A Rough Variant of Nontoxigenic *Vibrio Cholerae* O139 Imported From Vietnam

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Research Article

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Abstract

Purpose (stating the main purposes and research question)

Vibrio cholerae is classified in O-antigen polysaccharide outer membrane properties where O1 and O139 are strains that cause pandemics and epidemics while non-O1/non-O139 usually cause mild disease. The dynamic evolution of *Vibrio cholerae* involves new virulence factors through horizontal gene transfer and formerly nontoxigenic serogroups are being reported as causing increasingly severe forms of human disease. Our purpose was to investigate serogroup and virulence factors in one imported isolate from Vietnam and compare these to virulence factors seen in different strains of *Vibrio cholerae*.

Methods

We have serotyped one isolate of imported *Vibrio cholerae* from Vietnam to Denmark and performed whole genome sequencing to identify known virulence genes.

Results

We have identified virulence factors in our isolate that are recently discovered and one toxin, MakA, has not previously been reported from an Asian strain. The isolate was found to be a serogroup non-O1/non-O139 strain, however, sequence analysis gave a 96, 6% ID match of the *wbfZ* gene, stipulating our isolate to belong to either serogroup O22 or O139.

Conclusions

Looking at the combination of virulence factors of the isolate we suggest the finding of a rough variant of a nontoxigenic *Vibrio cholerae* O139 rather than a O22 serogroup version. This is a rare finding in a clinical isolate.

Introduction

Vibrio cholerae belongs to the family Vibrionaceae which naturally inhabits the marine and riverine microbiota (1–3). It is a motile gram-negative rod which was first described and named by Italian anatomist Filippo Pacini in 1854 (4), although the London-based physician John Snow, in a pamphlet published in 1849, preceded Pacini in arguing the theory of fecal contamination of water (5). Later, in 1883, Robert Koch isolated and detected the "Kommabacillus" using microscopy during an epidemic of cholera in Egypt, thus presenting evidence of the microbiological pathophysiology. The *Vibrio cholerae* serogroups were classified during the 1930's from outer membrane O-antigen polysaccharide composition and currently there are 206 known serogroups (6–9). There are two O1 biotypes; classical" or El Tor and three distinct serotypes named Ogawa, Inaba and Hikojima. The El Tor has properties in producing hemolysins and is the major cause of the current 7th cholera pandemic which emerged in Indonesia in 1961. The name El Tor derives from a quarantine camp in the Sinai desert, along the pilgrimage pathway from Mecca; the camp was initiated after the 19th century pandemics of cholera and

this is where the causative O1 hemolytic strain El Tor was first publicly reported on in 1906, although previously observed in 1897 in the Middle East (10–14).

The virulence of *Vibrio cholerae* has been extensively investigated, and dates back as far as 1884 when Robert Koch first suggested evidence of a cholera toxin, but the toxin was not proven until 1959 (15–17). The pathophysiology in cholera watery diarrhea is explained by an altered permeability of 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 *Vibrio cholerae* virulence (18–19). The exotoxin induces osmotic loss of fluid and electrolytes resulting in a rapidly progressing dehydration. The serogroups of *Vibrio cholerae* have traditionally been divided into toxigenic (O1 serogroup) and nontoxigenic (non-O1 serogroup), but in late 1992 a formerly unknown serogroup was identified during a cholera-like diarrhea outbreak in Bangladesh and India (20–21). This new serogroup, named O139 Bengal, was a potent cause of human disease and presented with different genetic composition and phenotype features than O1 El Tor. The O139 Bengal was the result of a mutation, substituting the 22kb rfb region in O1 with a 35kb region of DNA encoding the surface polysaccharide.(22). An altered variant named O139 Calcutta was later reported from an outbreak in 1996 (25–26). To this date the classification of serogroups O1/O139 and non-O1/non-O139 has stayed unchanged in the continuous 7th cholera pandemic. The evolutionary pathway of the 7th pandemic originates in the Bay of Bengal, with an estuarine aquatic environment which has made possible for *Vibrio cholerae* to genetically evolve into novel subtypes potentially harmful to humans (27). One hypothesis is that the *Vibrio cholerae* progenitor has acquired new characteristics by horizontal gene transfer, a way for genetic material to be passed "sideways", also to relatively unrelated species rather than to a descendant, having introduced several genomic islands (ie VPI-1, VPI-2 and GI-1 to GI-10, TLC) to become O1 and then additionally CTX and RS1 to further evolve into O1 El Tor and O139 strains. (23, 27) (Figure 1).

Most *Vibrio cholerae* serotypes are nonpathogenic but present with an extreme genomic plasticity which allows evolution of persistent heterogenous strains with a clonal intensity shifting with environmental salinity, temperature and suggested seasonal algal blooms (28). Non-O1/non-O139 strains do not normally possess the virulence factor(s) blocking host inflammation and consequently, when they cause human disease, occurrence of invasive bacteraemia is a more frequent finding. The common clinical findings in non-O1/non-O139 infections include gastroenteritis, wound infections, external otitis and bacteraemia (29). The non-O1/non-O139 were traditionally considered as nontoxigenic but have reportedly become commonly causative to both sporadic cases of human disease and smaller outbreaks of cholera (30–32). 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 such as *Pseudomonas aeruginosa*, *Haemophilus somnus*, *Vibrio vulnificus* and *Haemophilus influenzae* contributes to the genetic diversity in non-O1/non-O139 *Vibrio cholerae* (33). A major concern regarding non-O1/non-O139 strains is the development of resistance to antibiotics since antibiotics is a critical element of treatment of invasive disease in contrast to fluid and electrolyte resuscitation in cholera epidemics (34–35).

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 at high standards and he reported sole intake of bottled water, but food taken from street kitchens. He had no history of soft tissue wounds and no contact to 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 the loss of fluid, he was feeling increasingly unwell, had abdominal pains and on the 4th day after arrival to Denmark he contacted his general practitioner (GP). The GP sent stool cultures for analysis which grew *Vibrio 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 mmol/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 mmol/L (reference range, male over 50 years; 3,5 – 8,1 mmol/L). A stool sample was analysed using a Point-of-Care Test (FilmArray® Gastrointestinal panel, Biomérieux) with identification of *Vibrio cholerae* and enteroaggregative *E. coli* (EAEC). The same sample sent for routine stool culture at the local Microbiology Department grew only *Vibrio cholerae*. Further analysis at the national reference laboratory at Statens Serum Institut (SSI) demonstrated a non-O1/non-O139 serogroup. Prior to the results of the stool analysis the patient was prescribed oral metronidazole by the GP, which at the hospital was changed to a single dose of 1g of azithromycin. The patient also received 2 litres of iv Sodium-Potassium-Glucose fluid after which he quickly recovered from his diarrhea. He was discharged 16 hours after arrival to the hospital with Potassium-Chloride tablet treatment for xx days.

Methods

Data reported to SSI and *Vibrio* strain analysis performed at SSI was extracted from archives, for the years 2004 - 2019. Our reported isolate was tested at the North Zealand hospital using POCT procedures with BD FecalSwab TM (Copan Italia SpA) and PCR analysed in The Film Array GI Panel (BioFire Diagnostics, Biomérieux, Salt Lake City, Utah). The stool sample was routinely sent to the microbiological laboratory at Herlev University Hospital for cultivation and upon growth of *Vibrio cholerae* the pure culture was forwarded to SSI for serotyping for national surveillance. Serotyping was performed using O1 and O139 *Vibrio cholerae* specific antisera (Denka Seiken, Tokyo, Japan). The isolate was also subject to whole genome sequencing (WGS) using the Illumina NextSeq and WGS data 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 95% ID and min. 60% gene length). The Multi-locus sequence typing (MLST) was assigned using the webtool MLST 2.0 (<https://cge.cbs.dtu.dk/services/MLST/>).

Results

Denmark has a centralised mandatory clinical and microbiological reporting system for a list of pathogens and infections including cholera. Reporting is done to the SSI where only 25 cases have been registered from 2004 to 2019. Over the years 2004-2012 isolates were only tested for subtype O1 and from 2013 for both subtype O1 and O139. Analysis of all isolates sent to SSI found two serogroup O1 and 23 cases of non-O1/non-O139 isolates.

Serotyping of the analysed isolate was performed and was subsequently classified as *Vibrio cholerae* non-O1/non-O139. The sequence data revealed sequence type (ST) 558 and a match to the O139 *wbfZ* gene variant with 96, 6% ID. The isolate possessed the virulence genes: *makA*, *hlyA*, *toxR*, *rtxA* and *als*. Furthermore, markers from the genetic islands VSP-2 and VPI-2 were detected. 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.

Discussion

In recent years a modernised and rapid alternative to laboratory cultivation has been introduced in the infectious medicine clinician's daily work. The point-of-care-test (POCT) simplifies diagnostics on molecular test arrays (36–37). At the North Zealand hospital Emergency Unit we have introduced routine use of POCT for common infectious diseases such as influenza, RS virus and gastrointestinal disease. The point-of-care-test we use offers analysis of 22 bacteria, viruses and parasites with high sensitivity (98, 5%) and specificity (99, 2%) (38). Given the rare finding of an imported case of *Vibrio cholerae* from Vietnam to Denmark we decided to analyse the isolate and characterise its genetic and virulence properties.

Since the evolutionary change of O1 El Tor in late 1992 into the new *Vibrio 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 region *wbf* encoding the O139 O-antigen, involving homologous recombination or a suggested horizontal gene transfer from a non-O1/non-O139 strain (O22) (32, 39–42). The new O139 Bengal largely resembled the O1 El Tor but due to absent immunity in the population the new O139 Bengal infected susceptible adults rather than children, which O1 El Tor used to do (42). Over the years 1993-95 there was a local predominance of the O139 Bengal in reported cases of cholera disease in Bangladesh and parts of India, until a new clone of O1 El Tor reemerged. The two strains O1/O139 are now responsible for the ongoing cholera epidemic in Asia, still globally referred to as the 7th pandemic of cholera (41, 43).

Our initial serotyping into a non-O1/non-O139 serogroup was questioned when the WGS came out with a 96, 6% match on the serogroup O139 gene *wbfZ*. There are previously reported rough variants of the O1/O139 serogroups which present as O1/O139 on PCR analysis but lack the O1/O139 surface antigen and thus fail to agglutinate O1/O139 antisera; the analysed isolate suggesting a rough variant of nontoxic O139 (31). 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 (44). 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 (45). The absence of the expected 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 (46–47). The Vibrio Seventh Pandemic islands (VSP-1 and VSP-2) is a gene cluster identified in the 7th pandemic El Tor strains which is found absent in classical and pre-7th pandemic strains (48–49). The VSP-1 and VSP-2 are occasionally seen in non-O1/non-O139 strains but rarely together (31, 50). The role of VSP islands remains unestablished but are suggested to provide increased environmental fitness to those strains carrying the genes (50). VSP-2-like elements which have similar ORFs (Open Reading Frame) in non-cholera *Vibrios* indicate that they have a natural environment function (51). Recent genetic analysis has revealed VSP-2 subgroups with various geographical origins making it a tool in analysing genetic lineages of global cholera transmission (52). The analysed isolate possessed a complete VSP-2 and 86, 9% of VSP-1. 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 (53). The *hlyA*, *rtxA* and *toxR* are commonly found in both O1/O139 and non-O1/non-O139 strains (31, 48). The absence of CTX and Tcp in the analysed isolate indicate the known potency of other virulence factors, some which are recently described and not fully understood in mechanism. The Multifunctional Autoprocessing Repeats-in-Toxins (MARTX/RtxA) is considered to contribute significantly to the pathogenesis and cytotoxicity of *Vibrio cholerae* and is present in nearly all strains (54). The analysed isolate also presents with the novel toxin *MakA*, to our knowledge this is the first reported case from a strain of Asian origin. The *MakA* (motility associated killing factor A) is a flagella-mediated toxin secreted via the fT3SS. It was first described in 2018 as pathogenic in *Caenorhabditis elegans* and zebrafish later reported from Tanzania in reservoirs of *Vibrio cholera* O1 strains and from epidemics occurring 2015 - 2017 and also found in sequenced strains from Uganda O1 cholera outbreaks 2014 - 2016 (55–58). We conclude that the combination of virulence factors of our isolate suggests the finding of a rough variant of a nontoxigenic *Vibrio cholerae* O139 rather than a O22 serogroup version (Figure 2). Our ambition to more advanced serotyping and genetic analysis with phylogeny in a Japanese laboratory has failed due to European regulations (GDPR, General Data Protection Analysis) which has restricted an export of our isolate. Due to the limited number of publications on aquatic and reported clinical cases of *Vibrio cholerae* O139 in Vietnam we have not been able to perform a critical review on the genetic properties of the reported isolate (59). The importance of intensified reporting from Asia is reflected in findings that molecular strain changes during the 7th pandemic seem to evolve in South Asian regions and current reporting from the region are scarce and delayed (60–62).

Declarations

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Figures

Horizontal gene transfer

4 different ways for bacterial and archaeal species to share genetic material "sideways"

Transformation

Transduction

Conjugation

Gene transfer agents

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Figure 1

Horizontal gene transfer. Evolution of bacteria and archaeal species through horizontal gene transfer (or lateral gene transfer) means genetic material being transferred "sideways" instead of evolutionary vertical. It is seen within different variants of a species but also between neighboring species, as we describe in the case of *Caenorhabditis elegans* to *Vibrio cholerae*. There are three "classical mechanisms and a fourth hybrid (between transduction and transformation). Recently a fifth mechanism named

"transfection" has been introduced, but since it is artificially applied in laboratories i.e. with CRISPR/Cas9 technology it is not discussed in this article.

Figure 2

Schematic illustration of the *Vibrio cholerae* genetic evolution. The evolutionary pathway from an ancestral *Vibrio cholerae* strain towards the current 7th pandemic strain involves "milestone" genetic changes in virulent potency, reflecting the characteristics of a newly evolved strain in combination with the immunologic preparedness in the surrounding human population. *Vibrio cholerae* is prone to genetic change and analysis of historic events by horizontal gene transfer and recombination reveal the chronological pattern of virulence mechanisms. The plastic and genetically diverse pathway involves variant strains with shifting pathogenic potency. We report on a clinical *Vibrio cholerae* isolate where we have performed whole genome sequencing and found a rare O139 nontoxigenic rough variant imported to Denmark from Vietnam.