cholerae O1, which are closely related to the Asian and African seventh pandemic strains, have been reported from among the Latin American strains, detailed phenotypic and molecular studies seem to have been lacking. In the present study, we carried out retrospective phenotypic and molecular analyses of 104 *V. cholerae* O1 strains (both clinical and environmental), isolated in Mexico (1991 –1995), Peru (1991 – 1999), and Guatemala (1993); the aim being to determine their ancestry and the evolutionary trends to be able to develop unified global intervention and preventive measures. [Here we show that the epidemics in 1991 and subsequent endemic cholera in Latin America were attributed mainly to *V. cholerae* O1 El Tor in Peru, while El Tor, classical including hybrid-variants of both biotypes in Mexico, confirming two regional pools of *V. cholerae* O1, and also, Asia-independent emergence of hyb-ET from co-existing progenitors of both biotypes in Latin America.]

Materials and Methods

Bacterial strains. *V. cholerae* serogroup O1 strains characterized and compared in the present study with their source, place, and year of isolation are shown in Table 1 & 2.

Confirmation of the *V. cholerae* strains. *V. cholerae* strains used in the present study were of patents and surface water origins isolated in Latin America, between 1991-1995, Department of Public Health, Faculty of Medicine, Universidad Nacional Autonoma de Mexico. The bacterial strains shipped in soft agar were sub-cultured by streaking on Luria agar (Difco Laboratories, Detroit, Mich) followed by taurocholate tellurite gelatin agar (TTGA) (Difco Laboratories, Detroit, Mich.), and incubated at 37°C for 18 to 24 h. Colonies with the characteristic appearance of *V. cholerae* were confirmed by standard culture methods and identified by a combination of biochemical (**Tison, D.** 1999; 38) and molecular methods, as described previously (**Alam** 20061).

Serogrouping. The *V. cholerae* isolates that were identified using biochemical and molecular methods (**Alam *et al.*, 2006**; 1) were confirmed serologically by slide agglutination test using specific polyvalent antisera to *V. cholerae* O1 and O139, followed by a monoclonal antibody that is specific for both (**Alam *et al.*, 2006**; **Tison, *et al.*, 1999**; 38).

Biotyping. Biotype determination involved a number of tests: chicken erythrocyte agglutination; haemolysis of sheep erythrocytes; the Voges–Proskauer reaction; sensitivity to polymyxin B; and Mukerjee classical phage IV and Mukerjee El Tor phage V tests (**Basu** *et al.*, **1968**; 5). All tests were carried out as described in the Manual for Laboratory Investigations of Acute Enteric Infections, **WHO** (1987). To complement the biotype characterization by genetic traits, PCR assays targeted to detect *tcpA* (classical and El Tor variant) (**Keasler** *et al.*, **1993**; 20), and to the type of *rstR* gene encoding the phage transcriptional regulator were carried out using procedures described previously (**Kimsey** *et al.*, 1998; 21).

Storage of strains. *V. cholerae* strains confirmed by biochemical, molecular, and serological methods were sub-cultured on Gelatin Agar (GA) plates and a single representative colony from the GA was aseptically inoculated into T1N1 broth (1% Trypticase and 1% NaCl), incubated at 37°C for 3-4 hrs, and stored at -80°C with 15% glycerol until required.

Genomic DNA preparation. For the extraction of the genomic DNA, the harvested cells from 3 ml of overnight culture in LB broth (Miller) were subjected to alkaline lysis by 10% SDS in the presence of TE buffer (10mM Tris-HCl; 1mM EDTA, pH 8.0). The cells were then treated with freshly prepared Proteinase K (final concentration 100 µg/ml in 0.5% SDS) and incubated at 37°C for 1 h. After incubation, 1.0% CTAB/NaCl (cetyl trimethyl ammonium bromide in 0.7M NaCl) was added followed by incubation for 10 min at 65°C. RNA was removed by treating with RNase (final concentration 100 µg/ml) at 37°C for 1h. This was followed by phenol chloroform extraction and precipitation of the nucleic acid in the presence of isopropanol (**Chowdhury** *et al.*, 2000; 8). Excess salt was removed by 70% alcohol wash and the nucleic acid was air-dried, resuspended in sterile TE buffer and the purity was assayed using a spectrophotometer (Gene Quant, England) that self calculates the ratio of optical densities at 260 and 280 nm. The DNA was stored at -20°C for subsequent PCR analysis.

Confirmation of serogrouping by PCR assays. All the strains were reconfirmed using *V. cholerae*-species-specific *ompW* PCR as described previously (**Nandi** *et al.*, 2003; 27). The serogroup of these strains was further reconfirmed using polyvalent O1 and monovalent Inaba and Ogawa antisera, and by multiplex PCR targeted to identify genes encoding O1 (*wbeO1*) and O139 (*wbfO139*)-specific O biosynthetic genes and the cholera toxin gene (*ctxA*) as described previously (**Hoshino** *et al* 1998; 16).

Genome screening of *V. cholerae* by PCR assays. The genome of the strains was screened by PCR assays using 34 pairs of primers. The PCR primers used and the conditions under which the tests were carried out have been described previously (**O'shea** *et al.*, 2004; 28). PCR reagents and kits were obtained from either Perkin–Elmer or Invitrogen. During electrophoresis, DNA size of the known molecular weight marker was included to estimate the size of the amplicons. The PCR products were analyzed by electrophoresis in 1% agarose gels, stained with ethidium bromide, visualized under UV light, and recorded using a gel documentation system (Gel Doc 2000, BioRad).

MAMA-PCR assay for determination of *ctxB* gene type. The mismatch amplification mutation assay (MAMA) was recently developed to detect the sequence polymorphism between the classical and El Tor *ctxB* focused on nucleotide position 203 of the *ctxB* gene (**Morita** *et al.*, 2008; 24). MAMA-PCR assay was performed to test for the presence of the *ctxB* genes specific for classical and El Tor 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') (**Morita** *et al.*, 2008; 24) were used in this study. 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, and extension at 72°C for 30 sec; and final extension at 72°C for 2 min. *V. cholerae* O1 isolates O395 classical and N16961 El Tor were used as standard reference strains.

DNA sequencing of *ctxB* gene. Nucleotide sequencing of the *ctxB* genes of eight randomly selected strains of *V. cholerae* O1 representing El Tor, hybrid El Tor and classical strains the *ctxB* gene types of which were determined by MAMA-PCR was carried out using an ABI PRISM BigDye Terminator Cycle Sequencing Reaction kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) on ABI PRISM 310 automated sequencer as described previously (**Nair** *et al.*, 2006; 26). The nucleotide sequences of the reference strains were compared with the corresponding sequences of El Tor strain N16961 (GenBank accession no. NC-002505), and the classical strain 569B (GenBank accession no. U25679), which were retrieved from GenBank by BLAST search.

DNA sequence and proteomic analysis. The chromatogram sequencing files were inspected using Chromas 2.23 (Technelysium). Nucleotide sequences of the test isolates were compared with the corresponding sequences of the N16961 El Tor reference strain (NC_002505), the 569B classical reference strain (U25679), retrieved from GenBank using Basic Local Alignment Search Tool (BLAST) (**Altschul** *et al.*, 1997; 3). Multiple sequence alignments were developed using CLUSTALX 1.81.13, and DNA sequences were translated using GeneDoc version 2.6.002 alignment editor.

PFGE. The whole agarose-embedded genomic DNA from *V. cholerae* was prepared. Pulsed-field gel electrophoresis (PFGE) was carried out with a contour-clamped homogeneous electrical field (CHEF-DRII) apparatus (Bio-Rad) according to previously described procedures (**Cooper** *et al.*, 2004; 11). The conditions used for separation were as follows: 2 to 10s for 13h, followed by 20 to 25s for 6h. An electrical field of 6 V/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 destained. The DNA was visualized using a UV transilluminator and images were digitized via the 1D Gel documentation system (Bio-Rad). The images were then processed using Quantity One Software (version 4.4.1, Bio-Rad). The test fingerprint image was normalized according to the standard, and the molecular weights of the DNA fragments were determined using Quantity One Software (version 4.4.1, Bio-Rad). Digital images of PFGE fingerprint patterns were analyzed by Dice similarity coefficient and UPGMA clustering methods (Bio-Rad), as recommended by the manufacturer, and these were graphically represented as dendrograms.

Results

Microbiological and serological tests. All strains tested (n=104) produced characteristic colonies typical of *V. cholerae* when grown on microbiological selective agar media such as TTGA. The presumptive colonies gave biochemical reactions that were characteristics of *V. cholerae*, and all except two strains were primarily identified as serogroup O1 because they reacted to polyclonal and monoclonal antibodies specific for serogroup O1, but not to serogroup O139. All *V. cholerae* O1 strains, including the two non-O1/ non-O139 strains that were not recognized by the polyclonal and monoclonal O1 or O139 specific antibodies, reacted to monovalent Inaba and Ogawa antisera, suggesting that all belonged to serogroup O1 (**Alam *et al.*, 2006**;**Tison, *et al.*, 1999**; 1, 38).

Amplification of primers specific for *V. cholerae* serogroup O1 and *ctxA* by PCR assays. All *V. cholerae* strains tested amplified the primers for species-specific gene *ompW* by PCR (**Nandi** *et al.*, 2003; 27). All strains tested, except the two serologically non-O1/ non-O139, amplified the primers for the O biosynthetic gene *wbe*O1, but not *wbf*O139, and also the cholera toxin gene *ctxA*, confirming all to be *ctx*+ *V. cholerae* serogroup O1 (**Hoshino** *et al* 1998; 16).

Phenotypic and related genotypic characteristics. The results of the major phenotypic and related genetic characterizations of the *V. cholerae* O1 strains (n=104) are presented in Table 2. The *V. cholerae* O1 strains varied greatly in their phenotypic and related genetic characteristics. Sixty of the 104 strains showed sensitivity to chicken blood cells and El Tor-specific phage V, but were resistant to both polymixin B and classical-specific phage IV, in a manner that was typical of the El Tor reference strain N16961, and were thus recognized primarily as El Tor. All of these 60 phenotypic El Tor strains amplified the primers for the genes $tcpA^{ET}$ and $rstR2^{ET}$ but not $tcpA^{class}$ and $rstR1^{class}$, further confirming their El Tor background. Forty-nine of these 60 primarily identified El Tor represented all of the *V. cholerae* O1 strains of Peru, suggesting Peru as a pool of El Tor in Latin America. The remaining 11 primarily identified El Tor strains were however isolated in Mexico where *V. cholerae* O1 strains varied significantly in their biotype-specific phenotypic and genetic markers.

Forty of the remaining 44 *V. cholerae* O1 strains appeared to be hybrid variants of El Tor as they had major phenotypic classical markers that ranged from 1-4 in addition to having major El Tor traits and amplifying the primers for the El Tor-specific alleles of the genes, $tcpA^{El\ Tor}$ and $rstR2^{El\ Tor}$ (Table 2). Thirty-six of these El Tor variants amplified the CTX-prophage-encoded gene $rstR1^{Class}$ in addition to the resident $rstR2^{El\ Tor}$, and were thus designated as hybrid-El Tor. The remaining 4 El Tor variants had all the phenotypic and related genetic traits comparable to El Tor reference strains N16961 except that 3, including one of the two Guatemala, strains were sensitive to polymixin B, while one being resistant to El Tor specific phage V. These El Tor variants having phenotypic classical traits may have provided clue of the requisite genetic recombination in the emergence of hybrid-El Tor from the El Tor progenitors.

In sharp contrast, 4 of the remaining *V. cholerae* strains, including two that were not recognized by polyclonal or monoclonal O1- or O139-specific antibodies but reacted to monovalent Inaba antisera, had the major phenotypic markers of classical biotype (Table 2). Of these, the two serologically O1 strains possessing the major phenotypic and related genetic classical markers, except that one being resistant to the classical specific

phage IV while the other resistant to both polymixin B and the classical specific phage IV, amplified the primers for the genes *tcpA*^{Class} and *rstR1*^{Class}, but not *tcpA*$^{El\ Tor}$ or *rstR2*$^{El\ Tor}$, confirming them to belong to classical biotype, which is believed to remain extinct from all cholera endemic region since 1980s. Although the two serologically non-O1 strains had classical attributes such as they were sensitive to both Chicken blood cells and polymixin B, they were however resistant to both classical and El Tor-specific phages and did not amplify any of the primers for the biotype-specific genes *tcpA*, nor did they amplify the CTX-phage-encoded genes *ctxA* or *rstR*, suggesting that these two strains may have been originally toxigenic *V. cholerae* O1, and also biotype classical, but had their CTX-prophage excised.

The distribution of *V. cholerae* causing 1991 epidemic and subsequent endemic cholera in Latin America shows that all *V. cholerae* O1 irrespective of their spatio-temporal origins in Peru had all the phenotypic and related genetic markers of El Tor reference strain N16961, suggesting Peru to be a regional pool of prototype El Tor (Table 2). Conversely, *V. cholerae* strains isolated in Mexico, all of which were confirmed as serogroup O1, included both biotype classical and the El Tor occurring along with the hybrid variants, suggesting Mexico to be the other regional pool having diverse. Also, the hybrid-El Tor was found predominating among the El Tor strains in Mexico since 1991, showing the direct evidence of genetic transition from prototype El Tor to hybrid-El Tor in Latin America. Guatemala, a country geographically linked to Mexico, had one each of *V. cholerae* O1 belonging to *ctx*- classical, hybrid-El Tor, and phenotypic El Tor variant, all being isolated in 1993. Although this study was limited to analysing *V. cholerae* isolated in Peru, Mexico, and Guatemala, the results presented herein suggest that the emergence of hybrid-El Tor in Latin America may have occurred in the region having the Gulf of Mexico in common. Nonetheless, the extended survival of the classical biotype strains in Latin America, as were being isolated until 1995, was a significant event in the history of cholera.

MAMA-PCR. The mismatch amplification mutation assay (MAMA)-PCR using primers specific for classical or El Tor (**Morita** *et al.*, 2008; 24) offers a precise and accurate method for determining the type of the cholera toxin (CT) that cholera serogroup strains of *V. cholerae* O1 produce. The Latin American *V. cholerae* O1 strains including the reference classical (0395) and El Tor (N16961) strains were analysed for determining their CT-type by MAMA-PCR in the present study. As shown in Table 2, all the Peruvian *V. cholerae* O1 strains that were primarily recognized as biotype El Tor amplified the primers specific for the gene *ctxB*$^{El\ Tor}$, but not the *ctxB*^{class}, further confirming that they were prototype of the 7th pandemic El Tor in nature. Likewise, *V. cholerae* O1 strains that were primarily identified as El Tor in Mexico, including the variants having phenotypic classical markers, amplified the El Tor-specific gene *ctxB*$^{El\ Tor}$, but not *ctxB*^{class}, confirming their prototype El Tor traits. In contrast, the whole lot of *V. cholerae* O1 El Tor strains possessing the CTX^{class}-phage-encoded gene *rstR1*^{class}, and were thus designated hybrid-El Tor, including the two classical biotype strains in Mexico, amplified the gene *ctxB*^{class}, but not *ctxB*$^{El\ Tor}$, further confirming their CT to be of classical biotype. The results obtained in MAMA-PCR assays, while the reference classical (0395) and El Tor (N16961) strains serve as controls, confirm the co-existence of both classical and El Tor biotypes including the hybrid-El Tor in Mexico. Furthermore, the concurrence of CTX^{Class}- phage-encoded genes, *ctxB*^{CL} and *rstR1*^{CL}, in the hybrid-El

Tor of *V. cholerae* O1 may be suggestive of the incorporation of the entire CTX^{Class}-phage into the El Tor progenitor.

Multi-locus genetic screening. The multi-locus PCR screening for the vibrio pathogenicity island (VPI), biotype-specific major virulence and related genes and gene clusters provide an important tool to determine the biotypes of *V. cholerae* O1 (**Dziejman** *et al.*, 2002; **O'shea** *et al.*, 2004). Thirty four *V. cholerae* O1 strains of different spatio-temporal origins, covering the deduced biotype categorizations as determined by their phenotype and related genetic properties, were subjected to extensive multi-locus genetic screening by PCR assays in the present study; and compared with the reference El Tor (N16961) and classical (0396) strains. The 33 different genomic loci that were targeted in the present study include *ompU*, *toxR*, *ace*, the housekeeping genes encoding malate dehydrogenase (mdh) and chaperonin (groEL), major virulence marker genes encoding CTXΦ, RS1Φ, toxin-linked cryptic plasmid (TLC), repeat in toxin (RTX), Pilin E (PilE), hemolysin (Hly), and the mannose-sensitive hemolysin agglutinin (MSHA), which is a type IV pilus. In addition, the clusters included the most important El Tor biotype-specific marker genes, such as Vibrio seventh pandemic island-I (VSP-I) and VSP-II, Vibrio pathogenicity island-1 (VPI-1), and also Intl4 integron, which altogether covered more than 165 kb of the *V. cholerae* genome (**Dziejman** *et al.*, 2002; 12, **O'shea** *et al.*, 2004; 28). As shown in Table 3, the overall data of multi-locus genetic screening appeared in concordance with the sero-biotype categorizations made primarily based on the microbiological, phenotypic, and genetic data, except that the prototype ET had two distinct regional patterns in the VSP-II ORFs. Notably, all the Peruvian El Tor strains tested amplified all but two ORFs (512 and 514), while the prototype El Tor strains in Mexico failed to amplify the ORFs 512, 514, and 516 of the VSP-II gene cluster. This consistent clonal divergence between the *V. cholerae* O1 El Tor isolated in Peru and Mexico suggests that the 1991 epidemics in Mexico was presumably not an extension of Peru. In any case, the hybrid-El Tor strains however amplified the primers for all the 33 targeted genomic loci in a manner that was indistinguishable from the *V. cholerae* O1 El Tor reference strain N16961.

The results of the multi-locus genetic screening of two of the *V. cholerae* O1 classical biotype strains that were isolated in Mexico revealed that none of these two strains amplified any of the targeted ORFs of the VSP-I and VSP-II including that of the RTX and RS1, showing a pattern that was indistinguishable from that of the *V. cholerae* O1 classical reference strain O395, further confirming their classical attributes. In addition, the classical traits was further confirmed for the two serologically non-reacting but phenotypically O1 classical biotype strains because they had the multi-locus genetic pattern close to that of the classical reference strain O395, except that these two strains did not amplify any of the targeted genes of the VPI-I and CTX-prophage gene clusters.

PFGE and cluster analysis. The *Not*I-digested genomic DNA of *V. cholerae* O1 strains isolated in Peru (1991 – 1999), Mexico (1991 – 1995), and Guatemala (1992 – 1993), including one classical strain that was isolated in Mexico in 1983, were subjected to PFGE analysis to determine their genetic relatedness and clonal origin. As shown in Figure 1, the *Not*I restriction enzyme digested the genomic DNA of both the test as well as the reference control strains into 20 to 23 fragments; the molecular sizes of the restriction digested DNA fragments ranged between 20 to 350 kb. The overall banding

patterns obtained with the Latin American *V. cholerae* O1 strains were heterogeneous; a great deal of diversity was observed both regionally and within and between the two biotypes, including their hybrid genotypes that were analyzed.

When considered regionally, the PFGE patterns obtained with the Peruvian *V. cholerae* O1 El Tor strains were highly homogeneous except that two strains isolated in the year 1997 and 1998 varied in their PFGE patterns (Fig. 1), suggesting minor divergence. Nonetheless, the overall PFGE patterns of the Peruvian El Tor strains were by and large uniform and appeared analogous to that of the El Tor reference strain N16961, suggesting the El Tor ancestry. The Mexican *V. cholerae* O1 biotype El Tor strains, particularly their phenotypic and genetic variants showed significant diversity in the number and positioning of the bands in the PFGE (Fig. 1), although the overall banding patterns were comparable to that of the El Tor reference control, suggesting El Tor lineage for them. The variation in the banding patterns was maximal with the hybrid-El Tor strains having variable number of classical traits (Fig. 2). The Mexican progenitor El Tor strains were however related closely to the Peruvian El Tor in their PFGE patterns. A close interrelationship that was observed between the Peruvian and Mexican prototype El Tor, which resembled the prototype of 7th pandemic El Tor reference control (Fig. 2), suggested the common El Tor lineage for both. The classical biotype strains including their variants isolated in Mexico and Guatemala, which varied in their *Not*I restriction-digested patterns (Fig. 2) despite being typical of the classical, suggested the classical lineage for these strains.

To further understand the clonal relatedness between the *V. cholerae* O1 strains isolated in Latin America, cluster analysis was performed by dendrogram using the PFGE images of the *Not*I-digested genomic DNA of *V. cholerae* O1 strains isolated in Peru (1991 – 1999) and Mexico (1991 – 1995) separately, and also putting them together with the *V. cholerae* O1 strains of Asian and African origins that were available in our soft-database. The cluster analysis data obtained from dendrograms constructed with the *Not*I-digested PFGE patterns (Dice similarity coefficient and UPGMA clustering methods) revealed a high degree of homogeneity among the Peruvian prototype El Tor strains because they formed a compressed, major cluster of the closely related strains, irrespective of their spatio-temporal origins (Fig. 1). A couple of Peruvian El Tor strains that varied with the majority of the El Tor strains in their PFGE patterns however formed a separate but closely related cluster, suggesting divergence. Although clustering separated the Mexican El Tor from the classical biotype and the respective hybrid variant strains, a great deal of divergence was apparent from the clustering patterns because they formed large but loosely adherent clusters of genetically patchy strains (Fig. 2).

When the PFGE patterns of the Mexican *V. cholerae* O1 El Tor, classical, and the hybrid variants of both biotypes were subjected to cluster analysis together with that of the Peruvian El Tor for comparison, whilst the O395 and N16961 served as the classical and El Tor reference controls, respectively, major group-specific and regional clusters were obtained. As shown in Fig. 3, clustering separated the El Tor from the classical in one hand, the hybrids from their progenitor strains on the other, essentially indicating the application of the PFGE in determining the clonal lineage. Furthermore, a closely related major cluster that separated the Peruvian and Mexican prototype El Tor strains from the rests suggests a common ancestry for them. When the strains in this cluster was further analysed, the Peruvian El Tor strains formed a tight regional cluster

suggesting them to constitute a genetically homogeneous regional pool, independent of Mexico and Asia. The Mexican hybrid-El Tor strains showed a great deal of diversity by forming a loosely adherent but large cluster of many assorted small clusters (Fig. 3), which was shared by the Asian hybrid-El Tor strains, suggesting clonal interrelationships. The pre-existing classical progenitor including their hybrid variants that were isolated in Mexico and Guatemala formed a major separate cluster, suggesting genetic relatedness. The overall PFGE data and their analyses by dendrograms further suggest the 1991-epidemics and the emergence of hybrid-El Tor to be local, Asia-independent, events that involved locally existent *V. cholerae* O1 biotypes El Tor and classical in Latin America.

Discussion

V. cholerae O1 has two biotypes, classical and El Tor. The classical, which presumably caused six cholera pandemics in the Ganges Delta of Bay of Bengal, was introduced into the other parts of the world between 1817 and 1923 (**Dziejman** et al., 2002; 9, **Politzar** et al., 1959; 27). Ever since *V. cholerae* O1 biotype El Tor initiated the current seventh pandemic by resplacing classical biotype in early 1960s (**Faruque** *et al.*, 2003a), the classical had undergone slow extinction, since 1970s (**Samadi 1983, Siddique 1991**). The El Tor causing endemic cholera continued to spread in Asia, reached Africa (**Scracia 2006**) and finally in Latin America (**Mata,** L., 1994), in 1970 and 1991, respectively, and became endemic there.

When 1991-epidemics of cholera exploded into Latin America after about a century, conceivably the case fatality was very high due to the lack of adequate knowledge about the bacterium, appropriate intervention, and epidemiological management (**Mata,** L., 1994; **Wachsmuth** *et al.*, 1993). Although *V. cholerae* causing 1991-epidemics in Latin American was shown to be a clonal expansion of current 7th pandemic El Tor from Asia, after Africa (**Mata** 1994; **Wachsmuth** IK, 1993; **Wachsmuth 1994; Popovic** 1993; **Cameron 1994**), a significant recent development has been the emergence of a hybrid-El Tor possessing the cholera toxin (CT) encoding gene specific for the classical biotype, which remain extinct. While the hybrid-El Tor has already replaced prototype El Tor in Asia and Africa (**Nair** 2006, **Morita** 2008; **Safa** 2008), our knowledge of *V. cholerae* causing 1991-epidemics and subsequent endemic cholera in Latin America is limited; although understanding of the evolutionary trend of *V. cholerae* in this huge continent appears crucial for unified global intervention and preventive measures against cholera. This study, a retrospective one, shows toxigenic *V. cholerae* O1 El Tor, classical, and an array of hybrid-variants of both biotypes in 1991-epidemics and subsequent endemic cholera in Latin America. Our extensive phenotypic, molecular, and phylogenetic characterizations of the Latin American *V. cholerae* O1 strains and their comparison with that of Asia and Africa show Peru as a unique pool of prototype El Tor; while El Tor co-existing with classical and hybrid-variants (hyb-ET) in Mexico as the other pool. Based on the clonal divergence of *V. cholerae* O1 ET of Peru and Mexico, and the distinct clonal lineages of both classical and El Tor of Latin America with that of Asia, showed 1991-epidemics to be local event in Mexico, not an extension of Peru, and that the emergence of hyb-ET as local, Asia-independent events that involved locally existent *V. cholerae* O1 in Latin America.

The microbiological culture, biochemical, and serological tests confirmed that the cholera bacteria involved in Latin American epidemics, in 1991, and subsequent endemic cholera (**Mata** 1994, **Scracia 2006**) were *V. cholerae* serogroup O1, while two *V. cholerae* strains were serologically non-O1/ non-O139. These primary microbial results were further complemented by results obtained with the genetic screening using simplex and multiplex-PCR assays (**Alam** 2006), which confirmed that all of the test strains had *V. cholerae* species-specific gene *ompW* (**Nandi,** 2003), *ctxA* encoding the sub unit 'A' of the CT, and the surface antigen (serogroup O1-sepicific) encoding gene *wbe* (**Hoshino** 1998). These results confirmed our initial results that the epidemics, 1991, and the following endemic cholera in Peru (**Mata** 1994; **Wachsmuth 1994**) were caused by toxigenic *V. cholerae* belonging to serogroup O1 (**Popovic** 1993; **Cameron 1994**). The two serologically non-O1/ non-O139 strains that had the *V. cholerae* species-specific genes *ompW* (**Nandi,** 2003) and the serogroup-O1 encoding gene *wbe* (**Hoshino** 1998) but not the *ctxA*, suggesting that they were serologically non-O1/ non-O139 but *ctx*$^{-}$ *V. cholerae* O1 (**Kaper** 1995). The *ctx*$^{-}$*V. cholerae* O1 strains, which occur in the aquatic environment (**WHO** 1984), have been reported to result due to the loss of CTX prophage (**Alam** et al 2007 VBNC). Thus, the two serologically nonO1/ non O139 but *ctx*$^{-}$*V. cholerae* O1 strains that were isolated from the water sources in Mexico and Guatemala may have had their CTX Φ excised, as the toxigenic strains surviving in the aquatic environment can become non-toxigenic during the inter-epidemic periods (**Alam** et al 2007 VBNC).

V. cholerae O1 biotypes classical and El Tor are distinguished from each other based on differences in their phenotypic and genotypic characteristics (**Kaper** 1995; **Morita** 2008). The results of the phenotypic screening such as resistance to polymyxin B, sensitivity to chicken blood cells and El Tor-specific phage V, but not to classical phage IV, suggest the El Tor nature of the *V. cholerae* O1 strains that were isolated in Peru (**Chanda,** 1973; **Manning**, 1994). The El Tor traits of these Peruvian strains were further suggested by multiplex-PCR amplification of genes such as *ctxB*-El Tor (**Morita** 2008), *tcpA*-El Tor (**Keasler** 1993) and *rstR*2-El Tor (**Kimsey** 1998) encoding subunit B of CT, major pilin, and a repressor, respectively. Since these genes detected had their El Tor alleles with the specific DNA sequence compositions, our data appeared in agreement with the results reported earlier (**Suraia** 2009), confirming Peru as a pool of the 7th-pandemic El Tor that has been replaced by hybrid-ET in Asia and Africa (**Nair** 2006, **Morita** 2008; **Safa** 2008). Unlike Peru, where El Tor was the only biotype recognized, *V. cholerae* O1 in Mexico included both El Tor and classical together with strains that were neither El Tor nor classical, because they were hybrid of both biotypes. This study thus show two independent regional pools of *V. cholerae* O1 in Latin America, suggesting the epidemics, 1991, and the subsequent endemic cholera to be caused by biotype El Tor in Peru (**Mata** 1994; **Wachsmuth 1994; Popovic** 1993; **Cameron 1994**), while both El Tor and classical, together with the hybrid variants of both biotypes, in Mexico.

Although as many as seven cholera pandemics have been recorded globally, a little is known about the geographic distribution of *V. cholerae* beyond the Ganges Delta of the Bay of Bengal, where cholera is endemic for centuries (**Dziejman** et al., 2002; 9, **Politzar** et al., 1959; 27). Naturally, when cholera struck Latin America after about a century in 1991, the reservoir and transmission of the *V. cholerae* causing the epidemics was unknown; albeit, a handful of subsequent studies have proposed hypotheses that

were however contrasting (**Mata** 1994; **Wachsmuth 1994; Cameron 1994**). This study is the first to show the pre-1991 existence of the classical biotype in Latin America and that the 1991-epidemics and subsequent endemic cholera in Mexico were jointly caused by both classical and El Tor biotypes, which coexisted and were consistent in being isolated from both environmental and clinical sources of Mexico and Guatemala, since 1991. Despite the fact that the classical biotype strains were found pre-existing in Mexico, the ecological niche for *V. cholerae* is not well defined for Latin America, nor do we know of the pre-1991 existence of *V. cholerae* El Tor in Mexico. Nonetheless, the extended survival of the classical biotype strains and its co-existence with El Tor, as presented herein, support the supposition that the El Tor may also have pre-existed sharing niche with the classical in Latin America, as did classical and El Tor in the Ganges delta of Bay of Bengal until 1980s (**Siddique** 1994; **Siddique** 1996). The overall results presented herein may be interpreted to indicate the ecosystem close to the Gulf of Mexico as a persistent ecological niche for *V. cholerae* causing endemic cholera in Latin America, as does the Ganges delta of Bay of Bengal in Asiatic cholera (**Siddique** 1994; **Siddique** 1996).

Whereas the extinction of the classical biotype following the emergence El Tor biotype still remains largely a mystery, a significant recent development has been the emergence of a new hybrid-El Tor, having the gene *ctxB* of classical biotype, replacing the prototype El Tor. The corollary of such genetic shift is not fully understood, however the hybrid-El Tor as an epidemic clone is becoming increasingly significant for Bangladesh (**Siddique** 2007). While the world-wide distribution and the epidemiological significance made the hybrid-El Tor as the most thriving one in the cholera endemic regions of Asia and Africa (**Morita** 2008; **Safa** 2008), (**Alam** et al., unpublished data; **Siddique**, 2007; 35), a recent study has however shown the *V. cholerae* O1 El Tor prototype to be the cause of endemic cholera in Peru (**Suraia** 2009); albeit the study was partial and does not essentially reflect the other cholera endemic countries of Latin America. We herein provide further evidences, supporting the primary results of the prototype El Tor nature of *V. cholerae* O1 causing endemic cholera in Peru (**Suraia** 2009), showing that the genetic shift from prototype El Tor to hybrid-El Tor was not a local event limited only to Asia and Africa (**Morita** 2008; **Safa** 2008) but also true for Latin America. Furthermore, this study is the first to provide direct evidence of the extended survival of classical biotype in Latin America, showing how the hybrid variants, in particular the hybrid-El Tor, emerged from the concurrently occurring progenitors, the prototype El Tor and classical in Mexico. Since the *V. cholerae* O1 strains that were analyzed in this retrospective study was limited up to 1995 in Mexico, further molecular epidemiological and ecological study would be needed to understand the current scenario of the *V. cholerae* in Latin America.

Now that the emergence of hybrid-El Tor proves to be a global event extended also to Latin America, after it was first reported from Asia (**Nair** 2006) followed by Africa (**Morita** 2008; **Safa** 2008), naturally the valid questions would be that when did this change occur, and also, was this a single event that occurred in one of the endemic areas and then spread, or multi-clonal occurring globally? All *V. cholerae* O1 El Tor isolated in Bangladesh since 2001 were shown to be hybrid-El Tor, i.e., the *V. cholerae* O1 El Tor strains isolated before 2001 were of 7th pandemic El Tor prototype (**Nair**, 2006; 26). Subsequent studies have however proposed the transition to be 1995 for Asia

(**Safa** 2008). A very recent study has demonstrated the transition to be 1993 -1994 for Southeast Asia, while all *V. cholerae* O1 El Tor isolated before 1993 proved to be prototype El Tor (**Morita** 2008). The emergence and spread of the hybrid-El Tor thus occurred in around 1995 (**Safa** 2008), and the whole genetic shift presumably completed in the latter half of the 1990s (**Morita et al., US-Japan**). Nonetheless, this study is the first to demonstrate the genetic shift from prototype El Tor to hybrid-El Tor in Latin America, showing that it was a local event in Mexico, occurring in 1991, and was independent of Peru. Furthermore, evidence is provided here showing the hybrid variants in Mexico to be the results of direct genetic recombination between the progenitor El Tor and classical biotype strains that coexisted in Latin America.

The cholera toxin (CT) genes, *ctxAB*, are the integral part of a novel filamentous bacteriophage, CTXΦ, which is bio-specific and lysogenizes *V. cholerae* genomes (**Waldor** 1996), mostly of the O1 and O139 cholera strains. That is, the CTX-prophages of *V. cholerae* O1 are of two types, 'El Tor-specific CTXΦ' and the 'classical-specific CTXΦ'. Despite CTX-prophages CTX-prophages are biotype specific, *V. cholerae* O1 El Tor reported from Bangladesh was recently shown to be a hybrid of both classical and El Tor biotypes (**Nair** 2002). Such hybrid *V. cholerae* O1 El Tor strain, the "Matlab variants" described earlier, harbouring classical type CTX-prophage was also reported from Africa (**Ansar** 2004). Recent study shows that all of the *V. cholerae* O1 El Tor isolated in Dhaka since 2001 were hybrids, while those isolated before 2001 had the typical 7th pandemic El Tor attributes (**Nair** 2006). In the present study, the hybrid nature of the CT of the El Tor strains isolated in Mexico and Guatemala was demonstrated by the MAMA-PCR assay using classical-specific primers (**Morita** 2008), showing that although the host bacterium was El Tor, its CT was of a classical biotype, the prototype of which was reported from Bangladesh (**Nair** 2006, **Safa** 2006). While the Peruvian *V. cholerae* O1 El Tor strains did only respond to the El Tor-specific primers by MAMA-PCR assay (**Morita** 2008), suggesting El Tor nature of the *ctxB* genes. This may raise valid questions as to why until recently the Peruvian *V. cholerae* O1 El Tor strains did not switch from prototype to the hybrid type, while the El Tor strains like those of Asia and Africa (**Safa** 2008; **Morita** 2008) the El Tor strains in Mexico and elsewhere did so in as early as 1991. The answer may not be precise enough, but the likelihood of this transformation in Mexico, but not in Peru, may be genetic recombination promoted by the co-presence of the classical El Tor and biotypes strains in Mexico, as reported from Bangladesh until 1970s (**Samadi** 1983; **Siddique** 1994), but not in Peru.

There are two different suppositions in regard to the emergence and global spreading of the hybrid-El Tor in Asia and Africa (**Morita** 2008; **Safa** 2008). One claims the hybrid-El Tor to be the result of clonal expansion of a single ancestral El Tor that had acquired the *ctxB* gene of the classical biotype in one of the cholera endemic regions (**Morita** 2008). While, the other claims the emergence of the hybrid-El Tor to be multi-clonal events occurring independently in each region, although direct evidence supporting such hypotheses are lacking. The evidence is provided here showing that the emergence of the hybrid-El Tor in Latin America was a local event occurring presumably through genetic recombination between the progenitor classical and El Tor prototype that co-existed in Mexico. Although we do not have *V. cholerae* strains to analyze beyond 1995 in Mexico, the extended survival of classical biotype strains until 1995 appears to be a unique event in the present study because classical biotype is extinct even in

southern Bangladesh, the last of the niches where this biotype prevailed until 1970s (**Samadi** 1983; **Siddique** 1994). Nonetheless, the pre-1991 detection of classical biotype, as confirmed in the present study, and its co-presence with prototype El Tor and the hybrid variants of both biotypes since 1991 itself may have indicated a persistent niche for *V. cholerae* in Latin America. Even if data supporting the unified transition for El Tor to hybrid-El Tor in Asia is missing, this study, which demonstrates Asia-independent emergence of hybrid-El Tor in Latin America in 1991, suggests that the emergence of hybrid-El Tor may also be a local event in Asia (**Nair** 2002), although the hybrid-El Tor in Africa was proposed to be an extension of Asia (**Morita** 2008; **Safa** 2008).

In Latin America, the hybrid-El Tor was however found with the variant strains coexisting with the progenitor classical and El Tor strains only in Mexico, but not in Peru. The hybrid of each biotype, in particular the El Tor variants, varied significantly in the proportion of traits of each other that they possessed, suggesting disproportionate trading of genetic materials between the two. While the variant classical strains had only one or a few of the phenotypic El Tor traits, the El Tor phenotypic variants had in addition major classical traits, namely the *ctxB* and *rstR*1 genes of classical biotype (**Morita** 2008; **Kimsey** 1998), suggesting the latter to be analogue of the Matlab variant of *V. cholerae* O1 El Tor (hybrid-El Tor) reported from Bangladesh (**Nair** 2002; **Safa** 2005). Although the El Tor and its variants have already been replaced by the new hybrid-El Tor in Asia (**Nair** 2002; **Safa** 2005) and Africa (**Nair** 2006; **Morita** 2008; **Safa** 2008), it would not be possible for us to predict if the classical and El Tor progenitors that were found coexisting with the variants strains of both biotypes, until 1995, still exist or were replaced by hybrid-El Tor that predominated in Latin America. Nonetheless, what made the hybrid-El Tor of Latin America different from Asia (**Nair** 2002; **Safa** 2005) was that, all had $ctxB^{Class}$ and $rstR1^{Class}$ in addition to the resident $rstR2^{El\ Tor}$, suggesting that these CTX^{Class}-prophage-encoded genes may have been acquired concurrently, presumably through the incorporation of the CTX^{Class}-prophage into the El Tor progenitor.

Unlike the hybrid-El Tor, which carried CTX^{Class}-prophage-encoded genes, none of the variants classical in the present study harboured any $CTX^{El\ Tor}$-prophage-encoded genes other than that they had one or a few of the phenotypic El tor traits only. The reason may presumably be of evolutionary in nature, nonetheless a recent study has demonstrated the expulsion of CTX-prophage from naturally occurring *V. cholerae* O1 El Tor (**Alam** 2007), showing how a toxigenic *V. cholerae* O1 turns *ctx- in situ* (**Alam** 2007). Also, a more recent study has shown how a *ctx*- El Tor progenitor is transduced by CTX^{Class}-prophage from a toxigenic serogroup O141 (**Faruque** 2008, **Alam** 2007). So, we presume that the two serologically non-O1/O139 but phenotypically classical biotype strains representing Mexico and Guatemala, which lacked genes *ctxA* and *rstR*, and thus CTX^{Class}-prophage, may have been *ctx*⁻ *V. cholerae* O1 classical strains having lost the CTX-prophage from originally toxigenic classical strains. Even though the direct involvement of progenitor El Tor and classical in the process of genetic recombination, which likely resulted the hybrid-El Tor in Latin America and elsewhere (**Nair** 2002), cannot be ruled out, the results presented herein support the incorporation of CTX^{Class}-prophage into the progenitor El Tor, as reported elsewhere (**Faruque** 2008).

In addition to the conventional phenotypic and related genotypic traits that distinguish the classical and El Tor biotypes strains, they are distinguishable in many other genomic loci that show a high degree of genetic conservation (**Beltran**, 1999;

Karaolis, 1995; **Karaolis**, 2001). Multi-locus genetic screening by PCR of the virulence islands such as vibrio pathogenicity islands and the related genes, which constitute major gene-clusters that are biotype-specific and unique, provides an important tool for detecting genetic changes and characterizing new variants within and between the biotypes of *V. cholerae* (**Dziejman**, 2002; **Manning**, 1994; **Manning,** 1999; **O'shea** *et al.*, 2004). The Latin American *V. cholerae* strains showing variable results in major phenotypic and primary genetic characterizations were subjected to extensive multi-locus genetic screening by targeting different genomic loci, which involved 11 major clusters namely CTXΦ, RS1Φ, TLC, RTX, PilE, Hly, MSHA, VSP-I, VSP-II, VPI-1, and Intl4 integron, including the conserved genomic regions of the *V. cholerae* genome (**Dziejman** 2002; **O'shea** *et al.*, 2004). The data presented here provide important insights into the genetic traits of the *V. cholerae* O1 causing endemic cholera in Latin America, since 1991 (**Mata** 1994; **Popovic** 1993; **Cameron 1994; Wachsmuth 1994**). The data of the multi-locus genetic screening appeared in complete agreement with the primary biotype categorization made on the basis of the phenotypic and related genetic markers, except that *V. cholerae* O1 prototype El Tor strains, which are replaced by the hybrid-El Tor in Asia and Africa (**Nair** 2002; **Nair** 2006; **Safa** 2006; **Ansar** 2006; **Morita** 2008), varied regionally and also differed slightly with the Mexican hybrid-El Tor including El Tor control N16961 in their ability to amplify the VSP-II ORFs, suggesting unlike clonal lineages for them. The Peruvian *V. cholerae* O1 El Tor strains that consistently failed to amplify two particular VSP-II ORFs (**Nusrin** 2009) differed with that of Mexico, which also failed consistently to amplify three particular VSP-II ORFs, suggesting two distinct regional pool of clonally unlike *V. cholerae* O1 El Tor in Latin America. Such VSP-II genotypes among the El Tor in Peru was shown to be resulted by the acquisition of gene encoding hypothetical proteins homologous to those present in the nitrogen-fixing endophyte symbiotic *Azoarcus* sp. in the VSP-II region (ORF 20 and 21) by the progenitor of the Peruvian *V. cholerae* strains (**Nusrin** 2009). Nonetheless, the consistent clonal divergence that was observed between the El Tor of Peru and Mexico in the present study may suggest the 1991-epidemics in Latin America to be local events, caused by regional clones, and not an extension of Peru (**Mata** 1994; **Wachsmuth** IK, 1993; **Wachsmuth 1994; Popovic** 1993; **Cameron 1994**).

The *V. cholerae* O1 strains that were primarily identified as biotype classical in Mexico, in 1995, lacked the El Tor-specific ORFs of the VSP-I and VSP-II including that of the RTX and RS1 but had the multi-locus genetic profile comparable to that of the classical reference strain O395, further confirming their primary biotype categorization showing the extended survival of classical biotype strains in Latin America. Besides, the two other serologically non-O1/O139 but phenotypically classical strains, of which one isolated in Mexico (1992) and the other in Guatemala (1993), also showed their multi-locus genetic patterns close to classical, except that both failed to amplify any of the targeted genes of the VPI-I and CTX-prophage gene clusters, which may be suggestive of their classical ancestry but also, expulsion of virulence gene clusters such as CTX-prophage, as reported elsewhere (**Alam** 2007).

V. cholerae O1 El Tor initiating the Latin American epidemic was shown to be homogeneous initially (**Popovic** 1993, **Cameron 1994, Mata** 1994; **Wachsmuth 1994**), although divergence was reported later (**Dalsgaard** 1997; **Beltran** 1999). Likewise, the El Tor strains causing Peruvian epidemic were shown to be homogeneous, the multi-

locus enzyme electrophoresis (MLEE) type of which was determined to be ET4 (**Wachsmuth** et al., 1993), while the 7th pandemic El Tor in Asia and Africa was ET3 (**Wachsmuth**, 1993). Subsequent studies claimed at least two different clones distinguishable by MLEE, ribotyping, PFGE and antimicrobial resistance pattern (**Dalsgaard**, 1997; **Popovic**, 1993; **Evins**, 199). Also, the strains causing Latin American epidemic was shown to belong to ribotype 5 that was also reported from several other geographic locations (**Popovic,** 1993). A recent study however showed a variety of PFGE profiles suggesting genetic diversity among the El Tor strains causing Peruvian cholera epidemic (**Suraia** 2009). Except that two strains isolated in the year 1997 and 1998 varied in their PFGE patterns showing minor divergence in the present study, the homogeneity of the overall PFGE data revealed the Peruvian El Tor to be highly clonal. Besides, the El Tor ancestry was confirmed by their characteristic PFGE patterns of the El Tor reference control, suggesting possible clonal link with the 7th pandemic El Tor in Asia (**Mata** 1994; **Wachsmuth** IK, 1993; **Wachsmuth 1994; Popovic** 1993; **Cameron 1994**).

In sharp contrast to the Peruvian *V. cholerae* O1 strains, which were all typical El Tor, the PFGE patterns of the Mexican El Tor strains were highly variable suggesting divergence. Although maximum heterogeneity was observed with the hybrid-El Tor strains, as was the case for classical strains of Latin America and their Asian classical reference control showing clonal divergence, the prototype El Tor strains were however comparable to that of the Peruvian prototype El Tor, suggesting clonal relatedness and common El Tor ancestry for both. The classical strains that varied considerably among them and also with their Asian counterparts, suggested an Asia-independent pool of classical *V. cholerae* O1 in Latin America. The divergence that was observed between the hybrid-El Tor of Mexico may have been resulted due to inter-biotype genetic recombination and consequential genetic re-assortments.

Since *V. cholerae* O1 El Tor and in Peru differed with that of Mexico, where the classical strains were not homogeneous and also, the El Tor varied with hybrid-El Tor in their major phenotypic and genetic traits, the PFGE images of the El Tor, classical, and hybrid variant strains in the present study were subjected to cluster analyses by dendrogram to determine their clonal relatedness based on genetic signatures. The PFGE images of pre-1991 classical strain isolated in Mexico, Asian and African hybrid-El Tor including both El tor and classical control strains were also included for comparison in the present study. The clonal disparity, which was demonstrated between the prototype El Tor of Peru and Mexico, was further supported by the cluster analyses results, suggesting that they had clonal divergence, and thus unlike clonally. The clonal divergence between the Latin American and Asian classical strains was also indicated in the cluster analysis data, although all the classical strains formed a separate cluster suggesting classical, but not El Tor, ancestry for them. Since none of the two regional clusters of prototype El Tor, one for El Tor of Peru while the other for El Tor of Mexico, include any of the El Tor of Asia and Africa, which formed separate clusters or shared clusters with Mexican hybrid-El Tor showing interrelationship, the Latin American epidemics appear to be regional events, caused by *V. cholerae* O1 that are unlike clonally, and not an extension of Asia (**Mata** 1994; **Wachsmuth** IK, 1993; **Wachsmuth 1994; Popovic** 1993; **Cameron 1994**).

A number of contrasting hypotheses exist in regard to how *V. cholerae* O1 causing epidemic and endemic cholera reached in Latin America. While the epidemic

was proposed to be the result of dissemination of a single clone in one hand, the association of distinct O1 strains with cholera in Mexico and Brazil (**Coelho**, 1995; **Evins**, 1995) appeared in contrast. This study, which is a retrospective one on the detailed phenotypic and genetic characterizations of *V. cholerae* serogroup O1 strains isolated in Peru, Mexico, and Guatemala, and their comparison with that of Asia, provided data showing conclusively that the Latin American epidemics in 1991 and also the subsequent endemic cholera to be local events caused by unlike clones of *V. cholerae* that presumably evolved locally (**Dalsgaard** 1997; **Beltran** 1999). Since strains causing the Latin American epidemic were diverse clonally and also differed considerably with that of Asia and Africa, it is thus concluded that the Latin American *V. cholerae* O1 strains may have pre-existed but were undetected because of the lack of surveillance due to the fact that cholera was absent for a century (**Popovic** 1993).

Cholera, which has long been proposed to start at a point and then spread (Faruque, 2005), is caused by a single clone of either serogroup O1 or O139 (**Alam**, 2006), except for a few cases when both serogroups occur in the same location (Faruque, 2005). Recent molecular data however suggest that the cholera that starts at different endemic locations is initiated by one or a few clones that prevail locally (**Stine,** 2008). Although the source of the *V. cholerae* O1 initiating the Latin American epidemic in Peru still remains largely a question, the 'single introduction hypothesis' claims *V. cholerae* to have migrated by emptying of contaminated bilge waters of ships arriving from cholera endemic areas of Asia or Africa (**McCarthy**, 1994). Our data of the genetic divergence between the *V. cholerae* strains of Peru and Mexico, and their consistent disparity with those of Asia and Africa however appear in contrary to this hypothesis, as shown elsewhere (**Suraia** 2009).

The other hypothesis is that the outbreak in Peru and its rapid spreading was caused by the preexisting small population of pathogenic *V. cholerae* O1 that may have survived in association with the zooplankton or phytoplankton in Peruvian coastal waters (**Craig**, 1996). The data presented herein on the molecular characterizations of the *V. cholerae* O1 strains shows clearly that the El Tor strains that caused the Peruvian epidemic in 1991 were different from that of the El Tor strains causing epidemic in Mexico around the same time. Also, the pre-1991 existence of *V. cholerae* O1 classical biotype strains in Mexico and the clonal divergence of El Tor and classical of Latin America and Asia demonstrated in the present study suggest a persistent niche for *V. cholerae* in Latin America. The sea surface temperature and the location of the Bay of Bengal at sea level have shown a degree of correlation with the incidence of cholera in Bangladesh (**Colwell**, 1996), although little is understood as to how these factors contribute to seasonal cholera. Nonetheless, since cholera has been endemic in Latin America, and *V. cholerae* has long been established as an autochthonous flora of brackish waters and estuarine ecosystems (**Colwell**, 1992) where the bacterium survives in association with plankton (**Huq**, 1990; **Craig**, 1996), we presume that the South American epidemic that began in the coastal regions of Peru spreading to 21 countries including Mexico may have been triggered by environmental conditions related to the El Niño, which is a global event that was found linked to the incidence of cholera (**Colwell** 1996; **Seas** *et. al.* 2000).

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Running Title: Hybrid-El Tor co-exists with El Tor & classical since 1991 in Latin America

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References:

Alam, M., Hasan, N. A., Sadique, A., Bhuiyan, N. A., Ahmed, K. U., Nusrin, S., Nair, G. B., Siddique, A. K., Sack, R. B., Sack, D. A., Huq, A., Colwell, R. R. (2006). Seasonal Cholera Caused by Vibrio cholerae Serogroups O1 and O139 in the Coastal Aquatic Environment of Bangladesh. *Appl. Environ. Microbiol.* 72: 4096-4104.

Alam, N. H., Ashraf, H. Treatment of infectious diarrhea in children. *Pediatr Drugs* 2003;5:151-65.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25, 3389–3402.

Ansaruzzaman M, Bhuiyan NA, Nair BG, Sack DA, Lucas M, Deen JL, Ampuero J, Chaignat CL; The Mozambique Cholera vaccine Demonstration Project Coordination Group. Cholera in Mozambique, variant of *Vibrio cholerae*. Emerg Infect Dis. 2004 Nov;10(11):2057-9.

Basu S, Mukerjee S. Bacteriophage typing of Vibrio eltor. Experientia. 1968 Mar 15;24(3):299-300.

Beltran, P., G. Delgado, A. Navarro, F. Trujillo, R. K. Selander, and A. Cravioto. 1999. Genetic diversity and population structure of *Vibrio cholerae*. J. Clin. Microbiol. 37:581–590.

Chanda, P. K. and S. N. Chatterjee (1973). "Adsorption characteristic of a group I cholera phage." Bull Calcutta Sch Trop Med 21(1): 7-8

Chowdhury, N. R., Chakraborty, S., Ramamurthy, T., Nishibuchi, M., Yamasaki, S., Takeda, Y., & Nair, G. B. (2000). Molecular evidence of clonal *Vibrio parahaemolyticus* pandemic strains. *Emerg Infect Dis* 6, 631-636.

Colwell, R.R., and W.M. Spira. 1992. The ecology of *Vibrio cholerae*, p 107-127. In Barua, D., and Greenough III, W.B. (eds), Cholera. Plenum Medical Book Co., New York.

Colwell, R. R. 1996. Global Climate and infectious disease: the cholera paradigm. Science. 274:2025-2031.

Cooper J. E, Feil E. J. Multilocus sequence typing--what is resolved? Trends Microbiol. 2004 Aug;12(8):373-7.

Dziejman, M., E. Balon, Boyd, D., C. M. Fraser, J. F. Heidelberg, & J. J. Mekalanos, 2002. Comparative genomic analysis of *Vibrio cholerae*: genes that correlate with cholera endemic and pandemic disease. *Proc Natl Acad Sci U S A* 99, 1556–1561.

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Faruque, S. M., M. J. Islam, Q. S. Ahmed, A. S. G. Faruque, D. A. Sack, G. B. Nair, and J. J. Mekalanos. 2005. Self-limiting nature of seasonal cholera epidemics: Role of host-mediated amplification of phage. Proc. Natl. Acad. Science. 102: 6119-6124.

Finkelstein, R. A. 25 February 2006, posting date. Cholera, *Vibrio cholerae* O1 and O139, and other pathogenic vibrios. http://gsbs.utmb.edu/microbook/ ch024.htm.
Glass, R. I., M. I. Huq, B. J. Stoll, M. U. Khan, M. H. Merson, J. V. Leee, and R. E. Black. 1982. Endemic cholera in rural Bangladesh, 1966-1980. Am. J. Epidemiol. 116:959-970.

Hoshino, K., S. Yamassaki, A.K. Mukhodpadhyay, S. Chakraborty. A. Basu, S.K. Bhattacharya, G.B. Nair, T. Shimada, and Y. Takeda. 1998. Development and evaluation of multiplex-PCR assay for rapid detection of toxigenic *Vibrio cholerae* O1 and O139. FEMS Immunol. Med. Microbiol. 20: 201-207.

Huq, A., R. R. Colwell, R. Rahaman, A. Ali, M. A. R. Chowdhury, S. Parveen, D. A. Sack, and E. R. Cohen.1990. Detection of *Vibrio cholerae* O1 in the aquatic environment by fluorescent–monoclonal antibody and culture methods. Appl. Environ. Microbiol. 56:2370-2373.

Karaolis, D. K., R. Lan, and P. R. Reeves. 1995 The sixth and seventh cholera pandemics are due to independent clones separately derived from environmental, nontoxigenic, non-O1 *Vibrio cholerae*; *J. Bacteriol.* 177 3191–3198

Karaolis, D. K., R. Lan, J. B. Kaper, and P. R. Reeves. 2001. Comparison of *Vibrio cholerae* pathogenicity islands in sixth and seventh pandemic strains. Infect. Immun. 69:1947–1952.

Keasler SP, Hall RH. Detecting and biotyping *Vibrio cholerae* O1 with multiplex polymerase chain reaction. Lancet. 1993. Jun 26; 341(8861):1661.

Kimsey, H. H., G. B. Nair, A. Ghosh, and M. K. Waldor. 1998. Diverse CTXΦ and evolution of new pathogenic *Vibrio cholerae*. Lancet 352:457–458.

Manning, P. A., Strocher, U.H, and Morona, R. 1994. Molecular basis of O-antigen biosynthesis of *Vibrio cholerae* O1: ogawa- Inaba switching. P. 77-94. In K. Wachsmuth, P. A. Blake and O. Olsvik (eds.), *Vibrio cholerae* and cholera: Molecular to Global Perspective. ASM Press, Washington, D.C.

Manning, P. A., Clark, C. A. & Focareta, T. (1999). Gene capture in *Vibrio cholerae*. *Trends Microbiol* 7, 93-95

Morita, M., Ohnishi, M., Arakawa, E., Bhuiyan, N. A., Nusrin, S., Alam, M., Siddique, A. K., Qadri, F., Izumiya, H., Nair, G. B., & Watanabe, H. 2008. Development and validation of a mismatch amplification mutation PCR assay to monitor the dissemination of an emerging variant of *Vibrio cholerae* O1 biotype El Tor. *Microbiol Immunol* 52, 314-7.

Nair, G. B., Faruque, S. M., Bhuiyan, N. A., Kamruzzaman, M., Siddique, A. K. & Sack, D. A. 2002. New variants of *Vibrio cholerae* O1 biotype El Tor with attributes of the classical biotype from hospitalized patients with acute diarrhea in Bangladesh. *J Clin Microbiol* 40, 3296–3299.

Nair, G. B., Qadri, F., Holmgren, J., Svennerholm, A. M., Safa, A., Bhuiyan, N. A., Ahmad, Q. S., Faruque, S. M., Faruque, A. S., Takeda, Y., and Sack, D. A. 2006. Cholera due to altered El Tor strains of *Vibrio cholerae* O1 in Bangladesh J Clin. Microbiol. 44: 4211-4213

Nandi, S., Maiti, D., Shaha, A. & Bhadra, R. K. (2003). Genesis of variants of V. cholerae O1 biotype El Tor: role of the CTXΦ array and its position in the genome. *Microbiology* 149, 89-97.

O'Shea, A. Y., Reen, F. J., Quirke, A. M. & Boyd, E. F. (2004). Evolutionary genetic analysis of the emergence of epidemic *Vibrio cholerae* isolates on the basis of comparative nucleotide sequence analysis and multilocus virulence gene profiles. *J Clin Microbiol* 42, 4657–4671.

Responding to the 2007 floods: Record numbers of patients seek care at ICDDR,B's Dhaka Hospital. p.1-5, *Health and Science Bulletin(English)*. Vol. 5, No. 3 Sept. 2007.
Sack, R. B., A. K. Siddique, Ira M. Longini, Jr., A. Nizam, Md. Yunus, M. S. Islam, J. Glenn Moreris, Jr., A. Ali, A. Huq, G. B. Nair, F. Qadri, S. M. Faruque, D. A. Sack, and R. R. Colwell. 2003. A 4-Year Study of the Epidemiology of *Vibrio cholerae* in Four Rural Areas of Bangladesh.187: J. Infect Dis. 96-101.

Sack DA, Sack RB, Nair GB, Siddique AK. Cholera. Lancet. 2004 Jan 17;363 (9404):223-33.

Safa, A., N. A. Bhuyian,1 S. Nusrin, M. Ansaruzzaman, M. Alam, T. Hamabata, Y. Takeda, D. A. Sack, and G. B. Nair. 2006. Genetic characteristics of Matlab variants of *Vibrio cholerae* O1 that are hybrids between classical and El Tor biotypes. J. Med. Microbiol. 2006, 55, 1563–1569.

Samadi, A. R., Huq, M. I., Shahid, N., Khan, M. U., Eusof, A., Rahman, A. S., Yunus, M., and Faruque, A. S., 1983.Classical *Vibrio cholerae* biotype displaces El tor in Bangladesh. Lancet 1(8328): 805-7.

Schwartz, BS., Harris JB, Khan AI, Larocque RC, Sack DA, Malek MA *et al*. Diarrheal epidemics in Dhaka, Bangladesh, during three consecutive floods: 1988, 1998, and 2004. *Am J Trop Med Hyg* 2006; 74:1067-73.

Siddique, AK., Nair, GB., Alam, M., Sack, DA., Huq, A., Nizam, A., Longini, IM, Qadri, F., Faruque, SM., Colwell, RR., Ahmed, S., Iqbal, A., Bhuiyan, N., and R.B. Sack. 2007. El Tor Cholera with classical Toxin Causing More Severe Disease: A New Threat to Asia and Beyond. 42nd US-Japan Cholera and Other Bacterial Enteric Infections Joint Panel Meeting-2007. Austin, TX., December 5 – 7, 2007, p. 276.

Steine, C., Alam, M., L. Tang., GB., Nair., AK., Siddique., SM. Faruque., A. Huq., RR., Colwell., RB. Sack., JG., Morris, Jr. 2008. Cholera epidemics in rural Bangladesh are the result of multiple small outbreaks. *J. Infect. Dis.*, Vol. 14, No. 5, p-831-833.

Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol. 1995 Sep;33(9):2233-9.

Tison, D. 1999. Vibrios, p. 497-506. *In* P. Murray, E. Baron, M. Pfaller, F. Tenover, and R. Yolken (ed.), Manual of clinical microbiology, 7th ed. American Society for Microbiology, Washington, D.C.

Waldor, M. K., and J. J. Mekalanos. 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science 272:1910–1914.

Seas C, Miranda J, Gil AI, Leon-Barua R, Patz J, Huq A, et al. New insights on the emergence of cholera in Latin America during 1991: The Peruvian experience. Am J Trop Med Hyg. 2000; 62:513-517.

Kaper JB, Morris JG, Jr MM, Levine. Cholera. Clin Microbiol Rev. 1995; 8: 48–86.
Popovic T, Bopp C, Olsvik O, Wachsmuth IK. Epidemiologic application of a standardized ribotype scheme for *Vibrio cholerae* O1. J Clin Microbiol. 1993; 31:2474-2482.

Tauxe R, Mintz E, Quick R. Epidemic cholera in the New World: translating field epidemiology into new prevention strategies. Emerg Infect Dis. 1995; 1: 141-146.
Wachsmuth IK, Evins GM, Fields PI, Olsvik Ø, Popovic T, Bopp CA, et al. The molecular epidemiology of cholera in Latin America. J Infect Dis. 1993; 167:621– 626.
Dalsgaard A, Skov MN, Serichantalergs O, Echeverria P, Meza R, Taylor DN. Molecular evolution of *Vibrio cholerae* O1 strains isolated in Lima, Peru, from 1991 to 1995. J Clin Microbiol. 1997; 35:1151– 1156.

Evins GM, Cameron DN, Wells JG, Greene KD, Popovic T, Giono-Cerezo S, et al. The emerging diversity of the electrophoretic types of *Vibrio cholerae* in the Western hemisphere. J Infect Dis. 1995; 172:173-179.

Waldor MK, Mekalanos JJ. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science. 1996; 272: 1910-1914.

Kovach ME, Shaffer MD, Peterson KM. A putative integrase gene defines the distal end of a large cluster of ToxR-regulated colonization genes in *Vibrio cholerae*. Microbiology. 1996.142:2165-2174.

Glass, R. I., M. I. Huq, B. J. Stoll, M. U. Khan, M. H. Merson, J. V. Leee, and R. E. Black. 1982. Endemic cholera in rural Bangladesh, 1966-1980. Am. J. Epidemiol. **116:**959-970.

Dziejman, M., E. Balon, D. Boyd, C. M. Fraser, J. F. Heidelberg, J. J. Mekalanos. 2002. Comarative genomic analysis of *Vibrio cholerae*:Genes that correlate with cholera endemic and pandemic disease. Proc. Natl. Acad. Science. **99:**1556-1561.
Politzer, R. 1959. Cholera, Monograph Series, no. **43**. World Health Organization, Geneva.

Siddique, A. K., A. H. Baqui, A. Eusof, K. Haider, M. A. Hossain, I. Bashir, K. Zaman. 1991. Survival of classic cholera in Bangladesh. Lancet. **337:**1125-1127.

Faruque, S. M., N. Chowdhury, M. Kamruzzaman, Q. S. Ahmed, A. S. G. Faruque, M. A. Salam, T. Ramamurthy, G. B. Nair, A. Weintraub, and D. A. Sack. 2003. Reemergence of Epidemic *Vibrio cholerae* O139, Bangladesh. Emerging Infectious Disease. **9:**1116-1122.

Albert, M. J., A. K. Siddique, M. S. Islam, A. S. G. Faruque, M. Ansaruzzaman, S. M.Faruque, and R. B. Sack. 1993. Large outbreak of clinical cholera due to *Vibrio cholerae* non-01 in Bangladesh. Lancet: **341:**704.

Cholera Working Group, ICDDR, B. 1993. Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. Lancet **342:**387-390.
Ramamurthy, T., S. Garg, R. Sharma, S. K. Bhattacharya, G. Balakrish, T. Nair, T. Shimada, Y. Takeda, T. Karasawa, H. Kurazano, A. Pal, and Y. Takeda. 1993. Emergence

of novel strains of *Vibrio cholerae* with epidemic potential in southern and eastern India. Lancet. **341**:703-704.

Siddique, A. K., K. Zaman, K. Akram, R. Madsudy, A. Eusof, and R. B. Sack. 1994. Emergence of a new epidemic strain of *V. cholerae* in Bangladesh: an epidemiological study. J. Geog. Med. **46:**147-150.

Siddique, A. K., K. Akram, K. Zaman, P. Matsuddy, A. Eusof, and R. B. Sack. 1996. *Vibrio cholerae* O139: How great is the threat of a pandemic? Trop. Med. & Intl. Health. **1**:393-398.

Sack, R. B., Siddique, A. K., Longini, I. M., Nizam, A., Yunus, M., Islam, M. S., Morris, J. G., Ali, A., Huq, A., Nair, G. B., *et al.* (2003) *J. Infect. Dis.* **187,** 96-101.

Wachsmuth IK, Evins GM, Fields PI, Olsvik Ø, Popovic T, Bopp CA, et al. The molecular epidemiology of cholera in Latin America. J Infect Dis. 1993; 167:621– 626.
Dalsgaard A, Skov MN, Serichantalergs O, Echeverria P, Meza R, Taylor DN. Molecular evolution of *Vibrio cholerae* O1 strains isolated in Lima, Peru, from 1991 to 1995. J Clin Microbiol. 1997; 35:1151– 1156.

Nair, G. B., Faruque, S. M., Bhuiyan, N. A., Kamruzzaman, M., Siddique, A. K. & Sack, D. A. 2002. New variants of *Vibrio cholerae* O1 biotype El Tor with attributes of the classical biotype from hospitalized patients with acute diarrhea in Bangladesh. *J Clin Microbiol* 40, 3296–3299.

Nair, G. B., Qadri, F., Holmgren, J., Svennerholm, A. M., Safa, A., Bhuiyan, N. A., Ahmad, Q. S., Faruque, S. M., Faruque, A. S., Takeda, Y., and Sack, D. A. 2006. Cholera due to altered El Tor strains of *Vibrio cholerae* O1 in Bangladesh J Clin. Microbiol. 44: 4211-4213.

Kaper JB, Morris JG Jr, Levine MM. Cholera. Clin Microbiol Rev. 1995;8:48–86.