BRIEF REPORT

Second-Pandemic Strain of *Vibrio cholerae* from the Philadelphia Cholera Outbreak of 1849

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SUMMARY

In the 19th century, there were several major cholera pandemics in the Indian subcontinent, Europe, and North America. The causes of these outbreaks and the genomic strain identities remain a mystery. We used targeted high-throughput sequencing to reconstruct the *Vibrio cholerae* genome from the preserved intestine of a victim of the 1849 cholera outbreak in Philadelphia, part of the second cholera pandemic. This O1 biotype strain has 95 to 97% similarity with the classical O395 genome, differing by 203 single-nucleotide polymorphisms (SNPs), lacking three genomic islands, and probably having one or more tandem cholera toxin prophage (CTX) arrays, which potentially affected its virulence. This result highlights archived medical remains as a potential resource for investigations into the genomic origins of past pandemics.

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Cholera is a diarrheal disease caused by colonization of the intestines by cholera toxin–expressing strains of the waterborne enteric bacterium *V. cholerae*. An outbreak can arise suddenly, especially in vulnerable populations with compromised sanitation infrastructure, as in the devastating 2010 outbreak in Haiti.[1] In 2012 alone, *V. cholerae* infected 3 million to 4 million people, killing nearly 100,000.[2] Although all pathogenic *V. cholerae* strains possess a similar genomic backbone that may have facilitated adaptation to human intestinal mucosa,[3,4] the predominant pathogenic strain, serogroup O1, harbors two genetically distinct biotypes: classical and El Tor (for descriptions of these and other terms, see the Glossary). In the 20th century, for unknown reasons, El Tor replaced classical as the dominant biotype. There have been seven documented pandemics since 1817,[5] but the causal *V. cholerae* strains have been genetically characterized only for the two most recent outbreaks. Therefore, although most assume that the classical biotype was responsible for the earlier pandemics,[6] the identities of the strains that caused them remain unknown.

Diverse tissue specimens archived by medical practitioners at the time of an outbreak represent an essentially untapped genetic museum for pathogen research. One extraordinary collection includes a preserved intestine from a patient who died from cholera in the 1849 Philadelphia outbreak (Fig. 1), collected by Dr. John Neill. By coupling targeted enrichment with high-throughput sequencing,[7] we used trace degraded DNA from this specimen to reconstruct a mid–19th century *V. cholerae* genome and to test the hypothesis that the O1 classical biotype was responsible for the second cholera pandemic.

METHODS

We extracted DNA with the use of an organic extraction protocol and converted the extracted DNA into Illumina sequencing libraries[8] in dedicated ancient-DNA facilities. We then enriched the libraries for the O1 classical genome (strain O395; National Center for Biotechnology Information reference sequences NC_009456 and NC_009457) as well as for regions not found in classical strains (e.g., vibrio seventh-pandemic islands I and II [VSP-I and VSP-II]), human mitochondrial genome and amelogenin X and Y genes, and other regions. We mapped the sequencing reads to strain O395 with the use of Burrows–Wheeler Aligner, version 0.5.9rc1 (release 1561),[9] aligned the resulting consensus sequence to 31 full genomes (Table S2 in Supplementary Appendix 1, available with the full text of this article at NEJM.org) using Mauve,[10] called SNPs with a custom PERL script, and visualized features of the sequence in comparison with those of strain O395 with the use of Circos, version 0.36-4[11] (Fig. 2). We used Gblocks[12] to prune insertions, deletions, and potential misalignments, which resulted in a final alignment of 28,591 SNPs that we used in maximum-likelihood[13] and Bayesian[14] phylogenetic analysis.

Our full methods and results are available in Supplementary Appendix 1. Sequences can be found at the Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) under the BioProject accession number SRP029921. Ethics approval for the study was obtained from Hamilton Health Sciences and McMaster University.

Glossary

Ancient DNA: The term used to characterize nucleic acids isolated from an extinct or long-dead organism. Ancient DNA is typically highly degraded and damaged.

Cholera toxin phage (CTX): One of the main virulence factors of *Vibrio cholerae*, located on a genomic island acquired through the CTX prophage. Different pathogenic strains possess different CTX numbers and variants. Cholera toxin itself interferes with the membrane pores of gastrointestinal cells, inducing the water loss that leads to severe diarrhea.

Clade: A phylogenetic group that contains an ancestor and all its descendants.

Genomic islands (GI): Unique genome regions, frequently mobile elements, acquired through lateral gene transfer; these regions can confer specialized functions, such as antibiotic resistance or pathogenicity.

High-throughput sequencing: A suite of modern sequencing technologies that generate millions to billions of unique DNA sequences per run. Also known as next-generation sequencing.

Molecular-clock dating: Dating of divergence times between taxa by estimating the number of substitutions separating them and calibrating with either known times of sampling or fossil ages.

O1 classical: One of the two pathogenic cholera biotypes of the O1 serogroup. Classical cholera is believed to be responsible for the first six global pandemics (until the emergence of El Tor in the 20th century).

O1 El Tor: The second of the two pathogenic cholera biotypes of the O1 serogroup. El Tor strains have been responsible for most of the cholera outbreaks during the past approximately 50 years.

Phylocore genome (PG): The clade that includes all known pandemic cholera strains of serogroups O1 and O139, plus their close relatives. PG-1 and PG-2 are two subclades.

Prophage: A viral genome that has integrated into a bacterial plasmid or chromosomal genome.

Recombination: The process in which novel genetic sequences are formed through the exchange and combination of DNA from two original chromosomes.

RTX: Repeat-in-toxin gene cluster, located next to the CTX locus.

Single-nucleotide polymorphism (SNP): A DNA mutation that is a single-base-pair variant at a position (A, T, G, or C). A SNP is a form of point mutation.

Site saturation: Over evolutionary time, mutations in a DNA sequence can recur at the same site, in many cases reverting to a previous nucleotide. This can reduce, or saturate, the available signal for estimating rates of change or divergence between two sequences, leading to erroneous inferences.

Targeted enrichment: Method to "enrich" for DNA of a certain desired target (e.g., a pathogen genome) by the exposure of synthesized single-stranded "bait" molecules (RNA or DNA) to total prepared single-stranded sample DNA. Baits are either free-floating or fixed to a medium such as a glass slide. Baits and sample are exposed to each other for many hours, which allows for hybridization between any complementary sample and bait DNA sequences. The bait–sample mixture is subsequently washed to remove loosely or poorly bound sample molecules. Any hybridized sample molecules that remain bound to the baits are retrieved and sequenced with the use of high-throughput sequencing.

Vibrio pathogenicity islands (VPI): VPI-1 and VPI-2 are genomic islands (see above) in pathogenic *V. cholerae* strains that are known to function in cholera virulence through the addition of important adhesion and other genes.

Vibrio seventh-pandemic (VSP) islands: VSP-I and VSP-II are genomic islands (see above) found in seventh-pandemic *V. cholerae* strains (clade 7P, in PG-1) but not in other virulent *V. cholerae* strains (such as O1 classical).

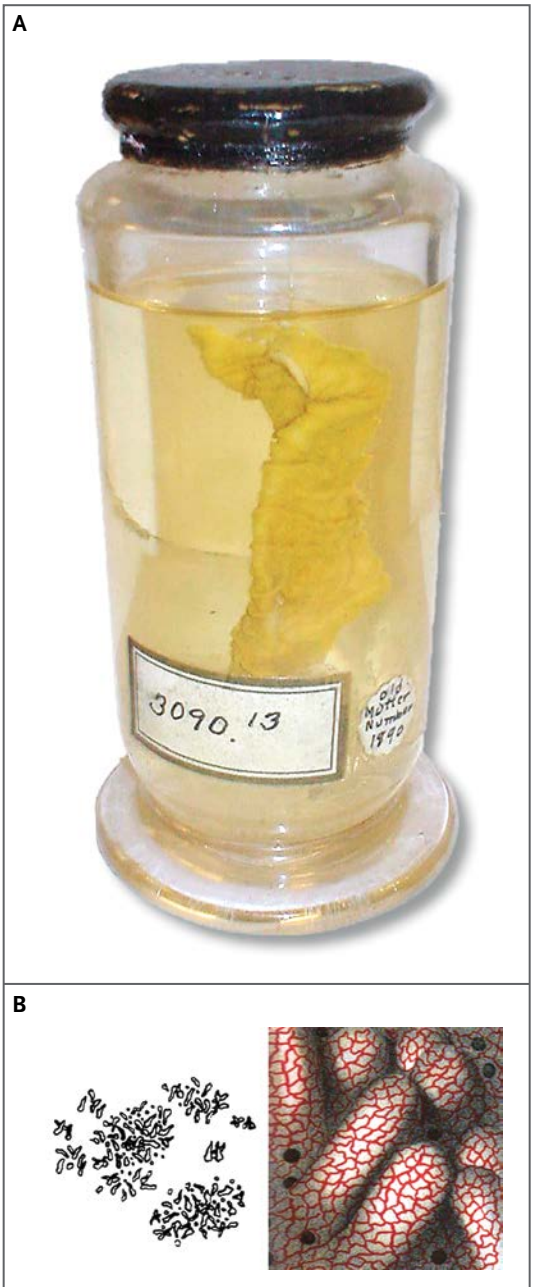

Figure 1. Historical Intestinal Specimen.

Panel A shows specimen 3090.13, a preserved portion of intestine from an 1849 cholera victim. The specimen was collected by Dr. John Neill and colleagues for the purpose of studying the effects of cholera on the intestinal lining. Their 1849 report was presented to the College of Physicians of Philadelphia, and the specimens were displayed, later becoming part of the Mütter Museum collections. Panel B shows examples of histologic and morphologic sketches of intestinal damage that accompanied the report; on the left is a sketch of villi structure, and on the right is a sketch of villi at greater magnification with injected capillaries visible.

RESULTS

HISTORICAL *V. CHOLERAE* GENOME

We reconstructed a draft *V. cholerae* genome (PA1849) at an average unique coverage depth of 15.0×, comprising 94.8% of the O395 reference strain with at least 1.0× coverage and differing by 203 SNPs (see Supplementary Appendix 2). If regions not present in PA1849 (see below) are excluded, then 97.4% of the reference sequence is present at an average coverage depth of 15.4×. Reference strain O395 regions not covered by our sequencing data could represent missing, rearranged, or highly divergent regions in the historical genome itself; could be the result of preservation or procedural biases (e.g., poorly preserved AT-rich regions, biased amplification, and uneven enrichment[7,15]); or both. The *V. cholerae* DNA fragments have typical ancient-DNA damage patterns (Fig. S4 in Supplementary Appendix 1).[16] Overall, coverage correlates strongly with GC content across most regions (Fig. 2, and Fig. S3 in Supplementary Appendix 1).

GENOMIC ISLANDS AND VIRULENCE FACTORS

Strain PA1849 shares the following phylocore genome (PG) genomic islands (GIs) with strain O395: O1, vibrio pathogenicity islands 1 and 2 (VPI-1 and VPI-2), and GI-1 through GI-10 (Table S3 in Supplementary Appendix 1); in addition, PA1849 possesses the PG-2 islands GI-23 (a putative prophage found today only in classical strains O395 and RC27) and GI-24 (a putative prophage with CRISPR [clustered regularly interspaced short palindromic repeat]–associated proteins) (Fig. 2).[4] Strain PA1849 does not have GI-11, GI-14, or GI-21; the absence of these GIs suggests that they were acquired after 1849 by the modern classical strains.

Strain PA1849 contains all known major virulence regions (e.g., VPI-1, VPI-2, and CTX prophage) common to classical *V. cholerae* but does not have nonclassical genomic regions or variants (e.g., VSP-I and VSP-II) (Tables S3 and S4 in Supplementary Appendix 1). The average GC content for these loci is not substantially lower than that in the successfully recovered genomic regions, suggesting that the absence of the loci is unlikely to be an artifact of preservation. VPI-1 has lower-than-average coverage (7.5×, vs. 15.0×), which is probably a result of its relatively low GC content (35%, vs. 46.7% for the entire genome) rather than its absence in the historical genome (Fig. 2).

Relative to the region in strain O395, VPI-1 in strain PA1849 contains one synonymous SNP (in *tcpA*), and VPI-2 contains four SNPs.

Like strain O395, strain PA1849 contains the classical *ctxB* and *rstR* variants and the expected deletion in the large-chromosome RTX element. It is also likely to possess the same CTX positions as strain O395, because it appears to have identical chromosomal flanking regions, albeit observed at low coverage. However, its CTX prophage configuration, which varies between strains,[4,17] has not been observed in classical strains to date; read assemblies indicate that there is a tandem CTX repeat span (Fig. S8 and S9 in Supplementary Appendix 1) on one or both chromosomes, with no read assemblies support-

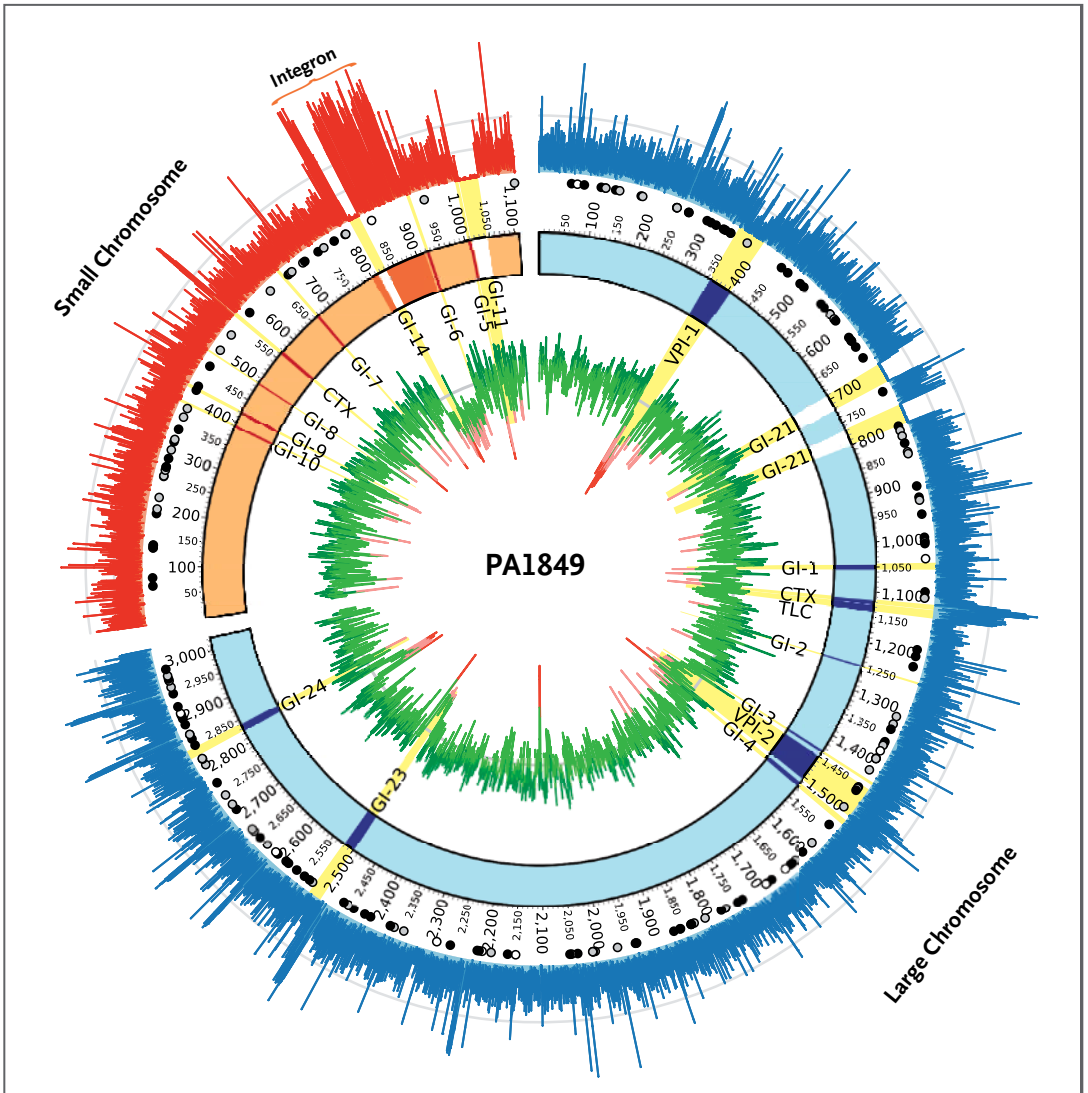

Figure 2. The PA1849 Genome.

Both chromosomes of the PA1849 draft genome, as compared with the O395 reference genome (NC_009456 and NC_009457), are illustrated. The ring closest to the center (in green and red) shows the GC content of O395 across 1000-bp consecutive windows (the gray axis line denotes the genomic average of 47.5%); the next ring shows the chromosomes (with the large chromosome shown in blue and the small chromosome shown in orange) measured in kilobases, with major genomic islands (GIs), vibrio pathogenicity islands (VPIs), cholera toxin phage (CTX), and toxin-linked cryptic (TLC) labeled; the next ring shows single-nucleotide polymorphisms (SNPs) as compared with O395 (black circles denote nonsynonymous, gray synonymous, and white noncoding SNPs); and the outer ring shows unique coverage across 100-bp consecutive windows (the gray axis lines indicate 25× and 50× coverage).

ing the presence of the truncated CTX prophage repeat that is typical of modern classical strains (Fig. S10 in Supplementary Appendix 1).

HUMAN MITOCHONDRIAL AND NUCLEAR DNA

The complete mitochondrial DNA genome from the patient with cholera was retrieved with a coverage depth of 149.0×, and reads exhibit a typical ancient-DNA damage profile (Fig. S4C in Supplementary Appendix 1).[16] The consensus sequence belongs to haplogroup L3d1b3, found today in sub-Saharan western Africa.[18] Reads matching amelogenin gene X and Y alleles suggest that this patient was male, although coverage across these regions was poor.

ORIGIN AND EVOLUTION OF *V. CHOLERAE*

Our phylogenetic analysis revealed a major division between the PG-1 and PG-2 lineages (Fig. 3). Most El Tor strains cluster in the seventh-pandemic (7P) clade, which also includes strain MO10.[4,19] Representatives of clades L6 (strain NCTC 8457), L3 (strain 2740-80), and L5 (strains M66-2 and MAK757), together with the 7P clade, make up the PG-1 clade, whereas the PG-2 clade comprises L7 (strain V52), L1 (classical strain), and strain PA1849. Strain PA1849 sits several SNPs away from the L1 clade node, with strong bootstrap support.

Our initial attempts to date the evolutionary history of *V. cholerae* were hindered by an inabil-

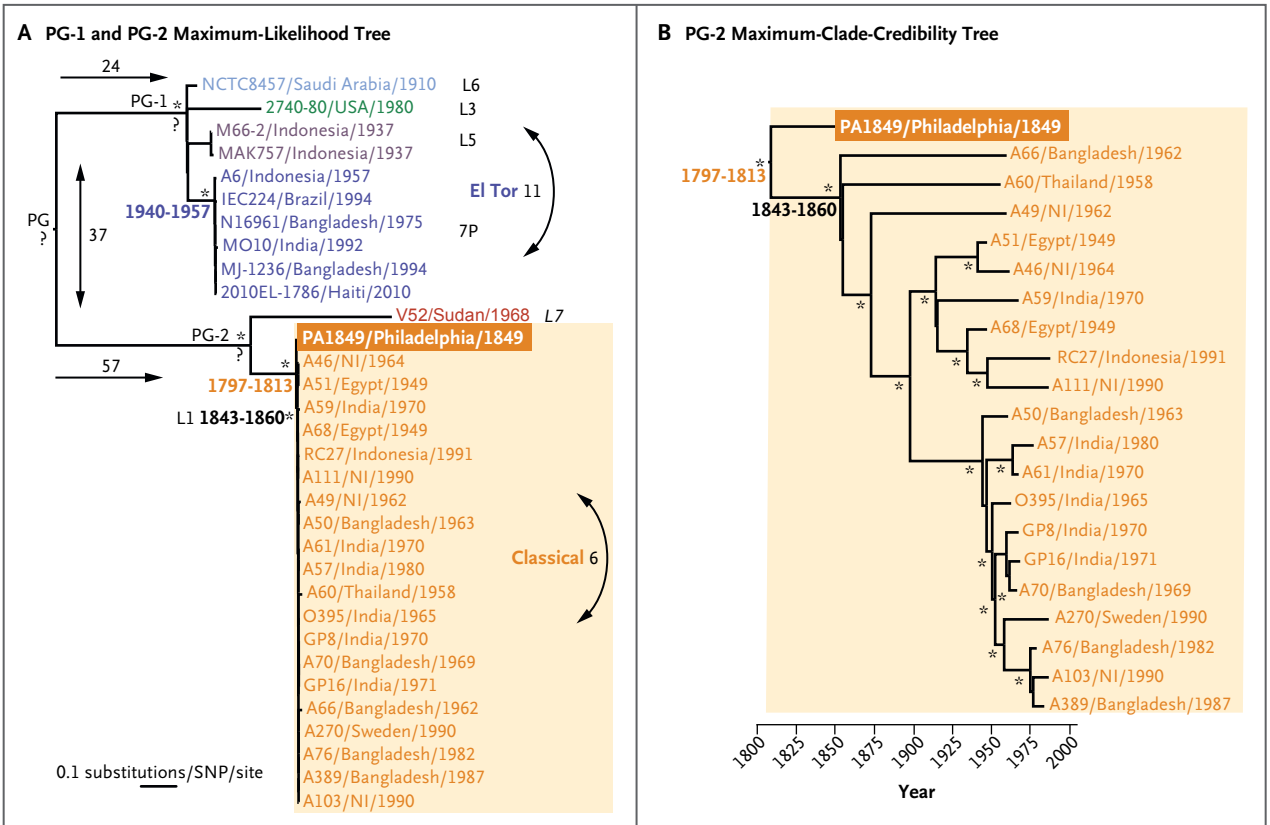

Figure 3. Evolutionary Analysis of *Vibrio cholerae*.

Panel A shows the maximum-likelihood phylogenetic tree of the El Tor and classical (including PA1849) strains (28,591 SNP sites) of *V. cholerae* present within the phylocore genome (PG) groups PG-1 and PG-2. Strain names are expanded to give the place and date of isolation. Branches are color-coded to match the geographic distribution of strains as shown in Figure S6 in Supplementary Appendix 1. Estimates of the divergence times (95% highest posterior density intervals) of key nodes obtained from a separate molecular-clock dating analysis are shown. Question marks indicate key nodes, particularly PG nodes, in which a combination of multiple substitutions and recombinations hindered attempts to accurately estimate divergence times. Branch lengths are scaled to the number of nucleotide substitutions per SNP site, and bootstrap support values greater than 90% at key nodes are indicated with an asterisk. The numbers of recombination events within (curved double-headed arrows) and between (vertical double-headed arrow) the PG-1 and PG-2 groups, as well as into PG-1 and PG-2 from unknown parents (horizontal arrows), are also shown. Panel B shows the maximum-clade-credibility tree for the evolution of the PG-2 classical strains (excluding L7) in inferred chronologic time. All tip times are set to the time of sampling, as shown on the x axis. Posterior probability values greater than 0.90 are indicated with an asterisk. 7P denotes the seventh-pandemic clade.

ity to estimate evolutionary rates from tip dates alone, even when PG-1 and PG-2 were analyzed separately. This is probably the result of a combination of site saturation and the extensive recombination that is typical of *V. cholerae*.[4,19,20] We confidently detected at least 37 recombination events between PG-1 and PG-2 (Fig. 3), as well as a number of intragroup recombination events and events that brought genetic diversity into PG-1 and PG-2 from unknown parental lineages. Such a recombination frequency makes it difficult to determine whether specific recombination events explain the recent predominance of El Tor strains.

To overcome these limitations, we imposed a strict molecular clock (1.3×10^{-3} nucleotide substitutions per SNP site per year) based on a reanalysis of a large El Tor data set.[19] Assuming this rate, we estimate that the El Tor 7P strains emerged between 1940 and 1957 (95% highest posterior density), in agreement with previous estimates.[19] Similarly, we estimate that the ancestor of the classical strains originated between 1843 and 1860, with divergence of the lineage leading to strain PA1849 occurring between 1797 and 1813, close to the time of the first recognized cholera pandemic, in 1817.[21] The combined topology and temporal estimations suggest that the first five cholera pandemics were caused by *V. cholerae* possessing a common core genome, each representing a clonal reemergence with few genome-scale mutational differences.

DISCUSSION

The PA1849 genome has a number of unique structural features but differs from strain O395 by only a few hundred SNPs across the entire 4-Mb genome. This suggests that modern *V. cholerae* evolution has been subjected to substantial selective constraint since the mid-19th century, similar to that of other pathogens that exhibit long-term core genome sequence conservation over a period of centuries (e.g., *Yersinia pestis*[7] and *Mycobacterium leprae*[22]). One of the striking features of the PA1849 historical genome is the tandem CTX configuration; this could indicate that it was capable of producing infectious CTX virions,[23] which potentially conferred greater pathogenic capacity.[24] Therefore, the suggestion that the absence of CTX virion production in classical strains may have contributed to their replacement by El Tor[24] may be unfounded. However, because there is a *V. cholerae* strain (B33) with tandem CTX repeats that does not produce replicating virions,[25] the functional implications of this structure in strain PA1849 cannot be confirmed without experimental expression in a model vibrio strain.

Previous attempts to date the origin of pandemic *V. cholerae* have returned very different results (e.g., approximately 130 to 50,000 years ago for the classical–El Tor split[26]). If a constant evolutionary rate of 1.3×10^{-3} substitutions per SNP site per year were applied across the entire phylogeny, then the common ancestor of all pathogenic *V. cholerae* (PG, potentially the ancestral strain first adapted to humans) would date to only 430 to 440 years ago. However, a combination of site saturation and recombination means that this date is an underestimate, and the date of PG is more likely to be on a time scale of millennia, predating all historically recognized pandemics and arguing against a post-medieval origin of pathogenic *V. cholerae*.[26] Our analysis therefore suggests that the PG-1 and PG-2 lineages cocirculated in humans and water sources for many centuries and potentially thousands of years before the 19th century pandemics, a finding compatible with the theory that cholera is a disease of the "first epidemiological transition," during which sedentary agriculture (beginning approximately 10,000 years ago) opened new disease niches.[27]

Collections of historical pathological specimens are invaluable resources for reconstructing pathogen evolution, yet the study of these collections remains a sensitive topic, because it was common for the bodies of marginalized minorities and the poor to be retained for medical research without consent.[28] We hope that by highlighting the intrinsic scientific, historical, and social value of these underappreciated collections, we can help to recognize and protect them in perpetuity.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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