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# Synergistic Effect of Bacteriophage and Ampicillin against *Shigella dysenteriae*

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**ABSTRACT: Bacteriophage therapy has been considered as a potential approach to control drug resistant bacteria that cannot be killed by conventionally used antibiotics. Currently, synergism between bacteriophages and antibiotics has been reported to be a more effective therapeutic approach than that using each of these agents alone. In this study, a lytic bacteriophage against *S. dysenteriae* DMST 15111, SD01, was isolated from a hospital wastewater sample. Host range study revealed that the bacteriophage was specific to genus *Shigella*. By using the spot test, the minimal inhibitory concentrations (MICs) of bacteriophage SD01 and ampicillin against *S. dysenteriae* DMST 15111 were $10^4$PFU/ml and 31.25 µg/ml, respectively. When they were used together the MICs of both agent were substantially reduced. The results suggested that the bacteriophage and ampicillin had a synergistic inhibitory effect against *S. dysenteriae* DMST 15111. Therefore, this approach has a potential as a therapeutic approach against *Shigella* spp.**

**Keywords:** Bacteriophage, ampicillin, *Shigella dysenteriae*

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## INTRODUCTION

*Shigella dysenteriae* is a rod-shaped, gram negative bacterial species and classified as a member of facultative anaerobes. It is considered as an important food borne pathogen especially in developing countries. Since it produces deadly Shiga toxin, its infection can be severe and live threatening (Niyogi, 2005). Infections with *S. dysenteriae* are normally treated by antibiotics especially ciprofloxacin, ampicillin, cotrimoxazole, erythromycin, tetracycline, streptomycin and chloramphenicol (Lolekha *et al.*, 1991). However, many drug resistant strains of *S. dysenteriae* have recently emerged which lessen the effectiveness of the traditional treatment of bacterial infections by antibiotics (Khan *et al.*, 2009, Rezaii *et al.*, 2015).

Currently, many research works have been conducted to use bacteriophages as alternatives to antibiotics to control bacterial infections. The approach is called bacteriophage therapy (Pelfrene *et al.*, 2016; Rattanachaikunsopon and Phukhachorn, 2017). Several bacteriophages have been reported to be able to kill *S. dysenteriae* including bacteriophage SF-9 that was isolated from a river in Dhaka, Bangladesh (Faruque *et al.*, 2003). ShigaShield is a *Shigella* specific bacteriophages cocktails available as a commercial product. It consists of 5 different bacteriophages specific to *Shigella* spp. commonly contaminated in foods including 4 strains of *S. dysenteriae*, 2013AM-2809, AM11413, AM17886 and AM25896 (Soffer *et al.*, 2017). Bacteriophages have many advantages over antibiotics when they are used to treat bacterial infections. Because of their high specificity to host cells, bacteriophages generally kill only target bacterial pathogens and leave normal flora untouched; thus, causing no side effect. Low treatment doses are required because bacteriophages can replicate when they infect their hosts (Pelfrene *et al.*, 2016).

The therapeutic approach using bacteriophages together with antibiotics have been proposed (Wolska *et al.*, 2012). With this approach, less amounts of both agents are

required. It also improves the effectiveness of bacterial infection treatment because escaping (unkilled) bacteria have to be resistant to both bacteriophages and antibiotics. Synergism between bacteriophages and antibiotics has been reported to successfully inhibit pathogenic bacteria. For examples, bacteriophages have been used together with amikacina and gentamycin to control *Pseudomonas aeruginosa* (Nouraldin *et al.*, 2016; Hagens *et al.*, 2006) and together with amoxicillin and ciprofloxacin to control *Klebsiella pneumoniae* (Bedi *et al.*, 2009; Verma *et al.*, 2010). Therefore, it is of interest to study bacteriophage-anibiotic synergism to control *S. dysenteriae*. In this study, a bacteriophage specific to *S. dysenteriae* was isolated from a hospital wastewater sample. Its host range against a variety of bacteria was examined. The inhibitory ability against *S. dysenteriae* was also determined when it was used individually and in combination with an antibiotic.

## MATERIALS AND METHODS

*A. Bacterial strains and culture conditions*

Bacterial strains used in this study are listed in Table 1. *S. dysenteriae* DMST 15111 was used as a host for bacteriophage isolation and purification. All of them were grown in Brian heart infusion (BHI) medium at 37°C. Stock cultures of all bacteria containing 20% glycerol (v/v) were maintained at -20°C.

*B. Bacteriophage isolation and purification*

A wastewater sample was collected from Pracharak Vejchakarn Hospital, Srisaket Province, Thailand. The sample (10 ml) was centrifuged at 3,500 rpm for 5 min and the supernatant was collected and filtered through a membrane filter with a 0.45 µm pore size. The filtrate was added to an equal volume of double strength LB broth. To the mixture, 100 µl of a log phase *S. dysenteriae* DMST 15111 culture was added. After incubation at 37°C for 24 h, the culture was centrifuged at 3,500 rpm for 5 min and the supernatant was filtered through a membrane filter with a 0.45 µm pore size. The resulting filtrate was examined for the presence of a bacteriophage by the spot test that was performed as follows. A log phase culture of each bacterial strain uniformly swabbed over the surface of a BHI agar. Ten µl of the bacteriophage suspension was spotted onto the bacterial lawn. The plate was incubated at 37°C for 24 h before observing the presence of a clear zone. A clear zone at the spot area, representing the lysis of host cells, indicated the lytic activity of the bacteriophage.

The presence of a bacteriophage in the filtrate was confirmed by plaque assay using *S. dysenteriae* DMST 15111 as a host (Phumkhachorn and Rattanachaikunsopon, 2018). After plaque assay, a single clear plaque was randomly selected and subjected to 2 more rounds of plaque assay and single plaque selection. The single plaque selected from the final round of plaque assay was transferred into a tube containing 10 ml of a log phase *S. dysenteriae* DMST 15111 culture. The tube was then incubated at 37°C overnight to allow bacterial cell lysis to occur. The bacteriophage lysate was centrifuged at 3,500 rpm for 5 min. The supernatant was filtrated through a membrane filter with a 0.45 µm pore size. The resulting filtrate or bacteriophage suspension was kept as a bacteriophage stock at 4°C.

Plaque assay was also used to determine the concentration of the bacteriophage in plaque forming unit (PFU)/ml (Phumkhachorn and Rattanachaikunsopon, 2018).

*C. Bacteriophage host range determination*

The inhibitory ability of the isolated bacteriophage against a variety of bacteria (listed in Table 1) was determined by using the spot test as mentioned above.

*D. Determination of the minimal inhibitory concentration of bacteriophage*

To determine the minimal inhibitory concentration (MIC) of bacteriophage against *S. dysenteriae* DMST 15111, ten-fold dilution of the bacteriophage suspension was performed to obtain the bacteriophage concentrations ranging from $10^9$ to 10 PFU/ml. Each bacteriophage concentration was examined for its ability to inhibit *S. dysenteriae* DMST 15111 by the spot test as mentioned above.

*E. Determination of the minimal inhibitory concentration of ampicillin*

To determine the MIC of ampicillin against *S. dysenteriae* DMST 15111, two-fold dilution of the antibiotic was performed to obtain the concentrations ranging from 1,000 – 31.25 µg/ml. Each ampicillin concentration was examined for its ability to inhibit *S. dysenteriae* DMST 15111 by the spot test as mentioned above.

*F. Study of bacteriophage-ampicillin synergism*

To study bacteriophage-ampicillin synergism to control *S. dysenteriae* DMST 15111, 5 µl of bacteriophage and 5 µl of ampicillin were combined to obtain a sub MIC range of both agents as shown in Table 2. Sterile distilled water was used as a negative control for both bacteriophage and ampicillin. Bacteriophage and ampicillin with the concentration of MICs were used as positive controls. Each bacteriophage-ampicillin combination was examined for its ability to inhibit *S. dysenteriae* DMST 15111 by the spot test as mentioned above.

## RESULTS AND DISCUSSION

*A. Bacteriophage isolation and purification*

A filtrate prepared from a hospital wastewater was shown by the spot test to have a bacteriophage specific to *S. dysenteriae* DMST 15111 because it produced an inhibition zone on the lawn of the bacterial host (Fig. 1a). The result was also confirmed by plaque assay. The filtrate produced uniform plaques with a diameter of about 0.1 mm (Fig. 1b). The bacteriophage was designated bacteriophage SD01. Since the inhibition zone and plaques produced by the bacteriophage were clear, it was likely to be a lytic bacteriophage. This type of bacteriophage is favorable for using as a therapeutic agent because it can kill the target bacteria.

The practical way to isolate a bacteriophage specific to a particular bacterial strain is to use the sample collected from where the bacterial host exists. Since the bacterial strain used as the host for bacteriophage isolation, *S. dysenteriae* DMST 15111, is a clinical strain isolate from a hospital, the ideal sample for bacteriophage isolation is a wastewater sample collected from a hospitals. Previously, several bacteriophages specific to hospital derived pathogens were isolated from hospital derived samples such as those specific to multidrug resistant *Pseudomonas aeruginosa*, *Klebseilla pneumonia*e, *Staphylococcus aureus* and *Escherichia coli* (Pallavali *et al.*, 2017), multidrug resistant *Acinetobacter baumannii* (Ghajavand *et al.*, 2017) and extended spectrum β-lactameses (ESBL) producing *E. coli* (Phumkhahcorn and Rattanachaikunsopon, 2015).

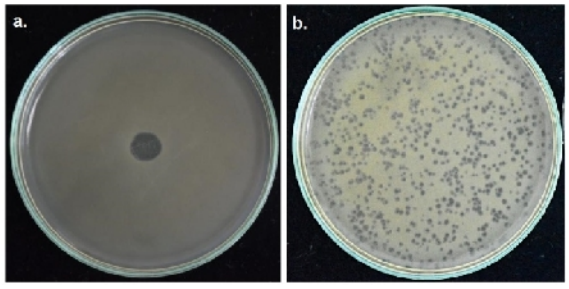


**Fig. 1.** Inhibition zone (a) and plaques (b) on lawn of *S. dysenteriae* DMST 15111 produced the bacteriophage SD01.

*B. Bacteriophage host range*

One parameter needed to be considered before using a bacteriophage as a therapeutic agent is its host range (Ross *et al.*, 2016). Bacteriophages with a broad host range may not be suitable for therapeutic use because they tend to inhibit beneficial normal flora residing in recipients. However, bacteriophages with a narrow inhibitory spectrum may cause limitation in their therapeutic use. This problem can be overcome by using cocktails of several bacteriophages (Chan *et al*., 2013) or combinations of bacteriophages and antibiotics (Wolska *et al.*, 2012). The bacteriophage SD01 isolated in this study was found to be genus specific because it inhibited all species of the genus *Shigella*, but not the rest of the bacteria used in this study (Table 1). Since it did not inhibit other genera of bacteria besides *Shigella*, it might be a safe therapeutic agent with no harm to normal flora.

**Table 1: Inhibitory ability of bacteriophage against a variety of bacteria.**

| Bacteria[a] | Inhibitory ability[b] |
|---|---|
| *Bacillus cereus* ATCC 11778 | - |
| *Escherichia coli* ATCC 25922 | - |
| *Klebsiella pneumoniae* ATCC27736 | - |
| *Proteus mirabilis* ATCC 12453 | - |
| *Pseudomonas aeruginosa* ATCC 27853 | - |
| *Salmonella typhi* ATCC 19430 | - |
| *Shigella boydii* DMST 28180 | + |
| *Shigella dysenteriae* DMST 15111 | + |
| *Shigella flexneri* DMST 4423 | + |
| *Shigella sonnei* DMST 561 | + |
| *Staphylococcus aureus* ATCC 25923 | - |

[a]ATCC, American Type Culture Collection; DMST, Department of Medical Sciences Thailand
[b] - = no inhibitory ability; + = having inhibitory ability

*C. MIC of bacteriophage SD01 against S. dysenteriae DMST 15111*

The different concentrations of bacteriophage SD01 ranging from $10^9$ to 10 PFU/ml were subjected to the spot test to examine their inhibitory ability against *S. dysenteriae* DMST 15111. The bacteriophage concentrations capable of inhibiting the bacterial host were $10^4$ PFU/ml and above while those produced inhibition zone against the bacterial host were $10^3$ PFU/ml and below. The results indicated that the MIC of bacteriophage SD01 against *S. dysenteriae* DMST 15111 was $10^4$ PFU/ml. Each MIC value is specific to each bacteriophage-host pair. When one party of the pair (bacteriophage or host) is changed, the MIC will alter. For example, the MIC values of bacteriophage SD01 against *S. sonnei* DMST 561 was $10^5$ PFU/ml (data not shown). Similar finding was also reported in the case of bacteriophage lambda. The bacteriophages T4 and T7 had different MIC values against the same *E. coli* host (Vipra *et al.*, 2013)

*D. MIC of ampicillin against S. dysenteriae DMST 15111*

The different concentrations of ampicillin ranging from 1,000–1.95 µg/ml were subjected to the spot test to examine their inhibitory ability against *S. dysenteriae* DMST 15111. The ampicillin concentrations capable of inhibiting the bacterial host were 31.25 µg/ml and above while those produced inhibition zone against the bacterial host were 62.5 µg/ml and below. The results indicated that the MIC of ampicillin against *S. dysenteriae* DMST 15111 was 31.25µg/ml. Similar to the bacteriophage-host relationship, each MIC value is specific to each antibiotic-host pair. When one party of the pair (antibiotic or host) is changed, the MIC will alter. For example, the MIC values of ampicillin against *S. sonnei* DMST 561 was 62.5 µg/ml (data not shown). Two different antibiotics, cefquinome and cephapirin, were found to have different MIC values against the same *E. coli* host (Sheldon *et al.*, 2004).

*E. Bacteriophage SD01-ampicillin synergism against S. dysenteriae DMST 15111*

When bacteriophage SD01 and ampicillin with concentration less than MIC values (sub MIC values) were used together to inhibit *S. dysenteriae* DMST 15111. At those concentrations, both bacteriophages SD01 and ampicillin alone could not inhibit the bacterial host. However, when they used together, they were able to inhibit *S. dysenteriae* DMST 15111 and the lowest concentrations of bacteriophages SD01 and ampicillin that could inhibit the host were $10^3$PFU/ml ($MIC_b$/10) and 7.81 µg/ml ($MIC_a$/4), respectively, which were in the sub MIC levels (Table 2). The results suggested that bacteriophage SD01 and ampicillin had a synergistic effect against *S. dysenteriae* DMST 15111. Similar findings were reported by Nouraldin *et al.* (2016) who used bacteriophages in combination with amikacin to control *P. aeruginosa* and Bedi *et al.* (2006) who used bacteriophages in combination with amoxicillin to control *K. pneumoniae*.

**Table 2: Inhibitory ability of bacteriophage and ampicillin against *S. dysenteriae* DMST 15111.**

| Ampicillin concentration | Bacteriophage concentration | | | |
|---|---|---|---|---|
| | No bacteriophage | $MIC_b$/100 | $MIC_b$/10 | $MIC_b$ |
| No ampicillin | -* | - | - | +* |
| $MIC_a$/4 | - | - | + | + |
| $MIC_a$/2 | - | - | + | + |
| $MIC_a$ | + | + | + | + |

$MIC_b$ = MIC of bacteriophage SD01; $MIC_a$ = MIC of ampicillin

* - = no inhibitory ability; + = having inhibitory ability

The use of bacteriophages and antibiotics combination have several advantages over using each one of these agents alone. Antibiotics can broaden the host ranges of bacteriophages; thus, improving inhibitory ability of bacteriophages. In addition, bacteriophages can reduce therapeutic doses of antibiotics; hence, reducing side effects causing by nonspecific inhibitory effect of antibiotics and lowering drug resistance emerge risks resulting from excessive use of antibiotics (Chaudhry *et al.*, 2017).

## CONCLUSIONS

Bacteriophage SD01 was a *Shigella* specific bacteriophage isolated from a hospital wastewater sample. It had inhibitory ability against *S. dysenteriae* DMST 15111 when used alone and used in combination with ampicillin. The synergism between these two agents substantially reduced their minimal inhibitory concentrations against *S. dysenteriae* DMST 15111. With further investigation, the bacteriophage, by itself or in combination with antibiotics, may be useful as a therapeutic agents in controlling *Shigella*spp.

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