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Prevalence and Antimicrobial Resistance of Gram-Negative Bacteria Isolates in Shellfish Samples from Two River Estuaries in South-South Nigeria

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Abstract

Antibiotic resistant bacteria pathogens remain the leading cause of shellfish borne diseases and a major health threat to humans worldwide. The objectives of this study were to isolate, identify, and determine the antibiotic resistance patterns of Gram-negative bacteria from shellfish. We analyzed a total of 540 shellfish (117 clams, 88 oysters, and 136 periwinkles) samples collected from different vendors at Iko and Douglas Creeks in Akwa Ibom State, South-South Nigeria. Conventional cultural techniques, morphological, biochemical characteristics, and PCR amplification were used to identify the bacterial isolates. Antibiotic susceptibility tests (Kirby-Bauer disk diffusion method) and ESBL phenotype (disk) of the isolates were performed. One hundred and thirty-five (135) Gram-negative bacteria comprising 5 genera and 14 species were detected at a prevalence of: *Alcaligenes faecalis* ***TRB-7*** 38 (28.2%), *Pseudomonas oryzihabitans strain* ***KCB005*** 16 (11.9%), *Paenalcaligenes retgerii strain* ***B5*** 12 (8.9%) *Pseudomonas aeruginosa* ***JB2*** 10 (7.4%), *Providencia stuartii* ***DMC-28b*** 9 (6.7%), *Alcaligenes species* ***TLT151*** 8 (5.9%), *Pseudomonas aeruginosa* ***CIFRI DTSB1*** 7 (5.2%), *Paenalcaligenes* species ***UN24*** 7 (5.2%), *Alcaligenes faecalis* ***BT10*** 7 (5.2%), *Vibrio species strain* ***PrVy108*** 6 (4.4%), *Pseudomonas xiamenensis* ***C10-2*** 5 (3.7%), *Providencia vemicola*

***Bu*15_38** 4 (2.9%), *Pseudomonas anguillisceptica* **4029** 3 (2.2%), and *Pseudomonas aeruginosa* ***N*15-01092** 3 (2.2%). All tested isolates showed various degrees of resistance to the thirteen antimicrobials evaluated. High levels of resistance (100%) to cefepime and imipenem were expressed by all isolates except the *Providencia* species. For the EBSL indicators, all isolates apart from *Alcaligenes* species were resistant (100%) to ceftriaxone. All *Vibrio* species were susceptible to norfloxacin, nalidixic acid, and ceftazidime. The identification of antibiotic resistant Gram-negative bacteria (GNARB) from shellfish in this study highlights the risk of disseminated multi-drug resistance—a serious public health concern.

Keywords

Shellfish, Gram-Negative Bacteria, ESBL-Indicators, Multi-Drug Resistance, Calabar

1. Introduction

Pathogens present in coastal waters, whether linked to anthropogenic activities or naturally occurring, are difficult to detect and therefore, can pose health threats to shellfish and shellfish consumers [1]. Numerous human pathogenic bacteria are documented to be present in coastal waters and shellfish [2] [3] [4]. They come from upstream catchment areas and are classified either as allochthonous or autochthonous. The allochthonous bacteria are those of faecal origin, such as members of Enterobacteriaceae (e.g., pathogenic *E. coli*, *Salmonella*), pathogenic enterococci, *Campylobacter*, and others from nearby aquatic and soil environments (*Aeromonas*, *Arcobacter*, and *Pseudomonas*), conversely, an example of autochthonous bacteria is the *Vibrio* spp. [4]. Human pathogen contaminating shellfish and water bodies are reportedly the sources of shellfish-borne or water-borne disease outbreaks [5] [6]. Seafood like crustaceans, shellfish, mollusks, and related products was implicated in 7.3% of foodborne disease outbreaks in 2013, all over the European Union [7]. This is strong evidence that seafoods are foodborne diseases and illnesses vehicles.

Shellfish are forms of aquatic or sea-life creatures (oysters, clams, scallops, mussel, periwinkle, lobsters, crabs, shrimps, crayfish, sea cucumber, sea urchins, sea stars, and sand dollars) that can be further processed for use as food by humans [8]. Shellfish cultivation, harvest, and sales serve as a source of income, occupation, and food source for the coastal settlers. Several reports indicate that areas where shellfish are harvested and cleaned are affected by possible faecal pollutions from humans, livestock, pets, and wildlife living within the catchment areas [1] [4]. Shellfish filter feed, allowing them to accumulate and concentrate microbial pathogens from surrounding waters. Therefore, people that consume raw or minimally processed shellfish products are exposed to harmful doses of bacterial and viral pathogens [4].

Antimicrobial resistance (AMR) remains a foremost global health crisis in both human and veterinary medicines, and is implicated in the ever-rising number of previously treatable bacterial infections [9] [10] [11]. Bacteria become resistant to antibiotics by either genetic mutations or by acquiring antibiotic resistant genes (ARGs) [12]. Antibiotic resistant bacteria (ARB) infections are projected to cause about 10 million deaths worldwide by the year 2050 [13]. The human-health threat posed by AMR is prevalent in low- to middle-income countries [14], especially in rural areas lacking adequate healthcare facilities. In these areas, there is a high propensity for community-acquired resistant infections and ease of transmissible disease burdens among the general populace. With poor access to health care services, there are increased morbidity rates, prolonged hospitalization, and increased healthcare costs [14] [15] [16]. These together should deter members of the public from consuming contaminated or unprocessed foods, as health burden brings economic burden on family units and the society in general.

Since the aquatic environments have been identified as an ideal setting for the acquisition and dissemination of antibiotic resistance, food harvested from contaminated areas is an additional health risk for humans, exposing them to antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) [12]. The cardinal drivers of antimicrobial resistance lie in humans, animals, plants, food, and environments [17]. Selective pressures exerted by the overuse or misuse of antibiotics in primary food production, genetically modified (GM) crops with antibiotic resistance marker genes, microorganisms added intentionally to the food chain (probiotic or technological) with potentially transferable antimicrobial resistance genes, food processing technologies used at sub-lethal doses (e.g., alternative non-thermal treatments), and the use of biocides [18] [19] [20] are among the main driving forces behind the selection and spread of antimicrobial resistance throughout the food chains.

The evaluation of antibiotic resistant bacteria pathogens in non-clinical environments is useful in assessing the risk levels and the scale of dissemination of resistant pathogens and genes. Accordingly, this study was aimed at isolating Gram-negative bacteria from shellfish and evaluating their antimicrobial susceptibility profile.

2. Materials and Methods

2.1. Sample Collection

A total of 540 shellfish (117 clams, 88 oysters, and 136 periwinkles) samples were sourced from different vendors at Iko and Douglas Creeks in Akwa-Ibom State, Nigeria (**Figure 1**) between the years 2015-2017 in the months of March to July (raining season) and August to February (dry season). Collected samples placed in sterile iced-packed coolers (4˚C) and transported to the Postgraduate Research Laboratory of the University of Uyo, Uyo, Nigeria for bacteriological examination and analysis within 5 hours collection time. Samples were assayed thereafter.

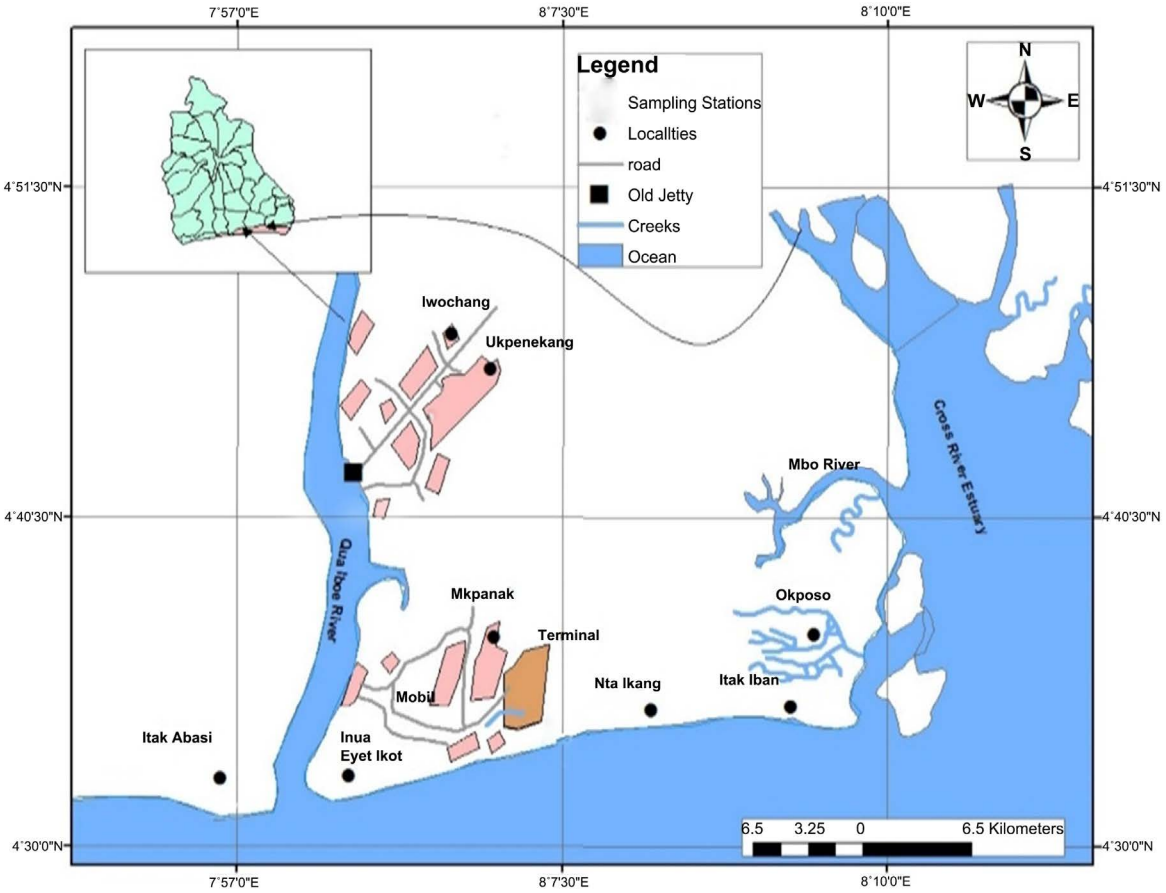

Figure 1. Map showing the sample collection areas along Douglas and Iko Creeks, Akwa Ibom State.

2.2. Sample Preparation

The shellfish were individually washed by scrubbing with sponge in sterile water, and then rinsed in 70% ethanol to remove external dirt and debris. Thereafter, the shellfish were aseptically shucked using a sterile shucking knife to remove the soft flesh. The flesh samples were individually dissected using sterile knives and scissors to separate body parts (flesh, intestines, and gills). Exactly 5 g of each shellfish part was homogenized (using Stomacher® 400 Circulator, Seward Ltd, UK) in 45 mL sterile Phosphate Buffered Saline (pH 7.3 - 7.4) for 5 minutes. Each homogenized sample was serially diluted ten-folds, spread plated on Tryptone Soya Agar (Thermo Scientific™ Oxoid™, United Kingdom) for total heterotrophic bacterial counts (THBC) and pour plated on to Membrane FC Agar (Thermo Scientific™ Oxoid™) for faecal coliform enumeration. The plates were incubated at 37˚C and at 44.5˚C for 24 hours for THBC and faecal coliforms enumeration, respectively.

2.3. Enrichment, Isolation and Bacterial Identification

Duplicates of 25 g of each shellfish parts (flesh, intestines, and gills) were homogenized in 225 mL of Alkaline peptone water (APW) and lactose broth (Oxoid™ Thermo Scientific™, UK) for pre-enrichment at 37˚C for 6 - 8 hours and 18 - 24

hours respectively. Afterwards, 100 µL and 1 mL of the pre-enriched APW, were transferred into 10 mL each of Rappaport-Vasilliadis Soya peptone broth (RVS), Oxoid™ Thermo Scientific™, UK, (UK) and Muller-Kauffman tetrathionate broth (MKTB) for selective enrichment for 24 hours at 41.5˚C and 37˚C, respectively. In addition, loopfuls of the Alkaline peptone water (APW) pre-enrichment were streaked on to Thiosulphate Citrate bile Salt Agar (TCBS, Oxoid™ Thermo Scientific™, UK). Similarly, several loopfuls of the lactose broth pre-enrichment were streaked on to Eosin Methylene Blue Agar (Modified) Levine and Violet Red Bile Glucose Agar (VRBG, Oxoid™ Thermo Scientific™, UK; ISO 21528 and ISO 11133:2014). Thereafter, loopfuls of the selective enrichment cultures were streaked on to selective agar plates: Xylose Deoxycholate Agar (XLD) and Brilliance *Salmonella* (Oxoid™ Thermo Scientific™, UK). Following the incubation of the plates at 37˚C for 24 hours, typical bacterial isolates were purified by streak plating on pre-solidified nutrient agar plates and incubated at 37˚C for 24 hours. Bacterial isolates were identified following previously described methods [21] [22]. Briefly, isolates were presumptively identified by cultural morphology and standard biochemical tests including: Gram reaction, motility tests, oxidase, urease, indole, Voges-Proskauer, hydrogen sulphide production, catalase, citrate utilization and sugar fermentation tests. Biochemical identification of bacterial isolates to the species level was performed using the API-20E and API-NE, (bioMerieux, Marcyl'Etoile, France). Confirmatory bacterial isolates identification was done by sequencing their 16S ribosomal RNA and matching a 100% similarity with that from the NCBI gene bank.

2.4. Antimicrobial Susceptibility Testing

The antimicrobial susceptibility of the 135 Gram negative bacterial isolates to 4 classes of antimicrobial agents namely: quinolones, aminoglycoside, sulfonamide, and cephalosporins (3rd generation) were evaluated using the Kirby-Bauer disc method in accordance with the guidelines by the Clinical and Laboratory Standards Institute [23]. Briefly, suspensions of purified bacterial isolates were made in 5.0 mL of Mueller Hinton broth (MHB, Oxoid™ Thermo Scientific™, UK). The optical density of the suspensions was adjusted to 0.5 McFarland standard (equivalent to 10^8 cfu/mL). Thereafter, Mueller Hinton agar plates (MHA, Oxoid™ Thermo Scientific™, UK) were aseptically inoculated with the respective suspensions using sterile cotton swabs and allowed to dry at room temperature for 5 minutes. Eight (8) selected antimicrobial discs (Oxoid™ Thermo Scientific™, UK): Trimethoprim/Sulfamethoxazole (25 µg), Cefepime (30 µg), Nalidixic acid (30 µg), Chloramphenicol (30 µg), Imipenem (10 µg), Amikacin (30 µg), Norfloxacin (10 µg), Ciprofloxacin (5 µg) were thereafter applied to the MHA plates using a disc dispenser (Oxoid™ Thermo Scientific™, UK) and the plates incubated at 35˚C for 18 - 24 hours. *Escherichia coli* strain ATCC 25922 and *Pseudomonas aeruginosa* strain ATCC 27853 were used as controls.

Detection of ESBL production was performed using the 5 antimicrobial disks

(Thermo Scientific™ Oxoid™, UK) which included: Cefotaxime (CTX 30 µg), Ceftriaxone (CRO 30 µg), Ceftazidime (CAZ 30 µg), Aztreonam (ATM 30 µg), and Cefpodoxime (PX 10 µg) according to CLSI guidelines. The diameter of the zones of inhibition on MHA plates for each isolate was measured with the aid of a caliper and susceptibility results were interpreted using criteria set by the Clinical and Laboratory Standards Institute [23]. Multiple antibiotic resistance (MAR) index was determined using methods described by Osundiya [24], namely the ratio of the total antibiotics used to the number of antibiotics to which the bacterium was resistant.

2.5. Statistical Analysis

Data were analyzed using the SPSS 24 statistical package (SPSS Inc., Chicago, U.S.A.). The Chi-square test and independent t-test were employed to assess differences in coliform loads from shellfish samples and to assess significant differences of variables at a P value < 0.05.

3. Results

The mean heterotrophic bacteria count of 540 shellfish samples analyzed relative to seasons were: Oyster = 3.27×10^8 - 3.6×10^4 (cfu/g), clams = 8.04×10^7 - 7.93×10^3 (cfu/g) and periwinkles = 2.83×10^7 - 1.45×10^5 (cfu/g) (**Table 1**). Oyster samples had higher heterotrophic bacteria count than other analyzed shellfish samples. The seasonal distribution of the mean heterotrophic bacteria counts varied; higher counts were recorded during dry seasons compared to counts during the rainy seasons. However, statistical difference between the seasonal heterotrophic bacteria counts was not significant.

In total, 135 Gram-negative bacteria pathogens were detected using the 16S rRNA gene sequences (**Figure 2**, representative gel). The Bacterial pathogens isolated from the shellfish samples comprised of 5 genera and 14 species that include human pathogens (**Table 2**). The most prevalent bacterial species isolated

Table 1. Bacterial colonization in shellfish types according to sampling locations.

Sample	Location	mean heterotrophic bacterial counts [MHBC]					
		Dry Season [cfu/g]			Rainy Season [cfu/g]		
		Intestine	Body	Gills	Intestine	Body	Gills
Oyster	Eastern Obolo	1.59×10^8	3.53×10^7	3.27×10^8	2.54×10^7	3.6×10^4	4.39×10^6
Oyster	Ibeno	3.65×10^7	6.58×10^6	8.25×10^7	3.11×10^6	3.12×10^6	2.55×10^7
Clams	Eastern Obolo	3.71×10^7	1.84×10^7	5.25×10^7	2.33×10^6	4.80×10^5	3.05×10^6
Clams	Ibeno	8.04×10^7	-	7.93×10^3	1.92×10^7	-	7.55×10^5
Periwinkle	Eastern Obolo	3.31×10^6	9.10×10^6	2.83×10^7	7.40×10^5	1.70×10^6	3.54×10^6
Periwinkle	Ibeno	-	1.45×10^5	2.64×10^7	-	1.50×10^6	4.51×10^6

Table 2. Identification of bacterial strains isolated from shellfish based on 16S rRNA gene sequence.

Sample codes	Isolated bacteria	Strain	Accession No.
B1 B3 B7	*Alcaligenes faecalis*	TRB-7 16S ribosomal RNA gene, partial sequence	MH109290.1
B2	*Pseudomonas anguilliseptica*	D4029 16S ribosomal RNA gene, partial sequence	FJ161260.1
B4	*Pseudomonas aeruginosa*	CIFRI DTSBI 16S ribosomal RNA gene, partial sequence	JF784011.1
B5	*Pseudomonas oryzihabitans*	KCBOO5 16S ribosomal RNA gene, partial sequence	FJ824120.1
B6	*Alcaligenes* sp.	JLT1515 16S ribosomal RNA gene, partial sequence	KX989249.1
B8	*Alcaligenes faecalis*	BT10 16S ribosomal RNA gene, partial sequence	KY066459.1
B9	*Vibrio* sp.	PrVy108 16S ribosomal RNA gene, partial sequence	MF948980.1
B10	*Providencia vermicola*	B u15_38 16S ribosomal RNA gene, partial sequence	KY671146.1
B11	*Pseudomonas xiamenensis*	C10-2 16S r RNA gene, partial sequence	NR_043533.1
B12, B17 B21	*Providencia stuartii*	DMC-28b 16S rRNA gene, partial sequence	MH150796.1
B13	*Pseudomonas aeruginosa*	JB2 chromosome, complete genome	CP028917.1
B14	*Pseudomonas aeruginosa*	N15-01092 Complete sequence	CP012901.1
B16	*Paenalcaligenes* sp.	UN24 16S ribosomal RNA gene, partial sequence	KP277115.1
B23	*Providencia rettgeri*	B5 16S ribosomal RNA gene, partial sequence	KY206744.1

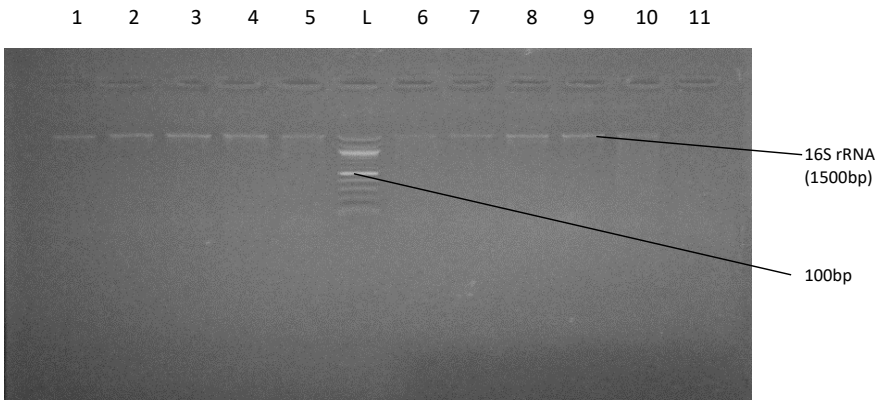

Figure 2. Agarose gel electrophoresis showing the amplified 16S rRNA gene of the bacterial isolates. Lane L represents the 100 bp molecular ladder.

from all shellfish types were *Alcaligenes faecalis* (38), *Pseudomonas oryzihabitans* (16), and *Paenalcaligenes retgerii* (12) (Table 3). The least dominant bacterial species detected from shellfish samples were *Pseudomonas anguillisceptica*

Table 3. Distribution of the bacterial isolates from the shellfish samples.

S/N	Bacterial Isolates	Number [%]
1	*Alcaligenes faecalis* TRB-7	38 [28.2]
2	*Pseudomonas anguillisceptica* 4029	3 [2.2]
3	*Pseudomonas aeruginosa strain* CIFRI DTSB1	7 [5.2]
4	*Pseudomonas oryzihabitans strain* KCB005	16 [11.9]
5	*Alcaligenes* sp. *strain* TLT1515	8 [5.9]
6	*Alcaligenes faecalis* BT10	7 [5.2]
7	*Vibrio* sp. *strain* PrVy108	6 [4.4]
8	*Providencia vemicola strain* Bu15_38	4 [2.9]
9	*Pseudomonas xiamenensis strain* C10-2	5 [3.7]
10	*Providencia stuartii strain* DMC-28b	9 [6.7]
11	*Pseudomonas aeruginosa strain* JB2	10 [7.4]
12	*Pseudomonas aeruginosa strain* N15-01092	3 [2.2]
13	*Paenalcaligenes* sp. UN24	7 [5.2]
14	*Paenalcaligenes retgerii strain* B5	12 [8.9]
	Total	**135**

(3) and *Pseudomonas aeruginosa* (3). Although there were observable seasonal variabilities in the mean heterotrophic bacteria counts and isolation rates, the variations were not statistically significant (Table 4).

The 135 Gram-negative bacteria isolates were tested against 13 selected antimicrobials agents (quinolones, aminoglycoside, sulfonamide, and cephalosporins-3rd generation) and exhibited varied antimicrobial susceptibility patterns, as shown in Table 5. *Alcaligenes* species were distinctly resistant to all the antimicrobial agents tested save for ceftriaxone and ceftazidime. Similarly, all the identified *Vibro* species were resistant to all the antimicrobial agents tested, save for norfloxacin and nalidixic acid. Apart from *Paenalcaligenes* species, all other isolates exhibited 100% resistance to at least three of the ESBL detection antibiotics tested.

4. Discussion

Discoveries of multi-drug resistant bacteria in consumed seafood have become a matter of great public health concern. Antimicrobial resistance (AMR) is a worrisome complex health issue globally, and humans, animals and the environment are implicated reservoirs that contribute the propagation of AMR in interconnected ecosystems [25]. Worldwide, the contribution of resistant microbes from various sources seems to be the major base of resistance in the environment [25]. Efforts directed at preventing the emergence and re-emergence of antibiotic-resistant bacteria strains and associated disease underscores the importance of routine antimicrobial susceptibility testing [26]. Shellfish and other aquatic organisms

Table 4. Seasonal variations in the frequency of bacterial isolates from shellfish.

Year	No. of samples examined	Frequency of bacterial isolates		Total Prevalence [%]	χ^2	[*P*-value]
		Dry Season [%]	Rainy Season [%]			
2014	80	9/49 [18.36]	6/31 [19.35]	15 [18.75]	**0.012**	**0.91**
2015	135	26/85 [30.58]	10/50 [20.00]	36 [26.66]	**1.30**	**0.25**
2016	185	26/116 [22.41]	11/69 [15.94]	37 [20.00]	**0.76**	**0.38**
2017	140	29/91 [31.86]	13/49 [26.53]	42 [30.00]	**0.22**	**0.64**
Total	**540**	**90/341 [26.40]**	**40/199 [20.10]**	**130 [24.07]**	**2.39**	**0.12**
χ^2		**4.83**	**1.97**	**6.11**		
P-value		**0.19**	**0.58**	**0.11**		

Table 5. Antimicrobial resistance rates of bacterial species isolated from shellfish.

BACTERIAL SPECIES	ANTIMICROBIAL RESISTANCE RATES [%]												
	CTX	CAZ	PX	CRO	ATM	FEP	IMI	AK	COT	CHL	CIP	NOR	NAL
***Alcaligenes* species n = 53**	100	0	100	0	100	100	100	86.8	84.9	100	100	86.8	86.8
***Pseudomonas* species n = 44**	56.8	100	100	100	72.3	100	100	75	50	100	77.3	88.6	93.2
***Providencia* species n = 25**	100	100	64	100	52	76	36	28	84	72	88	80	84
***Vibrio* species n = 6**	100	100	100	100	100	100	100	100	100	100	100	0	0
***Paenalcaligenes* species n = 7**	0	100	0	100	0	100	100	0	100	100	100	100	100

Key: **R** [Resistance in mm]. **Cefotaxime** [CTX 30 µg; R < 23], **Cefpodoxime** [PX 10 ug; R < 18], **Ceftriaxone** [CAZ 30 µg; R < 20], **Ceftazidime** [CRO30 µg; R < 18], **Cefepime** [FEP 10 µg; R < 19], **Nalidixic** acid [NAL 30 µg; R < 13], **Chloramphenicol** [CHL 30 µg; R < 12], **Imipenem** [IMI 10 µg; R < 18], **Amikacin** [AK 30 µg; R < 12], **Norfloxacin** [NOR 10 µg; R < 12], **Ciprofloxacin** [CIP 5 µg; R < 21].

are potential vehicles for the transmission of pathogenic microorganisms [27]. In most parts of Nigeria, shellfish-clams, oysters, periwinkles, etc., are liberally consumed as sources of dietary protein [28]. Although seafood is considered relatively free of human pathogens, except for *Vibrio* which are natural contaminants of seafood, the screening, monitoring, and surveillance for antimicrobial resistance patterns of pathogens from aquatic seafood sources is key in helping prevent human health risks from seafood consumption [28] [29].

In this study, high heterotrophic bacteria loads were observed in the 540 shellfish analyzed. Remarkably, a higher count was observed in clams than in other examined shellfish. The high heterotrophic bacteria count observed in clams may be attributed to the peculiar filter feeding mechanism in addition to their complex and fine-tuned symbiotic relationship with microbes [30]. It has been opined that many anthropogenic activities and rainfall influence the total heterotrophic counts in the region [31]. On the contrary, our seasonal distribution analysis of the mean heterotrophic bacteria counts revealed that higher counts were recorded during dry season compared to the rainy season. Our finding agrees with an earlier report by Silva-Neta *et al.* [32] that detected higher bacterial concentrations at the end of the dry season in certain seafood.

Essentially, bacteria found in the aquatic ecosystem can be indigenous or ex-

ogenous, persistent or transient as a result of shedding from animal, vegetal, or soil surfaces [9]. Therefore, microbial contamination of seafood can occur at various stages; exposures to the marine environment and weeds, during harvest, packaging, storage, transportation, and processing [33]. The level of seafood contamination is largely dependent on the amount of pollution in the environment [34]. We observed that 95% of the bacterial load measurements exceeded the acceptable contamination limits for shellfish as stipulated by the International Commission on Microbiological Specification for Food (ICMSF), Centre for food safety and applied nutrition (CFSAN) of the US Food and Drug Administration [34]. Apparently, the safest option for consumers is adequate cooking/processing before eating.

The antimicrobial susceptibility patterns of Gram-negative bacteria shellfish isolates vended around Iko and Douglas Creeks in Akwa Ibom State, Nigeria was determined. The 16S ribosomal RNA gene amplicon was used in sequencing and matching a 100% similarity with that from the NCBI gene bank to characterize the following 5 genera and 14 bacterial species that included human bacterial pathogens: *Alcaligenes faecalis* ***TRB*-7**, *Pseudomonas oryzihabitans*, *Paenalcaligenes retgerii*, *Pseudomonas aeruginosa* ***JB*2**, Providencia stuartii, *Alcaligenes species strain* ***TLT*1515**, *Pseudomonas aeruginosa* ***CIFRI DTSB*1**, *Paenalcaligenes species*, *Alcaligenes faecalis* ***BT*10**, *Vibrio species*, *Pseudomonas xiamenensis*, *Providencia vemicola*, *Pseudomonas anguillisceptica* and *Pseudomonas aeruginosa* ***N*15-01092**. Overall, the genus *Alcaligenes* was the most predominant bacterial pathogen isolated; it was followed by *Pseudomonas oryzihabitans* and *Paenalcaligenes retgerii*. The observed preponderance of the members of the genus *Alcaligenes* in this study is supported by similar reports from certain seafood products including seafood wastes, shrimp shells, prawn shells, crab shells, and other marine samples [35] [36]. On the other hand, the presence of *Alcaligenes* genus in seafood may have other biological value due to their good antagonistic and potentially probiotic/inhibitory ability against the growth of *vibrio* strains and other multidrug-resistant bacteria [37] [38] [39].

Seafood as carriers of multidrug-resistant bacteria has been highlighted as a growing danger leading to the wider dissemination of MDR-bacteria in the community [40]. In this study, all the Gram-negative bacteria isolates from shellfish evaluated exhibited varied high antimicrobial resistance rates to 13 different antimicrobials agents that included ESBL indicators. Predominantly, the *Alcaligenes species* (*Alcaligenes faecalis* ***TRB*-7**, *Alcaligenes species* ***TLT*151** and *Alcaligenes faecalis* ***BT*10**) distinctively showed high resistance to most of the antimicrobial agents tested except for ceftriaxone and ceftazidime. High antibiotic resistant rates as observed with the *Alcaligenes species* in this study conforms with the findings of Ayandiran and Dahunsi, [41] who reported high antibiotics resistance rates among *Alcaligenes faecalis* isolated from the indigenous fish (*Clarias species*) from River Oluwa, Nigeria. With decreasing susceptibility rate to commonly used antibiotics, *Alcaligenes* species particularly the *Alcaligenes faecalis* which is frequently implicated in infection sites such as the blood-

stream, urinary tract, skin and soft tissue, and middle ear, is a potentially emerging pathogen that usually causes opportunistic infections in humans and often very difficult to treat due to its increased resistance to several antibiotics [42].

We found high antimicrobial-resistant and potentially pathogenic *Vibrio* species in shellfish vended at Iko and Douglas Creeks in Akwa Ibom State. The antimicrobial susceptibility analysis confirmed that the *Vibrio* species isolated in this study were only susceptible to quinolone (norfloxacin and nalidixic acid), highly resistant to aminoglycosides, beta-lactams (including carbapenems and third-generation cephalosporins) and sulfonamides. Consumption of contaminated seafood or exposure to contaminated water is routes for *Vibrio* species infections, although self-limiting, it can often be fatal especially in immunocompromised patients or upon failure of antimicrobial therapy [43] [44]. Although cholera cases are frequently reported in areas with inadequate water quality and sewage treatment, other *Vibrio* species are relevant agents of seafood-borne infections on a global scale [45].

In this study, *Pseudomonas* species was among the major bacterial contaminants of shellfish and possible marine AMR-indicator candidate. The isolation of *Pseudomonas* species is consistent with the recent reports of *P. aeruginosa*—a predominant bacterial contaminant of frozen shellfish retailed within Lagos metropolis in Nigeria [46] [47]. The *Pseudomonas* species isolated in this study showed notable multiple resistant rates (100%) to ceftriaxone, cefpodoxime, ceftazidime, cefepime, imipenem, and chloramphenicol. The observed multiple resistant rates conformed with an earlier report by Maravić *et al.* [46] on the detection of multi-drug resistance in *Pseudomonas aeruginosa* in shellfish from human-impacted marine environment.

Interestingly, apart from the *Paenalcaligenes* species all the other bacterial isolates in this study exhibited 100% resistance to at least three of the ESBL detection antibiotics tested. The implication is that extended-spectrum β-lactam antibiotics such as cefotaxime, ceftazidime, cefpodoxime, ceftriaxone, aztreonam, and imipenem were widely used for the treatment of infections in the study area. The public health importance in antibiotic resistance studies of pathogens from seafood cannot be overemphasized. This is because aquatic bacteria indisputably contribute to increasing antibiotic selective pressure and facilitate the transfer of antibiotic-resistant determinants between microbial species, including fish and human pathogens; thus, allowing the residual antibiotic presence in commercialized fish and shellfish products [9] [48]. In addition, the discharge of effluents such as raw sewage and antimicrobials in waste water not only contaminate water bodies, but also shellfish harvested from such water bodies. This potentially contributes to the increasing emergence of antibiotic-resistant bacteria and antibiotic-resistant genes in water environments—an important environmental health issue [25]. The finding of antibiotic resistance in pathogenic bacteria from seafood is an invaluable indicator of the extent of alteration of water ecosystems by anthropogenic activities [9].

5. Conclusion

It was found that most of the vended shellfish from Iko and Douglas Creeks in Akwa Ibom State have considerably significant bacteria loads that exceeded the acceptable limits for consumable shellfish products. *Pseudomonas* species, a possible marine AMR-indicator candidate, was among the major bacterial contaminants of shellfish samples. Analysis of the antimicrobial susceptibility test confirmed that Gram-negative bacteria isolates from shellfish exhibited high antimicrobial resistance rates to different antimicrobials agents including ESBL indicators. *Alcaligenes* species distinctively showed high resistance to most of the antimicrobial agents tested except ceftriaxone and ceftazidime. Also, isolated *Vibrio* species were susceptible to quinolone, but highly resistant to aminoglycosides, beta-lactams (including carbapenems and third-generation cephalosporins) and sulfonamides. Overall, Gram-negative bacteria (GNARB) inherent in shellfish can further potentiate the risk of disseminating multi-drug resistance which is a serious public health concern.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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