/ml at disk potency of 30µg. The reduced susceptibility of *S. aureus* strains to vancomycin leaves clinicians with relatively few therapeutic options for treating these infections and therefore emphasizes the importance of prudent use of antibiotics and the use of infection-control precautions to prevent their transmissions.

**Keywords:** Antibiotic Susceptibility, VRSA, MRSA, HIV Positive Patients, *Staphylococcus aureus*, Plasmid Profile, *MecA* Gene, Haemolytic Activities

## 1. Introduction

Vancomycin continues to be an important antimicrobial agent for treating infections caused by *Staphylococcus* aureus strains that are resistant to oxacillin or methicillin (MRSA) and other antimicrobial agents. Human immunodeficiency virus (HIV) is a major health problem in Nigeria and HIV patients are vulnerable to several infections termed opportunistic infections because of their weakened immune system. Over the past two decades, *Staphylococcus aureus* has emerged as a significant opportunistic pathogen among HIV patients in both nosocomial and community settings, and recent studies have shown greater frequency and morbidity of this organism among HIV positive individuals [1, 2]. However, there is little data on the occurrence and antibiotic resistance of this organism among HIV positive patients in Imo state, Nigeria.

*S. aureus* normally localize in the skin and mucous membranes in the nose of healthy humans and about 30% of the normal healthy population are transiently colonised by the organism [3, 4] which has been associated with several syndromes such as skin infections, osteomyelitis, bacteraemia, septicaemia, diarrhoea, pneumonia and urinary tract infections [5 - 11]. These syndromes have been mostly described among HIV patients and are responsible for high rates of morbidity and mortality.

The prevalence of MRSA was about 10% in Tunisia, Malta, and Algeria and around 15% in Kenya, as compared to the

high prevalence of 21 to 30% in Cameroon and Nigeria when many African hospitals were studied [12], all MRSA isolates were sensitive to Vancomycin. In Uganda, about 10% of the surgical procedures become septic which account for an increasing morbidity and mortality with the commonest organism isolated being *S. aureus* [13]. Data from the national nosocomial infection surveillance network (USA) have shown that MRSA present more than 50% of *S. aureus* strains causing infection in patients in intensive care units [4]. In Europe, MRSA prevalence ranges from over 50% in Portugal and Italy to below 2% in Switzerland and the Netherlands, where infection control measures have been shown to work [13]. In Asia, the prevalence lies around 50%, with extremely high rates in Hong Kong (75%) and Japan (70%) [14]. These show that antimicrobial resistance is an increasing problem and a challenge worldwide [15]. The prevalence of resistance in *S. aureus* also is increasing globally. Staphylococcal resistance to penicillin is mediated by penicillinase production: an enzyme which breaks down the β-lactam ring of the penicillin molecule [16]. This study determined the antibiotic susceptibility and plasmid profile of vancomycin resistant *S. aureus* isolates from HIV positive patients as well as their haemolytic activities.

# 2. Materials and Methods

### *2.1. Study Subjects*

These were HIV positive outpatients, attending three general hospitals, namely; Specialist hospital Umuguma, General hospital Okigwe and General hospital Awo-Omama, from June 2011 to July 2012. The patients, already on HAART treatment and presenting symptoms for pneumonia, skin, oral as well as urinary tract infections (UTI) who were not admitted in the hospital three months before and during the course of this study were included in the study.

### *2.2. Ethical Considerations*

The written informed consent of the ethical committee of the hospital management board overseeing the three hospitals was obtained (Ref Number: HMB/AD/872/11/132) in accordance with ethical standards. Oral informed consents of all the leaders of the HIV support groups of the three hospitals were also obtained before sample collection. Written informed consent was also obtained from individual HIV patients before sample collection. For confidentiality the patients were given codes, which were in tandem with the samples collected.

### *2.3. Bacterial Isolation and Identification*

All cultures were grown on nutrient agar and blood agar media. Identification of the clinical isolates of *S. aureus* were performed by conventional biochemical tests, including catalase, coagulase, mannitol fermentation tests, and Gram staining [17]. Species identification was confirmed by PCR amplification of the 16S rRNA gene that is highly specific for *S. aureus*.

### *2.4. Molecular Confirmation of Staphylococcus Aureus Isolates*

The chromosomal DNA was extracted by boiling according to the method of Zhang *et al.* [18]. Purified DNA from isolates was quantified using a spectrophotometer (NanoDrop ND 1000). Molecular identity of the isolates was tested based on PCR, targeting the 16S rRNA according to McClure *et al*. [19]. The extracted bacterial DNA was prepared targeting a 756-bp internal fragment of the gene with primers as follows; forward: 5 - AAC TCT GTT ATT AGG GAA GAA CA - 3, and reverse: 5 - CCA CCT TCC TCC GGT TTG TCA CC -3 (Inqaba Biotechnical, South Africa). PCR reaction mixture was optimized by adding to each eppendorf tubes, 16.4 µl of sterile distilled deionised water, 5 µl of PCR buffer, 1.5 µl of 1x $MgCl_2$, 0.5 µl of dNTP, 0.25 ul each of the forward and backward primers and 0.1 µl of taq DNA polymerase (Promega USA) was added. To completely make it a 25 µl reaction mixture, 1 µl of extracted DNA from the processed clinical samples was added according to the labelling. PCR was carried out in a thermal cycler (Eppendorf Vapoprotect, Germany) with the reaction cycles consisting of an initial denaturation at 94ºC for 5 min; 34 cycles of 55ºC for 30 seconds, 52ºC for 1 min and 72ºC for 1 min. A final extension step at 72ºC was continued for another 10 mins. The PCR products were resolved on 2% agarose gels containing 0.5 µg/mL ethidium bromide and documented using a gel documentation system (Clinix Science, Japan).

### *2.5. Determination of Vancomycin Resistance*

Vancomycin-resistance was determined phenotypically by the Kirby-Bauer diffusion method using vancomycin disk (Oxiod, UK) of 30 µg. The vancomycin resistance was further confirmed using commercially prepared Brain Heart Infusion (BHI) agar plates (Remel ©, USA) containing 6 µg/ml of vancomycin by inoculating 10 µl of 0.5 McFarland standard bacterial suspension on the plates. The antibiotic-resistance profile of the VRSA isolates were determined by the Kirby-Bauer disk diffusion technique [20], using 9 antibiotic single disks. They were all obtained commercially from Atek UK except oxacillin (1 µg) antibiotic disk that was obtained from Oxoid, UK. The amounts (µg) of antimicrobial per disk were as follows: Gentamicin (30 µg), Streptomycin (25 µg), Erthromycin (5 µg), Cloxacillin (5 µg), Chloramphenicol (10 µg), Ciprofloxacin (5 µg), Cefuroxin (30 µg) and Augmentin (30 µg). The McFarland's standard of 0.5 was used for antibiotic susceptibility test by inoculation on Mueller- Hinton agar plates according to Cheesbrough [21].

### *2.6. Plasmid DNA Extraction*

The Plasmid DNA was extracted according to the modified method of Ehrenfeld and Clewell, [22]. Briefly overnight culture of 1.5 ml of the individual samples contained in eppendorf tubes was spined in a macro-centrifuge to pellet cells and the supernatant discarded. Solution A (200 µl) containing 10mg of lysozyme per ml to resuspend the cell and incubated for 30 minutes at 37ºC. After incubation, 400µl of

freshly prepared 1% sodium dodecyl sulphate in 0.2N NaOH was added and was mixed by inverting the tubes until the content is slimy to remove all impurities. A 300 µl solution of 30% potassium acetate (pH 4.8) was added, mixed by vortexing then incubated on ice for 5 minutes and centrifuged to remove debris which settled at the bottom. The supernatant was transferred into another 1.5 ml eppendorf tube and the plasmid DNA was extracted with 200 µl phenol-chloroform (1:1) mixture, vortexed and centrifuged at 12,000 rpm for 5 minutes with supernatant discarded. The plasmid DNA was precipitated with equal volume of isopropanol, then, the mixture was vortexed and incubated on ice for 30 minutes. After incubation the mixture was centrifuged at 14,000 rpm for 15 minutes and the supernatant discarded. The deposits were allowed to dry by slanting on a shelf and 200 µl tris EDTA was added to dissolve the plasmid DNA after drying. The various sizes of the plasmid DNA was separated by running on 0.8% agarose gel at 80 volts.

### *2.7. Determination of Haemolytic Activities*

The haemolytic activity testing of the *S. aureus* isolates was performed according to the method previously described by Jimenez *et al*. [23]. Briefly, Colombia agar was prepared and supplemented with 5% human blood. The microorganisms were inoculated on the Colombia blood agar and incubated at 37ºC. The plates were analyzed after 72 h and isolates were classified as non-haemolytic when no lysis was observed, beta (β) haemolytic when moderate lysis was observed and alpha (α) haemolytic when complete lysis of the blood in the media was observed.

### *2.8. Determination of the Presence of Mec A Gene and PVL Gene among the Isolates*

Polymerase chain reaction (PCR) assay according to the modified method of McClure et *al*. [19] was used. Amplification in a single PCR which produced PVL distinct bands corresponding to respective molecular sizes of 433bp and 310bp for *mecA* gene that were easily recognizable in agarose gel stained with ethidium bromide was first determined.

The *LUKS/F-PV* genes (which encoded the *PVL S/F* bicomponent proteins) with primers *luksF-PV-I* and *lukS-PV-2* and combined primers for *mec*A gene were combined. The conditions were optimized by assaying different primers concentrations and other PCR components as follows: 2 µl of template DNA preparations by previously boiling methods [18]. In a 25 µl final reaction volume containing, 9.5 µl of distilled deionised water, 12.5 µl of master mix, 0.25 µl of each *Luks/f-pv* and *Mec*A forward and backward primers, respectively with thermocycling conditions set at denaturation at 94ºC for 5 mins, followed by 30 cycles of denaturation at 94ºC for 45 secs, annealing at 55ºC for 45 secs and extension at 72ºC for 75 secs and a final extension step at 72ºC for 10 mins. The PCR products were also resolved on 2% agarose gels containing 0.5 µg/mL ethidium bromide and documented using a gel documentation system (Clinix Science, Japan).

## 3. Results

A total of 8 out of 59 isolates obtained from different types of samples were vancomycin resistant *S. aureus* (VRSA). All isolates were confirmed to be *S. aureus* by PCR by the observation of the specific bands as shown in Figure 1. Of these, 4 (50%) were from skin, 3 (37.5%) were from mouth and one (12.5%) from sputum. Of all the VRSA isolates, 7 (87.5%) were from female patients while one (12.5%) was from a male patient.

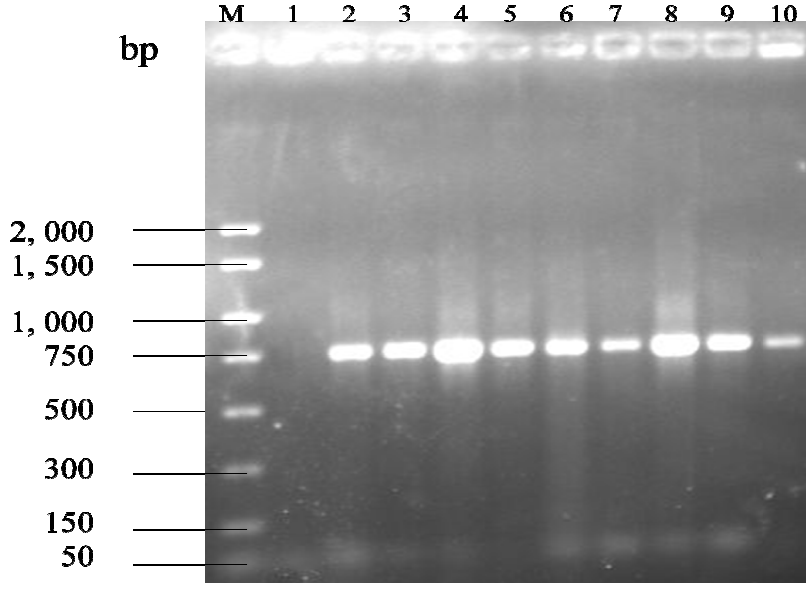


***Figure 1.*** *PCR amplification of the 16S rRNA gene, Lane M, 50-bp DNA ladder (Sigma Aldrich PCR Marker); Lane 1 is negative control while Lane 2 is the positive control. Lane 3 – 10 represents the VRSA isolates.*

### *3.1. Antibiotic Susceptibility/Resistance Profiles*

There were eight isolates phenotypically resistant to vancomycin (Figure 2 and 3). Five out of the eight (62.5%) VRSA isolates were resistant to all the antibiotics tested (gentamicin, streptomycin, erythromycin, cloxacillin, chloramphenicol, ciprofloxacin, cefuroxin, augmentin and oxacillin). However, one unusual strain (number 7) which was resistant to all the antimicrobial agents tested (Table 1), contained no plasmid (Figure 4), *Mec A* gene and PVL toxin gene (Figure 5). One VRSA isolate contained a large plasmid (~21.2 kb) and four small plasmids of ~5, 2.5, 1.2 and 0.8 kb respectively (Figure 4) when compared with the molecular size of plasmid markers of the reference strain promega G173A lamda. High resistance was observed among this group of isolates since none of the isolates was completely susceptible to all the tested antibiotics. Table 2 summarizes the antibiotic susceptibility profiles of the isolates against 9 antimicrobial agents.

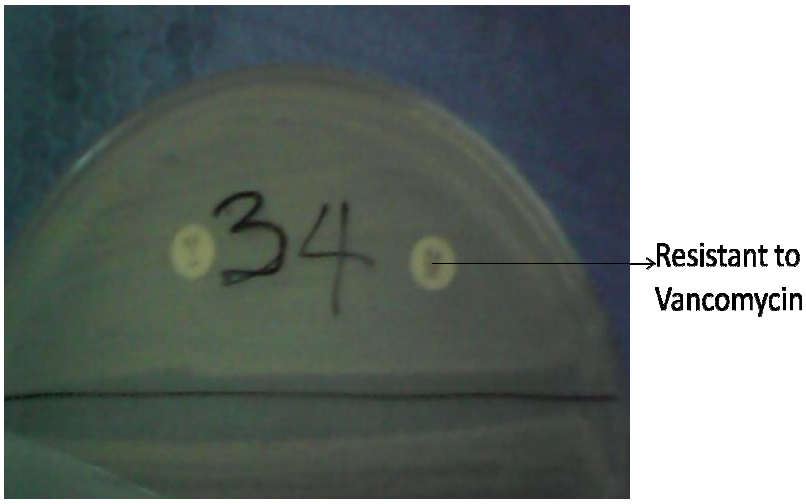


***Figure 2.*** *Showing isolate number 34 being resistant to both oxacillin and vancomycin.*


***Figure 3.*** *Showing the measured diameters of the antibiotics indicating sensitivity to vancomycin.*

***Table 1.*** *Characteristics of the vancomycin resistant isolates.*

| S/N | Sample Code | Age | Sex | Antibiotics tested | | | | | | | | | Number of Plasmids | Presence of *Mec A* gene | Presence of *PVL* gene | Source of Sample |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Gen | Str | Ery | Cxc | Chl | Cpr | Crx | Aug | Ox | | | | |
| 1. | 002/2623 | 24 | F | S | S | S | R | R | S | R | R | R | 1 | - | + | Skin |
| 2. | 002/02008 | 37 | F | R | R | R | R | R | R | R | R | R | 5 | + | - | Mouth |
| 3. | 002/2437 | 31 | F | R | R | R | R | R | R | R | R | R | 1 | - | - | Sputum |
| 4. | 003/1953 | 35 | F | R | R | R | R | R | R | R | R | R | 1 | - | - | Skin |
| 5. | 004/0987 | 24 | F | S | S | R | R | R | S | R | R | R | 1 | + | - | Skin |
| 6. | 002/00526 | 40 | F | R | R | R | R | R | R | R | R | R | 2 | + | - | Mouth |
| 7. | 002/0009 | 47 | M | R | R | R | R | R | R | R | R | R | - | - | - | Mouth |
| 8. | 002/02340 | 36 | F | S | S | R | R | S | S | S | R | R | 3 | - | - | Skin |

Abbreviations: GEN, gentamicin; STR, streptomycin; ERY, erythromycin; CXC, cloxacillin; CHL, chloramphenicol; CPR, ciprofloxacin; CRX, cefuroxin; AUG, augmentin; OX, oxacillin; R, resistant; S, sensitive.

### *3.2. Haemolytic Activity of the Isolates*

The haemolytic activities of the 8 VRSA isolates were also analysed (Table 2).

***Table 2.*** *The overall distribution of haemolytic activity of the isolates by sample type.*

| Haemolysis type | Sputum | Skin | Mouth | All samples (%) |
|---|---|---|---|---|
| α-haemolysis | - | 2 | 2 | 4 (50.0%) |
| β-haemolysis | - | 2 | 1 | 3 (37.5%) |
| γ-haemolysis | 1 | - | - | 1(12.5%) |

### *3.3. Presence of Plasmids, Mec A Gene and PVL Gene among the Isolates*

These are shown in figures 4 and 5 respectively.

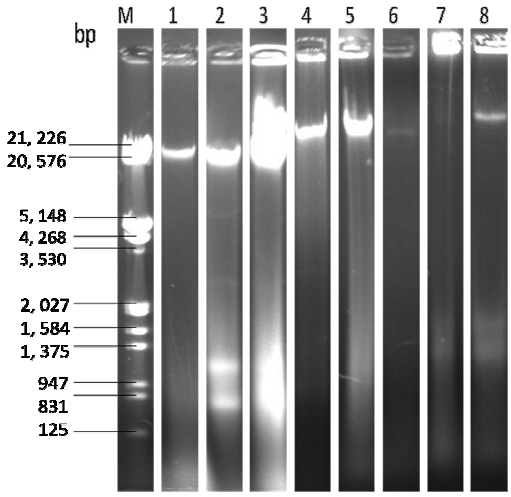


***Figure 4.*** *Agarose gel of the various plasmids present in the isolates. Lane 1 is the HIND III/ Eco R1 digest marker (Promega) while lanes 1-8 represents the isolates.*

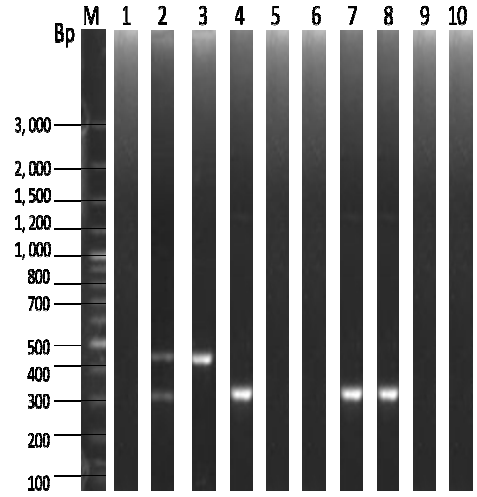


***Figure 5.*** *PCR amplification of the Mec A gene and the PVL gene, Lane M is the 100-bp DNA ladder (Thermo Scientific PCR Marker); Lane 1 is negative control while Lane 2 is the positive control showing bands for both Mec A gene (310bp) and the PVL gene (433bp). Lane 3 – 10 represents the isolates.*

## 4. Discussion and Conclusion

Antibiotic resistance represents a rapidly growing global health problem caused mainly by the use and misuse of antibiotics and spread of resistant bacteria as well as the lack of industrial development of new antibiotics [24]. Vancomycin has been the most reliable therapeutic agent against infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA). However, in 1996 the first MRSA to acquire resistance to vancomycin, was isolated from a Japanese patient [25]. The patient had contracted a post-operative wound infection that was refractory to long-term vancomycin therapy. Subsequent isolation of several vancomycin resistant *S. aureus* (VRSA) strains from USA, France, Korea, South Africa, and Brazil has confirmed that emergence of vancomycin resistance in *S. aureus* is a global issue [26] and Nigeria [27, 28] although the class of subjects involved were different. It has been reported that most *S. aureus* isolates with reduced susceptibility to vancomycin appear to have developed from pre-existing methicillin resistant *S. aureus* infection [29]. This was observed in this study as all the vancomycin resistant strains were also resistant to oxacillin.

A certain group of *S. aureus*, designated hetero-VRSA, frequently generate VRSA upon exposure to vancomycin, and are associated with infections that are potentially refractory to vancomycin therapy [25, 30]. Presence of hetero-VRSA may be an important indicator of the insidious decline of the clinical effectiveness of vancomycin in the hospitals. Vancomycin resistance is acquired by mutation and thickening of cell wall due to accumulation of excess amounts of peptidoglycan [30]. This seems to be a common resistance mechanism for all VRSA strains isolated in the world so far. Over the past decade, there has been an increase in the rate of infection and diseases caused by *S. aureus* particularly MRSA throughout the world [31]. The situation is even more alarming among patients with reduced immunity such as those undergoing chemotherapy or surgery, children, elders and patients with HIV and AIDS [1, 2]. *Staphylococcus* is an important cause of both community and hospital acquired infections resulting in high morbidity and mortality in tropical Africa [12]. Currently, there is little data available in Nigeria about the prevalence of vancomycin resistance of this pathogen particularly among HIV and AIDS patients. Understanding the vancomycin resistance and pathogenesis of *S. aureus* strains infecting HIV and AIDS patients in Imo State in particular and Nigeria in general is important for design of treatment and control strategies for the concerned patients since antibiotic prescription is part of their HAART regime. In this study, we found that isolates obtained from HIV patients recorded resistance rate above 90% to a number of antibiotics. All the vancomycin isolates recorded resistance to three (3) or more of all the antibiotics tested, with five being 100% resistant to all the antibiotics tested (Table 1). It appears that the site of infection plays an important role in relation to antibiotic resistance. This was

shown in the fact that all the isolates from mouth infections were 100% resistant to all the antibiotics tested therefore reducing the choice of antibiotics for the treatment of such infections.

Tn*1546* resides on plasmids in all VRSA strains, not on the chromosome (even though the chromosome is much larger and would seem to be a more probable insertion target) [32]. All the vancomycin resistant isolates were subjected to plasmid DNA isolation. Remarkably only one of the isolates, resistant to all the antibiotics tested, did not harbour plasmid DNA (Figure 4). Plasmids involved in Tn*1546* acquisition vary.

The reduced susceptibility of *S. aureus* strains to vancomycin leaves clinicians with relatively few therapeutic options for treating these infections and therefore emphasizes the importance of prudent use of antibiotics and the use of infection-control precautions to prevent their transmissions. In conclusion, the administration of methicillin, oxacillin or related drugs for treatment of *S. aureus* infection should be limited because most *S. aureus* isolates with reduced susceptibility to vancomycin appear to have developed from pre-existing methicillin or related drug resistant *S. aureus* infection [29]. Misuse of vancomycin could lead to the emergence of resistant strains in *S. aureus*. Hence, the overall antibiotic administration among HIV-positive patients should be reviewed in Imo State since antibiotics is part of their HAART regime to avoid community outbreak of MRSA. The monitoring and profiling of antibiotics susceptibility trend of *S. aureus* strains in these hospitals is important for revision and improvement of antibiotic guidelines.

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