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Characterization of EPS7-like Enterobacteria phage Isolated from Indonesia

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Bacteriophages are the most abundant entities in earth. The order *Caudovirales* is the largest and most widespread group among bacterial viruses. The purpose of this study was to characterize bacteriophages from Indonesian waters. During this experiment, we collected sample from Kuningan (West Java) and Buleleng (Bali), Indonesia. We used an overlay agar method with three strains of *E. coli* as a host (NBRC 13965, NBRC 12713 and NBRC 13168) combined with digestion profiling using three restriction enzymes (PvuII, EcoRV and HincII) and transmission electron microscope (TEM) to characterize the morphology of the phage from Indonesia. Our results showed that phage LIPI13-Bp006 is in a group of *Caudovirales* and highly similar to Enterobacteria phage EPS7.

Key words: bacteriophage, Bali, Enterobacteria phage EPS7, environment, *E. coli*

Bacteriophages merupakan organisme yang keberadaannya paling melimpah di muka bumi. Orde *Caudovirales* merupakan orde yang paling besar dan paling luas diantara orde *bacteriophages* yang lainnya. Penelitian ini bertujuan untuk karakterisasi *faga* dari perairan Indonesia. Pada penelitian ini, koleksi sampel dilakukan di Kuningan, Jawa Barat dan Buleleng, Bali. Karakterisasi *bacteriophages* pada penelitian ini dilakukan dengan menggunakan: metoda *overlay agar* menggunakan tiga strain *E. coli* sebagai host (NBRC 13965, NBRC 12713 and NBRC 13168) yang dikombinasikan dengan *digestion profiling* menggunakan tiga enzim restriksi (PvuII, EcoRV and HincII) dan *transmission electron microscope* (TEM). Hasil penelitian menunjukkan bahwa phage LIPI13-Bp006 termasuk dalam grup *Caudovirales* dan memiliki kemiripan yang tinggi dengan *Enterobacteria phage EPS7*.

Kata kunci: *bacteriophages*, Bali, *Enterobacteria phage EPS7, E. coli*

Bacteriophages are viruses that infect bacteria and the most abundant life forms on earth. The first bacteriophages attack documented by Frederick Twort (1915) and Felix d'Herelle (1917). An estimated viral population of bacteriophages is approximately 10 million per cubic centimeter of any environmental niche (Mc. Auliffe *et al*. 2007). According to the International Committee on Taxonomy of Viruses (ICTV), there are 17 families of phages, where their hosts are archaea and bacteria. The classification of bacteriophage is based on the morphology and the nucleic acids: from an enveloped head to a non-enveloped head, from a linear to a circular nucleic acid (Carstens 2012).

Research on bacteriophages developed from the study of DNA and the discovery of messenger RNA, until the basic molecular interactions and genetic regulation. Bacteriophage is valuable in the modern biotechnology industry, as diagnostic tools, vehicles for vaccines, for the detection of pathogenic bacterial strain, for genetic screening vectors and for therapeutics application. Phages can also be used as bio control agents in agriculture and petroleum industry (Clark and March 2006, Mc. Auliffe *et al*. 2007, Ul Haq *et al*. 2012).

The current state of bacteriophage genomics showed that the genetic diversity of the population is very high, actively evolving with active engagement of horizontal genetic exchange, and that their genomes are consequently pervasively mosaic in their architectures. Early indications from genomic sequencing and metagenomic analysis indicate that natural phage communities are reservoirs of the uncharacterized genetic diversity on Earth (Canchaya *et al*. 2007).

Indonesia is well known as mega biodiversity country, included the diversity of microorganism. However, information on the diversity of bacteriophages in Indonesia is limited to research on a lytic Pradovirus-like Ralstonia phage (Addy *et al*. 2018), a bacillus-phage (Handoko *et al.* 2019), and the bacteriophage family of Myoviridae (Sujonoputri *et al*. 2020, Wardani *et al*. 2020). Therefore, the purpose of

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this research is to characterize bacteriophages from Indonesia. This paper will elaborate detection, isolation and identification of bacteriophages from Indonesian environment, especially from Kuningan and Bali. We hope to find new type of bacteriophages isolated from Indonesia to enrich the database of Indonesian bacteriophages.

MATERIALS AND METHODS

Samples Collection. During the year 2012-2013, water samples were collected from Waduk Dharma, Kuningan (West Java) and Lovina Beach, Buleleng (Bali Island). The No. 802 broth media (Polypeptone 1%, Yeast Extract 0.2%, MgSO4-7H2O 0.1%) were added to the sample for bacteriophage enrichment. The supernatant for further isolation process were collected by centrifugation.

Bacteriophages Isolation, Host Specificity and Titer Test. The isolation methods were done using agar overlay methods, using the No. 802-broth medium, the No. 802-agar medium (No. 802 broth + 1.5 % agar) and the No. 802 agar top medium (No. 802 broth + 0.8 % agar). Three different strains of *E. coli* were used for host specificity test (*E. coli* B (NBRC 13168), *E. coli* K12 F^+ (NBRC 13965), and *E. coli* K12 (NBRC 12713). The serial dilution was used to determine the viral titer (pfu/ml). Purification of phage was performed by single plaque selection and filter centrifugation. Afterwards, the pure isolated phage was preserved in 7% DMSO and stored in -80ºC.

Phage Characterization. Restriction digestion analysis, DNA sequencing and electron microscope were done for the characterization of the phage. The morphology of phage was observed using TEM where the phage was negatively stained by uranyl acetate. For enzyme restriction analysis, the DNA phage was extracted using the Phage DNA isolation Kit (Norgen Biotek Corp. Canada). The DNA was digested using 3 restriction enzymes (*Pvu*II, *Hinc*II, and *Eco*RV). The restriction mixture was used to build a phage-cloning library. The DNA fragment was ligated in the *Hinc*II site of plasmid pUC118 and grown in *E. coli* JM109. The colony containing the insert was selected using the blue-white selection method. Colony direct PCR was performed using universal primer M13-M4 primer (5'-GTTTTCCCAGTCACGAC-3') and M13-RV primer (5'-CAGGA AACAGCTATGAC-3'). The PCR amplicon was used for DNA sequencing and the nucleic acid sequences were analyzed using the DNA Data Bank of Japan (DDBJ).

RESULTS

During the year of 2012-2013, we collected samples from West Java and Bali. Preliminary research showed that at least we have 8 candidates of bacteriophages isolated from Indonesian environments (Table 1).

Three different strains of *E. coli*: NBRC 13168, NBRC 13965 and NBRC 12713 were used as the host. Our result showed that the sample S2012-Bp003 and S2013-Bp005 formed plaque in every host, meanwhile the sample S2012-Bp002 formed plaque in only two of the host (NBRC 13168 and NBRC 13965). The viral titers were determined using serial dilutions. Table 2 showed the result of viral titer test among samples. The result showed that the titers were varies in range from 10^5 pfu/ml until 10^{10} pfu/ml. These imply that the amount of the phages among samples is quite high to be used for further characterization.

During phage purification, three different of single plaques (A, B and C) were chosen for specificity test against the three *E. coli* host (NBRC 13168, NBRC 13965 and NBRC 12713). However, no plaque observed in sample LIPI13-Bp006C and LIPI13-Bp008A, therefore in total we only have 66 sample phages. Table 3 showed the result of specificity test among three *E. coli* host (NBRC 13168, NBRC 13965 and NBRC 12713). Our specificity result (table 3) did not show the possibility of having specific *E. coli* host among phages, and only phages from Bali (LIPI13-Bp006A, LIPI13-Bp006B, LIPI13-Bp008C and LIPI13-Bp008C) that was not formed plaques while using host *E. coli* strain NBRC 13168.

However, the host specificity test (table 3) shown different profile compared to our pre-eliminary research (table 1). Table 1 showed that sample S2012-Bp003 and S2013-Bp005 formed plaque in all of the three of *E. coli* host (NBRC 13168, NBRC 13965 and NBRC 12713), meanwhile sample S2012-Bp002 formed plaque in only two of the host (NBRC 13168 and NBRC 13965). Meanwhile the specificity test (table 3) showed that sample from Kuningan (S2012-Bp002 and S2012-Bp003) formed plaque in all three *E. coli* hosts. Also, the specificity test of sample from Bali (S2013-Bp005) indicate that phage LIPI13-Bp007 formed plaque in all of the *E. coli* host, but phage LIPI13-Bp006 and phage LIPI13-Bp008 did not formed plaque in *E. coli* host NBRC 13168. This result indicate that at least we have two candidate of phages isolated from Kuningan and three candidate of phages isolated from Bali. Also, from this specificity profile

we can predict that samples from Kuningan might contain similar types of phages; meanwhile sample from Bali might have different types of phages.

Furthermore, the phage genomic DNA extracted from pure phage lysate and it was used for phage characterization. The phage genomic DNA digested with 3 restriction enzymes (*Eco*RV, *Hinc*II and *Pvu*II). The digestion profile (table 4) shown that the enzyme PvuII did not digested the DNA of the phages but LIPI13-Bp006A, LIPI13-Bp006B, and LIPI13-Bp007A. The enzyme HincII digested all of the phages, meanwhile the enzyme EcoRV digested all the phages but phage LIPI13-Bp007A and LIPI13-Bp007B. From the digestion profile, it showed that phage LIPI13-Bp007 has three different restrictions profile. Therefore, this result indicates that the phage LIPI13-Bp007 might contain three types of bacteriophages.

The enzyme HincII digested the DNA of all the sample phages; therefore it was used for further analysis (phage cloning library). The clone library was conducted using vector plasmid pUC118 and grown in *E. coli* JM109. Colonies selected from blue-white selection and used for sequence analysis. During this experiment, fifteen colonies of the phage LIPI13-Bp006 were used for sequencing. The result of sequence analysis (table 5) revealed that every colony with inserted DNA fragment of the phage LIPI13-Bp006 is highly similar with Enterobacteria phage EPS7. However, the nucleotide sequences analysis of the other seven phages did not show any similarity in the DNA databank. In our opinion, these other seven phages might be potential candidates for new bacteriophages from Indonesia. Therefore, further characterization using whole genome analysis can be used to gain more complete information among phages.

The morphology of phage LIPI13-Bp006 was observed using the electron microscope. Figure 1 confirms that phage LIPI13-Bp006 is in the group of order Caudovirales, identify with icosahedral head and long tail. This figure is in line with the result of our sequencing analysis (table 5), which shows that the phage LIPI13-Bp006 is highly similar to Enterobacteria phage EPS7, the group of order Caudovirales.

DISCUSSION

The Enterobacteria phage EPS7 first isolated from sewage sample in Korea. It is a T5-like group phage, grouped in the family of *Siphoviridae*, order of *Caudovirales* (tailed phages). The genome size of Enterobacteria phage EPS7 is 111,382 bp. It is a linier DNA phage with icosahedral head (diameter 65 nm) and non-contractile tail (185 nm). The original hosts of Enterobacteria phage EPS7 are *Escherichia coli*, *Salmonella typhimurium*, and *Salmonella enteritidis* (Hong *et al.* 2008). The Caudovirales (tailed phages) are the oldest virus and known as typical phages that are infected both Eubacteria and Archaea. The phages can be virulent (lytic) or temperate (Ackermann, 2005). Virulent (lytic) phages proceed with immediate replication after infecting the host cell. New viruses released in large numbers by lysis of the host cell after infection. Meanwhile, temperate (lysogenic) phages do not necessarily start replicating immediately. The phages may integrate their nucleic acid (genome) into the host cell until it induced to become autonomous again. Afterwards, it starts to replicate and lyse the host cell (Grabow 2001).

Phages are associated with almost all bacterial genera and grouped on the basis of a few characteristics: range, morphology, nucleic acid, strategies of infection, morphogenesis, phylogeny, serology, sensitivity to physical and chemical agents, and dependence on properties of hosts and the environment. A host is required to evaluate the existence of phages and various host strains have been used for phages detection. Most of these host strains detect groups of phages, in particular to somatic coliphages. The abundance and the distribution of phages are based on the existence of their host organisms. The host-specificity of phages can be a useful tool to classify certain bacteria. Phages may not only be specific for species of bacteria, but also for strains of bacteria, allowing typing beyond the level of species. However, no single method was established to detect phages in a specific host (Grabow 2001, Clokie *et al.* 2011).

Phages can be recovered and detected by many techniques and approaches. Major reasons for any inconsistencies are the host bacteria that are used for the detection of various groups of phages (Grabow 2001). Traditional and molecular approaches can be combined to have the overall picture of the viral community. The Epifluorescent microscopy or the flow cytometry can be used to determine the number of phages that infect all hosts. Meanwhile, the morphological diversity can be investigated using transmission electron microscopy (TEM). Appropriate hosts can be isolated specifically from the environment of interest, or a model permissive host can be used.

Table 1 Bacteriophages isolated from Kuningan and Bali

Sampling Area	Sample Number	Acc. Number	Bacteria Host *E. coli*
Kuningan, West Java	S2012-Bp002	LIPI13-Bp001	B (NBRC 13168)
		LIPI13-Bp002	K12 F^+ (NBRC 13965)
Kuningan, West Java	S2012-Bp003	LIPI13-Bp003	K12 (NBRC 12713)
		LIPI13-Bp004	B (NBRC 13168)
		LIPI13-Bp005	K12 F^+ (NBRC 13965)
Buleleng, Bali	S2013-Bp005	LIPI13-Bp006	K12 (NBRC 12713)
		LIPI13-Bp007	B (NBRC 13168)
		LIPI13-Bp008	K12 F^+ (NBRC 13965)

Table 2 Viral titer tests

No.	Acc. Number	*E. coli* host	Plaque forming unit (pfu)	
			10 µl	100 µl
1	LIPI13-Bp001	NBRC 13168	15×10^6	1×10^6
2	LIPI13-Bp002	NBRC 13965	12×10^8	38×10^8
3	LIPI13-Bp003	NBRC 12713	5×10^6	1×10^6
4	LIPI13-Bp004	NBRC 13168	4×10^6	45×10^6
5	LIPI13-Bp005	NBRC 13965	5×10^6	15×10^6
6	LIPI13-Bp006	NBRC 12713	2×10^{10}	18×10^8
7	LIPI13-Bp007	NBRC 13168	25×10^8	1×10^8
8	LIPI13-Bp008	NBRC 13965	4×10^{10}	2×10^{10}

Table 3 Host specificity test

No.	Sample number	Acc. Number	NBRC 13168			NBRC 13965			NBRC 12713		
			Plaque			Plaque			Plaque		
			A	B	C	A	B	C	A	B	C
1	S2012-Bp002	LIPI13-Bp001	√	√	√	√	√	√	√	√	√
2		LIPI13-Bp002	√	√	√	√	√	√	√	√	√
3	S2012-Bp003	LIPI13-Bp003	√	√	√	√	√	√	√	√	√
4		LIPI13-Bp004	√	√	√	√	√	√	√	√	√
5		LIPI13-Bp005	√	√	√	√	√	√	√	√	√
6	S2013-Bp005	LIPI13-Bp006	×	×	n/a	√	√	n/a	√	√	n/a
7		LIPI13-Bp007	√	√	√	√	√	√	√	√	√
8		LIPI13-Bp008	n/a	×	×	n/a	√	√	n/a	√	√

Note: (n/a) no samples, (√) plaques formed, (x) no plaques formed

Table 4 Digestion profile

No.	Acc. Number	*Pvu*II			*Hind*II			*Eco*RV		
		Plaque			Plaque			Plaque		
		A	B	C	A	B	C	A	B	C
1	LIPI13-Bp001	×	×	×	√	√	√	√	√	√
2	LIPI13-Bp002	×	×	×	√	√	√	√	√	√
3	LIPI13-Bp003	×	×	×	√	√	√	√	√	√
4	LIPI13-Bp004	×	×	×	√	√	√	√	√	√
5	LIPI13-Bp005	×	×	×	√	√	√	√	√	√
6	LIPI13-Bp006	√	√	n/a	√	√	n/a	√	√	n/a
7	LIPI13-Bp007	√	×	×	√	√	√	×	×	√
8	LIPI13-Bp008	n/a	×	×	n/a	√	√	n/a	√	√

Note: (n/a) no samples, (√) digested, (x) not digested

Table 5 Nucleotides BLAST analysis of phage LIPI13-Bp006 clone library

No	Source	Sequences ID	Number of Nucleotides	Blast analysis	Percentages
1	Bali	> JSAT13-2-Bp006A_01	114 letters	CP000917.1 Enterobacteria phage EPS7	99%
2	Bali	> JSAT13-2-Bp006A_02	634 letters	CP000917.1 Enterobacteria phage EPS7,	93%
3	Bali	> JSAT13-2-Bp006A_03	769 letters	CP000917.1 Enterobacteria phage EPS7	96%
4	Bali	> JSAT13-2-Bp006A_04	114 letters	CP000917.1 Enterobacteria phage EPS7	99%
5	Bali	> JSAT13-2-Bp006A_05	791 letters	CP000917.1 Enterobacteria phage EPS7	95%
6	Bali	> JSAT13-2-Bp006A_07	775 letters	CP000917.1 Enterobacteria phage EPS7	95%
7	Bali	> JSAT13-2-Bp006A_08	114 letters	CP000917.1 Enterobacteria phage EPS7	98%
8	Bali	> JSAT13-2-Bp006A_09	285 letters	CP000917.1 Enterobacteria phage EPS7	100%
9	Bali	> JSAT13-2-Bp006A_12	114 letters	CP000917.1 Enterobacteria phage EPS7	99%
10	Bali	> JSAT13-2-Bp006A_15	264 letters	CP000917.1 Enterobacteria phage EPS7	96%
11	Bali	> JSAT13-2-Bp006B_09	105 letters	CP000917.1 Enterobacteria phage EPS7	99%
12	Bali	> JSAT13-2-Bp006B_10	264 letters	CP000917.1 Enterobacteria phage EPS7	96%
13	Bali	> JSAT13-2-Bp006B_13	428 letters	CP000917.1 Enterobacteria phage EPS7	95%
14	Bali	> JSAT13-2-Bp006B_15	114 letters	CP000917.1 Enterobacteria phage EPS7	99%
15	Bali	> JSAT13-2-Bp006B_16	264 letters	CP000917.1 Enterobacteria phage EPS7	96%

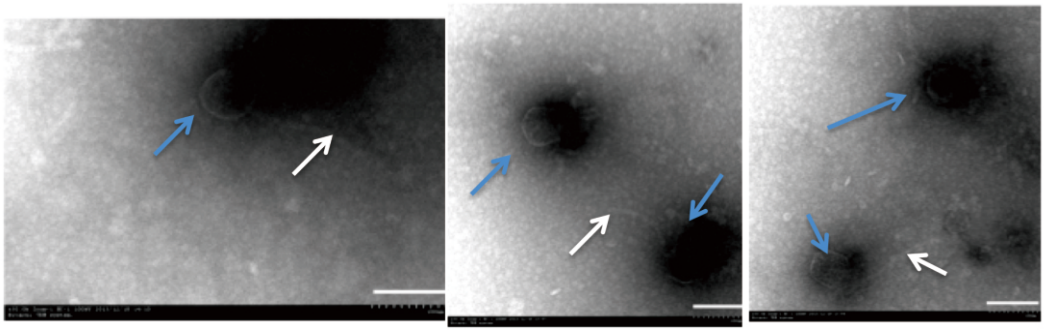

Fig 1 The morphology of Enterobacteria Phage EPS7 from Bali, Indonesia (bar: 100nm, blue arrow: head, white arrow: tail).

Furthermore, no universal molecular marker can be used to determine the phages, because no gene is suitably conserved within all phages. But restriction fragment length polymorphisms (RFLP) can also be used to assess the bacteriophage diversity (Clokie *et al*. 2011).

Studies of phages in environments have been reported from most parts of the world. During this research we characterize bacteriophages isolated from Indonesian environment using three *E. coli* model host (NBRC 13168, NBRC 13965 and NBRC 12713) combined with digestion profiling using three restriction enzymes *(Eco*RV, *Hinc*II and *Pvu*II) and transmission electron microscope (TEM). However, these approaches only identify phages that infect the specific *E. coli* strains that were being used as a host and did not show the actual proportion of the phage.

Bacteriophage is recently used in modern biotechnology as well as for the detection of pathogenic bacterial strain. A phage can be used individually to treat a bacterial infection by lysing the bacterial cell as it is having the lytic potential (Ul haq *et al*. 2012). Some phages have a global distribution while others may be endemic to particular environments. Phages and Bacteria (and Archaea) are probably co-existed and evolved together. Therefore it makes sense that the symbiotic relationship between bacteria and phage is advantageous because it may boost the bacterial ability to survive by encoding toxins and other useful genes (Clokie *et al.* 2011).

During the experiment, we identify the phage LIPI13-Bp006 isolated from Bali as Enterobacteria phage EPS7. Eight candidates of bacteriophage were isolated from Indonesian Environment, where five candidates isolated from Kuningan, West Java and three candidates isolated from Buleleng, Bali. The bacteriophage candidate isolated from Bali (LIPI13-Bp006) is highly similar to Enterobacteria phage EPS7. However, the other phages have no similarity with any phages in the databank. These unidentified phages are potential candidates as new bacteriophages isolated from Indonesian environment.

Little information is known about bacteriophages from Indonesia. Studies on the Indonesian bacteriophages were focusing on a lytic Pradovirus-like Ralstonia phage (Addy *et al*. 2018), a bacillus-phage (Handoko *et al.* 2019), and the bacteriophage family of Myoviridae (Sujonoputri *et al*. 2020, Wardani *et al*. 2020). the Pradovirus-like Ralstonia phage was isolated from soil, infecting *Ralstonia solanacearum*, an aerobic non spore Gram-negative bacterium (Addy *et al* 2018). The Bacillus-phage was isolated from soil samples, infecting *Bacillus* sp., a Gram-positive bacteria (Handoko *et al*. 2019). While. While the Myoviridae family were isolated from food and soil samples (Sujonoputri *et al* 2020), as well as liquid waste, cow and chicken intestines, chicken skin (Wardani *et al*. 2020). The present study isolated Enterobacteria phage EPS7 and unknown bacteriophages from water samples, infecting Escherichia coli, a Gram-negative bacteria. Therefore, we think that the information in this paper is valuable to support the research of bacteriophages in Indonesia.

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