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

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Five strains of *Vibrio cholerae* O1, one each from an Australian and a New Zealand tourist with gastrointestinal illness returning from an island resort in Fiji and the remaining three from water sources located in the same resort, were extensively characterized. Conventional phenotypic traits that are used for biotyping of O1 *V. cholerae* categorized all five strains as belonging to the El Tor biotype. Genetic screening of 11 regions that are associated with virulence in *V. cholerae* showed variable results. The absence of genes comprising *Vibrio* seventh pandemic island-I (VSP-I) and VSP-II in all the strains indicated that these strains were very similar to the pre-seventh pandemic *V. cholerae* O1 El Tor strains. The *ctxAB* genes were absent in all strains whereas *orfU* and *zot* were present in four strains, indicating that the strains were non-toxicogenic. Four strains carried a truncated CTX prophage. Although epidemiological and molecular studies suggested that these strains did not cause cholera amongst tourists at the resort, their similarity to pre-seventh pandemic strains, their prior association with gastrointestinal illness and their presence in the island resort setting warrant more attention.

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INTRODUCTION

Cholera is an acute secretory diarrhoeal disease caused by toxigenic strains of *Vibrio cholerae* O1 or O139. Currently more than 200 serogroups of *V. cholerae* are recognized based on the somatic O antigen but only strains belonging to serogroups O1 and O139 are associated with epidemic and pandemic forms. The O1 serogroup is further classified into two biotypes, namely classical and El Tor. The two biotypes are distinguished from each other on the basis of their differences in several phenotypic properties (Kaper *et al.*, 1995). More recent studies have identified genes unique to

the classical and seventh pandemic El Tor strains (Dziejman *et al.*, 2002).

Since 1817, cholera has spread into the world seven times in the form of pandemics. The first six of these pandemics originated from the Gangetic delta of Bengal whereas the ongoing seventh pandemic started from the Sulawesi island of Indonesia in 1961. Over the past four decades the seventh pandemic has spread into the Asian, African and American continents and continues to be endemic in these areas. There is firm evidence that the fifth and sixth pandemics of cholera were caused by the classical biotype while the seventh is

caused by the El Tor biotype. Between the sixth pandemic, which ended in 1923, and the seventh pandemic, which began in 1961, there was an intervening period of 38 years when cholera did not spread in the form of a pandemic but occurred as local outbreaks. Strains of *V. cholerae* O1 isolated during this period are referred to as the pre-seventh pandemic El Tor strains.

On 29 November 2004, the International Centre for Diarrhoeal Diseases Research, Bangladesh (ICDDR,B), a World Health Organization (WHO) Collaborating Centre for Diarrhoeal Diseases Research and training in Bangladesh, was approached by WHO, South Pacific Office, to characterize a number of non-toxicogenic strains of *V. cholerae* O1 originating from an island resort in Fiji in which there had been a large outbreak of gastrointestinal disease. The reason for this request was the recognition that non-toxicogenic *V. cholerae* O1 may have pathogenic potential for humans and there is some correlation between the presence of non-toxicogenic *Vibrio* in the environment and cholera outbreaks. The extensive characterization of the non-toxicogenic *V. cholerae* O1 El Tor strains indicated that these strains were unusual and very similar to the pre-seventh pandemic strains.

METHODS

Bacterial strains. Five strains of *V. cholerae* O1 were received by the Enteric Microbiology Unit of ICDDR,B: one (ER98-5538) from Auckland, New Zealand; three [W2796A(5), W2796C(1) and W2796C(5)] from Queensland, Australia; and one (V3MDU) from Melbourne, Australia. *V. cholerae* O1 strain 569B of the classical biotype isolated in 1948 in India and the whole genome sequenced O1 El Tor strain N16961 (Heidelberg *et al.*, 2000) isolated in 1971 in Bangladesh were used as reference strains.

Source of the strains. Seven passengers had gastrointestinal symptoms while returning from a resort on Malololailai Island in Fiji by Air Pacific flights from Nadi to Melbourne on 20 and 21 August 2004. Three passengers had stool specimens examined and in two of these the presence of pathogens was indicated. *V. cholerae* O1 was recovered along with norovirus RNA from a stool sample of a 54-year-old female (V3MDU), whilst norovirus RNA was isolated from this woman's partner, a 56-year-old male. As part of the subsequent outbreak investigation at the resort, a comprehensive environmental investigation was conducted, with detailed microbiological testing of samples. *V. cholerae* W2796C(1) and W2796C(5) were isolated from one of the tanks used to store water produced by desalinating plants for use by guests for washing purposes (not for drinking). A third isolate, W2796A(5), was obtained from a surface water pond at the resort which was not used as a water source. The above three strains were isolated by the Public Health Microbiology Laboratory, Queensland Health Scientific Services, Brisbane, Australia, where the samples collected at the resort in Fiji were packed and shipped in accordance with International Air Transport Association (IATA) requirements for air freighting. The New Zealand isolate of *V. cholerae* O1 (ER98-5538) was from a New Zealand male aged 28 years who also had a concurrent infection with *Salmonella enteritidis* phage type 23. These infections were detected in November 1998 on his return to New Zealand after he developed gastrointestinal symptoms whilst a guest at this same resort in Fiji.

PCR and serogrouping. The identity of these strains was first reconfirmed by *V. cholerae*-species-specific *ompW* PCR developed by Nandi *et al.* (2003). The serogroup of these strains was subsequently reconfirmed using polyvalent O1 and monovalent Inaba and Ogawa antisera and by a multiplex PCR targeted to identify genes encoding O1 (*rfbO1*) and O139 (*rfbO139*)-specific O biosynthetic genes and cholera toxin gene (*ctxA*) as described previously (Hoshino *et al.*, 1998). PCR reagents and kits were obtained from either Perkin-Elmer or Invitrogen. The PCR products were analysed by electrophoresis in 1% agarose gels, stained with ethidium bromide, visualized under UV light, and recorded by using a gel documentation system (Gel Doc 2000; Bio-Rad). Amplicons were sized with reference to standard molecular mass markers.

Biotyping. All strains were tested for susceptibility to polymyxin B (50 U), chicken red cell agglutination (CCA) and sensitivity to group IV classical and group 5 El Tor phages as described in World Health Organization (1987). To complement the biotype characterization by genetic traits, PCR assays targeted to detect *tcpA* (classical and El Tor variant) (Keasler & Hall, 1993) and the *rstR* gene encoding the phage transcriptional regulator were performed using procedures described elsewhere (Davis *et al.*, 1999; Kimsey *et al.*, 1998; Nusrin *et al.*, 2004).

PCR-based genetic screening. PCR assays were used to assay for 11 virulence-associated genes and/or gene clusters, namely VSP-I, VSP-II, MSHA pilin, RTX, RS1Φ, CTXΦ, toxin linked cryptic plasmid (*tlc*), *Vibrio* pathogenicity island-1 (VPI-1), a constituent gene of integron (*intI4*), *hlyA* and *pilE*, in the genome of the 7 *V. cholerae* isolates including classical and El Tor reference strains. Thirty-two sets of PCR primers were used and PCR conditions were described previously (Chow *et al.*, 2001; O'Shea *et al.*, 2004).

RESULTS AND DISCUSSION

Each of the five strains were positive for the species-specific *ompW* gene and were therefore confirmed as *V. cholerae*. Multiplex PCR showed that all strains harboured the *rfbO1* gene but were negative for the *rfbO139* gene, although one strain, W2796C(1), was rough. All five strains were of the El Tor biotype. They were resistant to polymyxin B (50 U), agglutinated chicken blood cells and were sensitive to El Tor phage 5, but were resistant to classical phage IV (Table 1). The serogroup and serotype of these strains using polyvalent and monovalent antisera are shown in Table 1. The genetic traits that are used for biotyping *V. cholerae* were, however, not informative since all the five strains were negative for *tcpA* and for *rstR*.

Given the interesting background of these strains of *V. cholerae* O1, we decided to extensively analyse several of the genetic regions associated with virulence in an attempt to understand the lineage and pathogenic significance of these strains. The eleven regions, namely TLC, CTXΦ, RS1Φ, RTX, Hly, MSHA, VSP-I, VSP-II, VPI-1, *pilE* and *intI4*, accounted for approximately 165 kb of the *V. cholerae* genome (Table 2). In the VPI-1 gene cluster the major virulence-associated genes, *toxT* and *tcpA* (Table 1), were negative in all the strains while *acfB*, which is another potential colonization factor in VPI-1, was positive. Previous studies have also shown the presence of VPI-1 in the sixth, seventh and pre-seventh pandemic strains (O'Shea *et al.*, 2004). The MSHA gene cluster could be amplified by

Table 1. Response of the test and reference strains of *Vibrio cholerae* O1 to the phenotypic and genotypic tests for determining serotype and biotype

Strain no.	Serogroup	Serotype	<i>ompW</i>	<i>rfbO1</i>	<i>rfbO139</i>	Phenotypic traits*				Genotypic traits			
						CCA	Polymyxin B (50 U)	Phage IV (classical)	Phage 5 (El Tor)	<i>tcpA</i> classical	<i>tcpA</i> El Tor	<i>rstR1</i> †	<i>rstR2</i> ‡
ER98-5538	O1	Ogawa	+	+	–	+	R	R	S	–	–	–	–
W2796A(5)	O1	Inaba	+	+	–	+	R	R	S	–	–	–	–
W2796C(1)	Rough	Rough	+	+	–	+	R	R	S	–	–	–	–
W2796C(5)	O1	Inaba	+	+	–	+	R	R	S	–	–	–	–
V3MDU	O1	Ogawa	+	+	–	+	R	R	S	–	–	–	–
Classical 569B	O1	Inaba	+	+	–	–	S	S	R	+	–	+	–
El Tor N16961	O1	Inaba	+	+	–	+	R	R	S	–	+	–	+

*S, Sensitive; R, resistant.

†*rstR1*, classical type.‡*rstR2*, El Tor type.

PCR in all the test strains. MSHA, a type IV pilus, is expressed by strains of the El Tor biotype but is only rarely expressed by strains of the classical biotype (Jonson *et al.*, 1991). Two other regions, *hlyA* and *pilE*, were also found in all the test strains and in the sixth and seventh pandemic reference strains. In the classical *V. cholerae* strain, there is a deletion of the *rtxC* gene in the RTX region, which encodes an acylation enzyme that activates the toxin (Lin *et al.*, 1999). In a more recent study, Dziejman *et al.* (2002) reconfirmed the presence of *rtxC* only in the El Tor but not in the classical biotype strains. In this study, we tested for two genes of the RTX gene cluster, *rtxC* and *rtxA*; both were present in all the test strains thereby reaffirming the El Tor biotype status of these strains. From the extensive analysis, it was concluded that the five test strains belonged to the El Tor biotype. Recently, Dziejman *et al.* (2002) identified

genes that are unique to El Tor strains of the seventh pandemic and termed them VSP-I and VSP-II. PCR examination for VSP-I and -II revealed that both the clusters were absent in all the test strains except for the presence of one ORF each, namely ORF185 in VSP-I and ORF502 in VSP-II (data not shown). The absence of VSP-I and VSP-II in the strains indicates their similarity to the pre-seventh pandemic strains that prevailed before the beginning of the seventh pandemic in 1961.

None of the *V. cholerae* O1 strains characterized here had the cholera toxin genes and they were therefore non-toxigenic. The pathogenic and epidemic potential as well as the clinical and public health significance role of non-toxigenic strains of *V. cholerae* O1 remain unclear but such strains have been isolated in several countries (Morris *et al.*, 1984; Rodrigue

Table 2. Response of the test and reference strains of *V. cholerae* O1 to PCR screening for different genes and/or gene clusters of *V. cholerae* O1

VSP-I, *Vibrio* seventh pandemic island-I; VSP-II, *Vibrio* seventh pandemic island-II; MSHA, mannose-sensitive haemolysin agglutination pilin; *hlyA*, haemolysin; VPI-1, *Vibrio* pathogenicity island; *toxT*, gene encoding a member of the AraC family of bacterial transcription activators involved in the ToxR regulatory system; *acfB*, accessory colonization factor B; *PilE*, fimbrial assembly protein; *rtxA*, gene present in RTX gene cluster; *rtxC*, gene present in RTX gene cluster; *rstC*, encodes antirepressor activity; *rstA*, involved in CTXΦ replication and predicted to encode a polypeptide of 359 amino acids; OrfU(p^{III_{ctx}}), minor coat protein; *tlc*, toxin-linked cryptic plasmid; *intI4*, constituent gene of integron.

Strain	VSP-I	MSHA	HlyA	VSP-II	VPI-1		PilE	RTX		RS1		CTX			<i>tlc</i>	<i>intI4</i>
	(VCO175– VCO185)	(VCO398– VCO406)	(489)	(VCO490– VCO517)	<i>toxT</i>	<i>acfB</i>	<i>pilE</i>	<i>rtxA</i>	<i>rtxC</i>	<i>rstC</i>	<i>rstA</i>	<i>orfU</i>	<i>zot</i>	<i>ctxAB</i>		
ER98-5538	–	+	+	–	–	+	+	+	+	+	+	+	+	–	+	+
W2796A(5)	–	+	+	–	–	+	+	+	+	–	–	–	–	–	–	+
W2796C(1)	–	+	+	–	–	+	+	+	+	+	+	+	+	–	+	+
W2796C(5)	–	+	+	–	–	+	+	+	+	–	–	+	+	–	–	+
V3MDU	–	+	+	–	–	+	+	+	+	+	+	+	+	–	+	+
Classical 569B	–	+	+	–	+	+	+	+	–	–	+	+	+	+	+	+
El Tor N16961	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

et al., 1994). However, the fact that these isolates are sporadically isolated from tourists complaining of gastrointestinal illness indicates that they may have some potential to initiate gastrointestinal symptoms, but not cholera. We further examined for other CTX prophage genes such as *rstA*, *orfU* and *zot* that are found adjacent to *ctxAB* to determine if the CTX prophage was present in the test strains. Strains W2796C(1), ER98-5538 and V3MDU were positive for all these three genes, whereas strain W2796C(5) was negative for only *rstA*. These strains seem to have a truncated CTX prophage. Only one strain, W2796A(5), was negative for all the three genes. Two other genes, namely *tlc*, which is present adjacent to the CTX prophage, and integrin (*int14*), were positive in all the test strains. None of the currently recognized alleles of the repressor gene *rstR* were found. However, PCR amplification of the *rstC* gene in the three strains, which were also positive for *rstA*, *orfU* and *zot* of the CTX prophage, indicates the presence of the RS1 element in their genome and thus confirmed them as the El Tor biotype. It also indicates the presence of a different allele of the *rstR* gene in the test strains.

In conclusion, we were able to show in this study the existence of non-toxicogenic El Tor biotype strains of *V. cholerae* O1 similar to pre-seventh pandemic strains in a resort setting in Fiji, indicating the presence of a silent reservoir at this resort. Although epidemiological and molecular studies suggested that these strains were unlikely to be playing a major role in the outbreak in this instance, with the characteristics of this outbreak more consistent with norovirus infection, the similarity of these strains to pre-seventh pandemic strains, their association with gastrointestinal illness and their presence in island resort settings warrant more attention.

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