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# Sub-Inhibitory Treatment of Gentamicin in *Escherichia coli* Decreases T7 Bacteriophage Infectivity and Cell Lysis

Mahta Amanian*, Selina D. Demetrick*, Jaime Gabriel G. Gana*, Tyler L. Tam*

**All authors contributed equally.* Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

**SUMMARY** Increasing rates of multidrug resistant *Escherichia coli* infections have become a growing concern worldwide. With our current repertoire of antibiotics becoming less effective against bacterial infections, novel antimicrobial therapies are needed. Bacteriophages have been proposed as an alternative therapy against antibiotic resistant bacteria. Previous literature has proposed that *E. coli* release outer membrane vesicles that can prevent bacteriophage infection. The presence of gentamicin has been shown to increase the production of outer membrane vesicles (OMVs). To determine if treating *E. coli* UB1005 with gentamicin decreases infectivity of T7 bacteriophage, we treated *E. coli* UB1005 with a sub-inhibitory concentration of gentamicin and measured T7 infectivity and the rate of cell lysis. We hypothesized that treating *E. coli* UB1005 with a sub-inhibitory concentration of gentamicin would result in reduced bacteriophage infectivity and cell lysis. We observed decreased T7 infectivity using both a plaque forming assay and a bacterial growth curve to observe cell lysis, a methodology developed in this study. Additionally, bacterial growth curve results indicate a greater decrease in T7 cell infectivity when *E. coli* is in exponential phase compared to stationary phase.

## INTRODUCTION

The emergence of multidrug resistant bacteria is occurring rapidly worldwide and places a major burden on public health systems. It has been reported that at least 2 million people acquire an antibiotic-resistant infection, and at least 23,000 people die each year in the United States alone (1). The number of deaths and the decreasing efficacy of the current repertoire of antibiotics highlight the necessity for alternative therapeutics to combat bacterial infections. Bacteriophage or phage therapy, refers to the intentional administration of phages to prevent and treat bacterial infections. This biological therapy has gained more attention over the years and has been proposed as an alternative to traditional antibiotics (2). Although antibiotics are often very effective at treating bacterial infections, phage therapy has several advantages over antibiotics. Phages are specific to a single species of bacteria, as opposed to antibiotics that target broader classes of bacteria. As such, they are unlikely to disturb beneficial commensal bacteria living in the human gut (3). Currently, no phage therapeutic approaches have been approved for human use in North America (4). With the inevitability of increasing numbers of multi-drug-resistant bacteria, phage therapy is a promising alternative to antibiotics. This research may provide insight towards potential drawbacks for phage and antibiotic cocktails used to treat infections.

Bacteriophages are viruses that are specific for bacteria. The T7 bacteriophage is a member of the *Podoviridae* family that employs a lytic life cycle that hijacks the host machinery of *Escherichia coli* (*E. coli*) (5). During T7's lytic life cycle, the bacteriophage

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Address correspondence to: https://jemi.microbiology.ubc.ca/

attaching to lipopolysaccharide (LPS) on the outer membrane of Gram-negative bacteria delivers its genome into the host cell (5). After undergoing viral replication in the cytosol using host machinery, new bacteriophage particles are produced and new virions escape through lysis of the bacterium (2). The lytic life cycle highlights the benefit of bacteriophage therapy, utilizing lytic viruses as self-amplifying "drugs" that target and kill susceptible cells which may be more efficient than antibiotics that are incapable of self-amplification (2).

Bacteria are exposed to a number of unpredictable stresses in the environment including antibiotics, changes in temperature, pH, and nutrient concentrations (6). When exposed to a non-lethal stress factor, bacteria are able to elicit cross-protection, a response induced by a single stressor that can confer increased resistance against the same or a different stress factor (7). Antibiotics are common antibacterial agents that induce a stress response in cells and cause them to activate a number of genes to cope with the environmental stress (8, 9).

A previous study conducted by Hardman *et al.* suggests that *E. coli* UB1005 exposed to gentamicin are less susceptible to bacteriophage infection (10). The *E. coli* UB1005 strain has been observed to possess hypersensitivity against a number of antibiotics, including kanamycin and streptomycin, which are also aminoglycosides like gentamicin (11). Gentamicin is a cationic aminoglycoside antibiotic that inhibits protein synthesis by binding to the bacterial 30S ribosomal subunit (12). Furthermore, previous research has found that gentamicin treatment results in increased release of outer membrane vesicles (OMVs), which are lipid vesicles released from the outer membrane. It has been proposed that prior treatment with gentamicin may reduce susceptibility to T7 bacteriophage infection in *E. coli* through OMVs acting as decoys for bacteriophage binding (13). More evidence is needed to confirm this observation.

In order to elucidate the mechanism by which a decrease in T7 cell infectivity occurs, we first hypothesized gentamicin treatment decreases T7 bacteriophage mediated lysis. In this study, we treated *E. coli* UB1005 with sub-inhibitory concentrations of gentamicin. Using a bacterial growth curve assay and a plaque forming assay (PFA) to observe T7 mediated lysis, we have confirmed results from previous literature by showing that gentamicin confers cross-protection to T7 bacteriophage (10). Additionally, bacterial growth curve results show reduced lysis is found in exponential phase *E. coli* UB1005, but not when culture is in stationary phase.

## METHODS AND MATERIALS

**Bacterial Strain and growth conditions.** *E. coli* UB1005 was donated by the Hancock Lab at the University of British Columbia. Cultures of *E. coli* UB1005 for experimentation were grown in Lysogeny Broth (LB) Miller (Per 1 L $dH_2O$, 10 g tryptone, 5 g yeast extract, 10 g NaCl) overnight at 37°C on a shaker at 200 rpm.

**Propagation of T7 bacteriophage and determination of phage titer.** *E. coli* UB1005 culture was grown to an $OD_{600}$ of 0.5 in LB supplemented with 0.001 M $CaCl_2$ and 0.001 M MgCl. T7 bacteriophage was isolated from a single 8 mm diameter plaque. Bacterial culture was inoculated with T7 bacteriophage and incubated on a shaker at 200 rpm at 37°C until clearing was observed. Phage lysate was filter-sterilized using a 0.22 μm filter. Filtered lysate

**TABLE 1. PCR primer sequences.**

| Gene Target | Primer Orientation | Sequence | Melting Temperature ($T_m$) |
|---|---|---|---|
| T7 *rpol* | Forward | 5'-CGA GGG CTT AGG TAC TGC-3' | 55°C |
| T7 *rpol* | Reverse | 5'-GGT GAG GTG CGG AAC TTC-3' | 56°C |
| T4 *gp23* | Forward | 5'-GCC ATT ACT GGA AGG TGA AGG-3' | 56°C |
| T4 *gp23* | Reverse | 5'-TTG GGT GGA ATG CTT CTT TAG-3' | 53°C |

was treated with 1% chloroform and incubated for 10 minutes at room temperature. Lysate was then centrifuged at 4,000 g for 5 minutes and the supernatant was transferred to a sterile glass container and stored at 4°C.

**Confirmation of stock purity.** T7 stock purity was determined by heating bacteriophage stock at 95°C for 10 minutes to denature the viral capsid. A polymerase chain reaction (PCR) was conducted using primers specific to the T7 *rpol* and T4 *gp23* (Table 1), yielding products of 294 bp and 378 bp. PCR was performed using the manufacturer's recommended conditions. Taq DNA polymerase (ThermoFisher) was used in the PCR. The presence of T7 and absence of T4 was observed using gel electrophoresis made of 1% agarose gel in TRIS/Borate/EDTA (TBE) (Per 500 ml $dH_2O$, 27 g of Tris base, 13.75 g of Boric Acid, 10 ml of 0.5 M EDTA [pH 8.0]) stained with SYBR SAFE (Invitrogen). The resulting gel was imaged on a ChemiDoc imaging system (Bio-Rad).

**Preparation of gentamicin stock solution.** Concentrated stock solution of Gentamicin (10 mg/mL) was prepared by dissolving gentamicin sulfate (MilliporeSigma) in distilled $H_2O$ ($dH_2O$) and filter sterilized using a 0.22 µm syringe filter (VWR). Stock solutions were stored at -20 °C.

**Minimal Inhibitory Concentration assay for E. coli UB1005 to gentamicin to determine sub-inhibitory gentamicin treatment.** Minimum inhibitory concentration (MIC) of gentamicin in *E. coli* UB1005 was determined by qualitative observation of growth in 13 x 100 mm cell culture tubes. The 10 mg/mL gentamicin stock was diluted in LB and serially diluted from 128 µg/mL to 2 µg/mL. Tubes were inoculated with *E. coli* UB1005 to a final concentration of $10^5$ cells/mL. Tubes were then incubated on a shaker at 200 rpm at 37°C for 24 hours, then analyzed visually for turbidity. MIC was determined to be 16 µg/mL. Based on the highest concentration of antibiotic showing growth, the sub-inhibitory concentration used for downstream experiments was determined to be 8 µg/mL. This concentration was used for all future experiments.

**T7 Plaque forming assay.** An overnight culture was subcultured and incubated for 3 hours and normalized to an $OD_{600}$ of 0.45. Culture was then incubated with or without gentamicin for 1 hour. 100 µl of T7 bacteriophage was added to 1 mL of *E. coli* UB1005 ($OD_{600}$ of 0.45) at a multiplicity of infection (MOI) of $1.1x10^{-5}$ and incubated for 8 minutes. Culture was centrifuged at 5,000 g for 2 minutes. Supernatant was aspirated and pellet was resuspended

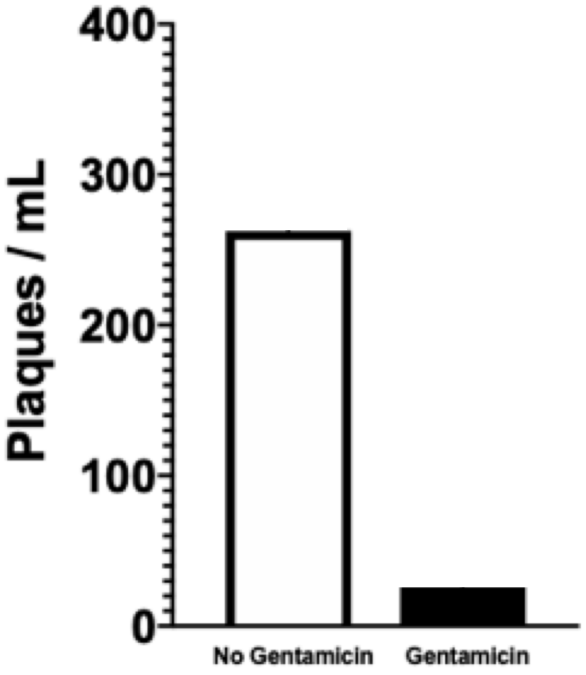


**FIG. 1 Treatment with sub-inhibitory concentrations of gentamicin reduces T7 plaque formation.** *E. coli* was incubated with 0 or 8 ug/mL gentamicin for 1 hour and phage was added at an MOI of $1.1x10^{-5}$. Culture was centrifuged, resuspended and plated on an LB plate. Plaques were counted after 6-hour incubation at 37°C. Each bar in the figure represents a single biological replicate that was plated in triplicate.

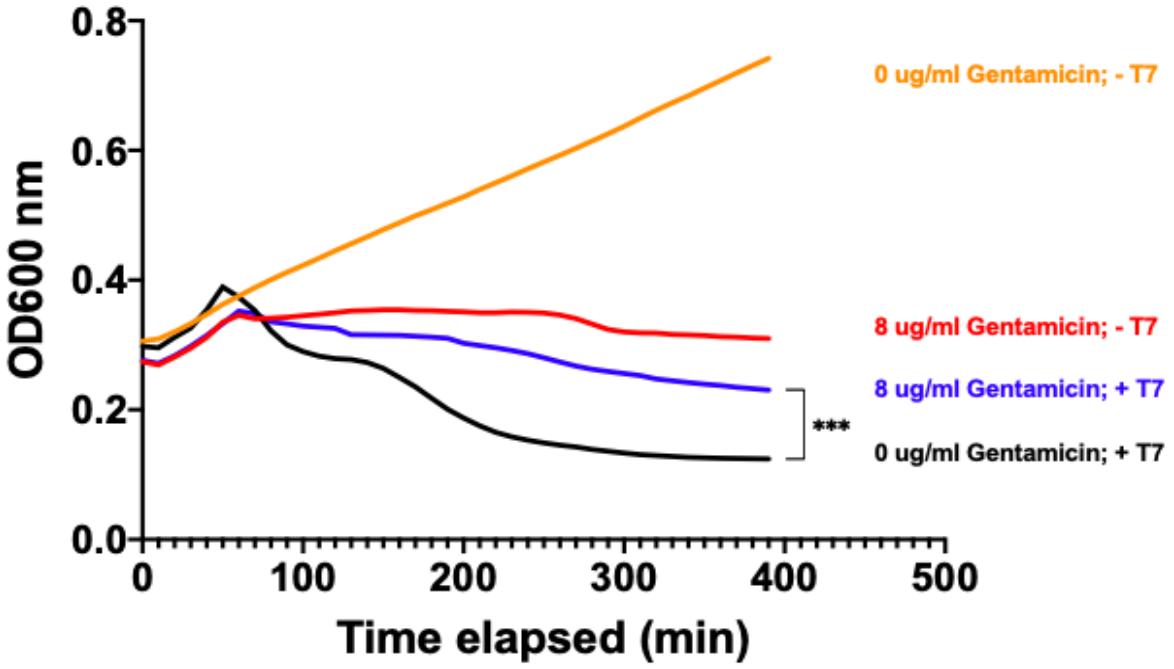


**FIG. 2 Sub-inhibitory gentamicin treatment reduces the rate of T7-induced phage lysis.** *E. coli* UB1005 was incubated with 8 $\mu$g/mL gentamicin or a $H_2O$ vehicle control for 1 hour and plated in triplicates on a 96-well plate. Phage and $H_2O$ control was then added at an MOI of $10^{-2}$ and OD was measured every 10 minutes for 400 minutes. Student's T-test; $p < 0.001$; $n = 3$ for every time point in each group.

in 1 mL of autoclaved $dH_2O$. Re-suspended cells were added to 3 mL of molten 9 g/L LB phage top agar (PTA) and vortexed to evenly distribute. PTA was poured onto LB plates and allowed to solidify. Plates were incubated at 37°C until bacterial lawn and plaques were visible. Plates with 3 to 300 plaques showing no overlapping due to overgrowth were enumerated. The final count is reported in plaques per mL of *E. coli* and bacteriophage that was added to the top agar.

**Generating stationary and exponential phase cultures.** *E. coli* UB1005 was incubated overnight at 37°C on a shaker at 200 rpm in LB. Exponential phase samples were prepared by diluting overnight culture 1:100 and incubating on a shaker at 200 rpm and 37°C until an $OD_{600}$ of 0.5 was measured. $OD_{600}$ 0.5 was at the boundary between lag and exponential phase (Fig. S1). Stationary phase samples were taken directly from the original overnight culture, after 16-hour incubation. Samples were normalized to an $OD_{600}$ of 0.5 with LB prior to gentamicin treatment.

**Lysis curve.** 5 mL cultures of *E. coli* UB1005 in exponential and stationary phase were incubated for 1 hour with 8 µg/mL of gentamicin, 4 µg/mL of gentamicin, or an autoclaved $dH_2O$ vehicle control. Samples were normalized to an $OD_{600}$ of 0.5 and plated on a microtiter plate. 20 µl of bacteriophage at an MOI of $10^{-2}$ or an autoclaved $dH_2O$ control was added to wells. The microtiter plate was incubated at 37°C on a BioTek Plate Reader for 6.5 hours. $OD_{600}$ was measured every 10 minutes, with 3 seconds of shaking before each reading.

**Statistics.** Statistical significance bacterial growth curve assay was evaluated using an unpaired Student's T-test. Data points consist of three biological replicates with three technical replicates each.

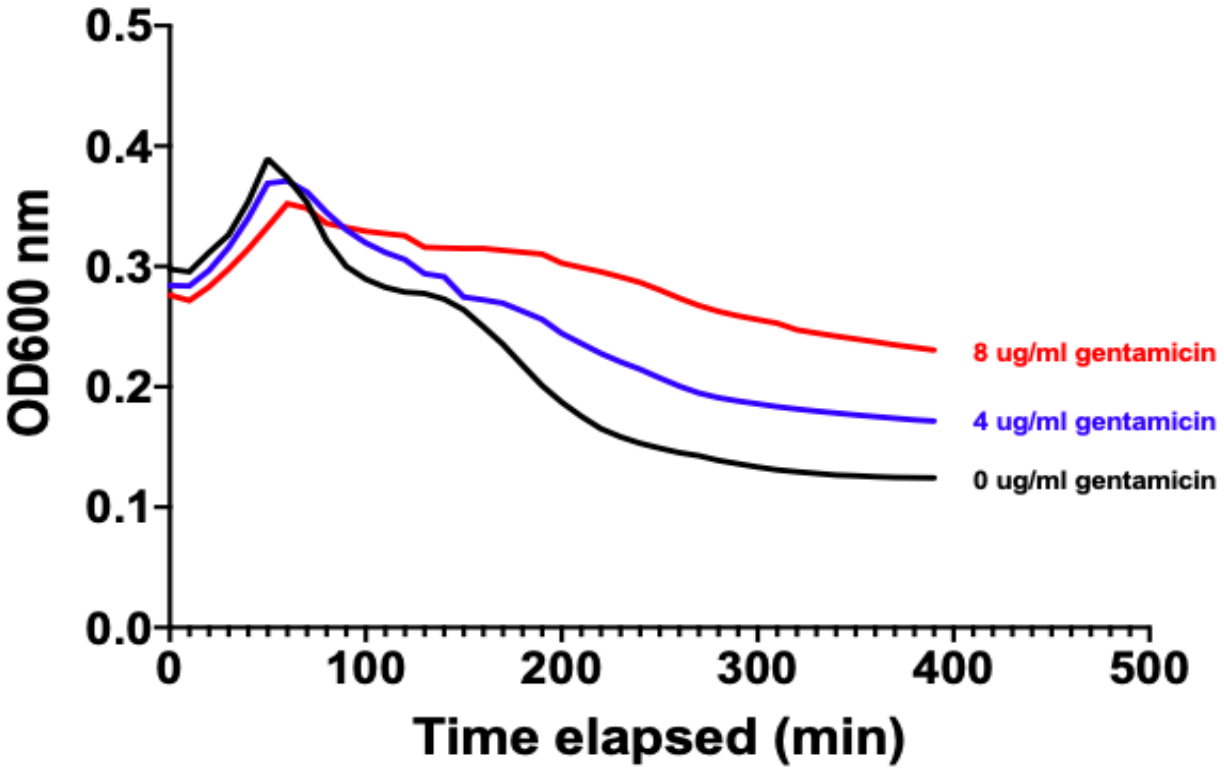


**FIG. 3 Delayed lysis and decreased T7 infectivity when treated with sub-inhibitory gentamicin is dose dependent.** *E. coli* UB1005 was incubated with gentamicin, 0 $\mu$g/mL, 4 $\mu$g/mL or 8 $\mu$g/mL and incubated for 1 hour. The culture was plated in triplicates on a 96-well plate and phage was added at an MOI of $10^{-2.7}$. $OD_{600}$ was measured every 10 minutes for 400 minutes.

## RESULTS

**Treating *E. coli* UB1005 with sub-inhibitory gentamicin reduces T7 plaque formation.** To validate the observations made by Hardman *et al.* that sub-inhibitory gentamicin treatment reduces T7 plaque formation, we conducted a plaque forming assay. Treatment with sub-inhibitory gentamicin, which was found to be 8 µg/mL, resulted in the formation of 10-fold fewer plaques, when compared to the number of plaques formed by T7 cell lysis of *E. coli* UB1005 cells treated with $H_2O$ alone (Fig. 1).

To screen for an appropriate MOI that would result in plates with a countable number of plaques, we performed the assay with 10 fold dilutions of bacteriophage ranging from an MOI of $10^{-2}$ to $10^{-7}$. Plates with an MOI of $10^{-2}$, $10^{-3}$, and $10^{-4}$ did not have a countable number of plaques. Plates with an MOI of $10^{-6}$ and $10^{-7}$ had a confluent lawn and no plaque formation. Plates with an MOI of $10^{-5}$ had between 3 and 300 plaques.

**Sub-inhibitory gentamicin treatment results in delayed T7 mediated cell lysis.** To measure phage mediated cell lysis over time, we constructed a bacterial growth curve through a real-time microtiter assay to observe cell lysis in response to sub-inhibitory gentamicin treatment (Fig. 2). Gentamicin treated *E. coli* UB1005 lysis began at the same time as no gentamicin controls. However, at all timepoints after the $OD_{600}$ began to decrease, gentamicin treated cells had a higher $OD_{600}$ than the autoclaved $dH_2O$. This data supports our result from the PFA. They both indicate sub-inhibitory gentamicin treatment decreases T7 cell infectivity.

**Delayed lysis of *E. coli* UB1005 to T7 when treated with gentamicin is dose dependent.** To further examine how the concentration of gentamicin can affect T7 mediated cell lysis, we performed a microtiter infectivity assay with *E. coli* UB1005 incubated with varying concentrations of gentamicin (4 µg/mL, 8 µg/mL, and 0 µg/mL control) to construct a

bacterial growth curve. Decreased lysis in response to T7 phage exposure was observed as sub-inhibitory concentration of gentamicin increased (Fig. 3). The degree of cell lysis for gentamicin treated *E. coli* UB1005 was lower compared to the control treatment (Fig. 3). These results show T7 cell infectivity is dose dependent.

**T7 cell infectivity is not observed in stationary phase *E. coli* UB1005.** Data from Figure 2 indicates treating *E. coli* UB1005 with gentamicin alters the rate of growth. This led us to question if the slower rate of cell lysis observed in Figure 2 is due to a reduced protein synthesis of *E. coli* UB1005 induced by gentamicin treatment. As bacteriophage depend on host machinery for protein synthesis, decreasing mRNA translation may impede propagation of T7 bacteriophage. Stationary phase culture has lower protein synthesis compared to exponential phase (16). Since gentamicin targets protein synthesis, we hypothesized stationary phase *E. coli* UB1005 would exhibit less T7 mediated cell lysis compared to exponential phase, because they have lower protein synthesis. To test this, stationary phase or exponential *E. coli* UB1005 with or without gentamicin was incubated with T7 bacteriophage with lysis measured over time (Fig. 4). Exponential phase *E. coli* UB1005 treated with gentamicin have delayed lysis compared to non-gentamicin treated. However, stationary phase *E. coli* UB1005 treated with and without gentamicin show the same lysis. When comparing non-gentamicin stationary and exponential samples, exponential has a more gradual decrease in $OD_{600}$ over time compared to stationary, which exhibit a sudden drop in $OD_{600}$ at 50 minutes.

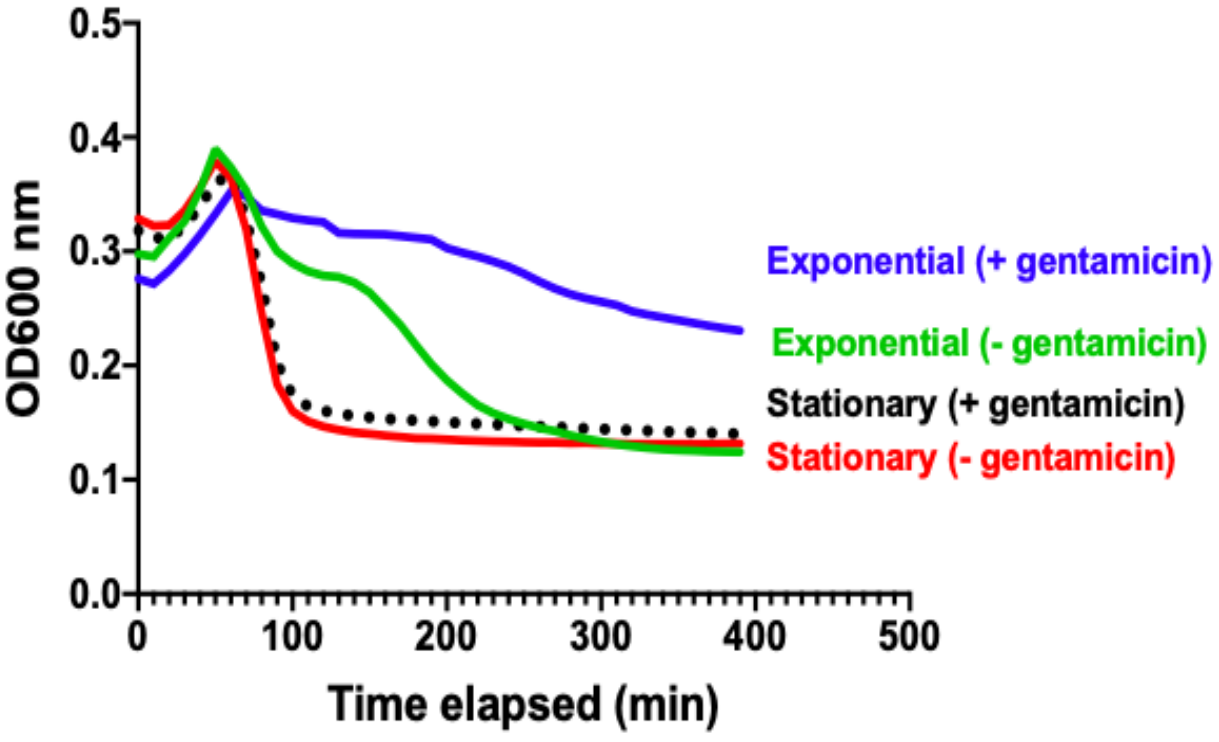


**FIG. 4 Decreased infectivity is only observed in exponential phase *E. coli* UB1005 and not stationary phase.** Exponential phase *E. coli* UB1005 prepared by making a 1:100 dilution of culture and incubating on a shaker at 37°C and 200 rpm to an $OD_{600}$ of 0.5. Stationary phase *E. coli* UB1005 prepared by incubating for 16 hours at 37°C and 200 rpm. Both cultures were normalized to an $OD_{600}$ of 0.5 and treated with 8 ug/mL gentamicin for 1 hour before adding phage at an MOI of $10^{-2}$. $OD_{600}$ was taken every 10 minutes for 400 minutes.

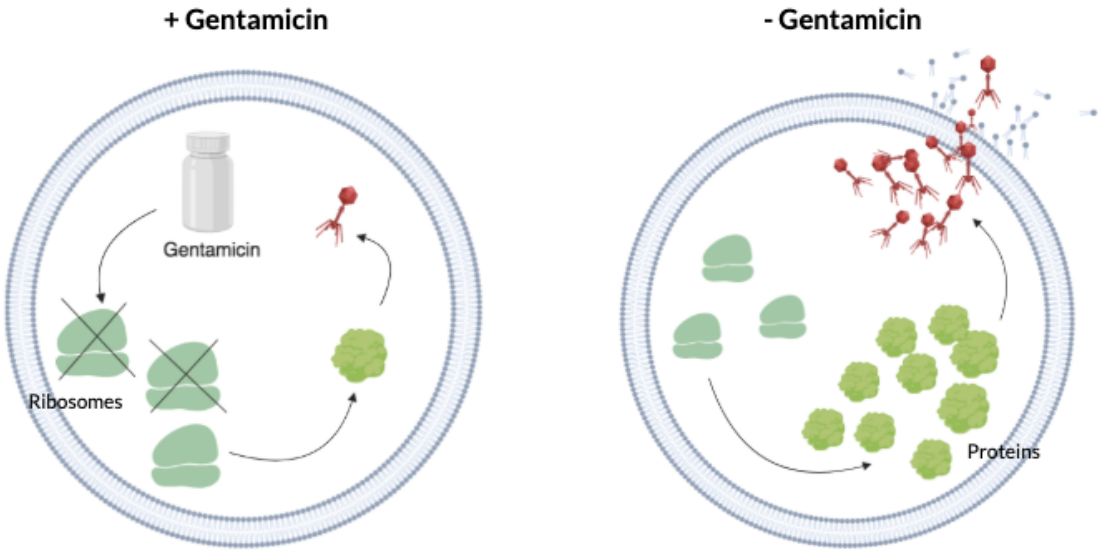


**FIG. 5 Proposed protein synthesis model for decreased T7 infectivity in gentamicin treated *E. coli* UB1005.** Treatment with sub-inhibitory concentrations of gentamicin leads to non-lethal inhibition of ribosomal activity, leading to subsequent decreased protein synthesis. This in turn leads to reduced concentrations of phage, which would lead to a lower ability to infect and lyse neighbouring cells (A). In the absence of gentamicin, the host cell machinery is hijacked by T7 phage (B). This results in composition of numerous phage particles.

## DISCUSSION

Phage therapy is an appealing therapeutic approach for the elimination of pathogenic bacterial infections in the skin, lungs, and gastrointestinal tracts (14). With rapid emergence of multidrug resistant bacterial infections, studying the efficacy of phage therapy has been gaining traction. Given the present preference of antibiotics as a first line treatment, our study, which investigates the possibility of antibiotics like gentamicin resulting in a decreased T7 cell infectivity is of great importance. We also investigated if protection is growth phase dependent in *E. coli.* Previous studies have shown gentamicin treatment has the capacity to activate cross-protection mechanisms against T7 mediated lysis (10). Other papers have focused on pre-treating multiple strains of *E. coli* with different antibiotics. They have found that β-lactams and aminoglycosides as the primary stressor can confer protection and delay T7 mediated lysis (10, 15, 16).

In order to elucidate the mechanisms by which a decrease in T7 cell infectivity occurs in *E. coli* UB1005 in response to gentamicin, we first investigated whether prior treatment with gentamicin can reduce T7 bacteriophage mediated lysis. The first approach made use of a PFA, which has been performed in prior studies (10). The PFA was conducted under the guidelines set by Hardman *et al.,* and we found that gentamicin treatment in *E. coli* UB1005 resulted in a ten-fold reduction in plaque formation when compared non-gentamicin treated *E. coli* UB1005. These results are in agreement with Hardman *et al*., which found a 2-fold decrease in the number of plaques when treated with gentamicin. The fold difference between our results and theirs may be due to modifications to their protocol. Hardman *et al.* used M9 minimal growth media, whereas our study was conducted using LB for cultures and to make top and bottom agar. M9 is a minimal media that is used to test stress factors on growing organisms (17). Consequently, we speculate the difference could be due to nutrient variability playing a role in the mechanism that causes the decreased T7 cell infectivity. Our bacterial growth curve also further supports the data from the PFA (Fig. 2). By observing a similar decline in $OD_{600}$ over time, the data indicates gentamicin treatment significantly delays T7 mediated cell lysis ($p < 0.001$). However, we only produced a single biological replicate for the PFA, which could call into question as to whether the data from Fig. 1 are reproducible. Moreover, Hardman *et al.* used an MOI of $7.0 \times 10^{-4}$, while we screened a variety of MOIs ($10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$ and $10^{-7}$) and determined an optimal MOI of $1.1 \times 10^{-5}$, which

formed an enumerable number of plaques. The difference in MOIs between the studies may have also played a role in the contrasting fold differences in plaques observed. Regardless, it is worthwhile to mention our results are in agreement with Hardman *et al.* in that sub-inhibitory gentamicin treatment decreases T7 infectivity. When comparing gentamicin treated and untreated controls without bacteriophage, we saw that sub-inhibitory gentamicin treatment slows growth (Fig. 2). Given that gentamicin inhibits ribosomal activity, we questioned whether the difference in cell lysis was primarily due to a decrease in protein synthesis and subsequently slower growth. This is represented in Figure 5. Here, we propose a second model to explain decreased infectivity in gentamicin treated cells. Gentamicin inhibits ribosomal activity and therefore protein synthesis. Since T7 bacteriophage relies on host ribosomes for viral protein synthesis, inhibition of ribosomal activity could lead to a prolonged intracellular life cycle and a longer period of time before the cell will lyse.

To determine if this was the case, we measured the rate of T7 mediated cell lysis in exponential and stationary phase cultures. Stationary phase cultures have lower rates of protein synthesis compared to exponential phase cells (18). If true, stationary phase cells would have a lower rate of cell lysis compared to exponential phase. However, when comparing the rate of lysis, stationary phase $OD_{600}$ decreased faster than exponential phase (Fig. 4). In addition, when treated with gentamicin, stationary phase cells do not exhibit a delay in T7 mediated cell lysis, which is only observed in exponential phase *E. coli* UB1005. These results suggest decreased infectivity is growth phase dependent in E. coli UB1005, and that lower protein synthesis in gentamicin treated cells is likely not the mechanism that decreases T7 cell infectivity levels. Taking into consideration the results from both the bacterial growth curve assay and the PFA, a plausible explanation is that the release of a soluble factor may prevent adsorption of bacteriophage. As we have previously mentioned, a greater fold difference resulted between gentamicin treated *E. coli* compared to Hardman *et*

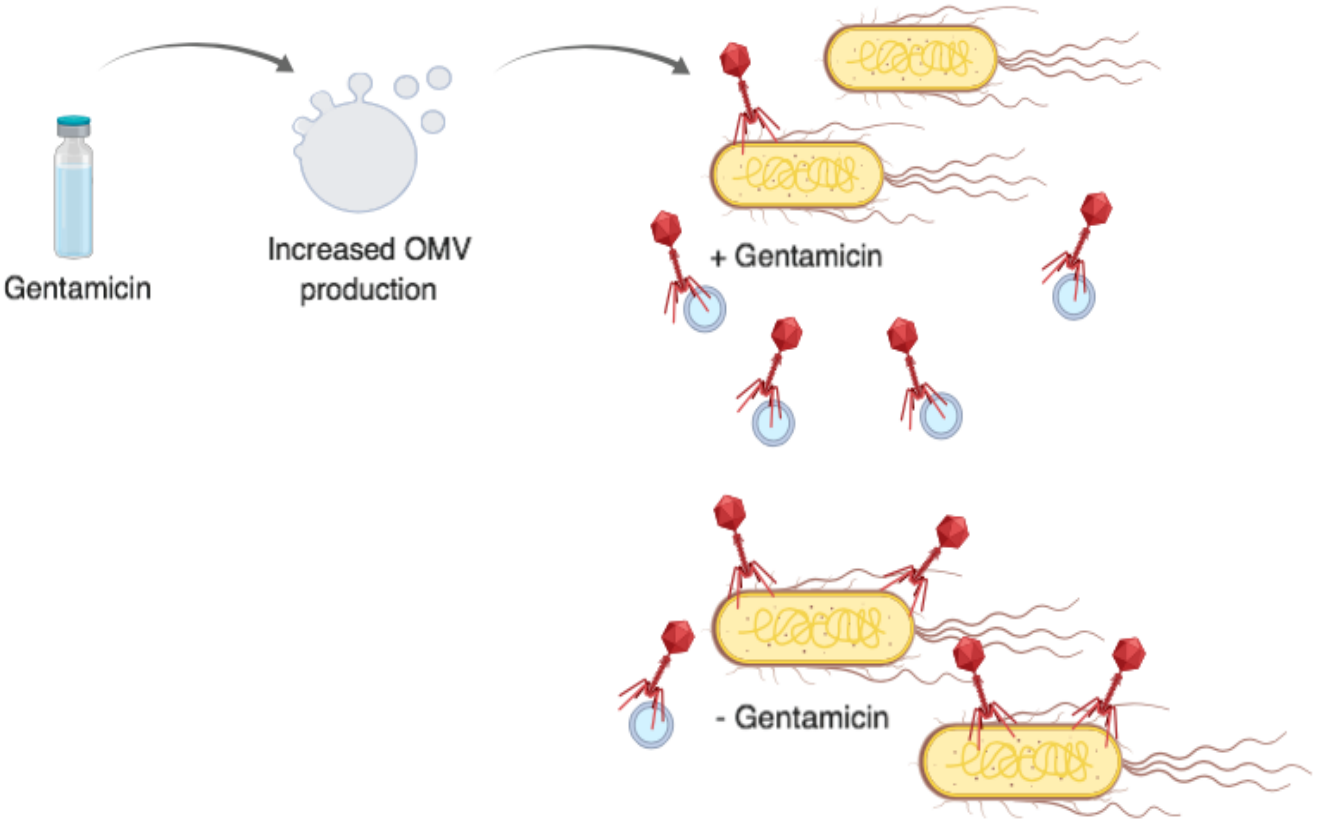


**FIG. 6 Proposed OMV model for decreased T7 infectivity in gentamicin treated *E. coli* UB1005.** Treatment with sub-inhibitory concentrations of gentamicin induces a stress response in *E. coli*, leading to an increase in production of OMVs. Vesicles then serve as binding sites for T7 phage, keeping the virus from binding to the host cell, thus conferring protection.

*al*. when using nutrient rich growth media. The production of a soluble factor is likely higher in cells that have sufficient growth conditions. Likewise, the release of soluble factors could also be dose dependent. Data in Fig. 3 shows increasing doses of sub-inhibitory gentamicin further delays cell lysis. It must also be acknowledged that the level of soluble factors secreted may also be dependent on the state of the cells. Stationary phase cells may not release as many secreted factors compared to exponential phase cells because of the reduced metabolism associated with stationary phase.

One potential released factor are OMVs. One study found OMV production to increase in exponential phase from increased production of peptidoglycan and LPS during cell division (19). OMVs form in gram negative bacteria when portions of the outer membrane bleb off, and release themselves from the cell (13). Additionally, OMVs have been shown to be produced when *E. coli* are exposed to gentamicin (20). A model for the protection following OMV production is outlined in Figure 6. We propose that upon treatment with sub-inhibitory gentamicin, *E. coli* produce a number of OMVs. These vesicles are then able to act as decoys for the phage, resulting in less T7 binding and infecting to the *E. coli*, leading to protection. Here we have focused on *E. coli*, however literature has shown OMVs are able to inhibit bacteriophage infection in *Vibrio cholerae* (21).

We proposed in our models that stationary phase cell had decreased cell lysis compared to exponential phase due to decreased protein synthesis. However, this difference between stationary and exponential cells may be mediated through other physiological mechanisms. E. coli's permeability to gentamicin may vary at different growth states. Another alternate explanation to this model is that gentamicin influences the production of phage lysis enzymes. A downregulation of phage lysis enzymes in the presence of gentamicin is a possible explanation for our observation.

**Conclusions** In conclusion, this study has confirmed sub-inhibitory treatment of *E. coli* UB1005 with gentamicin results in protection against T7 bacteriophage. We also show protection is proportional to the concentration of gentamicin. Using a PFA and a bacterial growth curve infectivity assay, we provide evidence that protection is primarily found in exponential phase and not in stationary phase. Our data cannot pinpoint the exact mechanism; however, we propose two plausible mechanisms worth studying. The release of OMVs acting as decoys for phage is based on previous literature (20, 21). Additionally, we show growth of *E. coli* UB1005 is affected by the presence of sub-inhibitory gentamicin, leading us to propose lower protein synthesis when treated by gentamicin may explain the delayed lysis in gentamicin treated cells (Fig 2).

These findings provide a foundation for future research in examining the use of phages in the treatment of bacterial infections. As there is an upward trend in using combinatorial therapeutics in treating bacterial infections, our results are important to understand the interplay between antibiotic treatment and bacteriophage mediated bacterial lysis. Based on our findings, the observed decreased level of T7 cell infectivity induced by gentamicin suggests a combination of gentamicin treatment and bacteriophage for antibiotic resistant *E. coli* infections may not be effective.

**Future Directions** Future studies should focus on elucidating the mechanism by which a decrease in T7 cell infectivity is occurring. We have proposed two mechanisms future researchers may decide to investigate, OMV production (Fig 5) or reduction in protein synthesis (Fig 6).

When testing the OMV model, we first propose isolation of supernatant from gentamicin treated *E. coli* UB1005. The supernatant will be used to resuspend a pellet of fresh *E. coli* UB1005. A PFA and bacterial growth curve infectivity assay can then be used to determine if a secreted factor is sufficient to decrease T7 cell infectivity. However, the results should be compared to samples with and without gentamicin that did not transfer supernatant. This comparison may show if multiple factors from the supernatant or associated with the cell is responsible for a decrease in T7 cell infectivity.

The overexpression of the *hlyF* gene in *E. coli* has also been implicated in an upregulation in OMV production (22). Consequently, a PFA could be performed on *hlyF* knockout *E. coli* UB1005 cells to see how this mutation affects the number of plaques that form. If an apparent

connection is observed, quantifying the expression of *hlyF* through qPCR when *E. coli* is treated with gentamicin may be performed. This would give insight into the possibility that gentamicin upregulates OMV production as the potential mechanism that decreases T7 infection and cell lysis.

Moreover, transmission electron microscopy on gentamicin treated *E. coli* cells incubated with T7 bacteriophage could be performed to visually confirm if T7 is adsorbing onto OMVs or any other potentially secreted products from gentamicin treatment.

Alternatively, we also propose further studies to determine if reduced protein synthesis is responsible for delayed lysis in *E. coli* treated with sub-inhibitory gentamicin compared to control. Here we show stationary phase *E. coli* did not exhibit a decreased level of T7 cell infectivity, whereas exponential cells did (Fig 4). We chose to compare stationary and exponential phase cells because stationary phase cells have lower protein synthesis. The first step in studying this possible mechanism is to determine if sub-inhibitory gentamicin decreases protein synthesis.

Although we have proposed two mechanisms, future researchers should actively consider alternate explanations. For example, an alternate explanation could be increased secretion of extracellular polysaccharide that forms a protective capsule around the outer membrane of E. coli (23). This coating has been proposed to be able to protect against T7 bacteriophage penetration (23).

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## CONTRIBUTIONS

All authors equally contributed to the experiments and writing of the paper.

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