ORIGINAL ARTICLE

Coliphage ASEC2202-Driven Eradication of Biofilm Matrix of *E. coli* Clinical Isolate

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ABSTRACT

Background: *Biofilm-producing multidrug-resistant (MDR) Escherichia coli remains a prominent global health concern and pose a formidable challenge to conventional antimicrobial therapies. Bacteriophage therapy has gained substantial attention as they target bacterial cells, often without disturbing the commensal microbiota, and can penetrate biofilm matrices, making them promising candidates for biofilm eradication.* ***Objective:*** *In this study, we evaluated anti-biofilm efficacy of ASEC2202, a novel lytic coliphage, isolated from sewage water in our lab.* ***Methodology:*** *The biofilms were produced using eight distinct E. coli MDR strains obtained from clinical specimens. The anti-biofilm activity of ASEC2202 bacteriophage was analysed using TCP method.* ***Results:*** *ASEC2202 exhibited a latent period of 30 minutes and a burst size of approximately 552 plaque-forming units (PFU) per infected cell. The phage demonstrated significant bactericidal activity, reducing E. coli population by approximately 3 logarithmic units (~99.9% reduction) within 10 hours of incubation. Coliphage ASEC2202 exhibited potent lytic activity against biofilm-producing MDR E. coli, with biofilm reduction varying by stage and phage concentration. In early-stage biofilms, reductions ranged from 68.66% to 66.52%, while mid-stage biofilms showed over 75% reduction at 54 hours post-infection. For mature biofilms, titrations of 10^5 and 10^7 PFU/mL achieved reductions of 70.16% and 69.75%, respectively.* ***Conclusion:*** *The study concludes that while mature biofilms exhibit increased resilience, targeted phage therapy with optimized concentrations remains effective for biofilm mitigation. However, further in vivo studies are necessary to fully explore therapeutic potential of coliphage ASEC2202.*

INTRODUCTION

Escherichia coli (E. coli), a versatile Gram-negative bacterium, is typically a gut commensal but can become highly pathogenic, causing both intestinal and extraintestinal infections. Pathogenic strains, or pathotypes, are classified into two groups: those causing enteric infections (e.g., EPEC, ETEC, EHEC, EIEC, EAEC, DAEC and those responsible for extraintestinal infections, termed ExPECs. Among ExPECs, uropathogenic *E. coli* (UPEC) causes urinary tract infections, while meningitis-associated *E. coli* (MNEC) leads to sepsis and meningitis[1]. The GLASS 2021 report highlights *E. coli*'s rising resistance to antibiotics, with alarming rates of resistance to third-generation cephalosporins (36.6% for BSIs) and cotrimoxazole (54.4% for UTIs), underscoring a significant public health threat[2,3,4].

E. coli's genetic tractability makes it an excellent model organism for studying biofilms across a range of molecular biology approaches, from classical methods to genome-wide analyses. Experiments involving *E. coli* in diverse static and dynamic biofilm setups, coupled with advanced microscopy, have uncovered numerous adhesins and revealed intricate regulatory networks governing biofilm formation[5]. Shifting from the widely used lab strain to wild commensal and pathogenic isolates has exposed novel physiological traits and untapped biological capabilities that enhance its adaptability within biofilms. These findings not only deepen our understanding of *E. coli*'s biofilm formation but also highlight its broader potential in biofilm research. Given its status as the most extensively studied organism, *E. coli* serves as an ideal choice for exploring the complexities of biofilm biology, particularly in the context of multispecies interactions and experimental molecular microbiology.

In the gastrointestinal tract, facultative species like *E. coli* may interact with anaerobic fusobacteria at the mucosal surface[6].Facultative bacteria likely play a key role as oxygen scavengers, consuming oxygen that diffuses from the host bloodstream into the lumen, thereby creating a more favourable environment for fastidious anaerobes. This interaction facilitates the

formation of mucosal biofilms, where facultative bacteria and fusobacteria co-aggregate to establish anaerobic conditions[7].These biofilms enable anaerobes to thrive by eliminating oxygen traces, highlighting a synergistic relationship between facultative and anaerobic bacteria in maintaining the microbial ecosystem of the gastrointestinal tract[8].

The evolution of pathogenic *E. coli* demonstrates how genetic elements can adapt a strain to specific host environments, forming distinct pathotypes capable of colonizing the gastrointestinal tract, urinary tract, or meninges. Through genomic plasticity, features such as pathogenicity islands (PAIs), plasmids, transposons, or phages can be added, while black holes or pseudogenes may be subtracted, enabling these strains to thrive and cause disease in immunocompetent hosts[9].This adaptability complicates the categorization of pathogenic *E. coli* into distinct pathotypes. These strains impact host cell functions, including signal transduction, protein synthesis, mitochondrial activity, and apoptosis, making their virulence factors valuable tools for studying fundamental microbial processes.

Biofilm-producing *E. coli*, especially antibiotic-resistant strains, pose a significant challenge in healthcare settings, particularly on medical equipment like catheters, ventilators, and prosthetics. Biofilms, composed of an extracellular polymeric matrix (EPM), provide bacteria with a protective environment, shielding them from antibiotics and the immune system. *E. coli* within these biofilms is notably difficult to eradicate, contributing to persistent infections. This matrix acts as a barrier that prevents antibiotics from penetrating the biofilm, leading to the persistence of infections. Biofilms are common on medical devices such as catheters and prosthetics, leading to nosocomial (hospital-acquired) infections and contributing to antibiotic-resistant bacterial strains[10]. *E. coli* is a leading cause of biofilm-associated infections, particularly in healthcare settings. The prevalence of *E. coli* biofilm formation on medical devices is alarming, as biofilms increase the risk of nosocomial infections. A recent global meta-analysis highlights that around 38.6% of *E. coli* isolates from hospital-acquired infections are weak biofilm producers, yet they are still associated with substantial antibiotic resistance. Infections associated with biofilms, such as urinary tract infections linked to catheter use, are particularly hard to treat due to their resistance to common antibiotics like cephalosporins and carbapenems[11]. Multidrug-resistant (MDR) strains of *E. coli* have become increasingly prevalent, especially extended-spectrum beta-lactamase (ESBL)-producing strains, which are difficult to treat with conventional antibiotics[12]. These MDR *E. coli* strains have shown a strong correlation between biofilm formation and resistance to multiple drugs, complicating treatment strategies.

The ability to form biofilms on indwelling medical devices complicates treatment, as traditional antibiotics often fail to eradicate biofilm-embedded bacteria. Biofilm-associated *E. coli* infections, including urinary tract infections (UTIs) and bloodstream infections, are difficult to treat due to the bacteria's ability to resist antibiotic action by hiding within the biofilm matrix. This poses a significant challenge for healthcare professionals, especially with the increasing prevalence of multidrug-resistant (MDR) *E. coli* strains. To address this, phage therapy has re-emerged as a promising alternative to antibiotics. Bacteriophages, viruses that infect and lyse bacteria, offer a potential solution to biofilm-associated infections. Bacteriophages are the most abundant organisms on Earth, with around $10^{\wedge 31}$ particles in the biosphere. This immense population offers a virtually unlimited resource for discovering new strains, making phages a valuable tool in combating antibiotic resistance and exploring microbial diversity[13]. Phages can penetrate biofilms due to their small size and unique mechanisms. They can replicate within bacterial cells and release enzymes, such as depolymerases, which degrade the biofilm matrix, exposing bacteria to phage attack[14].This process allows phages to infiltrate even dense biofilms, leading to the destruction of bacterial cells[15].Phage therapy is particularly attractive because bacteriophages are highly specific to their bacterial hosts, reducing the risk of off-target effects on the body's beneficial microbiota. Several studies have highlighted the effectiveness of phage therapy in targeting and disrupting *E. coli* biofilms. For example, phage cocktails have been shown to significantly reduce biofilm mass and bacterial viability in biofilm-forming MDR *E. coli* strains. Moreover, phages can be combined with antibiotics to enhance treatment efficacy, particularly when biofilms resist antibiotic penetration. Despite its potential, phage therapy remains underutilized due to the dominance of antibiotic treatments over the past century. However, with rising concerns over antibiotic resistance and limited treatment options, phage therapy is gaining attention as a viable alternative. Further research is needed to fully understand the interaction between phages and biofilms at the molecular level. If optimized, phage therapy could provide an effective solution for managing biofilm-associated infections and curbing the spread of antibiotic resistance.

Biofilms, microbial communities' adherent to each other, result in increased morbidity and persistent infections and are the main reason why bacterial diseases are hard to treat with antimicrobial agents[16, 17]. Some types of infection caused by *E.coli* are associated with biofilm formation which leads to an inability to eradicate the infection due to its intrinsic nature to resist high doses of antibiotics[18].

Objectives

The aim of the present work is to evaluate the potential of novel lytic coliphage, designated ASEC2202, for the mitigation of biofilms. Multidrug resistant clinical *E. coli* strains were used to produce biofilm in this study.

METHODOLOGY

***E. coli* clinical strains**

The study analysed 50 distinct *E. coli* strains obtained from clinical samples provided by the Department of Microbiology at Dr. Ram Manohar Lohia Institute of Medical Sciences (RMLIMS), Lucknow, India. The clinical isolates were mainly multidrug-resistant (MDR) strains, with the majority being extraintestinal pathogenic *E. coli* (ExPEC), of which 62% were uropathogenic *E. coli* (UPEC) originating from urinary tract infections (UTIs) or catheter-associated urinary tract infections (CAUTI). This suggests a significant prevalence of UTI-related infections in the sample population. Demographic data revealed a slight predominance of female patients (gender ratio 11:14 male to female), aligning with the higher frequency of UTIs in females as supported by previous research[19]. All *E. coli* strains were cultured in Luria-Bertani (LB) liquid medium (Hi Media, India) and grown on solid LB medium containing 1.5% bacteriological-grade agar (Hi Media, India). After 24hr incubation at 37^{o}C, the active host cultures were used for biofilm assay.

Bacteriophage isolation, and their titration

The bacteriophage ASEC2202 was isolated from wastewater taken from a sewage treatment plant, using the standard double-layer agar method as described elsewhere[20]. In brief, sewage water samples were enriched with LB media and *E. coli* (OD600 of 1 = 8 × 10^^{8} cells/ml), incubated at 37°C, lysed with 1% chloroform, centrifuged, and filtered to obtain phage-containing supernatant, which was serially diluted in PBS, mixed with *E. coli*, and plated using a 0.7% soft agar overlay for phage quantification.

After overnight incubation, plaques, which represent areas of bacterial lysis caused by phage, were counted. The concentration of functional bacteriophages in the original water sample was determined by counting the plaque-forming units (PFU) and expressing the concentration as PFU/ml.

Bacteriophage titers were determined using ten-fold serial dilutions in SM buffer, followed by the double-layer agar (DLA) method, where diluted phage suspensions were mixed with host bacteria, incubated, and plated for plaque counting, with titers calculated in PFU/mL.

Propagation of bacteriophage using double layer agar method

High phage titres were obtained by collecting plaques from higher dilutions, suspending them in SM buffer, and vertexing to release phages. The lysate was serially diluted up to 10-fold with SM buffer, incubated with *E. coli*, and analysed using a double agar overlay assay. After uniform plaque formation, the top agar layer from selected plates was scraped, resuspended again in SM buffer, centrifuged (10,000 rpm (17,000 g) for 25 minutes at 4°C) to remove bacterial debris, and filtered through a 0.22 µm membrane. The resulting purified phage lysate was stored for further applications in phage therapy or molecular research[21]. This method ensures the isolation of a clean, high concentration of bacteriophage stock, suitable for applications such as phage therapy or molecular research.

Biofilm formation

The *E. coli* biofilm formation assay was conducted using polystyrene flat-bottom 96-well microtiter plates, with each assay performed in triplicate and repeated for accuracy[22]. Fresh bacterial cultures were grown, diluted, and inoculated into wells containing ~10^{8} CFU/ml. Ten dilutions of ASEC2202 phage were tested, with controls ensuring sterility. Biofilm formation was monitored over eight time points (24–66 hrs with 6 hours interval). Wells were washed with PBS to remove non-adherent cells, allowing further analysis of biofilm formation, antimicrobial effects, and infection mechanisms. This washing step ensured that only the attached biofilm-forming bacteria remained, which could then be further analysed. This method is widely used to study bacterial biofilm development and clarifies biofilm-related infections and testing antimicrobial agents.

Detection of biofilm formation by tissue culture plate method (TCP)

The Tissue Culture Plate (TCP) assay is a widely used standard method for detecting bacterial biofilm formation[23]. In this study, all isolates were screened for biofilm-forming ability using a modified version of the TCP method, with extended incubation to 24 hours as suggested by O'Toole and Kolter[24]. After biofilm formation in microtiter plate wells, 25 µl of 1% crystal violet solution was added to each well. Crystal violet is a dye that selectively stains cells without binding to the polystyrene plate itself. The plates were incubated at room temperature for 15 minutes to ensure sufficient staining, followed by thorough rinsing with water to remove excess dye. The biofilm, which adheres to the well walls, was uniformly stained with crystal violet. To quantify the biofilm, the crystal violet-stained cells were solubilized in 200 µl of an ethanol-acetone solution (80:20 V/V), and 100 µl of the solubilized mixture was transferred to a new microtiter dish. These plates were

then subjected to a micro-ELISA reader at a wavelength of 570 nm. The optical densities (OD) measured at this wavelength reflect the amount of biofilm formed by the bacterial isolates. The OD values serve as indices of the bacteria's potential to adhere to surfaces and produce biofilms. Each experiment was performed in triplicate and repeated three times to ensure consistency. To control for background absorbance, the OD readings from wells containing ethanol alone were used as blanks and subtracted from the test results. Based on OD 570 readings, biofilm production was categorized as high, moderate, weak, or absent, according to established thresholds. The inhibition of biofilm formation was measured for each phage titre and at different incubation temperatures and represented as percent inhibition.

RESULTS

Selection of coliphage and its propagation

A total of twenty-seven bacteriophages were isolated, demonstrating lytic activity against clinical *E. coli* strains. Table 1 shows the isolated phage morphology along with the antibiotic sensitivity profile of all twenty-seven selected clinical MDR strains of *E. coli*. The ASEC2202 phage has shown good lytic activity against a clinical strain of *E. coli* (sample 36), isolated from catheter urine sample of pyelonephritis case. The bacterial strain was resistant to all the tested antibiotics, viz. aztreonam, ampicillin sulbactam, cefotaxime, cefoxitin, cefazolin, piperacillin tazobactam, levofloxacin, ciprofloxacin, imipenem, meropenem, cefepime, amikacin, gentamicin, tobramycin, nitrofurantoin, tetracycline, doxycycline, sulbactam, norfloxacin, ceftazidime. This resistant *E. coli* clinical isolate (sample 36) was effectively lysed by ASEC2202 with clear plaque morphology (figure 1). Therefore, ASEC2202 phage and eight resistant clinical strains of *E. coli* (sample no. 24, 25, 36, 46, 47, 48, 49 and 50 as given in table 1) were selected for further study of biofilm inhibition. The selected phage was propagated as described above to a titre of $1 \times 10^{\wedge 9}$ PFU/mL, made into different dilutions and used throughout the study.

Table 1. Antibiotic susceptibility and coliphage killing of *E. coli* clinical isolates.

Sample No.	Host spectrum	Age	Sex	Disease	Sample site	Phage Name	Plaque Morphology	Antimicrobial Susceptibility Testing																		
								Aztreonam	Ampicillin sulbactam	Cefotaxime	Cefoxitin	Cefazolin	Piperacillin tazobactam	Levofloxacin	Ciprofloxacin	Imipenem	Meropenem	Cefepime	Amikacin	Gentamicin	Tobramycin	Nitrofurantoin	Tetracycline	Doxycycline	Sulbactam	Norfloxacin	Ceftazidime
24	*E.coli*	6DAYS	F	Pyelonephritis	Catheter Urine	Coliphage 24R.	Clear	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25	*E.coli*	34Y	F	wound infection	Pus	Coliphage 25R.	Turbid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26	*E.coli*	28Y	F	Pyelonephritis	Catheter Urine	Coliphage 26R.	Clear	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	0	0	0
27	*E.coli*	58Y	F	Recurrent UTI	Urine	Coliphage 27R.	Clear	1	1	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0
28	*E.coli*	53Y	F	Urinary Tract Infection	Urine	Coliphage 28R.	Clear	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
29	*E.coli*	28Y	M	Urolithiasis	Urine	Coliphage 29R.	Clear with halo	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
30	*E.coli*	43Y	M	Percutaneous Nephrostomy	Pus	Coliphage 30R.	Clear with halo	1	1	0	0	0	1	1	1	1	1	1	1	1	1	0	1	0	0	0
31	*E.coli*	32Y	F	wound infection	Pus	Coliphage 31R.	Bull's eye morphology	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0
32	*E.coli*	36Y	M	Post operative Carcinoma	Pus	Coliphage 32R.	Clear	0	0	1	1	1	1	1	0	1	1	1	1	1	0	1	0	0	0	0
33	*E.coli*	49Y	F	Pyelonephritis	Catheter Urine	Coliphage 33R.	Clear	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
34	*E.coli*	32Y	M	Percutaneous Nephrostomy	Pus	Coliphage 34R.	Clear	0	0	0	1	0	0	0	0	1	1	0	1	0	0	1	0	0	0	0
35	*E.coli*	57Y	M	Renal Stone	Urine	Coliphage 35R.	Clear with halo	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
36	*E.coli*	49Y	F	Pyelonephritis	Catheter Urine	ASEC2202	Clear	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
37	*E.coli*	45Y	M	Urinary Tract Infection	Urine	Coliphage 37R.	Clear with halo	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
38	*E.coli*	53Y	F	Percutaneous Nephrostomy	Pus	Coliphage 38R.	Clear	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
39	*E.coli*	29Y	M	Pyelonephritis	Catheter Urine	Coliphage 39R.	Clear	1	1	0	1	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0
40	*E.coli*	26Y	F	Urinary Tract Infection	Catheter Urine	Coliphage 40R.	Clear	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
41	*E.coli*	54Y	M	Urolithiasis	Urine	Coliphage 41R.	Bull's eye morphology	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
42	*E.coli*	31Y	M	Recurrent UTI	Urine	Coliphage 42R.	Clear	1	1	0	0	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0
43	*E.coli*	2months	M	wound infection	Pus	Coliphage 43R.	Clear	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0
44	*E.coli*	27Y	F	Burn Infection	Pus	Coliphage 44R.	Turbid	0	0	1	1	1	1	0	0	1	1	1	1	1	0	1	0	0	0	0
45	*E.coli*	54Y	M	Urinary Tract Infection	Urine	Coliphage 45R.	Clear	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
46	*E.coli*	49Y	F	Urolithiasis	Urine	Coliphage 46R.	Turbid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
47	*E.coli*	59Y	F	Recurrent UTI	Urine	Coliphage 47R.	Clear	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
48	*E.coli*	21Y	F	wound infection	Pus	Coliphage 48R.	Clear	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
49	*E.coli*	14Y	M	Urinary Tract Infection	Catheter Urine	Coliphage 49R.	Bull's eye morphology	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
50	*E.coli*	26Y	M	Urinary Tract Infection	Urine	Coliphage 50R.	Clear	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

(In the heat map, Green represents bacterial strain's susceptibility for antibiotic, whereas Red represents resistance)

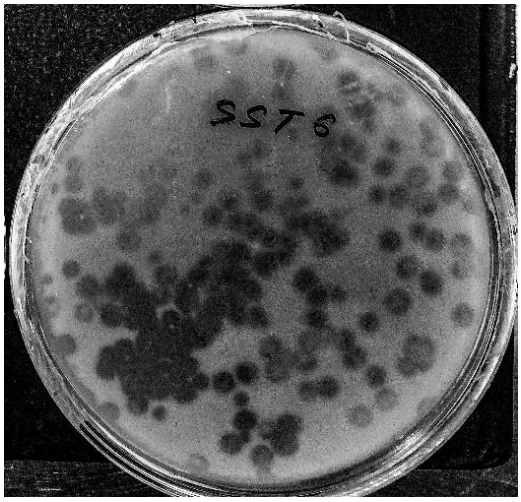

Fig. 1: *E. coli* MDR strain susceptible to phage ASEC2202

Bacteriophage latent time and burst size

One-step growth experiments were conducted to determine the latent period and burst size of the isolated phages. For ASEC2202, the latent period was found to be 30 minutes, with a burst size of 552 PFU (plaque-forming units) per cell (figure 2).

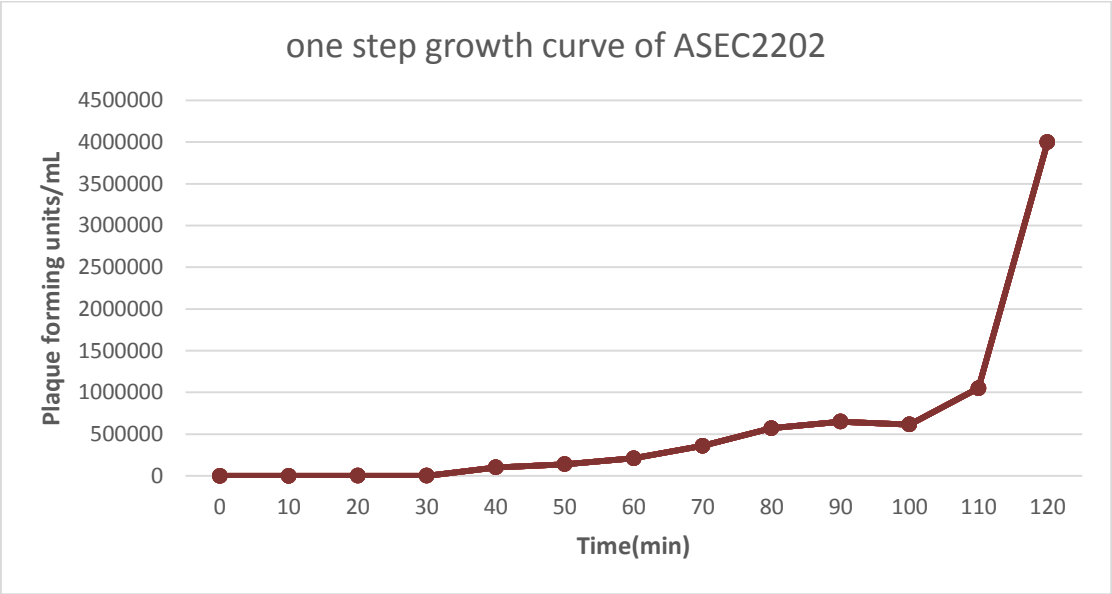

Fig. 2: One-step growth curve of coliphage ASEC2202

Bacteriophage lytic activity with host strains

For a bacteriophage to be effective in mitigating biofilm production in clinical equipment, it must maintain its lysis against the host *E. coli* strain. In this study, the performance of coliphage ASEC2202 has been evaluated in a liquid broth culture. Initially, their lytic activity was not significant during the first 6 hours post-inoculation. However, a notable decrease in bacterial numbers was observed at 8 hours after inoculation, indicating that ASEC2202 effectively reduced bacterial counts in LB broth (figure 3). The reduction reached approximately 10^3 CFU/mL, showcasing the potential of these bacteriophages as lytic agents against biofilm causing bacteria of clinical origin.

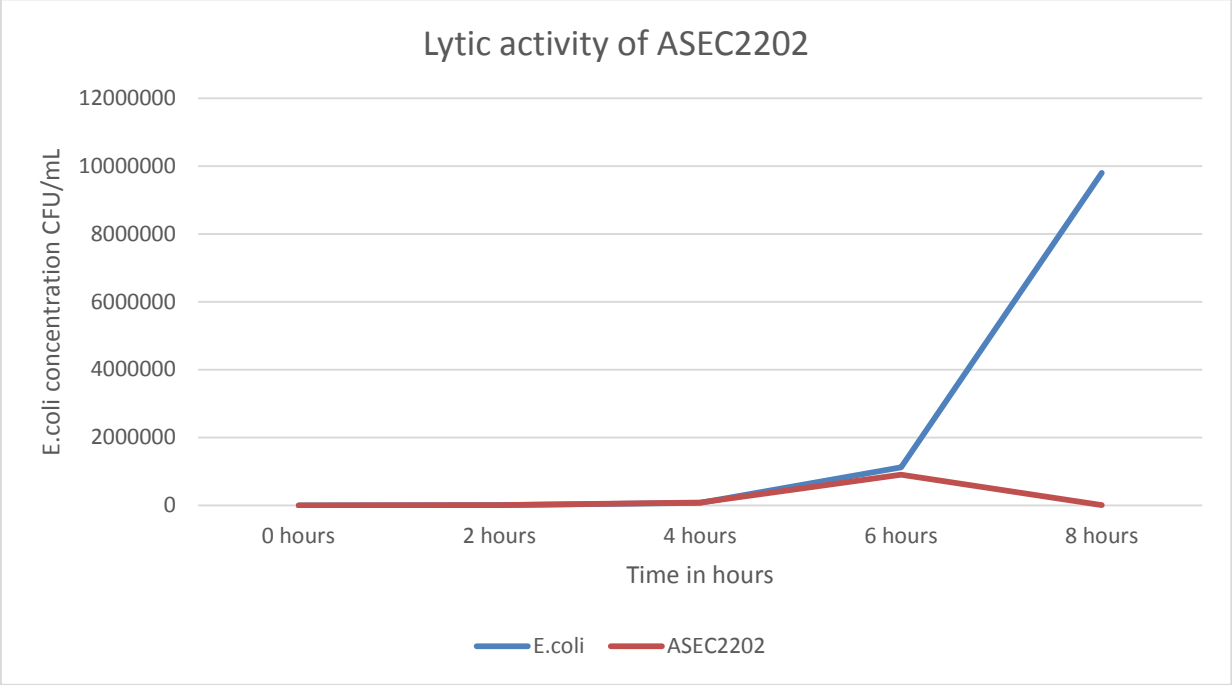

Fig. 3: Lytic activity of *ASEC2202* against MDR strain of *E. coli* at 37 °C at different intervals. The coliphage effectively reduced the viable bacterial cells within 8 hrs.

Biofilm formation and its lysis

The study evaluated biofilm formation in eight *E. coli* isolates using the TCP method, comparing *E. coli* alone with *E. coli* treated with coliphage ASEC2202 (figure 4). Results demonstrated that the phage strain exhibited varying levels of biofilm inhibition (figure 5). As given below, in the phage-treated group, coliphage ASEC2202 significantly improved biofilm control compared to the untreated group, highlighting its potential for biofilm disruption.

The *E. coli* biofilm titration data explores biofilm mitigation over different time points, crucial in clinical settings due to biofilms' resistance to antibiotics and disinfectants at different time periods. Biofilm formation begins quickly, thus the mitigation by phage was studied at various time intervals, starting from 24 hours till 66 hours. As represented in figure 4, varying degree of biofilm inhibition by phage was observed for selected clinical strain. At 48 hours the reduction in biofilm has been observed highest (68.66%, and 67.38%) via 10^{1} and 10^{2} ASEC2202 dilutions respectively, indicating requirement of high titre of phage for the weakening of early-stage biofilms. Whereas biofilm reduction percentage of 73.43%, 75.78%, 76.56%, 69.53% at phage dilutions of 10^{3}, 10^{4}, 10^{6}, and 10^{8} respectively was obtained at 54 hours. Mid-stage biofilms become more structured and resistant, and the identification of effective phage concentration at this point is crucial for preventing biofilm maturation, which is well represented by our selected phage ASEC2202. Even at 60 hours, the highest reduction of 70.16% and 69.75% in the *E. coli* biofilm, was found with phage dilutions of 10^{5} and 10^{7} respectively. This indicates the potential role of our phage in mitigating more mature biofilms, which are notoriously difficult to eradicate. This data also highlights the importance of optimizing titrations and treatment times to disrupt biofilm formation in clinical settings.

Despite the high tendency of the *E. coli* strains to produce biofilms, significant difference in biofilm production levels was observed between *E. coli* alone than those treated with coliphage ASEC2202. This suggests that while the phage is effective at controlling biofilm, it does directly influence the overall biofilm production tendency of the MDR bacterial strains.

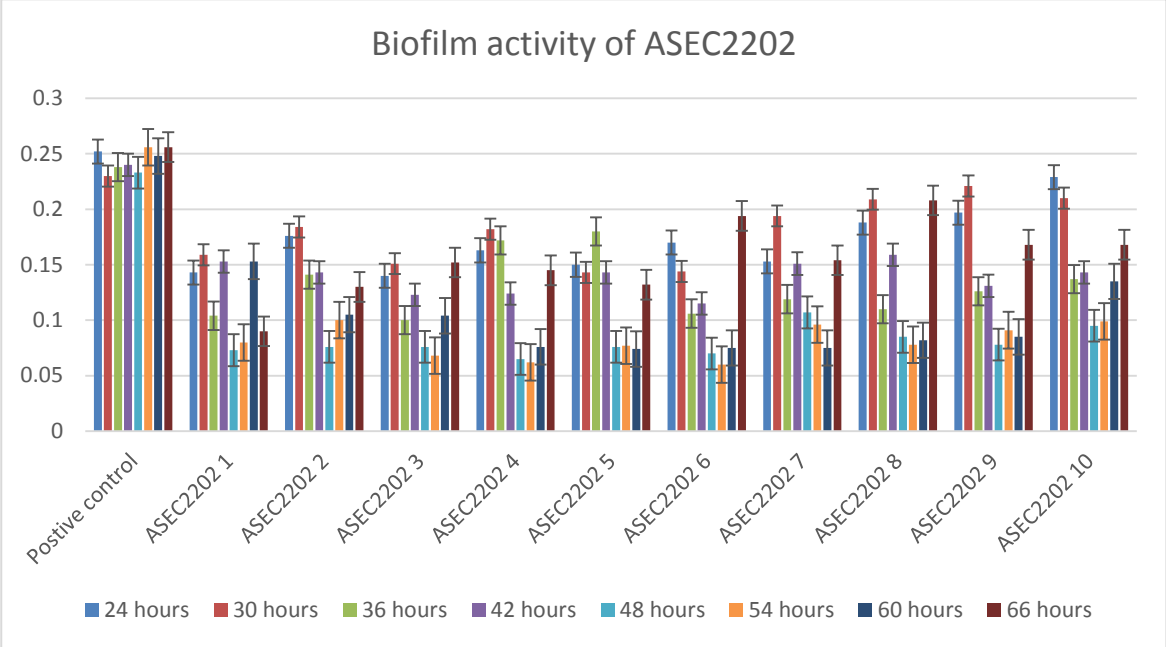

Fig. 4: ASEC2202 coliphage-based reduction of biofilms. Positive control is *E. coli* without ASEC2202. On x-axis from left to right, ASEC2202 1 to ASEC2202 10 are the dilutions of phage used in study.

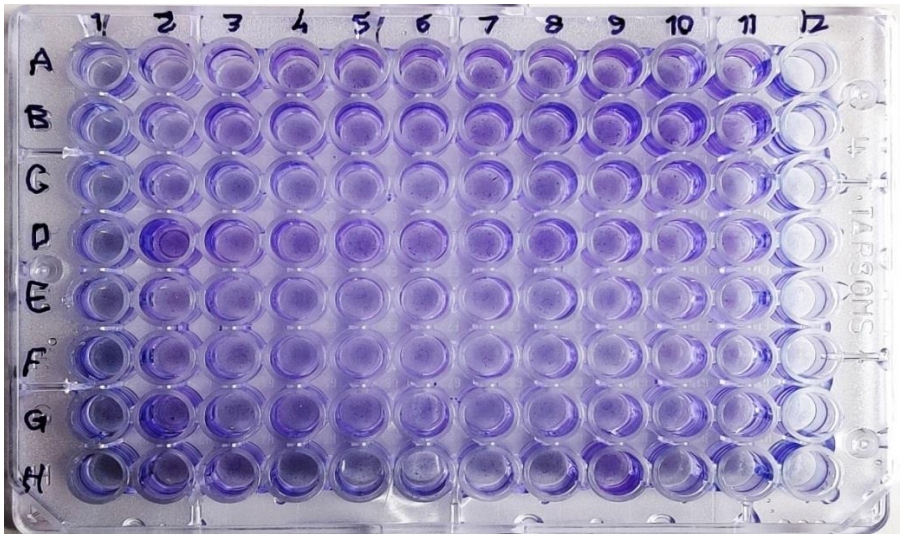

Fig. 5: The *E. coli* alone and *E.coli* treated with ASEC2202 phage were grown overnight in microtiter polystyrene flat-bottom plate containing LB media. After incubation, non-adherent cells were washed away, leaving behind the biofilm-forming cells. These adhered cells were visualized by staining with crystal violet, which binds to the biofilm matrix. The intensity of the crystal violet staining reflects the level of biofilm production.

DISCUSSION

A report by the UK's National Biofilms Innovation Centre (NBIC) estimates biofilms cause over $5 trillion in economic impact annually[25]. Studies have shown bacteriophages isolated from sewage can combat drug-resistant pathogens. Research by Chakraborty (2018) and Necel (2020) identified effective phage candidates against multidrug-resistant bacteria[26, 27]. while Montso's study demonstrated phages disrupting *E. coli* biofilms on contaminated beef[28]. Later in 2022, Alexyuk highlighted the clinical applicability of phages in their study, by using phage cocktails as treatment of multidrug resistant *E. coli* [29]. Vera-Mansilla and team also emphasized on versatility of phage as an alternative to traditional antibiotics, particularly for targeting resistant bacterial strains[30].

The increasing resistance of *E.coli* to multiple antibiotic classes, driven by genetic mutations, horizontal gene transfer, and biofilm formation,

complicates the management of its infections. These biofilms protect bacterial cells from high antibiotic concentrations, underscoring the urgent need for alternative or combinatorial therapeutic strategies.

The focus of this study was on hard-to-treat phenotypes of *E. coli.*, particularly biofilm-producing multidrug-resistant (MDR) strains. The efficacy of newly isolated bacteriophages hinges on their growth dynamics; a shorter latent period and larger burst size are generally advantageous. However, in chronic diseases, where bacterial densities are typically low (often below 10^**4** CFU/mL), phages with a longer latent period may also be beneficial. In this study, coliphage ASEC2202 exhibited a latent period of 30 minutes and a burst size of 552 PFU/cell, reflecting its high efficiency in lysing bacterial cells. This rapid replication and release of phage particles from infected bacterial cells underscore its effectiveness as a biological control agent. The phage was able to reduce the *E. coli* population by approximately 3 logs within 10 hours of incubation, even when exposed to stress conditions. This highlights the stability and robustness of coliphage ASEC2202 in various environments, which is critical for its potential use in real-world applications, such as treating infections in the human body or controlling bacterial contamination in agriculture and food production.

Coliphage ASEC2202 showed susceptibility against a completely resistant biofilm-producing *E. coli* strain. Biofilms, which are structured communities of bacteria encased in a self-produced matrix, present a formidable challenge to antibiotic treatment due to their resistance to both immune responses and antimicrobial agents. Phages like ASEC2202, which can penetrate and disrupt biofilms, offer a novel and much-needed approach to overcoming this challenge. For bacteriophages to be viable therapeutic agents in treatment of biofilm of *E.coli*, they must remain active during various pH, temperatures and osmotic stresses. These things can impede phage-bacteria interactions.

Some recent significant examples include report by Dakheel et al. showing two novel phages against 25 biofilm-producing MRSA strains[31]. An 80-100% biofilm removal of *K. pneumoniae* MDR strains by a Siphoviridae phage[32]. phage therapy for *P. aeruginosa* biofilm in the mouse model of cystic fibrosis and bacteriophages with high antimicrobial activity against biofilm-producing *A. baumannii* strains are some of the other significant examples of the same [33,34].

Therefore, the increasing incidence of antibiotic resistance in *E.coli* strains responsible for biofilm production necessitates the exploration of alternative therapeutic options, such as bacteriophage therapy. The promising results observed with isolated coliphage ASEC2202 in vitro suggest that it could be integrated into treatment strategies for mitigation of biofilm.

Continued research is critical to refine phage therapy, addressing potential resistance and optimizing efficacy within the complex environment of biofilm. As we expand our understanding of phage-bacteria interactions and develop effective phage preparations, the goal of improving biofilm mitigation management in clinical equipment and reducing reliance on antibiotics could be realized.

CONCLUSION

The isolated phages in this study demonstrated potential lytic activity against the targeted *E. coli* strains. However, further research is crucial to evaluate their efficacy in vivo and to assess their safety and potential interactions with existing treatments. With the increasing prevalence of antibiotic resistance, the integration of phage therapy into biofilm management strategies could provide a viable alternative for controlling infections and improving human health globally.

Conflict of Interest:
The authors declare no competing interests and no conflict of interest.

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