Emergence, ecology and dispersal of the pandemic generating *Vibrio cholerae* lineage

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Summary. Although cholera is an ancient disease that first arose at least half a millennium ago, it remains a major health threat globally. Its pandemic form is caused by strains from a single lineage of the bacterium *Vibrio cholerae*. The ancestor of this lineage harbored several distinctive characteristics, the most notable being the O1 antigen polysaccharide. This lineage generated two biotypes, first Classical, responsible for six pandemics, and later El Tor, responsible for the seventh and ongoing pandemic. Just as El Tor replaced Classical as the main cause of outbreaks in the last fifty years, several variants of El Tor have evolved and displaced their predecessors worldwide. Understanding the ecology, evolution and dispersal of pandemic *V. cholerae* is central to studying this complex disease with environmental reservoirs. Here, we present recent advancements of our knowledge on the emergence and spread of the pandemic generating lineage of *V. cholerae* in the light of established eco-evolutionary observations. Specific ecological interactions shape seasonal cholera, playing a role in the abundance and distribution of its causative agent. Both species-specific and lineage-specific genetic determinants play a role in the ability of *V. cholerae* strains to cause pandemics with seasonal outbreaks, having evolved gradually over centuries. On the basis of the current understanding, we outline future threats and changes in biogeographical and genomic-based investigation strategies to combat this global problem.

Keywords: *Vibrio cholerae* · cholera · pandemic · ecology · evolution

Introduction

Microorganisms causing human disease often have a complex dynamics of transmission, persistence and dispersion within their natural reservoirs [39]. *Vibrio cholerae*, the causative agent of cholera, is a unique model system to study the effect of such environment-human interactions in shaping a deadly infectious disease from aquatic origin [23]. Cholera has been endemic for centuries in many countries in South Asia and Africa, where it occurs almost every year, infecting people encountering the pathogen through consumption of untreated water [42]. According to World Health Organization (WHO) reports, there are roughly 1.3 to 4.0 million cases, and 21 000 to 143 000 deaths worldwide due to cholera every year (WHO cholera fact sheet, 2017). Cholera can also become pandemic and so

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far, seven recorded pandemics of this disease have shaken the world since 1817, killing millions of people worldwide [42, 32]. Unlike some other pandemic infectious diseases, cholera is unlikely to be eradicated. This is because its causative agent is an autochthonous member of marine and estuarine ecosystems around the world, and there is no clear transmission vector to serve as a means to control its human association cycle [23, 49]. Two main hypotheses have been proposed to explain the global spread of pandemic cholera. The first is that human travel has spread the bacteria from endemic countries to other parts of the world [61]. An alternative hypothesis is that ocean currents and maritime transfer of the pathogenic bacteria are responsible for the spread of cholera, with climatic events (i.e. El Niño, global warming) playing a major role in shaping pandemics [22, 35, 40]. Regardless of how *V. cholerae* spreads across the world, occurrence of the disease is significantly influenced by living standard of people in cholera prone regions as well as climatic conditions. For example, in countries with compromised water supply and sanitation infrastructure, excessive rainfall and

flooding can lead to massive cholera epidemics if pathogenic variants of *Vibrio cholerae* are present in the environment [42, 22,40]. Environmental links are evident in the dynamics of the disease, as cholera incidence in endemic areas usually show seasonal patterns, i.e. number of cholera cases increases and reaches a peak in specific months every year [33, 2].

In the environment, *V. cholerae* exists as a diverse species. However, a very small portion of the heterogeneous population of *V. cholerae* strains found in nature is capable of causing human disease [42]. The outer membrane lipopolysaccharides of *V. cholerae* have a region named O-antigen, the synthesis of which is encoded by a diverse combination of genes, giving rise to the remarkable diversity of more than 200 serogroups (starting from O1) of *V. cholerae* observed in nature [20]. Although isolated infections and outbreaks have been caused by various *V. cholerae* genotypes, pandemics are only caused by strains from a single lineage, most of which display the O1 antigen on their surface (Figure 1). Understanding cholera pandemics is therefore dependent on determining ecological characteristics of this specific lineage, not necessarily the entire *V. cholerae* species. This lineage has usually been referred to as *V. cholerae* O1/O139, which is misleading as it includes several strains displaying other serogroups (such as O37), and that many unrelated harmless strains display the O1 and O139 antigens [20]. Here we will refer to the monophyletic phylogenetic group containing all genotypes responsible for cholera pandemics as the Pandemic Generating (PG) lineage to avoid confusion. The factors affecting environmental persistence, survival during inter-epidemic periods, emergence and spread of pathogenic genotypes from this lineage are still poorly understood. This is because population-level analysis over a wide range of geographical locations and variety of potential niches requires large-scale sampling of a bacterium forming only a small proportion (usually <1%) of natural populations. Most of our current knowledge on *V. cholerae* ecology has been obtained from cultivation-based studies. Despite their limited sampling size, these studies have created a solid foundation to develop culture-independent population-level approaches that will enable investigations of pandemic *V. cholerae* local and global dynamics.

Seasonality of cholera is mediated by the ecological interactions of *V. cholerae*

V. cholerae has been detected in aquatic habitats from the tropics to temperate waters world-wide [50], underscoring it's highly adaptable and persistent nature over a broad range of environmental conditions [64]. However, cholera incidence patterns can vary greatly among geographic locations. It is firmly endemic in some South Asian countries, where it appears in distinct seasonal patterns [30]. Other regions, such as parts of South America and Africa, have historically had only sporadic epidemics of cholera [30]. In Bangladesh, cholera maintains an annual cycle with two infection peaks;

before monsoon and just after monsoon [1, 32, 2, 3]. This marked seasonality of cholera appears to be closely linked with the changes in flora and fauna of the coastal environment where pathogenic *V. cholerae* exists, mediated by micro- and macro-level environmental factors such as water temperature, salinity, organic matter concentrations, abundance of planktonic surface and water consumption [23, 30] and ultimately influenced by larger-scale climatic variables [49, 30]. In the aquatic environment, *V. cholerae* are known to be associated with phytoplankton, zooplankton, chitinous animals, aquatic plants, protozoa, bivalves, fish and water birds [50]. These associations could serve as environmental reservoirs where the pathogen can live over time, with the potential to be disseminated and cause cholera outbreaks in nearby human populations [9, 68]. Many of these studies looked at the entire *V. cholerae* species, but not specifically the PG lineage. Although the ecological parameters leading to high abundance of PG *V. cholerae* are not well understood, it is clear that during the annual cholera epidemic periods, the conditions in the coastal ecosystems are ideal for the multiplication and transmission of these bacteria. As a result, these water sources contain high enough concentrations of PG *V. cholerae* to cause human disease upon consumption of contaminated water or food that has come in contact with it [64, 38]. Those peak months display blooms of phytoplankton, which provide food for zooplanktons, both potential resources for growth of *V. cholerae* in water [27, 25, 66, 30]. Studies have found significant correlations with seasonal bloom in aquatic microorganisms and cholera incidence rates in nearby human populations [30, 37, 49]. During inter-epidemic periods, PG *V. cholerae* remains mostly undetectable by routine microbiological culture-dependent assays based on growth of the targeted bacteria on selective culture media [7]. However, using culture-independent techniques, including direct fluorescence antibody (DFA) assays and PCR, PG *V. cholerae* can readily be detected in the water year round, indicating a survival strategy making them either rare, unable to grow under laboratory conditions, or concentrated in specific reservoirs (host, sediments, particles, etc.) [7]. Indeed, *V. cholerae* possesses the ability to switch into a viable but non-culturable (VBNC) or dormant state in response to nutrient deprivation or other stresses [21, 24]. In its VBNC state, the *V. cholerae* cells become coccoid (as opposed to their normal curved rod shape) and do not respond readily to typical microbiological medium, hence cannot be detected by culture-based surveillance. These non-culturable cells have been found to retain pathogenic ability upon passage through animal intestine [21, 7]. Such a dormant state could serve as a survival strategy in inter-epidemic months, as resuscitation could occur once conditions are favorable again during epidemic months [22, 7].

In aquatic habitats, a possible reservoir of *V. cholerae* is chitin-containing organisms. Chitin can serve as a source of energy, carbon, and nitrogen and a substrate for biofilm formation for this microorganism [14, 70, 50]. Biofilms provide a
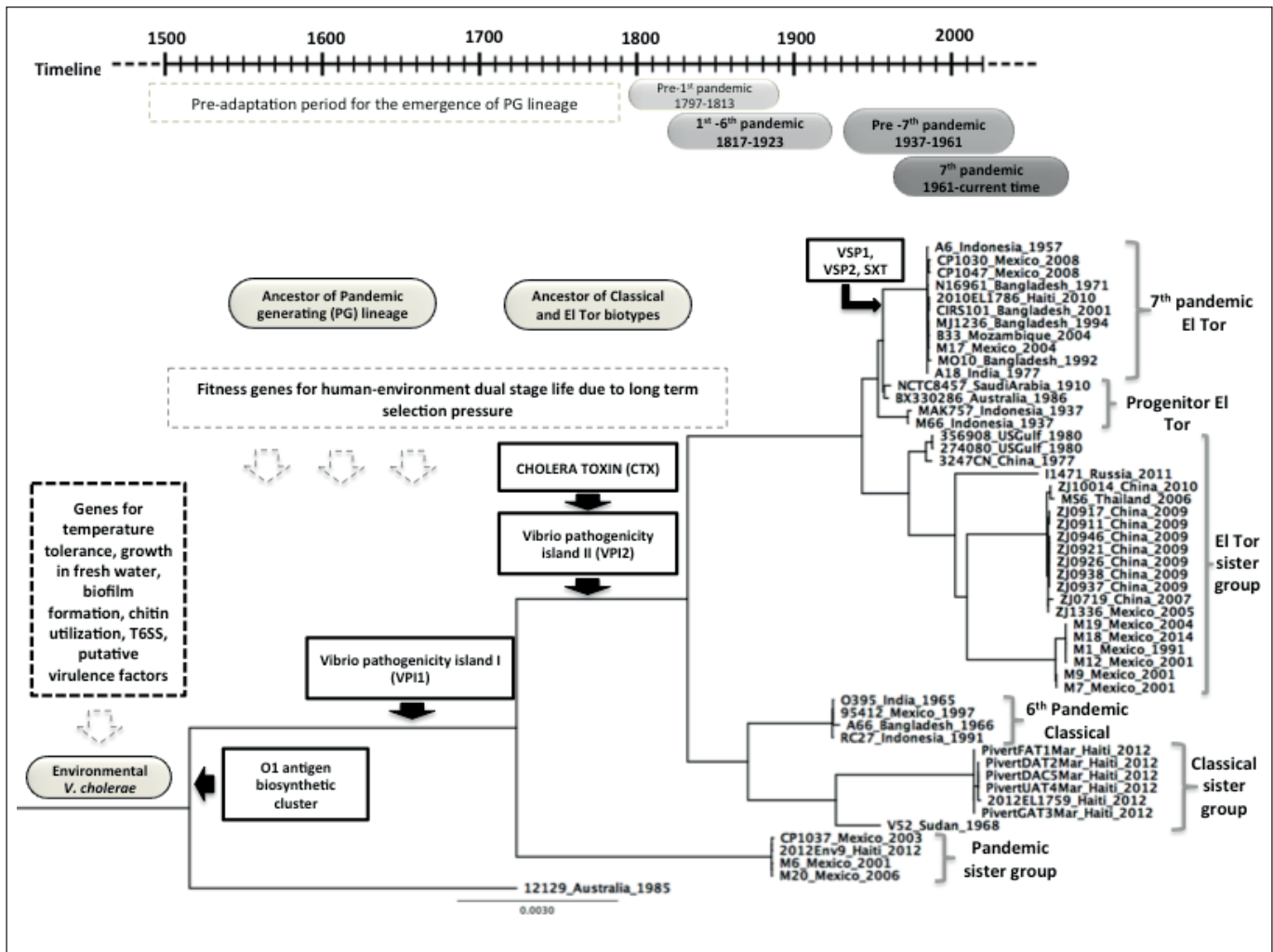
Fig. 1. Emergence and evolution of pandemic *V. cholerae* in a phylogenetic context. The maximum likelihood phylogenomic tree was constructed from the alignment of locally collinear blocks (2,784,396 bp) using GTR gamma substitution model with 100 bootstrap replicates (all nodes had >98% bootstrap support). Environmental non-toxigenic *V. cholerae* O1 strain 12129 was used as the outgroup to root the tree. The source and isolation year of the strains used are indicated next to the strain names. The timeline shows important events on the path of evolution toward pandemic *V. cholerae* from environmental origin. Important genetic elements including virulence factors are denoted in solid rectangles whereas putative genetic events are denoted in dotted rectangles. Stadium boxes denote for the emergence or appearance of lineages or pandemics.

microenvironment favoring survival and persistence, displaying increased resistance to various stresses, facilitating success in both of *V. cholerae* ecological niches, namely aquatic habitats and the human body [14, 70]. Association with plankton as biofilms could lead to the persistence of the pathogen during inter-epidemic periods [23], possibly an important part of the seasonal cycle of cholera in endemic areas. The infectious dose required to cause human disease is quite high (~ 10^4 to 10^{11}) for cholera, the bacterium needing to pass the acidic stomach to reach small intestine where the cholera toxin is effective [56]. The association of the bacterium with biotic or abiotic surfaces could lower the infectious dose, as indicated by the observation that ingestion of *V. cholerae* along with food products decreases the number required to cause infection [42, 9]. Biofilm forma-

tion can also simply increase the likelihood of ingesting a larger dose of *V. cholerae,* thus increasing chance of successful human infection [9]. Moreover, biofilm-derived *V. cholerae* was found to show hyper-infectious phenotypes, leading to a reduction of the infectious dose by orders of magnitude in contrast to the ingestion of planktonic cells [65].

These characteristics have particular significance in maintaining seasonal cycle of the bacteria in the aquatic environment, especially in cholera endemic areas. Most of the evidence for *V. cholerae* association with hosts in the environment remains anecdotal and no systematic study has been done so far. Moreover, studies of its presence in aquatic niches are rarely specific to the PG lineage, but look at the species as a whole, which is likely to display significant ecological vari-

ations below the species level. A culture-based study of a *V. cholerae* population in two connected water bodies at a single coastal location in northeastern USA, encompassing extensive sampling and phylogenetic analysis, revealed subspecies-level divergence between competing genotypes [46]. Clonal complexes, which are groups of closely related *V. cholerae* strains as defined by multi-locus sequence typing, showed distinct spatial distributions across adjacent water bodies and water column size fractions (free-living, small and large particle associated), indicating likely subspecies-level ecological differentiations [46]. If this finding is confirmed in other ecosystems, it is possible that pathogenic *V. cholerae* such as members of the PG lineage, differ from non-pathogenic strains in their ecological preferences. One possibility is that PG lineage strains could show significant association with zooplankton, helping to explain the seasonal cholera epidemics correlating with planktonic blooms. Such blooms could increase the number of bacteria in the water and trigger the epidemic cycle. It would also help explain why filtration of water with Sari cloth, which can trap larger particles and their associated microbes, can reduce the incidence of cholera [37].

The role of human hosts in the *V. cholerae* life cycle

In inter-epidemic periods, strains with pathogenic potential are rarely isolated in environmental surveillance [7, 2]. It seems that during the initiation of seasonal cholera epidemics, there is an enrichment period for pathogenic *V. cholerae* in the combined niche of aquatic habitats and human body [33, 9]. During inter-epidemic periods, most of the *V. cholerae* cells in nature have been found to be in the VBNC state [22, 7]. Passages in animal models showing resuscitation of *V. cholerae* from the VBNC state suggest a potential advantage for cells able to survive in a human/animal host [21, 9]. Furthermore, production of cholera toxin, which causes massive diarrhea, aids rapid spread/dissemination of the bacteria into the nearby water in very high concentrations [56]. This rapid increase is likely to be important to outnumber competing microbes, predators and lytic phages in natural reservoirs. Secreted bacteria from infected humans can also be hyper-infectious, requiring fewer bacteria to cause subsequent infection, fostering the epidemic cycle [53, 56]. Positive effect of human association was also evident in regulation of the type VI secretion system (T6SS), which has roles in competitive fitness of the bacteria in both the human host and the environment. Pandemic type *V. cholerae* strains were found to activate T6SS only inside the small intestine of animal host and not *in vitro* conditions [12]. Human association can also contribute to the survival and persistence of *V. cholerae* in the environment [48]. It was found that the transfer rate of CTXφ genetic element in *V. cholerae* was higher within the mice gastrointestinal tract than under laboratory conditions [69]. Baharoglu *et al.* have shown that the SOS response in *V.*

cholerae, triggered by pH changes, oxidative stress or exposure to DNA damaging antibiotics, can increase rate of gene cassette insertion in integrons, a gene capture element with the potential to acquire new advantageous genes [13]. The human intestine is an environment allowing for extensive interaction of diverse microorganisms at high densities, and could serve to induce acquisition of virulence and adaptive genes in *V. cholerae*. These observations make the human gastrointestinal tract a possible niche/hotspot for the exchange of crucial virulence or other advantageous gene clusters, which might have a significant role in the evolution of pathogenicity within *V. cholerae* populations.

Human hosts also serve as a vehicle to transfer the bacterium into new places through various forms of transportation. Asymptomatic carriers are of special interest in this case [56], as they could play an important role in transporting pathogenic *V. cholerae* to a new habitat [45]. Asymptomatic carriage was proposed to have role in initiating the recent cholera epidemic in Haiti [58], where pandemic *V. cholerae* were introduced by UN troops originating from a cholera endemic area [43, 57]. Asymptomatic infections are mild enough to go undetected and estimates of the ratio of asymptomatic to symptomatic infections have ranged from 3:1 to 100:1 [45]. Asymptomatic carriers usually posses a certain level of immunity to the disease, can be physically healthy individuals and thus travel anywhere unnoticed and shed approximately 10^3 bacteria per gram of stool [56]. Shed pathogenic *V. cholerae* can grow in numbers in the nearby environment if conditions are ideal; a scenario that would be consistent with the Haiti epidemic [40]. In areas where people do not have immunity to cholera at sufficient levels or at all (such as Haiti), disease can spread rapidly. Understanding the role of asymptomatic infections in the dynamics of endemic and epidemic cholera requires detailed investigation of a large number of people showing no symptoms, which as so far proved elusive.

Genetic factors influencing the dual stage life cycle of pandemic *V. cholerae*

Pandemic *V. cholerae* have both environmental and human stages in their life cycle in cholera endemic areas [56]. To maintain a successful seasonal epidemic cycle, these *V. cholerae* strains need to adapt with two different competitive niches. The ability of *V. cholerae* to survive in two drastically different niches is largely due to their inherent and/or acquired resistance to environmental shifts [50]. As a species, *V. cholerae* has certain attributes which makes it predisposed to survive in the human body. This includes the ability to live in freshwater, grow well at human body temperature, utilize human intestinal biopolymers with aquatic analogs, form biofilms, resist acidic passage in the stomach and evade the host immune system [9, 16]. In addition to these inherent abilities of the species, pandemic *Vibrio cholerae* harbor several genetic elements directly contributing

to virulence. These include the two major virulence factors cholera toxin (CT) and the toxin co-regulated pilus (TCP), as well as other genes thought to be associated with the infection process in humans, such as repeat in toxin (RTX), mannose sensitive hemagglutinin pillin (*mshA*), pillin gene (*pilE*), hemolysin gene (*hlyA*), and sialic acid degradation gene (*nanH*) [42, 32]. However, the exact role of all of these genes in the infection process is not clear. The gene set crucial for providing competitive advantage in environmental survival and human to environment transition to the pandemic *V. cholerae* are also not fully understood. However, regulation of virulence and fitness related genes are critical for the long-term viability of the bacterium in humans. A recent study using transposon mutagenesis combined with massively parallel sequencing (Tn-seq) revealed 133 genes including 76 genes previously unknown for having any role in human infection, as contributing to survival of pandemic *V. cholerae* O1 in the infant rabbit model [41]. When dissemination from host into the environment were studied, 165 genes were found to be important for survival in pond water, including genes having known or hypothetical roles in energy production and conservation, cell wall and outer membrane biogenesis, electron transport, flagellar biosynthesis, transcriptional regulation and transportation [41]. Fu *et al*. identified 400 genes potentially critical for the fitness of *V. cholerae* in the infant rabbit intestine [34]. Among these, genes for encoding outer membrane porin *ompU* were found important for the fitness of the bacterium inside the host and genes for glycogen utilization and storage were shown to be crucial for dissemination, survival and persistence of host released *V. cholerae* into the environment [41, 34, 18, 26]. Shapiro *et al*. also found evidence for allelic differentiations in *ompU*, linked to virulence and environmental survival; where there seems to be a trade-off between human adapted and environment specific roles of the gene among the *V. cholerae* populations [63]. One genetic system, which is believed to be significant in survival and fitness of pandemic *V. cholerae* in both human and environmental stages, is the type VI secretion system (T6SS) [59]. *V. cholerae* has been shown to contain three gene clusters, each harboring different combinations of effector–immunity proteins. In each effector-immunity module type, an effector gene encodes a protein that can kill other surrounding bacteria, and their corresponding immunity gene encodes a protein protecting the bacteria from their matching effector [67, 47]. Killing of non-compatible cells by T6SS might serve as source of readily available food and DNA from other bacteria with potentially beneficial or protective functions. In environments where *V. cholerae* deals with low nutrient conditions, type VI mediated killing could generate supplementary source of nutrients to maintain their physiological activities [59]. In the human intestine however, type VI mediated killing ability could give a selective advantage to a particular strain competing with the commensal host flora. It was found that mucin in human intestine can activate T6SS system in pandemic *V. cholerae*, whereas bile acids further modulate its activity [12]. After excretion with

diarrheal stool, *V. cholerae* with activated T6SS are potentially better equipped to fight against bacterial and eukaryotic predators in the aquatic environment [54]. Thus, T6SS might give significant advantage to pandemic strains inside the human host as well in the environment by outcompeting others for the colonization of a desired niche and providing energetic benefits from lysed cells upon entry into and before exiting the human host [59].

All known PG lineage *V. cholerae* strains consistently possess at least three genomic islands which are not shared with all other *V. cholerae*: CTXφ, Vibrio Pathogenicity Island I (VPI1) and Vibrio Pathogenicity Island 2 (VPI2) [32, 20] (Figure. 1). Maintenance of these genetic elements and coexistence in the same genome can be crucial for the disease-causing ability. They are part of a virulence gene repertoire that has been acquired progressively over centuries by horizontal gene transfer (Figure. 1) [20]. Acquisition of these factors can give advantage to PG lineage *V. cholerae* over benign environmental strains in surviving and exploiting the human gut as an ecological niche (Fig. 1). The toxin co-regulated pilus (TCP), one of the two main virulence factors of pandemic *V. cholerae*, is encoded within the horizontally acquired VPI1 [42] and serves as the essential colonization factor and receptor for the CTXφ phage, which carries the second major virulence factor, cholera toxin (CT). Phylogenetic analysis suggests that VPI1 was acquired long before CTXφ by the ancestors of modern pandemic *V. cholerae,* making them capable of integrating CTXφ in their genome to become cholera causing agents (Figure 1). Beside TCP, VPI1 also encodes metallo protease TagA, which can breakdown mucin glycoproteins, and cell-surface glycans, making them available as a source of nutrients for the bacterium [59]. Saccharides, mucins and the glycocalix on the surface of human gut epithelial cells provide energy sources necessary for the growth and multiplication of the bacterium during the early stages of infection [59]. VPI-2 encodes several genes for sialic acid transport and catabolism, some of which were found to provide *V. cholerae* with a competitive advantage against other bacteria in the mouse gut [9]. Two genomic islands present in 7th pandemic clade but mostly absent in other clades within the PG lineage, Vibrio Seventh Pandemic Island 1 and 2 (VSP1 and VSP2), encode yet to be fully described but potentially important functions for the pathogenesis and survival of the lineage. For example, VSP1 encodes a transcription factor required for efficient colonization of human epithelial cells [53, 9]. All these genetic elements likely provided advantages in aquatic populations before giving a fitness benefit inside the human host, which is a secondary niche for *V. cholerae* [16]. The toxin co-regulated pilus encoded in VPI-1 was shown to be crucial for bacterial interactions required for biofilm differentiation on chitinaceous surfaces and thus likely to have important role in ecological fitness [60]. As TCP also serves as a receptor for CTXφ, its expression during the formation of biofilms also fosters CTXφ transduction and thus represents an ecological setting outside the host in which selection for a

host colonization factor may take place [60]. We have already mentioned that T6SS can serve as a weapon in defense against predation by eukaryotic grazers or other competing bacteria in the aquatic environment [59]. Several studies have also found factors involved in pathogenesis to be expressed or required in association of the bacterium with algae, i.e. an increase in toxin production was observed in *V. cholerae* when in association with the green alga *Rhizoclonium fontanum* [38]. These findings support the idea that these pathogenicity factors have environmental functions and are useful for bacterial survival and persistence outside of the human host [9, 60].

Transcriptional profiling of *V. cholerae* secreted in stool from cholera patients revealed that genes involved in nutrient acquisition and motility were highly expressed whereas genes for chemotaxis were expressed at lower levels [41]. It appears that *V. cholerae* differentially regulates gene expression inside the human body and during passage to the environment, i.e. turns off expression of particular virulence genes as part of a program for dissemination to the environment [41, 53]. These changes in gene expression are thought to be linked to efficient exit from the host, re-entry to the aquatic environment and maintenance of an hyper-infectious state which enhances subsequent water borne spread of the cholera by lowering the infectious dose significantly [53]. When *V. cholerae* is shed into water, it is likely to encounter a drastic change in physiological conditions, i.e. drop in osmolarity, temperature and nutrient availability. *V. cholerae* transitioned into pond water was found to repress genes for protein synthesis and energy metabolism and induction of phosphate and nitrogen scavenging genes indicating a adaptive program in response to the low nutrient condition [56, 41]. The glycogen utilization and storage program was found to have a central role in this adaption to transition between to vastly different niches [41].

Although it is assumed that PG *Vibrio cholerae* survive inter-epidemic periods in aquatic reservoirs, they could potentially also reside in the human gut during that time. After ingesting PG *V. cholerae* at low concentrations from the environment, human carriers may not show disease symptoms but could still be colonized as asymptomatic carriers. These carriers could shed pathogenic clones into nearby water bodies, eventually facilitating the initiation of a seasonal epidemic [33]. Full-blown cholera can be considered helpful for bacterial dispersion in the environment in large numbers, cholera stool containing between 10^{10} and 10^{12} bacteria per litre [56]. This enables a particular type of bacteria to outnumber other types in the environment and with a continuous annual cycle, they get a selective advantage over other non-pathogenic type to sustain in an environment-human-environment life cycle. This kind of competitive advantage is not uncommon, which can explain how over time pathogenic *V. cholerae* becomes endemic within a population associated with a natural reservoir. But the ability to become a successful pandemic agent capable of going through a human infection and environmental survival cycle requires a constellation of virulence, regulatory and survival genes to be acquired and maintained for a long time. Environmental *V. chol-*

erae had to gradually change over a long period of time with continued selective pressure for such a genetic combination to evolve. The basic genetic backbone, which made the evolution of pandemic variants possible, apparently evolved a single time in the ancestor of the PG lineage (Figure 1). Actual pandemic variants evolved twice independently within this lineage, giving rise to two major pandemic biotypes [20, 16].

The rise and spread of a deadly pathogen

Lethal variants capable of causing pandemic cholera emerged twice independently from two branches of the pandemic generating lineage (PG), the Classical biotype (possibly in Asia between 1500 and 1800) and the El Tor biotype (Indonesia, between 1930 and 1960) [20, 55, 36, 61]. How, where and when did this PG lineage with the capacity to generate extremely virulent variants emerge from heterogeneous environmental *V. cholerae* populations?

Despite being a widespread aquatic bacterium, *V. cholerae* as a species has several characteristics that make it predisposed for survival in the human gut [9, 16]. These traits seemingly provide a basic genetic background that fortuitously makes survival in a human host more likely, but are not sufficient for *V. cholerae* to become a human pathogen, which requires virulence factors and other fitness genes to be added to this background and enhance their potential to cause human disease on a stable basis [42, 32, 41]. However, no lineage-specific genomic region or genes exclusively present in PGs but absent in environmental groups (EGs) were found in genome wide analysis [63]. Thus no particular gene or gene families could be linked to the emergence and evolution of the PG group. Hence, virulence adaptive polymorphism were proposed to play a vital role in the process, which implied that the environmental ancestor of the PGs had a particular genomic back-bone containing alleles of core genes that served as 'preadaptation' and enhanced its potential to give rise to the pandemic clones [63]. A proposed conceptual model states that a variety of virulence related genes circulate in a diverse, recombining environmental gene pool, which is maintained in the population through various biotic and abiotic selective pressures. Upon encountering a new ecological opportunity, such as human consumption or transient colonization of other animal hosts, proliferation and gradual expansion of the clones encoding an advantageous combination of vital genes for virulence and pandemicity is selected. These pre-adapted lineages can then serve as progenitors to acquire crucial virulence factors either in the environment or inside human body to mediate the emergence of pandemic *V. cholerae* [63].

Pandemic causing *V. cholerae* appears to have the optimized genetic systems to maintain a dual stage life cycle as opposed to most of their benign environmental counterparts. Epidemiological and genetic data suggests that only members of the PG lineage have been successful in evolving and maintaining these

adaptive traits [20]. Sporadic cholera cases are caused by *V. cholerae* strains outside this lineage throughout the world [42, 32, 20], but none of them could be established as a long-term etiological agent to cause consistent seasonal cholera episodes. This complex capability is unlikely to be created only in a single lineage without a consistent selection pressure over an extended period of time. This kind of evolutionary drive is most likely to have happened in Ganges delta, which has been endemic for cholera in at least the last three centuries and represents a unique ecosystem for *V. cholerae* [16]. It has been proposed that extensive contact between *V. cholerae* living in the coastal brackish waters and dense human population drinking from that water over centuries has created the circumstances for the emergence of pandemic lineage. Fecal-oral circulation of the bacterium in the local environmental reservoirs could have led to the selection and enrichment of variants capable of thriving both in the human gut and the environment [16]. This hypothesis implies that long-term association with human host is the driving factor for the emergence of *V. cholerae* with pandemic capabilities. Recent phylogenomic data suggests that the currently ongoing seventh pandemic of cholera might have originated from Bay of Bengal and from there spread to other parts of the world in several waves [55]. Hu hypothesized that the Middle East and Indonesia played essential roles in the evolution of seventh pandemic strains [36]. However, this hypothesis is based on the analysis of very few strains and remains highly speculative.

Even though this pandemic generating lineage, also termed phylocore genome (PG), is a distinct monophyletic group from an extremely diverse environmental pool, it can be divided into two main phylogenetic branches; PG1 and PG2 [20]. The PG1 branch contains strains of the El Tor biotype and the PG2 branch those of the Classical biotype. These biotypes differ from each other by certain phenotypic and molecular traits [62]. Strains of Classical biotype clade (PG2) are known to be responsible for the sixth and presumably the earlier pandemics, whereas strains from the El Tor clade (PG1) are the causative agent of the currently ongoing seventh pandemic of cholera starting in 1961 [62]. Classical biotype strains have not been isolated since the early nineties even from Southeast Asia, where they were last found and have thus been considered as completely outcompeted by the El Tor biotype both from clinical and environmental settings [62]. Expansion of the 7th pandemic has given rise to new variants of the prototype *V. cholerae* O1 El Tor regularly during the course of the pandemic. These variants include strains harboring Classical biotype features within an El Tor genetic backbone and/or other divergent genetic features including mutations in major virulence factors and hence are named atypical El Tor [62]. After the initial wave of the current pandemic spread prototype El Tor strains across the world, two additional waves spread the variants of El Tor strains, each wave mostly displacing bacteria from the preceding one and has been a feature of global cholera epidemiology [55].

The 7th pandemic of cholera struck South America in 1991 via the Peruvian coast and reached in Mexico the same year [5]. In 2010, one of the most devastating cholera epidemics in history occurred in Haiti, killing thousands of people [35, 43]. Cholera has now set residency in the local environment of Mexico and Haiti, even though both the countries did not have any recorded cases in 100 years before the recent epidemics happened [6, 10, 8]. Cholera epidemics leading to endemicity of PG *V. cholerae* in the affected area have prompted extensive environmental sampling of natural waters in Mexico and Haiti. These have revealed remarkable diversity of pandemic-related *V. cholerae* for countries, which did not have any known history of cholera until recently [6, 11]. In Mexico, a recent series of retrospective studies have reported the discovery of Classical, prototype El Tor, atypical El Tor, and non-toxigenic O1 strains with some unusual genetic features in *V. cholerae* strains isolated from 1983 to 2008 [4, 5, 6]. Along with this surprising diversity, there was presence of strains grouping at the base of the PG lineage that led to Classical and El Tor biotype strains, hence candidates for being considered as previously undetected descendants of the ancestor of the two biotypes [19, 17] (Figure 1). In Haiti as well, where cholera cases could clearly be attributed to the atypical El Tor strains introduced from Nepal [43], presence of this divergent lineage (termed pandemic sister group) in the water was evident [10, 17]. Azarian *et al.* estimated the time for the divergence of this lineage from the common ancestor of pandemic *V. cholerae* around 1548 C.E. [10], long before the report of the first pandemic in 1817. These observations are consistent with the historic records suggesting that descendants of the *V. cholerae* PG lineage common ancestor have been globally distributed for centuries and that this dissemination happened long before the first recorded pandemic [15, 16]. Presence of *V. cholerae* strains belonging to the PG lineage but clearly distinct from the Classical and El Tor strains have been isolated sporadically from around the world over the last few decades, including some non-endemic regions i.e., US Gulf coast, Australia, Russia, Thailand and China [20, 36, 17] (Figure 1) and have been reported very recently from Haiti and Mexico [19, 17, 10, 44]. Presence of these non-pandemic members of the PG lineage in wide geographical locations underscores that genomic database of *V. cholerae* today is extremely biased by clinical isolates and large-scale environmental sampling over wide geographical areas is needed to get a better picture of the diversity and global distribution of the PG lineage. Even though these close relatives of pandemic causing strains in most case lack the main virulence factor CT, they harbour TCP, which can act as the receptor for CT. The rest of their genetic backbone is also very similar to pandemic strains [19, 10, 17, 44]. Therefore, the possibility for the emergence of novel *V. cholerae* with pandemic potential from this globally spread lineage cannot be discounted.

Combating cholera epidemics in the future

Pathogenic bacteria with environmental reservoirs like PG *V. cholerae*, which has to survive in both host and environmental

conditions, need to maintain a delicate balance between two very contrasting life styles. The drastic transition from environment to human and vice versa requires adaptations for both human body and the aquatic environment. The currently ongoing 7th pandemic is the longest in duration and largest in geographical span. During the course of this pandemic, cholera has struck countries in virtually every continent except Antarctica and has even become endemic in countries other than Asia and Africa, surviving in those geographic settings successfully and causing regular cholera outbreaks [6, 11, 8]. El Tor biotype strains are known to have better survival in the environment than Classical biotype strains [32], whereas the Classical type toxin is found to cause more severe cholera than the El Tor type [42]. Currently found variants of prototype El Tor strains possess Classical type toxin in the El Tor genetic backbone, which is likely to make them more potent pandemic causing agents. 7th pandemic isolates contain two genomic islands, VSP-1 and VSP-2, which were not found consistently in other lineages. Even though exact function of these elements is not well understood, VSP1 encodes a transcription factor, which was shown to be required for efficient intestinal colonization [53]. Their consistent and exclusive presence in current 7th pandemic isolates implies that they might well have significant roles in environmental fitness and pathogenic capabilities [9]. From 1992 and onwards, most El Tor strains have been found to harbor a integrative conjugative element called SXT, which is known to serve as hotspot for acquisition of genes including resistance to certain antimicrobials and environmental persistence [55]. Acquisition of antimicrobial resistance can be crucial for the success of the modern El Tor strains as a long lasting disease-causing agent. The high fitness of the currently circulating strains might have selected for traits constraining their evolution. Most of the 7th pandemic clinical *V. cholerae* strains isolated since 2000, including strains causing epidemic cholera in Haiti, were found to harbor an integrative conjugative element (ICE) containing a gene encoding an endonuclease which inhibits the uptake of foreign genetic elements [29]. Studies have also observed remarkably lower recombination rate in 7th pandemic strains in comparison to other lineages supporting that idea [36].

These observations suggest that the 7th pandemic of cholera is likely to continue in the near future. New atypical variants of the El Tor biotype are likely to emerge and could trigger new waves of the pandemic. In 1992, *V. cholerae* O139 emerged in the Ganges delta region and caused severe cholera outbreaks in various parts of Asia and was even suggested as a possible "Eight pandemic of cholera" by some investigators [42, 32, 62]. Even though serogroup O139 became rare since 2005, it is still being isolated sporadically from environmental and clinical samples [2, 62]. Of concern is also the possibility that a novel pandemic biotype, separate from El Tor or Classical but still belonging to the PG lineage, would emerge. As the PG lineage has already generated two pandemic biotypes independently, a third one is a real threat, especially given ongoing global warming and rapidly changing climatic conditions. In ideal transmission and dissemination settings, these novel biotypes or variants

of current pandemic biotypes can adapt to the environment and spread to non-cholera endemic regions via human or environmental carriers to cause cholera outbreaks on a global scale.

Ecological niche modeling taking current and future climatic condition in consideration has predicted a latitudinal increase in potential areas of *V. cholerae* distribution in the future [31]. Effective methodologies to predict cholera outbreaks one to several months in advance would make controlling cholera outbreaks much easier. It is presumed that *V. cholerae* was originally a marine bacterium that could persist in estuarine, coastal waters over a broad range of environmental conditions [23, 49]. In cholera endemic areas, water current, flooding and human activity might carry the bacteria inland, where it can adapt and survive [40, 1] to infect human populations drinking contaminated water. Over the last decades, studies have identified potential environmental variables associated with *V. cholerae* occurrence. Ocean chlorophyll has been found to have the most consistent association with number of cholera cases in nearby populations and thus is thought to be a potential indicator for cholera outbreak prediction [28, 30]. However, prediction models for a complex and dynamic environmental disease like cholera would require more in-depth understanding of the ecology and biogeography of this pathogen, especially of its pandemic-generating lineage.

Concluding remarks

In 2010, cholera killed more than 8000 people in Haiti, a country that did not have any recent cholera history [57]. War torn Yemen is currently facing the devastation of cholera, one of the worst outbreaks on record, with nearly 2000 deaths and around 500,000 suspected cholera cases as of August 2017 (http://www.who.int/mediacentre/ news/releases/2017/cholera-yemen-mark/en/). The Haiti and Yemen episodes underscore the massive threat cholera poses even in this modern time, showing the need for more effective approaches to prevention and control of this deadly disease. Thus, a global scale coordination of biogeographical and genome based studies is warranted to improve prevention and management of future cholera epidemics.

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