Characterization of *Vibrio* spp. isolates from Germany by reference to human clinical isolates using phenotypic and WGS-assisted genotypic methods

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List of Abbreviations

ACD	actin cross-linking domain
Acf	accessory colonization factor
ADP	adenosine diphosphate
AI-2	autoinducer-2
BON domain	bacterial OsmY and nodulation domain
bp	base pair(s)
BPI	bactericidal/permeability-increasing protein
BWM	Ballast Water Management
Caco-2	human epithelial colon adenocarcinoma cell line
CAI-1	cholera autoinducer-1
cAMP	cyclic adenosine monophosphate
CDC	Centers for Disease Control and Prevention
CFU	colony forming unit(s)
Changliver	HeLa derivative
ChxA	cholix toxin
cps	gene encoding a putative capsule biosynthesis protein
CqsA	CAI-1 autoinducer synthase
CqsS	CAI-1 autoinducer receptor
CTX	cholera toxin
ctxA	gene encoding the A subunit of the cholera toxin
CTXΦ	lysogenic filamentous bacteriophage whose genome encodes the cholera toxin
dctM*, *dctP*, *dctQ	genes encoding proteins involved in sialic acid transport
δ-VPH	delta *V. parahaemolyticus* hemolysin
DLV	double-locus variant
DNA	deoxyribonucleic acid
DncV	*V. cholerae* dinucleotide cyclase
DT	diphtheria toxin
DTH	delta thermostable hemolysin
ECDC	European Centre for Disease Prevention and Control
eEF	eukaryotic elongation factor

HA/P; HapA	hemagglutinin/protease
Hcp	hemolysin-coregulated protein
HeLa	human epithelial cervix adenocarcinoma cell line
HHM	Hafen Hamburg Marketing e.V.
HisF	protein of the baseplate structure of the *V. cholerae* T6SS
HlyA	hemolysin A
HlyA^{ET}	El Tor variant of hemolysin A
HlyD	hemolysin D protein
HlyIII	hemolysin III protein
IfSG	Infektionsschutzgesetz
IMO	International Maritime Organization
ISO	International Organization for Standardization
kb	kilobase(s)
kbp	kilobase pair(s)
KLIWAS	Auswirkungen des Klimawandels auf Wasserstraßen und Schifffahrt in Deutschland (research program)
LDH	lecithin-dependent hemolysin
LuxPQ	AI-2 autoinducer receptor
LuxS	AI-2 autoinducer synthase
MARTX	multifunctional-autoprocessing repeats-in-toxin toxin
Mb	megabase(s)
MDa	megadalton
µm	micrometer
MLSA	multilocus sequence analysis
MLST	multilocus sequence typing
MPN	most probable number
MSHA	mannose-sensitive hemagglutinin
NaCl	sodium chloride
nagA*, *nanA*, *nanE*, *nanK	genes necessary for sialic acid catabolism
nanH	gene necessary for sialic acid scavenging
Neu5Ac	N-acetylneuraminic acid
O-antigen	surface antigen
O1	O1-antigen
O139	O139-antigen

OmpT	outer membrane protein T
OmpU	outer membrane protein U
ORF	open reading frame
OsmY	putative BON domain-containing hemolysin in *V. navarrensis*
PAAR-motif proteins	proline-alanine-alanine-arginine motif proteins
PCR	polymerase chain reaction
pilV*, *pilW	genes encoding proteins for type IV pilus biosynthesis
ppt	parts per thousand
pVH	plasmid VH
QMRA	quantitative microbial risk assessment
RadC	DNA repair protein
RAW 264.7	murine macrophage cell line
***rfb* O1**	O1-antigen-specific genomic region
***rfb* O139**	O139-antigen-specific genomic region
RNA	ribonucleic acid
RNase H1	ribonuclease H1
rpoB	gene encoding the β subunit of RNA polymerase
RstA	protein required for replication of CTXΦ
RstB	protein required for site-specific integration of CTXΦ
RstR	repressor of *rstA* and *rstB* transcription
rtx	repeats-in-toxin gene
SA	sialic acid
SLV	single-locus variant
SNP	single nucleotide polymorphism
SST	sea surface temperature
ST	sequence type
T1SS	type I secretion system
T4P	type IV pilus
T6SS	type VI secretion system
TarB	ToxT-activated sRNA B
TCP	toxin-coregulated pilus
TcpA	major pilin protein of the toxin-coregulated pilus
TcpB	minor pilin protein of the toxin-coregulated pilus
TcpP	transmembrane protein; activator of *toxT* transcription

TGD	Tiergesundheitsdienst Bayern e.V.
TLH	thermolabile hemolysin
TLV	triple-locus variant
tolC	gene encoding a type I secretion system protein
ToxR	transmembrane protein; activator of *toxT* and *ompU* transcription
ToxT	activator of *ctx* and *tcp* transcription
TRH	TDH-related hemolysin
TTSS	type III secretion system
US EPA	United States Environmental Protection Agency
VasA, VasB, VasE, VasJ	proteins of the baseplate structure of the *V. cholerae* T6SS
VasD, VasF, VasK	proteins of the membrane complex of the *V. cholerae* T6SS
VBNC	viable but nonculturable
VCC	*V. cholerae* cytolysin
VcsC2, VcsN2, VcsV2	structural proteins of the *V. cholerae* TTSS
VgrG	protein of the T6SS spike
VibrioNet	*Vibrio*-Infektionen durch Lebensmittel und Meerwasser in Zeiten des Klimawandels (research program)
VipA, VipB	proteins of the outer sheath of the *V. cholerae* T6SS
Vop	*Vibrio* outer protein
VPI-1/-2	*Vibrio* pathogenicity island-1/-2
VSP-1/-2	*Vibrio* seventh pandemic island-1/-2
VspD	structural protein of the *V. cholerae* TTSS
VspR	*V. cholerae* seventh pandemic regulator
VvhA	*V. vulnificus* hemolysin A
WGS	whole genome sequencing
WHO	World Health Organization
WHO-ORS	WHO-Oral Rehydration Solution

1 Introduction

1.1 *Vibrio cholerae* and *Vibrio navarrensis*

1.1.1 Taxonomy and Characteristics

Vibrio cholerae and *Vibrio navarrensis* are members of the genus *Vibrio*, which belongs to the six genera comprising family *Vibrionaceae* within the class Gammaproteobacteria (Gomez-Gil *et al*., 2014).

The genus *Vibrio* embraces asporogenous, Gram-negative, straight or curved rod-shaped bacteria with a size of 0.5-0.8 × 1.4-2.6 µm and polar, predominantly monotrichous flagellation. Vibrios tolerate a wide temperature range for growth (4°C-45°C), whereby the temperature requirements vary depending on the species (Farmer *et al*., 2005). While *V. navarrensis* can grow in a temperature range of 10°C to 42°C with a temperature optimum of 30°C to 37°C (Urdaci *et al*., 1991) and *V. cholerae* in a temperature range of 10°C to 43°C with a temperature optimum of 37°C (ICMSF, 1996), some species such as *V. tapetis* show optimal growth at lower temperatures (optimum: 18°C, range: 4°C-22°C) (Borrego *et al*., 1996). All *Vibrio* species grow well at 20±2°C (Farmer *et al*., 2005). With the exception of *V. mimicus* and *V. cholerae*, all *Vibrio* species have a salt requirement for growth, with many species tolerating NaCl concentrations ≥3.5% (Farmer *et al*., 2005). *V. cholerae* growth can occur in a salt range of 0-4% NaCl (ICMSF, 1996; Farmer *et al*., 2005). *V. navarrensis* can grow in a range of 0.5-7% NaCl, with a few strains showing weak growth at 0% NaCl (Urdaci *et al*., 1991). Most *Vibrio* species grow well in a salt range of 0.5-2% NaCl (Farmer *et al*., 2005). *Vibrio* bacteria have a low acid tolerance and grow well under neutral and alkaline conditions up to pH 9 (Tantillo *et al*., 2004). They are facultatively anaerobic and distributed in aquatic environments throughout the world (Thompson *et al*., 2004).

The genus *Vibrio* comprises over 100 species[1]. Among them, the following ten species have been repeatedly associated with human infections: *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. cincinnatiensis*, *V. fluvialis*, *V. furnissii*, *V. metschnikovii*, *V. mimicus*, and *V. harveyi* (Wilkins *et al*., 2008; Hundenborn *et al*., 2013; Gomez-Gil *et al*., 2014; Akram *et al*., 2015; Brehm *et al*., 2020). The most important human pathogenic *Vibrio* species are *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (Gomez-Gil *et al*., 2014).

[1] https://lpsn.dsmz.de/genus/vibrio

V. cholerae was first described in 1854 by Filippo Pacini, who discovered the bacterium in large numbers in the feces and intestinal mucosa of deceased cholera patients in Italy. In 1883 and 1884, *V. cholerae* was described for the second time by Robert Koch, who studied cholera disease in Egypt and India (Calcutta) and finally isolated the bacterium from the intestinal contents of cholera patients in pure culture (Lippi and Gotuzzo, 2014). Years of research on *V. cholerae* has been accompanied by the emergence of various classification schemes. One scheme that has been used in strain differentiation for many years is based on structural differences in the somatic O-antigen. To date, more than 200 serogroups have been identified, of which only serogroups O1 and O139 are associated with cholera epidemics and pandemics (Sozhamannan and Yildiz, 2011). Serogroup differentiation is complemented by biotype differentiation. On the basis of differences in phenotypic traits such as bacteriophage susceptibility, polymyxin B susceptibility, Voges-Proskauer test, agglutination of chicken erythrocytes, and hemolytic activity against sheep erythrocytes, serogroup O1 is subdivided into two biotypes: Classical and El Tor (Kaper *et al*., 1995). Between 1817 and 1926, there were six distinct cholera pandemics, all of which originated in the Indian Ganges delta region. The last pandemic was demonstrably caused by O1 strains of the classical biotype. Unlike the sixth pandemic, the seventh pandemic of cholera (1961 to the present) arose from the Indonesian island of Sulawesi and is associated with O1 strains of the El Tor biotype. In 1992, a non-O1 *V. cholerae* strain, later serogrouped as O139, was isolated against the background of cholera epidemics in India and Bangladesh (Faruque and Nair, 2006; Lekshmi *et al*., 2018). Genetic analyses of Comstock *et al*. (1996) revealed that the serogroup O139 originated from an O1 El Tor strain by a 22-kb deletion of the O1-antigen-specific genomic region (*rfb* O1) and simultaneous insertion of a 35-kb O139-antigen-specific genomic region (*rfb* O139) (Comstock *et al*., 1996). Serogroups other than O1 and O139 are commonly designated as non-O1, non-O139 *V. cholerae*. In 2000, the first complete whole genome sequence of *V. cholerae* was reported: The genome of *V. cholerae* El Tor N16961 consists of 4,033,460 base pairs (bp) that encode 3,885 open reading frames. It is split into two circular chromosomes, which contain 2,961,146 bp (chromosome 1) and 1,072,314 bp (chromosome 2), respectively. The large chromosome harbors most of the recognizable genes for bacterial pathogenicity (e.g., surface antigens, adhesins, and toxins) and essential cell functions (e.g., DNA repair and replication, transcription, translation, bacterial cell wall biosynthesis, and major metabolic pathways) (Heidelberg *et al*., 2000). In contrast, the small chromosome seems to carry more of the genes involved in the bacterial response on changing environmental conditions (Okada *et al*., 2005). While the proportion of hypothetical genes is

42% in the large chromosome, the proportion of this gene category in the small chromosome is distinctly higher (59%). Many of the hypothetical genes of chromosome 2 are localized in the integron island, a 125.3-kb large gene capture system that is still at the beginning of its exploration (Heidelberg *et al*., 2000). Meanwhile, complete or draft genome sequences of more than 1,000 *V. cholerae* strains are available[2].

During a survey on the distribution of pathogenic *V. cholerae* bacteria in aquatic environments of the Spanish province Navarra, a new *Vibrio* species was discovered in sewage and river water: *Vibrio navarrensis* (Urdaci *et al*., 1991). In 2007, further strains from the German Baltic Sea were reported, which were later classified as *V. navarrensis* biotype *pommerensis*. Differentiation of biotype *pommerensis* strains from *V. navarrensis* strains of the classical biotype is based on several biochemical properties (e.g., utilization of β-gentiobiose, 5-keto-D-gluconate, α-lactose, and lactulose as carbon sources) (Jores *et al*., 2007). In 2014, the Centers for Disease Control and Prevention (CDC) published results on the characterization of *V. navarrensis* isolates associated with human illness (Gladney and Tarr, 2014). A few weeks later, the first whole genome sequences of three *V. navarrensis* strains were reported: the genome sequence of a Spanish sewage strain, the type strain ATCC 51183 (identical to CIP 103381), as well as the genome sequences of two American human clinical strains, 0053-83 and 08-2462. The genome of *V. navarrensis* has an average size of 4.3 Mb, with an average number of predicted genes of approximately 3,800. It is split into two circular chromosomes, as it is characteristic for *Vibrio* species (Okada *et al*., 2005; Gladney *et al*., 2014). Analysis of the *V. navarrensis* genome sequences revealed the presence of many genes commonly associated with virulence in other human pathogens (Gladney *et al*., 2014). At the start of this cumulative dissertation, genome sequences of four *V. navarrensis* strains were available in public databases[3].

1.1.2 Isolation and Identification

A number of selective media for the enrichment and isolation of *Vibrio* bacteria have been developed. One of the most commonly used media for the selective enrichment of vibrios from environmental, food, and clinical samples is alkaline peptone water (APW). While the nutrients peptone and NaCl promote the growth of vibrios, the high pH value of the medium (pH 8.6) inhibits the growth of many background microorganisms (Oliver, 2012; Gomez-Gil *et al*., 2014).

[2] https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/Vibrio cholerae
[3] https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/Vibrio navarrensis

Selective isolation and purification of *Vibrio* spp. strains from clinical, environmental, or food sources is routinely performed on thiosulfate-citrate-bile salts-sucrose (TCBS) agar. On this medium, sucrose-fermenting *Vibrio* species like *V. cholerae*, *V. navarrensis*, and *V. alginolyticus* form yellow colonies, while sucrose-negative species such as *V. parahaemolyticus* and *V. vulnificus* grow as green to blue-green colonies. Gram-positive and coliform Gram-negative bacteria are strongly inhibited in their growth due to the presence of bile salts, which are commonly tolerated by *Vibrio* bacteria (Oliver, 2012; Gomez-Gil *et al*., 2014). Another plating medium recommended for the isolation of *Vibrio* species from environmental and food samples is CHROMagar^{TM} Vibrio (CVA). This chromogenic medium enables the differentiation between *V. cholerae*/*V. vulnificus* (green-blue to turquoise), *V. parahaemolyticus* (mauve), and *V. alginolyticus* (colorless) colonies (Oliver, 2012).

To confirm presumptive *Vibrio* spp. colonies, various molecular biological methods are used. In routine diagnostics, genus level identification and preliminary species level identification are commonly based on matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Dieckmann *et al*., 2010; Clark *et al*., 2013; Erler *et al*., 2015; Rychert *et al*., 2015). For confirmation, characterization, and subtyping of presumptive *V. cholerae* isolates, a multiplex PCR system has been established targeting *toxR* (virulence regulator gene, species identification), *ctxA* (cholera toxin gene), and *rfb* sequences specific for O1 and O139 serogroups (Schirmeister *et al*., 2014a; Bier *et al*., 2015). Another possibility for species identification or confirmation is partial sequencing of housekeeping genes. One of the most widely used genes for reliable species determination within the genus *Vibrio* and a promising gene for identification of recently discovered *Vibrio* species like *V. navarrensis* is *rpoB* encoding the β subunit of RNA polymerase (Tarr *et al*., 2007; Adékambi *et al*., 2009; Oberbeckmann *et al*., 2011; Schirmeister *et al*., 2014a; Schirmeister *et al*., 2014b).

1.1.3 Habitats and Ecology

Vibrios are primarily aquatic bacteria, which are distributed throughout the world.

V. cholerae bacteria naturally occur in marine coastal waters, estuarine (brackish) environments, and freshwater ecosystems (Farmer *et al*., 2005). In their natural habitats, they can be found as free-living cells in the water column (Kirschner *et al*., 2008), in sediments (Ceccarelli *et al*., 2015) as well as in association with plankton (Lizárraga-Partida *et al*., 2009), fish (Halpern and Izhaki, 2017), crustaceans (Sullivan and Neigel, 2018), and bivalve mollusks (Collin and Rehnstam-Holm, 2011). Their geographic range extends from tropical

waters (e.g., the Bay of Bengal) to temperate waters world-wide (e.g., Australia, South America, USA, Italy, and Sweden) (Lutz *et al*., 2013). In northern European waters, only non-O1, non-O139 *V. cholerae* strains occur (Böer *et al*., 2013; Huehn *et al*., 2014).

V. cholerae concentrations in aquatic habitats vary widely depending on water temperature, salinity, and source of isolation. In seawater and brackish water, the *V. cholerae* abundance is generally <50 (MPN-estimated) CFU per 100 ml (Vezzulli *et al*., 2009; Schuster *et al*., 2011; Böer *et al*., 2013; Léon Robles *et al*., 2013; Fang *et al*., 2019). Occasionally, elevated *V. cholerae* levels of 10^3 MPN-estimated CFU per 100 ml (Fang *et al*., 2019) or even 2.9×10^5 CFU per 100 ml (southern Californian coastal waters, summer 1999) (Jiang and Fu, 2001) were observed. In sediments, the population density of *V. cholerae* ranges from <1 to $>10^6$ CFU per 100 g depending on the study area (Murugesan *et al*., 2012; Böer *et al*., 2013). Due to their filter-feeding mode of life, bivalve mollusks can show up to 100-fold higher *V. cholerae* concentrations compared to the surrounding water (Vezzulli *et al*., 2010). Jones *et al*. (2014) reported a maximum *V. cholerae* level of $\sim 3 \times 10^3$ MPN-estimated CFU per 100 g in oysters harvested from Long Island Sound growing waters, a North Atlantic estuarine system, between July and September 2012. Similar maximum levels were reported by Fang *et al*. (2019) studying *V. cholerae* abundance in oysters from Apalachicola Bay, an inlet of the Gulf of Mexico, between 2012 and 2014. Quantitative studies on the occurrence of *V. cholerae* in fish or in association with zooplankton are rare. One study conducted in Israeli freshwater and marine habitats revealed *V. cholerae* numbers of 1.4×10^2 and 4.8×10^3 CFU per 1 g intestine content in Flathead gray mullet and Galilee St. Peter's fish, respectively (Senderovich *et al*., 2010). In another study conducted in the Chesapeake Bay from April to December, 10^5 to 10^7 *V. cholerae-V. mimicus* cells per m^3 of Choptank River water were found to be associated with zooplankton. Overall, *V. cholerae-V. mimicus* represented a large proportion (up to 60%) of the total zooplankton-associated *Vibrio-Photobacterium* population (Heidelberg *et al*., 2002).

As already mentioned, the occurrence of *V. cholerae* in aquatic habitats is strongly influenced by two abiotic environmental parameters: the water temperature and the salinity. Although *V. cholerae* bacteria have occasionally been found at water temperatures of 8-9°C (Hackbusch *et al*., 2020), they were commonly detected at water temperatures >17°C (Oliver *et al*., 2013). *V. cholerae* has been identified mainly in estuarine and marine coastal environments within a salinity range of 0-37 ppt (Jiang and Fu, 2001; Eiler *et al*., 2006; Griffitt and Grimes, 2013; Siboni *et al*., 2016). A meta-analysis of numerous studies revealed that *V. cholerae* occupies a temperature range of ca. 10-30°C in brackish water (1-10 ppt). An increase in salinity

(0-10 ppt → 40 ppt) is generally accompanied by a decrease in *V. cholerae* concentration. The highest *V. cholerae* abundance was found around a temperature of 20°C and in a salinity range of 0-10 ppt (ca. 10^5 cells per 100 ml), whereas abundances observed around this temperature at higher salinities were distinctly lower (ca. 10^2 cells per 100 ml) (Takemura *et al*., 2014).

One possible explanation for the low detection frequency of *V. cholerae* at water temperatures <10°C can be its entry into and persistence within the viable but nonculturable (VBNC) state. The VBNC state can be induced by various environmental stresses, such as low temperature or low nutrient availability. It is defined as a physiological state of dormancy, in which cells demonstrate strongly diminished metabolic activity and are no longer culturable on routine bacteriological media. Following favorable environmental stimuli, cells can resuscitate and regain their ability to cause infection (Colwell, 2000; Oliver, 2005; Oliver, 2010). Wai *et al*. (1996) reported resuscitation of *V. cholerae* from the VBNC state by temperature upshift. Colwell and colleagues observed resuscitation after injection of VBNC *V. cholerae* into rabbit ileal loops (Colwell *et al*., 1985) and ingestion of a VBNC *V. cholerae* suspension by human volunteers (Colwell *et al*., 1996).

Studies on the occurrence of *Vibrio* spp. in German coastal waters revealed specific spatial distribution patterns for major potentially human pathogenic *Vibrio* species. Along the Baltic Sea coast, non-O1, non-O139 *V. cholerae* was found to be the dominant species, whereas *V. vulnificus*, *V. alginolyticus*, and *V. parahaemolyticus* were less prevalent. The *Vibrio* population along the North Sea coast was dominated by *V. alginolyticus*, while *V. parahaemolyticus*, *V. vulnificus*, and non-O1, non-O139 *V. cholerae* were less frequent (Brennholt *et al*., 2014). The composition of the *Vibrio* populations in the North Sea and Baltic Sea may be influenced by the different salinity characteristics of the two coastal waters and mirrors the preference of *V. cholerae* for low to moderate salinity. The Baltic Sea is an epicontinental sea with lower salinity (<18 ppt), while the North Sea has higher salinity levels (up to 34 ppt) due to its direct connection to the Atlantic Ocean (Baker-Austin *et al*., 2012). Between 2010 and 2012, *V. cholerae* non-O1, non-O139 was detected with a frequency of 26% in water samples and 39% in sediment samples of the German Baltic Sea along the Mecklenburg-Western Pomeranian coastline (Huehn *et al*., 2014). During a survey on the prevalence of non-O1, non-O139 *V. cholerae* at recreational beaches along the central Wadden Sea coast and within the estuaries of Lower Saxony, *V. cholerae* was found with a frequency of 2% in North Sea water and 4% in sediment samples between 2009 and 2011 (Böer *et al*., 2013). Analysis of blue mussels (*Mytilus edulis*) of classified harvesting areas in

the German Wadden Sea revealed 16% and 11% non-O1, non-O139 *V. cholerae* positive samples in 2012 and 2013, respectively (Huehn *et al*., 2014). Along the German Baltic Sea coastline, no mussel production areas exist. When analyzing *Mytilus edulis* collected from near-shore rocks in the Sound (Öresund) on the south coast of Sweden during the summer 2006 with a mean water temperature above 20°C, 53% of samples were positive for non-O1, non-O139 *V. cholerae* (Collin and Rehnstam-Holm, 2011). In recent years, *V. cholerae* abundances in German coastal waters were generally low (Böer *et al*., 2013; Bartelt, 2014; Brennholt *et al*., 2014). However, increasing prevalences and concentrations of this human pathogenic *Vibrio* species must be assumed for future years due to climate change.

One of the major physical impacts of climate change is the rise of global sea surface temperature (SST) (EEA, 2017). European seas, such as the North Sea and Baltic Sea, are two of the most rapidly warming marine ecosystems of the world (Belkin, 2009). According to climate model projections of the German research program KLIWAS, an increase in the number of months with SST > 17°C from 1 month (1970-1999) to 2.5 months (2021-2050) is expected for the Baltic Sea coast, while an increase from 2-3 months (1970-1999) to 3.5-4.5 months (2021-2050) is predicted for the North Sea coast (Brennholt *et al*., 2014). Recent studies conducted in the German Bight revealed that extended warm periods with seawater temperatures above 17°C coincided with prolonged occurrences of potentially human pathogenic *Vibrio* spp. including *V. cholerae* (Hackbusch *et al*., 2020). In addition to rising sea surface temperatures, climate change is also associated with an increase in the intensity and frequency of extreme weather events (EEA, 2017). Severe heat waves and heavy precipitation events may result in warmer and less saline waters, especially in coastal areas, which may favor *V. cholerae* proliferation.

While *V. cholerae* has been extensively studied for decades, current knowledge on natural habitats of recently discovered *Vibrio* species such as *V. navarrensis* is still fragmentary. *V. navarrensis* was first isolated from sewage and river water of the Spanish province Navarra (Urdaci *et al*., 1991). Later, some strains from seawater of the Spanish Mediterranean coast (Macián *et al*., 2000) and the German Baltic Sea coast (Jores *et al*., 2003b; Jores *et al*., 2007) were reported, whereby species identification of the Spanish seawater strains was based exclusively on phenotypic characteristics (Arias *et al*., 1999; Macián *et al*., 2000). All *V. navarrensis* strains were found during surveys on the distribution of potentially human pathogenic *V. cholerae* (Urdaci *et al*., 1991) and *V. vulnificus* (Macián *et al*., 2000; Jores *et al*., 2007) in aquatic environments. Most of the strains confirmed by genotypic methods were recovered during summer months (Urdaci *et al*., 1991; Jores *et al*., 2003b; Jores *et al*., 2007).

In 2011 and 2015, first *V. navarrensis* strains were isolated from the German North Sea (Schwartz *et al*., 2017). Some time ago, our laboratory received veterinary *V. navarrensis* strains isolated from livestock in Germany that were found in aborted fetuses and/or placentas after miscarriages broadening the ecological range of habitats of this species. So far, however, no studies focusing on the occurrence and ecology of *V. navarrensis* have been performed.

1.1.4 Virulence Factors

In *V. cholerae*, a number of virulence factors and virulence-associated factors have been described. The major virulence factors of toxigenic O1/O139 *V. cholerae* strains are the cholera toxin and the toxin-coregulated pilus (Prouty and Klose, 2006). The cholera toxin (CTX) is a heterohexameric A-B toxin composed of a wedge-shaped toxic-active A subunit and a pentameric ring of cell-binding B subunits (Zhang *et al*., 1995). After binding of the holotoxin to G_{M1} ganglioside receptors in the small intestinal mucosa, the A subunit is translocated to the cytosol of the epithelial cells, where it catalyzes ADP-ribosylation of the α subunit of the G_S protein. As a result of this reaction, adenylate cyclase remains in an activated state leading to an increase in intracellular cAMP concentration and finally to hypersecretion of electrolytes and water into the small intestinal lumen (Sánchez and Holmgren, 2011; Clemens *et al*., 2017). The cholera toxin is encoded on CTXΦ, a lysogenic filamentous bacteriophage that integrated into the *V. cholerae* genome (chromosome 1 and/or 2) (Boyd, 2012). The CTXΦ genome consists of two distinct modules: RS2 region and core region. The RS2 region encodes proteins required for regulation (RstR), replication (RstA), and site-specific integration (RstB) of CTXΦ, with RstR acting as a repressor of *rstA* and *rstB* transcription. The core region includes the *ctxAB* operon (CTX) and encodes proteins that are important for phage morphogenesis and assembly (i.a., Ace and Zot) (Boyd, 2012; Pant *et al*., 2020). The toxin-coregulated pilus (TCP) is a type IV pilus composed of a polymer of repeating subunits of the major pilin protein TcpA and one or more copies of the minor pilin protein TcpB. TCP serves as the CTXΦ receptor and is involved in the colonization of the human small intestine by *V. cholerae* (Prouty and Klose, 2006; Gutierrez-Rodarte *et al*., 2019). Studies on the human intestinal cell line Caco-2 and the infant mouse model suggest that TCP mediates attachment of *V. cholerae* to the intestinal epithelial cells. Further, it has been observed that TCP mediates bacterial interactions, which facilitate microcolony formation and may thereby protect *V. cholerae* bacteria from host defenses (Kirn *et al*., 2000; Lim *et al*., 2010; Krebs and Taylor, 2011). The toxin-coregulated pilus is encoded on the *Vibrio* pathogenicity island-1 (VPI-1), a 39.5-kb mobile genetic element integrated into the

V. cholerae genome (Karaolis *et al*., 1998; Murphy and Boyd, 2008). The expression of the two major virulence factors CTX and TCP is controlled by a complex regulatory cascade, historically referred to as the ToxR regulon. In this virulence factor regulatory cascade, the transmembrane proteins ToxR and TcpP jointly activate *toxT* transcription and ToxT acts as a direct transcriptional activator of the *ctx* and *tcp* operons (Childers and Klose, 2007).

In addition to the major virulence factors CTX and TCP, multiple accessory virulence factors such as the mannose-sensitive hemagglutinin pilus, outer membrane proteins, the repeats-in-toxin toxin cluster, and hemolysins are known, which contribute to the infection process in a synergistic way (Schirmeister *et al*., 2014a).

The mannose-sensitive hemagglutinin (MSHA) pilus is a type IV pilus that facilitates environmental persistence of *V. cholerae* in aquatic habitats by promoting adherence to cell surfaces and contributing to biofilm formation (Watnick *et al*., 1999; Chiavelli *et al*., 2001; List *et al*., 2018). Similar to TCP, MSHA serves as a bacteriophage receptor (Boyd, 2008; Faruque and Mekalanos, 2012). While TCP plays an important role in human colonization, the role of MSHA still has to be clarified. The major pilin subunit of the mannose-sensitive hemagglutinin pilus is encoded by *mshA* within the MSHA pilus biogenesis gene cluster (Jonson *et al*., 1994; Marsh *et al*., 1996; Marsh and Taylor, 1999).

The pore-forming outer membrane protein OmpU contributes to the survival of *V. cholerae* in the human host by increasing *V. cholerae* resistance to bile. In a bile-containing environment like the human small intestine, the transmembrane protein ToxR (independent of TcpP and ToxT) activates *ompU* transcription while it represses *ompT* transcription. Due to the smaller channel size of OmpU compared to OmpT, the former porin prevents bile flux into the bacterial cell (Wibbenmeyer *et al*., 2002). In addition, *V. cholerae* OmpU confers resistance to antimicrobial peptides such as peptide P2 derived from the bactericidal/permeability-increasing (BPI) protein. BPI is an antimicrobial protein found on the surface of human intestinal epithelial cells as well as on the cell surface and in azurophilic granules of neutrophils (Mathur and Waldor, 2004; Mathur *et al*., 2007).

The *rtx* gene cluster of *V. cholerae* is composed of two divergent operons: *rtxHCA* and *rtxBDE*. The *rtxHCA* genes encode the multifunctional autoprocessing repeats-in-toxin toxin (RtxA, MARTX), a putative acyltransferase (RtxC), and a protein of unknown function (RtxH). The *rtxBDE* genes and the *tolC* gene outside the *rtx* locus encode a four-component type I secretion system mediating translocation of MARTX from the bacterial cytosol into the extracellular milieu (Linhartova *et al*., 2015). With a size of 4,545 amino acids (O1 biovar El Tor strain N16961), *V. cholerae* MARTX is among the largest RTX exotoxins. It consists

of conserved repeats-containing N-terminal and C-terminal regions and a central region with different effector domains (Satchell, 2007). *V. cholerae* MARTX of toxigenic El Tor strains contains three conserved effector domains, including an actin cross-linking domain (ACD). After binding of the MARTX toxin to the host cell and pore formation, the effector domains are translocated into the host cell cytosol. ACD-mediated actin cross-linking within neutrophil granulocytes leads to disruption of their cytoskeleton and finally to inhibition of their phagocytic ability. In this way, the MARTX toxin is involved in evasion of the human immune defense facilitating human colonization by *V. cholerae* (Satchell, 2015; Kim, 2018). The acyltransferase RtxC may contribute to MARTX activation by posttranslational modification (Linhartova *et al*., 2015).

In the human host, the amount of extracellular iron is very limited. Hemolysins, such as the pore-forming exotoxin HlyA, effect lysis of the erythrocyte membrane leading to a release of hemoglobin and consequently to an increase in the level of extracellular available iron, which fosters *V. cholerae* growth (Stoebner and Payne, 1988; Huntley *et al*., 1997). While El Tor *V. cholerae* strains have been described to be hemolytic on erythrocytes from a variety of animal sources, including chicken and sheep erythrocytes, classical *V. cholerae* strains are not capable of lysing sheep erythrocytes (Richardson *et al*., 1986). Differences in hemolytic activity among El Tor isolates and classical isolates seem to be the result of an 11-bp deletion in the *hlyA* gene of classical strains leading to the production of a truncated protein (Kaper *et al*., 1995). In addition to its role in tapping iron sources, the hemolysin HlyA, also referred to as *V. cholerae* cytolysin (VCC; Zhang and Austin, 2005), may be a major diarrheagenic factor for non-cholera-toxin-producing *V. cholerae* strains. *In vivo* studies have shown that injection of purified HlyA into rabbit ileal loops induced fluid secretion into the intestinal lumen, indicating a possible role in the pathogenesis of gastroenteritis (Ichinose *et al*., 1987).

DNA microarray-based comparative genomic analyses of *V. cholerae* revealed the presence of two additional pathogenicity island-like mobile genetic elements in *V. cholerae* strains of the seventh cholera pandemic: the *Vibrio* seventh pandemic islands (VSP)-1 and -2 (Dziejman *et al*., 2002; Murphy and Boyd, 2008). The 16-kb VSP-1 region encompasses 11 ORFs (VC0175-VC0185), which encode a deoxycytidylate deaminase-related protein (VC0175), a transcriptional regulator (VC0176), a patatin-related protein (VC0178), a putative transposase (VC0185), and seven hypothetical proteins of unknown function (Dziejman *et al*., 2002). The 26.9-kb VSP-2 region encompasses ORFs VC0490 to VC0516 encoding transcriptional regulators (VC0497, VC0513), a ribonuclease H1 (RNase H1, VC0498), a type IV pilin (VC0502), a DNA repair protein (RadC, VC0510), methyl-accepting chemotaxis proteins

(VC0512, VC0514), a bacteriophage-like integrase (VC0516), and also a number of hypothetical proteins (Dziejman *et al*., 2002; O'Shea *et al*., 2004). Recent studies identified a novel VSP-1 encoded transcriptional factor, the *V. cholerae* seventh pandemic regulator VspR (VC0177), whose ToxT-dependent TarB-mediated downregulation resulted in derepression of several VSP-1 genes including *dncV* (VC0179). DncV was found to be required for efficient *V. cholerae* intestinal colonization in the infant mouse model (Davies *et al*., 2012).

Genes encoding the major virulence factors of O1/O139 *V. cholerae* strains, *ctxA* and *tcpA*, are occasionally detected in non-O1, non-O139 isolates (Chatterjee *et al*., 2009; Hasan *et al*., 2013). Moreover, clinical non-O1, non-O139 isolates often possess *mshA* (Rahman *et al*., 2008; Chowdhury *et al*., 2016), *ompU* (Schirmeister *et al*., 2014a), as well as *rtxA* and *hlyA* (Chatterjee *et al*., 2009; Chowdhury *et al*., 2016). In contrast, VSP-1 and VSP-2 are rarely found in clinical non-O1, non-O139 isolates (Rahman *et al*., 2008; Schirmeister *et al*., 2014a).

Two factors with an apparently minor role in virulence of O1/O139 *V. cholerae* strains but a possibly central role in virulence of non-O1, non-O139 *V. cholerae* strains are the type III secretion system and the cholix toxin.

Virulence-associated type III secretion systems (TTSS) are wide-spread in Gram-negative pathogens. The TTSS apparatus is a 6 MDa nanosyringe-like structure that consists of about 20 different proteins. Upon contact to a eukaryotic host cell, translocator proteins mediate pore formation in the host cell membrane, which enables the injection of different bacterial effector proteins into the host cell cytosol. TTSS effectors promote infection in the eukaryotic host by modifying different cellular molecules, structures, and signaling pathways (Galán, 2009; Portaliou *et al*., 2016; Wagner *et al*., 2018). Genomic sequencing of *V. cholerae* AM-19226, a highly pathogenic non-O1, non-O139 strain lacking the major virulence factors of the toxigenic strains, revealed the presence of a type III secretion system that was related to the enterotoxicity-associated TTSS2 gene cluster of *V. parahaemolyticus* (Dziejman *et al*., 2005). The AM-19226 TTSS cluster consists of a central core region and 5'- and 3'-flanking regions. The core region encompasses genes encoding TTSS structural proteins (e.g., VcsC2, VcsN2, VspD, and VcsV2) as well as several translocated proteins (Vops) and is conserved among TTSS-harboring *Vibrio* strains. Further *vop* genes as well as genes encoding virulence-associated proteins (Acfs (accessory colonization factors), TRH (TDH-related hemolysin)) are located in the flanking regions that show considerable coding diversity between clades and species (Chaand *et al*., 2015). However, animal studies confirmed the necessity of the AM-19226 TTSS in intestinal colonization (Chaand *et al*., 2015) and causing fatal diarrhea (Shin *et al*., 2011). While TTSS-encoding genes are often found in clinical non-O1, non-O139

isolates (Chatterjee *et al*., 2009; Schirmeister *et al*., 2014a; Zeb *et al*., 2019; Arteaga *et al*., 2020), the gene cluster seems to be absent from toxigenic O1/O139 isolates (Rahman *et al*., 2008).

The cholix toxin (ChxA) is a 71-kDa exotoxin. Due to the common target molecule in eukaryotic cells, *Vibrio cholerae* cholix toxin, *Corynebacterium diphtheriae* diphtheria toxin, and *Pseudomonas aeruginosa* exotoxin A form a subgroup (diphtheria toxin (DT) group) within the cholera toxin-comprising mono-ADP-ribosyltransferase toxin family. DT group toxins catalyze ADP-ribosylation of the diphthamide residue of the eukaryotic elongation factor (eEF) 2 leading to inhibition of protein synthesis and finally to host cell death (Lugo and Merrill, 2015). The role of ChxA in human *V. cholerae* infections is unclear. Animal studies suggest that ChxA may be mainly associated with extraintestinal infections (Awasthi *et al*., 2013). Similar to the TTSS genes, the *chxA* gene is often detected in clinical non-O1, non-O139 isolates, while it is rarely found in O1 and O139 strains (Purdy *et al*., 2010; Awasthi *et al*., 2013; Schirmeister *et al*., 2014a; Hao *et al*., 2015).

Other virulence factors associated with *V. cholerae* strains from clinical sources include the type VI secretion system (Marin *et al*., 2013; Hasan *et al*., 2015; Irenge *et al*., 2020) and a hemagglutinin/protease (Jiang *et al*., 2018).

Type VI secretion systems (T6SS) gene clusters have been identified in about 25% of all Gram-negative bacteria sequenced to date (Bingle *et al*., 2008; Salomon and Orth, 2015). The T6SS apparatus is a bacteriophage tail-like structure composed of an outer sheath (VipA/VipB proteins in *V. cholerae*), an inner tube (Hcp proteins), and a spike (VgrG and PAAR-motif proteins). The tail complex is anchored on the cell membrane by a membrane complex (formed by VasD, VasF, and VasK in *V. cholerae*) and a baseplate structure (formed by VasA, VasB, VasE, VasJ, and HisF in *V. cholerae*) (Ho *et al*., 2014; Crisan and Hammer, 2020). Most structural proteins of the contact-dependent contractile *V. cholerae* T6SS apparatus are encoded in a major gene cluster that also contains genes for T6SS regulatory and toxin components. Further T6SS-associated genes are distributed across several small auxiliary clusters. Upon contraction of the T6SS outer sheath, the spike complex loaded with toxic effector proteins is translocated into adjacent prokaryotic or eukaryotic target cells (Joshi *et al*., 2017; Crisan and Hammer, 2020). Animal studies revealed that the T6SS contributes to elimination of the intestinal microbiota, which facilitates intestinal colonization by *V. cholerae* (Logan *et al*., 2018; Zhao *et al*., 2018). Further, the T6SS is associated with intestinal inflammation and diarrheal symptoms (Ma and Mekalanos, 2010). In addition, it is

involved in evasion of the host immune defense by disrupting the actin cytoskeleton of host macrophages (Pukatzki *et al*., 2007).

The *V. cholerae* hemagglutinin/protease (HA/P, HapA) degrades host proteins of intercellular tight junctions (e.g., occludin) between intestinal epithelial cells, which could promote paracellular fluid secretion (Benitez and Silva, 2016). Early studies confirm that HapA contributes to human diarrheal disease (Benítez *et al*., 1999).

In addition to the HlyA hemolysin, further hemolysins have been reported in *V. cholerae* that could contribute to the infection process in the human host. These include the heat labile TDH-related hemolysin (TRH) (Zhang and Austin, 2005; Chaand *et al*., 2015), the thermolabile hemolysin (TLH) (Fiore *et al*., 1997; Zhang and Austin, 2005), and the delta thermostable hemolysin (DTH), which shows strong homology to the δ-VPH hemolysin of *V. parahaemolyticus* (Fallarino *et al*., 2002). Whole genome microarray and quantitative reverse transcription PCR analyses revealed that *tlh* was upregulated in flagellar regulatory mutants similarly to genes with proven roles in virulence (Syed *et al*., 2009).

Another characteristic associated with several human pathogenic bacterial species is the presence of sialic acid catabolism components (Almagro-Moreno and Boyd, 2009a; Haines-Menges *et al*., 2015). Sialic acids (SAs) comprise a family of nine-carbon keto sugar acids, with N-acetylneuraminic acid (Neu5Ac) constituting the most prevalent SA in nature. SAs are widely distributed in mucus-rich environments like mucosa surfaces of the mammalian intestinal tract, where they are typically positioned at the terminal end of glycoproteins or glycolipids (e.g., gangliosides), collectively referred to as glycoconjugates (Vimr *et al*., 2004; Haines-Menges *et al*., 2015). In the human host, more than 65% of the intestinal mucin glycoproteins contain sialic acid residues. The ability to utilize sialic acids as alternative carbon, nitrogen, and energy sources confers a selective advantage on enteric pathogens, which have to succeed in a human environment where the competition for nutrients is high (Haines-Menges *et al*., 2015). In *V. cholerae*, genes necessary for the scavenging (*nanH* (VC1784)), transport (*dctQ* (VC1777), *dctP* (VC1778), *dctM* (VC1779)), and catabolism (*nanA* (VC1776), *nanE* (VC1781), *nanK* (VC1782), *nagA* (VC1783)) of sialic acids are located within a sialic metabolism gene cluster (*nan-nag* region) on the *Vibrio* pathogenicity island-2 (VPI-2), a mobile genetic element integrated into the *V. cholerae* genome (Jermyn and Boyd, 2002; Murphy and Boyd, 2008; Almagro-Moreno and Boyd, 2009b; Chowdhury *et al*., 2012).

VPI-2 was originally characterized as a 57.3-kb pathogenicity island encompassing 52 ORFs (VC1758-VC1809) (Jermyn and Boyd, 2002). Meanwhile, several variant VPI-2 islands have

been described, some of which contain genes for a type III secretion system in addition to the sialic acid metabolism gene cluster (Murphy and Boyd, 2008).

In many pathogens, virulence gene expression is connected to quorum sensing, a process by which bacteria monitor their cell population density via the extracellular concentration of signaling molecules (autoinducers). Two of the major autoinducers of *V. cholerae* are CAI-1 and AI-2, which are produced by the synthases CqsA and LuxS and detected by the receptors CqsS and LuxPQ, respectively. While the expression of CTX and TCP genes is upregulated at low CAI-1 and AI-2 autoinducer concentrations (i.e., low cell densities) and downregulated at high autoinducer levels (i.e., high cell densities), other virulence genes like T6SS genes show reciprocal expression patterns (Rutherford and Bassler, 2012; Herzog *et al*., 2019). Quorum sensing not only promotes coordinated gene expression within single bacterial cells but also allows synchronization of the population-wide virulence gene expression leading to efficient interaction of *V. cholerae* with the human host environment.

Knowledge on virulence factors associated with clinical *V. navarrensis* isolates is still fragmentary. Analyses of all whole genome sequences currently available revealed the presence of many genes associated with virulence in well-known human pathogens. These include genes encoding proteins for capsule (*cps*) and type IV pilus biosynthesis (*pilV*, *pilW*) as well as genes encoding hemolytic and cytolytic proteins (homologs of *tlh*, *osmY*, *vvhA*, *δ-vph*, *hlyIII*, and *hlyD* as well as *rtx*) (Gladney *et al*., 2014; Schwartz *et al*., 2017).

Bacterial polysaccharide capsules play various roles in pathogenicity. Due to the highly hydrated nature of the polysaccharide molecules, capsules can confer bacteria resistance to desiccation, which could be important during transmission from host to host. Furthermore, they can promote initial colonization of mucous surfaces and attachment to host cells, respectively. Finally, they can also confer resistance to the host immune defense (Taylor and Roberts, 2005). Several bacterial pathogens, such as *E. coli* K1, produce capsular polysaccharides that are structurally identical to host polysaccharides (molecular mimicry), contributing to evasion of the immune system (Cress *et al*., 2014).

Like polysaccharide capsules, proteinaceous type IV pili (T4P) can play varied roles in bacterial pathogenicity. T4P can be involved in adherence to host-cell epithelia as well as microcolony formation, with the latter contributing to both effective concentration of the bacterial cells (and associated toxins) at the site of infection and successful protection from the host immune defense. Further, T4P can mediate DNA uptake by natural transformation or serve as bacteriophage receptors as described earlier for *V. cholerae*. In many pathogens,

disruption of type IV pilus assembly results in attenuated virulence, emphasizing their importance in pathogenicity (Craig *et al*., 2004).

The *tlh* gene homolog in *V. navarrensis* encodes a putative thermolabile hemolysin. *Tlh* homologs have been reported in several human pathogenic *Vibrio* species, such as *V. cholerae*, *V. mimicus*, and *V. vulnificus* (Zhang and Austin, 2005). The thermolabile hemolysin TLH was originally discovered and characterized in *V. parahaemolyticus* (Taniguchi *et al*., 1985). Its role in pathogenicity is not clear. Besides its hemolytic effect on human erythrocytes, it seems to have additional functions in the host. Recent studies revealed that *tlh* transcription is strongly upregulated in *V. parahaemolyticus* under simulated intestinal conditions (Gotoh *et al*., 2010). Moreover, treatment of epithelial (HeLa, Changliver) and macrophage (RAW 264.7) cell lines with purified *V. parahaemolyticus* TLH, also often referred to as lecithin-dependent hemolysin (LDH) (Zhang and Austin, 2005), results in severe cytotoxic effects (Wang *et al*., 2012; Wang *et al*., 2015), which could indicate an important role in intestinal and extraintestinal infections.

The *osmY* homolog in *V. navarrensis* encodes a putative BON domain-containing hemolysin (GenBank Accession KGK 22069). According to current knowledge, BON domains seem to serve as binding domains for interaction with phospholipid membranes (Yeats and Bateman, 2003). In several human pathogens, such as the opportunistic foodborne pathogen *Cronobacter sakazakii*, the OsmY protein was indirectly associated with virulence. Quantitative real-time PCR and two-dimensional gel electrophoresis analyses revealed distinct upregulation of OsmY expression in the highly virulent *C. sakazakii* isolate G362 compared to the lower virulent isolate L3101 at 37°C (Ye *et al*., 2015).

The *vvhA* gene homolog in *V. navarrensis* encodes a putative cytolysin related to the potent *V. vulnificus* cytolysin-hemolysin VvhA that has been associated with a variety of pathological effects and lethality in animal studies (Jones and Oliver, 2009).

The *δ-vph* homolog in *V. navarrensis* encodes a putative thermostable hemolysin. δ-VPH was originally discovered in *V. parahaemolyticus* and found to be hemolytically active against a variety of mammalian erythrocytes including human erythrocytes (Taniguchi *et al*., 1990). Later, a homolog was reported in *V. cholerae* strains (Fallarino *et al*., 2002). However, the specific role of δ-VPH in pathogenicity is still unclear (Zhang and Austin, 2005).

The *hlyIII* homolog in *V. navarrensis* encodes a putative hemolysin III family protein. Some years ago, HlyIII of the closely related species *V. vulnificus* was studied in more detail. When administered intraperitoneally in mice, a *hlyIII* mutant of *V. vulnificus* displayed attenuated

virulence compared with the wild-type strain, indicating a possible role of HlyIII in virulence (Chen *et al*., 2004).

The *hlyD* gene homolog and the *rtx* gene in *V. navarrensis* encode a hemolysin D-like and a repeats-in-toxin protein, respectively. HlyD proteins are located in the bacterial inner membrane, where they contribute, as part of type I secretion systems (T1SS), to the translocation of hemolysins from the cytosol to the extracellular space (Pimenta *et al*., 2005; Kanonenberg *et al*., 2018). Secreted RTX proteins usually possess pore-forming activity. Pore formation in erythrocyte membranes is visible as hemolytic zone around bacterial colonies grown on blood agar plates (Linhartova *et al*., 2015).

In 2003, Jores *et al*. reported the discovery of a new 15.6-kb genomic DNA fragment in *V. navarrensis* CH-291, which seems to be specific for strains of the *pommerensis* biotype. Cloning of the DNA fragment into the plasmid pVH and subsequent introduction into *E. coli* K-12 DH5α conferred hemolytic properties to the recipient strain. Similar to *V. navarrensis* CH-291, *E. coli* DH5α (pVH) showed hemolytic activity against a variety of mammalian erythrocytes including human red blood cells. Further subcloning experiments revealed that the hemolytic properties were attributable to two neighboring regions of the 15.6-kb DNA fragment, with both regions comprising more than one ORF and ORF12 representing the largest ORF that confers hemolytic properties (Jores *et al*., 2003a).

1.1.5 Human Infections and Infection Epidemiology

V. cholerae and *V. navarrensis* bacteria are associated with different types of human infections.

Toxigenic serogroup O1/O139 *V. cholerae* strains can cause the epidemic disease cholera, which is characterized by profuse rice-water-like diarrhea (primarily attributable to cholera toxin activity) often accompanied by vomiting and muscle cramps in its most severe form. Massive loss of fluids and electrolytes can result in metabolic acidosis and life-threatening hypovolemic shock. The incubation period is 18 hours to 5 days (Clemens *et al*., 2017; Davis *et al*., 2018). Treatment of severely dehydrated cholera patients requires an initial intravenous rehydration therapy (Ringer's lactate or Cholera Saline) followed by administration of oral rehydration solutions (e.g., WHO-ORS) (Clemens *et al*., 2017). In addition, an antimicrobial therapy with tetracyclines, fluoroquinolones or macrolides is recommended (WHO, 2018b).

Non-O1, non-O139 *V. cholerae* strains can cause mild to severe gastrointestinal infections (Ballal *et al*., 2019; Arteaga *et al*., 2020) as well as extraintestinal infections like ear infections (Kechker *et al*., 2017; Díaz-Menéndez *et al*., 2018), wound infections (Andersson

and Ekdahl, 2006; Schirmeister *et al*., 2014a), soft tissue infections (Hirk *et al*., 2016; Homsy *et al*., 2020) and potentially fatal bacteremia (De Keukeleire *et al*., 2018; Shanley *et al*., 2019; Chen *et al*., 2020; Zhang *et al*., 2020). So far, the pathogenic mechanisms of non-O1, non-O139 strains are only poorly understood and there are no official guidelines for antimicrobial therapy (Deshayes *et al*., 2015). In the context of bacteremia, treatment with fluoroquinolones and third-generation cephalosporins has been reported. Tetracyclines or carbapenems have also occasionally been administered (Kaki *et al*., 2017; Zmeter *et al*., 2018; Shanley *et al*., 2019; Zhang *et al*., 2020). While cholera infections are primarily attributable to ingestion of fecally contaminated drinking water or food (Davis *et al*., 2018), non-O1, non-O139 *V. cholerae* infections are predominantly associated with the consumption of raw or undercooked seafood (such as oysters, shrimps) or contact to seawater or freshwater environments (e.g., during wading, bathing) (Huehn *et al*., 2014; Deshayes *et al*., 2015).

In many Asian and African countries with poor water, sanitation, and hygiene infrastructure, cholera is still an endemic disease (Davis *et al*., 2018). In European countries, cholera infections are rare and meanwhile all cases are imported from cholera-endemic areas (WHO, 2013-2015; WHO, 2016a; WHO, 2017; WHO, 2018a; WHO, 2019-2020). From 2000 to 2016, the WHO registered 21 cholera cases in Germany. In 2017, 2018 and 2019, no infections were reported, neither in Germany nor in other European countries (WHO, 2001a; WHO, 2001b; WHO, 2002-2015; WHO, 2016a; WHO, 2017; WHO, 2018a; WHO, 2019-2020).

While cholera only plays a subordinate role in Europe, non-cholera *Vibrio* infections, such as non-O1, non-O139 *V. cholerae* infections, gain attention as emerging infectious diseases (Le Roux *et al*., 2015). In the past 20 years, several locally acquired extraintestinal infections including ear infections, soft tissue infections, and bacteremia were reported from the Mediterranean region – some of them with severe clinical course of disease. Most infections were associated with previous water contact while bathing or fishing (Ottaviani *et al*., 2011; Dobrović *et al*., 2016; Maraki *et al*., 2016; Marinello *et al*., 2017; Díaz-Menéndez *et al*., 2018). Further non-O1, non-O139 *V. cholerae* infections were reported from coastal countries of the North Sea and Baltic Sea regions. These included some cases of wound infections in southeast Sweden associated with recreational exposure to seawater of the Baltic Sea (Andersson and Ekdahl, 2006) as well as sporadic cases of bacteremia in the Netherlands (among them one fatality) (Engel *et al*., 2016). During the summer months of the year 2014, 89 *Vibrio* spp. infections were registered in coastal counties of Sweden and Finland, with 70 infections being attributable to non-O1, non-O139 *V. cholerae* bacteria (among them another

case with fatal outcome). More than 50% of the 89 registered cases manifested as otitis or bacteremia. Many infections occurred after exposure to seawater (Baker-Austin *et al*., 2016). In Germany, the number of human *Vibrio* infections is still relatively low (Huehn *et al*., 2014). Between 1995 and 2017, a total of ten non-O1, non-O139 *V. cholerae* isolates were found that were associated with primarily extraintestinal infections after exposure to German coastal waters (Schirmeister *et al*., 2014a; Schwartz *et al*., 2019).

In contrast, no isolates of the species *V. navarrensis* had been known from German or other European clinical specimens until the start of this doctoral project. However, the Centers for Disease Control and Prevention (CDC) had recently reported on seven *V. navarrensis* isolates recovered from stool samples, ear swabs, and wound swabs of diseased American patients. Four more isolates had been associated with blood specimens, indicating more severe systemic disease progression (Gladney and Tarr, 2014). So far, pathogenic mechanisms of this *Vibrio* species are largely unknown. In spite of the repeated detection in human clinical specimens, there are no detailed case studies on *V. navarrensis*-associated human infections that could permit clear conclusions about the ways of transmission of this species or serve as references for the treatment of possible future infections.

1.2 Aims of the Study

While toxigenic O1/O139 *V. cholerae* strains do not occur in German coastal waters, non-O1, non-O139 *V. cholerae* strains are indigenous to the North Sea and Baltic Sea. So far, infections caused by these bacteria are rare in Germany. However, given rising sea surface temperatures due to climate change, the associated increase in non-O1, non-O139 *V. cholerae* prevalence and not least the popularity of German coastal regions as a travel destination, an increased exposure to strains of these serogroups must be assumed for future years. Therefore, in this doctoral project, a comparison of MLST sequence types and virulence factor profiles of environmental isolates and clinical isolates should be used to carry out a first risk assessment for non-O1, non-O139 strains from German coastal waters. In this context, a whole genome sequencing (WGS)-based approach should be established at the *Vibrio* consiliary laboratory that supplements PCR-based screening methods and could serve as a gold standard for investigating the pathogenicity potential of future non-O1, non-O139 *V. cholerae* isolates from environmental sources.

While *Vibrio* species such as *V. cholerae* have been intensively studied for decades as a result of the global disease burden, other *Vibrio* species such as the presumed environmental species *V. navarrensis* are still at the beginning of their exploration. Recently, *V. navarrensis* strains were detected in human clinical specimens as well as veterinary abortion specimens from livestock in Germany. For identification of new potential environmental reservoirs in Germany and against the background of a possible zoonotic potential, comparative genotypic analyses of *V. navarrensis* isolates from environmental, veterinary, and human clinical specimens should be performed. First, phylogenetic relationships will be evaluated based on SNP and MLSA analyses. In addition, WGS- and PCR-assisted analytical procedures for typing of virulence-associated genes in *V. navarrensis* isolates should be developed and established. In this context, suitable marker genes for rapid PCR-based species diagnostics of future potentially human pathogenic *V. navarrensis* isolates from environmental and animal sources should be identified.

Genotypic characterization of *V. navarrensis* and non-O1, non-O139 *V. cholerae* isolates from public health-relevant sources will be completed by phenotypic determination of the hemolytic activity, a property frequently associated with human pathogenic *Vibrio* strains.

2 Publications

2.1 List of Publications and Own Contribution

Publication 1

Environmental and Clinical Strains of *Vibrio cholerae* Non-O1, Non-O139 From Germany Possess Similar Virulence Gene Profiles

Keike Schwartz, Jens André Hammerl, Cornelia Göllner, and Eckhard Strauch

Frontiers in Microbiology, 2019, 10:733.

https://doi.org/10.3389/fmicb.2019.00733

I took parts in the study design and developed a WGS-assisted approach for risk assessment of non-O1, non-O139 *V. cholerae* isolates. I performed all experiments for genotypic and phenotypic characterization of the *V. cholerae* strains (with technical assistance by C. Göllner in whole genome sequencing). I analyzed the data (with initial support by J.A. Hammerl in the application of bioinformatical analysis tools), performed the statistical analyses, interpreted the results, and wrote the major part of the manuscript.

Publication 2

Diversity of *Vibrio navarrensis* Revealed by Genomic Comparison: Veterinary Isolates Are Related to Strains Associated with Human Illness and Sewage Isolates While Seawater Strains Are More Distant

Keike Schwartz, Cindy Kukuc, Nadja Bier, Karin Taureck, Jens André Hammerl, and Eckhard Strauch

Frontiers in Microbiology, 2017, 8:1717.

https://doi.org/10.3389/fmicb.2017.01717

I designed WGS- and PCR-assisted analytical procedures for typing of virulence genes in *V. navarrensis* isolates, developed a protocol for phenotypic determination of the hemolytic activity of the strains and was involved in the overall study design (selection of phylogenetic analyses). I performed experiments for genotypic and phenotypic characterization of the *V. navarrensis* strains (with technical assistance by C. Kukuc and N. Bier) and was responsible for final analysis and interpretation of the data. I wrote the major part of the manuscript.

2.2 Publication 1

frontiers in Microbiology

ORIGINAL RESEARCH
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Environmental and Clinical Strains of *Vibrio cholerae* Non-O1, Non-O139 From Germany Possess Similar Virulence Gene Profiles

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Vibrio cholerae is a natural inhabitant of aquatic ecosystems globally. Strains of the serogroups O1 and O139 cause the epidemic diarrheal disease cholera. In Northern European waters, *V. cholerae* bacteria belonging to other serogroups (designated non-O1, non-O139) are present, of which some strains have been associated with gastrointestinal infections or extraintestinal infections, like wound infections or otitis. For this study, environmental strains from the German coastal waters of the North Sea and the Baltic Sea were selected (100 strains) and compared to clinical strains (10 isolates) that were from patients who contracted the infections in the same geographical region. The strains were characterized by MLST and examined by PCR for the presence of virulence genes encoding the cholera toxin, the toxin-coregulated pilus (TCP), and other virulence-associated accessory factors. The latter group comprised hemolysins, RTX toxins, cholix toxin, pandemic islands, and type III secretion system (TTSS). Phenotypic assays for hemolytic activity against human and sheep erythrocytes were also performed. The results of the MLST analysis revealed a considerable heterogeneity of sequence types (in total 74 STs). The presence of virulence genes was also variable and 30 profiles were obtained by PCR. One profile was found in 38 environmental strains and six clinical strains. Whole genome sequencing (WGS) was performed on 15 environmental and 7 clinical strains that were ST locus variants in one, two, or three alleles. Comparison of WGS results revealed that a set of virulence genes found in some clinical strains is also present in most environmental strains irrespective of the ST. In few strains, more virulence factors are acquired through horizontal gene transfer (i.e., TTSS, genomic islands). A distinction between clinical and environmental strains based on virulence gene profiles is not possible for our strains. Probably, many virulence traits of *V. cholerae* evolved in response to biotic and abiotic pressure and serve adaptation purposes in the natural aquatic environment, but provide a prerequisite for infection of susceptible human hosts. These findings indicate the need for surveillance of *Vibrio* spp. in Germany, as due to global warming abundance of *Vibrio* will rise and infections are predicted to increase.

Keywords: *Vibrio cholerae*, North Sea, Baltic Sea, multilocus sequence typing, whole genome sequencing, virulence-associated factors, clinical isolates, Germany

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INTRODUCTION

The species *Vibrio cholerae* comprises Gram-negative bacteria which are distributed in aquatic ecosystems throughout the world. Strains of the serogroups O1 and O139 can cause the diarrheal disease cholera which affects millions of people in countries where the supply with clean drinking water is problematic. Poor sanitation as a result of damaged infrastructure occurring through natural catastrophes or human-caused disasters has led to cholera epidemics (Zuckerman et al., 2007). Major virulence factors of the toxigenic O1 and O139 strains are the cholera toxin (CTX) and the toxin-coregulated pilus (TCP), which are both encoded on mobile genetic elements integrated into the chromosome of the toxigenic strains (Harris et al., 2012). While the genes for CTX are part of the genome of the filamentous phage CTXΦ (McLeod et al., 2005; Bhattacharya et al., 2006), genes for the TCP biosynthesis are located within a pathogenicity island designated as *Vibrio* pathogenicity island VPI-1 (Karaolis et al., 1998; Murphy and Boyd, 2008).

However, most *V. cholerae* strains do not possess these two virulence factors and belong to other serogroups. On the basis of differences in the surface-expressed O antigen, more than 200 serogroups have been described and strains of these serogroups are commonly designated as *V. cholerae* non-O1, non-O139. A number of reports have been published revealing that some strains can cause intestinal infections and extraintestinal infections like wound and soft tissue infections, ear infections or bacteremia (Pang et al., 2007; Chatterjee et al., 2009; Octavia et al., 2013; Ceccarelli et al., 2015; Deshayes et al., 2015). There is no legal obligation to notify public health authorities of cases of non-O1, non-O139 *V. cholerae* infections in Germany so far, irrespective of the severity of the disease. In non-O1, non-O139 *V. cholerae* strains, a number of virulence factors can be present that are also found in the toxigenic strains and are known to contribute to the infection process in a synergistic way (Rivera et al., 2001; Singh et al., 2001). These accessory virulence factors include mannose-sensitive hemagglutinin pilus (MSHA), different hemolysins, repeats-in-toxin (RTX) toxin clusters, and outer membrane proteins (Schirmeister et al., 2014). However, the occurrence of these factors is diverse in the strains. More virulence factors are found only in some non-O1, non-O139 *V. cholerae* and contribute essentially to the pathogenicity of these strains. The type III secretion system (TTSS) was shown to be necessary for colonization and causing diarrheal disease in animal studies with pathogenic strains lacking the major virulence factors of the toxigenic strains (Dziejman et al., 2005; Shin et al., 2011).

Non-O1, non-O139 *V. cholerae* strains are present in marine environments of German coastal waters of the North Sea and Baltic Sea (Böer et al., 2013), whereas toxigenic *V. cholerae* strains do not occur in central Europe. Studies conducted in Austria revealed that Lake Neusiedl, a saline steppe lake, contained considerable numbers of non-O1, non-O139 *V. cholerae* bacteria. In other Austrian bathing waters, non-O1, non-O139 *V. cholerae* were also detected (Hirk et al., 2016). Phylogenetic analysis of the strains from Lake Neusiedl revealed a remarkable genomic diversity among these isolates and genetic similarities to strains from other European countries (Pretzer et al., 2017).

Climate change is known to have a great impact on *Vibrio* spp. occurrence in aquatic environments. Warming of water surface temperatures will lead to an increase of *Vibrio* spp. abundance and the incidence of human and animal infections caused by these bacteria is predicted to rise (Baker-Austin et al., 2012; Vezzulli et al., 2016). A number of infections and septicemia caused by non-O1, non-O139 *V. cholerae* and other *Vibrio* spp. have been reported by European countries of the Baltic Sea and North Sea region, although in Germany the number of infections is still quite low (Huehn et al., 2014). However, recent case reports of serious infections caused by non-O1, non-O139 *V. cholerae* from the Netherlands (Engel et al., 2016) and Austria (Hirk et al., 2016) emphasize the threat posed by these waterborne pathogens. Studies in Austria showed that temperature is the main predictor for *V. cholerae* abundance in lakes but the quantities of dissolved organic matter also influenced the *Vibrio* numbers (Bliem et al., 2018).

In Germany so far no studies in inland lakes were conducted. However, the occurrence of non-O1, non-O139 *V. cholerae* in marine and estuarine environments was already described several years ago (Bockemühl et al., 1986), although systematic studies were initiated only many years later in the framework of two research projects (Böer et al., 2013; Erler et al., 2015). These projects confirmed that such vibrios are indigenous to German coastal waters and their abundance underlies seasonal variations. A number of non-O1, non-O139 *V. cholerae* strains isolated in these projects was recently investigated for antimicrobial resistance (Bier et al., 2015). From this collection, we selected 50 strains from the North Sea region as well as 50 strains from the Baltic Sea for the current study in order to analyze the genetic relationships between the strains from the two seas. The Baltic Sea is an intracontinental ocean with low salinity, while the North Sea belonging to the Atlantic Ocean has reduced salinity near the estuarine regions. Therefore, *V. cholerae* strains of the North Sea were obtained only from recreational waters of the estuaries. In the current study, also clinical non-O1, non-O139 *V. cholerae* strains were included which came from patients who had contracted primarily extraintestinal infections from the coastal waters of Germany (Schirmeister et al., 2014).

By comparing MLST sequence types (STs) and virulence factor profiles, the aim of this study is to determine if environmental non-O1, non-O139 strains related to clinical strains are frequently present in the coastal waters of Germany. Rising water surface temperatures are expected to increase *Vibrio* abundance in future and data are needed for health authorities to carry out risk assessment for vibrios and to introduce measures to reduce the threat for the public.

MATERIALS AND METHODS

Bacterial Strains

The non-O1, non-O139 *V. cholerae* strains used in this study (n = 110) are summarized in **Table 1** and listed in detail in

TABLE 1 | Origin and source of *Vibrio cholerae* non-O1, non-O139 strains (n = 110) included in this study.

Origin	Geographical origin	Source	Source code
Environmental (E) (2009–2014; n = 100)	Baltic Sea (BS) (n = 50)	Seawater (sw) (n = 37)	E-BS-sw
		Sediment (sd) (n = 3)	E-BS-sd
		Seawater/sediment (sw/sd) (n = 10)	E-BS-sw/sd
	North Sea (NS) (n = 50)	Bivalve mollusks (bm) (n = 26)	E-NS-bm
		Seawater (sw) (n = 10)	E-NS-sw
		Seawater/sediment (sw/sd) (n = 14)	E-NS-sw/sd
Clinical (C) (1995–2017; n = 10)	Germany (G) (n = 10)	Extraintestinal (ext) (n = 8)	C-G-ext
		Intestinal (int) (n = 2)	C-G-int

Supplementary Table S1. Environmental strains (n = 100) from the Baltic Sea (n = 50) and North Sea (n = 50) were isolated by health authorities and nationally recognized scientific institutions within the German research programs KLIWAS[1] (n = 14) and VibrioNet[2] (n = 86) between 2009 and 2014. Seawater and sediment samples were mostly collected at bathing sites along the Baltic Sea and North Sea coastline as well as within the estuaries of the rivers Ems and Weser (Böer et al., 2013). Bivalve mollusk samples came from coastal areas of the North Sea. Clinical strains (n = 10) were isolated from German patients with extraintestinal or intestinal infections between 1995 and 2017. Strains were obtained from the State Office for Health and Social Affairs (LAGuS), Rostock, and the Robert Koch Institute (RKI), Berlin, or from the strain collection of the German Federal Institute for Risk Assessment (BfR), Berlin. Most of the clinical isolates had been characterized by Schirmeister et al. (2014). All environmental and clinical isolates (except VN-00533 and VN-00534) had been tested for antimicrobial resistances in previous studies (Bier et al., 2015; Hammerl et al., 2017).

Species Confirmation, Characterization, and Subtyping

Species confirmation of clinical and environmental *V. cholerae* strains was performed by whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (WC-MALDI-TOF MS) analysis and PCR analysis as previously described (Schirmeister et al., 2014; Bier et al., 2015). For PCR analysis, genomic DNA was extracted from 1 ml of an overnight culture in lysogeny broth using the RTP Bacteria DNA Mini Kit according to the manufacturer's protocol (Stratec Molecular GmbH, Berlin, Germany). Ten nanograms of genomic DNA served as template DNA. Primers, annealing temperature, and amplicon sizes of the *toxR-ctxA-rfb*O1-*rfb*O139 multiplex PCR assay are shown in **Supplementary Table S2**.

Multilocus Sequence Typing (MLST)

Genetic relationships of the environmental isolates and clinical isolates (**Supplementary Table S1**) were characterized by *V. cholerae* specific MLST. Using the genomic DNA, MLST was performed targeting seven housekeeping genes (*adk*, *gyrB*, *mdh*, *metE*, *pntA*, *purM*, *pyrC*) (Octavia et al., 2013) and the ST was identified using the non-O1, non-O139 *V. cholerae* MLST scheme[3]. MLST primers, annealing temperatures, and amplicon sizes are shown in **Supplementary Table S2**. Sequences were assembled using the Lasergene software SeqMan Pro version 12.0 (DNASTAR Inc., Madison, WI, United States). The consensus sequences of each locus were aligned and trimmed to reference length in Accelrys Gene version 2.5 (Accelrys Inc., San Diego, CA, United States). New allele sequences and new allelic profiles were submitted to the PubMLST database for assignment of new allele numbers and ST numbers, respectively. To visualize genetic relationships between the isolates, allelic profiles were analyzed with the software PHYLOViZ version 1.1a using the goeBURST Full Minimum Spanning Tree (MST) algorithm (Francisco et al., 2012). A single-locus variant (SLV) comprised two STs differing at one locus only, while the other six loci were identical. A double-locus variant (DLV) included two STs differing at two out of the seven loci. A triple-locus variant (TLV) contained two STs differing at three housekeeping loci. Isolates showing 100% identity in at least six of the seven loci were assigned to a single clonal complex (CC), with at least two isolates forming the CC. To elucidate the genetic relationship between the *V. cholerae* isolates from German coastal waters and from German clinical samples, a full MST encompassing all identified STs was generated using PHYLOViZ software. Closer genetic relationships between the environmental and clinical isolates were visualized by the identification of CCs, SLVs, DLVs, and TLVs comprising STs of both origins. To describe the genetic diversity of the environmental strains, Simpson's Index of Diversity D (Hunter and Gaston, 1988) was calculated for the strains from the Baltic Sea and North Sea using identified STs.

Hemolytic Activity Tests

The hemolytic activity of the environmental isolates was determined based on the procedure given in Schwartz et al. (2017). Blood agar plates were prepared from Mueller-Hinton agar CM0337 (Oxoid GmbH, Wesel, Germany) supplemented with 4% human erythrocytes (German Red Cross, blood donation service, Berlin, Germany) or 4% sheep erythrocytes (BfR, Berlin, Germany). For semiquantitative investigation of the hemolytic activity of the strains, 10 µl of an overnight culture in Mueller-Hinton broth CM0405 (Oxoid) were spotted on a blood agar plate and incubated for 22–24 h at 37°C. The hemolytic activity was characterized based on the diameter of the hemolysis zone around the macrocolony (**Supplementary Figure S1**). Isolates

[1]https://www.kliwas.de/

[2]http://www.vibrionet.de/

[3]https://pubmlst.org/vcholerae/

were divided into four categories: non-hemolytic (−), weak hemolytic activity (+), intermediate hemolytic activity (++), and strong hemolytic activity (+++). Breakpoints for interpretation are given in **Supplementary Table S3**. The hemolytic activity tests were performed twice. Strains that gave equivocal results in two assays were retested in a third assay.

PCR Typing of Virulence Genes

To recognize a possible pathogenic potential of the environmental isolates (**Supplementary Table S1**), the presence and absence of several virulence-associated genes and gene clusters was investigated according to Schirmeister et al. (2014). Prior to PCR typing of virulence-associated genes, bacterial strains were grown overnight and genomic DNA was extracted as described above. PCR amplification was performed in a total volume of 25 µl with 1 × PCR buffer (2 mM $MgCl_2$), 0.2 mM of each dNTP, 1 µM of each primer, 2 U of DreamTaq DNA Polymerase (Thermo Fisher Scientific Biosciences GmbH, St. Leon-Rot, Germany), and 10 ng of template DNA. PCR reactions were performed using a Mastercycler ep gradient (Eppendorf AG, Hamburg, Germany). The PCR running conditions were as follows: an initial denaturation step at 94°C for 2 min (*hlyA*, *ompU*, *tcpA*), 4 min (*mshA*, *rstR*, *rtxC*, *chxA* (VC-Cholix-fo/-re), TTSS) or 5 min (*chxA* (VC-chxA-F/-R), *rtxA*, VSP-1, VSP-2), 30 cycles of denaturation at 94°C for 30 s (*hlyA* and *ompU* for 1 min, *tcpA* for 2 min), primer annealing for 30 s (*hlyA*, *ompU*, and *tcpA* for 1 min) and extension at 72°C for 1 min per kb, and a final extension step at 72°C for 10 min (*chxA* (VC-chxA-F/-R), *rtxA*, VSP-1, and VSP-2 for 7 min). PCR primers, annealing temperatures, and amplicon sizes are shown in **Supplementary Table S2**. To ensure the quality of the PCR system, negative (HPLC grade water) and external positive amplification controls (**Supplementary Table S4**) were used. Most PCR assays were performed in simplex formats. The presence of the genes *hlyA* (alleles Classical and El Tor) and *tcpA* (alleles Classical and El Tor) was examined by separate multiplex PCR assays. In the *hlyA* PCR, two universal *hlyA* primers (hlyA-489F, hlyA-1184R) and one El Tor *hlyA*-specific primer (hlyA-744F) were used. In the *tcpA* PCR, one universal *tcpA* forward primer (tcpA-F_Class-ET) and two allele specific *tcpA* reverse primers (tcpA-R_class, tcpA-R_ET) were utilized. Strains that gave negative results with *chxA* primers published by Awasthi et al. (2013) were retested with a newly designed primer pair (VC-Cholix-fo/-re). PCR products (1.5 µl each) were separated in GelRed stained agarose gels. Selected PCR products were purified and sequenced for confirmation.

Whole Genome Sequence Determination and Bioinformatical Analysis

Whole genome sequence-based analyses were performed on a set of 22 non-O1, non-O139 *V. cholerae* strains. To gain deeper molecular insights into the genotypic traits of German environmental strains that are related to clinical strains in MLST STs, this included all SLVs, DLVs, and TLVs comprising both environmental and clinical STs (18 strains). In addition, four exemplary environmental strains showing an exceedingly low or high number of virulence-associated genes in PCR typing were selected for WGS-based analysis to confirm the PCR results and look for further virulence factors (**Table 5**). Preparation of genomic DNA and short-read whole genome sequencing (WGS; Illumina MiSeq, San Diego, CA, United States) was conducted as previously described (Schwartz et al., 2017). SPAdes *de novo* assemblies of raw reads were performed using the PATRIC database (release 3.5.21) (Wattam et al., 2017) following submission to the automated Prokaryotic Genome Annotation Pipeline of the NCBI website for genome annotation. Identification and assessment of putative prophage sequences was performed using the PHAge Search Tool (PHAST) according to the recommendations of the providers (Zhou et al., 2011). To detect specific genetic features within the genome sequences, different *in silico* analysis tools of the Center for Genomic Epidemiology (CGE), provided by the Danish Technical University, were used. Initial plasmid prediction was performed with the PlasmidFinder Web tool (release 2.0) (Carattoli et al., 2014). In addition, genomic contigs showing significantly higher sequence coverage levels than the rest of the contigs were screened for similarities to known plasmids using the BLASTN algorithm of the NCBI database[4]. For the initial detection of *Vibrio*-specific virulence determinants, the MyDbFinder Web tool (release 1.1) was used with a manually adapted database of the virulence factor database (VFDB[5]) (Chen et al., 2016). The database was derived from the VFDB DNA core dataset and only included *Vibrio* genes associated with experimentally verified virulence factors. CGE-based analyses were performed by using *de novo* assemblies of genomes. For *in silico* predictions, a minimum identity level of 50% (PlasmidFinder) and 30% (MyDbFinder), respectively, as well as a coverage level of at least 20% was used. To screen for *V. cholerae*-specific gene variants of the latter and of additional virulence determinants, the segmented genome fragments were applied to the BLASTN search of the NCBI database and compared to selected reference sequences using default settings. Accession numbers are given below. All isolates were analyzed for *V. cholerae*-specific sequences of *ompU*, *chxA*, *dth*, the NAG-ST gene (*stn*), *hapA*, *tlh*, *trh*, and *nanH*. *nanH*-positive isolates were further tested for the presence of genes of sialic acid metabolism enzymes as well as for the complete *Vibrio* pathogenicity island 2 (VPI-2) (Jermyn and Boyd, 2002). Isolates scored as potentially positive for sequences of the *Vibrio* seventh pandemic islands based on PCR results were screened for VSP-1 (Dziejman et al., 2002) and VSP-2 (O'Shea et al., 2004). All strains PCR-positive for the TTSS genes *vcsC2*, *vcsN2*, *vspD*, and *vcsV2* were further studied for the complete TTSS gene cluster (Dziejman et al., 2005; Chaand et al., 2015). In addition, *de novo* assemblies of the *mshA*-positive VN-00459 genome were analyzed for the presence of the MSHA gene cluster. To determine the phylogenetic relationship of the isolates, a CSI Phylogeny (version 1.4)-based SNP tree was prepared. The web-based tool was used under default settings and the exclusion of heterozygous SNPs. As reference genome, sequencing data

[4]https://www.ncbi.nlm.nih.gov

[5]http://www.mgc.ac.cn/VFs/

of the clinical *V. cholerae* isolate VN-00533 (MWZM00000000) were used. Nucleotide variations were predicted according to the specifications provided by Kaas et al. (2014).

Accession Numbers

Nucleotide sequences of new MLST alleles were deposited in *V. cholerae* PubMLST database sited at the University of Oxford under the numerical identifiers given in **Supplementary Table S5**. Genome sequences of *V. cholerae* isolates have been deposited in GenBank at the National Center for Biotechnology Information (NCBI) under the accession numbers given in **Supplementary Table S9**.

To screen the whole genome sequences for *V. cholerae*-specific variants of virulence determinants, reference sequences of virulence-associated genes and gene clusters were obtained from GenBank at NCBI. Accession numbers are CP000627.1 (*V. cholerae* O395; VC0395_A0162, *ompU*), NZ_GL989284.1 (*V. cholerae* BJG-01; VCBJG01_RS05475, *chxA*), NC_002506.1 (*V. cholerae* N16961; VCA1111, *dth*), M85198.1 (*V. cholerae* NRT-36; *stn*), CP028828.1 (*V. cholerae* N16961; N16961_VCA03457, *hapA*; N16961_VCA02936, *tlh*), NC_002505.1 (*V. cholerae* N16961; VC1784, *nanH*; VC1776-VC1779 and VC1781-VC1783, sialic acid metabolism homologs; VC1758-VC1809, VPI-2; VC0175-VC0185, VSP-1; VC0490-VC0516, VSP-2; VC0398-VC0411, MSHA cluster), AAKI03000011.1 (*V. cholerae* V51; VCV51_032643, *trh*), and DQ124262.1 as well as AATY02000000 (*V. cholerae* AM-19226; AATY02000003.1/AATY02000004.1, TTSS cluster and flanking regions).

RESULTS

High Diversity of Strains Revealed by MLST

The data on allelic diversity and ST diversity are summarized in **Supplementary Table S6** and listed in detail in **Supplementary Table S5**. Two *V. cholerae* strains from the Baltic Sea, VN-00455 and VN-00477, were excluded from data analyses due to failure of *pyrC* amplification.

In MLST analysis, a high number of new alleles and STs were found. The data revealed that all 108 strains (98 environmental strains and 10 clinical strains) possessed 74 different STs of which 71 were newly assigned STs based on the obtained sequencing data. The highest diversity of alleles was found in the *metE* and *pyrC* loci with 56 and 45 alleles, respectively. In the PubMLST database, these two loci show the highest genetic variability of the seven loci. The majority of STs (55) was present only once in each strain, 13 STs were found in two strains and only six STs in three or more strains. The genetic diversity is higher for strains of the Baltic Sea (D = 0.992; N = 48, s = 42) than for strains from the North Sea (D = 0.955; N = 50, s = 25). The STs of the 10 clinical strains included in this study also reveal a strong diversity between the strains (eight STs).

The genetic relationships of the different subsets of strains were analyzed using the goeBURST Full MST algorithm (**Figure 1**). The goeBURST Full MST analysis visualized the clonal relations between all strains and revealed no clear separation between strains isolated from coastal waters of the North Sea and Baltic Sea regions. The analysis yielded two clonal complexes at SLV level comprising clinical and environmental strains (**Figures 1A,B**) and four clonal complexes consisting only of environmental strains (indicated by a solid black line, **Figure 1C**).

Determination of Virulence Profiles by PCR

In a previous study (Schirmeister et al., 2014), we performed PCR genotyping to investigate the presence of virulence factors in clinical non-O1, non-O139 *V. cholerae* strains from German patients. The same set of primers was used to analyze the environmental non-O1, non-O139 strains with the aim to identify common virulence gene profiles between clinical and environmental strains.

Genes of the major virulence factors, the *ctxA* gene (CTX gene) and other elements of the CTX element (*zot*, *ace*, *rstR*) as well as the *tcpA* gene, were absent in both clinical and environmental *V. cholerae* non-O1, non-O139 strains (**Supplementary Table S7**). Besides the CTX element and the TcpA pilus, presence or absence of several factors was studied in the environmental strains. The genotyping of these virulence factors revealed a high diversity and a number of different virulence gene profiles were obtained.

In **Table 2**, the frequency of the presence of virulence factors is shown. The detailed results for every strain are shown in **Supplementary Table S7**. The *rtxC* gene and the *toxR* gene were the only genes detected in all strains. The second most abundant gene was the *hlyA* gene, as only in three strains of the same ST (ST336) no PCR products were visible. The products of the *hlyA* PCR displayed the El Tor variant of this gene ($hlyA^{ET}$) (Rivera et al., 2001; Schirmeister et al., 2014). The PCR for the genomic islands VSP-1 and VSP-2 revealed the absence of the islands when a PCR product of the expected size is obtained. In approximately 80% of the strains, either VSP-1 or VSP-2 or both elements were missing. In approximately 75% of the strains, the *ompU* PCR was positive. The primers of the *rtxA* PCR were designed to detect the MARTX toxin of the O1 reference strain N16961 which was only found in 22% of all strains. Four genes of the TTSS were detected in only 7% of all strains, whereas the cholix toxin gene (*chxA*) was found in 22% of the strains. The comparison of the distribution of virulence factors between North Sea and Baltic Sea strains did not show noteworthy differences.

A summary of all combinations of virulence factors obtained by PCR is shown in **Table 3**. We obtained a total of 30 profiles by PCR of which two profiles were also present in the German clinical strains. Strains were assigned to a different profile when no PCR products were obtained by using primers targeting the borders of VSP-1 and VSP-2 elements. One profile showed by far the highest frequency and was detected in 38 of the environmental strains (*toxR* $hlyA^{ET}$ *rtxC ompU* positive, absence of VSP-1 and VSP-2) and in six clinical strains.

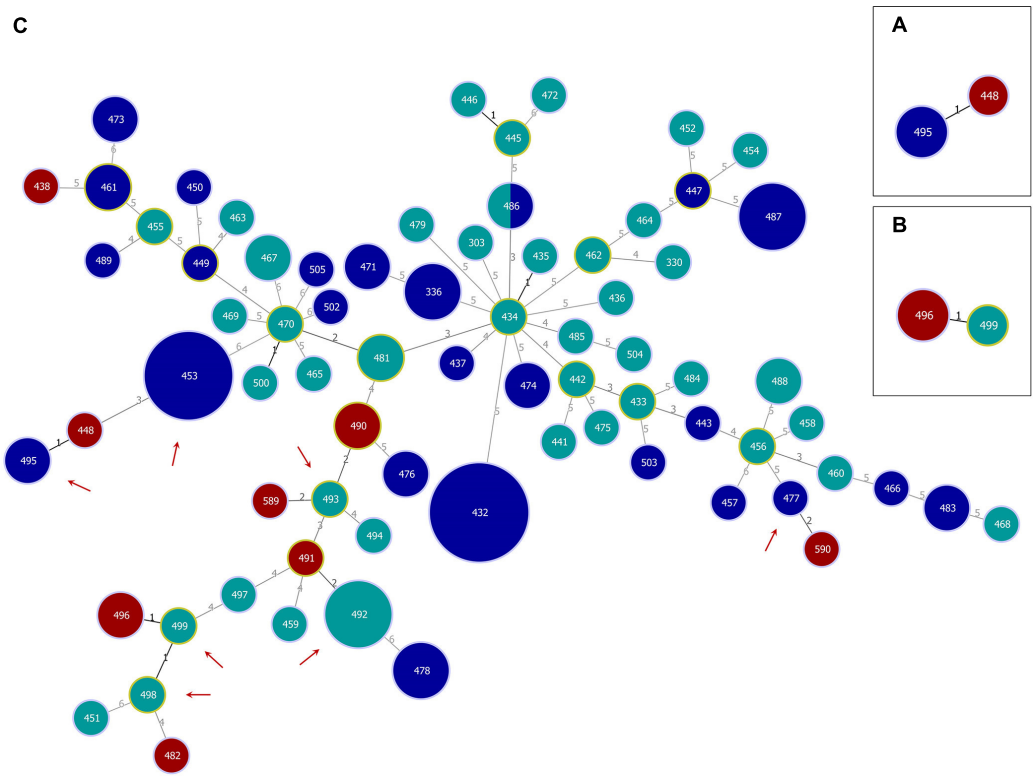

FIGURE 1 | Genetic relationships of *Vibrio cholerae* non-O1, non-O139 isolates from German coastal waters and from German clinical samples obtained by goeBURST Full MST analysis based on MLST allelic profiles using PHYLOViZ 1.1a. Each sequence type (ST) is displayed as a circle with a size proportional to the number of isolates by which it is represented. The coloring indicates the origin: environmental/North Sea (dark blue), environmental/Baltic Sea (turquoise), and clinical/Germany (dark red). Circles surrounded by a green halo are (sub-) group founders. Single-locus variants (SLVs) are connected via black lines. Gray lines indicate connections with a higher level. The number of differing loci between two STs is shown next to the connection lines. Clonal complexes (CCs) formed at SLV level by North Sea and German clinical (CC1; **A**) or Baltic Sea and German clinical (CC2; **B**) isolates, respectively, as well as the full Minimum Spanning Tree (MST; **C**) are shown. In the full MST, environmental STs forming SLVs to TLVs (triple-locus variants) with clinical STs are marked with a dark red arrow.

TABLE 2 | Presence/absence of virulence-associated genes and gene clusters in *Vibrio cholerae* non-O1, non-O139 isolates from German coastal waters based on PCR data.[a]

Origin	Virulence-associated genes and gene clusters														
	rfb O1	*rfb* O139	*ctxA*	*toxR*	*tcpA*[b]	*rstR*[c]	VSP-1 absent[d]	VSP-2 absent[d]	*hlyA*[e]	*mshA*	*ompU*	*rtxA*[f]	*rtxC*	*chxA*	TTSS[g]
Environmental (*n* = 100)	0%	0%	0%	100%	0%	0%	79%	80%	97%	6%	75%	22%	100%	22%	7%
Baltic Sea (*n* = 50)	0%	0%	0%	100%	0%	0%	80%	86%	100%	6%	74%	28%	100%	14%	8%
North Sea (*n* = 50)	0%	0%	0%	100%	0%	0%	78%	74%	94%	6%	76%	16%	100%	30%	6%

[a] *The analysis also includes isolates with weakly positive PCR results.* [b] *Alleles Classical and El Tor.* [c] *Alleles Classical, El Tor, and Calcutta.* [d] *The absence of VSP-1 and VSP-2 was demonstrated by the generation of 1.7 kbp and 800 bp PCR products, respectively, as the primers bind to flanking genomic sites of the pandemic islands. The generation of 3 kbp to 4 kbp PCR products indicated the presence of short VSP-1 fragments, while most of the pandemic island was absent. Strains showing none of the abovementioned PCR products were scored as potentially positive for VSP-1 and VSP-2.* [e] *Specific for V. cholerae El Tor hlyA. All V. cholerae strains were negative for Classical hlyA.* [f] *VC1451 of Vibrio cholerae O1 biovar El Tor str. N16961.* [g] *The presence of TTSS was demonstrated by the detection of vcsC2, vcsN2, vspD, and vcsV2.*

Hemolytic Activity Against Human and Sheep Erythrocytes

Hemolysis zones of strains were investigated on blood agar plates containing either sheep or human erythrocytes. The results of the hemolytic activity tests are summarized in **Table 4** and listed in detail in **Supplementary Table S8**. The vast majority of the strains showed hemolytic activity against both types of erythrocytes. Human erythrocytes were more susceptible to some strains than sheep erythrocytes. One strain was not hemolytic against sheep erythrocytes but showed hemolysis on human blood cells. Only

TABLE 3 | Virulence gene profiles of *Vibrio cholerae* non-O1, non-O139 isolates from German coastal waters compared to virulence genotypes of clinical non-O1, non-O139 isolates from German patients based on PCR data and profiles further analyzed based on WGS data.

No. of isolates			Virulence gene profile (PCR)[a]										Genome	
Environmental (*n* = 100)	**Baltic Sea/ North Sea (*n* = 50/*n* = 50)**	**Clinical (*n* = 10)**	**VSP-1[b]**	**VSP-2[b]**	***toxR***	***hlyA*[ET]**	***rtxC***	***rtxA*[c]**	***ompU***	***chxA***	**TTSS[d]**	***mshA***	**WGS performed**	**No. of sequenced genomes**
3	0/3	0	▒		■		■						X	3
6	6/0	1			■	■	■						X	1
2	0/2	0		▒	■	■	■							
38	20/18	6			■	■	■		■				X	11
3	0/3	0			■	■	■			■			X	1
0	0/0	1			■	■	■				■			
4	4/0	0	▒		■	■	■	■						
7	2/5	0	▒		■	■	■		■					
3	2/1	0		▒	■	■	■		■					
1	0/1	0	▒		■	■	■					■		
4	2/2	0			■	■	■	■	■					
1	0/1	0			■	■	■	■		■				
1	1/0	0			■	■	■	■			■			
5	3/2	0			■	■	■		■	■				
0	0/0	1			■	■	■		■		■		X	1
1	1/0	0	▒		■	■	■		■	■				
1	0/1	0	▒		■	■	■	■			■			
1	1/0	0	▒	▒	■	■	■		■				X	1
7	0/7	0		▒	■	■	■		■	■			X	2
1	0/1	0		▒	■	■	■	■		■				
1	1/0	0		▒	■	■	■	■			■			
1	1/0	0		▒	■	■	■		■			■		
1	1/0	0			■	■	■	■	■		■			
0	0/0	1			■	■	■		■	■	■		X	1
1	1/0	0			■	■	■	■	■			■		
2	1/1	0	▒		■	■	■	■	■	■				
1	1/0	0	▒	▒	■	■	■	■		■				
1	1/0	0		▒	■	■	■	■	■	■				
1	1/0	0			■	■	■	■	■		■	■	X	1
2	0/2	0		▒	■	■	■	■	■		■	■		

ET, El Tor. [a]*All isolates were negative for ctxA, tcpA (Classical, El Tor), rstR (Classical, El Tor, Calcutta) and hlyA (Classical) PCR. A gray/black box indicates the potential presence/presence of the marker.* [b]*The absence of VSP-1 and VSP-2 was demonstrated by the generation of 1.7 kbp and 800 bp PCR products, respectively, as the primers bind to flanking genomic sites of the pandemic islands. The generation of 3 kbp to 4 kbp PCR products indicated the presence of short VSP-1 fragments, while most of the pandemic island was absent. Strains showing none of the abovementioned PCR products were scored as potentially positive for VSP-1 and VSP-2.* [c]*VC1451 of Vibrio cholerae O1 biovar El Tor str. N16961.* [d]*The presence of TTSS was demonstrated by the detection of vcsC2, vcsN2, vspD, and vcsV2.*

TABLE 4 | Hemolytic activity of *Vibrio cholerae* non-O1, non-O139 isolates from German coastal waters analyzed in this study.

Type of erythrocytes	Environmental (n = 100)				Baltic Sea (n = 50)				North Sea (n = 50)			
	–	+	++	+++	–	+	++	+++	–	+	++	+++
Sheep	4%	26%	60%	10%	2%	26%	60%	12%	6%	26%	60%	8%
Human	3%	7%	71%	19%	0%	6%	78%	16%	6%	8%	64%	22%

The percentage of strains showing no (–), weak (+), intermediate (++), or strong (+++) hemolysis against sheep erythrocytes and human erythrocytes, respectively, is given.

three strains showed no lytic activity for both types of blood cells. The three strains belonged to the same ST (ST336) and did not yield any *hlyA* PCR products.

Whole Genome Sequencing (WGS)

The obtained genome sizes of the 22 sequenced strains vary between 3.9 and 4.2 Mbp and the average number of coding genes is approximately 3.74×10^3. Detailed information about the genomes is given in **Supplementary Table S9**. The results of the bioinformatic analysis concerning the presence of virulence genes are shown in **Table 5**.

The $hlyA^{ET}$ gene is present in all strains with the exception of the three strains that were hemolysis negative, showing that the gene was absent in these strains. Another hemolysin gene (*dth*) encoding the delta thermostable hemolysin (Fallarino et al., 2002) is also absent in the three strains, but is found in the remaining strains.

The *rtxC* gene was detected in all strains confirming the PCR results. The PCR targeting the *rtxA* gene was positive only in one strain (VN-00459). The applied primers were designed to amplify an internal fragment containing the ACD region (actin cross-linking domain) of the *rtxA* gene encoding the multifunctional autoprocessing repeats-in-toxin toxin (MARTX) of the O1 reference strain N16961 (gene VC1451) (Sheahan et al., 2004). The bioinformatic analysis confirmed that this region of the *rtxA* gene was highly similar to the corresponding region of gene VC1451 only in strain VN-00459. All other sequenced strains also harbor *rtxA* genes encoding MARTX toxins that differ in effector domains encoded in the central part of the *rtxA* gene (Satchell, 2015).

We observed that all strains possess *ompU* genes. In five of the sequenced strains which were negative in the *ompU* PCR (**Supplementary Table S7**) the absence of a PCR product can be explained by sequence variations in the primer binding sites. The environmental strains VN-02808, VN-02825, and VN-02923 possess an *ompU* variant which is only 80% similar to the *ompU* gene of the *V. cholerae* O1 strain from which the primers were derived (accession NC_009457, Singh et al., 2001). The bioinformatic analysis also revealed that in four strains, VN-00300, VN-10012, and two closely related environmental strains (VN-10143, VN-10191), the cholix toxin encoding *chxA* gene is present confirming the PCR results.

In some strains, no PCR product was obtained when using primer targeting sequences to the left and right of the seventh pandemic islands VSP-1 and VSP-2. Therefore, it was checked if parts of the islands might be present in the genomes. Four strains (VN-00470, VN-02808, VN-02825, VN-02923) did not yield a PCR product of the expected size of 1.7 kbp if VSP-1 is missing (Rahman et al., 2008; Schirmeister et al., 2014). There were additional sequences enlarging the size of the fragment to 3.8 kbp in VN-00470, but no sequences of the VSP-1 island were detected in this strain nor in the other three strains.

The BLASTN search for VSP-2 sequences gave a different result. In strain VN-00470, a VSP-2-like element (Haley et al., 2014) with a size of approximately 19.3 kbp was detected that harbored homologs (similarity > 92%) of the genes VC0495–VC0498 and VC0504–VC0510 of the VSP-2 of strain N16961. Between these regions, a 4.3 kbp fragment is present that carries two coding sequences of proteins with uncharacterized functions (**Figure 2A**). The integration of the element is in the same position of the chromosome as described for VSP-2 of strain N16961. A homolog of the phage integrase gene of VSP-2 (VC0516) is located at the right border of the VSP-2-like element and *attL* and *attR* sequences highly similar to the corresponding sites of VSP-2 of N16961 were identified (Murphy and Boyd, 2008). Investigation of the sequence data of the strains VN-10143 and VN-10191 revealed that only a short sequence (ca. 900 bp) is similar to a pseudogene region of VSP-2 (VC0501) which could encode a functional transposase.

In strain VN-00459, the PCR for the *mshA* gene yielded a product of the expected size. An alignment of the gene cluster encoding the MSHA pilus of strain N16961 to the genomic sequences revealed that the cluster is present in this strain (similarity > 99%).

Four genes of the TTSS of non-O1, non-O139 strains (Dziejman et al., 2005; Chaand et al., 2015) were detected by PCR in three strains used for WGS (VN-00300, VN-00533, VN-00459) (**Supplementary Table S7**). The bioinformatic analysis makes it likely that the complete TTSS cluster consisting of core region and 5′- and 3′-flanking regions of reference strain AM-19226 is present in strains VN-00300 and VN-00459 (Chaand et al., 2015). The cluster sequences possess a similarity of >95% to the TTSS of the reference and a *trh* gene encoding a thermostable direct hemolysin is also found in the 3′-flanking region (**Figure 2B**). The TTSS of strain VN-00533 comprises only the core region and few genes of the 3′-flanking region excluding the *trh* gene and another putative virulence gene (*acfD*) (Chaand et al., 2015).

The *nanH* gene which encodes a neuraminidase cleaving sialic acid residues from host gangliosides (Almagro-Moreno and Boyd, 2009) were also found in the genomes of two TTSS harboring strains (VN-00300, VN-00459). As the *nanH* gene is

TABLE 5 | Virulence gene profiles of *Vibrio cholerae* non-O1, non-O139 isolates from German coastal waters and from German clinical samples based on WGS data.

MLST group[a]		Strain[b]	Virulence gene profile[c]																	
			VSP-2*	*toxR***	*luxS*	*cqsA*	T6SS	*hapA*	*tlh*	*hlyA*^{ET}*	*dth*	*ompU**	*rtxC**	*rtxA**	TTSS*	*trh*	*nanH*	SA ME	VPI-2	*chxA**	*mshA**
SLV	ST495-ST448	VN-10012		■	■	■	■	■	■	■	■	■	■	■						■	
		VN-00300[d]		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	
	ST499-ST496	VN-00470	■	■	■	■	■	■	■	■	■	■	■	■							
		VN-00168[d]		■	■	■	■	■	■	■	■	■	■	■							
DLV	ST477-ST590	VN-10133		■	■	■	■	■	■	■	■	■	■	■							
		VN-00534		■	■	■	■	■	■	■	■	■	■	■							
	ST492-ST491	VN-00462		■	■	■	■	■	■	■	■	■	■	■							
		VN-00471		■	■	■	■	■	■	■	■	■	■	■							
		VN-00472		■	■	■	■	■	■	■	■	■	■	■							
		VN-05176		■	■	■	■	■	■	■	■	■	■	■							
		VN-00298[d]		■	■	■	■	■	■	■	■	■	■	■							
	ST493-ST490	VN-00473[d]		■	■	■	■	■	■	■	■	■	■	■							
		VN-00297		■	■	■	■	■	■	■	■	■	■	■							
		VN-00307		■	■	■	■	■	■	■	■	■	■	■							
	ST493-ST589	VN-00473[d]		■	■	■	■	■	■	■	■	■	■	■							
		VN-00533		■	■	■	■	■	■	■	■	■	■	■	■						
	ST498-ST496	VN-00461		■	■	■	■	■	■	■	■	■	■	■							
		VN-00168[d]		■	■	■	■	■	■	■	■	■	■	■							
TLV	ST453-ST448	VN-10143		■	■	■	■	■	■	■	■	■	■	■						■	
		VN-10191		■	■	■	■	■	■	■	■	■	■	■						■	
		VN-00300[d]		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	
	ST493-ST491	VN-00473[d]		■	■	■	■	■	■	■	■	■	■	■							
		VN-00298[d]		■	■	■	■	■	■	■	■	■	■	■							
Others	FVG,	VN-02808		■	■	■	■	■	■			■	■	■							
	CP-CRVc,	VN-02825		■	■	■	■	■	■			■	■	■							
	ST336	VN-02923		■	■	■	■	■	■			■	■	■							
	MVG, ST330	VN-00459		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■

*CP-CRVc, carbapenemase producing carbapenem-resistant Vibrio cholerae; ET, El Tor; FVG, few virulence-associated genes/gene clusters (according to PCR data); MVG, many virulence-associated genes/gene clusters (according to PCR data); SA ME, sialic acid metabolism. [a]The MLST groups are explained in section "Materials and Methods". Clinical STs are marked in red. [b]All strains of the MLST groups selected for whole genome sequencing were compared regarding sequence type, year of isolation, geographical origin, sample type, and PCR-based virulence gene profile. Presumably identical strains were summarized in subgroups. One strain from each subgroup was selected for whole genome sequencing. Clinical strains are marked in red. [c]All strains were negative for VSP-1 and stn. Reference sequences for WGS analysis are given in section "Materials and Methods". In general, strains showing >90% sequence similarity to a specific reference sequence were defined as positive for the respective virulence-associated trait. Further details are given in the "Results" section. *Virulence-associated gene/gene cluster initially investigated by PCR. **Virulence-associated gene only investigated by PCR. [d]Strain belonging to more than one MLST group.*

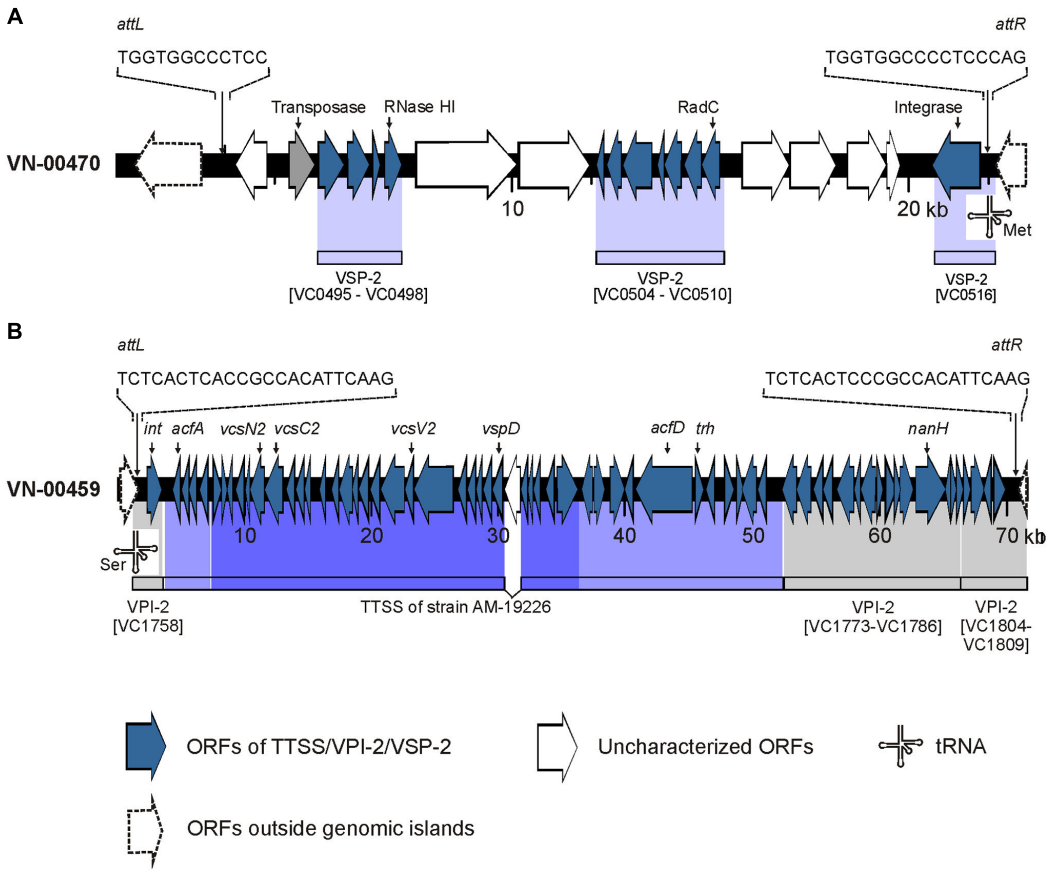

FIGURE 2 | Schematics of genomic islands of the *Vibrio cholerae* non-O1, non-O139 strains VN-00470 and VN-00459 from the German Baltic Sea. **(A)** *Vibrio* seventh pandemic island 2 (VSP-2)-like element identified in strain VN-00470. Light blue areas below the VN-00470 sequence indicate similarity to genes of VSP-2 of *V. cholerae* O1 reference strain N16961 (gene numbering according to NC_002505.1). **(B)** Variant *Vibrio* pathogenicity island 2 (VPI-2) present in strain VN-00459. Gray areas below the VN-00459 sequence indicate similarity to genes of VPI-2 of *V. cholerae* O1 reference strain N16961 (gene numbering according to NC_002505.1). Blue colored areas below the VN-00459 sequence indicate similarity to the type three secretion system (TTSS) of strain AM-19226 (accession number AATY02000000). Dark blue regions represent the core region of the TTSS. Light blue regions represent the 5′- and 3′-flanking regions of the TTSS. *attL*, left attachment site; *attR*, right attachment site; ORF, open reading frame.

part of the *Vibrio* pathogenicity island VPI-2 (Murphy and Boyd, 2008), an alignment of VPI-2 of strain N16961 was performed to the two genomes. In both strains, the genes of the sialic acid metabolism are present (VC1773–VC1786). In strain VN-00459, the TTSS and the sialic acid metabolism genes lie on the same contig and form a variant VPI-2 island with a size of 68.9 kbp (**Figure 2B**) (Murphy and Boyd, 2008). The element is integrated into the same chromosomal position as VPI-2 in the reference strain N16961 and possesses identical *attL* and *attR* sequences to VPI-2. In strain VN-00300, the variant VPI-2 sequences are distributed on several different contigs. However, the combined sizes of sequences and their high similarity to VN-00459 suggest that both strains harbor the same genomic island. In the third TTSS harboring strain, VN-00533, the TTSS sequences are adjacent to a truncated coding sequence (approximately 53%) of the *nanH* gene. This pseudogene is flanked by a 2.7 kbp sequence with high similarity to the right border of a VPI-2 element. However, no more sequences of a variant VPI-2 element are found.

The analysis also revealed that environmental and clinical strains are probably equipped with a functional type six secretion system (T6SS) as most of the genes of the core region and some effector proteins (genes *vipAB*, *vasA-vasK*, *vgrG-3*, *vgrG-2*) are present in all genomes (similarity > 95%) (Zheng et al., 2011; Unterweger et al., 2014).

The bioinformatic analysis was extended to include some virulence genes whose presence/absence has been studied in non-O1, non-O139 strains. The prevalence of the *stn* gene encoding the heat-stable enterotoxin (Ogawa et al., 1990) and the *tlh* gene encoding a heat-labile hemolysin (Syed et al., 2009), were analyzed. While the former gene was absent, the latter was found in all sequenced strains. In a process called quorum sensing, the expression of virulence genes of toxigenic *V. cholerae* strains is influenced by two major autoinducers CAI-1 and AI-2 (Higgins et al., 2007). The genes *cqsA* and *luxS* encoding synthases of the two autoinducers were found in all sequenced genomes. Another virulence gene, *hapA*, codes for the *V. cholerae* hemagglutinin/protease that degrades host proteins of

intercellular tight junctions (Wu et al., 2000). The gene was also detected in all strains.

PCR typing of virulence genes and whole genome sequence-based analyses revealed an overall matching degree of 84%. In 15% of cases, the virulence factor in question was only detected by WGS-based bioinformatical analyses. In 1% of cases, "positive" PCR results were not confirmed by WGS. This only concerned VSP-2 PCR assay, which involves primers binding to flanking regions of the genomic island and where strains are to be assessed as potentially VSP-2 positive when a PCR product of the expected size is absent.

SNP Analysis of Sequenced Genomes

All sequenced genomes were analyzed in an SNP analysis with the genome of strain VN-00533 as a reference. In total, 3,237,190 positions (corresponding to approximately 78.7% of the reference genome) were used in the analysis and the number of SNPs between the strains varied between 1 and 30,647 (**Supplementary Table S10**). The SNP tree is shown in **Figure 3**. Genomes of strains belonging to the same ST form clusters (SNP difference 1 to 847). Three clusters comprise only environmental strains (ST336 with strains VN-02808, VN-02825, VN-02923; ST492 with strains VN-00462, VN-00471, VN-00472, VN-05176; ST453 with the strains VN-10143 and VN-10191) and one cluster consisted of two clinical strains (ST490 with strains VN-00297 and VN-00307). The SNP tree shows that strains with single-locus variations in the MLST genes also group together, however, the SNP differences are distinctly higher. There are 11,892 SNPs between strains VN-10012 and VN-00300 and 4,657 between strains VN-00470 and VN-00168.

DISCUSSION

MLST and Virulence Gene Profiles Obtained by PCR

As a basis for comparison, an MLST analysis was performed for all strains. MLST defines strains from the sequences at housekeeping loci and has become the method of choice for molecular typing of many bacterial species. Sequence data are ideal for strain characterization as they are unambiguous and strains can readily be compared between laboratories via the internet (Aanensen and Spratt, 2005).

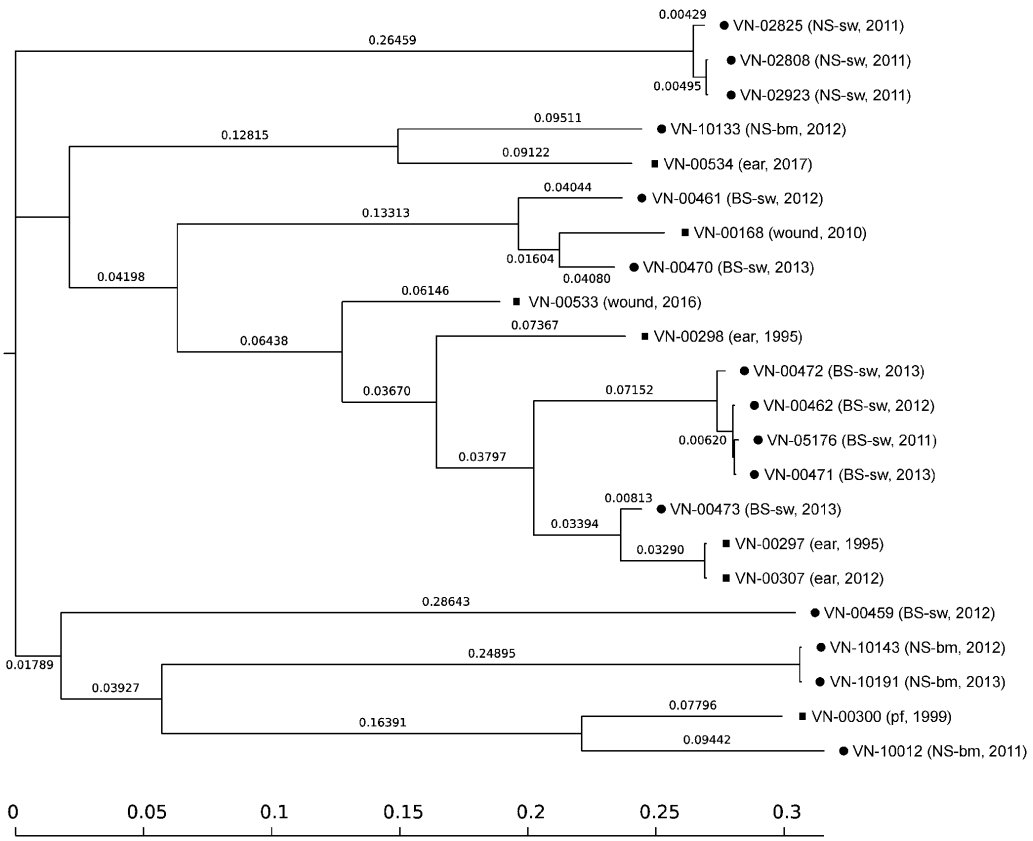

FIGURE 3 | SNP-based phylogeny tree of *Vibrio cholerae* non-O1, non-O139 isolates from German coastal waters (●) and from German clinical samples (■). SNP tree was conducted using CSI Phylogeny 1.4 under default settings and the exclusion of heterozygous SNPs. Single nucleotide polymorphisms (SNPs) were called by mapping to the *V. cholerae* VN-00533 genome as reference (MWZM00000000). Scale bar represents the number of nucleotide substitutions per site and numbers indicate branch length. Basic information about the sample type and the year of isolation are given after the strain identifier: North Sea/seawater (NS-sw), North Sea/bivalve mollusk (NS-bm), Baltic Sea/seawater (BS-sw), and peritoneal fluid (pf).

Our study revealed a significant diversity of *V. cholerae* non-O1, non-O139 strains from the German coastal waters. High genetic diversity of *V. cholerae* non-O1, non-O139 strains has been also observed in other studies in which environmental strains were analyzed by MLST (Octavia et al., 2013; Pretzer et al., 2017; Siriphap et al., 2017). The STs of five German *V. cholerae* non-O1, non-O139 strains which were already deposited in the PubMLST database were distinctly different. All deposited STs had at most one common locus with the STs of the strains analyzed by Octavia et al. (2013). Interestingly, Chinese serogroup O1 strains which were lacking the *ctxAB* genes displayed a high MLST diversity in contrast to toxigenic O1 strains (Zhou et al., 2014). Though the applied MLST scheme only partially overlapped with the standardized PubMLST scheme, this indicates that high genetic diversity can also be found within a serogroup (Zhou et al., 2014).

As a next step, the presence or absence of virulence factors in the environmental strains was studied by PCR. While genes of the CTX element and the TcpA pilus were not detected in any of the strains, some accessory virulence genes of toxigenic strains which play a synergistic role in the infection process were found. The investigated factors were the MSHA (Rahman et al., 2008), the *rtxA* and *rtxC* genes of the RTX toxin cluster (Chow et al., 2001; Satchell, 2007), an outer membrane protein (OmpU) (Mathur et al., 2007), the virulence regulator gene *toxR* and the hemolysin gene *hlyA* (Rivera et al., 2001; Rahman et al., 2008; Chatterjee et al., 2009). Additionally, the occurrence of two genomic islands of toxigenic *V. cholerae* strains of the seventh pandemic (VSP-1, VSP-2) was examined (Murphy and Boyd, 2008). Some virulence factors like the TTSS and the cholix toxin (*chxA*) that are not associated with toxigenic strains were also investigated.

In a study of non-toxigenic *V. cholerae* serogroup O1 strains (Zhou et al., 2014), the virulence profiles differed among each other. A number of strains harbored the *Vibrio* pathogenicity island-1 (VPI-1) that encodes the TCP (Murphy and Boyd, 2008) and all strains possessed a MSHA pilus. We did not find VPI-1 genes in the non-O1, non-O139 strains and detected the *mshA* gene encoding the major pilin subunit (Marsh and Taylor, 1999) only in 6% of the strains.

In total, we obtained 30 profiles by PCR (**Table 3**) of which two profiles were also present in the German clinical strains (Schirmeister et al., 2014). The most frequent virulence gene profile comprised the genes *toxR*, *hlyA*^{ET}, *ompU*, and *rtxC* and was found in 38 environmental and in six clinical strains.

It is remarkable that so many clinical strains belong to the group containing relatively few virulence genes. This could mean that many environmental strains with this profile could be potential human pathogens. While this conclusion seems plausible it also indicates the need for a deeper molecular analysis of the genetic makeup of the strains. For this reason, a number of strains with this profile were selected for WGS (see section "WGS Analysis of Virulence Factors and SNP Analysis").

Hemolytic Activity and Hemolysin Genes

Hemolysis of erythrocytes is a virulence trait widely distributed among pathogenic *Vibrio* species (Zhang and Austin, 2005) and is a phenotype that is routinely investigated in laboratories. We studied hemolytic activity of the strains against human and sheep erythrocytes to find out if the presence of the El Tor hemolysin gene is correlated to the lytic activity. Strains of the classical biotype carry a truncated hemolysin gene and do not lyze sheep erythrocytes (Alm et al., 1988). In a previous study (Rivera et al., 2001) it was observed that most non-O1, non-O139 strains possessed the El Tor *hlyA* gene, while few strains contained the classical *hlyA* gene with a premature stop codon and few strains lacked the gene.

The investigated environmental strains of our study possessed the *hlyA*^{ET} gene and were hemolytic on both types of blood cells. One strain showed hemolysis only on human cells but was not further studied. Three strains which had been negative in the *hlyA* PCR did not show hemolytic activity against both types of blood cells. This result could also be explained either by absence of the gene in the three strains or by PCR failure due to sequence variations to the applied primers. As *hlyA* has been used as a species marker for *V. cholerae* (Fykse et al., 2007; Jones et al., 2014) the three strains were included in the whole genome analysis and the absence of the gene was confirmed.

Remarkably, the *dth* gene related to the *δ-vph* gene of *V. parahaemolyticus* (Fallarino et al., 2002) was another hemolysin gene missing in the three strains, while detected in all remaining strains. However, it is not clear if under the tested conditions this gene contributes to the hemolytic activity at all. The *tlh* gene encodes a thermolabile hemolysin whose role in infection process is unclear though it was found to be upregulated in flagellar regulatory mutants in the same way as other virulence genes (Syed et al., 2009). The bioinformatic analysis showed that all strains possess this gene.

WGS Analysis of Virulence Factors and SNP Analysis

The WGS data were used to confirm the PCR results and to identify more virulence genes in the genomes of the strains. In the context of the bioinformatical analysis, the majority of PCR results (84%) were confirmed. In some strains that had shown negative results in the *ompU* PCR (5 strains) or the *rtxA* PCR (21 strains) in consequence of primer mismatches, variants of the virulence genes were found using WGS data. This underlines the higher resolving power of WGS-based analytical methods and emphasizes the need for completion of PCR-based virulence typing schemes by WGS-based analyses for a risk assessment of bacterial isolates.

To determine the genetic relationship of the sequenced strains in more depth, an SNP analysis was performed. The SNP tree (**Figure 3**) shows that strains of the same STs are closely related, whereas a clear separation in respect of SNP numbers is found between strains which differ only in one of the seven housekeeping loci used for MLST. The SNP tree also clearly reveals that most strains are not closely related with the exceptions of strains of the same ST. Mostly, more than 20,000 SNPs are calculated between the strains.

The bioinformatic analysis confirmed that in the German strains no sequences of the CTX element and the TCP genes are present. In other studies, few non-O1, non-O139 strains were

described that harbored *ctx*, *ace*, *zot*, or *tcpA* genes (Chatterjee et al., 2009; Ceccarelli et al., 2015).

A number of virulence genes present in all strains encode proteins that are probably primarily important for survival and niche adaptation in the natural aquatic environment (Sakib et al., 2018). An example is the *hapA* gene encoding a hemagglutinin/protease that affects epithelial tight junctions and contributes to diarrheal disease (Wu et al., 2000). In the natural environment, this protein is controlled by quorum sensing and degrades egg masses of insects (chironomids) which are a natural reservoir for *V. cholerae* (Halpern, 2010). Therefore, genes like *luxS* or *cqsA* encoding proteins involved in quorum sensing (Higgins et al., 2007) are also found in all genomes. The list of genes which are probably more important for survival in the natural aquatic environment rather than in a human host could be continued and explains why a number of virulence genes investigated in this study are present in all environmental strains (Sakib et al., 2018).

Another example is probably the *rtx* gene cluster. All strains harbor the *rtxC* gene encoding an acyltransferase which has been described as an activator of MARTX toxin. The role of the acyltransferase in infection is unclear, as the *rtxC* gene product is not necessary for MARTX function in an animal model (Cheong et al., 2010). While the *rtxC* gene is highly similar (identity > 91%) in all strains, the *rtxA* genes encoding MARTX vary considerably. MARTX proteins of *V. cholerae* strains are very large multifunctional proteins (4,565 amino acids in the toxigenic O1 strain N16961) with conserved N-terminal and C-terminal regions, whereas the central part of the toxin carries different effector domains (Satchell, 2015). The MARTX toxin of toxigenic El Tor strains possess three conserved internal domains and is probably involved in evasion of the host immune defense rather than in contributing to diarrheal disease.

In one sequenced strain (VN-00459) that was positive in the *rtxA* PCR a toxin gene highly similar to MARTX of the toxigenic O1 strain N16961 is present (identity of amino acid >98%). This MARTX toxin could be present in 21 more environmental strains that were positive in the *rtxA* PCR (**Supplementary Table S7**). With the exception of VN-00459, the remaining sequenced strains (clinical and environmental strains) harbored *rtxA* genes encoding MARTX variants differing in the central part of the coding region. It is known that environmental non-O1, non-O139 *V. cholerae* strains carry MARTX variants with different effector domains. These toxins can be active against eukaryotic cells from different organisms (mammals or fish or eels) and could play a role in adaptation to specific niches in the natural ecosystem (Satchell, 2015; Sakib et al., 2018).

The bioinformatic analysis of the genomes revealed that all strains probably harbor a functional T6SS. T6SS is a contact-dependent contractile apparatus resembling the spiked tail and tube of bacteriophages and is used for translocation of effector proteins mediating antagonistic interactions against many prokaryotic and eukaryotic organisms (Joshi et al., 2017; Sakib et al., 2018). In the environment, it protects against predators and helps in competition against antagonistic microorganisms and provides growth advantages. In diarrheal disease, it contributes to intestinal colonization against the gut microbiota and delivers effectors that are associated with intestinal inflammation and diarrheal symptoms in animal models. The role of T6SS in the environment and in infections explains why the secretion system is found in all clinical and environmental strains.

With the WGS sequence data, the genetic structure of islands and clusters detected by PCR in only few strains could be analyzed. The four TTSS genes as well as the *mshA* gene were identified as members of complete gene clusters encoding probably functional virulence factors. The MSHA pilus contributes to adhesion to cell surfaces and biofilm formation in the natural aquatic ecosystem (Chiavelli et al., 2001; List et al., 2018) while its role in human disease is not clear. The major pilin subunit gene *mshA* was present only in one of the sequenced strains. The TTSS is located within a variant VPI-2 genomic island together with more virulence factors (neuraminidase and enzymes of the SA metabolism). VPI-2 was shown to be a mobile genetic element (Murphy and Boyd, 2008).

Genes of the VSP-1 island are absent in all strains. However, in case of VSP-2, in one strain (VN-00470) a VSP-2-like element was identified (Haley et al., 2014). It is not clear if this element can contribute to virulence in humans. Similar elements are present in non-pathogenic *Vibrio* species indicating a role in the natural environment (Haley et al., 2014).

It is also possible that variations in promoter regions influence the expression of virulence genes. This could result in different manifestations of non-O1, non-O139 *V. cholerae* infections. In some *V. parahaemolyticus* strains harboring two hemolysin genes (*tdh*1 and *tdh*2), one gene product (TDH2) is found predominantly in culture supernatants. Analyses of the promoter sequences revealed differences in the -35 and -10 promoter sequences (Nishibuchi and Kaper, 1990; Nishibuchi et al., 1991). The question if variations in promoter sequences contribute to gene expression and hence influence the virulence of a strain can only be addressed in experimental studies.

CONCLUSION

The aim of our study was to determine if STs and virulence gene profiles of clinical and environmental *V. cholerae* non-O1, non-O139 strains originating from German coastal waters may be correlated and could be usable for a risk assessment of individual strains. The result of this study reveals that – given on the current knowledge about the potential pathogenicity of these strains – no such correlation is found. The MLST shows a high diversity of the bacteria, whereas a basic equipment with virulence genes is very similar between all strains. Due to horizontal gene transfer, in some strains the number of virulence genes is increased compared to other strains. TTSS harboring *V. cholerae* non-O1, non-O139 strains were discovered in diarrhea causing strains (Dziejman et al., 2005) and recent studies have confirmed the importance of these systems for gastrointestinal infections (Chaand et al., 2015). The clinical strains of our study were mostly isolated from extraintestinal infections (wound infection and otitis) and lacked the genes of the TTSS system with one

exception (strain VN-00533). This suggests that other virulence factors play a role in this type of infections.

Vibrio cholerae is a species that is a natural inhabitant of aquatic ecosystems. It is likely that most of the factors that contribute to virulence in a human host evolved as a response of these bacteria to challenges in their natural environment (Sakib et al., 2018). Thus, the genetic makeup of these bacteria is a result of biotic and abiotic stress in aquatic environments and adaptation to specific niches. This means that in the group of non-O1, non-O139 strains many of the so far recognized virulence genes are found in most isolates. It also means that a clear distinction between clinical and non-pathogenic environmental strains is probably not possible. The expected rise of *Vibrio* abundance in German coastal waters and the predicted increase of infections with these bacteria due to climate change demand measures and actions of the health authorities to reduce the risk for the public. For the time being, this could be the build-up of monitoring systems for vibrios at popular resort beaches and the establishment of surveillance systems for *Vibrio* infections by introducing a compulsory notification for diseases caused by these bacteria.

AUTHOR CONTRIBUTIONS

KS and ES designed the study. KS and CG performed the experiments. KS, JH, and ES analyzed the data, prepared the tables and figures, and wrote the manuscript. All authors edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00733/full#supplementary-material.

The datasets generated for this study can be found in PubMLST (**Supplementary Table S5**) and GenBank (NCBI; **Supplementary Table S9**).

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2.3 Publication 2

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Diversity of *Vibrio navarrensis* Revealed by Genomic Comparison: Veterinary Isolates Are Related to Strains Associated with Human Illness and Sewage Isolates While Seawater Strains Are More Distant

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Strains of *Vibrio navarrensis* are present in aquatic environments like seawater, rivers, and sewage. Recently, strains of this species were identified in human clinical specimens. In this study, *V. navarrensis* strains isolated from livestock in Germany were characterized that were found in aborted fetuses and/or placentas after miscarriages. The veterinary strains were analyzed using phenotypical and genotypical methods and compared to isolates from marine environments of the Baltic Sea and North Sea. The investigated phenotypical traits were similar in all German strains. Whole genome sequencing (WGS) was used to evaluate a phylogenetic relationship by performing a single nucleotide polymorphism (SNP) analysis. For the SNP analysis, WGS data of two American human pathogenic strains and two Spanish environmental isolates from sewage were included. A phylogenetic analysis of concatenated sequences of five protein-coding housekeeping genes (*gyrB*, *pyrH*, *recA*, *atpA*, and *rpoB*), was additionally performed. Both phylogenetic analyses reveal a greater distance of the environmental seawater strains to the other strains. The phylogenetic tree constructed from concatenated sequences of housekeeping genes places veterinary, human pathogenic and Spanish sewage strains into one cluster. Presence and absence of virulence-associated genes were investigated based on WGS data and confirmed by PCR. However, this analysis showed no clear pattern for the potentially pathogenic strains. The detection of *V. navarrensis* in human clinical specimens strongly suggests that this species should be regarded as a potential human pathogen. The identification of *V. navarrensis* strains in domestic animals implicates a zoonotic potential of this species. This could indicate a potential threat for humans, as according to the "One Health" concept, human, animal, and environmental health are linked. Future studies are necessary to search for reservoirs of these bacteria in the environment and/or in living organisms.

Keywords: *Vibrio* spp., pathogen, genome, diversity, whole genome sequencing, virulence-associated factors

INTRODUCTION

Vibrio navarrensis was first described as a species isolated from sewage and river water in the Spanish province Navarra in 1991 (Urdaci et al., 1991). Later, some strains from the Baltic Sea were reported that differed in some biochemical reactions to the Spanish strains. However, DNA-DNA hybridization and fatty acid analysis revealed them as *V. navarrensis* and they were classified as *V. navarrensis* biotype *pommerensis* (Jores et al., 2007). All *V. navarrensis* strains showed hemolytic activity on blood agar containing different types of erythrocytes, e.g., human, sheep, horse or cattle blood cells (Jores et al., 2003, 2007). The strains were regarded as environmental strains and found during surveys to determine the occurrence and distribution of pathogenic *Vibrio* species like *Vibrio cholerae* (Urdaci et al., 1991) and *Vibrio vulnificus* (Jores et al., 2007) in aquatic environments.

In 2014, the characterization of *V. navarrensis* isolates associated with human illness was reported in a publication of the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia (Gladney and Tarr, 2014). Most of the strains had been in the CDC strain collection for some time and could not be characterized to the species level by phenotypical methods at the time of isolation. By applying multilocus sequence analysis (MLSA), the strains could be placed in a phylogenetic framework and were assigned to the species *V. navarrensis* (Gladney and Tarr, 2014).

Our laboratory received *Vibrio* strains from a veterinary lab in Saxony, Germany, collected between 1990 and 2011 which contained strains that had been isolated from domestic animals like pig and cattle after abortions. The isolates had been found in animals intended for food production in farms of the German state Saxony that does not border on marine environments. The strains were recovered after abortions from placentas and aborted fetuses (Stephan et al., 2002; Schirmeister et al., 2014). At first, some of these strains had been classified as *V. vulnificus* by phenotypic characterization or were assigned *Vibrio* spp., but could be assigned to *V. navarrensis* by sequencing of the *rpoB* gene coding for the ß-subunit of RNA polymerase (Tarr et al., 2007; Adékambi et al., 2009; Dieckmann et al., 2010). As the animal source of these strains is an unusual source of *Vibrio* bacteria, we compared them to environmental *V. navarrensis* strains of the North Sea and Baltic Sea by studying genotypic and phenotypic traits to find out if the veterinary strains may originate from this environment. Additionally, by including published whole genome sequences of two human pathogenic strains (Gladney et al., 2014) the aim of the study was also to find out if the veterinary isolates are related to these strains which could indicate a pathogenic potential.

The occurrence of *V. navarrensis* strains in freshwater and seawater as well as the isolation from humans and domestic animals reveals a broad ecological range of habitats, which may show a wide genetic diversity of the species. For this purpose, WGS data and sequences of housekeeping genes were applied for constructing phylogenetic trees. For the analyses, whole genome sequencing (WGS) data of four published genomes of *V. navarrensis* strains consisting of two human pathogenic strains from the U.S. and two environmental strains from Spain were included. A number of genes associated with virulence in other human pathogens were found in the *V. navarrensis* genome sequence (Gladney et al., 2014). Presence or absence of some of these virulence-associated genes were investigated by genome comparison and confirmed by PCR analyses.

MATERIALS AND METHODS

Bacterial Strains

In total, 19 *V. navarrensis* isolates from German sources and one reference strain (CIP 103381 from Spain, isolate from sewage) were investigated in this study (**Table 1**). Ten strains were obtained from an official veterinary laboratory in Saxony (Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen, Dresden) and were mostly isolated from domestic animals after abortions. The veterinary strains were recovered from aborted fetuses or placentas or both. Two environmental seawater strains of the year 2011 were obtained from the Alfred Wegener Institute, Heligoland, and three environmental strains of the year 2015 from a university hospital (Medizinaluntersuchungsamt und Hygiene, Universitätsklinikum Schleswig-Holstein). *Vibrio navarrensis* biotype *pommerensis* strains came from the strain collection of the German Federal Institute for Risk Assessment (BfR). One environmental strain was isolated from a blue mussel harvested in the Wadden Sea of the North Sea.

TABLE 1 | *Vibrio navarrensis* strains used in this study.

Strain	Year of isolation	Source	Origin
CIP 103381*	1982	Reference strain/sewage	Spain
CH-271**	1996	Seawater	Baltic Sea
CH-280**	1996	Seawater	Baltic Sea
CH-291**	1996	Seawater	Baltic Sea
VN-0392	1999	Cattle/placenta	Saxony
VN-0413	2000	Cattle/placenta	Saxony
VN-0414	2000	Cattle/placenta	Saxony
VN-0415	2009	Cattle/fetus	Saxony
VN-0506	2000	Cattle/placenta	Saxony
VN-0507	2000	Cattle/placenta	Saxony
VN-0508	2000	Pig/placenta	Saxony
VN-0509	2001	Pig/fetus	Saxony
VN-0514	2007	Pig/placenta	Saxony
VN-0515	2007	Pig/placenta	Saxony
VN-0516	2015	Brackish water	Schleswig-Holstein
VN-0517	2015	Seawater	Schleswig-Holstein
VN-0518	2015	Seawater	Schleswig-Holstein
VN-0519	2011	Blue mussel	Lower Saxony
VN-3125	2011	Seawater	Kattegat
VN-3139	2011	Seawater	Kattegat

**Identical to ATCC 51183.*

***Strains of V. navarrensis biotype pommerensis. Strain CH-291 was deposited as DSM 15800.*

Biochemical Characterization

Vibrio navarrensis strains were routinely cultivated in LB medium (Merck KGaA, Darmstadt, Germany) at 37°C. Strains were characterized by biochemical tests used in routine diagnostics of the National Reference Laboratory (NRL) for Monitoring Bacteriological Contamination of Bivalve Mollusks located at the BfR. Tests included growth in 1% peptone water with 0, 3, 8, and 10% NaCl, cytochrome oxidase, sensitivity to the vibriostatic agent O/129 (10 and 150 μg), lysine decarboxylase, arginine dihydrolase, ornithine decarboxylase, nitrate reductase (all supplemented with 1% NaCl), and utilization of a number of carbohydrates (Farmer et al., 2003). Phenylalanine deamination was tested on phenylalanine agar IDM 31 (Mast Diagnostica GmbH, Reinfeld, Germany) supplemented with 1% NaCl. To ensure test results, the following Gram-negative bacterial strains served as controls: *Aeromonas hydrophila* ATCC 7966 (positive control for cytochrome oxidase test, resistance to O/129), *Escherichia coli* DSM 1103 (positive control for oxidative acid production from D-glucose, maltose, D-mannose, and trehalose; negative control for phenylalanine deaminase test, urease test, and citrate degradation test), *Klebsiella oxytoca* DSM 25736 (positive control for oxidative acid production from adonitol, L-arabinose, cellobiose, dulcitol, *myo*-inositol, lactose, D-mannitol, melibiose, raffinose, L-rhamnose, salicin, D-sorbitol, L-sorbose, sucrose, and D-xylose, esculin and citrate degradation tests), *Morganella morganii* DSM 30117 (negative control for oxidative acid production from cellobiose, trehalose, and D-xylose), *Proteus mirabilis* DSM 4479 (positive control for phenylalanine deaminase test, urease test, and H_2S production test; negative control for cytochrome oxidase test, oxidative acid production from adonitol, L-arabinose, dulcitol, *myo*-inositol, lactose, D-mannitol, D-mannose, melibiose, raffinose, L-rhamnose, salicin, D-sorbitol, L-sorbose, and sucrose, esculin degradation test), and *Shigella sonnei* DSM 25715 (negative control for oxidative acid production from D-glucose and maltose, H_2S production test). According to quality control standards of the NRL, functionality of liquid growth media supplemented with 1% NaCl was tested with the following *Vibrio* spp. strains: *V. alginolyticus* DSM 2171 (positive control for growth in 1% peptone water with 3, 6, and 8% NaCl), *V. cholerae* DSM 101014 (positive control for nitrate reductase test, lysine decarboxylase test, ornithine decarboxylase test, indole production test, growth in 1% peptone water with 0% NaCl; negative control for arginine dihydrolase test), *V. cholerae* ATCC 14035 (susceptibility to O/129), *V. furnissii* DSM 14383 (positive control for arginine dihydrolase test; negative control for lysine decarboxylase and ornithine decarboxylase tests), *V. metschnikovii* LMG 4416 (positive control for acetoin production test; negative control for nitrate reductase test, indole production test), and *V. parahaemolyticus* DSM 101031 (negative control for acetoin production test). Biochemical testing was repeated twice.

Hemolytic Activity Tests

Blood agar plates were prepared with Mueller-Hinton agar (Oxoid GmbH, Wesel, Germany) supplemented with 1% NaCl and contained 4% sheep (BfR, Berlin, Germany) or 4% human erythrocytes (German Red Cross, blood donation service, Berlin-Wannsee, Germany). Erythrocytes were washed three times in cold phosphate buffered saline and pelleted for 5 min at 400 × g and 10°C before use. Prior to hemolysis assay, bacteria were cultivated from glycerol stocks on Mueller-Hinton agar plates overnight at 37°C. Four milliliters of Mueller-Hinton broth were inoculated with one single colony and incubated for 12–14 h at 37°C with constant shaking (200 rpm). All culture media were supplemented with 1% NaCl. In order to investigate the hemolytic activity of the strains, 10 μl of the overnight cultures were spotted on a blood agar plate and incubated at 37°C to obtain macrocolonies. Zones of hemolysis around the macrocolonies were visually controlled and scored for up to 72 h. All experiments were performed twice. Strains which did not reveal hemolysis zones on sheep blood were recultivated from glycerol stocks, passaged three times on sheep blood agar plates consisting of Special Blood Agar Base DM101 (Mast Diagnostica GmbH, Reinfeld, Germany) supplemented with 5% defibrinated sheep blood (BfR, Berlin, Germany) and retested on the modified agar plates as described above.

rpoB Sequencing

Bacterial strains were grown overnight and genomic DNA was extracted using the RTP Bacteria DNA Mini Kit from Stratec Molecular, Berlin, Germany, according to the manufacturer's instructions. Analyses of the *rpoB* gene were performed using the PCR primers and sequencing primers described earlier (Mollet et al., 1997; Tarr et al., 2007; Table S1). Briefly, a 984 bp fragment of the *rpoB* coding sequence was amplified using the primers CM32b and 1110F. For sequencing of the amplification products, the PCR primers and two additional primers (1661F, 1783R) were used. PCR conditions were according to the protocol given in Tarr et al. (2007).

Whole Genome Sequencing (WGS) and Single Nucleotide Polymorphism (SNP) Analysis

Genomic DNA of *V. navarrensis* isolates was prepared using the PureLink Genomic DNA Mini Kit (Invitrogen, Karlsruhe, Germany). DNA libraries were generated with the Nextera XT DNA Sample Preparation Kit according to the manufacturer's protocol (Illumina Inc., San Diego, CA, USA). DNA sequencing using the MiSeq Reagent v3 600-cycle Kit (2 × 300 cycles) was performed on the MiSeq benchtop (Illumina Inc., San Diego, CA, USA). For *de novo* assembling of raw sequencing reads, the SPAdes (version 3.5.0) algorithm was used. Initial annotation of the genomes was performed by using the automated Prokaryotic Genome Annotation Pipeline of the NCBI website. Further genetic features and elements of the genomes were identified using the Bacterial Analysis Pipeline and the Phage Search Tool (Zhou et al., 2011). Putative prophage sequences were recorded based on clusters of more than six phage-like genes within a sequence region of the analyzed genome. Therefore, phage-like genes were identified according to their similarity against sequences of the NCBI database. Additionally, the genome annotation of the isolates was analyzed for phage specific terms like "protease," "integrase," and "tail fiber." Predicted prophage regions were assessed according to the recommendations (Zhou

et al., 2011). Initial plasmid prediction was performed by using *de novo* assemblies of genomes with the web-based tool "PlasmidFinder" of the Center for Genomic Epidemiology (Carattoli et al., 2014). Furthermore, contigs with significantly higher sequence coverage than the rest of the genomic contigs were applied to the BLASTN search of the NCBI database and screened for similarities to known plasmids.

The SNP tree was conducted by using CSI Phylogeny 1.4 (Center for Genomic Epidemiology) under default settings and the exclusion of heterozygous SNPs. To identify SNPs, all input sequences were mapped to the *V. navarrensis* 0053-83 genome as reference (JMCF01000001) and screened for relevant nucleotide variations as previously described (Kaas et al., 2014). The following criteria for high quality SNP calling and filtering were chosen: (I) a minimum depth of 10× at SNP positions, (II) a min. relative depth of 10% at SNP positions, (III) a min. distance of 10 bp between SNPs, (IV) a min. SNP quality of 30, (V) a min. read mapping quality of 25, and (VI) a min. *Z*-score of 1.96. Site validation for each SNP position was performed. SNPs that fail the necessary requirements were excluded in the final analysis. Based on concatenated alignments of high quality SNPs, maximum likelihood trees were created using FastTree version 2.1.7 (Price et al., 2010).

The concatenated sequences derived from whole genomes were used for screening on virulence-associated genes using the BLASTN algorithm of the NCBI database (https://www.ncbi.nlm.nih.gov). The web-based tool was used with standard settings.

Multilocus Sequence Analysis (MLSA) of Housekeeping Genes

Bacterial strains were grown overnight and genomic DNA was extracted as described above. MLSA was performed on four protein-coding housekeeping genes making use of the *Vibrio* spp. MLSA website (https://pubmlst.org/vibrio/info/Vibrio_primers.pdf) developed by Keith Jolley and sited at the University of Oxford (Jolley and Maiden, 2010). The 25 µl PCR mixtures contained 1 × PCR buffer (2 mM $MgCl_2$), 0.2 mM of each dNTP, 0.2 µM of each primer, 1.5 U DreamTaq DNA Polymerase (Thermo Fisher Scientific Biosciences GmbH, St. Leon-Rot, Germany), and 1 µl of genomic DNA. For amplification of *gyrB*, the primers VigyrBF and VigyrBR were used. Amplification of *atpA* was carried out with the primers Vi_atpAdg_F and Vi_atpAdg_R. PCR reactions were performed using a Mastercycler ep gradient (Eppendorf AG, Hamburg, Germany). PCR products were purified using the MSB Spin PCRapace Kit according to the manufacturer's instructions (Stratec Molecular GmbH, Berlin, Germany) and sequenced (Eurofins Genomics GmbH, Ebersberg, Germany). The sequences were assembled and analyzed using the Lasergene software SeqMan Pro version 12.0 (DNASTAR Inc., Madison, WI, USA) and the software Accelrys Gene version 2.5 (Accelrys Inc., San Diego, CA, USA). Allele sequences including the *rpoB* sequences were concatenated in the order of loci *gyrB-pyrH-recA-atpA-rpoB* to generate a 2,893 bp concatemer for each strain. A phylogenetic tree was constructed with MEGA version 6.0 (Tamura et al., 2013) based on the alignment of the concatenated allele sequences using the neighbor-joining method with the Kimura 2-parameter model. Bootstrapping with 1,000 replications was performed to verify the robustness of the tree.

PCR Typing of Virulence-Associated Genes

Extraction of genomic DNA and PCR reactions were performed as described above with 5 ng of template DNA. PCR primers, target genes, and amplicon sizes are shown in Table S1. Primer sequences were derived from whole genome contigs of three *V. navarrensis* strains (Gladney et al., 2014) using the software Accelrys Gene version 2.5. Accession numbers of contigs are given in Table S1. The PCR running conditions were as follows: an initial denaturation step at 94°C for 4 min, 30 cycles of denaturation at 94°C for 15 s, primer annealing at 55°C for 30 s and extension at 72°C for 45 s, and a final extension step at 72°C for 7 min. PCR products were analyzed in agarose gels to determine the product lengths. Selected PCR products were sequenced for confirmation.

Accession Numbers

Nucleotide sequences were deposited in the European Nucleotide Archive (ENA) with the following accession numbers: sequences of partial *rpoB* gene accession numbers LT546547-LT546563 (*rpoB* sequences of three strains already in database, see **Figure 1**), sequences of partial *atpA* gene accession LT546564-LT546583, sequences of partial *gyrB* gene accession LT546584-LT546603, sequences of partial *pyrH* gene accession LT546604-LT546623, and sequences of partial *recA* gene accession LT546624-LT546643.

Genome sequences of *V. navarrensis* isolates have been deposited in GenBank at National Center for Biotechnology Information (NCBI) under the accession numbers MPKB00000000 to MPKU00000000 (see **Table 3**).

For *rpoB* phylogeny presented in **Figure 1**, *rpoB* sequences of non-*V. navarrensis* strains and *V. navarrensis* strains 2232, 08-2462, CIP 103381 (identical to ATCC 51183), 0053-83, VN-3125, and VN-3139 were obtained from GenBank at NCBI. Accession numbers are FN423814 (LMG 3772), FN423805 (ATCC 27562), LMYA01000047 (DAL-79040), MCJC01000044 (ATCC 14181), FN423808 (LMG 7891), FN423806 (LMG 11664), HG794494 (DSM 14383), CP014035 (ATCC 33809), FN423804 (LMG 7896), FN423803 (NCTC 4711), CP013317 (NCTC 5395), AFWH01000033 (CIP 102891), FN423816 (CCUG 16330), FN423810 (DSM 6904), FN423812 (LMG 10935), FN423802 (ATCC 17749), LVYF01000041 (VN-7501), JNUL02000012 (CFSAN007457), MVKN01000048 (VN-0293), FN423813 (DSM 507), FN423801 (CCUG 13625), EF064429 (9046-81), JMCH01000016 (2232), JMCI01000045 (08-2462), JMCG01000002 (ATCC 51183), JMCF01000001 (0053-83), KJ647757 (VN-3125), and KJ647770 (VN-3139).

RESULTS AND DISCUSSION

rpoB Phylogeny

Determination of partial *rpoB* sequences has proved a reliable method for species identification for bacteria of the family *Vibrionaceae* (Tarr et al., 2007; Adékambi et al., 2009; Dieckmann

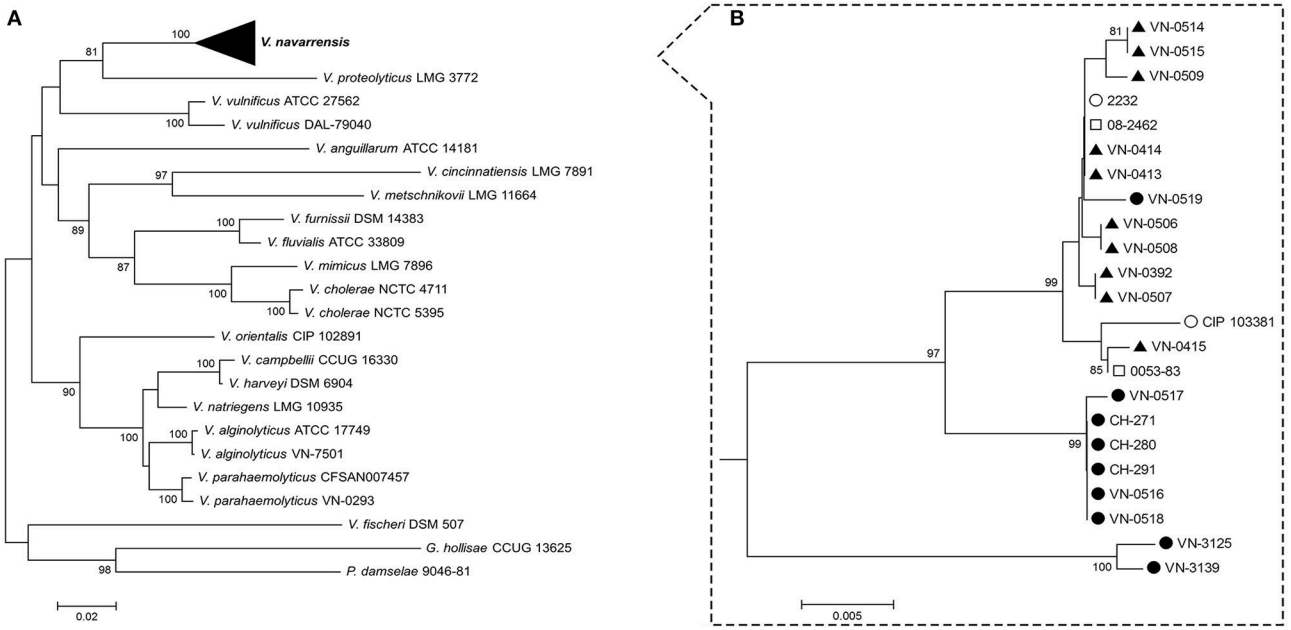

FIGURE 1 | Phylogenetic tree of different *Vibrionaceae* strains **(A)** and subtree of *V. navarrensis* strains **(B)** based on partial *rpoB* sequences (871 bp). The evolutionary history was inferred using the neighbor-joining method with Kimura 2-parameter distance model in MEGA version 6.0. Bootstrap values above 75% are shown next to the nodes ($n = 1{,}000$ replicates). Scale bars represent base substitutions per site. German veterinary (▲) and environmental (●), Spanish environmental (○), and human pathogenic *V. navarrensis* strains (□; from the U.S.) (Gladney and Tarr, 2014) form a distinct cluster within a number of different *Vibrionaceae* strains. *rpoB* sequences of non-*V. navarrensis* strains and *V. navarrensis* strains 2232, 08-2462, CIP 103381 (identical to ATCC 51183), 0053-83, VN-3125, and VN-3139 were obtained from GenBank at National Center for Biotechnology Information (NCBI). Accession numbers are given in Materials and Methods.

et al., 2010). The sequences of an 871 bp internal fragment of the coding sequence of the *rpoB* gene were identified for all isolates of this study. *rpoB* sequences of two Spanish environmental *V. navarrensis* strains [CIP 103381 (identical to ATCC 51183) and 2232] and two human pathogenic strains (0053-83 and 08-2462; Gladney et al., 2014) available in public databases were included for the construction of a phylogenetic tree (**Figure 1B**). All strains fell into a cluster that formed a distinct species among a number of different *Vibrio* species (**Figure 1**).

With the exception of two strains, the identity of the sequences of most *V. navarrensis* strains was greater than 98% in the sequenced region of 871 bp. In a previous study, we observed that in many *Vibrio* spp. the lowest sequence identity (determined by ClustalW) of this gene fragment was around 98% on species level (Dieckmann et al., 2010). The identity of the *rpoB* sequences of two *V. navarrensis* strains from seawater (VN-3125 and VN-3139) to the *rpoB* sequences of the remaining *V. navarrensis* strains was only ca. 96%. Only in three of the 40 polymorphic sites of the sequenced fragment, nonsynonymous substitutions leading to amino acid exchanges in the gene product were discovered. Two identical amino acid exchanges were observed only in the more distantly related strains VN-3125 and VN-3139.

The *rpoB* tree showed that the veterinary isolates from domestic animals clustered with two Spanish environmental strains [CIP 103381 (identical to ATCC 51183) and 2232] and two human pathogenic strains (Gladney et al., 2014; **Figure 1B**). Also one environmental strain, VN-0519 isolated from a blue mussel harvested from a mussel production area, fell into this cluster, while six environmental seawater isolates from Germany formed a separate subcluster.

Phenotypic Characteristics

All strains (**Table 1**) were phenotypically tested using a panel of standard biochemical reactions (**Table 2**). The biochemical properties were fairly homogenous with more than 90% of the strains showing the same result (only few variable reactions). Comparing to published results, biochemical characteristics were typical as described for *V. navarrensis* (Urdaci et al., 1991; Farmer and Janda, 2004; Jores et al., 2007; Gladney and Tarr, 2014; Farmer et al., 2015). The strains were negative for Voges-Proskauer test, arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase and were positive for sucrose fermentation and phenylalanine deaminase activity. The three strains of the biovar *pommerensis* (CH-271, CH-280, CH-291) were not studied in further details, as the biovar specific reactions were not part of our standard reaction panel (Jores et al., 2007).

Since the veterinary laboratory in Saxony, from which the strains were obtained, did not use molecular diagnostic techniques such as PCR and DNA sequencing, the isolates from animals had been phenotypically determined either as *Vibrio* sp. or as *V. vulnificus*. Misidentification by traditional methods happened, as phenotypic characteristics of *V. navarrensis* and *V. vulnificus* are very similar (Gladney and Tarr, 2014). The phenotypic characterization of all 20 strains was also done to

TABLE 2 | Phenotypic characterization of *V. navarrensis* strains.

Phenotypic test	No. of isolates (%)	
	+	−
Cytochrome oxidase	20 (100)	0 (0)
Nitrate reductase	20 (100)	0 (0)
Arginine dihydrolase	0 (0)	20 (100)
Lysine decarboxylase	0 (0)	20 (100)
Ornithine decarboxylase	0 (0)	20 (100)
Phenylalanine deaminase	18 (90)	2 (10)
Urease	0 (0)	20 (100)
Production of:		
Acetoin (Voges-Proskauer reaction)	0 (0)	20 (100)
H_2S	0 (0)	20 (100)
Indole	20 (100)	0 (0)
Oxidative acid production from:		
Adonitol	0 (0)	20 (100)
L-Arabinose	1 (5)	19 (95)
Cellobiose	19 (95)	1 (5)
Dulcitol	0 (0)	20 (100)
D-Glucose	20 (100)	0 (0)
myo-Inositol	0 (0)	20 (100)
Lactose	8 (40)	12 (60)
Maltose	20 (100)	0 (0)
D-Mannitol	20 (100)	0 (0)
D-Mannose	18 (90)	2 (10)
Melibiose	3 (15)	17 (85)
Raffinose	0 (0)	20 (100)
L-Rhamnose	2 (10)	18 (90)
Salicin	1 (5)	19 (95)
D-Sorbitol	0 (0)	20 (100)
L-Sorbose	0 (0)	20 (100)
Sucrose	20 (100)	0 (0)
Trehalose	20 (100)	0 (0)
D-Xylose	0 (0)	20 (100)
Degradation of:		
Esculin	4 (20)	16 (80)
Citrate (Simmons citrate reaction)	5 (25)	15 (75)
Growth in 1% peptone water		
+ 0% NaCl	0 (0)	20 (100)
+ 3% NaCl	20 (100)	0 (0)
+ 8% NaCl	0 (0)	20 (100)
+ 10% NaCl	0 (0)	20 (100)
Susceptibility to O/129 (10 μg/150 μg)	20 (100)	0 (0)
Hemolysis of:		
Human erythrocytes	19 (95)	1 (5)
Sheep erythrocytes	19 (95)	1 (5)

find out if distinct biochemical properties could be correlated to the source of the strains. The results of these investigations, however, did not reveal significant differences between the strains (see **Table 2**).

As *V. navarrensis* strains show hemolytic activity (Jores et al., 2003, 2007), we investigated all strains on agar plates containing sheep erythrocytes or human erythrocytes. Most strains did not show hemolytic activity within 24 h. However, after incubation for up to 72 h all strains but one were hemolytic against human erythrocytes. On sheep blood agar, six strains (two environmental and four veterinary strains) did not show hemolysis at first. However, after modification of the assay medium and repeated streaking on sheep blood agar also these strains (except one) displayed hemolysis zones. With sheep and human erythrocytes, hemolysis zones surrounding colonies were clear indicating a ß-hemolysis with complete degradation of hemoglobin (Zhang and Austin, 2005).

Whole Genome Sequencing

The results of genomes of ten German isolates (five veterinary and five environmental strains) are shown in **Table 3**. The genome sizes range from 4.14 to 4.90 Mbp and the GC contents of the genomes vary between 47.5 and 48.1%. The predicted number of coding sequences range from 3,559 to 4,247. The published genomes of two Spanish strains (ATCC 51183 and 2232) and two human pathogenic strains (0053-83 and 08-2462) vary between 4.2 and 4.4 Mbp (Gladney and Tarr, 2014). It was noted that the genomes of all five veterinary strains are also in this range, while the genomes of the five isolates from marine environments including the mussel isolate are larger (4.6–4.9 Mbp, **Table 3**). It is possible that the greater genome sizes of marine strains reflect a wider range of metabolic capabilities compared to the veterinary and human pathogenic strains. Some bacteria, especially those adapted to specific niches (e.g., pathogenic strains adapted to specific host environments) can lose metabolic capabilities leading to a reduction in genome size (Raskin et al., 2006). Further studies may address this question.

As bacteriophages are involved in horizontal gene transfer, the WGS data were analyzed with Phage Search Tool (Zhou et al., 2011). The search for phage sequences revealed the occurrence of several prophage sequences as expected (Table S2). Phages are one of the major forces driving horizontal gene transfer (Raskin et al., 2006). Most prophage sequences are related to giant viruses and of lower significance. In some strains, however, prophage sequences possibly encoding intact phages were detected (Enterobacteria phages HK630 and HK629, *Vibrio* phages martha 12B12 and VPUSM 8). However, no information about the phages except the genome sequences are available (Table S2). In two marine strains (VN-0516 and VN-3125), a possibly intact phage was found that is related to *Vibrio* phage VCY Φ. This phage is a small filamentous phage (approximately 7.1 kbp) and was found in association with environmental *V. cholerae* strains in ponds (Xue et al., 2012). Bioinformatics indicated the presence of a plasmid in only one strain (VN-3125). A small region of 638 bp was identified possessing high identity (>99%) to a replication region present in several *Enterobacteriaceae* plasmids [e.g., plasmid p8401 in *E. coli* (accession CP012198)]. In the four published *V. navarrensis* genomes, no plasmid sequences were reported so far.

TABLE 3 | Results of the whole genome sequence analysis of veterinary and environmental *V. navarrensis* strains.

Feature	CH-280	VN-0392	VN-0415	VN-0507	VN-0509	VN-0514	VN-0516	VN-0518	VN-0519	VN-3125
Genome size (bp)	4,899,705	4,287,414	4,138,545	4,271,170	4,356,049	4,278,964	4,605,884	4,684,360	4,788,163	4,765,427
GC content (%)	47.45	48.02	48.08	48.04	48.09	48.07	47.71	47.75	47.94	47.57
Genes (total)*	4,440	3,935	3,784	3,857	3,923	3,909	4,162	4,280	4,331	4,338
CDS (total)**	4,319	3,811	3,661	3,731	3,781	3,779	4,058	4,136	4,201	4,233
CDS (coding)	4,247	3,737	3,559	3,658	3,703	3,693	4,005	4,098	4,149	4,186
RNA genes (total)***	121	124	123	126	142	130	104	104	130	105
rRNAs (5S, 16S, 32S)***	7, 8, 7	9, 11, 10	5, 9, 7	7, 8, 7	8, 12, 11	7, 8, 11	4, 4, 4	5, 9, 1	7, 8, 9	8, 7, 7
tRNAs	95	90	98	100	107	100	88	85	101	79
ncRNAs	4	4	4	4	4	4	4	4	5	4
Pseudogenes (total)	72	74	102	73	78	86	53	38	52	47
CRISPR Arrays	2	3	3	1	1	1	1	0	1	0
Predicted prophages (no.)	7	3	7	7	2	9	1	2	4	4
intact	n.d.	1	n.d.	n.d.	1	n.d.	1	n.d.	1	3
incomplete	6	1	7	7	1	9	n.d.	1	2	1
questionable	1	1	n.d.	n.d.	n.d.	n.d.	n.d.	1	1	n.d.
Plasmids	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	ColRNAI, 88.39%
GenBank accession										
Bioproject	PRJNA353389	PRJNA353302	PRJNA353299	PRJNA353297	PRJNA353295	PRJNA353294	PRJNA353292	PRJNA353290	PRJNA353289	PRJNA353288
Biosample	SAMN06014880	SAMN06013691	SAMN06013689	SAMN06013684	SAMN06013686	SAMN06013681	SAMN06013683	SAMN06013678	SAMN06013679	SAMN06013680
Accession	MPKT00000000	MPKB00000000	MPKE00000000	MPKG00000000	MPKI00000000	MPKJ00000000	MPKL00000000	MPKN00000000	MPKO00000000	MPKP00000000

**Nucleotide sequences from the start codon (ATG) to the stop codon.*

***Nucleotide sequence that is translated to form proteins.*

****Including partial sequences of the respective element.*

n.d., not detected.

SNP Phylogeny of Whole Genome Sequences

For an SNP analysis, additional published WGS data of two human pathogenic strains (0053-83 and 08-2462) and two environmental Spanish strains (ATCC 51183 and 2232) were included. The human isolates were from human specimen isolated in the U.S. and the environmental strains were Spanish isolates from sewage (Urdaci et al., 1991). To identify SNPs, all input sequences were mapped to the *V. navarrensis* 0053-83 genome as reference (JMCF01000000) and screened for relevant nucleotide variations (Kaas et al., 2014). In total, the concatenated contigs used for the SNP analysis comprised approx. 3.688 Mbp and the number of SNPs between the strains varied between 10,000 to 63,000 (Figure S1). Using the concatenated alignments of high quality SNPs, maximum likelihood trees were created using FastTree 2 (Price et al., 2010; **Figure 2**). Based on the length of the branches, the five veterinary strains and the two Spanish environmental strains differ but are related. The two human isolates (0053-83, 08-2462) are closer related to each other (the SNP difference is around 16,000 between the two strains) but are more distant to the veterinary strains and the Spanish strains (approximately 30,000 SNPs). All marine isolates from Germany with the exception of the mussel strain VN-0519 are clearly separated from the other strains, but differ also from each other (SNP differences between 30,000 and 63,000). Only the seawater strains VN-0516 and CH-280 are closer related (difference approximately 10,000 SNPs) which indicates that VN-0516 may belong to the subspecies *V. navarrensis* biotype *pommerensis*.

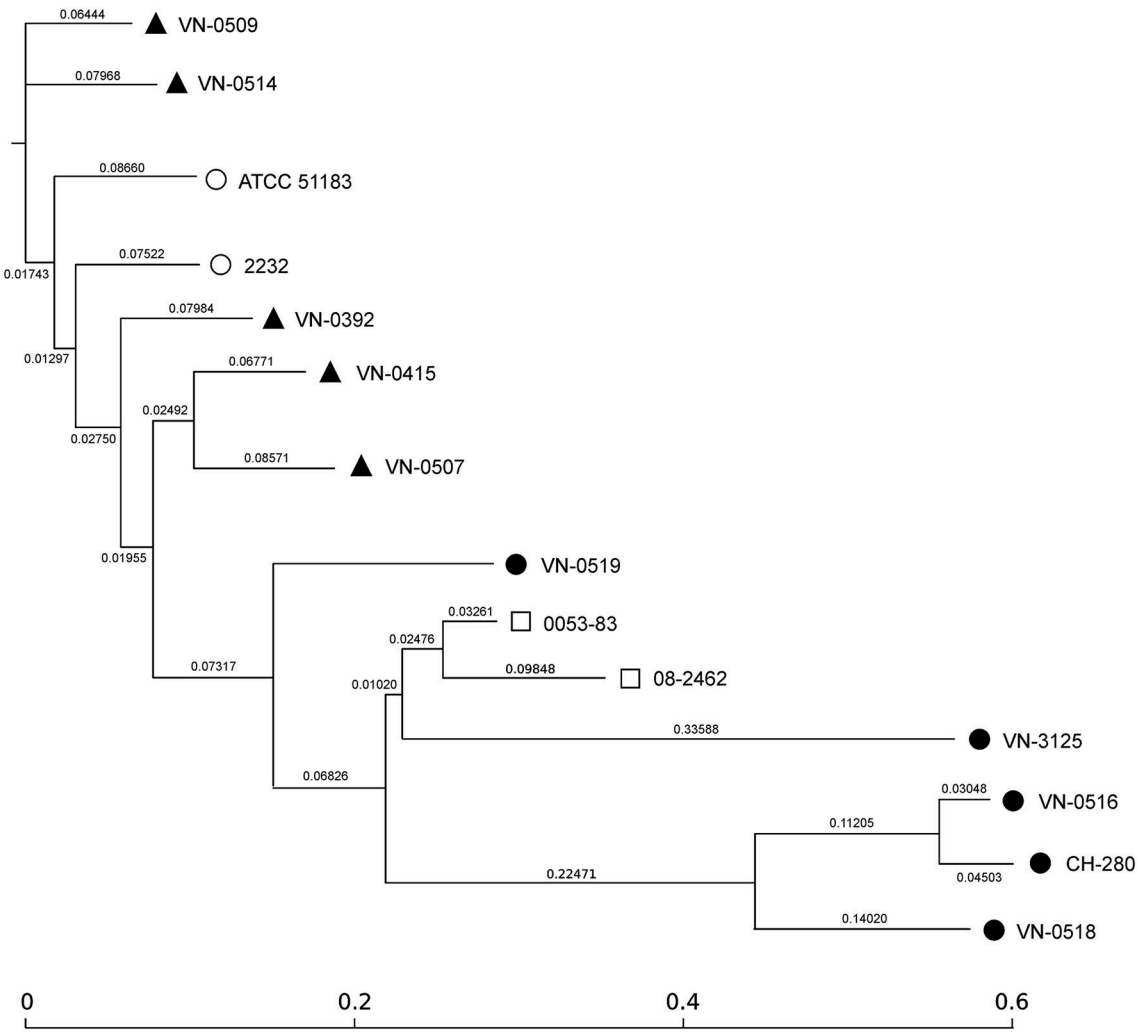

FIGURE 2 | SNP-based phylogeny tree of German veterinary (▲) and environmental (●) *V. navarrensis* isolates, two human (□) isolates from the U.S., and two Spanish environmental (○) strains [CIP 103381 (identical to ATCC 51183) and 2232 (see text)]. SNP-tree was conducted by using CSI Phylogeny 1.4 under default settings. Single nucleotide polymorphisms (SNPs) were called by mapping to the *V. navarrensis* 0053-83 genome as reference (JMCF01000000). Criteria for high quality SNP calling and filtering are described in Material and Methods. Based on concatenated alignments of high quality SNPs, maximum likelihood trees were created using FastTree version 2.1.7. Scale bar represents the number of nucleotide substitutions per site and numbers indicate branch length.

The SNP analysis revealed clearly that the seawater strains are distant to the remaining *V. navarrensis* strains. In *E. coli*, SNP analysis of the core genome consisting of 1,429 genes revealed 128,214 variable sites for a pathovar (Von Mentzer et al., 2014) and the average SNP differences between two related clades of isolates from different hosts were below 100 within a clade and below 1,800 between two clades (Schaufler et al., 2016). The SNP calculation for the *V. navarrensis* strains (without the seawater strains) vary from 18,000 to 32,000 variable sites. Thus, a close relatedness of all strains cannot be deduced from SNP data (Figure S1).

Phylogenetic Relationship from Multilocus Sequence Analysis of Housekeeping Genes

We applied the *Vibrio*-MLSA scheme available on the PubMLST website and included sequences from published WGS data of the four *V. navarrensis* strains (Gladney et al., 2014) into the phylogenetic analysis. To increase the depth of the analysis, the *rpoB* sequences were added and the sequences of the concatemer were arranged in the order *gyrB-pyrH-recA-atpA-rpoB*. Concatemers of sequences of housekeeping genes were created, as comparison of housekeeping genes is used for infra-species resolution and determination of the clonality of strains (Glaeser and Kämpfer, 2015).

The phylogenetic analysis of the concatemers displayed one subcluster containing the veterinary isolates, the mussel isolate, and the reference strain ATCC 51183 (identical to CIP 103381; **Figure 3**). The sewage strain 2232 and the two human isolates were also placed in this cluster. It should be noted that the two Spanish environmental strains were isolated from sewage in towns of the Spanish province Navarra away from the coast (Urdaci et al., 1991). The concatemers of the German seawater isolates split into three branches and were clearly distant to the subcluster formed by the remaining strains. The MLSA tree indicates a stronger relationship between the veterinary strains, the Spanish strains and the human pathogenic strains than the SNP phylogenetic tree. This discrepancy is likely to be explained by the fact that the housekeeping genes are encoding essential

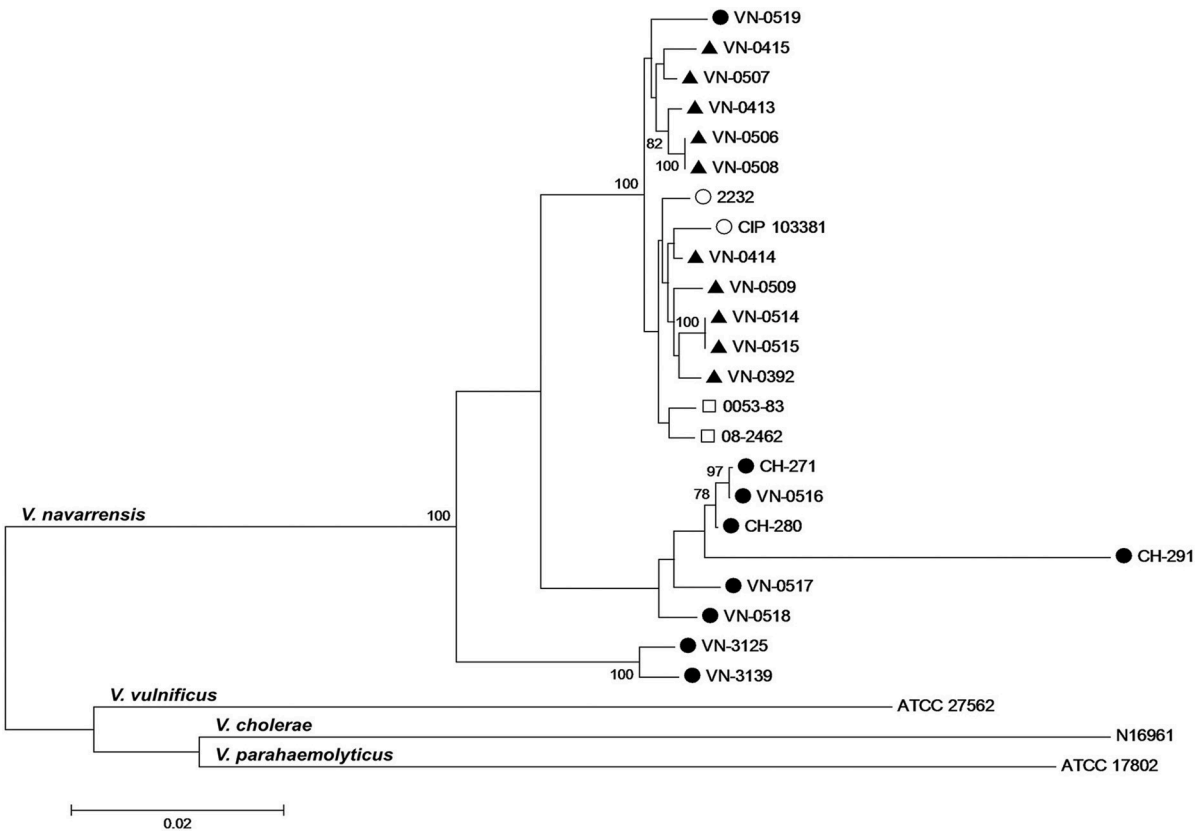

FIGURE 3 | Phylogenetic relationship of German veterinary (▲) and environmental (●) *V. navarrensis* isolates, human (□) isolates from the U.S., and Spanish environmental (○) strains based on concatenated sequences of five protein-coding housekeeping genes (*gyrB-pyrH-recA-atpA-rpoB*; 2,893 bp). Sequences of *V. navarrensis* 0053-83, 08-2462, CIP 103381 (identical to ATCC 51183), and 2232 were obtained from previous studies (Gladney and Tarr, 2014; Gladney et al., 2014). Sequences of *V. vulnificus* ATCC 27562, *V. cholerae* N16961, and *V. parahaemolyticus* ATCC 17802 from *Vibrio* spp. MLSA website (http://pubmlst.org/vibrio/) and GenBank at NCBI, respectively, were included for comparison. The evolutionary history was inferred using the neighbor-joining method with Kimura 2-parameter distance model in MEGA version 6.0. Bootstrap values above 75% are shown next to the nodes ($n = 1{,}000$ replicates). Scale bar represents 0.02 base substitutions per site.

cellular functions and are therefore more conserved and evolve relatively slowly (Hanage et al., 2006).

The observation that the environmental Spanish strains are related to the veterinary strains is a remarkable observation. The Spanish strains were isolated from low salinity aquatic environments away from the coast (Urdaci et al., 1991) in rivers and sewage. As bacteria of the genus *Vibrio* are regarded as environmental aquatic bacteria, it seems possible that *V. navarrensis* may also occur in freshwater in regions of Germany and that the veterinary isolates were ingested by the animals through uptake of surface water. This hypothesis is supported by the results of the phylogenetic analyses which show that the veterinary strains are distinctly different from the German seawater strains. Unfortunately, there is no knowledge concerning a possible origin of the veterinary strains as respective investigations were not undertaken. The phylogenetic studies also indicate that the human strains from the U.S. are more related to the veterinary ones. However, no further information concerning the American strains is available.

The mussel strain (VN-0519) is the only strain from a marine environment that shows a stronger relationship to the veterinary and human isolates. It should be noted that knowledge on natural habitats of *V. navarrensis* are fragmentary, as only few publications on this species are available. In one report from Thailand, partial sequences of 16S rDNA of uncultured bacteria recovered from the gut of marine shrimps were 99% identical to 16S rDNA sequences of *V. navarrensis* (Rungrassamee et al., 2013). In another recent paper, the occurrence of *V. navarrensis* in larval midgut of the date palm root borer *Oryctes agamemnon* in Saudi-Arabia (El-Sayed and Ibrahim, 2015) was detected based on sequence analysis of the gut microbiome. According to this paper, the endosymbiotic bacterial community was dominated by *Vibrionaceae* revealing that these bacteria can be prevalent in an insect environment.

Presence/Absence of Virulence-Associated Factors

To find out if environmental *V. navarrensis* strains can be distinguished from the veterinary strains and human pathogenic strains, the presence or absence of genes coding for virulence-associated factors were investigated by BLASTN searches of the WGS data and by PCR genotyping of the 20 available strains. A number of putative candidate genes were selected based on the published genomes of the reference strain ATCC 51183 (identical to CIP 103381) and the two human *V. navarrensis* strains 0053-83 and 08-2462. PCR primers were designed for a number of genes using the WGS data of these strains (Table S1). The genes targeted were coding for potential virulence factors with cytolytic or hemolytic activities and parts of secretion systems (Gladney et al., 2014). **Table 4** summarizes the results of these investigations for all *V. navarrensis* strains including the four published strains.

For genes encoding hemolytic and cytolytic proteins, we chose homologs of *vvhA*, *tlh*, δ-*vph*, *hlyIII*, and *osmY*. The *vvhA* gene of *V. vulnificus* encodes a potent cytolytic hemolysin whose role in pathogenicity has been under debate (Jones and Oliver, 2009; Lee et al., 2013). The gene is present in clinical and environmental *V. vulnificus* strains and is used for species identification (Campbell and Wright, 2003). Similarly, a *tlh* homolog encoding a putative thermolabile hemolysin is found in many *Vibrio* species. Its role in pathogenicity is unclear (Zhang and Austin, 2005) and it is used in *V. parahaemolyticus* for species identification (Jones et al., 2014). WGS data indicated the presence of the two genes in all genomes and PCR assays for gene homologs of *vvhA* and *tlh* were positive in the 20 *V. navarrensis* strains of this study, which indicates that these genes might also be suitable for identification of this species (**Table 4**).

A gene encoding a putative hemolysin III family protein (HlyIII) with a size of 214 amino acids is annotated in the WGS data of all *V. navarrensis* strains (except VN-0507). Due to nucleotide sequence variations, PCR amplicons of this gene were not obtained for all strains (data not shown). In case of *V. vulnificus*, a homolog of the HlyIII protein was investigated in more detail (Chen et al., 2004). As a *hlyIII* mutant of *V. vulnificus* exhibited attenuated virulence in a mouse model compared with the wild-type strain, a role of HlyIII in virulence was suggested (Chen et al., 2004; Zhang and Austin, 2005). Another thermostable hemolysin, δ-VPH, with unclear role in pathogenicity has been found in *V. parahaemolyticus* and *V. cholerae* (Zhang and Austin, 2005). In contrast to *tlh*, *vvhA*, and *hlyIII*, the putative δ-*vph* gene was only detected in strains from marine environments (seawater strains and blue mussel strain) and in the two human pathogenic strains (WGS data). The *osmY* gene homolog encoding a putative hemolysin (accession KGK22069) with a domain for attachment to phospholipid membranes was present in all strains (**Table 4**).

WGS data of all strains showed the presence of a *hlyD* gene encoding a hemolysin D protein and an *rtx* gene encoding a repeats-in-toxin protein. HlyD proteins are involved in transport of hemolysins through the bacterial inner membrane (Pimenta et al., 2005; Linhartová et al., 2010), while secreted RTX proteins mostly exhibit pore-forming activity visible as hemolytic halo surrounding bacterial colonies on blood agar (Linhartová et al., 2010).

Jores et al. cloned a 15.6 kbp DNA fragment of *V. navarrensis* biotype *pommerensis* CH-291 into the plasmid pVH that upon introduction into *E. coli* strain DH5α conferred hemolytic properties. DNA hybridization experiments of the whole fragment were positive only with strains of the biotype *pommerensis* and were suggested to be specific for the biotype. The hemolytic properties were found on two neighboring regions of the 15.6 kbp fragment, each containing more than one open reading frame (ORF) (Jores et al., 2003). ORF12, the largest ORF conferring hemolytic properties, was only present in four strains (CH-271, CH-280, CH-291, VN-0516; **Table 4**). The significance of this region for identification of a subpopulation of *V. navarrensis* strains requires the study of more strains.

Type IV pilins of Gram-negative bacteria play various roles in pathogenicity (Giltner et al., 2012). In toxigenic *V. cholerae*, a type IV pilus is a major virulence factor that functions as an essential colonization factor and acts as cholera toxin phage receptor (Karaolis et al., 1998; Rivera et al., 2001). Two genes, *pilW* and *pilV*, coding for type IV pilus assembly or pilus biosynthesis proteins were present in most strains. The *pilV* gene was absent

TABLE 4 | Presence/absence of virulence-associated traits in veterinary, human, and environmental *V. navarrensis* isolates based on WGS data.

Strain	Source code	Virulence-associated genotypic traits*													
		cps	T6SS *DUF877*	T6SS *DUF770*	T6SS *vasD*	*pilV*	*pilW*	*tlh*	*osmY*	*vvhA*	δ-*vph*	*hlyD*	*hlyIII***	*rtx***	ORF12
VN-0392	vet	■	■	■	■	■	■	■	■	■		■	■	■	
VN-0413	vet	■	■	■	■		■	■	■	■		■	■	■	
VN-0414	vet	■	■	■	■	■	■	■	■	■		■	■	■	
VN-0415	vet	■	■	■	■	■	■	■	■	■		■	■	■	
VN-0506	vet	■	■	■	■	■	■	■	■	■		■	■	■	
VN-0507	vet	■	■	■	■	■	■	■	■	■		■		■	
VN-0508	vet	■	■	■	■	■	■	■	■	■		■	■	■	
VN-0509	vet	■	■	■	■	■	■	■	■	■		■	■	■	
VN-0514	vet	■	■	■	■	■	■	■	■	■		■	■	■	
VN-0515	vet	■	■	■	■	■	■	■	■	■		■	■	■	
08-2462**	hum	■				■	■	■	■	■	■	■	■	■	
0053-83**	hum	■				■	■	■	■	■	■	■	■	■	
CIP 103381***	env-Sp	■	■	■	■	■	■	■	■	■		■	■	■	
2232**	env-Sp	■	■	■	■		■	■	■	■		■	■	■	
CH-271	env-G	■				■	■	■	■	■	■	■	■	■	■
CH-280	env-G	■					■	■	■	■	■	■	■	■	■
CH-291	env-G	■				■	■	■	■	■	■	■	■	■	■
VN-0516	env-G	■				■	■	■	■	■	■	■	■	■	■
VN-0517	env-G	■				■	■	■	■	■	■	■	■	■	
VN-0518	env-G	■				■	■	■	■	■	■	■	■	■	
VN-0519	env-G	■	■	■	■	■	■	■	■	■	■	■	■	■	
VN-3125	env-G	■	■	■	■		■	■	■	■	■	■	■	■	
VN-3139	env-G	■	■	■	■	■	■	■	■	■	■	■	■	■	

vet, veterinary; hum, human; env-Sp, environmental-Spain; env-G, environmental-Germany.

**In WGS analysis, gene sequences of V. navarrensis 08-2462 (cps, osmY, vvhA, δ-vph), 0053-83 (pilV, pilW, tlh, hlyD, hlyIII, rtx), CIP 103381 (T6SS vasD), and 2232 (T6SS DUF877, T6SS DUF770) as well as ORF12 of CH-291 were used as reference sequences. Strains showing 90–100% sequence similarity to the specific reference sequence were defined as positive for the respective virulence-associated trait. WGS data were confirmed by PCR assays. VN-0392, VN-0507, and VN-0519 were PCR-negative for cps, T6SS DUF770 and pilW, respectively. WGS data showed primer mismatches.*

***No verification of the WGS data by PCR assays.*

****Identical to ATCC 51183.*

in three out of the 20 strains (WGS and PCR), whereas the *pilW* gene was found in all strains, although in one strain (VN-0519), a PCR to confirm the gene failed. A gene coding for a putative protein of capsule biosynthesis (designated as *cps*) was detected in all strains (**Table 4**).

Type VI secretion systems (T6SS) have attracted attention, as they play important roles in virulence of a number of Gram-negative bacteria by translocating effector proteins into eukaryotic cells. Recently, T6SS have also been shown to transport proteins into prokaryotic cells showing bactericidal activity against competitors (Ho et al., 2014). Three genes of putative T6SS proteins were tested for presence in *V. navarrensis* strains. Two of the genes encode proteins associated with the baseplate containing domains of unknown functions (T6SS DUF877 and T6SS DUF770) and one gene encodes a VasD protein homolog which is a lipoprotein tethered to the outer membrane. Interestingly, six of eight seawater strains were negative for these genes indicating that the T6SS is not present in these environmental strains. In contrast, the mussel strain as well as the reference strain CIP 103381 from sewage and all veterinary strains harbor the T6SS (**Table 4**). The WGS analysis confirmed the PCR results. WGS data of the two human pathogenic strains 0053-83 and 08-2462 revealed the absence of the three selected genes and indicate the lack of the T6SS in these strains.

In summary, no clear discrimination based on virulence-associated factors was observed between the strains, as most of the investigated genes were present in all strains. Some

genes encoding virulence-associated traits may be useful for further analysis of strains from different origins. Candidate genes identified in this study are the δ-*vph* gene and genes encoding components of the T6SS and the hemolytic activity encoding region of biotype *pommerensis* strains. However, evidence if some of these genes contribute to a pathogenic potential do require additional research. It is feasible that discrimination of environmental and potentially pathogenic strains requires the identification of allelic variants of specific genes as it is the case for clinical strains of *V. vulnificus* (Jones and Oliver, 2009).

CONCLUSION

This study was initiated by a recent publication about *V. navarrensis* strains recovered from human specimens. The strains originated from diverse human sources (blood, wound, ear, stool) suggesting that this species is a human pathogen. The veterinary isolates of this study were isolated from animals intended for food production in farms of the German state Saxony that has no border to marine environments. The strains were recovered after abortions from placentas and some strains were isolated directly from inner organs of the aborted fetuses. A pathogenic potential of these isolates seems likely. However, it cannot be excluded that the strains were purely commensals, as no further investigations regarding pathogenicity were performed. The animal source of the strains is unusual, as *Vibrio* bacteria are mostly found in marine environments and are commonly associated with marine organisms. The uptake through feed of marine origin (seafeed) was discussed; however, no satisfying explanation for the occurrence of *Vibrio* strains in domestic animals was found. Cases of human vibriosis result either through contact to seawater or by uptake of contaminated seafood. The occurrence of possibly pathogenic *Vibrio* strains in mammalian hosts intended for food production is of great interest, as it could indicate a so far unrecognized source of *Vibrio* infections.

The isolation of *V. navarrensis* from domestic animals after miscarriages and from diseased humans suggests a pathogenic potential of these bacteria and could mean that this species is a so far unrealized zoonotic agent. The "One Health" concept acknowledges that human, animal, and environmental health are linked. Further research is necessary to identify reservoirs, sources, and ways of transmission of this species to determine a possible role as zoonotic agent.

AUTHOR CONTRIBUTIONS

KS, NB, KT, and ES designed the study. KS, CK, and NB performed the experiments. KS, CK, NB, JH, KT, and ES analyzed the data. KS, JH, and ES prepared the tables and figures, wrote the manuscript. All authors edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01717/full#supplementary-material

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3 General Discussion

3.1 Background

Since the late 1980s, several non-cholera *Vibrio* infections have been reported from European countries of the Mediterranean region – some of them with severe clinical course of disease and fatal outcome (Marne and Aznar, 1988; Reina *et al*., 1995; Tsakris *et al*., 1995; Farina *et al*., 1999; Fernández *et al*., 2000; Torres *et al*., 2002; Ottaviani *et al*., 2008; Thodis *et al*., 2009; Tena *et al*., 2010). Further infections have been reported from coastal countries of the North Sea and Baltic Sea regions such as Norway (Hardardottir *et al*., 1994), Sweden (Melhus *et al*., 1995; Andersson and Ekdahl, 2006), Finland (Lukinmaa *et al*., 2006); Denmark (Bock *et al*., 1994; Dalsgaard *et al*., 1996), and the Netherlands (Schets *et al*., 2006). Against this background, two research programs focusing on *Vibrio* research were established in Germany in 2009 and 2010: KLIWAS[4] and VibrioNet[5]. In the following years, systematic studies on the occurrence of *Vibrio* bacteria in German coastal waters were performed, which confirmed that the three most important human pathogenic *Vibrio* species *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* (non-O1, non-O139) are indigenous to the German North Sea and Baltic Sea regions (Böer *et al*., 2013; Brennholt *et al*., 2014). The projects not only provided valuable insights into spatial distribution patterns of potentially human pathogenic *Vibrio* species along the German coastline but also contributed to the successive establishment of an extensive strain collection of environmental isolates at the *Vibrio* consiliary laboratory. With the publication of first data on the characterization of clinical non-O1, non-O139 *V. cholerae* from German patients (Schirmeister *et al*., 2014a) and two more human cases of disease in 2016 and 2017 (Schwartz *et al*., 2019), *V. cholerae* non-O1, non-O139 bacteria came into focus. So far, in Germany, the number of human infections is still relatively low (Huehn *et al*., 2014). Between 1995 and 2017, a total of ten non-O1, non-O139 *V. cholerae* isolates were found that were associated with primarily extraintestinal infections after exposure to German coastal waters (Schirmeister *et al*., 2014a; Schwartz *et al*., 2019). However, given rising sea surface temperatures due to climate change, the associated increase in *Vibrio* numbers and the popularity of German coastal regions as a travel destination, an increased exposure to *Vibrio* bacteria, including non-O1, non-O139 *V. cholerae*, must be assumed for future years. In order to permit a first risk assessment for

[4] https://www.kliwas.de
[5] http://www.vibrionet.de

non-O1, non-O139 *V. cholerae* strains from German coastal waters, comparative MLST and virulence gene profile analyses of *V. cholerae* isolates recovered from environmental and clinical specimens were performed within the framework of this doctoral project.

During a survey on the distribution of *V. cholerae* bacteria in aquatic environments of the Spanish province Navarra, a new *Vibrio* species was discovered in sewage and river water: *Vibrio navarrensis* (Urdaci *et al*., 1991). Later, some strains from seawater of the Spanish Mediterranean region (Macián *et al*., 2000), the German Baltic Sea coast (Jores *et al*., 2003b; Jores *et al*., 2007) and the German North Sea (Schwartz *et al*., 2017) were reported. In 2014, the Centers for Disease Control and Prevention (CDC) published results on the characterization of *V. navarrensis* isolates associated with human illness (Gladney and Tarr, 2014). Some time ago, the *Vibrio* consiliary laboratory received veterinary *V. navarrensis* strains isolated from livestock in Germany. The isolates had been found in aborted fetuses and/or placentas after miscarriages, which are an unusual source for the primarily aquatic bacterial genus *Vibrio*. For identification of new potential environmental reservoirs in Germany and against the background of a possible zoonotic potential, comparative phylogenetic and virulence gene profile analyses of *V. navarrensis* isolates from environmental, veterinary, and human clinical specimens were performed in this work. Sections 3.2 to 3.4 summarize the major aspects and findings of the publications that are part of this cumulative dissertation and present a general discussion.

3.2 Characterization of Environmental *Vibrio cholerae* Isolates from Germany by Reference to Human Clinical Isolates

As a basis for a first risk assessment of non-O1, non-O139 *V. cholerae* strains from German coastal waters, a total of 100 environmental isolates including 50 isolates from the North Sea and 50 isolates from the Baltic Sea, were selected. All environmental isolates originated from the *Vibrio* spp. strain collection that was established at the *Vibrio* consiliary laboratory[6] within the framework of the research programs KLIWAS[7] and VibrioNet[8]. To ensure a broad spectrum of environmental strains, available non-O1, non-O139 *V. cholerae* isolates provided by KLIWAS and VibrioNet partner institutions were compared regarding sampling date, local origin, and sample type. Strains that were probably similar to each other were excluded from this study. Given the coordinated targeted sampling at registered bathing sites along the North

[6] https://www.bfr.bund.de/de/konsiliarlabor_fuer_vibrionen-245015.html
[7] https://www.kliwas.de
[8] http://www.vibrionet.de

Sea and Baltic Sea coastline as well as within important local estuaries over a six-year period (2009-2014), the environmental isolates analyzed in this work should provide a representative cross section for non-O1, non-O139 *V. cholerae* from German coastal waters relevant for public health. For comparison, a total of ten clinical isolates were included that were recovered from German patients who had contracted non-O1, non-O139 *V. cholerae* infections after exposure to German coastal waters between 1995 and 2017. Until the start of this study in late 2017, no systematic monitoring for *V. cholerae* infections had been carried out in Germany. According to the German Infection Protection Act (IfSG §§ 6,7 in the version applicable on July 25, 2017[9,10]), there was a legal obligation to inform public health authorities only in cases of O1/O139 *V. cholerae* infections (cholera). In contrast, non-cholera *Vibrio* infections including non-O1, non-O139 *V. cholerae* infections were not notifiable. With the aim of creating a solid basis for subsequent analyses, all available German clinical non-O1, non-O139 *V. cholerae* strains (Schirmeister *et al*., 2014a; Schwartz *et al*., 2019) were included in this study.

To characterize genetic relationships of the environmental isolates and clinical isolates, multilocus sequence typing (MLST) was performed on seven protein-coding housekeeping genes making use of the non-O1, non-O139 *V. cholerae* MLST scheme developed by Octavia *et al*. (2013). This scheme is well established up to bioinformatic level and has proven to be a reliable tool for sequence-based typing of non-O1, non-O139 *V. cholerae* isolates in previous studies (Luo *et al*., 2013; Li *et al*., 2014; Esteves *et al*., 2015; Siriphap *et al*., 2017; Jiang *et al*., 2018). Given the unambiguity, the high reproducibility and the easy portability of sequence data as well as the availability of web-based curated databases, MLST is an attractive method for characterizing and comparing isolates of clinically relevant bacterial species within the framework of public health research both at regional and global level (Sullivan *et al*., 2005; Maiden, 2006; Maiden *et al*., 2013). With a total of 74 STs (including 71 new STs), a considerable heterogeneity of sequence types was found in MLST analysis of the German non-O1, non-O139 *V. cholerae* isolates. Among the 100 environmental *V. cholerae* isolates from German coastal waters examined in this study, 66 different STs were identified indicating a high genetic diversity. The majority of STs were unique STs found only once and in one isolate. A few STs were represented by two or more isolates, with only one ST (ST486) showing occurrence in both investigation areas, the North Sea and Baltic Sea regions. The high genetic diversity of non-O1, non-O139 *V. cholerae* isolates from German

[9] https://www.buzer.de/gesetz/2148/l.htm

[10] https://www.rki.de/DE/Content/Infekt/IfSG/Meldepflichtige_Krankheiten/Meldepflichtige_Krankheiten_Erreger.html

coastal waters is in line with previous studies in which environmental isolates from other geographical regions were investigated by MLST (Li *et al*., 2014; Esteves *et al*., 2015; Pretzer *et al*., 2017; Siriphap *et al*., 2017). Interestingly, regional subdivision of the German environmental isolates with subsequent calculation of ST/strain ratio and Simpson's Index of Diversity revealed a higher genetic diversity for non-O1, non-O139 *V. cholerae* isolates from the Baltic Sea compared to isolates of the North Sea. This could result from the different salinity of both seas. Similar observations were made by Esteves *et al*. (2015) who studied non-O1, non-O139 *V. cholerae* populations in French Mediterranean coastal lagoons and reported a higher MLST diversity under low salinity conditions. Among the ten clinical *V. cholerae* isolates from German patients included in this study, eight different STs were identified indicating a high genetic diversity between these isolates as well. MLST sequence types associated with clinical strains were not found in the environmental isolates. However, some single-locus variants (SLVs), double-locus variants (DLVs), and triple-locus variants (TLVs) were identified comprising both clinical and environmental isolates. The only partial overlap between MLST allelic profiles of a few non-O1, non-O139 *V. cholerae* isolates from German coastal waters and clinical strains raised the question of the genetic makeup concerning virulence factors.

To investigate the pathogenicity potential of the environmental isolates, PCR-based typing of virulence genes was performed making use of a *V. cholerae*-specific typing scheme that had already been applied for the characterization of clinical non-O1, non-O139 *V. cholerae* strains (Schirmeister *et al*., 2014a). Analysis of PCR genotyping revealed the absence of the cholera toxin gene (*ctxA*) and the toxin-coregulated pilus gene (*tcpA*) in all 100 environmental isolates, which is in line with previous studies conducted in northern temperate waters (Collin and Rehnstam-Holm, 2011; Schuster *et al*., 2011; Böer *et al*., 2013; Brennholt *et al*., 2014). While the major virulence genes of pandemic O1/O139 strains were lacking, genes encoding other virulence-associated factors such as the virulence regulator ToxR, the El Tor hemolysin, the MARTX toxin, the outer membrane protein U, the cholix toxin, and the type III secretion system were detected with different frequencies, resulting in the identification of 27 different virulence gene profiles among the environmental isolates. Interestingly, the most frequently observed virulence gene profile (*toxR*$^{+}$ *hlyA*$^{ET+}$ *ompU*$^{+}$ *rtxC*$^{+}$) was found not only in more than one third of the environmental isolates but also in over 50% of all German clinical strains examined so far (Schirmeister *et al*., 2014a; Schwartz *et al*., 2019). This could indicate a human pathogenic potential of many environmental non-O1, non-O139 *V. cholerae* strains from German coastal waters. With the exception of three isolates, all environmental isolates

analyzed in this study possessed the El Tor hemolysin gene *hlyA*^{ET}. To determine if the *hlyA*^{ET} presence is correlated to the lytic activity of the isolates, semiquantitative tests for hemolytic activity against sheep erythrocytes and human red blood cells were performed. While the three *hlyA*^{ET} negative isolates did not show lytic activity, all remaining environmental isolates were distinctly hemolytic. Most isolates showed intermediate to strong hemolytic activity against both types of erythrocytes underlining the pathogenic potential of the investigated non-O1, non-O139 strains.

To confirm the PCR results and gain deeper insights into the genetic makeup of the strains, whole genome sequence-based analyses were performed on 15 environmental isolates and seven clinical isolates taking into account SLVs, DLVs and TLVs identified in MLST. Comparative analysis of WGS genotyping revealed that the virulence gene profile *toxR*$^{+}$ *luxS*$^{+}$ *cqsA*$^{+}$ *T6SS*$^{+}$ *hapA*$^{+}$ *tlh*$^{+}$ *hlyA*$^{ET+}$ *dth*$^{+}$ *ompU*$^{+}$ *rtxC*$^{+}$ *rtxA*$^{+}$ observed in some clinical isolates is also present in most environmental isolates irrespective of the MLST sequence type. In few environmental strains, additional virulence factors (TTSS, genomic islands) are present due to horizontal gene transfer. As the mentioned basic virulence gene profile is found in most environmental isolates examined by WGS – irrespective of the ST and the number of differing loci between MLST allelic profiles – it can be expected that this basic set of virulence genes is also present in many of the environmental isolates not further studied by WGS.

Several lines of evidence suggest that numerous virulence traits of *V. cholerae* evolved in response to abiotic and biotic pressures in the natural aquatic environment and are a result of bacterial adaptation to specific niches outside the human host (Sakib *et al*., 2018).

One example is probably the *V. cholerae* hemagglutinin/protease HA/P encoded by the *hapA* gene, which was found in all environmental isolates included in WGS analysis. This protease has been shown to play an important role in *V. cholerae* interaction with insects of the dipteran family *Chironomidae*. Chironomids are distributed throughout the world and are frequently the most abundant macroinvertebrate group in freshwater environments (Cranston, 1995), with representatives in brackish habitats like the Baltic Sea (> 200 species) (Brodin *et al*., 2013) and marine habitats such as the North Sea (Kaiser *et al*., 2010). The aquatic egg masses of these holometabolous insects were found to be a natural environmental reservoir for *V. cholerae* (Broza and Halpern, 2001). When *V. cholerae* cell density on chironomid egg masses is high, autoinducers CAI-1 and AI-2 of the *V. cholerae* quorum sensing system are upregulated leading to activation of HA/P enzyme expression. HA/P, in turn, degrades the gelatinous matrix of the chironomid egg masses which provides a nutritive source for

V. cholerae and thereby facilitates its survival and multiplication in the natural aquatic environment (Halpern *et al*., 2003; Halpern, 2010). The quorum sensing molecules CAI-1 and AI-2 are produced by CqsA and LuxS, respectively (Rutherford and Bassler, 2012), which explains why the genes of these two synthases were also found in all genomes.

Another virulence factor that probably evolved in response to abiotic and biotic stresses in the aquatic non-host environment is the *V. cholerae* type VI secretion system (T6SS). Besides *hapA*, *cqsA* and *luxS*, all environmental isolates harbored gene clusters encoding a likely functional T6SS. The bacteriophage tail-like, contact-dependent contractile T6SS apparatus serves translocation of toxic effector proteins into adjacent prokaryotic and eukaryotic target cells and thereby plays an important role in elimination of bacterial competitors in biofilms and *V. cholerae* escape from predatory amoebae in the natural aquatic environment (Joshi *et al*., 2017; Crisan and Hammer, 2020). In addition to facilitating *V. cholerae*'s survival through targeted killing of competing and predatory microorganisms, the T6SS contributes to *V. cholerae*'s evolution by fostering horizontal gene transfer. It has been shown that *V. cholerae* growth on chitinous surfaces (e.g., the exoskeleton of crustaceans) in densely packed biofilms can result in induction of natural competence and simultaneous activation of T6SS expression. Following T6SS-mediated killing of neighboring bacterial competitors, released fragments of DNA (with an individual maximum length of more than 150 kbp) can be absorbed by the competent *V. cholerae* killer cells and integrated into their genome if sufficient homology is present (Joshi *et al*., 2017; Matthey *et al*., 2019; Crisan and Hammer, 2020). The described transformation mechanism could explain why some environmental *V. cholerae* isolates analyzed in this study possessed virulence gene profiles exceeding the basic genetic makeup found.

As already mentioned, the basic virulence gene profile $toxR^{+}$ $luxS^{+}$ $cqsA^{+}$ $T6SS^{+}$ $hapA^{+}$ tlh^{+} $hlyA^{ET+}$ dth^{+} $ompU^{+}$ $rtxC^{+}$ $rtxA^{+}$ seems to occur in many German environmental strains and should therefore serve as the basis for a first differentiated risk assessment with regard to possible non-O1, non-O139 *V. cholerae* infections after recreational exposure to German coastal waters. The risk assessment will follow a qualitative approach and aims to estimate the risk for different *V. cholerae*-associated intestinal and extraintestinal types of infection. The development of a well-founded mathematical modeling approach for quantitative microbial risk assessment (QMRA) in seawater-related exposure settings according to the guidelines of the World Health Organization (WHO, 2016b) would require quantitative data on the concentration of non-O1, non-O139 *V. cholerae* strains with clinically relevant virulence factor profiles occurring in the North Sea and Baltic Sea. Furthermore, detailed

knowledge on dose-response relationships would be essential. At present, no information is available on the infectious dose that is required for a successful *ctx*$^-$ non-O1, non-O139 *V. cholerae*-associated intestinal or extraintestinal infection of a human host. Moreover, it is not known to what extent the infectious dose differs with regard to different population groups (age groups, immunocompetent vs. immunocompromised persons, individuals with or without underlying disease (e.g., intestinal diseases, skin diseases)). Given these fundamental gaps in knowledge, especially concerning dose-response relationships, a QMRA approach is currently less suitable for non-O1, non-O139 *V. cholerae* infections.

Based on the study results presented within the framework of this cumulative dissertation (Schwartz *et al*., 2019) as well as previous studies conducted in northern temperate waters (Collin and Rehnstam-Holm, 2011; Schuster *et al*., 2011; Böer *et al*., 2013; Brennholt *et al*., 2014), the risk of contracting a cholera-like diarrheal disease as a result of accidental water ingestion during bathing or other recreational activities in the German North Sea or Baltic Sea can be considered as very low at present. On the one hand, none of the analyzed non-O1, non-O139 *V. cholerae* isolates harbored the cholera toxin gene (*ctxA*). On the other hand, so far, no toxigenic O1/O139 *V. cholerae* strains have been found, which could serve as a *ctx* source in the context of horizontal gene transfer – neither in the North Sea nor in the Baltic Sea. Furthermore, studies with healthy adult volunteers and O1/O139 *V. cholerae* strains revealed that a high infectious dose (10^5 to 10^8 bacterial cells) is necessary to cause cholera diarrhea (Hornick *et al*., 1971; Cohen *et al*., 1999). However, it should be emphasized here that a future introduction of toxigenic *V. cholerae* strains by migratory waterbirds or shipping traffic cannot be excluded. A possible role of waterbirds as vectors for *V. cholerae* has been repeatedly pointed out (Halpern *et al*., 2008; Laviad-Shitrit *et al*., 2019). Depending on the bird species, waterbirds feed on small invertebrates and/or fish. A major dietary component of waterbirds living on insects and crustaceans are chironomids and copepods – two abundant invertebrate groups in aquatic ecosystems and natural reservoirs of *V. cholerae* (Halpern *et al*., 2008). Field studies conducted by Frisch *et al*. (2007) revealed that viable chironomids and/or copepods can be transported by waterbirds internally (in the digestive tract) or externally (on the feet and plumage) from one aquatic habitat to another. Furthermore, fecal specimens, cloacal swabs and intestine samples of waterbirds were tested positive for non-O1 as well as serogroup O1 *V. cholerae* (Lee *et al*., 1982; Ogg *et al*., 1989; Rodríguez *et al*., 2010; Laviad-Shitrit *et al*., 2017; Laviad-Shitrit *et al*., 2018). In a recent study conducted in Israel, the cholera toxin gene *ctxA* was detected in intestinal samples of *V. cholerae*-positive wild cormorants (Laviad-Shitrit *et al*., 2017). Subsequent feeding

experiments with captive, hand-reared cormorants demonstrated that *V. cholerae* can be transferred from naturally *V. cholerae*-colonized fish (such as tilapia) to waterbirds and survive for up to three days in the birds' digestive tract, a period of time during which great cormorants and other aquatic bird species are able to cover distances of up to 3,000 km (Laviad-Shitrit *et al*., 2017). In addition, increasing international shipping traffic could also result in the introduction of toxigenic *V. cholerae* strains to the North Sea and the Baltic Sea regions. Far more than 70% of the global trade is transported by oceans and seas (Hess-Erga *et al*., 2019), with the Port of Hamburg representing the third largest European and major German seaport concerning cargo handling from all over the world (Destatis, 2020; HHM, 2020). A few years ago, attention was drawn to the possible transport and discharge of epidemic serogroup O1/O139 *V. cholerae* strains by ships' ballast water (Dobbs *et al*., 2013). To reduce the risk of introducing harmful organisms and pathogens into new aquatic environments, the Ballast Water Management (BWM) Convention adopted by the International Maritime Organization recently entered into force globally (IMO, 2004; IMO, 2019). Standards on ballast water exchange away from coastal areas (BWM Convention, Regulations B-4 and D-1) as well as discharge limits for indicator microbes (e.g., toxigenic *V. cholerae* (O1/O139): < 1 CFU/100 ml) (BWM Convention, Regulation D-2) are likely to minimize risk for public health. However, following several ballast water treatment methods bacterial regrowth has been observed (Grob and Pollet, 2016; Hess-Erga *et al*., 2019) and environmental transport events could favor pathogen transfer to coastal areas.

Based on the study results presented in this cumulative dissertation (Schwartz *et al*., 2019), the risk of generally contracting a diarrheal disease as a result of ingesting non-O1, non-O139 *V. cholerae* containing water during swimming in the German North Sea or Baltic Sea also seems to be low. Genes encoding the type III secretion system (TTSS) were found only in a few of the analyzed environmental isolates while they were present in both analyzed German diarrheal isolates. Animal studies with *V. cholerae* AM-19226, another pathogenic non-O1, non-O139 strain lacking the major virulence factors of toxigenic strains (CTX, TCP) but harboring the TTSS (Dziejman *et al*., 2005), confirmed the necessity of the TTSS in intestinal colonization (Chaand *et al*., 2015) and causing diarrheal disease (Shin *et al*., 2011). So far, no reliable data are available on the infectious dose for a non-O1, non-O139 *V. cholerae* diarrheal disease. Starting from the assumption that the infectious dose resembles that of cholera (10^5 to 10^8 bacterial cells in healthy volunteers) (Hornick *et al*., 1971; Cohen *et al*., 1999), the risk for a swimming-associated diarrheal disease would remain low. According to a study conducted by the United States Environmental Protection Agency (US EPA), the

average water volume ingested by children and adults during recreational swimming activities is 47 ml and 24 ml per swim period, respectively (Evans *et al*., 2006). Given the low volumes of swallowed water calculated by the US EPA as well as generally low *V. cholerae* abundances reported for German coastal waters in recent years (Böer *et al*., 2013; Brennholt *et al*., 2014; Hackbusch *et al*., 2020), the above-mentioned high infectious dose is likely to be rarely reached. However, people suffering from a loss of or reduced gastric acid production (achlorhydria, hypochlorhydria) or showing an increased gastric pH due to medication (e.g., proton pump inhibitors) should exercise caution when swimming in the North Sea or Baltic Sea. For these persons, the number of non-O1, non-O139 *V. cholerae* bacteria for an intestinal infection will probably be several orders of magnitude lower (Bavishi and DuPont, 2011). Furthermore, the TTSS does not always seem to be essential for a non-O1, non-O139 *V. cholerae*-associated diarrheal disease. In a recent study, some TTSS-negative diarrhea-causing human isolates were reported (Schirmeister *et al*., 2014a). Interestingly, all of these isolates harbored the *hlyA*^{ET} gene, which was also found in almost all analyzed environmental isolates from coastal waters of the German North Sea and Baltic Sea. Data collection suggests that the El Tor hemolysin (HlyA^{ET}), also referred to as *V. cholerae* cytolysin (VCC; Zhang and Austin, 2005), may be another major diarrheagenic factor for non-cholera-toxin-producing *V. cholerae* strains. *In vivo* studies have shown that injection of purified HlyA^{ET} into rabbit ileal loops induced fluid secretion into the intestinal lumen, indicating a possible role in the pathogenesis of gastroenteritis (Ichinose *et al*., 1987). This result is supported, *inter alia*, by subsequent *ex vivo* electrophysiological studies in Ussing chambers showing that VCC promotes Cl^- secretion from intact human intestinal mucosa, which is crucial for intestinal fluid secretion (Debellis *et al*., 2009; Benitez and Silva, 2016). Moreover, WGS analysis revealed that many German environmental isolates harbored the *hapA* gene as well as a likely functional T6SS. The *V. cholerae* hemagglutinin/protease (HapA, HA/P) degrades host proteins of intercellular tight junctions (e.g., occludin) between intestinal epithelial cells, which could promote paracellular fluid secretion (Benitez and Silva, 2016). Early studies confirm that HapA contributes to human diarrheal disease (Benítez *et al*., 1999). The type VI secretion system (T6SS) also seems to play an important role in the development of diarrheal disease. Animal studies reveal that the T6SS contributes to elimination of the intestinal microbiota, which facilitates intestinal colonization by *V. cholerae* (Logan *et al*., 2018; Zhao *et al*., 2018). Additionally, the T6SS is associated with intestinal inflammation and diarrheal symptoms (Ma and Mekalanos, 2010). Given all these facts, many non-O1, non-O139 *V. cholerae* strains in German coastal waters could have the potential to cause diarrheal

disease in susceptible human hosts and especially above-mentioned risk groups and immunocompromised people should be careful when swimming in the North Sea or Baltic Sea.

Due to fundamental gaps in scientific literature, the risk of contracting a non-O1, non-O139 *V. cholerae*-associated ear or wound infection after exposure to German coastal waters (e.g., during swimming or wading) is also difficult to estimate. So far, it is not known which virulence factors are essential for these types of infection. Factors that are likely to play a role in such extraintestinal infections may include the outer membrane protein U (OmpU) and its regulator ToxR, the type VI secretion system (T6SS), the multifunctional autoprocessing repeats-in-toxin toxin (RtxA, MARTX) and its putative regulator RtxC as well as hemolysins (e.g., HlyA). *V. cholerae* OmpU confers resistance to antimicrobial peptides such as peptide P2 derived from the bactericidal/permeability-increasing (BPI) protein. BPI is an antimicrobial protein found, *inter alia*, on the cell surface and in azurophilic granules of neutrophils, which are part of the innate immune system (Mathur and Waldor, 2004; Mathur *et al*., 2007). The T6SS is involved in evasion of the host immune defense by disrupting the actin cytoskeleton of host macrophages (Pukatzki *et al*., 2007). Human colonization could be further facilitated by *V. cholerae* MARTX. MARTX of toxigenic El Tor strains was found to contain an effector domain mediating actin cross-linking within neutrophil granulocytes, which leads to disruption of neutrophils' cytoskeleton and finally to inhibition of their phagocytic ability (Satchell, 2015; Kim, 2018). Hemolysins, such as the pore-forming exotoxin HlyA, may also foster *V. cholerae* survival and growth within the human host by tapping iron sources (Stoebner and Payne, 1988; Huntley *et al*., 1997). WGS-based determination of virulence gene profiles within the framework of this cumulative dissertation (Publication 1) revealed the presence of hemolysin genes (*hlyA*, *tlh*, *dth*), *rtxA*, *rtxC*, *ompU*, *toxR* as well as genes necessary for a functional T6SS in all analyzed extraintestinal non-O1, non-O139 *V. cholerae* isolates from German patients. The same set of virulence genes was also found in most of the analyzed environmental isolates from German coastal waters. PCR-based screening of more environmental non-O1, non-O139 *V. cholerae* isolates from the German North Sea and Baltic Sea indicates a frequent occurrence of the MARTX El Tor variant. The similarity between virulence gene profiles of extraintestinal otitis and wound isolates from German patients and many environmental isolates from German coastal waters should be given attention. However, it cannot be ruled out that so far unrecognized virulence factors, which could be missing in several environmental strains, play an essential role in such human infections. Hitherto, no studies are available on the number of non-O1, non-O139

V. cholerae cells needed for a successful ear or wound infection. Considering dose-response data on other extraintestinally relevant human pathogens (Roser *et al*., 2014), an infectious dose of only a few cells seems likely. Given all these facts and first severe cases of otitis and wound infections after contact to German coastal waters (Handrick *et al*., 2004; Schirmeister *et al*., 2014a), people suffering from ear or skin lesions should avoid bathing in the North Sea or Baltic Sea as well as wading and walking at the water's edge if lower extremities are affected.

3.3 Characterization of Veterinary *Vibrio navarrensis* Isolates From Germany by Reference to Environmental and Human Clinical Isolates

The second study of this cumulative dissertation focused on first veterinary isolates of *V. navarrensis* that had been collected between 1999 and 2009 and were provided by the Land Saxony's Health and Veterinary Research Institute to the consiliary laboratory. All veterinary *V. navarrensis* strains had been isolated from livestock farms in Saxony, Germany, where they had been found in inner organs of aborted fetuses and/or placentas after miscarriages.
For identification of new potential environmental reservoirs in Germany and against the background of a possible zoonotic potential, the veterinary isolates were analyzed using phenotypical and WGS-based genotypical methods and compared to *V. navarrensis* strains from environmental samples and human clinical specimens. The reference group comprised all available German environmental *V. navarrensis* isolates, which had been isolated from marine environments of the North Sea and Baltic Sea between 1996 and 2015. In addition, WGS data of two Spanish environmental strains isolated from sewage and two American human pathogenic strains (wound, blood) were included, corresponding to all published whole genome sequences available at the beginning of this study. Originally considered an environmental species (Urdaci *et al*., 1991; Jores *et al*., 2003b; Jores *et al*., 2007), *V. navarrensis* has been only recently associated with human illness (Gladney and Tarr, 2014). Until the end of this study in late 2017, non-cholera *Vibrio* infections were not notifiable in Germany (IfSG §§ 6, 7 in the version applicable on July 25, 2017[11,12]) and neither German nor other European *V. navarrensis* isolates from human clinical specimens became known.

[11] https://www.buzer.de/gesetz/2148/l.htm
[12] https://www.rki.de/DE/Content/Infekt/IfSG/Meldepflichtige_Krankheiten/Meldepflichtige_Krankheiten_Erreger.html

Hemolytic activity tests revealed that almost all marine environmental strains and veterinary strains were hemolytic against human erythrocytes and sheep erythrocytes, properties frequently associated with pathogenic *Vibrio* strains (Restrepo *et al*., 2006; Schirmeister *et al*., 2014a; Hirsch *et al*., 2020; see also section 1.1.4). The lytic activity of the marine environmental isolates and veterinary isolates against human red blood cells could be attributed to the production of various hemolysins. WGS-based typing revealed that all strains harbored homologs of virulence-associated genes like *tlh* and *vvhA* as well as *rtx*. Further, most strains possessed an *hlyIII* homolog. *V. parahaemolyticus* TLH (thermolabile hemolysin; Taniguchi *et al*., 1985), *V. vulnificus* VvhA (*V. vulnificus* hemolysin A; Lee *et al*., 2013), and *V. vulnificus* HlyIII (hemolysin III; Chen *et al*., 2004), which have already been studied in more detail, have shown to induce lysis of human erythrocytes. Likewise, secreted RTX proteins are known to possess hemolytic properties (Linhartova *et al*., 2015). Moreover, all marine environmental and veterinary isolates of *V. navarrensis* harbored a *hlyD* gene homolog. HlyD proteins are located in the bacterial inner membrane where they contribute, as part of type I secretion systems (T1SS), to the translocation of hemolysins from the cytosol to the extracellular space (Pimenta *et al*., 2005; Kanonenberg *et al*., 2018).

To gain deeper insights into the genetic makeup of the strains and identify possible differences between environmental and potentially pathogenic strains, presence-absence analysis of virulence-associated genes was extended and published whole genome sequences of the aforementioned Spanish environmental strains and American human pathogenic strains were included. The WGS typing scheme was designed based on factors associated with virulence in well-known human pathogens, in particular the closely related *Vibrio* species *V. vulnificus*. The scheme comprised *V. navarrensis* genes encoding putative proteins for capsule (*cps*) and type IV pilus biosynthesis (*pilV*, *pilW*), genes of putative T6SS proteins as well as genes encoding putative hemolytic and cytolytic proteins (homologs of *tlh*, *osmY*, *vvhA*, *δ-vph*, *hlyIII*, and *hlyD* as well as *rtx* (see also section 1.1.4)). *V. navarrensis* ORF12 was also included. This genomic DNA fragment encoding a protein with hemolytic properties seems to be specific for the *pommerensis* biotype (Jores *et al*., 2003a). Genetic differences were observed for T6SS genes, which were detected in all veterinary isolates while they were absent from some environmental isolates. However, no clear pattern was found for the potentially pathogenic strains, i.e. veterinary and human clinical strains, versus environmental strains. The majority of the investigated genes were present in all *V. navarrensis* strains. In addition to the hemolysin gene homologs discussed above, some strains harbored a *δ-vph* (thermostable delta *V. parahaemolyticus* hemolysin; Taniguchi *et al*., 1990) homolog and all

confirmed environmental biotype *pommerensis* strains possessed ORF12. The similarity between the environmental strains and potentially pathogenic strains should be given attention. However, further research is necessary to demonstrate if some of the investigated *V. navarrensis* genes contribute to a pathogenic potential. Moreover, genomic analyses of future clinical isolates will be essential. It seems possible that identification of allelic variants of specific genes is required to recognize clinically relevant strains.

To evaluate a phylogenetic relationship, a single nucleotide polymorphism (SNP) analysis of whole genome sequences as well as a multilocus sequence analysis (MLSA) of housekeeping genes were performed. So far, well-established species-specific *Vibrio*-MLSA schemes are only available for the three most important human pathogenic *Vibrio* species, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, as well as the clam pathogen *V. tapetis* (https://pubmlst.org). Therefore, *V. navarrensis* MLSA analysis was based on the recently developed, four housekeeping genes-encompassing *Vibrio*-MLSA scheme (Rahman *et al*., 2014; Jolley *et al*., 2018). The scheme was complemented by *rpoB* sequences (Tarr *et al*., 2007) to increase the depth of the analysis. SNP analysis clearly revealed that the German seawater strains were phylogenetically distant to the remaining *V. navarrensis* strains with SNP differences between 41,000 and 57,000. MLSA of housekeeping genes gave similar results. The German seawater strains were clearly separated from the remaining *V. navarrensis* strains. Furthermore, veterinary, human pathogenic and Spanish sewage strains were placed into one well supported subcluster within the *V. navarrensis* cluster. The relationship to the Spanish sewage strains and the phylogenetic distance to the German seawater strains could indicate that the German veterinary strains originate from a low salinity aquatic environment. It seems possible that *V. navarrensis* also occurs in German freshwater environments. This could include rivers (such as the Elbe, the Mulde, the Elster, and the Spree) as well as other waterbodies in the German federal state of Saxony. *V. navarrensis* strains could have been ingested by the farm animals from any type of surface water including temporary standing waters. The possibility of *Vibrio* transport from one aquatic environment to another by waterbirds and/or insects has been repeatedly pointed out (Broza *et al*., 2005; Broza *et al*., 2008; Laviad-Shitrit *et al*., 2017; Laviad-Shitrit *et al*., 2019) and in a Saudi Arabian study, *V. navarrensis* was recently detected in an insect environment (El-Sayed and Ibrahim, 2015). So far, no systematic studies on the occurrence of *Vibrio* bacteria in German inland waters have been conducted. The relationship between the veterinary strains and the human pathogenic strains could indicate a human pathogenic potential of the former. The "One Health" concept recognized that human, animal, and environmental health are

interconnected (Mackenzie and Jeggo, 2019). Further research is necessary to identify environmental and/or animal reservoirs of *V. navarrensis* in Germany and to determine a possible role as a zoonotic agent.

3.4 *Vibrio* spp. Surveillance in Germany and Future Perspectives

Within the framework of this cumulative dissertation, valuable insights were gained into the genetic makeup of environmental non-O1, non-O139 *V. cholerae* strains from German coastal waters. Accumulating evidence suggests that many of these strains possess the potential to cause intestinal or extraintestinal infections in susceptible human hosts.

Climate change and the expected increase in *Vibrio* abundance in German coastal waters demand suitable monitoring systems for *Vibrio* bacteria at officially registered bathing sites along the North Sea and Baltic Sea coastline. A few years ago, Baker-Austin and colleagues emphasized that vibrios can be considered as a 'microbial barometer of climate change' in marine environments (Baker-Austin *et al*., 2017). Recent studies conducted in the German Bight confirmed that extended warm periods with seawater temperatures above 17°C coincided with prolonged occurrences of potentially human pathogenic *Vibrio* spp. including *V. cholerae* (Hackbusch *et al*., 2020). Against this background, a real-time model was developed that utilizes daily updated remotely sensed sea surface temperature and salinity data to identify and map coastal waters with environmentally suitable conditions for the growth of human pathogenic *Vibrio* species at a global scale (Baker-Austin *et al*., 2012; Semenza *et al*., 2017). The *Vibrio* Map Viewer is publicly available via the ECDC E3 Geoportal[13] and could serve as a first early warning system to reduce the risk of future *Vibrio* infections in the North Sea and Baltic Sea regions.

In order to further refine existing early warning systems, detailed knowledge on the pathogenic characteristics of *V. cholerae* bacteria from coastal waters is essential. Before the start of this work, scarcely anything was known about the genetic makeup of German environmental *V. cholerae* strains. As part of extensive PCR- and WGS-based analyses on strains collected at registered bathing sites along the North Sea and Baltic Sea coastline as well as within local estuaries over a six-year period, fundamental insights were gained into the virulence gene profiles of *V. cholerae* non-O1, non-O139 strains from these waters (Schwartz *et al*., 2019). The results of this study are likely to provide an important basis for further research questions in this field – especially in the course of climate change. Recent

[13] https://geoportal.ecdc.europa.eu/vibriomapviewer/

studies conducted in the German Bight indicate an increasing occurrence of the diarrhea-associated TTSS in non-O1, non-O139 *V. cholerae* strains from coastal areas (Hackbusch *et al*., 2020). It would be possible that environmental changes resulting from climate change foster *V. cholerae* strains with a higher infective risk. The WGS-based typing scheme that was developed in the context of this cumulative dissertation can serve as a helpful tool for monitoring and deeper investigation of virulence gene profiles of future non-O1, non-O139 *V. cholerae* isolates from environmental sources. In addition to TTSS presence, changes in the ChxA occurrence, a virulence factor that could favor severe extraintestinal infections (Awasthi *et al*., 2013), may be of particular interest. The establishment of an optimized *chxA* singleplex PCR at the *Vibrio* consiliary laboratory within the framework of this dissertation (Schwartz *et al*., 2019) enables efficient screening analyses in routine diagnostics and future research projects.

In a recent collaborative study between the German Federal Institute for Risk Assessment (BfR) and the Bavarian Animal Health Service (TGD), the developed WGS-based typing approach was already chosen for genotypic characterization of veterinary non-O1, non-O139 *V. cholerae* isolates (Hirsch *et al*., 2020). The strains had been found in inner organs of diseased domestic ducks from Bavarian and Saxonian poultry farms, among them some with significant animal losses. Similarities in the virulence gene profiles of German veterinary waterfowl and human pathogenic non-O1, non-O139 *V. cholerae* isolates (Schwartz *et al*., 2019; Hirsch *et al*., 2020) as well as the presence of El Tor-like MARTX variants in all veterinary isolates (Hirsch *et al*., 2020) could indicate a zoonotic potential of these isolates. The publication resulting from the TGD cooperation, in which I was considerably involved in data curation and software-based analyses, is included in the appendix of this cumulative dissertation. So far, no information is available on the occurrence and distribution of *V. cholerae* bacteria in German inland waters. However, the reasons and the findings of the aforementioned collaborative study between the BfR and the TGD underline the need to extend water monitoring to inland areas.

The repeated detection of potentially human pathogenic *Vibrio* species in the context of abortions in pigs and cattle in livestock farms in Germany requires additional attention (Müller and Kühn, 2001; Stephan *et al*., 2002; Weber *et al*., 2006; Jäckel *et al*., 2020). Within the framework of this cumulative dissertation, first insights were gained into the genetic makeup of veterinary *V. navarrensis* strains, a hardly explored *Vibrio* species that had never been reported in food-producing animals before (Schwartz *et al*., 2017). A role of *V. navarrensis* as a zoonotic agent seems possible emphasizing the necessity for further

research in this field to characterize and reduce a potential risk for public health. The detection of *V. navarrensis* in abortion material of domestic animals on farms away from the coast as well as the phylogenetic distance to German seawater strains suggests that this *Vibrio* species may also occur in German inland waters. Future studies on the occurrence and distribution of *V. navarrensis* bacteria in public health-relevant sources could be facilitated by the WGS-based typing approach developed and applied in the context of this work. While an ISO standard for the detection of potentially human pathogenic *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* has been available for some time (ISO 21872-1:2017), no standardized procedure for the identification of recently discovered *Vibrio* species such as *V. navarrensis* exists. WGS-based analyses performed within the framework of this dissertation revealed the presence of *vvhA* and *tlh* gene homologs in all *V. navarrensis* strains, two genes that are already used for species identification of *V. vulnificus* (Campbell and Wright, 2003) and *V. parahaemolyticus* (Jones *et al*., 2014), respectively. The subsequently developed *V. navarrensis*-specific *vvhA* and *tlh* singleplex PCRs (Schwartz *et al*., 2017) represent promising tools for cost-effective and time-efficient identification of this species in future research projects.

In summer 2020 for the first time, *V. navarrensis* was detected in blood cultures of a German patient hospitalized with an erysipelas-like leg infection (Schwartz *et al*., manuscript in preparation). This extraintestinal infection can result in severe complications like life-threatening sepsis and underlines the human pathogenic potential of this species, thus emphasizing the need for continued *Vibrio* spp. surveillance in Germany. Since March 2020, all confirmed acute infections with human pathogenic *Vibrio* spp. are notifiable in Germany (IfSG § 7 in the version applicable on March 1, 2020[14]). The legal obligation to inform public health authorities on the detection of human pathogenic *Vibrio* species in diseased patients should help to identify possible spatiotemporal clusters of cases and to take appropriate intervention measures in a short time.

[14] https://www.buzer.de/gesetz/2148/al85906-0.htm

4 Summary

V. cholerae bacteria are distributed in aquatic environments with low to moderate salinity throughout the world. Toxigenic serogroup O1/O139 *V. cholerae* strains can cause the epidemic disease cholera. In northern European waters, non-O1, non-O139 *V. cholerae* strains occur, some of which have been associated with gastrointestinal or extraintestinal infections. So far, infections caused by these bacteria are rare in Germany. However, due to climate change, an increase in non-O1, non-O139 *V. cholerae* prevalence is expected. Given the popularity of German coastal regions as a travel destination, an increased exposure of the population to strains of these serogroups must be assumed for future years. Therefore, by comparing MLST sequence types (ST) and virulence factor profiles of environmental isolates and clinical isolates, a first risk assessment for non-O1, non-O139 *V. cholerae* strains from German coastal waters was carried out in this work. The similarity of the virulence gene profiles of environmental strains and clinical strains - irrespective of the ST - as well as the hemolytic activity against human erythrocytes could indicate a human pathogenic potential of many environmental strains in the North Sea and Baltic Sea. Data comparison to a first follow-up study conducted in the German Bight suggests that environmental changes resulting from climate change possibly foster *V. cholerae* strains with a higher infective risk. The whole genome sequencing (WGS)-based typing scheme that was developed and established at the *Vibrio* consiliary laboratory in the context of this dissertation can serve as a tool for monitoring and deeper investigation of virulence gene profiles of future non-O1, non-O139 *V. cholerae* isolates obtained from public health-relevant sources.

While *Vibrio* species such as *V. cholerae* have been intensively studied for decades as a result of the global disease burden, other *Vibrio* species such as the presumed environmental species *V. navarrensis* are hardly explored. Recently, *V. navarrensis* strains were detected in human clinical specimens as well as veterinary abortion specimens from livestock in Germany. For identification of new potential environmental reservoirs in Germany and against the background of a possible zoonotic potential, comparative genotypic analyses of *V. navarrensis* isolates from environmental, veterinary, and human clinical specimens were performed in this work. The results of SNP and MLSA analyses suggest the occurrence of *V. navarrensis* in German freshwater environments, which would extend the range of this species from coastal waters of the North Sea and Baltic Sea to inland waters in Germany. Ingestion of *V. navarrensis* by farm animals through uptake of surface water seems possible. The presence of an MLSA subcluster encompassing all veterinary and human clinical strains

could indicate a human pathogenic potential of the former. The design of a WGS-based analytical procedure for typing of virulence-associated genes in *V. navarrensis* isolates provided deeper insights into the genetic makeup of all strains. Phenotypic traits such as the hemolytic activity of the investigated environmental and veterinary strains against sheep erythrocytes and human red blood cells underline the pathogenic character of this *Vibrio* species. A new case of a human infection in summer 2020 supports this assessment. The developed *V. navarrensis*-specific *vvhA* and *tlh* singleplex PCRs represent promising tools for rapid species diagnostics and may thereby facilitate future monitoring and research studies on *Vibrio* bacteria in the context of the “One Health” approach.

5 Zusammenfassung

V. cholerae-Bakterien sind in aquatischen Umwelten mit geringer bis moderater Salinität weltweit verbreitet. Toxigene *V. cholerae*-Stämme der Serogruppe O1/O139 können die epidemische Erkrankung Cholera verursachen. In nordeuropäischen Gewässern kommen non-O1, non-O139 *V. cholerae*-Stämme vor, von denen einige mit gastrointestinalen oder extraintestinalen Infektionen in Verbindung gebracht wurden. Bislang sind durch diese Bakterien verursachte Infektionen in Deutschland selten. Aufgrund des Klimawandels wird jedoch ein Anstieg in der non-O1, non-O139 *V. cholerae*-Prävalenz erwartet. Angesichts der Beliebtheit deutscher Küstenregionen als Reiseziel muss in den kommenden Jahren von einer erhöhten Exposition der Bevölkerung gegenüber Stämmen dieser Serogruppen ausgegangen werden. Daher wurde in dieser Arbeit über den Vergleich von MLST-Sequenztypen (ST) und Virulenzfaktorprofilen von Umweltisolaten und klinischen Isolaten eine erste Risikobewertung für non-O1, non-O139 *V. cholerae*-Stämme aus deutschen Küstengewässern durchgeführt. Die Ähnlichkeit der Virulenzgenprofile von Umweltstämmen und klinischen Stämmen - unabhängig vom ST - sowie die hämolytische Aktivität gegenüber humanen Erythrozyten könnten auf ein humanpathogenes Potential vieler Umweltstämme in der Nordsee und Ostsee hindeuten. Ein Vergleich der Daten mit denen einer ersten Folgestudie, die in der Deutschen Bucht durchgeführt wurde, legt nahe, dass sich aus dem Klimawandel ergebende Umweltveränderungen möglicherweise *V. cholerae*-Stämme mit einem höheren Infektionsrisiko fördern. Das whole genome sequencing (WGS)-basierte Typisierungsschema, das im Rahmen dieser Dissertation entwickelt und am *Vibrio*-Konsiliarlabor etabliert wurde, kann als Werkzeug zur Überwachung und tieferen Untersuchung von Virulenzgenprofilen künftiger non-O1, non-O139 *V. cholerae*-Isolate aus für die öffentliche Gesundheit relevanten Quellen dienen.

Während *Vibrio*-Arten wie *V. cholerae* infolge der globalen Krankheitslast seit Jahrzehnten intensiv untersucht werden, sind andere *Vibrio*-Arten wie die mutmaßliche Umweltspezies *V. navarrensis* kaum erforscht. Kürzlich wurden *V. navarrensis*-Stämme in humanen klinischen Proben sowie veterinären Abortproben aus Nutztieren in Deutschland nachgewiesen. Zur Identifizierung neuer potentieller Umweltreservoire in Deutschland und vor dem Hintergrund eines möglichen zoonotischen Potentials wurden in dieser Arbeit vergleichende genotypische Analysen von *V. navarrensis*-Isolaten aus Umweltproben, Veterinärproben und humanen klinischen Proben durchgeführt. Die Ergebnisse der SNP- und MLSA-Analysen deuten auf das Vorkommen von *V. navarrensis* in deutschen

Süßwasserumwelten hin, was das Verbreitungsgebiet dieser Spezies von Küstengewässern der Nord- und Ostsee auf Binnengewässer in Deutschland erweitern würde. Eine Aufnahme von *V. navarrensis* über Oberflächenwasser-Aufnahme durch Nutztiere scheint möglich. Das Vorliegen eines MLSA-Subclusters, welches alle veterinären und humanen klinischen Stämme umfasst, könnte auf ein humanpathogenes Potential der Ersteren hinweisen. Das Design eines WGS-basierten analytischen Verfahrens zur Typisierung Virulenz-assoziierter Gene in *V. navarrensis*-Isolaten bot tiefere Einblicke in die genetische Ausstattung aller Stämme. Phänotypische Merkmale wie die hämolytische Aktivität der untersuchten Umwelt- und Veterinärstämme gegenüber Schaferythrozyten und humanen Erythrozyten betonen den pathogenen Charakter dieser *Vibrio*-Art. Ein neuer Fall einer Humaninfektion im Sommer 2020 stützt diese Bewertung. Die entwickelten *V. navarrensis*-spezifischen *vvhA*- und *tlh*-Singleplex-PCRs stellen vielversprechende Werkzeuge für eine schnelle Speziesdiagnostik dar und dürften dadurch künftige Überwachungs- und Forschungsstudien zu *Vibrio*-Bakterien im Rahmen des “One Health”-Ansatzes erleichtern.

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7 Appendix

7.1 Supplementary Material

7.1.1 Publication 1

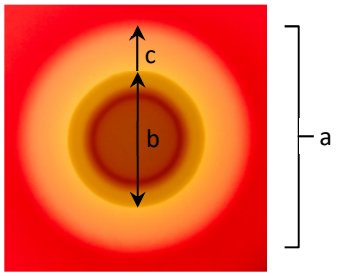

Figure S1. Elicitation of hemolytic activity data for *Vibrio cholerae* non-O1, non-O139 isolates from German coastal waters analyzed in this study (photograph: BfR, Friedmann-Marohn, 2015). Overnight cultures of *V. cholerae* non-O1, non-O139 strains were spotted on Mueller-Hinton agar containing 4% human erythrocytes or 4% sheep erythrocytes. After incubation at 37°C for 22-24 h, the hemolytic activity was characterized based on the diameter of the hemolysis zone around the macrocolony. To minimize measuring inaccuracies sometimes resulting from hemolytic halos without sharply defined edges, the diameter of the hemolysis zone (a) and the diameter of the macrocolony (b) were measured at the bottom of the Petri dish against the light and the mean diameter of the hemolytic halo (c) was mathematically calculated in millimeters [$\frac{(\boldsymbol{a-b})}{\boldsymbol{2}} = \boldsymbol{c}$]. Hemolysis zones also included parts on the outer edge of the hemolytic halo with incomplete lysis. Further details are given in Material and Methods.

Table S1. *Vibrio cholerae* non-O1, non-O139 strains from German coastal waters and from German clinical samples used in this study.

Strain	Year of isolation	Geographical origin	Source	Source code[2]	Institutional origin[4]
Environmental - Baltic Sea					
VN-00278	2011	Lubmin	Seawater	E-BS-sw	LAGuS
VN-00455	2011	Greifswald Bodden	Seawater	E-BS-sw	LAGuS
VN-00456	2012	Stresow	Seawater	E-BS-sw	LAGuS
VN-00457	2012	Wohlenberg Wiek/Niendorf	Seawater	E-BS-sw	LAGuS
VN-00458	2012	Karlshagen	Seawater	E-BS-sw	LAGuS
VN-00459	2012	Lubmin	Seawater	E-BS-sw	LAGuS
VN-00460	2012	Lubmin	Seawater	E-BS-sw	LAGuS
VN-00461	2012	Karlshagen	Seawater	E-BS-sw	LAGuS
VN-00462	2012	Lubmin	Seawater	E-BS-sw	LAGuS
VN-00463	2013	Lubmin	Seawater	E-BS-sw	LAGuS
VN-00464	2013	Warnemünde	Seawater	E-BS-sw	LAGuS
VN-00465	2013	Dranske/Libben	Seawater	E-BS-sw	LAGuS
VN-00466	2013	Binz/Prora	Seawater	E-BS-sw	LAGuS
VN-00468	2013	Spandowerhagen Wiek/Freest	Seawater	E-BS-sw	LAGuS
VN-00469	2013	Karlshagen	Seawater	E-BS-sw	LAGuS
VN-00470	2013	Lubmin	Seawater	E-BS-sw	LAGuS
VN-00471	2013	Lubmin	Seawater	E-BS-sw	LAGuS
VN-00472	2013	Stresow	Seawater	E-BS-sw	LAGuS
VN-00473	2013	Karlshagen	Seawater	E-BS-sw	LAGuS
VN-00474	2013	Stresow	Seawater	E-BS-sw	LAGuS
VN-00475	2014	Wohlenberg Wiek/Niendorf	Seawater	E-BS-sw	LAGuS
VN-00476	2014	Karlshagen	Seawater	E-BS-sw	LAGuS
VN-00477	2014	Lubmin	Seawater	E-BS-sw	LAGuS
VN-02995	2011	Schönhagen	Seawater	E-BS-sw	AWI
VN-03901	2011	Greifswald Bodden	Seawater/sediment	E-BS-sw/sd	LAGuS
VN-03902	2011	Darss-Zingst	Seawater/sediment	E-BS-sw/sd	LAGuS
VN-03903	2011	Wohlenberg Wiek/Niendorf	Seawater	E-BS-sw	LAGuS
VN-03907	2011	Darss-Zingst	Seawater/sediment	E-BS-sw/sd	LAGuS
VN-03908	2011	Darss-Zingst Bodden Chain	Seawater	E-BS-sw	LAGuS
VN-03911	2011	Darss-Zingst Bodden Chain	Seawater	E-BS-sw	LAGuS
VN-03916	2011	Darss-Zingst Bodden Chain	Sediment	E-BS-sd	LAGuS
VN-03918	2011	Darss-Zingst	Seawater/sediment	E-BS-sw/sd	LAGuS
VN-03939	2011	Darss-Zingst Bodden Chain	Sediment	E-BS-sd	LAGuS
VN-03942	2011	Darss-Zingst	Seawater/sediment	E-BS-sw/sd	LAGuS
VN-03944	2011	Darss-Zingst	Seawater/sediment	E-BS-sw/sd	LAGuS
VN-03949	2011	Darss-Zingst	Seawater/sediment	E-BS-sw/sd	LAGuS
VN-03951	2011	Darss-Zingst	Seawater/sediment	E-BS-sw/sd	LAGuS
VN-03954	2011	Darss-Zingst	Seawater/sediment	E-BS-sw/sd	LAGuS
VN-03955	2011	Darss-Zingst Bodden Chain	Sediment	E-BS-sd	LAGuS
VN-03958	2011	Darss-Zingst	Seawater/sediment	E-BS-sw/sd	LAGuS
VN-03963	2010	Binz	Seawater	E-BS-sw	LAGuS
VN-04241	2013	Graal-Müritz	Seawater	E-BS-sw	AWI
VN-04250	2013	Heringsdorf	Seawater	E-BS-sw	AWI
VN-05169	2011	Rügen	Seawater	E-BS-sw	IFH
VN-05172	2011	Rügen	Seawater	E-BS-sw	IFH
VN-05174	2011	Rügen	Seawater	E-BS-sw	IFH
VN-05176	2011	Rügen	Seawater	E-BS-sw	IFH
VN-05177	2011	Rügen	Seawater	E-BS-sw	IFH
VN-05185	2011	Rügen	Seawater	E-BS-sw	IFH
VN-05301	2012	Salzhaff/Pepelow	Seawater	E-BS-sw	IFH

Table continued					
Strain	**Year of isolation**	**Geographical origin**	**Source**	**Source code[2]**	**Institutional origin[4]**
Environmental - North Sea					
VN-02808	2011	Büsum	Seawater	E-NS-sw	AWI
VN-02825	2011	Speicherkoog	Seawater	E-NS-sw	AWI
VN-02923	2011	Schleswig-Holstein	Seawater	E-NS-sw	AWI
VN-03012	2009-2011	n.s.	Seawater	E-NS-sw	AWI
VN-03213	2014	n.s.	Seawater	E-NS-sw	AWI
VN-03301	2010	Dyksterhusen	Seawater/sediment	E-NS-sw/sd	KLIWAS
VN-03361	2010	n.s.	Seawater/sediment	E-NS-sw/sd	KLIWAS
VN-03377	2010	Neßmersiel	Seawater/sediment	E-NS-sw/sd	KLIWAS
VN-03405	2010	Dyksterhusen	Seawater/sediment	E-NS-sw/sd	KLIWAS
VN-03407	2010	Dyksterhusen	Seawater/sediment	E-NS-sw/sd	KLIWAS
VN-03428	2010	Norddeich	Seawater/sediment	E-NS-sw/sd	KLIWAS
VN-03460	2010	Dedesdorf	Seawater/sediment	E-NS-sw/sd	KLIWAS
VN-03469	2010	Dorum	Seawater/sediment	E-NS-sw/sd	KLIWAS
VN-03470	2010	Dorum	Seawater/sediment	E-NS-sw/sd	KLIWAS
VN-03471	2010	Dorum	Seawater/sediment	E-NS-sw/sd	KLIWAS
VN-03472	2010	Dorum	Seawater/sediment	E-NS-sw/sd	KLIWAS
VN-03475	2010	Wremen	Seawater/sediment	E-NS-sw/sd	KLIWAS
VN-03492	2011	n.s.	Seawater/sediment	E-NS-sw/sd	KLIWAS
VN-03503	2009	n.s.	Seawater/sediment	E-NS-sw/sd	KLIWAS
VN-04219	2013	Emden	Seawater	E-NS-sw	AWI
VN-04223	2013	Wangerland	Seawater	E-NS-sw	AWI
VN-04226	2013	Wilhelmshaven	Seawater	E-NS-sw	AWI
VN-04231	2013	Emden	Seawater	E-NS-sw	AWI
VN-04233	2013	Cuxhaven	Seawater	E-NS-sw	AWI
VN-10012	2011	Lower Saxony Wadden Sea	Oyster	E-NS-bm	IFF
VN-10013	2011	Lower Saxony Wadden Sea	Oyster	E-NS-bm	IFF
VN-10127	2012	Lower Saxony Wadden Sea	Oyster[1]	E-NS-bm	IFF
VN-10130	2012	n.s.	Oyster[1]	E-NS-bm	IFF
VN-10131	2012	n.s.	Oyster[1]	E-NS-bm	IFF
VN-10133	2012	n.s.	Oyster[1]	E-NS-bm	IFF
VN-10137	2012	n.s.	Blue mussel	E-NS-bm	IFF
VN-10143	2012	Lower Saxony Wadden Sea	Blue mussel[1]	E-NS-bm	IFF
VN-10144	2012	Lower Saxony Wadden Sea	Blue mussel[1]	E-NS-bm	IFF
VN-10145	2012	Lower Saxony Wadden Sea	Blue mussel[1]	E-NS-bm	IFF
VN-10146	2012	Lower Saxony Wadden Sea	Blue mussel[1]	E-NS-bm	IFF
VN-10150	2012	Lower Saxony Wadden Sea	Blue mussel[1]	E-NS-bm	IFF
VN-10156	2012	Lower Saxony Wadden Sea	Blue mussel	E-NS-bm	IFF
VN-10159	2012	Lower Saxony Wadden Sea	Blue mussel	E-NS-bm	IFF
VN-10162	2012	Lower Saxony Wadden Sea	Blue mussel	E-NS-bm	IFF
VN-10191	2013	Lower Saxony Wadden Sea	Blue mussel[1]	E-NS-bm	IFF
VN-10192	2013	Lower Saxony Wadden Sea	Blue mussel[1]	E-NS-bm	IFF
VN-10196	2013	Lower Saxony Wadden Sea	Blue mussel[1]	E-NS-bm	IFF
VN-10197	2013	Lower Saxony Wadden Sea	Blue mussel[1]	E-NS-bm	IFF
VN-10198	2013	Lower Saxony Wadden Sea	Blue mussel[1]	E-NS-bm	IFF
VN-10204	2013	Lower Saxony Wadden Sea	Oyster[1]	E-NS-bm	IFF
VN-10205	2013	Lower Saxony Wadden Sea	Oyster[1]	E-NS-bm	IFF
VN-10206	2013	Lower Saxony Wadden Sea	Oyster[1]	E-NS-bm	IFF
VN-10207	2013	Lower Saxony Wadden Sea	Oyster[1]	E-NS-bm	IFF
VN-10208	2013	Lower Saxony Wadden Sea	Oyster[1]	E-NS-bm	IFF
VN-10320	2014	Lower Saxony Wadden Sea	Oyster[1]	E-NS-bm	IFF

Table continued					
Strain	**Year of isolation**	**Geographical origin**	**Type of infection**	**Source code[2]**	**Institutional origin**
Clinical - Germany					
VN-00168	2010	Baltic Sea	Wound infection[3]	C-G-ext	LAGuS
VN-00169	2010	Baltic Sea	Wound infection[3]	C-G-ext	LAGuS
VN-00297	1995	Baltic Sea/Szczecin Lagoon	Otitis	C-G-ext	BfR
VN-00298	1995	Baltic Sea	Otitis	C-G-ext	BfR
VN-00300	1999	n.s.	Diarrhea, peritonitis	C-G-int	BfR
VN-00302	2012	North Sea/Bremerhaven	Diarrhea	C-G-int	RKI
VN-00305	2012	n.s.	Otitis	C-G-ext	RKI
VN-00307	2012	n.s.	Otitis	C-G-ext	RKI
VN-00533	2016	Baltic Sea/Ueckermünde Lagoon	Wound infection	C-G-ext	BfR
VN-00534	2017	Baltic Sea/Greifswald	Otitis	C-G-ext	BfR

AWI: Alfred Wegener Institute, Heligoland, Germany
BfR: Federal Institute for Risk Assessment, Berlin, Germany
IFF: Institute for Fish and Fishery Products, Cuxhaven, Germany (LAVES: Lower Saxony State Office for Consumer Protection and Food Safety)
IFH: Institute of Food Safety and Food Hygiene, Department of Veterinary Medicine, Free University of Berlin, Germany
KLIWAS: Research program “Impacts of climate change on waterways and navigation“
LAGuS: State Office for Health and Social Affairs, Rostock, Germany
RKI: Robert Koch Institute, Berlin, Germany

n.s., not specified.

[1] Organism from cultivation of bivalve mollusks (primary production).
[2] The source code is explained in Table 1.
[3] Isolates from the same patient.
[4] Environmental strains were isolated within the German research programs VibrioNet (AWI, IFF, IFH, LAGuS) and KLIWAS.

Table S2. Primers used for species confirmation, multilocus sequence typing (MLST), and PCR typing of virulence genes.

Gene/Target	Primer	Sequence (5‘ to 3‘)	Amplicon size (bp)	T_a (°C)	References
Species confirmation, characterization and subtyping					
toxR	UtoxF	GASTTTGTTTGGCGYGARCAAGGTT	640	59	Bauer et al. (2007)
	VctoxR	GGTTAGCAACGATGCGTAAG			
ctxA	ctxA1	CTCAGACGGGATTTGTTAGGCACG	301		Shirai et al. (1991);
	ctxA2	TCTATCTCTGTAGCCCCTATTACG			Chatterjee et al. (2009)
rfb O1 cluster	O1F	GTTTCACTGAACAGATGGG	192		Hoshino et al. (1998);
	O1R	GGTCATCTGTAAGTACAAC			Mantri et al. (2006);
rfb O139 cluster	O139F	AGCCTCTTTATTACGGGTGG	449		Shuan et al. (2009)
	O139R	GTCAAACCCGATCGTAAAGG			
Multilocus Sequence Typing (MLST)					
adk	V-chol-adk F	CATCATTCTTCTCGGTGCTC	592	59	Octavia et al. (2013)
	V-chol-adk R	AGTGCCGTCAAACTTCAGGTA			
gyrB	V-chol-gyrB F	GTACGTTTCTGGCCTAGTGC	749	58	Octavia et al. (2013)
	V-chol-gyrB R	GGGTCTTTTTCCTGACAATC			
mdh	V-chol-mdh F	ATGAAAGTCGCTGTTATTGG			Octavia et al. (2013)
	V-chol-mdh R1[a]	GCCGCTTGGCCCATAGAAAG	692	62	
	V-chol-mdh R2	TAGCTTGATAGGTTGGG	810	51	
metE	V-chol-metE F	CGGGTGACTTTGCTTGGT	827	56	Octavia et al. (2013)
	V-chol-metE R	CAGATCGACTGGGCTGTG			
pntA	V-chol-pntA F1[a]	CTTTGATGGAAAAACTCTCA	740	52	Octavia et al. (2013)
	V-chol-pntA F2	GGCCAGCCCAAAATCCT	758	52	
	V-chol-pntA R	GATATTGCCGTCTTTTTCTT			
purM	V-chol-purM F	GGTGTCGATATTGATGCAGG	734	58	Octavia et al. (2013)
	V-chol-purM R	GGAATGTTTTCCCAGAAGCC			
pyrC	V-chol-pyrC F	ATCATGCCTAACACGGTTCC	726	57	Octavia et al. (2013)
	V-chol-pyrC R	TTCAAACACTTCGGCATA			
PCR typing of virulence genes					
chxA	VC-chxA-F	TGTGTGATGATGCTTCTGG	2000	52	Awasthi et al. (2013)
	VC-chxA-R	TTATTTCAGTTCATCTTTTCGC			
	VC-Cholix-fo	GCAACAACACTGAAAACGAG	397	56	This study
	VC-Cholix-re	TCCTCATCAATCGCCAATTC			
$hlyA^{CL/ET}$	hlyA-489F	GGCAAACAGCGAAACAAATACC	727/738	60	Rivera et al. (2001);
$hlyA^{ET}$	hlyA-744F	GAGCCGGCATTCATCTGAAT	481		Singh et al. (2001)
$hlyA^{CL/ET}$	hlyA-1184R	CTCAGCGGGCTAATACGGTTTA			
mshA	VC0409-F	ATTCTCGGTATCTTGGCCGTC	459	62	Rahman et al. (2008)
	VC0409-R	ACAAGCAGTTCCAGCAACCC			

Table continued

Gene/Target	Primer	Sequence (5‘ to 3‘)	Amplicon size (bp)	T_a (°C)	References
PCR typing of virulence genes					
ompU	ompU-F	ACGCTGACGGAATCAACCAAAG	869	60	Rivera et al. (2001);
	ompU-R	GCGGAAGTTTGGCTTGAAGTAG			Singh et al. (2001)
rstR^{Calc}	VC-rstR-calc-F	CCAGCATTTCTGTTTCTTTG	104	56	Rahman et al. (2008)
	VC-rstR-calc-R	GGCAACAAAGCACATTAAAG			
rstR^{CL}	VC-rstR-class-F	CTCATCAGCAAAGCCTCCATC	241	62	Rahman et al. (2008)
	VC-rstR-class-R	TAGCAAATGGTATCGGCGTTG			
rstR^{ET}	VC1455-F	AGCCAACCAAAGAAAGGCAAT	186	62	Rahman et al. (2008)
	VC1455-R	TCATCTGTGGCCCATCTTCC			
rtxA[b]	VC1451-F	GATTCTTCCGTTCAAGCTCCG	2571	63	Schirmeister et al. (2014)
	VC1451-R	TGGTTCAGGCTGTTGCACAC			
rtxC	VC1450-F	TGCAAATCTCACATTAGCGCA	430	63	Schirmeister et al. (2014)
	VC1450-R	CCACTGCACCTTTCGGATACA			
tcpA$^{CL/ET}$	tcpA-F_Class-ET	CACGATAAGAAAACCGGTCAAGAG		60	Mantri et al. (2006)
tcpA^{CL}	tcpA-R_class	TTACCAAATGCAACGCCGAATG	620		
tcpA^{ET}	tcpA-R_ET	AATCATGAGTTCAGCTTCCCGC	823		
TTSS *vcsC2*	TTSS_vcsC2-A	CGTTACCGATGCTATGGGT	535	60	Chatterjee et al. (2009)
	TTSS_vcsC2-B	AGAAGTCGGTTGTTTCGGTAA			
TTSS *vcsN2*	TTSS_vcsN2-A	CAGTTGAGCCAATTCCATT	484	55	Chatterjee et al. (2009)
	TTSS_vcsN2-B	GACCAAACGAGATAATG			
TTSS *vcsV2*	TTSS_vcsV2-A	TTTGGCTCACTTGATGGG	742	55	Chatterjee et al. (2009);
	TTSS_vcsV2-B	GCCACATCATTGCTTGCT			Dziejman et al. (2005)
TTSS *vspD*	TTSS_vspD-A	AACTCGAAGAGCAGAAAAAAGC	422	55	Chatterjee et al. (2009);
	TTSS_vspD-B	CTTCCCGCTTTTGATGAAATG			Dziejman et al. (2005)
VSP-1	Vch-VspI-fo	GCTCTCGCCAGCAAGGAGCTG	1700	60	Rahman et al. (2008)
	Vch-VspI-re	CCGTCGAAGTGAACGGCGAAC			
VSP-2	Vch-VspII-fo	TGCCCATTCCGCTAAGTGTTC	800	60	Rahman et al. (2008)
	Vch-VspII-re	GCAAAAGCACTGCGTAAACTG			

Calc, Calcutta; CL, Classical; ET, El Tor; T_a, annealing temperature.

[a] alternative primer

[b] VC1451 of *Vibrio cholerae* O1 biovar El Tor str. N16961.

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Table S3. Criteria for the interpretation of hemolytic activity data of *Vibrio cholerae* non-O1, non-O139 isolates from German coastal waters analyzed in this study.

Mean diameter of the hemolytic halo (mm)	Category[a]
No hemolysis	-
Hemolysis only under the macrocolony or ≤ 1	+
> 1 and < 4	++
≥ 4	+++

[a] (-), non-hemolytic; (+), weakly hemolytic; (++), intermediately hemolytic; (+++), strongly hemolytic.

Table S4. Clinical *Vibrio cholerae* strains used as amplification controls in PCR typing of virulence genes.

Strain	Species	Year	Geographical origin	Type of infection	Reference	Genes/Features[1]
VN-00211	*Vibrio cholerae* non-O1, non-O139	2011	Italy	Diarrhea	RKI	*rtxA*[2]
VN-00300	*Vibrio cholerae* non-O1, non-O139	1999	Germany	Diarrhea, peritonitis	BfR	*chxA*
VN-00303	*Vibrio cholerae* non-O1, non-O139	2012	Hungary	Otitis	RKI	negative for $hlyA^{CL}$, $hlyA^{ET}$
VN-00308	*Vibrio cholerae* non-O1, non-O139	2012	Romania	Diarrhea	RKI	TTSS *vcsC2*/*vcsN2*/*vspD*/*vcsV2*; *rtxC*
VN-00315	*Vibrio cholerae* non-O1, non-O139	2011	Cuba	Diarrhea	AGES	VSP-2
1576	*Vibrio cholerae* O1	1959	Thailand	Diarrhea	BfR	classical biotype; $rstR^{CL}$
1360	*Vibrio cholerae* O1	1959	Thailand	Diarrhea	BfR	classical biotype; *toxR*; *ctxA*; O1 *rfb*; $hlyA^{CL}$; *ompU*; $tcpA^{CL}$, $tcpA^{ET}$; negative for VSP-1, VSP-2
MO45	*Vibrio cholerae* O139	1992	India	Diarrhea	BfR	El Tor biotype; *toxR*; *ctxA*; O139 *rfb*; $hlyA^{ET}$; *mshA*; $tcpA^{ET}$; VSP-1; $rstR^{ET}$

AGES: Austrian Agency for Health and Food Safety, Vienna, Austria
BfR: Federal Institute for Risk Assessment, Berlin, Germany
RKI: Robert Koch Institute, Berlin, Germany

CL, Classical; ET, El Tor.

[1] Only genes/features relevant to PCR typing of virulence genes are given.
[2] VC1451 of *Vibrio cholerae* O1 biovar El Tor str. N16961.

Table S5. MLST allelic profiles and resulting sequence types of *Vibrio cholerae* non-O1, non-O139 isolates from German coastal waters and from German clinical samples analyzed in this study.[a]

Strain	Source code[b]	Allelic profile							Sequence type
		adk	*gyrB*	*mdh*	*metE*	*pntA*	*purM*	*pyrC*	
Environmental - Baltic Sea									
VN-00278	E-BS-sw	**68**	**73**	14	**131**	**84**	**62**	**94**	**488**
VN-00455	E-BS-sw	**60**	**75**	**69**	**120**	**87**	1	n.d.[c]	n.d.
VN-00456	E-BS-sw	**72**	38	14	**126**	**79**	**60**	**92**	**497**
VN-00457	E-BS-sw	48	**84**	46	**127**	**88**	1	**105**	**467**
VN-00458	E-BS-sw	14	30	**66**	91	66	41	73	**454**
VN-00459	E-BS-sw	18	61	**67**	**156**	71	1	35	330
VN-00460	E-BS-sw	3	48	29	36	25	1	12[d]	**445**
VN-00461	E-BS-sw	**72**	**65**	11	**113**	**91**	**60**	**92**	**498**
VN-00462	E-BS-sw	**70**	36	14	**117**	**85**	**60**	**102**	**492**
VN-00463	E-BS-sw	**60**	**70**	**69**	**122**	**87**	1	**113**	**475**
VN-00464	E-BS-sw	**61**	55	14	**146**	**75**	1	**94**	**479**
VN-00465	E-BS-sw	14	49	15	47	**77**	1	**120**	**455**
VN-00466	E-BS-sw	2	23	57	9	59	1	35	**441**
VN-00468	E-BS-sw	**58**	48	**73**	**139**	47	**63**	**90**	**472**
VN-00469	E-BS-sw	**78**	**86**	14	**138**	1	**52**	**115**	**504**
VN-00470	E-BS-sw	**72**	**65**	45	**113**	**91**	**60**	**92**	**499**
VN-00471	E-BS-sw	**70**	36	14	**117**	**85**	**60**	**102**	**492**
VN-00472	E-BS-sw	**70**	36	14	**117**	**85**	**60**	**102**	**492**
VN-00473	E-BS-sw	**70**	**65**	14	**129**	**92**	**60**	**102**	**493**
VN-00474	E-BS-sw	**66**	**86**	14	**143**	47	1	**92**	**485**
VN-00475	E-BS-sw	49	1	**74**	**147**	**93**	**56**	**119**	**468**
VN-00476	E-BS-sw	14	5	14	**123**	**84**	**60**	**91**	**456**
VN-00477	E-BS-sw	**60**	**75**	**69**	**120**	**87**	1	n.d.[c]	n.d.
VN-02995	E-BS-sw	**68**	**73**	14	**131**	**84**	**62**	**94**	**488**
VN-03901	E-BS-sw/sd	18	5	14	43	71	**58**	35	**462**
VN-03902	E-BS-sw/sd	38	5	**75**	**152**	62	1	**121**	**465**
VN-03903	E-BS-sw	48	**84**	46	**127**	**88**	1	**105**	**467**
VN-03907	E-BS-sw/sd	3	48	29	36	25	3	12[d]	**446**
VN-03908	E-BS-sw	2	5	14	**122**	**91**	1	**103**[d]	**442**
VN-03911	E-BS-sw	**63**	5	14	**129**	6	1	67	**481**
VN-03916	E-BS-sd	1	38	14	**122**	**91**	**60**	**103**	**433**
VN-03918	E-BS-sw/sd	53	36	7	9	**90**	1	65	**469**
VN-03939	E-BS-sd	**63**	5	14	**129**	6	1	67	**481**
VN-03942	E-BS-sw/sd	**66**	5	8	36	6	1	**92**	**486**
VN-03944	E-BS-sw/sd	14	5	**74**	**137**	5	**64**	**110**	**458**
VN-03949	E-BS-sw/sd	18	5	15	48	17	1	45	**463**
VN-03951	E-BS-sw/sd	**73**	**85**	14	**149**	19	1	**96**	303
VN-03954	E-BS-sw/sd	1	5	14	**116**	6	1	**92**	**434**
VN-03955	E-BS-sd	1	5	14	**142**	6	1	**92**	**435**
VN-03958	E-BS-sw/sd	13	38	11	**148**	64	**59**	**118**	**451**
VN-03963	E-BS-sw	**70**	**65**	**73**	**116**	13	**60**	**117**	**494**
VN-04241	E-BS-sw	18	**81**	14	**130**	**86**	41	43	**464**
VN-04250	E-BS-sw	14	38	14	**117**	47	**60**	**116**	**459**
VN-05169	E-BS-sw	**65**	5	2	**131**	**80**	**60**	**103**	**484**
VN-05172	E-BS-sw	**74**	5	**62**	**129**	6	1	67	**500**
VN-05174	E-BS-sw	53	5	**62**	**129**	6	1	67	**470**
VN-05176	E-BS-sw	**70**	36	14	**117**	**85**	**60**	**102**	**492**
VN-05177	E-BS-sw	13	**77**	14	**151**	66	**61**	**122**	**452**
VN-05185	E-BS-sw	1	**70**	**74**	**157**	38	1	**111**	**436**
VN-05301	E-BS-sw	14	5	14	192	18	**60**	**96**	**460**

Table continued									
Strain	**Source code[b]**	**Allelic profile**							**Sequence type**
		adk	***gyrB***	***mdh***	***metE***	***pntA***	***purM***	***pyrC***	
Environmental - North Sea									
VN-02808	E-NS-sw	57	76	14	115	18	1	101	336
VN-02825	E-NS-sw	57	76	14	115	18	1	101	336
VN-02923	E-NS-sw	57	76	14	115	18	1	101	336
VN-03012	E-NS-sw	1	5	**74**	**119**	**80**	**53**	**106**	**432**
VN-03213	E-NS-sw	**65**	1	14	**140**	**93**	**62**	**90**	**483**
VN-03301	E-NS-sw/sd	14	**68**	**75**	19	9	**56**	**114**	**457**
VN-03361	E-NS-sw/sd	**76**	**82**	**76**	**150**	**83**	**65**	67	**502**
VN-03377	E-NS-sw/sd	13	5	15	59	**73**	1	67	**449**
VN-03405	E-NS-sw/sd	57	38	**70**	**125**	18	**54**	**95**	**471**
VN-03407	E-NS-sw/sd	57	38	**70**	**125**	18	**54**	**95**	**471**
VN-03428	E-NS-sw/sd	**79**	5	**68**	**159**	**81**	**57**	**109**	**505**
VN-03460	E-NS-sw/sd	1	5	**74**	**119**	**80**	**53**	**106**	**432**
VN-03469	E-NS-sw/sd	1	5	**74**	**119**	**80**	**53**	**106**	**432**
VN-03470	E-NS-sw/sd	13	1	**64**	**144**	5	1	**108**	**450**
VN-03471	E-NS-sw/sd	1	5	**74**	**119**	**80**	**53**	**106**	**432**
VN-03472	E-NS-sw/sd	1	5	**74**	**119**	**80**	**53**	**106**	**432**
VN-03475	E-NS-sw/sd	1	5	**74**	**119**	**80**	**53**	**106**	**432**
VN-03492	E-NS-sw/sd	1	5	**74**	**119**	**80**	**53**	**106**	**432**
VN-03503	E-NS-sw/sd	**77**	38	**65**	**154**	**91**	1	**93**	**503**
VN-04219	E-NS-sw	**58**	**78**	**63**	**128**	61	8	**100**	**473**
VN-04223	E-NS-sw	**69**	41	15	47	**74**	1	83	**489**
VN-04226	E-NS-sw	3	**79**	14	**153**	66	41	**98**	**447**
VN-04231	E-NS-sw	**58**	**78**	**63**	**128**	61	8	**100**	**473**
VN-04233	E-NS-sw	2	38	14	**123**	13	**60**	**103**	**443**
VN-10012	E-NS-bm	**71**	**74**	**76**	**133**	3	9	39	**495**
VN-10013	E-NS-bm	**71**	**74**	**76**	**133**	3	9	39	**495**
VN-10127	E-NS-bm	**66**	5	8	36	6	1	**92**	**486**
VN-10130	E-NS-bm	**60**	**65**	**71**	**136**	**80**	1	**104**	**476**
VN-10131	E-NS-bm	**60**	**65**	**71**	**136**	**80**	1	**104**	**476**
VN-10133	E-NS-bm	**60**	**83**	14	**126**	**76**	**63**	**91**	**477**
VN-10137	E-NS-bm	**65**	1	14	**140**	**93**	**62**	**90**	**483**
VN-10143	E-NS-bm	13	**71**	**62**	**124**	3	9	39	**453**
VN-10144	E-NS-bm	13	**71**	**62**	**124**	3	9	39	**453**
VN-10145	E-NS-bm	13	**71**	**62**	**124**	3	9	39	**453**
VN-10146	E-NS-bm	13	**71**	**62**	**124**	3	9	39	**453**
VN-10150	E-NS-bm	47	1	14	**160**	18	17	66	**466**
VN-10156	E-NS-bm	**60**	36	61	**134**	**82**	**56**	**89**	**478**
VN-10159	E-NS-bm	**60**	36	61	**134**	**82**	**56**	**89**	**478**
VN-10162	E-NS-bm	**60**	36	61	**134**	**82**	**56**	**89**	**478**
VN-10191	E-NS-bm	13	**71**	**62**	**124**	3	9	39	**453**
VN-10192	E-NS-bm	13	**71**	**62**	**124**	3	9	39	**453**
VN-10196	E-NS-bm	**59**	23	14	**135**	3	1	**97**	**474**
VN-10197	E-NS-bm	14	**64**	15	**121**	3	8	**99**	**461**
VN-10198	E-NS-bm	14	**64**	15	**121**	3	8	**99**	**461**
VN-10204	E-NS-bm	**67**	44	14	**132**	66	**55**	**88**	**487**
VN-10205	E-NS-bm	**67**	44	14	**132**	66	**55**	**88**	**487**
VN-10206	E-NS-bm	**67**	44	14	**132**	66	**55**	**88**	**487**
VN-10207	E-NS-bm	**67**	44	14	**132**	66	**55**	**88**	**487**
VN-10208	E-NS-bm	1	**69**	14	1	64	1	**93**	**437**
VN-10320	E-NS-bm	**59**	23	14	**135**	3	1	**97**	**474**

Table continued									
Strain	**Source code[b]**	**Allelic profile**							**Sequence type**
		adk	***gyrB***	***mdh***	***metE***	***pntA***	***purM***	***pyrC***	
Clinical - Germany									
VN-00168	C-G-ext	**72**	**65**	45	**114**	**91**	**60**	**92**	**496**
VN-00169	C-G-ext	**72**	**65**	45	**114**	**91**	**60**	**92**	**496**
VN-00297	C-G-ext	**70**	**65**	14	**129**	**92**	1	**107**	**490**
VN-00298	C-G-ext	**70**	38	14	**155**	**85**	**60**	**102**	**491**
VN-00300	C-G-int	13	**74**	**76**	**133**	3	9	39	**448**
VN-00302	C-G-int	2	44	11	64	3	8	43	**438**
VN-00305	C-G-ext	**64**	**65**	11	**141**	2	**60**	**112**	**482**
VN-00307	C-G-ext	**70**	**65**	14	**129**	**92**	1	**107**	**490**
VN-00533[e]	C-G-ext	**70**	**65**	14	**218**	5	**60**	**102**	**589**
VN-00534[e]	C-G-ext	**60**	**83**	14	**143**	**76**	**53**	**91**	**590**

n.d., not determined.

[a] Alleles and sequence types that were new to PubMLST database are in bold print.

[b] The source code is explained in Table 1.

[c] PCR amplification of *pyrC* failed.

[d] Fixed maximum length of *Vibrio cholerae pyrC* allele sequences in PubMLST database (449 bp) required the removal of a 6-nt insertion (GCCACA) between nucleotide 23 and 24. The resulting *pyrC* allele is given.

[e] MLST alleles were determined from *de novo* assemblies of genomes with the web-based tool "MLST 1.8" of the Center for Genomic Epidemiology (Larsen et al., 2012). New allele sequences were verified by PCR amplification and partial gene sequencing and finally submitted to the PubMLST database for assignment of new allele numbers (see Material and Methods).

References

Larsen, M. V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R. L., et al. (2012). Multilocus sequence typing of total-genome-sequenced bacteria. *J. Clin. Microbiol.* 50, 1355-1361. doi: 10.1128/JCM.06094-11

Table S6. MLST properties of the *Vibrio cholerae* non-O1, non-O139 populations analyzed in this study.

		Total[a] (n = 108)	Environmental[a] (n = 98)	Baltic Sea[a] (n = 48)	North Sea (n = 50)	Clinical (n = 10)	PubMLST[b]
Alleles							
adk	**n_t (p_t)**	32 (13.0%)	31 (13.1%)	22 (14.4%)	18 (14.0%)	6 (14.3%)	105 (9.9%)
	n_n (p_n)	20 (62.5%)	19 (61.3%)	12 (54.5%)	11 (61.1%)	4 (66.7%)	
gyrB	**n_t (p_t)**	30 (12.2%)	30 (12.7%)	18 (11.8%)	18 (14.0%)	5 (11.9%)	116 (10.9%)
	n_n (p_n)	17 (56.7%)	17 (56.7%)	8 (44.4%)	10 (55.6%)	3 (60.0%)	
mdh	**n_t (p_t)**	25 (10.2%)	25 (10.5%)	17 (11.1%)	14 (10.9%)	4 (9.5%)	121 (11.4%)
	n_n (p_n)	14 (56.0%)	14 (56.0%)	7 (41.2%)	10 (71.4%)	1 (25.0%)	
metE	**n_t (p_t)**	56 (22.8%)	51 (21.5%)	30 (19.6%)	25 (19.4%)	8 (19.0%)	270 (25.5%)
	n_n (p_n)	44 (78.6%)	40 (78.4%)	23 (76.7%)	19 (76.0%)	7 (87.5%)	
pntA	**n_t (p_t)**	38 (15.4%)	37 (15.6%)	28 (18.3%)	18 (14.0%)	7 (16.7%)	154 (14.5%)
	n_n (p_n)	19 (50.0%)	19 (51.4%)	13 (46.4%)	9 (50.0%)	4 (57.1%)	
purM	**n_t (p_t)**	20 (8.1%)	20 (8.4%)	12 (7.8%)	14 (10.9%)	5 (11.9%)	96 (9.1%)
	n_n (p_n)	14 (70.0%)	14 (70.0%)	9 (75.0%)	9 (64.3%)	2 (40.0%)	
pyrC	**n_t (p_t)**	45 (18.3%)	43 (18.1%)	26 (17.0%)	22 (17.1%)	7 (16.7%)	198 (18.7%)
	n_n (p_n)	34 (75.6%)	32 (74.4%)	19 (73.1%)	17 (77.3%)	5 (71.4%)	
total	**n_t (p_t)**	246 (100%)	237 (100%)	153 (100%)	129 (100%)	42 (100%)	1060 (100%)
	n_n (p_n)	162 (65.9%)	155 (65.4%)	91 (59.5%)	85 (65.9%)	26 (61.9%)	
Sequence types							
	n_t	74	66	42	25	8	718
	n_n (p_n)	71 (95.9%)	63 (95.5%)	40 (95.2%)	24 (96.0%)	8 (100.0%)	

n, number of isolates; n_t, total number of alleles/sequence types; p_t, proportion of n_t of locus X as a percentage of n_t of all loci of the respective *V. cholerae* population; n_n, number of new alleles/ sequence types; p_n, proportion of n_n as a percentage of n_t.

[a] Two *V. cholerae* strains from the Baltic Sea, VN-00455 and VN-00477, were excluded from the analyses due to failing *pyrC* amplification.

[b] PubMLST database as of November 5, 2018.

Table S7. Presence/absence of virulence-associated genes and gene clusters in *Vibrio cholerae* non-O1, non-O139 isolates from German coastal waters and from German clinical samples based on PCR data.

Strain	Source code[a]	Virulence-associated genes and gene clusters																					
		rfb O1	*rfb* O139	*ctxA*	*toxR*	*tcpA* CL	*tcpA* ET	*rstR* CL	*rstR* ET	*rstR* Calc	VSP-1	VSP-2	*hlyA* CL	*hlyA* ET	*mshA*	*ompU*	*rtxA*[g]	*rtxC*	*chxA*	TTSS *vcsC2*	TTSS *vcsN2*	TTSS *vspD*	TTSS *vcsV2*
Environmental - Baltic Sea																							
VN-00278	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
VN-00455	E-BS-sw	-	-	-	(+)	-	-	-	-	-	(+)[c]	-	-	+	-	+	-	+	-	-	-	-	-
VN-00456	E-BS-sw	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-00457	E-BS-sw	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	+	-	-	+	+	-	-	-	-	-
VN-00458	E-BS-sw	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	+	-	+	-	+	+	-	-	-	-
VN-00459	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	(+)[c]	+	+	+	+	-	+	+	+	+
VN-00460	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
VN-00461	E-BS-sw	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-00462	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-00463	E-BS-sw	-	-	-	(+)	-	-	-	-	-	(-)[b]	-	-	(+)[d]	-	+	(+)	+	-	-	-	-	-
VN-00464	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-00465	E-BS-sw	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	-	-	+	-	-	-	-	-
VN-00466	E-BS-sw	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	-	+	+	+	+	-	-	-	-
VN-00468	E-BS-sw	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-00469	E-BS-sw	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	-	+	-	+	-	-	-	-	-
VN-00470	E-BS-sw	-	-	-	+	-	-	-	-	-	(+)[c]	(+)[c]	-	+	-	+	-	+	-	-	-	-	-
VN-00471	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-00472	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-00473	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-00474	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-00475	E-BS-sw	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-00476	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-00477	E-BS-sw	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-02995	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
VN-03901	E-BS-sw/sd	-	-	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-
VN-03902	E-BS-sw/sd	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	+	-	(+)	+	+	+	-	-	-	-
VN-03903	E-BS-sw	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	+	-	-	(+)	+	-	-	-	-	-
VN-03907	E-BS-sw/sd	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
VN-03908	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-03911	E-BS-sw	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	+	-	-	+	+	-	-	-	-	-
VN-03916	E-BS-sd	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	-	+	-	+	-	-	-	-	-
VN-03918	E-BS-sw/sd	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	+	-	-	-	-
VN-03939	E-BS-sd	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	+	-	-	+	+	-	-	-	-	-
VN-03942	E-BS-sw/sd	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-03944	E-BS-sw/sd	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-03949	E-BS-sw/sd	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+	+	+
VN-03951	E-BS-sw/sd	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	+	+
VN-03954	E-BS-sw/sd	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-03955	E-BS-sd	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-03958	E-BS-sw/sd	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	+	-	-	-	-

Table continued

Strain	Source code[a]	Virulence-associated genes and gene clusters																				
		rfb O1	*rfb* O139	*ctxA*	*toxR*	*tcpA* CL	*tcpA* ET	*rstR* CL	*rstR* ET	*rstR* Calc	VSP-1	VSP-2	*hlyA* CL	*hlyA* ET	*mshA*	*ompU*	*rtxA*[g]	*rtxC*	*chxA*	TTSS *vcsC2*	TTSS *vcsN2*	TTSS *vspD*	TTSS *vcsV2*
VN-03963	E-BS-sw	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	+	-	+	-	+	-	-	-	-	-
VN-04241	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	+	-	-	-	-
VN-04250	E-BS-sw	-	-	-	+	-	-	-	-	-	(-)[b]	(+)[c]	-	+	+	+	-	+	-	-	-	-	-
VN-05169	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-05172	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-
VN-05174	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-05176	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-05177	E-BS-sw	-	-	-	+	-	-	-	-	-	(+)[c]	(+)[c]	-	+	-	-	+	+	+	-	-	-	-
VN-05185	E-BS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
VN-05301	E-BS-sw	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	-	-	+	+	-	+	+	+	+
Environmental - North Sea																							
VN-02808	E-NS-sw	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	-	-	-	-	(+)	-	-	-	-	-
VN-02825	E-NS-sw	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	-	-	-	-	(+)	-	-	-	-	-
VN-02923	E-NS-sw	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	-	-	-	-	(+)	-	-	-	-	-
VN-03012	E-NS-sw	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-03213	E-NS-sw	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	+	-	+	-	+	-	-	-	-	-
VN-03301	E-NS-sw/sd	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	+	+	-	-	-	-
VN-03361	E-NS-sw/sd	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	(+)[e]	-	-	+	+	-	+	+	+	+
VN-03377	E-NS-sw/sd	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	-	-	+	+	+	-	-	-	-
VN-03405	E-NS-sw/sd	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	-	-	-	+	-	-	-	-	-
VN-03407	E-NS-sw/sd	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	-	-	-	+	-	-	-	-	-
VN-03428	E-NS-sw/sd	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	(+)[d]	+	-	-	+	-	-	-	-	-
VN-03460	E-NS-sw/sd	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-03469	E-NS-sw/sd	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-03470	E-NS-sw/sd	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	-	-	+	+	-	-	-	-
VN-03471	E-NS-sw/sd	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-03472	E-NS-sw/sd	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-03475	E-NS-sw/sd	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-03492	E-NS-sw/sd	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	+	-	+	-	+	-	-	-	-	-
VN-03503	E-NS-sw/sd	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-04219	E-NS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	+	-	-	-	-
VN-04223	E-NS-sw	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	(+)	+	+	-	-	-	-	-
VN-04226	E-NS-sw	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	+	-	+	+	+	+	-	-	-	-
VN-04231	E-NS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	+	-	-	-	-
VN-04233	E-NS-sw	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-10012	E-NS-bm	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	-	-	+	+	-	-	-	-
VN-10013	E-NS-bm	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	-	-	+	+	-	-	-	-
VN-10127	E-NS-bm	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-10130	E-NS-bm	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	+	-	+	-	+	-	-	-	-	-
VN-10131	E-NS-bm	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	+	-	+	-	+	-	-	-	-	-
VN-10133	E-NS-bm	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-10137	E-NS-bm	-	-	-	+	-	-	-	-	-	(+)[c]	-	-	+	-	+	-	+	-	-	-	-	-
VN-10143	E-NS-bm	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	-	+	-	(+)	+	-	-	-	-
VN-10144	E-NS-bm	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	-	+	-	(+)	-	-	-	-	-

Table continued

Strain	Source code[a]	Virulence-associated genes and gene clusters																				
		rfb O1	*rfb* O139	*ctxA*	*toxR*	*tcpA* CL	*tcpA* ET	*rstR* CL	*rstR* ET	*rstR* Calc	VSP-1	VSP-2	*hlyA* CL	*hlyA* ET	*mshA*	*ompU*	*rtxA*[g]	*rtxC*	*chxA*	TTSS *vcsC2*	TTSS *vcsN2*	TTSS *vspD*	TTSS *vcsV2*
VN-10145	E-NS-bm	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	-	+	-	(+)	+	-	-	-	-
VN-10146	E-NS-bm	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	-	+	-	(+)	+	-	-	-	-
VN-10150	E-NS-bm	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-
VN-10156	E-NS-bm	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-10159	E-NS-bm	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-10162	E-NS-bm	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-10191	E-NS-bm	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	-	+	-	(+)	+	-	-	-	-
VN-10192	E-NS-bm	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	-	+	-	(+)	+	-	-	-	-
VN-10196	E-NS-bm	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	+	+	+	+	-	+	+	+	+
VN-10197	E-NS-bm	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	-	+	-	+	+	-	-	-	-
VN-10198	E-NS-bm	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	-	+	-	+	+	-	-	-	-
VN-10204	E-NS-bm	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	(+)	-	+	-	-	-	-	-
VN-10205	E-NS-bm	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	(+)	-	+	-	-	-	-	-
VN-10206	E-NS-bm	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	(+)	-	+	-	-	-	-	-
VN-10207	E-NS-bm	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	(+)	-	+	-	-	-	-	-
VN-10208	E-NS-bm	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-10320	E-NS-bm	-	-	-	+	-	-	-	-	-	-	(+)[c]	-	+	+	+	+	+	-	+	+	+	+
Clinical - Germany																							
VN-00168[f]	C-G-ext	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-00169[f]	C-G-ext	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	-	-	-	-	-
VN-00297[f]	C-G-ext	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-00298[f]	C-G-ext	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-00300[f]	C-G-int	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	+	-	+	+	+	+	+	+
VN-00302[f]	C-G-int	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	+	+	+
VN-00305[f]	C-G-ext	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-00307[f]	C-G-ext	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-
VN-00533	C-G-ext	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	(+)	+	+	+
VN-00534	C-G-ext	-	-	-	+	-	-	-	-	-	(-)[b]	-	-	+	-	-	-	+	-	-	-	-	-

Calc, Calcutta; CL, Classical; ET, El Tor.

[a] The source code is explained in Table 1.

The absence of VSP-1 and VSP-2 is demonstrated by the generation of 1.7 kbp and 800 bp PCR products, respectively, as the primers bind to flanking genomic sites of the pandemic islands.

[b] PCR product of ca. 3 kbp and 4 kbp, respectively.

[c] No visible or unspecific PCR products.

The presence of *hlyA*^{ET} gene is demonstrated by the generation of 738 bp and 481 bp PCR products.

[d] 738 bp PCR product only weakly visible.

[e] 481 bp PCR product only weakly visible.

[f] The presence/absence data of virulence-associated genes (except *chxA*) and gene clusters were obtained from Schirmeister et al. (2014).

[g] VC1451 of *Vibrio cholerae* O1 biovar El Tor str. N16961.

References

Schirmeister, F., Dieckmann, R., Bechlars, S., Bier, N., Faruque, S. M., and Strauch, E. (2014). Genetic and phenotypic analysis of *Vibrio cholerae* non-O1, non-O139 isolated from German and Austrian patients. *Eur. J. Clin. Microbiol. Infect. Dis.* 33, 767-778. doi: 10.1007/s10096-013-2011-9

Table S8. Hemolytic activity of *Vibrio cholerae* non-O1, non-O139 isolates from German coastal waters analyzed in this study.

Strain	Source code[a]	Hemolytic activity[b]	
		Sheep erythrocytes	Human erythrocytes
Environmental - Baltic Sea			
VN-00278	E-BS-sw	+++	+++
VN-00455	E-BS-sw	++	++
VN-00456	E-BS-sw	+	+
VN-00457	E-BS-sw	++	++
VN-00458	E-BS-sw	++	++
VN-00459	E-BS-sw	++	++
VN-00460	E-BS-sw	++	++
VN-00461	E-BS-sw	++	++
VN-00462	E-BS-sw	++	++
VN-00463	E-BS-sw	++	++
VN-00464	E-BS-sw	-	++
VN-00465	E-BS-sw	++	++
VN-00466	E-BS-sw	++	++
VN-00468	E-BS-sw	+	++
VN-00469	E-BS-sw	++	++
VN-00470	E-BS-sw	++	++
VN-00471	E-BS-sw	+	++
VN-00472	E-BS-sw	++	++
VN-00473	E-BS-sw	++	++
VN-00474	E-BS-sw	+	++
VN-00475	E-BS-sw	++	++
VN-00476	E-BS-sw	+	++
VN-00477	E-BS-sw	++	++
VN-02995	E-BS-sw	++	++
VN-03901	E-BS-sw/sd	+++	+++
VN-03902	E-BS-sw/sd	++	++
VN-03903	E-BS-sw	++	++
VN-03907	E-BS-sw/sd	++	++
VN-03908	E-BS-sw	+++	+++
VN-03911	E-BS-sw	++	++
VN-03916	E-BS-sd	++	+++
VN-03918	E-BS-sw/sd	++	++
VN-03939	E-BS-sd	+++	+++
VN-03942	E-BS-sw/sd	+	++
VN-03944	E-BS-sw/sd	+++	+++
VN-03949	E-BS-sw/sd	++	++
VN-03951	E-BS-sw/sd	++	+++
VN-03954	E-BS-sw/sd	+	++
VN-03955	E-BS-sd	+	++
VN-03958	E-BS-sw/sd	++	++
VN-03963	E-BS-sw	+++	+++
VN-04241	E-BS-sw	+	++
VN-04250	E-BS-sw	++	++
VN-05169	E-BS-sw	+	+
VN-05172	E-BS-sw	++	+
VN-05174	E-BS-sw	+	++
VN-05176	E-BS-sw	+	++
VN-05177	E-BS-sw	+	++
VN-05185	E-BS-sw	++	++
VN-05301	E-BS-sw	++	++

Table continued			
Strain	**Source code**[a]	**Hemolytic activity**[b]	
		Sheep erythrocytes	**Human erythrocytes**
Environmental - North Sea			
VN-02808	E-NS-sw	-	-
VN-02825	E-NS-sw	-	-
VN-02923	E-NS-sw	-	-
VN-03012	E-NS-sw	++	+++
VN-03213	E-NS-sw	++	++
VN-03301	E-NS-sw/sd	++	++
VN-03361	E-NS-sw/sd	++	++
VN-03377	E-NS-sw/sd	++	++
VN-03405	E-NS-sw/sd	+++	+++
VN-03407	E-NS-sw/sd	+++	+++
VN-03428	E-NS-sw/sd	+	++
VN-03460	E-NS-sw/sd	++	+++
VN-03469	E-NS-sw/sd	++	+++
VN-03470	E-NS-sw/sd	++	++
VN-03471	E-NS-sw/sd	++	+++
VN-03472	E-NS-sw/sd	++	+++
VN-03475	E-NS-sw/sd	++	+++
VN-03492	E-NS-sw/sd	++	+++
VN-03503	E-NS-sw/sd	+	+
VN-04219	E-NS-sw	++	++
VN-04223	E-NS-sw	++	++
VN-04226	E-NS-sw	++	++
VN-04231	E-NS-sw	++	++
VN-04233	E-NS-sw	+	++
VN-10012	E-NS-bm	+	++
VN-10013	E-NS-bm	+	++
VN-10127	E-NS-bm	+	++
VN-10130	E-NS-bm	+	++
VN-10131	E-NS-bm	+	++
VN-10133	E-NS-bm	++	++
VN-10137	E-NS-bm	+++	+++
VN-10143	E-NS-bm	++	++
VN-10144	E-NS-bm	++	++
VN-10145	E-NS-bm	++	++
VN-10146	E-NS-bm	++	++
VN-10150	E-NS-bm	++	++
VN-10156	E-NS-bm	++	++
VN-10159	E-NS-bm	+++	+++
VN-10162	E-NS-bm	++	++
VN-10191	E-NS-bm	+	+
VN-10192	E-NS-bm	++	+
VN-10196	E-NS-bm	+	++
VN-10197	E-NS-bm	++	++
VN-10198	E-NS-bm	++	++
VN-10204	E-NS-bm	+	+
VN-10205	E-NS-bm	+	++
VN-10206	E-NS-bm	+	++
VN-10207	E-NS-bm	++	++
VN-10208	E-NS-bm	++	++
VN-10320	E-NS-bm	++	++

[a] The source code is explained in Table 1.

[b] (-), non-hemolytic; (+), weak; (++), intermediate; (+++), strong. The categories of hemolysis are further explained in Table S3.

Table S9. Results of the whole genome sequence analysis of 22 *Vibrio cholerae* non-O1, non-O139 strains analyzed in this study.

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Table S10. SNP distance matrix of 22 *Vibrio cholerae* non-O1, non-O139 strains analyzed in this study.

Available online: https://doi.org/10.3389/fmicb.2019.00733

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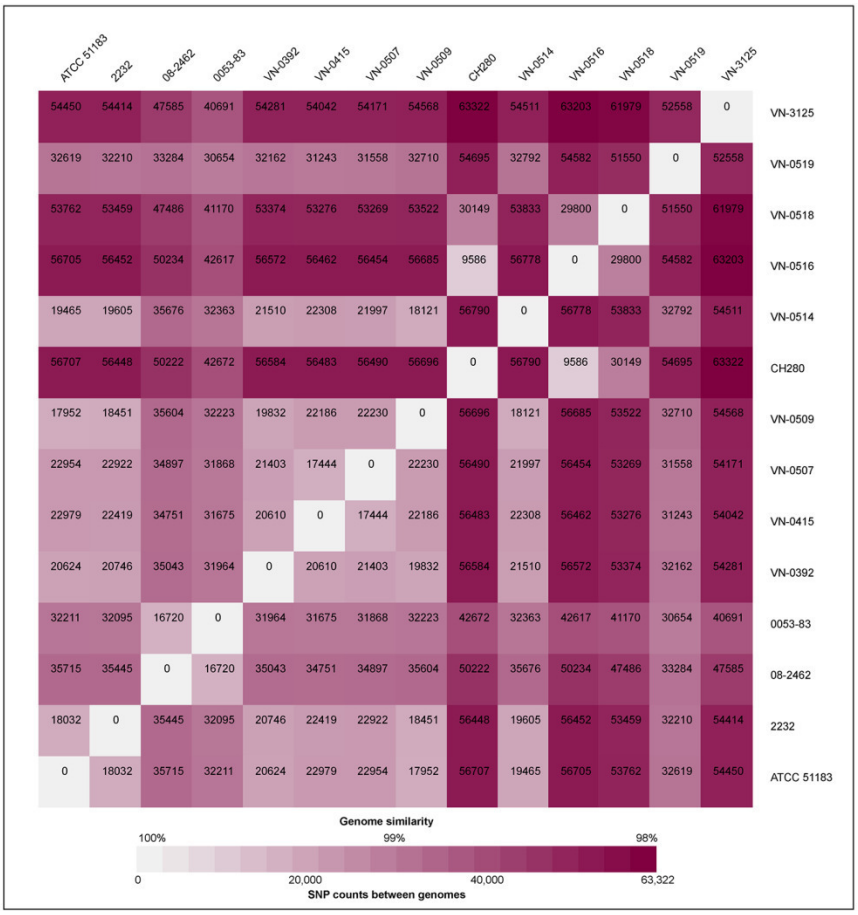

Figure S1. SNP distance matrix of 14 *V. navarrensis* strains analyzed in this study. The strains originated from different sources: veterinary/Germany (VN-0392, VN-0415, VN-0507, VN-0509, VN-0514), environmental/Germany (CH-280, VN-0516, VN-0518, VN-0519, VN-3125), human/U.S. (0053-83, 08-2462), and environmental/Spain (ATCC 51183 (identical to CIP 103381), 2232). SNP difference between each pair of strains was calculated by using CSI Phylogeny 1.4 (see Material and Methods) and is displayed numerically and graphically as a purple square with a color intensity proportional to the number of SNP differences, but inversely proportional to the percentage genome similarity.

Table S1. PCR primers used for *rpoB* sequence determination, MLSA, and genotyping of virulence-associated genes. Primer sequences were derived from whole genome shotgun contigs of the *V. navarrensis* strains 0053-83 (JMCF01000000), 08-2462 (JMCI01000000), and ATCC 51183 (JMCG01000000) or from the partial ORF12 sequence of *V. navarrensis* CH-291 (AJ314791).

Target gene	Primer	Sequence (5‘ to 3‘)	Amplicon size (bp)	Reference / Accession
rpoB	1110F	GTAGAAATCTACCGCATGATG	984	Tarr et al., 2007
	CM32b	CGGAACGGCCTGACGTTGCAT		
	1661F*	TTYATGGAYCARAACAACCC	-	Tarr et al., 2007
	1783R*	GGACCTTYAGGNGTTTCGAT		
gyrB	VigyrBF	GAAGGTGGTATTCAAGCGTT	570**	http://pubmlst.org/vibrio/info/Vibrio_primers.pdf
	VigyrBR	CGGTCATGATGATGATGTTGT		
pyrH	VipyrHdgF	CCCTAAACCAGCGTATCAACGTATTC	501**	http://pubmlst.org/vibrio/info/Vibrio_primers.pdf
	VipyrHdgR	CGGATWGGCATTTTGTGGTCACGWGC		
recA	VirecAF	TGCGCTAGGTCAAATTGAAA	462**	http://pubmlst.org/vibrio/info/Vibrio_primers.pdf
	VirecAdgR	GTTTCWGGGTTACCRAACATYACACC		
atpA	Vi_atpAdg_F	ATCGGTGACCGTCARACWGGTAAAAC	489**	http://pubmlst.org/vibrio/info/Vibrio_primers.pdf
	Vi_atpAdg_R	ATACCTGGGTCAACCGCTGG		
cps	Nav-cps-F	TGATGGTGGTGCTGGTAATG	349	JMCF01000086
	Nav-cps-R	TGTTCCAGACGTTCAAGCC		
T6SS *DUF877*	Nav-T6-DUF877-F	TCTCAGCAGAACAGCTAACC	345	JMCG01000001
	Nav-T6-DUF877-R	ACTCATCCCAAGTACAAGCC		
T6SS *DUF770*	Nav-T6-DUF770-F	GTCAAAACACCGAAGCTCTC	358	JMCG01000001
	Nav-T6-DUF770-R	CCAATCTTCAGTTCATCAGCC		
T6SS *vasD*	Nav-T6-vasD-F	GTTGTAGTGCGGCGAATATG	198	JMCG01000001
	Nav-T6-vasD-R	TATCTGGCCCGAGAACTTCAC		
tlh	Nav-tlh-F	GCACCGGAAACTCTCATAAC	425	JMCF01000074
	Nav-tlh-R	TTTCTCTTGGGCGATGTATTC		
osmY	Nav-osmY-F	ACACCAGAACCACCAAAGAG	301	JMCI01000041
	Nav-osmY-R	GCCAACAATGCGGATTTGAC		
vvhA	Nav-vvhA-F	GCCGATTTACATCACCAGCTC	438	JMCG01000001
	Nav-vvhA-R	GCCCCCACTTTACCATTCAC		

Table continued

Target gene	Primer	Sequence (5‘ to 3‘)	Amplicon size (bp)	Reference / Accession
δ-vph	Nav-vph-F	ACTTACATCGCTTTATGCCC	407	JMCI01000055
	Nav-vph-R	CGAGTGCTGATAGTAACTTCC		
hlyD	Nav-hlyD-F	AAGAACAAGCGATTCCACTC	430	JMCF01000099
	Nav-hlyD-R	AAACGACTATCCACAATGCC		
pilV	Nav-pilV-F	GAACAAAAAGCCGACTACGC	213	JMCI01000074
	Nav-pilV-R	TTTCAATGCCCCGGAGAGAG		
pilW	Nav-pilW-F	AGTAACCAACAATGCCGACCC	189	JMCF01000057
	Nav-pilW-R	TTGCCATTTTCGCCGACCAG		
ORF12	Vnav-pom-HemF	GAATACTTCCCACGGTGAAAC	547	AJ314791
	Vnav-pom-HemR	CAAACCAATCACGCTCAATG		

* Sequencing primer.
** Amplicon length for MLSA after trimming.

Table S2. Prophage analysis in *V. navarrensis* genomes of veterinary and environmental strains.

Predicted prophage	Length (kb)	Completeness	Score	Predicted CDS	Related phage (Accession No.)	GC content (%)
VN-0392_PP01	31.3	Questionable	80	21	*Vibrio* phage VP882 (NC_009016)	46.83
VN-0392_PP02	11.7	Intact	120	16	Enterobacteria phage HK630 (NC_019723)	51.46
VN-0392_PP03	15.2	Incomplete	20	22	*Shigella* phage SfII (NC_021857)	46.50
VN-0415_PP01	7.6	Incomplete	10	7	*Bacillus* phage G (NC_023719)	49.61
VN-0415_PP02	7.9	Incomplete	10	8	*Bacillus* phage G (NC_023719)	47.96
VN-0415_PP03	8.9	Incomplete	10	11	*Acanthamoeba* mimivirus (NC_014649)	49.37
VN-0415_PP04	7.3	Incomplete	10	9	*Burkholderia* phage phi1026b (NC_005284)	51.92
VN-0415_PP05	10.3	Incomplete	20	11	*P. dulcis* phage (NC_021858)	50.04
VN-0415_PP06	7.2	Incomplete	20	7	*Natrialba* phage PhiCh1 (NC_004084)	47.18
VN-0415_PP07	7.9	Incomplete	30	8	*Acanthamoeba* mimivirus (NC_014649)	47.07
VN-0507_PP01	7.3	Incomplete	10	9	*Burkholderia* phage phi1026b (NC_005284)	51.92
VN-0507_PP02	7.6	Incomplete	10	7	*Bacillus* phage G (NC_023719)	49.60
VN-0507_PP03	14.3	Incomplete	20	8	*Planktothrix* phage PaV-LD (NC_016564)	46.51
VN-0507_PP04	26.5	Incomplete	30	14	*P. dulcis* phage (NC_021858)	44.52
VN-0507_PP05	8.9	Incomplete	10	11	*Acanthamoeba* mimivirus (NC_014649)	49.44
VN-0507_PP06	7.2	Incomplete	20	7	*E. siliculosus* virus 1 (NC_002687)	47.16
VN-0507_PP07	7.9	Incomplete	10	8	*Bacillus* phage G (NC_023719)	48.02
VN-0509_PP01	10.8	Incomplete	20	21	*Shigella* phage SfIV (NC_022749)	46.99
VN-0509_PP02	20.6	Intact	150	20	Enterobacteria phage HK629 (NC_019711)	52.55
VN-0514_PP01	7.6	Incomplete	10	7	*Bacillus* phage G (NC_023719)	49.49
VN-0514_PP02	19.9	Incomplete	50	8	*Bacillus* phage vB_BanS-Tsamsa (NC_023007)	52.46
VN-0514_PP03	7.2	Incomplete	20	7	*E. siliculosus* virus 1 (NC_002687)	47.27
VN-0514_PP04	8.9	Incomplete	10	11	*Acanthamoeba* mimivirus (NC_014649)	49.42
VN-0514_PP05	13.6	Incomplete	20	7	*Planktothrix* phage PaV-LD (NC_016564)	46.18
VN-0514_PP06	7.9	Incomplete	10	8	*Bacillus* phage G (NC_023719)	47.95
VN-0514_PP07	10.3	Incomplete	20	11	*P. dulcis* phage (NC_021858)	50.09
VN-0514_PP08	7.3	Incomplete	10	8	*Burkholderia* phage phi1026b (NC_005284)	51.89
VN-0514_PP09	8.1	Incomplete	10	9	Enterobacteria phage phi92 (NC_023693)	44.60

Table continued

Predicted prophage	Length (kb)	Completeness	Score	Predicted CDS	Related phage (Accession No.)	GC content (%)
VN-0516_PP01	11.5	Intact	136	14	*Vibrio* phage VCY phi (NC_016162)	43.96
VN-0518_PP01	11.0	Questionable	83	10	*Vibrio* phage fs2 (NC_001956)	44.42
VN-0518_PP02	20.8	Incomplete	50	16	*P. dulcis* phage (NC_021858)	52.13
VN-0519_PP01	23.3	Incomplete	30	21	*Mannheimia* phage vB_MhS_1152AP2 (NC_028956)	48.67
VN-0519_PP02	41.3	Questionable	70	43	Enterobacteria phage VT2phi_272 (NC_028656)	48.83
VN-0519_PP03	46.6	Incomplete	60	20	*P. dulcis* phage (NC_021858)	49.10
VN-0519_PP04	37.0	Intact	110	46	*Vibrio* phage 12B12 (NC_021070)	50.00
VN-3125_PP01	10.9	Intact	136	15	*Vibrio* phage VCY phi (NC_016162)	45.17
VN-3125_PP02	20.6	Questionable	20	23	*Shigella* phage SfII (NC_021857)	46.42
VN-3125_PP03	20.4	Intact	140	20	Enterobacteria phage HK630 (NC_019723)	52.81
VN-3125_PP04	38.6	Intact	150	52	*Vibrio* phage 8 (NC_022747)	48.64
CH-280_PP01	14.2	Incomplete	20	7	*Planktothrix* phage PaV-LD (NC_016564)	46.63
CH-280_PP02	7.6	Incomplete	10	7	*Bacillus* phage G (NC_023719)	49.38
CH-280_PP03	7.9	Incomplete	10	8	*Bacillus* phage G (NC_023719)	47.78
CH-280_PP04	7.3	Incomplete	10	9	*Burkholderia* phage phi1026b (NC_005284)	52.12
CH-280_PP05	8.9	Incomplete	10	11	*Acanthamoeba* mimivirus (NC_014649)	49.21
CH-280_PP06	10.3	Incomplete	20	11	*P. dulcis* phage (NC_021858)	49.79
CH-280_PP07	10.1	Questionable	70	14	*Bacillus* phage G (NC_023719)	50.53

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microorganisms

MDPI

Article

Phenotypic and Genotypic Properties of *Vibrio cholerae* non-O1, non-O139 Isolates Recovered from Domestic Ducks in Germany

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Abstract: *Vibrio cholerae* non-O1, non-O139 bacteria are natural inhabitants of aquatic ecosystems and have been sporadically associated with human infections. They mostly lack the two major virulence factors of toxigenic *V. cholerae* serogroups O1 and O139 strains, which are the causative agent of cholera. Non-O1, non-O139 strains are found in water bodies, sediments, and in association with other aquatic organisms. Occurrence of these bacteria in fecal specimens of waterfowl were reported, and migratory birds likely contribute to the long-distance transfer of strains. We investigated four *V. cholerae* non-O1, non-O139 isolates for phenotypic traits and by whole genome sequencing (WGS). The isolates were recovered from organs of domestic ducks with serious disease symptoms. WGS data revealed only a distant genetic relationship between all isolates. The isolates harbored a number of virulence factors found in most *V. cholerae* strains. Specific virulence factors of non-O1, non-O139 strains, such as the type III secretion system (TTSS) or cholix toxin, were observed. An interesting observation is that all isolates possess multifunctional autoprocessing repeats-in-toxin toxins (MARTX) closely related to the MARTX of toxigenic El Tor O1 strains. Different primary sequences of the abundant OmpU proteins could indicate a significant role of this virulence factor. Phenotypic characteristics such as hemolysis and antimicrobial resistance (AMR) were studied. Three isolates showed susceptibility to a number of tested antimicrobials, and one strain possessed AMR genes located in an integron. Knowledge of the environmental occurrence of *V. cholerae* non-O1, non-O139 in Germany is limited. The source of the infection of the ducks is currently unknown. In the context of the 'One Health' concept, it is desirable to study the ecology of *V. cholerae* non-O1, non-O139, as it cannot be excluded that the isolates possess zoonotic potential and could cause infections in humans.

Keywords: diseased birds; virulence factors; phylogenetic analysis; antimicrobial resistance

1. Introduction

Vibrio cholerae are gram-negative bacteria found in aquatic ecosystems worldwide. Strains belonging to the serogroups O1 and O139 are the causative agent of cholera, which is a dreaded disease in developing countries and is responsible for thousands of illnesses and human deaths every year. The genes for the cholera toxin and the toxin-coregulated pilus are regarded as the major virulence factors of the toxigenic strains [1]. However, numerous *V. cholerae* strains exist, which do not possess these two virulence factors. The nontoxigenic strains belong to other serogroups and are commonly

designated as *V. cholerae* non-O1, non-O139. Some of these strains sporadically cause gastrointestinal infections or wound infections in humans [2].

Diseases of animals associated with *V. cholerae* non-O1, non-O139 are rarely reported. Though a number of *Vibrio* species are well-known pathogens for fish, a role of *V. cholerae* non-O1, non-O139 as a possible fish pathogen is reported less frequently. A recent review [3] gives an overview of the isolation of *V. cholerae* non-O1, non-O139 from diseased fish. Although, the authors suspected that, in some cases, the diseases might have been caused by other bacterial species or viruses. The increase in aquaculture production has led to a rise in infections by *V. cholerae* non-O1, non-O139 in farmed shrimps and infections in giant freshwater prawns and whiteleg shrimps being reported [4,5].

As *V. cholerae* is found worldwide in aquatic ecosystems, the occurrence of strains of this species in waterfowl is not surprising. Several years ago, *V. cholerae* were identified from cloacal swabs and feces taken from different species of aquatic birds [6,7]. In a recent study, several Vibrio species, including *V. cholerae*, were recovered from debilitated wrecked marine birds in Brazil [8]. Pretzer et al. (2017) [9] reported the occurrence of highly diverse *V. cholerae* non-O1, non-O139 populations in an Austrian lake (Neusiedler See) and migrating birds were hypothesized to play a key role in the transfer of the strains over long distances.

Diseases of water birds caused by *V. cholerae* non-O1, non-O139 have rarely been reported. *V. cholerae* NAG (non-agglutinable with antisera against O1 strains) were isolated from ducklings from Danish farms suffering from conjunctivitis. Isolates were found in the conjunctival fluid of puffins and were also present in the gut of the birds. Investigations of the environment made it likely that the bacteria were taken up from brackish water in their surroundings [10]. A subsequent investigation revealed that ducks inside the farm buildings had been free of *V. cholerae* and only after the birds were released into the open fields *V. cholerae* were detected in the cavum nasi and pharynx of the birds [11]. While in the first study some *V. cholerae* were isolated together with other bacteria from conjunctiva of diseased ducks, in the second study the animals were apparently healthy. A more likely association of a *V. cholerae* non-O1 infection in birds was reported [12] when a strain was isolated from the liver of a deceased goose. The goose was from a flock with six ill birds among 17 animals. Other poultry on the farm (chicken, mallards, and guinea fowls) were not affected. Another study reported two *V. cholerae* isolates among a number of other gram-negative bacteria that had been isolated from several cases of septicemia and airsacculitis in ducks [13]. As the study aimed to determine the antibiotic susceptibility of the bacterial isolates, questions concerning the cause of the infections were not addressed.

In a more recent paper, the antibiotic resistance patterns of *V. cholerae* isolates from local chicken of Bangladesh were determined. The bacteria were frequently found in animal samples, e.g., intestinal fluids, cloacal swabs, egg surfaces [14]. The strains presumably belonged to the toxigenic serogroups causing cholera, and the contaminated chickens were regarded as possible sources of infections for humans. In this study, no disease symptoms of birds were reported.

In 2016 and 2017, the reference laboratory for *Vibrio* hosted at the German Federal Institute for Risk Assessment received two *V. cholerae* non-O1, non-O139 isolates, which were obtained from diseased domestic ducks of a poultry farm in Bavaria. Both incidents caused significant losses of birds and the bacterial isolates were suspected of causing the disease. The unusual source of the isolates prompted this study. To obtain more information on strains from this host organism, two more *V. cholerae* isolates were investigated, which had been recovered earlier in the federal state of Saxony and originated from domestic ducks with serious disease symptoms (in 2011 and 1996).

We studied the four *V. cholerae* non-O1, non-O139 isolates using phenotypic and genotypic methods. Here, we report the results of the comparison of phenotypic traits including antimicrobial resistance patterns and a bioinformatics analysis of their whole genome sequences.

2. Materials and Methods

Isolates analyzed in this study are summarized in Table 1. The four investigated *V. cholerae* isolates were recovered in the German federal states Saxony and Bavaria. Isolate 16-VB00145 was identified

in a Bavarian poultry farm where ducklings died spontaneously. In two bacteriologically examined ducklings, *V. cholerae* was detected in the liver. Isolate 17-VB00405 was also found in the same farm approximately one year later. In livers of two birds, *V. cholerae* was isolated using a two media cultural approach (Columbia agar with 5% sheep blood and eosin methylene blue agar; Oxoid GmbH, Wesel, Germany). In this case, the incident was recorded by veterinarians and is presented in more details in the results section. The other two isolates were recovered from ducks of Saxonian farms. Isolate CH415 was identified in an incident where ducklings and adult ducks had shown several disease symptoms. Isolate T58 was recovered from a duck that suffered from pneumonia and peritonitis.

Table 1. *Vibrio cholerae* non-O1, non-O139 strains used in this study.

Strain	Year of Isolation	Source	Origin	Information on Disease
17-VB00405	2017	Duck/liver	Bavaria/Germany	Mild fibrinous-purulent hepatitis
16-VB00145	2016	Duckling/liver	Bavaria/Germany	Premature death/stunted growth/lameness
T58	2011	Duck/jejunum	Saxony/Germany	Pneumonia/peritonitis
CH415	1996	Duckling/lung	Saxony/Germany	Dyspnea/polyserositis/pneumonia/inability to stand

Histology. Samples from liver, spleen, brain, skeletal muscle and heart were fixed in 10% neutral buffered formaldehyde, paraffin embedded and sectioned with a microtome to obtain 4-µm-thick paraffin sections. H&E stain was performedusing hematoxylin (AppliChem GmbH, Darmstadt, Germany) and eosin (Merck KGaA, Darmstadt, Germany).

Species confirmation, PCR typing, and phenotypic characterization. Species confirmation of *V. cholerae* isolates was carried out by species-specific *sodB* PCR amplification as previously described [15]. The PCR was performed as multiplex PCR and included primers for the detection of the cholera toxin gene *ctxA* [16] and primers for detection of the serogroups O1 and O139 [17,18]. For PCR analysis, genomic DNA (gDNA) was extracted from 1 mL of an overnight culture in lysogeny broth (LB) using the RTP Bacteria DNA Mini Kit according to the manufacturer's protocol (Stratec Molecular GmbH, Berlin, Germany). Ten nanograms of gDNA served as template DNA for PCR analysis. The primers, annealing temperatures, and amplicon sizes are shown in Table 2.

Table 2. Multiplex PCR for species (*sodB*), cholera toxin gene (*ctxA*) and serogroups O1 and O139 detection.

Gene/Target	Primer	Sequence (5′ to 3′)	Amplicon Size (bp)	T_a (°C)	References
sodB	VcsodBf	AAGACCTCAACTGGCGGTA	248		[15]
	VcsodBR	GAAGTGTTAGTGATCGCCAGAGT			
ctxA	ctxA1	CTCAGACGGGATTTGTTAGGCACG	301		[16]
	ctxA2	TCTATCTCTGTAGCCCCTATTACG		59	
rfb O1 cluster	O1F	GTTTCACTGAACAGATGGG	192		[17]
	O1R	GGTCATCTGTAAGTACAAC			
rfb O139 cluster	O139F	AGCCTCTTTATTACGGGTGG	449		[18]
	O139R	GTCAAACCCGATCGTAAAGG			

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was conducted for initial species identification using the direct transfer method on a Microflex LT system mass spectrometer (Bruker Daltonik, Bremen, Germany) according to the manufacturer's recommendation [2]. Additionally, biochemical testing using API20E strips, phenotypic tests for NaCl tolerance, and hemolysis on sheep blood agar were performed.

Antimicrobial resistance testing. Antimicrobial susceptibility testing was performed by broth microdilution as previously described [19]. For the determination of the minimal inhibitory concentration the EUVSEC and EUVSEC2 microtiter plates (Trek Diagnostic Systems, East Grinstead, United Kingdom) were used according to the guidelines of the Clinical and Laboratory Institute (CLSI) [20]. The *Escherichia coli* isolate ATCC 25,922 was used as quality control for antimicrobial resistance testing. Interpretive categories of susceptible, intermediate and resistant were assigned according to CLSI clinical breakpoints for *Vibrio* spp. [20]. The agents were chosen according to the harmonized panel of antimicrobials of the European Union EFSA [21].

Whole genome sequence determination and bioinformatics analysis. Whole genome sequence (WGS)-based analyses were performed for the four non-O1, non-O139 *V. cholerae* isolates. The preparation of genomic DNA and short-read whole genome sequencing (MiSeq, Illumina, San Diego, CA, USA) was conducted as previously described [22]. SPAdes de novo assemblies of raw reads and genome annotation were performed using the Pathosystems Resource Integration Center (PATRIC) (release 3.5.39) [23] and the automated Prokaryotic Genome Annotation Pipeline (PGAP) of the National Center for Biotechnology Information (NCBI), respectively. The PHAge Search Tool Enhanced Release (PHASTER) was used for prediction of putative prophage sequences under default settings [24]. For the identification of a specific genetic element on the genome sequences of the different isolates, various tools of the Center for Genomic Epidemiology (CGE, Danish Technical University) were applied.

Average nucleotide identity (ANI) was determined with *V. cholerae* O1 El Tor N16961 as a reference genome (AE003852, AE003853) for pairwise comparison with each strain using the online tool ANIFinder (http://enve-omics.ce.gatech.edu/ani/index). The ANI calculations were performed using the default values given on the site (alignment options: minimum length 700 bp, minimum identity 70%, minimum alignments 50; fragment options: window size 1000 bp, step size 200 bp).

Multilocus sequence type (MLST) prediction was conducted using MLST Finder [25] making use of the non-O1, non-O139 *V. cholerae* MLST scheme (https://pubmlst.org/vcholerae/) [26]. Initial plasmid prediction was performed with the PlasmidFinder web tool (release 2.0, https://cge.cbs.dtu.dk/services/PlasmidFinder/) [27]. In addition, genomic contigs showing significantly higher sequence coverages than the rest of the contigs were screened for similarities to known plasmids using the BLASTN algorithm of the NCBI database (https://www.ncbi.nlm.nih.gov).

To determine the phylogenetic relationship of the isolates, a CSI Phylogeny (version 1.4; https://cge.cbs.dtu.dk/services/CSIPhylogeny/)-based single nucleotide polymorphism (SNP) tree was prepared. The tool was used under default settings and the exclusion of heterozygous SNPs. As a reference genome, sequencing data of the *V. cholerae* O1 El Tor strain N16961 (AE003852, AE003853) were used. Nucleotide variations were predicted according to the specifications provided [28]. To find out if closely related genomes of other *V. cholerae* strains were already deposited in public databases, the PATRIC service tool "Similar Genome Finder" (release 3.6.5) was applied. The most similar genomes were tested in pairwise ANI calculations and integrated into the SNP analysis with CSI Phylogeny described above.

For the initial detection of *Vibrio*-specific virulence determinants, the MyDbFinder web-based tool (release 1.1, https://cge.cbs.dtu.dk/services/MyDbFinder/) was used with a manually-adapted composition of determinants selected from the virulence factor database (VFDB; http://www.mgc.ac.cn/VFs/) [29]. The *in silico* prediction of plasmid replicon types and virulence factors and a sequence identity of >50% and >30%, respectively, were used. Furthermore, a minimum coverage of >20% was chosen. For the *in silico* analysis of *V. cholerae*-specific gene variants, the WGS were subjected to the NCBI database (BlastN) and compared to selected reference sequences as previously described [22].

Isolates were further tested for the presence of genes of the Vibrio pathogenicity island 2 (VPI-2) [30]. Isolates were screened for sequences of the Vibrio seventh pandemic islands VSP-1 [31] and VSP-2 [32]. All isolates were further studied for the complete type III secretion system (TTSS) gene cluster [33,34] and for the presence of the MSHA gene cluster. The accession numbers of reference gene clusters are NC_002505.1 (*V. cholerae* N16961; VC1758-VC1809 for VPI-2; VC0175-VC0185 for VSP-1; VC0490-VC0516 for VSP-2; VC0398-VC0411 for MSHA cluster), and DQ124262.1 as well as AATY02000000 (*V. cholerae* AM-19226; AATY02000003.1/AATY02000004.1 for TTSS cluster and flanking regions).

For the presence of specific determinants involved in antimicrobial resistance (AMR) development, ResFinder (release 3.1.0, https://cge.cbs.dtu.dk/services/ResFinder/) was used as specified before. For the prediction of *V. cholerae*-specific integron sequences, the WGS were subjected to NCBI BLASTN searches.

Accession numbers. Genome sequences of *V. cholerae* isolates have been deposited in GenBank at the National Center for Biotechnology Information (NCBI) under the accession numbers: PVFA00000000 (CH415), PVFB00000000 (T58), PVER00000000 (16-VB00145), and PVEX00000000 (17-VB00405).

3. Results

3.1. Case Study in 2017

In the Bavarian poultry farm, ducklings were kept for three weeks in rearing houses (rearing phase) and then fattened for 25–29 days (fattening period) in a mast compartment. In the described incident in the year 2017, 7.8% of ducklings (total 13,515 birds) died during the first three days of the rearing phase. The daily mortality rate decreased on the fourth and fifth day to eight animals (0.06%) and two animals (0.02%), respectively. From then on, daily losses were within acceptable limits (between two to six animals per day). At the end of the third week (day 21), the ducklings were moved to the mast compartment.

Starting at an age of 22 days, however, the losses increased up to 20 animals (0.15%) daily. Therefore, a veterinary examination was carried out on day 25. The animals displayed significant growth differences. The smallest animals could only run poorly and lay on the ground, sometimes they "crawled" through the stable with the help of the wings. A treatment with Suramox 100 mg/g (amoxicillin trihydrate 1000 mg; Virbac veterinary medicine GmbH, Bad Oldesloe, Germany) was immediately initiated with a dosage of 20 mg/kg body weight over four days.

In the first week post treatment, 143 animals died (1% mortality rate). In the following week, the mortality rate decreased to 0.7% (94 animals). All remaining birds were slaughtered shortly afterwards.

Three animals were sacrificed before the onset of antibiotic treatment and presented for necropsy. They were in moderate to poor physical shape. The livers were slightly swollen with rounded edges. Histologically, two livers showed moderate acute degeneration with cytoplasmic vacuolization and single cell necrosis (Figure 1). Additionally, a mild purulent hepatitis with predominantly heterophil granulocytes infiltration was observed. The third liver revealed mild fibrinopurulent hepatitis with perivascular infiltration of heterophil granulocytes and a mild focal precipitation of fibrin. One spleen showed mild fibrin deposits. Other organs, including the brain, heart and skeleton muscle, showed no specific lesions.

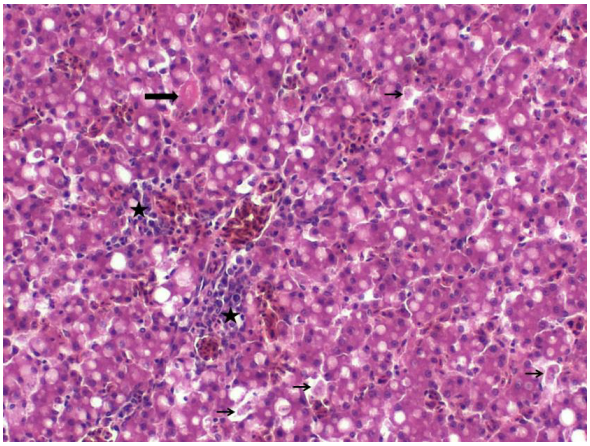

Figure 1. Liver, H&E stain, 200× magnification. Hepatitis with mild degeneration and vacuolation of hepatocytes, heterophilic to mixed inflammatory cell infiltration (stars), small deposits of fibrin (thick arrow), and individual cell necrosis (thin arrows).

A bacteriological examination was undertaken and *V. cholerae* were isolated from two livers and one spleen. In addition, a small number of *Aerococcus viridans* was detectable in one liver. As *A. viridans* is frequently encountered in bacteriological examinations of ducklings, the likely cause of the observed symptoms was attributed to *V. cholerae* by the responsible veterinarians. In the organs of the third bird, no bacteria were found. A pure *V. cholerae* culture (isolate 17-VB00405) was sent to the *Vibrio* reference lab for further investigations.

A systematic investigation of the environment or the water supply system of the farm was not conducted. The water pipes of the farm were disinfected with chlorine after the treatment of the birds with amoxicillin. Different water samples were taken a few days later and were free of *V. cholerae*.

3.2. Species Confirmation by PCR, MALDI-TOF MS and Phenotypical Characterization

The results of the multiplex PCR revealed that the four duck isolates belonged to the species *V. cholerae*. In all four isolates, the species PCR (*sodB* gene) yielded the expected PCR product, whereas the multiplex PCR assays were negative for the cholera toxin gene A (*ctxA* gene) and negative for genes specific for serogroups O1 and O139.

All isolates were further analyzed by MALDI-TOF MS using the MALDI Biotyper system with the evaluation criteria developed for species identification. The analyses of the obtained spectra confirmed the species *V. cholerae* (Table 3). Phenotypic characterization showed that the isolates were able to grow in the absence of NaCl and showed hemolytic activity on sheep blood agar. The API20E testing of all strains confirmed *V. cholerae* as probable species (data not shown).

Table 3. Species confirmation by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and average nucleotide identity (ANI).

Isolate	MALDI-TOF MS		Average Nucleotide Identity **
	Score *	Interpretation	
CH415	2.38	Highly probable identification to species level	98.26%
T58	2.28	Probable identification to species level	98.06%
16-VB00145	2.33	Highly probable identification to species level	98.06%
17-VB00405	2.30	Highly probable identification to species level	98.25%

* Quality of species identification according to Bruker instruction against Bruker main spectra (MSP) libraries. ** Pairwise comparison to genome of strain *V. cholerae* O1 El Tor strain 16961 (accession: NC_002505.1 and NC_002506.1, two-way ANI results): http://enve-omics.ce.gatech.edu/ani/.

3.3. Whole Genome Data

The whole genome sequencing results show that genome sizes of the four sequenced strains vary between 3.9–4.2 Mbp and the average number of putative coding sequences (CDS) is approx. 3.76×10^3. Detailed information of the genomes is given in Table S2.

For each genome, an ANI calculation was performed by comparison with the *V. cholerae* O1 El Tor reference strain N16961. In all cases, the ANI score was >98% (Table 3). A cut-off score of >95% indicates that two strains belong to the same species [35], thus confirming all duck isolates as *V. cholerae*. An MLST analysis of seven housekeeping genes revealed that a considerable difference exists between the strains. Only in one case did two strains share the same allele of one housekeeping gene. This indicates that none of the four duck isolates are genetically closely related. Two new alleles were identified (one *purM* and one *pyrC* allele; see Table S2).

The four genomes were analyzed for their phylogenetic relationship using SNP analysis with the genome of the strain *V. cholerae* O1 El Tor N16961 as a reference. To increase the information on phylogenetic relations, for each of the four strains a search was conducted to detect related *V. cholerae* strains based on genome similarity. For this purpose, the online service "Similar Genome Finder" (PATRIC Server) was applied (accessed 2020-06-14). The closest related genome for each strain was downloaded and included in the tree. ANI calculations, however, indicated that only in the case of strains 17-VB00405 and T58 were more closely related strains identifiable in the data base (data not shown).

In total, 3.327.189 positions (corresponding to approx. 82.5% of the reference genome) were used of the investigated genomes and the number of SNPs between the isolates varied between 4888 and 31,855 (Table S3). The differences between the genomes of the duck strains were more than 30,000 SNPs and confirmed that the strains are only distantly related. The SNP-derived tree reproduces these genomic differences (Figure 2). We additionally searched the PubMLST database for *V. cholerae* strains

that might be genetically closely related to our isolates; however, no isolate with identical MLST alleles in all seven genes were detected (data not shown).

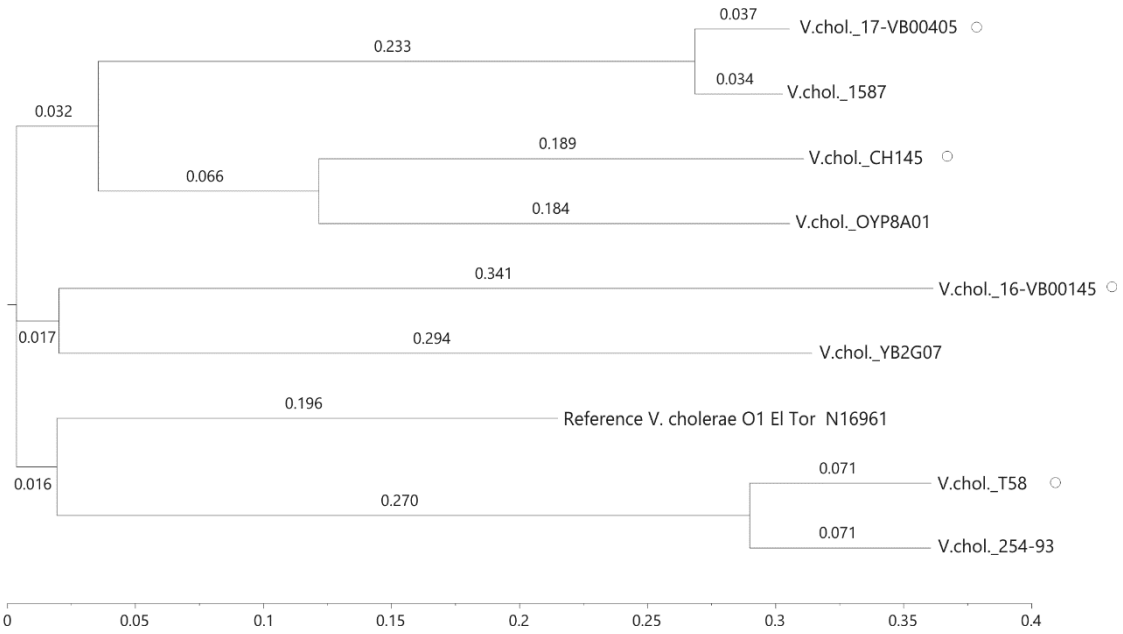

Figure 2. Single nucleotide polymorphism (SNP)-based phylogenetic relationships of *V. cholerae* non-O1, non-O139 isolates. Strains from ducks are marked with a circle. Four similar genomes of *V. cholerae* strains identified by bioinformatics were included in the tree. The SNP tree was conducted using CSI Phylogeny 1.4 under default settings and the exclusion of heterozygous SNPs. Single nucleotide polymorphisms (SNPs) were called by mapping to the *V. cholerae* O1 El Tor N16961 genome as a reference (accession NC_002505.1, NC_002506.1). The scale bar represents the number of nucleotide substitutions per site and numbers indicate branch length. Accession of *V. cholerae* (V.chol) genomes: LBGA01000000 (YB02G07), JMBP00000000 (V.chol 254-93), KQ410623 (V.chol 1587), NMSW00000000 (V.chol OYP8A01).

As *Vibrio* bacteriophages (phages) are common vehicles carrying virulence determinants, the genome sequences were also analyzed *in silico*. The search for prophage sequences revealed that no common prophages are present in the genomes of the four strains. In isolate CH415, a putative intact prophage could be identified using PHASTER. The prophage sequence shows significant similarity to phage K139 (accession NC_003313) (sequence identity >90%) associated with toxigenic *V. cholerae* strains [36]. Another putative prophage sequence of isolate 16-VB00145 revealed only short sequences of similarity to a *Vibrio* phage and was disregarded (data not shown).

3.4. Virulence Factors Revealed by WGS

The genome sequences of all strains were screened for virulence genes and the presence of pathogenicity islands. The results of the bioinformatics analysis concerning the presence of virulence genes are shown in Table 4. None of the isolates possesses the major virulence genes of toxigenic O1 and O139 strains that are located on the CTX prophage and the *Vibrio* pathogenicity island-I (VPI-1). The analysis showed that some virulence genes of *Vibrio* pathogenicity island-II (VPI-2) of toxigenic strains are present in strain 16-VB00145, which possess the gene cluster encoding genes for sialic acid metabolism and transport and the *nanH* gene which encodes a neuraminidase. In some non-O1, non-O139 strains, type three secretion systems (TTSS) are present. A TTSS consisting of core genes and flanking 5′ and 3′ regions highly similar to the *V. cholerae* strain AM-19226 T3SS genomic island [33] is found in isolate T58.

Table 4. Virulence factors found in duck isolates.

Virulence Factors/Function.	Related Genes	CH415	T58	16-VB00145	17-VB00405	Reference
CTX prophage/cholera toxin	*ctxAB*, *zot*, *ace*, *rstA*, *rstB*, *rstR*	n.d.	n.d.	n.d.	n.d.	[38]
Vibrio pathogenicity island 1 (VPI-1)/toxin-coregulated pilus accessory colonization factor	*tcp* cluster, *acf* cluster	n.d.	n.d.	n.d.	n.d.	[39]
Vibrio pathogenicity island 2 (VPI-2)/sialic acid (SA) metabolism	VC1776-VC1783 (SA transport, SA catabolism) VC1784 (*nanH*, neuraminidase)	n.d.	n.d.	VC1776-VC1784	n.d.	[40]
Type three secretion system * core region, 5′ and 3′ flanking region	*vsc/vsp* cluster, *vop* effectors, *acfA*, *acfD*, *trh*	n.d.	present	n.d.	n.d.	[33]
Vibrio seventh pandemic island 1 (VSP-1)/increased fitness	VC0175-VC0185	n.d.	n.d.	n.d.	n.d.	[40]
Vibrio seventh pandemic island 2 (VSP-2)/increased fitness	VC0490-VC0516	n.d.	VC0504-VC0510, VC0516	n.d.	VC0490-VC0516	[37]
Repeats-in-toxin (RTX) toxins/cytotoxin	*rtxA* (similar to VC1451), *rtxB*, *rtxC*, *rtxD*	present	present	present	present	[41]
Mannose-sensitive hemagglutinin pilus (MSHA pilus)/adhesion	*mshA*	present	n.d.	n.d.	n.d.	[42]
Hemolysin genes/cytotoxins	*hlyA*, *tlh*, *dth*	present	present	present	present	[43–45]
Outer membrane protein/defense	*ompU*	present	present	present	present	[46]
Type VI secretion system (T6SS) core genes, effectors/interaction	*vipAB*, *vasA-vasK*, *vgrG-2*, VCA0109, VCA0122	present	present	present	present	[47]
Quorum sensing/autoinducer	*luxS*, *cqsA*	present	present	present	present	[48]
Hemagglutination/protease	*hap*	present	present	present	present	[49,50]
Cholix toxin */ADP-ribosylating toxin	*chxA*	n.d.	n.d.	n.d.	present	[51]
Virulence gene expression/transcriptional activator	*toxR*	present	present	present	present	[52]
Heat-stable enterotoxin *	*stn*	n.d.	n.d.	n.d.	n.d.	[53]

* Factors present in non-O1, non-O139 strains. n.d.—not detected. Gene numbers starting with VC are from genome annotation of O1 El Tor reference strain N16961 (16961 (accession: NC_002505.1and NC_002506.1).

In toxigenic strains isolated since 1961 [37], two genomic islands are found that were designated *Vibrio* seventh pandemic islands 1 and 2 (VSP-1, VSP-2). Sequences related to these two islands were mostly absent in the four duck isolates. While no VSP-1 related genes were found at all, genes of VSP-2 were detected in isolate T58. Additionally, a complete VSP-2 island seems to be present in strain 17-VB00405.

A number of virulence genes can be present in *V. cholerae* non-O1, non-O139 that are also found in the toxigenic strains and are known to contribute to the infection process in a synergistic way [54,55]. The results of a bioinformatics analysis for the presence/absence of a number of these genes are displayed in Table 5. All isolates possess the genes *rtxA* and *rtxC* of the repeat-in-toxin (RTX) cluster. All *rtxA* genes encode a toxin highly similar to the multifunctional autoprocessing repeats-in-toxin toxin (MARTX) of the reference O1 strain N16961 [56]. One of the strains (CH415) may have a functional MSHA pilus, as the *mshA* gene is detected together with a number of secretory genes of the MSHA cluster. The hemolysin genes *hlyA*, *dth* and *tlh* were present in all isolates. The hemolysin gene *trh* associated with the TTSS was found only in strain T58 which harbors a TTSS. All isolates possess genes encoding key proteins involved in quorum sensing (*luxS*, *cqsA*) and a gene encoding the outer membrane protein OmpU. In all strains, genes for type VI secretion systems (T6SS) are found. The cholix toxin gene (*chxA*) is detected in isolate 17-VB00405.

Table 5. Phenotypic and genotypic results of antimicrobial resistance of *V. cholerae* non-O1, non-O139 strains.

Isolate	AMP	CHL	CIP	COL	FOX	GEN	NAL *	SMX	TAZCLA	TEMOCI *	TET	TMP	AMR Genes **
CH415	4	≤8	0.03	>16	8	2	16	512	≤0.12	4	≤2	>32	*aadA1*, *catB9*, *sul1*, *dfr1*, *gyrA* (p.D87G) *
T58	4	≤8	≤0.015	>16	8	≤0.5	≤4	≤8	≤0.12	2	≤2	0.5	-
16-VB00145	2	≤8	≤0.015	>16	4	1	≤4	≤8	≤5	2	≤2	0.5	-
17-VB00405	2	≤8	≤0.015	>16	8	≤0.5	≤4	≤8	≤0.12	2	≤2	0.5	*catB9*

Only selected phenotypes are shown (a complete list is given in Table S1). Gray boxes indicate resistance, MIC concentration in [µg/mL]. Interpretation criteria according to CLSIAbbreviations: AMP ampicillin, CHL chloramphenicol, CIP ciprofloxacin, COL colistin, FOX cefoxitin, GEN gentamicin, NAL nalidixic acid, SMX sulfamethoxazole, TAZCLA ceftazidime/clavulanic acid, TEMOCI temocillin, TET tetracycline, TMP trimethoprim. * no criteria specified by CLSI [20]. ** AMR genes derived from genome sequences are *aadA1* (coding for aminoglycoside nucleotidyltransferase), *catB9* (chloramphenicol acetyltransferase), *sul1* (sulfonamide resistant dihydropteroate synthase), and *dfr1* (dihydrofolate reductase). The *gyrA* gene (nalidixic acid resistance) has a mutation: aspartic acid to glycine in amino acid position 87.

3.5. Antimicrobial Resistance

Antimicrobial resistance testing was performed by broth microdilution. The tests were performed using 19 substances and two combinations (Table S1), whereby the selection of agents follows a standard of the European Union [21].

The isolates were susceptible to most of the tested antimicrobial agents (Table S1). Only one strain (CH415) displayed resistance to trimethoprim and sulfamethoxazole, two agents, which target enzymes of the folic acid metabolism. Furthermore, a high MIC value against nalidixic acid was observed in the same strain. All isolates displayed resistance to colistin. This phenotype is well known in *V. cholerae* and colistin is suggested for selection of this species (cellobiose-polymyxin B-colistin agar) [57].

The bioinformatics analyses using ResFinder revealed the presence of acquired AMR genes in isolate CH415 (Table 5), which harbored the genes *aadA1* (streptomycin/gentamycin resistance), *sul1* (sulphonamide resistance), *catB9* (chloramphenicol resistance), and *dfrA1* (trimethoprim resistance). In isolate 17-VB00405, only a *catB9* gene was present.

The nalidixic acid resistance of strain CH415 may be due to a mutation in the *gyrA* gene leading to an amino acid substitution (aspartic acid to glycine) in position 87.

The AMR genes *sul1*, *aadA1* and *dfrA1* of strain CH415 were physically linked to a class 1 integron integrase gene *intl1*.

4. Discussion

Few studies have been published in which a possible connection between *V. cholerae* and diseases of poultry have been described [10,12]. A disease of laying hens known as "avian vibrionic hepatitis" is a misnomer as the disease is likely to be caused by *Campylobacter* spp. [58].

In this study, isolates that had been recovered from diseased ducks, were characterized as non-O1, non-O139 *V. cholerae* isolates. Two isolates originated from birds of a Bavarian poultry farm, which suffered losses of ducks in 2016 and 2017. An investigation of two deceased ducks in 2016 revealed the presence *V. cholerae* bacteria in the liver of the birds. In 2017, *V. cholerae* was found again in the liver of diseased ducks. Only in the second incident was a limited outbreak investigation carried out. Both incidents point to a possible connection between the isolated bacteria and diseases in birds.

As disease symptoms in waterfowl associated with *V. cholerae* have rarely been reported, two more duck isolates, which had been found earlier in diseased ducks in Saxonian farms, were included in the study. These isolates were recovered from different inner organs (lung, jejunum) and infected animals showed different symptoms. In all cases, an uncertainty remains as to whether the isolates were responsible for the diseases and deaths, as further epidemiological studies were not carried out and an outbreak investigation was only performed once (case study 2017).

WGS was performed and bioinformatics analyses were done to determine possible genetic relationships of the isolates. A search for virulence genes or genomic islands was also conducted, which could indicate factors specific for this animal host. MLST Finder was used to predict sequences of seven housekeeping genes [59]. The MLST data are suited for the characterization of the isolates, as the sequence data are unambiguous and genomes can readily be compared via the internet [60]. Our study disclosed a distinct diversity of the alleles. The two isolates from the Bavarian farm aregenetically distantly related, which is also true for the other two isolates. An SNP analysis of the four genomes with the reference genome of O1 El Tor strain N16961 confirmed this result. Genes of the CTX phage and VPI-1 (toxin coregulated pilus) are missing in the four genomes. In other studies, some non-O1, non-O139 strains were described harboring *ctx*, *ace*, *zot*, or *tcpA* genes [16,61].

WGS were performed on environmental *V. cholerae* isolates from other regions of the world. Genomic features of strains from environmental sources from Uganda and Bangladesch [62,63] revealed a genetic relationship to pandemic cholera-causing O1 strains and the possession of the major virulence factors of pandemic strains. In Thailand [64], non-O1, non-O139 strains, which lacked the genes encoded on the CTX phage and for the toxin coregulated pilus, were also studied.

Prophages are frequently involved in the conversion of environmental strains to toxigenic strains. The most notorious case is the acquisition of the cholera toxin gene by integration of the CTX phage into the chromosome of *V. cholerae* O1 and O139 strains [38,65]. However, our analysis did not identify a prophage sequence common to all genomes. In strain CH415, a putative intact temperate phage is present that is related to *Vibrio* phage K139 (sequence identity >90%). K139 is a phage associated with toxigenic *V. cholerae* strains [36,66]. In an animal model, phage K139 was shown to harbor a virulence factor (*glo* gene). However, a *glo* related gene is missing in the prophage sequence of strain CH415.

The search for the presence of pathogenicity island VPI-2 and for two genomic islands associated with toxigenic strains of the seventh pandemic (VSP-1, VSP-2) gave mixed results. VPI-2 genes coding for enzymes of sialic acid metabolism involved in hydrolyzing the intestinal mucus [40] were found in one isolate. A probably complete VSP-2 sequence was present in isolate 17-VB00405 and parts of the island in isolate T58. The role of VSP islands in infection is unclear. It is hypothesized that they increase the fitness advantage of the toxigenic strains [37,67]. In strain T58, a complete TTSS system was identified. TTSS is only present in variants of the VPI-2 found in some non-O1, non-O139 strains and is a known virulence factor in diarrheal diseases caused by these strains [33,34].

In Table 4, the results of a bioinformatics analysis for more virulence genes which are thought to act synergistically in infections are displayed. As in a previous investigation [22], some genes were present in all strains and others were only detected in some strains. Genes of the latter category are, e.g., cholix toxin *chxA* gene (ADP-ribosylating toxin of eukaryotic elongation factor) [51] or the *mshA*

gene encoding the major pilin of the type 4 MSHA pilus [42]. The two virulence genes were present in isolate 17-VB00405 and CH415, respectively.

Virulence genes detected in all strains are likely to encode proteins that are probably primarily important for survival and niche adaptation in the natural aquatic environment of *V. cholerae* [68]. These factors comprise, e.g., hemolysin genes (*tlh, hlyA, dth*), genes for quorum sensing (*luxS, cqsA*), the hemagglutination/protease (*hap*), and genes of T6SS mediating antagonistic interactions against many prokaryotic and eukaryotic organisms [47,68]. The occurrence of these virulence factors was observed in all non-O1, non-O139 strains in a previous study [22]. In a study on isolates from Thailand [64], all *V. cholerae* non-O1, non-O139 strains were found to contain *hlya, rtxA* and *toxR* genes, whereas other virulence factors, such as TTSS, MSHA pilus and Elements of the VSP-1, and VSP-2 islands were present only in some strains.

While the bioinformatics analysis did not clearly indicate virulence factors that may be specific for avian hosts, two of the analyzed virulence factors present in all four strains may be of special interest for further research. The OmpU protein is the most abundant outer membrane protein in *V. cholerae*. It is an important virulence factor involved in host–cell interaction and recognition, as well as being critical for the survival in the host body and in harsh environments [69]. The OmpU protein is involved in resistance to antimicrobial peptides in the gut [52]. The primary structures of the four OmpU proteins showed remarkable differences. Whereas the OmpU proteins of CH415 and 17-VB00405 were identical, the proteins of isolates T58 and 16 VB00145 possessed only 77% and 94% identity, respectively. However, C-terminal regions of the protein involved in triggering a bacterial response by the activation of sigma factors were conserved [46]. The significant differences in the primary structures of the proteins may indicate a specific adaptation to different niches or environments.

Another interesting observation was the detection of *rtxA* genes encoding MARTX toxins with a high similarity (>97.7%) to the MARTX of toxigenic O1 El Tor strains. MARTX proteins of *V. cholerae* strains are very large multifunctional proteins (e.g., 4,565 amino acids in O1 strain N16961) with conserved N-terminal and C-terminal regions. The central part of the toxin carries different effector domains [41]. In O1 El Tor strains, the MARTX toxin has three conserved internal domains that are probably involved in the evasion of the host immune defense. In a previous study, we noticed that environmental *V. cholerae* strains frequently possessed MARTX variants differing in the central part of the coding region [22]. These MARTX variants possessed other effector domains and could be active against eukaryotic cells from different organisms (mammals or fish or eels). It was hypothesized that MARTX toxins play a role in adaptation to specific niches in the natural ecosystem [41,68]. Given the variability of MARTX toxins in environmental strains, it is remarkable that all duck isolates harbor the same variant. It was speculated that this variant toxin might be linked to human infections [41].

Acquired AMR genes were found in two isolates (17-VB00405, CH415). The *catB9* gene effective against chloramphenicol was detected and its coding region appeared to be functional. However, in the broth dilution tests, susceptibility to chloramphenicol was observed in the two strains. The *catB9* gene is widely distributed in toxigenic and in non-O1/non-O139 *V. cholerae* strains [64]. In isolate CH415, the AMR genes *sul1, aadA1* and *dfrA1* seem to be linked to a class 1 integron. Class 1 integrons are genetic assembly platforms that take up exogenous open reading frames via site-specific recombinations and are frequently found in *V. cholerae* [70].

5. Conclusions

V. cholerae non-O1, non-O139 strains are found worldwide in aquatic ecosystems and can cause diseases in aquatic organisms, such as fish and crustaceans. Diseases of waterfowl associated with *V. cholerae* have rarely been reported. For this reason, isolates from diseased birds were of special interest for this study. The analyzed isolates were recovered from different inner organs (liver, lung, jejunum) of domestic ducks and the infections showed different symptoms. Uncertainty remains as to whether the isolates were responsible for the diseases and deaths, as an outbreak analysis was only performed in one case. The great genetic heterogeneity between the isolates does not allow

any conclusion about factors contributing to the development in organs of this animal host. Future research is needed and more isolates are necessary to clarify this question. The observation that all four isolates possess MARTX toxins closely related to the MARTX of toxigenic O1 El Tor strains and show differences regarding the highly abundant OmpU protein could indicate a role of these virulence factors in the infection process.

In this context, it should be mentioned that information on the environmental occurrence of *V. cholerae* non-O1, non-O139 in Germany is sketchy. The occurrence of these bacteria in Germany is only documented for coastal waters of the Baltic Sea and some estuaries of the North Sea [71,72]. However, studies from Austria have clearly demonstrated that these bacteria can be present in lakes far away from marine coastal waters [9,73]. As *V. cholerae* non-O1, non-O139 is present in migratory water birds, these birds probably act as vectors for a long-distance transfer of the bacteria. The occurrence of bacteria in inland waters has not been investigated in Germany, so the source of the bird infections currently remains unknown. In the described incident from 2017, the water supply system was suspected to be contaminated (and was disinfected as a precaution). In the context of the 'One Health' concept, it is desirable to study the ecology of *V. cholerae* non-O1, non-O139, as it seems likely that the isolated strains from domestic ducks may be also pathogens for humans.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/8/1104/s1, Table S1: AMR of the duck isolates. Table S2: Results of the whole genome sequence. Table S3: SNP distance matrix of *V. cholerae* non-O1, non-O139 strains.

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7.3 Publications and Congress Presentations

Scientific journal articles

Hirsch, N., Kappe, E., Gangl, A., Schwartz, K., Mayer-Scholl, A., Hammerl, J.A., and Strauch, E. (2020). Phenotypic and genotypic properties of *Vibrio cholerae* non-O1, non-O139 isolates recovered from domestic ducks in Germany. *Microorganisms* 8:1104. doi: 10.3390/microorganisms8081104

Schwartz, K., Hammerl, J.A., Göllner, C., and Strauch, E. (2019). Environmental and clinical strains of *Vibrio cholerae* non-O1, non-O139 from Germany possess similar virulence gene profiles. *Front. Microbiol.* 10:733. doi: 10.3389/fmicb.2019.00733

Hammerl, J.A., Jäckel, C., Bortolaia, V., Schwartz, K., Bier, N., Hendriksen, R.S., Guerra, B., and Strauch, E. (2017). Carbapenemase VCC-1-producing *Vibrio cholerae* in coastal waters of Germany. *Emerg. Infect. Dis.* 23, 1735-1737. doi: 10.3201/eid2310.161625

Schwartz, K., Kukuc, C., Bier, N., Taureck, K., Hammerl, J.A., and Strauch, E. (2017). Diversity of *Vibrio navarrensis* revealed by genomic comparison: Veterinary isolates are related to strains associated with human illness and sewage isolates while seawater strains are more distant. *Front. Microbiol.* 8:1717. doi: 10.3389/fmicb.2017.01717

Bier, N., Schwartz, K., Guerra, B., and Strauch, E. (2015). Survey on antimicrobial resistance patterns in *Vibrio vulnificus* and *Vibrio cholerae* non-O1/non-O139 in Germany reveals carbapenemase-producing *Vibrio cholerae* in coastal waters. *Front. Microbiol.* 6:1179. doi: 10.3389/fmicb.2015.01179

Herrfurth, D., Oeleker, K., Pund, R.-P., Strauch, E., Schwartz, K., Kleer, J., Gölz, G., Alter, T., and Huehn, S. (2013). Uptake and localization of *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* in blue mussels (*Mytilus edulis*) of the Baltic Sea. *J. Shellfish Res.* 32, 855-859. doi: 10.2983/035.032.0329

Posters

Schwartz, K., Hammerl, J.A., and Strauch, E. (2019). Comparison of *Vibrio navarrensis* isolates from environmental and veterinary sources to human pathogenic strains. Junior Scientist Zoonoses Meeting 2019, Berlin, Germany, June 2019.

Schwartz, K., Bier, N., Schirmeister, F., Konietzny, A., and Strauch, E. (2017). Investigation of *Vibrio cholerae* non-O1, non-O139 isolated from environmental sources and from food in Germany. Microbiology and Infection 2017 – 5th Joint Conference of the DGHM and VAAM, Würzburg, Germany, March 2017.

Oral presentations

Schwartz, K. (2019). Comparison of *Vibrio navarrensis* isolates from environmental and veterinary sources to human pathogenic strains. Junior Scientist Zoonoses Meeting 2019, Berlin, Germany, June 2019.

Schwartz, K., Konietzny, A., and Strauch, E. (2018). Comparison of *Vibrio cholerae* non-O1, non-O139 from aquatic environments and from seafood in Germany with clinical strains. Annual Conference of the Association for General and Applied Microbiology, Wolfsburg, Germany, April 2018. ePoster presentation.

December 2, 2020

7.4 Curriculum Vitae

For reasons of data protection, the curriculum vitae is not included in the online version.

For reasons of data protection, the curriculum vitae is not included in the online version.

7.5 Eidesstattliche Erklärung

Ich versichere hiermit, dass ich die vorliegende Arbeit selbstständig verfasst und dazu keine anderen als die angeführten Behelfe verwendet habe.

Ort, Datum	Unterschrift