International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
DOI: 10.22192/ijarbs Coden: IJARQG (USA) Volume 7, Issue 7 -2020

Research Article

DOI: http://dx.doi.org/10.22192/ijarbs.2020.07.07.012

Occurrence and antibiotics susceptibility of *Vibrio* sp. from Penaeid shrimps from Kribi Coastal Water, Cameroon

Rhoda N. Bughe [1,3, 4*], Benedicta O. Oben[1], Akindeh M. Nji[2,4], Jean P. K. Chedjou[4], Aristid E. Mbange[4], Innocent M. Ali[4], Pius M. Oben[1] and Wilfred F. Mbacham[2,4*]

[1]Department of Fisheries and Aquatic Resources Management, Faculty of Agriculture and Veterinary Medicine, University of Buea, P.O. Box 63, Cameroon.
[2]Department of Biochemistry, Faculty of Science, University of Yaoundé 1, B.P. 812, Yaoundé, Cameroon.
[3]Department of Biological Sciences, Higher Training College, University of Yaoundé 1, B.P. 47, Cameroon.
[4]Food and Drug Safety (FODRUS) and the Laboratory for Public Health Research Biotechnologies, The Biotechnology Centre, Nkolbisson, University of Yaoundé 1, B. P. 3851, Cameroon.
*Correspondence: **Rhoda N. Bughe;** *rhodabughe@yahoo.com* or *rhoda.bughe@uy1.uninet.cm*, **Wilfred F Mbacham;** *wfmbacham@yahoo.com*

Abstract

Nutritionally, shrimps are rich in proteins to man and also contribute to income generation worldwide. Unfortunately, cases of mortality of shrimps have been reported in countries where shrimps aquaculture is practiced. *Vibrio* species have presented serious health problems to shrimps and humans. This study, sought to investigate the occurrence and antibiotics susceptibility pattern of *Vibrio* species from shrimps: *Parapaeopsis atlantica*, *Penaeus notialis* and *Penaeus kerathurus*. Shrimp samples (121) were obtained from the Kribi coastal water, Cameroon. 0.1 ml of 10^3 dilutions of a prepared homogenate was used to inoculate thiosulphate citrate bile salt sucrose agar plates. Presumptive identification of isolates was done through morphological and biochemical tests, genetically confirmed by polymerase chain reactions - restriction fragment length polymorphism (PCR-RFLP). Agar disc diffusion method was used to obtain inhibition zones of antibiotics. Of the 121 shrimp samples, 73 (60.03%) were contaminated with *Vibrio* spp. Five different *Vibrio* species were identified. *Vibrio alginolyticus* (46.2%) record the highest prevalence and *Vibrio* spp, the least (32.2%). *Vibrio parahaemolyticus* and *V. alginolyticus* were present in all shrimp samples. The PCR-RFLP identified all isolates analysed as *Vibrio* species. The phylogenetic tree showed *V. alginolyticus* strains split into 2 clearly different Clades which are Clade 1 and Clade 2 suggesting two population structures. Contrary to the banding pattern of *V. parahaemolyticus* 2 and *V. alginolyticus* 5, suggesting the same genotype, the two species branched in separate sub-trees. *Vibrio parahaemolyticus* and *V. alginolyticus* exhibited 100% susceptibility pattern to Ciprofloxacin, Netilmicin, and Chloramphenicol. *V. alginolyticus* varied in their susceptibility to Ceftazidime, Streptomycin, and Norfloxacin while *V. parahaemolyticus* showed 100%. 80% and 100% resistance to Penicillin G was recorded by *Vibrio parahaemolyticus* and *V. alginolyticus* respectively. *V. parahaemolyticus* and *V. alginolyticus* are implicated in vibriosis and gastroenteritis in shrimps and humans respectively. Their presence is of public health implication. The use of antibiotics: Ciprofloxacin, Netilmicin, and Chloramphenicol, and adequate processing of shrimps before consumption is recommended.

Keywords: *Vibrio*, *Shrimps*, Antibiotics susceptibility, PCR-RFLP, Coastal, Cameroon.

1. Introduction

Shrimps constitute healthy food and an important source of income in many countries. The emergence of new pathogenic bacteria in shrimps presents a serious health risk to shrimps(Lafferty et al., 2015), and consumers (Villena, 2003). Although *Vibrios* form part of the autochthonous microflora of the marine environment and are incorporated in live fish and shellfish. Vibriosis is a secondary bacterial disease caused by *V. parahaemolyticus*, *Vibrio harveyi*, and *V. alginolyticus* in immune suppressed shrimps (Jayasree et al., 2006) and other aquatic animals especially in aquaculture farms (Jiravanichpanisal et al., 1994; Jayasree et al., 2006; Karunasagar et al., 1994; Srinivasan and Ramasamy, 2009; Rao and Surendran, 2013).

Merwad et al. (2011) reported many *Vibrio* species in seafood: *V.parahaemolyticus*, *Vibrio. vulnificus*, *V. fluvialis*, *V. hollisae*, *V. furnissii*, *V. mimicus*, *V. alginolyticus* and *V. damsella* which were known to be related with human diseases. The clinical manifestations differ; varying from gastroenteritis, septicemia to wound infections (Gopal et al., 2005). *Vibrio parahaemolyticus* pathogenic potential have been demonstrated in the following studies of Merwad et al.(2011),Wang et al.(2012), Cruz et al.(2015), and Letchumanan et al. (2015).

Several outbreaks of seafood poisoning have been reported in many countries including USA, Japan, India, and Taiwan due to consumption of raw or improperly processed seafood that harbour certain strains of pathogenic *Vibrio* species (Abd-Elghang and Sallam, 2013). High nutritive content of seafood like lobsters, crabs, shrimps and cockles serve as a superb medium for the growth of microorganisms especially *Vibrio* species in the aquatic environment and are regularly reported there (Abd-Elghany and Sallam, 2013**)**.

Vibrio species have been isolated from seafood in Malaysia (Elhadi et al., 2004), Egypt (Merwad et al., 2011; Abd-Elghang and Sallam, 2013), Nigeria (Adebayo-Tayo and Okpo, 2010; Adebayo-Tayo et al. 2011a,b; Ashiru et al., 2012). In Cameroon, *Vibrio* spp have been reported in fresh water shellfish and fish (Ndip et al., 2002; Donkeng et al., 2012; Koji et al., 2015). Bughe et al. (2016)reported a high prevalence of *Vibrio parahaemolyticus* in *Penaeus monodon* from the Douala coastal water. Although *Vibrio* sp. had been reported in penaeid shrimps in Cameroon, there was however no report on the occurrence of *Vibrio* species in penaeid shrimps of the following species; *Penaeus notialis*, *P. kerathurus* and *Parapenaeopsis atlantica* of the Kribi coastal water. These shrimps are regularly consumed by the indigenous population and tourists who visit Kribi.

Various biochemical techniques have been used for the diagnosis of shrimp pathogens. Otta et al. (2000) used biochemical tests to identify *Vibrio* spp in *Penaeus monodon* in hatcheries in India. The Analytical Profile Index (API) 20E kit is one of such biochemical techniques (Montieri et al., 2010; Liang et al., 2013; Ransangan et al., 2013). However, biochemical techniques have setbacks like difficulties in identifying strains of the same species thereby rendering the procedure laborious and time consuming. Molecular technique such as polymerase chain reaction-restriction fragment length polymorphism (PCR - RFLP) has been recommended as the fastest, reliable and a very sensitive method to characterize microorganisms. Molecular characteristics are often used to distinguish species of the same genus. Each species of bacteria may have distinct molecular characteristics. One of such technologies can identify *Vibrio* species and targets and analyses the 16S rDNA gene of isolated strains (Felix et al., 2011).

Antibiotics are used in aquatic systems and livestock to treat or prevent the proliferation of disease causing bacteria. Most commonly used antibiotics are penicillin, Amoxicillin, Ampicillin, erythromycin, chloramphenicol, quinolones, tetracycline (Manage, 2018). Some of these antibiotics have developed resistance to the animal and human pathogens (Sudha et al., 2014; Molitoris et al., 1985). Any drug abuse in the environment ends up in the aquatic systems, the aquatic microbes for example *Vibrio* sp. are exposed to these antibiotic agents (Duran and Marshall, 2005). Thus, 'in appropriate used of antibiotic agents provide a favourable conditions for resistant bacteria to develop' (Manage, 2018). *Vibrio* sp. multidrug resistant incidences are reported in many parts of Cameroon and the world (Molitoris et al., 1985, Costa et al., 2015, Ndip et al., 2002). In Cameroon, drugs susceptibility testing was reported in *Vibrio cholerae*, *Vibrio parahaemolyticus* and *V. alginolyticus* from aquatic sources from Limbe and Douala (Ndip et al., 2002; Akoachere et al., 2013). However, it is more than a decade since drugs susceptibility was tested in *V. alginolyticus* and *V. parahaemolyticus* and given that these species were not from the same locality but

were obtained from a more cosmopolitan coastal area, it was of essence to revisit this aspect and check on the evolution of drug resistance. This study aimed at investigating the occurrence of *Vibrio* species and susceptibility to antibiotics in penaeid shrimps from Kribi in Cameroon for the purpose of ascertaining the quality of the shrimps consumed by the public.

2. Materials and Methods

2.1 Study site

Kribi, the capital city of the South region of Cameroon is located at latitude 02°56′06″N and longitude 09°54′36″E. According to Köpper's classification system, Kribi has a tropical monsoon climate (Foahom and Jonker, 1992) with four seasons: two rainy and two dry seasons. The main annual temperature is about 26º C. The annual rainfall is 36 ± 39 mm. The soil is well drained, very deep, yellowish brown clay, loams to light clays (Tomber, 1993). The vegetation in the study area form part of the domain of humid evergreen forest (Foahom and Jonker, 1992). The main activities in the area are fishing, hunting, subsistence agriculture, and small scale business. Shrimp samples were collected in all the shrimp landing sites of Kribi.

2.2 Sample collection

Following monthly collections of 9 to 11 shrimps per month, a total of 121shrimps of the *Penaeus* and *Parapenaeopsis* genera were sampled from Kribi coastal water from May 2014 to April 2015. Of the 121 shrimps, 44 were *Penaeus kerathurus*, 36 *Penaeus notialis*, and 41 *Parapenaeopsis atlantica*. Morphological identification of the shrimps were done following identification Guide for Fishery Purposes (Fransen, 2014) and confirmed by Fisheries experts in the University of Buea (FAREM). The shrimp samples were packed in sterile polythene bags, placed in an ice box, transported and analysed within 20 hours at the Food and Drug Safety Laboratory of the Biotechnology Center, Nkolbission, University of Yaoundé I, Cameroon.

2.3 Sample processing for bacteria isolation

Each shrimp was weighed. The gills were aseptically removed using a pair of sterile scissors and forceps and then weighed. They were put in a mortar macerated using a pestle. Five ml of sterile distilled water was poured into the macerated gills and a homogenate was formed. A volume of 1 ml homogenate was pipetted into a test tube containing 9 ml of alkaline peptone water and incubated at 37º C for 18 hours in an incubator (G-cell, Italy). A volume of 1 ml of the culture was pipetted and a tenfold serial dilution of up to 10^{-3} was performed. A volume of 0.1ml of the 10^{-3} dilution was used to inoculate thiosulphate citrate bile salt sucrose (TCBS) agar (Liofilchem, Italy) plate using the spread plate technique. The procedure was repeated for intestines and hepatopancreas, with the exception of disinfecting the skin surface by wiping with cotton soaked with 75% alcohol (Fawole and Oso, 2001).

2.4 Isolation, morphological and biochemical identification of bacteria isolates

Discrete green and yellow colonies were aseptically isolated and sub-cultured unto freshly prepared nutrient agar plates by streak technique to obtain pure colonies of the isolates. The discrete colonies were characterised macroscopically (colony morphology on culture plate), microscopically (motility test and Gram reaction), and biochemically; oxidase, catalase, salt tolerant test and the identity were confirmed by commercially available miniaturized systems; the Analytical Profile Index (API) 20 E kit (BioMerieux SA, Marcy L'Etiole France). The bacterial suspension for the API 20E test was prepared in 5 ml of 2% Sodium Chloride (NaCl) solution instead of the recommended 0.85% NaCl medium which failed until after this modification. The incubation time and temperature were maintained within the limits prescribed by the supplier (37 ± 2°C). The result was interpreted using the API 20E catalogue; version 4.0 data base. The isolates were stored at -20°C in cryotubes containing Tryptic soy broth medium supplemented with 20% glycerol for further analysis.

2.5 Molecular identification

In June, 2017, genotypes of 50 (fifty) *Vibrio* isolates: 14 *V. parahaemolyticus*, 16 *V. alginolyticus* and 20 of the non-specific *Vibrio* were analysed. *Vibrio fluvialis*, *Vibrio mimicus* and *Vibrio vulnificus* which were identified biochemically died before the genotypic analysis.

2.5.1 Chromosomal DNA extraction by chelex ® 100 resin

This study used the heat chelex extraction method of Nishiguchi et al. (2002). A volume of 200µl of chelex buffer was pipetted into 1.5ml labelled eppendorf tubes. A colony of young bacterium culture cell was isolated with a sterile plastic loop and inserted into the 1.5 ml tube. The tube was vortexed for 5 seconds after which 2µl of proteinase K was pipetted into the tube and incubated at 56ºC for one hour in a heat chamber.

The tube was then further incubated at 100ºC for 10 minutes. After incubation, the tube was vortexed for 5 seconds and centrifuged in a micro-centrifuge at 13000 rpm for 5 minutes. The supernatant containing chromosomal DNA was transferred into a new labelled tube and stored at -20 ºC as chromosomal DNA template.

2.5.2 Polymerase Chain Reaction

The 16SrDNA gene was amplified as described by Yoon et al. (2003). Briefly, the amplification was carried out using a T3 thermal cycler (Biometra, UK).

Each PCR cycle was performed in a total volume of 50µl containing 36.5 µl of nuclease free water, 5.0µl of 10X thermopol® buffer (New England Biolabs), 1µl of 10mM dNTPs (200µM of each deoxyribonucleotide), 0.5µl of 0.1M each primer (Universal primers; FD2, 5'-AGAGTTTGATCATGGCTCAG-3'; RP1, 5'-ACGGTTACCTTGTTACGACTT-3'), 0.5µl of 5Units/µl of Taq polymerase and 6µ of bacterium DNA extract. Amplification was performed under the following cycling conditions; an initial denaturation at 94º C for 5 minutes, 40 cycles of denaturation (94ºC, 1 min), annealing (65ºC, 1.5 minutes), extension (72ºC, 2minutes), and final extension at 72ºC for 5 minutes.

2.5.3 Gel Electrophoresis

Amplified DNA was examined by electrophoresis on a 1% agarose gel (Seakem, Lonza USA). With a micropipette adjusted to 10µl, the PCR product was gently mixed with 2.5µl of loading buffer and gently loaded into samples wells. A DNA ladder standard (100bp) was also loaded on the first lane at the same time to electrophorese simultaneously at 100volts for 40 minutes. The product was visualized under high performance UV transilluminator (UPV) and photographed using a digital camera.

2.5.4 Restriction fragment analysis

The 16S rDNA genes amplified by PCR were individually digested using three endonucleases (HinfI, NlaIII, and RsaI) according to the manufacturer's instructions by incubation at 37 ºC for 2 hours. The enzymes were chosen based on the report of Yoon et al. (2003). However, an isoenzyme; NlaIII was used instead of Hsp92II. The resulting restriction patterns were revealed using 2.0% (w/v) agarose gel electrophoresis. The distance migrated was visually monitored using tracking dyes (bromophenol blue and xylene cyanol) and the product was visualized under high performance UV transilluminator (UPV) and photographed using a digital camera.

The distance migrated was calculated against the standard DNA ladder and extrapolated from the standard curve. Table 1 was used to compare the genotype of the *Vibrio* isolates (Yoon et al., 2003).

Table 1: Signature restriction fragments and genotypes generated from 16S rDNA gene used as a reference

	Endonucleases					
Bacteria	HinfI		Hsp92 II		Rsa I	
V. alg.	942, 353, 180 bp	(1)[a]	370, 218 bp	(1)	673, 503 bp	(1)
V. par I	942, 353, 180 bp	(1)	370, 218, 180 bp	(2)	673, 503 bp	(1)
V. par II	942, 353, 180 bp		370, 218, 180 bp		673, 450 bp	(1')
V. flu	942, 353, 180 bp	(1)	370, 218, 180 bp	(2)	503, 450 bp	(2)
V. pro I	942, 353, 180 bp	(1)	370, 350, 180 bp	(3)	670 503 bp	(1)
V. pro II	942, 353, 160, 180 bp	(1)	370, 350, 180 bp		670 503 bp	
V. vul I	666, 322, 180 bp	(2)	370, 218, 180 bp	(2)	670 503 bp	(1)
V. vul II	942, 322, 180 bp	(2')	370, 218, 180 bp		670 503 bp	
V. cho	370, 270, bp	(3)	370, 350, 218 bp	(3)	430, 400 bp	(3)
A. hyd	600, 390 bp		790, 170 bp		530, 430 bp	
A. sal	600, 390 bp	(4)	790, 170 bp	(4)	530, 430 bp	(4)
P. shi	600, 390 bp		790, 170 bp		530, 430 bp	
P. pho	650, 320 bp	(5)	560, 350 bp	(5)	520, 490 bp	(5)
E. col	650, 320 bp		410, 370 bp	(6)	520, 490 bp	

Legend: V. alg.:*Vibrio alginolyticus*, V. par: *Vibrio parahaemolyticus*, V. flu.: *Vibrio fluvialis*, V. pro.: *Vibrio proteolyticus*, V. vul.: *Vibrio vulnificus*, V. cho.:*Vibrio cholerae*, A. hyd.: *Aeromonas hydrophila*, A. sal.: *Aeromonas salmonicida*, P. shi.: *Plesiomonas shigelloides*, P. pho.: *Plesiomonas phosphoreum*, E. col.: *Escherichia coli* , ((1)-(6), a); genotype, 1' and 2'; genotypes that are different from 1 or 2 by a band, bp: base pairs, Yoon et al. (2003).

2.5.5 Phylogeny Cladogram analysis

A rectangular binary matrix was used to score the absence ("0") or presence ("1") of bands generated from the enzymatic digestion of the 16S rDNA gene. Using this scoring system a genetic distance matrix was produced with PhylTools v1.32 software based on Jaccard's genetic distance formula described below.

The resulting distance matrix was exported in MEGA v6.0 for distance tree building.

$$Dij = 1-[a/(a+b+c)]$$

a = number of bands common to isolates i and j
b = number of bands present in i but absent in j
c = number of bands present in j but absent in i

The resulting distance matrix was used to infer the genetic distance tree based on the Neighbor-Joining algorithm implemented in MEGA version 6.0 software.

2.6 Antibiotics susceptibility test

The disc diffusion method of Bauer et al. in (CLSI, 2016) for antibiotics susceptibility test was used. A total of 40 *Vibrio parahaemolyticus* and 50 *Vibrio alginolyticus* were used for this study. Isolates were sub-cultured overnight at 37^{o}C on nutrient agar plates. Discrete colonies were picked from the plates and put into sterile tubes containing normal saline. Inoculum were made and adjusted to the turbidity of a 0.5 McFarland standard. Each of the inoculum was streaked on Mueller-Hinton agar plate to form a confluent grown. Antibiotics discs were placed on the plates which were incubated for 18h to 24 hours at 37^{o}C. The diameter of the inhibition zones was interpreted according to the Clinical and Laboratory Standards Institute (CLSI) criteria and *Escherichia coli* ATCC 25922 used as control. The different antibiotics used and breakpoints for inhibition zone diameter interpretive standards are listed in Table 2. Enterobacteriaceace breakpoint was used since none exist for *Vibrio* (CLSI, 2016).

Table 2: Antibiotics and breakpoint for zone diameter interpretive standards from CLSI, 2016 used as a reference

Antibiotics	code	Dose	Inhibition Zone diameter interpretive criteria		
			S	I	R
Penicillin G	P	10 unit	≥22	12-21	≤11
Ceftazidime	CAZ	30µg	≥21	18- 20	≤17
Streptomycin	S	10µg	≥15	12-14	≤11
Netilmicin	NET	30µg	≥15	13-14	≤12
Ciprofloxacin	CIP	5µg	≥21	16-20	≤15
Norfloxacin	NOR	10µg	≥17	13-16	≤12
Chloramphenicol	C	30µg	≥18	13-17	≤12

Legend: S= sensitive, I= intermediate, R= resistance, µg = microgram.

3. Results

3.1 Prevalence of *Vibrio* species in shrimp samples from Kribi coastal water

From the 121 shrimp samples collected, 73 (60.03%) shrimps were contaminated with bacteria. From the 266 *Vibrio* isolates identified,123 isolates of *Vibrio alginolyticus* were recorded, 48 isolatesof *Vibrio parahaemolyticus*, 5 isolates of *Vibrio vulnificus*, 3 isolates of *Vibrio fluvialis*, 1 isolate of *Vibrio mimicus*), and 86 other *Vibrio* sp.. The prevalence of *Vibrio* species obtained from the three Penaeid shrimps were as follow: 46.2% of *Vibrio alginolyticus*, 18.0% of *Vibrio parahaemolyticus*, 2.12% of *Vibrio vulnificus*, 1.1% of *Vibrio fluvialis*, 0.3% of *Vibrio mimicus* and 32.3% of other *Vibriospp* (Table 3). Among the five species,*Vibrio alginolyticus* was the most frequently isolated (46.2%) followed by *Vibrio parahaemolyticus* (18.0%). The overall prevalence of *Vibrio* species isolated from each of the three penaeid shrimps species in ascending order was 25.56% in *Parapenaeopsis atlantica*, 35.34% in *Penaeus notialis* and 39.10% in *Penaeus kerathurus* (Table 3).*Vibrio fluvialis* was absent in *Penaeus notialis* while *V. mimicus* and *V. vulnificus* were absent in *Parapenaeopsis atlantica* and in *Penaeus kerathurus* (Table 3).

Table 3: Prevalence of *Vibrio* isolates isolated from the different species of shrimps collected from the Kribi coastal water in Cameroon

Vibrio species	*Parapenaeopsis atlantica*	*Penaeus kerathurus*	*Penaeus notialis*	*Total*
V. alginolyticus	25(9.40%)	50 (18.80%)	48 (18.05%)	123 (46.2%)
V. parahaemolyticus	14(5.26%)	16(6.02%)	18 (6. 78%)	48 (18.09%)
Vibrio fluvialis	1 (0.38%)	2 (0.75%)	0	3 (1.1%)
Vibrio minicus	0	0	1(0.37%)	1 (0.3%)
Vibrio vulnificus	0	0	5(1.87%)	5 (2.1%)
Vibrio sp.	28 (10.52%)	36 (13.53)	22(8.27%)	86 (32.3%)
total	68(25.56%)	104(39.10%)	94 (35.34%)	266 (100%)

3.2 Molecular identification of the 16S rDNA gene and genotypes of *Vibrio* species in Kribi's Shrimps based on PCR- RFLP using three restriction endonucleases

All the 50 *Vibrio* isolates used for Polymerase chain reaction after chromosomal DNAs extraction produced a single band of about 1.5kb DNA that corresponded to the predicted size of the 16S rDNA gene of genus *Vibrio*.

Digestion of the amplified 16S rDNA gene with three endonucleases, gave rise to 1-5 restriction patterns for the 50 *Vibrio* isolates isolated from shrimps. Each unique restriction pattern consisted of 3 to 6 clearly distinct DNA bands. Among the enzymes used, *Rsa1* produced the most diverse restriction patterns from the isolates tested followed by *Nla111*. *Hinf1* produced only one pattern. The three restriction endonucleases total 15 signature restriction fragments and 6 genotypes from the fifty *Vibrio* isolates that were analysed (Fig. 1, 2, 3 and Table 4). Strain *V. alginolyticus* 4 and strain *Vibrio* sp. 1 had the same genotype (genotype 4), while strains *V. alginolyticus* 5, *Vibrio parahaemolyticus* 2 and *Vibrio* sp. 2 had the same genotype (genotype 5) (Table 4). On the contrary, strains of *V. alginolyticus*(1, 2, 3) and *V. parahaemolyticus* (1) had unique genotypes, 1, 2, 3 and 6, respectively (Table 4).

From the signature restriction fragment results, one of the non-specific *Vibrio* (strain No. 1) could be classified as *Vibrio alginolyticus* (strain No. 4) and strain No. 2 of *Vibrio sp*. could either be classified as *Vibrio alginolyticus* (strain No. 5) or as *V. parahaemolyticus* (strain No. 2) (Table 4).

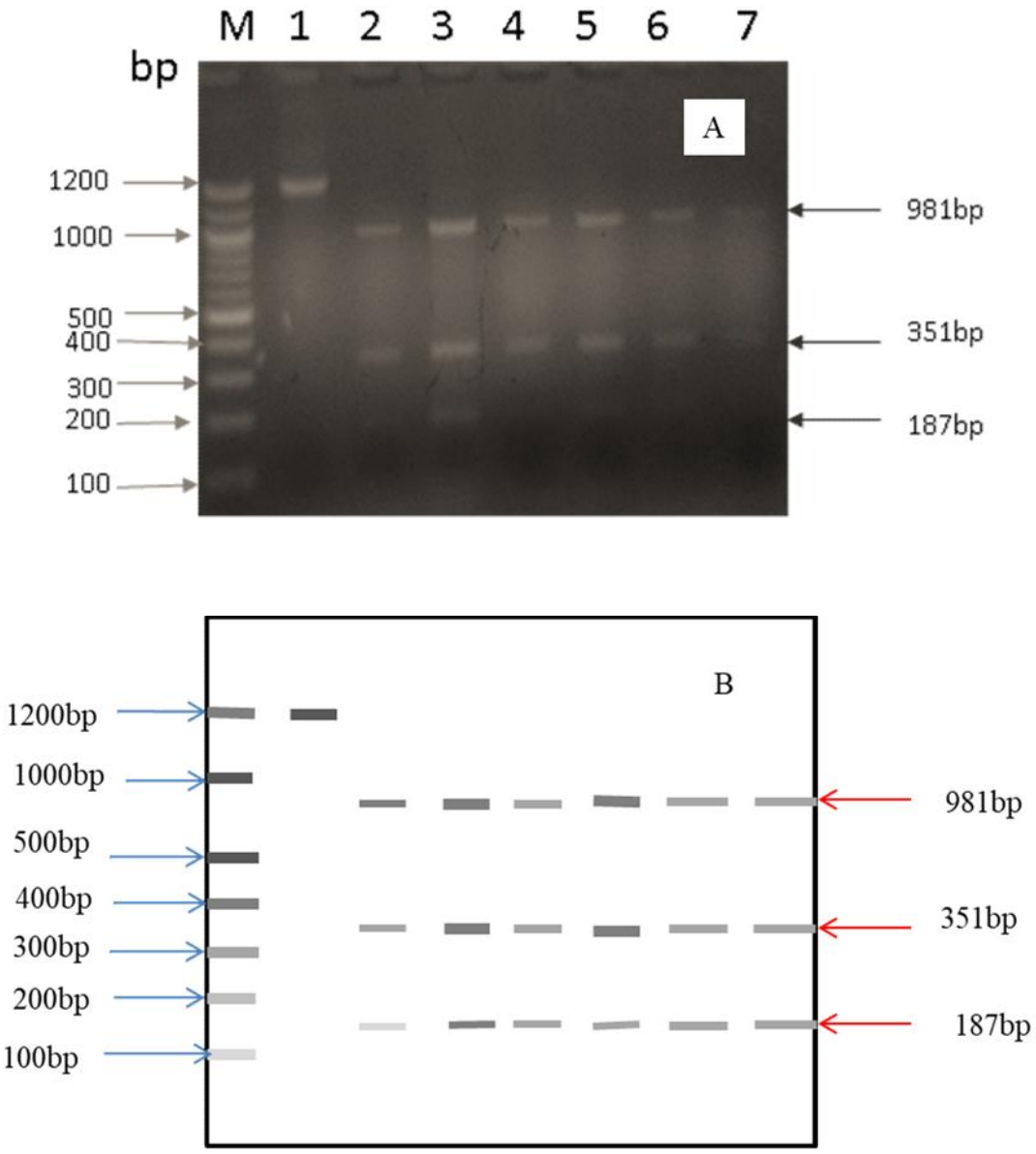

Fig.1. A: Electrophoregram showing the HinfI-RFLP of 16S rDNA gene of *Vibrio* isolates

Legend: lane M: molecular marker, lane 1: No restriction, lane 2: *V. alginolyticus*, lane 3: *V. parahaemolyticus*, lane 4: *V. alginolyticus*, lane 5: *V. parahaemolyticus*, lane 6: *V. alginolyticus*. Lane 7: *Vibrio* species, RFLP: restriction fragment length polymorphism.

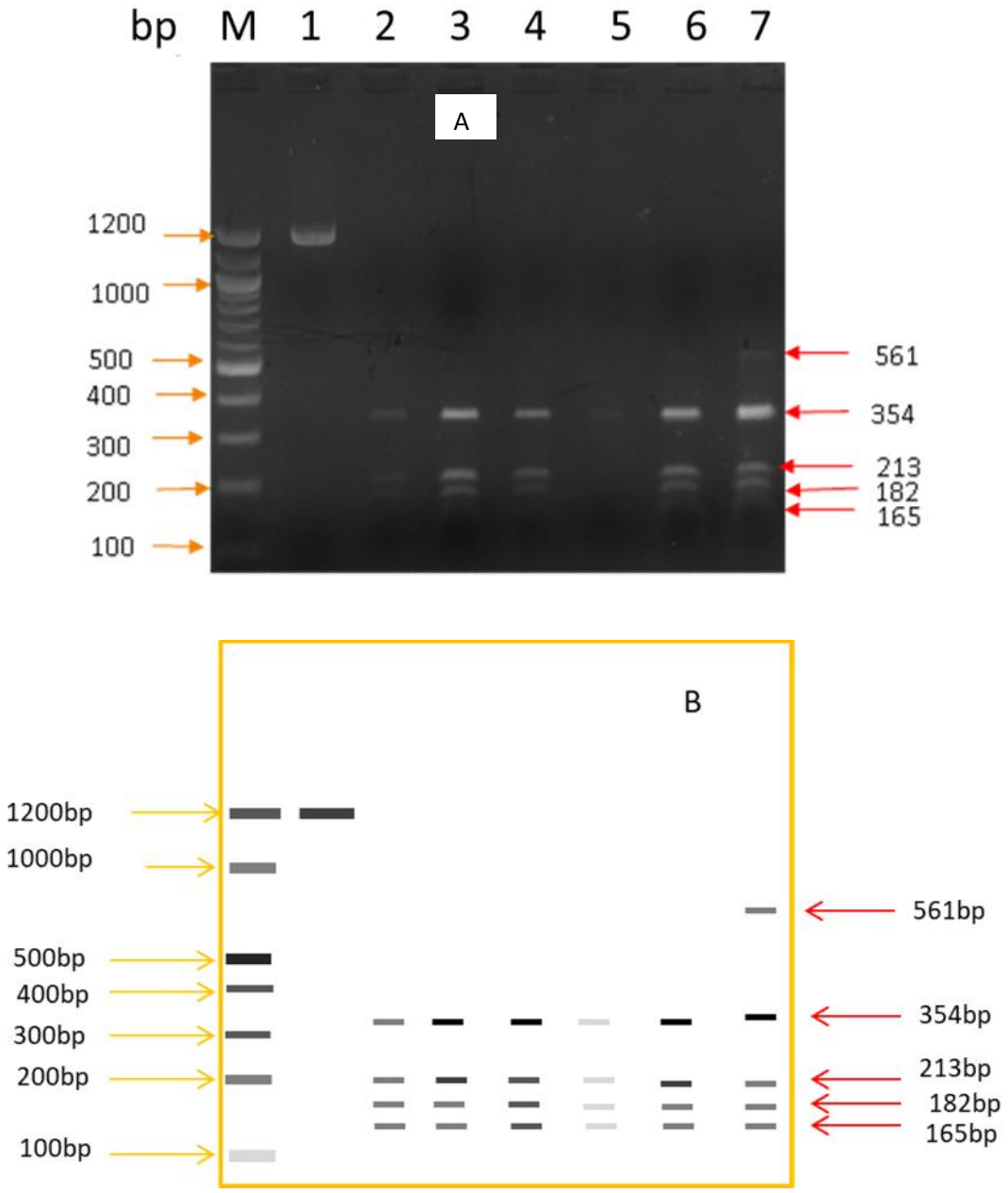

Fig. 2. A: Electrophoregram showing the NlaIII-RFLP of 16S rDNA gene of *Vibrio* isolates

Legend: lane M: marker, lane 1: No restriction, lane 2: *Vibrio*species, lane 3: *V. parahaemolyticus*, lane 4: *V. alginolyticus*, lane 5: *V. parahaemolyticus*, lane 6: *V. alginolyticus*. Lane 7:*V. alginolyticus*, RFLP: Restriction fragment length polymorphism.

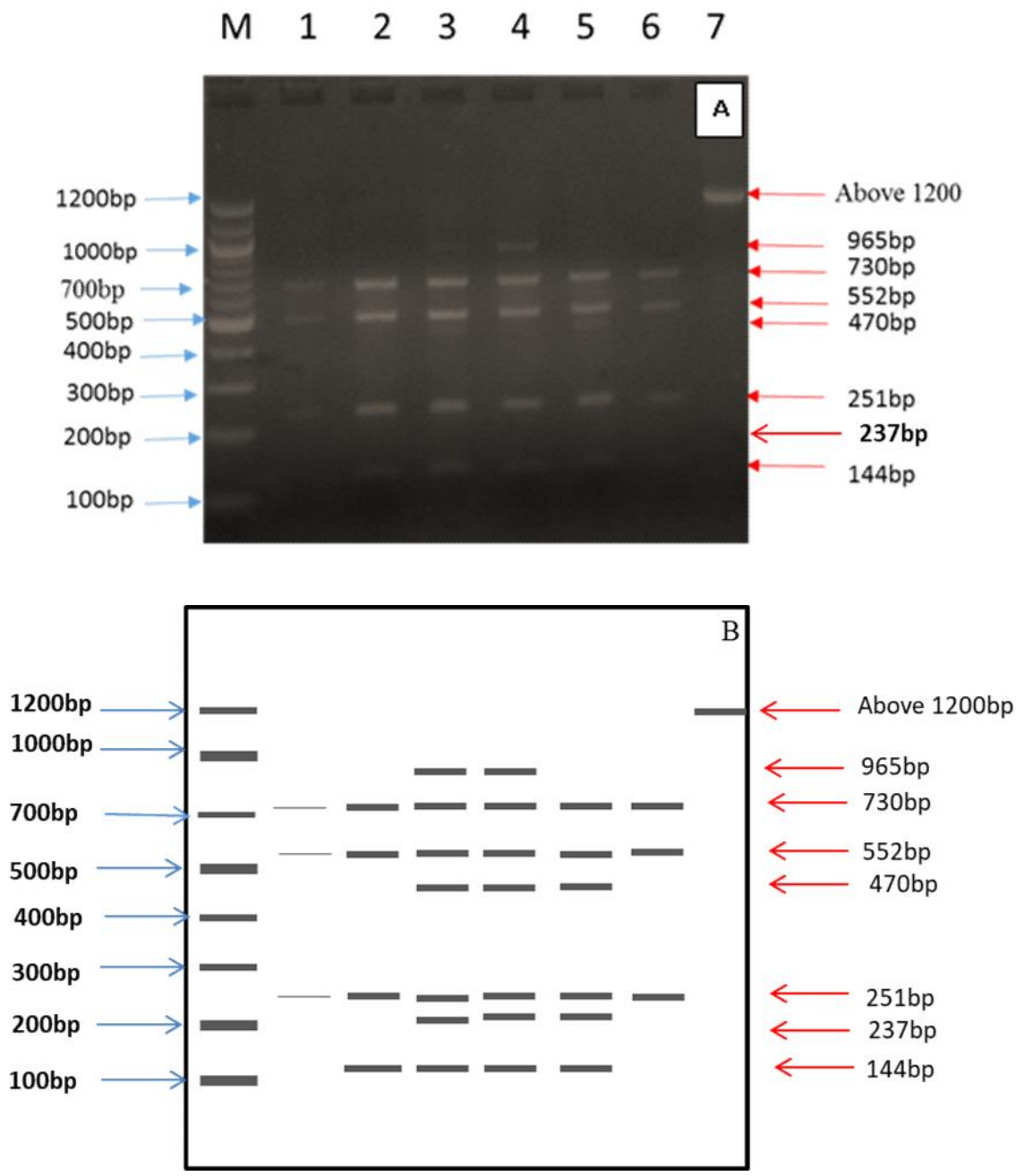

Fig. 3.Electrophoregram showing the different RsaI-RFLP of 16S rDNA gene of *Vibrio* isolates

Legend: lane M: marker, lane 1: *Vibrio* species, lane 2: *V. alginolyticus*, lane 3: *V.parahaemolyticus*, lane 4: *V. alginolyticus*, lane 5: *V. parahaemolyticus*, lane 6: *V. alginolyticus*. Lane 7: No restriction, Bp; base pair, RFLP: restriction fragment length polymorphism

NB: The 237bp band was visible with the naked eyes on this gel but the camera resolution was not sensitive enough to pick all the images on lane 3 (*Vibrio alginolyticus)* and lane 4 (*Vibrio parahaemolyticus*).

Table 4: Signature restriction fragments and genotypes generated from 16S rDNA of *Vibrio* species using three restriction endonucleases.

Vibrio strains	Endonucleases			Total Gen.
	HinfI	Nla III	Rsa I	
*V. alg.*1[b]	981, 351, 187 bp (1) [a]	561, 354, 213, 182, 165 bp (1)[a]	965, 730, 552, 470, 251, 237, 144 bp (1) [a]	1
2				2
3	981, 351, 187 bp (1)	561, 354, 213, 182, 165 bp (1)	965, 730, 552, 470, 251, 144 bp (2)	3
4				4
5	981, 351, 187 bp (1)	354, 213, 182, 165 bp (2) 354, 213, 182, 165 bp (2)	730, 552, 470, 251, 237 bp (3)	5
	981, 351, 187 bp (1)	354, 213, 182, 165 bp (2)	730, 552, 251, 144 bp (4)	
	981, 351, 187 bp (1)		730, 552, 251 bp (5)	
V. par. 1	981, 351, 187 bp (1)	354, 213, 182, 165 bp (2) 354, 213, 182, 165 bp (2)	965, 730, 552, 470, 251, 237, 144 bp (1)	6
2				5
	981, 351, 187 bp (1)		730, 552, 251 bp (5)	
V. sp. 1	981, 351, 187 bp (1)	354, 213, 182, 165 bp (2) 354, 213, 182, 165 bp (2)	730, 552, 251, 144 bp (4)	4
2				5
	981, 351, 187 bp (1)		730, 552, 251 bp (5)	

[a]Genotypes of this study, [b]show the different strains of the *Vibrio* species from shrimps of the Kribi coastal water.
Legend: V.alg.; *Vibrioalginolyticus*, V. par.; *Vibrio parahaemolyticus*, V. sp.; *Vibrio sp*., Gen.; genotypes.

3.3 Phylogenetic relationships among *Vibrio* species

The distance tree showed that *V. alginolyticus* species split into 2 different clear clusters (Clade 1) and (Clade 2) suggesting two population structures (Fig. 4). Contrary to the banding pattern of *V. parahaemolyticus* 2 and *V. alginolyticus* 5 suggesting the same genotype, the tree showed these two species branched in separate sub-trees (Fig. 4). Furthermore, unknown species of *Vibrio* (*Vibrio* sp. 1 and *Vibrio* sp. 2) are branched basal or are outlier to the *V. alginolyticus* 3/ *V. alginolyticus* 1/ *V. alginolyticus* 2 and *V. parahaemolyticus* 1/ *V. parahaemolyticus* 2 clusters, respectively.

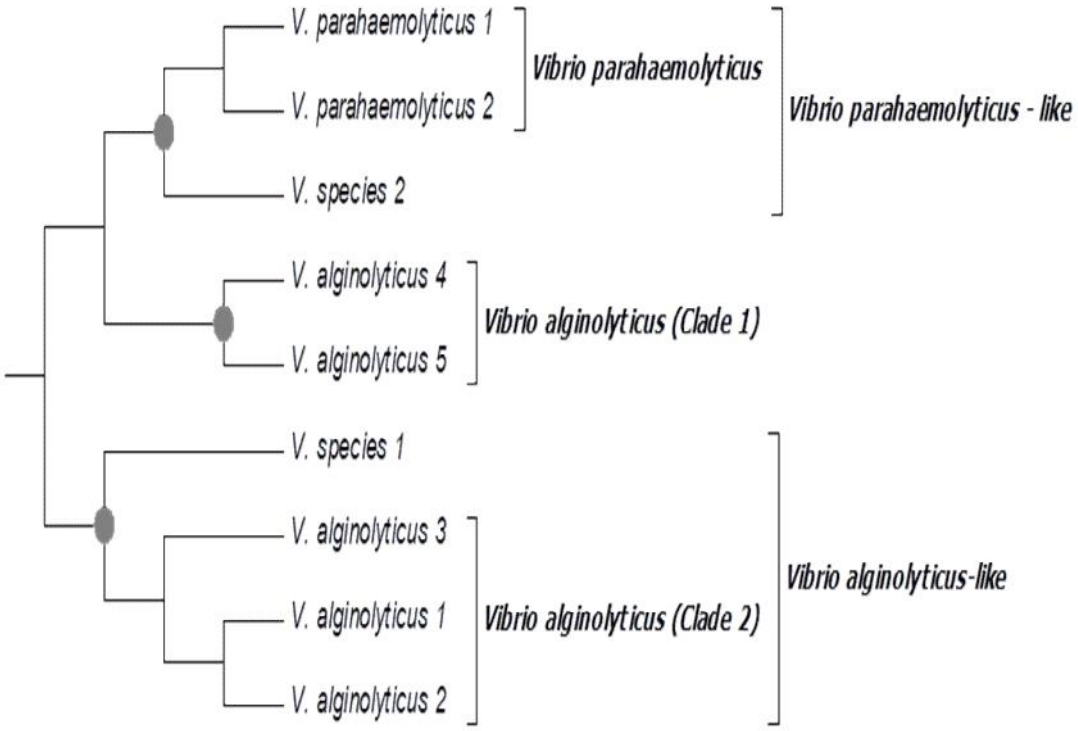

Figure: 4 Genetic distance tree inferring the relationship between *Vibrio* species/strains detected in Kribi, based on 16S rDNA RFLP Jaccard genetic distance. The tree shows three major clusters suggesting three different Vibrio genetic structures. *V. alginolyticus* groups into two different populations (Clade 1 and Clade 2). Grey nodes indicate the most recent common ancestor to members of the same cluster.

3.5 Antibiotics susceptibility test

Vibrio parahaemolyticus exhibited 100% susceptibility to Ceftazidime, Streptomycin, Netilmicin, Ciprofloxacin, Norfloxacin, and Chloramphenicol while 80% resistant was observed with Penicillin G. Majority of *Vibrio alginolyticus* isolates were susceptible to Ceftazidime, Streptomycin, and Norfloxacin while a few showed intermediate and resistant reactions. 100% of the isolates were susceptible to Netilmicin, Ciprofloxacin, Norfloxacin, and Chloramphenicol. 100% susceptibility was recorded with Penicillin G (Table 5). Two isolates of *Vibrio alginolyticus* recorded multidrug resistance pattern to Pencillin G, + Ceftrazidime + Norfloxacin in this study.

Table 5: Susceptibility pattern of *Vibrio parahaemolyticus* and *V. alginolyticus* to selected antibiotics

Antimicrobial agents	Dose	Vp., n=40			V. alg., n=50(%)		
		S	I	(R)	S	I	R
P	10 unit	3(7.5%)	5(12.5%)	32 (80%)	0	0	50(100%)
CAZ	30µg	40(100%)	0	0	46(92%)	0	4(8%)
S	10µg	40(100%)	0	0	48(96%)	2(4%)	0
NET	30µg	40(100%)	0	0	50(100%)	0	0
CIP	5µg	40(100%)	0	0	50(100%)	0	0
NOR	10µg	40(100%)	0	0	46(92%)	2(4%)	2(4%)
C	30µg	40(100%)	0	0	5(100%)	0	0

Legend: S: sensitive, I :intermediate, R: resistance, Vp.: *Vibrio parahaemolyticus*, V. alg.:*Vibrioalginolyticus*. P: Penicillin G, CAZ: Ceftazidime, S: Streptomycin, NET: Netilmicin, CIP: Ciprofloxacin, NOR: Norfloxacin, C: Chloramphenicol

Discussion

This is the first study in Cameroon to investigate the presence of *Vibrio* spp. in *Parapenaeopsis atlantica*, *P. kerathurus* and *P. notialis*. A high frequency of isolation of *Vibrio* from these shrimps was recorded (60.03%). Here five different *Vibrio* isolates and non-specific *Vibrio* isolates were macroscopically, morphologically and biochemically identified. Molecular identification produced sizes consistent with the expected size for 16S rDNA genes of *Vibrio* genus (Felix et al., 2007). Genotypically, some isolates were identified as *V. alginolyticus*, *V. parahaemolyticus*, and some strains as *V. parahaemolyticus* or *V. alginolyticus*. The non-specific *Vibrio* isolates were genotypically identified as outliers of *Vibrio alginolyticus* and *Vibrio alginolyticus* or *V. parahaemolyticus*. The signature restriction fragments and genotype generated from the three restriction endonucleases in this study differ with that of Yoon et al. (2003).

A key difference from that of Yoon et al. (2003) as observed in this study was that many more different band sizes were recorded when *V. alginolyticus* and *Vibrio parahaemolyticus* were digested with *Rsa1* and NlaIII restriction endonucleases. The band size could however be as a result of mutation in the genes over time. The ecological differences could also be the reason why the band sizes and genotypes of this study differ from that of Yoon et al. (2003). In the study by Urakawa et al. (1999), the authors also reported one restriction fragment pattern when the 16S rDNA of reference strains of these two species were digested with *Rsa1*.

From the signature restriction fragments, some *Vibrio* isolates were outlier of *V. alginolyticus* and *V. parahaemolyticus*. This observation is in accordance with the report of Oberbeckmann et al. (2011) who observed the close relationship between *V. alginolyticus* and *V. parahaemolyticus* group, reference strains of both species being positioned very close together on the gene tree. This study confirms the report of Kita-Tsukamoto et al. (1993) and Robert-Pillot et al. (2002) that *V. Parahaemolyticus* and *Vibrio alginolyticus* are hard to distinguish due to their close phylogenetic relationship. However, our phylogeny cladogram showed that *V. parahaemolyticus* and *V. alginolyticus* fall on clearly distinct clusters. Probably, a significant distinction between *V. alginolyticus* and *V. parahaemlyticus* isolates could not be achieved using RFLP 16Sr RNA gene analysis, indicating the need for more sensitive methods. Or, that RFLP and the phylogeny cladogram should be considered in combination.

Furthermore, phylogeny cladogram suggests that *Vibrio* sp. 2 and *Vibrio* sp. 1 share ancestral relationships with *Vibrio parahaemolyticus* and *Vibrio alginolyticus* respectively. Further characterization of *Vibrio* sp. from Kribi waters could increase the breadth of diversity of *Vibrio parahaemolyticus* and *V. alginolyticus*. In addition, *Vibrio* sp.2 and *Vibrio* sp. 1 could be more closely related to an ancestral *Vibrio* species yet to be sampled or no longer in circulation. Sequencing *Vibrio* isolates from Kribi will provide more insight into *Vibrio* genetic diversity and their phylogenetic relationship as which was simply a size polymorphism study.

Vibrio alginolyticus and *V. parahaemolyticus* that were identified in this study have previously been isolated from seawater, shrimp, sea sediment, pond sediment, shrimp culture ponds, crabs and fish (Ahangarzadeh et al., 2011; Alagappan et al., 2013; Arunagiri et al., 2013; Ashiru et al., 2012; Bughe et al., 2016; Gopal et al., 2005); Khamesipour et al., 2014; Mancuso et al., 2015;Raissy et al. 2015; Ransangan et al., 2013; Srinivasan and Ramasamy, 2009).*Vibrio alginolyticus* is implicated in shrimp diseases: shell disease, red disease, loose shell syndrome, white gut disease (Liu et al., 2004; Jayasree et al., 2006). Although *V. alginolyticus* is considered to be relatively non-pathogenic to human, it has been associated with human disease and reported to cause gastroenteritis (Lee et al., 1997). *Vibrio parahaemolyticus* was reported to be the dominant species in shrimp affected by red disease and tail necrosis (Jayasree et al., 2006). Recently, *V. parahaemolyticus* was reported to be the major agent of acute hepatopancreatic necrosis syndrome afflicting penaeid shrimp, and seriously damaging the shrimp aquaculture industry (Tran et al., 2013). Among the *Vibrio* species reported in this study, *V. parahaemolyticus*, which is one of them has been reported to cause gastro-intestinal problems following the consumption of contaminated seafood. *V. parahaemolyticus* isolated from *Penaeus monodon* in Cameroon demonstrated haemolytic property on blood agar (data not shown) indicating that, there are pathogenic strains of this *Vibrio* isolate in Cameroon. Regarding the zoonotic significance of *Vibrio vulnificus*, they are usually acquired through ingestion of shellfish or through contamination of open wounds during swimming, crabbing, shellfish cleaning and other marine activities (Heelan, 2001) and are implicated in human gastroenteritis, as well as wound

infections, both of which can progress to a fatal septicemiae in individuals who are immuno-compromised (Oliver and Kaper, 2001).

Two strains of *Vibrio alginolyticus* showed multidrug resistance in this study. There was just one multidrug resistance pattern for *Vibrio alginolyticus*. High resistance to Penicillin G by *Vibrio alginolyticus* (100%) and *V. parahaemolyticus* (80%) isolates in this study corroborated the findings of (Molitoris et al., 1985)) whose report records99.7% and 92% resistance of the two Vibrios respectively. Penicillin G resistance to Vibrios has also been reported by Srimivasan and Ramasamy, (2009) and Costa et al. (2015) in different penaeid culture areas and regions. This high prevalence of penicillin G resistance in these two Vibrios is indicative of antimicrobial abuse in the aquatics system and livestock in Cameroon. The findings of this study were similar to those of Jayasree et al. (2006) who registered a high prevalence of ciprofloxacin (100%), and chloramphenicol (100%,) susceptibility in *Vibrio alginolyticus* and in *Vibrio parahaemolyticus* respectively. This the antibiotic susceptibility pattern of these two isolate also corroborate the findings of Oh et al. (2011)who reported92.2% of chloramphenicol and 98.7% of ciprofloxacin susceptibilities in *V. alginolyticus,* and 96.7 % of chloramphenicol and 99.5% ciprofloxacin susceptibility in *Vibrio parahaemolyticus*. In Cameroon, from 2002 till the time of this study, there has not been any significant variation to chloramphenicol susceptibility of *V. alginolyticus* and *V. parahaemolyticus*. In this study 100% susceptibility of chloramphenicol was recorded in *Vibrio alginolyticus* and *V. parahaemolyticus* as opposed to 96.4% and 82.4% of the same antibiotic in *V. parahaemolyticus*and *Vibrio alginolyticus* respectively as shown by Ndip et al. (2002).These two antibiotics results demonstrate that they are still useful in solving infection problems of Vibrios.

Conclusions

This study revealed a high frequency of occurrence of *Vibrio* species (60.03%) in the different penaeid shrimps (*Parapenaeopsis atlantica*, *Penaeus notialis* and *Penaeus kerathurus)*. The different species were identified: *Vibrio alginolyticus* and *V.parahaemolyticus*. *Vibrio vulnificus*, *V. fluvialis*, and *V. mimicus*. These results are also suggesting that the *Vibrio* species of the Kribi coastal water, Cameroon could have different strains of *Vibrio alginolyticus* and *V. parahaemolyticus* or new emerging *Vibrio* species.

Penicillin G was resistance to all the isolates of the two different species of *Vibrio*. Thus, should be rejected for therapy against *Vibrio* infections while chloramphenicol, ciprofloxacin, and netilmicin should be recommended as treatment. The presence of *Vibrio parahaemolyticus* and *V. alginolyticus* in shrimps in this study is an indication of the risk associated with the consumption of shrimps in this location. We recommend proper processing and cooking of shrimps before consumption. These results are of public health significance.

To ascertain the pathogenic potential of these isolates, virulent genes investigation is recommended. Sequencing of the 16S rDNA of the different species of *Vibrio* is required to give a complete identification to the unidentified *Vibrio* isolates.

Conflicts of Interest

The authors declare that, the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. There was no conflict of interest.

Acknowledgments

The Department of Fisheries and Aquatic Resources Management, Faculty of Agriculture and Veterinary Medicine, University of Buea, Cameroon supported this work by purchasing part of the laboratory reagents. This work was carried out at the Food and Drug Safety Laboratory of the Biotechnology Center, University of Yaoundé 1. This Laboratory aided in buying other laboratory reagents and provided a good environment for the successful execution and analysis of this work.

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How to cite this article:

Rhoda N. Bughe, Benedicta O. Oben, Akindeh M. Nji, Jean P. K. Chedjou, Aristid E. Mbange, Innocent M. Ali, Pius M. Oben and Wilfred F. Mbacham. (2020). Occurrence and antibiotics susceptibility of *Vibrio* sp. from Penaeid shrimps from Kribi Coastal Water, Cameroon. Int. J. Adv. Res. Biol. Sci. 7(7): 96-111.
DOI: http://dx.doi.org/10.22192/ijarbs.2020.07.07.012