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Prevalence and Diversity of Coliphages in Dhaka, Bangladesh and Their Lytic Potentials against Pathogenic Bacteria

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Authors' contributions

This work was carried out in collaboration between all authors. Author MAU planned the experiments and supervised the research project. Author SS conducted the experiments, collected the data and produced the first draft of the manuscript, all three authors interpreted and analyzed the data and author RHK produced the final manuscript. All three authors approved the final manuscript.

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ABSTRACT

Aim: Dhaka is a flood-prone city with a high prevalence of diarrhoeal diseases. This study investigated the natural reservoirs and diversity of coliphages in Dhaka to establish phage strains of potential use in antidiarrhoeal therapy.
Materials and Methods: 296 surface water samples and 20 faecal samples of hospitalised diarrhoea patients were collected during April-September 2012 and phages isolated were used in infecting 67 strains of *Escherichia coli*, 20 strains of *Shigella* sp., and 10 strains of *Vibrio cholerae*. Phage isolates were grouped by plaque morphology and representative morphotypes were characterised by restriction fragment length polymorphism (RFLP) analysis and Southern blot hybridisation.
Results: Bacteriophage diversity and titers were higher during the beginning of the flood season (June-August) and lower in other months (April, May, and September), indicating that seasonal run-

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off affects phage diversity and abundance. The 98 phage isolates fell into nine distinct morphotypes designated as coliphage-Dhaka (CPD) 11-19. Host range, RFLP patterns, and the estimated genome size indicated that the morphotypes were distinct except that CPD13 and CPD19 have identical RFLP pattern. Southern blot analysis indicated that all the morphotypes except CPD14 and CPD15 are genetically related. A colony blot hybridisation screening of 500 different colonies of 97 different strains of three different bacterial species indicated that none of the phage isolates is lysogenic. Lytic infection of the 87 bacterial strains indicated that most morphotypes have a limited host range except CPD12 and CPD15. These two morphotypes infected and lysed 30-70% of the test bacterial strains.

Conclusions: Of the nine coliphage morphotypes characterised in this study, CPD12 and CPD15 have the potentials for developing therapeutic phage strains.

Keywords: Bacteriophages; coliphages; plaque morphotypes; phage therapy.

1. INTRODUCTION

Waterborne diarrhoeal diseases are among the major public health concerns in Bangladesh. Over 12 million of the 158 million citizens of the country live in Dhaka, and the population of the capital city increases by 5% per annum [1]. The city is located in a flood-prone area and flooding is an annual routine event in the country. In 2003, about 20% of the households in the municipal areas lacked access to latrines and 28% of the families only had access to unhygienic latrines [2]. The situation may have improved by 2015 but contamination of floodwater by human wastes remained a problem [3]. Many families, especially those living in the slums, depend on potentially faecal bacteria-contaminated surface water harvested by shallow tube wells. Treated municipal supply water is also susceptible to faecal contamination because the water supply infrastructure is frequently compromised by illegal water connections [4]. In recent studies, 58% of the water samples collected from municipal supply water were found positive for coliforms [5], and the coliform counts in water samples harvested at the consumer points were three-fold higher than that of the samples harvested at the supply point [6]. Thus anyone in Dhaka consuming surface or municipal supply water without post-harvest decontamination is potentially vulnerable to infections.

Globally, diarrhoeal diseases cause 1.7 million deaths/year and the second leading cause of deaths among children under five [7]. Diarrhoeal diseases account for 11% of all under-five deaths in Bangladesh and about 140,000 patients with severe diarrhoeal diseases are admitted to the hospitals in Dhaka every year [8]. People suffering from subclinical and chronic diarrhoeal diseases may not seek professional medical care but many of the patients treat the conditions through self-medication. Self-medication and inappropriate use of antibiotics are quite common in Bangladesh [9]. Enterotoxigenic *Escherichia coli* (ETEC), *Shigella* sp., *Salmonella* sp., and *Vibrio cholerae* are the major diarrhoeagenic bacterial pathogens in Bangladesh [10] but many strains of these bacteria have become resistant to the common antibiotics [11]. Bacterial antibiotic resistance has approached a crisis point in developing countries [12] because these nations have high incidences of infectious diseases but the cost of effective antibacterial drugs are getting out of the reach of the poor.

With the looming crisis of bacterial antibiotic resistance, bacteriophage therapy has gained more attention [13-15]. Phage therapy eliminates severe drug side effects, reduces the risk of opportunistic infections, and leaves the resident microbiota of the patient mostly unharmed [16-18]. Unlike chemotherapy, phage therapy is auto-dosing and effective against bacteria that form biofilms [17]. Finally, phage therapy is more cost-effective compared to antibiotic therapy in terms of development, efficacy testing and manufacturing [17]. The aim of the present study is to isolate and characterise coliphages in surface water and human faecal materials in order to establish a phage-based therapeutic agent and an environmental monitoring system for *E. coli* and *Shigella* sp. The results of the present study can be useful in tracing diarrhoea outbreaks, and in understanding evolution of bacteriophages.

2. MATERIALS AND METHODS

2.1 Strains of Bacteria

The reference strains of bacteria were a gift from the Molecular Genetics Laboratory, International

Center for Diarrhoeal Disease Research, Bangladesh (icddr, b). A total of 67 strains of pathogenic and environmental *E. coli* (including the laboratory *Escherichia coli* strains HB101, E190, DH5α, AP34378C1, AN33859, and AQ11806C2), 20 strains of *Shigella* sp., and 10 strains of toxigenic and non-toxigenic *Vibrio cholerae* (Table 1) were used in this study.

Table 1. Showing the strains of bacteria used, and the strains susceptible (+) or resistant (-) to lytic infections by the phages CPD11, 12, 13, 14, 15, 16, 17, 18 and 19

A. Laboratory *E. coli* strains									
Strain ID	CPD11	CPD12	CPD13	CPD14	CPD15	CPD16	CPD17	CPD18	CPD19
DH5α	+	+	-	-	-	-	-	+	+
B. Enterotoxigenic *E. coli* (ETEC) strains									
AP34378C1 (LT+VE)	+	+	-	-	-	-	+	-	+
AP30082C1 (LT+VE)	+	+	+	-	+	+	-	+	-
AP306C1(LT, AE+VE)	+	+	+	-	+	+	-	+	-
ETEC AN33859	+	+	-	-	-	-	+	+	+
E 118814 (LT+VE)	-	+	-	-	-	-	-	-	-
E118820	-	-	-	-	-	-	-	-	-
MQ127C2(LT+VE)	-	-	-	-	-	+	-	-	-
MQ127C1									
MQ1294C1(LT+VE)	-	-	+	-	+	-	-	+	-
MQ185C1(LT,AE+VE)	-	-	+	-	+	-	+	-	-
AQ11930 C2 (LT+VE)	-	-	-	-	-	-	-	-	-
AQ12230	-	-	-	-	-	-	-	-	-
Tx1	-	-	-	-	-	-	-	-	-
C. Enterohemorrhagic *E. coli* (EHEC) strains									
07-18:8227	-	-	-	-	-	-	-	-	-
C0207:7215	-	-	-	-	-	-	-	-	-
T10:7218	-	-	-	-	-	-	-	-	-
EDL 933:7213	+	-	+	-	-	-	-	+	-
EDL933:7200	-	-	-	-	-	-	-	-	-
C600(933J)	-	-	+	+	+	+	+	-	-
C600(933w)	-	-	+	-	-	+	-	-	-
N510	-	-	-	-	-	-	-	-	-
N511	+	+	+	-	+	-	+	+	-
D. Enteroaggregative *E. coli* (EAEC) strains									
JPN10	-	+	-	-	+	+	-	-	-
AN9678C1	-	-	-	-	-	-	-	-	-
AN9678C2	-	-	-	-	-	-	-	-	-
AN9677C1	-	-	-	-	-	-	-	-	-
E. coli 1	+	-	-	-	-	-	-	-	-
E. coli 2	+	+	-	-	+	+	-	-	-
E. coli 3	-	-	-	-	-	-	-	-	-
HB101	+	+	-	+	+	+	+	-	-
E. Enteropathogenic *E. coli* (EPEC) strains									
Strain ID	11	12	13	14	15	16	17	18	19
AQ1180362(AE+VE)	-	-	-	-	-	+	+	-	-
AQ1180365	-	-	-	-	-	-	-	-	-
B170	-	-	-	-	-	-	-	+	-
B171	-	-	-	-	-	-	-	-	-
AQ11827C1	-	-	-	-	-	-	-	-	-
AQ11827C2(AE+VE)	-	-	+	-	-	-	-	+	+
AQ126602 (AE+VE)	-	-	-	-	-	-	-	-	-
F. Environmental/ third generation cephalosporin-resistant *E. coli* strains									
E7	+	+	-	+	+	-	-	-	-
E132	-	+	-	-	-	-	-	-	-
E5	-	-	-	-	-	-	-	-	-
E115	-	-	-	-	-	-	-	-	-
E117	-	-	-	-	-	-	-	-	-
E3	-	+	-	-	-	-	-	-	+
E117	-	-	-	-	-	-	-	-	-

E30	-	+	-	-	-	-	-	-	+
E112	-	-	-	-	-	-	-	-	-
E4	+	+	-	-	-	-	-	-	-
E122	+	+	-	-	-	-	-	-	-
E116	-	-	-	-	-	-	-	-	-
E44	-	-	-	-	-	-	-	-	-
E33	-	-	-	-	-	-	-	-	-
E58	-	-	-	-	-	-	-	-	-
E90	-	-	-	-	-	-	-	-	+
E8	+	+	-	-	-	-	-	-	+
E74	-	-	-	-	-	-	-	-	-
E105	-	-	-	-	-	-	-	-	-
E60	-	-	-	-	-	-	-	-	-
E198/10	+	+	-	+	-	-	-	-	+
E108	-	+	-	+	+	-	-	-	-
E110	-	+	-	+	-	-	-	-	-
E128	-	+	-	+	-	-	-	-	-
E143/10	+	+	-	+	-	+	-	-	+
E199	-	+	-	-	-	-	-	-	-
E142/5	-	-	-	-	-	-	-	-	-
E190	-	+	-	-	-	+	-	-	+
E200	-	-	-	-	-	-	-	-	-
E194/10	-	-	-	-	-	-	-	-	-
G. *Shigella* sp.									
S. dysenteriae TII-26.AF87	-	-	-	-	-	-	-	-	-
S. dysenteriae T1-Vm.1064	-	+	-	-	+	+	-	-	-
S. dysenteriae TII-27.797	-	-	-	+	-	-	-	-	-
S. dysenteriae TII-27.798	-	-	-	+	-	-	-	-	-
S. dysenteriae -12-26868	-	-	-	-	-	-	+	-	+
S. dysenteriae -T2.26868	-	-	-	-	-	-	-	-	-
S. flexneri- 10.15695	-	-	-	-	-	-	-	-	-
S. flexneri -10.295C5	-	+	-	-	+	-	-	-	-
S. flexneri -10.295C1	-	+	-	-	+	-	-	-	-
S. *flexneri* -11.26126	-	-	-	-	+	-	-	-	-
S. boydii- 14.3C426	+	-	-	+	+	-	+	+	+
S. boydii- B14580	-	-	-	-	-	-	-	-	-
S. boydii- B14581	-	-	-	-	-	-	-	-	-
S. boydii- B2.17320	-	-	-	-	+	-	-	-	-
S. boydii-B19a919	-	+	-	-	+	+	+	-	+
S. boydii- B1.20299	-	-	-	-	+	-	-	-	-
S. sonnei-25.19784	-	+	-	-	+	+	-	-	-
S. sonnei-24.4484	-	-	+	-	-	-	-	+	-
S. sonnei- S1.28829	+	+	-	-	+	+	-	-	-
S. sonnei- S2.21377	+	-	-	-	-	-	-	-	-
H. *Vibrio cholerae* strains									
Inaba Classical-0395	-	-	-	-	-	-	-	-	-
Inaba Classical-596B	-	-	-	-	-	-	-	-	-
Inaba Classical- S224	-	-	-	-	-	-	-	-	-
Inaba Classical- L362	-	-	-	-	-	-	-	-	-
Environmental- RV508	-	-	-	-	-	-	-	-	-
Inaba El Tor- N916961	-	-	-	-	-	-	-	-	-
Inaba El Tor- C6706	-	-	-	-	-	-	-	-	-
Inaba El Tor- AF1471	-	-	-	-	-	-	-	-	-
Clinical- AH806	-	-	-	-	-	-	-	-	-
Ogawa El Tor-1471	-	-	-	-	-	-	-	-	-

2.2 Sample Collection

Water samples (100 ml) were collected in sterile glass bottles from nine different sites of two major rivers (the Turag and the Buriganga rivers), a lake (the Gulshan lake), and the sewerages of two hospitals, all located within the greater metropolitan area of Dhaka. Samples were collected from each of the sites once a week throughout the 6 months of the study period (April-September, 2012). Stool samples were collected from the Dhaka Hospital, Mohakhali, Dhaka; during the same study period from patients. Stools samples were acquired after obtaining signed informed consent to participate in the study and allow DNA extraction and analyses in accordance with the Helsinki Declaration. The stool samples (10 g) were suspended in 10 ml of sterile TS broth (0.85% NaCl and 0.01% tryptone, pH 7.3±0.2). All the samples were placed in an icebox and transported to the Advanced Biotechnology Laboratory, University of Dhaka, and were processed for detection of coliphages within three hours of collection.

2.3 Detection and Isolation of Bacteriophages

The environmental samples were cleared of plankton, bacteria, and environmental debris, initially by centrifugation (10,000xg for five minutes), and then by filtration using 0.22 micron filters (Millipore, Watford, UK). The faecal samples were suspended in 10 ml of sterile TS broth and then centrifuged for 15 minutes at 12,000xg to remove debris. The supernatant was filtered using a 0.22 micron filter. The filtrate was immediately used in infecting specific strains of bacteria (*Escherichia coli* strains HB101, E190, DH5α, AP34378C1, AN33859, and AQ11806C2) following standard protocols [19,20]. Briefly, 0.1 ml of the filtrate was spread on a bacterial lawn grown on 5.0 cm Petri dishes. Six Petri dishes were inoculated with each of the phage samples. The 54 plates were sealed with Parafilm and then incubated at 37ºC. Plaques developed on the plates were counted and the phage count per ml of surface water or per mg of faecal materials were determined.

2.4 Extraction of Phage DNA

A susceptible non-lysogenic strain of bacterium (*Escherichia coli* strains AN33859 for CPD11 and CPD17; E190 for CPD12 and CPD19, AP34378C1 for CPD13 and CPD18, DH5α for CPD14, HB101 for CPD15, and AQ11806C2 for CPD16) were grown in appropriate culture broth overnight at 37ºC on an orbital shaker set at 120 rotations/min. A small amount (0.2 ml) of the turbid culture was mixed with 10 ml of fresh broth in a 50 ml conical flask and incubated at 37ºC on the orbital shaker for 3-4 hours till the culture reached the log phase of growth (A_{600}= 0.3-0.6). At that point, $3x10^6$ phage particles (in 0.1 ml) were added to the culture flask and the flask was incubated in the orbital shaker at 37ºC for 9-12 hours till all the bacterial cells were completely lysed. The lysate was transferred to a 50 ml conical tube and centrifuged at 6,000xg for 10 minutes to remove cellular debris, and then pushed through a 0.22 micron filter. The filtrate (10 ml) was transferred to a sterile centrifugation tube and mixed with 2.5 ml of polyethylene glycol (PEG) precipitation solution (20% PEG 6000, 10% NaCl in distilled water). After briefly vortexing, the tube was incubated at 4ºC for two hours for the phage particles to precipitate. The tube was then centrifuged at 12,000xg for 50 minutes at 4ºC. The pellet was dried and then dissolved in 0.5 ml REact 2 buffer (20 mM Tris-Cl pH 7.5, 60 mM KCl, 10 mM $MgCl_2$ and 10 mM NaCl) and transferred into a fresh microfuge tube. The solution was treated with 20 units of pancreatic DNase I and 15 units of RNase A (Life Technologies, Grand Island, NY, USA) at 37 °C for 90 minutes, to remove any contaminating bacterial DNA and RNA. DNase I was inactivated by hating the mixture for 10 minutes at 75°C and the phage capsids were digested with 15 µl Proteinase K (20 mg/ml) enzyme (Life Technologies) in 1x Proteinase K buffer (0.5% SDS and 1.0 mM EDTA) by incubating the mixture at 65ºC for two hours. The mixture was cooled down to room temperature and then extracted with an equal volume of phenol and then with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) (Life Technologies). The aqueous phase was further extracted with pure chloroform and it was separated by centrifugation for 2 minutes at 10,000xg. DNA was precipitated from the aqueous phase by adding two volumes of ice-cold ethanol. The pellet was washed with 70% ethanol and then dried. The pellet was dissolved in a small volume of TE buffer (10 mM Tris-Cl, 1 mM EDTA pH 7.5) and DNA concentration was measured spectrophotometrically before storing the samples at -20ºC.

2.5 Restriction Fragment Length Polymorphism (RFLP) Analysis of Phage DNA

A sample of 1-5 µg of bacteriophage DNA was treated with 5-50 units of *Hin*DIII (New England Biolab, Hitchin, UK) in a total volume of 10-50 µl in the appropriate 1x reaction buffer for overnight at 37ºC. The reaction was stopped by adding 0.5M EDTA pH 8.0 to 1.0 mM, and then DNA loading dye (New England Biolab) was added to the mixture before resolving the DNA fragments along with 1.0 kb DNA ladder (Life Technologies) in 1.0% agarose gels. The gel was stained with ethidium bromide (1.0 µg/ml) and documented using a digital camera.

2.6 Southern Blot Hybridisation

Restriction endonuclease-treated bacteriophage DNA was resolved in 1.0% agarose gels and the bands were transferred onto a nitrocellulose membrane by the capillary transfer method following a standard protocol [20]. The wet membrane was exposed to UV light for two minutes to immobilise the transferred DNA molecules to the membrane. The membrane was washed and dried, and then stored at 4ºC until used. The radiolabeled DNA probes were derived from the whole genome of CPD17. The labeled probes were generated using Amersham Megaprime DNA Labeling System (GE Healthcare, Kolkata, India) in the presence of [α-^{32}P] dCTP following the protocol suggested by the manufacturer. The nitrocellulose membrane with the immobilised phase DNA bands were treated with a pre-hybridisation buffer (20x SSC, 10% SDS, 5% dextran sulfate and 100 µg/ml denatured salmon sperm DNA), and then with the hybridisation buffer (20X SSC, [α-^{32}P] dCTP-labeled denatured probe DNA, 0.5% SDS, 5% dextran sulfate and 100 µg/ml denatured salmon sperm DNA) following a standard protocol [20]. The hybridisation was conducted at 60°C for 12 hours, and then the membrane was washed with wash buffers, initially for 15 minutes with 2x SSC, 0.1% SDS, and then again for 15 minutes using 0.1x SSC, 0.1% SDS as described [20]. Finally the membrane was dried, wrapped in cellophane membrane and exposed to SuperRx X-ray film (Fujifilm, Dhaka, Bangladesh) under an intensifying screen for various time periods to obtain well-developed bands. The film was developed in an in-house facility and documented using a digital camera.

2.7 Colony Blot Hybridisation

The strains of bacteria were grown in appropriate culture medium (agar plates) and specific colonies were transferred onto a new agar plate in a grid. The plate was incubated at 37ºC for overnight and then the colonies were transferred onto a HyBond nylon membrane (Amersham, Ayelesbury, UK) by pressing a disc of the membrane over the plate. The membrane was treated with a denaturing solution (1.5 M NaCl, 0.5 M NaOH) and then with a neutralising solution (1.5 M NaCl, 1.0 M Tris-Cl, pH 8.0), and the liberated DNA was fixed onto the membrane by exposing the membrane to UV light for two minutes. The hybridisation procedure was the same as described in the Section 2.6. A mixture of equal amounts (100 ng each) of the genomic DNA of the nine CPD phages was used in synthesising the DNA probes to be used for colony blot hybridisation.

3. RESULTS

3.1 Phage Plaque Morphotypes

Out of the 296 water samples collected, 95 samples (32.10%) tested positive for the presence of coliphages. A total of 20 stool samples were also analysed during the same study period, and 3 samples (15%) collected in the month of June 2012 were tested coliphage-positive. The 98 bacteriophage isolates were categorised initially by plaque morphology and nine morphotypes designated as coliphage isolates (CPD)11-19 were established (data not shown). The 95 environmental isolates fell into eight plaque morphotypes CPD11-13, 15-19. The three phage isolates obtained from the clinical samples formed one plaque morphotype (CPD14).

3.2 Seasonal Abundance of the Phages

The concentration of the environmental phages varied apparently following a seasonal pattern (Table 2). The lowest abundance of the environmental bacteriophages was observed in the dry season (April and May). Highest phage titers were obtained during the beginning of the flood season (June). CPD14 was detected only in June (Table 2) and only in the clinical samples. Other phages were detected in two or more months during the observation period. CPD13 was detected in five of the six months of the observation period. Six of the nine phages were

detected in June and eight of the nine phages were detected in July and August, the peak of the flood season. Diversity and abundance of the phages dropped substantially by September, the end of the flood season (Table 2).

3.3 Phage Genomes and Interrelationships

Phage genomic DNA extracted from purified phage preparations was digested with *Hin*DIII and the digested DNA was resolved in 1.0% agarose gels (Fig. 1A). The size of the genomes of the nine morphotypes grossly approximated from the RFLP pattern ranged 25-105 kb (data not shown). Southern blot hybridisation using radiolabeled DNA probes derived from the genomic DNA of CPD17 indicated that all the isolates except CPD14 and 15 are related (Fig. 1B).

3.4 Lifestyle of the Phages

To investigate if any of the nine phages were lysogenic, radiolabeled DNA probes derived from a mixture of the nine phages were hybridised with 500 colonies of 97 different environmental and clinical strains of *V. cholerae*, *E. coli* and *Shigella* sp. (Table 1). A non-choleragenic *V. cholerae* strain served as the negative control and the phage-infected *E. coli* cells spotted on the membrane served as the positive control. Genomic DNA of none of the 500 colonies hybridised with the radiolabeled probes although the positive control hybridised with the DNA probe (data not shown), indicating that none of the tested strains of bacteria was a lysogen for any of the phages.

Table 2. The relative abundance (mean pfu/ml ± standard deviation) of the nine phage morphotypes during the months of April 2012 to September 2012

Months	CPD11	CPD12	CPD13	CPD14	CPD15	CPD16	CPD17	CPD18	CPD19
Apr	258±30	0	0	0	0	0	0	0	0
May	174±52	0	224±25	0	0	0	0	0	0
Jun	1±2	816±37	32±22	170±7*	67±21	0	49±8	0	541±50
Jul	21±8	302±218	70±10	0	105±7	42±12	109±50	464±248	198±55
Aug	41±3	299±33	85±5	0	33±19	242±80	56±35	233±44	114±20
Sep	19±6	143±29	107±13	0	1±2	207±116	4±6	257±54	9±14

**- expressed as plaque forming units/mg of faecal materials*

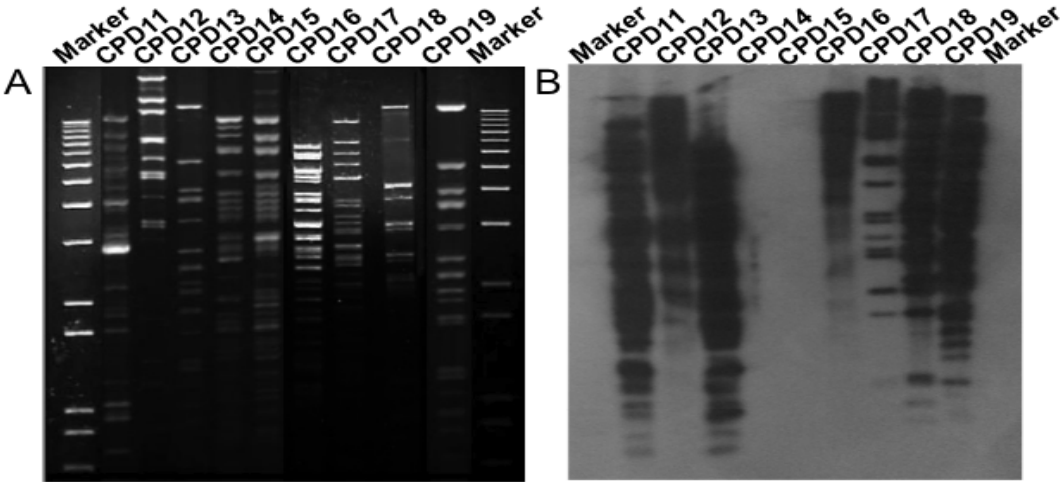

Fig. 1. The RFLP patterns and interrelationships of the nine coliphages. A. RFLP pattern. Shown is a photograph of an agarose gel indicating the DNA band pattern of the nine bacteriophages. The identity of the phage morphotypes is indicated at the top. The DNA samples were digested with *Hin*DIII. B. Interrelationships of the phages. A photoradiograph showing DNA binds hybridised to a radiolabeled DNA probes derived from the whole genome of CPD17. DNA bands of the gel (Fig. 1A) were transferred onto a membrane before hybridising with the radiolabeled DNA probes. The identity of the phages is indicated at the top. Marker- 1 kb DNA ladder

3.5 The Host-range of the Phages

The nine coliphages infected a large number of strains of *E. coli* including the enterotoxigenic *E. coli* (ETEC) strains, enterohemorrhagic *E. coli* (EHEC) strains, enteropathogenic *E. coli* (EPEC) strains and the third generation cephalosporin-resistant environmental *E. coli* strains but none infected any of the *V. cholerae* strains (Table 1). The phage CPD15 infected the highest numbers (36%) and CPD14 infected the lowest (10%) numbers of all of the pathogenic *E. coli* host strains tested (Fig. 2). Some of the phages also infected and lysed many of the environmental *E. coli* strains including several of the third generation cephalosporin-resistant strains. Of the nine phages, CPD12 infected the highest numbers (54%) of all the environmental *E. coli* strains tested, however, two of the strains (CPD13 and CPD17) infected none of the environmental *E. coli* strains (Fig. 2). The coliphages also infected several strains of *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* (data not shown). CPD15 infected and rapidly lysed the highest numbers (69%) of the *Shigella* strains tested (Fig. 2).

4. DISCUSSION

Bacteria and bacteriophages coexist and coevolve in their natural environments. In the natural environments, the ratio of phage counts to bacterial cell count is about 10:1, and the ratio of the numbers of phage species to the bacterial species is about 6-10:1 [21]. Thus every bacterial species serves as the host for multiple phage species. In theory, a lytic bacteriophage can be used to eradicate or significantly reduce the population size of a bacterial strain from a finite environment such as the alimentary canal. Felix d'Herelle, one of the discoverers of bacteriophages, proposed therapeutic / prophylactic use of phages in 1917. Phage therapy is a reality today although some concerns remained [22,23]. The present study screened bacteriophages from environmental and clinical samples to establish suitable phage strains to address a major public health problem of Bangladesh.

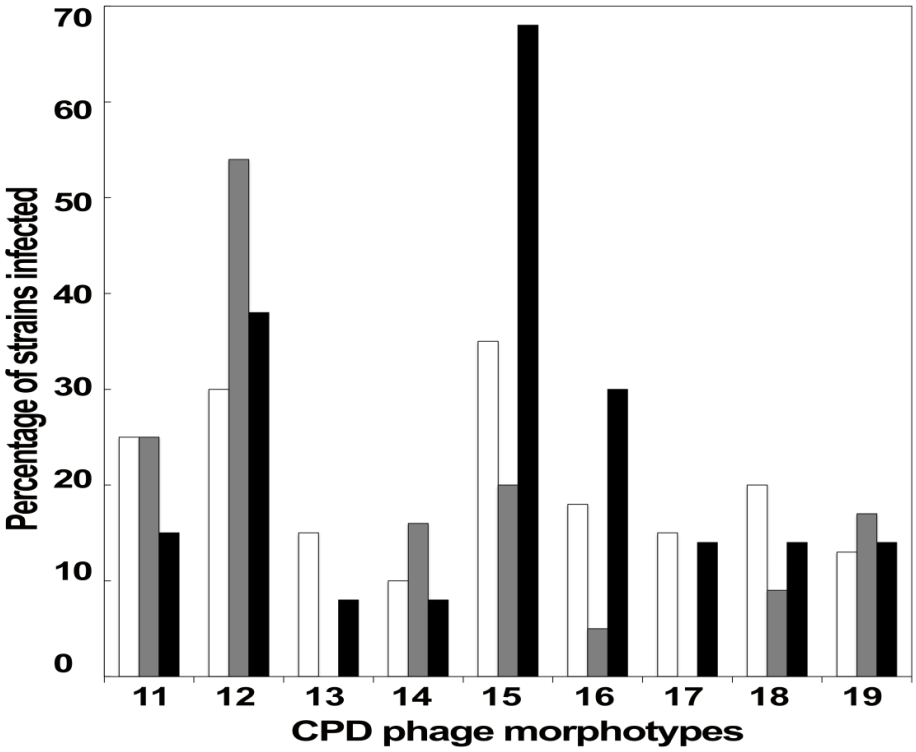

Fig. 2. A graphical overview of the host range of the nine coliphages as evidenced by lytic infection of the bacterial strains by the phages. Shown are the percentages of pathogenic *E. coli* strains (white bars), environment *E. coli* strains (gray bars) and pathogenic strains of *Shigella* sp. (dark bars) the indicated phages infected

This study recovered 98 isolates of lytic bacteriophages including three isolates from clinical samples and the isolates fell into nine plaque morphotypes. Both phage diversity and titers follow a seasonal pattern. The highest phage abundance was observed in June, the early part of the flood season when floodwater volume is low compared to the total volume of runoff water. The phage diversity increased slightly in the next two months but phage titers dropped (Table 1), most likely because of the dilution effect of the huge volume of rainwater. Both phage diversity and titers dropped by the end of the flood season, most likely due to lower runoff and lack of the mixing of the runoff water from various areas. Beside widespread sources of human wastes throughout the city, the surface water of Dhaka is also exposed to runoff from numerous waste dumps, fish markets, fish processing plants, slaughterhouses, and several leather plants, the additional sources of coliforms and coliphages. Other investigators have also observed seasonal variations in the abundance of coliforms and coliphages in the surface water of Dhaka and the surrounding areas [24-26]. Surface water was sampled only in the flood season (which also coincides with increased incidences of diarrhoeal diseases in the city) to maximise the chance of getting the highest numbers of strains of phages. In the dry season (October to March), the rivers and lakes of Dhaka almost dry up and get detached from the runoff, yet incidences of some of the diarrhoeal diseases increase in the dry season [10,24]. The lower count of bacteriophages in surface water is possibly a contributing factor to the dry season outbreaks of diarrhoeal diseases [24]. How bacteriophages survive during the dry season and rapidly increase in titers during the flood season remained to be fully elucidated. It is hypothesised that the phage particles are stable and the high seasonal titers reflect a buildup of the recalcitrant virions [27,28].

Lytic phages generally exhibit characteristic plaque morphology [29]. The nine plaque morphotypes isolated were quite different in host specificity, genome size, and RFLP patterns. However, Southern blot hybridisation using radiolabeled DNA probe derived from the genomic DNA of CPD17 indicated that all of the morphotypes except CPD14 and 15 are related. CPD12 and CPD15 infected the highest numbers of pathogenic and environmental *E. coli* strains and *Shigella* strains. Some of the environmental isolates of *E. coli* are resistant to the third generation cephalosporins. The third generation cephalosporins are the antibiotics of choice in Bangladesh because penicillins and fluoroquinolones are essentially ineffective in the subcontinent but increasing numbers of bacterial strains are becoming resistant to the third generation cephalosporins [30]. If the tendency matures, it will significantly curtail the ability of the poor to access antibiotic therapy. It puts more impetus on developing phage therapy but the therapy must be effective, easily deliverable, and price-competitive. Phage therapy is generally more cost effective in terms of the discovery phase, clinical trial, and manufacturing compared to chemotherapeutic agents [17]. The present study indicates that CPD12 and CPD15 are potential candidates for developing therapeutic strains. A recent study indicates that some *Shigella* strains have become multidrug-resistant [30]. The present study indicates that CPD11 and CPD16 infected many of the *Shigella* strains.

Lysogeny may limit the host range of the therapeutic phages [31]. The present study tested 500 different colonies of 98 different pathogenic and environmental strains including strains that were resistant to lytic infections by the nine coliphages but failed to identify any lysogens. It is possible that the Southern blot hybridisation was insufficient to detect single-copy genomic prophage. Studies are underway to conduct genome sequencing and polymerase chain reaction analyses to completely identify the viruses, and further investigate if any of the nine isolates of the bacteriophages is capable of lysogenic infection.

5. CONCLUSIONS

The present study characterised nine different morphotypes of coliphages from the environmental and clinical samples obtained at Dhaka city. The bacteriophage diversity and titers varied following a seasonal pattern, with higher diversity and titers during the early part of the annual flood season (June-August), indicating that the seasonal runoff carrying coliforms and coliphages from diverse sources to the drainages affect phage diversity and abundance. Molecular analysis indicated that most of the coliphage morphotypes are related and all the morphotypes are strictly lytic phages. Lytic infection of 87 pathogenic, environmental and laboratory stains of *E. coli*, *Shigella* sp., and *V. cholerae* indicated that most morphotypes have a limited host range, although two of the isolates infected and lysed 30-70% of the tested bacterial strains. The two strains could be

genetically manipulated to derive bacteriophages of therapeutic applications.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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