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Detection of *Vibrio parahaemolyticus* by polymerase chain reaction (PCR) technique

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Abstract

Vibrio parahaemolyticus causes gastroenteritis in humans, this bacterium lives in coastal water during the summer, repetitive extragenic palindromic PCR (REP-PCR), one of the fingerprinting technique that used to analyse the isolates of *V. parahaemolyticus* tested in the current study, the majority of clinical isolates produced a band at 1000 bp amplicon. The DNA from this band was cloned and sequenced and found to code for a peptide chain release factor 1. The specific PCR amplification results showed that all 100% of the all *V. parahaemolyticus* strains tested were positive for 400 bp products. None of the other *Vibrio* species and non-*Vibrio* produced this amplicon. A PCR method amplifying a 400 bp fragment of the *V. parahaemolyticus* peptide chain release factor 1 could be useful in the specific and rapid detection of the species.

Introduction

Vibrio parahaemolyticus is a Gram-negative bacterium, and virulent strains are considered a major human pathogen associated with consumption of fish and shellfish causing gastroenteritis (Feldhusen, 2000). The efficient analytical methods for the detection of *V. parahaemolyticus* in environmental and food samples must be available to reduce the risk of this organism infection and to ensure the safety of foods (Hara-Kudo et al., 2001). Identification of *V. parahaemolyticus* is usually achieved through a series of biochemical tests after their growth and isolation on a selective plating medium. Human pathogenic *Vibrio* species can be identified by commercial identification systems and biochemical tests, but may not always be accurate (O'Hara et al., 2003). However, Miyamoto et al., (1969) have demonstrated that the Beta haemolysis of human or rabbit erythrocytes on Wagatsuma agar as a marker of the existence of TDH, which correlates with human pathogenicity. Honda et al., (1980) have characterised immunological techniques as reproducible methods for detection of the haemolysin-producing pathogenic *V. parahaemolyticus*. Moreover, Honda et al., (1982) have reported immunological methods for identification of Kanagawa phenomenon in modified traditional selective media, bromthymol blue (BTB) - teepol agar and arabinose-ammonium sulfate-cholate agar, for *V. parahaemolyticus*. These methods are helpful for isolation of *V. parahaemolyticus* and identification of the Kanagawa phenomenon on a single plate.

Many PCR-based assays have been developed to assist in the specific detection of *V. parahaemolyticus* and in the detection of toxin producing genes. Furthermore, DNA gyrase subunit B encoded by the gyrase B (*gyrB)* gene that is responsible for DNA replication has been proposed by Venkateswaran et al., (1998) to distinguish *V. parahaemolyticus* from *V. alginolyticus*. The *gyrB*-targeted PCR for specific detection of *V. parahaemolyticus* in shrimp has been developed despite the homology of the *gyrB* sequences between both these bacteria (86.8%) (Venkateswaran et al., 1998).

There are PCR-fingerprinting techniques used primers that designed to known target sequence and will be complementary to repetitive sequence in the bacteria genome include enterobacterial repetitive intergenic consensus (ERIC) sequences, repetitive extragenic palindromic (REP) sequences, and BOX elements. These elements are highly conserved at the nucleotide level and their position in enterobacterial genome varies between different species and can be used as a genetic marker to differentiate bacterial species (Versalovic et al., 1991). REP elements were first described in *E. coli* as 35 bp sequences composed of a high conserved inverted repeat with the potential of forming a stem-loop structure (Stern, et al., 1984). Moreover, Aranda-Olmedo, et al., 2002 have found this element in *Pseudomonas putida* and repeated with a high degree of sequence conservation. REP-PCR and ERIC-PCR have been applied to study the genetic variation among non-O1 and non-O139 *Vibrio cholerae* at higher sensitivity levels (Rao and Surendran, 2010). The aim of study was to investigate the possibility that clinical isolates of *V. parahaemolyticus* may produce a unique REP band pattern that could be used to differentiate *V. parahaemolyticus* from other species.

Material and methods

Bacterial strains

A total of 32 *V. parahaemolyticus* strains were analysis in this study, 16 clinical strains and 16 environmental strains. A 9 *Vibrio* species and 6 non-*Vibrio* species were also used in this study. Clinical strains (from patients with diarrhea) and environmental strains (from seafood or environmental waters) of *V. parahaemolyticus*.

Repetitive extragenic palindromic PCR (REP-PCR)

Chromosomal DNA from all *V. parahaemolyticus* isolates was extracted from overnight culture of the organism in LB broth with 3% NaCl using DNeasy tissue kit (Qiagen, Ltd., UK) according to the manufacturer's instructions DNA used for REP-PCR. REP analysis was performed using 32 isolates of *V. parahaemolyticus.* REP-PCR primer sequences: REP1R-I, 5'-IIIICGICATCIGGC-3' and REP2-I. 5'-ICGICTTATCIGGCCTA-3' were used to amplify PCR. The PCR method was initially performed using 10 ng of template DNA. However, these reactions failed to produce a PCR product and it was necessary to increase the amount of template to 80 ng per reaction in order to produce visible amplicons. The PCR reaction mixture consisted of 5 µl of 10x reaction buffer (100 mMTris-HCl, 15 mM MgCl2, 500 mMKcl, pH 8.3), 1 µl of PCR Grade nucleotide (200 µM of each dNTP), 1.3 unit of *Taq* DNA polymerase, 50 pmol of primer and 80 ng of DNA template in a total reaction volume of 50 µl. Initially, we determined the effect of temperature on this primer in using the genome of the bacteria. The best

cycling conditions an initial denaturation step at 94°C for 7 minutes followed by 30 cycles of 94°C for 1 min, 38°C for 1 min, and 70°C for 2 min and a final extension at 70 °C for 8 min. Amplifications were performed on a thermal cycler (MWG, UK).

Polymerase Chain Reaction

PCR primers VPHK1 and VPHK2 were designed by using primer design software Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi), a lack of secondary structure and primer-dimer formation was assessed usingDNA calculator(Sigma-Genosys).Primer sequences were then run through Blast (http://blast.ncbi.nlm.nih. gov/Blast.cgi). Each 50 µl reaction contained 1.5 mM $MgCl_2$ (Roche), 50 pmol of each primer, each deoxynuclieaside triphosphate at a concentration of 0.2mM,1.3 U of *Taq*DNA polymerase, 40 ng of bacterial DNA and the reaction volume was made up to 50µl using PCR grade water. PCR conditions started with 1cycle of pre-denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 64°C for 1 min, and elongation at 70°C for 2 min. The amplification ended with a final elongation at 70°C for 5 min. A 15 µl aliquot of each PCR products were separated by electrophoresis on 2% agarose gels (w/v) and run in 1x Tris-borate buffer, pH 8.3. The ampilified DNA bands were visualised after ethidium bromide staining and photographed under ultraviolet light. A 100-bp ladder and 1 kb DNA markers (Invitrogen, UK) as standards was used as a marker in determining the size of the amplification products, and visualised by ethidium bromide stain. Band sizes were assigned by direct comparison to concurrently run DNA standards (100 bp). The PCR for each strain was performed in three separate experiments to confirm the pattern of amplified bands and to ensure the reproducibility of the patterns. For all PCR applications, a PCR mixture without DNA was used as negative control and to monitor for contamination. The stained bands were visualised with UV light (309 nm) using a trans-illuminator and gels were recorded as digital TIFF images using a gel documentation system (UVI-Tech).

DNA manipulation

Cloning, restriction endonuclease procedures, DNA ligation, and transformation of *E. coli* by plasmids were carried out using previously described standard protocols (Sambrook et al., 1989). As follow: The amplified DNA fragment with 1.09 bp by REP-PCR was ligated into pGEM-T easy vector (Promega, UK) after excised and purified from the gel. Then, the DNA was transformed into the competent cells of *E. coli* JM109. The transformants with pGEM-T carrying the 1.09 bp gene was selected on LB plate with Ampicilin. The transformants were cultivated in liquid LB medium with Ampicilin overnight at 37°C. The plasmids were isolated from the cultivated transformant cells 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. The recombinant plasmids containing the insert of expected size was submitted to MWG Laboratory (MWG, Germany) for sequencing. The nucleotide sequence data were analysed for homology to the published sequence in the GenBank database using theBasic Local Alignment Search Tool (BLAST) software provided by the National Centre for Biotechnology Information (NCBI).

Results and Discussion

Fingerprinting methods depend on the amplification of genomic sequences between the repetitive elements conserved in prokaryotes including repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC) and BOX elements (Versalovic et al., 1991). In this study, clinical and environmental *V. parahaemolyticus* isolates genetically analysed and identified the clinical *V. parahaemolyticus* specific DNA fragment by REP PCR. The fingerprints consisted of two to seven amplification bands, ranging in size from 250 bp to 2072 bp. The 1500-bp band was common to all *V. parahaemolyticus* isolates; and 800-bp was common band in most of the isolates. However, one band of 1000 bp was only present in most clinical isolates Figure 1. Patterns generated by REP PCR were reproducible in three different assays. REP PCR is quick, comparatively simple to achieve, and can be used as a single methods for typing.

After purification of the 1000 bp PCR products from the gel, the PCR products were ligated into plasmid pGEM-T easy vector and transformed into the competent cells of *E. coli* JM109. The positive transformants on LB plate including Amp, X-gal and IPTG were selected and the plasmids in the transformants were isolated. The gene was sequenced and the nucleotide sequence of the gene is shown in Figure 2. Using the BLAST program (www.ncb.nlm.nih.gov/blast/blast.cgi) to comparison with sequences available at GenBank and EMBL (Altschul et al., 1990) revealed that the 1.09 kp sequence of the obtained REP-PCR fragment was identical starting 780547 bp to 781635 bp of sequence of *Vibrio parahaemolyticus* RIMD 2210633 DNA, chromosome 1 (accession no.BA000031.2). The nucleotide sequence was analysed and found to be from a gene encoding a peptide chain release factor 1 (accession no.BA000031.2) 100% homology for *V. parahaemolyticus*, and it was found to be 92% homology to the hypothetical protein *Vibrio harveyi* (accession no. CP000789.1)

A PCR-based detection method for *V. parahaemolyticus* isolates was developed by using primer pair VPHK1 (5'GGCAAAAGAAGACGACGAAG-3') and VPHK2 (5'TGCACACGACCTTGAGATTC3'-3') that were designed by using this sequence information (accession no. BA000031.2) and screened against the sequenced of *Vibrio harveyi* (accession no.CP000789.1) to ensure specificity and protect against cross-reactivity. PCR was performed on genomic DNAs of 32 *V. parahaemolyticus* including 16 clinical and 16 environmental strains and 9*Vibrio* species; and 6 non-*Vibrio* species. The expected 400 bp amplicon could only be observed in the PCR profile of *V. parahaemolyticus* strains. The expected size amplicons were not observed from the PCR profiles of *Vibrio* species specifically *V. cholerae, V. mimicus, V. vulnificus, V. alginolyticus, V. harveyi, V. anguillarum, V. shiloi, Aeromonas hydrophila, and A. salmonicida*. Profiles from PCR using DNA template from non-*Vibrio* species specifically *Pseudomonas flouresecence, Staphylococcus aureus, Salmonella typhimurium, Proteus vulgaris, Klebsiella pneumonia* and *Eschericia coli* did not exhibit the 400 bp amplified fragment either Figure 3. It is possible that this fragment could be used as a probe to differentiate *V. parahaemolyticus* from *Vibrio* species. Brocchi et al., (2006) have used REP-PCR to differentiate between pathogenic and non-pathogenic of *Escherichia coli* strains. REP-PCR are quick, relatively simple to achieve, and cheap and were used in this study

In summary, this study aimed to use REP-PCR to assess whether *V. parahaemolyticus* could be differentiated from other species. The PCR assay developed can be used to specifically

identify *V. parahaemolyticus.* The findings of this study suggest that the REP-PCR method is a useful tool for identification of *V. parahaemolyticus* isolates but further work is needs to establish this possibility and needs to test a large number of the *V. parahaemolyticus* and *Vibrio* species.

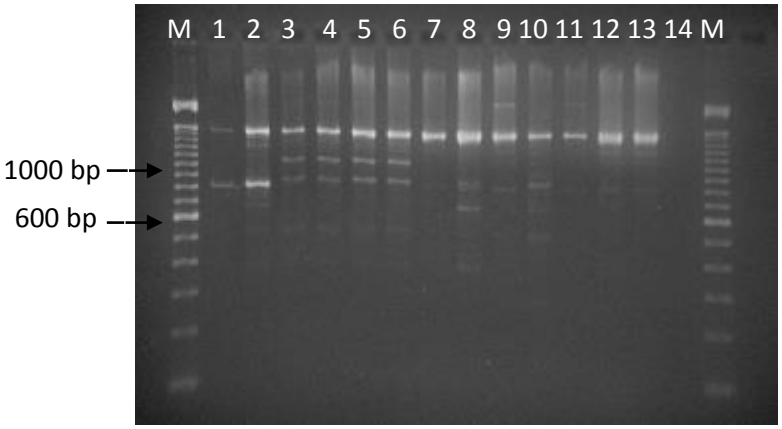

Fig 1 A unique REP-PCR (Band A) amongst clinical strains of *V. parahaemolyticus*. Lanes 1-6 clinical isolates; Lanes 7-13 environmental isolates; Lane 14 negative control; Lanes M, 100bp ladders with the sizes of selected markers indicated by the arrows.

5'- ATGAAAGCCTCTATTCTGACTAAGCTAGAAACGCTAGTAGAACGTTACGAAGAAGTTCAACATCTTCTTG
GTGATCCTGATGTAATTGGAGATCAGGACAAATTCCGTGCTCTATCAAAAGAGTACTCTCAGCTAGAAGA
AGTAACTAAGTGCTTCCAAGCTTACCAACAAGCTCAAGACGATCTTGCTGCCGCTGAAGAGATGGCAAAA
GAAGACGACGAAGAAATGCGTGAAATGGCTCAAGAAGAGATCAAAGACGCGAAAGAAGCGATTGAACGTC
TTGCTGATGAGTTGCAAATTCTTCTTCTTCCAAAAGATCCAAATGATGATCGTAACTGTTTCCTTGAAAT
CCGCGCTGGTGCTGGCGGTGATGAAGCAGGTATCTTCGCTGGCGACCTATTCCGTATGTACAGCAAATAC
GCAGAAAAGCGTGGCTGGCGCATTGAAGTGATGTCTTCAAACGAAGCGGAGCACGGTGGTTACAAAGAGA
TGATCGCAAAAGTGAGCGGCGATGGTGCATACGGCGTACTGAAGTTTGAATCTGGCGGTCACCGTGTACA
ACGTGTACCAGCGACAGAATCTCAAGGTCGTGTGCATACTTCTGCGTGTACAGTAGCGGTCATGGCGGAA
ATCCCAGAGGCGGATCTGCCAGAAATCAAAGCGGCAGACCTAAAAATCGATACGTTCCGTGCATCTGGCG
CGGGTGGTCAGCACGTTAACACCACGGATTCTGCAATCCGTATTACCCACTTACCAACGGGTACAGTGGT
AGAGTGTCAAGACGAGCGTTCACAGCATAAGAACAAAGCGAAAGCGATGGCAGTACTGGCTGCTCGTATC
GTTCAAGCGGAACAAGAGCGTCGTGCGGCAGAAGTGTCTGACACGCGTCGTAACCTACTAGGTTCTGGTG
ACCGCAGTGACCGTATCCGTACTTACAACTACCCGCAAGGCCGTGTTTCTGACCACCGAATCAACCTAAC
CATCTACCGTTTGAACGAAGTGATGGAAGGCGACCTACAAAGCTTGATTGACCCAGTGGTTCAAGAACAC
CAAGCAGACCAACTAGCGGCGCTAGCTGAGAACGCTTAA -3'

Figure 2. Nucleotide sequence of the REP-PCR of virulent *V. parahaemolyticus* product.

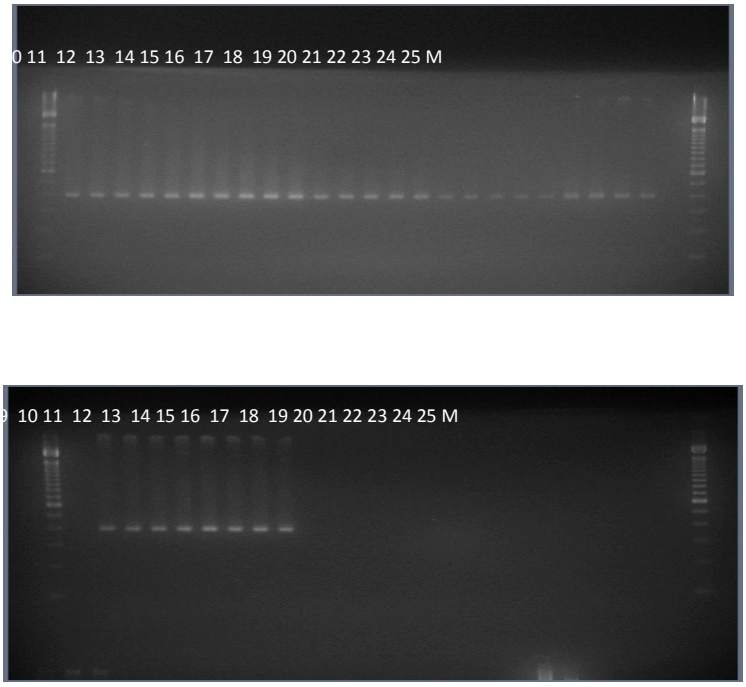

Figure 3. Agarose gel electrophoresis of PCR amplified products using specific primers, VPHK1 and VPHK2. Lanes M, 100-bp molecular mass marker. a Lane 1-12 clinical *V. parahaemolyticus*; Lanes 13-24 environmental *V. parahaemolyticus*; Lane 25, negative control; b Lane1, negative control; Lanes 2-5 clinical; Lanes 6-9 environmental *V. parahaemolyticus*; Lanes 10-25 other species.

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تشخيص بكتريا *Vibrio Parahaemoticus* بواسطة تقنية PCR

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الخلاصة

تسبب بكتريا *Vibrio parahaemolyticus* الالتهاب المعوي عند الانسان هذه البكتريا تعيش في المياه الساحلية خلال الصيف (PCR) Repetitive extragenic palindromic PCR. احدى التقنيات التي استخدمت في الدراسة الحالية وقد بينت النتائج ان اغلب العزلات السريرية تنتج حزمة عند 1000 bp وقد وجدت بانها تشفر peptide chain releaseوقد صمم PCRعلى اساس تتابعات قواعدها النيتروجينية وقد كانت جميع العزلات موجبة لهذا ال PCRالذي يمكن ان يكون مفيد في التشخيص السريع لهذا النوع من البكتريا