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SHORT COMMUNICATION

Molecular detection of *Vibrio cholerae* from human stool collected from SK Hospital, Mymensingh, and their antibiogram

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ABSTRACT

Objective: *Vibrio* spp., particularly, *Vibrio cholerae* is a major etiology of diarrhea in humans worldwide. In this study, we isolated and identified *V. cholerae* from the human stool of suspected cases along with antibiogram.

Materials and Methods: In total, 25 stool samples from cholera suspected patients were analyzed. Isolation and molecular detection of *Vibrio* species were performed based on staining, motility, cultural and biochemical characteristics followed by polymerase chain reaction (PCR) using *groEL* gene-specific primers.

Results: Among the 25 samples, seven showed growth of yellow color colonies on Thiosulfate-Citrate-Bile salts-Sucrose agar plates. The isolates were Gram-negative, curved shaped, and motile. Biochemically, they were found positive for indole and Methyl Red tests and negative for Voges–Proskauer test. Out of the seven positive samples, only three isolates were confirmed as *Vibrio* spp. using genus-specific primers. Subsequently, these three isolates were confirmed as *V. cholerae* by PCR using *V. cholerae groEL* gene-specific primers. Antibiotic sensitivity test revealed these three isolates as highly sensitive to azithromycin, chloramphenicol, gentamicin, and norfloxacillin while resistant to streptomycin, tetracycline, and oxacillin.

Conclusion: *Vibrio cholerae* were isolated from the stool of diarrheic human patients and confirmed by PCR targeting the *groEL* gene. The isolates were found resistant to streptomycin, tetracycline and oxacillin, and need further characterization to reveal the molecular basis of their origin and resistance.

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Introduction

The genus *Vibrio* is a member of the family Vibrioneceae. *Vibrio* is Gram-negative and porogenous rods having straight or curved rod shape. They are motile mostly by single polar flagellum when grown in liquid medium [1]. Currently, there are 72 species under this genus, of which 12 species occur in human clinical samples [2]. Among these 12 species, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* account for the majority of *Vibrio* infections in humans [3]. *Vibrio cholera* is the most important species in the genus *Vibrio*. Diarrhea is a global problem and frequently affects children in Bangladesh due to the geographical location. World Health Organization (WHO) has identified the diarrheal illness as the second leading factor causing 760,000 deaths annually in children of less than 5 years old, of which 10% of the population from low- and middle-income countries, including Bangladesh [4]. There are many infectious causes of diarrhea that may lead to death; among which *V. cholerae*, Adenovirus, *Campylobacter*, *Cryptosporidium*, Enterotoxigenic *Escherichia coli*, Rotavirus, *Salmonella*, and *Shigella* are the most known etiological agents. Among these, *V. cholerae* is considered as the most devastating agent [5]. Across the world, an estimated 1.3 billion people are at risk of cholera; of which Bangladesh jointly constitutes the largest share of the population at risk [6]. Recently, it has been reported that around 66 million people in Bangladesh are at risk for cholera with an incidence of about 1.64 per thousand people [7]. In Bangladesh, the estimated yearly case numbers are about 109,000, where the case fatality rate is 3% [7]. Two primary serogroups of *V. cholerae*, namely, O1 and O139, are linked with the epidemic outbreak of cholera [8]. Moreover, the seventh cholera pandemic was caused

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by drug-resistant *V. cholera* O1 El Tor biotype that spread rapidly from Indonesia to Bangladesh, India, Iraq, and Iran [9].

Vibrio cholerae is frequently found in the aquatic environment [10]. Riverine, coastal, estuarine, and freshwater environments represent the critical reservoirs of *Vibrio* species and play a role in its transmission and epidemiology [11]. A major proportion of human *Vibrio* infections are caused due to consumption of contaminated water and foods [12,13]. Consumption of undercooked or raw shellfish and shrimp also cause the *Vibrio* infection [14,15]. The lack of adequate practice of personal and domestic hygiene, as well as the dense population in Bangladesh, has been associated in the transmission of enteric diseases, like cholera [16,17]. *Vibrio cholerae* can be spread quickly in places where pathogens are introduced in poor water and sanitation system [13]. Outbreak occurs due to its short incubation period (a few hours–5 days for most subtypes) although cholera requires relatively higher infectious dose about 10^4 organisms [18].

Antimicrobial therapy plays a critical role in the recovery of cholera-infected patients. Large use of antibiotics in the 1970s and 1980s in Africa and in the 1990s in South America as prophylaxis during cholera outbreaks has resulted in the rapid development of resistance against tetracycline and doxycycline resistance in *Vibrio*. Subsequently, WHO recommended not to use mass antibiotic as prophylaxis for cholera [19]. *Vibrio cholera* resistance to antibiotics including ampicillin, gentamicin, kanamycin, streptomycin, tetracycline, trimethoprim, and sulfonamides has become a serious treatment problem in many countries of the world, including developing countries like Bangladesh [20]. In addition, recently multidrug resistance in *V. cholera* serogroup O1 biotype El Tor has also been reported globally [21,22]. Although several sets of primers are available to detect *Vibrio* for a long time, new primers are still under development and validation. *groEL* is a chromosomal house-keeping gene and is used as a molecular marker for the detection of many bacteria at the genus and species level [3]. The present study was set up to isolate and identify *V. cholerae* from stool samples by conventional bacteriological methods followed by polymerase chain reaction (PCR) with new *groEL* gene-specific primers that have not been used previously in Bangladesh. In addition, antibiogram of the isolates was also determined.

Materials and Methods

Ethical approval

Ethical permission was not required because the stool samples were collected from the individual pan used by the patients; however, before the collection of the samples, verbal permission was taken from each patient.

Sample collection

A total of 25 stool samples (18 from adult and 7 from children) were randomly collected from cholera suspected patients of SK Hospital, Mymensingh.

Isolation and identification of Vibrio species

Isolation and preliminary identification of *Vibrio* species from the stool samples were based on cultural on Thiosulfate-Citrate-Bile salts-Sucrose (TCBS) agar plates at 37°C for 18–24 hours aerobically followed by staining and biochemical tests (Methyl red, Voges–Proskauer, indol, catalase and oxidase tests), as described by Talukder et al. [23]. Hemolytic activities were also studied by growing each isolate on blood agar plates and motility activity by hanging drop method [24].

Molecular detection of V. cholerae

Molecular detection of the suspected *Vibrio* isolates at the genus and species level was carried out by PCR using two sets of primers targeting *groEL* genes one at genus level and another at species level amplifying 1117-bp and 418-bp amplicon, respectively [3,25]. DNA from pure broth culture was extracted by the boiling method as stated by Hossain et al. [26]. The PCR reactions were done in an Eppendorf Thermocycler (Eppendorf, USA) in a 25 µl reaction scale with 12.5 µl master mixture 2X (Promega, USA), 2 µl (50 ng) genomic DNA, 1 µl of each primer, and 8.5 µl nuclease-free water. Amplified products were analyzed by electrophoresis in 1.5% agarose gel. Amplified products were stained using ethidium bromide and finally visualized under ultraviolet trans-illuminator (Biometra, Germany).

Antibiogram

Nine commonly used antibiotics (HiMedia, India), namely, azithromycin (15 mg), chloramphenicol (30 mg), ciprofloxacin (5 mg), erythromycin (15 mg), gentamicin (10 mg), norfloxacin (10 mg), oxacillin (15 mg), streptomycin (10 mg), and tetracycline (30 mg) were selected for the sensitivity test. Antibiogram profile of the isolates was determined by the disk diffusion method on Mueller Hinton (HiMedia, India) agar plates, as described by Bauer et al. [27]. McFarland 0.5 standard was maintained for each culture suspension of bacterial isolates before the antibiogram study. As per the recommendations of CLSI [28], the results of the antibiogram were recorded as sensitive, intermediately sensitive, or resistant.

Results and Discussion

Bangladesh is a highly populated country. Most of the people in rural and urban areas are living in unhygienic

conditions [5]. Cholera is a disease mostly associated with poor sanitation. Diarrheal disease is known to be a major cause of child mortality in Bangladesh [29]. *Vibrio cholerae* is a major etiology of diarrhea leading to death. Present study was carried out to isolate and detect *V. cholerae* from suspected human stool samples. *Vibrio* specific selective media TCBS was used to culture and isolate *Vibrio* spp. On TCBS agar, seven out of 25 samples were found to produce yellow color button-shaped flattened colonies similar to the findings of Choopun et al. [30]. On blood agar, all the seven isolates produced hemolytic colonies suggesting their ability to produce infection. The isolated *Vibrio* spp. was observed as motile also reported by Kaper et al. [31].

PCR is a highly sensitive molecular technique. It is frequently used for the detection of certain bacteria targeting specific gene. Two sets of *groEL* gene primers were used of which one for *Vibrio* genus detection and another for *V. cholerae* detection. It is already proved that both primers are *Vibrio* genus and *V. cholerae* specific [25,32]. Among the seven culture-positive isolates, only isolates were confirmed as *Vibrio* species by PCR that showed a band of 1117 bp (Fig. 1). These three isolates were further screened by PCR using *groEL* gene-specific primers to detect *V. cholerae*, *V. parahaemolyticus*, or *V. vulnificus*. Interestingly, all the three isolates were confirmed as *V. cholerae* as evident by 418-bp PCR amplicon (Fig. 1). However, we do not rule out the possibilities of other four culture-positive isolates as *Vibrio* spp., without screening them with other primers available for the detection of *Vibrio* spp. *Vibrio* is the major cause of diarrhea in many countries of the world including Bangladesh [6,33]. A study conducted in Nepal showed that variation in geographical locations or primer used greatly influence the occurrence and detection of various *Vibrio* spp. [21]. Recently, a study found cholera at the point and source of drinking water in low-income settlement of Bangladesh [16]. Another study found cholera in 20% and 18% household, respectively, for a household rectal swab, stored drinking water, and 27% source of water [17]. These findings suggest that detection of cholera from suspected cases may be associated with intake of contaminated foods or water by the patients, as cholera is known to cause by contaminated foods and water. Cholera contamination in the community by feco-oral route is common in developing country due to the poor sanitation practice [12,13].

Antibiotic resistant is a major global public health concern. It is crucial to know the sensitivity and resistance pattern of any bacterial species for recommending effective drug of choice. The only way to tackle antibiotic resistance is the conscious use of antibiotics. Various factors are also responsible for the increase in bacterial resistance to antibiotics [34]. The main reason which is responsible for the occurrence of the expanding resistance is the easy availability of antibiotics and their widespread use in chemoprophylaxis leads resistance mainly through selective pressure apart from other factors [19]. *V. cholerae* is still considered as a notorious pathogen because of increasing resistance to a number of antibiotics [35]. *V. cholera* isolated in this study were subjected to antibiogram against nine commonly used antibiotics. All these three isolates were found highly sensitive to azithromycin, gentamycin, chloramphenicol, and norfloxacin (Table 1). But recently, resistant against azithromycin and chloramphenicol has appeared as an emerging phenomenon in some isolates of *Vibrio* such as *V. fluvialis* and

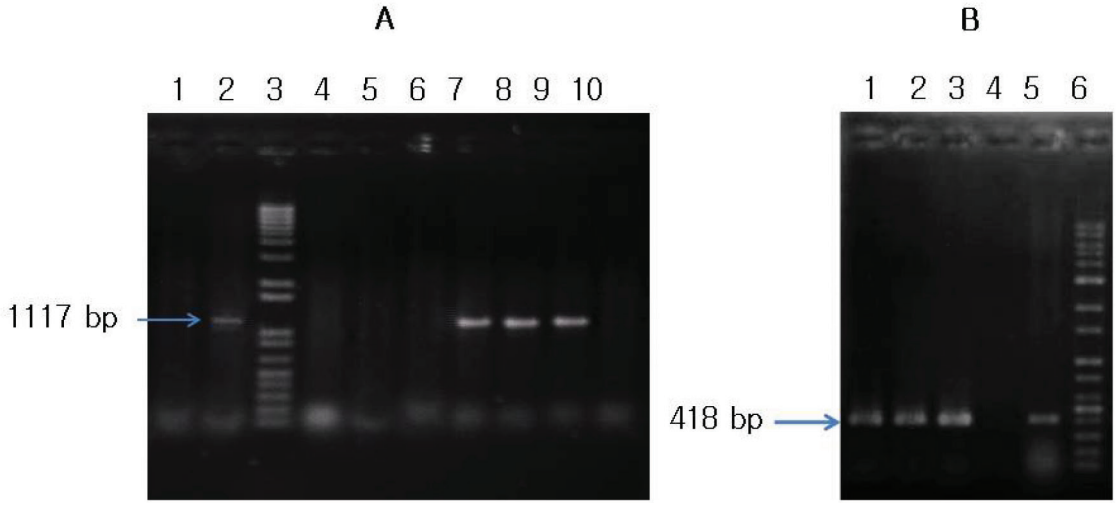

Figure 1. PCR-based detection of *Vibrio* spp., and *V. cholerae*. (A) Agarose gel electrophoresis of PCR products (1117-bp) of *Vibrio* spp. Lane 1: negative control, lane 2: positive control, lane 3: 100-bp DNA ladder, lane 4–10 samples analyzed. (B) Agarose gel electrophoresis of PCR products (418-bp) of *V. cholerae*. Lane 1–3: samples analyzed, lane 4: negative control, lane 5: positive control, and lane 6: 100-bp DNA ladder.

Table 1. Antibiotic sensitivity pattern of the isolated *V. cholerae* from stool samples of suspected cases of cholera.

Name of the isolate	Antibiotic sensitivity pattern (%)								
***V. cholerae* (*n* = 3)**	**Streptomycin**	**Erythromycin**	**Azithromycin**	**Tetracycline**	**Gentamicin**	**Chloramphenicol**	**Oxacillin**	**Norfloxacin**	**Ciprofloxacin**
Susceptible	1 (33.33%)	2 (66.67%)	3 (100%)	1 (33.33%)	3 (100%)	3 (100%)	0 (0%)	3 (100%)	3 (66.67%)
Intermediate	1 (33.33%)	1 (33.33%)	0 (0%)	%	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (33.33%)
Resistant	1 (33.33%)	0 (0%)	0 (0%)	2 (66.67%)	0 (0%)	0 (0%)	3 (100%)	0 (0%)	0 (0%)

V. cholerae, respectively [36,37]. Interestingly, all three (100%) isolates were found resistant to oxacillin followed by tetracycline (*n* = 2, 66.67%) and streptomycin (*n* = 1, 33.33%). However, it is difficult to discuss on the observed resistant pattern of the detected cholera due to the fewer number of isolates. Nevertheless, the findings of the present study will work as a reference for future elaborate work on *Vibrio*. The spread of antibiotic resistant *V. cholerae*, especially O139 strain, is known to be linked with the mobilization of drug resistance genetic elements [38]. In addition, class I integron has also been reported to be linked with the spread of antibiotic resistance in *V. cholera* [39]. Major limitations of the present study are the use of very few samples to analyze. In addition, minimum inhibitory concentration (MIC) of the antibiotic were not determined. Further studies are required for the detection and characterization of these mobile genetic elements in the isolated *V. cholerae*.

Conclusion

Present study detected *V. cholera* as the etiological agent of suspected cases of cholera using culture and *groEL* gene-targeted PCR. Majority of the isolates were found sensitive to commonly used antibiotics, except oxacillin, tetracycline, and streptomycin. Detailed studies are now required to reveal the origin of these isolates and the molecular basis of their resistance.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' contribution

FZ, MTH, and MTR designed the study. FZ and SA did the actual works. FZ, MAS, MTH and MTR drafted the manuscript. MTR and MTH critically checked and improved the manuscript. All the authors read the manuscript and approved the final version for publication.

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