



## A SIMPLE PROCEDURE FOR ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF *VIBRIO CHOLERAE* FROM CLINICAL SAMPLES

PARIMAL DUA<sup>1</sup>, AMIT KARMAKAR<sup>1</sup>, KUNAL DUTTA<sup>1</sup> AND CHANDRADIPA GHOSH<sup>\*1</sup>

<sup>\*1</sup>Department of Human Physiology with Community Health, Vidyasagar University, Paschim Medinipur, West Bengal, India.

<sup>1</sup>Department of Human Physiology with Community Health, Laboratory of Microbiology and Immunology, Vidyasagar University, Paschim Medinipur, West Bengal, India.

### ABSTRACT

Cholera is caused by *Vibrio cholerae*, which is a major health problem in several developing countries. Out of 147 diarrheal stool samples collected during one year period (2013) from patients with acute diarrhea admitted to the hospital, 71 cases were positive for *Vibrio cholerae*. In the present study, a direct multiplex PCR was developed targeting the 16S rRNA, *ompW*, *rfbO1* and *rfbO139* genes for confirmation of *Vibrio cholerae* and its serogrouping. Interestingly, all the clinically isolated strains of *Vibrio cholerae* belong to non-O1/non-O139 serogroup. The phenotypic characteristics of the clinical strains of *Vibrio cholerae* were examined. All the isolates of *Vibrio cholerae* were positive for most of the biochemical reaction. Variable results were obtained in the Voges-Proskauer test. The PCR assay can be very useful for rapid surveillance of *Vibrio cholerae* for confirmation of clinical isolates.

**KEYWORDS:** Diarrhea, cholera, *Vibrio cholerae*, Polymerase Chain Reaction, phenotype, serogroup



CHANDRADIPA GHOSH \*

Department of Human Physiology with Community Health, Vidyasagar University, Paschim Medinipur, West Bengal, India.

Received on: 18-05-2017

Revised and Accepted on: 09-08-2017

DOI: <http://dx.doi.org/10.22376/ijpbs.2017.8.4.b57-64>



[Creative commons version 4.0](https://creativecommons.org/licenses/by-nc-sa/4.0/)

## INTRODUCTION

Cholera is an acute infectious disease in countries with poor sanitation. The main clinical symptom of cholera is gastroenteritis but other clinical manifestations like septicemia and wound infections are also observed.<sup>1-3</sup> The clinical indication of the disease includes mild to moderate dehydration, vomiting, fever and abdominal pain.<sup>4</sup> Cholera continues to be a serious epidemic disease caused by a gamma proteobacterium, *Vibrio cholerae* acquired from contaminated water and food in many areas of Asia, Africa and America.<sup>4-5</sup> *Vibrio cholerae* is a gram-negative, oxidase positive, facultative anaerobic, motile bacterium.<sup>4</sup> The distribution of *Vibrio cholerae* is a global phenomenon and this organism is not only isolated from aquatic environments but also from human diarrhoeal patients.<sup>6</sup> *Vibrio cholerae* O1 and O139 strains are the causative agent of epidemic and endemic cholera although more than 200 serogroups of *Vibrio cholerae* exist on the basis of epitopic variations in cell surface lipopolysaccharides.<sup>7-9,18</sup> *Vibrio cholerae* O1 serogroup have been subdivided into El Tor and Classical biotypes.<sup>4</sup> *Vibrio cholerae* O1 belonging to El Tor biotype is the most common serogroups that caused the seventh pandemic while the O1 classical biotype were the cause of previous pandemics.<sup>4</sup> Rest of the serogroups other than O1 and O139 are collectively known as *Vibrio cholerae* non-O1/non-O139. *Vibrio cholerae* non-O1/non-O139 strains generally exist in aquatic environment. It has been reported that *Vibrio cholerae* non-O1/non-O139 strains are associated with sporadic cases of diarrhea and extra intestinal infections in different countries including India.<sup>10-13</sup> It has been also reported that *Vibrio cholerae* non-O1/non-O139 strains were isolated from diarrheal cases in Kolkata, India during 1996.<sup>14</sup> The incidence of *Vibrio cholerae* non-O1/non-O139 was also observed in the next two years (1997 and 1998) among the diarrheal patients admitted to the Infectious Diseases and Beliaghata General Hospital and BC Roy Children Hospital, Kolkata, India and the incidence of *Vibrio cholerae* non-O1/non-O139 was higher than the incidences of *Vibrio cholerae* O1 and O139 at that time.<sup>14-15</sup> The aim of this study was to elucidate the phenotypic characteristics of *Vibrio cholerae* strains isolated clinically from hospitalized patients of Paschim Medinipur, West Bengal, India in 2013-2014.

## MATERIALS AND METHODS

### Sampling site

Only patients with diarrheal illness admitted in the hospital were considered in this study. A total of 147 stool specimens collected from hospitalized patients at Paschim Medinipur in West Bengal, India during the year 2013 were included in the study and the patients were selected irrespective of age and sex.

### Bacterial strains

A total of 78 clinical strains of *Vibrio cholerae* were isolated from 147 stool samples of the diarrheal patients admitted in hospital according to WHO method.<sup>16</sup> Stool samples were collected in the plastic containers (HiMedia, Mumbai) from the diarrheal patients admitted to the hospital in each month during the year of 2013.

The containers containing samples were placed in the refrigerated boxes and transported to the Laboratory within 3 hours. 2 µl of stool were used to alkaline peptone water (APW). The samples were incubated at 37°C for 5 hours and then the surface growth was streaked onto Thiosulphate citrate bile salt sucrose (TCBS) agar (HiMedia, Mumbai) plates for selective isolation of *Vibrio cholerae*. After incubation overnight at 37°C, three single colonies from the cultures plate of each stool sample that produced bright yellow color on the selective agar were inoculated on to a gelatin agar (HiMedia, Mumbai) plates after an APW passage. Three reference strains were used as controls to validate the tests. One each of El Tor biotype (C6706), O1 classical biotype (O395) and O139 (MO10) serogroups were used in this study as controls.

### Enrichment and Plating

The tubes of APW containing water samples, sediment samples, and plankton samples were incubated (Shaking Incubator, Scigenics Biotech, India) at 37°C with shaking for 18-24 hours for the enrichment of the sample. The turbidity of the media was indicated by the bacterial growth.<sup>17</sup>

### Culture and Isolation

A loopful of inoculum from the enrichment cultures were streaked to thiosulphate citrate bile salts sucrose (TCBS) agar and incubated at 37°C for 24 hours. Developing yellow colonies were suspected to be *Vibrio cholerae* and were picked. Five to ten isolated colonies from each plate were randomly picked up for each sample and subsequently sub-cultured on fresh TCBS agar plate.

### Sub-Culture

The pure isolates were then subjected to gelatin agar plates and the isolates that produced a halo in gelatin agar plates after incubation (Incubator with thermostatic control, Indigenous) at 37°C for 24 hours were checked for other biochemical reactions.

### Thiosulfate citrate bile salts sucrose (TCBS) agar

Eighty eight gram of the TCBS medium (HiMedia, Mumbai) were suspended in one liter of purified water. The medium was heated with frequent agitation and boiled to completely dissolve the medium. Then the agar medium was cooled until cooled enough to pour (~50°C), leaving the lid and poured the medium into petri plates, then closed the lid. Prepared TCBS agar was green. However, it has a relatively short shelf life once prepared (3 days) unless plates are carefully protected against drying. The inoculum from APW was streaked on TCBS plate with a wire loop and incubated at 37°C for 24 hours.

### Gelatin agar (GA)

Ingredients (HiMedia, Mumbai) were added to the water and heated to boiling while stirring to dissolve the agar. Then pH was adjusted at 8.5 with a concentrated solution of sodium hydroxide. It was dispensed into screw-capped bottles and autoclaved at 121°C for 15 minutes. Selected several suspicious colonies from TCBS agar plate was streaked to GA plate with a wire loop and incubated at 37°C for overnight.

**Oxidase test**

At first we prepared freshly Kovac's oxidase reagent (1% *N, N, N', N'*-tetramethyl-*p*-phenylenediamine dihydrochloride) by dissolve the reagent in purified water. Prepared freshly before used. Two to three drops of Kovac's oxidase reagent (HiMedia, Mumbai) were placed on a piece of filter paper in a petri dish and allowed it to absorb; the filter paper were moist (but not wet) after the reagent had been absorbed. Bacterial colonies were picked using a platinum loop from non-selective media and rubbed it onto the moistened filter paper. Rapid appearance of a dark purple color was considered oxidase positive reaction.<sup>18</sup>

**Bile Esculin test**

Bile esculin media were prepared as recommended by the manufacturer. For the preparation of the medium, 64.5 grams of the bile esculin (HiMedia, Mumbai) was suspended in one liter of distilled water, mixed well and dissolved by heating with frequent agitation. The mixture was boiled for one minute to completely dissolve the medium. The medium was dispensed into test tubes and sterilize in autoclave at 121°C for 15 minutes. Tubes were allowed to cooling in a slanted position. The prepared dehydrated medium was inoculated by stabbing with a single colony then zigzag streaking on the surface of the slant. Cap was placed loosely on tube. The tubes were incubated for 24 hours at 37°C. After 24 hours incubation observed color change. Blackening of the medium indicates a positive reaction.<sup>18</sup>

**String test**

Sterile distilled water was added to sodium deoxycholate (HiMedia, Mumbai) and mixed well. The prepared reagent was stored at room temperature for up to used (maximum 6 months). The string test was performed on a glass microscope slide by suspending 24 hours growth from nonselective agar in a drop of 0.5% aqueous solution of sodium deoxycholate.

**Kligler's iron agar (KIA)**

Kligler iron agar slants were prepared as recommended by the manufacturer. For the preparation of the medium, 6.5 ml of the KIA medium (HiMedia, Mumbai) was dispensed in test tubes. Leave screw-caps loosed and autoclaved the medium. After autoclaving, allowed the slants to solidify in a manner such that the medium in the butt of the tube was about 3.5 cm deep and the slant was about 2.5 cm long. The tubes were inoculated by stabbing the butt and streaking the surface of the medium. Tubes were incubated at 37°C and then examined after 24 hours.<sup>18</sup>

**Triple sugar iron agar (TSI)**

For the preparation of the TSI medium, 6.5 ml of ingredients (HiMedia, Mumbai) was dispensed in the test tube to give a deep butt and a long slant. Leave screw-caps loosed and autoclaved the medium. After autoclaving, allowed the slants to solidify in a manner such that the medium in the butt of the tube was about 3.5 cm deep and the slant was about 2.5 cm long. TSI slants were inoculated by stabbing the butt and streaking the surface of the medium. Tubes were incubated at 37°C and examined after 24 hours. Caps on all tubes must be loosened before incubation.

**Lysine iron agar (LIA)**

For the preparation of the LIA medium, 6.5 ml of ingredients (HiMedia, Mumbai) was dispensed in test tubes to give a deep butt and a long slant. Leave screw-caps loosed and autoclaved the medium. After autoclaving, allowed the slants to solidify in a manner such that the medium in the butt of the tube was about 3.5 cm deep and the slant was about 2.5 cm long. LIA was inoculated by stabbing the butt and then streaking the slant. Tubes were incubated at 37°C and examined after incubation for 24 hours at 37°C.

**Glucose fermentation test**

For the preparation of the Glucose medium, 20 gram of glucose (HiMedia, Mumbai) was suspended in one liter of distilled water, mixed well and heated slightly until completely dissolved. It was dispensed into tubes with Durham fermentation (gas collection) tubes and sterilized at 118°C for 10 minutes. Glucose broth was inoculated lightly from fresh growth. The broths should be incubated at 37°C and read at 24 hours.

**Sucrose fermentation test**

For the preparation of the sucrose medium, 20 gram of sucrose (HiMedia, Mumbai) was suspended in one liter of distilled water, mixed well and heat slightly until completely dissolved. It was dispensed into tubes with Durham fermentation (gas collection) tubes and sterilized at 118°C for 10 minutes. Sucrose broth was inoculated lightly from fresh growth. The broths should be incubated at 37°C and read at 24 hours.

**Mannitol and arabinose fermentation test**

Mannitol or arabinose fermentation tests were performed with phenol red broth (HiMedia, Mumbai, India). Mannitol and arabinose were added at 1% concentrations to phenol red broths for the corresponding tests. The broth was sterilized by autoclaving and the pH was adjusted to 6.8 with sterile 10N NaOH. The inoculated mannitol or arabinose broths were incubated for 24 hours at 37°C. A result was considered positive when the broth turned to yellow from purple.<sup>18</sup>

**Arginine dihydrolase test**

The method used for the arginine dihydrolase test was based on Thornley's method.<sup>19</sup> In this test 1% (wt/vol) L-arginine was dispensed in 2-ml Luria-Bertani broth in test tubes. Phenol red powder (HiMedia, Mumbai) was added as an indicator. The broth was sterilized by autoclaving at 121°C for 15 minutes and the pH was adjusted to 6.8 with sterile 10N NaOH. After inoculation, the medium was covered with sterile mineral oil and incubated at 37°C for 24 hours. Appearance of a red color was considered a positive reaction.<sup>18</sup>

**Lysine and ornithine decarboxylase assays**

Lysine and ornithine decarboxylase assays were performed by using Decarboxylase Test Medium Base (HiMedia, Mumbai) amended with an amino acid (Lysine or ornithine) at a concentration of 1% (wt/vol). The medium was sterilized by autoclaving at 121°C for 15 minutes and the pH was adjusted to 6.8 with sterile 10N NaOH. The base medium, without addition of an amino acid, was inoculated as a control. After inoculation, the medium was covered with mineral oil and incubated at

37°C for 24 hours. Appearance of a dark purple color was considered a positive reaction compared to the color obtained with the base medium without an amino acid.<sup>18</sup>

#### Salt Tolerance Test

Different salt (HiMedia, Mumbai) concentrations (0, 3, 6, 8; wt/vol) were prepared in nutrient broth. Each of the isolates from fresh growth was inoculated into each of the salt concentration and incubated for 24 hours at 37°C. Turbidity of the media indicates growth of the tolerant *Vibrio cholerae*.<sup>18,20</sup>

#### Voges Proskauer (V-P) Test

The test organism was inoculated in 5 ml MR-VP broth and incubated at 37°C for 48 to 72 hours. 15 drops of reagent A (5% alpha-naphthol (HiMedia, Mumbai) in absolute ethanol) were added followed by 5 drops of reagent B (40% potassium hydroxide (HiMedia, Mumbai)). The tubes were shaken and the caps were loosened. The tubes were placed in a sloppy position. Development of a red colour starting from the liquid-air interface within one hour indicates a V-P positive test.<sup>21</sup>

#### The methyl red reaction

The methyl red reaction was tested by using MR-VP medium (HiMedia, Mumbai) incubated at 37°C for 48 h after inoculation.<sup>18</sup>

#### Motility test

Motility of the isolated *Vibrio cholerae* strains were tested by the method mentioned by Rashid et al. (2003) using swarm plate containing 0.3 % LB agar (HiMedia, Mumbai).<sup>22</sup> Migration of bacteria (showing increase

diameter of colony) were considered as positive swarming.<sup>23</sup>

#### PCR amplification

For development of the method, species identities of all presumptive isolates of *Vibrio cholerae* were confirmed by species-specific polymerase chain reaction (PCR). The PCR was performed as described by Chun et al. (1999).<sup>24</sup> DNA from *Vibrio cholerae* strains used for the PCR template was prepared from overnight L-broth cultures at 37°C. The culture was centrifuged at 10,000 g for 5 min and the pellet was suspended in 1ml sterile Milli Q water (Millipore-Synergy®, USA). The suspension was boiled for 10 min. and the boiled suspension was centrifuged at 12000 g for 5 min. After centrifugation the supernatant was stored at -20°C.<sup>25</sup> PCR was carried out in 20µl volumes containing 2µl template DNA, 2µl 10x concentrated PCR buffer [100 mM Tris/HCl, (pH 8.3), 500 mM KCl], 1.2 µl 15 mM MgCl<sub>2</sub>, 4 µl (5 pmol µl<sup>-1</sup>) each of appropriate primers, 2 µl DNA mixture (2.5 mM each dNTP), 0.5 µl (5 U µl<sup>-1</sup>) Taq DNA polymerase and 4.3 µl sterilized Millipore distilled water. The PCR primers and conditions are given in Table 1. All PCR assays were performed using an automated thermal cycler (Ependroff, Germany).

#### Gel Electrophoresis

The amplified products were then separated by agarose gel electrophoresis. PCR-products were run on 1% agarose gels (HiMedia, Mumbai, India) containing Ethidium Bromide stain (EtBr) (HiMedia, Mumbai, India) with 1x TAE buffer (40 mM Tris- HCl, 20 mM Na-acetate, 1mM EDTA, pH 8.4) and the bands were visualized under an UV transilluminator (Biometra, Germany). Images were captured with digital imaging system (Bio-Rad).

Table 1  
PCR primers used in this study

Target gene	Direction	Primer sequence (5'- 3')	Amplicon size (bp)	Annealing conditions	Reference
16S rRNA	F	CAG CMG CCG CGG TAA TWC	888	55 <sup>o</sup> C, 30 sec	40
	R	ACG GGC GGT GTG TRC			
ompW	F	CACCAAGAAGGTGACTTTATTGTG	588	58 <sup>o</sup> C, 1 min	33
	R	GAACCTTATAACCCACCCGCG			
rfbO1	F	GTT TCA CTG AAC AGA TGG G	192	54 <sup>o</sup> C, 45 sec	41
	R	GGT CAT CTG TAA GTA CAA C			
rfbO139	F	AGC CTC TTT ATT ACG GGT GG	449	55 <sup>o</sup> C, 45 sec	41
	R	GTC AAA CCC GAT CGT AAA GG			

F- Forward; R-Reverse

## RESULTS

Stool samples collected from hospitalized diarrheal patients admitted in hospital were analyzed for *Vibrio cholerae*. In our study 78 *Vibrio cholerae* strains were isolated from 147 stool samples of the patients

hospitalized with sporadic diarrhea during January, 2013 to December, 2013 (Figure 1). Important biochemical and physiological characteristics of the 78 *Vibrio cholerae* isolates in the study are presented in Table 2 and Table 3.

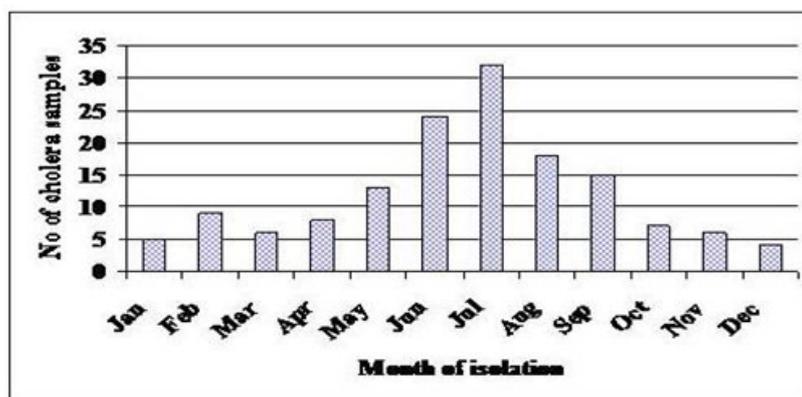


Figure 1

**Monthly isolation profile of diarrheal samples collected from hospitalized diarrheal patients admitted to Medinipur Medical College and Hospital in Paschim Medinipur, West Bengal, India in 2013.**

This work directed the demonstration of a simple, quick and accurate method to identify *Vibrio cholerae*. This method is comprised of two steps: (a) a screening procedure using certain biochemical assays and (b) a species-specific PCR assay for precise identification. The results of the phenotypic characterization for all strains are presented in Table 2 and Table 3. Alkaline peptone broth (pH 8.6) was the enrichment medium and thiosulfate-citrate-bile salts sucrose agar (TCBS; HiMedia, Mumbai) was the selective plating medium used throughout the study. In this study 85 yellow and shiny colonies were isolated from TCBS plate (Table 2). Then after inoculation of isolated strains on GA plate, smooth, opaque, white, colonies (2-4 mm in diameter) were obtained. In our study, of 147 samples, 78 sucrose-fermenting colonies isolated from TCBS agar were able to grow on nutrient agar without NaCl and

also gave an oxidase-positive reaction (Table 2). There was no strain found positive in the bile-esculin medium because *Vibrio cholerae* strains could not grow in this medium (Table 2). Other biochemical reactions of *Vibrio cholerae* were found to be presented in Table 3. All the *Vibrio cholerae* strains were found positive in string test. The reactions of *Vibrio cholerae* on KIA, which contains glucose and lactose, produced an alkaline (red) slant and acid (yellow) butt and no one produced gas and H<sub>2</sub>S. On TSI, *Vibrio cholerae* strains produced an acid (yellow) slant and acid (yellow) butt without production of gas or H<sub>2</sub>S. The LIA reaction for *Vibrio cholerae*, which contains lysine, gave typically an alkaline slant (purple), alkaline butt (purple) without production of gas or H<sub>2</sub>S. *Vibrio cholerae* ferments both glucose and sucrose but does not produce gas in either carbohydrate.

**Table 2**  
**Biochemical tests used to screen for *Vibrio cholerae* isolates from hospitalized cases of sporadic diarrhea**

No. of samples	No. of isolates from TCBS plate	Oxidase positive isolates	Esculin negative isolates	TCBS and Oxidase positive but Esculin Negative isolates
147	85	78	85	78

**Table 3**  
**Biochemical tests used to screen and characterize for *Vibrio cholerae* isolates from hospitalized cases of sporadic diarrhea**

Characteristic	a (b)
String test	78 (100)
Triple Sugar Iron Agar (TSI)	72 (92.31)
Kigler Iron Agar (KIA)	72 (92.31)
Lysine Iron agar (LIA)	75 (96.15)
Arginine Dihydrolase	0 (0)
Lysine Decarboxylase	73 (93.59)
Ornithine Decarboxylase	73 (93.59)
Gelatinase	78 (100)
Voges Proskaur (VP)	78 (100)
Methyle Red	54 (69.23)
Urease	0 (0)
NaCl tolerance (0%)	78 (100)
NaCl tolerance (1%)	78 (100)
NaCl tolerance (6%)	66 (84.62)
NaCl tolerance (8%)	0 (0)
Motility	74 (94.87)

<sup>a</sup> The numbers are the positive results of strains for the characteristic or test. <sup>(b)</sup> The numbers are the percentages of strains positive for the characteristic or test. V, Variable reaction; W, Weak reaction.

The genotypic characteristics of the *Vibrio cholerae* strains examined in this study are shown in Table 4. Table 4 shows that 91.03% (71) strains were positive for *Vibrio cholerae* specific 16S rRNA gene and produced an amplicon of 888 bp and 92.31% (72) strains were also positive for another *Vibrio cholerae* specific *ompW* gene and produced an amplicon of 588 bp. In PCR analysis 91.03% of these presumptive isolates were identified as *Vibrio cholerae* by 16S rRNA and *ompW*-based PCR. Thus, these results show that PCR can be used for confirmation of *Vibrio cholerae* after biochemical identification. It has been found that the 16S rRNA and *ompW*-based PCR seemed to be the best for species specific identification of *Vibrio cholerae*. The confirmation rate by 16S rRNA and *ompW*-based

PCR of good and excellent identifications with biochemical identification was 70.51%. PCR analysis also revealed that all *Vibrio cholerae* strains lacked *rfbO1* and *rfbO139* genes and these strains were considered to be *Vibrio cholerae* non-O1/non-O139. All the clinical isolates (71) were *Vibrio cholerae* non-O1/non-O139 (Table 4). Motility of *Vibrio cholerae* isolates were examined after overnight incubation at 30°C. Motility is indicated by the presence of diffuse growth (appearing as clouding of the medium) away from the line of inoculation (Table 3). Non-motile organisms did not grow out from the line of inoculation. Reactions were compared with positive and negative control strains. *Salmonella typhi* was used as a positive control.<sup>26</sup>

**Table 4**  
**Results of analysis by multiplex PCR with *Vibrio cholerae* strains**

Target gene	Results a(b)
16S rRNA	71 (91.03%)
<i>ompW</i>	72 (92.31%)
<i>rfbO1</i>	0 (0%)
<i>rfbO139</i>	0 (0%)

a; number of +ve strains, b; percentage of +ve strains.

## DISCUSSION

This study characterized 71 *Vibrio cholerae* strains that were isolated from hospitalized diarrhoeal patients in Paschim Medinipur, West Bengal, India in 2013. Although cholera-like outbreaks were reported from several places in India,<sup>27</sup> during our study period no outbreak occurred in Paschim Medinipur district in the state of West Bengal, India. A selective medium, such as TCBS agar, eliminates the non-target bacteria in clinical samples because TCBS agar is only recommended for the isolation of *Vibrio* spp.<sup>28-29</sup> Bacterial isolates who developed yellow colonies on TCBS agar plate were finally selected as *Vibrio cholerae* strains for further analysis.<sup>18</sup> Furthermore, a series of biochemical tests were designed for clinical samples in order to specifically detect *Vibrio cholerae* which were commonly used to identify *Vibrio cholerae*.<sup>30-32</sup> Four ordered traits (sucrose fermentation, non-requirement of added NaCl for growth, presence of oxidase and absence of esculin) are sufficient to distinguish *Vibrio cholerae* from the other species of *Vibrios*. Sucrose non-fermenting species are not taken into consideration for the sucrose fermenting *Vibrio cholerae* and thus eliminated the sucrose non-fermenting *Vibrio mimicus* species at the beginning of the test. Without addition of NaCl, growth on nutrient agar eliminated most sucrose-fermenting halotolerant or halophilic vibrio species that may be important in differential diagnoses (*Vibrio alginolyticus*, *Vibrio metschnikovii*, and *Vibrio fluvialis* and to a lesser extent *Vibrio furnissii*, *Vibrio cincinnatiensis*, *Vibrio anguillarum*, and *Vibrio carchariae*). Moreover, an oxidase-positive reaction eliminated *Vibrio metschnikovi* and esculin negative reaction eliminated gram negative bacteria. While these four taxonomic traits are insufficient to definitively identify the isolates as *Vibrio cholerae*, they do represent minimal traits which all members of the species must have. Thus, any isolates which do not meet these minimal criteria are deemed

not to be *Vibrio cholerae*. Besides conventional biochemical identification, alternative molecular method of PCR, which is now used by reference laboratories for the confirmation of presumptive identifications. PCR performed with primers specific for *Vibrio cholerae*, provide more reliable identification.<sup>33</sup> Generally 16S rRNA and *ompW*-based PCR gave a positive result for *Vibrio cholerae* without some exceptions.<sup>33-34</sup> Out of 78, 71 *Vibrio cholerae* strains were positive for the 588-bp PCR amplicon specific to *Vibrio cholerae ompW* gene and 72 strains were positive for the 888 bp PCR amplicon specific to *Vibrio cholerae* 16S rRNA gene.<sup>33-34</sup> The sequences of *ompW* and 16S rRNA genes are highly conserved among the *Vibrio cholerae* belonging to different serogroups and are targeted for the species-specific identification of *Vibrio cholerae*.<sup>35</sup> The identified *Vibrio cholerae* strains had been characterized as non-O1/non-O139 due to negative results obtained in PCR assay using specific primers for *rfbO1* and *rfbO139* genes. *Vibrio cholerae* non-O1/non-O139 strains isolated from diarrhoeal patients comprises 83.53% of the total cases (n=85) that were microbiologically positive for *Vibrio cholerae*; this is consistent with an earlier report on the prevalence of *Vibrio cholerae* non-O1/non-O139 strains isolated from acute diarrhoeal cases in Kolkata, India.<sup>36-37</sup> These results demonstrate the potential for strains of non-O1/non-O139 serogroups within the region where cholera is endemic (near to Kolkata, India) to cause sporadic diarrhea. Here, based on our data using 15 generally accepted biochemical tests and two pair primers for PCR for identification of clinical strains of *Vibrio cholerae*, we found that there is a little difference between the biochemical tests and the molecular methods used for confirming the presumptive identifications. From our results, it can be concluded that 16S rRNA and *ompW*-based PCR should be preferred.<sup>39</sup> Another two pair of primers were used for PCR to serogroup *Vibrio cholerae*. In our study period, an unusual event occurred in the Medinipur region. There was an unexplainable upsurge in the incidence of non-

O1/non-O139 *Vibrio cholerae* infections among hospitalized patients admitted to the hospital. From the diarrheal stool samples collected in this study area during this time point no *Vibrio cholerae* isolates belonging to O1 and O139 serogroups were identified.

## CONCLUSION

The proposed simple procedure for the identification of *Vibrio cholerae* is based upon a combination of phenotypic and genotypic testing methods. Various conventional biochemical assays were used for detection of *Vibrio cholerae*. However, many of these methods are time-consuming and laborious. Moreover, biochemical identification systems may not always be accurate as several *Vibrio* species display similar biochemical characteristics. Molecular methods have several advantages than the API 20E system; they are rapid, sensitive, and highly selective and do not require

## REFERENCES

1. Ninin E, Caroff N, El Kouri D, Espaze E, Richet H, Quilici ML, Fournier JM. Nontoxicogenic *Vibrio cholerae* O1 bacteremia: case report and review. *Eur J Clin Microbiol Infect Dis*. 2000 Jul 25;19(6):489-91.
2. Oliver JD, Kape JB. *Vibrio* Species. In: Doyle MP, Beuchat LR, Montville TJ, editors. *Food Microbiology: Fundamentals and Frontiers*. Washington DC: ASM Press; 1997. p. 228-64.
3. Ulusarac O, Carter E. Varied clinical presentations of *Vibrio vulnificus* infections: a report of four unusual cases and review of the literature. *South Med J*. 2004 Feb 1;97(2):163-9.
4. Kaper JB, Morris JG Jr, Levine MM. Cholera. *Clinical Microbiology Reviews*. 1995 Jan;8(1):48-86.
5. World Health Organization. Cholera. Cholera 2010; Weekly epidemiological record Relevé épidémiologique hebdomadaire. 2011 July 29;86:325-40.
6. Bakhshi B, Pourshafie MR, Navabakbar F, Tavakoli A, Shahcheraghi F, Salehi M, Faradjadegan Z and Zahraei SM. Comparison of distribution of virulence determinants in clinical and environmental isolates of *Vibrio cholerae*. *Iran Biomed J*. 2008 July 1;12(3):159-65.
7. Chakraborty S, Mukhopadhyay AK, Bhadra RK, Ghosh AN, Mitra R, Shimada T, Yamasaki S, Faruque SM, Takeda Y, Colwell RR, Nair GB. Virulence genes in environmental strains of *Vibrio cholerae*. *Appl Environ Microbiol*. 2000 Sep 1;66(9):4022-8.
8. Chatterjee SN, Chaudhuri K. Lipopolysaccharides of *Vibrio cholerae*: I. Physical and chemical characterization. *Biochim Biophys Acta - Molecular Basis of Disease*. 2003 Oct 15;1639(2):65-79.
9. Yamai S, Okitsu T, Shimada T, Katsube Y. Distribution of serogroups of *Vibrio cholerae* non-O1 non-O139 with specific reference to their ability to produce cholera toxin, and addition of

extensive time. In this study, we used a simple, sensitive and specific multiplex PCR that confirms the presence of *Vibrio cholerae* in clinical samples and exhibits its epidemic potential. The proposed screening procedure seemed to be very efficient since the identification confirmation rate was more than 90%. It is also easily adapted to the workflow in a routine microbiology laboratory.

## ACKNOWLEDGEMENTS

We thank all hospitalized cholera patients who were the part of this study.

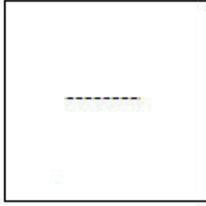
## CONFLICT OF INTEREST

Conflicts of interest declared none.

- novel serogroups. *Kansenshogaku zasshi. J J A Inf D*. 1997 Oct;71(10):1037-45.
10. Dalsgaard A, Albert MJ, Taylor DN, Shimada T, Meza R, Serichantalergs O, Echeverria P. Characterization of *Vibrio cholerae* non-O1 serogroups obtained from an outbreak of diarrhea in Lima, Peru. *J Clin Microbiol*. 1995 Oct 1;33(10):2715-22.
11. Dalsgaard A, Forslund A, Bodhidatta L, Serichantalergs O, Pitarangsi C, Pang L, Shimada T, Echeverria P. A high proportion of *Vibrio cholerae* strains isolated from children with diarrhoea in Bangkok, Thailand are multiple antibiotic resistant and belong to heterogenous non-O1, non-O139 O-serotypes. *Epidemiol Infect*. 1999 Apr;122(2):217-26.
12. Kamble TK, More SR, Chavan SS, Kulkarni ND, Lodha NS, Kamble AS. Clinical profile of non-O1 strain-O139 of *Vibrio cholerae* in the region of Ambajogai, Maharashtra. *J Assoc Physicians India*. 2000 May;48(5):505-6.
13. Mukhopadhyay AK, Saha PK, Garg S, Bhattacharya SK, Shimada T, Takeda T, Takeda Y, Nair GB. Distribution and virulence of *Vibrio cholerae* belonging to serogroups other than O1 and O139: a nationwide survey. *Epidemiol Infect*. 1995 Feb;114(1):65-70.
14. Sharma C, Thungapathra M, Ghosh A, Mukhopadhyay AK, Basu A, Mitra R, Basu I, Bhattacharya SK, Shimada T, Ramamurthy T, Takeda T. Molecular Analysis of Non-O1, Non-O139 *Vibrio cholerae* Associated with an Unusual Upsurge in the Incidence of Cholera-Like Disease in Calcutta, India. *J Clin Microbiol*. 1998 Mar 1;36(3):756-63.
15. Chakraborty S, Garg P, Ramamurthy T, Thungapathra M, Gautam JK, Kumar C, Maiti S, Yamasaki S, Shimada T, Takeda Y, Ghosh A. Comparison of antibiogram, virulence genes, ribotypes and DNA fingerprints of *Vibrio cholerae* of matching serogroups isolated from hospitalised diarrhoea cases and from the environment during 1997-1998 in Calcutta, India. *J Med Microbiol*. 2001 Oct 1;50(10):879-88.

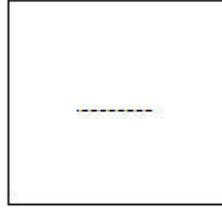
16. World Health Organization. Manual for the laboratory investigations of acute enteric infections. Geneva: World Health Organization; 1987. p. 111. (WHO/CDD/83.3/rev.1).
17. Cheesbrough M. District laboratory practice in tropical countries. Cambridge university press; 2006 Mar 2. p. 30-41.
18. Choopun N, Louis V, Huq A, Colwell RR. Simple procedure for rapid identification of *Vibrio cholerae* from the aquatic environment. Appl Environ Microbiol. 2002 Feb 1;68(2):995-8.
19. Taylor JJ, Whitby JL. Pseudomonas pyocyanea and the arginine dihydrolase system. J Clin Pathol. 1964 Mar 1;17(2):122-5.
20. West PA, Colwell RR. Identification and classification of Vibrionaceae-an overview. Vibrios in the Environment. In: Colwell R, editors. John Wiley & Sons, New York, NY; 1984. p. 285-363.
21. Harwood VJ, Gandhi JP, Wright AC. Methods for isolation and confirmation of *Vibrio vulnificus* from oysters and environmental sources: a review. J Microbiol Methods. 2004 Dec 31;59(3):301-16.
22. Rashid MH, Rajanna C, Ali A, Karaolis DK. Identification of genes involved in the switch between the smooth and rugose phenotypes of *Vibrio cholerae*. FEMS Microbiol Lett. 2003 Oct 1;227(1):113-9.
23. Biswas S, Sen S, Dua P, Mukherjee P, Bhunia S. A Study on Virulence and Signaling Mechanism Related to Biofilm Formation and Exopolysaccharide Expression in Certain *Vibrio cholerae* Environmental Isolates. Glob J Med Res. 2012 Jul 5;12(3).
24. Chun J, Huq A, Colwell RR. Analysis of 16S-23S rRNA intergenic spacer regions of *Vibrio cholerae* and *Vibrio mimicus*. Appl Environ Microbiol. 1999 May 1;65(5):2202-8.
25. Patrick GB, Nishibuchi M, Tunung R, Radu S. Molecular characterization of clinical isolate of *Vibrio cholerae* isolated from outbreaks cases in Malaysia. Int Food Res J. 2012; 19 (3):1267-74.
26. Chelvam KK, Chai LC, Thong KL. Variations in motility and biofilm formation of Salmonella enterica serovar Typhi. Gut Pathog. 2014 Feb 5;6(1):2.
27. Ramamurthy T, Garg S, Sharma R, Bhattacharya SK, Nair GB, Shimada T, Takeda T, Karasawa T, Kurazano H, Pal A, Takeda Y. Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. Lancet. 1993 Mar 13;341(8846):703-4.
28. Kobayashi T, Enomoto S, Sakazaki R, Kuwahara S. A new selective isolation medium for the Vibrio group; on a modified Nakanishi's medium (TCBS agar medium). Nihon saikingaku zasshi. Jpn J Bacteriol. 1963 Nov;18:387.
29. Elliot EL, Kaysner AC, Jackson L, Tamplin ML. *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and other Vibrio spp. USFDA Bacteriological Analytical Manual. 8th ed. AOAC International, Gaithersburg, MD; 2001. p. 901-27.
30. Baumann P, Schubert RHW. Family II. Vibrionaceae Veron 1965. Bergey's manual of systematic bacteriology. 1984;1:516-50.
31. Farmer JJ, Hickman-Brenner FW and Kelly MT. *Vibrio*. In: Lennette EH, Balows A, Hausler WJ Jr, Shadomy H.J. editors. Manual of clinical microbiology. 4th edn. American Society for Microbiology, Washington DC; 1985. p. 282-301.
32. Kay BA, Bopp CA, Wells JG. Isolation and identification of *Vibrio cholerae* O1 from fecal specimens. In *Vibrio cholerae* and Cholera, American Society of Microbiology. 1994 Jan 1 pp. 3-25.
33. Nandi B, Nandy RK, Mukhopadhyay S, Nair GB, Shimada T, Ghose AC. Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the gene of outer membrane protein OmpW. J Clin Microbiol. 2000 Nov 1;38(11):4145-51.
34. Vieira VV, Teixeira LF, Vicente AC, Momen H, Salles CA. Differentiation of environmental and clinical isolates of *Vibrio mimicus* from *Vibrio cholerae* by multilocus enzyme electrophoresis. Appl Environ Microbiol. 2001 May 1;67(5):2360-4.
35. Kingston JJ, Zachariah K, Tuteja U, Kumar S, Batra HV. Molecular characterization of *Vibrio cholerae* isolates from cholera outbreaks in North India. J Microbiol. 2009 Feb 1;47(1):110-5.
36. Sharma C, Thungapathra M, Ghosh A, Mukhopadhyay AK, Basu A, Mitra R, Basu I, Bhattacharya SK, Shimada T, Ramamurthy T, Takeda T. Molecular Analysis of Non-O1, Non-O139 *Vibrio cholerae* Associated with an Unusual Upsurge in the Incidence of Cholera-Like Disease in Calcutta, India. J Clin Microbiol. 1998 Mar 1;36(3):756-63.
37. Chatterjee S, Ghosh K, Raychoudhuri A, Chowdhury G, Bhattacharya MK, Mukhopadhyay AK, Ramamurthy T, Bhattacharya SK, Klose KE, Nandy RK. Incidence, virulence factors, and clonality among clinical strains of non-O1, non-O139 *Vibrio cholerae* isolates from hospitalized diarrheal patients in Kolkata, India. J Clin Microbiol. 2009 Apr 1;47(4):1087-95.
38. Baron S, Chevalier S, Lesne J. *Vibrio cholerae* in the environment: a simple method for reliable identification of the species. J Health Popul Nutr. 2007 Sep;25(3):312.
39. Vandamme P, Pot B, Gillis M, De Vos P, Kersters K, Swings J. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol Rev. 1996 Jun 1;60(2):407-38.
40. Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev. 1995 Mar 1;59(1):143-69.
41. Hoshino K, Yamasaki S, Mukhopadhyay AK, Chakraborty S, Basu A, Bhattacharya SK, Nair GB, Shimada T, Takeda Y. Development and evaluation of a multiplex PCR assay for rapid detection of toxigenic *Vibrio cholerae* O1 and O139. FEMS Immunol Med Microbiol. 1998 Mar 1;20(3):201-7.

## Reviewers of this article



**Dr. S. Dinesh Kumar**

Assistant Professor,  
Department of Microbiology,  
Karpagam University, Coimbatore,  
Tamil Nadu, India.



**Prof. Pratiti Ghosh, PhD**

HOD, Physiology, West Bengal State  
University, Barasat, Berunanpukuria,  
Malikapur, Kolkata-700126



**Dr. S. Swarnalatha M.Pharm., M.B.A.,  
Ph.D.(Pharmacology)**

HOD, Department of Pharmacology,  
Pallavan Pharmacy College,  
Iyyengarkulam, Kanchipuram, Tamilnadu,  
India



**Prof. Dr. K. Suri Prabha**

Asst. Editor, International Journal  
of Pharma and Bio sciences.



**Prof. P. Muthu Prasanna**

Managing Editor, International  
Journal of Pharma and Bio sciences.

We sincerely thank the above reviewers for peer reviewing the manuscript