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Molecular Studies on Vibrio Species Isolated from Imported Frozen Fish

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Abstract: A total of 150 imported frozen raw fish samples consisting of 20 sardine, 30 mackerel, 40 horse mackerel and 60 shrimp were collected and analyzed by bacteriological, antimicrobial and molecular methods. The main objectives of the present work were to study the occurrence and characteristics of *Vibrio* spp. in frozen marine fish. Nine isolates of *Vibrio* spp. were identified. They were divided into 3 isolates of *V. parahaemolyticus*, one isolate of *V. vulnificus* and 5 isolates of *V. fluvialis*. Biochemical confirmatory tests were performed using an automated phenotypic microbiology identification system utilizing colorimetric reagent cards (VITEK2C). The presence of virulence associated genes of pathogenic *Vibrio* spp. was investigated using standard PCR. The *16SrRNA* gene specific for the genus *Vibrio* was used to confirm the genus in all isolates. All the isolates, irrespective of the species, were positive for *16S rRNA* gene confirming the genus in all the isolates studied. *V. parahaemolyticus* isolates were positive for *tdh, trh* and *tlh* genes. On the other hand, *V. vulnificus* isolates were positive for virulence genes; *vvhA, SerE and Bt2*. Also, *V. fluvialis* isolates were positive for virulence genes toxR, vflu and vfh. Susceptibility of *Vibrio* spp. to antibiotics was studied using VITEK2C system that includes an Advanced Expert System (AES) that analyzes MIC patterns giving results in accordance to clinical and laboratory standards institute (CLSI guide) in MIC report. The high percentage of ampicillin-resistant *Vibrio* spp. isolates suggests low efficiency of Ampicillin in empirical treatment of infections caused by this organism. It could be concluded that there is a need for appropriate food safety practices when consuming these products. Moreover, continued monitoring of both the prevalence and antimicrobial susceptibility profile is needed to better safety.

Key words: Vibrio · PCR on vibrio · Frozen fish

INTODUCTION

Vibrio spp. is gram negative bacteria curved or rod-shaped and motile with one or more flagella. They are facultative anaerobes, with a respiratory or fermentative metabolism, oxidase positive, Na^+ stimulates their growth and they may be luminescent [1, 2]. In addition to their role in the global nutrient cycle, certain *Vibrio* spp. also may cause diseases in aquatic organisms and humans making them important from an economic and public health perspective [3].

There are three pathogenic *Vibrio* spp. of public health importance; *V. cholerae*, the causative agent of the disease cholera; *V. vulnificus*, which causes wound infections and primary septicemia and *V. parahaemolyticus*, which causes gastroenteritis [4]. Other *Vibrio* spp. while posing less threat to human health, can cause illnesses in marine life as well [5]. In 2005, there were 131,943 reported cases of cholera worldwide resulting in 2,272 deaths, which may only represent 5-10% of actual disease incidence [6].

Previous work examined how environmental parameters and water quality in aquatic systems impacted *Vibrio* spp. growth as variability in *Vibrio* concentrations has been associated with temperature, salinity, nutrient concentrations, sediments and the presence of other aquatic organisms such as plankton. Temperature and salinity have been recognized as the major predictive

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factors in *Vibrio* spp. abundance [7]. In addition to temperature, Fernandez *et al*.[8] found that increased rainfall corresponded to increased isolation of *V. cholera*, a trend likely linked to decreases in salinity.

Pathogenic strains of *V. vulnificus* and *V. parahaemolyticus* which are natural inhabitants of estuarine environments worldwide are often transmitted to humans through consumption of raw shellfish that flourish in the same estuaries [9].

Vibriosis is a major disease problem in shrimp aquaculture, causing high mortality and severe economic loss in all producing countries. Shrimp aquaculture is an important industry that experiences significant losses from *Vibrio* species, especially at the larval and juvenile stages [10].

Laboratory diagnosis of pathogenic *Vibrio* spp. has traditionally been based on phenotypic characteristics of these organisms, expressed as morphological, physiological and biochemical properties, including antigenic composition. Phenotypic identification of *Vibrio* spp. relies on time consuming techniques such as studies on the morphology and nutrition requirement that have limited discriminatory powers. Accurate phenotypic identification of *Vibrio* spp. is problematic, largely because of the great variability in biochemical characteristics [11].

These organisms can be detected directly through PCR assays in a much shorter time than conventional culture methods. Molecular methods that utilize the polymerase chain reaction and nucleotide sequence determination overcome many of the limitations of phenotypic methods. PCR can lead to identification of an isolate within hours and can be used on small quantities of cells, including those that are not viable or are otherwise uncultivable [12]

The main objectives of the present work were to study the occurrence and characteristics of *Vibrio* spp. in frozen marine fish, as well as the persistence of human pathogenic strains when encountering an aquatic environment that is clearly different from their human host. To achieve these aims, *Vibrio* spp. were determined in imported frozen fish by phenotypic methods and the recovered isolates were characterized by genotypic methods. In addition, the virulence and antibiotic resistance pattern in different strains of *Vibrio* spp. were also studied.

MATERIALS AND METHODS

Collection of Samples: A total of 150 imported frozen raw fish samples consisting of 20 sardine, 30 mackerel, 40 horse mackerel and 60 shrimp were used in this study. All samples were dissected and put in sterile stomacher bags and assigned an identification code in order to maintain a database of the isolates then, taken immediately in ice boxes to the laboratory and preserved at -20°C until further analysis. Flesh of examined fish was the principal tissue of investigation.

Preparation of Samples and Primary Isolation (According to ISO/TS 21872-1 & 21872-2): Two steps method for enrichment of *Vibrio* spp. were performed. Briefly, 25 g of each tissue sample was added to 225 ml alkaline saline peptone water-ISO (ASPW-ISO) as a first selective enrichment to form 1/10 initial suspension then incubated at 37°C for 6±1 h. After that, 1 ml of the initial suspension was transferred to a tube containing 10 ml ASPW-ISO representing the second selective enrichment, then incubated at 41.5°C for 18±1 h. Isolation and identification were started by streaking the surface of Thiosulphate Citrate Bile Sucrose agar (TCBS) and *Vibrio parahaemolyticus* Sucrose Agar (VPSA) plates with a loopful from the incubated ASPW-ISO to permit the development of the isolated colonies. Agar plates were then inverted and put in an incubator at 37°C. After 24±3 h of incubation, plates were examined for presence of typical colonies of presumptive pathogenic *Vibrio* spp. Suspected colonies were isolated and purified by streaking at least five typical colonies on saline nutrient agar plates and incubated at 37°C for 24±3 h. Pure colonies were stained with gram stain and tested for oxidase and microscopic motility.

Phenotypic Characterization of Vibrio Spp. *(Biochemical Identification)*: Obtained gram negative, oxidase positive and motile isolates were selected for further biochemical confirmatory tests using an automated phenotypic microbiology identification system utilizing growth-based technology through accommodating colorimetric reagent cards that are incubated and interpreted automatically (VITEK2 Compact System, BIOMERIEUX, France). Identification was started by transferring one pure colony using a sterile swab to a tube containing 3 ml sterile saline solution mixed till making a suspension between 0.5-0.63 Mcfarland turbidity range that was measured using a turbidity meter called Densichek TM device. Identification cards were then inoculated with microorganism suspensions using an integrated vacuum apparatus. A test tube containing the microorganism suspension was placed into a special rack (Cassette) and the identification card was placed in the neighbouring slot while inserting the transfer tube into the corresponding suspension tube.

Table 1: Biochemical reactions using (Vitek2c)

Vf	Vv	Vp	Abbreviation	Biochemical reaction	Vf	Vv	Vp	Abbreviation	Biochemical reaction
-	-	-	SAC	Saccharose/sucrose	-	+	+	APPA	Ala-Phe-Pro-arylamidase
-	+	-	dTAG	d-Tagatose	-	-	-	ADO	Adonitol
-	+	+	dTRE	d-Trehalose	-	+	+	PyrA	l-Pyrrolydonyl-arylamidase
-	-	-	CIT	Citrate (sodium)	-	-	-	IARL	l-Arabitol
-	-	-	MNT	Malonate	+	+	-	dCEL	d-Cellobiose
-	-	-	5KG	5-Keto-d-gluconate	-	+	-	BGAL	Beta-galactosidase
-	-	+	ILATK	l-Lactate alkalinisation	-	-	-	H2S	H2S production
-	-	-	AGLU	Alpha-glucosidase	-	+	+	BNAG	Beta-N-acetyl-glucosaminidase
-	-	+	SUCT	Succinate alkalinisation	-	-	-	AGLTp	Glutamyl arylamidase pNA
-	-	-	NAGA	Beta-N-acetyl-galosaminidase	+	+	+	dGLU	d-Glucose
-	-	-	AGAL	Alpha-galactosidase	-	-	-	GGT	Gamma-glutamyl-transferase
-	-	-	PHOS	Phosphatase	-	-	+	OFF	Fermentation/glucose
-	-	-	GlyA	Glyine arylamidase	-	+	-	BGLU	Beta-glucosidase
-	-	-	ODC	Ornithine decarboxylase	=	+	-	dMAL	d-Maltose
-	-	-	LDC	Lysine decarboxylase	-	+	+	dMAN	d-Mannitol
(+)	-	-	IHISa	l-Histidine assimilation	-	+	+	dMNE	d-Mannose
+	+	+	CMT	Courmarate	-	-	-	BXYL	Beta-xylosidase
-	-	-	BGUR	Beta-glucoronidase	-	-	-	BAlap	Beta-alanine arylamidase pNA
-	-	-	O129R	O/129 resistance (comp.vibrio)	-	+	+	ProA	l-Proline arylamidase
-	+	+	GGAA	Glu-Gly-Arg-arylamidase	-	-	+	LIP	Lipase
+	+	-	IMLTa	l-Malate assimilation	-	-	-	PLE	Palatinose
-	-	+	ELLM	Ellman	-	+	-	TyrA	Tyrosine arylamidase
+	+	-	ILATa	l-Lactate assimilation	-	-	+	URE	Urease
					-	+	-	dSOR	d-Sorbitol

Vp= Vibrio parahaemolyticus. Vv= Vibrio vulnificus. Vf= Vibrio fluvialis.

The filled cassette was placed into a vacuum chamber station. After the vacuum was applied and air was re-introduced into the station, the organism suspension was forced through the transfer tube into micro-channels that filled all the test wells. After that, the device (VITEK2C) performed about 47 biochemical reactions (Table 1) giving a final report of all applied biochemical reactions and the final identification of isolated colonies with its probability percentage after 6 hours.

Genotypic Characterization of Vibrio spp

DNA Extraction *of Vibrio* spp: DNA extraction was carried out using a boiled cell method. In brief, a 1 ml portion of each tube was subjected to centrifugation at 12000 rpm for 2 min to pellet the microorganisms. The pellet was resuspended in 500 µL of sterile distilled water and boiled for 10 min. The boiled cell lysate was immediately cooled at -20°C for 10 min and then centrifuged at 13000 rpm for 3 min. The boiled cell lysate was finally used as the DNA template for PCR [11].

Detection of Virulence Genes *of Vibrio* Spp. *By Polymerase Chain Reaction (PCR):* The presence of virulence associated genes of pathogenic *Vibrio* spp. was investigated using standard PCR. The *16SrRNA* gene [11], specific for the genus *Vibrio* was included to confirm the genus in all the isolates. The target genes oligonucleotide primers (Metabion International AG-Germany) and expected product sizes are listed in Table 2 for all species specific genes. The amplification was carried out in a 50 µL reaction mixture containing DNA master mix (Jena bioscience-Germany), template DNA, forward and reverse oligonucleotide primers (Metabion International AG-Germany) in determined volumes according to manufacturer's instructions and the final volume of the reaction mixture was adjusted to 50 µL with sterile deionised distilled water.

Amplification of DNA segment was performed in a thermal cycler (Applied Biosystems, USA) with the following temperature cycling parameters; initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 sec, primer annealing at 57°C for 30 sec, primer extension at 72 °C for 1 min and a final extension at 72 °C for 2 min. Ten µL of each amplified product was loaded in 1.5% agarose gel in 1X Tris-boric acid-EDTA buffer [TBE: 0.089M Trisbase, 0.089M boric acid and 0.002 M EDTA (pH 8.0)] at 100 volts for 40 minutes. After electrophoresis, amplification products were observed and digitalized by the Gel-Doc UV trans-illuminator (Bio-Rad, Hercules, CA, USA) after staining with ethidium bromide. A 100-DNA ladder (Jena Bioscience, Germany) was used as a molecular marker to indicate the size of the amplicons.

Table 2: List of the primers used in this study

Primer name		Sequence (/5-/3)	Target gene	Size of PCR Amplicon (bp)	References
Genus Primer	V16S- F	5'- GGCGTAAAGCGCATGCAGGT -3'	16S rRNA	663	[11]
	V16S- R	5'- GAAATTCTACCCCCCTCTACAG -3'			
Vibrio Parahaemolyticus	tlh-F	5' -AAAGCGGATTATGCAGAAGCACTG-3'	Tlh	450	[20]
	tlh-R	5' -GCTACTTTCTAGCATTTTCTCTGC-3'			
	tdh-F	5'-GTAAAGGTCTCTGACTTTTGGAC-3'	Tdh	269	[20]
	tdh-R	5'-TGGAATAGAACCTTCATCTTCACC-3'			
	trh-F	5'- TTGGCTTCGATATTTTCAGTATCT-3'	Trh	500	[20]
	trh-R	5'CATAACAAACATATGCCCATTTCCG3'			
Vibrio Fluvialis	toxR-F	5'- GACCAGGGCTTTGAGGTGGACGAC-3'	toxR	217	[23]
	toxR-R	5'AGGATACGGCACTTGAGTAAGACTC-3'			
	VFLU-F	5'- ATAAAGTGAAGAGATTCGTACC-3'	VFLU	278	[22]
	VFLU-R	5'-GTATTCCTGAATGGAATACAC-3'			
	vfh-F	5'- GCGCGTCAGTGGTGGTGAAG-3'	Vfh	800	[25]
	vfh-R	5'- TCGGTCGAACCGCTCTCGCTT-3'			
Vibrio Vulnificus	vvhA-F	5'- CGCCACCCACTTTCGGGCC-3'	vvhA	512	[21]
	vvhA-R	5'- CCGCGGTACAGGTTGGCGC-3'			
	SerE-F	5'- TGTTGTTCTTGCCCACTCTC-3'	SerE	665	[22]
	SerE-R	5'- CGCGCTTAGATTTGTCTCACC-3'			
	Bt2-F	5'- AGAGATGGAAGAAACAGGCG-3'	Bt2	344	[22]
	Bt2-R	5'- GGACAGATATAAGGGCAAATGG-3'			

Antimicrobial Susceptibility Test of Vibrio spp: Susceptibility of *Vibrio* spp. to antibiotics was studied using VITEK2c system that includes an Advanced Expert System (AES) that analyzes MIC patterns giving results in accordance to clinical and laboratory standards institute (CLSI guide) in MIC report for each antibiotic used in the test after 18 hours. This was applied following the same procedures for identification except using susceptibility cards instead of identification cards.

RESULTS

After examination of all tested samples, nine isolates of *Vibrio* spp. were identified. They were divided into 3 isolates of *V. parahaemolyticus*, one isolate of *V. vulnificus* and 5 isolates of *V. fluvialis*. This was confirmed by the following performed tests.

Phenotypic Characters of Vibrio spp

Colonial and Microscopical Picture: There were two typical morphologies for colonies of *Vibrio* spp. on TCBS agar. Typical colonies of *V. Parahaemolyticus and V. vulnificus* were smooth, green (Sucrose negative) and 2 to 3 mm in diameter. Typical colonies of *V. fluvialis* were smooth, yellow (sucrose positive) and 2 to 3 mm in diameter (Fig. 1). While Typical colonies of *V. Parahaemolyticus* only were smooth, green (Sucrose negative) and 2 to 3 mm in diameter on VPSA agar (Fig. 1). On microscopic examination, all selected colonies revealed gram negative comma shaped (Curved) bacilli, motile by single polar flagella non-spore forming and non-capsulated.

Biochemical Characters: Automated biochemical identification system using VITEK2C revealed the results summerized in Table 1.

Genotypic Characters of Vibrio Spp. *(Virulence Associated Genes):* *Vibrio parahaemolyticus* (3 isolates), *Vibrio vulnificus* (One isolate) and *Vibrio fluvialis* (Five isolates) were examined for the presence of genus gene and virulence associated genes. The distribution of virulence in these isolates was shown in Figs. 2, 3 & 4. All the isolates, irrespective of the species, were positive for *16S rRNA* gene confirming the genus in all the isolates studied. *V. parahaemolyticus* isolates were positive for *tdh, trh* and *tlh* genes (Fig. 2). On the other hand, *V. fluvialis* isolates were positive for virulence genes toxR, vflu and vfh (Fig. 3). Also, *V. vulnificus* isolates were positive for virulence genes; *vvhA, SerE and Bt2* (Fig. 4).

Antimicrobial Susceptibility Test of Vibrio spp: Susceptibility of *Vibrio* spp. to antibiotics was studied using VITEK2c system that includes an Advanced Expert System (AES) that analyzes MIC patterns giving results in accordance to clinical and laboratory standards institute (CLSI guide)[8] in MIC report for each antibiotic

Table 3: Demonstrating results of automated (VITEK 2 system) method for antimicrobial susceptibility test

Antimicrobial Class	Antimicrobial Agent	MIC (µg/mL) Interpretive Criteria			Result Vitek2In MIC					
		S	I	R						
Penicillins and Beta-lactam/beta-lactamase Inhibitor Combinations										
					Vp →	MIC	Vv →	MIC	Vf →	MIC
	Ampicillin	≤=8	16	≥32	R	>=32	R	>=32	R	>=32
	Ampicillin-sulbactam	≤8/4	16/8	≥32/16	S	4	S	4	S	4
	CEPHEMS									
	Cefazolin	≤8	16	≥32	I	16	I	16	S	16
	Cefepime	≤8	16	≥32	S	1	S	1	S	1
	Ceftriaxone	≤8	16-32	≥64	S	<=1	S	<=1	S	<=1
	CARBAPENEMS									
	Imipenem	≤4	8	≥16	S	<=0.25	S	<=0.25	S	<=0.25
	Meropenem	≤4	8	≥16	S	<=0.25	S	<=0.25	S	<=0.25
	AMINOGLYCOSIDES									
	Amikacin	≤16	32	≥64	S	<=1	S	<=1	S	<=1
	Gentamicin	≤4	8	≥16	S	<=1	S	<=1	S	<=1
	TETRACYCLINES									
	Tigecycline	≤4	8	≥16	S	<=0.5	S	<=0.5	S	<=0.5
	QUINOLONES									
	Ciprofloxacin	≤1	2	≥4	S	<=0.25	S	<=0.25	S	<=0.25
	Levofloxacin	≤2	4	≥8	S	<=0.25	S	<=0.25	S	<=0.25
	Moxifloxacin	≤2	4	≥8	S	<=0.25	S	<=0.25	S	<=0.25
	FOLATE PATHWAY INHIBITORS									
	Trimethoprimsulfamethoxazole	≤2/38	-	≥4/76	S	<=20	S	<=20	S	<=20
FURANS										
	Nitofurantoin	≤32	64	≥128	S	<=16	S	<=16	S	<=16

The above table is demonstrating results of automated (VITEK 2 system) method with MIC levels with referral to standards of Clinical and Laboratory Standards Institute for Zone diam Interpretive Criteria, MIC Interpretive Criteria and antibiotic concentration.

Where (MIC) means minimal inhibitory concentration, Vp=Vibrio parahaemolyticus, Vv= Vibrio vulnificus and Vf = Vibrio fluvialis. Also S= sensitive, I= Intermediate and R= Resistant

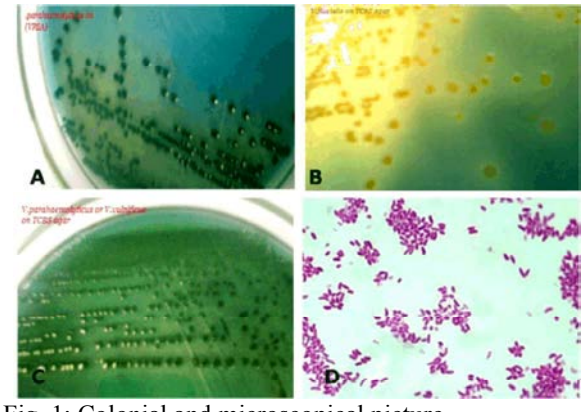

Fig. 1: Colonial and microscopical picture

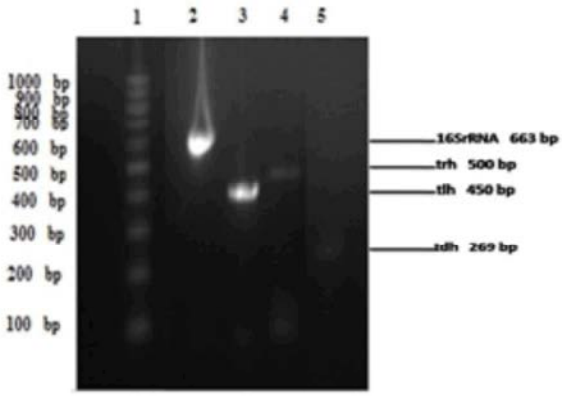

Fig. 2: Agarose gel electrophoresis of DNA fragments generated by PCR with *Vibrio* spp. Lane 1, DNA molecular weight marker (100 bp); Lanes 2 to 5, *V. parahaemolyticus* isolates. Genes corresponding to the amplified fragments are indicated on the left. Molecular sizes are given (In base pairs) at the left.

used in the test after 18 hours. This was applied following the same procedures for identification except using susceptibility cards instead of identification cards.

The results of automated (VITEK 2 system) method for antimicrobial susceptibility test are demonstrated in Table 3.

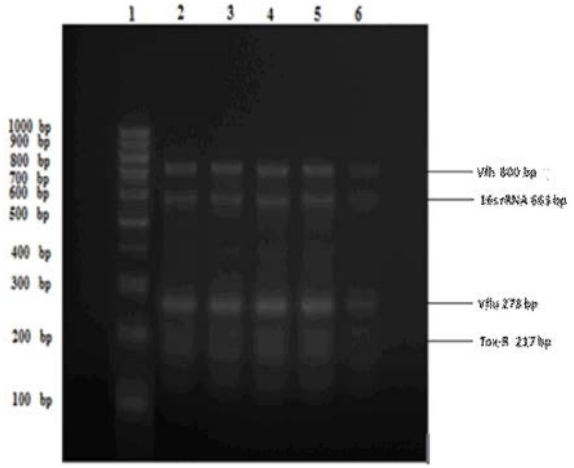

Fig. 3: Agarose gel electrophoresis of DNA fragments generated by PCR with *Vibrio* spp. Lane 1, DNA molecular weight marker (100 bp); Lanes 2 to 6, *Vibrio fluvialis* isolates. Genes corresponding to the amplified fragments are indicated on the left. Molecular sizes are given (In base pairs) at the left.

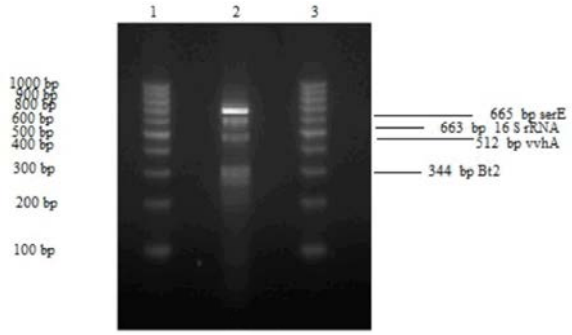

Fig. 4: Agarose gel electrophoresis of DNA fragments generated by PCR with *vibrio* spp. Lane 1and 3 DNA molecular weight marker (100 bp); Lanes 2 *Vibrio vulnificus* isolate; Genes corresponding to the amplified fragments are indicated on the left. Molecular sizes are given (In base pairs) at the left.

DISCUSSION

The present study confirmed the ability of growth and isolation of three different *Vibrio* spp. from marine fish and shrimp that were previously kept in deep freezing. Prior to May 2006, no standardized susceptibility testing method was available for noncholera *Vibrio* spp. Because of this, it was difficult to compare data from different laboratories due to variables involved in the testing. CLSI recently published the M45-A document which presented the most current information for drug selection, interpretation and quality control for MIC testing of infrequently isolated or fastidious bacteria, including non-Cholera *Vibrio* spp. [13]. All antimicrobials tested in the present study were in accordance with the guiines of the M45-A document and represent antimicrobials agents that may be used in the treatment of non-Cholera *Vibrio* spp. infections, particularly tetracycline, cefotaxime, ceftazidime and fluoroquinolones.

Our findings indicated that these first-line drugs remained highly effective against *Vibrio* spp.; however, the high prevalence of ampicillin-resistant *strains* suggested that ampicillin should not be used empirically to treat *Vibrio* spp. infection. This was in contrast to recommendations posted by CDC [14]. Interestingly, ampicillin resistance in *V. parahaemolyticus* is not a new phenomenon. Joseph *et al.* [15] reported that over 90% of 160 *V. parahaemolyticus* was resistant to ampicillin and exhibited β-lactamase activity [15]. Zanetti *et al.* [16] also reported unexpectedly high frequency (80%) of ampicillin resistance in eight *V. parahaemolyticus* and six *V. vulnificus*, mostly attributable to the production of β-lactamase [16]. Maluping *et al*.[17] found twelve out of fourteen *V. parahaemolyticus* were resistant to ampicillin [17]. Similarly, a study in India reported 100% ampicillin resistance among seven *V. vulnificus* and five *V. parahaemolyticus* tested by the disk diffusion method [18]. More recently, Akinbowale *et al*. [19] reported that an ampicillin resistance rate of 40% for *Vibrio* spp. however, only one *V. parahaemolyticus* and no *V. vulnificus* were included in that study [19]. The findings in the present study are in agreement with results from these earlier studies, which found high prevalence of ampicillin resistance in *Vibrio* spp.

The aim of the present study was to assess the virulence potential of *Vibrio* spp. isolated from various imported frozen fish samples. The isolates were analyzed for the presence of virulence associated genes. All isolates, irrespective of the species amplified the *16S rRNA* gene fragment (663 bp) confirming the genus in all the isolates studied, while species-specific genes could differentiate the three *Vibrio* spp. from each others. All the three *V. parahaemolyticus* isolates were positive for *tdh*, *trh* and *tlh* genes. On the other hand, the *V. vulnificus* isolate amplified these tested virulence genes vvhA, serE and Bt2. Also all of the five *V. fluvialis* isolates revealed presence of three different virulence genes vfh, vflu and toxR. Previous studies showed that in order to identify a potentially pathogenic strain, it is necessary to target multiple genes for PCR amplification. Species-specific tlh gene, pathogenic strain-specific tdh

and trh, were selected for the detection of *V. parahaemolyticus* in shellfish by PCR [20]. Similarly, species-specific vvhA [21], serE and Bt2 genes [22] were selected for the detection of *V. vulnificus* in addition toxR [23], vflu [24] and vfh [25] virulence genes specific for *V. fluvialis*.

CONCLUSION

These results illustrated the need for appropriate food safety practices when consuming these products. Moreover, the observed high percentage of ampicillin-resistant *Vibrio* spp. isolates suggests a potential for low efficiency of ampicillin in empirical treatment of infections caused by this organism. Continued monitoring of both the prevalence and antimicrobial susceptibility profile is needed to better ensure safety; particularly the retail survey could be expanded to the national level. In this study food poisoning *Vibrio* spp. have been isolated even from frozen sea food So that it is clear that special attention should given to proper cooking of sea foods either it was fresh or frozen and avoiding under cooked sea foods in order to minimize the risk of vibriosis.

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REFERENCES

1. Ansaruzzaman, M., M. Lucas, J.L. Deen, N.A. Bhuiyan, X.Y. Wang, A. Safa and A. Barreto, 2005. Pandemic serovars (O3: K6 and O4: K68) of *Vibrio parahaemolyticus* associated with diarrhea in Mozambique: spread of the pandemic into the African continent. Journal of Clinical Microbiology, 43(6): 2559-2562.
2. Lida, T., A. Hattori, K. Tagomori, H. Nasu, R. Naim and T. Honda, 2001. Filamentous phage associated with recent pandemic strains of *Vibrio parahaemolyticus*. Emerging Infectious Diseases, 7(3): 477.
3. Oliver, J.D., 2006. *Vibrio vulnificus*, p. 349-366. Edited by F. L. Thompson, B. Austin and J. Swings (ed.), the biology of vibrios. Washington, DC: ASM Press.
4. West, P.A., 1989. The human pathogenic vibrios–A public health update with environmental perspectives. Epidemiology and Infection, 103(01): 1-34.
5. Austin, B. and X.H. Zhang, 2006. *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. Letters in Applied Microbiology, 43(2): 119-124.
6. WHO, 2006. Weekly epidemiological record, 2006. 81, 297-308. Assessed online at http://www. who. int/wer/2006/wer8131.pdf. Assessed on 11/30/2009.
7. Wetz, J.J., A.D. Blackwood, J.S. Fries, Z.F. Williams and R.T. Noble, 2008. Trends in total *Vibrio* spp. and *Vibrio vulnificus* concentrations in the eutrophic neuse river estuary, North Carolina, during storm events. Aquatic Microbial Ecology, 53(1): 141-149.
8. Fernandez-Delgado, M., M.A. Garcia-Amado, M. Contreras, V. Edgcomb, J. Vitelli, P. Gueneau, *et al*., 2009. Vibrio cholerae non-O1, non-O139 associated with seawater and plankton from coastal marine areas of the caribbean sea. International Journal of Environmental Health Research, 19(4): 279-289.
9. Andrews, L.S., 2004. Strategies to control Vibrios in molluscan shellfish. Food Protection Trends, 24(2): 27-28.
10. Mohney, L.L., D.V. Lightner and T.A. Bell, 1994. An Epizootic of Vibriosis in Ecuadorian Pond-Reared *Penaeus vannamei* Boone (Crustacea: Decapoda). Journal of the World Aquaculture Society, 25(1): 116-125.
11. Thompson, F.L., T. Iida and J. Swings, 2004. Biodiversity of vibrios. Microbiology and Molecular Biology Reviews, 68(3): 403-431.
12. Robert-Pillot, A., A. Guénolé, J. Lesne, R. Delesmont, J.M. Fournier and M.L. Quilici, 2004. Occurrence of the tdh and trh genes in *Vibrio parahaemolyticus* isolates from waters and raw shellfish collected in two French coastal areas and from seafood imported into France. International Journal of Food Microbiology, 91(3): 319-325.
13. Jorgensen, J.H. and J.F. Hindler, 2007. New consensus guidelines from the Clinical and Laboratory Standards Institute for antimicrobial susceptibility testing of infrequently isolated or fastidious bacteria. Clin. Infect. Dis., 44: 280-286.
14. CDC, 2005. Summary of human Vibrio isolates reported. to CDC, 2004. Centres for Disease Control and Prevention. httpI//www. cd.c.gov/ foodborneoutbreaks/vibrio_,sum/CSTEVibrio2004.pdf
15. Joseph, S.W., R.M. DeBell and W.P. Brown, 1978. In vitro response to chloramphenicol, tetracycline, ampicillin, gentamicin and beta-lactamase production by halophilic Vibrios from human and environmental sources. Antimicrobial Agents and Chemotherapy, 13(2): 244-248.

16. Zanetti, S., T. Spanu, A. Deriu, L. Romano, L.A. Sechi and G. Fadda, 2001. In vitro susceptibility of *Vibrio* spp. isolated from the environment. International Journal of Antimicrobial Agents, 17(5): 407-409.
17. Maluping, R.P., C.R. Lavilla-Pitogo, A. DePaola, J.M. Janda, K. Krovacek and C. Greko, 2005. Antimicrobial susceptibility of *Aeromonas* spp. *Vibrio* spp. and *Plesiomonas shigelloides* isolated in the Philippines and Thailand. International Journal of Antimicrobial Agents, 25(4): 348-350.
18. Vaseeharan, B., P. Ramasamy, T. Murugan and J.C. Chen, 2005. In vitro susceptibility of antibiotics against *Vibrio* spp. and *Aeromonas* spp. isolated from *Penaeus monodon* hatcheries and ponds. Int. J. Antimicrob. Agents, 26: 285-291.
19. Akinbowale, O.L., H. Peng and M.D. Barton, 2006. Antimicrobial resistance in bacteria isolated from aquaculture sources in Australia. Journal of Applied Microbiology, 100(5): 1103-1113.
20. Bej, A.K., D.P. Patterson, C.W. Brasher, M.C. Vickery, D.D. Jones and C.A. Kaysner, 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of tlh, tdh and trh. Journal of Microbiological Methods, 36(3): 215-225.
21. Hor, L.I., C.T. Goo and L. Wan, 1995. Isolation and characterization o f *Vibrio vulnificus* inhabiting the marine environment of the south western area of Taiwan. Journal of Biomedical Science, 2(4): 384-389.
22. Lee, C.T., C. Amaro, E. Sanjuán and L.I. Hor, 2005. Identification of DNA sequences specific for *Vibrio vulnificus* biotype 2 strains by suppression subtractive hybridization. Applied and Environmental Microbiology, 71(9): 5593-5597.
23. Chakraborty, R., S. Sinha, A.K. Mukhopadhyay, M. Asakura, S. Yamasaki, S.K. Bhattacharya and T. Ramamurthy, 2006. Species-specific identification of *Vibrio fluvialis* by PCR targeted to the conserved transcriptional activation and variable membrane tether regions of the toxR gene. Journal of Medical Microbiology, 55(6): 805-808.
24. Lee, S.K., H.Z. Wang, S.H. Law, R.S. Wu and R.Y. Kong, 2002. Analysis of the 16S–23S rDNA intergenic spacers (IGSs) of marine vibrios for species-specific signature DNA sequences. Marine Pollution Bulletin, 44(5): 412-420.
25. Liang, P., X. Cui, X. Du, B. Kan and W. Liang, 2013. The virulence phenotypes and molecular epidemiological characteristics of *Vibrio fluvialis* in China. Gut Pathogens, 5(1): 6.