Species-specific identification of *Vibrio fluvialis* by PCR targeted to the conserved transcriptional activation and variable membrane tether regions of the *toxR* gene

Vibrio fluvialis has been reported to cause sporadic infections and outbreaks of diarrhoea in humans (Huq *et al.*, 1980; Lesmana *et al.*, 2002; Lai *et al.*, 2006) and has also been isolated from marine and estuarine environments (Seidler *et al.*, 1980; Lee *et al.*, 1981; Lockwood *et al.*, 1982). However, the public health significance of this pathogen has not been studied in detail due to the lack of simple and reliable diagnostic tests. Although the bacterium is known to produce several potent toxins, their role in pathogenesis is not well established (Lockwood *et al.*, 1982; Huq *et al.*, 1985; Kothary *et al.*, 2003; Chakraborty *et al.*, 2005). Information regarding virulence genes, and standard genetic markers for the identification of this organism, are not fully exploited. Despite the use of an array of biochemical tests including commercial identification systems, proper identification of *V. fluvialis* still remains a problem due to its phenotypic similarity with *Aeromonas* species (Seidler *et al.*, 1980).

toxR is an ancestral gene of the family *Vibrionaceae* which encodes a transcriptional activation domain (TAD), a transmembrane domain (TMD) and a periplasmic domain (PD) (Osorio & Klose, 2000). Among vibrios, there is a high level of homology within the TAD of the ToxR proteins and relatively conserved homology in the TMD and PD (Osorio & Klose, 2000). Interestingly, there is essentially no homology within the region between TAD and TMD. This region connects the TAD to the cytoplasmic membrane and it was therefore named the membrane tether region. *toxR*-based species-specific identification has been developed for *Vibrio parahaemolyticus* (Kim *et al.*, 1999) and *Vibrio hollisae* (Vuddhakul *et al.*, 2000). Here, we report the development of a PCR-based assay for the specific identification of *V. fluvialis* exploiting the

sequence divergence within the membrane tether region of the *toxR* gene.

Forty non-cholera *Vibrio* strains isolated from hospitalized patients with acute diarrhoea at the Infectious Diseases Hospital or B. C. Roy Memorial Hospital for Children, Kolkata, India, between 1998 and 2001 were included in this study. These strains were screened for *Vibrio cholerae* by biochemical and serological tests and by species-specific *ompW* PCR (Nandi *et al.*, 2000). The oxidase-positive and *ompW*-negative strains were further characterized using the API 20E identification system (bioMérieux). Salt tolerance was determined by growing the strains at 37 °C on nutrient agar (NA; Difco) plates containing 0 and 7 % NaCl. For identification of the strains, 16S rDNA sequencing (Microseq 500, 16S rDNA Bacterial Sequencing kit; Applied Biosystems) was performed following the manufacturer's instructions using an automated DNA sequencer (ABI Prism 310; Applied Biosystems).

To develop a PCR-based method for species-specific identification, primers targeted to the *toxR* gene of *V. fluvialis* were first tested with the strains that were previously identified as *V. fluvialis* by 16S rDNA sequencing (Table 1). The forward (5'-GACCAGGGCTTTGAGGTGGACGAC-3') and reverse (5'-AGGATACGGCACTTGAGTAAGACTC-3') primers were designed from the TAD and the membrane tether region of the *toxR* gene of *V. fluvialis*, respectively (accession no. AF170885; Osorio & Klose, 2000). The membrane tether region is highly variable, but unique for each *Vibrio* species. For PCR assay, the strains were grown in Luria broth (LB; Difco) containing 1 % NaCl at 37 °C with shaking at 200 r.p.m. overnight. For halophilic vibrios, the LB medium was supplemented with 3 % NaCl. Purified genomic DNA from these strains was used

as template in the PCR assay. Amplification was carried out in a thermal cycler (Applied Biosystems) with a standard PCR reaction mixture that contained 50 ng DNA as template, 2.5 µl 10 × PCR buffer with 15 mM MgCl₂, 0.20 µl (1 U) *Taq* Polymerase (Takara Shuzo), 2.0 µl 2.5 mM (each) deoxynucleoside triphosphate, 2.5 µl of each primer (10 pmol µl⁻¹) and sterile distilled water to make the volume to 25 µl. The amplification conditions were initial denaturation at 94 °C for 5 min, followed by 30 cycles consisting of denaturation at 94 °C for 40 s, annealing at 65 °C for 40 s and extension at 72 °C for 1 min. To detect the sensitivity, the VF-*toxR* PCR was performed with different concentrations of chromosomal DNA ranging from 10 to 60 ng. PCR products were electrophoresed through 2 % agarose gel to resolve 217 bp amplicons and visualized under UV light in a gel documentation system (Gel-Doc 2000; Bio-Rad) after ethidium bromide staining.

To confirm the specificity of the primers among members of the *Vibrionaceae* family, *Vibrio nereis* (ATCC 25917), *Vibrio furnissii* (ATCC 35016), *Vibrio anguillarum* (ATCC 19264), *Vibrio vulnificus* (ATCC 33816), *Vibrio proteolyticus* (ATCC 15338), *Vibrio aestuarianus* (ATCC 35048), 34 strains of *V. cholerae* including O1, O139 (10 strains each) and non-O1 non-O139 serogroups (14 strains) and 30 strains of *V. parahaemolyticus* were included in this study. As *V. fluvialis* shares biochemical properties with *Aeromonas* species, we included 60 clinical strains of *Aeromonas*, covering five species, viz. *Aeromonas caviae* (23 strains), *Aeromonas hydrophila* (18 strains), *Aeromonas trota* (6 strains), *Aeromonas veronii* (10 strains) and *Aeromonas schubertii* (3 strains).

All the 40 non-cholera vibrios grown in thiosulfate-citrate-bile salts-sucrose agar resembled sucrose-fermenting yellow

Table 1. Identification results of non-cholera vibrios by different identification systems

Strain	API 20E (% identity; profile number)	16S rDNA sequencing	VF-toxR
PG 39	<i>V. fluvialis</i> (81·1; 3046526)	<i>V. fluvialis</i>	+
PG 41	<i>V. fluvialis</i> (81·1; 3046526)	<i>V. fluvialis</i>	+
PL 45	<i>V. fluvialis</i> (83·9; 3046126)	<i>V. fluvialis</i>	+
PG 152	<i>V. fluvialis</i> (81·1; 3046526)	<i>V. fluvialis</i>	+
PL 78/7b	Match not found (2004126)	<i>V. fluvialis</i>	+
PL 169b	<i>V. fluvialis</i> (83·9; 3046126)	<i>V. fluvialis</i>	+
PL 171b	<i>V. fluvialis</i> (99·8; 3004126)	<i>V. fluvialis</i>	+
CRC 82	<i>V. fluvialis</i> (81·1; 3046526)	<i>V. fluvialis</i>	+
CRC 99	<i>V. fluvialis</i> (81·1; 3046526)	<i>V. fluvialis</i>	+
CRC 100	<i>V. fluvialis</i> (99·6; 3044526)	<i>V. fluvialis</i>	+
CRC 111	<i>A. hydrophila</i> (98·5; 3047527)	<i>V. fluvialis</i>	+
CRC 159	<i>A. hydrophila</i> (97·7; 3047526)	<i>V. fluvialis</i>	+
CRC 233	<i>A. hydrophila</i> (97·3; 3047126)	<i>V. fluvialis</i>	+
AN 48	Match not found (2046526)	<i>V. fluvialis</i>	+
GB 987A	<i>V. fluvialis</i> (83·9; 3046126)	<i>V. fluvialis</i>	+
RC 7	<i>A. hydrophila</i> (56·7), <i>V. fluvialis</i> (43·2) (3246526)	<i>V. fluvialis</i>	+
RC 30	<i>V. fluvialis</i> (73·3), <i>A. hydrophila</i> (26·6) (3046527)	<i>V. fluvialis</i>	+
RC 48	<i>V. fluvialis</i> (81·1; 3046526)	<i>V. fluvialis</i>	+
RC 50	<i>V. fluvialis</i> (99·6; 3044526)	<i>V. fluvialis</i>	+
RC 82	<i>V. fluvialis</i> (99·6; 3044126)	<i>V. fluvialis</i>	+
RC 110	ND	<i>V. fluvialis</i>	+
RC 152	<i>V. fluvialis</i> (99·6; 3044126)	<i>V. fluvialis</i>	+
NT 4189	<i>V. fluvialis</i> (81·1; 3046526)	<i>V. fluvialis</i>	+
VTE 624	<i>V. fluvialis</i> (83·9; 3046126)	<i>V. fluvialis</i>	+
GB 1136	<i>V. fluvialis</i> (83·9; 3046126)	<i>V. fluvialis</i>	+
CRC 2	<i>V. fluvialis</i> (81·1; 3046526)	<i>V. vulnificus</i>	+
CRC 15	ND	<i>V. vulnificus</i>	+
CRC 126	<i>V. fluvialis</i> (99·6; 3044526)	<i>V. vulnificus</i>	+
CRC 175	<i>V. fluvialis</i> (99·6; 3044126)	<i>V. vulnificus</i>	+
GB 898	<i>V. fluvialis</i> (83·9; 3046126)	<i>V. vulnificus</i>	+
GB 973	<i>V. fluvialis</i> (83·9; 3046126)	<i>V. vulnificus</i>	+
RC 6	<i>V. fluvialis</i> (98·3; 3244126)	<i>V. vulnificus</i>	+
RC 96	<i>V. fluvialis</i> (54·9), <i>A. hydrophila</i> (43·3) (2046126)	<i>V. vulnificus</i>	+
RC 108	<i>V. fluvialis</i> (81·8; 3046526)	<i>V. vulnificus</i>	+
RC 111	<i>V. fluvialis</i> (83·9; 3046126)	<i>V. vulnificus</i>	+
RC 112	<i>V. fluvialis</i> (98·9; 3044137)	<i>V. vulnificus</i>	+
RC 137	<i>V. fluvialis</i> (83·9; 3046126)	<i>V. vulnificus</i>	+
RC 203	ND	<i>V. vulnificus</i>	+
RC 218	ND	<i>V. vulnificus</i>	+
RC 237	ND	<i>V. vulnificus</i>	+

ND, Not done.

colonies of *V. cholerae*. Further characterization showed that they were oxidase-positive, grew on NA containing 7 % NaCl but not in the absence of NaCl, and were negative in the *ompW* PCR. The salt tolerance test is very important for differentiation of *V. fluvialis* from

Aeromonas species (Seidler *et al.*, 1980; Lee *et al.*, 1981) as *Aeromonas* species cannot grow in the presence of 7 % NaCl. When we tested 35 non-cholera vibrios by the API 20E system, there were variations in the identification result (Table 1). Overall, 27 (77·1 %) out of 35 tested strains were

identified as *V. fluvialis*, 3 (8·6 %) as *A. hydrophila* and for 2 strains the exact match was not found in the index of the manufacturer (Table 1). In addition, the API 20E system gave ambiguous identity (Table 1) with 3 strains (RC7, RC30 and RC96).

Table 2. Comparison of 16S rDNA sequencing results of non-cholera vibrios

Strain	16S rDNA sequencing (bit score; nucleotide match; % homology)		
	1st score	2nd score	3rd score
CRC 2	<i>V. vulnificus</i> (912; 463/464; 99)	<i>V. fluvialis</i> (904; 462/464; 99)	
CRC 15	<i>V. vulnificus</i> (981; 495/495; 100)	<i>V. fluvialis</i> (975; 494/495; 99)	
CRC 126	<i>V. vulnificus</i> (989; 499/499; 100)	<i>V. fluvialis</i> (975; 497/499; 99)	
CRC 175	<i>V. vulnificus</i> (1031; 520/520; 100)	<i>V. fluvialis</i> (1025; 519/520; 99)	
GB 898	<i>V. vulnificus</i> (1067; 538/538; 100)	<i>V. fluvialis</i> (1053; 536/538; 99)	
GB 973	<i>V. vulnificus</i> (937; 525/538; 97)	<i>V. fluvialis</i> (927; 511/522; 97)	
RC 6	<i>V. vulnificus</i> (906; 457/457; 100)	<i>V. furnissii</i> (898; 456/457; 99)	<i>V. fluvialis</i> (898; 456/457; 99)
RC 96	<i>V. vulnificus</i> (1059; 537/538; 99)	<i>V. fluvialis</i> (1045; 535/538; 99)	
RC 108	<i>V. vulnificus</i> (1060; 535/536; 99)	<i>V. fluvialis</i> (1046; 533/536; 99)	
RC 111	<i>V. vulnificus</i> (918; 493/499; 98)	<i>V. furnissii</i> (910; 492/499; 98)	<i>V. fluvialis</i> (904; 469/472; 99)
RC 112	<i>V. vulnificus</i> (1051; 536/538; 99)	<i>V. fluvialis</i> (1037; 534/538; 99)	
RC 137	<i>V. vulnificus</i> (1011; 510/510; 100)	<i>V. fluvialis</i> (1005; 509/510; 99)	
RC 203	<i>V. vulnificus</i> (1067; 538/538; 100)	<i>V. fluvialis</i> (1053; 536/538; 99)	
RC 218	<i>V. vulnificus</i> (1067; 538/538; 100)	<i>V. fluvialis</i> (1053; 536/538; 99)	
RC 237	<i>V. vulnificus</i> (1051; 536/538; 99)	<i>V. furnissii</i> (1043; 535/538; 99)	<i>V. fluvialis</i> (1037; 534/538; 99)

16S rDNA sequence analysis is based on the matching of the sequence from the amplified fragment with the sequences available in the data bank, which is represented by a score system, nucleotide match and per cent homology. The 16S rDNA sequencing identified 25 (62.5%) strains as *V. fluvialis* (Table 1) and 15 (37.5%) strains as *V. vulnificus* in the first identification score (Table 2). However, in the second and third identification scores, 12 and 3 strains, respectively, were identified as *V. fluvialis* (Table 2). The scores were based on the maximal match of sequenced nucleotides and a difference in 1 nucleotide altered the score value to 8. Due to such small variation in the nucleotides, 16S rDNA sequencing and the BLAST search (<http://www.ncbi.nlm.nih.gov>) identified 15 strains as *V. vulnificus* in the first score with 99–100% identity, of which 12 and 3 were *V. fluvialis* and *V. furnissii* in the second score with 97–99% and 98–99% identity, respectively (Table 2). The API profiles of 11 strains did not show any match with *V. vulnificus* or *V. furnissii* (Table 1).

The 16S rRNA nucleotide sequence comparison method is a useful molecular tool for the identification and taxonomy of different bacterial species. The minimal difference in the 16S rRNA sequences among vibrios (Kita-Tsukamoto *et al.*, 1993; Ruimy *et al.*, 1994) sometimes makes their identification inconclusive. Moreover, this time-consuming identification

method is not cost-effective, as it needs an automated sequencer and software.

Considering the association of *V. fluvialis* with disease (Huq *et al.*, 1980, 1985), it was important to develop a PCR-based identification method that would be useful for routine analysis. In the VF-*toxR* PCR, 25 *V. fluvialis* strains that were identified by 16S rDNA sequencing gave an expected amplicon of 217 bp (Table 1). Sequence analysis of the 217 bp amplicon from a representative strain confirmed the identity of *V. fluvialis toxR*, encoding the transmembrane regulatory protein (data not shown). Fifteen strains that were presumptively identified (based on API profile and/or NaCl tolerance test) as *V. fluvialis* were also positive in the VF-*toxR* PCR.

The VF-*toxR* PCR showed 100% sensitivity for all the *V. fluvialis* strains tested. Although 50 ng chromosomal DNA was used in the routine PCR assay, 10 ng DNA was found to give a positive result. More importantly, the VF-*toxR* primers did not give a positive amplicon with *Aeromonas* strains or with other vibrios. The observation that the VF-*toxR* primers can differentiate between *V. fluvialis* and *Aeromonas* strains is significant due to the fact that these two groups of organisms share common biochemical properties, which often leads to misidentification. This PCR method would be useful for rapid identification of *V. fluvialis* strains and may

play a role in establishing the public health and clinical significance of this organism. The utility of this PCR assay should be evaluated in terms of direct detection of *V. fluvialis* from stool specimens.

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