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# *E.coli* and Bacteriophage T4

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## Using lytic bacteriophages to prevent tissue culture contamination

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# Table of Abbreviations

Name	Abbreviation
Phosphate Buffered Saline	PBS
Gram Negative	G <sup>-</sup>
Gram Positive	G <sup>+</sup>
Roswell Park Memorial Institute	RPMI
Lysogeny Broth	LB
Multiplicity of Infection	MOI
KL25 strain of <u>E.coli</u>	KL25

## Abstract

Bacteria are commonly encountered cell culture contaminants. Due to the costly consequences of media contamination and the loss of research production, further research should be devoted to the prevention of contamination so time and money are not wasted. Phage have been demonstrated to prevent growth of pathogenic bacteria in food and animals, therefore this study determined how phages can be used to prevent media contamination. T4 was used to lyse contaminating E. coli bacteria, which showed as changes in OD600 levels in RPMI media. Results have demonstrated that T4 is significantly affective against growth of E.coli in RPMI media; however, not all E.coli growth was suppressed. This study provides further insight into decontamination via phage mediation in growth media.

Keywords: Contamination, E.coli, HeLa, Media, T4

## Introduction:

Bacteriophages are viruses that infect and reproduce within bacteria. Lytic bacteriophages kill the bacteria that they infect, therefore it is possible that phages could be used to combat antibiotic-resistant strains of bacteria by preventing further growth. The concept of using phages to prevent bacterial growth has been around since the discovery of phages in 1915. As early as 1971 Felix d'Herelle was convinced that the "lytic principle" of phage could be exploited clinically. Extensive research from this time produced promising results for treating cholera (Adams 1959). Initially, the therapeutic application of phages as antibacterial agents was hindered by several key factors (Merril et al. 1996): (i) the failure to recognize the relatively narrow host range of phages; (ii) the presence of toxins in crude phage lysates; and (iii) a lack of appreciation for the capacity of mammalian host defense systems, particularly the organs of the reticuloendothelial system, to remove phage particles from the circulatory system. As our understanding of phages developed, some hindrances were addressed through the use of toxin-free, bacteria-specific phage strains, which provided insight for the development of phage into therapeutically effective antibacterial agents. Such phages were typically isolated long-circulating mutants such as mutant *Escherichia coli*  $\lambda$  phage and *Salmonella typhimurium* phage P22 (Merril et al. 1996). Long-circulating mutants also have been reported to have greater capability as antibacterial agents than the corresponding parental strain in animals infected with lethal doses of bacteria.

Several studies have been conducted to determine the overall effectiveness of phages and their ability to lyse their corresponding host bacteria in food. Phages have been tested in foods contaminated with strains of *Campylobacter* (Goode et al. 2003, Loc et al. 2005), *Escherichia coli* (Abuladze et al. 2008, O'Flynn et al. 2004), *Enterobacter* (Kim et al. 2007), *Pseudomonas* (Ellis et al. 1973, Greer 1986), *Brochothrix* (Greer 2002), *Salmonella* (Leverentz et al. 2001 and 2006, Modi et al. 2001, Whichhard et al.

2003), and *Listeria monocytogenes* (Leverentz et al. 2003;2004, Carlton et al. 2005); however, a weakness of most approaches was the use of uncharacterized, sometimes temperate phages (Guenther et al. 2009). Taking this point into consideration, several authors (Bigwood et al. 2008, Leverentz et al. 2001, Leverentz et al. 2003, Whichard et al. 2003) have reported that treatments with lytic bacteriophages significantly decreased the levels of major food-borne pathogens in a wide variety of foods, with reductions ranging from 1.8 to 4.6 logs compared to those of untreated or placebo-treated controls. The FDA approved the use of phages for the treatment of ready-to-eat meat, and a mixture of six viruses was sprayed on ready-to-eat meat to eradicate strains of *L. monocytogenes* (Petty et al. 2007). With further extensive studies and careful selection of phage candidates, phages could eventually become one of the most effective antibacterial alternatives.

Due to the rise of antibiotic-resistant bacteria, a revitalization of interests on phage therapy has been observed in the last two decades in western countries (Brussow 2005; Skurnik and Strauch 2006). Treatment of *Escherichia coli* infections in experimental mice and calves (Smith et al. 1987), indicated that phages can be employed for clinical treatment or prevention of infectious diseases caused by both G<sup>+</sup> and G<sup>-</sup> bacteria (Bull et al. 2002; Stone 2002). Phage therapy has been pre-clinically tested with strains of *Escherichia coli* (Smith and Huggins 1982 and 1983; Barrow et al. 1998), *Pseudomonas* (Soothill et al. 1994), *Salmonella* (Topley et al. 1925), and *Shigella* (Dubos et al. 1943). The advantages of phage therapy over antibiotic therapy are summarized as follows: (i) phage are effective against multidrug-resistant pathogenic bacteria; (ii) phages do not create substitutions in the normal microbial flora because the selected phages kill only the targeted pathogenic bacteria; (iii) the frequency of phage mutation is significantly higher than that of bacteria, so there can be a quick response to the appearance of phage-resistant bacterial mutants because of this; (iv) the development costs for a phage treatment is cheaper

than that of new antibiotics; and (v) side-effects are very rare (Sulakvelidze et al. 2001; Matsuzaki et al. 2005).

Although phage therapy is a promising avenue of research there are still some concerns. These include: (i) lytic death results in the release of large amounts of bacterial membrane-bound endotoxins (Hagens et al. 2004); (ii) neutralization of phages by the host immune system may lead to failure of phage therapy, though this is typically occurs after several administrations and it is useful to note that a single administration is typically enough to eliminate the pathogenic bacteria (Wang et al. 2006); (iii) conversion of lytic phage to lysogenic phage (prophage) leads to bacterial immunity that will limit the infectivity the corresponding lytic phage and may also change the virulence of the bacteria, though this could be easily dealt with by properly selecting and characterizing long circulating lytic phage candidates such as the mutant  $\lambda$  and P22.

The main challenge for the expansion of phage use will be the necessary performance of large-scale clinical trials in accordance with U.S. FDA or European guidelines. Usually, these procedures are very expensive and take several years. Without profound interest from big pharmaceutical companies, which has not been expressed to date, it is difficult for phage therapy to gain widespread acceptance or application in the Western world in the near future. Since phage have been demonstrated to prevent growth of pathogenic bacteria in food and animals, further investigation should be required to determine further uses of them. Such uses might be in preventing bacterial contamination of cell media in laboratories. Bacteria are one of the most commonly encountered cell culture contaminants, and because of the costly consequences of media contamination, further research should go into means to prevent contamination so time and money are not wasted. The objective of this study is to determine how phage can be used to prevent media contamination by using T4 to kill contaminating *E. coli* bacteria.

## Methods:

### Bacterial and Viral Stocks

KL25 Escherichia coli strain served as the host for wild type T4 phage. T4 was supplied from Carolina Biological Supplier (#124330) and its host was supplied by Concordia University College of Alberta.

### Media

To prepare phage stocks for serial dilution, T4 was grown with E.coli in LB from Sigma Aldrich (#826150).

The media used to promote the maximum growth of KL25 E.coli for the plaque assays was a tryptone based top and base agar. The top agar contains 10g tryptone, 9g agar, 5g KCl, 1L H<sub>2</sub>O. Base agar contained 10g tryptone, 11g agar, 5g NaCl, 2ml 1M CaCl<sub>2</sub>, 1L H<sub>2</sub>O.

RPMI-1640 media from Life Technologies (11875-085) was used to promote the growth of E.coli for contamination.

Serial dilutions of T4 were performed with PBS containing MgCl<sub>2</sub> at pH 7.4.

### Phage Titer

Tubes of tryptone soft agar and five tryptone plates were labeled to indicate the various dilution of virus ( $10^{-5}$  through  $10^{-9}$ ), and placed into a water bath. A microwave was used to melt the agar, and then placed in a metal bead bath to maintain the melted agar at 60°C. Tubes of 0.1mL serially diluted phage were inoculated with 0.1ml of an overnight KL25 culture in LB. 4ml of top agar was added to each

tube then the tube. Immediately afterwards the tubes were mixed well and then poured onto a Petri plate with bottom tryptone agar, creating a thin layer of agar that has been inoculated with bacteria and viruses in each plate. The plates were all incubated in an inverted position for 24 hours at 37 °C. The data from this experiment was used to verify the viral titer, as well as see if T4 will lyse KL25.

#### Contaminated media without HeLa cells

Before testing contaminated cells in media, standalone media was tested once to observe if E.coli growth could be eliminated. A 96 well plate filled to 0.3ml was inoculated with the controlled concentration of E.coli (at 100 cells/mL). To see how incubation period of E.coli influences phage efficacy, KL25 was placed in an incubator at 37°C to incubate. After incubation time has elapsed, the various concentrations of T4 were added to the media (to match a 0.1, 1, and 10 MOI). The phage and bacteria were left to incubate for a day and then using a photo spectrometer, the absorbencies of the wells were measured to determine the amount of bacterial growth.

#### Statistical Analysis

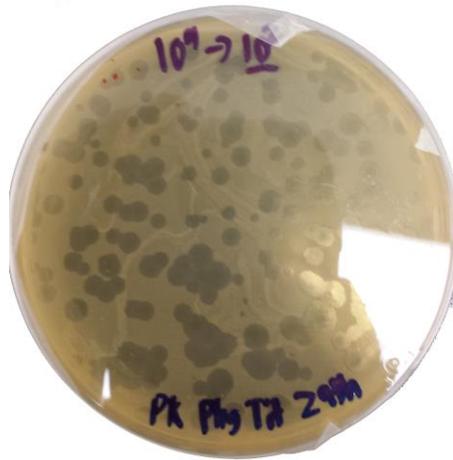
The significance that T4 had on preventing further contamination of cell media was determined by using a One-Way ANOVA. Natural variance in the spectrophotometer was estimated to be  $\pm 0.005$ nm. The blank mean was determined to be 0.102.

#### Contaminated media with HeLa cells

A 12 well plate filled to 1ml of RPMI was inoculated with the controlled concentration of E.coli (at 100 cells/mL). The various concentrations of T4 were added to the media (to match a 0.1, 1, and 10 MOI).

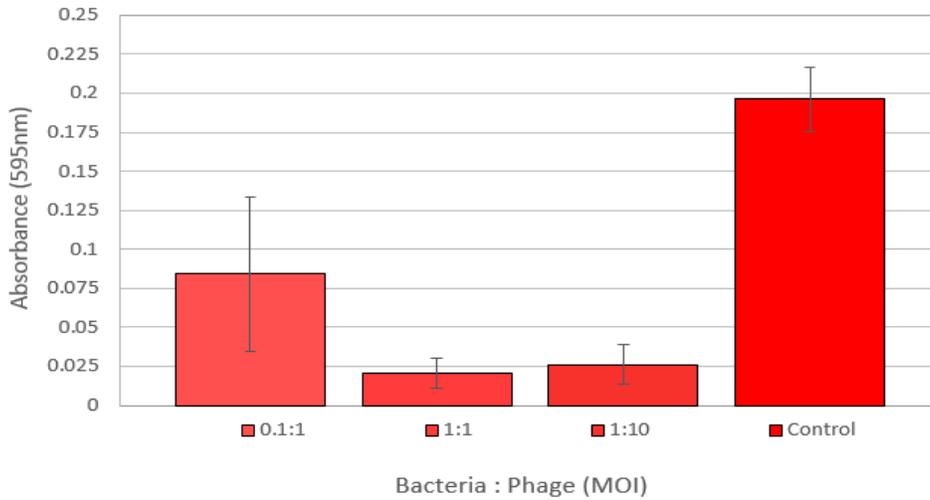
The phage and bacteria were left to incubate for a day and then analyzed under light microscopy. TSA plates were made for each well to qualitatively determine efficacy of T4 on KL25. The data collected was used to determine if T4 inhibited growth of HeLa cells, and how much KL25 growth was permitted.

## Results:

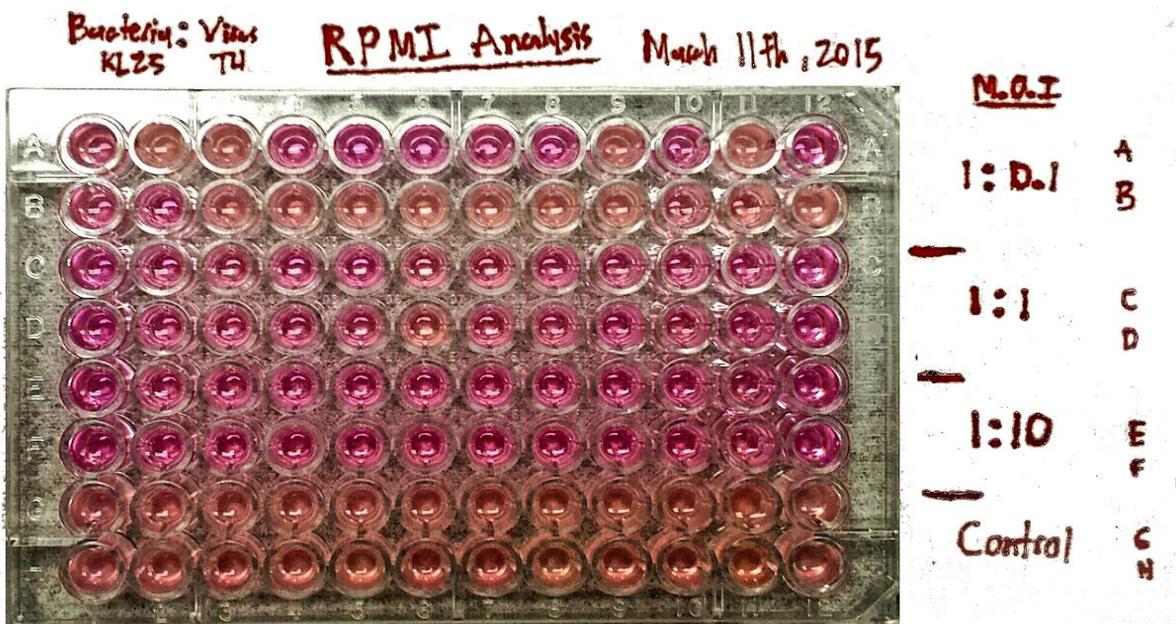


**Figure 1:** Plaque assay of phage titer at  $7 \times 10^2$  phage/mL

Five plates ranging from various titers ( $7 \times 10^{6-2}$  phage/mL) were recorded. The results of the phage titer demonstrated that T4 was successful at lysing the KL25 strain of E.coli. Only the  $7 \times 10^2$  phage/mL produced visible countable plaques (Figure 1). Many of the plaques were fused together, making an accurate count of the total number of units, therefore verification of the titer, difficult to determine.  $60^\circ\text{C}$  may have been too high of a temperature for most of the E.coli to grow successfully.

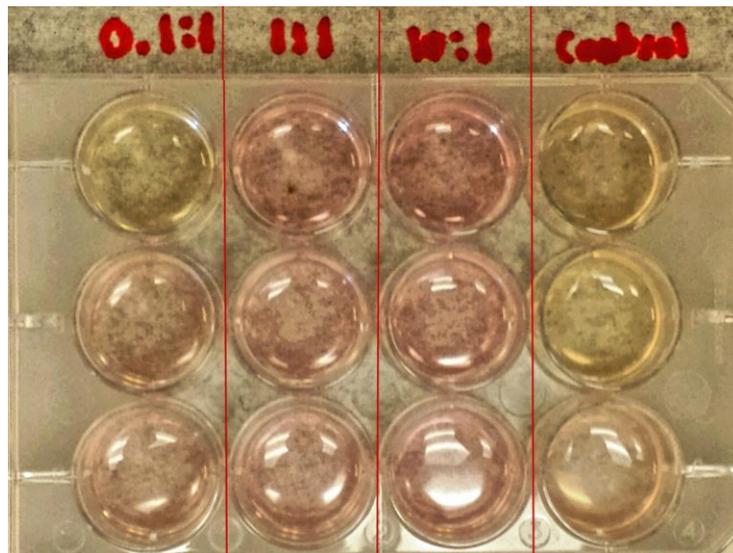


**Graph 1:** The mean absorbency readings for the MOI's conducted on 96-well plate analysis after exposure to 100 E.coli cells

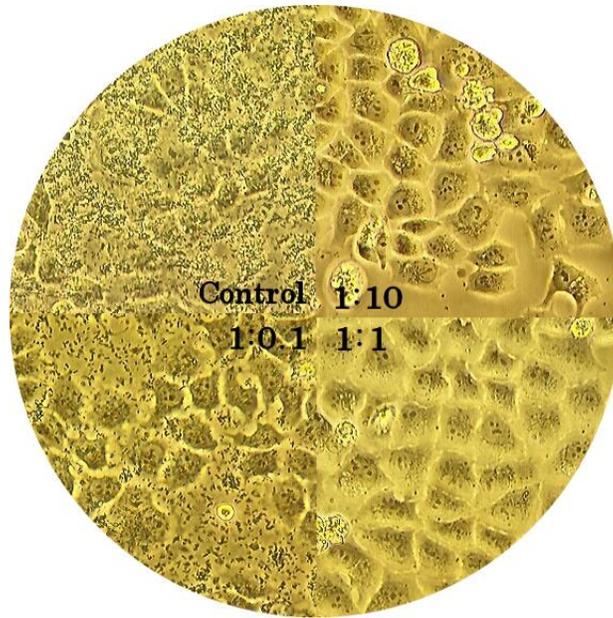


**Figure 2:** Dose response analysis of 1:0.1 (A & B), 1:1 (C & C), 1:10 (D & E), 4 (PGPP), along with a positive control (G & H) in a 96-well plate following addition of 100 E.coli cells. Orange indicates higher concentrations of E.coli due to insufficient mediation by T4 and purple indicates high mediation.

Since the titer could not successfully be verified, the stock titer of  $7 \times 10^9$  phage/mL was trusted as accurate. The KL25 was started on March 6<sup>th</sup>, 2015 used four day prior. The viability of the KL25 culture at time of use was calculated to be  $44\% \pm 5\%$ . The effect of various concentrations of T4 on the KL25 cell death is presented in [Figure 2](#). Optimal control appears to be observed at the MOI of 1:1 and 1:10 bacteria to phage ([Graph 1](#)). The cell death rate was significantly ( $F(3, 21) = 50.58, P < 0.001$ ) different in the treatment groups than the controls. This is expected since T4 should successfully lyse E.coli under proper conditions (As determined from [Figure 1](#)).



**Figure 3:** Dose response analysis of 1:0.1, 1:1, 1:10, along with a positive control (G & H) in a 12-well plate following addition of 100 E.coli cells. Orange-Green indicates higher concentrations of E.coli due to insufficient mediation by T4 and purple indicates high mediation.

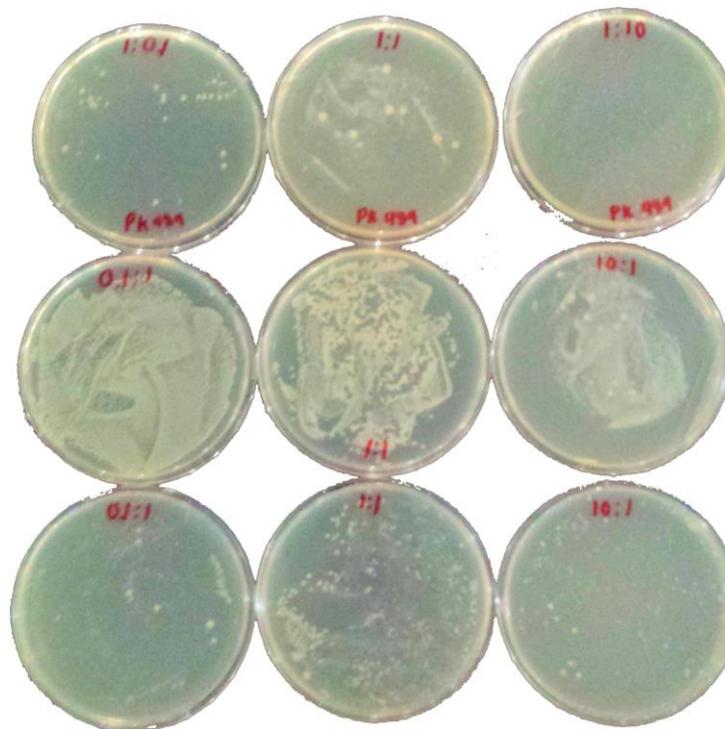


**Figure 4:** Microscopy analysis of 1:0.1, 1:1, 1:10, along with a positive control from the 12-well plate in Figure 3.

The KL25 was started on March 6<sup>th</sup>, 2015 used the day prior. The viability of E.coli was calculated to be  $50\% \pm 5\%$ . The confluence of the HeLa cells was approximately 90-95%. Highest amount of control is qualitatively observed at the MOI 1:1 and 1:10 bacteria to phage (Figure 3). Microscopy analysis was done to further analyze growth of E.coli in the wells (Figure 4), which shows clear reduction in the overall amount of KL25.



**Figure 5:** TSA plate of 50ul of the control from the 12-well plate.



**Figure 6:** TSA plate of 50ul of the 0.1:1, 1:1, and 1:10 MOI's from the 12-well plate.

TSA plates were conducted using 50ul from each of the 12 wells to further visualize the amount of growth from each plate ([Figure 5](#) and [Figure 6](#)). [Figure 5](#) demonstrates that complete mediation was not observed at the MOI of 1:1 and 1:10 bacteria to phage, which suggests that while reduction of KL25 population may be significant, it is not complete and some cells can continue to proliferate. [Figure 5](#) further suggests that the overall amount of growth is reduced as the multiplicities increase. It is possible that another bacteria other than KL15 created a lawn on the TSA, though this is unlikely due to the presence of plaques on some of the plates, as well as similarities in the colonies formed.

## Discussion:

The ability to establish and grow cell, organ, and tissue cultures has been widely exploited for basic and applied research. Regardless of whether the application is for research or commerce, it is essential that the cultures be established in vitro free of biological contamination and be maintained as aseptic cultures during manipulation, growth, and storage. The risks from microbial contamination are spurious experimental results due to the effects of latent contaminants or losses of valuable experimental or commercial cultures. The management of contamination in tissue culture involves three stages (Cassells 2012): disease screening of the culture with disease and elimination where detected; establishment and pathogen and contaminant screening of established initial cultures; observation, random sampling, and culture screening for micro-organism in multiplication and stored cultures. The critical control point management strategy for tissue culture laboratories is underpinned by staff training in aseptic technique and good laboratory practice. A lack of enforced aseptic techniques can lead to a host of problems for

laboratories that require tissues to be healthy for not only research, but also clinical purposes as well. While antibiotics are shunned because of increasing resistance, phage may have utilizations in mediations bacterial contamination, which could help save cultures, as well as time and money.

The proportion of bacterial cells that can be infected depends on primarily on two parameters (Kennedy and Bitton 1987). First, the binding of phages to their ligands on the bacterial surfaces is influenced by intrinsic factors, such as ionic strength, and pH. These factors are largely defined by the type of media itself and may change during the production, ripening, or storage of the media. Since it is difficult to predict the behavior of various types of phages in potentially complex media, further empirical data is required in different media types. Secondly, the concentration of phage at the time of application is crucial for efficacy, i.e., applying more phage generally resulted in greater inactivation. This is in accordance with the collected result from this study as well as the results of other studies, which also show that higher phage numbers yielded better results (Carlton et al. 2005; Leverentz et al. 2004). The results obtained throughout have further demonstrated that successful phage infection and subsequent killing of the host cells is strongly dependent on the initial inoculation dose of bacteria and the corresponding ratio of phage.

Though the results in [Figure 2](#) demonstrate a significance in the reduction in [E.coli](#) population, phage may not be an ideal candidate to completely inhibit bacterial growth in media (As demonstrated in [Figure 5](#)). It may however, have usage as a supplementary to antibiotics, with its primary purpose to target antibiotic resistant strains. A wide range of biochemical and physiological mechanisms may be responsible for resistance. In the specific case of antimicrobial agents, the complexity of the processes that contribute to emergence and dissemination of resistance cannot be overemphasized, and the lack of

basic knowledge on these topics is one of the primary reasons that there has been so little significant achievement in the effective prevention and control of resistance development. The emergence of phage-resistant bacterial mutants has been suggested to be a potential problem that might hinder the efficacy of phage treatment (reviewed in Leverentz et al. 2004); however, the results of numerous studies suggest that phage resistance is not a very frequent event and does not deleteriously affect the efficacy of phage treatment. This is a major contrast when comparing phage resistant bacteria to the increasing amounts of antibiotic resistant bacteria. It is vital that there should be absolutely no letup in the search for new or supplementary antimicrobial agents, especially with the ever-growing amount of antibiotic resistant strains (Boucher et al. 2009).

There is a major consequence for using antibiotics and/or phage; the consequence of overuse of antibiotics and phage is concealment of the poor aseptic technique and it is a major cause for possibly mycoplasma contaminated cultures. Though bacterial contaminants (especially the increasing amount of resistant antibiotic strains in laboratories) are typically a common risk when establishing of a tissue culture, they are usually obvious and easily detected. A serious problem is mycoplasma infection since these microorganisms are subtle and typically antibiotic resistant (Nikfarjam and Farzaneh 2011). The only truly successful method of get rid of mycoplasmas is to discard the contaminated cell cultures. This enforces that proper aseptic techniques should be used as the primary means of preventing tissue contamination, and that phage and antibiotics should only be used when necessary, such as maintaining tissue for transplant purposes.

## Conclusion:

At this point, we are just beginning to exploit the potential of phages for combating bacterial contaminations. Phage are cheap, effective alternative method to control bacterial populations in foods, but must be used as a supplement to antibiotics and aseptic technique in the laboratory. Phage be used in situations in which ensuring no bacterial contaminant is introduced, like in a tissue transplant facility. When used, phage are a significant mediator of target bacterial populations, though are not able to completely mediate target bacterial growth. They should be used as a supplement to antibiotics, which are a general means to control a large range of bacterial species. T4 has been shown to be a good, however incomplete mediator for E.coli contamination, and an ideal minimum MOI ratio of 1 bacteria to 10 phage should be enforced when using T4 as a mediator.

Proper aseptic technique, as well as maintaining a proper aseptic environment, was essential for obtaining the results shown, which further enforces the importance of using aseptic technique as the primary means to prevent bacterial growth in media. The erratic levels of growth shown in Figure 5 maybe from improper swirling of the 12-well plate, therefore causing an uneven 50ul solution of RPMI with phage and bacteria when plating on TSA. Results obtained from the 96-well plate are reliant upon choosing an appropriate reference value, ideally a negative control of standalone media. Instrumental drift should and has been considered in this report.

Because there is much potential to exploit in phages, much more research must be done to determine their potential. Different phage for different bacterial genus should be explored, as well as the use of a phage cocktail to mediate growth of multiple genii. Using different media with different intrinsic factors may yield a different sort of result compared to RPMI. Any of the mentioned would provide decent insight into further usage of phage in media.

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