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Isolation And Screening Of Specific Methicillin Resistant-*Staphylococcus Aureus* Bacteriophage From Hosiptal Waste At Banyumas

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Abstract The incidence of infections caused by Methicillin-resistant *Staphylococcus aureus* (MRSA) were increasing in many hospitals around the world every year. The use of antibiotics therapy became ineffective to control MRSA infection, because it was resistant to methicillin and many beta-lactam class of antibiotics. The use of bacteriophage as biological control agent was a promising strategy to control MRSA. Bacteriophage could infect specific bacteria and destroy their cells. Bacteriophages were found widespread in nature. The aim of this research were to isolate MRSA bacteriophages from hospital waste at Kabupaten Banyumas, and screen specific MRSA bacteriophages.The method was survey by using purposive random sampling to take the soil from waste management installation at Kabupaten Banyumas. Bacteriophages were isolated from the soil by bacteriophage particles precipitation technique and plaque assay. Isolated bacteriophages were henceforth tested for their specificity against MRSA and other pathogenic bacteria. Parameters that were observed encompass the presence of plaque, specific MRSA bacteriophages. Data were analyzed descriptively to interpret the presence of plaque bacteriophages, and the ability of bacteriophage to infect specific host cell (MRSA). The result reveals that the MRSA bacteriophages could be isolated from the hospital sewage, and found two types of bacteriophages were specific MRSA bacteriophages and unspecific MRSA bacteriophage.

Keywords: Bacteriophage, Hospital waste, Isolation, Methicillin Resistant *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus was Gram Positive Bacteria which known since 19th Century as the cause of local or systemic infections in humans and animals [7]. Cattles were infected with this bacterium suffer pus, mastitis, arthritis, and urinary tract infections, while in humans this bacterium could cause pneumonia, food poisoning, postoperative wound infections, bacteremia and nosocomial infections in hospitals [11].

The problem that appeared due to MRSA infection was nosocomial infections [16]. Nosocomial infections occurred throughout the world, particulary in developing countries. The prevalenceof MRSA infection in Southeast Asia varies from 7 % in the Philippines, 35 % in Malaysia, and 39 % in Singapore. While the prevalence of MRSA infection reached 25 % on 2016 in Indonesia [8]. Surgical site infection was the most common type of nosocomial infection [11].

Antibiotic therapy to overcome MRSA infection was less effective, so it needed other treatment alternatives. Therapeutic bacteriophage or phage therapy could be an alternative to overcome MRSA infection [17]. Bacteriophages used for phage therapy was a bacteriophage that had the lytic cycle and high specificity to a single host. Bacteriophages with lytic cycle effectively used for phage therapy [3][1].

The successful treatment using this method was shown by d'Herelle 1919 to treat dysentery patients at the Hospital des Enfants-Malades in Paris [20].Phage therapy had also successfully treat infectious diseases of lung, pleura, and skin diseases due to Staphylococcus genus [20]. The used of bacteriophages in food also have been applied to reduce contamination of Campylobacter jejuni in broiler chickens, thereby reducing the risk of gastroenteritis in humans [2].

Bacteriophages could be found in a variety of environments such as soil, sand, freshwater, waste, brackish waters and marine waters. Based on these descriptions, the purposes in this study were to find out the presence of MRSA bacteriophages from hospital waste at Banyumas, and screen specific MRSA bacteriophages.

MATERIAL AND METHODS

1. *Bacterial Strain and Growth Medium*

All strains used in this study were MRSA, *S. aureus, Staphylococcus epidermidis, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Vibrio* sp, *Salmonella thypii* dan *Shigella* sp. Strains were grown at optimum temperature in Nutrient Broth (NB) (Oxoid).

2. *Bacteriophage Isolation and Purification*

Nosocomial infection-causing strain Methicillin Ressistant *Staphylococcus aureus* was used as representative strain for phage isolation. Soil samples of 10 g hospital waste from Prof. Dr. Margono Soekarjo Hospital, Banyumas Hospital, and Ajibarang Hospital were obtained and added 100 ml sterile aquades. They were shake in shaker incubator at 80 g for 60 minutes. Samples were filtrared by multilevel filtration. Samples were filtrated by whatman filter paper and Millipore membrane filter. Filtrate was supplemented 15 ml buffer phage and incubated at room temperature for 5 hours. Filtrate centrifugatged 8000 g for 20 minutes. Supernatant was filtered with Millipore membrane filter 0.45 µm. Filtrate were precipated with PEG 8000 at 4°C for 24 hours and centrifugated 12000 g for 20 minute. Natant was resuspended by 50 µl buffer phage and stored it at 4°C. Natant known as stock crude phage. 200 µl MRSA were infected 100 µl stock crude phage, added 20 µl MgSO4 and 20 µl $CaCl_2$ and incubated at 37°C for s15 minutes. They are mixed with 7 ml Luria Bertani (LB) semi solid medium, cultivated to petridish, and incubated at 37°C for 48 hours. Purification step: Plaques were took and mixed 7 ml MRSA. Incubated at shaker incubator 80 g at 37°C for 48 hours. They are filtered using membrane filter 0.45 µm. 100 µl filtrate were added 200 µl MRSA, 20 µl MgSO4 and 20 µl $CaCl_2$ and incubated at 37°C for 15 minutes. They are cultivated using 7 ml LB semi solid medium and incubated at 37°C for 48 hours. Phage activity was observed with the formation of plaque. This step was repeated 3 times and precipitated phage from the last step.

3. *Specific MRSA Bacteriophage Screening*

Bacteriophage from the purification were tested their specificity to infect MRSA. MRSA, *S. aureus, Staphylococcus epidermidis, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Vibrio* sp, *Salmonella*

thypii dan *Shigella* sp were used to screen specific MRSA bacteriophage and cultivated in LB medium at optimum temperature for 12 hours. Each bacteria inoculum was mixed with 10 µl pure MRSA phage, 20 µl MgSO4, and 20 µl CaCl2 which incubated at 37°C for 6 hours. Bacterial growth inhibition caused phage infection measured using the absorbance with 600 nm wavelength.

RESULT AND DISCUSSION

A. *Bacteriophage Isolation and Purification*

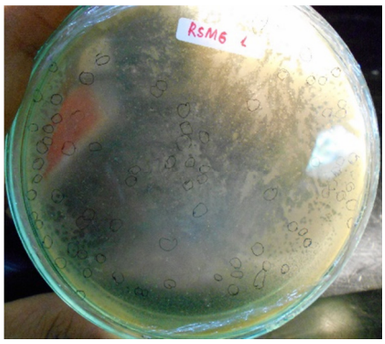
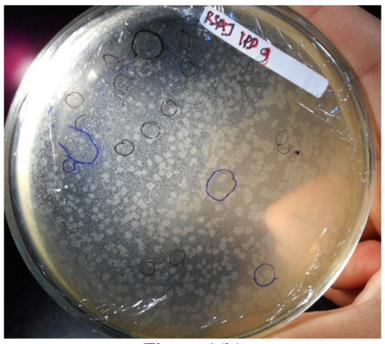


Figure 1(a)



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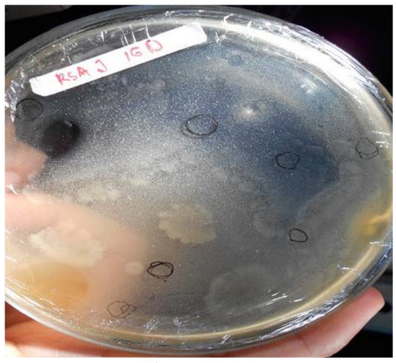

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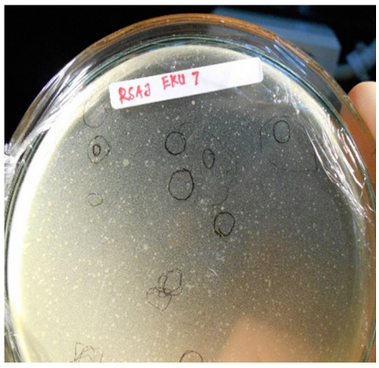

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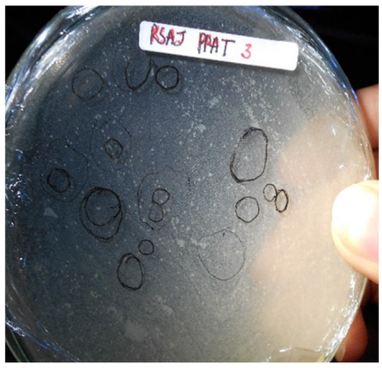

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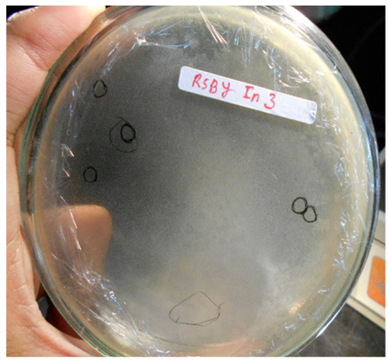

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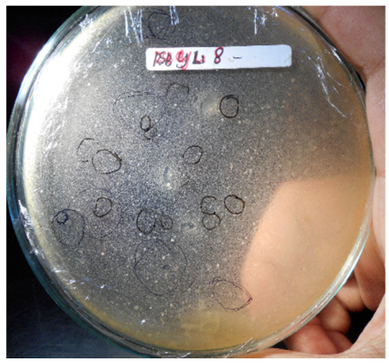

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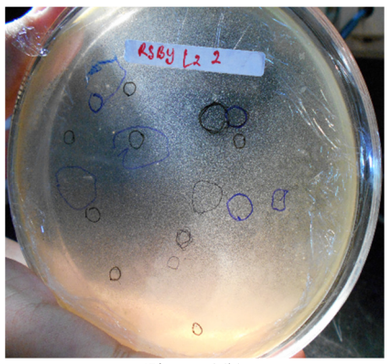

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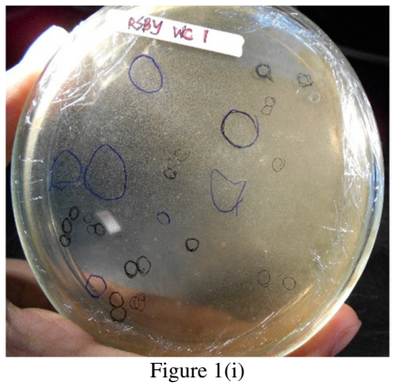

Figure 1(i)

Figure 1: Plaque Assay and Plaque Formation of MRSA Bacteriphage: (a) Hospital waste of RSUD. Prof. Dr.

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Margono, (b) Hospital waste of inpatient ward of RSUD Ajibarang, (c) Hospital waste of emergency room of RSUD Ajibarang, (d) Hospital waste of equalization of RSUD Ajibarang, (e) Hospital waste of pra-treatment of RSUD Ajibarang, (f) Hospital waste of Inlet of RSUD Banyumas, (g) Hospital waste of nutririon and radiology department of RSUD Banyumas, (h) Hospital waste of Surgical inpatient of RSUD Banyumas, (i) Hospital waste of toilet of RSUD Banyumas.

Table 1. Plaque forming of MRSA bacteriophage.

No	Location	Sample Code	Number of *Plaque*	*Plaque Type*
1	Prof. Dr. Margono Hospital	A-	50	4
2	Ajibarang Hospital			
	Inpatient unit	B-	6	2
	Emergency unit	C-	6	2
	Equalization	D-	16	4
	Pra-treatment unit	E-	12	2
3	Banyumas Hospital			
	Inlet unit	F-	18	4
	Nutrition and Radiology unit	G-	20	4
	Surgical inpatient unit	H-	14	5
	Toilet	I-	6	2

Bacteriophages were isolated by precipitate particles of a bacteriophage and plaque assay. Bacteriophage isolation results showed all hospital waste containing MRSA bacteriophages. The presence of bacteriophages indicated by plaque forming in the growth media. In general, bacteriophages were isolated by this method is a group of virulent bacteriophages. These bacteriophages capable of infecting and lysing the host cell, forming a clear zone plaque in growth media. The amount of plaque that can be isolated vary in each hospital waste. Highest number of plaque obtained from hospital waste of Prof. Dr. Margono hospital. Fifty plaques formed from there. This was occur because the environmental conditions each hospital waste was different. Environmental conditions affected by source of nutrients, pH, and temperature. Conditions of hospital waste of Prof. Dr. Margono Soekarjo had the character of the rest of the fresh food sourced from inpatient units. Other hospital waste had character along with the liquid form of sludge from various laundry drain, toilet, and inpatient at the hospital. This is supported by the statement Wichels et al. [25] bacteriophages had high density in the neighborhood. Number of bacteriophage very abundant in nature because of bacteriophages to function as controlling bacterial population. According Synott et al. [23] and Kaur et al [12], *Staphylococcus aureus* bacteriophage found in an amount not less in the areas of waste disposal. MRSA bacteriophages very easily isolated from the wastes of hospitals and farms. According to Voyles [24], plaque method was simple and accurate method for presenting the viability of the virus.

Plaque observations released some plaque morphology type. Physical plaque morphology that need to be observed were the diameter, form, edges and clarity. The different plaque morphology can show character and activity of bacteriophages when lysing the host. Bacteriophages isolated from hospital waste showed 5 types of plaque morphology. The first type had characteristics such as: a diameter of 2 mm, circular shape, flat edge, and clear. The second type had characteristics such as: a diameter of 1 mm, circular shape, flat edge, and clear. The third type had characteristics such as: a diameter of <1 mm, circular shape, flat edge, and clear. The fourth type had characteristics such as: a diameter of 1 mm, irregular shape, uneven edges, and turbid. The fifth type had characteristics such as: a diameter of <1 mm, irregular shape, uneven edges, and turbid (Table 1). This is supported by a statement of Li and Zhang [14] that the SPW virulent bacteriophages isolated from a cow suffering from mastitis capable of lysing S. aureus causes mastitis. Lysate activity produced an assortment of plaque diameter of 2-3 mm, circular shape, clear, and flat edge. According Sung-Sik et al. [22], the size of each bacteriophage plaque produced varies depending on the ability of bacteriophage infect host cells. Clear zone produced is proportional to the amount of particles released.

Plaque was formed on isolation process further purified to obtain a single plaque which had a uniform morphology. According to Nindita et al. [16] and Olievera et al. [19], the bacteriophage mixture isolation results need further purified in order to get a single plaque. Single plaque morphology observed purified uniformity of size and shape. Single plaque will determine the usefulness of bacteriophages in the field of phage therapy. Bacteriophages are derived from a single plaque has xthe accuracy and effectiveness of the strong to lyse the bacteria that cause disease.

B. Specific MRSA Bacteriophage Screening

No	Phage	MSO1	*S. aureus*	*S. epidermidis*	*Shigella* sp	*S. thypii*	*E. coli*	*B. cereus*	*K. pneumonia*	*Vibrio* sp
1	A-1	+	-	-	+	-	-	-	-	-
2	A-2	+	-	-	+	-	-	-	-	-
3	A-3	+	-	-	+	-	-	-	-	-
4	A-4	+	-	-	+	-	-	-	-	+
5	B-1	+	-	-		-	-	-	-	+
6	B-2	+	-	-	+	-	-	-	-	+
7	C-1	+	-	-	+	-	-	-	+	-
8	C-2	+	-	-	+	-	-	-	-	-
9	D-1	+	-	-	+	-	-	-	-	+
10	D-2	+	-	-	+	-	-	-	-	+
11	D-3	+	-	-	+	-	-	+	-	-
12	D-4	+	-	-	-	-	+	-	+	+
13	E-1	+	-	-	-	-	-	-	+	-
14	E-2	+	-	-	+	-	-	-	-	-
15	F-1	+	-	-	-	-	-	-	-	-
16	F-2	+	-	-	-	-	-	-	-	+
17	F-3	+	-	-	+	-	-	-	-	-
18	F-4	+	-	-	-	+	-	-	+	+
19	G-1	+	-	-	+	-	-	+	-	-
20	G-2	+	-	-	-	-	-	-	-	+
21	G-3	+	-	-	-	-	-	-	-	+
22	G-4	+	-	-	-	-	-	+	-	-
23	H-1	+	-	-	-	-	-	-	-	-
24	H-2	+	-	-	-	-	-	-	+	-
25	H-3	+	-	-	-	-	-	-	-	-
26	H-4	+	-	-	-	-	-	-	+	-

27	H-5	+	-	-	-	-	+	-	-	+
28	I-1	+	-	-	-	-	-	-	-	+
29	I-2	+	-	-	+	-	-	-	-	-

(+): infected; (-): not infected

Specificity test was performed by measuring the ability of the bacteriophage inhibits the growth of test bacteria. Inhibition of growth was indicated by the number of cell density was not increased as measured using a spectrophotometer at a wavelength of 600 nm. Based on test results obtained 3 specificity of bacteriophages specific to MRSA and 26 bacteriophages were not specific to MRSA. Bacteriophages were known as bacteriophages specific narrow spectrum because of its ability to infect specific bacterial species. Bacteriophages narrow spectrum consisted of bacteriophages F-1, H-1 and H-3. They were not able to infect bacteria alternative test consistently. Bacteriophages were not specific MS01 consisted of bacteriophage A-1, A-2, A-3, A-4, B-1, B-2, C-1, C-2, D-1, D-2, D-3 , D-4, E-1, E-2, F-2, F-3, F-4, G-1, G-2, G-3, G-4, H-2, H-4, H -5, I-1 and I-2. These type were known as bacteriophages broad spectrum. These bacteriophages able to infect bacteria cross-species, even across a group of bacteria based on the structure of the cell (gram-negative or gram-positive) (Table 2). One success was determined when the bacteriophage infected bacteria and attached to penetrate the bacterial cell wall. The success of bacteriophages on bacterial adhesion due to the bacterial receptor conformity with bacteriophage receptors.

Three specific bacteriophage to MRSA were thought to have receptors that very complementary to receptors located on the cell wall of the host bacterium, so that the bacteriophages infect suitable host cell specifically. This is supported by Rakhuba et al. [20] the ability of bacteriophage to infect specifically is determined by the specificity of adsorption. Adsorption specificity of bacteriophages to the bacteria depend on the nature and structure of the receptor on the surface of bacterial cells. In addition, the presence of the receptor on the cell wall, the number of receptors and receptor density in various sites on the cell wall also influence the adsorption process. The nature of bacteriophage receptors connect with the host cell is different depending on taxonomic relationships, the host cell wall composition and structure of the host cell wall.

The structure of the bacterial cell wall is one factor that determines the success of a bacteriophage infects host cells. The structure of the membrane of Gram-negative bacteria in contrast to the structure of Gram positive bacteria. This difference is seen from the high membrane permeability of Gram negative bacteria because of the increased transporter integral proteins form channels. In addition, differences can be seen is the presence of an external lipid layer or lipopolysaccharide. The proteins on the membrane and a variety of sites lipopolysaccharide bacteriophages to function as receptors. Both types of molecules is indispensable for adsorption. Gram positive bacteria cell wall structure and chemical composition was different from Gram negative bacteria. Gram-positive composed of 40-90% peptidoglycan. Peptidoglycan was heteropolymers compound of monomer disaccharide. These compounds which cause the peptidoglycan into a solid to protect the cell plasma membrane. Teichoic acids were essential compounds in Gram positive bacteria. This compound was a water-soluble polymer that was commonly found on the surface of bacterial antigens. Peptidoglycan and teichoic acid bacteriophages to function as receptors on Gram positive bacteria [20].

Bacteriophages which had a broad spectrum from specificity test showed the ability to infect other bacteria at the genus level. This is supported by a statement Grath [6], some bacteriophages were found to have a broad host range of cross-genus or even domain level despite it was thought that the presence of bacteriophages number of this type did not exceed 0.5% of the total bacteriophages in nature. Polyvalent bacteriophage was another term for a bacteriophage that had the ability to lyse more than one host cell. This ability was influenced by a lack of compatibility between the host receptor with receptors on the virus causing specific binding. Polyvalent bacteriophage had the ability to infect bacteria in the same family. Polyvalent bacteriophage found in families Pseudomodaceae, Actinomycetaceae, and Enterobactericeaceae [16]. The bacteria that includes family Enterobactericeaceae namely, Escherichia, Salmonella, Shigella, Klebsiella, and Seratia [5]. Some bacteriophage in nature have a wide host range to infect various species of bacteria, not limited to certain bacteria [1]. Bacteriophage SN-1, and D3 is able to infect *Sphaerotilus natans*, and *P. aeruginosa*. BHR1 bacteriophages capable of infecting P. aeruginosa and E. *coli.* BHR3 bacteriophages capable of infecting *Sp. natans* and *E.coli* [10]. Specificity to infect between various strains of *S. aureus* was also demonstrated by bacteriophages ϕSA023, ϕSA037, ϕSA039, ϕSA043 [23]. Bacteriophages K had the ability to infect bacteria in the same genus, namely, *S. aureus, S. epidermidis, S. capiris, S. caprae, S. haemolyticus, S. lugdunensis,* and *S. simultans* [9].

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CONCLUSION

In summary, this study revealed (i)MRSA bacteriophages could be isolated from the hospital waste at Banyumas and (ii) found two types of MRSA bacteriophages were specific MRSA bacteriophages and unspecific MRSA bacteriophage from hospital waste at Banyumas.

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