Isolation ~~Detection~~ and Molecular Characterisation of Vancomycin Resistant Bacteria Isolated from selected well water in Ula-ubie Community, Ahoada, Rivers State

ABSTRACT

The resistance of some microbial strains to vancomycin has made the treatment of some infections more difficult to handle. The aim of this study was to isolate and molecularly characterized vancomycin resistant bacteria in well water ~~was characterized~~ with a view of determining the level of resistance in the environment. ~~F~~Fifty (50) well water samples were collected from ten different stations in Ula-Ubie community, Ahoada, Rivers State for a period of five months. Standard microbiological methods were used to analyze the microbiological constituents of the water. 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. Out of 61 bacteria isolates obtained 34 were positive for vancomycin resistance. The agar rose gel electrophoresis showed the presence of *vanA* and *vanB* gene which had caused the resistance to the vancomycin. 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

Antibiotics are chemotherapeutic agents which are used in the treatment of infections or diseases caused by microorganisms especially bacteria. These chemotherapeutic agents could be cytostatic or cytotoxic to the bacteria thereby boosting the human immune system to eliminate the infectious bacteria 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 [4]. 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 [14]. There is no doubt that the emergence of antibiotics has reduced or helped man in the fight against infectious organisms. Though this war against infectious organisms with the help of antibiotics has witnessed serious setbacks due to the emergence of resistant microorganisms. This is because microorganisms 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 [9]. *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 [9]. The high rate or incidence of death and diseases globally is caused by pathogenic microorganisms [13] and

Comment [OP1]: Which month to which month? State it here clearly and which year?

Comment [OP2]: Summarize and present the Laboratory protocol scientifically.

Comment [OP3]: Results is not clear here. It should be elaborate because it is the bedrock of this study.

Comment [OP4]: Opening statement not capturing the manuscript title. It should either start with well water problem or vancomycin resistant bacteria as a public health concern.

the dissemination of resistant microbes is very significant in this regard [10]. 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 [8], 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 [6]. 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 dug wells which are the primary source of drinking water in Ula-ubie community for the presence of vancomycin resistant bacterial isolates.

Comment [OP5]: It should correlate with the aim in the abstract section.

Comment [OP6]: Look at the grammar and syntax errors in the paper and correct. Unnecessary statements observed here with few citations.

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 (Citations needed). 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.

UNDER PEER REVIEW

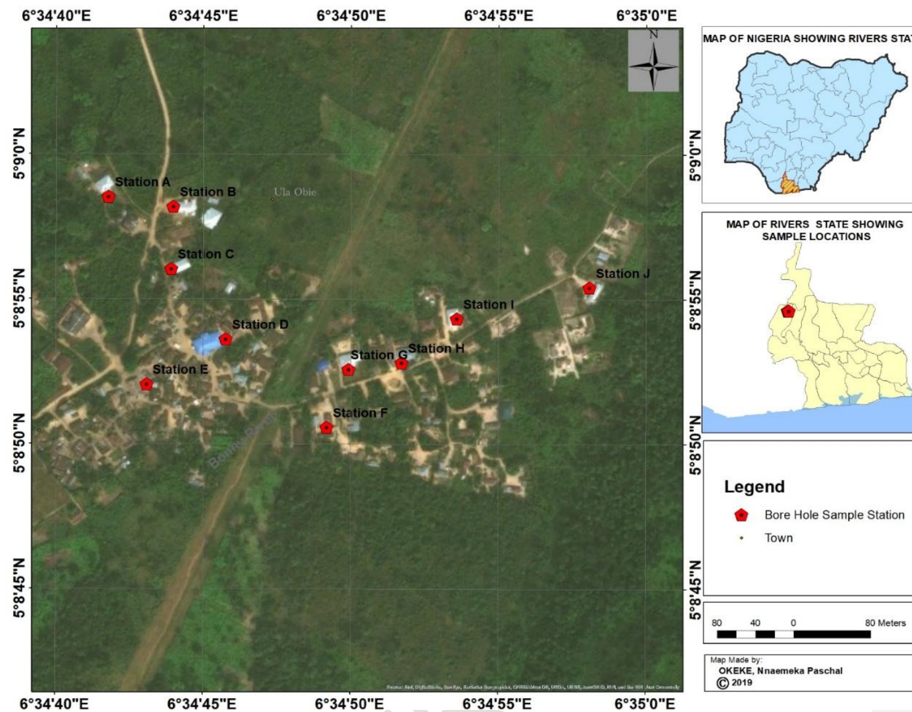

Fig.1. Map showing the various stations under study (Citations needed)

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) (Citation needed). 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.

2.3 Microbiological Analysis

The microbiological analysis of the stations involved enumeration and isolation of the bacteria present in the different stations. The microbial population in the soil and water samples were enumerated using the tenfold serial dilution of Harrigan and McCanc as described by [12]. 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 37ºC 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 both the soil and water stations. The morphological and biochemical characteristics of the bacterial isolates were determined using the method of [16]. ~~Further identification was done using the Polymerase chain reaction technique.~~

2.4 Screening for Vancomycin Resistance

The bacterial isolates were screened using the culture-based screening method as described by [5] with slight modification. In this method, nutrient agar was prepared in three

500mL conical flasks and sterilized by autoclaving at 121ºC for 15psi. After sterilization, the nutrient agar was supplemented with concentrations of 8 µg, 16 µg and 32µg 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 37ºC 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).

2.5 Polymerase Chain Reaction of genomic DNA and Plasmid Analysis

Genomic DNA of the bacterial isolates was carried out using the method described by [15] Amplification of Vancomycin genes were carried out on a Gene Amp System 9700 instrument (Perkin-Elmer Cetus, Norwalk, Conn.) at a final volume of 25µL. Amplifications were performed using 2x master mix from biolabs, UK, 100mol of each primer (vanAF: ATGAATAGAATAAAAGTTGCAATAC and VanAR: CCCCTTTAACGCTAATACGAT, VanBF: CCCGAATTTCAAATGATTGAAAA and VanBR: CGCCATCCTCCTGCAAAA, VanCF: GCTGAAATATGAAGTAATGACCA and VanCR: CGGCATGGTGTTGATTTCGTT) and 50 ng of the bacterial extracted DNA. The PCR program consisted of an initial denaturation step at 94 ºC for 3 min; this was followed by 30 cycles of DNA denaturation at 94 ºC for 30seconds, primer annealing at the appropriate temperature for each set of primers for 2 min, and DNA extension at 72 ºC for 2 min. After the last cycle, the reaction was terminated by incubation at 72 0C 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.

Comment [OP7]: How was the DNA extracted?

3. RESULTS AND DISCUSSION

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

Comment [OP8]: Where is the percentage occurrence of the isolates?

3.1 Molecular Characterization and Plasmid Analysis

The obtained 16S rRNA sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. 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 3I were closely related to *Staphylococcus scuiri, Providencia thailandensis* and *Enteroacter hormeachei* respectively (Fig. 2). The agar rose electrophoresis showing the bands of the vancomycin resistance is presented in Plates 1 and 2.

Comment [OP9]: Take to Protocol and not result section.

UNDER PEER REVIEW

Table 1. Response of Bacterial Isolates to Vancomycin

Isolates	**8** µg	**16** µg	**32** µg
Staphylococcus mascillensis	-	-	-
Bacillus cereus	-	-	-
Enterobacter homachei	+	+	+
Staphylococcus muscae	-	-	-
Staphylococcus gallinarum	+	+	+
Staphylococcus pettenkoferi	-	-	-
Solibacillus silverstris	-	-	-
Lysinibacillus macroides	+	+	+
Bacillus circulans	-	+	+
Salinicoccus samiensis	-	-	-
Streptococcus rupicaprae	-	-	-
Staphylococcus sciuri	+	+	+
Staphylococcus equorum	-	-	-
Bacillus pantiothenicus	-	-	-
Pectobacterium atrosepticum	+	+	+
Providencia thailandensis	+	+	+
Staphylococcus lentus	-	-	-
Bacillus baduis	+	+	+
Bacillus smithii	-	-	-
Bacillus samiensis	+	+	+
Staphylococcus jittensis	+	-	-
Bevibacillus laterosporus	-	-	-

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

UNDER PEER REVIEW

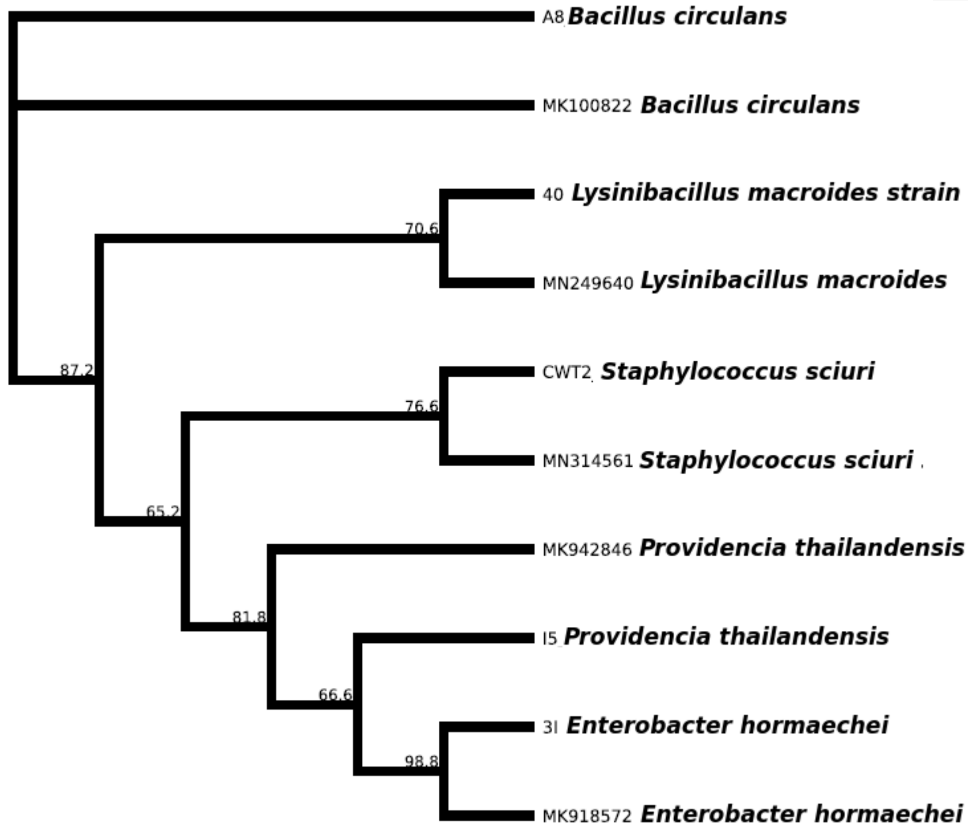

Fig 2. Evolutionary relationship between bacterial isolates

UNDER

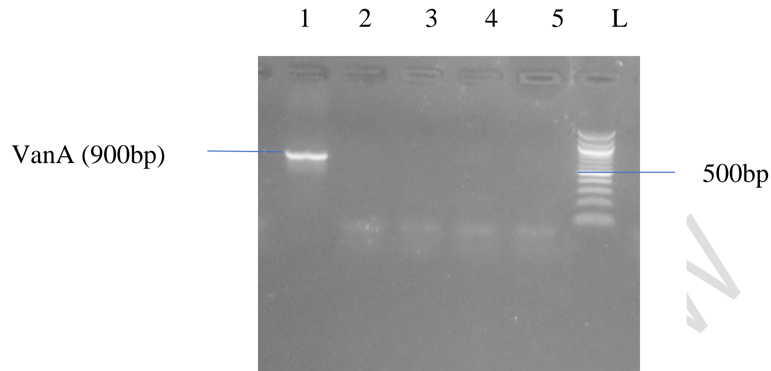

Plate 1. Agarose gel electrophoresis showing the amplified vanA gene. Lane 1 showing the van A gene band at 900bp. Lane L represents the 100bp molecular ladder

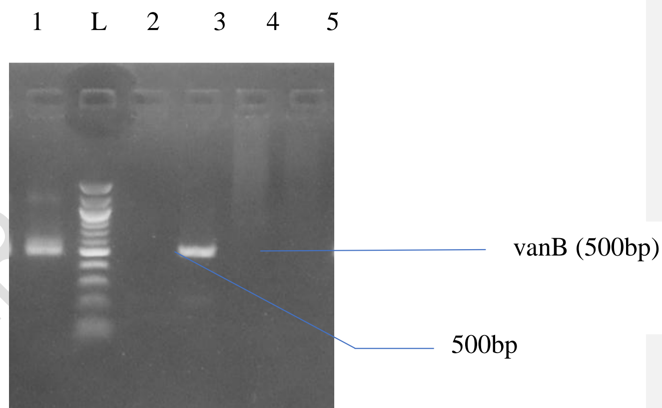

Plate 2. Agarose gel electrophoresis showing the amplified vanB gene. Lane 1 and 3 showing the van B gene band at 500bp. Lane L represents the 100bp molecular ladder

3.2 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 [1]. Previous study has defined that for susceptibility, the MIC should be ≤ 4µg/mL, while those whose MIC is 8-16 4µg/mL are within or should be reported as intermediate and those whose MIC is ≥ 32µg/mL are resistant [7]. In Japan, MIC values of 8µg/mL is considered resistant and these isolates have been reclassified in the United State as being intermediate [1]. 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 ≥ 256µg/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 ≥ 8-32µg/mL [3]. 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 [2;11]. 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-DSer), thereby orchestrating different patterns of resistance to glycopeptide antibiotics [6].

4. CONCLUSION

From the findings in this current study, 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.

Comment [OP10]: Limitation and way forward for this study.

UNDER PEER REVIEW

REFERENCES

Comment [OP11]: Recent references should be used. Always follow Journals style of referencing.
Few references noticed in this paper which is not standard to such work.

1. Arjun, S., James, D. D. and Trish, M. P. (2002). Vancomycin Resistance in Staphylococci. Clinical Microbiology Reviews. *American Society for Microbiology*; 15(3): 430-438.
2. Coombs, G. W., Pearson, J. C., Daley, D.A., Le, T., Robinson, O. J., Gottlieb, T., Howden, B. P., Johnson, P. D., Bennett, C. M., Stinear, T. P. and Turnidge, J. D. (2014). Australian Group on Antimicrobial Resistance. 2014. Molecular epidemiology of enterococcal bacteremia in Australia. J Clin Microbiol 52:897–905.

3. Faron, M. L., Ledeboer, N. A. and Buchan, B. W. (2016). Resistance mechanisms, epidemiology, and approaches to screening for vancomycin-resistant Enterococcus in the health care setting. J Clin Microbiol 54:2436–2447.
4. Levy, S. B. and Marshall, B. (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nat Med*; 10:122–129.
5. Matthew, L. F., Nathan, A. L. and Blake, W. B. (2016). Resistance Mechanisms, Epidemiology, and Approaches to Screening for Vancomycin-Resistant Enterococcus in the Health Care Setting. Journal of Clinical Microbiology; 54:2436–2447
6. Mohamed, O., Ahmed, and Keith E. B. (2018). Vancomycin-Resistant Enterococci: A Review of Antimicrobial Resistance Mechanisms and Perspectives of Human and Animal Health. *Microbial Drug Resistance;* 24: 5.
7. National Committee for Clinical Laboratory Standards. 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. NCCLS approved standard M7-A5. National Committee for Laboratory Standards, Wayne, Pa.
8. Pourakbari, B., Aghdam, K. M., Mahmoudi, S., Ashtiani. H. T. M., Sabouni, F. and Movahedi Z. (2012). High frequency of vancomycin resistant *Enterococcus faecalis* in an Iranian referral children medical hospital. *American Journal of Clinical Medicine*; 7:201-4.
9. Prescott, L.M., Harley, J. and klein, D.A. (2011). Microbiology 8th.ed, McGraw-Hill New York. pp. 809-811.
10. Rossolini, G. M. and Thaller, M. C. (2010). Coping with antibiotic resistance: Contributions from genomics. *Genome Medicine*; 2:15.
11. Schouten, M. A., Hoogkamp-Korstanje, J. A., Meis, J. F. and Voss, A. (2000). European VRE Study Group. 2000. Prevalence of vancomycin-resistant enterococci in Europe. Eur J Clin Microbiol Infect Dis 19:816–822
12. Wemedo, S.A., Obire O. and Akani, N.P. (2012). Bacterial Population of an Oilfield Wastewater in Nigeria. *Asian Journal of Biological Sciences;* 5: 46-51.
13. World Health Organization (2016). The Top 10 Causes of Death. Available from: http://www.who.int/mediacentre/ factsheets/fs310/en/. [Last accessed on 2016 Mar 13].
14. Zaman, S.B., Muhammed A. H., Rachel N., Varshil M., Kazi T.M and, Naznin H. (2017). A Review on Antibiotic Resistance: Alarm Bells are Ringing. *Cureus*; 9(6): 1403.
15. Robinson, V. K., & Wemedo, S. A. (2019). Molecular Characterization of Indoor air Microorganisms of a Model Primary Health Care in Port Harcourt, Rivers State,

UNDER PEER REVIEW

Nigeria. Asian Journal of Biotechnology and Genetic Engineering, 2(1), 1-9. Retrieved from http://www.journalajbge.com/index.php/AJBGE/article/view/30053

16. Chesbrough, M (2005). District Laboratory Practice in Tropical Countries, Part. Cambridge University Press U.K 55- 80.

UNDER PEER REVIEW