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Diversity of uropathogenic *Escherichia coli* lytic phage from Cisadane River, West Java, Indonesia based on morphology and protein molecular weight characteristics

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Abstract. *Sufa HI, Budiarti S, Rusmana I. 2018. Diversity of uropathogenic* Escherichia coli *lytic phage from Cisadane River, West Java, Indonesia based on morphology and protein molecular weight characteristics. Biodiversitas 19: 2359-2364.* The aimed of this study was to identify, isolate and characterized the lytic phage diversity which had a potential in reducing uropathogenic *Escherichia coli* (UPEC), antibiotic resistant-pathogenic bacteria infecting urinary tract from Cisadane river based on the morphology and protein molecule weight. To the best of our knowledge, this study has not yet been reported in Indonesia. Two novel lytic phages of UPEC, FU1, and FU3 were isolated from Cisadane River, Indonesia. Both of lytic phages were more closely related to Podoviridae, which had icosahedral capsid in 47.2 nm and 40 nm diameter length, respectively and short-noncontractile tail (< 40 nm). The FU1 and FU3 had different protein molecular weight ranges between 10-76 kDa and 8-120 kDa, respectively. In addition, the bacteriolytic activity of FU3 (1.6×10^7 PFU/mL) was able to reduce UPEC (1.7×10^7 CFU/mL) to become lower than the limitation value of infection (< 10^5 CFU/mL) in only 25 h of incubation. A better effect of FU3 phage in decreasing UPEC population in vitro is expected to be one of the further alternative biocontrol agents to reduce the abundance of UPEC population which grows in urinary tract or contaminates river water.

Keywords: Biocontrol, biodiversity, lytic phage, UPEC, river contaminant, urinary tract infection

INTRODUCTION

Uropathogenic *Escherichia coli* (UPEC) becomes one of the predominant pathogenic bacteria infecting human urinary tract (Kirecci et al. 2015). Some urine samples from different childhood's patient in Indonesia also showed the presence of culturable UPEC and unculturable *Escherichia* sp. based on 16S rRNA gene (Christine et al. 2018). Mechanism of UPEC in infecting urinary tract is commonly initiated by colonizing *E. coli* from anus to periurethral slot, then it moves to bladder and kidney by ascending. The dominance of UPEC as the highest bacteria presence in urinary tract is supported by genetic contents encoding virulent factors such as adhesion, motility, siderophore production, toxin production and host immune evasion (Nielubowicz and Mobley 2010). In addition, the ability of pathogenic *E. coli* to live and contaminate various habitat, including water source, become a serious problem that needs an effective solution. Interestingly, the previous study reported that more than 50% of UPEC isolated from 100 urine samples of infant and elderly UTI's patient had resistance to various types of antibiotics such as amikacin, aztreonam, chloramphenicol, cotrimoxazole, cefepime, cefpodoxime, ceftazidime, cefotaxime, ceftriaxone, cefoxitin, ciprofloxacin, gentamicin, tobramycin, piperacillin1, tetracycline, nitrofurantoin, and norfloxacin (Akram et al. 2007). The application of lytic phage can be a solution to accelerate the reduction of antibiotic-resistant pathogenic bacteria.

Lytic phage is a bacterial virus which specifically infects the host bacterial cell, then it lyses bacteria to produce many new mature lytic phages. A successful recognition of lytic phage to the host bacterial cell is determined by receptors compatibility in bacterial cell to ligand binding protein of phage (Madigan et al. 2015). The high specificity of lytic phage in infecting the bacterial cell give a good impact to lytic phage that becomes a safe biocontrol agent for human cell and normal microbiota in human body or in environment.

Some lytic phage studies from Indonesia have been reported for example lytic phage of enteropathogenic *Escherichia coli* (EPEC) isolated from domestic waste (Arivo et al. 2016), lytic phage from Ciapus River which is specific to *Bacillus pumilus* contaminating sewage water (Kusmiatun et al. 2015), and lytic phage from the dairy farm specific to *Staphylococcus aureus*, predominant bacteria causing bacteremia (Nugroho et al. 2016). There are some lytic phages which have been commercialized through long experiments. Three commercial lytic phages (Pyo, Intesti and Septaphage) were tested in vitro against various antibiotic-resistant pathogenic bacteria (*E. coli* and *Proteus* spp.). Each phage was able to lyse 61%, 67%, 9% of 70 *Escherichia coli* strains and 29%, 39%, 19% of 31 *Proteus spp* strains, respectively (Bernasconi et al. 2017). To best to our knowledge, phage lytic specific to UPEC has not been reported from Indonesia. In view of this, the novel study of discovery and identify the lytic phages diversity from Cisadane river can be developed to be a biocontrol

agent specific to UPEC which infects urinary tract and contaminates environment.

MATERIALS AND METHODS

Bacterial isolate preparation

UPEC used as phage host was isolated from catheter of clinical UTI patient by microbiology laboratory of Hasanuddin University, Indonesia in August 24th, 2015. The urine of patient had some characteristics which were indicated as UTI, such as the quantity of UPEC in urine patient was clinically enumerated $\geq 10^5$ and 10 leukocytes/mm^3 (Pappas 1991). UPEC was verified by culturing on Eosin Methylene Blue Agar (EMBA) (Oxoid, Basingstoke, UK), Gram staining, biochemical test by conventionally Indole-Methyl Red-Voges Proskauer-Citrate (IMViC) method (Zahera et al. 2011). Hemolysin production was analyzed on blood agar media. Bacterial stock was grown on Luria Bertani medium (LB) (Difco, Becton Dickinson and Company, USA).

Antibiotic sensitivity pattern test was determined by disk diffusion method based on the Clinical and Laboratory Standards Institute, with document number ISSN 2162-2914 page 53 guidelines (CLSI 2016). UPEC was incubated in LB broth at 37ºC for 24 h, then UPEC has dropped 100 µL into 5 mL LB soft agar in 40 ºC before pouring as the top layer of LB agar in 100 mm petri plate. The antibiotic sensitivity test used 6 disks of ampicillin 10 µg/mL; chloramphenicol 30 µg/mL; ciprofloxacin 5 µg/mL; clindamycin 30 µg/mL; doxycycline 30 µg/mL; cotrimoxazole 23.75 µg/mL. The disks were put on the top of double-layer agar and placed no less than 24 mm apart, center to center. The antibiotic sensitivity was observed after incubating at 37 ºC for 24 h by measuring the size of lytic zone around of the disk.

Lytic phage isolation and purification

Water sample from Cisadane River, Indonesia, was used in lytic phage isolation. The filtrate of centrifuged sample (10,000 ×g; 10 min) was treated by twice modified enrichment methods (Jensen et al. 1998). The first enrichment was carried out by mixing and incubating 2 mL filtrate of phage sample, 1 mL of LB [10×], 2 mL of UPEC culture (OD_{600nm}=1), collectively at 37 ºC for 24 h. Sample was centrifuged at 10,000 ×g for 15 min. Filtrate was treated for the second enrichment by adding 1 mL of LB [10×], 5 mL of UPEC culture (OD_{600nm}=1), collectively and incubated at 37 ºC for 24 h. Each filtration process was done by using a 0.22 µm membrane filter. The lytic phage presence was observed by the formation of plaques in double layer agar (Carey et al. 2006). The mixture of 100 µL of phage stock solution and 100 µL of UPEC culture (OD_{600nm}=1) was incubated for 10 min at room temperature then it was added into a tube containing 5 mL at 47°C soft agar (1% agar) before pouring it into LB agar. The double layer agar was incubated at 37 °C for 24 h. Phage was purified by taking a single clear plaque then it was treated by double layer plaque technique (Carey et al. 2006). Purified phage was stored at 4°C in a sterile tube contained phosphate buffer saline (PBS) pH 7.4 as stock solution.

Quantification of phage

Purified phage stock was diluted serially from 10^{-1} up to 10^{-8} in PBS pH 7.4, then it was overlaid with soft agar (1% agar) as the upper layer on LB agar. The plaque presence on a double layer agar with a number between 30-300 was enumerated as plaque forming units per milliliter (PFU/mL) (Carey et al. 2006).

Phage morphological observation by transmission electron microscope

Phage morphology was observed by using a transmission electron microscope JEM-1010 (JEOL, Tokyo, Japan), which was operated in 80 kV within 80,000× magnification in negative staining.

Analysis of protein molecule weight

The molecular weight of phage protein was analyzed by Sodium Dodecyl Sulphate-PolyAcrilamide Gel Electrophoresis (SDS-PAGE) (Laemmli 1970). Marker used in SDS-PAGE was 3-color broad range protein marker (SMOBIO, Hsinchu City, Taiwan) with variations of molecular weight (~5, 10, 15, 20, 25, 35, 45, 60, 72, 100, 140, 180 and 245 kDa). Separating gel with 10% polyacrylamide was allowed in order to make it solid as the bottom layer and the collecting gel with 4% polyacrylamide added as the top layer. Each phage sample and marker were mixed into sample buffer (4:1) then it was incubated for 24 h. The solution was boiled for 5 minutes before, 60 µL of it was loaded on SDS-PAGE wells. The electrophoresis process was operated in 25 mA and 70 V for 2.5 h and stopped when the sample dye reached at 0.5 cm from the end part of the gel. Gel was taken from glass and visualized by silver staining (Sigma-Aldrich, St. Louis, USA).

Phage effectivity in lysing bacterial population in vitro

The effectiveness of phage to lyse UPEC cells was analyzed by modified method (Budiarti et al. 2011). About 100 µL of UPEC cultures (OD_{600nm}=1) was infected by 100 µL purified phage solution, which then called as treated culture. The growth of 100 µL of UPEC culture without phage treatment was used as control. Both of controlled culture and treated culture were incubated in LB at 37 °C, then the UPEC population were monitored in serial time of incubation (0, 5, 10, 15, 20, 25 hours) by plating on LB agar. Plates were incubated at 37 ºC for 24 h.

RESULTS AND DISCUSSION

Host-bacterial isolate characteristic

The morphology and physiology of host bacteria (UPEC) was very similar to the characterization of *E. coli* (Table 1). UPEC could grow in selective and differential EMBA and result in purple blackish with green sheen colonies. UPEC produced β-hemolysin toxin which was able to lyse the erythrocyte contained on media, then

forming clear zone around the bacterial colony. Some tests of antimicrobial susceptibility showed that UPEC was resistant for some tested antibiotics such as ampicillin, chloramphenicol, ciprofloxacin, clindamycin, and cotrimoxazole, but it proved an intermediate effect to doxycycline (Table 2).

Lytic phage diversity based on plaque formation

Two selected plaques formed in double layer agar and then they were further mentioned as FU1 and FU3 (Figure 1). Phage capabilities and rate to replicate (lysis time) by FU1 and FU3 in bacterial cells were not too varied although they showed difference in plaque size. FU3 plaque was larger than FU1 plaque, namely 1.5 mm and 1 mm, respectively in 24 h incubation. Serial dilution test of purified phage stock showed that FU3 had a higher quantity than FU1, namely 1.4×10^7 PFU/mL and 1.6×10^7 PFU/mL, respectively.

Phage morphological characteristics

The morphological observation of FU3 phage using a transmission electron microscope showed FU1 and FU3 phage consists of icosahedral capsid (47.2 and 40) nm and very short non-contractile tail (12.5 and 20) nm, respectively (Figure 2). These characteristics had similarity with the members of Podoviridae family of Caudovirales order.

Molecule weight of phage protein

The protein molecule weight of FU1 and FU3 was measured using SDS-PAGE 10%. The comparison between marker band and sample band showed that FU1 and FU3 protein contained molecular weight ranges from 10-76 kDa and 8-120 kDa, respectively (Figure 3).

Table 1. The biochemical properties and characteristic of the isolate determined as UPEC

Kind of test	Result
Gram staining	Gram-negative, rod-shaped
Fermentation / oxidation (lactose)	Positive
Indol production	Positive
Citrate utilization	Negative
Methyl red (mix acid fermentation)	Positive
Voges Proskauer (fermentation of butanediol)	Negative

Table 2. UPEC antibiotic sensitivity pattern

Antibiotic	Disk content (µg/mL) *	Interpretation of zone diameter (nm)* S	I	R	Mean of zone diameter (nm)	Note
Ampicillin	10	≥17	14-16	≤13	0	R
Chloramphenicol	30	≥18	13-17	≤12	0	R
Ciprofloxacin	5	≥21	16-20	≤15	0	R
Clindamycin	30	≥15	12-14	≤11	10.7	R
Doxycycline	30	≥14	11-13	≤10	12.5	I
Cotrimoxazole	23.75	≥16	11-15	≤10	0	R

Note: *(CLSI 2016). S: sensitive, R: resistant, I: intermediate

Phage effectiveness to lyse host bacteria cells in vitro

The ability of FU3 phage in lysing host bacteria cells was very effective in decreasing UPEC population. The effectiveness of FU3 in multiplicity of infection value 0.9 between phages (1.6×10^7 PFU/mL) and UPEC (1.7×10^7 CFU/mL) was able to reduce UPEC population become lower than infected limitation value ($\leq 10^5$ CFU/mL) in only 25 h incubation (Figure 4).

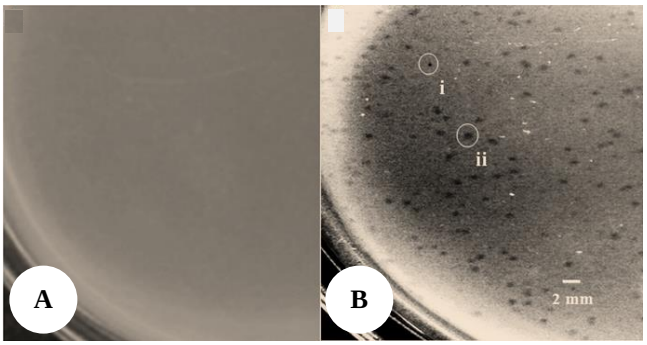

Figure 1. UPEC culture in double layer agar which was incubated at 37°C for 24 h: A. Without phage infection; B. With phage infection that resulted in various plaque size: FU1 (i), FU3 (ii)

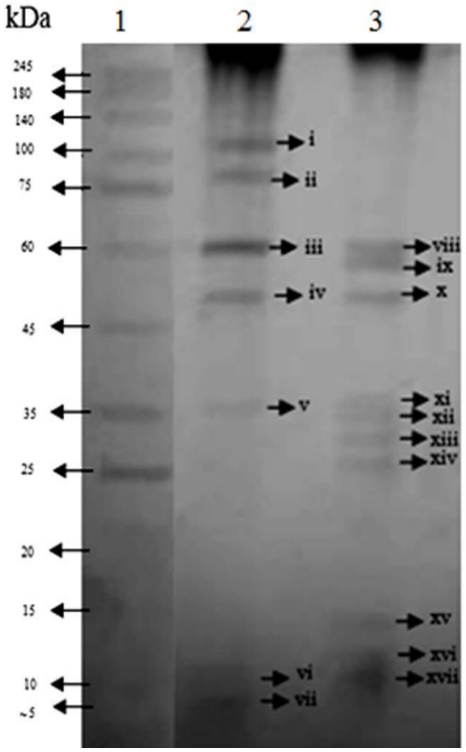

Figure 3. Protein molecule weight ranges in 10% concentration of SDS-PAGE: 1. Marker (SMOBIO PM 2700); 2. FU3: 120 kDa (i), 99 kDa (ii), 74 kDa (iii), 54 kDa (iv), 36 kDa (v), 11 kDa (vi), 8 kDa (vii); 3. FU1: 76 kDa (viii), 60 kDa (ix), 52 kDa (x), 37 kDa (xi), 32 kDa (xii), 30 kDa (xiii), 28 kDa (xiv), 13 kDa (xv), 11 kDa (xvi), 10 kDa (xvii)

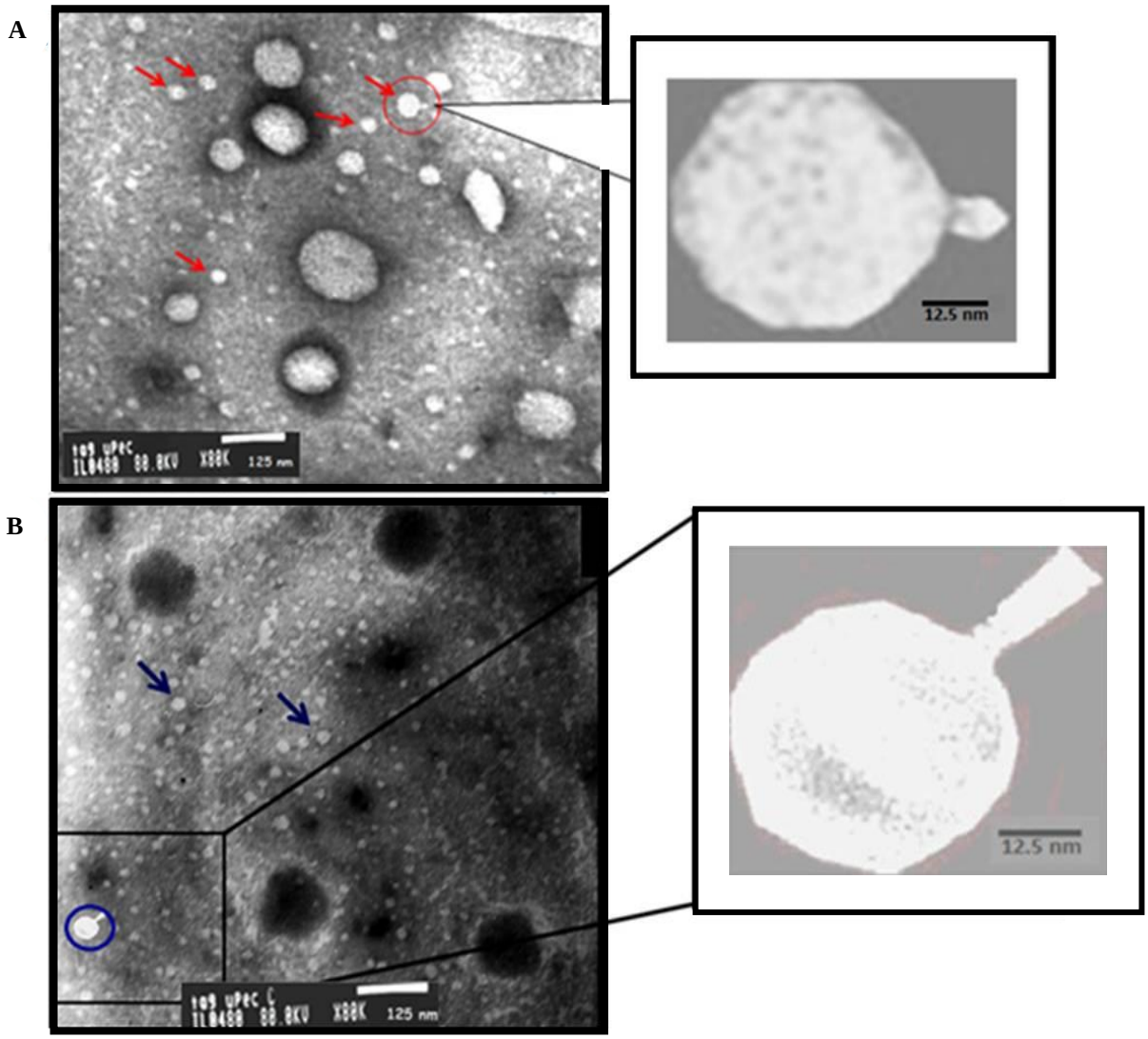

Figure 2. TEM image with negative staining by 2% *uranyl acetate* in magnification of 80000×: A. FU1 phage, B. FU3 phage

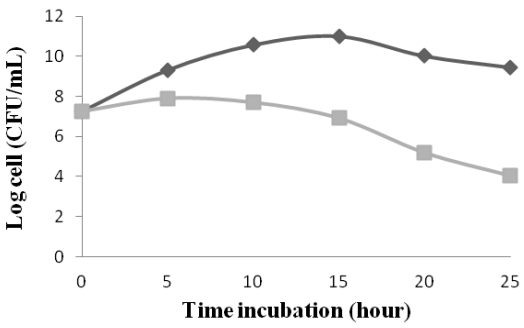

Figure 4. Growth pattern of UPEC; control (■), treatment by FU3 infection (■)

Discussion

Uropathogenic *E. coli* (UPEC) is the predominant antibiotic resistant-bacteria infected urinary tract (Kirecci et al. 2015). *Escherichia coli* belongs to the Enterobacteriaceae group which is known as one of human microbiome. In this study, the bacterial isolate has been reconfirmed and showed very identical with *E. coli* such as Gram-negative, lactose and mixed acid fermentor bacteria and it is able to produce tryptophanase (TnaA) to catalyze indole group of tryptophan. *E. coli* has these characteristics which differentiate it from other Gram-negative bacteria (Welch 2006). This bacterium could produce hemolysin toxin as its virulent factor which makes pathogenic *E. coli* different to the unpathogenic one. Haemolysin *in vivo* could take iron from red blood cell, damage phagocyte and it was a toxin for host tissue (Vandenesch et al. 2012). Therefore, UPEC population become one of the most dangerous bacteria that must be controlled.

Interestingly, from antimicrobial resistance test in vitro gives information that this UPEC has been resistant to five antibiotics in a different mechanism of action (ampicillin, chloramphenicol, ciprofloxacin, clindamycin,

cotrimoxazole). These antibiotics are commonly applied to UTI patients (Jancel and Dudas 2002). Based on the data from all WHO regions, *E. coli* that cause urinary tract infections have a very high rate of resistance (WHO 2014). Consequently, an alternative agent is very needed for controlling multidrug-resistant UPEC, for example, is by utilizing lytic phage.

Lytic phage specific to UPEC could be isolated by adding water sample from Cisadane river to the broth media contained UPEC host. This method aimed to facilitate the lytic phage contained in the water sample to reproduce inside its compatible host (UPEC) then it increased the quantity of new mature lytic phages. The presence of lytic phages was determined by the formation of a clear zone (plaque) in double layer agar media. The plaque size was in accordance to replication time of the phage and it affected to the phage quantity (Gallet et al. 2011). The lytic bacteria cell was caused by the destruction of β-1.4-glycosidic bond between *N*-acetyl glucosamine and *N*-acetyl muramic acid of peptidoglycan which was supported by the holin-lysine protein produced by lytic phage (Daniel et al. 2007). Lysed bacteria cells caused bacteria death so that lytic phage can be used as an alternative agent for killing UPEC population. High UPEC phage concentration in Cisadane river may indicate this river has been contaminated by UPEC so it will risk using this water directly before any sterilizing process.

Phage diversity based on the morphological characteristic can be observed using TEM with negative staining to give a contrast image between sample and dark background (Nugroho et al. 2016). Morphological characteristics can be used to classify a phage into a family based on the group members' similarities. Actually, tailed phages vary in size of tail length and head diameter, namely 10-800 nm for tail length and 30-160 nm for head diameter (Ackermann 2009). The tail length of FU1 and FU3 which was less than 40 nm becomes one of the parameters for grouping the phage into family Podoviridae (Jurczak-Kurek et al. 2016).

The unique information, although both FU1 and FU3 were indicated in the same family, they showed different based on the characteristic of the protein molecule weight. The variation of protein molecular weight of FU1 (10-76 kDa) and FU3 (8-120 kDa). These different ranges were still similar with protein molecular weight of T7-like phage protein which composed of at least 9 proteins from 13-150 kDa with a major capsid protein at 38 kDa (ICTV 2015).

In addition, our study chose FU3 phage to represent test about its capability to reduce UPEC population in vitro. FU3 phage was chosen because it showed bigger plaque size formation than FU1 phage at the same duration of incubation, moreover, their phage concentrations were not too different. Phage FU3 had given a great contribution in decreasing UPEC population since the first five hours' incubation (96.16%) but the line graph still showed a slight increased. This was caused by some bacteria that had not been infected by lytic phage still able to grow normally. They were presumed to have low latent time and slow introduction rate of FU3 phage to host receptors, so that the infection process was delayed. Another assumption is the host population was less dense so that the chance of phage receptors to stick to the host receptors become smaller.

The ability of lytic phage to infect and lyse a host cell were greatly varied (28.3-63.0 minutes) (Wang 2006). Physical and chemical factors such as temperature, pH, salinity, and ions also affected the effectiveness of phage infection (Jonczyck et al. 2011). However, the effectiveness of lytic phage (FU3) in 25 h incubation was able to decrease UPEC population become 10^4 CFU/mL (Figure 4). This value has been lower for tolerance limits of bacteria present in healthy urine because one of the characteristics of urine from patient which were indicated as UTI, namely the bacterial concentration in urine was clinically enumerated $\geq$ 10^5 CFU/mL and 10 leukocytes/mm^3 (Pappas 1991). The in vitro FU3 effectivity may give more yield promising result in further research to be developed become an alternative agent in controlling UPEC population in urinary tract or in other UPEC-contaminated environments such as in water river.

This manuscript becomes the first information about the UPEC lytic phage diversity in Indonesia, especially from Cisadane River. Two phages (FU1 and FU3) chosen from all plaque formations had different figure observed by TEM and also showed different morphology size and protein molecule weight. Interestingly, both of these lytic phages were still belonging to the same family and having capability to lyse the same host, namely UPEC. The abundance and densities of bacteriophages proposed as bacterial host indicators in the river waters (Lucena et al. 2003).

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