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Research Article
Isolation and Characterization of a Bacteriophage That Hosts on Avian-pathogenic *Escherichia coli* (APEC)

Haya Fakhouri, Orjowan Juneidi, Hana Al Mohtaseb, Murad Ishnaiwer and Fawzi Al-Razem

Applied Biology Program, Faculty of Applied Sciences, Palestine Polytechnic University, Hebron, Palestine

Abstract

Background and Objective: Infectious *Colibacillosis* disease caused by Avian-pathogenic *Escherichia coli* (APEC) commonly threatens poultry flocks worldwide. It limits productivity and causes severe economic losses to poultry sector. In addition, the emergence of multidrug resistance among APEC is an increasing concern since these resistant bacteria can infect humans and develop mechanisms that enable them to resist the commonly used antibiotics. The use of phages in treating pathogenic bacteria is considered a possible alternative to the conventional use of antibiotics. The aim of this study was to isolate and characterize APEC *E. coli* bacteriophage and to determine its cyclic replication through double layer test and one step growth curve. **Materials and Methods:** Bacteriophage was isolated from poultry feces samples using soft agar overlay method and was detected through the formation of clear zones on a lawn culture of APEC host bacterium on Luria Bertani (LB) agar plates. The phage total proteins analysis was carried out using SDS-PAGE through which samples were electrophoresed on a 10% polyacrylamide gel. The quantification of infectious viruses in bacterial suspension was determined through a one-step growth curve analysis using double layer test. The host range of the bacteriophage was further explored by performing spot tests with four APEC bacterial strains. **Results:** An APEC *E. coli* Bacteriophage was isolated. It was found to possess an apparently 17 kb genome size. The SDS-PAGE showed two major visible protein bands of 30 and 50 KDa. The phage was capable of lysing four tested APEC strains, an indicative of wide host range for the isolated bacteriophage. One step growth curve showed a phage latent period of about 24 h, burst period of 70 h and a burst size of about 2.4×10^4 plaque forming units (PFU) per plaque. **Conclusion:** A bacteriophage targeting four APEC strains has been isolated. It could be used to eliminate or reduce the scope of APEC infection in poultry and possibly used as an alternative to antibiotics.

Key words: APEC *Escherichia coli*, bacteriophages, burst period, burst size, *Colibacillosis*, latent period, plaque forming unit

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The poultry sector continues to be pressured by the increasing number of infections with multi-drug-resistant (MDR) bacteria. The emergence of antibiotic resistance in bacteria, particularly in the poultry sector, continues to challenge the treatment of these bacterial diseases, which carry concomitant risks for animal and human health. Many strains of bacteria have become increasingly resistant to the conventional therapeutic antibiotics due to the extensive increase in antibiotic consumption and the misuse of these antibiotics[1].

Several avian pathogenic *E. coli* (APEC) strains are intrinsically insensitive to antibiotics and cause severe and virulent infections in poultry. APEC has elevated the problems of the poultry industry due to several economic losses related to chronic respiratory diseases that may have been caused by APEC[2,3]. The APEC strains that are associated with severe fever and lesions of the airsacculitis/pericarditis and perihepatitis influence the growth rate and feed efficiency and reduce the egg laying and meat quality of infected individuals[2-4].

Avian colibacillosis is an opportunistic disease caused by APEC. It decreases production mainly by increasing the mortality rate in the poultry industry, which severely affects economic profits. Avian colibacillosis is characterized by extreme organ damage, such as salpingitis, synovitis, airsacculitis, pericarditis, osteomyelitis and peritonitis or yolk sac infection[5,6]. The symptoms of colibacillosis start with abdominal pain in birds, followed by watery diarrhea for approximately one day, which is then followed by bright red bloody stools for 2-5 days as an effect of the intestinal sores. In some cases, damage to the central nervous system may occur[5-7]. Several studies have connected APEC strains with extensive resistance to critically important antimicrobial compounds. These studies have demonstrated that resistant bacteria harbor extended-spectrum β-lactamase (ESBL) enzymes that provide resistance to most β-lactam antibiotics, including penicillin, cephalosporin and monobactam aztreonam[8-11].

In Palestine, a study confirmed the extreme resistance of *E. coli* to different combinations of antimicrobial agents[6]. A total of 66 APEC isolates from chickens with colibacillosis disease were used. The prevalence of resistance was 100% for tetracycline, 83.33% for ampicillin and amoxicillin, 80.3% for kanamycin, 72.72% for ciprofloxacin and 69.70% for neomycin. However, with the steady increase in the number of the MDR strains present among poultry pathogens, it has become quite difficult to eradicate these bacteria with commonly used antibiotics; this has driven the search for alternatives to combat antibiotic resistance. Bacteriophages have long been thought to be a possible alternative of conventional antibiotics in the treatment of bacterial infections[12-14]. Bacteriophages can be more specific than antibiotics and possibly be generated in a cost-effective manner. One advantage provided by phage therapy is the ability to specifically target only the host bacterial cells, while antibiotics may also kill a wide range of non-targeted bacteria in addition to the targeted harmful one[12,15].

Bacteriophages were first used as bactericidal agents between 1915 and 1917 when British and Russian bacteriologists first observed phage antibacterial activity against the *Vibrio cholera* bacterium, which causes cholera disease[16]. The discovery of antibiotics with well-understood mechanisms of action as opposed to the poorly understood mechanisms of the phage, caused the discontinuation of these studies[17]. However, Sulakvelidze *et al.*[18] successfully used phages to treat *E. coli* infections in mice and subsequent studies have demonstrated the ability of phages to fight human pathogenic bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus*, *Klebsiella*, *E. coli* and *Salmonella*[13,14,19,20]. Therefore, the discovery and isolation of new phages capable of lysing pathogenic bacteria represent a focal point of research interest. Furthermore, it is important to note that there are no reported cases of side effects associated with the use of phages unlike most synthetic and natural antibiotics which may cause side effects. The overall objective of this study was to isolate and characterize avian pathogenic E. coli bacteriophages that can lyse APEC poultry pathogens.

MATERIALS AND METHODS

Sample collection: The samples (sewage-chicken feces) were collected from Hebron City, Palestine and used for the isolation of bacteriophages. Four avian pathogenic *E. coli* (APEC) strains were obtained from the Palestine-Korea Biotechnology Research Center at Palestine Polytechnic University. The bacterial cultures were prepared by mixing 100 µL of the APEC stock cells that had been cultured overnight with 900 µL of LB broth (1 liter ddH$_2$O, 10 g tryptone, 5 g yeast extract and 10 g NaCl) to make the bacterial enrichment cultures; these were then used to host phage. From each sewage-feces sample, 30 mL was used to search for the presence of phages according to a previously published protocol[21].

Phage Isolation: The samples (sewage-chicken feces) were centrifuged for 10 min at 6000 rpm at room temperature and then the supernatant was filtered through a 0.22 µm

membrane filter to remove bacterial contaminants and debris before 20 mL of the filtered supernatant was mixed with 20 mL LB broth. To enrich the phage in the bacterial host, 1 mL of the APEC bacterial overnight culture from the stock was added and the culture was incubated overnight at 30°C in a shaker at 180 rpm[21,22].

Following incubation, each phage enrichment culture was filtered through a 0.45 μm membrane filter before 1 mL of the APEC stock and 10 mL of the LB broth was added to the 15 mL of the filtered enrichment culture. The mixture was incubated overnight at 30°C in a shaker at 160 rpm to enrich the phage a second time. To ensure efficient separation of the phage from bacteria during filtration, 200 μL chloroform was added to each stock for 10 m at room temperature before centrifugation for 5 min at 13000 rpm. The suspension was then centrifuged to remove the cell debris before filtration through a 0.45 μm membrane to remove bacterial cell debris, while phage particles were permitted to pass through the membrane[21,22]. The phage stocks were kept at 4°C during handling.

Bacteriophage lysis: The effects of the isolated bacteriophage were tested on four *E. coli* APEC strains by monitoring plaque formation to test whether the APEC strains exhibited phage resistance (no lysis) or sensitivity (lysis).

Each *E. coli* APEC strain was cultured on separate LB 1% agar plates (1 L ddH$_2$O, 10 g tryptone, 5 g yeast extract, 5 g NaCl and 10 g agar) and then 10 μl drops of the bacteriophage were spotted on the surface of the plates. As a control, separate plates containing only bacteria without phage filtrates was used. The tube contents were mixed, poured onto the surface of the LB agar plate and allowed to solidify. The plates were then incubated overnight at 37°C and examined for the presence of plaques. The lytic effects of the bacteriophage on the host cell were confirmed at least three times. Furthermore, the host range of the bacteriophage was investigated by testing the ability of the isolated phage to lyse four APEC strains (349, 519, 619 and 609) using the spot test.

One-step growth curve: The plaque-forming units (PFUs) were measured by the double-layer agar (DLA) method[23-26]. The plates contained a 1% agar layer coated with another soft layer (0.5%) of agar containing phage-bacteria stock. The number of phage particles released from bacteria was thereafter determined at different time periods via a plaque count protocol that included dilution of stock phage in SM buffer (Sodium chloride, Magnesium sulphate and gelatin Buffer) [5.8 g NaCl, 2 g MgSO$_4$ 7 H$_2$O, 50 mL 1 M Tris HCl (pH 7.4), 5 mL gelatin and 1 L ddH$_2$O]. This was done by mixing 100 μL of the isolated bacteriophage with 900 μL SM

buffer. Three different dilutions of phage sample (10^1, 10^2, 10^3) were prepared in sterile tubes for use in the second layer of the media plates. Each tube contained 100 μL APEC, 100 μL (used to make counting easier and clearer), 800 μL LB broth and 100 μL phage titer. Finally, 3 mL of molten LB agar was added to each tube after it was incubated for 40 min at 37°C. After mixing, the tubes were immediately poured onto pre-prepared 1% LB agar Petri dishes with one plate used as a control.

The phage-bacteria culture was first incubated at 37°C to ensure phage adsorption to the bacteria. Then, the soft agar was added under sterile conditions to avoid contamination with any other microorganism. Then, the plates were incubated and plaque formation was monitored and recorded. A plot of the plaque-forming units (PFUs) per ml against time in minutes was generated to establish a one-step growth curve using the following formula according to published, standard protocols:[27]

$$\text{PFU mL}^{-1} = \frac{\text{No. of plaques observed}}{\text{Dilution factor} \times \text{mL virus added}}$$

A one-step growth curve was generated to investigate the nature of the viral replication by infection of the host bacteria[25]. This approach is commonly used to measure the quantity of the infectious virus present. The titer of a virus stock can be calculated in plaque-forming units (PFUs) per milliliter. To test the virus titer, the plaques were counted and the titer calculation in PFU mL^{-1} was acquired by dividing the number of plaques observed by the product of the dilution factor and the inoculum volume[28].

Bacteriophage genome isolation: The genome of the bacteriophage was isolated according to a published protocol with minor modifications[29]. To isolate the genome, 500 μL of the culture containing the phage was mixed with 1000 μL of saturated ammonium sulfate containing 0.1% of 2-mercaptoethanol to protect the phage genome from degradation[30,31]. The supernatant was removed after centrifugation at 13,000 rpm for 8 min at 4°C. The phage genomes were liberated from DNA and RNA binding proteins by dissolving the resulting pellet in 200 μL of 1% SDS and 200 μL of 0.5 N NaOH and centrifuging at 13,000 rpm for 5 min to denature and precipitate the phage proteins.

To the clear supernatant (700 μL), 420 μL of 0.4 isopropanol was added to precipitate the genome. Then, 400 μL of 3 N sodium acetate buffer was added and the solution was left for 15 min at room temperature. To remove the binding proteins from the phage genome, the mixture was centrifuged at 13,000 rpm for 10 min and the resultant pellet

was incubated with 700 µL of proteinase K (100 µg mL^{-1}) at 37°C for 30 min. Finally, the phage genome was precipitated using 70% ethanol and the pellet was collected in 0.2 mL TE buffer after 2 min centrifugation at 8,000 rpm. The phage genome was then loaded onto an agarose gel with a 10 kb ladder and the electrophoresis apparatus was run at 80 V for 35 min.

SDS-PAGE: Bacteriophage total protein analysis: The SDS-PAGE was carried out according to Sambrook and Russel[32], with modifications as described by Ishnaiwer and Al-Razem[21] and Sangha *et al.*[33]. Briefly, the purified phage particles were subjected to SDS-PAGE and the proteomic patterns were detected following staining with 0.1% (w/v) Coomassie Brilliant Blue. The concentrated phage samples were equally mixed with 2×SDS loading buffer and 17 µL of mixture was heated and loaded on SDS-PAGE along with 5 L of a broad-range molecular weight protein ladder. The gel electrophoresis was allowed to run first at 80 V for 1 h and then at 120 V for 1:20 h. The protein sample was denatured and coated with detergent by heating in the presence of SDS and a reducing agent. The molecular weights of the proteins were estimated by comparing their migration to that of the protein standards in the molecular weight ladder.

RESULTS

Isolation of phage filtrates: When screened, the phage filtrates showed clear round plaques, each approximately 2 cm in diameter. The phage that was able to form plaques was named PPU3 (Palestine Polytechnic University 3) (Fig. 1).

The phage PPU3 isolate was plaque purified 3 times and shown to lyse all four of the tested antibiotic-resistant APEC strains (Table 1).

Characterization of the isolated bacteriophages genome: The genome of the phage was nearly 17-20 kb in size (Fig. 2).

One-step growth curve of the isolated bacteriophages: By monitoring the LB agar plates subjected to a double-layer plaque assay, plaques with different small transparent zone sizes were counted for analysis (Fig. 3).

Table 1: Host range of the isolated bacteriophage in four strains of avian pathogenic *Escherichia coli*

APEC strain	Lysis/No lysis
APEC 349	Lysis
APEC 519	Lysis
APEC 619	Lysis
APEC 609	Lysis

The data from the plaque assays were analyzed and the number of the PFUs per plate was plotted on the Y-axis against the time required for plaque formation on the X-axis. (Fig. 4).

The latent period for the PPU3 bacteriophage was approximately 12 h, the burst period was 70 h and the burst size was approximately 24,000 PFUs per plate.

SDS-PAGE: Bacteriophage total protein analysis: The whole-cell protein profiles of the bacteriophages showed two main visible protein bands at 30 kDa and 50 kDa (Fig. 5).
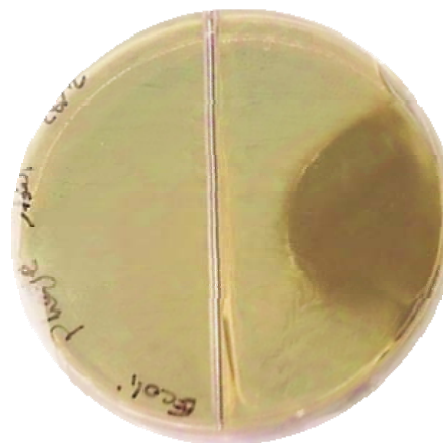

Fig. 1: Bacterial culture on LB agar plate showing bacteriophage lysis
Phage (PPU3) filtrate (10 µL) was added to each bacterial plate. Lysis appeared as a clear yellow circle (right side). The left side is a control with the host bacteria but no added phage
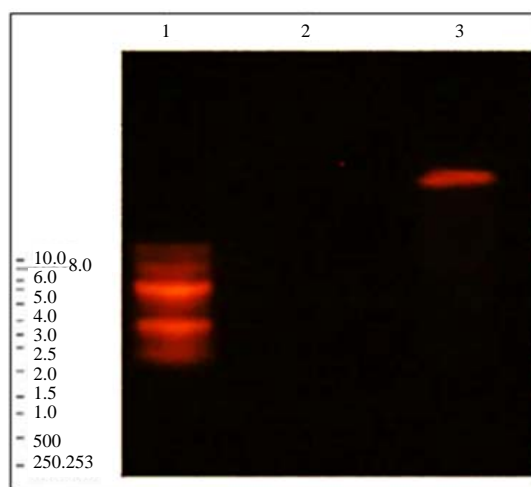

Fig. 2: Size of the PPU3 bacteriophage genome
The genome is approximately 17-20 kb. Lane 1: 10 kb ladder. Lane 2: negative control (master mix with no DNA template). Lane 3: Phage PPU3 genome
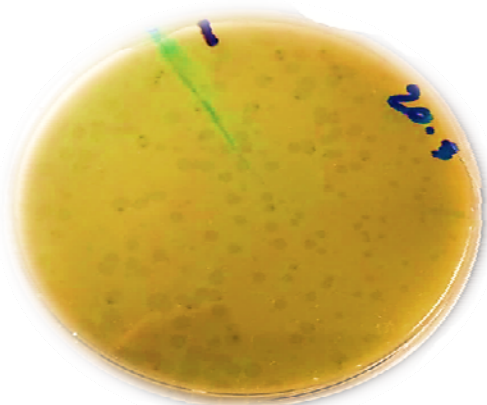
Fig. 3: Plaque assay results of the PPU3 bacteriophage using the double-layer method
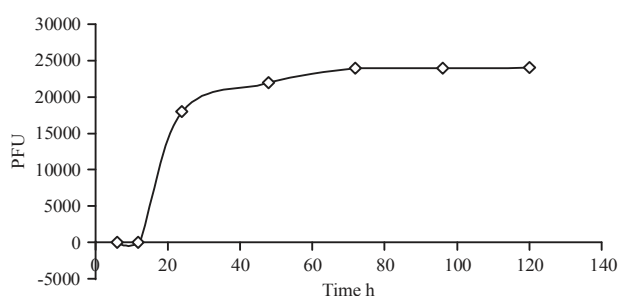The results shown were obtained from the first fold dilution

Fig. 4: The curve shows the burst period and burst size for the PPU3 phage during its replication in host bacteria
The curve was generated by counting the number of formed plaques per plate over time. The results are the measurements of three time trials
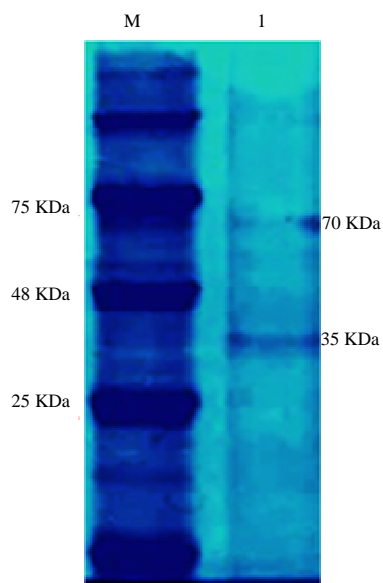

Fig. 5: SDS-PAGE analysis of the phage proteins
M: Protein ladder, Lane 1: PPU3 phage proteins

DISCUSSION

Characterization of bacteriophage: The high incidence and severity of colibacillosis diseases among poultry birds has become a major concern. This disease is caused by the avian pathogen APEC and has been an increasing and persistent problem in the poultry sector. This study describes the isolation of a novel antibacterial bacteriophage from poultry feces, which exhibited profound lytic effects on the APEC bacteria (Fig. 1). Many bacteriophages have been shown to effectively lyse target pathogenic bacteria, indicating high specificity for their respective host bacteria[34,35]. The isolated bacteriophage showed high efficacy in killing APEC bacteria during the *in vitro* experiments based on its ability to produce clear, circular plaque zones. Several studies of different phages have confirmed that phage therapy is effective against pathogenic bacteria in broilers, particularly against *E. coli*[36-38]. This study showed that it might be possible to use the isolated bacteriophage to treat colibacillosis in infected birds in future therapeutic applications.

Bacteriophages that use APEC as hosts are expected to be found in the pathogen's own environment. Poultry feces and sewage systems contain a large diversity of phages. To isolate specific APEC phages, filtered sewage samples should be incubated with bacterial cultures in the exponential phase of growth. This process of phage enrichment is critical to enhance the isolation and concentration of phages that can be tested against host bacteria. The host range tests carried out in these studies showed that the isolated phage had a broad host range and exhibited lytic activity against 4 different APEC strains. The specificity of the bacteriophage against target bacteria probably is a function of the ligands of phage receptors located on the bacterial host cell surfaces, which are easily recognizable for phage adsorption[39]. Similar studies have also shown broad effects of isolated bacteriophages against APEC. Jamalludeen *et al.*[40] reported the efficacy of two bacteriophages in lysing 100% of serotype O1, O2 and O78 strains, while *Oliveira et al.*[41] described the ability of two isolated phages, "phi258E and phiF61E", to lyse up to 45% of the 148 isolated APEC bacteria. Overall, the reported studies support a trend suggesting the use of such bacteriophages in the therapeutic treatment of poultry infected with APEC over the use of conventional antibiotics.

On the other hand, our results indicate that the PPU3 bacteriophage has a genome size of nearly 17-20 kb, as observed on agarose gel with a 10 kb ladder. Further differentiation tests need to be conducted to determine whether the genome is ssDNA/dsDNA or ssRNA/dsRNA. Several studies have demonstrated that *E. coli* bacteriophages

typically contain dsDNA. Several previous studies have addressed the isolation and characterization of the *E. coli* phage genome. Our PPU3 phage has a smaller genome compared with that of phages previously isolated from *E. coli* bacteria.

Jamalludeen *et al.*[40] described two Myoviridae (EC-*Nid1* and EC-*Nid2*) phages that were active against avian colibacillosis strains (O1, O2 and O78) with genome sizes of approximately 67.06 kb for EC-Nid1 and 68.04 kb for EC-Nid2. Shahrbabak *et al.*[42] characterized the *Escherichia coli* O157:H7-PhaxI DNA phage with a genome size of 156628 bp. Furthermore, a T4 phage that infects *E. coli* bacteria has been shown to possess a double-stranded DNA genome of 168,903 bp[43].

The latent period, burst period and burst size of the phage are commonly used for infected bacteria cell calculations[26,44-47]. We estimated such parameters by monitoring visible plaque formation. One viral particle triggers the formation of each plaque consisting of up to 10^9 virions[48]. Each plaque formed as a result of bacteriophage infection initiated its lytic reproductive cycle and spread overtime to the surrounding cells. Several factors have been described that influence plaque formation[49], including optimization of incubation and plating conditions. These conditions are required to allow bacteriophage adsorption to the host bacteria. The 12 h latent period of the isolated phage is apparently long. It represents the interval between the adsorption of the phages to the bacterial cells and the release of enough phage progeny to form a visible plaque. Shorter latent periods indicate effective host infection and strong phage lytic activity. There are reports of long latent periods for other phages, however, such as for those observed in adenoviruses[50]. It could be contributed to the slow adsorption of phage to host cells and by a subsequent slow release into the medium. In contrast, the burst size of the phage was determined as the ratio of the final number of plaque particles to the number of infected bacterial cells during the latent period[26]. Litt and Jaroni[46] isolated seven *E. coli* bacteriophages that showed a short latent period between 12 and 30 min and a large burst size (89-631 virions per infected cell). Another short latent period of 25 min was reported by Park *et al.*[47] for the SFP10 phage host in *E. coli* O157:H7. The burst size, in contrast, was apparently large (100 PFU per cell).

The SDS-PAGE detected only two major protein bands corresponding to the molecular weights of approximately 35 and 70 kDa and no clear minor proteins were detected. Although we detected well-separated proteins, it is not clear from this work whether the protein bands represented an aggregate of more than one polypeptide. In addition, the possibility of the existence of further minor phage proteins cannot be ruled out. Ayman *et al.*[50] described the protein profile of the *E. coli* bacteriophage and reported three major bands with molecular weight masses of 47, 35 and 16 kDa, while Ucan et al.[51] reported that whole-cell protein extracts of *E. coli* strains produced patterns of bands with molecular weights of 6, 500-200 kDa and 000 kDa. The 35 kDa band is a commonly shared between many bacteriophages and studies have also indicated that this band may correspond to a capsid protein[49,50].

The extensive application of antibiotics has resulted in large-scale resistance of APEC bacteria to antibiotics. One potential solution is the use of lytic phages to control bacterial infections. The isolated bacteriophage reported in this study can be used as a therapeutic biocontrol agent in poultry infected with APEC bacteria. This phage also has the potential for use in future biotechnology research as a specialized vector for efficient delivery of genomes and proteins into host cells. However, it is still not clear whether bacteriophages could completely replace antibiotics. Several limitations have affected phage *in vivo* tests. For example, the response of the immune system poses a serious concern as it might swiftly eliminate bacteriophages. Furthermore, the phages would need to be stable and bioactive in simulated and real gastrointestinal environments in order to serve as a successful therapeutic agent. Therefore, *in vivo* trials are needed to assess any drawbacks in the use of phages as therapeutic agents.

CONCLUSION

The uncontrolled use of antibiotics, particularly in the poultry sector, has increased multidrug resistance among poultry pathogenic bacteria. The newly isolated phage (PPU3) may be an effective agent in the effort to decrease the incidence of colibacillosis in poultry. Further characterization of the phage and determination of optimal conditions would significantly enhance the potential for the use of this phage *in vivo* trial. This study offers a base for future experiments with avian pathogenic *E. coli* phages and adds concomitant knowledge for the evaluation of phage activity for biocontrol treatments in broilers.

SIGNIFICANCE STATEMENT

There is a steady increase in the number of multi-drug resistance pathogens, particularly in livestock and poultry farms. This study supports the trend to search for alternatives to antibiotics. The isolated bacteriophage in this study was

tested on 4 avian pathogens and proven effective in lysing the bacterial cells. It is a first step that will be followed by farm trials to use this phage as an alternative to antibiotics and commercially producing it.

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