Evaluation of Bacteriophage as Biocontrol Agent Against Non-Typhoidal *Salmonella enterica* of Poultry Origin

Thesis

Submitted to the
DEEMED UNIVERSITY
ICAR-Indian Veterinary Research Institute
Izatnagar - 243 122 (U.P.), India

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Veterinary Science
(Veterinary Microbiology)

2022

Dedicated to…

My Beloved Family
and
Guide

भा.कृ.अनु.प.–भारतीय पशु चिकित्सा अनुसंधान संस्थान
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Dated: 26/12/**2022**

Certificate

This is to be certified that the research work embodied in this thesis entitled "**Evaluation of bacteriophage as biocontrol agent against non typhoidal *Salmonella enterica* of poultry origin**" *submitted by* **Dr. Lulu Gonmei, Roll No. M-6154,** *for the award of* **Master of Veterinary Science** *Degree in* **Veterinary Microbiology** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate herself under my supervision and guidance.*

It is further certified that **Dr. Lulu Gonmei, Roll No. M-6154,** *has worked for more than 21 months in the Institute and has put in more than 150 days attendance under me from the date of registration for the* **Master of Veterinary Science** *Degree in this Deemed University, as required under the relevant ordinance.*

(SOPHIA I.)
Chairperson
Advisory Committee

Certificate

We the undersigned members of Advisory Committee of **Dr. Lulu Gonmei, Roll No. M-6154,** *a candidate for the degree of* **Master of Veterinary Science** *with the major discipline in* **Veterinary Microbiology,** *agree that the thesis entitled* **"Evaluation of bacteriophage as biocontrol agent against non typhoidal *Salmonella enterica* of poultry origin"** *may be submitted in partial fulfilment of the requirement for the degree.*

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Master of Veterinary Science** *Degree of this Institute.*

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Master of Veterinary Science** *Degree of the Deemed University, Indian Veterinary Research Institute, Izatnagar.*

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ACKNOWLEDGEMENTS

During the course of my studies here, many known and unknown had lend me their helping hands and for that any formal acknowledgement would hardly be enough to convey my gratitude to them.

First and foremost, I would like to express my heartfelt gratitude towards my guide **Dr. Sophia I,** Scientist, Division of B&M, IVRI for her constant guidance, encouragement and patience towards me. This endeavor would not have been possible without her assistance and dedicated involvement in every step throughout the process. I am also immensely grateful for the wise counsel on spiritual and moral aspect apart from this research work.

I would like to express my deepest appreciation to the learned members of the advisory committee, **Dr. V.K. Chaturvedi**, Principal Scientist & Head, Division of Bacteriology and Mycology, IVRI, **Dr. Abhishek,** Senior Scientist, Division of Bacteriology and Mycology, IVRI, **Dr. Prasad Thomas**, Senior Scientist, Division of Bacteriology and Mycology, IVRI, **Dr. Karikalan M.**, Scientist (SS), Division of Pathology, IVRI and **Dr. G.V.P.P.S. Ravikumar**, Principal Scientist, Division of Biotechnology, IVRI for their valuable inputs, guidance with deliberative discussion, constructive criticism and for allowing me to use the facilities in their lab throughout the study period.

I am highly thankful to **Director, Joint Directors** (Acad.), **Joint Director** (Res.), ICAR-IVRI for providing me financial assistance and necessary facilities to successfully carry out my research work.

Words are inadequate to thank my batchmates who made my journey here so special **Dr. Santanu Pal, Dr. Jitendra Bagra, Dr. Sunil R.,** and **Dr. Lakshmi Prakashan.** I am deeply grateful for the love, encouragement and support they have showed me. I will always cherish the joyous company and the time we spent together.

I would like to extend my sincere thanks to **Dr. Sanjumon,** my batchmate from VPH division for always rending a helping hand whenever I needed.

Wholehearted thanks to my senior, **Dr. Himani Dhiman** for her pleasant attitude, always motivating, concerned and ever ready to help. Knowing her has been a great joy.

With special pleasure I acknowledge all my seniors and juniors in Bacteriology and Mycology Division for their friendly nature, for their wonderful company, for the support and all the help.

I extend my humble thanks especially to **Indrapal bhaiya** and **Jamalji**, for their constant help and assistance. Their helping hands played a huge role for completing my research on time.

It would have been impossible for me to complete this journey without my friends **Dr. Dengam, Dr. Lalrohlui, Dr. Rotluangkimi** Their constant love, support, encouragement and emphatic nature had been the greatest blessing in this journey. I am forever grateful for this beautiful friendship and will always cherish them.

I would also like to extend my special thanks to my friends (Hawreys) **Dr. Akum, Dr. Dima, Dr. Dolly, Dr. Febron, Dr. Iban, Dr. Shrilla and Dr. Tarakchi** for being my constant emotional support system, for the comfort and endless love.

It would be incomplete without thanking my wonderful family for their everlasting love and blessings. This success would not have happened without their encouragement, emotional support, and prayers, which gave me the strength to successfully complete this course.

Above all, I thank **Almighty God** for His grace which has always been sufficient for me and His strength which is perfect in my weakness.

There are so many others whom I have inadvertently left out and I sincerely thank all of them for their help and kindness.

Lulu Gonmei

Date: 26/12/22 ***(Lulu Gonmei)***

Place: *ICAR-IVRI, Izatnagar*

ABBREVIATIONS

°C	:	Degree centigrade
%	:	Percentage
ϕ	:	Bacteriophage
°C	:	Degree Celsius
BFF	:	Bacteria free filtrate
bp	:	Base pair
Cfu	:	Colony forming units
d	:	Day
DNA	:	Deoxy ribonucleic acid
DW	:	Distilled water
EDTA	:	Ethylene diamine tetra acetate
EP	:	Enriched phage
Fig.	:	Figure
G	:	Gram(s)
H	:	Hour(s)
HEA	:	Hektoen enteric agar
IVRI	:	India Veterinary Research Institute
kb	:	Kilo base
KCl	:	Potassium Choride
KH_2PO_4	:	Potassium Phosphate Monobasic
M	:	Molar
mg	:	Miligram(s)
$MgCl_2$	:	Magnesium Chloride
$MgSO_4$	:	Magnesium Sulphate
min.	:	Minute(s)
ml	:	Mililitre(s)
M	:	Molar
MOI	:	Multiplicity of infection
NaCl	:	Sodium Chloride
Na_2HPO_4	:	Disodium Hydrogen Phosphate
OD	:	Optical density
PAA	:	Peracetic acid
PBS	:	Phosphate Buffered Saline
PCR	:	Polymerase chain reaction
pfu	:	Plaque forming uints

pH	:	Log hydrogen ion concentration
RE	:	Restriction enzyme
RFLP	:	Restriction fragment length polymorphism
RNA	:	Ribonucleic acid
RT	:	Room temperature
Rpm	:	Revolutions per minute
s	:	Seconds
spp.	:	Species
SD	:	Standard deviation
TM	:	Tris magnesium buffer
TEM	:	Transmission electron microscopy
TAE	:	Tris-acetate-EDTA buffer
U	:	Unit
UV	:	Ultra violet
V	:	Volts
viz.	:	Namely
w/v	:	Weight by volume
µg	:	Micro gram
µl	:	Microlitre
µm	:	Micrometer

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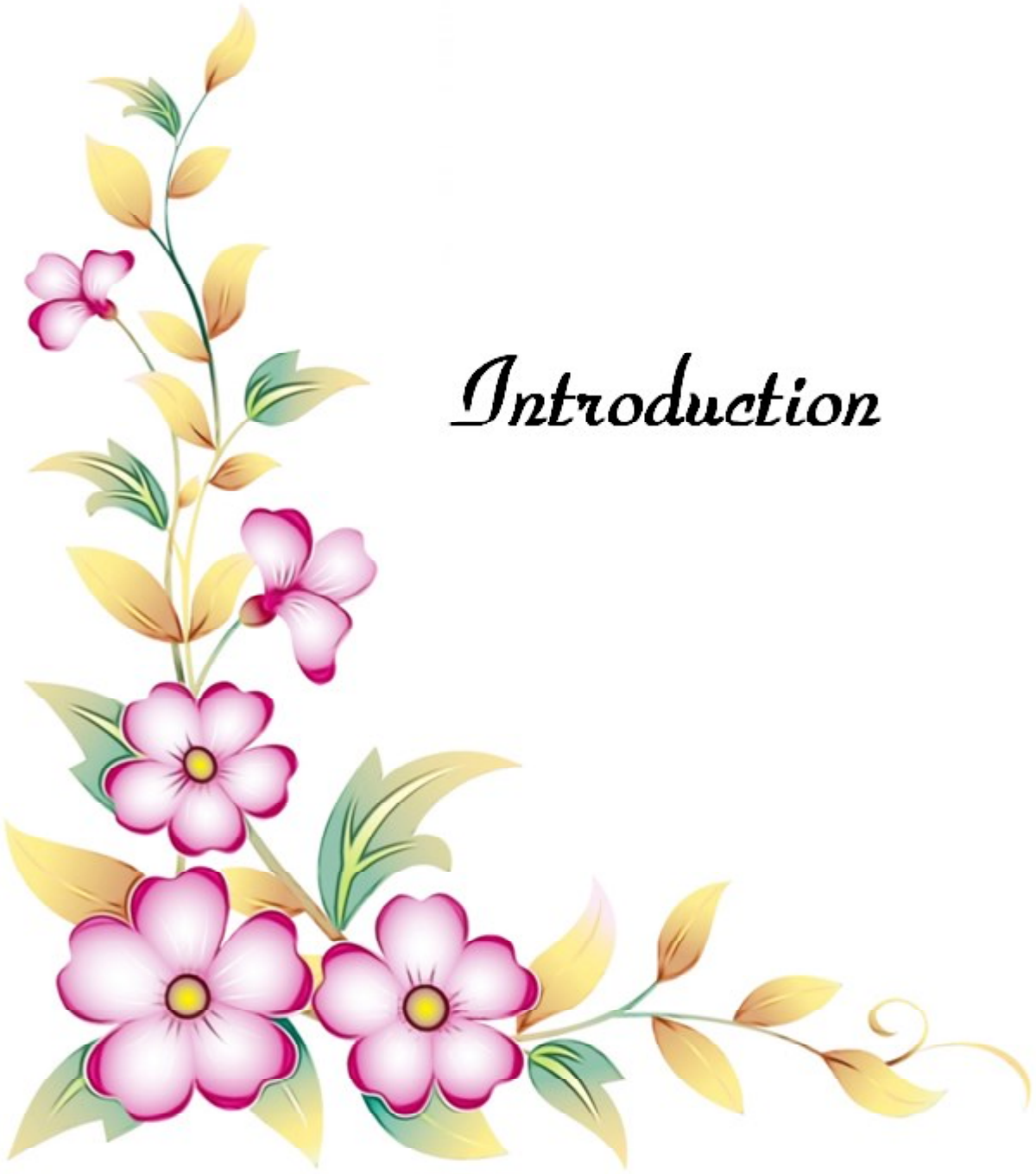


Introduction

1 INTRODUCTION

Poultry production is considered as a booming sector in animal husbandries in an industrial mode worldwide at present and the market value accounted to US$ 267,332.3 million in 2018 and is believe to grow annually by 3%. The Indian poultry market, which is driven by high demand for poultry meat (broiler) and eggs, was worth INR 1,750 billion in 2018. The market is further projected to reach INR 4,340 billion by 2024, growing at a CAGR of 16.2% during 2019-2024. Various infectious diseases hampered the growth of poultry sector and of public health concern. Salmonellosis is a food borne zoonotic infection affecting poultry and human population. Every year in the United States, it is estimated that 1.4 million *Salmonella* infections (salmonellosis), 26,500 hospitalizations, and 240 deaths (CDC, 2020). *Salmonella* was responsible for over 94,625 cases in Europe in 2015 (EFSA and ECDC, 2016). Non-typhoidal *Salmonella* (NTS) serovars are estimated to cause 93.8 million humans infections and 1,55,000 deaths each year (Majowicz *et al*., 2010). Non-typhoidal salmonellosis is a food borne zoonotic diseases transmitted from animals and poultry to humans. Non-typhoidal *Salmonella*e are a leading cause of food poisoning and enteric infections and has emerged as a major public health concern globally (Crump *et al*., 2011). Many serovars of *Salmonella* have been known to cause infection in both humans and animals (Tirado *et al*., 2001). The most common non-typhoidal *Salmonella* (NTS) serovars worldwide are *S*. Typhimurium and *S*. Enteritidis. *Salmonella* Typhimurium has a broad host range including humans, animals, birds and even reptiles. Lower Intestinal tract (ilium, caecum and colon) of all warm blooded animals particularly poultry is the reservoir of *Salmonella*. The main route of *Salmonella* Typhimurium transmission is through faeco-oral route. Consumption of

unpasteurized milk, vegetables and especially poultry meat contaminated during slaughtering from intestinal spillage is the main source of transmission to human population causing food poisoning although direct contact from farm animals, pets and many occupational outbreaks, have also been reported. *S.* Typhimurium infection is characterized by high morbidity but with low mortality. They cause enteritis and septicemia in most farm animals irrespective of their age but often subclinical and hence asymptomatic shedders. Acute enteritis is manifested by anorexia, fever, depression, foul smelling diarrhea often with blood, mucus and epithelial cast. Septicemic form is most commonly seen in young and debilitated animals. The clinical signs include high fever, depression, recumbency and die within 48 hrs if treatment is delayed. There is also report of abortion and stillbirth in farm animals. In poultry, infection cause by non-typhoidal serovars including *S.* Typhimurium is collectively known as fowl paratyphoid. In human population symptoms of salmonellosis include abdominal pain, diarrhea, fever, headache, nausea, vomiting, and is usually self-limiting but can cause *invA*sive type of salmonellosis in weakened immune systems or poor health which can be fatal (Boslaugh *et al*., 2016; LaRock *et al.,* 2015; Drozdz *et al*., 2021).

Extensive documentation of antimicrobial resistance in *S*. Typhimurium has been reported recently. In the recent days, non-typhoidal *Salmonella* found in United States were resistant to cephalosporins whereas NTS from European Union were resistant to fluoroquinolones. Both, cephalosporins and fluoroquinolones were considered as important antimicrobials in human medicine. Moreover, research in our lab (National *Salmonella* Center, ICAR-IVRI, Bareilly) with non-typhoidal *Salmonella enterica* isolated from animals and environment revealed recent emergence of antimicrobial resistance to fluoroquinolones in the isolates. Many studies have also reported multi-drug resistant *S*. Typhimurium. *Salmonella* Typhimurium antibiotic resistance patterns were frequently tetra-resistant ASSuT (ampicillin, streptomycin, sulfonamides, and tetracycline) and in penta-resistant pattern ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline) (Wang *et al*., 2019).

In 2010, India occupied 5th rank globally in antibiotic consumption by livestock and projected to being 4th by 2030 with consumption of 5,000 tons annually (Van Boeck *et al*., 2015). In 2020, SARS2-COVID 19 arose as a pandemic and threatened the entire globe.

But, antibiotic resistance is considered as the silent tsunami which threatened the treatment options of hospitalized patients worldwide in the recent past India can be called the hub of antimicrobial resistance with report of more than 60% *E. coli* ESBL positive, 13% *E. coli* carbapenem resistance and 28% fluoroquinolone resistance *Salmonella* Typhi. Inappropriate and increasing trends of antibiotic consumption viz. increased prescription of antibiotics, easy over-the-counter availability of antimicrobial drugs at affordable prices, prescription of antibiotics even for seasonal flu and vector-borne diseases outbreaks is contributed to antimicrobial resistance in humans. Majority of antibiotics prescribed in human medicine is also commonly used in poultry and livestock production. Antibiotics applications in livestock and poultry industries include therapeutics, prophylactics, metaphylactics against bacterial infections and as feed additives. Addition of antibiotics in subtherapeutic doses as feed additives in livestock and poultry production is the major reason for emergence of antibiotic resistance bacteria. These antimicrobial resistance bacteria (ARBs) or genes (ARGs) can spread to the environment and, eventually transmitted to human food chain through animals or animals products (Kumar *et al*., 2019). Also as India tries to achieve food security, food safety is the recent growing concern of the consumers. Therefore, replacement of antimicrobials with viable alternatives is necessary to battle antimicrobial resistance which needs a constant laboratory research. *Salmonella* Typhimurium is the leading cause of food poisoning worldwide as they can contaminate food such as poultry meat, pork, beef, milk and milk products.

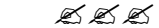
Hence, there arises the need to mitigate the antimicrobial resistance by using alternatives of antibiotic. The available alternatives to antibiotics are vaccines, probiotics, prebiotics, bacteriophages, phage endolysins, and phytobiotics. These alternatives to antibiotic can be used at various levels such as farm, prior to slaughter, as disinfectant in abattoir premises, in meat and meat products as bio preservatives. Bacteriophages are obligate parasites to their bacterial host with high host specificity. They are called the viruses of bacteria which may contain RNA or DNA based genetic material enclosed in protein coat called capsid. They are omnipresent organisms in the globe and co-evolve with target bacteria. The International Committee for taxonomy of viruses (ICTV) is the nodal organization for classification of bacteriophages based on nature of nucleic acid, morphology, physiochemical and genomic

data. More than 96% classified phages fall into the order *Caudovirales*, which possess icosahedral head and contractile tail, long and non-contractile tail and short tailed phages, respectively, belonging to *Myoviridae, Siphoviridae* and *Podoviridae* families.

Based on lifecycle, phages are divided into lysogenic or temperate and lytic or virulent phages. The temperate phages insert their DNA into bacteria which gets integrated with the bacterial DNA whereas the lytic phages exploit the host protein machinery to manufacture their own proteins, and ultimately phage progenies get released by bacterial cell lysis. Bacterial cell lysis is carried out by phage enzymes holin and endolysin. Unlike antibiotics, bacteriophages possess high target specificity so that they act only against target bacteria, do not harm other microbiota and also reported to be effective against bacterial biofilms formation. They are relatively stable under harsh environmental temperature, pH and chemicals. They are considered safe organisms for use in human medicine against drug resistant pathogens, veterinary medicine, prophylactics, biocontrol agents and as disinfectants. Phage stability maintenance under various environment, food matrices and animal gut remain challenging but can be improved by encapsulation techniques. The emerging bacterial resistance to phages is also an important limitation which can be addressed by targeting multiple phages against same pathogen

Bacteriophage is a promising alternative to combat antimicrobial resistance bacterial infections as therapy as well as biocontrol in food safety. Therefore, the current research aim is to isolate, characterize and experiment bacteriophages as alternatives to antibiotics against non-typhoidal *Salmonella enterica* and its application as biocontrol agent in poultry based meat products with the following objectives.

1. To isolate and bulk produce bacteriophage against non-typhoidal *Salmonella enterica*.
2. To characterize the isolated bacteriophage based on morphology, biological, sensitivity and stability and molecular characterization.
3. To evaluate the isolated bacteriophage as biocontrol agent in poultry meat.



Review of Literature

2 REVIEW OF LITERATURE

2.1 *Salmonella* and Salmonellosis

Salmonella is a widespread important foodborne pathogen belonging to family *Enterobacteriaceae. Salmonella is a major zoonotic pathogen that is the leading cause of reported food poisoning worldwide. They are rod shaped, gram negative, facultative anaerobic, non-spore forming, oxidase negative bacterium having a size of about 2.0 to 5.0 µm in length and 0.7 to 1.5 µm in width (*Fàbrega *et al*., 2013). The genus has two species are *Salmonella enterica* and *Salmonella bongori. Salmonella enterica* is further classified into 6 subspecies (*enterica* (I), salamae (II), arizonae (IIIa), diarizonae (IIIb), houtenae (IV), and indica (VI)) with over 2,600 serotypes (Gal-Mor *et al*., 2014). These serotypes can be grouped into typhoidal (*Salmonella* Typhi and *Salmonella* Paratyphi) and non-typhoidal. Typhoidal *Salmonellae* is the leading cause of morbidity and mortality however non-typhoidal *Salmonellae* are a leading cause of food poisoning and enteric infections and have emerged as a major public health concern globally (Crump *et al*., 2011). The most common non-typhoidal *Salmonella* (NTS) serovars worldwide are *S*. Typhimurium and *S*. Enteritidis with 26.1 percent in the United States, 68.3 percent in Europe causing salmonellosis including India and they show broad host range (Yildirim *et al*., 2018; Kumar *et al*., 2016; Choi *et al*., 2020). NTS serovars are estimated to cause 93.8 million human infections and 1,55,000 deaths each year *(*Majowicz *et al*., 2010). Every year in the United States, it is estimated that 1.4 million Salmonella infections (salmonellosis), 26,500 hospitalizations, and 420 deaths (CDC, 2020). *Salmonella* was responsible for over 94,625 cases in Europe in 2015 (EFSA and ECDC,

2016). Salmonella is found in the intestinal tract of warm-blooded animals, particularly chickens, and can thus be easily contaminate the meat during slaughter and processing (Antunes *et al.,* 2016). The consumption of Salmonella contaminated food products causes approximately 95% of salmonellosis. Salmonella zoonosis is typically caused by *S. enterica* serotypes *Typhimurium* and *Enteritidis.* Salmonellosis symptoms include abdominal pain, diarrhea, fever, headache, nausea, vomiting, and can be fatal in people with weakened immune systems or poor health (Boslaugh *et al*., 2016).

The vast majority of non-*invA*sive NTS are self-limiting gastrointestinal infections that do not require antibiotic treatment but in young and elderly people, as well as immunosuppressed individuals, they can cause invAsive type of salmonellosis (iNTS) known to associate with sepsis, septic aortic aneurysm, septic arthritis, meningitis, and is thought to result in death by the same serovars as NTS *(LaRock et al.,* 2015; Dróżdż *et al*., 2021).

2.2 Prevalence of *Salmonella* in poultry meat and its products

Poultry products are the most common source of human infection with non-typhoidal *Salmonella. Salmonella* can be transferred to the surface of chicken meat from intestinal contents, faeces, or cross-contamination during the slaughtering process (Rouger *et al*., 2017). As a result, despite other sources such as contact with animals, the environment, or person-to-person transmission, food-borne salmonellosis is the most relevant source with a high global impact on human health (Antunes *et al*., 2016).

Chen *et al*. (2021) screened 530 chicken meat for *Salmonella* randomly collected from market of China and found that 46.2% were contaminated with *Salmonella*. Siriken *et al*. (2015) tested 150 chicken meat and reported 42.66% to be positive for *Salmonella* with contamination rate higher in carcasses than in meat pieces. Balakrishnan *et al*. (2018) studied the prevalence of *Salmonella* in chicken meat collected from local market of Tamil Nadu. They reported the prevalence of 33.3% in the chicken meat and concluded that unhygienic handling during slaughtering contaminated the meat from the intestinal contents.

Yoon *et al*. (2014) examined 109 duck meat and 80 raw meat of chicken and found *Salmonella* contamination in duck meat 51.3% and 3.7% of chicken meat with predominant

serotypes as *Salmonella* Typhimurium and *Salmonella* Enteritidis. Out of 228 chicken meat, *Salmonella* was found in 23.7% and *Salmonella* Typhimurium was the dominant serotype in chicken meat with 59.2% (Kaushik *et al*., 2014). Gharieb *et al*. (2015) conducted a study on 100 poultry meat and reported *Salmonella* isolation from 10% and found 71.4% *S*. Typhimurium among the sample positive for *Salmonella*. Mejia *et al*. (2021) conducted a study on retail meat in Ecuador for *Salmonella* and reported 64.9% prevalence in chicken with *S*. Infantis as the dominant serovar.

Aung *et al*. (2020) analyzed the epidemiological distribution of *Salmonella* serovars from 2012-2016 and found that 60.8% *Salmonella* contamination in chicken and chicken products were with *Salmonella* Enteritidis and *Salmonella* Typhimurium as leading serovars. The study found that the prevalence of *Salmonella* was more in fresh meat than frozen chicken meat. The study also revealed that *Salmonella* Typhimurium was the most prevalent serovar in duck (43.9%) and 21.0 % in chicken. from fresh than frozen chicken meat.In contrast Bangera *et al*. (2019) reported *Salmonella* Infantis, 43.1% as the dominant serovar from the poultry. Gautam *et al*. (2017) screened frozen and chilled ready- to- cook poultry products for *Salmonella* and found 51% in chilled and 5% in frozen ready-to- cook chicken products with *S*. Typhimurium (75.2%) being the most prevalent serovar which is followed by S. Enteritidis (23%).

2.3 Antimicrobial resistance in *Salmonella*

Extensive documentation of antimicrobial resistance among NTS (non-typhoidal *Salmonella*) has been reported recently (Pandey *et al*., 2021).The rapid emergence of *Salmonella* isolates with antibiotic resistance is concerning because all these medications are essential for the effective treatment of *invA*sive infections (Parry *et al*., 2008).

S. Typhimurium DT104 has a high resistance rate to five antimicrobial agents, including ampicillin, streptomycin, chloramphenicol, sulphonamide and tetracycline (Mølbak *et al*., 1999). Report of increasing resistance to older antibiotics such as chloramphenicol, ampicillin and trimethoprime-sulfamethoxazole for years had led to include extended spectrum cephalosporins and fluroquinolones like ciprofloxacin for Salmonellosis. However emergence of resistance to

these important antibiotics has been reported and now the use of last resort antimicrobials like carbapenems is recommended but emergence of resistance to these has also been recorded in some *Salmonella* spp. (Antunes *et al*., 2016).

Gautam *et al*. (2017) isolated *Salmonella* from poultry products and found > 80% were multi drug resistant. Among them 90% *S*. Typhimurium isolates were resistant to nalidixic acid, chlortetracycline, kanamycin, oxytetracycline and tetracycline. All *S*. Enteritidis isolates were resistant to kanamycin and oxytetracycline, while more than 80% of isolates were resistant to nalidixic acid and chlortetracycline. Sohail *et al*. (2021) revealed 100% *Salmonella* isolates of poultry origin being resistant to at least one antibiotic. 94.34% of the isolates were resistant to doxycycline, followed by 84.91% cefpodoxime, ciprofloxacin (72.64%), gentamicin (65.09%), enrofloxacin (61.32%), colistin sulphate (40.42%), amikacin (34.91%), ampicillin (33.96%), neomycin (33.02), cefotaxime (30.19%) ,ceftazidime (29.25%), trimethoprim-sulfamethoxazole (23.58%), amoxicillin+clavulanic acid (21.70%), and chloramphenicol (12.26%). They also found 16.98% were extended spectrum β-lactamase (ESBL) producers, and 76.41% were multidrug resistant (MDR). In the United States, the percentage of *Salmonella* isolates resistant to ceftriaxone and ciprofloxacin has continued to increase year after year. The percentage of MDR *Salmonella* recovered from chicken cecal samples increased from 25 percent in 2017 to 32 percent in 2018. Similarly, the proportion of MDR *Salmonella* recovered from retail chicken increased from 17 percent in 2017 to 20 percent in *2018* (FDA, 2018).

The rising rate of antibiotic resistance in *Salmonella* Typhimurium is a major global concern. *Salmonella* Typhimurium antibiotic resistance patterns were frequently tetra-resistant ASSuT (ampicillin, streptomycin, sulfonamides, and tetracycline) and in penta-resistant pattern ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline) (Wang *et al*., 2019)

Irrational used of antibiotics in sub therapeutic doses in poultry feed to promote rapid growth is the major contribution of antimicrobial resistance development (Manyi-Loh *et al*., 2018). Antibiotic residues in poultry meat are a direct threat to public health. Antimicrobial resistance bacteria (ARBs) and genes (ARGs) can spread to the environment and, eventually to humans (Kumar *et al*., 2019).

2.4 Bacteriophage

Bacteriophages, also known as phages, are bacterial viruses that only infect bacterial cells. Phages can be found in a wide range of environment including faeces, sludge, sewage, seawater, soil and anywhere bacteria may grow and are the most abundantly found organism on earth, with 10^{31}-10^{32} bacteriophage particles on the planet's surface at any given time (Suttle, 2007; Keen, 2015).Phages size varies ranging from 24 to 400 nm in length with genomic size ranging from 18-400 kb (Orlova., 2012). A bacteriophage is composed of nucleic acid genome (40%) that is enclosed in protein coat known as capsid (60%) or is surrounded by a lipid membrane known as envelope (Paez-Espino *et al*., 2016). Its genome consist either DNA or RNA, but not both. The genome can be single-stranded or double stranded RNA or DNA, as well as circular or linear form. Structurally they composed of head packed with genetic materials, long tail with collar, a based plate with short spikes and tail fibers.

Phages, as naturally occurring bacterial parasites unable to independently reproduce (i.e., are non-living) and must rely on a bacterial host for survival (Lin *et al*., 2017). Bacteriophages are usually highly host-specific, attacking species specific or strains of bacteria (Hagens & Loessner, 2007). Bacteriophages undergo two types of replication strategies: lysogenic (temperate) and lytic (virulent) (Hobbs and Abedon, 2016). Phages bind to the complementary receptors on the surface of bacteria, incorporate the genetic material to bacterial cell. The lytic phages *invA*de the bacterial machinery, lyse the cell releasing new offspring. In lytic cycle, bacteriophages undergo adsorption involving adhesion to the susceptible bacterial cell surface. They bind with the receptors such as lipopolysaccharides in Gram negative, lipoteichoic and teichoic in Gram positive bacteria (Rakhuba *et al*., 2010). The penetration phase involves enzymatic rupture of the cell wall introducing genome to cytoplasm of host cell and uses the host's ribosomes to produce proteins. Resources of host cell are quickly converted to viral genomes and capsid proteins, which organize into numerous copies of the original phage while inhibiting bacterial DNA replication. The host cell wall and inner membrane lyse actively or passively by enzymes, endolysin and holin respectively, when phage progeny reach critical mass (few to 1000 viral particles). As it dies, it releases new phages to infect new bacterial cell, reinitiating the lytic cycle (Kasman and Porter, 2021; Weinbauer *et al*., 2004). The most common phages which follow lytic replication strategy are T1 and T4.

In lysogenic cycle, the phage genome is incorporated to bacterial chromosome or is kept as episomal element in which both is replicated and transferred to daughter cell from mother cell vertically without killing them. The phage genome integrated are known as prophages and lysogens are bacteria that contain them (Weinbauer *et al*., 2004; Kasman and Porter, 2021).When a lysogenic bacteriophage is under pressure or its survival is threatened, it has the ability to switch to lytic cycle, resulting in rapid replication of newly formed phages that burst out of cell of host (Hyman *et al*., 2009).

2.5 Classification of phages

Bradley proposed a classification of bacteriophages based on six morphological groups in 1967: Bacteriophage with hexagonal shaped head and tail with contractile sheath, Bacteriophage with hexagonal head and long, flexible tail, Bacteriophage with hexagonal head and short, non-contractile tail, Bacteriophage with only hexagonal shaped head in symmetry with big capsomere on, Bacteriophage with a simple regular hexagonal head, Bacteriophage with no head but with long flexible filament virion.

The Bacterial and Archaeal Viruses Subcommittee represents the prokaryotic virus community in the International Committee on Taxonomy of Viruses (ICTV). They classified phages on the criteria of morphology, nucleic acid and physicochemical properties of the virion particles. Majority of phages is double stranded DNA (dsDNA) but small percentage of phages has single stranded (ssDNA), single stranded RNA (ssRNA) or double stranded RNA (dsRNA) genome (Howard-Varona *et al*., 2017; Aiewsakun *et al*., 2018).

Bacteriophages are classified into 11 orders (*Belfryvirales*, *Kalamavirales*, *Caudovirales*, *Halopalevirales*, *Haloruvirales*, *Leviviriales*, *Mindivirales*, *Ligamenvirales*, *Petivirales*, *Tubulavirales* and *Vinavirales*). Further each order is classified under many family, subfamilies, and thousands of species. Morphologically, phages are grouped into tailed phages, filamentous, pleomorphic, polyhedral or cubic phages. The order *Caudovirales* represent the only order to which all tailed bacteriophages belong. Tailed phages form the majority (96 percent) of the phages discovered. This order consists of ten families, 44 subfamilies, 672 genera, and 1976 species. Of all the 10 families within the *Caudovirales*

order, bacteriophages belong mainly to 4 families: *Myoviridae*, *Siphoviridae*, *Podoviridae* and *Authographaviridae*. Family *Myoviridae*, possess a contractile tail. The viruses in the *Siphoviridae* family have a long noncontractile tail. *Podoviridae* and *Authographaviridae* phages earlier classified as same family, are distinguished by short tail (noncontractile) and can be distinguished only genetically because Authographaviridae phages have (RNAP) RNA polymerase encoded by virion (Adriaenssens *et al*., 2020).

2.6 Environmental condition for phage stability

Inspite of being abundance in environment, bacteriophage for commercial application is a challenge. Physicochemical factors influence bacteriophage survival and persistence at various pH, ions, temperature and pressure (Jończyk *et al*., 2011). Phages are composed of proteins that can change structure in harsh conditions. This may affect phage tail structure and its binding ability to host receptors, or it may even destroy the entire bacteriophage structure. The pH influences their infectivity, intracellular replication, and multiplication. In general, studies on lytic activity revealed that a neutral pH of 6–8 is optimal for most phages and their proteins, with values less than 5 and greater than 10 being less efficient (Kowalska *et al*., 2020). Garcia *et al*. (2009) showed that phage titer decreases gradually with decrease in pH. In the study, when the pH was reduced from 6.19 to 5.38 between 4 and 6 hr, the phage titer of *Staphylococcus aureus* was lowered by 2 log concentration. When the pH falls below 4.5, there is limited proliferation of several phages, and risk of pathogenic bacteria food contamination reduced. The T4 phage (*Myoviridae* family), for instance, is not stable at pH 5. After 1 hour at pH 5.0 and 37°C, phage PM2 (*Corticoviridae* family) loses all activity. In the case of oral injection of phage, there can lead to negative impact on phage survival, potentially leading to treatment failure (Watanabe *et al.*, 2007).

High temperatures may cause the proteins that make up the capsid to degrade. Furthermore, temperature influences bacteriophage viability, storage, and occurrence (Kowalska *et al*., 2020). Several studies have shown that pH stability and thermal stability is unique to each phage and varies depending on the phage isolate. When the phages are incubated at refrigeration temperatures, the latent period lengthens. Bacteriophages can survive at high

temperatures (40–90 degrees Celsius), and some *Lactococcus* phages can survive pasteurisation (Madera *et al*., 2004). Stalin *et al*. (2017) observed that the Vibrio phagesVHM1, VHM2, and VHS1 were capable of surviving at temperatures ranging from pH 3-11. Nakai *et al*. (1999) showed that anti-*Lactococcus garvieae* phages can survive in a variety of salinity solutions (NaCl 0–70 g/L) but have temperatures ranging from 5–37 °C and pH of 3.5, but up to 6–8) stability. Mylon *et al*. (2010) found that monovalent salts had no effect on phage titer in their study of MS2 phage stability taken in various salt solutions. Higher ionic strength, can increase phage aggregation (Langlet *et al*., 2008).

2.7 Advantages of phage therapy

- Phages multiply at infection site, allowing their presence during the most critical stages of infection (Payne *et al*., 2000).
- Phages are specific hence they may have no effect on the host's commensal bacteria (Miller *et al*., 2018). If broad spectrum is required, cocktails of phages can be used.
- Bacteriophages have been administered orally, dermally, rectally and parenterally (including intravenous & intramuscular and intra-peritoneally), and via aerosols in both animals and humans without causing harm.
- Low environmental impact: Because of their chemical composition and limited host range, phage eliminated after treatment, unlike broad-spectrum antibiotics, will affect only a small group of environmental bacteria in the worst-case scenario (Loc- Carrillo and Abedon 2011).
- Low cost, phage production primarily entails growth in its host and subsequent purification. Cost of growing the virus in its host varies depending on the type of bacteria, the cost of purification seems to be decreasing as technology advances.

2.8 Phage therapy in humans

The utility of bacteriophage viruses for treatment of bacterial infections is known as phage therapy. This practice has been around for over a century (d'Hérelle, 1931). They were first mentioned in 1915 by Frederick Twort, but their therapeutic applications were credited to

Félix d'Hérelle, who isolated them in the faeces of convalescing dysentery patients in 1917 (Pyle *et al*., 1926). But in the mid-20th century the advent of antibiotic surpassed the used of phage therapy. The discovery of antibiotics was a medical revolution that saved countless lives. Their indiscriminate use, however, led to emergence of multi-drug resistant (MDR) bacteria. This issue has now become a major global threat, posing a significant challenge in the look out for alternate therapies against MDR bacteria (Czaplewski *et al.,* 2016). The widespread rise of antimicrobial resistance (AMR), decline in antibiotic effectiveness has sparked renewed interest in revisiting this practice (Lin *et al*., 2017).

Felix d'Herelle isolated anti-shigella phage had used for shigellosis. He used phages to cure bubonic plague patients in Egypt and cholera patients in India, which lowered the mortality from 30% to 0% (Sulakvelidze *et al*., 2005). Bruynoghe and Maisin used bacteriophage to treat cutaneous furuncles and carbuncles by injecting staphylococcal-specific phage. They described clear evidence of clinical improvement in treated patients within 48 hours (Brunoghe *et al*., 1921). In 1938 clinical trial was conducted with 219 patients who were infected with bacterial dysentery and they were treated solely with a phage cocktail which contains a variety of phage targeting *Shigella shiga, Shigella flexneri, Proteus* spp*, E. coli, P. aeruginosa, Salmonella paratyphi A and B, Staphylococcus spp., Streptococcus, Salmonella* Typhi, within 24 hours, 28 percent of patients with blood in their stools were free of this symptom, and another 27 percent improved within 2-3 days. 74 percent of total patients improved or relieved of their symptoms (Chanishvili *et al*., 2008). Controversies about the application of phages due to the limited knowledge was cleared with the invention of electron microscopy, the structure and nature of infection were documented.

During a typhoid epidemic in 1974, 18577 children got enrolled in a prophylactic intervention study using typhoid phages and phage administration resulted in a 5-fold reduction in typhoid incidence (Kutateladze and Adamia, 2008). Emergence of antibiotic in 1930s remained active in Soviet countries but relegate phage therapy in Western countries. Eliava Institute, Georgia did an extensive study on phage and has developed many phages for preclinical and clinical treatment of common bacterial pathogens. Some of the phage preparation used includes Pyophage, composed of five components of phages against *Streptococcus,*

Staphylococcus aureus, *Pseudomonas aeruginosa, Proteus,* and *E. coli*. This phages mixture was used against wounds, dermatological complications, digestive tract infections such as stomatitis, gastritis, esophagitis, enterocolitis. Intestiphage, a phage mixture is reported to be effective for intestinal problems due to *Shigella, Proteus, Pseudomonas, Staphylococcus,* and *E. coli*, monoclonal preparations of staphylococcal bacteriophage, PhageBioDerm, a preparation of phages for wound infections. These preparations have shown to have effective result.

In Europe, phage therapy have been in used for treatment of many bacterial infection such as burns, tropical ulcers, wounds, diabetic foot ulcers (Morozova *et al*., 2018b). Multidrug resistant *Pseudomonas aeruginosa* infected burns wound was cured using bacteriophage (Abul-Hassan *et al*., 1990). Diabetic foot ulcers infected with *S. aureus* strains was effectively treated using *Staphylococcus* BP Sb-1 (Jault *et al*., 2018).

Wright *et al*. (2009) conducted a randomized placebo study in 24 patients in 2009 to test in the treatment of chronic otitis, the efficacy of a phage cocktail due to antibiotic-resistant *P. aeruginosa*. After a 42-day signs and symptoms were improved in phage treated group. Pyophage was recently used in cystic fibrosis case showed no response to other treatment. The treatment has shown good response when combined with antibiotic (Kutateladze and Adamia, 2008). For human Rhoads *et al*. (2009) published first phase 1 safety trial to treat venous leg ulcer caused by *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli.* They used WPP-201, a preparation consisting of 8 bacteriophages. This study has shown to have no adverse effect on the test group with the record of wound improvement. Schooley *et al*. (2017) developed and used cocktails of bacteriophages on a 68-year-old diabetic patient complicated with MDR *Acinetobacter baumannii* infection and had shown to recovered from the grave prognosis. Law *et al*. (2019) clinically resolved Cystic fibrosis by administering 4 lytic phages against *P.aeruginosa* intravenously in adjunct to antibiotic without adverse effect with no reccurent report in 100 days. Fish *et al*. (2016) healed diabetic toe ulcer wound caused by *Staphylococcus aureus* (MRSA) Using phage Sb-1 by topical application once weekly wound in average of 7 weeks. Lung transplant recipients with multidrug-resistant *P. aeruginosa* or *Burkholderia dolosa* infections were treated with lytic bacteriophages

and antibiotics (Aslam *et al*., 2019)

Despite its long history, phage therapy is not currently approved for routine human use in the western world. It is currently only approved in a few former Soviet republics, including Russia and Georgia, where commercial phage preparations are sold in pharmacies. However, numerous researches on animal model have proved the significance of phage therapy. Some worth mentioning are: mice challenged with sepsis caused by *Pseudomonas aeruginosa* was treated with phage oral administration and saved 66.7 percent of them from death, compared to 0% in the control group (Watanabe *et al*., 2007). In bacteremia models using vancomycin-resistant *Enterococcus faecium*, intraperitoneal administration of a single phage strain was sufficient to rescue 100% of the mice (Biswas *et al*., 2002).There is even evidence that phage can restore sensitivity of antibiotic in antibiotic-resistant bacteria, such as multidrug-resistant *Pseudomonas aeruginosa* (Chan *et al*., 2016).

Phages are also used for rapid detection of pathogen when molecular diagnostic techniques are not accessible. Due to their specificity, bacteriophages are suitable for rapid detection and their specificity for their target host cells and the speed of bacteriophage replication compared to host replication. Biotec Laboratories (Ipswich, UK) developed and marketed FASTPlaqueTB assay for Mycobacterium tuberculosis detection in sputum samples in human. This assay can detect the bacteriophage growth, indicated by plague formation in a lawn of bacteria. MicroPhage MRSA screening test was developed to identify methicillin-resistant *Staphylococcus aureus* (MRSA). The assay uses antibody capture to detect the growth of a specific bacteriophage after infection of the target cell, and results are available within 5 hours. The test is designed in such a way that the cells on a swab sample can be challenged with antibiotics, and bacteriophage will only grow if the cells are resistant to the antibiotics. Antibiotic-resistant isolates are quickly identified, and the assay is intended to be used as a practical, low-cost screening procedure for patients arriving at treatment facilities (Monk *et al*., 2010). Pathogenic bacteria can be detected using genetically modified phages from clinical, environmental, or food-related sources. Schofield *et al*. (2013) show that a (Wß::luxAB) engineered 'bioluminescent' reporter phage can detect *Bacillus anthracis* strains within 5 hours of transducing a bioluminescent phenotype.

2.9 Phage therapy in animals

Many pathogens of zoonotic importance colonize farm animals asymptomatically, potentially contaminating the cycle of foods and posing public health risks. Over the last 20 years, regular carcass sampling at retail by government authorities suggests that the prevalence of antibiotic resistance in foodborne pathogens has accelerated (Gigante and Atterbury, 2019). To face the antibiotic resistance problem possible effective alternative like bacteriophage therapy are applied in veterinary medicine and animal husbandry.

Most zoonotic pathogens encountered in livestock are *E. coli*, *Salmonella* spp., *Campylobacter* spp., *Listeria* spp., and *Clostridium* spp. (EFSA, 2018). D'Herelle pioneered bacteriophage therapy in animals, successfully treating fowl typhoid in chickens. Phage-treated birds survived 95–100 percent of the time, compared to 0–25 percent for untreated controls. Some following studies had shown inefficiency of the therapy (Pyle, 1926). Antibiotic discovery in 1920s had stopped extended studies for bacteriophage therapy. Phage therapy in animals has been re-discovered in 1980s. Smith and Huggins, (1982) has shown phages to be more significant than antibiotic for *Escherichia coli* infection in mice. Recently studies for bacteriophage therapy in companion animals and livestock have increased in quest to face antibiotic resistance.

2.9.1 Salmonellosis

Salmonella is a popular target for phage therapy because it infects a wide range of animals as well as humans and causes significant production losses in farm animals.

Administration of four phage cocktail (10^9 Plague Forming Units/g) to broiler chick in feed had shown to reduce colonization of *Salmonella* by 1 $\log_{10}$ CFU/g in gut when administered for 14 days (Sklar *et al*., 2001). Fiorentin *et al*. (2005) indicated that when exposed to seeder chicks of 7-day-old infected with 10^8 CFU of *Salmonella Enteritidis*, a single dose composed of 3 phages each (10^{11} PFU) when given orally reduced *Salmonella Enteritidis* colonisation by 3.5 log10 CFU/g in the caecum of the birds. On 10-day-old chicks, three specific *Salmonella* phages (10^8 PFU/mL/dose) were administered through drinking water or coarse spray 24 hours prior to the artificial infection with 9.6×10^5 CFU *S. Enteritidis*. Both

means of delivering phages via drinking water and coarse spray has reduced intestinal *Salmonella Enteritidis* counts by 1.6 $\log_{10}$ CFU/mL (Borie *et al*., 2008).

Wall *et al*. (2010) gavaged 5×10^9 CFU of *Salmonella Typhimurium* γ4232 and microencapsulated alginate beads containing 5×10^9 PFU of 16 cocktail phage to 3-4 week old pigs. The counts of *Salmonella* in the caecum, ileum and tonsils were reduced by 2 to 3 $\log_{10}$ CFU/g. In a different experiment, pigs were infected with 5×10^9 CFU of *S. Typhimurium* orally, then they were treated by introducing 10^{10} PFU of phage cocktail 48 hours later. *Salmonella* average caecal counts were reduced by 1.4 $\log_{10}$ CFU/mL in phage-treated pigs compared to untreated controls. Sorour *et al*. (2020) evaluated the efficacy of phage in prevention and treatment of salmonellosis caused by *Salmonella* Kentucky in broiler chicken. They isolated the bacteriophage against *Salmonella* Kentucky from environment and administered the phages (10^8 PFU ml^{-1}) on the test subjects after infecting with *S*. Kentucky orally (10^8 CFU ml^{-1}) and evaluated the effect on 9th, 16th and 23rd day. No clinical signs were observed on the test group but shows mild lesion which disappeared eventually. In the test group no mortality were observed compared to the 30 percent in the positive control. They also reported the reduction in *S*. Kentucky count in caecal sample in phage treated group in contrast to positive control which is increased by 0.67 $\log_{10}$ (x+1) CFUgm^{-1}. Seo *et al*. (2018) reduced colonization of *Salmonella* Typhimurium by introducing 10^9 PFU/mL bacteriophage cocktail in pigs.

2.9.2 Colibacillosis

Colibacillosis is caused by pathogenic strains of *Escherichia coli* in poultry causing high mortality rate by affecting the respiratory tracts. Huff *et al.* (2002) administered mixture of phages (DAF6, 2.35×10^9 PFU/mL and SPR02, 2.6×10^8 PFU/ mL) by spraying in broiler chicken followed by infecting them by pathogenic E. coli (5.6×10^4 CFU). Phage treatment showed a notable reduction in mortality, which was more when the phage was administered concurrently with the bacterial infection (30 percent mortality versus 60 percent mortality for untreated control birds). Huff *et al*. (2003) treat colibacillosis using phages DAF6 and SPR02. They administered the phages through intramuscular injection or aerosol spray. 5.96×10^4 CFU of *E. coli* were injected into the air sac of the seven day old chicks. The

aerosol spray phage contained 7.65×10^8 (DAF6) and 2.83×10^9 PFU/mL (SPR02). The study revealed significant protection to the birds, as evidenced by the low mortality of the treated group (20 percent) in comparison with the untreated group (50 percent). They also noted that when phage treatment is late by 24-48 hours after artificial infection, there was no therapeutic benefit. The intramuscular route of injection showed lower mortality than the aerosol route.

Smith and Huggins, (1983), studied the effectiveness of phages on calf, lamb and piglets. It has shown that mixture of phages used had cured the animals against *E. coli* diarrhea and had also shown to have lower bacterial count than the untreated. Jamalludeen *et al*. (2009) discovered that phages helped weaned pigs infected with enterotoxigenic *E. coli* O149:H10:F4. The pigs were given 10^{10} colony forming unit (CFU) of *Escherichia coli* orally, following treatment by 6 phages (GJ1-GJ7), either individually or in combination at a dose of 10^9 PFU each of experimental phages. The phages were given either prophylactically (15 minutes after challenge) or therapeutically (15 minutes following the challenge) (24 hours). In order to improve *E. coli* colonization, the antibiotic florfenicol was used prior to the bacterial challenge. The prophylactic use of the 6 phages individually significantly decreased the severity and duration of the diarrhoea, as evidenced by a clinical symptom score of 4 versus 10 in the challenged control. Furthermore, administration of the therapeutic two-phage cocktail significantly decreased symptoms, diarrhoea, and the excretion of pathogenic *E. coli* without affecting the numbers of commensal *E. coli*. Eid *et al*. (2022) reported no mortality compared to 26.7% in untreated birds when phages against *E. coli* O78 (10^8 PFU $ml^{-1)}$ was administered intrathecally.

2.9.3 Campylobacteriosis

Campylobacter spp. is a common cause of acute bacterial food poisoning. 95 percent of the reported cases were caused by *C. jejuni*. A study by Food Standards Agency (UK) revealed the isolation of many multidrug resistance from chicken hence considered to be of public concern.

Wagenaar *et al*. (2005) investigated the prophylactic and therapeutic efficacy of two lytic bacteriophages against *C. jejuni* intestinal colonization in broiler chicks. In the first trial,

the birds were given a phage mixture orally for 10 days, with doses ranging from $4x10^9$ to $2x10^{10}$ PFU; on day 5, the birds were given $1x10^{10}$CFU of *C. jejuni* orally. Although this treatment did not prevent c intestinal colonization, it did reduce it by two logs in comparison to the untreated infection group. Loc-Carrillo *et al*. (2005) investigated the effect of CP34 and CP8 bacteriophages on *C. jejuni* intestinal colonization. Broilers aged 20-22 days were orally administered with 2.7 to 7.8 $\log_{10}$ CFU of *Campylobacter jejuni* for this study. At 25 days, the chickens were given bacteriophages orally in an anti-acid suspension at a dose of 9.5 $\log_{10}$ PFU. The results showed that phage therapy reduced cecal bacterial counts by 0.5 to 5 $\log_{10}$ CFU/g of caecal content when compared to the untreated group.

2.9.4 Clostridiosis

Clostridium perfringens is the causative agent of necrotic enteritis, a chicken disease. Miller *et al*. (2010) demonstrated that giving a five-phage cocktail at 10^5 PFU/mL via drinking water or through orally to the experimentally challenged broiler chickens (0-42 days old) with *Clostridium perfringens* reported 92 percent decrease in mortality compared to non - treated control group. Endolysins from phages that target *C. perfringens* is also revealed as a promising route to reduce colonization or treat infection by this pathogen (Wernicki *et al*., 2017). Santos *et al*. (2011) demonstrated that the phages P5U5 and P2S2 had strong antimicrobial activity against twenty-six strains of *P. aeruginosa* isolated from keratitis dogs.

Many experiments were also done on the laboratory animals for safety as well for checking the efficacy issues. Abhishek *et al*. (2015) studied the therapeutic efficacy of Bacteriophage PSAE-1 against *Salmonella* Abortusequi using Guinea Pig as a Model. The study found that the phage treated group improved therapeutically without any adverse effect in comparison with the non-treated group, suggesting phage therapy can be used to reduce *Salmonella* infection or colonization.

2.10 Phage in food safety as biocontrol

During the process of milking, storage or packaging, fermenting or slaughtering there is every chance of bacterial contaminating. Many strategies have been explored to control the microbial contamination in raw food over few years. Several treatment methods including

physical treatment such as dry heat, steam and UV light have been applied but organoleptic deterioration is unacceptable. Therefore these methods are not applicable to vegetables, fresh fruits and ready-to-eat products. The phages application is widely accepted as natural antimicrobials inhibiting undesirable bacteria. Bacteriophage-based biocontrol assessments have the potential to significantly improve microbiological safety due to their long history of safe use, ease of handling, and high and specific antimicrobial activity (Garcia *et al*., 2008). Some of the evidences showing phage as a biocontrol for food safety are as follows:

2.10.1 *E. coli* O157:H7 control

Emergence of *E. coli* O157:H7 is a public health concern as ingestion as low as 10-100 cells can be fatal. Main reservoir of this strain are ruminants and spread through food chain. Therefore to avoid this, phage treatment is practice before slaughtering of these animals. Within two days of phage CEV1 administration in sheep orally, Raya *et al*. (2006) detected reduction in 2 log unit reduction in intestinal *Escherichia coli* O157:H7. Sheng *et al*. (2006) reported reduction of *Escherichia coli* O157:H7 in cattle when they were given a mixture of the phages SH1 and KHI rectally and also given in drinking water at 10^6 PFU ml-1, significantly fewer cell numbers were observed. To prevent pathogen development, phages also were used on the meat surface. O'Flynn *et al*. (2004) also reported that when combination of three different phages were applied on the beef contaminated with 10^3 CFU g^{-1} of *E. coli* O157:H7, no viable cells were recovered after storage at 37°C (O'Flynn *et al*., 2004).

2.10.2 *Listeria monocytogenes* control

Listeria monocytogenes is frequently associated with foods, such as or salads, dairy products, or stored foods at low temperatures. Leverentz *et al*. (2003) studied a cocktail of phages to control *Listeria* on fruits and had shown elimination of the bacteria in the fruits. Listex P100, a commercial product, was recently approved by the FDA as a food bio-preservative and granted GRAS status (Generally Recognised As Safe). The product employed virulent phage P100 previously used by Carlton *et al*. (2005) on cheese and eradicated complete target cells. Lang (2006) also developed phage preparation that can be food additive (LMP 102) composed 6 bacteriophages isolated from the environment. Cufaoglu *et al*. (2019) also

demonstrated that the application of the bacteriophages against *Listeria monocytogenes* on drumsticks achieved a reduction up to 3.3 log CFU/ml in *L. monocytogenes* count in 3 hr of incubation at 4°C

2.10.3 *Staphylococcus aureus* control

Staphylococcus aureus in curd was successfully inhibited by a dairy derived cocktail lytic phages added at MOI 10^2 (Garcia *et al*., 2007). Gill *et al.* (2006) examined the activity of *Staphylococcus aureus* bacteriophage K for mastitis but had revealed the inactivation of the phages within the mammary gland.

2.10.4 *Salmonella* control

Salmonella is one of major food borne pathogen causing food poisoning. With more than 10^7 PFU/g present in caecal content, *Salmonella* Enteritidis average faecal counts were reduced by 0.3–1.3 log units, but the complete eradication of pathogen was not observed (Sklar and Joerger, 2001).

Rivera *et al*. (2021) evaluated the effect of novel bacteriophage STGO-35-1 against *Salmonella* Enteritidis on chicken meat. The phages showed a signiûcant reduction of the bacterial load ($p < 0.05$) ie. 2.5 $\log_{10}$ at 4°C. Kumar *et al*. (2022) evaluated the efficacy of cocktail of phage against *S*. Typhimurium in chicken meat at different temperature (25 °C and 4 °C) and showed significant reduction in viable count in the phage treated group. Bigwood *et al*. (2008) also experiment the effect of *S.* Typhimurium PT160 phages on cooked and raw beef. Considerable host inactivation was observed with 2–3 log units at 5°C and more than 5.9 log units was achieved at 24°C. Kim *et al*. (2020) showed that used of cocktail phage against *Salmonella* spp., viable cell counts of bacteria were significantly reduced in raw chicken breast samples ($P < 0.05$) when examined after 7 days kept at 4°C. Aguilera and his coworkers (2022) used cocktail of 5 bacteriophages to reduce *S*. Typhimurium load in chicken meat by 1 log10 CFU/g reduction in 24 hr storage at 22°C and 30°C. Shang *et al*. (2021) also showed bacterial load reduction in milk and chicken meat using novel *Salmonella* phage vB_SalP_TR2. The phage was found to reduce 1.8 log10 CFU/ml and 0.9 log10 CFU/piece in milk and chicken meat respectively.

2.10.5 Commercial phage preparations used in food safety

Many preparations of phages are available commercially as biocontrol for many pathogens in food industries. The use of phage as a processing aid is legal in the United States, Canada, Switzerland, New Zealand, Australia, and Israel (Wessels *et al*., 2021). Commercial phage products have been approved on a regular basis in the United States (Ly-Chatain, 2014). Many commercial phage preparations are also approved by FDA.

Some commercial phages available to control bacteria in poultry, ready to eat meat prior to packing and vegetables are: Intralytix Inc. (USA) produced several phage products, ListShield™ which includes 6 phages to control *Listeria monocytogenes* (Perera *et al*., 2015), EcoShield™ for *E. coli* O157:H7 control (Abuladze *et al*., 2008), Ecolicide® for *E.coli* O157:H7 (Moye *et al*., 2018), SalmonFresh™ for *Salmonella* spp., ShigaShield™ for *Shigella* spp. (Soffer *et al*., 2017). EMB Food Safety (Netherlands) has also produced PhageGuard LISTEX™ for *L. monocytogenes* (Monk *et al*., 2010), PhageGuard S™ (Parveen *et al*., 2017), SALMONELEX™ for *Salmonella* spp. Another product SalmoPro® for *Salmonella* spp. control by Phagelux (Shanghai) (Moye *et al*., 2018).

2.11 Phages as Bio-sanitizer

Many opportunistic and foodborne pathogens persist on food surfaces, posing a public health risk. Various sanitizers can be corrosive and toxic, causing long-term damage to food contact surfaces. As a result, it is critical to develop alternative methods of disinfecting food processing equipment and surfaces.

2.11.1 *Listeria monocytogenes*

Many foodsafety studies focused on *Listeria monocytogenes* as they can form biofilms on many food contact surfaces, including stainless steel, glass and polystyrene. The commercially available phage products ListShield™ and Listex™ (now called PhageGuardL) were assessed for their efficacy in reducing the concentration of *Listeria monocytogenes* on contact surfaces. Under laboratory conditions, both products were successful in removing 48- and 72-h old biofilms from polystyrene and stainless steel surfaces after treatment for 4 hours at 12 °C and

32 °C. ListShield™ also inactivated biofilms of *Listeria monocytogenes* on stainless steel and rubber surfaces. After 2 hours of treatment, the biofilm on the stainless steel was significantly reduced ($P < 0.05$) by 1.9–2.4 log CFU/cm^2, whereas *Listeria monocytogenes* was reduced by 1.0 log CFU/cm^2 on the rubber surface (Sadekuzzaman *et al*. 2017).

2.11.2 *Escherichia Coli* O157:H7

Phages have been tested for their ability to reduce the presence of *E. coli* O157:H7, a pathogen linked to foodborne outbreaks of contaminated leafy greens, ground beef and other foods (CDC 2020). Abuladze *et al*. (2008) conducted the effect of EcoShield™, a three-phage cocktail on gypsum and glass. When EcoShield™ was applied on the surfaces after contaminated with three strain *E. coli* O157 mixture, there was significant reduction in bacterial count. When BEC8, a previously characterised phage mixture, was used, Viazis *et al*. (2011) demonstrated effective reduction of *E. coli* O157 isolates on food contact surfaces.

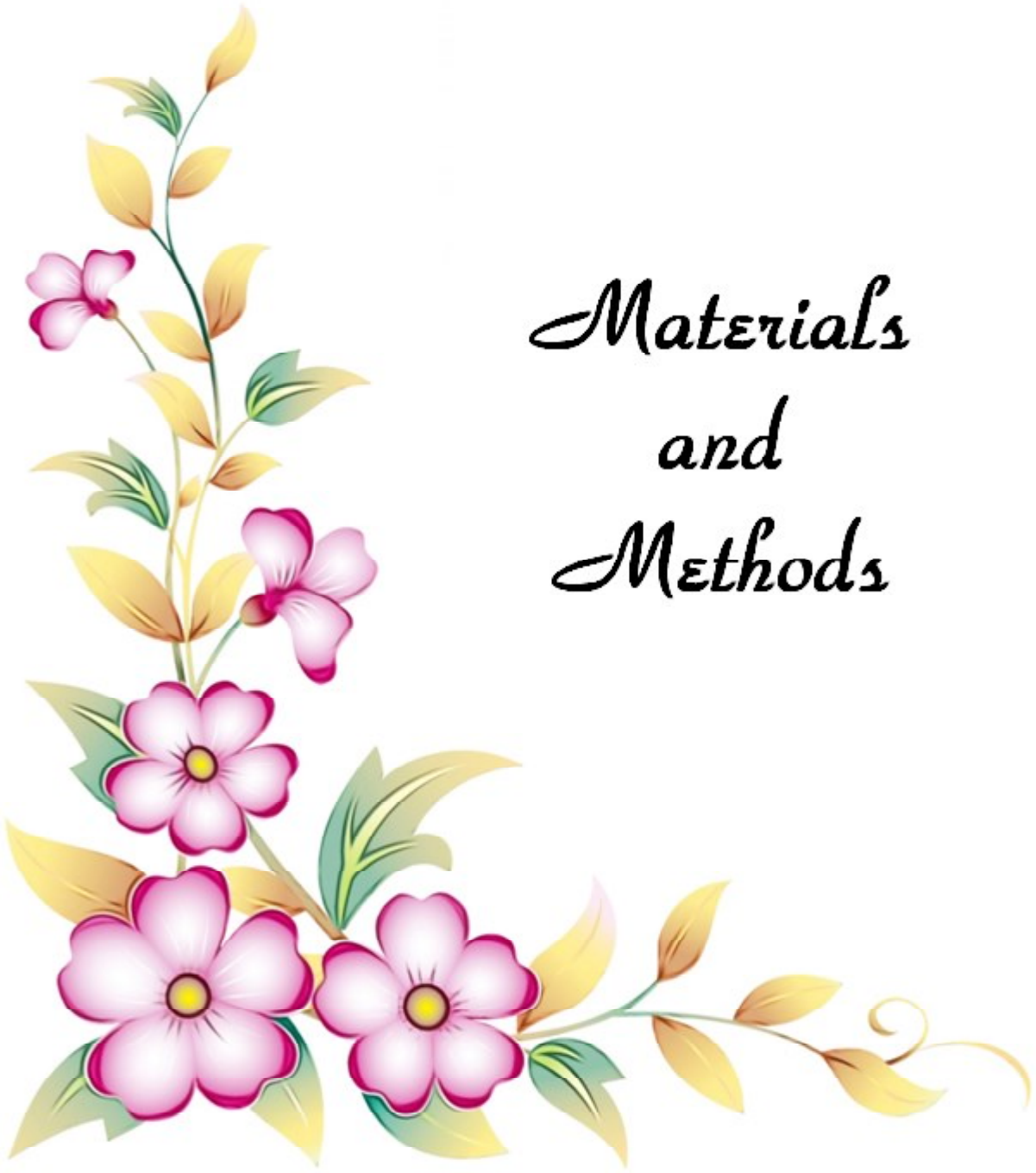
2.11.3 *Salmonella* spp.

Several approaches to reduce the bacterial load associated with poultry have been proposed. Bio-sanitizers based on phages can be used in hatcheries, farms, poultry processing plants, food contact surfaces and transport crates. SalmoFresh™, a commercial phage preparation, significantly (p 0.05) reduced *Salmonella* Brandenburg and Kentucky on glass surfaces and stainless steel by 2.1–4.3 logs (Woolston *et al*., 2013). Ferreira *et al*., (2014) investigated the antibacterial property of a bacteriophage mixture against *Salmonella Enteritidis* biofilms grown on stainless steel coupons at different ages (4, 8, and 12 days). The bacteriophage mixture was effective in reducing biofilms of all ages, with the most significant reduction observed on 8-day-old biofilms after 35 minutes of treatment with 10^7 PFU/ml bacteriophages.

2.12 Phages as biosanitizer in health care facilities

Phages have been used to sanitize hospital environment, including operating and critical care rooms. Over a six-month period, a trial was conducted in Tbilisi in three different hospitals to evaluate the phage's sanitation potential. The isolation frequencies of *Pseudomonas aeruginosa, Proteus,* and *Staphylococcus* nosocomial strains were initially 7.2 percent, 11.2 percent, and 13.6 percent, respectively, in 732 samples. Following phage sanitation, the

frequency dropped to 3.6 percent, 6.3 percent, and 8.2 percent after one month, 1.2 percent, 3.2 percent, and 3.3 percent after two months, and 0.3 percent, 1.8 percent, and 0.9 percent after six months, respectively (Kutter *et al*., 2010).There have also been many reports of phages for bio-sanitization of catheters. Curtin and Donlan (2006) investigated whether catheters coated by hydrogel pretreated with a coagulase negative bacteriophage reduced the formation of *Staphylococcus epidermidis* biofilms. Phage treatment significantly reduced *Staphylococcus epidermidis* and formation of biofilm. Similar study was conducted by Fu *et al*. (2010) by infecting catheters with *Pseudomonas aeruginosa* phages. Pretreatment of *Pseudomonas aeruginosa* phages reduces the biofilm viable count. These experimental findings are very relevant to the issue for implanted medical devices such as catheters, lenses, and prostheses, which frequently form biofilms and caused persistent infection (Lin *et al*., 2017).



Materials and Methods

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Media, Buffer and reagents

The bacteriological media were purchased from HiMedia Laboratories Pvt.Ltd., India. The appendix contains information about the preparation of media, buffers, and reagents used in this study.

3.1.2 Chemicals

All the chemicals were procured from Qiagen (Germany), Thermo Fisher Scientific (USA), and other reputed firms.

3.1.3 Kit and enzymes

The DNeasy blood and tissue kit and enzymes, DNase1, RNaseA, Proteinase K, *Eco*R1 and *Bam*H1 and RFLP employed for bacteriophage nucleic acid isolation and RFLP were procured from Qiagen, Germany and Thermo Fisher Scientific, USA.

3.1.4 Plasticware and glassware

Plasticwares such as centrifuge tubes, petri dishes, syringe filters (0.22 µ) were obtained from reputable companies such as Tarsons (India), Genaxy (India), Axiva (India) and Axygen (USA). Glasswares were obtained from Borosil (India) and Schott Duran (Germany). Plasticwares and glasswares were washed thoroughly and sterilised as needed as per the standard procedures.

3.1.5 Equipments

Some important equipment used in the study were 96 well thermal cycler(applied biosystems Thermo Fisher Scientific, US), refrigerated centrifuge (Sorvall ST 8R ThermoScientific, US) Biospectrometer (Eppendorf, Germany), ice flaking machine (Icematic F100 compact), deep freeze (-20°C) (Vestfrost, Denmark), gel documentation system (AlphaimagerTM 2200), hot air oven, incubator (Jain Scientific Glass works, India), pH meter (Corning, USA), variable pipette set (Thermo lab systems, USA), horizontal Gel electrophoresis apparatus (Geneilabs, Bangalore India), orbital shaker (Julabo-Shake Temp, SW22, Switzerland), water bath (Ambassador, India), highly sensitive balance (Danver Scales, India), autoclave (Scientronic, New Delhi, India), water distillation apparatus (Millipore India Pvt. Ltd., New Delhi, India) and Transmission Electron Microscope (JEM-1011, JEOL, Japan))

3.1.6 Oligonucleotide primers

Primers used in the study were custom synthesized from Eurofins Genomics India Pvt. Ltd. The details of the primers were given in Table 1.

Table 1: Oligonucleotide primers used in the study

Sl. No.	Primer code	Primer sequence 5' to 3'	Product size	Reference
1.	***invA* F**	ACAGTGCTCGTTTACGACCTGAAT	244bp	Elkhadragy *et al.* (2020)
	***invA* R**	AGACGACTGGTACTGATCGATAAT		
2.	***g23* F**	GATATTTGIGGIGTTCAGCCIATGA	600bp	FileéJ *et al.* (2005)
	***g23* R**	CGCGGTTGATTTCCAGCATGATTTC		
3.	***CapE* F**	TTCAGACCCACGGATGGTTG	511bp	Phothaworn *et al.* (2019)
	***CapE* R**	AGAAAGCGGCTACAACACGA		

3.1.7 Bacterial strains

All the *Salmonella enterica* serovars used in this study as host strains and for host susceptibility studies were obtained from National *Salmonella* Center (NSC). The list of *Salmonella enterica* serovars isolates was given in Table 2. Isolates of *Escherichia coli* used for host susceptibility in this study was provided by Division of Bacteriology and Mycology, IVRI, Izatnagar. List of the *E. coli* isolates is given in Table 3.

Table 2: List of *Salmonella* isolates used in the study

Isolates	Serovars	Origin
21UK04	Typhimurium	Pantnagar, Uttarakhand
21UK05	Virchow	Pantnagar, Uttarakhand
21UK06	Typhimurium	Pantnagar, Uttarakhand
21UK07	Typhimurium	Pantnagar, Uttarakhand
21UK08	Virchow	Pantnagar, Uttarakhand
21UK09	Typhimurium	Pantnagar, Uttarakhand
21UK10	Typhimurium	Pantnagar, Uttarakhand
21UK11	Typhimurium	Pantnagar, Uttarakhand
21UK13	Typhimurium	Pantnagar, Uttarakhand
21UK14	Virchow	Pantnagar, Uttarakhand
21JK01	Infantis	Jammu and Kashmir
21JK02	Infantis	Jammu and Kashmir
21JK03	Infantis	Jammu and Kashmir
21JK04	Infantis	Jammu and Kashmir
21JK05	Infantis	Jammu and Kashmir
21JK07	Agona	Jammu and Kashmir
21JK08	Agona	Jammu and Kashmir
21JK09	Agona	Jammu and Kashmir
21JK10	Agona	Jammu and Kashmir
21JK11	Kentucky	Jammu and Kashmir
21JK12	Kentucky	Jammu and Kashmir
20AN60	Kentucky	Jammu and Kashmir
22NSC004	Typhimurium	Izatnagar
ATCC13311	Typhimurium	Reference strain

Table 3: List of *E. coli* isolates used in the study

Sl. No.	Strain No	Serotype
1.	E-46d	*E. coli*
2.	E-48c	*E. coli*
3.	E-61a	*E. coli*
4.	E-65b	*E. coli*
5.	E-68d	*E. coli*
6.	E-154a	*E. coli*
7.	E-155a	*E. coli*
8.	E-156b	*E. coli*
9.	E-157c	*E. coli*
10.	E-158b	*E. coli*
11.	E-159b	*E. coli*
12.	E-160b	*E. coli*
13.	E-162c	*E. coli*
14.	E-163b	*E. coli*
15.	E-164a	*E. coli*
16.	E-165c	*E. coli*

3.2 METHODS

3.2.1 Revival of the target bacteria

Salmonella enterica isolates procured in glycerol stock were revived by inoculating into Luria Bertani (LB) then streaked onto *Salmonella* selective media, Hektoen enteric (HE) agar and tested biochemically by Triple sugar iron (TSI), Urease test and Simon Citrate test. From TSI slant, loopful of bacteria was inoculated into LB broth and incubated at 37 C for 16-18 hrs. The overnight broth culture was used for extracting DNA for molecular confirmation by PCR targeting *invA* gene. The culture was maintained for at 4°C in HE plates and TSI slant and at -20°C in glycerol stock for phage studies. Subculturing was done on every 10th day.

3.2.2 Characterization of *Salmonella enterica* isolates

3.2.2.1 Biochemical characterization

Salmonella colonies on Hektoen Enteric agar (HEA) gives transparent, smooth green or blue-green periphery colonies with black centered colonies. From HE agar plates, single colonies were inoculated on Triple sugar iron (TSI), and Simon Citrate slant for biochemical

confirmation. For further confirmation and to differentiate from *Proteus* sp. Urease test was performed.

3.2.2.2 Molecular characterization

3.2.2.2.1 Boiling-snap chilling method of DNA extraction

Salmonella strains which were used as target hosts (22NSC004 and ATCC13311) against which bacteriophage will be isolated were inoculated in LB broth. From this culture DNA was extracted by Snap-Chilling method following Anju *et al*. (2014) with slight modifications. Briefly, 1ml of bacterial overnight culture was transferred to 1 ml microcentrifuge tube and subjected to centrifugation at 8000 rpm for 8 minutes at 4°C to pellet the bacteria at the bottom of the tube. The supernatant was discarded and bacterial pellet was washed twice using 250 µl of PBS (Phosphate buffer Saline). After washing, the pellet was re-suspended in 200 µl of NFW (Nuclease Free Water) and kept in boiling water (100°C) for 15 minutes. After 15 minutes, the tube was transferred immediately to ice for 2 minutes. The content was then centrifuged at 8000 rpm for 5 minutes. Carefully, the supernatant was then transferred to new microcentrifuge tube and stored at -20°C until used.

3.2.2.2.2 Polymerase chain reaction (PCR)

Molecular characterization for further confirmation of the host strains was carried out by targeting *invA* gene which is a genus specific gene for *Salmonella*. The primer specific for *invA* produced a product size of 244bp. The detail of the primer used is in given table 1 and details of the PCR reaction mixture and PCR reaction condition is given in table 4 and 5 respectively.

Table 4: Reaction mixture of PCR for *invA* gene

Components	Volume (µl)
Master mix	10
Forward primer	1
Reverse primer	1
Template	1.5
Nuclease free water	11.5
Total	25

Table 5: Reaction conditions of PCR for *invA* gene

Reaction	Primer conditions	
Initial denaturation	95°C, 10 min	
Denaturation	95°C, 1 min	40 cycles
Annealing	60°C, 1 min	
Extension	72°C, 1 min	
Final extension	72°C, 15 min	

3.2.2.2.3 Agarose gel electrophoresis

The amplified PCR product was subjected to electrophoresis for detection of the product. 10 µl of the amplified product of PCR was loaded along with 4 µl of 100bp DNA ladder and electrophoresed on 1.25% of agarose gel containing ethidium bromide at the rate of 7µl/100 ml of agarose gel. The gel was run at 80V using 1X TAE buffer in electrophoresis apparatus (Bio-rad). Finally, gel documentation was done using gel documentation system (Alphaimager™).

3.2.3 Bacteriophage isolation

3.2.3.1 Sample collection

A sum of 81 farm waste from poultry, swine, cattle, buffalo farms, human sewages and hospitals waste water from in and around Bareilly were collected in sterile 50 ml centrifuged tubes. The samples were transported to the laboratory and kept at room temperature for 1-2 hours for the sediment in the samples to settle if present.

3.2.3.2 Phage enrichment

Approximately, 20 ml of supernatant from the samples were enriched with 200 µl of overnight culture of *Salmonella enterica* serovar Typhimurium strain ATCC 13311 and 22NSC004 separately and 2-3 ml of 10X BHI (brain heart infusion) broth was added to each sample and incubated at 37°C for 16-18 hrs. After overnight incubation, 10 ml of the supernatant was subjected to centrifugation at 9000 rpm for 9 minutes. After centrifugation, supernatants

were filtered using 0.22 µm syringe filter to avoid bacteria contamination. The filtrate was considered free from bacteria and was known as BFF (bacteria free filtrate).

3.2.3.3 Detection of bacteriophage activity using spot test

Lawn of bacteria viz. ATCC 13311 and 22NSC004 was made on NZCYM agar by spreading evenly using an L-shaped spreader. The plates were let to absorb the bacterial culture for 5minutes. The plates were labeled with sample numbers and 10 µl of BFF were spotted within the marked area. The plates were left undisturbed for 10-15 minutes for the phage lysate to get absorbed completely. Once dried, the plates were incubated overnight (12-18 hrs) at 37°C and observed for plaques (lysis zone) where enriched phage samples were spotted. Samples were considered positive for lytic phage when clear zone of lysis was observed and were considered negative, when no lysis was observed. The samples which exhibited lytic activity were further confirmed for presence of phages by double agar overlay method.

3.2.3.4 Double layer Overlay agar method

In double agar overlay method, NZYM agar plates were labeled with sample number, host bacteria and date of the experiment then, approximately 100-150µl of BFF and 70-100 µl of overnight host bacterial culture were added to the 7ml of NZCYM molten agar (0.7%) that was melted to 45-50°C and mixed gently. The mixture was poured on top of the NZYCM plate and gently swirled to make even lawn. The plate was left undisturbed for 20-30 minutes to get solidified. The plates were incubated overnight at 37°C and observed for plaque formation. Plaques morphology were observed and recorded. The well isolated plaques were picked to make homogenous phage lysate (Kropinski *et al*., 2009).

3.2.3.5 Phage picking

Phage picking was done to isolate the phages producing plaques of identical morphology (homogenous plaques). A sterile glass pasteur pipette or 100 µl micropipette tip was used to pick up well isolated plaques by piercing the soft agar at the center of the clear plaque and resuspended in 200 µl of TM buffer. The suspension was incubated overnight at 4°C for phage elution into the buffer.

3.2.4 Phage purification and concentration of phage

3.2.4.1 Soft-agar overlay method (Plate method)

Phages isolated were further purified by double agar overlay method. The top soft agar layer which showed complete lysis were harvested and re-suspended in TM buffer (2 ml per plates) and incubated at 4°C for 6 hours for phage elution into the buffer. After incubation, phages were recovered by centrifuging the suspension at 7000 rpm for 6 minutes refrigerated temperature (4°C). The supernatant was filtered through 0.22µm syringe filter. The phage lysate collected were passaged three to four times in order to obtain pure homogenous phage lysate.

3.2.4.2 Broth method (polyethylene glycol-$NaCl_2$ concentration method)

Overnight bacterial culture was inoculated to 200ml NZCYM broth supplemented with $CaCl_2$ and $MgSo_4$ at 1:100 ratio and incubated overnight at 37°C in shaker incubator at 160 rpm to get an OD_{600} of 0.1-0.2 ~$5X10^9$ CFU/ml.

The purified phage lysate was added at 1:100 ratio to the bulk bacterial culture and further incubated at 37°C in shaker incubator at 160 rpm until clearance of the phage-bacterial suspension takes place.

When phage-bacterial suspension showed clearance, chloroform was added at 1:100 ratio and mixed gently by vortexing for further clearing and the clear lysate was separated avoiding the chloroform.

To the clear lysate 6g $NaCl_2$ and 15g of PEG8000 was added and was allowed stirred to dissolve completely at 4°C, overnight using magnetic stirrer followed by centrifugation at 10,000 rpm for 25 min.

The pellet was resuspended in 8ml TM buffer and equal volume of chloroform was added, followed by gentle vortexing for 3-5 minutes. The mixture was centrifuged at 10,000 rpm for 10 minutes at 4°C to separate the aqueous and organic phase.

The aqueous phase (clear/bluish) which has phage was collected as phage stock and stored at 4°C for future use.

3.2.5 Estimation of bacteriophage concentration

The concentration of the bacteriophage was estimated by double agar overlay plaque assay (Kropinski *et al*., 2009). A serial 10-fold dilution was made from the phage lysate by diluting 100 µl of undiluted phage to 900 µl of TM buffer to make 10^{-1} dilution. The suspension was further diluted by transferring 100 µl from the 10^{-1} dilution to the second tube having 900 µl to make 10^{-2} dilution and so one till 10^{-10} dilution. Then for each dilution, phages were enumerated by double agar overlay method. Phage lysate of 100 µl from each dilution was titrated against 100 µl of host bacteria and concentrations of phages were enumerated. The concentration of the phage was calculated by the formula,

$$Phage\ concentration/\ titer\ (pfu/ml) = Number\ of\ plaques \times reciprocal\ of\ dilution\ factor \times reciprocal\ of\ vol.of\ initial\ phage\ used\ for\ serial\ dilution$$

3.2.6 Nomenclature of bacteriophages

The isolated phages were named following the recent ICTV (International Committee on Taxonomy of Viruses) nomenclature of bacteriophages (Adriaenssens *et al*., 2017).

3.2.7 Biological characteristic of bacteriophages

3.2.7.1 Morphological characterization

The high titer phage preparation of 6µl was placed on a carbon grid, allowed them to absorb for about 30 seconds then the sample was negatively stained with a drop of uranyl acetate 2% (w/v). The samples were allowed to set for 20 s, and the grid was rinsed with a drop of double distilled water. The grid was examined under Transmission Electron Microscope JEM 1011 (JEOL, Japan) at Division of Plant Pathology, ICAR-IARI, New Delhi. Multiple fields were observed and the measurement of the diameter and length of the phages were recorded.

3.2.7.2 Host range determination and lysis profile

The host ranges of the isolated phages were determined against 24 different isolates of *Salmonella enterica* belonging to different (Typhimurium= 9, Infantis= 5, Agona= 4, Virchow=

3, Kentucky= 3) and 16 isolates of *Escherichia Coli* using spot test described by Shang *et al* (2021) with some modifications. About 10 µl of undiluted phage stock solution (~10^9 pfu/ml) was spotted on lawn of each bacterial isolate. The plates were incubated overnight (12-18 hours) at 37°C and observed for plaque formation. Based on the clarity of spot ie. lysis zone, bacteria is assigned into two categories : presence of clear plaques as (+) indicating the ability of phages to infect and no lysis zone as (-) indicating inability of phages to infect the bacteria isolates.

3.2.7.3 Adsorption assay

Adsorption assay was done to determine the rate of adsorption of phage on the surface of host bacteria as described by Manohar *et al*. (2018). Aseptically, 750 µl of 10^{-1} phage lysate (~10^9 pfu/ml) and equal volume of exponentially grown overnight culture were mixed and incubated at room temperature (28°C). At every 5 minutes interval, 100 µl of the mixture were removed and diluted in pre cooled 4.4 ml of LB broth and 0.5 ml chloroform was incubated for another 15 minutes at room temperature (28°C). The procedure was repeated till 40 min. After incubation, 100 µl of the mixture was removed and diluted to 10^{-2} and the rate of adsorption was determined by double agar overlay method using 100 µl of diluted phage mixture with 100 µl of overnight grown bacterial culture. The experiment was repeated thrice and the adsorption curves were constructed from the plaque count reading (ratio of non-adsorbed phages) at different time interval and the duration at which plaque count showed least before the burst release was recorded as phage adsorption time. Adsorption rate constant was calculated from the formula given by Adams and Hancock, 1959.

$$Adsorption\ rate\ (K)\ =\ 2.3/\ (B)t\ X\ log\ (po/p)$$

Where,

B= conc. Of bacteria in cells per ml

p_o = phage at time 0(zero)

p = phage at time t (in minutes)

K= velocity constant (ml/ min)

3.2.7.4 One step growth experiment

One step growth experiment was done to determine the latent period and burst size of the bacteriophage as described by Manohar *et al*. (2018). 900 µl of the 10^{-5} dilution phage was mixed with 100 µl of overnight grown bacterial culture at an MOI 0.01. The mixture was diluted with 9 ml of LB broth and the mixture was incubated at room temperature for the phage to adsorb on the surface of the bacteria. For phage vB_StyS-PF79, incubation was done for 10 minutes and for phage vB_StyA-RH55, incubation was done for 30 minutes (adsorption time- differ from phage to phage). After incubation, the content was centrifuged at 10,000xg for 5 minutes at 4°C. The pellet was resuspended in 10 ml of LB broth and mixed well. The content was incubated at 37°C and aliquots of 100 µl were removed at every 5 minutes from initial (0 min) till 40 minutes and titrated against 100 µl of overnight grown host bacterium using double agar overlay method. The plaque counts for each durations were recorded and the experiment was conducted thrice. One step growth curve was constructed using mean ± standard deviation. Latent period was defined as the interval between phage adsorption to the bacterial host and the release of phage progeny. Burst sizes of the isolated phages were determined as ratio of final free phage to the initial free phage (Middelboe *et al*., 2010)

$$Burst\ size = final\ pfu/ml\ /\ initial\ pfu/ml$$

3.2.8 Sensitivity and stability of bacteriophage

3.2.8.1 Thermal stability

Thermal sensitivity of the phages was determined according to the method described by Yang *et al*. (2020) with slight modifications. The phages thermal stability was determined for the following temperatures, viz. -20°C, 9°C, 20°C, 37°C, 40°C, 50°C,60°C, 70°C and 80°C. The phage suspensions with titer 1×10^{8} pfu/ml was transferred to 1.5 ml microcentrifuge tubes and placed at the required temperature. The tubes were incubated at the test temperatures for one hour before being brought to room temperature for titration against the host bacteria. The titer of the phage was determined by double agar method in triplicates and the results were expressed as mean ± standard deviation.

3.2.8.2 pH stability

To determine the phage pH sensitivity, the phages were exposed to gradient of pH ranging from 3-10. Firstly, the required pH ranges (3-10) were derived from balancing 0.1M Acetic acid and 0.1 M Sodium Acetate following Anand and Bera (2019). In 1.5ml microcentrifuge tube, 300 µl of the phage with titer 1×10^8 pfu/ml was transferred and equal volume (300 µl) of pH adjusted buffer was added then the content was incubated for 1 hour at room temperature (28°C). After incubation, 100 µl of the pH treated suspension was titrated against 100 µl of the host bacteria by double agar method in triplicates. The results were expressed as mean ± standard deviation and the phage titer at different pH was compared with control sample that was suspended in TM buffer.

3.2.8.3 Chemical stability

To determine chemical stability of the phages, they were exposed to different chemicals such as chloroform, PAA (peracetic acid) and lactic acid. In 1.5 ml microcentrifuge tube, 400 µl of phage with titer 1×10^9 pfu/ml was transferred and equal volume of 10% Chloroform, 3% lactic acid and 0.15% were added separately and incubated for 60 minutes at room temperature (28°C) along with control which was suspended in TM buffer. After 60 minutes of incubation, 100 µl of the chemical treated suspension was titrated against 100 µl of host bacteria by double agar method in triplicates and the titer of the phages were expressed as mean ± standard deviation in pfu/ml were compared with control.

3.2.9 Molecular characterization of the bacteriophage

3.2.9.1 Bacteriophage nucleic acid isolation

Nucleic acid of the bacteriophages was isolated from broth harvested high titer phage lysate by DNeasy blood and tissue kit (Qiagen) following the manufacturer directions.

Prior to nucleic acid extraction, 15µl DNase1 and 15µl RNase A were added to the phage preparations to prevent contamination by the host genomic DNA. The enzyme activity was stopped by treatment with 0.5M EDTA followed by heat treatment at 70°C for 15-20 min.

1. Briefly, 200µl of highly concentrated phage harvested from broth method was transferred to 1.5 ml microcentrifuged tube and 20 µl of proteinase K was added.
2. To the suspension, 200 µl of buffer AL was added and vortexed to mix thoroughly then incubated at 56°C for 10 minutes at room temperature (15-25°C).
3. After incubation, 200 µl of ethanol (ninety-one hundred percent) is added and mixed thoroughly by vortexing.
4. The mixture was transferred into DNeasy Mini spin column and placed in 2 ml collection tube then, centrifuged at 8000 rpm for 1 minute at room temperature (15-25°C).
5. The flow-through collected in collection tube was discarded along with the collection tube.
6. The spin column was placed in a new 2 ml collection tube and 500 µl of AW1 buffer was added and subjected to centrifugation for 8000 rpm for 1 minute at room temperature (15-25°C).
7. The flow-through and collection tube was discarded.
8. The DNeasy Mini spin column was placed in a new 2 ml collection tube and 500 µl of AW2 buffer was added. The content was centrifuged at 14,000 rpm for 3 minutes at room temperature (15-25°C) and the flow-through and the collection tube was discarded.
9. The DNeasy Mini spin column was transferred to new sterile 2 ml centrifuged tube and 35 µl of AE buffer was added for DNA elution into the center of the spin column membrane.
10. Then, the content was subjected to incubation at room temperature (15-25°C) for 1 minute followed by centrifugation at 8000 rpm for 1 minute for DNA elution.
11. The elution step was repeated by adding fresh 20 µl of AE buffer followed by incubation and centrifugation to increase the yield of DNA.

The concentration and purity of the nucleic acid was checked using UV nano-drop spectrophotometer at OD_{260} and $OD_{280.}$

Purity of DNA was indicated by the value;

$$OD_{260} / OD_{280} = 1.6 - 1.8$$

3.2.9.2 Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism was used for molecular characterization of the isolated phage using the enzymes namely, *Eco*R1 and *Bam*H1. The reaction was prepared as mentioned in Table 7 and the mixture was incubated at 37°C for 60 minutes. After incubation, the enzyme treated samples were subjected to 0.7% agarose gel electrophoresis. The enzyme treated samples as well as untreated phage DNA were mixed with 6X loading dye and ran at 100V for 30 minutes. The gel was documented and the cut fragments were recorded.

3.2.9.3 Polymerase chain reaction

The isolated phages genomic DNA were subjected to PCR for major capsid gene *g23* and *capE*. The reaction mixture and reaction condition is given in table 6 and 7 respectively

Table 6: Reaction mixture of PCR for gene *g23* and *capE*

Component	Volume (µl) (g23)	Volume (µl) (capE)
Master mix (2x)	10	5.25
Forward primer	1	0.75
Reverse primer	1	0.75
Template DNA	4	7
Nuclease free water	9	11.5
Total	25	25

Table 7: Reaction conditions of PCR for gene *g23* and *capE*

Reaction	g23 PCR conditions		capE PCR conditions	
Initial denaturation	94°C, 90 sec		94°C, 3min	
Template denaturation	94°C, 45 sec	30cycles	94°C, 1 min	35cycles
Primer annealing	50°C, 1 min		60°C, 30sec	
Primer extension	72°C, 45 sec		72°C, 30 sec	
Final extension	72°C, 5min		72°C, 5min	

3.2.10 Multiplicity of infection (MOI) determination of the bacteriophages

The optimal multiplicity of infection (MOI) was determined according to the protocol described by Yang *et al*. (2020). Various MOIs ranging from 0.0001 to 100 were created by serially diluting ~10^{9} pfu/ml of bacteriophages and overnight grown host bacterial of concentration ~10^{6} cfu/ml. In microcentrifuge tubes, 100 µl of serially diluted overnight grown host bacteria were mixed with equal volume of serially diluted bacteriophages (MOI-0.0001, 0.001, 0.01, 0.1, 1, 10, 100) were added to 500 µl of LB broth. The mixtures were incubated at 37°C for 10 hours then centrifuged at 8000xg for 5 minutes at room temperature (28°C) to pellet out the bacterial cells. The supernatants were filtered using 0.45µm syringe filter and the filtrates were titrated against the host bacteria. The titer which first showed clearance/ confluent lysis was taken as optimal MOI.

3.2.11 Evaluation of the isolated *Salmonella* phages as biocontrol agent on artificially spiked chicken meat with *Salmonella* serovars

The effect of isolated *Salmonella* bacteriophages, vB_StyS-PF79 and vB_StyA-RH55 as biocontrol agent against *Salmonella enterica* serovar Typhimurium strain 22NSC004 on artificially spiked chicken meat was conducted following the protocol described by Aguilera *et al*. (2022). The two bacteriophages vB_StyS-PF79 and vB_StyA-RH55 were evaluated individually as well as in combination as phage cocktail as biocontrol agents on artificially spiked chicken meat. The effects of the two isolated phages were also evaluated in comparison with the commercially available chemical meat disinfectant.

3.2.11.1 MOI of inoculum

The optimal MOIs (ratios of phage to bacteria) earlier derived following the studies conducted by agar overlay method described in Yang *et al*. (2020) was implemented in this meat experiment.

3.2.11.1 Preparation of inoculum (Sample preparation and biocontrol assay in chicken meat)

1. Chicken breast was purchased from local meat shop in Bareilly and was aseptically cut into 5grams each using sterile knife and weighing balance.

2. The chicken pieces were exposed to UV light for about 15 minutes for each side in safety cabinet.
3. Five groups were considered for each experiment, group 1 as bacterial control (22NSC004), group 2 as vB_StyS-PF79 treatment, group 3 as vB_StyA-RH55 treatment, group 4 as phage cocktail (vB_StyS-PF79 and vB_StyA-RH55), group 5 as commercial chemical disinfectant (Chemtex) and 3 meat pieces were allotted to each group. Three random pieces were also included to screen for the presence of *Salmonella* prior to artificial spiking or contamination.
4. To each meat sample, 1 ml of the inoculum of bacteria (10^6 CFU/ml) were inoculated, 500 µl on each side of the meat. The pieces were left undisturbed for 30 minutes at room temperature, 15 minutes for each side.
5. Then the meat samples were immersed in 100 ml of distilled water containing phages (MOI=1) in group 2,3 and 4. The control samples were immersed in distilled water without any phages and in group 5, 100ml of distilled water containing 1ml of chemical meat disinfectant was added. The meat samples were treated for 5 minutes and were kept in sterile zip locked pouches. The samples were stored at 4°C for 0 day, 24 hours and 48 hours
6. Then the meat samples were taken out from the refrigerator at 0, 1 and 2 day and homogenized with 30 ml of sterile PBS in a meat homogenizer.
7. The homogenized samples (1ml) were then subjected to centrifugation at 10,000 xg for 3 minutes at 4°C.
8. The pellets were re-suspended in 1 ml of sterile PBS.
9. 100µl of aliquot was plated on HE plates using L-shaped spreader and incubated overnight at 37°C.
10. The target bacteria were then counted (cfu/g) and recorded.
11. The experiments were conducted in triplicates.

3.2.12 Statistical analysis

The data obtained from the experiments were analyzed and calculated as mean ± standard deviation using GraphPad Prism software version 9.3 by employing one-way and two-way ANOVA and Tukey's multiple range tests, and a P value of d" 0.05 were considered significant.

✍✍✍

Results

4 RESULTS

4.1 Revival and characterization of bacterial strains

When streaked on Hektoen enteric agar (HE), all the *Salmonella enterica* isolates formed smooth, transparent colonies with greenish periphery and black spot at the center (Fig. 1&2) When stabbed on Triple sugar iron (TSI) they showed red slant, yellow butt, with H_2S production indicated by blackening spot and gas production. In Simon citrate slant they developed blue color from green color slant. They also showed urease test negative, indicated by no change in color (Fig. 3)

4.2 Molecular characterization

Salmonella enterica serovar Typhimurium strain 22NSC004 and strain ATCC13311 were used as the targeted host against which bacteriophages were isolated. Genomic DNA isolation of the two isolates were done and PCR targeting *invA* gene was performed which yielded 244 bp gene product indicative of the genus *Salmonella* (Fig. 4)

4.3 Isolation, purification, propagation and titration of lytic bacteriophages

4.3.1 Isolation of lytic bacteriophages

In this study, bacteriophage against strain 22NSC004 was isolated from poultry farm waste water sample number 79 collected from poultry farm, Bareilly (PF79) and phage against ATCC13311 strain was isolated from sample number 55 collected from drainage of human hospital, Rohilkhand Medical Hospital, Bareilly (RH55). The enriched phage samples exhibited clear, transparent zone when spotted against the host strains indicating their lytic activity (Fig. 5).

4.3.2 Morphology of the plaques

Bacteriophage isolated against *Salmonella* Typhimurium strain 22NSC004 plaques formed clearly defined round plaques of about 1-2 mm in diameter (Fig. 6) whereas bacteriophage isolated against strain ATCC13311 formed plaques of clear and well-defined edges but with smaller diameter of about 0.1-1 mm (Fig. 7)

4.3.3 Purification, propagation and titration of phages

In bulk purification by PEG-NaCl method as well as plate method, the phages showed clear lysis of bacterial culture when incubated overnight ie. 22-16 hr (Fig. 8) and yielded concentration of $3.6x10^{12}$ pfu/ml and $2.1x10^{9}$ pfu/ml of phages PF79 and RH55 which was isolated against 22NSC004 and ATCC 13311, respectively.

4.4 Nomenclature of bacteriophage

Nomenclature of the two isolated bacteriophages was done in accordance to the rule of ICTV for naming bacteriophage following Adriaenssens and Brister (2017) after morphological characterization under transmission electron microscopy (TEM). The phage against 22NSC004 was named vB_StyS-PF79 indicating **v**irus of **B**acteria, infecting ***Salmonella*** *enterica* **Ty**phimurium, with ***S****iphovirus* morphology, isolated from Poultry Farm sample number 79 (**PF79**). The phage against ATCC13311 was named vB_StyA-RH55 denoting **v**irus of **B**acteria, infecting ***Salmonella*** *enterica* **Ty**phimurium, with **A**utographivirus virus morphology, isolated from **R**ohilkhand **H**ospital sample number **55** (**RH55**).

4.5 Characterization of the isolated bacteriophages

4.5.1 Morphological characterization

Transmission electron microscopy revealed that both phages belonged to the order *Caudovirales*. The phage PF79 against *S.*Typhimurium 22NSC004 was found to belong to family *Siphoviridae* with icosahedral head having diameter of 32.68nm and a long non-contractile tail of length 109.06nm (Fig. 9). The phage RH55 which was isolated against ATCC13311 strain belonged to *Autoghaphiviridae* family which revealed icosahedral head having diameter of 59.96nm and a short contractile tail of 22.54nm in length (Fig. 10)

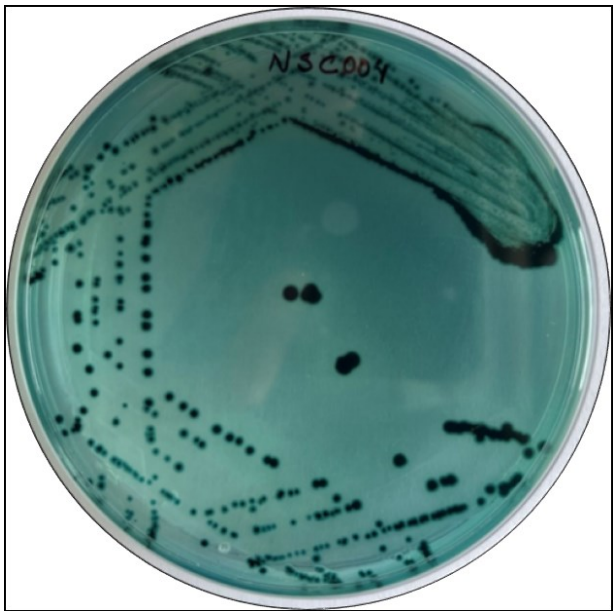


Fig. 1: Morphology of *Salmonella* Typhimurium 22NSC004 on Hektoen enteric agar

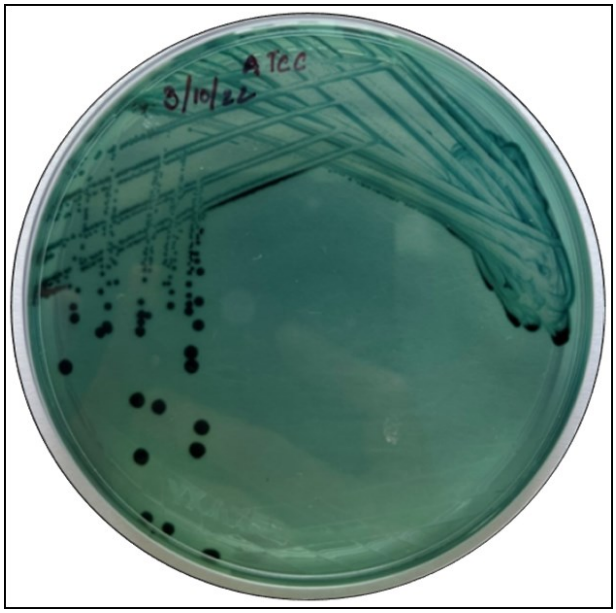
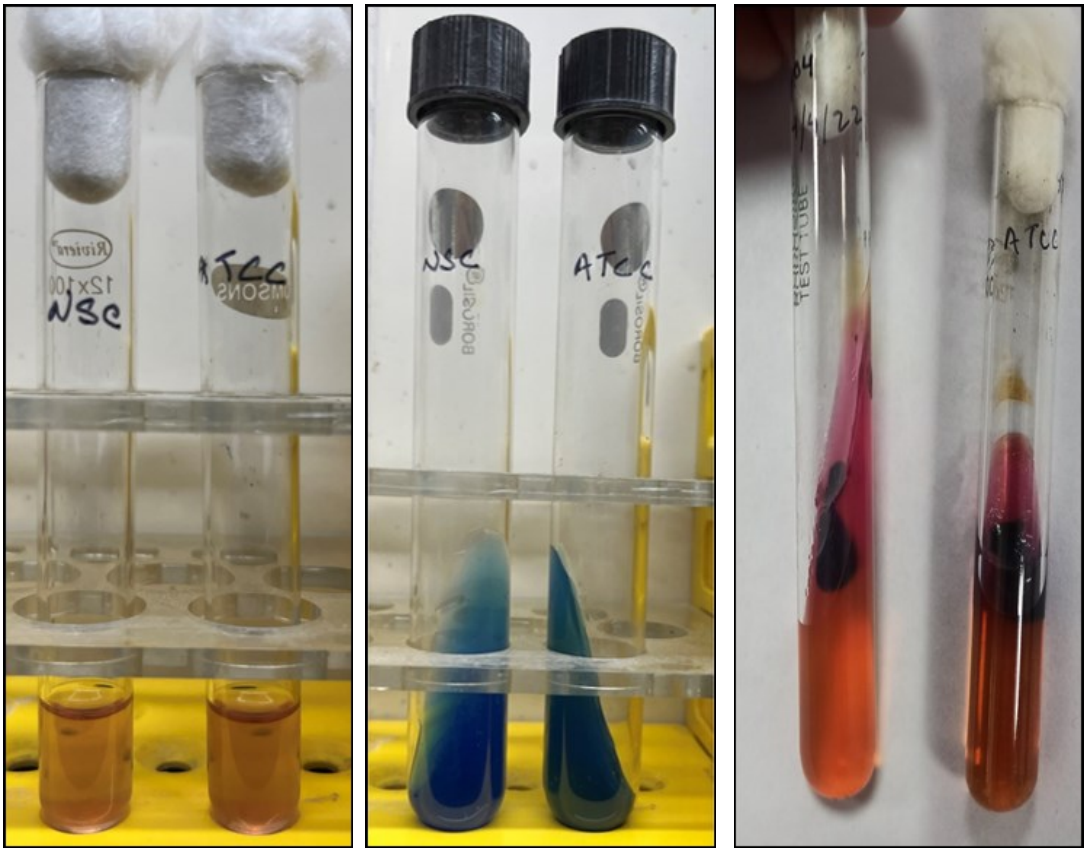


Fig. 2: Morphology of *Salmonella* Typhimurium ATCC13311 on Hektoen enteric agar



(A) **(B)** **(C)**

Fig. 3: Biochemical test of *Salmonella* Typhimurium 22NSC004 and ATCC13311

(A) : Urease Test

(B) : Citrate Test

(C) : Triple sugar iron Test

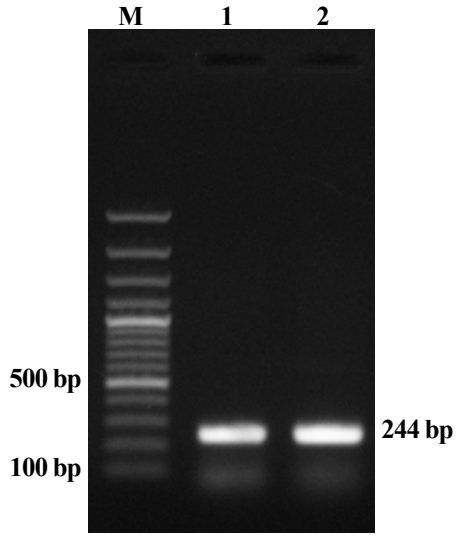
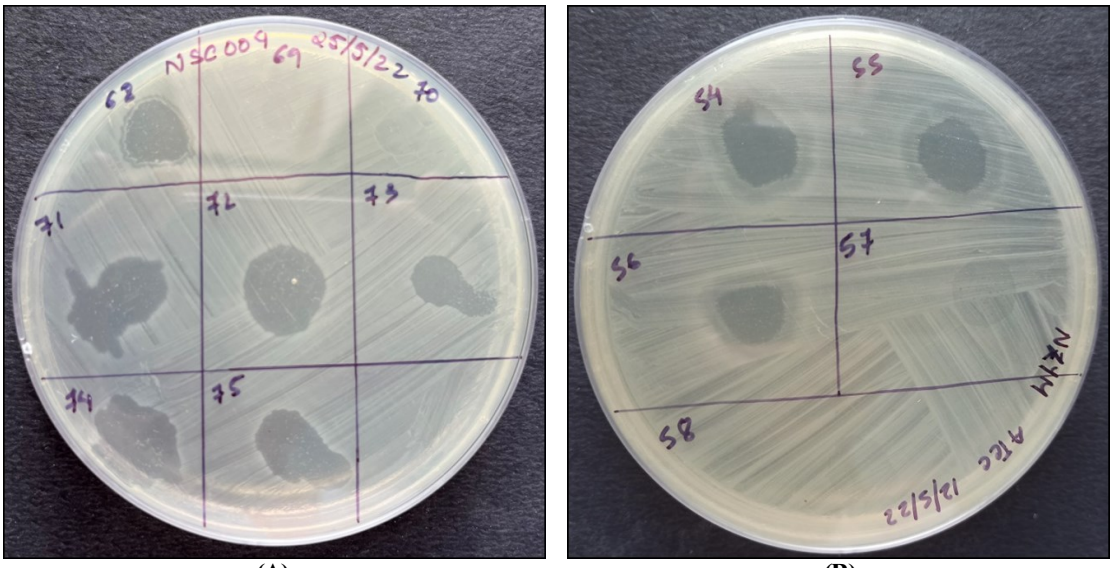


Fig. 4: PCR amplification of *invA*

Lane M : 100bp DNA ladder
Lane 1 : 22NSC004 (*Salmonella* Typhimurium)
Lane 2 : ATCC13311 (*Salmonella* Typhimurium



(A) **(B)**

Fig. 5: Isolation of lytic bacteriophages of *S*. Typhimurium by spot test on bacterial lawn

A : Samples BFF on a lawn of 22NSC004
B : Samples BFF on a lawn of ATCC13311

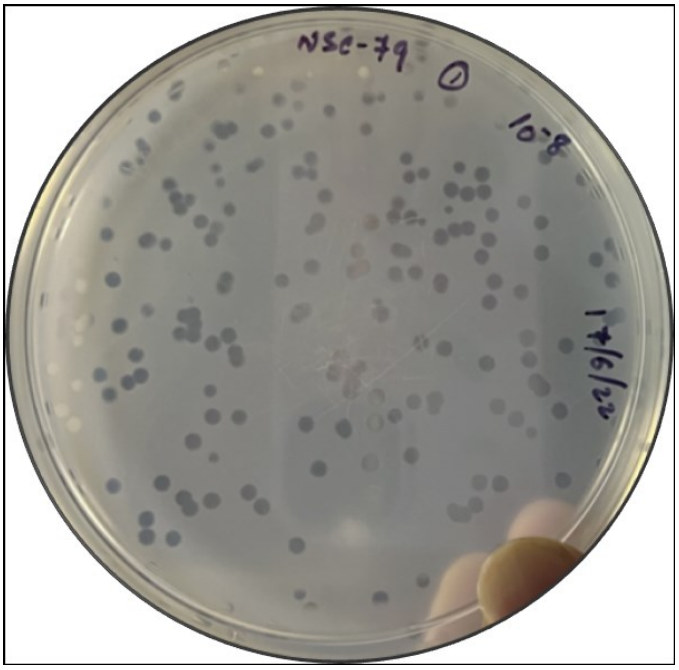


Fig. 6: Plaque morphology of ϕvB_StyS_PF79

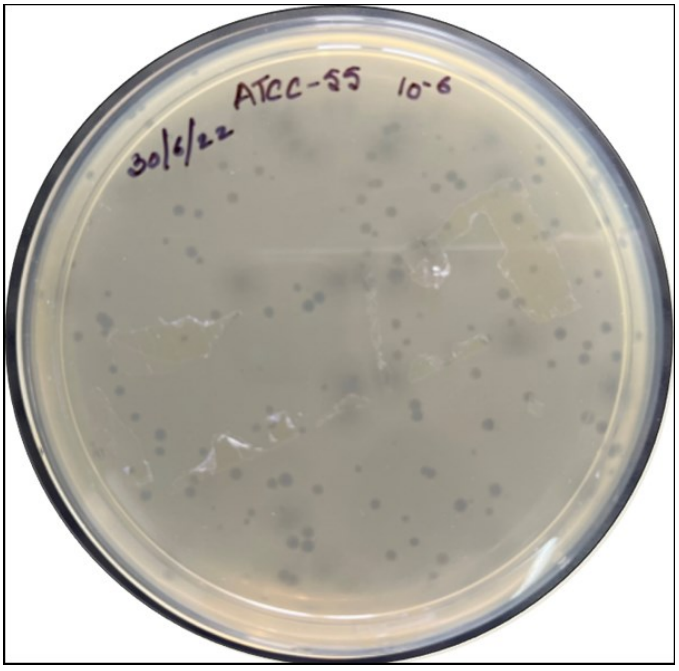


Fig. 7: Plaque morphology of ϕvB_StyA-RH55

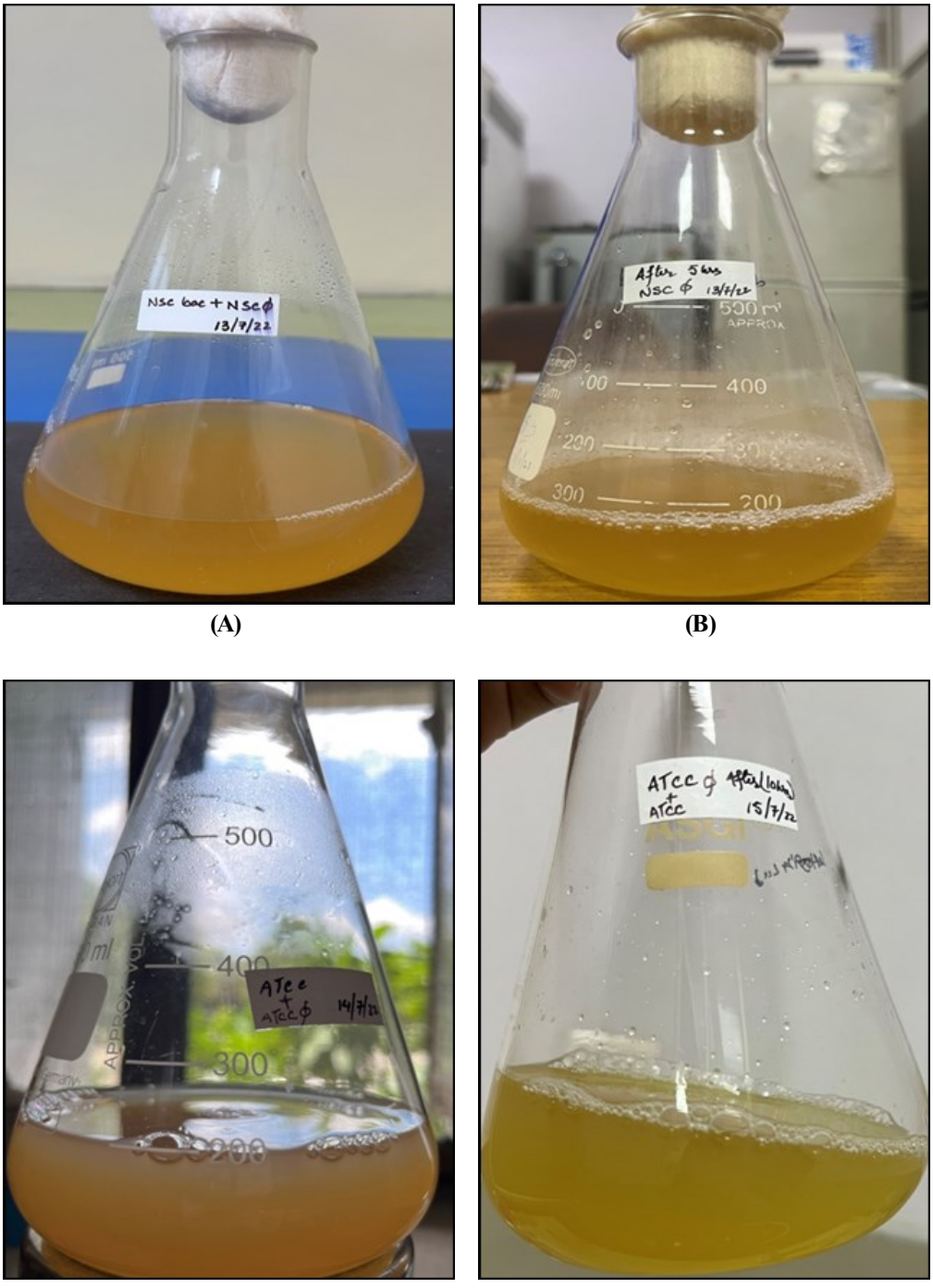


Fig. 8: Bulk purification of bacteriophages.

A: 22NSC004 culture with ϕvB_StyS_PF79, **B**: ϕvB_StyS_PF79 lysed 22NSC004 culture

C: ATCC13311 culture with ϕvB_StyA-RH55, **D**: ϕvB_StyA-RH55 lysed ATCC13311 culture

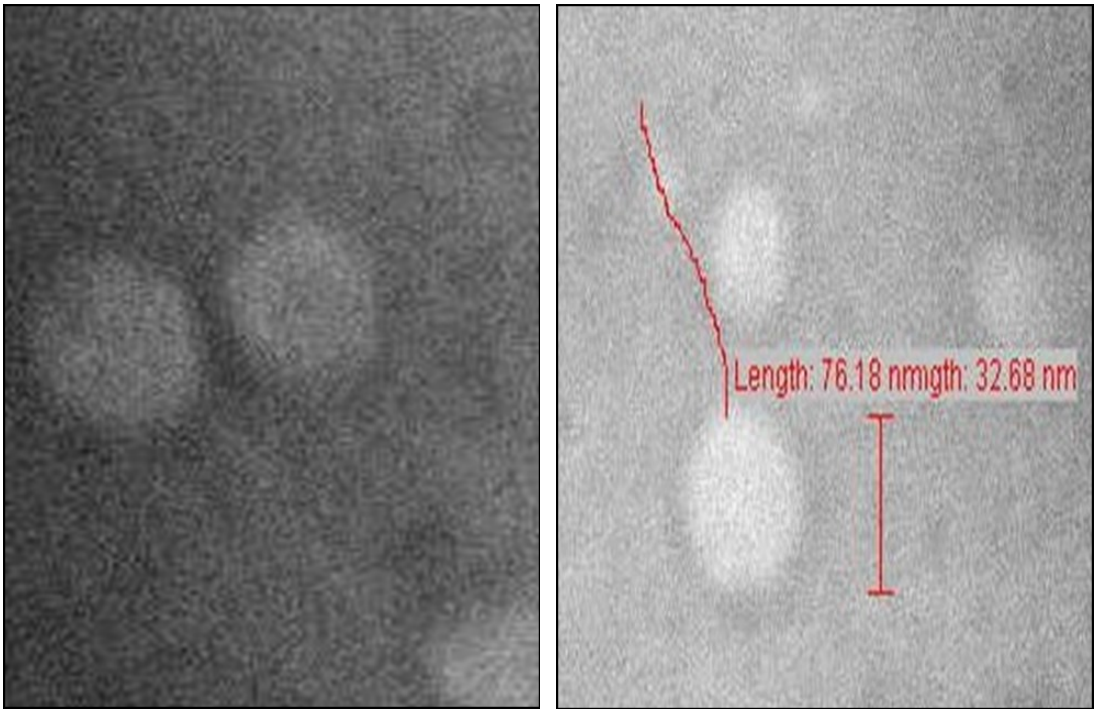


Fig. 9: Transmission electron micrograph of phage ϕvB_StyS_PF79

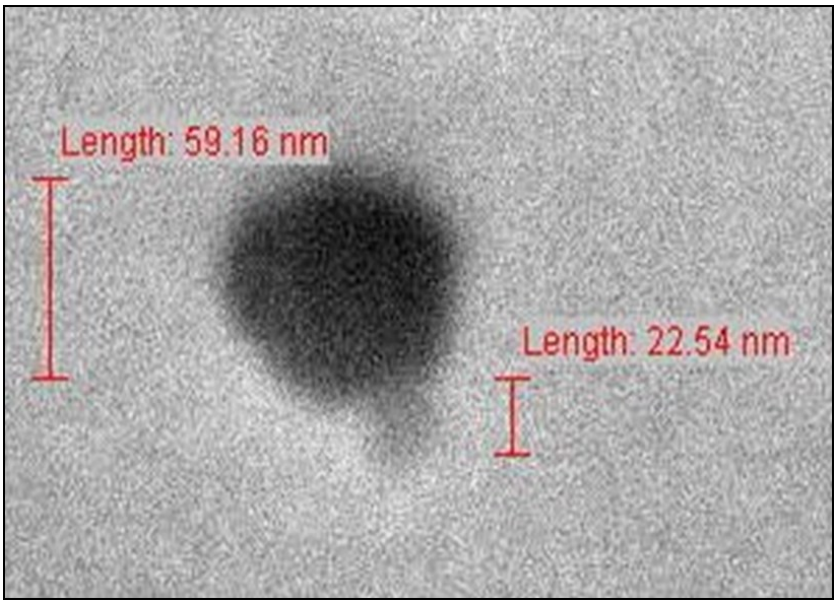


Fig. 10: Transmission electron micrograph of ϕvB_StyA-RH55

4.5.2 Biological characterization of isolated bacteriophages

4.5.2.1 Host range and lysis profile of the isolated bacteriophages

The phage vB_StyS-PF79 was found to lyse nine isolates belonging to *S.* Typhimurium and two isolates belonging to serovar *S.* Agona. Phage vB_StyA-RH55 could able to lyse 6 isolates belonging to different serovars, nine isolates of S. Typhimurium, three isolates of *S.* Virchow, four isolates of *S.* Agona, three isolates of *S.* Infantis and three isolates of *S.* Kentucky. However, neither of the isolated phages, vB_StyS-PF79 and vB_StyA-RH55 where able to lysed any isolates of *Escherichia coli.* The lytic spectrum of the phages is given in Table 8 and heat map representing the lytic spectrum is shown in fig.11.

Table 8: Host range lysis profile bacteriophages

Isolates	Vb_StyS-PF79	Vb_StyA-RH55
21UK04, Typhimurium	+	+
21UK05, Virchow	-	+
21UK06,Typhimurium	+	+
21UK07, Typhimurium	+	+
21UK08,Virchow	-	+
21UK09, Typhimurium	+	+
21UK10, Typhimurium	+	+
21UK11, Typhimurium	+	+
21UK13, Typhimurium	+	+
21UK14, Virchow	-	+
21JK01, Infantis	-	+
21JK02, Infantis	-	-
21JK03, Infantis	-	-
21JK04, Infantis	-	+
21JK05, Infantis	-	+
21JK07, Agona	+	+
21JK08, Agona	-	+
21JK09, Agona	+	+
21JK10, Agona	-	+
21JK11, Kentucky	-	+

21JK12, Kentucky	-	+
20AN60, Kentucky	-	+
NSC004, Typhimurium	+	+
ATCC13311, Typhimurium	+	+
E-46d, *E. coli*	-	-
E-48c, *E. coli*	-	-
E-61a, *E. coli*	-	-
E-65b, *E. coli*	-	-
E-68d, *E. coli*	-	-
E-154a, *E. coli*	-	-
E-155a, *E. coli*	-	-
E-156b, *E. coli*	-	-
E-157c, *E. coli*	-	-
E-158b, *E. coli*	-	-
E-159b, *E. coli*	-	-
E-160b, *E. coli*	-	-
E-162c, *E. coli*	-	-
E-163b, *E. coli*	-	-
E-164a, *E. coli*	-	-
E-165c, *E. coli*	-	-

+ lytic effect - no lytic effect

4.5.2.2 Adsorption assay

Adsorption time is the time period at which maximum amount of the phage adsorbed on the surface of the host strain before the burst. Adsorption curve revealed that the adsorption time taken by phage vB_StyS-PF79 where ~97% of the phage adsorbed on the host surface was within 10 minutes (Fig. 12 & Table 9) whereas a maximum of ~98% of vB_StyA-RH55 phage particles adsorb on surface of the host strain within 30 minutes (Fig. 13 & Table 9). The adsorption rate for phage vB_StyS-PF79 was $2.5x10^{-9}$ ml/min and for phage vB_StyA-RH55 was $1.4x10^{-8}$ ml/min.

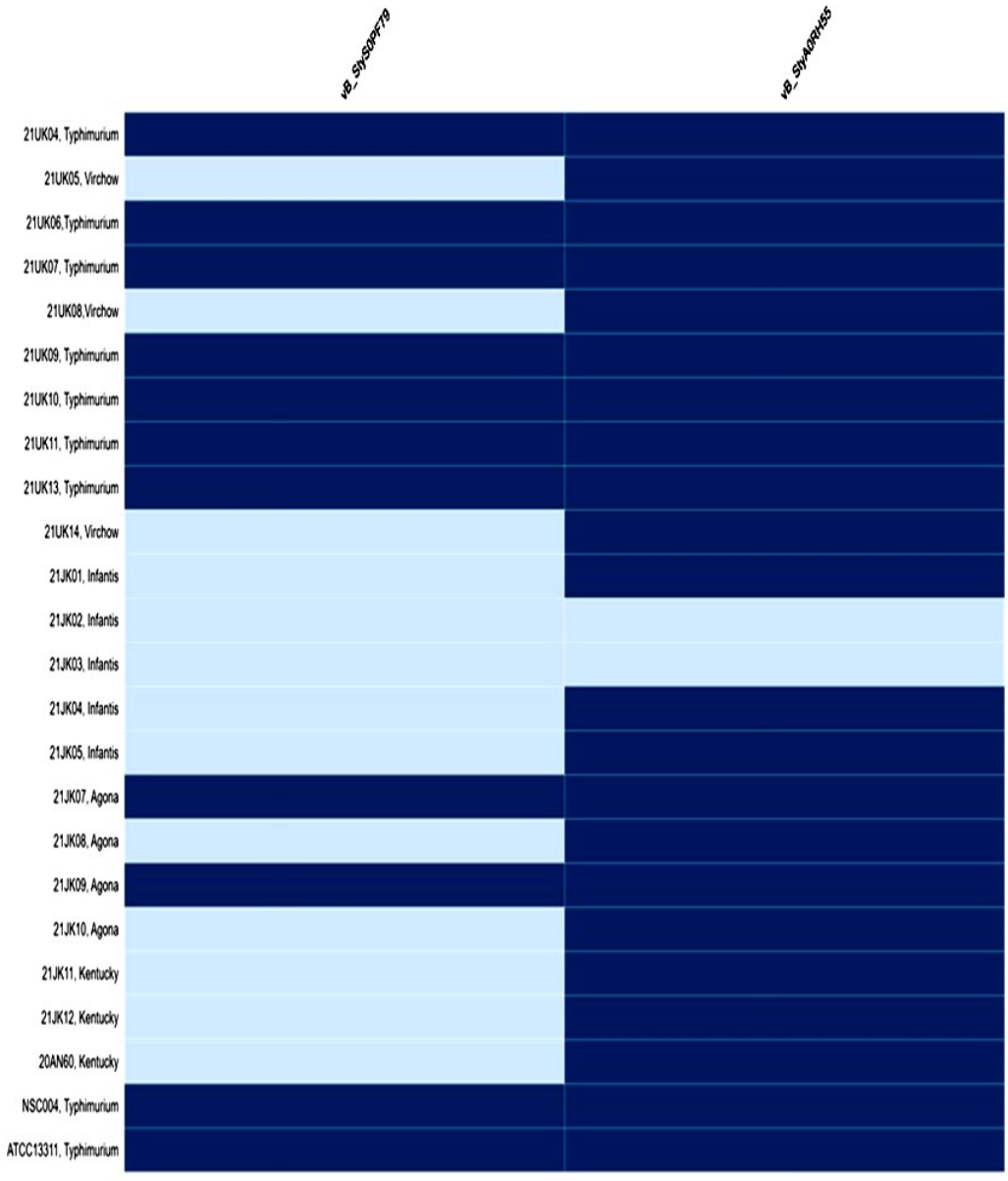


Fig. 11: Heat map representation of host range of *Salmonella* phages for *Salmonella* isolates. The left vertical axis lists the strain ID and top of the figure is labelled with the phages names. Lysis is represented as dark blue colour and no lysis is as light blue

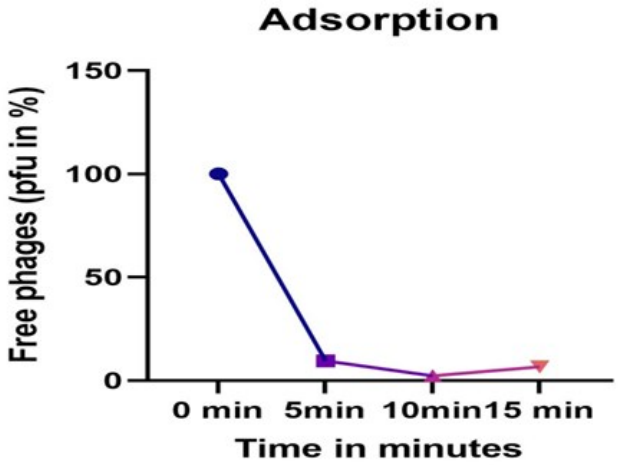


Fig. 12: Adsorption curve of ϕvB_StyS_PF79

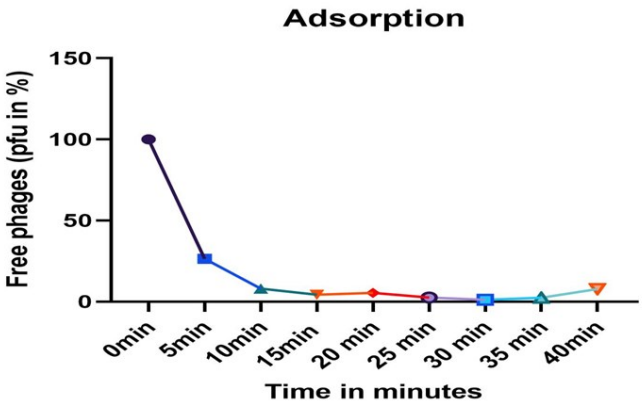


Fig. 13: Adsorption curve of ϕvB_StyS_RH55

Table 9: Adsorption assay of bacteriophages

Duration (min)	vB_StyS_PF79		vB_StyA-RH55	
	pfu/ml	% unabsorbed Φ	pfu/ml	% unabsorbed Φ
0	159.300±56.13	100	140.00±35.37	100
5	15.330±5.508	9.62	37.00±14.73	26.42
10	3.667±1.528	2.30	11.33±8.021	8.09
15	6.667±1.528	4.18	6.000±1.000	4.28
20	40.330±11.24	25.31	7.667±3.055	5.47
25	216.300±37.63	135.78	3.667±2.082	2.60
30	274.000±6.928	172.00	1.667±1.155	1.18
35	451.700±101.5	283.55	3.333±2.309	2.38
40	515.700±70.12	323.72	11.00±4.000	7.85

4.5.2.3 One-step growth curve

One-step growth curve was used to derive the latent period and burst size of the phages. Latent period is defined as the time interval between the adsorption and the beginning of the first burst of the phage. One-step growth curve revealed that the latent period for phage vB_StyS-PF79 was ~ 20 minutes (Fig. 14) and for phage vB_StyA-RH55 was found to ~10 minutes (Fig. 15).

Table 10: One-step growth kinetic of bacteriophages

Duration (min)	Plaque count (pfu/ml)	
	vB_StyS_PF79	vB_StyA-RH55
0	1.000± 0.000	3.667 ± 2.887
5	3.667±3.055	4.667±1.155
10	26.67±7.572	3.000±1.000
15	46.67±6.028	50.00±73.63
20	49.00±5.292	96.00±67.01
25	143.0±78.24	113.3±8.505
30	336.0±20.42	225.3±68.07
35	482.7±10.21	225.3±39.30
45	505.3±12.86	263.7±12.66
50	540.0±1833	269.3±56.58

The burst size for phage vB_StyS-PF79 from this assay was determined to be ~ 540 plaque forming units (pfu/cells) whereas for phage vB_StyA-RH55, the burst size was found to be ~ 75 pfu/cells under the laboratory growth conditions (Table. 10).

4.5.3 Physiochemical characterization of phage (Sensitivity and stability of the phage)

4.5.3.1 Thermal stability

The thermal stability studies of the two bacteriophages revealed different characteristics. vB_StyS-PF79 was able to maintain their activity and multiply at temperature ranges from -20°C to 60°C with no significant decrease in concentration. While slight increase in significant (p 0.040413) was shown between 20°C and 40°C from 154.0 ± 4.583X10^7 to 213.3± 10.79X10^7. However, when the phages were exposed to extreme temperature of 70°C the stability of the bacteriophages significantly reduced ($p \leq 0.000001$) to 31.67±1.155 X10^7.When the phages were incubated at 80°C complete inactivation was observed (Table 11 & Fig. 16).

As for phage vB_StyA-RH55, statistically there was no significant difference ($p \leq 0.4888$) in concentration of the phage when exposed to temperature ranging from -20°C to 60°C. The concentration of the phage was maintained at 158.7-190.3 X10^7. But when exposed to extreme temperature of 70°C and 80°C, no phage survival was observed (Table 11 & Fig. 17).

Table 11: Stability of bacteriophages at different temperature

Temperature	Plaque count (pfu/ml)	
	vB_StyS_PF79	vB_StyA-RH55
-20°C	198.7±42.16	158.7±11.85
4°C	190.7±11.93	174.7±29.96
20°C	154.0±4.58	175.7±20.01
37°C	163.3±32.52	174.3±15.5
40°C	213.3±10.79	173.3±14.64
50°C	199.7±3.78	190.3±17.1
60°C	183.0±13.23	163.0±8.88
70°C	31.67±1.15	0
80°C	0	0

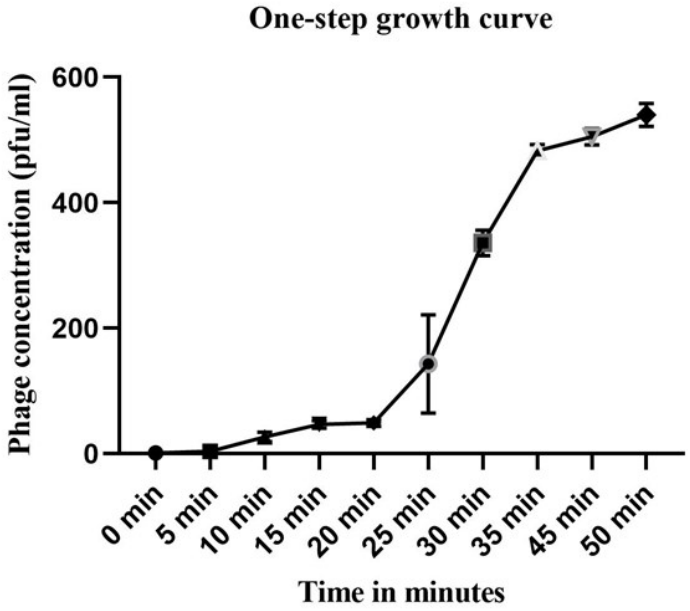


Fig. 14: One-step growth curve of ϕvB_StyS_PF79

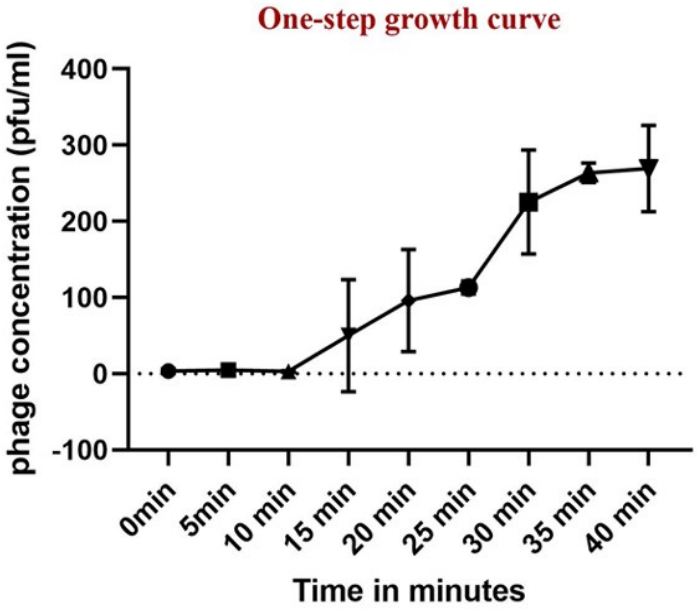


Fig. 15: One-step growth curve of ϕvB_StyA-RH55

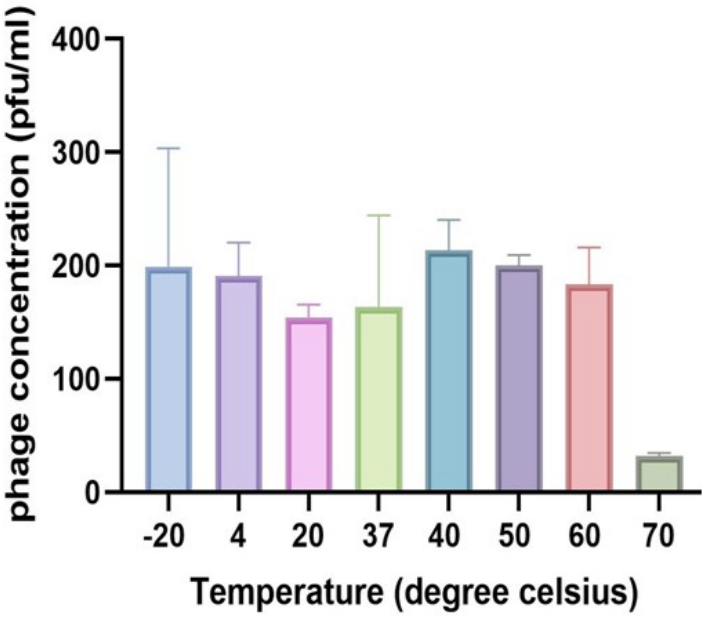


Fig. 16: Temperature stability of ϕvB_StyS_PF79

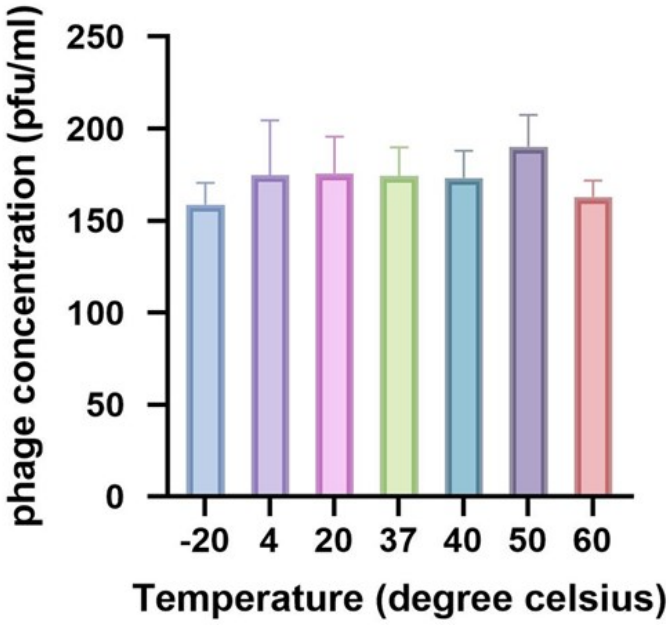


Fig. 17: Temperature stability of ϕvB_StyA-RH55

4.5.3.2 pH stability

Phage vB_StyS-PF79 revealed that there was no significant difference (p 0.2487) when exposed to different pH ranging from pH 3-10. At pH 5, 6 and 7 phage concentration were approximately similar to control (105.3±3.055, 109.3±3.786, 117.3±12.10X10^7) indicating their optimal activity to survive and multiply at this pH (Table 12 & Fig. 18)

Phage vB_StyA-RH55 has shown gradual increase in concentration from pH 3-7(101.3±23.44, 129.7±47.06, 156.3±18.50, 184.7±11.93, 190±7.937X10^7). There was significant reduction ($p \leq 0.0001, 0.001$) in concentration in comparison with control at pH 3-6. From pH 7-10, the titer remained almost constant (190.0-192.7X10^7). The highest concentration was seen in pH 10 ie.201.0±25.12 X10^7 with significance ($p \leq 0.0132$) (Table 12 & Fig. 19)

Table 12: Stability of phages at different pH

pH	Plaque count (pfu/ml)	
	vB_StyS_PF79	vB_StyA-RH55
Control	109.3±3.06	263.00±8.00
3	97.67±14.29	101.3±23.44
4	95.00±11.79	129.7±47.06
5	105.3±3.06	156.3±18.50
6	109.3±3.79	184.7±11.93
7	117.3±12.1	190.0±7.94
8	99.0±9.54	191.3±5.13
9	102.0±13.23	192.7±4.51
10	96.67±15.57	201.0±25.12

4.5.3.3 Chemical stability

Chemical stability study of the bacteriophages revealed that vB_StyS-PF79 decreases their activity when exposed to 2% lactic acid and 0.1% peracetic acid but no significant differences with 10% chloroform. There was significant decrease in phage concentration ($p \leq 0.0001$) when exposed to 2% lactic acid and with 0.1% peracetic acid exposure the concentration significantly decreased to 256.0±15.00 X10^7 ($p \leq 0.0001$). However, with 10%

chloroform no significant changes in phage concentration when compared to the control (Table 13 & Fig. 20).

Phage vB_StyA-RH55 showed significant reduction in concentration with all the chemicals used in the study. 2% lactic acid showed the most sensitivity with decrease in concentration of $9.333 \pm 6.028 X 10^7$ ($p \leq 0.0001$), 0.1% of peracetic acid revealed the sensitivity with decrease concentration of 74.33.0±37.63 ($p \leq 0.0002$) and 10% chloroform decrease the concentration to 69.33 ± 10.97 with $p \leq 0.0002$ (Table 13 & Fig. 21).

Table 13: Stability of phages with chemicals

Bacteriophages	Plaque count (pfu/ml)			
	Control	2% Lactic acid	0.1% Peracetic acid	10% chloroform
vB_StyS_PF79	529.00 ± 50.23	247.00 ± 18.04	256.00 ± 15.00	451.00 ± 4.29
vB_StyA-RH55	217.00 ± 25.24	9.33 ± 6.03	74.33 ± 37.63	69.33 ± 10.97

4.5.4. Molecular characterization of the isolated phages

RFLP was performed to determine genetic fingerprinting of the phage DNA using two restriction endonucleases namely *Eco*R1 and *Bam*H1. Out of the two enzymes used, only *Eco*R1 was able to digest DNA of phage vB_StyS-PF79 into 8 Fragments (Fig. 22) whereas no enzyme was able to digest DNA of phage vB_StyA-RH55 suggesting the absence of restricting endonuclease recognition site.

Both the phages showed no amplification when subjected to PCR for gene *g23* and *capE* (Fig. 23).

4.5.6 Multiplicity of infection (MOI) determination of the bacteriophages

When both phage vB_StyS-PF79 and vB_StyA-RH55 were infected with MOI of 1 it showed the earliest clear lysis in 6 hours indicating it was the optimal MOI for performing meat experiment.

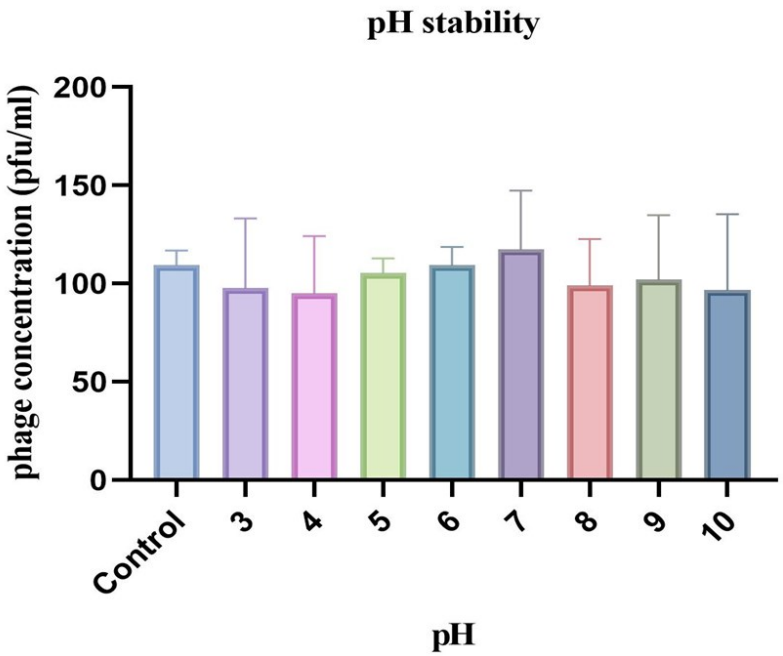


Fig. 18: pH stability of ϕvB_StyS_PF79

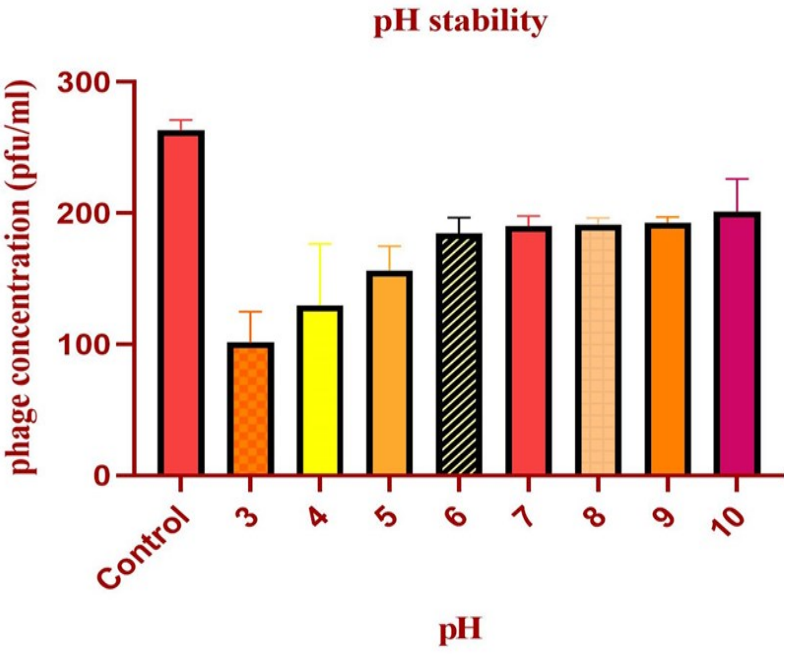


Fig. 19: pH stability of ϕvB_StyS_RH55

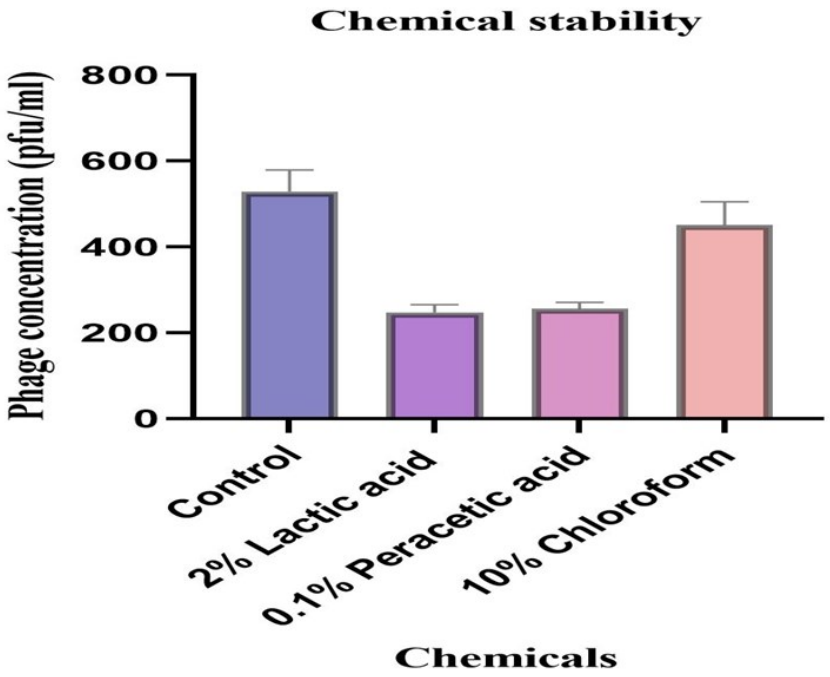


Fig. 20: Chemical stability of ϕvB_StyS_PF79

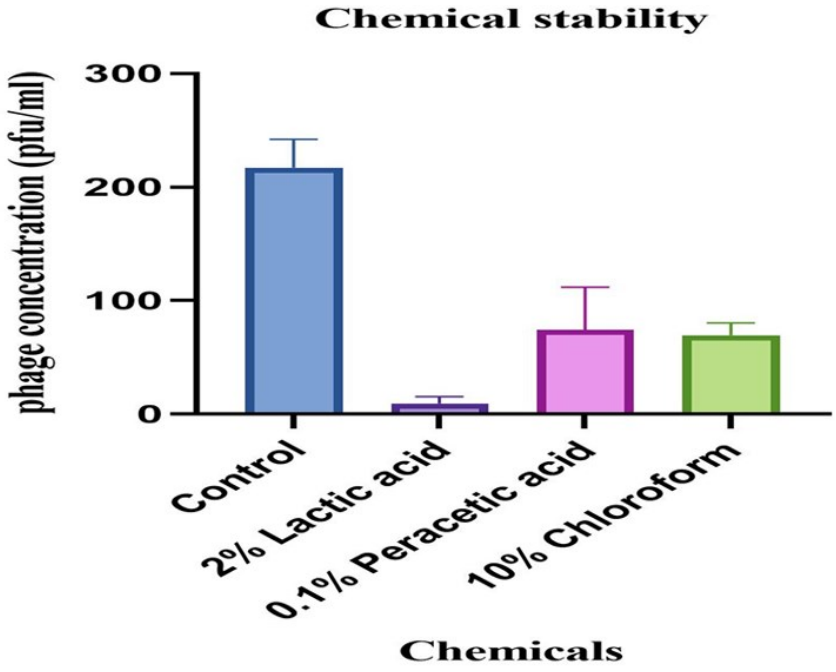


Fig. 21: Chemical stability of ϕvB_StyS_RH55

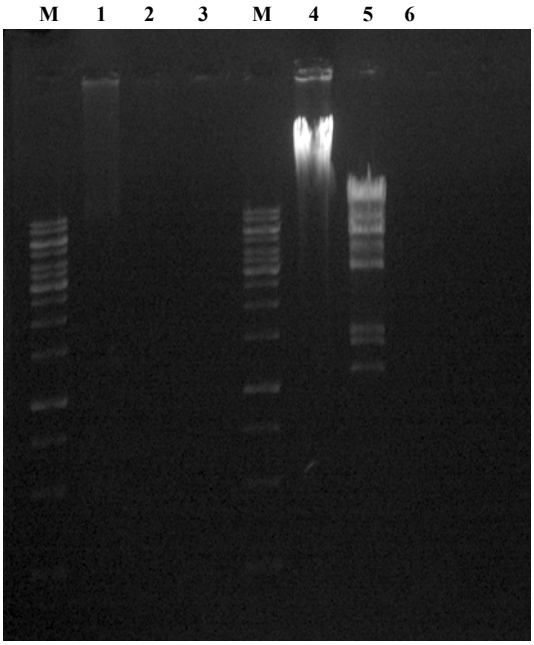


Fig. 22: RFLP profile of ϕvB_StyS_PF79 and ϕvB_StyA- RH55 DNA

Lane M : 1kb DNA Maker
Lane 1 : ϕvB_StyA-RH55 untreated DNA
Lane 2 : ϕvB_StyA-RH55 DNA with EcoR1 enzyme
Lane 3 : ϕvB_StyA-RH55 DNA with BamH1 enzyme
Lane 4 : ϕvB_StyS_PF79 untreated DNA
Lane 5 : ϕvB_StyS_PF79 DNA with EcoR1 enzym
Lane 6 : ϕvB_StyS_PF79 DNA with BamH1 enzyme

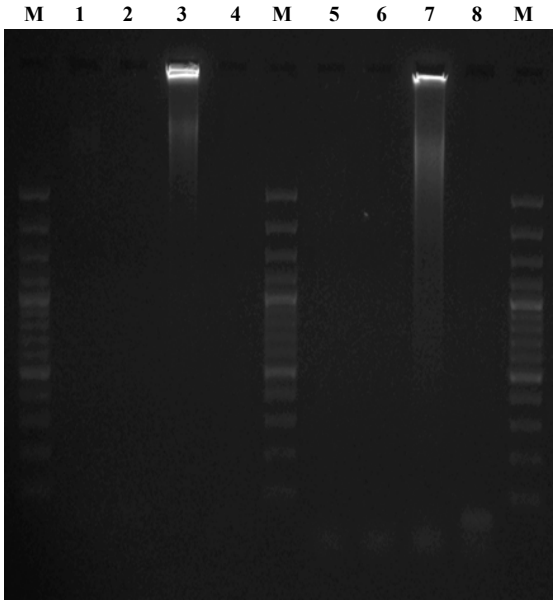


Fig. 23: PCR amplification of bacteriophage DNA for gene *g23* and *capE*

Lane M : 100bp DNA ladder,
Lane 1 : ϕvB_StyA RH55 (*g23*)
Lanes 2, 4, 6, 8 : non-template control
Lane 3 : ϕvB_StyS-PF79 (*g23*)
Lane 5 : ϕvB_StyA-RH55 (*capE*)
Lane 7 : ϕvB_StyS-PF79 (*capE*)

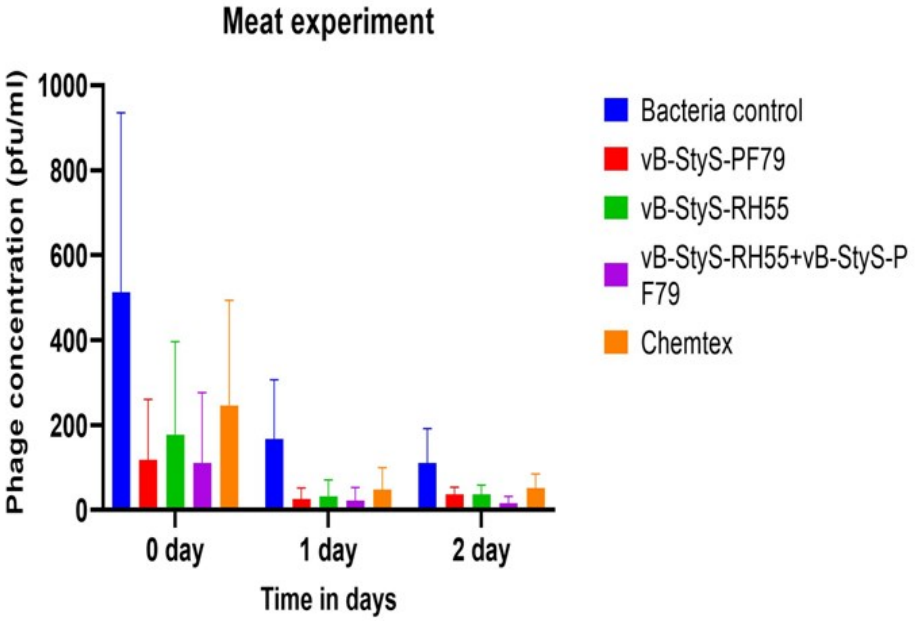


Fig. 24: Effect of bacteriophages and Chemtex (meat disinfectant) on chicken meat at 4°C

4.5.7 Effect of *Salmonella* bacteriophages on artificially spiked chicken meat with *Salmonella enterica* serovar Typhimurium

Chicken meat experiment was performed by employing *Salmonella* Typhimurium strain 22NSC004 as the host strain and phages vB_StyS-PF79 and vB_StyA-RH55 as biocontrol agents. The effect of the phages on the artificially spiked or contaminated meat were performed and evaluated at refrigerated temperature for a period of 0 day, 24 hours and 48 hours. The experiment was performed by inoculating a bacterial dose of $1x10^6$ cfu/ml and $1x10^6$ pfu/ml of phages for generating MOI of 1.

4.5.7.1 Effect of vB_StyS-PF79 phage on 22NSC004 artificially spike chicken meat at refrigerated temperature

On day-0, phage vB_StyS-PF79 showed no significant reduction in bacterial count as compared to bacterial control which was in the concentration $512\pm422.5x10^6$ cfu/g. However, after 24 hours i.e after 1 day, there was significant reduction ($p\leq 0.0485$) in bacterial count to 25.89±25.78x106 cfu/g (Table 14). After 2 days of incubation at 4°C, no significant reduction in bacterial count was observed as compared to control (Fig. 24).

Table 14: Effect of bacteriophages and Chemtex (meat disinfectant) on chicken meat at 4°C

Day	Plaque count (pfu/ml) Bacteriophages				
	Control	vB_StyS_PF79	vB_StyA-RH55	vB_StyS_PF79 +vB_StyA-RH55	Chemtex
0	512±422.5	117.7±142.9	177.0±219	110.7±165.7	246.7±247.4
1 (24 h)	167.3±139.6	25.89±25.78	31.89±39.18	21.67±31.23	48.33±51.68
2 (48 h)	110.9±27	37.11±16.82	36.56±22.08	15.78±16.41	51.67±33.13

4.5.7.2 Effect of phage vB_StyA-RH55 on 22NSC004 artificially spike chicken meat at refrigerated temperature (4°C)

The effect of phage vB_StyA-RH55 showed no significant reduction on day-0, 1 and 2 when compared with their respective control (Fig. 24).

4.5.7.3 Effect of phage vB_StyS-PF79 and vB_StyA-RH55 as cocktail on 22NSC004 artificially spike chicken meat at refrigerated temperature

On day-0 the phage cocktail does not show significant reduction statistically as compared to the bacterial control whereas after 24 and 48 hours of incubation at 4°C, significant reduction was observed in bacterial concentration viz. 21.67±31.23 x10^6 cfu/g ($p \leq 0.0481$) and 15.78±16.41 x10^6 cfu/g ($p \leq 0.0449$), respectively, in comparison to control (Table 12 & Fig. 24)

4.5.7.4 Effect of Chemtex (meat disinfectant) on 22NSC004 artificially spike chicken meat at refrigerated temperature

When Chemtex was used as a chemical disinfectant, no significant reduction was observed on day-0 and after 48 hours whereas significant reduction was observed after 24 hours of incubation at 4°C with bacterial concentration 48.33±51.68 x10^6 cfu/g (p 0.0427) in comparison to the control (Table 12 & Fig. 24)

Discussion

5 DISCUSSION

Salmonellosis is one of the most common zoonotic foodborne diseases reported worldwide (Rivera *et al*., 2022). Salmonellosis is caused by the genus *Salmonella*, which consists of only two species, *S. enterica* and *S. bongori*, but over 2600 different serotypes. The majority of which can infect both animals and humans (Lamas *et al*.,2021). *Salmonella enterica* serovar Typhimurium is one of the most common serovars responsible for human and animal salmonellosis around the world (Wang *et al*., 2019) It is estimated that *Salmonella* species globally reported to cause 115 million human infections and 370,000 deaths each year (Qin *et al*., 2022). *Salmonella enterica* can be transmitted to humans along the food chain from farm-to-fork, most commonly through contaminated animal-based foods especially poultry products like meat and egg (Mezal *et al*., 2014). As a result of *Salmonella*'s ability to persist in animal production systems and the surrounding environment, it is difficult to control possessing high risk to public health. In addition, the rising report of multi-drug resistant in *Salmonella* which can be transferred through food chain originating from livestock and poultry industries due to the unreasonable use of antibiotic is an emerging health issue that poses a serious threat to public health worldwide. Zhu *et al*. (2017) proposed that broiler chickens serve as a reservoir for multi-drug resistant *Salmonella*.

To reduce the spread of foodborne pathogens along the food chain, various methods has been in practiced such as physical, chemical and biological treatments. Chemical based antibacterial agents can be harmful to consumers when traces of chemical remain in the food. Physical treatment like heat, pressure and radiation can modify the nutritional value of foods. The need to address an alternative to antibiotic due to rising resistance makes bacteriophage

an attractive alternative for both bacterial controls serving both purposes in food safety preservation approaches as well as mitigation to antibiotic resistance.

Bacteriophages are naturally occurring parasite of bacteria and were said to be the most abundant forms of life on the planet, with a biome population of 10^{30}-10^{32} virions. They are reported to be isolated from wide range of environment including faeces, sludge, sewage, seawater, soil and anywhere bacteria may be present (Suttle, 2007; Keen, 2015). The capability of phage to grow and multiply only inside the specific host bacteria form the basis of their utility (Lin *et al*., 2017). Phage specificity of host varies; some are strain-specific, while others have demonstrated the ability to infect a wide range of bacterial strains and even genera (Koskella *et al*., 2013). The major advantages of phage are their negligible side-effect unlike antibiotic and other chemical disinfectants. Their ability to act on biofilms made them a suitable therapeutic candidate for use in humans, animals and plants, biopreservatives along the food chain, biosanitizers and biocontrol agents (D'Accolti *et al*., 2021; Lamas *et al*., 2021).

Considering the advantages of bacteriophage stated above, the objective of the research work was to isolate bacteriophages against, *Salmonella enterica* subsp Typhimurium of poultry origin, characterize them based on morphology, biological, sensitivity and stability, molecular characterization and to evaluate the isolated bacteriophage as bio control agent in poultry meat.*Salmonella* Typhimurium strains which were used for isolating bacteriophage for this study, ie. 22NSC004 and ATCC13311 were confirmed by streaking on Hektoen Enteric (HE) agar and other biochemical test which include, triple sugar iron (TSI), urease test and Simon citrate test. These are further confirmed by PCR detecting *invA*sion A *(invA*) gene which gives 244bp band visualized when gel was documented. This *invA* is considered unique to all *Salmonella* serovars and is widely used as marker for *Salmonella* sp. detection (Rahn *et al*., 1992; Abd-Elghany *et al*., 2015; Colello *et al*., 2018).

In this current study, using the confirmed host bacteria two bacteriophages was isolated from poultry farm wastewater and hospital drainage, Bareilly, indicating the presence of intended host bacteria because bacteriophages' existence is inextricably linked to that of their natural hosts (Suttle, 2007; Keen, 2015). However, phage enrichment of the sample was done by

supplementing media and indicator host to obtain suitable concentration for the phage to be detectable since in some environmental conditions, specific phages are present in very low concentration (Twest *et al*., 2009; Charante *et al*., 2021). Similar to this finding, many serovars of *Salmonella* phages were isolated from poultry farms (Nabil *et al*., 2018; Atterburry *et al*., 2020; Mhone *et al*., 2022; Pelyuntha *et al*., 2021).

The isolated bacteriophages were screened for their lytic activity indicated by clear lysis or plaque formation. The two phages showed different morphology of plaques indicating different phages. Phage isolated against the wild type 22NSC004 formed clearly defined round plaques of about 1-2 mm in diameter whereas bacteriophage isolated against strain ATCC13311 formed plaques of clear and well-defined edges but with smaller diameter of about 0.1-1 mm similar to the plaques formed by the well-known lytic bacteriophage of *Salmonella*, Felix 01 (O'Flynn *et al*., 2006; McLaughlin *et al*., 2008), phage st1 (Wong *et al*., 2014) and phage SAL 10 (Alharbi *et al*., (2022). However, many study reported *Salmonella* bacteriophages plaques size ranging from small pinpoint to ~6mm in diameter (Kumar *et al*., 2022) and with halo zone of lysis (Islam *et al*., 2020).

Host range for the isolated phages was determination using 24 isolates of *Salmonella enterica* belonging to different serovars (Typhimurium= 9, Infantis= 5, Agona= 4, Virchow= 3, Kentucky= 3) and 16 isolates of *Escherichia Coli*. The phages vB_StyS-PF79 and vB_StyA-RH55 revealed different host range profile. Phage vB_StyS-PF79 showed narrow host range, lysing only isolates of host serovar Typhimurium and two isolates of Agona whereas phage vB_StyS-PF79 exhibited wide host range according to Wongsuntornpoj *et al.* (2014) lysing 92% of the isolates, lytic to serovar Typhimurium, infantis, Virchow, Agona and Kentucky. The phages isolated in this study was able to lyse most common serovars involved in foodborne infections. However, neither of the isolated phages was able to lyse any of the *Escherichia Coli* isolates implying genus-restricted phages. *Salmonella* Typhimurium phages with narrow host range have also been widely isolated, for example, phage st1 (Wong *et al*., 2014), STA3, STA9, STA10 (Akhtar *et al*., 2014), P7 (Bigwood *et al*., 2008) and FGCSSa2 (Carey Smith *et al*., 2006). Broad host range phages against *Salmonella* Typhmurium phage Is_pst20, Ic_pst2 Ib_pst3 (Kumar *et al*., 2022), SEA1 and SEA2 (Akhtar *et al*., 2014) were also

isolated. In accordance with finding of this study, *S.* Typhmurium phage which was unable to lyse *Escherichia Coli* was reported by Phothaworn *et al*., 2019 and Islam *et al*., 2020. In contrast, *Salmonella* phage which cross-infect other genus such as *Escherichia Coli* and *Shigella flexneri* has been reported suggesting that they share similar receptor proteins with *S.*Typhimurium (Shin *et al*., 2012; Kumar *et al*., 2022). However, there is no clear explanation for different profile of host range within the same serotype, this may be due to the loss of receptor in the bacteria which can be acted upon by the bacteriophage due to different environmental origin.

Based on transmission electron microscopy documentation, the two isolated bacteriophage revealed distinct morphologies. Phage vB_StyS-PF79 revealed to have icosahedral head measuring 32.68nm in diameter and long non-contractile tail of length 109.06nm. In accordance with the ICTV, phage vB_StyS-PF79 was assigned to family *Siphoviridae* under order *Caudovirales*. Majority of reported *Salmonella* phages have long tail structures and are members of the *Siphoviridae* family (Yang *et al*., 2020). This finding was similar with many studies reported previously on *Salmonella* phages (Phothaworn *et al*., 2020; Shin *et al*., 2012; Kumar *et al*., 2022; Alharbi *et al*., 2022). However, phage vB_StyA-RH55 under TEM exhibited icosahedral head having diameter of 59.96nm and a short contractile tail of 22.54 nm in length. According to this finding, phage vB_StyA-RH55 was assigned to family *Autographiviridae* under family *Caudivirales*. *Autographiviridae* phages has also reported by many researcher such as anti-Pseudomonas syringae phages Eir4 and Eisa9 with icosahedral head (55-60 nm) with short tail (Korniienko *et al*., 2022) and phage Jarilo, anti-Pectobacterium phage with isometric head with 56.8±2.7 nm diameter (Pedersen *et al*., 2020). In contrast to the present findings, *Salmonella* phage belonging to *Myoviridae* family was reported by Hooton and his coworkers (2011) and *Podoviridae* family by Yang *et al*. (2020) and Islam *et al*. (2020).

Since efficacy of bacteriophage as biocontol for pathogenic microorganisms could be greatly influenced by pH and temperature the isolated phages were subjected to different ranges of temperature and pH (Ly-Chatain *et al*., 2014). When both phages vB_StyS-PF79 and vB_StyA-RH55 were exposed to temperature between -20°C to 60°C for 60 min, no

significant reduction in phage concentration indicating moderate heat resistance phages, the phages were able to maintain their activity at that temperature ranges. However, at 70°C vB_StyS-PF79 showed significant reduction n in phage concentration with $p<0.000001$ and phage vB_StyA-RH55 showed complete inactivation. Both the phages exhibited complete inactivation at 80°C. In contrast to this finding, *Salmonella* phage vB_SalP_TR2 could be recovered after exposure to 80°C (Sang *et al*., 2021). High temperature inactivation is due to denaturation of phages protein and nucleic acid, however, phages can also developed as heat resistant by strong protein interactions or mutation. The ability of phages to survive at temperature ranging from 30-50 C implies that they could be used as therapeutic agents in humans and animals. Stability at 4°C and -20°C indicated that storage at such temperatures did not affect the viability of phages (Mhone *et al*., 2022; Litt and Jaroni, 2017). Stability at high thermal temperature is applicable in food industries as high temperature exposure is required for processing of animal feed.

pH stability test revealed that phage vB_StyS-PF79 had no significant difference (p 0.2487) when exposed to different pH ranging from pH 3-10. At pH 5, 6 and 7 phage concentration was closest to control implying optimal pH for viability. Phage vB_StyA-RH55 has shown gradual increase in concentration from pH 3-7. At pH 7-10, the titer remained almost constant with slight significant difference with the control. The highest concentration was seen in pH 10 implying optimal pH for phage activity. Phages with stability at extreme pH ranging from 1-5 can be used to control foodborne pathogens. These phages which have ability to colonize gastrointestinal tract by withstanding the gastric pH and they can be used for oral administration. They can also be applied as biocontrol agents in acidic fruits, vegetables, pickles and meat which has generally low pH of 4~6 and also in fermented foods (Litt and Jaroni, 2017; Shang *et al*., 2021). Stability at pH between 8 and 9 implied that they can survive in intestinal environment, processing facilities and on food contact surfaces (Mhone *et al*., 2022)

When phage vB_StyS-PF79 was exposed to 10% chloroform, there was no significant reduction in phage titer but when exposed to 2% lactic acid and 0.2% peracetic acid, there was significant reduction in phage concentration as compared to control ($p \leq 0.0001$). Chemical

stability test for vB_StyA-RH55 revealed significant reduction in phage concentration when exposed to 2% Lactic acid ($p \leq 0.0001$), 0.1% Peracetic acid ($p \leq 0.0002$) and 10% chloroform ($p \leq 0.0002$) in comparison to control. The chemicals used for sensitivity test are meat disinfectants which are applied in multiple- hurdle strategy in combination with phages to control foodborne pathogens (Litt and Jaroni, 2017). The current experimental findings revealed that the phages vB_StyS-PF79 and vB_StyA-RH55 were not compatible with the chemicals and sensitivity of vB_StyA-RH55 to chloroform could indicate presence of lipophilic phage capsids.

Adsorption time of phage vB_StyS-PF79 and vB_StyA-RH55 was found to be 10 minutes and 30 minutes, respectively and rate of adsorption $2.5x10^{-9}$ ml/min and $1.4x10^{-8}$ ml/ min. This low adsorption time and high adsorption rate indicated high efficacy of infection. Phages with a higher adsorption rate encounter and attack host bacteria in less time (Shao *et al*., 2008). Latent period of the phages vB_StyS-PF79 and vB_StyA-RH55 were found to be 20 and 10 minutes respectively and their burst size revealed ~540 and 75 phages/ infecting cells respectively, which is considered large burst sized in comparison to many previous *Caudoviridae* family where the typical size is reported about 50-100 PFU $cells^{-1}$ (Bao *et al*., 2011). High burst size and short latent phages are preferred over other phages as they are considered an indicator of high lytic activity inactivating bacteria (Park *et al*., 2012). In contrast to this finding, late latent period of 50 minutes was also reported (Huang *et al*., 2018) and 100 minutes by Aguilera *et al*. (2022). Burst size contribute to plaque size, suggesting that increased burst size may contribute to bigger plaque size whereas smaller plaque will most likely be formed as a result of delayed cell lysis caused by the small burst size of progeny phages (Abedon *et al*., 2007; O'Flynn *et al*., 2006) This suggestion correlated to the plaque size formed in the present study by two phages isolated.

Restriction enzyme *Eco*R1 could digest phage vB_StyS-PF79 DNA into 8 fragments but *Bam*H1 was unable to digest. However, neither *Eco*R1 nor *Bam*H1 was able to digest DNA of phage vB_StyA-RH55. This finding implies that the phages are distinct from each other and consistent with those findings of the plaque assays, spot tests, adsorption assay and one-step growth kinetics. Neither of the phages amplify *g23* nor *capE* gene when subjected to PCR. *g23* and *capE* are major capsid proteins of *Myoviridae* and *Siphoviridae* family

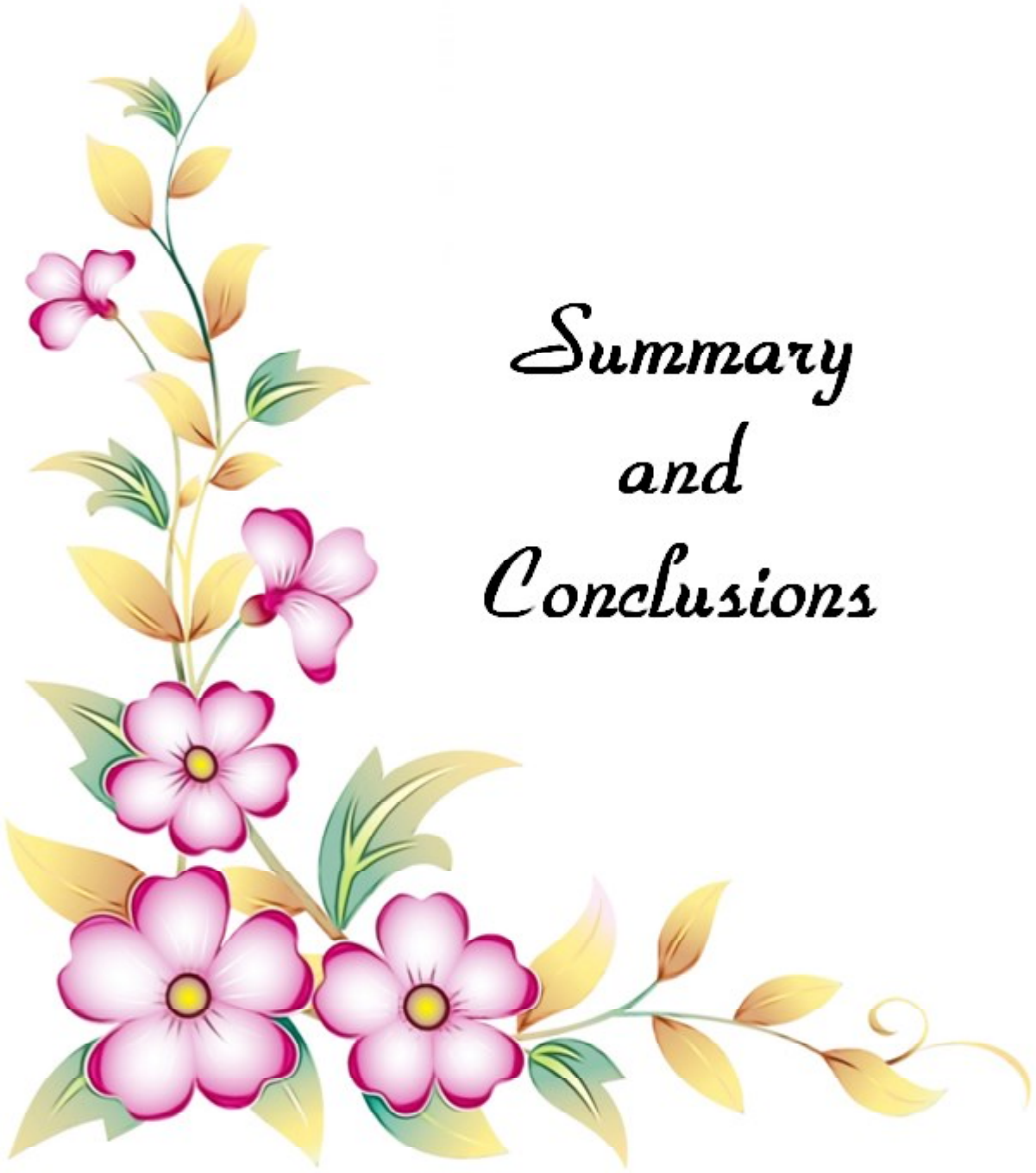
respectively but as viruses lack universal marker genes (Potavo *et al*., 2018; Filée *et al*., 2005), molecular characterization can confirm by whole genome sequencing.

The efficiency of the two isolated phages vB_StyS-PF79 and vB_StyA-RH55 has been demonstrated on chicken meat using MOI 1 by artificial spiking of *Salmonella* Typhimurium 22NSC004 as host bacteria at 4°C. On day 0, phages vB_StyS-PF79, vB_StyA-RH55 and cocktail of the two phages were unable to decontaminate the bacteria. This may be due to insufficient contact time between the phage and host bacteria or the latent period lengthens when the phages are incubated at refrigeration temperatures. On day 1, phage vB_StyS-PF79 alone could effectively reduce the bacterial count significantly with $p \leq 0.0485$. On the other hand, phage vB_StyA-RH55 could not significantly reduce the bacterial count individually. On day 2 it was found that both phages vB_StyS-PF79 and vB_StyA-RH55 were unable to decontaminate individually. However, combination of the phages as cocktail could effectively reduce the bacterial count to significant level $p \leq 0.0481$ on day 1 as well as on day 2 with $p \leq 0.0449$ at °C. This finding of better reduction in bacterial count in cocktail form in comparison to individual application suggested that the two phages might have different receptors for adsorption on the bacterial cell wall to infect the bacteria (Merabishvili *et al*., 2014). Similar findings have been reported by many researchers, although the time and temperature of analysis varies (Huang *et al*., 2018; Sang *et al*., 2021; Kumar *et al*., 2022). In contrast to many studies where high MOI (100, 1000, 10000) was utilized, this study used low MOI of 1 to successfully reduced the bacterial count which is in support of theoretical advantages where it was stated, large scale production and commercialization of phage products, would reduce the cost of preparation, purification and application of phage products (Wong *et al*., 2014). Howerver, many also suggested that an effective phage treatment regime in the in vivo therapeutic application and food biocontrol approach would be the rapid elimination of host cells in a short period of time using high MOI ratios (O'Flynn *et al*., 2004; Bigwood *et al*., 2008 and Atterbury *et al*., 2007)

It was also found that in comparison to the effect of chemical disinfectant to phages as bacterial control in chicken meat, no significant differences were observed (20% reduction differences). This finding is highly comparable to bacteriophage effect however, chemical

treatments have a negative impact on the organoleptic properties of the meat, making biological intervention with bacteriophages the more appealing option (Wessels *et al*., 2021).

Therefore this study concluded that bacteriophages can be used as biocontrol agent against non-typhoidal *Salmonella enterica* serovar Typhumirium at refrigeration temperature (4°C).



Summary and Conclusions

6 SUMMARY AND CONCLUSIONS

Foodborne illnesses remain the leading cause of deaths and hospitalizations globally. Among them, salmonellosis is one of the most commonly encountered. It is estimated that *Salmonella* species have reported to cause 115 million human infections and 370,000 deaths each year, globally. *Salmonella* Typhimurium is one of the most common serovar responsible for human and animal salmonellosis causing public health risk and economic loss. *Salmonella* is primarily spread through the consumption of contaminated food, particularly poultry and poultry-derived foods. Extensive documentation of antimicrobial resistance in *Salmonella* has been observed. The major contribution of antimicrobial resistance development is the irrational usage of antibiotics in sub therapeutic doses in poultry feed to promote rapid growth. Antibiotic residues in poultry meat are a direct threat to public health as the bacteria itself or the resistant genes can spread to the environment and, eventually to humans. Hence, there arises the urgent need to mitigate the antimicrobial resistance by using alternatives of antibiotic. Bacteriophage is a promising alternative to combat this antimicrobial resistance as therapy as well as biocontrol in food safety. Bacteriophages are ubiquitous in nature and are isolated from a variety of environmental sources such as soil, water as well as the gastrointestinal tracts of animals, and their specificity in nature render harmless to humans, animals, and plants. They have been recognized for their potential use in many expects such as biotherapy, biosanitation, biopreservation, and biocontrol agents along the food chain to improve food safety. Keeping in view the above stated advantages of bacteriophage, this present study was designed to isolate, characterized and evaluate the effect of bacteriophages on chicken meat as biocontrol agent against non-typhoidal *Salmonella enterica* of poultry origin.

Two bacteriophages were isolated from poultry farm and Rohilkhand hospital, Bareilly using *Salmonella* Typhimurium strain NSC004 and ATCC13311 respectively as isolating and propagating host. The isolated phages were subjected to transmission electron microscopy for morphological characterization and revealed that both the phages to belong to order *Caudovirales,* family *Siphoviridae* and *Autographiviridae,* respectively. The phages were named vB_StyS-PF79 and vB_StyA-RH55 according to ICTV rule of naming bacteriophage. Host range study revealed phage vB_StyS-PF79 as narrow host, specific to *S*. Typhimurium and *S*. Agona serovars whereas vB_StyA-RH55 was found to show wide host range lysis most serovars associated with foodborne infections. The phages were checked for their physical and chemical stability by exposing them to different temperature, pH and chemicals for 60 min. Both the phages vB_StyS-PF79 and vB_StyA-RH55 were exposed to temperature -20°C to 80°C. vB_StyS-PF79 phage decreases its titer to about 70% at 70°C but phage vB_StyA-RH55 was completely inactivated at 70°C. Complete inactivation of both the phages was observed at 80°C. vB_StyS-PF79 maintain their activity at 3-10 pH and vB_StyA-RH55 activity decrease to about 50-60% in acidic pH (3-5). The phage, vB_StyS-PF79 and vB_StyA-RH55 activity decreased when exposed to 2% lactic acid and 0.1 % peracetic acid. vB_StyS-PF79 was stable to 10% chloroform but vB_StyA-RH55 showed significant reduction suggesting their incompatibility.

Phage adsorption time of vB_StyS-PF79 and vB_StyA-RH55 was found to be 10 minutes and 30 minutes respectively and rate of adsorption $2.5x10^{-9}$ ml/min and $1.4x10^{-8}$ ml/min respectively. This low adsorption time and high adsorption rate indicated high efficacy of infection. Latent period of the phages vB_StyS-PF79 and vB_StyA-RH55 were found to be 20 and 10 minutes and their burst size revealed ~540 and 75 phages respectively which was consider high for *Caudovirales* order.

Phage vB_StyS-PF79 DNA could be digested by *EcoR1* into 8 fragments but unable to cut vB_StyA-RH55 phage. PCR showed no amplification for *g23*, *capE* genes for both the phages. On artificially contaminated chicken meat with strain 22NSC004, vB_StyS-PF79 phage alone could able to significantly reduce the bacterial count on day 1. Combination of the two phages as cocktails effectively reduced the bacterial count at 24 hours and 48 hours

incubation at 4 °C Chemical meat disinfectant also showed comparable effect with the bacteriophages in reducing bacterial count on day 1. Thus, the current study revealed that phage cocktails, vB_StyS-PF79 and vB_StyA-RH55 can effectively be used as biocontrol agents against non-typhoidal *Salmonella enterica* serovar Typhumirium at refrigeration temperature.


Mini Abstract

7 MINI ABSTRACT

Salmonellosis is one of the most commonly encountered foodborne illness worldwide caused by non-typhoidal *Salmonella enterica* serovars. *Salmonella enterica* serovar Typhimurium is one of the most common serovars responsible for human and animal salmonellosis around the world possessing both public health risk and economic loss. There is rising reports of multi-drug resistant *Salmonella* and broiler chicken is considered as the most important reservoir of this agent by which they can reach human population through food chain. Hence, to reduce foodborne pathogens, including *Salmonella*, lytic bacteriophages have emerged as a promising biocontrol intervention. Two bacteriophages were isolated against *Salmonella enterica* serovar Typhimurium strains namely, 22NSC004, ATCC13311 and named as vB_StyS-PF79 and vB_StyA-RH55, respectively. Phage vB_StyS-PF79 was found to be narrow host range, specific to serovar *S*. Typhimurium and *S*. Agona while phage vB_StyA-RH55 was found to have wide host range. Both phages in combination can infect most serovars of *Salmonella* causing foodborne infection. Temperature stability of the phages ranges from -20°C to 60°C. The phage, vB_StyS-PF79 can maintain their viability over a wide pH range ie. 3-10 but the phage, vB_StyA-RH55 has lower activity in acidic pH (3-5). Both the phages were observed to be sensitive to 2% lactic acid, 0.1% peracetic acid. vB_StyS-PF79 was stable to 10% but vB_StyA-RH55 showed very low tolerance for 10% chloroform. On RLFP, the phage vB_StyS-PF79 genomic DNA was digested into 8 fragments with *EcoR*1 but the phage vB_StyA-RH55 genomic DNA was resistant to *EcoRI*. Neither of the phage genomic DNA was able to be cut by *Bam*HI. The *g23* and *CapE* genes-based PCR yielded negative results. Phage adsorption time of PF79 and RH55 was found to be 10 minutes and 30 minutes, respectively and the rate of adsorption was $2.5x10^{-9}$ ml/min and $1.4x10^{-8}$ ml/min, for vB_StyS-PF79 and vB_StyA-RH55, respectively. Latent period of the phages vB_StyS-PF79 and vB_StyA-RH55 were found to be 20 and 10 minutes and their burst size revealed ~540 and 75 phages, respectively. The phage, vB_StyS-PF79 alone was able to reduce bacterial count on day 1. The phage cocktails yielded significant reduction in bacterial count on day 1 and 2 of storage at 4 C. Thus, the study concluded that the two isolated phages can be used as biocontrol agents against non-typhoidal *Salmonella enterica* serovar Typhimurium of poultry origin, effectively.

लघु सारांश

8 लघु सारांश

साल्मोनेलोसिस गैर–टाइफाइडल साल्मोनेला एंटरिका सेरोवार के कारण दुनिया भर में सबसे अधिक सामना की जाने वाली खाद्यय जनित बीमारियों में से एक है। साल्मोनेला एंटरिका सेरोवार टाइफिम्यूरियम दुनिया भर में मानव और पशु साल्मोनेलोसिस के लिए जिम्मेदार सबसे आम सेरोवर में से एक है, जिसमें सार्वजनिक स्वास्थ्य जोखिम और आर्थिक नुकसान दोनों हैं। बहु–दवा प्रतिरोधी साल्मोनेला और ब्रायलर चिकन को इस एजेंट का सबसे महत्वपूर्ण भण्डार माना जाता है जिसके द्वारा वे खाद्यय श्रृंखला के माध्यम से मानव आबादी तक पहुंच सकते हैं। इसलिए, साल्मोनेला सहित खाद्य जनित रोगजनकों को कम करने के लिए, लिटिक बैक्टीरियोफेज एक आशाजनक जैव नियंत्रण हस्तक्षेप के रूप में उभरा है। दो बैक्टीरियोफेज, vB_StyS-PF79 और vB_StyA-RH55 पृथक किए गए। फेज vB_StyS-PF79 को संकीर्ण मेजबान श्रेणी के रूप में पाया गया। जो सेरोवर एक टाइफिम्यूरियम और एस एगोना के लिए विशिष्ट था। जबकि फेज vB_StyA-RH55 में विस्तृत मेजबान रेंज पाया गया था। संयोजन में दोनों चरण साल्मोनेला के अधिकांश सेरोवर को संक्रमित कर सकते हैं जिससे खाद्य जनित संक्रमण हो सकता है। चरणों की तापमान स्थिरता –20 डिग्री सेल्सियस से 60 डिग्री सेल्सियस तक होती है। vB_StyS-PF79 एक विस्तृत पीएच रेंज यानी पर अपनी व्रूवहार्यता बनाए रख सकता है। 3–10 लेकिन फेज, vB_StyA-RH55 में अम्लीय पीएच (3–5) में कम गति विधि होती है। दोनों चरणों को 10% तक स्थिर था लेकिन vB_StyA-RH55 ने 10% क्लोरोफॉर्म के लिए बहुत कम सहनशीलता दिखाई। RLFP पर, vB_StyS-PF79 जीनोमिक डीएनए को *EcoRI* के लिए प्रतिरोधी था। *g23* और *capE* जीन–आधारित PCR ने नकारात्मक परिणाम दिए। vB_StyS-PF79 और vB_StyA-RH55 का फेज सोखना समय क्रमशः 10 मिनट और 30 मिनट पाया गया और सोखने की दर क्रमशः vB_StyS-PF79 और vB_StyA-RH55 के लिए 2.5×10^{-9} मिली/मिनट और 1.4×10^{-8} मिली/मिनट थी। फेज vB_StyS-PF79 और vB_StyA-RH55 की अव्रूक्त अवधि 20 और 10 मिनट पाई गई और उनके फटने का आकार क्रमशः ~540 और 75 फेज का पता चला। फेज, vB_StyS-PF79 अकेले दिन 1 पर बैक्टीरिया की संख्या को कम करने में सक्षम था। फेज कॉकटेल से 4 डिग्री सेल्सियस पर भण्डारण के दिन 1 और 2 पर बैक्टीरिया की संख्या में उल्लेखनीय कमी आई। इस प्रकार, अध्ययन ने निष्कर्ष निकाला कि दो अलग–अलग फेज हो सकते हैं पोल्ट्री मूल के गैर–टाइफाइड साल्मोनेला एंटरिका सेरोवर टाइफिम्यूरियम के खिलाफ बायोकंट्रोल एजेंटो के रूप में प्रभावी रू॰ से उपयोग किया जाता है।

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Appendix

APPENDIX

COMPOSITION OF THE MEDIAS AND GENERAL REAGENTS USED:

Composition	Grams/liter of DW
Casein enzymic hydrolysate	10.00
Yeast extract	5.00
MgSO4.7H_2O	2.00
Sodium chloride	5.00
Agar	15.00

NZCYM agar

Final pH 7.0 ± 0.2 at 25°C

Sterilized by autoclaving at 15 psi for 15 minutes.

New Zealand Casamino Yeast Medium (NZCYM) Overlay Agar

Composition	Grams
NZCYM broth	2.2
Agar powder Bacteriological grade	0.7
Distilled water	100 ml

Boil to melt the agar, dispense 7 ml in test tube and autoclave at 1210C (15 lb./sq. inch) for 20 min

NZCYM (New Zealand Casamino Yeast Medium) broth supplemented with extra $CaCl_2$ and $MgSO_4$

Composition	Grams/liter of DW
Tryptone	10.00
Yeast extract	5.00
Sodium chloride	5.00
Magnesium sulphate	0.98
$CaCl_2$	
$MgSO_4$	

Final pH 7.0 ± 0.2 at 25°C

Sterilized by autoclaving at 15 psi for 15 minutes.

TM buffer (Tris Magnesium buffer):

Composition	Volume (ml)
1M Tris HCl (pH7.5)	5.00
1M $MgSO_4$	1.00
Distilled water	Make up to 100ml

Sterilized by autoclaving at 15 psi for 15 min.

LB (Luria Bertani) broth

Composition	Grams/liter of DW
Tryptone	10.00
Yeast extract	5.00
Sodium chloride	10.00

Sterilized by autoclaving at 15 psi for 15 minutes.

10X BHI (Brain heart infusion) supplemented with $CaCl_2$ and $MgSo_4$

Composition	Grams
Calf brain, infusion from	200.0
Beef heart, infusion from	250.0
Proteose peptone	10.0
Dextrose	2.00
Sodium Chloride	5.00
Disodium Phosphate	2.50
$CaCl_2$	0.01
$MgSO_4$	0.246
Distilled water	100ml

Final pH (at 25ºC) 7.4±0.2

(Dispense 7 ml in test tubes and sterilized by autoclaving at 15 psi for 15 minutes)

Phosphate Buffer Saline (PBS)

Composition	Grams/liter of DW
NaCl	8.00
Na_2HPO_4	1.44
KCl	0.2
KH_2PO_4	0.2

Sterilized by autoclaving at 15 psi for 15 minutes

Hektoen Enteric (HE) Agar

Composition	Grams/liter of DW
Proteose peptone	12.00
Yeast extract	3.00
Lactose	12.00
Saccharose (Sucrose)	12.00
Salicin	2.00
Bile salt mixture	9.00
Sodium chloride	5.00
Sodium thiosulphate	5.00

Ferric ammonium citrate	1.50
Acid Fuchsin	0.10
Bromothymol blue	0.065
Agar	15.00

Final pH 7.5±0.2 at 25°C

Gently boiled to dissolve the medium completely (no autoclaving)

Urease test broth

Composition	**Grams/liter**
Urea	20.000
Potassium dihydrogen phosphate	0.091
Disodium hydrogen phosphate	0.095
Phenol red	0.010
Yeast extract	0.100

Final pH 6.8±0.2 at 25°C (no autoclaving)

Simmons Citrate Agar

Composition	**Grams/liter**
Magnesium Sulphate	0.200
Ammonium dihydrogen phosphate	1.000
Dipotassium phosphate	1.000
Sodium citrate	2.000
Bromothymol blue	0.080
Sodium chloride	5.000
Agar	15.000

Final pH 6.8±0.2 at 25°C.

Boil to dissolve the media. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Triple Sugar Iron (TSI) Agar

Composition	**Grams/liter of DW**
Peptone	20.00
HM extract	3.000
Yeast extract	3.000
Lactose	10.000
Sucrose	10.000
Glucose (Dextrose)	1.000
Iron (III) citrate	0.300
Sodium chloride	5.000

Sodium thiosulphate	0.3000
Phenol red	0.024
Agar	12.000

Final pH 7.4±0.2 at 25°C

Boil to melt the media, dispense 2ml to each 10 ml test tube and sterilized by autoclaving at 15 psi for 15 minutes

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

Tris-acetate-EDTA (TAE) buffer 10X

Composition	**Grams or ml/liter of DW**
Tris base	48.5
Glacial acetic acid	11.4ml
0.5 M EDTA (pH 8.0)	20ml

Ethidium bromide stock (10 mg / ml)

Composition	**mg or ml**
Ethidium bromide	10mg
Distilled water	1 ml

Stored at 4°C wrapped in aluminium foil. A concentration of 0.7µl/ ml was used for preparing agarose gel.

0.5 M EDTA (pH 8.0)

Composition	**Grams or ml**
EDTA (Disodium dehydrate salt)	95.05g
Distilled water	500ml

The pH was adjusted to 8.0 with NaOH pellets. The volume was made up to 500ml.

Loading dye (6X)

Composition	**% (w/v)**
Bromophenol blue	0.25
Xylene cyanol FF	0.25
Sucrose	40

Store at 4°C

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