



ISOLATION AND CHARACTERIZATION OF *Serratia marcescens* NBL 1001 BACTERIOPHAGES FROM SEWAGE WATER

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ABSTRACT – *Serratia marcescens* has been recognized as an opportunistic nosocomial pathogen. The emergence of multidrug-resistant *S. marcescens* strains, which pose threats to public health, prompts actions to control their growth and dissemination. One of the leading control strategies which has the potential to be used as either an alternative or a supplement to antibiotic treatment is phage therapy. This study was performed to isolate bacteriophages that can be used against *S. marcescens*, and characterize the isolated phages based on lytic activity and particle morphology. The bacteriophages were obtained from raw sewage through phage enrichment followed by double agar overlay plaque assay. Plaques exhibiting varying morphologies were isolated and purified. Phage isolates designated as P1, P2, P3 and P4 formed turbid plaques while phage P5 formed clear circular plaques surrounded by a large halo. Assessment of the lytic activity of the phages showed that the *S. marcescens* wild type and seven *S. marcescens* Tn5-insertional mutants were susceptible to the five phage isolates. Phages P1, P2 and P3 were able to infect *Escherichia coli* while only phage P4 was able to infect *Salmonella enterica* subsp. *enterica*. Unrelated genera which included *Bacillus megaterium*, *B. subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Micrococcus luteus* were not susceptible to all the phages. Based on the virion size and morphology as revealed by electron microscopic analysis, the possible identity of the phage isolates was deduced following the classification scheme of the International Committee on Taxonomy of Viruses. The five phages may belong to Order *Caudovirales*. Phages P1 and P2 having icosahedral-isometric heads with thin, long, non-contractile, flexible tail, may belong to Family *Siphoviridae*. Phage P3 has icosahedral-isometric head with no visible tail which may indicate that it belongs to Family *Podoviridae*. Phage P5 possesses an icosahedral-isometric head with a neck that is connected to a rigid contractile tail, which may classify it under Family *Myoviridae*. The results of this study suggest the possible use of the phages as bactericidal agents. Further characterization of the identified phages can be done to fully understand their potential application as biocontrol agents against *S. marcescens*.

Keywords: bacteriophages, electron microscopy, phage therapy, plaques assay, *Serratia marcescens*

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INTRODUCTION

Serratia marcescens is a Gram-negative, rod-shaped bacterium under the family Enterobacteriaceae. It is ubiquitous, saprophytic, and was historically, considered harmless and non-pathogenic (Hejazi and Falkiner 1997). Currently, it is recognized as an opportunistic pathogen, and is implied as an etiological agent for numerous nosocomial infections such as urinary tract infection (UTI), meningitis, and wound infections (Hejazi and Falkiner 1997; Mahlen 2011). It is frequently isolated from immunocompromised patients in intensive care units and pediatrics (Hejazi and Falkiner 1997; Khanna et al. 2013). Being a member of the family Enterobacteriaceae, it has a remarkable capacity to pick up and transfer multiple antimicrobial resistance genes and alter the expression of these genes (Hall et al. 2003). There are reported cases of *S. marcescens* infections caused by strains that exhibited resistance to different classes of antibiotics which include imipenem, cefepime, carbapenems, aminoglycosides, and quinolones (Yang et al. 2012; Merkier et al. 2013). In the UK and Ireland, the emergence of multidrug resistant (MDR) strain of *S. marcescens* which acquired resistance to different β -lactams, ciprofloxacin, and tetracyclines was reported (Moradigaravand et al. 2016). Moreover, a patient in Brazil was diagnosed of septic shock which was caused by *S. marcescens* enzymes (SME)-producing *S. marcescens* isolate (Cayo et al. 2017). SME carbapenemase-producing *S. marcescens* was also reported in the UK (Hopkins et al. 2017).

The emergence of MDR strains presents threats to public health. According to the World Health Organization, colistin is the last resort treatment for life-threatening infections caused by members of Enterobacteriaceae which are resistant to carbapenems. Since *S. marcescens*, a member of the Enterobacteriaceae group, is intrinsically resistant to colistin (Yao et al. 2016), it is therefore, necessary that effective means to control the dissemination of *S. marcescens* be developed. One of the leading control strategies being studied is phage therapy, which has the potential to be used as either an alternative or a supplement to antibiotic treatments.

Phage therapy is a control strategy that uses bacteriophages (or phages) in treating bacterial infections, where phages infect bacteria causing their lysis and subsequent death. Cell lysis is caused by the enzymes holins and lysins that degrade the bacterial cell wall (Veiga-Crespo et al. 2007). Phage therapy is not actually new in treating bacterial infections because as early as 1919, Felix d' Herelle successfully applied phage therapy by administering an oral suspension of the most potent anti-dysentery phage preparation to treat dysentery caused by *Shigella dysenteriae* (Summers 1999; Kutter and Sulakvelidze 2004). However, the discovery of penicillin which started the antibiotic era, and the obstacles encountered in developing phage therapy at that time, led to the abandonment of phage therapy (Matsuzaki et al. 2005). Now that the emergence and spread of antibiotic resistant pathogens is becoming alarming, the interest in using phage therapy in treating diseases has been renewed (Lin et al. 2017).

Cognizant of the potential contribution of phage therapy to combat certain pathogens, this study was aimed at isolating and identifying bacteriophages with bactericidal potential against *S. marcescens*. The source of the phages was raw sewage. The purified phages were characterized based on plaque morphology and lytic activity. The virion size and morphology were determined through transmission electron microscopy. The possible identification of the phages was deduced following the classification scheme of the International Committee on Taxonomy of Viruses (ICTV).

MATERIALS AND METHODS

Bacterial Strains Used and Growth Conditions

The *Serratia marcescens* NBL 1001 wild type (wt) and seven Tn5-insertional mutants derived from the *S. marcescens* wt were obtained from the Microbiology Division, Institute of Biological Sciences, University of the Philippines Los Baños (UPLB), College, Laguna, Philippines. The *S. marcescens* wt was grown on Luria-Bertani (LB) agar for 18-24 hours at ambient room temperature. Isolated colony exhibiting red color with metallic sheen was inoculated onto fresh LB plate and the purity was checked by Gram staining and microscopic observation. This *S. marcescens* wt was used in the isolation of bacteriophages from sewage water.

For the host range analysis/lytic activity assay, the following bacteria were used: *S. marcescens* NBL1001 Tn5-insertional mutants designated as SMM 05, SMM 17, SMM 21, SMM 24, SMM 49, SMM 80, and SMM 85; *Salmonella enterica* subsp. *enterica* JCM 1651; *Escherichia coli*; *Pseudomonas aeruginosa*; *Bacillus megaterium*; *B. subtilis*; *Staphylococcus aureus*; and *Micrococcus luteus*. *S. marcescens* mutants were kanamycin resistant, and therefore were grown on LB plate supplemented with 100 ppm kanamycin. All the other bacterial cultures were grown on LB plates. All the cultures were grown for 18-24 hours at ambient room temperature.

Phage Enrichment and Isolation

The raw sewage water samples were obtained from the raw sewage collection tank, (tank containing all raw sewage prior to treatment) located at the wastewater treatment facility at the College of Engineering and Agro-Industrial Technology, UPLB. Sample collections were done on July 5, 2017, and March 26, 2018. The raw sewage served as the source of bacteriophages. Ten milliliters of sewage water placed in a sterile screw capped tube was mixed with 1 mL of overnight culture of *S. marcescens* wt grown in LB broth. The mixture was incubated at ambient room temperature. After 24 hours, the mixture was centrifuged at $10,000 \times g$ at 4°C for 5 min to pellet out the bacterial cells and potentially large sewage debris. The pellet was discarded, and the supernatant was collected, and filtered through a $0.22 \mu\text{m}$ membrane filter. The filtrate was dispensed in microcentrifuge tubes and stored at 4°C . This served as the phage lysate or phage stock.

The concentration of the phage stock was determined through serial dilution from 10^0 to 10^{-7} followed by double agar overlay plaque assay as described by Kropinski et al. (2009) with modifications. Briefly, equal volumes (500 μL) of one of each of the dilutions and an overnight culture of *S. marcescens* wt were mixed and supplemented with 5 mM CaCl_2 . The phage and bacterial mixtures were incubated for 25 min at room temperature to allow maximum phage adsorption to the host bacterial cells. All the phage-host mixtures were individually transferred to tubes containing molten soft LB agar (0.7%) and carefully mixed. The mixture was overlaid onto a pre-poured LB agar (1.5%) plates. The plates were incubated at ambient room temperature until plaques were observed. The plaque-forming units (PFU) per mL was computed based on the number of plaques counted.

Phage Purification and Propagation

The plaques exhibiting varying morphologies as described by Jurczak-Kurek et al. (2016) were selected. The selected plaques were picked out from the plates using sterile pipette tips, and the agar containing phages was placed in sterile microcentrifuge tubes with 500 μL phage buffer (Bonilla et al. 2016). This was centrifuged at $10,000 \times g$ for 5 min, and the supernatant was inoculated onto a 10 mL

overnight culture of *S. marcescens* wt broth containing 5 mM CaCl₂. After 24-hour incubation, the broth was centrifuged at 10,000 × g for 5 min, and the supernatant was filtered through a 0.22 µm membrane filter. The phage lysate was stored at 4 °C until further use.

Further phage purification was carried out by single plaque picking which was done at least three times. The phage isolate was considered pure when the plaques that formed in the plate were uniform in size. Plaques were picked from the plates and placed in microcentrifuge tubes containing 1000 µL phage buffer, and vortexed for 5 min. The debris were pelleted out through centrifugation at 10,000 × g for 5 min. The supernatants were stored at 4 °C. The phages collected from the last step of purification were used to prepare a high-titer phage stock.

Propagation of phages was done following two methods as described by Bonilla et al (2016) with modifications. The first method was called liquid phage lysate which involved mixing of 500 µL phage lysate and 10 mL of overnight culture of *S. marcescens* and incubating the mixture overnight at ambient room temperature. The overnight mixtures were centrifuged at 10,000 × g for 5 min. The supernatant was filtered through a 0.22 µm membrane filter. The filtrate was placed in microcentrifuge tubes and stored at 4 °C until further use. The second method was through plate phage lysate. The dilution of phage that produced semi-confluent lysis was used in plating. After the adsorption period of 25 min, the phage and bacterial suspensions were mixed with 5 mL 0.7% LB agar containing 50 µL of 0.1 M CaCl₂, then overlaid onto a pre-poured 1.5% LB agar base plate. The inoculated plates were incubated at ambient room temperature until plaques were observed. One milliliter of phage buffer was added to the plate with semi-confluent lysis. The overlay agar was scraped and emulsified in a sterile 15-mL conical tube and centrifuged at 3,200 × g for 30 minutes to pellet out the agar and bacterial cell debris. The resulting supernatant was further centrifuged at 10,000 × g for 5 minutes. The supernatant was filtered using a 0.22 µm filter membrane. The lysate was stored at 4 °C until further use.

Host Range Determination of the Isolated Bacteriophages

The host range of the isolated bacteriophages was determined via spot-on-lawn assay (Mirzaei and Nilsson, 2015). The different hosts were separately inoculated in 5 mL Trypticase Soy Broth (TSB) and incubated overnight with shaking at 200 rpm at ambient temperature. Bacterial suspensions with turbidity equivalent to 0.5 McFarlands standards were prepared by harvesting the cells through centrifugation as described above and resuspending the cell pellet in 0.85 % saline solution. Bacterial lawns were prepared by dipping a sterile cotton swab in the bacterial suspension and streaking it on a pre-poured TSA plate. Approximately 4 µL of pure phage lysate was spotted on the bacterial lawn. Each bacteriophage was spotted in triplicates. After the spot has dried, the plates were incubated at ambient room temperature for 12 hours. Zones of lysis were observed and recorded.

Transmission Electron Microscopic (TEM) Analysis

The TEM analysis of phages P1, P2 and P3 were done by Albert Remus Rosana at the University of Alberta, Canada, while that of phage P5 was submitted at the Research Institute for Tropical Medicine (RITM) in Alabang City, Muntinlupa, Philippines. (Phage P4 was not anymore included in the TEM analysis since the plaque morphology was similar to phage P2, and it was assumed that they were the same phage). Both the TEM analyses employed negative staining. All phage lysates contain approximately 10¹⁰PFU/mL. For phages P1, P2, and P3, the following procedure was performed. A drop of phage suspension was deposited onto formvar-carbon coated grids and allowed to stand for 5 min. Then, it was blotted and stained with 2 % phosphotungstic acid (pH 7.4) for 15 s. Excess stain was wicked off and was

allowed to dry for 15 min before viewing under the TEM (Morgagni 268 TEM) (Philips, FEI, Hillsboro, OR, USA) that was equipped with GatanOrius CCD camera, and analyzed with Morgagni 268 v3.0 software.

The size of the whole virion, tail, neck and other structures were measured. Measurements were gathered using a screen ruler wherein the measurements were in pixels (Perfect Screen Ruler ver 3.0). The 100 nm scale bar was also measured for calibration purposes. Then, the average of the phage measurements in pixels was computed, and used in the conversion of phage measurements from pixels to nanometer following this formula:

$$\text{phage measurement (nm)} = \frac{100 \text{ nm}}{\text{ave. length of 100 nm bar in pixels}} \times \text{ave. phage measurement in pixels}$$

The classification of the isolated bacteriophages was deduced based on the virion morphology and measurements, following the International Committee on Taxonomy of Viruses (ICTV) classification scheme in determining the order, family and nucleic acid type (King et al. 2011). The group and morphological types were based on the scheme of Bradley (1967), and Ackermann and DuBow (1987).

RESULTS AND DISCUSSION

Phage Enrichment and Isolation

Raw sewage is a rich source of bacteriophages infecting enteric bacteria. In this study, raw sewage was used as source in the isolation of phages of *Serratia marcescens*. Prior to isolation, phage enrichment was carried out by adding *S. marcescens* in the sewage sample and then incubating it. This would then allow the phage present in the sample to infect a susceptible bacterial cell. The phage would then replicate to concentrations which can be detectable by plaque assay.

After enrichment of the raw sewage water, 3.9×10^9 PFU/mL were detected in the first raw sewage water sample and 6.4×10^6 PFU/mL in the second raw sewage water sample. The *S. marcescens* phages were obtained from the plates inoculated with the enriched sewage water samples from the two collections. Most of the plaques formed were small and turbid (Figure 1). It has been reported that *Serratia* phages can be successfully isolated from sewage, such as a novel *Podoviridae*-phage isolated by Xu et al. (2012), and a lytic phage SM701 which belongs to tailed family: *Siphoviridae* (Yu et al. 2008).

Phage enrichment is done to increase the concentration of phages in a given sample capable of infecting the susceptible host. In the study of Hernandez (2017), *Serratia* phages against fish spoilage bacteria were isolated by enriching the whey samples with actively growing bacteria, and incubating them at 20 °C for seven hours. Enrichment can also be done using multiple hosts for isolating specific phages such as coliphages, where sewage water was enriched with multiple strains of *E. coli* (Ackermann and Nguyen 1983). Enrichment technique is advantageous when isolating specific phages because it limits the diversity of bacteriophages from the source.

Phage Purification and Characterization Based on Plaque Morphology

Phage isolates were characterized based on plaque morphology which included size, type of clearing (turbid or clear plaques) and presence of halo (Jurczak-Kurek et al. 2016). Generally, a single plaque is assumed to be initiated by a single virus particle, and plaque morphology is specific to a certain

strain of bacteriophage thus, the presence of plaques with different morphologies indicates that there are several phage isolates present in a given sample (Kleczkowski and Kleczkowski 1951). Among the observed parameters for plaque morphology, the plaque diameter generally varies. This was observed during the purification of the isolated bacteriophages. Variations maybe due to bacterial host, phage, plating, and incubation conditions (Stent 1963).

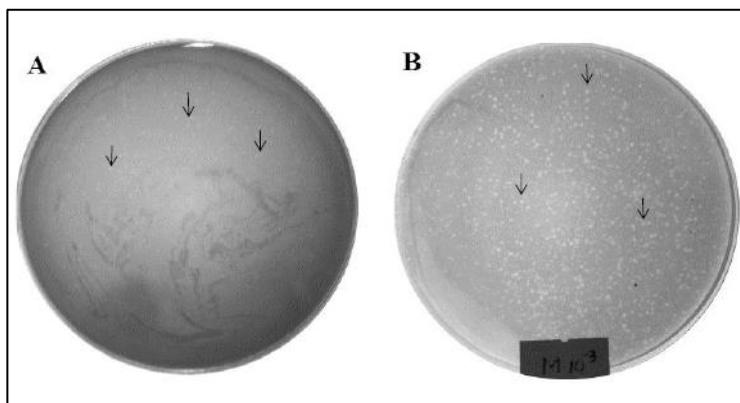


Figure 1. Double agar overlay plaque assay of *Serratia marcescens* enriched sewage water samples. (A) enriched raw sewage water sample 1 (B) enriched raw sewage sample 2. Plaques are shown (by the arrows) as translucent spots against an opaque bacterial lawn.

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The sizes of the formed plaques of the isolated bacteriophages were relatively small with plaque diameter ranging from 0.5 mm to 2 mm (Figure 1). Plaque size is one of the parameters used in the isolation

of phage isolates. Small plaque formation may indicate that the phage has a very low adsorption rate, which can also be blurry due to over-growth of bacterial cells. It may as well indicate short lysis time, and small burst size, which may mean less progeny available for diffusion (Gallet et al. 2011). The size of plaque may also signify the possible size of the bacteriophages isolated, where virions with smaller heads easily diffuse through the solid media than the virions with larger heads as observed in the study of Jurczak-Kurek et al. (2016).

Five different plaque morphologies were observed (Figure 2). The plaques were obtained and were designated as P1, P2, P3, P4 and P5. The first three plaque types (P1, P2 and P3) were isolated from the first collection sewage water, while, P4 and P5 were isolated from the second collection sewage water. Phage isolates P1, P2, and P3 produced turbid plaques (Figures 2A, 2B, 2C), but differ mainly on the size of the plaque formed. P1 has the largest plaque formed with size 1.5 – 2.0 mm, followed by P2 with plaque diameter of 1.0 – 1.5 mm and P3 with plaque diameter of 0.5 – 0.8 mm (Table 1). Phage P4 formed turbid plaques with plaque diameter of 1.0 – 1.5 mm (Figure 2D). Although P4 has the same plaque size as P2, it was isolated from a different sewage sample. Phage P5 was unique among all the phages because, it is the only phage isolate which exhibited clear circular plaques surrounded by a large halo (Figure 2E). The diameter of the clear area ranges from 1.0 – 1.5mm, and when measured with the halo, the plaque diameter ranges from 2.0 – 3.5 mm (Table 1).

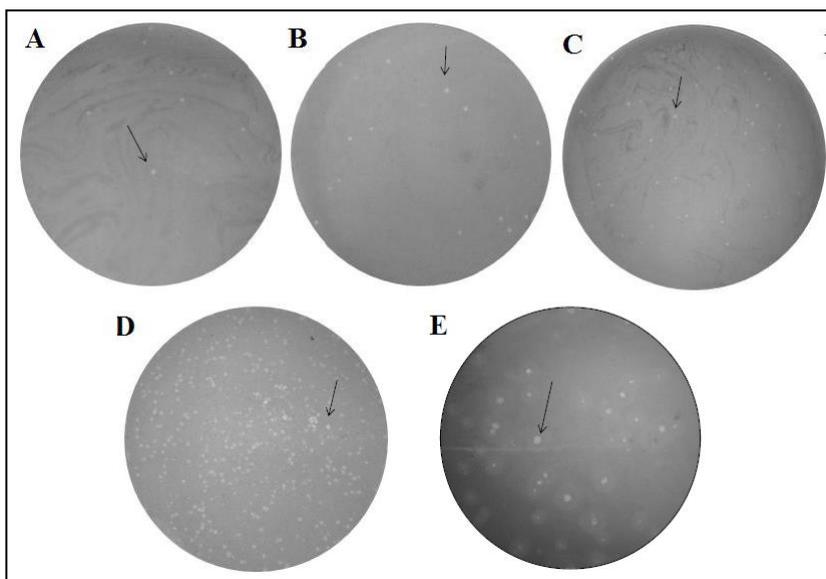


Figure 2. Plaque morphologies of the phage isolates. A. Phage P1; B. Phage P2; C. Phage P3; D. Phage P4; E. Phage P5. Plaques are shown (by the arrows) as translucent spots against an opaque bacterial lawn.

Formation of turbid plaques, as was observed in phage isolates P1, P2, P3 and P4, may indicate that the phage isolates are temperate phages (Jurczak-Kurek et al. 2016). Previous studies reported that temperate phages typically formed turbid plaques due to the growth of lysogenic bacteria (Levine and

Curtiss 1961; Jiang et al. 1998). Example of a temperate phage that forms turbid plaques is phage P22 of *Salmonella enterica* Ser Typhimurium (Zinder and Lederberg 1952). In the study of Jurczak-Kurek et al. (2016), nine phages forming turbid plaques were isolated from urban sewage. It was observed that the turbidity increases with incubation time (Kumar et al. 2008). The turbidity of the isolated plaques increased as it moves towards the peripheries of the plaques formed (Jurczak-Kurek et al. 2016). Lysogenic cells due to temperate phage infection have the ability to liberate phages with or without causing lysis to the infected cell and they are immune to their liberated phages (Bertani 1953).

On the other hand, phage isolate P5 formed clear plaques, which may indicate that it is a lytic phage due to its ability to lyse the infected cells. (Sundar et al. 2009; Jurczak-Kurek et al. 2016). Lytic phages are able to permanently disrupt the normal reproductive cycle of the host cell, use its machinery to produce virion parts, and finally, liberate the virions that lead to cell lysis (Bertani 1953).

Table 1. Phage morphology of phage isolates infecting wild type *Serratia marcescens*.

Phage Isolate	Size	Description
P1	1.5 – 2.0 mm	Turbid plaques
P2	1.0 – 1.5 mm	Turbid plaques
P3	0.5 – 0.8 mm	Turbid plaques
P4	1.0 – 1.5 mm	Turbid plaques
P5	1.0 – 1.5 mm (clear area); 2 – 3.5 mm (with halo)	Circular clear plaques with large halo

The formation of halo was observed in phage P5. The formed halo surrounding the clearing may be due to the diffusion of enzymes encoded by phage that act on the cell wall of the surrounding bacterial cells (Jurczak-Kurek et al. 2016). These enzymes are called extracellular polymeric substance depolymerases (Pires et al. 2016). The phages with depolymerase activity have better performance against strains forming biofilms, and therefore, are more preferred in phage therapy. Moreover, some phage isolates from different families of *Caudovirales* have the ability to encode depolymerases (Pires et al. 2016). In the study of Jurczak-Kurek et al. (2016), phages producing clear or turbid plaques formed halo. The formation of halo may also indicate a partial lysis due to lysis inhibition. This is a phenomenon which results from a cell's reinfection of the same phage, as was also observed in T4 plaque morphology (Birge 2013).

In the selection of candidate phages that can be used in phage therapy and biocontrol, obligatory lytic phages are usually the ones that are considered due to their ability to lyse the target bacterial cells, and they are unable to cause lysogenic problems like the temperate phages (Carlton et al 2005; Kutter 2014; Lin et al. 2017). Temperate phages which are capable of lysogeny are avoided due to their ability to transfer virulence determinants and toxin production by transduction to the host strains that may result to emergence of new pathogens that are more resistant (Saunders et al. 2001; Goodridge and Abedon 2003; Carlton et al. 2005; Lin et al. 2017).

Host Range Analysis

Host range determination indicates the genera, species and strains of bacteria that a particular phage can lyse. This method also allows the classification of phages as being polyvalent phages (wide-host

range) or with narrow host range (Kutter 2014). Results showed that all the *S. marcescens* mutants were susceptible to the isolated bacteriophages (Table 2). *E. coli* wild type was not infected, however, the *E. coli* rif^r kan^r mutant was infected by P1, P2 and P3. *S. enterica* subsp. *enterica*, on the other hand, was infected by P4 only (Figure 3). *P. aeruginosa*, *Staphylococcus aureus*, *Micrococcus*, *B. subtilis* and *B. megaterium* were not infected by any of the bacteriophages isolated (Table 2).

Phage adsorption plays a very important role in determining the lytic activity of phage against the host, because this step initiates the infection process (Bertozi-Silva et al. 2016). Phage and bacterial host interaction is made possible by the presence of receptors, where the phages bind. Receptors are generally found on the cell walls of both Gram-positive (Xia et al. 2011) and Gram-negative bacteria (Marti et al. 2013), as well as in bacterial capsules or slime layers (Fehmel et al. 1975) and appendages (Guerrero-Ferreira et al. 2011; Shin et al. 2012).

Table 2. Lytic activity of the phage isolates using different hosts.

Host	Bacteriophage Isolate				
	P1	P2	P3	P4	P5
<i>Serratia marcescens</i> wild type	+	+	+	+	+++
<i>S. marcescens</i> SMM 05	+	+	+	+	+++
<i>S. marcescens</i> SMM 49	++	++	++	++	+++
<i>S. marcescens</i> SMM 80	+	+	+	+	+++
<i>S. marcescens</i> SMM17	+	+	+	+	+++
<i>S. marcescens</i> SMM 21	+	+	+	+	+++
<i>S. marcescens</i> SMM 24	+	+	+	+	+++
<i>S. marcescens</i> SMM 85	+	+	+	+	+++
<i>Escherichia coli</i> wild type	-	-	-	-	-
<i>E. coli</i> rif ^r kan ^r	+	+	+	-	-
<i>Salmonella enterica</i> subsp. <i>enterica</i> JCM 1651	-	-	-	+++	-
<i>Pseudomonas aeruginosa</i>	-	-	-	-	-
<i>Staphylococcus aureus</i>	-	-	-	-	-
<i>Bacillus megaterium</i>	-	-	-	-	-
<i>Bacillus subtilis</i>	-	-	-	-	-
<i>Micrococcus luteus</i>	-	-	-	-	-

Legend: (-) – no lytic activity; (+) – partial lysis; (++) – few colonies with turbid background; (+++) – few colonies with clear background

Overall, the isolated bacteriophages exhibited lytic activity against *S. marcescens* wild type and Tn5-insertional mutants. Generally, bacteriophages infect only a specific bacterial host under one genus (Ackermann and Wegrzyn 2014). However, in this study, it was observed that the isolated phages P1, P2, P3 and P4, exhibited lytic activity against other Enterobacteriaceae belonging to different genera such as *Escherichia* and *Salmonella*. These phages maybe considered polyvalent phages, which are common among the members of Enterobacteriaceae because member genera are closely related (Krieg and Lockhart 1966; Ackermann 1998). In the study of Kurtböke et al. (2016), polyvalency of the isolated phages against *Escherichia*, *Klebsiella* and *Citrobacter* was also observed, not just within genus but also in other members

of Enterobacteriaceae family. Polyvalent phages are good candidates as biocontrol agents especially against biofilms and as phage therapeutics because there is no need to create phage cocktails (Kurtböke et al. 2016; Yu et al. 2017). The absence of phage lytic activity against *P. aeruginosa*, *S. aureus*, *B. subtilis*, *B. megaterium* and *Micrococcus* maybe attributed to their lack of phage receptors that are found only in the Enterobacteriaceae.

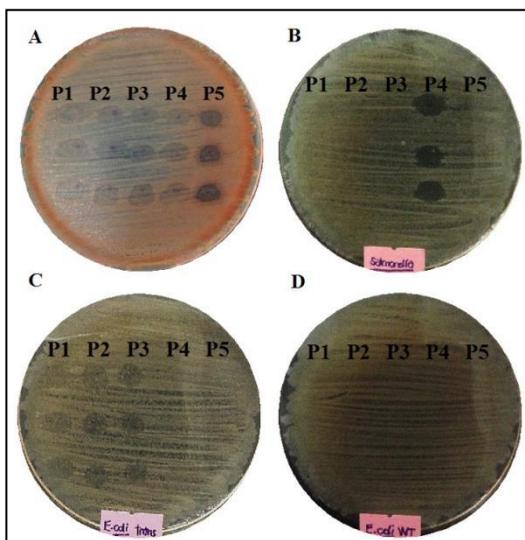


Figure 3. Lytic activity of the isolated phages on hosts (A) *Serratia marcescens* wild type (B) *Salmonella enterica* subsp. *enterica* (C) *Escherichia coli* rif^Rkan^R and (D) *E. coli* wild type.

Transmission Electron Microscopy Analysis and Virion Classification

The particle morphology of the isolated bacteriophages were determined through negative staining transmission electron microscopy (TEM) and the virion size were measured (Table 3). Results showed that phage P1 has icosahedral-isometric head with diameter of about 64 nm. It has thin, long, non-contractile, flexible tail measuring about 215 nm in length and has tail diameter of about 15 nm (Figure 4A). Total phage length is about 285 nm. Based from the data gathered from TEM analysis, phage P1 may belong to the order *Caudovirales* under the family of *Siphoviridae*, Group B, Morphotype B1, with double-stranded DNA (Bradley 1967; Ackermann and DuBow 1987; King et al. 2011).

Phage P2 was found to have icosahedral-isometric head with diameter of about 85 nm. Like phage P1, it has thin, non-contractile, flexible tail that measures about 215 nm in length and has tail diameter of about 15 nm (Figure 4B). Total phage length is about 305 nm. This bacteriophage may also belong to the Order *Caudovirales* under the Family *Siphoviridae*, Group B, Morphotype B1, and based on literature, it is a double-stranded DNA virus (Bradley 1967; Ackermann and DuBow 1987; King et al. 2011).

Phage P3 showed icosahedral-isometric head with diameter of about 65 nm. Based on the electron micrograph, the phage was associated with the flagella of the bacteria (Figure 4C). No phage tail was

observed but it is possible that it is a phage that has short tail which may also belong to the Order *Caudovirales* under the Family *Podoviridae*, Group C, Morphotype C1, and has also a double-stranded DNA (Bradley 1967; Ackermann and DuBow 1987; King et al. 2011).

Table 3. Phage isolates average measurements derived from electron micrographs.

Phage Isolate	Head length (nm)	Head diameter (nm)	Tail length (nm)	Tail diameter (nm)	Phage length (nm)
P1	71.84	64.70	214.61	15.37	285
P2	90.52	83.60	214.50	14.78	305
P3		65.61	-	-	65
P5	119.91	98.42	119.03*	85**	240

*contractile tail: 82.97; neck length:38.15

**neck width:15.23; middle tail width:19.73; top contractile tail width:74.55; bottom tail width/baseplate): 47.16

P5 has icosahedral-isometric head with diameter of about 100 nm. It has a neck of about 40 nm in length and has diameter of 15 nm that is connected to a rigid contractile tail that measures about 85 nm in length (Figure 4D). The total phage length is about 240 nm. The phage may belong to the Order *Caudovirales* under the *Myoviridae* family, Group A, Morphotype A1 and is also a double stranded DNA virus (Bradley 1967; Ackermann and DuBow 1987; King et al. 2011).

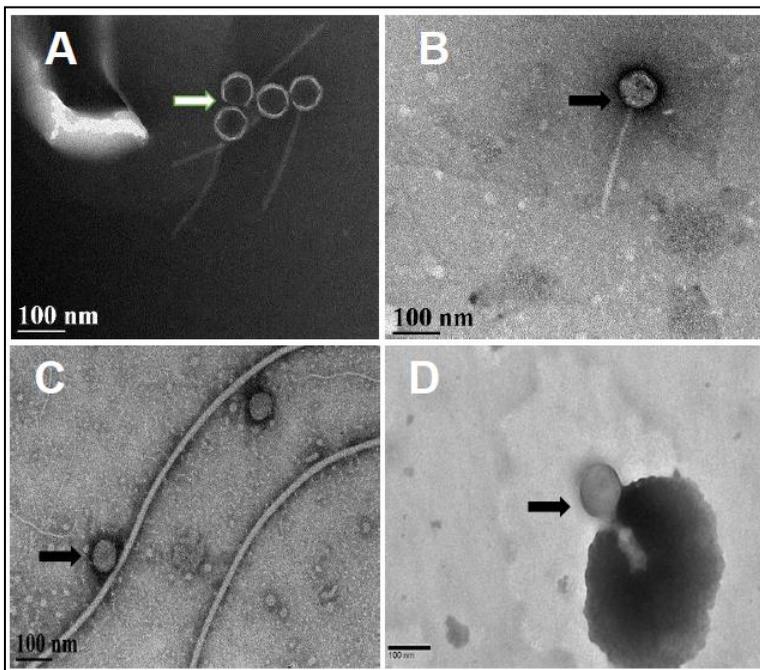


Figure 4. Electron microscopic images of the isolated phages. A. Phage P1. B. Phage P2. C. Phage P3. D. Phage P5. Arrow shows the virus particle.

TEM plays an important role in the classification of bacteriophages for it gives information of the bacteriophage much faster than the genome analysis (Giuseppe et al. 2018). Through the viral morphology, classification and the type of genetic material present can easily be determined (Giuseppe et al. 2018). Based on the electron micrographs, the isolated bacteriophages are tailed phages belonging to the Order *Caudovirales*. According to Ackermann (1998), tailed phages are the largest of all virus groups where approximately 96% of the phages are tailed phages. Classification is important in phage selection for phage therapy and phage biocontrol development (Giuseppe et al. 2018).

Among the isolated bacteriophages, P5 may be considered as a candidate that can be used against *S. marcescens*. It is probably an obligately lytic phage, as shown by the production of clear plaques, and it forms halo which may indicate that it has phage encoded enzymes called the extracellular polymeric substances depolymerases which can be effective against biofilm-forming strains of *S. marcescens*. It is recommended that the lytic activity of the phage isolates against other species of *Serratia* as well as against other strains of *S. marcescens* be carried out. More studies such as multiplicity of infection, *in vitro* challenge, genome sequencing, among others, can be conducted to further characterize the identified phages to be able to fully understand their potential as biocontrol agents against *S. marcescens*.

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STATEMENT OF AUTHORSHIP

All authors contributed to the form and content of this paper through data gathering, data analysis, and writing.

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