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Isolation and characterization of EPEC phage from domestic waste in Indonesia

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ABSTRACT

Aims: Enteropathogenic *Escherichia coli* (EPEC) K1.1 is one of pathogenic bacteria-causing diarrheal disease. This bacterium was resistant to tetracycline and ampicillin. The use of lytic phage can be a good solution to reduce antibiotic resistance of pathogenic bacteria. This study was to isolate and characterize phage that can lyse EPEC K1.1 cells.
Methodology and results: Lytic phage was isolated from domestic waste at Darmaga, Bogor, West Java, Indonesia. It uses double layer plaque technique. The kind of phages was determined by the plaque morphology, the structure, the host range, and the protein characteristics of phages as well as storage stability of phage in different buffer conditions. Lytic phage only infects the EPEC K1.1. It does not infect the other bacteria in this research. Electron microscope observation showed that phage FBd3 has icosahedral head with 57.14 nm in diameter and short-non contractile tail with 18.2 nm in length. It was grouped into family *Phodoviridae*. Lytic phage had good storage stability in *Ringers* buffer at low temperature (4 °C). It showed that viability of phage decreased by 28.04% after 9 days of storage. Phage FBd3 has six different proteins with various molecular weight: 33 kDa, 42 kDa, 49 kDa, 57 kDa, 77 kDa and 106.7 kDa. This study indicates that EPEC K1.1 can be infected by more than one type of lytic phages.
Conclusion, significance and impact of study: Lytic phage FBd3 reduces the population of PEC K1.1 effectively. It can be used as a bio-control agent to EPEC that contaminates food and water. Lytic phage found in this study potentially prevent bacterial contamination in food and beverage processing for diarrheal disease prevention.

Keywords: Lytic phage, EPEC, diarrhea

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) is one of foodborne disease bacteria which causes diarrheal disease transmitted by contaminated water and food (Miliotis and Bier, 2003; Newell *et al*., 2010; Osman *et al*., 2012). This bacterium attacks babies under five years old in a developing country for more than 50% cases of diarrheal disease in the world. A virulent factor of EPEC is due to their colonization on intestinal surface, and causes alternation of the intestine mucosal cells (Mearin *et al*., 2005). EPEC has extracellular proteolytic enzymes that can degrade mucin as a defensive system on human intestine (Budiarti and Mubarik, 2007). Antibiotics are commonly used to decrease exposure of pathogenic bacteria. However, the use of inappropriate antibiotics dose affects on bacterial resistance. This resistance was caused by mutations of bacterial chromosome or plasmid gene transferred from bacteria (Zhang *et al*., 2006).

The previous study reported that EPEC K1.1 isolated from diarrheal patient was resistant to tetracycline and ampicillin (Budiarti, 1998). Other studies reported that *E. coli* pathotypes causing diarrheal isolated from children in the age of six months to five years old in India more than 70% was resistant to norfloxacin, amoxicillin, cotrimoxazole, ampicillin, ceftriaxone, cefotaxime and metronidazole (Sudershan *et al.*, 2014). Resistance on antibiotics is not only found in pathogenic bacteria, but also it is found in normal flora bacteria. It was reported that *E. coli* as intestinal normal flora isolated from feces of new born children was antibiotic-resistant (Budiarti, 2011).

Lytic phage can be a good solution to reduce antibiotic-resistant of pathogenic bacteria (Hagens and Loessner, 2010). Phage is specific and effective to lyse targeted bacteria without disturbing normal flora (Brovko *et al*., 2012). The advantage of using the phage its own ability to replicate if its host bacterium exists and it will be inactive if its host bacterium does not exist (Fu *et al.*, 2010). The susceptibility of bacteria against phage infection is mostly related to the variation of receptor molecules, restriction modification system in host cells, or

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other phage tolerant systems such as abortive infection (Flynn *et al.*, 2004).

Based on previous study, in Indonesia, some phages that lyse pathogenic bacteria has been isolated. Bacteriophage isolated from Ciapus River was infected to *Bacillus pumilus* isolated from sewage water (Kusmiatun *et al.*, 2015). *Photobacterium damselae* infecting fish and human can be lysed by lytic phage isolated from environment (Novianti *et al*., 2014). An antibiotic-resistant bacterium called *Proteus mirabilis* isolated from feces of diarrheal patient can be lysed by lytic phage (Afriani *et al*., 2014). FB4 lytic phage can lyse 85% of EPEC K1.1 which is an antibiotic-resistant bacterium (Budiarti *et al*., 2011). The objective of this research was to isolate and characterize EPEC K1.1 phage to contribute in making phage cocktail as a bio-control agent.

MATERIALS AND METHODS

Bacterial stock preparation

EPEC K1.1 used in this study was an isolate bacterial collection at laboratory of Animal Biotechnology and Biomedicine, Bogor Agricultural University, Bogor, West Java, Indonesia. EPEC K1.1 isolate was grown on Eosine Methylene Blue Agar (Oxoid, Basingstoke, UK) plates showing good growth with metalic green sheen color. It was grown on Nutrient Agar medium (Difco, Becton Dickinson and Company, USA) as a bacterial stock.

Lytic phage isolation

Lytic phages were isolated from domestic waste at Darmaga, Bogor, West Java, Indonesia. About 1 mL of sample was diluted to 9 mL Nutrient Broth (Difco, Becton Dickinson and Company, USA) and centrifuged at 4000 *g* for 30 min. Supernatant was filtered using 0.45 µm pore size syringe filter. About 4.5 mL of filtrate was added to 0.5 mL of exponential phase culture (10^9 CFU/mL) of EPEC K1.1. The sample was incubated overnight at 37 °C. The culture was centrifuged at 4000 *g* for 30 min. Supernatant was filtered using 0.22 µm pore size syringe filter and was saved in sterile tube at low temperature (Atterbury *et al.*, 2007). Plaque assay was done using double layer plaque technique by adding 100 µL of filtrate to 100 µL exponential phase culture of EPEC K1.1 and incubated it at 37 °C for 30 min. The culture was added into 7 mL at 47 °C soft agar and poured it into Nutrient Agar medium. The plates were incubated at 37 °C for 24 h. Plaque formation was observed and counted (Clokie and Kropinski, 2009).

Phage morphology determination

Morphology of phages was determined using Transmission Electron Microscope (TEM) at Eijkman Institute. About 10 µL of each phage isolate of was dropped on grid (400 mesh) and left for 30 sec. About 5 µL of 2% Uranyl acetate was dripped on grid and dried using a filter paper. The grids were observed using JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) at magnification of 10,000-100,000× (Carey *et al*., 2006).

Determination of phage host range

Lytic phage suspension was tested to several bacterial strains i.e EPEC K1.1, *Salmonella* P38, *Salmonella* P84, *Proteus mirabilis*, *B. pumilus*, and *P. damselae*. Host range test was conducted using double layer plaque technique (Atterbury *et al*., 2007). A positive response was indicated by the formation of plaques.

Stability of lytic activity in buffer storage

Ringers buffer [8 g NaCl, 0.42 g KCL, 0.24 g $CaCl_2$, 0.20 g $NaHCO_3$ in 1 L H_2O], and Saline Magnesium (SM) buffer [5.8 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 50 mL Tris-Cl (pH 7.5), 5 mL gelatine in 1 L H_2O] were used as buffers for storing phage. Nutrient broth was used as a control. Two Petri dishes of phage plaque isolates were scraped and diluted in buffer. Each of suspensions was mixed thoroughly using a vortex for 30 sec and left at room temperature for an hour. Centrifugation was done at 4000 *g* and 4 °C for 20 min. Supernatant was filtered by 0.22 µm pore size syringe filter and the supernatant was stored at 4 °C and 27 °C. Viability of phage was determined by plating it using double layer plaque technique every three days (Phumkhachorn and Rattanachaikunsopon, 2010).

Analysis of phage protein

Proteins of phage were analysed by SDS-PAGE (Laemmli, 1970). The marker was a pre-stained protein (Pageruler Prestained Protein Ladder, Thermo Scientific Fermentas Technology, UK). Phage proteins were mixed with marker in buffers with ratio of 4:1 and incubated for 24 h. The mixtures were boiled for 5 min. The phage and sample markers were put in loading wells of 60 µL SDS-PAGE gel containing 12% of acrylamide. The electrophoresis was conducted at 20 mA, 50 Volt for 3.5 h. After the electrophoresis, the gel was removed from the glass plates and then silver staining was applied to visualize the result.

RESULTS

Lytic phage isolates

Host bacterium for lytic phage isolation has been confirmed as EPEC K1.1. It showed metalic green sheen bacterial colony on EMBA (Figure 1). Four phage isolates i.e. FBd1, FBd2, FBd3 and FBd4 have been isolated. They showed different morphology of plaque (Figure 2) and different concentration of phage: FBd1 (2.8×10^3 PFU/mL), FBd2 (1.1×10^4 PFU/mL), FBd3 (3.4×10^6 PFU/mL) and FBd4 (6×10^2 PFU/mL) (Table 1). From the plaque forming unit, data showed that FBd3 had the highest concentration indicating the effective phage to lyse EPEC K1.1.

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Host range of phage FBd3

There are six strains of bacteria EPEC K1.1. that have been tested as bacterial hosts of phage FBd3: *Salmonella* P38, *Salmonella* P84, *P. mirabilis*, *B. pumilus*, and *P. damselae*. Each of bacteria mixed with phage FBd3 using double layer plaque technique and incubated at 37 °C for 24 h. The result showed clear zones only appeared on EPEC K1.1 was infected by phage FBd3 on overlay agar (Table 2), while *Salmonella* P38, *Salmonella* P84, *P. mirabilis*, *B. pumilus*, and *P. damselae* in this research was not infected by phage FBd3.

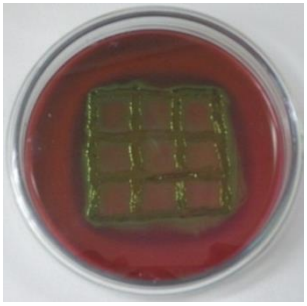

Figure 1: EPEC K1.1 on Eosine Methylene Blue Agar (EMBA).

Figure 2: Plaque morphology of EPEC K1.1 lytic phages isolated from domestic waste in Indonesia. (a) Lytic phage FBd1; (b) lytic phage FBd2; (c) lytic phage FBd3 and (d) lytic phage FBd4.

Table 1: Plaque characteristics and concentration of EPEC K1.1 lytic phages.

Sample location	Lytic phage isolate	Characteristics of plaque		Titer (PFU/ mL)
		Plaque morphology	Diameter of plaque (mm)	
Babakan Raya1	FBd1; FBd2	Clear; turbid	0.2; 0.7	2.8×10^3; 1.1×10^4
Babakan Raya	FBd3	Clear	0.5	3.4×10^6
Babakan Raya3	-	-	-	-
Jl.Darmaga	FBd4	Clear	0.3	6×10^2
Ciapus River	-	-	-	-

Table 2: Host range of phage FBd3.

Bacteria strains	Plaque formation
EPEC K1.1	+
Salmonella P38	-
Salmonella P84	-
Proteus mirabilis	-
Bacillus pumilus	-
Photobacterium damselae	-

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Phage morphology

Based on Transmission Electron Microscope micrograph (with 80,000× magnification), phage FBd3 has icosahedral head with 57.14 nm in diameter and short non-contractile tail with 33.3 nm in length (Figure 3). Based on its characteristic and according to *International Committee on Taxonomy of Viruses database* (ICTVdB, 2000), phage FBd3 can be grouped into *Phodoviridae*.

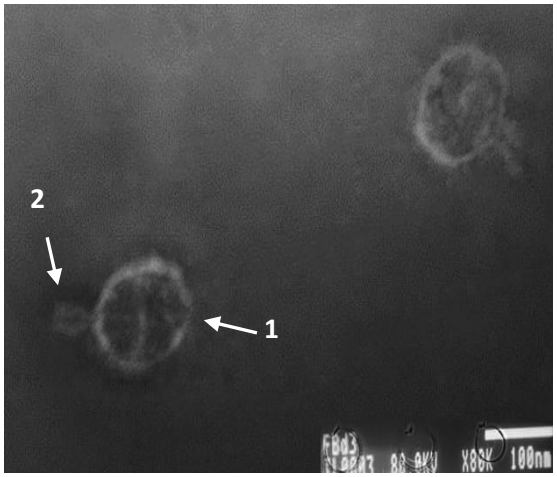

Figure 3: Transmission Electron Microscope micrograph of lytic phage FBd3 with 80,000× magnification. (1) Head of phage, and (2) Tail of phage.

Stability of phage in buffer storage

Phage FBd3 has good storage stabillity in *Ringers* buffer at low temperature (4 °C). Its viability decreased by 28.04% after 9 days of storage. If it was stored at 27 °C, the viability decreased by 7.18% after 9 days of storage. However, if the phage was stored in SM buffer at low (4 °C) and room temperature (27 °C), the viability decreased by 40.18% and 58.5% respectively. The control treatment stored in NB showed that the viability decreased by 94.7% at low temperature, moreover it was no viable phage when it was stored in NB at room temperature after 9 days of storage (Figure 4).

Phage protein characteristics

Protein analysis using SDS-PAGE showed that phage FBd3 had seven protein bands with different size of molecular weight. Based on the protein marker, the molecular weight of phage FBd3 proteins were 33 kDa, 42 kDa, 49 kDa, 57 kDa, 77 kDa, and 106.7 kDa (Figure 5).

DISCUSSION

Clear zone (plaque) on plate indicated the area of lysed bacteria which could be a parameter as a result of lytic phage infection to host bacterium (Carter and Saunders, 2007). Each of the phage isolate has different characteristics as well as their ability to lyse bacterial cells. Plaques morphology of

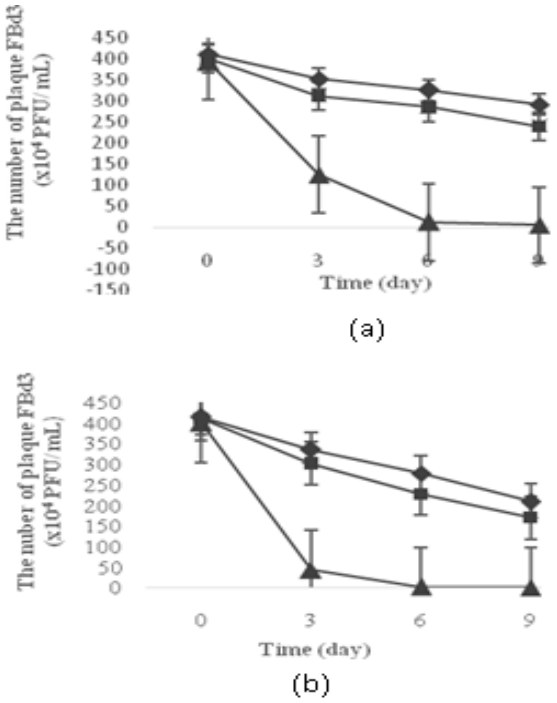

Figure 4: Storage stability of phage FBd3 in various buffers. (a) storage at low temperature, and (b) storage at room temperature. ♦: Ringers buffer, ■: SM buffer, ▲: Nutrient Broth. Bars indicate standard deviations.

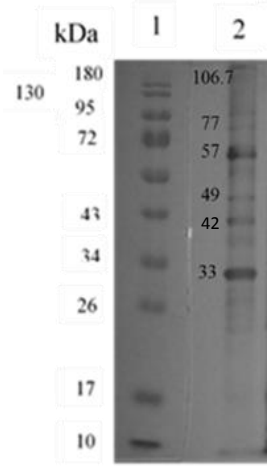

Figure 5: SDS-PAGE results of phage FBd3 proteins. (1) protein marker, and (2) proteins of phage FBd3.

phage FBd1, FBd2, FBd3, and FBd4 was 0.2 mm in diameter with clear plaque, 0.7 mm in diameter with turbid plaque, 0.5 mm in diameter with clear plaque, and 0.3 mm in diameter with clear plaque, respectively. Phage FBd3 can lyse more bacterial cells of EPEC K1.1.than the other phage isolates. Phage can lyse host cells because of its ability in synthesizing lysozyme in the bacterial cells. Lysozyme was synthesized by phage for damaging bacterial cell wall by cutting β-1,4–glycosidic bond between N-acetyl glucosamine and N-acetyl muramic acid of peptidoglycan (Madigan *et al.*, 2009). It causes the lysis of bacterial cells and newly formed phages are released (Tortora *et al.*, 2006).

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There are several factors that can affect plaque formation. Rapidity to lyse bacterial cells is one factor that can determine plaque morphology and size. This factor can make different appearing of plaque either in morphology or in size (Gallet *et al*., 2011). Rapidity of plaque formation was related to rapidity of phage multiplication (Pelczar and Chan, 2007). Phage FBd3 was infected EPEC K1.1. They can lyse bacterial cells of EPEC K1.1. from plaque forming unit, phage FBd3 showed the highest concentration of phage (Table 1). FB4 infecting EPEC K1.1 has phage concentration of 1.6 $\times 10^4$ PFU/mL (Budiarti *et al*., 2011). It means that phage FBd3 is different from phage FB4. So FBd3 can be applied as a bio-control agent of EPEC in contaminated water and food for diarrheal disease prevention.

Each phage has different receptors which determine its ability in infecting bacteria. The kinds of receptor specify the host range in phage infection (Bielke *et al*., 2007). Each phage recognizes different receptors on bacterial cell surface (Braunt and Hantke, 1997). Kinds of phage receptors are pili (Roncero *et al*., 1990), capsule (Suthereland *et al*., 2004), teichoic acid (Raisanen *et al*., 2007), lipopolysaccharide (Lindberg *et al*., 1978), and surface protein (Davison *et al*., 2005). Determination of host range (Table 2) showed that FBd3 was only lysed by EPEC K1.1 cells. It showed that among the tested bacteria in this study, the receptors of EPEC K1.1. may differ from others. This association is influenced by the specificity of receptor characteristic of each host. Phages identify their host bacterium by binding or adsorbing it to a specific structure on the surface of cells called as receptor. The basic of adsorption an interaction between specific structure on the surface of phage and a specific structure on the surface of bacterium (Kudva *et al*., 1999).

Transmission Electron Microscope micrographs proved that phage FBd3 has icosahedral head with 57.14 nm in diameter and short non-contractile tail with 33.3 nm in length (Figure 3). Based on its characteristic and according to *International Committee on Taxonomy of Viruses* (ICTV), phage FBd3 can be grouped into family *Phodoviridae*. However, there is a previous study reported that lytic phage FB4 infecting EPEC K1.1 having hexagonal icosahedral head with 81.56 nm in width and 103.11 nm in length was grouped into family *Siphoviridae* (Budiarti *et al*., 2011). It proved that EPEC K1.1 can be infected from different family of phage.

Several physical and chemical factors including temperature and ion determine viability of phage in environment. The inappropriate phage storage will cause a damage on tail, head, and DNA of phages (Jonczyk *et al*., 2011). Phage FBd3 had good storage stabillity in *Ringers* buffer at low temperature (4 °C) compared with SM buffers and nutrient broth. The composition of chemical compounds affects storage ability of lytic phage at different temperature in different buffer. The composition of Ca^{2+} and Mg^{2+} containing in ringers buffer could increase efficiency of phage adsorption in host cells (Cele, 2009). Denaturation and oxidation of phage proteins can be inhibited by storing the phage at low temperature which is needed to maintain the viability of phage protein (Fennema, 2008).

Proteins of phage capsid and tail are varied in size and functions which it depends on type of phages. Phage FBd3 has six different proteins with various molecular weight: 33 kDa, 42 kDa, 49 kDa, 57 kDa, 77 kDa and 106.7 kDa (Figure 5). The variety of protein molecular weight indicates the variety of its protein function. Protein of phage with molecular weight of 33 kDa and 42 kDa allegedly are capsid forming protein. This is similar to Conway's study (1995) who isolated phage protein that infects EPEC with capsid protein constituent ranges between 33 kDa-42 kDa. Proteins of phage FBd3 is larger in size than that of lytic phage FB4 which also infects EPEC K1.1. Lytic phage FB4 has proteins with molecular weight in the range of 35.9-40.3 kDa (Budiarti *et al.*, 2011). This result indicated that EPEC K1.1 can be infected by more than one type of lytic phages. Therefore there is a high possibility that phage cocktail can lyse bacterial cells of EPEC K1.1 more effective than single phage. Some of previous studies reported that the effectiveness of phage applications in fighting against pathogenic bacteria depends on several factors, such as the phages/targeted bacteria ratio, the model and moment of treatment, environmental conditions, the neutralization and accessibility of phage to targeted bacteria (Ly-Chatain, 2014). A phage cocktail is more effective than single phage in order to kill bacteria (Schmerer *et al*., 2014; Bourdin *et al*., 2014).

CONCLUSION

Four lytic phages i.e. phage FBd1, FBd2, FBd3, and FBd4 were successfully isolated from domestic waste in Indonesia. The FBd3 phage, which has the widest diameter plaques compared to three other phages can lyse EPEC K1.1. Phage has showed its best viability when it is stored at low temperature (4 °C) in Ringers buffer. Phage FBd3 has six different proteins with various molecular weights: 33 kDa, 42 kDa, 49 kDa, 57 kDa, 77 kDa and 106.7 kDa.

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