ARTICLE / INVESTIGACIÓN

Development of a multiplex polymerase chain reaction (m-PCR) for the detection and identification of virulent and avirulent forms of *Vibrio parahaemolyticus*

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Abstract: This study developed a new multiplex polymerase chain reaction (m-PCR) for rapidly detecting clinically essential strains of *V. parahaemolyticus*. This enables the detection of total and potentially virulent strains. The m-PCR was developed by targeting the species-specific transcriptional regulator *toxR* gene, and sequences for an outer membrane protein and a hypothetical protein encoded by omp and htp, respectively. The *omp* and *htp* sequences were discovered originally by randomly amplified polymorphic DNA (RAPD)-PCR. The m-PCR was performed on *V. parahaemolyticus* isolates, 23 clinical and 32 environmental. The toxR gene 367 bp fragment amplification was found in all *V. parahaemolyticus* tested; 17 out of 23 clinical isolates (73.91%) showed amplification of the *omp* and *htp*. Four isolates showed amplification of the *omp* gene sequence but not the *htp* gene and 2 isolates exhibited amplification for *htp* but not for *omp*. Therefore, both sequences for omp and htp must be targeted by PCR to detect all potentially virulent strains. Of the other species tested, no amplification was seen. This study confirms that RAPD-PCR helps differentiate virulent and avirulent forms. This allowed the development of an m-PCR for identifying *V. parahaemolyticus* and detecting virulent forms.

Key words: Multiplex PCR, RAPD-PCR, *Vibrio parahaemolyticus*.

Introduction

Vibrio parahaemolyticus is a gram-negative halophilic bacterium naturally present in marine and estuarine environments throughout the temperate zone[1]. Pathogenic strains of this organism cause gastroenteritis after they are consumed in raw or partially cooked seafood[2]. However, not all strains are considered pathogenic. The presence of virulent and avirulent forms of this bacterium has led to many attempts to distinguish them and investigate potential virulence factors. Takahashi *et al*.[3] have reported that conventional culture methods using biochemical and nutritional tests do not differentiate virulent and avirulent strains of *V. parahaemolyticus*. Kim *et al*.[4] and Sujeewa *et al*.[5] have demonstrated that PCR is a valuable and rapid method for identifying *V. parahaemolyticus* and detecting putative virulence genes. Using PCR, detection of the tdh gene or trh gene, or both, are considered diagnostic markers for pathogenic isolates of *V. parahaemolyticus*[6]. However, these virulence genes are absent in some pathogenic strains; thus, some *V. parahaemolyticus* isolates that lack TDH and/or TRH remain pathogenic[7-11]. Furthermore, *V. parahaemolyticus* strains lacking tdh and/or trh caused outbreaks in Southern Chile[12]; therefore, there is a need to investigate other markers of virulence. Simple and validated molecular methods are available for the identification of the species *V. parahaemolyticus*, such as PCR for the genes encoding the transcriptional regulator (toxR), the thermolabile hemolysin (tlh) and the pR72H fragment[4,13].

The distinction between virulent and avirulent strains of a species may require the use of molecular typing methods; for example, randomly amplified polymorphic DNA polymerase chain reaction (RAPD–PCR) is a technique that may be used to produce a unique banding pattern to differentiate virulent strains from avirulent strains. RAPD fingerprinting has been developed as a valuable tool for distinguishing pathogenic and non-pathogenic isolates of *V. harveyi*[14,15]. RAPD uses PCR primers under low specificity (stringency) conditions[16] to amplify small genome sections. An essential factor in RAPD analysis is selecting appropriate primers and optimizing PCR. The primers are simple, short, and used without prior knowledge of the template sequence of the target DNA[17]. RAPD-PCR may be performed with one or two primers, Hu *et al*.[18] used two primers in the RAPD reaction and found that the two-primer RAPD tends to produce smaller and more numerous fragments than the standard RAPD technique using only one primer. The usefulness of the two primer RAPDs is that it allows more reactions to be performed with a limited number of primers.

Previous work reported by Kadhim *et al*.[7] used RAPD-PCR with random primer set P1 (5'-d [GGTGCGGGAA]-3') and P5 (5'-d [AACGCGCAAC]-3') to reveal a unique band (approximately 600 bp) in the majority of *V. parahaemolyticus* clinical isolates tested, which corresponded to an outer membrane protein gene sequence (*omp*). This allowed the authors to develop a PCR test that amplified a 200 bp segment of omp, detecting 91% of virulent forms of *V. parahaemolyticus* tested. This study aimed to develop a rapid, reliable and straightforward method for differentiating virulent clinical and mainly avirulent environmental isolates of *V. parahaemolyticus*. This led to the development of a multiplex PCR (m-PCR) that can be used to reliably identify

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all isolates of the *V. parahaemolyticus* species and detect potentially virulent strains simultaneously. An RAPD method was used with novel sets of arbitrary primers to identify unique RAPD bands in virulent isolates. Their sequence information was used to develop the m-PCR-based method.

Materials and methods

Bacterial strains

All isolated bacteria used in the present study are listed in Table 1. The cultures of Vibrio were grown routinely on Tryptone soya agar (TSA) (Oxoid, Basingstoke, UK) supplemented with 3 % (w/v) sodium chloride at 37°C. Stock cultures were stored on slopes of marine salts agar (MSA) at room temperature. Other bacteria were grown at their optimum temperature on Luria-Bertani (LB) medium (Difco).

Genomic DNA extraction

According to the manufacturer's instructions, genomic DNA was extracted from bacteria using the DNeasy tissue kit (Qiagen, Ltd., UK). The concentration and purity of DNA were measured by absorbance at 260 and 280 nm using a NanoDrop 100 spectrophotometer (Lab-Tech, UK) and its integrity after extraction was determined using 0.8 % (w/v) agarose gels in TBE buffer. The DNA was stained with ethidium bromide (0.5 µg ml^{-1}), and photographed under UV light (309nm) with a Gel Documentation system (Uvi-Tech, UK).

RAPD-PCR

All PCR amplifications were performed in a Primus 96 plus thermal cycler (MWG Biotech). RAPD-PCR was performed using RAPD Analysis Beads (Amersham Biosciences). Reactions containing a bead resuspended in a 25 µl volume to give 1 unit Amplitaq DNA polymerase and Stoffel fragment, 0.4 mM of each dNTP, 2.5 µg BSA and buffer [3mM MgCl2, 30mM KCl and 10mM Tris, (ph 8.3)]. A 25pmol (5µl) amount of the appropriate primer and DNA template (10 ng) was added to each reaction. The following temperature-cycling parameters were used: 1 cycle at 95ºC for 5 min, followed by 45 cycles at 95 ºC for 1 min, 36 ºC for 1 min and 72 ºC for 2 min. RAPD-PCR was repeated at least two times to test for reproducibility.

Genomic DNA extraction

PCR primers VPHTP1 and VPHTP2 were designed using primer design software Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). DNA calculator (Sigma-Genosys) was used to test primers for secondary structure and primer-dimer formation. Primer sequences were then run through the Basic Local Alignment Search Tool (BLAST) software provided by the National Centre for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi)19. Primers were obtained from MWG Biotech (MWG, Germany). Each 50 µl PCR reaction contained 1x reaction buffer containing 1.5 mM MgCl2 (Roche Applied Science, UK), 50 pmol of each primer, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1 U of Taq DNA polymerase, 20 ng of bacterial DNA and the reaction volume was made up to 50 µl using PCR grade water. PCR conditions were as follows: one cycle consisting of 94°C for 5 min, 30 cycles consisting of 94°C for 1 min, 59°C for 1 min, and 70°C for 2 min, and a final cycle consisting of 70°C for 5 min. The PCR products were electrophoresed at 90 V for approximately 6 h using a 2 % (w/v) agarose gel. A 100-bp DNA ladder (Invitrogen, UK) as standard were used as a marker in determining the size of the amplification products. DNA bands were visualized after staining with ethidium bromide (0.5 µg ml-1) or Red gel stain and photographed under ultraviolet light (309 nm) with a Gel Documentation system (Uvi-Tech, UK., software). Band sizes were assigned by direct comparison to concurrently run DNA standards. For all tests, a PCR mixture without DNA was used as negative control and to monitor for contamination.

Multiplex PCR amplification

Multiplex PCR amplification was performed on genomic DNA from each of the isolates of V. parahaemolyticus, other Vibrio species and non-Vibrio species by the simultaneous addition of three primer pairs in a 50 µl reaction mixture consisting of 30 pmol of each of the oligonucleotide primers, 0.2 mM of each deoxynucleoside triphosphate, 1x reaction buffer containing 1.5 mM MgCl2 (Roche Applied Science, UK), 60 ng of DNA template, 2 U of Taq DNA Polymerase (Roche) and the reaction volume was made up to 50 µl using PCR grade water. The primer pairs used in this PCR were as follows:

VPOMP1 5'-GTCACGCGGCCAAACAAAGAGA-3' and VPOMP2 5'-ACCGCATATCACTGTTGGCTGGG-3'[17]

VPHTP1 5'-GACATCCAATCTGCACGCAAC-3' and VPHTP2 5'-CCTTTCGCTTCGAGCAATCA-3' was designed in this work; and toxR1 5'-GTCTTCTGACGCAATCGTTG-3' and toxR2 5'-ATACGAGTGGTTGCTGTCATG-3'[4].

The conditions for PCR were as follows: initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 59°C for 1 min, and primer extension at 72°C for 2 min. Following the amplification cycles, a final extension was performed at 72°C for 5 min. The m-PCR products were verified: 10 µl of the amplified DNA was separated in a 1.5 % (w/v) agarose gel using Tris-Borate/EDTA (TBE) electrophoresis buffer as described above.

DNA ligation and transformation

The unique RAPD band (Z) was excised and extracted from the gel using an agarose gel extraction kit (Roche Applied Science, UK) and purified using the 'High pure PCR Product Purification Kit' (Roche Applied Science, UK) according to the manufacturer's instruction. The purified DNA fragment was ligated into pGEM-T Easy vector (Promega, UK) using T4 DNA ligase and transformed into competent cells of E. coli JM109. The transformants with pGEM-T carrying the RAPD band Z fragment were cultured on LB agar containing ampicillin (100 µg ml-1), IPTG (0.5 mM), and X-Gal (80 µg ml^{-1}) overnight at 37°C. Recombinant plasmids were isolated from white colonies using the miniprep plasmid DNA extraction Kit (BioRad, Hemel Hempstead, UK). The presence of expected size inserts within the plasmids of transformants was verified by restriction enzyme analysis. Sequencing of both strands of selected recombinant plasmids containing the insert of the expected size was performed by MWG. Using BLAST, the nucleotide sequence data were analyzed for homology to deposited sequences in the GenBank database. The nucleotide sequence data presented in this paper has been deposited in GenBank nucleotide sequence database under accession number BA000031.2.

Sequencing of PCR products

To confirm amplicons were of the targeted sequence, selected PCR products were sequenced (MWG).

Results

RAPD-PCR

Initially, *V. parahaemolyticus* genomic DNA from two clinical isolates (C8 and C15) and two environmental isolates (E2 and E22) were screened with nine single primers OPK1-7, OPK12 and OPK18 in RAPD-PCR reactions. These primers failed to produce unique bands to differentiate the test clinical and environmental isolates of *V. parahaemolyticus*. However, when 30 different OPK primer pairs were used with the same selected isolates, only the OPK13 +15 pair produced a unique band with the clinical isolates Figure 1. This amplicon was named band Z. A unique band was found When the OPK13 +15 primer set was used on the complete collection of *V. parahaemolyticus* isolates. Most clinical isolates produced 300 bp, and most environmental forms did not produce this band (data not shown). Patterns generated by this RAPD-PCR were reproducible in assays on different occasions. The DNA from band Z was isolated and ligated into the pGEM-T Easy vector and transformed into *E. coli* JM109. Restriction enzyme analysis was used to verify the presence of expected size inserts within the transformants, and selected recombinant plasmids were sequenced. BLAST analysis and comparison with the genome of *V. parahaemolyticus* RIMD 2210633 DNA (accession no.BA000031.2) showed identity with a 312 bp segment from base pairs 1812453 to 1812764 on chromosome 1. The nucleotide sequence was analyzed and found from a gene encoding a hypothetical protein (htp). The 312 bp band Z sequence was identical to the htp gene from *V. parahaemolyticus*. At the protein level, HTP was similar to proteins from *V. alginolyticus* (accession no. ZP_01259705.1, 62% homology), and *V. harveyi* (accession no. YP_001444888.1, 39% homology).

htp-PCR

Based on the DNA sequence of htp, the specific primers VPHTP1 (5'-GACATCCAATCTGCACGCAAC-3') and VPHTP2 (5'-CCTTTCGCTTCGAGCAATCA-3') were developed in Figure 2. PCR was performed using this primer pair on the genomic DNA of *V. parahaemolyticus* isolates and other Vibrio and non-Vibrio species. The expected 300 bp product was found in 19 out of 23 (82.6%) clinical isolates of *V. parahaemolyticus*, including clinical NCIMB reference strain R1 and only 2 out of 32 (6.25 %) environmental isolates (Table 1). No band was seen with any other Vibrio and non-Vibrio species tested.

m-PCR

This new primer set and the primer set previously developed for *omp*[7] were used with the species-specific primer for toxR gene[4] to develop a multiplex PCR. This reliably identified all *V. parahaemolyticus* isolates tested and simultaneously distinguished between virulent and avirulent strains (Fig 3). The species-specific toxR gene 367 bp segment was amplified in all *V. parahaemolyticus* isolates tested. Of 23 clinical isolates, 23 (100%) showed the presence of either *omp* or *htp* presence. Of these, 17 (73.91%) showed amplification of both *omp* and *htp* target sequences, 21 (91%) showed amplification of the 200 bp fragment of the *omp* gene, and 19 (82.6%) showed amplification of the 300 bp fragment of the htp gene (Fig 3). This showed that most clinical *V. parahaemolyticus* isolates contained both genes. However, four clinical isolates showed amplification of the *omp* gene but not the htp gene, while two clinical isolates exhibited amplification of *htp* but not *omp*. Thus both *omp* and *htp* must be targeted in PCR tests to detect all virulent strains. Out of 32 environmental isolates, none contained both *omp* and *htp*. Only 3 out of 32 (9.37%) environmental isolates showed amplification for either *omp* (1 = 3.12%) or *htp* (2 = 6.25%) genes. No amplification of *omp* or *htp* sequences was seen in other Vibrio or non-Vibrio species tested.

Discussion

The results of this study indicate that RAPD shows a promising ability to differentiate between clinical (virulent) and environmental (predominantly avirulent) isolates of *V. parahaemolyticus*. It should be noted that some environmental isolates will have the capacity to cause disease. It may be that different RAPD primer sets might be able to distinguish virulent from avirulent isolates completely and be developed into a quick PCR-based test. In the meantime, this study developed a multiplex PCR to reveal potentially virulent forms by detecting either omp (200 bp) or htp (300 bp) sequences or both; and identification of all *V. parahaemolyticus* isolates by producing species-specific fragment toxR gene (367 bp). The multiplex PCR method was simultaneously used to identify *V. parahaemolyticus* and target both *omp* and *htp* genes in a single PCR reaction. Previous work by Kadhim *et al.*[7] tested *V. parahaemolyticus* isolates for cytotoxicity to the human Caco-2 cell line and found that positive isolates for omp were strongly associated with cytotoxicity. In the current study, it should be noted that isolates carrying htp were also associated with cytotoxicity (Table 1). Isolates that did not carry either *omp* or *htp*, i.e., most of the environmental isolates, showed low cytotoxicity, except for the NCTC reference strain R2. This is environmentally sourced, did not give a positive PCR for either *omp* or *htp*, but showed a level of cytotoxicity on a par with many of the clinical (virulent) isolates tested here. It is likely that either *omp* or *htp*, or both, act merely as markers for cytotoxicity, i.e., is not directly responsible for cytotoxicity. Perhaps they are located on a genetic region or element that carries a gene or genes for cytotoxic products when acquired. Maybe in R2, the marker genes have become detached and lost from any cytotoxic genes, but the latter remain and are active. Alternatively, R2 may contain a new or separate cytotoxic activity unrelated to omp or htp. Finally, it is possible that mutation at any of the primer-targeted regions for *omp* or *htp* sequences could have occurred, and therefore PCR amplification might fail.

Previously, between 6%[20] and 9%[21] of environmental *V. parahaemolyticus* isolates have been designated as potentially pathogenic, so the current study's findings align with others. Further work is required to investigate whether omp or htp are virulence factors or merely virulence markers.

Conclusions

The novel multiplex PCR developed in this study can target three gene segments simultaneously and enable the identification of V. parahaemolyticus and the detection of the potentially virulent forms of this species in a single reaction. Although the reliability of this m-PCR needs further evaluation with more clinical and environmental isolates, this PCR shows excellent promise to be used as a suitable tool to identify V. parahaemolyticus and detect potentially virulent forms. Moreover, it could be used in epidemiological

Strain	Obtained from *	Type and source (where known)	PCR product			Percent of Cytotoxicity #
			toxR(367bp)	*Omp*(200bp)	*htp*(300bp)	Caco-2
R1	PHLS	Clinical, NCIMB Reference Strain, UK	+	+	+	60.0
C1	NSVS	Clinical, Norway	+	-	+	40.0
C2	NSVS	Clinical, Norway	+	+	+	85.0
C3	NSVS	Clinical, Norway	+	+	+	81.0
C4	NSVS	Clinical, Norway	+	+	+	83.5
C5	NSVS	Clinical, Norway	+	+	+	84.0
C6	NSVS	Clinical, Norway	+	+	-	71.0
C7	NSVS	Clinical, Norway	+	+	+	72.0
C8	NSVS	Clinical, Norway	+	+	+	82.0
C9	NSVS	Clinical, Norway	+	+	-	74.5
C10	NSVS	Clinical, Norway	+	-	+	42.0
C11	KUMS	Clinical, Japan	+	+	+	83.6
C12	KUMS	Clinical, Japan	+	+	+	83.7
C13	KUMS	Clinical, Japan	+	+	+	65.0
C14	KUMS	Clinical, Japan	+	+	+	65.0
C15	PHLS	Clinical, UK	+	+	+	87.0
C16	USC	Clinical, Spain	+	+	+	64.6
C17	USC	Clinical, Spain	+	+	+	65.0
C18	USC	Clinical, Spain	+	+	+	61.9
C19	USC	Clinical, Spain	+	+	+	64.0
C20	USC	Clinical, Spain	+	+	+	60.0
C21	CEFAS	Clinical, Italy	+	+	-	55.8
C22	CEFAS	Clinical, UK	+	+	-	63.0
R2	PHLS	Environmental, NCTC Reference Strain, UK	+	-	-	60.8
E1	CEFAS	Environmental, Spain	+	-	-	0.6
E2	CEFAS	Environmental, Spain	+	-	-	19.8
E3	CEFAS	Environmental, Spain	+	-	+	39.0
E4	CEFAS	Environmental, Spain	+	-	-	0.5
E5	CEFAS	Environmental, Spain	+	-	-	0.6
E6	CEFAS	Environmental, Spain	+	-	-	0.5
E7	PHLS	Environmental, UK	+	-	-	20.5
E8	PHLS	Environmental, UK	+	-	-	0.4
E9	CEFAS	Environmental, UK	+	+	-	86.6
E10	CEFAS	Environmental, UK	+	-	-	0.6
E11	CEFAS	Environmental, UK	+	-	-	19.0
E12	CEFAS	Environmental, UK	+	-	-	0.6
E13	CEFAS	Environmental, UK	+	-	-	18.0
E14	CEFAS	Environmental, UK	+	-	-	19.6

Table 1. List of *Vibrio parahaemolyticus* isolates tested in this study and results of multiplex PCR.

E15	CEFAS	Environmental, UK	+	-	-		20.0
E16	CEFAS	Environmental, UK	+	-	-		25.9
E17	CEFAS	Environmental, UK	+	-	-		0.4
E18	CEFAS	Environmental, UK	+	-	-		24.5
E19	CEFAS	Environmental, UK	+	-	-		0.4
E20	CEFAS	Environmental, UK	+	-	+		28.0
E21	CEFAS	Environmental, UK	+	-	-		0.5
E22	CEFAS	Environmental, UK	+	-	-		0.1
E23	CEFAS	Environmental, UK	+	-	-		14.8
E24	CEFAS	Environmental, UK	+	-	-		22.0
E25	CEFAS	Environmental, UK	+	-	-		0.6
E26	CEFAS	Environmental, UK	+	-	-		26.7
E27	CEFAS	Environmental, Portugal	+	-	-		19.0
E28	CEFAS	Environmental, Portugal	+	-	-		27.0
E29	CEFAS	Environmental, UK	+	-	-		16.5
E30	CEFAS	Environmental, UK	+	-	-		24.7
E31	CEFAS	Environmental, UK	+	-	-		18.5

*CEFAS, Centre for Environment, Fisheries and Aquaculture Science, Weymouth, UK; PHLS, Public Health Laboratory Service, Southampton, UK (now Health Protection Agency); KUMS, Kyoto University Medical School, Japan; NSVS, Norwegian School of Veterinary Science, Oslo, Norway; USC, Universidad de Santiago de Compostela, Spain. #Results o: Cytotoxicity obtained from previous paper [7] 100% represents all cells lysed.

Table 1. List of *Vibrio parahaemolyticus* isolates tested in this study and results of multiplex PCR.

studies and investigations into the abundance of potentially virulent forms of V. parahaemolyticus in various environmental habitats.

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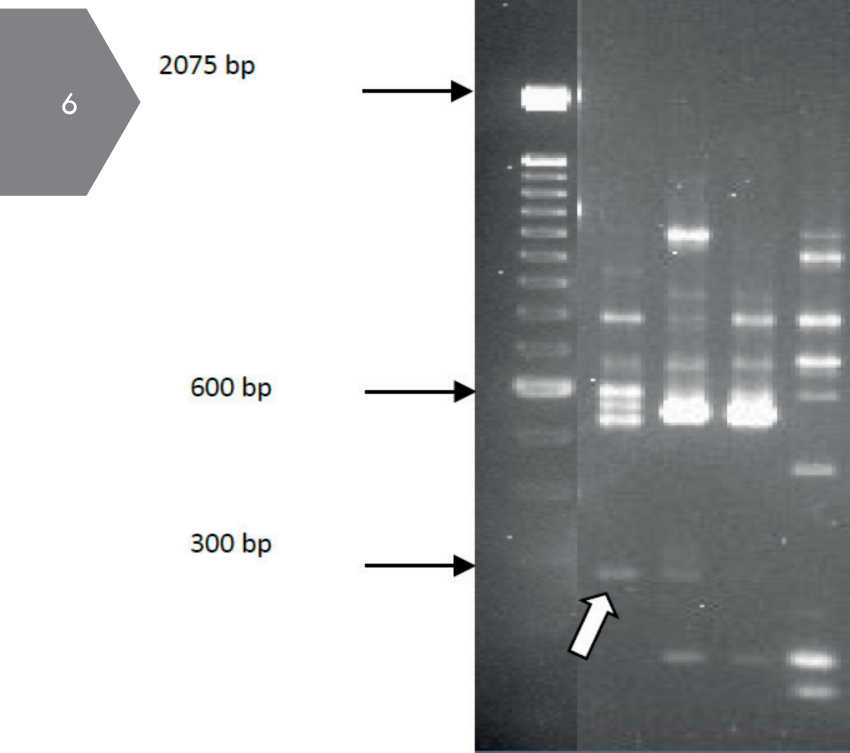

Figure 1. RAPD double primers screening of clinical and environmental isolates; Lane 1, C8; Lane 2, C15; Lane 3, E2; Lane 4, E22; Lane M, 100bp DNA ladder. Arrow indicates Band Z.

5'-
ATGTCTAAT**GACATCCAATCTGCACGCAAC**AGCCAACCCATTCACGGATTGGAGCA
AGTA
VPHTP1

GAACAACGTGAACTTGCGCCACAAGGAACGTTTCAAGGTCGCAAAGTGACCTTACTC
TCT

TCATCAGAAAACAAACAGGCACGCATGAGCTCAAAGCGAGAACTAAGCGAATGTTT
AAAC

CAGTTCGCGAGCCTTGAATCTTGCAATCAAGTCTTGGATTTTGAAAAGCCAACTGGAT
TT

GAGAAGCAAAGCGCTGCTATCGAAAAGCTTTTTGAGCGAAAGATAGATG**TGATTGC**
TCGA
VPHTP2

AGCGAAAGGTAA -3

Figure 2. Nucleotide sequence of clinical *V. parahaemolyticus* isolates that produced band Z and locations of PCR primers VPhtp1 and VPhtp2.

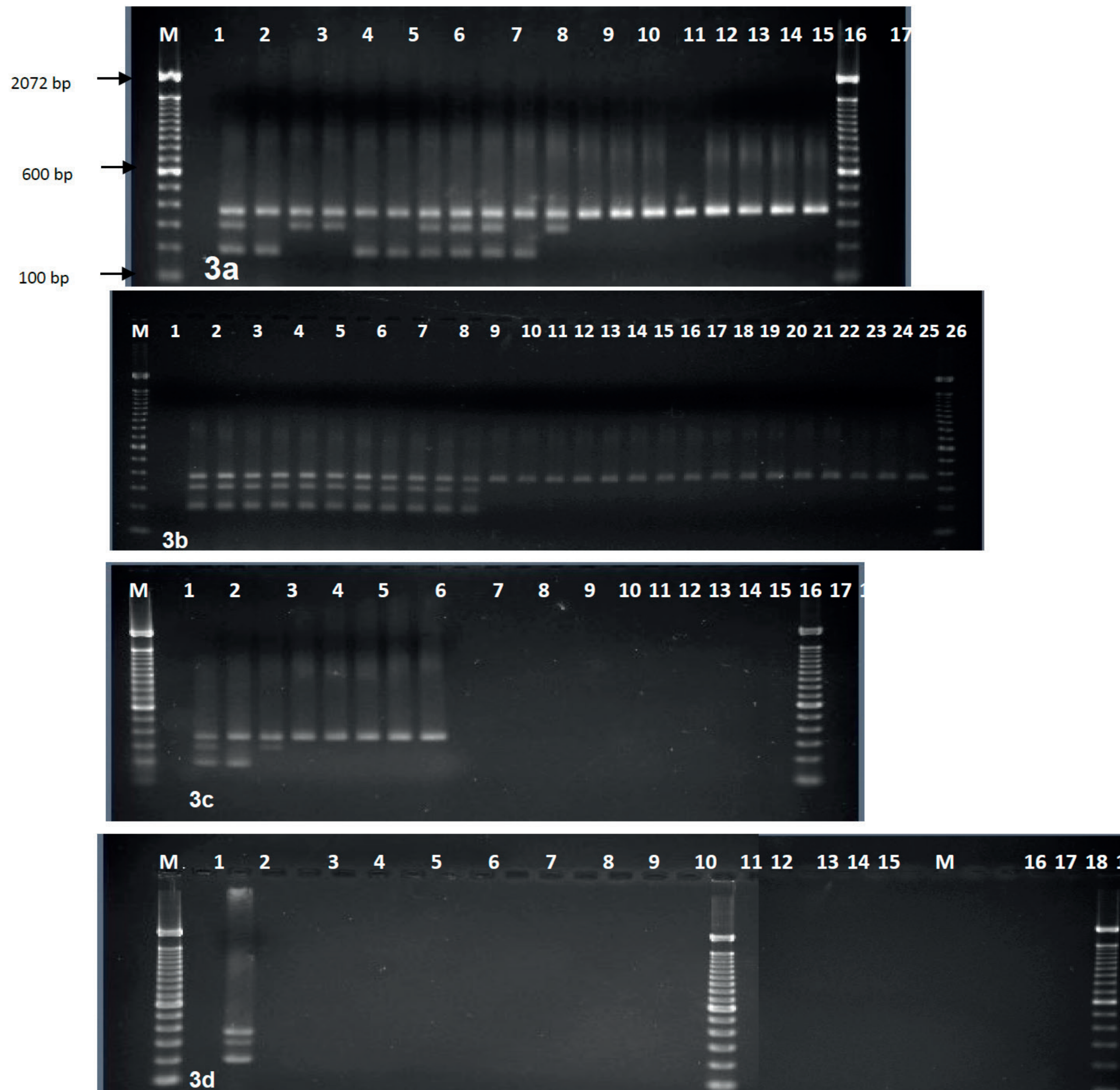

Figure 3. Agarose gel electrophoresis of multiplex PCR amplified products using three primer pairs, Lanes M, 100-bp DNA ladder. (a) Lane 1, control negative; 2-10 clinical isolates(2, R1;3, C9; 4, C1; 5, C10; 6, C21; 7, C22; 8, C7; 9, C18; 10, C2); 11-20 environmental isolates (11, E9; 12, E3; 13, E1; 14, E2; 15, E10; 16, E30; 17, E31; 18, E4; 19, E5; 20, E8). (b) Lane 1, control negative; Lanes, 2-12 clinical isolates (2, C20; 3, C19; 4, C11; 5, C12; 6, C13; 7, C14; 8, C17; 9, C16; 10, C3; 11, C4; 12, C5);Lanes, 13-28 environmental isolates (13, E11; 14, E12; 15, E16; 16, E21; 17, E22; 18, E23; 19, E24; 20, E25; 21, E26; 22, E27; 23, E28; 24, E29; 25, R2; 26, E13; 27, E14; 28, E15). (c) Lane 1, control negative; Lanes 2-3 clinical isolates (2, C8, 3, C6); Lanes 4-9 environmental isolates (4, E20; 5, E19; 6, E18; 7, E17; 8, E6; 9, E7) lanes 10-20, other Vibrio species (10, VC; 11, VM; 12-17, VV1-VV6; 18-19, VN1-VN2; 20, VS. (d) Lane 1, control negative without template DNA; 2, clinical isolate (C15); 3-13 other Vibrio species (lanes, 3-8, VA1-VA6; 9-13, VH1-VH5).; 14-17 Aeromonsa; 18-24 non Vibrio (lanes 18-19, Pf1-2; 20, St; 21, Pv; 22, Kp; 23-24, Ec1-Ec2); 25, control negative without primers.

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