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Genotypic Characterization of Antibiotic-Resistant Genes in Gram Negative Bacteria Isolated from Selected Fish Ponds Effluents Samples within Oyo State

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ABSTRACT

Extended-spectrum beta-lactamase (ESBL) and carbapenem-resistant bacteria are becoming a rising global public health risk, with food products serving as distribution channels and aquatic ecosystems as prospective storage. The focus of this research therefore is to isolate antibiotics resistant Enterobacteriaceae from selected fish pond effluents in Oyo State. A total of 129 effluents were collected from 42 fish ponds and were cultivated on MacConkey agar. Bacteria were isolated and antibiotic susceptibility of the isolates to Gentamicin, imipenem, meropenem, tetracycline, and cefepime was determined using the disc diffusion method. MDR bacteria were checked for *bla*CTX-M, *bla*SHV, *bla*TEM, *bla*KPC, *bla*OXA, and *bla*NDM resistance genes using polymerase chain reaction (PCR), and isolate with resistance genes were characterized using 16S rRNA sequencing. Forty-six point five (46.5) percent of the 270 Enterobacteriaceae isolates obtained from effluent samples were resistant to imipenem, meropenem (45.7%), and tetracycline (39.4%), cefepime (35.8%), and gentamycin (19.2%). *bla*SHV was the sole gene found in 13.33 % of the isolates examined by polymerase chain reaction. The detection of ESBL and carbapenemase-producing gram-negative bacteria from selected fish ponds in this study is confirmed and represents a major public health problem. As a result, regular surveillance of antibiotic-resistant bacteria in fish ponds is required to aid disease control and better understand their public health implications.

Keywords: Antibiotics resistance, Carbapenemese, ESBL, Fishpond effluents.

Introduction

A growing body of research has shown a connection between fish ponds and the emergence of antibiotic resistance. Fish farming has significantly increased globally, which is related to the heavy utilization of antibiotics to treat infectious diseases. [1-3] Other alternative reasons for the spread of antibiotics resistance include environmental runoff, other anthropogenic activities, contamination of the fish pond with antibiotic residues and bacteria that are resistant to antibiotics acquired from animals.[4-7] Fish do not often successfully digest antibiotics and transfer them in faeces, which is the main worry with antibiotic use in fish ponds.[8] This promotes the growth of antibiotic resistance in fish and surrounding environment bacteria.[1-2,9] When fish are harvested, bacteria and antibiotic residue build up and spread because fish farmers don't regularly change the water in their ponds. Antibiotic resistance genes can be exchanged between human and animal bacteria and fish bacteria, especially Enterobacteriaceae.[10-11] Of these, beta-lactam-resistant bacteria seem to be of particular interest because they are increasingly becoming resistant to almost all common antibiotics.[12-13] this Enterobacteriaceae include carbapenem-resistant (CRE) and extended-spectrum β -lactam (ESBL) strains. The wide range of β-lactamase enzymes found in ESBL can hydrolyze a variety of penicillin and cephalosporin antibiotics, but not carbapenems.[14] Nevertheless, carbapenemase enzymes present in carbapenem-resistant Enterobacteriaceae impart resistance to almost all β-lactam antibiotics, together with carbapenems.[15] The β-lactamase enzymes include 'TEM and SHV' families as well as 'CTX-M, OXA, and PER β-lactamases'.[16] The big five enzymes KPC, NDM, IPM, and OXA-48 are among the diverse group of β-lactamases that make up the carbapenemase enzymes.[17] Extended-spectrum β-lactamase (ESBL) and carbapenemase-producing Enterobacteriaceae have been found in clinical samples in numerous studies, but there is a dearth of information on their prevalence in nonclinical samples.[18,19] To determine their potential risk to human health, it is important to find and keep track of antibiotic-resistant genes in wastewater and other water sources. The current study examined the prevalence of antibiotic-resistant genes in non-clinical strains of gram-negative bacteria isolated from randomly selected earthen fish ponds in Oyo State and emphasized its health implications.

Materials and Methods

Sample collection

Effluent samples from 14 local government areas of Oyo state (Table 1) were collected between November 2019 to February 2020 as well as between August 2020 to October 2020 aseptically in sterile 1 L sterilized plastic bottles by specifically suspending the containers approximately 30 cm underneath the water surface.[20] In less than six hours, effluent samples were transported to Microbiology program, Bowen University, Iwo, Osun state Nigeria in ice for analysis.

Isolation of the bacteria

Aseptically diluted effluent samples were used to produce countable bacterial colonies on MacConkey agar plates. Dilutions 10^{-4} and 10^{-2} were plated for bacterial colony enumeration and isolation. Colonies of different morphologies could be seen on the plates, and they were streaked off onto fresh Nutrient agar plates for purification.

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Table 1: Sampling location

Location	Location Code	GPS
Akinyele	AK	7°33'32.499"N, 3°54'50.361"E
Ido	ID	7°27'39.327"N, 3°49'34.250"E
Egbeda	EG	7°26'39.788"N, 4°1'5.761"E
Ibadan south-east	IBSE	7 °22'26.244"N, 3 °50'47.109"E
Kajola	KJ	8 °01'24.292"N, 3 °21'14.076"E
Iseyin	IS	7 °58'27'491"N, 3 °35'15.638"E
Oyo east	OYE	7 °50'47.487"N, 3 °54'30.034"E
Oyo west	OYW	7 °49'25.935"N, 3 °56'26.556"E
Atiba	ATB	7 °51'17.853"N, 3 °56'11.579"E
Afijio	AF	7 °49'38.258N, 3 °57'33.719E
Ibarapa central	IBC	7 °34'53.913"N, 3 °26'46.737E"
Ibarapa east	IBE	7 °35'01.728"N, 3 °2647.552"E
Ogbomoso south	OS	8°04'23.52" N 4°14'11.69" E
Surulere	SU	8°1'27.39" N 4°26'18.86" E

Table 2: Primers for the detection of ESBL, carbapenem-resistant genes, and 16srRNA sequencing

Primer name	Sequence (5'-3')	Amplicon size	Ref
CTX-M-F	CGCTGTTGTTAGGAAGTGTG	569	24
CTX-M-R	GGCTGGGTGAAGTAAGTGAC		
TEM-F	TTTCGTGTCGCCCTTATTCC	403	24
TEM-R	ATCGTTGTCAGAAGTAAGTTGG		
SHV-F	CGCCTGTGTATTATCTCCCT	293	24
SHV-R	CGAGTAGTCCACCAGATCCT		
OXA-F	GCGTGGTTAAGGATGAACAC	438	25
OXA-R	CATCAAGTTCAACCCAACCG		
NDM-F	GGTTTGGCGATCTGGTTTTC	621	25
NDM-R	CGGAATGGCTCATCACGATC		
KPC-F	CGTCTAGTTCTGCTGTCTTG	798	25
KPC-R	CTTGTCATCCTTGTTAGGCG		
LhetF	AGAGTTTGATCCTGGCTCAG		
16srR	GCTGATCCGCGATTACTAGC		

Pure bacterial isolates were kept in a 20 percent glycerol broth that was frozen.[21]

Antibiotic sensitivity testing

Utilizing the Kirby-Bauer disc diffusion method, the test was run. The antibiotic sensitivity of the isolates was examined using gentamicin (CN: 30 µg), imipenem (IMP: 10 µg), Meropenem (MEM: 10 µg), tetracycline (TE: 30 µg), and cefepime (FEP: 30 µg). All of the tested antibiotics were provided by Oxoid in the UK. After incubation, the diameter of the inhibition zone was measured in millimeters, and the outcomes were contrasted with the CLSI specifications.[21]

DNA extraction

Plasmid DNA was extracted from a single colony of 60 multi-drug resistant (MDR) (i.e., resistant to three classes of antibiotics) using a plasmid DNA extraction kit (Presto™ Mini Plasmid Kit, Cat. No. PDH300) and chromosomal ISOLATION II Genomic DNA kit (Cat No: 52066) [18]

Molecular identification of resistance genes

PCR was used to predict the existence of the most reported antibiotics resistance genes coding for ESBL– *bla*CTX-M, *bla*TEM, *bla*SHV (Table 2),[22] as well as carbapenem resistance – *bla*KPC, *bla*OXA, and *bla*NDM (Table 2).[23] A PCR mixture was made as previously reported. [24] Electrophoresis was used to analyze the amplified PCR products on a 2 percent agarose gel. Sanger DNA sequencing was performed at GENEWIZ, Inc. South Plainfield, NJ to confirm blaSHV positive PCR results using forward and reverse primers. (Table 2).

A sequence for the blaSHV positive PCR products was submitted inside the GenBank database under the accession numbers provided in Table 3. The sequences of the acquired genes were compared to those in public databases using the NCBI BLAST service. ClustalW was used to align *bla*SHV gene sequences in Chromas (version 2.6.6.0). MEGA (version 11) was used to perform neighbour-joining phylogenetic analysis. The consensus tree was then estimated using 1000 bootstrap repetitions.[25]

Results and Discussion

Bacteria isolation and Antimicrobial Susceptibility Testing

A total of two hundred and seventy nonrepetitive bacteria belonging to eighteen genera namely*; Klebsiella* 12(4.44%), *Leminorella* 9(3.33 %), *Escherichia* 11 (4.07%), *Hafnia* 4(1.48%), *Serratia* 7(2.60%), *Yokenella* 6(2.22%), *Rahnella* 6(2.22%), *Kluyvera* 5(1.85%), *Citrobacter* 4(1.48%), *Ewingella* 15(5.56%), *Enterobacter* 35(12.96 %), *Proteus* 16(5.29%), *Pragia* 19(7.04%), *Providencia* 42(15.5%), *Edwardsiella* 14(5.19%), *Yersinia* 14(5.19%), *Shigella* 22(8.14%) and *Salmonella* 26(5.63%) were isolated from the ponds and their frequency of occurrence is presented in Figure 1. Igbinosa *et al*,[21] Ejikeugwu *et al*,[22] Tapela *et al*,[23] Falodun & Ikusika, [24] and Onuoha, [25] have all reported the occurrence of gram-negative bacteria from pond effluents from all around the globe. The presence of these microbes is a reason for worry because it is an opportunistic human disease that can attack patients with compromised immunity.[25-27]

Antibiotic susceptibility tests on isolated gram-negative bacterial strains revealed various degrees of resistance. Resistance to imipenem (46.5 %), meropenem (45.7%), tetracycline (39.4%), cefepime (35.8 %), and gentamycin (19.2%) was found among the therapeutically relevant medicines administered (Figure 2).

The isolates resistance assessments demonstrated 35.8 percent resistance to the 3rd generation cephalosporin (cefepime). This study resistance pattern to cefepime is greater than the previous observations by Ejikeugwu *et al*. [22] and Benie *et al*. [28] with 16.5 percent and 17 resistant rates, respectively, but lesser than the one of Tapela *et al*. [23] and Elhariri [29] with a 100 percent resistance rate. The increased resistance to cephalosporins, which are frontline antimicrobial drugs for the treatment of infections, must be a significant concern; high resistance towards this group of antibiotics would not be extremely helpful and therefore will lead to limited effective treatments.

This study discovered that 19.2 percent of the isolates were gentamicin resistant. A study conducted in Egypt found a higher amount of gentamicin resistance (28.5%). [29] Nevertheless, a study conducted in Nigeria,[22] South China, [30,] and India [31] found gentamicin resistance rates of 79 percent, 60 percent, and 72 percent, respectively. This difference may be found to differ in gentamicin prescription processes. [32] Tetracycline resistance was detected in 39.4% of the total isolates in this research, which is significantly lower than the findings of Manna *et al*,[33] who found 31% tetracycline resistance in gram-negative species isolated from a fish pond in India and almost similar to the findings of Bali *et al*.[34] who discovered 40% antibiotic resistance in *Shigella* strain obtained from Malaysian fish pond water samples throughout their examination. The resistance variation to tetracycline shown in this study could be attributed to the antibiotics' high stability in the environment, which causes them to stay there for long periods, exposing the isolates to the antibiotics and thus enhancing antibiotic resistance selection by the isolates.[35]

Resistance to carbapenems, such as imipenem (46.5%) and meropenem (45.7%), was also observed in the current study. This study's findings differed from those of Pollett *et al*,[36] Mohamed *et al*, [37] and Okoche *et al*, [38] who discovered a reduced percentage of sensitivity to meropenem and imipenem in their respective studies. The level of resistance to imipenem and meropenem detected in this study is astounding, considering that carbapenems are among the most

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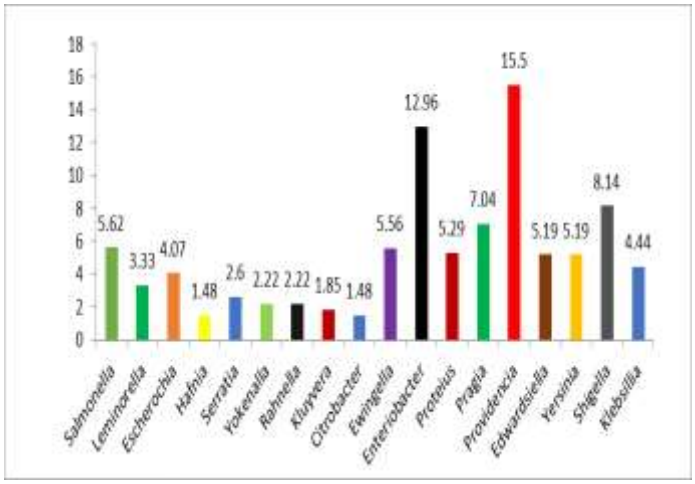

Figure 1: Percentage frequency of occurrence of Bacteria isolated

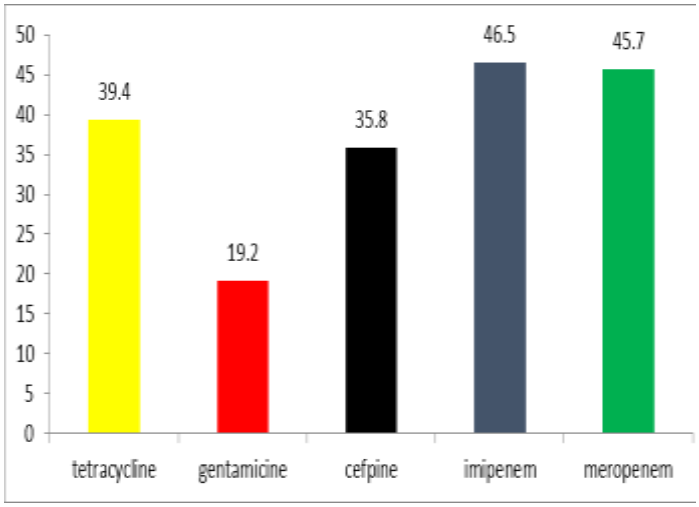

Figure 2: percentage of resistance to antibiotics

widely used and effective options for tackling gram-negative diseases, especially MDR illnesses. Carbapenem-resistant gram-negative bacteria are regularly associated with an increased rate of death owing to the enzyme carbapenemase facilitating resistance and the increased chance of resistance disseminating widely via mobile genetic elements. [39] Antibiotic-resistant bacteria and antibiotic-resistant genes, which can be transmitted to human pathogenic bacteria, are frequently spread as a result of the misuse of antibiotics in human and veterinary medicine. As a result of this transfer, some potentially fatal diseases eventually recur and new antibiotics become ineffective.[40]

Molecular Identification of resistant Genes and Evolutionary relationships Analysis

PCR assessment of ESBL and carbapenem-encoding genes revealed *bla*SHV amplification in several bacterial isolates. Figure 3 depicts the results of molecular identification of ESBL and carbapenem genotypes in the gram-negative bacteria using PCR. Eight (13.3 %) of the 60 multidrug gram-negative isolates tested positive for the *bla*SHV gene, whereas no isolates tested positive for *bla*CTX-M, *bla*TEM, *bla*KPC, *bla*OXA, or *bla*NDM. Following that, a phylogenetic tree with comparable sequences was constructed using the neighbor-joining technique for every isolated bacterial strain (Fig 4). The phylogenetic tree showed that EG-2A is 94% related to *Pedobacter mendelii* strain B34CS2014 (KP762221) isolated from the USA, IBC 1C 98% is similar to *Elizabethkingia meningoseptica strain BF 227* (MK491163) isolated from India, ATB 2a is 98% related to *Acinetobacter sp. strain NIOA328I* (MW487393) isolated from India, AF 3D has 99% closeness to *Escherichia coli strain FUA 1049* (GQ222388) isolated from Canada, OYE 2c and EG 3c was 79% similar to *Enterobacter sp. SGT-96* (KC455422) was isolated from China and IBC 2A and ID 2A were 100% related to *Stenotrophomonas sp. strain LSB20* (MK600536) isolated from China (Table 3).

The only resistant gene discovered by PCR in the present research was *bla*SHV. It was found in 8 different isolates (13.3 percent). This report agrees with the findings of several other researchers [41-44]. These statistics show that the ESBL encoding genes have been widely disseminated. There is cause for concern when multidrug-resistant bacteria, including ESBL producers, are released into aquatic environments. Because they carry mobile genetic material, these organisms can act as opportunistic pathogens whenever they persist in the environment, hastening the spread of antibiotic resistance.[45]

The phylogenetic tree was rooted by placing a root on the tree to demonstrate the phylogenetic route. The tree demonstrated the closeness of the isolates based on nucleotide sequence similarity.

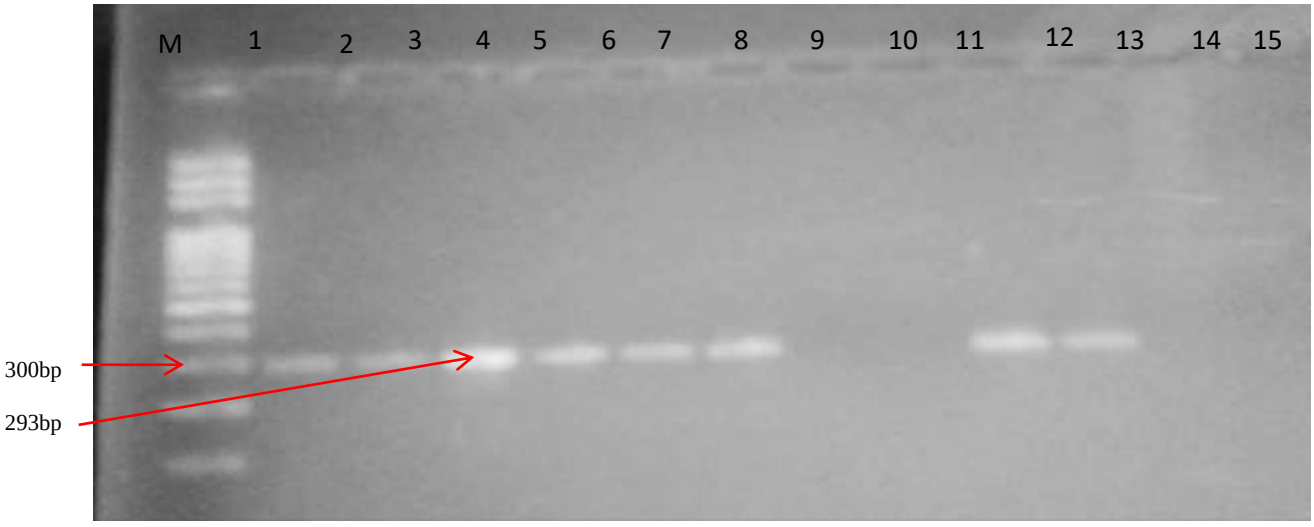

Figure 3: PCR Amplicons of bla_{SHV} gene amplified at 293bp in bacteria isolates. Lane: M=100bp DNA marker; 1: *Stenotrophomonas maltophilia*, 2: *Enterobacter sp, 3: Elizabethkingia meningoseptica, 4: Enterobacter cloacae, 5: Pedobacter mendelii, 6: Acinetobacter baumannii, 9: Stenotrophomonas maltophilia and 10: Escherichia coli*

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Table 3: The BLAST results of 16S rRNA sequences of 8 bacterial isolates

S/N	Isolate	Nucleotide acid size (bp)	Closeness of species	Geneback accession number	% of resemblance	E-Value	Query coverage %
1	IBC 2A	1552	*Stenotrophomonas maltophilia*	OM135509	99.72	0.0	100
2	OYE 2c	1613	*Enterobacter sp*	OM135508	98.77	0.0	97
3	IBC 1c	1507	*Elizabethkingia meningoseptica*	OM135510	98.97	0.0	100
4	ID 2A	1440	*Stenotrophomonas maltophilia*	OM135512	99.72	0.0	99
5	AF 3D	1521	*Escherichia coli*	OM135507	99.08	0.0	93
6	EG 2A	849	*Pedobacter mendelii*	OM372671	100	0.0	100
7	EG 3c	939	*Enterobacter cloacae*	OM372670	100	0.0	100
8	ATB 2A	1069	*Acinetobacter baumannii*	OM372669	100	0.0	100

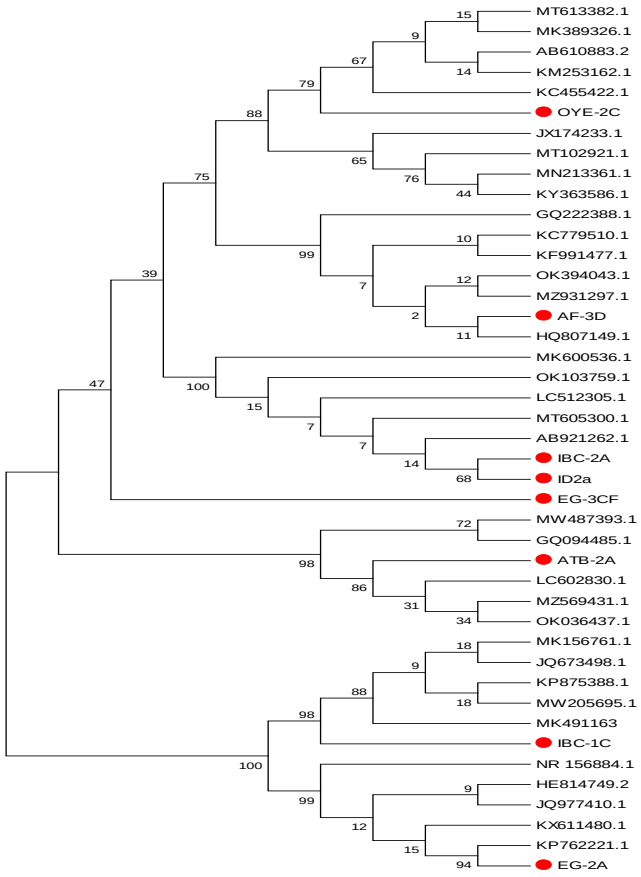

Figure 4: The phylogenetic relationship between bacteria isolates

Because it is based on the genetic variations of isolates, the molecular method of evaluating bacteria at the gene level is far more reliable than traditional approaches. This investigation's molecular data has proven to be quite relevant to evolutionary relationships. When the partial 16S rRNA gene sequences from the bacterial isolates were compared to those in the database, it was discovered that they belonged to three taxonomic lineages (Figure 4). Six of the Gama-proteobacteria divisions, one of the Sphingobacteria divisions, and one of the Elizabethkingia divisions are represented. They are all Eubacteria members, with gamma Proteobacteria (75%) dominating and *Sphingobacteriia* and *Elizabethkingia* accounting for a smaller proportion. The sequences of all of the isolates shared more than 95% similarity with other 16S rRNA sequences in the database. According to 16S rRNA analysis, all isolates shared similarities with the genera *Stenotrophomonas*, *Enterobacter*, *Elizabethkingia*, *Pedobacter*, *Acinetobacter*, *and Escherichia*.

Conclusion

This study revealed the existence of multidrug-resistant gram negative bacteria in the studied pond effluents and the presence of blaSHV resistant gene which can be easily transferred to other organisms in multiple niches. The outcomes of this study highlight the significance of drug resistance and resistance genes mostly in non-clinical settings, the necessity of collaborative monitoring at the national and sub-national levels, and the inclusion of the one health concept.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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