Role of Phages in the Epidemiology of Cholera

Shah M. Faruque

Abstract Understanding the genetic and ecological factors which support the periodic emergence of toxigenic *Vibrio cholerae* causing outbreaks of cholera in regions where the disease is endemic, is vital to develop preventive measures. Besides environmental factors which are not precisely defined, bacteriophages, and horizontally transmissible genetic elements are known to have a significant role in the epidemiology and evolution of the pathogen. Cholera epidemics are also known to be self-limiting, and hence identifying natural factors which contribute to the collapse of epidemics may have important implications in controlling the disease. Phages have been shown to play a crucial role in modulating cholera epidemics, and enhance *V. cholerae* evolution through a bactericidal selection process which favors the emergence of new clones.

Contents

S. M. Faruque (✉)
Centre for Food and Water Borne Diseases, International
Centre for Diarrhoeal Disease Research, Bangladesh,
68, Shaheed Tajuddin Ahmed Sharani,
Mohakhali, 1212 Dhaka, Bangladesh
e-mail: faruque@icddrb.org

Current Topics in Microbiology and Immunology (2014) 379: 165–180
DOI: 10.1007/82_2013_358

Published Online: 9 November 2013

1 Introduction

V. cholerae represents a group of bacteria which is autochthonous to coastal, river, and estuarine ecosystems, but at the same time, pathogenic for humans (Faruque et al. 1998; Kaper et al. 1995). Toxigenic strains of *V. cholerae*, cause the devastating diarrheal disease cholera, which frequently occurs in an epidemic form, particularly associated with poverty and lack of adequate clean water and sanitation. In recent times, remarkable progress has been made in identifying the major genetic determinants that account for virulence of this organism and understanding how virulent strains emerge in nature. Bacteriophages which act on *V. cholerae* (vibriophages) contribute to the evolution of this pathogen by mediating horizontal transfer of clusters of genes, genomic rearrangements, as well as by bactericidal selection (Brussow et al. 2004; Waldor and Mekalanos 1996; Faruque et al. 2005a). In this latter process, phage susceptible strains are selectively eliminated, whereas bacterial strains that are able to resist phage predation have a survival advantage. *V. cholerae* interacts with diverse phages, and the interaction can promote genetic diversity and/or cause selective enrichment of particular bacterial clones (Faruque et al. 2005a; Zahid et al. 2008, 2011).

Cholera is an ancient disease, and the occurrence of seven distinct pandemics has been recorded since the first pandemic began in 1817 (Faruque et al. 1998; Kaper et al. 1995). The 7th pandemic of cholera which originated in Indonesia in 1961 is the most extensive in geographic spread and duration, and the disease continues to affect an estimated 3–5 million people worldwide, and causes between 100,000 and 130,000 deaths, each year (World Health Organization 2010). Whereas the causative agent of the current 7th pandemic of cholera is *V. cholerae* O1 of the El Tor biotype, the 6th pandemic and presumably the earlier pandemics were caused by the classical biotype, which is now extinct (Siddique et al. 2009). The two biotypes of *V. cholerae* O1 differ in certain phenotypic and genetic characteristics (Kaper et al. 1995). The factors associated with the replacement of the classical biotype as the predominant epidemic strain by the El Tor biotype, and eventual disappearance of the classical strains have not been adequately explained. Possible contribution of particular groups of phages which selectively eliminated the classical biotype strains, while enriching the El Tor biotype (Zahid et al. 2011) has been proposed. However, the displacement of the classical strains by the El Tor might have also been driven by other environmental forces to which the El Tor strains adapted more efficiently.

Cholera epidemics occur most efficiently in regions of the world where transmission between humans occurs frequently due to inadequate sanitation and lack of access to clean water. Epidemics are often associated with the introduction of a fully virulent strain into a susceptible population. Rarely, strains of low pathogenicity acquire virulence genes and thus emerge as a unique new pathogenic clone. Perhaps the emergence of the El Tor biotype of *V. cholerae* to cause disease in Indonesia in 1961, was due to such an event (Faruque et al. 1998). Once endemicity is established in a region, cholera outbreaks appear to occur in a

seasonal pattern. For example, in the Ganges Delta region of Bangladesh and India, two cholera epidemics occur every year with almost predictable seasonal regularity. Factors associated with the maintenance of the periodicity of cholera epidemics are not clear, but a multitude of environmental, genetic, ecological, and socioeconomic factors may be involved.

The role of the human host in selective amplification of *V. cholerae* from the majority of environmental non-pathogenic *V. cholerae* prior to an epidemic has been proposed to be an important factor in cholera epidemiology. Asymptomatic infections of humans supposedly account for the pre-epidemic build up of pathogenic strains which then initiate the index case of cholera. This model of cholera epidemiology (Faruque and Mekalanos 2008) takes into account both environmental and host factors in the initiation of seasonal epidemics and also explains the limited clonality apparent in the strains causing these epidemics. Cholera epidemics are also known to be self-limiting in nature, since the epidemics subside after reaching a peak, even without any active intervention. Among other factors, bacteriophages that can kill *V. cholerae* (cholera phages) have been shown to play a significant role in modulating the course of epidemics (Faruque et al. 2005a, b; Faruque and Mekalanos 2008). In this chapter, we discuss the role of phages in modulating the prevalence of *V. cholerae*, as well as infectivity and transmission of the pathogen, and thus the effect of phages on the occurrence and magnitude of seasonal cholera epidemics.

2 Bacteriophages of *V. cholerae* (Vibriophages)

V. cholerae is the host for a variety of bacteriophages, which are generally referred to as "vibriophages". These phages include temperate phages represented by kappa-type phage produced by most El Tor biotype strains, and virulent phages, e.g., Mukherjee's cholera phages (Basu and Mukerjee 1968) which were popularly used for phage typing of *V. cholerae* O1. The use of phage susceptibility as a method of strain differentiation has contributed greatly to the understanding of the epidemiology of cholera. A phage typing scheme for *V. cholerae* O1 biotype E1 Tor (Basu and Mukerjee 1968) was efficiently used to study the initial spread of the E1 Tor biotype of *V. cholerae* O1. Later, new phage typing schemes were developed for *V. cholerae* O1 (Chattopadhyay et al. 1993), and O139 (Chakrabarti et al. 2000), in which new lytic phages were used. The lytic phages have a typical head and tailed structure, and usually kill the host *V. cholerae* strain in the process of phage multiplication. Another group of phages known as filamentous phages play important roles in the evolutionary biology of *V. cholerae*, and they usually do not kill the host bacterial cells. Whereas the lytic phages have a double stranded genome, the filamentous phages carry a single stranded genome and have been shown to be involved in horizontal transfer of genes among *V. choleare* strains (Faruque and Mekalanos 2003).

A remarkable discovery in recent times that cholera toxin (CT), the major virulence factor of toxigenic *V. cholerae*, is encoded by a lysogenic filamentous phage (CTXϕ), led to the exploration of other filamentous phages as a means of lateral gene transfer among *V. cholerae*. Accordingly, several other filamentous phages or satellite phages that lack genes required for phage morphogenesis have been described in *V. cholerae* O1 and O139 serogroup strains (Faruque and Mekalanos 2012). Possible roles of these phages in *V. cholerae* evolution have been studied (Hassan et al. 2010; Faruque and Mekalanos 2012), and several of these have been shown to cooperate in horizontal gene transfer, leading to increased evolutionary fitness of *V. cholerae*.

In contrast to filamentous phages, the lytic phages often kill the host cells and thus contribute a strong selective force in nature, for the emergence of bacterial clones which are resistant to one or more of these phages. Historically, in the nineteenth century it was recognized that certain elements in the waters of the Ganges and Yamuna rivers in India had marked antibacterial activity against *V. cholerae*, and could protect against cholera (Hankin 1896; Sulakvelidze et al. 2001; Boyd 2008). It was suggested that an unknown heat labile substance which passed through fine porcelain filters was responsible for this antibacterial activity, and for limiting the spread of cholera epidemics. Frederick Twort (1915) reported a similar phenomenon and hypothesized that it may have been due to, among other possibilities, a virus. However, bacteriophages were "officially" discovered 2 years later by Felix D'Herelle, at the Institut Pasteur in Paris (D'Herelle 1917). The discovery or rediscovery of phages by D'Herelle is often linked with an outbreak of hemorrhagic dysentery among French troops stationed on the outskirts of Paris in July–August 1915 (D'Herelle 1930). Phages were used to treat dysentery by D'Herelle, as the first attempt of a therapeutic application of phages. However, the role of phages in combating bacterial infections including cholera was eventually ignored mainly due to the discovery and availability of antibiotics.

The revived interest in cholera phages stems from observations that phage blooms coincide with the decline of *V. cholerae* in water in cholera-endemic areas (Faruque et al. 2005a, b; Jensen et al. 2006). These findings have provided an impetus to examine the potential application of lytic phages in developing control measures against cholera epidemics. A considerable number of lytic and temperate vibriophages have now been found, of which the JSF series of phages isolated from the environment or cholera patients in Bangladesh are of particular interest because of their role in the epidemiology and ecology of *V. cholerae*. At least 27 distinct phages (JSF1 through JSF 27) have been isolated in Bangladesh (Table 1) and efforts are under way to isolate and characterize more cholera phages (Faruque and Mekalanos 2012).

Table 1 Lytic vibriophages isolated from surface water or cholera patients in Bangladesh

Phage designation	Primary host strains	Alternative host strains	Plaque type	Isolation of lysogens
JSF-1	*V. cholerae* O1	Not found	Clear	–
JSF-2	*V. cholerae* O1[a]	Not found	Turbid	+
JSF-3	*V. cholerae* O139	Not found	Clear	+
JSF-4	*V. cholerae* O1	Not found	Clear	+
JSF-5, JSF-11, JSF-13, JSF-14, JSF-17, JSF-18	*V. cholerae* O1	Not found	Clear	–
JSF-6	*V. cholerae* O1	*V. cholerae* non-O1 non-O139	Clear	–
JSF-7	*V. cholerae* O1[a]	*V. cholerae* O141 strain V50; *V. cholerae* O139 strain AI1853	Clear on O1 strain; Clear/turbid on non-O1 strains	+
JSF-8	*V. cholerae* O1[a]	*V. cholerae* non-O1 non-O139 strains 3565, 3548; *V. mimicus* strains 957V1621, 778V1349, and 1016V1721	Clear on O1 strain; Clear/turbid on non-O1 strains	+
JSF-9	*V. cholerae* O1 classical biotype	*V. cholerae* O141 strain V50; non-O1 strains 79, 3565, 3548; *V. mimicus* strains 957V1621, 778V1349, and 1016V1721	Clear	–
JSF10	*V. cholerae* O1	*V. cholerae* O139 strain Arg-3 *V. cholerae* O141 strains V46 and V47	Clear	–
JSF12	*V. cholerae* O1[a]	*V. cholerae* non-O1 strains 79; *V. mimicus* strains 957V1621, 1016V1721	Clear	–
JSF-15	*V. cholerae* O1[a]	*V. cholerae* O141 strain V50; non-O1 strain 79, *V. mimicus* strains 957V1621, and 778V1349	Clear	–
JSF-16	*V. cholerae* O1	*V. cholerae* O141 strain V50	Clear/turbid	+

(continued)

Table 1 (continued)

Phage designation	Primary host strains	Alternative host strains	Plaque type	Isolation of lysogens
JSF-19	*V. cholerae* O1, classical biotype	Not found	Clear	–
JSF-20	*V. cholerae* O1	*V. cholerae* O139 strains MD01 and 1771	Clear on O1 strain; turbid on O139 strains	+
JSF-21	*V. cholerae* O1 classical biotype	*V. cholerae* O139 strain Arg-3	Clear/turbid	–
JSF23 JSF-24, JSF-25, JSF-27	*V. cholerae* O1[a]	Not found	Clear/turbid	–

[a] Recently isolated variants of El Tor biotype strains were found to be resistant to the *V. cholerae* O1-specific phages designated as JSF-2, JSF-7, JSF-8, JSF-12,JSF-15, JSF-17, JSF-19, JSF-25, and JSF-27

3 Prevalence of Phage and *V. cholerae* During the Epidemic Cycle of Cholera

A distinctive epidemiological feature of cholera is its appearance with seasonal regularity in endemic areas, such as the Ganges Delta region of Bangladesh and India. In Bangladesh, outbreaks usually occur twice during a year, with the highest number of cases just after the monsoon and a somewhat smaller number of cholera cases during the spring. The timing of the epidemics correlates with increased concentration of the causative *V. cholerae* in water (Khan et. al. 1984). Cholera epidemics are known to be self-limiting in nature, and interestingly lytic phages that can eradicate the epidemic strain of *V. cholerae* have been suggested to play a significant role in modulating the course of cholera epidemics (Faruque et al. 2005a, b; Jenson et al. 2006; Nelson et al. 2008, 2009).

In one of these studies, changing prevalence of lytic vibriophages in surface water and the number of cholera cases reporting to a nearby cholera hospital was monitored in Dhaka, Bangladesh. Over a nearly 3-year period between January 2001 and November 2003, the number of cholera patients was found to increase whenever the number of lytic vibriophages in water decreased. Similarly, cholera epidemics tended to end concurrent with large increases in the concentration of these phages in the water (Fig. 1). The dynamics of phage and *V. cholerae* concentration and its effect on the occurrence of cholera were also studied during an epidemic in Dhaka City in 2004 (Faruque et al. 2005b). The changing prevalence in the environment of the *V. cholerae* O1 strain causing an epidemic and a particular lytic cholera phage named JSF4 to which it was sensitive, was measured every 48–72 h for a period of 17 weeks. The incidence of phage excretion in stools of 387 cholera patients during the course of the epidemic was also monitored. This study showed that the peak of the epidemic was preceded by high *V. cholerae* prevalence in the environment and was followed by high JSF4 phage levels as the epidemic ended. Interestingly, the build up to high phage concentration in the environment coincided with increasing excretion of the same phage in the stools of cholera patients (Faruque et al. 2005b).

As mentioned above, choleraphages in the environment, and their amplification in cholera patients have been shown to remarkably influence the epidemiology of cholera. It has been proposed that when the balance of phage and bacteria tips in favor of the phage, there is a dramatic decline of numbers of bacteria to a point, where they are no longer able to sustain the epidemic. This prediction has been supported by a mathematical model that attempted to explain the dynamics observed for parameters such as cholera case load, density of *V. cholerae* and phage in the environment, and number of patients shedding *V. cholerae* alone, or shedding both *V. cholerae* and a phage to which the bacteria were susceptible (Jensen et al. 2006). As described in the following sections, phages appear to influence cholera epidemiology by interrupting transmission and by raising the infectious dose of the pathogen required to establish a productive infection.

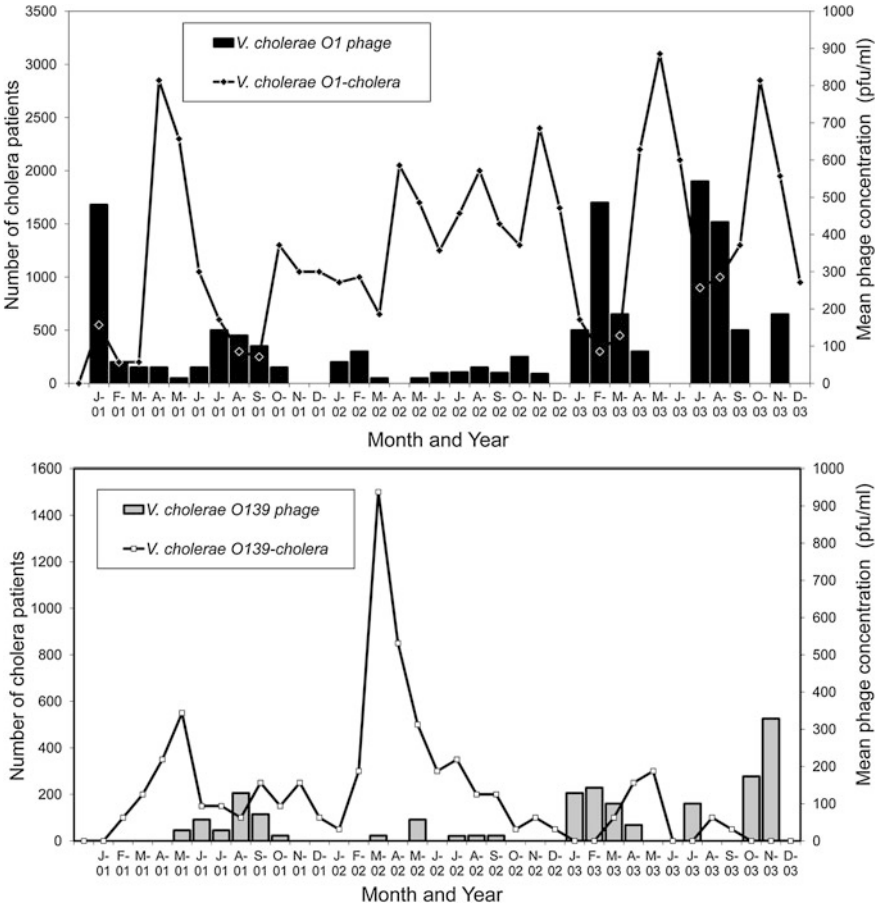

Fig. 1 Mean concentration of lytic vibriophages in the aquatic environment of Dhaka, Bangladesh, and the estimated number of cholera cases reporting to the Dhaka hospital of the International Centre for Diarrhoeal Disease Research, Bangladesh from 2001 to 2003. Number of cholera cases is extrapolated from a 2 % surveillance sample of all patients presenting for treatment

4 Effect of Phage on Transmissibility of Cholera

The factors which enhance water borne spread of cholera epidemics and sustain the epidemic strain in nature are somewhat unclear. It has been suggested that *V. cholerae* excreted in stools of cholera patients are hyperinfectious because the process of human colonization creates a hyperinfectious bacterial state (Merrel et al. 2002). It has been further proposed that the hyperinfectious state of *V. cholerae* in stools is maintained after dissemination into the aquatic environment and this phenomenon may contribute to the epidemic spread of cholera.

On the other hand, results of laboratory experiments have suggested that the rapid transmission of *V. cholera*e through water contaminated with stools of cholera victims to sustain an epidemic is quenched by lytic vibriophages (Nelson et al. 2008, 2009), which are often present in the same stools (Faruque et al. 2005b). In these studies, stools of cholera victims that naturally contained lytic phage or in vitro grown *V. cholerae* were incubated in a microcosm composed of pond water, and the culturability, infectious dose, and transcriptome were assayed over 24 h. The results showed that *V. cholerae* failed to colonize the small intestine after 24 h of incubation in pond water—the point when the phage titers were highest, suggesting that lytic phage block transmission (Nelson et al. 2008). This finding agreed with previous observations (Faruque et al. 2005b) suggesting that phages are most likely to antagonize the transmission of cholera during the late stage of an epidemic, when most patients excrete high titer of lytic phages together with the causative strain of *V. cholerae*.

It has been shown that stools from cholera patients contain a heterogenous mixture of biofilm-like aggregates and free swimming planktonic cells of *V. cholerae* (Faruque et al. 2006). Estimation of relative infectivity of these different forms of *V. cholerae* cells suggested that the enhanced infectivity of *V. cholerae* shed in human stools is largely due to presence of clumps of cells which disperse in vivo providing a high dose of the pathogen. It is widely accepted that cholera is rapidly transmitted through water contaminated with the pathogenic *V. cholerae* excreted in the feces of cholera patients. However, the pathogen has been found to exists in water mostly in a dormant state referred to as conditionally viable environmental cells (CVEC) alternatively known as the viable but non-culturable (VBNC) state. CVEC, the environmental survival form of pathogenic *V. cholerae* that resist cultivation by conventional techniques was found to exist in water as biofilm-like aggregates of partially dormant cells (Faruque et al. 2006). Such CVEC can be recovered as fully virulent bacteria by inoculation of water into rabbit intestines. More recently, it has been demonstrated that quorum sensing signal molecules called autoinducers can lead to resuscitation of the CVEC form of *V. cholerae* (Bari et al. 2013). Quorum sensing refers to regulatory responses in bacteria which are dependent on cell density.

V. cholerae shed in the stools of cholera patients, when inoculated in environmental water samples in the laboratory, exhibited characteristics similar to CVEC, suggesting that CVEC in nature may have been derived at least in part from human cholera stools. Thus these results support a model of cholera transmission in which in vivo formed biofilms contribute to enhanced infectivity and environmental persistence of pathogenic *V. cholerae* (Faruque et al. 2006). Further studies proposed that when the apparent concentration of *V. cholerae* in water is low by conventional enrichment and cultivation, certain human victims ingesting clumps of CVEC in the water might still get a fully infectious dose of the pathogen. In contrast to this scenario, when environmental phage concentration is high, even the clumped *V. cholerae* cells in water would fail to provide an infectious dose of the bacteria presumably due to predation by phages when the clumped

bacterial cells are dispersed in vivo. These assumptions led to further experimental studies of the effect of phages on the infectious dose of *V. cholerae*, as described in the following section.

5 Effect of Phage on the Infectious Dose of *V. cholerae*

To better understand the mechanisms involved in reducing the transmissibility of cholera by phages and phage-mediated collapse of epidemics, studies were conducted to examine the effect of phage on the infectivity of *V. cholerae* shed in stools of cholera patients. These investigations were conducted by using assays that enabled to study the dose of *V. cholera*e required to colonize infant mice. It was found that the infectious dose (ID_{50}) of *V. cholerae* cells in stools of cholera patients as well as that of laboratory-grown cells was higher in the presence of a phage, and ~10-fold more cells of *V. cholerae* would be required to cause a productive infection under the conditions in which cholera patients excrete *V. cholerae* together with phages in their stools (Zahid et al. 2008).

The sustainability of a cholera epidemic depends on amplification of the causative strain in each cholera victim, and waterborne spread of the pathogen to infect more individuals. The increase in the required infectious dose due to the deleterious effect of co-ingesting phage with *V. cholerae* therefore leads to a reduced number of new victims. Moreover, in the face of phage predation, the environment fails to support a heavy load of viable *V. cholerae*, and hence the epidemic ends. Thus these results provided a more clear understanding of the mechanisms involved in the conclusion of a seasonal epidemic of cholera.

6 Emergence of Phage Resistance in *V. cholerae*

The observed data and the theoretical model analysis strongly support a role for phage as a natural biological controller of epidemic cholera in Bangladesh. However, since epidemics reoccur in a seasonal pattern and often caused by the same or a similar strain, it's most likely that a proportion of the causative *V. cholerae* survive phage predation by various mechanisms, and these survivors seed the environment before the next epidemic season. Factors or genetic changes which enhance the ability of the bacteria to resist phage predation may also be a strong driving force in the evolution of *V. cholerae*. Therefore, studies were conducted to understand more about the interactions of *V. cholerae* with lytic phages and the emergence of phage-resistant derivatives.

Under laboratory conditions, co-culture of a phage with *V. cholerae* or culture of dilutions of phage-positive cholera stools were shown to cause emergence of phage-resistant derivatives of the concerned *V. cholerae* strains (Zahid et al. 2008). Frequently, these resistant derivatives were found to have lost their O1 antigen

which often acts as the receptor for these phages. While a proportion of the phage-resistant colonies were rough and agglutinated in normal saline, there were also phage-resistant derivatives that formed smooth colonies similar to the parent strains, but did not agglutinate with the O1 antiserum suggesting that these derivatives had undergone possible serotype conversion. However, it was observed that subsequent epidemics were in fact caused by strains which remain sensitive to the predominant vibriophage detected during the epidemic, and hence further studies were conducted to understand this phenomenon. Subsequently, challenge studies in mice with a mixture of *V. cholerae* and phage, suggested that the intestinal environment did not favor the emergence of phage-resistant derivatives that lost the O1 antigen (Zahid et al. 2008).

The observed resistance to phage may also be manifested by the metabolic status of the *V. cholerae* cells both inside the human host and in vitro, and other mutations that affect the ability of phages to replicate in the bacterial host. It's also likely that in the human host or in the environment a proportion of *V. cholerae* cells may survive phage predation by forming biofilms or cellular organization that becomes transiently inaccessible to the predatory phage or become unsuitable for phage replication due to transient dormancy of the bacterial hosts. Thus, the outcome of phage bacterial interactions is more complex in nature and involves a multitude of factors including environmental parameters, intrinsic properties of relevant phage and bacterial strains, and presumably the role of the human host.

7 Phages with Multiple Bacterial Hosts

The environment in a cholera endemic area (such as Bangladesh) not only promotes the persistence of pathogenic *V. cholerae,* but also enhances dramatic shifts in the strains that emerge to cause disease. It has been suggested that this process might be driven by bacteriophages either specific for *V. cholerae* of the epidemic serogroup or phages that can infect other aquatic bacterial species which act as alternative hosts. Such phages were hypothesized to grow in other *Vibrio* species or in *V. cholerae* non-O1 strains particularly in the absence of *V. cholerae* O1 strains, but are able to modulate the prevalence of the O1 serogroup strains when these become abundant. Environmental and clinical surveillance in Bangladesh has indeed identified certain phages that can grow on both *V. cholerae* O1 and non-O1 non-O139 strains as alternative hosts (Table 1). Some of these phages appear to exist as lysogens in the *V. cholerae* non-O1 non-O139 strains. However, it remains to be demonstrated what effect a "bloom" of these aquatic, non-cholera *Vibrio* species might have on epidemic strains of *V. cholerae*, whether it is direct (via competition for a limited aquatic niche) or indirect (through promoting phage replication that has a deleterious effect on the survival of pathogenic *V. cholerae*). It seems possible that vibriophages can also replicate in other common environmental species. These "non-*Vibrio* species" may be common to the aquatic environments, e.g., *Aeromonas*, *Pleiseomonas*, *Pseudomonas*, etc. Search for

alternative hosts for vibriophages which can act on epidemic *V. cholerae* strains is of particular importance, since identifying and characterizing such non-cholera *Vibrio* species may have predictive value in cholera epidemiology.

8 Co-evolution of Lytic Phages and *V. cholerae*

The ability of *V. cholerae* to evade phage predation constitutes important development in attaining evolutionary fitness. Conversely, phage resistance is often attained by altering expression of bacterial surface appendages such as flagella or pili or O antigens which may act as receptors for different lytic phages. Thus, there may also be a fitness cost associated with achieving phage resistance by *V. cholerae*, and the evolutionary success of the pathogen has presumably been dependent on a balance between these various factors.

The complete genomic sequences of a number of *V. cholerae* strains have been determined, and the crucial changes that define virulence altering traits are beginning to be revealed. Pathogenic strains typically differ from environmental strains in their virulence gene content and the lipopolysaccharide O1 or O139 antigens that are typically carried by the pathogens (Faruque et al. 1998; Kaper et al. 1995). Only strains belonging to these two serogroups (O1 and O139) are known to be associated with epidemic and endemic disease despite the fact that over 250 different serogroups of *V. cholerae* have so far been reported. Filamentous phages are known to be involved in some of the horizontal gene transfer events, that causes the emergence of strains carrying virulence genes. However, the role of lytic phages is also important because of the selection pressure they impart to different clones of *V. cholerae*. For example, the explanation for the evolutionary success of the 7th pandemic clone over the preexisting 6th pandemic strain remains largely a mystery since both classical and El Tor biotypes of *V. cholerae* O1 carry CTX and TCP. Notably, recent studies are beginning to suggest possible role of lytic phages in this process (Zahid et al. 2011). Phage resistance has also been associated with serotype conversion, suggesting that phages may provide a strong selective force for *V. cholerae* strains to undergo genetic or phenotypic alteration.

The ecological interactions of *V. cholerae* with chitin-rich zooplankton has been suggested to have an evolutionary significance and not just limited to deriving nutrients from this abundant carbon source. *V. cholerae* has been reported to become naturally competent to uptake exogenous DNA, while growing on a chitin substrate (Meibom et al. 2005). It has recently been suggested that free DNA released in the aquatic environment by phage-mediated lysis of *V. cholerae* can be harnessed and assimilated by surviving *V. choleare* strains (Udden et al. 2008). Thus, phages contribute to bacterial evolution in numerous ways and the full potential of these mechanisms in the evolution of *V. cholerae* as a pathogen is yet to be appreciated. As explained above, the role of phages in the evolution of *V. cholerae* involves not only a means of horizontal gene transfer among

Table 2 Predominant phages isolated from surface water and cholera patients in Bangladesh during 2002–2013

Year	Abundant Phages	Predominant Phage
2002–2004	JSF1 through JSF6	JSF4
2005	JSF1 through JSF9	JSF4
2006	JSF1, JSF4, JSF6 through JSF9	JSF4
2007	JSF1 through JSF11	JSF4/JSF11
2008	JSF1, JSF4 through JSF11	JSF11
2009	JSF1, JSF4 through JSF11	JSF11
2010	JSF1, JSF4 through JSF11	JSF11
2011	JSF1, JSF4 through JSF16	JSF11
2012	JSF11, JSF13	JSF13
2013	JSF11, JSF13	JSF13

V. choleare strains, but also provide a selection pressure for particular bacterial clones.

In keeping with changes in the host *V. cholerae* strains, temporal changes in the prevalence and properties of diverse lytic vibriophages have also been noticed. The environmental surveillance system to monitor phages and *V. cholerae* in the aquatic environment in Bangladesh yielded various phage isolates and data on phage prevalence. Different phage strains (JSF1 through JSF27) which interact with *V. cholerae* have been identified and partially characterized (Table 1). Fluctuation in the prevalence of the different predatory phages have been observed, with a temporal change in the most prevalent phage type (Table 2) which is a factor in the collapse of epidemics by phage predation (Faruque et al. 2005a; Faruque and Mekalanos 2012). Studies were also conducted to identify bacterial factors which mediate resistance to phage susceptibility, and thus provide a mechanism to survive predation by phages. As mentioned above, often phage-resistant strains were found to have lost their O antigen. The lipopolysaccharide O1 antigen is a major target of bacteriophages as well as the human immune system. Seed and colleagues (2012) showed that under the selective pressure of a lytic phage *V. cholerae* can modulate O antigen expression and exhibit intrastrain heterogeneity. Two phase variable genes *manA* and *wbeL* were found to be involved and these phase variants were found to be attenuated for virulence, providing functional evidence to support the critical role of the O1 antigen for infectivity. These authors suggested that the maintenance of the phase variable loci is an important means by which *V. cholerae* can generate diverse subpopulations of cells needed for infecting the host intestinal tract and for escaping predation by an O1-specific phage.

Incorporation of mutations in the *cyaA* or *crp* genes encoding adenylate cyclase or cyclic AMP receptor protein (CRP), respectively, into *V. cholerae* O1 strains was also found to cause alterations in their phage susceptibility patterns, and the susceptibility correlated with the ability of the bacteria to adsorb these phages. These results suggested that cAMP-CRP-mediated downregulation of phage

adsorption may contribute to a mechanism for the *V. cholerae* strains to survive predation by phages in the environment (Zahid et al. 2010).

Although the significance of the observed change in prevalence of particular phage types is not fully understood, occasional change in the predominant phage appears to maintain susceptibility of the epidemic strain to the most prevalent phage responsible for ending epidemics in a self-limiting manner. A bacterial adaptive immune system against phage invasion is the CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins) system, which provides sequence-specific protection from invading nucleic acids, including phage. Interestingly, a phage-encoded CRISPR/Cas system has also been documented, and this system is used to counteract a phage inhibitory chromosomal island of the bacterial host (Seed et al. 2013). Thus, *V. cholerae* and their phages co-evolve, and while a range of bacterial immunity mechanisms against phages has evolved, this in turn seems to have also resulted in the evolution of diverse phages with the immunity evasion strategies.

9 Conclusion

It has now been recognized that phage predation may not only play a role in the natural control of cholera epidemics, but that changes in pathogenic *V. cholerae* may have been driven to a large extent by phages. Although there are multiple other factors which can drive the evolution of *V. cholerae* and the epidemiological nature of the pathogen, phages have been shown to contribute extensively to the dynamics of cholera epidemics. Briefly, phage predation of *V. cholerae* influences the prevalence of the pathogen in the environment, and hence occurrence of cholera in Bangladesh, where a large proportion of the population use surface water due to inadequate access to safe water and sanitation facilities (Faruque et al. 2005a). The peak of the epidemic is preceded by high *V. cholerae* prevalence in the water and is followed by high levels of lytic choleraphages to which the epidemic strain is sensitive. Phage amplification occurs in the intestines of cholera victims, and eventually the build up of phage concentration in the environment might derive the collapse of an epidemic, thus causing cholera epidemics to be self-limiting in nature. Phages lead to the collapse of epidemics by modulating the required infectious dose of the bacteria, and interrupting transmission (Nelson et al. 2008; Zahid et al. 2008). Furthermore, although phage-resistant strains emerge in the process, these derivatives are often less virulent, and hence the dominance of phage-resistant variants due to bactericidal selective mechanism occurs rarely under natural settings, and the emerging variants are thus unable to sustain the ongoing epidemic. However, phages contribute to the evolution of *V. cholerae* in a variety of ways (Seed et al. 2012, 2013), and the occasional emergence of new or altered pathogenic strains may in a large part be driven directly or indirectly by diverse phages. On the other hand, phages also undergo

co-evolution to adapt to the genetic changes in the host *V. cholerae* strains, and maintain the ability to multiply on the host strains.

Acknowledgments Research in the laboratory of SMF were funded in part by National Institutes of Health grants 2RO1-GM068851, under a subagreement between the Harvard Medical School, and the International Centre for Diarrhoeal Disease Research, Bangladesh (Icddr, b). The Icddr, b is supported by countries and agencies which share its concern for the health problems of developing countries.

References

Bari SM, Roky MK, Mohiuddin M, Kamruzzaman M, Mekalanos JJ, Faruque SM (2013) Quorum-sensing autoinducers resuscitate dormant *Vibrio cholerae* in environmental water samples. Proc Natl Acad Sci USA 110:9926–9931

Basu S, Mukerjee S (1968) Bacteriophage typing of *Vibrio ElTor*. Experientia 24:299–300

Brussow H, Canchaya C, Hardt W (2004) Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. Microb Mol Biol Rev 68:560–562

Boyd F (2008) Filamentous phages of *Vibrio cholerae*. In: Faruque SM, Nair GB (eds) *Vibrio cholerae*: genomics and molecular biology. Caister Academic Press, Norfolk

Chakrabarti AK, Ghosh AN, Nair GB, Niyogi SK, Bhattacharya SK, Sarkar BL (2000) Development and evaluation of a phage typing scheme for Vibrio cholerae O139. J Clin Microbiol 38:44–49

Chattopadhyay DJ, Sarkar BL, Ansari MQ, Chakrabarti BK, Roy MK, Ghosh AN, Pal SC (1993) New phage typing scheme for *Vibrio cholerae* O1 biotype E1 Tor strains. J Clin Microbiol 31:1579–1585

D'Herelle F (1917) Sur un microbe invisible antagoniste des bacilles dysentériques. C R Acad Sci (Paris) 165:373–375

D'Herelle F (1930) The bacteriophage and its clinical applications. Charles C Thomas, Springfield

Faruque SM, Naser IB, Islam MJ, Faruque ASG, Ghosh AN, Nair GB, Sack DA, Mekalanos JJ (2005a) Seasonal epidemics of cholera inversely correlate with the prevalence of environmental cholera phages. Proc Natl Acad Sci USA 102:1702–1707

Faruque SM, Albert MJ, Mekalanos JJ (1998) Epidemiology, genetics and ecology of toxigenic *Vibrio cholerae*. Microbiol Mol Biol Rev 62:1301–1314

Faruque SM, Mekalanos JJ (2008) Molecular ecology of *Vibrio cholerae*. In: Faruque SM, Nair GB (eds) *Vibrio cholerae*: genomics and molecular biology. Caister Academic Press, Norfolk

Faruque SM, Mekalanos JJ (2003) Pathogenicity islands and phages in *Vibrio cholerae* evolution. Trends Microbiol 11:505–510

Faruque SM, Mekalanos JJ (2012) Phage bacterial interactions in the evolution of toxigenic *Vibrio cholerae*. Virulence 3:1–10

Faruque SM, Biswas K, Udden SN, Ahmad QS, Sack DA, Nair GB, Mekalanos JJ (2006) Transmissibility of cholera: in vivo-formed biofilms and their relationship to infectivity and persistence in the environment. Proc Natl Acad Sci USA 103:6350–6355

Faruque SM, Islam MJ, Ahmad QS, Faruque ASG, Sack DA, Nair GB, Mekalonas JJ (2005b) Self-limiting nature of seasonal cholera epidemics: role of host-mediated amplification of phage. Proc Natl Acad Sci USA 102:6119–6124

Hankin EH (1896) L'action bactericide des eaux de la Jumna et du Gange sur le vibrion du cholera. Ann Inst Pasteur 10:511

Hassan F, Kamruzzaman M, Mekalanos JJ, Faruque SM (2010) Satellite phage TLCϕ enables toxigenic conversion by CTX phage through dif site alteration. Nature 467:982–985

Jensen MA, Faruque SM, Mekalanos JJ, Levin BR (2006) Modeling the role of bacteriophage in the control of cholera outbreaks. Proc Natl Acad Sci USA 103:4652–4657

Kaper JB, Morris JG, Levine MM (1995) Cholera. Clin Microbiol Rev 8:48–86

Khan MU, Shahidullah M, Haque MS, Ahmed WU (1984) Presence of vibrios in surface water and their relation with cholera in a community. Trop Geogr Med 36:335–340

Meibom KL, Blokesch M, Dolganov NA, Wu C, Schoolnik GK (2005) Chitin induces natural competence in *Vibrio cholerae*. Science 310:1824–1827

Merrell DS, Butler SM, Qadri F, Dolganov NA, Alam A, Cohen MB, Calderwood SB, Schoolnik GK, Camilli A (2002) Host-induced epidemic spread of the cholera bacterium. Nature 417:642–645

Nelson EJ, Chowdhury A, Flynn J, Schild S, Bourassa L, Shao Y, LaRocque RC, Calderwood SB, Qadri F, Camilli A (2008) Transmission of *Vibrio cholerae* is antagonized by lytic phage and entry into the aquatic environment. PLoS Pathog 10:e1000187

Nelson EJ, Harris JB, Morris JG Jr, Calderwood SB, Cammilli A (2009) Cholera transmission: the host, pathogen and bacteriophage dynamic. Nat Rev Microbiol 10:693–702

Seed KD, Faruque SM, Mekalanos JJ, Calderwood SB, Qadri F, Camilli A (2012) Phase variable O antigen biosynthetic genes control expression of the major protective antigen and bacteriophage receptor in *Vibrio cholerae* O1. PLoS Pathog 8:e1002917

Seed KD, Lazinski DW, Calderwood SB, Camilli A (2013) A bacteriophage encodes its own CRISPR/Cas adaptive response to evade host innate immunity. Nature 494:489–491

Siddique AK, Nair GB, Alam M, Sack DA, Huq A, Nizam A, Longini IM, Qadri F, Faruque SM, Colwell RR, Ahmed S, Iqbal A, Bhuiyan NA, Sack RB (2009) El Tor cholera with severe disease: a new threat to Asia and beyond. Epidemiol Infect 14:1–6

Sulakvelidze A, Alavidze Z, Morris Jr JG (2001) Bacteriophage therapy. Antimicrob Agents Chemother 45: 649–659

Twort FW (1915). An investigation on the nature of ultramicroscopic viruses. Lancet 2:1241–1243

Udden SMN, Zahid MSH, Biswas K, Ahmad QS, Cravioto A, Nair GB, Mekalanos JJ, Faruque SM (2008) Acquisition of classical CTX prophage from *Vibrio cholerae* O141 by El Tor strains aided by lytic phages and chitin-induced competence. Proc Natl Acad Sci USA 23:11951–11958

Waldor MK, Mekalanos JJ (1996) Lysogenic conversion by a filamentous bacteriophage encoding cholera toxin. Science 272:1910–1914

World Health Organization (2010) Cholera vaccines. A brief summary of the March 2010 position paper. http://www.who.int/immunization/Cholera_PP_Accomp_letter_Mar_ 10_2010.pdf

Zahid MSH, Waise Z, Kamruzzaman M, Ghosh AN, Nair GB, Bashar SAMK, Mekalanos JJ, Faruque SM (2011) An experimental study of phage mediated bactericidal selection and emergence of the El Tor *Vibrio cholerae*. Indian J Med Res 133:218–224

Zahid MSH, Waise TM, Kamruzzaman M, Ghosh AN, Nair GB, Mekalanos JJ, Faruque SM (2010) The cyclic AMP (cAMP)-cAMP receptor protein signaling system mediates resistance of *Vibrio cholerae* O1 strains to multiple environmental bacteriophages. Appl Environ Microbiol 76:4233–4240

Zahid MSH, Udden SM, Faruque ASG, Calderwood SB, Mekalanos JJ, Faruque SM (2008) Effect of phage on the infectivity of *Vibrio cholerae* and emergence of genetic variants. Infect Immun 76:5266–5273