REVIEW

Non-toxigenic *Vibrio cholerae* – just another cause of vibriosis or a potential new pandemic?

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ABSTRACT

Although nontoxigenic *Vibrio cholerae* usually stands in the shadow of the two serogroups (O1 and O139) that cause pandemic cholera, its role in human pathology is increasingly recognized and described in the literature. The habitat of these pathogens is brackish seawater or even freshwater, and the infections caused by them include contact with these waters or consumption of seafood originating in this habitat, which is constantly expanding because of global warming. This habitat extension is a typical example of climate change's impact on infectious diseases. Although nontoxigenic *Vibrio cholerae* strains are rarely capable of producing the classical cholera toxin, they possess many other virulence factors, can secrete various other toxins, and thus produce illnesses that are sometimes even severe or life-threatening, more frequently in immunocompromised patients. Vibriosis may manifest as gastrointestinal illnesses, wounds, skin or subcutaneous tissue infections, or septicemia. To establish the correct etiological diagnosis for these infections, a high index of suspicion must be maintained, as the diagnostic techniques require targeted investigations and specific collection and transportation of the samples. Empiric treatment recommendations are available, but owing to the increasing resistance of this pathogen, susceptibility testing is needed for every diagnosed case. We intend to raise awareness regarding these infections, as they tend to be more frequent than they were in the past and to appear in areas where they had not been recognized before.

KEYWORDS: *Vibrio cholerae*; cholera; diarrhea; seafood; toxin; zoonosis

INTRODUCTION

The genus *Vibrio* is part of the family *Vibrionaceae* in the order *Vibrionales* of the class *Proteobacteria*, along with many other human pathogens. However, not all species in the *Vibrio* genus are pathogenic to humans. Among the more than 100 species, only 12 can cause human diseases [1]. The bacteria most commonly involved in human pathology are *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio alginolyticus*. The members of the *Vibrio* genus are Gram-negative, comma-shaped bacteria. They live in freshwater, estuarine, and marine waters and prefer warmer, low-salinity waters [2].

Species from the *Vibrio* genus are zoonotic agents associated with seawater fish, eels, and shellfish, such as oysters, clams, mussels, crabs, and shrimp [1]. It can also be found in seabirds, algae, corals, crustaceans, mollusks, and mammals. Sometimes, it is associated with plankton [3]. Human diseases caused by pathogenic *Vibrio* species can be described as cholera and noncholera infections (vibrioses). *Vibrio* infections are acquired by ingesting water (*Vibrio cholerae* may be found in freshwater) or consuming contaminated seafood. Other transmission routes include the exposure of wounds to infested seawater, injuries caused by contaminated objects in the water, or even shark or alligator bites [4]. The clinical manifestations of this disease depend on the *Vibrio* species, transmission route, and host susceptibility [1]. Three major types of clinical manifestations have been described as consequences of the above factors: gastroenteritis (diarrhea, abdominal cramps, nausea, vomiting, fever, watery and sometimes bloody stools, headache, and myalgia), septicemia (hypotension, fever, tachycardia, shock, multiple organ dysfunction, hypothermia) and wound infection (swelling, pain, erythema, bullae, necrosis, gangrene) [4,5]. Gastroenteritis and septicemia occur after the ingestion of the pathogen, and wound infection occurs after the exposure of a wound to contaminated water, in a wound produced by contaminated objects or in a contaminated environment. Other clinical manifestations include central nervous system involvement, such as meningitis or cerebritis, but these manifestations are rare [6].

The etiologic agent of cholera is *Vibrio cholerae*. Some of the serotypes (O1 and O139) of this species produce a specific toxin and severe gastroenteritis, which, without prompt

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treatment, can lead to severe dehydration and death [6]. Since 1817, seven cholera pandemics have begun in different areas of Asia and spread, affecting most of the world [7]. The "classical" O1 strain was responsible for the first six pandemics and was prevalent until the beginning of the seventh pandemic in 1961. In the pre-7th pandemic period (between 1923 and 1961), some sporadic outbreaks were caused by the O1 El Tor biotype, which is the cause of the current pandemic (the 7th) [8]. Serogroup O139 emerged in 1992 and caused a cholera epidemic in Southeast Asia, but it did not replace El Tor. The classical biotype has not been identified since the 1980s and is now considered extincted. Current waves of the 7th pandemic are caused by strains that are a mix of the Classical and El Tor biotypes. The other serotypes of *Vibrio cholerae* (non-O1, non-O139), the so-called nontoxigenic serotypes or nonagglutinable (NAG), may cause one of the above syndromes with various severities.

MICROBIOLOGY OF NONTOXIGENIC *VIBRIO CHOLERAE*

Vibrio cholerae is an oxidase-positive, gram-negative rod, comma-shaped bacterium that can be identified via various methods and cultured on selective media [9]. These bacteria are highly mobile and possess a unipolar sheathed flagellum. As mentioned above, *Vibrio cholerae* is a Proteobacteria (Gammaproteobacteria) class member. Other members in this class, such as *Escherichia coli* or *Shigella dysenteriae*, have a single circular chromosome, while the genome of *Vibrio cholerae* is multipartite. It consists of two unequal circular chromosomes [10]. The large chromosome, which is approximately three kb long, contains all the essential genes, including the genes involved in pathogenicity. In contrast, the small chromosome (approximately one kb long) contains genes with unknown functions. The bipartite genome of *Vibrio cholerae* is likely a consequence of an ancestor of this species that acquired a large plasmid that obtained essential genes and became a large chromosome [11]. The genes involved in pathogenicity are acquired later through lateral gene transfer from an unknown organism (probably a bacteriophage) [10].

More than 200 serogroups have been described based on the differences between their O-specific polysaccharide (OSP) chains of lipopolysaccharide (LPS) molecules [6]. Serogroups O1 and O139 are capable of producing large epidemics of cholera because they can secrete the cholera toxin. Serogroup O1 is further divided into two biotypes (El tor and Classical), closely related in their OSP structures but differ in different parts of their genomes [12]. The two *Vibrio cholerae* biotypes have distinct phenotypes, and they vary in terms of disease severity, resistance outside the host and seasonal dispersion. The El Tor biotype has three serotypes: Inaba, Ogawa and Hikojima. The differences between serotypes are even less significant, affecting only the antigenic determinants on the O side chain of the lipopolysaccharide (LPS) antigen [13]. Because the non-O1/non-O139 serogroups lack the cholera toxin, they usually do not cause large epidemics. However, they can cause small gastroenteritis outbreaks or the other clinical manifestations described above (bacteremia or wound infections) [13]. Non-O1 serotypes (including O139) have a capsule that determines virulence in extraintestinal infections [6]. Toxin coregulated pilus (TCP) colonization factor is another virulence factor of the O1 strains (permitting the colonization of the human intestine). Still, this factor can also be acquired by nontoxigenic strains of Vibrio cholerae, which become so-called CNTP (they do not have the gene that encodes toxin production (*ctxAB*) but are positive for the gene encoding TCP (*tcpA*)) [14]. CNTP isolates of non-O1 Vibrio cholerae are more frequently involved in the production of diseases than non-CNTP isolates (which are negative for both *ctxAB* and *tcpA*) [15]. Non-O1/non-O139 CNTP strains of *Vibrio cholerae* are frequently found in various environmental reservoirs, sometimes covering large areas, and they cause endemic or isolated cases of diarrhea, being the second most frequent cause of diseases caused by *Vibrio cholerae* [16]. The above classification is depicted in Figure 1. A genome-wide single-nucleotide polymorphism (SNP) study revealed that V. cholerae can be divided into eight principal lineages named L1-L8 [17]. According to the same study, the classical biotype of Vibrio cholerae belongs to lineage 1 (L1), and the El Tor biotype belongs to L2. Wang et al. described a ninth lineage, L9, which, along with L3b, contains CNTP isolates [18]. L4 and L7 are frequently found in environmental reservoirs and do not cause disease. Lines L3, L5, L6 and L8 have been associated with diseases in specific areas (Gulf of Mexico, Sulawesi, Saudi Arabia, and Australia) [19].

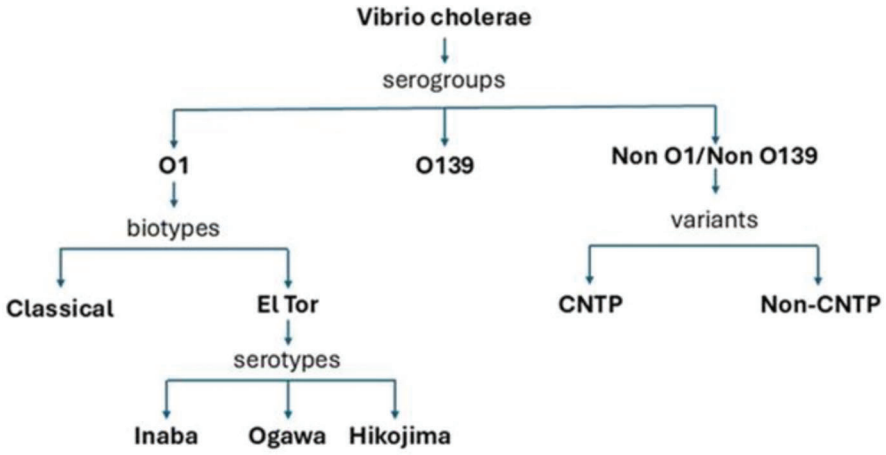

Fig. 1. Classification of *Vibrio cholerae* serogroups, biotypes, and serotypes.

PATHOGENESIS

The non-O1/non-O139 serogroups of *Vibrio cholerae* are highly diverse genetically. Their virulence and ability to cause disease depend on the virulence genes carried, and the resulting illness also depends on the host's immunity status. The virulence of serogroups O1 and O139 is determined by the presence of two major virulence factors, cholera toxin (CT) and TCP, which are encoded by the *ctxA*, *ctxB* and *tcpA* genes, which are encoded by a filamentous bacteriophage (CTXΦ), and are located within a pathogenicity island (VPI). Non-O1/non-O139 serogroups usually lack these significant virulence genes, but they may have other virulence mechanisms, such as the expression of different toxins, biofilm formation or secretion systems [20].

NAGs produce the following toxins: accessory cholera enterotoxin (*Ace*), zonula occludens toxin (*Zot*), heat-stable enterotoxin (*ST*), cholix toxin (*Chx*), multifunctional autoprocessing repeats-in-toxin (MARTX, also known as RTX), hemagglutinin protease (HAP), mannose-sensitive hemagglutinin (MSHA), and V. cholerae cytolysin (VCC) [20]. The main characteristics of these toxins are summarized in Table 1.

Once ingested, *Vibrio cholerae* reaches the stomach, where it must face the acidity of this site. For this purpose, vibrios undergo an acid tolerance response, an adaptive process that permits them to survive this hostile medium [27]. Despite this response, the number of vibrios that reach the small intestine is reduced. In a healthy person, a high infective dose (108 bacteria) is needed to produce cholera, while when given with proton pump inhibitors, a smaller dose is enough (105 bacteria) [28].

After passing the stomach acidity, vibrios reach the small intestine, the leading site of colonization. Here, they need to adapt to bile salts and various antimicrobial peptides, and they do so via a complex regulated signaling pathway named ToxR Regulon [20]. When stimulated by bile salts, ToxR changes the outer membrane proteins (OMPs) and protects bacteria by balancing the composition of OmpU and OmpT in the membrane [29]. Once protected against the mechanisms of defense in the small intestine, bacteria use the flagellum to penetrate the mucus layer and reach the epithelium [30]. HAP also helps penetrate the mucus layer through hydrolysis of the mucus barrier. The gut microbiota is another obstacle to *Vibrio cholerae* colonization. A system called the Vibrio cholerae type 6 secretion system (T6SS) is activated to overcome this obstacle. This system actually kills bacterial competitors via contact-dependent translocation of toxic effectors [31]. After overcoming all these obstacles, *Vibrio cholerae* attaches to intestinal epithelial cells, proliferates and forms microcolonies if they express TCPs (CNTP variants) [32].

Septicemia-causing non-O1/non-O139 *Vibrio cholerae* strains are all encapsulated. This capsule protects the germs against phagocytosis and serum bactericidal activity [33,34].

CURRENT AND PROJECTED EPIDEMIOLOGY

Vibrio cholerae usually inhabits aquatic environments such as estuaries, marine coastal waters, lakes, or aquaculture settings. They prefer brackish or slightly salted water [35]. Vibrios have also been isolated from birds, domestic animals, wild animals, and even insect eggs. Two factors are essential for the development of *Vibrio cholerae*: the temperature and salinity of the water [36]. *Vibrio* species can live at temperatures between 0.5°C and 48°C. In this range, some species can be pathogenic to marine animals (those that live at relatively low temperatures (22°C)) and species that are pathogenic to humans (37°C). In the last category, *Vibrio cholerae*, which can also be found at temperatures below 10°C and above 35°C, has a significant ability to adapt to temperature variations [37]. The optimum growth of Vibrio cholerae occurs between 23–30°C, while its maximum growth temperature is 37°C. At temperatures below 4°C, *Vibrio cholerae* enters a ''viable but nonculturable'' state, which can be recovered after reincubation at 20–23°C [38].

Table 1. Toxins produced by NAGs and their main characteristics [21-26].

Toxin	Mechanism of action	Results
Accessory cholera enterotoxin (*Ace*)	Stimulates Ca^{2+} -dependent Cl^-/HCO^{3-} cotransporters	Induces fluid secretion in the intestine
Zonula occludens toxin (*Zot*)	It affects the structure of the epithelial tight junction of the small intestine.	Increases the mucosal permeability and promotes the passage of the macromolecules through the paracellular route
Heat-stable enterotoxin (ST)	Signal cyclic guanosine monophosphate (cGMP) pathways to activate the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel	Elevates intracellular cGMP and thus induces anion secretion and diarrhea
Cholix toxin (*Chx*)	Prevents protein synthesis by altering the diphthamide residue of elongation factor 2 (eEF2)	Death of affected cells
Multifunctional autoprocessing repeats-in-toxin (MARTX)	Determines depolymerization of actin stress fibers and covalent cross-linking of cellular actin into multimers targeting the G-actin	Alter biological processes such as signaling or ytoskeletal structure
Hemagglutinin protease (HAP)	Acts through many global regulators, including cyclic AMP, receptor protein, and RNA polymerase sigma subunit	Degradation of the mucus barrier, modification of some of the toxins, and acting on junction-associated proteins.
Mannose-sensitive hemagglutinin (MSHA)	Hemagglutination	Helps biofilm formation and environmental survival of *Vibrio cholerae*
V. cholerae cytolysin (VCC)	Acts on the target cells by making transmembrane oligomeric β-barrel pores, leading to permeabilisation of the target cell membranes	Extensive vacuolation and death of cells creates an anion-selective channel in planar lipid bilayers and cells.

Other studies have also shown that if *Vibrio cholerae* is cultured at temperatures between 10°C and 30°C, there is a linear correlation between the increase in temperature and the abundance of bacterial growth [39,40]. Some studies have suggested that a temperature increase can also increase the virulence of *Vibrio* [41].

The other factor, salinity, influences the growth of *Vibrio* species; they can grow generally in waters with salinities between 1 g/l and 17 g/l [40]. The optimum salinity for *Vibrio cholerae* is between 5 and 25 g/L [36].

The pH of the water is also a factor that influences the growth of Vibrio cholerae. A higher pH is better tolerated and leads to a larger population of bacteria in the respective water [42]. An alkaline pH of 8.5 is optimal for the attachment and multiplication of *Vibrio cholerae* [43].

Vibrio cholerae was first recognized as an aquatic bacterium and only after a period as a human pathogen in 1977. However, John Snow established the relationship of cholera with contaminated water more than 100 years ago [44]. In areas where cholera is not endemic, most of the isolated strains are non-O1/non-O139 [45].

Vibrio cholerae, a toxigenic and nontoxigenic serogroup, can be found in many geographic areas, from the tropics (Bay of Bengal is still an epicenter for cholera outbreaks) to temperate climate areas [46] and North and Middle America (Maryland and Louisiana [47], California [48], Haiti [49], New Carolina [50]); South America (Peru [51], Brazil [52]); Africa (Mozambique [53], Angola, Democratic Republic of the Congo, Mozambique, Nigeria, Somalia, Tanzania, and South Africa [54]); Asia Pacific (Bangladesh [55], Fiji [56], Australia [57], Japan [58]); Middle East (Iran [59]), Azerbaijan [60], Georgia [61]) and Europe (Sweden [62], Italy [63], Austria [64] and Germany [65]). The above are examples of countries for which studies can be found in the literature; the number of *Vibrio cholerae*-populated areas is much greater.

Recent outbreaks of vibriosis in temperate areas have been reported. Alaska, the Northeast USA, northern Spain, Chile, the Baltic Sea, Sweden, and Finland are among these unusual regions affected by vibriosis, probably in the context of local warming [2,66-69]. Changes in sea surface temperatures are most likely responsible for the advance of vibrios in areas where they have not been observed before [70]. Other changes in climatic events, such as heat waves and flooding, are also causing more frequent and widely encountered vibriosis outbreaks [2].

The incidence of noncholera and nontoxigenic cholera vibriosis is expected to increase in the future. Global warming and extreme climate events will be responsible, as will socioeconomic and behavioral elements such as demographic changes, population growth in coastal areas, increasing use of water for recreational purposes, and changes in seafood consumption patterns [71]. The areas with the highest risk are northern Europe, the Atlantic Northeast, the Pacific Northwest, and southeastern China [71].

CLINICAL MANIFESTATIONS OF NONTOXIGENIC VIBRIO CHOLERAE INFECTIONS

Vibrio cholerae non-O1/non-O139 strains are more and more frequently implicated in human diseases. As toxigenic *Vibrio cholerae* has drawn the most attention of *Vibrio cholerae* infections, the clinical data for non-O1 *Vibrio cholerae* infections are limited [72]. Emerging infections caused by non-O1 *Vibrio cholerae* have become another unneglectable problem [72]. The most frequent clinical manifestation of non-O1 *Vibrio cholerae* infection is gastroenteritis. Still, sporadic cases of extraintestinal infections, such as bacteremia, sepsis, skin infections or cellulitis, pulmonary involvement, cholecystitis, endophthalmitis, spontaneous bacterial peritonitis, urinary tract infection, liver abscesses, splenic abscesses, intracerebral abscesses, meningitis, and cholangitis, have also been reported [4,5,72].

Gastroenteritis

A few Vibrio cholerae non-O1/non-O139 strains can produce cholera toxin (CT), the toxin responsible for severe diarrhea characteristic of epidemic cholera [73,74].

Other non-O1/non-O139 *Vibrio cholerae* strains are able to produce a heat-stable enterotoxin named NAG-ST, which is quite similar to the enterotoxin of enterotoxigenic *Escherichia coli* [73,74]. Patients with gastroenteritis caused by non-O1/non-O139 *V. cholerae* may present with the classical symptoms of diarrhea, abdominal pain, fever, nausea, and vomiting [74]. A study including 14 sporadic cases reported in the United States (CDC) showed that all patients had diarrhea (25% had blood in their stools), 71% presented with fever, and 21% with nausea and vomiting; the symptoms alleviated after a median duration of 6.4 days [74]. Some of the cases presented with a less severe form of illness, with a median incubation period of 12-24 hours and a duration of symptoms ranging from 12-24 hours [74].

Bacteremia /Septicemia

The clinical manifestations described in patients with non-O1/non-O139 *Vibrio cholerae* bacteremia include fever (100%), abdominal pain (70%), diarrhea (50%), skin lesions (45%), peritonitis (35%), hypotension (30%), encephalopathy (25%) and gastrointestinal bleeding (15%). The reported cases involve consuming common foods, such as prawns, shellfish and other seafood or fish. The mortality rate is 50% [74-76]. Cirrhotic patients seem to be more susceptible to developing bacteremia and other forms of enteroinvasive infections that are usually associated with significantly higher mortality [75, 76]. Patients at risk should be questioned about their eating behavior and administering empirical antibiotic therapy should be considered if suggestive symptoms occur [75,76].

Spontaneous bacterial peritonitis (SBP) is defined as the spontaneous infection of ascitic fluid without an intra-abdominal source of infection [75,77]. SBP is caused by the translocation of bacteria from the intestinal lumen to the lymph nodes, followed by a bacteremia episode and eventually seeding with germs of the ascitic fluid [77]. This is why cirrhotic patients develop bacteremia with non-O1/non-O139 *Vibrio cholerae if they have ascites, the ascitic fluid may become infected,* leading to spontaneous bacterial peritonitis [77,78]. The reason for the high prevalence of *Vibrio* spp. infection in cirrhotic patients may be due to impaired bactericidal activity, affected filtration function in the liver, or increased blood iron levels [78,79]. Serum complement tends to be lower in cirrhotic patients and negatively correlates with the Child-Pugh score [78,79]. Low concentrations of C3 and C4 also result in impaired opsonophagocytosis [79,80]. Non-O1/non-O139 *Vibrio cholerae* extraintestinal infections are rare but have an increased mortality rate.

This etiology should be considered in cases of peritonitis and bacteremia in compromised patients that have some particular eating habits or possible exposure to *V. cholerae*.

Septicemia caused by non-O1/non-O139 *V. Cholerae* is a not very frequent but life-threatening disease, especially in small children. There are few reports of infants who have developed septicemia due to non-O1/non-O139 *Vibrio cholerae*. These reports suggest that meningitis is a frequent complication of septicemia caused by non-O1/non-O139 *V. cholerae* since 6 of 7 infants developed it. Diarrhea was absent in all the infant patients. Factors such as *hap*, *PrtV*, and *chxA* are associated mainly with extraintestinal infections, thus explaining the absence of gastrointestinal symptoms in the reported cases. These virulence factors may act synergistically in the production of bacteremia and the invasion of the meninges [81,82].

Skin, soft tissues and wound infections

Non-O1/non-O139 *Vibrio cholerae* wound infections generally occur after wound exposure to seawater or brackish water. Cases have also been described as being associated with nonsaline water, including some reports of necrotizing fasciitis caused by non-O1/non-O139 strains in some Austrian bathing places [83]. Otitis or pneumonia have been described after near-drowning episodes [83]. The most frequently encountered soft tissue lesions are localized cellulitis, with or without bullous and hemorrhagic lesions (66.7%), whereas necrotizing fasciitis was not very frequent (29.2%). In Europe, only one necrotizing fasciitis case caused by non-O1/non-O-139 *Vibrio cholerae* (associated with Mediterranean Sea bathing) was reported in Italy in 2019 [83,84]. All these infections were associated with saline water, as were singular reports of *Vibrio cholerae* non-O1/non-O-139 necrotizing fasciitis from the United States and Taiwan [85,86]. Only the cases from Austrian bathing sites were not associated with saline water [83].

Endophthalmitis

Reported cases are sporadic in the literature, but few severe cases are related to patients with sepsis caused by *Vibrio cholerae* non-O1/non-O-139. Keratitis has also been reported as a rare manifestation [87]. In *Vibrio cholerae* non-O1, the hemagglutinin protease encoded by the *hap* gene can degrade the essential tight junction-associated protein occludin and disrupt the ZO-1 conformation [87,88]. Therefore, we may assume that Vibrio cholerae non-O1 can directly affect epithelial occludin and ZO-1 via this enzyme, digest collagens and lead to corneal opacification [88].

Otitis media and otitis externa

Otitis caused by *Vibrio cholerae* is rare. It is described in patients after exposure to freshwater or saltwater. The clinical manifestations include earache and purulent discharge from the ear. The condition may be complicated by hearing loss, inner ear, and/or facial nerve involvement, including vertigo, and extracranial and intracranial determinations [89,90].

DIAGNOSIS

Collection and Transport

When epidemiological data, the patient's geographical origin, contact with endemic areas, or contact with patients suffering from cholera are known, the laboratory is alerted for diagnostic preparation. Information regarding travel history, seafood consumption, events associated with marine activities (injuries, trauma), and aquatic hobbies is crucial.

Vibrionaceae are sensitive to dry conditions. The samples were transported in Cary Blair medium, alkaline peptone water, or any noninhibitory saline medium. Blood samples will be transported to the laboratory in special recipients for blood culture.

Direct examination

Direct examination is not recommended as a routine method, as pathogenic vibrios cannot be differentiated from other members of the enteric microbiota.

Direct detection of Vibrio cholerae O1 and O139 in feces

The oldest method is the microscopic immobilization test, which involves the loss of motility of *Vibrio cholerae* O1 and O139 after specific antibodies (O1 or O139) are added to fresh fecal samples [91]. Available commercial rapid tests include "Smart Cholera O1" (sensitivity 83% and specificity 88%) and "Bengal Smart O139" (sensitivity 100%, specificity 97%) (New Horizons Diagnostics Corp./Columbia, MD). There are also rapid immunochromatographic tests (dipstick) that detect the presence of O1 and O139 antigens in feces ("Span Diagnostics"/Surat, India and "Standard Diagnostics"/Kyonggi-do, South Korea). For "SD Bioline Cholera Ag O1/O139" (Alere Inc., Waltham, MA), the manufacturer presents a sensitivity of 95.4% for O1 and 99.2% for O139, with specificities of 94.1% and 98%, respectively.

Molecular detection in clinical specimens

Molecular methods for detecting species of the genus *Vibrio* have been described in the literature since the 1990s. One of the major advantages of molecular tests is the ability to store and freeze samples for further testing and subsequent epidemiological studies. Their utility has been highlighted in areas with low incidence rates, rare, unpredictable cases, and limited laboratory capabilities in terms of diagnostic experience or resources, leading to a substantial reduction in the turnaround time for results.

Currently, multiplex PCR nucleic acid amplification tests are used to detect *V. cholerae* directly in clinical samples. These tests include primers for the ctxA, rfb (for the O1 and O139 antigens), and tcpA genes (specific to the El Tor and classic biotypes) [92,93]. Commercial multiplex PCR gastrointestinal panels are available to detect pathogenic vibrations in fecal samples. Notably, these panels differ in the Vibrio species they detect (Table 2).

Bacterial isolation methods

Inoculation of biological samples is performed on specific media to isolate intestinal pathogens. The samples were inoculated onto MacConkey agar and Salmonella/Shigella agar (SS), and colonies belonging to the genus Vibrio appeared decolorized. Some strains of V. cholerae may be inhibited by these culture media. On media containing sucrose, such as Hektoen agar, *V. cholerae*, which is sucrose positive, cannot be differentiated from other Enterobacteriaceae that rapidly ferment sucrose. Inoculation of sheep blood agar can reveal beta-hemolytic colonies. A positive oxidase test for these colonies may suggest that they belong to the genus *Vibrio*. The selective thiosulfate-citrate-bile salts-sucrose (TCBS) medium is specific for the isolation of vibrios.

Table 2. Species of the genus *Vibrio* and target genes in commercial gastrointestinal multiplex panels.

Gastrointestinal Multiplex Panel	Detected *Vibrio* Species	*Vibrio* Target Gene	Comments
Luminex GPP x TAG	*V. cholerae*	*ctx*	Only strains expressing the *ctx* gene are detected.
Verigene enteric pathogens (Nanosphere)	*V. cholerae, V. parahaemolyticus*	*rfbL, trkH, tnaA*	Does not differentiate species.
BD MAX Extended Enteric Bacterial Panel	*V. cholerae, V. vulnificus, V. parahaemolyticus*	-	Does not differentiate species.
FilmArray Gastrointestinal Panel (Biofire)	*V. cholerae, V. vulnificus, V. parahaemolyticus*	*gyrB* *toxR*	Does not differentiate species.
	V. cholerae		Differentiates *V. cholerae* from other species.
QIAstat-Dx® Gastrointestinal Panel/QIAGEN GmbH	*V. cholerae, V. vulnificus, V. parahaemolyticus*	-	Differentiates *V. cholerae* from other species.

Ctx – cholera toxin, *trkH* – k transporter protein H, *tnaA* – tryptophanase A, *gyrB* – DNA gyrase B, *toxR* – virulence regulatory protein.

Sucrose-positive V. cholerae colonies develop on this medium as yellow, raised colonies [94].

Chromogenic media, such as CHROMagar Vibrio (CHROMagar Microbiology, Paris, France), can be used for isolating V. parahaemolyticus (in cases of food poisoning after seafood consumption) and other vibrios.

The morphology of *V. cholerae* colonies is highly variable: they can range from smooth (S), rough (R), convex, flat, or spread, to compact. Sometimes, *V. cholerae* develops very rough colonies with a highly wrinkled appearance on media lacking sugars, which are highly virulent to humans [95]. This phenotype is characterized by a unique extracellular polysaccharide involved in biofilm formation and resistance to chlorine, acidic pH, and the bactericidal activity of serum [95,96]. Some isolates of classic biotype *V. cholerae* possess the Vibrio polysaccharide synthesis (vps) gene, which encodes the rough phenotype. However, rough variants have only been described for the El Tor biotype [96].

Identification

1. Phenotypic tests – Commercial systems

Commercial identification systems are continually evolving as databases are updated. A comparative evaluation of six commercial systems used for identifying only the species listed in their databases was conducted: API20E (bioMérieux, Inc., Durham NC), Crystal E/NF (BD Biosciences), MicroScan Neg ID type 2 and 3 (Siemens, Healthcare Systems, West Sacramento, CA), and Vitek GNI+ and ID-GNB cards (bioMérieux). These systems correctly identify only 63–81% of microorganisms down to the species level. The accuracy of the identification of the three clinically significant *Vibrio* species varies. For *V. cholerae*, API 20E provides only 50% identification accuracy, whereas Crystal E/NF achieves over 97% identification accuracy. The latter accurately identified ⩾90% of *V. cholerae* and *V. vulnificus* strains, whereas API 20E and Vitek GNI+ and ID-GNB identified ⩾90% of *V. parahaemolyticus* strains [97].

2. Molecular methods

Molecular methods have become routine in epidemiological diagnostics and research but are not standard in laboratory diagnosis in areas where cholera is not endemic. The use of 16S rRNA sequencing is particularly useful for identifying Vibrionaceae, as interspecies differences are minimal, and a constant polymorphism in 16S rRNA genes has been described [98]. Tarr et al. developed multiplex PCR using primers from multiple species, including *sodB* (for V. cholerae), *sodB* (for *V. mimicus*), *flaE* (for *V. parahaemolyticus*), and *hsp* (for *V. vulnificus*), allowing accurate species identification, even for those misidentified by other methods [99]. Additionally, rpoB sequencing has been used to characterize strains that cannot be identified at the species level via phenotypic methods [100].

3. MALDI-TOF MS

"Matrix-assisted laser desorption ionization-time of flight mass spectrometry" (MALDI-TOF MS) is a rapid, cost-effective method for species-level identification. MALDI-TOF MS allows for phylogenetic classification with results comparable to those obtained from *rpoB* sequencing for *Vibrio* species [101]. A multicentric evaluation study using the VITEK MS v.2.0 (bioMérieux) system identified >90% of strains for the common species *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae*. However, data on the performance of MALDI-TOF MS for other species, such as *V. alginosus* and *V. mimicus*, are limited. While *V. parahaemolyticus* and *V. vulnificus* are included in FDA databases for both the Bruker Biotyper (Bruker Daltonics Billerica, MA) and VITEK MS systems, *V. cholerae* is included only in the VITEK MS database. There is a need to expand the database for the genus *Vibrio* [102].

4. Detection of Cholera Toxin

Historically, cholera toxin production was tested by detecting the cytopathic effect on hamster ovary cell cultures. Currently, a commercial test based on the principle of "reverse passive latex agglutination" (VET-RPLA, Denka Seiken) detects two types of toxins: cholera toxin and heat-labile enterotoxin produced by enterotoxigenic *Escherichia coli* (ETEC). Another accessible method for screening *V. cholerae* isolates for the presence of the ctxA gene is multiplex PCR [92,93]. Two genes, *tdh* and *tdh2x*, encode a thermostable hemolysin present in non-O1 *V. cholerae*. Like cholera toxin, this thermostable hemolysin can also be detected via latex agglutination (KAP-RPLA, Denka Seiken). PCR tests detecting genes for both toxins are under development and commercialization [93].

5. Serotyping

Commercial sera for serotyping are available only for *V. cholerae* and *V. parahaemolyticus*. BD Biosciences, Denka Seiken, Remel, and New Horizons produce polyclonal antisera (Inaba and Ogawa) against O1 and O139.

6. Molecular typing

Pulsed-field gel electrophoresis using *NotI* and *SfiI* enzymes is the standard traditional method for molecularly typing vibrios for epidemiological surveillance [95,103]. Genomic analyses have demonstrated that Haiti's cholera epidemic likely originated externally from Nepal [104,105].

■ TREATMENT AND PREVENTION

Treatment recommendations for nontoxigenic *Vibrio cholerae* infections are not standardized, and no controlled trials for gastroenteritis or sepsis with this etiology exist.

Table 3. Recommended antibiotics in the treatment of different forms of nontoxigenic Vibrio cholerae infections.

Infection type	Treatment regimens	Duration of treatment
Gastrointestinal	Monotherapy with	
	Doxycycline 300 mg once PO or	Once
	Doxycycline 100 mg bid PO or	3-5 days
	Azithromycin 1 g once PO or	Once
	Azithromycin 500 mg PO qd or	3-5 days
	Ciprofloxacin 500 mg bid po or	3-5 days
	Levofloxacin 500 mg qd po or	3-5 days
	Ceftriaxone 2 g/day IV or	3-5 days
	Cefotaxime 2 g IV tid or	3-5 days
	Ceftazidime 2 g IV bid	3-5 days
Wound infection	Combination therapy	
	Ceftriaxone 2 g/day IV or	
	Cefotaxime 2 g IV tid or	5-7 days
	Ceftazidime 2 g IV bid plus	
	Doxycycline 100 mg PO bid	
	Or	
	Ceftriaxone 2 g/day IV or	
	Cefotaxime 2 g IV tid or	
	Ceftazidime 2 g IV bid plus	5-7 days
	Ciprofloxacin 500 mg bid PO or	
	Levofloxacin 500 mg qd PO	
	Or monotherapy with	
	Ciprofloxacin 500 mg bid PO or	5-7 days
	Levofloxacin 500 mg qd PO or	Or adapted to the clinical evolution
Septicaemia	Combination therapy	
	Ceftriaxone 2 g/day IV or	
	Cefotaxime 2 g IV tid or	5-7 days
	Ceftazidime 2 g IV bid plus	
	Doxycycline 100 mg bid PO	
	Or	
	Ceftriaxone 2 g/day IV or	
	Cefotaxime 2 g IV tid or	
	Ceftazidime 2 g IV bid plus	5-7 days
	Ciprofloxacin 500 mg bid PO or	
	Levofloxacin 500 mg qd PO	
	Or monotherapy with	
	Ciprofloxacin 500 mg bid PO or	5-7 days
	Levofloxacin 500 mg qd PO or	Or adapted to the clinical evolution

The dosage is for adults. Doses in children are adjusted by age and weight.

Therefore, most treatment decisions are based on experience with toxigenic *Vibrio* cholerae infections and susceptibility testing of cultured isolates.

Patients who present with gastrointestinal forms of infections with non-O1/non-O139 *Vibrio cholerae* infections should initially benefit from volume repletion measures. In many cases, diarrhea is mild and self-limited, and antimicrobial therapy is unnecessary. In more severe forms, antimicrobial treatment is justified, as it is known to shorten the course of the disease and the excretion of viable germs [106]. Monotherapy is usually considered sufficient for treating gastroenteritis caused by nontoxigenic *Vibrio cholerae*. Based on current experience, doxycycline is a reasonable choice; macrolides and fluoroquinolones are alternatives. Because there has been an increasing number of cases of resistance in non-O1/non-O139 *Vibrio cholerae* to the above medications, susceptibility testing is recommended to confirm the correct choice of antibiotic [107,108]. Based on experience with cholera, the duration of therapy is between 1 and 5 days, according to the choice of antibiotic and the patient's response to therapy (Table 3).

Wound infections and bacteremia caused by nontoxigenic *Vibrio cholerae* require antibiotic therapy, as their evolution may be unfavorable without etiological treatment [109].

Wounds also need surgical care, such as early debridement of the infected wound or even fasciotomy in more profound trauma [7,109]. Patients with necrotizing fasciitis or compartment syndrome may deteriorate rapidly; therefore, surgical measures may be necessary as serial interventions [109]. Antibiotic treatment in these patients must be more aggressive and initiated immediately. The preferred regimen is a third-generation cephalosporin (e.g., ceftriaxone, ceftazidime, or cefotaxime) combined with a tetracycline antibiotic (e.g., doxycycline). An alternative would be a single-agent regimen with a fluoroquinolone (e.g., levofloxacin or ciprofloxacin). Most patients respond to one of the above regimens in 5–7 days, but the duration of treatment is established based on the clinical evolution of the wound [110,111]. Combinations of a third-generation cephalosporin plus a fluoroquinolone have also been tested and shown to be noninferior to the combination of a third-generation cephalosporin plus doxycycline. Tigecycline in combination with tigecycline plus ciprofloxacin has also been shown to be an efficient alternative in animal studies [112]. In children, a combination of trimethoprim-sulfamethoxazole plus

aminoglycoside may be used if tetracyclines or fluoroquinolones need to be avoided [110].

Sepsis caused by non-O1/non-O139 mortality rates varies between 24% and 61.5% in different cohorts [113]. Therefore, patients with such a suspicion of diagnosis must be treated aggressively, preferably in an intensive care unit, with empiric antimicrobial therapy and all the measures required to minimize the possible consequences of hypotension, septic shock, and multiple system and organ failure. The recommended regimens include combination therapy with a third-generation cephalosorin (e.g., cefotaxime or ceftriaxone) plus either a tetracycline (e.g., doxycycline or minocycline) or a fluoroquinolone (e.g., ciprofloxacin or levofloxacin) [110]. The duration of treatment is also not established but should be at least 14 days.

Avoiding the consumption of raw or undercooked seafood (particularly shellfish), especially during the warm summer months, prevents non-O1/non-O139 infections. Contact with brackish sea water should also be restricted primarily to immunocompromised patients. Any type of wound (e.g., cuts, abrasions) should not be exposed to seawater. Hand hygiene is important, but transmission occurs less often in this way because many microorganisms need to be ingested to cause the disease. Currently, vaccination is unavailable.

DISCUSSIONS

Because of its aggressive mode of manifestation, ability to produce pandemics, and not last, because of its historical importance, toxigenic *Vibrio cholerae* usually receives the most attention. Nevertheless, the so-called nontoxigenic serogroups (non-O1/non-O139) may sometimes be etiological agents of severe diseases, especially in immunocompromised patients and rarely in perfectly healthy patients. CNTP variants of the nontoxigenic serogroups of *Vibrio cholerae* are the second cause of diarrhea after toxigenic strains from this species of *Vibrio*. Nontoxigenic *Vibrio cholerae* (NTVC) can survive in highly varied and hostile media, thus overcoming most of the defense mechanisms of the human host. Although NTVCs are only exceptionally able to produce cholera toxin and induce well-known severe diarrhea, they can produce and use many other virulence factors that make them reputable pathogens for humans.

The area where *Vibrio cholerae* can be found is expanding toward the north because of global warming, and cases of vibriosis have been described in locations where these diseases were not found before, such as Alaska, the Northeast USA, Sweden, or Finland. Cases have been described even as close as 160 km from the Arctic Circle. A recent prediction of the extension of the favorable environment for *Vibrio* species shows that by the year 2100, the habitats of these pathogens will extend with 38,000 km of coastal borders [114]. Moreover, there is evidence that the extension of their habitat may also affect inland waters. NTVC expansion represents a clear example of how climate impacts the distribution of infectious diseases.

The identification of *Vibrio cholerae* and its serogroups requires a high level of suspicion. It is not as straightforward as other more common etiological diagnoses, requiring targeted maneuvers and investigations. Empirical recommendations for treatment are available, but susceptibility testing must always be performed because of the increasing resistance described recently.

CONCLUSION

Nontoxigenic serogroups (non-O1/non-O139) are increasingly identified as causative agents of human diseases. The forms of these diseases vary from mild to severe, sometimes even life-threatening. The clinical manifestations of NTCV infections can be grouped into gastrointestinal infections, wound infections, or septicemia, which may not always suggest this etiology. Since these microorganisms are continuously expanding in the context of current climatic changes, clinicians should be aware of the possibility of encountering *Vibrio cholerae* non-O1/non-O139 in regions where this microbe has not been described before. Therefore, raising awareness of these possible infections is essential, mainly because diagnosing such an infection is more complex and not as straightforward as diagnosing other diseases with similar manifestations.

Informed Consent Statement

Not applicable.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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