Population Genetics of \textit{Vibrio cholerae} from Nepal in 2010: Evidence on the Origin of the Haitian Outbreak


Population Genetics of Vibrio cholerae from Nepal in 2010: Evidence on the Origin of the Haitian Outbreak

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ABSTRACT Cholera continues to be an important cause of human infections, and outbreaks are often observed after natural disasters, such as the one following the 2010 earthquake in Haiti. Once the cholera outbreak was confirmed, rumors spread that the disease was brought to Haiti by a battalion of Nepalese soldiers serving as United Nations peacekeepers. This possible connection has never been confirmed. We used whole-genome sequence typing (WGST), pulsed-field gel electrophoresis (PFGE), and antimicrobial susceptibility testing to characterize 24 recent Vibrio cholerae isolates from Nepal and evaluate the suggested epidemiological link with the Haitian outbreak. The isolates were obtained from 30 July to 1 November 2010 from five different districts in Nepal. We compared the 24 genomes to 10 previously sequenced V. cholerae isolates, including 3 from the Haitian outbreak (began July 2010). Antimicrobial susceptibility and PFGE patterns were consistent with an epidemiological link between the isolates from Nepal and Haiti. WGST showed that all 24 V. cholerae isolates from Nepal belonged to a single monophyletic group that also contained isolates from Bangladesh and Haiti. The Nepalese isolates were divided into four closely related clusters. One cluster contained three Nepalese isolates and three Haitian isolates that were almost identical, with only 1- or 2-bp differences. Results in this study are consistent with Nepal as the origin of the Haitian outbreak. This highlights how rapidly infectious diseases might be transmitted globally through international travel and how public health officials need advanced molecular tools along with standard epidemiological analyses to quickly determine the sources of outbreaks.

IMPORTANCE Cholera is one of the ancient classical diseases and particularly prone to cause major outbreaks following major natural disasters, such as earthquakes and hurricanes, where the normal separation between sewage and drinking water is destroyed. This was the case following the 2010 earthquake in Haiti. Rumors spread that the disease was brought to Haiti by a battalion of Nepalese soldiers serving as United Nations peacekeepers. This possible connection has never been confirmed. Sequencing the genomes of bacteria can give detailed information on whether isolates from different sites share a common origin. We used this technology to sequence isolates of Vibrio cholerae from Nepal, identify single-nucleotide polymorphisms (SNPs), and compare these high-resolution genotypes to the complete genome sequences of isolates from the Haiti outbreak. We provide support for the hypothesis that the isolates were brought to Haiti from Nepal.
food, water, and aid workers. On 21 October 2010, the Haitian public health authorities confirmed a cholera outbreak. By 7 July 2011, 386,429 cases, including 5,885 deaths have been reported (3). The outbreak has also spread to the neighboring Dominican Republic and to Florida and the United States (4) where sporadic cases have been observed. In the early days of the outbreak, rumors spread that the disease was brought to Haiti by a battalion of Nepalese soldiers serving as United Nations peacekeepers (2, 5–8). Though not proven definitively, the putative link to United Nations peacekeepers from Nepal gained global media attention and sparked riots in Haiti that disrupted relief efforts.

Conventional and molecular characterization of bacterial isolates is useful in determining the relationship between strains and can assist in identifying the sources. Traditionally, *V. cholerae* strains are classified into serogroups based on their outer membrane O antigen and further subdivided into biotypes based on biochemical testing; however, most outbreaks during the seventh pandemic have been caused by the same serogroup and biotype, El Tor, limiting the utility of these analyses for outbreak investigations. Molecular typing using pulsed-field gel electrophoresis (PFGE) is commonly used to characterize strains but does not always provide sufficient discriminatory power. Single-nucleotide polymorphisms (SNPs) and insertions/deletions have been used to further resolve global transmission of El Tor, limiting the utility of these analyses for outbreak investigations.

A PFGE-based study by the U.S. Centers for Disease Control and Prevention indicated that the Haitian outbreak strain was related to contemporary strains circulating in South Asia and elsewhere (4). Another study using whole-genome sequencing has similarly shown that the Haitian outbreak strain is more closely related to recent strains from Bangladesh and Mozambique than to a strain from Peru (15); however, the Peruvian strain used in that study was more than 20 years old, which weakens their conclusions. So far, none of the published studies has included recent Nepalese *V. cholerae* isolates to evaluate their relatedness to the Haitian outbreak strain.

Cholera occurs in sporadic cases and outbreaks in Nepal each year. In 2010, a 1,400-case outbreak occurred in midwestern Nepal (http://www.irinnews.org/Report.aspx?ReportID=90231). The outbreak started around 28 July and was controlled by 13 or 26 August, just prior to the time the Nepalese soldiers left for Haiti. On the request by the public health authorities in Nepal and in our function as a World Health Organization Collaborating Centre, we conducted the current study to determine the genetic diversity of the most contemporary *V. cholerae* isolates. We then compared these data to the publicly available whole-genome sequence typing (WGST) is a powerful tool providing an almost complete picture of genetic polymorphisms for evolutionary and epidemiological investigations (11–14).

A PFGE-based study by the U.S. Centers for Disease Control and Prevention indicated that the Haitian outbreak strain was related to contemporary strains circulating in South Asia and elsewhere (4). Another study using whole-genome sequencing has similarly shown that the Haitian outbreak strain is more closely related to recent strains from Bangladesh and Mozambique than to a strain from Peru (15); however, the Peruvian strain used in that study was more than 20 years old, which weakens their conclusions. So far, none of the published studies has included recent Nepalese *V. cholerae* isolates to evaluate their relatedness to the Haitian outbreak strain.

DISCUSSION

Phylogenetic patterns indicate a close relationship between Haitian and Nepalese epidemic *V. cholerae* strains. Even with whole-genome sequencing, less than 100 SNPs were identified among these geographically disparate isolates; however, the few molecular characters that were available generated a robust and highly consistent phylogenetic topology with distinct subclade structure. The apparently identical Haitian genomes confirm the earlier findings that the Haitian outbreak originated from a single source (17). More importantly, one group that was well supported and had low diversity contained both
FIG 1 Genetic relationships among *V. cholerae* isolates from Nepal and Haiti. A single maximum parsimony tree was reconstructed using 752 SNPs from 34 whole-genome sequences. There were 184 parsimony-informative SNPs, of which 6 were homoplastic, resulting in a CI of 0.97 (excluding uninformative characters). The branch lengths are labeled in red, and for branches affected by homoplasy, minimum and maximum branch lengths are designated. Members of SNP genotypic group V (16) are indicated. SNP differences among the three most closely related Nepali groups and the Haitian group are shown and characterized in Table S1 in the supplemental material.

TABLE 1 Different point mutations observed among the three sequenced isolates from the Haiti outbreak and the three most closely related isolates from Nepal.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position</th>
<th>Reference strain</th>
<th>Haitian isolate</th>
<th>Nepalese isolate</th>
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<td>C</td>
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</table>

The reference strain is *Vibrio cholerae* O1 biovar El Tor strain N16961 (Bangladesh 1971). The NCBI reference sequences or accession numbers are NC_002505 for chromosome I and NC_002506 for chromosome II.
Nepalese and Haitian isolates. In addition, the next two basal sub-
clades were also Nepalese and more closely related to the Haitian 
outbreak strain than the Bangladeshi CIRS101 strain. Only a single 
SNP separates the Haitian and Nepalese isolates, providing strong 
evidence that the source of the Haitian epidemic was from this clonal 
group. This molecular phylogeny reinforces the previous epidemi-
ological investigation (2) that pointed towards United Nations peace-
keepers from Nepal as the source of the Haitian cholera epidemic. 
Given the implications of the epidemiological findings, it is impera-
tive to use empirical laboratory data to support such findings. By 
using WGST to compare the entire genomes of available V. cholerae 
sequences, including the 24 added Nepalese strains, definitive basal/ 
derived relationships have been established between and among these 
strains.

This study also showed that multiple clonal subclades were 
involved in the 2010 Nepalese outbreak, thus indicating that 
V. cholerae is prevalent in Nepal. Therefore, there is a general need 
for improved water hygiene and investment to reduce the occur-
rence of V. cholerae in Nepal.

Complete genomic analysis of pathogen populations is now a 
reality and is dramatically changing our approach to molecular 
epidemiology. With the cost and speed of new generation DNA 
sequencers improving exponentially, previously intractable prob-
lems can be resolved rapidly with modest expense. Outbreak 
pathogens will, almost by definition, have very little molecular 
diversity and may require comprehensive genomic analysis to dif-
frentiate and categorize isolates. In combination with evolution-
ary theory and advanced statistical methods, WGST represents the 
most powerful molecular approach imaginable and is setting a 
new standard for infectious disease epidemiology. While other 
descriptive and association-based epidemiological analyses (e.g., 
case control studies, geospatial analyses), along with limited-
resolution molecular tools (e.g., PFGE), may leave room for inter-
pretation on genetic linkage, WGST, as an empirical molecular 
epidemiological tool, does not (11, 13).

Infectious disease tracking requires global-scale information and 
cooperation. The current study was reliant upon genome analyses 
performed previously from other international studies. Future inves-
tigations will require high-quality genome databases that include rep-
resentative isolates and metadata from geographically distributed 
samples, representing both historical and contemporary epidemics. 
Such databases will provide the contextual framework necessary to 
make definitive conclusions regarding infective sources and action 
plans for controlling epidemics. While we have precisely defined the 
Nepal-4 V. cholerae clade and the Haitian membership in it, its geo-
graphic distribution needs continued work. It is possible that this 
genetic group will be discovered in countries other than Nepal and 
Haiti. Attribution of outbreak sources based upon WGST alone re-
quires comprehensive geographic strain collections. The current con-
clusion that Nepal is the source of the Haitian cholera outbreak can be 
reached only if both classical epidemiology and highly suggestive 
WGST are used together. Globally representative WGST databases 
will be available in the near future and increase our power to identify 
outbreak sources. It is now the charge of the world’s national health 
agencies and disease researchers to populate these databases with 
both sequences and rich metadata. Further, it must also be their mis-
tion to develop robust genomics and bioinformatics capabilities to 
rapidly generate and receive genomics-based data that can be turned 
into actionable public health knowledge.

Natural disasters such as the 2010 Haitian earthquake disrupt wa-
ter and sanitation systems, adding to the vulnerability of affected pop-
ulations. The United Nations, regional governments, and nongov-
ernmental organizations respond rapidly to such disasters to bring 
aid and reduce suffering. The putative link between the Haitian and 
Nepalese cholera outbreaks underscores the speed at which infectious 
diseases can be transported globally and forces us to reconsider relief 
deployment strategies. In the current study, we used advanced mo-
olecular techniques to retrospectively characterize isolates from a dev-
astating outbreak; in the future, we hope that rapid molecular diag-
nostics can be integrated into rapid screening programs for relief 
workers so their efforts will neither be delayed by ineffective diagnos-
tics nor tainted by infectious diseases.

MATERIALS AND METHODS

Isolates. A total of 45 V. cholerae isolates were identified at the National 
Public Health Laboratory (NPHL), Kathmandu, Nepal, from Nepalese 
patients with diarrhea in 2010. Of these, 24 were available for analysis. The 
isolates were obtained from 30 July to 1 November 2010 and originated 
from five different districts in Nepal (Fig. 2; Table 2). All isolates with the 
exception of one from Kathmandu, Nepal, were obtained during the rainy 
season (June to August). Fifteen isolates, including the first laboratory 
freshed case, were from a large outbreak in the municipality of Nepal-
gun) in Nepal that occurred in late July to mid-August. All isolates were 
identified as V. cholerae and serotyped at the NPHL. The isolates were 
shipped to the Technical University of Denmark (DTU) in February 2011.

Antimicrobial susceptibility testing. Antimicrobial susceptibility of the 24 V. cholerae isolates was determined utilizing MIC testing. The 
following antimicrobials were used: ampicillin, amoxicillin plus clavulanic 
acid, apramycin (veterinary approved aminoglycoside), ceftaxime, cef-
irax, chloramphenicol, ciprofloxacin, colistin, florfenicol, gentamicin, 
nalidixic acid, neomycin, spectinomycin, streptomycin, sulfamethoxa-
zole, tetracycline, and trimethoprim. Clinical and Laboratory Standards 
Institute guidelines and clinical breakpoints were utilized for the interpre-
tation of the MIC values (18–20). Exceptions were made for interpre-
tation of neomycin, where epidemiological cutoff values according to the 
EUCAST system were used (http://www.eucast.org/mic_distributions/).

Due to the absence of interpretation guidelines, exceptions were made for 
the interpretation of apramycin and streptomycin which were interpreted 
according to research results from DTU. Quality control using Esche-
richia coli ATCC 25922 was conducted according to Clinical and Labora-
ory Standards Institute (CLSI) recommendations.

PFGE. All of the V. cholerae isolates were analyzed for genetic related-
ness by pulsed-field gel electrophoresis (PFGE) using the SfiI and NotI 
enzymes (Fermentas, Sankt Leon-Rot, Germany) according to the CDC 
PulseNet protocol (http://www.pulsenetinternational.org/protocols/
/Pages/default.aspx) (21). Electrophoresis was performed with a contour-
clamped homogeneous electric field (CHEF) DR III System (Bio-Rad 
Laboratories, Hercules, CA) using 1% SeaKem gold agarose in 0.5 X Tri-
borate-EDTA. A two-block program was used consisting of block I with a 
pulse time of 2.0 to 10.0 s for 13 h and block II with a pulse time of 20.0 to 
25.0 s for 6 h; the gels in both blocks were subjected to 6 V/cm on a 
120° angle in 14°C TBE (Tris-borate-EDTA) buffer. A bundle file contain-
ing 14 pulsotypes of which 11 originated from the Haitian outbreak, 
including strain 201EL-1786, two from an early 1990s Latin American 
outbreak, and one from the U.S. Gulf Coast were sent to DTU by the United 
States CDC for comparison with the 24 isolates related to the Nepalese 
outbreak. The composite data set using both enzymes was evaluated by 
using BioNumerics software version 4.6 (Applied Maths, Sint-Martens-
Latem, Belgium) where the average similarity of the experiments was used 
as settings for similarity, the enzymes were weighted equally, and 
unweighted-pair group method using average linkages (UPGMA) was 
used to generate a dendrogram.

Sequencing.
The DNA samples were prepared for multiplexed, 
paired-end sequencing on the Illumina GAIIx genome analyzer (Illumina, 

Inc., San Diego, CA). For each isolate, 1 to 5 μg of double-stranded DNA (dsDNA) in 200 μl was sheared in a 96-well plate with SonicMan (catalog no. SCM1000-3; Matrical BioScience, Spokane, WA) to a size range of 200 to 1,000 bp with the majority of material at ca. 600 bp using the following parameters: prechill at 0°C for 75 s, 20 cycles, sonication for 10 s, 100% power, lid chill at 0°C for 75 s, plate chill at 0°C for 10 s, and postchill at 0°C for 75 s. The sheared DNA was purified using the QIAquick PCR purification kit (catalog no. 28106; Qiagen, Valencia, CA). The enzymatic processing (end repair, phosphorylation, A-tailing, and adaptor ligation) of the DNA was done following the guidelines in the Illumina protocol (22). The enzymes for processing were obtained from New England Biolabs (catalog no. E6000L; New England Biolabs, Ipswich, MA), and the oligonucleotides and adaptors were obtained from Illumina (catalog no. PE-400-1001). After ligation of the adaptors, the DNA was run on a 2% agarose gel for 2 h, after which a gel slice containing 500- to 600-bp fragments of each DNA sample was isolated and purified using the QIAquick

<table>
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<th>Strain</th>
<th>Case ID</th>
<th>Location in Nepal</th>
<th>Collection date (day-mo-yr)</th>
<th>Gender*</th>
<th>Age (yr)</th>
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* M, male; F, female.
gel extraction kit (catalog no. 28706; Qiagen, Valencia, CA). Individual libraries were quantified with quantitative PCR (qPCR) on the ABI 7900HT (catalog no. 4329001; Life Technologies Corporation, Carlsbad, CA) in triplicate at two dilutions, 1:1,000 and 1:2,000, using the Kapa library quantification kit (catalog no. KK4832 or KK4835; Kapa Biosystems, Woburn, MA). Based on the individual library concentrations, equimolar pools of no more than 12 indexed V. cholerae libraries were prepared at a concentration of at least 1 nM using 10 mM Tris-HCl (pH 8.0) plus 0.05% Tween 20 as the diluent. To ensure accurate loading onto the flow cell, the same quantification method was used to quantify the final pools. The pooled, paired-end libraries were sequenced on the Illumina GAIIx to a read length of at least 76 base pairs. The average genome coverage for these 24 isolates was greater than 100× with a minimum of 75×. Over 97.6% of the genomes were at 10× cover or better. The Illumina genome sequencing data were deposited in the Short Read Archive at the National Center for Biotechnology Information (NCBI) under the accession no. SRA039806. The three Haitian genome sequences generated by the CDC were obtained from NCBI under the following accession numbers: strain 1786, SRX031665 (Illumina) and SRX031636 (454); strain 1792, SRX032204 (Illumina) and SRX032203 (454); and strain 1798, SRX032202 (Illumina) and SRX032201 (454).

Alignment. Illumina WGS data sets were aligned against chromosomes I and II of the V. cholerae O1 biovar El Tor strain N16961 (NC002505 and NC002506) using the short-read alignment component of the BWA alignment tool (23). 454 data for the publicly available Haitian genomes was aligned with BWA-SW (23). Where appropriate, isolates that were sequenced on both 454 and Illumina platforms were merged with Picard tools after the alignments were completed (http://picard.sourceforge.net). Reads containing insertions or deletions and those mapping to multiple locations in the reference were removed from the final alignments.

Identification of single-nucleotide polymorphism. Each alignment was analyzed for SNPs using SolSNP (http://sourceforge.net/projects/solsnp/). SNPs were excluded if they did not meet a minimum coverage of 75× and if the variant was present in less than 90% of the base calls for that position. In parallel, publicly available genomes were aligned against both chromosomes of N16961 using MUMmer 3.22 (24). SNPs were extracted from the alignments using a custom script. Subsequently, regions found to be duplicated in the N16961 reference genome were identified using MUMmer version 3.22. SNPs residing within these repetitive regions were then removed. Loci that lacked reference sequence coverage data for one or more isolates were removed from the final analysis. This left us with a matrix of orthologous SNP loci shared across all genomes.

Phylogenetic analysis. Phylogenetic reconstruction was performed using parsimony criteria and a heuristic search in PAUP 4.0 (25); 1,000 generations were run for bootstrap analysis. Reference genome mapping and read depth statistics were determined using the Genome Analysis Toolkit (26) and Lasergene's SeqMan NGEN version 2.2 software (Lasergene, Madison, WI).

ACKNOWLEDGMENTS

We thank Christina Aaby-Svendsen for technical assistance determining MICS and performing PFGE and Peter Germer-Smidt for sharing the PFGE bundle file of the Haitian strains. This study was supported by the Center for Genomic Epidemiology (09-067103/DSF) and the WHO Global Foodborne Infections Network (WHO GFN) (http://www.who.int/gfn/en/).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00157-11/-/DCSupplemental.

Figure S1, DOC file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Table S1, XLSX file, 0.1 MB.

REFERENCES