Therapeutic & Prophylactic Evaluation of Phage Cocktail in Combating Mild & Severe Colibacillosis in Poultry

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2022

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A thesis submitted in the partial fulfillment of the requirement for the degree of

Master of Science in Industrial Biotechnology

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2022

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… To My Supportive Father & Loving Mother …

They are the pillars of my strength.

Acknowledgements

All praises to ALLAH Almighty, the Lord of the worlds, the most Gracious, the most Merciful. My limitless thanks to HIM for his countless blessings. I praise and thank **ALLAH** SWT for His greatness and for giving me the strength and courage to complete my research work.

I am extremely grateful to my supervisor, **Dr. Fazal Adnan** for his motivation, guidance and support. He welcomed me into his research group and I genuinely appreciate his helping, kind and humble nature. I have acquired various positive personality attributes from him unintentionally and he guided me to overlook what I lacked in my personality. I am confident that his training will be of great assistance to me in the future.

I would also like to thank the rest of my examination committee and my external examiner for helping me throughout the course of my research, encouraging my morale, and providing insightful feedback as GEC members. I appreciate **Ms. Fouzia Fazal** for facilitating me in Virology Lab-1 & **Mr. Fida Hussain** for his help at the animal lab house.

I would like to extend my huge and sincere thanks to my seniors, **Ms. Hira Niaz** & **Ms. Sidra** for their immeasurable support, encouragement and help. This endeavor would not have been possible without their love. I am eternally grateful to my best friend, **Ms. Sadia Masood** for being with me through thick and thin. The entire MS journey was filled with ups and downs, but it was lovely only because of her. She extended her shoulder in the moments of sadness and I really appreciate her kind and cheerful presence. These acknowledgements would not be complete without mentioning my friends **Ms. Maryam Zabeeh**, **Ms. Amina Basheer, Ms. Aeraj Amjad, Mr. Mohammad Noman** and **Ms. Saba Jahan.** Thank you all for your unwavering support and good wishes.

My deepest gratitude to my father, **Mr. Khizar Hussain** and my mother, **Ms. Imrana Bibi** for investing in me spiritually, morally and financially. Your immense love and support have made this journey possible. Your kindness and trust means more to me than you will ever know.

Last but not the least, I would like to extend my love to my annoying siblings, **Ms. Tahreem Khizar, Mr. Mohammad Haris** & **Ms. Adeesa Khizar** for their unconditional support, prayers and gentle nature. They have always been my cheerleaders.

I also acknowledge my colleagues and juniors for their assistance and contributions, directly or indirectly, to make this study a success.

Andleeb Khizar

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List of Abbreviations

APEC	Avian pathogenic *Escherichia coli*
MDR	Multi-drug resistant
PBM	Poultry bedding material
ExPEC	Extraintestinal Pathogenic *Escherichia coli*
TEM	Transmission Electron Microscopy
MOI	Multiplicity of Infection
O.D	Optical Density
PFU	Plaque Forming Unit
CFU	Colony Forming Unit
SD	Standard Deviation
FCR	Feed Conversion Ratio
IM	Intra-muscular
SC	Subcutaneous
ND	Nasal Drops
AT	Antibiotic Therapy
CT	Combination Therapy
PT	Prophylactic Treatment

Abstract

Avian pathogenic *Escherichia coli* (APEC) strains cause severe respiratory and systemic illnesses, endangering global food security and avian welfare. Progressive increase in multi-drug resistant bacteria have fueled interest in the use of bacteriophages to battle bacterial illnesses in humans and animals. The present study aimed at investigating the *in vivo* therapeutic and prophylactic performance of a phage cocktail to combat mild and severe colibacillosis in experimentally infected chicks. Three lytic coliphages isolated from the bedding material of poultry, were characterized and combined in a 1.47×10^{12} PFU/ml cocktail to be administered in 14-days old APEC O1 infected chicks. The mortality was reduced from 85.72% to 0% in prophylactic phage treatment and combination therapy, whereas therapeutic phage therapy decreased mortality to 33.3% in intramuscular-treated groups. Weight-gain and feed conversion ratios were significantly better in chicks treated with phage therapy than antibiotic therapy, combination therapy and untreated controlled birds. Absence of whitish fibrinous layers around the organs of the treated chicks emphasized that the severe damage in the positive control, corresponding to the score values of 2.7, 2.5 and 2.42 in the liver, heart and lungs respectively, had been reduced to mild damage after phage therapy. However, the lowest damage was recorded in all the organs of the combination therapy with the lesion score of 0.14, 0.28 and 0.28 in the liver, lungs and heart respectively. Histopathology of all the retrieved organs, heart, liver and lungs revealed necropsied degenerated cells with vascular congestion and oedema in the infected chicks however, such severe symptoms were not present in the treatment groups. The liver of the Positive control had 91.2% percentage in tissues, which was reduced to below 50% across all treatment groups, with combination therapy having the lowest damage at 10%. Damage to the heart was 86.1% in the positive control and was significantly decreased to 7.5% with the use of combination therapy. Lung damage was reduced from a mean of 75.5% to a range from 56.3% to 6.7% across all treatment groups, with the maximum reduction in the nasal-treated group. The data indicates that delivering phages either prophylactically, therapeutically or in combination with antibiotics via intramuscular, subcutaneous and nasal route may be an effective means for controlling colibacillosis caused by APEC O1 infection and may improve survival rate, weight gain, FCR, organ and tissue damage.

Chapter 1

Introduction

1. Introduction

Poultry infectious illnesses are a major threat to the health of animals, humans and the economy. In light of the widespread emergence of antibiotic-resistant bacterial strains and the restrictions on the use of antibiotics in poultry (Castro-Vargas et al., 2020), there is an urgent need to discover alternative methods for preventing and treating bacterial infections in poultry. Phage therapy is one of the most promising strategies in this field. (Gigante & Atterbury, 2019a).

Phages, also referred as bacteriophages, are viruses that kill and target selective bacteria. Phages are the most abundant living entity on the planet, with an estimated 10^{31}-10^{32} species present at any given moment (Baláž et al., 2020). They are also an essential component in controlling the bacterial population in different environments. Sewage water, soil, fresh water, marine water bodies and food products are among few source

s where phages are most abundant and can be easily isolated. The majority of phages are only infectious to bacteria that possess their appropriate receptor, restricting their host range. The method of action of "virulent" and "temperate" phages differ. Temperate phages have lysogenic life cycle, in which phage do not directly kill the host cell; rather, they integrate into the genetic material of the host or live as plasmids within it. Virulent phages infect and promptly kill their infected host cells during the lytic cycle (Clokie et al., 2011).

Despite the fact that bacteria have numerous intrinsic defences against lytic bacteriophage infection. However, phages have developed a variety of methods to circumvent this resistance. This can involve changes or loss of receptors, as well as integration of phage DNA into the clustered regularly interspaced palindromic repeats/CRISPR associated system (CRISPR/Cas) system, whereas for phages, it can include detection of new or changed receptors and anti-CRISPR genes (Lin et al., 2017).

Not all phages can be used for therapeutic purposes. The application of temperate phages, which have a lysogenic lifetime, as treatments can be difficult because of the encoding of bacterial virulence factors, in particular bacterial toxins. As a result, only lytic bacteriophages are favoured, and a wide variety of criteria, including phage stability, adsorption, life cycle, the absence of

pathogenic genes, and studies of phage genomes, are taken into consideration (Loc-Carrillo & Abedon, 2011).

Despite many benefits, serotype specificity of phages narrows the host–range and reduce the effectivity. To overcome this hurdle, multiple phages may be required to eliminate various strains of the same pathogen. Therefore, multiple phages with different host-range specificities can be incorporated in a cocktail. Bacteriophage cocktails are more preferred than individual phages because they are more effective, providing broader host-range and delaying the emergence of resistant strains.

The capacity of phages to infect only a certain bacterial species, serotype, or strain contributes to their high success rate and safety profile as compared to antibiotics. The use of antibiotics not only kills the pathogenic bacteria, but it also changes the normal microbiota of the gut, which can result in dysbiosis, immunological suppression, and subsequent infections. (Żbikowska et al., 2020). When antibiotics are combined with phages, a phenomenon known as phage-antibiotic synergy (PAS) occurs, which makes phages particularly effective against bacteria. This synergistic relationship was observed by administering sublethal dose of antibiotics in phage-infected bacteria, and an increase in the size of phage plaque was obtained. Interestingly, the phages delayed the resistance of bacteria against antibiotic and even restore the sensitivity of specific bacteria (Diallo & Dublanchet, 2022). Therefore, many studies have demonstrated that combined administration of phages and antibiotics is more effective than monotherapy (Huff et al., 2004, Rodriguez-Gonzalez et al., 2020, Li et al., 2021, Wang et al., 2021).

Escherichia coli is a Gram-negative bacillus, a normal inhabitant of the digestive tracts of birds which frequently spreads through feces. Although most strains are non-pathogenic, some are virulent and named as Avian Pathogenic *E. coli* (APEC). Strains of APEC are responsible for a variety of localized and systemic infections collectively known as avian colibacillosis. Primary or secondary infection may emerge as the disease. In the absence of proper sanitation practices, the primary route of infection is the inhalation of contaminated dust particles from the feces of an earlier flock. Secondary infection develops in immunocompromised chicks when the original pathogen has weakened the host immune system to the point where it is susceptible to other infections, resulting in a *E. coli* infection that is frequently associated with a greater death rate (*Colibacillosis - Microbewiki*, n.d.)

APEC Infections are prevalent in poultry of all ages and types and generally associated with high morbidity, mortality, lowered production, decreased egg production and carcass rejection, causing a significant economic loss to poultry industry worldwide. In Pakistan, poultry contributes 1.3% in national GDP and is the second largest industry of the country (Hussain et al., 2015). Pakistan is the 11th largest poultry producer in the world, producing 1,163 million broiler chicken annually. Poultry meat accounts for 28% of the nation's overall meat production (Jan et al., 2018). Considering the mortality rate of colibacillosis in chickens (1-10%) and even higher mortality in broilers (Omer et al., 2010), colibacillosis pose a significant economic threat. The disease is more common in young chicks of age 4-6 weeks with a prevalence rate of 36.73% (Rahman et al., 2004).

APEC strains recovered from colibacillosis-affected chickens primarily belong to serogroups O1, O2, and O78 (Schouler et al., 2012) . Phylogenetic and genome analysis show that these strains are closely related to human extra-intestinal pathogenic Escherichia coli (ExPEC) strains which cause meningitis, urinary tract infections and sepsis in humans. Because of these similarities, there exist concerns about the zoonotic potential of APEC strains. (Mora et al., 2013, Nandanwar et al., 2014, Ewers et al., 2009).

APEC is easily transmitted from chickens to humans where it colonizes the human gut causing infection. Many virulence traits have been identified that allow these pathogens to take over, colonize and cause infections in the body outside gastrointestinal tract. Humans get this infection by inhaling the contaminated dust or by eating poorly cooked poultry meat (Linton et al., 1977).

Two widely used treatments for colibacillosis are vaccination and antibiotics. Vaccines have been demonstrated to protect against some serogroups that cause colibacillosis. However, the diverse variety of APEC strains restricts the viability of an all-encompassing immunization (*Colibacillosis - Microbewiki*, n.d.). The live *E. coli* vaccination is 50% effective as a therapy for *E. coli* infections with significant high fatality rates.(*Colibacillosis in Layers: An Overview | The Poultry Site*, n.d.)

Although antimicrobials are frequently employed to treat and control colibacillosis, but their availability has decreased due to antibacterial resistance and lack of new drug development in the poultry industry. It has been reported that 90% of colibacillosis strains are resistant to tetracycline, and 60% are resistant to five or more antibiotics (*Colibacillosis - Microbewiki*, n.d.). Another antibiotic, enrofloxacin has been regarded as highly efficient treatment for colibacillosis in chickens, but it is a fluoroquinolone antibiotic, and fluoroquinolone related substances are critical

for treating bacterial infections in humans. In the light of ongoing concerns regarding the use of antibiotics in chicken production, there is a compelling need to identify safe and effective alternatives to prevent and treat avian infections (W. E. Huff et al., 2004).

The current study aims to characterize the phage (PBM-3), isolated from the bedding material of the chicken. The lytic potential, pH and thermal stability, adsorption to host (APECO1), latent period and burst size was evaluated by double layer agar assay. Two already characterized phages (PBM-1, PBM-2) and one phage characterize in this study (PBM-3) were mixed in equal proportions to prepare phage cocktail whose host specificity and resistance were compared to individual phages to test the hypothesis that mixture of phages has higher lytic potential, broader host-range and delayed resistance. Therapeutic and prophylactic efficiency of the phage cocktail individually and in combination with antibiotic (Enrofloxacin) was evaluated *in vivo* on experimentally infected chicken. Some factors, such as administration method and timing, were kept variable in order to determine the optimal timing and best administration route for phage therapy.

Chapter 2

Literature Review

2. Literature Review

2.1 Bacteriophages in nature:

Phages, also referred as bacteriophages, are viruses that kill and target selective bacteria. They are ubiquitous in nature being abundantly found in soil and water. Their size is ~50 times smaller than most bacteria (20–200 nm). Phages kill about 20% -40% of all sea surface bacteria every 24 hrs (*What Is Phage Therapy? - IPATH*, n.d.)

2.2 Phage Biology:

These non-living biological entities are composed of either DNA or RNA enclosed in a protein capsid. The phage structure comprises of 60% protein coat and 40% nucleic acid. Phages are structurally simple but diverse biologically. Due to their parasitic nature, they rely on bacterial host for reproduction and ultimately survival.

Most phages are only infectious to bacteria that carry their corresponding receptor, which limits the host range of phages. The host specificity of phages varies, some are strain-specific but others can infect a wide variety of bacterial strains. Bacteria have developed a diverse range of defensive mechanisms against lytic phage infection, while phages have created an equal array of strategies to defeat this defence. (Lin et al., 2017).

2.3 History:

Though the initial discoverer of bacteriophages is unknown, it is commonly accepted that Frederick Twort, an English bacteriologist, was the first to claim that prior discoveries of a "factor" that killed bacteria was caused by a virus in 1915.

Another microbiologist working at the Pasteur Institute in Paris at that time was Felix d'Herelle., who continued the work of Twort and offered phages as a treatment for human infections for the first time. In 1919, d'Herelle and other hospital interns tested the safety of a phage cocktail by consuming it, before administering it to a 12-year-old boy suffering from severe diarrhoea. After a single dose, the boy's symptoms disappeared, and he recovered completely within a few days (*Phage 101 - Bacteriophage Therapy - UC San Diego Health*).

2.4 Life Cycle of Bacteriophages:

During an infection, a phage binds to a bacterium and inserts its genetic material into the cell. After this, a phage will go through either the lytic (virulent) or the lysogenic (lysogenic) phase of its life cycle (temperate). Lytic phages take advantage of the machinery within the host cell in order to manufacture phage components. After that, they lyse the cell, which causes additional phage particles to be released into the environment. Lysogenic phages are those that insert their genetic material into the chromosome of their host cell and then multiply as a unit with it. This process does not result in the death of the host cell. Lysogenic phages are occasionally able to be coerced into following a lytic cycle if certain conditions prevail. (*Bacteriophage | Definition, Life Cycle, & Research | Britannica*)

2.5 The Dry Pipeline of Antibiotics: From the Golden Era to the Present

With the advent of penicillin in 1928, antibiotics made phage therapy outdated in the many parts of the world. During the so-called "golden period" of antibiotics, which began in the 1940s and lasted for more than four decades, more than forty different antibiotics were discovered and made available for use in clinical settings. During this time period, the appearance of resistance to a single antibiotic was met with a relatively low level of concern. This was due to the rapid discovery of additional compounds, many of which possessed enhanced pharmacokinetic and pharmacodynamic properties, fueling a cycle of antibiotic discovery, overuse, and the appearance of resistant bacteria. However, the discovery of new antibiotics sharply declined till 1990s, and the effects of overuse of available antibiotics became more prominent. The vast majority of the antibiotics that are currently available are either modified or combined forms of substances that were already known, which has led to a "dry pipeline" in the field of antibiotic discovery and development. (Gordillo Altamirano & Barr, 2019).

Multidrug-resistant (MDR) infections are the outcome of successive accumulation of antibiotic resistance characteristics. Antimicrobial resistance is considerably worsened by human activities, particularly therapeutic and industrial overuse of antibiotics. Antibiotics are used to boost animal growth, address diseases that affect crops and fish in agricultural and aquacultural settings, as well as, infections that affect people. A key contributor to the horizontal spread of antibiotic resistance genes are antibiotic-resistant bacteria that have been identified from the digestive tracts of people

and animals. Recent research indicates that by the year 2050, antimicrobial resistance will be responsible for the deaths of ten million people annually, which would result in a loss of $100 trillion to the worldwide economy. It poses a huge risk to the health of people all over the world and can inflict harm on people of any age, from any socioeconomic background, and in any region. (*Global Action Plan on Antimicrobial Resistance*, n.d.)

2.6 Weaponizing viruses to fight infections (phage therapy):

In 1980s, Western scientists "rediscovered" phage therapy. Since then, there has been a rise in interest in the use of phage therapy as a potential method of treatment for the problem of multidrug-resistant bacterial strains.

Phages and bacteria have a complex relationship and they co-evolve together. They are parasites that only live on bacteria. A phage will only attach to a specific bacteria or, in some cases, only to a few strains of that bacteria. The phage recognizes sugar or protein molecules on the bacterial cell wall that act as binding receptors. It then attaches itself to the cell. Specificity comes from the way these binding receptors work. At the end of each lytic cycle, each bacterial cell is destroyed, letting 10 to 200 virions out. The size of a phage's burst is based on how many virions it releases.

For therapeutic use, it is necessary to find active phages that kill pathogenic bacteria that are causing an infection in a patient, multiply these phages, and give them to their host so they can attack the bacteria. When phages are given to infected individuals, they will continue to multiply as long as their host bacteria is still alive. If the host bacteria dies, the phages will also die (Brives & Pourraz, 2020).

Temperate phages are not suitable for phage therapy as they integrate their genome in bacterial DNA. In this lysogenic cycle, bacteria may acquire new genes which may be pathogenic such as toxins encoded by phage which increase the virulence of bacteria or even antibiotic resistant determinants (Fortier & Sekulovic, 2013).

Treatment with phages is an attractive option for combating antibiotic resistance. Numerous studies have revealed the in vitro and in vivo therapeutic potential of phages, and while some clinical trials have been done in the past decade, additional data are required to create a solid regulatory case for their clinical usage. (Furfaro et al., 2018).

Instead of using a single phage, a **Phage Cocktail**, or combination of phages, can be used to combat bacterial infection. A single phage has a limited host range, limiting the efficacy of phage therapy. The use of phage cocktail overcomes the problem of narrow host range in monophage therapy, making it more effective.

2.7 Phage and antibiotic combination therapy: two is better than one

Phages used in conjunction with antibiotics have a greater therapeutic potential than either treatment alone and also help to prevent the emergence of resistant strains. Antibiotic resistant bacteria that are susceptible to phages develop resistance to phages as a result of selection pressure, but these phage resistant strains are also susceptible to antibiotics. In biology, this is known as "trade off," and it states that if an organism acquires genes, it must sacrifice some of its other genes. Hence, combination therapy is proven most effective than antibiotic therapy and phage therapy alone (Li et al., 2021).

Many mathematical models explore the relative relationship of antibiotics, phage therapy and host innate immune system in combating any infectious disease. Despite the fact that phages cannot promote innate immunity, bacteria-boosted innate immunity acts against phages, which is an important finding for understanding cases of phage ineffectiveness and recommending improved protocols for phage therapy.

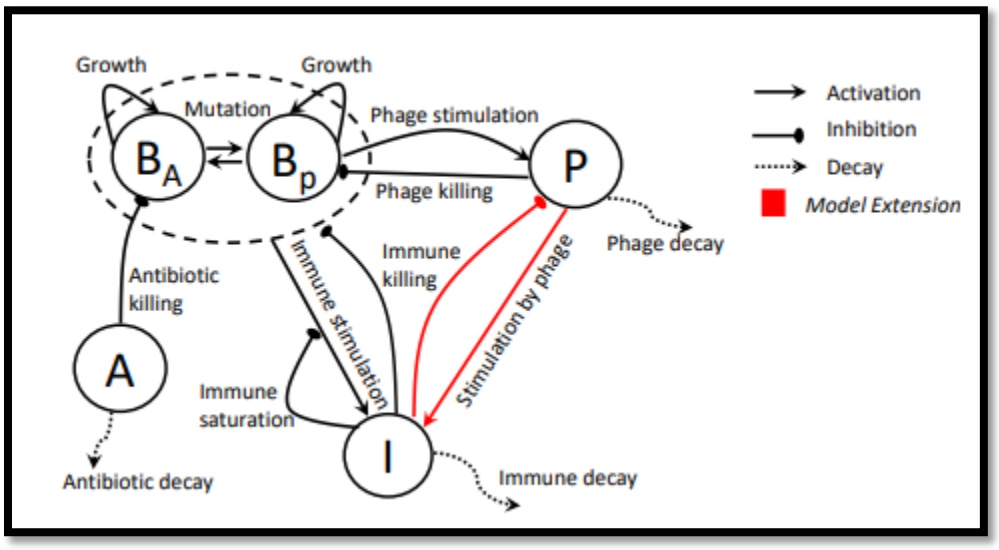


Figure 2.1: Mathematical model showing schematics of the phage-antibiotic combination therapy model (Banuelos et al., 2021)

In order to comprehend the role of the immune response in phage-antibiotic combination therapy, Banuelos et al. developed a model proposing that it is essential to comprehend the host immune system and that the frequency and dose of treatment are crucial factors in determining the efficacy of treatment. (Banuelos et al., 2021).

2.8 *In vivo* Testing of Phage Therapy:

In 2000, phage therapy was conducted on 42 patients with persistent leg ulcers, a cocktail of phages specific for *S. aureus, E. coli* and *Pseudomonas aeruginosa* was investigated for safety. The study only looked at safety, not clinical outcomes, because it was a phase 1 trial. There were no documented side effects from the phages (*Phage 101 - Bacteriophage Therapy - UC San Diego Health*, n.d.).

Recent animal researchers have examined into phage treatment against a variety of therapeutically important infections. When mice were challenged with *P. aeruginosa*-induced gut-derived sepsis, oral administration of phage saved 66.7% of them, compared to 0% in the control group. (Watanabe et al., 2007).

In a mouse model of Clostridium difficile, a single dose of phage given at the same time as C. difficile was enough to keep 11 of 12 mice from getting sick, but C. difficile and clindamycin-treated control animals died within 4 days. (Ramesh et al., 1999).

P. aeruginosa infections of the gut, lungs and skin have also been treated with phage cocktails in animal models. In humans, the pathophysiology of septicemia was very similar to the model developed in this study.

When the isolated lytic phage strain was given to mice via oral route, it increased the survival rate from 0% in saline-treated group to 66.7% in phage-treated group. Additionally, phage-treated animals had less viable P. aeruginosa cells in their blood, spleen, and liver. Additionally, the levels of inflammatory cytokines in the blood and liver of phage-treated mice were much lower than those of phage-untreated mice. (Watanabe et al., 2007).

In the EU and the US, Currently, no phage therapy products are approved for human use. However, the FDA has recognised a variety of commercial phage formulations used for bacterial pathogen biocontrol in the food industry as "generally considered as safe." These formulations are used to

treat infections caused by *Pseudomonas syringae, Mycobacterium TB, E. coli, Listeria monocytogenes, MRSA, Salmonella spp., and Listeria monocytogenes*. (Monk et al., 2010)

Food poisoning was reported in 48 million cases in the United States alone in 2011. There is evidence that phage biocontrol can reduce bacterial contamination in fruits, vegetables, and dairy products as well as enhance food safety at all stages of the production and processing of meat.

2.9 Phage Application in Poultry Industry:

The poultry industry is a major source of Salmonella and Campylobacter, two of the world's most common dietary pathogens that cause salmonellosis and Campylobacterosis in humans. The use of phages to control foodborne infections in chicken has received a lot of attention. *Campylobacter jejuni* colonisation in broiler chicks is frequent and difficult to avoid or control. Carrillo et al. (2005) explored the biologic effect of phages with the goal of preventing the spread throughout the flock. Two phages were chosen and delivered to 25-day-old broiler hens colonised with Campylobacter isolates at varied phage-strain combinations. In comparison to the control group, phage-treated birds had lower Campylobacter levels in their cecal contents (Loc Carrillo et al., 2005)

Bardina et al. (2012) investigated the potential of phage mixes as therapy against *S. Typhimurium* and their ability to lower bacterial populations in mice and chickens' intestines. *S. Typhimurium* infection in mice resulted in death in the absence of phage. When the phage mixture was given to the mice before infection and then given again at 6 hrs, 24 hrs, and 30 hrs after infection, the survival rate of the mice increased by 50%. Salmonella levels in chicken cecum contents decreased after several phage combination treatments pre- and post-infection. This research emphasizes the need of giving the phage before infection and the need to keep giving it after infection, to get considerable protection (Bardina et al., 2012).

Lim et al. (2012) explored the efficacy of utilizing a single virulent bacteriophage to reduce *S. Enteritidis* development in infected chickens' digestive tracts and its biotherapeutic effect in preventing horizontal transmission of the pathogen to uninfected cohabiting chicks. One-day old chicks were infected with 10^7 CFU of *S. Enteritidis* while the chicks in control group were uninoculated. After the introduction of the bacteria, all birds were given a bacteriophage preparation at 10^5, 10^7, and 10^9 PFU in feed for 21 days. All phage treatments resulted in a

significant decrease in S. Enteritidis intestinal colonisation relative to the control group in the chicks at weeks 1, 2, and 3. When bacteriophage concentrations of 10^7 and 10^9 PFU were utilized in the study, statistically significant reductions in colonization were observed. At the end of the three-week trial, 70% of the birds treated with 10^9 PFU of bacteriophage had no detectable levels of intestinal Salmonella, demonstrating that bacteriophages have the capacity to prevent horizontal transmission of the bacterium in chickens. These studies show that phages, particularly combinations, have the ability to biocontrol harmful bacteria in primary production. (Lim et al., 2012.)

2.10 Phage Therapy in the Treatment of Colibacillosis:

Escherichia coli, a Gram-negative bacillus, inhabits the digestive tracts of birds and is extensively transmitted via feces. The vast majority of strains are non-virulent, but some pathogenic serotypes, including avian pathogenic Escherichia coli (APEC), can cause serious illness and even death. This opportunistic pathogen is capable of acting as either a primary or secondary pathogen, depending on the circumstances. Infections caused by *E. coli* are common in chicks of all ages and categories (Lutful Kabir, 2010).

APEC is an etiologic agent of chicken extraintestinal infections known as colibacillosis. This disease is one of the leading causes of significant mortality and mobility, as well as economic loss in the poultry industry. Cellulitis, peritonitis, swollen head syndrome, airsacculitis, coli granuloma, omphalitis, enteritis, pericarditis, and septicemia are all risk factors for colibacillosis, which can lead to chicken death. Colibacillosis was found in 10.1 percent of poultry farms in Pakistan, particularly in Punjab, which has the majority of the country's poultry farms. In contrast, a 2012 study found colibacillosis to be the second most common poultry disease, with a prevalence rate of 18.61 percent (Munawar et al., 2013). Coliphages are bacteriophages that infect *E. coli*. Still no phage-based products for treating colibacillosis in chicken are available on the market. (Żbikowska et al., 2020).

Barrow et al. discovered that a bacteriophage R acquired from human sewage was efficient in preventing and treating cerebritis, meningitis and septicemia in chickens. *E. coli* was administered intramuscularly or intracranially to chickens, whereas phage preparations were administered intramuscularly (in the muscle of right leg). Nearly all of the 3-week-old and newly hatched chickens that were not treated in either way (control group) died. Phages were transmitted into the

brains of chickens that had previously been infected with *E. coli* by the intracranial route. They were able to rapidly reproduce, which led to a decrease in the total number of bacteria. In addition, the aforementioned authors proved that chickens can be protected by bacteriophage even when it is provided one to two days before they are challenged with *E. coli* or during the development of clinical symptoms. This finding suggests that phages can persist in tissues long enough to be employed for the treatment and prevention of colibacillosis. (Barrow et al., 1998)

Huff et al. observed that giving 7-day-old chickens an aerosol spray of bacteriophages before the triple *E. coli* challenge can prevent airsacculitis. In contrast, the aerosol spray of bacteriophages was ineffective route after the challenged with *E. coli.* The efficiency of bacteriophage treatment appears to depend on bacteriophage titers in circulation. Contrary to intramuscular bacteriophage injection, the aerosol spray could have produced only low amounts of bacteriophage in the blood, with just a few chickens exhibiting measurable levels. Further investigation demonstrated that bacteriophage therapy was as effective as enrofloxacin therapy. In addition, when bacteriophage and enrofloxacin were used simultaneously, their synergistic effect enhanced the therapeutic efficacy for colibacillosis. Combining the antibiotic with bacteriophage therapy could minimize the amount of antibiotics used to treat bacterial illnesses. (G. Huff et al., 2009).

Tawakol et al. showed that treating APEC infections with bacteriophages (by injecting them into the trachea) made them less severe and prevented chick's mortality. This was true for both single APEC infections and infections with both APEC and infectious bronchitis virus (IBV). Also, the bacteriophage treatment reduce the number of pathogenic *E. coli* and IBV greatly, in the group with mixed infection, but not in the group with only IBV infections (Tawakol et al., 2019).

A study was done to determine the effectiveness of bacteriophage and the antibiotic enrofloxacin in treating colibacillosis, both individually and in combination. In the birds of the positive control group, *E. coli* (10^4 CFU) were injected into the thoracic air sac when they were 7 days old. The birds were given a challenge, and the antibiotic treatment started immediately. For 7 days, 50 ppm of enrofloxacin was mixed in the drinking water. The bacteriophage treatment consisted of a single injection given intramuscularly of 10^9 PFU of two different bacteriophages right after the *E. coli* challenge. When *E. coli* was given to birds that weren't treated, 68 percent of them died, but bacteriophage and Enrofloxacin significantly decreased that mortality to 15 and 3 percent,

respectively. When both the bacteriophage and Enrofloxacin were given to birds, they were completely protected. This was an example of a significant synergy (W. E. Huff et al., 2004).

The purpose of this research was to examine how well three phages protected against highly virulent strains of *E. coli* in respiratory system of birds. Three lytic coliphages, phage 1, 2 and 3, belonging to the group Myoviridae, Siphoviridae, and Myoviridae were mixed into a 10^7 PFU/ml cocktail to be used in chicks that were naturally infected with APEC. Infected birds were given a single dose of phage 1 at either 10^7 PFU/ml or 10^9 PFU/ml. Phage 1 was administered orally and topically (by spray) in a single treatment. The scores for illness, death, and disease were compared to those of control birds that did not get phage therapy. The results showed that the success of phage therapy is depended on the dose. 10^7 PFU/ml of a single phage was insufficient to treat the infected chickens, but 10^9 PFU/ml of phage was sufficient to reduce mortality by 25% and morbidity by 43%. In the large-scale tests, the results showed that the low titer phage cocktail (10^7 PFU/ml) was very effective at reducing flock deaths to less than 0.5 percent in no more than three weeks, with no recurrence. Based on the results, it was predicted that treating chickens with phage is a good way to stop APEC infections (Oliveira et al., 2010).

Multiple studies have demonstrated that phages may be utilised to treat diseases that present a risk to humans and animals, which justifies their continued usage, particularly in view of rising drug resistance in bacteria and antibiotic limitations. The development of suitable phage preparations may prove to be one of the most successful strategies for combating harmful bacteria in humans and animals in the future, as well as making it feasible to acquire antibiotic-free products.

Despite the fact that a number of studies describe the use of bacteriophages to treat *E. coli* infections in chickens (Wernicki et al., 2017), previous researchers has only examined the outcomes of phage therapies on birds without comparing them to the effects of antibiotics. In our study, experimentally infected chickens were subjected to phage therapy, antibiotic treatment and a combination of two, with results comparing all the treatments.

Chapter 3

Materials & Methods

3. Materials & Methods

3.1 Media used for Bacteria and Phage Culturing:

The medium used for the isolation, enrichment, and plating of bacteria and bacteriophages are listed below. All media dissolved in distilled water and autoclaved before use.

Table 3.1: L-agar (400ml)

Sr. No	Ingredients	Quantity in g/L
1	Yeast extract	2
2	Tryptone	4
3	Sodium chloride	4
4	Agar	6

Table 3.2: L-Broth (400ml)

Sr. No	Ingredients	Quantity in g/L
1	Tryptone	4
2	Yeast extract	2
3	Sodium chloride	4

Table 3.3: Semi-solid Agar (400ml)

Sr. No	Ingredients	Quantity in g/L
1	Tryptone	4
2	Yeast extract	2
3	Sodium chloride	4
4	Agar	3

Table 3.4 EMB Agar (400ml)

Sr. No	Ingredients	Quantity
1	Eosin Methylene blue agar	15g
2	Distilled water	400ml

3.1.1 Preparation of Microbiological media's:

All the powdered ingredients were measured on weighing balance and added to the reagent bottle. The bottle was upto marked with 400ml distilled water, properly labelled, sealed and autoclaved at 121°C. The media was allowed to cool before pouring on autoclaved petri plates and incubated overnight at 37°C to be used the following day.

3.1.2 Solution and Buffer used for phage isolation and propagation

Following solution and buffers were used for isolation and propagation of phages.

Table 3.5: Phage Buffer (TM buffer)

Sr. No.	Ingredients	Quantity in ml/L
1	5M NaCl	3
2	1M Tris-Cl (pH=7.4)	4
3	1M $MgSO_4$	1
4	0.1M $CaCl_2$	1

3.1.3 Equipment used during the study

Equipments used during the study are enlisted below

Table 3.6: List of Equipments

Equipment	**Manufacturer**
Refrigerator	Caravell
Shaking incubator	JSR
Tabletop Balance	ShiMADZu
Hot Plate	Velp-Scientifica
Centrifuge Machine	Hermle
pH meter	WTW inoLab
Laminar Flow cabinet	Esco
Microcentrifuge	Sigma
Vortex Mixer	Heidolph
Spectrophotometer	Optima
Incubator	Memmert

3.1.4 Chemical Preparations:

Different chemicals were prepared as working solution from the stock solutions to use during animal trials.

- **Normal Saline:**

Normal saline is prepared by mixing 9g of NaCl (Sigma Aldrich, USA) per 1000ml of autoclaved distilled water. This is 0.9% NaCl solution.

- **10% formalin solution:**

27ml of Formaldehyde (37%) (Sigma Aldrich, USA) were diluted with 73ml autoclaved distilled water to make 100ml (10%) formalin solution.

3.2 Methods

3.2.1 Bacterial Strain:

Virulent and multi-drug resistant strain of *E. coli*, APECO1 was used to isolate its specific bacteriophages.

3.2.2 Isolation, Enrichment and Purification of Phages

3.2.2.1 Sample collection:

Chicken bedding material was collected in sterilized tubes from different sheds of a poultry farm located in Rawalpindi. Samples were processed for isolating bacteriophages.

3.2.2.2 Isolation of bacteriophages:

To isolate the phages, 20g of chicken bedding material was suspended in 40ml of 1/4th strength Ringer's buffer and incubated overnight in a shaking incubator to release the bacteriophages from faecal samples. The next day, suspensions were centrifuged at 6000rpm for 10 mins, and the supernatants were filtered with 0.22µm filters to ensure sterility. The filtrate contain bacteriophages. The isolated phage was named as PBM-3.

3.2.2.3 Enrichment of bacteriophages:

An overnight APEC O1 culture (1ml) and 1 ml of phage solution were added to approximately 40ml LB media and placed in shaking incubator at 37°C. After a period of 24 hrs, the flask was taken out from the incubator and 500µl of chloroform (final concentration of 1%) was added to eliminate bacterial cells. Some phages may get inactivated by chloroform, i.e. their lytic potential may be altered. T-phages are, however, unaffected. Let the bacterial debris settle down at the bottom of flask for 15-30 mins, at room temperature. Poured the suspension into falcons and centrifuged for 10 mins at 6000rpm to get rid of bacterial debris. Supernatant was taken with the help of sterile syringes and filter sterilized, first with 0.45µm and then with 0.22µm to further purify the suspension which is now called as phage lysate.

3.2.2.4 Assessment of lytic activity of phage by spot assay:

To determine the lytic potential of phages, plaque assay was performed using soft agar overlay method.

Take a pure colony of APEC O1, with the help of wire loop, from nutrient agar plate and culture in ~30ml LB broth in a flask. The flask was kept in shaking incubator at 120rpm and 37°C for growth. After overnight incubation, 100µl of bacterial culture was mixed with 3-4ml semisolid LB agar (LB agar with 0.6% agar) in a test tube and was poured onto LB agar plates to make a bacterial lawn. The plates were incubated at room temperature for 3-5 mins. 5µl of phage lysates were spotted on bacterial lawn, spots were air-dried followed by incubation of plates at 37°C for overnight.

3.2.2.5 Purification of bacteriophages:

To purify the phages, 1ml of exponential phase bacterial culture (APEC O1) was pipetted in 30 ml LB broth in 100ml flask. The area of phage spot was pricked with a sterile tip, then agar containing the phage was sucked on. The mixture containing bacteria, LB broth and phage was kept at 37°C at 120 rpm in shaking incubator for overnight growth.

After an overnight incubation, 1% chloroform was applied to remove bacterial debris, followed by 20 mins of centrifugation at 6000rpm. Supernatant was taken, poured into new falcon tube and was filter sterilized; first by 0.45µm and then later on by 0.22µm filters. The whole procedure was repeated 6 to 7 times to have a pure phage lysate.

3.2.3 Characterization of APEC O1 specific bacteriophage, PBM-3

3.2.3.1 Plaque Assay to determine pfu/ml:

Overnight culture of APEC O1 (1 ml) was cultured in 40ml LB broth and Optical density (OD_{600}) was measured using spectrophotometer. When it reached 0.5-0.7, culture was removed from shaking incubator.

Phages were serially diluted ten-folds (10^{-1} to 10^{-10}) by adding 100µl phage into 900µl TM buffer or LB in eppendorf tubes. Took 100µl of diluted phage and 100µl of exponentially growing bacteria and added to the falcon tubes followed by incubation at 37°C for 15-20 minutes so that the phage adhered to its host appropriately. After that, 3-4 ml of semisolid agar (at 50-60ºC) was added in the incubated falcon tubes, mixed and poured immediately on N-agar plates. The plates

were swirled a bit so that molten agar spread out evenly and allowed to solidify. Plates were placed in the incubator at 37°C for 24 hrs. Next day, plaques observed on the APEC lawn, indicating the lysis of bacteria, were counted and the pfu/ml of phage lysates were calculated by following formula:

Pfu/ml = No. of plaques/ Dilution factor × Volume of virus added to plate

3.2.3.2 Thermal and pH stability assay:

Exponential phase of APEC O1 was obtained by pipetting 1ml of overnight bacterial culture in 30ml of LB broth for 3-4 hrs until 0.8 OD is reached when measured through spectrophotometer. To measure the thermal stability of phages, known concentration
(2.4×10^{12} pfu/ml) of 1ml fresh phage lysates were mixed with 1 ml of LB broth (pH=7) and incubated at different temperatures including 4°C, 25°C, 37°C, 45°C, 60°C, and 70°C temperatures for 60 minutes. After 1 hr treatment, the falcon tubes were cooled down, 100 µl of phage lysate was taken and was serially diluted in TM Buffer or LB. Phage titers were calculated by double layer agar assay to enumerate the surviving phages.
To measure the pH stability of the lytic phages, LB media with different pH (3, 5, 7, 9, 11, 13) was prepared. 1ml fresh phage lysate was mixed in 1ml of different LB broth having different pH values and incubated at 37ºC for 60 mins. pH of LB broth was adjusted using 1M HCl and 1M NaOH solution prior to autoclaving. After 1 hr treatment of phages at different pH, 100 µl of phage lysates were serially diluted in TM buffer/LB and their titers were calculated by double layer agar assay.

3.2.3.3 Phage adsorption Studies:

Kim's study provided the protocol for phage adsorption investigations (Kim et al., 2019). Exponentially growing host culture (1.2×10^{8} CFU/ml) in LB broth was inoculated with phage lysate at ratio of 0.1 MOI. 100 µl of suspension was taken out at 0, 3, 6, 9, 12, 15 and 20 minutes, diluted in 900 µl ice cold TM buffer (phage buffer), centrifuged at 12000rpm, 4ºC for 5 minutes. Supernatant was transferred into the new tube and phage titer was measured using a double layer agar assay. Phage titer at 0 min was called as original titer (To). Rate of phage adsorption to APEC O1 was calculated by T/To.

3.2.3.4 One-step growth curve:

Following Kim's method, a single-step growth curve was evaluated (Kim et al., 2019) and Peng & Yuan (Peng & Yuan, 2018). 10 ml of exponentially growing host bacteria (1.2×10^8 CFU/ml) was injected with phage at a concentration of 0.1 multiplicity of infection (MOI) and incubated at 37°C and 120 rpm. Phage was allowed to absorb to host cells for the adsorption time determined above. After, the suspension was centrifuged at 6000rpm for 30 minutes. To eliminate unadsorbed free phages, the supernatant was discarded, and the phage-infected pellet was suspended in 10 ml of preheated LB broth. The mixture was incubated at 37°C and 120 rpm, 100µl aliquots were taken at 10 min interval for 1.5 hr. Phage titers were determined immediately by double layer agar assay.

3.2.4 Preparation of Phage Cocktail

One phage, PBM-3, characterized in this study and two already characterized phage's, PBM-1 and PBM-2 were used to made phage cocktail. The phage cocktail was made by mixing together each purified phage solution in equal volume.

3.2.4.1 Spot Assay & Plaque Assay:

The phage cocktail was subjected to spot assay and plaque assay on APECO1 lawn. It follows the same procedure as spot assay and plaque assay outlined above.

3.2.5 Comparative analysis of Phage Cocktail with Individual Phages

3.2.5.1 Phage Reduction Assay:

To assess the cell lysis kinetics of phage PBM-3, exponential phase bacterial cultures were infected with the phage at various multiplicities of infection (MOI). 1ml of overnight APEC O1 culture was inoculated in 20ml LB broth, placed in shaking incubator. The culture was grown till the bacteria is in exponential phase, having 10^8 CFU/ml. At this point, 100µl bacterial culture was mixed with 100µl phage at 0.01, 0.1, 1 and 10 MOIs and kept in shaking incubator at 37°C and 120rpm. OD_{600} was measured after every 1 hr interval up to 8 hrs (Chen et al., 2018).

3.2.5.2 Host Range Analysis:

Lytic activity of PBM-3 was determined against 14 bacterial strains by spot assay. Bacterial test strains of APEC were incubated in LB broth at 37°C. Following an overnight incubation, 3-4ml of semi-solid agar were combined with 100 µl of tested strains before being poured onto L-agar plates to make a double-layer agar medium. 5 µl of phage suspension PBM-3 was spotted onto L-agar plates containing the APECO1 strains. At 37°C, the plates were kept in an incubator overnight. Interaction between phages and tested bacterial strains was confirmed by presence of clear spots on the site of application of phage drop onto L-agar plates (Jasim et al., 2018).

Tested bacterial strains against which phages showed no lytic activity were scored as negative. Depending on spot morphology, results observed were differentiated into 3 groups: (+) clear spots and (-) for no spots.

3.3 *In vivo* testing of Phage Cocktail in Chickens

3.3.1 Ethical Statement:

Ethic statement was taken in the form of written consent from institutional review board (IRB) of National University of Sciences and Technology (NUST), Islamabad. The research was conducted in Animal house lab of Atta-ur-Rahman School of Applied biosciences (ASAB), NUST, Islamabad.

3.3.2 Animal Trials:

The goal of the present study was to test the preventive and therapeutic efficacy of phage cocktail in the treatment of mild and severe colibacillosis in chicken. New born, specific pathogen free (SPF) broiler chicks were purchased from the hatchery in Rawalpindi and kept under controlled temperature (27-30°C) and relative humidity (40-60%) conditions. The chicks were fed organic soybean meal and had unlimited access to water. No antibiotic based feed or supplementation was provided.

3.3.3 Grouping of Chicks:

We received 100 one-day-old chicks that were divided into 15 groups. There were 10 treatment groups, each with seven birds and 5 control groups, each with five birds.

Groups were labelled as

1. Negative control **(NC)** – Unchallenged and Untreated Birds - Given saline only
2. Positive Control **(PC)** – Challenged but Untreated - Injected with APECO1 o`nly, no treatment was given
3. Preventative Therapy **(PT)** – Treated & Challenged, Cocktail was injected before APECO1 infection
4. A: **(SC 0hr)** – Challenged & Treated. Treated via subcutaneous injections at 0hr
   B: **(SC 24hrs)** – Challenged & Treated. Treated via subcutaneous injections after 24hrs
5. A: **(IM 0hrs)** – Challenged & Treated. Treated via intramuscular injections at 0hr
   B: **(IM 24hrs)** – Challenged & Treated. Treated via intramuscular injections after 24hrs
6. A: **(ND 0hrs)** – Challenged & Treated. Treated via Nasal Drops at 0hr
   B: **(ND 24hrs)** – Challenged & Treated. Treated via Nasal Drops after 24hrs
7. Antibiotic Therapy **(AT):** Antibiotic was given immediately after challenge culture
8. A: Combination Therapy **(CT via water)**: Antibiotic and phage cocktail both injected in water
   B: Combination Therapy **(CT via injection)**: Phage cocktail was injected while an antibiotic was given in water.

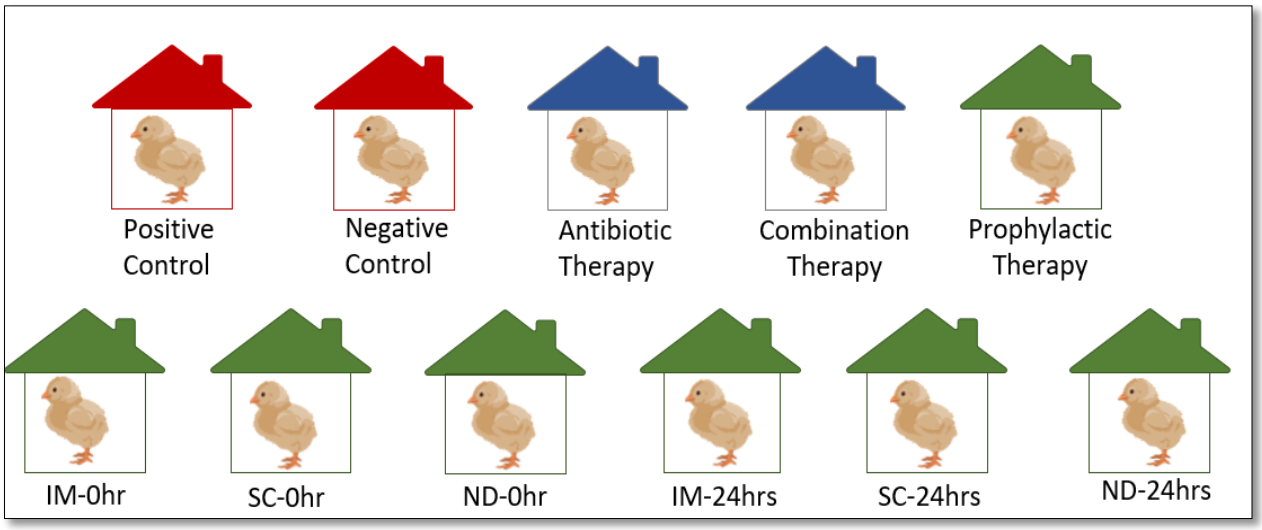


Figure 3.1: Grouping of chicken, 100 chicks were divided into 15 groups

3.3.4 Dose Calculations

APEC O1 was used to initiate symptoms of colibacillosis. After infection, cocktail and antibiotic (Enrofloxacin) was given as therapeutics. The dose of cocktail and antibiotic was calculated on the basis of body weight and feed intake.

3.3.5 Preparation of APEC O1 challenge culture:

To establish infection, the calculated dose of APEC O1 was given. To induce mild and moderate infection, two different APEC strains were employed. The bacterial strain was cultured in fresh LB broth to get a final concentration of 10^8 CFU/ml. 100µl APEC O1 culture were injected subcutaneously to induce the infection.

3.3.6 Cocktail Dose:

Fresh phage isolates were used to prepare phage cocktail and stored at 4°C. The cocktail dose was calculated by using the following formula:

$$\text{Required dose for 1 animal} = \text{Weight (kg)} \times \text{Optimized dose } (\frac{\text{mg}}{\text{ml}})$$

After mild infection, when chicks were two weeks-old, 140µl cocktail was given. After severe infection, when chicks were three-weeks old, 300µl cocktail was given.

3.3.7 Antibiotic Dose:

Enrofloxacin is generally used to treat bacterial infections in poultry chicks. Commercially available Enrofloxacin at a concentration of 200mg/kg was obtained from an authentic source. The dose required for 1 chick was calculated by using the formula:

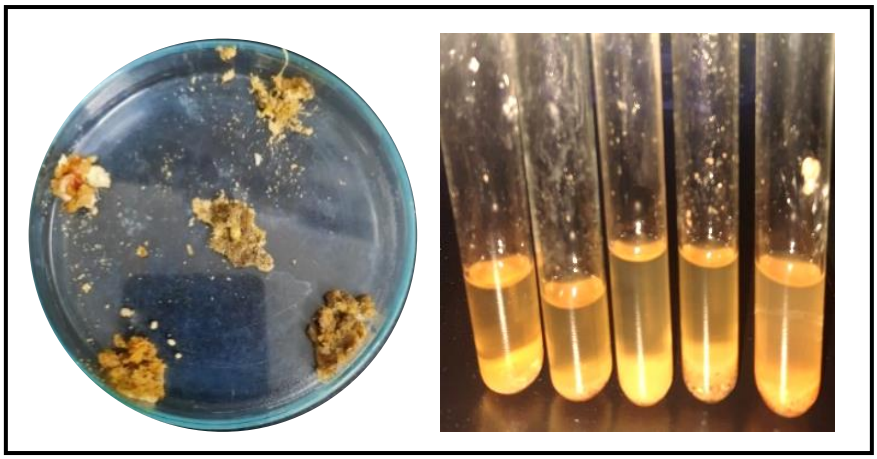
$$C_1V_1 = C_2V_2$$

3.3.8 Faecal Sample Testing for *E. coli* and its related Phages:

Before the experiments began, faeces samples from birds were collected. Individual samples were suspended in LB broth in falcon tubes. The samples were cultured overnight at 37°C in a shaking incubator, and streaking on EMB the following day. After 24hrs, plates were observed for green metallic sheen colonies which is a distinctive feature of *E. coli* growth on EMB.

The incubated faecal samples were also tested for APEC O1 related phages. For this, fecal samples were emulsified in LB broth and inoculated in a 3-4 hr grown culture of the APEC O1. The falcons were incubated overnight at 37°C while being shaken (120 rpm). The samples were centrifuged at

6000rpm for 15 minutes, filtered through 0.2µm filters and suspension was used for spot assay on APEC O1 lawn on LB agar plate, followed by incubation of plates at 37°C. Next day, plates were observed for the clear spots of phages (Oliveira et al., n.d.).



A B

Figure 3.2: (A) Fecal sample from the bedding material of chicken
(B) Fecal sample incubation in LB broth for 24hrs

3.3.9 Study Plan:

The most widely described means for administering poultry vaccinations, antibodies, and other formulations have been subcutaneous or intramuscular injections. After filling injection with inoculum of APEC O1 strain, the dose was injected in the chicks of respective groups. Chickens were taken out one by one and was given injection of 0.5 ml of the culture at a concentration of 10^8 CFU/ml in the skin or midway between head and body in the pectoral muscle of chicks. Two doses were given. The 7-day-old chicks were given a mild dose of APEC O1 through an injection subcutaneously. The lethal dose of APEC O1 was given to 14-day-old chicks.

The effect of preventative cocktail administration was assessed by administering the cocktail one day before giving APEC O1 infection.

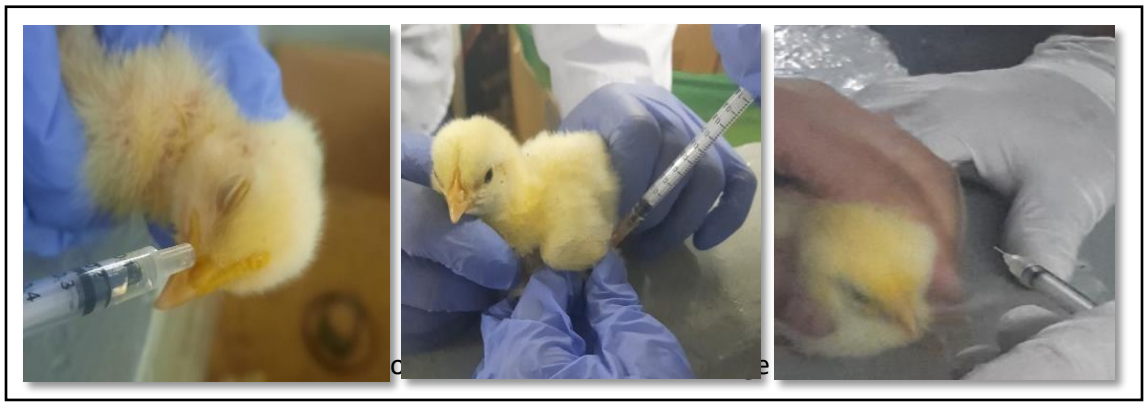
Therapeutic Cocktail Dose was given through three different administration routes, subcutaneous injections, intramuscular injections and nasal drops. Subcutaneous and intramuscular injections were given in the thigh of left leg of chick. Intramuscular injections are given deep in the muscles while subcutaneous are given on the skin. Nasal drops were administered with great care by

pouring three drops sequentially in the chicks' nostrils while holding their heads horizontally until the drop was absorbed (Montoto, 2018). From a total of 6 therapeutic cocktail groups, three groups were given dose immediately however, the other three groups received dose after an interval of 24hrs, next day after infection.

In the case of combination therapy, one group received both treatments by water, whereas the other received antibiotic in water while cocktail through subcutaneous injection. The same amount of the cocktail was added to the water as was injected into each bird. For a group of 7 birds, the cocktail was calculated accordingly.

3.3.10 Three Different Phage Therapy Routes:

The bacterial challenge was given on head region via subcutaneous injections. For the treatment with phage cocktail, three different administration routes were tested. i: **Subcutaneous injections** given on the head of the chicks, ii: **Intramuscular injection** given in the muscle of the thigh region, iii: **Nasal drops** administered in liquid form in the nasal cavity of the birds. While the antibiotic was added directly to the drinking water.



A: Nasal Drop **B**: Intra-muscular Injections **C**: Subcutaneous Injections

3.3.11 Feed preparation:

The chickens were given organic feed with vitamins and trace element supplements, but no antibiotics were used. Water was provided from tap. Both were given ad libitum.

3.3.12 Body Weight Evaluation:

Throughout the study, the birds were weighed early morning each day. To avoid cross-contamination, the weighing process began with the Control groups. After disinfecting the digital weighing scale with ethanol, the experimental birds were weighed. Every bird in each group was weighed on a daily basis.

3.3.13 Clinical signs and Mortalities:

Each day, all chicks were clinically assessed and monitored for any health-related issues. The chicks were observed for the following clinical signs:

- Fever
- Head down and isolated from the rest of group members
- Watery stool
- Inactive and lethargic
- Less water and feed intake

3.3.14 Dissection

Whole procedure of dissection was performed by taking into account the international guidelines (Guide for the Care and Use of Laboratory Animals. (8th Ed)., 2011; IACUC guidelines, 2019).

Any chick that died was immediately dissected and organs were stored. However, at the end of the study, alive chicks were euthanized using chloroform and then dissected.

For dissection, the chick was laid in ventral position, a few centimeter-long incision was made at the front of the neck by angling scissors 45 degrees towards the neck. Hold the skin tightly with forceps and cut through it in the forward direction through the abdomen. The upper skin is connected to the underlying tissue through connective tissue. Fold the skin and cut the underlying connective tissues with scalpel. Once the skin is sliced, the trachea, oesophagus, crop, and breast are visible. Make a cut at the lower end of the breast where it is connected to the rest of the body. Cut open the breast and observe underlying heart, liver, spleen and intestine condition. Hold the heart upward with the help of forceps and incise all the surrounding tissues until it comes out. Cut the esophagus located at the cranial of liver. Likewise cut all the connective tissues surrounding the liver and take it out. A healthy liver has sharp edges and a strong texture. A spotty liver with a

thick fibrinous layer around it show that it is infected with *E. coli*. If the liver is mushy, it is because it has been working hard. As lungs are delicate organs protected by rib cage, so to cut them out one need to cut the rib cage. The bones of young chicks are fragile so they are cut with the scissor, hold the lungs in forceps and cut their attachment to the bones. The spleen is exposed once the liver is removed, carefully cut the colon at the distal end and remove the entire intestines. Swollen, dark red/black coloured intestines are indicative of infection.

Figure 3.4: Images taken during the dissection of the chicks

Whole procedure of dissection was performed by taking into account the international guidelines (Guide for the Care and Use of Laboratory Animals. (8th Ed), 2011; IACUC guidelines, 2019).

3.3.15 Gross Pathology:

Chicken organs were observed for airsacculitis (air sacs inflammation), perihepatitis (inflammation of liver), pericarditis (heart inflammation) and enteritis (inflammation of intestines). A total of 80 chicken were necropsied and their organs were examined for gross pathology scoring.

A healthy liver with **no damage** is scored as 0. When lesions had just started to appear and thin fibrinous layer on various locations are observed, it is **mild damage** scored as 1. Thin layer of fibrinous exudate covering the whole organ is **moderate damage** with a score of 2. Thick and extensive layer of fibrinous exudate around the entire organ is a sign of **severe damage** and scored

as 3. All birds in each group were individually scored. The total score of one group was calculated by multiplying the score with the total number of birds having a specific pathological score. Then the final score for gross pathological changes in the flock was determined by dividing the overall score by the total number of birds(Antão et al., 2008) .

3.3.16 Preservation of Organs:

Retrieved organs were immediately preserved in 10% formalin. The falcon tubes were carefully labelled and stored at 4°C. The samples were sent for histopathology after the completion of trials.

3.3.17 Histopathology Analysis:

To further understand the impact of infection and treatment on the organ damage at tissue level, retrieved organs were subjected to histopathology. The section of liver, heart and lungs were collected and fixed by immersion in 10% neutral buffered formalin, dried in methanol, cleaned in xylene, trimmed, and embedded in paraffin. Hematoxylin and eosin (H&E) were used to stain 5µm sections for histopathological investigation under an optical microscope with a camera at magnifications of 10X and 40X.

3.3.18 Histopathological Analysis:

Gross pathology examination generally predicts the severity of disease at organ level. However, damage at tissue level is studied by histopathology. The microscopic results are studied in-depth to observe all the changes. The tissue damage is categorized into three stages based on these histopathological changes.

3.3.19 Degree of Tissue Damage:

By adapting the degree of tissue change (DTC) approach described by (Poleksić & MitrovićTutundžić, 1994), the severity of histopathological changes in the liver, lung, spleen, kidney, and heart of affected birds was assessed. For DTC analysis, the alterations in each organ were categorised according to consecutive stages of tissue damage: Stage I: Alterations that do not compromise normal tissue function Stage II: Changes that are more severe and impair normal tissue function Stage III: Extremely severe changes that inflict permanent damage.

Every organ was observed under microscope for each of these pathological changes which are categorized as following.

Degree I: Cloudy swelling, mottled greyish areas, Diffused enlargement

Degree II: Cellular Infiltration, Vascular congestion, Subserous congestion, Oedema

Degree III: Necrosis, Dilated sinusoids with leukocytes, Epithelial Necrosis

The following formula was used to determine the total amount of histopathological alterations that occurred in stages I, II, and III of the degree of tissue changes for each organ in each chicken:

$$\boldsymbol{DTC} = (\mathbf{1}\ \boldsymbol{x}\ \sum \boldsymbol{I}) + (\mathbf{10}\ \boldsymbol{x}\ \sum \boldsymbol{II}) + (\mathbf{100}\ \boldsymbol{x}\ \sum \boldsymbol{III})$$

Table 3.7: Scoring of histopathological changes in Liver, Heart and Lungs

Stage of DTC	Scoring	Description of Microscopic Lesions
		Liver
I	Mild	Congestion of blood vessels; odema in hepatic parenchyma; Hemorrhage in hepatic parenchyma; Sinusoidal fibrin thrombin
II	Moderate	Vascular congestion, cellular infiltration
III	Severe	Necrosis, Dilated sinusoids with leukocytes
		Heart
I	Mild	Cloudy swelling, Mottled greyish area, Diffused enlargement
II	Moderate	Vascular congestion, Subserous congestion
III	Severe	Epithelial Necrosis, oedema
		Lungs
I	Mild	Cloudy swelling, Fibrinous Degeneration
II	Moderate	Localized Inflammatory Cell Infiltration and Hemorrhage
III	Severe	Diffuse Alveolar And Interstitial Edema, Inflammatory Cell Infiltration, Hemorrhage, And Necrosis

DTC values ranging from 0 to 10 indicate normal organ function; values ranging from 11 to 20 indicate minor organ damage; values ranging from 21 to 50 indicate significant organ changes; values ranging from 50 to 100 indicate serious lesions; and values exceeding 100 indicate irreversible organ damage.

Degree of tissue change values were categorized as:

- Mild organ damage (1– 21)
- Moderate organ damage (22 – 50)
- Severe organ damage (≥ 51) (Camargo & Martinez, 2007)

3.3.20 Strain Confirmation analysis:

For the confirmation of *E. coli* in the infected organs, cotton swabs were used to streak from the liver, heart and lungs on EMB plates. The plates were incubated at 37 °C for 24hr and observed next day.

3.3.21 Statistical analysis:

The differences in the mean bodyweight, water intake, food consumption and lesion scores were calculated and statistical analysis including one way analysis of variance (ANOVA) were performed using GraphPad Prism.

Chapter 4

Results

4. Results

4.1 Phage characterization

4.1.1 Phage Titer & Plaque Morphology Determination:

The phage was isolated from the bedding material of the chickens by suspending the material in Ringer' buffer, followed by centrifugation and filtration through 0.22µm filters. The isolated phage was enriched and spotted on APEC O1 lawn. The phage gave a clear spot on APEC O1 lawn, this inhibition zone indicates lytic activity of phage. It was named as PBM-3.

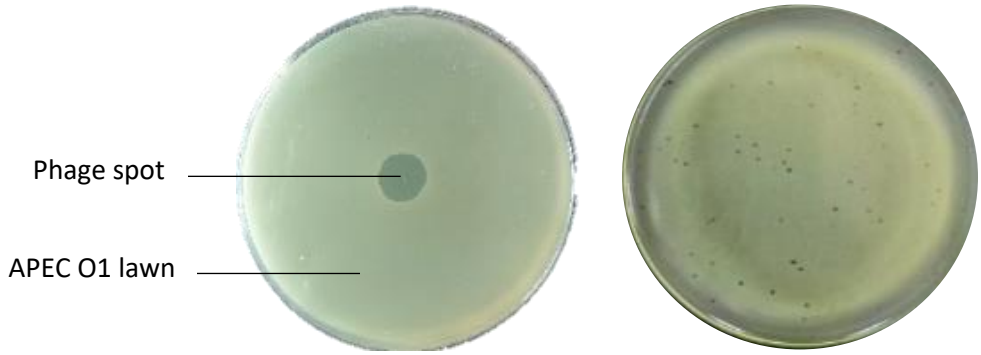


Figure 4.1: (A) Spot Assay of phage PBM-3 **(B)** PBM-3 Plaques appearing as transparent dots on APECO1 lawn

The observed plaques (Fig 4.1: B) were round, transparent and less than 1mm in size. Titer is manually calculated by counting the number of plaques. The titer of PBM-3 was 1.37×10^{10} pfu/ml.

4.1.2 Effect of temperature on the survival ability of phage PBM-3:

Thermal tolerance of the phage was studied by double layer agar assay by calculating phage titer at different temperatures. PBM-3 was thermally stable upto 45°C however, one-hr treatment at higher temperatures (60°C and 70°C) significantly reduced the activity ($p < 0.0001$).

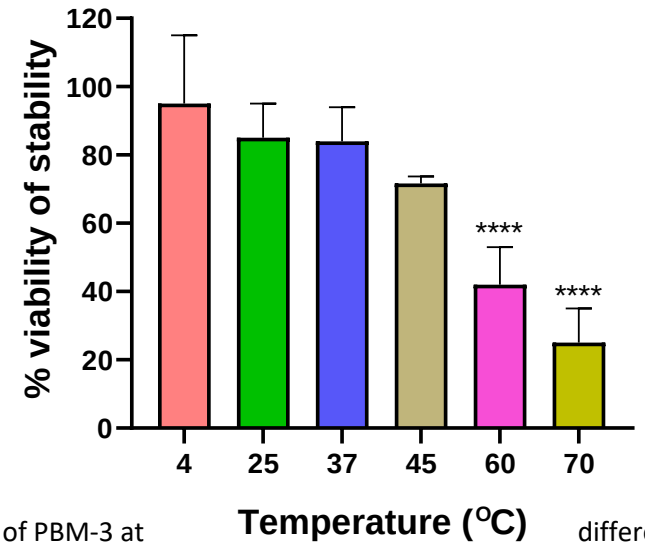


Figure 4.2: Stability of PBM-3 at different temperatures. Results are the mean values with SD indicated by error bars from three independent experiments

4.1.3 Effect of pH on the survival ability of phage PBM-3:

Viability of PBM-3 under different pH was evaluated by calculating phage titer by double layer agar assay.

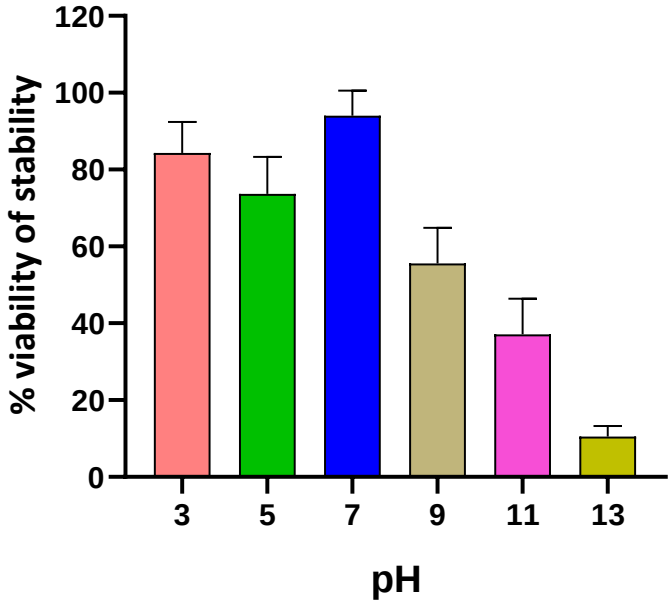


Figure 4.3: PBM-3 survival at different pH. Phages was incubated at different pH for one hr. Ear bar represents mean value with standard deviation as error bars from three independent replicates

PBM-3 was viable at acidic pH. However, at alkaline pH, viability was significantly ($p < 0.0001$) reduced to 37% and 10% at pH 11 & 13 respectively.

4.1.4 Phage adsorption assay:

Adsorption assay gives an estimation of time required by phage in encountering and attacking the host. It was observed that PBM-3 efficiently adsorbed to APECO1 host cells in comparatively short time.

Rate of phage adsorption was calculated by T/T_o (T = phage titer at different time intervals, T_o = phage titer at 0 time interval). Over 90% adsorption of PBM-3 was observed in 9 minutes.

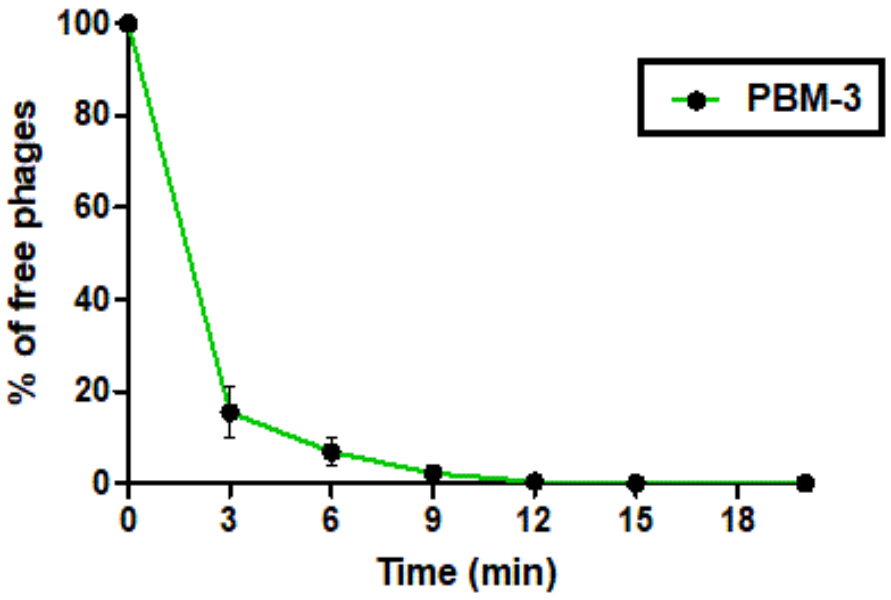


Figure 4.4: Rate of adsorption of PBM-3 to APEC-O1. At 0.1 MOI, the percentage of unadsorbed/free phages was calculated at different time intervals by T/T0×100. Results are the mean values with SD indicated by error bars.

4.1.5 One Step Growth Curve Analysis:

In order to understand the life cycle of a virus on a specific host, one-step growth curves are used. By tracking virus infection cycle, growth curve is constructed, and the burst size can be estimated. Average free phages before plateau A were used to determine burst size. By subtracting A from the average of free phages after plateau (B), C was determined. C denotes total burst/new phages produced. Burst size was calculated by dividing C with infecting phages (total number of phages at T_0- free phages at T_0). It was observed that PBM-3 has latent period of 20 mins and burst size of 92 per cell.

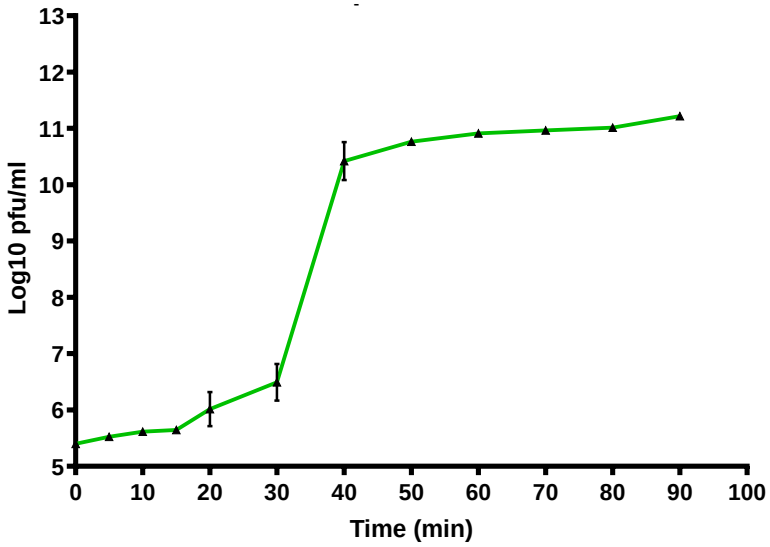


Figure 4.5: One-step growth curve of phage PBM-3. The results presented here are mean values with standard deviation from three independent experiments.

4.2 Phage Cocktail Preparation & Plaque Forming Unit (PFU) Calculation

Two phages (PBM-1 & PBM-2) already characterized by Saleha Masood (member of Anti-bacter) and one phage characterize in this study (PBM-3) was mixed in equal volume to prepare phage cocktail. The cocktail titer was determined to be 1.47×10^{12}.

4.2.1 Comparative analysis of Phage Cocktail & Individual Phages:

Combination of phages are reported to be more efficient with broader host range and delayed resistance as compared to individual phages. Therefore, host range and bacterial resistance towards cocktail were determined in comparison to individual phages.

4.2.2 Coverage Rate:

14 randomly sampled APEC isolates from the fecal material of chicken were collected from different pens of one farm. 5µl of bacteriophage cocktail suspension was spotted on the surface of APEC lawn and plates were incubated at 37°C for 24 hrs. Next day, lysis zone was observed.

Table 4.1: Host Range of PBM-3 against 14 Bacterial Strains:
Sign "+" represents clear plaques "-" represents no plaques

	P1	P2	P3	CT
38E	+	+	+	+
43E	+	-	-	+
46E	-	-	-	+
48E	-	-	-	-
50E	+	+	+	+
53E	-	-	-	-
57E	-	-	-	-
61E	+	-	+	+
65E	-	-	-	-
80E	+	+	+	+
81E	-	-	-	-
87E	+	+	+	+
92E	+	+	+	+
95E	-	-	-	-

Coverage rate of cocktail was measured using the formula:

$$\text{Coverage Rate} = \frac{\text{Number of bacterial isolates lysed by the phage cocktail}}{\text{Total number of bacterial isolates treated with the phage cocktail}} \times 100$$

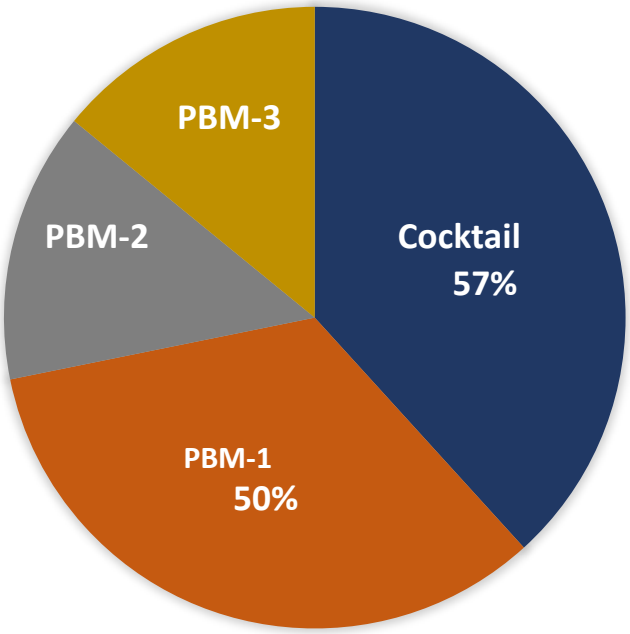


Figure 4.6: Comparison of Coverage Rate of Cocktail & Phages (PBM-1, PBM-2, PBM-3)

4.2.3 Growth Reduction Assay:

APEC-O1 when not inoculated with phages show a continuous increase in optical density OD_{600} upto 8 hrs. However, by co-incubating the bacterial strain with phages at different concentrations (0.01, 0.1, 1, 10), the phages significantly reduced the bacterial growth and as a result OD_{600} decreases. PBM-3 at all MOI reduced the bacterial growth for 5 hrs; after that OD begins to increase which shows the development of resistant strains. PBM-3 begin to show resistance in between 5-20hrs.

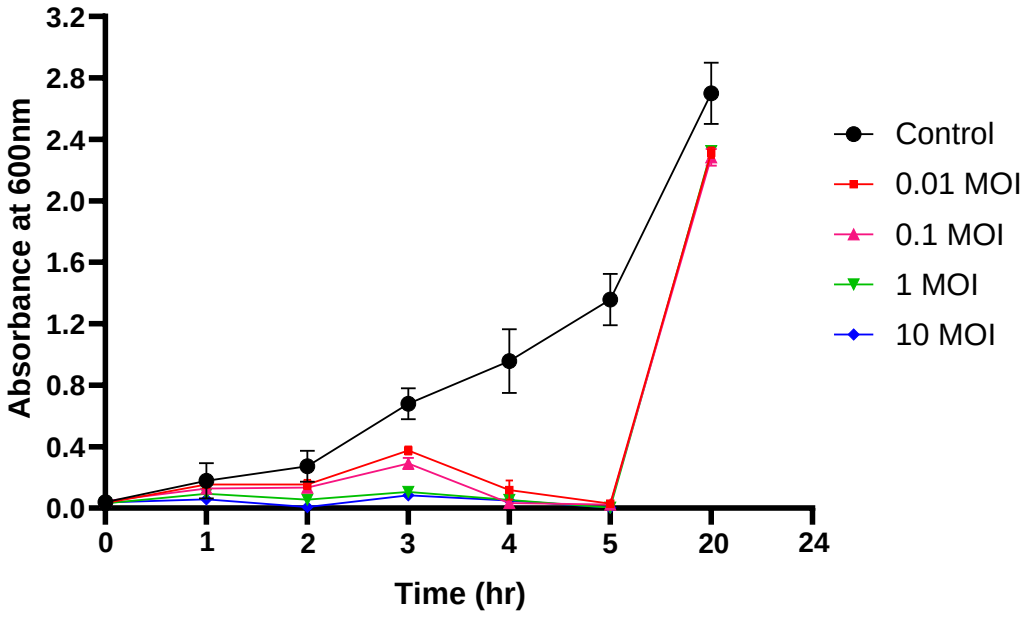


Figure 4.7: Planktonic cell lysis kinetics of **phage PBM-3** at an MOI of 0.01, 0.1, 1 & 10. Control represents APEC-O1 only (not incubated with phages)

The cocktail was also able to reduce bacterial growth for first 5 hrs however, the strains become resistant in between 5-24hrs.

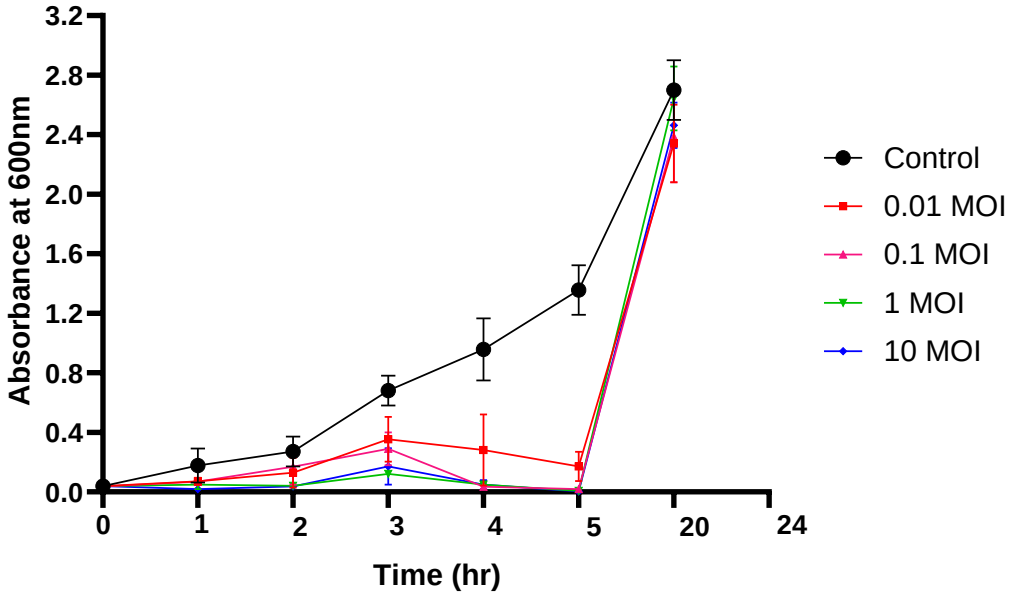
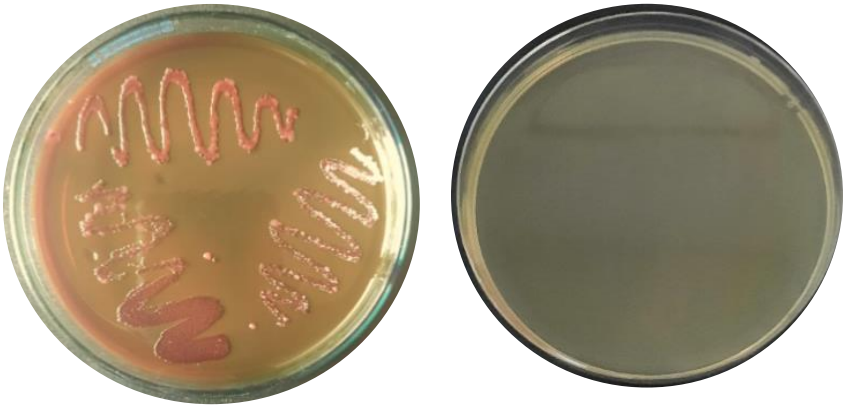


Figure 4.8: Planktonic Cell lysis kinetics of cocktail at an MOI of 0.01, 0.1, 1 & 10. Control represents APEC-O1 only (not incubated with phages)

4.3 Therapeutic & Prophylactic Efficacy Evaluation of Phage Cocktail in Chicken Models

4.3.1 Fecal Sample Testing for *E. coli* and related Phages:

Prior to start chicken infection with *E. coli* and treatment with phages, the fecal material was tested to check the presence of any *E. coli* and related phages. The fecal material after overnight incubation was streaked on EMB plate and spotted on APECO1 lawn for phages. However, green metallic shine colonies, characteristic of *E. coli* colonies, were not observed on EMB plate, indicating absence of *E. coli*. Likewise no spot were observed on APECO1 lawn indicating absence of phages.



A B

Figure 4.9: (A) Fecal samples streaked on EMB **(B)** Phage spot assay of fecal samples on APECO1 lawn

4.3.2 Clinical Signs & Symptoms:

All the groups were daily checked for clinical signs and mortalities. Fever, lethargicity, isolation, dullness, diarrhea, reduced feed consumption, wheezing sound during breathing and ruffled feathers were observed in diseased chicks. In treated groups, few of these symptoms were observed however, all the chicks of the positive control showed most of these symptoms.

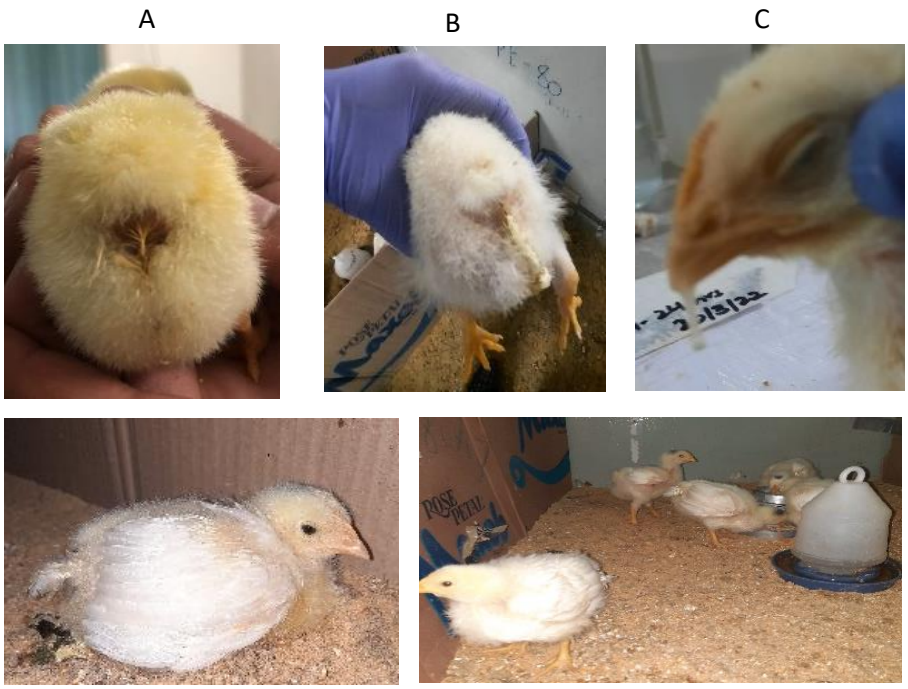


Figure 4.10: Birds showing symptoms of **(A)(B)** Diarrhea, **(C)** Nasal discharge **(D)** Ruffled feathers **(E)** isolation

4.3.3 Mortalities Evaluation:

Chickens were divided into 9 treatment groups and 5 control groups. When chicks were infected with mild pathogenic strain of APEC-O1 (on day 7), no mortality was observed till day 14. When chicks were infected with highly pathogenic strain of APEC-O1 (on day 14), 6 out of 7 mortalities were observed in the positive control on day 15. However, in other control groups, no mortalities were observed.

Chicks in three treatment groups including Prophylactic phage therapy, Antibiotic therapy and Combination therapy showed no mortality till day 21. In all the remaining treatment groups, mortalities were observed each day.

Table 4.2: Daily Death Record (Day 14 - Day 21). Each day mortalities were observed and recorded

Day	14-Mar	14th Mar	15th Mar	16th Mar	17-Mar	18-Mar	19-Mar	20-Mar	Total deaths
Time	3:00pm	3:30pm	Tue, 9:00am	Wed, 9:00am	Thu, 9:00am	Fri, 9:00am	Sat, 9:00am	Sun, 9:00am	
	Injections	Treatment							
Positive Control	APECO1	Nill	6 dead, 1 alive	1 alive & sick					6/7 deaths
Negative Control	Saline	Nill	All Alive & healthy						0/7 deaths
Phage Therapy IM	APECO1	Phage Cocktail	All Alive	All Alive	All alive	1 dead, 6 alive	6 alive	1 dead, 5 alive	2/7 deaths
Phage Therapy SC	APECO1	Phage Cocktail	1 dead, 5 alive	1 dead, 4 alive	1 dead, 3 alive	1 dead, 2 alive	2 alive	2 alive	3/7 deaths
Phage Therapy Nasal	APECO1	Phage Cocktail	2 dead, 5 alive	5 alive	5 alive	5 alive	5 alive	5 alive	2/7 deaths
Prophylactic Treatment	Phage Cocktail	APEC O1	All Alive						0/7 deaths
Antibiotic therapy	APECO1	Enrofloxacin	All Alive but organ damage was seen						0/7 deaths
Combination Therapy	APECO1	CT+Enrofloxacin	All Alive					1 sick	0/7 deaths
Phage Therapy IM	APEC O1	Phage Cocktail	All Alive	1 dead, 6 alive	1 dead, 5 alive	5 alive but 1 sick		5 alive, 1 sick	2/7 deaths
Phage Therapy SC	APEC O1	Phage Cocktail	2 dead, 5 alive	1 dead, 4 alive	2 dead, 2 alive	2 alive		2 alive	5/7 deaths
Phage Therapy Nasal	APEC O1	Phage Cocktail	4 dead, 3 alive	3 alive	1 dead, 2 Alive	2 alive		1 dead, 1 Alive	6/7 deaths

Among the 6 groups administered cocktail treatment via different routes, Intramuscular injection proved to be the most effective with the survival rate of 66.7% for both the injection at 0 hrs and

24 hrs. The survival percentage in the subcutaneous group at 0 hr was 57%, while for the group given subcutaneous injection 24 hrs after the administration of challenge was reduced to 28.57% when compared to the 14.28% survival in the positive control (challenged but untreated group).

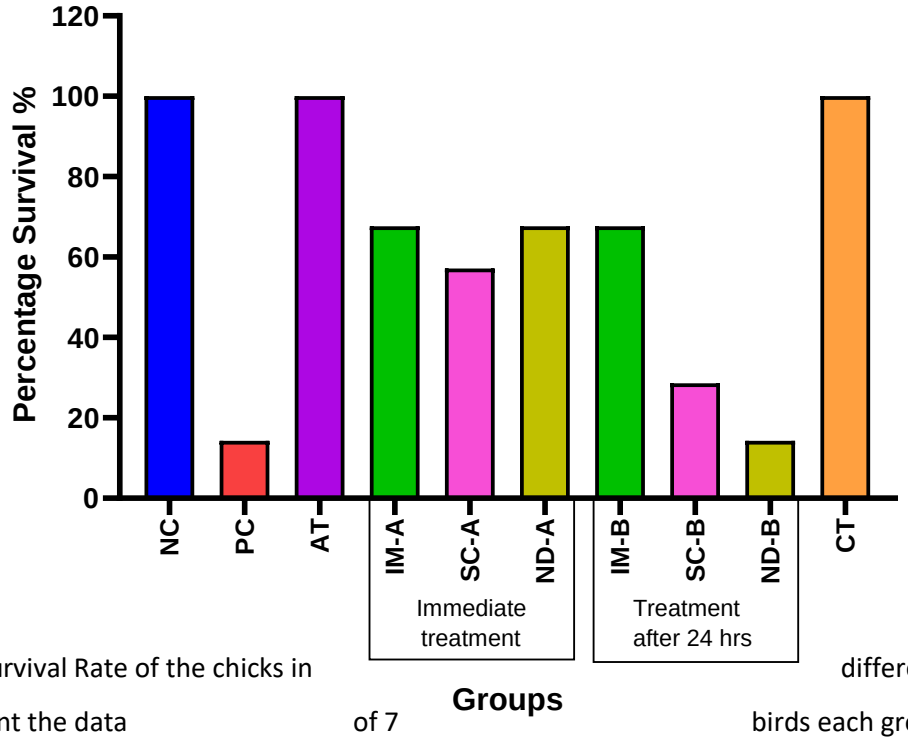


Figure 4.11: Survival Rate of the chicks in different groups. Values represent the data of 7 birds each group.

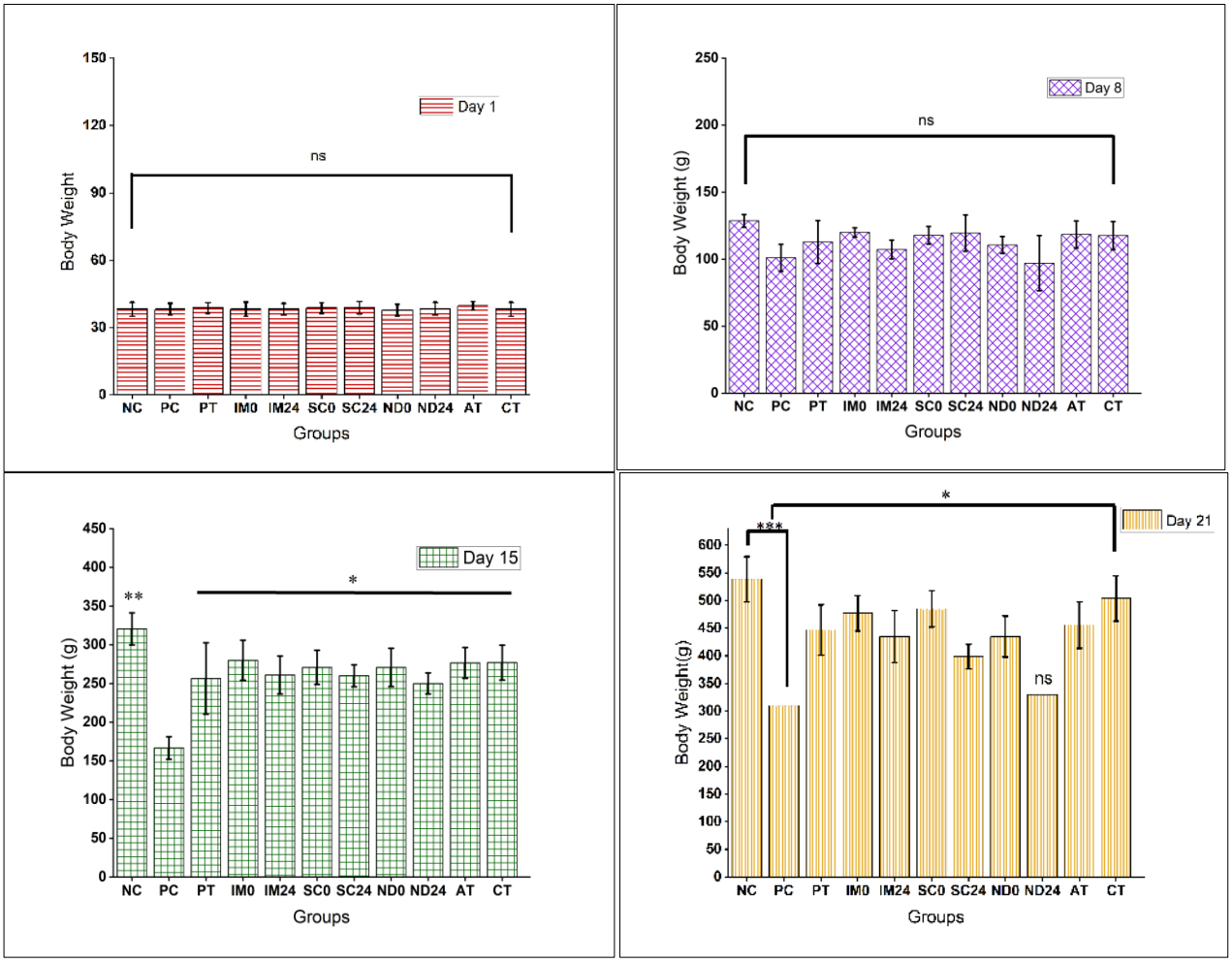
Nasal drops administered immediately following administration of challenge has the survival rate of 67.7% but when administered to a group at the 24 hrs i.e., one day mark did not manage to increase the survival even slightly and it was observed to be 14.28% which was the same as the positive control.

4.3.4 Body Weight Evaluation:

All birds had almost equal body weight when they were received on day 1. On day 8, immediate day after 1st dose administration (mild APEC O1 infection), slight non-significant

(p value > 0.05) weight loss was observed in positive control. The treatment groups also did not show significant body weight reduction.

A B



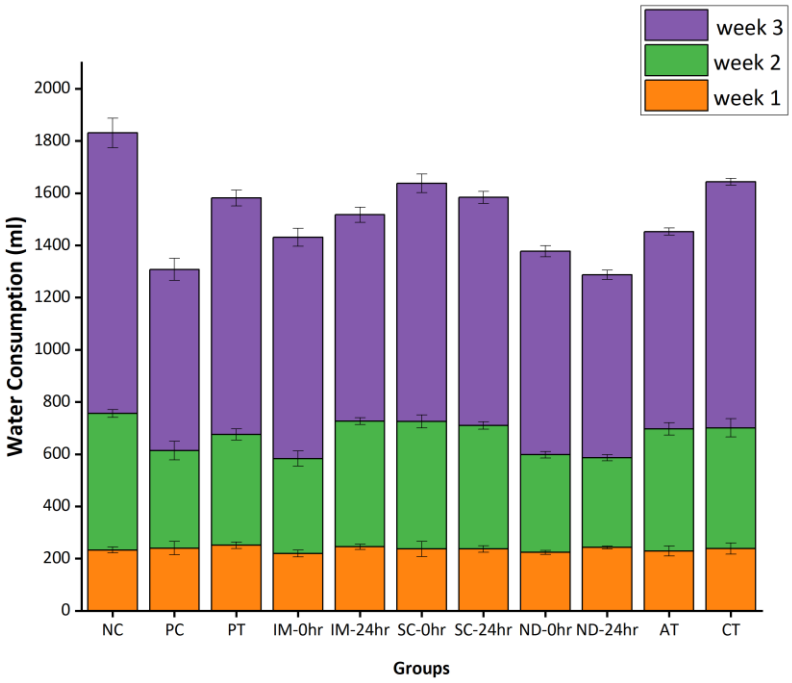
C D

Figure 4.12: Body weight data on different days. **A (Day 1)** One day old chicks of roughly equal weight were received. **B (Day 8):** Immediate day after mild APEC O1 infection, non-significant reduction in body weight was observed. **C (Day 15):** Immediate day after severe APEC O1 infection, significant reduction in the body weight of the positive control was observed as compared to treatment groups. **D (Day 21):** The same trend continues till day 21.

On day 15, immediate day after 2nd dose administration (severe APEC-O1 infection), a significant reduction in the body weight of positive control and treatment groups was observed. However, the treatment groups were able to restore body weight loss with each passing day unlike the positive control (infected but untreated). So, on day 21, the body weight of all the treated groups (except Nasal drops-24hrs) was significantly higher than untreated positive control.

4.3.5 Water Consumption:

The most crucial nutrient that an animal consumes is water. Birds can go weeks without food but only days without water. Water consumption is co-related with feed consumption which is directly linked with feed conversion ratio; an essential metric for calculating productivity rate.



NC: Negative Control
PC: Positive Control
PT: Prophylactic Treatment
IM: Intra-muscular
SC: Subcutaneous
ND: Nasal Drops
AT: Antibiotic Therapy
CT: Combination Therapy

Figure 4.13: Week-wise water consumption (ml) of the chicks. **Week 1** (orange color) represents data of acclimatization period when chicks were kept under normal conditions. **Week 2** (Green color) represents mild infection and treatment. **Week 3** (purple color) represents severe infection and treatment.

Figure 4.13 shows weekly water consumption pattern of chickens in each group. Each bar is a three week data of one group water consumption. As expected water intake increased over days in uninfected, untreated negative control group but intake become erratic after mild and severe infection in the positive control in the week 2 and 3 respectively. However, notice that the water consumption of prophylactic phage treatment, subcutaneous therapeutic phage treatment and combination therapy had best improved water consumption in week 2 and 3. Other treatments improved the consumption to some extent only with least improvement in the nasal treated groups.

Daily water intake (data not shown) reveals abrupt decrease in water consumption after infection which gradually increased with each passing day. Such inferences are difficult to made in week-wise data however, less water consumption in antibiotic therapy suggest the positive role of phage therapy in improving water consumption in combination therapy.

4.3.6 Feed intake:

All of the treatments affected the feed intake and the growth rate relative to those of the control group. Each bar represents data of one group and each color represents one week. When chicks were infected with *E. coli*, feed consumption was reduced significantly in the positive control.

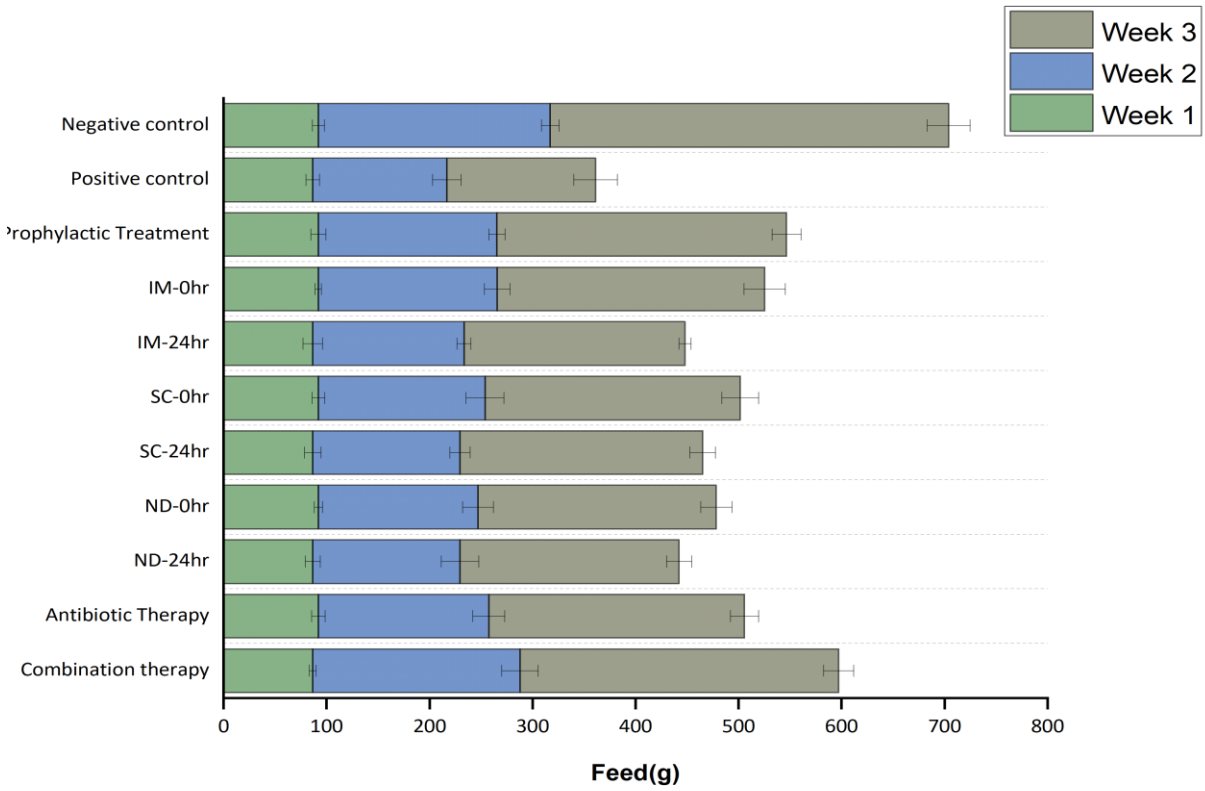


Figure 4.14: Week-wise feed consumption data of chicks. **Week 1** (green color) represents feed intake during acclimatization period. **Week 2** (Blue Color) represents feed intake after mild infection while **week 3** (Grey color) represents feed intake after severe infection.

The feed intake of the positive control from week 2 to week 3 increased to 11% while to 59.27% in combination therapy, 62.4% in prophylactic treatment, 80.13% in antibiotic treatment, 50.17% in IM-0hr, 53.31% in SC-0hr and 49% in ND-0hr. The compromised feed intake in the positive

control is associated with lesions which causes the damage of epithelium layer of organs ultimately decreasing nutrient absorption.

In conclusion, chicks that were challenged and treated with either antibiotics, phages (Prophylactic treatment only) or a combination of both, presented better feed consumption than those that were not treated.

4.3.7 Comparative Analysis of Feed Conversion Ratio (FCR):

The feed conversion ratio (FCR) measures how successfully a flock converts feed intake into live weight. It is calculated by the following formula:

FCR = Total feed consumed (kg) / weight of the chicks (kg)

Low FCR is better as it depicts that feed consumed is efficiently converted into weight. In this study, lowest FCR was observed in negative control and highest was observed in positive control. Among treatment groups, phage therapy (subcutaneous injections) has the lowest FCR followed by combination therapy.

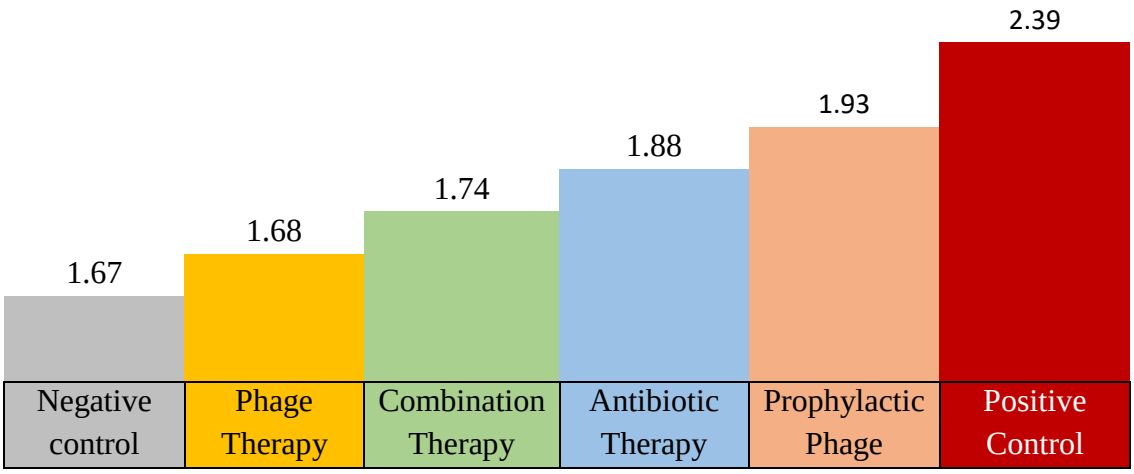


Figure 4.15: Comparative Analysis of FCR between Phage therapy, antibiotic therapy and combination therapy

4.3.8 Dissection & Lesion Scoring:

The dead chicks were dissected for analyzing infection associated organs. Multiple infected organs including liver, heart, lungs, intestines and trachea were studied however, only heart, liver and lungs were retrieved and stored in 10% formalin for lesion scoring and histopathological analysis. After each dissection, the organs were observed and individually scored. Chicks dissected within one week of mild infection show no visible symptoms of disease and no gross lesions on the organs. Therefore, pathomorphological studies were done on the chicks dissected after severe infection.

No pathogenesis was observed in negative control group. However, air sacculitis, perihepatitis and pericarditis were observed in the lungs, liver and heart of the positive control. A lesser degree of damage was observed in the organs of the treated groups.

Pathological changes in the organs were scored from 0-3 based on the severity of lesions. This categorization helped us in predicting the pathological condition of that organ per group for evaluating severity of disease based on organ appearance. The mean score value for each organ was calculated after each dissection.

Figure 4.16: Samples streaked from the surface of the organs on EMB plates. After 24hrs incubation, *E. coli* colonies were observed as Green with a characteristic appearance of metallic sheen

The chicks died immediately of septicemia did not show lesions, however, some of the surviving birds dissected at the end of trials showed thick severe lesions on their surface. The groups with

higher survival percentage had low lesion score while groups with high lesion score exhibited low survival rate. Figure 4.16 showed the samples streaked from the surface of the liver, heart and lungs, retrieved after the dissection of the dead chicken. Characteristics green metallic shine colonies were observed on EMB plates showing the presence of *E. coli* in the organs.

4.3.9 Gross Pathology Examination and scoring of Liver:

The liver of the negative control was normal with no lesions, so it was scored as 0. Most of the chicks in the positive control group died of septicemia the day after they were infected. And therefore, the liver obtained from these chicks had low lesion score. However, the remaining chicks that either died at later stages or dissected at the end of trials had severe damaged liver with high lesion score. The mean ± SD of the liver of the positive control was 2.7143 ± 0.48795 (Table 3) which indicates moderate to severe damage.

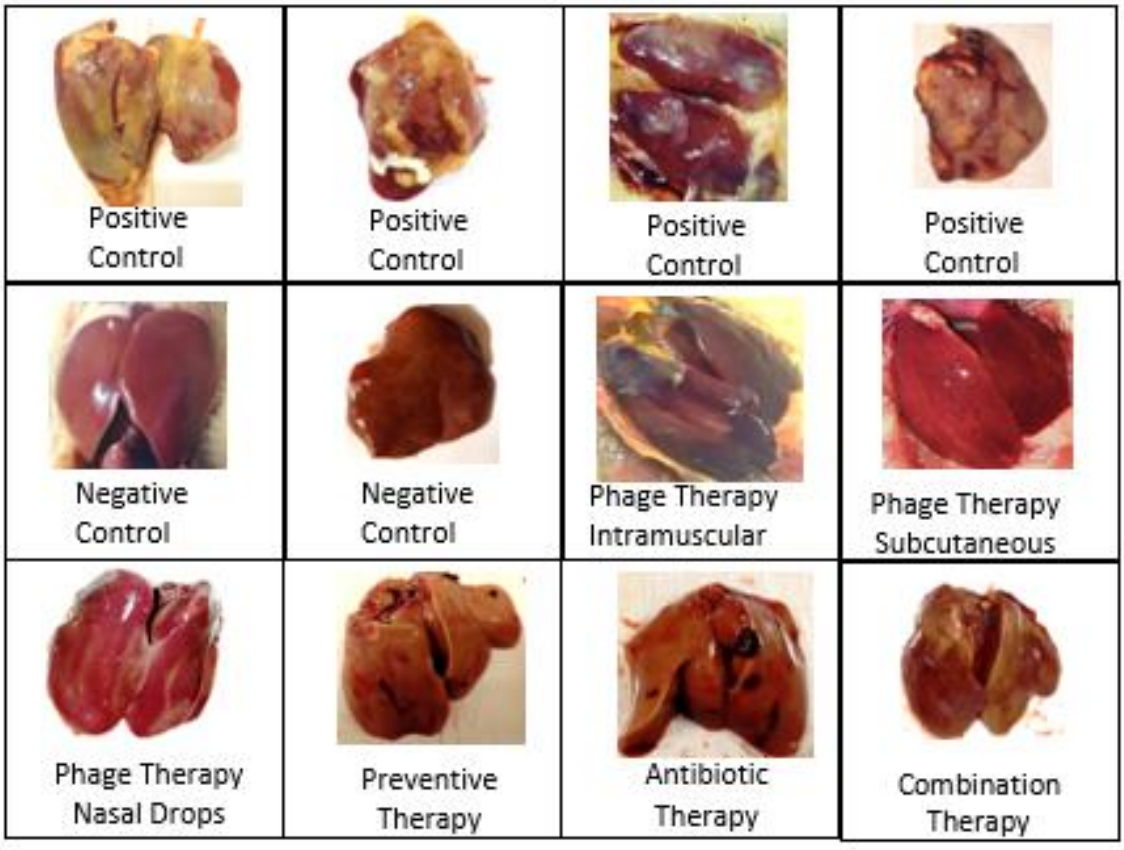


Figure 4.17: The liver of the positive control, negative control and different treatment groups. Normal liver has score 0, mild damage 1, moderate damage 2 while severely damaged liver is given a score of 3.

A similar relation between lesion scoring and survival percentage was observed in Intramuscular, subcutaneous, and nasal drop treated groups. In these groups, immediate mortalities were less and

most of the chicks survived up to 3 or 4 days producing thick lesions and therefore high pathomorphological score specifically in the intramuscular treated group. But both intramuscular treated groups (IM-0hr, IM-24hrs) had high survival percentage as compared to subcutaneous and nasal treated groups.

As no mortality was observed in prophylactic phage, antibiotic and combination therapy groups, their survival percentage was 100% but they also have low mean lesion score.

4.3.10 Gross Pathology Examination and scoring of Heart:

The post mortem examination of the negative control group chicks revealed a normal heart with no macroscopic lesions and was given a score of 0. Contrary to it, positive control, intramuscular and subcutaneous treated groups showed thick to very thick lesions on the heart revealing moderate to severe pericarditis.

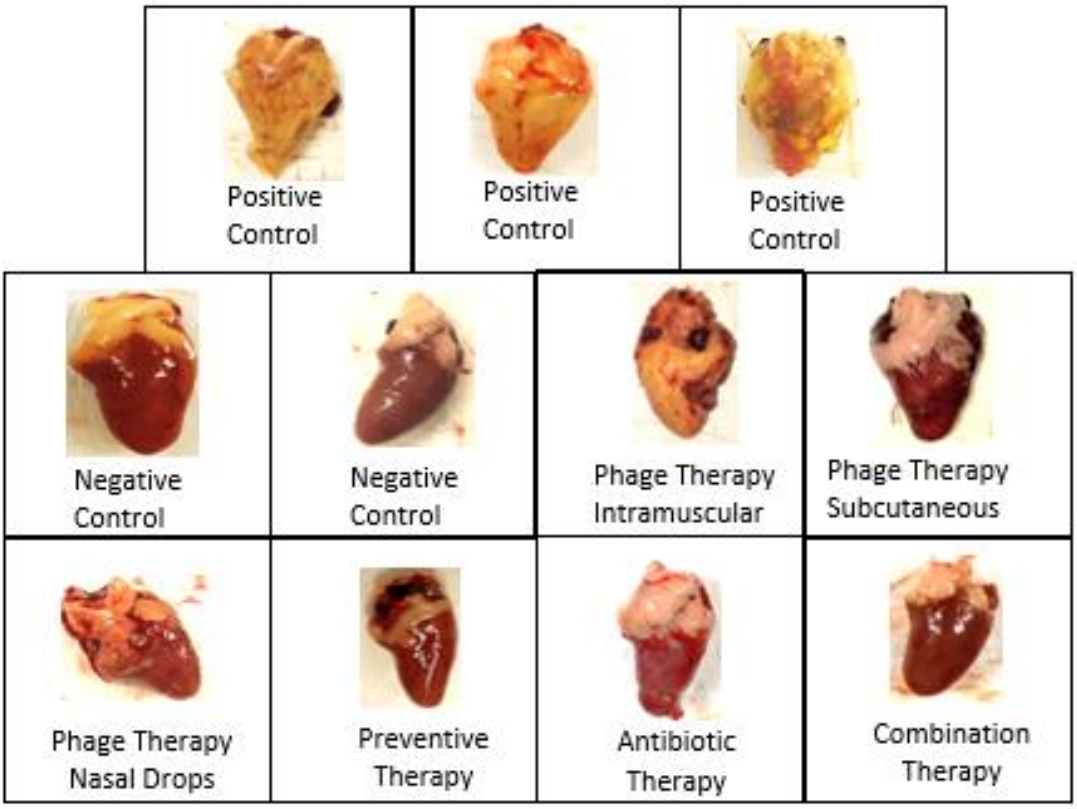


Figure 4.18: Gross Pathological Changes in the Heart. A normal heart with no lesions is given a score of 0, mild damage 1, moderate damage 2 while severely damaged liver is given a score of 3.

The lesions on the heart of prophylactic, antibiotic and combination therapy groups were mild and thin. Gross pathology examination generally predicts the severity of disease at organ level. Based

on this naked-eye examination, it was concluded that prophylactic phage and combination therapy better prevented damage of the heart.

4.3.11 Gross Pathology Examination and Scoring of Lungs:

Lungs of the negative control group show normal appearance. While the infected chicks (positive control) show congestion with traces of fibrin. The color and size of infected lungs differed from the healthy ones. Also the superficial covering become yellow to creamy thick and lungs become dark red to brown in the case of infection. Based on these pathological changes, lungs were scored from 0 to 3. Among treatment groups, the lungs of the prophylactic phage, Nasal drop groups and combination therapy were better protected showing very mild symptoms of disease than other groups.

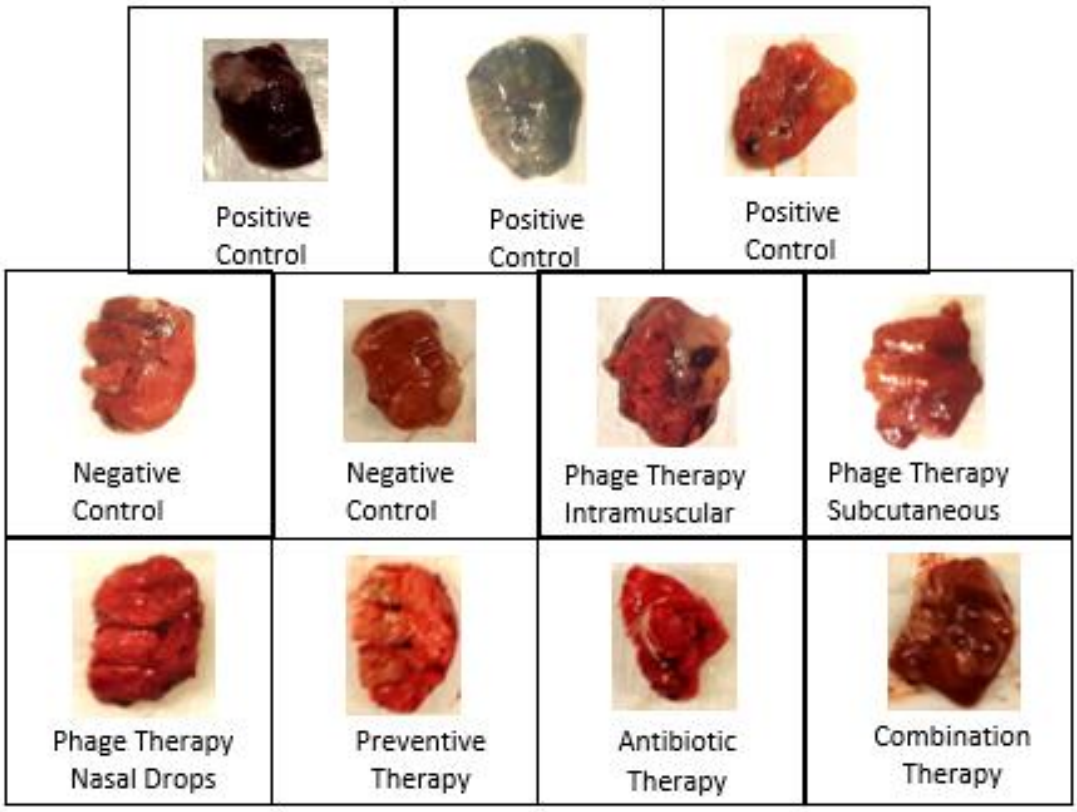


Figure 4.19: Gross pathological changes in the lungs. Negative control has normal lungs. The diseased lungs appear congested with white to yellow exudates covering the surface. Normal liver is scored as 0, mild symptomatic 1, moderate 2 and severely infected as 3.

It was observed that the lungs of the intramuscular treated groups (IM-24hrs) were most effected after positive control (infected but untreated) however, nasal drops in which phages are directly delivered to the air sacs, better protected the lungs.

4.3.12 Mean Scoring of organs based on Gross Pathological Examination:

The mean scoring of heart, liver and lungs of negative control is 0, corresponding to no damage to any organ. The mean scoring ± standard error mean of liver (2.7143 ± 0.48795), heart (2.5714 ± 0.534522) and lungs (2.4286 ± 0.534522) of the infected chicks in the positive control group was interpreted as moderate to severe damage. The mean score of intestine is 1.8929 ± 0.733955 which corresponds to moderate damage.

Table 4.3: The mean ± standard error mean pathomorphological scoring based on disease severity in the liver, heart, lungs and intestine. This shows significant result (P<0.01) of all treatment groups compared to the positive control.

	Groups	Liver	Heart	Lungs	Intestines
Controls	Negative control	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Positive control	2.7143 ± 0.487	2.5714 ± 0.5345	2.4286 ± 0.5345	1.8929 ± 0.7339

Phage Therapy	Prophylactic Treatment	0.1786 ± 0.374	0.6786 ± 0.965	0.4286 ± 0.553	0.3571 ± 0.537
	IM-0hr	1.3929 ± 0.497	1.1429 ± 1.069	0.7143 ± 0.713	0.2917 ± 0.4587
	IM-24hr	1.8571 ± 0.690066	2 ± 0.8164	1.1786 ± 1.0275	0.4286 ± 0.7867
	SC-0hr	1.2857 ± 0.48795	1.1429 ± 0.6900	0.5 ± 0.645497	0.3571 ± 0.626
	SC-24hrs	1.5714 ± 0.5345	1.4286 ± 0.534	0.8214 ± 0.624	0.5 ± 0.866
	ND-0hr	1.4286 ± 0.5345	0.8571 ± 0.8997	0.3571 ± 0.475	0.1429 ± 0.283
	ND-24hrs	1.5714 ± 0.5345	1.4286 ± 0.975	0.4643 ± 0.5850	0.61 ± 0.843
Antibiotic	Antibiotic Therapy	0.4286 ± 0.534	0.7143 ± 0.4879	0.8571 ± 0.690	0.1786 ± 0.374
Combination therapy	Combination Therapy	0.1429 ± 0.3779	0.2857 ± 0.4879	0.2857 ± 0.48795	0.1429 ± 0.377

Among treatment groups, mean scoring ± standard error mean of all the retrieved organs in the prophylactic treatment group is below 1, showing very mild damage. Immediate phage treatment groups (IM-0hr, SC-0hr, ND-0hr) showed mild damage to heart and liver while very little damage to lungs and intestine. Phage treatment after 24hrs (IM-24hrs, SC-24hrs, ND-24hrs) showed moderate damage to heart and liver while mild damage to lungs and intestines. In antibiotic therapy lungs were more effected than heart and liver but the mean scoring come within mild damage range. Combinatorial therapy, combining the effect of phage therapy and antibiotics, has the mean scoring ≤ 0.3, with little damage to all the retrieved organs.

Comparing the extent of damage to various organs, the positive control had severe signs of airsacculitis, pericarditis, and perihepatitis. However, in the therapy groups, more severe symptoms were observed for pericarditis and perihepatitis than air sacculitis.

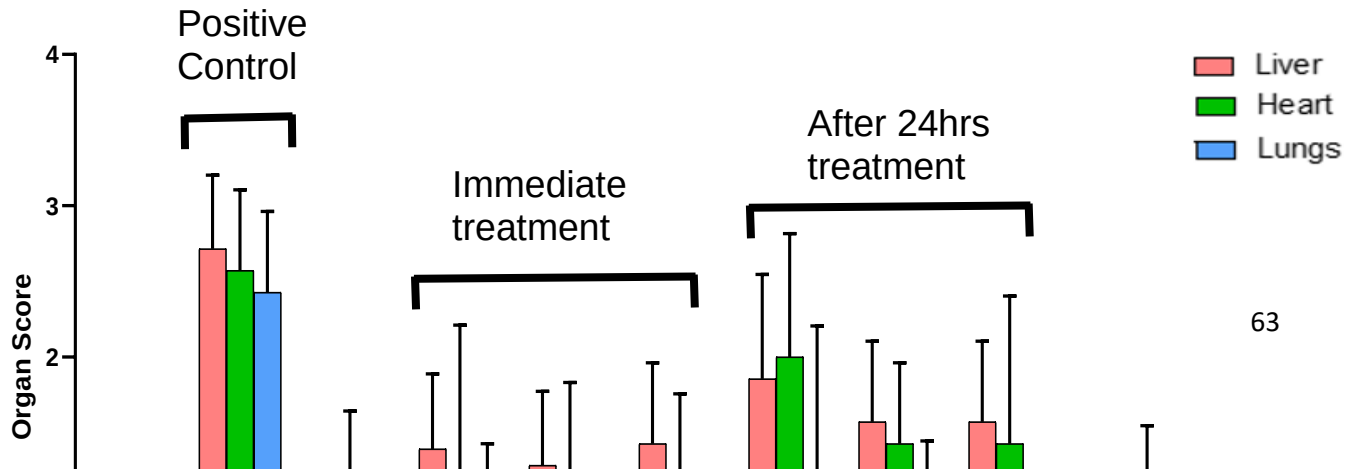


Figure 4.20: Combined lesion score of heart, liver and lungs of all the groups. The groups are NC: Negative Control, PC: Positive Control, PT: Prophylactic Treatment, IM: Intra-muscular, SC: Subcutaneous, ND: Nasal Drops, AT: Antibiotic Therapy, CT: Combination Therapy

Histopathological analysis of the damaged tissues:

After dissection, the infected organs were histopathologically evaluated to analyze the effect of pathogenesis at tissue level. This microscopic observation led to the evaluation of degree of tissue change in damaged tissue of all the dissected organs.

4.3.13 Histopathological changes in the liver:

Microscopic examination revealed that the hepatic parenchyma of chicken is composed of hepatocytes radially organized around a central vein. The liver of the negative control group showed no damage at tissue level when observed at 400X. However, positive control (infected but untreated) showed show severe vascular congestion (VC), cellular infiltration (CI), dilated sinusoid with leukocytes (DS) and necrosis (N). In prophylactic treatment less severe oedema (OD) and cellular infiltration (CI) was seen. Among therapeutic phage treatment groups treated immediately (IM-0hr, SC-0hr, ND-0hr) liver damage was much reduced with symptoms limited to damage of degree I.

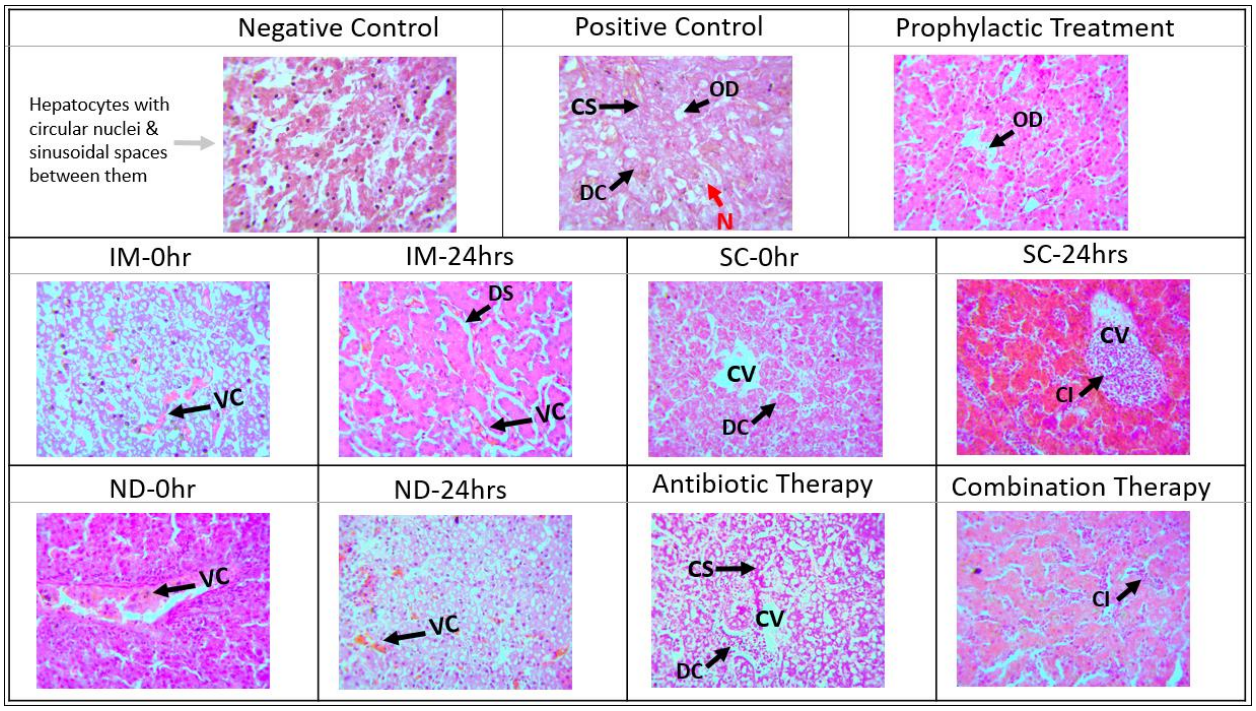


Figure 4.21: Histopathology of the liver tissue of chickens observed at 400X. H & E staining revealed Cellular degeneration (DC), cloudy swelling (CS), Vascular Congestion (VC), Necrosis (N), Oedema (OD) and Cellular Infiltration (CI).

Phage therapy groups, treated after 24hrs of infection (IM-24hrs, SC-24hrs, ND-24hrs) mostly revealed vascular congestion and cellular infiltration. However, in the antibiotic treatment group, degenerated cells and necrosis (degree III damage) was more visible than phage treated groups. The combination therapy better prevented the tissue damage where only cellular infiltration was seen as a result of high immune response.

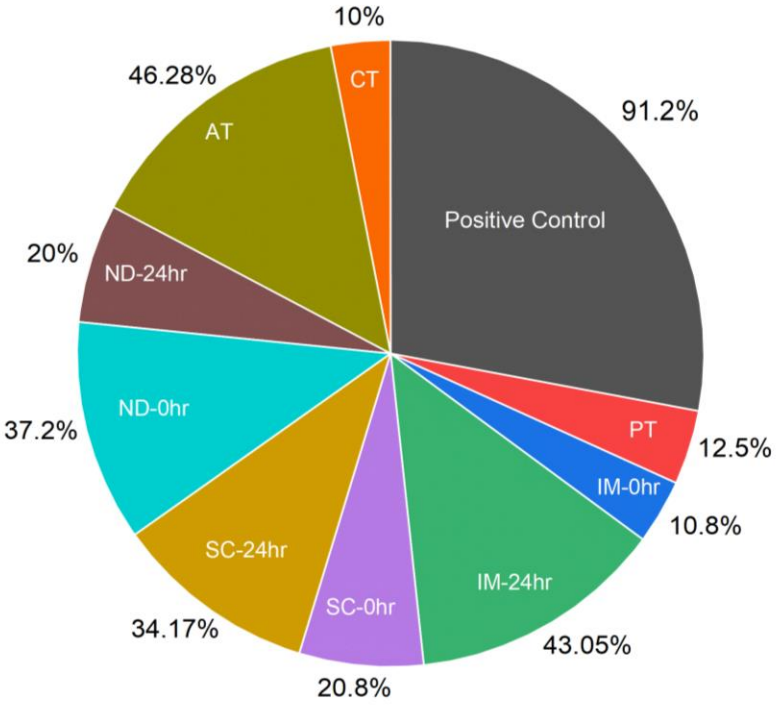
Table 4.4: The degree of tissue damage in Liver with mean ± SEM and level of damage categorized into mild (≤ 21), moderate (≤ 51) and severe (≤ 76).

Groups	DEGREE OF TISSUE DAMAGE	
	Values (mean ± SEM)	Interpretation
Positive Control	91.1 ± 6.819384	Severe Damage
Prophylactic Treatment	12.5 ± 12.144958	Mild Damage
IM-0hr	10.8333 ± 10.206207	Mild Damage
IM-24hrs	43.05 ± 23.212389	Moderate Damage

SC-0hr	20.8333 ± 9.174239	Mild Damage
SC-24hrs	34.1667 ± 31.530409	Moderate Damage
ND-0hr	37.2167 ± 33.55118	Moderate Damage
ND-24hrs	20 ± 18.439089	Mild Damage
Antibiotic Therapy	46.2833 ± 21.356069	Moderate Damage
Combination Therapy	10 ± 7.745967	Mild Damage

The damage at tissue level is observed under microscope at different magnifications, and type of damage is compared with the reference and individually scored. After that, the mean tissue damage is computed and analyzed. The mean ± standard error of the positive control was 91.1 ± 6.819384, interpreted as severe damage which is reduced to moderate damage in three phage therapy groups (IM-24hrs, SC-24hrs, ND-24hrs) and antibiotic treatment. Mild damage to liver was observed in prophylactic phage treatment and Therapeutic phage groups given treatment immediately (IM-0hr, SC-0hr, ND-0hr). The least damage score, however, was 10 ± 7.745967, which was observed in combination therapy.

The percentage damage of the liver of the positive control was 91.2% which has been reduced to 12.5% in prophylactic phage treatment and 46.28% in antibiotic therapy. Therapeutic phage treatment decreased the damage to different levels with minimum reduction in intramuscular treated group (IM-0hr). The least tissue damage was observed in



combination therapy group.

Figure 4.22: Percentage tissue damage in the liver retrieved from different groups.

4.3.14 Histopathological changes in the Heart:

A normal heart of the negative control has well-arranged nucleated muscle fibers with branching of cardiomyocyte. Also, flattened nuclei between myocytes can occasionally be seen. In the positive control (infected but untreated) cloudy swelling (CS) with cellular degeneration (DC), oedema (OD) and necrosis (Degree II & III Tissue damage) were observed. Immune cell infiltration was seen in different treatment groups related to degree I damage. Cloudy swelling (CS) and diffused enlargement were also observed at various locations in the heart histopathology of different treatment groups.

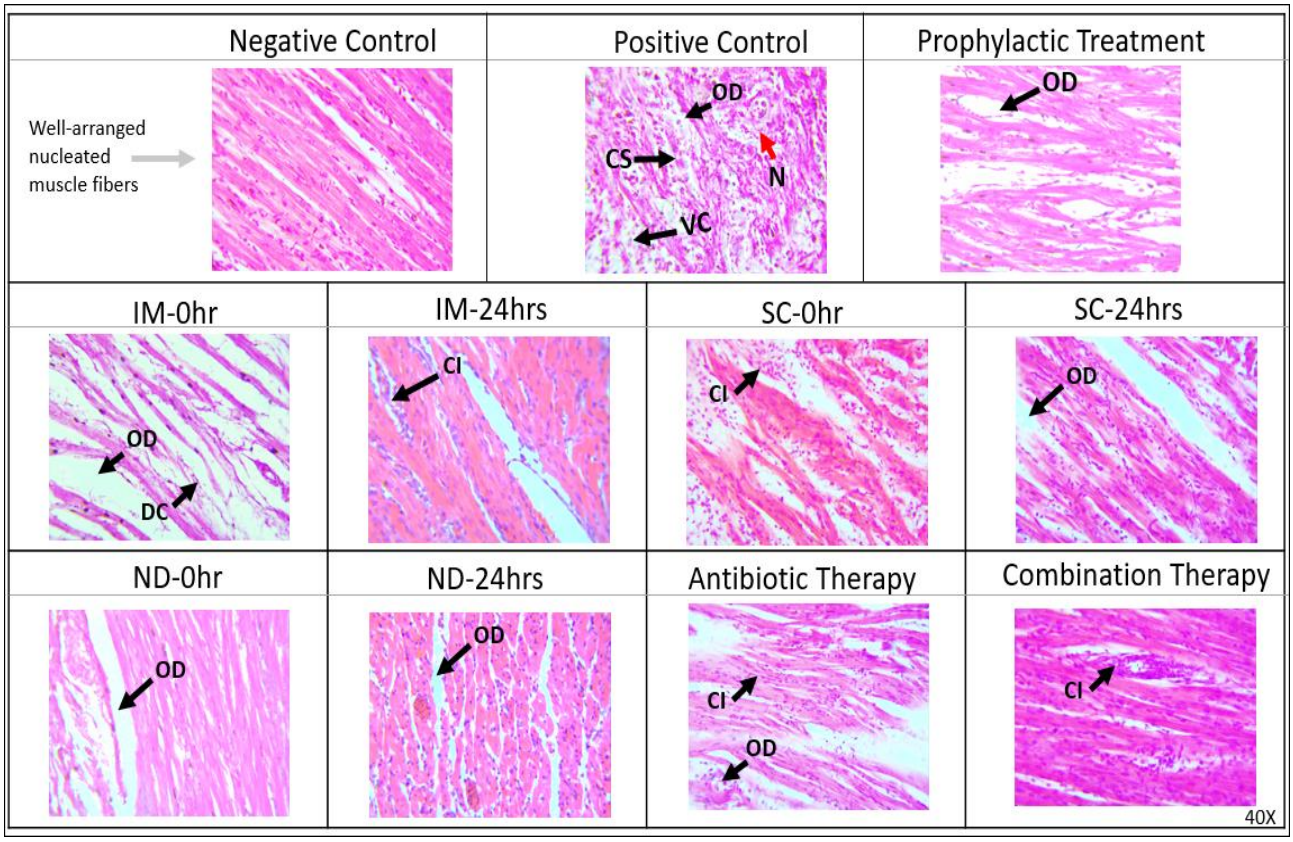


Figure 4.23: Histopathology of the Heart tissue of chickens observed at 400X. H & E staining revealed Cellular degeneration (DC), cloudy swelling (CS), Vascular Congestion (VC), Necrosis (N), Oedema (OD) and Cellular Infiltration (CI).

Each type of damage was individually observed under microscope at 400X and 100X. The damage was compared to the reference and then scored as percentage value. All the percentage damages were summed up and an average mean was calculated. Hence, mean ± SD is enlisted in the table. The mean value is interpreted as mild damage if the value is ≤ 21, as moderate damage if the value is ≤ 51 and as severe damage if the value is ≤ 76.

Percentage damage to the heart of the positive control was 86.1 ± 7.726836 and interpreted as severe damage. Antibiotic therapy, Nasal treatment and subcutaneous group has percentage damage lying within moderate range. And mild damage was observed in Prophylactic treatment, intramuscular treated group and combination therapy.

Table 4.5: The degree of tissue damage in Heart with mean ± SEM and level of damage categorized into mild (≤ 21), moderate (≤ 51) and severe (≤ 76).

Groups	DEGREE OF TISSUE DAMAGE	
	Values (mean ± SEM)	Interpretation
Positive Control	86.1 ± 7.726836	Severe Damage
Prophylactic Treatment	20.3333 ± 18.348479	Mild Damage
IM-0hr	18.05 ± 31.965841	Mild Damage
IM-24hrs	21.6667 ± 16.931233	Mild Damage
SC-0hr	53.6 ± 32.909026	Moderate Damage
SC-24hrs	21.6667 ± 22.060523	Mild Damage
ND-0hr	30.55 ± 30.623765	Moderate Damage
ND-24hrs	32 ± 24.647515	Moderate Damage
Antibiotic Therapy	41.6667 ± 19.407902	Moderate Damage
Combination Therapy	7.5 ± 4.1833	Mild Damage

The heart tissues of the positive control group was damaged above 80%, but comparatively less damage was observed in treatment groups. Lowest degree of organ and tissue damage, among treatment groups, was observed in combination therapy. The comparison between antibiotic therapy and phage treatment groups shows comparatively less tissue damage in the phage treated groups.

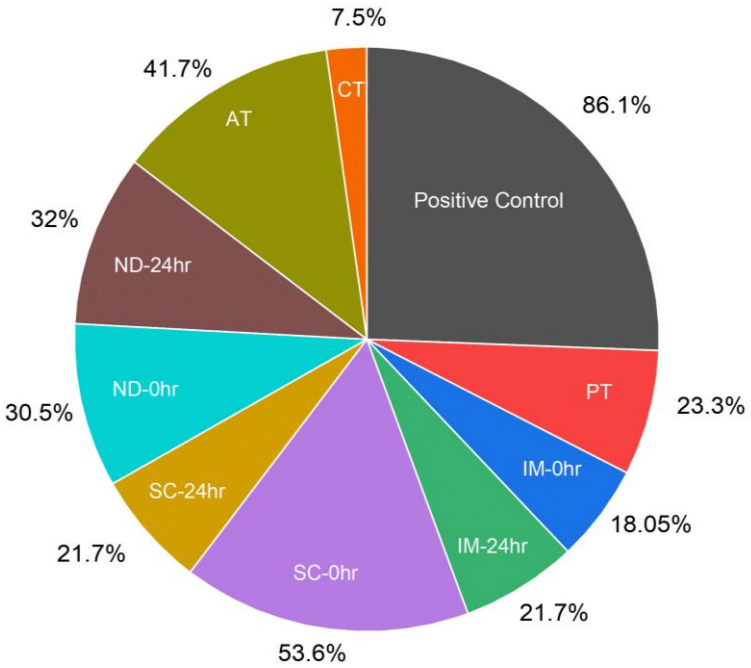


Figure 4.24: Percentage tissue damage in the Heart retrieved from different groups.

4.3.15 Histopathological changes in the Lungs:

Moderate edema, degenerated cells and infiltration of immune cells can be seen in the lungs of the positive control group. NC group chicks, on the other hand, has the proper structurally arranged alveoli. Cellular infiltration (CI) was observed in lungs epithelia of Prophylactic treatment chicks. Phage groups treated immediately (IM-0hr, SC-0hr, ND-0hr) has mild to no damage to alveoli. However, oedema (OD) was observed in the groups treated after 24hrs (IM-24hrs, SC-24hrs, ND-24hrs). Cloudy swelling (CS), oedema (OD) and infiltration of immune cells (CI) was observed at multiple sites of the lungs of the antibiotic treatment group. Filling of the respiratory lobule (tertiary bronchus, atria, and air capillaries) with fibrin, heterophils, macrophages, and cell debris is characteristic of pneumonia. One exception observed here is the damage in the lungs of combination therapy group in which disintegrated alveoli with cloudy swelling was observed.

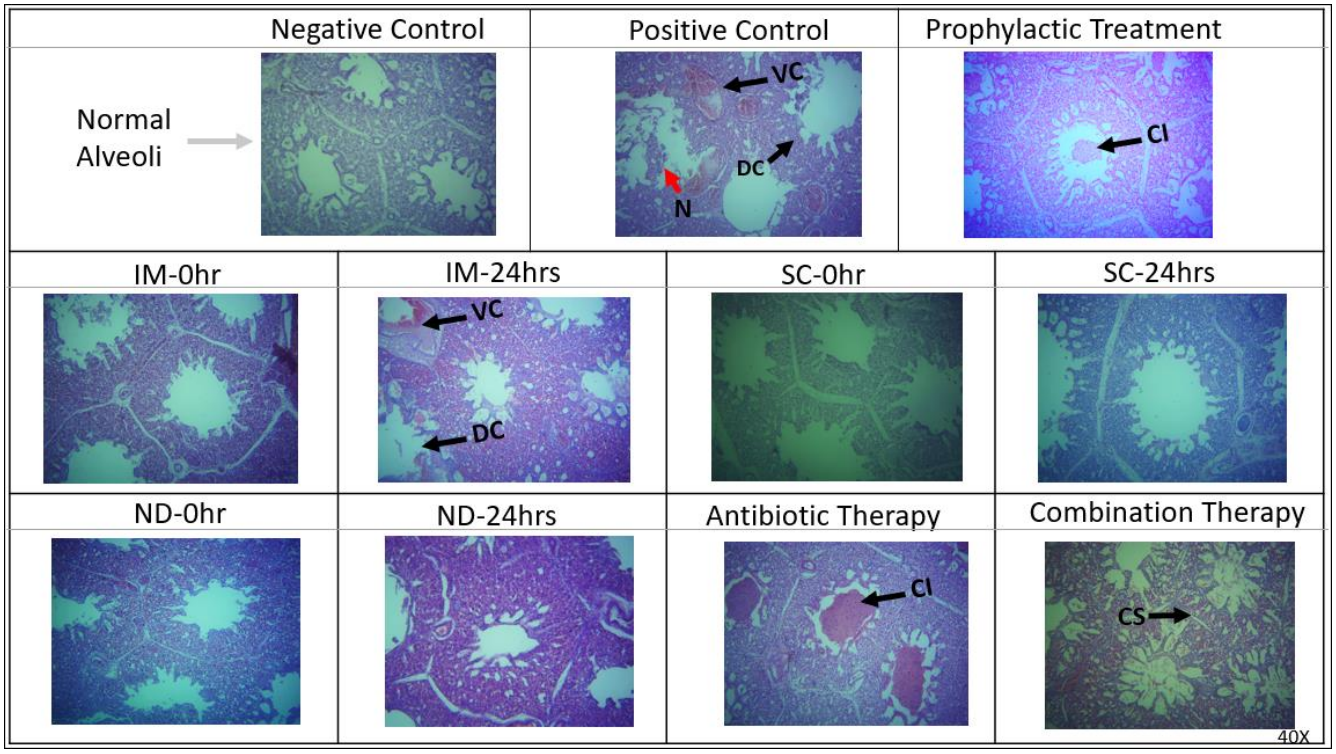


Figure 4.25: Histopathology of the Lungs tissue of chickens observed at 400X. H & E staining reveals H & E staining Cellular degeneration (DC), cloudy swelling (CS), Vascular Congestion (VC), Necrosis (N), Oedema (OD) and Cellular Infiltration (CI).

The damage score of 75.5%, a severe damage, was observed in the lungs of positive control group. Prophylactic treatment, intramuscular and nasal drops treated groups exhibited mild damage while remaining groups show moderate damage to the lungs.

Severe damage with a score of above 70% was observed in the positive control, however, mild damage was observed in most of the treatment groups. The lowest damage was seen in ND treated groups where phages were delivered via nasal route. Even the mean damage of ND-0hr (6.6667 ± 2.581989) was less than the combination therapy group (15.8333 ± 12.006942).

Table 4.6: The degree of tissue damage in Lungs with mean ± SEM and level of damage categorized into mild (≤ 21), moderate (≤ 51) and severe (≤ 76).

Groups	DEGREE OF TISSUE DAMAGE	
	Values (mean ± SEM)	Interpretation
Positive Control	75.55 ± 13.726434	Severe Damage
Prophylactic Treatment	14.1667 ± 12.416387	Mild Damage
IM-0hr	12.5 ± 8.215838	Mild Damage
IM-24hrs	56.3833 ± 28.772933	Moderate Damage
SC-0hr	27.5 ± 20.186629	Moderate Damage
SC-24hrs	37.5 ± 20.916501	Moderate Damage
ND-0hr	6.6667 ± 2.581989	Mild Damage
ND-24hrs	15 ± 10.954451	Moderate Damage
Antibiotic Therapy	48.3333 ± 16.02082	Moderate Damage
Combination Therapy	15.8333 ± 12.006942	Mild Damage

Severely damaged lungs of the positive control with the damage score of 75.5% is plotted against all the treatment group in the below graph.

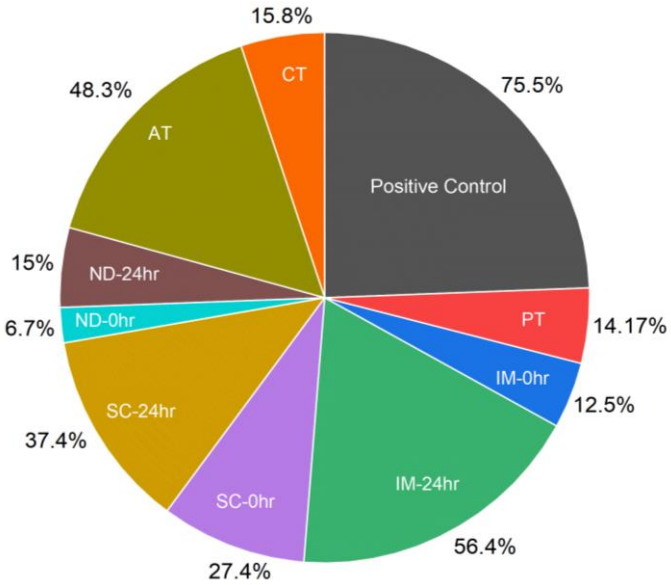


Figure 4.26: Percentage tissue damage in the lungs, retrieved from different groups

4.3.16 Degree of tissue change in Heart, Liver and Lungs:

The damage caused by APECO1 infection was much higher in untreated positive control groups as revealed by severe damage observed in the heart, lungs and liver. All the four different treatments were able to successfully reduce tissue damage.

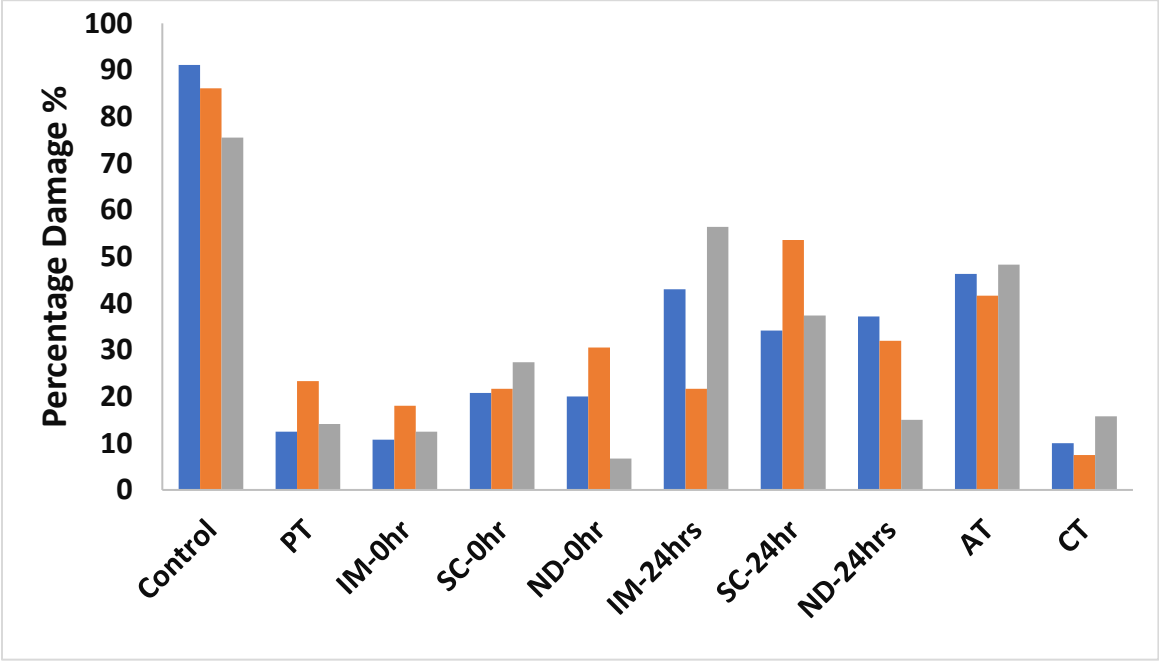


Figure 4.27: The degree of tissue damage in the liver, heart and lungs of all the groups. The lowest degree of damage was observed in combinatorial therapy while highest damage in the positive control.

Prophylactic Phage treatment, therapeutic phage treatment given immediately (IM-0hr, SC-0hr, ND-0hr) and combination therapy all prevented tissue damage better than antibiotic therapy, IM-24hrs, SC-24hrs and ND-24hrs (phage treatment after 24hrs). Histopathology results reveal the potential of phage therapy to reduce the tissue damage in major organs of chicks. The heart and liver of the dissected chicks were found to be more damaged than the lungs. However, in some groups, notably SC-0hr, IM-24hrs, and AT, lung damage was greater than heart and liver damage.

Chapter 5

Discussion

Discussion

In this study APEC O1-specific bacteriophages (BP) were isolated from the bedding material of the chickens. The phages named as PBM-1, PBM-2 and PBM-3 were combined in equal volumes to form a phage cocktail. Host range and resistance of the phage cocktail was compared with each individual phage and *in-vivo* therapeutic and prophylactic efficacy of phage cocktail were evaluated in chicken models.

APEC O1-specific bacteriophages were isolated from bedding material samples, all of them associated with the fecal material of the commercial chicken flocks. There are many studies which report the isolation of phages from the bedding material of chicken, wastewater treatment plants, sewage systems or cecal content of commercial chicken flocks, demonstrating their abundance in nature ***(Borie et al., 2008).***

Environmental parameters such as temperature and pH plays an important role in efficacy of phage therapy (Silva et al., 2014). The isolated phage PBM-3 was subjected to physiological characterization before utilizing in *in vivo* trials. PBM-3 showed maximum stability from 4-45ºC and after that significant reduction in the lytic ability of phages was predicted by fewer plaques. Temperature plays an important part in attachment of phage to its receptors. At higher temperatures, lysozyme enzyme is denatured and incapable of attachment with bacterial cell wall, resulting in decrease/loss of lytic ability. Stability of phage PBM-3 at 37ºC showed that it can work efficiently at body temperature.

pH also has an influence on phage multiplication, intracellular replication, infectivity and attachment (Jepson & March, 2004; Leverentz et al., 2001; Pirisi, 2000). pH can interfere with phage infectivity by disrupting the lysozyme enzyme and other capsid proteins thus, inhibiting the phage attachment to bacterial cell wall. PBM-3 tolerated pH range of 3-9 however, at alkaline conditions less titer was observed.

A phage must first adsorb onto the surface of a susceptible host cell in order to start an infection. Phage PBM-3 showed over 90% adsorption, relatively high adsorption rate, in 9 minutes suggesting that less time is required for the attachment of virion to the host cell. Adsorption rate has antagonist effect on lysis time because high adsorption rate leads to shorter lysis time. Lysis time, latent period and burst size are determined by one step growth curve. Burst size and latent

period have a progressive relationship, an optimal latent period give rise to efficient lytic ability of phage. Generation time of phage and burst size are positively linked; longer the generation time, higher will be the burst size and vice versa. A phage with a long lysis time will have a high burst size but will not cause many new infections; similarly, a phage with a less lysis time may infect other cells in the surroundings earlier but it will bear a smaller burst size (Shao & Wang, 2008). Having an intermediate lysis time is required for higher efficacy of phage therapy.

After characterization of phage, phage cocktail was developed and used to either prevent or treat avian pathogenic *Escherichia coli* (APEC) infection in chickens. The use of combination of phages is generally preferred in clinical settings for therapeutic purposes. Bacterial resistance to phages is sometimes attributed to the mutations of phage receptors present on host bacterial surface, which is a major cause of unsuccessful phage therapy. Phage cocktail, containing phages with different receptors, is therefore cited by many researchers as more effective tool against bacteria than individual phages (Chadha et al., 2016). In agreement with those authors, this study used the phage cocktail of three bacteriophages (PBM-1, PBM-2, PBM-3) and compared their resistant period and host-range, with individual phages.

Phage cocktail, as a combination of two phages, has been proved more effective than individual phages at preventing *E. coli* and *Salmonella* infections. Phage cocktail inactivation rates were faster and higher than those of single phage suspensions. (Costa et al., 2019). In our study, the cocktail was applied in a single-dose regime, to lessen the risk of developing resistance due to repeated dosing and to make this approach more feasible in a commercial setting.

Although phages replicate within their host cells, the most effective BP therapies seen in animal models make use of a high multiplicity of infection (MOI). The titer of the phage cocktail used, in our study, was 10^{12} PFU/ml as phage therapy at high concentrations of 10^{9} PFU or more than that could be as efficient as antibiotics (Gigante & Atterbury, 2019a). After *E. coli* challenge, administration of phage dose below 10^{4} PFU leads to *in vivo* proliferation but couldn't provide statistically significant protection against *E. coli* infection (Wernicki et al., 2017).

Single and multiple phage dosing is essential to consider to eradicate the bacteria as multiple phage dosing has been shown to be equally effective as single phage treatment in numerous studies. Our study suggests that single treatment with phage cocktail is effective enough to prevent the death of chicken. Earlier studies revealed that a single phage treatment leads to recovery in mice with

infections caused by *E. coli*, *P. aeruginosa*, MRSA, and vancomycin-resistant *Enterococcus faecium* (Watanabe et al., 2007). It has also been reported that in some cases multiple phage treatments were not more effective than single-phage regimen (Gigante & Atterbury, 2019). However, some studies reported that multiple doses are more effective than single doses. If *E. coli*-infected chickens are treated early, numerous doses of bacteriophages are preferable than a single treatment, according to one study. However, there is no noticeable difference between single and repeated doses if treatment is administered in the later stages of an infection (Huff et al., 2003).

Administration route plays a fundamental part in the phage therapy outcome, as it had a major effect on phage ability to penetrate into the system. Our study demonstrated that intra-muscular injections are more effective than subcutaneous and nasal drops administration. The possible reason might be the greater blood supply in muscle tissues which leads to faster adsorption (*Intramuscular Injection: Definition and Patient Education*, n.d.). The literature reports intramuscular injections in chicken and intraperitoneal mode of administration in mice to be more effective than others (Penziner et al., 2021) (Montoto, 2018). Comparison of bacteriophage aerosol spray and intramuscular injection in chicken model, after 24hr and 48hr of the bacterial challenge, showed that single intramuscular injection in thigh muscles was much more effective than aerosol spray (W. E. Huff et al., 2003b). While oral administration is the least effective of all administration methods among intraperitoneal (IP), intravenous (IV), and intramuscular (IM). The ability of phages to pass from the acidic conditions of the gastrointestinal tract to systemic circulation (phage penetration) is the major issue with phage administration via the oral route. Injections are not only most common but effective and faster in terms of phage delivery. Active phages are typically detected in the circulation within the first hr (even less than 5 minutes) ***(Krystyna, 2019).***

Phages have been reported to reduce the bacterial population densities and replication rates to a level where they can be controlled by the host immune system (Levin & Bull, 2004). This emphasizes treatment administration at right time before the bacterial infection overcomes the host immune system. Concerning time, the present study indicated that administration of phages immediately after bacterial challenge is more effective at reducing mortalities and organ damage as compared to late administration (after 24hrs). Similar findings were observed in many previous studies. One study reported no improvement in survival rate of mice phage-treated after 6 days of

gut derived sepsis caused by *P. aeruginosa*. The mortality in untreated control group (66.7%) significantly reduced to 0% in those animals which received phage treatment after one day of bacterial inoculation (Watanabe et al., 2007). However, many other studies emphasized the potential role of phage treatment within few hours of bacterial challenge, suggesting that phage therapy is more effective if treatment is given shortly after bacterial infection. The late administration of phages, either at day 2 or day 4, doesn't lead to efficient elimination of bacteria. In fact, the results were the same as when infected chickens were not given any anti-bacterial drugs (Kosznik-Kwaśnicka et al., 2022). When given immediately after bacterial challenge, phage treatment significantly reduced mortality, from 60% for untreated control birds to 30. However, no therapeutic benefit was observed when phage treatment was delayed for 24 or 48hrs following bacterial challenge (Gigante & Atterbury, 2019a). When given immediately, 24hrs, or 48hrs after challenge, IM injections of bacteriophages dramatically reduced mortality from 53 to 17%, 46 to 10%, and 44 to 20%, respectively (W. E. Huff et al., 2003b)

An increase in body weight is a crucial factor in broiler production, since a lower body weight correlates to a higher production cost for broiler meat (Gigante & Atterbury, 2019a). Colibacillosis, or in general, *E. coli* infection is associated with less feed and water consumption in poultry animals. In our study, all of the tested challenges affected the feed intake and the growth rate relative to those of the control group. From second to third week, intake of feed increased by 11% in the positive control group, but by greater amounts in all treatment groups, with the highest value being 59.27% in the combination therapy group. This suggests the beneficial role of all the treatments in maintaining health status of chicken. Lower feed intake in the positive control is related to the lesions present on the surface of vital organs. Severe damage to the liver and epithelium compromises nutrient absorption and thereby effect growth rate (Remus et al., 2014). A similar finding was reported in a study where infection with *E. coli* reduced the feed intake by 7% and consequently 10% reduction in growth rate (Remus et al., 2014). Broiler infection with *Salmonella* reduced feed intake by 9% and growth rate by 29% (Marcq et al., 2011) . In the untreated positive control, lowest feed intake after bacterial challenged is supported by a study which states that worsened performance of challenged broilers is attributed to the immune response requirements. Immune system activation and production of related proteins is negatively linked with growth performance of broilers (Humphrey & Klasing, 2019). It is estimated that 2.33 mg of muscle protein are catabolized to provide the amino acid needs for the synthesis of each

milligram of immune response protein (Reeds et al., 1994). In addition, the lymphoid organs of broilers with a high weight-gain potential are lighter. Cytokines production during immune response are accountable for the decrease in feed intake and weight during health problems. (Remus et al., 2014).

In our study, the day following infection was marked by decreased water and feed consumption in all groups; however, it progressively begins to increase in the treatment groups with each passing day. However, the untreated group (positive control) was unable to reverse the decreased water and food intake. Therefore, less water consumption was observed in the positive control and exceptionally in the antibiotic treatment group. Both of the groups have almost equal water consumption. The possible reason for this exception might be the taste of water as antibiotics were administered to animals via water. Despite having fewer taste buds than other animals, chickens have a well-defined sense of taste and will accept or reject specific flavors. Birds prefer water enriched with substances like thiamine and sugars (*Factors Affecting Water Intake and Its Utilisation by Chickens - All About Feed*, n.d.) Given the bitter taste of enrofloxacin (antibiotic used in this study) less water consumption is attributed to its addition in water in antibiotic treatment group.

Bacteriophages also have an established role as a growth promoter when added to feed (without bacterial challenge). In this study, bacteriophage injection has improved the body weight gain and consequently improved FCR. These findings are consistent with a study who found that increasing bacteriophage levels improved body weight increase and FCR (Kim et al., 2013). Other studies have also revealed a similar improvement in the layer's performance (Clavijo & Flórez, 2018) . However, Wang et al. observed that BP supplementation had no positive effect on the body weight and FCR of broiler chickens (J. P. Wang et al., 2013).

Lesion scores provide a measure of bacterial infection and associated gross pathological changes. Our study revealed thick macroscopic lesions on the organs of the chicks who survived septicemia and dissected at the end of the study. Whereas low lesion score was observed in the case of early death and dissection. Similar findings are reported in a study where different strains Newcastle disease virus induced lesions in multiple organs of the chicken (Hussein et al., 2018).

The mean lesion scoring of organs (Heart, Liver & lungs) based on pathomorphology show significant reduction of score value by treatment groups, specifically combination therapy,

antibiotic therapy, prophylactic therapy and immediate phage treatment groups (IM-0hr, SC-0hr, ND-0hr) in comparison to untreated positive control. On the other hand, three groups where phages were injected after 24hrs of bacterial challenge (IM-24hrs, SC-24hrs, ND-24hrs), high lesion score than other treatment groups were observed. The approach used for the pathomorphological organ scoring has been reported many times in the literature where researchers used the same way for calculating organ damage (Krishnegowda et al., 2020) (Talukdar et al., 2017) (Antão et al., 2008) (Abalaka et al., 2017). Septicemic lesions in various vital organs are usually associated with bacterial endotoxins. Chicks who survive acute septicemia usually develops severe lesions due to colonization of bacteria on serosal surfaces. Many of the organs showed prominent fibrinous exudation, a sign of severe acute vascular damage and is highly indicative of septicemia. Endotoxin could be responsible for the vascular damage. The fibrin exudates act as a chemotactic agent that attracts heterophils to the fibrinous deposits (Krishnegowda et al., 2020).

To analyze the damage at tissue level, histopathological analysis were performed. Clinical observations, organ damage and lesion scoring revealed histopathological alterations in infected chicken organs that were associated with the infection process. The observed generalized congestion in various organs might be due to vascular damage caused by the *E. coli* endotoxin. Acute inflammatory response microscopically marked by the oedema, vascular congestion, cell infiltration, and fibrinous inflammation marked by necrotic changes, were evident in the tissue histology of the positive control. Cellular degeneration and necrosis were more prominent in the tissues of positive control. In all the treatments, lesser degree damage was observed with the least damage observed in the liver, heart and lungs of the combination therapy. Phage-antibiotic synergy caused a significantly stronger bacterial reduction than the antibiotic alone. It is an established fact that phages and antibiotics are more effective when combined, because their mechanism of action complement each other.

Different studies performed *in vitro* showed synergistic effect for the use of phage with sublethal dose of antibiotic. Antibiotic cause's enlargement of plaque size, accelerates phage amplification, and increase burst size. Phages, on the other hand, degrades bacterial polysaccharides by enzymes allowing better antibiotic diffusion and cell penetration. This synergy also decreases phage/antibiotic resistant mutants by causing resensitization of bacteria to either treatments by trade-off principle. Recent human studies indicate that phages stimulate the production of IL-10,

an anti-inflammatory cytokine, which significantly reduces hepatocyte apoptosis and neutrophil infiltration, acting as a hepatoprotective agent. IL-10 has been reported to reduce haemorrhagic liver damage and also exhibit anti-fibrotic properties (Zhang & Wang, 2006). The moderate inhibitory effect of phages on the activation of nuclear factor kappa B (NF-κB) is known to inhibit liver inflammation and injury (Luedde & Schwabe, 2011).

Even if infections have not been completely eradicated, clinical phage therapy has been reported to have the potential to reduce inflammatory markers such as C-reactive protein (CRP)(Międzybrodzki et al., 2009). Immunosuppression caused by IL-10 and Kupffer cells (liver macrophages) co-relates with the increased body weight of phage treated chicken, as discussed earlier. These variables clarify the possible function of phages in tissue damage prevention, and the best outcomes are anticipated when they are paired with the tissue-protective properties of antibiotics.

Conclusion

5. Conclusion

All three phages (PBM-1, PBM-2, and PBM-3) possess numerous favorable characteristics, making them a viable option for *in vivo* research. It has been observed that phage mixtures are superior to single-phage preparations due of their lower resistance rate and broader host range. Following phage therapy, in-vivo trials revealed a substantial decrease in broiler mortality. Additionally, phage cocktail facilitated the growth of chickens and improved feed conversion ratio better than antibiotics. Systemic administration of phages was found to be safe with no prominent side effects. However, lesion scoring of organs and histopathology revealed more substantial difference in combination therapy where phages and antibiotics were delivered in synergy. In conclusion, the data suggests the dual potential of phages to be used as prophylactic as well as therapeutic, with the best outcome in combination therapy.

Future Prospects

6. Future Prospects

Absence of resistant and virulent genes can be confirmed by performing whole genome sequencing of PBM-1, PBM-2 and PBM-3. For further structural and functional characterization, TEM analysis, capsid protein extraction and mass spectrometry of PBM-3 can be done. As phages are biological entities, their pharmacodynamics and immunological aspects can be studied in another *in vivo* studies. Using the same cocktail, an oral route of administration via feed can be investigated in a parallel investigation. It is possible to develop and combine a cocktail against Salmonellosis (one of the major bacterial infections of poultry) with the current cocktail, and study synergistic effect.

7. References

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