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Isolation of vanconycin resistant *Staphylococcus aureus* from the wounds of hospital patients in Uyo, Akwa Ibom State, Nigeria

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ABSTRACT

Staphylococcus aureus is a very important human pathogen that generates a number of human infections. Isolation of vancomycin-resistant *Staphylococcus aureus* (VRSA) was carried out from wound samples of patients attending University of Uyo Teaching Hospital, Akwa Ibom state, Nigeria. A total of 45 wound samples from 15 patients were collected aseptically in triplicate, using sterile cotton swabs moistened in sterile normal saline. Microbiological analysis and susceptibility to vancomycin, including minimum inhibitory and minimum bactericidal concentrations (MIC and MBC), were carried out using standard methodologies. A total of 15 isolates were obtained and these showed varying MIC and MBC patterns. Out of the 15 *S. aureus* isolated, only one isolate had an MIC of approximately 4 µg/ml, while twelve isolates gave MIC values that ranged from 15.62 µg/ml to 250 µg/ml. The remaining 2 isolates gave MIC values that were ≥ 500 µg/ml. These 2 isolates exhibited alpha haemolysis on blood agar, unlike the others that were beta haemolytic. The results of the MBC also showed variations amongst the isolates. A total of 10 isolates gave MBC values that ranged from 62.5 µg/ml to 500 µg/ml, while the remaining 5 isolates gave MBC values that were ≥ 500 µg/ml. The high MIC and MBC values obtained showed that vancomycin-resistant *Staphylococcus aureus* is increasing at an alarming rate, and this accounts for the gradual decline in the effectiveness associated with the use of vancomycin. Given the widespread prevalence of VRSA, there is a need for newer therapeutics that can reverse this surge.

*Keywords***:** Methicillin, Vancomycin, antibiotic resistance, *Staphylococcus aureus*, Wounds

1. INTRODUCTION

For well over a century*, Staphylococcus aureus* has established itself as a well known pathogen and a major colonizer of over a third of the human population. It is capable of causing a wide range of human diseases such as superficial skin infections to overwhelming sepsis, severe necrotizing pneumonia and endocarditis. Furthermore, it is a leading cause of nosocomial infections [1, 2]. Its ability to spread resistance genes encoded on its plasmid has made them very successful in their spread of resistance [3-7].

Infections caused by this organism were treated effectively using penicillin. However, the first penicillinase-producing strains were recognized in 1944 and by the late 1950s, about half of all *Staphylococcal* isolates had become penicillin resistant [8]. Today, the level of resistance of *S. aureus* to penicillin is very high and spreading fast [8]. As a result, the semi-synthetic penicillin (methicillin) and cephalosporins were developed in the 1960s and 1970s to reverse the resistance trend. Sadly, these were also met with resistance by *Staphylococcus* in the mid-1970s making them less effective. Methicillin-resistant *Staphylococcus aureus* (MRSA) increased in prevalence from 2.4% in 1975 to 29% in 1991 [8]. MRSA has been reported on almost all the continents of the world in clinical and community settings.

Given the global spread of MRSA, the vancomycin eventually replaced methicillin and was tagged "the drug of last resort". Sadly, this versatile pathogen also acquired resistance to vancomycin in addition to other β-lactam antibiotics. Several authors have isolated MRSA that are also resistant to vancomycin from human clinical samples, environmental samples and wounds of livestock and companion animals [2, 9-19]. Wounds of patients may be due to burns, diabetes ulcers, pectic ulcers, post surgery and injuries [20]. In addition to *S. aureus* which was first implicated in post surgery mortality, *Pseudomonas aeruginosa* is also a frequent isolate from severe wounds [20]. Both isolates poses a great public health risk because of the ease with which they acquire resistance and spread it. Secondly, they make wound healing very difficult. Understanding the microbiology of wound is very important for designing therapies and in wound management [21].

In Nigeria and around the continent, a few studies have isolated VRSA from wounds samples. Thus, the primary aim of the study was to isolate VRSA from wound samples collected from a tertiary teaching hospital in Akwa Ibom State, Nigeria.

2. MATERIALS AND METHODS

2. 1. Study area

The research was carried out in the Microbiology Laboratory of the Department of Microbiology, Faculty of Natural and Applied Sciences, Obong University, Etim Ekpo, Akwa Ibom State, Nigeria from March to August 2018.

2. 2. Collection of samples and ethical approval

Based on a previous study, a total of 45 wound swab samples (3 per patient) were collected from the wounds of 15 patients attending the University of Uyo Teaching Hospital, Uyo City, and Akwa Ibom State, Nigeria. Sampling was done for 3 months (March to June, 2017). The samples were collected aseptically using a sterile cotton swab stick moistened with normal saline by gently rubbing it on the periphery of the wounds already cleaned with 70%

ethanol. The wound swabs were immediately covered, labelled appropriately and immediately taken to the microbiology laboratory for further processing. Ethical approval was sought and obtained from the management of hospital and as well informed consents from all patients before they were included in this study.

2. 3. Preparation of culture media

Nutrient, Mueller-Hinton, blood and mannitol salt agars and nutrient broth were used in this study. Both agar were purchased from HiMedia and prepared aseptically according to the manufacturer's instructions and as needed.

2. 4. Isolation and characterization of *Staphylococcus aureus*

The specimens were inoculated onto the freshly prepared mannitol salt agar (MSA) for 24 hours at 37 ºC. After overnight incubation, discrete colonies suspected to be *Staphylococcus aureus* were sub-cultured onto blood agar (BA), MSA and nutrient agar (NA) twice to purify the isolates. Colonies of diameter 1-2 mm with smooth glistering surface, and with golden yellow pigment were observed on mannitol salt agar. They were opaque and easily emulsifiable. With the exception of isolates 3 and 4 which exhibited alpha haemolysis, beta haemolysis was seen on blood agar with the rest of the isolates. The isolates were stored onto the various agar slants and also identified using Gram reaction, microscopy and a battery of biochemical (catalase and triple sugar iron) tests. These were all performed as previously described [22].

2. 5. Coagulase, catalase tests and Gram reaction

A total of 15 discrete colonies from Mannitol salt agar were further identified by the use of their biochemical profiles and Gram staining as described previously [23]. Coagulase and catalase tests were done as previously described [23-26]. Briefly, coagulase test was carried out by placing a drop of human blood plasma on a glass slide and a colony of the test organism was picked from MSA plates and added to the plasma. The mixture was then swirled gently and observed for coagulation against light. All the test isolates gave positive agglutination and were regarded as coagulase positive organisms. Catalase test was performed using 3% hydrogen peroxide (H_2O_2). A drop of H_2O_2 was put on a slide and a colony of the test organism from MSA was picked with a sterile wire loop and mixed with the H_2O_2. Gas bubbles were observed for all the test isolates indicating a catalase positive reaction. Gram reaction was done using the standard methodology descried previously [26]. On microscopy, the presence of spherical cocci with purple colour (Gram-positive), arranged in clusters was indicative of *Staphylococci*.

2. 6. Determination of the Minimum Inhibitory Concentration (MIC)

Following the producer's instruction, a bottle of 1g of vancomycin powder was dissolved in 20 ml of sterile water to obtain a concentration of 50 mg/ml of vancomycin. Exactly 1ml of 50 mg/ml of vancomycin was in turn diluted with 50 ml of water to obtained 1 mg/ml (1000 µg/ml) of vancomycin and was further subjected to a two-fold serial dilution in test tubes using sterile nutrient broth, as described previously [24]. The following graded concentrations of vancomycin were obtained: 0.95 µg/ml, 1.95 µg/ml, 3.9 µg/ml, 7.8 µg/ml, 15.63 µg/ml, 31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml. These were inoculated with the test organisms and incubated at 37 ºC for 24 hours. The inoculums were prepared using the

0.5 McFarland as standard. Three control culture tubes were also prepared containing sterile nutrient broth only, nutrient broth with the test organism only and nutrient broth with vancomycin (1000 µg/ml) only. These controls were designated as CA, CB and CC, respectively. After 24 hours of incubation, all the test tubes were examined for the presence of turbidity. Bacteria growth occurred when turbidity (cloudiness) appeared in the test tubes after incubation. Absence of turbidity in any test tube was an indication that growth was inhibited. The lowest concentration of vancomycin that inhibited the growth of the test organism was the minimum inhibitory concentration (MIC). For controls, growth occurred in the test tube containing nutrient broth with the test organism only. There was no evidence of growth in the tubes containing sterile nutrient broth only, and nutrient broth with vancomycin antibiotic only.

2. 7. Determination of the Minimum Bactericidal Concentration (MBC)

Exactly 0.01 ml of the broth culture from each MIC tube dilution test without turbidity was inoculated onto freshly prepared sterile nutrient agar at 37 ºC for 24 hours, after which the plates were observed for bacteria growth. The lowest concentration of vancomycin that killed 99.9% of the test organism was regarded as the minimum bactericidal concentration (MBC).

3. RESULTS

The results of the MBC and MIC for the 15 *Staphylococcus* isolates are presented in the Tables 1 and 2. Table 1 shows the various MIC for the *S. aureus* isolates. As can be seen in the table, the various graded concentrations used were 0.95 µg/ml, 1.95 µg/ml, 3.9 µg/ml, 7.8 µg/ml, 15.63 µg/ml, 31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, and 500 µg/ml. For the various concentrations, the number of isolates that were sensitive were 0, 0, 1, 1, 1, 3, 5, 10, 12, 13 and 13, respectively. As evident in the table, as the concentration of vancomycin increased, the number of sensitive isolates increased. Notably, isolates 3 and 4 were resistant across all the concentrations of vancomycin that were used in this study. They were the only isolates that were resistant to vancomycin at concentrations of 250 and 500 µg/ml. Isolate 1 was the most sensitive isolate as it was sensitive to vancomycin at concentrations that ranged from 3.9 to 500 µg/ml. A total of 12 isolates were susceptible to MIC range of 15.62 µg/ml to 250 µg/ml, while 2 isolates were susceptible to MIC values above 500 µg/ml (Table 1).

Table 1. Test isolates vancomycin MIC

Isolate	Concentration (µg/ml)										Controls		
	0.98	1.95	3.9	7.8	15.63	31.25	62.5	125	250	500	CA	CB	CC
1	+	+	-	-	-	-	-	-	-	-	-	+	-
2	+	+	+	+	+	+	+	-	-	-	-	+	-
3	+	+	+	+	+	+	+	+	+	+	-	+	-

4	+	+	+	+	+	+	+	+	+	+	-	+	-
5	+	+	+	+	+	+	-	-	-	-	-	+	-
6	+	+	+	+	+	+	-	-	-	-	-	+	-
7	+	+	+	+	+	-	-	-	-	-	-	+	-
8	+	+	+	+	-	-	-	-	-	-	-	+	-
9	+	+	+	+	+	+	-	-	-	-	-	+	-
10	+	+	+	+	-	-	-	-	-	-	-	+	-
11	+	+	+	+	+	+	-	-	-	-	-	+	-
12	+	+	+	+	+	+	-	-	-	-	-	+	-
13	+	+	+	+	+	-	-	-	-	-	-	+	-
14	+	+	+	+	+	+	+	-	-	-	-	+	-
15	+	+	+	+	+	+	+	+	-	-	-	+	-

Key: "+" = growth while "-" = no growth; CA, CB and CC = Controls containing sterile nutrient broth only, nutrient broth with the test organism only and nutrient broth with vancomycin (1000 µg/ml), respectively.

The 2 isolates exhibited alpha haemolysis on blood agar, unlike others that were beta haemolytic. The results of the MBC are as presented in Table 2. Isolates 5, 14 and 15 were first sensitive at 62.5, 125 and 250 µg/ml respectively but showed growth across all the concentrations used in the study in addition to isolate 2 and 3. A total of 10 isolates were susceptible at MBC range of 62.5 µg/ml to 500 µg/ml, while the remaining 5 isolates showed no MBC at 500 µg/ml.

Table 2. Test isolates vancomycin MBC.

Concentration (µg/ml)								
Isolate	3.9	7.8	15.63	31.25	62.5	125	250	500
1	+	+	+	+	-	-	-	-
2	+	+	+	+	+	+	+	-
3	+	+	+	+	+	+	+	+

4	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	-	-
7	+	+	+	+	+	+	-	-
8	+	+	+	+	+	-	-	-
9	+	+	+	+	+	+	+	-
10	+	+	+	+	+	+	-	-
11	+	+	+	+	+	+	-	-
12	+	+	+	+	+	+	-	-
13	+	+	+	+	+	+	-	-
14	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+

Key: "+" = growth while "-" = no growth.

Table 3. MIC and MBC values.

Concentration (µg/ml)	No. of isolate
MIC	
3.9	1
7.8	1
15.63	3
31.25	5
62.50	10
125	12
250	13
500	13
MBC	
62.50	1

125	2
250	8
500	10
500	5

4. DISCUSSION

S. aureus is frequently associated in wounds of hospitalised patients where they usually complicate the wound healing and prolong patient stay [20]. Isolation of vancomycin-resistant *Staphylococcus aureus* in this study made it clear that the organism is wide spread even in our study locality. A total of 15 clinical isolates of *Staphylococcus aureus* were obtained from wound swabs of patients in University of Uyo Teaching Hospital (UUTH), Uyo, Akwa Ibom State, Nigeria. *S. aureus* resistant to vancomycin is common place [9-19]. In an earlier study, a total of 500 *S. aureus* gave methicillin resistance of 47.4% (n = 237) [29]. A number of studies from across the world have shown that methicillin resistant isolates easily acquire vancomycin resistance [9-19].

The result of this study showed that only one isolate of *S. aureus* was susceptible to vancomycin at MIC of approximately 4 µg/ml. A total of 12 isolates gave MIC value that range from 15.62 µg/ml to 250 µg/ml, while 2 isolates had MIC values above 500 µg/ml. Following the recommendation by the Clinical and Laboratory Standards Institute 2006, vancomycin MIC of ≤ 2 µg/ml and ≥ 16 µg/ml for vancomycin sensitive and resistance *S. aureus* [27-28], it therefore means that all the organisms isolated and tested with vancomycin were vancomycin-resistant *Staphylococcus aureus*. Amongst the 5 isolates that were resistant even at MIC values greater than 500 µg/ml, two of these isolates were alpha-haemolytic on blood agar while all the other 3 isolates were beta haemolytic. Our findings (MIC values) for our isolated *S. aureus* were higher than the MIC values that ranged from 0.5 to 2 µg/ml previously isolated by Yadav *et al* (2018) in India from isolates obtained from clinical samples (pus, urine, blood, sputum, high vaginal swab, cerebrospinal spinal fluid and throat swab). A more agreeable result to our findings was that reported by Tiwari *et al* (2016) [9] from wounds of livestock and companion animals in India. In their study, the isolated 69 *S. aureus* isolates. Out of this, a total of 66 (95.65%) were found to be methicillin resistant. Out of the 66 found to be methicillin, 63 (95.45%) were vancomycin resistant. In another study, vancomycin MIC values that ranged from ≥ 8 and ≤ 512 µg/ml were reported for clinical samples [18]. In another study, a total of 34 out 95 isolates obtained from blood and wound samples that were ampicillin resistant were also resistant to vancomycin [1]. Furthermore they showed the presence of either vanA or vanB vancomycin resistance genes in the isolates. The results obtained in this study were also consistent with the work done by Hitoshi *et al.* (2011) which showed that 68.7% of *Staphylococcus aureus* studied were susceptible to vancomycin at MIC ≥ 2 µg/ml.

Furthermore, this study highlights the dangers of vancomycin-resistant *Staphylococcus aureus* which is now becoming a threat to the society. Vancomycin-resistant *Staphylococcus aureus* is ubiquitous, and the organisms colonize the wounds of infected patients. By their invasive nature, they are capable of haematogenous spread, resulting in bacteraemia which

could be more fatal. There is evidence to suggest that vancomycin bactericidal activity plays an influential role in the clinical outcomes of *S. aureus* bacteraemia, including methicillin-resistant *S. aureus* bacteraemia [30]. In other words, reduced vancomycin susceptibility may be associated with worse clinical outcomes resulting in mortality. Sakoulas *et al* (2004) [31] reported an association between elevated vancomycin MIC and treatment failure. He also noted that reduced bactericidal activity had a similar effect. Studies have shown that high MIC and MBC of vancomycin are associated with treatment failures, resulting in high mortalities [32-34]. Hitoshi *et al*. (2011) [33] in his study stated that 21.5% of patients with methicillin-resistant *S. aureus* treated with vancomycin died 28 days after the diagnosis of methicillin-resistant *S. aureus* bacteraemia, and noted that the organisms were resistant to vancomycin. The gradual reduction in susceptibility of methicillin-resistant *S. aureus* isolates to vancomycin occurring in recent times has been associated with increased treatment failure and mortality [32-33].

5. CONCLUSION

Other antibiotics should be employed as treatment options. The isolation of VRSA from the clinical specimens as demonstrated in this study is an indication that the organism is widespread in Akwa Ibom state. High MIC and MBC values obtained shows that VRSA is increasing with alarming rate, and this accounts for the gradual decline in the effectiveness of use of vancomycin antibiotic.

Recommendation

The major drawback of this study was the absence of molecular characterisation and plasmid profiling of the isolates which would have allowed for the detection of the various methicillin and vancomycin resistant genes. Further studied aimed it this will be carried out on the isolates. Furthermore, the findings in study is a clarion call for all stake holders to intervene on the indiscriminate use or abuse of vancomycin by way of continuous sensitisation workshops and seminars. Some agents have been recommended for effective treatment of VRSA infection such as daptomycin, linezolid, telavancin, ceftaroline, and quinupristin-dalfopristin and they should be used only after antibiotic sensitivity tests have been carried out.

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