O8:21 was also confirmed among other *tdh*+ serogroups (Alam *et al.*, Unpublished data). In this study, which is not a routine one but a follow-up carried out in two subsequent sampling carried out between late 2007 and early 2008 in the same coastal area, *V. parahaemolyticus* strains isolated from aquatic ecosystem showed the presence of *tdh*+ O8:K21 among other *tdh*+ serogroups, namely O9:KUT, O4:K46, and OUT:K33, but so far no pandemic serogroups namely O3:K6 was found among the strains isolated during 2007. Interestingly, when we carried out our subsequent environmental sampling in early 2008 in the same coastal region, pandemic pathogen O3:K6 was found to be the prevalent among the other pandemic seogroups O4:K68 and O1:KUT. Although other *tdh*+ serogroups namely OUT:KUT, O8:KUT, O3:KUT, O5:KUT, and O4:KUT were also found with the pandemic serogroup strains, the O8:K21, which was previously reported as being prevalent *tdh*+ serogroup having clinical link in the same aquatic ecosystem, was however not found during early 2008. The serogrouping data presented in this study thus clearly show a temporal serotypic shift of *V. parahaemolyticus*, suggesting the coastal aquatic ecosystem of the world's largest mangrove forest of Bay of Bengal as being an important niche where pandemic pathogen O3:K6 serogroup was first reported in 1996. Further study is in progress in our laboratory.

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Table 1. Serological and molecular characteristics of *V. parahaemolyticus* strains (n=60) isolated from the coastal aquatic ecosystem of Bangladesh during 2006 – 2008.

Year of Isolation	Source	Serogroup	Number	Species specific genes		Virulence gene content		Pandemic marker genes	
				toxR	*tlh*	*tdh*	*trh*	ORF8-PCR	GS-PCR
2006	Clinical	O8:K21	3	+	+	+	-	-	-
	Clinical	O3:K6	8	+	+	+	-	+	+
	Clinical	O4:K55	1	+	+	+	-	-	-
	Clinical	O1:K56	1	+	+	+	-	-	-
2007	Clinical	O1:KUT	1	+	+	+	-	+	+
	Clinical	O8:K22	1	+	+	+	-	-	-
	Clinical	O3:K5	1	+	+	+	-	-	-
2007	Environmental	O8:K21	3	+	+	+	-	-	-
	Environmental	O9:KUT	3	-	+	-	-	-	-
	Environmental	O9:KUT	1	+	+	+	-	-	-
	Environmental	O4:K46	3	+	+	-	-	-	-
	Environmental	OUT:K33	3	+	+	-	-	-	-
2008	Environmental	O4:K68	2	+	+	+	-	+	+
	Environmental	OUT:KUT	4	+	+	+	-	-	-
	Environmental	O5:K30	1	+	+	+	-	-	-
	Environmental	O8:KUT	3	+	+	+	-	-	-
	Environmental	O3:K6	16	+	+	+	-	+	+
	Environmental	O3:KUT	3	+	+	+	-	-	-
	Environmental	O5:KUT	2	+	+	+	-	-	-
	Environmental	O4:KUT	1	+	+	+	-	-	-
	Environmental	O9:KUT	1	+	+	+	-	-	-
	Environmental	O1:KUT	2	+	+	+	-	-	-

Characterization of Shigella like organism (SLOs) Isolated from Diarrhoeal Patients in Bangladesh

Investigators: Talukder KA, Azmi IJ, Salam MA, Islam MA, Ingrid Filliol, Thomas Cheasty, G. B. Nair, H. Watanabe, Françoois-Xavier Weill, Hubert P Endtz, and Alejandro Cravioto

Introduction:

Shigellosis is one of the major diarrhoeal diseases in Bangladesh and other developing countries, which accounts for a significant number of deaths especially among children less than 5 years of age (Kotloff *et al.,* 1999). Outbreaks due to *Shigella* infection are difficult to control because of their low infectious dose (Dupont *et al.,* 1989). Although more prevalent in developing countries, shigellosis is a worldwide problem. It has been estimated that more than 95,000 children <5 years of age die of shigellosis annually in Bangladesh (Kotloff *et al.,* 1999). *Shigella* is spread by direct fecal-hand-oral contact wherever personal hygiene is compromised (Weissman *et al.,* 1975). The genus *Shigella* is comprised of four subgroups that historically have been treated as species (Ent. sub. 1958): subgroup A is referred to as *S. dysenteriae*, subgroup B as *S. flexneri*, subgroup C as *S. boydii* and subgroup D as *S. sonnei.*

Until 1958, the time of the last major classification of the genus *Shigella* (*Enterobacteriaceae* Subcommittee of the Nomenclature Committee of the International Association of Microbiological Societies, 1958), *S. dysenteriae* consisted of 10 serotypes. Subsequently, Ewing et al. (Ewing *et al.,* 1958) described provisional serotypes 3873-50 and 3341-55, which were later proposed for addition to the *Shigella* scheme as *S. dysenteriae* serotypes 11 and 12, respectively (Brenner *et al.,* 1984 & Petrovskaya *et al.,* 1979). In 1985, Shmilovitz et al. (Shmilovitz *et al.,* 1985) isolated 31 strains of a new provisional serotype represented by the strain I9809-73. This provisional serotype was included in the *S. dysenteriae* scheme as serotype 13 after further characterization by Wathen-Grady et al. (Wathen-Grady *et al.,* 1990). Provisional serotypes E22383 and E23507 were originally reported in 1989 (Gross *et al.,* 1989). After extensive characterization and isolation from various geographical locations, Ansaruzzaman et al. (Ansaruzzaman *et al.,* 1995) recommended that they be designated

serotypes 14 and 15, respectively. Recently, seven non agglutinating strains have been reported as novel *S. dysenteriae* strain isolated from diarrhoeal patients (Talukder *et al.*, 2007). This potentially new serotypes were represented by strain KIVI 162.

Antigenic expression observed through serotyping remains the "gold standard" in identifying, subtyping and monitoring organisms such as *Shigella* (Melito *et al.*, 2005). In our previous study, we found that a significant number of strains isolated between 1999 and 2007 had biochemical properties typical of *Shigella* but could not be serotyped following the current serotyping scheme available for *Shigella* (Talukder *et al.*, 2007). These strains were designated as *Shigella*-like *organism* (SLO). It has been estimated that around 3% of *Shigella* infection at ICDDR,B hospital are caused by the SLO (Talukder *et al.*, 2007). Besides, the prevalence of SLO has been increasing over the years, which accentuates the necessity of an extensive characterization of these strains. The purpose of the present work was to characterize these non agglutinating *Shigella* isolates by biochemical tests, PCR, PFGE, *rfb*-RFLP and MLST. As a result, 9 Bangladeshi isolates tested were found to constitute a new serotype of *S. dysenteriae*. In addition, serotypic validation of these strains was done by French National Reference Centre for *E. coli* and *Shigella*, Institute of Pasteur, Paris, France.

Summary of activity findings:

During the period of January 2004 to December 2009, 14,278 isolates of *Shigella* had been isolated from the diarrhoeal patients attending the Dhaka treatment center of ICDDR, B. It has been estimated that around 4% of *Shigella* infection are caused by the SLO. None of the SLOs (n=24) identified in this study reacted to the established *Shigella* serovars. These isolates did not ferment lactose and showed typical biochemical characteristics of *Shigella* species. Of these isolates, two clusters were identified based on mannitol fermentation. The first cluster is biochemically identical with *S. dysenteriae*. The other cluster is biochemically identical to *S. boydii*. However in this study we mainly focused on the characterization of the first cluster, proposed new *Shigella dysenterae*.

We are regularly collecting *Shigella*-like organisms from clinical microbiology laboaratory and doing their characterization by phenotypic and genotypic techniques, such as antibiogram, PFGE and Plasmid profiling. Recently, we are raising antibody against the representative strains of these new group.

Strain no	Biotyping	Antibiotic susceptibility	Presence of virulence/invasive genes					Test for invasiveness			Plasmid typing	PFGE typing
			stx	*set1*	*set2*	*ial*	*ipaH*	140 MDa	Sereny test	CRB		
Proposed New S. dysenteriae												
KIVI 141	B1	R1	–	–	+	+	+	+	+	+	P1	A
KIVI 142	B1	R1	–	–	+	+	+	+	+	+	P1	A
KIVI 143	B1	R1	–	–	+	+	+	+	+	+	P1	A
KIVI 145	B1	R1	–	–	+	+	+	+	+	+	P1	A
KIVI 152	B1	R1	–	–	+	+	+	+	+	+	P1	A
KIVI 156	B1	R1	–	–	+	+	+	+	+	+	P1	A
KIVI 157	B1	R1	–	–	+	+	+	+	+	+	P1	A
KIVI 232	B1	R1	–	–	+	+	+	+	+	+	P1	A

TABLE 1. Characteristics of *Shigella*- like organisms studied

KIVI 316	B1	R1	–	–	+	+	+	+	+	+	P1	A

–, negative; +, positive.
B1, Glucose +, Arabinose +, Maltose +, Arginine +
R1, Amp^{s},Sxt^{s},Tet^{s},Mel^{s},Cro^{s},Nal^{s},Cip^{s}; ^{s}, sensitive.
P1, 140, 2.7, 1.8,1.4 MDa
CRB, Congo red binding ability

Characterization of SLOs isolated between 2004 and 2009

Shigella Like Organism (n=508)				
Published		**Unpublished**		**Under investigation**
***Shigella boydii* 20 (n=126)**	**Novel *Shigella dysenteriae* (n=43)**	**Proposed New *Shigella boydii* (n=47)**	**Proposed new *Shigella dysenteriae* (n=34)**	**258**

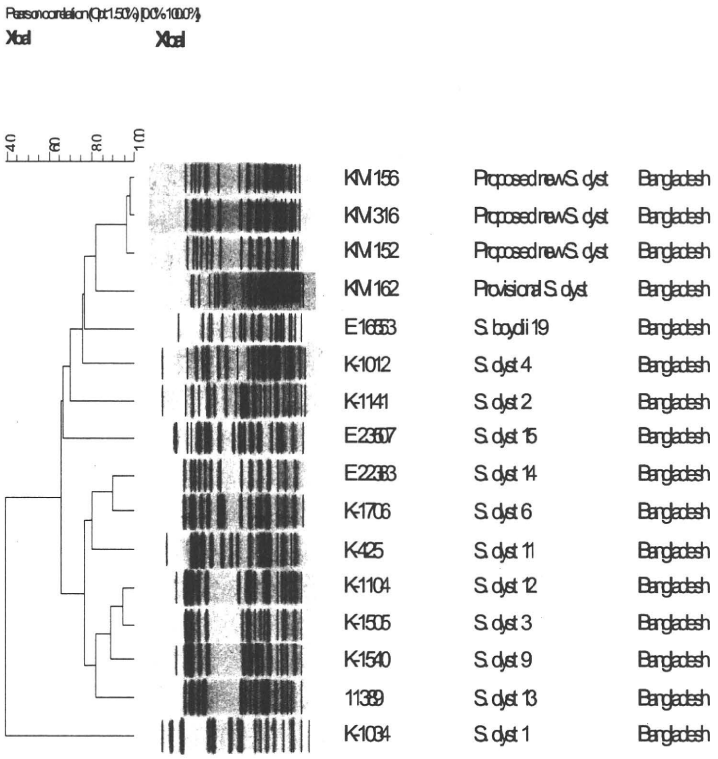
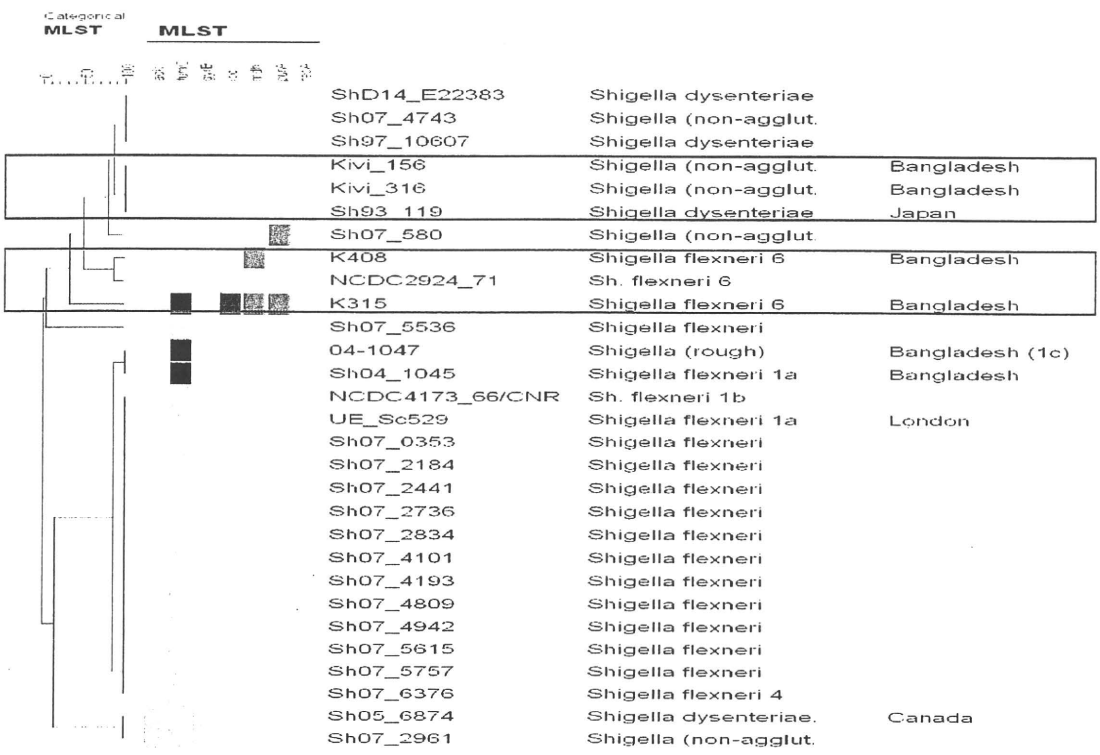
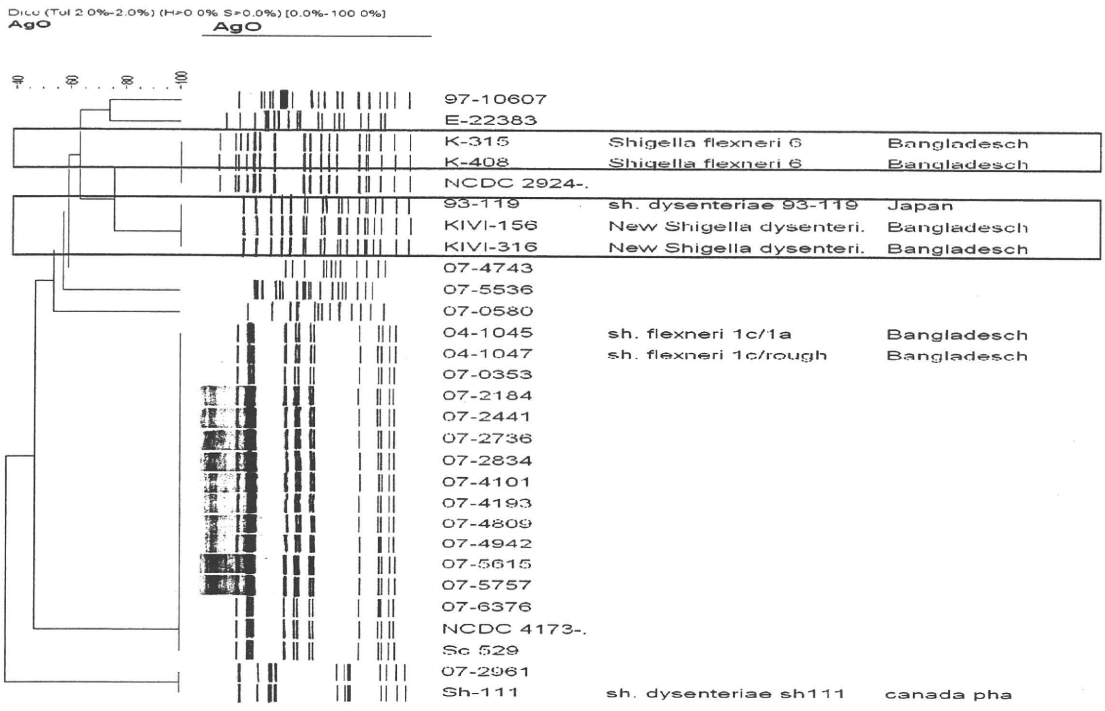


FIG. 1 Dendrogram showing the PFGE patterns of studied *S. dysenteriae* strains with other existing serotypes of *S. dysenteriae*. The dendrogram was constructed with Bionumerics 4.5 software using the unweighted pair group method with arithmetic means (UPGMA) method. Thin lines were added by the program and show the location (peak of densitometry curve) of less-intense bands included in the analysis.

FIG. 2 MLST Typing of new *Shigella*




Dice (Tol 2.0%-2.0%) (H=0.0% S=0.0%) [0.0%-100.0%]
AgO
AgO
97-10607
E-22383
K-315 Shigella flexneri 6 Bangladesch
K-408 Shigella flexneri 6 Bangladesch
NCDC 2924-.
93-119 sh. dysenteriae 93-119 Japan
KIVI-156 New Shigella dysenteri. Bangladesch
KIVI-316 New Shigella dysenteri. Bangladesch
07-4743
07-5536
07-0580
04-1045 sh. flexneri 1c/1a Bangladesch
04-1047 sh. flexneri 1c/rough Bangladesch
07-0353
07-2184
07-2441
07-2736
07-2834
07-4101
07-4193
07-4809
07-4942
07-5615
07-5757
07-6376
NCDC 4173-.
Sc 529
07-2961
Sh-111 sh. dysenteriae sh111 canada pha

Output:

Some of these data has been published in Journal Medical Microbiology in 2007.

Talukder KA, **Mondol AS,** Islam MA, Islam, Z, Dutta DK, Khajanchi BK, Azmi IJ, Hossain MA, Rahman M, Cheasty T, Cravioto A, Nair GB, Sack DA. 2007. A novel serovar of *Shigella dysenteriae* from patients with diarrhoea in Bangladesh. J Med Microbiol. 56: 654-8.

Future plan:

The rest of the isolates are being characterized following the methods as described above. At the same time we are looking for new SLOs for further characterization. All the characterized strains will be studied for pathogenicity in cultured-cell line and in animal models. O-antigen structures of all newly identified serotype of *Shigella* will be analyzed.

Paper presented in scientific conferences:

1. **Talukder KA**, Abdus. S. Mondol, M. Aminul Islam, Ishrat J. Azmi, Zhahirul Islam[1], Mohammad Aslam[1], Dilip K. Dutta[1], Mahmuda Akter[1], M. A. Hossain, Filliol I,Thomas Cheasty, Hubert P Endtz and Alejandro Cravioto. Identification and characterization of provisional serovar of *S. dysenteriae* from diarrhoeal patients in Bangladesh. 44th US-Japan Conference on Cholera and Other Bacterial Enteric Infections held October 12-14, 2009 in San Diego, California USA.

2. **Talukder KA**, D. K. Dutta, M. A. Islam, Z. Islam, I. J. Azmi, A. S. Mondol, M. Aslam, S. K. Niyogi, L. von Seidlein, I. Filliol, A. Nur-E-Kamal, T. Cheasty, G. B. Nair, D. A. Sack, H. P. Endtz, and A. Cravioto. New variants of *Shigella* species in Bangladesh and their molecular characterization. Presented in US-Japan cholera conference, April 06-10, 2009, Kolkata, India.

3. **Talukder KA**, Ishrat J. Azmi, Z. Islam, D. K. Dutta, M. A. Islam, Abdus S. Mondol, Lorenz von Seidlein, **Alam Nur-E-Kamal,** G. B. Nair, D. A. Sack, Hubert P Endtz, and Alejandro Cravioto. Shigella flexneri 1c, a new variant of Shigella: prevalence and their characterization at the molecular and cellular level. In: 6th PulseNet Asia Pacific Planning Meeting held in Nonthaburi, Thailand between 15-17 December, 2009.

Progress Report:

Title: Prototype El Tor Displaces Classical and Variant El Tor from Endemic Cholera in Mexico, 1998 – 2008

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Summary: *Vibrio cholerae* serogroup O1 biotype El Tor (ET), responsible for the current 7th pandemic of cholera, has recently been replaced in Asia and Africa by an ET variant possessing cholera toxin (CTX) of the classical (CL) biotype, which caused the 6th cholera pandemic before becoming extinct from Asia in the 1980's. In a recent retrospective study, biotypes CL, ET, and ET variants were shown to be involved in endemic cholera in Mexico between 1991 and 1997, although the CL biotype and ET variants have never been reported from Latin America, and ET prototype was the circulating biotype shown in Peru until recently. This study, a follow-up on the microbiological, molecular and phylogenetic analyses of *V. cholerae* isolated during 1998 – 2008 (n=83) shows the disappearance of CL and variant ET, including a series of genetic events that precede the restoration of ET prototype in endemic cholera in Mexico. According to sero-biotyping data, all *V. cholerae* tested had ET traits; the diverse strains of variant ET that had shown predominance in Mexico (1991 – 1997) were not found after 2000. All *V. cholerae* strains isolated during 2001 - 2003 lacked the targeted CTX-prophage marker genes, namely *ctxA*, *ctxB*, $rstR^{ET}$ or $rstR^{CL}$, but had *tcpA* alleles of either ET or CL, or both in the same ET host, showing a unique state of *V. cholerae* of the contemporary cholera. In 2004, ET possessing prototype CTX-prophage reemerged, displaced ET strains lacking CTX-prophage in 2005, and continued through 2008, displaying a rare evidence of inter- and intra-biotype competition involved in the evolution of prototype ET in Mexico. The transitional ET lacking CTX-prophage exhibited high heterogeneity in PFGE (*Not*I) patterns, while the pre- and post-2005 prototype ET showed high homogeneity and resembled with the prototype ET control (N16961), as confirmed by sub-clustering in dendrogram. The underlying molecular ecological basis for this clonal shift remains an area to be understood; nonetheless, the disappearance of ET variants and restoration of prototype ET in contemporary cholera in Mexico may be yet another turning point in the changing epidemiology of global cholera.

Purpose: *Vibrio cholerae* has more than 200 O serogroups, but cholera is caused by only toxigenic strains belonging to serogroups O1 and O139. *V. cholerae* O1 has two distinct biotypes, classical (CL) and El Tor (ET), the first of which caused first six of the seven recognized cholera pandemics. The ET biotype, which emerged as the cause of the seventh cholera pandemic by displacing the classical (CL) biotype from Asiatic cholera in 1960s, reached Africa in 1970 and then South America in 1991. The ET in Africa was claimed to be an extension of the 7th pandemic arriving from Asia. However, when cholera re-emerged after more than a century in South America, affecting Peru in January 1991, spreading rapidly to the countries of Latin America, reaching Mexico in June of the same year, an

extensive body of literature describes ideas about the source and transmission that were contrasting. The distinct characteristic features presented by the Latin American *V. cholerae* O1 biotype El Tor (ET) strains distinguished them from the *V. cholerae* ET causing the 7th pandemic in Asia. The clonal nature of the bacterium suggested, however, that the Latin American epidemic in 1991 was the extension of the 7th pandemic *V. cholerae* ET bacterium arriving through maritime transport plying to and from the Western hemisphere.

V. cholerae serogroup O139 first emerged as the cause of massive cholera outbreaks in the coastal villages of Bay of Bengal in 1992. This new cholera serogroup, which was shown to be of ET ancestry, was the result of exchange of the O antigen biosynthetic gene of a progenitor *V. cholerae* O1 ET with a non-cholera serogroup strain (Waldor M, 1994). Although *V. cholerae* O139 was thought to be the causal bacterium of the proposed 8th cholera pandemic, this serogroup was never reported from cholera in Latin America, and ET continues to remain the dominant strain causing cholera globally.

Historically, sporadic cholera cases occurred in the Americas in the 1830's and since then endemic cholera continued to be present there until 1895. Although *V. cholerae* CL biotype caused the first six of the seven recorded cholera pandemics, spreading from Asia between 1817 and 1923, with five of these affecting the American continent, CL biotype was never reported from America until recently. A significant recent development has been the emergence of a hybrid ET harboring the cholera toxin of the CL biotype, which has emerged by replacing the prototype ET that initiated the 7th pandemic by displacing *V. cholerae* CL biotype from its Asian habitats in the 1980's. Genetic changes among *V. cholerae* causing endemic cholera in Latin America were evident from many literature describing changing serotypes, electrophoretic types, ribotypes, and PFGE types. Nonetheless, while the new variant ET has completely replaced the prototype ET in Asia and Africa (29), *V. cholerae* O1 strain causing endemic cholera in Peru between 1991 and 2003 was shown to be ET of the 7th pandemic prototype. The Peruvian ET strains that seem to be linked clonally to the Asian and African ET prototype were, however, shown to have a distinct signature in the VSP-II region that distinguishes them from the ET prototype isolated worldwide (Nusrin 2008).

A recent retrospective study on *V. cholerae* isolated between 1991 and 1997 showed that the biotypes CL, ET, and ET variants were involved in endemic cholera in Mexico, although the CL biotype and ET variants have never been reported from Latin America before, and ET prototype was the circulating biotype reported from Peru until recently. In order to further understand the evolutionary trends of bacteria causing endemic cholera, we carried out a follow-up study on the microbiological, molecular and phylogenetic analyses of 89 *V. cholerae* strains isolated from cholera cases in Mexico between 1998 and 2008. Here we present evidence of the complete disappearance of *V. cholerae* biotypes CL and variant ET strains, showing a series of rare genetic events that preceded the re-emergence in 2004 of the prototype ET and its progression as the only biotype involved in endemic cholera in Mexico through 2008.

Methods:

Bacterial strains. *Vibrio cholerae* O1 strains included in the present study are shown in Table 1 together with their source, place, and year of isolation.

Identification of the *V. cholerae* strains. *V. cholerae* strains characterized in the present study were obtained from the Dept. of Public Health, Faculty of Medicine, National Autonomous University of Mexico (UNAM), and the Centro de Investigacio´n Científica y de Educacio´n Superior de Ensenada, Baja California, Mexico. These strains were of patient origin isolated as part of the

nationwide cholera surveillance between 1998 and 2008 (3). The bacterial strains shipped and received in soft agar were revived by standard culturing methods and identified by a combination of biochemical and molecular methods, as described previously (1).

Serogroup determination. *V. cholerae* strains identified using biochemical and molecular methods were screened serologically by slide agglutination test using specific polyvalent antisera to *V. cholerae* O1 and O139, followed by specific monoclonal antibody (1).

Biotype determination. *V. cholerae* O1 strains confirmed in the present study were screened further for their biotype characteristics. Biotyping involved the phenotypic tests such as: chicken erythrocyte agglutination; sensitivity to polymyxin B; and Mukerjee classical (CL)-specific phage IV and Mukerjee El Tor (ET)-specific phage V tests (15). Furthermore, the biotype-specific marker genes, such as *tcpA* (CL or ET), and *rstR* (CL or ET) (16), were targeted to complement the phenotypic tests and PCR assays were carried out using procedures described previously (Hoshino 1998).

Storage of the bacterial strains. *V. cholerae* strains identified by the abovementioned biochemical, serological and molecular methods were sub-cultured on Gelatin Agar (GA) plates and a single representative colony from the GA plate was aseptically inoculated into T1N1 broth (1% Trypticase and 1% NaCl), incubated at 37°C for 4-6 hrs, and stored at -80°C with 15% glycerol for future use.

Preparation of genomic DNA. Genomic DNA from each of the *V. cholerae* strains were extracted following previously described methods (24).

Complementation of the serogrouping results by PCR assays. *V. cholerae* strains identified by the abovementioned methods were further reconfirmed by primer extension of *V. cholerae*-species-specific gene *ompW* (24). The serogrouping results of these strains were further complemented with polyvalent O1 and monovalent Inaba and Ogawa antisera, and by multiplex PCR amplification of serogroup-specific O biosynthetic genes *wbe*O1 (serogroup O1) and *wbf*O139 (serogroup O139), and the cholera toxin gene (*ctxA*) (14).

Determination of *ctxB* gene type. The biotype-specific *ctxB* gene type was determined by recently developed PCR methods, mismatch amplification mutation assay (MAMA), which utilizes the sequence polymorphism between the CL and ET *ctxB* by focusing on nucleotide position 203 of the *ctxB* gene (21). MAMA-PCR assay was performed to test for the presence of the *ctxB* genes specific for CL and ET biotypes. A conserved forward primer (Fw-con, 5'-ACTATCTTCAGCATATGCACATGG -3') and two allele-specific polymorphism detection primers, Rv-cla (5'-CCTGGTACTTCTACTTGAAACG-3') and Rv-elt (5'-CCTGGTACTTCTACTTGAAACA -3') were used. PCR conditions were as follows: after initial denaturation at 96°C for 2 min, 25 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 10 sec, extension at 72°C for 30 sec; and final extension at 72°C for 2 min, the resulting *V. cholerae* O1 isolates O395 CL and N16961 ET were used as standard reference strains.

Pulsed-field gel electrophoresis (PFGE). The whole agarose-embedded genomic DNA from *V. cholerae* was prepared. PFGE was carried out with a contour-clamped homogeneous electrical field (CHEF-DRII) apparatus (Bio-Rad) according to procedures described elsewhere (6). The conditions used for separation were as follows: 2 to 10s for 13h, followed by 20 to 25s for 6h. An electrical field of 6V/cm was applied at an included field angle of 120°. Genomic DNA of test strains were digested by the *Not*I restriction enzyme (GIBCO-BRL, Gaithersburg, Md.) and *Salmonella braenderup* was digested by *Xba*I with the fragments being used as molecular size markers. The restriction fragments were separated in 1% pulsed-field-certified agarose in 0.5X TBE (Tris-borate-EDTA) buffer. In the post

electrophoresis gel treatment step, the gel was stained and de-stained. The DNA was visualized using a UV trans-illuminator and images were digitized via the 1D Gel documentation system (Bio-Rad).

Image analysis. The fingerprint pattern in the gel was analyzed using a computer software package, Bionumeric Software Package (Applied Maths, Belgium). After background subtraction and gel normalization, the fingerprint patterns were subjected to typing based on banding similarity and dissimilarity using Dice similarity coefficient and UPGMA clustering methods as recommended by the manufacturer; these were graphically represented as dendrograms.

Results: (if necessary, figures and tables will be included)

Microbiological and serological tests. All tested strains (n=89) had produced characteristic colonies typical of *V. cholerae* on TTGA. All *V. cholerae* strains reacted to polyvalent O1-specific antisera and agglutinated with the monoclonal antibody specific for O1, but not for O139, and reacted to either monovalent Inaba or Ogawa antisera, suggesting that they belonged to serogroup O1. Among 89 *V. cholerae* strains 47 were Inaba and the remaining 42 were Ogawa, as shown in Table 1.

Amplification of primers specific for *V. cholerae* serogroup O1 and *ctxA* by PCR assays. All of the tested strains (n=89) amplified the primers for the *V. cholerae* species-specific gene *ompW* (Table 1). The primers for the O-biosynthetic gene *wbe*O1 were amplified by all strains, complementing the serological data and further confirming all to be *V. cholerae* O1. When tested for the presence of *ctxA* gene encoding the 'A' subunit of cholera toxin (CTX), 58 strains amplified the primers specific for *ctxA* gene, confirming the *V. cholerae* O1 to be *ctx*+ (Table 1), while 31 *V. cholerae* O1 strains did not amplify the primers for *ctxA* gene, suggesting them to be *ctx*-.

Phenotypic and related genotypic characteristics. The data on *V. cholerae* O1 strains (n=89) showing variable phenotypic and related genetic characteristics are presented in Table 1. Although *V. cholerae* O1 strains varied in the reaction to ET-specific phage, and CL-specific phage could not be made available to test, all strains showed El Tor (ET)-specific phenotypic traits, such as chicken-cell-agglutination (CCA), resistance to both polymixin B (poly-B) and amplified the primers for the genes *rtxC*, an ET-specific maker, confirming their ET attribute (Table 1).

Seventy five strains having ET-specific phenotypic traits amplified the primers for gene $tcpA^{ET}$, of which 43 amplified $rstR^{ET}$ and $ctxB^{ET}$ genes (Table 1), confirming them to be *V. cholerae* ET of the 7th pandemic prototype. The remaining 14 strains possessing ET-specific phenotypic attributes amplified the primers for genes $tcpA^{ET}$ along with $ctxB^{CL}$ and $rstR^{CL}$, suggesting them to be *V. cholerae* altered ET, as reported from Mexico earlier. Thirty-one ET strains lacking major virulence gene *ctxA* also lacked *ctxB* and *rstR* genes, suggesting major genetic truncation that can result due to expulsion of the lysogenic CTX-prophage genome that transforms CTX-prophage^{+} *V. cholerae* into CTX-prophage^{-}. The CTX-prophage^{-} strains had either ET-specific allele $tcpA^{ET}$ only, CL-specific allele $tcpA^{CL}$ only, or both $tcpA^{ET}$ and $tcpA^{CL}$ under the ET biotype background, suggesting possible TCP^{CL}-phage mediated transduction in the evolution of TCP variants that were unique for the present study on *V. cholerae* strains isolated from diarrheal patients in Mexico.

Year-wise data show that the altered ET biotype strains that predominated among the CL and ET biotype strains in Mexico during 1991 -1997 existed with *V. cholerae* ET strains during 1998, 1999, and finally during 2000. The departure of altered ET from Mexico was coincidental with the emergence of a unique line of CTX-prophage^{-} *V. cholerae* ET that displaced the CTX-prophage^{+} prototype and altered ET in 2001, and continued to be involved with endemic cholera in Mexico. The CTX-prophage^{-} *V. cholerae* strains isolated during 2000 had the *tcpA* allele specific for the ET biotype strains. Subsequently, CTX-prophage^{-} strains harboring $tcpA^{CL}$ allele (specific for the CL biotype) or both

tcpA^{ET} and *tcpA*^{CL} alleles under the ET background emerged and became predominant during 2001. These TCP variant strains prevailed in Mexico during 2001- 2004, providing a unique example of the association of CTX-prophage^{-} *V. cholerae* with diarrhoea. Subsequently, an analogue of the ET

Table 1. Phenotypic and related genetic characteristics of *V. cholerae* O1 El Tor (ET) and its hybrid variants isolated in Mexico (n=89; 1998 - 2008).

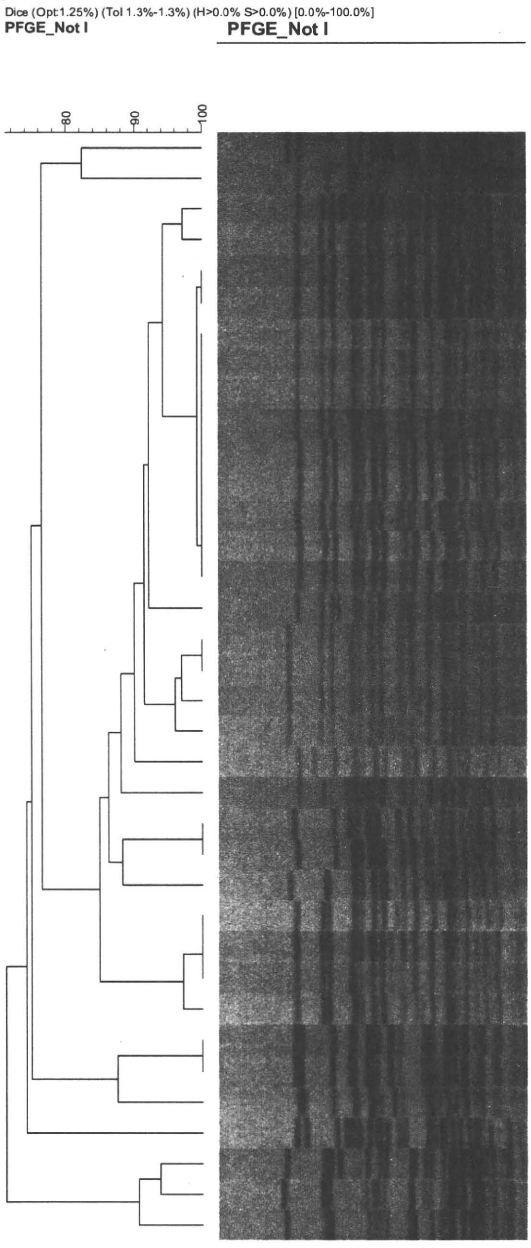
Country	Year of Isolation	No of Isolation	Serotype	Phenotypic properties				Genetic screening by PCR							Deduced
				Chicken Cell Aggl	Poly B (50U)	Phage IV Classical	Phage V El Tor	*ompW*	*wbeO1*	*ctxA*	**CtxB* type	*tcpA*	*rtxC*	*rstR* type	Biotype
Mexico	1998	3	Ogawa	+	R	ND	S	+	+	+	C	E	E	E,C	Alt-ET
	1999	3	Ogawa	+	R	ND	S	+	+	+	C	E	E	E,C	Alt-ET
		1	Ogawa	+	R	ND	S	+	+	+	E	E	E	E	ET
		5	Ogawa	+	R	ND	S	+	+	-	-	E	E	-	ET
		1	Ogawa	+	R	ND	R	+	+	-	-	E	E	-	ET
		1	Inaba	+	R	ND	S	+	+	-	-	E	E	-	ET
	2000	3	Ogawa	+	R	ND	S	+	+	+	E	E	E	E	ET
		8	Ogawa	+	R	ND	S	+	+	+	C	E	E	E,C	Alt-ET
		1	Ogawa	+	R	ND	R	+	+	-	-	E	E	-	ET
		4	Inaba	+	R	ND	R	+	+	-	-	E	E	-	ET
	2001	3	Ogawa	+	R	ND	S	+	+	-	-	E	E	-	ET
		2	Ogawa	+	R	ND	S	+	+	-	-	C	E	-	TCP-Var
		3	Ogawa	+	R	ND	R	+	+	-	-	E,C	E	-	TCP-Var
		1	Inaba	+	R	ND	S	+	+	-	-	E,C	E	-	TCP-Var
	2002	1	Ogawa	+	R	ND	S	+	+	-	-	E,C	E	-	TCP-Var
	2003	2	Ogawa	+	R	ND	S	+	+	-	-	E,C	E	-	TCP-Var
		1	Ogawa	+	R	ND	S	+	+	-	-	C	E	-	TCP-Var
		1	Inaba	+	R	ND	S	+	+	-	-	E,C	E	-	TCP-Var
	2004	1	Ogawa	+	R	ND	S	+	+	-	-	C	E	-	TCP-Var
		1	Ogawa	+	R	ND	R	+	+	-	-	E,C	E	-	TCP-Var
		3	Ogawa	+	R	ND	S	+	+	-	-	E	E	-	ET
		4	Inaba	+	R	ND	S	+	+	+	E	E	E	E	ET
	2005	10	Inaba	+	R	ND	S	+	+	+	E	E	E	E	ET
	2006	11	Inaba	+	R	ND	ND	+	+	+	E	E	E	E	ET
	2007	6	Inaba	+	R	ND	ND	+	+	+	E	E	E	E	ET
	2008	9	Inaba	+	R	ND	ND	+	+	+	E	E	E	E	ET
N16961		Control	Inaba	+	R	R	S	+	+	+	E	E	E	E	El Tor
O395		Control	Ogawa	-	S	S	R	+	+	+	C	C	-	C	Classical

* Determined by mismatch amplification mutation assay (MAMA) PCR (Morita et al., 2008) ; **Serologically non-O1/non-O139
Chicken cell aggl, chicken cell agglutination; Poly B, polymixin B; R, resistant; S, sensitive; ET, El Tor; E, El Tor; Alt, altered; C, classical

prototype harboring all the targeted ET-specific marker genes, including the CTX-prophage markers, re-emerged in 2004, completely displaced the CTX-prophage^{-} TCP variants, and continued through 2008, displaying a direct in situ evidence of intra-biotype competition that finally brought back *V. cholerae* ET of the 7th pandemic prototype as the clone causing endemic cholera in Mexico.

PFGE and cluster analysis. The genomic organization of *V. cholerae* O1 strains isolated during 1998 – 2008 in Mexico were examined by pulsed-field gel electrophoresis (PFGE) to determine genetic relatedness. The *Not*I restriction enzyme digested the genomic DNA of both the test and the reference control strains into 20 to 23 fragments; the molecular sizes of the DNA fragments ranged between 20 to 350kb. *V. cholerae* O1 strains of the prototype 7th pandemic ET biotype, which harbored *tcpA*^{ET} and all of the targeted ET biotype-specific CTX-prophage marker genes presented an overall banding pattern that was characteristic of the prototype ET of the 7th pandemic reference control N16961 (Fig. 1). The TCP-variant strains, which were truncated in CTX-prophage marker genes, varied greatly in their PFGE patterns and did not match with the signature banding patterns of *V. cholerae* ET or CL reference controls, suggesting them to represent a transitional and heterogeneous group that eventually lost niche with the prototype *V. cholerae* ET strain in Mexico.

Cluster analysis performed with dendrograms (Dice similarity coefficient and UPGMA clustering methods) obtained from the PFGE patterns of *Not*I-digested genomic DNA separated the transitional TCP-variants of ET from the pre- and post-2004 (1991 - 2008) strains of prototype *V. cholerae* ET isolated in Mexico. Unlike the TCP-variant strains, which showed high divergence and did not belong to any specific cluster, the prototype ET strains isolated in Mexico (1991 - 2008) formed a tight cluster that also included the ET reference control, suggesting clonal relatedness.



Key	Type	SB type	Source	Year	Country
2370	E&C tcpA	OGET	Clin	2001	Mexico
644	E&C tcpA	OGET	Clin	2002	Mexico
116068	Pro-ET	OGET	Clin	1991	Peru
N16961R	Pro-ET	INET	Clin	1972	Bangladesh
36	Pro-ET	INET	Clin	2006	Mexico
587	Pro-ET	INET	Clin	2006	Mexico
87151R	Pro-ET	INET	Clin	1992	Mexico
87397R	Pro-ET	INET	Clin	1993	Mexico
87409R	Pro-ET	INET	Clin	1995	Mexico
1488	Pro-ET	INET	Clin	2005	Mexico
354	Pro-ET	INET	Clin	2008	Mexico
504	Pro-ET	INET	Clin	2008	Mexico
584	Pro-ET	INET	Clin	2006	Mexico
830	Pro-ET	INET	Clin	2008	Mexico
985	Pro-ET	INET	Clin	2007	Mexico
1401	Pro-ET	INET	Clin	2004	Mexico
P-287	Pro-ET	OGET	Clin	1998	Peru
P-302	Pro-ET	OGET	Clin	1997	Peru
P-137	Pro-ET	OGET	Clin	1996	Peru
P-301	Pro-ET	OGET	Clin	1999	Peru
87275R	Pro-ET	INET	Clin	1991	Mexico
116054	Pro-ET	OGET	Clin	1991	Peru
1596	ctxAB (-)	OGET	Clin	2004	Mexico
2710	ctxAB (-)	OGET	Clin	2001	Mexico
1979R	Alt-ET	OGET	Clin	1998	Mexico
52	Alt-ET	OGET	Clin	1999	Mexico
64	Alt-ET	OGET	Clin	2000	Mexico
89	Pro-ET	OGET	Clin	2000	Mexico
54	Pro-ET	OGET	Clin	1999	Mexico
848	ctxAB (-)	INET	Clin	2000	Mexico
849	ctxAB (-)	INET	Clin	2000	Mexico
952	ctxAB (-)	INET	Clin	2000	Mexico
O395R	Class	OGET	Clin	1965	India
1474	Pro-ET	INET	Clin	2007	Mexico
1646	ctxAB (-)	OGET	Clin	2002	Mexico
2884	Pro-ET	INET	Clin	2007	Mexico

Fig. 1. Dendrogram of PFGE patters of *V. cholerae* O1 prototype ET and transitional CTX$^-$ ET possessing TCP of both ET and CL biotypes. Formation of closely related cluster of Mexican *V. cholerae* O1 prototype ET indicates their clonal nature, whereas no such tight clustering was seen for the CTX$^-$ ET and the transitional TCP variants of ET because they were highly heterogeneous in their

PFGE patterns, suggesting them to be clonally divergent. The Peruvian prototype ET strains formed a tight cluster that differentiated them from Mexican prototype ET, suggesting that they are not clonally identical because they have regional signatures in their patterns.

Discussion:

In *V. cholerae*, genetic divergence evolves through lateral gene transfer via transposable mobile genetic elements, bacteriophages, and acquisition of free DNA from the surrounding environment attributed to chitin induced competence of the bacterium of natural transformation (Meibom 2005). Recently, altered (variant) ET *V. cholerae* strains were shown to be predominant in Mexico, where both the ET and CL biotype progenitors co-existed by sharing niche, suggesting that the variant ET may have evolved via CTX^{CL}-phage-mediated transduction. The results presented herein provide rare in situ evidences of microevolution that involves the expulsion of lysogenic CTX-prophage from *V. cholerae* O1 ET biotype strains and the acquisition of $tcpA^{CL}$, a TCP gene specific for CL biotype strains, ^{CL}-prophage, in generating genetic divergence in strains, showing inter- and intra-biotype competition that led to the evolution of prototype ET in endemic cholera in Mexico. This study also provides important insights of how lysogenic CTX-prophage ejects out rendering the CTX^{+} host *V. cholerae* CTX^{-}, displaying a unique transition period when the CTX^{-} but TCP-variants of *V. cholerae* continued for four-years before the CTX^{+} *V. cholerae* O1 prototype ET emerged in endemic cholera in Mexico. The data presented support the supposition that the transitional CTX-prophage^{-} ET strains harboring tcp^{CL} allele, which were unique in the present study, may have been the result of TCP^{CL}-prophage-mediated transduction, since both the CL and ET biotype strains shared niche with ET variants in Mexico until recently. The underlying molecular-ecological bases that prompted the predominant variant ET strains to lose niche with CTX-prophage^{-} strains in endemic cholera in Mexico are yet to be understood; nonetheless, the disappearance of CL and ET variants, and the restoration of *V. cholerae* ET prototype in endemic cholera may be yet another turning point in the changing epidemiology of global cholera. Further study in this regard is in progress in our laboratory.

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