swiss medical weekly

Original article | Published 02 April 2024 | doi:https://doi.org/10.57187/s.3437
Cite this as: Swiss Med Wkly. 2024;154:3437

Genomic characteristics of clinical non-toxigenic *Vibrio cholerae* isolates in Switzerland: a cross-sectional study

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Summary

STUDY AIMS: Although non-toxigenic *Vibrio cholerae* lack the *ctxAB* genes encoding cholera toxin, they can cause diarrhoeal disease and outbreaks in humans. In Switzerland, *V. cholerae* is a notifiable pathogen and all clinical isolates are analysed at the National Reference Laboratory for Enteropathogenic Bacteria and Listeria. Up to 20 infections are reported annually. In this study, we investigated the population structure and genetic characteristics of non-toxigenic *V. cholerae* isolates collected over five years.

METHODS: *V. cholerae* isolates were serotyped and non-toxigenic isolates identified using a *ctxA*-specific PCR. Following Illumina whole-genome sequencing, genome assemblies were screened for virulence and antibiotic resistance genes. Phylogenetic analyses were performed in the context of 965 publicly available *V. cholerae* genomes.

RESULTS: Out of 33 *V. cholerae* infections reported between January 2017 and January 2022 in Switzerland, 31 were caused by *ctxA*-negative isolates. These non-toxigenic isolates originated from gastrointestinal (n = 29) or extraintestinal (n = 2) sites. They were phylogenetically diverse and belonged to 29 distinct sequence types. Two isolates were allocated to the lineage L3b, a *ctxAB*-negative but *tcpA*-positive clade previously associated with regional outbreaks. The remaining 29 isolates were placed in lineage L4, which is associated with environmental strains. Genes or mutations associated with reduced susceptibility to the first-line antibiotics fluoroquinolones and tetracyclines were identified in 11 and 3 isolates, respectively. One isolate was predicted to be multidrug resistant.

CONCLUSIONS: *V. cholerae* infections in Switzerland are rare and predominantly caused by lowly virulent *ctxAB*-negative and *tcpA*-negative strains. As *V. cholerae* is not endemic in Switzerland, cases are assumed to be acquired predominantly during travel. This assumption was supported by the phylogenetic diversity of the analysed isolates.

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Introduction

Vibrio cholerae causes the severe diarrhoeal disease cholera and has been responsible for seven major pandemics in the past two centuries. Although improved sanitation and hygiene have reduced the threat of cholera [1], it is still endemic in some countries and causes 95,000 deaths per year globally [2]. The WHO has reported an increase in cholera cases since 2021, mainly in Africa and the Eastern Mediterranean [3].

In routine diagnostics, *V. cholerae* isolates are usually initially characterised by serotyping, with pandemic *V. cholerae* belonging to O-antigen types O1 or O139. For epidemiological surveillance, serotype O1 isolates are further differentiated into biotypes El Tor and classical. Whole-genome sequencing (WGS) enables more accurate tracking of cholera outbreaks and transmission routes [4, 5]. Based on their genetic phylogeny, *V. cholerae* strains were divided into 9 major lineages (L1–L9) [6, 7]. Lineage L1 is assumed to have caused the first six pandemics and comprises serotype O1 classical isolates. Lineage L2 is responsible for the ongoing 7th pandemic and comprises serotype O1 El Tor and O139 isolates. Lineages L3, L5, L6 and L8 (serotype O1 El Tor) cause sporadic cholera cases in confined geographical regions [6, 7]. The L4 and L7 lineages comprise environmental isolates that rarely cause human disease [6]. Lineage L9 was recently described as intermediate between L1 and L4 [7].

The key virulence factors of pandemic *V. cholerae* strains are the cholera toxin and the toxin-coregulated pilus (TCP). The cholera toxin is a heat-labile enterotoxin and triggers the characteristic rice water stool in infected patients [8]. It is encoded by the *ctxA* and *ctxB* genes, which are located on the mobile prophage CTX. TCP plays a critical role in the colonisation of the host intestine and additionally acts as a CTXφ phage receptor [9]. It is encoded by the *tcp* gene cluster located on Vibrio Pathogenicity Island I (VPI-1) [10]. *V. cholerae* isolates often contain additional genomic regions enriched with virulence genes such as Vibrio Pathogenicity Island II (VPI-2) and Vibrio Seventh Pandemic Island I and II (VSP-1 and VSP-2).

While cholera outbreaks are mainly the result of poor hygiene conditions involving transmission via the faecal-oral

route, *V. cholerae* is also a natural inhabitant of aquatic ecosystems. Although most environmental *V. cholerae* isolates lack the *ctxAB* genes, they can cause mild human infections upon exposure to water or the consumption of seafood [11, 12]. Among *ctxAB*-negative (non-toxigenic) *V. cholerae*, *tcpA*-positive strains are associated with an increased risk of human infection and have occasionally been linked to local regional outbreaks, including a recent seafood-borne diarrhoea outbreak in China [13–19].

Besides causing mild disease, non-toxigenic *V. cholerae* are a concern for public health as they may transfer antibiotic resistance to toxigenic strains [20] or acquire CTXφ and transform into highly virulent toxigenic strains [13, 21, 22]. To date, few studies have taken a phylogenomic approach to investigating non-toxigenic *V. cholerae*. Although *V. cholerae* is not endemic in Switzerland, human infections are reported each year [23]. In this study, we genetically characterised all non-toxigenic *V. cholerae* isolates from human patients in Switzerland received between January 2017 and January 2022 at the National Reference Laboratory for Enteropathogenic Bacteria and Listeria (NENT).

Materials and methods

Patients

Cholera is a notifiable disease in Switzerland and all clinical *V. cholerae* isolates from inpatients and outpatients must be sent to the National Reference Laboratory for Enteropathogenic Bacteria and Listeria. Metadata available for this cross-sectional study included the patients' age, sex and place of residence. There were no data on the patients' travel history or symptoms. Institutional review board approval or informed consent was not required as this analysis was conducted as part of the tasks and duties of the NENT. A study protocol was not registered or published.

Bacterial isolates

Pure cultures were obtained on thiosulphate citrate bile salts sucrose (TCBS) agar. Colonies growing in yellow, flat to slightly convex colonies with a diameter of 2–3 mm on TCBS agar were considered *V. cholerae* candidates. Using ISO17025-accredited methods, these were further tested for the *ctxA* gene by PCR and O-antigen-serotyped with antisera (Denka Seiken Co.) against O1 El Tor Inaba, O1 El Tor Ogawa and O139.

Isolates with negative agglutination test results were considered as non-O1 non-O139 *V. cholerae*. The *ctxA* PCR reaction was performed according to "CDC Chapter 7: Detection of Cholera Toxin" (pp 62–88) with CTX2 and CTX3 as primers. The temperature programme was adapted as follows: initial denaturation at 94 °C for 15 minutes, 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds and elongation at 72 °C for 30 seconds. The final elongation was done at 72 °C for 7 minutes.

Whole-genome sequencing and genomic analyses

Genomic DNA was extracted using the DNeasy blood and tissue kit (Qiagen). Sequencing libraries were prepared with the Nextera DNA flex library preparation kit (Illumina) and sequenced on the Illumina MiniSeq platform (2 × 150 bp). Paired-end Illumina reads were trimmed with fastp v0.22.0 [24] and assembled using SPAdes v3.14.1 [25] implemented in the pipeline shovill 1.0.9 (https://github.com/tseemann/shovill). For quality control, assemblies were passed to CheckM v1.2.2 [26] using the lineage_wf workflow. For comparison, we downloaded 965 publicly available assemblies of global *V. cholerae* isolates from the National Center for Biotechnology Information (NCBI) (table S1, available for download as a separate file at https://doi.org/10.57187/s.3437). The downloaded genomes reflect the collection described by Wang et al. in 2020 [7] except for three assemblies that were flagged as low quality by NCBI.

Core genome alignments were generated with parsnp 1.5.6 [27,28]. For analyses including global isolates the "xtrafast" option was used. The generated alignments were used for construction of phylogenetic trees using IQ-Tree v2.2.0.3 with the generalised time-reversible (GTR) model and gamma distribution with 1000 bootstraps [29]. Trees were visualised using iTOL V5 [30] and annotated using Inkscape 1.2 [31]. SNP distances were determined from the core genome alignment using snp-dists v0.8.2 (https://github.com/tseemann/snp-dists). Lineages were defined based on phylogenetic clustering with isolates of known lineage affiliations. Multi-locus sequence types (MLST) were determined using the PubMLST suite and novel alleles and profiles submitted [32].

In silico O-antigen serotypes were determined with VicPred [33] to complement the laboratory-based results. Assemblies were annotated using Prokka v1.13 [34]. Virulence genes were detected using ABRicate V1.0.1 (https://github.com/tseemann/abricate) in combination with VFDB set B [35] (minimum coverage 70%; identity 70%). Using VicPred [33], we examined for the presence of virulence-associated islands (VPI-1, VPI-2, VSP-1 and VSP-2). A virulence island was considered present when at least 80% of the genes were identified and partly present when 50– 80% of the genes were identified. Genes associated with antibiotic resistance were identified using AMRfinder 3.11.2 [36] and ABRicate in combination with the ResFinder database (minimum coverage 50%, identity 90%) [37]. The presence of two mutations associated with fluoroquinolone resistance (*gyrA* S83I and *parC* S85L)[38] was manually investigated using CLC Main Workbench 22.0.2. Unless stated otherwise, default parameters were used for all analyses.

Results

Non-toxigenic *V. cholerae* isolates from Switzerland belong to the L3b and L4 lineages

Between January 2017 and January 2022, a total of 33 *V. cholerae* isolates were received at the NENT, of which 31 tested PCR-negative for *ctxA*. The 31 non-toxigenic isolates originated from human faeces (n = 28), urine (n = 1; isolate N18-0491), blood (n = 1; isolate N22-0171) and an unknown clinical sample (n = 1). The annual number of reported infections varied from 6 to 19 between 2017 and 2019 but dropped to one infection in 2020 (table S1) when international travel was restricted due to the

COVID-19 pandemic. Although the patients' travel history was not available, these data suggest that most cases were travel-acquired. Agglutination tests identified one isolate (N18-1211) as O1 El Tor Inaba, two isolates (N18-1982 and N18-1603) as O1 El Tor Ogawa and 28 isolates as non-O1/non-O139.

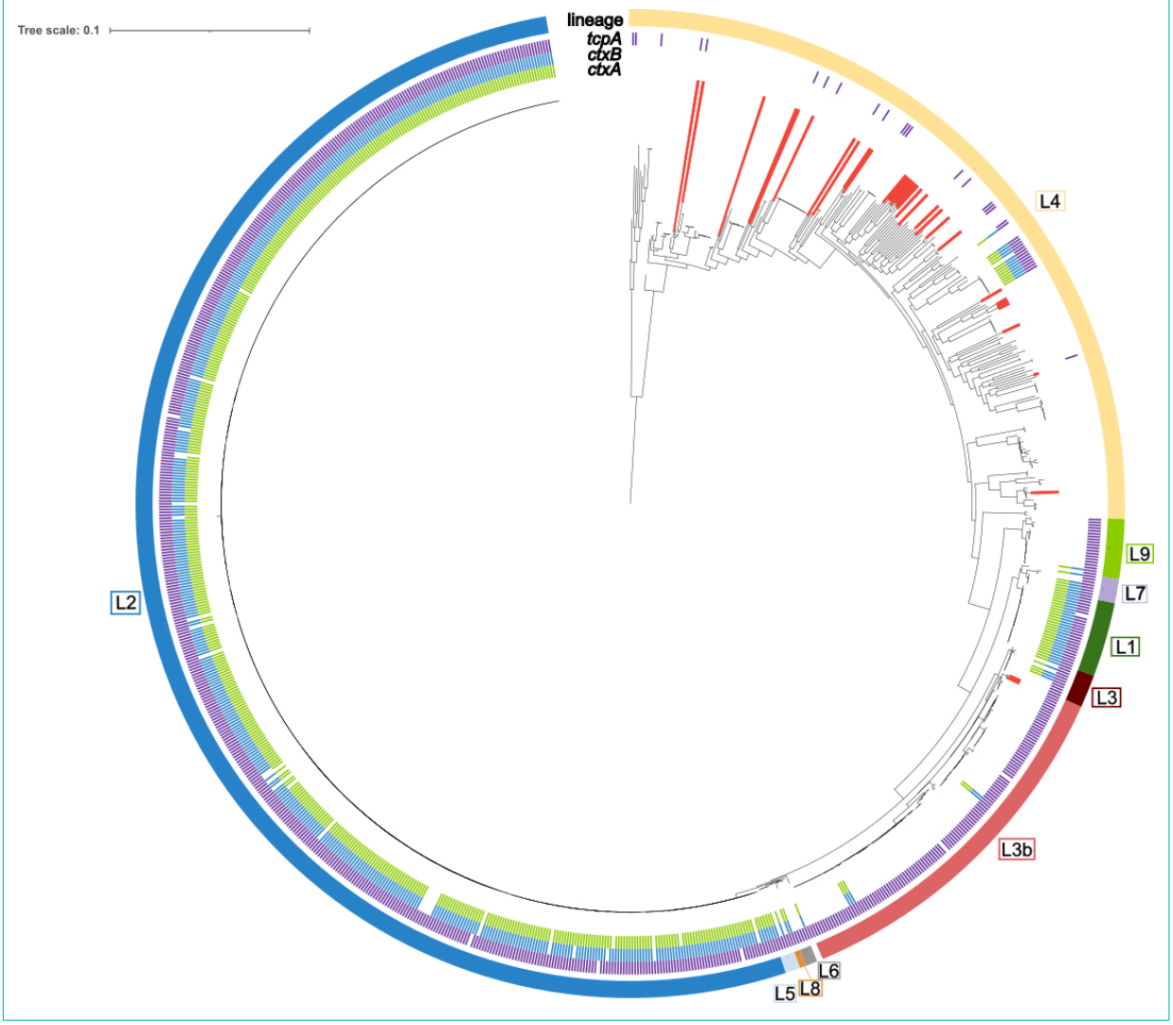
Whole-genome sequencing revealed substantial diversity, with all non-O1 isolates (n = 28) differing by at least 7215 pairwise SNPs in a core genome alignment. The two most closely related isolates (O1 isolates N18-1211 and N18-1982) differed by 1271 SNPs, suggesting that all isolates are epidemiologically unrelated. Multi-locus sequence typing assigned the isolates to 29 different sequence types (STs), of which 16 were novel. Only two STs occurred more than once: ST579 (comprising two O1 isolates) and ST1378 (comprising both O5 isolates) (table S1).

To determine lineage affiliations, a phylogenetic analysis was performed in the context of 965 additional *V. cholerae* isolates from global collections (figure 1). Of the 31 Swiss isolates, all non-O1 isolates (n = 28) and one O1 El Tor isolate belonged to lineage L4, a heterogeneous lineage lacking *ctxAB* and *tcpA* and associated with environmental and lowly virulent clinical isolates [6]. The remaining two isolates (N18-1211 and N18-1982, both O1 ST579) grouped in the L3b lineage. L3b is a subclade of L3 recently associated with a diarrhoeal epidemic of non-toxigenic strains in China [7] and otherwise comprised isolates from Asia or Latin America. The closest phylogenetic neighbours of N18-1211 and N18-1982 were collected in Russia and Turkmenistan (see figure S1 in the appendix). In silico serotyping with VicPred confirmed the O1 El Tor type of three isolates (figure 2). The most frequent predicted O-antigen type was O8 (n = 6). Other predicted types included O3, O4, O5, O7, O14, O37 and O49.

No indications of a recent CTX prophage loss

All global and Swiss *V. cholerae* genomes were screened for the presence of virulence genes. Importantly, all Swiss isolates belonged to branches consisting predominantly or exclusively of *ctxAB*-negative isolates (figure 1), suggesting that the absence of *ctxAB* was unlikely to be due to a potential (partial) loss of the CTX prophage during patient colonisation or subculturing in the laboratory. The *tcpA* gene (encoding a toxin-coregulated pilus subunit) was identified in three isolates: in both L3b isolates (N18-1211 and N18-1982) and one L4 isolate (N19-2973). Further, the two L3b isolates were the only isolates carrying the

Figure 1: Maximum-likelihood phylogenetic tree of 31 Swiss and 965 global *V. cholerae* isolates. Swiss isolates are indicated by red lines. The presence of *ctxA* (green), *ctxB* (blue) and *tcpA* (purple) is labelled (inner ring). The outer ring denotes lineage affiliations (L1–L9). The tree is based on 32,851 polymorphic sites identified in a multi-alignment-derived 0.45 Mbp core genome. The scale bar indicates the number of substitutions per site in the core genome alignment. The tree was visualised using iTOL.



CTX prophage-associated genes *zot* (an enterotoxin affecting intestinal tight junctions [39]) and *ace* (an enterotoxin causing fluid secretion in rabbit ileal loops [40]) (figure 2), suggesting the presence of a putative CTXφ precursor, as previously reported for L3b isolates [7]. The haemolysin gene *hlyA* was identified in all 31 isolates. The *rtxA* (cytotoxin) and *nanH* (neuraminidase) genes were found in 25 and 17 of the Swiss isolates, respectively. The *toxA* gene, which has been associated with environmental strains [41], was identified in five L4 isolates. The two L3b isolates contained the complete VPI-1 island (comprising *tcpA*). Other virulence pathogenicity islands or fragments thereof were identified in five L4 isolates, including the bloodstream isolate N22-0171, which carried fragments of VSP-2.

Several isolates contain genes associated with tetracycline and quinolone resistance

All Swiss isolates were screened for genes and mutations associated with decreased antibiotic susceptibility with a focus on the three first-line antibiotics doxycycline (a tetracycline), ciprofloxacin (a quinolone) and azithromycin (a macrolide). The two screening approaches used (AMRfinder and ResFinder) yielded identical results, except for one resistance gene (*varG*) that was not included in the ResFinder database. Three isolates harboured *tet* genes indicating potential tetracycline resistance (figure 2). Further, six isolates contained *qnrVC4* or *qnrVC5*, which are associated with ciprofloxacin resistance when occurring in combination with target mutations in *gyrA* and *parC* [38], as observed in one of the six isolates (N17-0919). An additional five isolates had mutations in quinolone-resistance determining regions of *parC* or *gyrA* but did not carry *qnrVC* genes. Isolate N19-1763 was identified as a potential multidrug-resistant strain, harbouring genes or mutations associated with resistance against aminoglycosides, beta-lactams, macrolides, phenicols, quinolones, sulphonamides, tetracycline and trimethoprim.

Discussion

Although saline aquatic environments – the natural habitat of *V. cholerae* – are absent in Switzerland, up to 20 infections caused by mostly *ctxA*-negative strains are reported annually. Because infections with non-toxigenic strains are usually mild and patients may not seek healthcare, the actual number of infections is likely higher. We assume that most cases are associated with travel abroad. This is supported by our investigation of 31 isolates, which demonstrated high genetic diversity suggesting distinct origins. In addition, the number of reported infections dropped to only one infection in 2020 when international travel was restricted. Notably, although *V. cholerae* occurs in the North Sea, a study of 836 *V. cholerae* infections in the UK could link >99% of the (mostly non-toxigenic) cases with available metadata to travel abroad [42, 43]. However contaminated imported seafood cannot be excluded as a potential infection source. Prevalence studies in Switzerland and neighbouring countries found non-toxigenic *V. cholerae* in 0.6% to 6.3% of the examined seafood products [44–47].

In line with other studies on non-toxigenic *V. cholerae* in Europe [48, 49], our isolates were phylogenetically diverse. Half of the isolates belonged to previously unknown

Figure 2: Maximum-likelihood phylogenetic tree and genetic characteristics of 31 Swiss non-toxigenic *V. cholerae* isolates. Lineage affiliations are indicated by the yellow (L4) or red (L3b) background. In silico-predicted O-antigen serotypes and the presence of virulence islands (filled square: complete presence; half-filled: partial presence [>50% of the genes]), virulence genes and antimicrobial resistance determinants are shown. The tree is based on 177,987 polymorphic sites identified in a multi-alignment-derived 2.8 Mbp core genome and was visualised using iTOL. The scale bar indicates the number of substitutions per site in the core genome alignment. AGly: aminoglycosides; Bla: beta-lactams; Flq: fluoroquinolones; MLS: macrolide-lincosamide-streptogramin; O1-EO: O1 EL Tor Ogawa; O1-EI: O1 El Tor Inaba; Phe: phenicols; PM: polymyxines; Sul: sulphonamides; Tet: tetracyclines; Tmt: trimethoprim.

sequence types, suggesting that the diversity of non-toxigenic strainsis largely unexplored. Further, these results indicate diverse infection sources. This contrasts with a recent genomic analysis of non-toxigenic *V. cholerae* from patients in China, which found that most of the 104 investigated *ctxAB*-negative isolates belonged to few phylogenetic SNP clusters and were epidemiologically linked [7, 13]. The source of this outbreak could be traced to aquatic food products. Whereas most of these outbreak-associated isolates were *tcpA*-positive and belonged to lineages L3b and L9, most Swiss isolates investigated here belonged to the environment-associated lineage L4. Among the Swiss isolates, the *tcpA* gene was only detected in one L4 and two L3b isolates, which did not belong to the Chinese outbreak clusters.

In recent decades, drug-resistant *V. cholerae* have emerged,with resistance patterns fluctuating with changing epidemiology and antibiotic use [20, 50–54]. Although antibiotics are not indicated for mild cholera infections, antimicrobial resistance genes have also been acquired by non-toxigenic strains [7, 55–57], possibly driven by inadequate treatment of patients or the increasing use of antibiotics in aquaculture [58, 59]. In our study, 10% of the isolates carried determinants associated with reduced susceptibility to both tetracycline and fluoroquinolone, two important first-line antibiotics. Information on the patients' treatment upon disease diagnosis was unavailable, nor was their travel history, limiting the interpretation of our findings.

In conclusion, our study provides insights into the prevalence and characteristics of non-toxigenic *V. cholerae* in Switzerland. Rising sea temperatures, intensified aquaculture production and global trade may lead to an increasing prevalence of *V. cholerae* infections in the future [60–62]. Continuous monitoring of the pathogen and antimicrobial resistance rates is important for informing public health management.

Data availability

Assemblies and read data were deposited in the NCBI repository under BioProject accession number PRJNA997795. Individual accession numbers are listed in table S1 (available for download as a separate file at https://doi.org/10.57187/s.3437).

Acknowledgments
We thank Andrea Diethelm from the National Reference Laboratory for Enteropathogenic Bacteria and Listeria (NENT) for her assistance in sample processing.

Financial disclosure
This work was supported by the Swiss Federal Office of Public Health, Division Communicable Diseases.

Potential competing interests
All authors have completed and submitted the International Committee of Medical Journal Editors form for disclosure of potential conflicts of interest. No potential conflict of interest related to the content of this manuscript was disclosed.

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Appendix

Figure S1: Maximum-likelihood phylogenetic tree of 122 L3b isolates. The country of isolation is indicated (outer ring). The tree is based on 39,702 polymorphic sites identified in a multi-alignment-derived 3.1 Mbp core genome and was visualised using iTOL. The scale bar indicates the number of substitutions per site in the core genome alignment.