BIODIVERSITAS
Volume 22, Number 12, December 2021
Pages: 5550-5554

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d221239

Short Communication: The potency of lytic bacteriophage isolated from various environments to control the growth of *Citrobacter braakii* causing urinary tract infection

SHEILAHRUSI, JEPRI AGUNG PRIYANTO, HENI RISMIYATI, IMAN RUSMANA, SRI BUDIARTI♥
Division of Microbiology, Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor. Jl. Agatis, Kampus IPB Dramaga, Bogor 16680, West Java, Indonesia. Tel./fax.: +62-251-8622833, ♥email: s_budiarti@yahoo.com

Manuscript received: 11 October 2021. Revision accepted: 26 November 2021.

Abstract. *Sheilahrusi, Priyanto JA, Rismiyati H, Rusmana I, Budiarti S. 2021. Short Communication: The potency of lytic bacteriophage isolated from various environments to control the growth of* Citrobacter braakii *causing urinary tract infection. Biodiversitas 22: 5550-5554. Citrobacter braakii* is one of the pathogenic bacteria causing urinary tract infection (UTI) in humans. Bacteriophages that are specifically infecting *C. braakii* could be the alternatives to combat antibiotics resistance cases of this bacterium. This study aimed to isolate lytic phages from various environmental samples (tofu factory wastewater, sewage water, rice field water, fishpond water, cattle farm wastewater, and goat farm wastewater), and to analyze their effectivity to reduce UTI-causing *C. braakii* population *in vitro*. Results exhibited that phages targeting this bacterium were found in goat and cattle farm wastewater and not present in other samples. Two phages, namely FC1 and FC2, had different plaque morphology characteristics. The number of phages in cattle farm wastewater was 2.8×10^5 PFU/mL and 1.32×10^4 PFU/mL in goat farm wastewater. It was observed that the phages found in these environments also indicate the presence of their host. After 8 h incubation, FC1 and FC2 phage reduced UTI-causing *C. braaakii* population *in vitro* from 0.23×10^8 CFU/mL to 0.03×10^8 CFU/mL and 0.02×10^8 CFU/mL, respectively. The phages isolated from these two samples can be further developed for the treatment of UTI caused by *C. braakii* and goat and cattle farm wastewater treatment to prevent contaminating other areas.

Keywords: Cattle, goat, lytic phage, UTI, wastewater

INTRODUCTION

Citrobacter braakii Brenner et al. 1993 is an aerobic Gram-negative bacilli that belongs to the Enterobacteriaceae family, and widely distributed in soil, water, food, the urinary and intestinal tract of humans and animals (Sami et al. 2017). This bacterium has been associated with nosocomial infection and one of the common species causing urinary tract infections (UTI) in women and men (Trivedi et al. 2016). However, UTI more frequently occurs in adult women, with a lifetime prevalence of 50-60% (Medina and Castillo-Pino 2019). Furthermore, this infection can be fatal and higher rate in hospitalized patients (Setyorini et al. 2019). *Citrobacter* strains are the third most common bacteria causing UTI in hospitalized patients besides *Escherichia coli* and *Klebsiella* strains up to 9.4% of all bacterial isolates obtained (Metri et al. 2013). A previous study through a culture-based approach also revealed that *C. braakii* has been found in urine from UTI-diagnosed children (Christine et al. 2018). Therefore, it is an emergence to find an effective treatment strategy to control the infection cases caused by this bacterium.

Currently, antibiotics-based treatment is commonly used as oral therapy to control *C. braakii* growth. But, unfortunately, this bacterium has been reported to be not sensitive to some antibiotics. Mostly *Citrobacter* spp. isolated from patients diagnosed with UTI were resistance to ampicillin, co-trimoxazole, norfloxaxin, ciprofloxaxin, gentamicin, netilmicin, amoxiclav, amikacin, cephalexin, cefatoxime, ofloxacin, nitrofurantoin, imipenem, and tobramicin (Metri et al. 2013). The resistance of this bacteria to commonly existing antibiotics is alarming us to develop a new treatment strategy to control this infection. One of the alternative treatments for treating bacterial infections is applying the bacteriophage. Conventionally, bacteriophage-based therapy refers to the use of naturally occurring phages (short call for bacteriophage) to infect and lyse specific bacteria in the infection site. However, biotechnological approaches have further developed new strategies using recombinant phage and purified phage lytic protein for specifically counter to multidrug-resistant bacterial growth (Lin et al. 2017). Therefore, it is clinically important to find the phage from natural sources that could specifically combat *C. braakii* for further development of non-antibiotics-based therapy.

The exploration of phages sources could be started from the existence of their host in the environment or in the pathogen-contaminated samples, since phages and their host coexist in the same environment. For example, lytic phages against pathogenic *E. coli* are found in hospital wastewater (Lingga et al. 2020), and *Bacillus pumilus*-targeting phages found in Ciapus River-Bogor, Indonesia (Kusmiatun et al. 2015). Nevertheless, the exploration of phages that specifically combats UTI-causing *C. braakii* was not studied yet, especially in Indonesia. Therefore, this study

investigated the existence of *C. braakii*-infecting phages from various water samples, such as tofu factory wastewater, pool water, sewage water, rice field water, fishpond water, cattle farm wastewater, and goat farm wastewater. This study aimed to isolate lytic phages from various environmental samples and to analyze their effectiveness in reducing UTI-causing *C. braakii* population in vitro.

MATERIALS AND METHODS

Bacterial isolate source and sample collection

Citrobacter braakii used in this study was obtained from the urine of UTI-diagnosed children, which has been isolated from our earlier study (Christine et al. 2018). Bacterial isolate was routinely culture in Luria Bertani agar (LA) medium. Various wastewater samples were collected from tofu factory wastewater, sewage water, rice field water (Pesanggrahan, South Jakarta, Indonesia), and from fishpond water (Dramaga, Bogor, West Java, Indonesia). In addition, cattle farm wastewater and goat farm wastewater were collected from cattle farm and goat farm, The Faculty of Animal Science, IPB University, Dramaga-Bogor, Indonesia. All samples were collected in a sterile falcone.

Sample preparation and lytic phage isolation

The phages were isolated from 4.5 mL water sample. This sample was mixed with 0.5 mL Luria Bertani broth medium and 0.5 mL *C. braakii* suspension (1×10^8 CFU/mL). The suspension was then incubated at 37°C for 24 h. Then, suspension was centrifuged at 5000 rpm for 20 minutes. The supernatant was collected using a sterilized syringe and filtered by 0.22 µm millipore filter. The phages were then isolated using double layer agar method. Then, 100 µl of filtrate was mixed with 100 µl of *C. braakii* (1×10^8 CFU/mL)-containing LB medium and incubated at room temperature (±28°C) for 30 minutes. The suspension was transferred into a new tube containing melted semisolid LA medium and homogenized using vortex. The homogenous suspension was poured into LA medium-containing petri dish and incubated at 37°C for 24 h. The plaque formation indicates the presence of phages in the sample.

Phage purification and quantification

Plaque obtained from the previous step was then collected using Pasteur pipette and transferred into Eppendorf containing 1.5 mL phosphate buffer saline solution (PBS). This suspension was then homogenized using vortex and left for 5-10 minutes at room temperature (±28°C). The suspension was then centrifuged at 5000 rpm for 20 minutes. The supernatant was filtered into sterilized tubes using 0.22 µm millipore filter, and stored at 4°C as phage stock suspension.

Phage quantification was carried out by serially diluted phage stock solution from 10^{-1} to 10^{-8} in phosphate buffer saline solution (PBS) with pH 7.4. A total of 100 µl of phage suspension from each dilution was mixed with 100 µl of *C. braakii*-containing melted semisolid LA medium (1% agar) and poured onto LA medium petri dishes. The inoculated plates were incubated at 37°C for 24 h. The plaque formed on the plate between 30-300 plaques were counted as plaque forming units per milliliter (PFU/mL). Then, the titer of plaque was determined using the following formula:

$$\text{Titer Phage (PFU/ml)} = \frac{\text{number of plaques per petri dish}}{\text{dilution factor} \times \text{volume phage suspension added (ml)}}$$

Bacteriolytic assay

Each phage isolate was assessed to their capability in lysing *C. braakii in vitro* as per Budiarti et al. (2011). 0.5 mL of *C. braakii* culture (2.3×10^7 CFU/mL) was inoculated with 0.5 mL of each phage suspension, including FC1 (1.3×10^4 PFU/mL), and FC2 (2.8×10^5 PFU/mL). *Citrobacter braakii* culture without phage suspension was served as a control. Each suspension was then inoculated into 50 mL LB medium and plated into LA medium at various incubation periods, i.e. 0, 2, 4, 6, and 8 h. The number of bacterial colonies was determined by the total plate count method.

RESULTS AND DISCUSSION

The presence of lytic phage in various water samples

The presence of lytic phages in the sample used in this study was indicated by the plaque formation in the upper layer of *C. braakii* inoculated medium. Of six water samples tested, 2 samples exhibited plaque formation, i.e from goat farm wastewater, and cattle farm wastewater (Figure 1). Plaques found in these samples have different morphological characteristics and diameters (Table 1). Plaque morphology found in goat farm wastewater was clear and irregular-shaped with diameters ranging from 1-3 mm, and coded as FC1, while plaque morphology in cattle farm wastewater was clear and regular-shaped with the diameter ranging from 2-4 mm, and coded as FC2.

Table 1. The presence of lytic plaque in various samples and its characteristics

Sources	Phage presence*	Plaque characteristics	
		Morphology	Diameter (mm)
Goat farm wastewater	+	clear, irregular-shaped	1 - 3
Cattle farm wastewater	+	clear, regular-shaped	2 - 4
Fishpond water	-	-	-
Sewage water	-	-	-
Rice field water	-	-	-
Tofu factory wastewater	-	-	-

*Note: +: present; - : absent

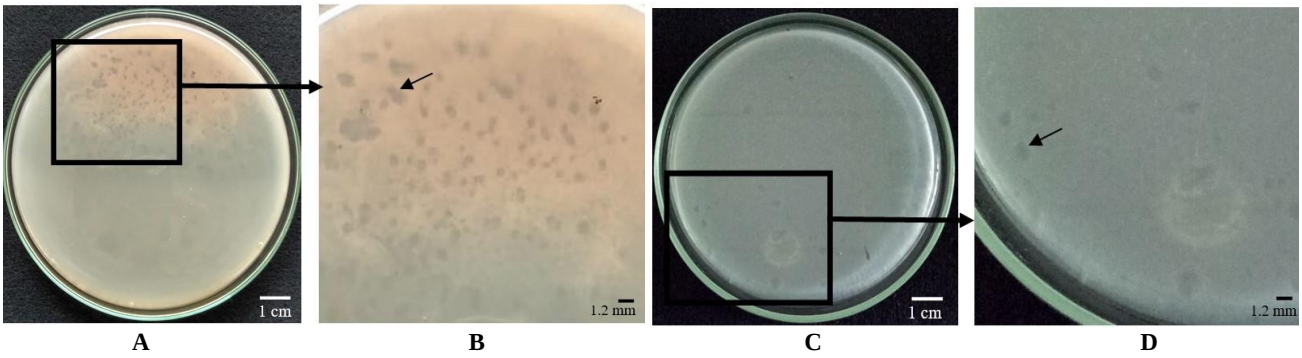

Figure 1. Plaque morphology in Luria Bertani Double Layer Agar isolated from: A. goat farm wastewater, B. zoom of A plate, C. cattle farm wastewater, D. zoom of C plate. Black arrow shows plaque morphology

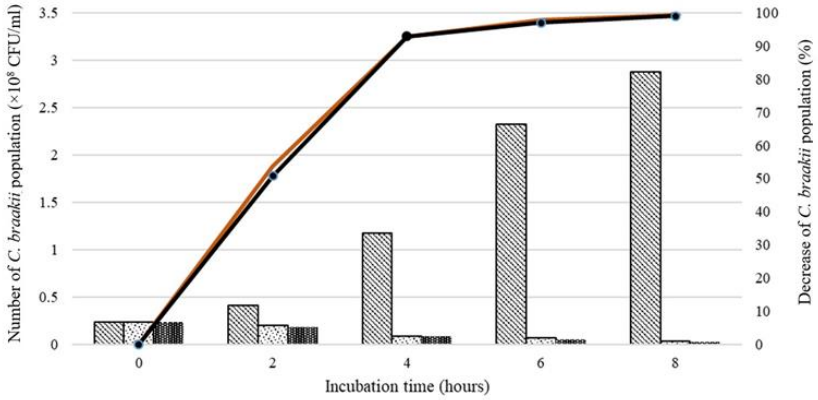

Figure 2. *Citrobacter braakii* population after inoculated with purified FC1 ( ) and FC2 ( ) phages compared to control ( ) and decreased population after treated with lytic phages FC1 ( ) and FC2 ( ) after 8 hours

The number of lytic-phage present in goat and cattle farm wastewater

Results showed that the number of phages present in cattle farm wastewater was higher than goat wastewater. The plaques number in cattle farm wastewater and goat wastewater were 2.8×10^5 PFU/mL and 1.32×10^4 PFU/mL, respectively.

Bacteriolytic activities of isolated phages against *Citrobacter braakii*

Phage isolated from goat farm wastewater (FC1) and cattle farm wastewater (FC2) decreased the number of *C. braakii* population *in vitro*. *C. braakii* population infected with phage FC1 and FC2 gradually decreased following the incubation time. After 8 h infected with FC1 and FC2, *C. brakii* population (0.23×10^8 CFU/mL) decreased to 0.03×10^8 CFU/mL and 0.02×10^8 CFU/mL. In contrast, *C. braakii* population in control treatment was gradually increased from 2.3×10^7 CFU/mL at 0 h incubation time to 2.88×10^8 CFU/mL after 8 h incubation. These two lytic phages effectively reduced *C. braakii* population up to 99% for FC1 and 99.3% for FC2 (Figure 2).

Discussion

Lytic phages infecting UTI-causing *C. braakii*, named as FC1 and FC2, have been isolated from two different wastewater sources, including from goat farm wastewater and cattle farm wastewater, respectively. The presence of these phages in the water samples is indicated by the clear plaque formation. The plaque formed because of the ability of phage to infect and lysis *C. braakii* cells specifically. Due to the specific phages coexisting with their specific host, these two environments also might be colonized by UTI-causing *C. braakii*. In contrast, no phage was found in other samples, such as tofu factory wastewater, pool water, sewage water, rice field water, indicating that UTI-causing *C. braakii* was not exist in these environments. In the earlier studies, phages infecting pathogenic bacteria were commonly found in clinical samples where their host was suspected to live in the same environment, such as hospital wastewater (Lingga et al. 2020), and the human body (Brown et al. 2016). Nevertheless, pathogenic bacteria-infecting phages also have been found in non-clinical samples, i.e, wastewater treatment plant (Khairnar et al. 2014), fishpond water (Jin et al. 2012), sewage (Jia et al. 2020), and river (Bhetwal et al. 2017). This is the first report that phages targeting UTI-causing *C. braakii* were

found in goat and cattle farm wastewater, as well as suggesting that *C. braakii* also exist in these environments. Therefore, good management of wastewater from these environments is necessary to prevent the spread of this pathogenic bacterium to other environments.

The number of phages present in cattle farm wastewater was higher than goat wastewater with plaques number of 2.8×10^5 PFU/mL and 1.32×10^4 PFU/mL, respectively. These phage numbers may be correlated with the number of hosts in wastewater sources. The results also indicated that these environments were favorable for the growth of phages and their hosts. As these areas were contaminated by cattle and goat fesses, and provide high nutrition from animal feed residues. *C. braakii* may originate from cattle and goat. As reported by previous studies, C*itrobacter* sp. asymptomatically associated with intestinal tract of ruminants (Seshadri et al. 2018) and also found as microflora in raw cow milk (Pukancikova et al. 2016).

FC1 and FC2 phage exhibited bacteriolytic activity and decreased UTI-causing *C. braakii* population *in vitro* up to 99% and 99.3%, respectively, after 24 h incubation. *C. brakii* culture infected with FC1 and FC2 phage contained 0.03×10^8 CFU/mL and 0.02×10^8 CFU/mL, significantly decreased from the early bacterial cell density (2.88×10^8 CFU/mL). Supporting these results, FU3 phage from Cisadane river also was able to reduce (10^4 CFU/mL) uropathogenic-*Escherichia coli.* (UPEC) after 25 h incubation (Sufa et al. 2018). The decrease of the host population is probably due to phage lytic enzyme production and activity. This enzyme was commonly known as lysin, an antimicrobial molecule produced by phage, which can degrade peptidoglycan of bacterial host and initiate cell lysis (Miguel et al. 2020). Supporting the present study, several earlier studies also found that phages originated from various animal farm wastes potentially have bacteriolytic activities against specific bacteria. Phage from goat farm waste was able to reduce shiga-toxin producing *E. coli* population (Lennon et al. 2020). Phage isolated from wastewater collection tanks of various animal species also showed bacteriolytic activities against most common bacteria, including *E. coli* and *Bacillus subtilis* (Shukla and Hirpurkar 2011). Phage originated from fecal cattle sample was able to infect *E. coli* O157:H7 (Hallewell et al. 2014). In addition, phage isolated from feedlot cattle also reduced *E. coli* O157:H7 population in cattle digestive tracts (Callaway et al. 2008). Fortunately, these lytic phages FC1 and FC2 specific to *C. braakii* may provide an alternative to antibiotics for the treatment of UTI caused by this bacterium. This phage can also be a biocontrol agent in goat and cattle farm wastewater treatment before releasing to other areas.

ACKNOWLEDGEMENTS

All authors thanks to Division of Microbiology, Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, Bogor, Indonesia for facilitating this research.

REFERENCES

Bhetwal A, Maharjan A, Shakya S, Satyal D, Ghimire S, Khanal PR, Parajuli NP. 2017. Isolation of potential phages against multidrug-resistant bacterial isolates: promising agents in the rivers of Kathmandu, Nepal. Biomed Res Intl 2017: 1-10. DOI: 10.1155/2017/3723254.

Brown TL, Petrovski S, Dyson ZA, Seviour R, Tucci J. 2016. The formulation of bacteriophage in a semi solid preparation for control of *Propionibacterium acnes* growth. Plos One 11 (13): e0151184. DOI: 10.1371/journal.pone.0151184.

Budiarti S, Pratiwi RH, Rusmana I. 2011. Infectivity of lytic phage to enteropathogenic *Escherichia coli* from diarrheal patients in Indonesia. J US-China Med Sci 8: 273-282.

Callaway TR, Edrington TS, Brabban AD, Anderson RC, Rossman ML, Engler MJ, Carr MA, Genovese KJ, Keen JE, Looper ML, Kutter EM, Nisbet DJ. 2008. Bacteriophage isolated from feedlot cattle can reduce *Escherichia coli* o157:h7 populations in ruminant gastrointestinal tracts. Foodborne Pathog Dis 5 (2): 183-191. DOI: 10.1089/fpd.2007.0057

Christine G, Budiarti S, Astuti RI. 2018. Diversity of urinary tract infection bacteria in children in Indonesia based on metagenomic approach. Biodiversitas 19: 1375-1381. DOI: 10.13057/biodiv/d190425

Hallewell J, Niu YD, Munns K, McAllister TA, Johnson RP, Ackermann HW, Thomas JE, Stanford K. 2014. Differing populations of endemic bacteriophages in cattle shedding high and low numbers of *Escherichia coli* O157:H7 bacteria in feces. Appl Environ Microbiol 80 (13): 3819-3825. DOI: 10.1128/AEM.00708-14.

Jia K, Yang N, Zhang X, Cai R, Zhang Y, Tian J, Raza SHA, Kang Y, Qian A, Li Y, Sun W, Shen J, Yao J, Shan X, Zhang L, Wang G. 2020. Genomic, morphological and functional characterization of virulent bacteriophage IME-JL8 targeting *Citrobacter freundii.* Front Microbiol 11: 1-12. DOI: 10.3389/fmicb.2020.585261.

Jin J, Li ZJ, Wang SW, Huang DH, Li YH, M YY, Wang J, Liu F, Chen XD, Li GX, Wang XT, Wang ZQ, Zhao GQ. 2012. Isolation and characterization of ZZ1, a novel lytic phage that infects *Acinetobacter baumannii* clinical isolates. BMC Microbiol 12 (156): 1-8. DOI: 10.1186/1471-2180-12-156.

Khairnar K, Pal P, Chandekar RH, Paunikar WN. 2014. Isolation and characterization of bacteriophages infecting nocardioforms in wastewater treatment plant. Biotechnol Res Intl 2014: 1-5. DOI: 10.1155/2014/151952.

Kusmiatun A, Rusmana I, Budiarti S. 2015. Characterization of bacteriophage specific to *Bacillus pumilus* from Ciapus River in Bogor, West Java, Indonesia. Hayati J Biosci 22: 27-33. DOI: 10.4308/hjb.22.1.27.

Lennon M, Liao YT, Slavador A, Lauzon CR, Wu VC. 2020. Bacteriophages specific to Shiga toxin-producing *Escherichia coli* exist in goat feces and associated environments on an organic produce farm in Northern California, USA. Plos One 15 (16): 1-16. DOI: 10.1371/journal.pone.0234438.

Lin DM, Koskella B, Lin HC. 2017. Phage therapy: an alternative to antibiotics in the age of multi-drug resistance. World J Gastrointest Pharmacol Theurapetics 8: 162-173. DOI: 10.4292/wjgptv8.i3.162.

Lingga R, Budiarti S, Rusmana I, Wahyudi AT. 2020. Isolation, characterization and efficacy of lytic bacteriophages against pathogenic *Escherichia coli* from hospital liquid waste. Biodiversitas 21: 3234-3241. DOI: 10.13057/biodiv/d210745.

Medina M, Castillo-Pino E. 2019. An introduction to the epidemiology and burden of urinary tract infections. Theurapeut Advin Urol 11: 3-7. DOI: 10.1177/1756287219832172.

Metri BC, Jyothi P, Peerapur BV. 2013. Antibiotic resistance in *Citrobacter* spp. isolated from urinary tract infection. Urol Annals 5: 312-313. DOI: 10.4103/0974-7796.120295.

Miguel TD, Rama JLR, Sieiro C, Sanchez S, Villa TG. 2020. Bacteriophages and lysins as possible alternatives to treat antibiotic-resistant urinary tract infections. Antibiotics 9 (8): 1-12. DOI: 10.3390/antibiotics9080466.

Pukancikova L, Lipnicanova S, Kacaniova M, Chmelova D, Ondrejovic M. 2016. Natural microflora of raw cow milk and their enzymatic spoilage potential. Nova Biotech et Chimica 15 (2): 142-155. DOI: 10.1515/nbec-2016-0015.

Sami H, Sultan A, Rizvi M, Khan F, Ahmad S, Shukla I, Khan HM. 2017. Citrobacter as a uropathogen, its prevalence and antibiotics susceptibility pattern. Virol J 4: 23-26. DOI: 10.4103/2348-3334.196037.

Seshadri R, Leahy SC, Attwood GT, The KH, Lambie SC, Cookson AL, Paez-Espino D, Hungate 1000 project collaborators, Perry R, Henderson G, Creevey CJ, Terrapon N, Lapebie P, Drula E, Lombard V, Rubin E, Kyrpides NC, Henrissat B, Woyke T, Ivanova NN, Kelly WJ. 2018. Cultivation and sequencing of rumen microbiome members from the Hungate1000 collection. Nat Biotechnol 36 (4): 359-367. DOI: 10.1038/nbt.4110.

Setyorini H, Tjempakasari A, Mardiana N. 2019. Risk factors for urinary tract infection in hospitalized patients. Biomol Health Sci J 2: 4-8. DOI: 10.20473/bhsjv2i1.11549.

Shukla S, Hirpurkar D. 2011. Recovery status of bacteriophages of different livestock farms of Veterinary College, Adhartal, Jabalpur, India. Vet World 4 (3): 117-119. DOI: 10.5455/vetworld.2011.117-119.

Sufa HI, Budarti 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 (6): 2359-2364. DOI: 10.13057/biodiv/d190646.

Trivedi MK, Branton A, Trivedi D, Nayak G, Mondal SC, Jana S. 2015. Phenotyping and 16S rDNA analysis after biofield treatment on *Citrobacter braakii*: A urinary pathogen. J Clin Med Genomics 3: 1-8. DOI: 10.4172/2472-128X.1000129.