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Occurrence of common core structure and genotype-specific signatures among the *Vibrio* of environmentally diverse vended crabs and shrimps from selected Lagos seafood markets, Nigeria

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Abstract. *Odeniyi OA, Olawuyi OJ, Ogunsanya O, Unuofin JO. 2022. Occurrence of common core structure and genotype-specific signatures among the* Vibrio *of environmentally diverse vended crabs and shrimps from selected Lagos seafood markets, Nigeria. Biodiversitas 23: 3081-3090.* This study investigated the occurrence of *Vibrio* species from the gastrointestinal tract of vended crabs and shrimps using Multiplex polymerase chain reactions (Multiplex PCR) method. Different primer pairs were used to detect the presence of *hsp*, *sodB*, *SodB-R*, *ctx*, *toxR*, and *Vchol* virulent genes in the samples. The phylogenetic relationships among the *Vibrio* species were determined and the polymorphic information content of selected *Vibrio* primer products was generated using Powermarker V3.25 Dendo-UPGMA program based on Jaccard's coefficient. The detection of *Vibrio* virulence genes confirmed the presence of *Vibrio* species in the DNA extracts of 19 out of the 60 crustacean samples. Among the *Vibrio* species, *Vibrio parahaemolyticus* was the most frequently identified species (47.4%), followed by *Vibrio cholerae* (36.8%), and *Vibrio mimicus* (15.8%), while the presence of *Vibrio vulnificus* was not detected in the crab and shrimp samples. Phylogenetically, the Vp.tdh primer constituted the highest allele number, gene diversity and polymorphic information content of 18.00, 0.9444 and 0.9415, respectively, while the mean of the Vp.tdh and Vp.trh primer sequence combination was polymorphic (0.7153). Among the 19 positive *Vibrio* strains, different major clusters and subclusters were identified, indicating the relatedness between the identified species. The result of this study indicates that the innocuously vended crab and shrimp samples are indeed reservoirs of pathogenic *Vibrio* species whose consumption by humans might affect the health of the general populace.

Keywords: Multiplex PCR, polymorphic information content, vended crustaceans, *Vibrio* species, virulence genes

INTRODUCTION

The changes in nutritional habits have brought an increase in consumption of undercooked or raw foods, especially seafood, such as shellfish, shrimps and some molluscs, thus exposing consumers to gastroenteritis and other diarrheagenic diseases (Newton et al. 2012; Bonnin-Jusserand et al. 2017; Abioye et al. 2021). *Vibrio* is Gram-negative rod-shaped bacteria that occur as part of the native microbiota in aquatic organisms and the surrounding marine environment (Adebayo-Tayo et al. 2011; Singhapol and Tinrat 2020), and their presence abounds in both temperate regions and the tropics. Oyelade et al. (2018) reported the retrieval of *Vibrio* species along some recreational shores in Nigeria while Igbinosa et al. (2021) also isolated the bacterium from ready-to-eat food (African salad). *Vibrio* is regarded as potential human pathogens and can concentrate and proliferate in shellfish gut, and may cause varieties of health issues when ingested by humans (Froelich and Noble 2016; Hikmawati et al. 2019; FAO and WHO 2020). *Vibrio* species, such as *Vibrio cholerae*, cause outbreaks and epidemics of cholera, a serious and potentially fatal gastrointestinal infection and a major health problem, especially in developing countries worldwide (Tack et al. 2020; Sampaio et al. 2022).

Foodborne infections associated with handling and or ingestion of *Vibrio* spp. and other Enterobacteriaceae are common in Africa, presenting a spectrum of clinical conditions, including septicemia, cholera and milder forms of gastroenteritis (FAO and WHO 2020). *Vibrio* species commonly associated with foodborne transmission include *V. vulnificus, V. cholerae* and *V. parahaemolyticus* (Tack et al. 2020; Sampaio et al. 2022). In Nigeria, *Vibrio* species have been consistently identified among the predominant bacterial species present in contaminated marine foods, making them a priority organism (Sangoyomi et al. 2012; Eyisi et al. 2015; Oramadike and Ogunbanwo 2015).

Other infections caused by *Vibrio* species could be transmitted to humans through contact with skin, mucosa or wounds exposed to marine water and consumption of certain undercooked or raw seafood, particularly shrimp, crabs, oysters and clams causing primary septicemia, gastroenteritis and wound infection (Oramadike and Ogunbanwo 2015). For instance, the toxigenic strain of *V. cholerae* could be acquired via the ingestion of contaminated food or water, and is usually associated with poor hygiene and polluted water supplies. *Vibrio*

parahaemolyticus was first implicated in an outbreak of food poisoning in Japan in 1950 (FAO and WHO 2020), and is still implicated in acute diarrhea in human beings (Abioye et al. 2021). The virulence properties of *V. parahaemolyticus* are characterized by the production of thermostable toxins (TDH) and/or TDH-related hemolysin (TRH) encoded by *tdh* and *trh* genes, respectively. Other species, such as shellfish-associated *V. vulnificus* cause septicemia and human vibriosis (Bonnin-Jusserand et al. 2017). Fatalities resulting from *Vibrio* infection have been linked with the production of toxigenic proteins, such as the cardiotoxic activity of thermostable direct hemolysin (TDH) (Takeda 1983). *Vibrio* species have been reported to possess two circular chromosomes of different sizes (Kumar et al. 2020) within which the multiple virulence factors, such as chemotaxis proteins, hemolysins, and antibiotic resistance proteins were detected (Guardiola-Avila et al. 2016). The longer of the two chromosomes carry genes for housekeeping functions, such as the genes for thermostability, while the other carry accessory genes (Kumar et al. 2020; Sampaio et al. 2022).

Phenotypic and biochemical identification of *Vibrio* spp. is fraught with many problems since their outcomes are overlapping or pseudo-positive in nature. Several molecular techniques such as random amplified polymorphic DNA (RAPD), repetitive extragenic palindromes (REP), and amplified ribosomal DNA restriction analysis (ARDRA), along with real-time PCR, DNA sequencing, and microarrays, which utilize nucleotide sequences, have been applied in the detection, identification and characterization of microorganisms. The same was applicable in the detection of *Vibrio* species for epidemiological purposes and to understand their pathogenicity and virulence (Oramadike and Ogunbanwo 2015; Singhapol and Tinrat 2020). Genomic information obtained through the application of Multiplex PCR approach is important from the epidemiological perspective because it provides fast, effective and less time-consuming strategy for identifying and tracking pathogenic *Vibrio* in aquatic samples (Beshiru et al. 2020; Abioye et al. 2021; Igbinosa et al. 2021).

Hence, our investigation aimed at detecting *Vibrio* species associated with the gastrointestinal tracts of retailed crabs and shrimps from two seafood markets in Lagos, Nigeria using Multiplex PCR for a fast identification of *Vibrio* species and screening for their associated virulence. Also the research sought to establish phylogenetic relationship among the *Vibrio* species contained in the sampled crabs and shrimps by consolidating the information on their polymorphism.

MATERIALS AND METHODS

Sample collection and processing

Thirty samples each of crabs (*Callinectes sapidus*) and shrimps (*Penaeus notialis*), sourced from the Lagos lagoon, were bought from Mushin (6º 31' 59.99" N 3º 20' 59.99" E) and Oyingbo (6º 29.1432' N 3º 23.2817' E), two major seafood markets in Lagos State, Nigeria. Three samples each were bought from individual vendors and collected into separate ziplock bags, labeled numerically and placed on ice cubes in a plastic bucket for immediate transfer to the laboratory. The crab and shrimp samples bought from Mushin market were labeled as Crab 1-15 and Shrimp 31-45, respectively. The samples bought from Oyingbo market were labeled as Crab 16-30 and Shrimp 46-60. The shrimp and crab samples were surface sterilized using 70% ethanol for 10 min and rinsed off with sterile distilled water (Darbyshire et al. 2019). The shells of the crustacean heads were aseptically removed using sterile forceps and knife, and the gastrointestinal tract was gently withdrawn using a sterile pair of forceps. One gram (1.0 g) of gut matter was weighed and used for further DNA extraction process (Abioye and Okoh 2018).

DNA extraction and primer sequences

The genomic DNA of the crab and shrimp gastrointestinal samples was prepared using a standard DNA extraction method according to Dellaporta et al. (2014) following the manufacturer's instructions. Briefly, 1 mL of phosphate buffer saline was added to the gut matter (1.0 g), centrifuged at 11,200 g for 5 min and the supernatant was discarded. The Dellaporta extraction buffer (750 µL) and 50 µL of 20% sodium dodecyl sulfate solution were added and mixed thoroughly. The tubes were incubated at 65ºC for 1 min, mixed with 250 µL of 5M potassium acetate, incubated at 65ºC for 10 min and centrifuged at 11,200 g for 20 min. The supernatant containing the genomic extract was transferred into another tube, mixed with 500 µL of isopropanol and incubated at -20ºC for 30 min. The tubes were centrifuged at 11,200 g for 15 min and the pellet obtained was re-dissolved in 700 µL of TE and centrifuged at 11,200 g to pellet insoluble debris. The supernatant was transferred to fresh Eppendorf tube(s), and extracted with equal volume of 1:1 phenol/ chloroform. Isopropanol (500 µL) and 75 µL of 3M sodium acetate were added to the aqueous phase, mixed and centrifuged. The extracted DNA quality was quantified using NanoDrop 2000 (Thermo Fisher, USA). The DNA pellet was washed using 1 mL of ice-cold ethanol, dried and re-dissolved in 100 µL of sterile water.

PCR amplification assay

The genes specific to the 16S rRNA variable regions of *Vibrio* species were employed in a polymerase chain reaction amplification process. This was performed in 25 µL of a reaction mixture to detect the presence of *Vibrio* species using specific primers. The reaction concentration was brought down from 5x concentration to 1x concentration containing 1x Blend Master Mix buffer. Buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphates (dNTP) (Solis Biodyne), 20 pMol of each primer (BIOMERS, Germany), 2 units of Hot FIREPol *DNA* polymerase (Solis Biodyne), Proofreading Enzyme, 5.0 µL of each sample's extracted DNA, and sterile distilled water was used to make up the reaction mixture. The reactions were performed with a PTC-100 thermal cycler (Eppendorf, Hamburg, Germany). The cycling conditions entailed a 5 min initial denaturation step

at 95°C, followed by 34 amplification cycles comprising a 30 s denaturation at 95°C, 30 s annealing at 60°C, with primer elongation at 72°C for 90 s, and a final elongation of 10 min at 72°C. The samples positive for *Vibrio* species were further subjected to another PCR assay to delineate the *Vibrio* species using *Vibrio* species specific primers as outlined in Table 1. The primer sets and their sequences with variable lengths used for the amplification of toxigenic *Vibrio* species-specific genes are shown in Table 1.

Gel electrophoresis and visualization

The amplicons (5 µL) obtained from the Multiplex reactions were separated by loading each lane of a 1.5% agarose gel containing 5 $\mu g.mL^{-1}$ of ethidium bromide in Tris-Borate-EDTA buffer. The extracted DNA from the crab samples was introduced into gel lanes 1-30, while lanes 31-60 contained those from the shrimp samples. The molecular marker (100-1500 bp DNA ladder) was loaded as a comparative standard and the molecules migrated in the electrophoresis system for 1 h at 100 V. The separated PCR amplicons on agarose gels were visualized and photographed using a UV transilluminator (BioDoc-IT System UV Printer by Mitsubishi) and Gel Documentation System.

Molecular statistical analysis

The amplicon bands on the gel were scored using 0 and 1 for the absence and presence of bands respectively. The data from the scores were generated based on binary similarity matrix adopting the Numerical Taxonomic System of Statistics (NTSYS) Version 2.21 statistical software**.** Dendrograms were constructed using Unweighted Pair Group Method of Arithmetic mean (UPGMA). The phylogenetic relationships among the *Vibrio* species and the virulence genes contained in the tested samples were analyzed using Powermarker V3.25 and Dendo-UPGMA program based on Jaccard's coefficient (Liu 2003). The Polymorphic Information Content of *Vibrio* primer products was also generated from the PowerMarker software version 3.25.

RESULTS AND DISCUSSION

The presence of *Vibrio* species was confirmed in the DNA materials of the crab and shrimp samples by PCR amplification of the 16S rRNA gene sequences. The target 663 bp band (Figure 1) revealed that some of the crab and shrimp samples were positive for the presence of *Vibrio* spp. Out of the 60 representative wells, about a third of all shellfish (equivalent to 31.6% of the sample size) observed in 19 wells, were positive for the genus *Vibrio.* The distribution of *Vibrio* positive samples was seen in twelve crab samples which occurred in lanes 1-8, 10, 15, 25, 28, and represented a 63% of the total 19 positive samples. Ten of these *Vibrio* occurred in crabs from Mushin market while the remaining two were found in crab samples from Oyingbo. The remaining seven *Vibrio* positive samples, identified in lanes 36, 37, 38, 49, 54, 58, and 60, all occurred in shrimp samples, three of which were obtained from Mushin and four from Oyingbo market. The shrimp samples (lanes 36, 38, 54, 58 and 60) were confirmed to contain *Vibrio* species, however, none of the target genes tested for was amplified therein. Oramadike and Ogunbanwo (2015) reported the isolation of different *Vibrio* species from seafoods bought from markets in Lagos island, Nigeria. Many researchers employed species-specific primers and Multiplex PCR to determine and identify *Vibrio* species in studied samples (Tarr et al. 2007; Abdelaziz et al. 2017; Oyelade et al. 2018; Igbinosa et al. 2021).

Table 1. Sequence of primers, amplicon size and target gene for *Vibrio* species of the primers

Target species	Targeting gene	Primer sequence (5'-3')	Amplicon size
Vibrio vulnificus	*Hsp*	Vv.*hsp*-326F GTC TTA AAG CGG TTG CTG C Vv.*hsp*-697R CGC TTC AAG TGC TGG TAG AAG	410 bp
Vibrio cholerae	*sodB*	Vc.*sodB*-F AAG ACC TCA ACT GGC GGT A Vc.*sodB*-R GAA GTG TTA GTG ATC GCC AGA GT	248 bp
Vibrio mimicus	*SodB-R*	Vm.*sodB*-F CAT TCG GTT CTT TCG CTG AT Vm.*sodB*-R GAA GTG TTA GTG ATT GCT AGA GAT	121 bp
Vibrio cholerae	*Ctx*	Vc_*ctx*-F AGT TCA TTT TTG CTT G Vc_*ctx*_R GGA AAC CTG CCC ATA A	897 bp
Vibrio cholerae	*ToxR*	Vc_*toxR*_F CGC AAT GAT TTG ACT T Vc_*toxR*_R TTG TGG GGA CTC GAA T	352 bp
Vibrio cholerae	*Vchol*	16S-*Vchol*-F AGA GTT TGA TYM TGG CTC AG 16S-*Vchol*-R GAA ATT CTA CCC CCC TCT AC	700 bp
Vibrio parahaemolyticus	*trh1*	Vp_*trh* 1_F AAA AGC GTT CTC AAT C Vp_*trh* 1_R CCA GAA AGA GCA TTG T	250 bp
Vibrio parahaemolyticus	*trh2*	Vp_*trh* 2_F CCC CAG TTA AAT TGT G Vp_*trh* 2_R AGG CGC TTA ATT TGA A	150 bp
Vibrio parahaemolyticus	*Tdh*	Vp_*tdh*_F CCA TCT GTC CTT CCT G Vp_*tdh*_R TAC GGT TTG TGT CAG A	180 bp
All *Vibrio* spp.		V.16S-700F CGG TGA AAT GCG TAG AGA T V.16S-325R TTA CTA GCG ATT CCG AGT TC	663 bp

Source: (Kim et al. 1999; Tarr et al. 2007; Oramadike and Ogunbanwo 2015)

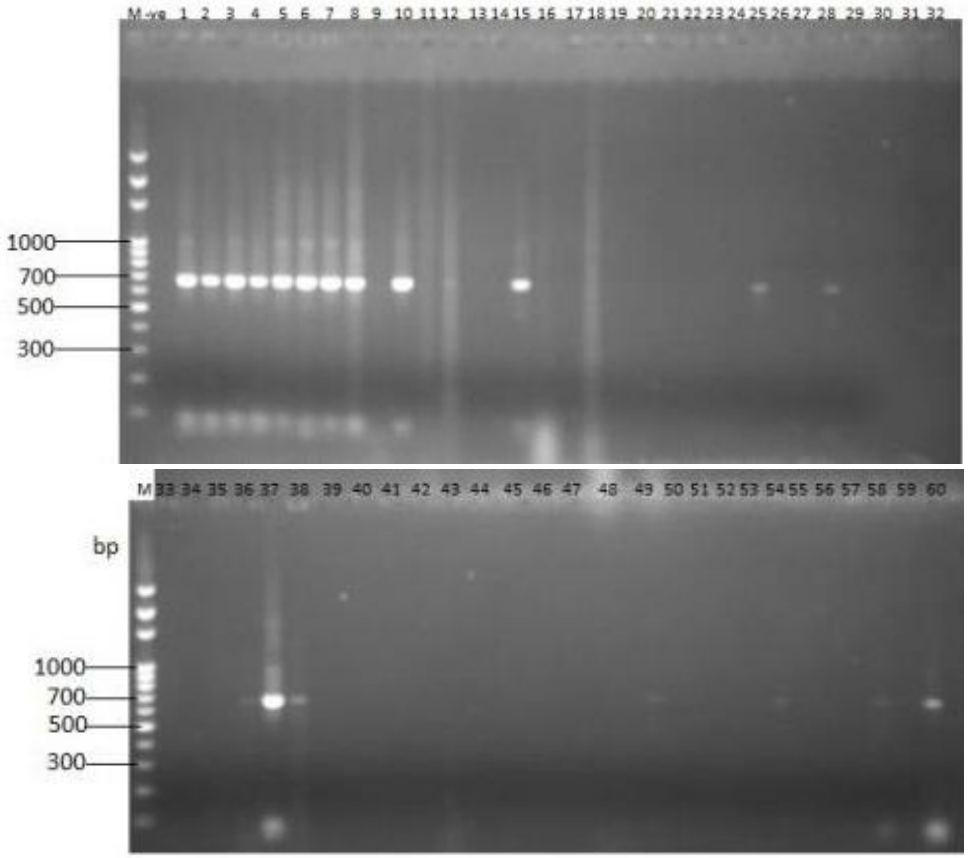

Figure 1. Agarose gel electrophoretogram of PCR products for detection of *Vibrio* species in DNA samples from *Callinectes sapidus* and *Penaeus notialis*

The presence of *tdh*-related hemolysin (*trh*) virulent genes were recorded in 47.4% of the 19 *Vibrio* positive samples and detected in lanes 2, 3, 4, 5, 6, 7, 8, 25 and 28. Three samples (in lanes 2, 5, and 6) demonstrated the presence of both *tdh* and *trh* virulent genes (Figure 2). In these shellfish samples, the presence of virulent strains of *V. parahaemolyticus* was confirmed, although the sequences for the *trh2* target genes were not amplified in any of the 60 samples. It was instructive that the presence of *V. parahaemolyticus* was recorded in only crab samples in this study, contributing to a 47.4% presence in *Vibrio* positive samples and 15% of all crab and shrimp samples tested. 77.8% was from crab samples obtained from Mushin fish market. The virulent *tdh* and *trh* genes amounted to 15.8% of the total *Vibrio*-positive samples.

The detection of the *trh* and both *tdh* and *trh* genes isolates from crabs in this study is quite disturbing as it suggests a public health challenge if these foods are consumed without sufficient proper preparation. This is mainly because these genes have been reported to be responsible for regulating virulence factors (hemolysis and cytotoxicity) in the host cells (Hasrimi et al. 2018), suggesting that these aquatic dwellers might not be safe for consumption directly, since the virulent *V. parahaemolyticus* proteins are able to inactivate the cell membrane and lyse red blood cells (tdh) and contribute in initiating pore-formation (trh), thereby allowing loss of cellular ions and water and cell death. Beshiru et al. (2020) also reported the presence of *trh* and *tdh* genes in the *Vibrio* samples they studied, recording these to be of highest occurrence compared to other *Vibrio* species isolated. Broberg et al. (2011) further reported that the acquisition of a Type III Secretion System (T3SS2) effector in conjunction with *trh* and *tdh* genes results in the diverse pathogenic abilities recorded in different strains of *Vibrio parahaemolyticus*. In addition, human cell lines infected by strains of *V. parahaemolyticus* lacking both *tdh* and *trh* genes still exhibited cytotoxicity (Broberg et al. 2011; Chung et al. 2018). Igbinosa et al. (2021) reported a higher number of *trh* compared to *tdh* genes in their *Vibrio* species as similarly recorded in this study. The occurrence of these genes in *Vibrio parahaemolyticus* from samples obtained from the marine environment is not new, as studies such as that of Hasrimi et al. (2018), reported the genes in samples from aquaculture water. While it should be well stressed that some *Vibrio parahaemolyticus* from environmental samples may lack the presence of virulent genes, thus making them incapable of causing illnesses in their hosts, some studies have also reported a low occurrence of the virulent genes in isolates of environmental origin (Paranjpye et al. 2012; Gutierrez West et al. 2013; FAO and WHO 2020). Singhapol and Tinrat (2020) reported the occurrence of a lecithin-dependent hemolysin gene-containing *V. parahaemolyticus* which lacked both the *tdh*

and *trh* genes and so exhibited less pathogenicity to humans. Although both or either of *tdh* and *trh* virulent genes were detected in the crab samples in this study, none was confirmed in the shrimp samples. This was however not the same with the report of Hossain et al. (2020) who documented 77% *tdh* and 85% *trh* genes specificities in shrimp.

The largest amplicon targeting *V. cholerae* was amplified by V*chol*, confirming the presence of *V. cholerae* in nine samples as shown in Figure 3. The *V. cholerae* occurred in both crab (*n=7*) and shrimp (*n=2*) samples in lanes 1, 4, 5, 7, 10, 15, 25, 37 and 49, representing a 47.4% of all the *Vibrio* species detected. (Beshiru et al. 2020) identified 1.67% *V. cholerae* among groups of ready-to-eat shrimp samples. *Vibrio cholerae* falls among one of the most pathogenic species of *Vibrio* which are pathogenic to human, closely followed by *Vibrio parahaemolyticus* and *Vibrio vulnificus* (Bonnin-Jusserand et al. 2017). Robert-Pillot et al. (2014) identified *V. parahaemolyticus* as the most common species in seafood samples analyzed using Multiplex *q*PCR. Ingesting raw or undercooked seafoods and related products may result in mild zoonotic infections and at times, death from cholera, a diarrheagenic situation generally accompanied with dehydration and vomiting as different toxins are released into the human system by the *Vibrio* species. The *V. cholerae* strains recorded in this work were not toxigenic since there was no amplification of the 248, 897 and 352 bp target Vc-*sodB*; Vc-*ctx* and Vc-*tox* genes in the nine *V. cholerae*-positive samples. Even though these serogroups were not identified, the detection of the presence of nine *V. cholerae* in these samples implied that ingesting these seafoods might cause gastroenteritis.

Vibrio mimicus was detected after amplification by the shortest amplicon (121 bp) containing the Vm.*sodB*-R gene in three crab samples which occurred in lanes 3, 6 and 8, and which represented 15.8% of the total *Vibrio*-positive samples (Figure 6). Biochemically differentiating between *V. cholerae* and *V. mimicus* as members of the *Vibrio* genus is difficult and they are often mistaken for one another (Bonnin-Jusserand et al. 2017), hence the PCR Multiplex amplification of the primer-specific sequences was able to successfully delineate and confirm which *Vibrio* species was actually present in each tested seafood sample. Beshiru et al. (2020) recorded a 8.33% presence of *V. mimicus* in their study on shrimp samples. *Vibrio vulnificus* was not detected in any of the 60 studied samples.

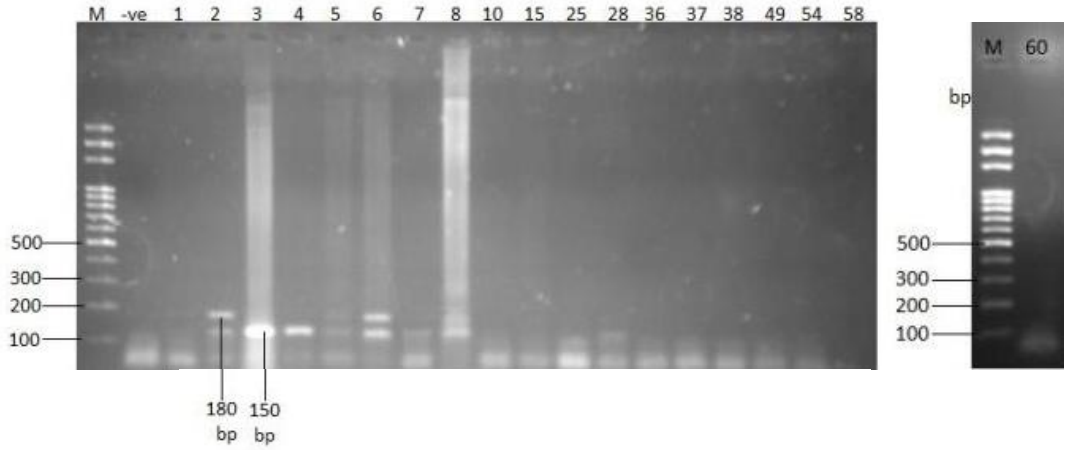

Figure 2. Agarose gel electrophoretogram of PCR products for detection of *tdh* and *trh* genes in DNA samples from *Callinectes sapidus* and *Penaeus notialis*

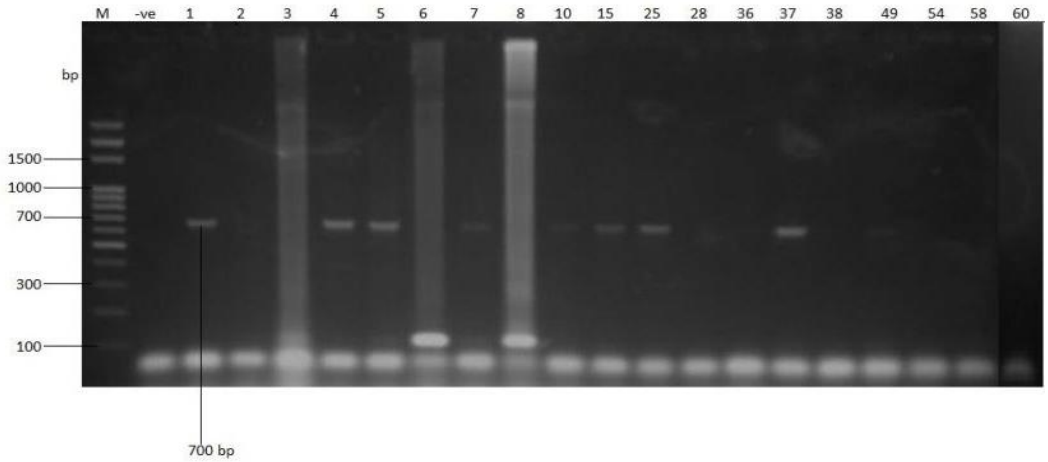

Figure 3. Agarose gel electrophoretogram of the DNA samples of the 19 positive *Vibrio* species in the *Callinectes sapidus* and *Penaeus notialis* samples after PCR amplification

Similarly, the research of Carvalho et al. (2016) also did not detect the presence of the *V. vulnificus* heat shock proteins in the crab samples studied, although authors such as Abdelaziz et al. (2017) and Li et al. (2019) reported the occurrence of *V. vulnificus* in their research involving some marine fishes and shrimps, respectively.

The Multiplex PCR procedure can reveal the presence of multiple species of a genus within samples. Some of the crab samples were found to contain more than one species of *Vibrio* (Table 2). Four crab samples reflected the simultaneous presence of *V. parahaemolyticus* (either or both *trh* and *tdh*) *and V. cholerae* (16S-*Vchol*), (21.1%); while 15.8% of *Vibrio* positive seafood samples containing both *V. parahaemolyticus* (either or both *trh* and *tdh*) and *V. mimicus* (*sodB-R*) strains (which occurred in crab samples). The Multiplex PCR amplified the occurrence of three different genes in crab samples in lanes 5 and 6. Multiplex PCR results by Liu et al. (2021) did not identify the presence of a combination of both *tdh* and *trh* at the same time in any of the *V. parahaemolyticus* strains studied.

The increasing human population and growing demand for more food has made many aquatic foods a competitive alternative source of dietetic protein (Parthasarathy et al. 2016). However, the presence of *Vibrio* species in aquatic animals such as fish, shrimp, lobster and crab, represent a possible health threat to humans during their handling and eating of the raw or undercooked seafoods. In Nigeria, and from different parts of the world, several *Vibrio* species had been reported from crustaceans, which include *V. cholerae*, *V. fluvialis*, *V. parahaemolyticus*, *V. harveyi*, *V. alginolyticus* and *V. vulnificus* (Adebayo-Tayo et al. 2011; Eyisi et al. 2015; Parthasarathy et al. 2016; Elbashir et al. 2018).

The findings of Nilsson et al. (2003) through 16S rRNA polymorphic analysis suggested the occurrence of sub-populations of *Vibrio* species with different virulence patterns which might be further distinguished using species-specific primers. When this was employed in this work, the adoption of PCR and Multiplex PCR diagnostic methods for *Vibrio* species using specific expected virulence factor genes as genetic markers complemented the detection and identification of *Vibrio* species and enhanced the identification of *V. parahaemolyticus, V. cholerae* and *V. mimicus.* Bonnin-Jusserand et al. (2017) also reviewed the accuracy of characterizing the pathogenicity of *Vibrio* species using Multiplex PCR, compared to the use of biochemical tests which might generate questionable interpretations, since this molecular method enlists targeted genes encoding specific toxin codes citing other authors.

Along with the occurrence of these pathogenic *Vibrio* species, the polymorphic information contents, generated from the electrophoretograms are as shown in Tables 3 and 4, while Figures 1-3 describe the gene diversity and the phylogeny of the *Vibrio* species encountered in the positive crab and shrimp samples. In Table 3, no polymorphism was recorded from the combined primer sequences used since the polymorphic information content of the nineteen confirmed *Vibrio* strains was lower than 0.5 (0.449). The results also revealed a gene diversity of 0.5 and an allelic number of 3.0 was realized from the gels of the primer sequences with a major allelic frequency of 0.6667.

Among the markers, the alleles ranged between 3.0 and 18.0 (Table 4) with a mean of 10.5. The highest allele number, gene diversity and polymorphic information content of 18.00, 0.9444 and 0.9415, respectively were recorded with Vp.*tdh* primer compared to Vp.*trh* which was non-polymorphic (0.489). This signified more diversity in the *Vibrio* species identified with the Vp.*tdh* gene primer. Unlike the non-polymorphism recorded in the V16s.70F and 325R primer combination, the mean of the Vp.*tdh* and Vp.*trh* primer sequence combination was polymorphic (0.7153). High polymorphism and gene diversities in primer Vp.*tdh* and primer combinations of Vp.*tdh* and Vp.*trh* were indications that the primers were good candidate genes to be selected and considered in consolidating information on the diversity of, tracking and detection of *Vibrio* species.

The phylogenetic relationship among the 19 *Vibrio*-positive samples revealed two major clusters A and B (Figure 4). The clade A comprised of two samples (58 and 60), while clade B was made up of three major subclades. Despite the close relatedness exhibited by the *Vibrio* species contained in samples 58 and 60 of clade A with one another, their genetic relationship with the remaining 17 samples was quite divergent, indicating that these *Vibrio* were possibly of different genetic background and were not related to the remaining 17 (contained in cluster B). The B_1 cluster comprised *Vibrio* species in the crab samples 1, 3, 4, 5, 6, 7, 8, 16 and 25; the B_2 was made up of *Vibrio* species present in one crab (sample 2), and three shrimp samples (37, 38 and 49), while the B_3 consisted of samples 10, 28, 36 and 54 from two crab and two shrimps, respectively. The clustering patterns exhibited in the subclades B_1, B_2, and B_3 showed the genetic relationship among the samples with respect to their closeness and showed outgroup sample 54 as the most distant among the subclades.

Table 2. Multiplex PCR amplified *Vibrio* target genes within the *Callinectes sapidus* and *Penaeus notialis* samples

Serial no. of V-16S-positive samples	Source	*Vp-trh1*	*Vp-tdh*	*SodB-R*	*16S-Vchol*
1*	Crab				+
2	Crab	+	+		
3	Crab	+		+	
4	Crab	+			+
5	Crab	+	+		+
6	Crab	+	+	+	
7	Crab	+			+
8	Crab	+		+	
10	Crab				+
15	Crab				+
25	Crab	+			+
28	Crab	+			
36	Shrimp				
37	Shrimp				+
38	Shrimp				
49	Shrimp				+
54	Shrimp				
58	Shrimp				
60	Shrimp				

Note: *All the V-16S-Positive seafood samples were devoid of the *Hsp, toxR, Ctx, trh2* and *sodB* target genes

Table 3. Combined Primer Sequence and Polymorphic Information Content of confirmed *Vibrio* Strains (19+)

Primer sequence	Major allele frequency	Sample size	Allele number	Gene diversity	Polymorphic information content
***V. 16S. 700*F and *V. 16S-325*R** 5'CGGTGAAATGCGTAGAGAT3' and 5'TTACTAGCGATTCCGAGTTC3'	0.6667	18.0000	3.0000	0.5000	0.4491

Table 4. Detection of *tdh* and *trh* oligonucleotides, and polymorphic information content of nineteen *Vibrio* species

Primer sequence	Major allele frequency	Sample size	Allele number	Number of polymorphic loci	Gene diversity	Polymorphic information content
Vp.*tdh*-5'-AAAAGCGTTCTCAATC-3' 5'-CCA GAA AGA GCA TTG T- 3'	0.0556	19.0000	18.0000	4.0000	0.9444	0.9415
Vp. *trh* -5'-CCC CAG TTA AAT TGT G- 3' 5'-AGG CGC TTA ATT TGA A- 3'	0.6111	19.0000	3.0000	4.0000	0.5494	0.4890
Mean	0.3333	19.0000	10.5000	4.0000	0.7469	0.7153

Figure 4. Genetic Relationship among the *Vibrio* species present in the *Callinectes sapidus* and *Penaeus notialis* samples

The *Vibrio* species also found in samples 10 (a crab) and 36 (a shrimp) were more closely related than those in the other two samples (28 and 54) of subclade B_3. In phylogenetic relationships, the outgroup (distance) observed in some of the *Vibrio* species is a distinct feature that could be as a result of their divergence more than other closely associated species. Furthermore, the relatedness of the isolates in cluster A might support the hypothesis that there is the influence of geographical and host specificity on the distribution of isolates, resulting in their genetic diversity compared to those in clade B. Our results are corroborated by Narayanan et al. (2020); whereas subcluster B_1 instead emphasizes the influence of host specificity irrespective of geographical orientation since the *Vibrio* species found in nine different crab samples from two markets (eight from Mushin and one from Oyingbo market) still clustered together in one subclade. The geographical distribution of the isolates might have been associated with gene transfer and clonal expansion that played significant role in evolution. The gene diversity recorded might be an indication of larger genomic differences of the distribution of *Vibrio* species. Liu et al. (2021) also suggested that lineage differences might be assumed to have occurred during evolution which resulted in the differences observed in the gene sequences of the strains of *V. parahaemolyticus* they studied.

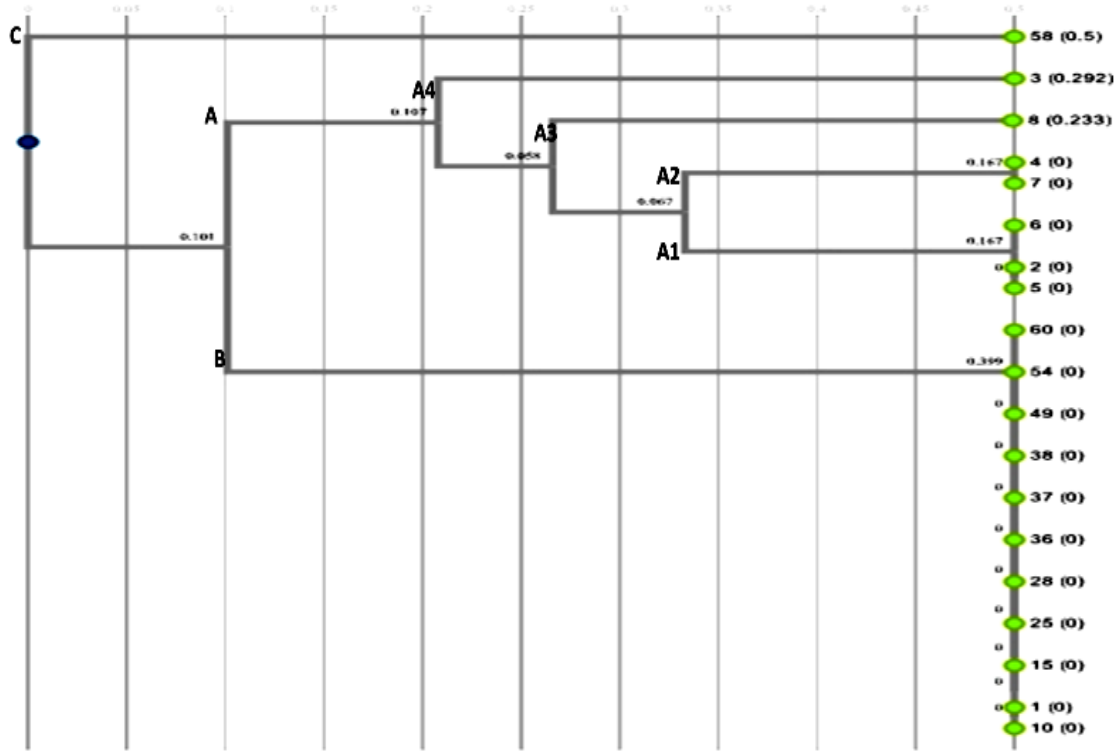

Figure 5. Genetic similarity of *Vibrio* strains from *Callinectes sapidus* and *Penaeus notialis* samples based on the presence of *tdh/trh* genes

The genetic relatedness or otherwise of the *tdh/trh* genes of *Vibrio* strains is shown in Figure 5. There were two major clusters, A and B, while the cluster C (reflecting sample 58 only) was an outgroup/outlier. The cluster A had 4 subclusters A_1, A_2, A_3 and A_4. Samples 2, 5 and 6, which formed subcluster A_1, were more closely related to one another than they were to samples 4 and 7 which formed subcluster A_2. These were, however, different from A_3 (sample 8) and A_4 (sample 3). Sample 58, (shrimp from Oyingbo market) in cluster C, was the most distant of all the groups indicating its genetic diversity from other samples. The distribution of the *tdh/trh* genes in cluster B showed the universality of the gene in different pathogenic *Vibrio* species, irrespective of seafood type and the source.

When the level of polymorphism was evaluated by determining the Polymorphic Information Content values for *Vibrio* strains after the results of the *hsp, sodB, SodB-R, ctx, toxR*, and *Vchol* primer combinations were studied, two major clusters A and B were observed (Figure 6). Subcluster A_1 comprised only sample 36, which was not related to samples 2, 25, 28, 38, 40, 54, 58 and 60 in subcluster A_2. A closer relationship was observed between amplified genes in samples 7 and 10 from subcluster B_1 compared to those in B_2 (samples 3, 6 and 8) even though the samples were obtained from different vendors. The case was however different with subcluster B_3 (1, 4, 5, 15 and 37) which was more distantly related to both subclusters B_1 and B_2.

The *Vibrio* positive shrimp and crab samples exhibited similar genetic characteristics regarding the amplification of the selected markers, however, the presence or absence of bands in some samples on the electrophoresis gels made it possible to analyse the genotypes in regards to their polymorphism based on how dominant the gene markers were. This also enabled the separation of the *Vibrio* into different clusters thus reflecting their profiles of genetic similarities. The phylogenetic relationships of *Vibrio* species were reported by Hossain et al. (2020) who found that the genes had a common ancestor and the clades formed presented with diverse traits and had low genetic distances. The variability of target genes and sequence inaccuracies were reported by other authors who also studied genetic relationships in *Vibrio* species using specific marker genes (Chonsin et al. 2015; Liu et al. 2015).

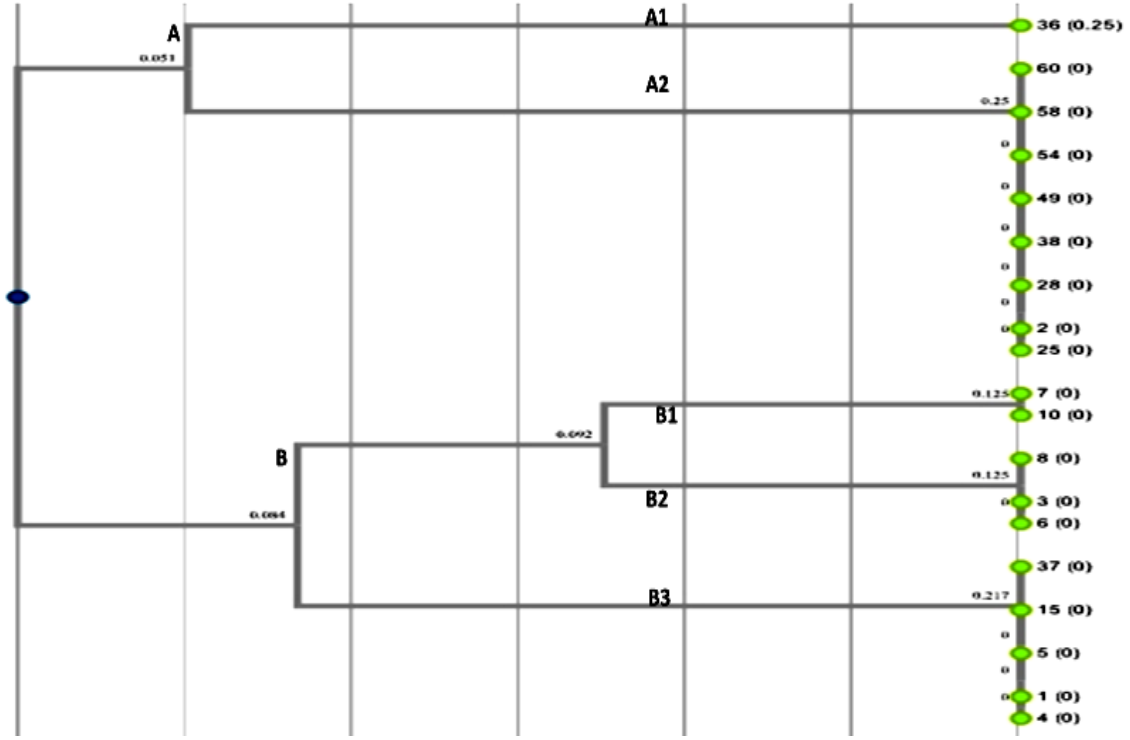

Figure 6. Genetic relationship of the *Vibrio* strains revealed by six primer combinations (*hsp, sodB, SodB-R, ctx, toxR,* and *Vchol*)

In conclusion, the present study demonstrates the presence of potentially pathogenic *Vibrio* spp. (*V. parahaemolyticus*, *V. mimicus* and *V. cholerae*) in shrimps and crabs distributed in retailed fish markets within Lagos, Nigeria using molecular target genes and statistical analysis. The findings suggested that the *Vibrio* strains amplified were potentially virulent and exhibited different genetic relatedness. More diversity was observed in the *Vibrio* species identified using the Vp.*tdh* gene primer and both the gene diversity and polymorphic information content were high for this gene (>0.94). This may represent a risk of seafood-borne illness in Lagos, Nigeria, and could serve as useful tracking tools in the epidemiological dynamics of *Vibrio* in Nigeria so as to adopt proper public health guidelines. The genetic unrelatedness identified in the two *Vibrio* species obtained from shrimp samples 58 and 60 when compared on the basis of their target core genes (*hsp, sodB, SodB-R, ctx, toxR,* and *Vchol*) also might pose a higher risk to the populace.

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