Detection of virulence-associated and regulatory protein genes in association with phage typing of human *Vibrio cholerae* from several geographical regions of the world

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Vibrio cholerae O1, O139 and occasionally non-O1/non-O139 serogroups are most often responsible for epidemic and pandemic cholera. This study used genotypic patterns of PCR-based detection of virulence-associated and regulatory protein genes, along with phage typing, to characterize 86 *V. cholerae* strains. Thirty-eight of 53 O1 biotype El Tor strains harboured both *tcpA* classical and *tcpA* El Tor genes, and three El Tor strains lacked the *V. cholerae* O1-specific gene (*Vc-O1*); three O139 strains contained both *Vc-O1* and *Vc-O139* genes and seven out of ten non-O1/non-O139 strains possessed the *Vc-O1* gene. The latter strains all harboured the virulence-associated genes *ctxA*, *zot*, *ace*, *RS1*, *hlyA*, *ompU*, *rtxA* and *sxt*. Two phage types, T27 and T25, were predominant in strains from different geographical regions of India, whereas more variation in phage susceptibility was observed for tetracycline-resistant strains from Kolkata. These results suggest that the pattern and distribution of virulence genes and phage types of *V. cholerae* are equally useful and discriminatory in tracing the origin of newly emerging strains.

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INTRODUCTION

Acute diarrhoea is a common cause of death in developing countries and is the second most common cause of infant deaths worldwide. Among the many causal organisms, only *Vibrio cholerae* has caused repeated pandemics (Wachsmuth *et al.*, 1994), dating from the pandemics of the 19th century to recent major epidemics in South America and Africa. *V. cholerae* is subdivided into more than 208 somatic O-antigen serogroups, but only two serogroups, O1 and O139, are recognized as pathogenic for man and as responsible for epidemic and pandemic cholera (Kaper *et al.*, 1995). In contrast, non-O1/non-O139 serogroups of *V. cholerae* may cause sporadic diarrhoeal episodes ranging from mild to severe morbidity (Kaper *et al.*, 1995; Mukhopadhyay *et al.*, 1995; Ramamurthy *et al.*, 1993).

The pathogenic variants of *V. cholerae* express genes encoding virulence-associated factors; in order to cause diarrhoea, strains require genes for the cholera toxin, the colonization factor TCP (toxin-coregulated pilus) and the central regulatory protein ToxR (Hall *et al.*, 1994;

Herrington *et al.*, 1988; Levine *et al.*, 1988; Miller *et al.*, 1989). It has become evident from earlier reports that a new epidemic *V. cholerae* clone could emerge by acquisition and combination of crucial virulence genes from the environmental pool through horizontal gene transfer (Faruque *et al.*, 2004). As a result, genotypic surveillance of pathogenic and virulence-associated genes in strains isolated from various localities nationally and internationally is monitored in order to identify the origin and circulation of strains with epidemic potential.

Among several typing methods, phage typing is one of the most important and useful methods for the identification and differentiation of *V. cholerae* strains. The two phage typing schemes for *V. cholerae* O1 and O139 were developed in, and are in routine use at, the National Institute of Cholera and Enteric Diseases (NICED) (Chakrabarti *et al.*, 1997; Chattopadhyay *et al.*, 1993). As a World Health Organization collaborating centre for diarrhoeal diseases research and training, NICED is a national reference laboratory and receives 700–1000 strains of *V. cholerae* per year from different parts of India and abroad for biotyping, serotyping and phage typing. We report here an analysis of the virulence gene cassettes of *V.*

Abbreviation: NICED, National Institute of Cholera and Enteric Diseases.

Table 1. Distribution of biotype, serotype and phage type of *V. cholerae* isolates from different parts of the world

Strain no.	Year of isolation	Geographical location	Biotype	Serogroup	Serotype	New phage type	O139 phage type	Group IV phage
PL-1	2005	Austria	—	O12	—	UT	UT	Resistant
PL-2	2005	Austria	—	O37	—	UT	UT	Resistant
PL-3*	Control	MAK 757	El Tor	O1	Ogawa	T27	UT	Resistant
PL-4	2005	Austria	—	O12	—	UT	UT	Resistant
PL-5	2005	Austria	—	O12	—	UT	UT	Resistant
PL-6	2005	Austria	—	O12	—	UT	UT	Resistant
PL-7	2005	Austria	—	O37	—	UT	UT	Resistant
PL-8	2005	Austria	—	O37	—	UT	UT	Resistant
PL-9	2005	Austria	—	O12	—	UT	UT	Resistant
PL-10	2000	Bangladesh	El Tor	O1	Ogawa	T27	UT	Resistant
PL-11	2000	Bangladesh	El Tor	O1	Ogawa	T27	UT	Resistant
PL-12	2000	Bangladesh	El Tor	O1	Ogawa	T25	UT	Resistant
PL-13	2000	Bangladesh	El Tor	O1	Ogawa	T27	UT	Resistant
PL-14	2000	Bangladesh	El Tor	O1	Ogawa	T27	UT	Resistant
PL-15	2000	Bangladesh	El Tor	O1	Ogawa	T25	UT	Resistant
PL-16	2000	Bangladesh	El Tor	O1	Ogawa	T27	UT	Resistant
PL-17	2000	Bangladesh	El Tor	O1	Ogawa	T27	UT	Resistant
PL-18	2000	Bangladesh	El Tor	O1	Ogawa	T25	UT	Resistant
PL-19	1996	Brazil	El Tor	O1	Inaba	T25	UT	Resistant
PL-20	1996	Brazil	El Tor	O1	Ogawa	T25	UT	Resistant
PL-21	1996	Brazil	El Tor	O1	Inaba	T25	UT	Resistant
PL-22	1996	Brazil	El Tor	O1	Inaba	T25	UT	Resistant
PL-23	1996	Brazil	El Tor	O1	Inaba	T25	UT	Resistant
PL-24	1996	Brazil	El Tor	O1	Inaba	T25	UT	Resistant
PL-25	1996	Brazil	El Tor	O1	Ogawa	T27	UT	Resistant
PL-26	1996	Brazil	El Tor	O1	Ogawa	T25	UT	Resistant
PL-27	1996	Brazil	El Tor	O1	Ogawa	T25	UT	Resistant
PL-28	1998	Chennai	El Tor	O1	Ogawa	T27	UT	Resistant
PL-29	1998	Maharashtra	El Tor	O1	Ogawa	T27	UT	Resistant
PL-30	1998	Karnataka	El Tor	O1	Ogawa	T27	UT	Resistant
PL-31	1998	Goa	El Tor	O1	Ogawa	T27	UT	Resistant
PL-32	1998	Gujrat	El Tor	O1	Ogawa	T27	UT	Resistant
PL-33	1998	Chennai	El Tor	O1	Ogawa	T27	UT	Resistant
PL-34	1998	Maharashtra	El Tor	O1	Ogawa	T27	UT	Resistant
PL-35	1998	Chennai	El Tor	O1	Ogawa	T27	UT	Resistant
PL-36	1998	Chennai	El Tor	O1	Ogawa	UT	UT	Resistant
PL-37	1970	Kolkata	Classical	O1	—	UT	UT	Sensitive
PL-38	1971	Assam	Classical	O1	—	UT	UT	Sensitive
PL-39	1971	Assam	Classical	O1	—	UT	UT	Sensitive
PL-40	1971	Assam	Classical	O1	—	UT	UT	Sensitive
PL-41	1980	Nagpur	El Tor	O1	Ogawa	T27	UT	Resistant
PL-42	1970	Patna	Classical	O1	—	UT	UT	Sensitive
PL-43	1970	Delhi	Classical	O1	—	UT	UT	Sensitive
PL-44*	Control	CL-154	Classical	O1	—	UT	UT	Sensitive
PL-45*	Control	O395	Classical	O1	—	UT	UT	Sensitive
PL-46	2003	Andrapradesh	El Tor	O1	Inaba	T27	UT	Resistant
PL-47	2003	Andrapradesh	El Tor	O1	Inaba	T27	UT	Resistant
PL-48	2003	Andrapradesh	El Tor	O1	Inaba	T27	UT	Resistant
PL-49	2003	Andrapradesh	El Tor	O1	Inaba	T24	UT	Resistant
PL-50	2003	Bombay	El Tor	O1	Inaba	T27	UT	Resistant
PL-51	2003	Bombay	El Tor	O1	Inaba	T27	UT	Resistant
PL-52	2003	Bombay	El Tor	O1	Inaba	T27	UT	Resistant
PL-53	2003	Bombay	El Tor	O1	Inaba	T27	UT	Resistant
PL-54	2003	Andrapradesh	El Tor	O1	Inaba	T27	UT	Resistant
PL-55	2002	Gujrat	—	O139	—	UT	UT	Resistant

Table 1. cont.

Strain no.	Year of isolation	Geographical location	Biotype	Serogroup	Serotype	New phage type	O139 phage type	Group IV phage
PL-56	2002	Gujrat	–	O139	–	UT	UT	Resistant
PL-57	2002	Gujrat	–	O139	–	UT	UT	Resistant
PL-58	2004	Gujrat	–	O139	–	UT	UT	Resistant
PL-59	2004	Gujrat	–	O139	–	UT	UT	Resistant
PL-60	2004	Chennai	–	O139	–	UT	UT	Resistant
PL-61	2002	Chennai	–	O139	–	UT	UT	Resistant
PL-62	2002	Chennai	–	O139	–	UT	UT	Resistant
PL-63	2004	Chennai	–	O139	–	UT	UT	Resistant
PL-64	2001	Goa	El Tor	O1	Ogawa	UT	UT	Resistant
PL-65	2001	Delhi	El Tor	O1	Ogawa	UT	UT	Resistant
PL-66	2001	Bikaner	El Tor	O1	Ogawa	UT	UT	Resistant
PL-67	2001	Delhi	El Tor	O1	Ogawa	UT	UT	Resistant
PL-68	2001	Goa	El Tor	O1	Ogawa	UT	UT	Resistant
PL-69	2001	Bikaner	El Tor	O1	Ogawa	UT	UT	Resistant
PL-70	2001	Baroda	–	Non-O1/non-O139	–	UT	UT	Resistant
PL-71	2001	Baroda	–	Non-O1/non-O139	–	UT	UT	Resistant
PL-72	2001	Baroda	El Tor	O1	Ogawa	T27	UT	Resistant
PL-73	2006	Delhi	–	O139	–	UT	T1	Resistant
PL-74	2006	Delhi	–	O139	–	UT	T1	Resistant
PL-75	2006	Delhi	–	O139	–	UT	T1	Resistant
PL-76	2006	Delhi	–	O139	–	UT	T1	Resistant
PL-77	2006	Delhi	–	O139	–	UT	T1	Resistant
PL-78	2006	Delhi	–	O139	–	UT	T1	Resistant
PL-79	2006	Delhi	–	O139	–	UT	T1	Resistant
PL-80	2006	Delhi	–	O139	–	UT	T1	Resistant
PL-81*	Control	NPR-4	–	O139	–	UT	T1	Resistant
PL-82	2007	Kolkata	El Tor	O1	Ogawa	T25	UT	Resistant
PL-83	2007	Kolkata	El Tor	O1	Ogawa	T16	UT	Resistant
PL-84	2007	Kolkata	El Tor	O1	Ogawa	T24	UT	Resistant
PL-85	2007	Kolkata	El Tor	O1	Ogawa	T25	UT	Resistant
PL-86	2007	Kolkata	El Tor	O1	Ogawa	T25	UT	Resistant
PL-87	2007	Kolkata	El Tor	O1	Ogawa	T25	UT	Resistant
PL-88	2007	Kolkata	El Tor	O1	Ogawa	T24	UT	Resistant
PL-89	2007	Kolkata	El Tor	O1	Ogawa	T27	UT	Resistant
PL-90	2007	Kolkata	El Tor	O1	Ogawa	T27	UT	Resistant

*These strains were used as controls in this study.

cholerae received from geographical regions of India and internationally over the last three decades and their correlation with phenotypic typing.

METHODS

Strains. Eighty-six *V. cholerae* strains, comprising 53 O1 biotype El Tor, six O1 biotype classical, 17 serogroup O139 and 10 non-O1/non-O139, isolated from diarrhoeal patients, were selected from strains submitted to NICED from different geographical regions of India and abroad from 1970 to 2007 for species confirmation, biotyping, serotyping and phage typing (Table 1). *V. cholerae* O1 biotype El Tor strain MAK 757 (ATCC 51352) (PL-3), two *V. cholerae* O1 biotype classical strains, CL-154 (PL-44) and O395 (PL-45), and *V. cholerae*

O139 strain NPR-4 (PL-81) were used as control strains. Strains were maintained in nutrient agar (HiMedia) stabs and stored in the dark in the repository of the Vibrio Phage Reference Laboratory, NICED.

Bacteriology. Strains were plated on thiosulfate-citrate-bile salt-sucrose agar (Eiken) and incubated at 37 °C for 16–18 h. *V. cholerae* was identified and confirmed by standard techniques (WHO, 1993). Strains were serotyped using polyvalent O1 and monospecific Inaba and Ogawa antisera, and monoclonal antibody O139 (Difco) and non-O1/non-O139 serogroup-specific antiserum (Thermo Fisher Scientific, Remel Products).

Phage typing. Phage typing was performed by the standard methodology of our laboratory (Sarkar *et al.*, 1994). After incubation, each reaction was recorded as positive if the number of plaques was

Table 2. Sequences of primers used for the detection of virulence and regulatory genes of *V. cholerae* isolates

Gene	Primer sequence (5'→3')	Amplicon (bp)	Annealing temp. (°C)	Reference
<i>ctxA</i>	CGGGCAGATTCTAGACCTCCTG CGATGATCTTGGAGCATTCCAC	564	60	Singh <i>et al.</i> (2002)
<i>zot</i>	TCGCTTAACGATGGCGCGTTTT AACCCCGTTTCACTTCTACCCA	947	60	Singh <i>et al.</i> (2002)
<i>sxt</i>	TCGGGTATCGCCCAAGGGCA GCGAAGATCATGCATAGACC	920	60	Bhanumathi <i>et al.</i> (2003)
<i>ace</i>	TAAGGATGTGCTTATGATGGACACCC CGTGATGAATAAAGATACTCATAGG	289	55	Singh <i>et al.</i> (2002)
<i>ompU</i>	ACGCTGACGGAATCAACCAAAAG GCGGAAGTTTGGCTTGAAGTAG	869	60	Singh <i>et al.</i> (2002)
<i>Vc-O1</i>	CAACAGAATAGACTCAAGAA TATCTTCTGATACTTTTCTAC	647	55	Binsztein <i>et al.</i> (2004)
<i>Vc-O139</i>	TTACCACTCTACATTGCC CGTTTCGGTAGTTTCTCTGG	741	55	Binsztein <i>et al.</i> (2004)
<i>tcpA</i> classical	CACGATAAGAAAACCGGTCAAGAG TTACCAAATGCAACGCCGAATG	620	60	Singh <i>et al.</i> (2002)
<i>tcpA</i> El Tor	CACGATAAGAAAACCGGTCAAGAG CGAAAGCACCTTCTTTCACACGTTG	453	60	Singh <i>et al.</i> (2002)
<i>toxR</i>	CCTTCGATCCCCTAAGCAATAC AGGGTTAGCAACGATGCGTAAG	779	60	Singh <i>et al.</i> (2002)
<i>hlyA</i> classical	GGCAAACAGCGAAACAAATACC CTCAGCGGGCTAATACGGTTTA	738	60	Singh <i>et al.</i> (2001)
<i>hlyA</i> El Tor	GAGCCGGCATTCATCTGAAT CTCAGCGGGCTAATACGGTTTA	481	60	Singh <i>et al.</i> (2001)
<i>rtxA</i>	GCGATTCTCAAAGAGATG CACTCATTCCGATAACCAC	1366	55	O'Shea <i>et al.</i> (2004), Lin <i>et al.</i> (1999)
<i>tcpI</i>	TAGCCTTAGTTCTCAGCAGGCA GGCAATAGTGTGCGAGCTCGTTA	862	60	Singh <i>et al.</i> (2001)
<i>RS1</i>	AACAGCTACTGGCTTATTC TGAGTTGCGGATTTAGGC	238	55	O'Shea <i>et al.</i> (2004)
<i>tagA</i>	KAR8: GGTGGTAAGATATTCACTC KAR9: GTCACAACAGGTACACC	1706	60	Karaolis <i>et al.</i> (1998)
<i>ssrA</i> right	KAR85: CGCCTGCGAACCACACGC KAR86: GCAGCAAGCCTCCACTCCG	600	55	Karaolis <i>et al.</i> (1998)
<i>ACF-ssrA</i>	KAR442: TTTCTCTCTAGGTTTGAGG KAR86: GCAGCAAGCCTCCACTCCG	908	55	Rajanna <i>et al.</i> (2003)
<i>ACF (int gene)</i>	KAR22: GATAAAGAGATCAAAGCC KAR23: ATCTGCTTCCATGTGGG	1237	55	Karaolis <i>et al.</i> (1998)
<i>aldA</i>	KAR3: AATAGCGAACTTCGAC KAR7: CCTCTAGGTCTATTTTA	1485	55	Karaolis <i>et al.</i> (1998)
<i>ssrA</i> left	KAR94: TATGATACTGAAAACACCTC KAR95: GATGCTAACAGCAGAGCATA	1455	50	Karaolis <i>et al.</i> (1998)

five or more. The 'classical' phage (ϕ 149 of Basu and Mukerjee classical group IV phage) was used to detect and confirm strains belonging to biotype classical. For O1 biotype classical strains, the reference strains CL-154 and O395 were used as controls, and for O1 and O139 strains, MAK 757 and NPR-4 were used, respectively, for confirmation of phage type.

Virulence-associated and regulatory protein genes. Strains were examined for the presence of the virulence-associated genes *ctxA*, *zot*, *ace*, *RS1*, *hlyA*, *rtxA*, *ompU*, *sxt* and *tcpA*, the regulatory genes for TCP expression (*tcpI*), and the central regulatory protein (*toxR*) and its two regulatory genes (*aldA* and *tagA*) (Chow *et al.*, 2001; Huhulescu *et al.*, 2007; Singh *et al.*, 2001). Integration of the *V. cholerae*

pathogenicity island into the chromosome of the strains was also investigated. Chromosomal DNA was extracted from each of the strains using a GenElute Bacterial DNA kit (Sigma-Aldrich). DNA was eluted with 50 μ l elution buffer and used as template for PCR. The primers used and the expected amplicon sizes are listed in Table 2. PCR was performed with a PTC 200 Peltier thermal cycler (MJ Research). RedTaq Ready Mix (2 $\times$; Sigma) was added to each sample in a 200 μ l PCR tube containing a reaction mixture volume of 25 μ l containing 1 μ l each forward and reverse primer (10 pmol μ l⁻¹) and 1 μ l DNA template. The PCR programme was initial denaturation 95 °C for 5 min, followed by 35 cycles of 95 °C for 15 s, the annealing temperature for respective primers (Table 2) for 35 s and 72 °C for 1.5 min, followed by maintenance at 4 °C. Reference strains MAK

Table 3. Co-occurrence of virulence traits of *V. cholerae* isolates

Gene	No. of strains of serogroup (biotype) containing each gene:			
	O1 (classical)	O1 (El Tor)	O139	Non-O1/non-O139
<i>tcpI</i>	6	53	17	10
<i>tcpA</i> (classical)	6	38	12	5
<i>tcpA</i> (El Tor)	6	52	17	10
<i>ssrAR</i>	6	53	17	7
<i>ACF-ssrA</i>	6	44	15	0
<i>ACF (int)</i>	6	52	17	1
<i>ssrAL</i>	1	43	12	0
<i>Vc-O1</i>	6	50	3	7
<i>Vc-O139</i>	0	0	17	0
<i>ctxA</i>	6	53	17	10
<i>zot</i>	6	53	17	10
<i>ace</i>	6	53	17	10
<i>RS1</i>	6	51	17	10
<i>toxR</i>	6	53	17	10
<i>tagA</i>	4	39	13	0
<i>aldA</i>	0	0	0	0
<i>hlyA</i> (classical)	6	53	17	10
<i>hlyA</i> (El Tor)	6	53	17	10
<i>ompU</i>	6	53	17	10
<i>rtxA</i>	6	53	17	10
<i>sxt</i>	6	52	17	10
Total no. of strains	6	53	17	10

Fig. 1. Virulence-associated and regulatory protein gene profiles within single phage types of *V. cholerae*.

757, NPR-4, CL-154 and O395 were used as positive controls. Amplified products were separated on a 1% agarose gel in 1 × TAE, stained with ethidium bromide (1.0 µg ml⁻¹) and visualized with a gel documentation system (Bio-Rad).

RESULTS AND DISCUSSION

The virulence and regulatory gene profiles of the *V. cholerae* strains are shown in Table 3. All 53 *V. cholerae* O1 biotype El Tor strains were positive for CTX genes (*ctxA*, *zot* and *ace*), virulence genes (*hlyA*, *ompU* and *rtxA*),

regulatory genes for TCP expression (*tcpI*) and the central regulatory protein gene (*toxR*). However, the regulatory genes *toxR* and *tagA* were also detected together in 38 El Tor strains and none harboured *aldA* (Table 3). ToxR is a transmembrane transcriptional activator that is part of a complex virulence gene regulon that regulates cholera toxin and TCP synthesis and expression of the outer-membrane protein OmpU (DiRita *et al.*, 1991; Huhulescu *et al.*, 2007).

All strains, irrespective of biotype, serotype, serogroup or phage type, were positive for *tcpA*, *ctxA*, *zot*, *ace*, *toxR*, *hlyA*,

ompU and *rtxA* by PCR, indicating that the genes comprising the virulence gene cassette and the genes encoding surface organelles required for intestinal adherence and colonization were intact. Thirty-eight El Tor strains were characterized by the presence of both *tcpA* classical and *tcpA* El Tor genes. Similar results were also obtained for the *V. cholerae* O139 strains. The ten non-O1/non-O139 strains harboured the virulence genes *ctxA*, *zot*, *ace*, *RS1*, *hlyA*, *ompU*, *rtxA* and *sxt*. One of the El Tor strains (PL-68) was negative for *tcpA*, possibly because of a polymorphism that could not be detected by primers specific for El Tor and the classical type of *tcpA*. These results indicated that *V. cholerae* O1, O139 and particularly non-O1/non-O139 strains of clinical origins have the ability to cause diarrhoea. Indeed, it is clear that the latter group are able to precipitate a cholera-like syndrome with the capacity to cause a localized outbreak (Sharma *et al.*, 1998).

Some interesting and unusual findings were observed in the PCR results. Three El Tor strains lacked the *V. cholerae* O1-specific gene (*Vc-O1*) and three O139 strains had both *Vc-O1* and *Vc-O139* genes. Moreover, of ten non-O1/non-O139 strains, seven harboured *Vc-O1* genes. These results suggested that *V. cholerae* O139 may have originated from an O1 ancestry through the lineage of non-O1/non-O139. It is noteworthy that four serogroups were identified and reported previously that have been derived from an O1 progenitor by serogroup conversion at the O-antigen (wb*) region (Blokesch & Schoolnik, 2007).

Phenotypic characterization of these *V. cholerae* strains by phage typing was also a point of interest in this study as phage typing is routinely performed in our laboratory. Phage typing results of *V. cholerae* O1 El Tor strains revealed that two types, T27 and T25, in particular were predominant among strains isolated from different geographical regions of India and from both Bangladesh and Brazil (Table 1). However, greater variation in the phage types was evident for tetracycline-resistant strains isolated from Kolkata than in other strains. Distribution of virulence-associated and regulatory protein genes into a single phage type was observed (Fig. 1); the *Vc-O1* gene was absent within phage type T27 in strain PL-47. Strains of phage types T25 and T24 did not show significant variation in their pathogenicity gene pattern. One (PL-68) of the seven phage-non-typable O1 El Tor strains was characterized as susceptible to trimethoprim, sulfamethoxazole and streptomycin due to the absence of the *sxt* gene. None of the non-O1/non-O139 strains had an intact *V. cholerae* pathogenicity island region.

Bhowmick *et al.* (2007) considered that strains of *V. cholerae* belonging to a single phage type originate from a single clone. This is corroborated by the present study where the distribution of virulence genes correlated strongly with phage types. Thus, regularly monitoring virulence gene patterns and genetic changes of pathogenic *V. cholerae* in conjunction with the phage typing pattern is

useful for tracing the origins of newly emerging clones of this species.

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