Molecular Characterization of Bacteria Isolated from Some Seafoods in Nembe Community, Bayelsa State, Nigeria

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ABSTRACT

The quest for better food quality has invariably increased cases of food-borne infections which in turn contribute to the problem of antibiotic resistance as a result of drug abuse. This study is aimed at characterizing bacterial isolates from some seafood sold in Nembe, Bayelsa State, Nigeria. A total of 200 fresh seafood samples (crab, shrimp, oyster and periwinkle) were collected randomly from Nembe, Bayelsa State. Isolates were obtained using the conventional microbiological methods and the pure cultures were screened by gram staining and biochemical test for preliminary identification. Isolates were further characterized for 16SrRNA using Polymerase Chain Reaction and Sequencing. The most dominant species isolated were *Staphylococcus gallinarum* 27(22.5%), *Vibrio rotiferanus* 17(14.2%), *Vibrio parahaemolyticus* 48(40%), *Klebsiella aerogenes* 10(8.3%) and *Klebsiella quasipneumoniae* 18(15%). Analysis of variance (ANOVA) by single factor was done to determine the variation in colony counts of isolates from the different seafood samples and P value was > 0.05 indicating that there is no significant difference in colony counts among the different sea foods. The presence of these bacterial species in these seafood samples renders the food unsafe for consumption. Adequate handling as well as proper cooking of seafood before consumption is highly recommended so as to reduce the incidence of food-borne infections.

Keywords: Sea foods, Molecular characterization, Bacterial isolates.

Introduction

The quest for better food quality has led to an exponential increase in the consumption of seafood because of the nutritional composition. Increased consumption of these foods without proper handling and preparation has led to an increase in the incidence of food borne infection and food poisoning thus posing such high public health risk (Adeleye *et al.,* 2010). There is therefore a need to ensure food safety at all levels by eliminating every source of contamination in these sea foods.

Bacteria of the genus *Vibrio, Klebsiella* and *Staphylococcus* have been implicated in seafood (Dib *et al.,* 2014). *Vibrio* species inhabit the estuarine/coastal regions and have been implicated in raw or undercooked seafood as well as account for a significant proportion of human infections such as gastroenteritis which is usually associated with consumption of raw or undercooked seafood, wound infections, septicemia and ear infections (Adeleye *et al.,* 2010). In Nigeria for instance, *V. parahaemolyticus* associated with gastroenteritis due to consumption of contaminated seafood has been reported (Adeleye *et al.,* 2010). They are widely distributed worldwide in seawater and are found commonly in association with resident aquatic organisms (Arungiri *et al.,* 2016).

Vibrio species are found in sea foods such as shell fish, bivalves mollusks, oysters, shrimps and other benthic animals and have been implicated in a number of gastro enteric diseases and pose a considerable public health threat and also as agent of sporadic and epidemic food borne infections associated with consumption of undercooked or raw sea foods (Arungiri *et al.,* 2016).

The *Vibrio* pathogenic species produce various virulence factors including enterotoxin, haemolysin, cytotoxin, protease, lipase, phospholipase, siderophore, adhesive factor and haemagglutinins (Zhang and Austin, 2005).The

pathogenicity of *V. parahaemolyticus* is associated with the production of thermostable direct haemolysis and TDH-related hemolysin encoded by the *tdh* and *trh* genes respectively, and these are responsible for the haemolysis of human red blood cells. It also possesses a thermolabile hemolysin encoded by the *tlh* genes (Zhang and Austin, 2005). These bacteria have been recognized as the main agents causing food borne diseases in many countries including Asia, United States, France, Mexico, Peru and Chile (Cabanillas *et al.,* 2006). Due to the increase in the consumption of sea foods worldwide there has been a drastic increase in food borne infection which in turn has led to the increase in gastro enteric diseases in humans (Cabanillas *et al.,* 2006). Sporadic cases of cholera have been reported in Nigeria as well as gastroenteritis caused by *V. parahemoluticus* resulting from the consumption of raw and undercooked seafood (Cabanillas *et al.,* 2006).

Staphylococcus species are usually associated with the skin and mucous membrane microflora of humans and animal such as chicken skin (Tibra *et al.,* 2010). They are gram positive, mesophilic, oxidase negative cocci organisms that have been implicated in several clinical and environmental samples (Tibra *et al.,* 2010). The diseases caused by staphylococcus species ranges from acute septicemia to osteomyelitis in poultry (Xu *et al*., 2018). *Staphylococcus gallinarum* are usually isolated from chicken skin but have also been isolated from seafood (Tibra *et al.,* 2010). *Staphylococcus* species possess virulence factors such as enterotoxin, coagulase, hemolysins, toxins etc (Kérouanton *et al*., 2007). Food poisoning arising from ingesting food infected by *Staphylococcal* enterotoxins is usually of high risk due to the presence of *Staphylococcal* enterotoxins (Omoe *et al*., 2005). *Klebsiella* species have been implicated in both human and animal samples where they play roles as pathogens widely distributed in the environment (Brisse *et al*., 2009; Shon *et al*., 2013). They are gram negative rod shaped oxidase negative bacteria that have been implicated in so many clinical infections such as pneumonia, wound infection, meningitis, urinary tract infections, nosocomial infections, etc (Holt *et al.,* 2015). The pathogenicity factors of *Klebsiella* are capsules or lipopolysachharide and are considered potential candidate for vaccination that will serve as infection immunological control measures (Holt *et al.,* 2015). Different species of *Klebsiella* are *Klebsiella pneumoniae, Klesiella aerogenes, Klebsiella quasipneumoniae, Klebsiella varicola* etc. (Brisse & Verhoef, 2001).

The people of Nembe live around the river banks and are known majorly for fishing as an occupation. There is constant discharge of domestic, agricultural and industrial sewage into the water bodies where these seafoods are harvested from and thus poses a high risk to the community who consume these foods undercooked or even raw.

Benthic organisms otherwise called benthos are community of organisms that live in, on or near the sea bed called the benthic zone. Benthic organisms such as sea stars, oysters, clams, sea cucumbers, brittle stars and sea anemones serve as important sources of food due to their nutritional properties (Ryan, 2007).

This study is aimed at screening seafood samples obtained from Bayelsa for the presence of bacterial species.

Methodology

Determination of ample size

Using a single proportion formula, the sample size is calculated thus:

$n = Z^2pq / d^2$

Where: n = desired sample size, when population is more than 10,000, Z = Critical value at 90% or 1.64 confidence interval, p = Prevalence or population with the desired attribute pegged at 25% i.e 0.25

$q = 1 - p$

d = degree of accuracy usually set at 0.05

Hence, Z = 1.64, p = 0.25, q = 0.75, d = 0.05, $n = 1.64^2$ x 0.25 x 0.75 / 0.052 n = 0.5043 / 0.0025 n = 201.72 n ≈ 200

Sample collection

Two hundred (200) seafood samples comprising of periwinkle, crab, oyster and shrimps were purchased from fisher men at the Nembe River, Bayelsa State, Nigeria. The samples were collected in sterile containers and transported in ice chest to the Microbiology Laboratory, Federal University of Technology, Owerri.

Sample preparation

Each of the samples collected were macerated using a sterile stomacher blender to increase surface area to volume ratio and to obtain a homogenous state of the sample. The samples were serially diluted (10 folds dilution) using peptone water and an aliquot (0.1 ml) of the diluted sample was collected and inoculated onto the already prepared sterile Thio-Citrate Bile salt Sucrose (TCBS) agar plates.

Inoculation and isolation

One-tenth milliliter (0.1 ml) of the aliquot from the required dilution factor (10^4) was aseptically inoculated onto the freshly prepared and surface dried medium using a sterilized pipette. An L-shaped spreader was used to evenly distribute the inoculums round the surface of the agar plate so as to obtain discrete and countable colonies after incubation. The agar plate was allowed to dry after which it was incubated at 35^0C for 24 h. The results of the colonies grown were recorded.

Phenotypic identification

One hundred and twenty (120) bacterial isolates were phenotypically identified by subjecting them to standard microscopic and biochemical test according to the method of Cheesbrough (2006).

Molecular analysis

DNA Extraction (Boiling method)

Five milliliters (5 ml) of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000 rpm for 3 min. The cells were re-suspended in 500 μl of normal saline and heated at 95^0C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000 rpm. The supernatant containing the DNA was moved to a 1.5 ml microcentrifuge tube and stored at -20^oC for other down-stream reactions (Tamura *et al.,* 2013).

DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2 μl of

sterile distilled water and blanked using normal saline. Two microlitres of the extracted DNA was loaded onto the lower pedestal; the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button (Tamura *et al.,* 2013).

PCR Amplification of the 16SrRNA gene (27F and 1492R)

The 16srRNA region of the rRNA gene of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTG GCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5 uM and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for   30 seconds for 35 cycles and final extention, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light transilluminator (Tamura *et al.,* 2013).

Agarose Gel electrophoresis

Electrophoresis of the DNA was carried out on a 1.5% agarose gel in a 0.5X concentration of Tris-Borate-EDTA (TBE) buffer. Agarose gel was prepared by boiling 1.5 g of agarose powder in 100ml of 0.5X TBE buffer. After boiling, the solution was allowed to cool and 10 µl of ethidium bromide was added to the cooled agarose solution. This was poured into a casting tray with a comb placed 2 meter from the rim to form wells. The gel was allowed to set for 30 minutes and the comb was gradually removed. 5 µl of the amplified PCR products were then loaded into the wells. A DNA 100 bp molecular weight marker (Solys biodyne) and a negative control (ultra pure water) were also loaded into one of the wells. The gel was thereafter electrophoresed in a horizontal tank at a constant voltage of 80 V for about 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining under a short wave ultraviolet light transilluminator and the photograph were taken using a digital camera (Tamura *et al.,* 2013).

Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10 µl, the components included 0.25 µl BigDye® terminator v1.1/v3.1, 2.25 µl of 5 x BigDye sequencing buffer, 10µM Primer PCR primer, and 2-10 ng PCR template per 100 bp. The sequencing conditions were as follows; 32 cycles of 96°C for 10 s, 55°C for 5 s and 60°C for 4 mins. The corresponding sequences were identified using the online blast search at, http://blast.ncbi. nlm.nih.gov/Blast.cgi (Tamura *et al.,* 2013).

Results

A total of 200 seafood samples were analyzed using the traditional microbiological methods. 120(60%) pure isolates were obtained from the seafood samples and characterized. 27(22.5%) of the isolates appeared as yellow, large, flat unbrate colonies. 65(54.2%) of the pure isolates appeared as bright yellow, round, shiny small colonies as well as green large round colonies on Thio Citrate Bile Salt agar while others 28(23.3%) were black flat colonies.

The total variation rates of *Staphylococcus* sp in the different seafood samples were 9(30%), 6(20%), 5(16.7%) and 7(23.3%) in Periwinkle, Crab, Oyster and Shrimps respectively. The total variation rates of *Vibrio* sp in the seafood samples were 16(53.3%), 16(53.3%), 15(50%) and 18(60%) in Periwinkle, Crab, Oyster and Shrimps respectively. The total variation rates of *Klebsiella* sp in the seafood samples were 5(16.7%), 8(26.7%), 10(33.3%) and 5(16.7%) in Periwinkle, Crab, Oyster and Shrimps respectively. The distribution rate of the different isolates in each of the seafood samples is shown in Figure 1.0 and Tables 1-4 below.

The isolates which had similar morphological and biochemical properties were grouped into five (A, B, C, D and E) as shown on Table 6. Representative isolates were molecularly characterized by 16SrRNA gene amplification using Polymerase Chain Reaction (PCR) as well as DNA sequencing using MEGA7.0 BLASTN. The bacterial isolates identified were *Staphylococcus gallinarum, Vibrio rotiferanus, Vibrio parahaemolyticus, Klebsiella aerogenes* and *Klebsiella quasipneumoniae*. The evolutionary history (phylogenic tree) was inferred using the Neighbour-Joining method to determine the relationship between strains as shown in Figure 4.
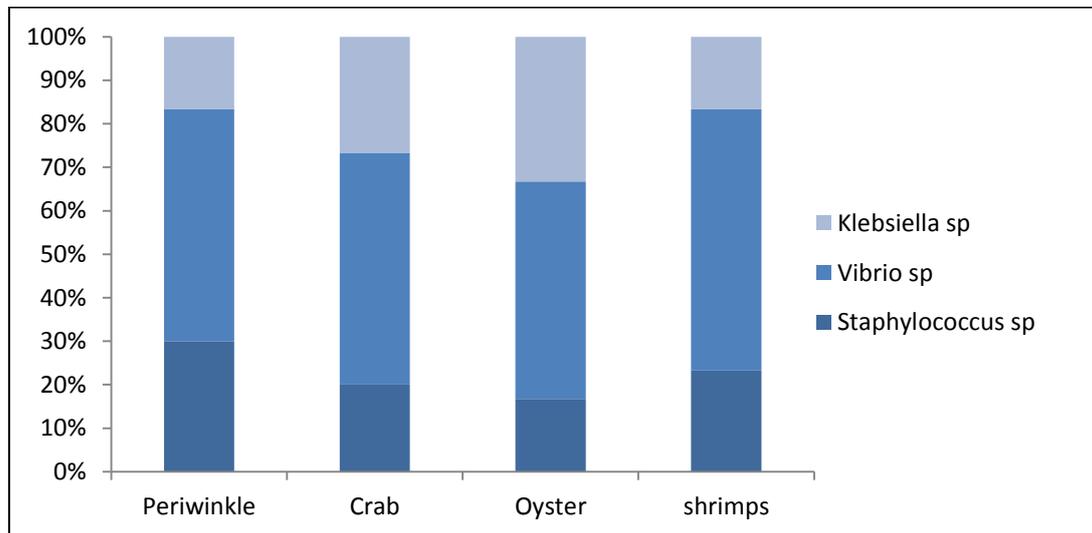

Fig.1. Distribution of bacterial isolates in the different seafood samples

Bacterial Isolates as Distributed in the Different Seafood Samples

Table 1. Distribution of *Staphylococcus* species in seafood samples

Seafood (n=200)	No of Samples collected	No of Isolates	No of *Staphylococcus sp* isolated	Prevalence (%)
Periwinkle	50	30	9	30
Crab	50	30	6	20
Oyster	50	30	5	16.7
Shrimps	50	30	7	23.3
Total	200	120	27	22.5

Table 2. Distribution of *Vibrio* species in seafood samples

Seafood (n=200)	No of Samples collected	No of Isolates	No of *Vibrio* sp isolated	Prevalence (%)
Periwinkle	50	30	16	53.3
Crab	50	30	16	53.3
Oyster	50	30	15	50
Shrimps	50	30	18	60
Total	200	120	65	54.2

Table 3. Distribution of *Klebsiella* species in seafood samples

Seafood (n=200)	No of Samples collected	No of Isolates (120)	No of *Klebsiella* sp isolated	Prevalence (%)
Periwinkle	50	30	5	16.7
Crab	50	30	8	26.7
Oyster	50	30	10	33.3
Shrimps	50	30	5	16.7
Total	200	120	28	23.3

Table 4. Group of bacterial isolates with similar biochemical characteristics

TEST	GROUP A 27	GROUP B 17	GROUP C 48	GROUP D 10	GROUP E 18
GRAM	+	-	-	-	-
SHAPE	Cocci	Rod	Rod	rod	Rod
HEMOLYSIS	-	-	+	-	-
CATALASE	+	-	+	+	+
OXIDASE	-	+	+	-	-
INDOLE	-		-	-	-
COAGULASE	-	ND	ND	ND	ND
GLUCOSE	+	+	+	+	+
SUCROSE	+	+	+	+	+
CITRATE	+	+	+	+	+

VOGUES PROSKEUR	ND	-	-	+	+
7% NaCl	ND	+	+	ND	ND

Key: - =Negative, += Positive, ND=Not Determined

**Table 5.** One Way Anova for Seafood Samples (Single Factor)

ANOVA

Source of Variation	SS	Df	MS	F	P-value	F crit
Between Groups	0.429608	3	0.143203	1.743	0.162073	2.682809
Within Groups	9.53042	116	0.082159			
Total	9.960029	119				

Table 6. List of bacterial isolates identified by 16sRNA gene sequence analysis

Genus	Group of isolates	Gene bank accession number	Identity (%)	Blast value
Staphylococcus gallinarum	A	MW308344	100	0.0
Vibrio rotiferanus	B	LR702061	100	0.0
Vibrio parahaemolyticus	C	MW142548	100	0.0
Klebsiella aerogenes	D	MW689239	100	0.0
Klebsiella quasipneumoniae	E	CP065841	100	0.0

Results from DNA Quantification
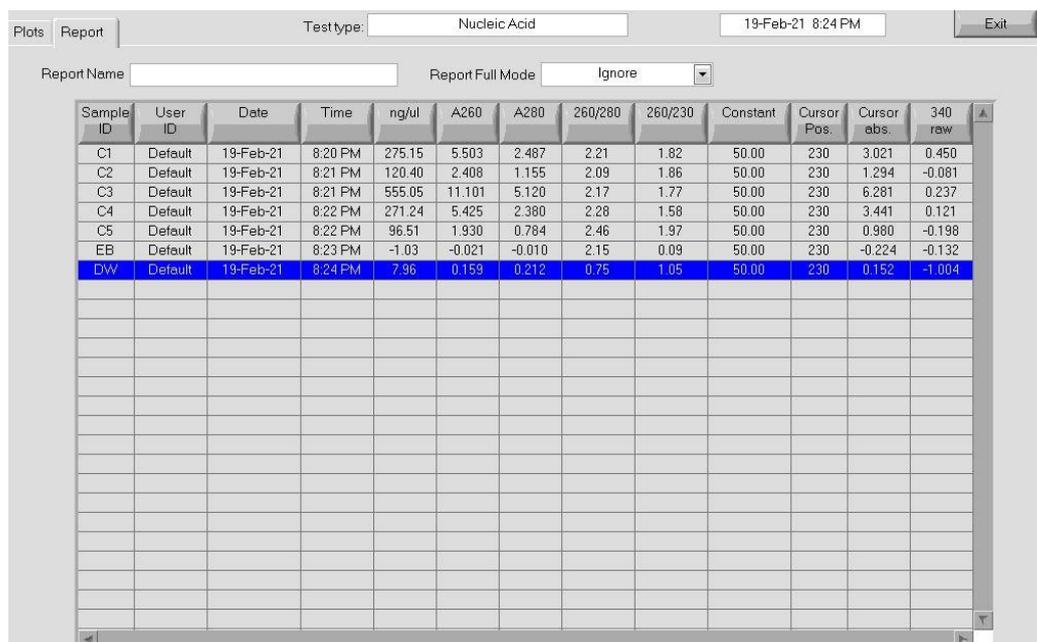
Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
C1	Default	19-Feb-21	8:20 PM	275.15	5.503	2.487	2.21	1.82	50.00	230	3.021	0.450
C2	Default	19-Feb-21	8:21 PM	120.40	2.408	1.155	2.09	1.86	50.00	230	1.294	-0.081
C3	Default	19-Feb-21	8:21 PM	555.05	11.101	5.120	2.17	1.77	50.00	230	6.281	0.237
C4	Default	19-Feb-21	8:22 PM	271.24	5.425	2.380	2.28	1.58	50.00	230	3.441	0.121
C5	Default	19-Feb-21	8:22 PM	96.51	1.930	0.784	2.46	1.97	50.00	230	0.980	-0.198
EB	Default	19-Feb-21	8:23 PM	-1.03	-0.021	-0.010	2.15	0.09	50.00	230	-0.224	-0.132
DW	Default	19-Feb-21	8:24 PM	7.96	0.159	0.212	0.75	1.05	50.00	230	0.152	-1.004

Fig.2. DNA Quantification figures on Nano 1000 (ng/µl)
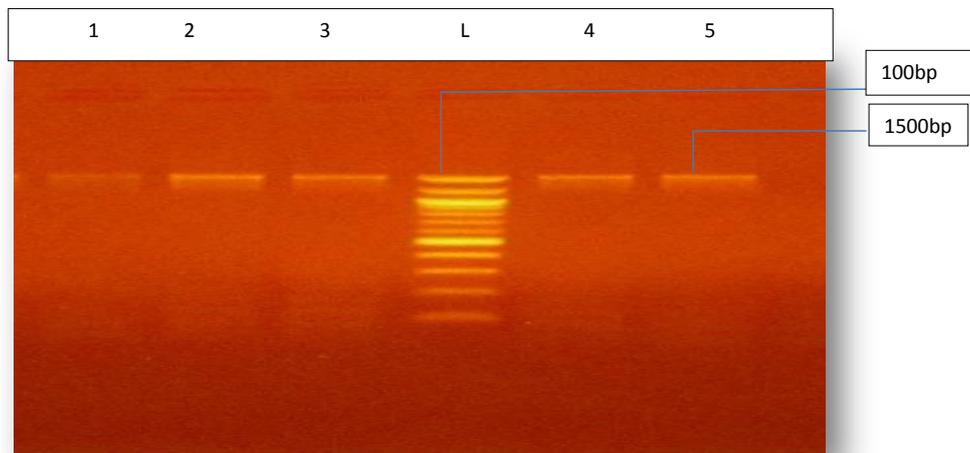
Results From Agarose Gel Electrophoresis

Fig.3. Agarose gel electrophoresis of some selected bacterial isolates. Lane 1,2,3,4 and 5 represents 16SrRNA gene bands (1500bp). Lane L represents the 100bp Molecular ladder
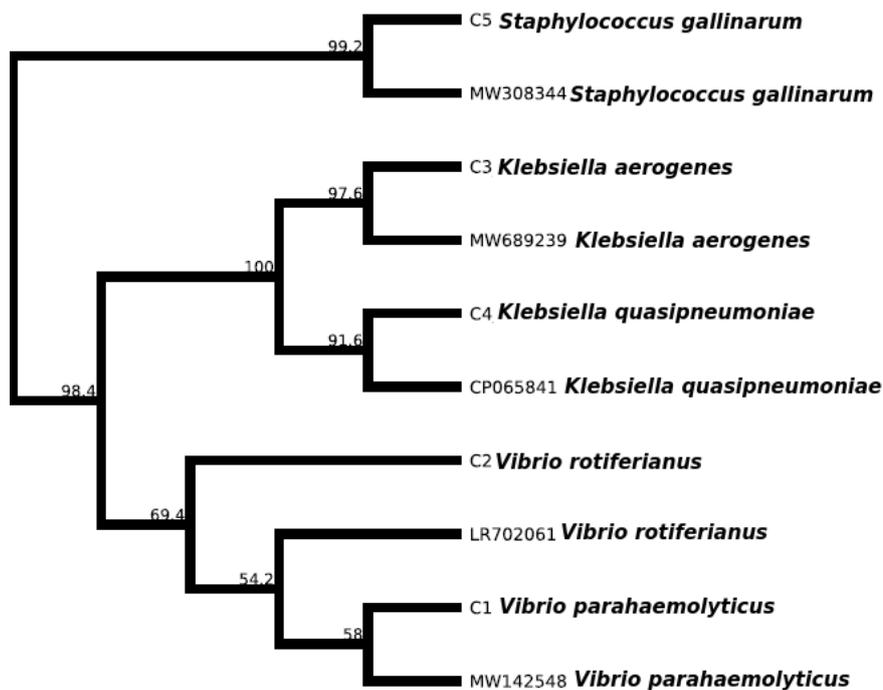

Fig.4. Evolutionary Distances between the Bacterial Isolates

Discussion

A total of 200 seafood samples were analyzed which produced 120 (60%) pure isolates after characterization. Seafood samples harbour a consortium of microorganisms ranging from pathogenic microorganisms to normal commensals. The community of microorganisms found in a particular habitat is dependent on a number of factors such as composition, weather, pollution, location, time, etc. (Farmer III *et al.,* 2005). Results obtained from this study showed that microorganisms of the genera *Staphyococcus, Vibrio,* and *Klebsiella* were present in the seafood sample. This corroborates the reports that seafoods harbour diverse groups of organisms such as *Vibrios, Listeria,* enteric virus etc (Papadopoulou *et al.,* 2006). The growth of *Staphylococcus* and *Klebsiella* clearly shows that

TCBS can indeed support the growth of other microorganisms other than *Vibrio* species. Out of the 120 pure cultures isolated, *Vibrio* species constitute 54.2% of the bacteria isolated while *Klebsiella* and *Staphylococcus* constitute 23.3% and 22.5% respectively. *Staphylococcus* species are not usually associated with marine environments but could perhaps be commensals in the sea-foods or the aquatic environment. The dominance of *Vibrio* species is evident of their normal commensals in seafood's (Cabanillas-Beltrán *et al.,* 2006*).*The presence of *Klebsiella* species could be attributed to the activities around the environment such as sewage and other effluent discharged into the water body from the communities (Dib *et al.,* 2014).

The analysis of variance (Anova) carried out shows that though the samples contain different isolates and different colony count, there is still no significant difference between the isolates in the samples as $p> 0.05$.

Staphylococcus gallinarum has been reported to be isolated from saliva of healthy humans, chicken, septic, and traumatic wounds of hospitalized patients (Ohara-Nemoto *et al*., 2008). It has also been isolated from blood cultures in patients with chronic hepatitis B infection thus its presence in seafood renders it unsafe for consumption. Its presence in the seafoods could be attributed to the human activities around the river banks where these seafoods are sold (Dib *et al.,* 2014).

Vibrio rotiferanus was isolated from the seafood samples. This result corroborates the reports of Gomez-Bill *et al.* (2003) that isolated of *V. rotiferianus* from the *rotifer* culture *(Brachionus plicatilis)*. Of all the bacterial species isolated, *Vibrio parahaemolyticus* was the most predominant and has been reported to be the top causative agent of food poisoning in Africa (Adeleye *et al.,* 2010).

The result obtained from this study with regards to the high contamination by *V. parahaemolyticus* in these seafoods was in tandem with the reports of Vincent *et al.,* (2015). The source of contamination could be from the water body, handlers of the seafoods or even as a result of the unhealthy practices by the people living around the river banks. *V. parahaemolyticus* is the major cause of food borne infection and its presence in food calls for urgent surveillance of contaminants in seafoods in order to ensure public health safety.

Klebsiella sp have been implicated in seafood samples due to the anthropogenic activities around the river banks as well as domestic activities by inhabitants of these areas. *K. quasipneumoniae* has been reported to be an opportunistic pathogen (Imai *et al.,* 2019). However, its presence in the seafood can be considered dangerous since it has been also implicated in clinical infections in humans as an opportunistic pathogen (Imai *et al.,* 2019).

The characterization of the isolates was done by PCR using 16srRNA as a target gene and all isolates possess the 16srRNA at 1500bp. The sequenced isolates has revolutionary relationship with *Staphylococcus gallinarum, Klebsiella quasipneumoniae, Klebsiella aerogenes, Vibrio parahaemolyticus and Vibrio rotiferianus* with 100% relatedness.

Conclusion and Recommendation

Although *Staphylococcus gallinarum* has not been reported to be implicated in food-borne infections or poisoning, the result of this study calls for a concern since the organism has been reported to be opportunistic. Also, there is risk for transfer of virulent and antibiotic resistant genes between the coagulase positive *Staphylococcus* and the

coagulase negative Staphylococcal species as well as other bacterial isolates such as *Vibrio* species. *Klebsiella* species reported in this study have been implicated in clinical as well as food borne infections, therefore, this calls for more investigative procedures to determine its presence in sea foods as well as proper handling of food by food handlers.

Vibrio species have been reported to be present in seafood as commensals and pathogens, however, new species are been implicated and this is of great concern as this could lead to the emergence of novel pathogenic strains which may lead to a global pandemic in the nearest future.

Health institutions and food processing firms should ensure food hygiene practices as well as proper food handling by food vendors. Public enlightenment on the need for proper cooking of food before consumption should be carried out especially in the rural areas of Southern Nigeria.

Consumption of raw sea foods such as Crab and Periwinkle by the Ijaws of the Niger Delta extraction should be strongly discouraged.

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