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

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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*, *rfb*O1and *rfb*O139 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***

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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.[1-3] The clinical indication of the disease includes mild to moderate dehydration, vomiting, fever and abdominal pain.[4] 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.[4-5] *Vibrio cholerae* is a gram-negative, oxidase positive, facultative anaerobic, motile bacterium.[4] 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.[6] *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.[7-9,18] *Vibrio cholerae* O1 serogroup have been subdivided into El Tor and Classical biotypes.[4] *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.[4] Rest of the serogroupes 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.[10-13] It has been also reported that *Vibrio cholerae* non-O1/non-O139 strains were isolated from diarrheal cases in Kolkata, India during 1996.[14] 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.[14-15] 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 Medinipore 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.[16] 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 streak 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 ElTor 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.[17]

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-ρ-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.[18]

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.[18]

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.[18]

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.[18]

Arginine dihydrolase test

The method used for the arginine dihydrolase test was based on Thornley's method.[19] 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.[18]

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.[18]

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*.[18,20]

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 shaked 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.[21]

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.[18]

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).[22] Migration of bacteria (showing increase diameter of colony) were considered as positive swarming.[23]

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).[24] DNA from *Vibrio cholerae* strains used for the PCR template was prepared from overnight L-broth cultures at 37^{o}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^{o}C.[25] 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 $MgCl_2$, 4 µl (5 pmol µl-1) each of appropriate primers, 2 µl DNA mixture (2.5 mM each dNTP), 0.5 µl (5 U µl-1) 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^{0}C, 30 sec	40
	R	ACG GGC GGT GTG TRC			
ompW	F	CACCAAGAAGGTGACTTTATTGTG	588	58^{0}C, 1 min	33
	R	GAACTTATAACCACCCGCG			
***rfb*O1**	F	GTT TCA CTG AAC AGA TGG G	192	54^{0}C, 45 sec	41
	R	GGT CAT CTG TAA GTA CAA C			
***rfb*O139**	F	AGC CTC TTT ATT ACG GGT GG	449	55^{0}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.

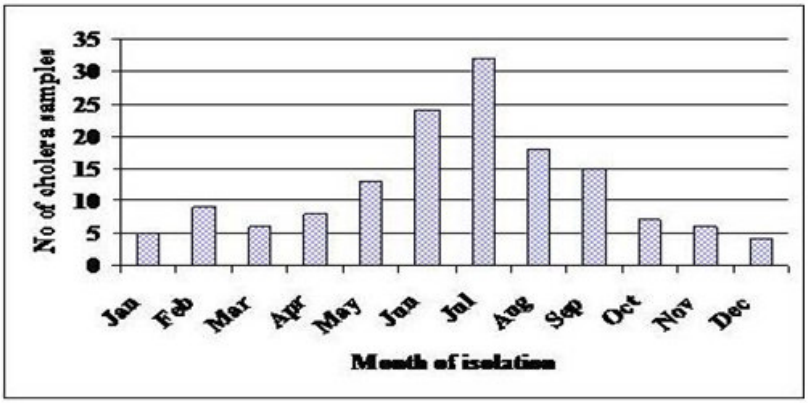

Figure 1
Monthly isolation profile of diarrheal samples collected from hospitalized diarrheal patients admitted to Medinipur Medical College and Hospital in Paschim Medinipur, West Bengal, India in 2013.

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_2S. On TSI, *Vibrio cholerae* strains produced an acid (yellow) slant and acid (yellow) butt without production of gas or H_2S. The LIA reaction for *Vibrio cholerae,* which contains lysine, gave typically an alkaline slant (purple), alkaline butt (purple) without production of gas or H_2S. *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)

[a] The numbers are the positive results of strains for the characteristic or test. [(b)] 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 *rfb*O1 and *rfb*O139 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.[26]

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

Target gene	Results a(b)
16S rRNA	71 (91.03%)
***omp*W**	72 (92.31%)
***rfb*O1**	0 (0%)
***rfb*O139**	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,[27] 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.[28-29] Bacterial isolates who developed yellow colonies on TCBS agar plate were finally selected as *Vibrio cholerae* strains for further analysis.[18] 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.*[30-32] 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.[33] Generally 16S rRNA and *ompW*-based PCR gave a positive result for *Vibrio cholerae* without some exceptions.[33-34] 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.[33-34] 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*.[35] 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 *rfb*O1 and *rfb*O139 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.[36-37] 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.[39] 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 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.

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CONFLICT OF INTEREST

Conflicts of interest declared none.

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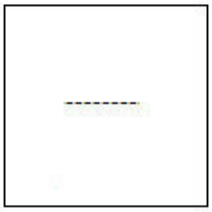

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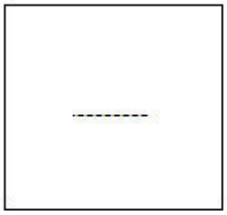

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