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ISOLATION OF BACTERIOPHAGE JH14 AND DETERMINATION OF ITS PROTECTION ABILITY AGAINST PATHOGENIC AMPICILLIN-RESISTANT *ESCHERICHIA COLI* ON MICE

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SUMMARY

Bacteriophage JH14 was isolated, named JH14. The plaques of the bacteriophage were clear with medium sized of 3.5 mm in diameter. Result from electron microscopy showed that phage JH14 belonged to the *Myoviridae* family. The phage possessed icosahedral heads, necks, and contractile tails, with tail fibers. JH14 belonged to the order *Caudovirales*. The head and tail dimension for JH14 was 100 × 80 nm and 130 × 30 nm, respectively. The phage was purified by a glycerol gradient procedure after purified particles were subjected to SDS-PAGE. There were many bands of protein, but three abundant bands of 50, 38 and 34 kDa were observed on the gel. Bacteriophage JH14 specifically lysed pig clinical isolates of O8 *E. coli*, whereas neither chicken and bovin *E. coli* strains, nor other O serogroup *E. coli* strains were sensitive. A high proportion of O8 ETEC strains isolates was sensitive to phage JH14. The LD_{50} of VN14 *E. coli* strain was 1.6×10^7 cells per 0.2 ml. Determination of toxicity of phage JH14 showed no toxicity of the phage in the experimented mice. The minimal dose of the JH14 phage for mice protection was 10^5pfu, significantly smaller than that of control. Only with 10^5pfu of JH14 phage could protect mice from infection $9 \times LD_{50}$ (1.4×10^8cfu) of a virulent strain of *E. coli*. A single intramuscular dose of phage JH14 was more effective than multiple intramuscular doses of Amikacin and Streptomycin. These studies support the view that bacteriophages could be useful in the treatment of animal infections caused by antibiotic- resistant strains of bacteria.

Keywords: *Bacteriophage, Enterotoxigenic, Escherichia coli, Therapy, Antibiotic.*

INTRODUCTION

Bacteriophages are non-hazardous self-replicating agents that increase their numbers as they destroy target bacteria. From the early 1920s, phage therapy has been considered as antimicrobial agents for the treatment of bacterial infectious diseases. However, the development of this therapy has been hampered by the advent of antibiotics (Sulakvelidze *et al.,* 2001). Due to the emergence of multidrug-resistant bacteria, phage therapy has been resurrected during the past few decades. In the 1980s, excellent studies on phage therapy were carried out by Smith and colleagues, using *E. coli* infection in mice and farm animals. Phage therapy might be a viable alternative to or complement conventional antibiotic therapy because it has already been proven to be advantageous as these are very specific, accurate and potent than antibiotics. Another advantage of using phages over antibiotics is that phages can replicate at the site of infection and thus become available in abundance at the desired site (Bai *et al.,* 2013). In addition, several recent and well-controlled animal studies have demonstrated the potential of phages for antibacterial therapy (Laslett, Canback, 2004). Pathogenic *E. coli* carrying F18 fimbriae colonizes at porcine small intestine and cause postweaning diarrhea or edema disease. Adherence of the bacteria to microvilli of small intestinal epithelial cells of the piglets is initiated by adhesins that are associated with F18 fimbriae. Colonization depends on the specific binding between adhesive fimbriae and receptors on the enterocytes. The F18 is composed of 2 closely related antigenic variants: F18ab, referred to as F107 and F18ac, also called 2134P and 8813. The F18ab is often expressed in strains producing Shiga toxins (STEC) causing edema disease belongs to serogroup O8, O139 and O16 whereas strains expressing F18ac fimbriae are enterotoxigenic *E. coli (ETEC)*.In the past, British scientists reported on

the successful veterinary application of *E. coli* phages in the 1980s (Sambrook and Russell, 2001; Smith and Huggins, 1982; Smith *et al.,* 1987).Only phages recognizing the K1 antigen were protective. Phages with highin vitro lytic activity were also the most effective in conferring pro-tection*in vivo*(Smith *et al.,* 1987). More recently and phage therapy is now back in the headlines. Present study was designed for the bacteriophage lysis experiment, determination of drug median lethal dose LD_{50} of host bacteria, safe bacteriophage and animal protective assessment.

MATERIALS AND METHODS

Culture and sampling medium and procedures

In vitro, liquid cultures of bacteria and phage were grown and maintained in Luria – Bertani broth (LB), LB agarose and LB top agarose were prepared as described previously (Jamalludeen *et al.,* 2007), SM buffer was prepared (5.8 g NaCl, 2 g MgSO4.7H2O, 50 ml/L of 1 M Tris pH 7.5, 5 ml/L of 2% gelatin in distilled water). Bacterial densities were estimated from colony counts on Petri dishes (plates) containing 25 ml of LB with 1.5% agar. Phage densities were estimated on these plates with 3 ml top (0.4%) agar containing LB and 100 µl culture of the bacteria (about 5×10^7 bacteria per ml). The antibiotics (Streptomycin, Amikacin) were given, in doses of 25 mg/kg body wt, every 12h for 6 day.

Bacterial strain

The strain for experimental infection of pigs was O8:F18: ETEC strain VN14 isolated from Vietnam, a hemolytic *E. coli* with genes for STa, STb and LT. This strain was isolated in 2010 from Vietnam pig with post-weaning diarrhea (PWD). The organisms were streaked on blood agar, checked for purity. O8 serotype and F18 fimbriae were PCR tested.

Preparation of bacterial inocula

As required, frozen stock cultures were plated overnight on MacConkey agar at 37°C and single colonies were cultured in LB for 12-16 h at 37°C with shaking at 150 rpm. For experimental infections, the ETEC strain VN14 in LB cultures were harvested by centrifugation at 5,000 × g, then resus-pended in 0.01 M phosphate-buffered saline at pH7.2 (PBS: Phosphate buffered saline) and adjusted spectrophotometrically to an OD600 of 1.4 equivalents to 10^9 cfu/ml approximately. The concentrations of the bacterial suspensions were confirmed by standard plate counts.

Phage isolation

Bacteriophages were isolated from fecal sample in Nanjing pig farm in 2011 as described previously (Xuan Hoa *et al.,* 2013). LB broth was inoculated with a mixture equal proportions of the six *E. coli* VN14-O8: F18 ETEC, VN2- O8: F4 ETEC, VN3-O8:F4 STEC, VN14-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 centrifuged before filtering through a 0.45-µm membrane filter to remove impurities and bacteria. 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 in a 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 performed as described previously (Kropinski *et al.,* 2009). Phage preparation were obtained as described elsewhere and stored at 4°C.

Preparation of bacteriophage suspensions

Broth cultures were made in 10 ml volumes of LB broth (Difco) in a 20 ml bottle incubated at 37°C for 24 h in a shaking incubator (150 rpm). A 30 ml volume of LB broth in a 100 ml conical flask was inoculated with aliquots of broth culture of VN4 to contain approximately 10^7 cfu/ml and phage JH14 preparation contained 10^6 pfu/ml. The cultures were incubated at 37°C in 3-4 h with shaking until the culture containing the phage had been cleared. At this point the flask was placed at 4°C overnight for additional lysis to be occurred. The culture was then centrifuged at 5,000 rpm for 30 min at 4°C, finally supernatant was filtered (0.45-µm pore size) (Xuan Hoa *et al.,* 2013).

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, tail length) (Bai *et al.,* 2013).

SDS-PAGE of phage JH14 particles

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 10^{10} pfu/ml) were boiled for 5 min and separated by SDS-PAGE loading buffer (50 mMTris-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).

Host range assay

To investigate the host range of the phage JH14, 32 O8 *E. coli* strains, 8 non-O8 *E. coli* strains(NT1-8), 20 bovine *E. coli* isolates (JV1-20) and 21 chicken *E. coli* strains (LYT 15-36) were tested as described elsewhere.

Animals

Mices (n= 108) were obtained from Changzhou University, weaned at 3 weeks of age (20-23g), transferred to the Department of Microbiology and Immunology, College of Veterinary Medicine, Nanjing Agricultural University and allowed to acclimatize for 2 days before commencement of experiments. The mice were housed in groups of up to four mice, fed a standard non-medicated ration. They were weighed at the commencement and end of three experiments. Fecal samples were collected from the rectum prior to and at intervals after infection and treatment. Bacteria, phage and control suspensions (0.25 ml) were injected intramuscularly. Simultaneous injection of bacteria and phage was done with a specially made holder which held two syringes (Soothill, 1992).

Determination of drug median lethal dose LD_{50}

During LD_{50} estimations, the mice were housed six/cage; all animals in each cage received the same inoculum (Reed, Muench, 1938). Account of death mice, Bliss software determination of drug median lethal dose was used. Death mouse are recorded from 5 h to 6 days after infection.

The toxicity of phage and control suspensions

The toxicity of phage and control suspensions for mice was investigated by injecting 0.25 ml of the suspension (SM) and phage JH14 (10^9pfu) into each of a group of four mice. Four uninjected mice were retained as normal controls. The mice were observed for signs of illness and temperatures were taken hourly during the first 5 h after injection and then daily during the next 4 days.

Protection studies

Doses of bacteria were used in the protection studies were established by LD_{50} measurements. Mice were given injections of four inoculate of bacteria, with four mice/inoculum level. Mice were killed when it was considered that they were terminally ill (reduced mobility, partially closed eyes, abnormal posture and an altered breathing pattern) (Paul *et al.,* 1998).In the first study, six groups of four mice received $9 \times LD_{50}$ of strain *E. coli-* VN14 five of the groups also received 0.25 ml of phage, the doses decreasing in five-fold dilution steps, the highest dose being 4.1×10^8pfu; the remaining group received 0.25 ml of control suspension. In the second pilot study, four mice were used; each received $6 \times LD_{50}$ of bacteria, four of the mice also received 0.25 ml of phage, the doses decreasing in 10-fold steps (one mouse/dose), the highest being 10^4 pfu; the remaining mouse received 0.25 ml of control suspension.Mice were inoculated into right gastrocnemius muscle with VN14 *E. coli* strain. Phage JH14 or antibiotic were inoculated into the opposite muscle. Bacteria and phage or bacteria and antibiotic were injected simultaneous into gastrocnemius muscle. The antibiotics were given in multiple dosages every 12 h for 6 day. Each dose of Streptomycin was 15 mg/kg body wt and Amikacin was 25 mg/kg body wt. In vitro,with 10^3pfu of phage JH14 was required to cause clearing of the standard broth cultures, demonstrating the high activity of these phages (O'Flynn *et al.,* 2004).

RESULTS

Isolation and morphology of bacteriophages

Bacteriophage was isolated, named JH14. The bacteriophage produced plaques that were clear and medium sized 3.5 mm in diameter. Electron microscopy confirmed that phage JH14 belongs to the *Myoviridae* family. Phage possessed icosahedral heads, necks, and contractile tails, with tail fibers. JH14 belong to the order *Caudovirales*. The head

and tail dimension for JH14 was 100 × 80 nm and 130 × 30 nm, respectively (Fig. 1). The phage was purified by a glycerol gradient procedure after purified particles were subjected to SDS-PAGE. There were many bands of protein, but three abundant bands of 50, 38 and 34 kDa were observed on the gel (Fig. 2).

Host range assay

Bacteriophage JH14 specifically lysed pig clinical isolates of O8 *E. coli*, whereas neither chicken and bovin *E. coli* strains, nor other O serogroup *E. coli* strains were sensitive. A high proportion (85 %, n=32) of O8 ETEC strains isolates was sensitive to phage JH14.

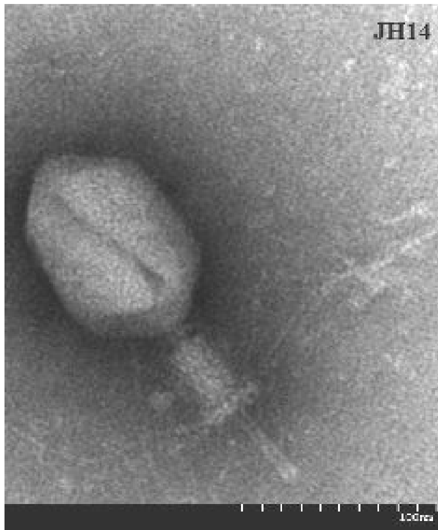

Figure 1. Electron microscopic appearance of phage JH14

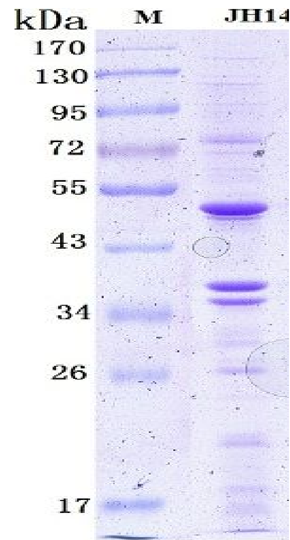

Figure 2. Structural proteins profiles of bacteriophage JH14 on SDS-PAGE gel

Determination of drug median lethal dose LD_{50}

During LD_{50} estimation, the mice were six in a cage; all animals in each cage received the same inoculum.

Six groups, each group 6 mice received 0.25 ml of *E. coli*-VN14 strain. The doses decreased in ten-fold dilution steps, the highest dose being 10^9 cfu; lowest dose being 10^5 cfu. Experiment control received 0.25 ml of LB solution. For annalysis, we used Bliss software to determination of drug median lethal dose (LD50), the results showedmedian lethal dose of VN14-*E. coli* $LD_{50} = 1.6 \times 10^7$ cfu/mouse.

Determination of toxicity of phage JH14 and control suspension

No signs of illness were observed in the mice four days after inoculation. It showed no toxicity of the phage in the experimented mice. There were no signs of illness and the temperatures of the mice did not differ significantly from those of controls (Table 2).

Table 1. Determination of median lethal dose LD_{50}

Dose	Animal number	Number death	Death percent (%)
10^9	6	6	100
10^8	6	5	83
10^7	6	3	50
10^6	6	0	0
10^5	6	0	0
Control	6	0	0

Table 2. Determination toxicity of phage JH14

Injected	Animal number	Number of survivors	Death percent (%)
Phage JH14(10^9pfu)	4	4	0
Suspension (SM)	4	4	0
Uninjected (Control)	4	4	0

Assessment of animal protection

Deaths occurred in groups of mice infected intramuscularly with VN14 strain and then treated intramuscularly with phage JH14 or antibiotic (The antibiotics were given in multiple dosages every 12 h for 6 day. Each dose of Streptomycin was 15 mg/kg body wt and Amikacin was 25 mg/kg body wt). Both studies showed clear protection (Table 3). In the study I, it indicated an effective dose of one phage particle for 103 bacteria. This dose (the highest dose used) protected the mice significantly as compared to the untreated controls (p = 0.0047).In the study II protected the mice was significantly compared with the untreated controls (p = 0.028). The significance testing by Chi-Square test ($P<0.05$), using SPSS 16.0. The lower doses did not confer protection. The dose, which protects mice 50% (PD50) was about 102 pfu. The Table 3 showed antibiotic can protect mice inoculated by at a dose of 6 × LD50 VN 14 strain did not protect againt strain at dose 9 × LD50. A single intramuscular dose of phage JH14 was more effective than multiple intramuscular doses of Amikacin and Streptomycin.

Table 3. Animal protection and evaluation of phage JH14 or antibiotic

Study I, 9 × LD50		Study II, 6 × LD50	
Phage dose (pfu)	Death rate	Phage dose (pfu)	Death rate
4.1×10^8	0/4	10^4	1/4
1×10^8	0/4	10^3	1/4
3.5×10^6	0/4	10^2	3/4
1.3×10^6	0/4	10	4/4
0.4×10^5	0/4		
Streptomycin	1/4	Streptomycin	0/4
Amikacin	1/4	Amikacin	0/4
Control	4/4	Control	4/4

$6\times LD_{50} = 9.3 \times 10^7$cfu; $9\times LD_{50} = 1.4 \times 10^8$cfu

DISCUSSION

Bacteriophages are ubiquitous in our world and extremely diverse. Although recent research of bacteriophage is mushrooming, it is still limited in some respects. To isolate and characterize more bacteriophage will facilitate the utilization of abundant bacteriophage resources. Due to their highly specific host recognition, phage has potential as therapeutic agents in the treatment of certain human, animal and plant bacterial infections. In this study, we isolated a novel bacteriophage named JH14 from fecal sample. Morphological characteristics were seen under an electron microscope. In the last 45 years, 96% of phages of the *Siphoviridae*, *Myoviridae,* and *Podoviridae* family were investigated (Kumari *et al.,* 2009). Based on morphological features and contractile tails, the phage JH14 against O8 *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 (Ackermann, 2011).

Phage JH14 was tested for its ability to lyse host ranges on the O8 ETEC, the predominant porcine

PWD *E. coli* strains. Most of the O8 *E. coli* strains were lysed by the phage JH14 but for the *E. coli* isolated from chicken and bovin there is a limitation of lysis by this phage. These variations might be caused by function of phage and physiological state of the host.

Highly lytic bacteriophage protects mice from effect of VN14 *E. col* strain. With low dose from 1 to 10^3 bacteriophage (pfu), the *E. coli* was lysed by bacteriophage *in vitro* and *in vivo*. The study of Smith showed that protection ability of bacteriophage can protect mice from *E. coli*. Besides the bacteriophages also have protection ability for calves, piglets and lambs. Recently, Jamalludeen et al showed that phages were effective in experimenting O149:H10:F4 ETEC diarrhea for weaned pigs in the process of prophylactically or therapy. It has applyed infection treatments by phages or streptomycin. In short, the high lysis ability of JH14 bacteriophage to VN14- *E. col* is useful for treating infections clinical, especially anti-biotic-resistant organisms. A single intramuscular dose of phage JH14 was more effective than multiple intramuscular doses of Amikacin and Streptomycin.

CONCLUSION

Phage JH14 against O8 *E. coli* in our study was a member of the *Myoviridae* family. A single intramuscular dose of phage JH14 was more effective than multiple intramuscular doses of Amikacin and Streptomycin to protect mice from *E.* coli infection. These studies support the view that bacteriophages could be useful in the treatment of animal infections caused by antibiotic- resistant strains of bacteria.

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PHÂN LẬP THỰC KHUẨN THỂ JH14 VÀ THÍ NGHIỆM KHẢ NĂNG BẢO HỘ CỦA NÓ ĐỐI VỚI CHUỘT GÂY NHIỄM BỞI *ESCHERICHIA COLI* KHÁNG AMPICILLIN

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TÓM TẮT

Chúng tôi đã phân lập được một chủng thực khuẩn thể JH14 trên nền vi khuẩn chủ *Escherichia coli* O8: F18 ETEC. Thực khuẩn thể này có khả năng phân giải vi khuẩn chủ tạo vết ban sáng, tròn, kích thước 3,5 mm. Thông qua kính hiển vi điện tử cho thấy JH14 có cấu trúc gồm phần đầu (hình khối 20 mặt, kích thước100 × 80 nm) và đuôi dài có thể co rút được130 × 30 nm. Căn cứ vào phân loại học thực khuẩn thể JH14 thuộc họ *Myoviridae,* loài *Caudovirales.* Thông qua kỹ thuật phân tích protein SDS-PAGE cho thấy vỏ bọc protein của JH14 chủ yếu được cấu trúc từ 3 tiểu phần có kích thước 50, 38 và 34 kDa. Thực khuẩn thể JH14 chủ yếu gây dung giải các chủng O8 *E. coli,* trong khi đó không gây dung giải với các chủng *E. coli* có nguồn gốc phân lập từ gia cầm và bò. Thông qua thí nghiệm kiểm tra tính an toàn cho thấy thực khuẩn thể JH14 an toàn, không gây độc cho chuột nhắt trắng. Liều gây chết 50% động vật của chủng vi khuẩn *E. coli* thí nghiệm là 1.6×10^7 tế bào bao hàm trong 0.2 ml. Thí nghiệm cho thấy JH14 không độc đối với chuột. Liều tối thiểu của thực khuẩn thể JH14 (10^5pfu) có khả năng bảo hộ động vật thí nghiệm khi chúng bị gây nhiễm bởi $9 \times LD_{50}$ (1.4×10^8cfu). Chỉ với một liều duy nhất thực khuẩn thể JH14 mang lại sự bảo hộ hiệu quả hơn so với liệu trình dùng kháng sinh Amikacin and Streptomycin trong 6 ngày liên tục.

***Từ khóa**: Thực khuẩn thể, Độc tố ruột, E. coli, Liệu pháp điều trị, Kháng kháng sinh*

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