The use of gene probes, immunoassays and tissue culture for the detection of toxin in *Vibrio cholerae* non-O1

BENGÜ SAID, SYLVIA M. SCOTLAND and B. ROWE

Laboratory of Enteric Pathogens, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT

Summary. *Vibrio cholerae* non-O1 strains were screened for the presence of cholera enterotoxin (CT) genes by means of digoxigenin-labelled polynucleotide CT_A and CT_B probes. In-vitro production of CT was investigated by the Y1 mouse adrenal cell assay, enzyme-linked immunosorbent assay (ELISA) and a commercial, reversed passive latex agglutination (RPLA) kit. Only two (0.25%) of 790 strains tested gave positive results with the CT_A and CT_B probes. The production of other bacterial cytotoxin(s) made it impossible to use the characteristic cell-rounding effect on Y1 cells for the detection of CT. CT production by the probe-positive strains was confirmed by the immunoassays. Two hundred and fifty-two of the 788 probe-negative strains were tested by both cell assay and immunoassays. Of these, 90% produced cytotoxin(s) in the cell assay. In addition, 37% gave positive results in CT-ELISA, but negative results with LT-ELISA and VET-RPLA. These results indicate the presumed presence of a toxin in *V. cholerae* non-O1 that is able to bind GM₁ and react with antisera to CT, but which is not identical to CT.

Introduction

Vibrio cholerae can be distinguished serologically on the basis of somatic (O) antigens. There are currently 82 known serogroups in addition to *V. cholerae* O1, the causative agent of epidemic cholera.¹ *V. cholerae* non-O1 strains are distributed world-wide, often in brackish surface waters. Some serogroups have been implicated in sporadic cases and outbreaks of gastrointestinal disease. The symptoms vary from bloody diarrhoea with fever to a severe watery diarrhoea indistinguishable from cholera.²

Cholera enterotoxin (CT) is a recognised virulence factor of *V. cholerae* O1 and a heat-labile enterotoxin identical or similar to CT has been found in some isolates of *V. cholerae* non-O1.^{3,4}

CT production in strains of *V. cholerae* non-O1 has been reported from both Bangladesh and India; Datta-Roy⁵ found 26% of a small sample of 34 clinical isolates and 10% of environmental isolates to give positive results in tests for CT production. However, the occurrence rate of CT in *V. cholerae* non-O1 in other parts of the world is lower. None of 44 human isolates and only 2% of environmental isolates from Thailand were reported to be CT positive.⁶ Similarly, in a study of 2500 environmental isolates from the Louisiana Gulf coast only 0.3% produced CT.⁷ However, in none of these studies have the serogroups of *V. cholerae* non-O1 been reported, nor has a correlation of CT production with serogroup been attempted.

The somatic antigens may be useful indicators of diarrhoeagenic potential within the non-O1 strains. In 1980, the WHO working group⁸ reported a prevalence of serogroups O5 in gastrointestinal disease and O8 in the environment, whereas Donovan⁹ found that serogroups, O2, O5, O7 and O37 predominated in gastrointestinal disease and O4 in the environment. Certain serogroups have also been linked to outbreaks of gastrointestinal illness: e.g., serogroup O5 is thought to have been responsible for a food-borne outbreak in Czechoslovakia in 1965¹⁰ and serogroup O37 for a water-borne outbreak in the Sudan in 1968.¹¹ Recent reports from India and Bangladesh have described large outbreaks due to CT-producing *V. cholerae* non-O1.^{12,13} The strains from India were untypable (O?) and, although serogrouping for the Bangladesh outbreak remains to be done, both outbreaks may have been caused by strains from the same clone.

The Laboratory of Enteric Pathogens (LEP) has a large collection of *V. cholerae* non-O1, belonging to many different serogroups, isolated from over 50 countries. To assess the correlation of CT with serogroup, strains from both environmental and human sources were examined for the presence of the CT genes and for the production of heat-labile enterotoxins with the Y1 cell test and immunoassays.

Materials and methods

Bacterial strains

Seven hundred and eight wild-type strains of

V. cholerae non-O1, from both human and environmental sources, were studied; all strains were taken from the culture collection of LEP. Of these, 310 strains belonged to 49 serogroups, 334 strains were untypable (O?) and 64 strains were rough (OR). The type strains of *V. cholerae* non-O1 (serogroups O2–O83) were kindly provided by Dr R. Sakazaki. The *V. cholerae* non-O1 control strain WBDV-101E (serovar O49, CT) was kindly provided by Dr P. Echeverria. The *V. cholerae* O1 control strain (E51116, CT) came from the LEP culture collection. All *V. cholerae* strains were maintained on nutrient-agar slopes at room temperature. The control *Escherichia coli* strain B7A (O148:H28), which produces both heat-labile enterotoxin and heat-stable enterotoxin, (LT⁺ STa⁺)¹⁴ was maintained on a Dorset's egg slope at room temperature.

Preparation and digoxigenin-labelling of polynucleotide CT_A and CT_B probes

Strains of *E. coli* K12 carrying plasmids with cloned CT gene sequences were used to prepare large quantities of covalently closed circular DNA.¹⁵ The CT gene sequence from *V. cholerae* El Tor 62746¹⁶ cloned into plasmid pBR325 (pCVD27) was provided by Dr J. B. Kaper. A 554-bp CT_A probe was cut from pCVD27 after digestion with *Eco*RI (Life Technologies Ltd, Uxbridge, Middlesex). The recombinant plasmid pCT19 is known to contain CT_B and part of CT_A;¹⁷ the gene sequences were cloned and subcloned from *V. cholerae* 1621 into pACYC184 as described by Gennaro *et al.*¹⁸ A 550-bp CT_B probe was cut from plasmid pCT19 after digestion with *Xba*I and *Hinc*II. Probe fragments were separated on and excised from agarose gels and purified with the GeneClean Bio 101 kit (Strattech Scientific Ltd, Luton, Bedfordshire). The cloned polynucleotide probes were labelled with digoxigenin according to the manufacturer's instructions (Boehringer Corporation Ltd, Lewes, East Sussex). Unincorporated nucleotides were removed with QIAGEN-tip 5 (Diagen, Dusseldorf, Germany) according to the manufacturer's instructions and the labelled probe was stored at –20°C.

Preparation of cultures for DNA hybridisation

Bacterial cultures, grown at 37°C with aeration for 18 h, were spotted on to Hybond N nylon membranes (Amersham International, Aylesbury, Buckinghamshire) placed on nutrient agar plates. Colonies were grown at 37°C for 6 h and the cells were lysed and DNA denatured as described by Maniatis *et al.*¹⁵

Hybridisation with polynucleotide probes

Hybridisations were performed in heat-sealed plastic bags which were placed in a water bath at the appropriate temperature with shaking. Hybridisation

was carried out at 68°C (high stringency conditions identifying sequences with > 80% identity) on colony blot membranes. In some experiments, lower stringency conditions (37°C) were used, allowing the hybridisation of sequences with only 60% identity.

Colony blot membranes were pre-washed with 10 ml of a hybridisation solution per 50 cm² membrane for 1 h at 68°C. The hybridisation fluid contains; five times concentrated SSC (where SSC consists of NaCl 0.88%, trisodium citrate 0.44%), blocking reagent (Boehringer) 0.5%, N-lauroylsarcosine 0.1%, SDS 0.02%.) The membranes were wiped and pre-hybridised with fresh solution for 2 h. Hybridisation was allowed to proceed for 18 h, with hybridisation solution 1.25 ml/50 cm² membrane, containing freshly denatured probe 26 ng/ml and freshly denatured herring sperm DNA 100 µg/ml. Membranes were washed with double strength SSC, SDS 0.1% for 5 min at room temperature followed by two washes of 15 min each of, tenth dilution SSC, SDS 0.1% at 68°C. Detection procedures were as described by Boehringer.

The protocol for hybridisation at 37°C did not differ from that described above, but the hybridisation solution was formamide 25%, five times concentrated SSC, Ficoll 0.01%, polyvinylpyrrolidone 0.01%, bovine serum albumin 0.01%, SDS 0.1%, and 1 mM EDTA. The post-hybridisation washes were with five times concentrated SSC, SDS 0.1% at 54.5°C for 1 h, followed by double strength SSC at room temperature for 30 s.

Bacterial culture and preparation of crude toxin

For routine testing of CT production by *V. cholerae* O1, bacteria were grown overnight in Syncase sucrose broth¹⁹ at 37°C with agitation. Initially these conditions were used to test *V. cholerae* non-O1 strains. For further assays of CT production, culture filtrates were prepared by growing the organisms in various media: Syncase sucrose broth, Syncase glucose broth,¹⁹ Trypticase Soy Broth (Becton Dickinson, Cockeysville, USA),²⁰ Brain Heart Infusion Broth (Unipath Ltd, Basingstoke, Hampshire),²¹ and caseamino yeast extract broth.^{22,23} Strains were inoculated into 10-ml volumes of the respective broths and incubated statically in a 250-ml flask at 30°C and 37°C, or with agitation (120 oscillations/min). The overnight culture was then centrifuged (17000 g for 30 min at 4°C) and the supernate was sterilised by filtration through a Millipore filter (pore size 0.2 µm). A sample of the culture supernate was also heated at 100°C for 15 min. The filtrate and the heated sample were used for both tissue culture tests and immunoassays. All tests were done in duplicate.

Y1 adrenal cell assay for toxin

Culture filtrates were tested for CT on monolayers of Y1 cells.^{21,24} CT and *E. coli* LT characteristically lead to a rounding of the Y1 cells. The effect of the

heated preparation was compared with that of the unheated to ensure that rounding was due to a heat-labile factor. Doubling dilutions of filtrate were used to determine titres of heat-labile enterotoxin and cytotoxin. Neutralisation with antiserum against CT or LT was performed to confirm the specificity of the rounding effect. For the neutralisations, 25- μ l volumes of filtrate and antiserum (1 in 200) were incubated together in 200 μ l of growth medium for 3 h at 37°C before being transferred on to Y1 cells.

Enzyme-linked immunosorbent assay

A modification of the GM₁-ELISA methods of Svennerholm and Holmgren²⁵ and Sack²⁶ was used. A solution of ganglioside GM₁ (2 μ g/ml Supelco, Bellefonte, USA) 100 μ l in PBS was added to micro-ELISA plates (Dynatech Laboratories Ltd, Billingshurst, Sussex) and incubated overnight at room temperature. Extra binding sites were blocked by adding 200 μ l of BSA (Sigma, Poole, Dorset) 1% w/v dissolved in PBS, for 30 min at 37°C. The steps of the procedure were as follows. After each step the plates were washed three times with PBS containing Tween 20 (PBST) 0.05% v/v; 100 μ l of the filtrate were added and the plates were incubated at room temperature for 2 h. One hundred μ l of the appropriate antiserum, diluted 1 in 200 in PBS were added: for CT-ELISA, rabbit anti-CT antiserum (LEP); for CT_B-ELISA, goat anti-CT_B antiserum (Calbiochem Novabiochem, Nottingham); for LT-ELISA, rabbit anti-LT antiserum (LEP). The plates were then incubated for 18 h at room temperature. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Sigma) 100 μ l or alkaline phosphatase-conjugated rabbit anti-goat immunoglobulin (Sigma) 100 μ l were added. Antibody preparations were diluted 1 in 7000 and 1 in 3000 in PBS respectively. The plates were incubated at room temperature for 2 h. Then 200 μ l of p-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer) were added. After 100 min in the dark at room temperature, the reaction was stopped with 25 μ l of 1 M NaOH and the absorbance was read at 405 nm. A control (strain E51116) was included on each plate.

Reversed passive latex agglutination

The VET-RPLA kit (Unipath Ltd) was used for the detection of CT and LT. Doubling dilutions of filtered supernates were tested according to the manufacturer's protocol.

Results

CT_A and CT_B probes at high stringency

The control strains (WBDV-101E, E51116 and B7A) and 790 *V. cholerae* non-O1 strains were tested by hybridisation at high stringency with poly-

nucleotide probes derived from both the CT_A and CT_B regions of the CT gene sequence. Strains WBDV-101E (serogroup O49) and E51116 hybridised with both the CT_A and CT_B probes. Strain B7A did not hybridise under high stringency conditions.

Only two of the 790 *V. cholerae* non-O1 strains were probe-positive. These were: a wild-type strain (E55879) serogroup O?, isolated from a 22-year-old male recently returned to the UK from Egypt; and strain 1322-69, the type strain of serogroup O37.

Of the 788 probe-negative *V. cholerae* non-O1 strains, five were serogroup O49 (all from environmental sources), four were O? (from patients returning from Egypt) and seven were serogroup O37 (all from human sources). The remaining typable strains belonged to 47 different serogroups.

CT_A and CT_B probes at low stringency

The heat-labile enterotoxin produced by *E. coli* is known to be related to CT.²⁷ However, in this study, hybridisation experiments showed that LT⁺ *E. coli* hybridised with the CT probes only at low stringency. *V. cholerae* non-O1 strains were also tested for hybridisation at low stringency with the CT_A and CT_B probes to determine if a CT/LT-related toxin was being produced. A total of 252 strains (representing 37 different serogroups from both human and environmental sources) was tested and all were probe-negative under low stringency conditions.

Y1 mouse adrenal cell assay

In all, 226 (89.6%) of 252 *V. cholerae* non-O1 strains tested in the Y1 cell assay produced a cytotoxic effect on Y1 cells within 24 h; the remaining 26 strains were completely negative. Of the identifiable serogroups producing cytotoxin, the most common were: O26 (23 of 26 strains tested were cytotoxin positive), O2 (10 of 10), O19 (9 of 9), O5 (7 of 7), O13 (4 of 4), O14 (4 of 4), O49 (4 of 4), O76 (4 of 4) and O37 (3 of 3). It is noteworthy that all strains from serogroups O2, O5, O13 and O37 produced cytotoxin, because these serogroups have been reported to be associated with human disease.^{8,9} The probe-positive *V. cholerae* non-O1 strains also produced cytotoxin(s) which made it impossible to distinguish true rounding from early cytotoxic effects. Even when filtrates were diluted to 1 in 156250, cytotoxin was still present. The cytotoxic effect present in the filtrates, even as CT, was destroyed by heating at 100°C for 15 min, but the effect was not neutralised by antiserum to cholera toxin. The control *V. cholerae* O1 strain did not produce cytotoxin under these conditions, but did cause typical rounding of the cells which was confirmed as CT by neutralisation. Similarly, *E. coli* strain B7A produced rounding typical of LT and this effect could be neutralised with anti-cholera antiserum.

The probe-positive *V. cholerae* strains and *E. coli* strain B7A were tested for CT (or LT) production

Table. CT production under different growth conditions

Culture medium	Aeration/temperature (°C)	ELISA* and VET-RPLA†					Y1 Cell Test‡				
		E55879 O?	1322-69 O37	WBDV-101E O49	E51116 O1	B7A <i>Escherichia coli</i>	E55879 O?	1322-69 O87	WBDV-69 O49	E51116 O1	B7A <i>E. coli</i>
Syncase sucrose broth	+ /37	+(32)	+(64)	+(64)	+(32)	+(128)	c	c	c	2560	156250
	- /37	+(8)	+(4)	+(8)	+(4)	+(32)	c	c	c	40	6250
	+ /30	+(16)	+(32)	+(32)	+(32)	+(16)	c	c	c	1280	250
	- /30	+(4)	+(4)	+(4)	+(4)	+(16)	c	c	c	40	250
Syncase glucose broth	+ /37	+(ND)	-(ND)	+(ND)	+(ND)	+(ND)	c	—	c	40	6250
	- /37	-(ND)	-(ND)	-(ND)	+(ND)	+(ND)	—	c	c	40	31250
	+ /30	+(ND)	+(ND)	+(ND)	+(ND)	+(ND)	c	c	c	1280	250
	- /30	+(ND)	+(ND)	+(ND)	+(ND)	+(ND)	c	c	c	320	1250
Trypticase soy broth	+ /37	+(32)	-(0)	+(8)	+(32)	+(128)	c	c	c	160	156250
	- /37	-(0)	-(0)	-(0)	+(32)	-(0)	—	c	—	320	250
	+ /30	+(16)	+(4)	+(4)	+(16)	+(16)	c	c	c	c	1250
	- /30	+(8)	-(0)	-(0)	+(8)	-(0)	c	c	—	40	250
Brain heart infusion broth	+ /37	+(ND)	-(ND)	+(ND)	-(ND)	+(ND)	c	c	c	20	6250
	- /37	+(ND)	-(ND)	-(ND)	-(ND)	+(ND)	c	c	—	—	1250
	+ /30	+(ND)	-(ND)	+(ND)	+(ND)	-(ND)	c	c	c	40	250
	- /30	+(ND)	-(ND)	+(ND)	+(ND)	+(ND)	c	c	c	40	250
Casamino yeast extract broth	+ /37	+(8)	+(8)	+(16)	+(16)	+(32)	c	c	c	160	31250
	- /37	+(4)	+(2)	+(4)	+(8)	+(32)	c	c	c	40	6250
	+ /30	+(8)	+(8)	+(8)	+(8)	+(4)	c	c	c	80	250
	- /30	+(2)	+(2)	+(4)	+(2)	+(4)	c	c	c	20	250

ND, not done; c, cytotoxin.

* ELISA positive OD 405 nm > 1.

† Figure in parenthesis is titre of reversed passive latex agglutination for CT and LT.

‡ The final titre is shown for cytotoxic response.

under various growth conditions (table). For the non-O1 strains, none of the conditions allowed the differentiation of CT from the cytotoxin in the Y1 test. For strain E51116, the highest CT titre (2560) was obtained after overnight incubation with agitation at 30°C in Syncase sucrose broth. *E. coli* B7A gave the highest titre (156250) after overnight incubation at 37°C with agitation with either Syncase sucrose broth or TSB.

ELISA

The probe-positive *V. cholerae* non-O1 strains, E51116 and B7A were tested in the CT, CT_B and LT-ELISA assays. All gave positive results in these three ELISAs.

Ninety-three (36.9%) of 252 probe-negative *V. cholerae* non-O1 strains tested in the CT-ELISA gave a positive result (OD₄₀₅ > 1). These 93 CT-ELISA positive strains also gave positive results in the CT_B-ELISA, but all gave negative results in the LT-ELISA. Fifty-six (60%) of these 93 strains could not be serogrouped. The commonest of the identifiable serogroups were O26 (eight strains) and O19 (seven strains); the remaining 22 strains belonged to 17 other serogroups.

VET-RPLA

The 93 *V. cholerae* non-O1 strains that gave positive results in the CT-ELISA all gave negative results in the VET-RPLA.

The probe-positive strains E55879, 1322-69, E51116 and B7A all gave positive results in this immunoassay. The effect of growth conditions on CT production by the probe-positive strains could be detected easily and

quantified in this immunoassay; there were conditions under which no toxin production could be detected by this immunoassay or by the ELISAs. CT production was optimal in Syncase sucrose broth and casamino yeast extract broth. The highest toxin titres—32 for strain E55879 (O?) and 64 for both type strain 1322-69 (O37) and control strain WBDV-101E (O49)—were obtained with Syncase sucrose broth at 37°C with aeration.

Discussion

In this study, *V. cholerae* non-O1 strains from 50 countries, isolated from both environmental and human sources, were tested for the production of CT by four methods.

Of the *V. cholerae* non-O1 strains which were tested with the CT probes, only three gave positive results, one belonging to serogroup O49 (this was the control strain WBDV-101E), one to O37 and one O? strain (from Egypt). There was no apparent correlation of serogroup with CT production. The low occurrence of CT probe-positive *V. cholerae* non-O1 strains (0.25%) is in agreement with previous reports.^{6,7}

CT and LT are part of a heterogeneous family of enterotoxins and different forms of LT have been described; i.e., LTh-I, LTp-I, LTIIa and LTIIb.²⁸ The suggestion has been made that there may be differences in molecular structure between enterotoxins produced by different serovars of *V. cholerae*.²⁹ In the present study, hybridisation at low stringency with CT_A and CT_B probes provided no evidence for genes in *V. cholerae* non-O1 encoding heat-labile enterotoxins related to CT or LT. As CT_A is the more conserved part of the toxin in the CT/LT family of toxins³⁰ it was

considered possible that some strains might hybridise with the CT_A probe only. However, no such strain was detected in this study.

All probe-positive strains were confirmed as CT-producers by immunoassays. However, the CT-ELISA and CT_B-ELISA also gave positive results with some strains which were negative with the CT probes. The ELISA assay depends on the ability of the GM₁ ganglioside to bind heat-labile enterotoxins, with bound toxin detected by specific antitoxin antibodies. The strains that gave positive CT-ELISA results may be producing a heat-labile enterotoxin distinct from CT,^{23, 31, 32} but nevertheless able to bind to ganglioside GM₁. This presumed toxin also appeared to share epitopes with CT, and more specifically with CT_B. Because of the production of cytotoxin, which masks cytotoxic effects on Y1 cells, it was not possible to show neutralisation of the putative toxin.

All CT-ELISA-positive strains produced cytotoxin in the Y1 cell assay. However, the CT-ELISA did not detect the cytotoxin, since most strains that gave negative results in the CT-ELISA produced cytotoxin on Y1 cells. Cytotoxin production may be important for virulence, as representatives of all the serogroups associated with diarrhoeal illness (O2, O5, O13 and O37) were found to produce cytotoxin.

The production of CT by *V. cholerae* O1 and LT by *E. coli* strain B7A was detected readily by the conventional Y1 cell assay and immunoassays. The probe-positive *V. cholerae* non-O1 strains produced cytotoxin which effectively masked the CT rounding in the Y1 cell assay and varying the growth conditions did

not affect the result. The immunoassays used could detect the presence of CT even in the presence of cytotoxin. The optimal condition for CT production, as detected by immunoassays, was growth in Syncase sucrose broth at 37°C with agitation.

Recent volunteer studies showed that some *V. cholerae* non-O1 strains, which did not produce CT, caused diarrhoea of a similar severity to that seen in cholera.³³ This suggests that factors other than CT, such as the presumed toxin identified in this study, may be important in the pathogenesis of diarrhoea caused by *V. cholerae* non-O1 strains.

In the present study, < 1% of *V. cholerae* non-O1 strains produced CT immunologically indistinguishable from the CT produced by serogroup O1. Because these few strains also produced cytotoxins, CT could be detected only by probe, or by immunoassay under specific growth conditions. Of the probe-negative strains, most (90%) produced cytotoxin(s) detected by Y1 cells. In addition, 37% of probe-negative strains gave positive results in the ELISA for CT but negative results in the ELISA for *E. coli* LT and VET-RPLA. The substance produced was distinct from the cytotoxin(s) detected in the Y1 cell assay. This substance could be detected only in the CT-ELISAs and may be a toxin structurally and antigenically similar to CT. The role in diarrhoeal disease of both the cytotoxin and the substance detected by the CT-ELISA needs to be evaluated.

The help of Dr G. A. Willshaw and Ms A. Thomas with the probe work, and of Mr T. Cheasty and Mr N. Stokes with the serogrouping was greatly appreciated.

References

1. Sakazaki R, Donovan TJ. Serology and epidemiology of *Vibrio cholerae* and *Vibrio mimicus*. *Methods Microbiol* 1984; **16**: 271–289.
2. Blake PA, Weaver RE, Hollis DG. Diseases of humans (other than cholera) caused by vibrios. *Ann Rev Microbiol* 1980; **34**: 341–367.
3. Yamamoto K, Takeda Y, Miwatani T, Craig JP. Purification and some properties of a non-O1 *Vibrio cholerae* enterotoxin that is identical to cholera enterotoxin. *Infect Immun* 1983; **39**: 1128–1135.
4. Shankar P, Agarwal RK, Sanyal SC. Experimental studies on enteropathogenicity of *Vibrio cholerae* serotypes other than O1. *Zentralbl Bakteriol Mikrobiol Hyg (A)* 1982; **252**: 514–524.
5. Datta-Roy K, Banerjee K, De SP, Ghose AC. Comparative study of expression of hemagglutinins, hemolysins, and enterotoxins by clinical and environmental isolates of non-O1 *Vibrio cholerae* in relation to their enteropathogenicity. *Appl Environ Microbiol* 1986; **52**: 875–879.
6. Hanchalay S, Seriwatana J, Echeverria P *et al.* Non-O1 *Vibrio cholerae* in Thailand: homology with cloned cholera toxin genes. *J Clin Microbiol* 1985; **21**: 288–289.
7. Roberts NC, Siebling RJ, Kaper JB *et al.* Vibrios in the Louisiana Gulf Coast environment. *Microbiol Ecol* 1982; **8**: 299–312.
8. WHO Scientific Working Group. Cholera and other vibrio-associated diarrhoeas. *Bull World Health Organ* 1980; **58**: 353–374.
9. Donovan TJ. Serology and serotyping of *Vibrio cholerae*. In: Colwell RR (ed) *Vibrios in the environment*. New York, Wiley Interscience. 1984: 83–101.
10. Aldová E, Lázníčková K, Štěpánková E, Lietava J. Isolation of non-agglutinable vibrios from an enteritis outbreak in Czechoslovakia. *J Infect Dis* 1968; **118**: 25–31.
11. Kamal AM. Outbreak of gastroenteritis by non-agglutinable (NAG) vibrios in the republic of the Sudan. *J Egypt Public Health Assoc* 1971; **46**: 125–173.
12. Ramamurthy T, Garg S, Sharma R *et al.* Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. *Lancet* 1993; **341**: 703–704.
13. Albert MJ, Siddique AK, Islam MS *et al.* Large outbreak of clinical cholera due to *Vibrio cholerae* non-O1 in Bangladesh. *Lancet* 1993; **341**: 704.
14. Dupont HL, Formal SB, Hornick RB *et al.* Pathogenesis of *Escherichia coli* diarrhea. *N Engl J Med* 1971; **285**: 1–9.
15. Maniatis T, Fritsch EF, Sambrook J. *Molecular cloning: a laboratory manual*. New York, Cold Spring Harbor Laboratory. 1982.
16. Kaper JB, Levine MM. Cloned cholera enterotoxin genes in study and prevention of cholera. *Lancet* 1981; **2**: 1162–1163.
17. Dallas WS, Gill DM, Falkow S. Cistrons encoding *Escherichia coli* heat-labile toxin. *J Bacteriol* 1979; **139**: 850–858.
18. Gennaro ML, Greenaway PJ, Broadbent DA. The expression of biologically active cholera toxin in *Escherichia coli*. *Nucleic Acids Res* 1982; **10**: 4883–4890.
19. Finkelstein RA, Atthasampunna P, Chulasamaya M, Charunmethee P. Pathogenesis of experimental cholera: biologic activities of purified procholera toxin. *J Immunol* 1966; **96**: 440–449.
20. Donta ST, Moon HW, Whipp SC. Detection of heat-labile *Escherichia coli* enterotoxin with the use of adrenal cells in tissue culture. *Science* 1974; **183**: 334–336.
21. Nishibuchi M, Seidler RJ. Medium-dependent production of

- extracellular enterotoxins by non-O1 *Vibrio cholerae*, *Vibrio mimicus* and *Vibrio fluvialis*. *Appl Environ Microbiol* 1983; **45**: 228–231.
22. Evans DG, Evans DJ, Gorbach SL. Identification of enterotoxigenic *Escherichia coli* and serum antitoxin activity by the vascular permeability factor assay. *Infect Immun* 1973; **8**: 731–735.
23. Craig JP, Yamamoto K, Takeda Y, Miwatani T. Production of cholera-like enterotoxin by a *Vibrio cholerae* non-O1 strain isolated from the environment. *Infect Immun* 1981; **34**: 90–97.
24. Scotland SM, Gross RJ, Rowe B. Laboratory tests for enterotoxin production, enteroinvasion and adhesion in diarrhoeagenic *Escherichia coli*. In: Sussman M (ed) *The virulence of Escherichia coli; reviews and methods*. London, Academic Press. 1985: 395–405.
25. Svennerholm AM, Holmgren J. Identification of *Escherichia coli* heat-labile enterotoxin by means of a ganglioside immunosorbent assay (GM₁-ELISA) procedure. *Curr Microbiol* 1978; **1**: 19–23.
26. Sack DA, Huda S, Neogi PKB, Daniel RR, Spira WM. Microtiter ganglioside enzyme-linked immunosorbent assay for *Vibrio* and *Escherichia coli* heat-labile enterotoxins and antitoxin. *J Clin Microbiol* 1980; **11**: 35–40.
27. Clements JD, Finkelstein RA. Demonstration of shared and unique immunological determinants in enterotoxins from *Vibrio cholerae* and *Escherichia coli*. *Infect Immun* 1978; **22**: 709–13.
28. Guth BEC, Twiddy EM, Trabulsi LR, Holmes RK. Variation in chemical properties and antigenic determinants among type II heat-labile enterotoxins of *Escherichia coli*. *Infect Immun* 1986; **54**: 529–536.
29. Finkelstein RA, Burks MF, Zupan A, Dallas WJ, Jacob CO, Ludwig DS. Epitopes of the cholera family of enterotoxins. *Rev Infect Dis* 1987; **9**: 544–561.
30. Moseley SL, Falkow S. Nucleotide sequence homology between the heat-labile enterotoxin gene of *Escherichia coli* and *Vibrio cholerae* deoxyribonucleic acid. *J Bacteriol* 1980; **144**: 444–446.
31. Shehabi AA, Richardson SH. Enterotoxigenicity of clinical isolates of non-O1 *Vibrio cholerae*. *Zentralbl Bakteriol Mikrobiol Hyg (A)* 1985; **260**: 311–318.
32. McCardell BA, Madden JM, Shah DB. Isolation and characterization of a cytotoxin produced by *Vibrio cholerae* serogroup non-O1. *Can J Microbiol* 1985; **31**: 711–720.
33. Morris JG, Takeda T, Tall BD *et al.* Experimental non-O group 1 *Vibrio cholerae* gastroenteritis in humans. *J Clin Invest* 1990; **85**: 697–705.