

RESEARCH ARTICLE

Characterization of *Pseudomonas aeruginosa* Infecting Bacteriophages from Swiftlet House Pond Water and Sewage in South Kalimantan, Indonesia

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Abstract: *Pseudomonas aeruginosa* is an opportunistic bacterium that may occur in animal-production environments and contribute to microbial contamination of animal-derived products. However, information on *P. aeruginosa*-infecting bacteriophages in artificial swiftlet-house environments remains limited. This study aimed to isolate and evaluate *P. aeruginosa*-infecting bacteriophages from the swiftlet-house environment in South Kalimantan, Indonesia. A total of 75 samples involving pond waters (n = 25), sewages (n = 25), and soil (n = 25) were obtained from four artificial swiftlet houses and screened for bacteriophage activity against *P. aeruginosa* FKH-AP (GenBank accession no. PZ351230), *Acinetobacter bereziniae* TK8 (PX000546.1), *Shigella flexneri* FB3 (PZ384610), *Escherichia coli* TJ9 (PZ384611), and *Klebsiella pneumoniae* TB4 (PZ384612), using spot tests and plaque assays. The bacteriophages were purified through three successive rounds of plaque purification. Plaque morphology and phage titer were evaluated using plaque assays, and virion morphology was examined using Transmission Electron Microscopy (TEM). Two of 75 environmental samples, designated AC2 (pond water) and FC2 (sewage), showed phage activity against *P. aeruginosa* FKH-AP, with initial titers of 5.1×10^7 PFU/mL and 4.1×10^7 PFU/mL, respectively. After purification, phage AC2 and FC2 reached titers of 1.2×10^{10} PFU/mL and 1.8×10^{12} . Based on the morphology test, phage AC2 produced small and clear plaques, while phage FC2 produced large and clear plaques. Further, TEM revealed virions with icosahedral capsids and elongated contractile tails, consistent with Myoviridae. This study provides evidence of *P. aeruginosa*-infecting bacteriophage activity from pond water and sewage associated with artificial swiftlet houses in South Kalimantan, Indonesia.

Keywords: Bacteriophage, *Pseudomonas aeruginosa*, Transmission Electron Microscopy, Veterinary Microbiology

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Introduction

Pseudomonas aeruginosa is well known as an opportunistic Gram-negative bacterium that is widely distributed in aquatic and livestock environments [1, 2]. In the animal-derived food industry, *P. aeruginosa* has been reported as a contaminant of animal products and may contribute to spoilage, greenish discoloration, and reduced product quality [3-5]. The bacterium can also form biofilms, which enhance its persistence on environmental surfaces and may complicate microbial control in animal-production environments, including artificial swiftlet houses [6].

Artificial swiftlet houses have been developed over decades in Indonesia, particularly in Kalimantan [7]. These houses provide habitats for swiftlets, particularly *Aerodramus fuciphagus*, which produces edible bird's nests [8]. Edible bird's nests are a valuable animal product exported to China, the United States, Canada, Australia, Singapore, Hong Kong, and Taiwan. International trade of edible bird's nests is subject to various requirements, including physical and microbiological quality standards [9]. Therefore, maintaining appropriate microbial conditions within swiftlet houses is important for production hygiene and product quality. However, microbial control in swiftlet houses remains largely based on conventional practices, highlighting the need to investigate alternative approaches for controlling undesirable bacteria.

Bacteriophages are viruses that specifically infect bacterial cells and have been investigated as potential biological tools for controlling bacterial populations in food, agricultural, poultry, and aquatic environments [10]. Their host specificity and ability to replicate in susceptible bacterial hosts make them attractive alternatives or complements to conventional antibacterial approaches. Unlike antibiotics, phages can target specific bacterial hosts and may provide a potential approach for reducing selected bacterial populations without directly affecting other microorganisms. Bacteriophages infecting *P. aeruginosa* have been isolated from various aquatic and environmental sources, particularly wastewater and sewage [11]. However, information regarding *P. aeruginosa*-infecting bacteriophages associated with edible bird's nest production remains limited, particularly in artificial swiftlet-house environments.

Therefore, this study aimed to isolate and characterize bacteriophages infecting *P. aeruginosa* from pond water and sewage associated with artificial swiftlet houses in South Kalimantan, Indonesia. The recovered bacteriophages were screened against selected Gram-negative bacterial strains using spot tests, characterized based on plaque morphology and Transmission Electron Microscopy (TEM), and determined phage titer. The findings provide baseline information on *P. aeruginosa*-infecting bacteriophage activity in swiftlet-house pond water and contribute to the understanding of bacteriophage occurrence in this relatively underexplored animal-production environment.

Materials and Methods

Bacterial Host Strains

Five bacterial isolates, including *P. aeruginosa* FKH-AP (GenBank accession no. PZ351230), *Acinetobacter bereziniae* TK8 (PX000546.1), *Shigella flexneri* FB3 (PZ384610), *Escherichia coli* TJ9 (PZ384611), and *Klebsiella pneumoniae* TB4 (PZ384612). The bacterial isolates were initially identified using conventional phenotypic microbiological methods and subsequently confirmed by PCR amplification and sequencing of the 16S rRNA gene [12, 13]. These isolates were selected for the spot test. All bacterial isolates were re-cultured on Tryptic Soy Agar (TSA) (Merck KGaA, Darmstadt, Germany) and incubated for 24 hours at 37°C before use.

Bacteriophage Isolation

Seventy-five environmental specimens, consisting of pond water, sewage, and soil samples, were obtained from established swiftlet habitats in South Kalimantan, Indonesia (4°02'23.6"S, 114°57'25.1"E). Sampling was conducted following standard procedures for environmental bacteriophage isolation [14, 15]. In summary, 50 mL of liquid specimens or 5 g of solid specimens were collected aseptically into sterile tubes. The specimens were delivered packed cold to the Laboratory of Molecular Genetics, Faculty of Veterinary Medicine, Universitas Wijaya Kusuma Surabaya, and analysed within 24 hours after retrieval. Each specimen underwent centrifugation at 10,000 x g for 10 minutes at 4°C, and the generated supernatants were passed through 0.22 µm pore-size Cellulose Acetate (CA) membrane filters (Millipore, USA). To enrich phages, 10 mL of every filtered sample was diluted with 10 mL of double-strength tryptic soy broth and 100 µL of an overnight culture from each bacterial host: *P. aeruginosa* FKH-AP (GenBank accession no. PZ351230), *Acinetobacter bereziniae* TK8 (PX000546.1), *Shigella flexneri* FB3 (PZ384610), *Escherichia coli* TJ9 (PZ384611), and *Klebsiella pneumoniae* TB4 (PZ384612). Each sample was incubated at 37

°C for 24 h. After incubation, 5 mL of each prepared sample underwent centrifugation at 15,000 x g for 10 minutes. The resulting supernatants were collected and filtered before being used for the spot test.

Screening Test

The screening test was performed with a spot test in accordance with standardized protocols for phage identification [16, 17]. Briefly, phage samples were serially diluted in SM buffer (pH 7.0) to a final dilution of 10^{-8} . Overnight cultures of *P. aeruginosa* FKH-AP (GenBank accession no. PZ351230), *Acinetobacter bereziniae* TK8 (PX000546.1), *Shigella flexneri* FB3 (PZ384610), *Escherichia coli* TJ9 (PZ384611), and *Klebsiella pneumoniae* TB4 (PZ384612) were cultivated overnight and subsequently combined with 0.7% soft agar, which was then applied onto TSA plates utilising the double-layer agar technique. In the spot test, 10 µL of each diluted phage sample was applied to the bacterial lawn and allowed to desiccate under aseptic conditions. The plates were thereafter subjected to incubation overnight at a temperature of 37 °C. Samples producing clear lysis zones were selected for subsequent plaque assays.

Plaque Assay and Plaque Morphology

A plaque assay was conducted to isolate the phages and evaluate their titers in accordance with an established bacteriophage methodology [18]. Samples that showed positive results in the spot test were selected for plaque purification. Each filtered phage sample was serially diluted to 10^{-8} in SM buffer (pH 7.0). For the plaque assay, 100 µL of a culture of bacteria cultivated overnight was included with 4 mL of 0.7% soft agar and incubated at 45°C. An appropriate dilution of the phage suspension was then added to the bacterial-soft agar mixture, mixed thoroughly, and immediately overlaid onto TSA plates. The plates were incubated at 37°C for 18–24 h. After incubation, individual and distinct plaques were visually examined. Plates containing approximately 20–200 plaques were selected for plaque morphology assessment using a digital microscope (Jiusion Technology Co., Ltd., Shenzhen, China). Individual plaques were subsequently selected for purification through successive plaque-picking and re-plating. Phage titers were expressed as plaque-forming units per milliliter (PFU/mL) and calculated using the following formula:

$$\text{Phage titer} = \frac{\text{Number of plaques counted (PFU)}}{\text{Volume plated (in mL)}} \times \text{dilution factor}$$

Phage Precipitation

The bacteriophage-containing supernatants were precipitated with 5% PEG 6000 to increase bacteriophage concentration. The filtrates were precipitated with PEG 6000 (5%, w/v) and NaCl (0.5 M), then incubated at 4°C for 24 hours to increase bacteriophage concentration. The precipitated bacteriophages were harvested by centrifugation at 15,000 rpm for 30 minutes at 4°C, then washed three times with sterile SM buffer (pH 7.0). The bacteriophage pellets were washed in SM buffer and preserved at 4°C. Then, plaque assays were performed by the double-layer agar technique. Briefly, 100 µL of phage lysate was mixed with 100 µL of host bacterial suspension and 4 mL of soft agar (0.7% agar), then spread onto TSA plates. Plates were incubated at 37°C under aerobic conditions for 18–24 hours. Final bacteriophage titers were calculated as plaque-forming units per milliliter (PFU/mL) by plaque counting with a colony counter. Bacteriophage isolates with titers of no less than 1×10^9 PFU/mL were selected for TEM analysis.

Transmission Electron Microscopy Analysis (TEM)

TEM observations were used to describe virion morphology according to the criteria described by [19]. A 20 µL aliquot of each plaque-purified phage suspension was adsorbed onto carbon-coated copper grids for 5 min. Excess liquid was removed using filter paper, and the grids were negatively stained with 2% uranyl acetate for 20 s before air drying. Virions were examined using a Talos L120C transmission electron microscope (Thermo Fisher Scientific, The Netherlands) operated at 120 kV. Images were acquired at a magnification of 73,000x and 120,000x. Morphometric measurements included head length, head diameter, tail length, and tail diameter.

Results

Isolation of Bacteriophages, Evaluation of Plaque Type, and Precipitation of Phages

The present study successfully isolated two distinct phages from pond water (AC2) and sewage (FC2) collected from the swiftlet habitats in South Kalimantan. No phage-positive samples were detected among the soil specimens under the

screening conditions used. No lytic activity was observed against *Acinetobacter bereziniae* TK8, *Shigella flexneri* FB3, *Escherichia coli* TJ9, or *Klebsiella pneumoniae* TB4 in the spot tests. Thus, under the screening conditions used in this study, the recovered phages showed detectable lytic activity only against the *P. aeruginosa* FKH-AP host strain (Figure 1). Both samples were subjected to further examination by a plaque assay. The plaque assay showed two distinct phages with high titers (Table 1) against *P. aeruginosa* FKH-AP.

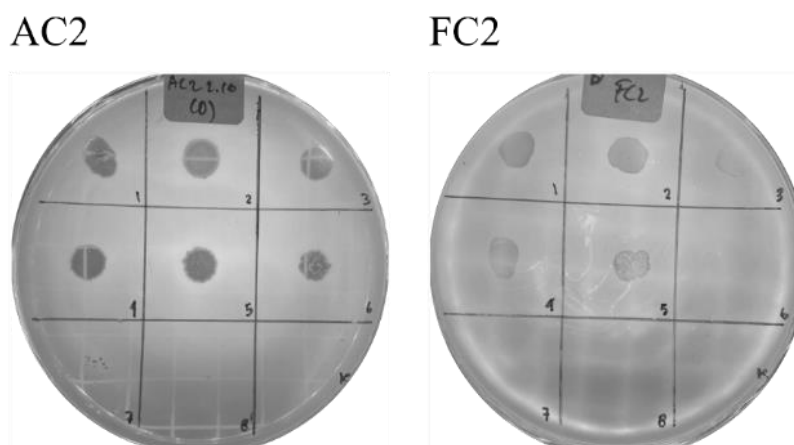


Fig. 1: The two positive samples of spot tests isolated from pond water (AC2) and sewage (FC2)

Table 1: Bacteriophage isolation from pond water and sewage of swiftlet environments in South Kalimantan

Sample ID	Specimen	Titer (PFU/mL)		Plaque type	Average plaque size (mm)
		T ₀	T ₁		
AC2	Pond water	5.1x10 ⁷	1.2x10 ¹⁰	Clear	2.1
FC2	Sewage	4.1x10 ⁷	1.8x10 ¹²	Clear	3.6

Note: titer before precipitation (T₀), titer after precipitation (T₁)

Plaque morphology differed between the two phage isolates (Table 1; Figures 2 and 3). AC2 produced smaller clear plaques, with an average diameter of 2.1 mm, whereas FC2 produced larger clear plaques, with an average diameter of 3.6 mm. Thus, the mean plaque diameter of FC2 was approximately 1.7-fold greater than that of AC2 under the assay conditions used. The clear plaque morphology observed for both isolates was consistent with lytic activity against *P. aeruginosa* FKH-AP. Figure 3 provides a comparative visualization of the plaque-size measurements for AC2 and FC2.

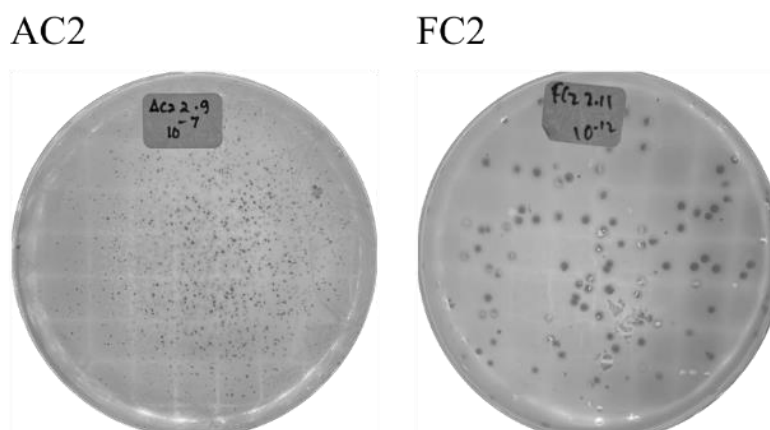


Fig. 2: The plaque assay results from AC2 and FC2 showed clear plaques

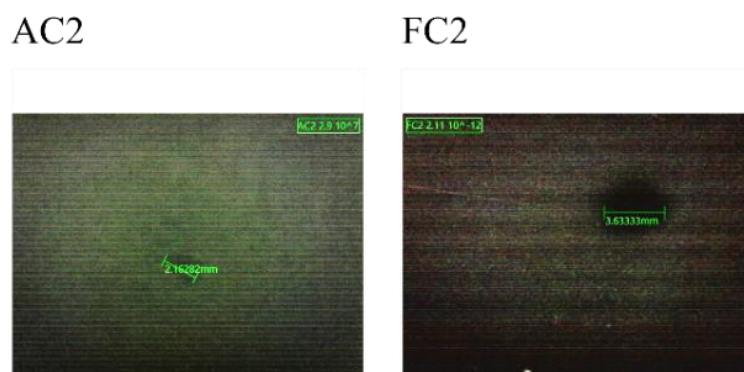


Fig. 3: Comparative measurement of plaque sizes produced by phage AC2 isolated from pond water and phage FC2 isolated from sewage on *P. aeruginosa* FKH-AP using digital microscopy. AC2 produced smaller plaques (mean diameter, 2.1 mm), whereas FC2 produced larger plaques (mean diameter, 3.6 mm)

TEM Analysis

Transmission Electron Microscopy (TEM) showed that phages AC2 and FC2 displayed similar virion morphology, defined by an icosahedral head and an elongated tail (Table 2, Figure 4). Phage AC2 showed a head length of 126.74 nm and a head diameter of 110.81 nm, as well as a tail diameter of 20.62 nm and a tail length of 177.63 nm. Phage FC2 showed a head length of 129 nm and a head diameter of 117 nm, as well as a tail diameter of 24.7 nm and a tail length of 170 nm. Based on TEM, phages AC2 and FC2 showed the typical morphology of Myoviridae.

Table 2: Virion morphology from pond water and sewage of swiftlet houses in South Kalimantan

Phages ID	hl (nm)	hd (nm)	td (nm)	tl (nm)	Morphotype
AC2	126.74	110.81	20.62	177.63	Myoviridae
FC2	129	117	24.7	170	Myoviridae

Note: head length (hl), head diameter (hd), tail diameter (td), tail length (tl)

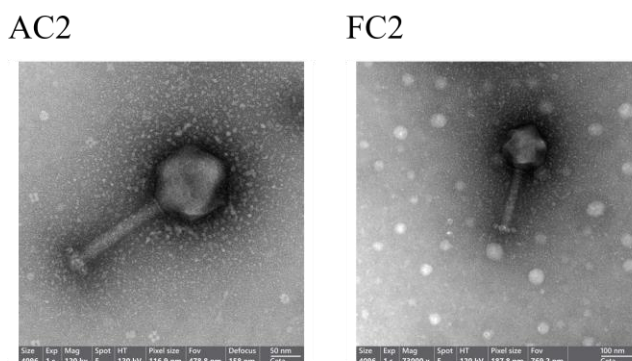


Fig. 4: Virion morphology of the representative phage isolates AC2 and FC2 using TEM

Discussion

The present study revealed the occurrence of bacteriophage activity against *Pseudomonas aeruginosa* in the environment of artificial swiftlet houses in South Kalimantan, Indonesia. From the 75 environmental samples analysed, two samples revealed phage activity (Figure 1) against *P. aeruginosa* FKH-AP. These positive samples originated from pond water (AC2) and sewage (FC2), indicating that *P. aeruginosa*-infecting bacteriophages can be detected in different aquatic compartments associated with swiftlet-house environments. Previous studies have also demonstrated that aquatic environments can harbor diverse bacteriophages infecting *P. aeruginosa* [20-22]. The recovery of phages from these environments is relevant because

artificial swiftlet houses represent a distinct animal-production ecosystem in which water, organic material, animal waste, and environmental surfaces may provide opportunities for bacterial persistence and phage host interactions [23]. Although the frequency of positive samples was relatively low, the findings provide baseline evidence of *P. Aeruginosa* infecting bacteriophages in an animal-production environment that has received limited attention in bacteriophage research.

The initial phage titers were 5.1×10^7 PFU/mL for AC2 and 4.1×10^7 PFU/mL for FC2. Following plaque purification and precipitation, the phage titers increased to 1.2×10^{10} PFU/mL for AC2 and 1.8×10^{12} PFU/mL for FC2 (Table 1). This finding is consistent with that of [24], which similarly reported that phage purification and concentration procedures yielded high-titer phage preparations suitable for subsequent characterization. The increase in phage titer following precipitation indicates that both phage isolates could be successfully enriched using *P. aeruginosa* FKH-AP as the host under the conditions used in this study. Both AC2 and FC2 produced clear plaques on the *P. aeruginosa* FKH-AP lawn (Figure 2, Table 1), with average plaque sizes of 2.1 and 3.6 mm (Figure 3, Table 1), respectively. The formation of clear plaques is compatible with active lytic replication under the assay conditions [25]. The larger plaques produced by FC2 compared with AC2 may indicate differences in plaque development; however, plaque size is influenced by several factors, including phage adsorption, replication rate, release of progeny virions, bacterial growth rate, agar concentration, and diffusion through the agar matrix [16]. In the present study, the plaque morphology primarily provides a phenotypic description of the recovered phage preparations.

The gold standard method to study bacteriophage characteristics is TEM [26]. In TEM morphological analysis, AC2 and FC2 belonged to the Myoviridae family of the Caudoviricetes (head size of 126.74-127x110.81-117 nm and a long contractile tail of 170-177.63 nm in length), which was similar to several other *P. aeruginosa* phages such as SL2 (head diameter: 120 nm and length tail: 170 nm) in previous study [27].

The recovery of *P. aeruginosa*-infecting bacteriophages from swiftlet-house environments is potentially relevant to veterinary microbiology and edible bird's nest production. *P. aeruginosa* can persist in aquatic environments and on environmental surfaces, and its ability to form biofilms may contribute to persistence in animal-production settings. The detection of naturally occurring bacteriophages targeting this bacterium indicates that phage-bacterium interactions may occur within the swiftlet-house ecosystem. In the future, genomic sequencing would be particularly valuable for determining whether the two isolates represent genetically distinct phages and for assessing their genome organization, taxonomic relationships, and potential safety characteristics. Overall, the findings provide phenotypic and morphological evidence of *P. aeruginosa*-infecting bacteriophages in pond water and sewage associated with artificial swiftlet houses in South Kalimantan.

Conclusion

This study confirmed the occurrence of *Pseudomonas aeruginosa*-infecting bacteriophage activity in pond water and sewage associated with artificial swiftlet houses in South Kalimantan, Indonesia. Two phage isolates, AC2 and FC2, were successfully recovered, producing clear plaques and high phage titers after purification and precipitation. TEM revealed icosahedral capsids and elongated contractile tails, consistent with the Myoviridae family of the Caudoviricetes. These findings provide phenotypic and morphological evidence of *P. aeruginosa*-infecting bacteriophages in the swiftlet-house environment and establish baseline information for further bacteriophage research in this animal-production system. Further studies involving broader host-range testing, one-step growth analysis, stability assays, and whole-genome sequencing are warranted to determine their biological characteristics, genetic diversity, taxonomy, biosafety, and potential applications.

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Author's Contributions

Siti Gusti Ningrum: Conceptualization, methodology, supervision, data curation, formal analysis, writing original draft, write review and edited.

Dyah Widhowati: Investigation, methodology, data collection, validation.

Novik Nurhidayat: Methodology, investigation, validation.

Titin Yulinery: Methodology, investigation, validation, data curation.

Evi Triana: Investigation, validation, data curation.

Yati Sudaryati Soeka: Methodology, validation, supervision, write review and edited.

Lusianawati Widjaja: Investigation, validation, write review and edited.

Arif Nur Muhammad Ansori: Formal analysis, validation, write review and edited.

Ethics

This study did not involve human participants or experimental animals and, therefore, did not require approval from an institutional ethics committee.

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