Emergence of *Vibrio cholerae* O1 Sequence Type 75, South Africa, 2018–2020

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We describe the molecular epidemiology of cholera in South Africa during 2018–2020. *Vibrio cholerae* O1 sequence type (ST) 75 recently emerged and became more prevalent than the *V. cholerae* O1 biotype El Tor pandemic clone. ST75 isolates were found across large spatial and temporal distances, suggesting local ST75 spread.

The seventh cholera pandemic, caused by *Vibrio cholerae* O1 biotype El Tor (7PET), arrived in Africa during 1970 and became endemic in many countries on the continent (1). Cholera was first reported in South Africa in 1974 (2). However, South Africa is not considered a cholera-endemic area; outbreaks typically are associated with importation, particularly from neighboring countries. The last cholera outbreak in South Africa was triggered by imported cases from an outbreak in Zimbabwe during 2008; South Africa reported 12,706 cases during November 2008–April 2009 (3).

Globally, 7PET isolates are genetically homogeneous and linked to the Bay of Bengal in South Asia (4,5). Most 7PET isolates are multidrug-resistant sequence type (ST) 69 (6). Rarely, 7PET has a single-locus variant, ST515, in isolates from Africa belonging to lineage T10 (7). As of September 2021, all cholera isolates from South Africa have been characterized as 7PET ST69 by multilocus sequence typing (MLST).

South Africa actively surveils for cholera. Since the 2008–2009 outbreak, few cases have been identified: 5 during 2010–2014, most of which were imported, and none during 2015–2017. During 2008–2009, large outbreaks occurred in 3 provinces, Mpumalanga, Limpopo, and KwaZulu-Natal (3), but all were caused by imported cases from neighboring Zimbabwe and Mozambique. Therefore, given their experience, healthcare workers and laboratorys in these provinces typically will test for cholera in all cases of acute watery diarrhea.

In South Africa, the National Institute for Communicable Diseases (NICD) is notified of suspected cholera cases. NICD’s Centre for Enteric Diseases supports case investigations and receives all human and environmental *V. cholerae* isolates for further investigation. The case definition for confirmed cholera is isolation of *V. cholerae* O1 or O139 from a person with diarrhea. We investigated the molecular epidemiology of *V. cholerae* in South Africa during 2018–2020.

The Study

During February 2018–January 2020, NICD received 102 *V. cholerae* isolates for testing; 9 were identified as *V. cholerae* O1. We characterized the bacteria by whole-genome sequencing, comparative genomics, and phylogenetic analysis (Appendix 1, https://wwwnc.cdc.gov/EID/article/27/11/21-1144-App1.pdf). The Human Research Ethics Committee of the University of the Witwatersrand (Johannesburg, South Africa) provided ethics approval for this study (protocol no. M160667).

Of 9 *V. cholerae* O1 isolates tested, we identified 2 ST69 (7PET) and 7 ST75 isolates. The ST69 isolates were collected in October 2018 from 2 cholera patients in a family cluster. The index case-patient had traveled to Zimbabwe, where an outbreak was ongoing (8), within the 7-day cholera incubation period before symptom onset. We confirmed these ST69 isolates belonged to the previously described highly antimicrobial-resistant Zimbabwe outbreak strain (8). The 7 ST75 isolates originated from KwaZulu-Natal and Limpopo Provinces. Five isolates were collected...
from patients with cholera, all adults 37–57 years of age; 2 isolates were from environmental samples collected during case investigations, 1 from sewage in Limpopo Province and 1 from river water in KwaZulu-Natal Province (Table 1). The 3 KwaZulu-Natal cases occurred ≥200–600 km apart; the first occurred in February 2018 and the last in January 2020. The 2 Limpopo cases occurred ≥70 km apart in the same district during November 2018. The Limpopo cases were ≥900 km from the KwaZulu-Natal cases. Epidemiologic investigations involved interviewing case-patients by using a standard case investigation form; visiting case-patients’ residences to inspect water and sanitation services and interview other household members; collecting stool samples from household members; and collecting environmental samples when indicated. Investigators found no evidence of importation from another country, epidemiologic links between cases, or secondary transmission.

The 7 ST75 isolates showed notable features (Table 2). In particular, all carried the cholera toxin (CTX) prophage resembling CTX-2 with ctxB1 genotype; Vibrio pathogenicity island 1 (VPI-1) encoding the toxin co-regulated pilus; and a variant form of Vibrio pathogenicity island 2 (VPI-2). However, isolates did not contain Vibrio seventh pandemic island 1 (VSP-I) and VSP-II. We noted several genomic islands (GLs), including VC-GI 119, but GI-05 was not present (Appendix 2, https://wwwnc.cdc.gov/EID/article/27/11/21-1144-App2.xlsx).

The only antimicrobial-resistance determinant found in all ST75 isolates was the qnrVC4 gene, located in the chromosomal superintegron. Various qnrVC alleles previously have been reported in the Vibrionaceae family and sometimes are associated with fluoroquinolone resistance (10,11). However, all ST75 isolates we analyzed showed fluoroquinolone susceptibility, MIC of ciprofloxacin 0.06 µg/mL, and susceptibility to all other tested antimicrobial drugs. This pansusceptibility sharply contrasts antimicrobial resistance trends observed in 7PET isolates from Africa, which reportedly became increasingly antimicrobial resistant over time; after the 2000s, none were susceptible to antimicrobial agents (5).

### Table 1. Clinical and demographic characteristics of 5 patients hospitalized with Vibrio cholerae O1 ST75 diagnosed from stool cultures and risk factors for V. cholerae infection, South Africa, 2018–2020*

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Province</th>
<th>Sample collection date</th>
<th>Patient age, y/sex</th>
<th>Clinical manifestations</th>
<th>Source of drinking water</th>
<th>Sanitation</th>
<th>Linked environmental samples</th>
<th>Type of environmental sample, isolate no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>YA00085869</td>
<td>KwaZulu-Natal</td>
<td>2018 Feb 8</td>
<td>37/F</td>
<td>Acute watery diarrhea, dehydration</td>
<td>Untreated river water</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>YA000132994</td>
<td>Limpopo</td>
<td>2018 Nov 9</td>
<td>38/M</td>
<td>Acute watery diarrhea, vomiting, dehydration</td>
<td>Untreated borehole water</td>
<td>Pit latrine and open defecation</td>
<td>N</td>
<td>NA</td>
</tr>
<tr>
<td>YA000134463</td>
<td>Limpopo</td>
<td>2018 Nov 20</td>
<td>45/M</td>
<td>Acute watery diarrhea, dehydration</td>
<td>Untreated borehole water</td>
<td>Flush toilets</td>
<td>Y</td>
<td>Sewage, OA01603367</td>
</tr>
<tr>
<td>YA000192016</td>
<td>KwaZulu-Natal</td>
<td>2019 Dec 29</td>
<td>49/M</td>
<td>Acute watery diarrhea, abdominal cramps, dehydration</td>
<td>Untreated river water</td>
<td>Pit latrine</td>
<td>Y</td>
<td>River water, CF00214281</td>
</tr>
<tr>
<td>YA000193061</td>
<td>KwaZulu-Natal</td>
<td>2020 Jan 12</td>
<td>57/F</td>
<td>Acute watery diarrhea, dehydration</td>
<td>NA</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
</tr>
</tbody>
</table>

*All cases were diagnosed from stool cultures. All patients survived. NA, Not available; ST, sequence type.
†Environmental samples tested positive for V. cholerae O1 ST75.

### Table 2. Features of Vibrio cholerae O1 ST75 isolates, South Africa, 2018–2020*

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Serotype</th>
<th>Biotype</th>
<th>AMR phenotype</th>
<th>AMR gene</th>
<th>Plasmids</th>
<th>ctxB allele</th>
<th>tcpA</th>
<th>wbeT mutation</th>
<th>Lineage</th>
</tr>
</thead>
<tbody>
<tr>
<td>YA00085869</td>
<td>Ogawa</td>
<td>El Tor</td>
<td>Pansusceptible</td>
<td>qnrVC4</td>
<td>None</td>
<td>ctxB1</td>
<td>tcpA&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>WT</td>
<td>L3b.1</td>
</tr>
<tr>
<td>YA00132994</td>
<td>Inaba</td>
<td>El Tor</td>
<td>Pansusceptible</td>
<td>qnrVC4</td>
<td>None</td>
<td>ctxB1</td>
<td>tcpA&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>WT</td>
<td>L3b.1</td>
</tr>
<tr>
<td>YA00134463</td>
<td>Inaba</td>
<td>El Tor</td>
<td>Pansusceptible</td>
<td>qnrVC4</td>
<td>None</td>
<td>ctxB1</td>
<td>tcpA&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>WT</td>
<td>L3b.1</td>
</tr>
<tr>
<td>OA01603367</td>
<td>Inaba</td>
<td>El Tor</td>
<td>Pansusceptible</td>
<td>qnrVC4</td>
<td>None</td>
<td>ctxB1</td>
<td>tcpA&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>WT</td>
<td>L3b.1</td>
</tr>
<tr>
<td>YA00192016</td>
<td>Ogawa</td>
<td>El Tor</td>
<td>Pansusceptible</td>
<td>qnrVC4</td>
<td>None</td>
<td>ctxB1</td>
<td>tcpA&lt;sup&gt;BT&lt;/sup&gt;</td>
<td>B08</td>
<td>L3b.1</td>
</tr>
<tr>
<td>CF00214281</td>
<td>Ogawa</td>
<td>El Tor</td>
<td>Pansusceptible</td>
<td>qnrVC4</td>
<td>None</td>
<td>ctxB1</td>
<td>tcpA&lt;sup&gt;BT&lt;/sup&gt;</td>
<td>B08</td>
<td>L3b.1</td>
</tr>
<tr>
<td>YA00193061</td>
<td>Ogawa</td>
<td>El Tor</td>
<td>Pansusceptible</td>
<td>qnrVC4</td>
<td>None</td>
<td>ctxB1</td>
<td>tcpA&lt;sup&gt;BT&lt;/sup&gt;</td>
<td>B08</td>
<td>L3b.1</td>
</tr>
</tbody>
</table>

*AMR, antimicrobial resistance; ST, sequence type; WT, wild-type.
†Nomenclature according to F.-X. Weill et al. (5).
‡Nomenclature according to H. Wang et al. (9).
**Figure.** Maximum-likelihood phylogenomic tree for *Vibrio cholerae* O1 sequence type (ST) 75 isolates collected from South Africa, 2018–2020. The tree represents phylogeny for 7 *V. cholerae* O1 ST75 isolates from South Africa (red text); 144 sequences from a global collection of ST75, or closely related ST169, ST170, and ST182 isolates; and 1 7PET *V. cholerae* O1 sequence. The 7PET genome N16961 (ST69) was used as an outgroup. For each genome, its name; year of collection, when known; and country of isolation, plus province of isolation for isolate from South Africa, are shown at the tips of the tree. The lineages, presence of CTXφ prophage or its variant form, and types of ctxB alleles are also shown. The 7PET outgroup genome, N16961, contains CTXφ with a ctxB3 allele (not represented in the figure). Red dots indicate bootstrap values ≥95%. Scale bar indicates the number of nucleotide substitutions per variable site. 7PET, seventh pandemic *V. cholerae* O1 El Tor; CTXφ, cholera toxin phi prophage; ctxB, cholera toxin B subunit gene.
DISPATCHES

We further compared the ST75 isolates from South Africa with a larger global collection of 144 ST75, or closely related ST169, ST170, and ST182, genomes (Appendix 2), and constructed a maximum-likelihood phylogeny by using 49,540 SNPs (Figure). Our phylogenetic analysis showed that the 7 isolates from South Africa clustered in the L3b.1 clade, defined by H. Wang et al. (9), with a maximum pairwise distance of 22 SNPs. Isolates from Limpopo Province had a maximum pairwise distance of 1–6, but KwaZulu-Natal Province isolates had no SNP differences. Core-genome MLST showed Limpopo Province isolates differed from the KwaZulu-Natal Province isolates by 4–5 alleles (Appendix 1 Figure). The closest related isolates were collected in Russia from Rostov Oblast in 2005 and Republic of Kalmykia in 2011 and from Turkmenistan in Central Asia in 1965, but none of those isolates contained the CTX prophage. L3b.1 isolates from Taiwan containing the CTX prophage ctxB3 allele were more distant.

Emergence of ST75 L3b.1 clade in South Africa is cause for concern. Recent studies on V. cholerae O1 isolated in Taiwan (12) and China (13) reported emerging and potential toxigenic ST75. Genomic signatures of these ST75 isolates closely resembled the US Gulf Coast V. cholerae O1 clone that emerged in 1973 (14). In particular, an investigation of V. cholerae O1 isolated during 2002–2018 in Taiwan showed that ST75 emerged there in 2009 and now is more prevalent than the ST69 pandemic clone (12). Our findings from South Africa align with the findings from Taiwan, showing that ST75 isolates outnumber ST69 isolates.

One limitation of our study is that we used reference laboratory data and a review of published V. cholerae O1 data to conclude that all previous cholera isolates in South Africa characterized by MLST were V. cholerae O1 biotype El Tor ST69. However, we cannot exclude the possibility that V. cholerae O1 isolates not characterized by MLST, particularly those from environmental samples, could have been non-ST69.

Epidemic 7PET lineage cholera demands an aggressive public health response to prevent outbreaks. In contrast, sporadic V. cholerae O1 infections mediated by other lineages, including those carrying toxin co-regulated pilus and CTX genes, typically are not epidemic-prone; most are associated with sporadic cases that rarely lead to secondary transmission (15). Tailoring the public health response to the degree of epidemic risk would be invaluable, especially in resource-limited settings.

In countries that are not cholera-endemic but are at high risk for cholera introductions, conventional laboratory determination of V. cholerae O1, even complemented by identifying ctxA or ctxB genes, might be insufficient. Typing resolution of genomics, which distinguishes between 7PET and nonepidemic lineages, can elucidate the local and global epidemiology of cholera and inform public health decisions.

Conclusions
The emergence and dominance of nonepidemic, non-7PET, V. cholerae ST75 L3b.1 in South Africa requires close monitoring. The spatiotemporal pattern suggests local spread, possibly indicating a geographically widespread risk for sporadic disease from this strain. South Africa should strengthen its disease and environmental surveillance systems to identify non-pandemic ST75 strains, define local epidemiology, and inform an appropriate public health response.

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EID Podcast

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Emergence of *Vibrio cholerae* O1 Sequence Type 75, South Africa, 2018–2020

Appendix 1

**Materials and Methods**

**Phenotypic Characterization**

We confirmed the identification of *Vibrio cholerae* isolates by using standard phenotypic microbiological identification and serotyping techniques. In brief, we subcultured bacteria onto 5% blood agar (National Health Laboratory Service, Diagnostic Media Products, https://www.nhls.ac.za) and thiosulfate citrate bile salts sucrose agar (Diagnostic Media Products) to check for purity of cultures. We identified bacteria by using the VITEK-2 COMPACT 15 (bioMérieux, https://www.biomerieux.com) automated microbial identification system. We determined serogrouping and serotyping by the slide agglutination method with polyvalent antisera and monospecific Inaba and Ogawa antisera (Mast Group Ltd, https://www.mast-group.com).

We performed antimicrobial susceptibility testing as follows. We determined the MIC of ampicillin, amoxicillin/clavulanate, cefepime, trimethoprim/sulfamethoxazole, chloramphenicol, ciprofloxacin, tetracycline, kanamycin, streptomycin, imipenem, and azithromycin by using the ETEST method (bioMérieux). We used the Clinical and Laboratory Standards Institute (CLSI) interpretative criteria for antimicrobial susceptibility testing of *Vibrio* species (M45) (1), when available. For antimicrobial drugs not listed on this *Vibrio* species in M45, we used the CLSI interpretative criteria for *Enterobacteriaceae/Salmonella* species (M100) (2). Interpretive criteria for streptomycin are not available in either CLSI document; thus, we used susceptibility ≤16 μg/mL and resistance ≥32 μg/mL.

**PCR for Toxin Detection and Biotyping**

We used real-time PCR to detect cholera toxin, *ctxA* gene (3). We used conventional PCR and analyzed PCR products using agarose gel electrophoresis to detect the presence of allelic variants of the toxin co-regulated pilus, *tcpA* gene, which determined the biotype as classical or El Tor type of *V. cholerae* O1 (4).
Genomic DNA Isolation from Bacteria and Whole-Genome Sequencing

We isolated genomic DNA from bacteria by using an Invitrogen PureLink Microbiome DNA Purification Kit (ThermoFisher Scientific, https://www.thermofisher.com). We performed whole-genome sequencing (WGS) by using NextSeq (Illumina, https://www.illumina.com) next-generation sequencing technology, and prepared DNA libraries by using a Nextera DNA Flex Library Preparation Kit (Illumina), before running 2 × 150 bp paired-end sequencing runs with ≈80 times coverage.

Additional Genomic Data

We downloaded and included raw sequence files from 71 *V. cholerae* genomes described by D. Domman et al. (5), A.R. Reimer et al. (6), Y. Luo et al. (7), S.S. Watve et al. (8), and Y.H. Tu et al. (9) from the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home) in the study (Appendix 2, https://wwwnc.cdc.gov/eid/article/27/11/21-1144-App2.xlsx). We downloaded and included 74 unpublished, complete, or assembled genomes described by J.F. Heidelberg et al. (10), K. Okada et al. (11), D. Hu et al. (12), K.V. Kuleshov et al. (13), H. Wang et al. (14) from GenBank in this study (Appendix 2).

Genomic Sequence Analyses

We used FqCleanER version 3.0 (https://gitlab.pasteur.fr/GIPhy/fqCleanER) to eliminate adaptor sequences (15), correct sequencing errors (16), and discard low-quality short-reads. We generated assemblies by using SPAdes version 3.15.0 (17).

We performed in silico multilocus sequence typing (MLST) on assembled sequences for the entire *V. cholerae* dataset by using MLST version 2.0 (https://cge.cbs.dtu.dk/services/MLST/) (18). We analyzed various genetic markers by using BLAST version 2.2.26 (https://blast.ncbi.nlm.nih.gov) against reference sequences of the CTX prophage (CTX-1, GenBank accession no. AE003852, coordinates 1566967–1573281; CTX-2, GenBank accession no. CP001486, coordinates 852233–858550), the *ctxB* gene (GenBank accession no. AE003852, coordinates 1566967–1567341), the toxin co-regulated pilus (TCP) genes (GenBank accession no. AE003852, coordinates 1566967–1567341), the toxin co-regulated pilus (TCP) genes (GenBank accession no. AE003852, coordinates 1566967–1567341), the toxin co-regulated pilus (TCP) genes (GenBank accession no. AE003852, coordinates 1566967–1567341), the toxin co-regulated pilus (TCP) genes (GenBank accession no. AE003852, coordinates 890449–891123), *Vibrio* pathogenicity island (VPI) 1 (VPI-1, GenBank accession no. AE003852, coordinates 873242–914124), VPI-2 (GenBank accession no. AE003852, coordinates 1896092–1952861), *Vibrio* seventh pandemic island (VSP) I (VSP-I, GenBank accession no. AE003852, coordinates 175343–189380), and VSP-II (GenBank accession no. AE003852, coordinates 523156–550021). We used VCGIDB (http://leb.snu.ac.kr/vcgidb/index) to determine the presence and type of genomic islands and ResFinder v4.0.1 (https://cge.cbs.dtu.dk/services/ResFinder) to determine acquired antimicrobial resistance genes.
**Phylogenetic Analysis**

We performed phylogenetic analysis on the 7 *V. cholerae* O1 ST75 isolates from this study and 145 additional genomes. We mapped the paired-end reads and genome assemblies onto the reference genome of *V. cholerae* O1 El Tor N16961, also known as A19 (GenBank accession nos. LT907989 and LT907990) by using Snippy version 4.1.0 and BWA version 0.7.17 (https://github.com/tseemann/snippy). We called SNPs by using Snippy version 4.6.0 and Freebayes version 1.3.2 (https://github.com/tseemann/snippy) under the following constraints: mapping quality of 60, minimum base quality of 13, minimum read coverage of 4, and a 75% read concordance at a locus for a variant to be reported. We aligned core genome SNPs in Snippy version 4.1.0 for phylogeny inference. We masked repetitive regions, such as insertion sequences and the TLC-RS1-CTX region, and recombinogenic VSP-II regions in the alignment (19). We built a maximum-likelihood phylogenetic tree from 49,540 aligned chromosomal SNPs, by using RAxML version 8.2.12, under the general time-reversible model with 200 bootstraps (20). The final tree was rooted on the N16961 genome, which we visualized by using Interactive Tree of Life (iTOL) version 6 (https://itol.embl.de) (21).

**Core-Genome Multilocus Sequence Typing**

We uploaded and investigated raw genomic sequencing data from FastQ files of paired-end reads to the EnteroBase web-based platform (http://enterobase.warwick.ac.uk/species/index/vibrio). We used the EnteroBase core-genome multilocus sequence typing (cgMLST) tool to perform a genomic comparison of isolates. The cgMLST scheme (Vibrio cgMLST + HierCC) incorporates 1,128 core genes. We displayed phylogenetic cluster analysis of cgMLST data by using a GrapeTree-generated minimum spanning tree with the MSTree V2 algorithm (https://bitbucket.org/enterobase/enterobase-web/wiki/GrapeTree) (22). We deposited short-read sequence data in the NCBI (https://www.ncbi.nlm.nih.gov) Sequence Read Archive under BioProject identification no. PRJEB39740.

All isolates showed ≤4 allele differences when compared against each other, indicative of highly genetically related isolates. We used the cgMLST hierarchical cluster tool of EnteroBase to further interrogate the isolates. At hierarchical cluster level 5 (HC5), where isolates are clustered at 5 allele differences, all isolates grouped into a single HC5:1000. At HC2, the isolates are split into 2 clusters: HC2:2146, which included isolates recovered in KwaZulu-Natal Province; and HC2:1003, which included isolates recovered in Limpopo Province. This finding suggests that the isolates from KwaZulu-Natal Province are slightly genetically different to those from Limpopo Province.
References


Appendix 1 Figure. Minimum spanning tree drawn by using cgMLST data from 7 *Vibrio cholerae* O1 sequence type 75 isolates collected in South Africa, 2018–2020. A) Isolate collection year; B) region of isolate collection. Circular nodes represent isolates having identical cgMLST profiles; the larger the node, the more isolates included. Number values between adjacent nodes indicate the number of allele differences between nodes. cgMLST, core-genome multilocus sequence typing.