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Coagglutination: A Rapid and Sensitive Assay Method for Detection of *Vibrio cholerae* O1 and O139 Serogroups Directly from Stool Specimens

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Abstract: Using monoclonal antibodies (MAbs), the coagglutination test detected *Vibrio cholerae* O1 and O139 serogroups both in the bacterial culture and in the stool specimens. Detection of *V. cholerae* O1 and O139 from the cultures of different *V. cholerae* O1 and O139 strains and non-O1 and non-O139 strains showed a specificity of 100%, a sensitivity of 100% and a 100% positive predictive value. Among the 146 stool samples tested by the coagglutination test, 36 were culture positive, but 33 were coagglutination test positive showing a specificity of about 92%. All the culture negative samples were negative in the coagglutination test indicating a specificity of 100%.

Key words: *Vibrio cholerae,* serogroups, serotypes, antibody, monoclonal, coagglutination

INTRODUCTION

V. 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* and infection occurs by faeco-oral route, usually via contaminating water or food and its infection is limited to the small intestine. The species *Vibrio cholerae* is dived 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 the 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 and 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]. Due to the devastating epidemic potential of *V. cholerae* rapid diagnosis and identification is specially important from the public health point of view. A devastating effect of such severely dehydrating diarrheal disease is the large decrease in the volume of extracellular fluids and electrolytes resulting in hypovolumic shock. Such hypovolumic shock can lead to death within 24 h if the infected persons remain untreated. To prevent or to control the spread of the disease, a surveillance program based on early and rapid detection is highly advantageous to coordinate implementation of treatment, education and sanitation measures. Additionally, due to the potential life-threatening nature of the disease, it is imperative to determine as quickly as possible, the causative agent from a patient exhibiting cholera-like disease.

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 a lot of methods, such as the quick one step test, the dipstick test, the coagglutination assay[8,9], the counterimmuno-electrophoresis, the latex agglutination assay[10] the Bengal SMART™[11], the darkfield microscopy[12], the PCR, the multiplex PCR[13,14], the IMS-PCR[15], the immunofluorescent staining[16,17], the ELISA, the ELISPOT, the immunostaining, the dot-blot assay, etc. for the diagnosis of enteric pathogens such as *V. cholerae, Bacteroids flagilis, Escherichia coli, Salmonella* spp. *Klebseilla pneumonae, Shigella dysenterae* and *Aeromonus caviae* etc.

All the rapid diagnostic tests mentioned require some sort of well-equipped laboratory. But in areas with minimal

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laboratory facilities, only the coagglutination assay to detect *V. cholerae* (also other pathogens) in faecal specimens is more applicable to assist the management of cases of severe diarrhea even without fully trained microbiology technicians.

MATERIALS AND METHODS

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 anti-*V. cholerae* O1 and O139 cell lines, ICL 12, ICL 17 and ICL 26 were produced, cloned and characterized in the Monoclonal Laboratory of the Immunology section of ICDDR,B during the study period. The mouse myeloma cell line, $SP_2O'^{92}$ was obtained from Sweden.

Culture of bacteria: Following standard procedures and maintaining sterile conditions throughout the process bacteria were grown in the laboratory. All media were sterilized in conical flasks plugged with non-absorbent cotton. Agar was used to store bacteria or for streaking the bacteria to identify single colonies. Single colonies were transferred to liquid media to obtain pure culture for the preparation bacterial suspension in normal saline (0.9% NaCl). All transferring steps of media and bacteria were performed under sterile conditions.

Production of monoclonal antibodies: The procedure followed for the production of monoclonal antibodies using the hybridoma technology included[18].

Immunization regimen: Female BALB/c mice (inbred) strains of 4-6 weeks were immunized with acetone treated extract of *V. cholerae* O1 and O139 at a weekly intervals for five times, first dose intravenously with Freund's complete adjuvant and others were intraperitonealy with Freund's incomplete adjuvant. Fusion was performed within 3-5 days after last dose administration (after booster dose).

Fusion protocol: The spleen and myeloma cells were mixed at a ratio of 4:1 in (-) FBS medium and their fusion was carried out using polyethyleneglycol4000 (PEG4000). Fused cells were then allowed to grow under Hypoxanthine-Aminopterine-Thymidine (HAT) selection followed by Hypoxanthine-Thymidine (HT) selection incubating in a humidified atmosphere of 5% CO_2 in air (CO_2 incubator). After day 21, HT was replaced by complete RPMI medium and continued onwards.

Screening and selection of hybridomas: Supernatant fluids harvested from hybridomas were tested in ELISA against acetone treated extract of *V. cholerae* O Inaba, O1 Ogawa and O139.

Preparation ascitic fluids: Ascites fluid were prepared by intraperitoneal injection of $1\text{-}2x10^6$ cloned cells per BALB/c mice that had been primed 10-14 days earlier of cell inoculation by Pristane (2, 6, 10, 14-tetramethyl pentadecane). Ascites were collected 8-10 days of injection of cell lines.

Characterization of ascites fluids: Immunoassays carried out for the characterization of monoclonal antibodies were Enzyme Linked Immunosorvent Assay (ELISA), Dot-Blot Assay, Immunostaining, FITC Immunostaining, Slide agglutination assay and Dark-Field Inhibition Assay (Table 1).

MAbs in the diagnosis of *V. cholerae*:

The coagglutination assay: The coagglutination assay for the detection of *V. cholerae* directly from the stool samples and from bacterial cultures according to the procedure described by Qadri *et al.*[18].

Preparation of test reagent: 200 µl of 10% *Staphylococcus aureus* suspension with 300 µl of PBS-azid, mixed well and centrifuged at 9000 rpm for 10 min. Pallets were resuspended in1 25 µl of PBS-azid add 100 µl of diluted antibodies (ICL 12 and ICL 17 at a dilution of 1:5 in PBS-azid), mixed well at room temperature on bi-directional shaker for 4 h and incubate overnight at 4°C. The Stap.-MAB complex was washed with PBS-azid at 8000 rpm for 10 minutes. Pellets were resuspended in 200 µl of PBS-azid and then the volume was made to 5.0 ml with PBS-azid. Centrifuged at 3000 rpm for 10 min at room temperature for 3 times. Pellets were resuspended in 200 µl of PBS-azid and kept at 4°C until use.

Coagglutination procedure: A small volume of the test reagent was placed on a clear microscope slide and same amount of the bacterial suspension or the stool sample to be tested was mixed well. Stirred and agglutination observed within one minute and noted.

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

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
ICL 12	Agglutination	-	+	-	*V. cholerae* O139	IgG2b
	ELISA titre	-	10116	-		
	Dot-blot	-	+	-		
	D/F inhibition*	-	+	-		
	Immunostaining	-	+	-		
	FITC-immunostaining	-	+	-		
	Coagglutination	-	+	-		
ICL 17	Agglutination	+	-	-	*V. cholerae* O1 Ogawa	IgG2a
	ELISA titre	51728	-	-		
	Dot-blot	+	-	-		
	D/F inhibition*	+	-	-		
	Immunostaining	+	-	-		
	FITC-immunostaining	+	-	-		
	Coagglutination	+	-	-		
ICL 26	Agglutination	+	-	-	*V. cholerae* O1 Ogawa	IgG2a
	ELISA titre	537195	-	-		
	Dot-blot	+	-	-		
	D/F inhibition*	+	-	-		
	Immunostaining	+	-	-		
	FITC-immunostaining	+	-	-		
	Coagglutination	+	-	-		

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

Table 2: Data obtained from the coagglutination assay

		Culture result		Coagglutination result			
Type of sample tested	Total sample	Positive	Negative	Positive	Negative	Specificity (%)	Sensitivity (%)
Culture of known biotypes/Serotypes	22	22	None	22	None	100	100
Culture of *V.cholerae* non-O1/O139 (reference strain)	146	None	146	None	146	100	100
Fresh stool	120	36	84	33	87	100	92

screened against the LPS of other enteric pathogens such as *S. dysenterae, Aerumonas caviae, E. coli,* etc.

Screening of hybridomas: Hybridomas showing the specificity only against the immunizing antigen were allowed to propagate further. Thereafter specificity of the hybridomas were also assessed using the agglutination, the immunostaining, the FITC-Immunostaining and the Quick Dot-Blot assays. The reactive and specific hybridomas produced were then propagated in BALB/c mice (1-2x10^6/mice) for the preparation of the ascites fluids. The tests described for the screening of hybridomas using the culture supernatant were also carried out for testing the specificity of the ascites fluids.

Specificity of the MAbs: The culture supernatants and their respective ascites fluids were assessed against the LPS of different strains of *V. cholerae, S. dysenterae, Aerumonas caviae, E. coli,* etc using the immunological techniques mentioned. Isotyping of the MAbs was carried out in ELISA using heavy chain class (IgG, IgA, IgM) and subclass (IgG1, IgG2a, IgG2b, IgG3, IgA1, IgA2) specific conjugates (Table 1).

Application of monoclonal antibodies and diagnosis of cholera:

The coagglutination assay: The coagglutination test was carried out to detect *V. cholerae* present both in the pure bacterial suspensions and in the watery stool samples. As control this test was also carried out with different *V. cholerae* strains and *V. cholerae* non-O1 and non-O139 strains (Table 2)

DISCUSSION

The present study showed that highly specific, reactive and stable monoclonal antibodies (MAbs) for use in the diagnostic or epidemiological studies could be produce, which could possibly replace the conventional antisera. In this study, the specific MAbs after characterization have been used as the diagnostic tool for the diagnosis of *V. cholerae* in stool specimens with finer specificity. The antibodies of IgG isotype were allowed to bind to the protein A of *Staphylococcus aureus.* Protein A with the bound Fc portion of IgG antibody providing the Fab portions available in to the outwards for the recognition of specific bacteria present in the specimens. Protein A is a quite bigger molecule and thus

agglutination of small number of bacteria with the IgG antibody coated on protein A causes a greater visualization of the precipitations.

The coagglutination assay is very rapid and straightforward immunoassay. There is an existing commercial rapid colorimetric immunoassay, the Bengal SMART™ for the detection of *V. cholerae* O139 in human feces. This Bengal SMART™ consistently detected suspensions containing at least $2x10^5$ cfu ml^{-1} of *V. cholerae* O139[11,19] and the reagent that are used are much more expensive in comparison to that used in the coagglutination assay, which was also shown to be highly specific, sensitive and rapid. The procedure of this study is much easier and the test reagent could be preserved for long time without affecting the activity at 4°C. In this assay system a diagnosis is available only within 2-5 min. A monoclonal antibody based coagglutination test for the detection of *V. cholerae* O139 directly from the watery stool samples had a 92% sensitivity, 100% specificity, 100% positive predictive value and 95% negative predictive value[11]. In the present study, the coagglutination assay was also used to detect for the detection of *V. scholerae* O1 and O139 in bacterial suspension and in watery stool specimens. From the culture of known bacterial strains (known culture of *V. cholerae* O1/O139 and non-O1/O139), this assay showed 100% sensitivity, 100% specificity, 100% positive predictive value and 100% negative predictive value. But with stool specimens, this assay showed 92% sensitivity, 100% specificity, 100% positive predictive value and a cross-reactivity of 8.9% and peculiarly with one of the fresh stool samples the assay gave a black precipitation. Among the 120 samples tested, 36 were culture positive and 33 of this were also coagglutination positive. It appears that further studies are needed for the modification of the procedure followed for the detection of *V. cholerae* directly from the stool samples. This assay can be used as a rapid test for the confirmation of the diagnosis cholera thus assisting clinicians in managing the disease. The other applications of this technique are as an inexpensive test, it could replace more expansive culture methods used in many diarrheal treatment and hospitals and as an aid of surveillance: it can assist epidemiologists for detecting *V. cholerae* in areas where bacteriological laboratories are not available. Finally, as a simple test, it can provide confirmation of cholera even without fully trained microbiology technicians.

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