

Full Length Research Paper

Isolation and characterization of two T4-like bacteriophages against pathogenic *Escherichia coli* of piglet

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This study, has described the isolation of two T4-like JH11 and JH14 phages. All bacteriophages produced clear plaques in double Luria Bertani (LB) agar, but JH11 produced the larger plaques. These phages lysed *Escherichia coli* O141: F18 (ETEC), they have icosahedral heads, necks and contractile tails, and belonged to the *Myoviridae* family. The genomes of these phages were a strand DNA, predicted to be larger than 2 kb. Titer of the two phages were unaffected by exposure to pH 5 - 9 for 16 h. Among the phages, JH11 and JH14 were resistance to enterotoxigenic *E. coli* O141: F18 (ETEC). Both phages contain three abundant protein bands with approximate molecular sizes of 80, 75 and 50 kDa for JH11, and 50, 38, and 36 kDa for JH14. According to the blast results, the phage JH11 random clones are in close relation with phage T4 gene 23 for major capsid protein MVSS (98%), ECML-134 (98%), T6(97%) and T4 (96% identity) identity. Phage JH14 random clones are in close relation with HX01 (96%), CEV1 (95%) and T4 (93%) identity. The two strains belong to T4-like phage genus. Gene of JH11 and JH14 were (93%) identical.

Key words: Bacteriophage, enterotoxigenic, *Escherichia coli*, T4-like phage.

INTRODUCTION

Bacteriophages or phages are enteric viruses that can replicate only inside susceptible bacteria, There are diverse forms of organisms in the world and exert a major influence on the microbial world (Hendrix et al., 1999). While phages have been proposed for the treatment of

bacterial diseases (Barrow et al., 1998), the nature of phage-host interactions, poor understanding of mechanisms of bacterial pathogenesis and introduction of antibiotics have hampered the investigations of their role in therapy (Soothill, 1992). Enterotoxigenic *Escherichia*

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coli (ETEC) are a cause of porcine diarrhea, that may occur sporadically or as outbreaks leading to substantial economic losses (Amezcuca et al., 2002). 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). Nevertheless, the potential use of phages as therapeutic agents in controlling human and animal disease has been recognized (Carey et al., 2006). Recently, there have been report on the isolation and application of phage in the treatment of animals with resistant *E. coli* infections (Jamalludeen et al., 2007). Bacteriophages were used to prevent and treat O149 colibacillosis in pig as also been explored by Jamalludeen et al. (2009a).

The goal of the present study was to isolate phages with lytic activity against enterotoxigenic *E. coli* (ETEC) and to characterize them according to their morphology, sequence, genome size, etc.

MATERIALS AND METHODS

Bacteria strain

E. coli strains (n=31) were isolated from pig farms located in various regions of central Vietnam. All strains were kept at -80°C Luria broth (LB) with 20% glycerol for preservation.

Samples

The bacteriophages were isolated from 30 individual fecal samples, collected from two pig farms located in Jiangsu Province of China farms (during the period of October 2011 to Jun 2012).

Media and chemicals

Luria Bertani (LB) broth, LB agar, and LB top agar (soft agar) were prepared as described by Sambrook et al. (1989). Each litre of bacteriophage broth contained tryptone 10 g, yeast extracts 5 g, and NaCl 10 g, pH 7.5. 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

LB broth was inoculated with mixture equal proportions of the VN11-O141:F18 ETEC and VN14-O141: F18 ETEC strains and incubated for 5 h at 37°C. The samples (5 g of fecal sample from pig farm in TS buffer) were centrifuged 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 10 ml of sample were then added to the flask incubated at 37°C for 24 h in a shaker to enrich *E. coli* bacteriophages. After incubation, the culture was added NaCl 5% for 30 min at 4°C, 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 performed as described previously by Kropinski et al. (2009). Phage preparations were obtained and stored at 4°C as described by Jamalludeen et al. (2009b).

Electron microscopy

Phage preparations 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 (capsid diameter and tail length) are as described by Bai et al., (2013).

Host range analysis and stability assays

To investigate the sensitivity of *E. coli* strains to phage JH11 and JH14, 31 piglet isolates (VN1-31), 10 bovine isolates (JV1-10) and 10 chicken strains (LYT 15-25) were tested as described elsewhere (Jamalludeen et al., 2009b).

One-step growth curve and adsorption of bacteriophage

The phage adsorption assay was carried out according to Shlyapnikov et al. (1984, 1985). 1 ml of phage in warmed water (37°C) suspension ($1-3 \times 10^5$ pfu/ml) was added in 9 ml of mid-log-phase bacterial culture and incubated at 37°C. At 2.5-min intervals, aliquots of 0.05 ml were removed and placed in chilled tubes containing 0.95 ml medium. Phage-cell complexes were removed by centrifugation (10,000 ×g, 10 min), and the titer of free un-adsorbed phage in the supernatant was then determined by the double-layer agar plate method (Kropinski et al., 2009). The one-step growth assay was carried out as described by Pajunen et al. (2002). In brief, 10 mL of the culture (containing 2×10^8 cfu/ml) was infected at a multiplicity of infection (MOI) of 0.1. Following incubation at 37°C for 2.5 min, the bacteria-phage mixture was diluted by 10^4 -fold to abruptly end adsorption. Samples were taken after various inoculation times and centrifuged at 10,000 ×g for 2 min, and the phage titer of the supernatant was determined by the double-layer agar plate method (Kropinski et al., 2009).

Assessment of bacteriophage resistance to acidity and alkalinity

Resistance to acidity and alkalinity in suspensions of each of the two 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). 100-µl bacteriophage suspension 107 (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.

Extraction of bacteriophage DNA

Bacteriophage DNA was extracted as described by Pickard (2009) and Sambrook et al. (1989). All bacteriophages were allowed to

completely lyse their 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 3-4 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 standing 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 phases were separated by centrifugation at 3000 ×g for 15 min at 4°C and the aqueous phase 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 in 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 min. 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 and 4°C for 30 min, the pellet was air dried and dissolved in 20-35 µL TE buffer (10 mM Tris, 1 mM EDTA, pH 8).

Agarose gel electrophoresis analysis

Genome sizes of undigested or digested bacteriophage were 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 and restriction enzyme *Dra* I before comparison by gel electrophoresis (Chakrabarti et al., 2000).

Protein of purified particles of phage

Bacteriophages particles were purified by centrifugation through a glycerol step gradient as determined by Sambrook and Russell (2001) after purified particles were subjected to SDS-PAGE on precast 4-15% gradient TRIS acrylamide gels (BioRad) along with protein molecular weight markers (Kropinski et al., 2012). The phage suspensions (approximately 10¹⁰ pfu/ml) were boiled for 5 min and separated by SDS-PAGE.

Denaturation, annealing and extension

The PCR and oligonucleotide primers were as described by Olsetart et al. (2001). The consensus primers that were used to amplify the central portion of gene gp23 of the various T4-type phages were: Mzia1 (5'-TGTTATATIGGTATGGTICGICGTGCTAT-3') and (5'-TGAAGT TACCTTACCACGACCG-3'). The primers that were initially used

to amplify the gens gp18 analogue of the T4-type phage were: FT18-N2 (5'-GGTAAATCCAATGGGGTCCAGCT T-3') and (5'-TATCAGCAGCCAACGGAACC CAA-3').

PCR sequencing

The PCR products were purified (Casjens et al., 2004) and sequenced with an Amersham Life Science Thermo Sequenase kit. The gene 18 and 23 nucleotide sequences of the various T4-type phages were determined by Mzia1 and FT18-N2 primer.

RESULTS

Bacteriophage isolation and morphology

Two bacteriophages were isolated from fecal sample and, named JH11 and JH14. All the bacteriophage produced similar plaques that were clear and medium sized 3.0-3.5 mm in diameter (Figure 1).

Electron microscopy confirmed that phages JH11 and JH14 belong to the *Myoviridae* family. Phages possessed icosahedral heads, necks and contractile tails, with tail fibers. JH11 and JH14 belong to the order *Caudovirales*. The head dimensions for JH11 and JH14 were 120 × 70 and 100 × 80 nm, and tail dimensions were 100 × 25 and 130 × 30 nm, respectively (Figure 2).

Host range analysis and stability assays

Phage JH11 and JH14 specifically lysed piglet clinical isolates of *E. coli*, whereas lysed neither bovine clinical isolates, nor other chicken. A high proportion (58.06%, n=31) of *E. coli* piglet isolates was sensitive to phage JH11 and JH14, thus confirming its broad host range (data not show). Both phages were found to be heat sensitive (Figure 3) as more than 50% of phage particles were killed after 30 min of incubation at 60°C, and only 10% of the phage particles were still alive after 120 min of incubation. Less than 10% phage particles survived after 30 min of incubation at 70°C.

Bacteriophage resistance to acidity and alkalinity

The two bacteriophages were highly susceptible to acidity at pH 1 - 2 and susceptible in varying degrees to overnight exposure to pH 3-4. All the bacteriophages were resistant to the range of pH 5-9. Phage JH11 appeared to be a lightly more acid resistant than the JH14 (data not show).

Nucleic acid of bacteriophages

Nucleic acid of all the two bacteriophages was a DNA. All the bacteriophages' DNA samples were digested with restriction enzyme *Dra* I (Figures 4 and 5).

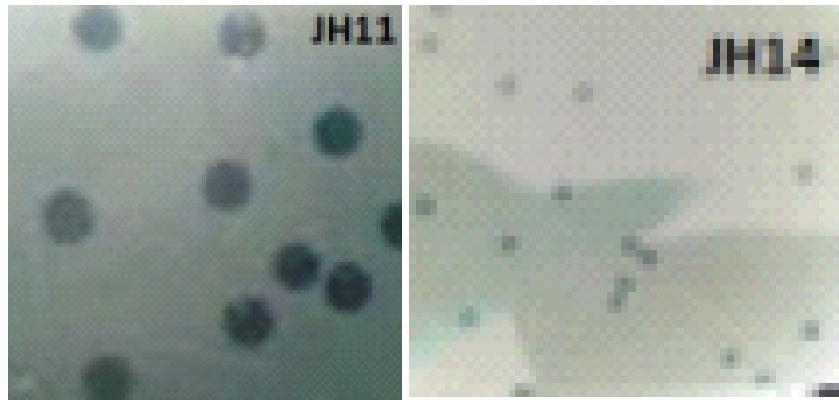


Figure 1. Plaques formed by phages JH11 and JH14 on O141 *E. coli*.

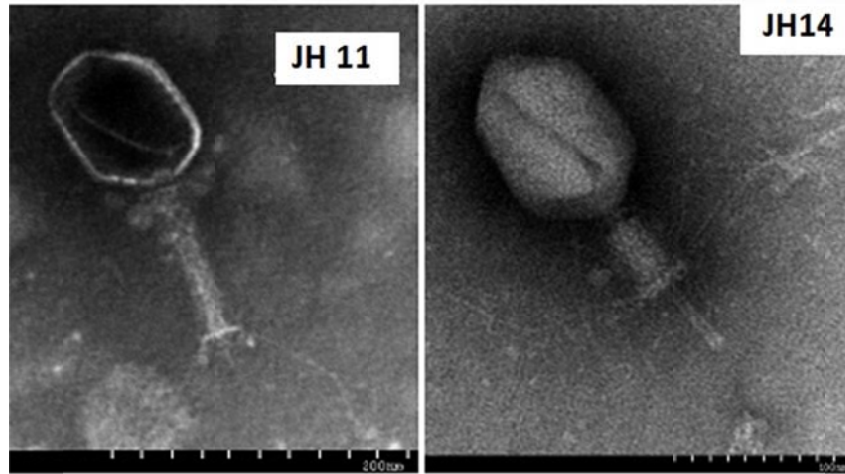


Figure 2. Appearance of phages JH11 and JH14 under electron microscopes.

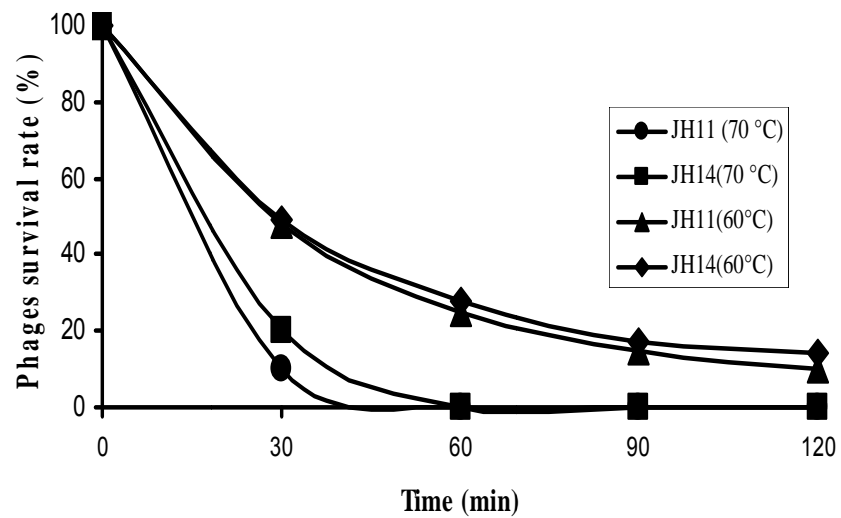


Figure 3. Thermostability of bacteriophages JH11 and JH14.

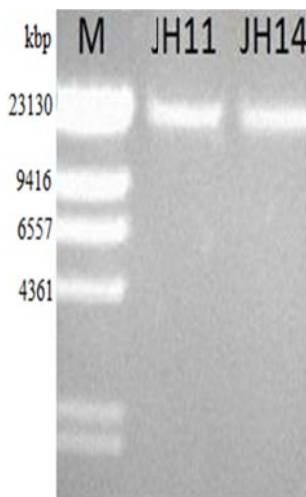


Figure 4. Electrophoresis of DNA of phages JH1 and JH14 on 0.7% agarose gels M = λ DNA marker.

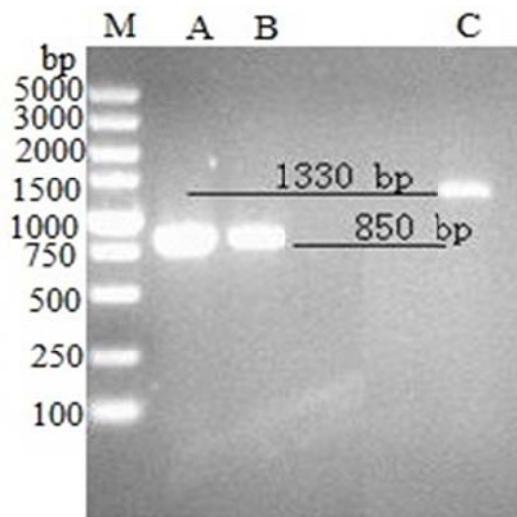


Figure 6. Agarose electrophoresis of PCR products, M: 5000 DNA marker, A: phage JH11 gene 23 PCR production, B: phage JHu14 gene gp23 PCR production, C: phage JH14 gene gp18 PCR production.

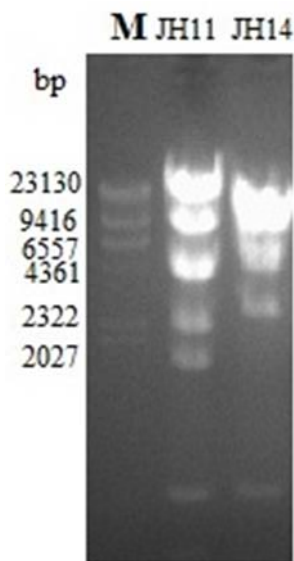


Figure 5. Analysis of phages genetic by enzyme *Dral* on 0.7% agarose gels M = λ DNA marker.

PCR sequencing of bacteriophage JH11 and JH14 of gp18, gp23 gene

Gene gp23 on the capsid surface structure and gene gp18 were structure protein of the T4 phage. The phage JH11 was gp23 positive, also the phage JH14 gp18 was gp23 positive, this is shown in Figure 6.

Blast analysis of the JH11, JH14 random clones

PCR products of gene 23 that resembled major capsid protein of JH11 and JH14 were dideoxynucleotide sequencing. Blast analysis results with the determined sequence on the NCBI website are demonstrated in Tables 1 and 2. According to the blast results, the gp23 of phage JH11 random clones were homologous with gene 23 of phage T4 MVSS (98%), ECML-134 (98%), T6 (97%) and T4 (96%) identity. Phage JH14 random clones are in close relation with HX01 (96%), CEV1 (95%) and T4 (93%). The two strains belong to T4-like phage genus. Gene of JH11 and JH14 were 93% identity (Figure 7).

Protein of purified particles of phage

Phage particles were purified and used to determine the structural protein content of each phage by SDS-PAGE analysis. Distinct profiles were observed among the two phages tested (Figure 8). Phage JH11 consisted of three abundant proteins (A, B and C) bands with approximate molecular sizes of 80, 75 and 50 kDa. Phage JH14 consisted of three abundant proteins (D, E and F) with estimated molecular size of 50, 38 and 36 kDa.

DISCUSSION

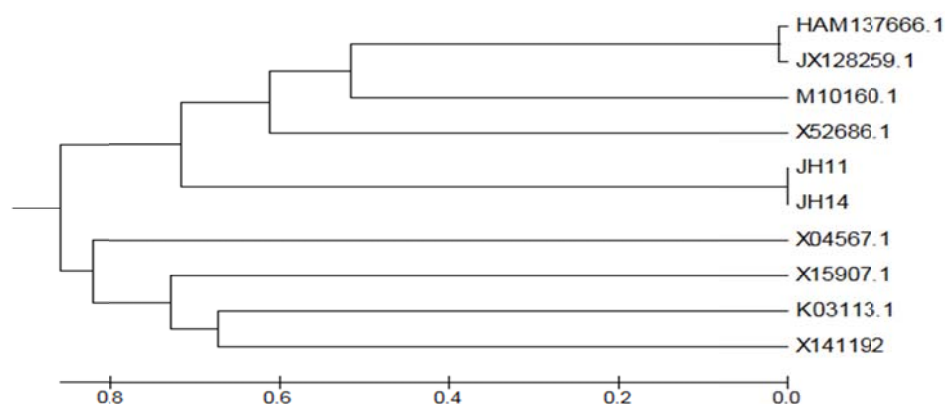
Bacteriophages are ubiquitous in our world and extremely diverse. Although recent research on bacteriophage is

Table 1. Blast analysis of bacteriophage JH11 random clones.

Accession	Random clone	Query coverage (%)	Max ident(%)
JX128259.1	Escherichia phage ECML-134, complete genome	96	98
Z78095.1	Bacteriophage T6 DNA (1506 bp)	96	97
JN202312.1	Enterobacteria phage ime09, complete genome	96	96
DQ485345.1	Enterobacteria phage MV SS major capsid protein gene, partial cds	90	98
HM137666.1	Enterobacteria phage T4T, complete genome	96	96
AF158101.6	Enterobacteria phage T4, complete genome	96	96
X01774.1	Bacteriophage T4 gene 23 for major capsid protein	96	96
K01765.1	Bacteriophage T4 genes 22 (partial) and 23 (complete cds)	96	96
DQ904452.1	Bacteriophage RB32, complete genome	96	96

Table 2. Blast analysis of bacteriophage JH14 random clones.

Accession	Random clone	Query coverage (%)	Max ident (%)
JX536493.1	Enterobacteria phage HX01, complete genome	94	96
AY331985.1	Bacteriophage CEV1 nonfunctional major capsid protein gp23	91	95
JX128259.1	Escherichia phage ECML-134, complete genome	94	93
HM137666.1	Enterobacteria phage T4T, complete genome	94	93
AF158101.6	Enterobacteria phage T4, complete genome	94	93
AY303349.1	Enterobacteria phage RB69, complete genome	94	93

**Figure 7.** Dendrogram showing relationship among phage JH11, JH14, and with the determined sequence on the NCBI website.

mushrooming, it is still limited in some respects. The isolation and characterization of more bacteriophage will facilitate the utilization of bacteriophage's resources. In this study, two novel bacteriophages named JH11 and JH14 were isolated from faecal pig farm. To identify the bacteriophages taxa, PCR method was applied with the sequencing blast analysis of bacteriophage. Preliminary study with this method revealed that bacteriophage JH11

and JH14 belonged to T4-like. The goal of this study was to isolate and characterize phages against the O141 *E. coli* with special emphasis on O141: F18 ETEC, which were reported as the most common sources of coliphages from sewage samples of domestic animal drainage (pigs). Pig sewage is a collection of waste drainage for the entire pig farms and was considered to be a good source from which to isolate phages against O141: F18 ETEC,

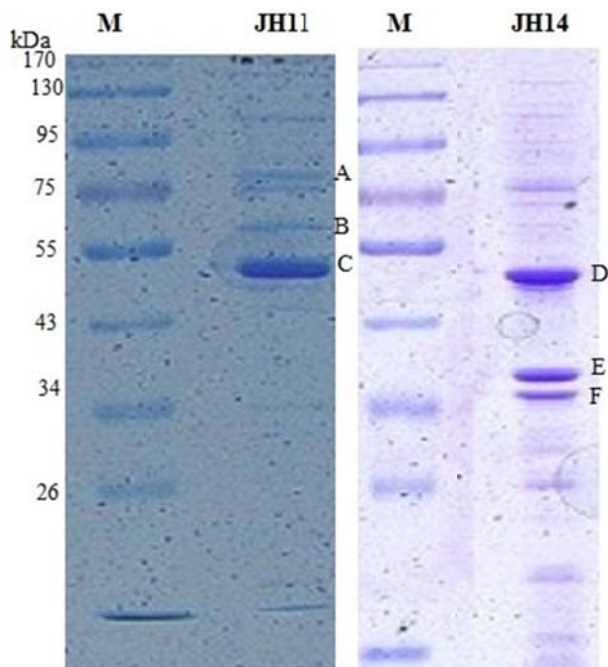


Figure 8. Structural proteins profiles of JH11 and JH14 bacteriophages by SDS-PAGE of constructural proteins of *E. coli* bacteriophages. Lanes: M: protein markers of high molecular sizes (in descending order from top to bottom, 170, 130, 95, 72, 55, 43, 34, 26, 17 and 10 kDa); 1, JH11; 2, JH12; polyacrylamide gel (12.5%wt/vol) was stained with Coomassie brilliant blue R-250.

especially when these farms had a record of infection with post-weaning *E. coli* diarrhea. To our knowledge this is the first report on isolation of phages against ETEC of any serogroup associated with PWD in pigs. Other study identified phages that were active against an O20: K101: F6 ETEC strain that causes diarrhea in neonatal pigs, and an O9: F5: ETEC that causes diarrhea in neonatal calves and lambs (Smith and Huggins, 1983). Jamalludeen et al. (2007) isolated 9 phages from raw sewage that lysed O149: H10: F4: ETEC in pig, while in chicken, 7 phages also isolated to lyse O1, O2 and O87 *E. coli* (Jamalludeen et al., 2009b). Interestingly, all the phages carried genes heat-stable enterotoxin of the *E. coli*. Morphological characteristics were seen under an electron microscope. In the last 45 years, 96% phages of the Siphoviridae, Myoviridae, and Podoviridae family were investigated (Kumari et al., 2009). Based on morphological features and contractile tails, the phages JH11 and JH14 against O141 *E. coli* in our study were members of the Myoviridae family. This family consists of six genes, and is characterized by having icosahedral or elongated head and contractile tails that are more or less rigid, long and relatively thick (ICTV, 1995). Most of T4-like phages specifically infected certain strains of *E. coli* or some other enterobacteria. In this study, phages JH11 and JH14

genome were in order to extract as a template. 1* PCR was performed by using gene gp18 and gene gp23 specific primers in amplification. Result of agarose gel electrophoresis showed that, PCR product size was about 1330 and 850 bp, and the expected size was 2* Chao et al. (2012) concluded that T4 phage are 160 T4-like phages and more 20 protein structure genes have gene gp23 structure of the capsid surface and gene gp18 is protein structure of tail sheath. Phages were tested for their ability to lyse host ranges on the O141, O8 and O2, the predominant porcine PWD *E. coli* strains. Most of the O141 *E. coli* strains were lysed by the phage JH11 and JH14. However, were resisted by O8 ETEC and O2 ETEC. These variations might be caused by function of phage and physiological state of the host (Flayhan et al., 2012).

These two phages were highly susceptible to acidity at pH 1-2, and susceptible in varying degree to overnight exposure to pH 3-4. Phage was often quite sensitive to protein denaturation in an acidic environment, which may result in a loss of viability of the phage. The ability to survive well over the pH range between 5 and 9 was a common feature for most phages. The pH in the stomach of weaned pigs may be as low as 1-2 before a meal and may rise quickly to 4-5 after the meal, depending on the diet and the feeding regime (Snoeck et al., 2004). The two phages were likely to undergo a marked reduction in titer following oral administration to pigs unless steps were taken to reduce their exposure to low pH in the stomach and upper small intestine.

Conclusions

In this study, two phages (JH11 and JH14) that lysed O141: F1 ETEC and belong to *Myoviridae* family were successfully isolated. Both phages were all highly active against O141: F18 ETEC. The genome sizes of these phages are larger than 20 kb. The phage JH11 was gp23 positive, while phage JH14 was gp18 and gp23 positive. The two bacteriophages belonged to T4-like bacteriophage genus. Both phages were resistant to pH 5-9, and three major protein bands were observed. These results indicated that the two phages have potential for prevention and as well as therapy for the porcine post-weaning diarrhea caused by O141: F18 ETEC.

Conflict of interest

The author(s) have not declared any conflict of interests.

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