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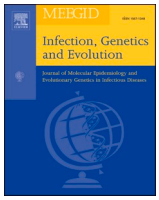

# German coasts harbor non-O1/non-O139 *Vibrio cholerae* with clinical virulence gene profiles

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ABSTRACT

Non-O1/non-O139 *Vibrio cholerae* (NOVC) are ubiquitous in aquatic ecosystems. In rare cases, they can cause intestinal and extra-intestinal infections in human. This ability is associated with various virulence factors. The presence of NOVC in German North Sea and Baltic Sea was observed in previous studies. However, data on virulence characteristics are still scarce. Therefore, this work aimed to investigating the virulence potential of NOVC isolated in these two regions. In total, 31 NOVC strains were collected and subjected to whole genome sequencing. *In silico* analysis of the pathogenic potential was performed based on the detection of genes involved in colonization and virulence. Phenotypic assays, including biofilm formation, mobility and human serum resistance assays were applied for validation. Associated toxin genes (*hlyA*, *rtxA*, *chxA* and *stn*), pathogenicity islands (Vibrio pathogenicity island 2 (VPI-II) and Vibrio seventh pathogenicity island 2 (VSP-II)) and secretion systems (Type II, III and VI secretion system) were observed. A maximum likelihood analysis from shared core genes revealed a close relationship between clinical NOVCs published in NCBI and environmental strains from this study. NOVC strains are more mobile at 37 °C than at 25 °C, and 68% of the NOVC strains could form strong biofilms at both temperatures. All tested strains were able to lyse erythrocytes from both human and sheep blood. Additionally, one strain could survive up to 60% and seven strains up to 40% human serum at 37 °C. Overall, the genetic virulence profile as well as the phenotypic virulence characteristics of the investigated NOVC from the German North Sea and Baltic Sea suggest potential human pathogenicity.

## 1. Introduction

*Vibrio* (*V.*) *cholerae* is a gram negative bacterium commonly found in aquatic ecosystems around the world (Bhandari et al., 2021). The O1 and O139 serogroups of *V. cholerae* are known as the causative agent of cholera and are regarded as the third most important foodborne pathogen of diarrheal diseases (Faruque et al., 1998; Faruque et al., 2000; Reidl and Klose, 2002). The world health organization (WHO) recorded 1.3 to 4.0 million cholera cases annually worldwide, leading to 21,000 to 143,000 deaths per year (Ali et al., 2015).

Apart from the O1 and O139 *V. cholerae*, there are 200 further serogroups based on the classification according to O-antigens which are grouped together under the name 'non-O1/non-O139 *V. cholerae* (NOVC)' and are less thoroughly investigated (Dutta et al., 2013). NOVC can also cause diarrheal diseases up to cholera-like symptoms after the consumption of contaminated raw or uncooked seafood. Nevertheless, a direct contact with water or sediment could also cause infections, such as extra-intestinal infections from wounds, ears and eyes (Deshayes et al., 2015). Sporadic extra-intestinal infections were recorded in some developing countries from Africa, Asia, and Latin America (Deshayes et al., 2015; Lan et al., 2014; Sachu et al., 2021; Shashindran et al., 2020). Rare cases have also been reported in Europe, particularly during summer heatwaves in 2014 and 2018: Seventy NOVC driven cases were reported in Sweden and Finland in 2014 (Baker-Austin et al., 2016). Brehm et al. (2021) reported 30 cases in Germany for 2018 and 2019 including 25 extra-intestinal infection cases. In northern Europe

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countries (Norway, Sweden, Finland, Denmark, Poland, Estonia), 100 NOVC cases, of which 89 were extra-intestinal infections, were reported in 2018 (Amato et al., 2022). Certain virulence factors found in the O1 and O139 serogroups, such as hemolysin, cholix toxin, repeats-in-toxin, secretion systems and pathogenicity islands, can also be detected in NOVC strains (Rajpara et al., 2013; Singh et al., 2001). In addition, virulence factors such as the type III secretion system (T3SS) (Dziejman et al., 2005), the type VI secretion system (T6SS) (Logan et al., 2018), sialidase and neuraminidase, whose coding genes are found in Vibrio pathogenicity island 2 (VPI-II), multivalent adhesion molecule 7, GlcNAc-binding protein, and flagellum-regulated haemagglutinin A leading to mucosal colonization in patients are also described for NOVC strains (Almagro-Moreno et al., 2015).

One of the most important niches of *V. cholerae* is brackish water in estuaries and coastal waters. Schwartz et al. (2019) compared the virulence-associated gene profiles between NOVC isolated from patients and the aquatic environment (from coastal areas of the German North Sea and Baltic Sea). It was not possible to distinguish between these two groups on the basis of the genetic profile. However, a virulence potential of NOVC could be assumed. Antimicrobial resistance of NOVC strains sampled in the same region was also tested, the authors concluded that some strains contain carbapenemase producing genes (Bier et al., 2015). Apart from the direct transmission of pathogenic environmental NOVC to humans *via* recreational water exposure (Crowe et al., 2016), the environment could also act as a reservoir of virulence-associated genes (Rahman et al., 2008). Non-toxigenic strains could acquire genes through horizontal gene transfer (HGT) and hence achieve a higher virulence potential, a broader spectrum of antimicrobial resistances to adapt more efficiently to both the environment and the human host (Dalia et al., 2015; Le Roux and Blokesch, 2018).

Increased water temperature caused by global warming positively correlates with detection and infection numbers of *Vibrio,* including NOVC (Amato et al., 2022; Baker-Austin et al., 2016; Fleischmann et al., 2022). In addition, the European Centre for Disease Prevention and Control (ECDC) annually reports a high to very high risk of infection along the German coastline during summer. Together with *V. vulnificus*, non-O1/non-O139 *V. cholerae* are the most important infective *Vibrio* species associated with direct water contact (Brehm et al., 2021). In this study, genotypic and phenotypic analysis methods were applied to evaluate the pathogenic potential of NOVC strains collected from German coastal regions in 2017 and 2018.

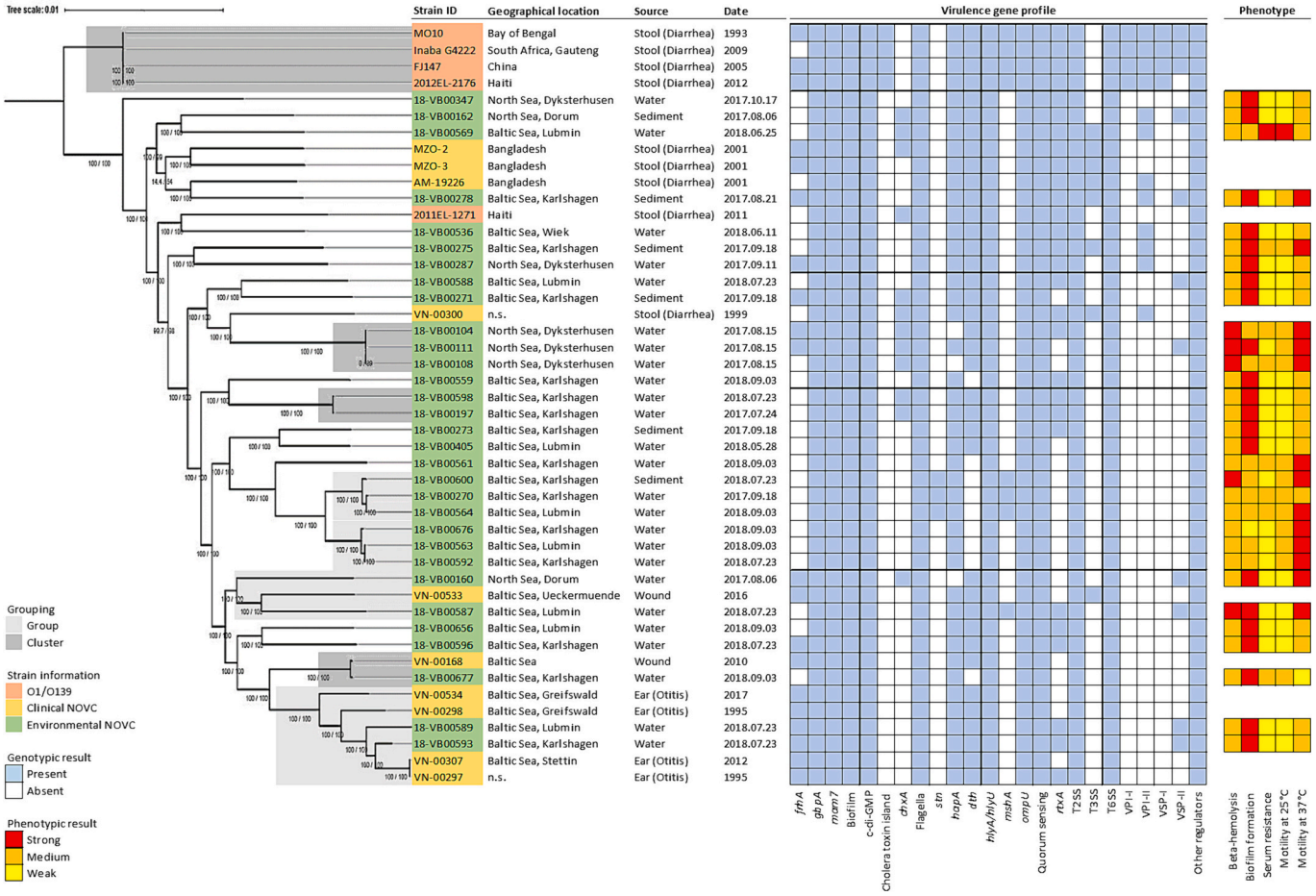


**Fig. 1.** Maximum-likelihood phylogenetic tree of 31 environmental NOVC strains, ten clinical NOVC strains and five O1/O139 pandemic strains based on a shared core genome (2.7 Mbp) combined with virulence profiling. The tree was rooted on the outgroup of four pandemic O1/O139 strains. Numbers in parentheses are SH -aLRT support (%) / ultrafast bootstrap support (%). The scale bar represents the average number of substitutions per site. Groups and clusters within the phylogenetic tree are shown in light gray and dark gray, respectively. Environmental NOVC originate from the North Sea and Baltic Sea are marked in pastel green, clinical NOVC originate from intestinal and extra-intestinal infections are marked in pastel orange, and O1/O139 *V. cholerae* originate from stool samples are marked in pastel red. For the results of the phenotypic analyses, yellow/orange/red square stands respectively for weak/medium/strong ability in hemolytic activity, biofilm formation, serum resistance or mobility assay. n.s. means, that the sampling location was not specified. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

## 2. Material and methods

### 2.1. Bacterial strains

Thirty-one NOVC strains originating from German coastal waters sampled in the North Sea (Dyksterhusen and Dorum) and Baltic Sea (Karlshagen, Lubmin and Wiek) were included in this study. Two *V. cholerae* O1 El Tor strains (serotype Ogawa and serotype Inaba) were used as control strains. General information about the *V. cholerae* strains (geographical location, source and collection date) is shown in Fig. 1. Strains were stored at −80 °C in cryotubes (CRY80, Mast Diagnostics, Reinfeld, Germany). The strains were re-cultured on Lysogeny Broth (LB) agar (Millipore, Merck, Darmstadt, Germany) at 37 °C for 18–24 h. To prepare an overnight culture, individual colonies were taken from the LB agar, transferred to liquid LB medium (Millipore, Merck, Darmstadt, Germany) and incubated for 18 h at 37 °C.

### 2.2. Genotypic assay

Whole genome sequencing (WGS) was applied to all strains to get the complete genetic profile. Briefly, DNA was extracted from a LB overnight culture using the MasterPure™ DNA Purification Kit (Lucigen, Biozym Scientific GmbH, Oldendorf, Germany). The DNA was quantified with the Qubit 2.0 fluorometer (Life Technologies, Darmstadt, Germany). Library preparation from genomic DNA was performed using Nextera XT™ (Illumina, Inc., San Diego, CA, USA) library preparation Kit according to the manufacture's instructions. Genome sequencing was performed on MiSeq™ system (Illumina, Inc., San Diego, CA, USA) with the Illumina MiSeq Reagent Kit v3 for paired-end sequencing (2 × 300 bp). Quality of paired-end Illumina reads was verified directly after sequencing with the FastQC software version 0.11.6. Raw read data were trimmed, filtered, error corrected, normalized, and paired using Geneious software (Biomatters, NZ, version v2022.1.1) at default settings. Paired reads were assembled into contigs by *de novo* assembly at default settings (Geneious assembler, highest sensitivity, slow speed).

The putative virulence potential of NOVC was deduced by the genetic information. Therefore, a list of relevant *V. cholerae* specific virulence-associated genes and their regulators were acquired from current literature (Table 1 and Table A.1). The resulting collection of genes were compared to the contigs from each strain *via* Geneious software (Geneious mapper, medium sensitivity, fast speed). Based on a match to the reference gene sequence between 80 and 100% identity by Geneious software using nucleotide BLAST algorithm (medium sensitivity, default setting parameters), the presence or absence of a gene was confirmed. Gene alignment of the T3SSs was also performed by Geneious software (Geneious alignment, 65% similarity) and visualized by Adobe Illustrator 2022.

To analyse the phylogenetic relationship of 31 environmental NOVC strains and draw conclusions about their relatedness to five pandemic O1/O139 strains (FJ147, Inaba G4222, MO10, 2011EL-1271, and 2012EL-2176) and ten clinical NOVC strains (AM-19226, MZO-2, MZO-3, VN-00168, VN-00297, VN-00298, VN-00300, VN-00307, VN-00533, and VN-00534), a phylogenetic study was conducted. First, based on the provided assemblies using panaroo v1.3.4 (Tonkin-Hill et al., 2020), 2752 core genes (99% ≤ strains ≤100%), 151 soft core genes (95% ≤ strains ≤99%), 1114 shell genes (15% ≤ strains <95%), 3987 cloud genes (0% ≤ strains <15%), and 8000 total genes (0% ≤ strains ≤100%) between all 46 mentioned strains were identified. The resulting shared core genome alignment produced by panaroo was further used as input to create a maximum-likelihood-based phylogenetic tree using IQ-TREE v2.2.0 (Nguyen et al., 2015). For phylogenetic tree construction, the GTR (General Time Reversible) model was used. Branch support values (Shimodaira-Hasegawa-like approximate likelihood ratio (SH-aLRT) test and ultrafast bootstrap) were calculated with 1000 replicates. The resulting phylogenetic tree was visualized using iTol on http://itol.embl.de updated May 15, 2023 (Letunic and Bork, 2021).

**Table 1**
Virulence-associated genes and their regulators of NOVC*

| Group | Gene |
|---|---|
| Adhesin | *frhA, gbpA, mam7* |
| Biofilm formation | *bap1, rbmA, rbmC, vpsA-vpsU* |
| c-di-GMP metabolism | *cdgC, cdgD, cdgH, cdgJ-cdgM, cdpA, rocS, vieA* |
| Cholera toxin island | *ace, ctxA, rstA, rstB, rstR, zot* |
| Cholix toxin | *chxA* |
| Flagella | *flhA, flhB, flgK, flgM, flgN, fliA, flrA-flrC, motA, rpoD, rpoN* |
| Heat stable enterotoxin | *stn* |
| Hemagglutinin protease | *hapA* |
| Hemolysin | *dth, hlyA, hlyU* |
| Mannose sensitive hemagglutinin | *mshA* |
| Outer membrane protein | *ompU* |
| Quorum sensing | *aphA, cqsS, hapR, luxO-luxQ, luxU, vqmA* |
| Repeat in toxin | *rtxA* |
| Type II secretion system | *epsC-epsN* |
| Type III secretion system | A33_1660-A33_1706 |
| Type VI secretion system | *hcp, vasH, vgrG1-vgrG3* |
| VPI-I | vc0817-vc0847 |
| VPI-II | vc1758-vc1809 |
| VSP-I | vc0175-vc0185 |
| VSP-II | vc0490-vc0516 |
| Other regulators | *crp, cyaA, hns* |

* Detail information about listed genes are shown in Table A.1.

### 2.3. Hemolysis assay

Hemolysis test was performed according to Bier et al. (2013). Both human blood and defibrinated sheep blood were used in this assay. Human blood was obtained from healthy volunteers by venipuncture using blood collection tubes containing dipotassium salts of ethylene diamine tetra acetic acid 139 (K2EDTA) as anticoagulant (DB Medical, Eysins, Switzerland). Defibrinated sheep blood was purchased from Thermo Scientific Oxoid. Erythrocytes were washed three times with 0.01 M phosphate-buffered saline (PBS) buffer (Medicago, Uppsala, Sweden) and separated by centrifugation (1000 *g*, 4 °C for 5 min). Afterwards, erythrocytes were concentrated by a second centrifugation step by 1000 *g* at 4 °C for 10 min. Blood agar plates were prepared using 5% erythrocytes and Mueller-Hinton agar (Oxoid GmbH, Wesel, Germany). *V. cholerae* strains were inoculated on blood agar plates (5 µL of LB overnight culture in triplicate), followed by 24 h incubation at 37 °C. Hemolytic activity (beta hemolysis) and the diameter of the hemolytic zone were determined. Strains causing a 1 cm hemolytic zone on human blood agar and/or a 2 cm zone on sheep blood agar were classified to have a medium hemolytic activity, strains with 1.5 cm hemolytic zone on human blood agar and/or 3.5 cm on sheep blood agar were classified to have a strong hemolytic activity.

### 2.4. Biofilm formation assay

To estimate biofilm formation, a combined assay from Mahoney et al. (2010) was used in this study with modifications. In brief, LB overnight cultures of *V. cholerae* strains were centrifuged at 5000 *g* and the obtained pellets were resuspended in PBS. Cell density was measured by spectrophotometer at 588 nm. Bacterial cells with an optical density (OD) of 0.7 were inoculated at hundredfold dilutions into 250 µl LB media per well (10 wells in a line) of a 96 well clear polystyrene, non-treated, flat bottom microplate (Corning, New York, NY, USA). The surrounding wells of a 96 well plate were filled with 250 µl LB only (negative controls). After 48 h incubation at 25 °C, the remaining liquid culture was removed, the remaining biofilm was washed twice with 300 µl PBS and dried overnight at room temperature. The biofilm was stained with 275 µl 0.1% crystal violet solution (*w/v* in distilled water) for 1 h and washed three times with 300 µl sterile water. After drying for 1 h at room temperature, the biofilm was resolubilized in 300 µl of 33%

acetic acid (w/v in distilled water) and incubated for 30 min. The biofilm formation was determined by absorbance at 595 nm and normalized to the absorbance of the LB negative controls. Based on $OD_{595}$ absorption, the biofilm formation capacity of each *V. cholerae* strain was classified into four groups: no biofilm formation (<0.5), weak (0.5–1.0), medium (1.0–3.0) and strong biofilm formation (>3.0). The biofilm formation assay was performed for each strain in three biological replicates.

### 2.5. Human serum resistance assay

Resistance to human blood serum was estimated in accordance to Bier et al. (2013). First, 12 µl of a *Vibrio* overnight LB culture were added into 600 µl LB broth and incubated at 37 °C for 5–6 h. Subsequently, 2 µl of this culture were transferred into each well of a 96-well microplate containing a gradient solution of 100 µl human serum (from healthy volunteers) and peptone-glucose broth (1% glucose, 0.0075% bromothymol blue, 1% peptone, 0.5% NaCl, pH 7.4). Therefore, human serum was mixed at different ratios in peptone-glucose solution to obtain the standard serum solution at gradient concentrations of 0 Vol%, 10%, 20%, 40% and 60%. All samples and two control strains (serum resistant *Escherichia coli* K-12 + pKT107; RS228 as positive control and serum sensitive *Escherichia coli* K-12 -pKT107; DSMZ423 as negative control) were incubated with those serum solutions at 37 °C for 24 h. Bacterial growth, indicating serum resistance, was detected by the colour change from blue to yellow due to glucose fermentation. Strains were classified as sensitive towards serum when growth was only observed in 0 to 20 Vol% human serum, as medium resistant when growth was observed in up to 40% human serum, and resistant when growth was observed in up to 60% human serum. Three biological replicates were performed for all tested strains.

### 2.6. Examining mobility

Bacterial mobility was examined using a soft agar assay according to the method published by Li et al. (2022) with minor modifications. For this purpose, 1 µl overnight LB culture of *V. cholerae* strains were directly injected into LB agar plates (0.3% agar). After 24 h and 48 h incubation at 37 °C, the diameter of the bacterial swarming zone was measured. Each strain was analyzed in six to nine biological replicates. Mobility was classified as following: weak mobile (diameter of swarming zone is <4 cm), medium (4–8 cm) and strong mobility (over 8 cm).

## 3. Results

### 3.1. Phylogenetic analysis and virulence-associated gene profiling

The analysis of the assemblies by panaroo revealed that the 31 NOVC strains from this study, the five pandemic O1/O139 strains (FJ147, Inaba G4222, MO10, 2011EL-1271, and 2012EL-2176), and the ten clinical NOVC strains (AM-19226, MZO-2, MZO-3, VN-00168, VN-00297, VN-00298, VN-00300, VN-00307, VN-00533, and VN-00534) shared a core genome of 2752 genes (≤ 99–100% of the strains). The alignment length of the filtered core genome was approximately 2.7 Mbp. The maximum-likelihood (ML) phylogenetic tree generated based on this alignment by IQ-TREE is shown in Fig. 1 along with strain grouping, detailed strain information, the virulence gene profile and phenotypic results.

Regarding the ML phylogenetic tree, a cluster of pandemic O1/O139 *V. cholerae* strains separated from clinical and environmental NOVC is shown. This separate cluster contains the O139 strain MO10 and the three O1 strains Inaba_G4222, FJ147 and, 2012EL-2176. All O1/O139 strains exhibit the cholera toxin island (*ctxA*, *ace*, *zot*, *rstA*, *rstB*, *rstR*) and all four pathogenicity islands (VPI-I, VPI-II, VSP-I and, VSP-II), expect VSP-II for strain 2012EL-2176. In the phylogenetic tree segment comprising clinical and environmental NOVC, there are two clusters and two groups of environmental NOVC as well as two groups and one cluster comprising clinical and environmental NOVC together. A grouping of environmental NOVC with clinical NOVC was only observed with clinical NOVC isolated from extra-intestinal infections (VN-00533, VN-00168, VN-00534, VN-00298, VN-00307 and, VN-00297). All clinical NOVC mentioned and the environmental NOVC grouped together with them originate from the Baltic Sea, with the exception of the North Sea strain 18-VB00160 and VN-00297, whose isolation location is unknown. However, a clinical NOVC originating from the North Sea was not available for this study. The two clusters containing only NOVC from the environment can be divided into NOVC isolated from the North Sea and NOVC isolated from the Baltic Sea and assigned to an isolation site. Whereas the two groups of environmental NOVC are formed exclusively from strains originating from the Baltic Sea, without a shared localization site. The cholera toxin island (*ctxA*, *ace*, *zot*, *rstA*, *rstB*, *rstR*), the main virulence factor of the O1 and O139 serogroups, was absent in all environmental and clinical NOVC. We also noticed the gene absence (*tcpP*-vc0826 and *toxT*-vc0838, located inside VPI-I) of an important modulation pathway of cholera toxin (CTX) and toxin coregulated pilus (TCP), which is formed by *aphAB-tcpP-toxRS-toxT* (Bina et al., 2018).

Quorum sensing (QS) system, including cAMP-CRP signaling pathway (*cyaA*) and histone like nucleoid structuring protein (*hns*), genes related for colonization, core genes for flagella and biofilm formation (see Table 1) are genetically encoded in all *V. cholerae* strains. The presence of mucus movement factors and adhesins was observed in O1 and O139 *V. cholerae* as well as in environmental and clinical NOVC with a prevalence rate of 94% for *hapA*, 100% for *gbpA*, 100% for *mam7*, 41% for *frhA* and 17% of the strains for *mshA*. A grouping of *mshA* containing strains were observed, formed by the cluster of the four pandemic O1/O139 *V. cholerae* strains (MO10, Inaba_G4222, FJ147, 2012EL-2176) and a group of three environmental NOVC strains (18-VB00600, 18-VB00270, 18-VB00564), respectively.

The essential outer membrane protein gene, *ompU* is present in all *V. cholerae* strains. Moreover, there is a wide distribution of additional toxin genes with different detection rates in all genomes; the hemolysin gene *hlyA* is presence in all of the strains, followed by *dth* with 80%, *rtxA* with 61% and *chxA* with 26%. The gene *stn*, that encode heat stable enterotoxin, was found only in the already mentioned group for *mshA* of the three environmental strains 18-VB00270, 18-VB00564 and, 18-VB00600. All *V. cholerae* strains contain a T2SS and a T6SS, while only five clinical (AM-19226, MZO-2, MZO-3, VN-00300 and, VN-00533) and three environmental NOVC (18-VB00275, 18-VB00278 and, 18-VB00569) contain a T3SS. Information on the genetic structure of the T3SS of the environmental NOVC compared to a clinical NOVC reference strain AM-19226 is shown in Fig. 2. Pathogenicity islands (PIs), VPI-II and VSP-II, are present in some environmental and clinical NOVC (see Fig. 1). Two of these environmental strains carry both PIs in their genome sequence (18-VB00278 and 18-VB00162). None of the environmental and clinical NOVC strains harbor the whole VPI-I sequence, only segments (vc0817, vc0818, vc0846, vc0847) can be found in nine environmental (18-VB00111, 18-VB00271, 18-VB00275, 18-VB00278, 18-VB00287, 18-VB00347, 18-VB00559, 18-VB00561, 18-VB00569) and five clinical strains (MZO-3, VN-279, VN-300, VN-307, VN-533), while the core functional part *tcpA* is missing. Similar results are observed for VPI-II, although 1 to 20 segments can be found in 20 environmental and nine clinical NOVC strains. Only six environmental and two clinical NOVC indicate a functional siladase and neuraminidase encoded in vc1773 to vc1784 based on their genetic information. The complete gene sequence for VSP-II (vc0490 to vc0516) could be found in seven environmental and one clinical NOVC strain.

T3SS gene sequence was identified in three environmental NOVC strains in this study. Comparing them with T3SS gene sequence of the clinical NOVC AM-19226, intrinsic regulator $vttR_A$ (A33_1664) was absent in the strains 18-VB00278 and 18-VB00569. In addition, another intrinsic regulator, $vttR_B$ (A33_1675) was absent in strain 18-VB00569. All structural protein encoding genes were detected in the strains 18-

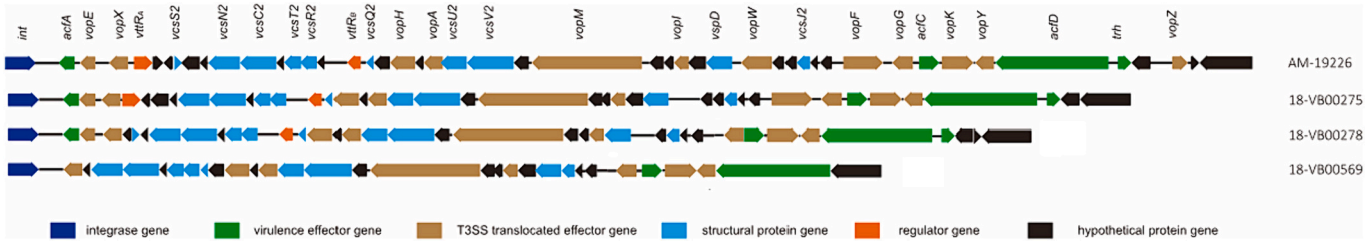


**Fig. 2.** Gene alignment of the T3SS of environmental NOVC strain 18-VB00275, 18-VB00278 and 18-VB00569 compared to reference *V. cholerae* strain AM-19226. Gene alignment of T3SS in *V. cholerae* was built according to Alam et al. (2011). Arrows stand for transcription initiation sites. Dark blue arrows indicate integrase gene *int*, green arrows indicate the homolog of accessory colonization factors and thermostable direct hemolysin in *V. parahaemolyticus*, brown arrows indicate T3SS translocated effector genes, light blue arrows indicate genes encoding structural components, orange arrows indicate regulator genes and black arrows show genes with a hypothetical function. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

VB00275 and 18-VB00278 while one inner membrane ring encoding gene, *vcsT*2 (A33_1672), cannot be identified in 18-VB00569. As for the translocation effector genes, four genes in the core region, including *vopH* (A33_1678), *vopA* (A33_1680), *vopM* (A33_1684) and *vopI* (A33_1687) were detected in all three strains, while *vopF* (A33_1696) was detected in 18-VB00275.

### 3.2. Phenotypic results

All environmental NOVCs and two O1 strains (classical Ogawa and El Tor) showed hemolytic activity on human and sheep blood agar with variable morphology. Compared to the O1 strains, 16% of the environmental NOVCs showed 1.5-fold larger hemolytic rings indicating strong hemolytic activity. Only the environmental NOVC strains 18-VB00569 showed strong and 23% of the remaining environmental NOVC showed medium resistance to human serum. All the other strains, including O1 classical Ogawa and El Tor, only grew in presence of a maximum of 20% human serum. A strong ability to form biofilms was observed in 68% of the strains, while only one strain formed a weak biofilm. As for the mobility test, the whole environmental NOVC collection is more mobile at 37 °C than 25 °C, with the exception of two strains (18-VB00569 and 18-VB00677). In total, 42% of the environmental strains showed a strong mobility at 37 °C, while only one strain showed weak mobility comparable to the O1 strains.

## 4. Discussion

Recently, NOVC-related infections have increased in the Baltic Sea and North Sea region (Baker-Austin et al., 2016; Brehm et al., 2021). The aim of this study was therefore to uncover the possible virulence potential of NOVC strains isolated from this region. A total of 31 NOVC strains were collected from five coastal waters of the German Baltic Sea and North Sea. Overall, the geographical distribution has no impact on the genetic and phenotypic virulence profiles (Fig. 1). Maximum-likelihood phylogenetic analysis of the core genome in comparison to virulence gene profiling confirm a high genomic diversity of the analyzed environmental NOVC strains. The study by Schwartz et al. (2019) also supports this point, suggesting virulence-associated genes could be distinguished into the water body and integrated into NOVC by HGT (Boyd and Waldor, 1999; Ceccarelli et al., 2013; Mohapatra et al., 2008). A distribution of various virulence-associated genes across all 31 environmental NOVC confirms this assumption. Nevertheless, a phylogenetic relationship was observed in the core genomes of clinical NOVC isolated from wound and ear infections and environmental NOVC strains resulting in two groups and one cluster, whereby the virulence gene profile varied (see Fig. 1). However, their geographical localization points are widely distributed along the Baltic Sea coast, except the clinical NOVC VN-00307 whose geographic location is unknown and the environmental NOVC 18-VB00160 isolated from the North Sea. Interestingly, the environmental isolates from this study appear to be closer related in their core genome to clinical NOVC from extraintestinal infections than to intestinal infections. On the other hand, the two groups and the cluster consisting of environmental NOVC originated from the Baltic Sea only, show closer genetic relationships to each other based on their core genome and their virulence gene profiles, although the sampling location was at two different sites on the Baltic Sea (Lubmin and Karlshagen). However, these findings are an exception because all other strains from this region are scattered across the phylogenetic tree. The environmental cluster consisting of NOVC from the North Sea shows variations in their virulence gene profile, although they were collected on the same day at the same sampling site and show high phylogenetic relatedness in their core genomes. As already mentioned, this in turn reflects as well the high genetic variability of NOVC. The O1 and O139 *V. cholerae* serotypes form a separate cluster in this study and differ clearly from the clinical and environmental NOVC in their core genome and virulence gene profiles. Only the O1 serovar 2011EL-1271 lies outside this cluster within the NOVC group, which is also reflected in its virulence gene profile. Neither the complete cholera toxin island nor PIs could be identified in this genome.

Genes required for flagella formation, biofilm formation and *c*-di-GMP regulators were identified in all *V. cholerae* genomes. However, eleven environmental NOVC strains showed less biofilm formation ability in the performed *in vitro* assay. One possible explanation could be the difference in *vpsR* gene sequence, a master regulator of polysaccharide and protein structure in biofilm formation (Hsieh et al., 2020). As previously mentioned, the homology of all biofilm relevant genes in Table 1 and additionally their predicted protein sequence was confirmed using Geneious software. Only VpsR variation could match with difference in biofilm formation assay among the environmental NOVC strains. Comparing with the VpsR reference sequence (AF221853.1), both single amino acid (aa) differences (18-VB00561: aa144, I instead of M; 18-VB00270, 18-VB00561, 18-VB00563, 18-VB00564, 18-VB00592, 18-VB00600, 18-VB00676: aa218 F instead of L) and larger gaps (aa209–234 in 18-VB00569) were detected in nine of the eleven environmental NOVC genomes, consistent with a medium and weak biofilm formation ability.

As for the virulence-associated genes, characteristic genes of O1 and O139 such as cholera toxin island (*ctxA*, *ace*, *zot*, *rstR* and *rstAB*) and toxin coregulated pilus (vc0817 to vc0847) were absent in all environmental and clinical NOVC. Nevertheless, all NOVC harbor the hemolysin gene *hlyA/hlyU* and 80% in addition the hemolysin gene *dth*. Hemolysin activity was confirmed by beta-hemolysis on sheep and human blood agar. We also observed the presence of toxin genes, including repeat in toxin (*rtxA*), heat stable enterotoxin (*stn*) and cholix toxin (*chxA*). The mannose-sensitive hemagglutinin gene (*mshA*), which is known to belong to the type 4 pili family, has also been detected in environmental NOVC. Type 4 pili have proven their importance as a colonization factor in the host during infections with *V. cholerae* serovar O1 (Marsh and Taylor, 1999). Schirmeister et al. (2014) investigated the virulence gene profile of NOVC isolated from German patients also with extra-intestinal

infections. These strains were also positive for *hlyA,dth* and *chxA,* but in contrast to environmental NOVC from this study negative for *rtxA* and *mshA*.

In all *V. cholerae* strains, the T2SS and T6SS could be identified in their genome sequences. The T3SS system could be only present in the genome sequences of five clinical and three environmental NOVC (Figs. 1 and 2). Previous studies showed that the T3SS is essential for epithelial cell colonization of NOVC strains, especially when VPI-I is absent in the genome, which play an important role in cell disruption (Chaand et al., 2015; Dziejman et al., 2005; Miller et al., 2016). Although T3SS sequence of the clinical NOVC AM-19226 not fully match to the environmental NOVC sequences (Fig. 2). Nevertheless, both of the two currently know functional effector genes, *vopM* and *vopF* were identified in all three and one environmental NOVC (18-VB00275) respectively, which might contribute to cell surface adhesion and tight junction damage during infection (Miller et al., 2019). Zeb et al. (2019) also reported presence of a T3SS in NOVC contributes to severe diarrhea symptoms than T3SS negative NOVC, indicating the relationship between the presence of a T3SS and pathogenicity. Therefore, there might be better colonization ability and higher virulence potential for those T3SS positive NOVCs compared to the T3SS negative strains.

Fragments of VPI-II (vc1773 to vc1784) and full VSP-II sequence (vc0490 to vc0516) was present in 20% of the clinical and environmental NOVC strains, respectively. Those NOVCs might have the ability of sialic acid utilization encoded in gene vc1784 on VPI-II, which might enhance their mucosal penetration ability, resulting in intestine epithelial cell surface localization (Jermyn and Boyd, 2002; O'Shea et al., 2004). Especially the identification of the toxins *chxA*, *hlyA* and *rtxA* in the genome of 18-VB00569 next to the T3SS and VPI-II in combination with a strong resistance to human serum strengthens the hypothesis that this strain could represent a human-pathogenic NOVC strain.

## 5. Conclusion

In conclusion, NOVC with multiple virulence-associated genes were isolated from the German North Sea and Baltic Sea suggesting a putative human pathogenic potential. Important virulence-associated genes, including *chxA, dth, hlyA*, *mshA*, *rtxA* and *stn* as well as pathogenicity islands (VPI-II and VSP-II) are present in their genomes. In addition to the T6SS, a T3SS, which plays an important role in infection, was also detected in 10% (3/31) of these strains. Compared to their virulence gene profile, hemolytic activity and serum resistance underline a possible pathogenic potential of the NOVC investigated. Therefore, there is the possibility that NOVC can cause infections if it comes to direct contact with seawater. As an increase in *Vibrio*-infections is observed in Northern Europe due to climate change, a *Vibrio*-monitoring including NOVC is beneficial for the German coasts to provide information for further risk control.

## CRediT authorship contribution statement

**Quantao Zhang:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Thomas Alter:** Writing – review & editing, Supervision, Conceptualization. **Eckhard Strauch:** Writing – review & editing, Conceptualization. **Inga Eichhorn:** Investigation, Writing – review & editing. **Maria Borowiak:** Writing – review & editing, Investigation, Formal analysis. **Carlus Deneke:** Writing – review & editing, Investigation, Formal analysis. **Susanne Fleischmann:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation, 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.

## Data availability

The assembled genomes of the 31 NOVC strains can be found in the NCBI database under the corresponding BioProject ID: PRJNA1031992.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2024.105587.

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