SPANDIDOS PUBLICATIONS

Therapeutic effectiveness of bacteriophages in the rescue of mice with extended spectrum ß-lactamase-producing *Escherichia coli* bacteremia

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Received October 11, 2005; Accepted November 24, 2005

Abstract. The emergence of multidrug-resistant bacteria has become a global crisis. Accumulating evidence shows that bacteriophages (phages) can rescue animals from a variety of lethal infections and be effective in treating drug-resistant infections in humans. Enterobacteriaceae, producing extended spectrum ß-lactamase enzymes (ESBLs), are resistant to a broad range of ß-lactamase antibiotics. One of the most common ESBL-producing gram-negative bacilli in Enterobacteriaceae is *Escherichia coli*. Since ESBL-producing *E. coli* poses a formidable challenge in the management of critically ill patients with bacterial infections, we undertook this study to explore the possible therapeutic utility of phages to control ESBL-producing *E. coli* infections. The phage Ø9882 used in this study was isolated from our hospital sewage and has lytic activity against a broad range of clinical isolates of ESBL-producing *E. coli*. ESBL-producing *E. coli* strains (n=30) were isolated in the clinic, and one of them was used to induce bacteremia in a murine model. Bacteremia was established by intraperitoneal (i.p.) injection of $3x10^7$ CFU/ml, the minimum lethal dose (MLD) of bacterium in this animal model. Mice infected with the MLD of this strain alone died within 14 h, whereas a single i.p. inoculation of Ø9882 (MOI $\geq 10^{-4}$) given 40 min after the bacterial challenge led to 100% survival at 24-168 h, compared to 0% survival of saline-treated controls. Protection was obtained even when administration of the phage was delayed up to 60 min after the bacterial infection and the survival rate of infected animals was 60% at 168 h. Furthermore, it was shown that the therapeutic efficacy of Ø9882 in lethal systemic infection in our model is due to the functional capability of the phage and not the nonspecific immune effects. Our data both *in vitro* and *in vivo* revealed that: i) the protection of mice from death occurred only in animals infected with selected bacterial strains and the virulent phage specific to them; ii) when the phages were heat-inactivated, survival of the infected mice was strikingly decreased to 0; and iii) the level of antibody against the phage was not substantially elevated when the bacteremic animals were protected by the phage. The present findings indicate that phages can effectively rescue our mouse model from bacteremia and death, and thus provide the rationale and framework to evaluate the therapeutical efficacy of lytic phages against fatal ESBL-producing *E. coli* infections in humans.

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Key words: phage therapy, animal model, mouse, *Escherichia coli*, bacteremia, antibiotic resistance, extended spectrum ß-lactamase enzymes

Introduction

In 1983, Knothe and colleagues reported the first isolation of Klebsiella and Serratia strains that had transferable plasmids encoding mutated enzymes that made the bacteria resistant to cefotaxime and structurally related ß-lactams (1). The major mechanism for the spread of antibiotic resistance among Enterobacteria and other bacterial species is through the transfer of resistant plasmids, which occurs between bacterial strains by the process of conjugation (2). The ability of conjugation to mediate resistant plasmid transfer among different bacterial species is of central clinical importance. Extended spectrum ß-lactamase enzymes (ESBLs) are believed to have mutated from a variety of plasmid-mediated

penicillinases and can inactivate third-generation cephalosporins (ceftazidime, cefotaxime, and ceftriaxone), as well as monobactams such as aztreonam (1,3). Such enzyme production often coexists with resistance to aminoglycosides, co-trimoxazole, tetracyclines and quinolones. Due to the carriage on plasmids and the promiscuous exchange of such material between bacteria, these resistance genes, such as TEM-1, TEM-2 and SHV-1, have spread widely and are also subject to mutation (2,4-7).

Escherichia coli is one of the most common ESBL-producing bacteria worldwide with a different degree of drug resistance in each country (8-11). The prevalence of ESBL-producing *E. coli* and other ESBL-producing Enterobacteriaceae is a dilemma for clinicians because multiple drug resistance is present in most of the clinical isolates of these organisms (12,13). Antibiotic resistance of *E. coli* contributes significantly to the problem of nosocomial infections, including urinary tract infections, respiratory tract infections and bacteremia, particularly in elderly or debilitated patients. Infections caused by ESBL-producing bacteria are difficult to detect by current susceptibility tests, and usually lead to high rates of morbidity and mortality in many areas of China (3,9,10). The options for antibiotic therapy are limited in clinical practice. Therefore, the development of alternative antibacterial approaches are necessary for the treatment of a broad array of antibiotic-resistant infectious diseases.

Phages (bacteriophages) are viruses that are harmless to humans but kill bacteria, and phage therapy is a method of harnessing phages as bioagents for the treatment of bacterial infectious diseases. Phage therapy was originally introduced ~80 years ago by Felix d'Herelle, one of the discoverers of phages, and showed promise but also aroused controversy (14). Early applications of antibacterial phage therapy (1920s to 1940s) were impeded by a number of factors, including: i) a paucity of understanding of the heterogeneity and ecology of the phages involved; ii) failure to select phages of high virulence against the target bacteria before using them in patients; iii) the use of single phages in infections by mixing several different bacteria; iv) emergence of resistant bacterial strains that occurred via a resistant mutation or lysogenization; v) failure to appropriately characterize or titrate phage preparations, in which some were inactive; vi) failure to neutralize gastric pH before oral phage administration; (vii) inactivation of phages by both specific and nonspecific factors *in vivo*; viii) liberation of endotoxins as a consequence of widespread lysis of bacteria within the body; and ix) lack of identification of availability or reliability of phage therapy. In addition, most early research into the therapeutic use of phages was poorly organized or uncontrolled, and the basic understanding of phage biology was immature (15). These factors in combination produced a negative outcome for phage therapy. Phage therapy was later abandoned in Western countries because the mass production of several effective antibiotics was clinically applied in the 1940s (16-19). However, extensive clinical research and implementation of phage therapy continued in Eastern Europe over the last 50 years (16-21). Although much work was done and some encouraging results were obtained, there was no confirmation of the validity of phage therapy in the clinic. In the past decade, there has been a revival in phage remedy because of the problem of antibiotic resistance (14,24-27). With the emergence of antibiotic-resistant bacteria such as ESBL-producing *E. coli*, VRE (vancomycin-resistant *Enterococcus*), and MRSA (methicillin-resistant *Staphylococcus aureus*), there is a need to explore the potential therapeutic applications of phages. We focused our efforts on ESBL-producing *E. coli* because it is a clinically important multidrug-resistant pathogen and the information regarding phage therapy for ESBL-producing *E. coli*-induced infections is scant. In this study, we report that phages are able to infect and kill the majority of clinical isolates of ESBL-producing *E. coli in vitro*. Our study also showed remarkable efficacy in phage therapy when treating mice with pernicious ESBL-producing *E. coli* infections without adverse effects, thereby suggesting a potential clinical application of phages to control ESBL-producing *E. coli*-induced infectious diseases in humans.

Materials and methods

Culture media. LB medium and SM buffer were prepared according to Sambrook *et al* (28). TSBM was TSB medium supplemented with 20 mM $MgCl_2$, and PEG/NaCl was 20% PEG-8000 (w/v) supplemented with 2.5 M NaCl. LB-based solid medium containing 1.5% agar and 0.7% agarose was used for the lower and upper layers, respectively. DNAse I, RNAse A, and proteinase K were purchased from Becbo, Sigma, and Amresco, respectively.

Bacterial strains. The bacterial strains used in our study included 30 ESBL-producing *Escherichia coli* strains. All samples of ESBL-producing *E. coli* strains were derived from clinical specimens obtained from patients at the Tongji Hospital. In the present study, ESBL-producing *E. coli* 9853 served as the experimental target of our phage Ø9882 (with a broad host range), unless otherwise stated, as ESBL-producing *E. coli* 9853 was sensitive to most of the isolated phages, including Ø9882 (see below). Three other ESBL-producing *E. coli* strains were established for the animal experiments. Bacterial growth was monitored by measuring turbidity using a UV-2000 spectrophotometer. A 0.5 OD_{600} value was assumed to be equivalent to $2x10^8$ *E. coli* cells/ml. This conversion formula was based on a previously standardized correlation between turbidity and bacterial cell numbers counted directly by quantitative plating.

Isolation and purification of phage strains. The 30 ESBL-producing *E. coli* strains mentioned above were used as hosts to isolate specific phages from raw sewage obtained from the Sewage Treatment Center of Tongji Hospital. The isolations, performed similarly but individually for each phage strain according to Biswas *et al*, were accomplished by adding salt (58 g of NaCl) to 1 liter of sewage, followed by centrifugation at 10,000 x g for 10 min (29). The supernatant was decanted into a separate container and mixed with polyethylene glycol (PEG; molecular weight, 8,000) to provide a final PEG concentration of 10% (w/v). The PEG-containing supernatant was precipitated overnight at 4˚C and centrifuged at 12,000 x g for 20 min. The resulting precipitate was dissolved in 5 ml of phage dilution buffer (SM) and extracted once with an equal volume of chloroform. An aliquot (300 μl) of this processed

was mixed with 200 μl of an overnight culture of ducing *E. coli* strains, incubated at 37˚C for 20 min, mixed with 3 ml of molten top agar (0.7% agarose) at 50˚C, and poured onto LB agar plates (1.5% agar; see above). Plates were incubated overnight at 37˚C. Phage plaques were harvested from the plate, and single plaques were purified 3 times on host strains using standard procedures described by Sambrook *et al* (28).

Large-scale amplification and purification of phage particles. Phage Ø9882 was purified according to the procedure described by Sambrook *et al*. ESBL-producing *E. coli* 9882 host cells suspended at $2x10^8$ cells/ml in 20 ml LB medium were exposed to a crude preparation of Ø9882 at an MOI of 0.01 and vigorously shaken for 4-5 h at 37˚C, resulting in the complete lysis of bacteria (28). After 10 min treatment with 1% chloroform at 37˚C, and treatment with 1 μg/ml DNase I and 1 μg/ml RNase A for 30 min at 37˚C, the culture fluid was centrifuged at 12,000 x g for 10 min at 4˚C to remove cell debris. PEG/NaCl was added to the supernatant to a final concentration of 1/6 (v/v) and kept overnight at 4˚C. The resultant precipitate containing the phage particles was collected by centrifugation at 10,000 x g for 20 min at 4˚C, resuspended in 500 μl SM buffer, and the phage suspension was placed on top of a discontinuous CsCl gradient (ϱ=1.3, 1.5, and 1.7), then centrifuged at 25,000 x g for 2.5 h at 4˚C (Beckman SW41 rotor). The phage band was collected and dialyzed against 10 mM saline that contained 50 mM Tris-Cl (pH 8.0) and 10 mM $MgCl_2$ for 2 h at 4˚C. CsCl-gradient separation and dialysis (1 h) were repeated. The purified phage suspension was divided into aliquots and stored at 4˚C until used. The samples were appropriately diluted with LB just before use for infections. The titers (PFU/ml) of purified samples were determined by inoculating them into *E. coli* 9882 strain.

Screening phage with broad host range. To select the phage with a broad host range *in vitro*, the 30 ESBL-producing *E. coli* strains were used to make lawns on a solid culture. The back of the plate was divided into 16-20 panes, and all panes were marked with the name of corresponding phages. Phages (1 μl) were dropped onto the plates and cultured at 37˚C for 12-16 h.

Biological characterization of phage Ø9882. The adsorption rate, latent period, and burst size of Ø9882 were determined according to the method of Adams (30). All incubations were carried out in LB medium at 37˚C. In brief, to examine the adsorption rate, Ø9882 ($5x10^9$ PFU/ml) was mixed with ESBL-producing *E. coli* 9882 cells ($5x10^7$ CFU/ml), and the number of free infectious phage virions was measured in the phage-cell mixture diluted 1,000 times. To determine the latent period and burst size, *E. coli* 9882 cells ($5x10^6$ CFU/ml) were exposed to Ø9882 ($5x10^7$ PFU) for 5 min, washed thoroughly with cold TSBM medium to remove unbound phages, and resuspended in fresh medium. An aliquot of the cell suspension was harvested regularly during incubation at 37˚C to be titrated for newly produced phages, both released and cell-associated phages, on a lawn of ESBL-producing *E. coli* 9882.

Animal experiments. Phage Ø9882, screened as having the broadest host range, was used to rescue bacteremic mice. ESBL-producing *E. coli* 9853, one of our 30 ESBL-producing *E. coli* strains, was used to infect mice as it was sensitive to most isolated phages, including Ø9882. Varying numbers of bacterial cells suspended in 0.5 ml saline were injected into the peritoneal cavities of mice through one side of the abdomen, and the purified phage suspensions in 0.5 ml LB medium were injected on the other side. As controls, equal volumes of saline or LB alone were injected intraperitoneally on all test occasions. The test animals were observed for 7 to 30 days.

Establishing the MLD in the mouse model. The experimental animals (6-8 weeks old; body weight 20±0.5 g), BALB/c female mice (5 mice in each group), were used for infection experiments. Preparation of the infecting bacteria was as follows. ESBL-producing *E. coli* 9853 were grown in 100 ml LB medium at 37˚C and centrifuged at 8,000 x g for 5 min at an early stationary phase (0.5 OD_{600} value). The cell pellet was washed with 100 ml saline, centrifuged again under the same conditions, and resuspended in 5 ml saline. After the appropriate dilution, turbidity was measured to determine bacterial cell numbers, as described above. To determine the minimum lethal dose (MLD), serial dilutions of *E. coli* 9853 were injected intraperitoneally (i.p.) into mice in 500 μl aliquots. The animals were observed for 7 days.

Protective effects of phage administration against ESBL-producing E. coli in mice. The effect of dosage on the ability of the phage to rescue mice from ESBL-producing *E. coli* bacteremia was determined. In the dose-ranging study, 11 groups of mice were challenged by i.p. injection of the MLD of ESBL-producing *E. coli* 9853. Each of these groups was treated with a single i.p. injection of phage Ø9882, administered immediately after the bacterial challenge at MOIs (multiple of infection) of 0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 100, and 200.

Effect of delay in treatment on the ability of the phage preparation to rescue bacteremic mice. In the experiment of delayed treatment, we studied the effect on outcome for various periods. The treatment (a single injection of phage Ø9882 at the highest dose) was initiated at 0, 20, 40, 60, 180, and 360 min after the bacterial challenge with the MLD of *E. coli* 9853. The health of these animals was monitored for 30 days.

Effects of heat-inactivated phage. An experiment was performed to determine whether phage rescue of mice with ESBL-producing *E. coli* bacteremia requires a phage that can grow on the bacterial host or might be associated with a nonspecific immune activation response. A sample of phage Ø9882 with a titer of $6x10^9$ PFU/ml was heat inactivated by incubation at 100˚C. Phages that had been heated for a total of 20 min, at which time no viable phage was detectable, were used to determine whether the phage rescue of mice with ESBL-producing *E. coli* bacteremia requires functional phage or might be associated with a nonspecific immune activation response. The mice in this study were divided into three groups of 5 mice each. All mice were challenged by

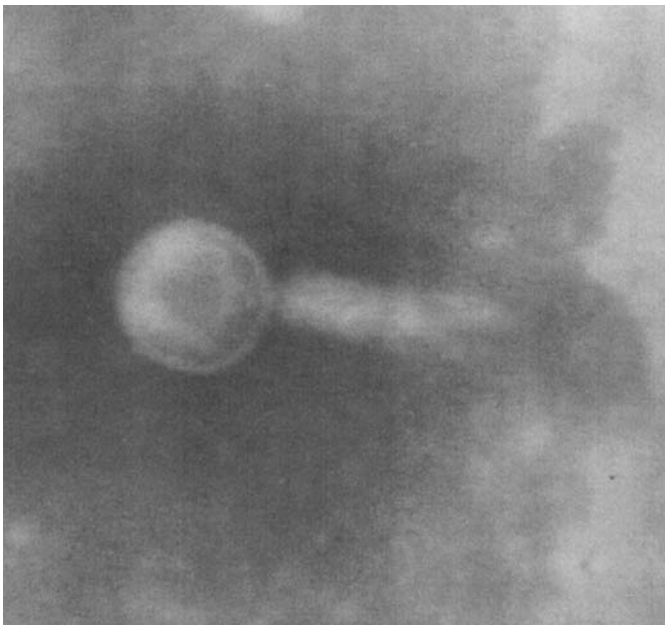

Figure 1. Electron micrograph of phage Ø9882 (bar, 100 nm). The ultrastructure under electron microscopy demonstrated that phage Ø9882 possesses a round-shaped head of 70 nm in diameter and a noncontractile tail of 100 nm in length, with a knob-like structure at its distal end.

i.p. injection of the MLD of ESBL-producing *E. coli* 9853. The first group was treated with a single i.p. injection of $6x10^9$ PFU/ml of phage Ø9882 immediately after the bacterial challenge. The second group was treated with an i.p. injection of $6x10^9$ PFU/ml heat-inactivated phage Ø9882 particles immediately after the bacterial challenge. The third group, used as a control, received a single i.p. injection of LB medium instead of phage, administered immediately after the bacterial challenge.

Titers of phage Ø9882 and E. coli 9853 in the bloodstream. Approximately 0.5 ml of blood was taken by puncturing the orbital plexus of test mice with a capillary tube and mixed immediately with 50 μl heparin (1,000 U/ml). After heparinization, the blood was diluted with saline, colony-forming units of ESBL-producing *E. coli* 9853 were measured on LB plates, and plaque-forming units of phage Ø9882 were measured by using ESBL-producing *E. coli* 9882 as the host on LB plates. Blood samples were also collected from untreated mice to ensure that mice used in the experiments were free of naturally or accidentally contaminated phages or bacteria.

Measuring the immune response to the phage. At various time points following a single i.p. injection of phage Ø9882 (10^9 PFU/ml), mouse serum was prepared, and indirect enzyme-linked immunosorbent assays (ELISAs) were performed. For these assays, 2-fold serial dilutions of serum were prepared in 96-well polystyrene microtiter plates precoated with 10^9 PFU/ml of phage Ø9882. Immunoglobulins were detected with goat anti-mouse IgG-specific antibodies conjugated to peroxidase and tetramethylbenzadine (TMB) peroxidase substrate.

Electron microscopy. Phage preparations purified by cesium chloride density centrifugation were deposited on 400 mesh copper grids, and stained negatively with 2% uranyl acetate (pH 4.0). Grids were air-dried, and electron micrographs were taken with a transmission electron microscope (Hitachi JEM-2100C; Hitachi).

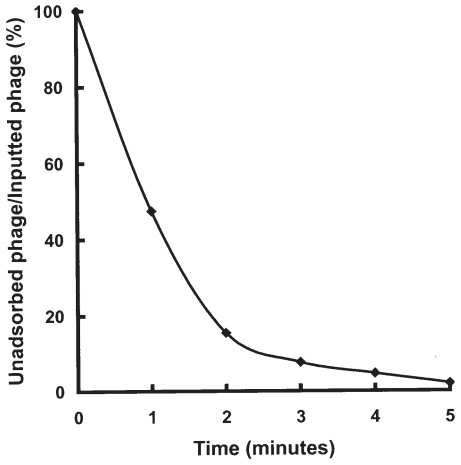

Figure 2. The adsorption rate of phage Ø9882. Phages were mixed with excess *E. coli* 9882 cells, and the nonadsorbed infectious phages were serially counted. Data are percentages of nonadsorbed Ø9882 relative to the initial input dose of phages.

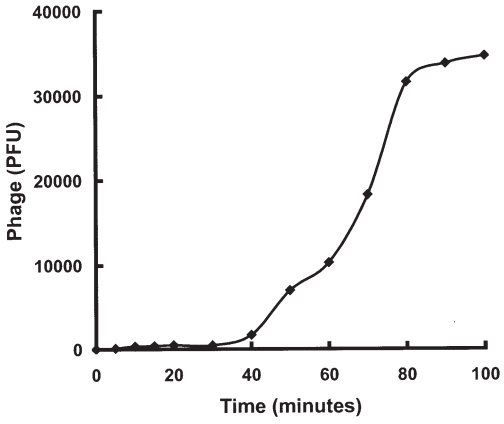

Figure 3. The one-step curve of phage Ø9882. After a 5-min adsorption of Ø9882 to a sufficient number of *E. coli* 9882 cells, the Ø9882-exposed bacterial cells were thoroughly washed with tryptic soy broth medium supplemented with 20 mM $MgCl_2$ at 4˚C to remove free phages and then resuspended in fresh medium. Newly synthesized phage virions, including both released and cell-associated virions, were then measured at regular intervals during incubation using harvested culture samples.

Results

Isolation and screening of E. coli-specific phages with therapeutic potential. A total of 30 phage strains were isolated from the Tongji Hospital sewage, and cultures of 30 ESBL-producing *E. coli* were subjected to the spot test. Of the phage strains isolated in these experiments, phage Ø9882 was found to form plaques on 36.67% of the ESBL-producing *E. coli* clinical isolates. Phage Ø9882, screened as having a broad host range, caused bacteriolysis of 11 ESBL-producing *E. coli* strains, 1068, 9716, 9719, 9539, 9853, 9854, 9860, 9882, 9730, 9739, and 9914.

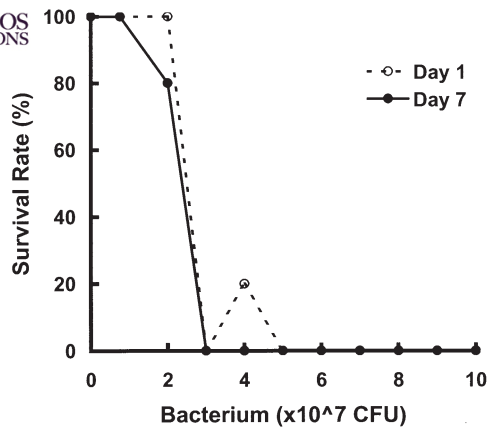

Figure 4. Determination of the lethal challenge dose of ESBL-producing *E. coli* 9853 to mice. Serially diluted suspensions of ESBL-producing *E. coli* 9853 cells were injected intraperitoneally into mice, and mouse fatalities were observed. White and black circles represent the survival rates at 1 and 7 days after injection, respectively. In the control experiment, denoted '0' on the horizontal axis, only 0.5 ml of saline was injected.

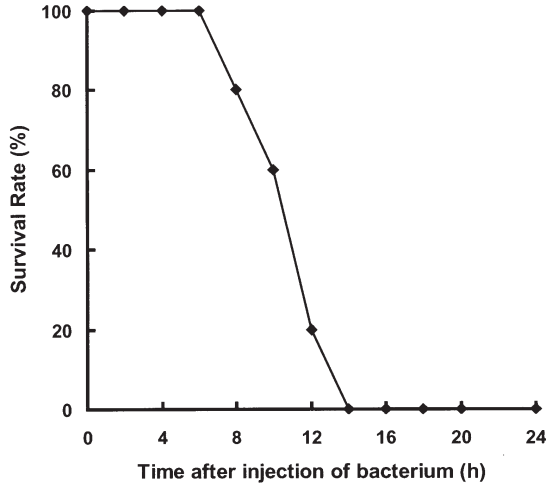

Figure 5. A precise time-chase analysis of *E. coli*-induced killing of mice. Five mice were each inoculated with a fixed number of ESBL-producing *E. coli* 9853 cells ($3x10^7$), and the resulting bacteremia was fatal within 14 h.

Morphological, biological, and genetic characterizations of phage Ø9882. Phage size measurements were performed directly on micrographs at a x70,000 magnification. As shown in Fig. 1, the ultrastructure under electron microscopy demonstrated that phage Ø9882 possesses a round-shaped head of 70 nm in diameter and a noncontractile tail of 100 nm in length, with a knob-like structure at its distal end. Restriction-enzyme (Ecor V) analysis revealed that a Ø9882 genome with an expected size of 30 kb. Biological studies clarified other features of Ø9882 (Figs. 2 and 3), such as: i) a rapid adsorption rate (when sensitive bacterial hosts were present in sufficient numbers, 98% of Ø9882 particles bound to them within 5 min); ii) a short latent period (30-40 min); and iii) a relatively large burst size (~112).

Based on the evidence cited above, Ø9882 was considered to be a suitable therapeutic phage candidate for the treatment of human ESBL-producing *E. coli* infections. Therefore, the following experiments were undertaken using Ø9882 in an animal model.

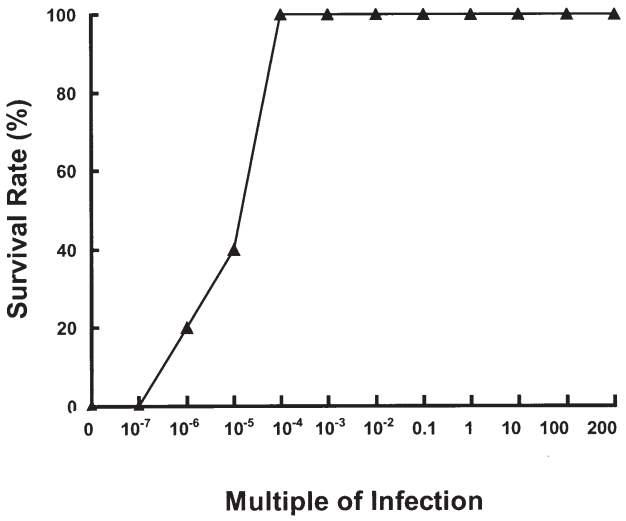

Figure 6. Dose-response effect of phage Ø9882 in rescuing mice from lethal ESBL-producing *E. coli* 9853 bacteremia. After injection of $3x10^7$ *E. coli* 9853 cells, Ø9882 was injected into the mouse peritoneal cavity at various MOIs, and the fate of the mouse was observed. Mice injected with only LB broth, which was used to prepare the phage suspensions, served as control mice and are represented by circles at an MOI of 0. There is a statistically significant difference in survival rates between mice treated with Ø9882 at an MOI ≥0.0001 (10^{-4}) and untreated control mice ($P<0.001$).

Mouse model of ESBL-producing E. coli 9853-induced disease. The dose of ESBL-producing *E. coli* lethal to mice was determined by injecting mice with varying numbers of ESBL-producing *E. coli* 9853, ranging from $1x10^7$ to $1x10^8$ CFU/ml (Fig. 4). Intraperitoneal (i.p.) injections of $1x10^7$-$2x10^7$ CFU/ml ESBL-producing *E. coli* 9853 did not reduce the survival rate of mice during the subsequent 7-day observation period. In contrast, injections of $3x10^7$-$1x10^8$ CFU/ml reduced the survival rate in a dose-dependent manner. Because the injection of $3x10^7$ ESBL-producing *E. coli* 9853 cells was fatal in 100% of mice within 24 h, this level of challenge was considered to be optimal for observing the phage effect on bacterial lethality (see below). Therefore, the dose of ESBL-producing *E. coli* at $3x10^7$ CFU/ml was chosen from the results mentioned above. A more precise time-chase analysis showed that i.p. injection of $3x10^7$ CFU/ml ESBL-producing *E. coli* 9853 cells killed most mice within 8 to 14 h after injection, with the associated preceding bacteremia (Fig. 5 and below). The dissection of mice that died from bacterial infection 6 h after injection revealed severe systemic congestion with splenomegaly and acute ascites (data not shown). As seen in Fig. 5, all mice i.p. inoculated with the MLD ($3x10^7$ CFU/ml) of the clinical isolate ESBL-producing *E. coli* 9853 died within 14 h.

Ability of the phage preparation to rescue mice from bacteremia. The phage preparations were purified by cesium chloride density centrifugation mentioned above. A single dose of phage Ø9882 was administered immediately after the challenge with the MLD of ESBL-producing *E. coli* 9853. By 24 h, a dose-response effect on the state of health of the infected animals was clearly visible. At higher phage doses, which achieved multiplicities of infection of 10^{-4} to 200, 100% of the animals survived, and only minimal signs of illness (mild lethargy) were seen in the first 24 h. As the

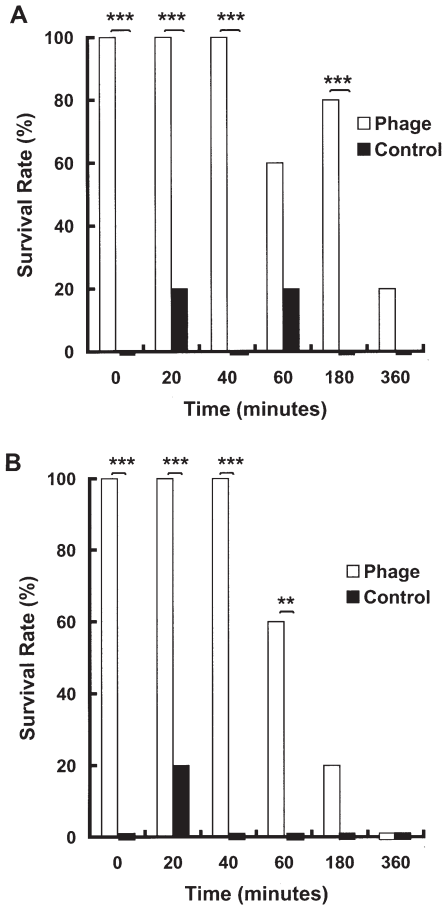

Figure 7. Protective effects with delayed administration of phage Ø9882. Purified Ø9882 (MOI, 200) was administered to 5 mice at the indicated time intervals, after challenge with ESBL-producing *E. coli* 9853 ($3x10^7$ cells). As a control, 0.5 ml of phage-free LB was injected into mice. Survival rates were determined after 1 (A) and 7 (B) days. White and black columns represent the phage-treated and untreated mouse groups, respectively. Asterisks indicate statistically significant differences compared with that of the controls: $^{**}P<0.01$ and $^{***}P<0.001$.

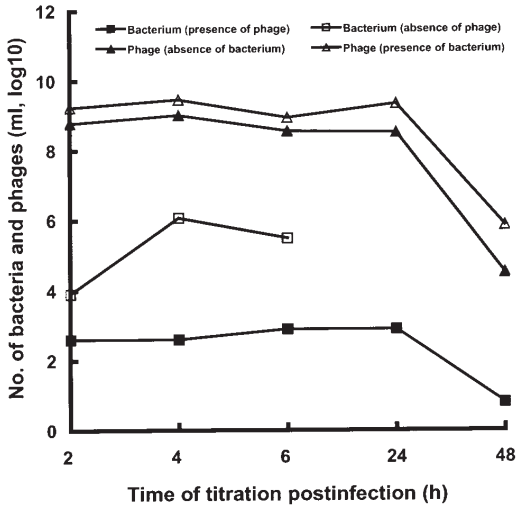

Figure 8. Rapid appearance of phage Ø9882 in circulation. Target bacteria ($3x10^7$ cells) and/or Ø9882 (MOI, 200) were i.p. injected into 3 groups of mice, each consisting of 5 mice. ESBL-producing *E. coli* 9853 was used as the target in this experiment. A peripheral blood sample was taken from 1 mouse of each group at 2, 4, 6, 24 and 48 h after injection and titrated to estimate the numbers of phages and bacteria in circulation. Black and white triangles represent plaque-forming units of Ø9882 in the presence and absence of ESBL-producing *E. coli* 9853, respectively. Black and white squares represent colony-forming units of the host bacteria with and without phage administration, respectively. All mice injected with only ESBL-producing *E. coli* 9853 died during the observation period within 6 h of injection.

phage dose decreased, resulting in lower multiplicities of infection (10^{-7} to 10^{-6}), the animals became critically ill, with survival rates of 0% and 20%, respectively, at day 7 and beyond (Fig. 6). All of the mice that were alive and healthy at day 7 remained this way for an additional 30 days, at which point the experiment was terminated.

Effect of delay in treatment on the ability of the phage to rescue mice from bacteremia. Purified Ø9882 was i.p. administered to mice at different times (up to 360 min) after the ESBL-producing *E. coli* 9853 injection (Fig. 7). Although Ø9882 administration proved to be effective at MOIs ranging widely from 0.0001 to 200 (Fig. 6), the highest MOI of 200 was applied, and subsequent investigations were conducted (see below) under the assumption that patients were in an extreme state of systemic ESBL-producing *E. coli* infection such as sepsis. All mice survived when treatment was delayed 40 min, and approximately 60% of mice survived even when the treatment was delayed for 1 h. When treatment was delayed 360 min, most of the mice treated with phage Ø9882 were dead. The therapeutic efficacy of Ø9882 was even discernible in mice treated 60 min after injection with bacteria, when all control mice injected with *E. coli* 9853 already exhibited signs of physical deterioration, such as reduced activity and ruffled hair. Although a few Ø9882-treated mice died in the following 6 days, the survival rates among mice treated with the phage at any time point were significantly higher than those of the untreated controls (Fig. 7).

Distribution of phage Ø9882 and E. coli 9853 in vivo. On the basis of the above results, the *in vivo* dynamics of the bacteria and phages were investigated in detail using ESBL-producing *E. coli* 9853 as the target cell. Mice were i.p. injected with $3x10^7$ CFU/ml *E. coli* 9853 alone, *E. coli* 9853 plus Ø9882 ($6x10^9$ PFU/ml), or Ø9882 alone, respectively. Bacteremia occurred within 2 h and persisted in the *E. coli* 9853-injected mice, regardless of phage treatment (Fig. 8). However, bacterial loads in the blood were significantly lower in Ø9882-treated mice than in untreated mice in our observations (Fig. 8). Compatible with the results shown in Fig. 8, all mice injected with only *E. coli* 9853 died within 12 h, whereas Ø9882-treated mice were invariably saved, concomitantly with subsiding septicemia within 24 h. After Ø9882 was injected into the peritoneal cavity, a significant number of infectious Ø9882 was readily detected 2 h later in blood specimens from both the *E. coli* 9853 infected and uninfected mice with titers of $6.0x10^8$ and $1.7x10^9$ PFU/ml, respectively. Under the circumstance of systemic dissemination of phage Ø9882, the circulating Ø9882 was sustained at a significant level until the target cells were eradicated, which must have counteracted the progression of bacteremia.

Effects of heat-inactivated phage. An experiment was performed to determine whether the phage rescue of mice with ESBL-producing *E. coli* bacteremia requires a functional phage or might be associated with a nonspecific immune

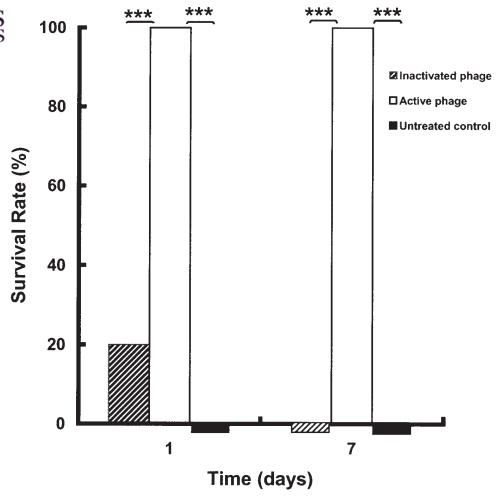

Figure 9. Comparison of the ability between active phage and nonfunctional heat-inactivated phage to rescue bacteremic mice. One group of 5 mice was treated with functional (plaque-forming) phage, while the other two groups were treated either with no phage or heat-inactivated phage. All bacteremic mice treated with functional phage survived. In contrast, only 20% of the mice in heat-inactivated groups survived at 24 h, and all of them died within 7 days; no mice from the control group survived. A statistically significant difference (P<0.001) in survival rates was observed between paired groups of active phage-treated mice and heat-inactivated phage-treated or phage-untreated control mice, as signified by horizontal brackets with asterisks.

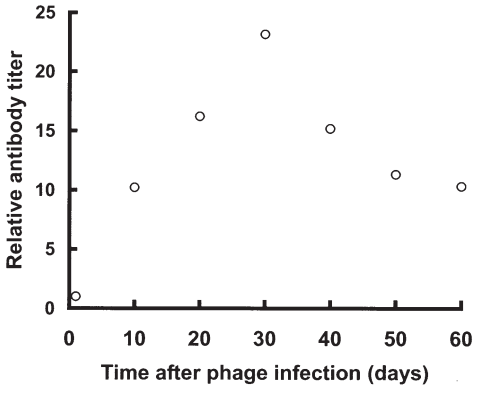

Figure 10. Antibody responses in mice to a single injection of phage Ø9882. Phage Ø9882 (10^9 PFU) was i.p. injected into mice. Mouse serum was collected at varying time points following a single i.p. injection of Ø9882, and indirect enzyme-linked immunosorbent assays (ELISAs) were performed. The titers of anti-Ø9882 IgG were detected with goat anti-mouse IgG-specific antibody and are indicated on the plot.

activation response. Heat-inactivated phages were used to test whether a functional phage and not a nonspecific immune response is responsible for the rescue of mice inoculated with a lethal dose of ESBL-producing *E. coli* 9853. Heating at 80˚C for 2 min decreased the phage titer by 100-fold, and no viable phage was detected after heating at 100˚C for 30 min. As illustrated in Fig. 9, only mice inoculated with plaque-forming phage had enhanced survival, with 100% survival at 7 days. By contrast, none of the PBS control mice and 0% of the mice injected with heat-inactivated phage survived.

Immune response to phage Ø9882. After a single injection of phage Ø9882, titers of IgG raised against the phage increased 23-fold above background (Fig. 10). IgG levels did not change substantially after 30 days. No anaphylactic reactions, changes in core body temperature, or other adverse events were observed in the mice injected with the phage.

Discussion

Pathogenic bacteria that are resistant to most, if not all, available antibiotics are responsible for increasingly serious problems in clinical settings, raising widespread fears of returning to a 'pre-antibiotic' era of untreatable infections and epidemics. As evidence, the prevalence of organisms producing ESBLs has been increasing worldwide. ESBL-producing *E. coli* is broadly resistant to third-generation cephalosporins. Based on the current experience, imipenem (Carbapenems) appears to be the best alternative choice for the treatment of severe infections caused by ESBL-producing *E. coli* (31). However, with the excessive and widespread use of imipenem, there exists the possibility of an emergence of resistant organisms to antibiotics in the near future. Confronted with the dilemma in the clinic, it is necessary to explore the potential therapeutic applications of phages. Matsuzaki *et al* (32) and Biswas *et al* (29) used phages to treat experimental MRSA and VRE infections in mice, respectively, and both achieved encouraging results, showing that phages are effective in treating bacterial infections in animal models and provide a reliable alternative therapeutic approach. These studies reported some important insights into the potential of phage therapy against various bacterial infectious diseases.

In the current study, we demonstrated that phage Ø9882, with its broad host range, is highly active and rapidly lytic *in vitro*, and effective in preventing and treating ESBL-producing *E. coli* infection in mice. Our data revealed that i.p. inoculation of Ø9882 given 40 min after the bacterial challenge led to 100% survival at 24-168 h, compared to 0% survival of saline-treated controls. Protection was also obtained when phage administration was delayed up to 60 min after the bacterial infection, and the survival rate of infected animals was 60% at 168 h. Even if treatment was delayed up to 3 h, approximately 20% of animals were rescued and recovered completely, suggesting that acute infections may be amenable to phage treatment. Matsuzaki *et al* (32) and Soothill (27) independently reported that a MOI of 1 was the minimum used in their studies to produce a fully protective effect in a mouse model. In contrast, our experiments showed that the mimimum MOI is 0.0001 (10^{-4}). The result demonstrates that phage Ø9882 possesses an astonishing bacteriolytic ability. An antibiotic therapeutic effect in infected mice requires an appropriate dosing interval of administration to maintain pharmacological concentrations in serum. Of interest, a single i.p. injection of a lytic phage(s) was sufficient for the complete clearance of invading *E. coli* from surviving mice. This property, referred to as therapeutic efficacy, provides a rapid and specific lytic activity, thus making these phages very promising candidates in current antimicrobial therapies.

Our study also showed that phage Ø9882 can rapidly enter circulation, accumulate in the tissues and blood to a sufficiently high concentration to be practically useful, and even cross the blood-brain barrier according to some reports (33), which implies a rapid systemic distribution of the phage

in vivo. Matsuzaki *et al* (32) and Merril *et al* (34) found that phage particles can be detected from different tissues and/or organs, such as the spleen, liver, kidney, brain and skeletal muscle. Our data also indicate that infectious Ø9882 was maintained at relatively higher levels in the bloodstream. Ø9882 ($6x10^9$ PFU/ml) retained a high titer of $1.7x10^9$ PFU/ml in bloodstream 24 h after i.p. injection, and was as high as ~10^6 PFU/ml until 48 h after injection. The levels of phage in the circulation are correlated with its antibacterial efficacy and therapeutic effectiveness. It was reported that phages persisted without a reduction in particle numbers on the skin for at least 48 h. Others showed that phages persisted long enough in the tissues to be effective when administered to chickens 1-2 days before the challenge with *E. coli*. Our data and those of others support the view that phage dosages can be used regardless of its rejection by the body, suggesting the possibility of prophylaxis (29,35,36).

Furthermore, our experiments demonstrate that ability of the phage to rescue bacteremic animals was the phage function and not a nonspecific immune activation response, although phage Ø9882 caused an immune response in mice, and IgG antibodies reached a maximum over the course of 20-30 days after a single phage injection. Our data both *in vitro* and *in vivo* revealed that: i) the protection of mice from death occurred only in animals infected with selected bacterial strains and the virulent phage specific to them; ii) when the phages were heat-inactivated, the survival of the infected mice was strikingly decreased to 0; and iii) the level of antibody against the phage was not significantly changed when the bacteremic animals were protected by the active phages. These results are consistent to those of Biswas *et al* (29). Additionally, Matsuzaki and co-workers confirmed that a 'mechanical' lysate of *S. aureus* did not induce an anti-bacterial effect, and phages had no therapeutic effect against infections with phage-lysogenic host bacteria (32). Lastly, bacterial antigens, such as endo- and exotoxins, must have been removed by repeated cesium chloride density centrifugation purification from the Ø9882 preparations (28), which are less prone to induce an immune response and cause side effects, as evidenced by the lack of any adverse effects in the control group mice inoculated with a high dose of the phage preparation.

Our results indicate that phages have the potential to prevent and/or treat certain bacterial infections in animals and, by extension, in humans. It is tempting to advocate investigation into several bacterial infections for which animal models are available and for which phages may be isolated. However, phage therapy as an alternative to antibiotics for bacterial infections could only become a practical measure under certain conditions and requires further study. First, most phages are highly host-specific and showed a close and narrow host range. A phage targets specific bacteria, usually a specific bacterial strain, and ignores other bacteria. Using a cocktail of lytic phages should minimize the risk of bacteria resistance to phage therapy. Second, phage biochemistry and gene characteristics were immature, resulting in the preparation of phage of variable quality, either contaminated with bacteria or their components. Sophisticated purification technology, such as purification of cesium chloride density centrifugation, can avoid the complications of earlier therapies. Finally, applying this strategy successfully will be technically difficult. The parenteral inoculation of phage could be used for mass treatment, but oral administration would be more convenient, and although there have been reports on the translocation of phages administered orally across the gut (37), it would seem unlikely that phages could accumulate in the tissues and blood to a sufficiently high concentration to be practically useful.

The present study reinforces the view that the potential of phage therapy is worth exploring. It is anticipated that bacteriophage therapy will be a powerful weapon against antibiotic-resistant bacteria and could provide a novel approach to the treatment of systemic bacterial infections. Given the increasing problems of bacterial disease and bacterial antibiotic resistance worldwide, it would appear timely to begin a search for lytic phages with potential therapeutic strength and evaluate the therapeutic effectiveness of phage treatment for systemic lethal ESBL-producing bacteria infections in humans.

Acknowledgements

This publication was made possible by the Cheng-Guang Projects for Young Scientists from the Department of Science and Technology of Wuhan, China (no. 20025001026 to J. Hu), and a grant from the National Institutes of Health, Bethesda, MD, USA (no. P20RR16440-010003 to Q.Q. Li).

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