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Characterization of virulence mechanisms of environmental and food-associated

non-O1/non-O139 *Vibrio cholerae*

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Non-O1/non-O139 *Vibrio cholerae*	NOVC
Cholera toxin	CT
Toxin coregulated pilus	TCP
Type III secretion system	T3SS
Type IV secretion system	T6SS
Antimicrobial resistance	AMR
Multidrug and toxic compound extrusion	MATE
Major facilitator family	MFS
Resistance-nodulation-cell division	RND
Small multidrug resistance	SMR
Integrating conjugative elements	ICE
Vibrio pathogenicity island	VPI
Vibrio seventh pathogenicity island	VSP
Reactive oxygen species	ROS
Reactive nitrogen species	RNS
Acid tolerance response	ATR
Quorum sensing	QS
Methyl-accepting chemotaxis protein	MCP
Hemolysin	Hly
Repeat in toxin	RTX
Cholix toxin	Chx
Heat stable enterotoxin	Stn
Two-component system	TCS
Histone-like nucleoid structuring protein	HNS
cyclic AMP-activated global transcriptional regulator	cAMP-CRP
Polymerase chain reaction	PCR
Reverse transcription polymerase chain reaction	RT-PCR

List of Abbreviations

Mobile genetic element	MGE
Whole-genome sequencing	WGS
core genome multilocus sequence typing	cgMLST
Horizontal gene transfer	HGT
Lactatdehydrogenase	LDH

Introduction

The pathogen *Vibrio cholerae* is the causative agent of cholera, an endemic and epidemic diarrheal disease that has affected populations for the past centuries (Reidl and Klose 2002). *V. cholerae* has been classified into over 200 serogroups through O-antigen serotyping (Shimada *et al.* 1994). Among these, two serogroups, O1 and O139, are responsible for the fatal diarrheal cholera, attributed to their ability to produce cholera toxin (CTX). The most recent pandemics, the seventh (occurring between the 1970s and 1990s) and the eighth (in the 1990s), were caused by the El Tor biotype O1 and O139, respectively (Faruque *et al.* 1998). According to the World Health Organization, cholera currently affects 47 countries, leading to an estimated 2.9 million cases annually (Legros 2018).

Unlike serogroups O1 and O139, other serogroups have been of lesser concern in public health due to their association with milder clinical symptoms and a lack of key virulence factors, cholera toxin (CTX) and toxin-coregulated pilus (TCP), the latter of which is critical for CTX production in O1 and O139 (Faruque *et al.* 1998). These less virulent groups are collectively referred to as non-O1/non-O139 *Vibrio cholerae* (NOVC) (Crowe *et al.* 2016). In recent years, however, several NOVC-related cases have been reported across different regions, particularly in developing countries (Morita *et al.* 2020; Zmeter *et al.* 2018). Further research has revealed that NOVC strains can produce a variety of toxins, including hemolysin (HlyA), hemagglutinin protease (HapA), repeats-in-toxin (RTX) toxins, sialidase (Nan), heat-stable enterotoxin (Stn), as well as components of the type III secretion system (T3SS) and type VI secretion system (T6SS) (Hirsch *et al.* 2020; Ceccarelli *et al.* 2015; Schirmeister *et al.* 2014).

In Germany, 95 NOVC infection cases were documented between 2011 and 2021 (Schmidt *et al.* 2023; Brehm *et al.* 2021; Schwartz *et al.* 2019). NOVC strains have been isolated from food sold in German retail chains and from surrounding water bodies, including the German North Sea and Baltic Sea (Chapters 3 and 4). Other studies have also found a positive correlation between the abundance of NOVC and global warming in water bodies worldwide (Escobar *et al.* 2015). Direct exposure to water and sediments may lead to infections, while

the consumption of NOVC-contaminated seafood represents another significant source of infection (Ceccarelli *et al.* 2015).

Due to the self-limiting nature of most NOVC-related cases and the incomplete understanding of its virulence mechanisms compared to O1 and O139, there are currently no standardized guidelines for NOVC monitoring and control. However, the occasional detection of NOVC in food and aquatic environments, combined with its potential virulence factors, raises public health concerns.

The aim of this doctoral thesis is to explore the virulence potential of NOVC and assess the necessity of establishing standards for its monitoring and control. NOVC strains used in this study were isolated from seafood and environmental sources. Chapter 2 provides a literature review of virulence-associated genes in *V. cholerae* and outlines a hypothetical infection route. Whole genome sequencing was performed on NOVC strains, and the presence of virulence-associated genes was analyzed *in silico* (Chapters 2.2 and 2.3). Based on these genetic findings, the type III secretion system (T3SS) was further investigated through expression analysis and cytotoxicity testing (Chapter 3).

1. Literature Review

1.1 *Vibrio cholerae*

Vibrio cholerae is a Gram-negative, curved rod-shaped bacterium belonging to the family *Vibrionaceae*. It has been classified into over 200 serogroups through O-antigen serotyping (Shimada *et al.* 1994). Among these, the O1 and O139 serogroups are facultative pathogens that cause cholera, a life-threatening disease characterized by severe watery diarrhea. The defining features of these serogroups are the genes encoding cholera toxin (*ctxA*) and toxin-coregulated pilus (*tcpA*) (Faruque *et al.* 1998).

There have been eight recorded cholera pandemics over the past two centuries. The first six occurred in the 19th century, affecting Europe, North America, and the Southern Hemisphere, and were caused by the classical biotype of O1 strains (Reidl and Klose 2002). The seventh pandemic began in 1961 in Indonesia and was caused by the 'El Tor' biotype of O1 strains. A key difference between the 'El Tor' and classical biotypes lies in their hemolytic activity, production of repeat in toxin, and ability to ferment acetoin (Hu *et al.* 2016). The differences in gene expression between these two biotypes have been studied extensively. Beyhan *et al.* (2006) identified a range of gene expression differences, including those related to amino acid synthesis, chemotaxis, and pathogenesis.

The outbreak of O139 Bengal strains, often described as the eighth pandemic, began in 1992. However, some studies suggest that this outbreak should be considered part of the seventh pandemic due to the high genomic similarity between O139 Bengal and O1 El Tor strains (Ramamurthy *et al.* 2022; Karaolis *et al.* 1994). Today, *V. cholerae* remains a significant health threat in developing countries, particularly in areas with poor sanitation (Charnley *et al.* 2023).

The remaining serogroups of *V. cholerae* are collectively referred to as non-O1/non-O139 *Vibrio cholerae* (NOVC). Since most NOVC strains cannot produce cholera toxin (*ctxA*) or toxin-coregulated pilus (*tcpA*), they have received less attention in public health. NOVC strains are widely distributed in the environment and have been detected in various regions

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(Igere *et al.* 2022). Although most NOVC strains lack the primary virulence factors of O1 and O139, they can produce several other toxins, including hemolysin, repeat-in-toxin, cholix toxin, and heat-stable enterotoxin (Schwartz *et al.* 2019). These toxins, along with large pathogenicity gene islands—such as *Vibrio* pathogenicity islands, *Vibrio* seventh pandemic islands, and secretion systems—contribute to the virulence potential of NOVC (Ramamurthy *et al.* 2020).

1.2 Non-O1/non-O139 *Vibrio cholerae*

1.2.1 Clinical Records

The virulence potential of non-O1/non-O139 *Vibrio cholerae* (NOVC) has been confirmed through numerous clinical reports of infections worldwide. Symptoms range from mild to severe, and both intestinal and extraintestinal diseases have been observed (Igere *et al.* 2022). The two main sources of infection are the consumption of NOVC-contaminated food and direct contact with contaminated water or sediment. In Germany, 63 cases of NOVC infection were reported between 2011 and 2021, with conditions including gastroenteritis, otitis, bloodstream infections, and wound infections (Schmidt *et al.* 2023). In 2018 and 2019, Brehm *et al.* (2021) reported 30 additional cases of NOVC infection, associated with wound infections, otitis, gastroenteritis, pneumonia, and primary septicemia. Furthermore, two NOVC strains isolated from wound and ear infections were identified by Schwartz *et al.* (2019) in 2016 and 2017, respectively.

1.2.2 Infection Route and Virulence-Associated Genes

The infection route of *V. cholerae* has been studied to better understand how these bacteria cause disease and manifest clinical symptoms. Understanding the infection pathway is also critical in this doctoral project for analyzing the genetic factors contributing to NOVC virulence.

As a gastrointestinal pathogen, *V. cholerae* follows a five-stage infection process: survival in

the gastrointestinal environment (Stage I), reaching epithelial cells (Stage II), attachment (Stage III), proliferation and production of virulence factors (Stage IV), and detachment (Stage V) (Figure 1).

In Stage I, genes involved in acid tolerance, resistance to reactive oxygen species, and resistance to reactive nitrogen species are crucial for survival. In Stage II, motility, biofilm formation, and chemotaxis genes play a significant role in reaching epithelial cells. Stage III involves three non-specific adhesins that have been identified as important for the initial adherence to epithelial cells.

In Stage IV, NOVC can produce a variety of toxins, such as hemolysin, repeat in toxin, cholix toxin, and heat-stable enterotoxin. These toxins disrupt the structure of intestinal cells, leading to diarrhea (Queen and Satchell 2012; Jørgensen *et al.* 2008; Krasilnikov *et al.* 1992; Arita *et al.* 1986). Additionally, the type III secretion system (T3SS) can damage the epithelial cell membrane, resulting in lethal diarrhea in mouse models (Miller *et al.* 2016).

Although *Vibrio* pathogenicity island 1 (VSP-1), which aids in colonization and proliferation, is absent in most NOVC strains, the presence of *Vibrio* pathogenicity island 2 (VSP-2) provides NOVC with an advantage in penetrating the gut mucosa. This is due to its neuraminidase, which cleaves sialic acid and alters the structure of mucin, aiding in infection (Jermyn and Boyd 2005). These virulence factors are regulated by a network of regulatory genes, such as those involved in quorum sensing, to ensure their proper function (Lo Scrudato and Blokesch 2012).

The infection route of NOVC and the associated virulence genes are systematically studied in Chapter 2.1. Additionally, regulators are integrated into this model due to their role in coordinating virulence activities. Global regulatory genes, such as quorum sensing, histone-like nucleoid structuring proteins, and cyclic AMP-activated global transcriptional regulators, are also included in the infection pathway. A large number of two-component system (TCS) regulators were reviewed, and virulence-associated TCS were identified through genetic analysis. Further details on the infection pathway are discussed in Chapter 2.1.
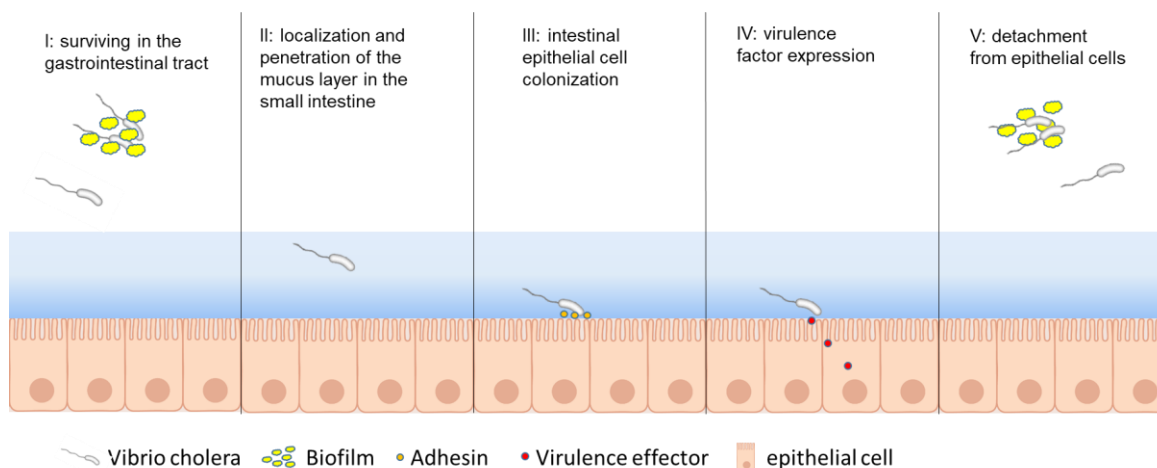
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Figure 1. ***Vibrio cholerae*** infection route in the human gastrointestinal tract.

The infection route of *V. cholerae* is divided into five stages: Stage I - survival in the gastrointestinal environment; Stage II - reaching the epithelial cells; Stage III - attachment to the epithelial cell surface; Stage IV - production of virulence factors; and Stage V - detachment.

1.2.3 Antimicrobial Resistance-Associated Genes

Vibrio cholerae employs three primary mechanisms for antimicrobial resistance (AMR): efflux pumps, point mutations, and enzyme-dependent inactivation (Das *et al.* 2020). Efflux pumps expel harmful molecules, including antibiotics, antimicrobial peptides, and heavy metals, from the bacterial cell. These pumps are powered by ATP hydrolysis and the proton-motive force. One example, VcaM, an ATP-binding cassette (ABC) transporter, is known to resist ofloxacin, streptomycin, ethidium bromide, tetraphenylphosphonium chloride, rhodamine 6G, and acridine orange (Huda *et al.* 2003). In addition, four types of proton-motive-force-driven pumps have been identified: MATE (multidrug and toxic compound extrusion), MFS (major facilitator superfamily), RND (resistance-nodulation-cell division), and SMR (small multidrug resistance) (Sharma *et al.* 2020).

Point mutations can alter the target sites of antibiotics, reducing their binding and effectiveness. Notable mutations include *parC/gyrB* (involved in DNA replication and repair, and the binding site for quinolones), *pbp* (for cell wall synthesis and beta-lactamase binding),

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katG (for resistance to reactive oxygen species), *vpsL* (for cell wall synthesis), and *rpoB* (for DNA transcription).

Another AMR mechanism is the hydrolysis or modification of antimicrobial molecules. *V. cholerae* has evolved genes such as *bla* (for beta-lactam hydrolysis), *ant* (for aminoglycoside nucleotidylation), and *fomA-C* (for fosfomycin phenylation) (Das *et al.* 2020). These AMR genes are often carried on mobile genetic elements such as integrating conjugative elements (ICEs), integrons, and plasmids (Verma *et al.* 2019). One common ICE in *V. cholerae*, SXT/R391, often carries various AMR factors, facilitating the horizontal transfer of resistance genes (Spagnoletti *et al.* 2014).

With advances in bioinformatics, complex AMR profiles have been documented, and two key online databases - ResFinder and the Comprehensive Antibiotic Research Database - provide valuable tools for tracking AMR information (Florensa *et al.* 2022; McArthur *et al.* 2013). Studies on NOVC phenotypic resistance reveal similar patterns. Sellek *et al.* (2012) collected NOVC isolates from Poland, Norway, and Spain, which demonstrated resistance to ampicillin, tetracycline/doxycycline, trimethoprim, gentamicin, nitrofurantoin, streptomycin, nalidixic acid, and colistin. In Iran, Bakhshi *et al.* (2009) found a comparable resistance profile, though some isolates remained susceptible to gentamicin.

1.2.4 Environmental NOVC and Extraintestinal Infections

As an aquatic commensal, NOVC has been detected in estuarine and coastal waters globally. *V. cholerae* typically thrives in temperatures between 20°C and 45°C (Martinez *et al.* 2010), explaining its frequent presence in warm waterbodies. Singh *et al.* (2001) reported NOVC isolates from environmental samples in India that were positive for *hlyA* and caused fluid accumulation in the rabbit gut. Crowe *et al.* (2016) documented toxigenic NOVC in the United States, highlighting cases linked to recreational water exposure. Similarly, NOVC isolates from estuarine environments in China were found to harbor virulence-associated genes such as *mshA*, *hlyA*, *rtxC*, *rtxA*, and the type III secretion system (T3SS) (Li *et al.* 2014). Other

studies also reported NOVC strains with virulence genes in Thailand (Ruenchit *et al.* 2018) and Chile (Arteaga *et al.* 2020).

In northern Europe, NOVC has been found in the North Sea and Baltic Sea. Schwartz *et al.* (2019) identified NOVC strains in the Baltic Sea, many of which carried virulence-associated genes such as *hlyA* and *rtxA*, as discussed in Section 1.2.2 (page 5). With global warming and increased recreational water use, environmental NOVC infections have risen (Baker-Austin *et al.* 2016). Exposure to environmental NOVC can result in extraintestinal infections such as wound infections, otitis, and bacteremia (Schmidt *et al.* 2023).

In cases of extraintestinal infection, NOVC can enter the bloodstream, where the host's immune system acts as a barrier to colonization and toxin production (Hickey and Kubes 2009). NOVC can form biofilms, which protect the bacteria from leukocytes and increase local concentrations. In these biofilms, hemolysin (*hlyA*) can lyse immune cells (Vidakovic *et al.* 2023), while *hlyA* also plays a critical role in damaging host target cells (Kechker et al. 2017). Additionally, the RTX toxin helps *V. cholerae* evade macrophages by crosslinking actin (Queen and Satchell 2012).

Publication 1: Non-O1/Non-O139 *Vibrio cholerae*-An Underestimated Foodborne Pathogen? An Overview of Its Virulence Genes and Regulatory Systems Involved in Pathogenesis (Review article)

Quantao Zhang, Thomas Alter, Susanne Fleischmann

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I developed the search strategy for the literature, performed independent title, abstract and full-text screening, created the infection model and the illustration, and wrote the first draft. In my function as first author, I considered and responded to the reviewer comments together with all co-authors.

Publication 2: Genetic and phenotypic virulence potential of non-O1/non-O139 *Vibrio cholerae* isolated from German retail seafood

Quantao Zhang, Thomas Alter, Eckhard Strauch, Jens Andre Hammerl, Keike Schwartz, Maria Borowiak, Carlus Deneke, Susanne Fleischmann

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I performed the literature research, the investigation, the data curation, created the illustration and wrote the first draft. In my function as first author, I considered and responded to the reviewer comments together with all co-authors.

Publication 3: German coasts harbor non-O1/non-O139 *Vibrio cholerae* with clinical virulence gene profiles

Quantao Zhang, Thomas Alter, Eckhard Strauch, Inga Eichhorn, Maria Borowiak, Carlus

2. Publications

Deneke, Susanne Fleischmann

I performed the literature research, the investigation, the data curation, created the illustration and wrote the first draft. In my function as first author, I considered and responded to the reviewer comments together with all co-authors.

Review

Non-O1/Non-O139 *Vibrio cholerae*—An Underestimated Foodborne Pathogen? An Overview of Its Virulence Genes and Regulatory Systems Involved in Pathogenesis

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Abstract: In recent years, the number of foodborne infections with non-O1 and non-O139 *Vibrio cholerae* (NOVC) has increased worldwide. These have ranged from sporadic infection cases to localized outbreaks. The majority of case reports describe self-limiting gastroenteritis. However, severe gastroenteritis and even cholera-like symptoms have also been described. All reported diarrheal cases can be traced back to the consumption of contaminated seafood. As climate change alters the habitats and distribution patterns of aquatic bacteria, there is a possibility that the number of infections and outbreaks caused by *Vibrio* spp. will further increase, especially in countries where raw or undercooked seafood is consumed or clean drinking water is lacking. Against this background, this review article focuses on a possible infection pathway and how NOVC can survive in the human host after oral ingestion, colonize intestinal epithelial cells, express virulence factors causing diarrhea, and is excreted by the human host to return to the environment.

Keywords: non-O1/non-O139 *Vibrio cholerae*; diarrheal disease; infection pathway; virulence genes; regulatory systems

1. Introduction

Vibrio (*V.*) *cholerae* is mainly known as the causative agent of the endemic and epidemic diarrheal disease cholera. However, *V. cholerae* is a globally distributed aquatic commensal that has been classified into more than 200 serogroups. Only two serogroups, O1 and O139, have the ability to cause pandemic cholera outbreaks. Since 1961, the *V. cholerae* serotype O1 biotype El Tor has been the predominant strain in the seventh pandemic, and since 1992, the *V. cholerae* serotype O139 has been the predominant strain in the eighth pandemic. Both pandemics are still ongoing today [1,2]. The World Health Organization (WHO) reported outbreaks of cholera in 30 countries in Asia, Africa, and America between 1 January and 15 December 2023, with over 667,000 cases and 4000 deaths [3].

Non-O1 and non-O139 *V. cholerae* (NOVC) serogroups are less in the focus of public health interest compared to O1 and O139 *V. cholerae* as they cause single-case disease or even localized outbreaks with milder and often self-limiting symptoms. In fact, one or both of the main virulence factors, cholera toxin (CT) and toxin-coregulated pilus (TCP), are missing in their genomes. Nevertheless, NOVCs are playing an increasingly important role in public health worldwide. Several studies have shown that the number of infections and outbreaks caused by NOVC has increased over time, being positively correlated with the progressive rise in seawater temperatures [4–7]. This is promoted by the anthropization of coastal regions, the increasing global trade of seafood, the trend towards the consumption of raw seafood (e.g., oysters and sushi), and the increasing number of immunocompromised people, especially older people with pre-existing diseases [7,8]. Particularly people with a compromised immune system can suffer from severe diarrhea with cholera-like symptoms. Bacteremia can also be caused by an orally acquired infection

via the infiltration of NOVC in the bloodstream through the portal vein and intestinal lymphatic system [8–10]. Individual infection cases that could be clearly attributed to the consumption of contaminated seafood have been described in Spain [11], Italy [12], Portugal [13], India [14], Australia [15], the USA [16], and Iran [17]. Localized NOVC outbreaks have been reported in India and Thailand in the past [18–23]. Meanwhile, NOVC outbreaks have also been described in the USA [24,25], China [26,27], and Chile [28] which have also been linked to seafood consumption.

Octavia et al., 2013, pointed out that a combination of virulence factors in the genome of clinical NOVC is a prerequisite for a successful infection process [29]. The combined virulence factors identified in the genomes of NOVC isolated from the above-mentioned infection cases and local outbreaks are the *Vibrio* pathogenicity islands VSP-2 and VPI-2, genomic islands (GI) encoding type III (T3SS) and type VI secretion systems (T6SS), enterotoxins (RtxA and Stn), and the hemolysin HlyA. We were able to show that genes encoding these virulence factors are also present in NOVC isolated from seafood and the environment in previous studies [30,31]. Further investigations showed that other virulence genes are also present in the NOVC genomes which could also play a role in the infection process, such as *hapA* for hemagglutinin protease; *mshA* for mannose sensitive hemagglutinin; and *frhA*, *gbpA*, and *mam7* for non-specific adhesins [30].

In addition to the presence of virulence factors, genes involved in host adaption and colonization are also required in the pathogenicity process. Before a successful infection, pathogens need to survive the host defense system such as acidic pH values in the stomach, anti-microbial peptides, reactive oxygen species (ROS), and an already predominant gut microbiota [32]. Mucosal penetration and epithelial cell attachment in the small intestine are also necessary for the final infection and proliferation of the bacteria [33]. *V. cholerae* has evolved a complex regulation system to ensure proper arrangement of various effective factors throughout the infection inside a human host, such as the quorum sensing system, two-component system, histone-like nucleoid structuring protein (Hns), small molecule signals (c-di-GMP), biofilm promotor and motility repressor modulation, and wide spectrum regulator (cAMP-CRP) [34].

Thus far, there are several comprehensive overviews about the virulence-associated genes in both O1/O139 *V. cholerae* and NOVCs [35,36]. Nevertheless, it should be noted that the relationship between virulence factors and a resulting infection is complex, and an interaction network rather than individual virulence factors must be considered at this point. The previous findings on virulence-associated genes and their interaction with other genetic features involved in the infection process will be discussed in this review article. Furthermore, a genetic model of a theoretical infection caused by NOVC inspired by the Kyoto Encyclopedia of Genes and Genomes (KEGG) mapping tool [32,37–39] was developed (see Figure 1). The whole infection workflow was divided into five stages as follows. Stage 1: survival in host gastrointestinal tract; stage 2: localization and penetration of the mucus layer in the small intestine; stage 3: intestinal epithelial cell colonization; stage 4: virulence gene expression; stage 5: detachment from the epithelial cells to return in the environment.
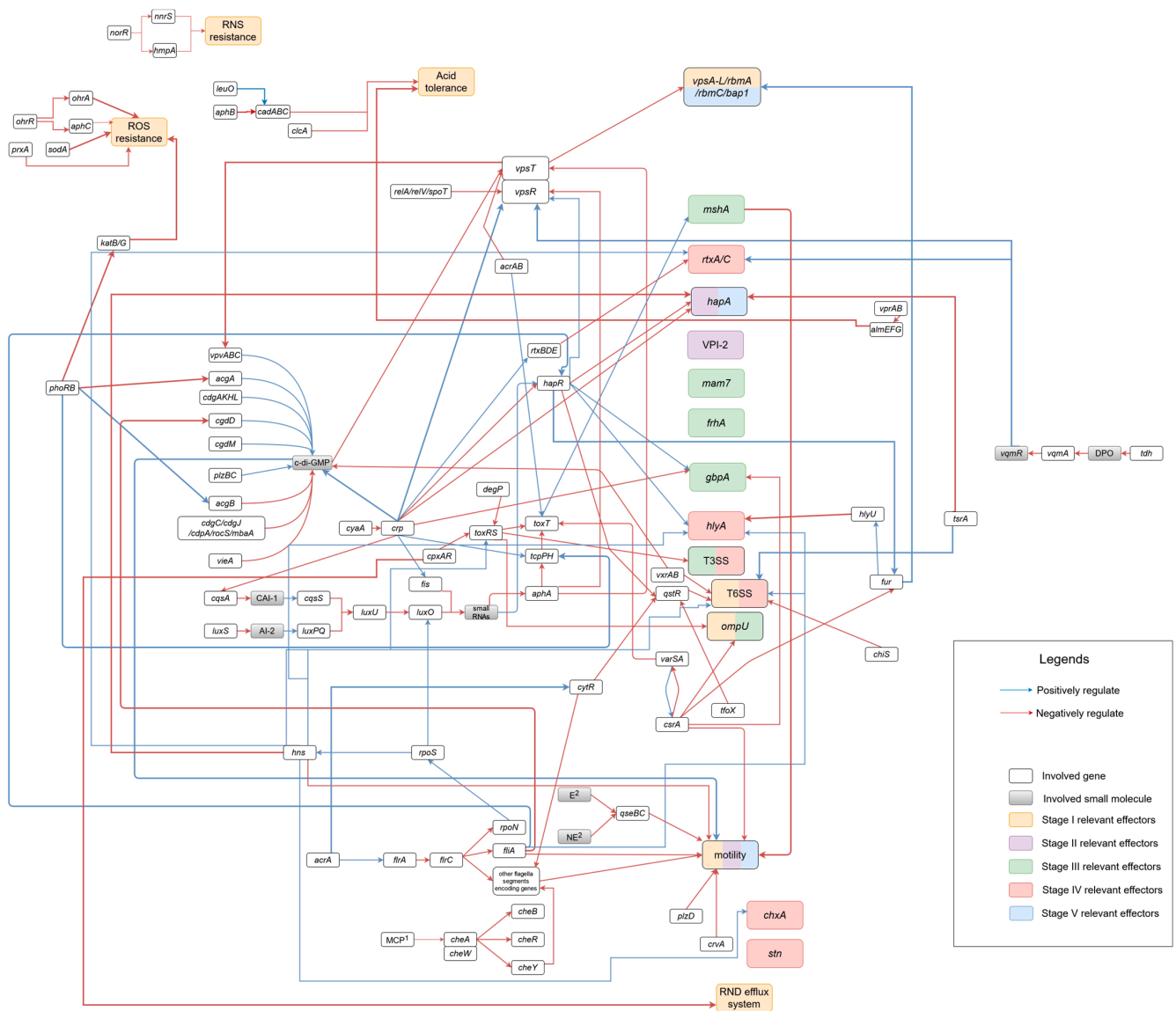
Figure 1. The map of virulence-associated genes and regulatory systems in NOVC: positive relationships are labeled with red arrows and negative relationships are labeled with blue arrows. The whole infection procedure is separated into five stages. Stage 1: survival in host gastrointestinal tract (in orange); stage 2: localization and penetration of the mucus layer in the small intestine (in purple); stage 3: intestinal epithelial cell colonization (in green); stage 4: virulence gene expression (in red); stage 5: detachment from the epithelial cells to return in the environment (in blue). The detailed information is shown in Table S1 in the Supplementary Files.

2. Stage 1: Survival in the Gastrointestinal Tract

After oral ingestion, pathogenic bacteria will encounter a set of host-derived defense systems, including chemical and biological barriers, when entering the stomach and arriving at the small intestine. Therefore, various genes involved in adaptation processes as a response to these conditions can be found in NOVCs to ensure that they reach the small intestine to interact with epithelial cells [40,41]. The stage 1 section therefore describes the adaptation to low pH values in the stomach [41,42]; the adaptation to reactive nitrogen and oxygen species in the stomach [43,44]; changes in porin channel size to prevent the diffusion of harmful molecules into the bacterial cell such as bile salts from the gallbladder in the duodenum [45]; efflux pumps to displace harmful molecules such as bile in the duodenum and antimicrobial peptides in the small intestine [46]; the formation of protective biofilms

to protect bacteria against antimicrobial substances from the stomach, duodenum, and the small intestine; and the T6SS to compete with the predominant gut microbiome [47]. All mechanisms and genes involved in stage 1 are shown in orange in Figure 1.

2.1. Acid Tolerance Response

A common feature of diarrheal pathogens is the acid tolerance response (ATR), necessary to survive the acidic pH environment in the stomach, which is a prerequisite for subsequent successful intestinal colonization [42]. In *V. cholerae*, the *cadABC* operon first described in *Escherichia* (*E.*) *coli* is important for protecting the bacteria from acid hydrolysis [40]. The genetic presence of the *cadABC* operon was identified in 90% of NOVC isolated from seafood and the environment in our previous studies, with genetic identities over 90% compared to the *V. cholerae* O1 El Tor biotype, suggesting a fully functional *cadABC* operon [30,31]. In particular, the *cadA* gene encodes a lysine decarboxylase that binds protons through the production of cadaverine and carbon dioxide. Finally, this antiporter system transfers protons out of the bacterial cell and neutralizes the pH value [40]. Kovacikova et al., 2010, mentioned that *cadC*, the regulator of the *cadABC* operon, can be directly activated by *aphB* encoding a cytoplasmic DNA-binding protein which will be upregulated during acid stress [48]. Additionally, the expression of *clcA*, a hydrochloric acid transporter, regulated by *aphB*, plays a role in neutralizing the pH value in the bacterial cell [49].

2.2. Adaptation to Reactive Nitrogen and Oxygen Species

In the stomach, nitrite from food and saliva that is exposed to the acidic milieu results in acidified nitrite, which can be reduced by reactive nitrogen species (RNS) to antimicrobially active nitric oxide. By the detoxification of RNS, the expression of the genes *nnrS* and *hmpA* plays an important role in *V. cholerae*, and these genes were also identified in NOVCs from seafood and the environment [30,31]. Both genes encode enzymes that are capable of destroying nitric oxide. It is assumed that the regulator for both genes is *norR*, although this regulator is not stimulated by nitric oxide [43].

In diarrheal diseases, the level of reactive oxygen species (ROS) in the host gastrointestinal tract increases, resulting in damage to the bacterial cell structure as an immune defense. In *V. cholerae*, genes with ROS resistance activity have been identified as part of ROS removal. Superoxide dismutases such as manganese-binding SodA, for example, convert superoxide into hydrogen peroxide and oxygen. Catalases such as KatB and KatG later detoxify peroxides into water and oxygen [50,51]. The organic hydroperoxidase OhrA and preoxiredoxins such as PrxA and AphC cleave organic (alkyl) hydroperoxides [52,53]. Two homologs of *ohrR*, the gene for hydrogen peroxide resistance in *E. coli*, were found as well in *V. cholerae*, namely *oxyR*1 and *oxyR*2, which have a modulating function on *prxA* and *aphC*, respectively [52].

2.3. Resistance Nodulation Division (RND) Efflux Pump

After passing through the stomach, pathogenic bacteria in the small intestine must resist against host-derived bile salts, organic acids, and antimicrobial peptides. The main systems which help the bacteria to pump numerous poisonous compounds out of the cell are efflux pumps [54]. The RND efflux pump is a multi-functional unit in both O1/O139 *V. cholerae* and NOVC [55], encoded by a vex gene cluster (*vexAB*, *vexCD*, *vexEF*, *vexGH*, *vexIJK*, and *vexLM*) and a shared outer membrane porin encoded by *tolC*. Using an infant mouse model, small intestine colonization deficiency was found in different RND mutants [46,56].

2.4. Outer Membrane Protein (OMP)

Another response to toxic components such as bile salts is the alteration of porins in the bacterial cell membrane. In *V. cholerae*, expression and upregulation of *ompU* take place when bile salts are present. OmpU is widely present in NOVC [9,57,58] and should have the same response to the presence of bile. Due to its smaller channel size which prevents

the influx of bile salts into the bacterial cell, the porin OmpU will be replaced instead of a larger channel porin such as OmpT [45,59].

2.5. Biofilm Formation

Biofilms provide a continuous protective cover around bacterial cells against multiple harmful components and play an important role in environmental adaptation and survival in the host [39]. In *V. cholerae*, *Vibrio* polysaccharides encoded by *vpsA* to *vpsK* and other biofilm-forming proteins encoded by the genes *rbmA*, *rbmC*, and *bap1* are the main structure components that build a stable biofilm [60,61], while *vpsR* and *vpsT* serve as transcriptional activators [62,63]. Another small molecule signal, c-di-GMP, also has a positive effect on biofilm formation through the upregulation of *vpsR* and *vpsT* [64]. The intracellular concentration of c-di-GMP can be increased in the presence of bile [65]. The presence of *vpsR* in NOVC was confirmed by Dua et al. (2018) [66], and variations in VpsR between O1/O139 *V. cholerae* and NOVC were identified in our previous studies, including point mutation and gene fragment deletions [30]. However, 99% of NOVCs could form stable biofilms in our previous studies, and all the biofilm-relevant genes were present in NOVCs [30,31].

2.6. Type IV Secretion System (T6SS)

Entering the small intestine, the T6SS plays an important role in competition between microorganisms, so that the distribution of commensals in the intestine is altered [47,67]. Therefore, it might act in stage 1 as well as in stage 4. In our previous studies, all NOVCs contained the T6SS [30,31]. In the *V. cholerae* strain O1 C6706, the T6SS is repressed at low cell density by quorum sensing (QS) molecules [68]. In contrast, N-acetyl glucosamine (GlcNac) can be sensed by the O1 *V. cholerae* serotype, which leads to *tfoX* (a major regulator of T6SS) expression followed by T6SS activation [69]. The regulation network of NOVCs is complex and not fully explored, but these regulators might have similar effects in NOVCs [70]. Three regulatory genes, *hapR*, *tfoX*, and *cytR*, achieve their T6SS regulation through the QS- and TfoX-dependent regulator (QstR) [34].

3. Stage 2: Localization and Penetration of the Mucus Layer in the Small Intestine

To cause diarrhea, *V. cholerae* need to reach the small intestinal epithelial cells to penetrate them. However, the intestinal epithelium is covered by a mucus layer (approximately 150 μm thick), making the ability to penetrate mucus important [32]. Motility is therefore necessary and responsible for a targeted direction [71], while the contribution of chemotaxis remains controversial [72,73]. In contrast to O1/O139 *V. cholerae* serotypes, whose fitness is supported by genetic features on *Vibrio* seventh pathogenicity islands 1 and 2 (VSP-1 and VSP-2), the movement of NOVC through the mucosa could be supported by the hemagglutinin protease and neuraminidase, which act as mucinases and are encoded on *Vibrio* pathogenicity island 2 (VPI-2) [1,74,75]. In addition, environmental NOVCs isolated from food and water sources carry not only the pathogenicity island VPI-2 but also the pathogenicity island VSP-2 in their genome [30,31]. All mechanisms and genes involved in stage 2 are shown in purple in Figure 1.

3.1. Motility via Flagella

The motility-related genes in NOVC were detected through Gene Oncology analysis and the KEGG pathway, which indicate the similar function of motility between NOVC and O1/O139 *V. cholerae* as these databases are mainly built based on research on O1/O139 *V. cholerae* [76]. All the motility-associated genes were identified to 100% in NOVC according to our previous studies [30,31]. As a highly motile bacterium, the driving force of *V. cholerae* is provided by a single polar flagellum. Motility is also functional in host environment adaptation, including nutrient acquisition and toxic component avoidance [77]. The flagellar motility of *V. cholerae* is important to move the bacteria through the mucus layer [78]. The structure of the flagellum and its four-hierarchy regulatory system was already described by Syed et al. (2009) [79]. The whole flagella system is regulated by sigma factor 54 FlrA, the

downstream activator FlrC, and the alternative sigma 28 factor FliA [79]. The motility of *V. cholerae* also declined due to c-di-GMP. Furthermore, several genes with motility regulation activity were reported. The multifunctional regulation gene *csrA* could upregulate *flrC* [80]. Besides, under a high-speed microscope, *arcA/cytR* and the O-antigen synthesis gene *cmd* were found to promote motility with an unclear mechanism [81].

3.2. Chemotaxis

The chemotaxis system can recognize chemical signals and regulate the motility and swimming behavior of *V. cholerae*. At first, a common chemotaxis model of *E. coli* was identified and subsequently applied in a chemotaxis study of *V. cholerae* [82] including methyl-accepting chemotaxis proteins (MCPs) encoded by *cheW*, *cheA*, *cheY*, *cheR*, and *cheB*. As the chemotaxis system in NOVC has not been explored and the chemotaxis-related genes in O1/O139 *V. cholerae* were identified in several NOVCs, we suspected that the chemotaxis system in NOVCs might play a similar role. In our previous study, we confirmed the presence of the genes *cheA*, *cheY*, and *cheR* in all analyzed NOVCs, while *cheW* and *cheR* were present in 32% of the strains [30]. Transmitted signals can be caught by the cytoplasmic linker protein *cheW* and transmitted to the two-component system *cheA/cheY*. Phosphate-activated *cheY* binds to the flagella motor and causes a reverse rotation direction, from left to right [72]. The genes *cheR* and *cheB* play a role in the transfer of methyl groups, which contributes to adaptation to a stable background level of attractants [82]. On the other hand, *V. cholerae* (both O1/O139 and NOVC) have a far more complex chemotaxis system than *E. coli*, with 68 related ORFs categorized into three clusters. Among those, cluster II seems to play a similar role in *E. coli* [83]. Later research reported that cluster I components are assembled into the supramolecular signaling complex in response to reduced cellular energy states, raising the possibility that the cluster I complex plays a role in sensing and signaling under microaerobic environments, such as in the host intestine [84]. The general stress regulator RpoS and autoinducer 1 in quorum sensing could regulate the expression of cluster III [85].

3.3. Vibrio Pathogenicity Island 2 (VPI-2)

VPI-2 (located between vc1758 and vc1809) was identified in O1/O139 *V. cholerae* and NOVCs. However, Jermyn and Boyd (2005), Haley et al. (2014), and Takahashi et al. (2021) studied the genetic variation of VPI-2 in NOVCs and showed that NOVCs could harbor an incomplete VPI-2 compared to O1/O139 [74,86,87]. This variation might result from the horizontal gene transfer of VPI-2 from the ancestors *V. mimicus* and O1/O139 *V. cholerae* [74]. VPI-2 contains the neuraminidase-encoding gene *nanH* [88] which plays a role in altering mucus structure by cleaving sialic acid groups (GM1 gangliosides) on the epithelial cell surface. Further studies on VPI-2 revealed that *nanA*, *nanE*, *nanK*, and *nagA1*, which are also localized on VPI-2, can catalyze the metabolism of N-acetylneuraminic acid, which is a component of mucin [6]. All of these functional genes in VPI-2 were identified in 33% of NOVCs isolated from seafood and the environment [30,31].

Vertebrate hosts could limit the zinc level for bacteria as a defense strategy. Zinc deficiency activates *Vibrio* energy taxis system A (VerA), which is also encoded on the pathogenicity island VSP-2. In addition, VerA could trigger the expression of *aerB* transcribing a methyl-accepting chemotaxis protein, which could bind *cheW* and affect the flagellum rotation and motility [89,90].

3.4. Hemagglutinin Protease HapA

The hemagglutinin protease HapA, encoded by *hapA*, is suggested to be responsible for altering the mucus layer and playing a role in mucus layer penetration during initial infection for both O1/O139 *V. cholerae* and NOVC [91,92].

4. Stage 3: Intestinal Epithelial Cell Colonization

After the localization of intestinal epithelial cells, *V. cholerae* must attach to their surface, whereby the type IV pili and the T3SS play a crucial role [93,94]. Subsequently, non-specific adhesins can be secreted via these systems [32]. In contrast to O1 and O139 *V. cholerae*, the T3SS plays an important role for NOVC in attachment and colonization when TCP is not present in the genome [94]. All mechanisms and genes involved in stage 3 are shown in green in Figure 1.

4.1. Type IV Pili

Type IV pili, encoded by *mshA*, play a role in the braking and anchoring function of *V. cholerae* during the landing process on the epithelial cell surface [95]. In addition, MshA pili cause an irreversible attachment and microcolony formation [93]. The presence of *mshA* in 27% of NOVCs was confirmed in our previous studies [30,31]. At the beginning stage after landing on the epithelial cell surface, several transient non-specific adhesins were secreted to bind the component of small intestine epithelial cells, including multivalent adhesion molecule 7 (*mam7*, binding with fibronectin and phosphatidic acid), GlcNAc binding protein A (encoded by *gbpA*), and flagellum-regulated hemagglutinin A (encoded by *frhA*, binding calcium) [79,96,97]. The presence rates of *mam7*, *gbpA*, and *frhA* in NOVCs were detected as 100%, 94%, and 22%, respectively, in our previous studies [30,31]. Sperandio et al., 1995, stated that a potential adherence factor to epithelial cells could be the outer membrane protein U (OmpU) [98]. This finding is supported by Potapova et al., 2024, who also mentioned that OmpU could also regulate the biofilm matrix assembly [99].

4.2. Type III Secretion System (T3SS)

The T3SS is suggested to have an important role in the colonization of intestinal epithelial cells by NOVCs when TCP is absent. Dziejman et al., 2005, showed using a rabbit and mouse model that the TCP-negative NOVC strain AM-19226 could colonize the intestinal epithelial cell surface through the T3SS [94]. The whole island contains 47 ORFs from A33_1660 to A33_1706. However, an exact mechanism of the T3SS in colonization has not yet been fully identified, although possible functions of several effectors have been addressed: VopF (A33_1696) and VopM (A33_1684) are two effectors in the core region with actin alteration activities, which could disrupt the cell structure and contribute to colonization [100]. VopM can bind F-actin and also plays an important role in colonization by remodeling the intestinal brush border, which facilitates bacterial adhesion [101]. The colonization activity of VopX (A33_1663) in AM-19226 was also stated by Alam et al. [102], and a contradictive result was reported by Chaand et al. [103]. Meanwhile, the T3SS is important for toxicity and toxin transfer; therefore, this part is explained further in stage 4.

5. Stage 4: Virulence Factor Expression

In contrast to the *V. cholerae* serovars O1 and O139, which express cholera toxin (CTX) and its accessory toxins within the CTX phage, various toxins can be produced by NOVCs after colonization of the small intestine. Currently, four secreted proteins with direct toxic effects shown on cell lines and in animal models have been identified: the hemolysin HlyA, repeats-in-toxin (RTX), heat-stable enterotoxin (ST), and cholix toxin (ChxA) [104–107]. The expression of these toxins leads to an alteration in the morphology of epithelial cells, cell damage, and subsequently to the death of the cells [35]. Similar to *V. cholerae* serotypes O1 and O139, whose virulence is supported by genetic features on VPI-1 and VPI-2 [1], VPI-2 was also identified in NOVC environmental isolates from food and water [30,31]. In addition to the toxin genes, the T3SS also plays an important role for NOVC toxicity by secreting virulence factors from the bacteria to the host cells when TCP is missing in the genome [94]. All mechanisms and genes involved in stage 4 are shown in red in Figure 1.

5.1. Toxin Expression

The hemolysin HlyA (also called *V. cholerae* cytolysin, VCC) could both lyse erythrocytes and form beta barrel pores on epithelial cells [104], followed by cytoskeleton damage, cell lysis, and diarrhea. The iron extracted from the cells in this way serves as a nutrient supplier for NOVCs [108,109]. The transcription of *hlyA* in *V. cholerae* is regulated by QS molecules, which regulate *hlyU*, resulting in the highest transcription of *hlyA* in the early mid-logarithmic growth phase [110].

Repeats-in-toxin (RTX) is a large protein (around 3500 to 5300 amino acids) widely present in many bacteria which could cause tight junction loss in lung and intestinal epithelial cells [111]. The in vivo toxicity of RTX in *hlyA*-harboring *V. cholerae* tends to present as innate immune evasion rather than diarrhea [105,112]. Three major functional units of RTX were found in *V. cholerae* O1 El Tor N16961. The actin cross-linking domain (ACD) is responsible for cytoskeleton disruption, the Rho GTPase-inactivation domain (RID) for cell rounding, and the alpha/beta hydrolase domain (ABH) for autophagic/endosomal trafficking inhibition. An additional cysteine protease domain is responsible for effectors' autoprocessing and distribution. The combination of RID and ABH could reduce the inflammatory response caused by ACD [113]. Compared to the El Tor O1 serogroup, NOVCs have more variations in their RTX domain [114]. A nucleotide cluster with five ORFs is responsible for the coding of RTX: the encoding toxin gene *rtxA*, the activator gene *rtxC*, and the associated ABC transportation gene cluster *rtxBDE* [113]. The whole RTX complex was identified in 61% of NOVCs in our previous studies [30,31].

Heat-stable enterotoxin (ST, encoded by *stn*) is a known toxin in *E. coli* and was also identified in the genome of NOVC [106,115]. The in vivo toxicity was attributed to fluid accumulation in mouse intestine [106]. The toxin consists of two domains, STa and STb. STa leads to anion secretion and calcium absorption, while STb could decrease the expression of the tight junction proteins ZO-1 and occludin [116].

The cholix toxin ChxA interacts with prohibitin and could therefore cause mitochondrial dysfunction and cytoskeletal remodeling. It is able to bind the lipoprotein receptors of the intestinal epithelial cells and inhibit protein synthesis by ADP-ribosylation. The in vivo toxicity presented as liver damage and final death through mouse assay [107,117]. Tangestani et al., 2020, also confirmed the presence of cholix toxin in NOVC [17].

5.2. Type III Secretion System (T3SS)

The T3SS plays an important role in NOVC after the colonization of intestinal epithelial cells in toxicity. Dziejman et al., 2005, suggested using a mouse model that the T3SS-positive NOVC strain AM-19226 causes mouse death in contrast to a T3SS-negative mutant strain [94]. The protein VopF contains three WASP homology 2 (WH2) actin-binding domains, which could remodel the actin cytoskeleton in eukaryotic host cells. The actin polymerization disorder triggered by VopF is essential for T3SS-mediated intestinal cell damage in AM-19226 [118]. The mechanism might be that VopF could induce cortical actin depolymerization and aberrant localization of the tight junction protein ZO-1, resulting in loosening of the tight junction between intestinal epithelial cells and causing diarrhea [119]. However, Miller et al., 2016, observed that cell death and disruption of the tight junction are independent of VopF [120]. It has been suggested that VopE (A33_1662) impairs mitochondrial dynamics and stimulates the innate immune pathway [121]. Furthermore, the in vivo toxicity of VopE was verified in an infant rabbit and mouse model [122]. The regulators VttRA and VttRB, which show homology with ToxR, can control T3SS activity both during colonization and pathogenesis [123].

Bacteremia Caused by NOVCs

When NOVCs enter and colonize the small intestine (as described in stages 1 to 3), they could enter the bloodstream through the portal vein and the intestinal lymphatic system [8,9]. The immune system, macrophages, and specific antibodies are involved in the blood defense system, indicating that genes for immune modulation are important for

NOVCs to cause blood infections [124,125]. Hemolytic properties, such as the presence of HlyA, suggest their ability to enter the bloodstream and lyse erythrocytes [104]. RTX could protect NOVCs from neutrophil-dependent clearance [105]. Biofilms could protect NOVCs from leukocytes of the human immune system. Additionally, NOVCs can form biofilms on the eukaryotic cell surface, causing a concentration of MshA and HapA, which could increase the local hemolysin level lysing immune cells [126].

6. Stage 5: Detachment from the Epithelial Cells

At the end of the infection cycle, NOVCs return to the environment through watery to bloody diarrhea. The symptoms of infection can be closely similar to those of the cholera caused by the serotypes O1 and O139. The starvation/stationary phase alternative sigma factor RpoS positively controls the expression of HapR, a gene involved in flagella assembly and chemotaxis. This enables the detachment and migration of NOVCs from the epithelial cells into the lumen of the intestine [127]. After the activation by RpoS, the hemagglutinin protease HapA encoded by *hapA* is responsible for detachment from intestinal epithelial cells [75]. Apart from the mucinase activity, HapA could degrade GbpA, the non-specific adhesin in colonization [128]. Furthermore, a set of potential biofilm degradation genes were also identified by Bridges et al., 2020 [129]. These genes include ribosome-associated GTPase encoded by *bipA*; c-di-GMP phosphodiesterases encoded by *cdgG*, *cdgI*, *rocS*, and *mbaA*; a polyamine transporter encoded by *potD*1; a peptidase encoded by *lapG*; a polysaccharide lyase encoded by *rbmB*; and a chemotaxis regulator encoded by *cheY*3. All genes were controlled by the two-component system *dbfS*/*dbfR* [129]. All the mentioned mechanisms and genes involved in stage 5 are shown in blue in Figure 1.

7. Multifunctional Regulation System

NOVCs have evolved several regulators to ensure the expression of genes that lead to successful colonization of the intestinal tract. In addition, NOVCs have evolved a number of adaptive mechanisms to adapt to both the environment and the human host as well as to the transition between host and environment [34]. One such multifunctional regulation system is quorum sensing. By cell-to-cell communication, NOVC is able to adjust the cell density. Three QS pathways through different chemical signals have evolved: cholera autoinducer 1 (CAI-1), autoinducer 2 (AI-2), and 3,5-dimethylpyrazin-2-ol (DPO) [130–132]. The downstream genes of CAI-1 and AI-2 are *cqsS* and *luxPQ*, respectively, followed by *luxO*, *aphA*, and *hapR* (Figure 1) [133]. DPO is the third QS signal mechanism, which can be sensed by *vqmA* [134], followed by the release of the small molecule *vqmR* to downregulate *rtxA* and *vpsR* [135,136].

Two-component systems are another set of regulators with a wide range of functions. Within the two-component system *varS*/*varA*, a receptor for QS and environmental signals represents, together with its downstream gene *csrA*, a multifunctional regulator in biofilm regulation, iron metabolism, virulence gene expression, and motility [80,137,138]. Another two-component system is *vprA*/*vprB*, which is involved in polymyxin and bile resistance [139], which also demonstrated a mutant strain showing colonization failure in host intestine in a mouse model. The gene set *vxrA*/*vxrB* could upregulate T6SS expression and biofilm formation [140,141]. The expression of *phoR*/*phoB* is activated by phosphate limitation, followed by repression of biofilm-related genes and upregulation of motility [142]. The *qseB*/*qseC* gene set is a receptor of the hormones epinephrine and norepinephrine and could affect bacteria motility through triggering *pomB* expression [143,144]. To adapt to oxygen-poor conditions, *acrB*/*acrA* could upregulate *toxT* and enhance biofilm formation and ROS resistance [145]. *chiS* is both the monitor and regulator of (ClcNac)2, which is important for intestinal epithelial cell adherence and gut fluid accumulation [146,147].

Two global regulators are histone-like nucleoid structuring protein (HNS) and cyclic AMP-activated global transcriptional regulator (cAMP-CRP). HNS acts as a mediator at the late stage of infection with a repressive effect on a large number of virulence-associated genes such as hemolysin *hlyA* [148], repeat-in-toxin *rtxA* [149], *Vibrio* polysaccharide

vps [51], and the T6SS [150], while it could also promote motility and the detachment-dependent protein HapA [151]. The global regulator CRP is the receptor of cAMP, the secondary messenger, and acts as a key regulator of many genes in response to lifestyle changes, including the genes *rtxBDE* and *hlyA* [152]. Moreover, CRP represses biofilm formation by repressing the genes *vpsR*, *vpsT*, and *vpsL* and, at the same time, activating the high cell density regulator HapR [153].

8. Schematic Infection Pathway of NOVC

Based on the research mentioned above, a schematic map of virulence-associated genes in NOVC was established and is summarized in Figure 1.

9. Conclusions

As this review article shows, the oral infection of human hosts by pathogenic NOVC is a complex process that depends on the infectivity of the bacterial cells and their ability to survive the harsh conditions in the host until they return to the environment.

It is known that the virulence profile of NOVCs varies, but among them, there are strains expressing all or most of the virulence genes and regulatory systems described in this review article, possibly leading to a pathogenesis ranging from self-limiting diarrheal diseases to cholera-like symptoms and/or bacteremia. Thus, this review article provides an overview of a variety of virulence-associated genes and regulatory systems supporting the understanding of how and why foodborne NOVCs can cause infections.

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Article

Genetic and Phenotypic Virulence Potential of Non-O1/Non-O139 *Vibrio cholerae* Isolated from German Retail Seafood

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Abstract: Non-O1 and non-O139 *Vibrio cholerae* (NOVC) can cause gastrointestinal infections in humans. Contaminated food, especially seafood, is an important source of human infections. In this study, the virulence potential of 63 NOVC strains isolated from retail seafood were characterized at the genotypic and phenotypic levels. Although no strain encoded the cholera toxin (CTX) and the toxin-coregulated pilus (TCP), several virulence factors, including the HlyA hemolysin, the cholix toxin ChxA, the heat-stable enterotoxin Stn, and genes coding for the type 3 and type 6 secretion systems, were detected. All strains showed hemolytic activity against human and sheep erythrocytes: 90% ($n = 57$) formed a strong biofilm, 52% ($n = 33$) were highly motile at 37 °C, and only 8% ($n = 5$) and 14% ($n = 9$) could resist $\geq 60\%$ and $\geq 40\%$ human serum, respectively. Biofilm formation and toxin regulation genes were also detected. cgMLST analysis demonstrated that NOVC strains from seafood cluster with clinical NOVC strains. Antimicrobial susceptibility testing (AST) results in the identification of five strains that developed non-wildtype phenotypes (medium and resistant) against the substances of the classes of beta-lactams (including penicillin, carbapenem, and cephalosporin), polymyxins, and sulphonamides. The phenotypic resistance pattern could be partially attributed to the acquired resistance determinants identified via in silico analysis. Our results showed differences in the virulence potential of the analyzed NOVC isolated from retail seafood products, which may be considered for further pathogenicity evaluation and the risk assessment of NOVC isolates in future seafood monitoring.

Keywords: non-O1/non-O139 *Vibrio cholerae*; seafood; genetic and phenotypic characterization; virulence potential; antimicrobial resistance

1. Introduction

Vibrio cholerae is a water-borne, Gram-negative bacterium found in aquatic ecosystems worldwide [1]. The *V. cholerae* serogroups O1 and O139 are considered to be human pathogens that lead to epidemic and pandemic cholera outbreaks. Those two serogroups act as the causative infectious agents of the intestinal disease cholera by producing cholera toxin (CTX) and toxin-coregulated pilus (TCP) as their main virulence factors [2,3].

However, the species *V. cholerae* is divided into more than 200 serogroups. Serogroups other than O1 and O139 are collectively referred to as non-O1/non-O139 *V. cholerae* (NOVC). In contrast to O1 and O139 *V. cholerae*, whose virulence mechanisms and metabolic pathways have largely been clarified [4], only limited knowledge is available regarding the virulence factors implicated in the pathogenicity of this large NOVC cluster. Therefore, in recent years, scholarly attention has been directed to further exploration of the pathogenic ability of NOVC.

NOVC infection history can be traced back to the 1990s, with several cases reported in South America and Southeast Asia [5,6]. Since then, virulence-associated factors have been identified in NOVC, including hemolysin HlyA, hemagglutinin protease HapA, repeats-in-toxin (RTX) toxins, sialidase Nan, heat-stable enterotoxin Stn, and type 3 (T3SS) and type 6 (T6SS) secretion systems [7]. Currently, there are several reports of NOVC infections, such as case reports and literature reviews [8]. The U.S. Centers for Disease Control and Prevention (CDC) reported 52 NOVC infection cases in the United States from 1998 to 2014. Seafood consumption was attributed to 89% of these cases. In all cases, symptoms resulted in diarrhea with abdominal cramps and nausea. Only 9% of patients had bloody stools. Overall, 38% of affected people were hospitalized, and none of them died. Due to the self-limiting disease process, it is currently assumed that there are around 100 cases per year, with further unreported and undiagnosed cases likely occurring [9]. In Europe, NOVC-associated cases were also reported [10]. In Germany, 30 NOVC cases were registered between 2003 and 2020, of which 17% caused gastroenteritis after oral ingestion [11].

Seafood consumption is an important source of NOVC infections. In Germany, NOVC are detected in approximately 6% of seafood products offered in retail markets [12]. However, studies focusing on NOVC strains isolated from seafood have previously been rather generally compared to isolates from patients and the environment. Ottaviani et al., 2009, analyzed 17 NOVC strains from different seafood sources. They found that all strains were positive for *hlyA* (Hemolysin encoding gene, El Tor biotype variant) [7], while the CTX phage (the cholera toxin encoding island, which contains *ctxA*, *ace*, *zot*, *rstA*, and *rstB*) was absent. Their virulence was further confirmed using Vero cells and an-mouse model [13]. The virulence potential of NOVC isolated from many kinds of seafood was also reported in other studies [14,15]. However, those studies paid much attention to the characteristics of pandemic strains such as CTX phage presence and hemolytic activity, while other genes related to virulence, regulation, and colonization were not addressed. Nevertheless, those genes might also contribute to the virulence performance of NOVC [7].

In addition to the presence of virulence factors, antimicrobial resistance (AMR) is an important trait [16]. Three mechanisms were identified as enabling *V. cholerae* to escape antibiotic treatment: point mutations of chromosomal genes affecting resistance development, efflux systems, and drug degradation enzymes. Efflux systems and drug degradation enzymes are physically linked to mobile genetic elements (MGEs), such as integrating conjugative elements (ICEs) and plasmids [17]. There are several commonly reviewed AMR related genes in NOVC, including *sul1/2* (sulfonamides resistance) and *strA/B* (streptomycin resistance), *int* (integrons of MGE), *setR*, *dfr* (dihydrofolate reductase), and *qnrVC* (quinolone resistance) [18–20]. Due to the mobile and interchangeable nature of MGEs, aquatic environments might become reservoirs for AMR [21].

Due to the limited number of cases, self-limiting disease, and unclear pathogenic properties, NOVC strains have not been included in current food safety standards by the U.S. Food and Drug Administration (FDA) or the European Food Safety Authority (EFSA) [22,23]. Therefore, the aim of this study is to characterize the virulence and antibiotic resistance potential of seafood-associated NOVC from a German market by analyzing different phenotypic and genotypic traits.

2. Materials and Methods

2.1. Strain Collection

In this study, a collection of 63 NOVC strains from German retail seafood was provided by the "Consultant Laboratory for *Vibrio* spp. in Food" hosted by the German Federal Institute for Risk Assessment (BfR). The strains originated from food matrices collected and investigated between 2014 and 2019, in accordance with two International Organization for Standardization methods, namely ISO/TS 21872-1/2007 and 21872-1/2017. In addition, two *V. cholerae* O1 El Tor strains, namely serotype Ogawa (DSMZ 100200) and serotype Inaba (DSMZ 106276), were included as positive controls. Further information on bacterial strains is given in Table S1. Prior to analysis, isolates were cultivated on lysogeny broth

(LB) agar (Millipore, Merck, Darmstatd, Germany) for 18–24 h at 37 °C. Subsequently, a single colony was selected and cultivated overnight in LB medium (Millipore, Merck, Darmstatd, Germany) for 18 h at 37 °C. Strains were stored, based on the manufacturer's instructions, at −80 °C in CRY80-tubes using 1 mL of the overnight culture (Mast Diagnostics, Reinfeld, Germany).

2.2. Whole-Genome Sequencing and Bioinformatics Analysis

All 63 NOVC strains were subjected to whole-genome sequencing (WGS). DNA was extracted from liquid overnight LB culture using the MasterPure™ DNA Purification Kit (Lucigen, Biozym Scientific, Oldendorf, Germany). DNA was quantified using the Qubit 2.0 fluorometer (Life Technologies, Darmstadt, Germany). Library preparation from genomic DNA was performed using the Nextera DNA Flex library preparation kit (Illumina, Inc., San Diego, CA, USA). Paired-end sequencing was performed via a MiSeq benchtop sequencer (Illumina, Inc., San Diego, CA, USA) in 2 × 300 cycles using the MiSeq Reagent Kit v3 (Illumina, Inc., San Diego, CA, USA). The AQUAMIS Pipeline (version 1.3.7) [24] was used for assembly and quality control. Raw reads were trimmed using fastp (version 0.20.1) [25] and assembled using shovill (Seemann 2020, version 1.1.0, https://github.com/tseemann/shovill (accessed on 12 October 2022)). All samples passed the quality criteria, as implemented in AQUAMIS; therefore, the samples showed sufficient base quality and coverage depth. The assemblies' genome length (3,907,054 to 4,290,325 bp) and GC content (47.1% to 47.7%) were in their expected ranges, and there were no signs of contamination (https://bfr_bioinformatics.gitlab.io/AQUAMIS/report_ test_data/assembly_report.html#thresholdt (accessed on 12 October 2022)).

In order to identify the phylogenetic relationships between the strains, core genome multilocus sequence typing (cgMLST) was employed using the chewieSnake pipeline (version 3.1.1) [26] and by applying the PubMLST cgMLST scheme for *V. cholerae* [27]. In total, the cgMLST schema includes 2443 loci. In all strains, 97–99% of the cgMLST loci could be identified. Four clinical NOVC sequences (AM-19226, MZO-2, MZO-3, and VN-300) and six O1/O139 pandemic strains (2010EL-1786, 2011EL-1271, 2012EL-2176, FJ147, Inaba G4222, and MO10) were included to draw conclusions about possible relatedness. A minimum spanning tree was inferred using grapetree [28] and visualized using Geneious software (v2022.1.1).

The presence or absence of virulence-associated genes for all strains was confirmed via Geneious software (v2022.1.1) using the nucleotide BLAST algorithm (medium sensitivity, default setting parameters) based on a match to the reference sequence with an identity of between 80 and 100%. The *V. cholerae* O1 serovar El Tor FJ147 (NZ_CP009041, NZ_CP009042), the *V. cholerae* O139 serovar MO10 (NZ_CP060094, NZ_CP060095), the environmental strain O1 Env390 (NZ_CP013013, NZ_CP013014), and the WGS of the available clinical T3SS positive NOVC strains AM-19226, MZO-2, MZO-3 [29], and VN-300 [7] were included as positive controls for virulence marker determination.

2.3. Hemolytic Test

For hemolytic tests, blood agar based on Mueller–Hinton agar (Oxoid GmbH, Wesel, Germany) supplemented with 5% human or sheep erythrocyte were prepared. Human blood was taken from healthy volunteers via venipuncture. The blood was collected using blood collection tubes containing dipotassium salts of ethylene diamine tetra acetic acid (K2EDTA) for use as an anticoagulant (DB Medical, Eysins, Switzerland). Defibrinated sheep blood was ordered from Thermo Scientific Oxoid, Landsmeer, Netherlands. Erythrocytes were washed three times using 0.01 M phosphate-buffered saline (PBS) buffer (Medicago, Uppsala, Sweden). PBS was removed as a supernatant after centrifugation (1000× *g* at 4 °C for 5 min). After washing, the isolated erythrocytes were compacted via a final centrifugation step at 1000× *g* for 10 min at 4 °C before being added to the agar.

Hemolytic tests were performed by inoculating 5 µL of LB overnight culture of *V. cholerae* strains in triplicate on the prepared blood agar plates. After 24 h of incubation at

37 °C, hemolytic activity was visually assessed, and the diameter of the complete hemolytic zone (beta hemolysis) was determined.

2.4. Biofilm Formation

The biofilm formation assay was performed using the method described by Mahoney et al., 2010 [30], albeit with minor modifications. In total, 3 mL of each of the LB overnight culture of *V. cholerae* strains was centrifuged at $5000 \times g$ for 3 min, and the pellet was resuspended in 500 μL PBS. The bacterial cell density was employed to the optical density (OD) of 0.7 at 588 nm. *V. cholerae* cells (125 μL) were then inoculated in 125 μL of LB broth in a 96-well clear polystyrene, non-treated, and flat-bottom microplate (Corning, New York, NY, USA). After 48 h of incubation at 25 °C, LB media was carefully removed via pipetting, and the plates were washed twice, using 300 μL of PBS buffer per well. The remaining biofilm was dried overnight at room temperature. For biofilm staining, 275 μL of 0.1% crystal violet solution (*w/v* in distilled water) was used. After 1 h of incubation, crystal violet was carefully removed via pipetting, and the stained complex was washed three times using sterile water and dried at room temperature for 1 h. The stained biofilm was dissolved in 300 μL of 33% acetic acid (*w/v* in distilled water) for 30 min. The OD of the staining solution was measured at 595 nm and normalized to the absorbance of LB as a negative control. According to OD595 absorbance, *V. cholerae* strains were assigned to four groups due to their biofilm formation ability: none (OD < 0.5), weak (OD 0.5–1.0), medium (OD 1.0–3.0), and strong biofilm formation (OD > 3.0). For all the strains tested, three biological replicates were analyzed, with each having ten technical replicates.

2.5. Serum Resistance

Serum resistance tests were performed according to a method previously described by Bier et al., 2013 [31]. In total, 12 μL of LB overnight culture was transferred in 600 μL of LB broth and incubated at 37 °C for 5–6 h, and 2 μL per well was transferred to 96-well microplates containing gradient mixtures consisting of 100 μL of human serum (pooled from healthy volunteers) and peptone–glucose broth (1% glucose, 0.0075% bromothymol blue, 1% peptone, 0.5% NaCl, pH 7.4). The blood serum concentrations were 0%, 10%, 20%, 40%, and 60%, respectively. Plates were incubated at 37 °C for 24 h, and the serum resistance was examined based on a color shift from blue to yellow, indicating metabolic activity via glucose fermentation. In each 96-well plate, a positive control of *Escherichia* (*E.*) *coli* K-12 + pKT107 (RS228) carrying the serum resistance plasmid and a negative control *E. coli* K-12 − pKT107 (DSMZ423) was used. Bacterial serum resistance was classified as none (bacterial growth was below 20% serum), weak (bacterial growth in up to 20% serum), medium (bacterial growth in up to 40% serum), or strong (bacterial growth in 60% serum). Three biological replicates were performed for all the tested strains.

2.6. Motility Determination

To determine the motility of *V. cholerae* isolates, a soft agar plate assay was performed based on the method of Li et al., 2022 [32], albeit with modifications. In brief, 1 μL of overnight LB broth cultured *V. cholerae* strains was injected directly into fresh 0.3% LB agar plates. The diameters of the motility zones were measured after 24 and 48 h of incubation at both 25 °C and 37 °C. For each strain, 6 to 9 replicates were performed. Motility was classified based on the diameter of the motility zone on the plate: none (0 cm), weak (0–4 cm), medium (4–8 cm), or strong (≥8 cm).

2.7. Antimicrobial Resistance Evaluation

Both in silico and in vitro approaches were employed to verify the antimicrobial resistance (AMR) pattern of all tested strains. Two online search engines, namely ResFinder (v4.1) [33] and Resistance Gene Identifier (v5.2.1) from the Comprehensive Antibiotic Resistance Database [34], were used for the screening of AMR-related genes with default settings. Moreover, a collection of recently identified AMR genes in NOVC was examined

via Geneious software: the multidrug and toxic compound extrusion (MATE) efflux system (*vcmA*, *vcmB*, *vcmD*, *vcmH*, *vcrM*, and *vcmN*), the ATP binding transporter (vcaM), and the conserved region of SXT/R391 ICE (SXT/R391) [18–20].

As for the in vitro tests, antimicrobial susceptibility testing (AST) of *V. cholerae* was performed via broth microdilution based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI) M07, 11th ed. M07-A10 [35,36]. We used a harmonized European panel of antimicrobials combined in a commercial plate format (EUVSEC, ThermoScientific, Meerbusch, Germany) containing 15 antimicrobial substances of 12 antimicrobial classes: ampicillin (AMP), azithromycin (AZI), cefepime (CEFEPI), cefotaxime (FOT), ceftazidime (TAZ), and clavulanic acid (CLA), as well as in combination with FOT (TAX-CLA) or TAZ (TAZCLA), cefoxitin (FOX), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), ertapenem (ERTAPE), gentamicin (GEN), imipenem (IMIPEN), meropenem (MERO), nalidixic acid (NAL), sulphamethoxazole (SMX), temocillin (TEMOCI), tetracycline (TET), tigecycline (TGC), and trimethoprim (TMP). Antimicrobial substances were used in concentration ranges described in the European Commission implementing decision 2020/1729/EU [37]. The *E. coli* isolate ATCC 25922 was used as a quality control and reference strain during AST. The minimal inhibitory concentration (MIC) values of all strains are listed in Table S2. Multidrug resistance was defined based on combined resistance to ampicillin (AMP), cefotaxime (FOT) and/or ceftazidime (TAZ), and ciprofloxacin (CIP) (der KRINKO, 2012) [38].

3. Results

3.1. Genetic Characterization

The initial screening of food isolates was achieved via PCR using the gene-specific primers of *ompW* for *V. cholerae* species confirmation based on the method of Nandi et al., 2000 [39]. Primers detecting *ctxA* [40] and gene fragments of *rfbO*1 and *rfbO*139 [41] were then used to identify the cholera toxin, as well as the O1 and O139 serogroups. All strains are positive for *ompW* and negative for the other three target genes, which confirms that the collected strains are NOVC. In order to acquire a clear view of the sub-species population level of our NOVC seafood isolates and the relationships between different strains, cgMLST was performed based on the method in [42]. The minimum spanning tree, as shown in Figure 1, exhibits two highlighted clusters (shown in orange). These clusters represent the grouping of five seafood-associated NOVC with an O1 serotype (2011EL-1271) and the grouping of eight seafood-associated NOVC with a clinical NOVC (MZO-3). There is also a cluster comprising only *V. cholerae* of serotypes O1 and O139 (shown in red), as well as six clusters that exclusively encompass seafood-associated NOVC (shown in green).

Genotypic results were acquired by comparing the virulence-associated genes (Figure 2a,b and Table S3) of positive control strains to the collected seafood-associated NOVC; the results are shown in Figure 2a,b.

All tested seafood-associated NOVC are positive for most genes conferring adaption in host environments. In summary, genes related to acid tolerance, reactive oxygen species (ROS) resistance, resistance nodulation cell division (RND) efflux systems, and outer membrane vesicle (OMV) regulators were identified in all strains, including reference strains. However, other genes were found in only parts of the seafood-associated NOVC strains studied. These include the lysine decarboxylation mediator *cadA* (90% presence rate) and the catalase encoding gene *katB* (32%). Two genes of the chemotaxis system, namely *cheW* and *cheB*, acting as transporters between chemical receptors and the stabilizer of chemical noise, have a 32% prevalence in the investigated seafood-associated NOVC. Nevertheless, all strains including reference strains harbor other chemotaxis genes (*cheA*, *cheY*, and *cheR*). The two c-di-GMP modulators, namely *acgAB* and *vieSAB*, were absent in six and four strains of the seafood-associated NOVC, respectively. However, other genes with putative functions in biofilm formation and motility were present in all samples, including reference strains. To identify possible motility and biofilm deficiency in the
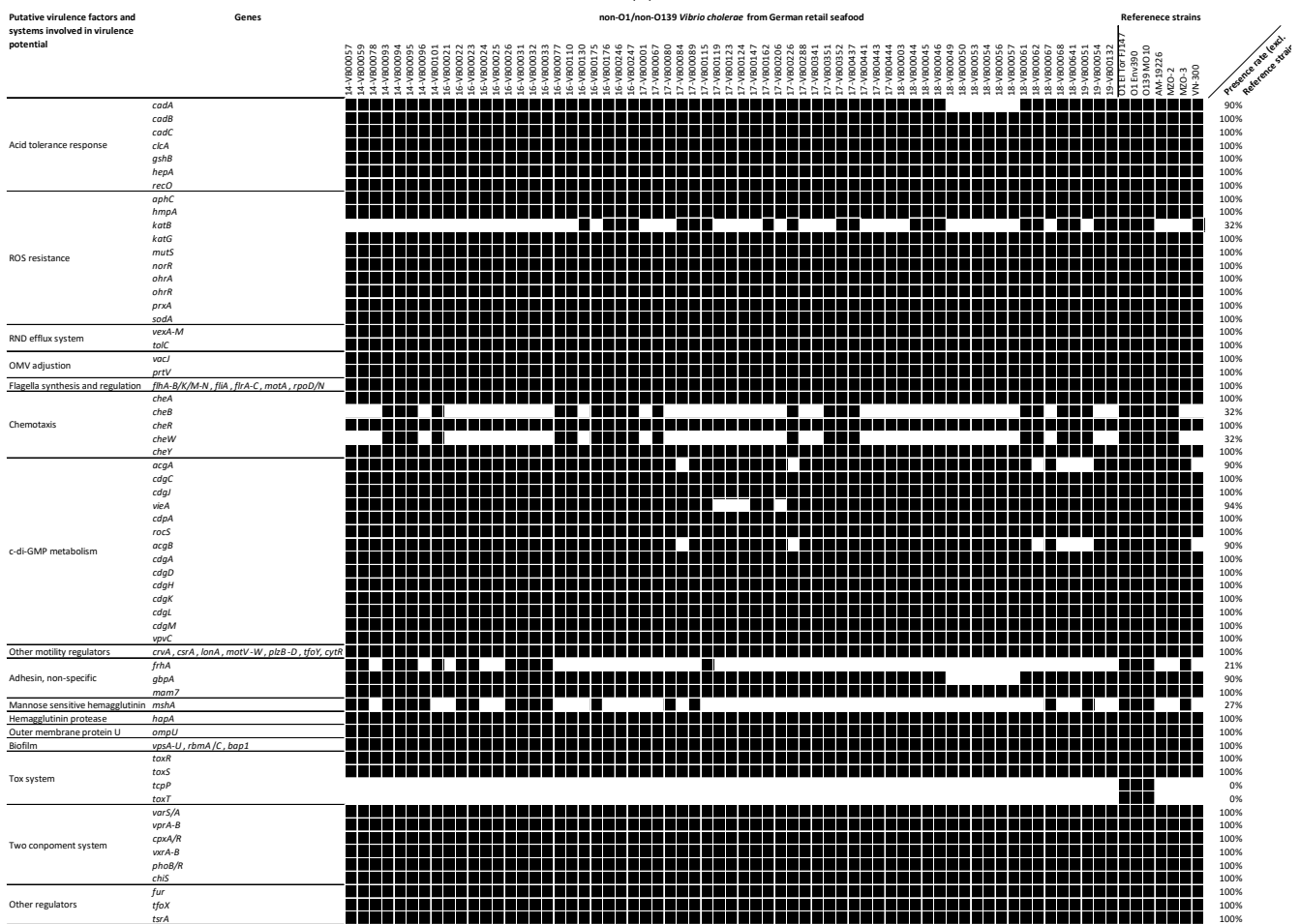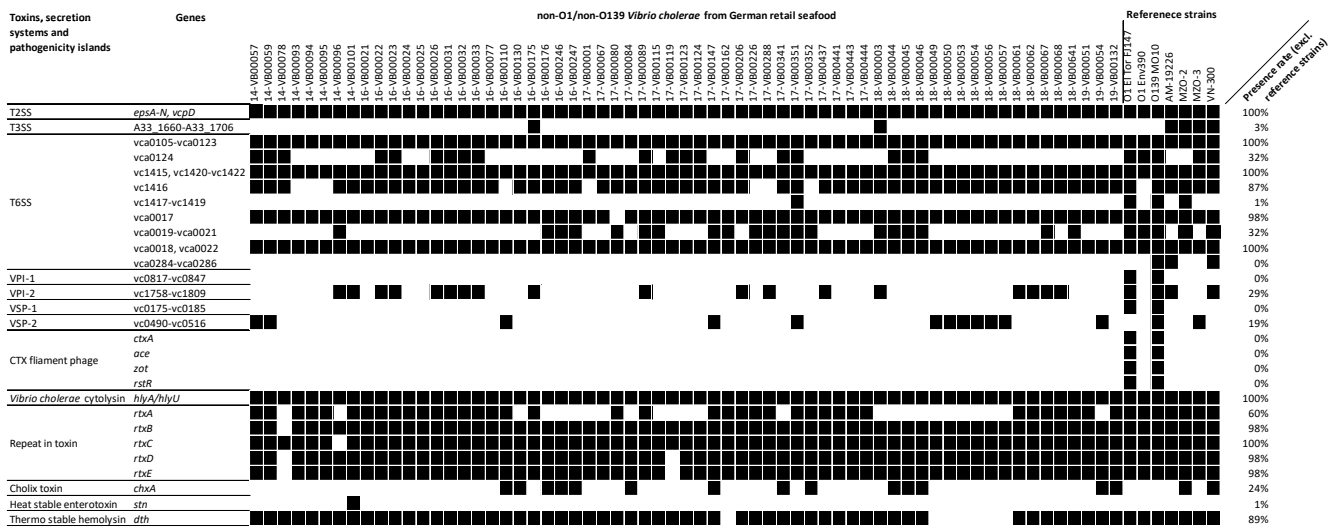
seafood-associated NOVC based on the absence of genes that have not been considered or are not known so far, we performed in vitro motility and biofilm formation tests.

Three non-specific adhesins, namely *gbpA* (90% prevalence), *mam7* (100% prevalence), and *frhA* (21% prevalence), were identified in the seafood-associated NOVC. Another colonization-involved gene, namely *mshA*, which is the MSHA pili-encoding gene, was also present in 27% of the seafood-associated NOVC. Both VPI-1 and VSP-1 are absent in these isolates, while several ORFs of the two other pathogenicity islands, namely VPI-2 and VSP-2, are present in 29% and 19% of the seafood-associated NOVC, respectively. Although none of the seafood-associated NOVC possessed a full VPI-2, we observed the occurrence of the integrase-, sialidase-, and neuraminidase-encoding segments (vc1758, vc1773-vc1783, and vc1784) among those VPI-2 positive strains [43].

Figure 1. cgMLST analysis of seafood-associated NOVC, clinical NOVC, and pandemic O1/O139 *V. cholerae* strains. Seafood-associated NOVC are labeled in green, clinical NOVC are labeled in orange, and pandemic O1/O139 *V. cholerae* are labeled in red. A green background shows a clustering of only seafood-associated NOVC. An orange background shows clusters containing clinical NOVC or pandemic O1/O139 *V. cholerae* and seafood-associated NOVC. A red background shows a clustering of only pandemic O1/O139 *V. cholerae*. The clusters were defined based on a clustering threshold ranging from 1 to 1000 allelic differences to identify a phylogenetic relationship according to Liang et al., 2020 [42]. The minimum spanning tree was created using grapetree [28] and visualized using Geneious software (v2022.1.1).

(a)

(b)

Figure 2. (**a**). The presence/absence of toxin genes, genes involved in secretion systems, and genes included in pathogenicity islands of seafood-associated NOVC (*n* = 63) based on WGS data. Black boxes represent gene presence, and white boxes represent gene absence. Detailed information about the genes can be found in Table S3. (**b**). The presence/absence of genes that play roles as putative virulence factors or genes involved in systems that play roles in the virulence of seafood-associated NOVC (*n* = 63) based on WGS data. Black boxes represent gene presence, and white boxes represent gene absence. Detailed information about the genes can be found in Table S3.

All tested seafood-associated NOVC are negative for cholera related genes, including the CTX phage (*ctxA*, *ace*, *zot*, *rstA*, and *rstB*), *tcpA*, and *toxT*. As for the secretion systems, all samples contained T2SS, while two strains (16-VB00175 and 18-VB00003) possessed whole T3SS when using the *V. cholerae* strain AM-19226-derived T3SS clusters as references (GenBank ID: AATY02000004 and AATY02000003). While most of the T6SS related genes were present in all strains, vca0124 was detected in 32% of strains. The *vgrG*1 encoding gene in auxiliary cluster 1 with actin-crosslinking activity (vc1416) was detected in 87% of strains. The *vasX* encoding gene in auxiliary cluster 2 with pore formation effect on bacterial cells (vca0020) was detected in 68% of strains. Only auxiliary cluster 3, i.e., vca0284 to vca0286, was absent in all strains. The detachment determining factor encoding gene *hapA* was found in all samples. The prevalence rate of *hlyA* (together with its direct regulator *hlyU*) was 100%, which is much higher than of the other additional toxin encoding genes for the thermostable hemolysin (*dth*; 89%), the multifunctional autoprocessing RTX toxins (*rtxA-rtxE*; 60%), the cholix toxin (*chxA*; 24%), and the heat-stable enterotoxin (*stn*; 1%).

3.2. Phenotypic Characterization

Phenotypic tests were designed to verify the bioinformatic data and further explore the virulence potential of the collected strains. Table 1 shows the percentage of seafood-associated NOVC isolates that can form a biofilm; are resistant to human blood serum, hemolyze human, and sheep erythrocytes; and are motile. Over 90% of the investigated NOVC can form strong biofilms at 37 °C (Table 1 and Table S4). Serum resistance was examined through a gradient mixture of human serum and standard protein–peptone solution. O1 strains were sensitive to human serum under our experimental conditions [7]. For NOVC, five strains were able to withstand 60% human serum, nine strains were able to withstand 40% human serum, and the remaining 49 strains were sensitive and grew only in 0 to 20% human serum. All strains could lyse sheep and human erythrocytes with variation in the hemolysis ring. Five strains have the same hemolytic zone as the control O1 El Tor strain and are shown in Table 1 and S4 to possess strong hemolytic activity: 17-VB00123, 17-VB00124, 18-VB00049, 18-VB00056, and 18-VB00057.

Table 1. Phenotypic characterization of seafood-associated NOVC (*n* = 63).

Classification	Biofilm Formation	Serum Resistance	Hemolytic Activity	Motility
Strong	90% (*n* = 57)	8% (*n* = 5)	8% (*n* = 5)	52% (*n* = 33)
Medium	8% (*n* = 5)	14% (*n* = 9)	92% (*n* = 58)	40% (*n* = 25)
Weak	0% (*n* = 0)	22% (*n* = 14)	0% (*n* = 0)	8% (*n* = 5)
None	2% (*n* = 1)	56% (*n* = 35)	0% (*n* = 0)	0% (*n* = 0)

As for the motility characteristics on soft agar plates (Table 1 and Table S4), 92% of strains have medium-to-strong motility. Almost all strains showed a medium to high motility at 37 °C.

3.3. Antimicrobial Resistance Profile

In total, the non-wildtype phenotypes of seven antibiotics were noticed in the NOVC collection: AMP (11%), CIP (2%), COL (87%), FOX (2%), IMIPEN (78%), NAL (5%), and TMP (6%). Although the official *Vibrio* spp. breakpoint is not available for COL, NAL, and TMP, strains that could grow under the maximum MIC value were considered as resistant [44]. No resistance against AZI, CEFEPI, CHL, ERTAPE, FOT, GEN, MERO, SMX, TAXCLA, TAZ, TAZCLA, TEMOCI, TET, and TGC was observed in any of the investigated strains. Five seafood-associated strains were found to be simultaneously resistant to three antibiotic classes (beta-lactams: IMIPEN; polymyxins: COL; and sulphonamides: TMP): 16-VB00021, 16-VB00024, 16-VB00025, 17-VB00441, and 19-VB00051.

Moreover, all strains are positive for the multidrug and toxic compound extrusion pump (MATE) efflux system (*vcmA*, *vcmB*, *vcmD*, *vcmH*, *vcrM*, and *vcrN*), the ABC trans-

porter (*vcaM*), and the genes involved in antibiotic resistance: *parE*, *dps*, *almG*, and *bcr*. The quinolone resistance factors *qnrVC*4, *qnrVC*5 and *qnrVC*7 (27% prevalence), the Ambler class B metallo-beta-lactamase gene *varG* (52% prevalence), the CARB beta-lactamase *blaCARB*7 (6% prevalence), the chloramphenicol acetyltransferase *catB*9 (13% prevalence), the elfamycin resistance gene EF-Tu (86% prevalence), and the dihydrofolate reductase dfrA31 (5% prevalence) were also detected in the strain set. The resistance genes for tetracycline (*tet*), florfenicol (*floR*), streptomycin (*strA/B*), sulfamethoxazole (*sul1* and *sul2*), bleomycin (*ble*), and aminoglycoside (*aadA*1, *aadA*5, and *aac*(6′)) were absent in all seafood-associated NOVC. Fourteen strains had a conserved region of ICE SXT/R391 (Figure 3), which represents the presence of MGE. However, only a few segments were identified, compared to the 60 segments identified in SXT/R391 in *V. cholerae* [45].
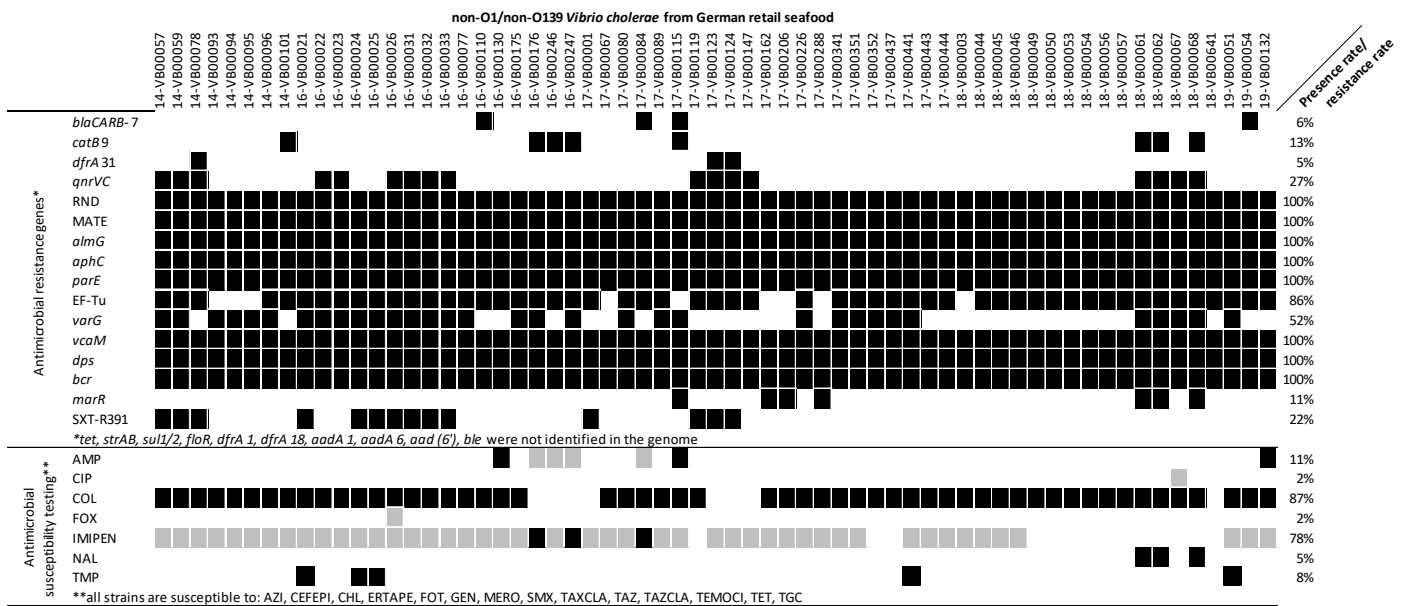

Figure 3. Antibiotic resistance pattern of NOVC strains (*n* = 63) at genetic and phenotypic level. SXT/R391: conserved region of SXT/R391. *bcr*: Bcr/CflA family efflux transporter. *dps*: member of ferritin-like diiron-carboxylate proteins encoding gene. *vcaM*: ATP binding cassette encoding gene. *varG*: VarG beta-lactamase Subclass B1 encoding gene. EF-Tu: elfamycin resistance gene. *parE*: fluoroquinolone resistance gene. *almG*: lipid A acyltransferase encoding gene. MATE: multidrug and toxic compound extrusion pump encoding gene. *qnrVC*: quinolone resistance gene. *dfrA*31: trimethoprim resistant dihydrofolate reductase encoding gene. *catB*9: chloramphenicol acetyltransferase encoding gene. *blaCARB*-7: beta-lactamase encoding gene. In genetic result, black boxes represent gene presence, and white boxes represent gene absence. In phenotypic result, black boxes represent resistance, grey boxes represent intermediate, and white boxes present susceptibility. Detail information about breakout points is shown in Table S2.

4. Discussion

In this study, the virulence and antimicrobial resistance potential of seafood-isolated NOVC strains were investigated through genotypic and phenotypic approaches. In cgMLST, thirteen seafood-isolated NOVC have close relationships with clinical NOVC (Figure 2), indicating a similar core genome between seafood isolated and clinical NOVC.

4.1. Adaption between Environment and Host

Apart from toxin-producing genes and genes strongly associated with them, which have been the focus of previous studies of the virulence potential of NOVC [7,46], other genes not directly related to toxicity also play roles in the infection process [47]. Acid tolerance-related genes are present in most seafood-isolated NOVC, while six strains are negative for *cadA*, which could encode lysine decarboxylase and play important roles in

host gastrointestinal surveillance [48]. As for the ROS resistance, 32% of *katB* and 100% of other relevant genes were detected in the strain collection. The catalase-encoding gene *katB*, associated with H_2O_2 reduction activity [49], is absent in some strains. *katB*, in *V. cholerae*, seems to make a limited contribution to catalase activity when *katG* (an additional catalase-encoding gene) is active [50].

Other studies have highlighted the relationship between the motility and toxicity of *V. cholerae*. Motility could contribute to localization on biotic and abiotic surfaces, including the successful colonization of the gut, mucus penetration, and further systemic infections [51]. Genes required for flagella synthesis and regulation were detected in all NOVC considered in this study. Potential motility-modulating genes are also located in all the analyzed NOVC except *acgAB*, i.e., a pair of genes in c-di-GMP (an important life-mode switching signal) synthesis [52]. The subsequent motility assay revealed that most NOVC are motile, whereas the less motile strains could not be explained by the presence or absence of specific motility-modulating genes (see Figure 2b). All strains are more motile at 37 °C than at 25 °C, which is consistent with other studies defining increased c-di-GMP levels at low temperature as a modulator of life adaption [53]. This study and other studies show that O1 strains can be less motile than NOVC [54].

In addition to motility, the formation of biofilms has advantages in the infection process, as biofilms are an adaptation not only to harsh environmental conditions but also to host conditions [55]. The genes required for polysaccharide and essential protein recruitment were present in all strains, whereas the absence of *acgAB* and *vieSAB* might have an impact through c-di-GMP alteration [52]. The results of the in vitro biofilm assay showed that 90% of strains form strong biofilms, highlighting their adaption ability [56]. On the other hand, there was one strain which was not able to build a biofilm and five strains with medium biofilm formation ability, suggesting that these strains might be more susceptible to unfavorable environment and host conditions because they are not protected by biofilms [55].

The chemotaxis system might be relevant to chemical sensing and the following reaction. This system might be incomplete in our investigated NOVC strains because of the basic gene in the chemotaxis working frame, namely *cheW*, was only identified in 32% of strains [57]. Early reports mentioned that chemotaxis mutant *V. cholerae* strains have different moving behaviors, leading to them having a wider distribution in intestines than wild-type strains [58].

4.2. Adherence and Toxin Production Ability

The attachment and proliferation on intestinal epithelial cells is the prerequisite of toxin production [47]. We noticed that several non-specific adhesins were missing in our seafood isolates, such as *gbpA* and *frhA*, indicating the intestinal epithelial cell attachment deficiency for the respective strains [59,60]. VPI-1, the critical colonization determinant, was missing in all isolates. The complete T3SS of the reference strain AM-19226 (a clinical NOVC) was identified in two strains, namely 16-VB00175 and 18-VB00003, and those two strains were closely related to clinical NOVC strains with T3SS (AM-19226 and VN-300), according to the cgMLST result (Figure 1). This result suggests that these two strains might have better intestine adherence and proliferation ability during infection compared to other strains [29,61]. According to a large-scale genomic search for *V. cholerae*, all NOVC with human infection record are T3SS-positive [62]. We also observed that two pathogenicity islands (PIs) were partially (VPI-2, VSP-2) present, which unveiled the putative function of sialic acid utilization (VPI-2) and di-nucleotide molecule production (VSP-2) for relevant strains [43,63]. VPI-1, which might have impact on environment adaption, was completely missing in our collection [64]. However, NOVC could grow and show infectivity without these three PIs in spite of their contributive functions in the infection process [29]. The large cluster of T6SS was identified in all strains, while the auxiliary clusters 1 and 2 (encoding *vgrG*1 and *vasX* respectively) were partially present, which is similar to the clinical NOVC gene-screening result from Arteaga et al., 2020 [62]. Previous works suggested that *vgrG*1

has a toxic effect on eukaryotic cells by forming pores or disrupting cellular structures, *vasX* has the potential to disrupt the gut microbiome and hemostasis, and *vgrG*3 plays a role in antibacterial activity that allows *V. cholerae* to establish itself as the dominant species [65]. Auxiliary cluster 3 of T6SS (vca0284 to vca0286) is missing in all NOVC strains, which could encode amidase TseH and degrade the peptidoglycan cover of bacteria [66]. Genes with regulation effect on T6SS were also found in all NOVC strains (*tfoX*, *tfoY*, and *tsrA*), indicating the proper expression of T6SS [65].

The filamentous CTX phage was not detected in any of the analyzed seafood-associated strains, which is consistent with the characteristics of most NOVC [46]. All strains are positive for the hemolysin *hlyA* and its regulator *hlyU*, whose functionality have been demonstrated via blood agar hemolysis for sheep and human blood [67]. Other researchers also found the high occurrence rate of *hlyA* in NOVC [46]. After the consumption of contaminated seafood, a transition of motile NOVC into the bloodstream through the portal vein or intestinal lymphatic system is possible [68]. In this study, 14 NOVC strains were identified as having medium-to-high resistance against human serum, showing their ability to survive in human blood and cause bacteremia. For 13 of these serum resistant strains, a correlation with medium-to-high biofilm formation ability was identified (Table S4). King et al., 2009, reported this relationship in *Acinetobacter baumannii*, a Gram-negative pathogen, suggesting that either the better biofilm formation results from serum resistance or the biofilm substance is beneficial for serum resistance.

In addition, several other toxin-encoding genes were detected, including *chxA*, *stn*, and *rtxA*. The heat-stable enterotoxin-encoding gene *stn* was only present in one strain: 14-VB00101. The ADP-ribosylation factor, namely cholix toxin *chxA*, was detected in 24% of strains, which could induce mitochondrial dysfunction and cytoskeletal rearrangement. The toxicity of these accessory toxins was previously confirmed via a mouse model [69–71]. Furthermore, *hlyA*, *rtxA*, *chxA*, and T3SS were detected in clinical NOVC isolated from German and Austrian patients by Schirmeister et al., 2014 [7], while higher *hlyA* prevalence was noticed in our strain collection. The fact that these virulence-associated genes are also present in NOVC isolated from German retail seafood in this study confirms the assumption that these strains have a pathogenic potential.

4.3. Antimicrobial Resistance Ability

Fourteen strains with conserved region of SXT/R391 were compared to a SXT/R391 collection [45], and the presence of fragments was confirmed. Moreover, a whole integron (integrase *intI* and recombination site *attI/attC*) was detected in 60% of the seafood-associated NOVC via IntegronFinder [72]. These results suggest the potential for the HGT transition of isolated NOVC in coastline seawater [21]. Antibiotic resistance could be simultaneously shown against three antibiotic classes (beta-lactams, for example, penicillin, cephalosporin, and carbapenem; polymyxins; and sulfonamides) for five seafood-associated NOVC (16-VB00021, 16-VB00024, 16-VB00025, 17-VB00441, and 19-VB00051). Relevant gene distribution could explain some results from antibiotic in vitro assays, including *blaCARB* presence and AMP resistance [73,74], *almG* presence and COL resistance [75], *qnrVC*s presence and quinolone (CIP and NAL) resistance [76], *varG* and IMIPEN resistance [77], and *dfrA*31 presence and TMP resistance [78]. However, AST result cannot be fully explained by genetic information: the COL resistance rate is 87%, while all NOVC strains used in this study contain *almG*; *blaCARB* was not found in the three AMP resistant strains; and all *catB*9 harboring strains have no in vitro resistance to CHL. The reason for this trend might be gene variation, while incomplete databases could lead to discrepancies, and there might be other genes that relativize the phenotype [19]. Doxycycline (a second-generation TET), AZI, and CIP are recommended for cholera treatment by the CDC. In this study, only resistance against CIP (2%) was detected.

5. Conclusions

In summary, the putative virulence of seafood-associated NOVC was confirmed, in particular by the presence of virulence genes, such as *hlyA*, *rtxA*, *chxA*, and the T3SS. In total, 13 of 63 NOVC originally isolated from seafood products clustered together with clinical *V. cholerae* strains in cgMLST, indicating a possible relationship between them. A strong capacity for biofilm formation, motility, and hemolytic activity were confirmed via phenotypic experiments. Antibiotics recommended by the CDC for cholera treatment are effective against infection with seafood-associated NOVC. Only 2% of strains are resistant to ciprofloxacin.

Nevertheless, seafood-associated NOVC have the genetic and phenotypic potential to cause human infections through the consumption of contaminated seafood and should be included in future seafood surveillance.

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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

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.
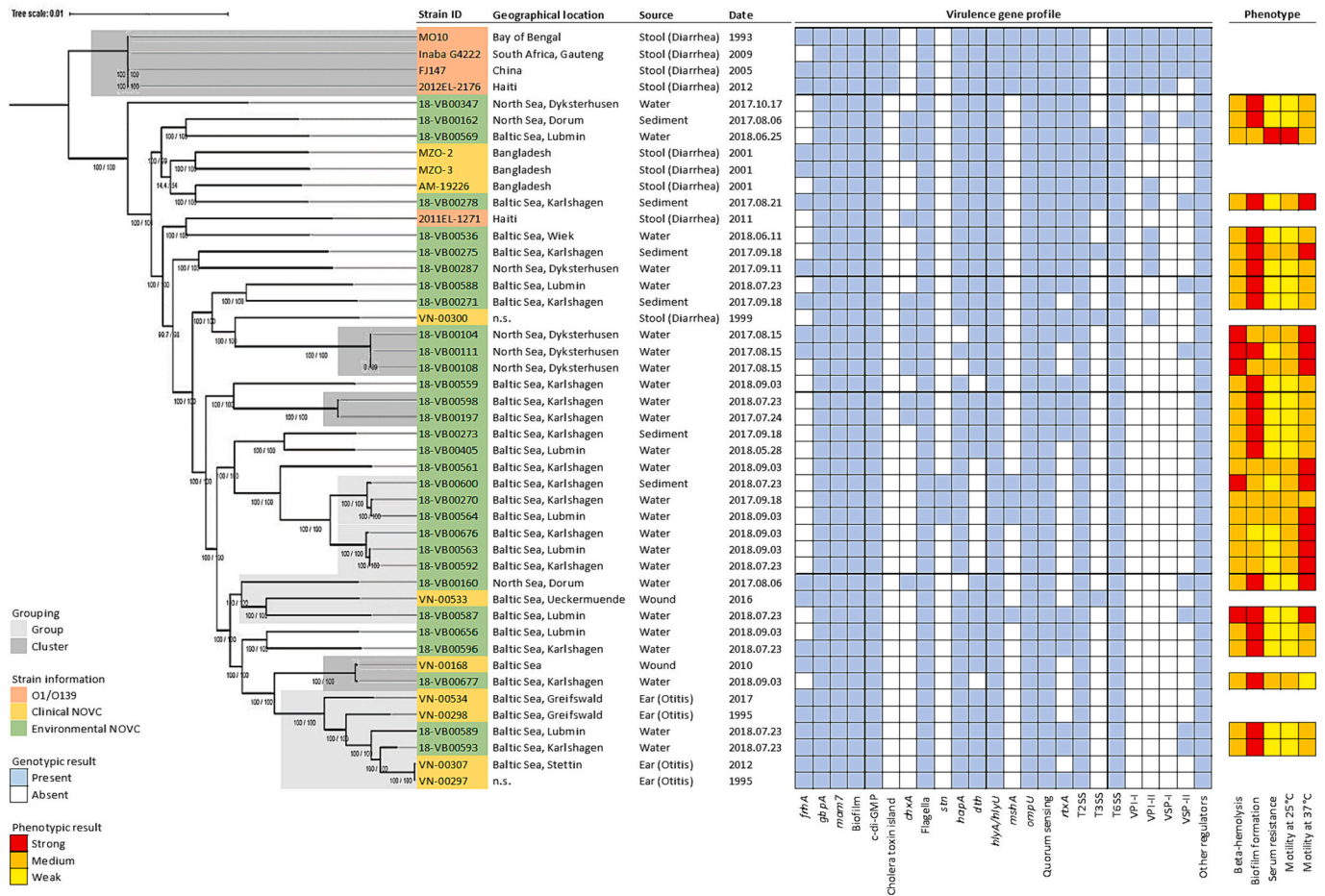
Fig. 1. Maximum-likelihood phylogenetic tree of 31 environmental NOVC strains, ten clinical NOVC strains and five O1/O139 pandemic strains based on a shared core genome (2.7 Mbp) combined with virulence profiling. The tree was rooted on the outgroup of four pandemic O1/O139 strains. Numbers in parentheses 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).

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%

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, vgrG*1-*vgrG*3
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.

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-
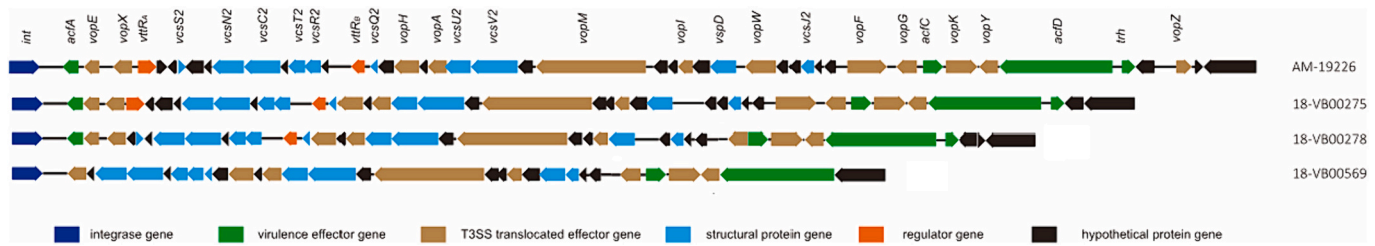
Fig. 2. Gene alignment of the T3SS of environmental NOVC strain 18-VB00275, 18-VB00278 and 18-VB00569 compared to reference *V. cholerae* strain AM-19226. Gene alignment of T3SS in *V. cholerae* was built according to Alam et al. (2011). Arrows stand for transcription initiation sites. Dark blue arrows 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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3. Discussion

This doctoral project involved a comprehensive investigation into the infection pathways of NOVC, comprising literature research, two characterization studies to identify virulence factors in NOVC isolated from seafood and environmental sources, and an analysis of T3SS pathogenicity. The infection route was outlined, and virulence-associated genes of NOVC were compiled from the literature (Chapter 2.1). Based on these findings, the characterization studies confirmed the putative virulence of NOVC strains isolated from German retail seafood (Chapter 2.2) and the coastal regions of the German North Sea and Baltic Sea (Chapter 2.3), using genetic and phenotypic assays. Furthermore, the significant role of T3SS in pathogenicity was examined through gene expression and cytotoxicity analyses, although some unclear gene functions require further exploration (Chapter 3.5, page 51).

3.1 Can NOVC isolates progress through all five stages of the genetic infection model?

Whole genome sequencing (WGS) was performed on all NOVC strains isolated from seafood and environmental sources. The WGS data were compared to the virulence-associated genes identified in Chapter 2.1, using the nucleotide BLAST algorithm in Geneious software (v2022.1.1). The seafood study included 63 strains, and the environmental study included 31 strains. Genotypic and phylogenetic analyses were conducted to assess the virulence potential of these strains. A close core-genome relationship was observed between eight seafood strains (14-VB00057, 14-VB00059, 16-VB00022, 16-VB00023, 16-VB00026, 16-VB00031, 16-VB00032, 16-VB00033) and clinical diarrhea-associated NOVC (MZO-3), as determined by core genome multi-locus sequence typing (cgMLST) (Chapter 2.2, page 29). Additionally, seven environmental strains (18-VB00160, 18-VB00587, 18-VB00589, 18-VB00593, 18-VB00596, 18-VB00596, 18-VB00677) clustered with clinical NOVC isolated from extraintestinal infections (VN-00533, VN-00168, VN-00534, VN-00298, VN-00307, and VN-00297) (Chapter 2.3, page 40).

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3.1.1 Stage I: Survival in the gastrointestinal tract

NOVC has developed several mechanisms to survive in the host's harsh gastrointestinal environment, which includes exposure to stomach acid, reactive species, and nutrient deprivation. All strains from both studies (seafood, Chapter 2.2; environment, Chapter 2.3) possessed acid tolerance genes, while the catalase-encoding gene *katB* was found in 32% of seafood isolates (Chapter 2.2, page 30) and 76% of environmental isolates (Chapter 2.3, page 40). However, *katB* was not essential for resistance to reactive oxygen species (ROS) in NOVC (Fernandez and Waters 2019).

At the genetic level, all strains possessed biofilm formation and motility genes, though phenotypic assays revealed that some strains exhibited weaker biofilm formation and motility (Chapter 2.2, page 30; Chapter 2.3, page 40), likely due to differences in gene expression, sequence variation, or other unidentified factors. The biofilm formation genes (*vps* cluster, *vpsR*, *vpsT*) and other regulatory genes (Chapter 2.1, page 12) were present in all strains, but variations in biofilm strength were observed (Chapter 2.2, page 30; Chapter 2.3, page 40). This may be due to sequence variations in *vpsR*, a global regulator of biofilm formation (Hsieh *et al.* 2020). Alignment analysis of the *vpsR* sequence supported this hypothesis for environmental strains (Chapter 2.3, page 43).

Although the main cluster of T6SS genes was found in all NOVC strains, the presence of auxiliary gene islands varied, indicating gene variation (Crisan and Hammer 2020).

3.1.2 Stage II: Localization and penetration of the mucus layer in the small intestine

The chemotaxis system, which enables bacteria to sense chemical signals and modulate gene expression for toxin production, motility, biofilm formation, and metabolism, plays a role in the localization and penetration of the mucus layer (Butler and Camilli 2004). However, the chemotaxis system was incomplete in both seafood and environmental NOVC, as the key component *cheW* was absent in 68% of seafood isolates and 81% of environmental isolates. Despite this, chemotaxis is not essential for intestinal colonization in *V. cholerae*, suggesting that these chemotaxis-deficient strains may still function similarly (Millet *et al.* 2014).

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The presence of sialidase and neuraminidase genes in the VPI-2 genomic island (*vc1773* to *vc1784*) was detected in 29% of seafood strains and 19% of environmental strains, indicating enhanced mucus penetration capability in these strains (Jermyn and Boyd 2005).

3.1.3 Stage III: Intestinal epithelial cell colonization

Adhesion to the intestinal epithelial surface is critical for colonization. Non-specific adhesins such as *mam7* and *gbpA* were present in 100% and 94% of strains, respectively, while the *frhA* gene was present in 22% of seafood isolates and 41% of environmental isolates. Besides, FrhA has been studied in O1 El Tor strains, where it plays a role in calcium binding on intestinal epithelial cells (Syed *et al.* 2009). The absence of *frhA* in some strains may complicate epithelial attachment, and further research is needed to explore the interaction between these adhesins.

3.1.4 Stage IV: Virulence factor expression

Four key toxins - hemolysin, repeat-in-toxin (RTX), cholix toxin, and heat-stable enterotoxin - are well-established virulence factors in clinical NOVC. These toxin genes were detected at varying frequencies in the strains studied. The *hlyA* gene (encoding hemolysin) was present in all strains, correlating with blood agar assays. The RTX gene cluster (*rtxABCDE*) was found in 60% of seafood isolates and 61% of environmental isolates. The *chxA* gene (cholix toxin) was present in 24% of seafood strains and 26% of environmental strains, while the *stn* gene (heat-stable enterotoxin) was present in only 1% of seafood strains and 8% of environmental strains (Chapter 2.2 and 2.3). Thus, NOVC strains in this study are likely capable of producing toxins at the genetic level.

Pathogenicity islands, such as VSP-2 and T3SS, were also identified in both studies. VSP-2 was present in 19% of seafood strains and 20% of environmental strains, indicating enhanced colonization ability. T3SS was found in six strains (two seafood strains and four environmental strains), suggesting these strains may exhibit higher colonization and infection efficiency (Dziejman *et al.* 2005).

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3.1.5 Stage V: Detachment from epithelial cells

At the end of the infection cycle, NOVC detaches from host epithelial cells to return to the environment. The *hapA* gene, which encodes hemagglutinin protease, was present in 100% of both seafood and environmental strains, facilitating detachment (Chapter 2.1). Flagella and motility-related genes, discussed in Chapter 3.1.2 (page 4), are also crucial in this stage.

3.1.6 Regulatory systems

Several regulatory systems, including quorum sensing, two-component systems, histone-like nucleoid structuring protein (HNS), and cyclic AMP-activated global transcriptional regulator (cAMP-CRP), were identified in NOVC strains. These regulators are responsible for controlling biofilm formation, motility, T6SS, adherence to epithelial cells, and pathogenicity (Chapter 2.1, page 16). Except for the O1/O139-specific regulators (e.g., *toxT*, *rstR*), all key regulators were present in the NOVC strains, suggesting robust self-regulation for survival in various environments. Quorum sensing enables cell-density communication, while HNS represses toxin gene expression in the later stages of infection. The cAMP-CRP system regulates hemolysin and RTX expression.

In summary, toxin-encoding genes were present in the isolated strains, and the absence of certain genes does not appear to act as a bottleneck in the infection process. Notably, some clinical NOVC strains contain only a subset of these virulence-associated genes, such as *rtxA*, *chxA*, and T3SS (Schirmeister *et al.* 2014). Clinical NOVC strains share a similar genetic makeup with those from food and environmental sources, a topic that will be further explored in Chapters 3.3 (page 51) and 3.4 (page 52).

3.2 Antimicrobial Resistance

The overuse and misuse of antimicrobial agents against *Vibrio cholerae* have contributed to the development of antimicrobial resistance (AMR), posing a growing threat to public health (Das *et al.* 2020). In this study, AMR genes were screened in NOVC strains isolated from

3. Discussion

seafood, and a microplate-based assay was conducted to assess phenotypic resistance. At the genetic level, all NOVC strains were found to carry efflux pumps, including resistance-nodulation-cell division (RND) and multidrug and toxic compound extrusion (MATE) systems. Additionally, specific AMR genes were identified, including *qnrVC* (quinolone resistance), *catB9* (chloramphenicol resistance), *blaCARB* (ampicillin resistance), *almG* (colistin resistance), *varG* (imipenem resistance), and *dfrA31* (trimethoprim resistance).

The conserved region of the SXT-R391 mobile genetic element (MGE) was detected in 14 strains, suggesting the presence of this transferable element. However, full integrons were identified in over 60% of the strains using the integron finder tool. Both complete integrons (with integrase *intI* and recombination sites *attI* /*attC*) and incomplete integrons (lacking one or both components) were found, but these integrons were not always located near AMR genes. This suggests that while integrons may be present in these NOVC strains, they may not be directly involved in conferring antimicrobial resistance in this context. The phenotypic AMR results aligned partially with in silico analysis, and incomplete AMR databases may explain some discrepancies, such as the absence of *blaCARB* in three ampicillin-resistant NOVC strains. In these cases, resistance might be mediated by efflux systems instead (Li *et al.* 2019).

AMR in NOVC has been reported globally. For example, *qnrVC* genes were found in NOVC isolated from shrimp in China (Xu *et al.* 2024), and this project also detected *qnrVC* in shrimp (chapter 2.2), suggesting the gene's prevalence in NOVC strains. Additionally, four multidrug-resistant NOVC strains were reported in Italian seafood (Ottaviani *et al.* 2018). A recent meta-analysis (Wu *et al.* 2023) highlights AMR in NOVC strains isolated over the last decade, with resistance to a wide array of antibiotics, including polymyxin B, norfloxacin, neomycin, kanamycin, gentamicin, streptomycin, ampicillin, cotrimoxazole, nalidixic acid, chloramphenicol, tetracycline, furazolidone, erythromycin, and ciprofloxacin. In this project, resistance to colistin, trimethoprim, and imipenem was newly observed (chapter 2.2, page 30).

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3.3 Virulence Potential of Food-Isolated NOVC

As previously discussed, foodborne NOVC infections have been reported, and these isolates often harbor a variety of virulence-associated genes. Although most NOVC strains do not carry the *ctxA* and *tcp* genes required for cholera toxin production, non-toxigenic NOVC can acquire these genes via phage-mediated horizontal gene transfer, potentially becoming toxigenic (Boyd *et al.* 2000). While the *ctxA* gene was absent in most NOVC strains in this study, NOVC of serotypes O75 and O141 have been shown to produce cholera toxin and cause severe watery diarrhea in the U.S. (Crowe *et al.* 2016).

All four toxin-encoding genes identified in clinical NOVC strains - *hlyA*, *rtxA*, *chxA*, and *stn* - were also present in the seafood-isolated NOVC in this study (chapter 3, page 30). The *hlyA* gene, which encodes hemolysin, was found in all strains, a pattern consistent with other food-associated NOVC worldwide (Tulatorn *et al.* 2018). The presence of *rtxA* and *rtxC* genes has been reported in other studies linking foodborne NOVC to human illness (Jiang *et al.* 2018; Luo *et al.* 2013; Chatterjee *et al.* 2009). The *chxA* gene, which encodes the cholix toxin, was detected both in this project and in foodborne clinical NOVC from countries such as Iran (Tangestani *et al.* 2020), Mexico (López-Hernández *et al.* 2015), and the U.S. (Purdy *et al.* 2010).

The Type III Secretion System (T3SS) also plays an important role in NOVC pathogenicity. The presence of T3SS-positive NOVC strains has been reported in multiple countries, including the U.S. (Dziejman *et al.* 2005), China (Luo *et al.* 2013), Pakistan (Zeb *et al.* 2019), and Chile (Arteaga *et al.* 2020). These studies consistently show that T3SS-positive NOVC strains are associated with more severe clinical symptoms compared with T3SS-neagtive NOVC strains. Functional T3SS genes, such as *vopE*, *vopF*, *vopM*, *vopW*, and *vopZ*, were identified across various studies, though gene alignments often differ, suggesting possible variations in T3SS morphology and pathogenicity, which warrant further investigation (chapter 3.5, page 51). Overall, the widespread distribution of virulence genes in food-associated NOVC highlights their potential to cause illness. Although NOVC is currently considered low-risk (Díaz *et al.* 2024), continued monitoring and research are essential.

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3.4 Virulence Potential of Environment-Isolated NOVC

The virulence potential of NOVC isolated from the German North Sea and Baltic Sea was analyzed in chapter 2.3, based on the presence of virulence-associated genes, genetic similarities to clinical NOVC, and phenotypic assays. A study by Schmidt *et al.* (2023) confirmed the presence of NOVC in German surface waters, with a genetic profile similar to the strains analyzed in this project, including the presence of *hlyA*, *rtxA*, and the Type VI Secretion System (T6SS). Studies from South Asia over the past 20 years have also reported NOVC strains with hemolytic activity and biofilm formation capabilities in surface waters (Halder *et al.* 2017; Bag *et al.* 2008; Singh *et al.* 2001). Similar findings have been observed in regions such as China (Li *et al.* 2020) and the U.S. (Ceccarelli *et al.* 2015), with a high prevalence of *hlyA* and *rtxA* in environmental NOVC strains, suggesting the global spread of these virulence genes.

Extraintestinal infections caused by NOVC have also been reported, with clinical strains harboring toxin-encoding genes such as *hlyA*, *rtxA*, and *chxA*. Interestingly, Schirmeister *et al.* (2014) found NOVC strains from otitis and wound infections that contained *hlyA* and *chxA* but were negative for *rtxA*.

This doctoral project confirmed the presence of virulence-associated genes in environment-isolated NOVC, indicating their pathogenic potential. Previous studies have linked increasing seawater temperatures to the geographic expansion of *Vibrio cholerae*, with warming waters facilitating the growth of NOVC and increasing the likelihood of horizontal gene transfer events that could enhance virulence (Baker-Austin *et al.* 2024; Vezzulli *et al.* 2020). The rising prevalence of antimicrobial-resistant genes in NOVC further complicates treatment options following infection, highlighting the need for continued research and monitoring (Verma *et al.* 2019).

3.5 Outlook: type three secretion system in NOVC

3. Discussion

The Type Three Secretion System (T3SS) was selected as the focus of this study due to its unique role and essential function in the colonization of the intestinal epithelium by NOVC (Dziejman *et al.* 2005). While other associated toxins such as hemolysin, repeats-in-toxin (RTX), cholix toxin, and heat-stable enterotoxin have been extensively studied (reviewed in Chapter 2.1, page 14), the T3SS is of particular interest due to its importance in NOVC toxicity. Despite its significance, the precise mechanism of T3SS action and the functions of its translocated proteins (Vops) remain under debate.

For example, VopF has been reported to disrupt tight junctions between host cells (Tam *et al.* 2007), but later research suggested that this disruption occurs independently of VopF (Miller *et al.* 2016). Similarly, VopE has been implicated in mitochondrial interference during colonization (Suzuki *et al.* 2014), but other studies argue that VopE is not required for colonization in infant mice (Chaand *et al.* 2015). Furthermore, many genes within the T3SS have yet to be fully understood (Chaand *et al.* 2015). Given these uncertainties, this study aimed to investigate the role of T3SS in NOVC toxicity, with preliminary results already available.

For gene expression analysis, reverse transcription polymerase chain reaction (RT-PCR) was employed, with the delta-delta-Ct method used for data standardization. Bovine bile (0.4%) was added to the culture medium as a trigger for T3SS expression, a method supported by a previous study (Alam *et al.* 2010). T3SS gene expression was confirmed in all six T3SS-positive NOVC strains, consistent with genetic data. The stimulatory effect of bovine bile on T3SS expression was also validated by Alam *et al.* (2011) and Miller *et al.* (2016).

However, differences in expression levels were observed between this study and others. For instance, Alam *et al.* (2011) reported an upregulation of *vttRA* following treatment with 0.4% crude bovine bile extracts, whereas in this study, *vttRA* expression was unaffected by bile treatment. This discrepancy could be due to strain-specific differences in T3SS expression, similar to the strain-specific expression of the *trh* gene (encoding thermostable-related

3. Discussion

hemolysin) in the T3SS2 of *Vibrio parahaemolyticus*, a homolog of T3SS in NOVC (Odaka *et al.* 2009).

Interestingly, none of the NOVC strains, including the T3SS-positive control strain VN459, harbored the *vopW* gene. Previous studies have identified VopW as a hydrophilic translocator involved in pore formation in host cells (Miller *et al.* 2019; Zhou *et al.* 2012). Another key gene, *vopF*, which is associated with colonization and pathogenicity (Tam *et al.* 2007), was detected in only one environmental NOVC strain, 18-VB00275. The other five NOVC strains showed lower sequence identity (70%–79%) to the control strain AM-19226, suggesting variations in *vopF* gene alignment and potentially different levels of gene expression and toxicity. Furthermore, expression levels of T3SS structural genes (*vcsS2, vcsN2, vcsC2, vcsR2, vcsV2, vcsJ2*) and effector genes (*vopH, vopA*) were higher in the strain 18-VB00287 compared to other T3SS-positive NOVC strains, indicating a potential advantage in colonization and infection (Dziejman *et al.* 2005).

In summary, T3SS genes were actively transcribed in the seafood- and environment-isolated NOVC strains in this study, supporting the functional role of T3SS in these strains.

To further investigate the functionality of T3SS, a cytotoxicity test was conducted based on the observed gene expression. The lactate dehydrogenase (LDH) assay was used to measure cell death rates, and electron microscopy was planned to observe morphological changes in the infected cells. HT-29B6 cells were selected as the target, and 0.4% bovine bile extract was added to stimulate T3SS gene expression. The results showed that bile treatment significantly increased both T3SS gene expression and cell death rates, consistent with findings from other studies (Miller *et al.* 2016).

Summary

Non-O1/non-O139 *Vibrio cholerae* (NOVC) is a natural inhabitant of aquatic ecosystems. It can cause both intestinal and extraintestinal infections in humans through the consumption of contaminated food or direct contact with contaminated surfaces, such as seawater or sediment. Previous studies have identified virulence-associated genes in NOVC that may contribute to its ability to cause infections. This doctoral thesis aimed to characterize the virulence mechanisms of NOVC isolated from food and environmental sources.

A literature review was conducted to explore the infection routes of NOVC and its virulence-associated gene profiles. The genes were then compared with whole genome sequences of NOVC isolates from seafood (63 strains) and the German sea coastline (31 strains) to determine the presence of virulence-associated genes. Phylogenetic analysis was also performed. Additionally, phenotypic tests, including biofilm formation, hemolytic activity, motility, and serum resistance, were carried out to further validate the genetic findings. The antimicrobial resistance (AMR) of NOVC was also examined at both the genetic and phenotypic levels.

Virulence-associated genes were classified into five stages, representing the infection process from ingestion to detachment from the human host. Most of these genes were detected in NOVC isolates from both food and environmental sources, including key toxin-encoding genes (*hlyA, rtxA, chxA, stn*), pathogenicity islands (VPI-2 and VSP-2), and secretion systems (T3SS and T6SS). A close core genome relationship was observed between the NOVC strains in this study and clinical NOVC strains. Notably, all NOVC strains exhibited increased motility at 37°C compared to 25°C, and most were capable of forming strong biofilms at both temperatures. All strains showed hemolytic activity against both human and sheep erythrocytes, but only a small subset of (6%) NOVC could survive in 60% human serum. In antimicrobial susceptibility tests, five NOVC strains developed non-wildtype resistance to antimicrobials across three different classes (penicillin, carbapenem, cephalosporin).

Summary

In conclusion, this study characterized the virulence profiles of NOVC isolates from seafood and German coastal waters, highlighting the need for further monitoring and research.

Zusammenfassung

Non-O1/non-O139 *Vibrio cholerae* (NOVC) sind weltweit in aquatischen Ökosystemen verbreitet und sind in der Lage sowohl intestinale als auch extraintestinale Infektionen beim Menschen auszulösen. Die Übertragung erfolgt über den Verzehr kontaminierter Lebensmittel oder den Kontakt mit kontaminiertem Wasser oder Sedimenten. Frühere Studien haben bereits virulenzassoziierte Gene in NOVC identifiziert, die potenziell mit Infektionen in Zusammenhang gebracht wurden. Ziel dieser Dissertation war es, die Virulenzmechanismen von NOVC-Isolaten aus Lebensmitteln und der Umwelt umfassend zu charakterisieren.

Um mögliche Infektionswege von NOVC sowie das Vorhandensein virulenzassoziierter Gene aufzudecken, wurde eine weitreichende Literaturrecherche durchgeführt. Anschließend fand ein Genescreening aller bisher publizierten virulenzassoziierten Gene in den Ganzgenomensequenzen aus Fisch und Meeresfrüchten (63 Stämme) sowie der deutschen Ost- und Nordseeküste (31 Stämme) isolierten NOVC statt, um die Präsenz relevanter Virulenzfaktoren zu bestimmen. Darüber hinaus wurden phylogenetische Analysen zur Bestimmung der genetischen Verwandtschaft zu klinischen Isolaten durchgeführt. Phänotypische Untersuchungen wie die Analyse der Biofilmbildung, hämolytischen Aktivität, Motilität und Serumresistenz dienten der Validierung der genetischen Ergebnisse. Die antimikrobielle Resistenz (AMR) der NOVC-Isolate wurde sowohl auf genetischer Ebene als auch mittels phänotypischer Tests analysiert.

Anhand der vorangegangenen Literaturrecherche wurde ein Infektionsmodell für NOVC erstellt, welches die benötigten Gene im Infektionsverlauf nach oraler Aufnahme in den humanen Organismus bis zur Ausscheidung in die Umwelt beschreibt. Virulenzassoziierte Gene wurde in NOVC-Isolaten aus Lebensmitteln und Umweltproben nachgewiesen, darunter wichtige Toxin-kodierende Gene (*hlyA*, *rtxA*, *chxA*, *stn*), Pathogenitätsinseln (VPI-2 und VSP-2) sowie Sekretionssysteme (T3SS und T6SS). Die phylogenetische Analyse zeigte eine enge Kern-Genom-Verwandtschaft zwischen NOVC-Isolaten aus Infektionsgeschehen, Lebensmitteln und der Umwelt. Auffällig war, dass alle NOVC-Stämme bei 37°C eine erhöhte

Zusammenfassung

Motilität im Vergleich zu 25°C zeigten und bei beiden Temperaturen Biofilmbildung aufwiesen.

Alle Stämme zeigten eine hämolytische Aktivität gegenüber Human- und Schaf-Erythrozyten, wobei jedoch nur 6% der Isolate in 60% menschlichem Serum überleben konnten. Antimikrobielle Empfindlichkeitstests ergaben, dass fünf NOVC-Isolate eine nicht-wildtypische Resistenz gegenüber antimikrobiellen Wirkstoffen aus drei verschiedenen Wirkstoffklassen (penicillin, carbapenem, cephalosporin) entwickelten.

Diese Arbeit liefert eine umfassende Charakterisierung der Virulenzprofile von NOVC-Isolaten aus Fisch und Meeresfrüchten sowie deutschen Küstengewässern und betont die Notwendigkeit einer verstärkten Überwachung und weiterführenden Forschung.

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Conflict of Interest

In the context of this work, there are no conflicts of interest due to contributions from third parties.

Declaration of independence

I hereby certify that I have prepared this thesis independently. I certify that I have used only the sources and aids indicated.

Berlin, 10/10/2024, Quantao Zhang