Mitigation of Biofilm Produced by *E. coli* Clinical Strain using Coliphage - ASEC2201

Padmesh Sudhaker[1]*, Saeed Humaira[1], Singh Aditi[1], Sen Manodeep[2] and Singh Sujeet Pratap[1]
1. Amity Institute of Biotechnology, Amity University Uttar Pradesh, Lucknow Campus, Gomti Nagar Extension, Lucknow, 226028, INDIA
2. Department of Microbiology, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, 226010, INDIA
*sudhakarparadox@gmail.com

Abstract

Biofilm-producing multidrug resistant (MDR) strains of Escherichia coli (E. coli) pose severe health and economic challenges. Bacteriophage or phage therapy offers a promising alternative to conventional antibiotics. This study evaluated the anti-biofilm activity of a novel coliphage - ASEC2201, which is isolated in our lab from sewage water. We observed that the phage exhibited a latent period of 30 minutes and a burst size of 615 PFU/cell, indicating its high efficiency in lysing bacterial cells. The coliphage ASEC2201 reduced E. coli population by approximately 3 logs within 10 hours of incubation. It showed specific lytic activity against biofilm-producing MDR E. coli strains. The titration data demonstrates the importance of timing and treatment in biofilm mitigation. Higher reductions of more than 31.82% at mid stage were observed at 54 hours. Mature biofilms reductions of 18.42% and 17.50% via phage titrations of 10^1 and 10^2 showed that mature biofilms, while more resistant, can still be mitigated with optimized concentrations.

The present study indicates that ASEC2201 can be applied independently or in combination with antibiotics to enhance its efficacy. However, further in-depth and in vivo studies are necessary to fully explore the therapeutic potential of coliphage ASEC2201 and to ensure its safety and efficacy in real-world applications.

Keywords: Bacteriophage, Antimicrobial resistance, Biofilm, MDR, *E. coli*.

Introduction

The global rise of antimicrobial-resistant bacteria is a major health concern, especially in low- and middle-income countries with high morbidity and mortality rates. Biofilm formation plays a critical role by shielding bacteria in a protective matrix, enhancing resistance to antibiotics and harsh treatments. These biofilms often form on medical devices like catheters and implants, leading to persistent infections. Their presence also promotes the spread of resistance genes, posing both clinical and environmental challenges in managing bacterial infections[22]. Among microorganisms, Gram-negative bacteria *E. coli* shows great potential in forming biofilms. Within biofilms, *E. coli* is shielded by the EPM (extracellular polymeric substance) which acts as a physical and chemical barrier against antibiotics and immune responses. This protection makes biofilm-associated bacteria particularly difficult to eradicate, contributing to the persistence of infections and increasing the likelihood of treatment failure. Many strains of *E. coli* are harmless and play an essential role in gut health, others can evolve into potent pathogens, capable of causing a broad spectrum of diseases. These pathogenic strains, referred to as pathotypes, are categorized based on the infections they cause[3]. Enteric pathotypes include enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteroaggregative (EAEC) and diffusely adherent *E. coli* (DAEC), all of which typically lead to gastrointestinal disorders.

On the other hand, extraintestinal pathogenic *E. coli* (ExPEC) is associated with infections outside the intestinal tract. Among these, uropathogenic *E. coli* (UPEC) is the primary cause of urinary tract infections (UTIs) while meningitis-associated *E. coli* (MNEC) can lead to life-threatening conditions like neonatal meningitis and sepsis[10].

The Global Antimicrobial Resistance and Use Surveillance System 2021 report underscores a disturbing trend in antibiotic resistance among *E. coli* strains. The report found resistance rates as high as 36.6% to third-generation cephalosporins in bloodstream infections and 54.4% to cotrimoxazole in urinary tract infections. These figures reflect the growing threat of multidrug-resistant (MDR) *E. coli* strains, making treatment increasingly difficult and highlighting the urgent need for alternative therapeutic strategies[33]. In the human gastrointestinal tract, facultative anaerobes like *E. coli* play a vital ecological role, especially through their interactions with strict anaerobes such as *Fusobacteria* at the mucosal surface[15]. By consuming oxygen that diffuses from the bloodstream into the intestinal lumen, *E. coli* helps to maintain the anaerobic conditions necessary for the survival of fastidious anaerobes[11]. This oxygen-scavenging activity promotes the development of complex mucosal biofilms where facultative and anaerobic bacteria co-aggregate, supporting gut microbiome stability. While these biofilms are essential for gastrointestinal homeostasis, their disruption can lead to disease progression[16].

Pathogenic *E. coli* strains demonstrate remarkable genomic plasticity, enabling them to adapt to various host environments[4]. They acquire virulence factors through mobile genetic elements such as pathogenicity islands (PAIs), plasmids, transposons and bacteriophages. At the same time, unnecessary genes may be lost, forming pseudogenes or genomic “black holes” to streamline their

pathogenic potential. These genetic changes allow *E. coli* to colonize niches like the urinary tract, bloodstream and central nervous system. The virulence factors they express, can disrupt host cell processes including signal transduction, mitochondrial function, protein synthesis and apoptosis, making them valuable tools for studying host-pathogen interactions[24,28].

A global meta-analysis revealed that approximately 38.6% of hospital-derived *E. coli* isolates are weak biofilm producers. However, even these strains show strong links to antibiotic resistance. Infections like catheter-associated urinary tract infections (CAUTIs) are difficult to treat, particularly due to resistance to antibiotics such as cephalosporins and carbapenems. The growing prevalence of multidrug-resistant (MDR) and extended-spectrum beta-lactamase (ESBL)-producing strains has further complicated treatment efforts[9,23].

With traditional antibiotics losing efficacy, phage therapy is gaining renewed attention. Bacteriophages, viruses that specifically target bacteria, offer a highly selective treatment approach. Their small size and lytic proteins, like endolysins and enzymes like depolymerases, allow them to penetrate biofilms and lyse bacterial cells[13,17,20,27]. Phage cocktails have shown effectiveness in reducing biofilm mass and killing MDR *E. coli*. Though still underutilized, phage therapy presents a promising alternative for managing chronic, biofilm-related infections and combating antibiotic resistance.

Biofilms are structured microbial communities that adhere to biotic or abiotic surfaces and to one another, exhibiting enhanced resistance to antimicrobial agents[26]. Their formation is a major contributing factor to increased morbidity and the persistence of infections, often complicating treatment and eradication efforts. Infections caused by *E. coli* are frequently associated with biofilm development, which confers a heightened tolerance to antibiotic therapy[30]. This resistance is primarily due to the protective extracellular matrix and altered physiological state of biofilm-embedded cells, making conventional antimicrobial treatments largely ineffective[25].

Material and Methods

***E. coli* clinical strains:** The study investigated 50 distinct *Escherichia coli* strains isolated from clinical samples provided by the Department of Microbiology at Dr. Ram Manohar Lohia Institute of Medical Sciences (RMLIMS), Lucknow, India. Most isolates were multidrug-resistant (MDR), predominantly extraintestinal pathogenic *E. coli* (ExPEC). Among these, 62% were identified as uropathogenic *E. coli* (UPEC), associated with urinary tract infections (UTIs) and catheter-associated UTIs (CAUTIs), indicating a notable prevalence of UTI-related infections in the sampled population. Globally, *E. coli* remains the leading cause of community-acquired UTIs, responsible for approximately 70–95% of cases[12]. Demographic analysis showed a slight predominance of female patients (male-to-female ratio of 11:14), consistent with previous findings highlighting higher UTI incidence in females[20]. All isolates were cultured in Luria-Bertani (LB) liquid medium (Hi Media, India) and on LB agar plates containing 1.5% bacteriological-grade agar. After 24 hours of incubation at 37°C, active cultures were used for subsequent biofilm formation assays.

Isolation and Titration of Bacteriophage: Bacteriophages were isolated from wastewater samples collected from a sewage treatment plant using the standard double-layer agar (DLA) method, as previously described[20]. To enrich for phages, water samples were supplemented with 10% (v/v) of 10× Luria-Bertani (LB) medium and actively growing *E. coli* cultures (OD_{600} = 1.0, approximately 8×10^8 cells/mL). This mixture was incubated overnight at 37°C with shaking to allow phage replication within host cells. After incubation, 1% chloroform was added to induce bacterial lysis and the mixture was chilled at 4°C and centrifuged to remove bacterial cell debris. The supernatant containing phage particles was then filtered through a 0.22-µm nitrocellulose syringe filter to eliminate any residual bacteria.

For quantification, 10-fold serial dilutions of the filtered lysate were prepared in phosphate-buffered saline (PBS). Each dilution was mixed with *E. coli* and 0.7% molten top agar, then overlaid onto solid LB agar plates. After overnight incubation at 37°C, clear plaques, zones of bacterial lysis were counted. Phage titres were also determined using dilutions in SM buffer (50 mM Tris–Cl, 100 mM NaCl, 8 mM $MgSO_4$). The concentration of functional bacteriophages was expressed in plaque-forming units per milliliter (PFU/mL), calculated using the formula: number of plaques × 10 / dilution factor.

Propagation of bacteriophage using double-layer agar (DLA) method: To obtain high-titre bacteriophage stocks, plaques from higher dilutions were carefully collected using a sterile glass pipette and transferred into microcentrifuge tubes containing 500 µL of SM buffer. The tubes were gently vortexed to release phages from the agar and the resulting lysate was serially diluted up to 10-fold using SM buffer. Each dilution was mixed with 500 µL of *E. coli* culture and incubated for 30 minutes to facilitate phage adsorption followed by analysis using the DLA method.

Once uniform plaques are formed, the top agar from the three most well-defined plates was scraped off and suspended in 15 mL of SM buffer. This suspension was centrifuged at 10,000 rpm (17,000 g) for 25 minutes at 4°C to remove bacterial debris.

The supernatant containing phage particles was filtered through a 0.22 µm membrane to yield a purified lysate. This procedure facilitates the generation of a highly purified and concentrated bacteriophage stock, suitable for a range of

downstream applications including therapeutic interventions (phage therapy), molecular biology experiments and host range determination studies.

Biofilm formation: The described *E. coli* biofilm formation assay utilizes sterile 96-well polystyrene microtiter plates, a standard and reliable method for studying biofilm development. Fresh bacterial isolates were cultivated in nutrient broth under stationary conditions, then diluted 1:50 before inoculation into the wells, each containing ~10^8 CFU/ml. The experiment included ten different dilutions of high-titre ASEC2201 phage, introduced to assess their effect on biofilm formation. Negative controls ensured sterility and excluded non-specific media binding. Plates were incubated at 37°C and biofilm formation was monitored at eight time intervals: 24, 30, 36, 42, 48, 54, 60 and 66 hours. After each incubation period, wells were gently washed with PBS (pH 7.2) to remove planktonic cells, retaining only the adherent biofilm.

Performing each assay in triplicate and repeating them three times improved accuracy and reproducibility. This method provides valuable insights into the kinetics of biofilm formation, the influence of bacteriophages like ASEC2201 and potential antimicrobial strategies. It is particularly relevant in medical microbiology for understanding chronic infections where biofilms play a key role in antibiotic resistance and persistent colonization.

The tissue culture plate (TCP) assay, a standard method for assessing bacterial biofilm formation[7], was employed in this study with modifications based on the protocol proposed by O'Toole and Kolter[19]. All bacterial isolates were evaluated for their biofilm-forming capacity using 96-well microtiter plates. Following 24-hour incubation to allow for biofilm development, 25 µl of 1% crystal violet solution was added to each well. Crystal violet selectively stains adherent bacterial cells without binding to the polystyrene surface. After 15 minutes of staining at room temperature, excess dye was removed through multiple washes with distilled water.

The retained biofilm, now stained, was solubilized in 200 µl of ethanol-acetone (80:20 v/v). From this, 100 µl of the solution was transferred to a fresh microtiter plate and optical density (OD) was measured at 570 nm using a microplate reader. OD_{570} values served as a quantitative measure of biofilm biomass. To correct for background absorbance, wells containing only ethanol-acetone were used as blanks and subtracted from sample readings. Experiments were performed in triplicate and repeated three times to ensure reproducibility. Biofilm-forming ability was classified as high, moderate, weak, or absent, based on OD thresholds. The inhibitory effect of different phage titres and incubation temperatures on biofilm formation was calculated and expressed as percentage inhibition.

Results

Selection of coliphage and its propagation: A total of twenty-seven bacteriophages were isolated, demonstrating lytic activity against clinical *E. coli* strains. The ASEC2201 phage has shown good lytic activity against a clinical strain of *E. coli*, 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 was effectively lysed by ASEC2201 with clear plaque morphology (figure 1).

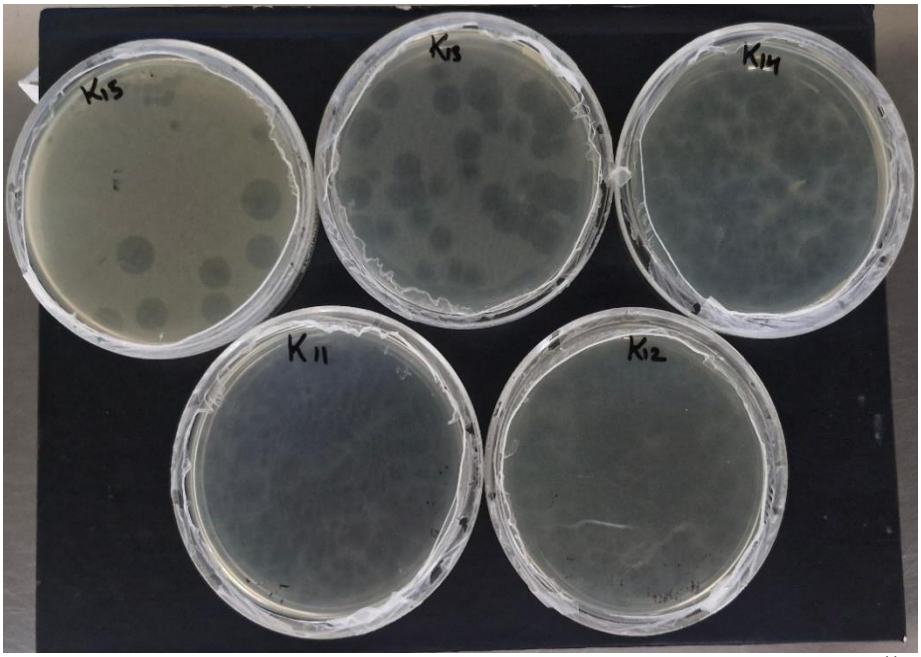

Figure 1: *E. coli* MDR strain susceptible to bacteriophage - ASEC2201 (Titre 10^{11} to 10^{15})

Therefore, in further studies, the same clinical strain of *E. coli* and ASEC2201 phage were selected for further evaluation 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.

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 ASEC2201, the latent period was found to be 30 minutes with a burst size of 615 PFU (plaque-forming units) per cell (figure 2).

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 different dilutions of coliphage ASEC2201 (Figure 3 A to J). Results demonstrated that the phage strain exhibited varying levels of biofilm inhibition. As given below, in the phage-treated group, coliphage ASEC2201 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. The time-based classification of ASEC2201-treated biofilms into strong, mid and weak categories, alongside percentage reduction, highlights the compound's progressive anti-biofilm activity. At 24 hours, most samples (ASEC2201 1–5) displayed strong biofilm (absorbance ≥ 0.160), with minimal reduction.

However, over the 66-hour treatment period, absorbance values declined substantially. For instance, we observed 18.42% reduction (0.190 to 0.155) in 10^1, 17.50% reduction (0.200 to 0.165) in 10^2, 19.44% reduction (0.180 to 0.145) in 10^3 and 31.82% reduction (0.110 to 0.075) at 10^{10} dilution. By 42 hours, samples transitioned to the mid biofilm category (0.120 to 0.159), showing partial EPS breakdown and early detachment. By 54 to 66 hours, all samples reached weak biofilm status (absorbance ≤ 0.119), with up to 31.82% biomass reduction. This trend reveals ASEC2201's consistent and time-dependent degradation of mature biofilms. The percentage reductions align with enzymatic or signalling interference mechanisms, validating ASEC2201 as a promising agent for biofilm reduction.

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 ASEC2201. 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.

Discussion

E. coli is a major infectious pathogen responsible for biofilm formation in clinical apparatus, leading to spread in more severe infections. 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[27].

Bacteriophages have emerged as promising alternatives to combat difficult-to-treat *E. coli* phenotypes, particularly multidrug-resistant (MDR) and biofilm-forming isolates. While recent studies have concentrated on isolating phages targeting *E. coli*, there has been limited investigation into their efficacy against MDR strains associated biofilms. The current study successfully isolated one bacteriophage demonstrating lytic activity against biofilm of *E. coli* resistant strain.

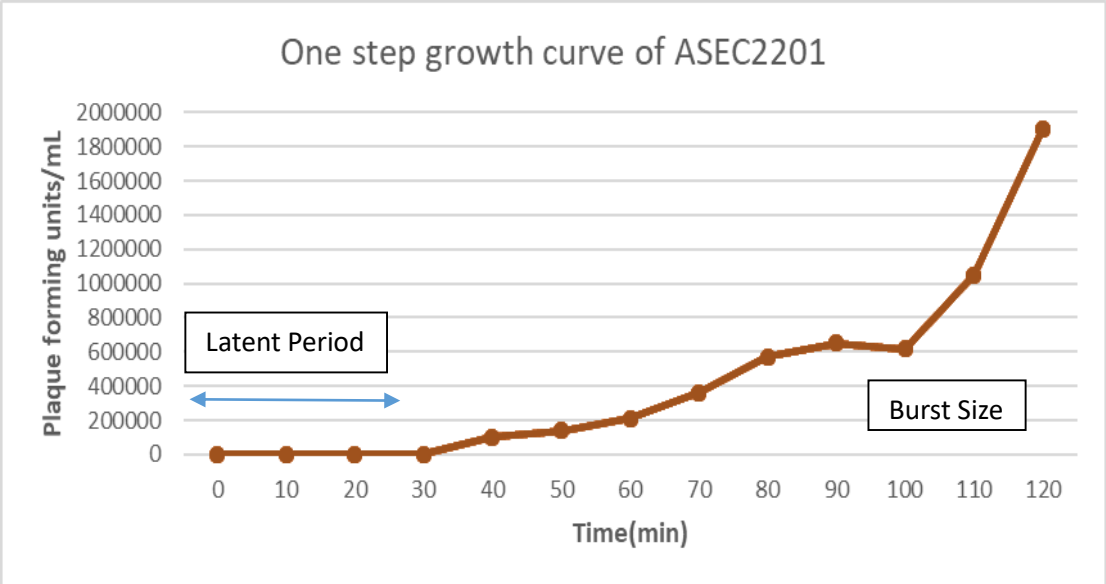

Figure 2: One-step growth curve of coliphage ASEC2201

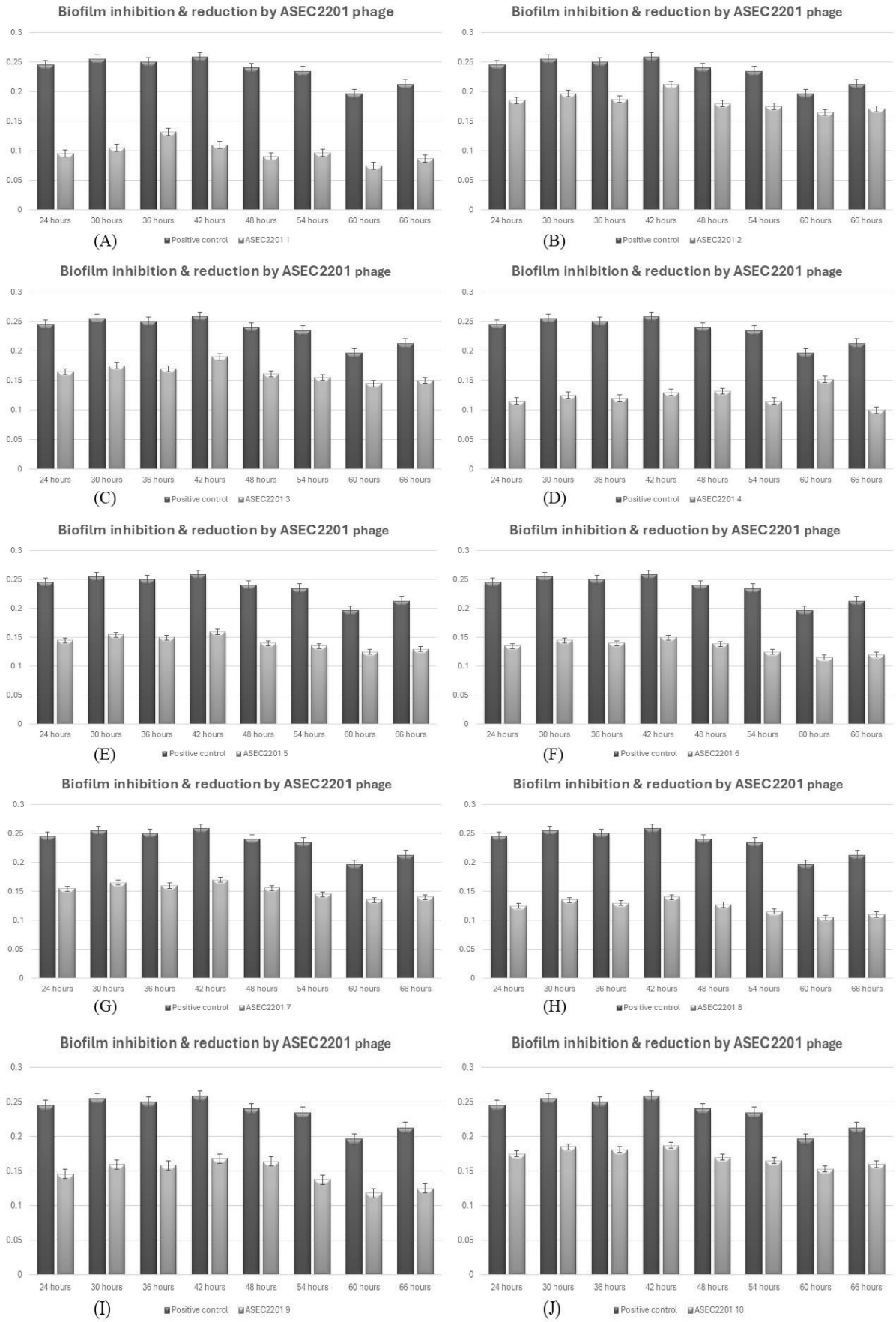

Figure 3: Reduction of bacterial biofilm caused by ASEC2201 coliphage at different dilutions from 24 to 66 hours. Graphs from A to J represent dilution of ASEC2201 from 10^1 to 10^{10} respectively. Positive control is *E. coli* without ASEC2201. X-axis represents the dilution of ASEC2201 and Y-axis represents the OD at 570nm.

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 ASEC2201 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 ASEC2201 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 ASEC2201 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 ASEC2201, which can penetrate and disrupt biofilms, offer a novel and much-needed approach in 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.

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 ASEC2201 *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 aim of the present study was to characterize bacteriophage for treating biofilms caused by antibiotic-resistant *E. coli*. Biofilms remain a significant concern globally, with *E.coli* being one of the leading pathogens implicated in this condition. According to a recent report compiled through a market analysis conducted by the UK's National Biofilms Innovation Centre (NBIC), biofilms can have an economic impact exceeding $5 trillion annually[5]. Several studies have demonstrated the isolation of bacteriophages from sewage water, revealing their potential in combating drug-resistant pathogens and showing their strong therapeutic potential.

Research by Chakraborty and team[6] and Necel and team[18] reported effective phage candidates against multidrug-resistant bacteria. Montso et al[14] found that phage can disrupt *E. coli.* biofilms on artificially contaminated beef. Alexyuk et al[1] highlighted the clinical applicability of phages in their study, by using phage cocktails as treatment of multidrug resistant *E. coli*. Vera-Mansilla and team[31] also emphasized on versatility of phage as an alternative to traditional antibiotics, particularly for targeting resistant bacterial strains. These studies collectively support phage therapy as a promising solution to antibiotic resistance.

The focus of this study was on hard-to-treat phenotypes of *E. coli.*, particularly biofilm-producing multidrug-resistant (MDR) strains. In this context, bacteriophages emerge as promising biological control agents that can be used alone or in combination with antibiotics to mitigate bacterial biofilm. Some recent significant examples include report by Dakheel et al showing two novel phages against 25 biofilm-producing MRSA strain[8]. An 80-100% biofilm removal of *K. pneumoniae* MDR strains by a Siphoviridae phage[29], phage therapy for *P. aeruginosa* biofilm in the mouse model of cystic fibrosis[32] and bacteriophages with high antimicrobial activity against biofilm-producing *A. baumannii* strains[2] are some of the other significant examples of the same.

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.

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