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SHORT COMMUNICATION

Characterization of *Vibrio* species isolated from freshwater fishes by ribotyping

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Abstract Three *Vibrio* species from the resident microflora of gastrointestinal tract of freshwater carps and prawns were isolated and confirmed biochemically as *V. fluvialis* from *Cyprinus carpio/Labeo rohita; V. parahaemolyticus* from *Macrobrachium rosenbergii* and *V. harveyi* from *Macrobrachium malcomsoni*. The genetic relationship among these *Vibrio* species was carried out by polymerase chain reaction (PCR) amplification of 16S rRNA gene followed by restriction digestion with *Hae* III, *Bam* HI and *Pst* I. Dendogram based on ribotyping showed the isolated *Vibrios* were differentiated into three clusters. *V. harveyi* was closely related to *V. vulnificus* (reference Microbial type Culture Collection (MTCC) strain) and distantly related to *V. parahaemolyticus* as well as *V. fluvialis*.

Keywords Freshwater *Vibrios* · 16S rRNA gene · Ribotyping

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Vibrios are important fish pathogens causing mortality in fish and shellfish and play important roles in nutrient cycling in aquaculture [1]. To characterize different strains of *Vibrios*, ribotyping and 16S rRNA gene sequencing are considered to be superior to conventional typing methods [2]. Therefore, present study was carried out to discriminate different species of *Vibrios* isolated from freshwater carps and prawn by ribotyping.

The gastrointestinal tract of rohu, common carp and freshwater prawns were collected from the local markets of Bhubaneswar, Orissa. The intestinal contents were flushed into sterilized peptone water and serially diluted. From each sample, 50 µl of diluted material (10^{-2} to 10^{-4} dilutions) was plated in thiosulphate citrate bile salts sucrose (TCBS) agar by spread plate technique and incubated at 37°C for 24 h to obtain well-separated colonies. Again a single colony was picked up and inoculated into alkaline peptone water and incubated at 37°C in a shaker water bath for 16–18 h. Identification of the *Vibrio* species were carried out by observing the morphological properties, colony characteristics and biochemical tests [3]. Pure cultures were preserved in 20% (v/v) glycerol. Two *Vibrio* species *viz*., *V. parahaemolyticus* (MTCC – 451) and *V. vulnificus* (MTCC – 1146) obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India, were employed as reference strains in this study.

The bacterial cultures were grown in alkaline peptone water (pH 8.4) at 37°C for 18 h with shaking and the genomic DNA was extracted from these cultures following the methods as previously described [4]. To eliminate RNA contamination, DNA was digested with 2 µl of RNAse (10 mg/ml). Finally the DNA was stored at –20°C till further use. For PCR, forward (5′-AAG AGT TTG ATC CTG GCT CAG-3′) and reverse primers (5′-GGT TAC

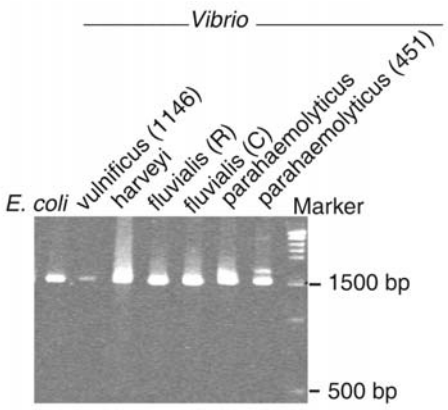

Fig. 1A Analysis of PCR-amplified 16S rRNA gene in 5% PAGE.

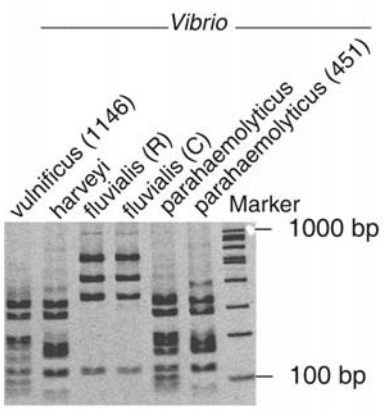

Fig. 1B Analysis of *Hae* III digested 16S rRNA gene product in 8% PAGE.

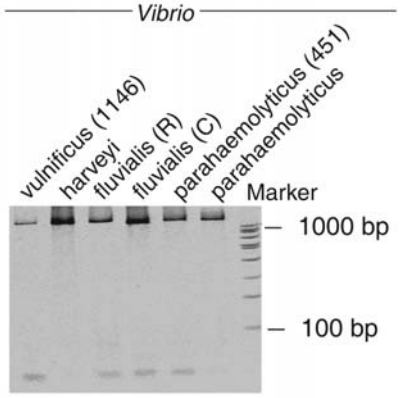

Fig. 1C Analysis of *Bam* HI digested 16S rRNA gene product in 8% PAGE.

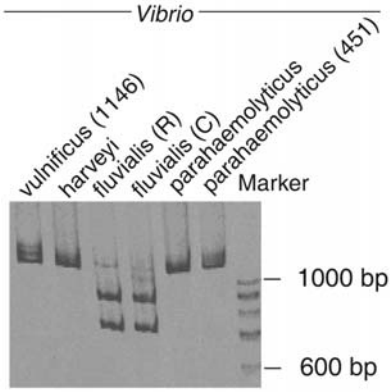

Fig. 1D Analysis of *Pst* I digested 16S rRNA gene product in 8% PAGE.

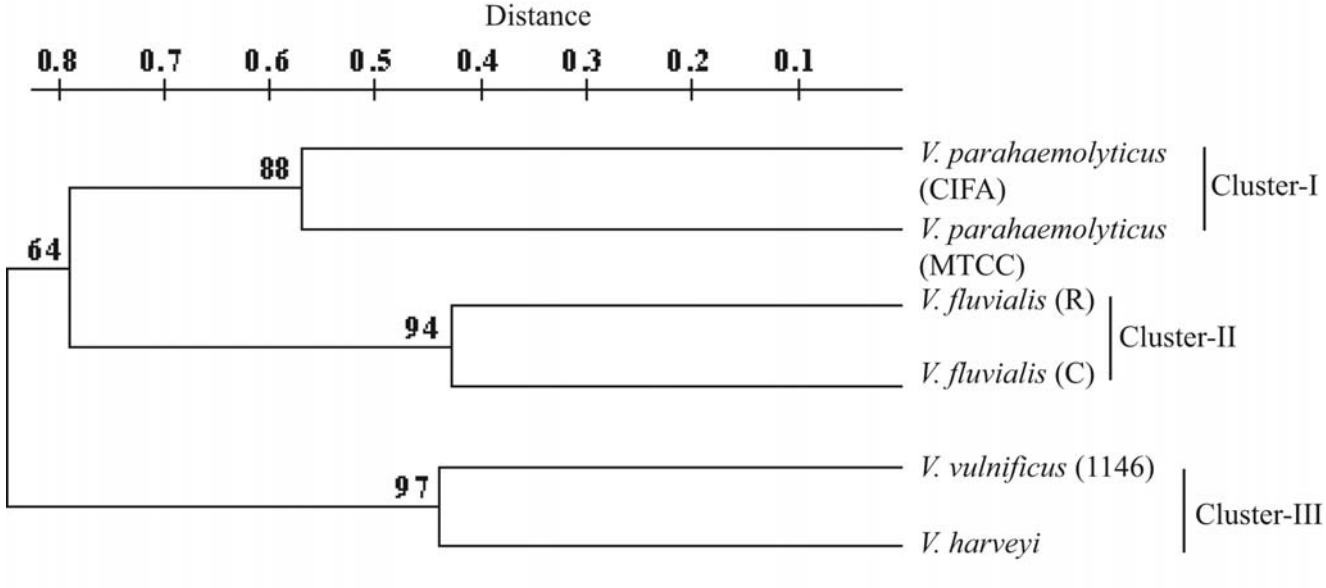

Fig. 1E Dendogram showing genetic relationship among *Vibrio* species.

CTT GTT ACG ACT T-3′) designed from *E. coli* 16S rRNA gene sequence using Qβ gene program (97% score) and were obtained from GeNei, Bangalore, India. PCR cycle was as per the following: initial step of 94°C for 5 min, 35 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 1 min 30 sec (primer extension) and one step final extension at 72°C for 7 min followed by cooling at 4ºC. PCR products were analyzed in 5% polyacrylamide gel electrophoresis.

Restriction enzyme (RE) digestion of the 16S rRNA PCR products were carried out with *Hae* III/*Bam* HI/*Pst* I. The RE mixture (20 µl) containing 5 µl PCR product, 2 µl 10 × RE buffer, 1.5 µl (1 U/µl) restriction enzyme and 11.5 µl of water was incubated at 37°C for overnight and then heat inactivated at 65°C for 10 min. RE digested products were analyzed in 8% polyacrylamide gel electrophoresis for 2 h at 100 V. The photograph was taken in a gel documentation system (Alpha Innotech Corporation, USA). Phylogenetic analysis was performed by using Gene Profiler rev 10.98 (Scanlytics, Fairfax, VA) and Tree Con software [5]. Restriction fragment length polymorphism (RFLP) restriction bands were converted to estimate distances [6]. The un weighted pair group method using arithmetic averages (UPGMA) was used for cluster analysis [7]. The method was performed with 100 bootstrap iterations [8].

On the basis of growth on *Vibrio* specific media, Gram staining, cultural characteristics, sensitivity to 0/129, morphological characteristics and a panel of biochemical tests (results not shown) *Vibrio* isolates were tentatively identified as *V. parahaemolyticus* from *Macrobrachium rosenbergii* and *V. harveyi* from *Macrobrachium malcolmsoni*. *V. fluvialis* isolated from *Cyprinus carpio* and *Labeo rohita* were further differentiated by RAPD-PCR (results not shown) to two different strains designated as *V. fluvialis* (C) and *V. fluvialis* (R), respectively. Although, they did not differ in their biochemical properties, on TCBS agar, *V. fluvialis* and *V. harveyi* exhibited large and small yellow colonies, respectively and *V. parahaemolyticus* produced green colonies. Amplification of the 16S rRNA gene of all the *Vibrio* species were carried out using 16S rRNA gene primer pair designed from *E. coli* 16S rRNA gene sequence. PCR products of different *Vibrio* species were analyzed in 5% polyacrylamide gel electrophoresis (PAGE) and revealed a single fragment of 1.5 kb (Fig. 1A). Further, PCR-RFLP of the 16S rRNA with *Hae* III showed five bands each of almost similar size in *V. parahaemolyticus* of Central Institute of Freshwater Aquaculture (CIFA) strain and MTCC-451 strain. Whereas, in *V. fluvialis* isolates from *L. rohita* and *C. carpio* revealed five common bands of about 910 bp, 600 bp, 400 bp, 328 bp and 110 bp. The banding pattern of *V. vulnificus* (MTCC – 1146) showed homology with *V. parahaemolyticus* (CIFA strain and MTCC strain) and *V. harveyi* (Fig. 1B). PCR-RFLP with *Bam* HI revealed one common band of ~1,040 bp in all the isolates. Additionally, a band of ~35 bp was common in all the isolates except *V. harveyi* and *V. parahaemolyticus* (CIFA strain) (Fig. 1C). PCR-RFLP with *Pst* I revealed a common band of ~1,100 bp in all the isolates except *V. fluvialis* (C and R) which differed from the rest revealing two extra bands of 870 bp and 726 bp (Fig. 1D). A dendogram based on the results of PCR-RFLP of 16S rRNA with *Hae* III, *Bam* HI and *Pst* I revealed three clusters on the basis of similarity percentage. *V. parahaemolyticus* CIFA strain and MTCC strain formed cluster I with 45% similarity level; *V. fluvialis* (C) and *V. fluvialis* (R) formed cluster II with 55% similarity level; *V. vulnificus* (MTCC – 1146) and *V. harveyi* formed cluster III with 55% similarity level. Inter-species relationship of *V. parahaemolyticus* with *V. fluvialis* showed 20% similarity (Fig. 1E).

The results indicate prevalence of *Vibrio* species in the gastrointestinal tract of carps and prawns in freshwater ecosystems.

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