Molecular Characterization of Isolates and Antibiotic Resistance Genes Associated with Fish Pond

Niyi-David, C.C, Ogbonna, D.N, Akani, N.P, Douglas, S.I.

Department of Microbiology, Rivers State University, P.M.B 5080, Port Harcourt, Nigeria

***Abstract*: Characterization of isolates from the fish pond water collected from Port Harcourt, in Rivers State, was done using 16S rRNA gene sequencing method to identify their phylogenetic relationship. The presence of the resistance gene was investigated by polymerase chain reaction followed by agarose gel electrophoresis of the amplification products. A total of 10 bacteria were identified, they include *Escherichia coli, Klebsiella quasipnemoniae, Morganella morgani, Vibrio parahaemolyticus, Pseudomonas xiamenesis, Staphylococcus sp, Bacillus flexus, Lysinibacillus macroides, Myoides odoratimimus* and *Chryseobacterium cucumeris*. The *Lysinibacillus macroides* and *Vibrio parahaemolyticus* were found to be carriers of the CTX-M gene, while *Escherichia coli* carried the QNRB gene. SHV gene was also found in the *Vibrio parahaemolyticus.* The resistance gene found in these bacteria can be transferred to other microbes to cause antibiotic pollution which is a major health challenge.**

I. INTRODUCTION

There are several microorganisms found in ponds including bacteria, fungi, algae, protozoa, nematodes and virus (Ajayi and Okoh, 2014). Bacteria are important microorganisms in ponds, some are friend while some are foes. Freshwater and brackish water fishes can harbor human pathogenic bacteria like coliform group (Adedeji *et al*., 2011). According to Pillay (1990) fish living in natural environment are known to harbor pathogenic enterobacteriaceae. Several bacteria species have been identified in great lakes and other water bodies (Udeze *et al*., 2012). Fish pond under a controlled condition also habour good number of microorganisms including pathogenic and non pathogenic organisms. Several researchers have reported the presence and occurrence of bacteria flora in pond waters (Abu and Wondikom, 2018; Abu and Egenonu, 2008; Naim and Ahmed, 2012). In their finding it was revealed that gram-negative rod shaped bacteria inhabited a cultured population. Continuous intensification of fish farming, increasing the risk of disease, has resulted in the need for treatment with antibiotics (Bostock *et al.,* 2010). Many literature data clearly indicate that the ponds in which antibiotics are used are a reservoir of ARGs, and therefore pose a great risk to the health and life of humans (Kemper, 2008). The characteristic bacterial genera in aquacultures found with a high frequency, *Aeromonas* spp. and *Vibrio* spp., are responsible for fish diseases. What is interesting is that some of the *Aeromonas* strains causing disease in humans, as the specific vector, may transfer MGEs carrying resistance genes to pathogenic or opportunistic bacteria in the human microbiome, and thus pose a threat to public health. This study aims at identifying the bacteria isolates and their resistance gene from fish pond water in Port Harcourt, using molecular methods.

II. MATERIALS AND METHODS

Study Area

This research was carried out in a private fish farm (Sokari Integrated Farms and Services) located in Sangana Street, Mile One Diobu within Port Harcourt metropolis, within longitude 4.4735^{o}N, and latitude 6.5954^{o}E, 20m Elevation and Rivers State University Nkpolu Oroworukwo in Port Harcourt, located within longitude 4.4754^{o}N, and latitude 6.5846^{o}E, 10m Elevation both in Rivers State, Nigeria. Rivers State is one of the coastal states in Nigeria with one of the highest rainfalls and the climate is that of tropical wet climate with long and heavy rainy seasons and very short dry season according to Gobo *et al.* (2008).

Sample Collection

Wastewater samples were collected from fish tanks fertilized with poultry waste, Ash and periwinkle shells and also from tanks treated with *Lemna minor (Duckweed), Eichhornia crassipes (Water Hyacinth)* and *Pistia stratiotes* (Water Lettuce). These were collected with sterilized plastic bottles. Each sample bottle was rinsed three times with the appropriate sample before it was finally collected according to the standard methods (APHA, 2001). When collecting the water sample, the base of each sterilized sample bottle was held with one hand, and plunged about 30cm below the water surface with the mouth of the sample bottle positioned in an opposite direction to water flow (APHA, 2001). After the samples were collected, they were labeled and immediately carried in a cooler packed with ice blocks for analysis.

Isolation of the Bacteria

Ten-fold Serial dilutions of all the samples were made according to the methods described by Oliveira *et al.* (2016). 1 ml of the wastewater was aseptically introduced into 9ml of sterile distilled water giving an initial dilution of 1:10ml. Then Aliquots (0.1 ml) of 10^{-2} and 10^{-3} were inoculated in duplicate onto sterile solidified Nutrient agar (NA), Cetrimide (CA), Thiosulphate citrate bile salt agar (TCBS), MacKonkey Agar (MA), Eosin Methylene Blue Agar (EMB) and Salmonella-Shigella Agar (SSA) and evenly spread out with a sterile flamed glass spreader. These were incubated at 37^{o}C for 24 hours and observed for growth and that of EMB were incubated at 44 ^{o}C for 24 hours. The colonies on the plates

were counted and recorded using colony forming unit of the sample (Guo *et al.,* 2013; Hussain *et al.,* 2013).

After the colonies were counted the mean for each sample was expressed as cfu/ml Counts for each sample were then calculated using the formula below (Nrior and Odokuma, 2015).

$$\text{THB (cfu/g)} = \frac{\text{Number of Colonies}}{\text{Dilution x Volume plated (0.1ml)}}$$

Representative colonies were sub-cultured onto sterile Nutrient agar (NA) plates to obtain pure cultures for further characterization.

Molecular Identification

Discrete colonies were molecularly identified to ascertain the genetic makeup of the organisms. This was done involving five (5) major steps.

DNA extraction (Boiling method)

Boiling method is used in DNA extraction and separation. Pure culture from nutrient agar was aseptically inoculated into Luria Berta (LB) contained in sterile bijou bottles with the aid of an inoculating pin and incubated for 24hours. After 24 hours, 5ml (Five milliliters) of the broth culture from LB was dispensed into epidorf or microcentrifuge tubes, stuck into the centrifuge and spun at 14000rpm for 3minutes. The cells were re-suspended in 500µl of normal saline and stuck in the heating block to lyse the cells at 95^{o}C for 20 minute. The heated bacterial suspension was fast cooled on ice for 10minutes and spun for 3 min at 14000rpm. The supernatant (300µm) containing the DNA was transferred to a 1.5ml micro-centrifuge tube with the aid of a micropipette and stored at -20^{o}C for other downstream reactions.

DNA quantification

This was done to purify and quantify the DNA. The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2ul of sterile distilled water and blanked using normal saline. Two micro-liters (2 µl) of the extracted DNA was loaded onto the lower pedestral, the upper pedestral was brought down to contact the extracted DNA on the lower pedestral. The DNA concentration was measured by clicking on the "measure" button.

16S rRNA Amplification

The 16s rRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 25 micro-liters for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; anealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 20 minutes and visualized on a blue transilluminator.

Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10µl, the components included 0.25ul Big Dye® terminator v1.1/v3.1, 2.25µl of 5 x Big Dye sequencing buffer, 10µM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing condition were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4minute.

Phylogenetic Analysis

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. These sequences were aligned using MAFFT. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969).

Plasmid Profile

Plasmids are known as circular deoxyribonucleic acid (DNA) molecules found in bacteria that are usually independent of the chromosome. Plasmids have the ability to encode antibiotic resistance or virulence factor genes and also serve as markers of various bacterial strains during plasmid finger printing or plasmid profile. Thereby, the knowledge and study of plasmids is very essential to medical microbiologist. Into 1.5ml microcentrifuge tube 600*ul* of bacterial culture grown in Luria Bertani medium was dispensed. 100*ul* of 7X Lysis Buffer (Blue) was added then mixed by inverting the tube 4-6 times, and incubated for 1-2 minutes. After the addition of 7X Lysis Buffer, the solution should change from opaque to clear blue, indicating complete lysis. 350*ul* of cold neutralizing buffer (Yellow) was added and thoroughly mixed (not vortex).

Note: Neutralization is said to be complete when the sample is yellow throughout and precipitate has formed.

Centrifuge for 2-4 minutes at ≥11,000 × *g* then place zymo-spin™ IIN column in a collection tube and transfer the supernatant from step 4 into zymo-spin™ IIN column.

NOTE: Avoid disturbing the pellet containing cell debris. Centrifuge the Zymo-spin™ IIN for 15 seconds at ≥ 11,000 × *g*. Discard the flow-through and return the zymo-spin IIN column to the same Collection Tube.

NOTE: Ensure the flow-through does not touch the bottom of the column. 200ul of Endo-Wash Buffer was added to the column and centrifuged for 30 seconds at ≥ 11,000 × *g*. 400ul of Zyppy™ Wash Buffer was also added to the column and centrifuged for 1 minute at ≥11,000 × *g*. The column was transferred into a clean 1.5ml microcentrifuge tube then ≥30ul of Zyppy™ Elution Buffer was added directly to the column matrix and incubated for one minute at room temperature then centrifuge for 30 seconds at ≥11,000 × *g* to elute the plasmid DNA.

Amplification of QnrB genes

QnrB genes from the isolates were amplified using the qnrBF: 5′-GATCGTGAAAGCCAGAAAGG-3′ and qnrBR: 5′-CGATGCCTGGTAGTTGTCC-3′primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 58ºC for 40 seconds; extension, 72ºC for 50 seconds for 35 cycles and final extention, 72ºC for 5 minutes. The product was resolved on a 1% agarose tinted with E-Z vision gel at 120V for 25 minutes and blue light transilluminator for a 800bp product size.

Amplification of SHV genes

SHV genes from the isolates were amplified using the SHV F: 5' CGCCTGTGTATTATCTCCCT-3' and SHV R: 5'-CGAGTAGTCCACCAGATCCT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 56ºC for 40 seconds; extension, 72ºC for 50 seconds for 35 cycles and final extention, 72ºC for 5 minutes. The product was resolved on a 1% agarose tinted with E-Z vision gel at 120V for 25 minutes and blue light transilluminator for a 281bp product size.

Amplification of CTX-M genes

CTX-M genes from the isolates were amplified using the CTX-MF: 5'-CGCTTTGCGATGTGCAG-3' and CTX-MR:5'-ACCGCGATATCGTTGGT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extention, 72ºC for 5 minutes. The product was resolved on a 1% agarose tinted with E-Z vision gel at 120V for 25 minutes and blue light transilluminator for a 550bp product size.

III. RESULTS

A total of 194 bacterial isolates belonging to eleven genera were identified from the eight fish ponds with concrete (4 tanks) having total heterotrophic bacteria count (THB), total coliform count (TCC), total salmonella shigella count (TSS), total feacal coliform count (TFCC), total vibroid count (TVC), and total pseudomonad count (TPC) ranged from 4.78 ± 0.5×10^4 to 5.74 ± 0.39×10^5, 4.06 ± 0.06 $\times10^4$, to 5.8 ± 0.43 $\times10^5$, 4.3 ± 0.24×10^4 to 4.99 ±0.42 $\times10^4$, 4.18 ± 0.39 $\times10^4$ to 5.08 ± 0.43 $\times10^5$, 4.08±0.35 x 10^5 to 5.24±0.46 x 10^5 and 4.1 ± 0.3 × 10^4 to 5.15 ± 0.44 $\times10^4$ cfu/ml, respectively and plastic (4 tanks) having total heterotrophic bacteria count (THB), total coliform count (TCC), total salmonella shigella count (TSS), total feacal coliform count (TFCC), total vibroid count (TVC), and total pseudomonad count (TPC) ranged from 4.55 ± 0.46×10^4 to 5.74 ± 0.4×10^5, 4.43 ± 0.23 $\times10^4$, to 5.78 ± 0.36 $\times10^5$, 4.00 ± 0.5×10^4 to 5.00 ±0.47 $\times10^5$, 4.18 ± 0.39 $\times10^4$ to 5.17 ± 0.45 $\times10^5$, 3.78±$0.35x10^3$ to 5.24 ± $0.46x10^5$ and 3.81 ± 0.26 × 10^3 to 5.15 ± 0.44 $\times10^5$ cfu/ml, respectively. The bacteria genera includes, *Staphylococcus, Micrococcus, Bacillus, Enterococcus, Proteus, Pseudomonas, E.coli, Salmonella, Klebsiella, Vibrio and Shigella* sp.

Table 1: Bacterial isolates and its occurrence in the concrete and plastic tanks

Bacterial Isolates	Concrete tanks n(% occurrence)	Plastic tanks n(% occurrence)
Staphylococcus spp	26 (26.26)	25 (26.59)
Micrococcus spp	15 (15.15)	14 (14.89)
Bacillus spp	15 (15.15)	14 (14.89)
Enterococcus spp	4 (4.04)	4 (4.25)
Proteus spp	7 (7.07)	6 (6.38)
Pseudomonas spp	6 (6.06)	5 (5.31)
*E.coli*spp	6 (6.06)	6 (6.38)
Salmonella spp	5 (5.05)	5 (5.31)
Klebsiella spp	4 (4.04)	5 (5.31)
*Vibrio*spp	5 (5.05)	5 (5.31)
Shigella spp	6 (6.06)	6 (6.38)

Ten isolates coded with K8, 10, OA1, 18, OA2, OA4, C8, D10, U8 and OA3 were identified as *Escherichia coli*, *Klebsiella quasipnemoniae, Morganella morgani, Vibrio parahaemolyticus, Pseudomonas xiamenesis, Staphylococcus sp, Bacillus flexus, Lysinibacillus macroides, Myoides odoratimimus* and *Chryseobacterium cucumeris,* respectively as shown in Table 2 with their accession numbers. The phylogenetic tree showing the relatedness of the isolates to their relatives in the Gene bank is shown in Fig 1.

Table 2 Molecularly Identified Isolates with their Accession Numbers

Isolate code	Molecular Identification	Accession Number
K8	*Escherichia coli*	MN097134
10	*Klebsiella quasipnemoniae*	MN097133
OA1	*Morganella morgani*	MN094330
H8	*Vibrio parahaemolyticus*	MN097132
OA2	*Pseudomonas xiamenesis*	MN094331
OA4	*Staphylococcus sp*	MN094333
C8	*Bacillus flexus*	MN097130
D10	*Lysinibacillus macroides*	MN097131
U8	*Myoides odoratimimus*	MN097135
OA3	*Chryseobacterium cucumeris*	MN094332

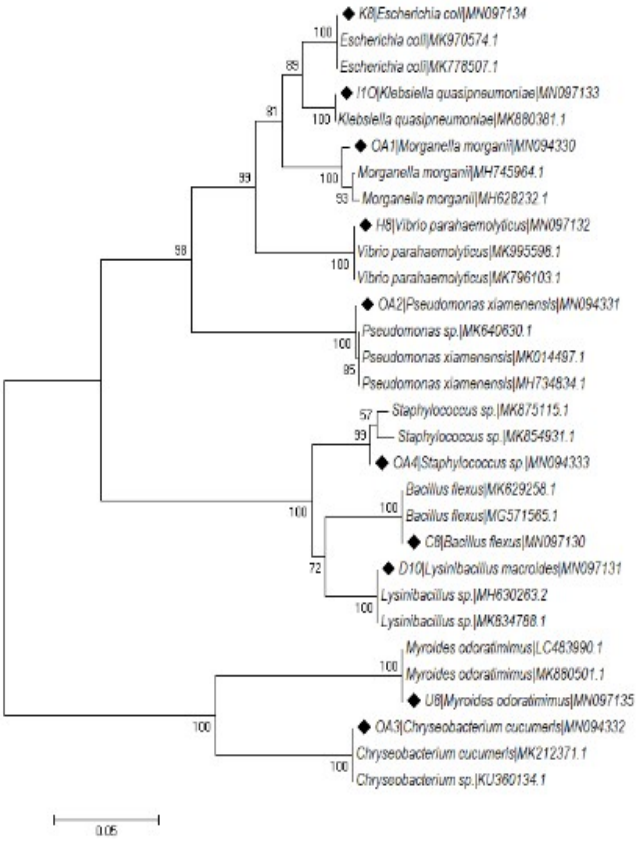

Fig.1: Phylogenetic tree showing the evolutionary distance between the bacterial isolates

Plate 1-3 shows the resistant gene of isolate in the primers used. For Plate 1, the CTX-M gene were identified in D10 and H8 (*Lysinibacillus macroides* and *Vibrio parahaemolyticus*). Plate 2 identified the QNRB gene in K8 (*Escherichia coli*) while plate 3 identified the SHV gene in H8 (*Vibrio parahaemolyticus*).

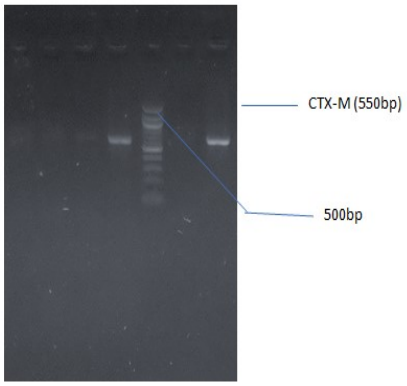

Plate 1: Agarose gel electrophoresis showing the CTX-M gene band. Lane D10 and H8 showing the CTX-M gene at 550bp while lane L represents the 100bp molecular ladder

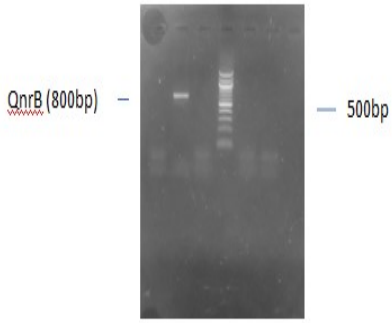

Plate 2: Agarose gel electrophoresis showing the QnrB gene band. Lane K8 showing the QnrB gene at 800bp while lane L represents the 100bp molecular ladder

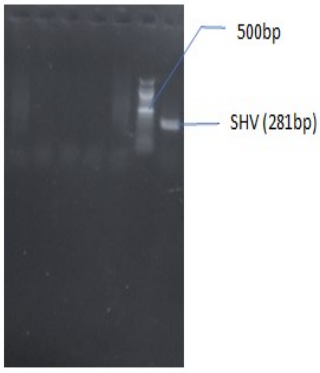

Plate 3: Agarose gel electrophoresis showing the SHV gene band. Lane H8 showing the SHV gene at 281bp while lane L represents the 100bp molecular ladder

IV. DISCUSSION

The pond 3 recorded highest heterotrophic bacteria count which was followed by pond 1 and then 2. The Salmonella shigella and total fecal coliform count recorded zero count in pond 2 and highest count was recorded in pond 3. Total vibriod and pseudomonas count also recorded highest count in pond 3. The results of this investigation showed high counts. However there were variations in the ponds. High bacteria load recorded could be attributed to rainfall or runoff from land during the rainy season which could have washed off animal excretes or other agricultural waste into the ponds. Bano (2006), Douglas and Isor (2015) also observed a similar trend in their studies involving streams where they recorded higher microbial load in the rainy season. The bacteria isolated from the pond during the study includes *Escherichia coli, Klebsiella quasipnemoniae, Moeganella morgani, Vibrio parahaemolyticus, Pseudomonas xiamenesis, Staphylococcus sp, Bacillus flexus, Lysinibacillus macroides, Myoides odoratimimus* and *Chryseobacterium cucumeris*. *Escherichia coli* in pond are an indicator of fecal pollution which could be attributed to human activities or excrete from the fishes in the pond. The presence of such bacteria indicates the possible presence of fecal material (Shekha *et al*., 2013; Uzoigwe and Agwa, 2012). Bacteria from *Enterobactericaea* family such as *E.coli, Klebsiela* and *Pseudomonas* sp isolated from the fish water sample can cause human diseases (Oliveria *et al*., 2017). *Vibrio* sp occur naturally in aquatic environments and are one of the most commonly occurring bacteria in shrimp farming. Occurrence of Vibrio parahaemolyticus in fish pond has been reported by several researchers (Abraham *et al*., 1997; Sanjeev, 2002). *Staphylococcus* species in fish pond water may pose a threat to the health of the fishes and consumers (Torimiro *et al*., 2014). The presence of *Bacillus* species in aquatic environment are commonly found as probiotics in fish culture (Sarjito *et al*., 2019). Other bacteria isolated such as *Lysinibacillus macroides*, *Myoides odoratimimus* and *Chryseobacterium cucumeris* have little or no information as regards to fish pond water. The *Lysinibacillus macroides* and *Vibrio parahaemolyticus* were found to be carriers of the CTX-M gene, while *Escherichia coli* carried the QNRB gene. SHV gene was also found in the *Vibrio parahaemolyticus.* One of the most noted consequences of antibiotic misuse and pollution is the increase in frequency of bacteria harbouring antibiotic resistance gene in different environment (Kummerer, 2009). It is worth nothing that these bacteria with resistance gene can transfer these genes to other susceptible bacteria in the environment via horizontal gene transfer (Chopra and Roberts, 2001). The antibiotic resistance gene found could be attributed to the indiscriminate use of antibiotics in the fish farm.

V. CONCLUSION

The results showed that antibiotic resistance genes were present in some of the bacterial isolates which can spread and pose serious antibiotic resistance pollution to human and animal health.

REFERENCES

[1] Abraham, T.J., Manley, R., Palaniappan, R. and Dhevendran, K. (1997). Pathogenic and antibiotic sensitivity of luminous Vibrio harveyi isolated from diseased penaeid shrimp. *J. Aquaculture Trop.12,* 1-8.

[2] Abu, G.O. and Egenonu, C. (2008). The current pollution status of the new Calabar River in the Niger Delta region of Southern Nigeria: A survey of antibiogram profiles of its bacterial isolates. *African Journal of Environmental Science and Technology, 2 (6),* 134-141.

[3] Abu, G.O. and Wondikom, A.C. (2018). Isolation, Characterization and antibiotic resistance profile studies of bacteria from an excavated pond in Port Harcourt Metropolis, Nigeria. *Journal of Applied Science and Environmental Management, 22 (8),* 1177–1184.

[4] Adedeji, O., Tiamiyu, A,M. and Emikpe, B.O. (2011). Isolation and identification of aerobic bacterial flora of the skin and stomach of wild and cultured *Clarias gariepinus* and Oreochromis niloticus from Ibadan, Southwest Nigeria. *Journal of Applied Sciences Research, 7(7),* 1047-1051.

[5] Ajayi, O.A. and Okoh, I.A. (2014). Bacteriological study of pond water for aquaculture purpose. *Journal of Food, Agriculture and Environment, 12(2),* 1260-1265.

[6] APHA (American Public Health Association) (2001). Revision to Standard Methods for the Examination of Water and Wastewater. 20th Edition. American Water Works Association, Washington DC.

[7] Bano, K. (2006). Nutrient load and pollution study of some selected station along Ogunpa river in Ibadan, Nigeria. MSc Dissertation, University of Ibadan, Nigeria.

[8] Bostock J., Mcandrew, B., Richards, R., Jauncey, K., Telfer, T., Lorenzen, K., Little, D., Ross, L., Handisyde, N., Gatward, I., Corner, R. (2010). Aquaculture: global status and trends. *Philos Trans R Soc Lond B Biol Sci.* 365, 2897.

[9] Chopra, I. and Roberts, M. (2001). Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews, 65(2),* 232-260.

[10] Douglas, S.I. and Isor, F.N. (2015). Bacteriological investigation of pond water quality from Ogoniland, Nigeria. *Journal of Environmental Science, Toxicology and Food Technology, 9(2),* 36-41.

[11] Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, *39*, 783-791.

[12] Gobo A. E., Ubong I. U. and Ede P. N. (2008). Relationship between rainfall trends and flooding in the Niger-Benue river basins. *Journal of Metrology,* 13:13.

[13] Guo M., Yuan, Q. and Yang, J. (2013). Microbial selectivity of UV treatment on antibiotic-resistant heterotrophic bacteria in secondary effluents of a municipal wastewater treatment plant. *Journal of Water Research, 47,* 6388-6394.

[14] Hussain T., Roohi, A., Munir, S., Ahmed, I., Khan, J. and Edel-Hermann, V. (2013). Biochemical characteristics and identification of bacterial strains from drinking water sources of Kohat, Pakistan. *African Journal of Microbiology Research,*7(6): 1579-1590.

[15] Jukes, T.H. and Cantor, C.R. (1969).Evolution of protein molecules. In Munro HN, editor, Mammalian Protein Metabolism, pp. 21-132, Academic Press, New York.

[16] Kemper, N. (2008). Veterinary antibiotics in the aquatic and terrestrial environment. *Ecol Indic. 8,* 1.

[17] Kummerer, K. (2009). Antibiotics in the aquatic environment – a review – part II. *Chemosphere,* 75, 435.

[18] Naim, U. and Ahmed, H.A. (2012). Bacterial flora of polycultured common carp (Cyprinus carpio) and African catfish (Clarias gariepinus). *International Aquatic Research, 4*:10 DOI10.1186/2008-6970-4-10.

[19] Nrior, R.R. and Odokuma, L.O. (2015). Comparative toxicity of drilling fluids to marine water shrimp (Mysidopsis bahia) and brackish water shrimp (Paleamonetes africanus). *Journal of Environmental Science Toxicology and Food Technology, 9,* 73-79.

[20] Oliveira, M. A. S., Malinger, G., Ximenes, R., Szejnfeld, P. O., Alves, S. S. and Bispo, F. A. M. (2016). Zika virus intrauterine infection causes fetal brain abnormality and microcephaly: tip of the iceberg? *Ultrasound Obstetrics and Gynecology,* 47, 6-7.

[21] Oliveira, R.V., Oliveira, M.C. and Pelli A. (2017). Disease infection by Enterobacteriaceae family in fishes: A Review. *Experimentation,* 4:5.

[22] Pillay, T.V.R. (1990). Fish and public health and disease. *In: Aquaculture principle and practices*. Pillay, T.V.R (Editors). Fishing new book. Farrihan, New York, 174-215.

[23] Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution,4*, 406-425.

[24] Sanjeev, S. (2002). Pathogenic halophilic vibrios in sea foods. *In*: *Quality assurance in seafood processing* (T.S. Gopalakrishna lyer, M.K. Kandoran,Mary Thomas and P.T. Mathew, eds.). CIFT and Society of Fisheries Technologists (India), Cochin, 252.

[25] Sarjito, A.H.C., Haditomo, R.W., Ariyati, A., Sabdaningsih, D. and Prayitno, S.B. (2019). Screening of potential isolate candidates probiotic against Aeromonas hydrophila from Boyolali, Indonesia. Journal of Physics, 1217, 012147.

[26] Shekha, Y.A., Hero, M., Ismael, Z. and Ahmed,A.A. (2013). Biotechnological and mycological assessment for water quality of Duhok reservoir, Iraq. *Jordan Journal of Biological Sciences, 6(4):* 308-315.

[27] Torimiro, N., Bebe, P.T.,Ogundipe, F.E., Esan, D.M. and Aduwo, A.I. (2014). The bacteriology and physicochemical analysis of fresh water fish ponds. *International Journal of Microbiology, 5(3),* 28-32.

[28] Udeze, A.O., Talatu, M., Ezediokpu, M.N., Nwanze,J.C., Onoh, C. and Okonko, I.O. (2012). The effect of *Klebsiella pneumonia* on catfish (*Clarias gariepinus*). *Researcher, 4(4),* 51-59.

[29] Uzoigwe, C.I. and Agwa,O.K. (2012). Microbiological quality of water collected from boreholes sited near refuse dumpsites in Port Harcourt, Nigeria. *African Journal of Biotechnology, 11(13):* 3135-3139.