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Development of a Plaque Reduction Assay as an Antiphage Activity Evaluation Method

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Abstract

Background: Antiviral screening of newly isolated or synthesized compounds is an important matter which requires a reliable antiviral test. In order to address this issue, development of a rapid antiphage test has been conducted. To achieve this goal, the antiphage activity of three antiviral drugs (Acyclovir, Lamivudine and Trifluridine) against phage CP51 which infects Bacillus cereus (ATCC 10876) was investigated.

Materials and Methods: Phage lysate was prepared by inoculation of bacterial culture with few phage plaques. The number of phage has to be about one million units per milliliter. The antiviral drugs were dissolved in suitable solvents and different concentrations of each drug were prepared. Phage lysate (0.1ml) mixed with appropriate amount of each drug. After 30 minutes incubation at ambient temperature or without any incubation, 500 µl inoculum of 5 hours old liquid culture of B.cereus was added to the mixture. Then, melted top agar (1.4-1.9 ml) was subjoined at the end and the final admixture was immediately seeded on the solid PA agar. After 24 h, plaques were counted.

Results: Out of three drugs, only trifluridine significantly decreased the plaque forming unit ratio ($p<0.05$) at higher concentration (133 µg/ml). The results of preincubation and non-incubation methods did not show any significant differences ($p>0.05$).

Conclusion: This study supported the development of one rapid qualitative and quantitative antiphage assay.

Keywords: Plaque reduction assay; Antiphage assay; Trifluridine

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Introduction

Development of an inexpensive, rapid and easy antiviral susceptibility bioassay is a valuable research work due to pre-screening of a lot of antiviral candidates in a short time. There is some phenotypic antiviral evaluation methods in which the activity of bioactive compounds against viral growth is determined. These include: plaque assay (1-4), Hybriwix assay (2, 5), ELVIS assay (2, 6, 7) and recombinant phenotypic assay (2, 8).

Interest on the antiphage assay as an initial tool for further antiviral evaluation study of potential bioactive compounds mainly rise from the fact that there is no need for living mammalian cells in the bio-assay procedure. In addition, the bacteria are easy to treat, grow fast and need cheap culture media. Phage analysis could be performed by evaluation of some techniques including plaque forming unit (PFU) (9, 10), the efficiency of plating (EOP) (11-13) and the multiplicity of infection (MOI) (14-16).

There exist a lot of antiviral drugs which are divided into six groups: (a) agents to treat herpes simplex virus (HSV) and varicella-zoster virus (vzv) infections (17, 18), (b) agents to treat cytomegalovirus (CMV) infections (17), (c) anti- retroviral agents (17, 19), (d) antihepatitis agents (17, 18), (e) anti-influenza agents (17, 18), and (f) other antiviral agents (17, 18).

Acyclovir, lamivudine and trifluridine have been selected as positive controls to develop the method.
In this study, the ability of an *in vitro* method that could be used to screen the antiviral activity of compounds either natural or synthetic was evaluated. In order to make sure that the negative results were not due to the inaccuracy of the method developed, three antiviral drugs were adopted. Trifluridine and acyclovir inhibit viral DNA synthesis (17, 18). Acyclovir is active against HSV-1 (0.02-0.9 µg/ml), HSV-2 (0.03-2.2 µg/ml) and VZV (0.8-4 µg/ml) (17, 18). Lamivudine inhibits HBV replication in vitro at 4-7 ng/ml (17, 18). *Bacillus cereus* (ATCC 10876) and its phage (CP51) as an indicator were employed for the test and the antiviral activity was assessed by comparing plaque forming unit ratio (%).

Materials and Methods

Antiviral drug stock solutions

2 mg acyclovir and 30 mg lamivudine were dissolved in 1 ml of 5 mg/ml sodium bicarbonate solution and 1 ml distilled water, respectively. Trifluridine eye drop (3 mg/ml) was used as a stock solution.

Culture media

Phage Assay Broth (PA Broth) was prepared by adding following salts to 13 g/l nutrient broth (Merk, Germany): NaCl: 5 g/L (Merck, Germany), $MgSO_4.7H_2O$: 0.020 g/L, $MnSO_4.H_2O$: 0.010 g/L, $CaCl_2.2H_2O$: 0.015 g/L In all the protocols pH was adjusted to 5.6-6.0.Afterwards, PA broth medium was mixed with 15 g/L and 7 g/L of agar (Merk, Germany) to prepare Phage Assay agar (PA agar) and Phage Assay top agar (PA top agar), respectively. Soybean Casein Digest Agar (SCDA) was purchased from Himedia, India.

Microorganisms

Bacteriophage CP51 (20) which infects *Bacillus cereus* was used in this investigation. *Bacillus cereus* (ATCC 10876) was stored at -20 °C in glycerol and revived by streaking the bacteria onto agar plate (SCDA) and incubating at 37 °C for one day.

Phage Lysate preparation

Bacillus cereus streaked onto PA agar plates using a kind of quadrant method (21). The Bacteriophage spread on already-inoculated plate with the help of inoculating loop. After one-day incubation at 25 °C, 5 ml of 2-hours old *Bacillus* culture in liquid medium together with 5 ml of PA broth were poured to the surface of the growing plate over the lysed colonies to make a microbial suspension using a microbial loop. The resulted mixture was transferred to a test tube and vortexed. The bacterial/viral admixture was incubated at 25 °C to enrich the phage concentration. After 1-2 days, the suspension was centrifuged at 3000 *g* for 30 min, then, the supernatant was sterile-filtered by letting it flow through a 0.45 µm membrane filter. The final filtrate was stored at 4 °C as a phage stock solution.

Phage titration

The number of phage in the phage stock solution was quantitatively titered using a modified over layer method. In a test tube containing 0.9 ml PA broth, 0.1ml aliquot of phage stock solution was added and mixed to make the first dilution. In the same way, 8 ten-fold serial dilutions of phage lysate solutions were prepared by withdrawing 0.1 ml aliquot of one dilution and mixed with 0.9 ml PA broth in next test tube with the help of air displacement micropipette. After preparing phage dilutions, 0.5 ml freshly-cultivated bacterial culture (OD_{570nm}= 0.7) was mixed with 100 µl of each phage dilution in separated vials. Finally, 2.4 ml of melted PA top agar (45-50 °C) subjoined to the mixture and, immediately, seeded on the plate which had contained 10 ml PA agar as a base layer. After incubation at 37 °C for 24 hours, the number of plaques appeared on the bacterial lawn was counted. The phage dilution that generated between 30-300 numbers of well-isolated plaques (Figure 1) was reliable to calculate the PFU.

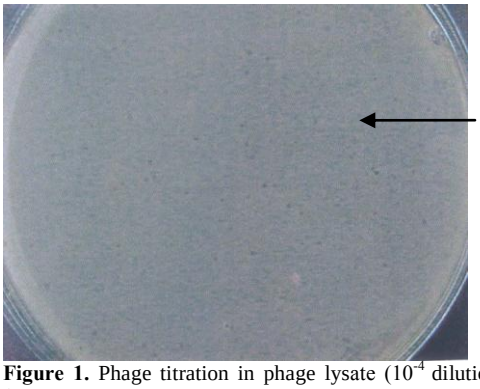

Figure 1. Phage titration in phage lysate (10^{-4} dilution). Plaque forming units are being seen as clear zones on the surface of the bacterial lawn.

Plaque reduction assay

Preincubation protocol: 0.1 ml aliquot of selected-diluted phage lysate was mixed with the desired concentration of each antiviral drug solution and maintained at ambient temperature for 30 min. Then, 0.5 ml of 5 hours old bacterial culture was added to the treated phage lysate. Finally, melted top PA agar was mixed with the admixture to the final volume of 3 ml and, immediately, overlaid onto PA agar and incubated at 37 °C. After 24 h, the number of distinctively-formed plaques was counted. In the samples of negative control, the phage lysate was

treated in the same way without the presence of antiviral drugs in the solvents.
Non preincubation protocol: The antiviral analysis was performed according to the pre-incubation method except the elimination of 30 min incubation of phage lysate with antiviral drugs at room temperature. All of the experiments were done in duplicate.

Statistical analysis
Experiments were accomplished in duplicate. The results of the plaque reduction assays expressed in PFU ratio (%).

$$\text{PFU ratio}(\%)=\frac{\text{the number of plaque forming unit of the treated sample}}{\text{the number of plaque forming unit of the negative control}}\times 100$$

The arithmetic mean ± standard error of mean (SEM) of control and experimental results and statistical analysis were estimated using the Dunnett′s test and Student′s t-test. $P< 0.05$ was regarded as statistically significant.

Results
The enrichment procedure of phage was performed properly until the results of phage titration in phage lysate showed enough number of phage (Table 1).
The 10^{-4} and 10^{-5} dilutions seemed to be acceptable for calculating the number of phage in the phage stock solution. The phage titer estimated to be around 2.32×10^6 and 2.65×10^6 calculated from fourth and fifth 10-fold dilutions, respectively.
Acyclovir and lamivudine did not show any antiviral activity against phage CP51 using this method (results not shown). In contrast, trifluridine significantly decreased the number of plaque forming unit (Figure 2). PFU ratio (%) declined to 5.8 ± 3.1 % and 36.2 ± 9.3 % using preincubation and non preincubation methods, respectively, at concentration of 133 µg/ml trifluridine.

Table 1. Phage titration in this study

Phage dilution	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
PFU [1] (Mean)	NC[2]	NC	NC	1347 ± 190	232 ± 25	26.5 ± 9	2.5 ± 2	0	0

Discussion
In this study, a modified antiphage assay has been developed using plaque reduction assay. In order to rely on the results of antiphage assay, phage lysate should be enriched sufficiently and appropriate dilution should be selected for subsequent experiments. According to Table 1, 10^{-4} diluted phage lysate was chosen for further antiviral analysis. It was observed that a new phage lysate had to be prepared around every 25 days because of the reduction in the number of living phages present. One limitation of this method lies in the representation of the plaques. One plaque (Figure 1) regarded as one virus in the phage calculation, but each plaque could be the result of two or more joined viruses.
During the titration, the 4-5 hours old culture of *Bacillus cereus* in which the absorbance of 0.7-0.75 at 570 nm obtained, was found to be the best time to observe clear plaques on the bacterial lawn with distinctive borders. Cultivation period of bacteria around two hours resulted in irregular plaque morphology with mixed borders because the bacteria were in initial logarithmic stage of growth and therefore bacteria were still fresh and active. If the phage lyses the bacteria and forms a plaque, the bacteria grow around the plaque borders and make it irregular. On the contrary, if this period is too long like 7 hours and more, unclear plaques will be formed. Acyclovir and lamivudine did not change the plaque forming unit ratio ($P>0.05$) even by increasing the drug-concentration to 1000 µg/ml (data not shown) whereas trifluridine showed changes in the PFU ratio (Figure 2).

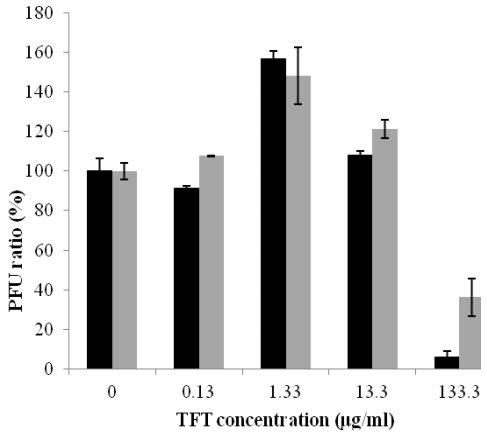

Figure 2. Antiphage method development using trifluridine as an active candidate against phage CP51. TFT concentration calculated in the final volume of seed layer. TFT (trifluridine) decreased the plaque forming unit ratio (PFU %), significantly, in both pre-incubation (■) and non-incubation (■) protocols.

At low concentration, it increased the plaque forming unit ratio significantly ($P<0.05$) around 40% at 1.33 µg/ml in preincubation method that could be due to the stimulation of phage separation. At higher concentration, trifuridine provided a substantially

inhibitory effect. According to the one way ANOVA test, the reduction of PFU% at 133 µg/ml TFT was considered statistically significant.

Trifluridine is a fluorinated pyrimidine nucleoside that inhibits the viral DNA synthesis (17, 18). Trifluridine triphosphate competes with thymidine triphosphate as a substrate for DNA polymerase. In addition, thymidylate synthetase is inhibited irreversibly by trifluridine monophosphate (22). Based on statistical analysis, there was no significant differences ($P>0.05$) between the results of preincubation and non-incubation methods in TFT containing samples at various concentrations using student's t-test. Although plant extract are toxic to virus capsid (23) and logically their preincubation with phage can lead to a better antiphage activity compared with non-incubation method, but the effect of preincubation is unknown for the synthetic antiviral derivatives that affect the replication stage, specifically. Maybe, the first incubation facilitates the entry of drugs into the bacterial cell.

Atta-ur-Rahman et al. (24) proposed a protocol for antiphage activity assay that has some differences with our developed method. First they performed only a qualitative test while the here-developed method provides antiphage evaluation both qualitatively and quantitatively. Secondly, the amount of bacterial inoculums could be standardized in our method. Finally, the effect of bioactive compound on the phage before lytic cycle initiates and during this cycle could be studied using newly-developed method whereas the method developed by Atta-ur-Rahman et al. shows the toxicity of active compound on the virus only before incubating the phage with the bacteria.

The present study has developed an inexpensive, rapid and easy antiphage assay that can be adopted both qualitatively and quantitatively.

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Authors' contributions

DA carrying out most of the experiments, writing and finalizing the manuscript. BJ and RM designing the study and revising the manuscript. SZ participating in some part of experiments.

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Conflict of interest

The authors declare that they have no conflict of interest in this work.

Role of sponsor

The sponsor provided the grant for financial supporting and facilities.

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