Isolation, characterization and *in vivo* evaluation of therapeutics and prophylactic efficacy of bacteriophage(s) against pathogenic *Escherichia coli* in chickens

BY
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CERTIFICATE - I

This is to certify that this thesis entitled "**Isolation, characterization and *in vivo* evaluation of therapeutics and prophylactic efficacy of bacteriophage(s) against pathogenic *Escherichia coli* in chickens**" submitted for the degree of **Doctor of Philosophy** in the subject of **Veterinary Public Health and Epidemiology** to the **Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar** is a bona fide research work carried out by **Dr. Punit Jhandai,** Admission No. **2019V02D** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of this investigation have been fully acknowledged.

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CERTIFICATE- II

This is to certify that this thesis entitled "**Isolation, characterization and *in vivo* evaluation of therapeutics and prophylactic efficacy of bacteriophage(s) against pathogenic *Escherichia coli* in chickens**" submitted by **Dr. Punit Jhandai**, Admission No. **2019V02D**, to the **Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in the subject of **Veterinary Public Health and Epidemiology**, has been approved by the Student's Advisory Committee after an oral examination on the same.

MAJOR ADVISOR **EXTERNAL EXAMINER**

HEAD OF THE DEPARTMENT

DEAN, POST-GRADUATE STUDIES

Dedicated
To My
Beloved Parents

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ABBREVIATIONS

- ve	:	Negative
%	:	Per cent
+ ve	:	Positive
µg	:	Microgram
µl	:	Microlitre
µM	:	Micromolar
ºC	:	Degree Celsius
AMR	:	Antimicrobial Resistance
APEC	:	Avian pathogenic *E. coli*
AST	:	Antimicrobial Susceptibility test
ATCC	:	American Type Culture Collection
BHI	:	Brain Heart Infusion broth
BIS	:	Bureau of Indian Standard
blaTEM	:	Beta lactamase Temoniera gene
bp	:	Base pairs
CA	:	Clavulinic acid
CFU	:	Colony Forming Unit
CLSI	:	Clinical and Laboratory Standards Institute
d	:	Days
DNA	:	Deoxyribonucleic acid
dNTP	:	deoxynucleotide triphosphate
E. coli	:	*Escherichia coli*
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme-linked Immunosorbent Assay
EMB	:	Eosine methylene blue agar
ESBL	:	Extended spectrum beta lactamase
et al.	:	Et alii/alia
etc.	:	Et cetera
ExPEC	:	Extra-intestinal pathogenic *E. coli*
Fig	:	Figure
fimH	:	Adhesive subunit of Type1 fimbriae
g	:	Gram
GN	:	Gram negative
h	:	Hour/ Hours
hlyF	:	Putative avian haemolysin
intI	:	Integron integrase
iroN	:	Salmochelin siderophore receptor gene
iss	:	Episomal increased serum survival gene
iutA	:	Aerobactin siderophore receptor gene
LB	:	Luria-Bertani broth
lit	:	Liter
MCT	:	Micro Centrifuge Tube

MDR	:	Multidrug resistant
mg	:	Milligram
MHA	:	Mueller Hinton agar
MIC	:	Minimum Inhibitory Concentration
min	:	Minute
ml	:	Milli litre
MLA	:	MacConkeys agar
mm	:	Milli molar
NaCl	:	Sodium Chloride
NaOH	:	Sodium Hydroxide
NSS	:	Normal saline solution
OD	:	Optical Density
ompT	:	Episomal outer membrane protease gene
PAIs	:	Pathogenicity Associated islands
PBS	:	Phosphate buffer saline
PCR	:	Polymerase Chain Reaction
PFU	:	Plaque Forming Units
rpm	:	Revolutions per minute
RT	:	Room Temperature
SM	:	Suspension Medium
TE	:	Tris EDTA
TEM	:	Transmission Electron Microscopy
UV	:	Ultra violet
V	:	Volts
v/v	:	Volume by volume
VGs	:	Virulence genes
viz.	:	Videlicet
w/v	:	Weight by volume
WHO	:	World Health Organization
µg	:	Microgram
µl	:	Micro liter
µm	:	Micrometer

CHAPTER-I

INTRODUCTION

Poultry meat including chicken is a popular food all over the world. The poultry industry has developed rapidly in India since the late seventies (Muduli *et al.*, 2019). However, this industry remains under constant threat of various infectious diseases caused by bacteria, viruses, fungi and parasites like colibacillosis, salmonellosis, campylobacteriosis, avian influenza, coccidiosis, etc (Cutler, 2002). Among these infections, colibacillosis is one of the most common causes of death which result in massive economic losses and condemnations of carcass (Huff *et al*., 2002). It refers to any localized or systemic infection caused by avian pathogenic *Escherichia coli* (APEC) and includes colisepticemia, air sac disease (chronic respiratory disease), venereal colibacillosis, coliform cellulitis (inflammatory process) and peritonitis. The bacterium, APEC is an important cause of economic losses to the poultry industry in terms of morbidity, mortality, condemnation of carcasses and costs incurred in treatment and disinfection (Kathayat *et al*., 2021). Further, a large number of intestinal pathogenic *Escherichia coli* can be transmitted to other animals or humans directly or indirectly which pose a significant risk to animal and human health (Doyle and Erickson, 2006). The virulence factors, serotype, phylogenetic group and drug resistance of avian pathogenic *E. coli* can be used to classify the bacteria. Various virulence-associated genes i.e., *iutA, hlyF, iss, iroN* and *ompT* in avian pathogenic *E. coli* allow extraintestinal survival. Moreover, these genes have been proposed as the minimum criteria for identification of an APEC strain with the highest pathogenicity (Johnson *et al*., 2008; Kazibwe *et al*., 2020). Further, various *E. coli* serotypes have been identified based on the somatic (O) antigen serogroups, although only a few numbers are relevant to avian colibacillosis. According to previous research conducted in other countries, the most prevalent APEC serogroups are O1, O2 and O78, but others do occur. (Dou *et al*., 2015; Paixao *et al*., 2016; Halfaoui *et al*., 2017).

For many years, antibiotics against *E. coli* have been continuously used to control colibacillosis in poultry. Of late, the research findings have suggested that inappropriate or excessive abuse of antibiotics can lead to the emergence and

spread of more virulent resistant bacterial strains which result in complex and costly treatment regimens (Gorski *et al*., 2009; Ngu *et al*., 2020). In India, the data collected from small as well as large poultry establishments indicates the development of significant resistance against the most commonly used antimicrobials due to their indiscriminate use for prevention or therapeutics of various bacterial poultry diseases (Kumar *et al.,* 2019). Due to its weak bio-containment, *E. coli* could represent a risk to the environment and humans (Nguyen *et al*., 2015). However, these concerns may result in regulations and laws that severely restrict the use of antibiotics in animal and poultry production. The treatment for *E. coli* has been increasingly complicated by the emergence of resistance to most first-line antimicrobial agents. Therefore, there is a real need to find alternatives to antibiotics for both the prevention and/or therapeutics of *E. coli* infections in poultry.

Though several alternatives are available to tackle the AMR problem, however, among those bacteriophages are considered as one of the best remedies. The term "bacteriophage" meaning "to destroy bacteria" is considered to be originat from the words "bacteria" and "phagein". Furthermore, bacteriophages are natural bacteria killers and are the most abundant and widely distributed microbiological organisms in soil, sea and sewage water; amounting to approximately 10^{31} phage virions on earth (Batinovic *et al*., 2019). Bacteriophage was discovered by Frederick William Twort from University of London in 1915, whereas Felix d'Herelle coined the term ‘bacteriophage’ (Twort, 1915; d'Herelle, 1917). For investigating the efficiency of phages to treat bacterial illnesses, researches were undertaken even before antibiotics were discovered.

Furthermore, phage treatment has certain additional advantages over antibiotic therapy as phages are naturally present in the environment, simple and inexpensive to isolate (Abdelrahman *et al*., 2022). Furthermore, unlike antibiotics, these are specific to bacterial targets and have no negative impacts on the normal microbiota (Abdelsattar *et al*., 2019; Mu *et al*., 2021). It is noteworthy to mention that the phages may be an ideal choice for treatment of pathogenic bacteria due to their ability in killing target bacterial cell (Batinovic *et al.,* 2019). It could be possible to use these phages to prevent and treat the bacterial diseases. Further, phage can exhibit specific patterns of host infectivity as they prevent healthy flora destruction. Such a lytic mechanism prevents bacterial dysbiosis (microbial

imbalance), which may prevent secondary infections (Sulakvelidze *et al.,* 2001). Moreover, the phages are also able to multiply exponentially at the site of infection. These qualities make bacteriophages "a valuable candidate as therapeutic agent" against pathogenic bacteria (Elbreki *et al.,* 2014).

Bacteriophages are divided into two categories based on their interactions with bacteria and life cycle: lytic (virulent, productive) and lysogenic (temperate, inactive). Certain bacteriophages can undergo both lytic and lysogenic stages (e.g., phage lambda of *Escherichia coli*). A bacteriophage infects a target bacterium (living bacterial target cell), replicates, kills the bacterium by lysis and releases multiple daughter cells from 20 to 1000 (Huff *et al*., 2009; Fortier and Sekulovic, 2013) throughout the lytic cycle. The phage protein (holin) creates pores through the cytoplasmic membrane toward the end of the lytic cycle, allowing the phage-encoded endolysin (named 'lysin') to gain access and hydrolyze the peptidoglycan layer. As a result, cell lysis occurs and offspring phages (made by the host bacterium) are released, infecting neighbouring bacterial cells and restarting the cycle (Zbikowska *et al*., 2020). The duration of the entire cycle varies, although it normally lasts for 20-40 min and may be up to 12 h.

Further, the lytic phages can be used in multiple ways. In human and veterinary medicine phages are employed commonly nowadays. Modern phage medicine relies on virulent phages as they can act against antibiotic resistant bacteria, potentially lowering the number of multidrug-resistant bacterial illnesses due to their multifaceted actions (Fu *et al*., 2010; Mapes *et al*., 2016). Bacteria can acquire resistance to phages alike to antibiotics. However, unlike antibiotics, phages are dynamic and may co-evolve with bacteria in an ever-escalating arms battle (Donlan, 2009). Because of concerns about resistance, phages are generally used as cocktail directed against a specific pathogen. Bacteriophages could be a future alternative to antibiotic treatment of bacterial infections as in recent years, where the drug-resistant strains of bacteria are showing increasing trend. Bacteriophage therapy has been widely attempted to treat different animal diseases caused by various types of bacteria (Oliveira *et al.,* 2010).

Moreover, phages have been used to control colibacillosis-causing pathogens of poultry, in different parts of the world (Huff *et al*., 2002; Jamalludeen *et al*., 2009; Kazibwe *et al.,* 2020). Phage therapy, therefore, appears

to be an effective approach for prophylaxis or treatment of bacteria associated colibacillosis in poultry.

Hence the present study was planned with the following objectives:

1. **Isolation and characterization of pathogenic *E. coli* from chickens.**
2. **Isolation and characterization of lytic bacteriophage(s) against isolated pathogenic *E. coli.***
3. ***In vivo* study on therapeutic and prophylactic activity of isolated bacteriophage(s) against pathogenic *E. coli* in chickens.**

CHAPTER-II

REVIEW OF LITERATURE

Theodor Escherich in 1885 isolated an organism, *Bacterium coli* commune while attempting to isolate the etiologic agent of cholera from the stool of neonates. This organism is now known as *Escherichia coli* (*E. coli*). *E. coli* is one of the most important pathogens which causes infection in a wide range of animal species. There are several subtypes of *E. coli* and many of those are harmless, but six pathotypes can cause intestinal illness including enterotoxigenic *Escherichia coli* (ETEC), enterohemorrhagic *Escherichia coli* (EHEC), enteropathogenic *Escherichia coli* (EPEC), enteroaggregative *Escherichia coli* (EAEC), enteroinvasive *Escherichia coli* (EIEC) and diffusely adherent *Escherichia coli* (DAEC) (Kaper *et al.*, 2004). The pathogenic variants of *E. coli* cause intestinal and extra-intestinal infections including gastroenteritis, urinary tract infection, meningitis, peritonitis and septicemia. Commensals, diarrhoeagenic *E. coli* and extraintestinal pathogenic *E. coli* (ExPEC) are various types of *E. coli* based on their pathogenic potential. ExPEC infects the gut asymptomatically but they have a variety of virulence characteristics capabling them to colonize in extraintestinal tissues of humans, pets and poultry (Valiatti *et al*., 2020). Colibacillosis is an extraintestinal illness in poultry caused by a sub-pathotype of ExPEC strains called avian pathogenic *E. coli* (APEC). The symptoms include septicemia, air vasculitis, pericarditis, perihepatitis, splenomegaly and cellulitis (Foster-Nyarko *et al*., 2021).

Avian colibacillosis is one of the most common diseases caused by *E. coli* that affects the poultry industry worldwide. High morbidity and mortality expenditures (treatment, vaccination etc.), declines in feed conversion efficiency, growth rate, egg production and carcass condemnation at slaughter facilities are various factors that contribute to significant economic losses (Nolan *et al*., 2013).

To infect chickens, APEC uses a variety of virulence and pathogenesis factors, including adhesins, invasins, protectins, iron acquisition mechanisms and toxins (Dziva and Stevens. 2008). These factors of APEC facilitate the establishment of infection in chickens by colonization, proliferation and systemic dissemination in the host (Kathayat *et al*., 2021). Recent research suggests that APEC (especially isolates from sequence types ST95 and ST131 or serogroups O1, O2 and O18) are potential foodborne zoonotic pathogen as well as a cause or reservoir of extraintestinal infections in humans (Belanger *et al*., 2011; Mellata, 2013; Markland *et al*., 2015; Liu *et al*., 2018). In particular, APEC shares genetic similarities with human ExPECs, uropathogenic *E. coli* (UPEC) and

newborn meningitis *E. coli* (NMEC). APEC contains virulence genes able to cause urinary tract infections (UTI) and meningitis in mice and rat models (Tivendale *et al*., 2010).

2.1 Isolation and Identification of *E. coli* from Poultry

Pathogenic *E. coli* is normally found in the intestinal tract of birds along with commensal *E. coli* strains (Awawdeh, 2018). In the presence of predisposing factors APEC is responsible for variety of localized and systemic infections in poultry called as avian colibacillosis. APEC can infect all types of birds of any age group (Grakh, 2019).

Joshi *et al*. (2012) examined tissue samples from 35 poultry birds affecting from colibacillosis in Pantnagar, India. They recovered a total of 20 *E. coli* isolates from these samples.

Sahoo *et al*. (2012) collected and examined 317 swab samples from colibacillosis suspected birds in Odisha. The found that a total of 105 samples were positive for *E. coli.*

Messai *et al*. (2013) examined a total of 200 colibacillosis-affected birds from May-August 2010 in Algeria and found 180/200 (90%) of liver samples positive for *E. coli*.

Dadheech *et al*. (2016) conducted a study to determine the distribution of *E. coli* in visceral organs of 12 colibacillosis affected chickens in Ajmer. The results revealed that from 12 suspected birds, all birds (100%) were found to be positive for *E. coli* infection.

Ozaki *et al*. (2017) collected a total of 83 swab samples from pericarditis and perihepatitis lesions in broiler chickens from four commercial farms in Japan. The findings revealed 100% prevalence of *E. coli* isolates from collected swab samples.

Magray *et al*. (2018) examined 600 tissues sample collected from colibacillosis suspected chickens in Srinagar. The author found that a total of 145 samples carried *E. coli.*

Azam *et al*. (2019) investigated 84 diseased/dead birds affected with colibacillosis in the Faisalabad region of Pakistan. The *E. coli* isolates were recovered from 75 (89.2%) samples diagnosed on the basis of colony morphology and biochemical characteristics.

Grakh (2019) collected 114 tissues and environmental samples from poultry farms situated in the Haryana state of India. He isolated a total of 62 *E. coli* isolates and out of that, 22/22 were from diseased birds tissues, 6/10 were from cloacal swabs and 34/82 were from different environmental sources of these farms. The prevalence of *E. coli* isolates from colibacillosis-affected birds was 87.5% (28/32).

Adil (2020) investigated 135 outbreaks in various poultry farms of Srinagar and found that 1088 cases were found positive for colibacillosis causing *E. coli* from a total of 4255 necropsied carcasses.

Awad *et al*. (2020) examined a total of 54 field broiler flocks (n = 54) in the northern delta of Egypt, wherein 51.85% of these flocks were found to be positive for *E. coli* by bacterial culture and biochemical identification.

2.2 Antibiotic Sensitivity of *E. coli* Isolates

Antimicrobial medications are frequently used to control the illness and reduce the financial losses caused by *E. coli* infection in poultry (Nolan *et al*., 2013). An increase in resistance to commonly used antibiotics is a global concern for the poultry industry as well as the general public (Aarestrup, 2005).

Salehi and Bonab (2006) examined 50 *E. coli* isolates cultured from colibacillosis affected birds for antimicrobial susceptibility testing in Iran. High resistance was observed for various antibiotics mainly tetracycline (94%), streptomycin (81%), ciprofloxacin (67%), chloramphenicol (67%), nitrofurantoin (56%) and ampicillin (47%).

Koo and Woo (2012) isolated 61 isolates of *E. coli* from poultry meat in Korea. A total of 88.5%, 78.7% and 80.3% were resistant to tetracycline, streptomycin and ampicillin, respectively. All isolates were found to be sensitive for imipenem.

Saidi *et al*. (2012) subjected 103 *E. coli* isolates to antimicrobial susceptibility testing using disc diffusion method in Zimbabwe. The maximum resistance was observed for tetracycline (100%) followed by ampicillin (94.1%). The maximum sensitivity was observed for gentamicin (97.1%).

Ahmed *et al*. (2013) studied the antimicrobial sensitivity of 73 *E. coli* isolates from septicemic broilers of different farms located in different geographic areas of Egypt. The maximum resistance was found for ampicillin (97.3%) followed by tetracycline (95.9%) and streptomycin (93.2%).

Oosterik *et al*. (2014) studied the antimicrobial susceptibility pattern of 97 APEC isolates in Belgium and the highest percentages of resistance were detected for tetracycline (53.6%) followed by ampicillin (35.1%). Resistance to third-generation cephalosporins or monobactams (ceftriaxone, ceftazidime, cefotaxime, aztreonam) was found to be varying from 0.0 to 7.2%.

Dou *et al*. (2015) subjected 243 APEC isolates to antimicrobial susceptibility testing in China and found more than 60% resistance for ampicillin, streptomycin and tetracycline. The resistance to gentamicin, amikacin, ciprofloxacin and nitrofurantoin ranged from 6.6% to 63.8%. Lower resistance (0.0-21.0%) was observed for cefotaxime, ceftriaxone, ceftazidime and aztreonam.

Magray *et al*. (2018) subjected antimicrobial-sensitivity test for APEC isolates from colibacillosis suspected chickens in Srinagar. Resistant pattern observed were; ampicillin (63.30%), amoxycillin-clavulanic acid (46.60%), gentamicin (15.50%), streptomycin (68.80%) and tetracycline (96.60%).

Kumar and Gupta (2019) tested 106 *E. coli* isolates isolated from tissue samples from colibacillosis suspected birds in the Hisar district of Haryana. The results revealed that 90 (84.9%), 86 (81.1%), 83 (78.3%), 82 (77.3%), 72 (67.9%), 67 (63.2%), 65 (61.3%), 63 (59.4%), 60 (56.6%) and 55 (51.8%) isolates were resistant to ampicillin, tetracycline, ciprofloxacin, levofloxacin, piperacillin, cefotaxime, streptomycin, ceftriaxone, cefoperazone and gentamicin, respectively.

Grakh *et al*. (2022) studied antimicrobial sensitivity patterns in 47 APEC isolates obtained from poultry farms in Haryana state. The results indicated resistance among 89.4%, 66%, 61.7% and 23.4% isolates against tetracycline, ampicillin, piperacillin and imipenem, respectively; 25.5% resistance each for amoxicillin/clavulanic acid and cefpodoxime; and 12.8% each for amikacin, gentamicin and nitrofurantoin. However, all the APEC isolates were sensitive to Polymyxin B.

2.3 APEC Characterization

Johnson *et al*. (2008) in the USA conducted a study intending to identify a minimum number of virulence genes that could identify APEC. The authors identified five virulence genes (*iutA, iss, ompT, iroN* and *hlyF*) that were significantly associated with APEC strains. The virulence gene prevalence obtained for these genes was 84% (*iroN*), 80.7% (*iutA*), 80.5% (*iss*), 78.6% (*ompT*) and 78.2% (*hlyF*).

Hussein *et al*. (2013) characterized 219 *E. coli* strains to determine the prevalence of eight ExPEC virulence-associated genes (*cvaC*, *iroN*, *ompT*, *hlyF*, *iss*, *iutA*, ireA and *papC*) in Egypt. The prevalence of *iroN*, *ompT*, *hlyF*, *iss* and *iutA* ranged from 89.5%-94.7% among APEC strains More than 90% of the total APEC examined possessed *iroN*, *ompT*, *hlyF*, *iss* and *iutA* genes.

Dissanayake *et al*. (2014) in Sri Lanka observed that four virulence genes (*sitAP*, *ompT*, *hlyF* and *iroN*) out of 17 genes studied were more significantly associated with APEC. The detection rate for the four genes was 78% (*iroN*), 74% (*ompT*), 72% (*hlyF*) and 72% (*SitAP*) among 30 APEC isolates.

De Carli *et al*. (2015) in Brazil used ten virulence genes for the characterization of 138 *E. coli* isolates cultured from colibacillosis-affected chickens (74 isolates) and turkey (64 isolates). The authors identified 81 APEC isolates and prevalence obtained for five virulence genes (VGs) was 98.8% (*iroN*), 81.5% (*iutA*), 96.3% (*iss*), 100% (*ompT*) and 100% (*hlyF*).

Mbanga and Nyararai (2015) characterized *E. coli* isolates, cultured from colibacillosis-affected birds based on 12 virulence genes in Zimbabwe (*iutA*, *hlyF*, *ompT*, *frz, sitD, fimH, kpsM, sitA, sopB, uvrY, pstB* and *vat*). None of the isolate was found to be having more than six genes. The three most prevalent virulence genes detected in the studied 45 APEC were *iutA* (80%), *fimH* (33%) and *hlyF* (24%), whereas, genes *kpsM*, *pstB* and *ompT* were detected in only 2.2% of the isolates.

Awawdeh (2018) from Australia studied virulence genes related to APEC, 93.1% of *E. coli* isolates cultured from colibacillosis-affected birds harbored all the five virulence genes (*iutA*, *iss*, *ompT*, *iroN* and *hlyF*) using protocol and criteria described by Johnson *et al*., 2008. All the *E. coli* isolates from colibacillosis-affected birds were characterized as APEC using this criterion.

Subedi *et al*. (2018) in Nepal characterized APEC strains using 11 APEC-associated virulence genes. A total of 45 (90%) out of 50 isolates were characterized as APEC. The genes used along with their detection rate among APEC isolates were as follows: *iss*, *ompT*, *hlyF* and *iroN* (100% each) and *iutA* (82%) gene.

Azam *et al*. (2019) characterized 75 *E. coli* isolates, isolated from 84 diseased and dead birds affected with colibacillosis in the Faisalabad region of Pakistan. All the *E. coli* isolates were found to be APEC and 32 (42.6%) isolates harbored more than five Virulence associated genes (VAGs). Most commonly found genes were increased serum survival (*iss*; 84%), *iroN* transport (*iutA*; 74.6%) and colicin V (*ColV*; 60%). Twenty-two isolates (29.3%) were found to possess a combination of VAGs i.e., *iss, tsh*, *iroN* and *iutA* in addition to other VAGs.

Grakh *et al*. (2022) characterized 62 *E. coli* isolates for APEC from tissues and environmental samples collected from poultry farms in Haryana state. The researchers found that the prevalence of *hlyF*, *ompT*, *iroN*, *iutA* was 100% among 47 APEC isolates, whereas, *iss* prevalence was 95.7%. The results revealed that all the *E. coli* isolates isolated from poultry tissue samples were APEC. The prevalence of the five virulence genes was found to be significantly associated with APEC isolates using the chi-square test ($p<0.01$).

2.4 ESBL Characterization

Hasan *et al*. (2011) in Bangladesh isolated 101 *E. coli* from the tissue of colibacillosis-infected birds. They subjected five *E. coli* isolates that exhibited resistance against more than six antibiotics (including beta lactam antibiotics) for ESBL detection. None of the isolates were found to be ESBLs.

Saeed (2014) isolated a total of 311 *E. coli* from 460 healthy chickens in Iraq from April to October 2013. Out of 311 *E. coli* isolates, 264 (84.9%) produced beta

lactamase in chickens and 76.1% (201/264) of beta lactamase isolates were found to be ESBL producers. The author reported a 43.7% prevalence of ESBL producers among 460 healthy chickens.

El-Shazly *et al*. (2017) characterized the resistance mechanisms to expanded spectrum cephalosporins among resistant veterinary *Escherichia coli* isolates in Egypt. Only Three strains (6%) were confirmed as ESBL among 50 clinical multi-resistant *E. coli* strains. Confirmation of *E. coli* was done by the presence of ESBL genes of *blaTEM-57, blaSHV-12* and *blaCTX-M-1*.

Mohsin *et al*. (2017) in Pakistan studied the prevalence and phylogenetic relationships of ESBL *E. coli* from wild birds. They analyzed ESBL *E. coli* phenotypically using the Vitek-2 automated system and the results indicated that 17.3% (26/150) of birds were carriers of ESBL *E. coli*.

Jhandai (2019) characterized 63 *E. coli* isolates isolated from chicken meat in Hisar based on ESBL production. The author identified that 68.25% (43/63) isolates were found to be ESBL producers.

2.5 Presence of *BlaTEM* Gene in *E. coli* Isolates

Yuan *et al*. (2009) conducted a study to determine the prevalence and molecular characterization of ESBL-producing *E. coli* in poultry from China. Thirty-one of the 51 tested isolates were positive for an ESBL phenotype and 29 of these isolates carried one or more *bla* genes. A total of 22 (43%) isolates harbored *blaTEM* genes and 15 (29%) isolates carried *blaCTX-M* genes.

Kawamura *et al*. (2014) in Japan characterized 52 *E. coli* isolates from different animal foods for ESBL production. A total of 43 (82.69%), 8 (15.39%) and 1 (1.92%) isolate out of a total of 52 positive ESBL isolates harbored *blaCTX, blaSHV* and *blaTEM* group genes, respectively.

Zarfel *et al*. (2014) analyzed 50 samples of chicken from Austrian slaughterhouses to isolate multi-drug resistant bacteria. A total of 26 *E. coli* isolates were recovered from samples and all were resistant to cefotaxime. Among 26 ESBL-producing *E. coli blaTEM* gene were detected in three isolates.

Le *et al*. (2015) studied a total of 350 food samples including poultry (n = 143) collected in July and November 2013 from a local market in Vietnam. The prevalence of ESBL-producing *E. coli* from poultry (58.7%) was significantly higher than those of pork (32%) and shrimp (18.3%). The isolated ESBL-producing *E. coli* carried predominantly genes of the *CTX-M* class (131/142, 92.3%), followed by the *TEM* (59.9%) and *SHV* (2.1%).

Randall *et al*. (2017) determined the prevalence and types of extended-spectrum β- lactamase (ESBL)-producing *Escherichia coli* in chicken meat (n=397) collected from the UK. All *E. coli* from the chicken samples that were presumptive ESBL producers were confirmed to have at least one ESBL gene. Among all genes, *blaTEM* was detected in 3.8% of samples.

Jhandai (2019) identified 63 *E. coli* isolates from chicken in Hisar and further characterized those isolates based on the carriage of the *blaTEM* gene. The author observed that 73% (46/63) isolates were found to harbour the *blaTEM* gene.

2.6 Presence of Integron Integrase 1 in *E. coli* Isolates

Vasilakopoulou *et al*. (2009) studied the prevalence of class 1 integrons and the gene-cassette content of class 1 integrons in *Escherichia coli* of poultry origin in Greece from a total of 65 *E. coli* isolates in farm poultry. The integron was carried in 49.2% of poultry isolates. Six integron types ranging in size from 663 to 2674 base pairs were identified.

Dessie *et al*. (2013) assessed 99 *Escherichia coli* and 33 *Salmonella* isolates from apparently healthy chicken faeces for the presence of integrons. The author concluded that a total of 70 (70.7%) and 3 (9.1%) integrons were detected in *E. coli* and *Salmonella* isolates, respectively. In the *E. coli* isolates, class 1 integrons (n = 54; 54.5%), class 2 integrons (n = 6; 6.1%) and class 1 and class 2 integrons mutually (n = 5; 5.1%) were detected.

Adelowo *et al*. (2014) isolated 36 *E. coli* from poultry farms which were collected in May and June, 2008 in Nigeria. Out of 36 *E. coli* isolates, class 1 integron was detected in five (14%) isolates and class 2 integrons in six (17%) isolates, while one isolate had both classes of integrons.

Kheiri and Akhtari (2016) isolated and characterized 200 *E. coli* isolates from chickens, humans, cattle and sheep. Class I integron was detected in 50%, 38%, 6% and 16% of chicken, human, cattle and sheep *E. coli* isolates, respectively.

Kumar and Gupta (2019) tested 106 *E. coli* isolates isolated from tissue samples from colibacillosis suspected birds in the Hisar district of Haryana. The results revealed that 37 (34.90%) were found positive for class 1 integrons.

Kumar (2021) identified 225 *E. coli* from poultry farms that were collected from November, 2019 to February, 2020 in Haryana. Class I integron was detected 55.56% of isolates in the study.

2.7 Biofilm Formation by *E. coli* Isolates

Skyberg *et al*. (2006) in the USA studied the biofilm production ability of 105 APEC and 103 AFEC strains on the plastic surface of a microtitre plate. The authors used

three different media for assessing the biofilm formation and found that most of the isolates (75% AFEC and 55.2% APEC) were able to form moderate or strong biofilms in at least one type of media.

Oosterik *et al*. (2014) studied 93 APEC isolates for their biofilm forming potential on different surface materials used in poultry houses in Belgium. The authors observed that the biofilm-forming ability of the same strain varied from 18%-44%. The findings indicated that the materials used in poultry houses were prone to biofilm formation.

Dou *et al*. (2015) in China determined the biofilm forming potential of 243 APEC strains and found that 7.4%, 28.8% and 25.9% of the isolates were strong, moderate and weak biofilm producers, respectively but 92 (37.9%) isolates were unable to form biofilms.

Branco *et al*. (2016) isolated biofilm-producing *E. coli* from drinkers in poultry houses in Brazil. A total of eight out of the fifteen strains of *Escherichia coli* that were detected in drinkers were biofilm producers.

Wang *et al*. (2016) from China found that out of 256 *E. coli* studied, 65 (25.39%) had a strong biofilm-forming ability, 80 (31.25%) produced moderate biofilm, 74 (28.90%) produced weak biofilm and 47 (18.36%) were not able to form any biofilm.

Rodrigues *et al*. (2019) in Brazil determined biofilm formation ability of 238 APEC isolates. A total of 133 strains (55.8%) were classified as biofilm producers. Out of these 133 strains, 55% were from bedding material and 48% from the lesions of the affected birds.

Grakh *et al*. (2022) studied 47 APEC isolates for their biofilm-forming ability from Haryana. The author found that 30 (63.8%) APEC isolates were moderate to strong biofilm producers, of which six were moderate biofilm producers and 24 were strong biofilm producers, while 17 (36.2%) APEC isolates were weak to non-producers.

2.8 Serotyping of *E. coli* Isolates

Blanco *et al*. (1998) determined serogroups of 625 avian *E. coli* isolated between 1992 and 1993 in Spain. According to the author serogroups O12, O14, O18, O53, O78, O81, O102, O115, O116 and O132 were almost exclusively identified among septicemic *E. coli*. They also reported O120 and O17 isolates from poultry.

Wani *et al*. (2004) serotyped 401 *E. coli* isolates from 500 chicken samples in Srinagar. The results revealed that O9, O8, O60 and O25 serogroups were the most predominant strains. The author also found that four and six isolates belonged to O11 and O120 serotypes, respectively.

Irino *et al*. (2005) in Brazil examined 153 fecal samples of cattle for Shiga toxin (*Stx*) production and further characterized those isolates for serotyping. The author

concluded that 25.5% of animals were colonized with STEC and serotypes O113:H21, O178:H19 and O79:H14 were the most frequently associated with STEC while others such as O77:H18, O88:H25 and O98:H17 serotypes also occurred.

Sekhar *et al*. (2017) recovered *E. coli* from fecal swabs of different poultry species (n=150) and farm workers (n=15) in Andhra Pradesh. The author found that 12 virulent *E. coli* isolates belonging to nine different serotypes, of which O2 and O60 were predominant (two each), followed by O49, O63, O83, O101 and O120 (one each).

Awad *et al*. (2020) examined a total of 54 field broiler flocks (n=54) in Egypt, 51.85% of these flocks were positive for *E. coli* by culture and biochemical identification. Further, these isolates were investigated to determine their serotype and the presence of virulence genes. The most common isolated serotype was O115 (14.2%); followed by O142 (10.7%); O158, O55, O125 and O114 (7.1% each); and O27, O20 and O15 (3.5% for each); whereas, O1, O2 and O78 serotypes represent 0%.

Kim *et al*. (2020) isolated 79 APEC isolates from colibacillosis affected chickens from 60 commercial broiler farms in Korea. They further investigated to determine their serotype and the results revealed that the most predominant serogroup was O78 (16 isolates, 20.3%), followed by O2 (7 isolates, 8.9%) and O53 (7 isolates, 8.9%).

Temmerman *et al*. (2020) from Belgium studied 125 APEC strains from broilers with clinical colibacillosis collected from November 2017 to June 2018 and the result revealed that O78 was the most prevalent determined serotype (19% of the strains). Further, 5% and 8% belonged to the O1 and O2 serotypes, respectively.

2.9 Phylogrouping of *E. coli* Isolates

Rodriguez-Siek *et al*. (2005) recognized phylogroup of 524 *E. coli* isolates isolated from avian colibacillosis that was obtained from various locations within the USA. The majority of APEC fell into group A and a substantial number of both APEC (29.6 %) were assigned to group D.

Ramadan *et al*. (2016) in Egypt obtained 29/108 *E. coli* isolates from chicken viscera. The author further characterized *E. coli* isolates based on serotyping, phylogroups and the presence of virulence genes. Phylogrouping revealed a high occurrence of pathogenic phylogroup D (51.7%) followed by A (24.1%), B2 (13.8%) and B1 (10.3%).

Logue *et al*. (2017) in the USA performed a comparative analysis of phylogenetic assignment of human and avian ExPEC *Escherichia coli* using the (Previous and Revised) Clermont phylogenetic typing methods. The author found that according to the revised protocol majority of APEC fell into group C (125/452) followed by F (87/452) and B1 (84/452).

Mittal *et al*. (2022) studied phylogrouping of 64 *E. coli* isolates comprising APEC (n=50) and non-APEC (n=14) in Haryana. The results revealed that 28.1%, 12.5%, 10.9%, 7.9%, 6.2%, 1.6% and 1.6% *E. coli* isolates belonged to phylogroup A, B1, D, F, B2, E and Clade I, respectively. A total of 25% of isolates were of unknown phylogroup and none of the *E. coli* isolates belonged to phylogroup C.

2.10 Isolation of Bacteriophages

Huff *et al*. (2002) isolated a bacteriophage SPR02 against *E. coli* from water samples of municipal sewer treatment plants and a poultry processing plant in Arkansas, USA. Bacteriophage was isolated from bacterial and environmental samples enrichment tubes by serially diluting the supernatant and preparing soft agar overlay plates.

Bacteriophage Esc-A was isolated from sewage samples using the intestinal pathogenic *Escherichia coli* as the host by Xie *et al*. in 2005 in China. Esc-A grew into clear plaques at 37°C, with a plaque of size up to 6 mm.

Jamalludeen *et al*. (2009) discovered seven phages belonging to *Myoviridae* that are effective against *E. coli* serogroups O1, O2 and O78, which are the most common cause of avian colibacillosis from wastewater and fecal samples collected from poultry processing plants in Ontario during the montsh of August and September, 2007. The phages were recovered from wastewater samples collected from chicken processing plants and a mixture of *E. coli* serogroups was used as the host.

PhiF78E, phiF258E, phiF2589E, phiF61E and phiF5318E were isolated from poultry sewage collected randomly from Portuguese poultry houses by Oliveira *et al*. (2009). To screen for the presence of phage, a sewage sample was processed and a spot test method was performed as a screening test. For phage isolation, a procedure based on the modified double layer plaque method was used, followed by single plaque isolation. Two T4-like phages and one T1-like phage were detected among the isolated phages.

Kumar *et al*. (2017) isolated and purified five different bacteriophages (PSP1, PSP4, PSP5, PSP6 and PSP7) against *Salmonella* Typhimurium from sewage where both poultry and swine waste accumulates from five different locations of Pantnagar, Uttarakhand (India). Bacteriophages named PSP 4, PSP 5 and PSP 7 belonged to the family *Siphoviridae* and PSP 1 and PSP 6 were a member of the family *Podoviridae*. Bacteriophage sensitivity was tested by double layer plaque assay and spot-on test.

Ribeiro *et al*. (2018) identified a bacteriophage, EcoM017, from sewage network samples in Brazil and employed in tests to prevent and degrade *E. coli* biofilms. They discovered that it belongs to the family *Myoviridae* and the greatest phage titer decreased *E. coli* bacterial growth and biofilm amount by 90.0% and 87.5%, respectively.

Tawakol *et al*. (2019) isolated bacteriophages specific to APEC O78 from sewage samples. The study was conducted between late 2017 and early 2018 and samples were collected in northern Egypt. They tested the presence of bacteriophages by the spot test method based on the double layer plaque technique.

Vijay (2019) isolated a lytic bacteriophage (*Escherichia* phage BoMT4LUVAS) against *E. coli* from a wastewater sample in Hisar. The author classified the phage under the order *Caudavirales* and the family *Myoviridae* based on the icosahedral head and tail observed by electron microscopy.

Kazibwe *et al*. (2020) purified seven lytic bacteriophages (UPEC01, UPEC03, UPEC04, UPEC06, UPEC08, UPEC09 and UPEC10) against APEC isolates from effluent and droppings from poultry houses in Uganda. The researcher checked their lytic activity against 56 APEC isolates which varied from 1.8% to 17.9%.

Sjahriani *et al*. (2021) discovered eight Lytic bacteriophages against *E. coli* O157:H7 from sewage samples in Indonesia. The author identified bacteriophage belonging to the family *Siphoviridae* and *Myoviridae*.

Abdelrahman *et al*. (2022) isolated three phages identified as ZCEC10, ZCEC11 and ZCEC12 against *E. coli* O18 as a bacterial host from hospital sewage water samples in Egypt. The researcher found that phages under study had typical morphology of the *Siphoviridae* family with an icosahedral head and long thin tail.

2.11 Host Range Determination of Bacteriophages

Jamalludeen *et al*. (2009) tested the spectrum of virulence of two discovered phages EC-Nid1 and EC-Nid2 against 75 *E. coli* strains. Phage EC-Nid1 and EC-Nid2 lysed all the strains that were tested. These included 4 O1, 5 O2 and 3 O78 isolates.

Kulikov *et al*. (2012) studied the characteristics of bacteriophage G7C isolated from horse feces against *Escherichia coli* in Russia. The author determined the host range of bacteriophage G7C against nearly 50 *E. coli* strains isolated from the same specimen of feces and other animals and found that the only strain supporting its growth was *E. coli*.

Huang *et al*. (2013) isolated a virulent *Acinetobacter baumannii* bacteriophage Abp1 belonging to the family *Podoviridae* in China. The author further determined the host range of Abp1 and found that out of the tested 80 *A. baumannii* clinical strains it infected only two strains, thus, Abp1 had a high specificity for the host bacteria AB1.

Kumar *et al*. (2017) studied the host range of five different bacteriophages (PSP1, PSP4, PSP5, PSP6 and PSP7) by spot-on test using a total of 58 *Salmonella* strains. A spot-on test revealed that most bacteriophages exhibited lytic activity against all the *Salmonella* strains used in the study. Host range analysis revealed that bacteriophages

PSP 4, PSP 5 and PSP 7 were able to lyse the maximum number of *Salmonella* strains, whereas bacteriophages PSP 1 and PSP 6 were selective in lysing bacterial strains.

Kazibwe *et al*. (2020) examined the lytic activity of seven isolated bacteriophages (UPEC01, UPEC03, UPEC04, UPEC06, UPEC08, UPEC09 and UPEC10) against 56 APEC isolates in Kampala, Uganda. The results revealed that phage UPEC04 had the broadest host range, inhibiting 10 (17.9%) APEC isolates followed by UPEC06 and UPEC10 at 6 (10.7%) isolates each, then UPEC03 at 5 (8.9%) isolates, UPEC01 and UPEC08 at 4 (7.1%) isolate each; while UPEC09 had the narrowest host range of 1 (1.8%) isolate and only 14 (25%) APEC isolates out of the 56 were sensitive to any one phage and the combined lytic spectrum of UPEC04 and UPEC10 phages includes all the total APEC isolates that were sensitive

Abdelrahman *et al*. (2022) studied the host range of three isolated bacteriophages in Egypt against a variety of *E. coli* and *Salmonella* spp. strains and found that five pathogenic *E. coli* reference strains (*E. coli* O157:H7, *E. coli* O2, *E. coli* O127:H6, *E. coli* ATCC 8739 and *E. coli* O18 (indicator host) exhibited susceptibility toward the individual bacteriophage.

2.12 Biophysical Characterization of Bacteriophages

2.12.1 Thermal sensitivity of bacteriophages

Jepson and March (2004) checked the stability of lambda bacteriophage at different temperatures in the UK. Liquid phage λ stocks were tested at 42°C, 37°C, 20°C and 4°C for up to 181 days and results revealed that titer dropped most rapidly at the higher temperatures, with no viable phage detected after 84 days at 42°C and after 120 days at 37°C. Some loss was observed at ambient temperature, while the liquid phage stock was essentially stable over the period tested at 4°C.

Yang *et al*. (2010) carried out thermal stability testing of Bacteriophage AB1 of *Acinetobacter baumannii* in China. The study revealed that phage AB1 stock solution retained almost 100% infection activity after incubation at 37°C for one month. The results exhibited phage AB1 was extremely heat stable, 73.2% and 64.1% of phages remained alive after 60 min incubation at 50°C and 60°C, respectively; only 0.52% of phages were alive after 60 min incubation at 70°C; while more than 99% phages lost their infection ability in 15 min at 80°C, or 5 min at 90°C.

Hammeri *et al*. (2014) examined the thermal sensitivity of eight isolated bacteriophages at temperatures between 4°C and 70°C for 8 h. The results revealed that at temperatures up to 40°C, the phages remained fully active while higher temperatures caused a gradual loss of activity. The long-term stability of the phages was studied for up to three years. Storage at 4°C and 25°C resulted in a decrease of activity by

approximately 1 $\log_{10}$ unit after one year and by two to three $\log_{10}$ units at the end of the experiment (after three years). Freezing at −20°C decreased the phage titer by four and seven $\log_{10}$ units after one and two years of incubation, respectively, whereas no activity was detectable after three years.

Chen *et al*. (2016) in China assessed the thermal stability of two phages (P483 and P694) against APEC. The results revealed that more than 50% of the phages survived at 50°C or 55°C for 30 min and less than 1% of the phage particles remained active at 60°C but no phage particles survived after incubation at 65°C for 30 min.

Xu *et al*. (2016) isolated a virulent phage QL01 belonging to the family *Myoviridae* against *Escherichia coli* in China. Further assessment of thermal stability revealed that more than 80% of phages survived at 50°C for 40 min, but less than 1% of the phage particle remained active at 70°C.

Kumar *et al*. (2017) determined the thermal sensitivity of five different bacteriophages (PSP1, PSP4, PSP5, PSP6 and PSP7) at temperatures between 30°C and 70°C for 15, 30 and 60 min. Maximum stability was observed at the temperature ranging from 30°C to 50°C. As the temperature increased beyond 60°C, bacteriophage activity started declining and at 70°C no bacteriophage activity was observed.

Kazibwe *et al*. (2020) determined the thermal sensitivity of two bacteriophages (UPEC04 and UPEC10) out of 7 purified phages for temperatures ranging from 20°C-70°C in Uganda. The results revealed that after 30 min of incubation phages were stable to heat with only slight reductions in titers up to 50°C, followed by a steep decline up to 70°C; beyond which they were undetectable. The highest titers were obtained between 20°C-50°C making this the range of temperature at which the two phages are most stable.

Abdelrahman *et al*. (2022) examined the thermal sensitivity of three isolated bacteriophages in Egypt at −20, 4, 37, 50, 60, 70, 75 and 80°C. The results revealed that the titer of the three phages ZCEC10, ZCEC11 and ZCEC12 were stable at approximately 10^8 PFU/mL for 60 min at temperatures of −20, 4, 37, 50 and 60°C. However, when the phages were incubated at 70°C, the titer was stable at 10^8 PFU/mL for ZCEC12 and decreased to 10^7 PFU/mL for both ZCEC10 and ZCEC11. At 75°C incubation, the titer of the phages decreased rapidly to approximately 10^5 PFU/ml. Upon incubation at 80°C, all the titers drastically dropped below the detection limit.

Mozaffari *et al*. (2022) determined the thermal sensitivity of bacteriophages isolated against *E. coli* O157: H7 in Iran. The results of various incubations (4, 25, 37, 45, 65 and 80°C) for 1 h indicated that the bacteriophage stability decreased following increase in temperature. The phage concentration was not significantly affected at 25°C and 37°C incubations at which the phage titer reduced by 1.2 $\log_{10}$ (PFU/mL) in

comparison to 4°C. The bacteriophage concentration was notably diminished following the incremental temperatures of 45°C, 65°C and 80°C. The bacteriophage concentration decreased from 10^9 PFU / mL to 10^6 PFU / mL at 65°C and 10^4 PFU/mL at 80°C. The results indicated a decrease in phage levels to 4.7 $\log_{10}$ at 80°C in comparison to 4°C. Furthermore, there was no change in the bacteriophage titer after three months of storage at 4°C and the phage retained its stability at this temperature.

2.12.2 pH sensitivity of bacteriophages

Jepson and March (2004) checked the stability of lambda bacteriophage at different pH (2-14) for 24 h in the UK. Over this period, phage was stable in the pH range 3-11, with no significant drop in titer observed. Between pH 11.2 and 11.8, a 2 $\log_{10}$ drop in titer was observed over the 24 h while no viability was seen at all above pH 11.8. At very low pH values a rapid drop in viability was observed, with a 9 $\log_{10}$ drop in titer after 5 min of storage at pH 2.0, although for the same period at pH 2.2 the drop in titer was only 1 $\log_{10}$, marking a sudden transition in phage stability between pH 2.0 and 2.2.

Yang *et al*. (2010) carried out pH stability testing of Bacteriophage AB1 isolated against *Acinetobacter baumannii* in China. The study revealed that no reduction in titer of phage at pH 6.0 after one-hour incubation, while different reduction percentages were obtained at other pH ranges, with only 42.9% recovery of infectious phage AB1 at pH 5.0. These results suggested that extreme pH ranges might affect phage AB1 stability.

Niu *et al*. (2012) studied acid tolerance of bacteriophage AKFV33, a T5-like phage of the family *Siphoviridae*. The results revealed that titers of phage AKFV33 dropped by 1.99 $\log_{10}$ PFU/ml at pH 3.0 within 15 min and were undetectable after 2 h.

Kumar *et al*. (2017) determined the pH sensitivity of 5 different bacteriophages (PSP1, PSP4, PSP5, PSP6 and PSP7) at pH 3 to 11, adjusted with NaOH or HCl, for 1 h at 37°C. The activity of 5 bacteriophages was relatively stable at pH 6 to 9 and declined dramatically at high as well as low pH. According to their results, no bacteriophage activity was detected at pH 3 and 11.

Kazibwe *et al*. (2020) determined the pH sensitivity of two bacteriophages (UPEC04 and UPEC10) out of seven purified phages for pH ranging from 2-12 at both 25°C and 40°C. The findings revealed that phages retained viability across the different pH values with the lowest titers registered at the extremes of pH (2 and 12), while the highest titers were registered between pH 4 and 8. The changes in the titers followed a similar pattern at the two temperatures, though the titers were consistently higher at 25°C compared to 40°C.

Abdelrahman *et al*. (2022) examined the pH sensitivity of three isolated bacteriophages in Egypt. The results indicated that out of three phages, ZCEC12

remained viable at a pH range of 4.0-11.0 at approximately 10^8 PFU/mL, while the titers of ZCEC10 and ZCEC11 decreased to 10^7 PFU/mL. When incubated at pH 2, 3 and 12, the phages were utterly inactive. Thus, the optimum pH range for phages ZCEC10, ZCEC11 and ZCEC12 was found to be 4.0-11.0.

2.13 One-Step Growth Studies of Bacteriophages

Fan *et al*. (2012) in China isolated 18 *E. coli* phages from hospital sewage. The author further characterized the broad-spectrum phage, belonging to *Myoviridae* family and the growth curve suggested the latent time was 30 min and the burst size was 43.

Aljarbou and Aljofan (2014) isolated a phage against *Neisseria* and determined the latent period and burst size by one step growth curve method. They found that the latent period was 25 min and the average burst size of the three experiments was calculated to be 24±2 virus particles per bacterial cell.

Lee and Park (2015) isolated two phages of *Escherichia coli* O157:H7 from a sewage sample in South Korea. The author identified isolated phages BECP2 and BECP6, which belonged to the *Myoviridae* and *Podoviridae*, respectively. Further characterization of these phages concluded that burst sizes were 33 PFU/cell for BECP2 and 51 PFU/cell for BECP6 after a latent period of 25 min.

Dalmasso *et al*. (2016) isolated and characterized three coliphages (ϕAPCEc01, ϕAPCEc02 and ϕAPCEc03) in Ireland from human fecal samples. The author found that ϕAPCEc01 belonged to *Myoviridae* and had a latent period and burst size of 60 min and 90.3±1.4 phage particles, respectively. Further characterization of ϕAPCEc02 and ϕAPCEc03 revealed that they belonged to *Siphoviridae* and had small burst sizes of 30.8±1.9 and 47.4±11.3 phage particles, respectively.

Liu *et al*. (2019) in Beijing isolated a phage ST20 from a wastewater sample from a sewage treatment plant. The author characterized the phage as a member of the family *Siphoviridae* based on morphological observations. Further, they performed a one-step growth curve experiment to determine the generation time, including the burst size and latent period (38±2 min) of phage ST20 at 37°C.

2.14 Whole Genome Sequencing of Bacteriophages

Kim *et al*. (2013) studied the genome of PBECO4 infecting *Escherichia coli* O157:H7 belonging to the family *Myoviridae* in Korea and found that genomic DNA contains 348,113 base pairs with a GC content of 34.09 %. A total of five hundred fifty-one putative open reading frames (ORFs) and six tRNAs were found within the genome sequence. The author functionally annotated 61 ORFs among the total of 551 ORFs and the rest 490 didn't match any known protein.

Hendrix *et al*. (2015) in the USA studied the genome sequence of *Salmonella* Phage X belonging to the *Siphoviridae* family. The results revealed that the *Salmonella* Phage X genome was 59,578 bp long and had 56.5% G/C content. The phage X genome contained 75 predicted protein-coding genes and no tRNA genes.

Amarillas *et al*. (2016) characterized bacteriophage phiC119 against *Escherichia coli* O157:H7 in Mexico. The study revealed that the genome sequence of phiC119 consisted of 47,319 bp with a GC content of 44.20% and it encoded 75 putative proteins, but lysogeny and virulence genes were not found in the phiC119 genome.

Tabassum *et al*. (2018) in Lahore analyzed the genome of bacteriophage TSK1 exhibiting potent lytic activity against *K. pneumoniae* Author found that the genome was 49836 bp long with the GC content of 50.44% and comprised 75 predicted open reading frames (ORFs), while no tRNA was found.

Amarillas *et al*. (2020) isolated and characterized a new *Siphoviridae* phage, named "*Pseudomonas* phage vB_PsyS_Phobos" in Mexico. The study revealed that the genome of Phobos was dsDNA of 56,734 bp with a GC content of 63.3%, containing 65 ORFs. The author further assigned the predicted ORFs based on sequence similarities and function could only be assigned to 26 of the predicted ORFs, 37 ORFs shared sequence similarity with phage proteins of unknown function,

Sorensen *et al*. (2020) isolated, sequenced and characterized 38 *Escherichia coli*-infecting phages (coliphages) from poultry feces in Belgian. The results revealed that all the phages belonged to either the *Siphoviridae* or *Myoviridae* family and their genomes ranged between 44,324 and 173,384 bp, with a G+C content between 35.5 and 46.4%.

Kumar *et al*. (2021) in India performed the whole-genome analysis of a bacteriophage HCF1 infecting *Citrobacter* and the results revealed that the HCF1 genome has a double-stranded linear DNA molecule of 45.8 kbp size with a GC content of 44.5%. Based on RAST analysis, the author identified 71 putative ORFs, However, the tRNA gene was not found in the HCF1 genome.

2.15 Restriction Endonucleases Profiling of Bacteriophages

Goodridge *et al*. (2003) in Canada characterized two coliphages, AR1 and LG1, based on their morphological, host range and genetic properties. The author suggested that both phages belonged to *Myoviridae* family based on TEM and phage LG1 and AR1 DNA were completely digested by *SspI* and *TaqI* upon restriction endonucleases digestion.

Kakoma (2009) isolated nine bacteriophages from sewage samples and five from chicken faecal matter against *E. coli* K12 in Africa. The author carried out restriction endonucleases profiling using REs *viz*. *EcoRI*, *AluI*, *Acc651*, *BamHI*, *BgIII*, *HaeIII*,

HindIII, *PstI*, *XbaI*, *SalI* and *PotI* and none could cleave the phage DNA. The author explained that a possible reason for this is that the nucleic acid is methylated and therefore, the coliphages remained insensitive to enzyme activity.

Bhagya Raj (2014) in India characterized 2 phages ϕ EC26 and ϕ SI55 isolated against *Escherichia coli* and *Salmonella*, respectively. The author found that based on morphology they belonged to the order *Caudavirales* and family *Siphoviridae* and their restriction endonucleases profiling suggested that they produced more than eight and ten restriction sites for ϕ EC26 and ϕ SI55 DNA with restriction enzyme *HaeIII*, respectively and partially digested by *EcoRI* enzyme.

Torabi *et al*. (2021) characterized bacteriophage Pϕ-Bw-Ab which was isolated against *Acinetobacter baumannii* in Iran. The author compared enzyme digestion patterns of different REs and found that phage was completely digested by *HindIII* and *EcoR1*.

2.16 LD50 Dose Rate of APEC *E. coli*

Giovanardi *et al*. (2005) in Italy obtained 59 *E. coli* isolates from 4 flocks that exhibited signs of colibacillosis. The author further characterized those isolates for serotyping and performed a chicken pathogenicity test, using O78 and O139 strains of *E. coli* in unvaccinated day-old chicks. The author observed 100% mortality in day-old birds when inoculated subcutaneously with 10^8 CFU of each strain.

Nagano *et al*. (2012) performed lethality tests using parent and attenuated mutant of APEC serovar O78 on 5-week-old SPF chickens in Japan. The result of LD50 testing revealed that LD50 for the parent and the mutant strain was 8.0×10^7 CFU/bird and 6.2×10^8 CFU/bird, respectively when administered through the i/v route.

Li *et al*. (2016) in China determined the LD50 of the wild-type APEC (DE205B), deletion mutant (DE205BδireA) and complementary strain (DE205BCΔireA) using 7-day-old ducklings. LD50 calculated for wild-type, deletion mutant and complementary strains were 1.74×10^5, 2.45×10^5 and 3.16×10^5, respectively. The author observed no significant difference between the LD50 of the wildtype and deletion mutant strains.

Hussain *et al*. (2017) performed *in vivo* experiment using two-day-old broiler chickens to determine the LD50 of three highly virulent and resistant *E. coli* isolates (112, 357 and 381) in China. The author observed that for isolate 381, LD50 was 5.6×10^6 and 2.3×10^5 with oral and intraperitoneal administrations, respectively; for isolate 112, LD50 was 1.3×10^8 and 1.2×10^8 during oral and intraperitoneal administrations, respectively; for isolate 357, LD50 was 1.3×10^8 and 2×10^8 with oral and intraperitoneal administrations.

Liu *et al*. (2020) in China studied the median lethal dose (LD50) of *E. coli* APEC strain DE205B and its mutant Prophage phiv205-1 through chick challenge test in Seven-

day-old chicks. The author calculated LD50 when inoculated intramuscularly of the mutant strain and wild strain as 3.13×10^5 CFU and 3.86×10^4 CFU, respectively.

Landman *et al*. (2021) studied the LD50 of three EPS (*E. coli* peritonitis syndrome) strains isolated from bone marrow in hens in the Netherlands. Author calculated LD50 of strain 1 (serogroup O1), strain 3 (serogroup O78) and strain 6 (serogroup O2) as <2.7 $\log_{10}$ CFU (5×10^2), 3.8 $\log_{10}$ CFU (6.3×10^3) and 5.3 $\log_{10}$ CFU (2×10^5), respectively.

2.17 Bacterial Challenge Test

O'Flynn *et al*. (2004) performed *in vitro* challenge trials of phage e11/2, e4/1c, pp01 and their cocktail against *E. coli* O157:H7 in Ireland. The results revealed at MOI 100, a reduction in viable numbers of the culture to below detectable levels were observed. The author observed phage e4/1c caused a 3-log-unit reduction in the number of viable cells within 2 h and a 5-log-unit reduction was seen in 1 or 3 h after treatment with the cocktail or phage pp01.

Han *et al*. (2013) in Korea assessed the bacteriolytic activity of phage SAH-1 against the *S. aureus* N315 strain at MOIs of 0, 0.01, 1 and 100. The results revealed that the bacterial strain was not infected by phage at MOI 0.01, the absorbance (OD600) continued to increase during the incubation but bacterial growth was strongly prohibited at MOI 100 after phage infection.

Wang *et al*. (2016) performed *in vitro* bacteriolytic activity of phage SLPW against *S. aureus* in China. The phage bacteriolytic activity was assessed by monitoring the cell absorbance of the culture solution (OD600) at 30 min intervals for up to 4hr at MOI of 0.01, 1 and 100 and the result revealed that the growth of these strains steadily declined at an MOI 1 and was completely inhibited at MOI 100. However, the author observed the absorbance (OD600) continued to increase during the incubation when the culture was administered using phage SLPW at MOI 0.01.

Amarillas *et al*. (2016) performed a bacterial challenge test of phage isolated against *E. coli* O157:H7 at an MOI of 0.1, 1.0 and 100 in Mexico. The author monitored bacterial growth by turbidity measurements at every 30-min interval for 4 h using OD600 nm and the result revealed that significant decreases in the viability of bacterial strains were observed, mainly in cells infected with an MOI of 1.0 and 100.

Pereira *et al*. (2017) studied the lytic activity of phage Φ241 against its natural host *E. coli* O157:H7 B0241 and investigated at MOI of 10, 3, or 0.3, respectively. The results indicated that phage infection at MOI of 3 or 10 there was a 3 or 4.5-log decrease in the cell concentration within 1 h. It was observed that at MOI of 0.3, there was less

than 0.5-log reduction during the first hour, but rapid cell lysis (3-log reduction) during the second hour.

Mozaffari *et al.* (2022) investigated the lytic activity of the VaT-2019a bacteriophage with MOI of 1 against *E. coli* O157: H7 in Iran. They observed that after two hours of incubation, the bacterial concentration decreased by 2.65 $\log_{10}$ in the B.P (bacteria + phage) group compared to the B.C (bacteria) group. Based on the results of the independent t-test author concluded that VaT-2019a bacteriophage at MOI of 1 reduced the amount of *E. coli* to an acceptable level.

2.18 Phage Therapy

Barrow *et al*. (1998) determined the effect of bacteriophage administration on mortality in chickens at United Kingdom. The result of the study revealed that in the absence of phage administration, the *E. coli* produced almost 100% mortality in both 3-week-old and newly hatched chickens irrespective of *E. coli* administration route i.e., intramuscular as well as intracran routes of inoculation. During *E. coli* and phage adminstration by the intramuscular route (in different muscles) in equal numbers of both (10^6 CFU and PFU), no morbidity or mortality was observed in chickens.

Park *et al*. (2000) in Japan studied the kinetics of orally administered *Pseudomonas plecoglossicida* and phage in cultured ayu fish (*Plecoglossus altivelis*) after oral administration in form of pellets. The study revealed that in infected control groups, fish began to die seven days after bacterial challenge, and the mortality was 65.0% during initial two weeks period. However, the fish receiving phage-impregnated feed, died later and the mortality was less (22.5%) in this group. The results indicated that mortality in phage-treated fish was significantly less as compared to the mortality in control group.

Huff *et al*. (2002) performed a study to determine the efficacy of a cocktail of two bacteriophages, SPRO2 and DAF6 via aerosol spray to protect broiler chickens from air sac challenge of *E. coli*. The results indicated that there was 30% mortality in the birds sprayed with the phage and challenged with *E. coli* as compared to 60% mortality in the birds challenged with *E. coli* only at 7 d of age.

Huff *et al.* (2004) conducted a study to evaluate the therapeutic efficacy of cocktail of two bacteriophages (DAF6 and SPR02) and enrofloxacin individually and also in combination of phages with enrofloxacin to treat colibacillosis. The results revealed that mortality in the birds challenged with *E. coli* and untreated was 68%, however, cocktail of bacteriophages and enrofloxacin individual treatments significantly decreased mortality to 15% and 3%, respectively. It was observed that total protection in birds that received both the bacteriophage and enrofloxacin representing a significant synergy.

Huff *et al*. (2006) conducted a study to evaluate the therapeutic efficacy of bacteriophage (SPR02) through the intramuscular route. The study indicated bacteriophage treatment at 10^8 PFU titer significantly reduced mortality from 48% in the birds challenged with *E. coli* (positive control) to 7% in the treated group.

Oliveira *et al*. (2009) determined the *in vivo* efficacy of bacteriophage (phi F78E). The experimentally infected birds were treated with phi F78E both orally and by spray at two different titers (10^7 PFU/ml and 10^9 PFU/ml). The results revealed that use of highly concentrated phage suspension reduced the mortality by 25% and morbidity by 41.7%.

Lau *et al*. (2010) determined the *in vivo* efficacy of bacteriophage EC1 at MOI 100 against *E. coli* O78 in chicks in Malaysia. The authors observed that the mortality rate of birds during the three-week experimental period decreased from 83.3% in the untreated *E. coli* challenged birds to 13.3% in birds treated with bacteriophage EC1. Further effect of phage on the body weight of chicks was also observed it was found that the body weight of *E. coli* challenged birds treated with bacteriophage EC1 was 15.4% higher as compared to the untreated *E. coli* challenged birds on 21st day post-infection.

Mohammad *et al*. (2019) studied *in vivo* performance of phages in treating colibacillosis in quails in Iran. The study indicated that total mortality rate decreased from 46.6% in the untreated *E. coli* challenged group to 26.5% and 13.6% in the *E. coli* challenged groups treated with single phage TM3 or cocktail of 4 phages, respectively.

Tawakol *et al*. (2019) attempted *in vivo* evaluation of bacteriophage treatment after challenging the chicks with APEC O78 and infectious bronchitis virus (IBV). They compared the clinical lesions and mortality in bacteriophage treated and untreated groups after individual challenge with APEC O78, IBV or both. The study revealed that bacteriophage treatment delayed the onset of the clinical signs to six days post-challenge (dpc) and in addition markedly reduced the severity of clinical sign in both groups. Further, bacteriophage treatment was not found to be associated with mortality in single APEC or mixed APEC and IBV infected groups. In contrast, birds challenged with APEC alone and mixed APEC and IBV infection without bacteriophage treatment indicated a 16% and 29% mortality rate at eight- and seven-days post-infection, respectively.

Ngu *et al*. (2022) evaluated the efficacy of bacteriophage B1 and B2 against *Salmonella enterica* subsp. enterica serovar Typhimurium infected Noi chicken, a native Vietnamese broiler breed in Vietnam. The study indicated that the mortality in phage treated group was lesss (11.1%) as comared to untreated group (51.1%) after four weeks of infection. The authors also reported that bacteriophage administration had resulted in increased weight gain particularly on inclusion of both phages in the treatment.

CHAPTER-III

MATERIALS AND METHODS

The present study was carried out at Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, LUVAS, Hisar (Haryana). The samples for *E. coli* isolation were collected from poultry birds presented for disease diagnosis at the Department of Veterinary Public Health and Epidemiology, COVS, LUVAS. For bacteriophage isolation, the samples were collected from poultry farm waste and excreta, biogas plant, sewage treatment plant and Ganges water, Haridwar.

3.1 MATERIALS

3.1.1 Media and Reagents for Isolation and Identification of *E. coli* and Bacteriophage

The molecular biology grade reagents and chemicals were used for preparation of various solutions using autoclaved distilled water. The nutrient agar (NA), MacConkey lactose agar (MLA), brain heart infusion (BHI) broth, eosin methylene blue (EMB) agar, Luria-Bertani (LB) agar and broth, Muller Hinton agar (MHA), nutrient broth (NB), phosphate buffered saline (PBS, pH = 7.2), Simmon's citrate agar, Kovac's reagent, methyl red (MR) reagent, Barritt's reagents, oxidase disc, MR-VP medium (glucose phosphate broth), sodium chloride, calcium chloride (0.1 M), magnesium chloride (0.1 M), SM buffer, glycerol etc. were procured from the Hi-Media Laboratories Pvt. Ltd., Mumbai. All the media were sterilized by autoclaving at 15 psi at 121°C for 15 min. Sterility of media was checked by incubating at 37°C for 24 h. The contamination free media were used for further study.

3.1.2 Glassware/ Plastic Ware

Sterile nuclease free 0.5 ml, 1.5 ml and 2 ml micro centrifuge tubes (MCT), 0.2 ml PCR tubes, 15 ml and 50 ml centrifuge tubes, micropipette tips; sterile sample container, cryo vials 1.8 ml (Tarsons Products Ltd.), petri dish disposable (HiMedia Laboratories Pvt. Ltd.), 250 ml, 500 ml, 1 L and 5 L conical flasks (Borosil Glass Works Ltd.) were procured from suppliers as indicated. Disposable sterile syringes (5 ml and 10 ml) and 0.22 µ millipore syringe driven membrane filters (Merck) were used for sterilization of buffers and purification of phages. L-spreader, sterile cotton swabs and sterile inoculation loops (HiMedia Laboratories Pvt. Ltd.) were used for bacterial culture.

3.1.3 Equipments

The major equipments used for conducting the research work were refrigerator (Samsung Electronics Co., Ltd.), deep-freeze (Celfrost Innovations Pvt. Ltd.), horizontal laminar air flow (Thermo Fisher Scientific - IN.), water-bath (Grant Instruments India Pvt. Ltd.), water distillation system (Borosil Glass Works Ltd.), UV-transilluminator (Anachem Laboratories and Consultancy Pvt. Ltd.), gel documentation system (Biozen laboratories), submarine horizontal electrophoresis system (Biometra GmbH), electronic weighing balance (Mettler-Toledo International Inc.), incubator (Memmert GmbH + Co.), hot air oven (Scientific Instrument India Ltd), micropipettes (200-1000 µl, 20-200 µl, 10-100 µl, 0.5-10 µl) (Eppendorf AG), thermal cycler (Applied Biosystem, Thermo Fisher Scientific corporation), versaMax ELISA reader (Molecular Devices, LLC), spinix vortex mixer (Tarsons Products Ltd.), VITEK®2 Compact system (BioMerieux India Pvt. Ltd.), Refrigerated microcentrifuge (Thermo Fisher Scientific - IN.), ultrapure water filteration unit (Merek Millipore Pvt. Ltd.), biophotometer (Eppendorf AG), ultracentrifuge (Beckman Coulter Life Sciences) etc.

3.1.4 Antimicrobial Discs

The antimicrobial discs listed in Table 3.1 were obtained from HiMedia Laboratories Pvt. Ltd., Mumbai and were used as per recommended protocol of manufacturer.

Table 3.1 - List of antimicrobials used for antimicrobial sensitivity of *E. coli*

Antimicrobials	**Disc conc.**	**Antimicrobials**	**Disc conc.**
Penicillin's		**Aminoglycosides**	
Ampicillin (AMP)	10 mcg	Amikacin (AK)	30 mcg
Pipercillin (PI)	100mcg	Gentamicin (GEN)	10 mcg
Beta lactam		Streptomycin (S)	10 mcg
Amoxyclav (AMC)	30 mcg	**Tetracycline**	
Cephalosporin's		Tetracycline (T)	30 mcg
Ceftriaxone (CTR)	30 mcg	**Lipopeptide**	
Cefpodoxime (CPD)	10 mcg	Colistin (CL)	10 mcg
Cefotaxime (CTX)	10 mcg	Polymyxin B (PB)	
Cefotaxime/Clavulanic acid	30/10 mcg	**Quinilones**	
Ceftazidime (CAZ)	30 mcg	Ciprofloxacin (CIP)	5 mcg
Ceftazidime/Clavulanic acid	30/10 mcg	Levofloxacin (LE)	5 mcg
Monobactam		**Folate pathway**	
Aztreonam (AT)	30 mcg	Trimethoprim (TR)	5 mcg
Carbapenems		**Nitrofurans**	
Imipenem (IPM)	10 mcg	Nitrofurantoin (NIT)	300 mcg
Macrolides			
Azithromycin (AZM)	15 mcg		

3.1.5 Reference Strain

E. coli positive strain (ATCC 25922), beta lactamase positive strain of *Klebsiella pneumoniae* (ATCC 700603) and *Staphylococcus aureus* (ATCC 25923) (Hi-Media Laboratories Pvt. Ltd.) available in the department were used.

3.1.6 Commercial Kits

Gram's staining kit (HiMedia) was used for identification of *E. coli*. Wizard DNA cleanup kit (Promega) was used for isolation of bacteriophage genome.

3.1.7 Polymerase Chain Reaction Chemicals

Tris acetate EDTA (TAE) (50X) (Thermo), TE buffer (Takara), agarose molecular biological grade (Thermo Fisher), ethidium bromide (Sigma, USA), master mix (Sapphire fast PCR- hot start master mix 2X), gene ruler 100 bp DNA ladder (GeNei, India), loading dye (Thermo Scientific), nuclease free water (Thermo Scientific) used in the study were procured from the mentioned reputed firms.

3.1.8 Oligonucleotide Primers

Highly purified salt free (HPSF) oligonucleotide primers in lyophilized form were procured from Sigma-Aldrich Chemical Pvt. Ltd, Bangalore. The primers were reconstituted with nuclease free water to make a stock solution of 100 pmol/µl and working solutions of 10 pmol/µl and stored at -20°C. The detail of different primers used is given in Table 3.2.

Table 3.2 - Detail of primers used for confirmation and characterization of *E. coli*

Gene	Primers used	Primer's sequence	Amplicons size (base pairs)	Reference
Alkaline phosphatase	*PhoA*	F-5'- GGTAACGTTTCTACCGCAGAGTTG-3'	468	Shome *et al.*, 2011
		R-5'-CAGGGTTGGTACACTGTCATTACG-3'		
Beta lactamase	*blaTEM*	F-5'-TTGGGTGCACGAGTGGGTTA-3'	504	Ozgumus *et al.,* 2008
		R-5'-TAATTGTTGCCGGGAAGCTA- 3'		
Integron integrase 1	*IntI1*	F-5'- ACGAGCGCAAGGTTTCGGT-3'	565	Kumar and Gupta, 2019
		R-5'-GAAAGGTCTGGTCATACATG- 3'		
Salmochelinsiderophore receptor gene	*iroN*	F-5'-AATCCGGCAAAGAGACGAACCGCCT-3'	553	Johnson *et al.*, 2008
		R-5'-GTTCGGGCAACCCCTGCTTTGACTTT-3'		
Episomal outer membrane protease gene	*ompT*	F-5'-TCATCCCGGAAGCCTCCCTCACTACTAT-3'	496	
		R-5'-TAGCGTTTGCTGCACTGGCTTCTGATAC-3'		
Putative avian Hemolysin	*hlyF*	F-5'-GGCCACAGTCGTTTAGGGTGCTTACC-3'	450	
		R-5'-GGCGGTTTAGGCATTCCGATACTCAG-3'		
Episomal increased serum survival gene	*iss*	F-5'-CAGCAACCCGAACCACTTGATG-3'	323	
		R-5'-AGCATTGCCAGAGCGGCAGAA-3'		
Aerobactinsiderophore receptor gene	*iutA*	F-5'-GGCTGGACATCATGGGAACTGG-3'	302	
		R-5'-CGTCGGGAACGGGTAGAATCG-3'		
Quadruplex	*chuA*	F-5′-ATGGTACCGGACGAACCAAC-3′	288	Clermont *et al.*, 2013
		R-5′-TGCCGCCAGTACCAAAGACA-3′		
	yjaA	F-5′-CAAACGTGAAGTGTCAGGAG-3′	211	
		R-5′-AATGCGTTCCTCAACCTGTG-3′		
	TspE4.C2	F-5′-CACTATTCGTAAGGTCATCC-3′	152	
		R-5′-AGTTTATCGCTGCGGGTCGC-3′		
	arpA	F-5′-AACGCTATTCGCCAGCTTGC-3′	400	
		R-5′-TCTCCCCATACCGTACGCTA-3′		
Group E	*ArpAgpE*	F-5′-GATTCCATCTTGTCAAAATATGCC-3′	301	Lescat *et al.,* 2013
		R-5′-GAAAAGAAAAAGAATTCCCAAGAG-3′		
Group C	*trpA*	F-5′-AGTTTTATGCCCAGTGCGAG-3′	219	
		R-5′-TCTGCGCCGGTCACGCCC-3′		

3.2. METHODS

3.2.1 Isolation, Identification and Confirmation of *E. coli* Isolates from Colibacillosis Infected Chickens

3.2.1.1 Preparation of glassware for culture work

All the glassware were cleaned, washed and sterilized by standard procedures. The glassware used were dipped in chromic acid solution overnight, rinsed with tap water, dried in hot air oven at 45°C for 1 h, wrapped with aluminium foil and brown paper after cooling and kept again in hot air oven at 160°C for 1 h for sterilization.

3.2.1.2. Collection of tissue samples

The tissue samples were collected from 50 poultry flocks presented for disease diagnosis at Veterinary Public Health and Epidemiology, COVS, LUVAS, Hisar. One poultry bird exhibiting typical colibacillosis lesions like pericarditis, perihepatitis, air sacculitis etc (Fig.3.1) was selected from each flock. The various tissue samples like liver, lung and spleen were collected aseptically from such bird for further processing. The consent of farmers was obtained before collection of samples. All the collected samples were kept in icebox at 4°C and brought to the laboratory of VPHE, department for further processing on the same day.

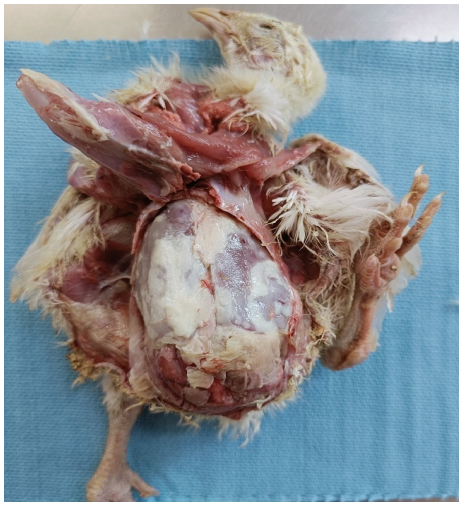


Fig. 3.1 - Typical lesions of colibacillosis in chick

3.2.1.3 Processing of tissue samples for bacterial isolation

All the collected samples were processed for isolation and identification of *E. coli* using standard methods as described earlier (Holt *et al*., 1994) with certain modifications. The collected tissue samples were inoculated in MacConkey broth (1:10), thoroughly mixed and incubated at 37°C for 24 h. A loopful of enriched inoculum was streaked on MacConkey lactose agar (MLA) and incubated at 37°C for

24 h. After incubation a loopful of small, round and pink colored colonies from MLA were streaked on eosin methylene blue (EMB) agar and incubated at 37°C for 24 h. The colonies on EMB agar with metallic sheen were further identified and confirmed by Gram staining, biochemical and molecular methods.

3.2.1.5 Preliminary biochemical confirmation of *E. coli* isolates

Presumptive identification of *E. coli* was carried out by colony characteristics, Gram staining and IMViC test (Cowan and Steels, 1974).

All the 50 presumptive *E. coli* isolates were subjected to Gram's staining. The isolates were smeared over a clean grease free glass slide and subjected to Gram's staining procedure. The isolates having Gram negative and pink colour with rod shape were further examined by IMViC test. For indole test, a loopful of culture was inoculated in a test tube containing tryptone water and incubated at 37°C for 24 h. Later on, Kovac's reagent (0.5 ml) was added to the culture tube and shaken gently. A positive reaction was indicated by appearance of a red-coloured ring on the top of culture tube, whereas no change in colour of reagent indicated a negative reaction. For methyl red (MR) test, a loopful of culture was inoculated in a test tube containing MR-VP broth (glucose phosphate broth) and incubated at 37°C for 24 h. Later 2-3 drops of methyl red indicator were added to the incubated broth. A positive reaction was indicated by appearance of distinct red color. No change in the colour of the broth indicated a negative reaction. Voges-Proskauer (VP) test was carried out by Barritt's method, in which a loopful of culture was inoculated in a test tube containing MR-VP broth (glucose phosphate broth) and incubated at 37°C for 24 h followed by addition of few drops of 5 per cent solution of α-naphthol in absolute ethanol (Barritt A) and 40% potassium hydroxide (Barritt B). Development of a pinkish-red colour at the surface of the tube was indicative of a positive test. No change in the colour of the broth indicated a negative reaction. In citrate utilization test, slant of Simmon's citrate agar was prepared and streaked with the culture, this inoculated slant was left for incubation at 37°C for 24-48 h. Bacteriological growth with the development of intense blue colour in the medium was considered as a positive reaction for citrate utilization, whereas, no change in slant colour i.e., green colour indicated negative reaction.

The bacterial exhibiting positive reaction for indole and MR test and negative reaction for VP and citrate test were considered as biochemically confirmed *E. coli.*

3.2.1.6 Molecular confirmation of presumptive *E. coli* isolates

3.2.1.6.1 DNA extraction

The genomic DNA extraction from tentatively confirmed *E. coli* colonies was carried out using boiling method (Englen and Kelley, 2000) with some modification. Briefly, loopful of colonies were suspended in 100µl TE buffer (pH-8.3) followed by centrifugation at 1000 rpm for 3 min. The suspensions were then boiled at 100°C for 10 min, snap chilled immediately on ice and centrifuged at 10000 rpm for 10 min. The supernatant was transferred to clean nuclease free tubes and stored at -20°C till further use.

3.2.1.6.2 PCR confirmation of *E. coli*

DNA sample extracted from tentatively confirmed colonies were subjected to *E. coli* specific PCR targeting alkaline phosphate gene (*PhoA gene*). The PCR was performed using primer pair (Shome *et al.*, 2011) as detailed in Table 3.2. The DNA extracted from ATCC 25922 was used as positive control for *E. coli*. The PCR reactions were carried out in 25 µl volume consisted of 12.5 µl of master mix, 0.5 µl of 10 µM solution of each primer (forward and reverse), ~200 ng of the DNA sample and remaining nuclease free water (NFW) to make 25.0 µl in thermocycler. The thermal cycler conditions used for PCR are described in Fig. 3.2.

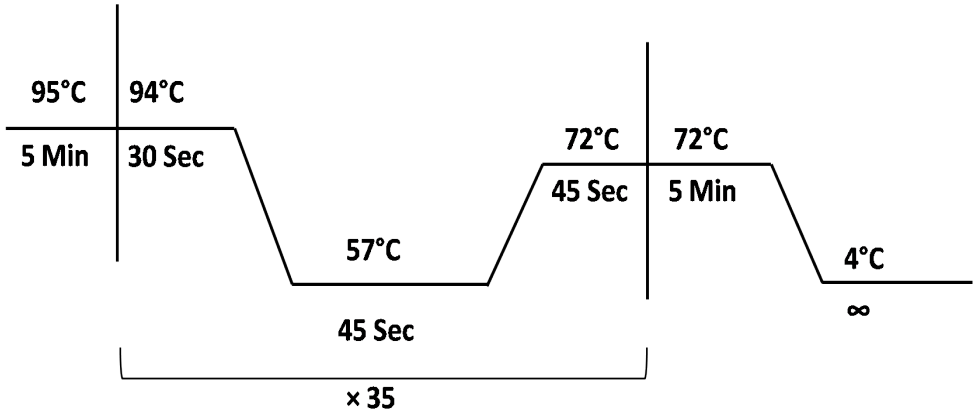


Fig. 3.2 - PCR conditions for *PhoA* gene of *E. coli*

3.2.1.6.3 Procedure for agarose gel electrophoresis

Agarose at a final concentration of 1.5% was suspended in 1-X Tris-acetate EDTA (TAE) buffer and heated to dissolve it completely. The solution was allowed to cool at 55°C and ethidium bromide was added to a final concentration of 0.5µg/ml. A gel tray was prepared using the gel cast. Upon solidification, the comb was removed and the gel was immersed in 1-X TAE buffer in horizontal submarine electrophoresis tank. A total of 10 µl of amplified product was loaded into agarose gel well and electrophoresed in 1X TAE at 70 V for 2 h. The size of the amplified product was

ascertained by comparison with standard 100 bp DNA marker. The amplified products were observed and photographed by gel documentation system.

3.2.1.7 Vitek confirmation of presumptive *E. coli* isolates

The Vitek 2 Compact system was used to confirm the provisionally confirmed *E. coli* isolates. Gram negative (GN) reagent cards were used to identify gram-negative bacteria according to the manufacturer's guidelines. Briefly, using a 12×75 mm polystyrene test tube, *E. coli* colonies were suspended in 3.0 ml of sterile saline solution (0.50% NaCl, pH 4.5 to 7.0). The McFarland turbidity range was adjusted to 0.50-0.63 using a turbidity metre (DensiCheckT™). The Vitek 2 Compact system was loaded with cassettes containing tubes and reagent cards and the findings were analysed according to the manufacturer's instructions.

3.2.2 Antibiotic Susceptibility Profiling of *E. coli* Isolates

The bacterial isolates were subjected to *in vitro* antibiotic sensitivity test as per the method of Bauer *et al*. (1966). Commercially available antibiotic discs were used to test sensitivity of the isolated bacteria against the different class of antibiotics as per CLSI guidelines (Table 3.1). The isolates were grown in nutrient broth until the turbidity attaining similar to 0.5 McFarland standard. The MHA plates, pH 7.2-7.4 were inoculated with inoculum with the help of a sterile cotton swab. The surface of media was uniformly inoculated with the help of swab to ensure even distribution. Then antibiotic discs were placed with the help of sterile forceps on the MHA plates at appropriate distance from each other. The plates were incubated at 37°C for 6-8 h and diameters of the zones of inhibition were measured. The results were interpreted as sensitive, intermediate and resistant based on the zone size interpretative chart provided by the manufacturer.

3.2.3 APEC Characterization of *E. coli* Isolates by PCR

The DNA of the *E. coli* isolates confirmed by PCR were subjected to amplification of five virulence genes (*iron, ompT, hlyF, iss* and *iutA*) using primer pairs as described in Table 3.2. The positive strain of APEC (APEC 9/2/L/BW) available in the Department of VPHE was used as positive control (Grakh *et al*., 2022). Pentaplex PCR was carried out for detection of all the five virulence genes together as per method of Johnson *et al.,* 2008. The PCR reactions were carried out in 25 µl volume consisted of 12.5 µl of master mix, 2.5 µl of 10 µM solution of each pooled primer set of 5 genes (forward and reverse), ~200 ng of the DNA sample and

remaining nuclease free water (NFW) to make 25.0 µl in thermocycler. The thermal cycler conditions used for pentaplex PCR are described in Fig. 3.3.

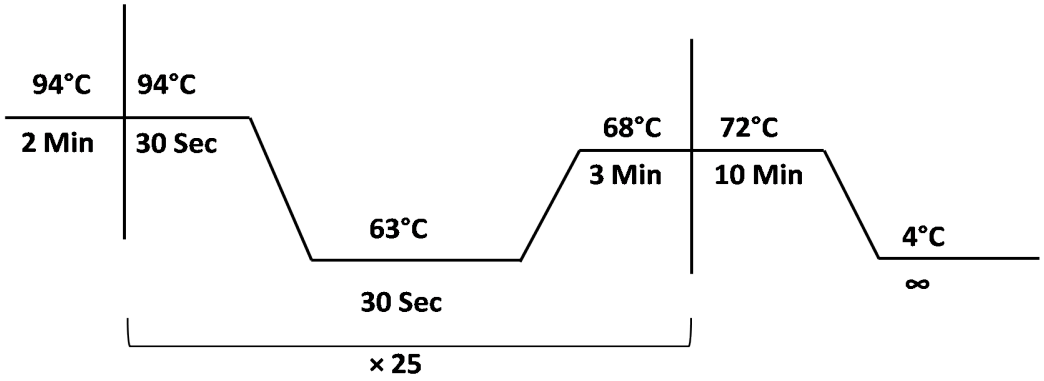


Fig. 3.3 - Pentaplex PCR reaction conditions for detection of APEC associated virulence genes

3.2.4 Detection of Extended Spectrum Beta Lactamases (ESBLs) in *E. coli* Isolates

ESBL screening was based on cefpodoxime, ceftazidime, aztreonam, cefotaxime and ceftriaxone sensitivity. Using a fresh pure culture, a suspension of the test organism equal to 0.5 McFarland standard was prepared. Then using a sterile cotton swab, the adjusted suspension was smeared over the entire area of a Mueller Hinton agar plate. Discs of cefpodoxime, ceftazidime, aztreonam, cefotaxime and ceftriaxone were applied onto the inoculated plate, ensuring sufficient distance between individual discs and incubated at 37°C for 18-24 h. The *Klebsiella pneumoniae* (ATCC 700603) strain which was available in department was used as beta lactamase positive reference strain. In Combination disc test, discs containing cephalosporin alone (cefotaxime, ceftazidime) and in combination with clavulanic acid were applied (Table 3.3). The inhibition zone around the cephalosporin disc combined with clavulanic acid was compared with the zone around the disc with the cephalosporin alone. The test is positive, if the inhibition zone diameter is ≥ 5 mm larger with discs containing cephalosporin with clavulanic acid than discs containing cephalosporin alone (CLSI, 2012).

Table 3.3 - Criteria for ESBL confirmation

Method	Antibiotic Disc		ESBL Confirmation criteria
Combination Disc Test (CDT)	Cefotaxime	CTX-30 mcg	Inhibition zone of cephalosporin with clavulanic acid was ≥ 5 mm than cephalosporin alone
	Cefotaxime with Clavulanic acid	CEC-30/10 mcg	
	Ceftazidime	CAZ-30 mcg	
	Ceftazidime with Clavulanic acid	CAC-30/10 mcg	

3.2.5 PCR Detection of Beta Lactamase (*blaTEM*) and Class 1 Integron Intergrase (*intI1*) Genes in *E. coli* Isolates

Polymerase chain reaction was carried out on extracted DNA from *E. coli* isolates for the detection of Class 1 integron integrase (*Int1I*) gene as well as beta lactamase gene (*blaTEM*). The detail of the primers used for study is given in Table 3.2.

The PCR reactions were carried out in 25 µl volume consisted of 12.5 µl of master mix, 0.5 µl of 10 µM solution of each primer (forward and reverse), ~200 ng of the DNA sample and remaining nuclease free water (NFW) to make 25.0 µl in thermocycler. The PCR amplification was achieved with initial denaturation of DNA then followed by 35 cycles of denaturation, annealing and extension and final extension. The PCR products were visualised under horizontal gel electrophoresis using 1.5% agarose. The detail of thermal profile of PCR for detection of different genes is given in Fig. 3.4.

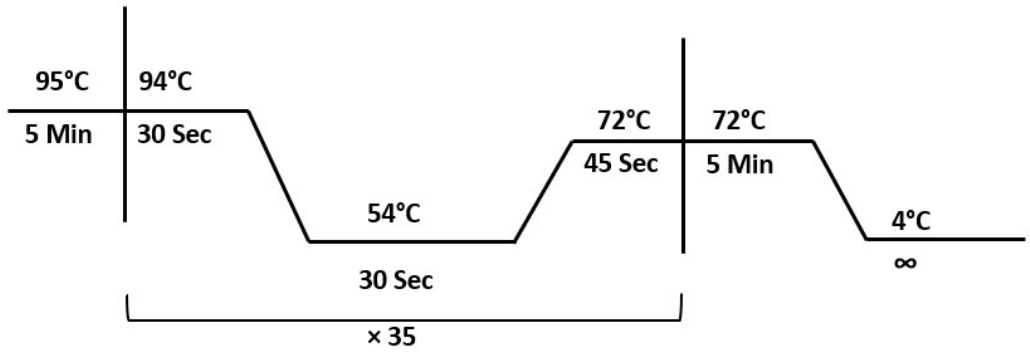


Fig. 3.4 - PCR conditions for *blaTEM* and *intI1* gene of *E. coli*

3.2.6 Detection and Quantification of Biofilm Forming Ability of *E. coli* Isolates

Biofilm formation and its quantification were carried out using 96-well polystyrene microtitre plates as per the method described by Skyberg *et al.*, 2006 with minor modifications. Briefly, the *E. coli* colonies were inoculated in 5 ml of Luria-Bertani (LB) broth and incubated overnight at 37°C. Turbidity of the broth was adjusted

to OD_{600} = 0.05 by diluting the overnight culture LB broth. A volume of 200 µl aliquots of the dilution were then dispensed into microtitre plate wells in triplicate. Then uninoculated LB broths were used as negative control and ATCC 25922 was used as a positive control for biofilm producer (Branco *et al.,* 2016). Three sets of plates were inoculated aerobically without shaking at 37°C for 72h. The contents of the plates were gently poured off and the plates were washed with sterile double distilled water. The microtitre plates were then stained with 200 µl of 0.1% Crystal Violet for 30 min. The microtitre plates were washed three times with sterile double distilled water to remove excess stain followed by air drying of plates for 30 min. After drying, adherent cells were resolubilized using 250 µl solution of ethanol and acetone (80:20). A volume of 150 µl of this resolubilized solution was then transferred to a new microtitre plate and the OD of each well was measured at 570 nm (OD_{570}) using an automated micro plate reader. All tests were carried out in triplicates and the average value of results was taken. The cut off OD (ODc) was calculated as ODc= average OD of negative control + 3 (SD of negative control, SD= Standard deviation). Based on the OD produced by bacterial biofilms, bacterial isolates were classified into the following categories as described by Stepanovic *et al*., 2004.

$OD \leq ODc$	= Isolates with no biofilm forming ability
$ODc < OD < (2 \times ODc)$	= Isolates with weak biofilm forming ability
$(2 \times ODc) < OD \leq (4 \times ODc)$	= Isolates with moderate biofilm forming ability
$(4 \times ODc) < OD$	=Isolates with strong biofilm forming ability

3.2.7 Phylogenetic Typing of *E. coli* Isolates using PCR

All *E. coli* isolates were subjected to phylogenetic typing using the protocols described (Clermont *et al*., 2013). The primer pairs used for phylogenetic typing were *chuA*, *yjaA*, *TspE4.C2, arpA, ArpAgpE* and *trpA.* The PCR reaction was carried out in a 25 µl reaction volume with the following PCR conditions: initial denaturation for 4 min at 94℃ followed by 30 cycles of denaturation for 5 s at 94℃; annealing for 30 s each at 52℃, 60℃ and 62℃ for group E, quadruplex and group C, respectively; and extension for 30 s at 72℃ with a final extension at 72℃ for 5 min. PCR products generated were subjected to gel electrophoresis as described earlier. A 100 bp molecular weight marker was used as the size standard. Gels were stained with

ethidium bromide and bands corresponding to each gene present were digitized using a gel documentation system.

3.2.8 Bacteriophage Isolation against Colibacillosis causing APEC

The bacteriophage was isolated against colibacillosis causing APEC. One of the multidrug resistant APEC isolates was used as host for bacteriophage isolation. The detailed procedure for phage isolation is as detailed below.

3.2.8.1 Collection and processing of samples for phage isolation

The presence of phage(s) is expected to be more in areas where bacterial load is high, hence waste water and excreta samples were collected from a variety of locations *viz*. waste water of poultry farm, LUVAS; sewage sample from sewage water treatment plant and slurry from biogas plant, CCSHAU. For phage(s) isolation, Ganges water from Haridwar was also collected. A total of five samples were processed for bacteriophage isolation.

Each sample (10 ml) was mixed with 100 ml distilled water. All the five samples were poured in separate 250 ml conical flasks, labelled and allowed to stand undisturbed at room temperature (RT) for 1h so that large particulate matter get settled down. From these flasks, 45 ml of supernatant was collected in fresh 250 ml sterile conical flasks. To each flask, 5 ml of 10-X LB broth was added along with 1 ml of overnight grown APEC-P02 strain. The flasks were then incubated at 37°C overnight in a shaking incubator at 120 rpm. A total of 30 ml of this solution was taken in a centrifuge tube and centrifuged at 8000 rpm for 15 min. The supernatant was collected and filtered through 0.22 µ millipore syringe driven membrane filters (Fig. 3.5). The filtrate was stored at 4°C till further used.

3.2.8.2 Spot test

To check the presence of bacteriophages in the filtrate against APEC-P02, spot test was carried out (Kumar *et al.,* 2020). The 100 µl aliquot of previously isolated *E. coli* culture of mid-exponential phase was taken and spread on LB agar plate with the help of sterilized L-loop. Afterwards, 10 µl of sample filtrate was added as a drop to these plates and incubated overnight at 37°C. Clear zone of inhibition of bacterial growth on the place of drop indicates the presence of bacteriophage in the filtrate. The phage filtrate that passed the positive spot test was further processed to isolate the phage.

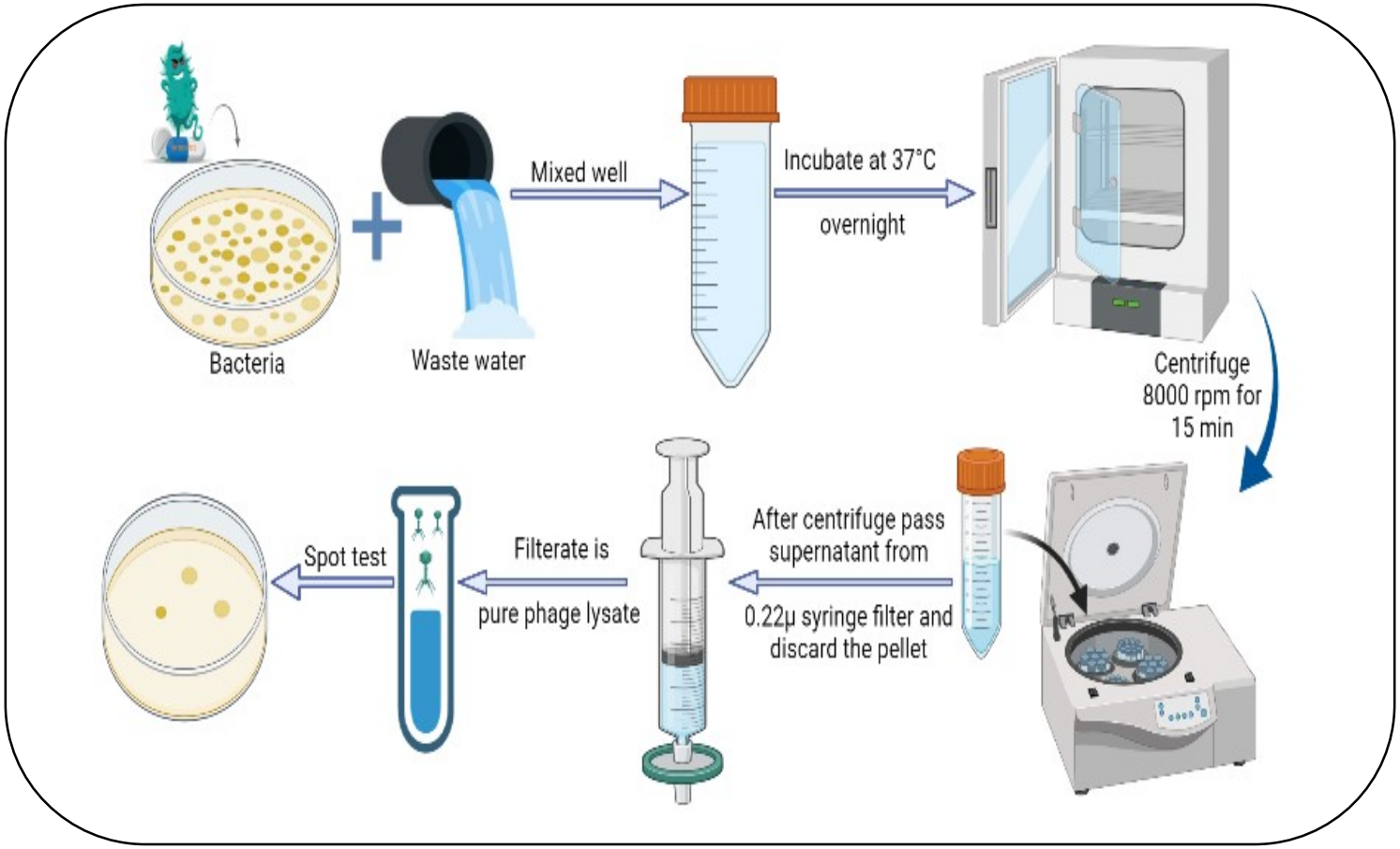


Fig. 3.5 - Isolation of bacteriophage

3.2.8.3 Bacteriophage selection and enrichment using double agar overlay technique

3.2.8.3.1 Double agar overlay assay

For bacteriophage isolation, 10-fold dilution double agar overlay method was used to isolate bacteriophages (Adams, 1959). From 10^{-1} to 10^{-9} dilutions, the phage filtrate was diluted 10-fold in SM buffer. Nine 2 ml MCTs labelled as 1 to 9 were taken. For serial dilution of phage, 450 µl of SM buffer was taken in each tube. In the first tube, 50 µl of phage filtrate was placed and gently mixed. A 50 µl portion of this mixture was transferred to the next tube and the process was repeated until the phage dilution 10^{-9} was obtained. Afterwards, nine centrifuge tubes of 15 ml capacity were taken and labelled as 1 to 9.

A 100 µl of respective phage dilution (10^{-1} to 10^{-9}) was transferred into these 15 ml tubes. A volume of 100 µl of bacterial APEC-P02 culture grown at 37°C to mid-exponential phase was added to these tubes followed by addition of 3 ml molten top agar (0.7% LB broth) in each tube. The tubes were gently mixed at a steady room temperature to prevent the agar from solidifying and then transferred onto LB agar plates labelled 1 to 9. For phage isolation, the plates were incubated at 37°C overnight in an incubator and checked next day for plaque formation.

3.2.8.3.2 Plaque picking

The sterile micropipette tip (1000 µl) with a broad end was used to pick plaques from LB agar petri plates. The picked plaques were poured in a sterile 1.5 ml MCT containing 500 µl SM buffer. For leaching, the plaques were kept at 4°C overnight. The MCT was centrifuged at 8,000 rpm for 8 min and the supernatant was transferred to a new sterile MCT.

3.2.8.3.3 Phage enrichment

In a conical flask, 10 ml of LB broth and 100 µl of overnight grown *E. coli* bacterial culture was taken as described by Bonilla *et al.,* 2016. The flask was incubated for 1 h at 37°C. Subsequently, 100 µl of phage filtrate was added and cultured overnight at 37°C in a shaking incubator at 120 rpm. The flask's contents were transferred to a 15 ml tube and centrifuged at 10,000xg for 10 min at 4°C. A 0.22 µm membrane syringe filter was used to filter the supernatant. To get pure bacteriophages, the double agar overlay method was carried out using the filtrate to isolate plaques. The phage isolation and enrichment process were repeated three time

to obtain purified single phage. The isolated pure phage was given the nomenclature '*Escherichia* phage PJDM' as per the guidelines (Adriaenssens and Brister, 2017).

3.2.8.3.4 Determination of plaque forming units (PFU)

A serial dilution of *Escherichia* phage PJDM lysate in SM buffer from 10^{-1} to 10^{-9} was made to estimate the plaque forming unit (PFU) of the phage preparation as described by Jothikumar *et al*., 2000 with minor modifications. Briefly, an equal amount of dilution and an *E. coli* culture grown up to mid-exponential phase were combined and subjected to the double agar overlay procedure. The PFU was estimated by counting the plaques present in the maximal dilution of phage preparation and then applying the following formula.

PFU count = Number of plaques counted X 1/Dilution factor X 1/Volume of phage taken

All the assays were performed in triplicates.

3.2.9 Morphological Characterization of Bacteriophage using Transmission Electron Microscopy

The size and morphology were determined using transmission electron microscopy (TEM). For sample preparation, the *Escherichia* phage PJDM particles were precipitated overnight using polyethylene glycol (PEG-8000) followed by ultracentrifugation at 50,000 RPM for 2 h at 4°C at Plant Pathology Department, CCS HAU, Hisar. The pellet was dissolved in 500 µl of SM buffer. Afterwards, 20 µl of phage suspension was spotted on top of a 400-mesh sized carbon coated grid. Phages were allowed to adsorb for 2-3 min and then washed with distilled water. The negative staining was carried out using 2% uranyl acetate. The grid was dried with the help of Whatman filter paper 1 strip and viewed under electron microscope (JEM-1011) at different magnification using TEM available at the Division of Plant Pathology, ICAR-IARI, PUSA, New Delhi.

3.2.10 Host Range and Specificity Testing of Bacteriophage

To check the host range and specificity of *Escherichia* phage PJDM, bacterial strains available in the Department of VPHE, LUVAS i.e., *Klebsiella pneumoniae* (ATCC 700603), *Staphylococcus aureus* (ATCC 25923), *Salmonella* Typhimurium along with *E. coli* strains isolated from colibacillosis affected poultry tissue samples were taken. These bacterial strains were grown in LB broth and spot test was performed to know the efficacy of phage for different bacterial strains and serotypes of *E. coli*.

3.2.11 Serotyping of Susceptible *E. coli* Isolates

All the *E. coli* isolates were tested for their susceptibility to the isolated phage. The *E. coli* isolates exihibiting positive spot test were selected for serotyping and sent to Central Research Institute, Kasauli for this purpose.

3.2.12 Biophysical Characterization of Bacteriophage

3.2.12.1 Thermal sensitivity of bacteriophage

Thermal stability tests were carried out for the *Escherichia* phage PJDM. Briefly, the phages (1.2×10^{10} PFU/ml) were kept at different temperatures (4°C 30°C, 37°C, 42°C, 50°C, 70°C and 90°C) and studied for different interval of times (15 min, 30 min, 60 min, 120 min, 6 h, 24 h, 7 d, 30 d, 60 d, 120 d and 300 d). The phage was checked for effect of temperature by measuring titre using plaque counting (based on double agar overlay procedure) in presence of APEC-P02 (host bacterium). All the assays were performed in triplicates.

3.2.12.2 pH sensitivity of bacteriophage

The pH stability of the phage was evaluated using SM buffer solution adjusted to different pH (pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13). The pH was adjusted using 1 M NaOH or 1 M HCl (Jepson and March, 2004). The change in phage PFU (1.2×10^{10} PFU/ml) was measured by keeping the phage at above stated pH for different interval of times (15 min, 30 min, 60 min, 120 min, 6 h and 24 h) at 25°C. After pH treatment, the presence of viable phage was quantified using the double agar overlay method. All assays were performed in triplicates.

3.2.13 Determination of Latent Period and Burst Size of Bacteriophage

To determine the life cycle of *Escherichia* phage PJDM in the APEC-P02, one step growth curve test was conducted. The *E. coli* culture was grown in LB broth at 37 °C for 3-4 h (Capra *et al*., 2006). A 5 ml of bacterial culture was centrifuged at 4000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in PBS till turbidity matching to 0.5 Mcfarland (containing 10^8 CFU/ml) was attained. The phage lysate was added at a conc. of 10^7 PFU/ ml at multiplicity of infection (MOI) equal to 0.1. The mixture was then centrifuged at 13,000 rpm for 1 min and the supernatant decanted. The pellet was resuspended in 50 ml LB broth and incubated at 37°C. The 2 ml aliquot from incubated LB broth was taken every 10 min in a sterile MCT. The aliquots were centrifuged at 10,000 rpm for 5 min. Afterwards filtration using 0.22 µm membrane syringe filter (Millipore) was carried out and stored at 4°C till further use. The double agar overlay technique was performed to calculate the

number of plaques and graph was plotted against time (X-axis = Time; Y-axis = PFU/ml) to get the one step growth curve. Using curve, the latent period i.e., duration between the phage adsorbed until the release of phage virions was calculated. The burst size of phage was also calculated by taking ratio of final number of free phage particles to the initial number of phages.

3.2.14 Whole Genome Sequencing of Bacteriophage

3.2.14.1 Phage genome extraction

The phage PJDM genome was extracted with minor changes in the protocol described by Gill, 2015. Briefly, using the phage enrichment procedure, a fresh phage lysate with a concentration of 10^{10} PFU/ml was prepared. In a centrifuge tube, 50 ml of phage lysate was added, along with 0.5 µl of nuclease solution per ml of lysate. The precipitant solution (30 percent w/v PEG-8000, 3 M NaCl in distilled water) was added to the lysate at a rate of 1:2 precipitant: lysate after 30 min of incubation at 37ºC. The tube was gently mixed, incubated at 4°C overnight and centrifuged at 10,000xg for 15 min at 4°C. The supernatant was decanted and the pellet was gently pipetted up and down in 500 µl of SM buffer. The resuspended phage was then placed in a 1.5 ml MCT and centrifuged for 5-10 sec to pellet any insoluble particles. The supernatant was transferred to a fresh 2 ml MCT.

Wizard DNA cleanup kit was used to extract the genome. One ml of the Wizard kit purification resin was added to the phage suspension and gently mixed. The mixture was transferred to a 3 ml syringe and passed through a Wizard minicolumn into a clean 1.5 ml MCT. The flow through was discarded and the minicolumn washed by passing 2 ml of 80 % isopropanol using the same syringe. To dry the resin, the minicolumn was put in a fresh 1.5 ml MCT and centrifuged at 13,000xg for 2 min at room temperature. The mini column was again placed in a new 1.5 ml MCT. Then 100 µl of NFW (preheated to 80ºC) was added on the top of column and centrifuged immediately at 13,000xg for 1 min at room temperature (RT) to elute the DNA. The eluted DNA concentration and purity measured with help on Biophotometer and DNA was stored at -20ºC till further used.

3.2.14.2 Whole genome sequencing

The whole genome sequencing and bioinformatics analysis were got performed commercially using the service of Eurofins Scientific. Extracted DNA sample of *Escherichia* phage PJDM was sent to the company for genome sequencing and bioinformatics analysis. The vendor carried out whole genome sequencing using

NextSeq 500 with 2x150 bp chemistry. The sequenced raw data was processed to obtain high quality clean reads using Trimmomatic v0.39 to remove adapter sequences, ambiguous reads and low-quality sequences. The high-quality reads were aligned to the reference genome of *Escherichia coli str. K-12 substr. MG1655* (NC_000913.3), downloaded from NCBI, using BWA MEM (version 0.7.17), MEM is an algorithm works by seeding alignments with maximal exact matches (Align 70bp-1Mbp query sequences) and then extending seeds with the affine-gap Smith-Waterman algorithm (SW). The unmapped reads were extracted using Samtools. The above-mentioned unmapped reads of bacteriophage sample were assembled into scaffolds using CLC Genomics Workbench version 9.5.2. Genes in bacteriophage sample were predicted using Prokka and searched against NCBI non redundant protein database (Nr) using Diamond (BlastX mode) at an E-value threshold of 1e-05. Genome mapping of functional annotated ORFs was performed using SnapGene. The brief work flow of bioinformatics is depicted in Fig. 3.6.

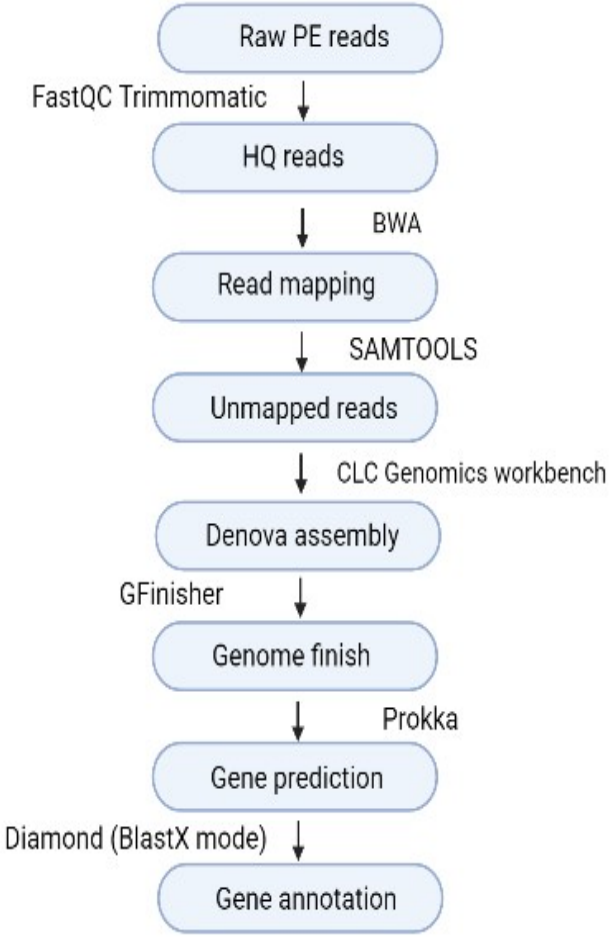


Fig. 3.6 - Bioinformatics workflow

3.2.15 Restriction Endonuclease Profiling of Phage PJDM Bacteriophage

Escherichia phage PJDM DNA sequence obtained after whole genome sequencing were subjected to in silico restriction endonucleases using NEBcutter V2.0 cutter tool.

3.2.16 Determination of *in vivo* Mean Lethal Dose (LD50) of Isolated Avian Pathogenic *E. coli* in Chicks

Permission from Institutional Animal Ethics Committee (IAEC) was obtained prior to *in vivo* experimental studies vide letter no. VCC/IAEC/566-82, dated 18/03/2021.

To calculate mean lethal dose (LD50) of isolated APEC-P02 in chicks, *in vivo* challenge study was carried out. The unvaccinated day-old sixty broiler chicks were procured from a local hatchery and kept in cage system (Fig. 3.7A). These chicks were divided randomly into six groups (n=10) (Table 3.4). For the mean lethal dose (LD50) test, a series of 10-fold dilutions of overnight grown *E. coli* culture (in PBS) having 10^4 to 10^8 cfu/0.5 ml (2×10^4 to 2×10^8 cfu/ml) were orally administered into chicks (Fig. 3.7B). The group 1a was control group in which no infection was given. The chicks were observed for up to 10 days for any mortality and weight gain was recorded. The LD50 was calculated by the method of Reed and Muench, 1938.

A **B**

Fig. 3.7 - (A) Housing and (B) Handling of chicks during experimental study

Table 3.4 - Grouping of chicks (n=60) for determination of LD50 for APEC isolate

Experiment			
Group (In each group n=10)	***E. coli* infection**		
	Dose	**Administration route of administration**	**Administration age (days)**
1a	nil	NA	NA
2a	2×10^4 cfu/ml	oral	1
3a	2×10^5 cfu/ml	oral	1
4a	2×10^6 cfu/ml	oral	1
5a	2×10^7 cfu/ml	oral	1
6a	2×10^8 cfu/ml	oral	1

3.2.17 Bacterial Challenge Test

Bacterial challenge test was performed as described by Wang *et al.* (2016) with some modification to investigate the ability of *Escherichia* phage PJDM to lyse *E. coli* in *in vitro* conditions. Overnight culture of APEC-P02 was diluted 1:100 in fresh BHI broth incubated at 37°C until OD_{600}=0.11 (calculated LD50- 1.12×10^8 CFU/ml equal to OD_{600} 0.11) was achieved. The phage was added at MOI of 0.01, 1 and 100 using APEC-P02 culture was used as the host. The mixture was grown at 37°C along with shaking at 120 rpm. The phage bacteriolytic activity was assessed by monitoring the cell absorbance of the culture solution (OD_{600}) and by calculating CFU/ml of sample at 30-min intervals for up to 3 h. The assay was performed in triplicates. The MOI at which phage lysed the host efficiently i.e., during *in vitro* bacterial challenge test was selected for further *in vivo* therapeutic and prophylactic studies of phage.

3.2.18 *In vivo* Therapeutic and Prophylactic Study of Isolated Bacteriophage in Chicks Challenged with Pathogenic *E. coli*

Permission from Institutional Animal Ethics Committee (IAEC) was obtained prior to *in vivo* experimental studies vide letter no. VCC/IAEC/566-82, dated 18/03/2021.

To determine the therapeutic and prophylactic efficacy of *Escherichia* phage PJDM against APEC-P02 in chicks *in vivo* challenge study was carried out. The unvaccinated day old (n=140) broiler chicks were procured from hatchery and were divided randomly into seven groups with 20 birds in each group (Table 3.5).

Table 3.5 - Determination of *in vivo* therapeutics and prophylactic efficacy of *Escherichia* phage PJDM

Experiment						
Name of group (n=20)	***E. coli* infection**			**Bacteriophage treatment**		
	Dose	**Route of administration**	**Administration age (days)**	**Dose**	**Route of administration**	**Administration age (days)**
A	Nil	NA	NA	Nil	NA	NA
B	Nil	NA	NA	*In vitro* calculated dose of bacteriophage**	Oral	(2, 7, 14)
C	Calculated LD50*	Oral	1	Nil	NA	NA
D	Ten-fold less dose than that of calculated LD50*	Oral	1	*In vitro* calculated dose of bacteriophage**	Oral	(2, 7, 14)
E	Calculated LD50*	Oral	1	*In vitro* calculated dose of bacteriophage**	Oral	(2, 7, 14)
F	Ten time more dose than that of calculated LD50*	Oral	1	*In vitro* calculated dose of bacteriophage**	Oral	(2, 7, 14)
G	Calculated LD50*	Oral	5	*In vitro* calculated dose of bacteriophage**	Oral	(1, 2, 3, 4)

* Calculated LD50 as per experiment described in section 3.2.16

** In vitro calculated dose of bacteriophage from test described in section 3.2.17

The group A represented healthy untreated control group; group B represented healthy treated control group; group C represented infected control group; group D, E and F were used to determine the therapeutic efficacy of bacteriophage(s) at different lethal dose of *E. coli* and group G was used to determine the prophylactic efficacy of bacteriophage(s) against LD50 determined *E. coli* isolate. Efficacy of bacteriophage was assessed on the basis of mortality and clinical manifestations. The carcasses of dead birds were dissected to know the cause of death. The collected data was analyzed statistically to determine the therapeutic and prophylactic activity of isolated bacteriophage(s) against pathogenic *E. coli* in chickens.

3.2.19 Storage of Bacterial Isolates and Phage Lysate

All confirmed *E. coli* isolates were inoculated into 5ml BHI (brain heart infusion) broth and incubated at 37°C for 6 h. Then 500µl of each culture was mixed with 500 µl of 100 % glycerol in a 2 ml micro-centrifuge tube (MCT). The MCT were sealed using paraffin film and kept at 4°C for 24 h followed by storage at -20°C for storage. The phage lysate was stored at 4°C for long term storage.

3.2.20 Statistical Analysis

Data was analysed statistically using STATA™IC/15.1. Various statistical tests applied were Chi square/ Fisher's exact analysis and logistic/ conditional regression.

CHAPTER-IV

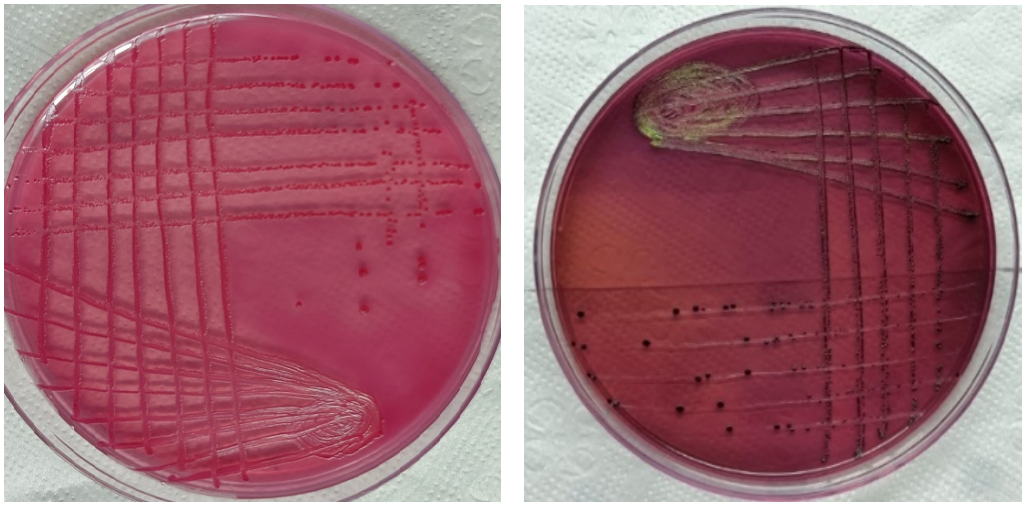
RESULTS

The present study was undertaken with the objectives to isolate and characterize pathogenic *E. coli* and bacteriophage along with *in vivo* study on therapeutic and prophylactic activity of isolated bacteriophage. Initially isolation of *Escherichia coli* was carried out from colibacillosis suspected poultry birds. The isolated *E. coli* were phenotypically and molecularly characterized. Out of these isolates, one isolate was selected and used for further *in vivo* determination of LD50. The *in vivo* evaluation of therapeutic and prophylactic efficacy of isolated bacteriophage against isolated *E. coli* was studied in experimental broiler chicks. The due permission for *in vivo* experimental trials was obtained from Institutional Animal Ethics Committee. The results of the present study are detailed as under.

4.1 Isolation, Identification and Confirmation of *E. coli* Isolates from Colibacillosis Infected Chickens

4.1.1 Isolation and preliminary confirmation of *E. coli* isolates

The bacterial colonies producing pink color on MLA plates and greenish metallic sheen on EMB agar plates were identified as presumptive *E. coli* (Fig. 4.1). A total of 50 *E. coli* isolates were isolated from 50 tissue samples (liver) of colibacillosis suspected poultry birds. The IMViC pattern of theses bacterial isolates were indicated of *E. coli* (++--).



A **B**

Fig. 4.1 - *E. coli* colonies on (A) MLA (pink color colonies) and (B) EMB (green metallic sheen)

4.1.2 Molecular confirmation of presumptive *E. coli* isolates

For confirmation, isolates were subjected to PCR targeting *E. coli* specific *PhoA* gene. The PCR of all the 50 presumptive *E. coli* isolates and positive control (ATCC-25922 *E. coli* strain) yielded amplification band of 468bp specific to *PhoA* gene confirming them as *E. coli* (Fig. 4.2).

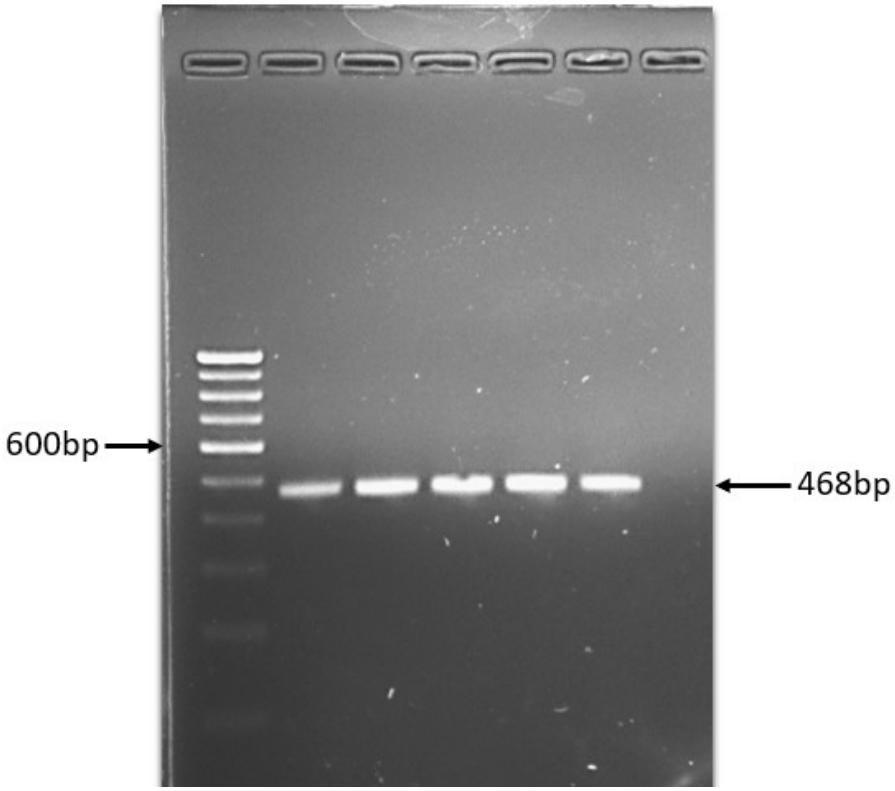


Fig. 4.2 - *E. coli* specific *PhoA* gene PCR amplification (468bp)

Lane M– 100bp DNA ladder; Lanes 1-4– *E. coli* isolates; Lanes 5– Positive control (ATCC-25922); Lane 6– Negative control

4.1.3 Vitek confirmation of presumptive *E. coli* isolates

The Vitek 2 Compact system also confirmed all the 50 bacterial presumptive colonies as *E. coli.* The results obtained from Vitek 2 Compact system using Gram negative (GN) reagent cards (Fig. 4.3) were with excellent confidence identification (99% probability).

Food Safety Laboratory, VPHE, LUVAS

bioMerieux Customer:
System #:

Laboratory Report

Printed Jun 1, 2020 16:08 IST
Printed by: LabAdmin
Report Version: 1 of 1

Isolate Group: Punit-1

Card Type: GN Testing Instrument: 00001899ECFA (16209)

Bionumber: 0405610450424610
Organism Quantity:

Comments:	

Identification Information	**Card:** GN		**Lot Number:** 2410317103	**Expires:**	Oct 9, 2020 12:00 IST
	Completed: Jun 1, 2020 16:10 IST		**Status:** Final	**Analysis Time:**	4.00 hours
Selected Organism	99% Probability	**Escherichia coli**			
	Bionumber: 0405610450424610			**Confidence:**	Excellent identification
SRF Organism					
Analysis Organisms and Tests to Separate:					
Analysis Messages:					
Contraindicating Typical Biopattern(s)					

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	-
40	ILATk	-	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	+	53	lHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Installed VITEK 2 Systems Version: 07.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

Page 1 of 1

Fig. 4.3 - VITEK-2 result report of presumptive *E. coli* isolates

4.2 Antibiotic Susceptibility Profiling of *E. coli* Isolates

The antibiotic susceptibility testing of all the 50 isolates using 20 antibiotic disc representing different classes of antibiotics revealed that maximum sensitivity was present against colistin (62%), followed by trimethoprim (50%) (Fig. 4.4). The high antibiotic resistance was observed for imipenem, tetracycline, ciprofloxacin, levofloxacin (96% each), piperacillin (92%), cefpodoxime and cefotaxime (90% each) (Table 4.1 and Fig. 4.5).

All the 50 isolates were multiple drug resistant (MDR) as these were resistant for three or more classes of antibiotics. The MDR pattern indicated existence of 38 resistotypes (Table 4.2).

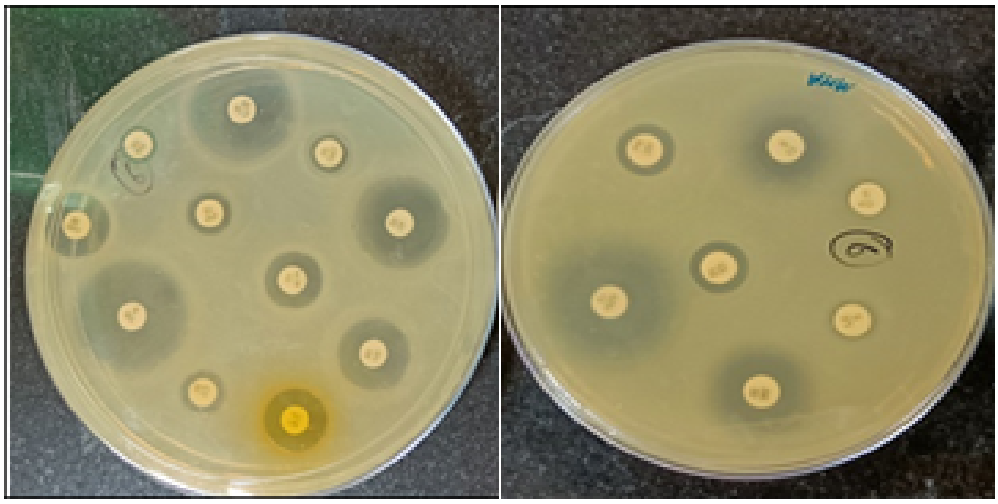


Fig. 4.4 - Antibiotic susceptibility testing of *E. coli* isolates on MHA

Table 4.1 - Antibiotic susceptibility profile of *E. coli* isolates (n=50)

Antibiotics	Antibiotic susceptibility profile					
	Resistant (R)		Intermediate (I)		Sensitive (S)	
	Number (%)	95% CI	Number (%)	95% CI	Number (%)	95% CI
Ciprofloxacin	48 (96)	86.29-99.51	0 (0)	0-7.11	2 (4)	0.49-13.71
Imipenem	48 (96)	86.29-99.51	2 (4)	0.49-13.71	0 (0)	0-7.11
Levofloxacin	48 (96)	86.29-99.51	0 (0)	0-7.11	2 (4)	0.49-13.71
Tetracycline	48 (96)	86.29-99.51	0 (0)	0-7.11	2 (40)	0.49-13.71
Piperacillin	46 (92)	80.77-97.78	4 (8)	2.22-19.23	0 (0)	0-7.11
Cefpodoxime	45 (90)	78.19-96.67	3 (6)	1.25-16.55	2 (4)	0.49-13.71
Cefotaxime	45 (90)	78.19-96.67	2 (4)	0.49-13.71	3 (6)	1.25-16.55
Nitrofurantoin	44 (88)	75.69-95.47	6 (12)	4.53-24.31	0 (0)	0-7.11
Polymyxin B	37 (74)	59.66-85.37	0 (0)	0-7.11	13 (26)	14.63-40.34
Streptomycin	37 (74)	59.66-85.37	13 (26)	14.63-40.34	0 (0)	0-7.11
Gentamicin	35 (70)	55.39-82.14	13 (26)	14.63-40.34	2 (4)	0.49-13.71
Amoxyclav	33 (66)	51.23-78.79	14 (28)	16.23-42.49	3 (6)	1.25-16.55
Ampicillin	32 (64)	49.19-77.08	18 (36)	22.92-50.81	0 (0)	0-7.11
Ceftazidime	30 (60)	45.18-73.59	18 (36)	22.92-50.81	2 (4)	0.49-13.71
Trimethoprim	25 (50)	35.53-64.47	0 (0)	0-7.11	25 (50)	35.53-64.47
Amikacin	22 (44)	29.99-58.75	16 (32)	19.52-46.7	12 (24)	13.06-38.17
Ceftriaxone	22 (44)	29.99-58.75	17 (34)	21.21-48.77	11 (22)	11.53-35.96
Colistin	15 (30)	17.86-44.61	4 (8)	2.22-19.23	31 (62)	47.17-75.35
Azithromycin	13 (26)	14.63-40.34	18 (36)	22.92-50.81	19 (38)	24.65-52.83
Aztreonam	5 (10)	3.33-21.81	26 (52)	37.42-66.34	19 (38)	24.65-52.83

* CI= confidence interval

Table 4.2 - Multi drug resistant resistotypes of *E. coli* isolates (n=50)

Number of resistant antibiotics	Resistotypes	No. of isolates
18	A-P-AC-C-CP-CZ-CT-AT-I-AK-S-AZ-T-PM-CF-LP-TP-N	**1**
17	A-P-AC-C-CP-CZ-CT-I-G-S-T-CL-PM-CF-LP-TP-N	**3**
	A-P-AC-C-CP-CZ-CT-I-G-AK-S-T-PM-CF-LP-TP-N	**1**
	A-P-AC-C-CP-CZ-CT-I-G-AK-S-T-PM-CF-LP-TP-N	**1**
	A-P-C-CP-CZ-CT-I-G-AK-S-AZ-T-PM-CF-LP-TP-N	**1**
16	A-P-AC-C-CP-CZ-CT-I-G-AK-T-CL-PM-CF-LP-N	**1**
	A-P-AC-C-CP-CZ-CT-I-G-S-AZ-T-PM-CF-LP-N	**1**
	A-P-AC-C-CP-CZ-CT-I-AK-S-AZ-T-CF-LP-TP-N	**1**
	A-P-CP-CZ-CT-I-G-AK-S-AZ-T-PM-CF-LP-TP-N	**2**
	P-AC-C-CP-CZ-CT-AT-I-G-S-T-CL-PM-CF-LP-N	**1**
	P-AC-C-CP-CT-I-G-AK-S-AZ-T-PM-CF-LP-TP-N	**1**
15	A-P-AC-C-CP-CZ-CT-I-S-T-PM-CF-LP-TP-N	**1**
	A-P-AC-CP-CZ-CT-I-G-AK-T-CL-PM-CF-LP-N	**1**
	A-P-AC-CP-CZ-CT-I-AK-S-AZ-T-CF-LP-TP-N	**1**
	A-P-AC-CP-CZ-CT-I-G-S-T-CL-PM-CF-LP-N	**1**
	A-P-AC-CP-CZ-CT-I-G-S-T-PM-CF-LP-TP-N	**1**
	A-P-CP-CZ-CT-I-G-AK-S-T-PM-CF-LP-TP-N	**2**
	P-AC-CP-CT-I-G-AK-S-AZ-T-PM-CF-LP-TP-N	**1**
14	A-P-AC-CP-CZ-CT-I-S-T-PM-CF-LP-TP-N	**1**
	A-P-AC-CZ-CT-I-G-AK-T-CL-PM-CF-LP-N	**1**
	A-P-AC-C-CP-CZ-CT-I-S-T-CF-LP-TP-N	**1**
	A-P-CP-CZ-CT-I-G-AK-S-AZ-T-CF-LP-N	**1**
	A-P-CP-CZ-CT-I-G-S-T-CL-PM-CF-LP-N	**2**
	P-C-CP-CZ-CT-AT-I-G-T-CL-PM-CF-LP-N	**3**
13	A-P-AC-C-CP-CT-I-S-T-CF-LP-TP-N	**1**
	P-AC-CP-CZ-CT-I-G-T-CL-PM-CF-LP-N	**2**
12	A-P-AC-CP-CT-I-T-PM-CF-LP-TP-N	**2**
	A-P-AC-CP-I-S-T-PM-CF-LP-TP-N	**2**
	A-P-AC-CP-I-AK-S-T-PM-CF-LP-N	**1**
	A-P-AC-C-CT-I-T-PM-CF-LP-TP-N	**1**
11	P-AC-CP-CT-I-G-AK-S-T-CF-LP	**1**
	P-AC-C-CP-CT-I-G-S-T-CF-LP	**1**
	CP-CT-I-G-AK-S-AZ-T-CF-LP-N	**2**
10	C-CP-CT-I-G-AK-S-T-CF-LP	**1**
9	CP-CT-I-G-AK-S-T-CF-LP	**1**
	P-AC-CP-I-G-T-CF-LP-N	**1**
	P-AC-C-I-G-T-CF-LP-N	**1**
4	P-CT-S-PM	**2**

* AK- Amikacin, A- Ampicillin, AC- Amoxicillin-clavulanic acid, AZ- Azithromycin, AT-Aztreonam, CP- Cefopodoxime, CT- Cefotoxime, CZ-Ceftazidime, C- Ceftriaxone, CF- Ciprofloxacin, CL-Colistin, G- Gentamicin, I- Imipenem, LP- Levofloxacin, N- Nitrofurantoin, P- Penicillin, PM-Polymyxin B, S- Streptomycin, T- Tetracyclin and TP- Trimethoprim

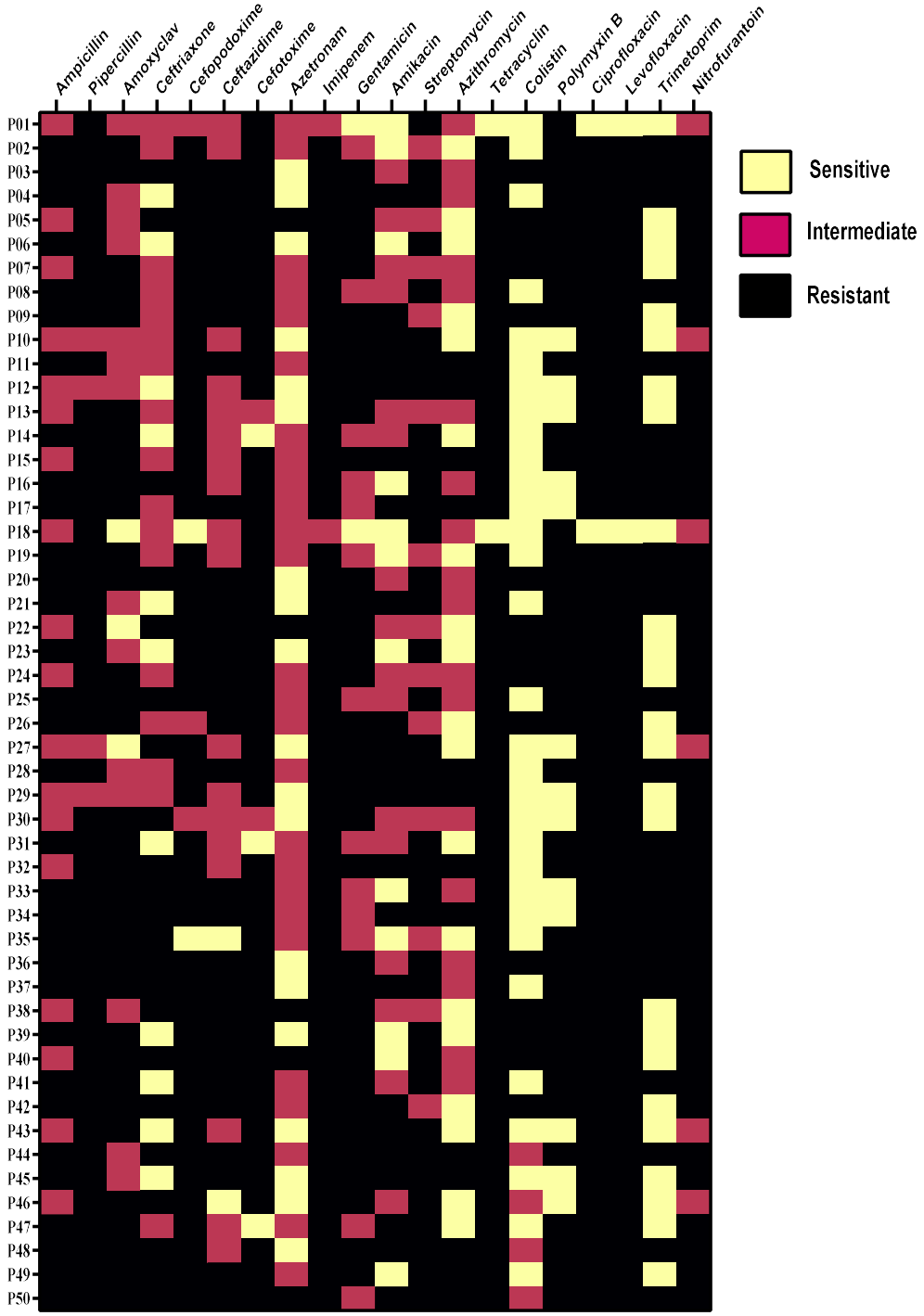


Fig. 4.5 - Heat map of antibiotic susceptibility profile of *E. coli* isolates (n=50)

4.3 APEC Characterization of *E. coli* Isolates by PCR

The characterization of *E. coli* isolates (n=50) as APEC and non-APEC by using a set of five virulence genes (*iroN, ompT, hlyF, iss* and *iutA*) pentaplex PCR indicated the existence of both APEC as well as non-APEC strains of *E. coli*. All the *E. coli* isolates were compared to positive control (APEC 9/2/L/BW) that yielded expected PCR products of sizes 553bp, 496bp, 450bp, 323bp and 302bp during gel electrophoresis. By pentaplex PCR, 45 (90%) isolates were characterized as APEC and 5 (10%) as non-APEC.

The multiplex PCR (Fig. 4.6) of all the 50 isolates exhibited presence of five VGs in 40 isolates, four VGs in five isolates and two VGs in five isolates (Table 4.3). The occurrence of the virulence genes among APEC isolates was 100% each for *iroN, ompT, hlyF, iutA* and 88.9% for *iss* (Table 4.4).

Table 4.3 - Characterization of *E. coli* isolates as APEC by multiplex PCR

Sr. No.	Total isolates tested (n=45)		
	Genes present (*iroN, ompT, hlyF, iss, iutA*)	**Number of isolates harboring genes**	**Characterization**
1	All 5 genes	40	APEC = 45 (90%)
2	4 genes (*iroN, ompT, hlyF, iutA*)	5	
3	2 genes (*iroN, iss*)	5	APEC = 5 (10%)

Table 4.4 - Presence of APEC associated virulence genes in *E. coli* isolates (n=45)

Sr. No.	Gene	APEC (%)	Non-APEC (%)	Total (%)
1	*iroN*	45 (100)	5 (100)	50 (100)
2	*ompT*	45 (100)	0 (0)	45 (90)
3	*hlyF*	45 (100)	0 (0)	45 (90)
4	*iss*	40 (88.9)	0 (0)	40 (80)
5	*iutA*	45 (100)	5 (100)	50 (100)

The analysis of APEC and non-APEC isolates regarding their antimicrobial resistance indicated that APEC (n=45) isolates exhibited resistance to imipenem, tetracycline, levofloxacin and ciprofloxacin (100% each) followed by cefpodoxime (93.33%), piperacillin, ceftazidime and nitrofurantoin (91.11% each). The resistant pattern comparison of different antibiotics in relation to APEC and non-APEC revealed statistically significant difference for cefpodoxime, imipenem, tetracycline, levofloxacin and ciprofloxacin (Table 4.5)

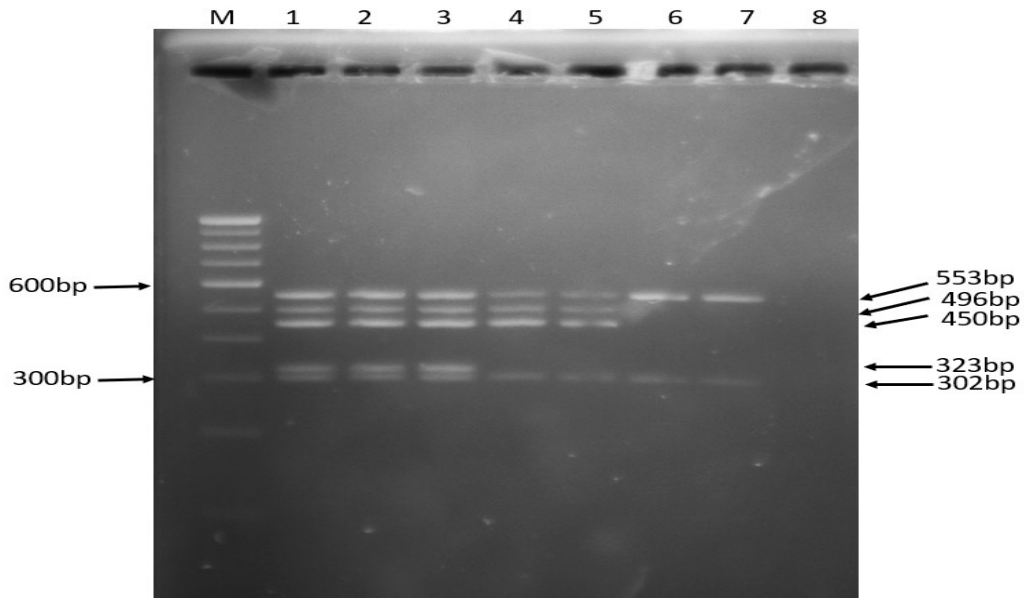


Fig. 4.6 - APEC characterization of *E. coli* isolates by pentaplex PCR

Lane M– 100bp DNA ladder; Lanes 1– Positive control; Lanes 2-5– APEC isolates; Lanes 6-7– Non-APEC isolates; Lane 8– negative control

Table 4.5 - Positivity of APEC isolates among antimicrobial resistant isolates

Antibiotics (No. of resistant isolates)	**APEC + (45)**		**APEC - (5)**		**p-value**
	n (%)	95% CI	n (%)	95% CI	
Ampicillin (32)	29 (64.4)	48.78-78.13	3 (60)	14.66-94.73	0.844
Piperacillin (46)	41 (91.11)	78.78-97.52	5 (100)	47.82-100	0.487
Amoxyclav (33)	30 (66.67)	51.05-80	3 (60)	14.66-94.73	0.765
Ceftriaxone (22)	20 (44.44)	29.64-60	2 (40)	5.27-85.34	0.849
Cefpodoxime (45)	42 (93.33)	81.73-98.6	3 (60)	14.66-94.73	**0.018**
Cefotaxime (45)	29 (64.44)	48.78-78.13	1 (20)	0.51-71.64	0.054
Ceftazidime (30)	41 (91.11)	78.78-97.52	4 (80)	28.36-99.49	0.432
Aztreonam (5)	5 (11.11)	3.71-24.05	0 (0)	0-52.18	0.432
Imipenem (48)	45 (100)	92.13-100	3 (60)	14.66-94.73	**0.000**
Gentamicin (35)	33 (73.33)	58.06-85.4	2 (40)	5.27-85.34	0.123
Amikacin (22)	20 (44.44)	29.64-60	2 (40)	5.27-85.34	0.849
Streptomycin (37)	32 (71.11)	55.69-83.63	5 (100)	47.82-100	0.162
Azithromycin (13)	11 (24.44)	12.88-39.54	2 (40)	5.27-85.34	0.452
Tetracycline (48)	45 (100)	92.13-100	3 (60)	14.66-94.73	**0.000**
Colistin (15)	15 (33.33)	20-48.95	0 (0)	0-52.18	0.123
Polymyxin B (37)	32 (71.11)	55.69-83.63	5 (100)	47.82-100	0.162
Ciprofloxacin (48)	45 (100)	92.13-100	3 (60)	14.66-94.73	**0.000**
Levofloxacin (48)	45 (100)	92.13-100	3 (60)	14.66-94.73	**0.000**
Trimetoprim (25)	24 (53.33)	37.87-68.34	1 (20)	0.51-71.64	0.157
Nitrofurantoin (44)	41 (91.11)	78.78-97.52	3 (60)	14.66-94.73	**0.042**

4.4 Detection of Extended Spectrum Beta Lactamases (ESBLs) in *E. coli* Isolates

The detection of ESBL producing ability by combine disc test (cephalosporin with clavulanic acid vs cephalosporin alone) indicated that 10 *E. coli* isolates (20%) were ESBL producers out of 50 tested isolates (Fig. 4.7).

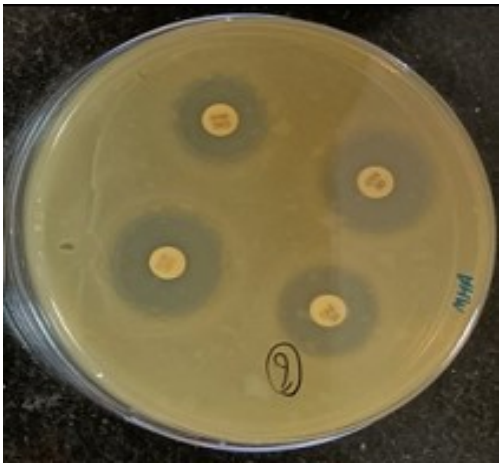


Fig. 4.7 - Combined disc test for confirmation of extended spectrum beta lactamase *E. coli*

The *E. coli* isolates producing ESBLs were compared with the isolates not producing ESBLs for resistance towards different antimicrobials (Table 4.6). *E. coli* with ESBL phenotype (n=10) displayed resistance to cefotaxime, piperacillin and imipenem (100% each) followed by cefpodoxime (90%), ceftriaxone, amoxyclav and ampicillin (80% each). No statistically significant difference was observed in resistance to different beta lactam antibiotics except ceftriaxone (odds ratio- 7.43) with respect to ESBL production.

Table 4.6- Relationship between ESBL production in *E. coli* isolates and resistance to beta lactam antibiotics.

Antibiotics (No. of resistant isolates)	**ESBL + *(10)***		**ESBL – *(40)***		**p-value**
	n (%)	95% CI	n (%)	95% CI	
Ampicillin (32)	8 (80)	44.39-97.48	24 (60)	43.33-75.1	0.239
Piperacillin (46)	10 (100)	69.15-100	36 (90)	76.34-97.21	0.296
Amoxyclav (33)	8 (80)	44.39-97.48	25 (62.5)	45.8-77.27	0.296
Ceftriaxone (22)	8 (80)	44.39-97.48	14 (35)	20.63-51	**0.01**
Cefpodoxime (45)	9 (90)	55.5-99.75	36 (90)	76.34-97.21	1
Cefotaxime (45)	10 (100)	69.15-100	35 (87.5)	73.2-95.81	0.239
Ceftazidime (30)	6 (60)	26.24-87.84	24 (60)	43.33-75.1	1
Aztreonam (5)	2 (20)	2.52-55.61	3 (7.5)	1.57-20.39	0.239
Imipenem (48)	10 (100)	69.15-100	38 (95)	83.08-99.39	0.470

4.5 PCR Detection of Beta Lactamase (*blaTEM*) and Class 1 Integron Intergrase (*intI1*) Genes in *E. coli* Isolates

The detection of *blaTEM* and *intI1* genes by PCR indicated that out of 50 *E. coli* isolates, 62% isolates (31) were positive for *blaTEM* gene, whereas 68% isolates (34) carried *intI1* gene. The positive isolates yielded amplification band of 504 bp and 565 bp specific to *blaTEM* and *intI1* genes (Fig. 4.8 and 4.9). The detection of *blaTEM* and *intI1* genes by PCR in ESBL positive isolates (n=10) indicated that both *blaTEM* and *intI1* were present in eight ESBL positive isolates (80%). The strain of *E. coli* positive for *blaTEM* and *intI1* (Jhandai, 2019) available in the VPHE, Department was used as positive control.

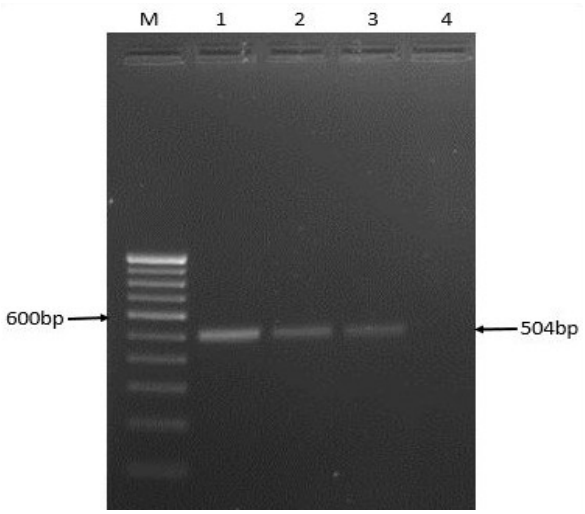


Fig. 4.8 - Detection of *blaTEM* (504bp) gene of *E. coli* isolates by PCR
Lane M– 100bp DNA ladder; Lanes 1-2– *E. coli* isolates; Lanes 3– Positive control; Lane 4– Negative control

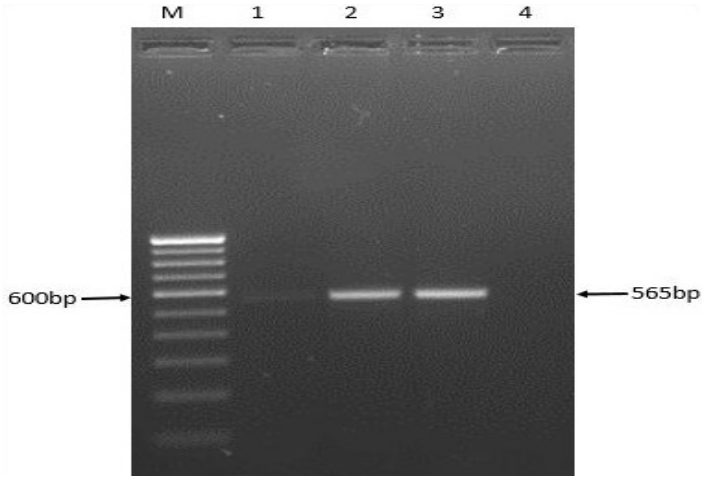


Fig. 4.9 - Detection of *intI1* (565bp) gene of *E. coli* isolates by PCR
Lane M– 100bp DNA ladder; Lanes 1-2– *E. coli* isolates; Lanes 3– Positive control; Lane 4– Negative control

The *E. coli* isolates harboring *blaTEM* were compared with the isolates not containing *blaTEM* gene for resistance towards different beta lactam antimicrobial (Table 4.7). *E. coli* having *blaTEM* gene (n=31) exhibited resistance to imipenem (100%) followed by piperacillin (93.5%), cefpodoxime (90.3%) and cefotaxime (83.87%). There was no statistically significant difference in resistance to different beta lactam antibiotics except ampicillin (odds ratio- 7.14) with carriage of *blaTEM* gene.

Table 4.7- Relationship between carriage of *blaTEM* gene by *E. coli* isolates and resistance towards different beta lactam antibiotics

Antibiotics (No. of resistant isolates)	***blaTEM* + (31)**		***blaTEM* – (19)**		**p-value**
	n (%)	95% CI	n (%)	95% CI	
Ampicillin (32)	25 (80.65)	62.53-92.55	7 (36.84)	16.29-61	**0.002**
Piperacillin (46)	29 (93.55)	78.58-99.21	17 (89.47)	66.86-98.7	0.606
Amoxyclav (33)	22 (70.97)	51.96-85.78	11 (57.89)	33.5-79.75	0.344
Ceftriaxone (22)	14 (45.16)	27.32-63.97	8 (42.11)	20.25-66.5	0.833
Cefpodoxime (45)	28 (90.32)	74.25-97.96	17 (89.47)	66.86-98.7	0.923
Cefotaxime (45)	26 (83.87)	66.27-94.55	19 (100)	82.35-100	0.065
Ceftazidime (30)	18 (58.06)	39.08-75.45	12 (63.16)	38.36-83.71	0.721
Aztreonam (5)	2 (6.45)	0.79-21	3 (15.79)	3.38-39.58	0.285
Imipenem (48)	31 (100)	88.78-100	17 (89.47)	66.86-98.7	0.065

The *E. coli* isolates harboring class 1 integron intergrase (*intI1*) were compared with the isolates not containing class 1 integron intergrase (*intI1*) for resistance towards different antimicrobial (Table 4.8). The study on these criteria indicated that *E. coli* isolates with *intI* gene (n=34) exhibited resistance to tetracycline, levofloxacin, ciprofloxacin and imipenem (100% each). There was no statistically significant difference in resistance to different antibiotics except amikacin (odds ratio- 5.49), tetracycline, levofloxacin, ciprofloxacin and imipenem with respect to carriage of *intI1* gene.

Table 4.8- Relationship between carriage of class 1 integron intergrase (*intI1*) and resistance towards different antibiotics

Antibiotics (No. of resistant isolates)	*intI1* + (34)		*intI1* – (16)		p-value
	n (%)	95% CI	n (%)	95% CI	
Ampicillin (32)	21 (61.76)	43.56-77.83	11 (68.75)	41.34-88.98	0.631
Piperacillin (46)	30 (88.24)	72.55-96.7	16 (100)	79.41-100	0.153
Amoxyclav (33)	22 (64.71)	46.49-80.25	11 (68.75)	41.34-88.98	0.778
Ceftriaxone (22)	15 (44.12)	27.19-62.1	7 (43.75)	19.75-70.1	0.981
Cefpodoxime (45)	31 (91.18)	76.32-98.1	14 (87.5)	61.65-98.45	0.686
Cefotaxime (45)	30 (88.24)	72.55-96.7	15 (93.75)	69.77-99.84	0.544
Ceftazidime (30)	19 (55.88)	37.89-72.81	11 (68.75)	41.34-88.98	0.386
Aztreonam (5)	4 (11.76)	3.3-27.45	1 (6.25)	0.16-30.23	0.544
Imipenem (48)	34 (100)	89.72-100	14 (87.5)	61.65-98.45	**0.035**
Gentamicin (35)	26 (76.47)	58.83-89.25	9 (56.25)	29.88-80.25	0.146
Amikacin (22)	19 (55.88)	37.89-72.81	3 (18.75)	4.05-45.65	**0.014**
Streptomycin (37)	25 (73.53)	55.64-87.1	12 (75)	47.62-92.73	0.912
Azithromycin (13)	11 (32.35)	17.39-50.53	2 (12.5)	1.55-38.35	0.135
Tetracycline (48)	34 (100)	89.72-100	14 (87.5)	61.65-98.45	**0.035**
Colistin (15)	9 (26.47)	12.88-44.36	6 (37.5)	15.2-64.57	0.427
Polymyxin B (37)	23 (67.65)	49.47-82.61	14 (87.5)	61.65-98.45	0.135
Ciprofloxacin (48)	34 (100)	89.72-100	14 (87.5)	61.65-98.45	**0.035**
Levofloxacin (48)	34 (100)	89.72-100	14 (87.5)	61.65-98.45	**0.035**
Trimetoprim (25)	19 (55.88)	37.89-72.81	6 (37.5)	15.2-64.57	0.225
Nitrofurantoin (44)	30 (88.24)	72.55-96.7	14 (87.5)	61.65-98.45	0.941

4.6 Detection and Quantification of Biofilm Forming Ability of *E. coli* Isolates

The detection of biofilm formation by *E. coli* isolates (n=50) and its quantification in LB media after 72h of incubation indicated that out of 50 isolates, 13 isolates (26%) were strong biofilm producers, nine isolates (18%) were moderate biofilm producers, 28 isolates (56%) were weak biofilm producers (Fig. 4.10).

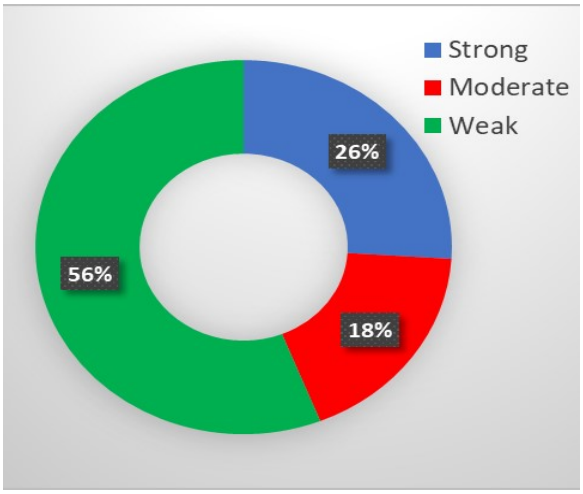


Fig. 4.10 - Biofilm forming potential of *E. coli* isolates

4.7 Phylogenetic Typing of *E. coli* Isolates using PCR

The phylogenetic typing of all the 50 *E. coli* isolates using *chuA* (288bp), *yjaA* (211bp), *TspE4.C2* (152bp), *arpA* (400bp), *ArpAgpE* and *trpA* genes primers indicated that phylogroup B1 (38%) was dominant group followed by A (28%), B2 (10%), unknown/untypable group (8%), F, Clade I/II (6% each) and D (4%) (Fig. 4.11- 4.14). Similar results were also obtained during quadreplex PCR (Fig. 4.15). None of the *E. coli* isolates belonged to phylogroup C in the prsent study. The assignments and distribution of different phylogroups among APEC and non-APEC isolates is summarized in Table 4.9.

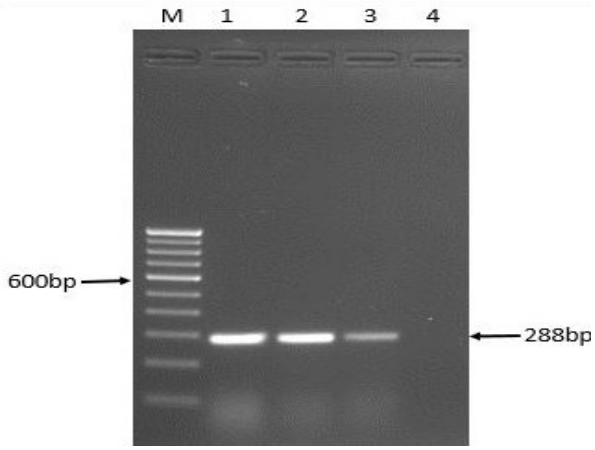


Fig. 4.11- Phylogenetic typing of *E. coli* isolates using *chuA* (288bp) gene by PCR

Lane M– 100bp DNA ladder; Lanes 1-2– *E. coli* isolates; Lanes 3– Positive control; Lane 4– Negative control

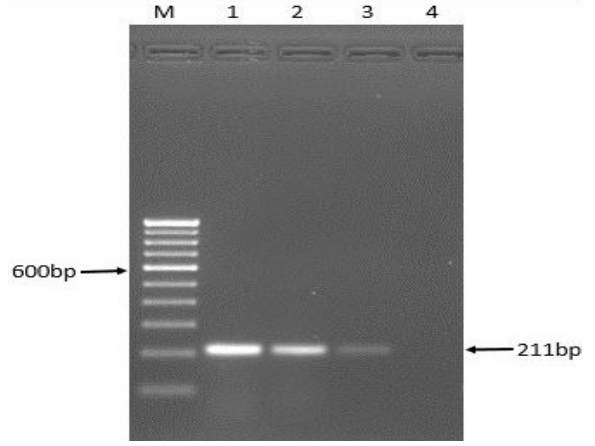


Fig. 4.12 - Phylogenetic typing of *E. coli* isolates using *yjA* (211bp) gene by PCR

Lane M– 100bp DNA ladder; Lanes 1-2– *E. coli* isolates; Lanes 3– Positive control; Lane 4– Negative control

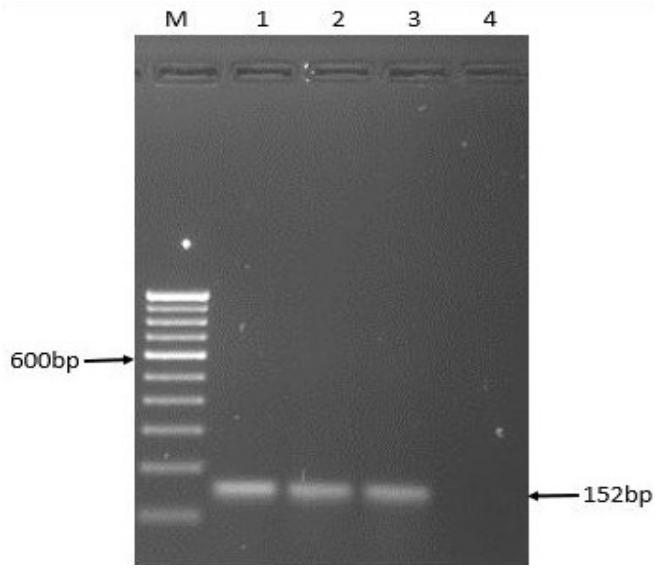


Fig. 4.13 - Phylogenetic typing of *E. coli* isolates using TspE4.C2 (152bp) gene by PCR

Lane M– 100bp DNA ladder; Lanes 1-2– *E. coli* isolates; Lanes 3– Positive control; Lane 4– Negative control

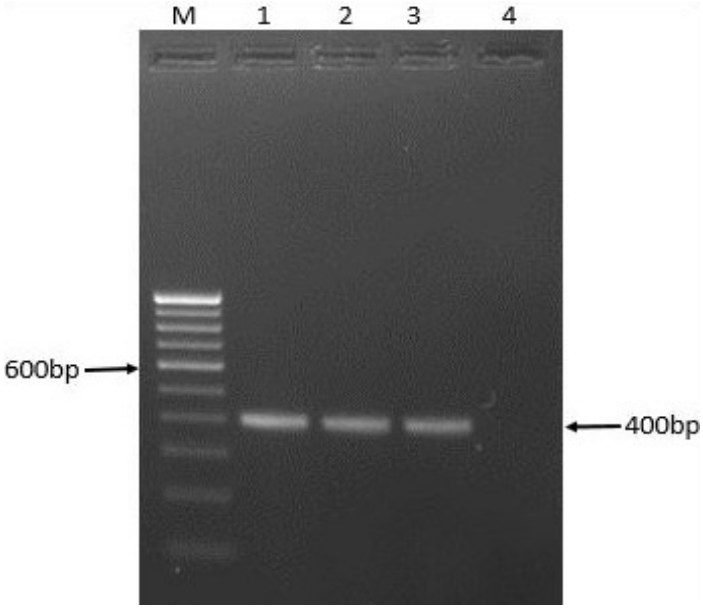


Fig. 4.14 - Phylogenetic typing of *E. coli* isolates using arpA (400bp) gene by PCR

Lane M– 100bp DNA ladder; Lanes 1-2– *E. coli* isolates; Lanes 3– Positive control; Lane 4– Negative control

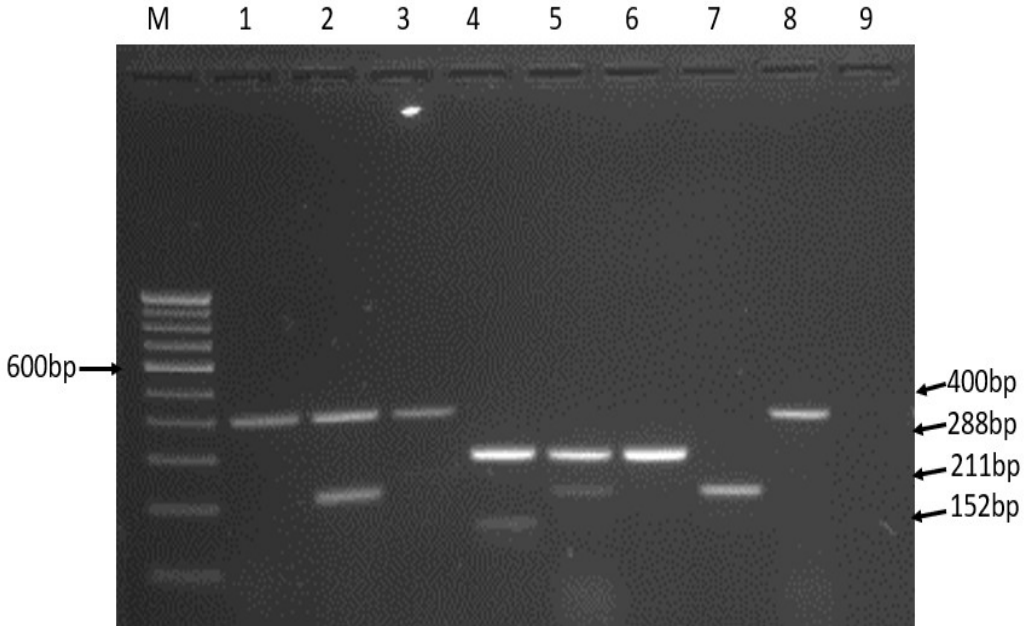


Fig. 4.15 - Phylogenetic typing of *E. coli* isolates using quadruplex phylogroup genes by PCR

Lane M– 100bp DNA ladder; Lanes 1-8– *E. coli* isolates with different gene combination; Lane 9– Negative control

Table 4.9 - Association of genes (*arpA, chuA, yjA, TspE4.C2*) and phylogroups

Phylogroup/Category/Clade	Genotype combination	Number of isolates
A (14)	*arpA* + *chuA* - *yjA* + *TspE4.C2* -	9
	arpA + *chuA* - *yjA* - *TspE4.C2* -	5
B1 (19)	*arpA* + *chuA* - *yjA* - *TspE4.C2* +	19
B2 (5)	*arpA* - *chuA* + *yjA* + *TspE4.C2* +	5
	arpA - *chuA* + *yjA* - *TspE4.C2* +	0
Clade I/II (3)	*arpA* - *chuA* - *yjA* + *TspE4.C2* -	3
D (2)	*arpA* + *chuA* + *yjA* - *TspE4.C2* +	2
F (3)	*arpA* - *chuA* + *yjA* - *TspE4.C2* -	3
Unknown (4)	*arpA* + *chuA* - *yjA* + *TspE4.C2* +	4

4.8 Bacteriophage Isolation against Colibacillosis causing APEC

4.8.1 Spot test

The spot test was conducted to check the presence of bacteriophage against colibacillosis causing *E. coli* from the filterates of all the five samples viz. wastewater of poultry farm, LUVAS; sewage sample from sewage water treatment plant and slurry from biogas plant, CCSHAU, Ganges water. The bacteriophages were found to be present in all the five filterates as evident from presence of different types of zones

of lysis. The clear zone of lysis are indicative of lytic bacteriophage and filterate of wastewater sample from poultry farm exhibited clear zone of lysis on *E. coli* bacterial lawn onto LB/EMB/MLA agar plate (Fig. 4.16) indicating the existence of desired phage. The isolated *E. coli* strain APEC-P02 was used as host to isolate bacteriophage. No clear zone of lysis were observed in rest of the samples *viz,* sewage water, biogas plant slurry and Ganga water filtrates hence taken as negative for phage.

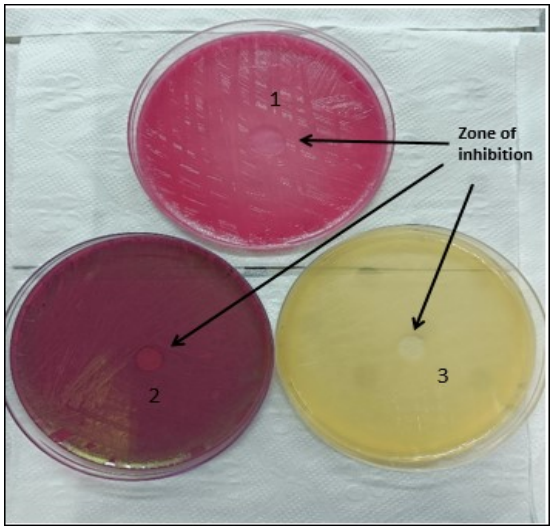


Fig. 4.16 - Detection of phage by spot test against APEC 1. MLA; 2. EMB; 3. LB agar

4.8.2 Bacteriophage selection and enrichment using double agar overlay technique

In the double agar overlay technique, bacteriophage at different dilutions produced circular clear area of cell lysis i.e., plaques indicating presence of pure phage using LB agar plates. These plaques were having clear cut edges (Fig. 4.17).

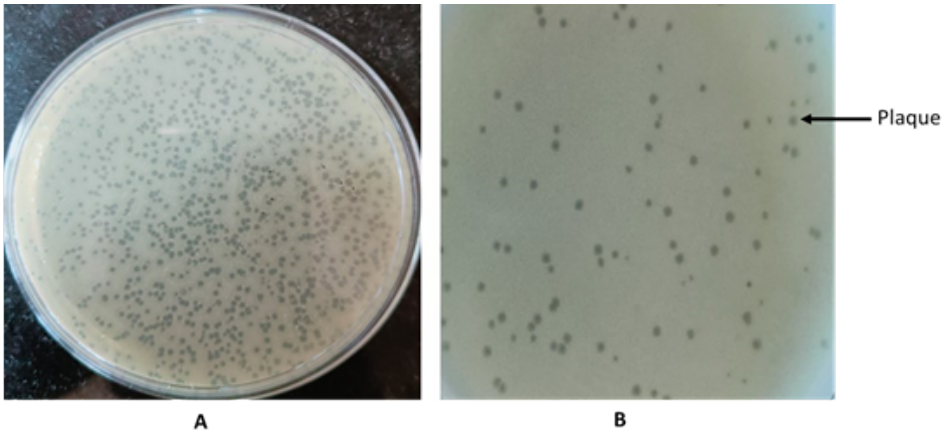


Fig. 4.17 - *Escherichia* phage PJDM plaque morphology (A) 10^{-6} dilution (B) 10^{-7} dilution

4.8.3 Determination of plaque forming units

The number of plaques in 10^{-7} dilution of phage filtrate were found to be 180. Further, calculation of PFU using the recommended formula as detailed below indicated that phage titer obtained was $1.2x10^{10}$ PFU/ml. The calculation as follow

PFU/ml= Number of plaques counted X 1/Dilution factor X 1/ Volume of phage taken

PFU/ml= 180 plaques X 1/ 10^{-7} dilution X 1/150 µl

PFU/ml=$1.2x10^{10}$ PFU/ml.

4.9 Morphological Characterization of Bacteriophage using TEM

The morphological characterization of isolated bacteriophage carried out at TEM facilities at Plant Pathology Department of ICAR-IARI, PUSA, New Delhi indicated that the size of phage was approximately 80 nm head and 140 nm tail (Fig. 4.18). The shape of the phage i.e., icosahedral head, non-contractile tail and further comparison of size and shape of isolated phage with previously reported phages (Harper, 2011; ICTV, 2011) revealed that the isolated phage was of family *Siphoviridae* (non-contractile tail) and order *Caudovirales* (tailed phage). The isolated phage was named as '*Escherichia*, phage PJDM' as per the guidelines (Adriaenssens and Brister, 2017).

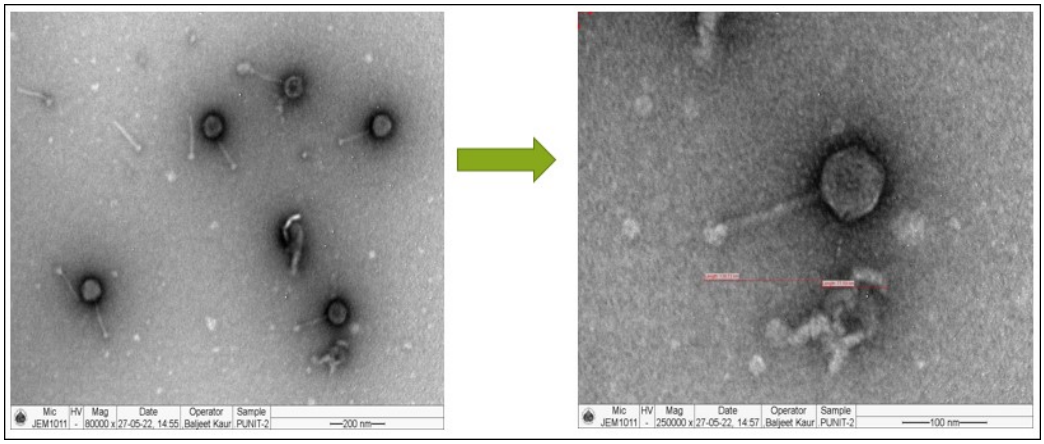


Fig. 4.18 - Transmission electron microscopy of *Escherichia* phage PJDM

4.10 Host Range and Specificity Testing of *Escherichia* Phage PJDM

The examination of host range and specificity testing of *Escherichia* phage PJDM against all the 50 *E. coli* isolates, *Klebsiella pneumonia*, *Salmonella* Typhimurium, *Staphylococcus aureus* by spot test indicated that the isolated phage PJDM was specific to 25 *E. coli* isolates, as these 25 isolates exhibited positive spot test against PJDM. The spot test was negative for *Klebsiella pneumonia*, *Salmonella*

Typhimurium, *Staphylococcus aureus* (Fig. 4.19) indicating phage PJDM was ineffective against these bacterias.

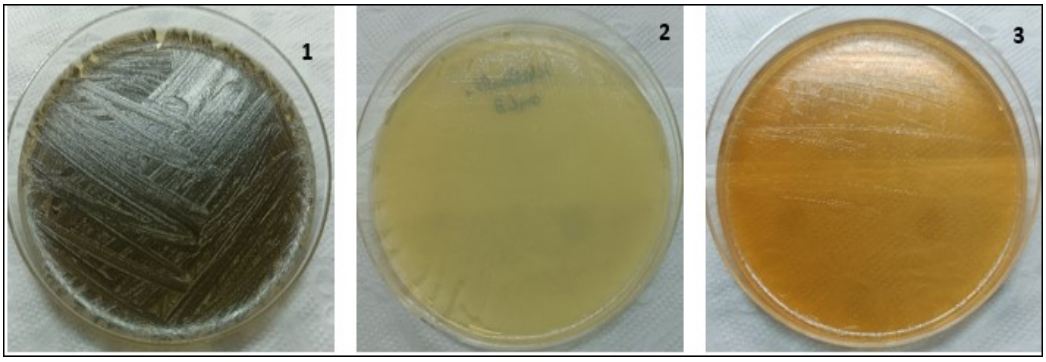


Fig. 4.19 - Host range and specificity tesing of *Escherichia* phage PJDM against different bacteria by spot test. (1) *Staphylococcus* on Baird Parker agar (2) *Klebsiella* on LB agar (3) *Salmonella* on MLA

4.11 Serotyping of Susceptible *E. coli* Isolates

The serological identification of 25 isolates exhibiting positive spot test for isolated bacteriophage revealed that 24/25 isolates of *E. coli* were typable, whereas one isolate was untypable. The serotype identified were O26 (9/25, 36%), O98 (8/25, 32%), O120 (3/25, 12%), O11 (2/25, 8%) and O135 and O17 (one each).

4.12 Biophysical Characterization of Bacteriophage

The study on biophysical characterization of *Escherichia* phage PJDM in terms of thermal and pH stability revealed that isolated phage resisted the temperatures 30°C, 37°C and 42°C for 24 h. However, with the passage of time, titer of phage gets reduced but phage remained viable up to 300 days at these three temperatures. The phage also remained viable up to 6h at 50°C, whereas, it became inactive within 120 min and 15 min at 70°C and 90°C, respectively (Table 4.10 and Fig. 4.20). The study on pH stability indicated that the isolated phage exhibited negligible change in titer at pH 5 to 11. The phage titer gets reduced by one log and seven log point at pH 12 and 13 within 24h, respectively. The titer gets reduced to half at pH 4 in 24 h and it became inactive within 120 min and 60 min at pH 3 and pH 2, respectively (Table 4.11 and Fig. 4.21).

Table 4.10 – Thermal stability of *Escherichia* phage PJDM

Temperature	**30°C**	**37°C**	**42°C**	**50°C**	**70°C**	**90°C**
Time	PFU (10^8)	PFU (10^8)	PFU (10^8)	PFU (10^8)	PFU (10^8)	PFU (10^8)
0 min	120	120	120	120	120	120
15 min	120	120	120	100	15	0
30 min	120	120	110	100	13	0
60 min	120	110	107	33	10	0
120 min	120	110	40	20	0.3	0
6 h	120	110	33	1.5	0	0
24 h	100	100	33	0	0	0
2 d	30	20	20	0	0	0
7 d	20	20	20	0	0	0
20 d	18	18	15	0	0	0
60 d	10	10	10	0	0	0
120 d	10	2	0.2	0	0	0
300 d	2	0.1	0.0002	0	0	0

Table 4.11 - pH stability of *Escherichia* phage PJDM

Temperature	**pH 2**	**pH 3**	**pH 4**	**pH 5**	**pH 6**	**pH 7**	**pH 8**	**pH 9**	**pH 10**	**pH 11**	**pH 12**	**pH 13**
Time	PFU (10^8)	PFU (10^8)	PFU (10^8)	PFU (10^8)	PFU (10^8)	PFU (10^8)	PFU (10^8)	PFU (10^8)	PFU (10^8)	PFU (10^8)	PFU (10^8)	PFU (10^8)
0 min	120	120	120	120	120	120	120	120	120	120	120	120
15 min	1	10	100	100	100	120	100	100	100	100	20	0.02
30 min	0.02	0.2	100	100	100	120	100	100	100	100	10	0.02
60 min	0	0.1	100	100	100	100	100	100	100	100	10	0.01
120 min	0	0	90	90	100	100	100	100	100	100	10	0.002
6 h	0	0	80	80	90	100	90	80	70	70	10	0.00002
24 h	0	0	60	75	90	10	85	80	70	70	10	0

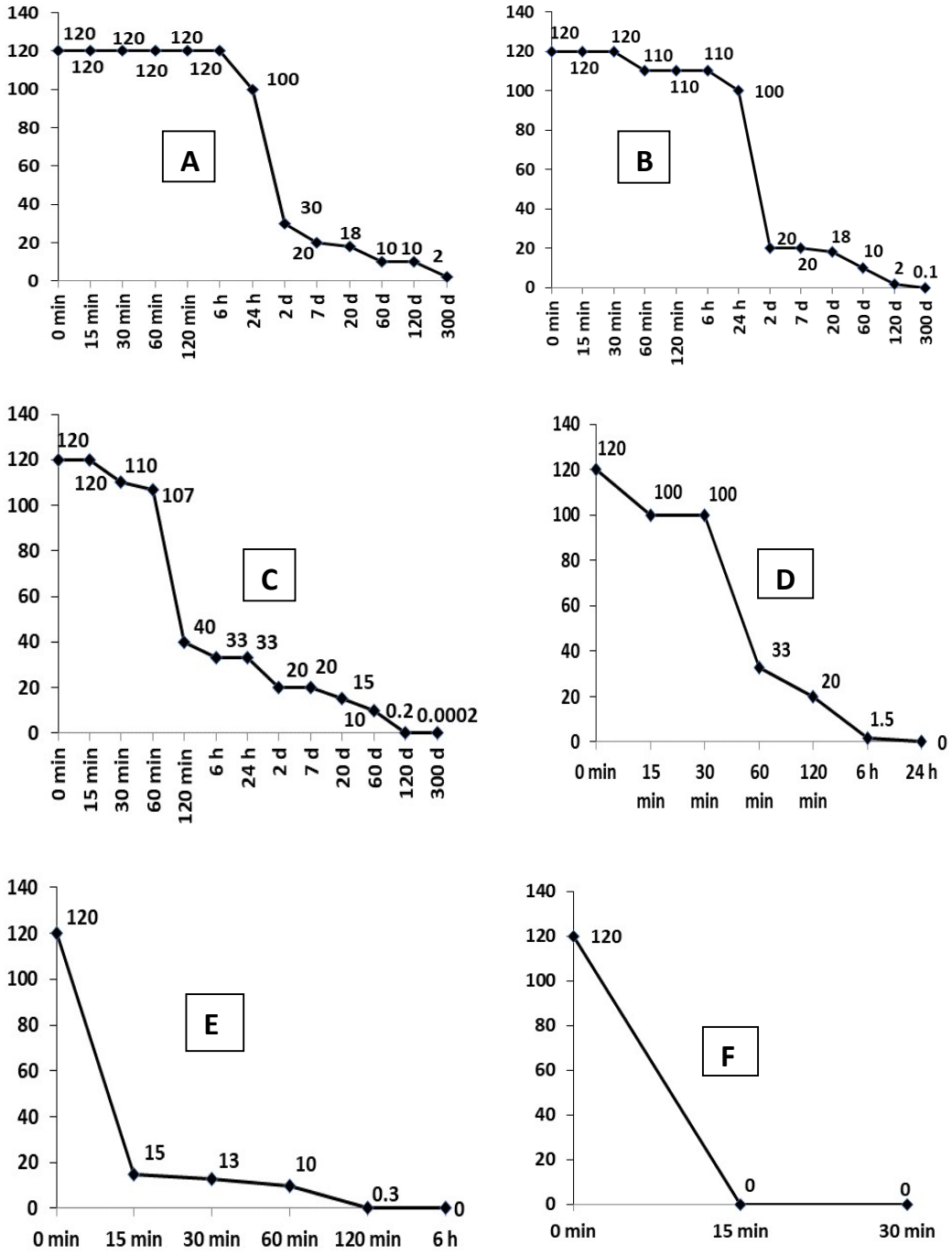
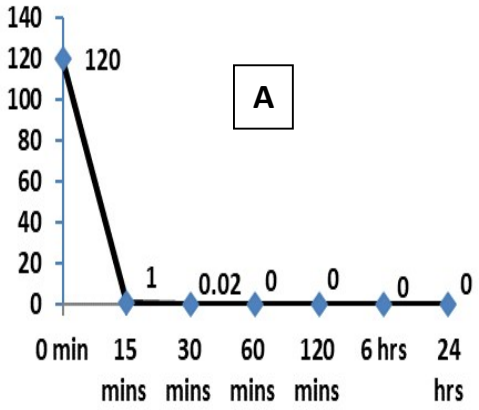


Fig. 4.20 - Thermal sensitivity of *Escherichia* phage PJDM at different temperature

A- 30°C; B- 37°C; C- 42°C; D- 50°C; E- 70°C; F- 90°C

(X axis- PFU/ml (10^8) and Y axis- Time)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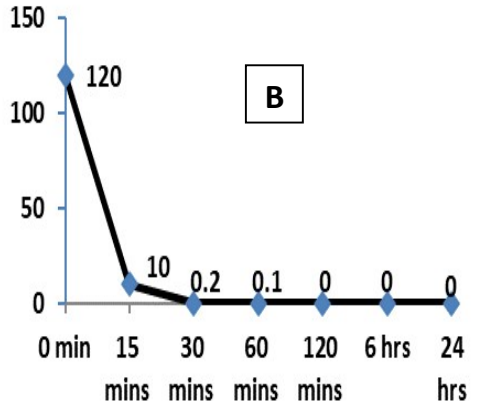
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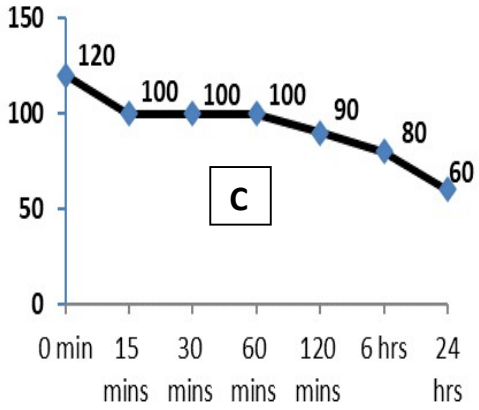
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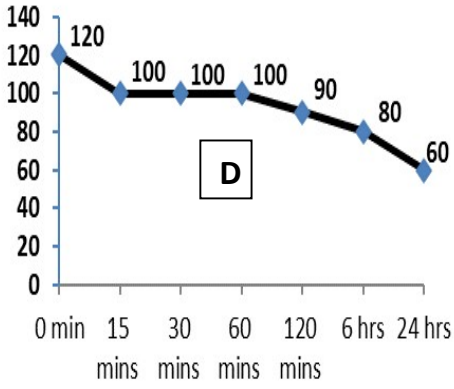
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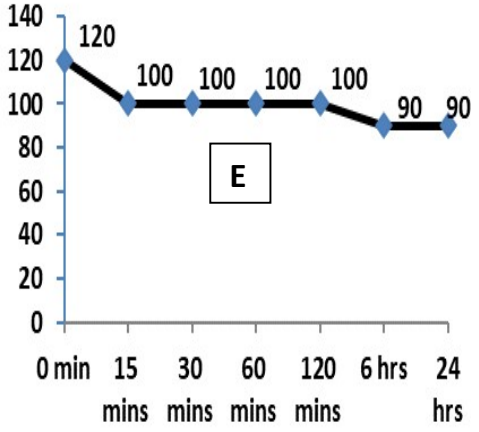
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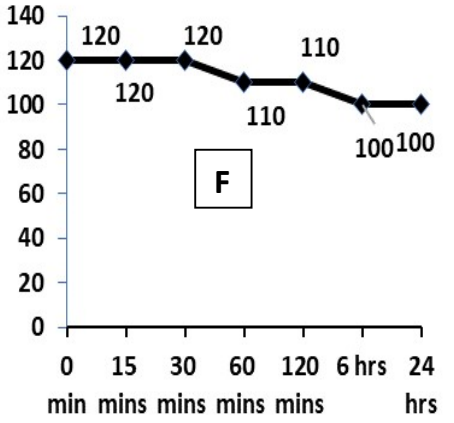
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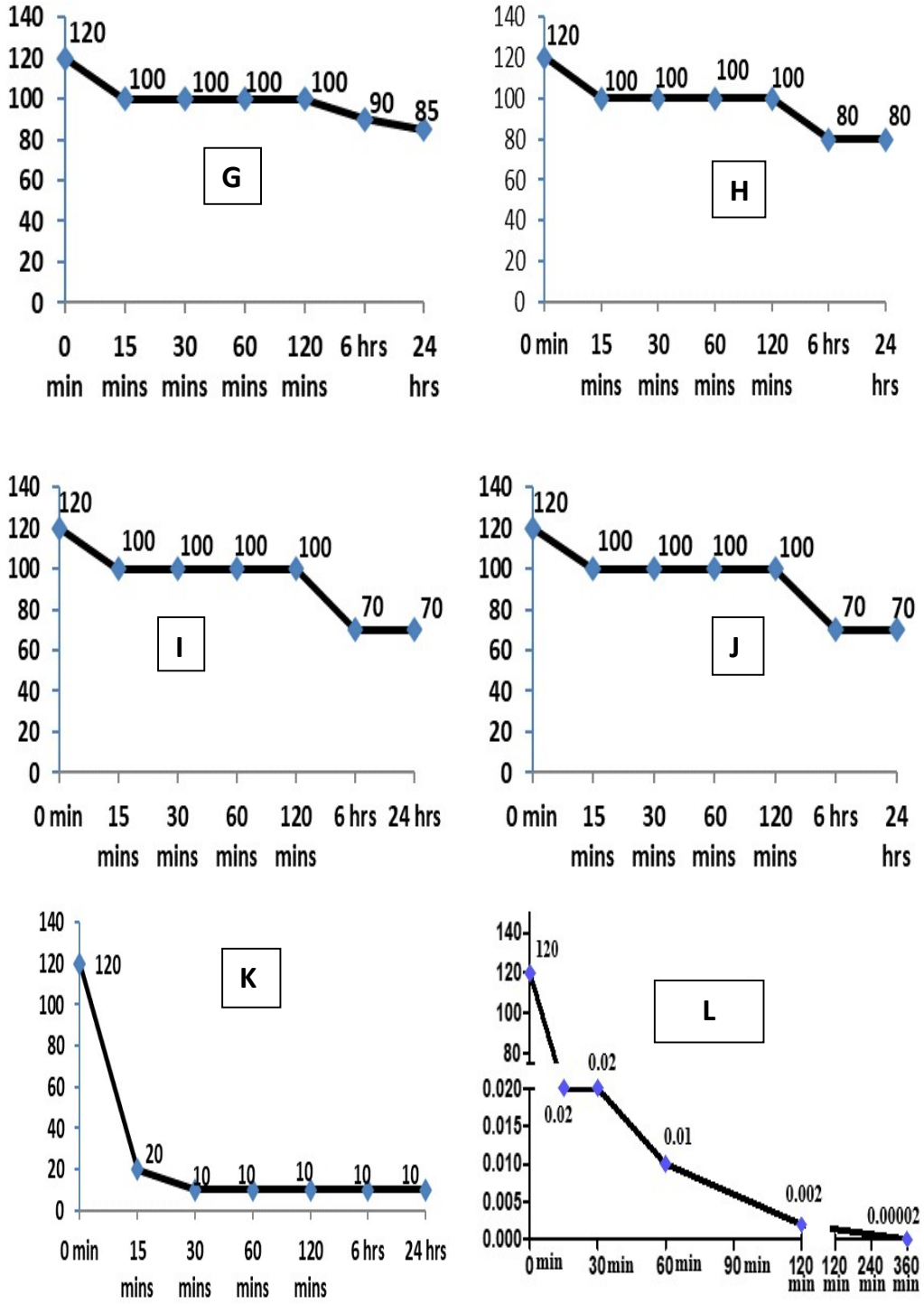

Fig. 4.21 - pH sensitivity of *Escherichia* phage PJDM at different pHs

A- pH 2; B- pH 3; C- pH 4; D- pH 5; E- pH 6; F- pH 7; G- pH 8; H- pH 9; I- pH 10; J- pH 11; K- pH 12; L- pH 13

(X axis- PFU/ml (10^8) and Y axis- Time)

4.13 Determination of Latent Period and Burst Size of *Escherichia* Phage PJDM

The one step growth curve of *Escherichia* phage PJDM studied at 37°C to calculate phage titer by testing samples for PFU at an interval of 10 min for 80 min revealed that the latent period of phage was 20 min (Fig. 4.22). After 30 min of infection, the progeny formation increased drastically. The one step growth curve analysis further revealed that the burst size of phage was 26.5 phages per bacterial cell.

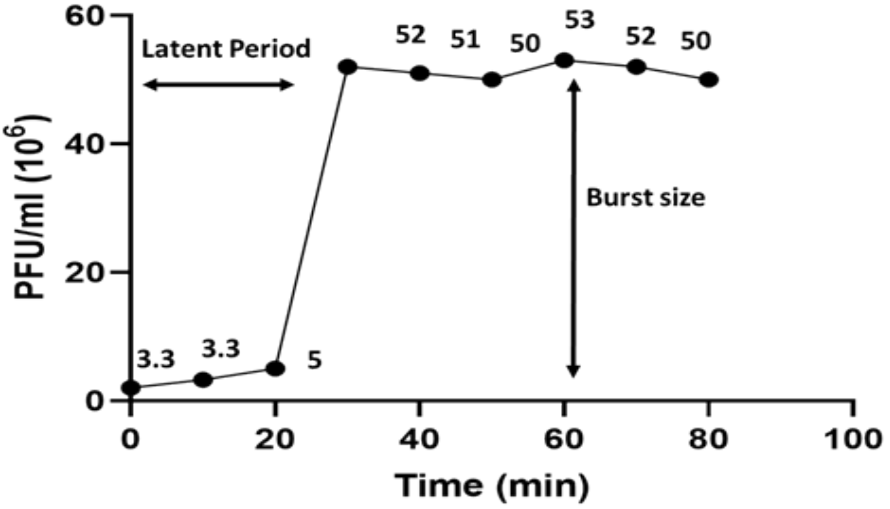

Fig. 4.22 - Latent period and burst size of *Escherichia* phage PJDM by one Step growth curve

4.14 Whole Genome Sequencing of Phage PJDM

Whole genome analysis of bacteriophage results revealed that the phage PJDM genome has a double-stranded linear DNA molecule and its genomic DNA contains 57,756 base pairs with a GC content of 43.58 %. The sequence similarity results by BLASTN search revealed that the phage PJDM was related to *Escherichia* phage vB_EcoS_AVIO78A (GenBank accession number MZ726797) with 93.29% similarity, after covering 84% query. The genome assemblies had a coverage of 49,864.95X and *Escherichia* phage PJDM genome consisted of 98 predicted putative ORFs and there were no putative tRNA genes in genome. Gene annotation using Diamond (BlastN mode) resulted in the annotation of 89 genes in *Escherichia* phage PJDM sample, whereas nine genes had no significant BLAST hits/annotations. The majority of hits were found to be against *Escherichia* phage vB_EcoS_AVIO78A followed by *Siphoviridae* spp. isolate ctZVb9 (Fig. 4.23). The blastN analytic results of 98 genes are detailed in Table 4.12.

The open reading frames (ORFs) of *Escherichia* phage PJDM identified by blastX search predicted a total of 98 ORFs. Among these genes, 34 genes were predicted to have known functions (Table 4.13 and Fig. 4.24) and were grouped into three functional modules as follows: phage packaging and lysis (ORFs 1, 14, 26, 36, 63, 64 and 66), phage structure and assembly (ORFs 15, 45, 46, 51, 52, 53, 54, 57, 61 and 62) and DNA metabolism and replication (ORFs 6, 34, 56, 60, 71, 73, 76, 77, 78, 79 and 80). Additionally, six auxiliary metabolic genes (AMGs) (ORFs 31, 59, 69, 70, 84 and 95) were predicted and 64 ORFs were also predicted to encode hypothetical proteins.

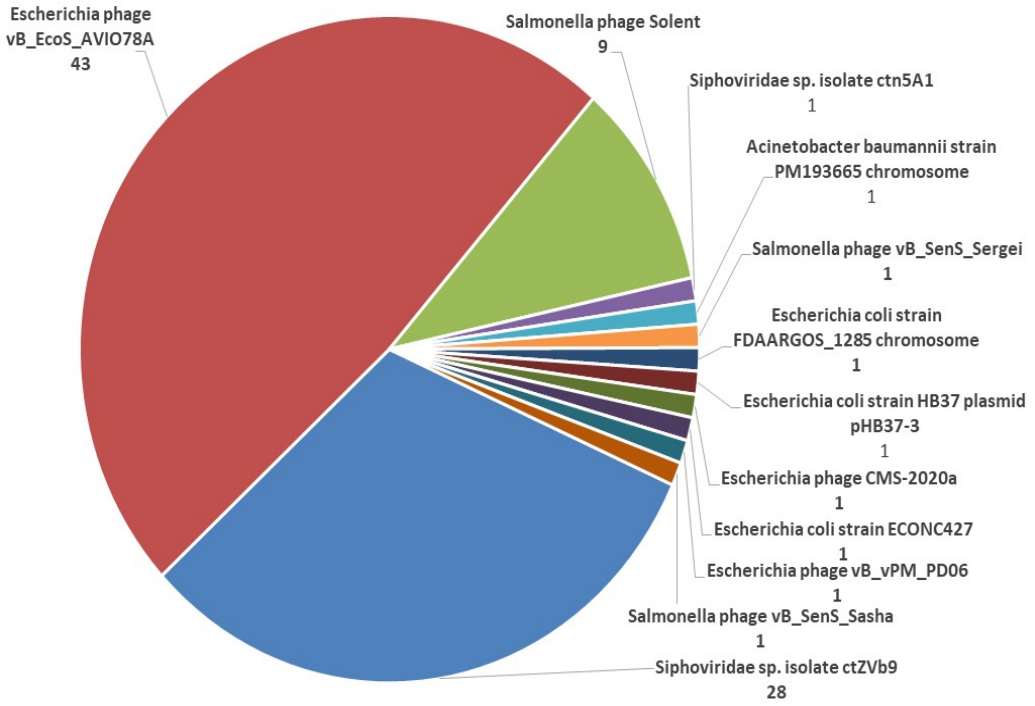

Fig. 4.23 - Top hit species distribution for *Escherichia* phage PJDM genes by blastN analysis (n=98 genes)

Table 4.12 - Detail annotation of *Escherichia* phage PJDM genes (n=98)

Sr No.	Gene	Start	End	Sequence length	Strand	Hit desc.	Hit ACC	E-Value	Similarity
1	01	324	959	636	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_ asm: *Siphoviridae* sp. isolate ctZVb9	28616168	0	96.53
2	02	1075	1311	237	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	4.5751E-108	98.67
3	03	1308	1460	153	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	7.93098E-69	98.69
4	04	1528	1860	333	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	3.1181E-111	91.14
5	**05**	**1857**	**2024**	**168**	**-**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**
6	06	2021	2935	915	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	0	95.08
7	07	2932	3129	198	-	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	4.91271E-87	96.96
8	08	3126	3323	198	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	2.26955E-90	97.97
9	09	3320	3475	156	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	3.8641E-52	91.66
10	10	3472	3738	267	-	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	1.4303E-123	97.37
11	11	3774	3980	207	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	5.11113E-97	98.5
12	12	4052	4441	390	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	9.4593E-172	95.34
13	**13**	**4483**	**4677**	**195**	**-**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**
14	14	4742	6505	1764	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	0	96.14
15	15	6667	7365	699	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	0	95.08

Sr No.	Gene	Start	End	Sequence length	Strand	Hit desc.	Hit ACC	E-Value	Similarity
16	16	7416	7775	360	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	8.6241E-177	98.33
17	17	7792	8295	504	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	0	98.61
18	18	8282	8536	255	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	6.3551E-117	97.25
19	19	8748	9023	276	-	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	3.171E-130	97.82
20	20	9029	9169	141	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	9.69315E-33	96.70
21	21	9163	9492	330	-	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	1.7679E-143	95.15
22	22	9510	9710	201	-	gnl|BL_ORD_ID|70251260MH586730.1 *Salmonella* phage Solent	70251260	1.06777E-93	98.50
23	23	9714	9860	147	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	3.60445E-47	91.15
24	**24**	**9857**	**10177**	**321**	**-**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**
25	25	10425	10619	195	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	1.94227E-06	95.23
26	26	10761	12116	1356	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	0	96.17
27	27	12149	12481	333	-	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	3.7315E-165	98.79
28	28	12497	12841	345	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	3.9093E-160	96.81
29	29	12904	13152	249	-	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	1.3476E-108	95.98
30	30	13149	13478	330	-	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	6.1812E-163	98.78

Sr No.	Gene	Start	End	Sequence length	Strand	Hit desc.	Hit ACC	E-Value	Similarity
31	31	13475	13978	504	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	0	98.21
32	32	14086	14745	660	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	3.7129E-148	96.06
33	33	14735	14917	183	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	9.72185E-84	98.36
34	34	14917	15111	195	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	2.22778E-90	98.46
35	35	15098	15241	144	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	1.58692E-65	99.30
36	36	15284	16243	960	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	0	95.31
37	37	16240	16470	231	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	2.6342E-115	100
38	**38**	**16470**	**16679**	**210**	**-**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**
39	**39**	**16639**	**16890**	**252**	**-**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**
40	40	16896	17171	276	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	3.1486E-135	98.91
41	**41**	**17336**	**17443**	**108**	**-**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**
42	42	17957	18304	348	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	3.8917E-170	98.27
43	43	18301	18711	411	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	0	99.02
44	44	18711	19970	1260	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	0	98.25
45	45	19970	20380	411	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	0	97.32
46	46	20384	21403	1020	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	0	98.33

Sr No.	Gene	Start	End	Sequence length	Strand	Hit desc.	Hit ACC	E-Value	Similarity
47	47	21467	21820	354	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	8.525E-172	98.02
48	48	21752	22189	438	-	gnl|BL_ORD_ID|28615340BK024293.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctn5A1	28615340	5.0689E-160	96.06
49	49	22574	22984	411	+	gnl|BL_ORD_ID|70251260MH586730.1 *Salmonella* phage Solent	70251260	0	99.51
50	50	22984	23358	375	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	0	98.93
51	51	23360	23791	432	+	gnl|BL_ORD_ID|70251260MH586730.1 *Salmonella* phage Solent	70251260	0	99.07
52	52	23791	24213	423	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	0	98.10
53	53	24250	25707	1458	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	0	97.05
54	54	25813	26235	423	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	0	98.58
55	**55**	**26624**	**27235**	**612**	**-**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**
56	**56**	**27237**	**27416**	**180**	**-**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**
57	57	27551	30118	2568	+	gnl|BL_ORD_ID|70251260MH586730.1 *Salmonella* phage Solent	70251260	0	96.79
58	58	30118	30621	504	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	0	98.21
59	59	30618	31124	507	+	gnl|BL_ORD_ID|70251260MH586730.1 *Salmonella* phage Solent	70251260	0	96.64
60	60	31121	31480	360	+	gnl|BL_ORD_ID|70251260MH586730.1 *Salmonella* phage Solent	70251260	4.0409E-170	97.22
61	61	31473	34001	2529	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	0	94.61
62	62	33998	36037	2040	-	gnl|BL_ORD_ID|70544648NC_052653.1 *Escherichia* phage vB_vPM_PD06, complete genome MH816848.1 *Escherichia* phage vB_vPM_PD06	70544648	0	94.375
63	63	36064	36387	324	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	7.8889E-157	98.14

Sr No.	Gene	Start	End	Sequence length	Strand	Hit desc.	Hit ACC	E-Value	Similarity
64	64	36384	36773	390	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	9.3926E-177	95.89
65	65	36784	37014	231	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	2.6529E-110	98.70
66	66	37001	37399	399	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	0	96.24
67	67	37464	37697	234	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	2.7126E-105	97.00
68	68	37704	38144	441	-	gnl|BL_ORD_ID|81072210CP050415.1 *Acinetobacter baumannii* strain PM193665 chromosome, complete genome	81072210	1.05659E-07	89.28
69	69	38110	38649	540	-	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	0	97.25
70	70	38642	38995	354	-	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	1.8716E-158	95.76
71	**71**	**38992**	**39219**	**228**	**-**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**	**No Blast Hit**
72	72	39212	39748	537	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	0	96.99
73	73	39788	41644	1857	-	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	0	96.98
74	74	41725	42588	864	-	gnl|BL_ORD_ID|58981124KY002061.1 *Salmonella* phage vB_SenS_Sergei	58981124	0	95.83
75	75	42604	43749	1146	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	0	97.81
76	76	43749	44501	753	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	0	96.41
77	77	44677	47355	2679	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	0	97.17
78	78	47397	47888	492	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	0	98.1

Sr No.	Gene	Start	End	Sequence length	Strand	Hit desc.	Hit ACC	E-Value	Similarity
79	79	47885	48160	276	+	gnl|BL_ORD_ID|70251260MH586730.1 *Salmonella* phage Solent	70251260	9.59758E-71	99.3
80	80	48164	50518	2355	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	0	96.85
81	81	50609	50821	213	+	gnl|BL_ORD_ID|70251260MH586730.1 *Salmonella* phage Solent	70251260	5.2521E-102	99.06
82	82	50823	51020	198	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	1.06341E-83	95.95
83	83	51013	51228	216	+	gnl|BL_ORD_ID|58981123NC_047786.1 *Salmonella* phage vB_SenS_Sasha, complete genome KX987158.1 *Salmonella* phage vB_SenS_Sasha, complete genome	58981123	5.37858E-97	97.22
84	84	51225	51905	681	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	0	95.74
85	85	51902	52297	396	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	4.5073E-165	93.6
86	86	52369	53136	768	+	gnl|BL_ORD_ID|70251260MH586730.1 *Salmonella* phage Solent	70251260	0	97.39
87	87	53133	53312	180	+	gnl|BL_ORD_ID|23826175CP070152.1 *Escherichia coli* strain FDAARGOS_1285 chromosome, complete genome	23826175	3.45052E-78	97.22
88	88	53324	53521	198	+	gnl|BL_ORD_ID|81405228CP053083.1 *Escherichia coli* strain HB37 plasmid pHB37-3, complete sequence	81405228	4.84369E-97	100
89	89	53523	53786	264	+	gnl|BL_ORD_ID|82557550CP054387.1 *Escherichia* phage CMS-2020a, complete genome	82557550	6.5221E-127	98.48
90	90	53797	54465	669	+	gnl|BL_ORD_ID|25648808CP071133.1 *Escherichia coli* strain ECONC427 chromosome	25648808	0	99.20
91	91	54462	54632	171	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	7.05841E-70	95.88
92	92	54722	54946	225	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	2.62684E-95	95.57
93	93	54947	55270	324	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	1.56438E-34	95.91

Sr No.	Gene	Start	End	Sequence length	Strand	Hit desc.	Hit ACC	E-Value	Similarity
94	94	55257	55499	243	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	4.6778E-113	98.32
95	95	55502	56071	570	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	0	98.77
96	96	56074	56295	222	+	gnl|BL_ORD_ID|28616168BK020197.1 MAG TPA_asm: *Siphoviridae* sp. isolate ctZVb9	28616168	1.20285E-93	95.51
97	97	56288	56542	255	+	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	5.1622E-83	96.39
98	98	56672	57469	798	-	gnl|BL_ORD_ID|45512187MZ726797.1 *Escherichia* phage vB_EcoS_AVIO78A, complete genome	45512187	0	94.86

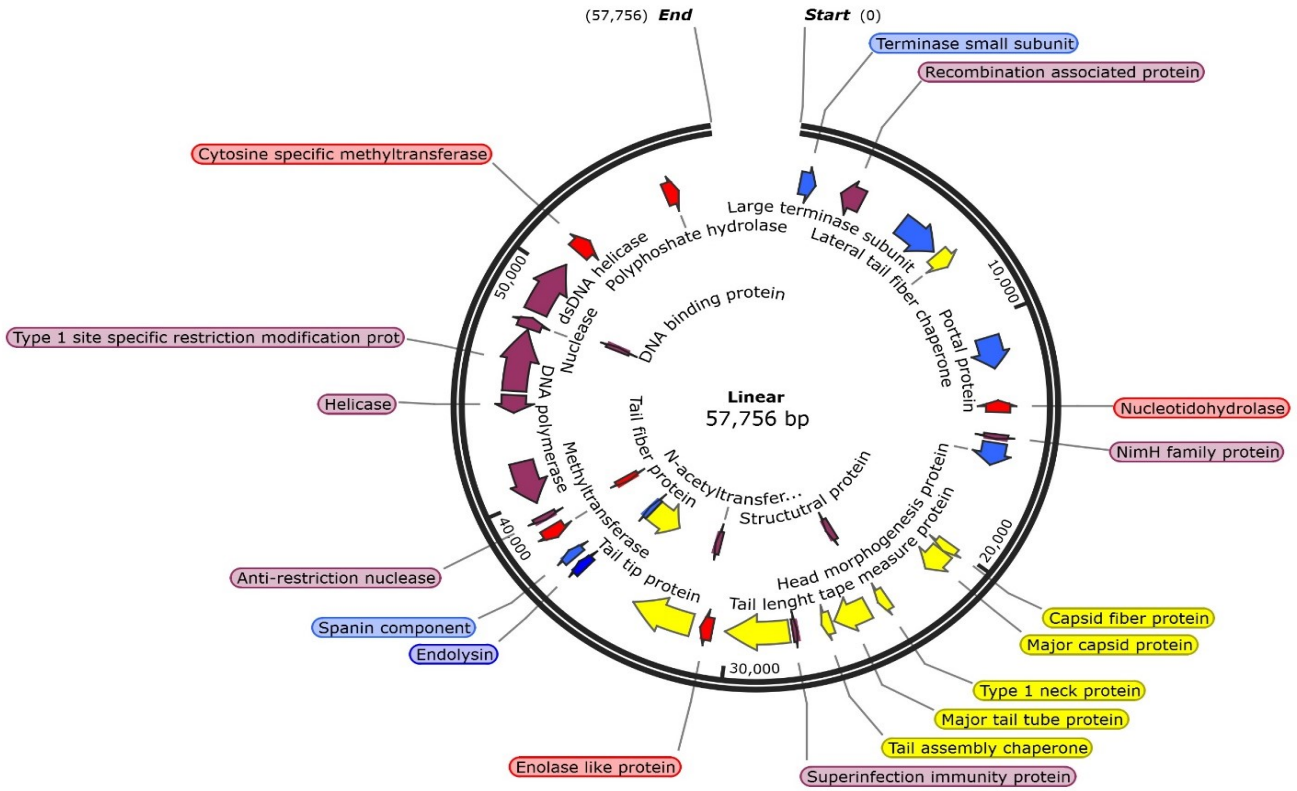

Fig. 4.24 - Functional annotation of *Escherichia* phage PJDM using SnapGene

Yellow arrow represents phage structure and assembly ORFs

Blue arrow represents phage packaging and lysis ORFs

Purple arrow represents DNA metabolism and replication ORFs

Red arrow represents auxiliary metabolic ORFs

Clockwise direction of arrow represents ORFs on positive strand of DNA

Anticlockwise direction of arrow represents

ORFs on negative strand of DNA

Table 4.13 - Genomic annotation of *Escherichia* phage PJDM

ORF	Start	End	Strand	Function
1	324	959	+	Terminase small subunit
6	2021	2935	-	Recombination-associated protein
14	4742	6505	+	Large terminase subunit
15	6667	7365	+	Lateral tail fiber chaperone/hypothetical
26	10761	12116	+	Portal protein
31	13475	13978	-	Deoxyuridine 5'-triphosphate nucleotidohydrolase
34	14917	15111	+	NinH family protein
36	15284	16243	+	Head morphogenesis protein
45	19970	20380	+	Capsid fiber protein
46	20384	21403	+	Major capsid protein
51	23360	23791	+	Type I neck protein
52	23791	24213	+	Structural protein
53	24250	25707	+	Major tail tube protein
54	25813	26235	+	Tail assembly chaperone
56	27237	27416	-	Superinfection immunity protein
57	27551	30118	+	Tail length tape measure protein
59	30618	31124	+	Enolase-like protein
60	31121	31480	+	N-acetyltransferase tail protein
61	31473	34001	+	Tail tip protein
62	33998	36037	-	Tail fibers protein
63	36064	36387	+	Holin
64	36384	36773	+	L alanyl D glutamate peptidase endolysin
66	37001	37399	+	i-spanin component
69	38110	38649	-	DNA N-6-adenine-methyltransferase
70	38642	38995	-	2'-deoxynucleoside 5'-phosphate N-hydrolase
71	38992	39219	-	Anti-restriction nuclease
73	39788	41644	-	DNA polymerase
76	43749	44501	-	Helicase
77	44677	47355	+	Type I site specific restriction modification protein
78	47397	47888	+	Nuclease
79	47885	48160	+	DNA binding protein
80	48164	50518	+	dsDNA helicase
84	51225	51905	+	Cytosine specific methyltransferase
95	55502	56071	+	Polyphosphate hydrolase

4.15 Restriction Endonuclease Profiling of Phage PJDM

Escherichia phage PJDM DNA sequence obtained after whole genome sequencing were subjected to *in silico* restriction endonucleases NEB cutter tool and results revealed that phage PJDM had 17, 20 and 26 restriction sites for restriction enzymes *EcoRI, SspI* and *VspI,* respectively.

4.16 Determination of *in vivo* Mean Lethal Dose (LD50) of APEC-P02 in Chicks

Using oral administration route, the LD50 of multidrug resistant APEC isolate (APEC-P02) was determined by i*n vivo* in unvaccinated day-old broiler chickens. Different mortality rates were observed in different groups. All the dead birds during the experiment showed pathological lesions of colibacillosis during post mortem examination. No mortalities were observed in groups 1a (control group), 2a, 3a, 4a during the study period of 10 days. The LD50 was calculated by the method of Reed and Muench. The analysis of mortality and survivability of chicks in each group during 10 d trial period indicated that the studied APEC-P02 was having mean LD50 of 1.12×10^8 CFU/ml during oral administration (Table 4.14).

Table 4.14 - Mortality in chicks and determination of LD50 dose of APEC-P02

Group	Dose	Mortality	Survivability	Cumulative mortality	Cumulative survivability	Total	% Mortality
6a	2×10^8 CFU/ml	6	4	7	4	11	63.63
5a	2×10^7 CFU/ml	1	9	1	13	14	7.14
4a	2×10^6 CFU/ml	0	10	0	23	23	0
3a	2×10^5 CFU/ml	0	10	0	33	33	0
2a	2×10^4 CFU/ml	0	10	0	43	43	0
1a	Nil	0	10	0	53	53	0

Log_{10} 50% end point dilution = Log_{10} of lower dilution + (proportionate distance×Log_{10} of dilution factor)

$$\text{Proportionate distance} = \frac{\text{50 per cent} - \text{Mortality at dilution next below}}{(\text{mortality next above}) - (\text{mortality next below})} \rightarrow \frac{50-7.14}{63.63-7.14} = 0.75871$$

Log_{10} 50% end point dilution = $Log_{10}(2\times10^7) + (0.75871 \times Log_{10} 10) = 7.30103 + 0.75871 = 8.05$

LD50 = antilog of 8.05974 = **1.12×10^8 CFU/ml**

4.17 Bacterial Challenge Test

The investigation of *Escherichia* phage PJDM to lyse APEC-P02 in *in vitro* at multiplicity of infection (MOI) of 0.01, 1.0 and 100 to APEC-P02 turbidity (OD_{600} =0.11; calculated LD50- 1.12×10^8 CFU/ml) challenge study was performed. The significant decreases in the viability of bacterial strains were observed, primarily in cells infected with an MOI of 100, however, the reduction of bacterial cells was not significant at an MOI of 0.01 and 1 (Fig. 4.25). After a period of three hours of phage

addition at an MOI of 100, a 3-log unit (1,000-fold) reduction in the number of viable bacteria was observed as compared to negative control i.e., without addition of phage. Hence, the *Escherichia* phage PJDM were used at an MOI 100 against APEC-P02 for further *in vivo* therapeutic study.

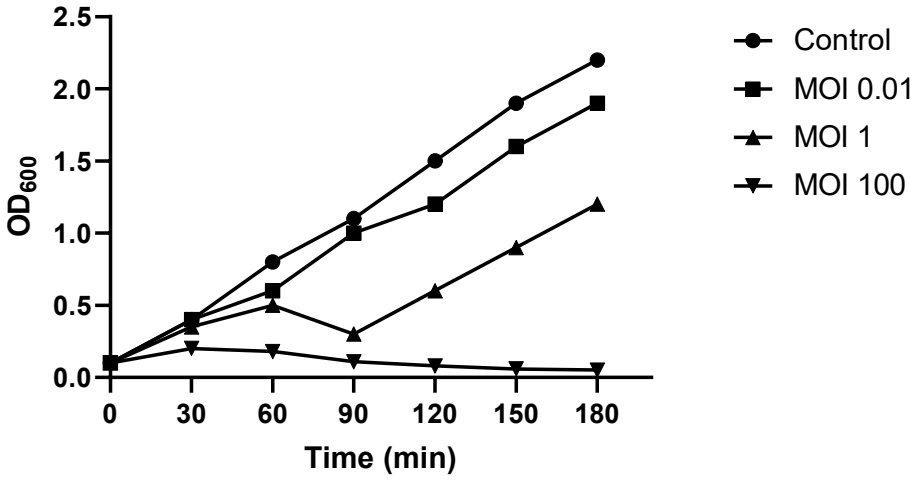

Fig. 4.25 - Bacterial challenge test at different time interval and MOIs

4.18 *In vivo* Evaluation of Therapeutics and Prophylactic Efficacy of Phage PJDM

Using oral administration route, therapeutics and prophylactic efficacy of bacteriophage PJDM was determined by *in vivo* in unvaccinated day-old broiler chickens. Different mortality rates were observed in different groups. It was observed that chicks of group E treated with phage PJDM dose (1.12×10^{10} PFU/ml; MOI=100) had lower mortality (30%) as compared to mortality of 50% in untreated group C at same level of infection. The results also revealed that phage PGDM can be efficiently used as prophylactic agent against APEC as 30% reduction in mortality was observed in group G as compared to infected untreated control (Group C). The death rate was 5% in group D and 60% in group F i.e., administration of ten-fold less dose than calculated LD50 and ten time more dose than calculated LD50 of APEC-P02, respectively during this experiment (Table 4.15). During post mortem examination pathological lesions suggestive of colibacillosis were observed in all the birds died during *in vivo* trial study.

Table 4.15 - Effect of oral administration of *Escherichia* phage PJDM on mortality in *E. coli* challenged broiler chicks

Experiment							Mortality (%)
Name of group (n=20)	APEC-P02 infection			*Escherichia* phage PJDM treatment			
	Dose	Administration route	Administration age (days)	Dose	Administration route	Administration age (days)	
A	Nil	NA	NA	Nil	NA	NA	Nil
B	Nil	NA	NA	*In vitro* calculated dose of bacteriophage**	oral	(2, 7, 14)	Nil
C	Calculated LD50*	oral	1	Nil	NA	NA	10 (50)
D	Ten-fold less dose than calculated LD50*	oral	1	*In vitro* calculated dose of bacteriophage**	oral	(2, 7, 14)	1 (5)
E	Calculated LD50*	oral	1	*In vitro* calculated dose of bacteriophage**	oral	(2, 7, 14)	6 (30)
F	Ten times more dose than calculated LD50*	oral	1	*In vitro* calculated dose of bacteriophage**	oral	(2, 7, 14)	12 (60)
G	Calculated LD50 *	oral	5	*In vitro* calculated dose of bacteriophage**	oral	(1, 2, 3, 4)	4 (20)

* Calculated LD50 of APEC-P02 from experiment described in section 4.16

** *In vitro* calculated dose of bacteriophage from test described in section 4.17

The weekly chicks body weight recording revealed that all chicks challenged with *E. coli* at one day of age had significantly lower body weight than healthy untreated and treated control group throughout the experiment (Table 4.16). The result also revealed that there was significantly higher weight gain in birds of group E (treated group) as compared to group C (untreated group) when same dose of infection was administered (Fig. 4.26). The determination of prophylactic efficiency of phage indicated that weight gain was significantly higher in birds of group G compared to untreated group (group C) at same dose of infection.

Table 4.16 - Effect of oral administration of *Escherichia* phage PJDM on weight gain in *E. coli* challenged broiler chicks

Group	Weight of birds (gm)				
	0 week	1st week	2nd week	3rd week	4th week
A (Healthy untreated control)	46.2	163.83^{a}	407.67^{a}	795.83^{a}	1320.33^{a}
B (Healthy treated control)	46.2	150.50^{ab}	400.83^{ab}	793.17^{a}	1309.83^{a}
C (Infected untreated control)	46.2	94.00^{c}	241.83^{c}	531.67^{b}	759.17^{b}
D (Therapeutic effect on ten-fold less infectious dose than LD50)	46.2	114.17^{c}	353.83^{bd}	700.00^{c}	1190.00^{c}
E (Therapeutic effect on LD50 infectious dose)	46.2	103.00^{c}	301.17^{e}	647.33	1020.00
F (Therapeutic effect on ten times more infectious dose than LD50)	46.2	95.00^{c}	273.67^{ce}	564.67^{b}	790.67^{b}
G (Prophylactic activity)	46.2	138.83^{b}	378.50^{ad}	714.33^{c}	1136.80^{c}

Same (a, b, c, d) alphabet represent no significant difference

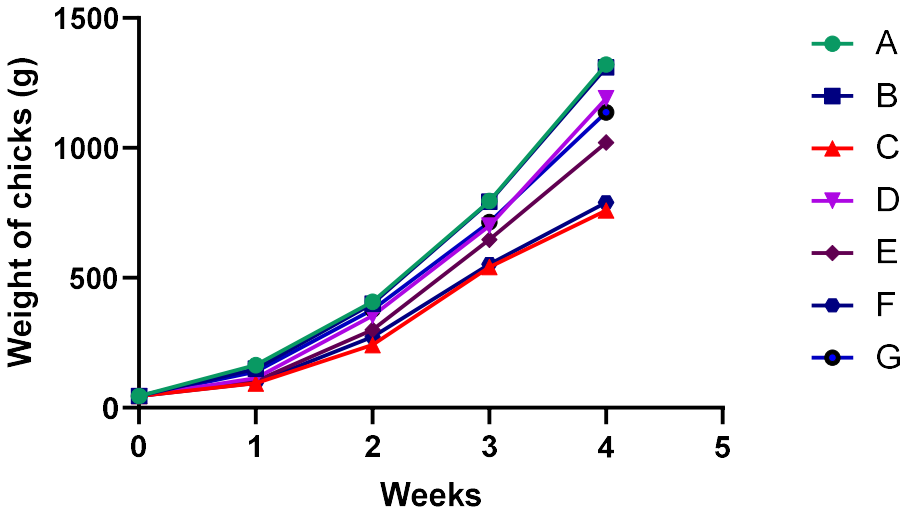

Fig. 4.26 - Effect of *Escherichia* phage PJDM treatment on chicks body weight

CHAPTER-V

DISCUSSION

Colibacillosis is regarded as one of the primary issues for the poultry industry which accounts for losses in several million dollars yearly worldwide (Nolan *et al*., 2013; Guabiraba and Schouler, 2015 and Awawdeh, 2018). In the present study, bacteriological investigation for characterisation of APEC was performed on 50 broiler flocks exhibiting pathological lesions of colibacillosis *viz*., air sacculitis, pericarditis, perihepatitis and splenomegaly (Huff *et al*., 2006, Raji *et al*., 2007, Jamalludeen *et al*., 2009, Oliveira *et al*., 2009, Khoo *et al*., 2010, Kika *et al*., 2013, Zakeri and Kashefi, 2014). Further, characterization of isolates for serotyping, virulence traits, biofilm-formation ability and antibiotic susceptibility was carried out. Then, *in vivo* study was carried out to determine LD50 of APEC-P02 multidrug resistant strain of *E. coli*. This APEC strain was then used as a host to isolate *Escherichia* phage PGDM from poultry farm waste water followed by phenotypic and molecular characterization of the isolated bacteriophage. Finally, *in vivo* therapeutic and prophylactic efficacy was determined against the APEC isolate.

5.1 Isolation, Identification and Confirmation of *E. coli* Isolates from Colibacillosis Infected Chickens

In the present study, all tissue samples (n=50) from lesions of colibacillosis-affected birds were found to harbour *E. coli* based on characteristic colonies and biochemical identification. The isolated bacterial cultures were identified using the Automated VITEK2 Compact System (Manohar *et al*., 2018, Grakh *et al*., 2022). Further, molecular analysis was performed by amplifying the *PhoA* gene specific to *E. coli* as reported in previous literature (Jhandai *et al*., 2019; Kumar, 2021). For confirmation of *E. coli* isolates conventional PCR may be preferred over the VITEK2 Compact system in terms of economics. The results obtained in the present study were in adjunct with other studies which reported 90-100% positivity of *E. coli* from the tissue of colibacillosis infected poultry (Messai *et al*., 2013; Ozaki *et al*., 2017; Azam *et al*., 2019 and Grakh, 2019).

5.2 Antibiotic Susceptibility Profiling of *E. coli* Isolates

Antimicrobial medications are frequently used to control the illness and reduce the financial losses brought on by avian colibacillosis (Nolan *et al*., 2013). As per the

available literature, the most frequently used antibiotics in poultry farms for the prevention and treatment of colibacillosis were enrofloxacin, sulphamethoxazole/ trimethoprim, gentamicin, amikacin, amoxicillin, tetracyclines, ampicillin, colistin and tylosin, whereas, antimicrobials such as ceftiofur, ceftriaxone and nitrofurantoin were among the less frequently used antibiotics (Subedi *et al*., 2018; Grakh *et al*., 2022). However, the emergence of antimicrobial-resistant bacteria is now affecting the way colibacillosis is being treated (Nolan *et al*., 2013). The majority of current treatment strategies focus on early prevention of the disease in the broiler flocks rather than treatment after infection (Kabir, 2010). Increased antimicrobial resistance among pathogenic and commensal *E. coli* strains in poultry has been caused by the continued use of antibiotics as prophylactic and growth promoter. It results in raising serious concerns for human health and the poultry industry's bottom line (Allocati *et al*., 2013).

Most of the *E. coli* isolates were resistant to piperacillin (90%) and moderately resistant to ampicillin (64%). Piperacillin is a broad-spectrum β-lactam antibiotic, high resistance to this drug can be due to its degradation by β-lactamases. Piperacillin is used for the treatment of critical, hospital-acquired infections. The resistance to piperacillin in the present study was 90% but earlier studies reported a relatively lower resistance such as 61.7% (Grakh *et al*., 2022) and 67.9% (Kumar and Gupta, 2019). However, piperacillin is not commonly used in the poultry sector, but the use of other penicillin derivatives might have caused co-resistance of this drug. The resistance against ampicillin resistance was 64% in the present study which is in accordance with other studies (Magray *et al*., 2018; Grakh *et al*., 2022), which reported resistance between 64-66% of the isolates. However, the level of resistance varied to a great extent between studies. Some of the studies reported a higher level of resistance ranging between 87% and 97% (Saidi *et al*., 2012; Ahmed *et al*., 2013; Dou *et al*., 2015 and Kumar and Gupta, 2019), whereas others reported a lower resistance of 35% to 47% (Salehi and Bonab, 2006 and Oosterik *et al*., 2014). Ampicillin is used to prevent and treat various bacterial infections, such as respiratory tract infections, urinary tract infections, meningitis and endocarditis in humans (WHO, 2016). WHO includes ampicillin among the list of most effective and safe medicines needed in a health system (WHO, 2016).

In this study cefpodoxime and cefotaxime (90% each), ceftazidime (60%) and ceftriaxone (44%) exhibited high resistance among *E. coli* isolates. Cefotaxime is

useful in treating significant infections in various parts of the body but is most frequently used to treat birds with serious susceptible bacterial infections in the brain (Animal DVM, 2022). The results are somewhat in accordance with Kumar and Gupta, 2019, who observed 59.4% resistant to ceftriaxone but less resistant to cefotaxime (63.2%) and ceftriaxone (56.6%). The results are on higher side as compared to previous reports in the region for cefpodoxime (Grakh *et al*., 2022: 25%). Oosterik *et al*. (2014) observed very low resistance to third-generation cephalosporins ranging from 0.0 to 7.2%. Third-generation cephalosporins exhibit greater effectiveness against Gram-negative pathogens as well as a broad spectrum of activity. These might be especially helpful in treating infections acquired in hospitals (Klein and Cunha, 1995). As observed in the present study, the high level of resistance against these antibiotics may restrict their use in human medicine in the future.

Around 96% of isolates in the present study were found to be resistant to tetracycline. The similar findings have been observed in other studies, which ranged between 81% - 98.3% (Kumar, 2005; Salehi and Bonab, 2006; Ahmed *et al*., 2013; Kumar and Gupta, 2019). Nevertheless, studies with a lesser prevalence of resistance against tetracycline ranged between 29.7% and 53.6% in India and other parts of the world (Oosterik *et al*., 2014; Amadi *et al*., 2015). Tetracyclines exhibit broad-spectrum bacteriostatic activity against intracellular organisms as well as Gram-positive and Gram-negative bacteria. Tetracyclines are a popular class of antibiotics because of its qualities like the rarity of serious adverse effects and their low price (Nelson and Levy, 2011). Tetracycline is added as a growth promoter, which might be responsible for higher resistance in the field isolates (Bhardwaj *et al*., 2021).

Notably, isolates were most resistant to fluoroquinolones with 96% for both ciprofloxacin and levofloxacin. Several researchers observed resistance to fluoroquinolones ranging from 60% to 80% (Salehi and Bonab, 2006; Dou *et al*., 2015; Kumar and Gupta, 2019). While the average resistance rates of resistant *E. coli* are above 40% in nations like Brazil, China and the EU where the use of fluoroquinolones is legal, however, fluoroquinolone resistance rates to both quinolones and fluoroquinolones are below 5% in US, as fluoroquinolones are not registered for use (Roth *et al*., 2019). A water additive containing levofloxacin (10% w/w) are used in farms as a prophylactic measure against *E. coli* in various part of

India (Bhardwaj *et al*., 2021). This may be the probable reason for high resistance observed against fluoroquinolones in India.

The resistance to the aminoglycosides class of antibiotics observed in the present study varied from 44% in amikacin to 74% in streptomycin among *E. coli* isolates. There has been a wide variation in the resistance profile against these antimicrobials as evident from various studies (Ahmed *et al*., 2013; Tripathi *et al*., 2017; Kumar and Gupta, 2019; Grakh *et al*., 2022). Aminoglycoside antibiotics exhibit *in vitro* activity against a wide variety of clinically important Gram-negative bacilli such as *E. coli*, *Salmonella* spp., *Shigella* spp., *Acinetobacter* spp., *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp., *Morganella* spp., *Proteus* spp., *Klebsiella* spp. and *Pseudomonas* spp. as well as Gram-positive *Staphylococcus aureus* and some *Streptococci* (Vakulenko and Mobashery, 2003). The increased resistance among organisms of animal origin against aminoglycosides may have an adverse effect on human welfare (WHO, 2016). Farmers use streptocycline, a 90:10 mixture of streptomycin and tetracycline, indiscriminately in crops along the banks of the Yamuna in Delhi, Hisar, Haryana and Fazilka, Punjab (Narain, 2020). This might be one of the reasons for the higher level of resistance observed against aminoglycosides in this part of India, particularly to streptomycin (74%) as compared to antimicrobials of the same class.

In India, nitrofurantoins are commonly used to treat uncomplicated lower urinary tract infections (UTIs) in humans (Huttner *et al*., 2015). Very high (88%) resistance against nitrofurantoin was observed in the current study. However, several researchers observed lower resistance up to 56% in chicks for nitrofurantoin (Salehi and Bonab, 2006; Dou *et al*., 2015). The high resistance against antibiotic that is only used in humans or rarely used in poultry is of great concern. Illegal use of nitrofurans in food animals can be the reason for such high resistance (Antunes *et al*., 2006).

Furthermore, around 96% of isolates in the present study were resistant to imipenem. The findings obtained in the study are in concordance with earlier studies in India, which reported a resistance level against the imipenem ranging between 90-100% (Bhushan *et al*., 2017 and Tripathi *et al*., 2017). An earlier study in Korea reported that all the *E. coli* isolates were sensitive to imipenem (Koo and Woo, 2012). Earlier studies in India reported a lesser degree of resistance against imipenem, such as 6.22% and 23.4% (Kumar, 2021; Grakh *et al*., 2022). The imipenem, an antibiotic of the carbapenem class is used in hospitals as last drug of resort, is regarded as a high

priority critically important (CI) antibiotic class and is only to be used in the treatment of Gram-negative multidrug-resistant bacteria. The high resistance found in this study suggests that this kind of medication, which should not be used on animals, is employed in the production of poultry. The presence of resistance to these vital medications, including imipenem, raises public health concerns. In present study 10% of isolates were resistant to aztreonam and various researchers reported similar results where 0-8% of the isolates were resistant against the antibiotic (Koo and Woo, 2012 and Rasheed *et al*., 2014). Aztreonam has clinically useful potency against aerobic gram-negative bacteria, including those expressing Ambler class B metallo-beta-lactamases (MBLs). Although, it is currently being re-examined as a therapeutic agent in light of the global spread of carbapenems resistance in aerobic gram-negative bacilli (Ramsey and MacGowan, 2016).

The World Health Organization (WHO) compiled a list of critically important antimicrobials for human medicine, which suggests that these should be the only or one of the few therapies used to treat serious bacterial infections in humans. These infections should be caused by either: (1) bacteria that may transmit to humans from non-human sources, or (2) bacteria that may acquire resistance genes from non-human sources. Some critically important antimicrobials have been further classified as "Highest priority critically important" (WHO, 2016), meaning that they meet all three prioritization criteria, including a high proportion of patients with serious infections in healthcare settings for which the antimicrobial class is the only or one of a few alternatives to treat serious infections in humans (P1). Furhter, high proportion of patients with serious infections treated with the antibiotic class regularly in medical settings may promote the development of resistance in both situations (P2) and when infections in humans for which there is evidence of transmission of resistant bacteria (such as non-typhoidal *Salmonella* and *Campylobacter* spp.) or resistance genes (high for *E. coli* and *Enterococcus* spp.) from non-human sources are treated with antibiotics (P3).

All the isolates in the present study were resistant to three or more classes and designated as multidrug-resistant (MDR). Such a high proportion of *E. coli* being MDR and specifically to antimicrobials of critical importance is perturbing and is of public health concern. The MDR positivity in the present study are more than the previous study carried out by Hussain *et al*. (2017), in different parts of India that the later study reported a 64% of the isolates from broiler caeca were MDR. The reason

behind all the *E. coli* isolates being MDR in the present study may be attributed to the sampling of birds which were already treated for commonly used antibiotics in the field. The findings show that antimicrobials are being used carelessly in commercial chicken farming. Furthermore, due to cross-contamination of poultry carcasses with the bird's faecal flora during various stages following slaughter, such MDR pathogens may end up in entering our food chains (Mensah *et al*., 2022).

5.3 APEC Characterization of *E. coli* Isolates by PCR

For characterization of *E. coli* isolates as APEC, a set of five virulence genes as described by Johnson *et al*. (2008) was used. Out of all 50 isolates, 90% of isolates were characterized as APEC and 10% of isolates were found to be non-APEC. APEC is responsible for high mortality (up to 53.5%) in young chickens (Kathayat *et al*., 2021). Subedi *et al*. (2018) in Nepal and Azam *et al*. (2019) in Pakistan reported similar results from diseased and dead birds affected by avian colibacillosis. Awawdeh, 2018 and Grakh, 2019 reported a higher prevalence (100%) of APEC among *E. coli* isolates from lesions of affected colibacillosis birds. Such high prevalence of APEC in *E. coli* isolates may be attributed to the fact that all isolates in this study obtained from colibacillosis affected flock.

The subset of virulence genes (VGs), including *iutA, hlyF*, episomal *iss, iroN* and episomal *ompT*, exhibited correspondence to APEC pathotypes and appeared to be able to distinguish APEC isolates from non-APEC isolates (Johnson *et al*., 2008; Dissanayake *et al*., 2014; De Oliverira *et al*., 2015; Mbanga and Nyararai, 2015; Awawdeh, 2018; Subedi *et al*., 2018 and Grakh *et al*., 2022). The current study reported that 88.9% (40/45) of all APEC carried the five VGs (*iutA, iss, ompT, iroN* and *hlyF*). The maximum prevalence (100% each) was observed in the case of four genes *viz., hlyF, ompT, iroN, iutA* among APEC isolates. Non-APEC isolates carried *iroN* and *iutA* genes in this study. *Iss* gene was present in 88.9% (40/45) of total APEC isolates. Studies in India (95%) and Egypt (90%) have reported a similar frequency of these five VGs among APEC isolates cultured from lesions of birds with colibacillosis (Hussein *et al*., 2013; Grakh *et al*., 2022). According to their functions and contributions to the APEC pathogenicity mechanisms, the combination of APEC-associated VGs found in the current study play roles in various elements of the extraintestinal pathogenesis of APEC (Nolan *et al*., 2013; Awawdeh, 2018).

VGs may have a role in the pathogenicity mechanism of APEC at various phases of infection, including intracellular survival (*ompT*), Toxins (*hlyF*), iron

acquisition (*iutA, iroN*) and serum complement resistance (*iss*) (Kathayat *et al*., 2021). In the present study, among APEC isolates 100% positivity was detected for *iroN, iutA, ompT* and *hlyF* genes, whereas 88.9% positivity for *iss* gene. The gene *iroN* encodes salmochelin siderophore receptor, which was prevalent among all the APEC isolates which are similar to findings reported by several researchers (Awawdeh, 2018; Subedi *et al*., 2018; Grakh *et al*., 2022). However, slightly lower prevalence of 78% to 90% has been reported by other researchers (Johnson *et al*., 2008: Hussein *et al*., 2013: Dissanayake *et al*., 2014). Further, the gene *iutA* encodes for aerobactin siderophore receptor and it was observed in all the APEC isolates under study. A lower prevalence ranging from 69%-90% was reported by several researchers in their findings (Johnson *et al*., 2008; Hussein *et al*., 2013; Dissanayake *et al*., 2014; Subedi *et al*., 2018). The findings for the *ompT* gene, which encodes for outer membrane protease for APEC corroborates with the findings of Subedi *et al*. (2018) and Grakh *et al*. (2022). However, lower prevalence was reported by Hussein *et al*. (2013); Johnson *et al*. (2008); Dissanayake *et al*. (2014); Mbanga and Nyararai (2015) who found the positivity of 94%, 78%, 74% and 2.2% for *ompT,* respectively. The gene *hlyF* helps bacteria to deliver bacterial virulence factors into the host by the production of outer membrane vesicle which was prevalent in all the APEC isolates. A hundred percent positivity was found for *hlyF* gene among APEC isolates by De Carli *et al*. (2015) in Brazil; Subedi *et al*. (2018) in Nepal and Grakh *et al*. (2022) in India. Whereas, a lower prevalence of 90%, 78%, 72% and 24% for *hlyF* gene was reported by Hussein *et al*. (2013) in Egypt; Johnson *et al*. (2008) in the USA; Dissanayake *et al*. (2014) in Sri Lanka and Mbanga and Nyararai (2015) in Zimbabwe. Resistance to serum complement can be facilitated by *iss*, which encodes for a lipoprotein of the bacterial outer membrane (McPeake *et al*., 2005, Nolan *et al*., 2013). In the current study, the positivity of *iss* in APEC was 88.9%, which is slightly lower than the findings of Grakh *et al*. (2022) (95.7 %) and variable as compared to other countries such as the USA (80.5%), Egypt (89%) and Brazil (96%) (Johnson *et al*., 2008; Hussein *et al*., 2013; De Carli *et al*., 2015). Different geographical conditions, sources and years of isolation may be the cause of the variance in gene prevalence between countries.

In the current study, all the APEC isolates were found to be multidrug resistant. Several researchers have previously reported on the diversity in antimicrobial resistance among APEC isolates from various places (Dziva and

Stevenes, 2008; Hussein *et al*., 2013). The use of different antimicrobials at different farms and countries, purpose of use, dose of drug used, biosecurity measures and presence of other resistant bacterial species are possible reason for variation (Awawdeh, 2018). Interestingly, many APEC isolates were resistant to piperacillin, cefpodoxime, nitrofurantoin and imipenem despite the fact that they are not commonly used in poultry production in Haryana. This observation suggests that the resistance may have developed because of similarities among these antimicrobials with other antimicrobials that are commonly used or are from same antimicrobials classes (Zhao *et al.*, 2005; Hussein *et al.*, 2013).

5.4 Detection of Extended Spectrum Beta Lactamases (ESBLs) in *E. coli* Isolates

Extended-spectrum-lactamases (ESBLs), are a group of diverse, complex and quickly changing enzymes that are mediated by plasmids. Of late, it presents a significant therapeutic challenge in the care of hospitalized, community-based patients and animals. Additionally, ESBL-producing microbes exhibit co-resistance to numerous additional antibiotic classes, which restricts the range of available therapy options (Rawat and Nair, 2010). In the present study, 10 (20%) isolates were found to be phenotypically positive for ESBL. It could be due to the use of beta lactams and even 4th generation cephalosporins - in veterinary medicine (Geser *et al*., 2012). The ESBL positive isolates were significantly more resistant to cephalosporins i.e., ceftriaxone. The findings of the present study are not in agreement from various studies conducted in the different parts of the globe as the authors have reported the varying degree (0, 43.7%, 6% and 68%) of ESBL prevalence among *E. coli* of poultry broilers (Hasan *et al*., 2011; Saeed, 2014; El-Shazly *et al*., 2017; Jhandai, 2019). Nearly similar prevalence (17%) of ESBL among APEC isolates has been reported by Mohsin *et al.* (2017). The variation in the ESBL positive isolates in different studies might be due to the source of isolation and antimicrobials commonly used at the poultry farm (Hussein *et al*., 2013). The investigation conducted by Falgenhauer *et al*. (2019) on human and poultry *E. coli* isolates in Ghana revealed that meat products or poultry farms may be a significant source of ESBL-producing bacteria which cause non curable infections in humans and thus are of serious concern. The presence of ESBLs in this investigation warrants restrictions on the use of antibiotics in poultry.

The *E. coli* with ESBL phenotype (n=10) exhibited resistance to cefotaxime, piperacillin and imipenem (100% each) followed by cefpodoxime (90%), ceftriaxone, amoxyclav and ampicillin (80% each). The resistance towards penicillins and 3rd

generation cephalosporins by ESBL producing isolates may be due to the fact that these are beta lactam antibiotics and are used against these ESBLs producing strains. ESBL producer APEC isolates also exhibited resistance towards imipenem, which is a carbapenem. Carbapenems are the drug of choice against ESBL-producing organisms. The similar results of imipenem resistance have been reported in human medicine also, which has further limited the therapeutic options available for treatment of these multi drug resistant microorganisms (Rawat and Nair, 2010). As reviewed by Rawat and Nair (2010), plasmid carrying ESBL genes also carry various genes cassettes for resistance to many antimicrobial agents such as aminoglycosides, trimethoprim, tetracyclines and chloramphenicol and this may be the reason of multidrug resistant characteristics of ESBL producing *E. coli* isolates. ESBLs can be easily transferrable among *E. coli* strains including human strains because they are plasmid mediated (Grakh, 2019). WHO has listed ESBL positive and carbapenem resistant *E. coli* as of critical priority for human population. The presence of ESBL producer isolates in our study along with carbapenem resistance thus raises serious public health concerns.

5.5 Characterization of *Escherichia coli* for Presence of *BlaTEM* and Class 1 Integron Intergrase (*intI1*) Genes

5.5.1 Presence of *blaTEM* in *E. coli* isolates

In the present study, 62% of the total isolates were positive for *blaTEM.* The similar findings have been reported by Le *et al*. (2015) from Vietnam and Jhandai, (2019) from India who had reported 59.9 and 73% positivity for *blaTEM* from poultry *E. coli* isolates, respectively. Many other studies have reported lesser carriage of the gene in isolates of poultry origin ranging from 1.92% to 43% (Yuan *et al*., 2009; Kawamura *et al* 2014; Zarfel *et al*., 2014; Randall *et al*., 2017). Increasing ESBLs prevalence rate is an alarming situation, which accounts for their public health significance.

5.5.2 Characterization of *Escherichia coli* for presence of integron

Out of the tested 50 isolates, 68% were found positive for class 1 integron integrase (*IntI*1). Class 1 integron is the most efficient mechanism, involved in the horizontal antimicrobial resistance transfer. It is involved in the recruitment, maintenance and spreading of resistance genes among Gram-negative isolates (Chamosa *et al.*, 2017). Various researchers found presence of *intI1* in *E. coli* isolates ranging from 14% - 70% (Vasilakopoulou *et al.*, 2009; Dessie *et al.,* 2013; Adelowo *et al.,* 2014; Kheiri and Akhtari, 2016; Alam *et al.,* 2020).

E. coli isolates with *intI* gene (n=34) exhibited resistance to tetracycline, levofloxacin, ciprofloxacin and imipenem (100% each). There was statistically significant difference in resistance to different antibiotics amikacin (p=0.014), tetracycline, levofloxacin, ciprofloxacin and imipenem (p=0.035 each) under study among class 1 integron positive and negative isolates. Further, studies with a greater number of samples may provide better insight into this relationship. Amin *et al*. (2017) and Kumar (2021) in their study also have observed positive relationship between carriage of Class 1 integrons and resistance to fluoroquinolones. Ou *et al*. (2017) in their study in China have concluded that class 1 integron in carbapenem resistant *Klebsiella pneumoniae* (CRKP) have strong ability to capture the genes resistant.

High carriage of Class 1 integron as observed in our study is an alarming situation and calls out for immediate attention to regulate use of antimicrobials in poultry production, particularly those belonging to classes of critical importance, specifically fluoroquinolones.

5.6 Detection and Quantification of Biofilm Forming Ability of *E. coli* Isolates

The biofilm formation in LB media (72h) indicated that all the *E. coli* isolates produced strong, moderate, or weak biofilm. Among 50 isolates, 26% (13) isolates were strong biofilm producers, 18% (9) isolates were moderate biofilm producers and 56% (23) isolates were weak biofilm producers.

The lower number of biofilm-producing APEC isolates has been reported by Oosterik *et al*. (2014); Dou *et al*. (2015); Branco *et al*. (2016) and Wang *et al*. (2016) who reported that 44%, 36.2%, 53% and 30% APEC isolates were biofilm producer, respectively. Grakh *et al*. (2022) from India reported that a total of 30/47 (63.8%) APEC isolates were moderate to strong biofilm producers. These findings collectively entail that a larger proportion of APEC isolates are capable of developing biofilm. The amount of nutrients available for the bacteria in the media diminishes with time, resulting in nutrient depletion, media utilized and encouraging the production of biofilms. The extracellular polymeric substance (EPS) synthesis rises in response to nutrient deprivation or dilution in the media, which also causes bacterial cells to adopt the biofilm phenotype (Jayathilake *et al*., 2017). The amount of nutrients fed to the colony can determine the critical thickness, which is the thickness at which matrix synthesis begins to grow in bacterial colonies (Zhang *et al*., 2014).

5.7 Phylogenetic Typing of *E. coli* Isolates using PCR

The distribution of phylogroups significantly differed for resistant towards 3rd generation cephalosporins and carriage of *intI1* gene. Additionally, the presence of ESBLs in isolates correlated with the phylogroups in the present study. Interestingly, there were notable differences in virulence trait distribution among phylogenetic groups, although the isolates had been recovered from the same types of infection. Similar results have been reported by Rodriguez-Siek *et al*. (2005) and Mittal *et al*. (2022), wherein phylogroup A is dominating phylogroup among APEC isolates. Phylogroup B2 was significantly associated with non-APEC isolates in the present study. Among 50 isolates of *E. coli* recovered from the lesions of poultry with colibacillosis, phylogroup B1 was the most common (19 isolates), followed by A (14), B2 (5), F (3), F (3) and D (2). None of the isolates in the present study belongs to phylogroup C and E. While the majority of APEC isolates fall into phylogenetic types A, B1 and D, whereas the majority of human ExPEC isolates mostly fall into phylogenetic types B2 and, to a lesser extent, D. (Rodriguez-Siek *et al*., 2005). Further, the pathogenicity is caused by plasmid-mediated pathogenicity-associated islands (PAIs) and other extrachromosomal and mobile elements. It cannot be accounted for phylogenetic categorization, which depends on recognizing specific chromosomal markers. Since extra chromosomally positioned PAIs are a characteristic of the APEC pathotype and seem to be essential for APEC virulence (Tivendale *et al*., 2004; Johnson *et al*., 2008; Skyberg *et al*., 2008). Nevertheless, these isolates originated from chickens suffering from perihepatitis and other colibacillosis syndromes (Logue *et al.*, 2017). Phylogroups A and B1 have been identified as sister groups (Lecointre *et al*., 1998). Murase and Ozaki (2022) suggested that *E. coli* isolates assigned to phylogroups A and B1 from colibacillosis lesions have a pathogenic potential based on virulence genotyping. The findings of this study are different from the previous findings by Murase and Ozaki, 2022 who found 37 isolates belonged to phylogroup F recovered from 56 colibacillosis-infected broiler birds. It may be attributed to geographical differences and/or any other selection pressure due to climatic conditions.

5.8 Bacteriophage Isolation

In practically every environment where bacteria occur, phages live as obligatory parasites on their bacterial hosts (Naureen *et al*., 2020). It has been reported that the bacteriophages could be used as biocontrol agents to reduce or

eradicate disease-causing bacteria (Alomari *et al*., 2021). The fact that host-specific bacteriophages are self-limiting and only replicate on the particular bacterium gives them an edge (Connerton and Connerton, 2005). These benefits, along with the use of APEC-P02 multidrug resistant strain of *E. coli* isolated from poultry, allowed for the isolation of the host-specific bacteriophage. The search for alternatives to the use of antibiotics sheds light on lytic bacteriophages.

APEC-P02 multidrug resistant strain of *E. coli* was used as a host candidate for the isolation of bacteriophages. It was also evident that while isolating the bacteriophages for therapeutic and prophylactic use, it is necessary to confirm that they should be lytic phages not the lysogenic forms prophage. Lysogenic phages may be responsible for the transmission of antibiotic resistance (Wendling *et al*., 2021).

Many bacteriophages (lytic and lysogenic) were isolated from different samples including poultry farm wastewater and excreta, sewage treatment plant wastewater, slurry and biogas plants slurry. Additionally, the sewage was removed from the top levels in the early morning since high quantities of hazardous chemicals and large amounts of organic detritus were present in deep layers of sewage water.

A lytic bacteriophage which was isolated from poultry farms wastewater sample was further purified by employing the double agar overlay method using the avian pathogenic *Escherichia coli* as a representative strain. In the present study no phage was recovered in Ganges water but Khairnar (2016) revealed the presence of bacteriophages against putrefying and pathogenic bacteria in the waters of Ganges. It may be attributed that presence of phage is more expected where host bacteria are harboured. The phage recovered in the present study is isolated from poultry farm excreta as host bacteria of poultry origin. Oliveira *et al.* (2009) also employed the same method for the isolation of bacteriophages. During the isolation procedure, 0.8% top agar and 2.5% bottom agar instead of the top (0.6%) and bottom layers (1.5%) as described by Oliveira *et al.* (2009) were used as the high prevailing environmental temperature in this area prevents proper solidification of agar. In the present study, several phages were isolated including both lytic and lysogenic from different samples. Thereafter, a lytic bacteriophage was isolated from the poultry wastewater by using APEC-P02 multidrug resistant strain of *E. coli* as the host. Following pre-enrichment treatment, the filtrate was collected and tested using the spot test method for the presence of bacteriophage. Pre-enrichment and spot tests have also been reported by other researchers (Oliveira *et al*., 2009; Tawakol *et al.,* 2019; Vijay,

2019). After confirming the presence of phage in the filtrate, a double agar overlay technique (Adams, 1959) was used for isolation and purification of a single phage. A number of researchers have reported the use of double agar overlay technique for phage isolation (Oliveira *et al*., 2009; Tawakol *et al.,* 2019; Vijay. 2019). Similarly, several other researchers also isolated bacteriophage against pathogenic *E. coli* from various sources (Huff *et al*., 2002; Xie *et al.,* 2005; Jamalludeen *et al*., 2009; Oliveira *et al*., 2009; Ribeiro *et al*., 2018; Tawakol *et al.,* 2019; Kazibwe *et al.,* 2020; Abdelrahman *et al.,* 2022). One isolated lytic bacteriophage against APEC-P02 was selected based on plaque morphology and lytic pattern. Then it was purified by sequential single plaque isolation for three times and propagated to $1.2x10^{10}$ PFU/ml.

5.9 Morphological Characterization of Bacteriophage

The morphological characterization of bacteriophages is essential to determine the order and family of bacteriophages. For this purpose, transmission electron microscopy was carried out at ICAR-IARI, Pusa, New Delhi. Along with its morphological structure, the size of a single phage was also measured and the findings revealed that it had an icosahedral head of size 80 nm and a total length of 220 nm and a non-contractile tail. According to the International Committee on Taxonomy of Viruses' (9th Report, 2011) findings, the distinctive morphological characteristics of phages belonging to the family *Siphoviridae* and order *Caudovirales* which is the commonly isolated bacteriophage group against *Escherichia coli* (Oliveira *et al*., 2009; ICTV, 2011; Sjahriani *et al*., 2021; Abdelrahman *et al*., 2022). The *Caudovirales* account for more than 95% of the phages reported in the scientific literature and majority of bacteriophages (60%) that have been described are phages with long, flexible tails that belong to the *Siphoviridae* family (Yazdi *et al.,* 2020). In accordance, the phage belongs to this taxonomic classification. The isolated phage was given the nomenclature '*Escherichia* phage PJDM' as per the guidelines (Adriaenssens and Brister, 2017).

5.10 Host Range and Specificity Testing of Bacteriophage

The characterization of lytic bacteriophages is essential in the medical exploration of the antibacterial potential. Hence under microbiological characterization the host range of *Escherichia* phage PJDM was observed and results revealed that it shows lytic nature for serotype- O11, O26, 017, O98, 0120 and O135 but exhibited no lysis for *Klebsiella pneumonia*, *Salmonella* Typhimurium, *Staphylococcus aureus.* The observed lytic effect was against different strains of *E. coli*, this is in occurrence with Jamalludeen *et al*. (2009); Kazibwe *et al.* (2020). Determination of the lytic

range of bacteriophages is critical for the selection of phages as a candidate for a specific purpose (therapeutic application and bio sanitation) (Fong *et al*., 2021). Mozaffari *et al*. (2022) isolated VaT-2019a phage from the wastewater of a livestock slaughterhouse which exhibited lytic spectrum against many serotypes of *E. coli* along with its natural host *E. coli* O157:H7. Phage vB_EfaS-DELF1 isolated against *Enterococcus faecalis* by Soleimani-Delfan *et al*. (2021) was only sensitive to its host and exhibited no lytic behaviour against other strains and bacteria. Abp1 phage, isolated from wastewater by Huang *et al*. (2013), was able to only affect the two *Acinetobacter* spp. strains, proving that some bacteriophages only affect one bacterial species and do not have a broad host range. The N4-like phages category includes the G7C bacteriophage, which possesses restricted lytic activity toward only its initial host, *E. coli* (strain 4 s) (Kulikov *et al*., 2012). Due to differences in the proteins in the fiber and tail spikes which are specific receptor of their bacterial hosts, bacteriophage possess high genetic diversity which results in high specificity (Sorensen *et al*., 2021).

5.11 Serotyping of *E. coli* Isolates

Serological identification of the isolates (n=25) revealed that 24/25 isolates of *E. coli* were typable, whereas one isolates was untypable. The most common isolated serotype was O26 9/25 (36%); followed by O98 (32%); O120 (12%); O11 (8%); O135 (4%) and O17 (4%) whereas, 0% sero-positivity was detected for O1, O2 and O78 serotypes. The serotypes O98, O11 and O135 were not reported previously from poultry; however, O98 was reported from cattle responsible for haemorrhagic colitis and haemorrhagic uremic syndrome (Irino *et al*., 2005). The other serotypes O11, O17 and O120 were reported in poultry by various researchers (Blanco *et al*., 1998; Wani *et al*., 2004; Sekhar *et al*., 2017), which may result in systemic diseases in poultry. It has been reported by many researchers that the most often prevalent APEC serogroups were O1, O2 and O78 (Kim *et al.,* 2020; Temmerman *et al.,* 2020). Generally, the recorded *E. coli* serotypes are more than 1,000 but fewer have been implicated in poultry diseases. However, the geographical localization of a flock may affect the prevalence of certain serotypes. The observable decreased rate of O78 incidence may be attributed to the use of vaccines containing this serotype (Awad *et al*., 2020). The high prevalence of O98, O120, O11, O135 and O17 isolates was not expected and may indicate the emergence of new serogroups associated with avian colibacillosis are not yet reported.

5.12 Biophysical Characterization of Bacteriophage

The biophysical characterization of *Escherichia* phage PJDM revealed that it was stable at temperatures 30°C, 37°C and 42°C for 24h, whereas it was not able to survive at 50°C, 70°C and 90°C. The temperature ranges were selected as it encompasses both the room temperature and body temperature of chicken among other temperatures (Kazibwe *et al.,* 2020). The findings were in accordance with previous findings (Jepson and March, 2004; Yang *et al*., 2010; Hammeri *et al*., 2014; Kumar *et al*., 2017; Kazibwe *et al.,* 2020; Abdelrahman *et al.,* 2022). Further, longer time survivability of phages storage at 30°C, 37°C and 42°C make them a reliable candidate for biocontrol agents in the sub-tropical area like Haryana in shortage of cold chain. Other phages matching *Escherichia* phage PJDM include two coliphages P483 and P694 and phage QL01 which were destroyed at 65°C (Chen *et al*., 2016; Xu *et al*., 2016). According to Ahmadi *et al*. (2017) the amount of DNA a phage contains determines its thermal stability. Therefore, the longer phage DNA consisting of denser capsid is responsible to greater temperature tolerance. Additionally, the tailed phages are more temperature resistant than the tailless variants and the relationship could be correlated between thermal stability and the presence of a long and flexible tail (Jonczyk *et al.,* 2011; Mozaffari *et al*., 2022). *Escherichia* phage PJDM was found to be stable at pH range from 4 to 11 and labile at pH 2 and 13. These findings were in concurrence with findings of previous researchers (Kumar *et al*., 2017; Kazibwe *et al.,* 2020; Abdelrahman *et al.,* 2022). Niu *et al*. (2012) revealed that *E. coli* phage AKFV33's titer at pH 3 decreased by 1.9 logs PFU/ml after 15 min and became undetectable after two hours. According to Nobrega *et al*. (2016), phages can develop irreversible mutations at low pH, which may account for the phage's survival at low pH in the current investigation. A linear link between the rate of phage mutation and incubation at low pH was established by Strack *et al*. (1964), indicating that phages can undergo mutation to live in an acidic environment. Irreversible coagulation and precipitation at very low pH might be also the factors that limit phage activity (Jonczyk *et al.,* 2011). The dissociation of the capsid protein due to the high hydrogen and hydroxyl ion concentrations may be the cause of reductions in phage titers at pH 13 (Feng *et al*., 2003). The survival ability to these phages at low pH make potential candidate could to use them as control pathogens through oral administration (Filho *et al*., 2007). By taking into account the aforementioned fact, it is vital that these bacteriophages are effective against the host bacteria in the real-world scenario where

the pH of the proventricular secretions would be around four and the temperature would be around 42°C which will mimic the *in vivo* circumstances.

5.13 Determination of Latent Period and Burst Size of Bacteriophage

The one-step growth studies were conducted to investigate the different phases of the phage infection process such as the latent period and the burst size of *Escherichia* phage PJDM. According to the one-step growth experiment, the latent period of phage was 20 min. After 30 min of infection, the progeny formation increased drastically. The timing of phage-induced host cell lysis, which is normally regulated by a phage protein complex known as a holin, determines the length of the phage latent period. Endolysins that break down cell walls are inhibited by holins and changes in holin genes can significantly modify the timing of host cell lysis (Abedon *et al.,* 2001). The burst size of the phage was 26.5 plaque-forming units (PFU) per infected cell. The findings of the present study suggest lytic nature of the *Escherichia* phage PJDM and higher Lytic activity than the previously published lytic *E. coli* phages (Lee and Park, 2015; Dalmasso *et al*., 2016; Liu *et al*., 2019) belonging to the same family *siphoviridae* but less than Fan *et al*., 2012 who isolated the phage belonging to family *Myoviridae* with a burst size of 43 PFU/infected cell. Population dynamics characteristics of *siphoviridae* are slightly lower than a member of the family *Myoviridae* (Dalmasso *et al*., 2016). Burst size will vary monotonously with phage latent period, whereas shorter latent period results in a proportionately shorter burst size (Abedon *et al.,* 2001).

Phages with strong lytic activity against large numbers of bacterial target cells are needed for wide-scale biocontrol of bacterial pathogens and this property is connected with the big burst size. The burst size is regarded as one of the primary properties of an efficient bacteriophage as an antimicrobial agent since burst size is closely related to phage proliferation (Gallet *et al*., 2011). Since they can rapidly multiply the original dose of phages by several hundred times, phages with a large burst size may have a selective advantage as antibacterial agents (Choi *et al*., 2010; Amarillas *et al*., 2017). As a result, *Escherichia* phage PJDM's burst size can be a distinct benefit for its use as a bio-control agent against bacterial infections.

5.14 Whole Genome Sequencing of Bacteriophage

For molecular characterization of bacteriophages, the first phage nucleic acid was extracted using the Wizard DNA cleanup kit. Before extraction, phage lysate was digested with DNase and RNase to avoid bacterial host nucleic acid contamination (Gill, 2015). Whole genome sequencing of *Escherichia* phage PJDM revealed that it

has nucleic acid of size 57.7 kbp. These findings were in concurrence with Hendrix *et al.* (2015), Tabassum *et al.* (2018), Amarillas *et al.* (2020) and Sorensen *et al.* (2020). It was also indicated that the molecular weight of phage DNA was around 21-134 kbp which belong to the family *Siphoviridae* of order *Caudovirales* (ICTV, 2011). The open reading frames (ORFs) of *Escherichia* phage PJDM were identified by blastX search and a total of 98 ORFs were predicted and there were no putative tRNA genes in genome implying its dependence on host tRNA for protein synthesis. Among these genes, 34 genes were predicted to have known functions (Table 4.13) and are grouped into three functional modules as follows: phage packaging and lysis (ORFs 1, 14, 26, 36, 63, 64 and 66), phage structure and assembly (ORFs 15, 45, 46, 51, 52, 53, 54, 57, 61 and 62) and DNA metabolism and replication (ORFs 6, 34, 56, 60, 71, 73, 76, 77, 78, 79 and 80). Additionally, six auxiliary metabolic genes (AMGs) (ORFs 31, 59, 69, 70, 84 and 95) were predicted and 64 ORFs were predicted to encode hypothetical proteins.

A majority of the genes involved in phage packing are found at the start of the *Escherichia* phage PJDM genome. The terminase small (TerS) and large (TerL) subunits, respectively, were encoded by ORFs 1 and 14. The terminase and DNA recognition proteins mediate the packaging of dsDNA virus concatemers and this requires interaction between the prohead and the virus DNA (Ortega and Catalano, 2006). It is believed that nucleoprotein structure at the packaging initiation point formed when the TerS joins with the TerL (Black, 1989). The portal protein regulates the size of the assembled viral genome which is encoded by ORF 26 and it prevents the escaping of DNA from capsid during assembly (Isidro *et al*., 2004). Phage portal proteins are essential viral components of the DNA-packaging complex which initiate capsid assembly and also play a major role in tail assembly (Cuervo *et al*., 2019). NinH protein, which is encoded by ORF 34, influences cellular and viral DNA processing and gene expression and has possible positive impacts on phage propagation (Chakraborti *et al*., 2020). The head morphogenesis protein, ORF 36, is located either internally or toward the C terminus of the *Caudovirales* tailed bacteriophages. The minor head protein according to Becker *et al*. (1997), binds to the viral DNA inside the capsid and the portal protein may regulate the viral DNA exit from the virion which is necessary for viral head morphogenesis (Vinga *et al*., 2006). Holin, a tiny hydrophobic protein that is encoded by ORF 63, produces a hole in the host by oligomerizing in the cytoplasmic membrane. Endolysin, which cleaves the peptidoglycan in the cell wall, is encoded by ORF 64. They together form the

traditional holin-endolysin lysis system. Endolysin enters the cell wall through the pore that holin has created and then completes the host cell wall lysis (Catalao *et al*., 2013). Genes related to the structure are mainly located in the middle of the *Escherichia* phage PJDM genome. ORF 45 and 46 encode the capsid fibers and major capsid protein (MCP), respectively, which synthesize the protein coats of viruses that encapsulate their genetic material (Wang *et al*., 2021)

ORFs 53, 54 and 60 encode the tail tubular protein, without compromising the integrity of the cell this allows phages to introduce their genomes into bacterial cytoplasm (Cuervo *et al*., 2013). ORF 52 was similar to ORF 14 of alteromonas phage ZP6 (Wang *et al*., 2021), identified as a structure-related gene with an unknown role. ORF 15, 57, 61 and 62 encode the tail fiber protein, which helps the phage in identifying host cells (Salmond and Fineran, 2015). Genes related to the replication and regulation of bacteriophage DNA were mainly located in the downstream region of the *Escherichia* phage PJDM genome. ORF 56 was a predicted superinfection immunity protein, which plays imp role in superinfection exclusion. The virus develops inhibitory mechanisms to block their competitors to compete with one another for hosts. A superinfection exclusion mechanism, which stops a subsequent infection, is one of these mechanisms mainly during the adsorption and replication processes (Biggs *et al*., 2021).

ORFs 71 and 78 contained a virus nuclease (NUC) domain. It is associated with restriction-modification enzymes (Kinch *et al*., 2005). ORF 73 encodes a DNA polymerase that is similar to the DNA polymerase encoded by the host bacteria which plays an important role in the replication of the phage genome in the host cell. ORF 76 and 80 encode DNA helicase, which is required for the unwinding of dsDNA, which is a prerequisite for DNA replication, expression, recombination and repair (Ryan and Owen-Hughes, 2011). ORF 31 encodes for deoxyuridine 5'-triphosphate nucleotidohydrolase, which helps in providing an appropriate supply of deoxyribonucleotides for phage to achieve a high rate of DNA synthesis by preventing the incorporation of uracil into DNA (Warner *et al.,* 1979). ORFs 70 and 95 encode nucleotide pyrophosphohydrolase, which enables the host cells to stop programmed cell death by hydrolyzing (p)ppGpp which increases the survival rate of the host in nutrient-depleted environments (Wang *et al*., 2021)

ORF 6 encodes for a recombination-associated protein, it supports replication, circularization of genomes with terminal repetitions and genome repair (Lopes *et al*., 2010). ORF 77 encodes for type I site-specific restriction modification protein which

serves as the phage's defensive mechanism against host type I restriction endonucleases (REases) (Vasu and Nagaraja, 2013). Enolase inhibitors are proteins encoded by the ORF 59 gene, they may have two advantages for the phage: first, they promote dNTP synthesis for phage progeny production by causing a hunger response due to compromised host glycolysis and the phosphotransferase system; second, it may aid in promoting rupture bacterial cell wall. The enolase dissembling strategy might serve as a starting point for the creation of new antibiotics and perhaps even anti-cancer medications (Zhang *et al*., 2022). ORF 69 and 84 encode for methyltransferases (MTases) that protect REases of their bacterial host. This ability of phage to overcome restriction-modification (R-M) systems and other phage-targeting resistance systems can be useful in phage therapy. Phages with additional resistance to host defenses may increase the effectiveness of phage therapy (Murphy *et al*., 2013). ORF 66 encodes for Spanins, a lysis protein that is required for the final step of Gram-negative host lysis as it lysed the outer membrane of the host. Two-component spanin like Rz-Rz1 from phage lambda consists of an integral inner membrane protein: i-spanin and an outer membrane lipoprotein: o-spanin, that form a complex spanning the periplasm (Kongari *et al*., 2018)

5.15 Restriction Endonucleases Profiling of Bacteriophage

The nucleic acid genome of *Escherichia* phage PJDM's under restriction endonuclease analysis revealed the presence of 17, 20 and 26 restriction sites for *Escherichia* phage PJDM with *EcoRI, SspI and VspI* enzymes. These results coincided with Goodridge *et al*. (2003) who isolated coliphage AR1, whose DNA was completely digested by *SspI* restriction enzyme. However, restriction endonuclease profiling results has varied greatly between studies indicating a vast diversity and specificity of phage in nature. In some of the studies the isolated phage belongs to the same group but is not digested by *EcoRI* (Bhagya Raj, 2014 and Kakoma, 2009). Whereas, another study exhibited complete digestion by *EcoRI* by bacteriophage Pϕ-Bw-Ab (Torabi *et al*., 2021).

5.16 *In vivo* Mean Lethal Dose (LD50) of APEC-P02 Strain

To determine the therapeutic and prophylactic efficacy of bacteriophage, the first *in vivo* study to determine LD50 dose rate of APEC-P02 strain was conducted. For this purpose, day-old chicks were administered with APEC strain which was sensitive to isolated phage under *in vitro* experiments. The chicks observed for 10 days and mortality was recorded. The typical signs of colibacillosis were characterized by multiple organ lesions, i.e., pericarditis, air sacculitis, perihepatitis and septicemia

(Giovanardi *et al*., 2013). Oral inoculation appears to promote a more gradual evolution of the infection which is closer to the real conditions as the oral route of *E. coli* infection represents the normal route of infection as reported by Kabir, 2010.

The mean LD50 of representative APEC-P02 strain observed was 1.12×10^8 CFU/ml during oral administration. Various studies were performed in past to determine the lethal dose of *E. coli* in chickens with different virulent strains and obtained heterogeneous results (Giovanardi *et al*., 2005; Nagano *et al*., 2012; Li *et al*., 2016; Hussain *et al*., 2017; Liu *et al*., 2020; Landman *et al*., 2021).

The findings from this study when compared with other studies, it was noticed that the mean lethal dose of the same species of bacteria in the same type of host under similar environmental conditions varied a lot. The similar results have also been observed in the findings of previous researchers that LD50 for APEC ranges from 10^2 to 10^8 CFU (Nagano *et al*., 2012; Hussain *et al*., 2017; Liu *et al*., 2020; Landman *et al*., 2021). These variations depend on many factors i.e., pathogenicity of strain, age of birds, or route of administration. Giovanardi *et al*. (2005) observed 100% mortality in day-old birds when inoculated subcutaneously with 10^8 CFU of each O78 and O139. Li *et al*. (2016) studied the mean lethal dose of APEC strain DE205B in seven-day-old ducks and found the dose as 1.74×10^5 CFU). Serogroups O1, O2 and O78 are predominating virulent *E. coli* strain isolated during different years and at different geographic locations among common serogroups (O1, O2, O18, O35, O36, O78 and O111), which were previously associated with disease (Barbieri *et al.,* 2015; Landman *et al.,* 2021). However, other serogroups were also found to be associated with disease in chickens (Blanco *et al*., 1998; Zhao *et al*., 2005), questioning the value of serogroup as a virulence marker. Also, the serogroups are of hardly any value in epidemiological studies as genetic differences occur within serogroups. The high prevalence of O11, O126, O98, O120 and O135 isolates was not expected and may indicate the emergence of new serogroups associated with avian pathogenic *E. coli* not yet reported.

5.17 Bacterial Challenge Test Study of Bacteriophage

To investigate the ability of *Escherichia* phage PJDM to lyse APEC in *in vitro* culture conditions, challenge tests were performed which include the addition of phage at an MOI of 0.01, 1.0 and 100. Bacterial growth is strongly prohibited at MOI 100 directly after phage infection and the findings obtained are in accordance with other researchers (O'Flynn *et al*., 2004; Han *et al*., 2013; Wang *et al*., 2016; Amarillas *et al*., 2016). Whereas, phage VaT-2019a and Φ241 at MOI of 1 and 0.3,

respectively, reduced the amount of *E. coli* to an acceptable level (Pereira *et al*., 2017; Mozaffari *et al*., 2022). The formation of phage-resistant cell lines within the target strain may also be the cause of bacterial growth. By employing phage cocktails against a certain bacterial species, the likelihood of mutation may be decreased (Lu and Breidt, 2015). The *Escherichi*a phage PJDM could be used to inactivate strains of pathogenic *E. coli* and has the potential to be utilized as a biocontrol agent *in vivo*, according to the results of the *in vitro* challenge test. An MOI 100 was used for the therapeutic investigation *in vivo*.

5.18 *In vivo* Therapeutic and Prophylactic Study of Bacteriophage

Phage therapy can be used in the treatment of severe *E. coli* infections in birds and has been considered a reliable alternative to antibiotics (Oliveira, 2009). It has been proposed that phages might be able to lower the bacterial load and dissemination of the infecting bacteria to levels at which the host immune system may be able to control them (Levin and Bull, 2004). Phages will also be able to control localized diseases by replicating in the infected areas, that are relatively inaccessible via the circulatory system as, for example, the air sacs in chickens. Theoretically, a bacteriophage should be effective in eliminating the infection, if it reaches the site of a bacterial infection (Carlton, 1999; Huff *et al*., 2006; Oliveira, 2009). These attributes make phages powerful antimicrobials alternatives. Several phage researchers shared the same opinion that these viral particles are effective in treating bacterial diseases. Indeed, reports of successful studies indicate that phages provide substantial levels of protection against infections (Park *et al*., 2000; Huff *et al*., 2002; Huff *et al*., 2004; Huff *et al*., 2005; Oliveira *et al*., 2009; Mohammad *et al.*, 2019; Tawakol *et al.*, 2019; Ngu *et al*., 2022).

In the present study, *in vivo* efficiency of *Escherichia* phage PJDM as a therapeutic and prophylactic agent in treating chickens with colibacillosis was evaluated. Before the *in vivo* phage efficacy trials, preliminary experiments were conducted to calculate LD50 dose rate of the selected APEC strain that was able to cause chickens colibacillosis. Oral inoculation appeared to promote a more gradual evolution of the infection which is closer to the real conditions as the oral route of *E. coli* infection represents the normal route of infection (Kabir, 2010). For *in vivo* trial day-old unvaccinated chicks were procured, as phages are only able to treat the chickens that were in an early stage of the infection in experimental circumstances (Oliveira, 2009). In the *in vivo* phage efficacy trials, *Escherichia* phage PJDM was able to reduce mortality, on average by 20%.

Regarding the *Escherichia* phage PJDM efficiency performance, when it was administered at 1.12×10^{10} PFU/ml, this phage was able to reduce the mortality. A significant reduction in mortality was observed in group G (20%; where phage was used as a prophylactic agent) when compared with the infected control group (50%). A significant increase in the weight gain of birds at different weeks was noticed in the phage treated groups than in untreated groups. These results might explain the effectiveness of phage against colibacillosis causing *E. coli*. Similar prophylactic results of phages were observed by Huff *et al*. (2002) against colibacillosis when these authors sprayed a cocktail of phages before administration of infection. It is important to emphasize that, in contrast to experimental settings, naturally occurring infections involve the gradual horizontal spread of bacteria from one chicken to another and do not include synchronized infection stages in the birds (Huff *et al*., 2005). Therefore, it might be speculated that the tested phage can be more efficient in treating natural colibacillosis, by controlling infection at earlier stages and avoiding the progressive transmission to the flock.

Group F of the experiment in which phage was administered as MOI 100 shows 60% mortality this may be because the effectiveness of phage therapy highly depends on the phage titer administered and whether it provides sufficient number of particles to the site of the infection or not (Sajjad *et al*., 2004; Oliveira, 2009). The treatment efficacy in a systemic infection could be improved by ensuring that sufficiently large numbers of phages are available in the bloodstream so it can be concluded that phage therapy is dose-dependent therapy. Oliveira, 2009 demonstrated that phage was able to reach the lungs and air sacs when administered orally, so the high concentration of phage (MOI=100), was probably enough to control a severe infection.

The protecting capacity of phages against APEC was found to be similar in earlier study (Oliveira *et al*., 2009; Tawakol *et al*., 2019). It must be stressed that some differences in mortality between control groups from different experiments (Barrow *et al*., 1998: 100% reduction in mortality; Huff *et al*., 2004: 53% reduction in mortality; Lau *et al*., 2010: 57% reduction in mortality) were noticed. The success rates were different and depended on many factors, such as the type of phages administered, chicken's age, dose and route of phage administration between experiments (Huff *et al*., 2003). Further, timing and concentration of administered phages are also known to have an important effect on the success rate of phage therapy (Ryan and Owen-Hughes, 2011), with successful treatment even seen after administration of phages within one or

two days after the bacterial infection (Tsonos *et al*., 2014). An early administration after infection, wherein a lower bacterial concentration is there, it may result in the clearing of phages before they can actively replicate *in vivo*. (Payne *et al*., 2000). Phages administered at low doses or intracellular host strain multiplication (for example, in macrophages or heterophils), prevent phages from reaching their receptor on the bacterial host and are likely to be responsible for the failure of some of the phage treatments reported (Tsonos *et al*., 2014).

Overall, the results of the phage *in vivo* performance demonstrated that the phage can be used as a therapeutic and prophylactic agent against APEC.

Phage therapy has shown some incredibly encouraging outcomes, but there are still some concerns to its application in clinical practice. Some of the main drawbacks of phage therapy that restrict its widespread use include the quick emergence of bacterial resistance to phage (Principi *et al.*, 2019), the immune response that triggers the production of antibodies that neutralize phage action (Lusiak-Szelachowska *et al*., 2014), the safety concerns regarding phage preparations that might contain bacterial endo-and exotoxins (Hietala *et al*., 2019), as well as the narrow spectrum of activity. The development of molecular biology methods that permit the genetic modification of phage genomes has already started to resolve some of these obstacles. For example, phages have been genetically engineered to enhance antibiotic activity (Lu and Collins, 2009; Edgar *et al*., 2012), improve their performance against biofilms (Lu and Collins, 2007) and expand their host ranges by forming recombinant phages (Yoichi *et al.,* 2005; Mahichi *et al*., 2009). For therapeutic applications, the utilization of specific, purified phage preparations is essential (Pires *et al*., 2015). In addition, a thorough understanding of phage-host interaction, phage dynamics, phage diversity and genome function is essential for figuring out the limitations of phage therapy and developing innovative approaches to overcome them.

CHAPTER-VI

SUMMARY AND CONCLUSION

Poultry industry is the one of the most organized and rapidly growing animal agriculture sectors in India. There has been phenomenal growth of broiler industry in the state of Haryana during last few years. Despite the intensification and modernisation of poultry industry, the challenges of infectious diseases (viral and bacterial) are still hampering the production potential to be achieved to its maximum. The bacterial diseases alone or in combination with other etiological agents are causing major economic losses to poultry farmers in the state of Haryana. Avian colibacillosis is one such disease which is caused by avian pathogenic *Escherichia coli* (APEC).

Due to intensive nature of chicken production and the farmer's desire to maximize profits in a short span of time, some farmers are using antimicrobials as growth promoters, prophylactic agents and for the treatment purposes. The non-judicious use of antimicrobials in such scenario is leading to development of antibiotic-resistant bacteria.

The development of resistance to the majority of first-line antibiotics has made the treatment of widely prevalent avian pathogenic *E. coli* more challenging. So, in this connection, finding alternatives to antibiotics is very much important for both prevention and therapeutics purpose for *E. coli* infections in poultry.

Therefore, the present study was envisaged with an effort to explore the alternatives to antibiotic usage against *E. coli* infections. For this purpose, a bacteriophage was isolated against colibacillosis causing APEC. Initially, isolation and characterization of APEC isolates was carried out from tissues of 50 colibacillosis affected flocks. Using PCR and Vitek 2 Compact system, a total of 50 isolates were confirmed as *E. coli*. Then, the confirmed *E. coli* isolates were subjected to *in vitro* antimicrobial sensitivity test as per the method of Bauer *et al.* (1966). The antimicrobial susceptibility testing of *E. coli* isolates revealed a maximum sensitivity for colistin (62%) followed by trimethoprim (50%), whereas, high antibiotic resistance was detected for imipenem, tetracycline, ciprofloxacin, levofloxacin (96% each), piperacillin (92%), cefpodoxime and cefotaxime (90% each). All the 50 isolates were found to be multiple drug resistant (MDR) and antimicrobial profiling

indicated that these isolates can be classified into thirty-eight resistotypes. For molecular studies, the DNA was extracted from *E. coli* isolates using snap chill method. The multiplex PCR using a set of five virulence genes was performed. A total of 45 (90%) isolates were detected as APEC, while 5 (10%) were found to non-APEC as these isolates were not harbouring four or more virulence genes. The study on antimicrobial profiling of APEC and non-APEC isolates indicated that there was statistically significant difference in resistance for cefpodoxime, imipenem, tetracycline, levofloxacin and ciprofloxacin. The phenotypic confirmation of ESBLs isolates was carried out by combined disc method and it was found that out of 50 *E. coli* isolates, 10 (20%) isolates were phenotypically ESBL producers. Further, molecular characterization for detecting presence of beta lactam resistance gene (*blaTEM*) and class 1 integron integrase (*intI1*) responsible for resistance in bacterial isolates was performed. Out of the tested 50 isolates, 62% were found to harbour *blaTEM* gene, whereas 68% isolates carried *intI1* gene. However, out of 45 APEC isolates, 29 (64.44%) and 34 (75.55%) APEC isolates harboured *blaTEM* and *intI1* gene, respectively. Moreover, out of 10 ESBL positive isolates, both *blaTEM* and *intI1* was detected in 8 (80%) ESBL positive isolates. The statistical analysis indicated that significant difference in resistance to different antibiotics *viz*. amikacin (odds ratio- 5.49), tetracycline, levofloxacin, ciprofloxacin and imipenem with respect to carriage of *intI1* gene was observed. Further, assessment on biofilm formation at 72 h incubation, thirteen (26%) isolates were strong biofilm producers, nine (18%) and twenty-eight (56%) isolates were found to be moderate and weak biofilm producers, respectively. The strong biofilm producing APEC (n=13) exhibited resistance to pipercillin (100%), followed by cefotaxime (92.3%). Statistically significant difference was observed for resistance towards different antibiotics i.e., ampicillin, gentamicin, amikacin, polymyxin B and nitrofurantoin with respect to type of biofilm production. The phylogenetic typing of all the 50 *E. coli* isolates using the Clermont *et al*. (2013) protocol indicated that phylogroup B1 (38%) was dominant among *E. coli* isolates, followed by A (28%), B2 (10%), unknown/untypable group (8%), F, Clade I/II (6% each) and D (4%).

Further, one of the isolated *E. coli* from poultry which exhibited typical lesion of colibacillosis and also found to be resistant to most of the antibiotics tested in this study was selected for *in vivo* characterization to calculate its LD50 dose in chicks through oral administration designated as APEC-P02. The LD50 for APEC-P02 in

day old chicks was calculated by using the method of Reed and Muench and it was observed as mean 1.12×10^8 CFU/ml.

Further, attempts were made to isolate bacteriophage(s) against APEC-P02 isolate as host bacteria. For this purpose, the samples from poultry farm wastewater (LUVAS), sewage slurry (sewage treatment plant, CCSHAU), biogas plant slurry (CCSHAU) and Ganges water (Haridwar) were tested for presence of bacteriophage against APEC-P02. The desired lytic bacteriophage against APEC-P02 was obtained from poultry farm wastewater, LUVAS as the filtrate of this source yielded positive spot test i.e., clear zone of lysis against APEC-P02. The bacteriophage filtrate was further processed for its purification and enrichment using double agar overlay technique. The bacteriophage was enriched to $1.2x10^{10}$ PFU/ml and produced desired clear plaques on LB agar indicating its high specificity for host bacteria. Morphological characterization was carried out using TEM facility available at ICAR-IARI, PUSA, New Delhi. On the basis of morphology, the phage isolated against *E. coli* belonged to family *Siphoviridae*, order *Caudovirales* (tailed phage) as the isolated phage was having approximately 80 nm icosahedral head and 140 nm non contractile tail. The isolated phage was given the nomenclature '*Escherichia* phage PJDM' as per the guidelines (Adriaenssens and Brister, 2017). Subsequently, on determination of host range of phage PJDM, the phage exhibited lytic activity against *E. coli* (O11, O26, 017, O98, 0120 and O135) and no effect was documented against other tested bacteria, i.e., *Klebsiella pneumoniae*, *Salmonella* Typhimurium and *Staphylococcus aureus*. The biophysical characterization of phage PJDM in terms of its sensitivity towards different pHs and temperatures indicated that isolated phage remained viable up to 300 days at 30°C, 37°C, 42°C and up to 24 h at pH 5 to 11 with negligible change in titer. The determination of latent period and burst size of *Escherichia* phage PJDM by one step growth curve at MOI 0.1 revealed that the isolated phage exhibited a latent period of 20 min with a burst size of 26.5 phages per bacterial cell. The *in vitro* study on bacteriophage activity against APEC-P02 using bacterial challenge test at different MOIs (0.01, 1 and 100) indicated that there were significant decreases in the viability of bacterial strains mainly in cells infected with an MOI of 100. However, the reduction of bacterial cells was not significant at an MOI of 0.01 and 1. On the basis of *in vitro* study, the further *in vivo* characterization of phage PJDM was carried out at MOI 100 to know its therapeutic and prophylactic efficacy against APEC-P02 in broiler chickens.

The results indicated that the isolated phage was able to reduce mortality on an average by 20% on administration of same dose of infection (APEC-02) by oral route to broiler groups under study. Whereas, the prophylactic efficacy study indicated that there was 30% reduction in mortality of broiler chicks as compared to infected control group. It was also observed that there was significantly more weight gain in phage treated chicks as compared to untreated group at same dose of infection.

Following conclusions can be drawn from the present study-

- The study is first in terms of phage isolation and characterization against colibacillosis causing *E. coli* from Haryana. The isolated *Escherichia* phage PJDM was found to be of order *Caudovirales,* family *Siphoviridae* on the basis of its morphology using transmission electron microscopy and whole genome sequencing.
- The study is first of its kind in estimation of LD50 of APEC isolate in the day-old chick by oral challenge.
- The biophysical characterization of isolated phage indicated its sturdy nature and easy to store at room temperature as it was able to withstand broad range of temperature (30°C - 42°C) for 300 d and up to 24 h at pH (5-11).
- The characterization of *E. coli* isolated from tissue samples of broilers indicated existence of multidrug resistant APEC in the environment of Haryana stressing the need of alternatives to antibiotic therapy.
- *In vitro* and *in vivo* study on phage PJDM indicated its potential for therapeutics and prophylactic use against multidrug resistant avian pathogenic *E. coli* to combat antibiotic resistance.

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ABSTRACT

Title of thesis	**Isolation, characterization and *in vivo* evaluation of therapeutics and prophylactic efficacy of bacteriophage(s) against pathogenic *Escherichia coli* in chicken**
Name of the degree holder	**PUNIT JHANDAI**
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The bacterial diseases alone or in combination with other pathogens are causing major economic losses to poultry farmers in the state of Haryana. Avian colibacillosis is one among these diseases which is caused by avian pathogenic *Escherichia coli* (APEC). The development of resistance to the majority of first-line antibiotics has made the treatment of widely prevalent avian pathogenic *E. coli* more challenging. So, in this connection, finding alternatives to antibiotics is very much important to control *E. coli* infections in poultry. Though several alternatives are available to tackle the antimicrobial resiatance problem, but bacteriophages are considered as one of the best remedies. By keeping this in view, the present study was envisaged with the objectives to isolate and characterize pathogenic *E. coli* and bacteriophage along with *in vivo* study on therapeutic and prophylactic activity of isolated bacteriophage. Using PCR and Vitek 2 Compact system, a total of *E. coli* 50 isolates were recovered from tissues of 50 colibacillosis affected flocks. Out of these 45 (90%) isolates were characterized as APEC by multiplex PCR using a set of five virulence genes. The antimicrobial susceptibility testing of *E. coli* isolates revealed that high antibiotic resistance were observed against imipenem, tetracycline, ciprofloxacin and levofloxacin (96% each). All the 50 isolates were found to be multiple drug resistant (MDR) and the antimicrobial profiling indicated that these isolates could be classified into thirty-eight resistotypes. Moreover, phenotypic characterization of *E. coli* isolates revealed that 10 (20%) isolates were ESBL producers. On genotypic characterization, 62% isolates were positive for *blaTEM* gene, whereas, 68% isolates carried *intI1* gene. Further, assessment on biofilm formation at 72 h incubation, thirteen (26%) isolates were strong biofilm producers, nine (18%) and twenty-eight (56%) isolates were found to be moderate and weak biofilm producers, respectively. Phylogenetic typing of all the 50 *E. coli* isolates indicated that phylogroup B1 (38%) was dominant among *E. coli* isolates followed by A (28%), B2 (10%), unknown/untypable group (8%), F, Clade I/II (6% each) and D (4%). Furthermore, *in vivo* characterization of multidrug resitant APEC-P02 isolate revealed mean LD50 of 1.12×10^8 CFU/ml during oral challenge study in day old chicks. Subsequently, a novel lytic phage *Escherichia* phage PJDM against avian pathogenic *E. coli* was isolated from poultry farm wastewater and further characterized *in vitro* and *in vivo*. The TEM analysis of phage PJDM exhibited an icosahedral head and long non contractile tail, which can be classified as a member of the *Siphoviridae* family and *Caudovirales* order. Further, on determination of host range of phage PJDM, the phage exhibited specific lytic activity against several *E. coli* serotypes (O11, O26, 017, O98, 0120 and O135). Biophysical characterization of phage PJDM indicated that isolated phage remained viable up to 120 days at 30°C, 37°C, 42°C and up to 24 h at pH 5 to 11 with negligible change in its titer. Moreover, one step growth at MOI 0.1 kinetics revealed a latency period of about 20 min and a burst size was 26.5 phages particles/infected cell. Further, the phage PJDM genome has a double-stranded linear DNA molecule and its genomic DNA contains 57,756 base pairs with a GC content of 43.58 % on the basis of whole genome sequencing. *Escherichia* phage PJDM genome consisted of 98 predicted putative ORFs in genome. Among these 98 genes, 34 genes were predicted to have known functions. The *in vitro* study on bacteriophage activity against APEC using bacterial challenge test at different MOIs (0.01, 1 and 100) indicated that there were significant decreases in the viability of bacterial strains when infected with an MOI of 100. Then, *in vivo* evaluation of the efficacy of phage PJDM in day old chicken through oral route at MOI 100 to control *E. coli* infections revealed that the phage was able to reduce mortality on an average by 20 % and 30% in therapeutic and prophylactic group. The study is first in terms of phage isolation and characterization against colibacillosis causing *E. coli* from Haryana.

MAJOR ADVISOR **DEGREE HOLDER**

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