Genomic insights into the 2016–2017 cholera epidemic in Yemen

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Yemen is currently experiencing, to our knowledge, the largest cholera epidemic in recent history. The first cases were declared in September 2016, and over 1.1 million cases and 2,300 deaths have since been reported1. Here we investigate the phylogenetic relationships, pathogenesis and determinants of antimicrobial resistance by sequencing the genomes of Vibrio cholerae isolates from the epidemic in Yemen and recent isolates from neighbouring regions. These 116 genomic sequences were placed within the phylogenetic context of a global collection of 1,087 isolates of the seventh pandemic V. cholerae serogroups O1 and O139 biotype El Tor2–4. We show that the isolates from Yemen that were collected during the two epidemiological waves of the epidemic—the first between 28 September 2016 and 23 April 2017 (25,839 suspected cases) and the second beginning on 24 April 2017 (more than 1 million suspected cases)—are V. cholerae serotype Ogawa isolates from a single sublineage of the seventh pandemic V. cholerae O1 El Tor (7PET) lineage. Using genomic approaches, we link the epidemic in Yemen to global radiations of pandemic V. cholerae and show that this sublineage originated from South Asia and that it caused outbreaks in East Africa before appearing in Yemen. Furthermore, we show that the isolates from Yemen are susceptible to several antibiotics that are commonly used to treat cholera and to polymyxin B, resistance to which is used as a marker of the El Tor biotype.

We investigated the bacterial populations that caused the cholera epidemic in Yemen by sequencing 42 V. cholerae O1 serotype Ogawa isolates that were recovered during this epidemic. Thirty-nine of these isolates were collected from patients with cholera who lived in three different governorates of Yemen (Fig. 1a, b). They span both waves of the epidemic, having been collected between 5 October 2016 and 31 August 2017. The three remaining isolates were collected from patients from a temporary refugee centre on the Saudi Arabia—Yemen border on 30 August 2017 (Fig. 1b). We also sequenced 74 7PET isolates from South Asia, the Middle East, and Eastern and Central Africa (Extended Data Fig. 1 and Supplementary Table 1). We placed these new isolates in the context of a global collection of 1,087 7PET genomic sequences2–4 (Supplementary Table 1) and constructed a maximum-likelihood phylogeny of 1,203 genomes, using 9,986 single-nucleotide variants (SNVs) that were evenly distributed across the non-repetitive, non-recombinant core genome (Fig. 2a).

We also detected a strong temporal signal, making it possible to estimate time-scaled phylogenies (Fig. 2b and Extended Data Figs. 2–4), which showed that the epidemic in Yemen originated from a recently emerged 7PET wave 3 clade5, which contains the cholera toxin subunit B gene variant ctxB7 (Fig. 2). All of the isolates from Yemen clustered together (median pairwise SNV difference of 3 (range, 0–13)), confirming that the two epidemiological waves that were observed during the epidemic in Yemen, which had very different clinical attack rates6, were produced by a single clone rather than arising from two separate introductions. We estimated the date of the most recent common ancestor of the isolates from Yemen to January 2016 (95% Bayesian credible interval, September 2015 to June 2016) (Fig. 2b, Extended Data Fig. 3 and Extended Data Table 1). Our phylogenetic analysis shows that the isolates from Yemen are different from those that have been circulating in the Middle East over the last decade, such as those isolated in Iraq in 2007 and 2015, and in Iran from 2012 to 2015 (Fig. 2a). These isolates from the Middle East also belong to 7PET wave 3, but are attributed to different sublineages on the phylogenetic tree and were imported from South Asia on two separate occasions. The isolates from Yemen are most closely related to isolates that were collected from outbreaks in Eastern Africa (Kenya, Tanzania5 and Uganda1) from 2015 to 2016 (Fig. 2). Collectively, these isolates belong to a new sublineage (T13), which corresponds to the most recent, newly identified introduction of 7PET into East Africa. All of these T13 isolates are different from those previously recovered in West or East Africa (sublineages T12 and T10, respectively) (Fig. 2). Our data suggest that this 7PET wave 3 clade, which contains all isolates with the ctxB7 allele, first emerged in South Asia in the early 2000s (Fig. 2b), consistent with the first detection of ctxB7 isolates in Kolkata, India in 20065. This ctxB7 clade has been exported to areas outside Asia on at least three separate occasions: West Africa (T12 introduction event)7 in 2008 (estimates with 95% credible interval), Haiti in 20102,8 and East Africa (T13 introduction event)9 between 2013 and 2014 (estimates with 95% credible interval) (Fig. 2b, Extended Data Fig. 3 and Extended Data Table 1).

In addition to the ctxB7 allele, all of the analysed isolates from Yemen had the following genomic features (Table 1): (1) the toxin-coregulated pilus gene subunit A gene variant tcpA1810; (2) a deletion (∆VC0495–VC0512) within Vibrio seventh pandemic island II (VSP-II); and (3) an SXT/R391-integrating conjugating element (ICE) called ICEVchInd5/I CEVchBan5, which is associated with multiple-drug resistance.

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Consistent with the genomic evidence, all of the isolates from Yemen have a similar narrow phenotype of antimicrobial drug resistance to nalidixic acid, the vibriostatic agent O/129 and nitrofurantoin (Table 1). Mutations in the DNA gyrase gene gyrA that resulted in an S83L amino acid substitution and mutations in the topoisomerase IV gene parC that resulted in an S85L substitution explain the resistance of the isolates from Yemen to nalidixic acid and their decreased susceptibility to ciprofloxacin. An approximately 10-kb deletion in ICE variable region III resulted in the loss of four genes that encode resistance to streptomycin (strA and strB), chloramphenicol (floR) and sulfonamides (sul2). The fifth gene of this region, which encodes resistance to the vibriostatic agent O/129 (dfrA1), is present in the isolates from Yemen. This deletion is not unique, as similar deletions that encompass the strA, strB, floR and sul2 genes, flanked by transposase genes, have independently arisen several times in 7PET wave 3 isolates\(^2,9\). The resistance of *V. cholerae* to nitrofurans is due to the loss of expression of a reductase enzyme that converts the drug into its active form\(^10\). By combining phenotypic and genotypic data, we found lesions in the VC0715 and VCA0637 genes of nitrofurantoin-resistant isolates (Extended Data Table 2). VC0715 and VCA0637 encode orthologues of the NfsA (52% amino acid identity) and NfsB (58% amino acid identity) proteins of *Escherichia coli* K12 (GenBank accession number NC_000913), respectively. In *E. coli*, disruption of the nitroreductases that are encoded by these genes confers nitrofurantoin resistance\(^11\). In all 7PET wave 3 isolates, including the isolates from Yemen, the observed mutations in VC0715 led to a R169C amino acid substitution and the mutation in VCA0637 introduced a premature stop codon (Q5Stop) that probably abolishes protein function.

The isolates from Yemen were also susceptible to polymyxins. This is an important finding, because resistance to polymyxin B has been used as a marker of the *V. cholerae* O1 El Tor biotype since the beginning of the seventh cholera pandemic in 1961\(^12,13\). Unlike the El Tor biotype, the classical biotype (responsible for the six previous pandemics)\(^14\) is susceptible to polymyxin B. Polymyxin resistance is conferred by changes to the lipid A domain of the surface lipopolysaccharide, thereby altering its charge\(^12,13\). The *vprA* (VC1320) gene, disruption of which is known to restore susceptibility to polymyxin in 7PET isolates, is required for expression of the *aimEFG* operon that encodes the genes that are required for the glycine modification of lipid A\(^12\). A specific non-synonymous SNV in *vprA* genes (predicted to result in a D89N substitution, Extended Data Fig. 5) was present in 97% (63 out of 65) of polymyxin B-susceptible isolates (Extended Data Table 2), including all of the isolates from Yemen. The first polymyxin-susceptible 7PET isolates with this VprA D89N substitution in our dataset were identified in Kolkata, India, where polymyxin B-susceptible *V. cholerae* O1 isolates emerged in 2012 and replaced polymyxin-resistant strains after 2014\(^15\).

7PET isolates from the ctxB7 clade have been associated with the two largest cholera epidemics in recent history. In addition to the current Yemeni epidemic, the introduction of this sublineage into Haiti in 2010 in the wake of a devastating earthquake, resulted in one million cases and almost 10,000 deaths by 2017\(^16,17\). These two major events highlight the threat that cholera continues to pose to public health in vulnerable populations. The UN (United Nations) estimates that 16 out of 29 million people in Yemen lack access to clean water and basic sanitation because of the destruction of public and health infrastructures during the years of civil conflict\(^18\). The complexity of the situation in Yemen before the epidemic was set against a backdrop of large acute watery diarrhoea or cholera outbreaks across the Horn of Africa (Extended Data Fig. 1), which serves as a major hub of migration into Yemen\(^19,20\).
Fig. 2 | Phylogenetic relatedness of the V. cholerae O1 El Tor isolates from the 2016–2017 epidemic in Yemen. a, Maximum-likelihood phylogeny of 1,203 genomic sequences. M66 was used as the outgroup. The scale bar denotes substitutions per variable site (SNVs). Branches are coloured according to geographical location, inferred by stochastic mapping of the geographical origin of each isolate onto the tree. The inferred introduction events into Africa are indicated by the letter 'T'. The sublineage labelled AME (Asia/Middle East) contains the most recent Middle Eastern isolates. b, Maximum clade credibility tree produced with BEAST for a subset of 81 representative isolates of the distal part of genomic wave 3 (that is, those with the ctxB7 allele). Geographical location of the isolates is indicated in the same colours as in a. Selected nodes supported by posterior probability values ≥0.8 are shown. Acquisition of the polymyxin susceptibility-associated non-synonymous SNV in VCI320 (vpra) is indicated. c, The geographical distribution of selected 7PET sublineages. An asterisk denotes data from a previous study. The date ranges shown for introductions are the 95% credible interval estimate of the most recent common ancestor in years. Dashed lines and the grey area in T13 indicate that the sublineage was detected in East Africa before its appearance in Yemen. This does not represent a precise route of transmission. The maps were created with Tableau Desktop version 10.1.5 using the base map from © OpenStreetMap contributors (https://www.openstreetmap.org), available under an Open Database License.

Table 1 | Characteristics of the 2016–2017 cholera epidemic strain from Yemen

| Species | Vibrio cholerae |
|---------|-----------------|
| Serogroup, serotype and biotype | O1, Ogawa, El Tor |
| Genomic wave | 3 |
| Genetic markers | ctxB7, tcpA<sup>CRS101</sup>, rtxA<sup>1</sup>, VSP-ii<sup>3</sup> |
| AMR profile | POL<sup>5</sup> (1–2 mg l<sup>−1</sup>), COL<sup>5</sup> (2–8 mg l<sup>−1</sup>), O129<sup>5</sup>, NAL<sup>5</sup> (64 to >256 mg l<sup>−1</sup>), CIP<sup>5</sup> (0.25–0.5 mg l<sup>−1</sup>), FT<sup>R</sup> |
| Horizontally acquired AMR element, acquired AMR gene (AMR phenotype) | ICEVchhn55,ICEVchhBan5<sup>4</sup>, dfrA1 (O129<sup>5</sup>) |
| Mutated chromosomal genes (AMR phenotype) | gyrA<sup>S83I</sup> and parC<sup>S85L</sup>, NAL<sup>5</sup>, CIP<sup>5</sup>, rfsA<sup>R169C</sup> and rfbB<sup>Q5Stop</sup> (FT<sup>R</sup>) |

MIC range values are indicated in parentheses. The superscript R, S and DS indicate resistant, susceptible and decreased susceptibility, respectively. VSP-ii<sup>3</sup> indicates a deletion that encompasses VCO4595–VCO0912, according to GenBank accession AE003852. ICEVchhn55,ICEVchhBan5<sup>4</sup> indicates a deletion that encompasses ICEVchhNod50011–ICEVchhNod50021 according to GenBank accession GQ463142. O129<sup>5</sup> denotes cross-resistance to the vibriostatic agent 0/129 and trimethoprim. AMR, antimicrobial resistance; CIP, ciprofloxacin; COL, polymyxin E; FT, nitrotetran-toin; NAL, nalidixic acid; POL, polymyxin B.

The available genomic data for the historical and current importations of the 7PET sublineage into Africa are not consistent with a local origin, but instead highlight the importance of human-mediated spread of the epidemic 7PET lineage from South Asia. An inability to obtain samples from countries in this region hampered our efforts to reconstruct the routes of transmission in East Africa before the appearance of this strain in Yemen more precisely.

In summary, a single recent 7PET sublineage with an unusual antimicrobial resistance phenotype is responsible for the cholera epidemic in Yemen. Our study illustrates the key role of genomic microbial surveillance and cross-border collaborations in understanding the global spread of cholera, the evolution of virulence and determinants of antibiotic resistance.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-018-0818-3.

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Author contributions F.-X.W. and D.D. designed the study. A.A.A., M.N., S.S.N., A.R., A.M.A., N.C.S., S.K., M.R.P., A.A., J.Y.C., J.F.W., C.S., B.B., H.H.N.A., D.K., S.M.N., M.R.M., J.K., F.J.L., A.S.A., T.R. and M.-L.Q. collected, selected and provided characterized isolates and their corresponding epidemiological information. J.R. performed the DNA extractions and phenotyping and molecular typing experiments. T.M. analysed protein data. C.B. performed the whole-genome sequencing. F.-X.W. and D.D. wrote the manuscript, with major contributions from N.R.T. All authors contributed to the editing of the manuscript.

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Phylogenetic analysis. Repetitive (insertion sequences and the TLC-RS1-CTX region) and recombogenic (VSP-II) regions were masked from the alignment; Putative recombogenic regions were detected and masked with Gubbins26 version 1.4.10. A maximum-likelihood phylogenetic tree was built from an alignment of 9,986 chromosomal SNVs, with RAxML27 version 8.2.8 under the GTR model with 100 bootstraps.

BEAST28 version 1.10.1 was used to estimate time-resolved phylogenies for a spatially and temporally representative subset of 81 7PET isolates under the GTR nucleotide substitution model. We tested a combination of molecular clock and tree prior models to identify the best fit (Extended Data Table 1). Both path and stepping-stone sampling showed the best fit to be an uncorrelated relaxed clock (lognormal distribution of rates) model with a Bayesian skyline coalescent tree prior. Priors were kept at default values, with the exception of the ‘constant.popSize’ value, which was set to a lognormal distribution (initial value = 1, mu = 1, sigma = 10) under the constant population coalescence tree prior. The choice of model had little influence on the dating of key nodes in this analysis (Extended Data Table 1). For each model, we ran three independent Markov chain Monte Carlo chains over 50 million steps, sampling every 2,000 steps. We used a burn-in of 5 million steps for each chain and then combined chains, resampling every 10,000 steps. The effective sample size for all estimated parameters was greater than 200. We tested for an adequate temporal signal, using TempEst29 version 1.5, by calculating the linear regression between the root-to-tip distance and isolation date for each sample. We also performed 20 date-randomization tests with the R package TipDatingBeast29 to assess the mean rate under the uncorrelated lognormal relaxed molecular clock (ucld.mean parameter).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The whole-genome alignment for the 1,203 genomes and other files that support the findings of this study have been deposited in FigShare: https://figshare.com/s/b70a9efa9c9e2f265480. Short-read sequence data were submitted to the ENA, under study accession numbers PRJEB24611 and ERP021285 and the genome accession numbers are provided in Supplementary Table 1. Phylogeny and metadata can be viewed interactively at https://microreact.org/project/globalcholera.


citation
Extended Data Fig. 1 | Geographic location of the sequenced *V. cholerae* O1 El Tor isolates and number of reported cholera cases. **a**, Geographic location of the 116 *V. cholerae* O1 El Tor isolates sequenced. The number of isolates collected per country is indicated. The three isolates collected in Jizan, Saudi Arabia (denoted by an asterisk) were from Yemeni refugees originating from Hajjah District. The map is a cropped version of the one available at https://commons.wikimedia.org/wiki/File:BlankMap-World.png. **b**, Number of cholera cases per country reported to the WHO (World Health Organisation) between 2014 and 2016. The total number of cholera cases reported to the WHO by the countries was 268,337. The maps were created using Paintmaps, a free online map generating tool (http://www.paintmaps.com/).
Extended Data Fig. 2 | Assessment of the temporal signal within the dataset. 

(a) Linear regression of the root-to-tip distance against sampling time obtained with TempEst\(^{29}\) using a maximum-likelihood phylogeny of 81 representative seventh pandemic *V. cholerae* O1 isolates (that is, those used for the BEAST analysis). Bars on nodes indicate the precision of the isolation date (for example, if only the year of isolation is known, the bar spans the entire year). 

(b) Comparison of the ucld.mean parameter estimated from 20 date-randomization BEAST experiments and the original dataset. The rate for the correctly dated tree is shown in red. The median and 95% Bayesian credible interval for the ucld.mean parameter are provided.
Extended Data Fig. 3 | Timed phylogeny of the ctxB7 clade. Maximum clade credibility tree produced with BEAST for a subset of 81 representative isolates of the distal part of the genomic wave 3 (that is, those with the ctxB7 allele). The nodes supported by posterior probability values ≥0.5 are indicated.
Extended Data Fig. 4 | Visualization of the posterior distribution of trees from the BEAST Markov chain Monte Carlo analysis. The opacity of the branches is scaled according to the number of times a clade is seen in the distribution. There is high support for the East Africa/Yemen clade. The uncertainty in the placement of the node for the Indian/East African isolates is the reason for the low posterior support value for this node in Extended Data Fig. 3.
Extended Data Fig. 5 | Multiple sequence alignment of VprA (VC1320) with two-component response regulators. A non-synonymous mutation at position 89 of VC1320 that resulted in a D-to-N amino acid change was associated with a phenotype of polymyxin B susceptibility.
Extended Data Table 1 | Summary of the Bayesian models used for BEAST analyses

| Model                        | Median | Upper 95% HPD | Lower 95% HPD | Median | Upper 95% HPD | Lower 95% HPD |
|------------------------------|--------|---------------|---------------|--------|---------------|---------------|
| Yemeni isolates tMRCA        |        |               |               |        |               |               |
| Strict, Constant             | 2016.04| 2016.34       | 2015.72       | 2014.14| 2014.62       | 2013.59       |
| Strict, Bayesian skyline     | 2016.03| 2016.35       | 2015.71       | 2014.16| 2014.63       | 2013.61       |
| UCLN, Bayesian skyline       | 2016.05| 2016.44       | 2015.67       | 2014.38| 2014.86       | 2013.74       |

| Model                        | log MLE Path Sampling | Rank | log MLE Stepping Stone Sampling | Rank |
|------------------------------|-----------------------|------|--------------------------------|------|
| Strict, Constant             | -5481314.81           | 3    | -5481312.99                    | 2    |
| Strict, Bayesian skyline     | -5481314.47           | 2    | -5481313.00                    | 3    |
| UCLN, Bayesian skyline       | -5481313.28           | 1    | -5481311.50                    | 1    |

Analyses were carried out on a subset of 81 representative *V. cholerae* O1 isolates of the distal part of the genomic wave 3. HPD, highest posterior density region; MLE, marginal likelihood estimate; tMRCA, time to the most recent common ancestor; UCLN, uncorrelated lognormal relaxed clock.
Extended Data Table 2 | Gene alteration frequencies in isolates susceptible or resistant to certain antibiotics

| Gene alteration frequencies in isolates susceptible or resistant to certain antibiotics |
|----------------------------------------|-------------------------------|
|                                        | Number (%) of isolates:       |
|                                        | FT\textsuperscript{a} | FT\textsuperscript{n} |
| VCA\_0637                              |                             |
| Wild-type                               | 258 (94.2) | 0 (0) |
| Genetic alteration                      | 16 (5.8)  | 446 (100) |
| Total                                   | 274   | 446   |
|                                         | 720*  |
| VC\_0715                                |                             |
| Wild-type                               | 264 (96.4) | 5 (1.1) |
| Genetic alteration                      | 10 (3.6)  | 441 (88.9) |
| Total                                   | 274   | 446   |
|                                         | 720*  |
| VCA\_0637 and VC\_0715                  |                             |
| Wild-type                               | 258 (94.2) | 0 (0) |
| Genetic alteration                      | 16 (5.8)  | 446 (100) |
| Total                                   | 274   | 446   |
|                                         | 720*  |
| VC\_1320                                |                             |
| Wild-type                               | 2 (3.1)    | 40 (97.6) |
| D80N                                    | 63 (96.9)  | 1 (2.4)  |
| Total                                   | 65    | 41    |
|                                         | 106**  |

\textsuperscript{FT, nitrofurantoin; POL, polymyxin B.}
\textsuperscript{*720 genomes with antimicrobial susceptibility testing data (this study and published previously).}
\textsuperscript{**106 genomes with antimicrobial susceptibility testing data (this study).}
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|-----------------|---------------------|
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- A description of any restrictions on data availability

Short-read sequence data were submitted to the European Nucleotide Archive (ENA) (http://www.ebi.ac.uk/ena), under study accession numbers PRJEB24611 and
ERP021285, and the genome accession numbers are provided in Supplementary Table 1. The whole-genome alignment for the 1203 genomes and other files that support the findings of this study have been deposited in FigShare: https://figshare.com/s/b70a9efac9cf2625480e

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size                        | We have sequenced all the 45 Yemeni V. cholerae isolates sent by two different laboratories in Yemen and Saudi Arabia. |
|------------------------------------|-------------------------------------------------------------------------------------------------------------------|
| Data exclusions                    | Three genomes out of 45 were excluded due to low coverage or mixup determined by the quality control step. The threshold for an appropriate coverage was predefined (15X or more). The mixup was discovered by analysing a discrepancy between phenotypic testing and genome content. |
| Replication                        | The Yemeni genomes have been analysed by two different phylogenetic approaches (Maximum Likelihood and Bayesian). This was replicated two times during the revision process by including 14 new genomic sequences then by including three genomes. All attempts at replication were successful as we have obtained a same tree topology. |
| Randomization                      | It is not applicable as for the phylogenetic tree we have analysed all the genomes that have passed the sequencing quality control (42/45). |
| Blinding                           | The phylogenetic trees were initially drawn without any geographic information associated with the genomes. |

Reporting for specific materials, systems and methods

| Materials & experimental systems   | Methods |
|-----------------------------------|---------|
| n/a                               | n/a     |
| ☒ Involved in the study           | Involved in the study |
| ☒ Unique biological materials     | ☒ ChIP-seq |
| ☒ Antibodies                      | ☒ Flow cytometry |
| ☒ Eukaryotic cell lines           | ☒ MRI-based neuroimaging |
| ☒ Palaeontology                   |         |
| ☒ Animals and other organisms     |         |
| ☒ Human research participants     |         |