Shigella dysenteriae Type 1-Specific Bacteriophage from Environmental Waters in Bangladesh

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Shigella dysenteriae type 1 is the causative agent of the most severe form of bacillary dysentery, which occurs as epidemics in many developing countries. We isolated a bacteriophage from surface water samples from Bangladesh that specifically lyses strains of _S. dysenteriae_ type 1. This phage, designated SF-9, belongs to the _Podoviridae_ family and has a 41-kb double-stranded DNA genome. Further screening of water samples for the prevalence of the phage revealed 9 of 71 (12.6%) water samples which were positive for the phage. These water samples were also positive in PCR assays for one or more _S. dysenteriae_ type 1-specific genes, including _ipaBCD_ and _stx1_, and live _S. dysenteriae_ type 1 was isolated from three phage-positive samples. The results of this study suggest that phage SF-9 may have epidemiological applications in tracing the presence of _S. dysenteriae_ type 1 in environmental waters.

Bacillary dysentery caused by *Shigella dysenteriae* type 1 is a major public health problem in many developing countries, including Bangladesh (8, 15, 19). There are four known species of *Shigella* which are pathogenic, and infection with *S. dysenteriae* type 1 usually progresses to the most severe form of dysentery with life-threatening complications (8). The most common underlying cause of death in fatal shigellosis is severe colitis, and the immediate associated causes are septicemia and pneumonia (20). Infection with *S. dysenterieae* type 1 can occur in an epidemic form, and *Shigella*-contaminated food and drink are often the source of epidemic spread. Very little was known about the presence of the pathogen in the aquatic environment until a recent study indicated that *Shigella* can survive and persist in surface waters (10). This suggested that environmental monitoring for the presence of the pathogen and its possible spread through environmental waters may provide new insights regarding the epidemiology of *Shigella* infection.

Shigella is ordinarily identified in stool specimens by in vitro culture of the organism and by several biochemical tests and agglutination assays. DNA probes and PCR assays directed against the large invasion plasmid have also been used to detect *Shigella* in stools (12, 18). However, identification of *Shigella* in environmental samples, in which the number of organisms is likely to be small, is limited mainly by the lack of a suitable enrichment technique. Shigellae have previously been identified in surface waters by a combination of PCR and culture techniques (10). For effective environmental monitoring, there is a need to develop more convenient and inexpensive alternative techniques. The presence of specific bacteriophages in water sources may serve as an indicator for the

presence of the host bacteria, and this approach has been used in a previous study for epidemiological surveillance of *Vibrio cholerae* (16). Thus, detection of phages specific for *Shigella* strains may be a useful tool for predicting the presence of *Shigella* in environmental waters. Identification and characterization of specific phages are prerequisites for establishing phage-based methods of environmental monitoring. In this study, we isolated and characterized a bacteriophage specific for *S. dysenteriae* type 1 and studied its distribution in environmental waters in Bangladesh. Furthermore, we analyzed the phage-positive surface water samples for the presence of *S. dysenterieae* type 1.

MATERIALS AND METHODS

The *S. dysenteriae* type 1-specific bacteriophage SF-9 was initially isolated from a sample of river water collected in Dhaka City, Bangladesh. To study the prevalence of the phage in the environment, 71 water samples collected from 10 different sites in two major rivers and a lake in Dhaka were tested. Samples were collected during a 6-month period between March and September 2002. All water samples were obtained in sterile containers, and the initial processing of the samples for detection of *S. dysenteriae* type 1-specific phages, PCR, and culture of *Shigella* was done within 3 h of collection.

Detection and isolation of bacteriophage. Aliquots (20 ml) of water samples were centrifuged at a low speed (1,000 × *g*) to precipitate debris, and the supernatants were filtered through 0.22-μm-pore-size filters (Millipore Corporation, Bedford, Mass.) to exclude bacteria. Twenty-five different *Shigella* strains (Table 1) were used as potential recipients to detect the presence of possible *Shigella* phages. Logarithmic-phase cells (500 μl) of each bacterial strain in nutrient broth (Difco, Detroit, Mich.) were mixed with 3.5-ml aliquots of soft agar (nutrient broth containing 0.8% Bacto Agar [Difco]), and the mixtures were overlaid on nutrient agar plates. Aliquots (10 μl) of each bacterium-free filtrate were inoculated onto the plates. Six samples of filtrates were inoculated per plate, and the plates were incubated for 16 h at 37°C. A sample was positive for phage when a lytic or lysogenic plaque type was observed on the plates. Negative samples were retested after concentration of possible phage particles as follows. Aliquots (10 ml) of the sterile filtrates were mixed with 2.5 ml of a solution containing 20% polyethylene glycol 6000 and 10% NaCl and centrifuged at 12,000 × *g*. The precipitates were dissolved in 100 μl of SM buffer (100 mM NaCl, 8.1 mM $MgSO_4$, 0.05 mM Tris-Cl [pH 7.5], 0.01% gelatin). These con-

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TABLE 1. Susceptibility test results for various bacteria with
Shigella phage SF-9 isolated from a river water sample
in Bangladesh

Organism	No. of isolates	Source of isolates	No. of isolates susceptible to phage SF-9
S. dysenteriae type 1	9	Patient, Bangladesh	9
S. dysenteriae type 1	2	Surface water, Bangladesh	2
S. dysenteriae type 1	5	Patient, India	5
S. dysenteriae type 2	3	Patient, Bangladesh	0
S. dysenteriae type 3–10	4	Patient, Bangladesh	0
S. flexneri type 1a	2	Patient, Bangladesh	0
S. flexneri type 1b	2	Patient, Bangladesh	0
S. flexneri type 2a	6	Patient, Bangladesh	0
S. flexneri type 2b	3	Patient, Bangladesh	0
S. flexneri type 3a	2	Patient, Bangladesh	0
S. flexneri type 3b	1	Patient, Bangladesh	0
S. flexneri type 3c	1	Patient, Bangladesh	0
S. flexneri type 4a	1	Patient, Bangladesh	0
S. flexneri type 5	1	Patient, Bangladesh	0
S. flexneri type 6	2	Patient, Bangladesh	0
S. flexneri type X	1	Patient, Bangladesh	0
S. flexneri type Y	1	Patient, Bangladesh	0
Schigella boydie type 1–6	4	Patient, Bangladesh	0
S. boydie type 7–11	3	Patient, Bangladesh	0
S. boydie type 12–15	4	Patient, Bangladesh	0
Shigella sonnei	3	Patient, Bangladesh	0
V. cholerae O1	15	Patient, Bangladesh	0
V. cholerae O139	12	Patient, Bangladesh	0
Enterotoxigenic *E. coli*	20	Patient, Bangladesh	0
Enteropathogenic *E. coli*	18	Patient, Bangladesh	0
Providencia alcalifaciens	3	Patient and surface water, Bangladesh	0
STEC	2	Patient, Bangladesh	0
E. coli HB101	1	Laboratory collection	0

centrated preparations were then retested for the presence of the phage as described above.

Phage production and test for host specificity. A single discrete plaque was purified three times by the soft agar (0.7%) overlay method (17) with control *S. dysenteriae* type 1 strain SD33891 (13). To grow the phage in liquid medium, an overnight culture of strain SD33891 was diluted 1:100 in fresh nutrient broth and grown at 37°C for 4 h. The culture was then inoculated with phage from a single plaque. The bacterium-phage culture was incubated at 37°C for 16 h, when lysis of most of the bacteria occurred. The culture was centrifuged at $10,000 \times g$ for 20 min, and chloroform (final volume, 1%) was added to the supernatant to kill any unlysed bacteria. The supernatant was kept at 4°C for 20 h, and the chloroform was evaporated. The number of phage particles was determined by testing serial dilutions of the supernatant by the soft agar overlay method with propagating strain SD33891. The host range of the phage was tested at a titer of 10^3 PFU/ml.

Stability of phage. The effects of temperature, pH, and salinity on the stability of phage SF-9 were assessed as described previously (11). Briefly, defined numbers of phage particles were added to SM buffer preadjusted to different salinity and pH values and were incubated at different temperatures. The titer of phage particles remaining after 6 h was expressed as a percentage of the original titer.

PCR assays. PCR primers used in this study have been described previously (10) and were based on previously published sequences of the relevant genes (22, 23). Aliquots (20 ml) of water were centrifuged at a low speed to precipitate debris, and the supernatants containing bacterial cells were used to isolate total nucleic acids by previously described methods (10) One to five microliters of the extracted nucleic acids was used in a multiplex PCR assay for the genes encoding invasive plasmid antigens (*ipaBCD*) and Shiga toxin (*stx1*). All PCR primers were synthesized commercially by Oswel DNA Service (University of Edinburgh, Edinburgh, United Kingdom). PCR reagents and kits were purchased from Perkin-Elmer Corporation (Norwalk, Conn.) and were used in accordance with the manufacturer's instructions. The thermocycling parameters for the PCR assays were denaturation at 94°C for 2 min, annealing of primers at 55°C for 2 min, and primer extension at 72°C for 3 min. Each PCR was conducted for 36

cycles, and the sizes of the PCR amplicons were ascertained by agarose gel electrophoresis.

Culture of environmental samples. Water samples were analyzed for the presence of *Shigella* by previously described methods (6, 10). Briefly, 50-ml samples of water were filtered as described above, and the filters with residue were incubated in nutrient broth for 4 h at 37°C with shaking. Aliquots of each suspension were streaked onto MacConkey agar plates (Difco, Becton Dickinson and Company) and xylose lysine deoxycholate agar plates (Plasmatec Laboratory Products Ltd., Bridport, Dorset, United Kingdom). Suspected colonies were picked and subjected to biochemical and serological tests to identify *Shigella*. Culture-confirmed isolates were analyzed further by PCR for the presence of *Shigella*-specific virulence genes.

Electron microcopy of phage particles. A high-titer phage preparation ($\sim10^{10}$ PFU/ml) was obtained by using the plate lysis procedure as described previously (4). The phage particles were negatively stained with 2% uranyl acetate and were examined with a Philips transmission electron microscope (model 420T) as described previously (4).

Isolation and analysis of phage nucleic acid. For isolation and analysis of phage nucleic acid, culture supernatants containing phage particles were filtered through 0.22-μm-pore-size filters (Millipore). Each filtrate was mixed with 0.25 volume of a solution containing 20% polyethylene glycol 6000 and 10% NaCl and centrifuged at $12,000 \times g$ to precipitate the phage particles. The precipitate was dissolved in a solution containing 20 mM Tris-Cl (pH 7.5), 60 mM KCl, 10 mM MgCl, and 10 mM NaCl and was digested with pancreatic DNase I (100 U/ml) and RNase A (50 μg/ml) at 37°C for 2 h. The solution was extracted with phenol-chloroform, and the total nucleic acids were precipitated with ethanol. The phage nucleic acid was digested with restriction endonucleases (Invitrogen Corporation, Carlsbad, Calif.) and analyzed by agarose gel electrophoresis by using standard procedures.

RESULTS AND DISCUSSION

Phage SF-9 was initially isolated from a sample of river water from Dhaka, Bangladesh. This phage produced clear plaques with a diameter of approximately 1 mm on a lawn of an *S. dysenteriae* type 1 strain. When grown in nutrient broth with control host strain SD33891 (10), the phage produced had a titer of $\sim10^8$ PFU/ml. The specificity of the phage was examined by using a panel of strains belonging to different species or serogroups (Table 1). Only *S. dysenteriae* type 1 strains were susceptible to the phage, and all other strains tested were clearly resistant. Previously described *Shigella* phages infected a broader spectrum of *Shigella* strains belonging to different species or serotypes (5, 14). Important examples include phages associated with serotype conversion of *Shigella flexneri* and phages encoding Shiga-like toxins (1, 5, 14). Shiga toxin-converting phages have been found to be distributed widely among *Shigella* and *Escherichia coli* strains (9, 14). The *stx* prophages carried by Shiga toxin-producing *E. coli* (STEC) strains can also multiply and lyse their bacterial hosts upon induction and are able to infect recipient strains. Although phage-specific DNA sequences are also found in the regions adjacent to *stx* genes in the chromosomes of *S. dysenteriae* type 1 strains, this prophage is assumed to be defective, and intact Shiga toxin-converting phages are not produced by *S. dysenteriae* type 1 (9).

Electron microscopic examination of phage SF-9 revealed that the phage particles had hexagonal heads and short tails and thus belonged to the family *Podoviridae* (2) (Fig. 1). Analysis of nucleic acids derived from the phage preparation by using a number of restriction enzymes revealed that the genome of the phage consisted of approximately 41 kb of double-stranded DNA. The genome of SF-9 was considerably smaller than the genomes of lamboid phages containing genes for Shiga-like toxins (9).

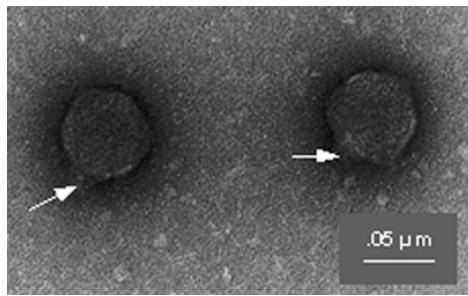The phage was fairly stable at pHs ranging from 6.0 to 9.0

FIG. 1. Electron micrograph showing the morphology of *S. dysenteriae* type 1-specific phage SF-9. Note the hexagonal head and short tail (arrows). Magnification, ×180,000.

TABLE 2. Analysis of 71 surface water samples for the presence of *S. dysenteriae* type 1-specific phage, SF-9 and *S. dysenteriae* type 1

No. of samples[a]	Presence of SF-9 phage[b]	PCR assay for:		Isolation of *Shigella* species
		ipaBCD	*stx1*	
1	+	+	+	*S. dysenteriae* type 1
1	+	+	+	Negative
5	+	−	+	Negative
2	−	−	−	Negative
56	−	−	−	Negative
1	−	+	−	*S. flexneri*
3	−	−	−	Negative
2	+	−	+	*S. dysenteriae* type 1

[a] Samples were collected from 10 different sampling sites in two rivers and a lake in Dhaka city, Bangladesh.
[b] All nine water samples which were found to be positive for phage SF-9 were from the two rivers.

and at temperatures below 37°C. As determined after 6 h of incubation, 65 to 82% of the original phage particles remained infectious. At temperatures above 45°C, however, the majority of the phage particles were rapidly inactivated. Phage particles remained infectious for more than 4 weeks when they were stored at room temperature in SM buffer containing at least 0.5% NaCl. These findings suggest that SF-9 particles may persist in the aquatic environment as infectious agents depending on these and possibly other parameters.

SF-9 was detected in 9 of 71 water samples (12.6%) tested for the presence of the phage. The phage isolates were first tested for host specificity and later were compared by using the restriction endonuclease cleavage patterns of the phage DNA. Phage isolates from the nine different positive samples produced similar DNA restriction patterns, confirming that the phage isolates were the same strain (Fig. 2). The same water samples were also analyzed by *Shigella*-specific PCR and by conventional culture.

The most important components of virulence in *Shigella* infection include adherence, invasiveness, and toxigenicity (19). The genes for these virulence factors reside either on
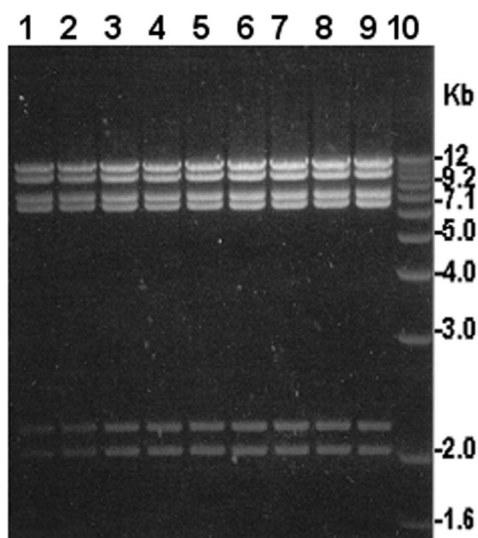

FIG. 2. Restriction analysis of DNA isolated from SF-9 phage isolated from different water samples. Lanes 1 through 9, *Mlu*I restriction profiles of phage DNA from different samples; lane 10, 1-kb Plus DNA ladder (Invitrogen) used as a molecular size marker.

plasmids, on the bacterial chromosome, or on lysogenic bacteriophages. The major virulence genes of *S. dysenteriae* type 1 include plasmid-encoded genes for invasiveness and intracellular movement, whose products are referred to as invasive plasmid-associated antigens (Ipa), and the gene for Shiga toxin, which is encoded by a chromosomally integrated phage genome (22, 23). In the present study, all nine water samples that were positive for the SF-9 phage were also positive for one or more *Shigella*-specific genes in PCR assays (Table 2). Two of these samples were positive for both the *ipaBCD* genes encoding invasive plasmid antigens and the *stx1* gene encoding Shiga toxin, whereas the remaining seven samples were positive for *stx1* and negative for *ipaBCD*. PCR assays specific for *stx1* failed to produce an amplicon from SF-9 phage DNA, indicating that SF-9 did not carry the *stx1* gene. One possible reason for the presence of *stx1* alone in these seven samples is that *S. dysenteriae* type 1 strains lost the large plasmid encoding the *ipaBCD* genes. It has previously been demonstrated that *Shigella* strains in the environment tend to lose the plasmid-encoded virulence genes, whereas the chromosomal genes are more conserved (10). The presence of STEC strains could also be a reason for positive *stx1* and negative *ipaBCD* results in the PCR assay. Although *S. dysenteriae* type 1 produces the prototypical Shiga toxin, different variants of Shiga toxin are known to be produced by STEC strains, which carry temperate lambdoid phages encoding Shiga toxin (21). However, previous epidemiological surveillance has shown that diarrhea due to STEC strains is extremely rare in Bangladesh (3, 7). Since all seven water samples were positive for phage SF-9, which was shown to be specific for *S. dysenteriae* type 1 and failed to lyse all other strains tested, including STEC strains (Table 1), the *stx1* PCR product was most likely derived from *S. dysenteriae* type 1 strains which had lost the plasmid encoding *ipaBCD*. This conclusion was further supported by the isolation of live *S. dysenteriae* type 1 strains from three SF-9 phage-positive water samples. PCR analysis confirmed the presence of the *stx1* gene but the absence of the *ipaBCD* genes in two of these isolates (data not shown). All *S. dysenteriae* type 1 isolates from environmental water samples were also found to be susceptible to SF-9 phage in subsequent assays.

It was demonstrated previously that a preliminary screening by PCR followed by culturing substantially improves the chance of isolating *Shigella* from environmental samples (10).

The present study indicated that the presence of SF-9 phage in water may also be used as a possible indicator of the presence of *S. dysenteriae* type 1. However, it should be noted that live *Shigella* was not isolated from six of nine water samples which were positive for the phage, although the samples were positive for one or more *Shigella*-specific genes as determined by PCR assays. Nevertheless, the presence of the phage indicates the presence of live or dead organisms and may indicate possible fecal contamination of surface water by patients with shigellosis. In addition, since the SF-9 phage was found to be fairly stable at temperatures below 37°C, the presence of SF-9 may also indicate possible fecal contamination of water in the recent past.

At least three periods of epidemic outbreaks of dysentery due to *S. dysenteriae* type 1 were recorded between 1972 and 1994 on the Indian subcontinent (8, 15, 19). However, the mechanism associated with periodic outbreaks of shigellosis and the factors associated with the emergence or decline of epidemics are not clear. Shigellae are generally believed to have only a human or primate host. Although *Shigella*-contaminated food and drink are often the source of epidemic spread, a recent study indicated that *Shigella* can persist and possibly spread through environmental waters (10). This finding has epidemiological significance, particularly since in developing countries with inadequate sanitation, fecal contamination of environmental waters by enteric pathogens is very common. Since phage SF-9 was isolated from a considerable proportion of water samples in the present study, we assume that the presence of *S. dysenteriae* type 1 in surface water is more common than previously appreciated. It is, therefore, important to monitor the presence of *S. dysenteriae* type 1 in the environment to better understand the epidemiology of shigellosis. In a previous study the presence of vibriophages in water was demonstrated to be a potential tool for predicting the presence of *V. cholerae* (16), which causes periodic epidemics of cholera in many developing countries. In view of inadequacies in convenient techniques to detect *Shigella* in environmental samples, monitoring for the presence of bacteriophages specific for *Shigella* may prove to be a useful epidemiological tool for predicting outbreaks and the spread of shigellosis. Detailed analysis of a large number of water samples for the presence of *Shigella* by conventional methods is also impractical. Although PCR is a sensitive detection technique, use of this technique in remote areas for environmental monitoring is not yet practical, particularly in developing countries with inadequate laboratory facilities. The phage-based detection technique, on the other hand, may be easier to perform in such situations and has the potential to be adapted to field settings. The identification of phage SF-9 in this study may contribute significantly to this effort.

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