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Cholera: Environmental Reservoirs and Impact on Disease Transmission

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ABSTRACT *Vibrio cholerae* is widely known to be the etiological agent of the life-threatening diarrheal disease cholera. Cholera remains a major scourge in many developing countries, infecting hundreds of thousands every year. Remarkably, *V. cholerae* is a natural inhabitant of brackish riverine, estuarine, and coastal waters, and only a subset of strains are known to be pathogenic to humans. Recent studies have begun to uncover a very complex network of relationships between *V. cholerae* and other sea dwellers, and the mechanisms associated with the occurrence of seasonal epidemics in regions where cholera is endemic are beginning to be elucidated. Many of the factors required for the organism's survival and persistence in its natural environment have been revealed, as well as the ubiquitous presence of horizontal gene transfer in the emergence of pathogenic strains of *V. cholerae*. In this article, we will focus on the environmental stage of pathogenic *V. cholerae* and the interactions of the microorganism with other inhabitants of aquatic environments. We will discuss the impact that its environmental reservoirs have on disease transmission and the distinction between reservoirs of *V. cholerae* and the vectors that establish cholera as a zoonosis.

INTRODUCTION

Cholera is a severe and sometimes fatal diarrheal disease caused by the comma-shaped bacterium *Vibrio cholerae*. The disease is acquired through the consumption of food or water contaminated by this microorganism. Cholera has virtually disappeared from developed countries due to high hygiene standards and water quality; however, many developing countries that lack the needed infrastructure and have poor sanitation continue to endure the menace of the disease (1). Disease outbreaks are often associated with and accentuated by floods and conflict that allow increased fecal contamination of water supplies.

There are more than 200 known serogroups of *V. cholerae*, yet only 2 of them are known to cause cholera in humans (choleragenic): serogroups O1 and O139 (2). The two major pathogenicity factors of choleragenic *V. cholerae* are the cholera toxin (CT), the enzymatic source of the watery diarrhea; and the toxin-coregulated pilus (TCP), an essential colonization factor (3, 4). Nonetheless, there are several other serogroups of *V. cholerae* that, even though they do not cause cholera, can cause bloody diarrhea, gastroenteritis, and extraintestinal infections (5–8). These strains use an alternative set of virulence factors than those used by choleragenic *V. cholerae*, such as type III and type VI secretion systems (9–11).

V. cholerae belongs to the family *Vibrionaceae*, a highly varied group that encompasses both pathogenic and nonpathogenic bacteria (12). The *Vibrionaceae* are part of the marine and riverine microbiota and can be found both free living and in association with biotic and abiotic surfaces (12). Like other members of the *Vibrionaceae*, *V. cholerae* can be found associated with numerous components of its native ecosystem (Fig. 1).

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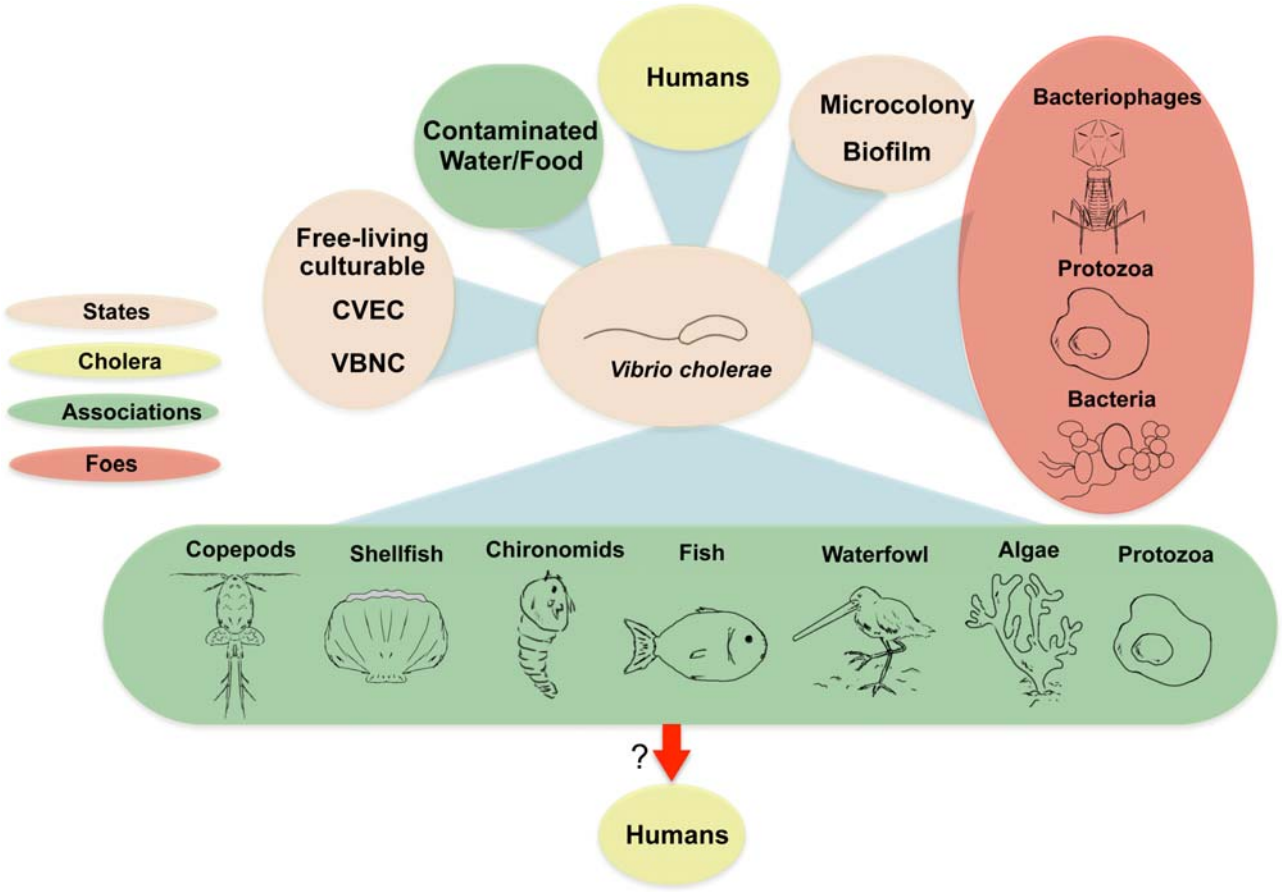
V. cholerae has been found associated with invertebrate members of the zooplankton such as crustaceans, dipterae, and shellfish; with vertebrates such as fish and waterfowl; and with other microorganisms such as *Acanthamoeba castellanii* (Fig. 1) (12–18). Also, a handful of studies found *V. cholerae* attached to the mucilaginous sheath of the blue-green algae *Anabaena* sp. (Fig. 1) (19, 20).

The infectious dose of *V. cholerae* required to cause cholera in healthy individuals is quite high; nonetheless, when the low pH of the stomach is buffered with sodium bicarbonate prior to the oral administration, the required dose to elicit the diarrhea decreases several logs (1). These results indicate that it is unlikely for *V. cholerae* in the free-living state to be the major source of epidemic cholera, as the stomach barrier appears to be a major hindrance to its survival. Thus, the association of the bacterium with other organisms and/or abiotic surfaces facilitates the ability of *V. cholerae* to cause the disease. Numerous findings support this hy-

FIGURE 1 *V. cholerae* life cycle and interactions.The life cycle of *V. cholerae* is complex and includes numerous physiological states and interactions with natural inhabitants of brackish riverine, estuarine, and coastal waters. *V. cholerae* can be directly isolated from the water (free-living culturable) or found in a VBNC state, as CVEC, or in the form of biofilms on diverse surfaces. It has been shown that the stools of patients with cholera still contain microcolonies of pathogenic *V. cholerae*. *V. cholerae* has several known natural predators, such as bacteriophages and protozoa. These predators are thought to play a crucial role in the dynamics of cholera epidemics by thriving on choleragenic *V. cholerae* when their numbers flourish. Also, some bacteria have antagonistic interactions with *V. cholerae*, preventing its growth on solid surfaces. Cholera can be acquired not only through the consumption of contaminated water containing choleragenic *V. cholerae* but also through the ingestion of foods contaminated with the bacterium. *V. cholerae* has been found associated with several sea and riverine dwellers such as algae, shellfish, chironomids and their egg masses, fish, waterfowl, amebae, and crustaceans, most critically copepods. The role of some of these environmental reservoirs of *V. cholerae* in cholera epidemics remains to be clarified. Nonetheless, several novel findings discussed in the text point to *V. cholerae* naturally requiring some of these hosts as vectors to cause cholera in humans, which establishes the disease as a zoonosis. doi:10.1128/microbiolspec.OH-0003-2012.f1



pothesis; for instance, it was found that ingestion of *V. cholerae* with food products decreases the infectious dose required to cause cholera, and the removal of particulate matter through filtration with a sari cloth reduced the incidence of the disease (1, 21–23). Whether *V. cholerae* interacts with other inhabitants of the aquatic environment and some of those interactions are involved in pathogenesis remains to be elucidated. Nonetheless, it is clear that *V. cholerae* can be transmitted through a set of vectors, indicating that cholera is a zoonosis.

What are the ecological factors affecting the life cycle of *V. cholerae* and its interactions with other inhabitants of the aquatic environment? What are the differences between organisms that act as reservoirs of *V. cholerae* and those that act as vectors of the bacterium?

VIBRIONACEAE

The family *Vibrionaceae* encompasses eight genera, of which the best studied are *Vibrio* and *Photobacterium*. The *Vibrionaceae* have an astonishingly wide array of hosts and they range from pathogens to symbionts. Some of the pathogenic members of the *Vibrionaceae* include *Vibrio vulnificus*, which causes fulminant septicemia in humans and is acquired through wounds mainly caused while handling shellfish. *Vibrio parahaemolyticus* causes an acute gastroenteritis in humans and is acquired primarily through the consumption of raw or undercooked seafood. Some members of the *Vibrionaceae* can be pathogenic in species other than humans. Many of those are of particular significance as they sicken and kill species related to aquaculture, causing major economic losses to the industry. For instance, *Vibrio anguillarum* causes vibriosis in farmed salmon, *Vibrio tubiashi* kills Pacific oysters, and *Vibrio harveyi* causes vibriosis in shrimp. Moreover, some members of the *Vibrionaceae* family affect the food industry negatively by establishing a symbiosis with their hosts. This is the case of the pufferfish and its *Vibrio* symbiont. The pufferfish meat is highly toxic to humans with the exception of some parts; however, the edible parts are a delicacy in countries such as Japan, where it is known as fugu. Some pufferfish species are poisonous due to a toxin produced by its *Vibrio* symbiont, which provides protection to its host from predators in its natural environment. Some members of the *Vibrionaceae* also pose ecological risks. This is the case with *Vibrio mediterranei*, *Vibrio shiloi*, and *Vibrio corallilyticus*, which are thought to be major causes of coral bleaching. These vibrios attack the endosymbionts (zooxanthellae) of the coral, and the loss of these pigmented symbionts results in bleaching of the coral. Many species belonging to the *Vibrionaceae* are bioluminescent, such as *V. harveyi* and *Vibrio fischeri*. *V. fischeri* establishes a symbiotic relationship with the Hawaiian squid *Euprymna scolopes*. *V. fischeri* colonizes the light organ of the squid and provides luminescence that is thought to confer protection from predators to its host by negating its shadow from the moon. Other species such as *Photobacterium phosphoreum* are the bioluminescent symbionts of jellyfish. None of these species are either obligate pathogens or obligate symbionts, as they can be found as free living in their natural environment.

V. CHOLERAE AND CHOLERA

Cholera causes death due to dehydration and electrolyte loss. Historically, only strains belonging to serogroups O1 and O139 have been known to cause the disease (1, 24). O1 strains can be subdivided into two biotypes: classical and El Tor. O1 classical was the cause of the first six recorded pandemics of cholera, which lasted from 1817 until 1923 (1, 24). O1 El Tor is the cause of the current seventh pandemic, which started in 1961. Since 1993 El Tor has virtually displaced classical (24). Serogroup O139 emerged in 1992, causing an explosive outbreak. That was first reported in India and Bangladesh. Serogroup O139 was originally thought to be a potential source for the eighth cholera pandemic; however, just a few years after the 1992 outbreak, cholera cases due to strains belonging to serogroup O139 steadily dwindled in number and are now virtually nonexistent (24). Recently, a series of El Tor clinical isolates have been identified that possess several classical traits (25–29). These El Tor variants have, among other characteristics, classical cholera toxin genes and increased CT production (25–29). Cholera remains endemic in parts of Africa, Latin America, and southern Asia, where seasonal epidemics occur frequently (1). Cholera still affects hundreds of thousands of people every year; for instance, a recent outbreak of cholera in Haiti killed more than 7,000 Haitians and sickened more than 500,000, affecting approximately 5% of the population (30, 31). Nonetheless, a simple effective treatment for cholera patients is fluid replacement with the appropriate electrolyte composition. To shorten the recovery time, antibiotics can be given to patients with severe cholera symptoms.

How *V. cholerae* regulates its virulence genes has been extensively studied. Briefly, two inner membrane-

localized regulators, ToxR and TcpP, are required to transcribe the *toxT* gene, which encodes the master regulator of virulence in choleragenic *V. cholerae* (32, 33). ToxT is a transcriptional regulator required for the expression of the *ctxAB* operon, which encodes the two subunits of CT; and the *tcp* operon, which encodes TCP. Nonetheless, little is known about what role these virulence factors may have in the environment, if any.

NONCHOLERAGENIC, PATHOGENIC *V. CHOLERAE*

Several non-O1, non-O139 strains have been identified as the cause of sporadic cases of gastroenteritis, bloody diarrhea, and extraintestinal infections (5–9, 34, 35). Non-O1, non-O139 strains are very heterogeneous by nature, so it is highly likely that they have developed independent ways of colonizing the intestine and causing disease (36). In particular, the mechanisms of two non-O1, non-O139 strains have recently been elucidated. *V. cholerae* V52 belongs to the O37 serogroup and encodes a type VI secretion system that allows it to be virulent toward amebae, mice, and other bacteria such as *Escherichia coli* (37–40). *V. cholerae* AM-19226 is a non-O1, non-O139 strain that belongs to the O39 serogroup (10). In 2005 it was found that AM-19226 encodes a type III secretion system that might confer pathogenic properties to the microorganism (10). Recently, it was shown that *V. cholerae* AM-19226 requires the type III secretion system to produce diarrhea and epithelial damage in rabbits (11, 41). Further research will likely uncover novel mechanisms of pathogenesis for some of the yet unstudied virulent non-O1, non-O139 strains.

EPIDEMIOLOGY AND ECOLOGY OF CHOLERA

The vast majority of *V. cholerae* strains isolated in their natural environment, brackish rivers, estuaries, and coastal areas, are nonpathogenic. In one study of an area of cholera endemicity, only 0.8% of the strains isolated encoded TCP and carried the phage encoding CT, CTXϕ (36). Furthermore, in areas of endemicity such as the Ganges Delta region, cholera is strongly seasonal, with outbreaks occurring twice a year. Typically there is one major outbreak right after the monsoon and another one during the spring. Its marked seasonality and association with a small number of pathogenic strains make the epidemiology of cholera quite puzzling and complex. In the natural environment of *V. cholerae* there are numerous factors that affect its persistence, survival, and pathogenic potential (Fig. 1).

Some studies have shown that *V. cholerae* can be directly cultured from water samples (42). However, in most cases *V. cholerae* has been found to persist in its natural environment mainly in two forms: viable but not culturable (VBNC) and conditionally viable environmental cells (CVEC) (42, 43). VBNC is a dormant state that *V. cholerae* enters in response to nutrient deprivation and other environmental conditions (42). These forms cannot be recovered by culture techniques but are still able to cause infection and under certain conditions can revert to the culturable form (42). It was recently shown that *V. cholerae* can also enter a CVEC state in which it can be recovered after the appropriate enrichment culture techniques are applied (43).

Several physicochemical conditions affect *V. cholerae* populations in the natural environment, such as water temperature, salinity, oxygen tension, sunlight, rainfall, pH, and the availability of trace elements and chemical nutrients (42, 44). Although there are strong correlations between the changes in the physicochemical conditions in the environment of *V. cholerae*, the mechanisms by which some of them affect the population dynamics of *V. cholerae* remain unknown.

The known environmental hosts of *V. cholerae* include algae, shellfish, chironomid egg masses, fish, waterfowl, amebae, and most ubiquitously, copepods (15–18, 42, 45–53). Nonetheless, it is very possible that *V. cholerae* associates with a larger number of dwellers of its natural environment as the field is still young and some of these associations were found recently. Some associations might allow the bacterium to persist during interepidemic periods and act as a reservoir for *V. cholerae*. Nevertheless, there are several instances where *V. cholerae* is transmitted through a vector due to the consumption of fish or shellfish, indicating that cholera can perhaps more accurately be described as a zoonosis (54–60).

Prior to a full epidemic outbreak, several factors have to be met. From the environmental standpoint, there need to be changes in the physicochemical conditions that have been linked with algal blooms, where copepods thrive. Two major drivers of phytoplankton abundance have been found: the upwelling of cold, nutrient-rich deep ocean waters and, more recently, river discharges with terrestrial nutrients (61, 62). *V. cholerae* in turn establishes a commensal association with the copepods by forming biofilms on their chitinous surfaces, thus also multiplying during the algal blooms (13, 42, 44). Since the number of toxigenic strains during

interepidemic episodes is very limited, it is thought that there is a period of enrichment for choleragenic strains both in the human host and in the environment prior to an epidemic of cholera (36). Briefly, intestinal passage of a mixed population of *V. cholerae* allows pathogenic clones to colonize and multiply, going through a selective enrichment period. However, due to low initial concentrations of choleragenic *V. cholerae*, the carriers can show no symptoms of the disease. Nonetheless, these asymptomatic carriers will shed pathogenic clones in their stools, further enriching the water sources with virulent bacteria and facilitating the initiation of an epidemic (36). In the early epidemic period a similar process happens; this time the patients will show symptoms of cholera and will shed strongly adapted and highly virulent epidemic clones (36). It is thought that when the number of predators of *V. cholerae* significantly outnumbers the total of toxigenic clones, the epidemics come to a collapse (63, 64). For example, an increase in the number of bacteriophages that thrive on *V. cholerae* in both water and stools is directly correlated with the termination of cholera epidemics (63, 64). Nonetheless, other environmental factors likely also play a role in the self-limiting nature of cholera epidemics, which we will discuss in further sections.

GENOME EVOLUTION OF CHOLERAGENIC *V. CHOLERAE*

Only O1 and O139 isolates of *V. cholerae* have been reported to be choleragenic. Interestingly, their two major virulence factors are encoded within mobile genetic elements that were acquired through horizontal gene transfer (65, 66). CT is encoded within the filamentous phage CTXϕ (65). The CTXϕ phage has been demonstrated to be transferable between *V. cholerae* strains, with TCP acting as the phage receptor (65). Interestingly, the transfer rate was higher within the gastrointestinal tracts of mice than under laboratory conditions (65). These findings place the human gastrointestinal tract of asymptomatic carriers not only as a vehicle for multiplication of toxigenic *V. cholerae* but also as a possible niche where the acquisition of virulence genes might occur. TCP is encoded within the *Vibrio* pathogenicity island-1 (VPI-1) (66). Like CTXϕ, VPI-1 is able to excise from its host chromosome and form circular intermediates (67). This would potentially allow for the transfer of the TCP operon to "naïve" strains of *V. cholerae*.

Other mobile genetic elements have been associated with virulence in choleragenic isolates of *V. cholerae*: the SXT integrative conjugative element, VPI-2, and *Vibrio* seventh pandemic island-1 (VSP-1) and -2 (VSP-2) (68–70). The SXT element is self-transmissible and confers to *V. cholerae* isolates resistance to streptomycin, sulfamethoxazole, and trimethoprim (68). VPI-2 is confined to pathogenic isolates of *V. cholerae* and encodes a cluster of genes involved in the transport and catabolism of sialic acid (71, 72). The ability to utilize sialic acid as a carbon source confers a competitive advantage to choleragenic vibrios in the mouse intestine (71). VSP-1 and VSP-2 were identified a decade ago using microarray technology to identify regions that were unique to El Tor strains; however, not until recently has a putative function for VSP-1 been described (70, 73). VSP-1 encodes a transcription factor, VspR, that is under the control of a ToxT-regulated small RNA (73). VspR modulates the expression of several genes encoded within VSP-1, one of which encodes a new class of dinucleotide cyclase, DncV (73). DncV synthesizes a hybrid cyclic AMP-GMP molecule, which is required for efficient intestinal colonization and downregulates *V. cholerae* chemotaxis, a phenotype that is associated with hyperinfectivity (73, 74). To date, no putative function has been associated with VSP-2. The four pathogenicity islands that choleragenic *V. cholerae* encodes, VPI-1, VPI-2, VSP-1, and VSP-2, can excise from their host's genome and form circular intermediates, which could hypothetically allow the transfer of virulence genes to other nonpathogenic *V. cholerae* strains (67, 75, 76).

The fact that the major pathogenicity factors of *V. cholerae* are encoded within mobile genetic elements suggests that there might be hybrid strains that have acquired only a subset of these elements. Interestingly, it has been repeatedly found that some non-O1, non-O139 environmental strains carry virulence genes (77–80). These strains have the potential of acting as reservoirs of virulence genes for noncholeragenic strains of *V. cholerae*.

It was recently found that the major component of the shell of crustaceans, chitin, induces natural competence of *V. cholerae* (81). *V. cholerae* has been found associated with copepods in its natural environment, where it forms biofilms while attached to their chitinous surface. The remarkable finding that *V. cholerae* becomes naturally competent when thriving on chitin, together with the existence of numerous hybrid strains of *V. cholerae* that encode some of the mobile genetic elements associated with virulence, strongly points to the shell of copepods being a crucial place where exchange of genetic material occurs among *V. cholerae* strains and where novel pathogenic isolates might arise.

INTERACTIONS OF *V. CHOLERAE* WITH ITS MULTIPLE ENVIRONMENTAL HOSTS

V. cholerae establishes complex interactions with a plethora of sea and riverine dwellers (Fig. 1).

Crustaceans

Of the many associations in which *V. cholerae* has been found, the most widely studied and feasibly critical one is that with copepods (13, 42, 44). Copepods, from the Greek for "oar feet," encompass a group of small crustaceans that are natural inhabitants of sea- and freshwater. Copepods feed on microscopic algae and, in turn, critically serve as food for millions of other invertebrates and fish, as they tend to be dominant members of the zooplankton. The population size of copepods is strongly associated with phytoplankton blooms from which they graze.

The exoskeleton of copepods and other crustaceans is composed of chitin. Chitin is a polymer of *N*-acetylglucosamine and is the most abundant polysaccharide found in aquatic environments. However, chitin is insoluble, and without bacterial activity that returns the polysaccharide into a soluble and thus biologically useful form, seawater would become depleted of carbon (82). *V. cholerae* has been found associated with the exoskeleton of copepods in large numbers (16, 51). *V. cholerae* is able to utilize chitin as a carbon source and has a complex chitin utilization program consisting of three sets of differentially regulated genes (83). The commensal relationship between *V. cholerae* and chitinaceous hosts provides several advantages to the bacterium other than nutrients. It has been found that when attached to copepods *V. cholerae* cells can withstand changes in salinity and pH that are detrimental to the organism in its free-living state (14, 84). *V. cholerae* forms biofilms while associated with copepods; this facilitates its growth, survival, and persistence in aquatic ecosystems. Biofilm formation requires the presence of the mannose-sensitive hemagglutinin type IV pilus and the flagellum (85). The mannose-sensitive hemagglutinin type IV pilus contributes to the attachment of *V. cholerae* to the copepod *Daphnia pulex* (52). The chitin-regulated pilus is also involved in the attachment of *V. cholerae* to chitin (83). Interestingly, one colonization factor, GbpA, mediates attachment to the exoskeleton of *D. pulex*, epithelial intestinal cell lines, and the mouse intestine (86). This finding provides a link between environmental survival and the pathogenesis of *V. cholerae*, indicating that the functions of some pathogenicity factors do not have to be exclusively related to virulence (86). Furthermore, when *V. cholerae* grows on chitin, it becomes naturally competent; that is, it can take up DNA from its environment (81). Therefore, the possibility of horizontal gene transfer, that is, the bacterial acquisition of genes or gene clusters that might confer pathogenic potential, is greater when *V. cholerae* is attached to the chitinous surface of the copepods. It was recently found that *V. cholerae* also enters a hyperinfectious state when growing on biofilms (87). Tamayo et al. showed that the infectious dose required to colonize the mouse intestine was orders of magnitude lower for biofilm-derived *V. cholerae* than for planktonic cells (87).

The association with copepods provides at least four crucial advantages to *V. cholerae*: its exoskeleton can be used as a carbon source; and it provides protection, induces the transfer and acquisition of genes, and promotes *V. cholerae* entry into a hyperinfectious state. Overall, these findings show that the association with copepods is critical in the epidemiology of cholera. Several findings support this hypothesis. First, the infectious dose of *V. cholerae* needed to cause cholera in healthy individuals decreases several logs when the low pH of the stomach is buffered with sodium bicarbonate prior to oral administration or when the bacterium is found associated with food products (1). As aforementioned, the association of *V. cholerae* with copepods confers resistance to low pH and might be a requirement to go through the stomach. This hypothesis seems to be supported by recent findings (21–23). It was found that the removal of particulate matter from drinking water through filtration with a traditional Indian garment termed a sari yielded a 48% reduction in the incidence of cholera in some areas of endemicity in rural Bangladesh (23). A subsequent study showed a sustained decrease in the incidence of cholera in those villages that kept using the sari filtration method (21).

V. cholerae also associates with other crustaceans such as shrimp and blue crab (48, 49, 88). Nonetheless, the direct association between the presence of *V. cholerae* attached to these crustaceans and its survival through the stomach remains to be elucidated.

Shellfish

V. cholerae has recurrently been isolated from raw oysters at a wide variety of locations around the world, including the United States, Australia, Brazil, and India (47, 50, 53, 59, 89–92). The bacterium has also been found associated with clams and other mollusks (93). Strikingly, there are several reported cases of cholera and severe diarrhea due to ingestion of raw oysters harboring *V. cholerae* (58–60). Several of those cases occurred

in the United States in places such as Texas, Florida, and Louisiana, where regular inspections occur and hygiene standards are high, stressing the difficulty of detecting *V. cholerae* and preventing it from establishing itself within its host (58, 59). The presence of choleragenic *V. cholerae* poses a potential threat to public health that might be on the rise as waters warm up due to climate change and *V. cholerae* populations migrate to previously inhospitable new niches (94, 95).

Arthropods

In 2001, Broza and Halpern showed that *V. cholerae* also associate with egg masses of the nonbiting midge *Chironomus* sp. (51, 96–99). They found that the egg masses acted as the sole carbon source for *V. cholerae*, allowing the bacterial population to be sustained solely on that substrate. This finding introduced a novel natural reservoir for *V. cholerae*, which is also highly abundant as chironomids are the most widely distributed insect in freshwater (51). Additionally, *V. cholerae* can colonize the fly intestine in a biofilm-dependent manner (100). Overall, these findings clearly reveal that arthropods act as major reservoirs of *V. cholerae*.

Fish

Isolated cases of cholera have been associated with the consumption of sardines, salt fish, and dried fish in places such as Australia, Peru, India, Italy, and Tanzania (54–57, 101). *V. cholerae* was isolated from fish, *Sciaena deliciosa*, that were caught in Peru during a Peruvian epidemic (55). It has even been postulated that the endemicity of *V. cholerae* in areas of India and Bangladesh might be due to its association with hilsa fish (15). Only recently has *V. cholerae* been directly isolated from fish samples (15). Senderovich et al. found that several fish species from different habitats contained *V. cholerae* in their digestive tract, with concentrations as high as 5×10^3 CFU per gram of intestine (15). Among them was *Tilapia* spp., which is known to consume copepods and chironomids, known reservoirs of *V. cholerae* (15, 18). These findings demonstrate that fish act both as a reservoir and vector for the transmission of *V. cholerae*, facilitating colonization of humans and also dispersal of the bacterium and its migration to novel habitats.

Waterfowl

Recently, attention has been directed toward the role that waterfowl have in the dispersal of *V. cholerae* into novel areas (18). Residential and migratory waterfowl thrive on chironomids and copepods, which can survive within the gut of water birds (18, 102). There is also evidence that viable copepods and chironomids can be found associated with the feet and feathers of waterfowl (18). These two findings indicate that waterfowl could potentially disseminate two major reservoirs of *V. cholerae*. As previously mentioned, fish also act as reservoirs of *V. cholerae* (15). *Tilapia* spp., from which *V. cholerae* has been isolated, are regularly consumed by numerous species of waterfowl such as cormorants, pelicans, seagulls, egrets, and herons (103). Waterfowl also consume other potential reservoirs of *V. cholerae* such as shellfish and crustaceans (18).

Both O1 and non-O1, non-O139 strains of *V. cholerae* have been isolated from a wide variety of birds (18). *V. cholerae* was detected in cloacal swabs taken from gulls in England and from the feces of aquatic birds in Colorado and Utah (104, 105). It is noteworthy that the incidence of isolations followed a strong seasonal pattern, with the highest numbers of *V. cholerae* being isolated in spring and fall (105). The seasonality in the isolations of *V. cholerae* from waterfowl follows a similar pattern as that of cholera outbreaks. Overall, these findings strongly support the hypothesis that migratory waterfowl act as disseminators of *V. cholerae* across water bodies.

Protozoa

In its natural environment, *V. cholerae* becomes the prey of several inhabitants of the aquatic ecosystem. *V. cholerae* has been found to establish two kinds of associations with different amebae species: as prey and as a putative symbiont. It was recently shown that *V. cholerae* can survive and multiply intracellularly inside the free-living amebae *A. castellanii* and *Acanthamoeba polyphaga*, which indicates that these protozoa may act as reservoirs of *V. cholerae* in the aquatic environment (17, 106, 107). It is possible that the association of *V. cholerae* with amebae and other protozoa might favor its transmission and survival within the human host, in a similar fashion to how *A. polyphaga* acts not only as a reservoir but also as a vector for *Legionella pneumophila* (108).

Algae and Water Plants

Using immunofluorescence, several studies have found *V. cholerae* associated with a wide variety of algal species and water plants. *V. cholerae* attaches to the mucilaginous sheath of cyanobacteria (*Anabaena variabilis*), diatoms (*Skeletonema costatum*), and phaeophytes (*Ascophyllum nodosum*) and to freshwater vascular aquatic plants such as water hyacinths and duckweed (20, 45, 46, 109, 110). Several studies have found some

factors involved in pathogenesis to be expressed or required while *V. cholerae* associates with algae. Islam et al. detected an increase in toxin production when *V. cholerae* is in association with the green alga *Rhizoclonium fontanum* (45). Also, a mucinase (HapA) that is part of the intestinal escape response of *V. cholerae* was found to play an important role in the association of *V. cholerae* O1 with *Anabaena* sp. (109, 111). HapA is additionally involved in the chemotactic response of *V. cholerae* toward the mucilaginous sheath of the green alga (112). These findings not only show that algae and other water plants can act as reservoirs of *V. cholerae* but also that some pathogenicity factors might have an environmental function and are useful for the bacterium outside of the human host.

THE FOES OF *V. CHOLERAE*

In its natural environment *V. cholerae* encounters two main predators: bacteriophages and protozoa. It has also been found that some antagonistic bacteria inhibit the growth of *V. cholerae* (Fig. 1).

Bacteriophages

There are more than 200 identified species of bacteriophages that can infect *V. cholerae*, known as vibriophages (113). Vibriophages can be lytic and/or lysogenic. The best-characterized vibriophage, CTXϕ, is a filamentous lysogenic phage that harbors the CT genes (65). In the last few years, the close relationship between the abundance of vibriophages and the seasonal nature of cholera epidemics has been revealed (63, 64, 114). Faruque et al. found that during a 3-year period (2001 to 2003) in Dhaka, Bangladesh, the number of cholera patients increased whenever the number of lytic vibriophages in water decreased (63). The study also found that the number of patients decreased and the overall cholera epidemics ended at the same time that the population of the vibriophages in the water increased to large numbers (63). Likewise, prior to the peak of the epidemic in Dhaka in 2004 there was high prevalence of *V. cholerae* in the environment, and as the epidemic ended the numbers of the lytic vibriophage JSF4 increased (64). Furthermore, there is a correlation between the peak in the numbers of the vibriophages and an increase in the presence of JSF4 in patients' stools (64). Mathematical models predict that if a cholera outbreak originates through an increase of *V. cholerae* in the environment, then the number of vibriophages will consequently proliferate, eventually leading to the decline and termination of the outbreak (114). It is likely that other factors are also involved in the termination of a cholera outbreak; however, their nature and role remain to be determined.

Protozoa

The relationship between *V. cholerae* and protozoa is puzzling, as some studies have found that *V. cholerae* can kill amebae but also survive and persist inside amebae or be consumed by them (37, 39, 106). The factors that modify and affect the nature of their relationship are beginning to be elucidated. For instance, it was recently found that the virulence regulator ToxR in *V. cholerae* is required for survival inside *A. castellanii*, providing an environmental function for a master regulator that is involved in the virulence of *V. cholerae* (115). This study highlights how just one factor can erase the thin line between being a commensal and becoming prey (115). Amebae such as *Dictyostelium discoideum* thrive on *V. cholerae* O1; however, it was recently found that some non-O1, non-O139 strains encode a mechanism that prevents *D. discoideum* from grazing on them (39). Pukatzki et al. determined that a type VI secretion system was responsible for killing *D. discoideum* in the strain V52, which belongs to the O37 serogroup (39). How grazing by amebae affects epidemics of cholera has yet to be determined; however, it seems likely that they might act synergistically with phages in the termination of cholera epidemics along with other factors such as changes in the environment.

Other Bacteria

Little is known about the relationship of *V. cholerae* with other marine bacteria, in particular regarding antagonistic interactions. Long et al. found that some marine bacteria inhibit the growth of *V. cholerae* on surfaces (116). Interestingly, they found that bacterial isolates derived from the surface of particles made of marine agar show a greater frequency of *V. cholerae* inhibition than free-living bacteria (116). *V. cholerae* is less susceptible to inhibition at higher temperatures, such as those measured during El Niño–Southern Oscillation and other seasonal events such as the monsoon. The mechanism of inhibition was linked to the biosynthesis of andrimid, an antibacterial agent produced by the antagonistic bacteria. The production of andrimid is decreased at higher temperature, which correlates with the lower susceptibility of *V. cholerae* at these temperatures (116). Overall, these findings corroborate the increased competitiveness of *V. cholerae* under warmer conditions and substantiate the hypothesis that many factors act in conjunction during cholera epidemics.

CONCLUDING REMARKS

V. cholerae associates with numerous dwellers of its natural environment, and its relationships with the different hosts vary widely. In this article we have presented a comprehensive description of these diverse associations. As can be gathered from the available data, an important distinction must be made between these associations: reservoirs and vectors of *V. cholerae*. A reservoir is a habitat in which an infectious agent generally lives, grows, and multiplies and can include humans, animals, and environmental niches. From this description we can contend that algae, arthropods, waterfowl, and protozoa act as reservoirs of *V. cholerae*, as the bacterium has been found associated with them but, so far, there is no evidence of cholera cases directly associated with these reservoirs. We argue that there is a distinction between reservoirs of *V. cholerae* and organisms that act as its vector—an epidemiological term that describes any living organism that carries and transmits an infectious pathogen into another living organism. There is significant supporting evidence that fish and shellfish act as vectors of *V. cholerae*, as they can directly transmit the disease. A very interesting association is that of *V. cholerae* with copepods, because those crustaceans may be one of the major vectors of cholera. However, linking the consumption of *V. cholerae* associated with copepods and the appearance of cholera is not trivial, as the crustaceans are microscopic and the patient is often unaware of ingesting them. Nonetheless, from these facts one can propose that cholera is a zoonosis with a diverse group of vectors and reservoirs, since a zoonosis is an infectious disease that can be transmitted from animals to humans by a vector. These advances in the ecoepidemiology of cholera and the subsequent changes in the terminology used to describe the disease will allow us to better understand how *V. cholerae* behaves in its natural environment and, thus, help researchers foresee and eventually prevent cholera outbreaks in areas of endemicity.

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