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EFFECT OF A LYTIC BACTERIOPHAGE ON RABBITS EXPERIMENTALLY INFECTED WITH PATHOGENIC *ESCHERICHIA COLI*

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Abstract: Pathogenic *Escherichia coli* (*E. coli*) is severely threatening the rabbit industry in China, and the concern over antibiotic-resistant bacteria has given rise to an urgent need for antibiotic alternatives. In this study, a member (ZRP1) of the *Myoviridae* family was isolated from rabbit faeces using a strain of rabbit atypical enteropathogenic *E. coli* (ZR1) as host. The one-step growth curve indicated that the latent period was around 25 to 30 min and the burst size was 144±31 plaque-forming unit/cell. The rate of phage-resistant mutation was $7\times10^{-5}\pm4\times10^{-5}$. When the bacteriophage input at the multiplicity of infection (MOI) was 0.1, 1 or 10, the growth of host *E. coli* in broth was inhibited for 5 h. A single intravenous injection of ZRP1 at MOI 0.1, 1 or 10 significantly prolonged the survival time of rabbits which simultaneously received a lethal dose of ZR1.

Key Words: atypical enteropathogenic *Escherichia coli*, bacteriophage, phage therapy, rabbit.

INTRODUCTION

The number of commercial rabbits raised in China is the highest in the world. Based on the FAOSTAT data, the production quantity of rabbit meat of China was 727 000 tons and accounted for more than 40% of the world's total production in 2013. However, development of the Chinese rabbit industry is hindered by some infectious diseases in rabbit, including bacterial enteritis and diarrhoea. Pathogenic *Escherichia coli* strains were reported to be isolated frequently from diarrhoeic rabbits (Penteado *et al.,* 2002) and often have a fatal effect on rabbits.

Antimicrobial drugs have been used to cure bacterial infection of animals, but the abuse and misuse of antibiotics resulted in the emergence of drug resistant bacteria. Because research into new antibiotics was scarce in European Union and United States (Wittebole *et al.,* 2014), widespread appeals for alternatives to antibiotics have been voiced (Cunha *et al.*, 2017).

Bacteriophages or phages are viruses that can kill host bacteria in a different way from antibiotics. The first successful application of phage therapy dates back nearly 100 yr, but it was soon eclipsed by the advent of antimicrobial agents (Chan *et al.,* 2013). However, over the last two decades, phage therapy has been reconsidered as a potentially viable alternative to antibiotics and the UK government was encouraging its development in 2000 (Henein, 2013). By now, there were some works in the literature that described the characteristics of bacteriophages (Chibani-Chennoufi *et al.,* 2004; Jamalludeen *et al.,* 2007) or the efficacy of bacteriophages applied in livestock and poultry (Huff *et al.,* 2005; Zhang *et al.,* 2015), but similar works in the field of rabbit industry were notably absent.

The aim of this study was to isolate and identify a pathogenic *E. coli* strain from a diarrhoeic rabbit and its specific bacteriophage. Experiments *in vitro* and *in vivo* were carried out to assess whether the lytic bacteriophage has the potential for controlling the infection of this pathogenic *E. coli* in rabbits.

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MATERIALS AND METHODS

Isolation and identification of pathogenic E. coli

One pathogenic *E. coli* strain (ZR1) was isolated from the liver of a dead rabbit which came from a diarrhoea-prevalent commercial rabbit farm in Yuyao, Zhejiang Prov., China. Genome of ZR1 was extracted by QIAamp DNA mini kit (QIAGEN, Germany) and 2 multiplex polymerase chain reaction (PCR) were conducted to detect virulence genes (*eaeA*, *bfpA*, *stx1*, *stx2*, *ipaH*, *ST*, *LT* and *aatA*) of the purified strain with a 16S rRNA PCR as control (Chacon-J. *et al.,* 2012). The serogroup of *E. coli* ZR1 was assessed with 17 O antisera, including O1, O18, O26, O44, O55, O86, O88, O111, O114, O119, O125, O127, O128, O142, O146, O151 and O158 (Tianjin Biochip Corporation, China). The susceptibility to 17 (see Table 1) antimicrobials (Hangzhou Microbial Reagent Co., Ltd., China) was tested by Kirby-Bauer disc diffusion test following the procedure of CLSI M100-S25. Biochemical reactions including VP test, H_2S test, utilisations of citrate, lactose, sucrose and D-sorbitol were tested by commercial methods (Hangzhou Microbial Reagent Co., Ltd., China) according to the specifications.

Bacteriophage isolation and purification

Approximately 2 kg of rabbit faeces were collected from the farm and suspended in sterile saline buffer for 10 min. The mixture was then filtered through sterile gauze followed by centrifuging for 20 min at 8000×*g*. One mL of the supernatant was added to 5 mL of tryptic soya broth (TSB; Oxoid Ltd., UK) with *E. coli* ZR1 and incubated overnight at 200 rpm and 37°C. The culture was clarified by centrifugation (20 min; 8000×*g*) and then passed through a 0.22 µm filter (Surevent®, Merck Millipore, USA). The filtrate was properly diluted to examine whether it contained bacteriophages by the double-layer agar method (Zhang *et al.,* 2013). Plaques appearing on the plate were stabbed with a tip and eluted in 0.1 mL PBS. Each bacteriophage sample was serially amplified and isolated for 5 times by the method mentioned above. However, only one phage named ZRP1, which formed the clearest and biggest plaque, was selected for further study.

Table 1: Results of antimicrobial susceptibility tests on *E. coli* ZR1.

	Results (R/I/S)
Ampicillin	R
Cefazolin	R
Gentamicin	I
Tobramycin	S
Cefuroxime	R
Cefepime	S
Cefoxitin	S
Cefotaxime	I
Ceftriaxone	S
Ciprofloxacin	S
Levofloxacin	S
Piperacillin	R
SXT[1]	R
Aztreonam	S
Ceftazidime	S
Chloramphenicol	I
Tetracycline	R

R=Resistant, I=Intermediate, S=Susceptible.
[1] Sulphamethoxazole-Trimethoprim

Electron microscopy

A droplet of the concentrated bacteriophage ZRP1 suspension (1×10^{10} plaque-forming units [PFU]/mL) was applied to a Formvar-carbon-coated copper grid for 10 min. Excess liquid was then removed by a piece of filter paper. The remaining sample on the grid was dyed with 2% phosphotungstic acid (PTA) and examined in a Philips CM100 transmission electron microscope (TEM) at a voltage of 80 kV.

One-step growth curve

One hundred µL of phage ZRP1 (5×10^7 PFU/mL) was added to 0.9 mL of log-phase *E. coli* ZR1 (5×10^7 CFU/mL) suspension and allowed to absorb for 10 min at 37°C. Then it was transferred to a tube with 9 mL of ZRP1 antiserum dilution (1:10) and left to stand for 5 min to neutralise the free phages. The ZRP1 antiserum was prepared by hypodermic injection of 1 mL purified phage suspension (>1×10^{10} PFU/mL) into a healthy laboratory rabbit weekly for 4 wk. Blood was taken from the marginal ear vein and centrifuged for collecting serum after coagulation. After that, the mixture was diluted by 400 times and incubated at 37°C with shaking at 200 rpm. Aliquots were taken at 10 (or 5) min intervals. Titre of each sample was

measured immediately by the double-layer agar method. Burst size is the ratio of the plaque count at the plateau phase to the plaque count at latent phase (Zhang *et al.,* 2013). The trial was repeated for 2 times and averages were calculated.

Determination of the phage-resistant mutant rate

Six sets of 50 µL of *E. coli* ZR1 culture which contained 10^9, 10^8, 10^7, 10^6, 10^5 and 10^4 colony forming units (CFU) each were mixed with 50 µL ZRP1 stock solution (1×10^{11} PFU/mL) respectively and stored in 37°C for 10 min to allow absorption. The mixture was poured over a tryptone soya agar (TSA; Oxoid Ltd., UK) plate surface. After incubation overnight at 37°C, isolated colonies that were considered bacteriophage-insensitive mutants were counted. The frequency of emergence of phage-resistant mutants was calculated as the ratio of the number of surviving colonies to the number of original *E. coli* (O'Flynn, *et al.,* 2004; Pouillot, *et al.,* 2012). All assays were performed in triplicate.

Bacteriostasis of phage at different MOIs in vitro

Bacteriophage ZRP1 and log-phase host *E. coli* ZR1 suspensions were mixed to set the multiplicity of infection (MOI) at 10, 1, 0.1, and 0.01, and the final concentration of bacterial cells was 1×10^8 CFU/mL. Each MOI group had 3 replicates. Mixed suspensions were incubated at 37°C with shaking of 200 rpm for 7 h and aliquots were taken at 1 h interval. The OD_{600} of samples were measured and recorded by a multifunctional microplate reader (Spectramax® M5, Molecular Devices, LLC., USA). In this study, a ZR1 culture (1×10^8 CFU/mL) without phages was used as the control group. Data were analysed by One-Way ANOVA of SPSS 17.0 and least significant difference (LSD) was used as post-hoc multiple comparisons.

Protection against E. coli *experimental infection in rabbits*

Thirty-two 5-week-old rabbits with an average weight of 0.88 kg were equally assigned to 4 groups. The rabbits were injected via marginal ear vein with bacteriophage ZRP1 at different MOIs (10, 1 and 0.1) or sterile saline (Control), respectively, immediately after they received a lethal dose of *E. coli* ZR1 (1×10^{10} CFUs) via intravenous injection. Deaths of experimental rabbits were observed and recorded every hour after infection. Necropsy of each dead rabbit was performed and *E. coli* was re-isolated from livers of dead rabbits by MacConkey agar. Re-isolated *E. coli* was identified by multiple PCR and biochemical reactions as mentioned above. The statistical software SPSS 17.0 was used to analyse data by Kaplan-Meier method of Survival analysis.

Ethics

All animal studies were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996) and were approved by the Animal Care and Use Committee of Institute of Animal Husbandry and Veterinary Science, Zhejiang Academy of Agricultural Sciences.

RESULTS

Characteristics of E. coli *ZR1*

The purified bacteria formed small round red colonies on MacConkey agar. The detection of 16S ribosomal RNA gene of *E. coli* (V3 and V6 regions) was positive, but only a 384 bp band representing *eaeA* gene emerged on the gel when 2 multiplex PCR were performed (Figure 1). *E. coli* ZR1 did not agglutinate with 17 sorts of common

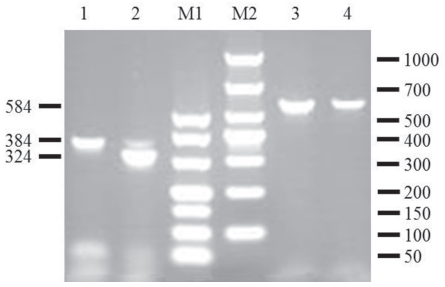

Figure 1: Multiplex PCR and 16S rRNA PCR of *E. coli* ZR1 and a typical EPEC strain (CVCC 1396). Lane M1, DL 500 DNA marker (Takara); Lane M2, DL 1000 DNA marker (Takara); Lane 1, ZR1 (*eaeA*$^+$, 384bp); Lane 2, CVCC 1396 (*eaeA*$^+$ *bfpA*$^+$, 384bp and 324bp); Lane 3, ZR1 (16S rRNA PCR$^+$, 584bp); Lane 4, CVCC 1396 (16S rRNA PCR$^+$, 584bp).

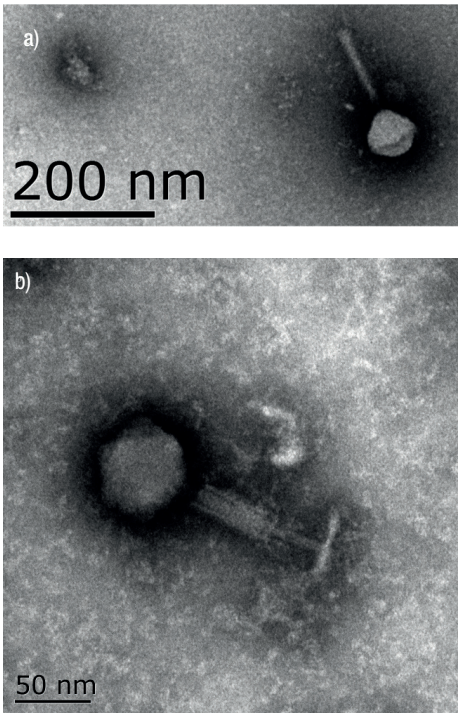

Figure 2: Transmission electron microscopic appearance of bacteriophage ZRP1. (a) Electron micrograph of a phage ZRP1. (b) Electron micrograph of a phage ZRP1 showing its contractile tail.

enteropathogenic *E. coli* (EPEC) O antisera. The results of antimicrobial susceptibility tests demonstrated that ZR1 was sensitive to quinolones, aminoglycosides and the third- or fourth-generation cephalosporins. However, ZR1 was resistant to the first- and second-generation cephalosporins, sulphamethoxazole-trimethoprim (SXT), tetracycline and ampicillin (Table 1). The results of VP, H_2S, citrate and sucrose tests were negative, but the tests on lactose and D-sorbitol were positive.

Isolation and morphology of bacteriophage ZRP1

The ZRP1 plaques were round and clear with 0.5 to 1 mm in diameter on average. Under transmission electron microscopy, the virion (Figure 2a, 2b) had a hexagonal head (65 nm in diameter) and a contractile tail (95 nm in length and 10 nm in width) with a sheath (15 nm in width in contraction).

Latent period, burst size and rate of phage-resistant mutation

The one-step growth curve of bacteriophage ZRP1 was shown in Figure 3. Latent period was defined as the timeframe between the onset of incubation and the initiation of rise period. It was estimated to be approximately 25 to 30 min in the 2 tests, and the mean burst size of ZRP1 was 144±31 (mean± standard deviation [SD]) PFU per infected cell. The mean fraction of ZR1 resistant to ZRP1 was $7\times10^{-5}\pm4\times10^{-5}$ (mean±SD).

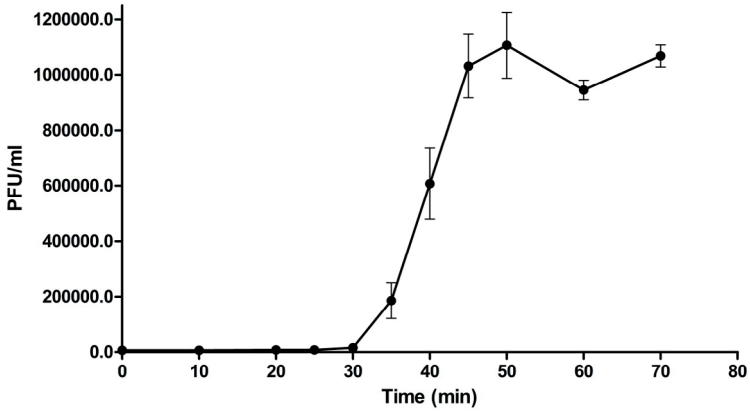

Figure 3: One-step growth curve of phage ZRP1 on *E. coli* ZR1. Titres at different sample times are shown in plaque-forming unit (PFU)/mL. Each point is the mean of 2 independent experiments, and the bar represents standard deviation.

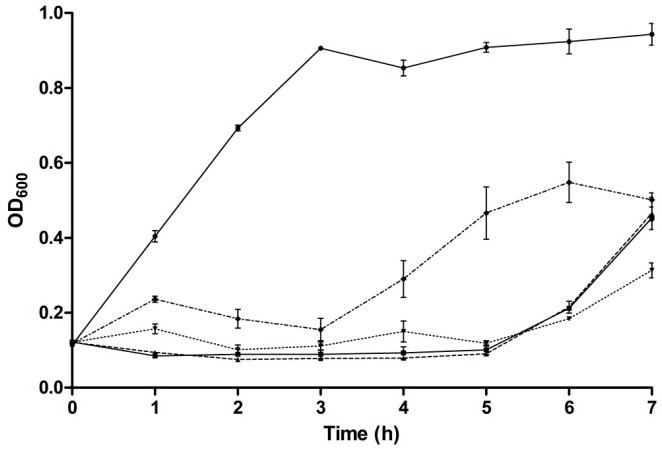

Figure 4: OD_{600} development of *E. coli* ZR1 (1×10^8 colony forming units/mL at the beginning) infected with or without ZRP1 at different MOIs. Each point on the figure is shown as mean±standard deviation of 3 replicates. The initial OD_{600} value was 0.120. OD: Optical density; MOI: Multiplicity of infection. —●— Control; —■— MOI 10; - -▲- - MOI 1; ···▼··· MOI 0.1; -·-◆-·- MOI 0.01.

Growth inhibition of E. coli *at different MOIs* in vitro

The growth of *E. coli* ZR1 without infection reached stationary phase in 3 h, but the growth of ZR1 infected with ZRP1 at any MOI was retarded. The result of One-Way ANOVA and LSD showed significant differences between the non-infected group and infected groups from 1 h to 7 h post-infection (P=0.000). At MOI 0.01, the OD_{600} started to decrease after 1 h but increased again at 4 h post-infection. However, OD_{600} values of MOI 0.1, 1 or 10 remained stable before 5 h post infection but quickly increased after 6 h (Figure 4). The statistical results indicated that OD_{600} values were significantly higher in the MOI 0.01 group than in other MOIs before 6 hr post-infection (P<0.05).

Efficacy of phage therapy in vivo

The experiment was finished at 14 h after the infection and all rabbits died except 2 which survived. One of them was treated with MOI 10 and the other was treated with MOI 0.1. However, both rabbits were dying at that time and showed symptoms such as trembling, depression and clenching teeth tightly. They were humanely killed to alleviate the pain they suffered. Statistical analysis showed that survival times were significantly different between the control group and phage-treated groups, but no significant difference was found among phage-treated groups (Table 2). Necropsy of rabbits in the experiment revealed haemorrhages in the caecal and rectal mucosae. Results of biochemical reactions and PCR identification of *E. coli* re-isolated from all rabbits were as the same as those for ZR1.

Table 2: Survival time of rabbits treated with or without different levels of bacteriophage (mean±standard deviation).

	Control	MOI 0.1	MOI 1	MOI 10
Weight (kg)	0.87±0.25	0.90±0.26	0.87±0.26	0.88±0.26
Survival Time (h)	6.25±0.16[a]	8.37±0.93[b]	9.50±1.12[b]	9.38±1.17[b]

[a,b]Means with no common superscript letter differ significantly (P<0.05).

DISCUSSION

E. coli ZR1 should be classified as atypical EPEC for its *eae*$^{+}$ *bfp* genetic profile (Nataro and Kaper, 1998). It can adhere to the enterocyte membrane, producing the characteristic attaching and effacing (A/E) lesion (Kaper *et al.,* 2004) and therefore be a common cause of diarrhoea in rabbits (Penteado, *et al.,* 2002; Swennes, *et al.,* 2012). Most atypical EPEC strains were reported belonging to traditional EPEC serogroups such as O26, O55, O86, O111, O119, O125 and O128, but quite a few strains could not agglutinate in the usual set of O antisera (Trabulsi, *et al.,* 2002).

Ampicillin and piperacillin could be inactivated by β-lactamase produced by penicillin-resistant *E. coli*, and resistances to tetracycline or SXT were generally found in diarrhoeagenic *E. coli* in south China (Zhang *et al.,* 2017) which might be induced by a selective pressure of antimicrobial misuse.

Analysis of the TEM images suggested that bacteriophage ZRP1 belongs to the family *Myoviridae* for its hexagonal head and contractile sheathed tail (Zhang *et al.,* 2013). Burst size was considered as a critical parameter that affected phage therapy (Skurnik and Strauch, 2006) because the more the progeny were released, the more advantage the phage had. The burst size of ZRP1 was larger than those of coliphages such as the *Myoviridae* family Bp7 (Zhang *et al.,* 2013), *Siphoviridae* family ECP4 (Lee and Park, 2013) and *Podoviridae* family EC200pp (Pouillot, *et al.,* 2012).

It was highly likely that phage-resistant mutants would emerge in a bacterial population of 10^6 to 10^8 (Skurnik and Strauch, 2006). Our result was similar to those reporting rates such as from 3×10^{-4} to 1.9×10^{-6} (O'Flynn *et al.,* 2006) or 7×10^{-6} (Pouillot *et al.,* 2012).

OD_{600}-based assay was commonly used to assess or to select bacteriophages before attempting in animal trials (Alam *et al.,* 2011; Han *et al.,* 2013). Obviously, *in vitro* experiments demonstrated that using ZRP1 above a certain MOI threshold immediately inhibited the growth of host bacteria, and the results indicate that ZRP1 performed better against ZR1 at a higher MOI than a lower one. However, the regrowth observed after several hours indicated the substitution of phage-resistant bacteria (Tanji, *et al.,* 2004). Intravenous injection of pathogenic *E. coli* ZR1 caused haemorrhages of the large intestine and led to a systemic infection involving the liver. Bacteriophages injected intravenously could rescue experimental animals (Cerveny *et al.,* 2002) and MOIs between 0.01 and 100 are often used in *in vivo* experiments (Mai Huong Ly-Chatain, 2014). A dose-response effect was observed (Wang *et al.,* 2005) and it was suggested that phage should be given as large a dose as possible if it were not for side effects in phage therapy (Payne and Jansen, 2003). Nevertheless, increasing the phage level did not show a significant advantage in our *in vivo* experiment. Unfortunately, although survival time of rabbits was prolonged by phage treatment, almost all rabbits died in the experiment. One possible explanation might be that the infection was too serious to save. Phage-resistant mutants should also be taken into account, as they might cause the failure of phage therapy (Smith and Huggins, 1983).

In general, ZRP1 proved to be effective against ZR1 *in vitro* and *in vivo*. This paves the way for further research into the efficacy of ZRP1, considering different infecting doses and pathways of *E. coli* ZR1.

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