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An improved plating assay for determination of phage titer

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Phage is a virus that is parasitic on bacteria. It is very important to determine the titer of test sample in the study of phage. In this study, an improved plating assay was developed for detection of the number of recombinant phage Cap-T7 present in a test solution at a certain dilution point by counting the plaque forming units. The data demonstrated that the improved plating assay is fast, useful, and convenient for the determination of the phage titer in a sample.

Key words: Phage Cap-T7, detection method, plaque forming units.

INTRODUCTION

Bacteriophage is a virus that infects and replicates within a bacterium; it was discovered independently by Frederick Twort and Félix d'Hérelle, respectively, in 1915 and 1917 (Kaur et al., 2012). There are a wide variety of phages and they are among the most common and diverse entities in the biosphere (Clokie et al., 2011). Every bacterium is likely to have their own specific phage viruses (Flores et al., 2011; Örmälä and Jalasvuori, 2013), and phages play an important role in bacterial evolution (Labrie et al., 2010).

Phages infect bacteria very selectively; their growth and proliferation are in a specific host without harming commensal bacterial flora (Loc-Carrillo and Abedon, 2011). Phages have significant potential in the resistance to bacterial infection; they have been used for many years as the alternative to antibiotics in the former Soviet Union and Central Europe, as well as in France (Clark and March, 2006). While the role of phage anti-bacterial infection has been ignored for many years due to all kinds of reasons, including the discovery of the antibiotics (Cui, 2015). In recent years, antibiotic-resistant bacterial infection has become a global concern (Zhang et al., 2015); more and more countries presently ban the use of antibiotics to control bacterial infections in swine (Thacker, 2014). Phage therapy is re-valued by researchers to combat the growing menace of antibiotic-resistant infections (Torres-Barceló and Hochberg, 2016).

Determination of phage titer in a sample is a key step in the study of the phage involved. It is very important to select suitable dosage in the phage therapy for bacterial infection, to analyze the efficiency of packaging reactions *in vitro*, to detect the number of primary recombinant

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phages in a constructed library, and to monitor the enrichment of phage during biopanning. As a common method of detection, a plating assay at present is widely used to detect phage number present in the test samples at various diluted points (Sambrook and Russell, 2001); the results are then further used to calculate phage titer. According to the plating assay, the test sample from every one dilution at least needs one agarose plate. This may lead to the need of a lot of plates, waste large amount of reagent and consumptive materials, increase the experimental funds and labor. The aim of the current study was to develop an improved plating assay for the determination of phage titer in a fast, useful and effective manner.

MATERIALS AND METHODS

Phage and host strain

The purified recombinant phage Cap-T7, conserved in Chongqing Academy of Animal Science Veterinary Research Institute, was used in the present investigation. *Escherichia coli* BLT5403, the host strain of phage Cap-T7, was bought from Novagen, Inc. (Darmstadt, Germany).

The plating assay of phage Cap-T7 by the fixed method

The plating assay is used to calculate the number of phage Cap-T7 present in samples at various points according to the fixed protocol described briefly as follows: a series of dilutions of the Cap-T7 were prepared by adding 100 µl of the sample to 900 µl of sterile Luria-Bertani (LB) medium. 250 µl of the host cells (OD_{600} = 0.8) were pipetted into 4 ml sterile tubes. Starting with the highest dilution, 100 µl of the Cap-T7 dilution was added into each tube. After that, 3 ml of autoclaved and cooled to 45°C top agarose (Bacto tryptone 1 g, yeast extract 0.5 g, NaCl 0.5 g, agarose 0.6 g, per 100 ml) was added to the tubes. The contents were mixed and then poured onto a pre-warmed LB/carbenicillin agar plate. On the agarose spreading evenly and hardened, the plates were inverted and incubated at 37°C for 4 h. Phage number in test sample was calculated by counting the plaque forming units (pfus) on the plate.

Improved plating assay of phage Cap-T7

Preparation of the improved agarose plate

LB liquid culture (4 ml) of *E. coli* BLT5403 (OD_{600} = 0.8) were added into 100 ml triangular flask containing 50 ml autoclaved and cooled to 45°C top agarose as described earlier. The contents were immediately mixed and then 4 ml were taken out from the flask, and poured in a standard 100 × 15 mm Petri dish containing 20 ml of hardened LB/carbenicillin agar pre-warmed at 37°C. The plate was swirled gently to spread the agarose evenly. The plate was allowed to sit undisturbed until the top agarose was hardened, and then it was inverted and stored at 4°C.

Phage incubation and its titer

A series of the phage Cap-T7 dilutions were carried out as ten-fold dilution described earlier. After that, 5 µl solutions were taken out from every phage dilution and 4 different dilution samples were uniformly put in separate spots onto the surface of the improved agarose plate. The plate was allowed to sit undisturbed for more than 30 min until the 5 µl solutions become dry. Then, it was inverted and incubated at 37°C. Phage number in test solutions were calculated as described earlier.

Statistical analysis

The data was expressed as mean ± standard deviation (SD). Statistical significance of the difference was analyzed using Student's T-Test. P-value less than 0.05 ($P < 0.05$) was considered as statistically significant.

Plaque polymerase chain reaction (PCR)

The pfus were randomly selected out to be used for subsequent PCR assay according to the protocol of T7Selected® System Manual (Novagen, Darmstadt, Germany). A set of specific primers were designed using software GeneTool, the forward primer is 5'-ggc tgc agg aat tca tga ctt-3', reverse primer is 5'-tcg ata agct tgt acg ggt t c-3'. Amplifications were performed with 0.5 µM of each primer, 0.2 mM of each dNTP and 2.0 U *Taq* DNA polymerase (TaKaRa, Dalian, China) with the following cycling parameters: denaturation at 94°C, 4 min; 35 cycles (94°C, 45 s; annealing at 55°C, 40 s; 72°C, 60 s); final elongation at 72°C, 10 min. PCR products were then electrophoresed and the sequences were identified (Sangon Biotech, Shanghai, China).

RESULTS AND DISCUSSION

Plaque assay

T7 phage, one of the best-studies bacterial viruses (Häuser et al., 2012), is a lytic phage. Its phage particle assembly takes place in *E. coli* cytoplasm and mature phages are released by cell lysis (Dunn and Studier, 1983). Therefore, when the Cap-T7 was present in the test sample, pfu (clear area) will be observed in the lawn of its host *E. coli* BLT5403 which corresponds to individual phage infection event produced by single Cap-T7. In the experiments, ten-fold dilutions of the Cap-T7 ranging from 1×10^{-16} to 1×10^{-20} solutions were used to affect bacteria for pfus counting. With the extension of culture time, pfus appeared to become larger and larger. A series of plaques were observed on the plates incubated at 37°C for ~4 h. The pfus in the improved agarose plates vary from mutual fusion to clearly distinguishable and numerable single unit with the increase of gradient dilution up to no pfus appearing at last. More specifically, some pfus can be found at 10^{-17}, 10^{-18}, and 10^{-19} dilution points, with 3 clearly distinguishable pfus that can be counted at the 10^{-19} dilution point (Figure 1a). In contrast, the pfus from one of the plates of the fixed plating assay reflected the phage number of the test solution at only one dilution point. In this case, there were only 73 pfus in the 100 µl solution at the 10^{-19} dilution point (Figure 1b). Besides, some additional tests were also conducted to perform the plaque assay using the same methods described earlier,

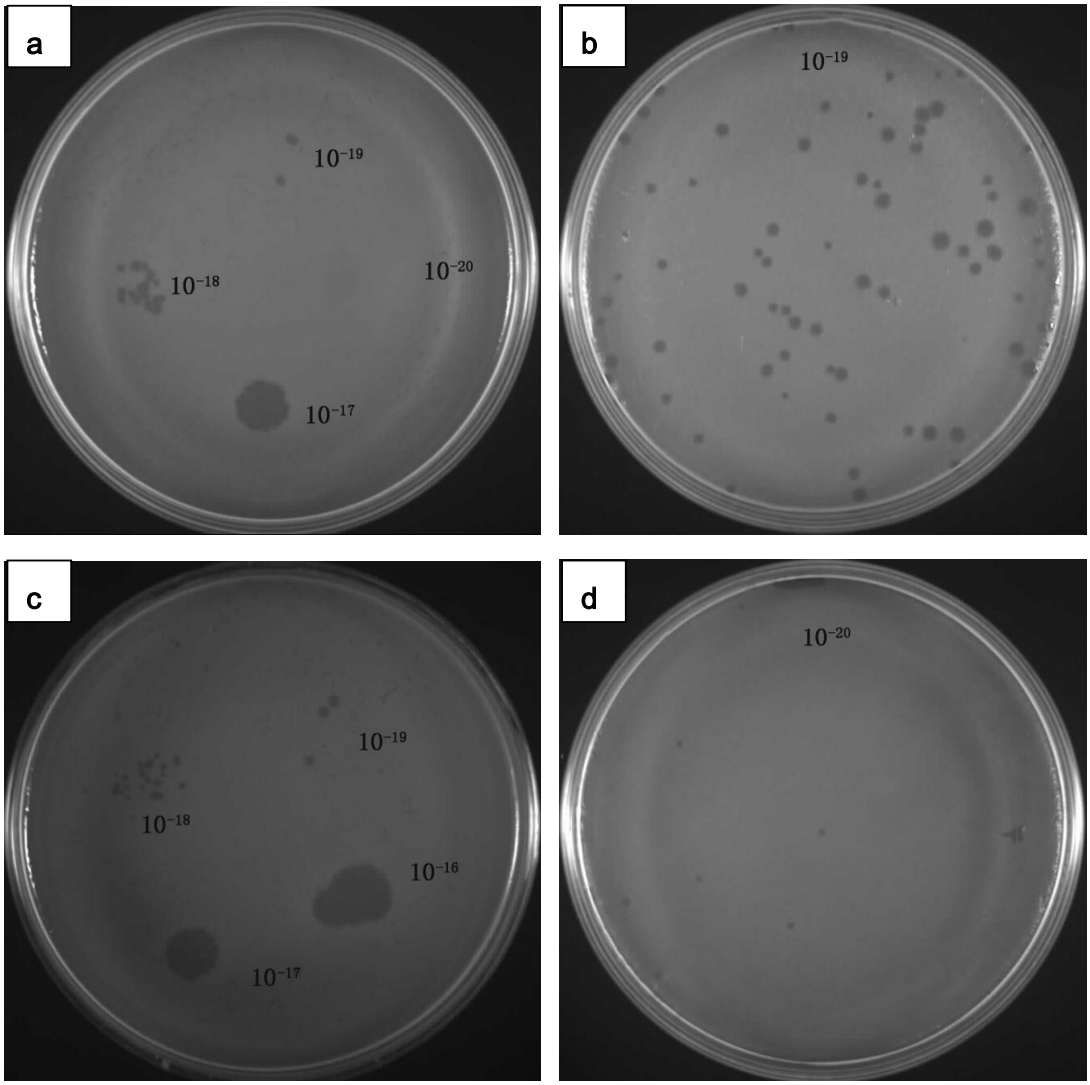

Figure 1. The pfus in the agar plates incubated at 37°C for ~4 h at different dilutions. Ten-fold dilutions of the test sample Cap-T7 ranging from 1×10^{-16} to 1×10^{-20} solutions were used for pfus counting. As indicated in the agar plates, a and c showed the plaques in the improved plate at 10^{-17}, 10^{-18}, 10^{-19}, 10^{-20} and 10^{-16} 10^{-17}, 10^{-18}, 10^{-19} dilutions, respectively, and 3 single plaques were observed in the 5 µl at 10^{-19} dilutions. b and d showed the plaques in the plate of fixed method at 10^{-19} and 10^{-20} dilutions, 73 and 7 single countable plaques can be observed in the 100 µl solutions, respectively.

and similar results were obtained. For instance, they also have a series of plaques at different dilutions including the 3 single pfus at the 10^{-19} dilution point in the improved plate (Figure 1c) and 7 plaques at the 10^{-20} dilution point in the control plate (Figure 1d). Obviously, it is more convenient for the plaque assay using the improved method than that using the fixed method.

Determination of phage titer

According to the number of the single countable plaques on the plate, the volume of the detection, and the dilution of the test sample, phage Cap-T7 titer can be easily calculated. For example, there were 3 plaques on a plate from a 10^{-19} dilution in 5 µl volume (Figure 1), then the titer of the sample was $3\times200\times10^{19} = 6\times10^{21}$ pfu/ml based on the improved method. Through three separate experiments, the titer of the phage Cap-T7 in the test sample was finally determined (Table 1). The phage titer values obtained from the improved plating assay and the fixed plating assay were not identical, showing certain degrees of variation. However, the two values ($6.67 \pm 1.15 \times 10^{21}$ and $6.93 \pm 0.40 \times 10^{21}$) have the same order of magnitude, and there was no statistically significant difference ($P > 0.05$) for the two test methods to determine

Table 1. Statistical analysis of the phage titer assays.

Group	Dilution	Spotting solutions (µl)	Phage titer (pfus/ml)	*P*-value
The improved plating assay	10^{-19}	5	$6.67\pm1.15 \times 10^{21}$	$P > 0.05$
The plating assay	10^{-19}	100	$6.93\pm0.40 \times 10^{21}$	

The values of phage titer are the average values of three determinations with standard deviation. P < 0.05, significantly different after Student's T-Test analysis.

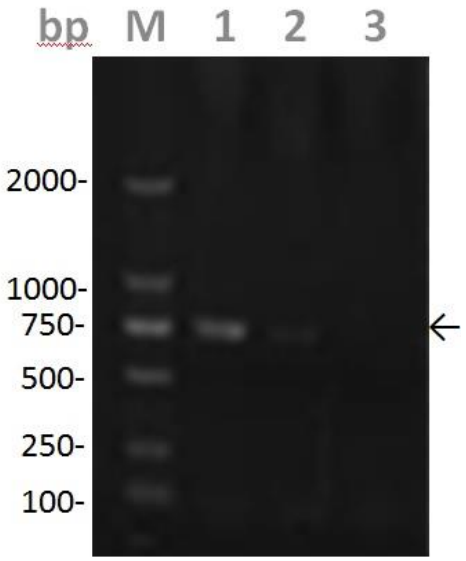

Figure 2. The electrophoresis results of the plaque PCR production. Product length was 727bp. M. Stand for DNA Marker, 1. PCR production of plaque from the plate of the improved plating assay, 2. PCR production of plaque from the plate of the common plating assay, 3. Negative control.

the phage titer. At the same time, the statistical analysis also proved that it was credible using the improved plating assay to determine phage titers.

Identification of plaque forming units

In order to determine the plaque forming unit authenticity of the recombinant phage particle, some plaques were selected out from the plates randomly and PCR analysis was conducted. The agarose gel electrophoresis map of the 2 plaques' PCR production is as shown in Figure 2. The result showed that there was a clear DNA band located at about 750 bp in the gel and the molecular weight (MW) of PCR production of the plaques from the improved plating assay group and the fixed plating assay group was consistent with the expected length size. In addition, sequence identified results also verified that the DNA bands had the same DNA sequence (Sequence 1). All proved that the phage plaques obtained from the improved method were the target plaques rather than others.

Conclusions

In this paper, recombinant phage Cap-T7 was taken as the research target to improve the common plating assay for the detection of the number of phages present in a test solution by counting pfus at a certain dilution and easily determined the titer of the phage Cap-T7. When compared with the common plating assay, the improved plating assay showed some advantages in the determination of the phage titer in a sample, such as using less agarose plates, saving labor and time. In a word, improved plating assay is feasible and worth spreading. Finally, it should be emphasized that in order to obtain the clear, distinguishable and countable pfus

Sequence 1. The DNA sequence corrosponding to the PCR production for identifying plaques.

GGCTGCAGGAATTCATGACTTATCCGCGTCGTCGCTATCGTCGTCGCCGTCACCGCCCA
CGTTCCCATCTGGGTCAGATCCTGCGTCGTCGTCCGTGGCTGGTACATCCGCGCCACCG
CTACCGTTGGCGCCGTAAAAACGGTATTTTCAACACCCGTCTGTCTCGTACCTTCGGTT
ACACTATCAAACGTACCACCGTGAAGACCCCTTCTTGGGCAGTTGACATGATGCGCTTC
AACATCAACGACTTCCTGCCGCCAGGCGGTGGTTCCAACCCGCGTTCCGTTCCGTTTGA
ATACTACCGCATTCGTAAAGTGAAAGTTGAGTTCTGGCCGTGCTCTCCGATTACGCAGG
GCGACCGTGGTGTTGGCTCTTCTGCGGTTATCCTGGATGATAATTTCGTAACGAAAGCT
ACTGCTCTGACCTACGACCCGTACGTCAATTACTCTAGCCGCCACACCATTACCCAACC
GTTCTCTTACCACAGCCGTTACTTCACGCCGAAACCGGTACTGGATTCCACCATCGACT
ATTTTCAGCCGAACAATAAGCGTAACCAGCTGTGGCTGCGTCTGCAAACTGCCGGTAAC
GTGGATCACGTTGGCCTGGGCACTGCGTTTGAGAACAGCATCTATGACCAGGAATATAA
CATCCGTGTGACCATGTACGTCCAGTTCCGCGAATTTAACCTGAAAGATCCTCCACTGA
ACCCGTACAAGCTTATCGA

using the improved plating assay, more attention should be given to well distribute the mixture of host cells and melt top agarose, select appropriate volume amount of spotting solutions, control the incubation time, and be sure to replace the pipette tips between samples of different phage dilution to avoid cross contamination.

Conflict of Interests

The authors declare that they have no conflict of interests.

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