Volume 8 Issue 1 June 2020

pISSN 2302-1616
eISSN 2580-2909

BIOGENESIS

Jurnal Ilmiah Biologi

DEPARTMENT OF BIOLOGY
FACULTY OF SCIENCE AND TECHNOLOGY
UNIVERSITAS ISLAM NEGERI ALAUDDIN
MAKASSAR

Volume	Issue	June	Page	pISSN 2302-1616
8	1	2020	1-95	eISSN 2580-2909

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BIOGENESIS Jurnal Ilmiah Biologi
pISSN 2302-1616, eISSN 2580-2909
Vol 8, No. 1, June 200, pp. 22-28
Available online http://journal.uin-alauddin.ac.id/index.php/biogenesis
DOI https://doi.org/10.24252/bio.v8i1.11280

Synergism of Phage ϕPT1b and Antibiotics for Reducing Infection of *Escherichia coli*

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Received 30 November 2019; Received in revised form 07 March 2020;
Accepted 14 May 2020; Available online 30 June 2020

ABSTRACT

Foodborne disease caused by *Escherichia coli* contamination is increasing every year. It also followed by elevating of drug-resistance of *E. coli*. Bacteriophage can be an alternative for therapy infection. This study aimed to determine synergism effect of bacteriophage ϕPT1b which has a high rate virulence to *E. coli* and phage-antibiotics (tetracycline and amoxicillin) synergy. The indigenous bacteria isolates were KR, MJ, KP, PT, PR. Five bacteriophages used namely ϕKR1b, ϕKR2, ϕPT1a, ϕPT1b, and ϕMJ1b. Virulence test was used to determine the ability of each phage in reducing *E. coli*. Treatment to examine synergism of phage ϕPT1b and antibiotics were P1: amoxicillin, P2: ϕPT1b, P3: ϕPT1b + Amx = 1:1, P4 : ϕPT1b + Amx = 2:1, P5: ϕPT1b + Amx = 1:2, P6 : tetracycline, P2: ϕPT1b, P7: ϕPT1b + Tet = 1:1, P8 : ϕPT1b + Tet = 2:1, and P9: ϕPT1b + Tet = 1:2. The virulence test showed that isolate ϕPT1a with 106 CFU/ml had the highest ability in reducing *E. coli*. While, the result of synergism test indicated that the synergism of bacteriophage and antibiotics differ significantly ($P \leq 0.05$). The best ratios of synergism were 1:1 (ϕPT1b+tetracycline) and 2:1 (ϕPT1b+amoxicilline). In summarize, phage-antibiotic synergy (ϕPT1b with tetracycline/amoxicilline) can reduce the level of antibiotic resistance in isolated *E. coli*.

Keywords: amoxicillin; bacteriophage; phage-antibiotic synergy; tetracycline; virulence abilities

INTRODUCTION

Foodborne disease is caused by contaminated food consumption. Contamination happens during preparation and serving process; it poses threats to health in general (Ameme *et al*., 2016). One type of food prone to bacterial contamination is vegetables (Quinlan, 2013). *Campylobacter* (Verhoeff-Bakkenes *et al*., 2011; Mohammadpour *et al*., 2018), *Salmonella* (de Freitas Neto *et al*., 2010; Golberg *et al*., 2011), *Shigella* (Ranjbar *et al*., 2010; Magnone *et al*., 2013), *Listeria* (Ponniah *et al*., 2010; Oliveira *et al*., 2011), *Yersinia* (Xanthopoulos *et al*., 2010; Tirziu *et al*., 2011) and *Escherichia coli* (Harapas *et al*., 2010; Ijabadeniyi *et al*., 2011; Uzeh & Adepoju, 2013) are among the common bacteria contaminate vegetables.

Pathogenic *E. coli* are the dominant cause of diarrhea which is the primary indicator of food poisoning.The symptoms can be severe and fatal. Diarrhea-caused *E. coli* can be divided into several types, including enterotoxic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), and enterohaemorrhagic *E. coli* (EHEC). In addition, a uropathogenic *E. coli* can infect extraintestinal and causing urinary tract infections. Some of these strains can cause foodborne disease (Caine *et al*., 2014).

Typical treatment for foodborne disease is using antibiotics, but now antibiotics pose a new problem, which is an increase in the incidence of antibiotics resistance. Antibiotic resistance is characterized by failure inhibition of bacterial growth (Andersson & Hughes, 2010; Humaida, 2014; Brown & Wright, 2016). *E. coli* show antibiotic resistance in all β-lactam antibiotics, tetracycline, and in cephalothin and cefoxitin from cephalosporins (Sidik *et al*., 2016). Another alternative treatment for foodborne disease is using bacteriophages

(phages). Bacteriophage is a virus that can infect and kill bacterial cells. The presence of bacteriophages that can kill these hosts can be applied in the technology of the lytic process for biofilm-forming bacteria (Buana & Wardani, 2013). Bacteriophages have a tiny size, in nanometers, and live in the same place as the bacterial host. Bacteriophages have species-specific properties and even in strains that can only infect bacteria that are like their hosts (Hamdi *et al*., 2017).

Phage therapy has proven effective for controlling pathogens that cause foodborne disease both pre- and post-harvest. Administration of phages in pre- and post-harvest can control the contamination of bacteria such as *E. coli* O157: H7, *Salmonella*, and *Campylobacter* in food products. The use of phages in pre-harvest to reduce pathogens is carried out directly by providing phages to the plants, while the administration of phages in the post-harvest is carried out directly to eliminate unwanted contaminants in food products that are ready to be consumed (Teng-Hern *et al*., 2014).

Phages as biocontrol have several advantages such as the ability to attack bacteria and zero influence on other cell types including human, animal and plant cells. This is as reported in the ϕECP100 and ϕAHFAHEc1 tests carried out as natural preservatives of fresh vegetables and meat products contaminated by *E. coli* O157: H7. Test results showed that bacteriophage ϕECP100 and ϕAHFAHEc1 could eliminate contamination of *E. coli* in raw beef (Lee *et al*., 2017) . In other studies, ϕVCEV1 can also infect *E. coli* O157: H7 where this bacteriophage has a 70 nm icosahedral head shape and a long flexible tail measuring 175 nm in length and 10 nm in width with a genome size of 120 kb (Raya *et al*., 2015).

Another advantage of bacteriophages is that it can reduce the resistance of pathogenic bacteria to antibiotics. Combinations between bacteriophages and antibiotics usually referred to as phage-antibiotic synergy (PAS). In ϕKS12 and ϕKS14 with a combination of ciprofloxacin, meropenem, and tetracycline can kill *Burkholderia cepacia* thus it can reduce the resistance properties of *Burkholderia cepacia* against antibiotics (Kamal & Dennis, 2015; Sfeir, 2018). In a previous study, isolated five bacteriophages from *E. coli* contaminating vegetables in the Jember Regency (Narulita *et al*., 2018). The five bacteriophages have not been known of the ability to perform PAS including bacteriophage ϕPT1a that have a high virulence rate. This study aimed to determine synergism effect of bacteriophage ϕPT1a which has a high rate virulence to *E. coli* and phage-antibiotics (tetracycline and amoxicillin) synergy.

MATERIALS AND METHODS

Sample Preparation. Bacteria were grown in eosin methylene blue agar (EMBA) to determine whether the bacteria used were isolates of *Escherichia coli* PT (Tanjung Market), KR (Kreongan Market), MJ (Peddler), KP (Kepatihan Market), PR (Patrang Market). In EMBA, *E. coli* PT (Tanjung Market), KR (Kreongan Market), MJ (Peddler), KP (Kepatihan Market), PR (Patrang Market) give characteristics of metallic green colonies. For further treatment luria bertani (LB) medium was used. The incubation was carried out for 24 hours at 37°C. Particles of phages were multiplied by plaque assay techniques following. A 100 µl of each filtrate bacteriophage of ϕKR1a, ϕKR1b, ϕPT1a, ϕPT1b, ϕMJ1b and ϕKR2 suspension added into 250 µl of *E. coli* suspension which had been incubated for 2 hours at 37°C. The suspension was mixed with warm (around 50°C) top agar medium, and then poured on LB. The petri dish was incubated at 37°C for 24 hours. The colonies of phage were indicated in the formation of plaques. SM buffers as much as 5 ml were added to acquire the phages. The media were shaken for 4 hours at 4°C and then were centrifuged for 10 minutes at 10000 rpm. The phages were harvested using a membrane filter with a pore membrane density of 0.2 µm. The filtrate served as bacteriophage stock (Askora *et al*., 2009).

Virulence Assay. The test was carried out using the plaque assay technique (Askora *et al*., 2009). A 300 µl suspension of 5 hours *E. coli* and, 3 µl phage suspension was added to the

0.3% warm top medium. The mixture then was poured on the surface of LB agar media in a petri dish (double layer method). Incubation carried out at 37°C for 24 hours. The growth of phages was observed by counting the formation of plaque. Different concentrations were used for each type of phage suspension i.e 102, 104, and 106 of phages suspension.

Virulence Assay of phage + antibiotic to the growth of *E. coli*. The technique to find out the strength of phage+antibiotic virulence was using spot test technique according to (Narulita *et al*., 2018). A 300 µl suspension of 5 hours *E. coli* (0.1 U of OD600) and 3 µl bacteriophage suspension was added to the 0.3% warm top medium. The mixture then was poured on the surface of LB agar medium in a petri dish (double layer method). Next, 3 µl of the phage+3 µl antibiotic suspension was spotted on the LB agar medium and incubated at 37°C for 24 hours. Growth was observed with the formation of plaque with six replications. Serial of phage concentration was used which gave the maximum results in the previous test (virulence test without antibiotics).

Bacteriophages used in the phage-antibiotic synergy (PAS) test was ϕPT1a that has a highest virulence rate in infecting *E. coli* isolates. The antibiotics used were amoxicillin (Amx) and tetracycline (Tet). The treatment was divided into P1: amoxicillin, P2: ϕPT1b, P3: ϕPT1b + Amx = 1:1, P4 : ϕPT1b + Amx = 2:1, P5: ϕPT1b + Amx = 1:2, P6 : tetracycline, P2: ϕPT1b, P7: ϕPT1b + Tet = 1:1, P8 : ϕPT1b + Tet = 2:1, and P9: ϕPT1b + Tet = 1:2. The effect of this test results determined by analysis of variants (ANOVA) of 95% confidence level ($p < 0.05$), then followed by Duncan's test.

RESULT AND DISCUSSION

Each phage has different virulence abilities. From three different phage concentrations of 10^2, 10^4, 10^6 (CFU/ml) of each tested bacteriophage, ϕPT1b gave the highest result of infection to *E. coli* at concentration of 10^6 CFU/ml (Figure 1 and 2) compared to other bacteriophages.

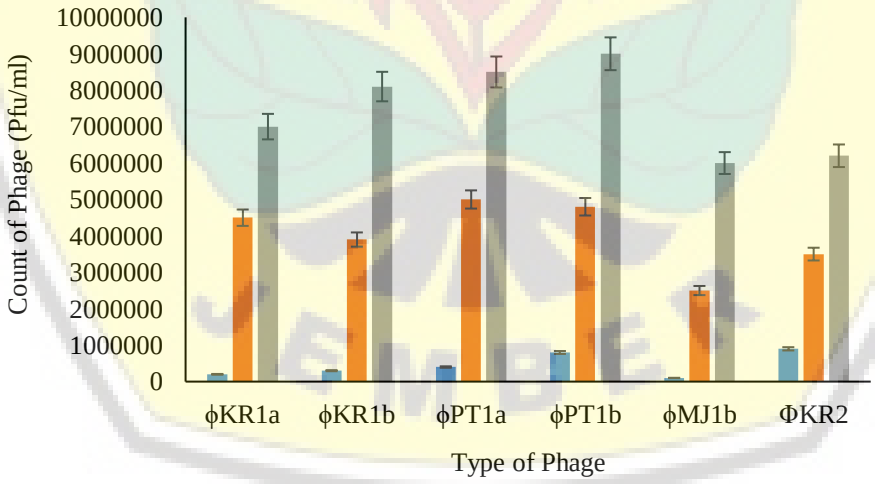

Figure 1. The number of phage on plaque assay with *Escherichia coli*

The combination between phage and amoxicillin showed different results that in treatment P4 gave higher efficiency than the others, P2 had a higher level of infection than amoxicillin whereas P7 was a combination of phage and tetracycline indicating that P7 had a higher effectiveness (Table 1 and 2).

Virulence test used amoxicillin and tetracycline because both types of antibiotics performed resistance to *E. coli*. In the treatment area that only used antibiotics, no plaque was formed. This result showed no growth inhibition in *E. coli* (resistance occurred). The resistance occurs due to biochemical modifications that alter the nature of bacteria from those that are usually sensitive to antibiotics to become insensitive.

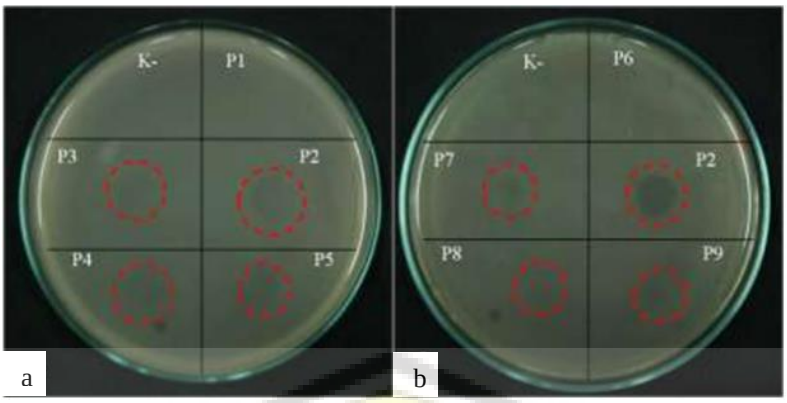

Figure 2. Virulence test results of phage combination: a. amoxicillin antibiotics with treatment P1: amoxicillin, P2: ϕPT1b, P3: ϕPT1b + Amx = 1:1, P4 : ϕPT1b + Amx = 2:1, P5: ϕPT1b + Amx = 1:2; b. tetracyclin with treatment P6 : tetrasiklin, P2: ϕPT1b, P7: ϕPT1b + Tet = 1:1, P8 : ϕPT1b + Tet = 2:1, P9: ϕPT1b + Tet = 1:2.

The chemical modification that occurs can be an enzyme that deactivates antibiotic receptors and changes in protein in the cell wall that inhibit the absorption of these antibiotics. The existence of genetic mechanisms in the form of mutations and transformations can also trigger antibiotic resistance. Mutations may occur naturally during cell division. The more cells undergo replication, the higher the chance for mutation. Mutations that occur may encode a new resistance gene to deactivate antibiotic receptors (Andersson & Hughes, 2014; Blount, 2015). Plaque can be found in the treatment area that uses a combination of antibiotics and phages (Figure 2). On the other hand, the bacteriophage virulence test showed that ϕPT1b was able to infect bacteria higher than the other phages at a concentration of 10^6 CFU/ml (Figure 1).

Table 1. The result of virulence assay for phage + amoxicillin combination to *E. coli*

Treatments	Average of Plaque Diameter (mm)
P1	0.00 ± 0.0^{a}
P2	1.50 ± 0.22^{c}
P3	1.31 ± 0.29^{bc}
P4	1.41 ± 0.21^{bc}
P5	1.19 ± 0.14^{b}
K-	0.00 ± 0.0

Notes: P1 : amoxicillin (Amx), P2 : ϕPT1b, P3 : Phage + Amx= 1:1, P4 : Phage + Amx= 2:1, P5: Phage + Amx= 1:2. The same letter in the same column indicates not significantly different by Duncan test using α 5%

Table 2. The result of virulence assay for phage + tetracyclin combination to *E. coli*

Treatments	Average of Plaque Diameter (mm)
P6	0.00 ± 0.00^{a}
P2	1.50 ± 0.22^{c}
P7	1.36 ± 0.21^{b}
P8	1.30 ± 0.12^{b}
P9	1.23 ± 0.25^{b}
K-	0.00 ± 0.00

Notes: P2 : ϕPT1b, P6 : Tetracyclin (Tet), P7 : Phage + Tet = 1:1, P8 : Phage + Tet = 2:1, P9 : Phage + Tet = 1:2. The same letter in the same column indicates not significantly different by Duncan test using α 5%

The results of virulence test of phage-antibiotics combinations gave different results between the two types of antibiotics. The treatment of P2, P3, P4, P5, P7, P8 and P9 were significantly different from P1 and P6 (Table 1 and Table 2). In the combination of phage with amoxicillin, it showed that P4 gave higher virulence effectiveness than P3 and P5 (Table 1). While the combination of phage with tetracycline indicated that the P7 treatment has higher effectiveness compared to P8 and P9 (Table 2). P2 treatment which was phage

ϕPT1b only, it turned out to have the highest infection rate compared to the combination with amoxicillin and tetracycline (Table 1 and 2). Large clear zones formed for both types of antibiotic combinations can be seen in Figure 2.

The difference in plaque size is due to the replication process of phages that produce new plaques (Bhardwaj *et al*., 2015). The more phages produced will have larger plaque sizes, and vice versa, if the fewer phages are produced, then the size plaque will get smaller too. The resulting plaque size is determined by the number of virions produced in each infected cell and how long it takes for bacteriophages to lyse infected cells (Comeau *et al*., 2007).

When phages and antibiotics are combined, there is a possibility of phage mutant that lyses bacteria faster and can make plaque formation more quickly than the parent bacteriophage. Antibiotics also have the same working time as bacteriophages, hence a combination of bacteriophages and antibiotics can inhibit the growth of pathogenic bacterial cells (Golkar *et al*., 2014; Tagliaferri *et al*., 2019). Similar research was conducted, regarding bacteriophage therapy to control pathogenic *Pseudomonas aeruginosa* by combining antibiotics and bacteriophages (Torres-Barceló *et al*., 2014). The results showed that the combination of streptomycin and bacteriophages results in a positive synergy that inhibits the growth of *Pseudomonas aeruginosa*. T4 type phages could also be combined with cefotaxime type antibiotics Comeau *et al*. (2007) found there was positive synergy marked by the reduction of *E. coli* cell production. The presence of phage-antibiotic synergy (PAS) response is seen with the formation of clear zones which indicate that chemicals in antibiotics stimulate phage growth at the same level as the conditions of antibiotics when inhibiting bacterial cell division.

CONCLUSION

Phage-antibiotic synergy (ϕPT1b with tetracycline/amoxicilline) can reduce the level of antibiotic resistance in isolated *E. coli*.

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