Beneficial Effect of Lytic Pasteurella Bacteriophage and Its Potential Use as A Biocontrol against *Pasteurellamultocida* Infection in Septic Mice Model

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ABSTRACT

The present study aimed to evaluate the isolate phages that were active against *Pasteurellamultocida*and to characterize to explore its possibility as therapeutic and prophylactic agents. The lytic range of the phage was determined against several bacterial isolates. Studies on a phage growth curve and its survival rate with respect to temperature and pH were carried out. *In-vivo* study was conducted to explore the levels of phage in the liver, spleen, kidney, lung, and blood at 24 h post-administration.The isolated phage was confirmed asthefamily Podoviridae. The estimated genome size was about 21 kb. On restriction enzyme digestion only 7 fragments in ranges of 3000 to 10000bp were seen. Phage was stable 5-50^{o}C and survival pH was 4-9. In the safety and therapeutic study challenged mice treated with phage was showing mild clinical signs and died one or two mice after 72 hrs and the survival rate increased significantly ($P \leq 0.05$). Poultrychallenged group 7 birds survived in the subcutaneously treated groupat end of 72 hrs and5 birds survived at the end of 72 hrnasal route treatment. In conclusion a beneficial effectsof phage therapy on treating the mice infected with different *P. multocida*strains was observed.

Keywords: LyticBacteriophage, *P.multocida*, Mice, Poultry, Therapeutic Application.

1. INTRODUCTION

Pasteurellamultocida organisms are classified into five capsular types (A, B, D, E, and F) (Carter, 1955).Pasteurelloses in animals and poultry, is an acute, febrile and fatal disease, causing death in susceptible animals in less than 36 to 48 hours after exposure to the organism (Jabbari and Moazeni, 2005). Many researchers have reported that the infection of *P. multocida* isolates exhibits host-specific and specific capsular types associated withdisease occurrence (Wilkie et al, 2012). Chemotherapy can be done, however, the results are not satisfactory due to the resistance of *P. multocida*to most antimicrobial drugs used (Muller et al, 2001). Vaccination has been shown to greatly reduce the incidence of hemorrhagic septicemia (HS) in endemic areas. Broth bacterin, oil adjuvant vaccine, double emulsion vaccine and a live vaccine have been reported to be the most significant control measure (Benkirane and De Alwis, 2018).

P. multocida phages were first reported in 1956 (Kirchner and Eisenstark, 1956) however, the lytic *P. multocida* phages had not been characterized until 2018 (Chen et al, 2018; Qureshi et al, 2018). Application of phage in livestock and poultry involves using them to prepare bacterial lyates that can be used as vaccines and treatment of infection has been widely studied for bacteria such as *E. coli, Pseudomonas* and*Klebsiella*. In the recent past, the emergence of antibiotic-resistant bacteria has gained much research attention. Previous studies exhibit isolated *P. multocida* strains, showing resistance to chloramphenicol, quinolones and tetracycline group of antibiotics, from various animals, including chicken, ducks, turkeys, quails, and geese (Shivachandra et al, 2004; Sarangi et al, 2015; Maynou et al, 2017). Hence, there is a need to identifythe thrust areafor new therapeutic strategies against these multidrug-resistant *P. multocida* strains. The goal of this research was therefore to isolate phages that were active against *Pasteurellamultocida*and to characterize the morphology, genome size and therapeutic activity before, exploring their possibility as therapeutic agents.

2. MATERIALS AND METHODS

2.1. Bacterial strains andpasteurellaphage: The study was conducted from July 2010 to June 2012 at Indian Veterinary Research Institute,Izatnagar, India. The lyophilized culture of *Pasteurellamultocida* P52 and A: 1 strain were revived by standard methods. All 7 cultures of *P. multocida* strains as *P. multocida*strain P52 (B:2) and, FC strains- LKO and Madras strain are maintained from the Type Culture Lab of the Division of Biological Standardization, Indian Veterinary Research Institute. *P. multocida*A1, A:1,4 and F:3,4 were procured from the Division of Bacteriology and Mycology, Indian Veterinary Research Institute.The identity of each *P. multocida*strain was confirmed by morphological, biochemical and serological examinations. The strains were maintained by periodic sub-culturing on Blood agar (Difco) slopes throughout the period of the study.

2.2. Ethical statement: All the experimental protocols carried out on laboratory animals were approved by (Letter no:25 dated 2 Dec 2011, IVRI IAEC proceedings) the Institute Animals Ethics Committee (IAEC) of Indian Veterinary Research Institute (IVRI), Izatnagar-243122 (India). Animals were kept in IAEC-approved facilities and provided water and food ad-lib. Blood was collected through the cardiac puncture of the animal.

2.3. Phage isolation: The lyophilized culture of *Pasteurellamultocida* P52 and A: 1 strain were revived by standard methods. Bulk stocks of phages were prepared by conventional liquid culture methods (Rawat and Verma, 2007). Briefly 1 litre sterilized NZCYM broth in a flat bottom flask (3-litre capacity Hafkins flask) was inoculated with 50µl of 18-hour pure broth culture of *Pasteurellamultocida* serotype A: 1 and incubated at 37ºC for 2 hours. To each flask, which contains the young culture sufficient quantity of a previously available stock suspension of phage was inoculated to attain a 1:100 final phage bacterial ratio. The phage bacteria mixture was incubated at 37ºC with vigorous intermittent shaking until complete lysis was obtained. The bacterial lysate was filtered through a 0.22-micron membrane filter and collected aseptically in sterile bottles. The stocks were stored at 40ºC for 1 month to eliminate residual lytic activity attributed to phage-induced enzymes. The sterility of phage lysate stock was tested by standard methods (Indian Pharmacophoea, 2010).

2.4. Lytic range of phage: The lytic range of the phage was studied against *Pasteurellamultocida*B: A:F serogroups, *Salmonella Gallinarum, S. Thyphimurium, S. Enteritidis*and field isolates of *Escherichia coli* and *Staphylococcus aureus*, using the spot-inoculation technique (Zhang et al, 2016).The optimum phage: bacteria ratio required to achieve maximum lysis of the indicator A:1 within the shortest period of incubation was determined.

2.5. Preparation of pure phage stock: To about 250 ml of the NZCYM broth culture of *P. multocida*indicator strain in exponential phase in a 500 ml Erlenmeyer flask, such volume of the purified phagewas added aseptically so that the phage-bacteria ratio of the culture was about

1:100.The mixture was incubated at 37°C with intermittent shaking for a period until complete clearance of the turbidity could be observed indicating complete lysis of the organism. The lysate was filter sterilized, and the phage count of the preparation was evaluated by agar overlay. The procedure was repeated 2 times with about 500 ml broth cultures of the organism using the fresh phage preparations obtained during this step.The sterilized lysates obtained by different cycles were pooled and stored at 4°C. The phage counts on the pooled Master Lot (ML IVRI-Pm1/I) was evaluated once again.

2.6. Electron microscopy of phage: Pure suspension of phage in SM diluent (10^9pfu/ml) was fixed in 0.1% M Sodium cacodylate containing 2% Glutaraldehyde. The method is two percent Phosphotungstic acid (PTA) in double distilled water or 0.1 to 1.0% ammonium acetate solution was prepared, and the pH of the solution was adjusted to 6.8 to 7.4 with 1 N potassium hydroxide. The resultant potassium phosphotungstate formed an electron-dense background for negative staining. A drop of suspension of the particulatespecimen was placed on the coated grid and the excess fluid was allowed to drain off.A drop of PTA was placed and allowed to stand for 2 minutes.The stained grid was allowed to dry and was observed.

2.7. Phage DNA: size and restriction enzyme analysis: DNA of IVRI*Pm*-1was isolated using a lambda DNA extraction kit (Akusobei et al, 2018).The quality and molecular size of the DNA wereanalysed by agarose gel electrophoresis in 0.7% horizontal slab submarine gel containing ethidium bromide (10mg/ml), using 1x TAE buffer (Sambrook and Russsell, 2001).The genomic DNA of the phage were subjected to digestion with *EcoR*1 and *BamH*1 to compare the restriction pattern of the genomic DNA.

2.8. Effects of temperature and pH: The effect of temperature functional lytic efficacy was determined by exposing it to different temperatures for at least 30 min and conducting plaque counts on agar overlay plates methods (Rawat and Verma, 2007).900µl of sterile deionized water was preheated to desirable temperatures ranging between 25ºC and 90ºC. 100 µl of phageadjusted to 10^9 pfu/ml was added to the preheated water and kept for 30 min. The heat-treated phage samples were transferred to the ice bath and surviving phage was assayed by the agar overlay method. The effect of pH (2, 3, 4,5,6, 7, 8, 9, 10, 11 and 12) of the medium on the lytic activity of phage was determined (Chenet al, 2018).The desired pH of the growth medium was achieved by varying the concentration of mono and dibasic phosphates. 900 µl of medium 100 µl of Phage (10^9 pfu/ml) was added and incubated overnight at 37ºC. The phage titre was determined by agar overlay.

2.9. Composition of lysate batches: The protein concentrations of the 3 phage lysate batches were determined by Nanodrop Technique in the Division of Animal Genetics and Breeding, IVRI.The carbohydrate content of each batch was determined by the phenol, sulphuric acid method. For the determination of the endotoxin contents of the batches, limulus amoebocyte lysate (LAL) assay was carried out using a commercially available Kit of GCC Biotch.

2.10. Safety test: A safety test was conducted in mice as per Indian Pharmacopoeia 2010 for safety testing. The toxicity of phage, six 5-week-old female mice were randomly divided into two groups with six mice in each group. Mice in each group received an intraperitoneal injection of 0.5 ml (10^9pfu/mL), and 0.5 ml PBS buffer, respectively. All mice were housed under the same conditions and were observed for 10days.

2.11. Therapeutic efficacy: Three groups of mice, namely group I (intramuscular treated), group II (infected untreated control) and group III (normal control) consisted of 10 mice/group. Mice were injected with the culture of *P. multocida* serotype A:1 and B:2 with the lethal dose of 100 µl (10^{-8} and 10^{-6} dilutions respectively). After 6 h of challenge infection, group I, II and III each consisting of 10 mice was inoculated with the phage containing approximately $2x10^8$ pfu as per techniques recommended. No treatment was given to control animals. Mice were observed for any mortality for up to 7 days. Dead animals were used for the isolation organism to confirm the death

caused by *P. multocida*.The health statuses of mice were monitored atleast three times a day for 21 days.About 4 days old chicks were procured from the CARI section of IVRI and they were kept in the Divisional animal shed under the ideal condition of management for 6 weeks. The experimental trial was conducted in the 7th week.Two groups of birds, namely group I (intramuscular treated), group II (subcutaneously treated) and group III (normal control) consisting of 10 birds/group. Birds were injected with the culture of *P. multocida* serotype A:1 with the lethal dose of 1ml of 10^{-8} dilutions. After 6 h of challenge infection, group I and II each consisting of 10 birds was inoculated with the phage containing approximately $1.6x10^8$ pfu as per techniques recommended. No treatment was given to control birds. Birds were observed for any mortality for up to 7 days. Dead birds were used for the isolation organism to confirm the death caused by *P. multocida*.

2.12. Statistical analysis: Survival was analyzed using the test of significance t-test (statisticallysignificant at $p < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Morphological characteristics of phage: Sewage samples were collected and processed for the isolation of bacteriophage against *P. multocida*. The lytic phages against the highly virulent *P. multocida* A1 serotypes were isolated (Fig.1). The lysate content wasdepicted in table 2.The number of plaque-forming units of bacteriophage in therapeutic stock was $2x10^8$ pfu/ml and above on the day of treatment as agar overlay methods. The plaques were approximately 0.5–1.3 mm, the phages were lytic to other serotypes like F (3,4) and F (3,11) and P_{52}serotypes of the *P. mutocida*. The phage:bacteria ratio and optimum lytic efficacy were found to be $1:10^2$ (Table 1).

Fig. 1: Presence of lytic phage against *P. multocida*of the strain asobserved bypresence of plaque forming units.

Table 1: Determination of multiplicity of infection (MOI) of phage

Phage:bacteria ratio	Total viable counts (CFU/ml) in NZCYM medium			
	30 min	**60 min**	**90 min**	**180 min**
$1:10^4$	$2x\ 10^9$	$6.6x10^7$	$8x10^7$	$2x10^6$
$1:10^3$	$1.2x10^9$	$7.6x10^7$	$7x10^6$	$2.5x10^6$
$1:5x10^2$	$1x10^8$	$4x10^7$	$6x10^6$	$9x10^5$
$1:10^2$	$1x10^9$	$2x10^7$	$3x10^6$	$3x10^4$
Control	$4x10^9$	$6x10^9$	$8x10^{10}$	$7x10^{10}$

Table 2: Determination of contents of phage lysate

Preparations	Protein content (µg/ml)	CHO content (µg/ml)	Endotoxin con (ng)

PL-I	2.35	2.5	<125 ng
PL-II	2.68	3.0	<125 ng
PL-III	2.14	4.0	<125 ng
Mean	2.39	3.16	<125 ng

3.2. Electron microscopy and phage DNA: The Electron microscopy of the phage after negative staining revealed that the phage possessed a hexagonal head and well-marked tail. The molecular size of the genomic DNA of the phage was found to be 21 kb. Restriction endonuclease digestion of genomic DNA of phages of genomic DNA done using *EcoR*1and*BamHI*. Several restriction sites for *EcoR*Iand*BamHI*were observed and clear-cut restriction pattern difference was revealed by agarose gel electrophoresis. *EcoR1*, digestion produced 9 fragments ranging from 3000 to 10000bp, whereas *BamH*produced 7 fragments rangingfrom 3000 to 10000bp. The tailed phage based on these morphological characteristics was determined as a member of the family Podoviridae, and the order Caudovirales(Fig. 2,3).

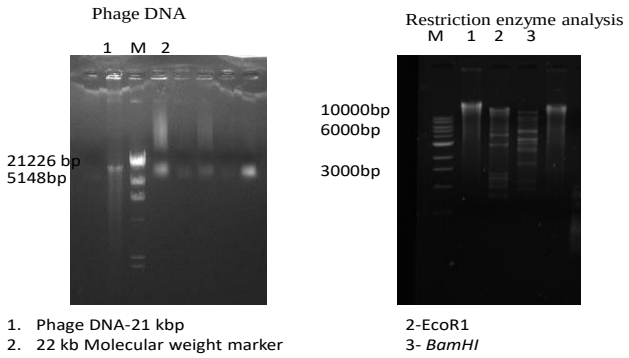

Fig. 2: Phage DNA: molecular size and restriction enzyme analysis

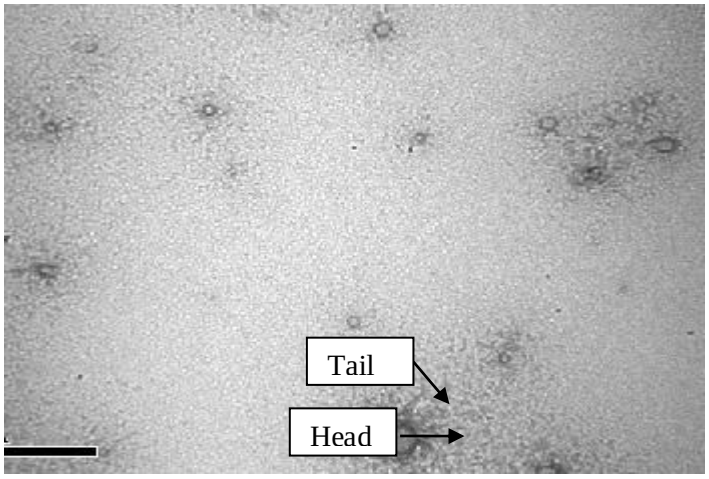

Fig. 3: Morphology of phage in electron microscopy

3.3. Life cycle properties ofphage: Thermolability tests showed that phage was stablebetween 5-50ºC.The temperature increases phage titre decreases were observed when temperature used above 60ºC and above. The phage titre was reduced by 50, 60, and 85% at 60, 70, and 80ºC respectively (Fig.4). Plaque-forming units however increased exponentiallyfrom 25˚C toa maximum of 35˚C. The infective capability of the phages was between pH 4–9. Plaque forming units however decreased at pH 2, 3 and 10, 11 (Fig.5). The lytic cycle of phage showed maximum infectivity during the 0-9h (Fig.6).

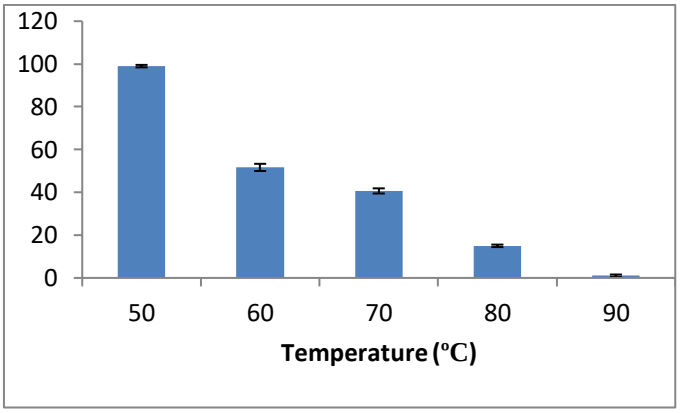

Fig. 4: Determination of temperature of phage

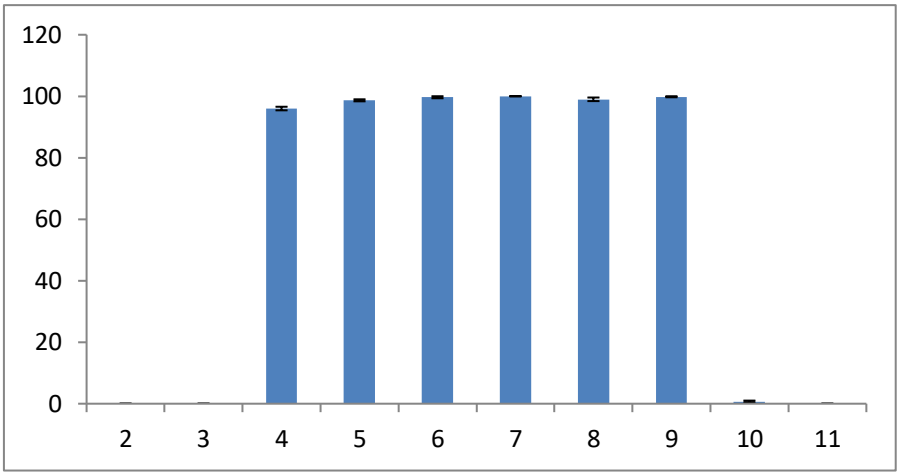

Fig. 5: Effect of pH on functional stability of phage

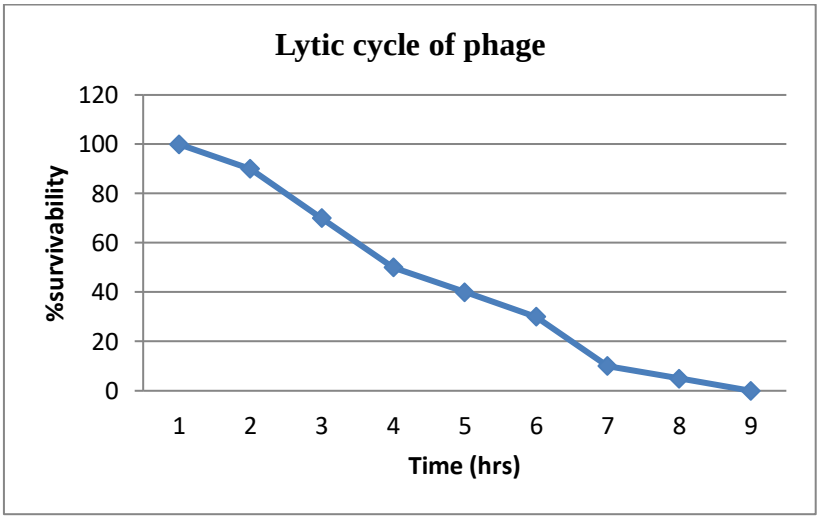

Fig. 6: Life cycle of phage

3.4. Safety and therapeutic effect ofphage: In order to determine the therapeutic efficacy of specific lytic phage, all the challenge-infected mice and birds of group I were treated with phage preparation containing $2x10^8$pfu/ml through the subcutaneous route in mice and Subcutaneous routeand intranasal route in poultry. Challenged mice treated with phage was showing mild clinical signs and died one or two mice after 72 h (Table 3) and the survival rate increased significantly ($P \leq 0.05$) whereas those challengedmice in the control group all died in 72 h. of challenge with virulent strains of P52 and A:1.The number of phage in the liver (pfu/g), lungs (pfu/g), spleen (pfu/g), kidney (pfu/g), and blood (pfu/mL) of the injected mice decreased as time increased; no phages were detected in thekidney blood of the injected mice at 86 hours post-injection (Fig.7). In poultry, the result was as in challenged group 8 birds survived after 24hrs but attheend of 72 hrs. 7 birds survived in the subcutaneously treated group. In the case of nasal route treatment, only 5 birds survived at the end of 72 h, the survival rate decreased as time increased and survival rate were increased significantly ($P \leq 0.05$) whereas those challengedbirds in the control group all died in 72 hof challenge with virulent strains of A:1 (Table 4). The number of phages in the liver (pfu/g), lungs m(pfu/g), spleen (pfu/g), kidney (pfu/g), and blood (pfu/mL) of the injected birds decreased as time increased; no phages were detected in the liver, kidney and blood of the injected birds at 86 h post-injection (Fig.8).

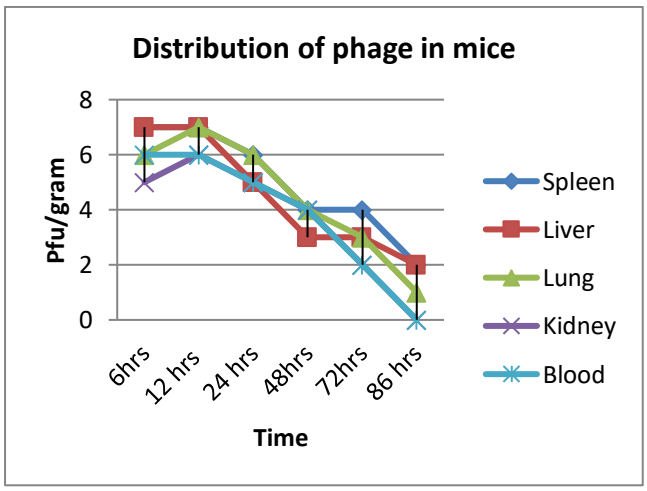

Fig. 7: Distribution of phage in mice (pfu/gram)

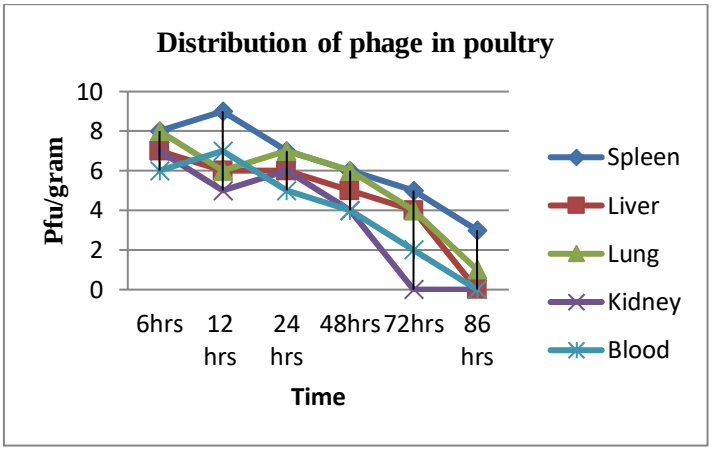

Fig. 8: Distribution of phage in mice (pfu/gram)

Table 3: Therapeutic efficacy of phage in mice (2x 10^8pfu/ml)

Treatment	No. mice survived					
	24 h		48 h		72 h	
	A:1	P52	A:1	P52	A:1	P52
Subcutaneous route	10/8	10/8	10/7	10/7	10/7	10/6
Untreated infected control	10/4	10/2	All died		All died	
Normal control	No dead		No dead		No dead	

Table 4: Therapeutic efficacy of phage in poultry (2x10^8pfu/ml)

Treatment	No. Birds Survived		
	24 h post	48 h	72 h
	A:1	A:1	A:1
Subcutaneous route	10/8	10/7	10/7
Intranasal Route	10/7	10/6	10/5
Normal control	All dead	All dead	All dead

The first reported *Pasteurellamultocida*phage was by Kirchner and Eisenstark.Since there have been scanty reports regarding lytic phages against*P. multocida* (B:2 and A:1) in India. To our knowledge, this is the first study to demonstrate thatlytic effect phage for pasteurellosis in animals.In this report, the phage specific for *P. multocida* capsular type B, and A was isolated and characterized and therapeutic uses were studied for the first time, which helps us understand more about *P. multocida* phages.More than 95% of all phages described in the past belong to the order Caudovirales (tailed ds-DNA phages), which comprises three families: the families Myoviridae (viruses with contractile tails), Siphoviridae (viruses with long, noncontractile tails), and Podoviridae (viruses with short noncontractile tails) (Lu et al, 2013; Pushpa et al, 2017).The bacteriophage isolated was showing the presence of a tail and head in electron microscopy by

negative staining. The tailed phage comprises 96% of currently identified bacteriophages out of which the *Siphoviridae* constitutes 62%, *Myoviridae* 25% and *Podoviridae* approximately 14% (Verma et al, 2013). Electron microscopy of the phage showed a somewhat rounded head with a long tail, consistent with theprevious report that most Pasteurella phage hasa long tail in Pasteurella phages in the family Siphoviridae, which have long noncontractile tails (100 nm) (Vermaet al, 2013)**.** Atiter of phage used in therapy was 10^8PFU/ml. It comes in the range of concentration that was comfortable for phage therapy (Ryan et al, 2011). Host range tests showed that phage-displayed good specificity for killing *P. multocida* capsulartype A,B,D and F isolates. Biosafety issues such as phage, LPS, carbohydrates and proteins in this study showing good enough to use fortreatment purposes.The genome of isolated phage DNA size is 21 kb. The DNA has the restriction sites for the *EcoRI* and *BamHI*. Bacteriophage genomes consist of either single or double-stranded DNA or RNA with extremely variable sizes ranging from 4-725 kb (Ackerman, 1998) which may be circular or linear. Temperate phages are generally had 20 kb and ds DNA.In vivo tests using mouse and poultry models showed that the administration of phage was safe for the mice and poultry and had a good effect on treating the mice infected with A, and B strains including virulent strains and indicator strains. The therapeutic effect was significantly ($P \leq 0.05$) increases day by day.Simultaneous treatment with phage is not necessary for survival following *P. multocida* infection (Chang et al, 2005). Therefore, it is feasible to administer phage at some time after the initial infection.These findings suggest that phage has potential use in therapy against *P. multocida* infections after 6 h of initial challenges with pathogenic strains. In addition to antibacterial actions, phages stimulate some anti-inflammatory activities (Chang et al,2005;Jo´nczyk-Matysiak et al, 2017; Miedzybrodzki et al, 2017). Therefore, the number of inflammatory cytokines may be used as markers for monitoring treatment efficacy. The application of phage resulted in a down-regulation in the levels of proinflammatory cytokines such as particularly TNF-α in mice infected with *Staphylococcus aureus* (Zimecki et al, 2009; Chadha et al, 2017; Cao et al, 2015). Treatment with a phage cocktail resulted in a significant reduction of inflammatory cytokine levels, including IL-1β and TNF-α, when compared with the infection control group in a model of *K. pneumonia*infection (Wang et al, 2015; Sharp et al, 2001). Therefore, further research work should be carried out to reveal host cytokine and inflammatory responses following phage inoculation, such as quantifying the release of IL-6 and TNF-α.

4. CONCLUSIONS

A novel lytic bacteriophage IVRI-Pm-1 specific for *P. multocida* was characterized in the present study. Both morphological and genetic analysis indicated that this phage was a member of the subfamily family *Siphoviridae* within the order *Caudovirales.* Identified phage-displayed good ability on killing most of *P. multocida* type A,and D strains, and it also had a good therapeutic and prophylactic effect on the infections caused by *P. multocida* type A,B. This phage has a potential for application against *P. multocida* capsular type A in poultry.

Funding

Not Applicable.

Conflict of Interest

The authors declare that they have no Conflict of Interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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