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Original Article

Effect of Phage-Antibiotic Synergism (PAS) in increasing antibiotic inhibition of bacteria caused of foodborne diseases

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Abstract

Introduction: Food contaminated with pathogenic bacteria is one of the most harmful things that can even threaten human life. Over time, these pathogenic bacteria are increasingly resistant to antibiotics. Continuous use of synthetic preservatives will also have an adverse effect. This study was conducted to evaluate the synergy of bacteriophage and antibiotics in increasing antibiotics inhibition to the bacteria that cause foodborne disease.
Methodology: The test was performed by plaque assay and paper disc diffusion on NA medium in the same petri dish. The combination was incubated for 24 hours at 37ºC. An antibiotic inhibition on a non-bacteriophage test showed cefadroxil could only inhibit P21B bacteria.
Results: Cefadroxil inhibition in the PAS test showed that these antibiotics could inhibit some other foodborne disease bacteria (*Salmonella* spp., *Staphylococcus aureus*, and *Escherichia coli*). The inhibitory observed from the clear zone located around the disc paper.
Conclusion: These results provide useful information to reduce the risk of antibiotic resistance in humans and foods.

Key words: Antibiotics; bacteriophage; PAS.

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Introduction

Food is an essential requirement for humans. Frequently the need for this commodity obtained without thinking about hygiene and other health aspects. As essential needs, food should be guaranteed to be free from a variety of biological, chemical, physical, and other hazardous substances that may interfere with health. The presence of contamination in these foods can cause foodborne disease, which is a disease in humans caused by contaminated food and beverages.

Biological contaminants found in foods can be bacteria, viruses, parasites, molds, or fungi. The most dangerous organic contaminants that can cause human disease outbreaks are pathogenic bacteria, including *Salmonella* spp., *Escherichia coli*, *Bacillus anthracis*, *Clostridium* spp., *Listeria monocytogenes*, and *Shigella* [1]. According to WHO data (2015), there are approximately 582 million cases of 22 different enteric diseases in food and 351,000 related deaths. The agents of enteric disease responsible for most deaths are *Streptococcus pneumonia* [2], *Salmonella typhi* (52,000 deaths), enteropathogenic *E. coli* (37,000) and norovirus (35,000). The African region recorded the highest disease burden for enteric foodborne illness, followed by Southeast Asia. The use of synthetic preservatives often may be toxic and carcinogenic [3]. The problem of increasing bacterial resistance to antibiotics indicates the need for new alternative solutions for the removal of pathogenic bacteria [4]. For that, it takes control of pathogens in foods or foods that are not harmful to humans. One restriction of some pathogenic bacteria that are considered useful is bacteriophage [5]. The purpose of this study was to determine the effect of a combination of bacteriophage with antibiotics against bacteria that cause foodborne diseases.

Methodology

Bacteria and culture conditions

Salmonella spp. (P21A, P21D1, P21D2) isolates were obtained from Microbiology Laboratory of Biology Education, University of Jember. *Salmonella typhosa*, *Staphylococcus aureus*, and *Escherichia coli* were collected from the Microbiology Laboratory of Biology Department, University of Jember. *Salmonella*

spp. and *Salmonella typhosa* were reactivated on Salmonella-Shigella Agar (Sigma-Aldrich, Inc., St. Louis, USA) medium. *Staphylococcus aureus* and *Escherichia coli* are rejuvenated on Nutrient Agar (Sigma-Aldrich, Inc. St. Louis, USA) medium.

Isolation Bacteriophage

Bacteriophages were isolated from shrimp and fish samples. Shrimp were obtained from Traditional Market, while fish samples were collected from TPI (fish center) Puger Jember. The obtained sample was washed using sterile aquadest and soaked for 5 minutes. 1 mL of each washing water was cultured on LB medium which had previously been inoculated with 24-hour-old *Salmonella* bacteria and incubated at 37 ºC for 24 hours. The next process is a spot test that aims to determine the presence of bacteriophages in each sample obtained. The incubated samples (3 µL) were dropped in double layer medium with *Salmonella* spp. bacteria on top of it. The formed plaque is then purified using the plaque assay method to obtain a single plaque [6].

Virus Titer

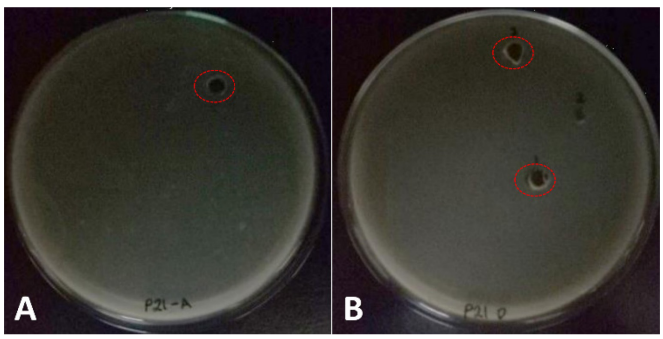
A plaque assay technique was carried out by counting the number of the clear zone. The number of phage particles was calculated on the plate having a plaque between 10-100 [7]. The formula used is as follow:

$$PFU/mL = \frac{total\ plaques \times dilution\ factor}{volume\ of\ bacteriophage\ used}$$ [8]

Virulence Test

The virulence test was performed by spot test. The suspension of bacterial pathogens added to the lukewarm (50ºC) medium of top Agar 0.5% (Sigma-Aldrich, Inc. St. Louis, USA) the medium then poured onto the surface of NA medium in the petri dish. Subsequently, after the medium solidified, 3 µL of bacteriophage suspension (10^8, 10^7, and 10^6 pfu/mL) added by dripping on the surface of the medium and incubated at 37ºC for 24-48 hours. The growth of bacteriophage observed by the formation of plaque (clear zone).

Figure 1. Circled in red: plaque bacteriophage taken for purifying process; A) shrimp sample, B) fish sample.



Antibiotics Test

The antibiotic test was performed by diffusion method [9]. Paper discs soaked in 3 µL antibiotics solution, left for 5 minutes and placed on the surface of NA medium that contains bacteria. Seven types of antibiotics used in antibiotic tests, namely amoxicillin, ampicillin, cefadroxil, ciprofloxacin, chloramphenicol, cefixime, and tetracycline. Aquadest used as negative control.

PAS (Phage-Antibiotic Synergy) Test

The PAS test was carried out by diffusion and double-layer method [10]. Precisely 300 µL of ± 5 hours bacterial suspension (OD 0.3) and 3 µL of bacteriophage on lukewarm (50ºC) Top Agar (0.5% agar) added onto the petri dish filled with NA medium. After solidified, paper discs with various types of antibiotics were put on top of the double layer medium and incubated at 37ºC for 24-48 hours.

Results

Isolation of Bacteriophages

Three bacteriophages collected namely ϕSZUT, ϕSZIP1, and ϕSZIP2. ϕSZUT obtained from shrimp sample, while ϕSZIP1 and ϕSZIP2 from fish samples (Figure 1). Bacteriophage isolation with positive results indicates a clear zone that has varying turbidity levels. The resulting plaque shows that bacteriophages can infect the bacteria. Infection can occur because most bacteriophages degrade the cell wall of bacteria through endolysin enzymes encoded in the bacteriophage genome that hydrolyze the peptidoglycan layer and are followed by cell wall disturbance caused by osmotic pressure. The diameter of the isolated plaque is tiny ± 0.1 cm.

Virulence Test

The bacteriophage virulence test showed that all types of bacteriophages (ϕSZUT, ϕSZIP1, and ϕSZIP2) might be virulent in all bacteria tested but with varying levels of infection at each or bacteriophage concentration. The virulence test showed that bacteriophage with a titer of 10^8 pfu/mL had the highest virulence level compared with the other titers. The higher the bacteriophage ratio indicates a relatively rapid bacterial mortality during infection.

Table 1. Antibiotics Test Result.

Bacteria	The average diameter of the clear zone (cm)							
	Negative Control (1)	*Amoxicillin* (2)	*Ampicillin* (3)	*Cefadroxil* (4)	*Ciprofloxacin* (5)	*Chloramphenicol* (6)	*Cefixime* (7)	*Tetracycline* (8)
P21A	0	0.44	0.5	0	0.11	0.33	0.43	0.52
P21B	0	0.37	0.41	0.41	0.89	0	0.67	0.65
P21D	0	0.21	0	0	0.93	0.44	0.64	0.6
St	0	0	0	0	0.98	0	0.2	0.77
Sa	0	0.93	0.91	0	0.77	0	0	0
Ec	0	0	0.34	0	0.15	0.38	0.39	0.41

Antibiotic Test

The results of the antibiotic test showed that ciprofloxacin had higher rates of infection due to the higher average appearance of clear zones compared to other types of antibiotics, whereas the lowest infection rate was cefadroxil antibiotic, where the clear zones appeared only in P21B bacteria (Table 1).

PAS test

PAS test was performed by combining bacteriophage with antibiotics. The bacteriophage used has a concentration of 10^8 pfu/mL. The result of clear zone sizes varies on each of the bacteria tested (Table 2). The largest diameter zone is *Salmonella* spp. P21D. While the diameter of the most explicit zone with the most narrow size is *Staphylococcus aureus* (*S. aureus*) (Figure 2). Differences are also shown from the plaque on the surface of the medium. An antibiotic test alone does not show any plaque/clear zone on the surface of the medium except around the disc paper. It indicates that antibiotic infection is limited to discs that have been sprayed with antibiotics as much as 3μL. In this study, the effect of PAS may be strongly considered if the sublethal concentration of ciprofloxacin was used for the paper disc diffusion test.

The PAS effect is detectable only in the zone adjacent to the disc paper where the drug does not completely inhibit bacterial growth. Sub lethal levels of antibiotics can synergistically stimulate bacteriophage growth. Thus a relatively large plaque is observed in the zone close to the ciprofloxacin disk paper. Antibiotics with the lowest inhibitory resistance during antibiotic testing, cefadroxil may inhibit some of the tested bacteria (*Salmonella* spp., *Staphylococcus aureus*, and *Escherichia coli*) during PAS testing. The increased clear zone may be caused by a plaque produced by bacteriophage burst release.

Discussion

The rise of many drug-resistant bacteria has become a global problem because of the lack of newly develop antibiotics. New therapeutic strategies are needed to

Table 2. PAS Test Result.

Bacteria	ϕ	The diameter of the clear zone (cm)							
		1	2	3	4	5	6	7	8
	P21A-U	0	1.08	0	0	2.98	0.64	0.71	0.88
St	P21D1-I	0	0.79	0	0	2.78	0.65	0.88	0.96
	P21D2-I	0	0.88	0	0	1.98	0.68	0.63	2.21
	P21A-U	0	1.5	0	0.92	0.8	0	0.66	1.5
Sa	P21D1-I	0	1.85	0.61	0.98	0.93	0	0	1.33
	P21D2-I	0	1.92	0.59	1.4	0.95	0.63	0.63	1.36
	P21A-U	0	0.61	0.68	0.58	1.4	0.68	0.79	1.21
Ec	P21D1-I	0	0.86	0.7	0.59	1.56	0.82	1.1	1.22
	P21D2-I	0	0.81	0.71	0.61	1.6	0.8	0.96	1.16
	P21A-U	0	0.71	0.67	0.73	0.95	0.6	0.65	0.88
P21A	P21D1-I	0	0.77	0.73	0.71	1.03	0.73	0.7	0.81
	P21D2-I	0	0.76	0.87	0.7	0.91	0.72	0.66	0.8
	P21A-U	0	0.79	0.75	0.63	2.7	0.84	0.77	1.2
P21B	P21D1-I	0	0.77	0.75	0.76	2.67	0.74	0.78	1.06
	P21D2-I	0	0.64	0.62	0.69	2.28	0.68	0.75	0.9
	P21A-U	0	0.67	0.75	0.7	2.06	0.85	0.68	1.25
P21D	P21D1-I	0	0.69	0.78	0.74	2.25	1.2	0.79	1.15
	P21D2-I	0	0.67	0.6	0.68	2.53	0.87	0.63	1.15

1) Negative Control (sterile Aquadest); 2) Amoxicillin; 3) Ampicillin; 4) Cefadroxil; 5) Ciprofloxacin; 6) Chloramphenicol; 7) Cefixime; 8) Tetracycline.

solve clinical and public health problems associated with infection caused by drug-resistant bacteria. The application of bacteriophage has been recognized as one of the potential alternatives [11]. This study explains the possibility of using the synergism between bacteriophage and antibiotics as an alternative method to reduce bacterial resistance levels.

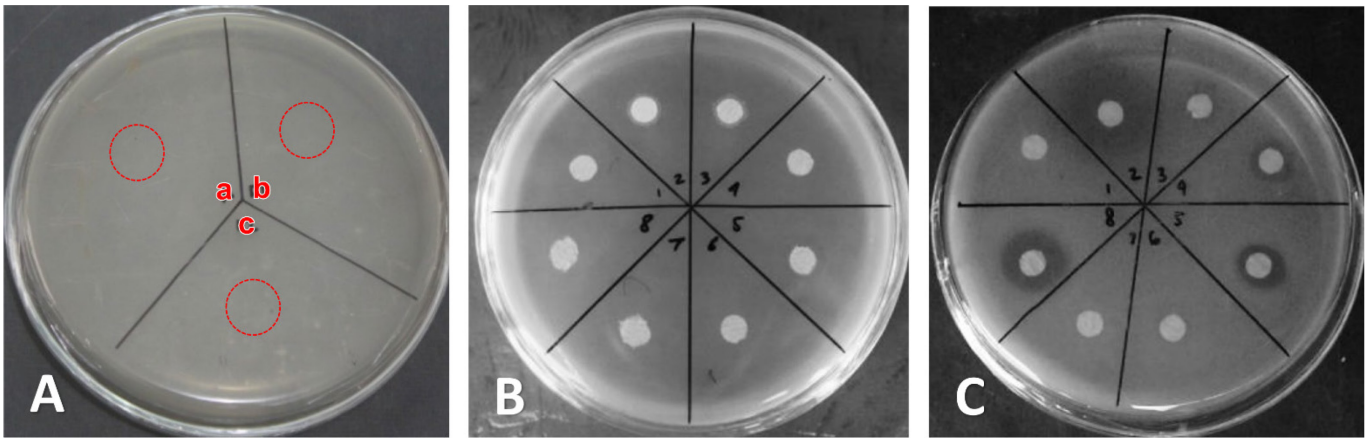
Pathogenic bacterial pathogens include *Staphylococcus aureus* and *Escherichia coli*. *S. aureus* in NA medium has a circular shape and yellowish white colonies. In nutrient agar, *S. aureus* produces dull yellow or yellow colonies [12]. *E. coli* colonies on NA medium are round, glossy white on the surface. The colonies of *E. coli* are white or creamy with a glossy texture and often look like mucus or cloudy films all over the plate surface [13]. *S. typhosa* on SSA medium has a transparent colony with blackish color in the center after incubation 37ºC for 24 hours. Bacteria *Salmonella* spp. (P21A, P21B, and P21D), rejuvenated on the SSA medium, showing the same morphological colony with transparent colors and blackish in the middle. *Salmonella* is round, transparent or translucent [14]. *Salmonella* colonies are transparent, black or colorless in the SSA [15].

Bacteriophage isolation with positive results indicates a clear zone that has varying turbidity levels. The resulting plaque shows that bacteriophages can infect the bacteria. Infection can occur because most bacteriophages degrade the cell wall of bacteria through endolysin enzymes encoded in the bacteriophage genome that hydrolyze the peptidoglycan layer and are followed by cell wall disturbance caused by osmotic pressure [16]. Based on the previous research, there are three kinds of positive bacteriophages, namely ϕSZUT, ϕSZIP1, ϕSZIP2. The diameter of the isolated plaque is tiny ± 0.1 cm. The width of the *Salmonella* bacteriophage is 0.569 ± 0.172 cm [17]. The three isolated bacteriophages have almost the same level of turbidity in which they are not too cloudy or somewhat turbid so it can be seen with the help of light. Bacteriophages that have a clear plaque can be indicated that the bacteriophage is lytic bacteriophage (virulent), a cloudy plaque may indicate a type of lysine bacteriophage (temperate) plaque [6].

Bacteriophage titers with plaque quantities between 10-100 can be calculated on the 14th dilution. The bacteriophage titer used in the virulence test is 10^8, 10^7, 10^6 pfu/mL. The customized bacteriophage suspension contains 10^8 pfu/mL per spot [18]. The bacteriophage virulence test showed that all types of bacteriophages (ϕSZUT, ϕSZIP1, and ϕSZIP2) might be virulent in all bacteria tested but with varying levels of infection at each or bacteriophage concentration. The virulence test showed that bacteriophage with a titer of 10^8 pfu/ml had the highest virulence level compared with the other titers. The higher the bacteriophage ratio indicates a relatively rapid bacterial mortality during infection [19].

Bacteriophage with main host *Salmonella* spp. can infect other pathogenic bacteria by showing a plaque with varying diameter. The resulting plaque size variation did not differ significantly in each of the bacteria tested. Isolate ϕSZUT and ϕSZIP2 can infect *E. coli* at a titration of 10^8 pfu/mL. Bacteriophages with 10^8 pfu/mL titer can also infect *Staphylococcus aureus*. Bacteriophage Vb_SenS_394 [16] with the primary host *Salmonella* spp is also broad-range by affecting other bacteria such as the genus *Escherichia* and also *Shigella*. Not all bacteriophages are specific hosts such as host bacteria [20]. Bacteriophages can have a broad

Figure 2. A) Spot test zone on *Sa*, i) ϕSZUT, ii) ϕSZIP1, iii) ϕSZIP2; B) Antibiotic paper disc on *Sa* without bacteriophage; C) Antibiotic paper disc on *Sa* with ϕSZIP1; Description: 1) K- (sterile Aquadest); 2) Amoxicillin; 3) Ampicillin; 4) Cefadroxil; 5) Ciprofloxacin; 6) Chloramphenicol; 7) Cefixime; 8) Tetracycline.



host range and infect more than two-thirds of the tested strains [21]. The virulence properties of bacteriophages that can affect some pathogenic bacteria of this disease can be a breakthrough for natural control in overcoming foodborne diseases. The specificity of bacteriophages for certain bacteria can range very narrowly and widely and depends on bacteriophage titer [22]. The availability of bacteriophages with a wide range of hosts will allow potential applications for bacterial infection or in the treatment of foodstuffs [20]. Host species vary among bacteriophages, some of which are strain-specific, whereas others have demonstrated infectious abilities in various bacterial strains and even genera [22]. The plaque produced in this study showed a slightly turbid color. The strength of bacteriophages to infect receptor molecules on the surface of the host cell will affect the plaque formation. Different plaque turbidity levels may indicate the cycle type of bacteriophage itself [6].

Table 1 indicates that ciprofloxacin has the highest known inhibitory of the clear zone diameter formed on all bacteria tested. Among *Salmonella* serovars tested in the study [14], 100% were highly sensitive to ciprofloxacin. Ciprofloxacin is known to be clinically useful antibiotic to induce an emergency response caused by bacteria [24]. The lowest inhibitory was performed by cefadroxil with the clear zone appeared in one type bacteria only. Most antimicrobial compounds are naturally produced molecules, so bacteria have evolved mechanisms to overcome their actions to survive. Thus, these organisms are often considered intrinsically resistant to one or more antimicrobials. The development of acquired resistance may be the result of mutations in the chromosomal genes or by the acquisition of an external genetic determinant of resistance, possibly derived from intrinsically resistant organisms present in the environment [25].

The mechanism of synergism between bacteriophages and antibiotics occurs when bacteriophages first infect or weaken the bacterial defenses. Bacteriophage adsorption for host receptors is an early step in infection and may be one of the most complicated steps; bacteriophage must recognize specific host specific cell components [26]. The presence of an external lipid layer is a unique glycolipid (lipopolysaccharide) in the outer membrane of a bacterial cell containing a protein that acts as a bacteriophage receptor [27]. Once the bacterial receptor is recognized by bacteriophage, the bacterial defense begins to interfere; antibiotics can inhibit bacterial growth through various mechanisms. The β-lactam group antibiotics such as amoxicillin, ampicillin, cefadroxil, and cefixime inhibit bacterial cell wall synthesis by mimicking D-alanyl D-alanine parts of the peptide chains commonly associated with penicillin-binding proteins (PBPs). PBP interacts with β-lactam and is not available for the synthesis of new peptidoglycan. Disorders of the peptidoglycan lead to bacterial lysis. Tetracycline antibiotics inhibit by targeting the 30s subunit of bacterial ribosomes while chloramphenicol targets the 50s subunit of bacterial ribosomes [28].

Conclusions

Three samples of bacteriophages namely ϕSZUT, ϕSZIP1, and ϕSZIP2 had virulence ability against pathogenic bacterial pathogens tested (*Salmonella* spp., *Staphylococcus aureus* and *Escherichia coli*). Cefadroxil that has low inhibitory power in antibiotic tests has a higher inhibitory effect and may infect other bacteria during PAS testing.

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