Characterization of *Proteus mirabilis* Lytic Phage from Situ Letik River, Bogor, Indonesia

Rachmi Afriani[1], Iman Rusmana[2], Sri Budiarti[3*]
Graduate Student[1], Lecturer[2], Lecturer[3]
Bogor Agricultural University – Biology Department
Bogor – Indonesia

*s_budiarti@yahoo.com

Abstract-***Proteus mirabilis*** **isolated from the feces of diarrhea patients was resistant to ampicillin, amoxicillin and trimethoprim-sulfomethaxazole. Therefore, to reduce potential infection of antibiotic-resistant *P. mirabilis*, alternative solutions such as application of lytic phage were needed. The aims of this study were to isolate and characterize phage that can lyse *P. mirabilis* cells. Phages were isolated from poultry waste and river water in Situ Letik village. Phage isolates were determined by their ability to form plaques, specificity, characteristics of protein and morphology. Phage was specific to *P. mirabilis* as its host. Sodium Dodecyl Sulphate-Poly Acrilamide Gel Electrophoresis (SDS-PAGE) analysis showed that the phage virion contains 8 proteins with molecular weight between 20-91 kDa. Transmission electron microscope observation showed that the phage was the member of *Myoviridae* family with icosahedral hexagonal head, the diameter about 68.185 nm, the length of tail about 109.019 nm, and the diameter of the tail about 17.89 nm width.**

Keywords: **diarrhea, lytic phage, *Proteus mirabilis***

I. INTRODUCTION

Diarrheal is one of the most common disease leading cause of death in developing countries, including Indonesia. Diarrhea can be caused by the variety of microorganisms such as viruses, protozoa and bacterial enteropathogens. *Proteus mirabilis* is a Gram-negative bacteria (enteropathogens) which can cause diarrhea. There are 8 bacterial enteropathogens including *Proteus* spp that were isolated from stool samples of 100 children with diarrhea aged under 5 years [14]. The most of treatment stage to control diarrheal disease caused by *P. mirabilis* is using antibiotics. However, several studies reported that the current use of antibiotics to control bacterial pathogens becomes ineffective due to the emergence antibiotics resistance of *P. mirabilis.* Some isolates of *P. mirabilis* multiresistant to antibiotics, namely ampicillin, gentamicin, ceftazidime, cefotaxime, cefuroxime, cefalothin, cefepime, piperacillin, trimethoprim-sulfamethoxazole and ciprofloxacin [15]. Some isolates of *P. mirabilis* have ESBL (Extended Spectrum β-Lactamases) that play an important role to increase the resistancy of *P. mirabilis* to the β-lactam group antibotic [18].

Antibiotic-resistant bacteria *P. mirabilis* can contaminate food and water. Along with the rapid development of science and technology, many researches were done to look for the biocontrol agents that are eco-friendly in purpose to control water and food pollution by *P. mirabilis*. One of the alternative is by using lytic phages. The use of lytic phages in controlling the growth of human pathogenic bacteria is a natural alternative methods that are environmentally friendly. The most common source of phage to reduce pathogenic bacterial contaminants of water and food can be isolated from the stool, a wide variety of food, water, wastewater, soil and infected tissue. Some phages have been applied as biocontrol of food contamination, including *Enterobacter sakazaki* phage lytic applied to infant formula and lytic phage of *Salmonella* and *Campylobacter* on chicken skin [11].

Lytic phages as biocontrol bacterial pathogens has been applied to the cheese address to control the listeriosis disease caused by *Listeria monocytogenes* [6]. Phage has ability to lysis indigenous *Salmonella* P38 from feces of diarrheal patient [23]. Lytic phage infectivity presents against enteropathogenic *Escherichia coli* (EPEC K.1.1) antibiotic-resistant isolates from patients with diarrhea in Indonesia [4]. Research characterization of lytic phages against antibiotic-resistant bacteria *P.mirabilis* pathogens that can contaminate water, food and the cause of diarrhea has not been done. Therefore, the objective of this research are to characterize lytic phage of *P. mirabilis* to be used as biocontrol water and food contamination caused by *P. mirabilis*.

II. MATERIALS AND METHODS

Identification and Pathogenicity Test of *Proteus mirabilis*

Identification of *P. mirabilis* was determined using gram staining test and API 20E KIT. Pathogenicity test was conducted at *blood agar* plates. Observations of media discoloration blood was done on 3 hours and 5

hours first, then incubation was continued up to 24 hours to determine the virulence factors that play a role in the pathogenicity of *P. mirabilis* bacteria.

***Proteus mirabilis* Resistance Test against Antibiotics**

The sensitivity of *Proteus mirabilis* was tested by 4 types of antibiotics, namely ampicillin, amoxicillin, ciprofloxacin, and trimethoprim-sulfomethoxazole. The method use disapplying the paper disc diffusion. Paper discs containing antibiotics placed on the surface of the media and incubated at 37 °C for 24 hours. The diameter of inhibition zone formed was measured and compared with the diameter of inhibition zone according to the *Clinical and Laboratory Standards Institute* [7] to determine the level of *P.mirabilis* resistance to antibiotics ampicillin, amoxicillin, ciprofloxacin and trimethoprim-sulfomethoxazole.

Phage Isolation

Phage samples were isolated from several sources, such as domestic wastewater, Situ Letik Village river, Ciparigi river, chicken feces, feces goat and chicken farm waste. 4.5 mL of sample was introduced into a centrifuge tube containing 0.5 ml *P. mirabilis* culture (OD_{600nm} =1 or 10^8 CFU/mL), then added by 5 mL of *Nutrient Broth* (NB) and incubated at 37 °C for 24 to 48 hours [19] with modification. The sample was centrifuged for 20 minutes. Supernatant was filtered using membrane filters milipore 0.22 µm. Filtered supernatant was stored in sterile tubes at 4 °C.

Phage Purification

Plaques (clear zone) formed was transferred into a centrifuge tube that has been filled with 5-7 mL of buffer *Saline Magnesium* (SM). Phage suspension was homogenized for 5-10 minutes at room temperature. The suspension was centrifuged for 20 minutes 2 times replications. The supernatant was filtered. Filtered supernatant was stored as a stock phage at room temperature in a sterile tube [12].

Plaque Assay

The number of phages was determined by counting the number of plaques formed (Plague forming units(pfu/mL)). Stock phage diluted to10^{-8}, 100 µL of each dilution was taken and added to 100 mL culture of *P. mirabilis* that had been grown in NB medium. Suspension were incubated at 37 °C for 30 minutes then put in soft agar temperature 47 °C, poured on NA medium and incubated at 37 °C for 24 hours. The number of plaques formed were counted after 24 hours of incubation [10].

Phage Host Range Test

Phage host range test conducted by modification techniques of double layer [5]. 100 mL of each culture *P. mirabilis*, EPEC K.1.1, *Salmonella* sp., *Bacillus pumilus* and *Photobacterium damselae* which had been grown in NB (OXOID) was mixed with 100 mL of stock phage and incubated at 37 °C for 15-30 minutes. Each mixture was put in soft agar at 47 °C and incubated at 37 °C for 24 hours.

Phage Morphology Observation by Transmission Electron Microscope (TEM)

Phage stock was dropped 10 µL on the grid (400 mesh) and then left for 1 minute. A total of 5 mL of 2% *uranyl acetate* solution was dripped into the top grid. The grid was then dried using filter paper and allowed to dry. The dried grids were observed using the *transmission electron microscope* JEOL JEM-1010 models at magnification 10000-100000x [5].

Characterization of proteins

The protein concentration was measured based on the stock phage method of [3]. Analysis of the phage protein molecular weight was determined using Sodium Dodecyl Sulphate-Poly Acrilamide Gel Electrophoresis (SDS-PAGE) [16]. Marker used was Presstained Protein Molecular Weight Markers with molecular weights by respectively 20, 25, 35, 50, 85, 120 kDa. Separator gel concentration used was 12.5% polyacrylamide gel while stacking gel concentration was 4%, then silver staining was applied for visualization the result.

III. RESULTS AND DISCUSSION

Identification and pathogenicity Proteus mirabilis

The results of the API 20E identification with KIT showed that the bacteria isolated were *Proteus mirabilis* (99.9%). *P. mirabilis* seems not ferment mannose, glucose and arabinose. Urease test and gelatin showed positive results, whereas indole and lysine decarboxylase test showed negative results (Table 1).

TABLE 1. CHARACTERIZATION PHYSOLOGY OF *P.mirabilis*

Uji	Hasil uji	Uji	Hasil uji
ONPG (Ortho Nitrofenil-β-Galaktopiranosidase)	-	Sitrat	+
Ornitin dekarboksilase	+	Glukosa	-
Lisin dekarboksilase	-	Manosa	-
H_2S	+	Inositol	-
Urease	+	Sorbitol	-
Indol	-	Rhamnosa	-
Voges Proskauer	-	Sukrosa	-
Gelatin	+	Arabinosa	-

+: positive reaction; -: negative reaction

Gram staining results showed that *P. mirabilis* is a gram-negative and rod-shaped bacterium. Colonies of *P. mirabilis* is black when grown on media *Salmonella Shigella* Agar (SSA) with black color on the center of the colony and clear on the edge of the colony. Based on the test results of the *P. mirabilis* pathogenicity on blood agar media, it is known that *P. mirabilis* is able to lyse blood cells and produce alpha hemolysin. Alpha hemolysin is an important virulence factors in pathogenic bacteria that can lead to a variety of infections because it can form pores that trigger cellular responses and long-term effects for the host organism such as a mammal [22].

Proteus mirabilis **Resistance to Antibiotics**

The test results showed that the antibiotic resistance of *P. mirabilis* was resistant to the antibiotic ampicillin, amoxicillin and trimethoprim-sulfametahoxazole (Table 2). Resistance in bacteria can be caused by several factors such as the presence of antibiotic resistant genes in the chromosome or a plasmid, modification of antibiotic target compounds and genetic material as a result of changing in methylation and insertion [17].

TABLE 2 *P. mirabilis* RESISTANCE TO SOME ANTIBIOTICS

Antibiotic	Disk content (µg/mL)	Zone diameter interpretive criteria nearest whole (mm)			Zone diameter of inhibition (mm)	Results
		Sensitif	Intermediet	Resisten		
Ampicillin	10	≥17	14-16	≤13	0	Resisten
Amoxicillin	20	≥18	14-17	≤13	0	Resisten
Trimethoprim-sulfomethaxazole	5	≥16	11-15	≤10	0	Resisten
Ciprofloxacin	5	≥21	16-20	≤15	26	Sensitif

Phage Isolation

Phages were isolated from sewage of chicken farm and Situ Letik river, Darmaga, Bogor (Table 3). Phages that lyse bacterial cell will form a clear zone on medium called plaque. Plaque or clear zone is parameter of the damage caused by phage infection [1].

TABLE 3RESULTS OF PHAGE ISOLATION FROM VARIOUS SOURCES PHAGE

Sources	The number of sample	The number of replicates	The number of phage-containing sample	The number of phage isolates	Description
Liquid waste house	2	4	-	-	-
Chicken feces	2	3	-	-	-
Goat feces	2	3	-	-	-
Chicken farm waste	2	4	1	1	Phage 1
Ciparigi river	2	2	-	-	-
Desa Situ Letik river	2	4	1	2	Phage 2, Phage 3
Total sample	12	20	2	3	

The results showed that the phage plaques formed by 3 phage isolates are 2 mm in diameter. Plaque morphology of each isolate phage showed different characteristics. Phage isolates 2 were more dense than the phage isolates 1and 3, ie $1.3x10^8$ PFU/mL (Table 4).

TABEL 4 CHARACTERISTICS OF *P. mirabilis* LYTIC PHAGE

Phage isolates	The number of plaque (PFU/mL)	Plaque morphology
Phage 1	1×10^{4}*	Plaque round, clear, 2 mm in diameter, has a ring around the plaque
Phage 2	1.3×10^{8}	Plaque round, faint, diameter 2 mm, no rings around the plaque
Phage 3	2×10^{7}*	Plaque round, clear, 2 mm diameter, no ring around the plaque

*Phage isolates cannot be grown

The results of purification and production of the three phage isolates showed that isolate phage that can be purified and grown back is isolates phage 2, while phage isolates 1and 3 can not be produced because both these isolates can not be grown back when the culturing process of the phage. It may be caused by the differences of stability level in each phage isolate. Stability factor on the phage is closely associated with the phage proteins constituent [8].

Phage Host Range

Host range test showed that *P. mirabilis* phage belongs to specific phages. Phage of *P.mirabilis* can only infect *P.mirabilis* and cannot infect other bacteria such as *B. pumilus*, *P. damselae*, *Salmonella* sp. and EPEC K.1.1 (Table 5). The nature of the host-specific phage has more advantage compared than antibiotics. Phages is more secure because it only infect a specific host and reduce bacteria without disturbing the normal intestinal microflora.

TABLE 5 PHAGE HOST RANGE OF *P. mirabilis*

Host bacteria	Description
Proteus mirabilis	Plaques present
Salmonella sp	No plaques
EPEC K.1.1	No plaques
Bacilluspumilus	No plaques
Photobacterium damselae	No plaques

Specificity of *P. mirabilis* phage formed due to specific receptor molecules on *P. mirabilis* that can be recognized by its phage. *P.mirabilis* phage can not recognize the receptor molecules found in other bacteriasuch as *B.pumilus*, *P.damselae*, *Salmonella* sp. and EPECK.1.1. The receptor molecules can be in flagella, polysaccharide, lipopolysaccharide, capsule, proteins of cell wall, phili, fimbria, theicoic acid form [13]. Differences in receptor molecules on the bacterial species differ greatly affect the nature of the phage specificity [20]. Different bacteria have different sensitivities toward each phage due to the differences in receptor molecules that are recognized by the phage [9].

Phage Morphology

Based on observations by *Transmission electron microscope*, *P.mirabilis* phage has a hexagonal shaped icosahedral head and a long tail tubular helix (Figure 1).

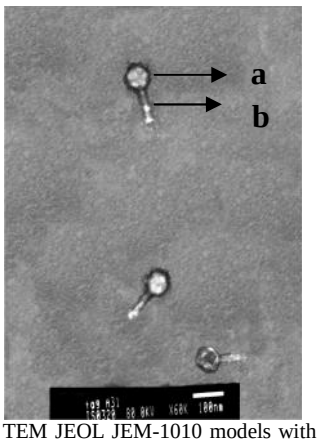

Fig. 1 Morphology of phages of *P. mirabilis* using TEM JEOL JEM-1010 models with 2% uranyl acetate staining; a) phage head and b) phage tail, magnification 60.000x, bar = 100nm.

The characteristics possessed by *P.mirabilis* phage is included in *Caudovirales* order and *Myoviridae* family. Virus that includes the Caudovirales order *Myoviridae* family have double stranded DNA enclosed by the hexagonal-shaped capsid and tail contractile [21]. *P. mirabilis* phage head has 68.185.nm of diameter, 109.091nm of tail length and18.182 nm phage tail a diameter.

Characteristics of *Proteus mirabilis* phage proteins

Based on the analysis using SDS-PAGE known that there are protein band that has 90.50, 82.03, 74.35, 43.31, 37.37, 30.70, 27.83 and 20.72 kDa in size, respectively (Figure 2). Protein molecule with a certain weight indicates constituent components such as phage heads, tails and tail fibers [2]. The results of the concentration of *P. mirabilis* phage proteins showed that the protein concentration of 316.198 mg/mL.

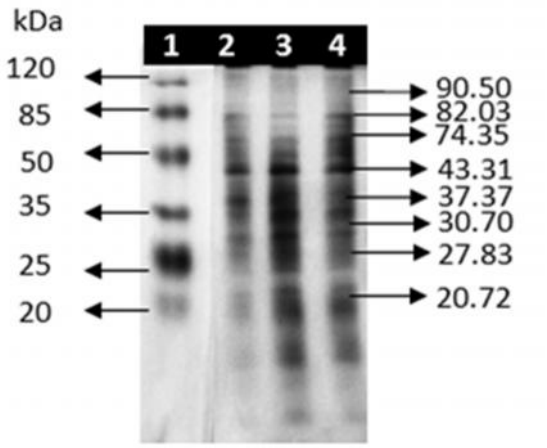

Fig. 2 Molecular weight range of *P.mirabilis* phage proteins on SDS-PAGE. 1) Marker, 2), 3), 4) *P.mirabilis* phage proteins

IV. CONCLUSION

Lytic phages isolated from Situ Letik water can infect bacteria *P.mirabilis.* Plaque is round with a diameter of 2 mm. *P.mirabilis* lytic phage is specific to *P. mirabilis*. This phage has a hexagonal head icosahedral 68.185 nm in diameter, the relative length of the phage tail reaches109.091nm and18.182 nm in diameter. Phage protein molecular weight ranging from 20-91 kDa.

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