

REVIEW

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# Quorum regulated latent environmental cells of toxigenic *Vibrio cholerae* and their role in cholera outbreaks

Shah Nayeem Faruque<sup>1,2</sup>, Shinji Yamasaki<sup>3,4</sup> and Shah M. Faruque<sup>5\*</sup>

## Abstract

**Background** Diverse bacterial group behaviors are controlled by quorum sensing, a regulatory network of bacterial gene expression based on cell density, and involving communication through chemical signal molecules called autoinducers. Multidisciplinary research in toxigenic *Vibrio cholerae* the etiologic agent of cholera, appear to suggest group behavior in the ecology, epidemiology, pathogenesis and transmission of the pathogen. This review summarizes latest advances and known aspects of quorum regulated environmental survival form of *V. cholerae*, and their role in cholera outbreaks, as well as the significance of this knowledge in tracking the pathogen for prevention of cholera.

**Main body** Pathogenic *V. cholerae* naturally exists in aquatic reservoirs, and infects humans, often leading to epidemic outbreaks of cholera. Effective detection and monitoring of the pathogen in surface waters have been a research focus in preventing cholera outbreaks. However, in the aquatic reservoirs, *V. cholerae* persists mostly in a quiescent state referred to as viable but non-culturable (VBNC), or conditionally viable environmental cells (CVEC), which fail to grow in routine bacteriological culture. The presence of CVEC can, however, be observed by fluorescent antibody based microscopy, and they appear as clumps of cells embedded in an exopolysaccharide matrix. Current studies suggest that CVEC found in water are derived from in-vivo formed biofilms excreted by cholera patients. The transition to CVEC occurs when dilution of autoinducers in water blocks quorum-mediated regulatory responses that would normally disperse the cellular aggregates. Consequently, CVEC are resuscitated to actively growing cells if autoinducers are replenished, either in the laboratory, or naturally by other environmental bacteria or the intestinal microbiota when CVEC are ingested by humans or aquatic animals.

**Conclusion** Quorum sensing plays a crucial role in the environmental persistence of toxigenic *V. cholerae* in a latent state, and their periodic emergence to cause cholera outbreaks. Furthermore, the autoinducer driven resuscitation of these cells may be a basis for improving the detection of *V. cholerae* in water samples, and monitoring *V. cholerae* in their aquatic reservoirs in cholera endemic areas.

**Keywords** Cholera, *Vibrio cholerae*, Biofilms, Quorum sensing, Autoinducers, Conditionally viable environmental cells (CVEC)

\*Correspondence:  
Shah M. Faruque  
shah.faruque@iub.edu.bd

Full list of author information is available at the end of the article



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## Background

Epidemics of cholera due to infection with toxigenic *Vibrio cholerae* O1 continue to be a significant public health problem in many developing countries of Asia, Africa and Latin America [1, 2]. *V. cholerae* as a species encompasses more than 200 serogroups which exist as a part of the normal bacterial flora of the aquatic ecosystems, although some strains are pathogenic and infect humans. *V. cholerae* belonging primarily to the O1 or O139 serogroups can cause cholera epidemics, and generally carry a set of virulence genes which encode the factors required for pathogenesis in humans [1, 3]. The serogroup O1 has two major serotypes, Ogawa and Inaba as well as a rarely reported serotype Hikojima. These serotypes have been further classified into two biotypes, classical and El Tor based on several biochemical properties [1]. The major virulence genes carried by the epidemic strains include a prophage encoded enterotoxin, cholera toxin (CT), and a pathogenicity island encoding a pilus colonization factor, toxin coregulated pilus (TCP). Besides, these strains are also known to carry genes for a number of putative accessory virulence factors such as mannose sensitive hemagglutinin (MSHA) pilus, hemolysins, and the RTX toxin [1]. *V. cholerae* O1 has caused all historically known pandemics of cholera, whereas *V. cholerae* O139 emerged in late 1992 as a new highly pathogenic strain causing explosive cholera epidemics, and continued to be associated with epidemics during a few subsequent years [3, 4]. However, *V. cholerae* O139 serogroup has not been reported to cause any cholera epidemic since the last reported O139 outbreak in Bangladesh in 2002 [5]. The virulence associated gene clusters in *V. cholerae* appear to have a structure that is capable of being propagated horizontally [6], and the genes for the major enterotoxin cholera toxin (CT) is transferred through the filamentous phage CTX $\phi$  [7]. The ecosystem comprising of diverse bacterial strains, CTX $\phi$  and other phages and genetic elements, and the intestinal environment of the host population may all contribute to the emergence and enrichment of new pathogenic *V. cholerae* strains through horizontal acquisition of virulence genes by precursor strains with lower virulence potential [1, 8, 9].

When a pathogenic *V. cholerae* strain becomes endemic in a region, it can persist in aquatic reservoirs, and cholera outbreaks may appear to occur periodically. For example, in the regions of the Ganges Delta of Bangladesh and India, seasonal epidemics are known to occur almost every year. Complex interactions of a multitude of genetic, ecological, and socioeconomic factors may drive the periodicity of the cholera epidemics. In these areas, viable toxigenic *V. cholerae* O1 can be readily isolated from surface water during cholera epidemics. However, during inter-epidemic periods, it becomes very challenging to culture these bacteria from water

using conventional methods, because of their persistence in a quiescent state often referred to as viable but non-culturable (VBNC) or conditionally viable environmental cells (CVEC) [10, 11]. It has been suggested that CVEC are derived from bacterial biofilms formed in vivo during cholera pathogenesis, and the conversion to CVEC occurs when stools of cholera patients contaminate surface water [11, 12].

In cholera endemic regions, the dormant form of pathogenic *V. cholerae* O1 that exists in aquatic reservoirs is known to periodically revive and amplify prior to occurrence of seasonal epidemics [13]. The mechanism triggering this revival was however, unknown until resuscitation of CVEC in water was demonstrated in the laboratory by exposure to exogenous autoinducers (AIs) [14], which are bacterial signal molecules involved in quorum sensing [15]. Recently, natural resuscitation of CVEC has been proposed to occur in aquatic niches through exposure to AIs produced by *V. cholerae* non-O1 non-O139, or by the gut microbiota when CVEC are ingested by humans or other animals [16, 17]. The primary purpose of this review is to discuss and summarize the advances in our knowledge of the persistence of toxigenic *V. cholerae* O1 in a latent form in their reservoirs when there are no apparent cholera cases around, and the mechanism of their resuscitation prior to initiation of an outbreak of cholera.

## Persistence of pathogenic *V. cholerae* in the environment

*V. cholerae* as a species belongs to a group of organisms which normally reside in the aquatic ecosystems [18], even though nonpathogenic strains are more commonly found in the environment, than the strains which are capable of causing cholera epidemics. This observation is explained by the fact that there are more than 200 different serogroups of *V. cholerae*, of which only two serogroups namely *V. cholerae* O1 and O139 carry the full array of virulence genes needed to cause epidemic disease in humans. However, some of the putative virulence genes or their homologues are also dispersed among non-epidemic environmental strains of *V. cholerae* belonging to diverse serogroups with varying degree of virulence potential, suggesting that the virulence associated factors or their homologues may have alternative function in the aquatic environment [19–21].

Like numerous other bacteria in the aquatic environment, *V. cholerae* are known to form surface associated bacterial communities known as biofilms. Formation of biofilms on chitin surfaces of crustaceans is enhanced by the TCP pili [19]. Chitin associated *V. cholerae* have been reported to use chitinase, an enzyme secreted by the bacteria to decompose chitin and obtain nutrients [22]. Furthermore, it has been found that *V. cholerae* biofilms

formed on chitin surfaces are resistant to grazing by protozoa [23]. These observations suggested that while TCP functions as a major intestinal colonization factor during pathogenesis of *V. cholerae*, it may also promote persistence of *V. cholerae* O1 in the aquatic environment where TCP facilitates adhesion of the bacteria to other aquatic organisms. Similarly, the Type III secretion system (T3SS) carried by some *V. cholerae* strains may also have important functions in the environment [24], since *V. cholerae* come in contact with eukaryotic cells in the marine environment where the bacteria can associate with plankton, copepods, shrimp and insects. Furthermore, *V. cholerae* in their aquatic reservoirs, have to survive predation by unicellular eukaryotes, and it has been suggested that the type VI secretion system (T6SS) in *V. cholerae* is involved in the process [25]. The virulence associated secretion (VAS) genes of the T6SS in *V. cholerae* are essential for their contact-dependent cytotoxicity toward *Dictyostelium discoideum*, an aquatic amoebae species [25]. Presumably, because of the alternative roles of virulence factors in the environment, particular strains of pathogenic *V. cholerae* can persist for a long time in their aquatic reservoirs even in the absence of any known incidence of cholera due to that strain, but can potentially reemerge and cause sporadic disease in humans. Likewise, although the O139 serogroup of *V. cholerae* seems to have disappeared, it is not possible to ascertain whether strains of this epidemic serogroup still persist in any environmental niche. Notably, the classical biotype of *V. cholerae* O1 which was replaced by the El Tor biotype in 1973, reemerged in Bangladesh in 1982 and coexisted with the El Tor biotype until 1992 [26, 27].

In view of ample evidence from environmental and laboratory studies, the survival and persistence of pathogenic *V. cholerae* and possible alternative roles of virulence associated factors in the aquatic environment is now widely accepted. Recent studies are also beginning to provide insights of the metabolic state in which toxigenic *V. cholerae* O1 persist in the aquatic ecosystems, and suggest possible mechanisms which regulate their periodic emergence causing outbreaks of cholera [16, 17]. Originally, various survival states and conditions such as specific association of *V. cholerae* with aquatic plants or animals, and/or the existence in a dormant but viable form was proposed [10, 28]. It was suggested that in response to stress under unfavorable environmental conditions *V. cholerae* are converted to a metabolically less active VBNC state, in which the bacteria fail to grow in culture by conventional techniques, but remain potentially viable and infectious [10]. There were considerable debates on the precise nature and metabolic state of VBNC, since their resuscitation beyond reasonable uncertainty could not be demonstrated. Understandably, the ecological and public health significance of any

dormant state depends on whether these cells are reconvertible to normal infectious bacteria. Subsequently, as described below a somewhat similar survival state referred to as conditionally viable environmental cells (CVEC) of *V. cholerae* that respond to quorum sensing autoinducers, and resuscitate to actively growing cells has been described [11, 12, 14]. However, for clarity both the terms VBNC and CVEC have been used interchangeably in this review.

### Conditionally Viable Environmental Cells (CVEC) of *V. cholerae*

As described above, pathogenic *V. cholerae* O1 can be readily isolated from nearby environmental water sources during a cholera outbreak, whereas isolation of the bacteria from water when there is almost no incidence of cholera is challenging. However, with the development of modified enrichment and culture methods, it has become evident that environmental reservoirs indeed contain considerably more viable pathogenic *V. cholerae* O1 cells even during the absence of reported cholera cases, than are detected by conventional culture [11, 14, 29]. Since these latent environmental *V. cholerae* cells could actually be grown in culture by the modified methods [14, 29], for clarity the terminology “conditionally viable environmental cells (CVEC)” has been introduced to describe these cells [11].

Initially, certain simple modifications incorporated in the methods for recovering *V. cholerae* from water samples began to provide a better understanding of the environmental prevalence of pathogenic *V. cholerae*. In one of these early methods termed Antibiotic Selection Technique (AST), after usual enrichment for vibrios, the culture was subjected to selection on an antibiotic (to which the target *V. cholerae* strain was known to be resistant) in order to suppress the growth of numerous other strains of mostly nonpathogenic *V. cholerae* normally found in water [29]. This modification significantly enhanced isolation of *V. cholerae* O1 from water samples that were found to be negative for the organism by conventional culture. In another novel method, it was demonstrated that when water samples which were apparently negative for *V. cholerae* in usual culture were inoculated in the intestines of adult rabbits, culturable *V. cholerae* O1 could be isolated from the intestinal fluids after several hours [30]. These findings suggested that VBNC or CVEC present in water samples could convert to actively growing culturable cells either on exposure to replenished nutrients or passage in the intestinal environment. Taken together, several studies involving passage in rabbit models, AST, fluctuation analysis, and fluorescent-antibody based microscopy established that CVEC are clumps of partially dormant cells, and these cells can be

revived under appropriate in vitro and in vivo conditions [11, 14, 30].

### Relationship between CVEC and biofilms

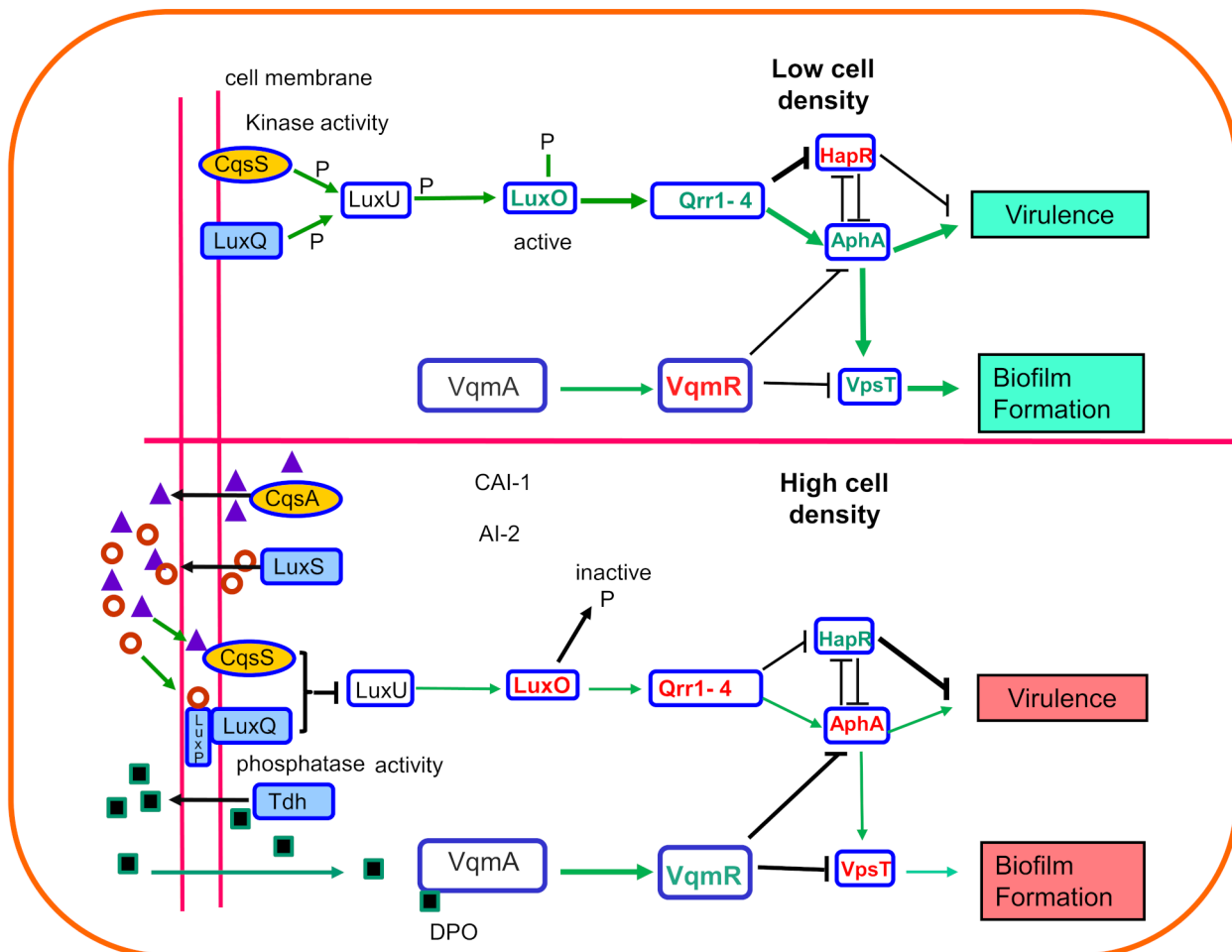
*V. cholerae* is known to form biofilms, and the metabolic pathway involved in this process has been described [31, 32]. Biofilms are surface associated communities of bacteria, in which the cells are embedded in an exopolysaccharide matrix and protected from adverse environmental conditions. The formation and dispersion of biofilms are regulated by quorum sensing through detection of autoinducers and involving an intricate signal transduction cascade, which are components of the quorum sensing system [31–33]. A number of studies have suggested that quorum sensing also controls expression of virulence genes in *V. cholerae*, and that the bacteria form biofilms under in-vivo conditions as well while colonizing the host intestine [11, 15, 34, 35]. Consequently, microscopic analysis of stool samples from cholera patients, revealed that the stools contain both free swimming planktonic cells as well as large clumps of *V. cholerae* cells resembling biofilms [11, 36]. In agreement with these observations, experiments in the rabbit model involving inoculation of pathogenic *V. cholerae* cells into the ileal loops of adult rabbits, showed the presence of both free swimming planktonic cells and biofilm-like clumped cells in the ileal loop fluids recovered after 16 h of inoculation [12]. It was further demonstrated that when cholera stools are diluted in filter sterilized environmental water samples, CVEC are formed from the clumps of *V. cholerae* cells excreted in the stools. Comparative genetic fingerprinting of *V. cholerae* isolates derived from naturally occurring CVEC, and isolates from cholera patients suggested that *V. cholerae* cells that enter into the CVEC state typically exist as clumps of cells that comprise a single clone closely related to isolates causing the most recent local cholera epidemic. These findings are consistent with the suggestion that in cholera endemic areas, contamination of water by stools of cholera patients may lead to the formation of CVEC, which are also the abundant environmental survival form of the pathogen [11, 12].

### Evidence for association of quorum sensing with CVEC

Attempts have been made to understand the mechanisms involved in the transition of *V. cholerae* to the CVEC state. Formation of surface associated biofilms, or cellular aggregates in bacterial suspension, and colonization of the intestine have been found to be influenced by the *V. cholerae* quorum sensing system [31–34, 37]. Fluorescent antibody based microscopic examination of surface water samples revealed the presence of naturally occurring CVEC, and these cells appeared to be organized in aggregates embedded in an exopolysaccharide material [11].

The genes responsible for production of *Vibrio* extracellular polysaccharides are controlled through the quorum sensing pathway that includes autoinducers (AIs) and their cognate receptors, as well as a signal transduction cascade [31–33].

In the *V. cholerae* quorum sensing system (Fig. 1), AI signal molecules are sensed by their respective receptors and the signals from the sensory circuits converges to LuxO, the response regulator which negatively controls HapR, a transcriptional regulator that in turn controls several genes involved in the formation or dissolution of biofilms or in intestinal colonization. Considering the clumped organization of cells observed in CVEC, possible association of quorum sensing with CVEC formation has been further explored by creating and analyzing genetic mutants with altered quorum sensing ability. Subsequently, it has been shown that mutations in crucial genes required for quorum sensing and biofilm formation also impair CVEC formation by *V. cholerae*, when such mutants are added into environmental water samples following intestinal infections [12]. For example, in laboratory experiments, an isogenic strain carrying a mutation in the *hapR* gene produced more robust biofilms than the wild type strain, and formed CVEC when inoculated in water after passage in the rabbit intestine. On the other hand, a *luxO* mutant produced very little biofilm as compared to the wild type strain, and also failed to form CVEC under the above condition. Generally, mutations which enhance or block the formation of quorum regulated biofilms were found to similarly enhance or block the formation of CVEC. Mutants that produced more robust biofilms under in vitro conditions, also produced more compact and larger clumps of cells when inoculated in the intestine of adult rabbits. These mutants were more capable in forming CVEC when introduced into water samples after passage in the rabbit ileal loops [12]. The above studies concluded that CVEC are in fact derived from in vivo-formed bacterial biofilms, and are formed when aquatic reservoirs are contaminated by stools of cholera patients. It has been further proposed that while in the water, rapid dilution of AIs resulting in an inability to sense these signals by the clumped cells enhance the process of CVEC formation [12]. The biofilm associated cells enter into a quiescent metabolic state as dilution of AIs blocks quorum-mediated regulatory responses that would normally disperse the cellular aggregates excreted in the stools of cholera patients [11, 14]. In agreement with these observations, Wu et al. [38] demonstrated that a *hapR* mutant of *V. cholerae* O1 rapidly enters into a dormant VBNC state as compared to the wild type strain when incubated at 4°C in artificial sea water, whereas upregulation of *hapR* results in a prolonged culturable state.



**Fig. 1** Quorum sensing systems in *Vibrio cholerae*. Quorum sensing in *V. cholerae* involves at least three autoinducers (CAI-1, AI-2 and DPO) and their respective receptors CqsS, LuxP, and VqmA together with intricate signal transduction cascades. The autoinducers CAI-1 and AI-2 are produced by autoinducer synthase CqsA and LuxS respectively, whereas DPO is derived from threonine catabolism, and requires threonine dehydrogenase (Tdh). The CAI-1 and AI-2 are sensed by the membrane-bound receptors CqsS and LuxPQ which channel information into a common regulatory pathway. Conversely, DPO is detected by the VqmA receptor-transcription factor in the cytoplasm. VqmA in turn activates expression of *vqmR* gene that encodes the VqmR small RNA (sRNA) which regulates target mRNAs. At low autoinducer concentrations (low cell concentrations), CqsS and LuxPQ act as kinases to phosphorylate LuxU. Phosphorylated LuxU transfers the phosphate to LuxO, and LuxO-P induces the expression of the Qrr1–4 sRNAs. The Qrr sRNAs repress hapR and activate aphA, promoting virulence gene expression and biofilm formation. AphA also activates the transcription of *vpsT*. At high cell concentration (autoinducer concentrations), on binding of CAI-1 and AI-2 to CqsS and LuxPQ, respectively, the receptors act as phosphatases, which reduces LuxO-P levels and inhibits *qrr1–4* expression. Under these conditions, aphA is repressed and hapR is activated. The VqmA-DPO complex induces the transcription of the VqmR sRNA. VqmR inhibits biofilm formation by repressing VpsT and virulence gene expression by inhibiting AphA. In summary, virulence and biofilm formation are upregulated at low cells density (shown in green). At high cell density, all three QS systems repress virulence and biofilm formation (shown in red). Active key factors are shown in green whereas repressed or inactive factors are shown in red font

**Response of CVEC to exogenous autoinducers**

In an attempt to verify the above assumption that CVEC are formed because the clumped cells fail to sense sufficient concentration of AIs which are vastly diluted in the water, it was explored whether supplementation of the enrichment culture medium for isolation of *V. cholerae* from water samples with exogenous AIs would reverse the process, and lead to the dispersion and/or resuscitation of CVEC. It was found that the quiescent form of pathogenic *V. cholerae* that exist in aquatic reservoirs

can be converted to actively growing ‘culturable’ cells by exposure to either of two different AIs, namely cholera autoinducer-1 (CAI-1) and AI-2 produced exogenously [14, 16]. The observations that CVEC formation is enhanced in an AI depleted state (due to dilution in water) [12], and the ability of these clumped cells to respond to AIs and resuscitate into actively growing planktonic cells suggest that the pathways for formation and dispersion of biofilms of *V. cholerae*, and that of CVEC are linked and both involve quorum sensing.

In another study with *V. vulnificus*, it was reported that autoinducer AI-2 could induce resuscitation of the VBNC form of *V. vulnificus* [39], and VBNC cells of a mutant unable to produce AI-2 were resuscitated when cultures were supplemented with exogenous AI-2. Furthermore, a quorum sensing inhibitor cinnamaldehyde delayed the resuscitation, thus confirming the importance of quorum sensing in the resuscitation process [39].

### Resuscitation of CVEC under natural conditions

The CAI-1 is produced by a narrow range of vibrio species [40], whereas AI-2 is produced by numerous bacterial species in the environment [41, 42]. Therefore, it is possible that fully viable cells of pathogenic *V. cholerae* can emerge from the CVEC state in the environment in response to AIs produced by the same or other bacterial species. It was explored whether possible genetic variants of *V. cholerae* which overproduce one or more AIs exist in nature, since hypothetically such variants could produce AIs at a sufficiently high concentration to enhance the resuscitation of CVEC in water. As a proof of concept, initially screening of a mutant library of a typical *V. cholerae* O1 strain C6706 [43] was conducted to identify possible mutants which could overproduce AIs, and indeed a derivative of the parent strain was identified which markedly overproduced AI-2 compared to the parent strain [16]. Furthermore, environmental surveillance confirmed the abundance of AI-2 hyper-producing variants of *V. cholerae* in natural waters [16, 17]. Remarkably, AI-2 produced in the culture supernatants of these variant strains were also found to enhance resuscitation of CVEC in environmental water samples in the laboratory. Notably, natural *V. cholerae* O1 isolates with different quorum sensing functions and constitutive expression of quorum sensing genes with a variety of abilities to remain culturable or enter into the dormant state have been described [38].

### Role of non-cholera vibrios

*V. cholerae* belonging to the non-O1 non-O139 serogroups (non-cholera-vibrios) are abundant in aquatic ecosystems, and naturally occurring variant strains of *V. cholerae* non-O1 non-O139 which overproduce AI-2 have also been identified [17]. Similar to the *V. cholerae* O1 strains, the AI-2 produced in the culture supernatant of the non-O1 non-O139 variant strains has been found to resuscitate CVEC in water samples. Temporal variation in environmental prevalence of such AI-2 hyper-producing non-O1 non-O139 strains and their co-occurrence with *V. cholerae* O1 in water samples have been monitored [17]. Increased prevalence of *V. cholerae* O1 in surface was found to coincide with an increase in AI-2 overproducing *V. cholerae* non-O1 non-O139 strains. These observations suggest that *V. cholerae*

non-O1 non-O139 variant strains overproducing AI-2 presumably contribute in resuscitation of the latent pathogen, prior to seasonal cholera epidemics.

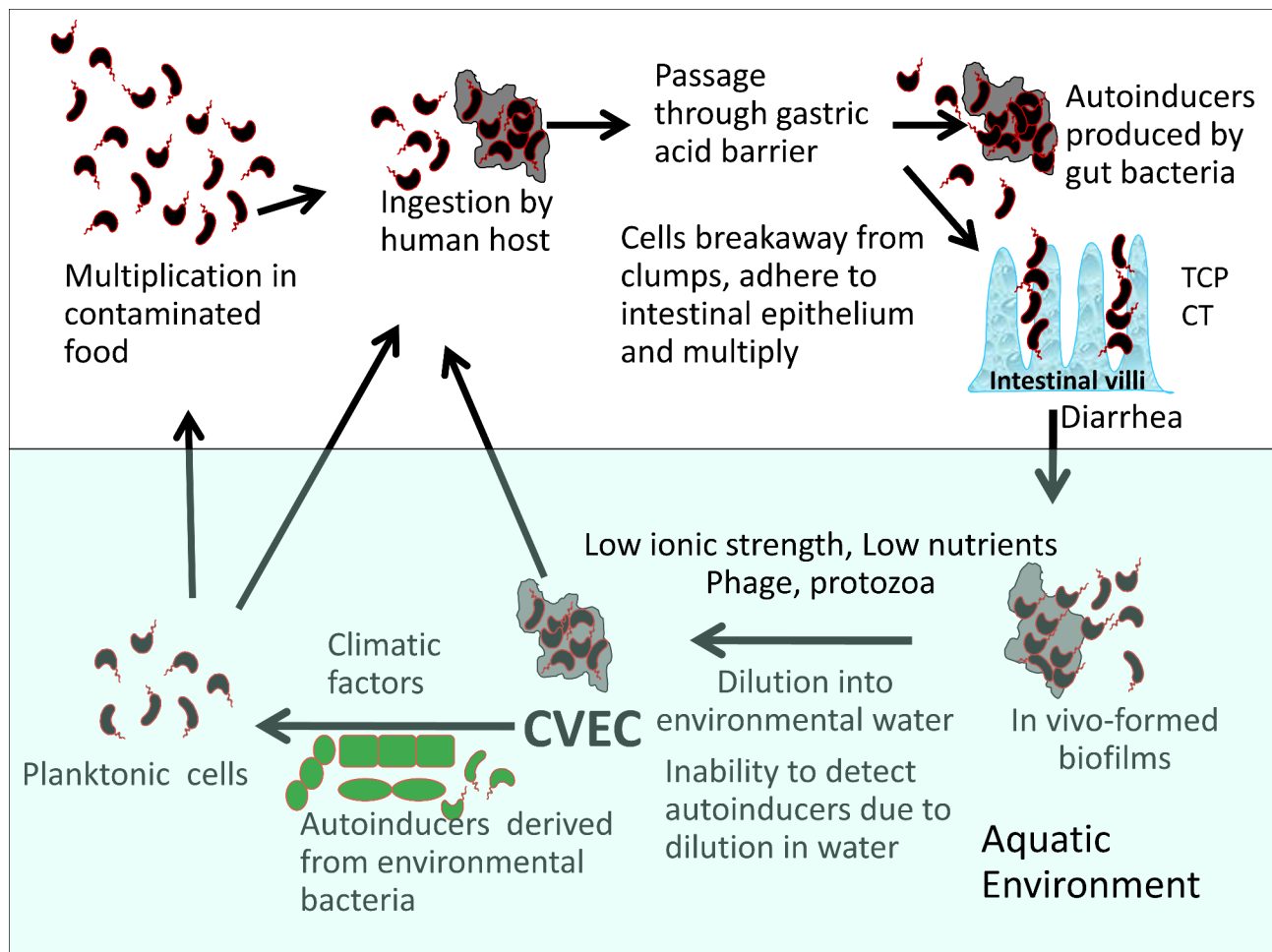
### Role of host intestinal environment

It is now recognized that the dormant environmental form of toxigenic *V. cholerae* O1 are potentially infectious, and ingestion of these forms can cause disease. In agreement with this assumption when water samples which were apparently negative for *V. cholerae* O1 in conventional culture, were introduced into rabbit intestine, actively growing toxigenic *V. cholerae* O1 could be recovered from the intestinal fluids [30]. The recent demonstration of the effect of exogenous AIs on the resuscitation of CVEC sheds light on how CVEC might be revived in the intestinal environment. It follows that since AI-2 is produced by numerous bacterial species, the resuscitation of CVEC in response to AIs could also occur inside the host through exposure to AIs produced by the normal intestinal microbiota. Therefore, CVEC could also be resuscitated into actively growing cells when ingested by humans or aquatic animals.

Example of one such aquatic host could be the vertebrate fish species *Danio rerio*, widely known as zebrafish. Both *V. cholerae* and zebrafish are abundant in natural waters in the Indian subcontinent where cholera is endemic, and the zebrafish can be rapidly colonized by *V. cholerae* [44]. Moreover, intestinal colonization of zebrafish by *V. cholerae* leads to bacterial multiplication, and development of diarrhea excreting *V. cholerae* [44, 45]. The zebrafish is likely to be a natural aquatic host for *V. cholerae*, as colonization of the zebrafish intestine occurs via a natural process when exposed to *V. cholerae*, without requiring any manipulation. Given that the zebrafish is known to have a core gut microbiota [46], it seems possible that the intestinal microbiota might contribute to the resuscitation of CVEC when ingested by zebrafish. There may be a natural association between zebrafish and *V. cholerae* in certain settings overlapping regions of cholera endemicity, and the habitat of the zebrafish [47].

### CVEC resuscitation and seasonal cholera outbreaks

An important aspect of cholera epidemiology in endemic areas is the periodic occurrence of epidemics and persistence of the pathogen in the aquatic reservoirs predominantly in a 'nonculturable' or CVEC state during the inter-epidemic period. The model described in Fig. 2, seeks to explain likely natural mechanism of CVEC resuscitation and its association with the occurrence of cholera outbreaks. Prior to the epidemic, environmental parameters (e.g., temperature, salinity, presence of aquatic hosts, etc.) may cause blooms of diverse bacterial species that produce AIs, particularly AI-2 which is



**Fig. 2** Role of CVEC and diverse environmental and host factors in cholera outbreaks. Diagrammatic representation of the formation and resuscitation of CVEC, and their involvement with environmental and host factors in the occurrence of periodic cholera outbreaks. Stools of cholera patients contain *V. cholerae* as a heterogeneous mixture of active planktonic cells, and biofilm-like clumped cells. Upon introduction of cholera stools into environmental water which vastly dilutes extracellular autoinducers (AIs), a temporary loss of quorum sensing occurs. As a result, quorum-mediated regulatory responses that would normally disperse biofilm-associated cells are blocked and the cells exist embedded in thick exopolysaccharides in a dormant form referred to as CVEC. Presumably, the CVEC form of *V. cholerae* survives phage predation, and persists in water during the inter-epidemic time. The CVEC can provide a large dose of the pathogen when ingested by a potential victim, and may be resuscitated in the intestine in response to AIs produced by the gastrointestinal microbiota. On the other hand, CVEC may be resuscitated in the environment when climatic factors lead to multiplication of diverse environmental bacteria many of which produce AIs. The resulting planktonic cells seed the environment for rapid spread and amplification of the strain, together with selective enrichment of the pathogen in the human gastrointestinal environment. The gradual build up of the pathogenic clone in the environmental water and consumption of contaminated food or drinks may cause an index case of cholera and may eventually initiate an epidemic

known to be produced and sensed by numerous bacterial species including non-cholera vibrios which are abundant in natural waters [17, 48, 49]. In response to the AIs, nearby CVEC form of *V. cholerae* may resuscitate to the actively growing state. Moreover, the presence of AI-2 overproducing variants of environmental bacteria [17] might enhance this process, and lead to an index case of cholera (Fig. 2).

Resuscitation of CVEC might also occur in the human gut through the influence of AIs produced by the human gastrointestinal microbiota, when CVEC in water are ingested by a potential victim. Epidemics may be preceded by a gradual enrichment of the pathogenic *V.*

*cholerae* through passage in humans who consume surface water. The role of the human host in selective amplification of pathogenic *V. cholerae* O1 from the majority of environmental non-pathogenic *V. cholerae* has been proposed to be a crucial step that leads the onset of an epidemic [30]. Studies have also suggested that passage in the human intestine is linked with the generation of a hyperinfectious form of the pathogen [50], and that cholera transmission dynamics involve multiple host and environmental factors [51]. Because of the hyperinfectivity, cholera spreads rapidly through water contaminated with the stools of cholera patients. Moreover, it has been shown that stools of cholera patients contain large

clumps of *V. cholerae* cells, and so ingestion of a clump of cells facilitates the delivery of a high dose of the pathogen to a potential victim. Once the epidemic subsides due to predation of the causative bacteria by phages [51–53] or other interventions, the remnant clumps of epidemic *V. cholerae* cells presumably continue to persist in the water as aggregates of latent cells in the CVEC state. This model of cholera epidemiology appears to unify several observations involving both environmental and host factors in the initiation of periodic epidemics and also explains the limited clonality apparent in the strains causing the cholera epidemics.

### Conclusions and future directions

The findings from numerous studies discussed here can explain the mode of environmental persistence of pathogenic *V. cholerae* in which CVEC originate from aggregates of *V. cholerae* cells excreted in the stools of cholera patients, upon introduction into environmental water [11, 12]. During an epidemic both free-swimming planktonic form and clumped CVEC form of *V. cholerae* are found in water. On the other hand, during the inter-epidemic periods the pathogen predominantly exist in aquatic reservoirs in the CVEC state, and therefore, the prevalence of the pathogen in water can not be accurately estimated using conventional culture methods when there are no reported cholera cases in the area. Several studies could further explain aspects of reappearance of the pathogen in an actively growing state leading to cholera outbreaks in endemic regions such as the Ganges Delta [16, 17]. The seasonal epidemics may be caused by pathogenic strains resuscitated from the CVEC state caused by exogenous AIs produced by diverse environmental bacteria or by the intestinal microbiota, if CVEC are ingested by animals or humans. Asymptomatic infections of humans may account for the pre-epidemic buildup of epidemic strains which then initiate the index case of cholera.

Cholera is known to spread through water contaminated with *V. cholerae* excreted in the stools of cholera patients. Therefore, increased survival of the pathogen in water is likely to favor epidemic or pandemic spread of the pathogen, as well as its persistence in aquatic reservoirs after the epidemic, as a potential threat of future cholera outbreaks. Monitoring of the presence and concentration of the pathogen in surface water is essential to track the pathogen and control the initiation and spread of an epidemic in a community. Enrichment techniques incorporating exogenous AIs CAI-1 or AI-2 have been shown to remarkably improve the assays for detecting *V. cholerae* in environmental water samples [14, 16, 17]. Therefore, use of modified techniques using exogenous AIs (either chemically synthesized or produced by cloning relevant genes) may be recommended for more

accurate detection of pathogenic *V. cholerae* in water. Since quorum sensing systems are present in numerous bacterial species, the applicability of the improved enrichment culture might also be useful for detection of other waterborne bacterial pathogens beyond *V. cholerae* as such. Further studies could be designed and conducted to test this possibility. Lastly, despite significant advances in understanding the survival state in which pathogenic *V. cholerae* persist in aquatic reservoirs during inter-epidemic periods, and their resuscitation prior to epidemics, there is scope for further investigations to more completely appreciate the role of diverse natural factors and the precise genetic regulation that lead to the formation and reactivation of the CVEC state of pathogenic *V. cholerae*.

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### Author contributions

S.N.F. wrote initial draft of the manuscript text and prepared Figure-1; S.Y. provided academic feedback and contributed to scientific editing; S.M.F. provided overall guidance and concept of the review, and proposed the model in Figure-2. All authors reviewed the manuscript.

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### Data availability

No datasets were generated or analysed during the current study.

### Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

All authors read and approved the manuscript for publication. The manuscript does not contain any individual person's data in any form.

### Competing interests

The authors declare no competing interests.

### Author details

<sup>1</sup>Laboratory Sciences and Services Division, International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka 1212, Bangladesh

<sup>2</sup>School of Medical Sciences, Faculty of Medicine and Health, University of Sydney, Sydney, NSW, Australia

<sup>3</sup>Department of Veterinary Science, Graduate School of Veterinary Science, Osaka Metropolitan University, Osaka 598-8531, Japan

<sup>4</sup>Osaka International Research Centre for Infectious Diseases, Osaka Metropolitan University, Osaka 545-8585, Japan

<sup>5</sup>School of Environment and Life Sciences, Independent University, Bangladesh, Bashundhara R/A, Dhaka 1229, Bangladesh

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