

BIOGENESIS

Jurnal Ilmiah Biologi

Stem Biomass Lignation of *Urochloa* *urochloa* 5,7, Diakn
Mufarroti Masriyah, Juliana Dwiy, Alita Alita, Zak Alimul Himmah

Isolation and Molecular Characterization of Gelatinase-Producing Bacteria from Mangrove Sediment
Rup Anandito Priyanto, Mohamad Mulyawan, Akhik Aza Fauzi, Hadia Nurul Dzakri

Voluntary Diving Exercise Improves Hippocampus-dependent Learning in Rats
Rahmawati Yudo Hartawan, Heru Eko Nugro, Ayu Yohana Tega Anang, Eka Nurulita Pratiwi, Mufarroti

Synergism of Phage ϕ PT1b and Antibiotics for Reducing Infection of *Escherichia coli*
Aulia Sherina, Gilda Ernawati, Ayu Watiyana, Siti Nurhidayah, Rizki Nurliana

Enhancing the productivity performance of *Cyprinus carpio* L. by *Manihot* *ubellissimo* Pohl. leaves supplementation
Tria Ayu Eka Nurulita, Ulia Effiana Sari, Hanania Hanania, Rizki Nurulita Nurulita

Indonesia Black Cumin (*Nigella arvensis* L.) Seeds Extract as Anticancer Reproductive Function in Type-2 Diabetes Mellitus
Rizka Santiaji, Alif Nurrahmah Alif Nurrahmah, Nurulita Nurulita, Tri Nurulita

Performance Comparison of Data Sampling Techniques to Handle Imbalanced Class on Prediction of Compound-Protein Interaction
Alif Nurrahmah Alif Nurrahmah, Tri Nurulita Nurulita, Nurulita Nurulita, Nurulita Nurulita

Antifungal Activity of *Maranta arvensis* leaf extracts against *Colletotrichum acutatum*
Dhika Nurulita, Rizki Nurulita Nurulita, Nurulita Nurulita

New distributional records of *Cleome chelidonii* L. and *Cleome ravidosperma* DC. (Cleomaceae) in Madura Island
Alif Nurrahmah Alif Nurrahmah, Nurulita Nurulita, Nurulita Nurulita, Nurulita Nurulita

Genetic Variation at Microsatellite Loci in *Odonnottia* *basis* (Boulenger, 1891)
Dhika Nurulita, Nurulita Nurulita, Nurulita Nurulita

Hematological response of *Tilapia* (*Oreochromis niloticus*) in Laundry wastewater
Sapardi, Nurulita Nurulita, Nurulita Nurulita, Nurulita Nurulita

Diversity of Aquatic and Terrestrial Molluscs from Simeulue Island, with Notes on Their Distribution and Some New Records
Dhika Nurulita, Nurulita Nurulita, Nurulita Nurulita

Beak Line and Eye Alignment as Phenotypic Sexing for Domestic Canaries (*Serinus canaria*)
Alif Nurrahmah Alif Nurrahmah, Nurulita Nurulita, Nurulita Nurulita, Nurulita Nurulita

Determining Phytocomponent of Vetiver Grass (*Chrysopogon zizanioides*) Under Drought Stress
Ani Susantiyanti, Syarifuddin, Nurulita Nurulita

Magainin as an Antiviral Peptide of SARS-CoV-2 Main Protease for Potential Inhibitor: An In Silico Approach
Taufik Mufarroti Fauzi, Mufarroti Fauzi, Nurulita Nurulita

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

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

RESEARCH ARTICLES

<u>Stem Biomass Equation of Eucalyptus urophylla S.T. Blake</u>	PDF
 Melewanto Patabang, Julianus Dising, Adrin Adrin, Aah Ahmad Almulqu	1-9
 Abstract - 325	
<u>Isolation and Molecular Characterization of Gelatinase-Producing Bacteria from Mangrove Sediment</u>	PDF
 Asep Awaludin Prihanto, Hidayatun Muyasyaroh, Abdul Aziz Jaziri, Nada Itorul Umam	10-14
 Abstract - 230	
<u>Voluntary Diving Exercise Improves Hippocampus-dependent Learning in Rats</u>	PDF
 Rahadian Yudo Hartantyo, Helen Eko Putro, Epa Yohana Toga Torop, Laksmindra Fitria, Mulyati Mulyati	15-21
 Abstract - 203	
<u>Synergism of Phage ϕP1b and Antibiotics for Reducing Infection of Escherichia coli</u>	PDF
 Eria Narulita, Gerda Permata Aji, Bevo Wahono, Siti Murdiyah, Ria Yulian	22-28
 Abstract - 131	
<u>Enhancing the productivity performance of Cyprinus carpio L. by Manihot utilissima Pohl. leaves supplementation</u>	PDF
 Teuku Reza Efianda, Uci Elfana Sari, Humeira Humeira, Kiki Rishki Ananda	29-34
 Abstract - 217	
<u>Indonesia Black Cumin (Nigella sativa L.) Seeds Extract as Ameliorant Reproductive Function in Type-2 Diabetes Mellitus</u>	PDF
 Retno Susilowati, Nailirrohmah Hidayatin, Amalia Rizka Diana, Tri Kustono Adi	35-40
 Abstract - 201	
<u>Performance Comparison of Data Sampling Techniques to Handle Imbalanced Class on Prediction of Compound-Protein Interaction</u>	PDF
 Akhmad Rezki Purnajaya, Wisnu Ananta Kusuma, Medria Kusuma Dewi Hardhienata	41-48
 Abstract - 271	
<u>Antifungal Activity of Morinda citrifolia leaf extracts against Colletotrichum acutatum</u>	PDF
 Oktira Roka Aji, Larasati Hallimah Roosyidah	49-54
 Abstract - 254	
<u>New distributional records of Cleome chelidonii L.f. and Cleome rutidosperma DC. (Cleomaceae) in Madura Island</u>	PDF

-  Arifin Surya Dwipa Irsyam, Muhammad Rifqi Hariri, Ashari Bagus Setiawan, Rina Ratnasih Irwanto, Asih Perwita Dewi 55-61
 Abstract - 278
- [Genetic Variation at Microsatellite Loci in *Odorrana hosii* \(Boulenger, 1891\)](#) [PDF](#)
 Djong Hon Tjong, Anugrah Viona Agesi, Dewi Imelda Roesma 62-68
 Abstract - 265
- [Hematological Response of Tilapia \(*Oreochromis niloticus*\) in Laundry Wastewater](#) [PDF](#)
 Saparuddin Saparuddin, Yanti Yanti, Salim Salim, Harish Muhammad 69-78
 Abstract - 317
- [Diversity of Aquatic and Terrestrial Molluscs from Simeulue Island, with Notes on Their Distribution and Some New Records](#) [PDF](#)
 Nova Mujiono, Ristiyanti M Marwoto, Heryanto Heryanto 79-88
 Abstract - 209
- [Beak Line and Eye Alignment as Phenotypic Sexing for Domestic Canaries \(*Serinus canaria*\)](#) [PDF](#)
 Afif Muhammad Akrom, Soedarmanto Indarjulianto, Yanuartono Yanuartono, Trini Susmiati, Alfarisa Nururrozi, Slamet Raharjo, Rief Ghulam Satria Permana, Puveanthan Nagappan Govendan 89-93
 Abstract - 175
- [Determining Phytocomponent of Vetiver Grass \(*Chrysopogon zizanioides*\) Under Drought Stress](#) [PDF](#)
 Ani Sulistiyani, Syamsul Falah, Triadiati Triadiati 94-103
 Abstract - 201
- [Magainin as an Antiviral Peptide of SARS-CoV-2 Main Protease for Potential Inhibitor: An In Silico Approach](#) [PDF](#)
 Taufik Muhammad Fakhri, Mentari Luthfika Dewi, Eky Syahroni 104-110
 Abstract - 340

Synergism of Phage ϕ PT1b 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 ϕ PT1b 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 ϕ KR1b, ϕ KR2, ϕ PT1a, ϕ PT1b, and ϕ MJ1b. Virulence test was used to determine the ability of each phage in reducing *E. coli*. Treatment to examine synergism of phage ϕ PT1b and antibiotics were P1: amoxicillin, P2: ϕ PT1b, P3: ϕ PT1b + Amx = 1:1, P4 : ϕ PT1b + Amx = 2:1, P5: ϕ PT1b + Amx = 1:2, P6 : tetracycline, P7: ϕ PT1b, P8 : ϕ PT1b + Tet = 1:1, P9 : ϕ PT1b + Tet = 2:1, and P10: ϕ PT1b + Tet = 1:2. The virulence test showed that isolate ϕ PT1a 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 (ϕ PT1b+tetracycline) and 2:1 (ϕ PT1b+amoxicillin). In summarize, phage-antibiotic synergy (ϕ PT1b with tetracycline/amoxicillin) 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 enterotoxigenic *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 ϕ ECP100 and ϕ AHFAHEc1 tests carried out as natural preservatives of fresh vegetables and meat products contaminated by *E. coli* O157: H7. Test results showed that bacteriophage ϕ ECP100 and ϕ AHFAHEc1 could eliminate contamination of *E. coli* in raw beef (Lee *et al.*, 2017). In other studies, ϕ VCEV1 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 ϕ KS12 and ϕ KS14 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 ϕ PT1a that have a high virulence rate. This study aimed to determine synergism effect of bacteriophage ϕ PT1a 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 μ l of each filtrate bacteriophage of ϕ KR1a, ϕ KR1b, ϕ PT1a, ϕ PT1b, ϕ MJ1b and ϕ KR2 suspension added into 250 μ l 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 μ m. 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 μ l suspension of 5 hours *E. coli* and, 3 μ l 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 10², 10⁴, 10⁶ (CFU/ml) of each tested bacteriophage, φPT1b gave the highest result of infection to *E. coli* at concentration of 10⁶ CFU/ml (Figure 1 and 2) compared to other bacteriophages.

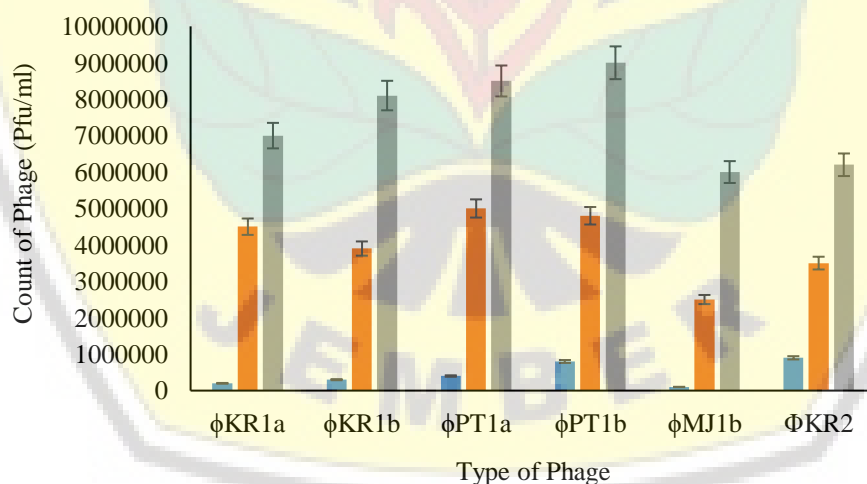


Figure 1. The number of phage on plaque assay with *Escherichia coli*

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.

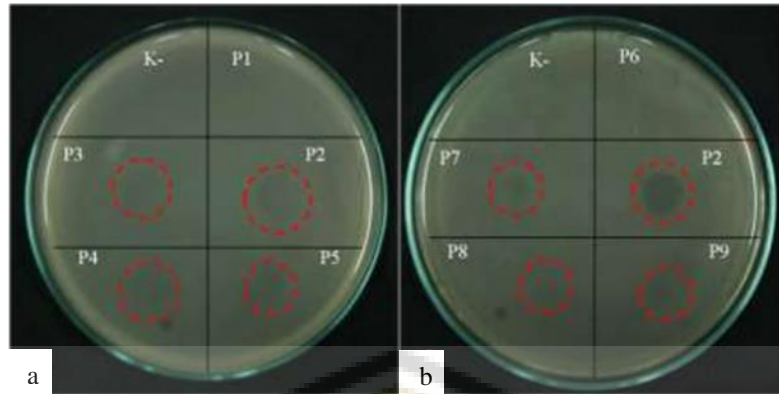


Figure 2. Virulence test results of phage combination: a. amoxicillin antibiotics with treatment P1: amoxicillin, P2: ϕ PT1b, P3: ϕ PT1b + Amx = 1:1, P4 : ϕ PT1b + Amx = 2:1, P5: ϕ PT1b + Amx = 1:2; b. tetracyclin with treatment P6 : tetrasiklin, P2: ϕ PT1b, P7: ϕ PT1b + Tet = 1:1, P8 : ϕ PT1b + Tet = 2:1, P9: ϕ PT1b + 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 ϕ PT1b 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 : ϕ PT1b, 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 : ϕ PT1b, 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

ϕ PT1b 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 (ϕ PT1b with tetracycline/amoxicilline) can reduce the level of antibiotic resistance in isolated *E. coli*.

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