Rapid detection of *V. cholerae* 01

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Summary

Monoclonal antibodies directed against group specific antigen A of *Vibrio cholerae* 01 were obtained through hybridoma technology which involved fusions of Sp 2/0 myeloma cells with splenocytes of Balb/c mice immunized with whole cell lysates of *V. cholerae* 01. Specificity and crossreactivity of the monoclonal antibodies were determined against whole cell lysates prepared from 38 strains of *V. cholerae* 01, six strains of *V. cholerae* non-01, five strains of other vibrios, 45 strains of enterobacteria and *Entamoeba histolytica* by indirect and dot-blot enzyme-linked immunosorbent assay (ELISA). The monoclonal antibodies (MAb) reacted specifically to the whole cell lysates of the *V. cholerae* serogroup 01 and did not react to the antigens prepared from other organisms. The MAb were used in a dot-blot ELISA for detecting *V. cholerae* 01 antigen in 335 seafood specimens and in stools and rectal swabs of patients with acute watery diarrhoea. The dot-blot ELISA performed on rectal swab specimens enriched in alkaline peptone water gave 96.0% sensitivity, 99.3% specificity, 94.7% positive predictive value, 99.5% negative predictive value and 98.9% efficacy, respectively, when compared to the conventional culture method. The two methods showed excellent agreement beyond chance, on the basis of a k coefficient value of 94.7%. No *V. cholerae* 01 was isolated from the 335 seafood samples and the dot-blot ELISA for *V. cholerae* 01 antigen was also negative, consistent with a high specificity of the assay. The dot-blot ELISA is easy to perform, relatively inexpensive, highly sensitive and specific. It permits multisample analysis at a single time, requires no special equipment and does not pose any disposal problem (compared with the culture method). Most of all, diagnosis of cholera cases could be made accurately within 1–3 h (the dot-blot ELISA takes 1 h, while the culture method takes 2 days). The method is recommended for rapid detection of *V. cholerae* 01 in contaminated foods, in environmental samples and in stools of diarrhoeic patients.

Key words: *V. cholerae* 01, cholera, rapid diagnosis, hybridomas, monoclonal antibodies, specific antigen, ELISA

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Introduction

Rapid detection of cholera is necessary for controlling an explosive outbreak which usually occurs within a day or two after a single unrecognized case. Cholera vibrios are commonly and rapidly disseminated by either a patient, convalescent carrier or individual with inapparent or mild infection whose faecal matter contaminates water supplies, food, or household contacts who are under poor personal hygiene[1,2].

Rapid detection of cholera could be performed by dropping the patient's watery stool on a glass slide and observing under dark-field illumination. Live *Vibrio cholerae* have a 'darting' movement characteristic which is inhibited by adding specific antiserum[3]. However, the procedure works well only in the hands of experienced personnel. In common practice, when a cholera case is suspected in an area remote from an available microbiology laboratory, a few drops of stool or rectal swab are put in a suitable transport medium,

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e.g. Cary-Blair medium. The *V. cholerae* organisms survive in this medium for several days[4]. The specimen is then sent to the laboratory for further processing, i.e. growing in alkaline-peptone water (APW) at 37° C for 6–8 h and culturing on specific media. The APW allows much more rapid growth of *V. cholerae* than of Enterobacteriaceae. After 6–8 h, smears of the culture can be stained with fluorescein-labelled specific *V. cholerae* antiserum for quick presumptive identification. The requirements of an expensive fluorescence microscope and fluorescein-antibody conjugate limit the method from widespread use, especially in the field in underdeveloped or developing areas where the health problem due to cholera is real. The 6–8 h culture in APW or the diarrhoeic stool may be streaked on thiosulphate-citrate bile salt sucrose (TCBS) agar or taurocholate gelatin agar (TGA). The colonies of *V. cholerae* on TCBS are yellow. At this stage, smears may be stained by Gram stain and biochemical reactions are performed. *V. cholerae* are gram-negative, curved rods that are oxidase positive and give rise to acid butts/acid slants in TSI agar. They are lysine and ornithine decarboxylase positive. For final identification, the organisms in typical colonies are subjected to an agglutination test against *V. cholerae* polyvalent 01 antiserum[5]. The whole process of *V. cholerae* isolation and identification takes, at a minimum, 2 days and by that time the disease might have spread explosively. Therefore rapid detection of cholera is needed for controlling the disease.

In this report, a rapid, simple, specific and sensitive monoclonal antibody-based dot-blot enzyme-linked immunosorbent assay (ELISA) has been developed using specific monoclonal antibody directed against *V. cholerae* 01 antigen A to detect this antigen in stools of patients with watery diarrhoea and in various kinds of seafoods.

Materials and methods

Preparation of antigens

Lipopolysaccharides (LPS) were prepared from *V. cholerae* El Tor Ogawa 295/33 and Inaba 92/34 which were isolated from cholera patients and from a laboratory strain, Ogawa 17SR. The phenol-water extraction method of Westphal and Jann[6] was followed throughout. The single extracted LPS were re-extracted two more times until no proteins could be detected by Lowry's method[7].

Whole cell lysates (Ly) were prepared from the organisms listed in Table 1. Bacterial cells obtained from growth at 37° C for 4 h in trypticase soy broth (TSB) were lysed in distilled water to give an optical density (OD) of 2.0 at 540 nm. The preparations were subjected to ultrasonication at 20 kHz for 10 min. Dry weights and protein contents of all preparations were determined. Extract of *Entamoeba histolytica* trophozoites was kindly provided by Assistant Professor W Maleewong, Department of Medicine, Khon Kaen University, Thailand. All of the Ly and the *E. histolytica* extract were used for specificity and crossreactivity checking of the monoclonal antibodies.

Log phase cultures of *V. cholerae* 01 were obtained by inoculating a loopful of the organisms from an overnight culture plate into 10 ml of alkaline tryptone broth and incubating at 37° C for 4 h in a shaking incubator. Viable counts were performed by dilution plating.

Immunization of mice

Young adult Balb/c mice (2–4 months old) were kindly supplied by the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok. Mice were divided into four groups of four to eight mice in each group. Each mouse was immunized intraperitoneally with 100 µl of 70 µg of Ly (Ogawa 17SR for group 1; Ogawa 295/33 and Inaba 92/34 for group 2; Ogawa 17SR and Inaba 92/34 for group 3; and Inaba 92/34 for group 4, respectively) emulsified in 100 µl of complete Freund's adjuvant (CFA).They were reimmunized 14 days later by the same route and the same amount of the respective antigens but the incomplete Freund's adjuvant (IFA) was used in the booster dose. Two weeks after the second injection, each mouse was bled to collect serum for antibody assessment by an indirect ELISA[8] against the homologous Ly and LPS. Mice showing high antibody titres were chosen as spleen cell donors for hybridoma production. Three days before collecting their splenocytes, they were given an intravenous booster with 35 µg of the respective antigens in 200 µl of normal saline solution (NSS).

Hybridoma technology

Before each mouse was killed by cervical dislocation, the animal was bled via the retro-orbital plexus. The serum was collected and used as positive control (immune mouse serum; IS). The spleen of the mouse was removed aseptically, washed in three changes of 5 ml RPMI 1640 medium, then placed on a fine wiremesh submerged in the RPMI 1640 medium. The spleen was homogenized with a sterile syringe plunger. Single spleen cells were collected in RPMI 1640 medium and washed twice by centrifugation at 200 × ***g*** for 10 min at room temperature. Viability of the cells was checked by the trypan blue exclusion method. These immune spleen cells were fused with Sp 2/0 myeloma cells by using polyethylene glycol 3350 as a fusogen as previously described[8,9]. The fused cells were suspended in hypoxanthine-azaserine selective medium and approximately 2.5×10^5 cells were distributed in 200 µl aliquots into wells of 96-well tissue culture plates. All plates were placed in a humidified 5% CO_2 incubator at 37° C. The old culture medium in wells was replaced with the fresh culture medium at an appropriate time. Supernates from wells containing growing hybrids were collected and screened for antibodies against Ly of *V. cholerae* Ogawa 295/33 and Inaba 92/34. Cells from the positive wells were

Table 1. Bacterial strains and *Entamoeba histolytica* used for preparing whole cell lysates (Ly)

1. *Vc* Classical 569B Inaba
2. *Vc* El Tor Ogawa 241/33
3. *Vc* Ogawa 17SR
4. *Vc* Ogawa 295/33
5. *Vc* K23/7
6. *Vc* Ogawa 339/33
7. *Vc* Ogawa 231/33
8. *Vc* Ogawa (laboratory strain)
9. *Vc* Ogawa 54/35
10. *Vc* Ogawa 58/35
11. *Vc* Ogawa 61/35
12. *Vc* Ogawa 263/35
13. *Vc* Ogawa 90/35
14. *Vc* Ogawa 285/35
15. *Vc* Ogawa 286/35
16. *Vc* Ogawa 138/35
17. *Vc* Ogawa 216/35
18. *Vc* Ogawa 117/35
19. *Vc* Ogawa 287/35
20. *Vc* Ogawa 215/35
21. *Vc* Ogawa 29/34
22. *Vc* Ogawa (laboratory strain)
23. *Vc* Ogawa (laboratory strain)
24. *Vc* Ogawa (laboratory strain)
25. *Vc* Ogawa 1228/34
26. *Vc* Ogawa 339/35
27. *Vc* Inaba 316/33
28. *Vc* Inaba 257/33
29. *Vc* Inaba (laboratory strain)
30. *Vc* Inaba (laboratory strain)
31. *Vc* Inaba 213/35
32. *Vc* Inaba 1271/34
33. *Vc* Inaba 1018/34
34. *Vc* Inaba 609/34
35. *Vc* Inaba 35/35
36. *Vc* Inaba 92/35
37. *Vc* Inaba 1856/34
38. *Vc* Inaba 1019/34
39. *Vc* non 01 63/33
40. *Vc* non 01 48/33
41. *Vc* non 01 46/33
42. *Vc* non 01 Heiberg group I
43. *Vc* non 01 Heiberg group II
44. *Vc* non 01 Heiberg group III
45. *Vibrio parahaemolyticus*
46. *V. furnissii*
47. *V. alginolyticus*
48. *V. anguillarum*
49. *V. fluvialis*
50. *Aeromonas hydrophila*
51. *A. sobria* 82/36
52. *A. caviae* 173/36
53. *Plesiomonas shigelloides*
54. *Hafnia alvei*
55. *Salmonella typhi* 0901
56. *S. paratyphi* A
57. *S. paratyphi* B
58. *S. paratyphi* C
59. *S. typhimurium*
60. *S. blockley*
61. *S. weltevreden*
62. *S. montevideo*
63. *S. agona*
64. *S. panama* 01
65. *S. panama* E4
66. *S. cholerae-suis*
67. *Shigella dysenteriae*
68. *Sh. flexneri*
69. *Sh. sonnei*
70. *Sh. boydii*
71. *Edwardsiella tarda*
72. *Yersinia enterocolitica*
73. *Serratia marcescens*
74. *Escherichia coli* 54/32
75. *E. coli* 324/34 0:125
76. *E. coli* 320/31 0:44
77. Enteroinvasive *E. coli* 1277/33
78. *E. coli* 305/33, ST^+
79. *E. coli* 461/31 0:28
80. *E. coli* 78/32 LT^+
81. *E. coli* 350/31 0:86
82. Enteroinvasive *E. coli* (laboratory strain)
83. *Proteus vulgaris*
84. *P. mirabilis*
85. *Providencia stuartii*
86. *Enterobacter cloacae*
87. *Enterobacter aerogenes*
88. *Pseudomonas aeruginosa*
89. *Citrobacter freundii*
90. *C. diversus*
91. *Klebsiella pneumoniae*
92. *Providencia rettgeri*
93. *Morganella morganii*
94. Campylobacter spp. (10 isolates)
95. *Entamoeba histolytica*

Vc = Vibrio cholerae.

subjected to cloning by the limiting dilution method. Supernates from these clones (hybrid clones) were retested for antibody activities by the indirect ELISA[8] and for antigenic specificities by Western blot analysis against *V. cholerae* Ly. Crossreactivity with heterologous Ly and *E. histolytica* extract was also determined by indirect ELISA and dot-blot ELISA. Hybrid clones which produced antibodies specific to *V. cholerae* 01 antigens were expanded in the *in vitro* culture for bulk production of the specific monoclonal antibodies.

Indirect ELISA

This method was used for determining antibody titres of sera from immunized mice (IS) and cell cultured fluids in order to screen for hybrids producing *V. cholerae* 01 specific antibodies. The technique was also used for determining specificity vs crossreactivity of the monoclonal antibodies. The microtitre plates were coated with 10 μg ml^{-1} of Ly of the organisms listed in Table 1 or LPS of *V. cholerae* Ogawa 295/33, Ogawa 17SR or Inaba 92/34 in carbonate-bicarbonate buffer, pH 9.6. The sensitized plates were incubated at 37° C in a humidified chamber for 2 h and at 4° C overnight. The unbound antigens were washed off using phosphate buffered saline (PBS) containing 0.5% Tween 20 (PBST). The empty sites on plates were blocked with 1% bovine serum albumin (BSA) in PBS and incubated at 37° C for 1 h. The excess BSA was washed off and 100 μl of serially diluted antibody

preparation (IS, cell cultured fluid or fresh culture medium which served as a blank) were placed into an appropriate well. The antigen-antibody reaction was allowed to take place for 1 h at 37° C. After washing thoroughly with PBST, 100 µl of 1 : 1000 dilution of rabbit anti-mouse immunoglobulin horseradish peroxidase conjugate (Dakopatts, Denmark) in PBS containing 0.2% BSA and 0.2% gelatin was added to each well and incubated at 37° C under humid condition for 1 h. The unbound conjugate was removed by washing with PBST. The enzyme substrate was added to all wells (100 µl per well). The reaction was allowed to take place in the dark for 30 min then stopped by adding 50 µl of 1 N NaOH per well. The OD of each well was measured at 492 nm by ELISA reader (Titertek Multiskan MCC/340, USA) against the blank. The ELISA titre of the antibody preparation was the highest dilution of the antibody which gave an OD >0.05. One indirect ELISA unit was the smallest amount of the antibody which gave a positive indirect ELISA reaction.

Dot-blot ELISA

Dot-blot ELISA was performed as described by Chaicumpa et al.[10] with modifications. Three microlitre aliquots of specimens or antigen were applied onto a nitrocellulose (NC) strip and the strip was air dried. The positive control was 3 µl of *V. cholerae* 01 LPS in PBS; the negative control was 3 µl of distilled water or PBS. The NC strip was then blocked with 0.5% BSA in PBS, pH 7.4, incubated at room temperature for 10 min, and then washed with 0.1 M Tris-HCl, pH 7.5. The strip was incubated with the monoclonal antibody specific to *V. cholerae* 01 A antigen (80 ELISA units ml^{-1}) on a rocking platform for 10 min, washed and then incubated with rabbit anti-mouse immunoglobulin alkaline phosphatase conjugate (1 : 1000 in PBS, pH 7.4, containing 0.2% BSA, 0.2% gelatin). After 10 min at room temperature, the strip was washed and then placed in a substrate solution (5 mg of nitroblue tetrazolium and 2.5 mg of 5-bromo-4 chloro-3 indolyl phosphate in 20 ml of 0.1 M Tris-HCl containing 0.1 M NaCl and 50 mM $MgCl_2$) for 10 min at room temperature. The reaction was stopped by washing the NC strip with distilled water, the strip was then air dried. Positive reactions appeared as blue spots while negative reactions appeared as brown (non-specific reaction) or clear areas.

Collection of specimens

Stool specimens

Three batches of stool specimens were collected from patients with acute watery diarrhoea admitted to Bamrasnaradura Infectious Diseases Hospital, Nonthaburi Province, Thailand. The first batch were 194 freshly passed stools collected between the months of April and June 1992. One gram of each specimen was mixed with 4 ml of 0.1 M citrate buffer, pH 5.0 containing 5% BSA. The preparation was centrifuged at 850 × ***g*** for 10 min, the supernate (stool extract) was collected, boiled for 15 min and kept at –70° C for dot-blot ELISA. The specimens of the second batch, i.e. 147 freshly passed stools were collected during January to May, 1993. The stool samples (diarrhoeic stools) were boiled for 15 min before subjecting them to the dot-blot ELISA. Specimens of the third batch included 643 rectal swabs which were enriched in APW for 6–8 h (rectal swab cultures), boiled for 15 min and assessed by the dot-blot ELISA.

The specimens of each batch were subsequently divided into three groups according to the results of stool examination. Group 1 included specimens of patients whose stool examination revealed *V. cholerae* 01 with or without other enteric pathogens. Group 2 were specimens which contained pathogenic bacteria other than *V. cholerae* 01 and/or parasites while specimens from group 3 revealed no pathogenic bacteria or parasites (possibly viral or chemical diarrhoea). Stools of healthy, parasite-free individuals were included as specimens of group 4 of the first batch. Bacteria and parasites found in stool specimens of groups 1 and 2 included *V. cholerae* non-01, *V. parahaemolyticus*, *V. fluvialis*, *Shigella flexneri*, Salmonella groups B, C, D and E, *Aeromonas hydrophila*, *A. sobria*, *Plesiomonas shigelloides*, Campylobacter spp., *Entamoeba histolytica* cysts and trophozoites, *Giardia lamblia* cysts and trophozoites, *Trichomonas hominis* trophozoites, hookworm eggs, *Strongyloides stercoralis* larvae, *Opisthorchis viverrini* eggs and tapeworm segments. A summary of specimen collection and classification is given in Table 2.

Stool examination

Stool examination for enteropathogenic bacteria and parasites was performed in the Microbiology and Parasitology Laboratories, Department of Pathology, Bamrasnaradura Infectious Diseases Hospital, Nonthaburi Province, Thailand. Stool specimens and rectal swabs were collected from the patients upon hospital arrival and prior to any treatment. For bacterial culture, three rectal swabs were taken and one each placed in alkaline-peptone water for *V. cholerae* isolation, selenite F broth for Salmonella culture and Cary–Blair medium for isolation of Salmonella, *Escherichia coli*, Shigella, other vibrios, Campylobacter and other bacteria, respectively. Bacterial isolation and identification were performed according to the procedures previously described[11–13].

Freshly passed stool specimens of the patients were also examined for parasites macroscopically and microscopically. A wet mount was made from freshly passed stool by placing a drop of physiological saline on one part of a microscopic slide and a drop of iodine solution on another part. Aliquots of stool sample were emulsified in the saline and iodine, respectively. Cover slips were placed on the suspensions and the slide was examined under the microscope for parasites or their products such as eggs.

Table 2. Summary of specimen collection and processing

Batch	*Group*	*Pathogen(s) found in stool*	*No.*	*Treatment of specimens*
1	1	*V. cholerae* 01	101	1 g of stool was mixed with 4 ml of 0.1 M citrate buffer pH 5.0 containing 5% BSA, centrifuged at 850 × ***g***, 10 min; supernate was boiled for 15 min before testing by dot-blot ELISA
	2	Bacteria other than *V. cholerae* 01 and/or parasites	35	
	3	No pathogens (perhaps viral or chemical diarrhoea)	50	
	4	None (healthy controls)	8	
2	1	*V. cholerae* 01	115	Watery stool was boiled for 15 min before testing by dot-blot ELISA
	2	Other pathogens	16	
	3	No pathogens	16	
3	1	*V. cholerae* 01	74	Rectal swab was enriched in alkaline-peptone water for 6–8 h, then boiled for 15 min before testing by dot-blot ELISA
	2	Other pathogens	569	
	3	No pathogens	0	

Seafoods

A total of 335 seafood samples were obtained from various fresh markets in Bangkok metropolis. There were 50 green mussels (*Perna viridis*), 40 undulated surf clams (*Paphia undulata*), 50 rainbow cuttle fish (*Sepia pharaonis*), 40 giant tiger prawns (*Panaeus monodon*), 55 white shrimps (*Penaeus merguiensis*), 50 diamond-scale grey mullet (*Lisa vaigiensis*) and 50 greasy groupers (*Epinephelus tauvina*).

Seafood samples weighing approximately 10 g were each placed in 100 ml of APW and incubated at 37° C for 6–8 h with constant shaking (135 rpm). An aliquot of this enrichment culture was boiled for 15 min and 3 µl was dotted onto the nitrocellulose strip for dot-blot ELISA. A loopful of the enrichment culture was also streaked onto TCBS agar for vibrio culture. The colonies obtained which were thought to be vibrios were sent to the Enteric Bacteriology Section, Division of Clinical Pathology, National Institute of Health, Thailand for identification.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis (WB)

The SDS–PAGE was carried out in a vertical slab gel apparatus (Bio-Rad Laboratories, USA) according to the method of Laemmli[14]. A 4% stacking gel and 10% acrylamide separating gel were used in the process. Western blotting was performed by transblotting the SDS–PAGE separated *V. cholerae* Ly or LPS from the gel to a nitrocellulose paper[15]. The unoccupied sites on the NC paper were blocked by soaking in a solution of 0.5% gelatin, 0.2% BSA in PBS for 1 h. After washing with PBST, the NC paper was treated with antibody preparation (IS or monoclonal antibodies) at room temperature for 1 h. After washing thoroughly, the NC paper was put in a solution of rabbit anti-mouse immunoglobulin horseradish peroxidase conjugate (Dakopatts, Denmark) at a dilution of 1 : 1000 in PBS, pH 7.4 containing 1% BSA and 1% gelatin for 30 min at 26° C. The NC paper was washed with PBST and finally was washed with phosphate buffer, pH 7.6 before being placed in a substrate solution containing 0.2 g of 2,6-dichlorophenol indophenol (Sigma Chemical Co., USA) and 0.01% H_2O_2 in 100 ml phosphate buffer, pH 7.6 for 1 min, washing thoroughly with distilled water, then air dried.

Statistical analysis

Sensitivity, specificity, predictive values and efficacy of the dot-blot ELISA were evaluated in comparison with the culture method using the method of Galen[16]. The k coefficient[17] was used to measure the degree of agreement of the association between the outcomes of cholera diagnosis using the dot-blot ELISA and bacterial culture. The probability of k, introduced by Fleiss[18] was also used to indicate the extent of agreement beyond chance of the culture and the dot-blot ELISA. If the probability of the k is statistically significant, it indicates that the degree of agreement of the two tests (culture vs. dot-blot ELISA) did not occur by chance (beyond chance). In other words, an association between the outcomes of the two tests existed. Landis and Koch[19] have characterized different ranges of values for k with respect to the degree of agreement. They suggested that for most purposes, values greater than 75% or so may be taken to represent excellent agreement beyond chance, while values below 40% or so may be taken to represent poor agreement and values between 40 and 75% represent fair to good agreement beyond chance.

Results

Antibody titres against *V. cholerae* 01 Ly and LPS of the immunized mice assessed by the indirect ELISA at

Table 3. Antigens used for mouse immunization and the antibody titres of blood samples collected from each mouse 2 weeks after the second intraperitoneal immunization

Group	*Antigen source*	*Mouse no.*	*Reciprocal indirect ELISA titre against*	
			Ly	*LPS*
1	O:17SR	1	20480	1280
		2*	40960	40960
		3	81920	5120
		4	5120	320
2	O:295/33	1	40960	1280
		2	40960	320
		3*	40960	1280
3	O:295/33/I:92/34	1	20480/20480	5120/2560
		2*	40960/40960	2560/1280
		3	20480/20480	640/2560
		4	20480/20480	160/2560
		5	20480/20480	1280/640
4	I:92/34	1	3200	400
		2	3200	800
		3	3200	400
		4	3200	800
		5	3200	400
		6	800	200
		7*	3200	6400
		8	1600	800

*Mice used as spleen donors in hybridizations.
O = Ogawa; I = Inaba.

14 days after the last intraperitoneal immunization are shown in Table 3. One mouse from each group with high antibody titres, namely mouse no. 2 from group 1, mouse no. 3 from group 2, mouse no. 2 from group 3 and mouse no. 7 from group 4 were used as splenocyte donors in four cell fusions. The ratio of immune spleen cells: Sp 2/0 myeloma cells in each fusion was 10 : 1. From the four cell fusions, six hybrid clones which produced antibodies to *V. cholerae* 01 antigens (Ly and LPS) as tested by the indirect and dot-blot ELISA were obtained. These hybrid clones were designated 4D3 from fusion 1, 8G8 and 12B4 from fusion 2, 26F8 from fusion 3 and 27E2 and 27E10 from fusion 4, respectively. The immunoglobulins produced by the six hybrids were isotyped using the mouse typer sub-isotyping kit (Bio-Rad Laboratories, USA). The isotypes were IgM, IgA, IgG2a, IgG1, IgG3 and IgG1, respectively. Their antigenic type of light chain was k.

Unfortunately, the hybrid 8G8 which produced IgA antibodies died and only a small amount of its cultured fluid was obtained, which was just enough for Western blot analysis (see below). The antibodies from the remaining five clones were tested for their specificities and crossreactivities against Ly from all of the bacteria listed in Table 1, and against *E. histolytica* extract by indirect and dot-blot ELISA. It was found that the antibodies from all clones reacted specifically only to *V. cholerae* 01 Ly and LPS and did not react with the antigens of *V. cholerae* non-01 (except the antibodies produced by the clone 12B4 which reacted to Ly of one strain of *V. cholerae* non-01), other bacteria or *E. histolytica* extract. The antibodies produced by the clones 27E2 and 27E10 reacted to the Ly and LPS of all *V. cholerae* 01 tested; the antibodies from the clone 26F8 reacted only to the antigens prepared from *V. cholerae* 01 serotype Ogawa while the antibodies produced by the clones 4D3 and 12B4 reacted to antigens from only some strains of *V. cholerae* 01 (Table 4).

Figures 1 and 2 illustrate the reaction patterns in Western blot analysis of the antibodies produced by the six hybrid clones against Ly prepared from *V. cholerae* 01 serotypes Ogawa and Inaba, respectively. The antibodies produced by clones 4D3 and 12B4 reacted to the low molecular weight antigen of the Ly from both serotypes. The antibodies from clones 8G8, 27E2 and 27E10 reacted to the fast moving antigens of Ly from both serotypes and showed a ladder appearance. The antibodies from the clone 26F8 gave a similar reaction to the Ly of serotype Ogawa but did not react to the Ly of almost all strains of serotypes Inaba except one strain, against which the MAb gave a very weak reaction (lane f, Figure 2). When the *V. cholerae* 01 LPS was used in the Western blot analysis instead of the Ly, similar results were obtained. However, the reaction patterns appeared as diffused bands instead of the ladder characteristic.

From the above findings, it could be concluded that the antibodies produced by the clones 27E2 and 27E10 were directed to the group-specific 'antigen A' of all *V. cholerae* 01, while the antibodies from the clone 26F8 were directed to the type-specific 'antigen B' of *V. cholerae* 01 serotype Ogawa. The hybrid clone 27E10 was also healthy and produced antibodies with high indirect ELISA titre (1 : 256) in the *in vitro* culture, thus it was expanded for bulk production of the specific antibodies (MAb 27E10). The antibodies

Table 4. Reactivities of monoclonal antibodies produced by the five clones against the Ly of some strains of *V. cholerae* 01 and non-01

Strain	*Monoclonal antibodies from clone*				
	4D3	*12B4*	*26F8*	*27E2*	*27E10*
1. *Vc* Classical 569B Inaba	++	+++	–	+	+
2. *Vc* El Tor Ogawa 241/33	+	+	+++	+++	++++
3. *Vc* El Tor Ogawa 17SR	+	+	+	+	+
4. *Vc* El Tor Ogawa 295/33	+	+	++++	+++	++++
5. *Vc* El Tor Ogawa K237	++	+	+++	++	++++
6. *Vc* El Tor Ogawa 339/33	+	++	+++	++	++++
7. *Vc* El Tor Ogawa 231/33	+	+	++++	++	++++
8. *Vc* El Tor Ogawa lab. strain	–	+	+++	++	+++
9. *Vc* El Tor Ogawa 54/35	+	+	++++	++	++++
10. *Vc* El Tor Ogawa 58/35	+/–	+	++++	+++	++++
11. *Vc* El Tor Ogawa 61/35	+/–	+	++++	+++	++++
12. *Vc* El Tor Ogawa 263/35	+/–	++	++++	+++	++++
13. *Vc* El Tor Ogawa 90/35	–	+	++++	++	++++
14. *Vc* El Tor Ogawa 285/35	–	+	++++	++	++++
15. *Vc* El Tor Ogawa 286/35	–	+	++++	+++	++++
16. *Vc* El Tor Ogawa 138/35	–	+	++++	++	++++
17. *Vc* El Tor Ogawa 216/35	–	+/–	++++	++	+++
18. *Vc* El Tor Ogawa 117/35	–	+	++++	++	++++
19. *Vc* El Tor Ogawa 287/35	–	+	++++	++	++++
20. *Vc* El Tor Ogawa 215/35	–	+	++++	++	++++
21. *Vc* El Tor Ogawa 29/34	–	+	++++	+++	++++
22. *Vc* El Tor Ogawa 1228/34	–	+	++++	++	++++
23. *Vc* El Tor Ogawa 339/35	+	+	++++	++	++++
24. *Vc* El Tor Inaba 316/33	–	+/–	–	+	++
25. *Vc* El Tor Inaba 257/33	+/–	+	–	++	++++
26. *Vc* El Tor Inaba 213/35	+/–	+	–	+++	++++
27. *Vc* El Tor Inaba 1271/34	–	+	–	++	++++
28. *Vc* El Tor Inaba 1018/34	–	+	–	+++	++++
29. *Vc* El Tor Inaba 609/34	+/–	+/–	–	+++	++++
30. *Vc* El Tor Inaba 35/35	–	+	≠	+++	++++
31. *Vc* El Tor Inaba 92/34	+	+	–	+++	++++
32. *Vc* El Tor Inaba 1856/34	–	+	–	+++	++++
33. *Vc* El Tor Inaba 1019/34	–	+	–	++	++++
34. *Vc* non-01 63/33	–	–	–	–	–
35. *Vc* non-01 48/33	–	–	–	–	–
36. *Vc* non-01 16/33	–	–	–	–	–
37. *Vc* non-01	–	–	–	–	–
38. *Vc* non-01	–	+	–	–	–
39. *Vc* non-01	–	–	–	–	–

O = Ogawa; I = Inaba.
– = Negative reaction (OD <0.05); +/– = weakly positive reaction (OD = 0.05–0.09); + = OD = 0.10–0.19; ++ = OD = 0.20–0.29; +++ = OD = 0.30–0.39; ++++ = OD >0.40.

were used in the dot-blot ELISA for the detection of *V. cholerae* 01 antigen in all specimens.

Dot-blot ELISA performed on the stool extracts of the first batch of stool specimens revealed that among 101 *V. cholerae* 01 culture positive samples, 55 samples were positive by the dot-blot ELISA. The ELISA was also negative when performed on the 93 specimens which were culture negative. The sensitivity, diagnostic specificity, positive predictive value, negative predictive value and the efficacy of the dot-blot ELISA[16], when compared to the culture method, were 54.5%, 100%, 100%, 66.9% and 76%, respectively. The k coefficient value[17] was 53% which indicates good agreement (beyond chance) of the two tests. When the dot-blot ELISA was performed on the boiled, diarrhoeic stools of the second batch, it gave positive results to 72 samples from 115 samples which were *V. cholerae* 01 positive by the culture method. Among 32 culture negative diarrhoeic stools, one of them was positive by the dot-blot ELISA. The sensitivity, diagnostic specificity, positive predictive value, negative predictive value and efficacy of the dot-blot ELISA were 62.6%, 96.9%, 98.6%, 41.9% and 70.1%, respectively. The two methods, i.e. dot-blot ELISA and culture were in good agreement (k coefficient value 40%).

From 74 culture positive samples of the third batch of stool specimens, 71 of them were positive by the dot-blot ELISA. The ELISA was also positive when tested on four of the 569 rectal swab cultures which were *V. cholerae* 01 negative by the culture method. The sensitivity, diagnostic specificity, positive predictive value, negative predictive value and efficacy of the dot-blot ELISA for the detection of *V. cholerae* 01

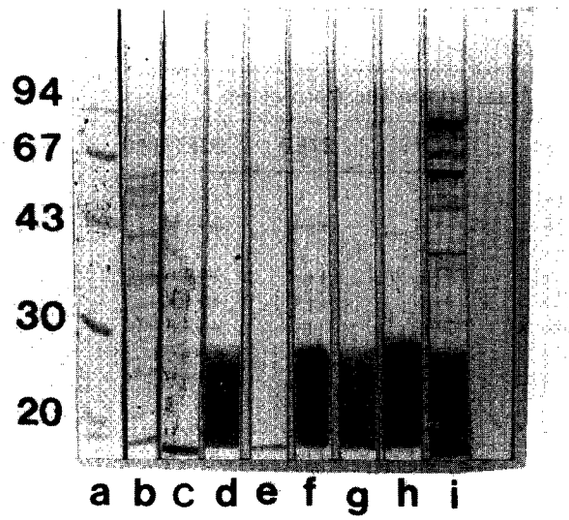

Figure 1. Patterns of Western blot analysis for determining antigenic specificities of the monoclonal antibodies produced from the six hybrid clones and IS to Ly prepared from *V. cholerae* 01 Ogawa strain. Lane a = mol. wt. markers; lane b = electrophoresed Ogawa Ly stained with Amido black; lane c = WB of MAb from 4D3; lane d = WB of MAb from 8G8; lane e = WB of MAb from 12B4; lane f = WB of MAb from 26F8; lane g = WB of MAb from 27E2; lane h = WB of MAb from 27E10 and lane i = WB of IS.

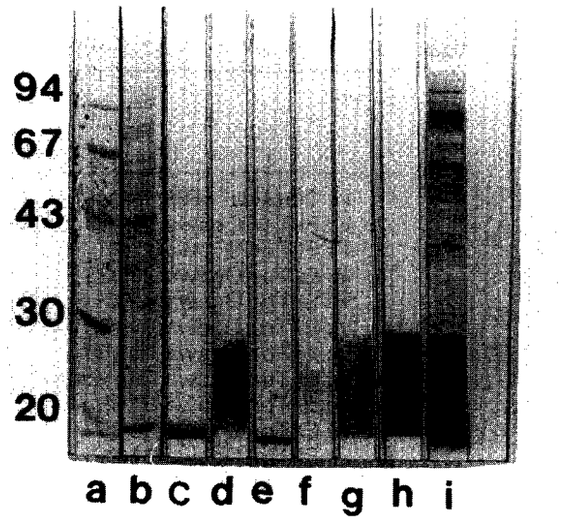

Figure 2. Patterns of Western blot analysis of the monoclonal antibodies produced by the six hybrid clones and IS against Ly prepared from *V. cholerae* 01 serotype Inaba. Lane a = mol. wt. markers; lane b = electrophoresed Ly stained with Amido black; lane c = WB of MAb from 4D3; lane d = WB of MAb from 8G8; lane e = WB of MAb from 12B4; lane f = WB of MAb from 26F8; lane g = WB of MAb from 27E2; lane h = WB of MAb from 27E10 and lane i = WB of IS.

antigen in the APW-enriched rectal swab specimens compared to the culture method were 96.0%, 99.3%, 94.7%, 99.5% and 98.9%, respectively. The two methods showed excellent agreement beyond chance as the k coefficient value was 94.7%. Table 5 summarizes the results of *V. cholerae* 01 antigen detection in stool specimens by dot-blot ELISA and the culture method.

From the above findings it could be concluded that the 6–8 h APW-enriched rectal swab cultures are the most suitable specimens for the detection of cholera in the patient's stool by the MAb-based dot-blot ELISA. However, the enrichment time of up to 6–8 h might be too long and the test might not be regarded as a rapid test. Deliberate experiments were therefore performed to test for the shortest time of enrichment in which the MAb-based dot-blot ELISA would give positive results on the rectal swab cultures. Rectal swabs from patients of acute watery diarrhoea with various numbers of *V. cholerae* 01 in stools were enriched in APW for 1, 2, 3, 4 and 5 h. Aliquots of the cultures at each time were boiled and subjected to the dot-blot ELISA. It was found that when the patient's stool contained high numbers of *V. cholerae* 01 (10^8–10^9

Table 5. Results of the dot-blot ELISA performed on three batches of the stool specimens compared with the results of the culture method

	First batch (stool extract) %	*Second batch (stool)* %	*Third batch (rectal swab)* %
Sensitivity	55	63	96
Diagnostic specificity	100	97	99
Positive predictive value	100	99	95
Negative predictive value	67	42	99
Efficacy	76	70	99
Statistical interpretation	Good degree of agreement between the two tests beyond chance (k = 53%)	Good degree of agreement between the two tests beyond chance (k = 40%)	Excellent degree of agreement between the two tests beyond chance (k = 95%)

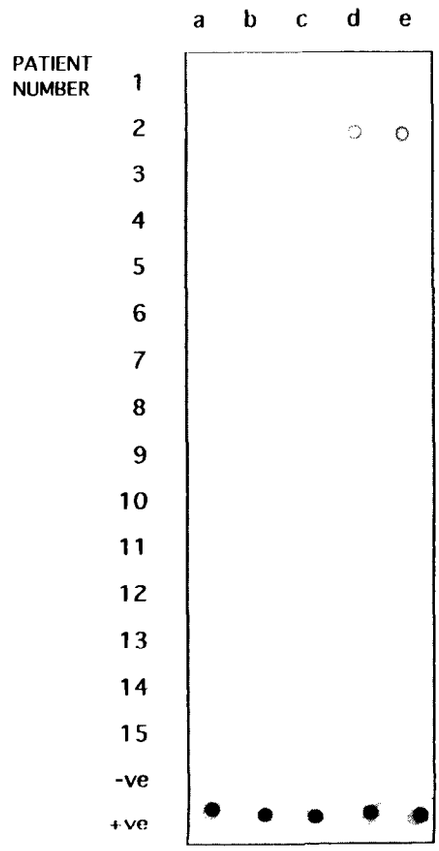

Figure 3. Dot-blot ELISA of the rectal swab cultures from patients with acute watery diarrhoea enriched in alkaline peptone water for 1(a), 2(b), 3(c), 4(d) and 5(e) h, respectively. Patient no. 2 was a cholera patient whose stool specimen contained $<10^6$ *V. cholerae* 01 ml^{-1} of stool. The dot-blot ELISA was positive after the stool was enriched for 2 h. Patient nos. 1 and 3–15 were stool culture negative for *V. cholerae* 01. –ve = negative control (APW); +ve = positive control (*V. cholerae* 01 LPS).

vibrios ml^{-1} of stool) there was no need to incubate the rectal swab culture at all. The rectal swab absorbed about 0.1 ml of stool, hence enough vibrios were transferred to the APW and gave a positive dot-blot ELISA. However, when the numbers of the vibrios in stools were low ($<10^6$ cells ml^{-1} of stool) the ELISA was positive only after the cultures were incubated for about 2 h (Figure 3). Depending upon the numbers of vibrios present in the stool, the detection of cholera would therefore take about 1–3 h (the dot-blot ELISA itself takes about 1 h).

When the dot-blot ELISA was performed on known amounts of *V. cholerae* 01 LPS and *V. cholerae* 01 whole cells, it was found that the smallest amounts of the LPS and whole cells which gave positive results were 1.95 ng and 3000 vibrios (10^6 cells ml^{-1}), respectively.

Pathogenic bacteria which were recovered from the 335 seafood samples are listed in Table 6. *V. cholerae* 01 were not isolated from the food samples and the dot-blot ELISA was also negative. The specificity of the test was therefore 100%.

Discussion

V. cholerae 01 can spread rapidly and cause explosive outbreaks. Conventional culture methods to detect cholera take at least 2 days, by which time the disease might have spread widely. The rapid detection of cholera is therefore a perceived necessity for public health surveillance.

V. cholerae 01 is characterized by the group-specific antigen A. Serogroup 01 is divided into serotypes Ogawa, Inaba and Hikojima which depend on antigens A, B and C. Serotype Ogawa contains antigens A and B, serotype Inaba contains antigens A and C while serotype Hikojima possesses all three antigens. The three antigens are located in the carbohydrate portion of *V. cholerae* 01 LPS and they are thermostable. *V. cholerae* other than serogroup 01 are designated as non-agglutinable (NAG) vibrio strains or *V. cholerae* non-01. The serogroups of *V. cholerae* non-01 are 02–0140[20].

There have been several reports on the use of polyclonal antibodies in the detection of *V. cholerae* 01 antigens and the differentiation of strains between the Ogawa and Inaba serotypes. These immunoassays include indirect immunofluorescence[21], slide agglutination[22] and coagglutination tests[23]. The preparation of the specific polyclonal antiserum depends on the prolonged immunization and the sequential absorption of the antiserum with related (crossreactive) antigens. It was found that when crossreacting antibodies have been removed by such absorption, the titre of specific antibodies was decreased. Therefore, monoclonal antibodies directed against a unique determinant of *V. cholerae* 01 should represent a more reliable source of highly specific reagent for the immunological assays[24]. However, crossreaction may also occur with monoclonal antibodies if they recognize related antigenic determinant on different antigens and such crossreaction cannot be eliminated by absorption (since antibodies derived from one clone are identical and are mono-epitope specific; removal of the cross-reactivity would remove all of the antibody activity). The best monoclonal antibody should therefore be the one that reacts only to a unique *V. cholerae* 01 antigen, e.g. antigen A.

Applications of monoclonal antibodies specific to *V. cholerae* 01 have been partly outlined by Holme and Gustafsson[25]. The monoclonal antibodies can be used instead of the polyclonal antibodies in the final step of culture and identification of *V. cholerae* by the culture method and in the rapid detection of *V. cholerae* 01 in diarrhoeic stool by the motility inhibition test under dark-field microscopic examination. The monoclonal antibodies may be used in an immunoassay for rapid detection of *V. cholerae* 01 antigen in stools or other specimens, e.g. contaminated food and the environment. Both of the above rapid detection methods are of great epidemiological importance as action for prevention of cholera spread and control of epidemics can be promptly arranged. Moreover, monoclonal

Table 6. Pathogenic bacteria found in the seafood samples by the culture method

Pathogens		*Seafood samples*						
		Green mussels	*Undulated surf clamp*	*Rainbow cuttle fish*	*Giant tiger prawns*	*White shrimps*	*Greasy groupers*	*Diamond-scale grey mullet*
A.	*V. alginolyticus*, *V. fluvialis* and *V. parahaemolyticus*	27	22	25	24	15	20	13
B.	*V. alginolyticus* and *V. parahaemolyticus*	4	–	1	4	15	9	13
C.	*V. fluvialis* and *V. parahaemolyticus*	8	3	11	3	25	13	13
D.	*V. alginolyticus*, *V. fluvialis* and *V. vulnificus*	3	2	0	1	0	0	0
E.	*V. alginolyticus*, *V. fluvialis*, *V. parahaemolyticus* and *A. sobria*	1	0	0	0	0	0	0
F.	*V. fluvialis*, *V. parahaemolyticus*, *V. cholerae* non-01	0	0	11	2	0	3	4
G.	*V. alginolyticus*, *V. parahaemolyticus* and *V. cholerae* non-01	1	0	0	1	0	0	2
H.	*V. fluvialis*	2	3	1	1	0	1	0
I.	*V. alginolyticus* and *V. fluvialis*	4	10	1	0	0	4	5
J.	*V. alginolyticus*, *V. fluvialis*, *V. parahaemolyticus* and *V. vulnificus*	0	0	0	4	0	0	0
Total		50	40	50	40	55	50	50

V = Vibrio; A = Aeromonas.

antibodies have proved to be useful in the study of chemical structures of O-antigenic determinants of *V. cholerae* 01. They can also be used for checking stability and efficacy of vaccines at different stages of production or in the final products. Live, killed or refined antigen cholera vaccine is usually composed of various immunogenic components. Determination of the presence of such important immunogens in adequate concentrations would be necessary for controlling the quality of the vaccine. In these instances, monoclonal antibodies offer a superior possibility to the conventional polyclonal antisera. Besides, monoclonal antibodies can also be used as a tool for screening of mutants or genetically modified organisms.

In this study, six hybridomas were obtained from four cell fusions. It was noticed that there was no relationship between the antibody titres of the immune mice used as splenocyte donors and the percentages of hybrids obtained. Moreover, the percentages of growing hybrids were not correlated at all with the numbers of positive hybridomas. The numbers of positive clones from each fusion were rather low when compared with other reports on hybridoma production[8,9,25]. This may be due to several different factors such as the immunogens, immunization schedules, myeloma cell lines used, conditions of fusion, different media and other details of the techniques.

It is well accepted in hybridoma technology that the production of monoclonal antibodies depends very much on the sources, forms and nature of antigens used to immunize the animals. Pure antigens are not always essential or even preferred. In this study, the mice were immunized with the whole cell lysates (crude antigens) of *V. cholerae* 01 and antibodies directed against the homologous antigens and LPS were obtained. Gustafsson et al.[26] could elicit high anti-LPS titres against *V. cholerae* 01 LPS in mice by using crude LPS which contained some proteins prepared from Ogawa and Inaba strains. In our study, it was found that when the protein-free LPS (triple extracted LPS) was used as an immunogen, anti-LPS could be detected at low titre and no memory response was observed in the mice (data not shown). We therefore did not use the pure LPS for preparing immune spleen cells.

In order to obtain clones producing antibodies against the group and type specific LPS of *V. cholerae* 01, Gustafsson and Holme[24] used whole cells of the vibrios killed by either heat or formaldehyde in the mouse immunization[27]. In our study, the whole cell lysates were used and the clones which produce monoclonal antibodies directed against the group (clones 27E2 and 27E10) and Ogawa type (clone 26F8) specific LPS were obtained but not against the Inaba type-specific clone. The finding that the monoclonal

antibodies produced by the clone 26F8 reacted to all Ogawa strains and also gave a weak reaction to one of the Inaba strains by Western blot analysis but not by the indirect ELISA indicates higher sensitivity of the former and the mistyping of such an 'Inaba' strain by the original serotyping procedure which used the absorbed rabbit antisera in slide agglutination. In fact, this particular 'Inaba' strain should be classified as Hikojima strain as it possessed both antigens B and C. It is known that the presence of both antigens in Hikojima strains is not only a qualitative but also a quantitative matter and may require highly specific antibodies (e.g. monoclonal) and a highly sensitive assay to reveal them[25].

Isotypes of monoclonal antibodies produced by all hybrid clones were characterized. All of them secreted k type light chains. This is not surprising as it is known that the occurrence of k light chains in mouse immunoglobulins is 10 times higher than that of the λ type. The isotypes of heavy chains were different. The IgG1 nature of the monoclonal antibodies produced by the clone 27E10 (MAb 27E10) makes the antibodies more suitable as a diagnostic reagent since IgG1 usually has high affinity to its epitope.

The MAb 27E10 were used in a dot-blot ELISA for detecting purified LPS and *V. cholerae* 01 whole cells by the dot-blot ELISA. The lowest amounts of the LPS and of the cells which could be detected by the ELISA were 1.95 ng and 3000 cells (1×10^6 vibrios ml^{-1}), respectively. This level of analytical sensitivity was lower than those reported by Sugiyama et al.[28] who found that the lowest amounts of *V. cholerae* 01 which could be detected by monoclonal antibody latex agglutination tests were $5 \times 10^6 - 3 \times 10^7$ cells ml^{-1}. The MAb 27E10-based dot-blot ELISA should be sensitive enough to detect *V. cholerae* 01 in stools of most of the cholera patients as the patients tend to excrete about 10^6 vibrios ml^{-1} or higher. For a few patients, whose stools contained less than 10^6 vibrios ml^{-1}, only 1–2 h enrichment of the rectal swabs in alkaline-peptone solution is required prior to subjecting the specimens to the dot-blot ELISA.

The results of *V. cholerae* 01 cultures of seafood samples were all negative. The finding that dot-blot ELISA was also negative implies high specificity of the assay.

The sensitivity, specificity and efficacy of the dot-blot ELISA compared to the culture method when performed on rectal swab cultures of the patients were 96%, 99% and 99%, which indicates excellent agreement between the two tests. The ELISA is easy to perform, relatively inexpensive, sensitive and specific. It permits multisample analysis at a single time, requires no special equipment and does not pose any disposal problem (compared with the culture method). Most of all, it reduces the test time from at least 2 days for the culture method to 1–3 h (the dot-blot ELISA procedure itself takes about 1 h). This method is therefore recommended as a rapid screening test of cholera cases. Validity of the dot-blot ELISA in the detection of cholera carriers is reported elsewhere[29].

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