



Isolation and Molecular Characterisation of Vancomycin Resistant Bacteria Isolated from Selected Well Water in Ula-Ubie Community, Ahoada, Rivers State

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Authors' contributions

This work was carried out in collaboration among all authors. Author NPA designed the study and performed the statistical analysis. Author MO managed the analyses of the study, wrote the protocol, managed the literature searches and wrote the first draft of the manuscript. Author RRN supervised the study. All authors read and approved the final manuscript.

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ABSTRACT

The resistance of bacteria to vancomycin has made the treatment of some infections more difficult to handle. The aim of this study was to isolate and molecularly characterize vancomycin resistant bacteria in selected well water with a view of determining the level of resistance in the environment. fifty (50) well water samples were collected from ten different stations in Ula-Ubie community, Ahoada, Rivers State for a period of six months (January 2019 to June, 2019). Bacteria diversity was analysed using standard microbiological techniques. In this technique, aliquots of 10^{-1} , 10^{-2} and 10^{-3} dilutions were seeded into prepared Nutrient agar and Brain Heart infusion agar (BHI). Microbial loads were enumerated and distinct bacterial colonies were picked and inoculated on freshly prepared nutrient agar. Purification of isolates was done by streaking on freshly prepared nutrient agar plates until isolates were void of mixed culture. The isolates were further

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characterized using biochemical and molecular methods and preserved in bijoux bottles containing 5 ml of 10% (v/v) glycerol. Isolates from the glycerol were subcultured on fresh nutrient agar plates before each use. The vancomycin resistant bacteria were screened using the culture-based screening method. In this method, sterile nutrient medium was supplemented with different concentrations of the vancomycin antibiotics. Inoculation of bacterial isolates on the vancomycin supplemented agar followed before plates were incubated. The ensuing bacterial isolates were characterized using biochemical and molecular methods. Ninetene (19) bacteria isolates were screened for vancomycin and of this numbers, six (6) were positive for vancomycin resistance. The agarose gel electrophoresis showed the presence of *vanA* and *vanB* gene which could be responsible for the resistance to the vancomycin observed in most isolates. The presence of vancomycin resistant genes in these isolates is of public health concern due to the transfer of resistance to other isolates. Standard hygienic practice is therefore recommended in the use of well water.

Keywords: Vancomycin-resistant bacteria; well water; molecular characterization of bacteria.

1. INTRODUCTION

Vancomycin resistant microbes are microorganisms (gram positive) that are not susceptible to the vancomycin antibiotics. Antibiotics are chemotherapeutic agents used in the treatment of infections caused by microorganisms especially bacteria. These chemotherapeutic agents could be cytostatic or cytotoxic to the bacteria. They could also boost the human immune system to enable it eliminate the disease-causing agent. Most of these chemotherapeutic agent function by preventing key steps in the life cycle of the bacteria. For instance, some function by preventing the synthesis of the cell wall, synthesis of protein, nucleic acids as well as other functions [1]. Another way through which antibiotics work is by utilizing the energy-dependent transport mechanisms in ribosomal sites of the bacteria when they penetrate their cell wall [2]. The emergence of antibiotics has helped man in the fight against infectious organisms. This war against infectious organisms using antibiotics has witnessed serious setbacks due to the emergence of resistant microorganisms which have developed mechanisms or substances which confers or protect them from the effect of antibiotics. Thus, the emergence of antibiotic resistant bacteria.

Vancomycin is one of the antibiotics which is synthesized from *Streptomyces orientalis*. The antibiotic is a glycopeptide cup shaped molecule composed of a peptide which is linked to a disaccharide. The antibiotic is a cell wall synthesis inhibitor and it functions by binding to the D – alanyl – D – alanine terminal sequence on the pentapeptide portion of the bacteria peptidoglycan there by inhibiting the

transpeptidation reaction [3]. *Staphylococcus* species, some members of *Clostridium*, *Bacillus*, *Streptococcus* and *Enterococcus* are very sensitive to the antibiotic and the drug is bactericidal. The drug can be administered both orally and intravenously and has been very vital in the treatment of infections or diseases caused by antibiotics resistant *Staphylococcus* and *Enterococcus* species [3]. The high rate or incidence of death and diseases globally is caused by pathogenic microorganisms [World Health Organization [4] and the dissemination of resistant microbes is very significant in this regard [5]. Vancomycin resistant bacteria especially *Staphylococcus* and *Enterococcus* species have emerged and it is indeed a public health threat as the dreaded antibiotic have become less effective against these bacterial pathogens. In a previous study by [6], vancomycin resistant *Enterococcus* (VRE) species were reported to cause a global outbreak in hospitals due to the inappropriate use of the drug and has led to high incidence of diseases caused by strains of the genus.

Well water (ground water) is a drinking water source which is readily assessible to rural communities in Ahoada. Studies have evaluated the microbial consortium of well water as well as the antimicrobial properties. The genetic and molecular basis of vancomycin resistance have been described with evidence that vancomycin resistant microbes especially the VREs could be reservoirs and sources of other antimicrobial-resistant genes [7]. Vancomycin Resistant *Enterococcus* (VRE) has become a major problem of Public Health because its colonization of the colon makes it present in faecal materials. Passage of feces on the ground or pit toilet in the villages could lead to the contamination of the

environment with the intestinal microflora which could flow into underground water during rainy season through erosion. Thus, this study was carried out to evaluate the vancomycin resistant bacterial isolates in dug wells which are the primary source of drinking water in Ula-Ubie community for the presence of vancomycin resistant bacterial isolates.

2. METHODOLOGY

2.1 Description of Study Area

The study was carried out in Ula-Ubie community. Ula-Ubie is one of the communities located in Ahoada, Ahoada West Local Government Area of Rivers state, Nigeria. Ahoada is a city in Orashi Region of Rivers State, Nigeria, found northwest of Port Harcourt. The Orashi Region used to be known as the Ahoada Local Government Area, yet has since been brought up in rank to district status and is currently separated into two Local Government Areas: Ahoada East, with its seat in the city of

Ahoada and Ahoada West, with its seat in Akinima. The people of Ahoada speak ekpeye language. The wells in this community were selected because they are the only source of drinking water. People from this community also use the well water for domestic activities including cooking and bathing. As a drinking source, some of the wells are left open with the scooping bucket (fetching bucket) not properly kept. The map of the stations where stations were collected is presented in Fig. 1.

2.2 Collection of Water Samples

Fifty (50) well water (underground water) samples were collected in sterile containers from ten different stations in the community. The well water was drawn out of the well using a fetching bucket (a bucket that has a rope tied to it; used in scooping water from the well). The collected samples were placed in ice pack container and sent to the microbiology laboratory of the department of Microbiology, Rivers State University for analysis.

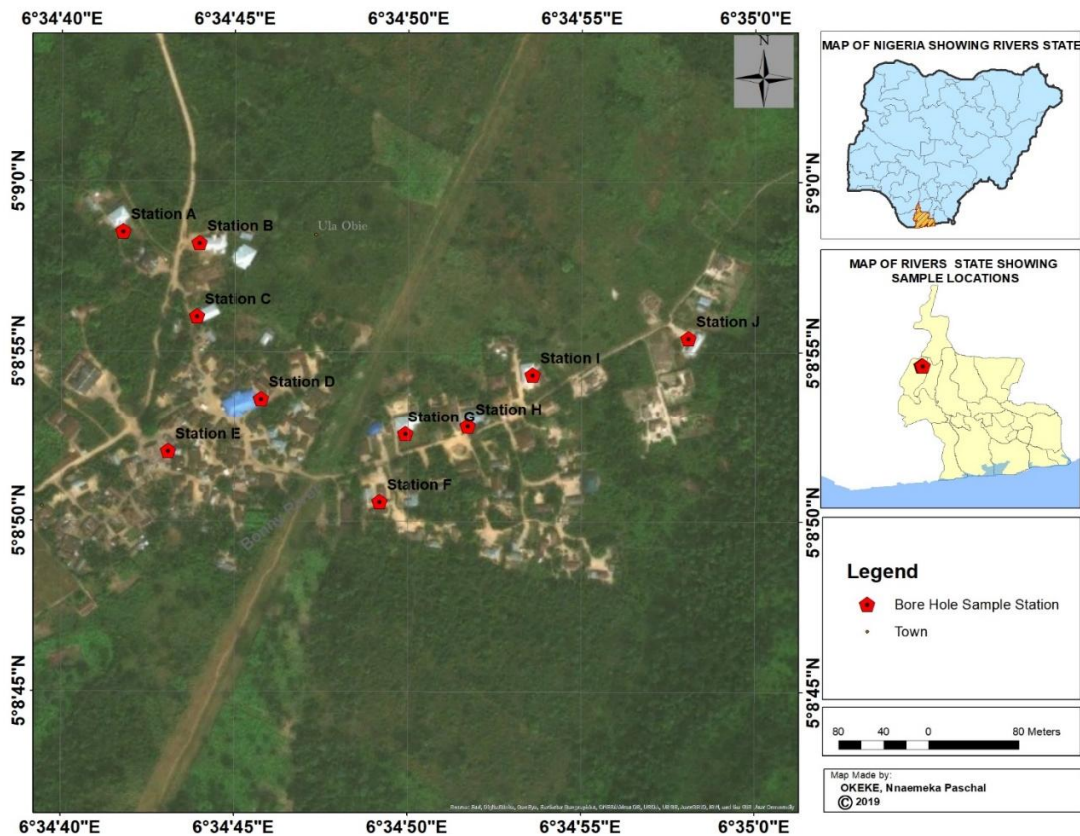


Fig. 1. Map showing the various stations under study

2.3 Duration of Study

The study was carried out and completed within one year. Six months study. Which started from the month of January 2019 to the month of June 2019.

2.4 Microbiological Analysis

The microbial population of the water samples were enumerated using the tenfold serial dilution of Harrigan and McCanc as described by Wemedo [8]. After the serial dilutions, aliquots of 10^{-1} , 10^{-2} and 10^{-3} dilutions were seeded into prepared Nutrient agar and Brain Heart infusion agar (BHI) (TM media india) plates. Swabs from the fetching buckets were inoculated directly on the respective agar plates. Plates were incubated at 37°C for 24-48 hours. After incubation, plates were observed for microbial growth. Counts were made for the respective plates and colonies were characterized morphologically and were subcultured on freshly prepared nutrient agar plates. The counts from the different plates were used in enumerating the microbial load present in the water stations. The morphological and biochemical characteristics of the bacterial isolates were determined using the method of [9].

2.5 Screening for Vancomycin Resistance

The bacterial isolates were screened using the culture-based screening method as described by [10] with slight modification. In this method, nutrient agar was prepared in three 500 mL conical flasks and sterilized by autoclaving at 121°C for 15psi. After sterilization, the nutrient agar was supplemented with concentrations of 8 µg, 16 µg and 32 µg of the vancomycin antibiotics. This was homogenized by swirling before they were poured into petri dishes. The Petri dishes were allowed to solidify before they were dried. The test isolates which have been standardized were inoculated on the vancomycin agar plates according to their respective labels. Plates were later incubated for 24 hours at 37°C in the incubator. After twenty-four hours, the plates were observed for the presence of growth. The absence of growth in the least concentration (8 µg) is read as vancomycin susceptible bacterium (VSB), while the presence of growth is read as vancomycin resistant bacterium (VRB).

2.6 Extraction of DNA and Plasmid Analysis

Genomic DNA of the bacterial isolates was extracted and quantified using the method

described by [11]. The 16s rRNA region of the rRNA gene of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. After sequencing, the obtained sequences were edited using the bioinformatics algorithm Trace edit. Similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN.

The Amplification of Vancomycin resistant genes were carried out on a Gene Amp System 9700 instrument (Perkin-Elmer Cetus, Norwalk, Conn.) at a final volume of 25 µL. Amplifications were performed using 2x master mix from biolabs, UK, 100mol of each primer (vanAF: ATGAATAGAATAAAAAGTTGCAATAC and VanAR: CCCCTTTAACGCTAATACGAT, VanBF: CCCGAATTTCAAATGATTGAAAA and VanBR: CGCCATCCTCCTGCAAAA, VanCF: GCTGAAATATGAAGTAATGACCA and VanCR: CGGCATGGTGTGATTTTCGTT) and 50 ng of the bacterial extracted DNA. The PCR program consisted of an initial denaturation step at 94°C for 3 min; this was followed by 30 cycles of DNA denaturation at 94°C for 30 seconds, primer annealing at the appropriate temperature for each set of primers for 2 min, and DNA extension at 72°C for 2 min. After the last cycle, the reaction was terminated by incubation at 72°C for 6 min. The PCR products were resolved on 1.5% agarose gel electrophoresis stained with E-Z vision dye and visualized on a blue light trans illuminator.

3. RESULTS AND DISCUSSION

3.1 Vancomycin Screened Isolates

The result for the response of the bacterial isolates to Vancomycin showed that *Staphylococcus gallinarum*, *Lysinibacillus macrolides*, *Bacillus circulans*, *Staphylococcus sciuri*, *Bacillus circulanss*, *Pectobacterium atrosepticum*, *Bacillus baduis*, and *Bacillus samiensis* were highly resistant to vancomycin while other listed isolates were completely sensitive to the vancomycin antibiotics (Table 1).

3.2 Molecular Characterization and Plasmid Analysis

The 16S rRNA of the isolate A8 and 40 showed a percentage similarity to other species at 100%.

The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolate A8 and 40 within the *Bacillus* sp and revealed a closely relatedness to *Bacillus circulans* and *Lysinibacillus macroides*, CWT, I5

and 31 were closely related to *Staphylococcus sciuri*, *Providencia thailandensis* and *Enterobacter hormaechei* respectively (Fig. 2). The agarose electrophoresis showing the bands of the vancomycin resistance is presented in Plates 1 and 2.

Table 1. Response of bacterial isolates to vancomycin

Isolates	8 µg	16 µg	32 µg
<i>Staphylococcus mascillensis</i>	-	-	-
<i>Bacillus cereus</i>	-	-	-
<i>Staphylococcus muscae</i>	-	-	-
<i>Staphylococcus gallinarum</i>	+	+	+
<i>Staphylococcus pettenkoferi</i>	-	-	-
<i>Solibacillus silverstris</i>	-	-	-
<i>Lysinibacillus macroides</i>	+	+	+
<i>Bacillus circulans</i>	-	+	+
<i>Salinicoccus amiensis</i>	-	-	-
<i>Streptococcus rupicaprae</i>	-	-	-
<i>Staphylococcus sciuri</i>	+	+	+
<i>Staphylococcus equorum</i>	-	-	-
<i>Bacillus pantiothenicus</i>	-	-	-
<i>Staphylococcus lentus</i>	-	-	-
<i>Bacillus baduis</i>	+	+	+
<i>Bacillus smithii</i>	-	-	-
<i>Bacillus samiensis</i>	+	+	+
<i>Staphylococcus jittensis</i>	+	-	-
<i>Bevibacillus laterosporus</i>	-	-	-

Keys: +: resistant (growth of organism), -: not resistant (no growth of organism)

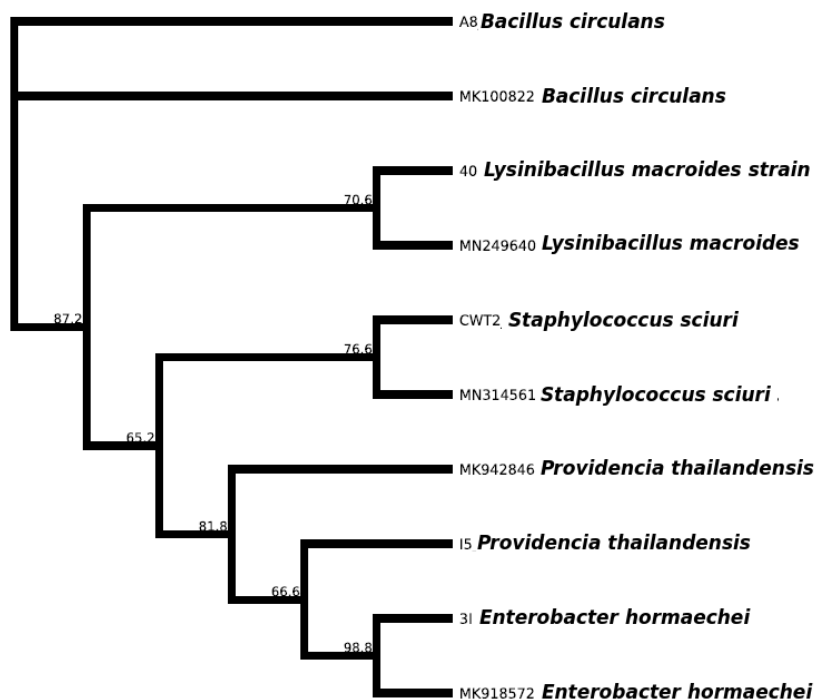


Fig. 2. Evolutionary relationship between bacterial isolates

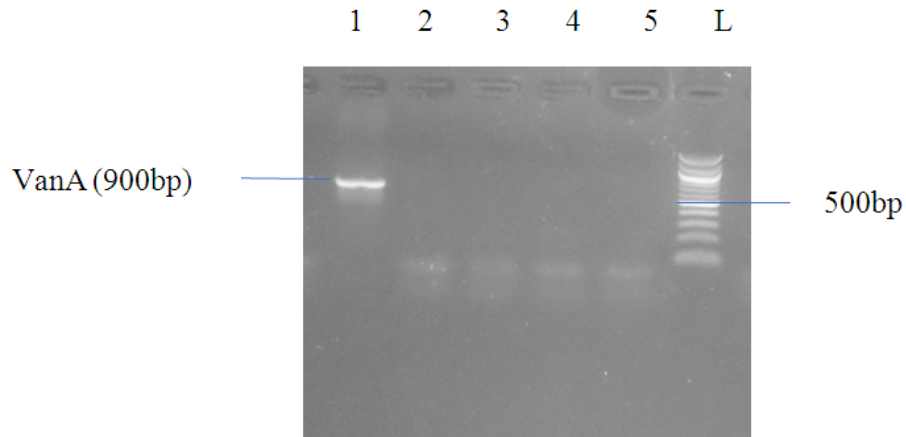


Plate 1. Agarose gel electrophoresis showing the amplified vanA gene. Lane 1 showing the van A gene band at 900bp. Lane L represents the 100 bp molecular ladder

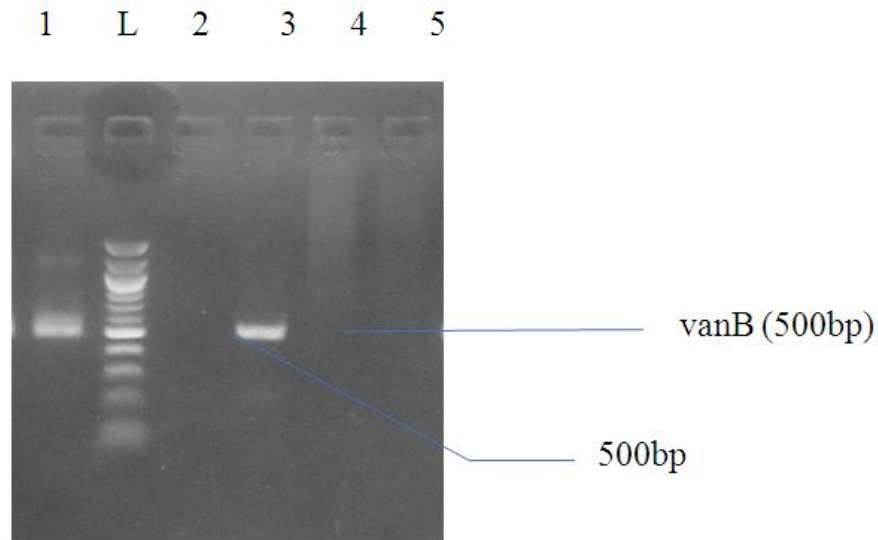


Plate 2. Agarose gel electrophoresis showing the amplified vanB gene. Lane 1 and 3 showing the van B gene band at 500 bp. Lane L represents the 100 bp molecular ladder

3.3 Vancomycin Resistance

Vancomycin resistance could be referred to the inability of vancomycin antibiotic to inhibit or eliminate the growth of bacteria pathogens which it usually has effects on. These pathogens develop resistance either through modification or development of substances that aid in resisting the antibacterial activity of Vancomycin. The response of the bacterial isolates in Table 1 showed that *Staphylococcus gallinarum*, *Bacillus subtilis*, *Staphylococcus hyicus*, *Pectobacterium atrosepticum*, *Bacillus baduis* and *Bacillus samiensis* could be referred to being highly

resistant to vancomycin. There is still lack of clarity to the proper definition of vancomycin resistance by previous studies due to the different breakpoints in the susceptibility to vancomycin adopted in different countries where Vancomycin resistant *Staphylococcus* have been documented [12]. Previous study has defined that for susceptibility, the MIC should be $\leq 4 \mu\text{g/mL}$, while those whose MIC is $8-16 \mu\text{g/mL}$ are within or should be reported as intermediate and those whose MIC is $\geq 32 \mu\text{g/mL}$ are resistant [13]. In Japan, MIC values of $8 \mu\text{g/mL}$ is considered resistant and these isolates have been reclassified in the United State as being

intermediate [12]. Thus, isolates in this study (Table 1) are resistant to Vancomycin. Furthermore, 14.3% of the staphylococcal isolates from the water stations were resistant to vancomycin, while three of the streptococcal isolates were completely resistant to vancomycin. Resistance to vancomycin by Gram positive cocci (*Enterococcus* and *Staphylococcus*) are well documented. High level and low-level resistance have been described to be associated with the possession of vancomycin resistant genes which confers the bacterial isolates immunity against vancomycin. High level Vancomycin resistance bacterial isolates whose MIC is $\geq 256 \mu\text{g/mL}$ are said to possess *Van A* gene (which are plasmid borne), while low level resistance are those that possess the *Van C* gene and MIC is $\geq 8\text{-}32 \mu\text{g/mL}$ [14]. In this current study, the bacterial isolates assayed for *Van* genes showed the presence of *Van A* (Plate 1) and *Van B* (Plate 2) genes. The *Van B* gene confers varied form of resistance to the isolates ranging from moderate to high level resistance but are less prevalent. They are mostly found in *Enterococcus faecium* and occurrence has been confirmed in Australia [15,16]. Thus, the presence of these genes in this current study could be responsible for the level of resistance observed. These genes could be responsible for the alteration of the route of synthesis of the peptidoglycan, via the replacement of D-Alanine-D-Alanine (D-Ala-D-Ala) with either D-Alanine D-Lactate (D-Ala-D-Lac) or D-Alanine-D-Serine (D-Ala-D-Ser), thereby orchestrating different patterns of resistance to glycopeptide antibiotics [7].

4. CONCLUSION

From the findings in this current study, bacterial isolates showed high resistant to vancomycin. Thus, we could resolve that there is an emergence of vancomycin resistant bacterial isolates amongst the *Bacillus*, *Staphylococcus* and *Streptococcus* with the presence of *Van A* and *Van B* genes. The water from the different well stations are therefore not safe for consumption. More so, the presence of *Van* genes amongst these isolates rings an alarm as this could pose a serious health problem not just to those who consume the water but to the general public especially if there is a transfer of *Van* genes to other bacterial isolates. Though this current study considered only the vancomycin resistant bacterial isolates, more studies on antibiotic screening of microorganisms isolated from dug wells or drinking water sources

should be carried out. Water purification methods including boiling of water before consumption is recommended.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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