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Characterization of lytic phage S*taphylococcus aureus*from dairy farm cows in Indonesia

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Abstract : *Staphylococcus aureus* is one of pathogenic bacteria causing multitude of diseases. This bacterium was resistant to ampicillin, tetracycline and chloramphenicol. Therefore, to reduce potential infection of antibiotic resistant *S. aureus*, alternative solutions such as application of lytic phage were needed. The aims of this study were to isolate and characterize phage that can lyse *S. aureus* cells. Phages were isolated from waste water from local dairy farm. Phage isolates were determined by their ability to form plaques, specificity, morphology, and effectiveness of bacteriolysis. In this study, 3 phages (FSb, FSs, and FSk) were isolated to replicate *S. aureus* isolates as a potential way to control these infections. Phage was specific to *S. aureus* as its host. Transmission electron microscope observation showed that the FSb and FSk can be classified as a member of *Myoviridae* family, while FSk was the member of *Siphoviridae* family. FSb had an icosahedral head in 85.71 nm diameter, long tail of 126.67 nm, and the diameter of the tail about 20.00 nm width; FSs had an icosahedral head in 53.33 nm diameter, long tail of 106.67 nm, and the diameter of the tail about 13.33 nm width; FSk had an icosahedral head in 66.67 nm diameter, long tail of 120.00 nm, and the diameter of the tail about 16.67 nm width. All lytic phage reduced the population of *S. aureus* effectively. Lytic phages found in this study can be used as an alternative therapy agent to *S. aureus* infected.
Keywords: Characterization, lytic phage, *Staphylococcus aureus*.

Introduction

Bacteremia case between 2014-2015 in Great Britain especially was caused by infection of *E. coli*, *S. aureus*, and *C. difficile*[1,2], and about 24.4% of infection cases at the patients in Indonesia was caused by *S. aureus*[3].Based on surveillance program of resistant bacteria, it was known that 28% isolate of *S. aureus*from Indonesia already resistant toward various antibiotic such as ceftriaxone, clindamycin, doxycycline, erythromycin, levofloxacin, meropenem, oxacillin, and trimethoprim-sulfamethoxazole[4],furthermore WHO reported that more than 50% pathogenic bacteria of infection cause at the patients in hospital already resistant toward antibiotic used[5].

Bacteriophage is used as one of alternative therapies toward antibiotics resistant bacteria[6]. Phage is a virus that infect bacteria only and make the bacteria as host. Phage abundance in the nature is high, about 10^{31} mL^{-1}or10 times than bacteria[7]. Phage life cycles consist of lysogenic and lytic cycle. Lytic phage is developed as antibacterial because it causes bacteria lysis after infection[8].

Previous study in Indonesia reported some phages wasspesific to infected bacteria pathogen EPEC K1.1[9], *Photobacterium damsalae*[10], *Proteus mirabilis*[11], and *Bacillus pumillus*[12]. The research aimed at

obtaining the characteristics of *S. aureus*lytic phage that has prospect as antibacterial therapy toward infection of *S. aureus*.

Materials and Methods

Verification and antibiotics resistant test of *S. aureus*

*S. aureus*was used as host for lytic phage.*S. aureus*1787, *S. aureus*1656, *S. aureus*4734, *S. aureus*8212, and*S. aureus* ATCC 25923were used in this study from microbiology laboratory Medical Faculty of the University of Indonesia, andwas verified conventionally methods[13] and confirmed automatically by instrument of vtek 2 *system* (Biomerieux, Marcy-l'Etoile, *France*). Agardisc diffusion method[14,15]and automation method with vtek-2 *System* were used for resistance test of *S. aureus*towardantibiotic.

Sample collection and phage isolation

Phage samples were liquid waste of liquid cow manure taken from cowshed, sewer, and final processing of waste at diary farm cows center of PondokRanggonof East Jakarta, Indonesia. Phage isolation described briefly 5 mL samples were centrifuged at 4513.14 x *g*for 30 minutes (Centurion K240, West Sussex, UK) then the supernatant was filtrated by using *syringe filter* nylon 0.22 µm (Grace, Maryland, USA). Sample filtrates were mixed in same amount with host culture of 18hours BHI broth ($CaCl_2$ 5mM) OD_{600nm} = 1 (10^8 mL^{-1}) of 3-5 mL.The mixture then be incubated for 24 hours on 37°C. The culture then be centrifuged at speed of 4513.14 x *g*for 30 minutes, then the supernatant was filtered by using *syringe* nylon 0.22 µm. The process was replicated three times[16].Sample filtrates then be analyzed to know the phage existence by using double layer agar technique (DLA)[17]. About 500 µL sample filtrates then be added by host culture of 18 hours OD_{600nm} = 1 (10^8 mL^{-1}) of 500 µL then be incubated for 20 minutes on 37 ^oC.About 100 µL sample mixture and incubated host culture then be entered into 6 mL BHI soft agar (0.7% agar) temperature 45-50°C and be homogenized by using vortex then immediately poured on the 7 mL BHI hard agar (1.2% agar). Sterile BHI be used as control. All petri were incubated for 24 hours on 37°C, then be observed the formation of plaque. Plaque is the bacterial lysis results by phage. The morphological characteristic of the plaque then be observed, including the size, form, and clarity[18]. Each plaque that had different characteritict was purified then be stored as the phage stock in the *Phospate Buffered Saline* (PBS) (137 mMNaCl, 2.7 mMKCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4)[17].

Production and phage titer

Host culture 18 hours OD_{600nm} = 1 (10^8 mL^{-1}) was entered of 100 µL into BHI broth of 5 mL, then be added by phage stock of 100 µL. The culture mixture was incubated for 4-8 hours on 37°C. The change from turbid become clear in the culture was observed during incubation. The clear culture then be centrifuged at4513.14 x *g*for 30 minutes. Supernatant then be separated and filtered by using *syringe filter* nylon 0.22 µm[19]. Phage titer was calculated by DLA.The formed plaque amount then be calculated and stated as the phage titer(PFU mL^{-1}).

Phage host range

Host range activities of phage isolate done by using *spotted test*[20]. Bacteria for host range test including all isolate of *S. aureus*, Gram negative and Gram positive bacteria. Gram positive bacteria for host range test were *S. epidermidis*, *S. hominis*, *S. mutans*and *Bacillus*and the Gramnegative bacteria of *K. pneumoniae*, *Salmonella*, *E. coli*, and *P. aeruginosa.* Sterile BHI broth without phage addition was used as control.

Phage morphology

Phage morphology was observed by negative staining by using uranyl acetate 2%[21]and observed on magnification of 20000-80000x by using transmission electron microscope JEM-1010 (JEOL, Tokyo, Japan) with voltage of 80.0 kV.

Bacteriolytic Effectiveness

Each culture of *S. aureus* (18 h) for 1.2 x 10^5 CFU mL^{-1} was infected by 2.1 x 10^6 PFU mL^{-1} Fag FSb; 1.5 x 10^6 PFU mL^{-1} FSs; and 5.0 x 10^6 PFU mL^{-1}FSk. Control by using culture of *S. aureus*of 1.2 x 10^5 CFU

mL^{-1} without infection by phage. All mixture and control were incubated on 37 °C, then the population amount of *S. aureus*was determined by *Total Plate Count* (TPC)[22]on hour of 0, 2, 4, 6, and 8 by using agar*Mannitol Salt*[23]. Bacteriolytic effectiveness was determined based on the decrease of control bacteria cell amount.

Results and discussion

Verification and resistance of *S. aureus*

The results of partial physiological test by using conventional method gave positive results to all catalase tests, mannitol fermentation, hemolytic, and coagulation and characterization results of host bacteria physiology by using instrument of vtek 2 *system* (table 1) showed 99.0% identical with *S. aureus***.**

Table 1 Physiological characteristic of host bacteria

Results	Physiological test
Positive	Catalase*, mannitol*, coagulase*, hemolysis*, D-amygdalin, arginine hydrolase, α-glycosidase, alkaline phosphatase, urease, polymixin B resistance, D-galactose, D-ribose, lactose, *N*-acetyl-D-glucosamine, D-maltose, bacitracin resistance, growth in 6.5% NaCl, D-mannitol, D-mannose, O/129 resistance, saccharose, D-trehalose, arginine dihydrolase 2, optochin resistance
Negative	Phosphatidylinositol phospholipase, D-xylose, β-galactosidase, alanine-phenylalanine-prolinearylamidase, α-cyclodextrin, L-aspartate arylamidase, β-galactopyranosidase, α-mannosidase, leucinearylamidase, L-prolinearylamidase, β-glucuronidase, α-galactosidase, L-pyrrolidonyl-arylamidase, β-glucuronidase, allaninearylamidase, tyrosine arylamidase, D-sorbitol, L-lactate alkalinization, novobiocin resistance, methyl-β-D-glucopyranoside, pullulan, D-raffinose, salicin

*conventional method

The four host bacteria showed sensitivity pattern differences toward antibiotic. Control culture of *S. aureus* ATCC 25923 and host culture of *S. aureus*8212 showed sentitivity toward all antibiotic (table 2).

Table2 Resistance pattern of host bacteria toward antibiotic

Antibiotic types	***S. aureus***				
	ATCC 5923	**8212**	**1656**	**1787**	**4734**
Chloramphenicol	S	S	S	S	**R**
Gentamicin	S	S	**R**	S	S
Erythromycin	S	S	**R**	S	S
Ciprofloxacin	S	S	**R**	**R**	S
Tetracycline	S	S	**R**	**R**	S
Benzyl penicillin	S	S	**R**	**R**	S
Oxacillin	S	S	**R**	S	S
Levofloxacin	S	S	**R**	S	S
Moxifloxacin	S	S	**R**	S	S
Clindamycin	S	S	**R**	S	S
Dalfopristin	S	S	S	S	S
Linezolid	S	S	S	S	S
Vancomycin	S	S	S	S	S
Tigecycline	S	S	S	S	S
Nitrofurantoin	S	S	S	S	S
Rifampicin	S	S	S	S	S
Trimethoprim sulfamethoxazole	S	S	S	S	S

S: Sensitive; R: Resistant

Resistance pattern of host bacteria almost similar with previous study[24,25], that *S. aureus*in Indonesia had been resistant toward antibiotic of penicillin, gentamicin, erythromycin, ciprofloxacin, levofloxacin, and trimetropim-sulfametoksazol. *S. aureus*that had been resistant toward antibiotic will make difficult the healing of diseses, then lytic bacteriophage can be used as alternative therapy to medicate the antibiotic resistant bacteria. *S. aureus*that had been verified and known as resistant toward antibiotic used as host for isolation lytic phage of *S. aureus.*

Phage isolation

Three phage isolates were successfully isolated from liquid waste of sewer, but phage unable to be isolated from liquid waste near the cowshed and waste final processing. The presence of phage was marked by the plaque formation as the results of infection and lysis of bacteria at the lawn agar.

The phage absence at the taken sample from the cowshed because the population growth of *S. aureus*as the host for the phage is depressed by the growth of dominant anaerobic bacteria in the feaces, while aeration in the sewer support the growth of aerobic *S. aureus*. Anaerobic condition of waste final processing depress the growth of aerobic bacteria growth, such as *S. aureus*.

Three phage isolates (FSb, FSs, dan FSk) were selected based on the formed plaque morphological characteristic. The three phage isolates were lytic phages shown by the formation of clear plaque (figure 1). FSb phage had the biggest diameter, between 2-3 mm with irregular form[26], while FSs and FSk plaque had regular form with 1-2 mm diameter, and <1 mm (table 3). The plaque diameter size and phage titer showed the phage virulence, and influenced by several factors, such as the phage adsorption rate, phage diffusion at media, and latent phase[27]. The three phage isolates then were purified and stored in PBS buffer on 4 °C.

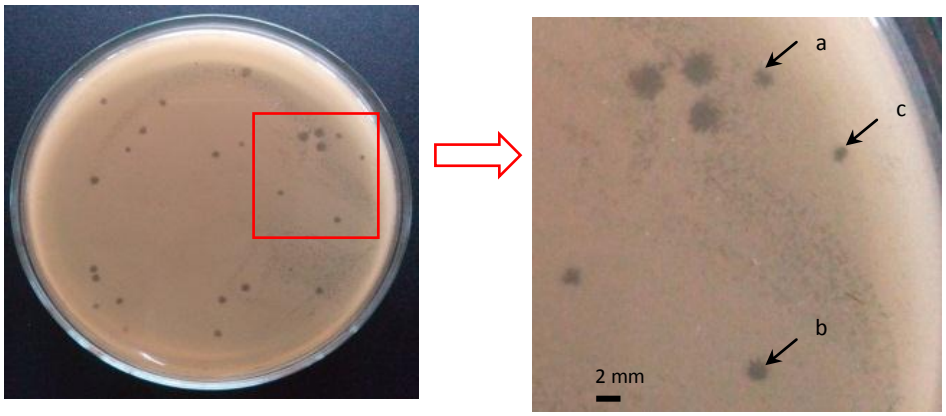

Figure 1Morphology of phage isolate plaque:FSb, clear big plaque 2-3 mm (a); FSb, clear plaque 1-2 mm (b); FSk, clear small plaque <1 mm. Bar: 2 mm

The three phage isolates showed different titer after quantification by using double layer agar with same comparison between phage and host bacteria. FSs phage had the highest titer with phage amount 33.10^7 PFU mL^{-1},then the FSbphage with amount of $11. 10^7$ PFU mL^{-1}, and FSk phage with amount of 5.10^7 PFU mL^{-1} (table 3).FSband FSs phage similar with SaPh3 page (3.10^8 PFU mL^{-1})indicated as had strong virulence[28]. Phage that had strong infection will rapidly adsorb, replicate, and come out from host bacterial cell to infect other bacteria and form plaque at the media.

Table3 Characteristic of plaque morphology and phage isolate titer

Isolates	Plaque morphology	Size (mm)	Titer (x 10^7 mL^{-1})
FSb	Clear iregular plaque	2-3	11
FSs	Clear regular plaque	1-2	33
FSk	Clear regular plaque	<1	5

Phage host range

Determination of host range showed phage specificity that infect bacteria. Phage specificity was very influenced by receptor at the bacterial cell. Teichoic acid and capsule were the phage receptor at the Gram positive[29]. All phage isolates showed high specificity, that only infect the host bacteria of *S. aureus*and did not infect other bacteria including normal microbiota of *S. hominis*and *S. epidermidis* (table 4). However, all phage isolates not infected tostrain *S. aureus* 1656. All phage isolates unable to recognize and adhere at the receptor site of *S. aureus* 1656 as the initial process of infection, it was supposed because the *S. aureus* 1656 produced protein A that cover the receptor so phage unable to recognize and adhere to the receptor[30].

Table 4 Range results of phage isolate host

Host bacteria	Phage isolates		
	FSb	FSs	FSk
Control	-	-	-
*S. aureus*ATCC 25923	+	+	+
*S. aureus*1758	+	+	+
*S. aureus*4713	+	+	+
*S. aureus*8212	+	+	+
*S. aureus*1656	-	-	-
S. epidermidis	-	-	-
S. hominis	-	-	-
S. mutans	-	-	-
Bacillus	-	-	-
E. coli	-	-	-
K. pneumoniae	-	-	-
P. aeruginosa	-	-	-
Salmonella	-	-	-

+: plaque formed; -: plaque was not formed

All phage isolates were appropriate to be candidates to decrease the pathogens *S. aureus*bacteria because their high specificity and did not infect normal microbiota population

Phage morphology

Characterization results of phage morphology by using transmission electron microscope with negative staining showed morphological characteristic differences from each isolate. Uranyl acetate used in the negative staining was useful to get contrast between object and dark background, so the phage morphology can be observed clearly. The three phage isolates known had varied size and had tail. The phage tail was served for adsorption at the receptor site of bacteria, and genetical material injection. FSb phage had bigger head and tail size, while FSs phage was the smallest (table 5 and figure 2).

Table5 Morphological characteristic of phage isolate

Iso	**Head shape**	**Tail type**	**Ø head (nm)**	**Ø tail (nm)**	**Tail length (nm)**	**Family**
FS	Icosahedral	Contractile	85.71	20.00	126.67	*Myoviridae*
FS	Icosahedral	Non ontractile	53.33	13.33	106.67	*Siphovirida*
FS	Icosahedral	contractile	66.67	16.67	120.00	*Myoviridae*

FSband FSs phage isolates were identified in *Myoviridae*family because had icosahedral head shape and contractile tail while FSkphage included into *Siphoviridae*family that had icosahedral head and non contractile tail[31](figure 2). Isolate morphology of FSband FSkphage were almost similar with Stau2[32]and SAH-1[33]. Phage from *Myoviridae*and *Siphoviridae*families were bacteriophage with the highest abundance in nature. *Myoviridae*and *Siphoviridae*also were reported as the most researched phage at the study of *S. aureus*. But the phage from *Siphoviridae*family had lysogenic gene rarely, so will make difficult if be used as biocontrol

pathogenic bacteria. Different with *Myoviridae*family as obligate lytic phage. The bacteriolytic of phage as biocontrolto inhibit growth or decrease the bacteria population can be known from the phage bacteriolytic effectiveness test.

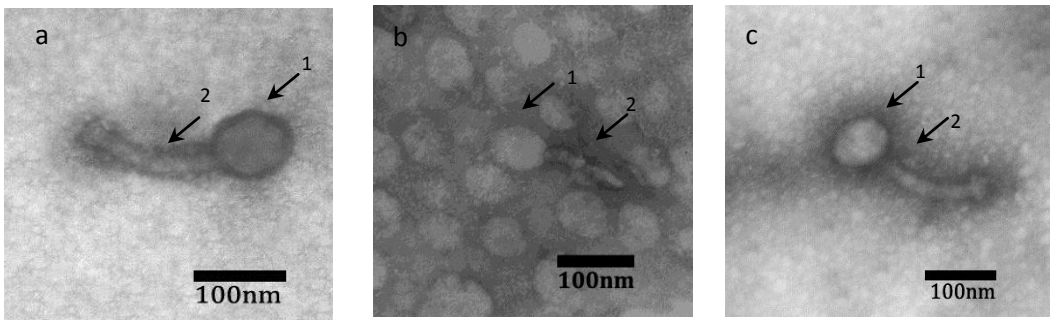

Figure 2 Phage morphology observed by TEM 60000x with 2% staining of uranyl acetate; Fag FSb (a), FSs (b), FSk (c). 1: head , 2: tail. Bar: 100 nm.

Bacteriolytic Effectiveness

The results in figure 3 showed that at the second hour after infection process, the *S. aureus*amount infected by FSs phage decreased. The bacteriolytic activity pattern of FSs phage same with JS25[34]and SAH-1[33], but the bacteriolytic activity of FSs phage lower than Stau2 phage that able to decrease the *S. aureus*population after one hour infection[32]. Bacteria population infected by FSb and FSk phages increase after two hour infection, although the increase lower than the control bacteria. Population of control bacteria increase from beginning to end of observation (Figure 4). The increase of all population bacteria at the first two hours after infection by phage considered because all phage isolates had low adsorption rate. Phage receptor with low introduction and adherence response rate caused the nucleic acid entrance into host cell become slower. The entrance retardation of nucleic acid caused process series of phage protein assembly up to the emergence of progeny become delayed. Host cell density also influence the phage growth. It was caused by the more host cells the more opportunities phage adsorption at receptor, so the infection and lysis process become higher.

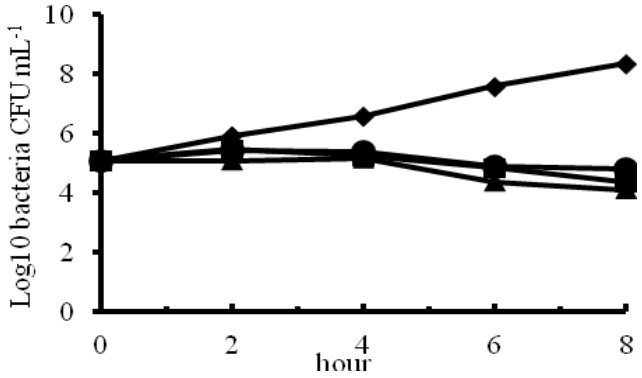

Figure 3 Bacteriolytic effectiveness by phage; Control bacteria ( ), *S. aureus*infected by FSb phage ( ), *S. aureus*infected byFSs phage ( ), *S. aureus*infected by FSk phage ( ).

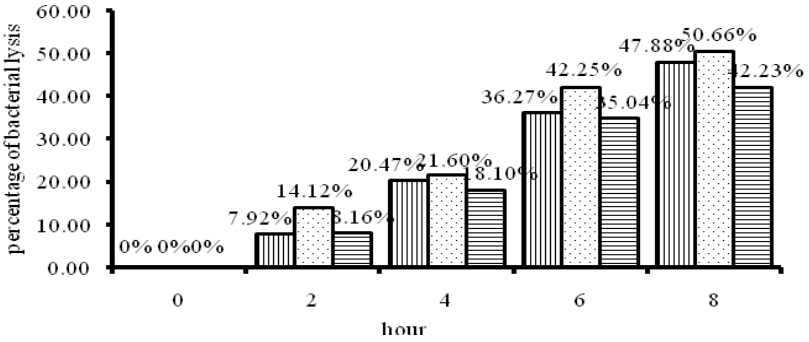

Figure 5 Percentage of bacterial lysis of *S. aureus*by phage; lysis percentage by FSb (■), FSs (■), FSk(■).

Bacteriolytic effectiveness by phage showed based on the decrease of bacterial cell amount by phage infection toward control bacterial amount. FSs phage had the highest bacteriolytic effectiveness toward *S. aureus*, then be followed by FSband FSk phages. FSs phage decreased50.66% of *S. aureus* population, FSband FSkphage decreased 47.88% and 42.23% *S. aureus*population after 8 hours (figure 5). Phage bacteriolytic effectiveness was influenced by several factors, such as phage concentration, environmental condition (pH, aw, and temperature), and specificity[35].

Conclusion

Three lytic phages of *S. aureus* (FSb, FSs, and FSk) of the isolation results from dairy farm cowsspesificto infect *S. aureus*. FSband FSkphages included in *Myoviridae* family, FSs phage included into *Siphoviridae* family. All phages were effective in decreased the *S. aureus* population. Molecular characterization and bacteriolyticactivities in vivo are in further studies.

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