

CHOLERA

Integrated view of *Vibrio cholerae* in the Americas

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Latin America has experienced two of the largest cholera epidemics in modern history; one in 1991 and the other in 2010. However, confusion still surrounds the relationships between globally circulating pandemic *Vibrio cholerae* clones and local bacterial populations. We used whole-genome sequencing to characterize cholera across the Americas over a 40-year time span. We found that both epidemics were the result of intercontinental introductions of seventh pandemic El Tor *V. cholerae* and that at least seven lineages local to the Americas are associated with disease that differs epidemiologically from epidemic cholera. Our results consolidate historical accounts of pandemic cholera with data to show the importance of local lineages, presenting an integrated view of cholera that is important to the design of future disease control strategies.

Cholera is an acute intestinal infection that leads to a rapid and severe dehydrating diarrhea, and is caused by serogroup O1 and O139 *Vibrio cholerae*. The global disease burden of cholera is estimated to be between 1.3 and 4 million cases a year with 21,000 to 143,000 deaths (1). The current seventh pandemic (7P) of cholera began in 1961 and is attributed to a *V. cholerae* O1 biotype El Tor lineage, which is different from the Classical biotype *V. cholerae* O1 thought to be responsible for previous pandemics. Aside from being a prominent human pathogen, exploratory analyses have demonstrated since the 1970s that *V. cholerae* is an integral member of many coastal, estuarine, and brackish water ecosystems, as are other *Vibrio* species, in which it is often associated with copepods and zooplankton (2). Accordingly, a view of *V. cholerae*

epidemiology emerged in the following decades, which posits that locally evolving, but globally distributed, *V. cholerae* populations are responsible for cholera outbreaks, which occur when climatic or environmental stimuli provide favorable bacterial growth conditions in these environs (3, 4). This perception has had profound effects on all levels of global public health; cholera is now considered to be ineradicable because its etiological agent is ubiquitous in aquatic ecosystems (3, 5).

Despite advances in our understanding of the global epidemiology of cholera, we still face unanswered fundamental questions about the relationships between local and global *V. cholerae* populations. Latin America presents a notable opportunity to investigate these relationships. Although this region has local foci of endemic *V. cholerae*, such as on the Gulf Coast of the United States and Mexico (6), pandemic cholera was absent from Latin America for nearly 100 years. In January 1991, a cholera outbreak occurred along the coast of Peru and spread rapidly to nearly every country in Latin America, causing 1.2 million disease cases and 12,000 deaths by 1997 (7). More recently, pandemic cholera was introduced into Haiti (8), where the resultant epidemic has affected more than 797,000 people and caused over 9400 deaths (9). In response to these two large-scale epidemics, regional and national surveillance systems in Latin America were hyperalert for cholera outbreaks, and as a result, their sampling framework captured a diverse collection of *V. cholerae* during both epidemic and interepidemic periods (10, 11). Coupling these precise epidemiological data, which describe the beginning of the epidemic, to increased sampling within Latin America, allows studies within this region to offer unprecedented opportunities to address the relationships be-

tween local populations and globally circulating pandemic lineages of *V. cholerae*.

The relationships between these bacterial populations have been difficult to characterize until now, primarily due to the molecular methods used to assess the relatedness of *V. cholerae* isolates to one another (10, 12–14). Inconsistency in applying and interpreting results generated using these methods, such as the use of different restriction enzymes for pulsed-field gel electrophoresis and ribotyping, and the lack of standardized nomenclatures, has further complicated comparisons between studies. However, it is possible now to unify these results by using whole-genome sequencing.

To examine the relationship between *V. cholerae* lineages in Latin America, we sequenced a collection of 252 isolates. Phylogenetic analysis showed that 164 strains were of the 7P El Tor (7PET) cholera lineage and 88 were strains distinct from 7PET (collectively referred to as non-7PET) (15). This collection is geographically and temporally broad, and includes representative isolates from 14 countries spanning 1974 to 2014, including pre-epidemic, epidemic (isolated and typed during the 1991 and Haitian epidemics), and interepidemic periods. Critically, this collection includes serogroup O1 and non-O1 isolates, both clinical and environmental isolates (figs. S1 to S3 tables S1 to S3, and supplementary text note 1), isolates typed by early molecular approaches (table S4) (11, 12), and several key additional 7PET isolates from Africa [see companion analysis of Weill *et al.* (16)].

We placed these isolates into a phylogenetic framework, and determined the evolutionary relationships between lineages in Latin America. A global phylogeny, comprising a total of 665 isolates, revealed a marked diversity of *V. cholerae* lineages present in this region (Fig. 1 and figs. S2 and S3). Representative isolates from both the 1991 and 2010 epidemics clustered within the 7PET lineage (table S4). The phylogeny also revealed that isolates sampled in different years, and in some cases across multiple countries, comprise 11 lineages in Latin America (Fig. 1 and fig. S4). These lineages include Classical *V. cholerae* isolated from Mexico during the mid-1990s, as well as several *V. cholerae* O1 local lineages, such as the Gulf Coast lineage (17), or those containing isolates of Mx1 to Mx3 ribotypes (MX-1 to MX-3 lineages) described in Mexico (11). The Tucumán variant from Argentina (18) and the Amazonia variant from Brazil (19) form a single lineage, named Endemic Latin American I (ELA-I), in which these isolates remain clearly separated phylogenetically by their country of origin (Fig. 1A and fig. S2). Although 7 of the 18 samples sequenced from the Tucumán and other regions of Argentina belong to ELA-I, the other samples are distributed among five other lineages. More than 30 additional isolates sampled across Latin America do not belong to any previously known lineage and comprise at least eight different serotypes (fig. S5 and table S2).

Local *V. cholerae* O1 lineages in Latin America harbor a wide range of genetic determinants that are associated with pandemic disease (figs. S6 and

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S7 and tables S2 and S3). For instance, the genes encoding the bipartite cholera toxin (*ctxAB*), the primary virulence determinant of cholera borne by the lysogenic CTX ϕ bacteriophage, are present in several isolates from the Gulf Coast lineage and the MX-2 lineage (fig. S7 and tables S2 and S3). Typing based on the *ctxB* locus revealed that these lineages harbor different variants of the cholera toxin. Several isolates unaffiliated with lineages sampled from Argentina and the U.S./Mexican Gulf Coast also harbor *ctxAB*. Notably, the genes encoding the toxin co-regulated pilus (TCP), which allows *V. cholerae* to colonize the human intestine successfully, are present in all ELA-3, Gulf Coast, and MX-2 isolates, whether or not they are CTX ϕ ⁺ (fig. S7 and tables S2 and S3). The presence of TCP (if expressed) confers upon CTX ϕ -negative strains

the potential to be infected and lysogenized by CTX ϕ to become cholera toxin producers.

Our results support previous descriptions of isolates from the Classical lineage in Mexico between 1995 and 1997 (20, 21). This finding is notable because it was thought that this biotype had disappeared, globally, in the late 1980s (20). A recent study in Thailand, however, recovered Classical strains isolated as late as 2000 (22), indicating that this lineage persisted longer than previously thought. It has been proposed that Classical cholera isolates were present in Mexico more than a decade earlier and persisted there until the mid-1990s (21) because of a single case, imported into the United States from Cancún, Mexico, in 1983 (23, 24). This isolate was subsequently shown to be a member of the Gulf Coast

lineage by ribotyping and electrophoretic typing (ET) (23–25). Our phylogenetic data show that Classical biotype isolates in Mexico in the mid-1990s are part of the Classical lineage, and not derived from the Gulf Coast lineage (Fig. 1 and fig. S2).

To understand better the relationships within the 7PET lineage, we calculated a robust maximum likelihood phylogeny from 518 7PET genomes. We detected a strong temporal signal, which allowed us to estimate dated phylogenies (Fig. 2 and fig. S8). These data show that the Latin American cholera epidemics were the result of multiple intercontinental introductions (Fig. 2), which we refer to as LAT (Latin American transmission) 1 to 3. Our phylogeny reveals that two independent intercontinental introductions of 7PET *V. cholerae* into Latin America contributed to the 1991 epidemic (Fig. 2). The major epidemic clone during the 1991 epidemic is represented by the LAT-1 sublineage, corresponding phylogenetically to the previously described 7PET pandemic wave 1 isolates that form the West-African South American (WASA) lineage (26); these were originally typed as serotype Inaba, ribotype 5, and ET 4 (table S4) (12). We identified a frame-shift mutation at position 165 in the *wbeT* gene, consistent with the Inaba serotype in this lineage (table S1). LAT-1 isolates carry the El Tor variant of *ctxB* (*ctxB3*). The direct ancestors of LAT-1 in our phylogeny are isolates from Western and Central Africa (Angola, Cote d'Ivoire, Sao Tome) from the late 1980s. These African isolate genomes and the LAT-1 sublineage are separated by only 13 nonrecombinant single-nucleotide polymorphisms (SNPs). Further support for an African ancestry is the placement of African isolates (Uganda 1992, Nigeria 1997) within the LAT-1 sublineage. Several lines of evidence suggest that this introduction occurred close in time to the recorded start of the epidemic. First, our time-resolved phylogenies date this introduction to between 1985 and 1989, and the most recent common ancestor of LAT-1 isolates to 1989 (Fig. 2A). Second, our data show that genetic features that define the LAT-1 sublineage—the VSP-II gene variants (insertion between VC_0510 and VC_0516) and the WASA-1 genomic island—were acquired successively and in Africa during the late 1980s prior to this introduction (Fig. 2A and fig. S9). Moreover, Western Africa experienced cholera outbreaks immediately before the Peruvian epidemic (fig. S10). Tests for vibriocidal antibodies in stored sera from Lima, Peru, in 1990 indicate that *V. cholerae* was not present at this time (25). LAT-1 isolates were isolated in Mexico until 2010 (Fig. 2C). The more recent LAT-1 isolates collected between 2004 and 2010 in Mexico harbored a truncated CTX ϕ duplication and represent localized adaptations of these 7PET strains (27) (Fig. 2A).

The second clone introduced into Latin America in 1991 was described as serotype Ogawa, ribotype 6a, ET3 (12, 13), and resistant to furazolidone, sulfisoxazole, and streptomycin (13). This clone was first detected in a mountainous village near Mexico City, Mexico, in June 1991 and is believed to have been imported via coca smugglers using

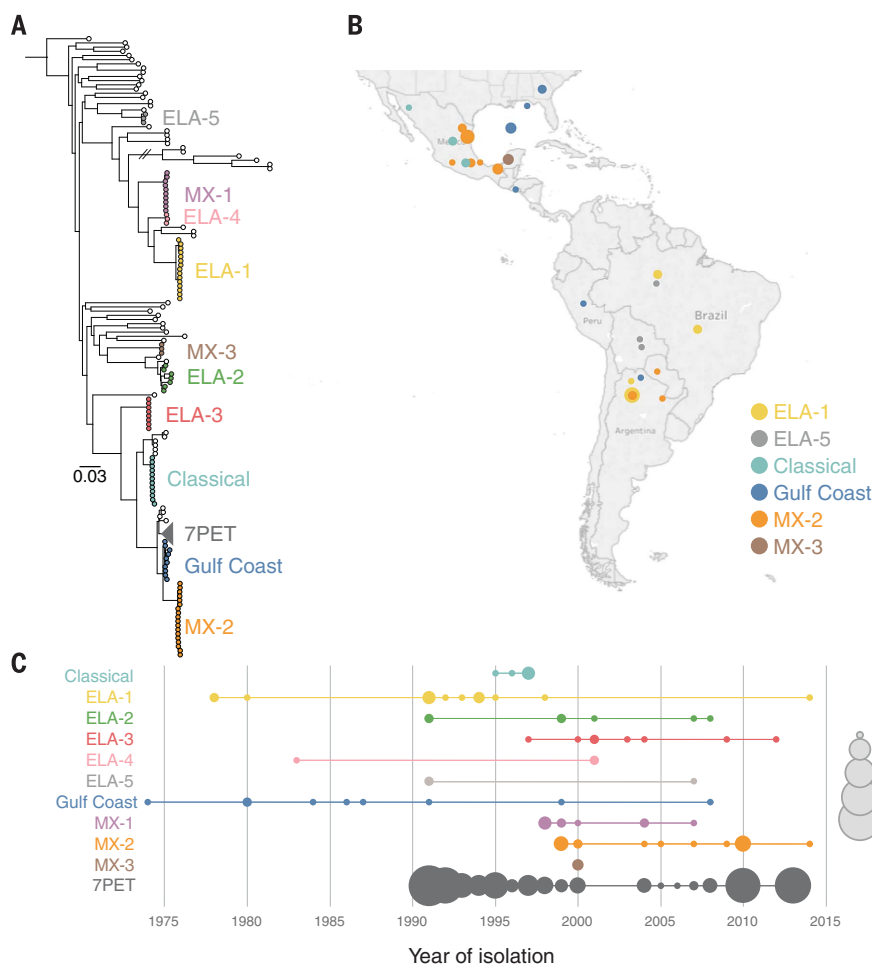


Fig. 1. Multiple lineages of *V. cholerae* are present in Latin America. (A) Maximum likelihood phylogeny of 148 *V. cholerae* genomes. Local lineages present in Latin America are highlighted. The 7PET lineage is shown as a collapsed triangle. Four 7PET genomes (reference genome N16961, and examples of LAT-1 to -3) were used as representatives of the 518 7PET genomes in this study. The scale bar denotes substitutions per variable site. The hash mark denotes a branch that was artificially shortened; the full tree is shown in figs. S2 and S3. (B) Geographical distribution of selected local *V. cholerae* lineages in Latin America. The size of the circle denotes the number of genomes analyzed from that area. Only isolates annotated with explicit geographic information are shown. (C) Temporal distribution of genomes sampled from *V. cholerae* lineages present in Latin America. The size of the circle scales with the number of genomes in our study for each lineage.

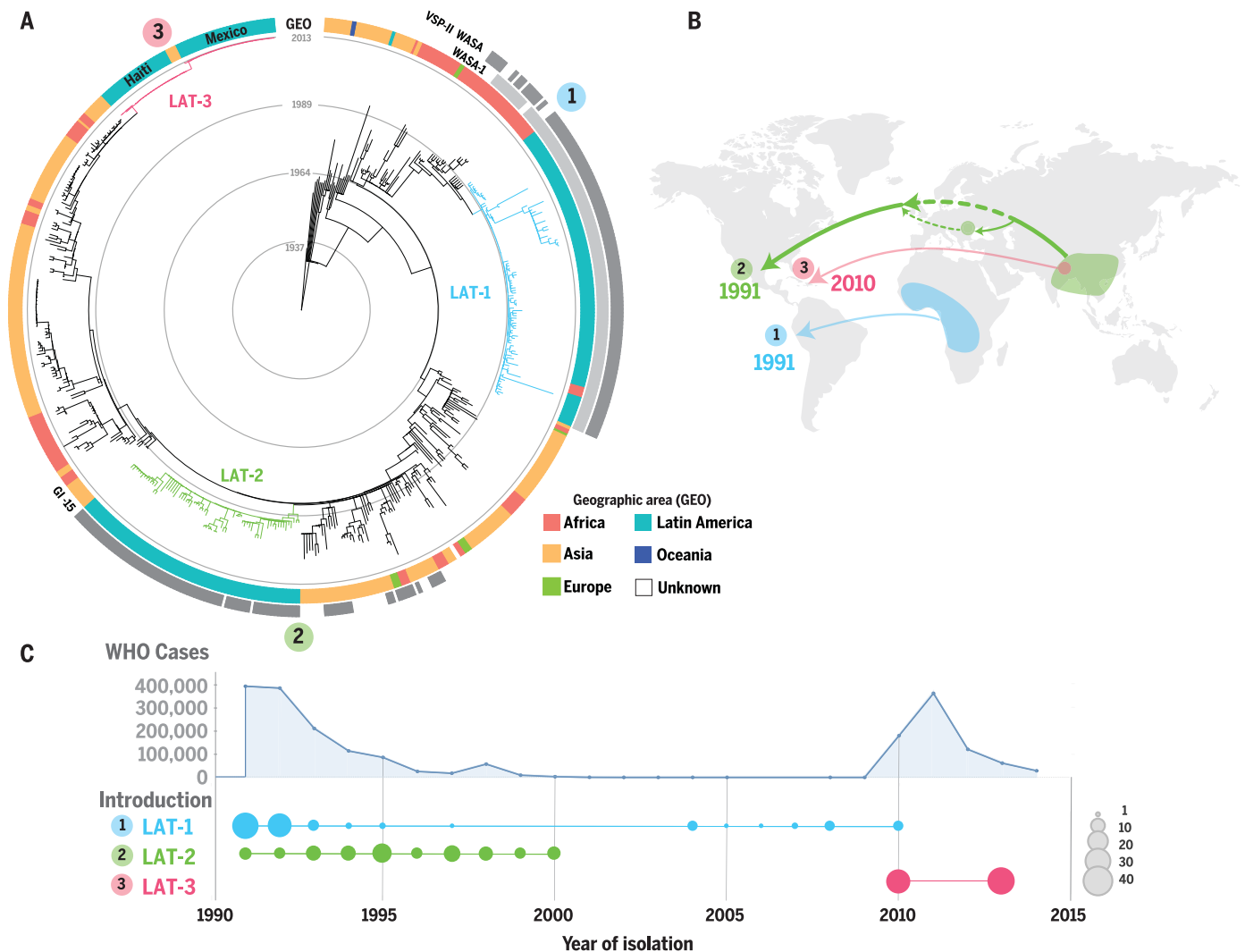


Fig. 2. Intercontinental introductions of seventh pandemic *V. cholerae* El Tor into Latin America. (A) Time-scaled maximum likelihood phylogeny of 518 7PET genomes. Inner rings denote time in years. Colored branches correspond to sublineages introduced into Latin America and are labeled LAT-1 to -3. The geographic location of the isolates corresponds to the colored block in the GEO legend. Key genomic features that define lineages are shown in gray in outer bands. The introduction events are numbered on the outside of the circle. **(B)** Intercontinental introductions of seventh pandemic cholera

into Latin America. Introductions are indicated by solid lines. The direct introduction of the LAT-2 sublineage from South Asia or China or introduction via Eastern Europe is uncertain and is denoted by dashed lines. The year of the first appearance of these lineages in Latin America is indicated. **(C)** Temporal distribution of the number of cases reported to the World Health Organization (WHO) in Latin America and number of genomes sampled in this study per LAT sublineage. The size of the circle scales with the number of genomes in our study for each sublineage.

nearby private airstrips (28). This clone subsequently spread throughout Central America (13). After 1993, this was the major clone circulating in Mexico, where it persisted until 2000 (Fig. 2C) (11). Our phylogeny shows that the introduction of this second sublineage (LAT-2) occurred between 1987 and 1989, making this event concurrent with that of the LAT-1 introduction (Fig. 2A). The distinctive drug resistance profile of the LAT-2 sublineage was linked to the presence of a genomic island (GI-15) (Fig. 2A and figs. S9 and S11). GI-15 harbors the genes responsible for streptomycin (*aadA*) and sulfisoxazole (*sulI*) resistance. With the exception of a few sporadic isolates, LAT-1 isolates were pan-susceptible to antimicrobials, and all lacked GI-15. The most

closely related isolates to those of LAT-2 are those that were collected in South and Southeast Asia, Western Asia (Lebanon), and Eastern Europe (Romania) (Fig. 2A and table S1), many of which also harbor GI-15. These globally circulating wave 2 isolates, including the LAT-2 sublineage, also harbor *ctxBI* (fig. S9) with CTX ϕ integrated into the smaller chromosome. Thus, the previously identified 7PET lineage harboring *ctxBI* in Mexico (20) was not a local lineage, but was derived from the LAT-2 introduction. Our phylogeny indicates that this lineage originated from South or Southeast Asia (Fig. 2), from where it radiated globally. However, we cannot rule out that the introduction into Mexico came via secondary site(s) and not directly from Asia (Fig. 2, A and B).

The third introduction (LAT-3) involved the import of a South Asian strain into Haiti in 2010 and has been well documented (8, 29, 30). The Haitian clone has been imported into surrounding countries, including Cuba, the Dominican Republic, the United States, and Mexico (31, 32) (Fig. 2). An outbreak in 2013 within the Mexican region of Hidalgo was suspected to be the result of an import of the Haitian clone (32, 33). Our phylogeny indicates that these isolates descended from the Haitian (LAT-3) sublineage (Fig. 2) and share key genomic features, including the *ctxB7* variant and a characteristic deletion within VSP-II (*AVC_0495-VC_0512*) (29) (fig. S9).

Combined, we observe that *V. cholerae* lineages are associated with three distinct patterns of

diarrheal disease within Latin America. First, there are lineages responsible for sporadic cases or limited outbreaks, in which secondary infections are rare or nonexistent (Fig. 1C) (17). Second, lineages that occupy long-term environmental reservoirs (such as the Gulf Coast lineage) cause illness over longer periods of time and across larger geographic areas (Fig. 1C and fig. S4). The third pattern, caused by pandemic *V. cholerae*, is visibly distinct. Pandemic lineages are responsible for massive, explosive epidemics that occur over short periods of time. The epidemiological distinction between local and pandemic lineages is stark—nearly 20,000 cases per week were seen at the beginning of the 1991 epidemic in Peru (28), and more than 250,000 cases were seen over 6 months at the beginning of the 2010 Haitian epidemic. By contrast, only 65 infections reported over a 20-year period in the USA were associated with the Gulf Coast reservoir (34). We expand upon these definitions in supplementary text note 2.

We show conclusively that both historical cholera epidemics within Latin America were the result of intercontinental introductions of globally circulating 7PET lineages and were not derived from indigenous local lineages. These data (i.e., the introduction of LAT-1 from Africa) also do not support the hypothesis that El Niño was responsible for the introduction of cholera in Peru in 1991 by potentiating the long-distance transport of aquatic pathogens from Asia through a biological corridor (35) or due to a surge in preexisting local lineages (36, 37) (Figs. 1 and 2). Our data are instead consistent with descriptions of how cholera was introduced into Haiti in 2010 (i.e., through carriers or patients from endemic regions) (8, 30). We have shown that over a 30-year span, several local lineages are present at relatively constant levels (Fig. 1C). This underlines that local and pandemic lineages exhibit different epidemiological behaviors, and may occupy different ecological niches in Latin America.

We show that there are local foci of diverse *V. cholerae* lineages that cause sporadic outbreaks across Latin America. Local lineages share many characteristics with pandemic clones, such as being toxigenic and of serogroup O1 (table S3). Disease caused by these lineages would thus be defined as cholera by both the World Health Organization (38) and U.S. Centers for Disease Control and Prevention (39). However, these local lineages show markedly different patterns of dis-

ease to that of the 7PET pandemic *V. cholerae* lineage. The potential of a *V. cholerae* isolate to cause disease is best understood by studying its genomics, whether by whole-genome sequencing or a polymerase chain reaction-based typing scheme, as well as considering clinical symptoms, epidemiological context, and basic pheno- and serotyping data.

In this study, we have unified previous accounts of cholera within Latin America into a cohesive genomic framework that correctly emphasizes the relative contributions of different bacterial lineages to this diarrheal disease. An appreciation of the differences between pandemic and local lineages should inform the design of disease control strategies in Latin America. Measured and graded public health responses could be designed based on an understanding of which lineages are responsible for outbreaks of cholera. *V. cholerae* lineages can be prioritized as public health concerns if they deviate from patterns associated with local lineages.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/358/6364/789/suppl/DC1
Materials and Methods
Supplementary Text
Tables S1 to S4
Figs. S1 to S11
References (40–86)

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Integrated view of *Vibrio cholerae* in the Americas

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Wave upon wave of disease

The cholera pathogen, *Vibrio cholerae*, is considered to be ubiquitous in water systems, making the design of eradication measures apparently fruitless. Nevertheless, local and global *Vibrio* populations remain distinct. Now, Weill *et al.* and Domman *et al.* show that a surprising diversity between continents has been established. Latin America and Africa bear different variants of cholera toxin with different transmission dynamics and ecological niches. The data are not consistent with the establishment of long-term reservoirs of pandemic cholera or with a relationship to climate events.

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