Characterization of pathogenicity island prophage in clinical and environmental strains of *Vibrio cholerae*

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In this study 86 isolates of *Vibrio cholerae* were analysed for their adhesive properties and the presence of pathogenicity island genes. With the exception of three isolates, all of the other clinical isolates (92.5 %) contained an intact TCP (toxin-co-regulated pilus) gene cluster. In contrast, 95 % of all environmental non-O1-non-O139 isolates were negative for the TCP gene cluster. The majority of clinical isolates (82.5 %) possessed the complete vibrio pathogenicity island (VPI) gene cluster and had a similar RFLP pattern, while only a single environmental strain possessed an almost complete VPI cluster (lacking 0.4 kb in the *tcpA* and *toxT* region). The result showed that the isolates with *tcpA*⁺/*toxT*⁺ had a strong attachment for HT-29 and Vero cells, whereas isolates with *tcpA*⁺/*toxT*⁻ or *tcpA*⁻/*toxT*⁻ genomic characteristics showed no autoagglutination and weak attachment for the cell lines. Two environmental strains (*tcpA*⁻/*toxT*⁻) showed strong adhesive properties to the cell lines, indicating that non-fimbrial adhesive factors are involved in the environmental *V. cholerae* strains in the absence of TCP.

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INTRODUCTION

Vibrio cholerae O1 causes acute gastrointestinal disease, which is reported annually in Iran with differing frequency (Bakhshi *et al.*, 2008). However, non-O1/non-O139 strains are not associated with epidemics, but several cases of sporadic diarrhoea, gastroenteritis and skin infections have been reported (Restrepo *et al.*, 2006; Rozemeijer *et al.*, 2009).

The vibrio pathogenicity island (VPI) is a primary genetic factor required for the emergence of epidemic *V. cholerae*. The VPI has several features such as a low mol% G+C content (35 mol%), phage-like attachment (*att*) sites at its termini, insertion at the *ssrA* site, a phage-like integrase gene (*int*) and a transposase-like gene (*vpiT*) (Heidelberg *et al.*, 2000). ToxT is a soluble transcriptional activator that amplifies its own expression, directly regulates the expression of cholera toxin (CT) encoded by integrated CTX phage (DiRita *et al.*, 1991) and five other genes, *aldA*, *tagA*, *acfA*, *acfD* and *tcpI*, in the VPI cluster (Peterson & Mekalanos, 1988). Other virulence factors such as toxin co-regulated pilus (TCP), encoded by phage, mediate the intestinal colonization (Kirn *et al.*, 2000). TcpP and TcpH have been shown to regulate CT and TCP expression possibly through ToxT (Carroll *et al.*, 1997; Häse & Mekalanos, 1998). The majority

of *V. cholerae* serotypes do not contain genes for CT and TCP (Taylor *et al.*, 1988; Bakhshi *et al.*, 2009), whereas the gene for ToxR is ubiquitously present (Nandi *et al.*, 2000).

Other proteins within the VPI segment are AldA and TagA, which encode aldehyde dehydrogenase and metalloprotease activities, respectively (Parsot & Mekalanos, 1991; Szabady *et al.*, 2011). ACF (accessory colonization factor), which is located in the right segment of the VPI, controls the expression of CT and AcfB, playing a role in chemotaxis, thereby assisting in intestinal colonization (Hughes *et al.*, 1995).

In the present study, we compared the entire VPI loci of clinical and environmental *V. cholerae* isolates to address the following questions: (i) whether all clinical *V. cholerae* strains possessed an identical VPI gene content; (ii) the difference between the VPI gene content of the environmental and clinical isolates; and (iii) the correlation between VPI gene content and the bacterial attachment pattern.

METHODS

Bacterial strains. A total of 86 *V. cholerae* isolates were included in this study. The 46 environmental *V. cholerae* isolates were obtained from surface water, and were biochemically identified and confirmed using intergenic 16srRNA–23srRNA specific primers (Choopun *et al.*,

Abbreviations: CT, cholera toxin; L-PCR, long template PCR; TCP, toxin co-regulated pilus; VPI, vibrio pathogenicity island.

Fig. 1. Schematic presentation of VPI (39.5 kb) in *V. cholerae* El Tor strain N16961. The ORFs and gene clusters are shown. Black arrowheads indicate the location of the PCR primers listed in Table 1.

2002). The 40 clinical isolates were randomly selected from our *V. cholerae* culture collection, and were obtained from patients in different provinces in Iran during the years 2004–2006. *V. cholerae* ATCC 14035 was used as a positive control throughout the experiments.

PCR, long template PCR (L-PCR) and RFLP analysis. Fig. 1 shows the general structure of the VPI cluster gene content. Classical PCR was used for the analysis of *aldA-tagA* and *acf* gene clusters. DNA was extracted using a QIAamp DNA mini kit (Qiagen) and PCR was performed in a reaction mixture with total volume of 25.5 µl, containing 5 µl template DNA (20 ng), 2.5 µl 10× *Taq* polymerase buffer [100 mM Tris/HCl (pH 8.3), 500 mM KCl and 15 mM MgCl₂], 0.25 µl (100 pmol µl⁻¹) each primer, 0.25 µl (10 mM) dNTPs, 0.2 µl (5 U µl⁻¹) *Taq* DNA polymerase and 16.8 µl sterilized distilled water.

PCR was carried out as follows: initial denaturation step at 94 °C for 5 min; followed by 30 cycles consisting of denaturation (94 °C for 1 min), annealing (58 °C for 1 min, separately adjusted for each set of primer pairs) and extension (72 °C for 1 min); followed by a final extension step at 72 °C for 3 min.

The Expand L-PCR system (Roche Diagnostic) and two set of primers, *tcpI-F/tcpQ-R* and *tcpQ-F/tcpF-R* (targeting between the *tcpI* through *tcpF*) were used (Fig. 1). L-PCR was carried out in a volume of 25 µl, with 2.5 µl each primer (300 nM), 2 µl dNTP (350 µM), 2.5 µl 10× PCR buffer with 27.5 mM MgCl₂, 5 µl genomic DNA up to 500 ng and 0.75 µl DNA polymerase. For L-PCR, the following program was used: a first run of 2 min at 94 °C; followed by 10 cycles of 15 s at 95 °C, 30 s at 59 °C and 8 min at 72 °C; followed by a second run comprising 20 cycles of 20 s at 95 °C, 30 s at 61 °C and 7 min at 72 °C (increasing 20 s for each cycle), and a final extension for 7 min at 72 °C. The primers and restriction enzymes used are shown in Table 2. RFLP-PCR was performed in a volume of 25 µl containing 2.5 µl 10× buffer specific for each enzyme, 0.6–1 µl restriction enzyme and 2 µg each PCR product.

The VPI gene content of the clinical isolates was analysed using 17 primer pairs with overlapping sequences (Table 2). The presence of VPI cluster genes in the environmental isolates was investigated using eight primer pairs specifically chosen to amplify within separated segments of the whole VPI cluster. The positive PCR product was then subjected to further analysis using 17 overlapping primer pairs.

Autoagglutination. For autoagglutination, all strains were grown in LB (pH 6.8) supplemented with 1% (w/v) NaCl and incubated at 37 °C for 18 h (Chakraborty *et al.*, 2000).

Cell culture. HT-29 and Vero cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco-BRL) with 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. The stock cultures were passaged at an interval of 2 and 4 days for Vero and HT-29 cell lines, respectively. The cultured cells were transferred to coverslips (Ø=12 mm; Corning) in the 24-well-culture plates, prior to the experiment to assess adhesion of *V. cholerae*.

Adherence assay. Fifteen strains of *V. cholerae* belonging to different VPI PCR patterns were chosen for the adherence assay. The strains were grown in tryptic soy broth (Difco Laboratories) at 37 °C for 18 h with shaking. They were then harvested and washed with PBS (pH 7.4). The micro-organisms were suspended in DMEM without fetal bovine serum or antibiotics. *V. cholerae* ATCC 14035 was used as the positive control.

The number of vibrios to adhere to Vero and HT-29 was examined by the method of Taguchi *et al.* (1997). Briefly, semi-confluent cultures of Vero and HT-29 (to a final concentration of 0.5–1.5 × 10⁶ cells ml⁻¹) grown on plastic coverslips were placed in 24-well-culture plates. The turbidity of each bacterial suspension in DMEM was separately adjusted to 0.5 McFarland, which was confirmed by plating on nutrient agar to reach about 1 × 10⁸ c.f.u. ml⁻¹. DMEM (1 ml) containing *V. cholerae* strains was added to each cell line and then incubated at 37 °C for 1 h in the presence of 5% CO₂. The coverslips were then washed three times with PBS, fixed with 70% methanol and stained with a 10% Giemsa solution. The stained coverslips were then mounted on glass slides and examined with a light microscope. A total of 100 Vero cells and 30 HT-29 cells were selected randomly

Table 1. Clinical *V. cholerae* strains studied, year of isolation and serogrouping

Year of isolation	Serotype		
	O1-Ogawa	O1-Inaba	Non-O1-non-O139
2004	+ (9/9)		
2005		+ (18/18)	
2006		+ (11/13)	
Total	9/40 (22.5%)	29/40 (72.5%)	2/40 (5%)

Table 2. Oligonucleotide primers used in this study and restriction enzymes used for RFLP analysis

Primer designation	Primer sequence	Reference	PCR product size (bp)	Nucleotide no.	Digested by	Applied in:	
						Environmental strains	Clinical strains
LJ-F	5'-GTGAATCTTGATGAGACGCTCTG-3'	Mukhopadhyay <i>et al.</i> (2001)	1010	872908	ND	+	
LJ-R	5'-GGTGAGCCAGGCTTATTTGGG-3'			873918			
LJ-F	5'-GTGAATCTTGATGAGACGCTCTG-3'	Mukhopadhyay <i>et al.</i> (2001)	2783	872908	<i>EcoRI</i>		+
VPI1-R	5'-TTGGTGGGCATCCTAACAA T-3'	This study		875691			
aldA-F	5'-GCATCTGATTATGACCAAAGAATAGG-3'	Mukhopadhyay <i>et al.</i> (2001)	1115	875605	ND	+	
aldA-R	5'-GTCAATGGATGAAGCCACACAGTG-3'			876720			
tagA-F	5'-GGTGGTAAGATATTCACTCTTAATG-3'	Sarkar <i>et al.</i> (2002)	350	877009	ND	+	
tagA-R	5'-GAGACATCTATAGAATACTGGCTG-3'			877359			
aldA-F	5'-GCATCTGATTATGACCAAAGAATAGG-3'	Mukhopadhyay <i>et al.</i> (2001)	1754	875605	ND		+
tagA-R	5'-GAGACATCTATAGAATACTGGCTG-3'	Sarkar <i>et al.</i> (2002)		877359			
VPI2-F	5'-TCGATGATTTAAAACCTAACAGC-3'	This study	2605	877317	<i>DdeI</i>		+
VPI2-R	5'-ATCGAGGTAACCAGCACCAT-3'			879922			
VPI3-F	5'-CATTTTATGGTGCTGGTTACCTC-3'	This study	2522	879897	<i>EcoRV</i>		+
VPI3-R	5'-AATGCAGCAGCATCCTTTTT 3'			882419			
VPI4-F	5'-GGTTTCGTGTAGGGAAGGAT-3'	This study	2557	882393	<i>Clal</i>		+
VPI4-R	5'-GCCCAGTAGGTATGCTCTGC-3'			884950			
VPI5-F	5'-TGGCTAAGCTAACTGGTGGA-3'	This study	2630	884855	<i>EcoRV</i>		+
VPI5-R	5'-CGCAGGCCTATAAAATTCCA-3'			887485			
tcpI-F	5'-GCCGTCTCCGCATTAAGCTCTGCAC-3'	Mukhopadhyay <i>et al.</i> (2001)	~5400	887219	<i>EcoRV, BglI, Clal</i>		+
tcpQ-R	5'-GAGGACTGTTCTGCAATCTGCTCAT-3'			892604			
tcpQ-F	5'-ATGAGCAGATTGCAGAACAGTCTC-3'	Mukhopadhyay <i>et al.</i> (2001)	~7100	892580	<i>EcoRI, EcoRV</i>		
tcpF-R	5'-GCCACGAATGTGGCTGTTATCTTATC-3'			899724			
tcpA-F	5'-CACGATAAGAAAACCGTCAAGAG-3'	Mukhopadhyay <i>et al.</i> (2001)	500	890480	ND	+	
tcpA-R	5'-ACCAAATGCAACGCCGAATGGAGC-3'			890951			
toxT-F	5'-ACTGTATAGCAAAGCATATTCAGAGA-3'	Sarkar <i>et al.</i> (2002)	1124	899653	ND	+	+
toxT-R	5'-CAGTGATACAATCGAAAAATAGGATC-3'			900777			
VPI6-F	5'-TCTGGGTTTGCTAGGCTTTC-3'	This study	1449	900622	<i>Clal</i>		+
VPI6-R	5'-CGGCATCAAATGGATTGTAG-3'			902071			
acfB-F	5'-TTTGTCTGAGCCGTATGTCG-3'	Sarkar <i>et al.</i> (2002)	1992	901913	<i>EcoRV</i>	+	+
acfC-R	5'-CACTATTGGGGCAAAAACG-3'			903905			
VPI7-F	5'-TCATTGATAGTTTTGGAATC-3'	This study	2655	903753	<i>EcoRV</i>		+
VPI7-R	5'-GCTTACAACCTATAAGAAAACCCAAA-3'			906408			
VPI8-F	5'-TCATTGATAGTTTTGGGTTTTCTT-3'	This study	2717	906397	<i>EcoRI</i>		+
VPI8-R	5'-GCATTGGCTTCAGACTCTCC-3'			909113			
VPI9-F	5'-TGGTTTGTATGTACCCCGATA-3'	This study	2121	909057	<i>EcoRV</i>		+
VPI9-R	5'-CCAACCTCAATATTAACCCTTAGGA-3'			911177			
Int-F	5'-GAAGTAATGAAACCGATAAGTGG-3'	Mukhopadhyay <i>et al.</i> (2001)	311	913235	ND	+	
Int-R	5'-TGCTTTGTACCAGTCACAGATAG-3'			912924			
VPI10-F	5'-GGGGGATCTAGTTTGCTTCC-3'	This study	2281	910968	ND		+
Int-R	5'-GATAGACACTGACCATGTTTCGT-3'			913248			
RJ-F	5'-TGCTTTGTACCAGTCACAGATAG-3'	Mukhopadhyay <i>et al.</i> (2001)	1475	912902	ND	+	+
RJ-R	5'-TCGTTAGCGTGTCCGTTCCGAGG-3'			914377			

ND, Not done.

Table 3. Different patterns of VPI gene content in clinical strains

No. (percentage) of strains with the given gene profile	VPI gene profile																	
	LJF-VPIR	aldAF-tagAR	VPI2F-R	VPI3F-R	VPI4F-R	VPI5F-R	tcpIF-QR	tcpQF-FR	tcpAF-R	toxTF-R	acfAF-R	VPI6F-R	VPI7F-R	VPI8F-R	VPI9F-R	intF-R	VPI10F-intR	RJF-R
2/40 (5)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1/40 (2.5)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1/40 (2.5)	+	+	+	+	+	+	+	+	+	—	+	+	+	+	+	+	+	+
3/40 (7.5)	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
33/40 (82.5)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Percentage of strains with each VPI gene	85	92.5	92.5	92.5	92.5	92.5	92.5	92.5	92.5	90	92.5	92.5	92.5	92.5	92.5	95	92.5	95

and the number of adhering micro-organisms was counted (Taguchi *et al.*, 1997). Each experiment was performed three times.

RESULTS AND DISCUSSION

Bacterial strains

A total of 40 *V. cholerae* strains were isolated from different provinces of Iran during 2004–2006. From 40 *V. cholerae* isolates, 72.5 and 22.5 % isolates were identified as *V. cholerae* O1 El Tor Inaba and Ogawa serotypes, respectively. Five per cent of the clinical and all of the environmental strains were non-O1-non-O139 (Table 1).

PCR, L-PCR and RFLP analysis

Out of 40 clinical *V. cholerae*, 33 strains (82.5 %) harboured a complete set of VPI-associated genes. Two strains (5 %) were negative for the whole VPI cluster, three (7.5 %) were negative for LJ-F/VPI1-R, one (2.5 %) was positive for *int* and *RJ*, and one (2.5 %) was negative for *toxT* (Fig. 1). The distribution of different VPI-associated genes between the clinical and environmental *V. cholerae* isolates is presented in Tables 3 and 4, respectively.

Initially for the search of VPIs, we examined the presence of *aldA-tagA*, TCP and *acf* subclusters. An L-PCR assay of the core TCP cluster of the clinical isolates with the primers flanking *tcpA* (*tcpI-F* and *tcpQ-R*; *tcpQ-F* and *tcpF-R*) yielded products of the expected sizes (~5.4 and ~7.1 kb), identical to *V. cholerae* ATCC 14035. The RFLP of L-PCR products of the clinical strains were identical and indistinguishable from *V. cholerae* ATCC 14035 (Fig. 2).

Three (7.5 %) clinical strains of *V. cholerae* were negative for the TCP cluster. Two and one isolates belonged to non-O1-non-O139 and O1 Inaba serotypes, respectively.

The majority of the clinical isolates (86 %) contained *acf* and *aldA-tagA* gene clusters. PCR analysis also showed that 36 *V. cholerae* O1 strains (90 %) contained the *toxT* regulon in VPI cluster (Table 3).

The *aldA-tagA*, TCP and *acf* cluster genes were absent in 98 % of the environmental strains. Only one environmentally isolated non-O1-non-O139 *V. cholerae* strain (EVC4) contained the entire VPI cluster of genes (lacking only *tcpA* and *toxT*). RFLP analysis of the L-PCR product of EVC4 with *tcpQ-F/tcpF-R* primers was identical to that of *V. cholerae* ATCC 14035. The *BglI* endonuclease did not digest the amplified product of the *tcpI-tcpQ* region of the EVC4 isolate. The 5.0 kb amplified product of *tcpI-tcpQ* region of EVC4 was 0.4 kb smaller in the *tcpA* region than the clinical and ATCC 14035 isolates, which were 5.4 kb in size. The smaller size of *tcpA* could be due to the absence of the *BglI* restriction site within the *tcpA* of EVC4 as the result of point mutation or gene deletion (up to 400 bp). Amplification of the *tcpA* gene using *tcpA-F/TcpA-R* primers did not yield any products, supporting the gene deletion hypothesis.

Table 4. Different patterns of VPI gene content in environmental strains

No. (percentage) of strains with the given gene profile	VPI gene profile							
	<i>Int</i>	<i>LJ</i>	<i>aldA</i>	<i>tagA</i>	<i>tcpA</i> El Tor	<i>toxT</i>	<i>acfB-C</i>	<i>RJ</i>
1/46 (2.2)	+	+	+	+	–	–	+	+
2/46 (4.3)	–	+	–	–	–	–	–	–
1/46 (2.2)	+	+	–	–	–	–	–	–
13/46 (28.3)	+	–	–	–	–	–	–	+
16/46 (34.8)	+	–	–	–	–	–	–	–
13/46 (28.3)	–	–	–	–	–	–	–	–
Percentage of strains with each VPI gene	67	8.6	2	2	2	0	2	30

EVC4 was considered as being of *tcpA*[–]/*ctxB*⁺ genotype supporting the suggestion that not all CT positive *V. cholerae* strains are TCP positive, since TCP is known to be receptor for CTX phage (Chakraborty *et al.*, 2000). The findings reported here are supported by those of Karaolis *et al.* (2001) who demonstrated that the central region of the VPI of *tcpA* contains most of the divergence between the VPIs of the 6th (classic) and the 7th (El Tor) pandemic strains. Previous

studies by others have described that the genetic variants of the TCP island were carried by a number of environmental *V. cholerae* strains belonging to non-epidemic serogroups (Boyd & Waldor, 2002; Mukhopadhyay *et al.*, 2001; Tay *et al.*, 2008). Overall, the consensus all published reports have indicates mutational events in the *tcp* region. To the best of our knowledge this is the first report suggesting deletion in the *tcpA* region.

Among the environmental strains, six different patterns of the VPI gene cluster were obtained, among which 30, 8.6 and 67% of isolates contained the *RJ*, *LJ* and *int* gene, respectively. The implication from these results may be that: (i) some environmental strains could have acquired VPI phage; however, due to phages' size limitation they did not acquire the whole phage genomics; and (ii) the ability of *V. cholerae* to acquire, integrate and excise the VPI cluster is mediated by the VPI-encoded recombinase *int* and *vpiT* (Karaolis *et al.*, 1998; Rajanna *et al.*, 2003). Generation of amplicons with right and left junction primers suggests that VPIs have been integrated in the large chromosome of these isolates at sites identical to those of O1 strains (Fasano *et al.*, 1991). Murphy & Boyd (2008) showed that all three pathogenicity islands can excise from the chromosome, which is likely a first step in their horizontal transfer. Boyd *et al.* (2000) suggest that *Vibrio mimicus* may be a significant reservoir for VPI phage and may play an important role in the emergence of new toxigenic *V. cholerae* isolates.

Fig. 2. (a) L-PCR amplification of *tcpI*-F/*tcpQ*-R in the TCP cluster. Lanes: 1, representative of clinical *V. cholerae* strains; 2, EVC4; 3, *V. cholerae* ATCC 14035. (b) RFLP pattern of amplified products using *tcpI*-F/*tcpQ*-R primers. Lanes: 4, 5 and 6, representatives of *Bgl*I digests of clinical *V. cholerae* strains, EVC4 and *V. cholerae* ATCC 14035, respectively; 7, 8, 9 and 10, representatives of *Eco*RV digests of two clinical *V. cholerae* strains, *V. cholerae* ATCC 14035 and EVC4, respectively. (c) L-PCR amplification of *tcpQ*-F/*tcpF*-R in TCP cluster. Lanes: 11 and 12, *Eco*RV digests of EVC4 and clinical *V. cholerae* strains (identical to ATCC 14035), respectively; 13 and 14, *Eco*RI digests of EVC4 and clinical *V. cholerae* strains (identical to ATCC 14035), respectively; lane 15, L-PCR product of *tcpQ*-F/*tcpF*-R in *V. cholerae* ATCC 14035. Ma, 1 kb DNA size marker; Mb, 100 bp DNA size marker.

Autoagglutination and adherence assay

To determine the *tcpA* gene expression, all of the TCP cluster positive strains ($n=37$) were examined for autoagglutination. It was found that 36 (97%) of the clinical strains exhibited the autoagglutination phenotype. Autoagglutination was not observed in any other broth medium or culture condition in the remaining clinical strains (3%) and the single environmental strain tested.

The adherence assay on Vero and HT-29 cells was carried out for five environmental and ten clinical *V. cholerae* strains including *V. cholerae* ATCC 14035 (Fig. 3). Similar

Fig. 3. Giemsa-stained bright-field micrographs of Vero and HT-29 cells inoculated with *V. cholerae* strains. Adherence of bacteria with weak attachment pattern on Vero (a) and HT-29 (b) cells. Adherence of bacteria with strong attachment pattern on Vero (c) and HT-29 (d) cells.

adherence results (dispersed or diffused) were obtained when *V. cholerae* isolates were incubated with either HT-29 or Vero cell lines. Amongst the *V. cholerae* strains, CVC1 and

EVC4 showed the strongest adhesion (about 16 bacteria per cell). However, the CVC8 and EVC3 strains exhibited the weakest adhesion (about 7 bacteria per cell) (Table 5).

Table 5. Adherence of *V. cholerae* strains to Vero and HT-29 cells assessed by light microscopy

Strain	Relevance characteristics	No. of bacteria per Vero cell	No. of bacteria per HT-29 cell	Adhesion pattern*
ATCC 14035	Classic, Inaba, <i>tcpA</i> ⁺ , <i>toxT</i> ⁺ , autoagglutination ⁺	4.4 ± 1.4†	3.8	+
CVC1	El Tor, Inaba, <i>tcpA</i> ⁺ , <i>toxT</i> ⁺ , autoagglutination ⁺	15.93 ± 2.1	16.4	++++
CVC2	El Tor, Inaba, <i>tcpA</i> ⁺ , <i>toxT</i> ⁺ , autoagglutination ⁺	13.46 ± 5.4	14.3	++++
CVC3	El Tor, Inaba, <i>tcpA</i> ⁺ , <i>toxT</i> ⁺ , autoagglutination ⁺	11.40 ± 3.01	ND	++++
CVC4	El Tor, Inaba, <i>tcpA</i> ⁻ , <i>toxT</i> ⁻ , autoagglutination ⁻	7.6 ± 4.16	6.1	++
CVC5	El Tor, Inaba, <i>tcpA</i> ⁺ , <i>toxT</i> ⁺ , autoagglutination ⁺	14.6 ± 0.7	12.4	++++
CVC6	El Tor, Inaba, <i>tcpA</i> ⁺ , <i>toxT</i> ⁻ , autoagglutination ⁻	7.2 ± 1.8	6.6	++
CVC7	El Tor, Inaba, <i>tcpA</i> ⁺ , <i>toxT</i> ⁺ , autoagglutination ⁺	12.6 ± 0.6	14	++++
CVC8	El Tor, Inaba, <i>tcpA</i> ⁻ , <i>toxT</i> ⁻ , autoagglutination ⁻	7 ± 2	7.2	++
CVC9	El Tor, Inaba, <i>tcpA</i> ⁺ , <i>toxT</i> ⁺ , autoagglutination ⁺	13.0 ± 5.5	12	++++
EVC1	Non-O1 non-O139, <i>tcpA</i> ⁻ , <i>toxT</i> ⁻ , autoagglutination ⁻	7.73 ± 3.5	ND	++
EVC2	Non-O1 non-O139, <i>tcpA</i> ⁻ , <i>toxT</i> ⁻ , autoagglutination ⁻	7.1 ± 1.9	ND	++
EVC3	Non-O1 non-O139, <i>tcpA</i> ⁻ , <i>toxT</i> ⁻ , autoagglutination ⁻	6.2 ± 1.0	8	++
EVC4	Non-O1 non-O139, <i>tcpA</i> ⁻ , <i>toxT</i> ⁻ , autoagglutination ⁻	16.3 ± 3.2	15.4	++++
EVC5	Non-O1 non-O139, <i>tcpA</i> ⁻ , <i>toxT</i> ⁻ , autoagglutination ⁻	11.33 ± 3.3	9.6	++++

CVC, Clinical *Vibrio cholerae*; EVC, environmental *V. cholerae*; ND, not done.

*Strong adhesion, ≥ ++++; weak adhesion, ≤ ++.

†Values were recorded as mean ± SEM each in triplicate.

Amongst the total clinical strains, four (10 %) did not contain *toxT*, and one of which contained all other VPI-associated genes. The strain, CVC6, showed no autoagglutination but a weak attachment on HT-29 and Vero cells. Two other *toxT*⁻ strains (CVC4 and CVC8) did not contain any genes of the VPI cluster nor show an autoagglutination phenotype. They presented, however, a weak attachment on Vero and HT-29 cell lines. *tcpA*⁺/*toxT*⁺ strains showed a typical autoagglutination and strong attachment to the cells. Chakraborty *et al.* (2000) studied environmental strains of *V. cholerae* and demonstrated that strains carrying both *tcpA* and *toxT* showed an autoagglutination phenotype, whereas strains positive for *tcpA* but negative for *toxT* did not autoagglutinate. However, they did not associate the autoagglutination with the cellular adhesion. Based on the results presented in our study we propose the hypothesis that the strong bacterial/cell attachment is a multi-factorial phenotype due to the presence of different colonization factors including TcpA. Weak attachment to cells was seen in strains with no Tcp expression and autoagglutination (either *tcpA*⁻ *toxT*⁻ or *tcpA*⁺ *toxT*⁻), possibly due to colonization factors other than Tcp or to non-specific attachment mediated by the bacterial cell surface.

In this study, *V. cholerae* ATCC 14035 (the positive control) showed a weak attachment to Vero and HT-29 cells (characteristic of weak virulent strains), which is consistent with the study done by Pruzzo *et al.* (2003), revealing the possibility of development of atypical or mild clinical forms of cholera. Data presented by others (Baselski *et al.*, 1978; Smirnova *et al.*, 2004) also support this hypothesis.

In the environmental strains, the cellular attachment followed a different pattern than that observed in the clinical isolates. Two of our environmental strains (EVC4 and EVC5) with *tcpA*⁻/*toxT*⁻ genotype showed strong adhesive properties to the cells. Non-fimbrial adhesive factors such as MSHA (mannose sensitive haemagglutinin antigen) and 53 kDa CBP (chitin binding protein) have been suggested as colonization factor candidates (Kirn *et al.*, 2005; Zampini *et al.*, 2005; Vezzulli *et al.*, 2008). Other colonization factors such as TcpF, TcpI and AcfB have recently been identified (Kirn *et al.*, 2003; Chaparro *et al.*, 2010). Other unknown colonization factors may also play role in the TCP⁻ non-O1-non-O139 environmental *V. cholerae* strains as suggested by Faruque *et al.* (2004).

In conclusion, this study shows that the strong attachment of the clinical isolates of *V. cholerae* to the cell lines could be due to the intact structure of the pathogenicity island. However, the mosaic structure of the VPI in the environmental strains might be attributed to selective pressure of the environmental signals that render them to express and use adhesive molecules other than TCP.

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