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Original Article

Evaluation of Direct Plating and Enrichment Methods for Isolation of *Vibrio cholerae* O139 from Faecal Samples

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In this study, direct-plate culture method alone, or in combination with enrichment culture for isolation of *Vibrio cholerae* O139 from faecal samples at a Diarrhoea Treatment Centre in Dhaka, Bangladesh had been evaluated. Faecal samples of 528 patients with acute phase of diarrhoea, attended the Centre, were cultured directly onto thiosulfate-citrate-bile-salts-sucrose agar (direct-TCBSA) and tellurite-taurocholate-gelatin agar (direct-TTGA) and after 6-h and overnight enrichment in alkaline-bile-peptone-broth for *V. cholerae* O139 (ABPB) [6h-ABPB-TCBSA and 6h-ABPB-TTGA, and overnight-ABPB-TCBSA and overnight-ABPB-TTGA]. Direct-TTGA-plating and overnight-ABPB-TTGA methods (latter for samples yielding negative results by overnight-direct-TTGA cultures) were also performed for another 18,647 faecal samples submitted for culture in our laboratory and 1,552 rectal swabs transported in Cary-Blair medium during investigation of acute diarrhoea outbreaks in the country. During acute phase of diarrhoea, direct-TCBSA, or direct-TTGA cultures equally supported O139 growth, while direct-plating plus 6-h, or overnight enrichment cultures were not superior as compared to the direct-plating. Direct-TTGA-plating plus overnight-ABPB-TTGA increased O139 isolation from transported rectal swabs. Thus, direct-plating provides optimum stool culture results for *V. cholerae* O139 during acute phase of diarrhoea. However, a combination of direct-TTGA-plating and overnight-ABPB-TTGA cultures was useful for isolation of *V. cholerae* O139 from transported rectal swab samples.

Keywords: Cholera, *Vibrio cholerae* O139, enrichment cultures, direct-plate culture

Introduction

Cholera has re-emerged recently as a major public health problem with significant morbidity and mortality in many developing countries of the world imposing a health threat to nearly all countries[1-4]. Since 1990, an unhindered spread of the current seventh cholera pandemic caused by *Vibrio cholerae* O1 El Tor Ogawa has been observed. Cholera has also been reported to World Health Organization (WHO) implicating new countries and continents than in the previous decade[4-5]. The situation has worsened since 1992 with the emergence and rapid spread of newly detected second aetiological agent of cholera designated as *V. cholerae* O139 in the Indian subcontinent[6-8] and adjacent countries such as Pakistan, Nepal, China, Thailand, Kazakhstan, Afghanistan and Malaysia[8]. The new epidemic strain infected many people and caused explosive outbreaks along Bay of Bengal replacing serotype O1 in many areas[6-7,9]. More than 150,000 cases were reported from India and Bangladesh between October 1992 and January 1994 with over 1,000 fatalities only in Bangladesh[6-7].

Currently, *V. cholerae* O139 has now become an endemic cholera strain in the Indian subcontinent like O1 El Tor[6,8-13]. In 1999, serogroup O139 accounted for approximately 17% of laboratory-confirmed cholera cases in Asian countries in which the disease is endemic[4]. The number of cholera cases caused by O139 is increasing as epidemics of O139 cholera continue to occur more frequently in recent years in the Indian subcontinent indicating a shift in trends in the outbreak propensity of O139[8,14]. In July 2002, cholera outbreak caused by *V. cholerae* O139 was detected in Karachi, Pakistan[15] and in March 2005, *V. cholerae* O139 emerged as the sole cause of a significant outbreak of cholera in southern costal area of Bangladesh[16]. It is now obvious that O139 caused more outbreaks and spread more rapidly during the first twelve years of its emergence compared to O1 El Tor during the same period of time since its emergence in 1906[1,5, 9,11]. The rapid spread of *V. cholerae* O139 to the Indian subcontinent and neighbouring Asian countries, the detection of cases in the United States, the United Kingdom, Germany and Japan, and the repeated outbreaks in India, Bangladesh and other countries raised concern that the new epidemic strain O139 could become a global threat by causing eighth pandemic starting its journey from the Indian subcontinent[4,6,11,14]. Thus, efforts to monitor the spread of O139 at a global level, particularly in high-risk areas, need to be strengthened by isolation and surveillance of this new potential pandemic strain of cholera by appropriate laboratory methods.

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Though, O139 differs from O1 in many ways, laboratory protocols for the isolation and identification of *V. cholerae* O1 are adopted by WHO and Centre for Disease Control and Prevention (Atlanta, GA, USA) for the detection of *V. cholerae* O139 from clinical samples and widely practiced by microbiology laboratory[7,17]. However, it has never been systematically evaluated by studies[7,17].

Recent reports of trimethoprim-sulfamethoxazole-susceptible and pteridine (O/129)-susceptible *V. cholerae* O139 (in contrast to trimethoprim-sulfamethoxazole-resistant *V. cholerae* O139 detected in 1992 and pteridine-resistant O1 El Tor), and sucrose-negative O139 isolates further stress the importance of evaluation of laboratory protocol for optimum isolation of O139 from faecal samples[10-11,18]. Direct plating technique on thiosulfate-citrate-bile-salts-sucrose agar (TCBSA) or tellurite-taurocholate-gelatin agar (TTGA) in combination with enrichment in appropriate broth is recommended by the WHO and commonly used for maximum recovery of vibrios from clinical samples[2,19]. Thus, we compared direct-TCBSA plating with direct-TTGA alone, or in combination with subculture results of 6-h and/or overnight enrichment in alkaline bile peptone broth (ABPB) onto TCBSA and TTGA plates for recovering *V. cholerae* O139 from faecal samples during acute phase of diarrhoea[20]. As TCBSA and TTGA equally supported O139 growth, the direct-TTGA plating and overnight enrichment for isolation of O139 from faecal samples, ignoring consistency (diarrhoeal, or non-diarrhoeal stools) that were submitted for culture to Dhaka Clinical Microbiology Laboratory, ICDDR,B, and rectal swabs that were collected from patients with acute diarrhoea during outbreaks in the community and transported in Cary-Blair medium, were evaluated in this study.

Materials and Methods

Study areas and population

The study was carried out with faecal samples collected in 1993-1994 during first cholera epidemic caused by *V. cholerae* O139 among three different group of study population with diarrhoea in ICDDRB: Centre for Health and Population Research, Dhaka, Bangladesh. The first group included 528 consecutive patients with acute phase of diarrhoea, who attended the Dhaka hospital for treatment during the last 6 months of 1993. The second group included 18,647 patients who submitted faecal samples (excluding 528 samples of the first batch), diarrhoeal and non-diarrhoeal, for culture to Dhaka Clinical Microbiology Laboratory between July 1993 and June 1994. The third group included 1,552 patients of diarrhoea outbreaks in different parts of the country who provided rectal swabs that were transported in Cary-Blair medium to Dhaka clinical microbiology laboratory within 3 to 5 days of collection by the physicians during 1993-1994.

Collection of faecal samples and bacteriological analyses

Faecal samples were collected from 3 different groups of study populations and cultured for isolation of *V. cholerae* O139. In the first group, faecal samples were inoculated directly onto TCBSA and TTGA. It was also inoculated into a tube containing 3 ml of ABPB (1% NaCl, 1% peptone, 0.5% sodium taurocholate, pH 8.8)[21] and incubated aerobically at 37°C, and was sub-cultured onto TTGA and TCBSA plates after 6 h (6-h-ABPB-TTGA or TCBSA), and overnight (overnight-TTGA, or TCBSA) incubation. All TTGA and TCBSA plates were incubated aerobically overnight at 37°C. Colony morphology resembling *V. cholerae* (halo with or without a black centre) on TTGA were identified as *V. cholerae* O1 or O139 by gelatinase production, positive oxidase test and slide agglutination using *V. cholerae* O1 and O139 specific antisera[2,7,10,14,21]. Yellow colonies from TCBSA were sub-cultured onto gelatin agar plate and colonies with surrounding halo zones were identified as *V. cholerae* O1, or O139 as described earlier[7,14].

In the second group, faecal samples were inoculated directly onto TTGA and, thereafter into 3 ml of ABPB. Cultures were incubated aerobically overnight at 37°C. If colonies of *Vibrio* were not present on TTGA plate, the ABPB enrichment cultures were plated on one half of a TTGA plate and incubated overnight aerobically at 37°C. Suspected *Vibrio* colonies were identified by colony morphology, positive oxidase test, conventional serological and biochemical, and salt tolerance tests[2,7,10,14,17].

In the third group, rectal swabs were streaked directly onto TTGA plate and thereafter inoculated in to 3 ml ABPB for enrichment and incubated at 37°C for overnight aerobically. Then the laboratory procedure of the second batch was followed to isolate and identify *V. cholerae* O1, 139 and other vibrios from swab samples.

Statistical analyses

Statistical comparisons were carried out by Chi-square test.

Results

Table 1 showed that *V. cholerae* O139 was detected from 112 of 528 (21.2%) faecal samples from patients with acute diarrhoea. Both direct-TTGA and direct-TCBSA plating methods identified O139 in each of 107 of 112 (95.5%) positive samples. 6-ABPB-TTGA, or 6-h-ABPB-TCBSA provided almost the same isolation rate of O139 as that of overnight-ABPB-TTGA, or overnight-ABPB-TCBSA ($p > 0.05$). The combinations of direct-TTGA and 6-h-ABPB-TTGA, or overnight-ABPB-TTGA marginally increased the isolation of O139 compared to direct-TTGA alone ($p > 0.05$). Similar results were obtained with TCBSA. The combination of all three methods (direct-TTGA, 6-h-ABPB-TTGA and overnight-ABPB-TTGA, or direct-TCBSA, 6-h-ABPB-TCBSA and overnight-ABPB-TCBSA) was not better than any two combined methods (Table 1). With TTGA, all three methods yielded O139 in 102 of 112 (91.1%) positive samples. However, direct-TTGA, 6-h-ABPB-TTGA and overnight-ABPB-TTGA increased the number of O139 isolation by 5, 7 and 4 respectively (data not shown). With TCBSA, all three methods were positive for O139 in 96 of 112 positive (85.7%) samples and direct-TCBSA, 6-h-ABPB-TCBSA and overnight-ABPB-TCBSA increased isolation number by 11, 12 and 8 isolates. A total of 91 (17.2%) *V. cholerae* O1 was isolated from 528 diarrhoeal faecal samples.

Table 1. *Results of direct-plating and enrichment* cultures for Vibrio cholerae O139 and O1 isolation from faecal samples collected during acute phase of diarrhoea (n = 528)*

	Culture method	Isolation rate, No. (%)	
		V. cholerae O139	*V. cholerae* O1
A.	Direct-TTGA	107 (20.3)	86 (16.3)
	6-h-ABPB-TTGA	109 (20.6)	86 (16.3)
	Overnight-ABPB-TTGA	106 (20.1)	84 (15.9)
	Direct-TTGA + 6-h-ABPB-TTGA	109 (20.6)	91 (17.2)
	Direct-TTGA + Overnight-ABPB-TTGA	111 (21.0)	91 (17.2)
	Direct-TTGA + 6-h-ABPB-TTGA + Overnight-ABPB-TTGA	111 (21.0)	91 (17.2)
B.	Direct-TCBSA	107 (20.3)	84 (15.9)
	6-h-APBB-TCBSA	108 (20.4)	85 (15.5)
	Overnight-ABPB-TCBSA	104 (19.7)	84 (15.9)
	Direct-TCBSA + 6 h-ABPB-TCBSA	112 (21.2)	89 (16.8)
	Direct-TCBSA + Overnight ABP-TCBSA	111 (21.0)	90 (17.0)
	Direct-TCBSA + 6-h-ABPB-TCBSA + Overnight-ABPB-TCBSA	112 (21.2)	90 (17.0)

*Enrichment broths were sub-cultured onto TTGA and TCBSA at 6 h and overnight incubation. TTGA = Tellurite-taurocholate-gelatin agar, ABPB = Alkaline-bile-peptone broth, TCBSA = Thiosulfate-citrate-bile-salts-sucrose agar

Table 2 showed that direct-TTGA plating detected O139 in 1,111 of 1,197 (92.8%) positive samples detected by combination of direct-TTGA and overnight-ABPB-TTGA cultures. Overnight-ABPB-TTGA method isolated additional 86 (7.2% of 1,197) O139 from 16,247 direct-TTGA negative faecal samples. The effect of enrichment of faecal samples in ABPB was similar for *V. cholerae* O1 where 120 (10% of 1,214) additional isolates were detected from direct-TTGA negative samples. The overnight-ABPB-TTGA also increased isolation of *V. cholerae* non-O1, non-O139 serogroups and other diarrhoeagenic *Vibrio* species.

In case of rectal swabs from acute diarrhoeal patients that were transported in Cary-Blair transport medium from the remote area, overnight-ABPB-TTGA method detected additional 45 *V. cholerae* O139 (14% of 321 positive samples) from 1,040 rectal swabs (Table 3) that were negative by direct-TTGA cultures showing the importance of pre-culture enrichment. Similar results were also obtained by overnight-ABPB-TTGA for O1 isolation from transported rectal swabs.

Table 2. *Culture results of faecal samples by direct tellurite-taurocholate-gelatin agar (TTGA) plating and subculture of overnight alkaline-bile-peptone broth (ABPB) cultures on to TTGA for Vibrio cholerae O139 and other vibrios*

Vibrio isolate	No. (%) of isolates (n = 2,620)	No. (%) of isolates by group/species identified by	
		Direct-TTGA cultures (n = 18,647)	Overnight-ABPB-TTGA cultures[a] (n = 16,247)
All isolates	2,620 (100.0)	2,364 (90.2)	256 (9.8)
V. cholerae O139	1,197 (45.7)	1,111 (92.8)	86 (7.2)
V. cholerae O1	1,214 (46.3)	1,094 (90.0)	120 (10.0)
V. cholerae Non-O1, Non-O139	162 (6.2)	130 (80.0)	32 (20.0)
V. parahaemolyticus	24 (0.9)	15 (62.5)	9 (37.5)
V. fluvialis	12 (0.5)	7 (58.3)	5 (41.7)
V. mimicus	5 (0.2)	4 (80.0)	1 (20.0)
V. furnissi	4 (0.15)	2 (50.0)	2 (50.0)
V. damsela	2 (0.07)	1 (50.0)	1 (50.0)

[a]Overnight ABPB enrichment cultures were sub-cultured on to TTGA only when TTGA plates were negative for vibrios after overnight incubation at 37ºC.

Table 3. *Culture results of rectal swabs transported in Cary-Blair medium obtained by direct tellurite-taurocholate-gelatin agar (TTGA) plating and subculture of overnight alkaline-bile-peptone broth (ABPB) cultures onto TTGA for Vibrio cholerae O139 and other vibrios*

Vibrio	No. (%) of isolates (n = 602)	No. (%) of isolates by group/species identified by	
		Direct-TTGA cultures (n = 1,552)	Overnight-ABPB-TTGA cultures[a] (n = 1,040)
All isolates	602 (100.0)	512 (85.0)	90 (15.0)
V. cholerae O139	321 (53.3)	276 (86.0)	45 (14.0)
V. cholerae O1	229 (38.0)	197 (86.0)	32 (14.0)
V. cholerae Non-O1, Non-O139	20 (3.3)	16 (80.0)	4 (20.0)
V. fluvialis	30 (5.0)	21 (70.0)	9 (30.0)
V. parahaemolyticus	2 (0.3)	2 (100.0)	0 (0.0)

[a]Overnight ABPB enrichment cultures were sub-cultured on to TTGA only when TTGA plates were negative for vibrios after overnight incubation at 37ºC.

Discussion

The findings of the present study suggest that both direct-TCBSA and direct-TTGA have good utility for isolation of *V. cholerae* O139 from faecal samples during acute phase of diarrhoea. An enrichment of faecal samples for 6 h, or overnight in ABPB before culturing on to selective agar media recommended by the WHO, such as TCBSA, or TTGA, did not offer significant benefit over direct plating for the isolation of this pathogen during acute phase. Fennels *et al*.[24] reported good sensitivity of direct-TCBSA for isolating *V. cholerae* from acute diarrhoeal stool specimens where an enrichment broth detected an additional 4% of all *V. cholerae* isolates. In this study, similar results were obtained for the isolation of *V. cholerae* O139 from faecal samples collected during acute phase of diarrhoea by direct-TCBSA, or direct-TTGA plating and ABPB enrichment cultures. However, when consistency of stool (diarrhoeal, or non-diarrhoeal) was ignored and the faecal samples were routinely enriched overnight in ABPB before inoculation onto selective agar, such as TTGA (overnight-ABPB-TTGA) in addition to direct-TTGA plating, the combination of two methods was found useful for maximizing the recovery of O139 from faecal samples (diarrhoeal and non-diarrhoeal) cultured. More importantly, it observed that direct-TTGA culture appeared less effective for isolation of O139 from transported rectal swabs collected during acute phase of diarrhoea where overnight-ABPB enrichment was essential for optimum isolation offering increased case detection (14%). These results are similar to those who evaluated the utility of alkaline peptone water enrichment for isolating *V. cholerae* serogroups, other than O139, from rectal swabs of diarrhoeal patients transported in transport media and demonstrated that the combination of direct plating and pre-culture enrichment was superior to direct plating alone[19-20].

The type of enrichment broth and the duration of incubation of broth after inoculation of faecal samples have been mentioned as important contributing factors in isolation efficiency of *V. cholerae*. ABPB as enrichment broth for the isolation of O139 was used in this study since it was reported to offer better results for O1 than alkaline peptone water[20]. Also, many investigators have expressed concern that alkaline peptone water enrichment for more than 6 to 8 h may promote overgrowth by competing organisms[14,19-22]. For overnight ABPB enrichment, overgrowth was not a problem in this study as was also reported by others[20]. In this study, the comparison of alkaline peptone water with ABPB for isolation of O139 from faecal samples was not done. Further work is necessary for evaluation of enrichment broths.

V. cholerae O139 is an organism with great epidemic potential like serogroup O1[7-8,11,13]. Thus, a laboratory procedure offering highest number of isolation from faecal samples should be adopted. Since the combination of direct plating (TTGA, or TCBSA) and 6-h-ABPB-TTGA, or 6-h-ABPB-TCBSA enrichment was similar to the combination of direct plating (TTGA, or TCBSA) and overnight-ABPB-TTGA, or overnight-ABPB-TCBSA for the isolation of both *V. cholerae* O139 and O1 serogroups during the acute phase of diarrhoea, therefore the later procedure has been practicing routinely in the Clinical Microbiology Laboratory using TTGA. This procedure has been found practical and rewarding for processing faecal samples in cholera endemic country like Bangladesh. Hence, the combination of direct-plating and overnight-ABPB enrichment followed by subculture of enrichment broth cultures onto a selective agar (such as TTGA that are negative by direct-plate culture, for maximum recovery of O139 from faecal samples, particularly in cholera endemic areas) is recommended. Spira and Ahmed[20] also found 18-h enrichment in alkaline bile peptone water before inoculation onto TTGA, an efficient method for isolation of *V. cholerae* O1 from contaminated surface water.

Both TTGA and TCBSA were found to have good utility and selectivity for isolation of *V. cholerae* O139. On TTGA, O139 colonies were relatively small (1 to 2 mm after overnight incubation and 3 to 4 mm after 48 h), translucent and circular, or occasionally lunette or irregular shaped because of bacteriophage infection with a cloudy zone surrounding colonies. Colonies develop dark centre usually after 24 h. TTGA was also found suitable for identifying and serogrouping of suspected *V. cholerae* isolates into O1, O139 and non-O1, non-O139 serogroups (serogroups O2 to O138, and O140 to O206) in 24 h by oxidase and serological tests, which was an advantage of TTGA. On TCBSA, *V. cholerae* O139 isolates produced large, yellow colonies. The frequency of sucrose-negative *V. cholerae* O139 on TCBSA (green colonies) does not appear to be common in Bangladesh and less than 1% of 112 isolates was sucrose-negative in this study. Earlier report on sucrose-negative strains might represent an unusual occurrence as suggested by this study and others[2,18,25].

The results of overnight-ABPB enrichment before inoculation onto selective agar and direct-plating, showed that a significant number of *V. cholerae* O139, O1 and non-O1, non-O139 serogroups and few other clinically important diarrhoeagenic *Vibrio* species were isolated only by overnight-ABPB enrichment of faecal samples. An overnight enrichment in ABPB increased the isolation of *V. parahaemolyticus* and *V. fluvialis* from faecal samples. Similar results were reported by Lesmana *et al*.[21] for isolation of *V. parahaemolyticus*.

In conclusion, faecal samples during acute phase of diarrhoea could be cultured directly on TTGA, or TCBSA alone for isolating *V. cholerae* O139. However, a combination of direct-plating on selective agar such as TTGA and overnight-ABPB enrichment before TTGA inoculation provides optimum results for transported rectal swabs from patients with acute diarrhoea. It might also maximize the overall isolation of *V. cholerae* O139 and other vibrios when all faecal samples are cultured by this method in a Clinical Microbiology Laboratory in a cholera endemic area.

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