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Isolation and characterization of lytic bacteriophages against multidrug resistant *Escherichia coli*

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Abstract

The aim of this study was to isolate and characterize *Escherichia coli*-specific lytic bacteriophages from different wastewater sources. Three bacteriophages, named JC01, JC02, and JC03, were successfully isolated from wastewater samples collected from the wastewater treatment plants of Kudprakhow community, Sanprasitthiprasong Hospital, and Khong Chiam Hospital, respectively. Host range determination revealed that all bacteriophages had only *Escherichia coli* as a host, indicating a high specific host range property. The inhibition of clinical isolates of multidrug resistant *E. coli* showed that bacteriophages JC01, JC02, and JC03 inhibited the growth of *E. coli* at the percentages of 51.7 (138/267), 52.4 (140/267), and 28.5 (76/267), respectively. All bacteriophages were able to tolerate normal saline and distilled water for up to 40 min but were not able to tolerate ethanol and hydrogen peroxide at every time point tested. Heat stability showed that bacteriophage JC01 had the highest resistance at 60°C after 60 min. Meanwhile, bacteriophages JC02 and JC03 showed resistances at 60°C after 45 min. Bacteriophage classification by genome analysis demonstrated that all the bacteriophages were double-stranded DNA viruses. Viral particle morphology observed by transmission electron microscope found that all bacteriophages had the viral particle composed of a head with a hexagonal shape and long tails with contractile. The size from head to tail of all the bacteriophages was approximately 200 nm. Therefore, based on the International Committee on Taxonomy of Viruses *(*ICTV*)* classification, bacteriophages JC01, JC02, and JC03 belonged to Family *Myoviridae*, Order *Caudovirales.* Therefore, the bacteriophages derived through this study can be further used to study their potential use in advanced research steps, such as in cell cultures and animal models.

Keywords: *bacteriophage, Escherichia coli, lytic bacteriophage, multidrug resistant bacteria, phage, phage therapy*

1. Introduction

Effective treatment of infectious diseases caused by pathogenic bacteria can still be obtained by using appropriate antibiotics. However, it has been discovered that pathogenic bacteria were able to resist any groups of antibiotics called multidrug resistant bacteria (MDR bacteria). Most reported MDR bacteria are ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) (Rice, 2008). Recently, a Gram negative bacterium *Escherichia coli* was reported as a major public health concern due to this bacterium showing resistance to any antibiotics mediated by extended-spectrum β-lactamases, resulting in serious diseases in patients (Alhashash et al., 2016; Hadifar et al., 2017). For these reasons, untreatable bacterial infectious diseases may become problematic in the near future. Alternative approaches for treatment of bacterial infectious diseases become interesting, and one of these approaches is bacteriophage therapy.

Bacteriophage or phage is the virus that has a bacterial cell as a host for its propagation. Bacteriophage has been isolated from various environments, including soil, human feces, and water sewage (Galtier et al., 2016). In general, bacteriophage is usually presented in the environment where the bacterial host is predominant (Janez & Loc-Carrillo, 2013; Weber-Dabrowska et al., 2016). The administration of bacteriophage into patients for treatment has been reported (Lin, Koskella, & Lin, 2017). The success of using bacteriophage therapy in terms of "efficacy and safety" for treatment of *E. coli* infection has been already reported in both animal models (Galtier, et

al., 2016) and clinical human trials (Sarker et al., 2012), and the use of phages as an effective alternative has been developed for medical use in both Europe and United states of America (Biswas et al., 2002).

Based on these previous researches, it is interesting to seek effective bacteriophages against particular pathogens. The bacteriophages derived from this study may be used to evaluate their efficacy in further advanced steps of research, including the elimination of MDR *E. coli* in cell cultures and animal models.

2. Objectives

The aim of this study was to isolate and characterize bacteriophages against MDR *E. coli*.

3. Materials and methods

3.1 Bacterial strains and growth conditions

Bacterial strains used in this study are shown in Table 1. The bacteria strains including *Escherichia coli* strains, *Pseudomonas aeruginosa* No.40, *Klebsiella pneumoniae* ATCC1705, *Salmonella* Typhimurium DMST5784, *Shigella dysenteriae* DMST2137, *Vibrio cholerae* DMST2873, *Enterococcus faecalis*, and *Staphylococcus aureus* ATCC29213, were grown in nutrient broth (NB) media (HiMedia Laboratories, Nashik, India) at 37°C for 24 h in aerobic conditions. *Lactobacillus casei* TISTR1341 and *Lactobacillus sakei* CECT906 were cultured in Man Rogosa Sharpe (MRS) media (LAB, Lancashire, UK). *Bifidobacterium breve* CMP4 was grown in MRS broth supplemented with 0.25% cysteine (MRSC) in anaerobic jars under anaerobic conditions generated with an oxygen absorber-CO_2 generator system (AnaeroPack-Anaerobe, Mitsubishi, Japan) and incubated at 37°C for 24-48 h. Agarified media was prepared by adding bacteriological-graded agar at 1.5% (w/v) to the corresponding media.

Table 1 Bacterial strains used in this study

Bacteria	Relevant properties	Source or reference
Escherichia coli No.40	Strain resisted to ampicillin, cefotaxime, ceftazidime, ceftriaxone, cefuroxime, ciprofloxacin, and gentamicin	Strains were kindly provided by Sanprasitthiprasong hospital
Escherichia coli EM1 to EM267	Clinical isolates and MDR strains	
Pseudomonas aeruginosa No.40	Strain resisted to amikacin, ceftazidime, ampicillin, and ciprofloxacin	
Vibrio cholerae DMST2873	Pathogenic strain	DMST
Salmonella Typhimurium DMST5784	Pathogenic strain	
Shigella dysenteriae DMST2137	Pathogenic strain	
Klebsiella pneumoniae ATCC1705	Clinical isolate	ATCC
Escherichia coli ATCC25922	Antibiotic sensitive strain, CLSI and EUCAST control for antimicrobial susceptibility testing	
Staphylococcus aureus ATCC29213	Oxacillin-sensitive strain	
Enterococcus faecalis	Clinical isolate	CMP
Bifidobacterium breve CMP4	Probiotic strain isolated from human	
Lactobacillus casei TISTR1341	Probiotic strain	TISTR
Lactobacillus sakei CECT906	Probiotic strain	CECT

Key of culture collections :TISTR Thailand Institute of Scientific and Technological Research, ATCC American Type Culture Collection, CECT Colección Española de Cultivos Tipo Spanish Type Culture Collection, CMP College of Medicine and Public Health, CLSI Clinical Laboratory Standards Institute, EUCAST European Committee on Antimicrobial Susceptibility Testing, DMST Department of Medical Sciences Thailand.

3.2 Collection of wastewater samples and isolation of *Escherichia coli*-specific bacteriophages

Three wastewater samples were collected from wastewater treatment plants in Ubon Ratchathani Province, Thailand. One wastewater sample was collected from Khong Chiam Hospital, and another two samples were collected from wastewater treatment plants located in Sanprasitthiprasong Hospital and the Kudprakhow community. Each wastewater sample was used separately for bacteriophage isolation as follows. Ten ml of wastewater sample was centrifuged at 3000 rpm for 5 min. The supernatant was collected and filtered with a 0.45 µm-membrane filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany), designated as filtrate_1. Five ml of filtrate_1 was added to 5 ml of a double strength nutrient broth supplemented with overnight culture in NB of *E. coli* No.40, an *E. coli* strain was isolated from a urethritis patient and verified as MDR strain. The mixture was

then incubated at 37°C for 24 h. After centrifuging at 3000 rpm for 5 min, the culture was filtered using a 0 a 0.22 µm-membrane (Sartorius Stedim Biotech GmbH, Goettingen, Germany) and then collected through the filter paper, and designated as filtrate_2. The filtrate_2 derived from each wastewater sample was kept in 70% glycerol and stored at 4 °C until use.

3.3 Detection of bacteriophage by spot test

A single purified colony of *E. coli* No.40 was grown in NB for 18 to 24 h. Bacterial cell suspension was adjusted so that the concentration equaled 0.5 McFarland (Biosan SIA, Riga, Latvian), which was equal to $1x10^8$ cfu/ml. One ml of adjusted bacterial cell suspension was spreaded on NA plate. 10 µl of each filtrate_2 was dropped on the center of the NA plate. The plate was further incubated at 37°C for 18 to 24 h. After incubation, the cultured plate was checked for the presence of a clear zone in the area in which filtrate_2 was dropped, indicated the presence of *E. coli*-specific bacteriophage.

3.4 Purification and quantification of bacteriophage

Individual filtrate_2 was used to prepare a ten-fold serial dilution. A 100 µl of appropriate diluted filtrate_2 was added to 3 ml of pre-warmed semisolid Brain Heart Infusion (BHI) media supplemented with 100 µl of $1x10^8$ cfu/ml *E. coli* No.40. The mixture was further incubated at 37°C for 18 to 24 h. The appearance of clear halo indicated the lytic activity of bacteriophage, hereafter called plaques, was counted and recorded. The quantity of bacteriophage in the unit of plaque-forming unit/ml (pfu/ml) was calculated as the following equation: Plaque-forming unit/ml (pfu/ml) is equal to Plaque×10×dilution.

For purification, the sterile micropipette tips-based purification method was performed. A bottom side of sterile micropipette tip was pressed into the plaque area in NA media. The agar containing bacteriophage was added into NB media and incubated at 37°C by shaking for 1 h. The mixture was centrifuged at 3000 rpm for 20 min at 4°C. The solution was filtered by the use of 0.22 µm-pore size membrane filters. The filtered solution was used in bacteriophage characterization.

3.5 Host range determination

Bacterial host range of bacteriophage was performed by spot test method as previously described with slightly modification. One ml of $1x10^8$ cfu/ml bacterial cell suspension was spreaded on NA plate. The purified bacteriophage suspension with quantity of 10^8 pfu/ml was dropped on the center of the NA plate. The plate was incubated at 37°C for 18 to 24 h and observed for the presence of a clear zone. Bacterial strains used as indicators are listed in Table 1.

3.6 Stability of bacteriophage

Tolerances to chemical solutions and heat were tested in this study. Three chemical solutions, 0.85% (w/v) normal saline, 10% (v/v) ethanol, and 1% (v/v) hydrogen peroxide, were used in this test. Distilled water was used as the control. A 100 µl of bacteriophage solution was added to 900 µl of each chemical solution. The solutions were then incubated at 37°C for 10, 20, 30, and 40 min. For the heat stability test, NB media were pre-incubated at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60°C for 15 min. A 100 µl of bacteriophage solution with quantity of 10^8 pfu/ml was added to 900 µl of each pre-warmed NB in microcentrifuge tubes. The solutions were further incubated at the same previous temperature of pre-warmed NB. After incubation, the stability of the bacteriophages was examined by spot tests as previously described.

3.7 Bacteriophage genome isolation

A 100 µl of purified bacteriophage suspension with the quantity of 10^8 pfu/ml was mixed with 10% sodium dodecyl sulfate and incubated at 65°C for 15 min. An equal volume of phenol:chloroform:isoamyl alcohol (1:1:24) was added and mixed by inversion. The upper phase of the supernatant of the mixture was collected by centrifugation at 13,000 rpm for 10 min at 4°C. The suspension was added to 300 µl of 3 M sodium acetate (pH 4.8) and mixed by inversion. An equal volume of isopropanol was added to the suspension and incubated at -20°C for 1 h. The genomic DNA was collected by centrifugation at 13,000 rpm for 10 min at 4°C. Finally, the DNA pellet was rinsed with 1 mL of 70% ethanol followed by air-drying and suspension in 50 µl of sterile distilled water or TE (pH 8.0). The genomic DNA was kept at -20°C for further analysis.

3.8 Bacteriophage genome analysis by restriction enzyme digestion

The purified phage genome was digested by restriction enzyme *Eco*RI, *Nco*l, *Pae*l, *Hind*III, RNase A, and DNase. The procedure was performed

according to the instructions of the manufacturer, Thermo Fisher Scientific, Carlsbad, USA (Dobnik et al., 2013; Nivas et al., 2015).

3.9 Determination of bacteriophage morphology

Ten µl of the purified bacteriophage suspension was transferred to a copper grid and incubated for 5 min to allow the copper plate to absorb the bacteriophages. Two percent phosphotungstic acid (pH7.0) was dropped onto the copper grid and incubated for 15 min. The dye was dropped onto the copper grid and incubated for 2 h. The electron microscope (JEOL Ltd, Tokyo, Japan) operated at 80 kV.

4. Results and discussion

Phage therapy is an alternative approach for the treatment of bacterial infections, especially, bacteria with multidrug-resistance not eliminated by antibiotics (Lin, et al., 2017). Compared to antibiotic treatment, bacteriophage has a high specificity to destroy bacteria at the strain-level, thus only the real target bacterium is affected and eliminated from the host. Different effective bacteriophages have been isolated from various environments, and it was suggested that appropriate sources for bacteriophage isolation for use in bacteriophage therapy were sewage from hospitals and communities due to the presence of many pathogenic bacteria (Janez & Loc-Carrillo, 2013; Weber-Dabrowska, et al., 2016).

In this study, three wastewater samples were collected, one each from Kudprakhow community, Sapprasitthiprasong Hospital, and Khong Chiam Hospital. The MDR *E. coli* No.40 was initially used as the host for bacteriophage amplification. Based on spot tests, it was found that the three bacteriophages against MDR *E. coli* No.40, designated as JC01, JC02, and JC03, were isolated from the wastewater treatment plants of Kudprakhow swamp, Sanprasitthiprasong Hospital, and Khong Chiam Hospital, respectively (Figure 1). This result was in accord with Mattila and colleagues (2015) who demonstrated that sewage was a reservoir of bacteriophage against MDR bacteria, including *Escherichia coli* (Mattila, Ruotsalainen, & Jalasvuori, 2015), indicating that wastewater can be a good source of bacteriophage specific to particular pathogenic bacteria.

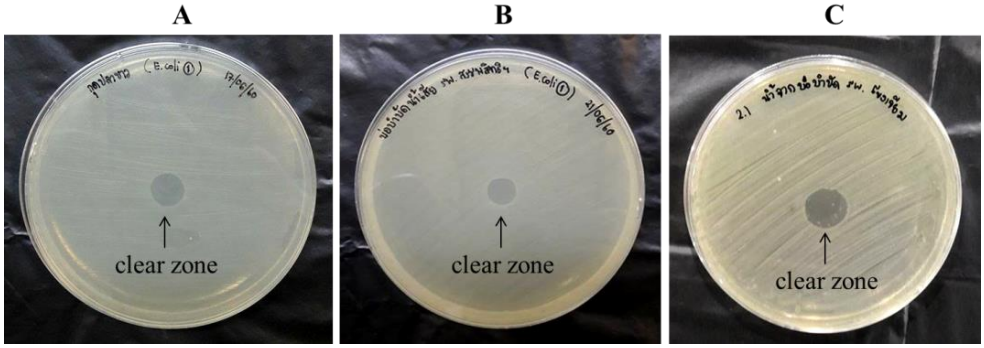

Figure 1 Bacteriophage detection by spot test. Nutrient agar plate was supplemented with *E. coli* No.40. The clear zone indicates the presence of bacteriophage in wastewater samples collected from Kudprakhow swamp (A), Sanprasitthiprasong Hospital (B), and Khong Chiam Hospital (C).

In the determination of the quantity of purified bacteriophage in terms of plaque forming unit per millitter (pfu/ml), it was found that bacteriophages JC01, JC02, and JC03 were 2.66 x 10^8, 6.98 x 10^7, and 2.23 x 10^7 pfu/ml, respectively, indicating the high viral titer sufficient for further analysis and application. This result was similar to a previous report showing that bacteriophage at the titer of 10^5 pfu/ml was able to be utilized to improve symptoms in MDR *Pseudomonas aeruginosa*-infected patients, and no adverse effects were observed, indicating safety for bacteriophage treatment (Wright, Hawkins, Anggard, & Harper, 2009).

One advantage of using bacteriophages as therapeutic agents is the high specificity to their hosts. This is different to that of treatments by antibiotics which not only target pathogenic bacteria

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but also other bacteria including commensal bacteria could be affected that may cause host dysbiosis and diseases such as antibiotic-associated diarrhea (Rea, Dinan, & Cryan, 2016; Tsonos et al., 2014). To determine the bacterial host range of the bacteriophages JC01, JC02, and JC03, spot test methodology involving nine bacterial strains and three strains of probiotics was employed. As shown in Table 2, all bacteriophages showed inhibition of only *Escherichia coli* strains, *E. coli* ATCC25922, and *E. coli* No.40, but not for all other bacteria genera tested. These results suggest that bacteriophages JC01, JC02, and JC03 had highly specific for *E. coli* species.

Table 2 Host range determination by spot test method

Bacteria	JC01	JC02	JC03
E. coli No.40	+	+	+
E. coli ATCC25922	+	+	+
P. aeruginosa No.40	-	-	-
K. pneumoniae ATCC1705	-	-	-
S. Typhimurium DMST5784	-	-	-
S. dysenteriae DMST2137	-	-	-
V. cholerae DMST2873	-	-	-
E. faecalis	-	-	-
S. aureus ATCC29213	-	-	-
L. casei TISTR1341	-	-	-
L. sakei CECT906	-	-	-
B. breve CMP4	-	-	-

(-) represents no clear zone on NA plate
(+) represents clear zone on NA plate

In addition to the tests of the specific host ranges of the bacteriophages, the potential for inhibition of the multidrug resistant *E. coli* was investigated by the inclusion of two hundred and sixty-seven clinical isolates of *E. coli* with multidrug resistant properties in this study. The clinical *E. coli* isolates were kindly provided by Sanprasitthiprasong hospital. Based on spot test methodology, it was found that bacteriophages JC01, JC02, and JC03 showed the inhibition of clinical isolates *E. coli* at the percentages of 51.70 (138/267), 52.4 (140/267), and 28.5.4 (76/267), respectively. These results suggested that the bacteriophages were not only specific to bacterial species levels but also showed strain-specific levels by the indication that some MDR *E. coli* isolates were eliminated. There has been research suggests that the host ranges of bacteriophages were dependent on the presence of bacteriophage receptors on the bacterial cell surfaces (Casey, van Sinderen, & Mahony, 2018). Other research found that Gram-negative bacteria, including *E. coli*, had different receptors for particular bacteriophage which may reflect the bacteriophage host range (Mc Grath, & van Sinderen, 2007).

For therapeutic application, a bacteriophage should be evaluated for its stability in storage conditions, including storage solutions and appropriate temperatures. It was reported that the stability of bacteriophage is phage-dependent, thus the candidate bacteriophage's stability must be tested before further advanced application (Vandenheuvel et al., 2013). To determine the stability of the bacteriophages in different solutions at different time points (10, 20, 30, and 40 min), solutions of ethanol, hydrogen peroxide, and normal saline and sterile water were used in this study.

Using spot test methodology, all three bacteriophages were tolerant in normal saline and distilled water for up to 40 min after incubation but were unable to resist ethanol and hydrogen peroxide at every time point (Table 3). This indicated that normal saline and distilled water were able to be used as storage solutions in bacteriophage preparation. In addition, ethanol and hydrogen peroxide with that concentration may be used to control the spread of bacteriophages, especially in the process of bacteriophage preparation. The stability of the bacteriophages at different temperatures was also determined in this study. As shown in Table 4, it was demonstrated that bacteriophage JC01 showed the ability to resist a temperature of 60°C after 60 min of incubation. Bacteriophages JC02 and JC03 showed the ability to resist a temperature of 60°C after 45 min of incubation.

Table 3 Stability of bacteriophages in chemical solutions at different time points

Bacteriophages	Chemical solutions and time points (min)															
	Distilled water				85% (w/v) Normal saline				10% (v/v) Ethanol				1% (v/v) Hydrogen peroxide			
	10	20	30	40	10	20	30	40	10	20	30	40	10	20	30	40
JC01	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
JC02	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
JC03	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-

Remark: - and + symbol indicate the negative and positive result, respectively, by Spot test method

Table 4 Heat stability of bacteriophages

	Bacteriophages and temperatures (°C)											
	JC01				JC02				JC03			
Time (min)	30	40	50	60	30	40	50	60	30	40	50	60
5	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+	+	+	+	+
35	+	+	+	+	+	+	+	+	+	+	+	+
40	+	+	+	+	+	+	+	+	+	+	+	+
45	+	+	+	+	+	+	+	+	+	+	+	+
50	+	+	+	+	+	+	+	-	+	+	+	-
55	+	+	+	+	+	+	+	-	+	+	+	-
60	+	+	+	+	+	+	+	-	+	+	+	-

Remark: - and + symbols indicate the negative and positive results, respectively, by spot test methodology

Analysis of bacteriophage genome and viral particle morphology was performed to classify the bacteriophages. For viral genome analysis, the extracted viral DNA was digested with six bases cutter endonucleases, RNase, and DNase. As shown in Figure 2, gel electrophoresis revealed that only *Hind*III digested the bacteriophage JC01 genome (Figure 2, panel A), indicating that the bacteriophage JC01 genome could be double stranded-DNA (dsDNA), due to *Hind*III enzyme having double stranded-nucleotide AAGCTT/TTCGAA as specific recognition site for digestion. In addition, the bacteriophage JC01 genome was digested with DNase but not with RNase, indicating that the bacteriophage genome was DNA type. This result was the same with the cases of bacteriophage JC02 (Figure 2, panel B) and bacteriophage JC03 (Figure 2, panel C). Therefore, the bacteriophage genomes of JC01, JC02, and JC03 were dsDNA viral type.

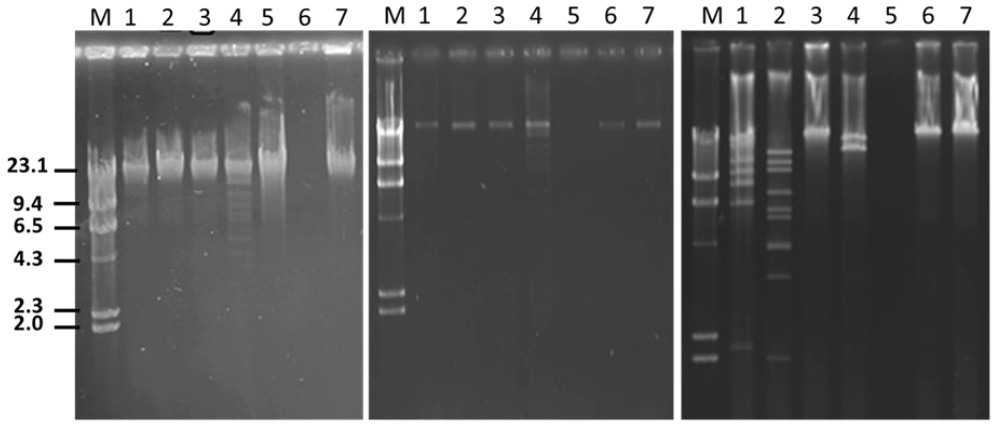

Figure 2 Ethidium staining gel of enzymes-digested bacteriophage genomes JC01 (A), JC02 (B), and JC03 (C).

Viral particle morphology analysis, based on transmission electron micrographs (Figure 3), revealed that all three bacteriophages, JC01, JC02, and JC03, had hexagonal-shaped heads and long tails with contractile. The size from head to tail was approximately 200 nm for all bacteriophages.

According to the International Committee on Taxonomy of Viruses (ICTV) (Mattila, et al., 2015), bacteriophages JC01, JC02, and JC03 could be classified in Family *Myoviridae*, and Order *Caudovirales*. The genus of these bacteriophages were not classified in this study.

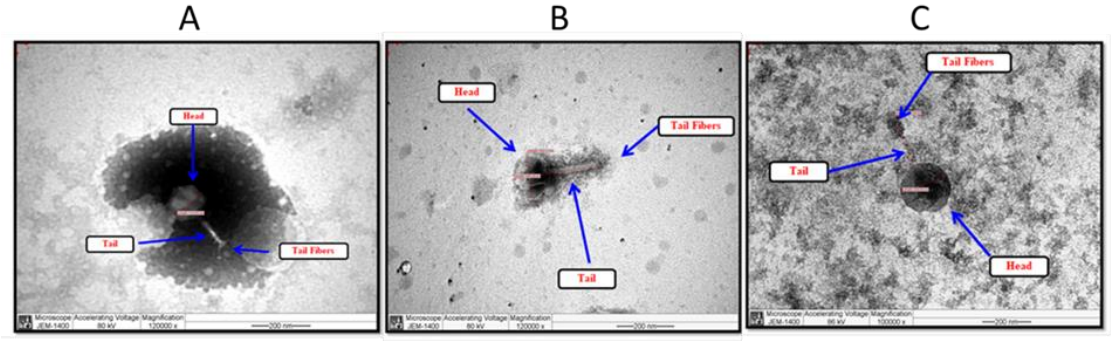

Figure 3 Transmission electron micrographs of bacteriophage isolates JC01 (A), JC02 (B), and JC03 (C). Scale bar indicates 200 nm.

5. Conclusion

This study successfully isolated and characterized three bacteriophages isolated from wastewater from two hospitals and one community against multidrug-resistant *E. coli* strains. These bacteriophages, JC01, JC02, and JC03, showed high specificity to only *E. coli* strains, and had enough stability in particular conditions. Therefore, bacteriophages derived from this study could be suitable for advanced research, such as the verification of viral complete genome and analysis, and the determination of safety and efficacy when using bacteriophages in cell cultures and animal models.

6. Acknowledgements

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