ISSN: 2475-6296

Research Article

Journal of Clinical & Experimental Immunology

Plasmid Profiling of Antibiotic Resistant Organisms Isolated From Hospital Effluents Discharged Into Nworie River Imo State

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Submitted: 12 Feb 2020; **Accepted**: 19 Feb 2020; **Published**: 29 Feb 2019

Abstract

The emergence of multiple antibiotics resistant in bacteria and the indiscriminate use of antibiotics contribute to the dissemination of resistant pathogen in the environment. Hospital effluents are potential sources of antibiotic resistant bacteria, which if released into the rivers leads to the contamination of the water by the resistant strains which are potential threat to human health as they might have direct access to man or transported from sea animals to man through food. Plasmids are major mechanism for the spread of antibiotic resistant gene in bacteria population. Plasmid profiling is one of the methods used to determine and characterize antibiotic resistance traits in bacteria. In this study, Samples were collected using sterile sample bottles at three different locations of Nworie River (Two Federal Medical Center and the third behind Umezuruike hospital) in Imo State. A total of eighteen isolates were screened for antibiotic susceptibility. The isolates were tested against ten (10) different antibiotics using the disc diffusion method. Eight (8) isolates were found to be resistant to at least five antibiotics. While the plasmid DNA were extracted using the TENS extraction method and separated by agarose gel electrophoresis. Four of the resistant strains had plasmid DNA.

Keywords: Plasmid, Antibiotics, Resistant, Isolates, Nworie River, Profiling, Hospital

Introduction

All human irrespective of their social, economic status have the right to clean water in quantities required for their basic needs. Hospitals should be a source of solutions to human health related issues and not the other way round. Waste water generation from hospitals is an inevitable process even in the foreseeable future, therefore proper management of these waste are required because hospital waste usually contain pathogens, human tissues, fluids and pharmaceuticals substances with genotoxic properties, heavy metals, and radio-active wastes which are detrimental to human health and when exposed pose a great danger to public health. Hospital effluents and most public wastewaters eventually end up in streams, rivers, lakes and oceans, where they often have devastating effect on the environment and interfere with the legitimate use of the water resources. Usually these effects are more pronounced in communities where waste water treatment is inadequate or completely lacking.

Nworie River is a first order stream that runs about a 5km course across Owerri metropolis in Imo State, whose watershed is subject to intensive human and industrial activities resulting in the discharge of a wide range of pollutants. The river is used for various purposes by inhabitants of Owerri especially when the public water supply fails, the river further serves as a source of direct drinking water, especially for the poorer segment of the city [1]. The river also supports recreational and part-time fishing for youth.

Nworie River is vulnerable to various source of pollution. All through its course, there is a steady input of large quantities of detergents from laundry activities [1]. And at several points, the river receives large quantities of sewage and solid wastes, especially plastic water sachet. In like manner, when it rains, a large volume of run-off carrying agricultural and human waste are discharged directly into the river, which pollutes the water and renders it unusable for virtually all its purpose.

Studies of water quality parameters are therefore necessary to determine the extent of pollution so as to monitor likely danger, not only to the human population but also to the aquatic life and the entire ecosystem. In rivers where hospital wastes are disposed, it is expected to be highly polluted with series of microorganisms which are capable of causing nosocomial infections and other kinds of diseases both to man and other forms of life in the water. Usually most of these organisms have evolved to posses the potential of antibiotic resistance, which are sometimes determined by the presence of plasmid.

Plasmid being a non-chromosomal double stranded DNA located at the cytoplasm of cells and which is capable of replicating itself

within a competent cell equally has the ability of carrying genes that encode traits for antibiotic resistance and other capabilities, such as digesting pollutants from the environment. This study is aim at isolating and identifying various microorganism from hospital discharge and profiling of the plasmids to determine the various strains of organisms resistant to antibiotics so as to prevent water borne and sea food borne infections.

Materials and Methods

Area of Study

Nworie River is a first order stream that runs about a 5km course across Owerri metropolis in Imo State locate. The area of study lies between latitudes 5˚25′ – 5˚30′N and longitudes 7˚00′ – 7˚05′E. It covers an area of 1135 square kilometers; Pysiographically, the terrain is characterized by two types of landforms; high undulating ridges and a nearly flat topography. The ridges trend in the N – S direction and have an average elevation of about 122m above the sea level. Between these ridges is Valley of Nworie River. River Nworie flows in the NE – SW direction with average hydraulic gradient between 0.005 and 0.02 [2].

The river is of enormous economic importance to inhabitants of Owerri metropolis as it serves as a water source for various domestic uses and substantial recreational and part-time fishing for youths. Some segments of the human population in Owerri use it as direct source of drinking water especially during failures of the public water supply. Nworie watershed is subject to intensive human and industrial activities resulting in the discharge of a wide range of pollutants including hospital wastes. Studies of water quality parameters and microorganisms present are therefore necessary to determine the extent of pollution so as to monitor likely danger, not only to the human population but also to the aquatic life.

Nworie River takes its sources in Ohii and joins Otamiri River at Nekede. Its total length is 9.2km. This watershed is covered by depleted rain forest vegetation with an annual rainfall of 2500mm. It flows through the Federal Medical Centre, Owerri (FMC), Alvan Ikoku Federal College of Education (AIFCE) and Holy Ghost College [2].

Sample Collection

Samples were collected using sterile sample bottles at three different locations of Nworie river. Two were behind FMC and the third behind Umezuruike hospital both in Owerri Imo state. The samples were kept in an ice packed cooler before transportation to the laboratory for analysis

Sterilization of materials

All glass wares used in the study was properly washed with detergent, rinsed with tap water and finally with sterile distilled water, while the glass wares together with bijou bottles were sterilized using the autoclave at a temperature of 121˚c for 15minutes.

Media Preparation

All media used was prepared according to the manufacturer's prescription, which includes. Nutrient agar(NA), Mac Conkey agar(MAC), Salmonella and Shigella agar(SSA), Thiosulphate citrate-bilesalt -sucrose agar(TCBS), Simon Citrate agar.

Serial Dilution of Samples

This is a stepwise dilution of a substance in a solution, which could be sterile distilled water or peptone water so as to avoid dense culture of cells. To obtain an appropriate concentration of cells, serial dilution was carried out using peptone water and 9mls each was dispensed into 10 arranged bijou bottles labeled 10^{-1}, 10^{-2}, 10^{-3},10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8},10^{-9}, 10^{-10}. Then 1ml of the undiluted sample was pipette into the first bijou bottle (10^{-1}) and from there 1ml into the next bottle (10^{-2}), then in such order serially diluting into the respective bijou bottles up to the 10th bottle (10-10).

Inoculation of Samples

After serial dilution, 0.1ml of dilution 8 (10^{-8}) each of the three samples were inoculated on the prepared Nutrient, salmonella and shigella (S.S), Mac Conkey and Thiosulphate citrate-bilesalt -sucrose agar (TCBS) agar media, while 0.1ml of dilution 10 (10^{-10}) each of the three samples were inoculated into the Nutrient and MacConkey agar media using spread plate method to which a sterile spreader was used to spread the inoculum evenly on the media. The Nutrient and Mac Conkey agar was incubated inverted in the incubator at the temperature of 37˚c while the S.S and TCBS was incubated at room temperature for 24hours and colour change, morphology, growth pattern and the number of colonies was observed. Distinct colonies from the growing culture were sub-cultured aseptically using a wire loop into a newly prepared nutrient agar medium.

Characteristics and Identification of Microbial Isolates

- **Gram Staining**

This differentiates between gram positive and gram negative organisms. Using a 48 hours old culture, a smear of the test organism was made on a clean grease free glass slide which was heat fixed by passing it severally over flame of a bunsen burner followed by flooding of the smear with crystal violet solution (primary stain) for 45seconds and was rinsed with distilled water. The smear was then flooded again with lugol's iodine solution for 45 seconds and was rinsed with distilled water and then decolorized with acetone alcohol for 5seconds. It was immediately rinsed off with distilled water. It was counter stained with safranin for one (1) minute and was finally rinsed with water. Then the slide was air dried and examined microscopically under oil of immersion. Gram-positive cells appeared purple while gram-negative cells appeared red or pink in colour.

- **Oxidase Test**

This test demonstrates the ability of organisms to utilize oxidase enzyme in breaking down indophenolsoxidase which helps to transform electrons. A colony of 48 hours culture of test organism was picked and robbed on an oxidase-impregnated filter paper. A change in colour to purple indicates positive reaction.

- **Catalase Test**

This test demonstrates the ability of microorganisms to degrade hydrogen peroxide with the release of water and oxygen using the catalase enzyme.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Using a sterile pasteur pipette, a drop of freshly prepared 3% hydrogen peroxide was placed on a clean slide and the 24 hours old test organism were picked with a sterile wire loop and mixed with the reagent. An effervescence caused by the liberation of free oxygen indicates the presence of the enzyme, hence a positive reaction.

- **Citrate Utilization Test**

This test was used to test the ability of microorganisms to utilize citrate as the sole source of carbon and energy, and ammonium salts as the source of nitrogen. Inoculum from an overnight slant culture of each test organism was inoculated into Simon's citrate agar medium and examined after 24 hours. The presence of turbidity and blue coloration gave a positive result.

- **Methyl Red Test**

The methyl red test was employed to detect production of sufficient acid during fermentation of glucose. The test organisms in broth culture was inoculated in prepared methyl red broth medium after which it was incubated for 24hours at 37˚c. At the end of the period, three (3) drops of methyl red was added to the tube for each isolates. The presence of red coloration gave a positive test, while the yellow coloration gave a negative result.

- **Voger Proskauer Test**

The test organisms in broth culture was inoculated in prepared methyl red broth medium after which it was incubated for 48hours at 37˚c.1ml of alpha-naphtol solution was added to the tube followed by 1ml of 40% potassium hydroxide solution. The tubes were shaken and allowed to incubate for 1hr standing on the table. The presence of red coloration showed the formation of methyl-carbinol, which is the Voger Proskauer positive test while a reddish brown coloration gave a Voger Proskauer negative result.

- **Indole Production Test**

Indole test was employed to determine the ability of certain bacteria to decompose the amino acid tryptophan to indole, which accumulates in the medium. The isolate were inoculated in typtone broth and was incubated overnight at a temperature of 37°C for 48 hours. Then few drops of Kovac's reagent were added and the tubes observed for result. A red coloration indicated positive test while no coloration indicates negative test.

- **Motility test**

This test is used to differentiate motile bacteria from non-mortile bacteria. A semi-solid (half solid) nutrient agar was dispensed 10ml into test tubes and sterilized. On cooling, the medium was allowed to set in an upright position. The medium was inoculated by stabbing with a sterile straight wire to a depth of about 2cm. The inoculated tubes were then plugged with cotton wool and incubated at 37˚c for 24hours. After incubation, motility was indicated by spreading growth of the organism from the stab line in the medium.

Antibiotic Susceptibility Testing of Isolates

This was done to determine the level of susceptibility or resistance of the isolates to the different antibiotics used. A colony of the organism was picked and sub-cultured into LB broth medium, which was incubated for 24 hours. 0.1 ml was inoculated into nutrient agar medium using the micropipette and with a sterile swab, stick was streaked repeatedly back and forth and from edge to edge while rotating the plate to obtain even distribution of organism on the media. The media was left for 15minutes, and then multidisc containing different antibiotic was inserted on the medium. The disc was pressed down firmly after insertion to ensure complete level contact with the agar after which it was incubated inversely at a temperature of 35˚C for 24hours. The presence of a clear zone of inhibition around the disc indicates the susceptibility of the organism to the particular antibiotics, while the absence of a clear zone denotes the resistant of the organism to the antibiotic.

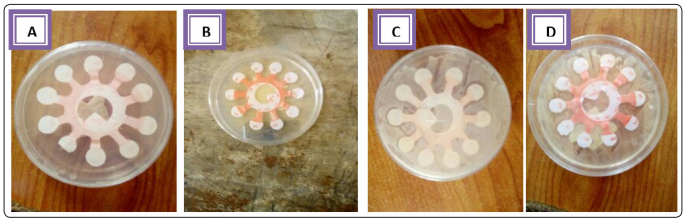

Plate 1: shows antibiotic resistant and susceptible plates

Plasmid Isolation

1.5ml of overnight bacterial culture was spinned for 60 seconds in a micro-centrifuge to pellet cells. The supernatant was decanted, leaving 50-100 µl in the tube. Then it was vortexed to resuspend the bacteria pellet completely. 300µl of TENS solution was added. It was mixed by inverting tubes several times and then vortexed again for 5 seconds to mix properly. 150 µl of 3.0M sodium acetate was added and vortexed for 5 seconds to mix properly. It was then centrifuged for 5 minutes in a micro-centrifuge to pellet cell debris and chromosomal DNA.

Then the supernatant was transferred to a fresh tube and 900µl of pre-chilled 100 % ethanol added. It was again centrifuged for 10 minutes in a micro-centrifuge. White pellet, containing plasmid DNA and bacterial RNA, was formed at the bottom of the tube.

The supernatant was discarded and 1 ml of 70 % ethanol was used to rinse the pellet twice. The sample was then air-dried and re-suspended in 30µl of TE buffer at a temperature of 4˚C.

Agarose Gel Elctrophoresis

This was carried out to visualize and separate plasmid DNA by size, using a gel matrix and an electric current. The agarose gel used was produced by dissolving 0.8g of agarose powder in 100mls TBE after and was heated to near boiling points. The melted agarose was allowed to cool down and 10 µl of ethidium bromide was added to the mixture. It was swirled and the mixture was poured into the electrophoresis tray with placed comb. The mixture was left for 20 minutes to polymerize and the comb was removed. The tray was placed in the electrophoresis tank. X1 TBE buffer was poured into the tank ensuring it covers the gel surface and then 20µl of the each of the samples were mixed separately with 2µl loading dye and were carefully loaded into the wells created by the comb with the marker in the last lane. The electrodes of the tanks were connected to the power pack with the negative terminal at the well side where the samples were loaded. The electrophoresis was run at 75volts until the loading dye was seen three quarter of the gel field. The power pack was turned off and the electrodes disconnected. The gel was brought out from the tank and observed under a UV-trans illuminator.

Results

The result shows that a total of eighteen isolates were identified and they include three *Escherichia spp spp*, four species of *Salmonella*, and two species of *Shigella*, four Citrobacter spp. one *Pseudomonas* spp, *Aeromonas* spp, *Enterobacter* spp, *Bacillus* spp and *Klebsiella* spp. Antibiotic susceptibility test result, revealed that three *Escherichia spp* were susceptible to all the ten antibiotics used, while two *Salmonella* spp were resistant to antibiotics 5 and 8. *Shigella* spp were resistant to antibiotics 7 and 10. The *enterobacter*, *bacillus* and *Klebsiella* isolates were susceptible to the entire antibiotics used

while the *Pseudomonas* and *Aeromonas* spp were resistant to antibiotics 9 and 7 respectively. Also two *Citrobacter* spp were resistant to antibiotics 6 and 5. Thus, a total of eight resistant isolates. As shown in Table 1and 2.

Table 1: Antibiotics Susceptibility Test Result

Isolates	SXT	CH	SP	CPX	AM	AU	CN	PEF	OFX	S
Escherichia spp	++	++	+++	++	++	++	++	++	+++	++
Enterobacter spp	++	++	++	++	++	+	++	+++	++	++
Salmonella spp	++	R	R	R	R	R	R	+	R	R
Shigella spp	R	R	R	R	R	R	+	R	+	+
Pseudomonas spp	R	R	R	+++	R	R	R	R	R	R
Aeromonas spp	R	R	R	R	R	R	+	+	+	R
Escherichia spp	++	++	++	++	+++	++	++	++	++	++
Salmonella spp	++	++	+++	+++	++	++	++	++	++	++
Escherichia spp	+	++	++	+++	++	+++	++++	++	++	++
Salmonella spp	++	+++	+++	+++	+++	+++	+++	+++	++	++
Shigella spp	R	R	R	R	R	R	R	R	R	R
Salmonella spp	+++	+++	R	+	R	R	R	R	+	+

R = resistant, SXT = septrin 30µg, CH= chloranphenicol 30µg, SP= sparfloxacin 10µg, CPX ciprofloxacin 10µg, AM=amoxacillin 30µg, AU = augmentin 30µg, CN=gentamycin 10µg, PEF=pefloxacin 30µg, OFX= tarivid 10µg and S= streptomycin 30µg,

Table 2: Antibiotic Susceptibility Test Result of Gram Positive Isolates

Isolates	CPX	NB	CN	AML	S	RD	E	CH	APX	LEV
Bacillus spp	+++	++	+++	+++	++	+++	+++	+++	+++	+++
Citrobacter spp	++	+	++	+	+	++	++	++	++	++
Klebsiella spp	++	++	++	+	++	++	++	++	++	+++
Citrobacterspp	+	++	++	+++	++	+++	+	+	++	+
Citrobacter spp	++	R	++	R	R	R	+++	R	R	++
Citrobacter spp	+++	R	+	R	+	R	+	R	R	+++

R = resistant, CPX =ciproflox 10mcg, NB = norfloxacin 10mcg, CN = gentamycin 10mcg, AM=amoxil 20mcg, S = streptomcin 30mcg, RD = rifampicin 20mcg, E= erythromycin 30mcg, CH = chloramphenicol 30mcg, APX= ampiclox 20mcg, LEV = levofloxacin 20mcg

The gel electrophoresis, when viewed under U.V transimillator, revealed that four out of the eight resistant strains of the isolates contained plasmid DNA. These isolates include *Aeromonas spp* which is resistant to antibiotic 7, the *Shigella spp* which is resistant to all ten antibiotics used, the *Salmonella spp* is resistant to antibiotics 5 and the *Citrobacter* spp is resistant to antibiotics 6, as shown in Figure 2.

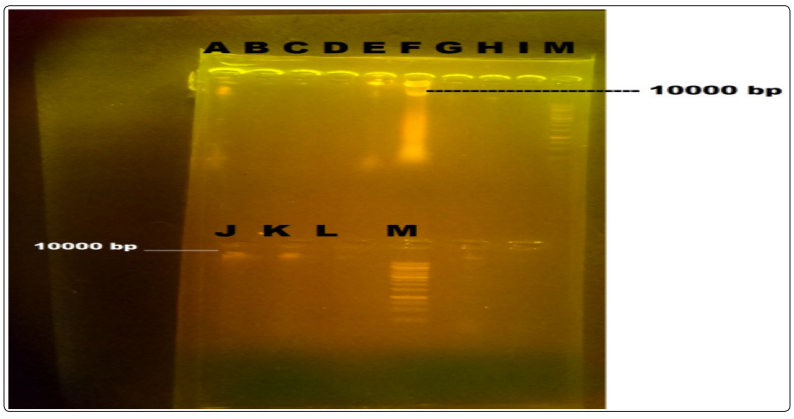

Figure 2: Plasmid Profile Result of Resistant Strains

E= Shigella spp, F = Salmonella spp, G = Salmonella spp, H = Shigella spp, I = Pseudomonas spp, J = Aeromonas spp,K = Citrobacter spp L = Citrobacter spp M = Marker

Discussion

Multiple antibiotic resistances in bacteria population is currently one of the greatest challenges in the effective management of infections [3]. Total of eighteen (18) isolates were identified from samples and these include four *Salmonella spp*, two *Shigella spp*, three *Escherichia spp*, four *Citrobacter spp*, one *pseudomonas spp*, *Aeromonas spp*, *Enerobacter spp*, *Bacillus spp*, and *Klebsiella spp*. Eight isolates were found to have multiple resistances to at least five or more out of the ten antibiotics, used against the test organisms. This shows that hospital effluents are full of assorted loads of antibiotic resistant plasmids with genes that supplement the resistome of receiving aquatic environments. This supports the report of Xia et al, Varela et al and Alouache et al, that Urban rivers, provide an ideal setting for the acquisition and spread of plasmid-mediated antibiotic resistance (PMAR) because of continuous pollution from anthropogenic activities e.g., effluents from municipalities, hospitals, and aquaculture [4-6]. The high rate of resistance found in this study can be explained by the wide use of antibiotics in hospitals for treatment of infections.

The constant use of antibiotic drugs in hospitals promotes development of resistant strains with R plasmids. It is of significant public health concern that multi antibiotic resistant organisms from hospital effluents discharged into rivers constitute a potential reservoir of resistant plasmid as seen from the result that could be transferred to pathogenic bacteria [7]. This is also in line with Poirel et al report that Hospital effluents discharged into Rivers may alter biodiversity in the ecosystem, as well as the characteristic microbiota of water. Pathogens with antibiotic resistant limit the available option in treating infectious diseases of animals and humans. The high prevalence of multiple antibiotic resistance that exist among the isolates from hospital effluent discharged into Nworie river suggest the urgent need to prevent hospital waste discharge into rivers and improve education and communication on the issue of antibiotics used in the hospitals and among individuals, as these isolated organisms depicts to be causative organisms for diseases like typhoid, diarrhea, meningitis, and other various form of urinary and respiratory tract infections.

Antibiotic resistance plasmids are bacterial extrachromosomal elements that carry genes conferring resistance to one or more antibiotics. Results from the plasmid profiling, shows that four of the isolates *Aeromonas spp*, *Shigella spp*, *Salmonella spp* and *Citrobacter spp* with resistant strains has plasmid DNA. Using a comparative metagenomic approach, it has shown that effluents entering a river catchment contain antibiotic resistance genes (ARGs) and that the abundance of these ARGs is greater than that of the receiving environment, suggesting that effluents are contributing ARGs to the resistome [8-10]. McEneff et al and Bruchmann found that pharmaceutical antibiotic residues present in effluents are selective to pressures that can directly influence ARGs in the environment. Plasmid profiles have been used to verify the identity of bacterial isolates and show that they are different, despite the fact that they might be of the same species, as seen in *Salmonella* and *Citrobacter* spp that had two resistant strains each with just one each having plasmid DNA. Several aquatic bacterial species have intrinsic genes mixing with allochthonous species from different sources and tends to promote genetic exchange, which may aggravate clinical drug resistance and reduce environmental quality [7].

Conclusion

Although Antibiotics has helped to control several human diseases, there is a growing awareness of public health concern associated with the use of antibiotics [11, 12]. Fang et al report has proven that Plasmid is major mechanism for the spread of antibiotics resistant gene in bacterial population. This research revealed multiple antibiotic resistant organisms present in hospital effluents discharged into Nworie Rivers and possible transfer of such resistance strains from one organism to the another. Therefore, cautions should be taken on disposal of hospital effluents into Nworie River and other fresh water bodies to control the contamination it poses to aquatic life and dangers it may pose to human and animal that depend aquatic lives as a source of food. From the plasmid profile result, it possible that the resistant strains lacking plasmid may house resistant genes in their chromosomal DNA rather than in plasmid DNA. This is in line with the findings of Aja et al [13].

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