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Rapid Method for Species-Specific Identification of *Vibrio cholerae* Using Primers Targeted to the Gene of Outer Membrane Protein OmpW

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The distribution of genes for an outer membrane protein (OmpW) and a regulatory protein (ToxR) in *Vibrio cholerae* and other organisms was studied using respective primers and probes. PCR amplification results showed that all (100%) of the 254 *V. cholerae* strains tested were positive for *ompW* and 229 (~98%) of 233 were positive for *toxR*. None of the 40 strains belonging to other *Vibrio* species produced amplicons with either *ompW*- or *toxR*-specific primers, while 80 bacterial strains from other genera tested were also found to be negative by the assay. These studies were extended with representative number of strains using *ompW*- and *toxR*-specific probes in DNA dot blot assay. While the *V. cholerae* strains reacted with *ompW* probe, only one (*V. mimicus*) out of 60 other bacterial strains tested showed weak recognition. In contrast, several strains belonging to other *Vibrio* species (e.g., *V. mimicus*, *V. splendidus*, *V. alginolyticus*, *V. fluvialis*, *V. proteolyticus*, *V. aestuarianus*, *V. salmonicida*, *V. furnissii*, and *V. parahaemolyticus*) showed weak to strong reactivity to the *toxR* probe. Restriction fragment length polymorphism analysis and nucleotide sequence data revealed that the *ompW* sequence is highly conserved among *V. cholerae* strains belonging to different biotypes and/or serogroups. All of these results suggest that the *ompW* gene can be targeted for the species-specific identification of *V. cholerae* strains. The scope of this study was further extended through the development of a one-step multiplex PCR assay for the simultaneous amplification of *ompW* and *ctxA* genes which should be of considerable value in the screening of both toxigenic and nontoxigenic *V. cholerae* strains of clinical as well as environmental origin.

The diarrheal disease cholera in the epidemic form is caused by the organism *Vibrio cholerae* belonging to the O1 or O139 serogroup (10). *V. cholerae* organisms belonging to non-O1/non-O139 serogroups, which can be isolated in abundance from aquatic or estuarine sources, cause sporadic cases or limited outbreaks of diarrhea in humans (17). Identification of *V. cholerae* is usually achieved through a series of biochemical tests after their growth and isolation on a selective plating medium e.g., TCBS agar (25). The process, however, is laborious and time-consuming and may be quite expensive for a laboratory handling a large number of clinical and/or environmental samples. Further, close relatedness among *V. cholerae* and certain other members of the *Vibrio* spp. (e.g., *V. mimicus*) or *Aeromonas* spp. with respect to their biochemical properties has often made unambiguous identification of the organism quite difficult. Although the commercial (or otherwise) availability of O1 and/or O139 antisera has considerably helped in the identification of epidemic causing strains of *V. cholerae*, it may not be true for the non-O1/non-O139 strains which, until recently, were known to range from the O2 to O138 and from the O140 to O193 serogroups (32). The problem has been accentuated recently as the non-O1/non-O139 strains of diverse serogroups have been implicated as the causative agents of a large number of diarrheal cases in the Indian subcontinent (23, 28) and elsewhere (2, 4). As a matter of fact, the majority of these strains do not contain virulence markers such as cholera toxin, toxin-coregulated pilus (TCP), etc. (10, 17), that are known to be associated with the pathogenic strains of *V. cholerae* O1 or O139, thereby making their identification more difficult.

Attempts to identify *V. cholerae* strains on the basis of their 16S rRNA sequences have not been successful so far due to the lack of appreciable differences between these sequences occurring in *V. cholerae* and other members of *Vibrionaceae* family (13, 24). Other approaches were directed toward the identification of toxigenic strains of *V. cholerae* through the use of cholera toxin gene (*ctxAB*) probes or appropriate primers for the amplification of toxin genes by PCR assay (21). A multiplex PCR has also been developed to identify epidemic-causing strains of *V. cholerae* containing the *ctxA* and TCP protein subunit (*tcpA*) genes (11). *V. cholerae* strains belonging to the O1 or O139 serogroups could be detected by using probes or primers designed from their *rfb* regions responsible for "O" antigen biosynthesis (1, 7). However, none of these methods are applicable for the identification of all *V. cholerae* strains. Recently, a PCR based method targeted to the *toxR* gene was developed for the species-specific identification of *V. parahaemolyticus* (12). No information, however, is available in the literature regarding the identification of *V. cholerae* using *toxR* primers, although *toxR* genes (sharing sequence homology) are distributed among certain other *Vibrio* species, including *V. cholerae* (12, 15, 18, 22).

In the present study, we have evaluated the use of *toxR* primers and/or probes for the identification of *V. cholerae* strains. The study is extended using primers and/or probes targeted to a gene encoding an outer membrane protein

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TABLE 1. Summarized results of PCR and DNA dot blot analyses of strains belonging to *Vibrio* spp. using primers or probes for *ompW* and *toxR* genes

Analysis no(s).	Strain	Source[a]	Detection[b] of:			
			ompW by:		*toxR* by:	
			PCR	Dot blot	PCR	Dot blot
1	*V. cholerae* O1	14035 (ATCC)	+	+	+	+
2	*V. cholerae* O1	39315 (ATCC)	+	+	+	+
3–8	*V. cholerae* O1	Lab stock	+	+	+	+
9–11	*V. cholerae* O139	Lab stock	+	+	+	+
12–13	*V. cholerae* (rough)	Lab stock	+	+	+	+
14	*V. cholerae* non-O1/non-O139	25872 (ATCC)	+	+	+	+
15	*V. cholerae* non-O1/non-O139	25874 (ATCC)	+	+	+	+
16–18	*V. cholerae* non-O1/non-O139	Lab stock	+	+	+ (2/3)[c]	+
19	*V. mimicus*	33653 (ATCC)	−	+W	−	+
20	*V. mimicus* E13072	NICED	−	−	−	−
21	*V. mimicus* 4208	NICED	−	−	−	+
22	*V. mimicus* NT 2310	NICED	−	−	−	−
23	*V. mimicus* 1	NICED	−	−	−	−
24	*V. mimicus* 4053	NICED	−	−	−	−
25	*V. vulnificus*	33816 (ATCC)	−	−	−	−
26	*V. vulnificus*	27562 (ATCC)	−	−	−	−
27–28	*V. vulnificus*	NICED	−	−	−	−
29	*V. alginolyticus*	17749 (ATCC)	−	−	−	+
30	*V. aestuarianus*	35048 (ATCC)	−	−	−	+
31	*V. fluvialis*	33809 (ATCC)	−	−	−	+
32	*V. furnissii*	35016 (ATCC)	−	−	−	+W
33	*V. furnissii*	NICED	−	−	−	−
34	*V. proteolyticus*	15338 (ATCC)	−	−	−	+W
35	*V. anguillarum*	19264 (ATCC)	−	−	−	−
36	*V. anguillarum* 943	43305 (ATCC)	−	−	−	−
37	*V. anguillarum* 944	43306 (ATCC)	−	−	−	−
38	*V. anguillarum*	NICED	−	−	−	−
39	*V. carchariae*	35084 (ATCC)	−	−	−	−
40	*V. tubiashii*	19109 (ATCC)	−	−	−	−
41	*V. natriegens*	14048 (ATCC)	−	−	−	−
42	*V. salmonicida*	43839 (ATCC)	−	−	−	+W
43	*V. splendidus*	33125 (ATCC)	−	−	−	+
44	*V. nereis*	25917 (ATCC)	−	−	−	−
45	*V. parahaemolyticus*	2210001 (RIMD)	−	−	−	−
46–51	*V. parahaemolyticus*	NICED	− (6/6)	− (6/6)	− (6/6)	− (5/6)[d]
52	*V. tyrogens*	RS	−	−	−	−
53	*V. tyrogens*	RS	−	−	−	−
54	*V. metschnikovii*	RS	−	−	−	−
55	*V. metschnikovii*	NICED	−	−	−	−
56	*V. metschnikovii*	STM	−	−	−	−
57	*V. hollisae*	NICED	−	−	−	−
58	*V. proteus*	RS	−	−	−	−

[a] Lab stock, strains available as stock culture at the Bose Institute, Calcutta, India; NICED, National Institute of Cholera and Enteric Diseases, Calcutta, India; RIMD, Research Institute of Microbial Diseases, Osaka, Japan; RS, R. Sakazaki, Tokyo, Japan; STM, School of Tropical Medicine, Calcutta, India.
[b] +, Detectable; +W, weakly detectable; −, not detectable.
[c] One strain gave a negative result.
[d] One strain gave a weakly positive result.

OmpW of *V. cholerae* whose nucleotide sequence was first reported by Jalajakumari and Manning (8). We demonstrate that although the *toxR* primers are sensitive and specific for *V. cholerae* strains, the primers targeted to *ompW* are better suited for the purpose due to the unique presence of the gene with conserved sequence in *V. cholerae*. Finally, a multiplex-PCR assay has been developed for the detection of toxigenic strains of *V. cholerae* through simultaneous amplification of *ompW* and *ctxA* genes.

MATERIALS AND METHODS

Bacterial strains. A total of 254 *V. cholerae* strains isolated from both clinical and environmental sources were included in this study. The majority of these were isolated locally over the years and kept in our collection. Others were standard or type (American Type Culture Collection [ATCC]) strains which had been initially isolated and used by various groups of workers around the world and made available to us. Organisms were grown on a selective medium (TCBS agar) and subsequently characterized by standard biochemical procedures (25). *V. cholerae* strains were subjected to serogroup analysis using O serogroup-specific antisera. Of the 254 *V. cholerae* strains included in this study, 36 and 22 strains belonged to serogroups O1 and O139, respectively. Two of these O1 strains had ATCC designations. A total of 176 strains belonged to non-O1/non-O139 serogroups on the basis of agglutination reactions using O2 to O141 specific antisera. Of these, 96 non-O1/non-O139 strains were, in fact, reference strains used to raise O-specific antisera (29), while two others were ATCC type strains. Of the remaining 20 *V. cholerae* strains which could not be typed by the existing O1 to O141 antisera, 16 belonged to the rough variety and were agglutinable by the rough antiserum. The remaining four strains were classified under O untypeable category.

A total of 40 strains belonging to other *Vibrio* species were also included in the study (Table 1). Of these, 17 were type strains with ATCC numbers. The sources of the others are indicated in Table 1. Other bacteria included in this study were

TABLE 2. Sequences of primers used in this study

Primer no.	Target gene	Sequence (size)	Source or reference
1	*ompW* (terminal sense)	5′-CACCAAGAAGGTGACTTTATTGTG-3′ (24-mer)	This study
2	*ompW* (terminal antisense)	5′-GAACTTATAACCACCCGCG-3′ (19-mer)	This study
3	*ompW* (internal sense)	5′-CCACCTACCTTTATGGTCC-3′ (19-mer)	This study
4	*ompW* (internal antisense)	5′-GGTTTGTCGAATTAGCTTCACC-3′ (22-mer)	This study
5	*toxR* (sense)	5′-ATGTTCGGATTAGGACAC-3′ (18-mer)	6, 15
6	*toxR* (antisense)	5′-TACTCACACACTTTGATGGC-3′ (20-mer)	6, 15
7	*ctxA* (sense)	5′-CTCAGACGGGATTTGTTAGGCACG-3′ (24-mer)	11
8	*ctxA* (antisense)	5′-TCTATCTCTGTAGCCCCTATTACG-3′ (24-mer)	11

Aeromonas spp., *Escherichia coli*, *Shigella* spp., *Salmonella* spp., *Pseudomonas* spp., *Klebsiella* spp., and *Staphylococcus aureus*.

PCR assay. Amplification of the target gene was carried out by PCR assay using bacterial cell lysate as the source of template DNA. Briefly, bacterial cells were grown overnight at 37°C on Luria agar (LA) plates. For strains belonging to certain *Vibrio* species, the LA medium was supplemented with 3% NaCl. Next, isolated colonies were picked up and mixed with 100 μl of normal saline, and bacterial cells were pelleted by centrifugation. The cell pellet was resuspended in 100 μl of double-distilled water and boiled for 10 min. Cell debris was removed by centrifugation, and the supernatant containing the template DNA was taken into a fresh microfuge tube for PCR assay.

Four different primers were designed and synthesized on the basis of the available nucleotide sequence data of *ompW* (8). The primer sequences are indicated in Table 2. Three combinations of *ompW* primer pairs (1-2, 1-4, and 2-3) were used to generate amplicons of three different sizes in separate PCR tubes. Primers used for the amplification of the *toxR* gene are also indicated in Table 2. The sense primer corresponds to nucleotides 1 to 18 of the *toxR* gene, while the antisense primer was complementary to nucleotides 865 to 884 of the *toxR* gene (6, 15). The primers used for the amplification of *ctxA* are listed in Table 2.

PCR amplification of the target DNA was carried out in a thermal cycler (Perkin-Elmer) using 200-μl PCR tubes with a reaction mixture volume of 25 μl. Each of the reaction mixtures contained 3 μl of template DNA (lysate), 2.5 μl of each primer (10 pmol/μl), 2.5 μl of 2.5 mM deoxynucleoside triphosphates, 0.3 μl (5 U/μl) of *Taq* DNA polymerase (Takara Shuzo Co., Ltd.), 2.5 μl of 10× reaction buffer containing 20 mM $MgCl_2$ (Extaq; Takara), and 11.8 μl of distilled water. The reaction mixture was subjected to an amplification of 30 cycles, each of which consisted of three steps in the following order: denaturation of template DNA at 94°C for 30 s, annealing of the template DNA at 64°C for 30 s, and extension of the primers at 72°C for 30 s. Before initiation of the first cycle, the reaction mixture was heated at 94°C for 5 min to allow complete denaturation of the template. PCR products, thus obtained, were electrophoresed through 1.5% (wt/vol) agarose gel to resolve the amplified products which were visualized under UV light after ethidium bromide staining.

A multiplex PCR assay was carried out by the simultaneous addition of primer pairs for *ompW* (primers 1 and 2, Table 2) and *ctxA* (primers 7 and 8, Table 2) in the same reaction mixture. In initial experiments, the *ctxA* primer concentration was varied between 1.0 and 0.15 pmol/μl, keeping the *ompW* primer concentration fixed either at 1.0 or at 1.2 pmol/μl in the final reaction mixture of 25 μl. Optimum results were obtained with primer concentrations of 1.2 and 0.25 pmol/μl for *ompW* and *ctxA*, respectively. Other conditions for PCR amplification remained as described earlier.

DNA dot blot assay. Genomic DNA of bacterial strains was isolated using a miniscale preparation method (31) with minor modifications. For this, organisms were grown overnight at 37°C in 5 ml of Luria broth (supplemented with 3% NaCl to support the growth of certain noncholera vibrios). Next, 1.5 ml of the bacterial culture was centrifuged, and the pellet thus obtained was resuspended in 567 μl of TE buffer (10 mM Tris-HCl containing 1 mM EDTA; pH 8.0) followed by the addition of 30 μl of 10% (wt/vol) sodium dodecyl sulfate and 3 μl of a proteinase K solution (20 μg/μl) (Sigma Chemical Co.). The mixture was incubated at 50°C for 90 min to obtain a clear lysate. Next, 100 μl of 5 M NaCl and 100 μl of 10% (wt/vol) cetyltrimethylammonium bromide (CTAB) in 0.7 M NaCl were added to the lysate and kept at 65°C for 10 min. Thereafter, the DNA material was extracted by adding an equal volume of a mixture of chloroform-isoamyl alcohol (24:1). After centrifugation, the aqueous phase containing nucleic acids was collected and reextracted with phenol-chloroform-isoamyl alcohol (25:24:1) to remove proteinaceous material. The aqueous phase was transferred to a fresh 1.5-ml microfuge tube, and DNA was precipitated by the addition of 0.6 volume of isopropanol at room temperature. The precipitate was collected by centrifugation, washed with 70% (vol/vol) ethanol, and finally reconstituted in 100 μl of TE buffer. The concentration of DNA was measured spectrophotometrically (26).

Purified bacterial DNA preparations were used for dot blot assay using *ompW* and/or *toxR* probes. The probes were prepared by PCR amplification of target genes *ompW* and/or *toxR* using primer pairs 1 and 2 (for *ompW*) and 5 and 6 (for *toxR*) (Table 2). Appropriate amplicons thus obtained were purified by using the QIAQuick purification kit (Qiagen) and labeled with horseradish peroxidase by the glutaraldehyde conjugation method using the Direct Nucleic Acid Labeling Kit ECL (Amersham Lifesciences). Briefly, 50 ng of DNA sample (amplicon) was taken in 10 μl of water and denatured by heating for 10 min in a boiling water bath, followed by immediate chilling. The cooled DNA was mixed with equal volumes of the labeling reagent and glutaraldehyde solution (supplied with the kit). Following incubation at 37°C for 20 min, the labeled probe was taken in hybridization buffer (5 ml) and used. For the dot blot assay, the target DNA was denatured by boiling in a water bath for 10 min and spotted onto a nylon membrane presoaked with 2 N NaOH and 2× SSC (30 mM trisodium citrate plus 0.3 M NaCl) buffer. The spotted DNA was linked to the membrane by UV irradiation, prehybridized at 42°C for 1 h, and hybridized with the labeled probe (10 ng/ml) for 14 h at 42°C. After hybridization, the membrane was washed thoroughly with primary (twice at 42°C) and secondary (twice at room temperature) wash buffers under highly stringent conditions. Next, the membrane was exposed to the detection solution and autoradiographed using X-ray film.

Restriction fragment length polymorphism (RFLP) analysis of *ompW* PCR amplicons. The 588-bp PCR amplicons of *ompW* obtained from representative *V. cholerae* strains using primers 1 and 2 (Table 2) were purified by the Gel Extraction Purification kit (Qiagen) and subsequently digested with the three restriction enzymes *Hin*dIII, *Nde*I, and *Hpa*I (Gennei) in separate reactions. Digested materials were run on a 2% (wt/vol) agarose gel, stained with ethidium bromide, and viewed under UV light.

Sequencing of *ompW* amplicons. The nucleotide sequence of *ompW* amplicons obtained by PCR using the primer pair 1 and 2 (Table 2) from *V. cholerae* strains was determined using an automated DNA sequencer (Perkin-Elmer 310).

RESULTS

PCR assay using *ompW* and *toxR* primers. PCR amplification of *ompW* gene of *V. cholerae* using three combinations of primers (primers 1 and 2, primers 1 and 4, and primers 2 and 3; Table 2) yielded amplicons of 588, 304, and 336 bp, respectively. Initial experiments were carried out with about 50 *V. cholerae* and other strains. Representative data are shown in Fig. 1. It should be noted that the bacterial strains other than *V. cholerae* did not produce any amplified product under the experimental conditions used.

Subsequent experiments were carried out with the *ompW* primer pair 1 and 2 because this produced an amplicon (588 bp) which differed considerably in size from the *ctxA* amplicon (301 bp) generated with the primers 7 and 8 (Table 2). PCR amplification data obtained with 254 strains of *V. cholerae*, 40 strains of other *Vibrio* spp., and 80 bacterial strains from other genera are presented in a summarized form (Table 3). While all *V. cholerae* strains were found to be positive by the *ompW*-based PCR assay, other organisms tested, including those belonging to other *Vibrio* species, were found to be negative. When these strains were subjected to PCR using *toxR* primers, only 4 of 233 *V. cholerae* strains tested were found to be negative. All four strains belonged to typeable non-O1/non-O139 serogroups. Noncholera vibrios and other bacterial species failed to yield any *toxR* amplicon when tested under comparable conditions.

DNA dot blot analysis using *ompW* and *toxR* probe. Strains belonging to different bacterial species were subjected to DNA dot blot analysis using *ompW* and *toxR* probes. The results presented in Table 1 show that all of the 18 *V. cholerae* strains

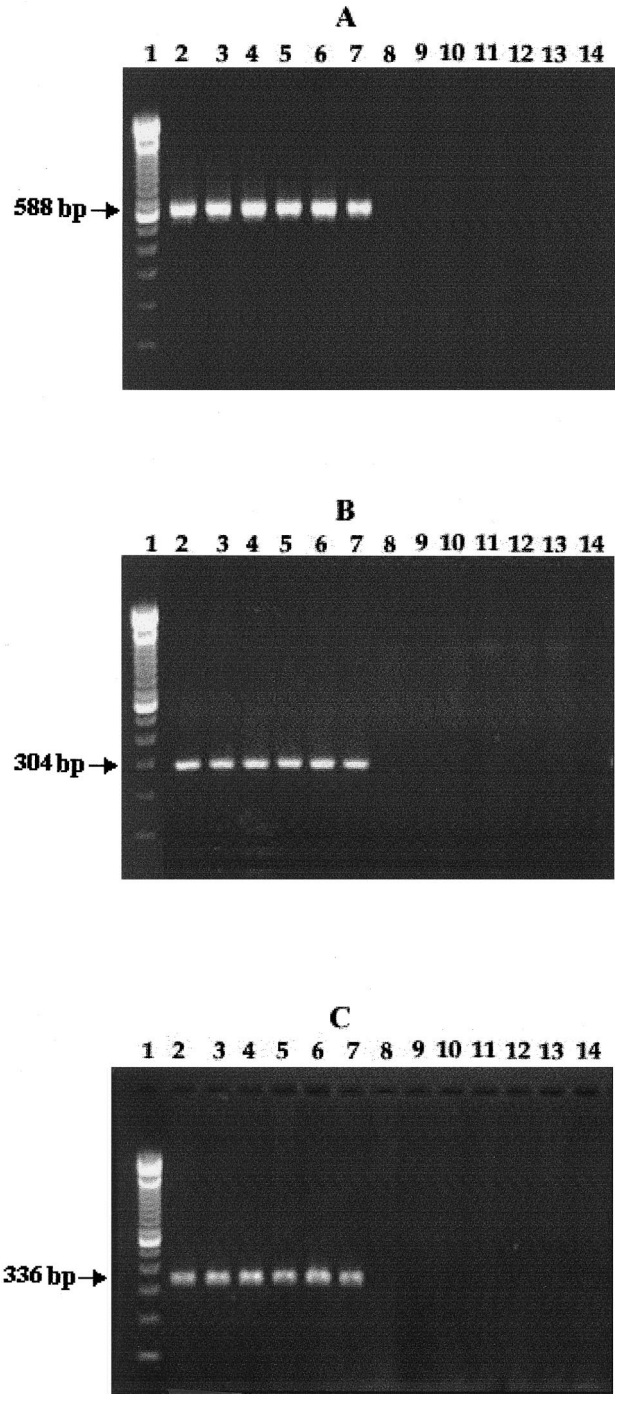

FIG. 1. PCR amplification results obtained with bacterial strains using *ompW*-specific primer pairs 1 and 2 (A), 1 and 4 (B), and 2 and 3 (C). The bacterial strains used were *V. cholerae* O1 classical (lane 2), O1 El Tor (lane 3), O139 (lane 4), rough (lane 5), and non-O1/non-O139 (lanes 6 and 7). Other bacteria used were *V. parahaemolyticus* (lane 8), *V. mimicus* (lane 9), *V. anguillarum* (lane 10), *V. alginolyticus* (lane 11), *V. furnissii* (lane 12), *Aeromonas* spp. (lane 13), and enteroaggregative *E. coli* (lane 14). Lane 1 represents marker DNA of known molecular weights. The amplicon sizes are indicated by arrows.

tested produced positive signals with both of the probes. In contrast, of 40 strains belonging to other *Vibrio* species tested, only 1 *V. mimicus* strain gave a weak signal against the *ompW* probe, while 10 strains, e.g., *V. mimicus* (two strains), *V. splendidus*, *V. alginolyticus*, *V. fluvialis*, *V. proteolyticus*, *V. aestuarianus*, *V. salmonicida*, *V. furnissii*, and *V. parahaemolyticus* gave weak to strong signals with *toxR* probe (Table 1, Fig. 2). Twenty strains belonging to other bacterial species (e.g., *Aeromonas* spp., *E. coli*, *Shigella* spp., *Salmonella* spp., *Klebsiella* spp. *Pseudomonas* spp., and *S. aureus*) were found to be negative for both *ompW* and *toxR* genes in the dot blot assay (data not shown).

TABLE 3. Summarized PCR results obtained with *V. cholerae* and other bacteria using *ompW* and *toxR*-specific primers

Strain	No. of strains positive/total no. of strains tested	
	ompW[a]	*toxR*[b]
V. cholerae O1[c]	36/36	36/36
V. cholerae O139	22/22	22/22
V. cholerae non-O1/non-O139[c]	176/176	155/159
V. cholerae (rough/OUT[d])	20/20	16/16
Other *Vibrio* spp[e]	0/40	0/40
Aeromonas spp.	0/7	0/7
E. coli[f,g]	0/28	0/28
Shigella spp.	0/19	0/19
Salmonella spp.	0/16	0/16
Pseudomonas spp.[g]	0/7	0/7
Klebsiella spp.	0/2	0/2
S. aureus[g]	0/1	0/1

[a] Using the primers 1 and 2 (Table 2).
[b] Using the primers 5 and 6 (Table 2).
[c] Includes two ATCC strains.
[d] O untypeable.
[e] Strains are listed in Table 1.
[f] Strains include enteropathogenic, enteroinvasive, enterotoxigenic, enterohemorrhagic, and enteroaggregative types.
[g] Includes one ATCC strain.

RFLP analysis of *ompW* amplicons. The 588-bp *ompW* amplicons obtained from different *V. cholerae* strains using the primer pair 1 and 2 (Table 2) were digested with the restriction enzymes (*Hin*dIII, *Nde*I, *Hpa*I). The RFLP patterns presented in Fig. 3 demonstrate identity among the *V. cholerae* strains with respect to these restriction sites in the *ompW* gene.

Nucleotide sequence analysis of *ompW* amplicons. The *ompW* amplicons generated from five *V. cholerae* strains belonging to different serogroups and/or biotypes (O1/El Tor, O34, O37, O53, and O139) were subjected to nucleotide sequence analysis, and the data were compared with each other as well as with the published sequence data of an O1 classical strain 569B (8). The results (Table 4) showed only minimum variation (ranging between 0.2 and 2.2%) in the *ompW* sequence among these strains.

Multiplex-PCR assay using *ompW* and *ctxA* primers. Simultaneous amplification of *ompW* and *ctxA* genes in a given reaction mixture generated amplicons of both *ompW* (588 bp) and *ctxA* (301 bp) in toxigenic *V. cholerae* strains belonging to the O1, O139, and non-O1/non-O139 serogroups (Fig. 4). As expected, a nontoxigenic strain yielded only the *ompW* amplicon (lane 6), while a *V. mimicus* strain failed to yield any amplicon under comparable conditions (lane 7).

DISCUSSION

The *toxR* gene was shown to be involved in the regulation and expression of several genes of *V. cholerae* (19). Subsequent studies demonstrated the presence of *toxR*-related gene sequences in other organisms belonging to *Vibrio* spp., although their sequences showed considerable variations (14, 18, 22). As a matter of fact, the *toxR* gene was recently used as a probe for

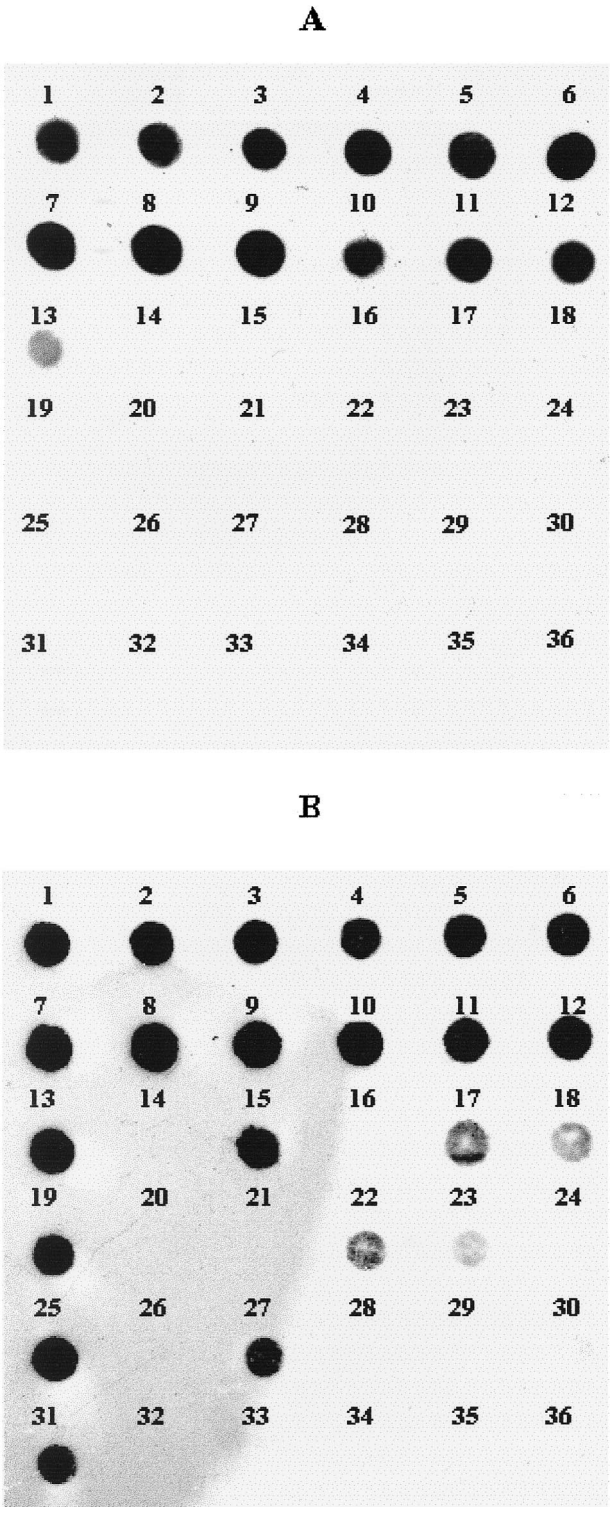

FIG. 2. DNA dot blot hybridization test carried out with *V. cholerae* strains using *ompW* (A) and *toxR* (B) gene probes. The test strains used were *V. cholerae* O1 classical (O395 [blot 1], 569B [blot 2], and ATCC 14035 [blot 3]), O1 El Tor (PG27 [blot 4] and ATCC 39315 [blot 5]), O139 (Arg3 [blot 6] and SG25 [blot 7]), rough ALO46 (blot 8), non-O1/non-O139 (ATCC 25872 [blot 9], ATCC 25874 [blot 10], V5 [blot 11], and S7 [blot 12]), *V. mimicus* ATCC 33653 (blot 13), *V. tyrogens* (blot 14), *V. alginolyticus* ATCC 17749 (blot 15), *V. anguillarum* ATCC 19264 (blot 16), *V. furnissii* ATCC 35016 (blot 17), *V. proteolyticus* ATCC 15338 (blot 18), *V. mimicus* (blot 19), *V. vulnificus* (ATCC 33816 [blot 20] and ATCC 27562 [blot 21]), *V. salmonicida* ATCC 43839 (blot 22), *V. parahaemolyticus* (121 [blot 23] and RIMD 2210001 [blot 24]), *V. splendidus* ATCC 33125 (blot 25), *V. carchariae* ATCC 35084 (blot 26), *V. aestuarianus* ATCC 35048 (blot 27), *V. nereis* ATCC 25917 (blot 28), *V. natriegens* ATCC 14048 (blot 29), *V. tubiashii* ATCC 19109 (blot 30), *V. fluvialis* ATCC 33809 (blot 31), *Aeromonas* sp. (blot 32), *E. coli* ATCC 25922 (blot 33), *Shigella* sp. (blot 34), *Salmonella* sp. (blot 35), and *P. aeruginosa* ATCC 27853 (blot 36).

TABLE 4. Variations in *ompW* sequences among *V. cholerae* strains

V. cholerae strain (serogroups/biotypes)	Sequence variations[a]					
	O1/classical	O1/El Tor	O139	O34	O37	O53
O1/classical		12/588	13/588	13/588	7/588	11/588
O1/El Tor	2.0		1/588	3/588	7/588	11/588
O139	2.2	0.2		2/588	6/588	10/588
O34	2.2	0.5	0.3		6/588	10/588
O37	1.2	1.2	1.0	1.0		4/588
O53	1.9	1.9	1.7	1.7	0.7	

[a] Values in the upper-right triangle indicate the numbers of nucleotide differences/total number compared. The values in the lower-left triangle indicate the percentage of sequence variations.

the species-specific identification of *V. parahaemolyticus* (12). Interestingly, this gene probe developed for *V. parahaemolyticus* failed to detect *V. cholerae* despite 52% identity in their *toxR* gene sequences. These results appear to be somewhat consistent with our data since the *toxR* probe for *V. cholerae* recognized only one, though weakly, of the seven *V. parahaemolyticus* strains tested (Table 1). However, as observed with the *V. parahemolyticus toxR* probe (12), the *toxR* probe for *V. cholerae* recognized organisms belonging to certain other *Vibrio* species with variable level of reactivities (Fig. 2). Incidentally, the organism *V. alginolyticus* was the common species recognized by both of the *toxR* probes.

The *toxR* primers used in this study were found to be quite specific (~98%) for *V. cholerae* and were able to differentiate these from other *Vibrio* spp. (Table 3). These results suggest that one or both the primer sequences of *V. cholerae toxR* are likely to differ from the corresponding *toxR* sequences of other vibrios. That this may indeed be the case is supported by the information, though limited, available on *toxR* sequences (14, 18).

The *ompW* primers showed 100% specificity for all *V. cholerae* strains tested (Table 3). More importantly, the *ompW* gene probe did not hybridize with target DNAs of other bacteria except for showing a weak reaction with only one of six *V. mimicus* strains examined (Table 1, Fig. 2). This observation and the fact that *ompW* primers can differentiate between *V. cholerae* and *V. mimicus* strains assume considerable significance in view of the report that these two groups of organisms share common biochemical properties and serological markers (5). Therefore, the presence of *ompW* in *V. cholerae* strains, coupled with the fact that its nucleotide sequence remained practically unchanged among different *V. cholerae* strains, makes it a highly suitable genetic marker for the organism.

A literature survey showed that genes partially homologous to *ompW* of *V. cholerae* are present in certain other bacteria, e.g., *E. coli*, *Aeromonas* spp., etc. (9, 16). Several functions were proposed for the OmpW-related proteins in these bacteria, including their pore- or channel-forming (9) and colicin receptor properties (20). Although the precise function of the OmpW protein in *V. cholerae* is not yet known, it may play a role in the adherence process, which is likely to facilitate the survival of the organism within the host or in the environment or both (27). Preliminary genome data available (30) demon-

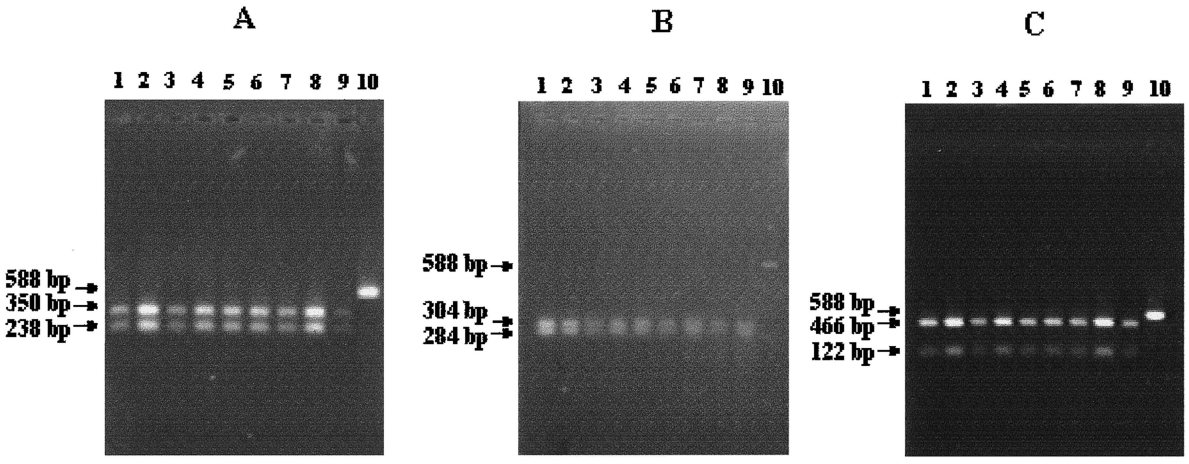

FIG. 3. RFLP analysis of *ompW* amplicons of different *V. cholerae* strains using the restriction enzymes *Hin*dIII (A), *Nde*I (B), and *Hpa*I (C). The bacterial strains used were *V. cholerae* O1 classical (lane 1), O1 El Tor (lane 2), O139 (lane 3), and non-O1/non-O139 (lanes 4 to 9). Lane 10 represents the uncut *ompW* amplicon shown for a comparison. The fragment sizes are indicated by arrows.

strate the presence of two chromosomes in *V. cholerae*. It is important to note that while the *ompW* gene is present in the smaller chromosome, the *toxR* gene is located in the larger chromosome of the organism.

Epidemic-causing strains of *V. cholerae* belong to the O1 or O139 serogroups and produce cholera toxin, which is the major contributing factor for profuse diarrhea (cholera gravis) (10). However, genes related to *ctxAB* have also been demonstrated in a number of strains of non-O1/non-O139 *V. cholerae* (6, 17, 21) that are responsible for diarrheal episodes in humans, causing a considerable public health problem. The multiplex PCR described here is likely to facilitate the rapid detection of toxigenic *V. cholerae* strains and therefore play a key role in the cholera surveillance program.

The rRNA nucleotide sequences have provided valuable information for the identification and taxonomy of different bacterial species. Unfortunately, the 16S rRNA sequences of different *Vibrio* spp. show minimal differences, making species-specific identification difficult (13, 24). In a recent study, Chun et al. (3) were able to design primers based on subtle differences in the nucleotide sequences of 16S-23S rRNA intergenic spacer regions of *V. cholerae* and *V. mimicus*. PCR amplification using the primer pair was able to generate amplicons, though of variable sizes (295 to 310 bp), from several *V. cholerae* strains tested. The data presented in this study, however, demonstrate that PCR primers 1 and 2 designed on the basis of *ompW* sequence (uniquely present in *V. cholerae*) generate amplicons of identical sizes (588 bp) from all *V. cholerae* strains, which should provide a very rapid and reliable method for the species-specific identification of *V. cholerae* and for their differentiation from other bacteria. Further, identification of *V. cholerae* strains harboring genes for cholera toxin by one-step multiplex PCR assay (Fig. 4) is likely to enhance the scope of this method significantly toward the screening of both toxigenic and nontoxigenic *V. cholerae* strains of clinical as well as environmental origin.

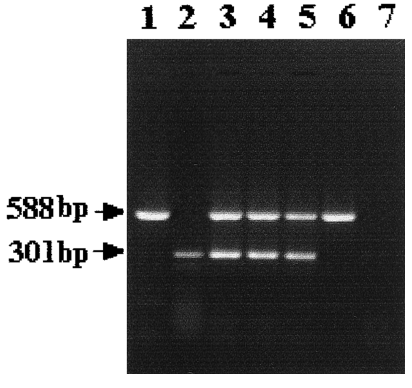

FIG. 4. Multiplex-PCR analysis of *V. cholerae* strains using primers 1 and 2 for *ompW* and primers 7 and 8 for *ctxA*. Bacterial strains used were *V. cholerae* O1 (lane 3), O139 (lane 4), non-O1/non-O139 (lane 5), a nontoxigenic non-O1/non-O139 (lane 6), and a *V. mimicus* strain (lane 7). The positions of the *ompW* and *ctxA* amplicons generated by the use of individual primer pairs are shown in lanes 1 and 2, respectively.

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