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Full Length Research Paper

A fast method for large-scale isolation of phages from hospital sewage using clinical drug-resistant *Escherichia coli*

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With the continued emergence of multi-drug resistant genes and drug-resistant bacteria, the functions and applications of traditional antibiotics are challenged. Thus, products that can be combined with existing antibiotics or replace them are of great interest to the research and medical communities. As a natural parasite of bacteria, phages have been reconsidered for use in treating super or drug-resistant bacterial infections. The main aim of this study was to isolate new phages from hospital sewage, especially those with broad-spectrum infectivity for the establishment of an *Escherichia* phage library. According to differences in their infection spectrum, bacteriophages were identified using a plaque method. In total, 18 *E. coli* phages were isolated from hospital sewage. The characteristics of the wide host spectrum *E. coli* bacteriophage E12P1 were further studied. Electronic micrographs indicated that phage E12P1 had a cubic symmetry and a long tail. The growth curve suggested the incubation time was 30 min, the burst phase was 20 min, and the burst size was 43. This plaque-forming method could be adopted to isolate *E. coli* phage easily, rapidly and in large quantities. Among the 18 isolated *E. coli* phages, 10 of them had a broad host range in *E. coli* and warrant further study.

Key words: *Escherichia coli* phages, large-scale isolation, drug resistance, biological properties.

INTRODUCTION

Phage, first discovered in 1915 by d'Herelle, is a class of virus whose host is a type of prokaryote (including bacteria, fungi, actinomycetes, or spiral). Later, d'Herelle discovered that phages could crack bacteria, and named them "bacteriophages" (DuckworthDH, 1987). The phage mainly consists of capsid (proteins) and nucleic acid with no cellular structure and only replicates in living microbial cells, making it a parasitic microorganism that is widely distributed in nature. As the phage participates in bacteria-lysis and strict species-specific infection, it can be used for bacterial classification and identification, prevention and treatment of some infectious diseases, especially in conjunction with antibiotic treatment of multidrug-resistant bacterial infections (Collins, 2009). With the emergence of multi-drug resistant genes such as NDM-1 (Marisa et al., 2003) and super drug-resistant bacteria such as German *Escherichia coli* (Frank et al., 2011), the functions and applications of traditional antibiotics are challenged (Spellberg et al., 2004). As a natural parasite of bacteria, phage may be a likely candidate useful in treating super bacterial infection. The main aim of this study was to isolate some novel phages from hospital sewage especially those with broad-spectrum infection capabilities.

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MATERIALS AND METHODS

Main reagents and *E. coli* strains

Nutrient agar and nutrient broth were provided by the Equipment Office of the Academy of Military Medical Sciences; 0.2 μm disposable sterile filters were from Pall (USA) and a micro-centrifuge was from Sigma-Aldrich (Germany). Thirty clinical isolates of drug-resistant *E. coli* collected from sewage obtained from the Affiliated Hospital, Academy of Military Medical Sciences, were used as the indicator bacteria for isolating phages. These isolates of *E. coli* were confirmed through biochemical methods and 16 S rDNA sequencing (data not shown).

Isolation and titration of *E. coli* phage

Five hundred milliter (500 ml) of untreated sewage was collected from Wastewater Treatment Division of the Affiliated Hospital, Academy of Military Medical Sciences. The sewage was centrifuged twice to remove the pellet, and then the supernatant was collected and filtered with a 0.2 μm filter. Ten milliter (10 ml) of filtrate was added to 5 ml of sterile 3×LB, and the mixture were inoculated respectively with the above 30 isolates of *E. coli* at 37°C for 12 h, then at room temperature for 60 min. The culture was centrifuged at 12000 rpm for 10 min to collect the supernatant, and then filtered through a 0.2 μm filter. The filtrate was diluted serially in the range of 10^{-1} to 10^{-8}. One hundred microliter (100 μl) of the diluted solution was mixed with 500 μl of indicator bacteria in the logarithmic phase and they were added to the semi-solid agar medium (0.8%), and then incubated at 37°C for 24 h after solidification. Number of plaques was calculated and the phage titer was determined according to its original dilution. Single plaques were picked from the plates and the plaque was inoculated to the corresponding bacilli solution that was in the logarithmic phase. When the bacilli were completely lysed at 37°C, the supernatant was collected through centrifugation and filtration. After freeze-dried treatment, the filtrate was stored at 4°C.

Determining the multiplicity of the optimal infection (MOI) of E. *coli* phage

Phage and host bacteria were mixed at 0.1, 1, 10, and 100 MOI respectively and the LB medium was added to make the same final volume. The mixture was cultured at 37°C for 6 h with rotating at 200 rpm. Meanwhile, the host bacteria without phage and phage without host bacteria were used as the controls. The phage titer was determined as described previously, and the MOI was defined as the multiplicity of optimal infection after the phage was grown in culture for 6 h.

Determination of host range and growth pattern with one-step growth curve

A spot test was adopted to determine the host range of the phages. Briefly, the 30 kinds of indicator bacteria were inoculated respectively on semi-solid agar medium (0.8%). After solidification, each culture plate was divided into 30 grids. A 2 μl aliquot of phage solution from a single plaque was dropped onto the center of every grid, and then the culture plate was incubated at 37°C for 12 h after the liquid was absorbed. When the plaque was formed, the host range of each phage isolate could be determined. In turn, when the host range was determined, the growth properties of phages in its host bacteria were measured using a one-step growth curve. Adsorption of phage to its host bacteria was performed with 0.1 MOI, then the bacteria was cultured at 37°C, at 200 rpm. An aliquot of 1 μl of culture medium was collected at the indicated time and the phage titer was measured by serial dilution.

Identification of the phage genome and morphology

The phage genome was extracted using the protease K/SDS method (Sambrook and Maniatis 2001). A volume of 1 μl of phage genome was treated respectively with DNase I, RNase A and restriction endonuclease enzyme (EcoRI), incubated at 37°C for 2 h, then the mixture was loaded on a 0.7% agarose gel for electrophoresis. For the observation of phage morphology, 100 μl of a phage suspension was fixed with 10 μl fixative (10% formaldehyde and 5% glutaraldehyde in deionized water), and then about 10 μl of fixed phage suspension was applied onto the grid and left for 30 s to 1 min. Before sample was dried, 10 μl of 2% aqueous phosphotungstic acid (adjusted pH to 7.3 using 1 N NaOH) was added and incubated for 30 s, and then removed from the edge of the grid with filter paper. The grid was placed directly into a grid box and allowed to air-dry for 30 min before observation.

RESULTS

Isolation and titration of phage

Using 30 strains of clinical drug-resistant *E. coli* as the indicator bacteria, T4, IME08 and IME09, were isolated by our laboratory previously (Huanhuan Jiang et al., 2011a, bWang Sheng et al.,2010, Xiaofang Jiang et al.,2011) as the control phages, a total of 18 strains of phage from hospital sewage were isolated (Figures 1 and 2). According to the lysis spectrum, each bacterial strain has several corresponding bacteriophages and each bacteriophage also has many host bacteria. Among them, 10 strains of the newly isolated phages including E12P1 could lyse 10 or more of the indicator bacteria, indicating that these phages had a broad host range with potential applications in treating drug-resistant bacterial infection.

The multiplicity of optimal infection

After serial dilution and inoculation, the multiplicity of optimal infection of phage E12P1 was identified with 1 MOI. When cultured on the double agar overlay plaque assay plate, the plaque induced by E12P1 appeared at 4 h after inoculation, with the plaque diameter reaching the maximum at 7 h with a clear edge.

Host range of the phages

In this assay, 30 clinical isolates of *E. coli* were used as the indicator bacteria, and 18 strains of phage isolated from hospital sewage were tested in order to further evaluate their host spectrum.

The 30 strains of *E. coli* were inoculated on double overlay agar plate and the plates were divided equally into 18 grids and each strain of phage was inoculated on one grid. T4, IME08 and IME09, which were isolated by

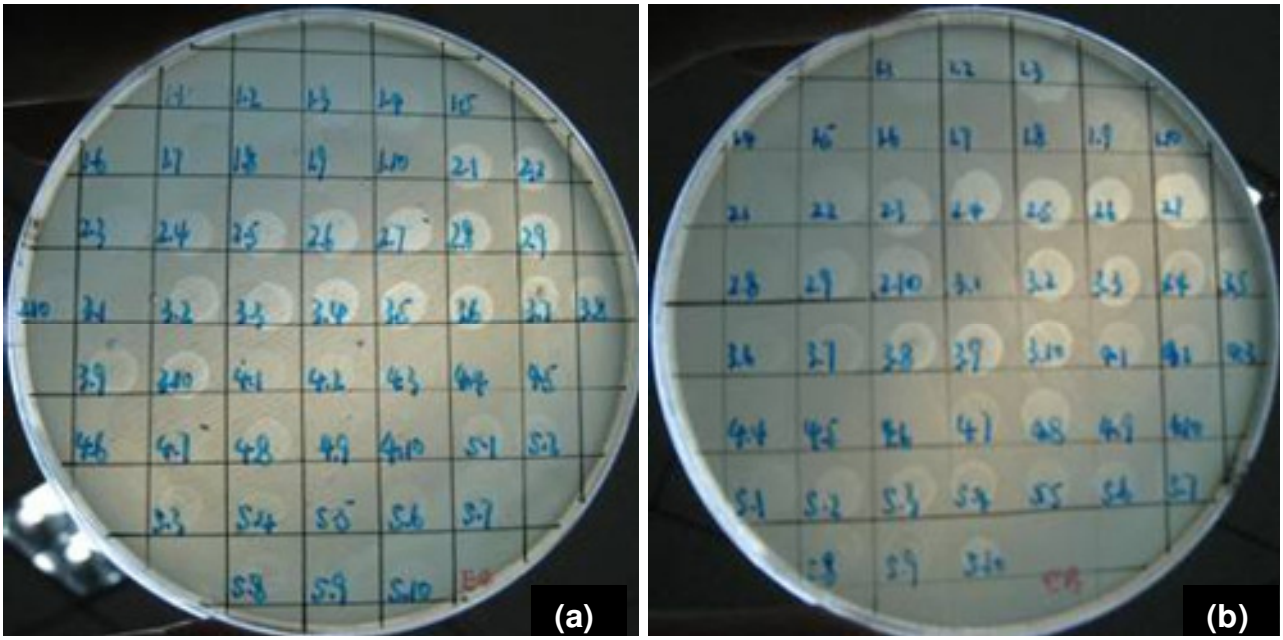

Figure 1. Two example of the spot test; (a) the isolation of phages using *E(. coli)*4, and (b) the isolation of phages using *E(. coli)* 13.

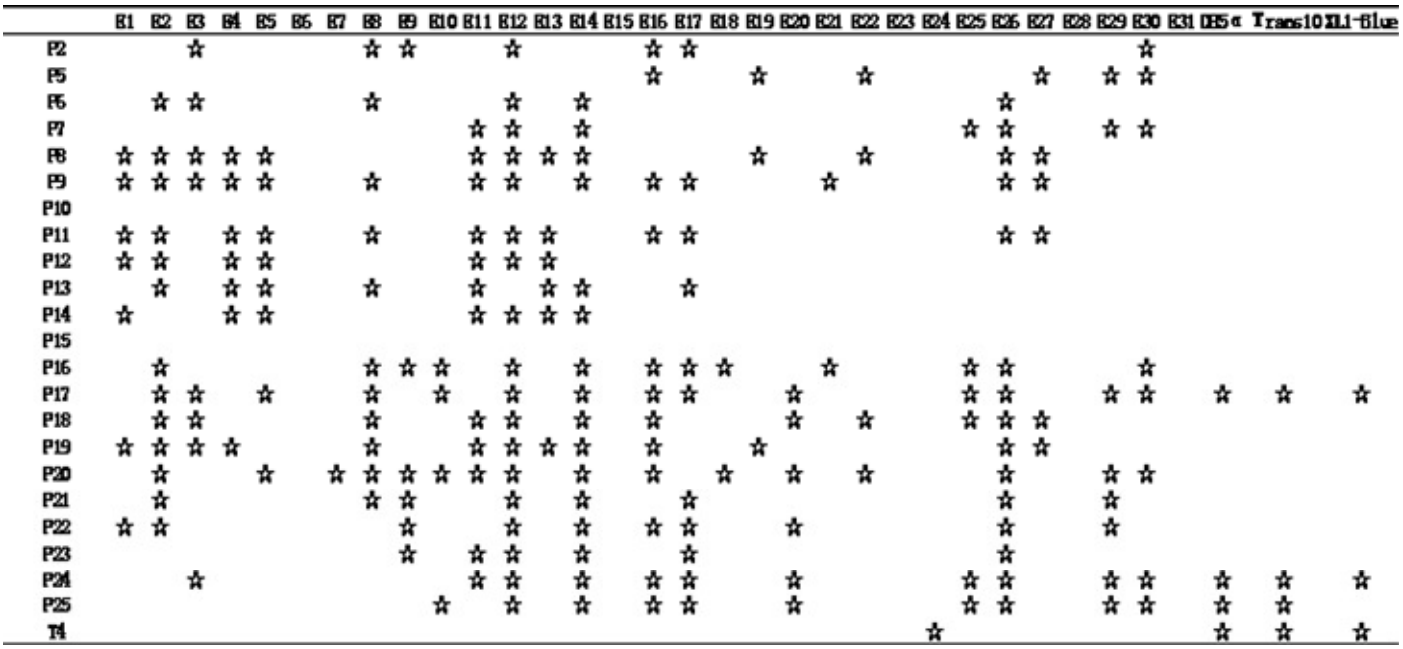

Figure 2. The host spectrum of the newly phage isolates. Thirty clinical isolates of *Escherichia coli*, DH5α, trans 10 and XL-1 blue were the indicator bacteria, T4 and IME08 and IME09, which were separated by our laboratory previously, were the control phages. (E means *E.coli,*and P means phage)

our laboratory previously, were the control phages. Using this method, we successfully isolated 18 types of phage from the hospital sewage as shown in Figure 2. Of these, 10 phages could crack 10 or more of the indicator bacteria, indicating that these phages had a broad host range, with potential uses in the phage therapy.

Determination of a one-step growth curve

As shown in Figure 3, during the period (5 to 15 min) after the adsorption started, the number of plaques did not increase, indicating that the phage replication and assembly was not completed (we called this the phage

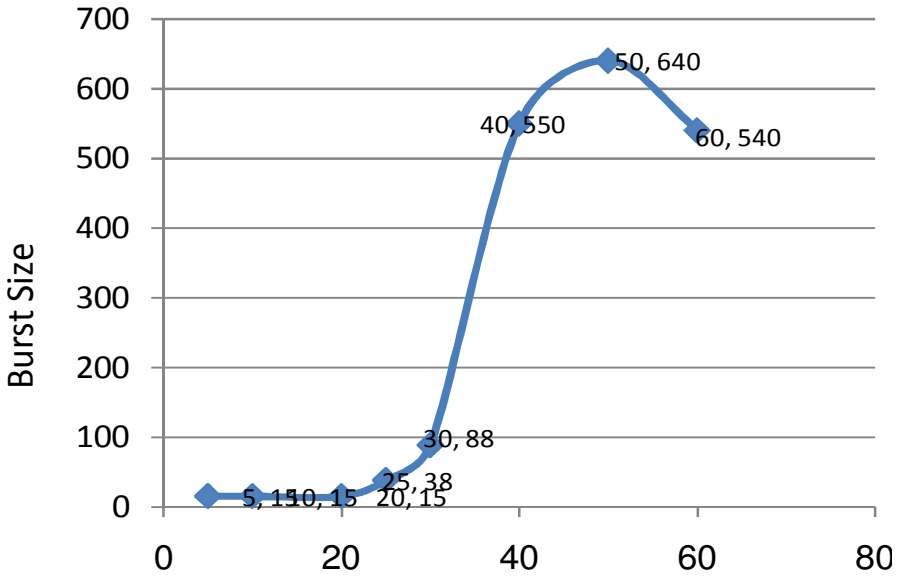

Figure 3. The one-step growth curve of *E. coli* phage E12P1 in E12.

incubation period). During a subsequent period of time (15 to 30 min after infection), the number of plaques on the plate suddenly soared, releasing phage host cells (the cracking period). The average amount of phages each infected bacterium released was the amount of cracking. When all the host cells were cracked, the titer of the phage in the solution reached the highest point, the stationary phase. According to the one step growth curve, the phage cracking-related parameters were obtained: An incubation period of 30 min, a cracking period of 20 min, and the amount of cracking, namely, the average number of phage particles that each infected bacteria produced was 43 ± 1 pfu.

Identification of the phage genome

As shown in Figure 4, after DNase I and RNase A digestion of the phage genome, electrophoresis results showed that the genetic material was digested completely by DNase I but no significant changes resulted by RNase A digestion. Further restriction enzyme treatment of the DNA demonstrated that the genetic material was dsDNA.

Phage E12P1 morphology by electronic microscopy

Purified phage E12P1 particles by negative staining were observed under transmission electron microscopy (TEM) as shown in Figure 5. The phage showed a typical icosahedral structure of the head and tail, with a head width of 60 nm, length of 80 nm, and a tail length of about 55 nm, and with several tail wires, leading us to conclude that the phage belongs to the Myoviridas family.

DISCUSSION

Phages are the most abundant living entities on earth-the estimates range from 10^{30} to 10^{32} in total-and play key role in regulating the microbial balance in every ecosystem where this has been explored (Kutter, 2005). However, given this large number of phage species, only very few have been isolated and characterized, thus isolation of new phages is an ongoing task. Phages are widely distributed, and are almost always found alongside bacteria in sea water, soil, human and animal intestines among other places. To enrich the phage resources, we attempted to isolate phages from untreated hospital sewage, and identified 18 strains of phages using clinical drug-resistant *E. coli* as the indicator bacteria. An enzyme digestion assay determined that the phage's genomic nucleic acid was dsDNA. Electronic microscopy showed the phage had a twenty-polyhedral head and a long retractable tail, a typical morphology of phage that belongs to the family Myoviridae. Based on these characteristics, the phages were identified as a T4-like phage from the family Myoviridae, order Caudovirales. Among the isolated phages, we identified 10 strains of phages that have a broader host range than the others and could lyse more than 10 strains of the indicator bacteria, indicating they have potential applications in the prevention and treatment of drug-resistant *E. coli* bacteria.

We also wanted to develop several "phage cocktail" agents that would be capable of antagonizing clinical drug-resistant bacteria. To do so, we mixed 5 strains of phages to observe their inhibition of clinical drug-resistant bacteria. The results in Figure 2 showed that although the cocktail mixture can lyse most of the clinical *E. coli* strains, the strains that could not be lysed originally were

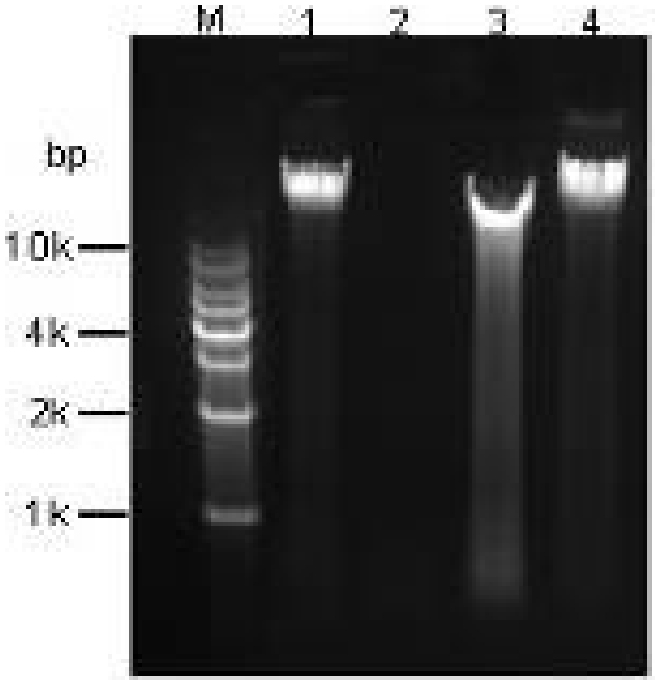

Figure 4. The genetic material of phage E12P1 digested by RNase A, DNase I and restriction endonuclease enzyme. Lane M: 1 kb marker; lane 1, RNase A digestion; lane 2, DNase I digestion; lane 3, EcoRI restriction enzyme digestion; lane 4, untreated.

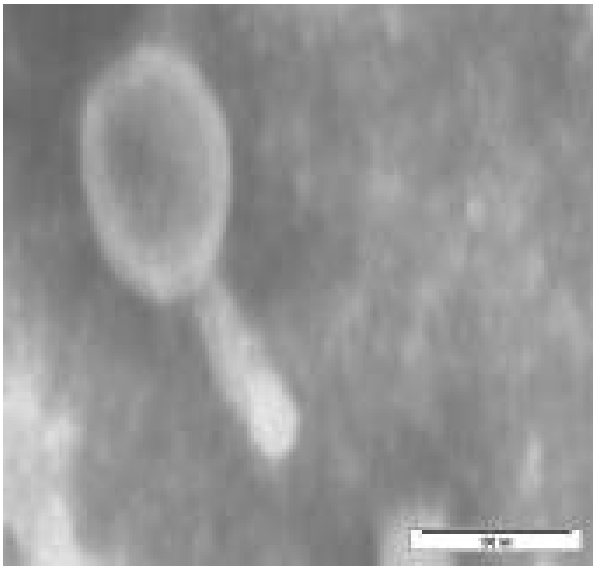

Figure 5. Electronic microscopy picture of Phage E12P1 morphology.

not lysed when subjected to the cocktail mixture. In order to increase the host range of the mixture, it will be necessary to isolate more phages from hospital sewage using above mentioned, "mixture-resistant" bacteria.

As a result of these experiments, some conclusions could be drawn about the phage culturing conditions and identification of the host range. For example, it is important that the bacillus should be in logarithmic phase if the aim is to obtain abundant phage of high quality. When identifying the phage host range, to avoid the superiority of bacteria in the "battle between the phage and its bacteria", an appropriate amount of phage solution (approximately 3 µl) should be added to the corresponding grid immediately after the semi-solid medium dries, and before the bacteria have reached logarithmic phase.

Some limitations of this method as shown in Figure 2, include that although in theory, it is likely to find the corresponding phage for each type of bacteria identified in the same environment, in this case, it was not easy to find the corresponding phages of all clinical bacterial sewage isolates such as E6, E15, E23, E28. This result prompted us to expand the screening range to identify the phages of the above bacteria. When we cultured the phage E12P1 in E12, an intriguing phenol-menon that we call the "flora alternating phenomenon" (Alisky and Rapoport 1998) was identified. That is, when the bacteria in logarithmic phase were incubated with their corresponding phage, the culture medium became gradually muddy, and then slowly transparent, but then muddy again and more and more muddy at last. More commonly, the bacterial solution should be gradually transparent during the period after it is incubated with its lytic phage. The reasons why the culture of the host bacteria becomes "transparent" first and then "turbid" might be related to the following factors: The physiological condition of the host bacteria and the phage itself, such as the nutritional conditions, growth rate, cell size and surface area of bacteria, the synthesis of phage components and speed of assembly, and the cleavage period (Hilla Hadas et al., 1997). Exploring the mechanism of phage infection, such as how the phage invades the bacteria and how the bacteria avoid being cracked should provide insight into the above phenomenon.

ACKNOWLEDGMENTS

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REFERENCES

Alisky JIK, Rapoport A (1998). Bacteriophages show promise as antimicrobial agents. J. Infect. 36(1): 5-15.

Collins TKLaJ (2009). Engineered bacteriophage targeting gene networks as adjuvents for antibiotic therapy. PNAS, 106(12): 4629-

4634.
DuckworthDH (1987). History and basic properties of bacterial viruses. Phage Ecol. 1-44.
Frank C, Askar MSF, Bernard H, Fruth A, Gilsdorf A, Höhle M, Karch H, Krause G, Prager R, Spode A, Stark K, Werber D (2011). Large and ongoing outbreak of haemolytic uraemic syndrome, Germany, May 2011. Eur. Surveill 16(21): 1-3.
Hilla Hadas ME, Itzhak F, Arieh Z (1997). Bacteriophage T4 development depends on the physiology of its host *Escherichia coli*. Microbiology 143:179–185.
Huanhuan Jiang , Xiaofang J, Sheng W, Cun L, Bin C, Xiaoping A, Zhiqiang M, Jiankui C, Yigang T (2011b). The complete genome sequence of a novel T4-like bacteriophage. Arch. Virol. 156(8): 1489-1492.
Huanhuan Jiang, Sheng W, Cun L, Dabin L, Changming Y, Xiaoping A, Zhiqiang M, Jiankui C, Yigang T (2011a). Rapid genetic characterization of a novel Enterobacteria phage and identification of its host recognizing genes. Chinese J. Biotechnol. 27(6): 884-890.
Kutter ESA (2005). Bacteriophages Biology and Applications. CRC Press.
Marisa L, Pedulla MEF, Tharun K, Jennifer MH, Roger WH, Graham FH, Anthony RP, Eddie BG, Danella AW-S, Sherwood RC (2003). Corrected Sequence of the Bacteriophage P22 Genome J. Bacteriol. 85(4): 1475-1477.
Sambrook JFE, Maniatis T (2001). Molecular cloning: a laboratory manual (Third Edition). New York: Cold Spring Harbor Laboratory Press.
Spellberg BPJ, Brass EP, Miller LG, Edwards Jr. JE (2004). Trends in antimicrobial drug development: Implications for the future. Clin. Infect. Dis. 38: 1279-1286.