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Changing facades of *Vibrio cholerae*: An enigma in the epidemiology of cholera

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Cholera, caused by the Gram-negative bacterium *Vibrio cholerae*, has ravaged humanity from time immemorial. Although the disease can be treated using antibiotics along with administration of oral rehydration salts and controlled by good sanitation, cholera is known to have produced mayhems in ancient times when little was known about the pathogen. By the 21st century, ample information about the pathogen, its epidemiology, genetics, treatment and control strategies was revealed. However, there is still fear of cholera outbreaks in developing countries, especially in the wake of natural calamities. Studies have proved that the bacterium is mutating and evolving, out-competing all our efforts to treat the disease with previously used antibiotics and control with existing vaccines. In this review, the major scientific insights of cholera research are discussed. Considering the important role of biofilm formation in the *V. cholerae* life cycle, the vast availability of next-generation sequencing data of the pathogen and multi-omic approach, the review thrusts on the identification of suitable biofilm-inhibiting targets and the discovery of anti-biofilm drugs from nature to control the disease.

Key words Cholera - integrons - multi-omic approach - rapid diagnostic tests - serotypes - *Vibrio cholerae* - whole genome

Introduction

Cholera (meaning being 'a gutter') is the right name for the disease, caused by the ingestion of contaminated water. The causative organism is a comma-shaped, flagellated bacterium, *Vibrio cholerae*. From 1817, there have been seven pandemics of cholera till date. High mortality rate and its spread in all major continents such as Asia, America, Europe and Africa make it important for research. The Indian subcontinent is considered to be the homeland of cholera, and from here, it had spread rapidly to other countries of the world. Symptoms that distinguish cholera from other diarrhoeal illnesses are the typical rice watery stool and severe vomiting that can lead to dehydration and death within 48 h if left untreated. According to the World Health Organization (WHO) report, 3.5 million people get infected and 100,000-120,000 people die due to cholera each year in the developing countries[1]. In 2014, the case fatality due to cholera was 1.17 per cent in 42 countries, which represented a 47 per cent increase as compared to 2013[1]. Cholera pandemics are ranked sixth amongst the world's worst ten pandemics. The huge death rate is particularly reported by countries that suffer from poor sanitation facilities and lack of

proper water distribution systems. Cross-border cases of cholera have been a serious issue in the Sub-Saharan African countries since the past many years raising the case-fatality rate to 5 per cent[2]. School-going children and children below five years are most affected in cholera-endemic countries. Cholera cases and deaths in African and South Asian countries have accounted for 99 per cent of the total cholera cases worldwide[2].

Serotypes and biotypes

Over 200 serogroups of *V. cholerae* have been recognized so far, and among them, only a few produce the cholera toxin (CT), which is responsible for the characteristic clinical symptoms of the disease. The most common serogroups are O1 and O139 which cause epidemic cholera. The O1 serogroup is further classified into Ogawa, Inaba and Hikojima based on the mutation on *wbeT* gene and type of somatic O antigen formed. Generally, the non-O1/non-O139 strains do not cause the disease, but there have been rare cases of diarrheal infection caused by these[3]. In non-CT-producing *V. cholerae*, virulence factors such as heat-stable enterotoxin (Stn), haemolysin (Hly A), repeat in toxin (RTX) and type 3 secretion systems play major roles in causing infections[4]. Serogroup O1 is further subdivided into Classical and El Tor biotypes based on several phenotypic properties[5]. The classical strains produce larger amounts of CT than the prototype El Tor. The difference between classical and El Tor strains of *V. cholerae* is primarily based on phenotypic traits such as susceptibility to polymyxin B, chicken cell (erythrocytes) agglutination, haemolysis of sheep erythrocytes, Voges-Proskauer test, and phage susceptibilities where El Tor strains are susceptible to bacteriophage V but are resistant to bacteriophage IV; similarly, classical strains show the reverse characteristics in phage typing which is a gold standard test to differentiate classical and El Tor strains. Ogawa and Inaba serotypes are found to be common both in the classical and El Tor biotypes. *V. cholerae* belonging to the serogroup O139 was identified as a derivative of the serogroup O1 El Tor[6]. Till today, O1-specific antiserum is the best way to identify *V. cholerae* in the field. Major differences between El Tor and classical biotypes are also observed at the molecular level. Single nucleotide polymorphisms (SNPs) have been observed between the biotypes in the B subunit of CT (*ctxB*), toxin-coregulated pili (*tcpA*) and *rtx*. Previously, methods for identification of the pathogen were restricted to conventional biochemical and serological techniques. Later on, advancements in genomics and transcriptomics have helped us understand the pathogen better at the molecular level, however, the number of cases and high mortality rate caused by the pathogen still pertain in resource-poor countries. This makes cholera a disease that still deserves attention. In this review, the major scientific insights of cholera research are discussed with a view to develop an update of events to the scientific community in the concerned field.

Environmental reservoirs

The aquatic environments, including fresh, marine and brackish water bodies, have been identified as the natural reservoirs for cholera pathogen. A definite pattern of regular seasonal resurgence is characteristic of cholera[7]. Although studies have proven the existence of vibrios in association with copepods, shellfish, algae and in biofilm stage[8], a clear and established understanding about the pathogen's inter-epidemic reservoir is not yet known. It has been observed that there are cholera outbreaks in September-October after a phytoplankton and zooplankton bloom in the water bodies of Bangladesh, a major epidemic-prone area[8]. Colwell *et al*[9] demonstrated that a simple method of preventing the disease using inexpensive cloth (*sari*) folded four to eight times could greatly reduce the *V. cholerae* attached to plankton and also in biofilm state, thereby cutting down cholera cases drastically. It has also been shown that the bacterium enters into a dormant phase which is a viable but non-culturable (VBNC) state, accounting for why it could not be found in between epidemics using culture-dependent techniques[10]. Apart from planktons, *V. cholerae* also colonizes soft turtles and marine fishes which can be attributed as one of the major reasons for global dissemination of the pathogen[11]. Molecular subtyping of *V. cholerae* strains isolated from patient's stool samples and these soft turtles and fishes has shown high similarity, suggesting that these could serve as vehicles for the pathogen. Furthermore, this increases the incidence of cholera cases in those countries where sea foods are eaten raw or half cooked[11]. Cholera outbreaks have been reported in Vietnam after consumption of iced tea, showing the ability of *V. cholerae* to survive at low temperature and revive back in suitable conditions[12].

History of cholera and *Vibrio cholerae*

Although there had been recorded cases of cholera in the 16th century, the knowledge about the causative organism improved only after two centuries. The first

cholera case was well documented from India in 1563 by Garcia de Orta, a Portuguese physician[13]. Initially, it was believed that cholera was caused by bad air, arising from decayed organic matter or miasmata. Even after the invention of the microscope in 1660, the cause of cholera remained unknown to physicians of that time. It was in 1854, an Italian Anatomist Dr Filippo Pacini linked *V. cholerae* to the disease cholera by observing the pathogen in the intestine of corpses of the cholera epidemic at Florence[13]. With these observations, he described the disease as a massive loss of fluid and electrolytes from intestinal mucosa due to the bacteria and recommended the intravenous injection of saline in extreme cases of dehydration which went unnoticed until many years after his death[14,15]. Another major event in 1854 was that John Snow announced polluted water supply as the major cause for the spread of cholera in London[16]. Until then, the importance of clean water for human use and proper sanitation to prevent diarrhoeal diseases were not known. Snow's classical epidemiological research on the London cholera outbreak has helped in understanding the potential role of the contaminated water supply through public water pump on Broad Street as the source of cholera[16]. Through the epidemiological studies the first proofs to challenge the 'miasma theory' emanated. Later, only after two more lethal pandemics of cholera quivering the world from 1841 to 1875, Koch[17] isolated the bacterium as a pure culture in 1883. The major events of cholera during 16th to 18th century and 19th century are summarized in Figure (A) and (B), respectively. At the end of the 19th century, there was a surge of vaccine trials in many cholera-stricken nations[21,22]. The vaccine trials existed over many decades, but the disease could not be contained, and the world saw the fifth and the sixth pandemics of cholera seizing many lives.

The 20th century witnessed major discoveries in cholera research. In 1959, De, in Kolkata, India, identified CT to be responsible for the abysmal symptoms of cholera[23]. He performed experiments in rabbit ileum to replicate the disease and established CT as an enterotoxin[23]. This was a turning point in cholera research as it helped in understanding the pathogenicity of *V. cholerae*. Since then, there have been extensive studies on this pathogen, which led to better knowledge about the natural habitat, mode of infection, virulence and transmission of the bacteria. These discoveries modified the preventive measures used to control the disease. However, overcoming all discoveries, the pathogen evolved mechanisms to persist in the environment as well as withstand antimicrobial treatment by acquiring genes that conferred resistance. It is estimated that 40 per cent of the *V. cholerae* genes is part of mobile genetic elements which can be transferred from one strain to another distributing drug resistance[24-26]. Unlike the first six pandemics, the seventh pandemic was caused by the El Tor strains[27]. These new strains which were less virulent compared to the classical strains had the advantage of environmental persistence. In 1992, a novel strain of *V. cholerae* with epidemic potential was isolated from south India. It was designated as *V. cholerae* serogroup O139 Bengal since the first outbreak of the disease was reported from the coastal areas of the Bay of Bengal[28]. Since 1995, there have been variations in the clinical isolates of *V. cholerae* such that El Tor strains have been found to produce CT of the classical biotype. These strains are capable of being dominant in transmission as well as in virulence. One major development of 20th century was the identification of oral rehydration therapy to replenish lost fluid in cholera patients[29]. The milestones of cholera research in the 20th century are given in Figure (C).

Modern façade of *Vibrio cholerae* through era of multi-omics

The development of molecular techniques such as 16S-23S intergenic spacer region amplification made it easier to differentiate *V. cholerae* from other vibrios[34]. The genomic analysis of *V. cholerae* revolved around understanding the differences between the two biotypes, classical and El Tor. Septaplex and multiplex polymerase chain reactions have been developed for detecting major virulence and resistance genes, which could be used to differentiate these biotypes[35,36]. Important variations in the genetic organization of the evolving strains of *V. cholerae* are reported in TCP and CT. Such genetic changes would have played significant roles in the difference in their pathogenicity[37,38]. The genes that encode CT (*ctxAB*) exist within the genome of a lysogenic filamentous bacteriophage CTXφ, first discovered by Waldor and Mekalanos[20]. The CTXφ is integrated at one chromosome or both the chromosomes within El Tor and classical, respectively. The major differences between the biotypes of *V. cholerae* at the CTX gene cluster and fine tuning of *V. cholerae* strains based on the type of CtxB they harbour are described in the Table.

Applications of next-generation sequencing

Whole genome sequencing of *V. cholerae* by Heidelberg *et al*[39] was a milestone in understanding

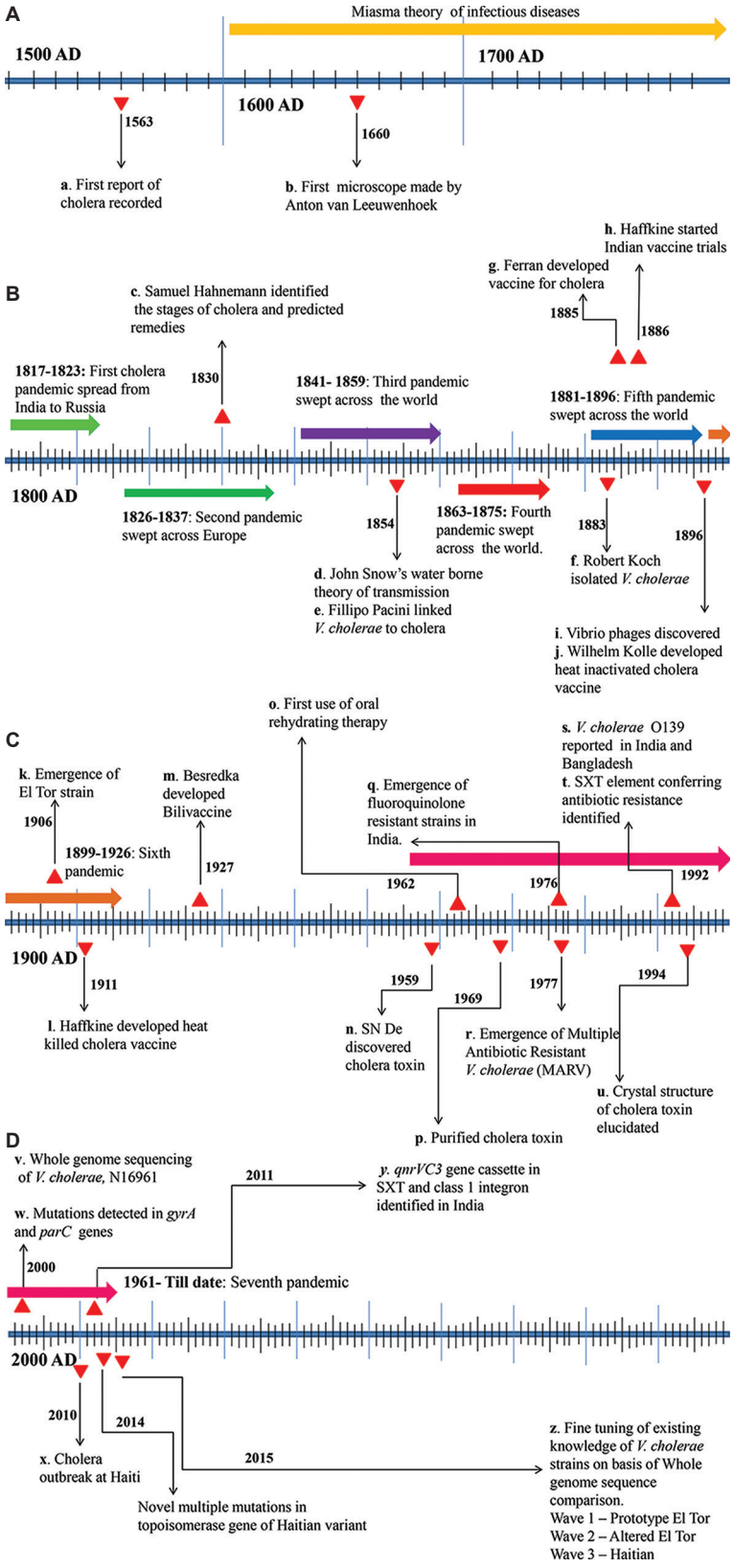

Figure. Timeline showing the major events and breakthroughs in cholera research. (**A**) 16th to 18th century, (**B**) 19th century, (**C**) 20th century, (**D**) 21st century till 2015 are represented.
Source: Refs. a[13]; b[18]; c[19]; d[16]; e[14,15]; f[17]; g[21]; h[22]; i[20]; j[21]; k[27]; l[22]; m[21]; n[23]; o[29]; p[30]; q[25]; r[31]; s[28]; t[32]; u[33]; v[39]; w[44]; x[65]; y[66]; z[67].

Table. Major differences between virulence, regulatory and quinolone resistance-determining region of *Vibrio cholerae* epidemic strains at nucleotide level (A, G, T and C)

Pandemics	Strain	Wave	Nucleotide positions													
			rstA			*rstB*					*ctxB*			*tcpA*		*gyrA*	*parC*
			927	933	942	74-76	87	93	105	189	58	115	203	266	277	248	2033
One to six (1817-1926)	Classical	-	C	T	T	Δ^{GTT}	T	C	A	G	C	C	C	C	A	G	G
Seven (1961-ongoing)	El Tor	Wave 1	T	C	G	GTT	A	T	G	A	C	T	T	A	T	G	G
	Atypical El Tor	Wave 2	C	T	T	GTT	A	T	G	A	C	C	C	A	T	G	G
		Wave 3 (early Wave 3)	C	T	T	GTT	A	T	G	A	C	C	C	A	T	G	G
	Haitian	Current Wave 3	C	T	T	Δ^{GTT}	T	C	A	G	A	C	C	G	T	T	A

A, adenosine; G, guanine; T, thymine; C, cytosine; Δ, deletion mutation. *Source*: Modified from Ref. 67.

the pathogen in the genomic level. It was revealed that *V. cholerae* possesses two circular chromosomes of 2,961,146 bp and 1,072,314 bp collectively coding for 3885 open reading frames. The majority of genes for essential cell functions and pathogenicity are located on the large chromosome and small chromosome contains majorly hypothetical genes and genes usually present in the plasmids[39]. *V. cholerae* genome sequence initiated the understanding of how an environmental non-toxigenic bacterium emerged and evolved to become a significant human bacterial pathogen. It is through changes in global patterns of transcription that *V. cholerae* adapts to different environmental conditions. These transcriptional changes alter the organism's protein repertoire which in turn changes its metabolic status[40]. Thus, information on global changes in the *V. cholerae* transcriptome in both *in vitro* as well as *in vivo* conditions had been helpful in the analysis of the adaptive processes. It also provided potential insight for therapeutics. A notable difference is seen in the genes being transcribed in *in vivo* and *in vitro* conditions. Finally, *V. cholerae* comparative genomics was also studied using microarray[41]. Global proteome profiles of pandemic strains of *V. parahaemolyticus* and *V. cholerae* in planktonic and biofilm stage have provided an understanding of the importance of genes such as mannose-sensitive haemagglutinin and chitin-regulated pilus in biofilm formation[42]. The outer membrane vesicle of *V. cholerae* has been studied through high-throughput lipidomics[43]. All these studies have informed that the genomic plasticity of the pathogen is very high and acquisition of new genes in *V. cholerae* enhances its chances of survival by allowing growth in a previously hostile environment. Notable genetic changes such as transposable elements and site-specific recombination systems such as integrons and super-integrons have been identified in *V. cholerae.* These genetic elements can mobilize genes from one replicon to another, thus establishing conditions for interspecies and intraspecies gene transfer. The current variants have revealed a *gyrA* mutation for quinolone resistance and also possessed integrating conjugative elements that confer multiple drug resistance, to trimethoprim, streptomycin and chloramphenicol[44,45]. The currently circulating strains also possessed multiple novel mutations in the topoisomerase gene, which is further attributed to multidrug resistance[46]. Although genomics has revealed a lot about the genetic variations in *V. cholerae*, each new outbreak instigates a fresh demand for more studies as the pathogen is still evolving.

Fighting the disease

At present, cholera is managed with oral rehydration solution (ORS) to correct dehydration and supplemented with antibiotics in extreme cases. ORS is one of the greatest discoveries in cholera prophylaxis which is cheap and easy to prepare even in the most remote areas. The currently used cholera vaccines do not give complete protection against the disease for a lifetime. The WHO recommends the use of cholera vaccines only in combination with other preventive measures among those at high risks. Shanchol® (manufactured by Shantha Biotech in India) and Dukoral® (manufactured by SBL Vaccines) are the two WHO prequalified oral cholera vaccines used against cholera[47]. In 2016, the WHO approved a single-dose live oral cholera vaccine, Vaxchora® in the United States for adults aged 18-64 yr travelling to a cholera-endemic area[48]. Several live attenuated cholera vaccines

that have the ability of providing long-term protection with a single dose are under development, of which none are expected to be marketed within the following few years. Mass vaccination against cholera is not recommended in cholera-endemic countries. However, cholera vaccination in high-risk population such as children below five, school-going children, pregnant women, old age groups and immunocompromised individuals is a requisite in such countries.

Rapid diagnostic tests

Simple and reliable methods for detecting *V. cholerae* from patients' stool samples and the environment are of great value for epidemiologists, clinicians and health officials in instigating control measures before an outbreak. Rapid diagnostic tests (RDTs) require minimum laboratory infrastructure, less time and basic technical skills as compared to the routine culture techniques. Currently, more than 20 RDTs are marketed which have been field tested by the WHO and Global Task Force on Cholera Control (GTFCC) Surveillance Laboratory Working Group[49]. Efficacy, mode of detection, structure of RDTs and their use differ from region to region. Cholera RDTs in the market are either lateral flow units or dipsticks and work by detecting either CT[50,51] or antigenic lipopolysaccharide (LPS) of *V. cholerae*[52]. Some of the RDTs such as Sensitive Membrane Antigen Rapid Test (SMART) II (developed by New Horizons Diagnostics Corporation, Columbia, MD) utilizes a calorimetric gold-based immunoassay with monoclonal antibody specific for A antigen of O1 LPS of either *V. cholerae* O1 or O139[53]. There are a few RDTs that utilize the polyclonal antibody-based systems that can detect both *V. cholerae* O1 and O139 with the same kit. A few commonly encountered problems in using RDTs are that (*i*) these tests may detect both viable and non-viable bacteria. In addition, a shortly vaccinated person's stool sample gives positive results as many of the vaccines have LPS as its major component, and (*ii*) some RDTs show cross-reactivity to closely related species of microorganisms. However, Crystal VC (Span Diagnostic, Surat, India) has a high sensitivity of 92-100 per cent and has been used for several field studies in India[54].

Identification of anti-biofilm targets and compounds

As *V. cholerae* has dual life cycle both within the host body and in aquatic environments, the bacterium has been used as a model organism to understand the molecular mechanisms and signals underlying biofilm formation, assembly and dispersal for last 20 years[55]. Biofilm assembly is initiated by change in global transcriptome profile downregulating genes for motility and chemotaxis and upregulating genes important for the synthesis of its extracellular exopolysaccharide matrix. The major exopolysaccharide *Vibrio* polysaccharide is encoded by a cassette of *vps* genes, which is in turn controlled by *rpoS*. Further it has been demonstrated that c-di-GMP (cyclic diguanylate monophosphate) signalling regulates transition of cells between the planktonic and biofilm state[56]. The increase and decrease of the levels of c-di-GMP within the *V. cholerae* cell are regulated by diguanylate cyclase (DGC) and phosphodiesterases, respectively. These regulations for biofilm development and dispersal at the genetic level are in turn associated with the environmental cues the bacteria receive. Among the conditions that affect the biofilm development are temperature, *p*H, O_2 levels, hydrodynamics, osmolarity, presence of specific ions, nutrients and factors derived from the biotic environment[57]. The role of biofilms in the environmental persistence, dissemination, tolerance to antibiotics and transmission of *V. cholerae* has been well established. Increasing cholera outbreaks spanning different continents and emergence of multidrug resistance in *V. cholerae* necessitate an urgent need to look for alternative strategies to combat the infections by reducing virulence rather than by killing the bacteria. Thus, discovering potential anti-biofilm compounds against *V. cholerae* and finding their anti-biofilm targets are important.

A single biofilm inhibitor can theoretically control both virulence, biofilm formation and dispersal of the pathogen. Anti-biofilm drugs from nature, such as phytochemicals, frog skin peptides, human breast milk-derived small molecules and probiotic microbes that have anti-biofilm activity against vibrios, are fast-gaining popularity as these have fewer side effects. In addition, the pathogen's ability to develop resistance against these natural products is considered to be limited[58-60]. Probiotic strains, such as *Ruminococcus obeum* that are non-infectious and indigenous to human gut, are known to limit colonization of *V. cholerae* and other enteric pathogens[61]. Transcriptomic and proteomic approaches to identify conserved biofilm inhibiting targets are also promising strategies to strengthen the fight against cholera.

The metagenomic approach in cholera research has produced unexpected results, showing the presence of *V. cholerae* in the gut of healthy children,

which requires detailed investigation[62]. The presence of *V. cholerae* in the VBNC state has been understood by metagenomics[63]. In spite of having substantial knowledge about the pathogen in the 21st century and novel strategies developed to fight the disease, there is a dearth of access to an effective therapy for cholera in developing countries[64]. Cholera research is expanding fast in the 21st century, as represented in Figure (D).

Conclusion

Today, the perception of *V. cholerae* as an uncontrollable ferocious pathogen lurking in polluted waters, causing large-scale morbidity and mortality, is changed. We have now several effective measures in controlling the infection and spread of the disease. However, *V. cholerae* prevails in the environment during inter-epidemic periods unnoticed out-competing our efforts to control the disease during natural disasters such as floods and earthquakes that lead to unhygienic environment and overcrowding. The best we could do is to understand the evolving strains and avoid another major outbreak that has epidemic and pandemic potential. Although there have been a few initiatives to identify a potential biofilm inhibiting drug target in the pathogen[60,68], there is still a vacuum in this area. The variations and difference in pathogenicity of the evolved and emerging strains are to be studied. To date, 26 complete genomes and more than 220 draft genomes of *V. cholerae* are available in the National Center for Biotechnology Information (NCBI) database (*http://www.ncbi.nlm.nih.gov/genome/genomes/505*). A detailed spatiotemporal investigation of the genomic diversity of outbreak strains is warranted to study the evolutionary adaptation and transmission disparity of the pathogen. An interpretation compiling the transcriptomic, proteomic and metabolomic data available in the respective databanks to identify a suitable drug target in the *Vibrionaceae* family at large is not an unattainable task. Further, updated knowledge on the history of cholera and pathogenicity of *V. cholerae* in relation to its epidemiology and evolution is essential. Hence, the coordination of all cholera research groups and strong establishment of international networking is warranted for the battle against cholera.

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References

1. Cholera, 2014. *Wkly Epidemiol Rec* 2015; *90* : 517-28.
2. Bwire G, Mwesawina M, Baluku Y, Kanyanda SSE, Orach CG. Cross-border cholera outbreaks in Sub-Saharan Africa, the mystery behind the silent illness: What needs to be done? *PLoS One* 2016; *11* : e0156674.
3. Chen YT, Tang HJ, Chao CM, Lai CC. Clinical manifestations of non-O1 *Vibrio cholerae* infections. *PLoS One* 2015; *10* : e0116904.
4. Chakraborty S, Mukhopadhyay AK, Bhadra RK, Ghosh AN, Mitra R, Shimada T, *et al.* Virulence genes in environmental strains of *Vibrio cholerae*. *Appl Environ Microbiol* 2000; *66* : 4022-8.
5. Svennerholm AM, Holmgren J. Identification of *Escherichia coli* heat-labile enterotoxin by means of a ganglioside immunosorbent assay (GM1-ELISA). *Curr Microbiol* 1978; *1* : 19-23.
6. Raychoudhuri A, Mukhopadhyay AK, Ramamurthy T, Nandy RK, Takeda Y, Nair GB, *et al.* Biotyping of *Vibrio cholerae* O1: Time to redefine the scheme. *Indian J Med Res* 2008; *128* : 695-8.
7. Wu X, Lu Y, Zhou S, Chen L, Xu B. Impact of climate change on human infectious diseases: Empirical evidence and human adaptation. *Environ Int* 2016; *86* : 14-23.
8. Huq A, Sack RB, Nizam A, Longini IM, Nair GB, Ali A, *et al.* Critical factors influencing the occurrence of *Vibrio cholerae* in the environment of Bangladesh. *Appl Environ Microbiol* 2005; *71* : 4645-54.
9. Colwell RR, Huq A, Islam MS, Aziz KM, Yunus M, Khan NH, *et al.* Reduction of cholera in Bangladeshi villages by simple filtration. *Proc Natl Acad Sci U S A* 2003; *100* : 1051-5.
10. Alam M, Sultana M, Nair GB, Siddique AK, Hasan NA, Sack RB, *et al.* Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. *Proc Natl Acad Sci U S A* 2007; *104* : 17801-6.
11. Wang J, Yan M, Gao H, Lu X, Kan B. *Vibrio cholerae* colonization of soft-shelled turtles. *Appl Environ Microbiol* 2017; *83*. pii : e00713-7.
12. Nguyen TV, Pham QD, Do QK, Diep TT, Phan HC, Ho TV, *et al.* Cholera returns to Southern Vietnam in an outbreak associated with consuming unsafe water through iced tea: A matched case-control study. *PLoS Negl Trop Dis* 2017; *11* : e0005490.
13. Sen S. Indian cholera: A Myth. *IJHS* 2012; *47* : 345-74.
14. Nardi MG. Discovery of *Vibrio cholerae* by Filippo Pacini, of Pistoia, established in the initial phases of microbiological thought and judged after a century. *Minerva Med* 1954; *45* : 1024-9.

15. Subba Rao M, Howard-Jones N. Original observations of Filippo Pacini on *Vibrio cholera*. *Bull Indian Inst Hist Med Hyderabad* 1978; *8* : 32-8.

16. Snow J. *On the Mode of Communication of Cholera*. London: John Churchill; 1855.

17. Koch R. An address on cholera and its *Bacillus*. *Br Med J* 1884; *2* : 403-7.

18. Egerton FN. Leeuwenhoek as a founder of animal demography. *J Hist Biol* 1967; *1* : 1-22.

19. Hahnemann S. Appeal to Thinking Philanthropist Respecting the Mode of Propagation of Asiatic Cholera. In: *The Lesser Writings of Samuel Hahnemann*; 1831. p. 753-63.

20. Waldor MK, Mekalanos JJ. Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* 1996; *272* : 1910-4.

21. Kabir S. Cholera vaccines: The current status and problem. *Rev Med Microbiol* 2005; *16* : 101-16.

22. Hawgood BJ. Waldemar Mordecai Haffkine, CIE (1860-1930): Prophylactic vaccination against cholera and bubonic plague in British India. *J Med Biogr* 2007; *15* : 9-19.

23. De SN. Enterotoxicity of bacteria-free culture-filtrate of *Vibrio cholerae*. *Nature* 1959; *183* : 1533-4.

24. Jesudason MV, Saaya R. Resistance of *Vibrio cholerae* 01 to nalidixic acid. *Indian J Med Res* 1997; *105* : 153-4.

25. Mukhopadhyay AK, Basu I, Bhattacharya SK, Bhattacharya MK, Nair GB. Emergence of fluoroquinolone resistance in strains of *Vibrio cholerae* isolated from hospitalized patients with acute diarrhea in Calcutta, India. *Antimicrob Agents Chemother* 1998; *42* : 206-7.

26. Sack DA, Lyke C, McLaughlin C, Suwanvanichkij V. *Antimicrobial resistance in shigellosis, cholera and campylobacteriosis*. Geneva: World Health Organization; 2001.

27. Hu D, Liu B, Feng L, Ding P, Guo X, Wang M, *et al*. Origins of the current seventh cholera pandemic. *Proc Natl Acad Sci U S A* 2016; *113* : E7730-9.

28. Ramamurthy T, Garg S, Sharma R, Bhattacharya SK, Nair GB, Shimada T, *et al.* Emergence of novel strain of *Vibrio cholerae* with epidemic potential in Southern and Eastern India. *Lancet* 1993; *341* : 703-4.

29. Ruxin JN. Magic bullet: The history of oral rehydration therapy. *Med Hist* 1994; *38* : 363-97.

30. Finkelstein RA, LoSpalluto JJ. Pathogenesis of experimental cholera. Preparation and isolation of choleragen and choleragenoid. *J Exp Med* 1969; *130* : 185-202.

31. Faruque AS, Alam K, Malek MA, Khan MG, Ahmed S, Saha D, *et al*. Emergence of multidrug-resistant strain of *Vibrio cholerae* O1 in Bangladesh and reversal of their susceptibility to tetracycline after two years. *J Health Popul Nutr* 2007; *25* : 241-3.

32. Thungapathra M, Amita, Sinha KK, Chaudhuri SR, Garg P, Ramamurthy T, *et al*. Occurrence of antibiotic resistance gene cassettes *aac(6')-Ib, dfrA5, dfrA12*, and *ereA2* in class I integrons in non-O1, non-O139 *Vibrio cholerae* strains in India. *Antimicrob Agents Chemother* 2002; *46* : 2948-55.

33. Merritt EA, Sarfaty S, van den Akker F, L'Hoir C, Martial JA, Hol WG. Crystal structure of cholera toxin B-pentamer bound to receptor GM1 pentasaccharide. *Protein Sci* 1994; *3* : 166-75.

34. Chun J, Huq A, Colwell RR. Analysis of 16S-23S rRNA intergenic spacer regions of *Vibrio cholerae* and *Vibrio mimicus*. *Appl Environ Microbiol* 1999; *65* : 2202-8.

35. Hoshino K, Yamasaki S, Mukhopadhyay AK, Chakraborty S, Basu A, Bhattacharya SK, *et al.* Development and evaluation of a Multiplex PCR assay for rapid detection of toxigenic *Vibrio cholerae* O1 and O139. *FEMS Immunol Med Microbiol* 1998; *20* : 201-7.

36. Mantri CK, Mohapatra SS, Ramamurthy T, Ghosh R, Colwell RR, Singh DV, *et al.* Septaplex PCR assay for rapid identification of *Vibrio cholerae* including detection of virulence and int SXT genes. *FEMS Microbiol Lett* 2006; *265* : 208-14.

37. Häse CC, Mekalanos JJ. TcpP protein is a positive regulator of virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci U S A* 1998; *95* : 730-4.

38. Mukhopadhyay AK, Chakraborty S, Takeda Y, Nair GB, Berg DE. Characterization of VPI pathogenicity island and CTXphi prophage in environmental strains of *Vibrio cholerae*. *J Bacteriol* 2001; *183* : 4737-46.

39. Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ, *et al.* DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* 2000; *406* : 477-83.

40. Beyhan S, Tischler AD, Camilli A, Yildiz FH. Differences in gene expression between the classical and el tor biotypes of *Vibrio cholerae* O1. *Infect Immun* 2006; *74* : 3633-42.

41. Dziejman M, Balon E, Boyd D, Fraser CM, Heidelberg JF, Mekalanos JJ, *et al.* Comparative genomic analysis of *Vibrio cholerae*: Genes that correlate with cholera endemic and pandemic disease. *Proc Natl Acad Sci U S A* 2002; *99* : 1556-61.

42. Dharmaprakash A, Mutt E, Jaleel A, Ramanathan S, Thomas S. Proteome profile of a pandemic *Vibrio parahaemolyticus* SC192 strain in the planktonic and biofilm condition. *Biofouling* 2014; *30* : 729-39.

43. Kulkarni HM, Jagannadham MV. Biogenesis and multifaceted roles of outer membrane vesicles from Gram-negative bacteria. *Microbiology* 2014; *160* : 2109-21.

44. Beaber JW, Hochhut B, Waldor MK. SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature* 2004; *427* : 72-4.

45. Hochhut B, Lotfi Y, Mazel D, Faruque SM, Woodgate R, Waldor MK, *et al.* Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins. *Antimicrob Agents Chemother* 2001; *45* : 2991-3000.

46. Divya MP, Sivakumar KC, Sarada Devi KL, Remadevi S, Thomas S. Novel multiple mutations in the topoisomerase gene of Haitian variant *Vibrio cholerae* O1. *Antimicrob Agents Chemother* 2014; *58* : 4982-3.

47. Lopez AL, Gonzales ML, Aldaba JG, Nair GB. Killed oral cholera vaccines: History, development and implementation challenges. *Ther Adv Vaccines* 2014; *2* : 123-36.

48. Cabrera A, Lepage JE, Sullivan KM, Seed SM. Vaxchora: A single-dose oral cholera vaccine. *Ann Pharmacother* 2017; *51* : 584-9.

49. Ontweka LN, Deng LO, Rauzier J, Debes AK, Tadesse F, Parker LA, *et al*. Cholera rapid test with enrichment step has diagnostic performance equivalent to culture. *PLoS One* 2016; *11* : e0168257.

50. Almeida RJ, Hickman-Brenner FW, Sowers EG, Puhr ND, Farmer JJ 3rd, Wachsmuth IK, *et al.* Comparison of a latex agglutination assay and an enzyme-linked immunosorbent assay for detecting cholera toxin. *J Clin Microbiol* 1990; *28* : 128-30.

51. Yam WC, Lung ML, Ng MH. Evaluation and optimization of a latex agglutination assay for detection of cholera toxin and *Escherichia coli* heat-labile toxin. *J Clin Microbiol* 1992; *30* : 2518-20.

52. Carillo L, Gilman RH, Mantle RE, Nunez N, Watanabe J, Moron J, *et al.* Rapid detection of *Vibrio cholerae* O1 in stools of Peruvian cholera patients by using monoclonal immunodiagnostic kits. Loyaza Cholera Working Group in Peru. *J Clin Microbiol* 1994; *32* : 856-7.

53. Hasan JA, Huq A, Tamplin ML, Siebeling RJ, Colwell RR. A novel kit for rapid detection of *Vibrio cholerae* O1. *J Clin Microbiol* 1994; *32* : 249-52.

54. Ley B, Khatib AM, Thriemer K, von Seidlein L, Deen J, Mukhopadyay A, *et al.* Evaluation of a rapid dipstick (Crystal VC) for the diagnosis of cholera in Zanzibar and a comparison with previous studies. *PLoS One* 2012; *7* : e36930.

55. Berk V, Fong JC, Dempsey GT, Develioglu ON, Zhuang X, Liphardt J, *et al.* Molecular architecture and assembly principles of *Vibrio cholerae* biofilms. *Science* 2012; *337* : 236-9.

56. Valentini M, Filloux A. Biofilms and cyclic di-GMP (c-di-GMP) signaling: Lessons from *Pseudomonas aeruginosa* and other bacteria. *J Biol Chem* 2016; *291* : 12547-55.

57. Teschler JK, Zamorano-Sánchez D, Utada AS, Warner CJA, Wong GCL, Linington RG, *et al.* Living in the matrix: Assembly and control of *Vibrio cholerae* biofilms. *Nat Rev Microbiol* 2015; *13* : 255-68.

58. Ammons MC, Copié V. Mini-review: Lactoferrin: A bioinspired, anti-biofilm therapeutic. *Biofouling* 2013; *29* : 443-55.

59. Brackman G, Defoirdt T, Miyamoto C, Bossier P, Van Calenbergh S, Nelis H, *et al.* Cinnamaldehyde and cinnamaldehyde derivatives reduce virulence in *Vibrio* spp. by decreasing the DNA-binding activity of the quorum sensing response regulator LuxR. *BMC Microbiol* 2008; *8* : 149.

60. Augustine N, Goel AK, Sivakumar KC, Kumar RA, Thomas S. Resveratrol - a potential inhibitor of biofilm formation in *Vibrio cholerae*. *Phytomedicine* 2014; *21* : 286-9.

61. Hsiao A, Ahmed AM, Subramanian S, Griffin NW, Drewry LL, Petri WA Jr., *et al.* Members of the human gut microbiota involved in recovery from *Vibrio cholerae* infection. *Nature* 2014; *515* : 423-6.

62. Monira S, Nakamura S, Gotoh K, Izutsu K, Watanabe H, Alam NH, *et al.* Metagenomic profile of gut microbiota in children during cholera and recovery. *Gut Pathog* 2013; *5* : 1.

63. Materna AC, Friedman J, Bauer C, David C, Chen S, Huang IB, *et al.* Shape and evolution of the fundamental niche in marine *Vibrio*. *ISME J* 2012; *6* : 2168-77.

64. Ghosh A, Ramamurthy T. Antimicrobials & cholera: Are we stranded? *Indian J Med Res* 2011; *133* : 225-31.

65. Chin CS, Sorenson J, Harris JB, Robins WP, Charles RC, Jean-Charles RR, *et al.* The origin of the Haitian cholera outbreak strain. *N Engl J Med* 2011; *364* : 33-42.

66. Kumar P, Thomas S. Presence of *qnrVC3* gene cassette in SXT and class 1 integrons of *Vibrio cholerae*. *Int J Antimicrob Agents* 2011; *37* : 280-1.

67. Kim EJ, Lee CH, Nair GB, Kim DW. Whole-genome sequence comparisons reveal the evolution of *Vibrio cholerae* O1. *Trends Microbiol* 2015; *23* : 479-89.

68. Dharmaprakash A, Thandavarayan R, Joseph I, Thomas S. Development of broad-spectrum antibiofilm drugs: Strategies and challenges. *Future Microbiol* 2015; *10* : 1035-48.

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