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Detection of *Vibrio cholerae* O1 and O139 Serogroups Directly from Stool Specimens by Combined Immunomagnetic Separation and Polymerase Chain Reaction

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Abstract: Studies were carried out on monoclonal antibodies (MAbs) ICL12, ICL26 and ICL31, which had been produced against *Vibrio cholerae* O1 and O139. These MAbs of IgG isotypes were used for the development of the Immunomagnetic Separation together with Polymerase Chain Reaction (IMS-PCR). Using MAbs ICL12, ICL26 and ICL31, the IMS-PCR procedure detected *V. cholerae* in the pure cultures and in the stool samples. The detection limit of this method was 10^1 cfu mL⁻¹ for *V. cholerae* O139 while for the detection of *V. cholerae* O1 Ogawa it was 10^2 cfu mL⁻¹ and for *V. cholerae* O1 Inaba it was 10^3 cfu mL⁻¹. Of the 25 fresh stool samples tested, 15 were culture positive but 18 were IMS-PCR positive. Of the 55 frozen stool samples tested, 46 were culture positive but only 34 were IMS-PCR positive. However, when the 71 stool samples were frozen with Bovine Serum Albumin (BSA) added subsequently tested, 37 were culture positive while 49 were IMS-PCR positive. This showed that when stools were frozen with added BSA, the IMS-PCR procedure was much more specific and sensitive than the conventional culture method.

Key words: Monoclonal antibodies, immunomagnetic separation, polymerase chain reaction, *Vibrio cholerae*, serogroups

INTRODUCTION

Vibrio cholerae was discovered by Robert Koch in 1884^[1], which is a gram negative comma-shaped rod of 2-4 nm length. The human being is the only known host for *V. cholerae*. The species *Vibrio cholerae* is divided into different serogroups on the basis of the O-antigen polysaccharide^[2]. It is divided into more than 159 O-serogroups; however only the organisms of O1 and O139 serogroups are the most common causative agents of cholera in humans. The O1 serotype exists as two biotypes, classical and El Tor. *V. cholerae* O1 strains of both biotypes have further been subdivided into three serotypes, designated Inaba, Ogawa Hikojima according to the structure of O-antigens on the lipopolysaccharide (LPS). The three serotypes have in common antigenic determinant referred to as A antigen. In addition, there are two specific antigens, B and C, that are expressed to various degree on different serotypes; Inaba strains express only C, while Ogawa strains express both C and B but C in very reduced amount^[2-6]. The serotype Hikojima is rare and unstable and has been described as expressing all three antigens in high amount^[7].

The homogeneity of Mabs reduces the reaction time and there is less likelihood of non-specific cross-reactions occurring. A consequence of these advantages namely consistency, specificity and speed, there are lots of methods, such as the quick one step test, the dipstick test, the coagglutination assay^[8-10] the counter immunoelectrophoresis, the latex agglutination assay^[11] the Bengal SMARTTM^[12], the dark field microscopy^[13], the PCR^[14], the multiplex PCR^[14,15], the IMS-PCR^[16], the immunofluorescent staining^[17-19].

Due to the immuno-specificity of Mabs and sensitivity of PCR, the IMS-PCR procedure has already been used for the confirmed diagnosis of Johne's disease^[20] for the detection of *Salmonella typhimurium*^[21], *Cryptosporidium parvum* oocysts^[22], *Cryptosporidium parvum*^[23], *Shigella dysenteriae* type 1 and *Shigella flexneri* in feces^[24], *Chlamydomyxa abortus* in Ovine Milk^[25], *Mycobacterium avium* subsp. paratuberculosis in milk^[26,27], *Mycobacterium paratuberculosis* from milk^[28], *Enterocytozoon bienersi* Spores^[29], Enterotoxigenic *Bacteroides fragilis*^[30], intestinal spirochaetes *Brachyspira pilosicoli* and *Brachyspira hyodysenteriae* from porcine faeces^[31].

Several MAbs have been produced against the diarrheal pathogens, such as *V. cholerae* with an aim of using these for immunodiagnostic purposes^[14,15,32]. These MAbs have been useful for serotyping of different *V. cholerae* strains and their diagnosis.

MATERIALS AND METHODS

Bacterial strains: Strains of different serotypes of *V. cholerae* and other vibrios but non O1 and non O139 and strains of other enteric pathogens were from culture collection of the immunology laboratory of International Centre for Diarrheal Disease Research, Bangladesh.

The Female BALB/c mice (inbred) strains of 4 to 6 weeks were immunized with acetone treated extract of *V. cholerae* O1 and O139 at a weekly intervals for five times with Freund's adjuvant^[12]. Fusion of the sensitized spleenocytes and myeloma cells was carried out in FBS free medium using polyethyleneglycol 4000 (PEG4000). Fused cells were grown under Hypoxanthine-Aminopterin-Thymidine (HAT) selection followed by Hypoxanthine-Thymidine (HT) selection in a humidified atmosphere of 5% CO₂ in air. After day 21, HT was replaced by complete RPMI medium and continued onwards. Supernatant fluids harvested from hybridomas were tested in ELISA against acetone treated extract of *V. cholerae* O1 Inaba, O1 Ogawa O139 at weekly intervals. Ascites fluids were prepared by intraperitoneal injection of 1-2x10⁶ cloned cells per BALB/c mice that had been primed 10-14 days earlier with Pristane (2, 6, 10, 14-tetramethyl pentadecane). Mabs were characterized through various immunoassays such as Enzyme Linked Immunosorbent Assay (ELISA), Dot-Blot Assay, Immunostaining, FITC Immunostaining, Slide agglutination assay, Coagglutination assay Dark-Field Inhibition Assay.

Procedure followed for IMS-PCR^[16,30] was that 420 µL of Dynabeads Pan Mouse Immunoglobulin G (magnetic bead) for each of three samples *V. cholerae* O1 Ogawa Mab (ICL26), *V. cholerae* O1 Inaba Mab (ICL31) *V. cholerae* O139 Mab (ICL12)] were mixed separately at 4°C overnight with bi-directional shaking. Beads from three tubes were mixed together and washed once with PBS-Tween 20. Twelve hundred and sixty µL of PBS was added to the coated beads, mixed and kept at 4°C. Twenty µL of this coated beads was mixed with 250 µL of bacterial suspension or watery stool sample on rotatory shaker at 37° for 2 h. Bacteria attached to the coated beads were separated by Magnetic Particle Concentrator and washed once with PBS-Tween 20. Separated bacteria

with coated beads were incubated at 37° for 4 h at 100 rpm in LB broth.

Bacteria separated by centrifuging at 12,000 rpm for 5 min were resuspended in 50 µL of deionized water and boiled at 100° for 10 minute and 10 µL of this DNA template was used for PCR using the primers; *V. cholerae* O139-*rbf* specific primer; sense strand [5' AGC CTC TTT ATT ACG GGT GG 3 and antisense strand [5' GTC AAA CCC GAT CGT AAA GG 3'], *V. cholerae*.. O1-*rbf* specific primer: O1F2-1 sense strand, [5' GTT TCA CTG AAC AGA TGG G 3'] anitsense strand, [5' GGT CAT CTG TAA GTA CAA C 3'], the cholera toxin gene (*ctxA*) primer: VCT-1 sense strand [5' ACA GAG TGA GTA CTT TGA CC 3'] antisense strand [5' ATA CCA TCC ATA TAT TTG GGA G 3']. PCR protocol followed was with a denaturation temperature of 94° for 5 min for 1 once followed by a denaturation at 94° for 1 min, primer annealing at 55° for 1 min polymerization at 72° for 1 min following a total of 35 cycles an extension cycle at 72° for 7 min. Three percent agarose in TBE buffer was used in the gel electrophoresis for the detection of PCR product(s).

RESULTS

Cell fusion: Several fusions with sensitized spleenocytes were carried out. Within 10-14 days after fusion the growth of hybridomas was observed in more than 60% of the well of the microtitre plates. All positive hybridomas were cloned to have single colonies, expanded and again screened against the LPS of other enteric pathogens such as *S. dysenteriae*, *Aerumonas caviae*, *E. coli* etc. The reactive and specific hybridomas produced were then propagated in BALB/c mice (1-2x10⁶/mice) for the preparation of the ascites fluids. The culture supernatants and their respective ascites fluids were assessed against the LPS of different strains of *V. cholerae*, *S. dysenteriae*, *Aerumonas caviae* and *E. coli* etc. using the immunological techniques mentioned. Isotyping of the MAbs was carried out in ELISA (Table 1).

The IMS-PCR procedure: The IMS-PCR procedure was carried out to detect *V. cholerae* present both in the pure bacterial suspensions and in the watery stool samples. The experimental procedure showed that the IMS-PCR procedure could be able to detect the presence of *V. cholerae* O139 in the stool samples when it was present only at a concentration of 10 cfu mL⁻¹. The detection limits for *V. cholerae* O1 Ogawa and *V. cholerae* O1 Inaba were 10² and 10³ cfu mL⁻¹,

Table 1: Data obtained from the characterization of the monoclonal antibodies (MAbs)

MAb ID	Test name	Name of the antigen (s) (Strains or LPS)			Specificity	Isotype
		X-25049 (VC O1 Ogawa)	4260 B (VC O139)	4306 (VC O1 Inaba)		
ICL 12	Agglutination	-	+	-	<i>V. cholerae</i> O139	IgG2b
	ELISA titre	-	10116	-		
	Dot-blot	-	+	-		
	D/F* inhibition	-	+	-		
	Immunostaining	-	+	-		
	FITC-immunostaining	-	+	-		
	Coagglutination	-	+	-		
ICL18	Agglutination	+	-	+	<i>V. cholerae</i> O1 Ogawa and Inaba	IgM
	ELISA titre	45231	-	25161		
	Dot-blot	+	-	+		
	D/F* inhibition	+	-	+		
	Immunostaining	+	-	+		
	FITC-immunostaining	+	-	+		
	Coagglutination	+	-	+		
ICL26	Agglutination	+	-	-	<i>V. cholerae</i> O1 Ogawa	IgG2a
	ELISA titre	537195	-	-		
	Dot-blot	+	-	-		
	D/F* inhibition	+	-	-		
	Immunostaining	+	-	-		
	FITC-immunostaining	+	-	-		
	Coagglutination	+	-	-		
ICL 31	Agglutination	-	-	+	<i>V. cholerae</i> O1 Inaba	IgG3
	ELISA titre	-	-	6095		
	Dot-blot	-	-	+		
	D/F* inhibition	-	-	+		
	Immunostaining	-	-	+		
	FITC-immunostaining	-	-	+		
	Coagglutination	-	-	+		

* = D/F for Darkfield, + = for reactivity - = for no reaction

Table 2: A Comparison between the IMS-PCR and direct PCR for the detection of *V. cholerae* from diarrheal stools*

Methods	Total samples	Results		Specificity (%)	Sensitivity (%)	Positive predictive value
		Positive	Negative			
Culture	33	13	20	100	100	100
IMS-PCR	33	18	15	100	100	100
Direct PCR	33	13	20	100	100	100

* Stools were preserved with BSA (400 ng/Fl)

Table 3: Summary of the IMS-PCR procedure

Samples	Total	VC-O1 samples	VC-O139 samples	D/F result		Culture result		IMS-PCR result	
				(+) Ve	(-) Ve	(+) Ve	(-) Ve	(+) Ve	(-) Ve
Fresh	25	8	7	6	19	15	10	18	7
Frozen	55	36	10	10	45	46	9	34	21
Frozen with BSA	71	25	12	3	68	37	34	49	21
Total samples	151	69	29	19	132	98	53	101	50

respectively. The specificity of this procedure was found to 100, 80 and 100% for fresh, frozen and frozen with BSA added samples (Table 3).

A comparison of the IMS-PCR and the Direct PCR procedures showed that among the 33 samples tested, all 13 culture positive samples were also positive in Direct PCR but 18 were IMS-PCR positive (Table 2). On comparison the IMS-PCR procedure using the fresh and frozen stool from the same specimens, it was found that among the 8 specimens tested, 4 were culture positive but

5 were IMS-PCR positive on using fresh samples, whereas 4 were IMS-PCR positive while using only frozen samples. A comparison of the IMS-PCR procedure was also carried out on using frozen with added BSA and frozen without BSA of the same specimens; among the 20 samples compared 13 were culture positive. On using frozen samples 13 were IMS-PCR positive while using samples frozen with added BSA 19 were IMS-PCR positive. In addition, 6 samples that were culture negative were positive on IMS-PCR (Table 3).

DISCUSSION

The hybridomas ICL12, ICL26 ICL31 were with high specificity and sensitivity recognizing only the *V. cholerae* O139, *V. cholerae* O1 Ogawa and *V. cholerae* O1 Inaba, respectively. These specific MABs after characterization have been used as the diagnostic tool for the diagnosis of *V. cholerae* in stool specimens with finer specificity and sensitivity through immunomagnetic separation in combination with polymerase chain reaction (IMS-PCR). The method IMS-PCR instead of direct PCR was chosen for the detection of *V. cholerae* in stool specimens taking the advantage of the MABs that bind specifically only to the surface antigen(s) of specific type of bacteria and thus concentrating the bacteria in solution and enabling their separation. The beads used, the Dynabeads Pan Mouse IgG is designed for cell separation with any subclass of mouse IgG. The primary antibody of IgG class can bind the Dynabeads Pan Mouse IgG through Fc portion making the antigen-binding (Fab) portion available outwards. As a result if there is any respective bacteria present in the specimens it can be captured by the antibodies coated on the beads. Again one of the interesting advantages of this technique is that it can detect even the presence of dead bacteria in the stool samples showing negative results through the culture method (Fig. 1). PCR inhibitors present in the feces seem to affect the sensitivity of the direct PCR and the culture methods. This may be also responsible for false negative results. Therefore the procedure concerned with a combined immunomagnetic separation and polymerase chain reaction for the detection of *V. cholerae* with higher specificity and sensitivity. Mixture of coated beads (individually coated with ICL12, ICL26 ICL31) was used to increase the capability to capture any serotypes of *V. cholerae* present. With the aid of magnetic particle concentrator the captured *V. cholerae* strains were separated and other accessory substances that might be inhibitory to the PCR analysis were effectively removed. In this procedure, the *V. cholerae* O139 specific primers yielded a *rfb*-specific DNA amplicon of 449 bp and *ctxA* specific DNA amplicon of 308 bp from all pure culture of *V. cholerae* O139. The O1 specific primers yielded a *rfb* specific DNA amplicon of 192 bp and a *ctxA* specific DNA amplicon of 308 bp from all pure cultures of *V. cholerae* O1 Ogawa and *V. cholerae* O1 Inaba (Fig. 1). The *ctxA* specific DNA amplicon was common both to *V. cholerae* O1 and O139. No other bacterial cultures yielded such amplicons.

During the study period, the IMS was carried out from fresh diarrheal stool samples and PCR of the

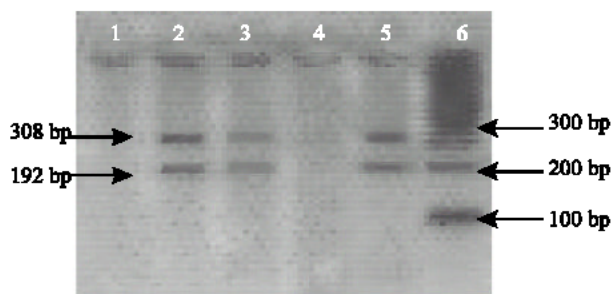


Fig. 1: Showing the detection of *V. cholerae* directly from stool by IMS-PCR. Lane 1 and 4 for culture negative samples, lane 2, 3 and 5 for culture positive samples and lane 6 for 100 bp DNA ladder

separated bacteria after 4 h incubation. It detected the presence of *V. cholerae* in the specimens with finer sensitivity, i.e. with a 100% positive predictive value. But it is not always possible to work with fresh samples. Thus attempts were taken for the diagnosis of *V. cholerae* in frozen (-70°C) samples by IMS-PCR. But the results obtained showed a degree of false negative prediction, which might be due to the presence of fully undefined inhibitors of growth and survival of *V. cholerae* in the stool specimens. To inhibit these undefined substances stools were preserved with bovine serum albumin (BSA) at a concentration of 400 ng μL^{-1} of stool. The IMS-PCR from these stool samples (frozen with added BSA) decreases the prediction of false negative results; rather some samples negative by culture method were positive by IMS-PCR. In order to evaluate the sensitivity and specificity of this method, study with a large number of fecal samples from patients and control strains were conducted. From this study it was the IMS-PCR procedure that was more sensitive than the gold-standard culture method. This IMS-PCR procedure could be applicable for the diagnosis of cholera directly from stool samples with higher degree of specificity and sensitivity than any other existing methods. It is very sensitive technique to be used for the detection of very low numbers of bacteria and for epidemiological studies in laboratories with limited facilities. Thus the IMS-PCR procedure uses the immunospecificity of the MABs and sensitivity of the PCR and becomes more specific and sensitive method than any other existing immunological assay methods.

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