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Isolation and characterization of lytic bacteriophages against biofilm forming *E. coli* from livestock and poultry sewage samples

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Abstract
Uropathogenic *Escherichia coli* (UPEC) are among the most prevalent pathogens responsible for urinary tract infections (UTIs), accounting for up to 90% of community-acquired and 50% of hospital-acquired UTIs. The ability of UPEC to form biofilms and their role in bladder colonisation play crucial roles in the pathogenesis of UTIs. Bacteriophages are the most potential alternative to antibiotics for clinical usage due to the rise of resistant UPEC strains and a decline in the rate of novel antibiotic development. The lytic bacteriophages were isolated from livestock and poultry sewage samples using *E. coli* as indicator strain. Phage were characterized based on the morphology using TEM, host range determination, thermal and pH stability. A total of 09 lytic phage were isolated against Uropathogenic *E. coli* (UPEC) and purified. The morphological characterization revealed that the isolated *E. coli* phage belong to the family *Siphoviridae*, *Podoviridae* and *Myoviridae*. Phage thermal stability revealed that the phage titre was stable at temperature 37 °C and pH 7.

Keywords: Bacteriophage, antibiotic resistance, phage therapy, UPEC, UTIs

Introduction
Phages are among the most suitable substitute to antibiotics, which can be used for medicine, agriculture, and related fields. The therapeutic application of bacteriophages for potentially life-threatening conditions is known as phage therapy. Phages are preferred over broad-spectrum antibiotics because, these are highly effective and safe due to their specificity for a particular bacteria and ability to infect only one species or serotype or strain (Wernicki *et al*., 2017) [32]. They are known to kill cells via different molecular mechanisms. They act without infecting eukaryotic cells or disrupting the commensal intestinal microflora (Bhattacharjee, 2015) [5]. The self-replication ability of phages, which eliminates the need for repeated administration, is one of its advantages. Phages have also been used as a promising approach to control and eradicate biofilm forming bacteria (Lee *et al*., 2013) [14].

Phage therapy has been used to control the infectious diseases caused by *Escherichia coli*, *Vibrio cholerae*, *Staphylococcus aureus*, and *Salmonella* spp. with the right dosing (Adebayo *et al*., 2017) [2]. In the early 20th century, phage therapy was developed, promoted and used extensively in the Soviet Union. Phage therapy was marginalized as a result of the post-war expansion of the pharmaceutical industry and the efficacy of antibiotics. But as a result of resurgence of antibiotic resistance and a steady decline in the discovery of novel classes of antibiotics, once again it has become more important to find alternative therapeutic measures to the traditional antibiotics. Phage therapy is one of the most promising substitutes to antibiotics which can be used for successful treatment of infectious diseases that are resistant to many antibiotics.

Among many infectious diseases, UTIs are one of the most common infections caused by pathogenic microorganisms, predominantly caused by facultative Gram-negative bacteria *Escherichia coli* which belong to the Enterobacteriaceae family within the class *Gammaproteobacteria*. The most prevalent pathogen causing UTIs, UPEC, is a diverse group of extraintestinal pathogenic *E. coli* (ExPEC) (Rezatofighi *et al*., 2021) [22]. According to Terlizzi *et al*. (2017) [30] and Flores-Mireles *et al*. (2015) [12], UPEC is the major cause of community-acquired UTIs (approximately 80–90%) and a significant fraction of nosocomial UTIs, which result in significant medical expenses, mortality, and morbidity globally. The capability of UPEC to induce symptomatic UTIs is linked with the expression of a wide range

of virulence factors. During UTIs, UPEC pathogenesis includes colonization, ascending and growth as planktonic cells in urine, adherence to the surface, biofilm formation, invasion and replication by forming bladder intracellular bacterial communities which reside in the underlying urothelium resulting in kidney colonization and host tissue damage with increased risk for bacteraemia/septicaemia.

Biofilms are complex microbial colonies consisting of planktonic and dormant bacteria bound to surface. The bacterial cells within the biofilm are embedded within the extracellular polymeric materials which poses a major challenge against common treatment option due to extensive antibiotic treatment. Patients in a nosocomial situation face a special problem because of the extreme resistance of biofilms to antibiotics, which is primarily connected to lower respiratory and urinary tract infections. Phages are capable of destroying the bacterial host and therefore preventing the formation of biofilm. Phages could also penetrate existing biofilm and eliminate biofilm structure with or without killing resident bacteria (Domingo-Calap & Delgado-Martínez, 2018) [9].

Therefore, considering the involvement of *E. coli* in UTIs and their ability to form biofilm along with significantly increasing antibiotic resistance worldwide, the present study aimed to isolate and characterize lytic bacteriophages against biofilm forming *E. coli* from livestock and poultry sewage samples.

Materials and Method

The study was conducted in the year 2018-2019 at the Department of Veterinary Microbiology, Bihar Veterinary College, Patna, Bihar.

Bacterial isolates

In the present study, 20 isolates of *E. coli* from the repository of Department of VPHE, BVC, Patna were used for enrichment of sewage samples and isolation of *E. coli* bacteriophages. A total of 40 isolates of urine samples were used for determining host range, pH and thermal stability of bacteriophages. Molecular characterization of the isolates was done by PCR targeting species specific 16SrRNA gene (Sabat *et al*., 2000) [23]. The biofilm forming ability of *E. coli* was determined by Stepanovic *et al.,* (2004) [28] with some modifications and all the confirmed isolates of *E. coli* were checked for biofilm formation in this assay. The absorbance was measured at optical density of 595 nm.

Isolation of bacteriophages against *E. coli*

Agar overlay technique was used to isolate bacteriophages against *E. coli* (Adams, 1959) [1]. A total of ten sewage samples were collected from poultry and livestock farms of BASU, Patna and processed for isolation of phage. To the 5 mL of 10X BHI (Brain Heart Infusion) broth which was already supplemented with 1M $MgSO_4$ and 10 mM $CaCl_2$, 45 mL of supernatant sewage sample was added. 1 mL of an early log-phase indicator strain was then added. After 18-24 h incubation at 37 °C, the medium was centrifuged at 10,000 rpm for 10 min, filtered through 0.22 µm syringe filter and stored at 4 °C till further use, which was then tested for lysis against indicator strain of *E. coli* and then subjected to plaque count.

Elution of bacteriophage plaque

The isolated plaques were picked with sterile pipette tip, suspended in 1 ml SM Buffer and kept overnight at 4 °C in SM buffer. Then it was filtered using 0.22 µm syringe filter. The phage suspension was ten-fold serially diluted and 100 µl of host bacteria along with 100 µl of diluted phage was mixed with 5 ml NZCYM soft agar (0.7% agar, w/v). The mixture was instantly poured over the solidified nutrient agar plate (3% agar, w/v) and incubated overnight at 37 °C.

Host range determination

Spot tests on agar overlay against 60 *E. coli* isolates using the conventional method were used to determine the host range of all isolated bacteriophages, and the results were interpreted according to the lysis.

Transmission electron microscopy (TEM)

TEM was performed at the Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi using Electron Microscope (JEOL JEM-1011, Japan Electronics and Optics Laboratory, Tokyo, Japan) to identify the shape of the bacteriophage. Before visualization in TEM, phage samples were negatively stained with 5 µl of 2% (w/v) uranyl acetate over carbon-coated grids.

Temperature and pH stability

The physiochemical characterization of all 9 bacteriophages were done by determination of their stability at various temperatures of 37 °C, 42 °C and 65 °C. 100 µl of the bacteriophage suspension (10^{10} PFU/ml) was subjected to temperature of 37 °C, 42 °C and 65 °C for a period of 60 min in a water bath. The PFU count of bacteriophage before and after exposure to the above mentioned temperature was calculated. Similarly, to determine the pH stability, pH of SM buffer was adjusted by addition of buffers like glycine-HCl (pH 2) and citric acid-Na_2HPO_4 (pH 6) in order to set a particular pH value. 100 µl of the bacteriophage suspension (10^9 PFU/ml) was observed for changes in PFU count at different pH ranges of 2, 6 and 7 for a period of 60 min at 37 °C.

SDS-PAGE

SDS-PAGE of the phage samples was carried out according to the Laemmli method (1970) with modifications. The destaining was done overnight for best visibility of the protein bands and photographed using a scanner.

Genomic DNA isolation of Bacteriophage

The isolation of nucleic acid from broth harvested bacteriophage suspensions was performed using DNeasy blood and tissue kit (Qiagen) for the purpose of PCR amplification. The phage nucleic acid was run as per standard protocol (Sambrook and Russell, 2001) [24] on 0.8% w/v agarose gel prepared in 0.5X TAE for submarine agarose gel electrophoresis (at potential difference of 5 volt/cm) to check the integrity and to determine approximate genomic size of nucleic acid using 1 kb plus DNA ladder. Ethidium bromide was added @ 0.5 µg/ml in melted agarose. The bands were seen with a U.V. transilluminator and photographed using gel documentation system.

Results and Discussion

UPECs are reported as the leading cause of UTIs, being responsible for approximately 90% of global UTI cases (Mao *et al*., 2012) [17]. They are the primary cause of community acquired and nosocomial UTIs causing cystitis and

pyelonephritis leading to urosepsis (Kaper *et al*., 2004) [19]. These bacteria form biofilm, which are defined as structured community of cells encased in a self-produced polymetric matrix adherent to each other and to surfaces or interfaces (Soto *et al*., 2011) [27]. The complex mechanism of biofilm formation involves switching from a planktonic cell to a sessile mode of growth. They have bacteria in close contact which helps in the transfer of genetic information such as antimicrobial resistance plasmids and transposons (Eberly *et al*., 2017) [10].

Tetracycline resistance was found in 92.86% of UPEC isolates, followed by sulfonamide (71.43%), ampicillin (52.38%) and 28.57% to chloramphenicol, erythromycin, and streptomycin. (Rahman *et al*., 2022) [21]. The significantly high antimicrobial resistance of UPEC isolates harbouring large number of virulence genes has made UTI therapy extremely challenging (Zeng *et al*., 2021) [34]. As a result, effective alternative therapeutic measures are required to combat the threat posed by biofilm-forming bacteria.

Bacteriophages, the viruses infecting bacteria comprise the most abundant element of life on the planet and frequently isolated from various environmental sources and gastrointestinal tract of animals (Higgins *et al*., 2008) [33]. The bacteriophages have been identified as having the potential to be used in biotherapy, bio sanitation, biopreservation and biocontrol agents (Coffey *et al*., 2010; Mahony *et al*., 2011; Sillankorva *et al*., 2012) [8, 16, 26]. Bacteriophages also have ability to combat biofilm forming bacteria either by destroying the host bacterium or by penetrating and eliminating biofilm structure.

Keeping in view the above factors, this study was designed with the objective to isolate and characterize lytic bacteriophages against *E. coli* and to evaluate bacteriophage(s) as a biocontrol candidate of planktonic and biofilm forming cells of *E. coli*.

In the current study, different *E. coli* associated with the human and animal urinary tract infections were obtained as well as isolated and confirmed by biochemical and molecular methods. All the characterized isolates of *E. coli* were used as indicator strain for bacteriophages. Further they were also characterized for their biofilm forming ability. The finding of microtiter plate assay showed that out of 60 *E. coli* isolates, 40 (66.67%) were moderate biofilm producers followed by 18 (30.00%) weak biofilm producers and 2 (3.33%) were non-biofilm producers. Subsequently, *E. coli* bacteriophages were isolated from sewage sample and characterized by biological, morphological, physiochemical and molecular methods. The distribution of lytic phage (09) among different livestock/ poultry farm included 04 from cattle/buffalo farm and 05 from poultry farm. All 09 samples showing lytic activity against the indicator strain were then purified and propagated using the soft agar overlay method **(**Fig.1). The plaques produced varied in size from 0.2 to 0.5 mm (Fig. 2). All isolated bacteriophages were named as recommended by Adriaenssens and Brister (2017) [3]. All lytic phage isolated against *E. coli* from BASU poultry farms were named as Pbpec1, Pbpec2, Pbpec3, Pbpec4 and Pbpec5. The phage isolated from BASU dairy cattle/buffalo farm included Pbpec6, Pbpec7, Pbpec8 and Pbpec9.

All the isolated bacteriophages (n=09) were tested on *E. coli* isolates (n=60). Depending upon the number of *E. coli* isolates lysed by a particular bacteriophage, they were further classified into wide host range and narrow host range. The *E. coli* phage were found lytic to a minimum of 8 and maximum of 19 different isolates of *E. coli.* Particularly phage from poultry farm namely Pbpec1, Pbpec2, Pbpec3, Pbpec4 and Pbpec5 showed their lytic activity against 13 (21.67%), 19 (31.67%), 19 (31.67%), 9 (15.00%) and 17 (28.33%) of tested *E. coli* isolates respectively. Whereas phage isolated from livestock farms namely, Pbpec6, Pbpec7, Pbpec8 and Pbpec9 showed lysis against 8 (13.33%), 17 (28.33%), 9 (15.00%) and 10 (16.67%) *E. coli* isolates, respectively. The phage tested against *E. coli* isolates showed an overall lysis efficiency of 13.33 -31.67%. Based on the lysis pattern, the phage Pbpec2, Pbpec3, Pbpec5 and Pbpec7 that showed lysis against 28.33-31.67% of tested isolates were considered as broad host range. In concordance with the results of this study, 66.67% lysis of biofilm forming *E. coli* with the phage ACG-C40 was reported by Chibeu *et al*., (2012) [7]. The lysis efficiency of phage determined in this study was supported by the reports of Viazis *et al.* (2011) [31] who reported 40-50% lytic efficiency of lytic bacteriophages against enterohemorrhagic *E. coli.*

The morphology was determined by transmission electron microscopy, which revealed that all 09 *E. coli* phage belonged to tailed phage group of order *Caudovirales*. According to the International Committee on Taxonomy of Viruses, tailed phage are classified into three families including *Siphoviridae*, *Myoviridae* and *Podoviridae*. The morphological categorization of *E. coli* phage isolated revealed that the majority of isolated phage belonged to *Siphoviridae* except Pbpec5 and Pbpec6 which belonged to *Podoviridae* and *Myoviridae* respectively (Table1, Fig. 3). The finding of present study was in concordance with the established fact that more than 61% of all bacteriophages described in the literature belonged to the *Siphoviridae* family (Niu *et al*., 2014) [20].

The stability study of phage for their heat and pH resistance would be beneficial in minimising phage loss and maintaining phage viability under varied settings, which plays a function in numerous elements of phage therapy (Li and Zhang, 2014) [15]. Hence, the pH and thermal stability of phage was assessed in this study which revealed that the phage titre was stable at 37 °C while showing complete inactivation at 65 °C. The survivability of *E. coli* phage was seen at pH 7 with a complete inactivation at pH 2 within an hour. The results are in concordance with the earlier studies of different researchers (Chandra *et al*., 2011; Ateba and Akindolire, 2019) [6, 4].

The gel electrophoresis of the phage nucleic acid revealed the presence of genome size of more than 20 kb which was in accordance with the finding of Ngangbam and Devi (2012) [18].

The SDS-PAGE analysis of structural proteins of all 09 phage of *E. coli* allowed the identification of 7-13 protein bands ranging from 16 to 137 kDa. All isolated *E. coli* phage showed different banding pattern with some major structural proteins as well as minor proteins. Most of the phage had a minimum 03 major structural proteins detected between 42-51 kDa. Along with major structural proteins, several (4-10) minor structural proteins of 16-137 kDa were also present. The presence of structural proteins in phage of this study was supported by the findings of Ngangbam and Devi (2012) [18] who reported 8 to 11 structural proteins. However, in contrast to present finding Santos *et al*. (2011) [25] resolved the phage particles by SDS-PAGE and reported at least 21 protein bands.

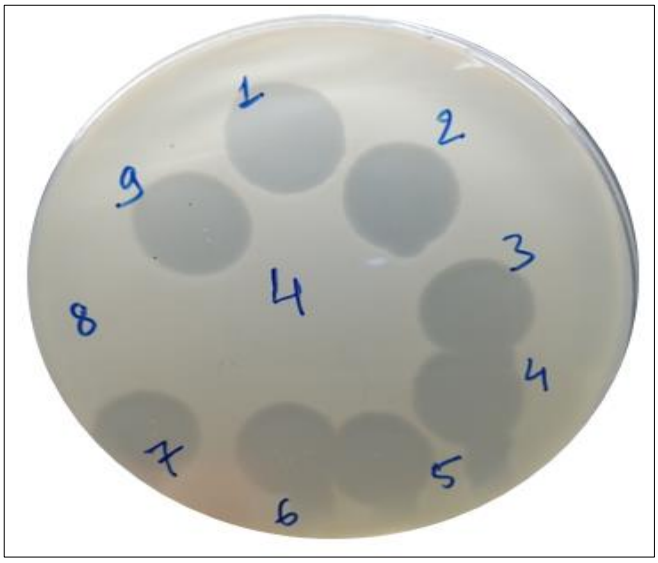

Fig 1: Isolation of lytic phage of *E. coli* by spot test on overlay bacterial lawn

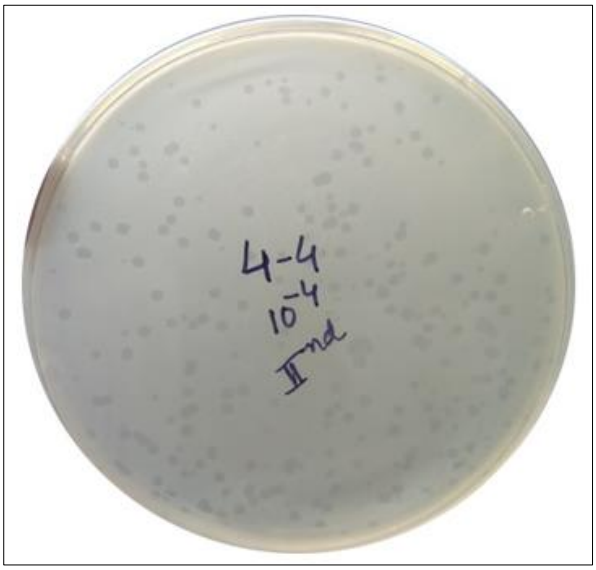

Fig 2: Plaque morphology of *E. coli* phages

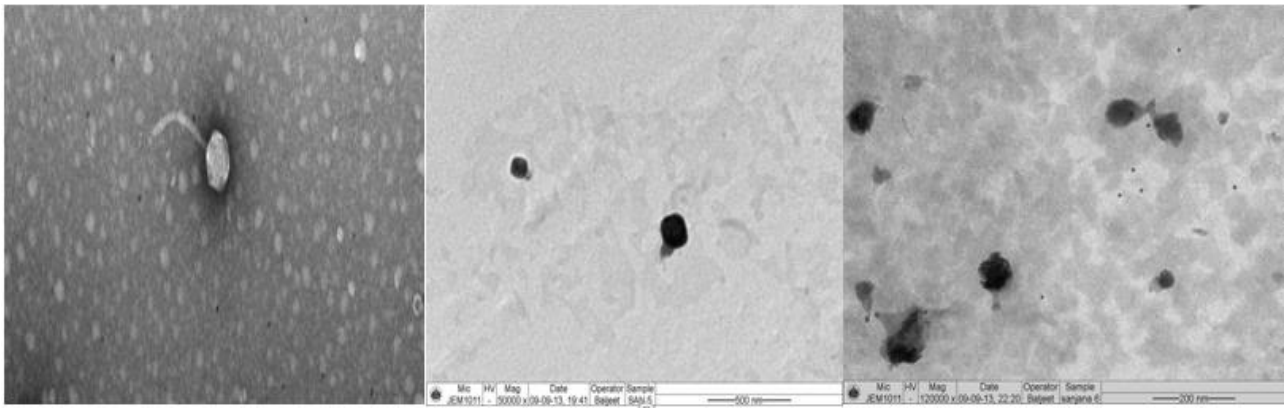

Fig 3: TEM images of *E. coli* phages Pbpec9 (*Siphoviridae*), Pbpec5 (*Podoviridae*) and Pbpec6 (*Myoviridae*)

Table 1: Average Head and Tail size of *E. coli* phage

S. No.	Bacteriophage	Avg. Head diameter (nm)	Avg. Tail size (nm)	Proposed Family
1	Pbpec1	88.35	262.43	*Siphoviridae*
2	Pbpec2	74.97	171.64	*Siphoviridae*
3	Pbpec3	56.21	173.27	*Siphoviridae*
4	Pbpec4	113.98	247.60	*Siphoviridae*
5	Pbpec5	165.35	81.55	*Podoviridae*
6	Pbpec6	198.07	268.58	*Myoviridae*
7	Pbpec7	61.22	182.12	*Siphoviridae*
8	Pbpec8	60.78	172.66	*Siphoviridae*
9	Pbpec9	85.79	66.76	*Siphoviridae*

Conclusion

In conclusion, this study indicates that phage isolate Pbpec1-Pbpec9 are stable at 37 °C, pH range 06-07 and show an overall lysis efficiency of 13.33-31.67% which can be utilized in future after testing its efficacy in laboratory animal models against UPEC which can further be extrapolated to be utilized in animals as well as human.

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