Isolation and Molecular Characterization of Important Fish Pathogens from River Niger and Two Other Major Rivers in Anambra State, Nigeria

***[1]Oko, A. O., [1&2]Agwu, S. C., [3]Sethi, S. K., [1]Ali, F. U., [1]Ebenyi, L. N. and [1]Ominyi, M. C.**
1. Department of Biotechnology, Ebonyi State University, Abakaliki
2. Nigerian Institute for Oceanography and Marine Research, Lagos
3. School of Biotechnology Gangadhar Meher University Amruta Vihar, Odisha, India
* Correspondence: okpanioko@gmail.com

Abstract: This study was designed to isolate and characterize pathogenic bacteria from fish samples from Niger, Otuocha and Amansea rivers using polymerase chain reaction (PCR) and sequencing approaches. Thirty-two fishes (16 tilapia and 16 catfish) were sampled from the three rivers. The intestines (one gram each) of all the fish samples were screened for the presence of pathogenic microbes of green colonies using *Aeromonas* agar base (Sigma) enriched with Ampicillin for selectivity. The extraction and sequencing of 16s rRNA of the pure isolates was done in GeneWiz laboratory USA. Polymerase chain reaction was used to assay for haemolysin gene and microscope for their morphological characteristics respectively. Basic local ailment tool (BLAST) compared the 16s rRNA sequences with the ones in the National Centre for Biotechnology Information (NCBI) Database. Results showed that 99% of the catfish samples grew with green colouration and were resistant to ampicillin, while none of the tilapia samples showed any form of growth on the selective media. BLAST analysis of the 16s rRNA sequences showed pathogenic *Enterobacteriaceae spp*. (*Citrobacter freundi*, *Enterobacter cloacae* and *Serratia rubidaea*) and *Ochrobactrum anthropi* bacteria in the catfish samples which were 99% identical with 16s rRNA sequences. They were haemolysin negative and microscopic result showed the isolates to be rod shaped and motile. The study showed that 99% of the catfish samples contained more than one pathogenic microorganism. These findings serve for awareness creation to fish consumers, handlers and processor in order to guide against such infectious microbes.
Keywords: Fish Pathogens, Molecular Characterization, Sequencing, River Niger

INTRODUCTION

Fish is a crucial source of animal protein in many countries of the world including Nigeria and known as a nice protein and mineral sources globally (Oko, 2019; Oko, et al., 2019; Adebayo Tayo *et al.*, 2012). In the developing countries of the world, 50% farmed and captured fishes which are consumed as local delicacy (Adebayo-Tayo *et al*., 2012). Microorganisms easily catabolise the protein content of fish which leads to fish spoilage (Udeze *et al*., 2012).

Fishes are infected with pathogenic microbes through the contaminated water, feed, and through the handling, processing, transportation, etc. (Adebayo-Tayo *et al.,* 2012). It is established that fishes from both rivers and ocean water bodies contain different human pathogens (Adebayo-Tayo *et al.,* 2012).

Enterobacteriaceae spps and *Ochrobactrum anthropic* bacteria are widespread in the environment and many mesophilic species contaminate food in low numbers (Garcia-Armesto *et al*., 1993). The psychotropic taxa can multiply in refrigerated foods such as meat, fish and milk. *Hafnia alvei, Serratia spp.* and *Enterobacter spp*. have been reported to occur in such high numbers that they actually spoil the food (Lund *et al.,* 1992). Mesophilic taxa of *Enterobacteriaceae* such as *Salmonella spp*., *Shigella* spp. and certain *Escherichia coli* may cause severe diarrhoea, and so are the psychrotrophic *Yersinia enterocolitica.* However, food spoiling *Enterobacteriaceae*, capable of growth in refrigerated foods, may also be of clinical concern. This is partly because they can act as opportunistic pathogens in immunocompromised patients and partly because shiga-like toxins (verotoxins) and other enteropathogenic toxins as well as other virulence factors have been found in *Enterobacteriaceae* genera outside the traditionally pathogenic taxa (Albert *et al*., 1992).

The strains of Ochrobactrum anthropi are known to be rod-shaped, Gram-negative, non-pigmented, and motile (Louws *et al.,* 1996). They produce acid in ammonium salt medium when grown in an aerobic environment using carbohydrate (glucose, arabinose, fructose, ethanol, etc.) however, they don't use lactose. According to the report of Holmes *et al.,* (1988), *Ochrobactrum anthropi* can reduce nitrate and nitrite, and slime in 24–48 hours when grown on culture medium containing carbohydrate. They show positive result in catalase and oxidase biochemical test, grows at 37^{o}C and 18-22 ^{0}C temperature. Different culture media used for culturing *Ochrobactrum anthropi* are: β-hydroxy-butyrate, MacConkey and atrazine (2-chloro-4-ethylamino-6-isopropyl-amine-s-triazine) (Louws *et al*., 1996).

Strains of *O. anthropi* are of clinical importance according to Holmes *et al., (*1988) who reported an aged woman infected with *O. anthropic* which was the first human case of such infection, since then they have been recognised as a human pathogen. They have been frequently identified in different clinical samples (blood, urine, vagina, pus, ear, etc.) (Holmes *et al*., 1988) but haven't been often implicated in systemic illness. There is scanty information on illness they cause like the infection of the urinary track, wound osteochondritis, etc (Holmes *et al*., 1988). There are reports of outbreak *O. anthropi* nosocomial bacteremia in five organ transplant patients that were injected with infected rabbit antithymocyte globulin that was contaminated with *O. anthropi* during production (Holmes *et al*., 1988).

In the family of the *Enterobacteriaceae, is Citrobacter freundii* which is an important opportunistic infection associated with neonatal meningitis (fatality rate 25-50%) (Julie *et al.,*1999) and brain abscess with high death rate. The mortality and morbidity rate of *Citrobacter* meningitis is unacceptably high (Julie *et al.,* 1999). According to the report of Alfredo *et al*., (1987) who stated that *Citrobacter freundii* synthesizes heat-stable enterotoxin and repeatedly implicated in children's excreta that can either be potential pathogen or normal intestinal micro flora. Other reports of Alfredo *et al*., (1987) stated that they are enteric pathogens that can cause diarrhoea in children.

Enterobacter Cloacae Blood Infection outbreak has been reported by Fraser *et al*., (2007) in his researches in which 52% and 12.5% cases were recorded respectively. Generally, *Enterobacteriaceae* family had been found, in big percentage, in fish, minced meat and canned milk which contains toxin encoding genes especially in *Serratia liquefaciens* and *Citrobacter freundii*. They are also found in stored canned milk at 78 ^{0}C, 31% retailed fish and in 100% minced meat. In minced meat, Strains of *Serratia liquefaciens* and *Citrobacter freundii* were repeatedly isolated from minced meat and milk products. Yu *et al*. (2004) reported isolation of serratia specie- *Serratia marcescens* from fish sampled from Kong waters that is non-sporing, non-acid-fast and can produce tetrodotoxin -neurotoxin.

Different methods of detection fish pathogens like polymerase chain reaction (PCR) and sequencing approaches in screening of virulent gene(s) has been reported to be the most effective way for diagnosis and assay for their virulent gene(s) Sarker, *et al.* (2013). Contamination and infections caused by these pathogens in man also may be due to catching, transporting, handling, etc.

Work of Sakar *et al.* (2013) reported the detection of 232bp virulent gene of pathogenic *Aeromonas hydrophila* through PCR based technique and bioinformatics tool like BLAST (crustalw software) for the analysis 16s rRNA to accurately identify the presences of the pathogenic microbes in fish Sakar *et al.* (2013).

The aim of this study is accurately screen for the presence of fish pathogens using sequencing and polymerase chain reaction (PCR) are highly crucial for targeted treatment of infections caused by these dangerous microorganisms.

MATERIALS AND METHODS

Experimental Site

The six month study was carried out in Awka, Anambra State of Nigeria between January and June.

Sample Collection

The fish samples were purchased from fishermen at Rivers Niger, Amansea and Otuocha all in Anambra State. They were transported to the laboratory and sterilized with ethanol before use. A total of thirty-two (32) fish samples were used, eight tilapia and eight catfish from two locations along River Niger and eight (four tilapia and four cat fish) fish samples each from Amansea and Otuocha rivers. Samples used were taken from the intestine of the fish samples.

Screening for Pathogenic Microbes of Green Colonies

The thirty-two (32) fish samples (sixteen tilapia and sixteen catfish) that were sampled from the rivers were subjected to screening for pathogenic microbes of green colonies using Aeromonas agar base selective media from Zigma. Ampicillin antibiotics were added appropriately to the media to make it more selective according to the direction of the manufacturer.

Sample Processing and Stock Culture Preparation

The fish samples were dissected using a surgical blade. Subsequently, samples (part of the intestines) were taken from the intestinal tracts of both the tilapia and catfish samples respectively. About one gram (1g) each was weighed and pulverized in sterile mortar and pestle and transferred into 8 test tubes. About 4ml of alkaline peptone water was added into each sample in a test tube and incubated at 37^{o}C for 24 hours to get a stock solution.

Serial dilution, plating and pure culture preparation

Serial Dilution Preparation

Exactly 10^3 Serial dilution of the stock solution was prepared for each sample and the maximum dilution was used in the plating on the *Aeronomas* Agar base media according to the laboratory standards. Procedure: A 0.9ml of distilled water was transferred into 3 (autoclaved) test tubes and 0.1ml of stock culture was transferred to the first test tube; 0.1ml is transferred from the first test tube to the second test tube and down to the third test tube.

Plating and Evaluation of the Suspected Colonies

A 0.1ml aliquot was aseptically transferred from the third diluted test tube ($10^{\wedge 3}$) of each sample unto sterile petri dishes containing *Aeromonas* agar base using spread plate method. The plates were incubated at 37^{o}C for 24hrs and observed for green colonies. Pure culture preparation was made through sub-culturing the green colonies on nutrient agar using streak plate method and later on the *Aeromonas* agar-base selective media.

Extraction of RNA

Extraction of RNA was carried out according to the method of Sarkar *et al.* (2013).

Procedure: culture medium containing the isolates was centrifuged at 12,000 x 5min at 4 ^{O}C following modification of the protocol of Sambrook *et al.* (1889). The pellets were re-suspended in 0.2ml of TE-1 buffer and 50ul DNase added with 50ul proteinase k (15mg/ml) and incubate at 56 ^{O}C for 3-4 hours in a water bath. RNA was extracted by adding equal volume of saturated phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged.

To the supernatant, 200ul chloroform was added and supernatant collected after centrifugation. About 250ul of chilled absolute alcohol was added and RNA was collected after wash with 70% alcohol and dried. Pure RNA was dissolved in TE buffer, converted to cDNA using reversed transcriptase and stored at 4 ^{O}C for PCR.

Polymerase Chain Reaction and 16s rRNA Sequencing

Polymerase chain reaction and 16s rRNA sequencing were carried out in Gene Wiz Laboratory in USA using the method of Sarkar *et al.* (2013). Procedure: Polymerase chain reaction was used to detect 232bp gene in Isolates. Primers specific for heamolysin gene (232bp product) was used as target genes for PCR amplification. Primers used were AHCR1:5aac tga cat cgg act c-3 3'and AHCR2:5'acc tga cat cgg cct tga act c-3', A 25ul PCR mixture contained 2.5mm $MgCl_2$, 2.5ul reaction buffer, 10 nmole dNTPs, 10 nmole each primer, 2 units of taq polymerase (Bomega) and 20ng template cDNA. PCR was carried out on PCR system using the cycle as initial denaturation at 95 ^{O}C for 5mins followed by 30 cycles at 95 ^{O}C for 2mins, 55 ^{O}C for 1min and 72 ^{O}C for 11min. 7mins final e1xtension at 72 ^{O}C. Amplicons was examined and visualized by electrophoresis in 1.5% agarose gel in TE buffer. The gel was stained with EtBr (sigma) and viewed.

RESULTS

Media isolation

All the Isolates of catfish from Rivers Niger, Amansea and Otuocha produced green colonies and were resistant to ampicillin (Plate1).

Microscopic analysis:

Microscopic analysis result showed that all the isolates were rod shaped and motile.

Blast analysis of the 16s rRNA gene sequences

Table 1 shows the identified pathogenic microorganism *Citrobacter freundii* strain B38 after the 16s rRNA Gene sequences were subjected to microbial blast in NCBI. Table 2, table 3, table 4 and table 5 also show the identified microorganism *Enterobacter cloacae* strain ECNIH4, *Ochrobactrum anthropi* strain OAB chromosome 1, *Enterobacter cloacae* mbrl1077 and *Serratia rubidaea* strain 1122 respectively.

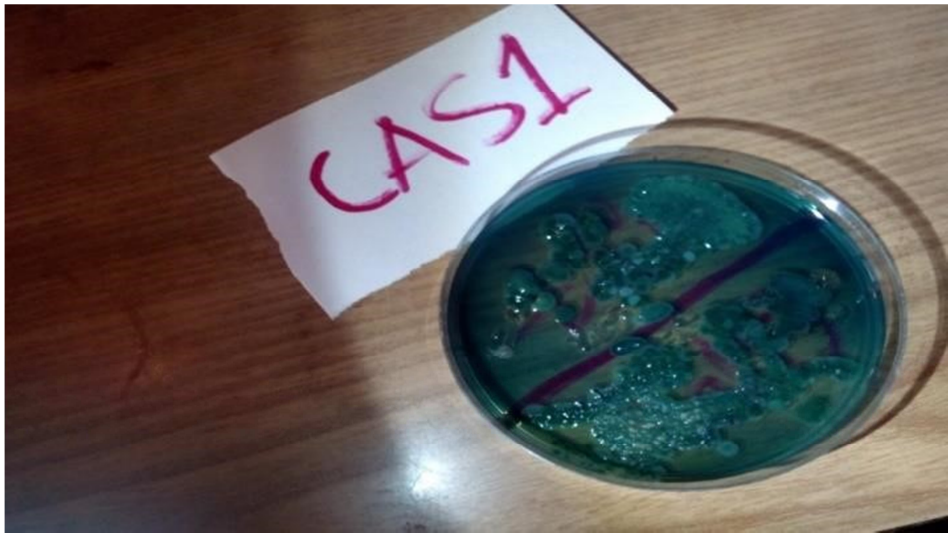

Plate 1: Pure culture Image of the isolates after 24 hours observed in this study.

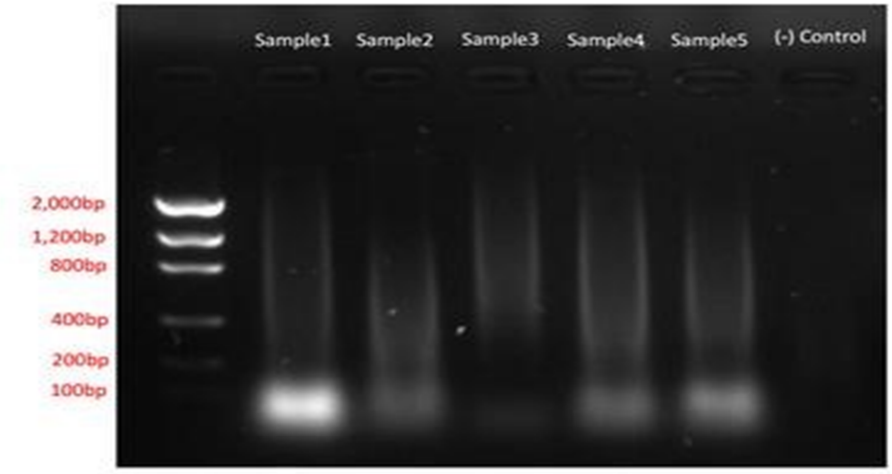

Figure 1: Gel Picture as observed in this study

Table 1: Blast analysis of 16s rRNA gene sequences for sample 1, pure isolate from Niger River. Identified organism: *Citrobacter freundii* strain B38 that was ninety-nine percent identical and resistant to antibiotic.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Citrobacter freundii strain B38, complete genome	1864	14826	94%	0	99%	NZ_CP016762.1
Enterobacter kobei strain DSM 13645, complete genome	1825	14527	92%	0	99%	NZ_CP017181.1
Enterobacter cloacae complex 'Hoffmann cluster IV' strain DSM 16690, complete genome	1825	14438	92%	0	99%	NZ_CP017184.1
Enterobacter ludwigii strain EN-119, complete genome	1825	12646	92%	0	99%	NZ_CP017279.1
Enterobacter cloacae complex sp. 35734 chromosome 1, complete sequence	1825	14410	92%	0	99%	NZ_CP012162.1

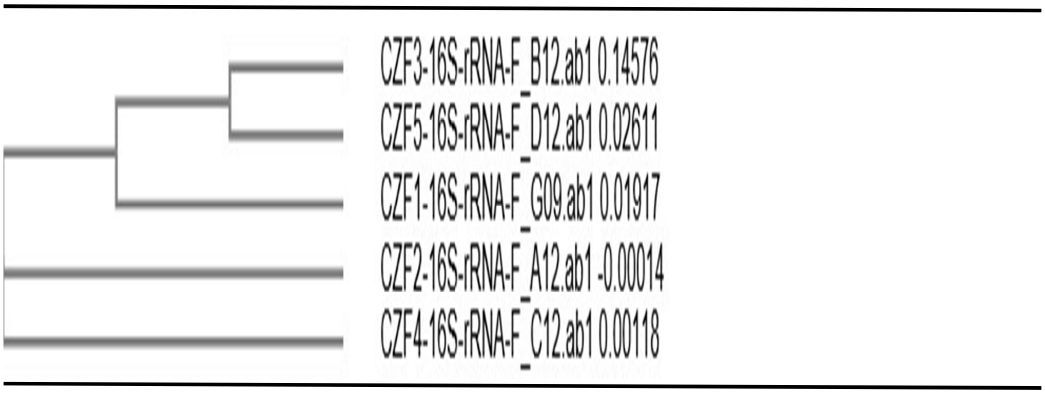

Figure 2: The phylogenetic tree of the 16s rRNA sequences

Table 2: Blast analysis of 16s rDNA gene sequences for sample 2, pure isolate from Otuocha river. Identified organism: *Enterobacter cloacae* strain ECNIH4 that was ninety-nine percent identical and resistant to antibiotic.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Enterobacter cloacae strain ECNIH4, complete ge	1810	14792	75%	0	99%	NZ_CP009850.1
Enterobacter asburiae L1, complete genome	1810	14464	61%	0	99%	NZ_CP007546.1
Enterobacter cloacae subsp. cloacae ENHKU01, complete genome	1810	14809	75%	0	99%	NC_018405.1
Enterobacter cloacae complex 'Hoffmann cluster IV' strain DSM 16690, complete genome	1810	14442	61%	0	99%	NZ_CP017184.1
Enterobacter sp. HK169, complete genome	1810	14414	61%	0	99%	NZ_CP017087.1

Table 3: Blast analysis of 16s rDNA gene sequences for sample 3, pure isolate from Niger River. Identified organism: *Ochrobactrum anthropi* strain OAB that was ninety-nine percent identical and resistant to antibiotic.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Ochrobactrum anthropi strain OAB chromosome 1, complete sequence	1799	3599	97%	0	99%	NZ_CP008820.1
Ochrobactrum anthropi strain OAB chromosome 2, complete sequence	1799	3599	97%	0	99%	NZ_CP008819.1
Ochrobactrum anthropi ATCC 49188 chromosome 2, complete sequence	1799	3599	97%	0	99%	NC_009668.1
Ochrobactrum anthropi ATCC 49188 chromosome 1, complete sequence	1799	3599	97%	0	99%	NC_009667.1
Ochrobactrum pseudogrignonense strain K8 chromosome 2, complete sequence	1727	1727	97%	0	98%	NZ_CP015776.1

Table 4: Blast analysis of 16s rDNA gene sequences for sample 4 pure isolate from Amansea River. Identified organism: *Enterobacter cloacae* strain mbrl1077 that was ninety-nine percent identical and resistant to antibiotic.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Enterobacter cloacae isolate mbrl1077	1779	14200	97%	0	99%	NZ_CP009850.1
Enterobacter cloacae strain UW5, complete genome	1779	14152	97%	0	99%	NZ_CP011798.1
Enterobacter sp. ODB01, complete genome	1779	14202	97%	0	99%	NZ_CP015227.1
Enterobacter cloacae subsp. cloacae ENHKU01, complete genome	1779	14213	97%	0	99%	NC_018405.1
Enterobacter cloacae strain AA4, complete genome	1779	14174	97%	0	99%	NZ_CP018785.1

Table 5: Blast analysis of 16s rDNA gene sequences for sample 5 pure isolate from Niger River. Identified organism: *Serratia rubidaea* strain 1122 that was ninety-nine percent identical and resistant to antibiotic

Description	Max score	Total score	Query cover	E value	Ident	Accession
Serratia rubidaea strain 1122, complete genome	1578	10853	97%	0	99%	NZ_CP014474.1
Yersinia pseudotuberculosis strain 1, complete genome	1439	10044	97%	0	97%	NZ_CP009786.1
Yersinia pestis strain PBM19, complete genome	1439	8599	97%	0	97%	NZ_CP009492.1
Yersinia pestis strain Harbin35, complete genome	1439	10022	97%	0	97%	NZ_CP009704.1
Yersinia pestis Pestoides F, complete genome	1439	10044	97%	0	97%	NZ_CP009715.1

Polymerase chain reaction analysis

PCR analysis showed that none of *Enterobacteriacea spp* and *Ochrobacterium spp.* from Niger, Amansea and Otuocha rivers had 232 bp heamolysin sequence gene in 2% agarose gel. Therefore, they are haemolysin negative (Figure 1)

Phylogenetic analysis: Phylogenetic tree of 16s-rRNA genes of the *Enterobacteriaceae* strains and *Ochrobactrum anthropi* strain were subjected to microbial phylogenetic using Clustalw2 to ascertain their evolutional relatedness. And czf3 and czf5 genes are closely related than czf1, czf2 and czf4 respectively. Hence: *Ochrobacterium anthropic* strain OAB and *Serratia rubidaea* are closely related than *Citrobacter freundii, Enterobacter cloacea* strain ECNIH4 and *Enterobacter cloacea* mbrllo77 respectively figure 2

DISCUSSION

In this study, the pure isolates were green, gram negative and resistant to ampicillin antibiotics and this result is in line with the report of Brenner *et al.* (2005), Gram, (1884) and Donnenberg *et al.* (2015) who reported the common characteristics of the *Enterobacteriaceae spp.*

Microscopic analysis showed that they are rod shaped and motile, the motility and shape may contribute to its wide distribution. BLAST analysis of 16s rRNA showed pathogenic *Enterobacteriaceae spp.* (*Citrobacter freundi*, *Enterobacter cloacae*, *Serratia rubidaea*) and *Ochrobactrum anthropi* bacteria in the catfish samples which are resistant to ampicillin antibiotics but did not harbour haemolysin gene, this observation is in line with the reports of Bekele (2019) who included these pathogens in the list of microbial pathogens isolated from tilapia in Uganda and Ethiopia respectively. However, Oko *et al,* (2018) reported *Aeromonas hydrophila* isolates from tilapia and catfish other than the pathogens implicated in this work. However, Agwu *et al,* (2021) observed an absence of *Enterobacteriaceae spp.* and *Ochrobactrum anthropi* in tilapia which is similar to the findings in this study. These observed differences in the distribution of the fish pathogens maybe as a result of changing climatic conditions in the regions and the immunity of various fishes.

Sakar *et al.* (2013) reported the detection of 232bp virulent gene of pathogenic *Aeromonas hydrophila* through PCR based

technique. This study contradicted this report as the pathogenic *Enterobacteriaceae spp*. (*Citrobacter freundi*, *Enterobacter cloacae*, *Serratia rubidaea*) and *Ochrobactrum anthropi* bacteria in the catfish samples from the rivers showed absence of virulent gene. The screening of 232 bp heamolysin virulent gene using PCR showed absence of the gene from all the samples drawn from the rivers. This observation may be as a result of the fact that pathogenic microbes can either have virulent heamolysin gene or aerolysin virulent gene which has not been clearly established.

CONCLUSION

Pathogenic *Enterobacteriaceae spp*. (*Citrobacter freundi*, *Enterobacter cloacae*, *Serratia rubidaea*) and *Ochrobactrum anthropi* bacteria are established by this research work to affect catfish from the rivers studied, with PCR analysis showing that the microbes are not haemolysin positive but resistant to ampicillin antibiotics . Nevertheless, they may contain gene(s) that is in-charge of their pathogenicity. The catfish in these rivers harbour more than one pathogenic microorganism that are resistant to antibiotics.

Recommendation

Further research work needs to be carried out to establish if *Enterobacteriaceae* species and *Ochrobactrum anthropi* bacteria fresh water fishes in Anambra State contain any other virulent gene(s) other than heamolysin gene.

REFERENCES

Adebayo-Tayo, A.C., Odu, N.N., Michael, M.U. and Okonko, I.O. (2012). Multi-Drug Resistant (MDR) Organisms isolated from Sea-foods in Uyo, South-South Nigeria. *Journal of Nature and Science* **10**(3): 61-70.

Agwu, S. C.,Oko, A. O., Afiukwa, C. A., Ogbanshi, M.E.,Ebenyi, L.N., Nwezza, S.N and Akinrotimi, O. A. (2021). Biochemical Characterization of Fish Pathogens from River Niger and Other Major Rivers in Anambra State. Poceedings of the 37th Annual Conference of the Fisheries Society of Nigeria (FISON).

Albert, M.J., Barque, S.M., Ansaruzzaman, M., Islam, M.M., Haider, K., Alam, K., Kabir, I and Robins-Browne, R. (1992). Sharing of virulence associated properties at the phenotypic and Genetic levels between enteropathogenic *Escherichia coli* and *Hafnia alvei*. *Journal of Medical Microbiology,* **37**: 310–314.

Bekele, B., Workagegn, K. B and Natarajan, P (2019). Prevalence and Antimicrobial Susceptibility of Pathogenic Bacteria in Nile Tilapia, Oreochromis niloticus. International Journal of Aquaculture Fish Science 5(4): 022-026. DOI: 10.17352/2455-8400.000047

Brenner, D. J., McWorter, A. C., Leete Knutson, J. K and Steigerwalt, A. G. (2005). *Escherichia vulneris*: a new species of Enterobacteriaceae associated with human wounds. *Journal of Clinical Microbiology,* 15: 1133–1140.

Chelikani, P., Fita, I and Loewen, P.C. (2004). "Diversity of structures and properties among catalases". *Journal of Cellular and Molecular Life Sciences,* **61** (2): 192–208.

Donnenberg, M (2015). "*Enterobacteriaceae*". Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 8th Edition pp. 2503–2517.

Fraser, S.L. (2007). Enterobacter Infections. eMedicine.

http://www.emedicine.com/med/topic678.htm, 2017.

Garcia-Armesto, M.-R., Prieto, M., Alonso, C., Garcia-Lopez,M.-L., Garcia Fernandez, M.-C., Otero, A.(1993). Numerical taxonomy of psychrotrophic bacteria isolated from raw ewes' milk. *Journal of Dairy Resourses,* **60**:371-383.

Gram, H. C (1884). Oberdie Isolierte Farbungder Schizomyceten in Schnittund Trockenpraparatten". Fortschritte der Medizin Germine. *International Journal of System Bacteriology,* **2**:185-189.

Hirono, I and Aoki, T (1991). Nucleotide sequence and Expression of ab Extra cellular

Hemolysin Gene of Aeromonas hydrophila. *Journal of Microbial Pathogenesis,* **11** (5): 189-197.

Holmes, B., Popoff, M., Kiredjian, M and Kersters, K. (1988). Ochrobactrum anthropi. from human clinical specimens and previously known as group Vd. *International Journal of System Bacteriology*, 38: 406–416.

Louws, F. J., Schneider, M. and de Bruijn, F. J. (1996). Assessing genetic diversity of microbes using repetitive sequence-based PCR (rep-PCR). In Nucleic Acid Amplification Methods for the Analysis of Environmental Samples. *Journal of Molecular biology,* **23**: 63–94.

Lund, A.M., Mostert, J.F., Carelsen, M.A. and Lategan, B. (1992). Proteolytic and lipolytic psychotropic Enterobacteriaceae in pasteurised milk and dairy products. S. *African Journal Dairy Sciences,* **24**:7-10.

MacFaddin, J.F., (2000). Biochemical Tests for Identification of Medical Bacteria. 3rd ed. Philadelphia: Lippincott Williams and Wilkins pp. 363-7

Oko, A. O. (2019). Comparative Analyses of the Nutritional Composition of Fish, Fish Eggs and Fowl Eggs. *American-Eurasian Journal of Agriculture and Environmental Sciences,* 19 (4): 242-248

Oko., A. O., Agwu S. C., Eluu, S. C., Oko, E. E. and Ehihia, L.U (2018). Antibiotics Resistance and Plasmid Profile of *Aeromonas hydrophila* Isolates from Cultured Tilapia and Catfish. *Middle East Journal of Scientific Research* 26 (5): 536-542.

Oko, A.O, Eluu, SC, Ugwu, DO, Okoye, ED, Ngele, KK and Oluwole, A. O (2019). Amino Acid Analysis of three Imported Fish Species Consumed in Abakaliki, Nigeria. *Middle-East Journal of Scientific Research,* **27**(5): 371-378, 2019

Sarkar, A., Mousumi, S and Parnab, R (2013). Detection of 232bp Virulent AHCYTONE Gene of Pathogenic *Aeromonas spp*. Through a rapid Molecular Diagnostic Approach. *Journal of Biotechnology,* **3**:83-87.