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Isolation, Identification and Antimicrobial Susceptibility Profile Analysis of *Vibrio cholerae* O1 from Stool Samples of Bangladesh

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Abstract

Cholera is a severe diarrheal disease which is usually caused by toxigenic strain of *Vibrio cholerae* O1 and O139. Cholera is still one of the major health concerns in developing countries like Bangladesh due to poor sanitation and unavailability of safe drinking water. This experiment was confronted to identify *V. cholerae* O1 from stool samples as well as to determine the antibiotic susceptibility pattern of the isolated strains. A total of 140 stool samples from people infected with diarrheal disease were collected from July 2016 to December 2016. Among all, 58 samples were found positive for *V. cholerae* which were further subjected to sero-grouping by specific anti-sera and antimicrobial susceptibility test by Kirby Bauer disc diffusion method. The zones of inhibition were measured and interpreted by following the recommendations of the criteria of Clinical and Laboratory Standards Institute (CLSI). It was found that 43 (74.1%) isolates of *V. cholerae* were O1 serogroup of Ogawa serotype and the rest 15 (25.9%) were O1 serogroup of Inaba serotype. People aged between 41 - 50 were most susceptible to *V. cholerae* O1 having about 39.7% of positive cases. The isolates were highly susceptible to Ciprofloxacin and Gentamicin with 100% susceptibility whereas 100% resistant was found towards Nalidixic acid. Though most of the isolates in our study were susceptible against tested antibiotics, the continuous surveillance is required to see the changing pattern of serogroups or serotypes and antimicrobial profile

in this region.

Keywords

Cholera, Antibiotic Resistant, *Vibrio cholerae*

1. Introduction

Cholera is an acute and life-threatening diarrheal disease. It occurs in epidemic form in many developing countries like Bangladesh. It is usually associated with unavailability of safe drinking water, poor socio-economic conditions, rudimentary sanitary systems and public hygiene [1].

The causative agent of cholera is *Vibrio cholerae,* a gram negative organism. This bacterium has more than 200 serogroups. But only the serogroup *V. cholerae* O1 and O139 produce cholera toxin (CT) and have been associated with epidemic and pandemic cholera [2]. The other non-O1, non-O139 serogroups are usually associated with sporadic gastroenteritis [3].

Cholera is one of the major public health burdens with a high morbidity and mortality rate around the globe [4]. Every year millions of people die of this deadly disease. Though much efforts have been made to identify the actual number of cholera cases, 90% - 95% cases of cholera remain unnoticed due to insufficient surveillance system and poor socio-economic condition. According to World Health Organization (WHO), every year about 3 to 5 million positive cases of cholera are reported which represents only 5% - 10% of the actual number of cholera cases [5] [6] [7] [8].

Over the years, a number of cholera outbreaks were reported by WHO which cost millions of lives. In 2008-2009, Zimbabwe experienced 8 months long cholera outbreak with estimated 96,591 positive cases and 4201 deaths [9] [10]. In 2010, Nigeria faced epidemic form of cholera around the country costing 352 lives with 6400 positive cases [11]. After a long absence of cholera, Haiti experienced a deadly form of it in 2011 that killed 4533 people and made some 234,303 people sick [12]. In 2012, Sierra Leone fought against cholera but lost 290 lives in 21,500 positive cases [13]. African country Ghana experienced this deadly cholera outbreak for several times from 2011 to 2014. According to WHO in 2014, approximately 14,411 cases of cholera were found in Ghana and 127 died due to it [14] [15] [16]. Recently, cholera outbreak in Yemen is reported as one of the most severe cholera outbreaks in the history which surpassed about 200,000 positive cases with an increase of 5000 new cases each day [17]. Bangladesh is one of the most vulnerable countries of South East Asia for cholera outbreak. As reported by experts, every year an estimated 352,000 cholera cases are recorded in Bangladesh among which 3500 to 7000 death [18].

The purposes of present study were to isolate *V. cholerae* O1 from patients stool sample collected from a tertiary care hospital of Bangladesh and to deter-

mine their antimicrobial susceptibility towards locally available antibiotics to provide information to develop empirical treatment of cholera.

2. Material and Methods

2.1. Sample Collection and Processing

Present study was conducted in Microbiology laboratory of Primeasia University, Banani, Dhaka-1213. Total 140 stool samples were collected from patients with diarrheal symptoms admitted in a hospital located at middle Badda in Dhaka city of Bangladesh. Samples were collected during the month of July 2016 to December 2016. All the collected samples were transported inside an insulated foam box with ice bags to the laboratory by maintaining temperature within 4˚C to 6˚C to avoid contamination. Microbiological examination was done promptly to avoid undesirable change. Stool samples were directly inoculated onto MacConkey and Thiosulphate Citrate-Bile-Salt Sucrose (TCBS) agar and then incubated at 37˚C for 24 hours. Following incubation, colonies that produced lactose negative or slightly pink colors on MacConkey and yellow color on TCBS were initially suspected as *V. cholerae* and were isolated as pure culture by sub-culturing single colonies onto Nutrient agar.

A battery of biochemical tests was performed following the standard procedure for further confirmation of *V. cholerae* [19] [20]. These are Oxidase test, Catalase test, Gelatinase test, Kligler's iron agar (KIA) test, Motility indole urease (MIU) test, Fermentation of carbohydrates (glucose, inositol, mannitol, sucrose, mannose, arabinose), lysine and ornithine decarboxylase, arginine dihydrolase, and salt tolerance are tested using broths with 0%, 6.5% and 8% NaCl concentrations.

Serogroup of *V. cholerae* was determined by slide agglutination assay with *V. cholerae* O1 and O139 antisera. One drop of phosphate buffered saline (PBS) was added in microscope slide. A loopful of fresh bacterial culture grown on Nutrient agar was suspended into drop of PBS. A drop of equal sized of group O1 polyvalent antiserum was added to the drop. The antiserum-culture suspension was mixed by tilting the slide back and forth. Presence of agglutination within 1 minute indicated a positive result. Isolates those reacted with anti-O1 were further sub typed using antisera specific for Ogawa, Inaba and Hikojima strains.

2.2. Antibiotic Sensitivity Assay

Antimicrobial susceptibility testing was done using the Kirby-Bauer disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) recommendations [21]. Briefly, a single colony of *V. cholerae* O1 were lightly touched with a loop and inoculated in a tube containing 2 ml of Mueller Hinton broth and incubated for a few hours at 37˚C until the suspension became slightly turbid. Then the suspension was diluted with sterile saline to adjust with 0.5 MacFarland standard (3×10^8 CFU/ml). A sterile non toxic cotton swab was

dipped into the standardized suspension and streaked uniformly on Mueller Hinton agar plate after squeezing off extra fluid on the walls of the tube. The inoculated plate was allowed to dry for about five minutes and the appropriate antibiotic discs were then applied using sterile forceps and incubated overnight at 37˚C. The commercial antibiotic discs (HiMedia, Mumbai, India) used included: Kanamycin (30 µg), Imipenem (10 µg), Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Azithromycin (15 µg), Nitrofurantoin (300 µg), Gentamicin (10 µg), Tetracycline (30 µg), Ceftriaxone (30 µg), Doxycycline (30 µg), Amoxicillin (10 µg) and Nalidixic Acid (30 µg). After the incubation, the inhibition zone diameters were measured and interpreted by following the recommendations of the criteria of the Clinical and Laboratory Standards Institute (CLSI) [21]. *E. coli* (ATCC 25922) was used as control organism in our study.

3. Results

Among 140 stool samples, 58 *V. cholerae* were isolated. Based on serological confirmation, it was found that 43 (74.1%) isolates of *V. cholerae* were *V. cholerae* O1, serotype Ogawa and the rest 15 (25.9%) were serotype Inaba.

Significant difference was found in case of relationship between age and *V. cholerae* O1 infection. It was observed that the majority of people infected with *V. cholerae* O1 were between the ages of 41 - 50 years which was about 39.7% of all cases. People aged between 11 - 30 years were at least risk of infection having a percentage of only 1.7 (Table 1).

Antimicrobial susceptibility tests revealed that *V. cholerae* O1 showed 100% susceptibility to Gentamicin and Ciprofloxacin followed by 96.6% susceptibility to Kanamycin, 94.8% to Chloramphenicol and 91.4% susceptibility to Imipenem (Table 2). The isolated strains showed 82.8% susceptibility to Azythromycin and

Table 1. Antimicrobial susceptibility patterns of *V. cholerae* O1 isolated from stool sample.

Antibiotics name	Susceptibility (%)	Resistance (%)
Kanamycin	56 (96.6)	2 (4.4)
Imipenem	53 (91.4)	5 (8.6)
Ciprofloxacin	58 (100)	(0)
Chloramphenicol	55(94.8)	3 (5.2)
Azithromycin	48 (82.8)	10 (17.2)
Nitrofurantoin	33 (56.9)	25 (43.1)
Gentamicin	58 (100)	(0)
Tetracycline	41 (70.7)	17 (29.3)
Ceftriaxone	43 (74.1)	15 (25.9)
Doxycycline	48 (82.8)	10(17.2)
Amoxicillin	17 (29.3)	41 (70.7)
Nalidixic Acid	(0)	58 (100)

Table 2. Percentages of *V. cholerae* O1 positive patients according to age.

Age	Positive patients (%)
0 - 10	7 (12.1)
11 - 20	1 (1.7)
21 - 30	1 (1.7)
31 - 40	15 (25.9)
41 - 50	23 (39.7)
51 - 60	3 (5.2)
61 - 70	8 (13.8)

Doxycycline followed by 70.7% and 74.1% to Tetracycline and Ceftriaxone respectively. Besides, they showed least level of susceptibility to Nitrofurantoin (56.9%) and Amoxicillin (29.3%). All the isolates were found resistant to Nalidixic acid (Table 2).

4. Discussion

Cholera is an acute enteric disease that not only confined to developing countries but also spread throughout the world. This epidemic disease is still one of the major health concerns in many parts of Latin America, Africa and Southeast Asia [22].

Cholera is quite common in Bangladesh as seasonal outbreak of cholera is like annual occurrence in this country [23]. Though fluid and ORS (oral rehydration solution) are generally prescribed for the treatment of cholera, antibiotics are also needed in severe cases [24].

Effective use of antibiotics in diarrheal disease could potentially reduce the duration of diarrhea as well as excretion of pathogenic bacteria but may also increase the risk of generating multi drug resistant strains [25] [26].

Present study showed that, among 58 *V. cholerae* O1 isolates, 43 were of Ogawa serotype which was about 74.1%, whereas about 25.9% were of Inaba serotype. This result indicated the prevalence of *V. cholerae* O1 serotype Ogawa in the region of Bangladesh but disagreed with the findings of Pal *et al.* (2006) where they found the dominancy of *V. cholerae* O1 serotype Inaba which was about 66.07% in Orissa, India in 2005 [27]. Our study correlated the prevalence of *V. cholerae* O1 among diverse aged people. Middle aged people, age ranging from 31 - 50 years were mostly affected with *V. cholerae* O1, having 38 positive cases among 58 cases. Children and older people those are most vulnerable were at medium risk in our study which differs with the result of Adagbada *et al.* (2012) where they found less stomach acid producers like young children and older people are at great risk in having *V. cholerae* O1 mediated cholera [28].

In our study, all the isolates showed 100% sensitivity towards both Ciprofloxacin and Gentamicin which support the result of Ukaji *et al.* (2015) and Urassa *et al.* (2000) in case of Ciprofloxacin but Shukla *et al.* (2008) found opposite re-

sult from us where they reported that 100% of *V. cholerae* O1 were resistant against Ciprofloxacin in a study in East Delhi, India [26] [29] [30]. However, most of the isolates were sensitive to Kanamycin, Imipenem and Chloramphenicol which was about 96.6%, 91.4% and 94.8% respectively. This result corroborates the findings of Das *et al.* (2011) where they showed that 98.3% of *V. cholerae* O1 isolates were sensitive to Chloramphenicol [31]. Both Doxycycline and Azithromycin that are commonly recommended for the treatment guideline of cholera, showed 82.8% sensitivity in our study. This finding is quite similar with the result of Barati *et al.* (2015) where 84.7% sensitivity towards Doxycycline was observed in Alborz Province, Iran in 2011 [32]. Tetracycline which was accepted by Word Health Organization as drug of choice for cholera outbreak, in our study had 70.7% sensitivity, followed by Ceftriaxone of 74.1% sensitivity.

V. cholerae O1 expressed highest resistance against Amoxicillin and Nitrofurantoin after Nalidixic acid where they showed 70.7% and 43.1% resistance respectively. Resistance pattern against Amoxicillin is similar with the findings of Murhekar *et al.* (2013), where 75.8% of *V. cholerae* isolates were resistant against Amoxicillin [33]. Only Nalidixic acid was found to be 100% resistant by all the isolates of *V. cholerae* O1 which ultimately supports the result of Rahbar *et al.* in 2007 [34]. Resistance to Nalidixic acid might be due to over use to human and animal feed, spontaneous mutation in *V. cholerae*, and transfer of resistance genes between gut coliforms or other co-existing microflora and *Vibrio* spp. [35] [36].

Our study had some inherent limitations. All the samples were collected from a specific hospital and the number of samples are quite small. If a bigger amount of samples could be collected then the outcome would be more clear and vivid. This study also lacks molecular data that could support our result more strongly.

5. Conclusion

Our study revealed that, prevalence of Ogawa serotype of *V. cholerae* O1 was higher than Inaba serotype among cholera cases and people aged between 41 - 50 years were found more susceptible to cholera. From this study, it can be concluded that Nalidixic acid and Amoxicillin should be avoided empirically for treating cholera. Moreover, Ciprofloxacin and Gentamicin would be a better option for the treatment of cholera.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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