Epidemiol. Infect. (2002), **129**, 245–251. © 2002 Cambridge University Press
DOI: 10.1017/S0950268802007392 Printed in the United Kingdom

Preferential association of the heat-stable enterotoxin gene (*stn*) with environmental strains of *Vibrio cholerae* belonging to the O14 serogroup

B. SARKAR[1], T. BHATTACHARYA[1], T. RAMAMURTHY[1], T. SHIMADA[2], Y. TAKEDA[3] AND G. BALAKRISH NAIR[4]*

[1] *National Institute of Cholera and Enteric Diseases, Beliaghata, Calcutta 700 010, India*
[2] *National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162, Japan*
[3] *Faculty of Human Life Sciences, Jissen Women's University, Tokyo 181-8510, Japan*
[4] *International Centre for Diarrhoeal Diseases Research, Bangladesh* (*ICDDR,B*), *Mohakhali, Dhaka-1212, Bangladesh*

(*Accepted 24 April 2002*)

SUMMARY

Toxigenic *Vibrio cholerae* O1 and O139 serogroups have the capacity of causing epidemic and pandemic cholera but are infrequently found in the environment. The other serogroups are abundant in aquatic environments but do not possess the virulence genes necessary for causing the disease. Of the 559 environmental strains of *V. cholerae*, collected during different periods from environmental samples in Calcutta, 9 (1·6%) harboured the heat-stable enterotoxin gene (*stn*). Six of the 9 strains belonged to the O14 serogroup. Thus, *V. cholerae* strains carrying the *stn* gene revealed preferential association with the O14 serogroup. Three of the six strains harboured the *tcpA* gene of the E1 Tor type, which is an unusual feature among environmental *V. cholerae* strains. A strain that possessed the E1 Tor type *tcpA* also had the CTX prophage. Pulsed field gel electrophoresis (PFGE) revealed that the *stn* gene positive O14 strains of *V. cholerae* were not clonal.

INTRODUCTION

Vibrio cholerae is an autochthonous inhabitant of brackish waters and estuarine systems [1]. Among the 200 currently recognized serogroups of *V. cholerae* [2], only the O1 and O139 serogroups have the capacity of causing epidemic and pandemic cholera. After colonizing the small intestine, *V. cholerae* O1 and O139 produce copious amounts of cholera toxin (CT), which is responsible for the major symptoms associated with the disease cholera. The genes encoding CT are the component of the genome of an unusual lysogenic filamentous phage, CTXΦ [3]. The receptor for CTXΦ is shown to be a type IV pilus, the toxin co-regulated pilus (TCP) and *V. cholerae*, cells that do not express TCP are resistant to CTXΦ infection [3]. The *tcpA* gene is part of *V. cholerae* pathogenicity island (VPI) [4]. The structural features of VPI are suggestive of bacteriophage origin, and there is a report describing the production of a bacteriophage designated VPIΦ [5]. However, what is still not clear is why CTXΦ and VPI are selectively associated with the O1 and the O139 serogroups.

V. cholerae strains belonging to serogroups other than O1 or O139 are ubiquitous in natural aquatic environments such as bays and estuaries [6]. The role played by these strains in human disease is imperfectly understood, although in recent years a variety of virulence factors such as cholera toxin-like enterotoxin [7], Kanagawa haemolysin [8], non-membrane-dam-

* Author for correspondence.

aging cytotoxin (9) and a heat-stable enterotoxin (NAG-ST) [10] have been proposed to explain clinical manifestation of non-O1 gastroenteritis. The NAG-ST, a 17-amino-acid peptide, encoded by the *stn* gene which exhibits remarkable similarity, especially in the carboxyl-terminal toxic domain, to the heat-stable enterotoxins (STs) produced by enterotoxigenic *Escherichia coli* (ETEC) [10], is the other well-studied virulence factor of *V. cholerae*. This gene was found to occur in a small proportion of both environmental and clinical *V. cholerae* non-O1 isolates from diverse geographic areas such as Calcutta [11], Thailand [12], Mexico and the United States [13]. A human volunteer study has clearly demonstrated that in presence of adequate colonization factors, a NAG-ST-producing strain of *V. cholerae* non-O1 caused diarrhoea of a severity comparable to that of typical clinical cholera [14]. Another study established the production of heat-stable enterotoxin by a CT-producing environmental isolate of O1 which was originally isolated from a river in Australia [15].

Analysis of strains of *V. cholerae* collected over a period from different environmental studies conducted in Calcutta revealed preferential association of the *stn* gene with the O14 serogroup. The O14 *stn* gene positive strains were not clonal as determined by PFGE.

MATERIALS AND METHODS

Bacterial strains

The *V. cholerae* strains examined in this study were from two environmental studies conducted during different times. A total of 378 strains of *V. cholerae* non-O1, non-O139 collected from water, sediment, and plankton from a lake during an ecological survey conducted between June 1984 and July 1985 in Calcutta were included in this study. In addition, another 181 strains of *V. cholerae* strains isolated between June 1998 and September 1999 during a survey of sewage water from the outfall channels of the city were also included in this study. These strains were isolated and identified using procedures detailed previously [6, 16]. *V. cholerae* O1 biotype E1 Tor Inaba serotype (strain number GP156), an environmental isolate from a riverine source of Australia [17], carrying the *stn* and *ctx* genes [15] was used as the reference strain for all experiments in this study. *V. cholerae* strains examined in this study were serotyped by the somatic O-antigen serogrouping scheme for *V. cholerae* developed at the National Institute of Infectious Diseases, Tokyo, Japan.

Antibiotic susceptibility testing

The *V. cholerae* strains were examined for resistance to ampicillin (10 μg), chloramphenicol (30 μg), co-trimoxazole (25 μg), ciprofloxacin (5 μg), furazolidone (100 μg), gentamicin (10 μg), neomycin (30 μg), nalidixic acid (30 μg), norfloxacin (10 μg), streptomycin (10 μg) and tetracycline (30 μg) using commercially available disks (Hi-Media, Mumbai, India) as previously described [16]. Characterization as susceptible, intermediate or resistant was based on the size of the zone of inhibition around each disk according to the manufacturer's instructions. *E. coli* strain ATCC 25922 that is sensitive to all drugs was used for quality control.

Suckling mouse assay

The biological activity of *stn* gene positive strains of *V. cholerae* non-O1, non-O139 were examined by the conventional suckling mouse assay following the procedure detailed previously [15]. The culture supernatant of strains, which gave a negative response were re-examined using supernatant concentrated by ammonium sulphate precipitation as described previously [10]. The fluid accumulation ratio (FA) was calculated as the ratio of intestinal weight to remaining carcass weight and values $> 0{\cdot}08$ were considered as a positive test.

PCR assay

A multiplex PCR assay for *ctxA* and *tcpA* (both classical and E1 Tor variants) and a uniplex one for the *stn* gene were performed to identify strains of *V. cholerae* carrying these genes [18, 19]. The multiplex PCR was performed in a reaction volume of 25 μl using 10 ng of genomic DNA, 10 pmol of each of the primers, 1 unit of Taq DNA polymerase (Takara, Shuzo Co. Ltd, Japan) and the following cycling conditions: denaturation at 94 °C for 1 min 30 s, annealing at 60 °C for 1 min 30 s and extension at 72 °C for 1 min 30 s for 30 cycles in an automated thermal cycler (PerkinElmer 2400). For the uniplex PCR, the conditions were same except for annealing

Table 1. *PCR primers used in this study*

Primer	Primer sequence (5′–3′)	Target gene	Amplicon (bp)	Reference
NST-F	CCTATTCATTGCATTAATG	*stn*	215	[19]
NST-R	CCAAAGCAAGCTGGATTGC			
CtxA-F	CTCAGACGGGATTTGTTAGGCACG	*ctxA*	301	[18]
CtxA-R	TCTATCTCTGTAGCCCCTATTACG			
TcpA-F (classical)	CACGATAAGAAAACCGGTCAAGAG	*tcpA* (classical)	617	[18]
TcpA-R (classical)	ACCAAATGCAACGCCGAATGGAGC			
TcpA-F (E1 Tor)	GAAGAAGTTTGTAAAAGAAGAACAC	*tcpA* (E1 Tor)	471	[18]
TcpA-R (E1 Tor)	GAAAGGACCTTCTTTCACGTTG			

temperature, which was reduced to 55 °C. The primers used in this study are shown in Table 1.

DNA extraction

A modification of the method of Murray and Thompson was used for DNA extraction [21]. Briefly, cells from 18-h LB culture were harvested and suspended in TE buffer [10 mM Tris–HCl, 1 mM EDTA (pH 8)], treated with 10% (wt/vol), sodium dodecyl sulphate and freshly prepared proteinase-K (Sigma Chemical Co., St. Louis, MO) followed by incubation at 37 °C for 1 h. Following this, 10% cetyl trimethyl ammonium bromide (CTAB) in 0·7 M NaCl was added and the preparation incubated at 65 °C for 10 min. The aqueous phase was treated with 70% ethanol. The extracted nucleic acid was suspended in TE and treated with RNase at 37 °C for 30 min.

DNA probes and hybridization

The DNA fragment used as the probe for the *stn* gene for restriction fragment length polymorphism (RFLP) studies was generated by PCR using primers specific for *stn* gene with *V. cholerae* strain GP156 as the template. The 215 bp amplicon was excised from the gel and purified using the Qiagen gel extraction kit. RFLP of the *stn* gene was performed using genomic DNA from the *V. cholerae* strains after digesting with *Eco*RI and *Hin*dIII and the fragments were electrophoretically separated in a 0·8% agarose gel using TAE buffer (40 mM Tris acetate EDTA, Amersham International, plc, Buckinghamshire, England). Southern-transfer was done using Hybond™-N + Nucleic acid transfer membrane. This membrane was exposed to the *stn* gene probe conjugated with horseraddish peroxidase to allow hybridization, which was detected by use of a chemiluminescent substrate (Amersham). The membrane was then exposed to an X-Ray film (Fuji film, Fuji, Japan), which was developed to detect the DNA bands that had hybridized. For *ctxA*–RFLP, the *ctxA* probe consisting of a 540 bp *Xba*I–*Cla*I fragment of *ctxA* cloned in pKTN901 using *Eco*RI linkers was employed [16]. For preparing this probe, the plasmid was isolated and digested with appropriate enzymes and electrophoresis was performed with a λ *Hin*dIII (Takara, Shuzo Co. Ltd, Japan) marker and the 540 bp fragment was excised and purified using the Qiagen gel extraction kit. The *ctxA*–RFLP was performed using genomic DNA digested with *Pst*I, *Bgl*I and *Bgl*II (Takara) following procedures outlined for *stn* gene.

PFGE

PFGE agarose plugs of the genomic DNA were prepared as described previously [21]. These blocks were equilibrated in restriction enzyme buffer for 1 h at room temperature and then cleaved with 50 U of *Not*I in fresh buffer (Takara, Shuzo Co. Ltd, Japan) overnight at 37 °C. PFGE of the *Not*I digested inserts was carried out in a contour-clamped homogeneous electric field on a CHEF Mapper system (Bio-Rad, USA) using 1% PFGE grade agarose in 0·5× TBE (44·5 mM Tris–HCl, 44·5 mM boric acid, 1·0 mM EDTA, pH 8·0) for 40 h 24 min. A DNA size standard (lambda ladder, NEB UK) was used as the molecular mass standard, and a minichiller (model 1000; Bio-Rad, USA) maintained the temperature of the buffer at 14 °C. Run conditions were generated by the auto-algorithm mode of the CHEF mapper PFGE system based on the DNA size range of 20–300 kb. After electrophoresis, the gel was stained in ethidium bromide solution (1 μg/ml) for 30 min, destained in water for 15 min, then photographed under UV-light. Banding patterns were analysed and comparisons made using diversity database fingerprinting software (Bio-Rad), employing the Dice similarity coefficient in conjunction with the unweighted pair group method using arithmetic averages (UPGAMA) for clustering.

Table 2. *Serotype, antibiogram, virulence genotype and biological activity of NAG-ST of* V. cholerae *strains carrying the* stn *gene*

Strain no.	Date of isolation	Source/sample	Serotype	Virulence genes		Restriction fragment length (in kb)					Anti-biogram	Suckling mice assay (FA ratio)
						ctxA			*stn*			
				ctxA	*tcpA* El Tor	*Pst*I	*Bgl*I	*Bgl*II	*Eco*RI	*Hin*dIII		
X4	6.6.98	Sewage Water	O14	−	−				> 23·1	14	AFzNST	0·092*
X26	24.7.98	Sewage Water	O14	−	−				> 23·1	16	AFzNST	0·095*
X67	15·10·98	Sewage Water	O6	+	+	5·7, 7·2	6·9, 7·2	7·2, 8	9·1	8·5	AFzNST	0·085
X119	3.5.99	Soil	O14	−	+				9·3, > 23·1	4·6, 7·2	AFzN	0·1
X140	15.5.99	Sewage Water	R†	−	−				3·2	3·8	–	0·096†
X142	15.5.99	Sewage Water	O14	−	−				> 23·1	16·9	AFzN	0·087
VCE89	1984–5	Lake Plankton	O43	−	−				14·1	4·1	AFzS	0·091
VCE101	1984–5	Lake Plankton	O14	−	+				> 23·1	19·1	Fz	0·098
VCE196	1984–5	Sewage Water	O14	−	−				> 23·1	18·4	AFzN	0·095
GP156	Not known	Albert/Logan river, Australia	O1	+	+	5·8	7, 12	7·8	9·2	3·8	AS	0·1

* After concentrating; † Rough. Abbreviations: A, ampicillin; Fz, furazolidone; N, neomycin; S, streptomycin; T, tetracycline.

Bacterial isolates differing by a single genetic event, reflected as a difference of 1–3 bands, are closely related. Isolates differing by 4–6 bands, representing 2 independent genetic changes, were possibly related and more than 6 band differences were considered unrelated and typed as distinct type as proposed by Tenover et al. [22].

RESULTS

Two different collections of *V. cholerae* strains isolated from the aquatic environment of Calcutta were examined by PCR for the presence of the *stn* gene. Out of 559 strains, only 9 (1·6%) were positive for the *stn* gene. Three of the 9 strains (X67, X119, VCE101) were positive for the E1 Tor variant of *tcpA* and 1 (X67) of the 3 was also positive for *ctxA*. Of the 9 *stn*-positive strains, 6 (66·7%) belonged to the O14 serogroup and the remaining 2 (22·2%) belonged to serogroups O43 (VCE89) and O6 (X67). One strain (X140) was identified as a rough strain. As shown in Table 2, six strains expressed NAG-ST with the heat-treated culture supernatants in the suckling mice assay while for strains X4, X26, and X140, the culture supernatant gave positive fluid accumulation (FA) ratios only after concentration by ammonium sulphate precipitation, indicating that all the strains produced biologically active toxin. The antibiotic susceptibilities of the *stn*-positive strains of *V. cholerae* are given in Table 2. All the strains, with the exception of one, were resistant to furazolidone and 7 of the 9 strains were resistant to ampicillin also (Table 2).

The results of the RFLP analyses of the *stn* gene by use of the restriction enzymes *Hin*dIII and *Eco*RI are shown in Table 2. Eight strains and the reference strain GP156 contained a single copy of the *stn* gene whereas X119 had two copies of this gene. RFLP of *ctxA* using restriction enzymes *Pst*I, *Bgl*I and *Bgl*II revealed two bands for the strain X67. A single band after *Pst*I and *Bgl*II digestion and two bands after *Bgl*I digestion was observed in the reference strains GP156 (Table 2).

The *Not*I PFGE profiles of the *V. cholerae* strains revealed dissimilar patterns (Fig. 1) indicating that the *stn* gene positive *V. cholerae* strains are phylogenetically unrelated as shown in the Figure 2 with the exception of the strains X4 (O14) and X142 (O14) (differ 2–3 bands). The level of similarity between the strains X4 and X142 was 73%.

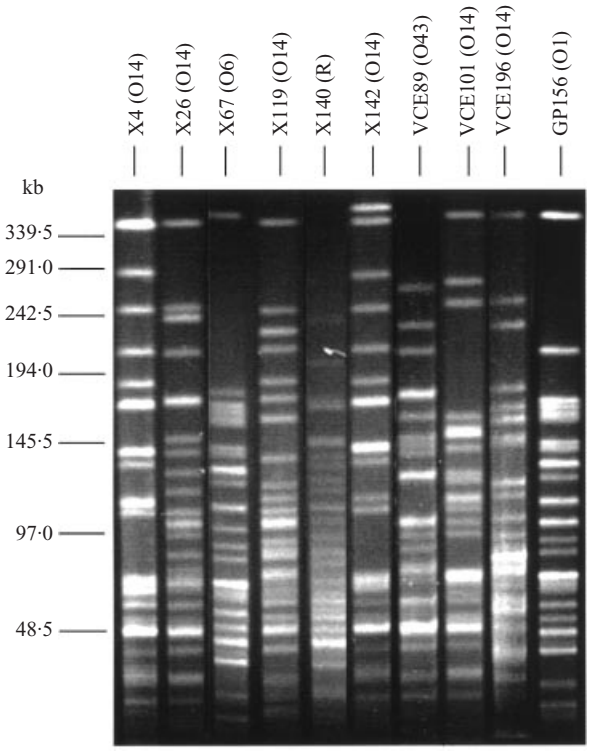

Fig. 1. PFGE profiles of *stn* gene positive environmental strains using *Not*I enzyme.

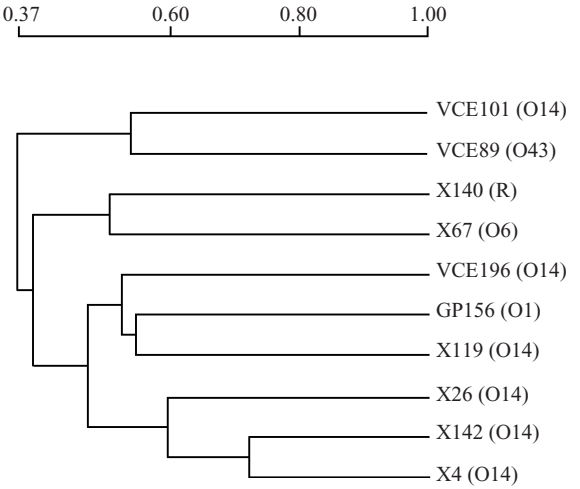

Fig. 2. Results of phylogenetic analysis derived from PFGE profiles of *stn* gene positive strains. The similarity scale is above the dendrogram.

DISCUSSION

V. cholerae is a diverse species consisting of both pathogenic and non-pathogenic strains. The pathogenic strains of *V. cholerae* belong to serogroups O1 and O139 and possess specific virulence determinants (CT, TCP and others) encoded within a filamentous bacteriophage (CTXΦ) or pathogenicity island (VPI). Presence of toxin genes in defined serogroups of *V. cholerae*, like CT with O1 and O139, is a phenomenon not completely understood so far. It is now known that CT genes are part of the genome of a lysogenic filamentous bacteriophage whose receptor is the *tcpA* which, in turn, is the product of a cluster of genes located in the VPI. The importance of heat-stable enterotoxin of *V. cholerae* non-O1 as a virulence factor was clearly established in a human volunteer study [14]. When tested in the suckling mouse assay, we found that all the *stn* harbouring *V. cholerae* O14 strains caused fluid accumulation, confirming their virulence. In this study, we fortuitously observed another association of sorts with the presence of the *stn* gene being linked with the O14 serogroup of *V. cholerae* strains isolated from Calcutta aquatic environments. Likewise, about 50% of the 14 *stn* gene positive strains of *V. cholerae* non-O1, non-O139 isolated from a major shrimp production area in southern Thailand belonged to the O14 serogroup [23].

We went on to investigate whether the O14 strains, which harboured the *stn* gene, are clonal by using PFGE. By this technique, it was clear that most of the O14 strains were not clonal as reflected by the different PFGE profiles. A phylogenetic tree based on the PFGE profiles was also made to analyse the relationship between these O14 strains. This cluster analysis also confirmed the finding that none of the O14 serotype strains were clonal but some of them were closely related to each other. In spite of the diverse genetic background and *stn* polymorphism, we found the preferential association of *stn* gene with O14 serogroup of *V. cholerae* isolated from the environment. This preferential selection is some what similar to the one reported for *E. coli* strains belonging to the same serotype that did not necessarily fall into the same cluster nor were they clones belonging to the same disease category [24]. Southern blot analysis of the *stn* gene positive strains indicated the genetic unrelatedness within the coding sequence of the *stn* gene in *V. cholerae* non-O1, non-O139 strains recovered from the environment.

We examined all the strains that harboured *stn* for other established virulence genes of *V. cholerae* (those present in the CTXΦ) to determine the potential of these strains to cause diarrhoeal disease. By PCR it was found that the strain X67 harboured *ctxA*. This strain produced copious fluid accumulation in the rabbit ileal loop test confirming production of cholera toxin *in vivo* (data not shown). The two environmental strains of *V. cholerae* X67 and GP156 (Australian

isolate) are distinct by themselves in their genomes as shown in the PFGE analysis and in the *ctxA* RFLP. Presence of E1 Tor *tcpA* and the CTX prophage in an environmental *V. cholerae* non-O1, non-O139 strain is uncommon. TCP is an important virulence factor of *V. cholerae* which has a role in colonization of the organism in human gut mucosa [25]. In addition, TCP acts as a receptor for CTXΦ, which can infect *V. cholerae*, leading to conversion of non-toxigenic to a toxigenic strain [3].

ACKNOWLEDGEMENTS

This work was supported in part, by the Council of Scientific and Industrial Research (project no. 37 (1019)/99/EMRII) and the Japan International Co-operation Agency (JICA/NICED project No. 054-1061-E-0). The award of a Senior Research Fellowship to T. Bhattacharya by CSIR, New Delhi, India is gratefully acknowledged.

REFERENCES

1. Colwell RR. Global climate and infectious disease: the cholera paradigm. Science 1996; **274**: 2025–31.
2. Yamai S, Okitsu T, Shimada T, Katsube Y. Distribution of serogroups of *Vibrio cholerae* non-O1 non-O139 with specific reference to their ability to produce cholera toxin, and addition of novel serogroups. Jpn J Assoc Infect Dis 1997; **71**: 1037–45.
3. Waldor MK, Mekalanos JJ. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science 1996; **272**: 1910–4.
4. Karaolis DKR, Johnson JA, Baily CC, Boedker EC, Kaper JB, Reeves PR. A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. Proc Natl Acad Sci USA 1998; **95**: 3134–9.
5. Karaolis DKR, Somara S, Maneval Jr. DR, Johnson JA, Kaper JB. A bacteriophage encoding pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. Nature 1999; **399**: 375–9.
6. Nair GB, Sarkar BL, De SP, Chakrabarti MK, Bhadra RK, Pal SC. Ecology of *Vibrio cholerae* in the freshwater environs of Calcutta, India. Microb Ecol 1988; **15**: 203–15.
7. Yamamoto K, Takeda Y, Miwatani T, Craig JP. Evidence that a non-O1 *Vibrio cholerae* produces enterotoxin that is similar but not identical to cholera enterotoxin. Infect Immun 1983; **41**: 896–901.
8. Yoh M, Honda T, Miwatani T. Production by non-O1 *Vibrio cholerae* of hemolysin related thermostable direct hemolysin of *Vibrio parahaemolyticus*. FEMS Microbiol Lett 1985; **29**: 197–200.
9. Saha PK, Nair GB. Production of monoclonal antibodies to the non-membrane damaging cytotoxin (NMDCY) purified from *Vibrio cholerae* O26 and distribution of NMDCY among strains of *Vibrio cholerae* and other enteric bacteria determined by monoclonal-polyclonal sandwich enzyme-linked immunosorbent assay. Infect human 1997; **65**: 801–5.
10. Arita M, Takeda T, Honda T, Miwatani T. Purification and characterisation of *Vibrio cholerae* non-O1 heat-stable enterotoxin. Infect Immun 1986; **52**: 45–9.
11. Pal A., Ramamurthy T, Bhadra RK, et al. Reassessment of the prevalence of heat-stable enterotoxin (NAG-ST) among environmental *Vibrio cholerae* non-O1 strains from Calcutta, India, by using a NAG-ST DNA probe. Appl Environ Microbiol 1992; **58**: 2486–9.
12. Bagchi K., Echeverria P, Arthur JD, Sethabutr O, Serichantalergs O, Hoge CW. Epidemic of diarrhoea caused by *Vibrio cholerae* non-O1 that produced heat-stable toxin among Khmers in a camp in Thailand. J Clin Microbiol 1993; **31**: 1315–7.
13. Hoge CW, Sethabutr O, Bodhidatta L, Echeverria P, Robertson DC, Morris Jr. JG. Use of a synthetic oligonucleotide probe to detect strains of non-serovar O1 *Vibrio cholerae* carrying the gene for heat-stable enterotoxin (NAG-ST) J Clin Microbiol 1990; **28**: 1473–6.
14. Morris Jr. JG, Takeda T, Tall BD, et al. Experimental non-O1 *Vibrio cholerae* gastroenteritis in humans. J Clin Invest 1990; **85**: 697–705.
15. Takeda T, Peina Y, Ogawa A, et al. Detection of heat-stable enterotoxin in a cholera-toxin gene positive strain of *Vibrio cholerae* O1. FEMS Microbiol Lett 1991; **80**: 23–8.
16. Mukhopadhyay AK, Garg S, Mitra R, et al. Temporal shifts in traits of *Vibrio cholerae* strains isolated from hospitalized patients in Calcutta: a 3-year (1993 to 1995) analysis. J Clin Microbiol 1996; **34**: 2537–43.
17. Mallard KE, Desmarchelier PM. Detection of heat-stable enterotoxin genes among Australian *Vibrio cholerae* strains. FEMS Microbiol Lett 1995; **127**: 111–5.
18. Keasler SP, Hall RH. Detecting and biotyping *V. cholerae* O1 with multiplex polymerase chain reaction. Lancet 1993; **341**: 1661.
19. Ogawa A, Kato J, Watanabe H, Nair GB, Takeda T. Cloning and nucleotide sequence of a heat-stable enterotoxin gene from *Vibrio cholerae* non-O1 isolated from a patient with traveler's diarrhea. Infect Immun 1990; **58**: 3325–9.
20. Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. Nucl Acids Res 1980; **8**: 4321–5.
21. Yamasaki S, Nair GB, Bhattacharya SK, Yamamoto S, Kurazono H, Takeda Y. Cryptic appearance of a new clone of *Vibrio cholerae* O1 biotype E1 Tor in Calcutta, India. Microbiol Immunol 1997; **41**: 1–6.
22. Tenover FC, Arbeit RD, Goering RV, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 1995; **33**: 2233–9.
23. Dalsgaard A, Serichantalergs O, Shimada T, Sethabutr O, Echeverria P. Prevalence of *Vibrio cholerae* with heat-stable enterotoxin (NAG-ST) and cholera toxin

genes; restriction fragment length polymorphisms of NAG-ST genes among *V. cholerae* O serogroups from a major shrimp production area in Thailand. J Med Microbiol 1995; **43**: 216–20.

24. Pupo GM, Karaolis DKR, Lan R, Reeves PR. Evolutionary relationships among pathogenic and nonpathogenic *Escherichia coli* strains inferred from multilocus enzyme electrophoresis and *mdh* sequence studies. Infect Immun 1997; **65**: 2685–92.
25. Herrington DA, Hall RH, Losonsky GA, Mekalanos JJ, Taylor RK, Levine MM. Toxin, toxin-corregulated pili, and the *toxR* regulon are essential for *Vibrio cholerae* pathogenesis in humans. J Exp Med 1988; **168**: 1487–92.