

## Multi-Drug Resistance Genes associated with some Gram-Negative Bacterial Isolated from Shellfish in Iko and Douglas River Estuaries, in Nigeria

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### ABSTRACT

**Background:** Multi drug resistant bacterial agents that contaminate seafood cause several diseases in humans and are widely documented as a global public health challenge.

**Methods:** This study evaluated the microbiological and antimicrobial resistance genes profiles of bacterial Isolates from shellfish vended at Iko and Douglas Creeks of Cross River State, Nigeria. A total of 540 shellfish (117 clams, 88 oysters, 136 periwinkles) samples were collected from various vendor at the two Creeks were analyzed. The samples were processed using standard microbiological methods to identify bacterial pathogens. Antimicrobial susceptibility was assayed using the Kirby-Bauer disk diffusion method. Isolates were screened for antimicrobial resistant genes using polymerase chain reaction.

**Results:** Overall, a total of 135 bacteria isolates were identified. The most common isolate was *Alcaligenes* species 53(39.2%) followed by *Pseudomonas* species 44(32.6%), *Providencia* species 25(18.5%), *Vibrio* species 6(4.4%), and *Paenalcaligenes* species 7(5.2%). The isolates showed varying susceptibilities to Imipenem (36%) and amikacin (28%) but were all resistant to Trimethoprim-Sulfamethoxazole. Fifty-three isolates had a multiple antibiotic resistance index (MARI) of  $\geq 0.9 - 1.0$ . Most of the bacterial isolates were detected with TEM genes (82.2%), SHV (51.8%), VIM (50.3%) resistance genes. None of the isolates expressed Veb gene. Only 40.7% of the isolates expressed QnrB gene while none expressed QnrA and QnrS.

**Conclusion:** The detection of these multidrug resistant clinically relevant bacterial species suggests a significant linkage of commonly consumed seafood in the community and environmental spread of MDR bacteria.

**Keywords:** Shellfish, aminoglycoside, MARI, phenotypic, Gram-negative bacteria, PCR

### INTRODUCTION

Multi-drug resistant bacteria among foodborne pathogens has become a matter of great public health concern [1]. It is estimated that unsafe food containing harmful bacteria, viruses, parasites or chemical substances, causes more than 200 diseases – ranging from diarrhoea to cancers. [2].

The world Health Organization, reported in 2017 that the global burden of food borne diseases each year was as many as 600 million,

or almost 1 in 10 people in the world, fall ill after consuming contaminated food. Of these, 420,000 people die, including 125,000 children under the age of 5 years [2].

Globally, various bacterial pathogens affect a wide range of aquatic species and are responsible for considerable economic losses [3]. Foodborne bacterial infections caused by resistant bacteria are difficult to treat because of it being unsusceptible to even last resort antibiotics [4]. It is estimated that by the year 2050 about 10 million people globally will die

from resistant infections [5]. World-wide, the rate of spread of antimicrobial resistance has become worrisome with alarming increase in the number and types of environmentally disseminated antimicrobial resistances (AMR) which has become a major risk to human health [5,6,7,8]. Notably, due to their distinctive structure, the Gram-negative bacteria (GNB), in non-negligible numbers, manifest concomitant resistance more than Gram-positive bacteria to all commonly used classes of antimicrobials leading to significant morbidity and mortality [9].

Antibiotics used therapeutically and prophylactically in animal production for different purposes are the main route for its dissemination in the environment [10]. The use of antibiotics in aquaculture is well-known to results in the selection of proliferated antibiotic-resistant bacteria possessing mobile genetic elements such as transposons, plasmids, integrons and gene cassettes which through horizontal gene transfer can be transferred to the whole environment [11]. Environmental dissemination of antibiotics particularly in aquatic environments potentiates antibiotic pressure and, promotes the dissemination of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) [4, 10, 12]. As has been proven over time, drug resistant genes transfers can spontaneously occur from non-human pathogenic bacteria to human pathogens co-existing in the same environment [13].

Antimicrobial resistance by multi-drug resistant Gram-negative bacteria (MDRGN) is mostly plasmid mediated and several different species are involved [14]. These resistance traits can be transferred from individual bacteria to other individual bacteria, and in rare cases even between species [15,16]. The mechanism of resistance especially in Gram negative bacilli arises from the expression of antibiotics activating enzymes and non-enzymatic pathways [17]. Often, these results from the intrinsic resistance due to mutations in chromosomal genes (such as increasing the expression of antibiotic-inactivating enzymes, efflux pumps, permeability or target modifications) or acquisition by transfer of mobile genetic elements carrying resistance genes such as plasmid encoding  $\beta$ -lactamases, aminoglycosides modifying enzymes, or non-enzymatic mechanisms like Qnr (plasmid-borne quinolone resistance gene) for fluoroquinolone (FQ) resistance in Enterobacteriaceae [18].

Though integral to the epidemiology of antimicrobial resistance, the aquatic ecosystem has not been adequately studied [19]. Secondary contamination of fresh seafood with enteric bacteria resistant to multiple antibiotics may implicate seafood as a potential carrier of antibiotic resistant bacteria and emphasizes an urgent need to prevent environmental contamination and dissemination of such bacteria [20]. Commonly consumed seafood as carriers of multidrug-resistant bacteria has been underscored as a growing human threat causing wider dissemination of MDR-bacteria in the community and potentially can result in the transfer of resistant determinants to other clinically important bacteria [1, 20, 21, ]. Seafood is relatively free of human pathogens, except for Vibrios which are natural contaminants of seafood from the marine environment [20]. In most part of Nigeria, these shellfish types - clams, oysters, periwinkles as a good source of dietary protein. Even so, not much is known regarding the health implications of human consumption of these shellfish vended at Iko and Douglas Creeks of Cross River State, Nigeria. In this sense, the need to assess and investigate the potential risk posed to human health by consuming these shellfish cannot be overemphasized. Much more efforts are required to fully explore the connectivity of the aquatic environment and hence the intrinsic linkages between humans, animals and the environment in the global AMR dissemination [22]. The “One Health” approach, a holistic and multisectoral approach, is also needed to address AMR’s rising threat [23]. Therefore, screening, monitoring and surveillance for antimicrobial resistance pattern of bacterial pathogens derived from seafood from aquatic sources is key in helping to prevent human health risks from seafood consumption. This study evaluated the microbiological and antimicrobial resistance genes profiles of bacterial Isolates from shellfish vended at Iko and Douglas Creeks of Cross River State, Nigeria.

## **METHODOLOGY**

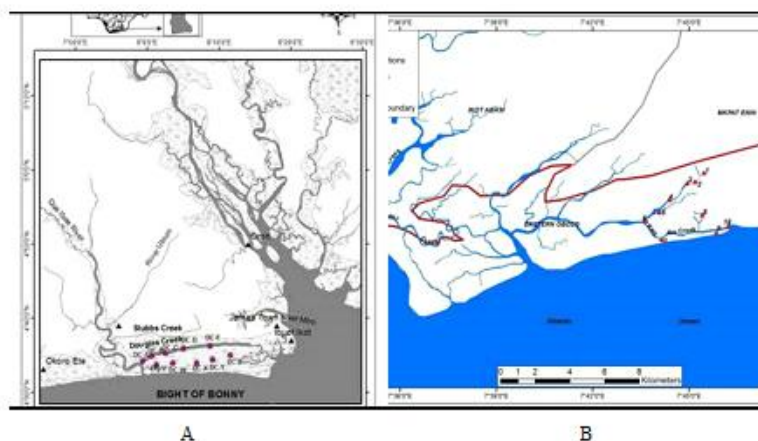
### **Study Area**

The study is an experimental analysis carried out within the catchment of Iko and Douglas Creeks, Akwa Ibom State, in the South-South region of Nigeria. It is located between Latitude 4°32' and 5°33' North and Longitudes 7°25' and 8°25' East. The State has a population of over 5 million people, with a total area of

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7,081km<sup>2</sup> (2,734 sq mi). The main occupational lines of the populace are farming, trading and fishing. It has two major seasons: wet and dry seasons. The State has the mean annual temperature of 80°F or 25°C and annual relative

humidity of 70 to 80%, with a mean annual rainfall of 3017 millilitres which favours agricultural practices. The State is drained by three major rivers; Qua Iboe River, Imo and Cross River respectively [24].



Map Showing Douglas (A) and Iko Creeks (B) Akwa Ibom State. Lfish

### Sample Collection/Processing

In total, 117 clams, 88 oysters, 136 periwinkles were harvested from Iko and Douglas Creeks of Akwa Ibom State, Nigeria, during the rainy (March-July) and dry seasons (August-February) of the years 2015-2017. These samples were collected and placed in sterile iced-packed coolers and transported to the post graduate research laboratory, University of Uyo for bacteriological examination and analysis within 5 hours of each collection time. Initial processing of the shellfish included individual washing and scrubbing with a sponge in sterile water. These were then rinsed in 70% ethanol to remove adhered but external specks of dirt and debris. The shellfish were then shucked aseptically with a sterile shucking knife to remove the soft flesh. Subsequently, the flesh samples were individually dissected into body parts (flesh, intestine and gills) using sterile knives and scissors and homogenized using sterile plastic mortar and pestle. The protocols as in International Standard Organization [25] and Farmer *et al.* [26] were adopted for the bacteriology.

### Bacterial isolation (Pre-enrichment, Enrichment and Plating).

Briefly, twenty-five 25 g of each shellfish homogenate were pre-enriched in 225ml of DEV lactose peptone broth (Merck Darmstadt, Germany) at 37°C in an incubator for 24 hours. An aliquot (0.1 ml) of the pre-enriched culture were transferred using a sterile pipette into 10 ml of Mueller-Kauffmann tetrathionate broth

(MKTB), 10 ml of Modified Semi-solid Rapaport-Vassilidis Agar (MSRV) and 1ml of same was copiously inoculated onto Thiosulphate Citrate Bile Salt Agar (TCBS). The inoculated tubes and plates were incubated at 37°C and 41°C for 24 hours respectively for further selective enrichment. The selectively enriched cultures were inoculated by spreading 0.1mL of the incubated selective enrichment broth onto Xylose Lysine Deoxycholate Agar (XLD), Brilliant Green agar (BGA) and nutrient agar plates respectively and the plates were incubated at 37°C for 24 hours. Typical bacterial isolates were purified by streak plating on nutrient agar and incubated at 37°C for 24 hours. The discrete colonies formed were maintained on nutrient agar slants after sub-culturing as pure cultures and preserved in the refrigerator at 4°C for further analysis.

### Characterization and identification of the isolates

Purified typical colonies were characterized for identification using cultural, morphological and standard biochemical tests for identification of medical bacteria namely: Gram reaction, motility tests and standard biochemical tests such as; oxidase, urease, indole, Voges-Proskauer, hydrogen sulphide production, catalase, citrate utilization and sugar fermentation tests [27]. Biochemical Tests for Identification of Medical Bacteria. 3rd edition. New York, USA, Lippincott Williams & Wilkins[28]. Bacterial isolates were further confirmed by molecular identification using the

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universal 16S rRNA bacterial primers for microbiological identification of common bacterial pathogens. out using the universal 16S rRNA bacterial primers [29].

### Antibiotic Susceptibility

Identified bacterial strains we were tested for susceptibility to the following antibiotics: cefepime (10µg), nalidixic acid (30µg), ciprofloxacin (5µg), amikacin (30µg), imipenem (30µg), norfloxacin (10µg), trimethoprim-sulfamethoxazole (35µg) and chloramphenicol (30µg), using the standard Kirby Bauer disk diffusion susceptibility testing method [30, 31]; *Escherichia coli* ATCC 25922 was used as a control. Detection of ESBL production was done using the following disks: cefotaxime (30µg), ceftriazone(30µg), cefpodoxime (10µg), ceftaxidime (30µg) and aztreonam (30µg). The zones of inhibition of each isolate were tested on Mueller-Hinton agar plates (Oxoid, Basingstoke, UK). Interpretive criteria established by the CLSI were used to categorize the results of antimicrobial susceptibility testing and ESBL production [32].

### Multiple Antibiotic Resistance Index (MARI)

The multiple antibiotic resistance was calculated as follows:

$$\text{MAR} = \frac{\text{Ratio of number of antibiotics an isolate is resistant to (a)}}{\text{Total number of antibiotics to which the organism is exposed to (b)}}$$

Take for an example; if A1 is resistant to 6 out of 8 antibiotics tested, the MAR index will be

$$\text{MAR} = a/b;$$

Where **a** is the aggregate antibiotic-resistant score of an isolate and **b** the total number of antibiotics tested.

### DNA Extraction/Sequencing

The bacterial genome was extracted using the method of Dashti *et al.* [33], with slight modifications as follows: the genomic extraction was carried out using the boiling method with a heating block at 95°C for 20 minutes. Plasmid DNA extraction was done using a Plasmid miniprep kit (Inqaba Biotechnology, Pretoria, South Africa). The sequencing of the amplicons was carried out according to the manufacturer's instructions on a 3510 AB1 sequencer using the Big Dye Terminator Kit (Inqaba Biotechnology, Pretoria, South Africa).

### Amplification of the 16SrRNA genes

The amplification of the 16S rRNA region of the isolates' rRNA genes of the isolates was carried out using the universal 16S rRNA

bacterial primers 27F (5'-AGAGTTTG ATCC TGGCTCAG -3') and 1392R (5'-GGTTA CCT TGTTACGAC TT-3') on an AB1 9700 Applied Biosystems thermal cycler with a final volume of 50 µl for 35 cycles. The PCR mix used for the amplification was: X2 Dream Taq Master Mix supplied by Inqaba, South Africa (Taq polymerase, dNTPs, MgCl), a 0.4M concentration of the primers and the extracted DNA being the template. The conditions required for the PCR amplification included: Initial denaturation at 95°C for 5 minutes; denaturation at 95°C for 30 seconds; annealing at 52°C for 30 seconds, extension at 72°C for 30 seconds for 35 cycles and a final extension at 72°C for 5 minutes. The product of this amplification was transferred onto a 1.0% agarose gel at 120V for 20 minutes and a UV trans-illuminator was used for visualization of the bands.

### Amplification of *qnr* Genes

The *qnrA*, *qnrB* and *qnrS* genes each were amplified on an AB1 9700 Applied Biosystems thermal cycler at a final volume of 50µl for 35 cycles. The PCR Mix was the X2 Dream TaqMaster mix supplied by Inqaba, South Africa (Taq Polymerase, DNTPS, Mg Cl), 0.4M concentration of Primers were used, and lastly, the extracted DNA was used as a template. The cycling conditions for amplification were as follows: Initial denaturation for 5 minutes at 95°C; denaturation for 30 seconds at 95°C; annealing at 52°C for 30 seconds; extension at 72°C for 30 seconds for 35 cycles and a final extension at 72°C for 5 minutes. The resulting PCR products were analysed by electrophoresis with 1.0% agarose gels in Tris-borate-EDTA buffer (TBE;Gibco, NT, USA) at 120 V for 20 minutes. Each gel was stained with ethidium bromide and bands produced were photographed on an ultraviolet light transilluminator. A molecular weight standard (100 bp and 1000 bp ladder, Promega, Madison, USA) was included on each gel. The primer sequence used for the amplification of *qnrA*, *qnrB* and *qnrS* genes were:

qnr A/F: 51- TTCACGAAGATTTCTCA-31

qnr A/R: 51-GGCAGCACTATTACTCCCA-31

qnr B/F: 51-CCTGAGCGGCACTGAATTTAT-31

qnr B/R: 51-GTTTGCTGCTTGCCAGTCGA-31

qnr S/F: 51-CAATCATACATATCGGCACC-31

qnr S/R: 51-TCAGGATAAACAACAATACC C-31

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### ESBL genes amplification

The *TEM*, *SHV* and *VIM* genes were amplified using AB1 9700 Applied Biosystems thermal cycler at a final volume of 50 µl for 35 cycles. The PCR Mix was the X2 Dream Taq Master Mix supplied by Inqaba, South Africa (Taq Polymerase, dNTPs, MgCl<sub>2</sub>), 0.4M concentration of primers was used, and lastly, the extracted DNA was used as a template. The cycling conditions for amplification were as follows: initial denaturation for 5 minutes at 95°C; another 30seconds at 95°C; annealing at 52°C for 30 seconds; extension at 72°C for 30 seconds for 35 cycles and a final extension at 72°C for 5 minutes. The resulting PCR products were analysed by electrophoresis with 1.0% agarose gels in Trisborate-EDTA buffer (TBE; Gibco, NT, USA) at 120 V for 15 minutes. The gels were stained with ethidium bromide and bands produced were photographed on an ultraviolet light transilluminator. A molecular weight standard (100 bp and 1000 bp ladder, Promega, Madison, USA) was included on each gel. The primer sequence used for the amplification of *TEM*, *SHV* and *VIM* genes were:

*TEMF*: 5<sup>1</sup> TCCGCTCATGAGACAATAACC-3<sup>1</sup>

*TEMR*: 5<sup>1</sup>ATAATACCGCACCACATAGCAG-3<sup>1</sup>

*SHVF*: 5<sup>1</sup> –TACCATGAGCGATAACAGGG 3<sup>1</sup>

*SHVR*: 5<sup>1</sup> – GATTTGCTGATTTTCGCTCGG-3<sup>1</sup>

*VIM F*: 5<sup>1</sup> TTATGGAGCAGCCAAGCAGTG 3<sup>1</sup>

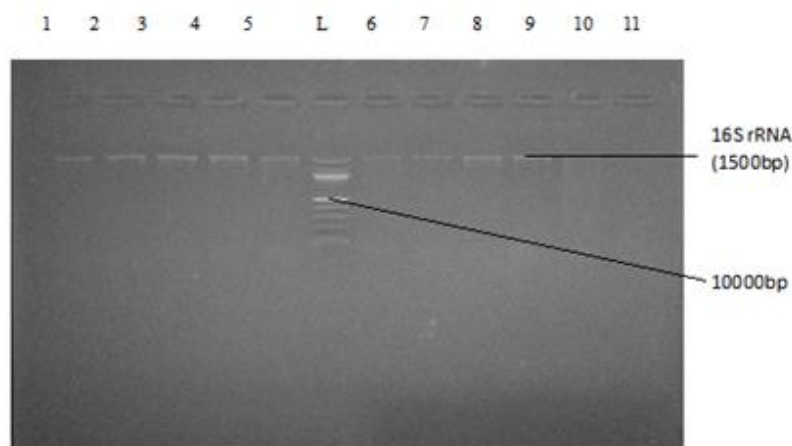
*VIMR*: 5<sup>1</sup> – CGAATGCGCAGCACCAGG

### DATA ANALYSIS

The Statistical Package for Social Sciences (SPSS 22.0 Inc., Chicago, US) was used to analyze the data generated from this study. P-values of less than 0.05 (P <0.05) were considered statistically significant.

### RESULTS

One hundred and thirty-five bacterial agents isolated from the shellfish samples were identified to the genus level using standard microbiological methods including biochemical tests. Furthermore, molecular confirmation of all isolates was done by sequencing the PCR amplification of species-specific 16S rRNA gene and the results compared with the data in the NCBI gene Table 1.



**Fig1.** Agarose gel electrophoresis showing the amplified 16S rRNA gene of the bacterial isolates. Lane L represents the 10,000bp molecular ladder

**Table1.** Identities of the bacterial isolates

Sample codes	Isolated bacteria	Strain	Accession No.
B1 B3 B7	<i>Alcaligenes faecalis</i>	TRB-7 16S ribosomal RNA gene, partial sequence	MH109290.1
B2	<i>Pseudomonas anguilliseptica</i>	D4029 16S ribosomal RNA gene, partial sequence	FJ161260.1
B4	<i>Pseudomonas aeruginosa</i>	CIFRI DTSBI 16S ribosomal RNA gene, partial sequence	JF784011.1
B5	<i>Pseudomonas oryzihabitans</i>	KCBOO5 16S ribosomal RNA gene, partial sequence	FJ824120.1
B6	<i>Alcaligenes</i> sp.	JLT1515 16S ribosomal RNA gene, partial sequence	KX989249.1

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B8	<i>Alcaligenes faecalis</i>	BT10 16S ribosomal RNA gene, partial sequence	KY066459.1
B9	<i>Vibrio</i> sp.	PrVy108 16S ribosomal RNA gene, partial sequence	MF948980.1
B10	<i>Providencia vermicola</i>	B u15_38 16S ribosomal RNA gene, partial sequence	KY671146.1`
B11	<i>Pseudomonas xiamenensis</i>	C10-2 16S r RNA gene, partial sequence	NR_043533.1
B12, B17 B21	<i>Providencia stuartii</i>	DMC-28b 16S rRNA gene, partial sequence	MH150796.1
B13	<i>Pseudomonas aeruginosa</i>	JB2 chromosome, complete genome	CP028917.1
B14	<i>Pseudomonas aeruginosa</i>	N15-01092 Complete sequence	CP012901.1
B16	<i>Paenalcaligenessp.</i>	UN24 16S ribosomal RNA gene, partial sequence	KP277115.1
B23	<i>Providencia rettgeri</i>	B5 16S ribosomal RNA gene, partial sequence	KY206744.1

Figure 1 is a photograph of the agarose gel electrophoresis amplifying the 16S rRNA gene of the bacterial isolates. Lanes 2, 3, 4, 5, 6, 7, 8, 9 and 10 on the gel picture shows the bacteria that harbour the 16S rRNA genes with base pair 1500.

All the 135 of the pathogenic Gram-negative bacterial isolates -were tested against thirteen

commonly used antibiotics in Table 2. The least potent antibiotics against all the Gram-negative bacteria were cefepime, imipenem, ciprofloxacin, chloramphenicol and trimethoprim- sulfamethoxazole. -Amikacin and the quinolones had significant activity against *Pseudomonas*, *Alcaligenes* and *Vibrio* species.

**Table2.** Antimicrobial resistance profiles of the bacteria isolated from shellfish

Antibiotics	Bacterial Isolates														
	Alcaligenes spp. n = 53			Pseudomonas spp. n = 44			Providencia spp. n = 25			Vibrio sp. n = 6			Paenalcaligenes sp. n = 7		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
	(% )			(% )			(% )			(% )			(% )		
<b>ESBL indicators</b>															
CTX	-	-	53(100)	19(43.2)	-	25(56.8)	-	-	25(100)	-	-	6(100)	7(100)	-	-
CAZ	53(100)	-	-	-	-	44(100)	-	-	25(100)	-	-	6(100)	-	-	7(100)
PX	-	-	53(100)	-	-	44(100)	9(36.0)	-	16(64.0)	-	-	6(100)	7(100)	-	-
CRO	53(100)	-	-	-	-	44(100)	-	-	25(100)	6(100)	-	-	-	-	7(100)
ATM	-	-	53(100)	10(27.7)	-	34(72.3)	12(48.0)	-	13(52.0)	-	-	6(100)	7(100)	-	-
<b>Other antibiotics</b>															
FEP	-	-	53(100)	-	-	44(100)	6(24.0)	-	19(76.0)	-	-	6(100)	-	-	7(100)
IMI	-	-	53(100)	-	-	44(100)	16(64.0)	-	9(36.0)	-	-	6(100)	-	-	7(100)
AK	7(13.2)	-	46(86.8)	11(25.0)	-	33(75.0)	18(72.0)	-	7 (28.0)	-	-	6(100)	7(100)	-	-
C	8(15.1)	-	45(84.9)	22(50.0)	-	22(50.0)	4(16.0)	-	21(84.0)	-	-	6(100)	-	-	7(100)
CLO	-	-	53(100)	-	-	44(100)	7(28.0)	-	18(72.0)	-	-	6(100)	-	-	7(100)
CIP	-	-	53(100)	10(27.7)	-	34(77.3)	3(12.0)	-	22(88.0)	-	-	6(100)	-	-	7(100)
NOR	7(13.2)	-	46(86.8)	5(11.4)	-	39(88.6)	5(20.0)	-	20(80.0)	6(100)	-	-	-	-	7(100)
NA	7(13.2)	-	46(86.8)	3(6.8)	-	41(93.2)	4(16.0)	-	21(84.0)	6(100)	-	-	-	-	7(100)

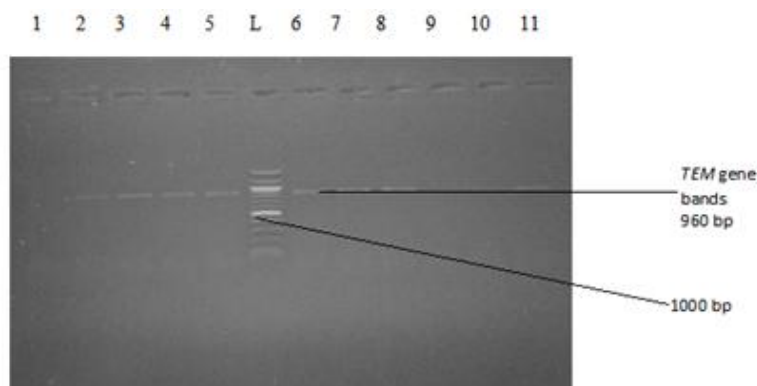
Key: R - Intermediate I - Intermediate S - Sensitive  
 CTX (30 µg) - Cefotaxime < 23 23-25 > 25 PX(10 µg) - Cefpodoxime <18 18-20 > 20  
 CAZ (30 µg) - Ceftriazone < 20 20-22 > 22 CRO(30 µg) - Ceftazidime <18 18-20 > 20  
 ATM (30 µg) - Aztreonam <18 18-20 > 20 C (35 µg) - Cotrimoxazole <12 12-16 > 17  
 FEP (10 µg) - Cefepime <19 19-23 > 20 NA (30 µg) - Nalidixic acid <13 13-17 > 18  
 CLO (30µg) - Chloramphenicol <12 12-16 >17 IMI (10 µg) - Imipenem < 18 18- 21 > 22  
 AK(30 µg) - Amikacin < 12 13- 15>16 NOR (10 µg) - Norfloxacin <12 12-15 > 16  
 CIP (5 µg) - Ciprofloxacin < 21 20-29 >30  
 I - Intermediate R - Resistant S - Sensitive

Twelve of the isolates had MARI >0.6 - 0.7, 34 isolates had >0.7-0.8, 35 to >0.8-0.9 and 54 had >0.9-1.0 (Table 3). *Alcaligenes* spp. had MARI of 38 (70.4%) and the least was 5(41.70%) for *Pseudomonas* spp.

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**Table3.** Multiple antibiotic resistance index of the isolates in shellfish by genera

Isolated bacteria	0.5 – 0.6	>0.6 – 0.7	>0.7 – 0.8	>0.8 – 0.9	>0.9 – 1.0
<i>Pseudomonas</i> spp.	0(0.00)	5(41.70)	12(35.30)	10(28.60)	15(29.63)
<i>Alcaligenesspp.</i>	0(0.00)	7(58.33)	0(0.00)	8(22.70)	38(70.40)
<i>Providencia</i> spp.	0(0.00)	0(0.00)	16(47.10)	10(28.60)	0(0.00)
<i>Vibrio</i> sp.	0(0.00)	0(0.00)	6(17.70)	0(0.00)	0(0.00)
<i>Paenalcaligenes</i> sp	0(0.00)	0(0.00)	0(0.00)	7(20.00)	0(0.00)
<b>Total</b>	<b>0(.00)</b>	<b>12(8.90)</b>	<b>34(25.20)</b>	<b>35(26.00)</b>	<b>53(39.30)</b>



**Fig2.** Agarose gel electrophoresis showing the amplified *TEM* genes of the bacterial isolates. Lane L represents the 1000bp molecular ladder, lanes 2-8, 12, 13, 17, 21 and 23 showed *TEM* bands (960 bp).

As shown in Figure 2 -the agarose gel electrophoresis for the amplification of - the *TEM* gene of the bacterial isolates, - Lane L represents the 1000 bp molecular ladder, lanes

2, 3, 4, 5, 7, 7, 8, 12, 13, 17, 21 and 23 show the amplified *TEM* bands (960 bp). The bacterial isolates positive for the *TEM* gene- are presented in Table 4.

**Table4.** *ESBL/PMQR* genes among the bacterial isolates in shellfish

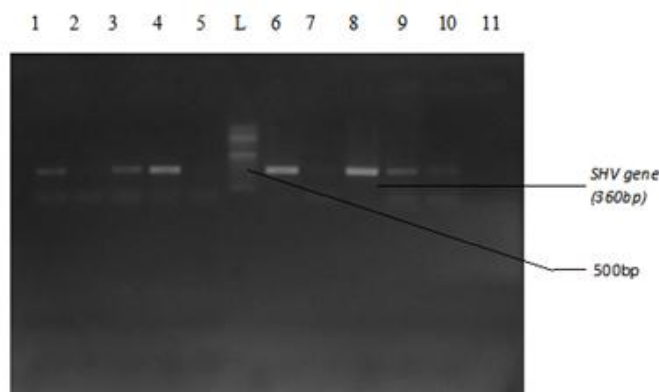
Genes	Bacterial Isolates					Total no/ % positive
	<i>Alcaligenesspp.</i> (%) n = 53	<i>Pseudomonas</i> spp. (%) n = 44	<i>Providencia</i> spp. (%) n = 25	<i>Vibrio</i> sp.(%) n = 6	<i>Paenalcaligenes</i> sp. n = 7 (%)	
<i>VIM</i>	15(28.30)	15(34.10)	25(100)	6(100)	7(100)	68(50.3)
<i>TEM</i>	53(100)	37(84.10)	21(84.00)	-	-	111(82.2)
<i>SHV</i>	53(100)	7(15.90)	4(16.00)	6(100)	-	70(51.8)
<i>VEB</i>	-	-	-	-	-	-
<i>QnrB</i>	15(28.20)	16(36.30)	11(84.00)	6(100)	7(100)	55(40.7)
<i>QnrS</i>	-	-	-	-	-	-
<i>QnrA</i>	-	-	-	-	-	-

Extended spectrum beta-lactamase genes Key:

*VIM* – Vimentin, *TEM* – Temoneria, *SHV* - sulfhydryl reagent variable

*QnrS* - Quinolone resistance genes *SQnrB* - Quinolone resistance genes B

*PMQR* - Plasmid-mediated quinolone resistance - = Negative result



**Fig3.** Agarose gel electrophoresis showing the amplified *SHV* genes (360bp) of the bacterial isolates. Lane L represents the 500bp molecular ladder, lanes 1, 3, 4, 6, 8, 9 and 10 showed *SHV* bands (360 bp).





zole, cefepime and imipenem. This is in consonance with previously reported resistance of Gram-negative bacteria in shellfish to chloramphenicol, ciprofloxacin, tetracycline and trimethoprim-sulfamethoxazole [41]. We detected 72% resistance by Gram-negative bacterial isolates from shellfish to chloramphenicol. The observed diminished potency and resistance of some Gram-negative bacterial strains to antibiotics such as chloramphenicol, ciprofloxacin, tetracycline and trimethoprim-sulfamethoxazole is comparable to other earlier report of very low susceptibility rates of 8.6% for chloramphenicol and 3% to ciprofloxacin and trimethoprim-sulfamethoxazole by some Gram-negative bacteria [42].

Carbapenems were reported to have high activity against Gram-negative bacteria and had at a time remained the drug of choice for treating stubborn infections [43]. Contrarily, resistance of Gram-negative bacteria to carbapenems has recently become very widespread [42]. Marked resistance to imipenem was observed in this study, only *Providencia* species had demonstrable susceptibility of 36% to the imipenem antibiotics. This is in conformity to the designation of *Providencia* species as one of the members of the Enterobacteriaceae species that have intrinsic imipenem resistance [45].

From our results, *Pseudomonas* species showed varying multiple-drug resistance to nalidixic acid (93%), chloramphenicol (100%) and trimethoprim -sulfamethoxazole (50%) agreeing therefore with prior report from Nigeria on the resistance of *Pseudomonas* species to nalidixic acid, chloramphenicol and trimethoprim-sulfamethoxazole [46]. We observed that *Vibrio* species were susceptible (100%) to the norfloxacin (quinolones), nalidixic acid and ceftazidime but equally resistant (100%) to the ESBL indicators and all other antibiotics tested. The observed susceptibility to ceftazidime by *Vibrio* species in this study differ from an earlier report of the resistance of pathogenic *Vibrios* in shellfish to ceftazidime from a similar study [47].

In this study, the Gram-negative bacterial isolates from shellfish exhibited multiple resistance to many commonly used antibiotics and most of them tested positive for ESBL-production. While the *Pseudomonas* species were resistant (100%) to Ceftriazone (CAZ (30 µg) and Ceftazidime (CRO 30 µg), the *Alcaligenes* species on the other hand were

susceptible (100%) to both antimicrobial agents. The antimicrobial pattern for the *Pseudomonas* species in this study is at variance with a similar reported resistance pattern of *Pseudomonas* species [42]. Although the *Alcaligenes* species and *Vibrio* species were susceptible (100%) to Ceftazidime, resistance (100%) to the same antimicrobial agent were shown by *Providencia*, *Paenalcaligenes* and *Pseudomonas* species. Our result shows that ceftazidime was the most potent antibiotics (susceptibility 100%) for *Vibrio* species and is in agreement with previous documented excellent activity of ceftazidime against *Vibrios* species [48]. Furthermore, we detected resistance to cefotaxime (CTX 30 µg) amongst the *Vibrios*, *Alcaligenes* and *Providencia* species. Interestingly, the *Vibrio* isolates from shellfish in this study exhibited cefotaxime and ceftazidime antimicrobial patterns distinct from that of an earlier comparable study [49].

It has been opined that the spread of antibiotic-resistant bacteria in the aquatic environment may have originated from the indiscriminate use of antibiotics, the release of untreated sewage containing antibiotic residues and from resistant bacteria from humans and animals [50]. Also, of notable concern is the fact that aquatic bacteria interact and may exchange resistance genes freely with their close neighbours and distant relatives which may lead to the acquisition of multiple antibiotic-resistant mechanisms by human pathogens [51]. This means that resistance genes can be transferred to other clinically important bacteria making antibiotics treatments ineffective as a result of acquired resistance by pathogens [4, 10, 12, 52].

In the present study, all the isolates from our shellfish samples had MAR indices > 0.6, portraying high antibiotics use and also high selective pressure in the environment. MAR index of 0.2 is the cut-off point normally used to discriminate between low and high-risk regions in areas where antibiotics are overused [53].

We found that 12 isolates had MARI >0.6 - 0.7, 34 isolates had >0.7-0.8, 35 to >0.8-0.9 and the remaining 54 isolates had >0.9-1.0 (Table 3). *Alcaligenes* species had MARI of 38 (70.4%) whereas, *Pseudomonas* species had the least MARI of 5 (41.70%). These results agree with the reported high antibiotic resistance, multiple antibiotic resistance index (MAR1) and the possession of MAR genes by some of the bacterial isolates [54, 55].

## Multi-Drug Resistance Genes associated with some Gram-Negative Bacterial Isolated from Shellfish in Iko and Douglas River Estuaries, in Nigeria

This study observed that *Alcaligenes* and *Pseudomonas* species expressed VIM, TEM and SHV genes, *Vibrio* species expressed VIM and SHV, *Providencia* species expressed TEM and SHV genes while *Paenalcaligenes* species expressed only VIM gene. However, VEB gene was not detected in any of the isolates. The Gram-negative bacteria showed resistance to cefotaxime, ceftriazone, ceftazidime, monobactam (aztreonam). Similarly, this study also showed that 82.2% of the isolates expressed TEM gene, 51.8% expressed SHV and 50.3% for VIM genes. No VEB genes were detected among the isolates. Most ESBL-producing organisms inhabit the bowel, blood, skin, wounds, urine, sputum and the ESBL-genes are capable of spreading directly by person-to-person contact and indirectly by contaminated surfaces to a person [56, 57]. ESBL genes production is considered a public health menace because the genes are known to be capable of conferring resistance to the beta-lactam antibiotics and thereby rendering the drugs ineffective for treatment.

Among the *qnr* positive isolates we detected, 40.7% expressed *qnrB* gene while none expressed *qnrS* and *qnrA* genes. *Providencia*, *Pseudomonas*, *Vibrio*, *Alcaligenes* and *Paenalcaligenes* species were all *qnr* positive. The *qnr* has been incriminated as key determinants in mutation and alteration in the synthesis of DNA gyrase and topoisomerase iv capable of altering the binding sites of quinolones, making the quinolones/fluoroquinolones antibiotics ineffective [58]. Our detection of the *qnr* in the *Providencia* species corroborates a previously first reported detection of PMQR gene in *Providencia rettgeri* [59]) and the PMQR gene is now known to significantly correlate with other antimicrobial genes such as ESBL genes [60, 61].

Remarkably, the antibiotic resistance exhibited by the most of the Gram-negative bacterial isolates from shellfish as observed from the ESBL indicators susceptibility tests may have been conferred by a mechanism for  $\beta$ -lactam resistance as evidenced by higher ESBL genes detection rates in comparison with other published report [50, 62].

### CONCLUSION

This study identified amikacin and imipenem as the most effective antibiotics against *Providencia* spp. while cefepime, chloramphenicol, ciprofloxacin and

trimethoprim-sulfamethoxazole were the least effective drugs. The multiple antibiotics resistance indices of the isolates showed that with exceptions to *Paenalcaligenes* spp. all other isolated Gram-negative bacteria were resistant to all the antimicrobials tested. Fifty-four shellfish isolates in this study showed MARI greater than 0.9 while none of the isolates showed MARI of 0.5-0.6 or less. The most prevalent ESBL genes from this study were TEM gene 82.2%, SHV had 51.9% and 50.4% for VIM gene. Nonetheless, none of the Gram-negative bacteria expressed VEB gene. This study also showed that 40.7% of the bacterial isolates expressed *qnrB* genes which conferred resistance to quinolones, fluoroquinolones antibiotics. The genera isolated in this study were multi-drug resistant bacteria. The contamination of the shellfish with human pathogens indicates direct faecal or other body wastes contamination of the creeks, unhygienic method of harvesting, handling, transportation, processing. The extensive use, misuse and abuse of antibiotics is a clear pointer to the high MARI observed in this study which leads to high mortality and treatment failure.

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