Original Article
Isolation of lytic bacteriophages against pathogenic
Escherichia coli strains in poultry in the northwest of Iran

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ABSTRACT
In this study, 90 internal organ samples of poultry with symptoms of colibacillosis were obtained from Maragheh poultry farms in East Azerbaijan, Iran. In total, 70 bacterial isolates were confirmed as Escherichia coli (E. coli) strains using standard biochemical tests, and antibiotic sensitivity was determined by the disk diffusion method. Antibiotics used in this study included ampicillin, penicillin, nitrofurantoin, tetracycline, amoxicillin, ciprofloxacin, nalidixic acid, and sulfamethoxazole (n=8). Ciprofloxacin showed the highest susceptibility, while the lowest susceptibility was observed with penicillin and amoxicillin. Among the bacterial isolates, 50% showed resistance to at least five antibiotics, and 10 isolates with multidrug resistance were selected for bacteriophage (phage) isolation against recent E. coli isolates using spot test and double-layer agar overlay technique. In addition, water samples for phage isolation were provided from rivers, poultry farm sewages, and an urban sewage treatment center. In total, eight phages were successfully isolated from the urban sewage treatment center (total: 10). After enrichment, purification and titration, phages were further concentrated by polyethylene glycol precipitation. Lowest and highest bacteriophage titers were determined to be $1.05 \times 10^6$ and $1.9 \times 10^9$ PFU/ml, respectively. Host range of the isolated phages was assayed by spot testing, and antibacterial effects against four E. coli isolates were observed in one of the isolated phage suspensions, which was introduced as the most potentiated agent for phage therapy. In the morphological analysis of the selected phage using an electron microscope, we observed a hexagonal head with a diameter of 95 nm and contractile tail length of 90 nm, which indicated its similarity to the Myoviridae family. In conclusion, results of this study showed that bacteriophages could be appropriate alternatives to combat pathogenic E. coli strains with antibiotic resistance in poultry. Considering the changeable antibacterial effects of bacteriophages against different isolates of extraintestinal avian pathogenic E. coli, it is suggested that future investigations be conducted regarding the efficacy of lytic phages against different bacterial strains for the effective control of the associated infections in this region of Iran.

Keywords: Bacteriophage, Colibacillosis, Antibiotic resistance, Escherichia coli, Poultry

Isolation des bactériophages lytiques agissant contre les souches pathogènes d’Escherichia coli chez les volailles du nord-ouest de l’Iran

Résumé: Dans cette étude, 90 prélèvements d’organes internes de volailles montrant des symptômes de colibacillosse ont été obtenus des exploitations avicoles de la ville de Maragheh, située dans l’Est Azerbaïdjan (Iran). Au total, 70 isolats bactériens ont été identifiés comme étant des souches d’Escherichia coli (E. coli) par des tests biochimiques standard. La sensibilité aux antibiotiques des isolats a été ensuite déterminée par la méthode de diffusion sur disque. Les antibiotiques utilisés étaient l’ampicilline, la pénicilline, la nitrofurantoin, la tétracycline, l’amoxicilline, la ciprofloxacine, l’acide nalidixique et le sulfaméthoxazole (n=8). Les isolats étudiés montraient une sensibilité plus importante vis-à-vis de la ciprofloxacine alors que le penicilline et le amoxicilline représentaient les deux antibiotiques les moins performants. Parmi les isolats bactériens, 50% étaient résistants à au moins 5 antibiotiques. Dix isolats multi-résistants ont été sélectionnés pour l’isolement des bactériophages par le biais de test ponctuel (spot test) et par la méthode de double
**Introduction**

*Escherichia coli* (E. coli) are commensal gram-negative bacteria in human and animal intestine, which are considered harmless to their host. However, colibacillosis is a severe infectious disease occurring outside chicken intestine, which is triggered by some strains of these bacteria, known as extraintestinal avian pathogenic *E. coli* (APEC) (Zhao et al., 2005). Colibacillosis has been associated with a heavy economic burden, high treatment costs, and reduced production (Roy et al., 2006). To control colibacillosis in poultry, in addition to antibiotic treatment in case of infectious diseases, subtherapeutic antibiotic doses are commonly fed in order to promote growth and compensate for stressful conditions. However, inappropriate or excessive use of antibiotics could lead to the emergence and spread of resistant bacterial strains. On the other hand, APEC strains isolated from poultry are closely related to other extraintestinal pathogenic *E. coli* strains causing infections in humans, such as uropathogenic *E. coli* and neonatal meningitis-causing *E. coli* (Ewers et al., 2007). Presence of APEC strains forms a reservoir of virulence and resistance genes for other *E. coli* strains, rendering it a major health concern in humans. Since the antibiotic era, these antimicrobial agents have been used as the primary defense against bacterial infections. Recently, several reports have been published focusing on the reduced effectiveness of antibiotics in the treatment of common infections, especially in urgent cases (Laxminarayan et al., 2015). Considering the emergence of drug-resistant bacteria causing infectious diseases, as well as the growing concern regarding the failure of antibiotic drug discovery pipeline, introducing proper alternatives to conventional antibiotics is of paramount importance (Sulakvelidze et al., 2001). Bacteriophages (phages) are viruses that infect bacteria. These agents are non-hazardous and self-replicating, which increase in number with the destruction of the target bacteria. Bacteriophages are the most abundant entities on earth (Clark and March, 2006), and extensive research has been conducted regarding their applications. Potentiality to kill bacteria could undoubtedly candidate phages as major therapeutic agents. For instance, virulent phages, which are classified as natural antimicrobial controlling agents, are remarkably effective in the treatment of bacterial infections and removal of bacterial biofilms (Jassim et al., 2012). Considering the advantages of phages over antibiotics, rising trend of antibiotic resistance in bacterial pathogens, and low rate of discovering new and clinically effective antibiotics, biocontrol programs have confirmed the strong potential of phages as effectual antibacterial agents in the prevention and treatment of infectious diseases in humans and animals. Furthermore, several available phage products are applied for the prevention and treatment of intestinal infections (Krylov et al., 2012).
while phage cocktails are used against *Listeria* strains in the food industry (Housby and Mann, 2009). This study aimed to isolate and enrich lytic bacteriophages against antibiotic-resistant APEC strains in Maragheh, located in East Azerbaijan province (Iran), and evaluate their antibacterial efficacy and host range in order to design an effective phage therapy package to control colibacillosis in this region.

**MATERIALS AND METHODS**

**Isolation of avian pathogenic *E. coli* (APEC) strains.** In this study, 90 internal organ samples were collected from different broiler farms in Maragheh, Iran during August 2014-2015. All *E. coli* strains were isolated from broiler carcasses with typical postmortem lesions of colibacillosis. Samples from the heart, blood, liver and lungs of all the broiler chickens were immediately plated on MacConkey agar and blood agar. Cultured plates were incubated overnight at the temperature of 37 °C, and bacterial isolates were confirmed as *E. coli* using standard biochemical tests (Quinn et al., 1994). Afterwards, *E. coli* isolates were stored at the temperature of -20 °C.

**Antibiotic susceptibility and selection of bacterial isolates.** All the collected *E. coli* isolates were evaluated in terms of antibiotic susceptibility using the disk diffusion method (Bauer et al., 1966). Antibiotic disks used in this study included ampicillin, penicillin, nitrofurantoin, tetracycline, amoxicillin, ciprofloxacin, nalidixic acid, and sulfamethoxazole (Padtan Teb, Iran). Bacterial isolate suspensions in trypticase soy broth (TSB) were adjusted to a 0.5 McFarland standard. Bacteria were spread on the entire surface of Mueller-Hinton agar plates (Merck, Germany) using sterile cotton-wool swabs to stir the bacterial suspensions. In addition, antibiotic disks were placed on cultured Mueller-Hinton agar. After incubation at the temperature of 37 °C (24 h), growth inhibition zones were measured on the plates, and the isolates were determined as resistant, intermediate or sensitive to antibiotics in accordance with the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS). Finally, 10 multi-resistant bacteria were subjected to phage isolation assay and stored at the temperature of -20 °C.

**Isolation, detection and titration of bacteriophages.** Isolation of phages against 10 *E. coli* isolates was performed using water samples collected from different sources (rivers, poultry farms sewages, and an urban sewage treatment center) during August-March 2015. Initially, 50 ml of each sample was centrifuged at 1000 g for 5 min. After the filtration of centrifuged samples using a 0.45-µm Millipore Membrane Filter, 5 ml of concentrated TSB medium and 5 ml of 6-h incubated antibiotic-resistant bacterial suspension were added to 45 ml of the filter-sterilized sample. For the optimal attachment of the bacteria to phages, 70 µl of 1% MgSO4 (v/w) was added, and the mixture was shaken at the temperature of 37 °C for 24 h. The solution was mixed with 3 ml of chloroform, shaken for 20 min, and placed at room temperature for 2 h. In the next step, centrifugation was carried out (30 min, 1500 g), followed by the isolation and filtration of the supernatant with a 0.45-µm membrane. To increase the phage titer, the procedure was repeated twice from cocultivation to filtration (Karamoddini et al., 2011). Phage activity against 10 *E. coli* isolates was investigated based on a spot test (Chang et al., 2005) and plaque assay using a double-layer agar overlay technique as previously described (Adams, 1959). In the double-layer agar overlay technique, 100 µl of the filtered suspension and 1 ml of the 6-h incubated *E. coli* culture were added to 3 ml of soft trypticase soy agar (TSA) with 0.7% agar, and mixed and transferred to TSA medium plates. In the spot test, 10 µl of each phage lysate was dropped on a lawn of fresh bacterial suspension in soft TSA medium (0.7%). Purification of the isolated phages was performed based on the study by Jun et al. (2013) with some modifications. In this process, a single phage plaque was harvested from the agar overlay plate using a sterile Pasteur pipette. Afterwards, 5 ml of TSB medium was added to the harvested single plaque, which was incubated for 2 h at the temperature of 37 °C. The mixture was centrifuged
at the temperature of 4 °C for 15 min in 14000 g and filtered through a 0.45-µl filter. Co-cultivation of the phages and host *E. coli* isolates was performed by adding 100 µl of the filtered suspension to the 6-h *E. coli* culture (1 ml) and incubation of the resultant at the temperature of 37 °C for 24 h. Moreover, single plaque isolation steps were repeated three times. For phage suspension titration, phage samples were diluted 10x in tubes containing TSB, and double-layer agar overlay technique (Adams, 1959) was carried out as previously described. Following that, the plates were incubated at the temperature of 37 °C for 24 h. Number of the phages was calculated in the plates containing 30-300 phage plaques, and the titer was expressed as PFU/ml.

After the filtration of the obtained phage suspension, the phages were further concentrated and purified by polyethylene glycol (PEG) precipitation. To do so, PEG 8000 and NaCl (7/5 ml 20% PEG 8000, 2/5 M NaCl) were added to the phage suspension, which was incubated at the temperature of 4 °C overnight and centrifuged in 4000 g for 5 min. Finally, purified bacteriophages were stored in SM buffer (50 mmol/L Tris-HCl, pH: 7.5; 0.1 mol/L NaCl; 8 mmol/L MgSO4) with 0.01% gelatin (pH: 7.2) at the temperature of 4°C (Ahmadpour et al., 2016).

**Morphological examination of bacteriophages via electron microscopy.** One isolated phage with the highest host range was selected for morphological examination via electron microscopy. Phage morphology was assessed using a transmission electron microscope (TEM) with negatively stained preparations. In addition, a drop of approximately $10^{10}$ PFU/ml was deposited on the surface of a formvar-coated grid (200 mesh copper grid) stained with 2% uranyl-acetate and examined using a Zeiss LEO 906 TEM operating at 100 kV (Zeiss Company, Germany).

**RESULTS**

In this study, 77 gram-negative and lactose-fermenting colonies were distinguished from 90 samples with colibacillosis in broiler flocks. In total, 70 isolates were confirmed as *E. coli* based on standard biochemical tests, which were subjected to antibiotic sensitivity test. According to the results of antibiotic susceptibility, the lowest and highest isolate resistance was observed with ciprofloxacin (0%) and penicillin and amoxicillin (100%), respectively. Antibiotic resistance percentage of the studied bacterial isolates is depicted in Figure 1.

![Figure 1. Drug resistance percentage of *E. coli* isolates to antibiotics](image)

According to the results of this study, the majority of bacterial strains were resistant to more than three antibiotics. Moreover, 50% of the *E. coli* isolates were resistant to five or more antibiotics, and 10 bacterial isolates were selected from distinctive poultry farms.
with resistance to more than four antibiotics. Patterns of multidrug resistance of the selected bacterial isolates are shown in Table 1.

Table 1. Antimicrobial resistance patterns of selected E. coli isolates (n=10) (*resistance patterns)

<table>
<thead>
<tr>
<th>Resistance patterns*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Am, P, TE, AMX, FM, NA, SXT</td>
</tr>
<tr>
<td>2 Am, P, TE, AMX, NA, SXT</td>
</tr>
<tr>
<td>3 Am, P, TE, AMX, NA, SXT</td>
</tr>
<tr>
<td>4 P, TE, AMX, NA, SXT</td>
</tr>
<tr>
<td>5 P, TE, AMX, NA, SXT</td>
</tr>
<tr>
<td>6 P, TE, AMX, NA, SXT</td>
</tr>
<tr>
<td>7 Am, P, TE, AMX, SXT</td>
</tr>
<tr>
<td>8 Am, P, TE, AMX, SXT</td>
</tr>
<tr>
<td>9 P, TE, AMX, SXT</td>
</tr>
<tr>
<td>10 P, AMX</td>
</tr>
</tbody>
</table>

In the next step, phage isolation against the selected bacteria was carried out, and 8 out of 10 phages were isolated from the samples collected from the urban sewage treatment center, labeled P1-P10. After phage isolation by spot testing and double-layer agar technique, the plaques (diameter: 1-2 mm) appeared on the plates (figures 2a & 2b). Results of phage titration to determine the number of phages in each suspension after enrichment are presented in Table 2. Purification of the phages was performed to prepare the effective suspension against E. coli isolates in poultry if needed. Afterwards, all the isolated phages (n=10) were precipitated using PEG, and obtained pellets were stored at the temperature of 4 °C. According to our findings, P1, P2, P3, and P10 had bactericidal effects against one bacterial isolate. Furthermore, antibacterial characteristics of P5, P6 and P7 were observed against two E. coli strains, while P4 and P9 exhibited antibacterial effects against three bacterial strains, and P8 showed this property against four bacterial isolates. The selected bacteriophage was examined by TEM, with head measuring diameter of 90-95 nm, a hexagonal outline, a contractile tail of 10 nm in diameter and length of 90 nm. Based on the morphological features, the phage was classified as the Caudovirales order and Myoviridae family (Figure 3).

Table 2. Titration results of isolated phages against E. coli isolates

<table>
<thead>
<tr>
<th>Phages</th>
<th>E. coli isolates</th>
<th>PFU/ml</th>
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<tbody>
<tr>
<td>Phages</td>
<td>P1</td>
<td>Iso1</td>
</tr>
<tr>
<td>P2</td>
<td>Iso2</td>
<td>1.9×10^8</td>
</tr>
<tr>
<td>P3</td>
<td>Iso3</td>
<td>1.05×10^6</td>
</tr>
<tr>
<td>P4</td>
<td>Iso4</td>
<td>3×10^6</td>
</tr>
<tr>
<td>P5</td>
<td>Iso5</td>
<td>2.6×10^7</td>
</tr>
<tr>
<td>P6</td>
<td>Iso6</td>
<td>10^6</td>
</tr>
<tr>
<td>P7</td>
<td>Iso7</td>
<td>10^6</td>
</tr>
<tr>
<td>P8</td>
<td>Iso8</td>
<td>3×10^7</td>
</tr>
<tr>
<td>P9</td>
<td>Iso9</td>
<td>2×10^6</td>
</tr>
<tr>
<td>P10</td>
<td>Iso10</td>
<td>1.3×10^7</td>
</tr>
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DISCUSSION

Colibacillosis is a significant problem in poultry flocks, starting as a respiratory infection and rapidly turning into a systemic complication. It is one of the
most fatal diseases in the northwest of Iran, as well as many other regions around the world. Despite the scarce data regarding the unresponsiveness of antibiotics in the treatment of colibacillosis in this region, this disease is associated with a heavy economic burden annually. Recent emergence of new antibiotic-resistant strains of bacteria has become a major clinical and public health concern. Although distribution mechanism of antibiotic resistance from animals to humans is controversial, historical evidence suggests animals as the main reservoir for human E. coli (Cooke et al., 1971). Furthermore, several reports have been indicative of the spread of antibiotic resistance in E. coli plasmids from chicken to human, noting the significant risk of E. coli transmission to human health, which requires the use of antibiotics (e.g., phages) (van den Boggaard et al., 2001). Emergence of antibiotic-resistant bacterial strains in poultry is considered a substantial risk to the poultry industry, as well as a critical healthcare concern in humans. Given the possibility of failure in the discovery of antibiotic drugs and growing interest in the investigation of biological antimicrobial agents due to the awareness of harmful chemical agents, development of new alternatives to conventional antibiotics is of paramount importance (Sulakvelidze et al., 2001). Based on our findings, E. coli bacteria were isolated from most of the poultry cases with colibacillosis. High frequency of multidrug-resistant isolates of E. coli is an alarming public health issue. In the current research, numerous bacterial isolates showed resistance to four or more antibiotics. Consequences of this antibiotic resistance would pose great risk to poultry farms and human health due to the possibility of its direct or indirect transfer to humans. In the scientific literature, phages have been described as biological therapeutic agents for the treatment of clinical bacterial infections (Goodrige and Abedon, 2003). The first evidence to reveal the efficacy and safety of therapeutic phages was published in 2009 to combat antibiotic resistance in Pseudomonas aeruginosa in a case of chronic otitis (Wright et al., 2009). These valuable antimicrobial candidates have unique features, such as the ability to bind to specific surface receptors to enter the bacteria, capacity to disrupt bacterial metabolism, being harmless for animals, plants and environment, preventing the destruction of healthy flora, and inexpensive and easy preparation process. Among the other prominent characteristics of phages are immunomodulatory effects (Mankiewicz et al., 1974) and inhibitory properties against tumor growth (Dabrowska et al., 2004). In animal husbandry, remarkable success has been achieved with regard to the antibacterial features of phages (Biswa et al., 2002; Hawkins et al., 2010). For instance, phages have been shown to be significantly effective in the control and management of food-borne diseases. Moreover, some studies have indicated that fatal E. coli respiratory infections could be prevented through phage therapy in broiler chicken (Huff et al., 2002a; Huff et al., 2002b). One of the most notable advantages of phage therapy is the reduced use of chemical agents against pathogens (Fujiiwa et al., 2011). Recent approaches of phage therapy involve the increasing counts of these agents in response to the incidence of pathogens, effectiveness against antibiotic-resistant bacteria, capability to remove bacterial biofilms, food bioprocessing (Jassim et al., 2012), and low toxicity (Merril et al., 2003; Jassim et al., 2012). Furthermore, researchers have focused on the application of phages for various therapeutic and non-clinical purposes (Kutter et al., 2010). Lytic life cycle of phages does not involve horizontal gene transfer (Hendrix, 2003). As such, use of the lytic cycle of bacteriophages is befitting to phage therapy against bacterial infections. Recently, the dried forms of phages have been successfully applied as respirable powders in the treatment of human pulmonary infections (Matinkhoo et al., 2011). Phages have been proposed as remarkable biological control agents of animal bacterial infections, particularly in the prevention of E. coli respiratory infections in broiler chicken (Biswa et al., 2002; Hawkins et al., 2010). Moreover, phages specific to antigens have been used to control E. coli O157:H7 (Kudva et al., 1999). Concentrations of Campylobacter
Salmonella in poultry have been shown to decrease with phage therapy (Atterbury et al., 2007; Kittler et al., 2013). The current study is the first experiment to isolate and characterize the lytic phages against multidrug-resistant *E. coli* strains in poultry in East Azerbaijan province (Iran). Among the three groups of the collected environmental samples used in the current study, samples of the urban sewage treatment center were the most potentiated source for phage isolation. Failure of phage isolation in samples of the broiler farm sewage, in contrast to the successful isolation of phages from human urban sewage samples, suggests the possibility of a close relationship between poultry and human *E. coli* strains, which significantly affects the bacteriophage ecology within poultry farms (Owens et al., 2013). Therapeutic application of phages is identified with some challenges. Phage therapy is an effective method in the treatment and prevention of APEC in poultry farms (Lau et al., 2010; Oliveira et al., 2010). However, some recent reports have denoted that efficient in-vitro phages do not guarantee in-vivo therapeutic results against avian colibacillosis (Tsonos et al., 2014). Therefore, other factors (e.g., titers of phages used in vivo) play a pivotal role in the effectiveness of phages against bacterial infections (Huff et al., 2005). Isolation, purification and titration of different lytic phages against gram-negative (e.g., *Escherichia coli, Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae, Vibrio vulnificus,* and *Salmonella spp.*) and gram-positive bacterial strains (e.g., *Enterococcus faecium* and *Staphylococcus aureus*) have confirmed their efficacy in the treatment of infectious diseases in vitro (Karamoddini et al., 2011). However, such therapeutic properties in vivo remain controversial. Variable behaviors of phages against different field isolates of bacterial species are considered another challenge in this area (Owens et al., 2013), which has been commonly disregarded in the majority of investigations. Meanwhile, few studies have evaluated the isolation and purification of bacteriophages against field isolates of bacteria (*Campylobacter jejuni* (Owens et al., 2013). Results of the recent mentioned study confirmed our findings regarding the different behavior of phages against the isolates of the same bacterial species. For the successful treatment of infectious diseases and effective prophylaxis, further survey is required on the effectiveness of the phages used against circulating field isolates. In the present study, the phages used against each of the bacterial isolates were not effective in case of other field isolates, which indicates the high specificity of phages in combating bacterial strains. In order to design effective phage therapy cocktails, phages with broad host range must be selected. According to the results of TEM in the current research, the most potentiated phage for phage therapy belonged to the *Myoviridae* family, which is in congruence with the results of some of the previous studies in this regard (Chibani-Chennoufi et al., 2004; Hudson et al., 2013). Despite the advantages of phages as alternatives to antibiotics, these agents could not be used as routine antibiotics in the treatment of bacterial infections. Proper use of phages requires comprehensive knowledge of each of these agents for successful phage therapy. As cocktails against bacterial pathogens, multiple phages could be operant against numerous field strains of bacteria. Furthermore, lysis of a wide range of bacteria yields a combination of bacteriophages, which could be applied as a commercial control agent against some bacteria (e.g., *Listeria monocytogenes*) in food production processes (Lang, 2006). However, Use of phage cocktails could be restricted by the environmental parameters needed for the persistence of each phage. Viability and storage of bacteriophages are determined based on various external factors that are likely to cause damage to the structural elements of these agents. In general, a profound comprehension of the intrinsic properties of phages is crucial to designing therapeutic interventions. With respect to the biocontrol applications of phages in poultry farms, appropriate administration based on a specific strategy and comprehensive methodology, detailing phage-host interactions, and optimization of doses in different conditions are necessary (Jassim and
Effective in-vivo control of pathogenic bacteria using phage therapy requires a comprehensive phage control program. On the other hand, the ability to isolate bacteriophages via routine methods may vary under different rearing conditions in poultry farms. Unfortunately, no scientific report is available regarding the validity of phage therapy against poultry E. coli strains in Iranian farms. Moreover, no former research has assessed the effectiveness of phages in the lysis of antibiotic-resistant E. coli isolates in poultry in the northwest of Iran. The majority of studies in this regard have focused on only one E. coli strain to evaluate the lytic properties of phages against bacteria. Findings of the current study confirmed the potential efficacy of phage therapy against cases of colibacillosis in poultry where the disease could not be controlled due to the emergence of multidrug-resistant bacterial strains. On the other hand, results of the present study regarding the different behavior of various wild isolates of E. coli against the isolated phages in poultry suggest that administration of a phage package against an infection requires adequate information on the circulating isolates of bacteria in broiler flocks. Considering the various aspects of implementing phage therapy against different bacterial infections (e.g., environmental factors, administration routes, and drug doses), it is recommended that further investigations be conducted in this regard. In conclusion, results of this study indicated that phage therapy could be an effective alternative to antibiotics in controlling APEC infections in poultry. Meanwhile, effectiveness of the biological control strategy largely depends on the identification of circulating bacterial strains, recognition of the properties of isolated phages, accurate selection of phages, and successful in-vivo experiments.

**Ethics**

I hereby declare all ethical standards have been respected in preparation of the submitted article.

**Conflict of Interest**

The authors declare that they have no conflict of interest.

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