Simultaneous direct detection of toxigenic and non-toxigenic *Vibrio cholerae* from rectal swabs and environmental samples by sandwich ELISA

Urmil Tuteja,¹ Sanjay Kumar,¹ Jyoti Shukla,¹ Joseph Kingston¹ and Harsh V. Batra²

Correspondence
Harsh V. Batra
hvbatra@mail.com

¹Division of Microbiology, Defence Research and Development Establishment, Jhansi Road, Gwalior, Madhya Pradesh, India

²Division of Microbiology, Defence Food Research Laboratory, Siddharth Nagar, Mysore, Karnataka, India

A mAb-based simple, specific and rapid two-tip dipstick ELISA was developed for simultaneous detection of toxin- and non-toxin-producing strains of *Vibrio cholerae*, and for direct detection of *V. cholerae* from rectal swabs of patients and from environmental water samples. Rabbit polyclonal antibodies and murine mAbs were raised against recombinant protein (r-protein) antigens of cholera toxin B (CtxB) and outer membrane protein W (OmpW). Rabbit polyclonal antibodies to both r-proteins were coated individually onto the tips of nitrocellulose (NC) membranes of a two-tipped NC dipstick as capture antibodies and a mixture of two mAbs was used for the detecting antibodies. The test was found to be specific for *V. cholerae* strains O1, O139, non-O1 and non-O139, and did not show any cross-reaction to closely related bacterial strains. The test was evaluated on rectal swabs collected at the bedside of 75 hospitalized diarrhoeal patients and on 50 environmental water samples after enrichment for 4 h in alkaline peptone water. The mAb two-tip dipstick ELISA detected *V. cholerae* in 52/75 rectal swabs and 2/50 environmental water samples for CtxB antigen, and in 1/50 environmental water samples for the non-toxin OmpW antigen of *V. cholerae* within 1.5 h. These findings were identical to those observed using PCR and conventional culture methods. Thus, this mAb-based two-tip dipstick ELISA could be used for early and reliable simultaneous detection of toxigenic and non-toxigenic strains of *V. cholerae* from clinical and environmental water samples.

Received 12 January 2007
Accepted 13 June 2007

INTRODUCTION

In recent years, cholera has become endemic in a large number of geographical areas with immense global implications. It is a life-threatening diarrhoeal disease caused by *Vibrio cholerae* serogroups O1 and O139. The non-O1/non-O139 serogroups of *V. cholerae* also cause sporadic cases of diarrhoea in humans, and can be isolated in abundance from aquatic and estuarine sources (Blake *et al.*, 1980; Sanyal, 1986).

Conventional laboratory methods depend on the isolation and identification of the agent by biochemical tests, which are costly, time-consuming and tedious (WHO, 1974). The limited facilities available in small laboratories to undertake isolation and identification make the detection of this organism difficult. Moreover, the severity and epidemiology of the disease is a serious problem. It is therefore

important to detect this agent as quickly as possible in clinical and environmental specimens, so that appropriate monitoring and effective preventive measures can be undertaken. Consequently, efforts have been made to develop simple, specific and rapid diagnostic tests that would allow the detection of *V. cholerae* in the field. Specific antibodies to serogroup O1 do not react with O139 or non-O1/non-O139 strains of *V. cholerae* (Chaicumpa *et al.*, 1998; Hisatsune *et al.*, 1993). It is necessary, therefore, to have anti-*V. cholerae* group-specific antibodies, either polyclonal or monoclonal, for use in the detection of *V. cholerae* organisms. Co-agglutination tests (Colwell *et al.*, 1992; Qadri *et al.*, 1994; Rahman *et al.*, 1987) and ELISA systems (Ramamurthy *et al.*, 1990, 1992; Uesaka *et al.*, 1992) have been reported for field use. Dipstick ELISAs have also been utilized for the detection of *V. cholerae* O1 and O139 from stool samples (Bhuiyan *et al.*, 2003; Nato *et al.*, 2003). These tests have shown various degrees of success in the diagnosis of cholera. However, to date, none has become widely available for direct use on clinical or environmental water samples for the simultaneous

Abbreviations: DAB, 3,3-diaminobenzidine tetrahydrochloride; HRP, horseradish peroxidase; NC, nitrocellulose; r-protein, recombinant protein.

detection of toxigenic and non-toxigenic strains of *V. cholerae*.

The sequence of the outer membrane protein W encoding gene (*ompW*) is conserved among *V. cholerae* strains belonging to different serogroups and is used for species-specific identification of *V. cholerae* (Nandi *et al.*, 2000), whilst the presence of the cholera toxin genes *ctxAB* in *V. cholerae* identifies them as toxigenic. A multiplex PCR based on these genes has been reported for the detection of toxigenic strains of *V. cholerae* through the simultaneous amplification of the *ompW* and *ctxA* genes (Nandi *et al.*, 2000). However, these PCR methods require trained personnel and sophisticated equipment, and are poorly suited for use in general diagnostic laboratories. In the present study, attempts were made to develop a rapid, simple, sensitive and specific mAb-based two-tip dipstick ELISA for confirmatory and simultaneous detection of toxigenic and non-toxigenic strains of *V. cholerae* from clinical and environmental water samples.

METHODS

Bacterial strains. The bacterial strains used in this study are shown in Table 1. The standard strains of *V. cholerae* were obtained from the National Institute of Cholera and Enteric Diseases, Kolkata, India. Other organisms used for specificity testing were procured from the Microbial Type Culture Collection, Chandigarh, India.

Samples. Rectal swabs were collected from 75 patients suffering from acute watery diarrhoea hospitalized at the District Hospital, Bhind, Madhya Pradesh, and the Municipal Corporation of Delhi Hospital, Delhi, India. Fifty environmental water samples were collected in and around the hospital areas and patients' residence.

Isolation and identification of *V. cholerae*. The rectal swabs of diarrhoeal patients and environmental water samples were streaked on thiosulphate citrate bile salts sucrose (TCBS) agar after 4 h enrichment in alkaline peptone water (Farmer & Hickman-Brenner, 1992) and incubated at 37 °C for 18–24 h for the isolation of

organisms following the standard World Health Organization protocol (WHO, 1974). Suspected colonies of *V. cholerae* were tested by biochemical tests for the following: oxidase activity; indole production; citrate utilization; H₂S production; urea hydrolysis; fermentation of glucose, lactose, mannitol, sucrose, maltose, arabinose, xylose, rhamnose, adonitol and dulcitol; decarboxylation of lysine and ornithine; and hydrolysis of arginine.

PCR for *V. cholerae*. Genomic DNA from bacterial strains and rectal swabs was extracted using a DNeasy tissue kit and a QIAamp DNA stool kit (Qiagen), respectively. Based on published sequences (cholera toxin B encoding *ctxB* gene sequence GenBank accession no. X00171 and *ompW* sequence GenBank accession no. X51948), PCRs were performed on standard *V. cholerae* O1 DNA using primers for the *ompW* gene (forward primer 5'-CACCAAGAAGGTGACTT-TATTGTG-3'; reverse primer 5'-GAACCTTATAACCCCGCG-3'; Nandi *et al.*, 2000) and primers designed in-house for the *ctxB* gene (forward primer 5'-ACATGGATCCACACCTCAAAATATTACTG-3'; reverse primer 5'-TTAAGGTACCATACTAATTGCGGCAATCGC-3', *Bam*HI and *Kpn*I restriction sites underlined). DNA from *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Plesiomonas* sp., *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus* was used for negative controls. PCR amplification of the target DNA was carried out in a thermal cycler (Bio-Rad) using 200 µl PCR tubes with a reaction volume of 25 µl. Each reaction mixture contained 3 µl template DNA, 2.5 µl each primer (10 pmol µl⁻¹), 2.5 µl 2 mM dNTPs, 0.3 µl (5 U µl⁻¹) *Taq* DNA polymerase (MBI Fermentas), 2.5 µl reaction buffer, 1.5 µl 25 mM MgCl₂ and 10.2 µl distilled water. The reaction mixture was subjected to 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 or 50 °C for 50 s (for *ompW* and *ctxB*, respectively) and extension at 72 °C for 60 s. Before initiation of the first cycle, the reaction mixture was heated at 94 °C for 5 min to allow complete denaturation of the template. PCR products were electrophoresed through a 1% agarose gel and visualized under UV light after ethidium bromide staining.

Cloning, expression and purification of recombinant proteins (r-proteins). The purified PCR product for the *ctxB* gene (300 bp) was directionally cloned using *Bam*HI and *Kpn*I restriction enzymes into pQE-30, whereas the *ompW* fragment (588 bp) was cloned using *Sma*I restriction enzyme into the pQE-32 expression vector, and the constructs used to transform CaCl₂-treated *E. coli* SG13009 host cells following a standard protocol (Maniatis *et al.*, 1982). Transformants were selected and confirmed by restriction digestion of their plasmid preparations, as well as by PCR. The positive clones were induced to express r-proteins by induction with IPTG. Optimal expressions of r-proteins were achieved 5 h after the addition of 1.5 mM IPTG. SDS-PAGE revealed the expected 13 kDa band for r-CtxB and a 24 kDa band for the r-OmpW proteins. The His-tagged r-proteins were purified by Ni-NTA chromatography according to the manufacturer's protocol (Qiagen). Protein concentrations were determined by the method of Lowry (1951) to be 20 µg r-OmpW ml⁻¹ and 25 µg r-CtxB ml⁻¹.

Hyperimmune sera. Two female BALB/c mice and one rabbit were immunized at weekly intervals with 50 and 100 µg, respectively, of r-CtxB protein, and another set was immunized similarly with r-OmpW protein. The injections were administered on day 0 after emulsifying the r-proteins with complete Freund's adjuvant. Booster injections with the same amount of r-proteins emulsified with incomplete Freund's adjuvant were given on days 14, 21 and 28. The titres were checked by dot-ELISA using a 12-tipped nitrocellulose (NC) comb (MDI) coated with purified r-proteins. Rabbit or mouse polyclonal serum was used as the primary antibody and anti-rabbit or anti-mouse horseradish peroxidase (HRP) conjugate (Dako Cytomation) as the secondary antibody. 3,3'-diaminobenzidine tetrahydrochloride (DAB)-H₂O₂ (Sigma) was used as the chromogen.

Table 1. Bacterial strains used in this study

Bacterial strain	Source/strain ID
<i>Vibrio cholerae</i> O1	NICED
<i>Vibrio cholerae</i> O139	NICED
<i>Vibrio cholerae</i> non-O1	NICED
<i>Vibrio cholerae</i> non-O139	NICED
<i>Pseudomonas aeruginosa</i>	MTCC 7904
<i>Escherichia coli</i>	MTCC 6365
<i>Salmonella typhimurium</i>	MTCC 3214
<i>Plesiomonas</i> sp.	MTCC 1737
<i>Aeromonas hydrophila</i>	MTCC 1739
<i>Staphylococcus aureus</i>	MTCC 7443
<i>Escherichia coli</i> SG13009	Qiagen

MTCC, Microbial Type Culture Collection, Chandigarh, India; NICED, National Institute of Cholera and Enteric Diseases, Kolkata, India.

mAbs. Immunized mice were sensitized by successive intraperitoneal injections each day for a period of 3 days prior to fusion. The sensitized spleen cells were fused with the mouse myeloma cell line Sp2/O-Ag 14 following the general protocol of Kohler & Milstein (1975) with slight modifications. Briefly, myeloma cells were harvested from two confluent (3–4 days growth) 25 cm² tissue culture flask cultures and fused directly with the cells recovered from one immunized spleen without resorting to red blood cell lysis or counting of splenocytes. Fused cells were added with hypoxanthine-aminopterin-thymidine (HAT) medium in three 96-well flat-bottomed tissue culture plates. The medium was changed the following day with addition of fresh HAT medium (~300 µl per well). After 8–10 days without any further change in medium, the supernatants of the wells containing hybrids were checked for antibody production. The specificity of mAbs was checked by dot-ELISA using sonicated antigen preparations of standard strains of *V. cholerae* (O1, O139, non-O1 and non-O139), *P. aeruginosa*, *A. hydrophila*, *Plesiomonas* sp., *E. coli*, *Salmonella typhimurium* and *Staphylococcus aureus*. For this, bacterial cells harvested from brain heart infusion agar plates, were washed three times with sterile PBS and suspended in sterile PBS containing PMSF (20 µg ml⁻¹). The cells were sonicated using a Microson sonicator (Misonix). The protein content was estimated and 200 ng was coated onto each tip of a 12-tipped NC comb. All mAbs were tested as primary antibodies, using anti-mouse HRP conjugate as the secondary antibody. DAB-H₂O₂ was used as the chromogen.

mAb-based two-tip dipstick ELISA. Two microlitres of a 1:100 dilution (in PBS) of rabbit polyclonal antiserum to r-OmpW was coated onto the upper tip and 2 µl of a similar dilution of r-CtxB onto the lower tip of a two-tipped NC dipstick (MDI) for capturing the antigens of *V. cholerae*. Blocking of non-specific sites of the NC dipsticks was carried out using 5% skimmed milk powder in PBS with overnight incubation at 4 °C. The dipsticks were then incubated in samples (diluted 1:3 in PBS) at room temperature for 30 min. Thereafter, the dipsticks were washed three times with PBST (0.05% Tween 20 in PBS, pH 7.2) for 5 min. This washing step was carried out after each step. Further incubation of the dipstick was carried out with a mixture of two highly reactive and specific mAbs (C1024 ctxB and O1002 OmpW) to r-CtxB and r-OmpW as the detecting antibodies for 20 min at room temperature. This was followed by incubation with rabbit anti-mouse HRP conjugate (diluted 1:1000) for 20 min at room temperature. The dots were developed with DAB (5 mg in 10 ml) in PBS containing 0.015% H₂O₂. The appearance of brown-coloured dots was considered a positive reaction.

The sensitivity of the two-tip dipstick ELISA was determined by testing tenfold serial dilutions of *V. cholerae* O1 culture ranging from 10⁹ to 10² c.f.u. ml⁻¹ and twofold serial dilutions of purified r-CtxB (1000 ng ml⁻¹ to 15 pg ml⁻¹) by the method described above.

RESULTS AND DISCUSSION

The mAb-based two-tip dipstick ELISA described here is likely to facilitate the rapid and reliable detection of toxigenic and non-toxigenic strains of *V. cholerae* directly from clinical and environmental water samples. mAbs against the group- and type-specific antigens of *V. cholerae* have been used in conventional culture methods for identifying the serogroups of isolated organisms in diarrhoeic stool samples (Holme & Gustafsson, 1985). Development of mAb-based rapid tests for the detection of *V. cholerae* O1 and O139 strains in clinical specimens, and in food and water, have been reported (Garg *et al.*, 1994;

Fig. 1. PCR amplification of standard bacterial cultures using *ompW* primers. Lanes: 1, *P. aeruginosa*; 2, *A. hydrophila*; 3, *Plesiomonas* sp.; 4, *E. coli*; 5, *Salmonella typhimurium*; 6, *Staphylococcus aureus*; 7, *V. cholerae* O1; 8, *V. cholerae* O139; 9, *V. cholerae* non-O1; 10, *V. cholerae* non-O139; M, 1000 bp marker.

Hasan *et al.*, 1994a, b, 1995), but are yet to be evaluated for their application in routine laboratories.

For the development of the two-tip dipstick ELISA described in the present study, specific nucleotide sequences of the toxin gene *ctxB* and the group-specific gene *ompW* from *V. cholerae* O1 strain were cloned in expression vectors after PCR amplification. *V. cholerae* O1 showed the expected PCR amplification fragments of 300 and 588 bp for the *ctxB* and *ompW* genes, respectively (Figs 1 and 2). In addition, the *ompW* gene was also amplified in non-O1 and non-O139 strains (Fig. 1). However, amplification of *P. aeruginosa*, *A. hydrophila*, *Plesiomonas* sp., *E. coli*, *Salmonella typhimurium* and *Staphylococcus aureus* gave negative results. After obtaining optimal expression of the r-proteins, the His-tagged r-proteins were purified by Ni-NTA chromatography.

Rabbits and mice immunized with purified r-OmpW and r-CtxB elicited high antibody titres (Table 2). In Western blots, rabbit and mouse polyclonal antisera to r-CtxB and r-OmpW detected bands of the expected sizes with cell lysate antigens of IPTG-induced recombinant clones, the

Fig. 2. PCR amplification of standard bacterial cultures using *ctxB* primers. Lanes: M, 1000 bp marker; 1, *V. cholerae* O1; 2, *V. cholerae* O139; 3, *Plesiomonas* sp.; 4, *E. coli*; 5, *Salmonella typhimurium*; 6, *Staphylococcus aureus*; 7, *P. aeruginosa*; 8, *A. hydrophila*.

Fig. 3. Specificity testing of mAb O1002 ompW generated against r-OmpW. Reaction with the 24 kDa band is indicated with an arrow. Lanes: M, molecular mass marker; 1, *V. cholerae* O1; 2, *V. cholerae* O139; 3, *V. cholerae* non-O1; 4, *V. cholerae* non-O139; 5, *E. coli*; 6, *Salmonella typhimurium*; 7, *Staphylococcus aureus*; 8, *P. aeruginosa*; 9, *A. hydrophila*; 10, *Plesiomonas* sp.

Fig. 4. Specificity testing of mAb C1024 ctxB generated against r-CtxB. Reaction with the 13 kDa band is indicated with an arrow. Lanes: M, molecular mass marker; 1, *V. cholerae* O1; 2, *V. cholerae* O139; 3, *V. cholerae* non-O1; 4, *V. cholerae* non-O139; 5, *E. coli*; 6, *Salmonella typhimurium*; 7, *Staphylococcus aureus*; 8, *P. aeruginosa*; 9, *A. hydrophila*; 10, *Plesiomonas* sp.

respective purified r-proteins and *V. cholerae* O1 and O139 lysates (not shown). In addition, the polyclonal antiserum to r-OmpW showed a reaction with the expected 24 kDa band in non-O1 and non-O139 strains (not shown). Host cells and uninduced clones of recombinant *E. coli*, *P. aeruginosa*, *A. hydrophila*, *Plesiomonas* sp., *E. coli*, *Salmonella typhimurium* and *Staphylococcus aureus* did not exhibit a reaction with any of these polyclonal antisera.

Table 2. Antibody titres of hyperimmune sera raised against r-OmpW and r-CtxB

Animal	r-OmpW	r-CtxB
Mouse	1:64 000	1:64 000
Rabbit	1:256 000	1:128 000

A total of 10 mAbs against r-CtxB (C1020 ctxB to C1029 ctxB) and 12 against r-OmpW (O1000 ompW to O1011 ompW) were obtained using murine hybridoma technology. All of the mAbs to r-CtxB and r-OmpW showed specific reactions with the O1 and O139 strains of *V. cholerae* in Western blots. Representative blots are shown in Figs 3 and 4 for mAbs O1002 ompW and C1024 ctxB, respectively. Like the polyclonal antibodies, the mAbs to r-OmpW also reacted with non-O1 and non-O139 strains (Fig. 3). However, none of the mAbs reacted with *P. aeruginosa*, *A. hydrophila*, *Plesiomonas* sp., *E. coli*, *Salmonella typhimurium* or *Staphylococcus aureus*. Thus, these results correlated with those of the PCR (Figs 1 and 2).

A mixture of two mAbs showing high affinity and specificity (C1024 ctxB and O1002 ompW) was used for the detecting antibodies in the mAb-based two-tip dipstick ELISA. When tested for specificity, this ELISA gave positive results only with strains of *V. cholerae*, and did not cross-react with any of the other bacterial strains tested (Fig. 5). Following determination of sensitivity, the two-tip dipstick ELISA consistently detected 10^4 c.f.u. ml⁻¹ and 60 pg CtxB ml⁻¹. This ELISA was then evaluated by directly testing 75 rectal swabs of suspected cholera patients and 50 environmental water samples after 4 h of enrichment in alkaline peptone water. The samples were also subjected simultaneously to PCR and a conventional culture method. Both the dipstick ELISA and PCR identified 52/75 rectal swabs and 2/50 environmental water samples as having toxin-producing strains of *V. cholerae*, and 1/50 environmental water samples as a non-toxin-producing strain. On culturing, *V. cholerae* organisms were also recovered from the same 52 rectal swabs and the 3 environmental water samples on TCBS media (Table 3). Therefore, the mAb raised against the r-OmpW protein was able to detect specifically both the toxigenic and the non-toxigenic strains of *V. cholerae* directly from the samples. As the *ompW* gene is present universally in all *V. cholerae* strains (Nandi *et al.*, 2000), the mAb to r-OmpW protein, when mixed with a

Fig. 5. Reaction of the two-tip dipstick ELISA with *V. cholerae* O1 (a), *V. cholerae* O139 (b), *V. cholerae* non-O1 (c), *V. cholerae* non-O139 (d), *E. coli* (e), *Salmonella typhimurium* (f), *Staphylococcus aureus* (g), *P. aeruginosa* (h), *A. hydrophila* (i) and *Plesiomonas* sp. (j).

Table 3. Detection of *V. cholerae* in clinical and environmental water samples by mAb-based two-tip dipstick ELISA and PCR compared with conventional culture

Sample type	No. of positive results from:				
	mAb two-tip dipstick ELISA		PCR		Culture
	OmpW	CtxB	ompW	ctxB	
Rectal swabs (<i>n</i> =75)	52	52	52	52	52
Environmental water samples (<i>n</i> =50)	3	2	3	2	3

specific mAb to r-CtxB as the detecting antibodies in a two-tip dipstick ELISA, could successfully be used for simultaneous identification and differentiation of toxigenic and non-toxicogenic strains of *V. cholerae* from clinical as well as environmental water samples. The identical results observed by PCR and culture give confidence of the suitability of this mAb-based two-tip dipstick ELISA as a possible rapid, reliable and economical test in routine investigation procedures, even in remote parts of the world where cholera is endemic but laboratory facilities are less sophisticated.

ACKNOWLEDGEMENTS

The authors are thankful to Dr K. Sekhar, Director, and Dr R. Vijayaraghavan, Head, Division of Microbiology, DRDE, Gwalior, India, for their keen interest and constant support in this study.

REFERENCES

- Bhuiyan, N. A., Qadri, F., Faruque, A. S. G., Malek, A. A., Salam, M. A., Nato, F., Fournier, J. M., Chanteau, S., Sack, D. A. & Nair, G. B. (2003). Use of dipsticks for rapid diagnosis of cholera caused by *Vibrio cholerae* O1 and O139 from rectal swabs. *J Clin Microbiol* **41**, 3939–3941.
- Blake, P. A., Weaver, R. W. & Hollins, D. G. (1980). Diseases of humans (other than cholera) caused by vibrios. *Annu Rev Microbiol* **34**, 341–367.
- Chaicumpa, W., Srimanote, P., Sakolvaree, Y., Kalampaheti, T., Chongsa-Nguan, M., Tapchaisri, P., Eampokalap, B., Moolasart, P., Nair, G. B. & Echeverria, P. (1998). Rapid diagnosis of cholera caused by *Vibrio cholerae* O139. *J Clin Microbiol* **36**, 3595–3600.
- Colwell, R. R., Hasan, J. A. K., Huq, A., Loomis, L., Siebeling, R. J., Torres, M., Galvaez, S., Islam, S. & Bernstein, D. (1992). Development and evaluation of a rapid, simple, sensitive monoclonal antibody based co-agglutination test for direct detection of *Vibrio cholerae* O1. *FEMS Microbiol Lett* **76**, 215–219.
- Farmer, J. J., III & Hickman-Brenner, F. W. (1992). The genera *Vibrio* and *Photobacterium*. In *The Prokaryotes*, pp. 2952–3011. Edited by A. Balows, H. G. Truper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer-Verlag.
- Garg, S., Ramamurthy, T., Mukhopadhyay, A. K., Deb, B. C., Nair, G. B., Shimada, T., Takeda, T., Huq, A., Colwell, R. R. & Takeda, Y. (1994). Production and cross reactivity pattern of panel of high affinity monoclonal antibodies to *Vibrio cholerae* O139 Bengal. *FEMS Immunol Med Microbiol* **8**, 293–298.
- Hasan, J. A. K., Bernstein, D., Huq, A., Loomis, L., Tamplin, L. & Colwell, R. R. (1994a). Cholera DFA: an improved direct fluorescent-monoclonal antibody staining kit for rapid detection and enumeration of *Vibrio cholerae* O1. *FEMS Microbiol Lett* **120**, 143–148.
- Hasan, J. A. K., Huq, A., Tamplin, M. L., Siebeling, R. J. & Colwell, R. R. (1994b). A novel kit for rapid detection of *Vibrio cholerae* O1. *J Clin Microbiol* **32**, 249–252.
- Hasan, J. A. K., Huq, A., Nair, G. B., Garg, S., Mukhopadhyay, A. K., Loomis, L., Bernstein, D. & Colwell, R. R. (1995). Development and testing of monoclonal antibody-based rapid immunodiagnostic test kits for direct detection of *Vibrio cholerae* O139 synonym Bengal. *J Clin Microbiol* **33**, 2935–2939.
- Hisatsune, K., Kondo, S., Isshiki, Y., Iguchi, T., Kawamata, Y. & Shimada, T. (1993). O-antigenic lipopolysaccharide of *Vibrio cholerae* O:139 Bengal, a new epidemic strain for recent cholera in the Indian subcontinent. *Biochem Biophys Res Commun* **196**, 1309–1315.
- Holme, T. & Gustafsson, B. (1985). Monoclonal antibodies against group and type specific antigens of *Vibrio cholerae* O:1. In *Monoclonal Antibodies Against Bacteria*, vol. 1, pp. 167–189. Edited by A. J. L. Macario & E. C. de Macario. New York: Academic Press.
- Kohler, G. & Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495–499.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with folin phenol reagent. *J Biol Chem* **193**, 265–273.
- Maniatis, T., Fritsch, F. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Nandi, B., Nandy, R. K., Mukhopadhyay, S., Nair, G. B., Shimada, T. & Ghose, A. C. (2000). Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the gene of outer membrane protein OmpW. *J Clin Microbiol* **38**, 4145–4151.
- Nato, F. A., Boutonnier, M., Rajerison, M., Grosjean, P., Dartevelle, S., Guenole, A., Bhuiyan, N. A., Sack, D. A., Nair, G. B. & other authors (2003). One-step immunochromatographic dipstick tests for rapid detection of *Vibrio cholerae* O1 and O139 in stool samples. *Clin Diagn Lab Immunol* **10**, 476–478.
- Qadri, F., Chowdhury, A., Hossain, J., Chowdhury, K., Azim, T., Shimada, T., Nasirul Islam, K. M., Bradley Sack, R. & John Albert, M. (1994). Development and evaluation of rapid monoclonal antibody-based coagglutination test for direct detection of *Vibrio cholerae* O139 synonym Bengal in stool samples. *J Clin Microbiol* **32**, 1589–1590.
- Rahman, M., Sack, D. A., Mahmood, S. & Hossain, A. (1987). Rapid diagnosis of cholera by coagglutination test using 4-h fecal enrichment cultures. *J Clin Microbiol* **25**, 2204–2206.
- Ramamurthy, T., Pal, T. A., Nair, G. B., Pal, S. C., Takeda, T. & Takeda, Y. (1990). Experience with toxin bead-ELISA in cholera outbreak. *Lancet* **336**, 375–376.
- Ramamurthy, T., Bhattacharya, S. K., Uesaka, Y., Horigome, K., Paul, M., Sen, D., Pal, S. C., Takeda, T., Takeda, Y. & Nair, G. B. (1992). Evaluation of the bead enzyme-linked immunosorbent assay for

detection of cholera toxin directly from stool specimens. *J Clin Microbiol* **30**, 1783–1786.

Sanyal, S. C. (1986). Vibrio toxin. In *Pharmacology of Bacterial Toxins. International Encyclopedia of Pharmacology and Therapeutics*, vol. 119, pp. 207–255. Edited by F. Dorner & J. Drews. Oxford: Pergamon Press.

Uesaka, Y., Otsuka, Y., Kashida, M., Oku, Y., Horigome, K., Nair, G. B., Pal, S. C., Yamasaki, S. & Takeda, Y. (1992). Detection of cholera toxin by a highly sensitive bead-enzyme linked immuno-sorbent assay. *Microbiol Immunol* **36**, 43–53.

WHO (1974). *Guidelines for the Laboratory Diagnosis of Cholera*. Geneva: World Health Organization.