

2006

Bacteriophage for the elimination of methicillin-resistant staphylococcus aureus (MRSA) colonization and infection

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Bacteriophage for the Elimination of Methicillin-Resistant *Staphylococcus aureus*
(MRSA) Colonization And Infection

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
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Date of Approval:
February 27, 2006

Keywords: nosocomial infection, antibiotic resistance, bacterial pathogens, alternative therapies, bacterial viruses

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Angela Clem

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is among the most important pathogens affecting the human race in our time. In spite of recent medical advances, our therapeutic choices for MRSA infections remain limited due to the propensity of this organism to develop resistance to antimicrobials. Therefore, there is a continuing need to develop newer methods of treating MRSA infections.

This dissertation examines the effects of bacteriophages 88 and 92 on ten clinical isolates of MRSA from the central Florida area. . For the majority of the MRSA isolates, bacteriophages 88 and 92 were unable to induce lysis. However, bacteriophage 88 was found to lyse MRSA Sample 94. Reduced cytotoxicity and apoptosis due to MRSA Sample 94 was also observed. This protective effect was most notable in the 1:10⁻⁶ concentration of MRSA 94 and bacteriophage 88. In addition, this effect was observable with both immediate inoculation of the cell culture with the MRSA concurrent with the bacteriophage and with bacteriophage applied one hour after initial inoculation of the MRSA. This effect was likely due to the increased replication of the bacteriophage in the actively growing bacteria found in the 1:10⁻⁶ samples. The bacteria in the 1:10⁻⁶

concentration were likely more able to replicate in comparison to the higher bacterial concentrations because of less competition between the bacteria for the limited nutrients in the 1:10⁻⁶ concentration.

The long-term goal of this study is the development of a bacteriophage-containing ointment for the control of MRSA nasal carriage. In addition, the concept of bacteriophage therapy may open a new horizon in controlling infections such as those caused by MRSA.

Finally, as for future studies, it would be informative to be able compare these results with other MRSA isolates and bacteriophages samples to examine the effects across a wider sample of bacteria and bacteriophages. In addition, it would be interesting to examine the possibility of being able to modify the bacteriophage in order to allow lysis of the previously resistant bacterial strains.

Introduction

Staphylococcus aureus (Microbiology - Virulence factors and Resistance)

Staphylococcus aureus is a Gram-positive bacterium. It is nonmotile and nonsporulating. It is also coagulase positive and a mannitol fermenter [14]. Methicillin-sensitive *Staphylococcus aureus* (MSSA) are ubiquitous denizens of the human skin and nares. *S. aureus* possess several intrinsic enterotoxins and exotoxins which can produce various clinical syndromes [15].

At the cellular level, these enterotoxins and exotoxins can induce cellular damage. The microorganism itself can induce cellular apoptosis after invasion of host cells. This invasiveness of *Staphylococcus* is mediated by staphylococcal fibronectin-binding proteins which allow the microorganism to bind to hosts cells. In addition, these cytotoxic strains of *S. aureus* could survive within host cells by avoiding degradation by the endolysosomal pathway and that these cytotoxic strains could produce a greater bacterial load and greater lethality than noncytotoxic Staphylococcal strains [52].

Methicillin-resistant *S. aureus* (MRSA) are more therapeutically challenging primarily due to their resistance to commonly used antimicrobial agents. The *mecA* gene is responsible for the increased antibiotic resistance of MRSA. The *mecA* gene encodes for PBP2a, which is a penicillin-binding protein with low-binding affinity and which mediates methicillin resistance [28]. The *mecA* gene is chromosomally encoded, and has been shown by ribotyping to have integrated into at least three distinct MSSA

backgrounds, known as A, B, and C. The *mecA* gene is polymorphic and has three types, I, II, and III. These types vary in (1) the number of base pairs, (2) the genetic organization, (3) the number of insertion sequences, and (4) the resistance determinants. All three of the *mec* types have integrated into cluster A; while, only Type II has integrated into cluster B and cluster C. Therefore, five distinct lineages of MRSA have been identified since the first MRSA strain was recognized in the United Kingdom in 1961 [27].

Two genes, known as *ccrA* and *ccrB*, have been shown to be capable of mobilizing *mecA*. These two genes are cassette chromosomal recombinases genes A and B. These genes are homologous to the DNA recombinases of the invertase-resolvase family. Even though these genes exist, the horizontal transfer of *mecA* is still considered relatively rare [28].

MRSA Prevalence: Global, Healthcare and Community Prevalence

The overall prevalence rate for MRSA in Tampa Bay is difficult to determine because there is no mandatory reporting of MRSA in Florida. One study [97] found that the prevalence rate for MRSA in urgent care centers averaged 12 per 10,000 visits, 24.5 per 10,000 visits in emergency room patients, and 158 per 10,000 hospital in-patients. Further, this study found that MRSA comprised 42% of the *S. aureus* isolates from urgent care centers, 52% of the *S. aureus* isolates from emergency rooms, and 69% of the *S. aureus* isolates from hospital in-patients.

Nationwide, prevalence rates may vary considerably. According to the 1990 to 1995 National Nosocomial Infections Surveillance System (NNIS) data, 16 % of

hospital-acquired bacteremia was due to *S. aureus* [7]. Approximately 25 % of all these nosocomial infections from *S. aureus* were due to MRSA [3, 7, 88]. In recent years, MRSA has accounted for approximately 80 % of all *S. aureus* isolates reported to NNIS [17].

There has been an increasing trend in the MRSA prevalence rate in the U.S. in the past 20 years. In U. S. hospitals, one literature source noted that MRSA had increased from 2.4 % in 1975 to 29 % in 1991 [75]. Another source indicated that MRSA has increased from 2 % to 39.7 % over the past two decades [101]. According to the 1994 - 1998 NNIS data, the MRSA prevalence in selected United States intensive care units (ICUs) ranged from 32 to 45 % [24]. A 1995 CDC estimate indicated that 34 % of *S. aureus* isolates from nosocomial bacteremia were actually MRSA [7]. The Intensive Care Antimicrobial Resistance Epidemiology (Project ICARE) (1996) study found that their MRSA rate was 35.9% [107]. These sources indicated overall prevalence rate of approximately 30 % for MRSA in the early to mid-1990s in the U.S. More recent figures may indicate even higher prevalence levels of MRSA in this country.

For example, some sources noted that MRSA rates in U. S. hospitals were as high as 40 % [90, 101]. By the end of 1998, the NNIS data were indicating the MRSA isolate rate was near 50 % [28]. The 1999 NNIS data indicated a higher figure of 52.3 % for the number of the *S. aureus* isolates that were identified as MRSA in participating U. S. ICUs [43].

Globally, the MRSA prevalence rates vary greatly. In northern European countries such as the Netherlands prevalence rates are approximately 1 % [27, 75, 101]

due to very stringent patient isolation procedures. In Denmark, the MRSA rate has decreased dramatically from approximately 20 to 30 % down to 3 % during the past decade because of decreased antibiotic usage [112]. However, MRSA prevalence rates are about 6 % in Switzerland and the other Scandinavian countries [101].

The rates of MRSA prevalence in central and southern European countries such as France, Spain, Italy, and Greece are around 30 to 40 % [27, 47, 75, 101]. However, some hospitals have high-risk units with MRSA rates as high as 90 % [75]. As for the United Kingdom, a British study indicated that the MRSA rates increased from 1.7 % in 1990 to 3.8 % in 1993 to 32 % in 1997 to 34 % in 1998 [43].

In Japan, one source reported that the rate ranged from 50 to 70 % [6]. However, other sources indicated that the prevalence rate may be greater than 80 % [75, 101].

Finally, a study indicated that MRSA isolates increased from 1 % in 1995 to 6 % in 1999 in Canadian hospitals [101]. The literature however did not provide any definitive rates for South and Central America; although, the literature did indicate that MRSA is also an issue in these regions of the world.

MRSA History

Before antibiotics, the fatality rate from *S. aureus* bacteremia was approximately 90 % [59]. In the 1940s, this rate dramatically decreased with the introduction of penicillin G. However, by 1942, *S. aureus* was already showing resistance to penicillin G. By 1944 to 1945, the *S. aureus* resistance rate for penicillin had risen to 12 % to 22 % [59]. This rate continued to rise until the 1960s when semisynthetic penicillins like methicillin and oxacillin were introduced. However, resistance was noted almost

immediately. By 1961, 0.04 to 0.2 % of *S. aureus* isolates were resistant to methicillin and oxacillin. Although increasing *S. aureus* resistance to methicillin was noted in Europe, Australia, and the U. S. [59], there were few MRSA isolates in the U. S. before 1967. However, by the 1970s, there was an increase in MRSA in the U. S., especially in large teaching facilities. MRSA slowly spread because the *mecA* gene, which encodes for an altered penicillin-binding protein, causes low-level resistance and is not plasmid-mediated. By the 1980s and the early 1990s, MRSA had become endemic in U. S. hospitals and long-term care facilities [90].

Concurrent with the increases of MRSA acquired in the healthcare setting, there has also been a recently recognized phenomenon: “community-acquired MRSA”. “Community-acquired MRSA” has been defined as a MRSA-positive specimen that has been obtained outside the hospital setting or within 2 days of hospital admission, and with the codicil that the patient has not been hospitalized within 2 years of the date of the positive specimen [81]. Community-acquired MRSA isolates, still sensitive to vancomycin, are usually only resistant to beta-lactam antibiotics in contrast to the resistance to multiple antibiotics commonly detected in healthcare-acquired MRSA isolates [28].

Although, vancomycin is the drug of choice against MRSA, the number of *S. aureus* isolates developing resistance to vancomycin has been increasing. In the 1990s, vancomycin intermediate *Staphylococcus aureus* (VISA) (Minimum Inhibitory Concentration (MIC) 8 to 16 ug/mL) had emerged in Japan and the U. S. (in 1997) [88, 103]. More recently, vancomycin resistant *S. aureus* (VRSA) (MIC greater or equal to

32 ug/mL) in the United States has been reported [89].

Staphylococcus aureus: Mode of Transmission, Colonization vs. Infection

S. aureus is a resilient bacterium that makes its appearance on healthy humans (skin and nasopharynx) soon after birth, and then may reappear throughout life [14]. Mucin appears to be critical in this colonization process [104]. During this process, there are interactions between the staphylococcal protein(s) and the mucin carbohydrate [104].

Colonization indicates that microorganisms are present on the skin and mucous membranes without any clinical signs or symptoms [36]. Colonization is much more common than infection [28].

The anterior nares (nasal cavity) are the main reservoir for *S. aureus* [3, 14, 17, 30, 33, 59, 77, 104] and this colonization may persist for years in 10 to 20 % of carriers [3]. One study indicated that other than the anterior nares, the skin can also be a primary reservoir for *S. aureus* [33]. *S. aureus* skin carriage occurs on the hands, axilla, perineum, nasopharynx, and oropharynx [3, 17, 107] in 25 % to 50 % of individuals [3].

Colonization in other parts of the respiratory tract and other body sites may occur and can result in *S. aureus* persistence. These other sites can provide an available source of *S. aureus* for nasal recolonization [32]. Therefore, it may be difficult to eliminate nasal *S. aureus* carriage [14] because of repeated autoinoculation. One study using multisite culturing (pharynx, perineum, groin, axilla) found that 16 % of carriers had negative anterior nares cultures, but 66 % had positive cultures of extranasal sites [105].

As mentioned above, nasal carriage of *S. aureus* may spread to the individual's skin and eventually to other people [33]. The nares may be the primary reservoir for hand carriage [30]. Interestingly, the carriage of *S. aureus* in the nares approximates the hand

carriage [17]. According to the literature, two-thirds of chronic *S. aureus* carriers carry it on their hands at least once during the year [30]. Therefore, colonized healthcare workers can be a potential source of MRSA transmission in a medical facility.

The literature varied greatly regarding the carrier rates of *S. aureus*. Among healthy adults, the carrier rates of *S. aureus* can range between 11 % to 50 % [28, 29, 58, 88]. As for healthcare workers (HCWS), these rates can range from 20 % to 90 % for the nares and approximately 50 % on the hands [14, 17, 59].

At least 10 % to 20 % of healthy people have chronic *S. aureus* nasal carriage and 60 to 90 % have transient nasal carriage [31, 50, 77, 104]. Another article indicated that *S. aureus* colonizes the skin and mucous membranes in 30 to 50 % of healthy adults and children; although, it was not clearly defined whether this was chronic or transient colonization [63].

Among the chronically ill with such conditions as diabetes, human immunodeficiency virus infection, and/or chronic dialysis, 40 to 60 % of these patients admitted to a hospital will become *S. aureus* carriers [75]. Furthermore, 30 % to 41 % of ICU patients will become *S. aureus* carriers [75].

Colonization may be transient or persistent, possibly lasting for years [28]. One study observed a median duration of 3.5 years for MRSA colonization [19]. It has been found that *Staphylococcus aureus* adheres better to the nasal epithelia of carriers and patients with eczema [105]. A previous history of MSSA nasal colonization was noted in 25 % of MRSA carriers and indicates that certain individuals maybe at a greater risk for colonization [76]. Persistent carriage is more common in children than in adults [101].

Young children tend to have higher colonization rates because of frequent contact with respiratory secretions [28].

A study found that 6 to 24 % of newborns are colonized by *S. aureus* after staying 3 to 4 days in a well-baby nursery [59]. In the 1950 to 1960s, it was shown that infants with hospital-acquired *S. aureus* colonization could carry it for 6 to 12 months after discharge [19]. Furthermore, 13 to 53 % of these infants were still colonized with the hospital strain 6 months after being discharged, and a small proportion still had nasal or throat colonization 1 year after discharge [2].

Nosocomial transmission of *S. aureus* occurs primarily from the hands of health care workers (HCWs) due to contamination by colonized and infected patients [17]. These infected and colonized health care workers can then serve as reservoirs and disseminators of *S. aureus* [3, 17]. Further, a recent study found that medical personnel are more likely to be colonized with antibiotic-resistant microorganisms, than non-medical personnel [26].

Approximately 20 % to >93% of patients were infected or colonized before/upon hospital admission and up to 25 % of patients admitted to hospitals will become nasal *S. aureus* carriers [19, 47, 48, 76, 105]. Finally, patients with MRSA nasal colonization undetected by clinical culture may account for up to 1/3 of all patients with MRSA in a hospital at any given time [19]. Therefore, these patients may be inadvertent disseminators of MRSA to health care workers and to other patients.

Colonization Can Lead to Infection

There are numerous factors that are associated with MRSA infection. First, nasal colonization increases the risk of *S. aureus* skin infections [76, 77], and infections after

surgical procedures [3]. Next, any conditions that compromise (or potentially compromise) the body's immune status such as invasive surgical procedures [3, 19, 47, 50, 104], severe underlying conditions/ immunocompromised [17, 50, 76], elderly status [17, 50, 76], invasive devices (intravenous catheters, prosthetics, mechanical ventilation, tracheostomy) [3, 28, 50, 76], dermatological conditions (*S. aureus* skin infections, burns, desquamating skin disorders, pressure ulcers, chronic skin lesions) [39], and frequent needle use (diabetes, hemodialysis, peritoneal dialysis, IV drug use, allergy shots) [3, 28, 30, 47, 50, 76, 77, 104] increased the risk of colonization and potential infection.

Furthermore, the use of antibiotics in the recent past [3, 19, 39, 50], currently [76], or in combinations [76] increases the risk of colonization and infection. In addition, the use of ciprofloxacin and other fluoroquinolones greatly increases this risk. Sub-MIC (minimum inhibitory concentration) levels of ciprofloxacin increase the adhesion of quinolone-resistant MRSA [63]; and, fluoroquinolones allow increased MRSA adhesion and carriage [43] as well as directly enhancing the expression of high-level oxacillin-resistant *S. aureus in vitro* [63].

Further, potential contact with a colonized or infected patient [19] may also increase this risk of colonization and infection. Therefore, this is a risk factor for health care workers [28, 50] and for those individuals with a history of hospitalizations within the past 12 months [19], current hospitalization [46], frequent (hospitalizations) [39, 47], prolonged hospital stays [3, 14, 19, 50, 76], and high-risk hospital areas (including the ICU, dermatology wards, burn unit [3, 14, 19, 39, 50, 76]).

Nosocomial Infection

According to the 1979 - 1995 NNIS data, *S. aureus* accounted for up to 13 % of all isolates from nosocomial infection [104]. MRSA colonization represents a much greater risk for bacteremia than MSSA colonization [7, 76]. At least 30 % of inpatients, who become colonized with MRSA, will develop a severe infection (such as pneumonia, bacteremia, and/or a wound infection) [75].

Among ICU patients, *S. aureus* nasal carriers are at a greater risk for *S. aureus* bacteremia than noncarriers [76]. Also, one-third to two-thirds of colonized ICU patients will ultimately develop an nosocomial MRSA infection [14]. Further, preoperative patients with nasal *S. aureus* carriage are more likely to develop a surgical site infection [3, 48]. In those individuals with head trauma, *S. aureus* nasal carriers were more likely to develop *S. aureus* pneumonia than noncarriers [3]. As for skin colonization, heavy colonization of the skin at the insertion site is the primary predictor of catheter-related infections for short-term catheters [3]. Hemodialysis patients with *S. aureus* skin colonization are at a six-fold increase for vascular-access device bacteremia compared to noncarriers [3].

Current Guidelines for Infection Control

Although the recommendations in the literature were relatively comparable, there were minor differences between sources. The literature emphasized that the focus should be on the identification of the pathogen source and its eradication [14]. Barrier (contact) precautions are preferred over Standard precautions to prevent the spread by colonized patients [43]. However, the Centers for Disease Control (CDC) indicated that the Standard precautions (including hand washing, gloving, masking, gowning, appropriate

device usage, and appropriate laundry handling) are usually sufficient unless it is determined that the infection is of “special clinical or epidemiological significance” [24]. According to the CDC recommendations, only in these “instances” should Contact precautions be considered.

Further, although one study indicated that swabs of skin lesions provide the best indicator of MRSA presence [49], another study indicated that active surveillance is more cost effective than personnel culture surveys [17]. Healthcare workers should be restricted from patient care activities if the worker(s) has draining *S. aureus* skin lesions until after antibiotic treatment [16]. However, no work restriction or antibiotic treatment need be given for *S. aureus* carriers unless there is an epidemiological link to an outbreak [17, 23].

Elimination of *S. aureus* nasal colonization may decrease *S. aureus* infections, and thus can be cost effective. For example, one study found that the mean excess medical and total costs attributable to nosocomial MRSA infection were approximately \$ 3500 and \$ 9275 respectively, while the cost of the control measures to stop an outbreak were less than 10 % of the cost of treating the infection [27].

Mupirocin

Mupirocin (Pseudomonic acid A) is a topical antibiotic produced by the fermentation of *Pseudomonas fluorescens* NCIB10586 [33, 64]. It has a unique chemical structure that is active *in vitro* primarily against Gram-positive organisms [33]. Although, mupirocin is highly active against *S. aureus*, it is inactive against normal skin flora [14]. It competitively inhibits bacterial isoleucyl-tRNA synthetase thus interfering with protein

synthesis [60]. Its effect is primarily bacteriostatic; but, with increasing concentrations, its effect becomes bactericidal [33].

The dosage is 0.5 grams to each (or one inch) to each nostril twice a day for 5 days. However, there is no increased effect by using the ointment longer than 5 days [14]. The most common adverse effects are respiratory symptoms including rhinitis, nose erythema, swelling, burning/stinging, pruritis, and dryness [14].

Intranasal application of mupirocin kept the nares sterile for relatively long periods [76]. It was found that intranasal application may also reduce surgical site infections and bronchopulmonary infections [108]. There was also a decreased incidence of *S. aureus* infection after cardiothoracic, general, and neurosurgery following mupirocin use [14]. In addition, there were decreased *S. aureus* infections in adults with long-term hemodialysis or ambulatory peritoneal dialysis with intermittent and/or continuous intranasal mupirocin [14].

However, with heavy colonization at multiple sites and/or with skin breaks, intranasal mupirocin is unlikely to eliminate colonization [44, 107]. This is readily understandable due to the potential for autoinoculation of the recently decolonized site. It has also been found that it is difficult to eliminate *S. aureus* from chronic wounds and dermatological conditions [7], since skin damage (from minor lesions, eczema, psoriasis, foreign body insertion) actually increases the risk of nasal carriage [105].

Mupirocin Resistance

Mupirocin-resistant MRSA was first isolated from patients with long-term mupirocin use for skin infections [108]. Mupirocin resistance may emerge after the use of oral and/or topical mupirocin for *S. aureus* colonization eradication [7, 17, 50, 107], and

that inappropriate mupirocin use may result in a persistent carrier state [33]. For example, low-level mupirocin-resistant MRSA emerged quickly in Japan after the introduction of mupirocin for nasal MRSA decolonization [108]. It was also found that intranasal mupirocin eradicated MRSA in the nasal cavity but not in the pharynx [108]. Instead, the MRSA in the pharynx was transformed into low-level mupirocin-resistant MRSA (MIC 8 ug/mL) (without an alteration in the strain after the mupirocin treatment) [108].

Further, there is both low and high level resistance to mupirocin [30, 64, 108]. Resistance has been predominantly low-level and an infrequent occurrence. Low-level resistance is due to a chromosomally encoded, modified *ileS* gene, which codes for a modified isoleucyl-tRNA synthetase [3, 14, 37, 56, 108]. Low-level resistant MRSA is still susceptible to 2 % mupirocin usually [3, 14].

In contrast, high-level resistance (MIC greater or equal to 512 mg/mL) is a plasmid-encoded gene, *mupA* (or *ileS-2*), which codes for a isoleucyl-tRNA synthetase without any affinity for mupirocin [12, 14, 44, 56, 108]. The gene, *mupA*, has minimal homology with the staphylococcal *ileS* gene; and, therefore, it is believed to have originated in another species [56].

Other Agents Used to Reduce Colonization

Before mupirocin, bacitracin was used to decrease colonization [103]. However, one study found variable results with bacitracin application in the anterior nares and rapid recolonization after the treatment ended [77].

Systemic agents may impact *S. aureus* colonization. Rifampin is considered the most effective systemic agent for MRSA. It eliminates nasal carriage in 79 to 95 % of patients; however, 57 % of health care workers became recolonized within 4 weeks after

treatment [14]. Furthermore, rifampin resistance develops when it is used as a single agent; therefore, the literature recommended that it not be used as a single agent [103].

Treatment

Intravenous vancomycin is the drug of choice for MRSA [35]. However, resistance to vancomycin is developing. Vancomycin intermediate *S. aureus* (VISA) (MIC 8 ug/mL) appeared in Japan in 1996 [103, 89]. By June of 2002, there were eight cases of VISA infection confirmed in the United States; and, the first case of vancomycin-resistant *S. aureus* (VRSA) (MIC greater or equal to 32 ug/mL) was reported in the United States in July of 2002 [89]. Other currently available FDA approved antimicrobials effective against MRSA include linezolid, daptomycin, quinupristin/dalfopristin, and tigecycline [3, 17, 77, 80, 103].

Phages

Phages are viruses that attack bacteria. There are probably more phages in the world than any other organism [99]. They are ubiquitous in the biosphere; and, their numbers are directly related to the number of bacterial hosts present. It has been estimated that there are 10^6 tailed phages per milliliter of coastal seawater and an estimated 10^9 tailed phages per milliliter of fresh water. Furthermore, there may be as many as 10^{30} tailed phages in the world [4].

There are at least 12 distinct groups of phages [68]; and, each phage species is specific to its bacterial host. The exact morphology and genetic material (DNA or RNA) varies according to the phage species. The typical structure of a phage is a hollow head filled with phage DNA or RNA and a tunnel-like tail for injecting the genetic material into the bacteria [67]. However, other morphological forms of phage exist.

The two major types of phage are lytic and lysogenic. Lytic phages invade the bacterial host, rapidly replicate themselves, and then burst the host cell. In contrast, lysogenic phages integrate their genetic material into the host DNA. For phage therapy, only lytic phages are employed in order to rapidly kill the targeted microorganism [67].

In phage therapy, specific samples of bacteriophages are given to a patient in order to treat an infection by a particular microorganism. The underlying assumption of the bacteriophage treatment is that the treatment contains a particular bacteriophage to which the microorganism is susceptible.

In phage therapy, there are also active versus passive treatments. Active treatments involve providing enough phage to overwhelm the infection in one initial dose. On the contrary, the passive method involves providing a lower dose of phage that eradicates the infection as the phages multiply [72].

In this study, phage 92 and phage 88 were used. These two phages have been shown to lyse methicillin-resistant *S. aureus* (MRSA) [79, 84, 85, 86, 87] and are available from American Type Culture Collection (ATCC). These two phages are lytic phages belonging to the family Siphoviridae, which includes phages containing DNA and having long, thin, noncontractile tails [106].

Phage Immunology

Phages will not infect animals [32]. Phages lack the ability to enter mammalian cells. This makes phages less effective at lysing intracellular microorganisms. However, in one article [22], this deficiency was circumvented by first introducing the phages into *Mycobacterium smegmatis*, a nonvirulent mycobacterium. Next, these treated mycobacteria were used to infect a cell culture previously infected with *Mycobacterium*

avium and *Mycobacterium tuberculosis*. The phages were then effective in lysing the infected cells.

Although phages are unable to produce disease in animals, when phages are injected into them, the phages will elicit antibody production that will eventually inactivate the phages [32]. The main side effects of phages to humans are due to the liberation of bacterial toxins as the phages lyse the bacterial cells rather than due to the bacteriophages [54, 67]. In addition, bacteriophages can produce effects in other aspects of the immune system. For example, it was found that patients treated with phages had lower levels of neutrophils, an increased turnover of neutrophils, and a decreased ability of these neutrophils to engulf bacteria [111]. These effects may be due to an increased number of immature neutrophils in the circulation and that these effects were associated with a successful recovery. It has been hypothesized that this increase in the number of immature neutrophils is due to the destruction of the microbes by the phages which stimulates the release of proinflammatory cytokines that lead to neutrophil degranulation and lactoferrin release which in turn stimulate several cytokines including colony-stimulating factors. These colony-stimulating factors then stimulate the release of neutrophil precursors from the bone marrow thus leading to an increase in the number of immature neutrophils [111].

Finally, the reticulo-endothelial system normally rapidly clears the phages from the circulation [61]. In another study [16], the immune response to phages was examined in mice. Although the mice did develop IgG and IgM to the phage, there were no anaphylactic reactions, no alterations in body temperature, and no other adverse effects.

In summary, although there is a potential for antibody production with intravenous bacteriophage treatment, bacteriophages have not been shown to have a detrimental effect on human health. Instead, phage treatment has the potential to stimulate the production of neutrophils while concurrently eliminating susceptible microorganisms.

Phage History

In 1896, Hankins noted that existence of “anti-infectious agents” in the Ganges and Jumna rivers [54, 95]. He did preliminary research on the effects of these agents on *Vibrio cholerae*; however, he did not continue the research further.

F. W. Twort published the first account of phages in 1915. He had noticed in his experiments that colonies of *Micrococcus* had undergone a “glassy transformation” that was transferable to other colonies [32].

In 1917, Felix d’Herelle independently discovered phages while at the Pasteur Institute in Paris [96]. He believed that “invisible microbes” were present in bacteria-free filtrates of dysentery stool samples. Emile Roux, director of the Institute, supported this endeavor and tested phages on avian typhosis and *Shigella* dysentery [96]. Experiments were also performed on infected sheep, bovine hemorrhagic septicemia in Indochina, and *Pasturella multocida* in water buffalos. In addition, d’Herelle successfully treated four patients with bubonic plague using bacteriophages [96].

The first reported phage trial was in 1921 by Bruynoghe and Maisin in France [58, 67]. The phages were used successfully against *Staphylococcus*; and, the overall reported phage success rate was reported at 80 to 95 % [58, 67]. However, many of the

later studies in the 1930s to 1940s using phages for staphylococcal abscesses were unsuccessful because the phages were unable to enter the walled-off abscesses [96].

During the 1920s to 1940s, enthusiasm overwhelmed scientific methodology in the experiments with phage therapy. Lytic phages, without sufficient *in vitro* purification, were tested on animals and directly in (poorly controlled) clinical trials. The most extensive phage trials occurred during the Bacteriophage Inquiry during the time period of 1927 to 1936. However, these phage trials had variable results [54].

Exaggerated claims were occurring during the 1930s to 1940s. For example, one treatment, “Enterofagos” could supposedly cure any type of diarrhea, tuberculosis, and mental disorders [10] as well as herpes and eczema [29]. Other studies tested phages against *Vibrio cholerae* and *Shigella dysenteriae* in Southeast Asia by pouring phage solutions into village wells [10].

However, phages were also used with some success during this time period. The Red Army used phage preparations in the 1930s Finnish war and World War II to protect against dysentery and gangrene [29]. And, the British army used phages in 1935 in India during a cholera epidemic [29]. Also, in 1939, Igor Asheshov had good results with anti-Vi phage against *Salmonella typhimurium* in mice; but, this was due to having mixed the phages and bacteria *in vitro* prior to administration to the mice [10]. And, in 1943, Dubos and colleagues protected mice against an experimental infection with *Shigella dysenteriae* [10].

Phages were even licensed for sale in the United States in the 1930s by such companies as Parke-Davis and Lilly [95, 96]. In the 1940s, Eli Lilly produced seven

phage products for humans [95]. And, since 1934, phages have been used successfully at the Eliava Institute of Bacteriophage, Microbiology, and Virology in Tbilisi, Georgia. A study by the Institute reported a 90 % success rate against *S. aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli* [2]. In addition, other eastern European countries and the former Soviet Union also had phages available on the market for years [66].

But, by 1941, phages were considered unreliable and being abandoned by the West [29]. And, in 1959 - 1960, the World Health Organization (WHO) discarded phage therapy due to the success of tetracycline [10]. However, in the 1970s, a WHO-sponsored study in Pakistan found that high dose phages seemed “equivalent to tetracycline in certain aspects of the clinical control of cholera” [96].

By the 1980s and 1990s, renewed interest in phage therapy was occurring. In 1982, Smith and Huggins worked with phage specific to the K-1 capsule of *Escherichia coli*. They found that the phages were generally more effective than streptomycin, tetracycline, ampicillin, and TMP-SMX [10].

Phages Benefits and Challenges

Phages have numerous characteristics that can make them effective therapeutic agents. They are specific to their target, the bacterial host [54, 58, 74, 83, 95, 96]. They are self-replicating at the site of infection [54, 95, 74, 96] but are also self-limiting [54, 58] because phages only multiply in response to the presence of their bacterial host [54, 74].

Further, there are minimal side effects [54, 74]; and, they may be useful either as

adjuncts to antibiotics [54] or as alternatives for when the patient had antibiotic allergies [54]. The literature noted that phages may also be useful for prophylaxis of certain diseases [54].

In addition, they are naturally abundant [74] and relatively inexpensive to propagate [54, 67]. The literature noted that it is relatively easy to select the appropriate phage [67, 95]. They also can not produce disease in plants and animals [96]. Finally, they mutate in conjunction with their bacterial hosts [74], which decrease the potential for resistance [58]; and, when resistance does develop, there is usually an attenuation of the bacterial virulence since the phage targets the virulence determinants on the bacterial membrane for entry [54, 67].

Many of the challenges to phage therapy have arisen due to methodological problems in the original studies. There was a lack of information on all aspects of phages [54, 67]. There was minimal understanding of phage specificity so phages were not selected for virulence against the correct host [54, 67]. And, single phage species were used incorrectly against multiple bacterial strains [54, 74, 95]. In addition, it is more effective if the pathogen is known and then the proper phage can be selected.

Further, there were problems in the processing of the phages. Laboratories were unreliable [54]. Titering of the phage was not done [54]. And, after impurities were removed, phage viability was not determined [74].

In addition, considerations were not given to the effects that the body would have on the phages. For orally administered phages, the gastric acid was not reduced first and this may have inactivated the phages [54, 67]. Furthermore, immune system responses to the phages were not considered [54, 74, 95].

As mentioned previously, enthusiasm overcame scientific proof. There was “exaggerated claims of success” [95] without firm scientific proof of efficacy [74, 95]. These unsubstantiated claims affected the credibility of phage therapy in the West. Eventually, phage therapy was relegated to second-class science in the West [83]. And, finally, as might be expected, bacteria developed resistance to the phage preparations [54].

Recent Phage Experiments

In recent years, phage therapy has been used for a variety of microorganisms, albeit, seldom against *S. aureus*. Phages have been used to treat experimental infections. For example, one study attempted use of phages to prevent infection in rabbits with an enteropathogenic strain of *E. coli* 0103 [78]. Another study was more successful in using phages that attached to the K1 capsular antigen of *E. coli* to prevent septicemia and a meningitis-like infection in chickens and delayed blood bacteremia in colostrums-deprived calves [11].

Also, another study used *E. coli* O157 antigen specific phages, KH1, KH4, and KH5, to control *E. coli* O157 *in vitro* with affecting other strains [53]. Next, another study used *Lactococcus garvieae* phages to prevent an experimental *Lactococcus garvieae* infection in yellowtail fish. Their study found that both intraperitoneal and oral administration prevented infection [65]. While, another study used phage specific to *Pseudomonas plecoglossicida* which causes bacterial hemorrhagic ascites disease in cultured ayu fish to control an experimental infection in the fish. Even more interestingly, the bacteria that became resistant to the phage became less virulent (as had been discussed in a previous section) [70].

Next, the University of Florida examined *Vibrio vulnificus*-specific phages from seawater and experimented on iron-overloaded mice infected with *Vibrio*. All the untreated mice died within 24 hours of infection; while, none of the phage treated mice died [74]. While, a study used a murein hydrolase from streptococcal phage C1 called lysine, which is specific for groups A, C, And E *Streptococci*. The lysine had a rapid lethal effect both *in vivo* and *in vitro* on Group A *Streptococci* [66]. Finally, another study successfully used phages to treat a potentially fatal vancomycin-resistant enterococcus infection in mice [16].

Phages have also been used to treat actual infections in humans. One study used phages to treat septic patients [92]. Another study used phages to treat suppurative bacterial infections [91]. A different study used phages to treat infection in cancer patients [109]. In a further study, phage treatment was administered from 1981 to 1986 to 550 cases of suppurative bacterial infections due to *Staphylococcus* and Gram negative bacteria with an improvement observed in 92.4 % of the patients. They found that their phages were very effective in the treatment of *S. aureus* with a sensitivity of 95 % [110].

Phages have also been used experimentally to control environmental microorganism contamination. A review of an American Society for Microbiology meeting in Los Angeles indicated that phages have been used for the treatment of *Vibrio vulnificus*, anthrax, wound and burn infections, as well as meat and poultry contamination [98, 16]. For example, one study used phages to decrease *Pseudomonas* growth on beef, but the phages were unable to extend the retail shelf life because of the narrow specificity of the phages used [40]. And, a more recent study, phages were used to decrease *Salmonella* on honeydew melon slices. The levels of *Salmonella* were decreased by 3.5

logs at 5 to 10 C and by 2.5 logs at 20 C. But, they were unable to accomplish this on apple slices due the acidity of the apple slices [57].

Finally, experiment(s) have been done to overcome one of the major challenges to phage therapy, which is the body's immune response to the phages. One study developed a "serial passage" method for the isolation of long-circulating phage strains. They used the serial-passage technique to select for long-circulating *E. coli* phage lambda mutants and *Salmonella typhimurium* phage 22 mutants [61].

Hypothesis

Currently available antimicrobials against MRSA have innate limitations including emergence of resistance and side effects. Therefore, there is a need for effective alternatives for prophylaxis and treatment of MRSA infection. Bacteriophage (phage) therapy is one such method. The ultimate goal of this project is the creation of a phage-containing ointment that can readily be used to prevent the spread of MRSA in the hospital setting and in the community.

This dissertation examines the effects of bacteriophages 88 and 92 on methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from the central Florida area in an epithelial cell culture model. It is being hypothesized that these two phages from the American Type Cell Culture that are specific for MRSA will be effective in eliminating the MRSA isolates from the cell cultures. The specific questions being asked are: (1) Will phages 88 and 92 be able to lyse samples of MRSA from the central Florida area? and (2) Will phages 88 and 92 be able to neutralize the detrimental effects of the MRSA isolate(s) on an epithelial cell culture? Finally, the experimental procedures for this dissertation are summarized in Table 1.

Table 1

Flowchart of Experimental Protocol

Controls (HEp-G2 + MRSA isolates) → ELISA testing (cytotoxicity and apoptosis)



Plaque Assays (MRSA isolates + Bacteriophage samples)



Treated (HEp-G2 + MRSA isolates + Bacteriophage samples) → ELISA testing
(cytotoxicity and apoptosis)

Methods

HEp-G2 Cell Culture

Samples of HEp-G2 cells were obtained from The Moffitt Research Institute of USF campus. The HEp-G2 cells were propagated in DMEM media (Fisher Scientific) containing 10 % fetal calf serum (Fisher Scientific) and 50,000 units of Penicillin-Streptomycin (Fisher Scientific). The cells were incubated at 37°C and in a humidified atmosphere of 5 % carbon dioxide and ambient oxygen. The cells were being used to study the effects of MRSA on epithelial cells.

MRSA Propagation and Maintenance

Ten isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) were obtained on agar stabs from an anonymous local healthcare system. Antibiotic resistance of the MRSA isolates was determined by minimum inhibitory concentration (resistant to Oxacillin MIC >2 mcg/ml) as well as growth on Mueller Hinton agar with 4% NaCl and 6 ug/ml Oxacillin at the local healthcare system. All ten isolates were determined to be resistant to Penicillin (PCN), Oxacillin (Oxa.), Cefazolin (Cef.), and Erythromycin (Ery.). In addition, all isolates except sample 53 were determined to be resistant to Augmentin (Aug.), Ciprofloxacin (Cipro.), and Levofloxacin (Levo.). Samples 51, 46, 41, 94, and 53 were determined to be resistant to Unasyn. And, isolates 22, 77, 49, 38, and 39 were determined to be resistant to Clindamycin (Clind.).

Table 2

Antibiotic Sensitivity of MRSA Samples

MRSA Sample #	PCN	Oxa.	Cef.	Ery.	Aug.	Cipro.	Levo.	Unasyn	Clind.
41	R	R	R	R	R	R	R	S	R
38	R	R	R	R	R	R	R	R	S
46	R	R	R	R	R	R	R	S	R
49	R	R	R	R	R	R	R	R	S
51	R	R	R	R	R	R	R	S	R
22	R	R	R	R	R	R	R	R	S
77	R	R	R	R	R	R	R	R	S
53	R	R	R	R	S	S	S	S	R
94	R	R	R	R	R	R	R	S	R
39	R	R	R	R	R	R	R	R	S

Pulse-field gel electrophoresis (PFGE) was also carried out at the anonymous local healthcare system in order to determine the genotypic patterns of the ten MRSA isolates. The patterns for these MRSA isolates can be seen in Figure 21. Although, there are various methods to determine both the genotypic patterns and antibiotic resistance patterns of Staphylococcal strains, PFGE is considered the “gold standard” for typing MRSA.

The MRSA isolates were propagated in an incubator at 37 °C in ambient oxygen and then maintained on Mueller-Hinton plates (Fisher Scientific) and stored at 4 °C prior to experimentation. Approximately 20 hours prior to experimentation, a selected sample from an isolated colony was inoculated into Mueller-Hinton broth (Fisher Scientific) and incubated in a shaker overnight at 37 °C in ambient oxygen.

Bacteriophages

Vials of bacteriophages 88 and 92 were obtained from the American Type Culture Collection (ATCC). The bacteriophage 88 sample arrived lyophilized and bacteriophage 92 arrived frozen from ATCC. One milliliter of Mueller-Hinton broth was added to the lyophilized bacteriophage 88 sample and 0.5 ml of Mueller-Hinton broth was added to the frozen 0.5 ml sample of bacteriophage 92. These samples were then stored at -20 °C in the Mueller-Hinton broth.

Ten µl samples of each bacteriophage were applied to a soft agar overlay of Mueller-Hinton plates of each MRSA isolate to determine the capability of the bacteriophages to form plaques on each MRSA isolate. Each MRSA isolate had been grown for 20 hours at 37 °C with ambient oxygen.

For each MRSA sample, the process of inoculation with each bacteriophage sample was replicated on three different occasions to confirm the ability of the particular bacteriophage to lyse the MRSA isolate. Each plate was then observed for plaque formation. This process of bacteriophage application was also replicated on plates of host MRSA identified by (and purchased from) ATCC as being specific for bacteriophage 88 and bacteriophage 92.

Plaque formation on the inoculated plates was indicative of bacterial lysis and bacteriophage propagation. MRSA samples that showed no plaque formation were excluded from further study.

Cellular DNA Fragmentation ELISA

The cytotoxic and apoptotic effects of each of the MRSA samples on HEp-G2 cell samples were examined using the Cellular DNA Fragmentation ELISA from Roche Molecular Biochemicals. The Cellular DNA Fragmentation ELISA provides a quantitative measure of cytotoxicity and apoptosis. Approximately 20 hours prior to experimentation, the HEp-G2 monolayer is inoculated with DMEM containing 5'-Bromo-2'-deoxy-uridine (BrdU) (final concentration 10 μ M) and incubated aerobically overnight at 37 °C in 5% carbon dioxide. BrdU is a non-radioactive thymidine analogue used by the nuclear DNA of the HEp-G2 cells as a metabolic labeling agent and that can be detected by monoclonal antibodies against BrdU fragments.

The media collected from the experimental samples contains the remnants of cellular debris generated by the MRSA toxin damage to the HEp-G2 cells. In contrast, the cellular lysates from the experimental samples provide an indication of the amount of induced cellular death that has been generated by the bacterial invasion of the HEp-G2 cells.

Experimental Procedure

Initial testing was carried out on 24-well plates of HEp-G2 cells determine the effects of varying dilutions of the selected MRSA samples. The Hep-G2 cells were counted via microscopy. Trypan blue was used to confirm the viability of the cells. After

trypan blue staining, dead cells will appear blue because of the inability of the cells to pump the trypan blue out of the cells. Approximately five hundred thousand HEp-G2 cells were added to each well. The HEp-G2 cells were incubated overnight for approximately 20 hours at 37 °C with 5 % carbon dioxide and ambient oxygen. The HEp-G2 cells in each well were grown in 1 ml of DMEM with 10 % fetal calf serum and Penicillin-Streptomycin and BrdU (final concentration 10 µM) during the 20 hour incubation period.

Concurrently, samples of selected MRSA isolates were incubated in Mueller-Hinton broth at 37 °C in a shaker in ambient oxygen. After 20 hours of incubation, the MRSA isolates were aliquoted into 1 ml samples and pulsed centrifuged into pellets. The Mueller-Hinton broth was then decanted and 1 ml of DMEM (without antibiotics) was added to the MRSA samples. The MRSA samples were mixed into this DMEM.

Concurrently, DMEM media was removed from the HEp-G2 monolayers; and, the Hep-G2 monolayers were washed with 1 ml of PBS. The PBS was decanted; and, 1 ml of fresh DMEM without antibiotics was applied to each negative control well.

Next, the camptothecin-treated wells had camptothecin added to DMEM (without antibiotics) to a final concentration of 3.4 mcg/ml. The camptothecin-treated cells were used as positive controls for cellular damage and induced cellular death, since camptothecin is a known inducer of apoptosis.

For the MRSA treated HEp-G2 monolayers, three different dilutions of the MRSA were used. The MRSA had been grown overnight in Mueller-Hinton broth at 37 °C; and, then, broth samples were aliquoted and pulse spun for the dilutions. The broth

was then decanted; and, one ml of DMEM was added to each sample for further dilution. These full-strength samples were used for the 1:1 concentrations. The dilutions used in the experiments were 1:1, $1:10^{-3}$, and $1:10^{-6}$ for the MRSA samples. Approximately 300 cfu/ml of MRSA were in each $1:10^{-6}$ dilution. This was determined by the plating of isolates on Mueller-Hinton agar and then counting the colonies. Finally, for the positive controls for the assay, 1 ml of DMEM containing Triton X-100 (10% solution) was added to each positive control well.

The experimental testing on these 24-well plates of inoculated HEp-G2 cells was carried out over a 3-hour duration. At the end of the 3-hour incubation, the cell media and cell lysates were removed as described by the manufacturer's procedures for the Cellular DNA Fragmentation ELISA. The next steps of the ELISA were followed according the procedures in the Cellular DNA Fragmentation ELISA manual available online from Roche.

Procedures described above were then repeated using combinations of bacteriophage and MRSA samples. As with the initial experimentation, the MRSA sample and the HEp-G2 monolayer were incubated 20 hours prior to inoculation with 10 μ l of bacteriophage.

Finally, combinations of MRSA-phages were retested with the application of 10 μ l of bacteriophage delayed 1-hour after the MRSA application to the HEp-G2 wells. This delayed time duration was used compare the effects of delayed bacteriophage application with the results of the simultaneous phage/MRSA combinations.

Table 3

Summary Table of Experimental Combinations

HEpG2 + MRSA isolates
HEpG2 + MRSA+ Bacteriophage 88 (Immediate Treatment)
HEpG2 + MRSA+ Bacteriophage 88 (Delayed Treatment)

Results

ELISA Results of HEpG2 Cells Treated with Selected MRSA Samples

These averaged results are the compiled results of three different sample sets for each methicillin-resistant *Staphylococcus aureus* sample. Due to time and resource limitation, these sample sets were carried out on different days then compiled into these results in order to provide an average result for each MRSA isolate. For each sample set, there are three individual samples for each experimental condition. The bars on each graph represent the standard error for the averaged results.

Methicillin-resistant Staphylococcus aureus Sample 41

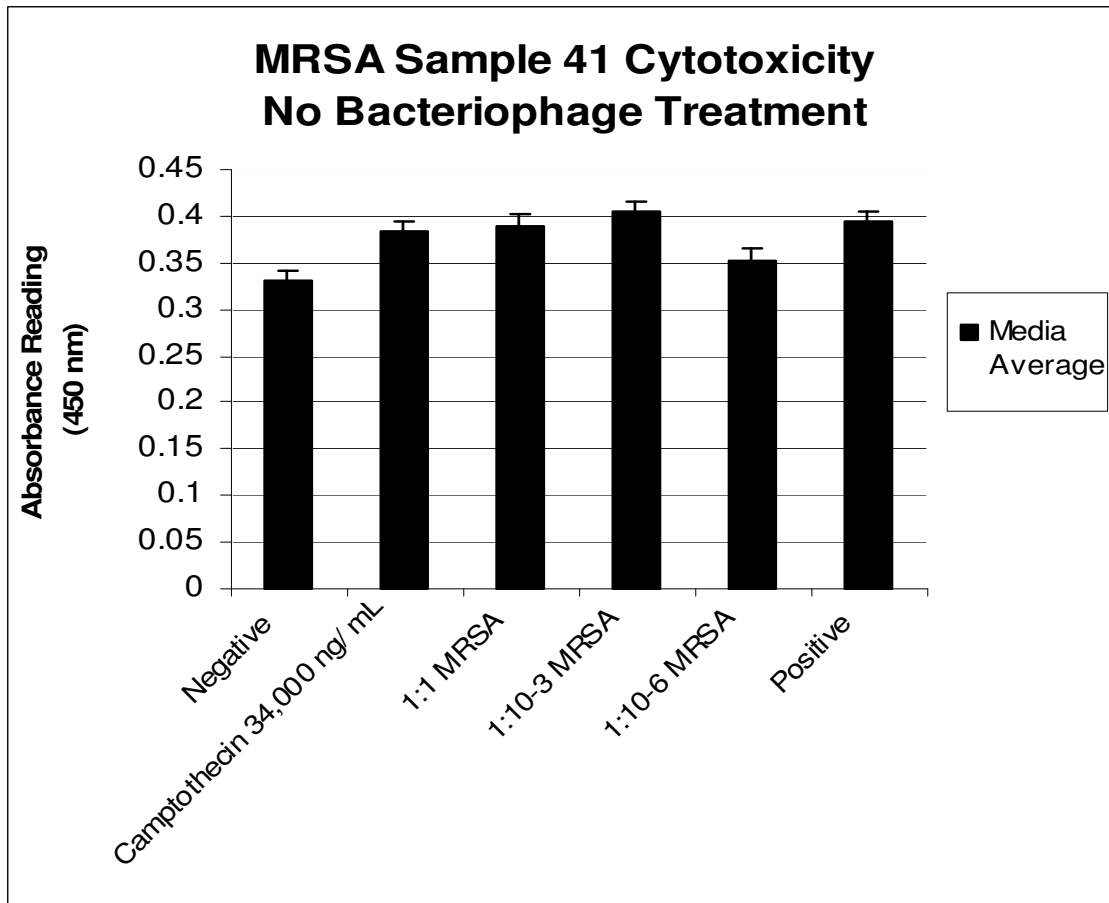


Figure 1. MRSA Sample 41 cytotoxicity in HEpG2 cells without bacteriophage treatment

In Figure 1, overall, MRSA treatment of HEpG2 cells did not induce significant cellular damage; however, the HEpG2 samples containing $1:10^{-3}$ concentration of MRSA Sample 41 showed the highest levels of cellular damage. The $1:10^{-3}$ sample appears to have had the greatest production of toxins that resulted in the cellular damage; although, this was not statistically significant.

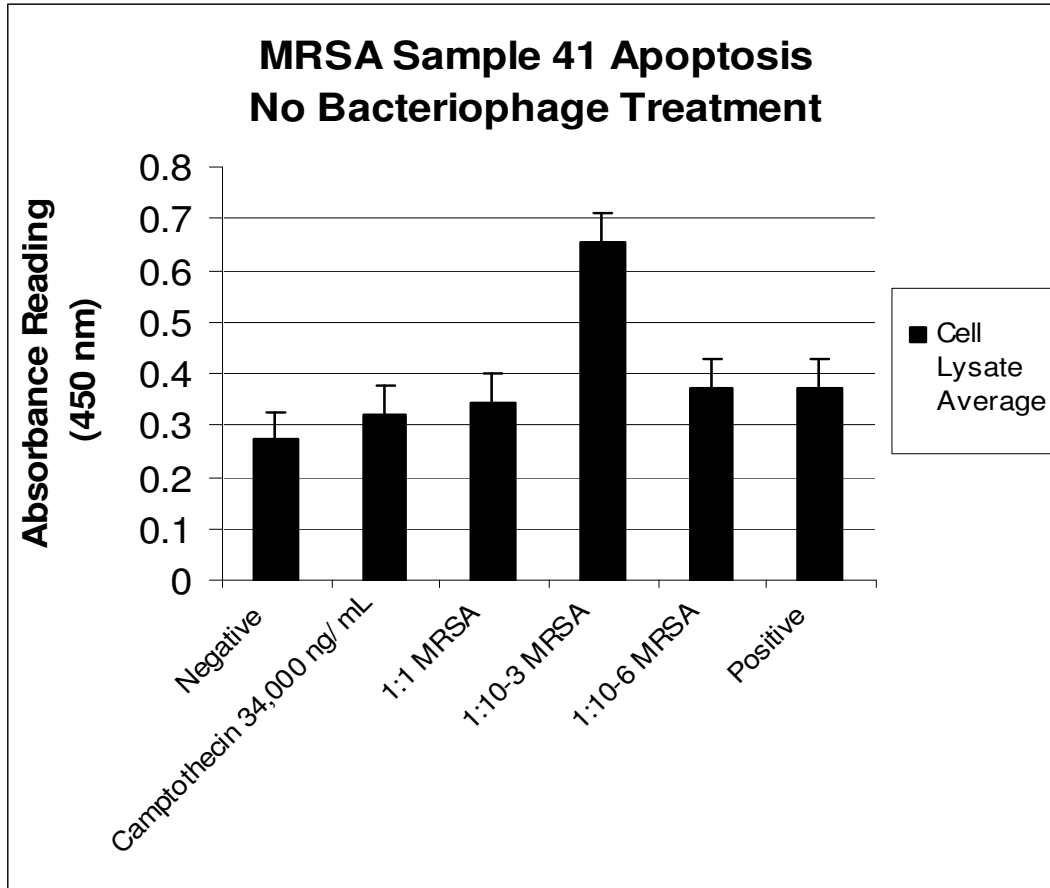


Figure 2. MRSA Sample 41 apoptosis in HEpG2 cells without bacteriophage treatment

In Figure 2, the HEpG2 samples containing 1:10⁻³ concentration of MRSA Sample 41 showed the highest levels of induced cellular death. Comparable to the results for the media for Sample 41, it appears that the 1:10⁻³ concentration had the greatest replication of MRSA during the 3-hour experimentation. The full-strength concentration

of MRSA likely had less replication due to the already concentrated nature of the sample (i.e. reduced log-phase growth); and, the $1:10^{-6}$ concentration had insufficient bacteria to provide as strong an effect as the $1:10^{-3}$ concentration.

Methicillin-resistant Staphylococcus aureus Sample 38

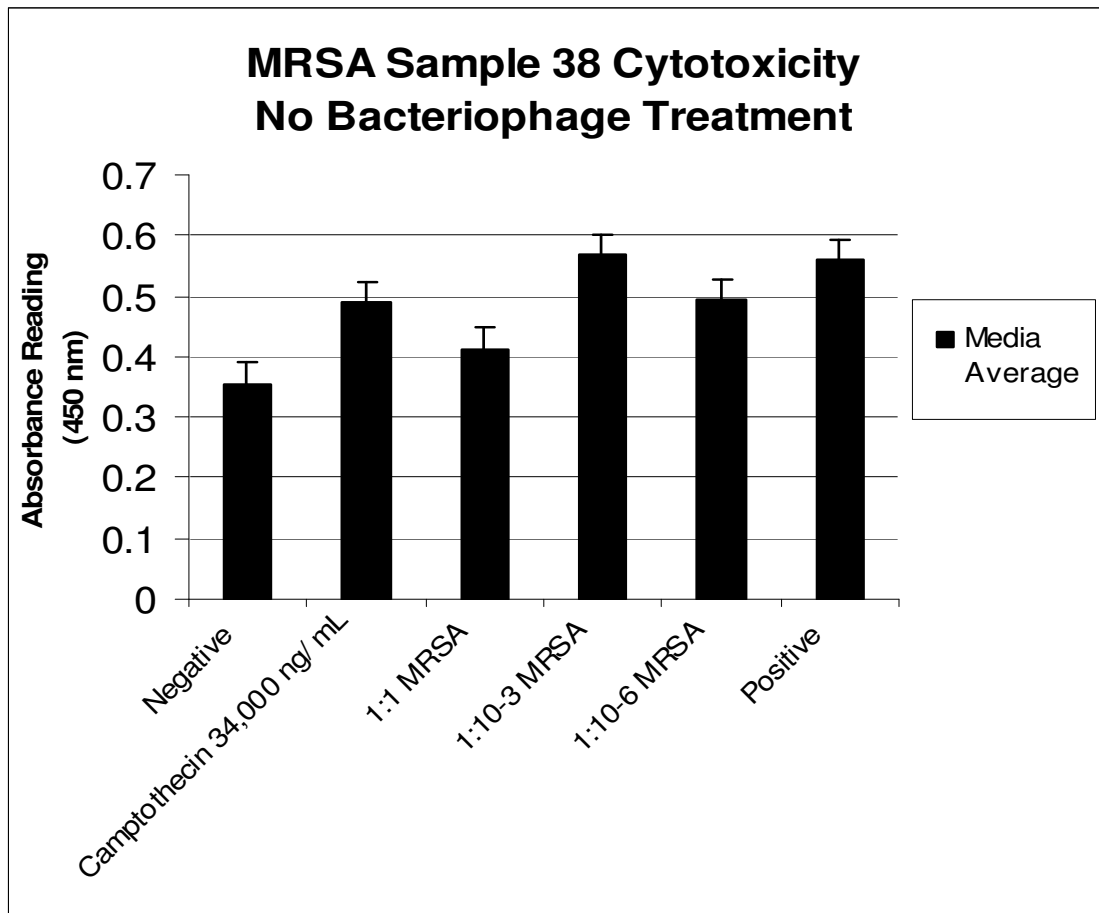


Figure 3. MRSA Sample 38 cytotoxicity in HEPG2 cells without bacteriophage treatment

In Figure 3, the HEPG2 samples containing $1:10^{-3}$ concentration of MRSA Sample 38 showed the highest levels of cellular damage. The full-strength concentration possibly had less replication due to the already concentrated nature of the sample. With less replication, there would likely have been less toxins produced in the full-strength

samples. With this sample, the $1:10^{-6}$ concentration does exhibit a greater level of toxin damage (and thus toxin production) than the full-strength sample but less than the $1:10^{-3}$ samples.

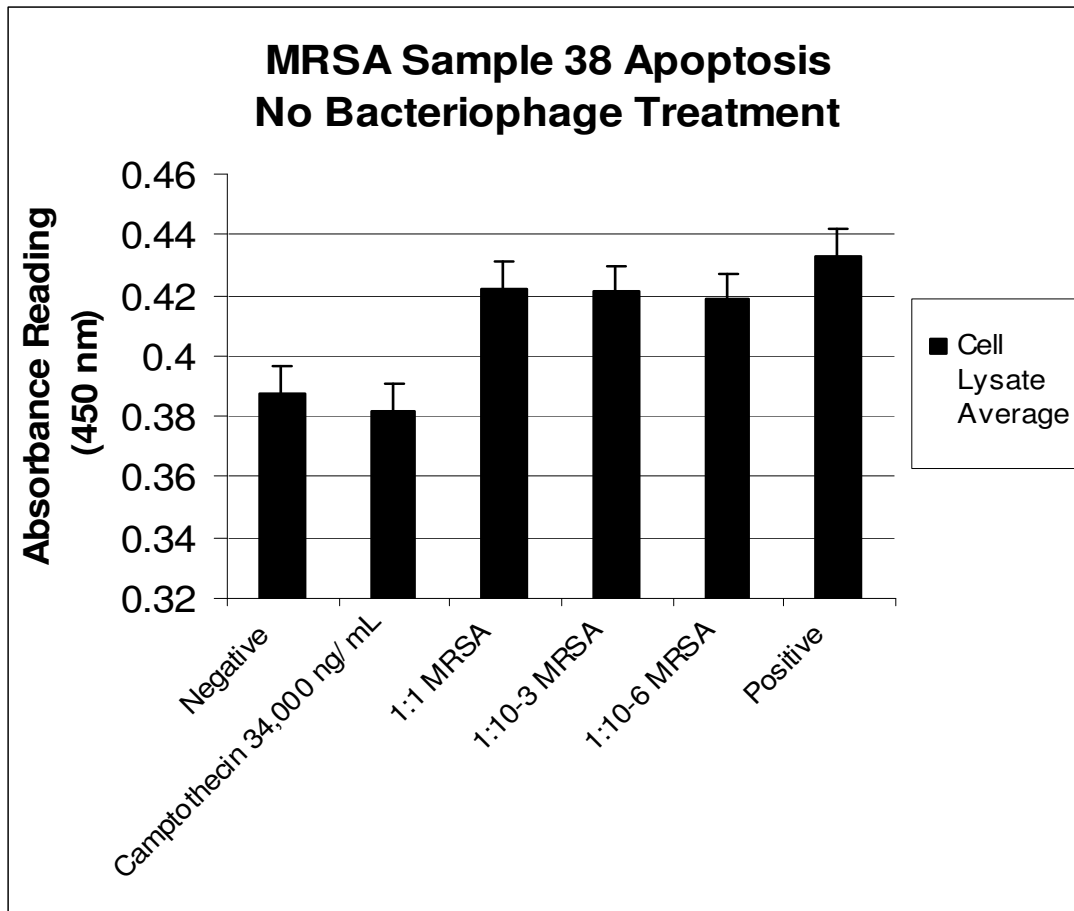


Figure 4. MRSA Sample 38 apoptosis in HEPG2 cells without bacteriophage treatment

In Figure 4, the HEPG2 samples containing 1:1 concentration of MRSA Sample 38 showed the highest levels of induced cellular death; although, the levels of apoptosis were similar with both $1:10^{-3}$ and $1:10^{-6}$ concentrations of MRSA Sample 38. For this particular sample, it appears that the concentration of MRSA did not have a significant on the amount of apoptosis.

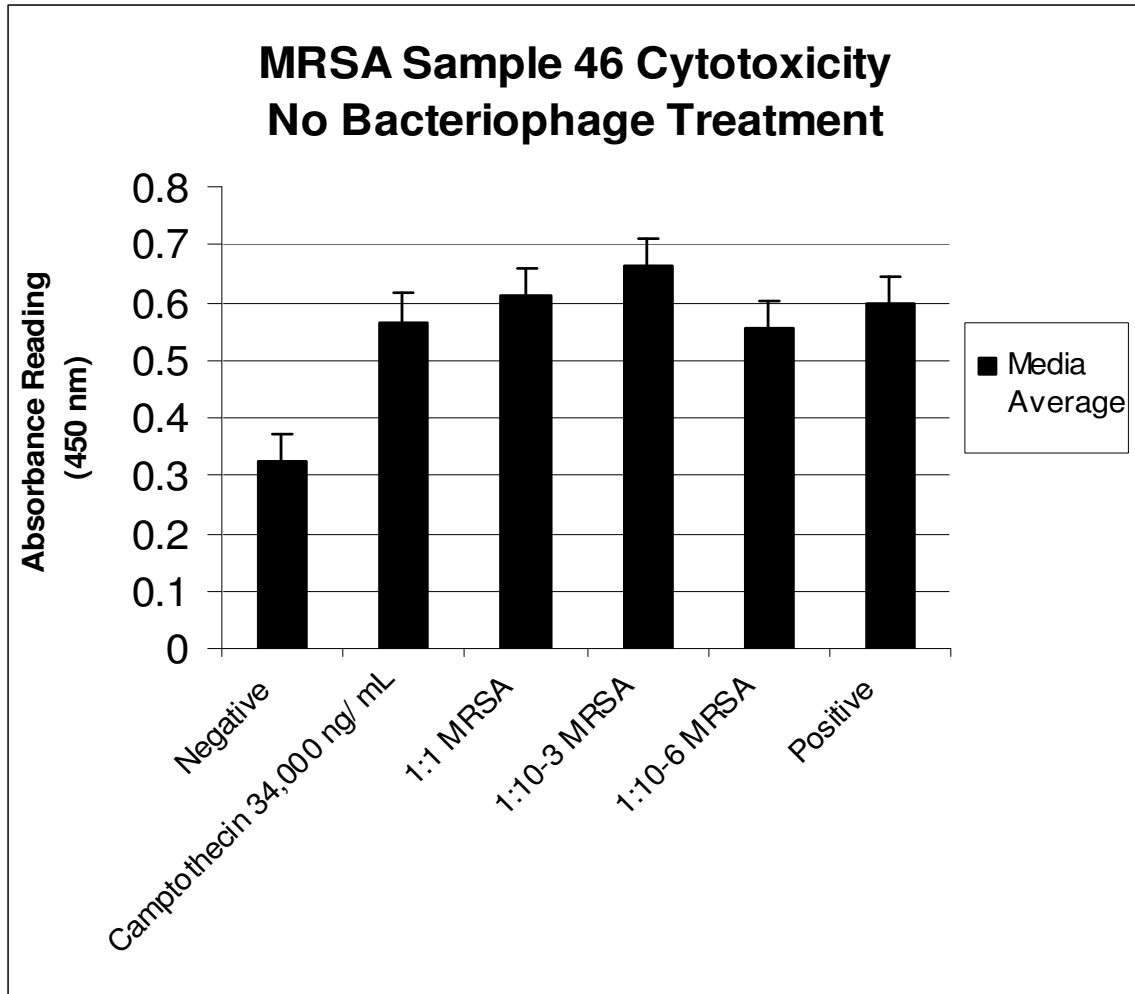


Figure 5. MRSA Sample 46 cytotoxicity in HEPG2 cells without bacteriophage treatment

In Figure 5, the HEPG2 samples containing $1:10^{-3}$ concentration of MRSA Sample 46 showed the highest levels of cellular damage. Comparable to MRSA Samples 41 and 38, the $1:10^{-3}$ concentration had the greatest amount of toxin production and likely the greatest amount of MRSA replication. It is possible with the $1:10^{-6}$ concentration that there were insufficient bacteria to produce an effect comparable to the other two concentrations.

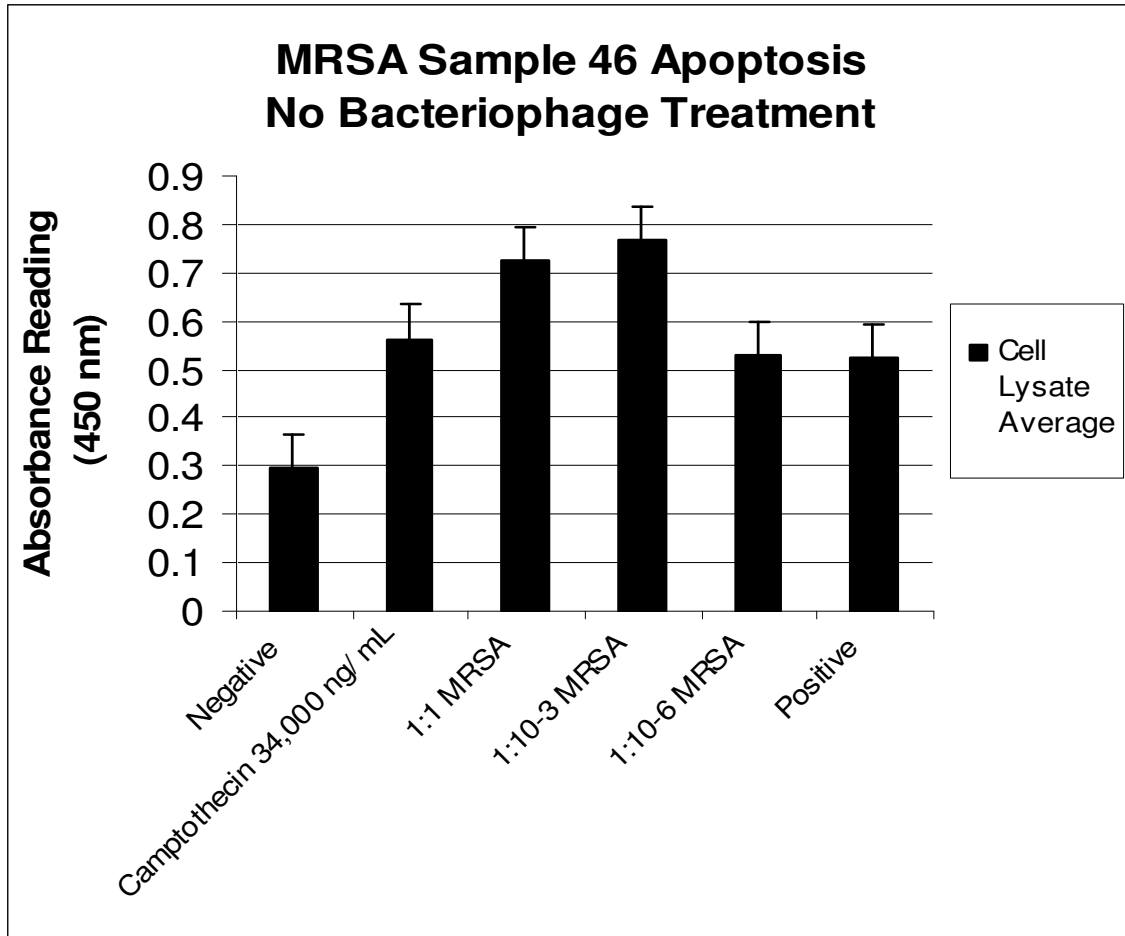


Figure 6. MRSA Sample 46 apoptosis in HEpG2 cells without bacteriophage treatment

In Figure 6, the HEpG2 samples containing 1:10⁻³ concentration of MRSA Sample 46 showed the highest levels of induced cellular death. For the 1:10⁻⁶ concentration, it is likely that there were an insufficient number of bacteria to produce an effect comparable to the other two concentrations. Of all the MRSA isolates, Sample 46 showed the highest levels of apoptosis.

Methicillin-resistant Staphylococcus aureus Sample 49

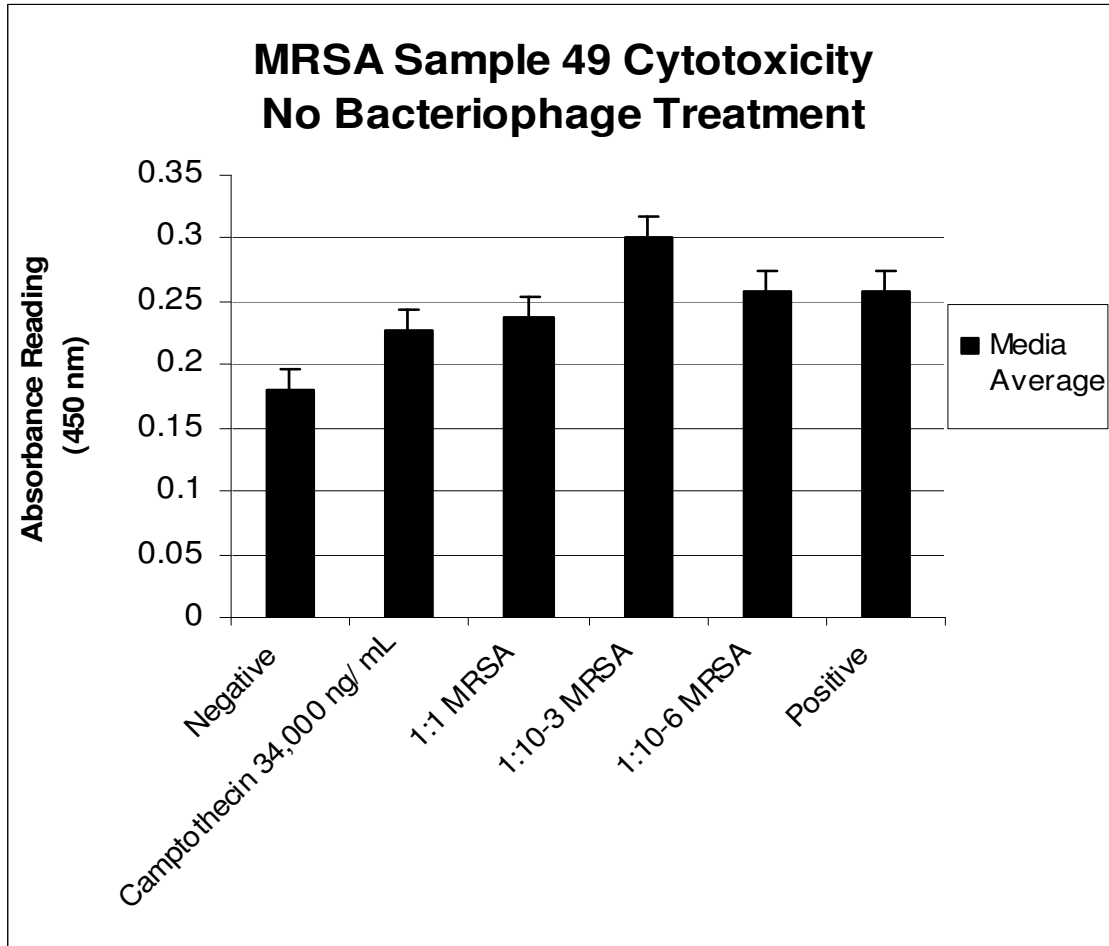


Figure 7. MRSA Sample 49 cytotoxicity in HEPG2 cells without bacteriophage treatment

In Figure 7, the HEPG2 samples containing $1:10^{-3}$ concentration of MRSA Sample 49 showed the highest levels of cellular damage. Comparable to the previous samples, the $1:10^{-3}$ concentration had the highest level of toxin production which likely was concurrent with the replication of the bacteria. The $1:10^{-6}$ concentration also showed greater toxin production than the full-strength concentration yet less than the $1:10^{-3}$ concentration. This effect can be explained by the likelihood that the $1:10^{-6}$ concentration

would have greater nutrients (than the full-strength concentration) to allow rapid replication yet have less initial bacteria than the $1:10^{-3}$ concentration.

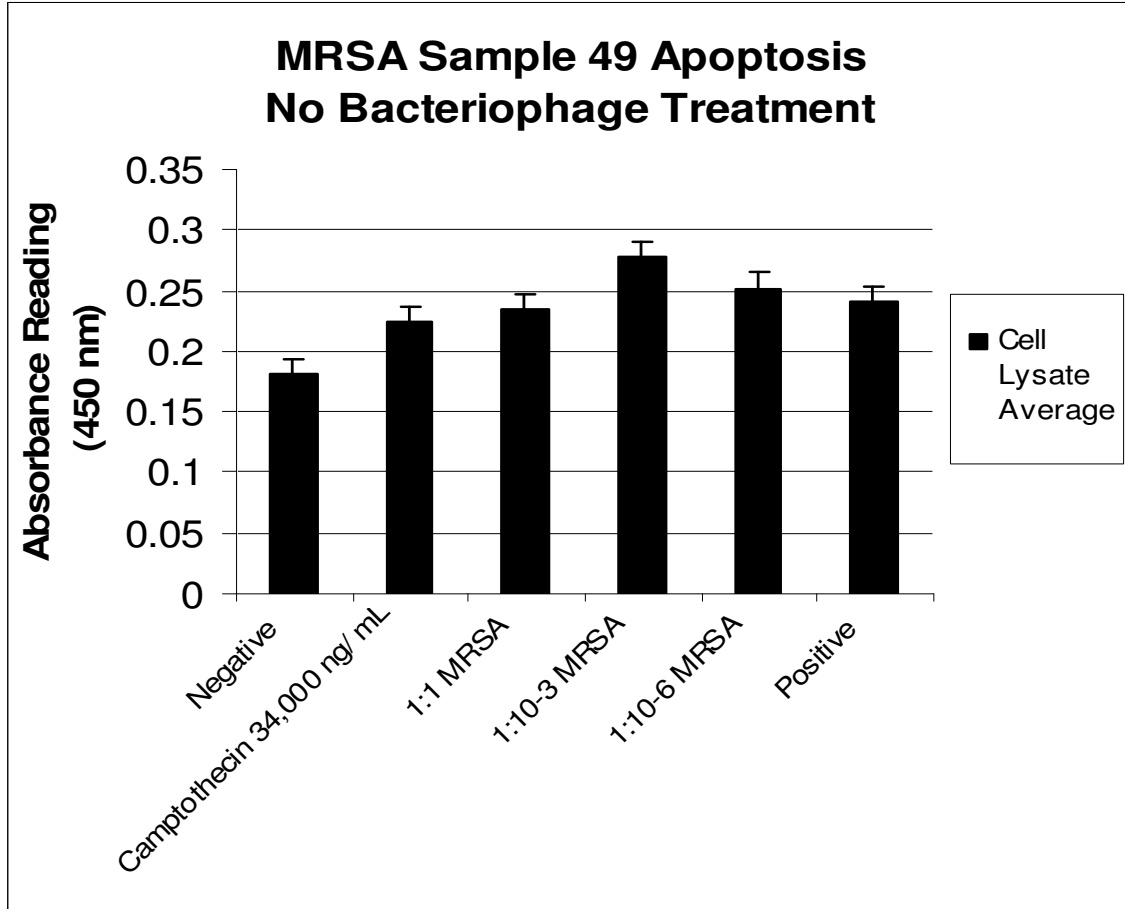


Figure 8. MRSA Sample 49 apoptosis in HEPG2 cells without bacteriophage treatment

In Figure 8, the HEPG2 samples containing $1:10^{-3}$ concentration of MRSA Sample 49 showed the highest levels of induced cellular death. Comparable to the previous samples, the $1:10^{-3}$ concentration has the highest level of apoptosis which likely occurred concurrent with the replication of the bacteria. The $1:10^{-6}$ concentration also showed greater induced cell death than the full-strength concentration yet less than the

1:10⁻³ concentration. This effect can be explained by the likelihood that the 1:10⁻⁶ concentration would have greater nutrients (than the full-strength concentration) to allow rapid replication yet have less initial bacteria than the 1:10⁻³ concentration.

Methicillin-resistant Staphylococcus aureus Sample 51

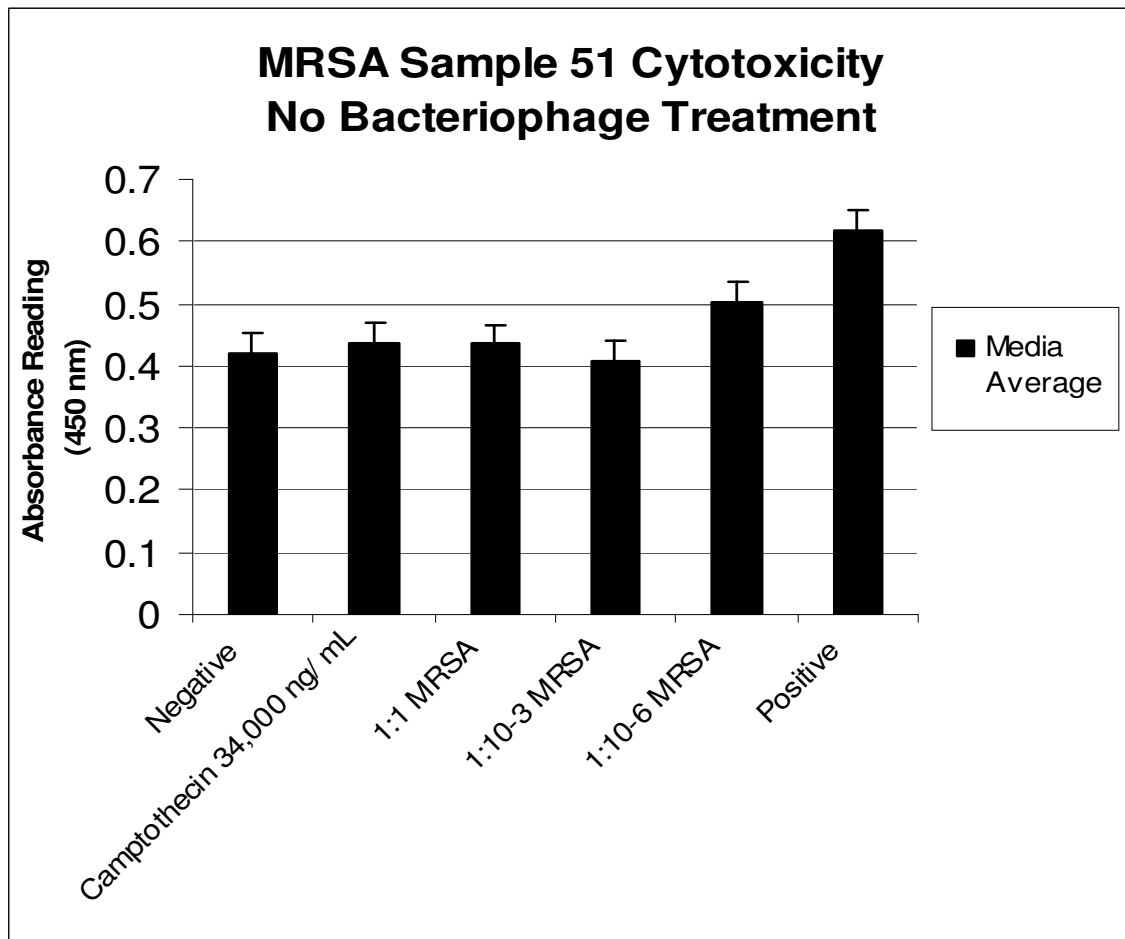


Figure 9. MRSA Sample 51 cytotoxicity in HEPG2 cells without bacteriophage treatment

In Figure 9, the HEPG2 samples containing 1:10⁻⁶ concentration of MRSA Sample 51 showed the highest levels of cellular damage. For this sample, it is likely that the 1:10⁻⁶ concentration had sufficient nutrients to allow rapid replication of the bacteria.

It is possible for this sample that the other concentrations had insufficient nutrients to allow as rapid a replication as the $1:10^{-6}$ concentration.

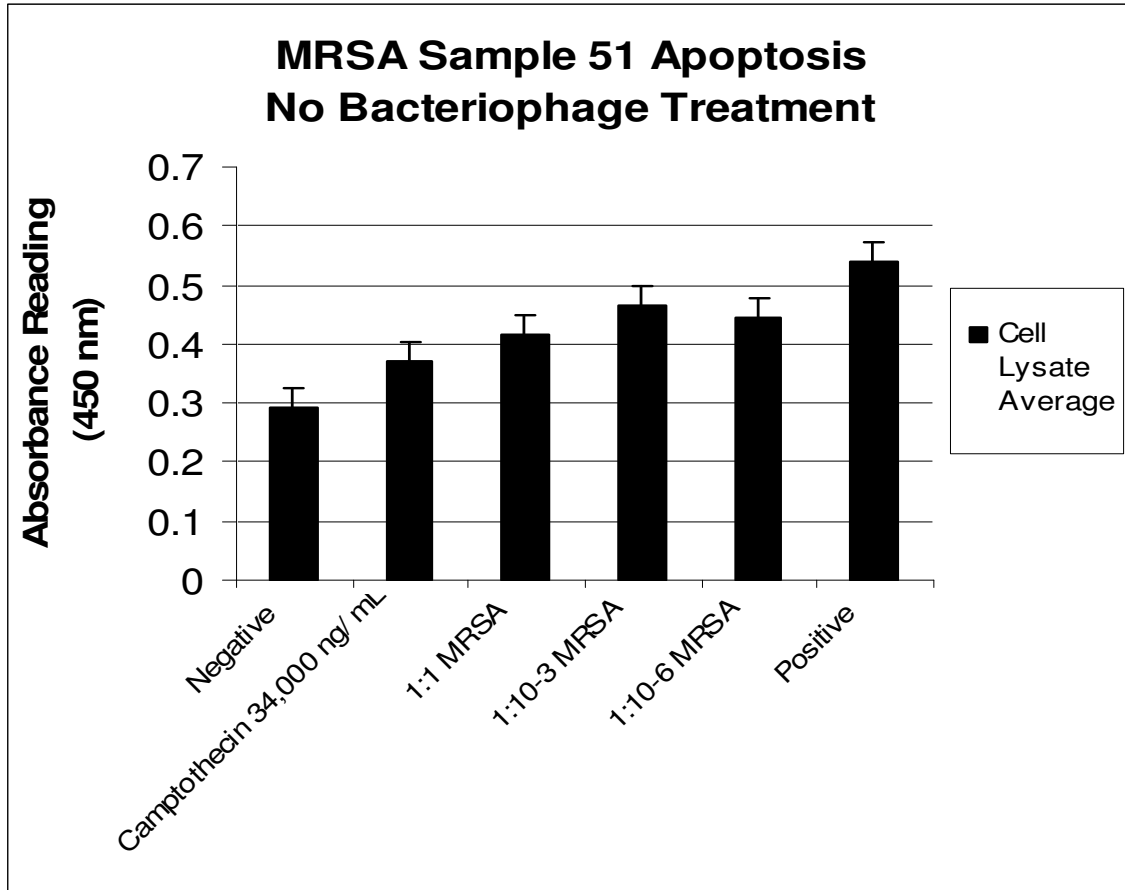


Figure 10. MRSA Sample 51 apoptosis in HEpG2 cells without bacteriophage treatment

In Figure 8, the HEpG2 samples containing $1:10^{-3}$ concentration of MRSA Sample 51 showed the highest levels of induced cellular death. Although the $1:10^{-3}$ concentration for Sample 51 did not produce as much cellular damage as the $1:10^{-6}$ concentration, the $1:10^{-3}$ was capable of producing the most apoptosis; although, these results were within the standard error of the $1:10^{-6}$ concentration.

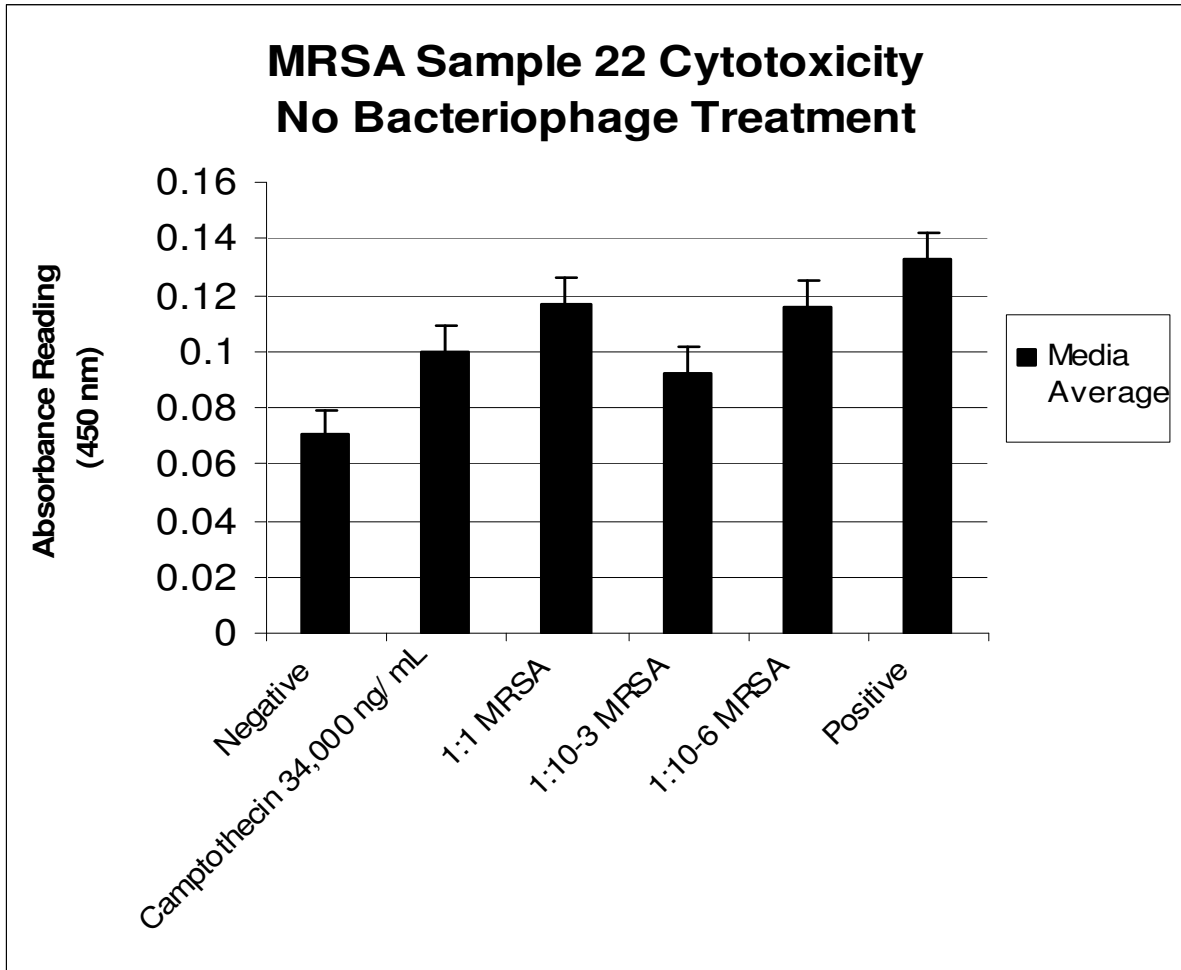


Figure 11. MRSA Sample 22 cytotoxicity in HEpG2 cells without bacteriophage treatment

In Figure 11, the HEpG2 samples containing 1:1 concentration of MRSA Sample 22 showed the highest levels of cellular damage; although, the 1:10⁻⁶ concentration of MRSA Sample 22 showed a nearly comparable level of cellular damage. For this sample, the full-strength concentration appeared to have sufficient nutrients to allow the greatest level of replication and toxin production.

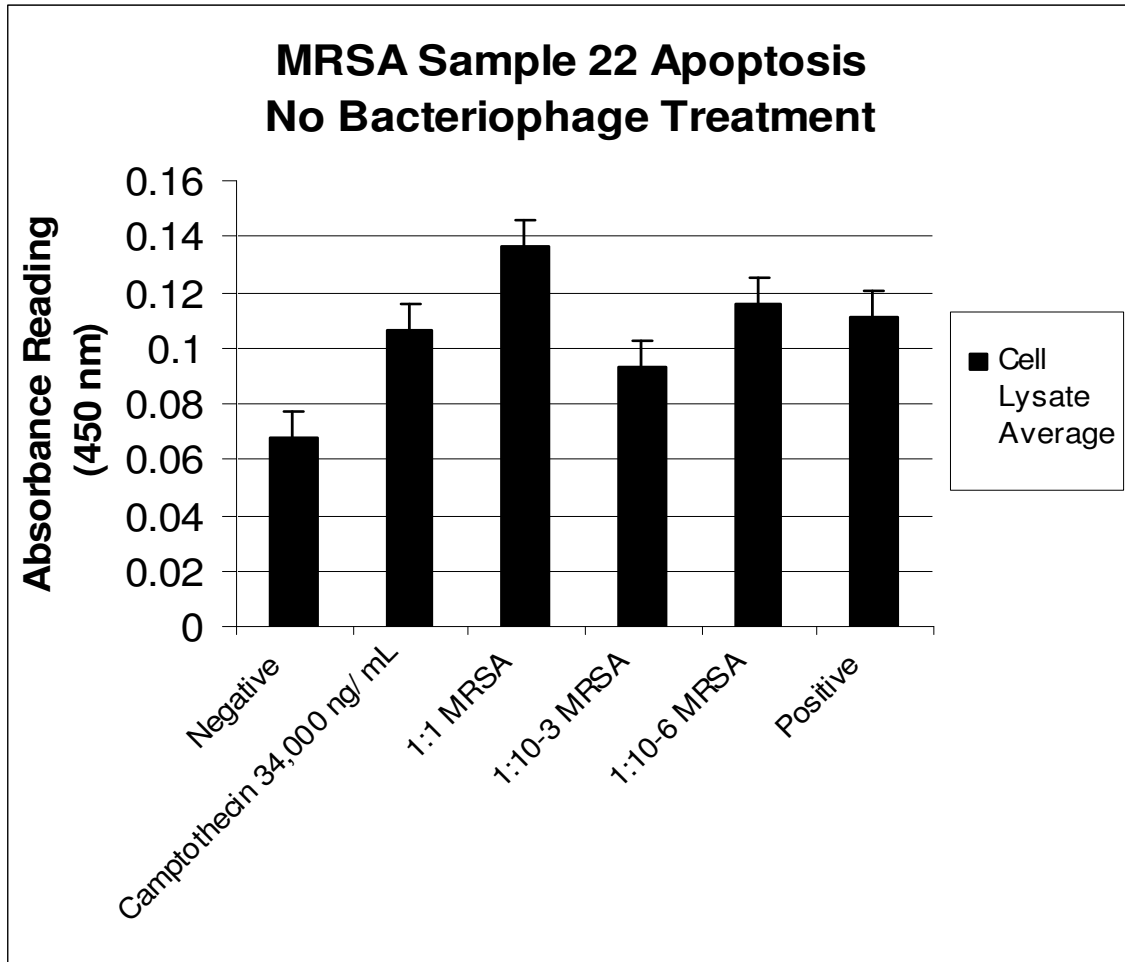


Figure 12. MRSA Sample 22 apoptosis in HEpG2 cells without bacteriophage treatment

In Figure 12, the HEpG2 samples containing 1:1 concentration of MRSA Sample 22 showed the highest levels of induced cellular death. Comparable to the cytotoxicity results, the full-strength concentration appeared to have sufficient nutrients to allow the greatest level of replication and toxin production.

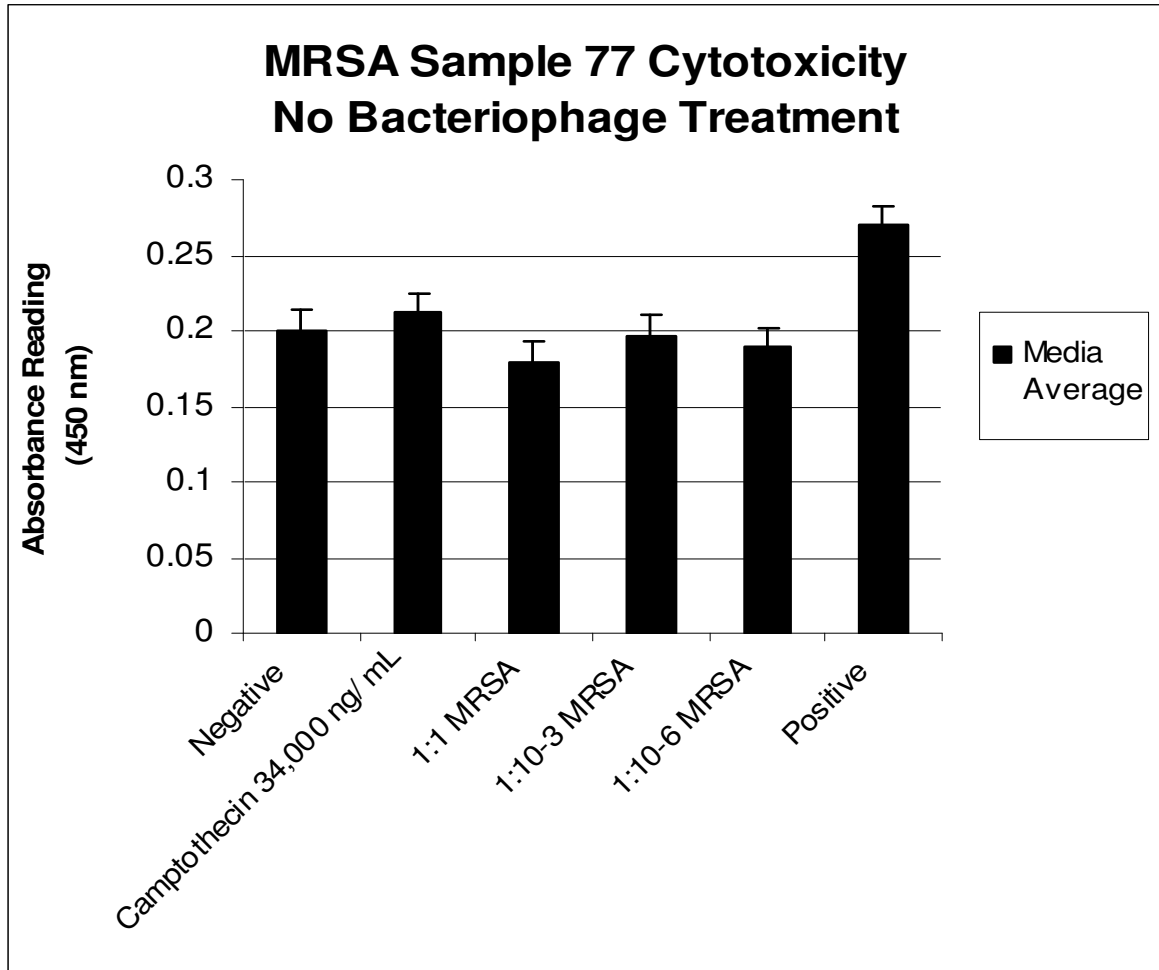


Figure 13. MRSA Sample 77 cytotoxicity in HEpG2 cells without bacteriophage treatment

In Figure 13, the HEpG2 samples containing $1:10^{-3}$ concentration of MRSA Sample 77 showed the highest levels of cellular damage; although, this effect was comparable to the effects of the other two concentrations. It appears that the concentration of bacteria did not have a significant effect on toxin production.

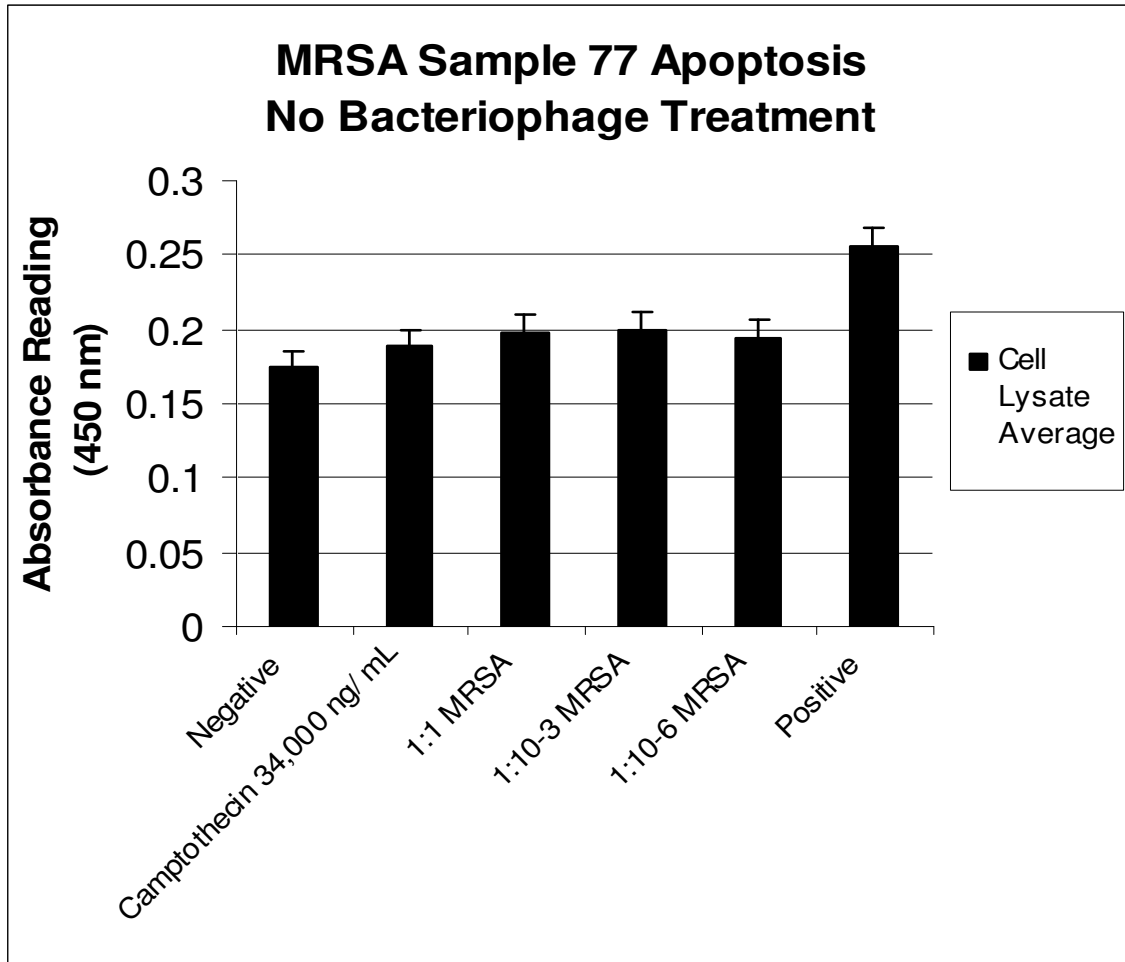


Figure 14. MRSA Sample 77 apoptosis in HEpG2 cells without bacteriophage treatment

In Figure 14, the HEpG2 samples containing $1:10^{-3}$ concentration of MRSA Sample 77 showed the highest levels of induced cellular death; although, the 1:1 and $1:10^{-6}$ concentrations of MRSA Sample 77 showed nearly comparable levels of apoptosis. It appears that the concentration of bacteria did not have a significant effect on induced cellular death.

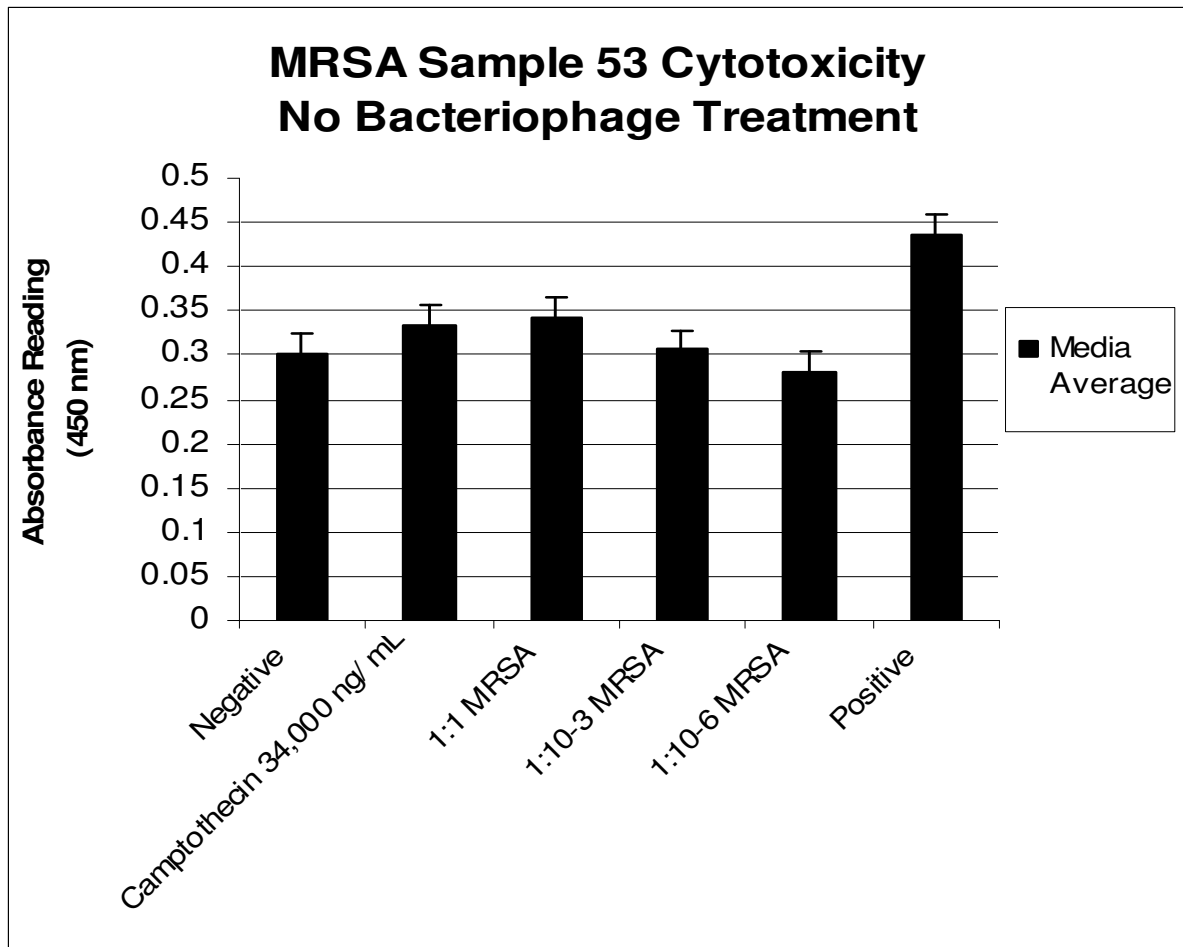


Figure 15. MRSA Sample 53 cytotoxicity in HEPG2 cells without bacteriophage treatment

In Figure 15, the HEPG2 samples containing the 1:1 concentration of MRSA Sample 53 showed the highest levels of cellular damage. It appears that the full strength concentration had sufficient nutrients to allow rapid replication; and, it is likely that the other concentrations had insufficient bacteria to produce a comparable effect.

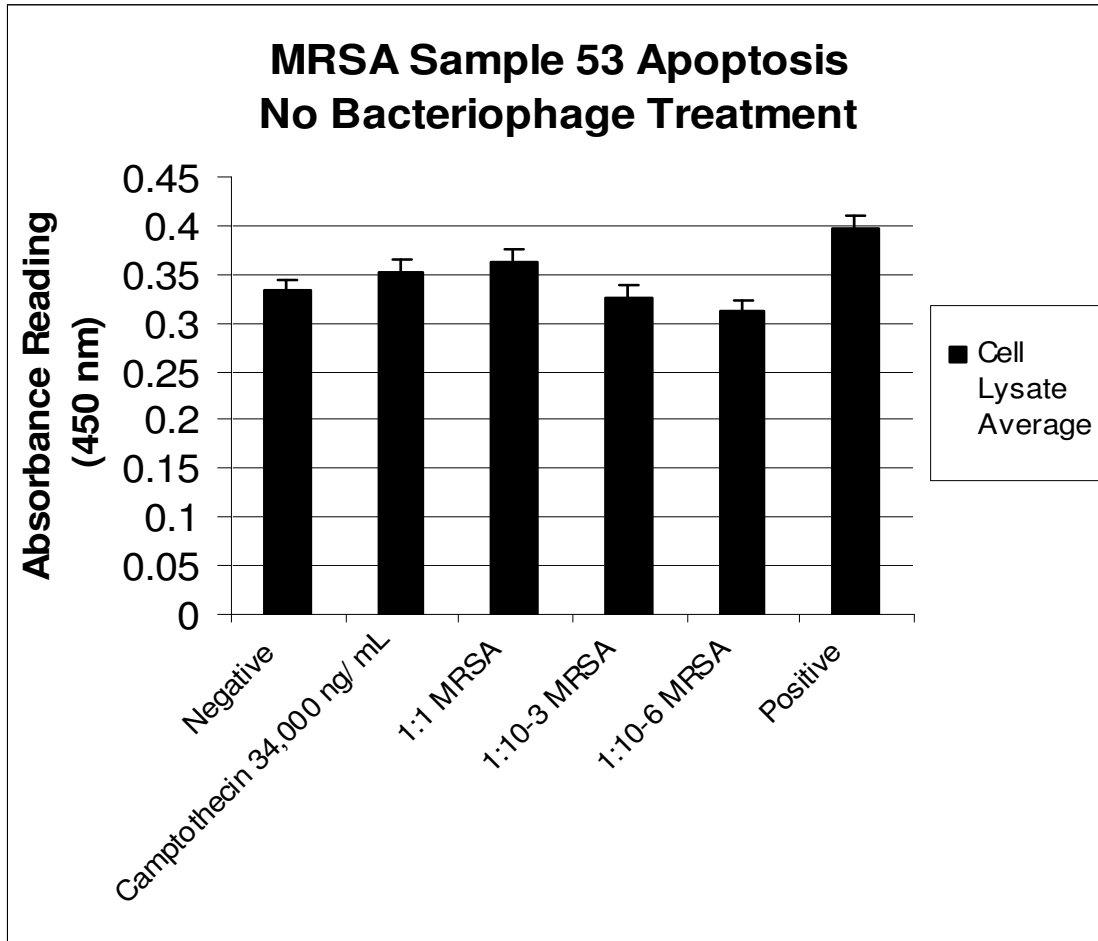


Figure 16. MRSA Sample 53 apoptosis in HEpG2 cells without bacteriophage treatment

In Figure 16, the HEpG2 samples containing the 1:1 concentration of MRSA Sample 53 showed the highest levels of induced cellular death. It appears that the full strength concentration had sufficient nutrients to allow rapid replication; and, it is likely that the other concentrations had insufficient bacteria to produce a comparable effect.

Methicillin-resistant Staphylococcus aureus Sample 94

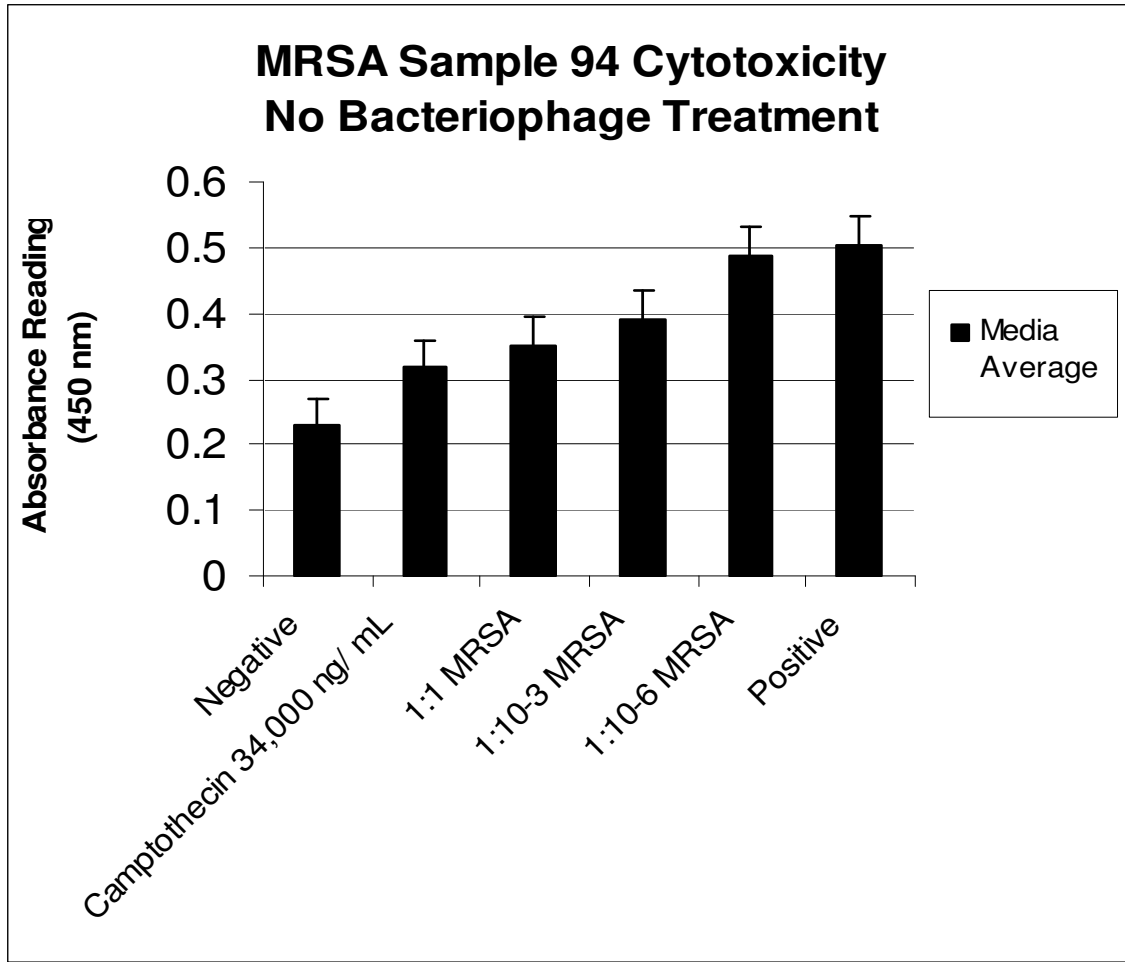


Figure 17. MRSA Sample 94 cytotoxicity in HEpG2 cells without bacteriophage treatment

In Figure 17, the HEpG2 samples containing the $1:10^{-6}$ concentration of MRSA Sample 94 showed the highest levels of cellular damage. For this sample, it is likely that the $1:10^{-6}$ concentration had sufficient nutrients to allow rapid replication of the bacteria. It is possible for this sample that the other concentrations had insufficient nutrients to allow as rapid a replication as the $1:10^{-6}$ concentration.

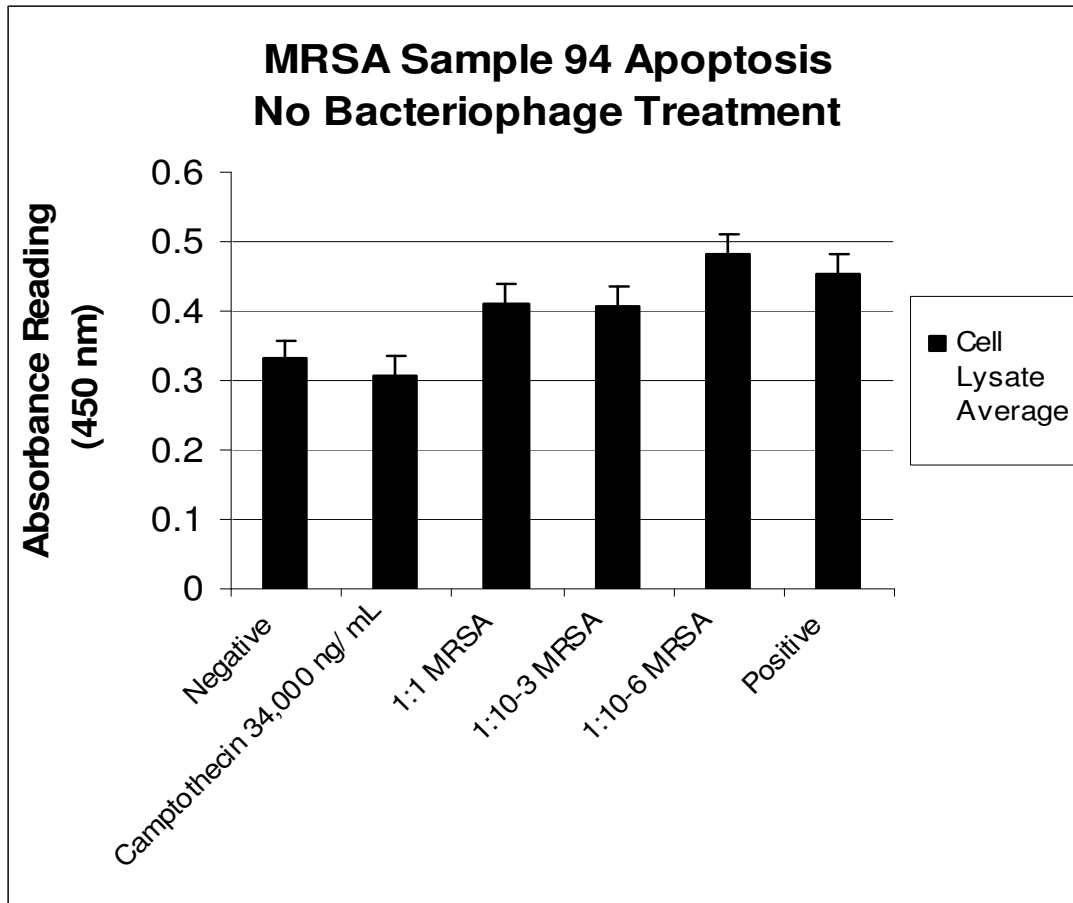


Figure 18. MRSA Sample 94 apoptosis in HEpG2 cells without bacteriophage treatment

In Figure 18, the HEpG2 samples containing the $1:10^{-6}$ concentration of MRSA Sample 94 showed the highest levels of induced cellular death. For this sample, it is likely that the $1:10^{-6}$ concentration had sufficient nutrients to allow rapid replication of the bacteria. It is possible for this sample that the other concentrations had insufficient nutrients to allow as rapid a replication as the $1:10^{-6}$ concentration.

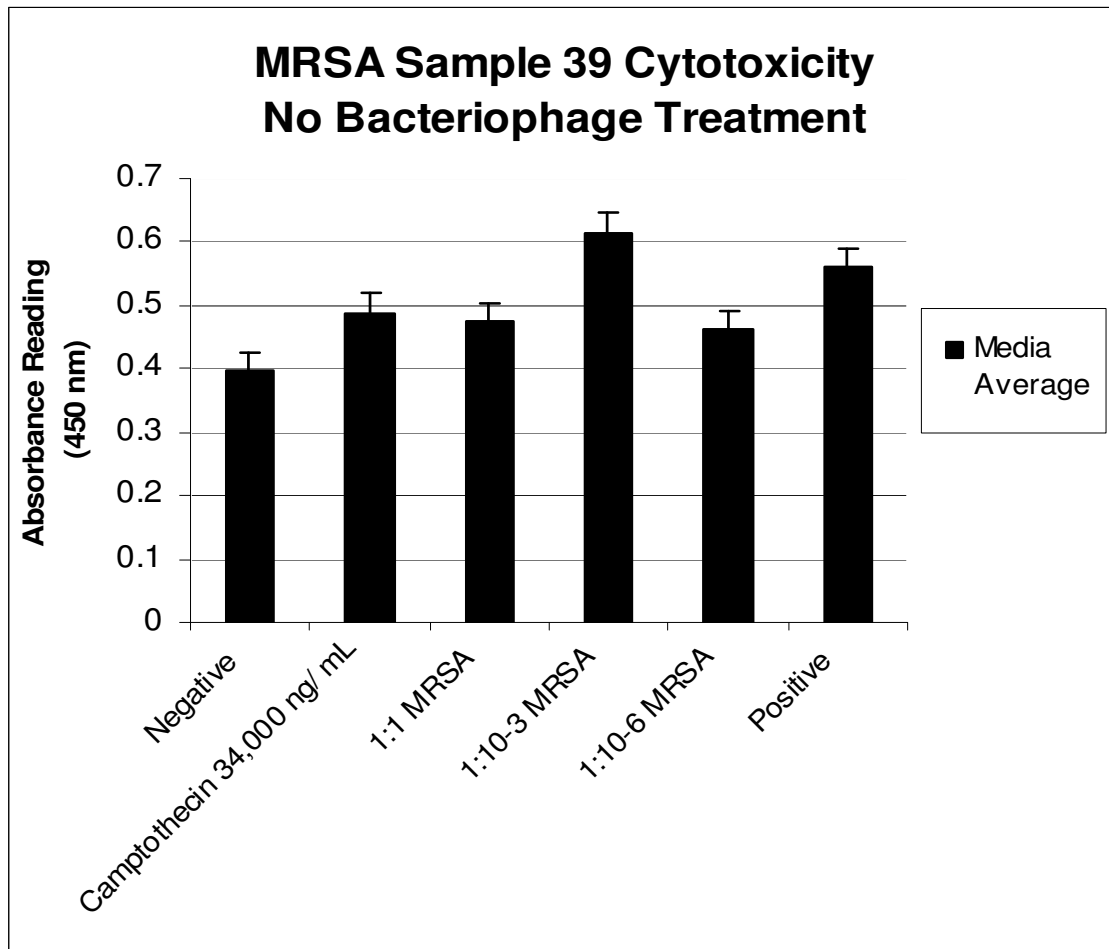


Figure 19. MRSA Sample 39 cytotoxicity in HEpG2 cells without bacteriophage treatment

In Figure 19, the HEpG2 samples containing $1:10^{-3}$ concentration of MRSA Sample 39 showed the highest levels of cellular damage. Comparable to MRSA Samples 41 and 46, the $1:10^{-3}$ concentration had the greatest amount of toxin production and likely the greatest amount of MRSA replication. For the $1:10^{-6}$ concentration, it is likely that there were an insufficient number of bacteria to produce an effect comparable to the other two concentrations.

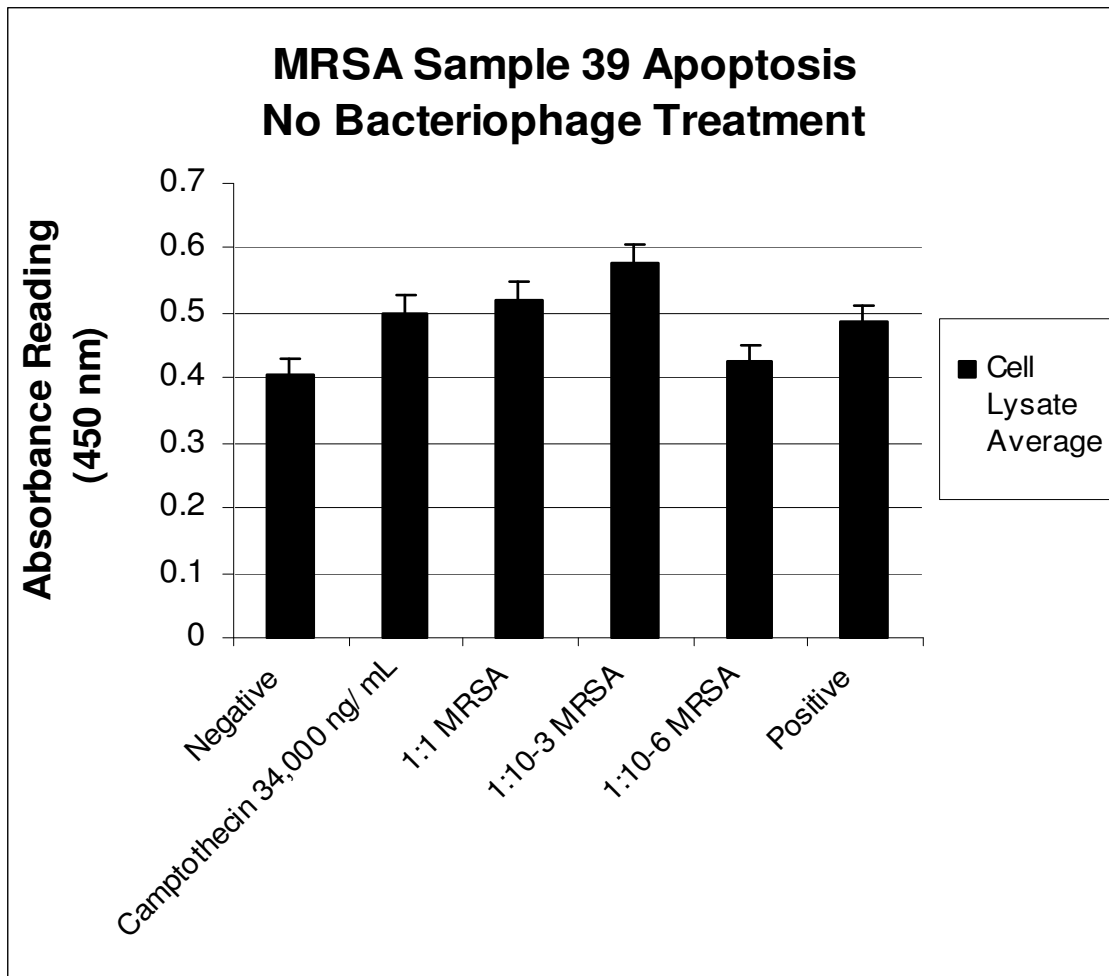


Figure 20. MRSA Sample 39 apoptosis in HEpG2 cells without bacteriophage treatment

In Figure 20, the HEpG2 samples containing 1:10⁻³ concentration of MRSA Sample 39 showed the highest levels of induced cellular death. For the 1:10⁻⁶ concentration, it is likely that there were an insufficient number of bacteria to produce an effect comparable to the other two concentrations.

Summary of Cytotoxicity and Apoptosis Results for MRSA Samples

The cytotoxicity results of the MRSA samples are summarized in Table 4; and, the apoptosis results of the MRSA samples are summarized in Table 5. The levels of cytotoxicity and apoptosis are indicated by the number of + signs: +++ (high), ++ (medium), and + (low).

Table 4

Summary Table of Cytotoxicity Results for MRSA Samples

MRSA Sample #	1:1	1:10³	1:10⁶
41	++	+++	+
38	+	+++	++
46	++	+++	+
49	+	+++	++
51	++	+	+++
22	+++	+	++
77	+	+++	++
53	+++	++	+
94	+	++	+++
39	++	+++	+

Table 5

Summary Table of Apoptosis Results for MRSA Samples

MRSA Sample #	1:1	1:10³	1:10⁶
41	+	+++	++
38	+++	++	+
46	++	+++	+
49	+	+++	++
51	+	+++	++
22	+++	+	++
77	++	+++	+
53	+++	++	+
94	++	+	+++
39	++	+++	+

Bacteriophage Treated HEp-G2 Samples

Table 6

Bacteriophage Treatment

MRSA Sample	Bacteriophage 88 Plaque Formation	Bacteriophage 92 Plaque Formation
Sample 22	-	-
Sample 38	-	-
Sample 39	-	-
Sample 41	-	-
Sample 46	-	-
Sample 49	-	-
Sample 51	-	-
Sample 53	-	-
Sample 77	-	-
Sample 94	+	-

Each MRSA sample was tested in three separate trials carried out on different days with 10 µl of full-strength bacteriophage-containing Muller-Hinton media. This

procedure was also carried out on plates of the host MRSA specified by ATCC. Only on the plates of host MRSA did visible and widespread plaques form within three days. With the experimental MRSA isolates, plaques developed only on the plates of Sample MRSA 94 inoculated with bacteriophage 88. This plaque formation by bacteriophage 88 on Sample 94 was limited and slower to develop (within seven days of bacteriophage inoculation). Because of this bacterial resistance to the bacteriophage, further testing with the bacteriophages on the 24-well plates was limited to experimentation with MRSA Sample 94 and bacteriophage 88.

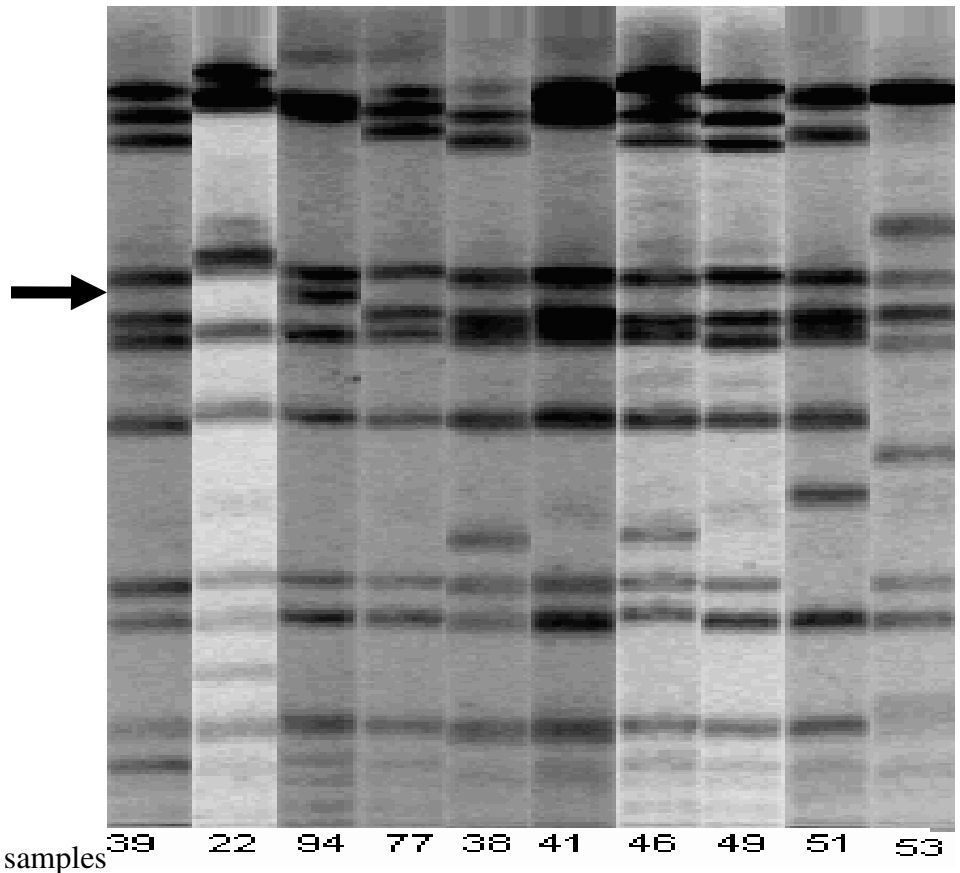


Figure 21. Pulsed-Field Gel Electrophoresis of MRSA Samples

Pulsed-field gel electrophoresis (PFGE) was carried out to determine the genotypic patterns of each MRSA isolate. Figure 21 provides these PFGE patterns of the selected MRSA samples used in the experiments. Comparable patterns for the original host MRSA strains from the American Type Culture Collection were unavailable for comparison with these above PFGE patterns. MRSA Sample 94 appears to have an additional band (marked with an arrow) that is unique from the other MRSA samples and may account for the susceptibility of MRSA sample 94 to bacteriophage 88.

Immediate Bacteriophage Treatment

After the completion of the ELISA testing of the HEp-G2 monolayers treated with the selected MRSA isolates, plaque assay testing of bacteriophages 88 and 92 was carried out with the MRSA isolates. From Table 6, only the combination of bacteriophage 88 and MRSA sample 94 showed plaque formation; therefore, only MRSA sample 94 was used for further experimentation. This plaque formation was minimal in comparison to the amount of plaque formation that occurred with the inoculation of bacteriophage 88 on its host MRSA.

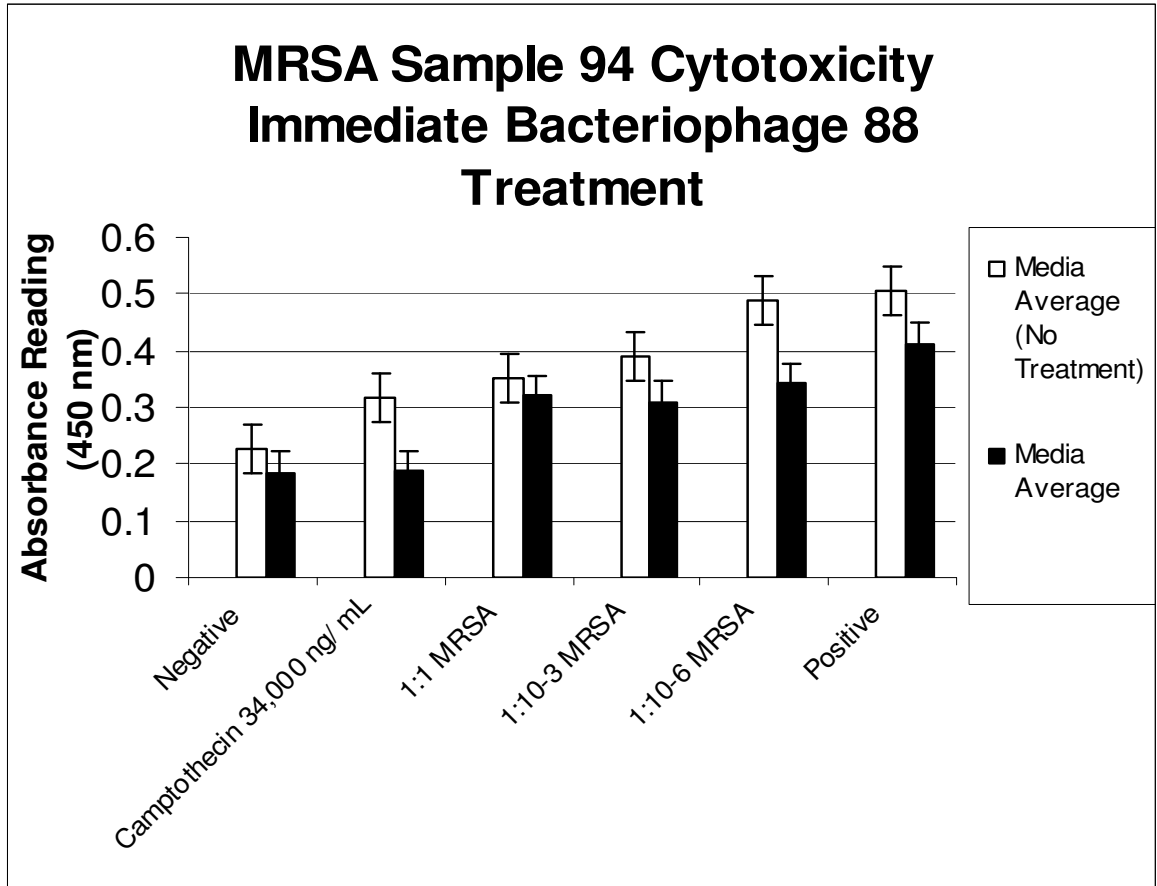


Figure 22. MRSA Sample 94 cytotoxicity in HEpG2 cells with immediate bacteriophage treatment compared with untreated samples

In Figure 22, the HEpG2 samples immediately treated with bacteriophage 88 showed decreased levels of cellular damage. This effect appeared greatest with the $1:10^{-6}$ concentration of MRSA Sample 94 and the $1:10^{-6}$ concentration of bacteriophage 88. Without treatment, the $1:10^{-6}$ treatment exhibited the greatest level of cellular damage which was likely indicative of the greatest bacterial replication. Because bacteriophages replicate in active growing cells, it is likely that their effect would be greatest in the samples containing the greatest number of actively replicating bacteria which would be

the 1:10⁻⁶ samples. This effect was seen with the 1:10⁻⁶ samples treated with the bacteriophages.

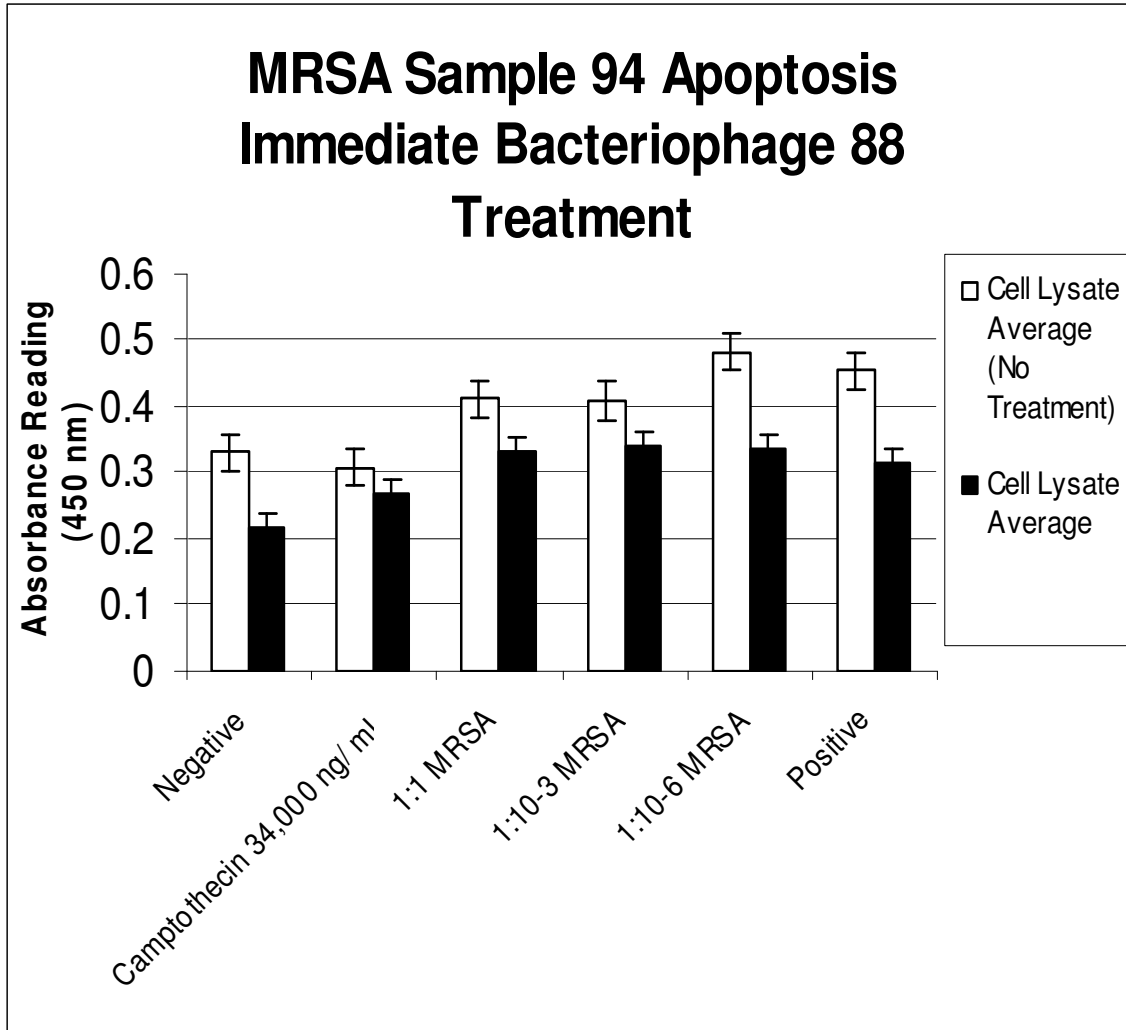


Figure 23. MRSA Sample 94 apoptosis in HEpG2 cells with immediate bacteriophage treatment compared with untreated samples

In Figure 23, the HEpG2 samples immediately treated with bacteriophage 88 showed decreased levels of induced cell death. This effect appeared greatest with the $1:10^{-6}$ concentration of MRSA Sample 94 and the $1:10^{-6}$ concentration of bacteriophage 88. Without treatment, the $1:10^{-6}$ treatment exhibited the greatest level of apoptosis which was likely indicative of the greatest bacterial replication. Because bacteriophages replicate in active growing cells, it is likely that their effect would be greatest in the samples containing the greatest number of actively replicating bacteria which would be the $1:10^{-6}$ samples. This effect was seen with the $1:10^{-6}$ samples treated with the bacteriophages.

At the completion of the three hour incubation, samples of the MRSA were not removed from the 24-well plates and plated to determine if there was complete eradication of the MRSA. It is unlikely that there was complete eradication of the MRSA in this limited time period. The three hour incubation period was used to allow time for the MRSA and bacteriophage to interact within the cell culture while minimizing the effects of the natural apoptosis of the HEp-G2 cells due to decreasing nutrients and increasing cellular waste products.

The cytotoxicity and apoptosis results for immediate bacteriophage treatment are summarized in Table 7. The levels of cytotoxicity and apoptosis are indicated by the number of + signs: +++ (high), ++ (medium), and + (low).

Table 7

Summary Table of Results for MRSA Sample 94 (Immediate Bacteriophage Treatment)

	1:1	1:10⁻³	1:10⁻⁶
Cytotoxicity	+	++	+++
Apoptosis	++	+	+++

Statistical Results for Immediate Bacteriophage Treatment

Due to the variations between the results for the no treatment samples in comparison to the immediate treatment samples, the results were normalized against the positive controls for each sample set. For each of the three sample sets, the three results for the positive control were averaged. Next, each individual result for the other conditions (negative, camptothecin 3.4 mcg/ml, MRSA 1:1, MRSA 1:10⁻³, MRSA 1:10⁻⁶) within the sample set were normalized against the average of the three positive control results for that particular sample set. This process was completed for both the immediate treatment samples and the no treatment samples (to serve as a comparison).

A Tukey statistical test (alpha = 0.05, Q = 2.88849) was performed on these results. There was no significant difference between the treatments for cytotoxicity. In addition, there was no significant difference between the conditions for the cytotoxicity results; although, there was a significant difference (p<0.0001) between the cytotoxicity results for the negative controls and for the camptothecin results for the delayed treatment results.

Both a Tukey statistical test (alpha = 0.05, Q = 2.88849 for the conditions and Q = 2.36823) and a Student's t test (alpha = 0.05, t = 1.97658) were performed for the

apoptosis results. Although there was no significant difference between the conditions, there was a significant difference ($p < 0.0001$) between the apoptosis results for the no treatment and immediate treatment groups for the negative controls and for the camptothecin results.

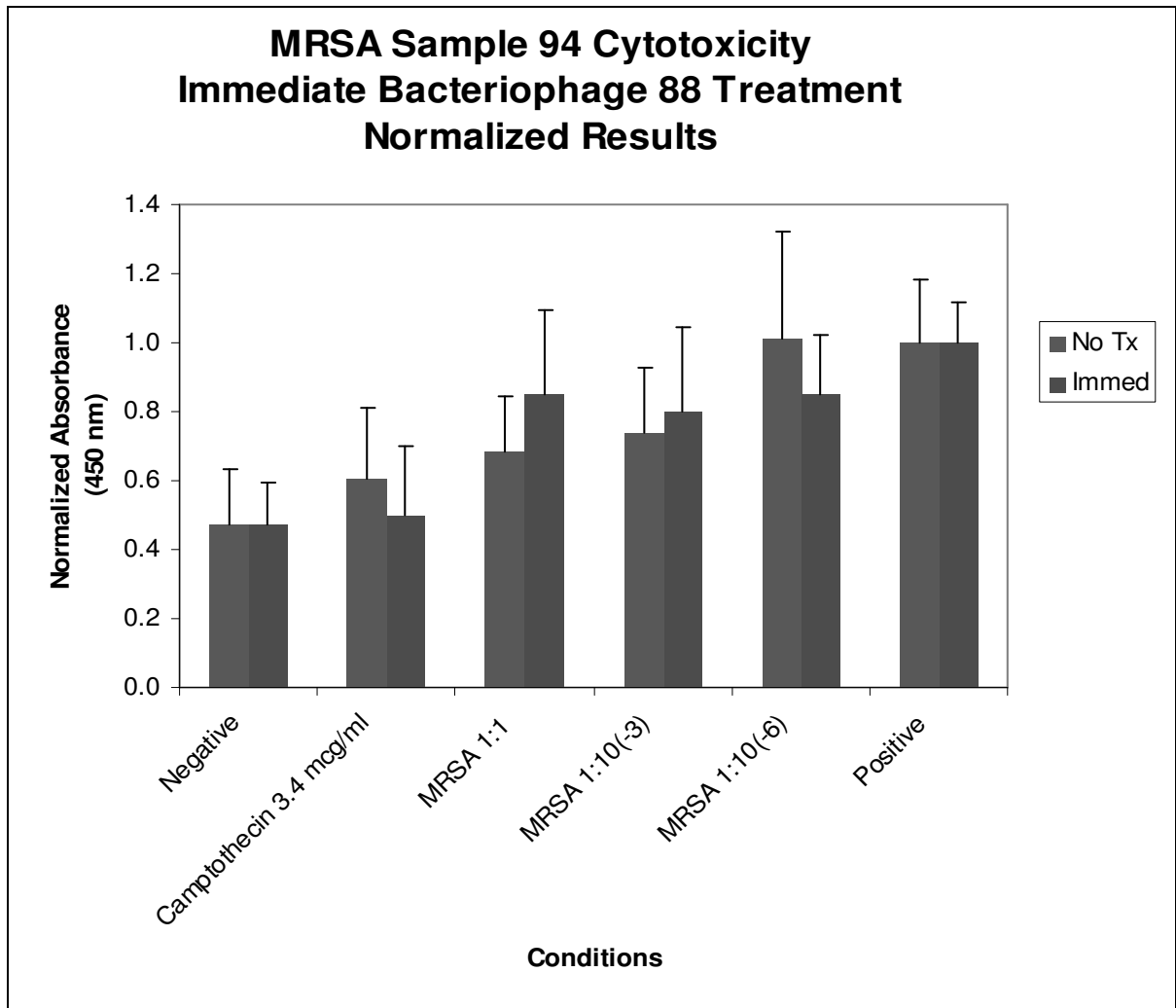


Figure 24. Normalized results of MRSA Sample 94 apoptosis in HEpG2 cells with immediate bacteriophage treatment compared with untreated samples (The error bars indicate the standard deviation for each condition.).

From Figure 24, the normalized results did not indicate any significant differences between the no treatment and immediate treatment results; although, the MRSA 1:10⁻⁶

condition for the immediate does appear lower than the no treatment results. In contrast, the results for the other MRSA concentrations actually appear higher than the no treatment results.

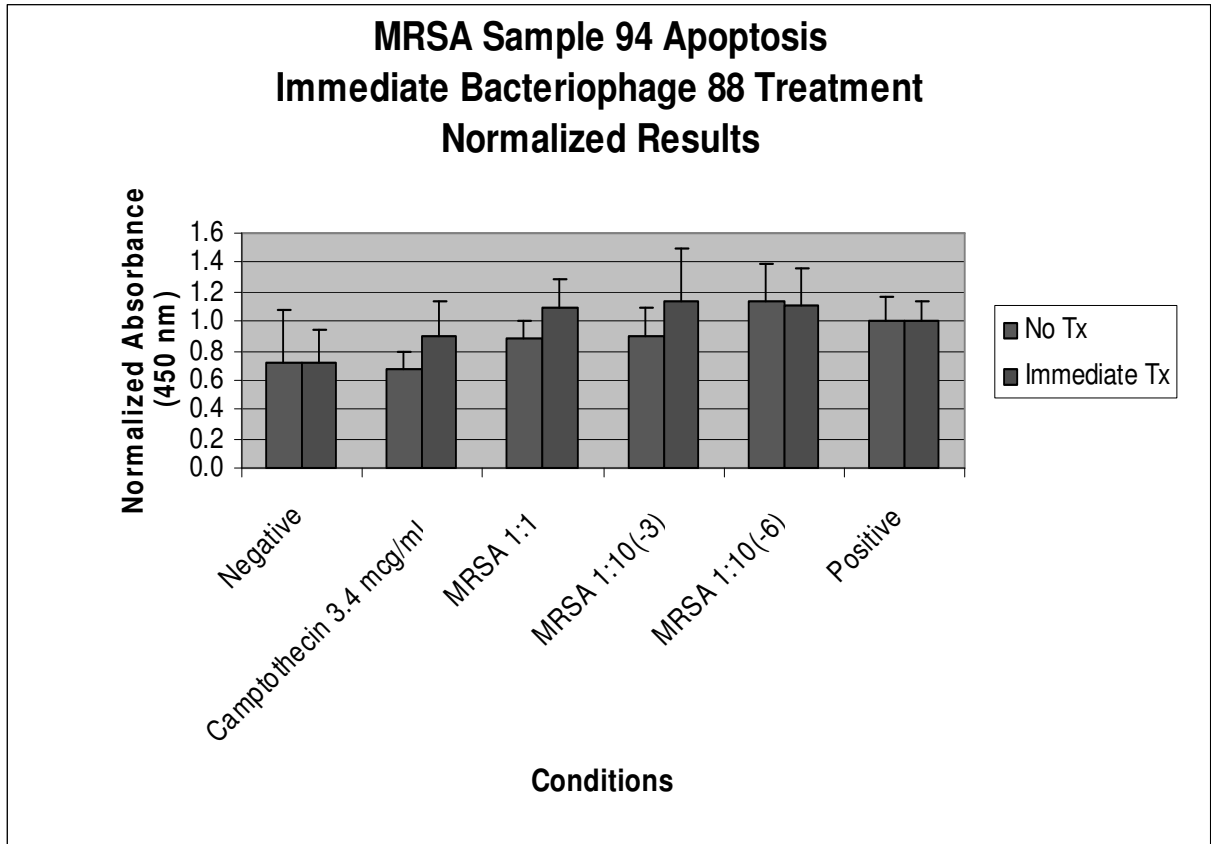


Figure 25. Normalized results of MRSA Sample 94 apoptosis in HEpG2 cells with immediate bacteriophage treatment compared with untreated samples (The error bars indicate the standard deviation for each condition.).

From Figure 25, the normalized results for both the no treatment and immediate treatment groups appear comparable; although, the higher concentrations of the MRSA appear higher in the immediate treatment groups.

Delayed Bacteriophage Treatment

Concurrent with the experimentation involving bacteriophage 88 applied simultaneously with MRSA sample 94 onto HEp-G2 monolayers, experimentation was carried out with MRSA sample 94 applied to the HEp-G2 monolayer but with bacteriophage 88 applied one hour after the initial inoculation of MRSA sample 94 onto the HEp-G2 monolayer. This delayed treatment with bacteriophage 88 was to determine if the bacteriophage would be capable of neutralizing the effects of the MRSA even with delayed bacteriophage treatment.

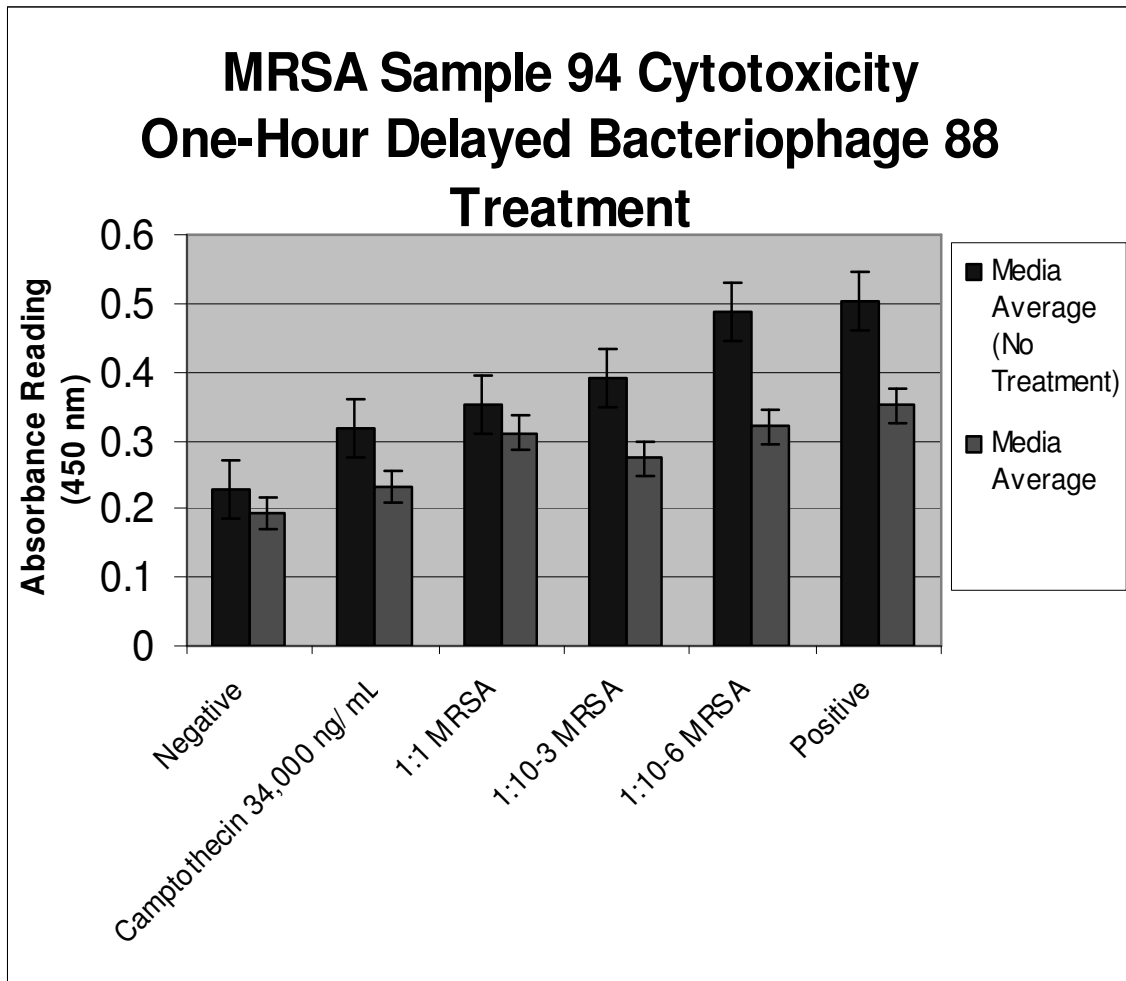


Figure 26. MRSA Sample 94 cytotoxicity in HEpG2 cells with one-hour delayed bacteriophage treatment compared with untreated samples

In Figure 26, the HEpG2 samples with delayed treatment with bacteriophage 88 showed decreased levels of induced cytotoxicity. This effect appeared greatest with the $1:10^{-6}$ concentration of MRSA Sample 94 and the $1:10^{-6}$ concentration of bacteriophage 88. As comparable with the immediately treated samples, the $1:10^{-6}$ MRSA treatment (without bacteriophages) exhibited the greatest level of cellular damage which was likely indicative of the greatest bacterial replication. Because bacteriophages replicate in active growing cells, it is likely that their effect would be greatest in the samples containing the greatest number of actively replicating bacteria which would be the $1:10^{-6}$ samples. This effect was seen with the $1:10^{-6}$ samples treated with the bacteriophages.

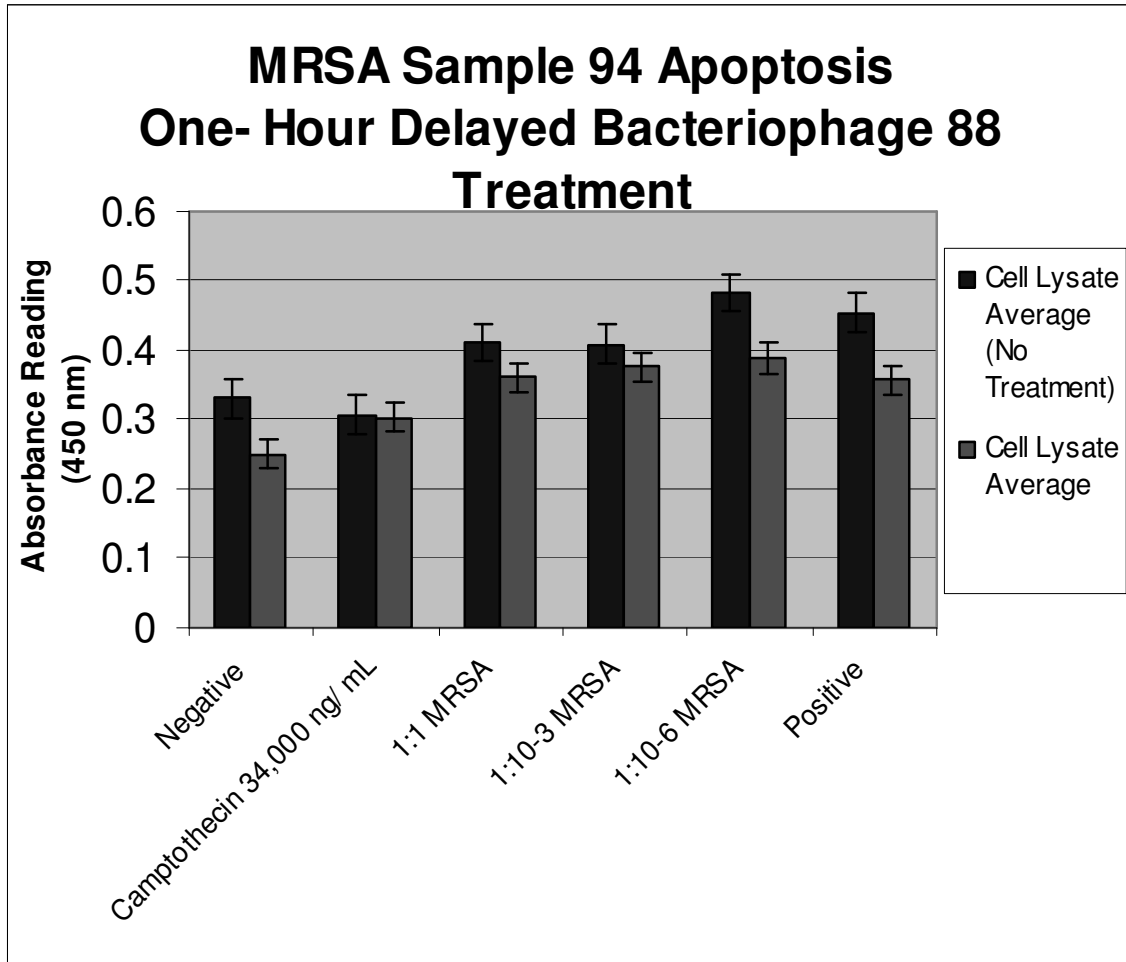


Figure 27. MRSA Sample 94 apoptosis in HEpG2 cells with one-hour delayed bacteriophage treatment compared with untreated samples

In Figure 27, the HEpG2 samples with delayed treatment with bacteriophage 88 showed decreased levels of induced cell death. This effect appeared greatest with the $1:10^{-6}$ concentration of MRSA Sample 94 and the $1:10^{-6}$ concentration of bacteriophage 88. As comparable with the immediately treated samples, the $1:10^{-6}$ MRSA treatment (without bacteriophages) exhibited the greatest level of apoptosis which was likely indicative of the greatest bacterial replication. Because bacteriophages replicate in active growing cells, it is likely that their effect would be greatest in the samples containing the

greatest number of actively replicating bacteria which would be the $1:10^{-6}$ samples. This effect was seen with the $1:10^{-6}$ samples treated with the bacteriophages.

At the completion of the three hour incubation, samples of the MRSA were not removed from the 24-well plates and sampled in order to determine if there was complete eradication of the MRSA. It is unlikely that there was complete eradication of the MRSA in this limited time period. The three hour incubation period was used to allow time for the MRSA and bacteriophage to interact within the cell culture while minimizing the effects of the natural apoptosis of the HEp-G2 cells due to decreasing nutrients and increasing cellular waste products.

The cytotoxicity and apoptosis results for immediate bacteriophage treatment are summarized in Table 8. The levels of cytotoxicity and apoptosis are indicated by the number of + signs: +++ (high), ++ (medium), and + (low).

Table 8

Summary Table of Results for MRSA Sample 94 (Delayed Bacteriophage Treatment)

	1:1	1:10⁻³	1:10⁻⁶
Cytotoxicity	+	++	+++
Apoptosis	++	+	+++

Statistical Results for Delayed Bacteriophage Treatment

As with the immediate treatment samples the results were normalized against the positive controls for each sample set. For each of the three sample sets, the three results for the positive control were averaged. Next, each individual result for the other

conditions (negative, camptothecin 3.4 mcg/ml, MRSA 1:1, MRSA 1:10⁻³, MRSA 1:10⁻⁶) within the sample set were normalized against the average of the three positive control results for that particular sample set. This process was completed for both the delayed treatment samples and the no treatment samples (to serve as a comparison).

A Tukey statistical test (alpha = 0.05) was performed on these results. There was no significant difference between the treatments for apoptosis. There was a significant difference (p<0.0001) between the cytotoxicity results for the negative controls and for the camptothecin results for the delayed treatment results.

Both a Tukey statistical test (alpha = 0.05) and a Student's t test (alpha = 0.05) were performed for the apoptosis results. There was no significant difference between the apoptosis results for the no treatment and delayed treatment group results.

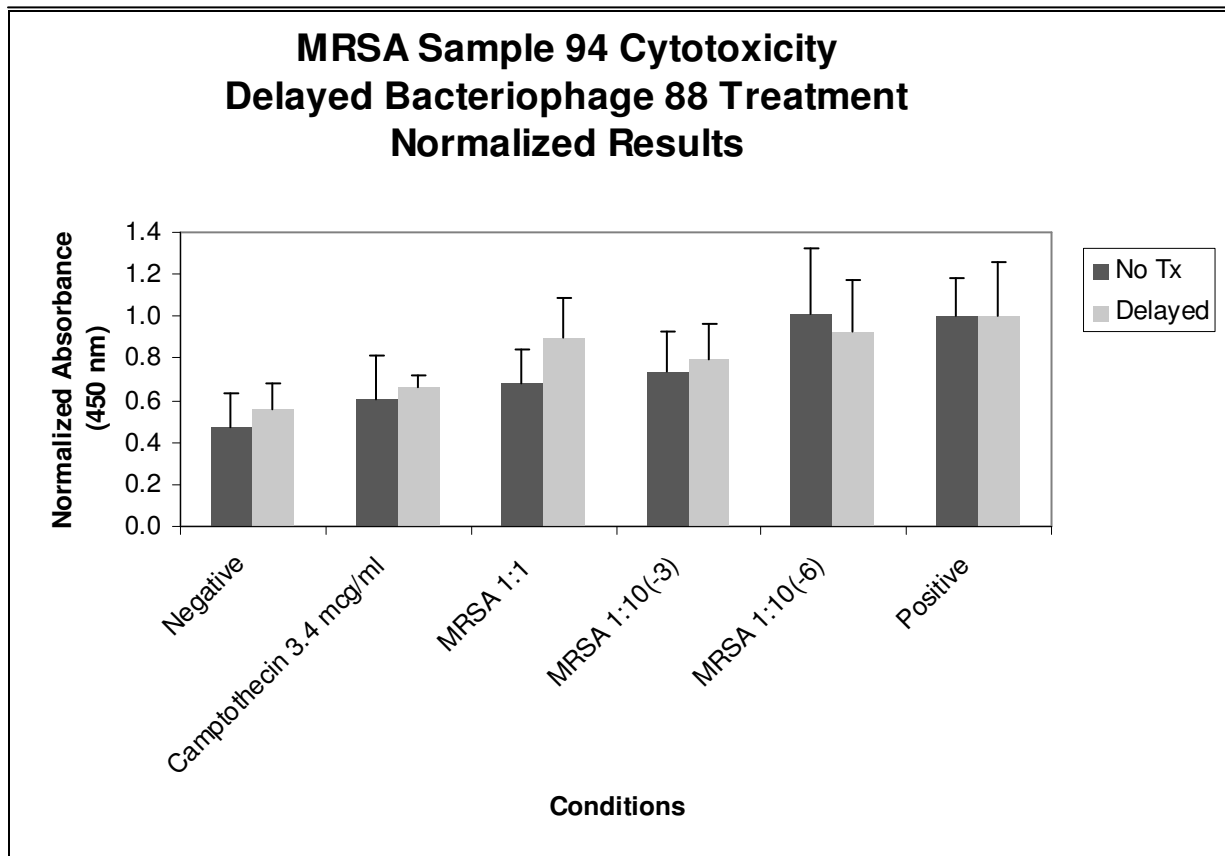


Figure 28. Normalized results of MRSA Sample 94 apoptosis in HEpG2 cells with delayed bacteriophage treatment compared with untreated samples (The error bars indicate the standard deviation for each condition.).

From Figure 28, the normalized results appear comparable for both the no treatment conditions and the delayed treatment conditions; although, the MRSA results for the delayed treatment MRSA 1:10⁻⁶ concentration appear slightly lower than for the no treatment condition.

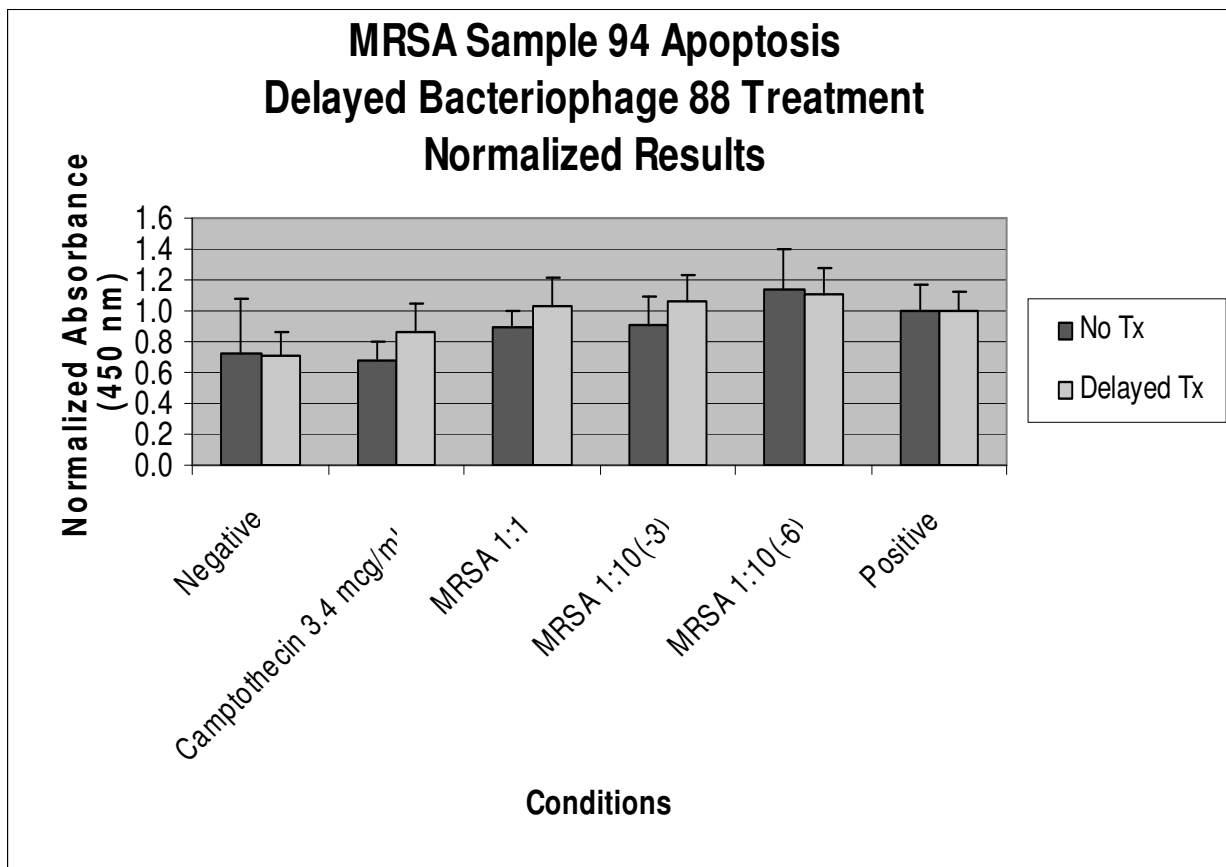


Figure 29. Normalized results of MRSA Sample 94 apoptosis in HEpG2 cells with delayed bacteriophage treatment compared with untreated samples (The error bars indicate the standard deviation for each condition.).

From Figure 29, the normalized results for both the no treatment and immediate treatment groups appear comparable; although, the higher concentrations of the MRSA appear higher in the delayed treatment groups.

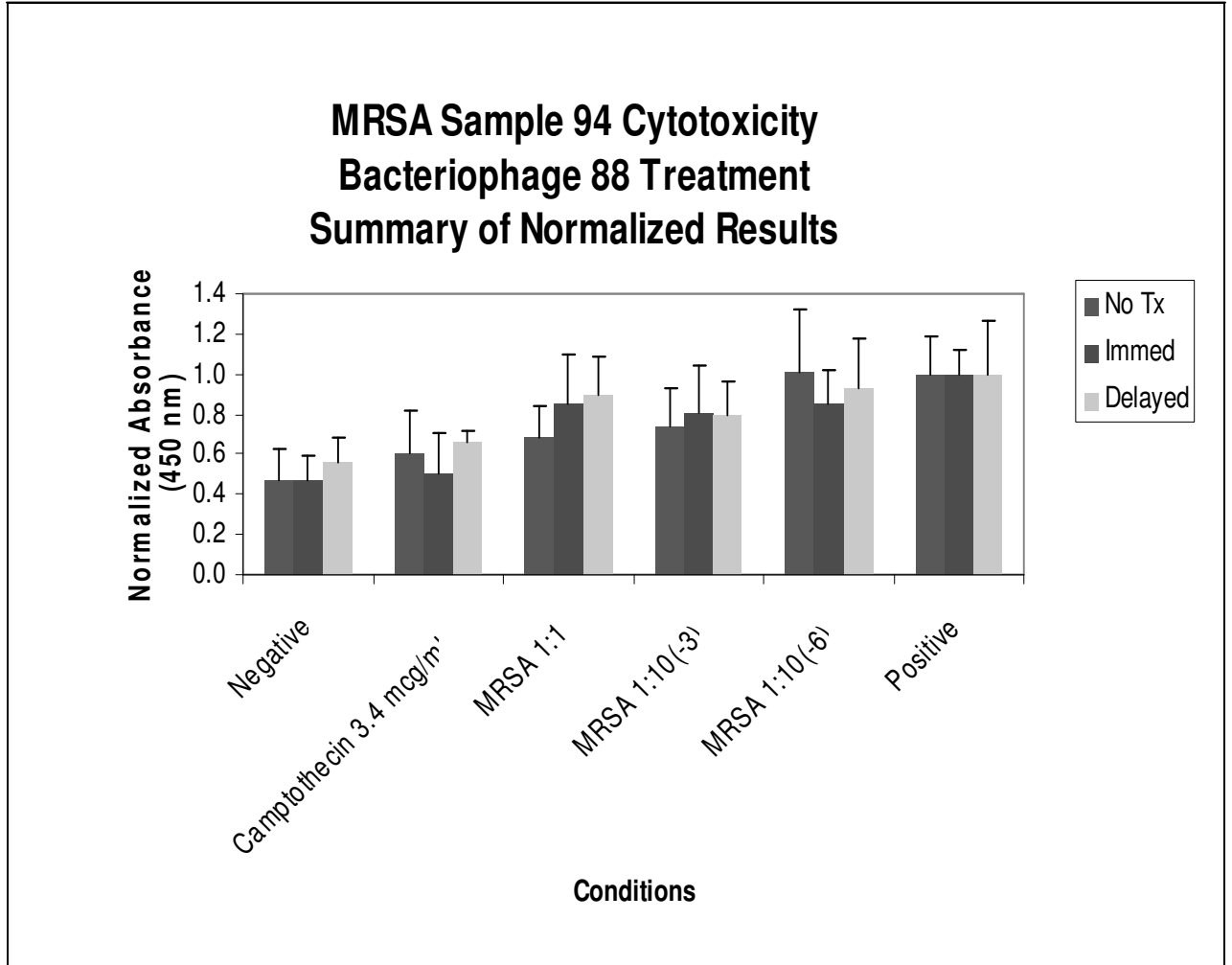


Figure 30. Normalized results of MRSA Sample 94 apoptosis in HEpG2 cells with immediate and delayed bacteriophage treatment compared with untreated samples (The error bars indicate the standard deviation for each condition.).

From Figure 30, it appears that the normalized results for all the conditions appear comparable; although, both the immediate and delayed results for the MRSA 1:10⁻⁶ concentration appear slightly lower than the no treatment results.

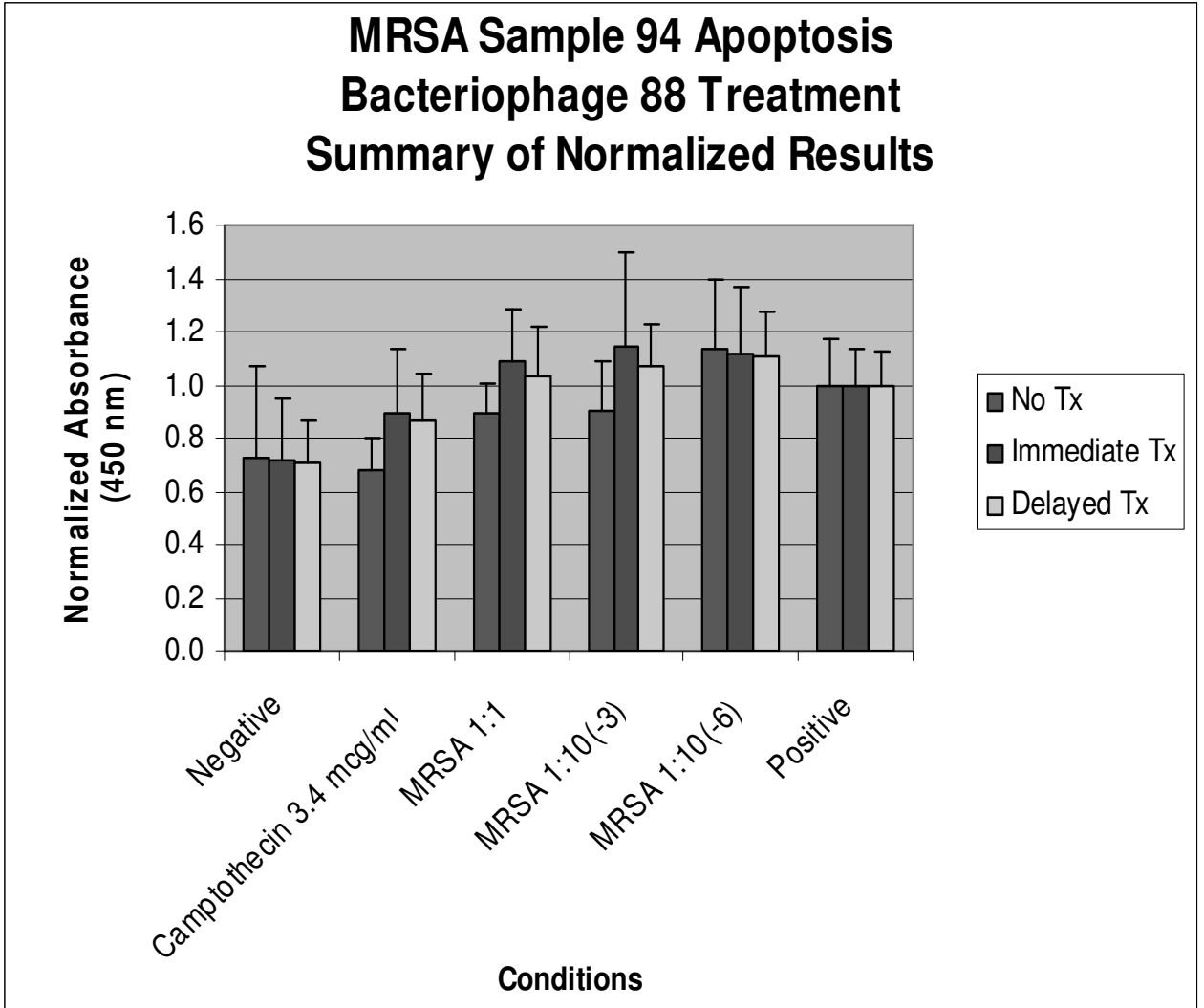


Figure 31. Normalized results of MRSA Sample 94 apoptosis in HEPG2 cells with immediate and delayed bacteriophage treatment compared with untreated samples (The error bars indicate the standard deviation for each condition.).

From Figure 31, it appears that the normalized results for all the conditions appear comparable. There does not appear be any difference in the level of apoptosis between the no treatment, immediate treatment, and delayed treatment groups for the MRSA 1:10⁻⁶ concentration.

Discussion

There were two questions regarding the use of bacteriophages for the elimination of MRSA that were explored in these experiments. In regards to the first question, Will phages 88 and 92 be able to lyse samples of MRSA from the central Florida area?, put forth by this dissertation, bacteriophages 88 and 92 were unable to induce lysis in the majority of the selected MRSA samples. MRSA sample 94 was the exception. The susceptibility of MRSA sample 94 to bacteriophage 88 may have been due to the particular surface receptors on MRSA sample 94.

The literature indicates that numerous surface proteins have been identified on *S. aureus* strains. These surface proteins include clumping factor A, clumping factor B, Fibronectin binding protein A, Fibronectin binding protein B, collagen adhesion, SdrC, SdrD, SdrE, Protein A, Methicillin resistance surface protein, phosphoglucomutase, multiple cell wall surface anchor proteins, 5-nucleotidase family protein, extracellular adherence protein, extracellular matrix and plasma binding protein, cell wall-associated fibronectin binding protein, fibrinogen binding-related protein, fibrinogen binding protein, elastin binding protein, and biofunctional autolysin. However, there was no information available identifying which of these particular surface proteins can be used by bacteriophage as receptors to enter Staphylococcal strains [38].

From Figure 21, the pulsed-field gel electrophoresis (PFGE) patterns of the ten MRSA samples were shown. MRSA sample 94 appears to have a band that is unique from the other MRSA samples; and, it is likely that this band accounts for the susceptibility of MRSA sample 94 to bacteriophage 88.

Although the American Type Culture Collection was contacted regarding the genotypic patterns of their bacteriophage hosts, the genotype patterns of the MRSA hosts for bacteriophages 88 and 92 were unavailable. In addition, ATCC indicated that the company had no information available on the effects of these two bacteriophages on other MRSA strains because the two bacteriophages were propagated on their particular host MRSA strains.

This limited the comparison of the host MRSA genotype patterns with the genotype patterns of the selected MRSA isolates to determine if the particular band seen on the PFGE pattern on MRSA Sample 94 also occurred on the genotype pattern for the bacteriophage 88 host. If this particular band was shared by both MRSA isolates, it could indicate that this particular band is connected to the susceptibility of these two MRSA isolates to bacteriophage 88. Also, it may indicate that this band is linked to the particular receptor that allows bacteriophage 88 to invade and lyse this MRSA isolate.

There are other potential factors that may have affected the ability of bacteriophages 88 and 92 to lyse the MRSA isolates. For instance, bacteriophages are known to be species specific in regards to their host range; however, it has also been noted in the literature that bacteriophages can even be strain specific for their particular bacterial host [1].

Further, lysogenic bacteriophages may also have affected the ability of phages 88 and 92 to lyse the MRSA isolates. Lysogenic bacteriophages are known to integrate into bacterial genomes and prevent infection of the same bacteria by a homologous bacteriophage. However, there are mutant bacteriophages, known as *vir* (short for

virulent) mutants, which can circumvent this resistance due to a lysogenic bacteriophage. [1]

Another consideration in regards to the resistance of the MRSA isolates to the bacteriophages 88 and 92 is that the MRSA isolates originally cultured with the bacteriophages 88 and 92 came from the same geographic area and time period as the bacteriophages. [86, 87] In addition, it should be noted that only one particular MRSA host was noted by ATCC for each bacteriophage rather than a selection of potential MRSA hosts. This would likely indicate that these were the bacterial isolates that were predominantly lysed by bacteriophages 88 and 92.

Also, MRSA isolates have continued to evolve over time. There are at least eight different MRSA (oxacillin-resistant) lineages, known as USA100 through USA800, in the U.S. currently. USA100, USA200, USA500, USA600, and USA800 are typically found in healthcare settings. In contrast, USA300 and USA400 are more commonly found in community settings; and, USA700 can be found in both healthcare and community settings. Further, USA100 isolates are the most commonly cultured oxacillin-resistant lineage; and, these isolates are usually multidrug resistant and include most of the VISA and VRSA isolates in the U.S.. USA200, USA500, and USA600 are also multidrug resistant and have similar PFGE patterns to epidemic strains from Europe and Australia. In contrast, USA300 and USA400 typically contain community-acquired isolates that are only resistant to beta-lactam drugs and erythromycin [60]. With this continued evolution, the present day MRSA isolates have varying levels of dissimilarity to the MRSA from previous decades as evidenced by the differential lysis patterns of the phage hosts versus the MRSA isolates examined in this study.

In regards to the second question, Will phages 88 and 92 be able to neutralize the detrimental effects of the MRSA isolate(s) on an epithelial cell culture?, put forth by this dissertation, bacteriophage 88 does appear to have had some protective effect on the HEp-G2 samples from the cellular damage and apoptosis that had been previously induced by MRSA sample 94 inoculation. This protective effect of the bacteriophages appeared greatest in the HEp-G2 samples containing $1:10^{-6}$ concentrations of MRSA sample 94 and bacteriophage 88.

In conclusion, the effects of bacteriophage 88 on the MRSA sample 94 inoculated on the HEp-G2 wells does indicate that bacteriophages can provide a protective effect from MRSA cellular damage and apoptosis when used against susceptible MRSA strains. However, it has to be noted that due to the strict specificity of the bacteriophages for particular bacterial strains, there must be a good match between the bacteriophages and the bacterial strains in order to have the intended effect of bacterial destruction. For example, in one study [41], it was found that twelve samples of vancomycin-intermediate *S. aureus* in general had altered bacteriophage susceptibility patterns in contrast to the bacteriophage susceptibility patterns of their original seven vancomycin-sensitive parent strains; however, the particular receptor alterations that changed the bacteriophage susceptibility patterns were not identified. Also, this study noted that major genetic changes were not required for MRSA to develop into vancomycin intermediate-resistant *S. aureus* (VISA). In fact, *Sma*I chromosomal RFLPs could remain quite similar between the vancomycin-sensitive parent strains and the altered VISA strains.

From another study, it is entirely possible to correlate antibiotic resistance patterns with PFGE patterns; however, antibiotic resistance patterns and PFGE patterns

may not be indicative of bacteriophage susceptibility patterns for Staphylococcal strains [46].

This difficulty may be overcome by using multiple bacteriophages strains against the particular bacterial strain. With the availability of genotypic testing for MRSA, it would be possible to determine particular problematic MRSA strains in a community (or communities) that could be targeted with different bacteriophages known to lyse these particular MRSA strains. It may be necessary that the bacteriophages used in treatment would have to be in varied combinations specified by the prevailing MRSA strains in a particular community. However, it is highly probable that these bacteriophage combinations would change over time as the prevailing MRSA strains changed.

Low doses of bacteriophage in different preparations such as ointments may increase in sufficient numbers when applied in the anterior nares where *S. aureus* is commonly found and may allow eradication of the targeted bacterial host. Further, from a recent study, it was shown that bacteriophages propagated on weakly susceptible bacterial strains could result in modified bacteriophages that were then capable of lysing the original bacterial strains [69].

As for future studies, it would be informative to be able to compare these results with other MRSA isolates and bacteriophages. With the change in receptors over time, it may be possible that other staphylococcal phages that have not previously been associated with the lysis of MRSA may be able to lyse some MRSA strains.

Also, it would be useful to study the PFGE patterns of the host MRSA for bacteriophage 88 and bacteriophage 92. Because these host MRSA were originally cultured in the late 1970s, ATCC was unable to provide PFGE patterns for their MRSA

hosts. In addition, it would be useful to do a prospective study of MRSA isolates from specific clinical syndromes that would be used in further studies in order to compare specific bacteriophage susceptibilities.

Techniques such as Fluorescent Amplified-Fragment Length Polymorphism Analysis and *staphylococcal chromosome cassette* (SCC) typing may also be used to examine the genotypes of the different staphylococcal strains tested against the bacteriophages. In addition, it would be interesting to examine the possibility of being able to modify the bacteriophage in order to allow lysis of the previously resistant bacterial strains [69].

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