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Isolation and characterization of bacteriophages against enterotoxigenic *Escherichia coli* strains causing swine diseases

Nguyen Xuan Hoa[1,2], Fang Tang[1], Qinqin Bai[1], Chanjuan Shen[1], Thanh Thin Vo[3] and Chengping Lu[1,*]

[1]Department of Microbiology and Immunology, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China.
[2]Hue University of Agriculture and Forestry, 102 Phung Hung St, Hue, Vietnam.
[3]Central Vietnam Veterinary Institute, km4 Dong De Street, Nha Trang, Vietnam.

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Six lytic bacteriophages (JH1-JH6) lytic against O8 ETEC were isolated from 40 individual fecal samples and 30 sewage samples from pig farms in Nanjing, China. Two of the bacteriophages (JH1 and JH2) with icosahedral heads, necks, contractile tails belonged to the *Myoviridae* family. The other four bacteriophages (JH3, JH4, JH5 and JH6) had relatively thin, long, non-contractile and belonged to the *Siphoviridae* family. Unlike typical *Siphoviridae* family members' morphologyes had flexible tails, whereas JH5 had the straight tails and fibers. All bacteriophages produced clear plaques in double agar, with JH6 producing the largest plaques. Phages JH1 to JH6 lysed 50 to 100% of 12 O8: F18 ETEC strains, 50 to 75% of 20 O8:F4 ETEC, 5 to 42.5% of 40 non-O8 ETEC and 4.76 to 9.52% of 21 chickens *E. coli* strains. No lysis of *E. coli* strain from cows, as well as *Escherichia coli* in other O serogroups (O2, O5, O9, O11, O138, O139, O141 and O149), was observed. All bacteriophages titers were unaffected by exposure to pH 5 to 9 for 16 h. Sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) obtained from proteins was extracted from the various bacteriophages. The proteomes of JH1 and JH2 appear to be identical while that of the other four phages are all different indicating they are probably different. These six bacteriophages are potential candidates for prophylaxis and therapeutic strategies against diarrhea in piglets caused by O8 ETEC.

Key words: O8 serogroup, bacteriophage, enterotoxigenic, *Escherichia coli*, diarrhea in piglets.

INTRODUCTION

Bacteriophages are diverse organisms in our world and exert a major influence over the microbial world (RW et al., 1999). While phages have been proposed for the treatment of bacterial diseases (Abhilash et al., 2009), the nature of phage-host interactions, poor understanding of mechanisms of bacterial pathogenesis and introduction of antibiotics have hampered the investigations into their role in therapy (Soothill, 1992). Antibiotics have been commonly used in the treatment of infectious diseases, but their wide spread and improper use has led to antibiotic resistance in porcine colibacillosis (Lu and Koeris, 2011). Recently, scientists have reported on the isolation and application of phage in the treatment of animals with resistant *Escherichia coli* infections (Jamalludeen et al., 2009a).

The goal of this present study was to isolate phages with lytic activity against enterotoxigenic *E. coli* (ETEC) serotype O8, to characterize their morphology and biologi-

*Corresponding author. E-mail: lucp@njau.edu.cn. Tel: +86 25 84396517. Fax: +86 25 84396517.

cal file.

MATERIALS AND METHODS

Bacterial strains

Six strains: VN1-O8: F18 ETEC, VN2- O8: F4 ETEC, VN3-O8:F4 STEC, VN4-O8:F18 ETEC, VN5-O8 ETEC and VN6-O8 ETEC were isolated from Vietnamese pig farms. Eight strains: O2 ETEC, O5 ETEC, O9 ETEC, O11 ETEC, O138 ETEC, O139 ETEC, O141 ETEC and O149 ETEC *E. coli* chicken (21 strains) and *E. coli* bovin (20 strains) were obtained from the Central Vietnam Veterinary Institute and the Department of Microbiology and Immunology, Nanjing Agricultural University, China.

Media and chemicals

Luria Bertani (LB) broth, LB agarose, and LB top agarose (soft agarose) were prepared as described by Sambrook et al. (1989). Each litre of bacteriophage broth contained trypton 10 g, yeast extract 5 g and NaCl 10 g, pH 7.5 and TS buffer (8.5 g of NaCl and 1 g tryptone per litre). The following reagents were also used: RNase I, DNase I (Roche, Basel, Switzerland, cat no: 10104159001), proteinase K, and ethidium bromide (Invitrogen, Carlsbad, CA, USA).

Bacteriophage isolation and purification

Bacteriophages were isolated from 40 individual fecal samples and 30 sewage samples from four Nanjing pig farms (during the period of October 2011 to Jun 2012). LB broth was inoculated with a mixture of equal proportions of the six *E. coli* VN1-O8: F18 ETEC, VN2- O8: F4 ETEC, VN3-O8:F4 STEC, VN4-O8:F18 ETEC, VN5-O8 ETEC and VN6-O8 ETEC strains and incubated for 5 h at 37°C. The samples (fecal sample in TS buffer, or sewage samples) were centrifuge the samples before filtering through a membrane filtered (0.45-µm membrane) to remove impurities and bacteria before being added to the host suspension. Twenty milliliters of LB broth and 20 ml of a suspension of *E. coli* strains in broth culture (OD600 = 1.4) and sample were then added to the flask incubated at 37°C for 24 h shaking to enrich *E. coli* bacteriophages. After incubation, the culture was centrifuged twice at 4,000 × *g* for 15 min at 4°C, the supernatant was collected into a sterile flask and filtered through a sterile 0.45-µm membrane filter (Fisher Scientific). To detect the presence of phage in the filtrate, spot testing was perfored as described previously (Kropinski et al., 2009). Phage preparation were obtained as described (Jamalludeen et al., 2009b) elsewhere and stored at 4°C.

Electron microscopy

Phage pareparations were applied to a carbon film and fixed to a copper grid being negatively stained with phosphotungstic acid (PTA, 2% w/v). Electron micrographs were taken with an H¯7650 (HITACHI, Japan) transmission electron microscope (TEM) operating at 80 kV. Both phage morphology and dimension were capsid in diameter and having a lengthy tail (Bai et al., 2013).

Host range assay

One hundred and twenty one (121) *E. coli* strains were tested in this study, including 12 strains of O8:F18 ETEC, 20 strains of O8:H7 ETEC and 40 strains of non-O8 *E. coli* piglet strains. Eight (O2 ETEC, O5 ETEC, O9 ETEC, O11 ETEC, O138 ETEC, O139 ETEC, O141 ETEC and O149 ETEC), 21 *E. coli* chicken and 20 *E. coli* bovin strains were used to test the spectrum of virulence of phages JH1 to JH6 according to the spot test procedure previously described (Kutter, 2009).

Extraction of bacteriophage DNA

Bacteriophage DNA was extracted as described previously (Pickard, 2009; Sambrook et al., 1989). All bacteriophages were allowed to completely lyse host *E. coli* strains in a soft agar overlay. The overlay was added to SM buffer, and bacteriophages were allowed to diffuse into the buffer at 4°C for 3 to 4 h with gentle shaking as previously described. After the suspension was centrifuged at 4000 × *g* for 15 min, the supernatant was collected. Solid NaCl was added to a final concentration of 1 M and dissolved by swirling. Following incubation on ice for 1 h, the suspension was centrifuged at 11,000 × *g* for 10 min at 4°C (Beckman Coulter, J2-MC Centrifuge). The supernatant was collected, solid polyethylene glycol (PEG 8000) was added to a final concentration of 10% (w/v), and the mixture was stirred slowly at room temperature. After cooling in ice water and left to stand for 1 h on ice, the mixture was centrifuged at 14,000 × *g* for 10 min at 4°C. The bacteriophage pellet was then resuspended in 1 mL of SM buffer. An equal volume of chloroform was added to the phage suspension and mixed by vortexing for 30 s. The phages were separated by centrifugation at 3000 × *g* for 15 min at 4°C and the aqueous phages was recovered. Pancreatic DNase I and RNase I were added to a final concentration of 5 and 1 µg/mL, respectively, and allowed to digest substrates for 30 min at 37°C. EDTA, pH 8.0 was added to a final concentration of 20 mM. Proteinase K was added to a final concentration of 50 µg/mL, then sodium dodecyl sulfate (SDS, 10%) was added to a final concentration of 0.5% and the mixture was inverted several times prior to incubation at 56°C for 2 h. An equal volume of phenol: chloroform:isoamyl alcohol (25:24:1, v/v/v) was mixed into the sample. The aqueous phase was collected after centrifugation at 10,000 × *g* for 10 min and extracted with an equal volume of chloroform:isoamyl alcohol (24:1, v/v). Centrifugation was repeated and the aqueous phase was collected. Two volumes of ice-cold 95% ethanol were added and the mixture was kept at room temperature for 20 mins. The precipitate was collected by centrifugation at 10,000 × *g* for 10 min at 4°C and the pellet was washed with cold 70% ethanol. Following centrifugation at 10,000 × *g* at 4°C for 30 min the pellet was air dried and dissolved in 20 to 35 µL Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

Assessment of bacteriophage resistance to acidity and alkalinity

Resistance to acidity and alkalinity in suspensions of each of the six bacteriophages were evaluated by exposure to various pH conditions ranging from 1 to 11 and checked for survival over a 16-h period as described by Jamalludeen et al. (2007). In brief, a 100-µl bacteriophage suspension 10^7 (pfu/ml) and 900 µl of normal saline, pH 7.2, was also incubated at 37°C for 16 h. After incubation, a 100-µl volume of the bacteriophage suspension was serially diluted 10-fold, mixed with 100 µl of host bacterium (10^8 cfu/ml) and incubated for 15 min at 37°C before being added to 4 ml of soft agar and spread over an LB plate. Titers of the surviving bacteriophage were determined by plaque assays with 10-fold dilutions using the soft agar overlay method.

Agarose gel electrophoresis analysis of bacteriophage DNA

Genome sizes of undigested or digested bacteriophage were

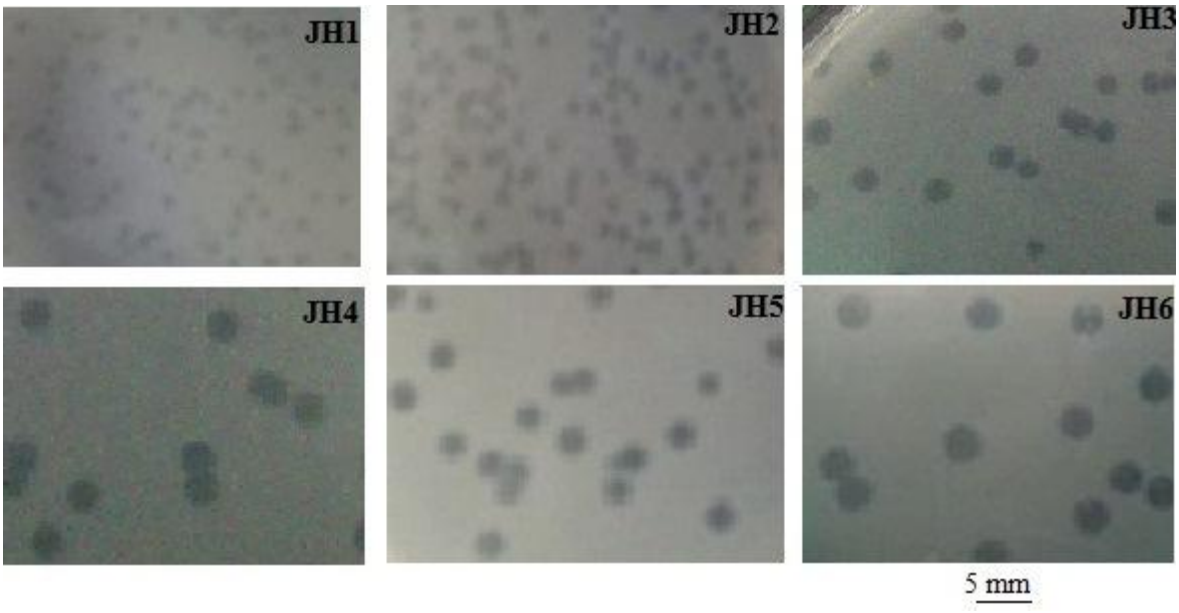

Figure 1. Photograph of plaques produced by phages JH1-JH6 on lawns *E. coli* VN1-VN6.

determined by electrophoresis (Sambrook and Russell, 2001). The bands were stained with ethidium bromide (EB) and compared against a λ phage DNA digested with *Hind* III marker; where indicated, purified bacteriophage DNA samples were digested with restriction enzyme *Dra* I before comparison by gel electrophoresis (Chakrabarti et al., 2000).

Protein of purified particles of phage

Bacteriophages particles by centrifugation through a glycerol step gradient were determined by Sambrook and Russell. (2001) after purified particles were subjected to SDS-PAGE on precast 4 to 15% gradient TRIS acrylamidegels (BioRad) along with protein molecular weight markers (Kropinski et al., 2012). The phage suspensions (approximately 10^{10} pfu/ml) were boiled for 5 min and separated by SDS-PAGE loading buffer (50 mM Tris-HCl, 3% SDS, 1% β-mercaptoethanol, 20% glycerol, 0.7% bromophenol blue pH 6.8) on 12.5% acrylamide gel. Electrophoresis was initiated at 80 V until samples had run through the stacking gel (approximately 30 min). The voltage was subsequently increased to 120 V and electrophoresis was continued until the tracking dye had reached the bottom of the gel (approximately 2 h). Proteins were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich).

RESULTS

Isolation of bacteriophages

Six bacteriophages were isolated using a mixture of *E. coli* causing piglet diseases strains as hosts. The bacteriophages (designated JH1, JH2, JH3, JH4, JH5 and JH6) were characterized according to their morphological features. Two bacteriophages (JH5 and JH6) were isolated from sewage samples (5%) and four bacteriophages (JH1, JH2, JH3 and JH4) were isolated from raw fecal samples (13%) from Nanjing pig farms.

Plaque characteristics and morphology of bacteriophage

All bacteriophages produced similar plaques that were clear and medium-sized, ranging from 1.5 to 3.5 mm in diameter. The approximate diameter of plaques formed by the bacteriophages was: JH1 and JH2, 1.5 mm; JH3 and JH4, 2.5 mm; JH5, 2 mm; JH6, 3.5 mm (Figure 1). Electron microscopy confirmed that two of the six bacteriophages (Figure 2); JH1 and JH2 showed icosahedral heads, necks and contractile tails belonged to the *Myoviridae* family. The other four bacteriophages (JH3, JH4, JH5 and JH6) had relatively thin, long, non-contractile and belonged to the *Siphoviridae* family. Unlike typical *Siphoviridae* family members' morphologyes had flexible tails, whereas JH5 had the straight tails and fibers (Ackermann, 2009). All six lytic bacteriophages (JH1 to JH6) belonged to the order *Caudovirales* (Ackermann, 2011). Table 1 shows the measurements of the heads and tails of the bacterio-phage obtained from electron microscopic images. The diameter of its capsid was estimated at ranging from 50±1.58 to 75 ±0.84; while all of the bacteriophages had long tails, ranging from 90 to 160 nm.

Nucleic acid of bacteriophages

Nucleic acid of all the six bacteriophages had a DNA. All the bacteriophages DNA samples were digested with restriction enzyme *Dra* I (Figure 3).

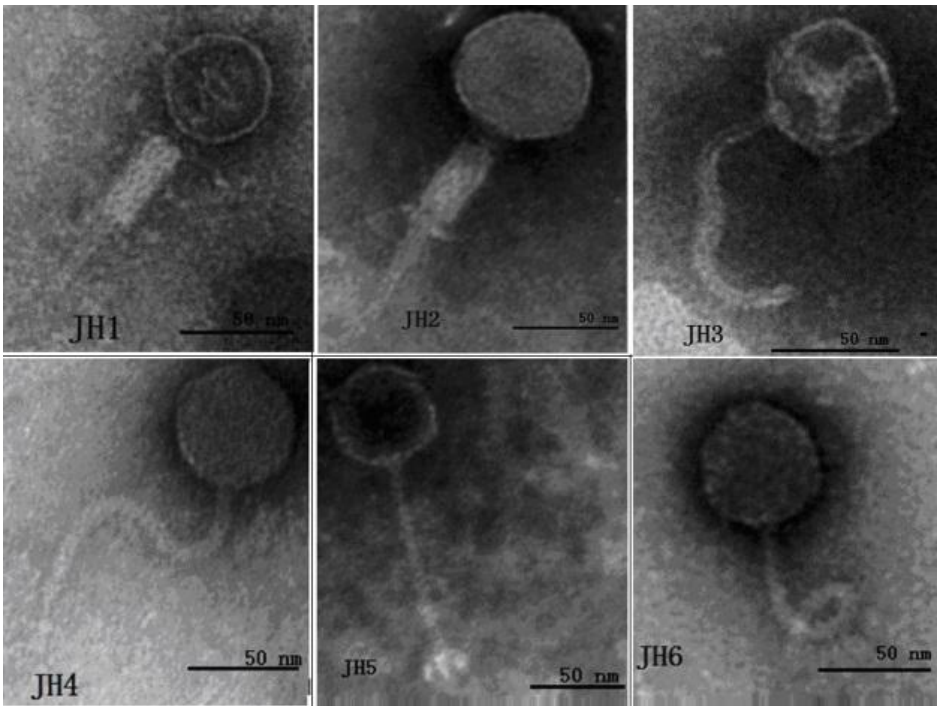

Figure 2. Morphological examination of phages by TEM.

Table 1. Estimated dimensions[a] and genome sizes[b] of six bacteriophages from pig sewage or feces.

Phage	Head dimension (nm)		Tail dimension (nm)	
	Length	Width	Length	Width
JH1	50±1.58	50±1.14	90±4.12	20±1.58
JH2	75±0.84	70±3.19	100±2.24	22±1.58
JH3	50±1.3	52±1.3	140±1.92	10±0.71
JH4	60±0.89	60±0.17	150±1.58	10±0.84
JH5	60±0.71	58±1.14	120±3.61	10±0.71
JH6	60±1	55±2.3	160±3.54	10±1.41

[a]Determined from TEM micrographs of negatively stained phage particles from concentrated phage lysates. [b]Determined by restriction endonuclease digestion and electrophoresis.

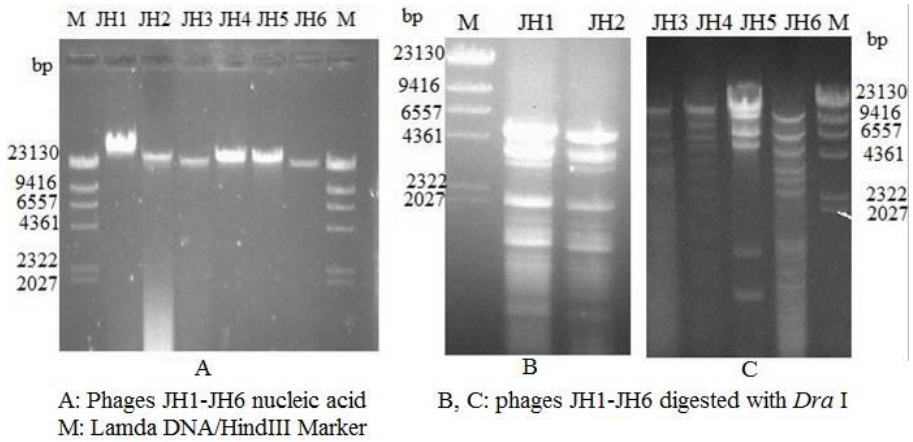

Figure 3. Electrophoresis of DNA of phages JH1 to JH6.

Table 2. Summary of lytic activity of six bacteriophages against porcine O8 ETEC and other *E. coli* strains.

E. coli strain (*n*)	Phage activity (% of strains lysed)					
	JH1	JH2	JH3	JH4	JH5	JH6
O8:F18 ETEC (12)*	50.00	83.33	58.33	83.33	75.00	100
O8:F4 ETEC (20)*	50.00	60.00	75.00	55.00	50.00	75.00
Non-O8 ETEC (40)*	20	12.5	10	12.5	42.5	5
E. coli chicken (21)[a]*	0	9.52	4.76	0	0	0
E. coli bovine (20)[a]*	0	0	0	0	0	0
(O2, O5, O9, O11, O138, O139, O141 and O149)(8)*[b]	0	0	0	0	0	0

*Strain number. [a]Obtained Department of Microbiology and Immunology, Nanjing Agricultural University, China. [b]Obtained from the Central Vietnam Veterinary Institute.

Table 3. Survival of phages JH1-JH6 following exposure to varying pH levels (1 to 11).

pH	Titre of surviving, viable phage (pfu/ml)					
	JH1	JH2	JH3	JH4	JH5	JH6
1 and 2	ND	ND	ND	ND	ND	ND
3	10^3	10^3	10^3	10^4	10^2	10^4
4	10^5	10^4	10^5	10^5	10^5	10^5
5-9	10^7	10^7	10^7	10^7	10^7	10^7
10	10^5	10^4	10^5	10^6	10^5	10^5
11	10^3	10^2	10^3	10^3	10^2	10^3
Control	10^7	10^7	10^7	10^7	10^7	10^7

ND, Not detected.

Host range

The results show that bacteriophages JH1 to JH6 could lyse 50 to 100% of the 12 O8:F18 ETEC/STEC, 50-75% of the 20 O8:F18 ETEC, 5 to 42.5% of the non-O8 porcine ETEC and 0 to 9.52% of *E. coli* in chickens. No lysis of *E. coli* strain from cows, as well as *E. coli* in other O serogroups (O2, O5, O9, O11, O138, O139, O141 and O149) was observed (Table 2).

Bacteriophage resistance to acidity and alkalinity

Survival of bacteriophages over the range of pH 1 to 11 is shown in Table 3. The six bacteriophages were highly susceptible to acidity at pH 1 to 2 and susceptible in varying degrees to overnight exposure to pH 3 to 4. All of the bacteriophages were resistant to the range of pH 5 to 9. Bacteriophages JH4 and JH6 appeared to be slightly more resistant to acidic conditions than the others, as they survived at pH 3.

Protein of purified particles of phage

Distinct profiles of the particles were observed among the six bacteriophages tested (Figure 4). Bacteriophage particles derived from two of the bacteriophages (JH1 and JH2) contained four abundant protein bands with approximate molecular sizes of 92, 55, 43 and 9 kDa; lanes 1 and 2). Two bacteriophages (JH3 and JH4) consisted of three abundant proteins with estimated molecular sizes of 40, 38 and 34 kDa (Figure 4, lanes 3 and 4). The remaining two bacteriophages consisted of three abundant proteins with slightly different estimated molecular sizes (JH5, 32, 26 and 15 kDa; JH6, 32, 28 and 20 kDa) (Figure 4, lanes 5 and 6).

DISCUSSION

The goal of this study was to isolate and characterize bacteriophages against O8 *E. coli*, with special emphasis on those infecting O8:F18 ETEC, which were reported to be the most common in feces. In this study, sewage was collected from waste drainage of entire pig farms, which was considered to be a good source from which to isolate bacteriophages against O8 ETEC, especially since these farms had a record of post-weaning *E. coli*-related diarrhea. The percentages of bacteriophages isolated from sewage samples and fecal were 13 and 5%, respectively. This difference may be likely due to the pooling of feces from large numbers of animals that occurs in sewage and the use of 100 ml sewage samples

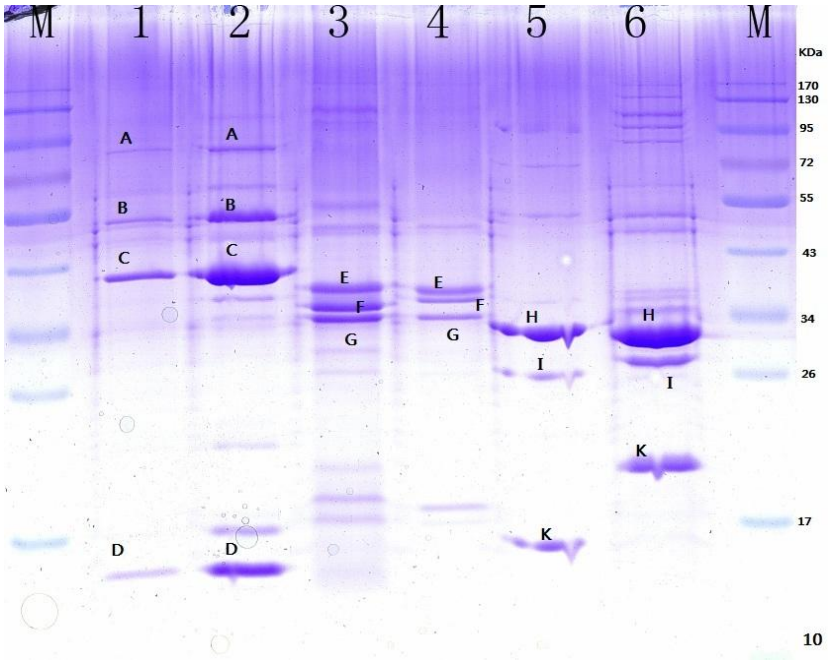

Figure 4. SDS-PAGE profiles of structural proteins of O8 *E. coli* bacteriophages JH1-JH6. Lane M, High molecular weight protein marker; lane 1, JH1; lane 2, JH2; lane 3, JH3; lane 4, JH4; lane 5, JH5; lane 6, JH6.

Table 4. Survival of phages JH1–JH6 following exposure to varying pH levels (1 to 11)

pH	Titre of surviving, viable phage (pfu/ml)					
	JH1	**JH2**	**JH3**	**JH4**	**JH5**	**JH6**
1 and 2	ND	ND	ND	ND	ND	ND
3	10^3	10^3	10^3	10^4	10^2	10^4
4	10^5	10^4	10^5	10^5	10^5	10^5
5-9	10^7	10^7	10^7	10^7	10^7	10^7
10	10^5	10^4	10^5	10^6	10^5	10^5
11	10^3	10^2	10^3	10^3	10^2	10^3
Control	10^7	10^7	10^7	10^7	10^7	10^7

ND: not detected

versus 5 g feces samples. In this study, the rates of recovery of bacteriophages from sewage samples were 50% from March until June and 16.7% in November and December suggesting a substantial seasonal effect on the isolation of bacteriophages. Furthermore, higher isolation rates in the warmer months have been reported for other bacteriophages (Comeau and Krisch, 2005).

Morphological characteristics seen under an electron microscope are considered important in bacteriophage taxonomy and used to identify 96% of all bacteriophages investigated in the last 45 years, especially members of the *Siphoviridae*, *Myoviridae* and *Podoviridae* families (Kumari et al., 2009). Of the six isolated bacteriophages (JH1, JH2, JH3, JH4, JH5 and JH6) which lysed O8 *E. coli* in this study, JH1 and JH2 were determined to be members of the *Myoviridae* family based on their morphological features and their contractile tails. This family consists of six genera, characterized by having icosahedral or elongated heads and contractile tails that were more or less rigid, long and relatively thick (80 to 455 nm × 16 to 20 nm). The four bacteriophages (JH3, JH4, JH5 and JH6) were found to belong to the *Siphoviridae* family of bacteriophages, each consisting of a head, a tail and no envelope.

The six bacteriophages were highly susceptible to acidity at pH 2 and susceptible to varying degrees to overnight exposure to pH 3 to 4 and ability to survive well over the pH range between 5 to 9 is a common feature in bacteriophages. JH4 and JH6 bacteriophage were susceptible to low pH while bacteriophage JH5 was susceptible than the others. Hazem (2002) had reported that bacteriophages are often quite sensitive to protein

denaturation in an acidic environment, which may result in a loss of viability of the bacteriophages. He found that many bacteriophages are stable at pH 3 or 4. The pH in the stomach of weaned pigs may be as low as one to two before a meal and may rise quickly to four to five after the meal, depending on the diet and the feeding regime (Snoeck et al., 2004). The six bacteriophages will likely undergo a marked reduction in titer following oral administration to pigs unless steps are taken to reduce their exposure to low pH in the stomach and upper small intestine (Jamalludeen et al., 2007).

Conclusion

Among the six bacteriophages isolated and characterized in this study, JH1 was related to JH2, and JH3 was related to JH4, based on the spectrum of activity against O8:F18 ETEC, morphology and restriction enzyme digestion patterns. These bacteriophages all exhibited high activity against O8:F18 ETEC and genomic analyses will provide further indication of the extent of their relationships. The information gained from the present study provides a basis for selection of bacteriophages for their potential therapeutic and prophylactic use against O8:F18 ETEC infections in piglets.

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