Multilocus genetic analysis reveals that the Australian strains of *Vibrio cholerae* O1 are similar to the pre-seventh pandemic strains of the El Tor biotype

Ashrafus Safa,¹ Nurul A. Bhuiyan,² Denise Murphy,³ John Bates,³ Suraia Nusrin,² Richard Y. C. Kong,¹ Manas Chongsanguan,⁴ Wanpen Chaicumpa⁵ and G. Balakrish Nair⁶

Correspondence

Ashrafus Safa

mrpsafa@yahoo.com

¹Department of Biology and Chemistry, City University of Hong Kong, 83 Kowloon Tong, Kowloon, Hong Kong SAR

²International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka 1212, Bangladesh

³Public Health Microbiology Laboratory, Forensic and Scientific Services, Cooper, Coopers Plains, Queensland, Australia

⁴Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Bangkok 10400, Thailand

⁵Molecular Microbiology and Molecular Immunology Laboratories, Faculty of Allied Health, Thammasat University, Pratumtance 12121, Thailand

⁶National Institute of Cholera and Enteric Diseases, Kolkata, India

Episodes of cholera stemming from indigenous *Vibrio cholerae* strains in Australia are mainly associated with environmental sources. In the present study, 10 *V. cholerae* O1 strains of Australian origin were characterized. All of the strains were serogroup O1 and their conventional phenotypic traits categorized them as belonging to the El Tor biotype. Genetic screening of 12 genomic regions that are associated with virulence in *V. cholerae* showed variable results. Analysis of the *ctxAB* gene showed that the Australian environmental reservoir contains both toxigenic and non-toxigenic *V. cholerae* strains. DNA sequencing revealed that all of the toxigenic *V. cholerae* strains examined were of *ctxB* genotype 2. Whole genome PFGE analysis revealed that the environmental toxigenic *V. cholerae* O1 strains were more diverse than the non-toxigenic environmental O1 strains, and the absence of genes that make up the *Vibrio* seventh pandemic island-I and -II in all of the strains indicates their pre-seventh pandemic ancestry.

Received 21 June 2008

Accepted 5 September 2008

INTRODUCTION

Vibrio cholerae is an autochthonous inhabitant of the aquatic environment. It colonizes the human intestine and causes a severe form of diarrhoea known as cholera. *V. cholerae* strains are subclassified primarily based on the diversity of their somatic 'O' antigen structure and to date, more than 200 serogroups of *V. cholerae* have been documented. Of these serogroups, it is mostly *V. cholerae* O1 and O139 strains that have been linked to epidemic and pandemic cholera. Based on distinct biochemical and phenotypic traits, *V. cholerae* O1 strains have been further

classified into two biotypes, classical and El Tor. The complete genome sequence of El Tor strain (N16961) has provided additional information about the structural and functional features of the *V. cholerae* genome (Heidelberg *et al.*, 2000). The critical role played by the *ctxAB*-encoded cholera toxin in causing the disease and the origin of its phage (CTXΦ) is now known (Waldor & Mekalanos, 1996). Allelic variations in the different virulence-associated genes of integrated CTXΦ and chromosomal origin in *V. cholerae* strains have already been reported and used as important markers to differentiate *V. cholerae* at the strain level (Rader & Murphy, 1988; Faast *et al.*, 1989; Kimsey *et al.*, 1998; Bhattacharya *et al.*, 2006). The discovery of the self-transducing ability of the RS1 satellite phage and the exclusive presence of the *rtxC* gene of the RTX cluster in El Tor strains are a few of the other significant observations made in previous years (Lin *et al.*, 1999; Faruque *et al.*,

Abbreviations: CCA, chicken blood cell agglutination; MSHA, mannose-sensitive haemagglutinin; VSP, *Vibrio* seventh pandemic island.

The GenBank/EMBL/DDBJ accession numbers for the *ctxB* gene sequences of *V. cholerae* O1 and O139 are EU828583–EU828588.

2002). Recently, two genomic islands, *Vibrio* seventh pandemic island (VSP)-I and -II, were shown to be unique to the El Tor strains of the seventh pandemic, that is, they were absent from both the pre-seventh pandemic El Tor strains and the classical biotype strains (Dziejman *et al.*, 2002). These two islands are believed to be associated with the pandemic tendency of the seventh pandemic El Tor *Vibrio* strains (Faruque & Mekalanos, 2003).

The first six pandemics of cholera were recorded between 1817 and 1923. The sixth and, presumably, the fifth pandemics were caused by *V. cholerae* O1 of the classical biotype. In 1961, the seventh pandemic, caused by the *V. cholerae* O1 El Tor biotype, began on the island of Sulawesi in Indonesia (Kaper *et al.*, 1995) and has continued until today. Between the end of the sixth pandemic and the start of the seventh, there was an intervening period of 38 years during which time cholera did not spread in the form of a pandemic, but occurred intermittently or caused local outbreaks. The *V. cholerae* O1 strains isolated during this period are referred to as pre-seventh pandemic El Tor strains (Byun *et al.*, 1999). The second cholera-causing serogroup, O139, was identified in late 1992 (Shimada *et al.*, 1993). *V. cholerae* O139 initially spread rapidly and caused several outbreaks of cholera on the Indian subcontinent, but cholera due to O139 strains has recently declined, although they continue to coexist with strains of the O1 serogroup (Albert & Nair, 2005).

Australia recorded cholera just 15 years after the first pandemic began in India in 1831 (Barua, 1992), although the disease only persisted for a short time. During the seventh pandemic, apart from a few imported cases, Australia had no indigenous cases of cholera until the mid-1970s (Sutton, 1974). Following the first indigenous cholera case in south-east Queensland in 1977, Australia started to report sporadic isolation of toxigenic El Tor strains of *V. cholerae* O1 from patients suffering from diarrhoea (Rao & Stockwell, 1980; Rogers *et al.*, 1980). Epidemiological investigations showed that all of these cases were directly or indirectly associated with the river waters of Australia and suggested that these water bodies may serve as a reservoir of *V. cholerae* (Blake, 1994). Molecular typing using techniques such as multilocus enzyme electrophoresis, ribotyping and the DNA sequencing of the *ctxB* gene showed that the Australian toxigenic *V. cholerae* strains were indigenous and likely to be clonal (Olsvik *et al.*, 1993; Popovic *et al.*, 1993; Wachsmuth *et al.*, 1993).

Given the indigenous nature and genetic disparities of the Australian strains, compared with other clones of *V. cholerae*, we adopted a PCR-based multilocus gene profiling approach using the most current knowledge from *V. cholerae* research to characterize Australian *V. cholerae* strains of environmental and clinical origin.

METHODS

Bacterial strains. This study was performed on 10 *V. cholerae* O1 strains collected in Australia (available from the culture collection of

the Public Health Microbiology Laboratory, Forensic and Scientific Services, Queensland) between 1977 and 1986. *V. cholerae* O1 strains BX330286, 12129(1), 220386, 120186, 366(1), 362(2), P358, 6732/80 and 365(2) were obtained from water sources, while strain Cis77 was isolated from human blood. *V. cholerae* O1 classical 569B and El Tor N16961 strains were used as references.

PCR and serogrouping. All strains were confirmed as *V. cholerae* by *ompW*-specific PCR as described by Nandi *et al.* (2003). Serogrouping and serotyping were confirmed by serology using polyvalent O1 and monovalent Inaba- and Ogawa-specific antisera, and by using the O1 (*rfbO1*), O139 (*rfbO139*) and cholera toxin (*ctxAB*) gene-specific multiplex PCR assay described by Hoshino *et al.* (1998). PCR reagents and kits were obtained either from Perkin-Elmer or Invitrogen. PCR products were analysed by electrophoresis in 1% agarose gels, which were stained with ethidium bromide and visualized using the Gel Doc 2000 gel documentation system (Bio-Rad).

Biotyping. All strains examined were tested for susceptibility to polymyxin B (50 U), chicken blood cell agglutination (CCA) and sensitivity to group IV (classical) and group 5 (El Tor) phages using standard procedures (WHO, 1987). To complement the biotyping tests, PCR assays targeting the *tcpA* (classical and El Tor variants) (Keasler & Hall, 1993) and *rstR* genes were performed using procedures described previously (Nusrin *et al.*, 2004; Safa *et al.*, 2006).

Isolation of genomic DNA. Genomic DNA was extracted from overnight cultures in Luria-Bertani (LB) broth using standard methods (Sambrook *et al.*, 1989) with slight modifications. Briefly, cells were harvested by centrifugation and the pellets were resuspended in TES buffer (10 mM Tris/HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 10% SDS) and incubated at 65 °C for 10 min. Following proteinase K treatment at 50 °C for 18 h, DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated in ethanol and DNA dissolved in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). RNase treatment was performed at 37 °C for 1 h, and ethanol-precipitated DNA was dissolved in TE buffer and stored at -20 °C.

Multilocus PCR analysis. PCR-based multilocus genetic screening was performed on 12 *V. cholerae* O1 isolates, including classical and El Tor reference strains, by PCR amplification of internal fragments of 12 virulence-associated genes and/or gene clusters [VSP-I, VSP-II, mannose-sensitive haemagglutinin (MSHA) pilin, HlyA, VPI-1, VPI-2, *pilE*, RTX, RS1Φ, CTXΦ, toxin-linked cryptic plasmid (*tlc*) and class 1 integrin (*int14*)]. The PCR conditions and 32 pairs of primers used in this analysis were previously described by Chow *et al.* (2001) and O'Shea *et al.* (2004).

Nucleotide sequencing of *ctxB*. PCR amplification of *ctxB* was performed in a 25 µl reaction mixture in a Peltier Thermal Cycler (PTC-200; M. J. Research). The PCR primers and conditions used were as described by Mitra *et al.* (2001). PCR products were purified with a Microcon centrifugal filter device (Millipore) according to the manufacturer's instructions, and cycle sequencing was performed in a 20 µl reaction mixture containing 40 ng purified PCR product, 4 µl ready reaction mixture and 3.2 pmol each primer. After 25 cycles of amplification, unincorporated nucleotides were removed by ethanol precipitation. Dried template was resuspended in template suppression reagent and sequencing was performed using an automated 310 DNA sequencer (Applied Biosystems). DNA chromatograms were inspected using Chromas 2.23 (Technelysium). Nucleotide sequences of six representative test isolates (data not shown) were compared with the corresponding sequences of the N16961 El Tor and 569B classical reference strains (GenBank accession numbers NC_002505 and U25679, respectively) using BLAST (Altschul *et al.*, 1997). Multiple sequence alignments were developed using CLUSTAL_X version 1.81.13

and DNA sequences were translated using GeneDoc version 2.6.002 alignment editor.

PFGE. Intact agarose-embedded chromosomal DNA was prepared from *V. cholerae* O1 isolates and PFGE was performed using a contour-clamped homogeneous electric field (CHEF-Mapper) apparatus (Bio-Rad) according to the PulseNet *V. cholerae* subtyping protocol (Cooper *et al.*, 2006). Genomic DNA was digested with *NotI* (10 U μl^{-1} stock; Invitrogen) and DNA fragments were separated in 1% SeaKem Gold agarose in 0.5× Tris/borate/EDTA buffer. Genomic DNA of *Salmonella braenderup* H9812 digested with 40 U *XbaI* (Invitrogen) was used as a size marker. Following electrophoresis, gels were stained with ethidium bromide (50 $\mu\text{g ml}^{-1}$) for 30 min and destained with reagent grade water. Images were captured using the Gel Doc 2000 and Gel Doc XR systems (Bio-Rad).

RESULTS AND DISCUSSION

Cholera due to indigenous strains of *V. cholerae* was reported in Australia in late 1977, more than 15 years after the seventh pandemic El Tor biotype began spreading around the globe in 1961. The *V. cholerae* strains isolated from patients during that time were of environmental origin and were toxigenic (Olsvik *et al.*, 1993; Rao & Stockwell, 1980; Rogers *et al.*, 1980). In this study, we aimed to analyse the environmental and clinical strains of Australian *V. cholerae* to gain a better understanding of their virulence and genetic lineage based on recently available information about the genome of *V. cholerae* O1. All 10 Australian strains were positive for the species-specific *ompW* gene and were therefore confirmed as *V.*

cholerae (data not shown). Multiplex PCR showed that all of the strains harboured the *rfbO1* gene, but were negative for the *rfbO139* gene and thereby confirmed them as serogroup O1 (data not shown). The serogroup and serotypes of these strains determined using polyvalent and monovalent antisera are shown in Table 1. Except for the clinical isolate, Cis77, all of the strains were resistant to polymyxin B (50 U) and agglutinated with chicken blood cells, which are typical traits of the El Tor biotype. Eight of the 10 strains were resistant to classical phage IV but sensitive to El Tor phage 5, and the remaining two were resistant to both phages (Table 1). The PCR-based multilocus genetic analysis of the 12 regions, namely VSP-I, VSP-II, MSHA pilin, *hlyA*, VPI-1, VPI-2, *pilE*, RTX, RS1Φ, CTXΦ, *tlc* and *int4*, accounted for approximately 165 kb of the *V. cholerae* genome. In the VPI-1 gene cluster, the major virulence-associated genes, *tcpA*, *toxT* and *acfB*, were present in seven isolates but absent in three (Tables 1 and 2). These genes are mainly associated with the colonization that occurs prior to infection. Most of the pathogenic strains of *V. cholerae* that were isolated during the sixth, seventh, and pre-seventh pandemics were shown to possess these genes (O'Shea *et al.*, 2004). The MSHA cluster encodes a type-IV pilus and is expressed by strains of the El Tor biotype, but rarely by those of the classical biotype (Jonson *et al.*, 1991). All of the genes of the MSHA gene cluster were present in all of the Australian strains (data not shown). The *stn* gene of the VPI-2 gene cluster encodes a heat-stable enterotoxin in *V. cholerae* strains, which is believed to be present only in non-O1 strains of *V.*

Table 1. Phenotypic and genotypic traits of *V. cholerae* O1 strains isolated in Australia, 1977–1986

Strains	Year of isolation*	Source	Serotype	Phenotypic traits†				Genotypic traits‡					
				Polymyxin B (50 U)	CCA	Phage IV (classical)	Phage 5 (El Tor)	<i>tcpA</i> ‡	<i>tcpA</i> §	<i>rstR</i> ‡	<i>rstR</i> §	<i>rstR</i>	<i>rstR</i> ¶
BX330286	1986	Water	Inaba	–	+	R	S	–	+	+	–	–	+
12129(1)	1985	Water	Inaba	–	+	R	S	–	–	–	–	–	–
220386	1986	Water	Inaba	–	+	R	S	–	–	–	–	–	–
120186	1986	Water	Inaba	–	+	R	S	–	+	+	–	–	–
366(1)	NK	Water	Inaba	–	+	R	S	–	+	+	–	–	+
362(2)	NK	Water	Inaba	–	+	R	S	–	+	+	–	–	+
P358	1986	Water	Ogawa	–	+	R	R	–	–	–	–	–	–
6732/80	1980	Water	Inaba	–	+	R	S	–	+	+	–	–	–
365(2)	NK	Water	Inaba	–	+	R	S	–	+	+	–	–	+
Cis77	1977	Clinical	Inaba	–	–	R	R	–	+	+	–	–	–
Classical 569B (reference strain)	1948	Clinical	Inaba	+	–	S	R	+	–	+	–	–	–
El Tor N16961 (reference strain)	1971	Clinical	Inaba	–	+	R	S	–	+	–	+	–	–

*NK, Not known.

†+, Positive; –, negative; S, sensitive; R, resistant.

‡Classical type.

§El Tor type.

||Calcutta type.

¶Environmental type.

Table 2. Prevalence of 12 virulence-associated genes or gene clusters among 10 Australian isolates of *V. cholerae* O1

Other virulence-associated genes or gene clusters that tested positive in all strains examined in this study included MSHA, *hlyA*, *pilE*, *rtxA*, *zot* and *int14* (data not shown in Table 2). +, Positive; –, negative.

Strains	VSP-I					VSP-II	VPI-1		VPI-2	RTX	RS1	CTXΦ			TLC
	VCO175	VCO178	VCO180	VCO183	VCO185	(VCO490-VCO517)	<i>toxT</i>	<i>acfB</i>	<i>stn</i>	<i>rtxC</i>	<i>rstC</i>	<i>rstA</i>	<i>orfU</i>	<i>ctxAB</i>	<i>t1c</i>
BX330286	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
12129(1)	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
220386	–	+	+	+	+	–	–	–	–	+	–	–	–	–	–
120186	–	–	–	–	–	–	+	+	+	+	–	+	+	+	+
366(1)	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
362(2)	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
P358	–	+	+	+	+	–	–	–	–	+	–	–	–	–	–
6732/80	–	–	–	–	–	–	+	+	–	+	–	+	+	+	+
365(2)	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
Cis77	–	–	–	–	–	–	+	+	–	+	–	+	+	+	+
Classical 569B (reference strain)	–	–	–	–	–	–	+	+	–	+	–	+	+	+	+
El Tor N16961 (reference strain)	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+

Fig. 1. *NotI*-restricted PFGE patterns of chromosomal DNA of 10 Australian *V. cholerae* O1 strains. Lanes: 1, *XbaI*-restricted chromosomal DNA of *S. braenderup* strain H9812 as size marker; 2, Cis77 (pulsotype A3); 3, 365(2) (pulsotype A4); 4, 6732/80 (pulsotype E); 5, P358 (pulsotype D); 6, 362(2) (pulsotype A1); 7, 366(1) (pulsotype A1); 8, 120186 (pulsotype A2); 9, 220386 (pulsotype C); 10, 12129(1) (pulsotype B); and 11, BX3302 (pulsotype A1).

cholerae. Later study showed that *stn* can also be present in toxigenic *V. cholerae* O1 strains, although this occurs less frequently in O1 strains than it does in non-O1 strains (Takeda *et al.*, 1991). Interestingly, most of the *ctxAB*-positive *V. cholerae* O1 strains were positive for *stn*, except for 6732/80 and Cis77. The RTX gene cluster encodes one kind of cytotoxin, and toxin activity is regulated by the *rtxC* gene (Lin *et al.*, 1999). It was demonstrated that some regions of the RTX gene cluster, including *rtxC*, are absent in the genome of the classical strains, whereas the cluster is intact in strains of the El Tor biotype (Lin *et al.*, 1999; Dziejman *et al.*, 2002). We tested two genes of this cluster, *rtxC* and *rtxA*, and found that both were present in all of the strains, including the VPI-1-negative strains (Table 2). RS1 usually flanks the entire CTXΦ genome, i.e. the core and the RS2 region. RS2 and RS1 are almost the same; the major difference between them is that the latter possesses an additional gene, *rstC*. This gene is unique to strains of the El Tor biotype; it is absent in classical strains (Waldor *et al.*, 1997). In this study, only four strains were positive for *rstC*. The remaining six, including the clinical strain, were negative (Table 2). The *rstC* gene provides anti-repressor activity to the phage replication process, but the impact of its absence on the cellular process is not currently well-understood (Davis & Waldor, 2003). Overall observations indicate that these strains are virulent and have similarities to the *V. cholerae* strains of the El Tor biotype.

Analysis of the integrated CTXΦ genome showed that the major toxin-producing gene, *ctxAB*, is present in the seven strains and thereby classified as toxigenic. Other CTXΦ-encoded genes were also present in these seven toxigenic strains (Table 2), which suggests that there is an intact core region. In contrast, most of the genes, including *ctxAB*, were absent in three strains, 12129(1), 220386, and P358, representing a truncated phage genome. These non-toxigenic strains possess only the *zot* gene, which is known to play an important role in phage assembly. Such non-toxigenic strains are sporadically isolated from tourists

with gastrointestinal illness, which indicates that they may have the potential to initiate gastrointestinal symptoms but not cholera (Kaper *et al.*, 1995). The diversity of CTXΦ among the biotypes is mainly due to variations in the repeat sequence elements, particularly in the *rstR* gene region. This gene encodes a regulatory aspect of phage replication, and four variants have been reported to date, *rstR*^{classical}, *rstR*^{El Tor}, *rstR*^{Calcutta} and *rstR*^{environmental}. These variants were named after the biotype, source or location of the strains in which they were first identified. An assortment of different alleles of the *rstR* gene in a single *V. cholerae* strain was first reported in 1998 (Kimsey *et al.*, 1998). In this study, all seven toxigenic strains were positive for the *rstR*^{classical} and/or *rstR*^{environmental} type, and the remaining two types of the *rstR* gene were not found in any of the strains tested (Table 2). Since the beginning of the seventh pandemic, classical strains have gradually been replaced by the El Tor biotype, and none has been reported recently. It is thus interesting to find classical-type *rstR* genes in El Tor-like strains. These may have been horizontally transferred from the classical strains either before their extinction or from an unknown gene pool. The presence of *rstR*^{environmental} in the *V. cholerae* strains of environmental origin may be considered to be further confirmation of their source of isolation. Finally, we made an effort to analyse these strains using the two most recently identified gene clusters, VSP-I and -II (Dziejman *et al.*, 2002), which are unique to the seventh pandemic El Tor strains but absent in pre-seventh pandemic El Tor strains. PCR analysis revealed that both of the clusters were absent in most of the test strains, including the classical reference strain. They were present only in the N16961 El Tor reference strain (Table 2). The absence of VSP-I and -II in most of the strains indicates their similarity to the pre-seventh pandemic strains that prevailed after the sixth pandemic.

To understand the clonal relationship, all 10 of the *V. cholerae* O1 strains isolated from Australia were analysed by PFGE. *NotI* digestion restricted the chromosomal genome

to 17–22 fragments, and these fragments ranged from 6 to 350 kb. Based on the banding pattern, five major pulsotypes, designated A, B, C, D and E, were observed among the 10 strains (Fig. 1). Pulsotype A was further divided into four subtypes, designated A1–A4, based on minor banding differences (Fig. 1). A1 was the most prevalent subtype of the four, whereas only the clinical isolate Cis77 showed subtype A3. PFGE analysis revealed that the environmental toxigenic *V. cholerae* O1 strains were less diverse than the non-toxigenic environmental O1 strains (Fig. 1). The clinical strain, Cis77, showed significant similarity to those of environmental origin, which is an indication of possible genetic relatedness between strains from two different sources. Heterogeneity within the *ctxB* gene and translated protein was first reported in the early 1990s and based on this, three *ctxB* genotypes of the *V. cholerae* O1 strains have been identified (Olsvik *et al.*, 1993). Based on amino acid residue substitution at positions 39, 46 and 68, all of the classical and US Gulf Coast El Tor strains have been categorized as genotype 1, the Australian El Tor strains as genotype 2 and the El Tor strains of the seventh pandemic and Latin American epidemic as genotype 3. The *ctxB* sequencing data in this study are consistent with previous findings (Olsvik *et al.*, 1993) that Australian *V. cholerae* strains are local inhabitants of the Australian environment and display genotype 2, which is different from genotypes 1 and 3.

Nair *et al.* (2006) recently reported pre-seventh pandemic-like strains of *V. cholerae* in water bodies in Fiji, which neighbours Australia. In this study, we also found that the Australian *V. cholerae* toxigenic strains of environmental origin closely resembled the pre-seventh pandemic El Tor strains and were comparable to the seventh pandemic strains. Due to the small number of total strains examined in this study, it is difficult to speculate on whether the cholera episodes in Australia are isolated incidences or an extension of seventh pandemic cholera. However, given that the Australian aquatic environment harbours both toxigenic and non-toxigenic *V. cholerae* that closely resemble the pre-seventh pandemic clone, indigenous Australian *V. cholerae* strains may have resulted from genetic exchange between the pre-seventh pandemic precursor strains and the toxigenic and/or non-toxigenic *V. cholerae* strains.

ACKNOWLEDGEMENTS

This study was funded by the International Centre for Diarrhoeal Disease Research, Bangladesh: Centre for Health and Population Research and its donors, who provided unrestricted support to the Centre for its operations and research. We gratefully acknowledge the support of the following donors and their commitment to the Centre's research efforts: the Australian International Development Agency (AusAID), Government of Bangladesh, Canadian International Development Agency (CIDA), The Kingdom of Saudi Arabia (KSA), Government of the Netherlands, Government of Sri Lanka, Swedish International Development Cooperative Agency (Sida-SAREC), Swiss Development Cooperation (SDC) and Department for International Development, UK (DFID).

REFERENCES

- Albert, M. J. & Nair, G. B. (2005). *Vibrio cholerae* O139 Bengal–10 years on. *Rev Med Microbiol* **16**, 135–143.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Barua, D. (1992). History of cholera. In *Cholera*, pp. 1–36. Edited by D. Barua & W. B. Greenough, III. New York: Plenum.
- Bhattacharya, M. K., Bhattacharya, S. K., Garg, S., Saha, P. K., Dutta, D., Nair, G. B., Deb, B. C. & Das, K. P. (1993). Outbreak of *Vibrio cholerae* non-O1 in India and Bangladesh. *Lancet* **341**, 1346–1347.
- Bhattacharya, T., Chatterjee, S., Maiti, D., Bhadra, K. R., Takeda, Y., Nair, G. B. & Nandy, K. R. (2006). Molecular analysis of the *rstR* and *orfU* genes of the CTX prophages integrated in the small chromosomes of environmental *Vibrio cholerae* non-O1, non-O139 strains. *Environ Microbiol* **8**, 526–534.
- Blake, P. A. (1994). Endemic cholera in Australia and the United States. In *Vibrio Cholerae and Cholera: Molecular to Global Perspectives*, pp. 309–319. Edited by I. K. Wachsmuth, P. A. Blake & Ø. Olsvik. Washington, DC: American Society for Microbiology.
- Byun, R., Elbourne, L. D., Lan, R. & Reeves, P. R. (1999). Evolutionary relationships of pathogenic clones of *Vibrio cholerae* by sequence analysis of four housekeeping genes. *Infect Immun* **67**, 1116–1124.
- Chow, K. H., Ng, T. K., Yuen, K. Y. & Yam, W. C. (2001). Detection of RTX toxin gene in *Vibrio cholerae* by PCR. *J Clin Microbiol* **39**, 2594–2597.
- Cooper, K. L., Luey, C. K., Bird, M., Terajima, J., Nair, G. B., Kam, K. M., Arakawa, E., Safa, A., Cheung, D. T. & other authors (2006). Development and validation of a PulseNet standardized pulsed-field gel electrophoresis protocol for subtyping of *Vibrio cholerae*. *Foodborne Pathog Dis* **3**, 51–58.
- Davis, B. M. & Waldor, M. K. (2003). Filamentous phages linked to virulence of *Vibrio cholerae*. *Curr Opin Microbiol* **6**, 35–42.
- Dziejman, M., Balon, E., Boyd, D., Fraser, C. M., Heidelberg, J. F. & Mekalanos, J. J. (2002). Comparative genomic analysis of *Vibrio cholerae*: genes that correlate with cholera endemic and pandemic disease. *Proc Natl Acad Sci U S A* **99**, 1556–1561.
- Faast, R., Ogierman, M. A., Stroehner, U. H. & Manning, P. A. (1989). Nucleotide sequence of the structural gene, *tcpA*, for a major pilin subunit of *Vibrio cholerae*. *Gene* **85**, 227–231.
- Faruque, S. M. & Mekalanos, J. J. (2003). Pathogenicity islands and phages in *Vibrio cholerae* evolution. *Trends Microbiol* **11**, 505–510.
- Faruque, S. M., Asadulghani, Kamruzzaman, M., Nandi, R. K., Ghosh, A. N., Nair, G. B., Mekalanos, J. J. & Sack, D. A. (2002). RS1 element of *Vibrio cholerae* can propagate horizontally as a filamentous phage exploiting the morphogenesis genes of CTXΦ. *Infect Immun* **70**, 163–170.
- Heidelberg, J. F., Eisen, J. A., Nelson, W. C., Clayton, R. A., Gwinn, M. L., Dodson, R. J., Haft, D. H., Hickey, E. K., Peterson, J. D. & other authors (2000). DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* **406**, 477–483.
- Hoshino, K., Yamasaki, S., Mukhopadhyay, A. K., Chakraborty, S., Basu, A., Bhattacharya, S. K., Nair, G. B., Shimada, T. & Takeda, Y. (1998). Development and evaluation of a multiplex PCR assay for rapid detection of toxigenic *Vibrio cholerae* O1 and O139. *FEMS Immunol Med Microbiol* **20**, 201–207.
- Jonson, G., Holmgren, J. & Svennerholm, A. M. (1991). Identification of a mannose-binding pilus on *Vibrio cholerae* El Tor. *Microb Pathog* **11**, 433–441.

- Kaper, J. B., Morris, J. G., Jr & Levine, M. M. (1995). Cholera. *Clin Microbiol Rev* 8, 48–86.
- Keasler, S. P. & Hall, R. H. (1993). Detecting and biotyping *Vibrio cholerae* O1 with multiplex polymerase chain reaction. *Lancet* 341, 1661.
- Kimsey, H. H., Nair, G. B., Ghosh, A. & Waldor, M. K. (1998). Diverse CTXΦ and evolution of new pathogenic *Vibrio cholerae*. *Lancet* 352, 457–458.
- Lin, W., Fullner, K. J., Clayton, R., Sexton, J. A., Rogers, M. B., Calia, K. E., Calderwood, S. B., Fraser, C. & Mekalanos, J. J. (1999). Identification of a *Vibrio cholerae* RTX toxin gene cluster that is tightly linked to the cholera toxin prophage. *Proc Natl Acad Sci U S A* 96, 1071–1076.
- Mitra, R. K., Nandy, R. K., Ramamurthy, T., Bhattacharya, S. K., Yamasaki, S., Shimada, T., Yamasaki, S., Shimada, T., Takeda, Y. & Nair, G. B. (2001). Molecular characterization of rough variants of *Vibrio cholerae* isolated from hospitalized patients with diarrhoea. *J Med Microbiol* 50, 268–276.
- Nair, G. B., Safa, A., Bhuiyan, N. A., Nusrin, S., Murphy, D., Nicol, A., Valcanis, M., Iddings, S., Kubuabola, L. & Vally, H. (2006). Isolation of *Vibrio cholerae* O1 strains similar to pre-seventh pandemic El Tor strains during an outbreak of gastrointestinal disease in an island resort in Fiji. *J Med Microbiol* 55, 1559–1562.
- Nandi, S., Maiti, D., Shaha, A. & Bhadra, R. K. (2003). Genesis of variants of *V. cholerae* O1 biotype El Tor: role of the CTXΦ array and its position in the genome. *Microbiology* 149, 89–97.
- Nusrin, S., Khan, G. Y., Bhuiyan, N. A., Ansaruzzaman, M., Hossain, M. A., Safa, A., Khan, R., Faruque, S. M., Sack, D. A. & other authors (2004). Diverse CTX phages among toxigenic *Vibrio cholerae* O1 and O139 strains isolated between 1994 and 2002 in an area where cholera is endemic in Bangladesh. *J Clin Microbiol* 42, 5854–5856.
- O'Shea, Y. A., Reen, F. J., Quirke, A. M. & Boyd, E. F. (2004). Evolutionary genetic analysis of the emergence of epidemic *Vibrio cholerae* isolates on the basis of comparative nucleotide sequence analysis and multilocus virulence gene profiles. *J Clin Microbiol* 42, 4657–4671.
- Olsvik, Ø., Wahlberg, J., Petterson, B., Uhlén, M., Popovic, T. & Wachsmuth, I. K. (1993). Use of automated sequencing of polymerase chain reaction-generated amplicons to identify three types of cholera toxin subunit B in *Vibrio cholerae* O1 strains. *J Clin Microbiol* 31, 22–25.
- Popovic, T., Bopp, C. A., Olsvik, Ø. & Wachsmuth, K. (1993). Epidemiologic application of a standardized ribotype scheme for *Vibrio cholerae*. *J Clin Microbiol* 31, 2474–2482.
- Rader, A. E. & Murphy, J. R. (1988). Nucleotide sequences and comparison of the hemolysin determinants of *Vibrio cholerae* El Tor RV79 (Hly1) and RV79 (Hly2) and Classical 569B (Hly2). *Infect Immun* 56, 1414–1419.
- Rao, A. & Stockwell, B. A. (1980). The Queensland cholera incident of 1977. 1. The index case. *Bull W H O* 58, 663–664.
- Rogers, R. C., Cuffe, R. G. C. J., Cossins, Y. M., Murphy, D. M. & Bourke, A. T. C. (1980). The Queensland cholera incident of 1977. 2. The epidemiological investigation. *Bull World Health Organ* 58, 665–669.
- Safa, A., Bhuiyan, N. A., Nusrin, S., Ansaruzzaman, M., Alam, M., Hamabata, T., Takeda, Y., Sack, D. A. & Nair, G. B. (2006). Genetic characteristics of Matlab variants of *Vibrio cholerae* O1 that are hybrids between classical and El Tor biotypes. *J Med Microbiol* 55, 1563–1569.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sutton, R. G. A. (1974). An outbreak of cholera in Australia due to food served in flight on an international aircraft. *J Hyg (Lond)* 72, 441–451.
- Takeda, T., Peina, Y., Ogawa, A., Dohi, S., Abe, H., Nair, G. B. & Pal, S. C. (1991). Detection of heat-stable enterotoxin in a cholera toxin gene-positive strain of *Vibrio cholerae* O1. *FEMS Microbiol Lett* 64, 23–27.
- Wachsmuth, I. K., Evins, G. M., Fields, P. I., Olsvik, Ø., Popovic, T., Bopp, C. A., Wells, J. G., Carrillo, C. & Blake, P. A. (1993). The molecular epidemiology of cholera in Latin America. *J Infect Dis* 167, 621–626.
- Waldor, M. K. & Mekalanos, J. J. (1996). Lysogenic conversion by a filamentous bacteriophage encoding cholera toxin. *Science* 272, 1910–1914.
- Waldor, M. K., Rubin, E. J., Pearson, G. D. N., Kimsey, H. & Mekalanos, J. J. (1997). Regulation, replication, and integration functions of the *Vibrio cholerae* CTXΦ are encoded by region RS2. *Mol Microbiol* 24, 917–926.
- WHO (1987). *Manual for Laboratory Investigations of Acute Enteric Infections*. Geneva: World Health Organization.