Full Paper

Bacteriophage Esc-A is an efficient therapy for *Escherichia coli* 3-1 caused diarrhea in chickens

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The bacteriophage Esc-A was isolated from sewage by using the intestinal pathogenic *Escherichia coli* 3-1 as the host. Toxicity in chickens showed its safety as a bio-product. Phage therapy against diarrhea in chickens indicated that Esc-A could decrease the death rate more efficiently compared with antibiotic treatments.

Key Words——bacteriophage therapy; pathogenic *Escherichia coli*

Introduction

The discovery of bacteriophage by Twort in 1915 and d'Herelle in 1917 led to an enthusiasm for the use of bacteriophage in bacterial disease prophylaxis and therapy, but early studies were poor and uncontrolled. With the rapid development of chemotherapy, the investigation of phage therapy seemed to have no reason to continue (Barrow and Soothill, 1997). Although chemotherapy is a rapid and effective method to treat and prevent bacterial infections, frequent use of chemotherapeutic agents has allowed drug-resistant strains of bacteria to develop. In particular, the problem of the growing drug-resistant bacteria population caused by antibiotic treatments have become more and more serious in China since 9 drugs were licensed for aquaculture use from 1995 to 1997 (Table 1).

In the 1980's Smith and colleagues studied phage

therapy against *Escherichia coli* infection in mice and farm animals (Smith and Huggins, 1982, 1983). Thereafter, a series of successful results for phage therapies against drug-resistant strains of bacteria have been reported by using various models, especially in Poland and the Soviet Union (Alexander et al., 1988).

Potential advantages of phage treatment over antibiotics are: 1) the narrow host range of phage prevents harm to the normal intestinal microflora; and 2) the self-perpetuating nature of phages in the presence of susceptible bacteria insures fast and long-lasting treatment with low titer bacteriophage, and potentially avoids multiple administrations (Nakai et al., 2002).

Chickens and other animals on modern farms, are constantly threatened by microbial attack and the occurrence of drug-resistant bacteria. No study on phages had been made with a view toward preventing bacterial infections without incubating pathogenic *E. coli* in chicken until our recent works. In this paper, we report our studies on phage Esc-A and its effects against the diseases caused by *E. coli* infection in Roman chickens.

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Table 1. Sensitivity of *Escherichia coli* to antibiotics from 1995 to 1997 (Tian et al., 1997).

Antibiotics	Degree of sensitivity (%)		
	1995	1996	1997
Colistin	90.3	30.7	4.5
Neomycin	54.5	41.2	24.3
Chloramphenicol	44.9	46.3	34.2
Gentamicin	56.8	48.1	38.8
Streptomycin	16.7	11.5	15.7
Kanamycin	36.1	35.7	15.3
Enrofloxacin	—	45.0	18.1
Norfloxacin	—	48.7	38.7
Ofloxacin	—	40.2	33.3

—, not examined.

Materials and Methods

Organism. The enteropathogenic *E. coli* strain 3-1, was isolated from an outbreak of neonatal diarrhea in chickens.

Selection media. LB Broth (1% peptone, 0.5% yeast extracts and 1% NaCl, pH 7.2) and agar plate (1.5% Agar) were used for growing *E. coli* 3-1. Cultivation was carried out at 37°C, under aerobic conditions.

Bacteriophage isolation, propagation and preparation. Sewage samples were centrifuged at 12,000 rpm for 30 min and the supernatants were incubated at 37°C with an exponential growth-phase culture of *E. coli* 3-1. After overnight shaking (90 rpm), the bacteria were removed by centrifugation, and the supernatant was plated on *E. coli* 3-1 by using the double agar layered method of Adams (Van, 1992). Plaques which formed on the plates were stabbed with a needle and eluted with a small volume of phage buffer. Each phage suspension was serially propagated twice on the same strain. Phage Esc-A, whose plaques were clear and big (Fig. 1), was selected for further study in our work.

10^8 PFU/ml phage Esc-A of 100 μ l were incubated with an exponential grow-phase culture of *E. coli* 3-1 of 100 ml at 37°C. For 30 min without shaking, then shaken (90 rpm) until visible lysis. The phage titer of 10^{12} PFU/ml was obtained.

The lysate was centrifuged at 12,000 rpm for 30 min. NaCl and PEG6000 were added to the supernatant to reach the final concentration of 0.5 M and 40% (w/v) respectively and phage particles were precipitated at

Fig. 1. The plaque of phage Esc-A.

–20°C overnight. The phage pellet was dissolved in SM buffer (0.05% NaCl, 0.2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% glutin, 0.005 M Tris-HCl, pH 7.5).

Electron microscope and DNA characterization. Fresh cultured phage plaque was prepared using the double agar layer method. Grids were placed on it for about 5 min and 2% uranyl-acetate was used to negatively stain the phage for 2 min. After the excess staining solution was drained with filter paper, the grids were examined with a JEM-100CXII electron microscope (Japan Electron Optics Laboratory Co., Ltd., Mitaka, Tokyo). Phage was identified using the morphological criteria outlined by the International Committee of Taxonomy of Virus (ICTV) (Sagar et al., 1987).

The DNA of phage Esc-A was prepared according to the procedures of Brown et al. (1994). Four restriction enzymes were selected to digest the genome DNA of the phage. The chosen enzymes were *Eco*II, *Hind*III, *Bam*HI, and *Pst*I. The digestions were performed for 2 h at 37°C in 20 μ l reaction volumes containing 8 μ l of genome DNA solution, 2 μ l of the commercially supplied incubation buffer, 9 μ l of water, and 1 μ l (10 U/ μ l) of the restriction enzyme. DNA fragments were resolved in 1% (w/v) agarose gels with $1 \times$ Tris-acetate-EDTA buffer. DNA was visualized by transillumination with UV light after the gels were stained with ethidium bromide.

MIC tests. MICs were determined by standard microdilution procedures, as recommended by the National Committee for Clinical Laboratory Standards (Alzoreky and Nakahara, 2003). Antibiotics were tested at final concentrations (prepared from serial twofold dilutions) ranging from 0.01 to 250 μ g/ml. The MIC was defined as the lowest antibiotic concentration that yielded no visible growth. The test medium was LB broth; the inoculum was 10^6 CFU/ml; the inoculated

trays were incubated for 16 h at 37°C.

Toxicity experiment of phage Esc-A. Three hundred twenty-day-old chickens weighing approximately 450 g were given 10^8 PFU (g body wt) $^{-1}$ daily (group 1). The second group received SM buffer (group 2). Chickens were housed separately and the toxicity experiment was tested in 2 weeks by death rate and weight.

Experimental animals and treatment procedure. Newly hatched Roman chicks were divided into three groups, of 250 chickens each, and raised in separate houses. One group was examined for the protective effects of phage (group 1); another group was examined for the protective effects of antibiotic of chloromycetin (group 2). Each chicken in group 1 was given 10^5 phage orally every day. Group 2 was given chloromycetin to protect against the diarrhea of chickens at a dose of 1 mg (10 g body wt) $^{-1}$. Group 3 was given neither antibiotics nor phage therapy. The rate of death and diarrhea were recorded after 3 weeks.

Results

Bacteriophage isolation and DNA characterization

Bacteriophage Esc-A was isolated from one of the six sewage samples tested. It grew into clear plaques at 37°C, with a plaque of size up to 6 mm (Fig. 1). An electron micrograph of phage Esc-A showed that it had a long tail and an isometric head (Fig. 2). Based on the morphology, the phage belongs to the bacteriophage family of B2. No restriction site was found in genome DNA of phage Esc-A for the first three enzymes. But for the last enzyme, there were six of them, which created seven fragments of 21 kb, 16.5 kb, 6.5 kb, 5.1 kb, 2.9 kb, 2.3 kb and 1.4 kb (Fig. 3).

MIC tests

Among the 9 tested antibiotics, Chloromycetin and neomycin showed low MIC values (3.9 µg/ml and 7.8 µg/ml, respectively). *E. coli* 3-1 exhibited serious resistance against streptomycin, ciprofloxacin and erythromycin (MIC>1 mg/ml, each). Chloromycetin was selected to do the cure experiment for its low MIC (Table 2).

Toxicity experiment

In 2 weeks of trial, no chicken fed with or without phage died. The weight of the chickens in group 1 increased 66.7% on average, compared with 67.5% in

Fig. 2. Electron micrograph of phage Esc-A ($\times 100,000$).

Fig. 3. The patterns obtained after restriction of genomic DNA with *Pst*I (the left is a marker).

Table 2. Comparative activities (MICs) of 9 antibiotics against *E. coli* 3-1.

Antimicrobial agent	MIC (µg/ml)
Chloromycetin	3.9
Gentamicin	>125
Streptomycin	>1,000
Kanamycin	41.5
Neomycin	7.8
Ciprofloxacin	>1,000
Erythromycin	>1,000
Amikacin	>256
Enrofloxacin	>256

group 2. We could conclude that phage was nontoxic and caused no disease in the chickens.

Effect of phage Esc-A therapy on chicken diarrhea

Chicken treated with phage Esc-A had a lower incidence of diarrhea (26% vs. 51.6% in the untreated group, $p < 0.01$). The death rate was 14% in group 3 and 1.2% in group 1 during the first week.

Table 3. The statistical result of different therapies.

	Group ^a	First week		Second week		Third week	
		Number	Rate	Number	Rate	Number	Rate
Infection	1	65	26.00	0	0.00	0	0.00
	2	90	36.00	31	12.40	1	0.40
	3	129	51.60	63	25.20	0	0.00
Death	1	3	1.20	7	2.80	0	0.00
	2	15	6.00	20	8.00	0	0.00
	3	35	14.00	37	14.80	0	0.00
Death caused by bursal disease	1	0	0.00	0	0.00	0	0.00
	2	2	0.80	5	2.00	0	0.00
	3	8	3.20	13	5.20	0	0.00

^a Group 1 was given 10^5 phage orally every day; Group 2 was given Chloromycetin at a dose of 1 mg $(10 \text{ g body wt})^{-1}$; Group 3 was given neither antibiotics nor phage therapy.

Chloromycetin (group 2) was less effective than the phage, exhibiting a rate of diarrhea of 36%, and a death rate of 6%.

In the second week, no diarrhea was found in group 1, while there were still 12.4% and 25.2% diarrhea rates observed in groups 2 and 3. The death rate in the second week was 14.8% in group 3, which was 2 times and 5 times more than that of group 2 and group 1 respectively (Table 3).

In the third week, the diarrhea was only found in group 2 and no death occurred in any other groups.

Discussion

The initial aim of this work was to study whether the effect of phage treatment was comparable to that of antibiotics. The results of this study showed that the phage Esc-A had obvious advantages in treatment compared with antibiotic Chloromycetin.

Phage treatment also had some unique advantages over antibiotics. In our following study, we found chicken treated with phage showed resistance to other intestinal diseases, such as infectious bursal disease. The reason may be that phage treatment had high specificity without affecting other profitable microbes, thus keeping a favorable intestinal micro-ecological balance. In another experiment, we also found that compared with antibiotic treatment, phage treatment could enhance the conversion rate of feedstuff and increase the weight of chickens.

On the basis of the results of the present work, phages lytic to other pathogenic microbes could also be isolated, and used together with phage Esc-A to make this new bio-therapy more efficient and applicable. Because of the restriction site of four enzymes, the phage was very special, and future study should focus on the phage.

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