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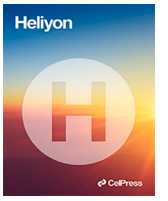

Research article

Characterising virulence in a nontoxigenic non-O1/non-O139 *Vibrio cholerae* isolate imported from Vietnam

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ABSTRACT

Vibrio cholerae is a major human pathogen that can cause life-threatening acute diarrhea. *V. cholerae* are classified according to O-antigen polysaccharide outer membrane properties, where the serotypes O1 and O139 are strains that cause pandemics and epidemics while non-O1/non-O139 usually cause mild disease. The dynamic evolution of *V. cholerae* involves acquisition of new virulence factors through horizontal gene transfer and formerly nontoxigenic serogroups are increasingly being reported to cause severe forms of human disease.

In this study we have serotyped one isolate (ST588-CPH) of imported *V. cholerae* from Vietnam to Denmark and performed whole genome sequencing to identify known virulence genes and furthermore studied the pattern of virulence in closely related pathogenic strains of *V. cholerae*.

ST558-CPH was found to be a non-O1/non-O139 strain. Initial analysis from the whole genome sequencing gave a 96,6 % match to the O139-specific *wbfZ* gene, but in a second analysis with a higher identification threshold, the *wbfZ* gene was absent. We suggest a "de novo" display of a database misannotation, which explains the conflicting results. The MLST analysis revealed that the isolate belongs to the nontoxigenic non-O1/non-O139 sequence type ST558. ST558 has recently been reported as a sequence type forming a cluster of ST's that should be monitored, as it has shown to have virulence causing moderate to severe illness. Our analysis of virulence genes identified *MakA*, a recently discovered toxin, which seems to be generally present in both toxigenic and nontoxigenic strains.

1. Introduction

Vibrio cholerae belongs to the family *Vibrionaceae* which naturally inhabitate within the marine and riverine microbiota [1–3]. It is a motile Gram-negative rod first isolated by Robert Koch in 1883 [4]. Classifying *V. cholerae* serogroups from outer membrane O-antigen polysaccharide composition was introduced in the 1930's and currently there are 206 known serogroups [5–7]. There are two O1 biotypes; "classical" or El Tor and three distinct serotypes named Ogawa, Inaba and Hikojima. Since the evolutional change of O1 El Tor in 1992 into the new *V. cholerae* serogroup O139 Bengal, the cholera epidemic has persisted and spread in Asia. The O139 Bengal had acquired new genetic properties by a deletion of the *wbe* region of O1 and an insertion of the *wbf* region encoding the O139 O-antigen, involving homologous recombination or a suggested horizontal gene transfer from a non-O1/non-O139 strain (O22) [8–12]. Still globally referred to as the 7th pandemic of cholera, starting in Indonesia in 1961, the two strains O1/O139 are

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continuously responsible for the ongoing cholera epidemic in Asia [11,13]. The pathophysiology of cholera watery diarrhea is explained by an altered permeability of the small intestine epithelial cells, caused by the cholera toxin, which together with the colonising factor toxin-coregulated pilus (Tcp) represents the two major components of *V. cholerae* virulence [14,15]. The exotoxin induces osmotic loss of fluid and electrolytes resulting in rapidly progressing dehydration.

Most *V. cholerae* serotypes are nonpathogenic but the species is characterised by an extreme genomic plasticity which allows evolution of heterogenous strains, with clonal intensity shifting with environmental salinity, temperature and suggested seasonal algal blooms [16]. Non-O1/non-O139 strains do not normally possess the virulence factors which block host inflammation and consequently, when they cause human disease, occurrence of invasive bacteremia is a more frequent finding. The common clinical presentations of non-O1/non-O139 infections include gastroenteritis, wound infections, external otitis and bacteraemia [17]. The non-O1/non-O139 are traditionally considered as nontoxigenic but have increasingly been reported to cause both sporadic cases of human disease and smaller outbreaks of cholera [18,19]. Recent studies of the evolutionary development show that horizontal gene transfer of virulence factors, not only within the *Vibrionaceae* family, but also from neighboring species; *Pseudomonas aeruginosa*, *Haemophilus somnus*, *Vibrio vulnificus* and *Haemophilus influenzae* contributes to the genetic diversity in non-O1/non-O139 *Vibrio cholerae* [8]. A major concern regarding non-O1/non-O139 strains is the development of antimicrobial resistance, since antibiotics is critical in treating invasive disease, in contrast to treatment in cholera epidemics where fluid and electrolyte resuscitation is of major importance [20,21]. In this study we aim to describe the genetic and virulence properties of non-O1/non-O139 *V. cholerae* and present the findings of a clinical isolate from a MLST cluster which seem to be causing more serious human disease than other non-O1/non-O139 *V. cholerae* strains.

1.1. Case presentation

We report on a previously healthy person in the mid-60's who developed severe gastroenteritis upon returning to Denmark from Vietnam after a three-week vacation.

All accommodations were of high standard and he reported intake of solely bottled water, but food taken from street kitchens. He had no history of soft tissue wounds and no contact with animals. Prior to the departure to Vietnam the patient had a Di-Te booster, HAV and HBV vaccinations. The patient had a history of hypertension. During the return flight to Denmark the patient developed watery diarrhea and over the following six days he had bowel movements up to 30 times daily. Even though the patient tried to compensate for the loss of fluid, he was feeling increasingly unwell, had abdominal pains and on the 4th day after arrival to Denmark contacted his general practitioner (GP). The GP sent stool cultures for analysis which grew *V. cholerae* and the patient was referred to the Department of Pulmonary and Infectious Diseases at the local teaching hospital. The patient had a normal physical examination. Blood tests showed slightly low potassium (3, 3 mol/L; reference range 3, 5–4, 4 mmol/L) and signs of dehydration, with creatinine 137 μmol/L (reference range, male over 18 years; 60–105 μmol/L) and urea 8, 7 μmol/L (reference range, male over 50 years; 3, 5–8, 1 mmol/L). A stool sample was furthermore analysed using a Point-of-Care Test (FilmArray® Gastrointestinal panel, Biomérieux) with identification of *V. cholerae* as well as enteroaggregative *E. coli* (EAEC). The same sample sent for routine stool culture at the local Microbiology Department grew only *V. cholerae* (Selenite, SOS medium plate and Cholera plate with TCBS; Thiosulfate Citrate Bile Salts Sucrose). Serotyping at the national reference laboratory at Statens Serum Institut (SSI) demonstrated a non-O1/non-O139 serogroup. Prior to the results of the stool samples the patient was prescribed oral metronidazole by the GP, which at the hospital was changed to a single dose of 1 g of azithromycin. The patient also received 2 L of iv Sodium-Potassium-Glucose fluid, after which he quickly recovered from his diarrhea. He was discharged 16 h after arrival to the hospital with Potassium-Chloride tablet treatment. Given the rare finding of an imported case of *V. cholerae* from Vietnam to Denmark we decided to analyse the isolate further.

2. Materials and methods

2.1. Epidemiological data

Denmark has a centralised mandatory clinical and microbiological reporting system for a list of pathogens and infections including *V. cholerae*. Data on all registered cases of *V. cholerae* including serotype for the years 2004–2019 was collected from SSI. In the period 2004–2012 isolates were only tested for subtype O1, whereas from 2013 isolates are tested both for subtype O1 and O139.

2.2. Detection and serotyping of V. cholerae isolate

The reported *V. cholerae* isolate was first detected at the North Zealand hospital using POCT procedures with BD FecalSwab TM (Copan Italia SpA) and Film Array GI Panel (BioFire Diagnostics, Biomeriux, Salt Lake City, Utah). The stool sample was routinely sent to the microbiological laboratory at Herlev University Hospital for cultivation with growth of *V. cholerae*. The pure culture was forwarded to SSI for serotyping for national surveillance. Serotyping was performed using O1 and O139 *V. cholerae* specific antisera (Denka Seiken, Tokyo, Japan).

2.3. Whole genome sequencing and bioinformatic analyses

The isolate was subjected to whole genome sequencing (WGS) using Illumina NextSeq 500. The genome was assembled using SKESA v. 2.2, this produced an assembly of 3,956,791 bp with a GC of 47,6 % consisting of 123 contigs and an N_{50} of 122,972 bp [22].

The genome was analysed for the presence of virulence genes using the CholeraeFinder webtool located at the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/CholeraeFinder/), with minimum 95 % identity and 60 % gene length. Multi-locus sequence typing (MLST) was performed using the webtool MLST 2.0 (https://cge.cbs.dtu.dk/services/MLST/). Raw reads were uploaded to European Bioinformatics Institute (EBI) under Biosample SAMEA7534127. Further gene sequence analyses on *V. cholerae* key markers were performed at the Department of Veterinary and Animal Sciences at University of Copenhagen with raw reads assembly with Unicycler and BLAST/MyDBFinder.

All available CC558 genome sequences (n = 28) and three O1/O139 genomes were downloaded from PubMLST with associated metadata (serogroup, biotype, sequence type, country and continent) [23]. The presence of genetic islands, virulence and resistance genes were identified using the CholeraeFinder webtool. All sequences were then annotated using Prokka and a core genome alignment produced using Roary [24,25]. A core genome phylogeny was then inferred using FastTree with a GTR model [26]. The phylogeny with strain-associated metadata was visualized using iTOL [27]. A Minimum Spanning Tree was produced using goeBURST with the 43 available CC558 isolates in the PubMLST database.

The *wbfZ* reference sequence (AB012956) from the CholeraeFinder webtool database was compared to the O-antigen biosynthetic loci of 187 non-O1/O139 *V. cholerae* isolates using nBLAST comparisons and MAUVE alignment [28,29].

3. Results

In Denmark, only 25 cases of *V. cholerae* have been registered from 2004 to 2019. Serotyping of all isolates found two serogroup O1 and 23 cases of non-O1/non-O139.

From initial serotyping our isolate was classified as *V. cholerae* non-O1/non-O139. The sequence data revealed that the isolate was sequence type 558 and had a match to the O139 *wbfZ* gene variant with 96, 6 % ID. The isolate (named ST558-CPH) possessed the virulence genes *makA*, *hlyA*, *toxR*, *rtxA* and *als*. Furthermore, markers from the genetic islands Vibrio Seventh Pandemic island 1 and 2 (VSP-1 and VSP-2) and Vibrio Pathogenicity Island 2 (VPI-2) were detected (Table 1). No acquired resistance genes were present in the strain, while a *ParC* gene variant encoding a mutated topoisomerase IV subunit A (resistance to quinolone) was found.

In the second analysis the genome was further analysed for *V. cholerae* key markers including genes *ompW* (100 % match), *ctxA* (not found), *ctxB* (not found), *gyrA* (99 % match), *gyrB* (97 % match), *parC* (98 % match), *parE* (99 % match), *rfbO139* (not found), *rfbO1* (not found), *tcpA* (not found), *wbeT* (not found) (Table 2). With a threshold of 98 % ID and 60 % coverage the second analysis (BLAST/MyDBFinder) the *wbfZ* gene was not identified.

We present a core genome phylogeny of 28 *V. cholerae* isolates from CC558 (Fig. 1), of which 11 were ST558, illustrating the global dissemination of CC558. The phylogeny shows that the strain we describe clusters most closely with other ST558 strains, originating mainly from Asia (Fig. 1). Furthermore, the ST558-CPH strain is similar to other ST558 strains regarding presence of pathogenicity islands, resistance and virulence genes (Fig. 2). In the two phylogenies we have included three isolates from O1/O139 strains to illustrate the difference in present virulence genes from the CC558 and also the homogenous virulence within this group.

4. Discussion

Our initial serotyping into a non-O1/non-O139 serogroup was questioned when the whole genome sequencing came out with a 96, 6 % match to the serogroup O139 gene *wbfZ,* but then again supported in our second analysis with a higher 98 % ID threshold and 60 % coverage, where the *wbfZ* was absent. Upon more detailed investigation, we found an almost universal match of the *wbfZ* reference sequence (AB012956) to the gene annotated as *rjg* when analysing the O-antigen biosynthetic loci of 187 non-O1/O139 *V. cholerae* isolates. Possibly, the *rjg* gene has been misannotated as *wbfZ* in the *V. cholerae* software tool database (CholeraeFinder), thus giving a false match. This is a “de novo” display which we wish to highlight. Recent advances in *V. cholerae* genetics indicate that the *wbfZ* is shown to be unspecific to O139 as it is also partially present in other serogroups, therefore it is suggested to rely on the more specific rfbO139, which was absent in our analysis [30].

There are previously reported rough variants of the O1/O139 serogroups which present as O1/O139 in PCR analysis but lack the O1/O139 surface antigen and thus fail to agglutinate O1/O139 antisera; our attempts to find a commercial “rough antiserum” for O139

Table 1
Results from whole genome sequencing on Illumina NextSeq 500.

WGS gene	Match
wbfZ	99,6 %
makA	98,5 %
hlyA	98,6 %
toxR	98,6 %
rtxA	98 %
Als	99 %
VSP-1	86,9 %
VSP-2	100 %
VPI-2	99,6 %
ParC	100 %

Table 2
Results from Unicycler and BLAST/MyDBFinder

Vibrio key markers	Match
ompW	100 %
ctxA	Negative
ctxB	Negative
gyrA	99 %
gyrB	97 %
parC	98 %
parE	99 %
rfbO139	Negative
tcpA	Negative
wbeT	Negative

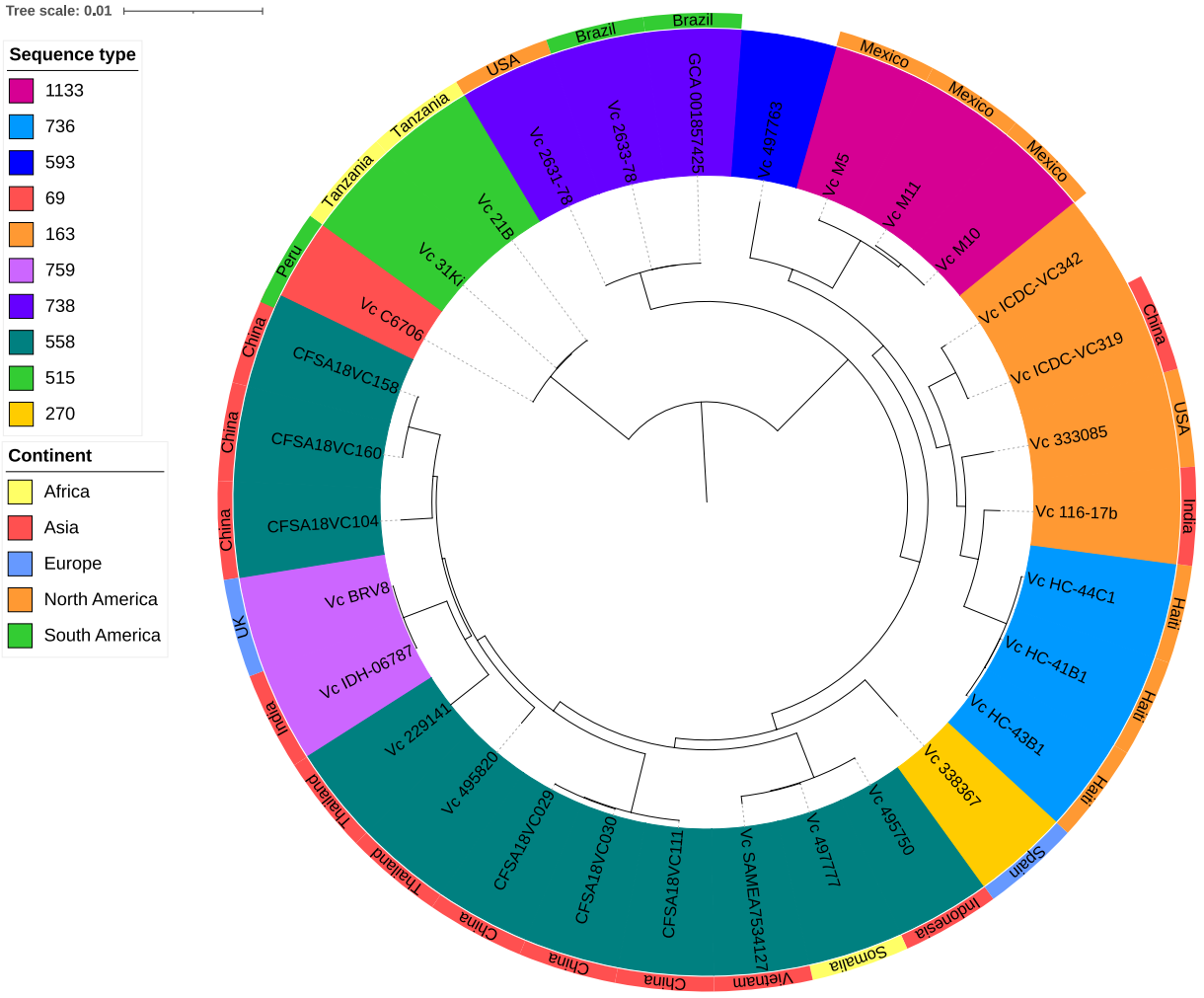

Fig. 1. Core genome phylogeny of CC558 with comparison of geographical dissemination. Three O1/O139 strains and 28 non-O1/non-O139 CC558 isolates were included.

serogroup failed [31]. The rough variants are hypothesised to represent strains which are more prone to genetic change as they are commonly seen on epidemiological and surveillance data during periods preceding a change in serogroup, from Inaba to Ogawa or vice versa [32]. However, there is also reported misidentification due to cross-agglutination with commercial O139 antisera due to related O antigens in O139, O155 and O22 serogroups [33]. The absence of the major virulence factors CTX and toxin-coregulated pilus (Tcp) is previously presented as unconventional O139 nontoxigenic strains, reported in two publications from 2019; a Shanghai study of clinical cases from the 7th pandemic and in a genetic analysis of four O139 strains from Bangladesh [34,35].

The ST558-CPH possessed a complete Vibrio Seventh Pandemic island 2 (VSP-2) and 86, 9 % of the Vibrio Seventh Pandemic island

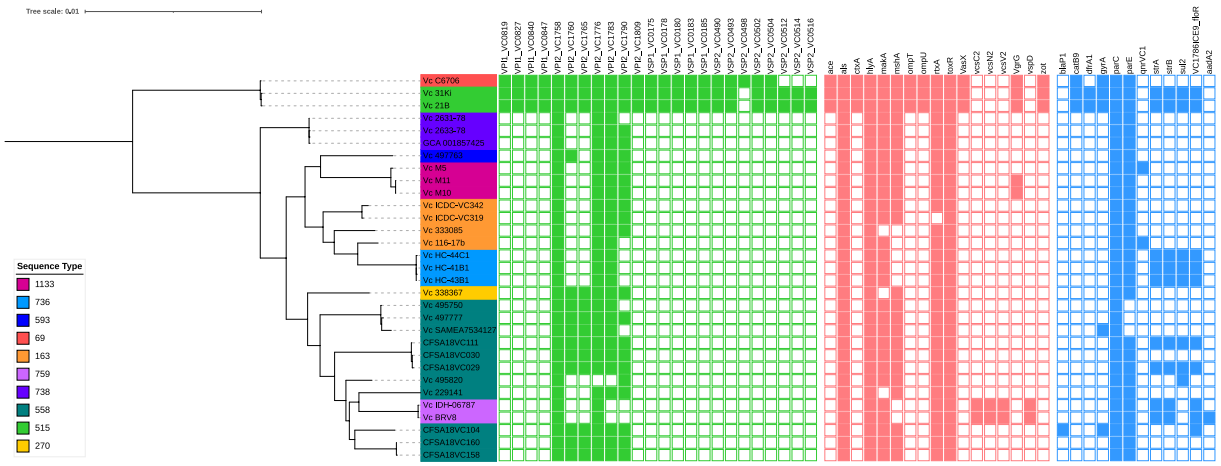

Fig. 2. Core genome phylogeny of CC558 with comparison of genetic islands, virulence and resistance genes. Three O1/O139 strains and 28 non-O1/non-O139 CC558 isolates were included.

1 (VSP-1). VSP-1 and VSP-2 are gene clusters identified in the 7th pandemic El Tor strains which are absent in classical and pre-7th pandemic strains [36,37]. VSP-1 and VSP-2 are occasionally seen in non-O1/non-O139 strains but rarely together [19,38]. The role of VSP islands remains unclear, but they are suggested to provide increased environmental fitness to those strains carrying the genes [38]. VSP-2-like elements which have similar ORFs (Open Reading Frames) in non-cholera *Vibrio* spp. indicate that they have a function in the natural environment [39]. Recent genetic analysis has revealed VSP-2 subgroups with various geographical origins, making it a marker for genetic lineages of global cholera transmission [40]. The common finding of incomplete VSP islands in environmental strains may represent either precursors that are intermediates in the process of clustering into functional VSP islands or the result from gene deletion events [41].

The virulence factors hlyA, rtxA and toxR detected in the analysed isolate are commonly found in both O1/O139 and non-O1/non-O139 strains [19,35]. The absence of CTX and Tcp in the analysed isolate indicates the known potency of other virulence factors, some which are recently described and their mechanism not fully understood. The Multifunctional Autoprocessing Repeats-in-Toxins (MARTX/RtxA), also detected in this isolate, is considered to contribute significantly to the pathogenesis and cytotoxicity of *V. cholerae* and is present in nearly all strains [42]. The analysed isolate also presents with the gene encoding the novel toxin MakA (motility associated killing factor A) and when compared to other strains it seems to be a generally present virulence factor with matches in both the three O1/O139 and 25 out of 28 CC558 isolates (Fig. 1). MakA is a flagella-mediated toxin secreted via the flagellar type III secretion system (fT3SS). It was first described in 2018 as pathogenic in *Caenorhabditis elegans* and zebrafish, and later reported in reservoirs of *V. cholerae* O1 strains in Tanzania. It has also been reported from epidemics occurring in 2015–2017 and found in sequenced strains from O1 cholera outbreaks in 2014-2016 in Uganda [43–46].

While the *V. cholerae* O139 strains normally belong to the ST69 group, the ST558-CPH analysed belonged to ST558 [47]. This sequence type belongs to clonal complex (CC) 558, which is a rare non-endemic complex with global dissemination; a recent study of 152 isolates submitted to the Gastrointestinal Bacteria Reference Unit (GBRU) in England from 2015 to 2018 identified 18 isolates

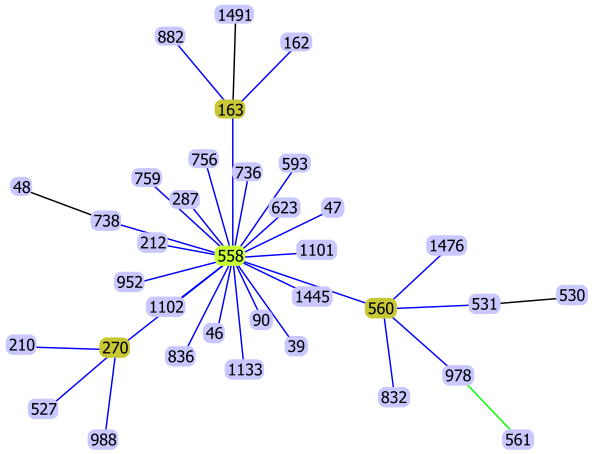

Fig. 3. Minimum spanning tree of *V. cholerae* CC558 (from Flach EJ et al., 2020; reference 49).

belonging to CC558, with reported travel to the Caribbean, South East Asia, Africa, Europe and Central America [48]. In a study from ZSL London Zoo, 239 isolates received at Public Health England from 2015 to 2018 were sequence typed showing that CC69, CC558 and CC616 were the main groups associated with human disease [49]. A publication from China furthermore describes two cases of septic shock caused by non-O1/non-O139 *V. cholerae* strains [50]; the two cases were from the inland Guizhou Province and one of the isolates belonged to the not previously described ST985 while the other was a ST558. The authors performed goeBURST analysis, describing a large CC558 consisting of 28 different STs, of which most originate in China.

We present a *V. cholerae* isolate lacking the *rfbO139* gene, which in addition to serotyping results and with the combination of virulence genes, supports the finding of a nontoxigenic non-O1/non-O139 strain rather than a rough O139 or O22 serogroup strain. Our initial results were based on an annotation error in the CholeraeFinder webtool and our report represents a "de novo" display of this probable misannotation. The ST558-CPH belongs to the CC558, a globally disseminated non-endemic cluster with potential for severe disease in humans, we support the opinion of other researchers that CC558 should be monitored. The CC558 now includes 43 different STs, as shown in the Minimal Spanning Tree (Fig. 3).

Due to the limited number of publications on aquatic and reported clinical cases of *V. cholerae* in Vietnam we have not been able to perform a comprehensive critical review on the genetic properties of the ST558-CPH in comparison with previously reported cases from the same region [51]. The importance of intensified reporting from Asia is reflected in findings that molecular strain changes during the 7th pandemic seem to evolve in South East Asian regions and current reporting from the region are scarce and delayed [52–55].

The limitations of this observational and descriptive study include the inclusion of a single isolate, which was therefore compared to a collection of available genomes of the same sequence type. Furthermore, we were unable to perform a confirmatory PCR investigation to verify the expected absence of *rfbO139* due to an applied GDPR legislation decision at one of our collaborators' departments (SSI, QA Compliance).

Ethical approval statement

Review and/or approval by an ethics committee was not needed for this study because the study did not involve animal or human experiments and did not address other issues in need of ethical consideration. According to Danish law and regulations including the Ethics Committee system and Danish Data Protection Authorities anonymised case reports should not be reported or approved.

Consent statement

The patient in this case history has given a written consent to anonymised scientific publication.

Consent to publish

NA.

Funding

NA.

Data availability statement

Data availability: Yes
The datasets used and/or analysed during the current study is available at:
ENA database (ebi.ac.uk)
Type: Accession
Sample: ERS5290590
(SAMEA7534127)
Experiment: ERX4671768
Run: ERR4802243

CRediT authorship contribution statement

Pontus Westerström: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Christina Gabrielsen Ås:** Writing – review & editing, Software, Resources, Investigation, Formal analysis, Data curation. **Ulrik Bak Dragsted:** Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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