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Full Length Research Paper

Categorization of two T4-like acteriophages aligned with 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.

INT RODUCTION

Ba cteriophages or phages are enteric viruses that can replicate only inside susceptible bacteria, There are div erse forms of organisms in the world and exert a major infl uence on the microbial world (Hendrix et al., 1999). Wh ile 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 pathogen esis and introduction of antibiotics have hampered the investigations of their role in therapy (Soothill, 1992). Enterotoxigenic *Escherichia*

coli (ETEC) are a cause of porcine diarrhea, that may occur sporadically or as outbreaks leading to substantial economic losses (Amezcua 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 acrylamidegels (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 Olsetetart 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 TACCTTCACCACGACCGG-3´). The primers that were initially used to amplify the gens gp18 analogue of the T4-type phage were: FT18-N2 (5´-GGTAAATTCCAATGGGGTCCAGCT 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 sensivitive (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).

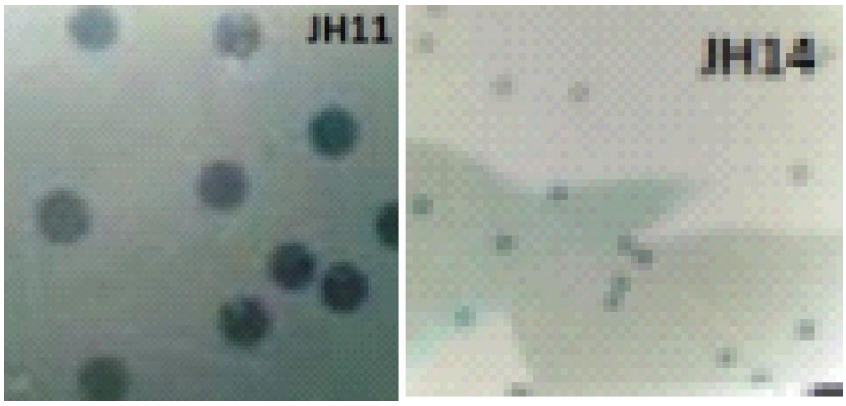


Figure 1. Plaques formed by phages JH11 and JH 14 on O141 *E. coli.*

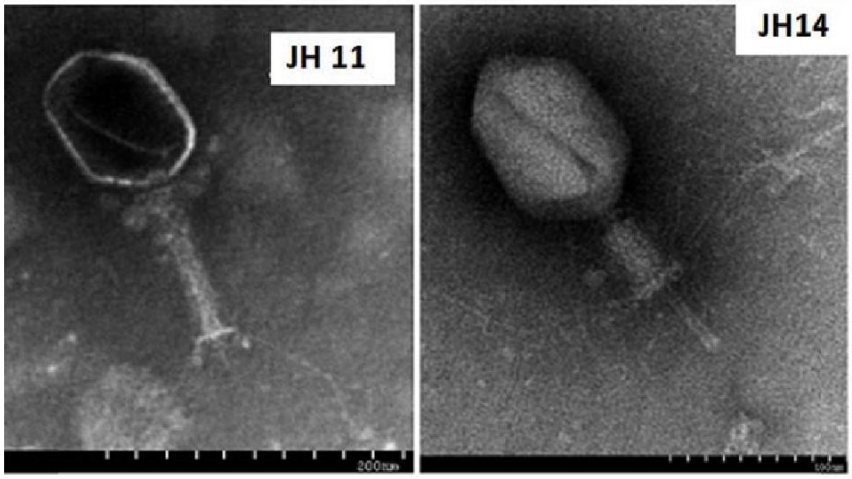
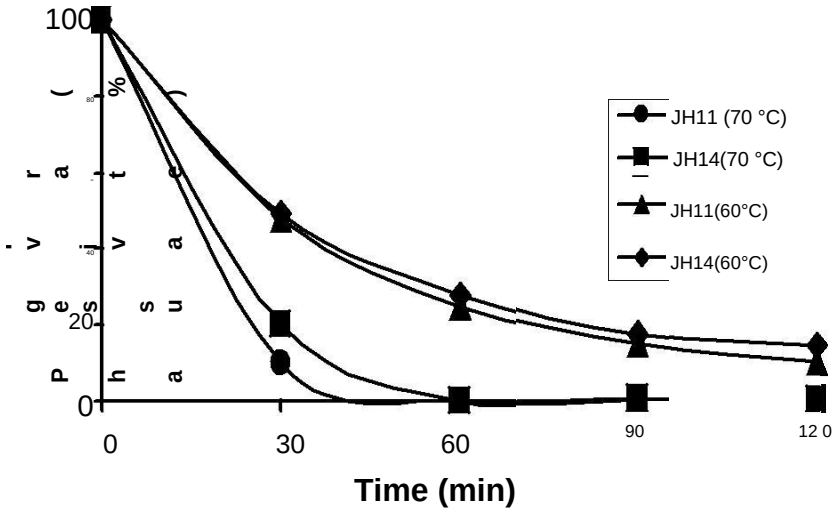


Figure 2. Appearanc e of phages JH 11 and JH14 under electron m icroscopes.



Figu re 3. Thermostability of bacteriophages JH11 and JH14.

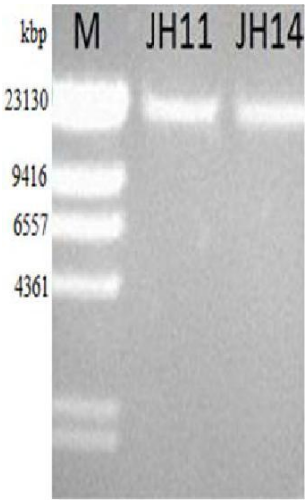


Figure 4. Electrophoresis of DNA of phages JH1 and JH14 on 0.7% agarose gels M = λ DNA marker.

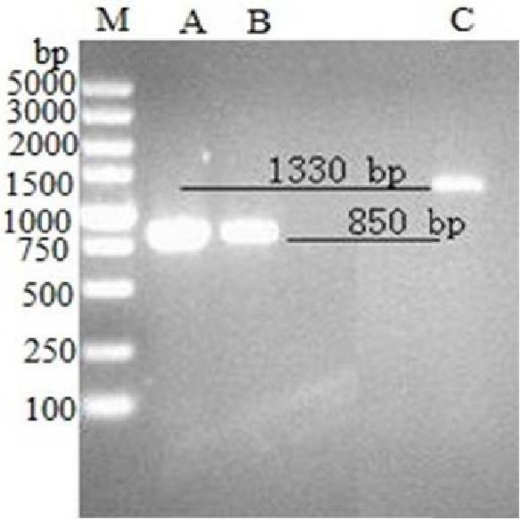


Figure 6. Agarose electrophoresis of PCR products, M : 5000 DNA arker, A: pha ge JH11 gene *23* P CR production, B: phage JHu14 gene gp*23* PCR production, C: phage JH14 gene gp*18* PCR produ ction.

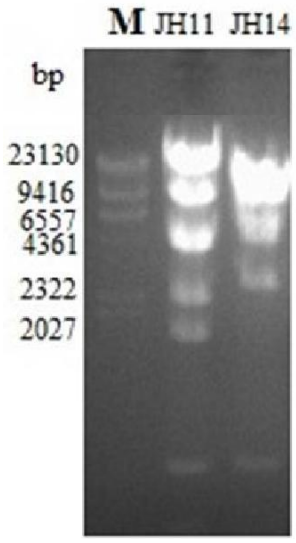


Figure 5. Analysis of phages genetic by enzyme *Dra*I on 0.7% agarose gels M = λ DNA m arker.

PCR sequenci g of bacteriophage JH1 1 and JH14 of gp 18, gp23 gen e

Gene gp23 on the capsid surface stru ture and ge ne gp 8 were struc ture protein o f the T4 ph age. The pha ge JH 11 was gp23 positive, als o the phage JH14 gp18 w as gp 3 positive, th is is shown i n Figure 6.

Blas t analysis of the JH11, J H14 random clones

PCR products of gene 23 tha t resembled major capsid protein of JH11 and JH14 were dideoxynucleotide sequ encing. Blast analysis results with the determined sequ ence on the NCBI website are de monstrated in Table s 1 and 2. A ccording to th e blast resul s, the gp23 o f phage JH11 rand om clones w ere homolog ous with gene 23 of phage T4 M VSS (98%), E CML -134 (9 8 %), T6 (97%) and T 4 (96%) ide ntity. Phage H14 random clones are in close relation with HX01 (96%), CEV1 (95%) and T4 (93%) . The two s trains belon g to T4-like phage genus . Gene of JH11 and JH14 were 3% identity (Figure 7).

Prot ein of purified particles o f phage

Phage particles w ere purified and used to determine the struc tural protein content of each phage by SDS-PAG E analy sis. Distinct profiles were observed among the two phages tested (Fi g ure 8) . Pha ge JH11 con s isted of three abundant proteins (A, B and) bands wit approximate mole cular sizes o f 80, 75 and 50 kDa. Phage JH14 consisted of thre abundant p roteins (D, E and F) with estim ated molecular size of 50, 38 and 36 kDa.

DISC USSION

Bact riophages a e ubiquitous in our world and extremely diver se. Although recent re earch on ba cteriophage i

Table 1. Bla st analysis of bacteriophage J H11 random clo nes.

Accession	Random clone	Query covera ge (%)	Max ident(%)
JX128259.1	Escherichia phage ECML- 134, complete genome	96	98
Z78095.1	Bacteriopha ge T6 DNA (1506 bp)	96	97
JN202312.1	Enterobact ria phage ime09, complete g enome	96	96
DQ485345.1	Enterobact ria phage MV SS major cap id protein gene, partial cds	90	98
HM137666.1	Enterobact ria phage T4T, complete gen ome	96	96
AF158101.	Enterobact ria phage T4, complete gen me	96	96
X01774.1	Bacteriopha ge T4 gene 2 for major capsid protein	96	96
K01765.1	Bacteriopha ge T4 genes 2 2 (partial) and 23 (complete cds)	96	96
DQ904452.1	Bacteriopha ge RB32, com plete genome	96	96

Table 2. Blast analysis of bacteriophag e JH14 random clones.

Accession	Rand m clone	Query coverage (%)	Max ide nt (%)
JX536493.1	Entero bacteria phag e HX01, complete genome	94	96
AY331985.1	Bacteriophage CEV1 nonfunctional major capsid protein gp23	91	95
JX128259.1	Escherichia phage ECML-134, com plete genome	94	93
HM137 666.1	Entero bacteria phag e T4T, complete genome	94	93
AF158101.6	Entero bacteria phag e T4, complete genome	94	93
AY303349.1	Entero bacteria phag e RB69, complete genome	94	93

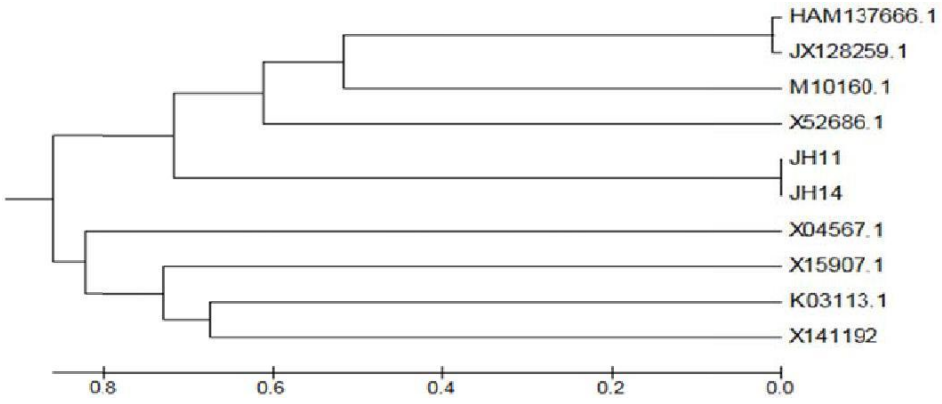


Figure 7. Dendrogram showing relationship among phage JH11, JH14, and with the determined sequence on the NCBI website.

mushrooming, it is still limited in some respects. T he iso ation and characterizatio n of more bacteriophage will facilitate the utilization of b cteriophage's resources. In this study, two novel bacteriophages named JH11 a nd JH 14 were isol ted from faecal pig farm. To identify he ba teriophages taxa, PCR ethod was applied with the seq uencing bla st analysis of bacteriopha ge. Prelimin ary study with this ethod revealed that bact eriophage JH 11 and J H14 belonge d to T4-like. The goal of this study was to is late and ch aracterize phages agains t 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 dr ainage (pigs . Pig sewage is a c ollection of w aste drainag for the entire pig fa rms and was considered to be a goo d source fro whic to isolat e phages against O141: F18 ETEC,

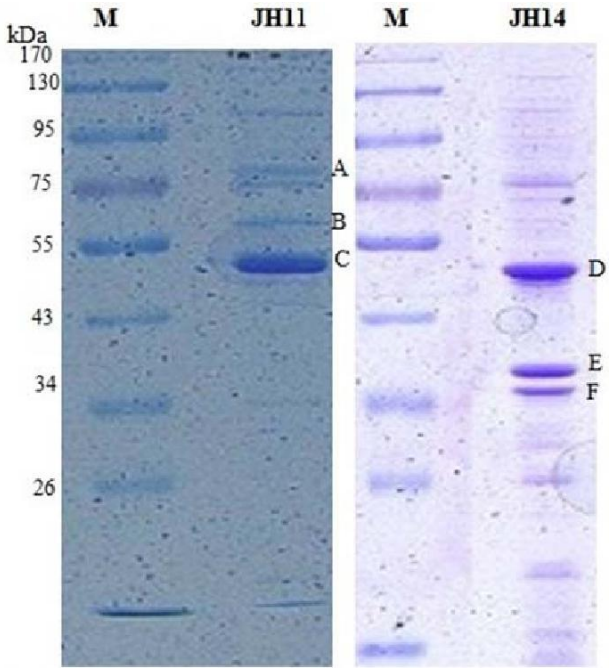


Figure 8. Str ctural proteins profiles of JH 1 and JH14 bacteriophages by SDS-PAGE of contructur al proteins of *E. coli* bacteri ophages. Lanes : M: protein ma rkers of high molecular size s (in descendi g order from t p to bottom, 170, 130, 95, 72, 55, 43, 34, 6, 17 and 10 k Da); 1, JH11; 2, JH12; polyacrylamide gel (12.5%wt/vol) was stained with Coomassie brilliant blue R-250.

esp ecially when these farms had a record of infection w ith po t-weaning *E. coli* diarrhea. To our knowledge thi s is the first report o n isolation of phages agai st ETEC of a ny serogroup asso ciated with PWD in pigs. Other stu dy identified phages that were a ctive against an O20: K1 01: F6 ETEC strain that causes d iarrhea in ne onatal pigs, a nd an O9: F5: ETEC that cause s diarrhea in neonatal calv es an lambs (Smi h and Huggi ns, 1983). Jamalludeen et al. (2007) isolated 9 phages from raw sew age that lys ed O1 49: H10: F4: ETEC in pig, while in chicken, 7 phag es als o isolated to lyse O1, O2 a nd O87 *E. c li* (Jamallude en et al., 2009b). I nterestingly, all the phages carried gen es he t-stabile enterotoxin of the *E. coli*. Morphological cha racteristics ere seen under an electr on microsco pe. In the last 45 years, 96% phages of th e Siphovirid ae, Myoviridae, an d Podoviridae family were investiga ted (Ku mari et al., 2009). Base d on morphological featu res an contractile ails, the phages JH11 an d JH14 against O1 41 *E. coli* in o ur study wer e members of the Myovirid ae fam ily. This f mily consists of six genes, and is cha racterized by having icosahedral or elongated he ad an contractile ails that are more or less rigid, long a nd rel tively thick (ICTV, 1995). Most of T4-like phag es spe cifically infe c ted certain strains of *E. co li* or some ot her enterobacteria. In this study, phages JH11 and JH14 genome were in order to extr act as a tem plate. 1* PC R was performed b y using ge ne gp18 an d gene gp23 specific primers in amplificati on. Result of agarose ge l elect rophoresis sh owed that, PCR product size was abou t 1330 and 850 bp, and the expected size was 2* Chao e t al. (2012) conclu ded that T 4 phage are 160 T4-like phages and more 20 protein s tructure genes have gene gp23 structure of the capsid surface and gene gp18 is protein structure of tail sheat . Phages were tested for their ability to lys e host ranges on the O14 , O8 and O2 , the predominant p orcine PWD *E. coli* strains. Most of the O141 *E. coli* strains were lyse d by the ph ge JH11 and JH14. However, were resiste d by O8 ETEC and O2 ETE C. These variations might be caused by function o f phage and physiological state of the host (Flayhan et al , 2012).

Th ese two phag es were hig ly susceptible to acidity a t pH 1-2, and susc eptible in va rying degre to overnigh t expo sure to pH 3-4. Phage was often quite sensitive to protein denaturati on in an acidic environment, which may resul t in a loss o f viability of the phage. The ability to survi ve well over the pH range between 5 and 9 was a com mon feature f or most phages. The pH i n the stomach of w e aned pigs may be as low as 1-2 befo re a meal and may rise quickly to 4-5 after th e meal, depending on the diet a nd the feedin g regime (Sn oeck et al., 2004). The two phages were likel y to undergo a marked re duction in titer follow ing oral ad ministration to pigs unless steps were taken to reduce t eir exposure to low pH i the stomach and u pper small in testine.

Conc lusions

In this study, two phages (J 11 and JH1 4) that lysed O141: F1 ETEC and belong to *Myovirida e* family were succ essfully isolated. Boths phages were all highly active again st O141: F18 ETEC. T h e genome s izes of these phages are larger than 20 kb. The phage J H11 was gp23 positive, while ph age JH14 w a s gp18 and gp23 positive . The two bacteriophages belonged to T4-like bacte riophage ge nus. Both phages were r esistant to p H 5-9, and three major protein bands were ob served. These resul ts indicated that the two phages hav e potential for preve ntion and as well as therapy for the porcine post-weaning diarrhea caused by O141: F1 8 ETEC.

Conflict of interest

The uthor(s) have not declared any conflic of interests.

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