PHYSICOCHEMICAL AND MOLECULAR CHARACTERIZATION OF BACTERIOPHAGES ΦSP-1 and ΦSP-3, SPECIFIC FOR PATHOGENIC *SALMONELLA* AND EVALUATION OF THEIR POTENTIAL AS BIOCONTROL AGENT

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By

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CERTIFICATE

This is to certify that the research work presented in the thesis entitled **"Physicochemical and molecular characterization of bacteriophages ΦSP-1 and ΦSP-3, specific for pathogenic *Salmonella* and evaluation of their potential as biocontrol agent"** is based on the original research work carried out by Ms. Jeena Augustine under my guidance and supervision at the Department of Biotechnology, Cochin University of Science and Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any degree.

SARITA G BHAT

DECLARATION

I hereby declare that the thesis entitled **"Physicochemical and molecular characterization of bacteriophages ΦSP-1 and ΦSP-3, specific for pathogenic *Salmonella* and evaluation of their potential as biocontrol agent"** is the authentic record of research work carried out by me for my doctoral degree, under the supervision and guidance of Dr. Sarita G Bhat, Associate Professor and the Head, Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has previously formed the basis for the award of any degree or diploma, associateship or other similar titles or recognition.

Cochin - 682022 **Jeena Augustine**

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LIST OF ABBREVATIONS

WHO	World Health Organization
FAO	Food and Agriculture Organization
%	Percentage
MDR	multidrug-resistant
NARMS	National Antimicrobial Resistance Monitoring System
CDC	Centre for disease control
USDA	United States Department of Agriculture
KW	Kauffman White
TTSS	type-III-secretion system
SPI	*Salmonella* Pathogenicity Island
MPa	Megapascal Pressure
log	logarithm
EDTA	Ethylenediaminetetraacetic acid
TSP	Trisodium phosphate
ICTV	International Committee on Taxonomy of Viruses
Fig	Figure
LPS	lipopolysaccharides
mRNA	messenger ribonucleic acid
DNA	deoxy ribonucleic acid
RM	Restriction Modification
Abi	abortive infection
kDA	kilo Dalton
EIBMV	Eliava Institute of Bacteriophage, Microbiology and Virology
HIIET	Hirszfeld Institute of Immunology and Experimental Therapy

MHRA	Medicines and Healthcare products Regulatory Agency
COREC	Central Office for Research Ethics Committee
EPA	Environmental Protection Agency
GRAS	Generally Recognized As Safe
kb	kilobase
UV	Ultraviolet
NCBI	National Center for Biotechnology Information
G+C	guanine + cytosine
A+T	adenine + thymine
MOI	multiplicity of infection
^{o}C	Degree Celsius
NCAAH	National Centre for Aquatic Animal Health
NICED	National Institute for Cholera and Enteric Diseases
FDA	Food and Drug Administration
BAM	Bacteriological Analytical Manual
ml	milliliter
XLD	Xylose– lysine–deoxycholate
HE	Hektoen enteric
BS	Bismuth sulfite
NA	Nutrient agar
TSI	Triple Sugar Iron agar
LIA	Lysine Iron agar
PCR	polymerase chain reaction
mM	millimolar
TE	Tris-EDTA
SDS	sodium dodecyl sulfate
ng	nanogram

bp	base pair
dNTP	Deoxyribonucleotide triphosphate
µl	microliter
TAE	Tris-acetate-EDTA
µM	micrometer
BLAST	Basic Local Alignment Search Tool
TTC	2,3,5-triphenyltetrazolium chloride
rpm	revolution per minute
PFU	plaque forming units
CFU	colony forming units
PEG	Polyethylene glycol
v/v	volume/volume
w/v	weight/volume
PBS	Phosphate buffered saline
TEM	Transmission Electron Microscopy
ANOVA	Analysis of Variance
PFGE	Pulsed-Field Gel Electrophoresis
MCP	Major Capsid Protein
PAGE	polyacrylamide gel electrophoresis
NCIM	National Collection of Industrial Microorganisms
NGM	Nematode Growth Medium
LB	Luria Bertani
nm	nanometer
TD	time to death
min	minutes
sec	seconds
Da	dalton
M	Molar
N	Normality

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Chapter 1

INTRODUCTION

Bacterial infection is a leading cause of mortality worldwide (O'Connell *et al.,* 2005) and among the bacterial infections, food borne pathogens are the principal cause of illness and death in less developed countries, killing approximately 1.8 million people annually (Faruque, 2012).

The Members of the Food and Agriculture Organization of the United Nations (FAO) and of the World Health Organization (WHO) have expressed concern regarding the level of safety of food both at the national and the international levels.

Salmonella is prominent among the bacterial pathogens. In recent years problems related to *Salmonella* have increased significantly, both in terms of incidence and severity of the cases of human salmonellosis. Non typhoidal *Salmonella* ranks second (11% of the total diseases) among the causative agents of domestically acquired food borne illnesse. It ranks first in contributing to hospitalization (35%) and cause of death (28%) resulting from domestically acquired food borne illnesses. (http://www.cdc.gov/foodbor neburden/2011-foodborne-estimates.html).

Each year, approximately 40,000 *Salmonella* infections are culture-confirmed, serotyped and reported to the United States Centers for Disease Control and Prevention (CDC). Of the total salmonellosis cases, an estimated 96% are caused by contaminated food (Mead *et al.,* 1999). Illnesses caused by the majority

of *Salmonella* serotypes range from mild to severe gastroenteritis, and in some patients, bacteremia, septicemia and a variety of associated long-term conditions. Extensive varieties of food have been implicated in food borne illness attributable to *Salmonella enterica*. Food of animal origin, especially poultry, poultry products and raw eggs, are often implicated in sporadic cases and outbreaks of human salmonellosis (Bryan and Doyle, 1995; Humphrey, 2000). Poultry is widely acknowledged as a reservoir of *Salmonella* infections in human due to the ability of *Salmonella* to proliferate in the gastrointestinal tract of chicken (Poppe, 2000) and subsequently survive on commercially processed broiler carcasses.

The beginning of the 1980s, saw the emergence of *Salmonella* strains resistant to a range of antimicrobials, including first-choice agents for the treatment in human, which became a serious public health problem. Multi-drug resistance to "critically important antimicrobials" are further compounding the problems (Ribot *et al.,* 2002; Faldynova *et al.,* 2003). Antimicrobial resistance or the ability of microorganisms to withstand treatment with drugs, they were once susceptible to, is a significant and multifaceted public health problem. In addition, the scarcity of new antimicrobial agents and the dearth of new agents in the drug development pipeline limit treatment options, particularly for patients with infections caused by multidrug-resistant (MDR) organisms. The societal and financial cost of treating antimicrobial-resistant infections place a significant human and economic burden on society, as individuals infected with drug-resistant organisms are more likely to remain in the hospital for a longer period of time and to have a poor prognosis (Lee *et al.,* 1994).

Antimicrobial agents are currently used for three main purposes: (1) to treat infections in human, animals and plants; (2) prophylactically in human, animals, and plants; and (3) subtherapeutically in food animal as growth promoters and for feed conversion (Angulo *et al.,* 2000). When antibiotic use became the norm in both

human and animal medicine, selection pressure increased the bacterial advantage of maintaining and developing new resistance genes that could be shared among bacterial populations (Matthew *et al.,* 2007). The first suggestion that antibiotic use in livestock led to antibiotic-resistant bacteria was in 1951. Starr and Reynolds (1951) reported streptomycin resistance in generic intestinal bacteria from turkeys that were fed with antibiotic.

MDR strains of *Salmonella* are encountered more frequently now and the rates of multidrug-resistance have increased considerably in recent years. Even worse, some variants of *Salmonella* have developed multidrug-resistance as an integral part of the genetic material of the organism, and are therefore likely to retain their drug-resistant genes even when antimicrobial drugs are no longer used, a condition wherein other resistant strains would typically lose their resistance.

The use of antibiotics not only selects for antimicrobial-resistant bacteria, but may also increase the likelihood of disease transmission. In 2006, Bauer-Garland and group researched the transmission of MDR *Salmonella* Typhimurium in broiler chicken under selective-pressure. MDR *S.* Typhimurium strain had significantly increased transmission when chicken were treated with tetracycline, demonstrating that antimicrobial use influences transmission of antimicrobial-resistant pathogens in poultry (Bauer-Garland *et al.,* 2006). *Salmonella* Enteritidis is one of the most common serotypes of *Salmonella* causing human illness that is associated with consumption of egg-containing products and chicken (Voetsch *et al.,* 2009). Since 1996, the number of nalidixic acid (a drug closely related to ciprofloxacin the most commonly prescribed antibiotic for *Salmonella* infections) resistant *S.* Enteritidis, submitted to the National Antimicrobial Resistance Monitoring System (NARMS) is increasing. Of these resistant isolates, 90% also showed decreased susceptibility to ciprofloxacin (2009).

Investigations for new alternative anti-microbials, effective against bacterial pathogens including *Salmonella,* have become increasingly relevant for both human and veterinary applications, among which bacteriophage are a potential candidate (Boyle *et al.,* 2007).

OBJECTIVES OF THE STUDY

Bacteriophages (phages) are obligate intracellular parasites that multiply inside bacteria and in doing so make use of the host biosynthetic machinery. In fact, right from the discovery of phages, their potential as a therapeutic agent was introduced by Félix d'Herelle, a French-Canadian microbiologist. But research in this direction was abandoned by the Western scientific community with the discovery of antibiotics. But now, the emergence of antibiotic resistance is one of the motivating factors, pushing the scientists to go back to this long forgotten cure (Matsuzaki *et al.,* 2005). Thus, after several years of abandonment, the use of phages for killing bacteria has drawn recent attention and reappraisal. This has led to a vast phage research, in varied fields, with impressive outcomes (Sulakvelidze *et al.,* 2001). Recent review also hints the potential of phages as alternatives to antibiotics (Thiel, 2004; Greer, 2005; Skurnik and Strauch, 2006).

The first and foremost advantage of phage as an alternative biocontrol is that they are active against bacteria that have become resistant to antibiotics (Alisky *et al*., 1998; Carlton, 1999; Górski *et al*., 2007; Skurnik *et al*., 2007; Kutter *et al*., 2010) and second is their specificity, thereby preventing secondary bacterial infection which is commonly observed in antibiotic therapy (Kutter and Sulakvelidze , 2005).

They are relatively harmless supplemente consume them every day (Skurnik *et al*., 2007; Ab *et al*., 2010). When well-purified phages are used, not many side effects have been described, for all types of administration (Alisky *et al*., 1998; Carlton, 1999; Górski *et al*., 2007; Kutateladze and Adamia, 2010).

Unlike antibiotics, phages are an 'intelligent' drug. They multiply at the site of the infection until there are no more bacteria (Inal, 2003). In addition, bacteria that have become resistant to a certain type of phage continue to be destroyed by other types of phages. They are easy to isolate as they are found throughout nature (Skurnik *et al*., 2007). Thus the search to find new phages when bacteria become resistant to them is effortless as evolution drives the rapid emergence of new phages that can destroy bacteria resistant to existing phages as well as the antibiotic resistant forms (Sulakvelidze *et al*., 2001). This means that there will be an 'inexhaustible' supply of bacteriophages available to exploit. Finally phages can be genetically modified in order to make up for some of their disadvantages as evidenced by the several works (Geier *et al*., 1973; Cao *et al.,* 2000; Hagens *et al*., 2004; Fischetti *et al*., 2006; Yacoby *et al*., 2007; Lu and Collins, 2009). In addition, individual components of phages (e.g. lysins) can also be used as antimicrobial substances (Borysowski *et al*., 2006).

The ability of *Salmonella* phages to effectively reduce the bacterial load in food products have given promising results (Leverentz *et al*., 2001; Goode *et al*.,2003; Fiorentin *et al*.,2004, 2005; Pao *et al*.,2004; Jianxiong *et al.,* 2009; Ye *et al.,* 2010).

Thus the aim of the present study was to isolate and characterize *Salmonella* and *Salmonella* specific lytic bacteriophages from the intestinal content of broiler chicken where they are most likely to be found (Goyal *et al.,* 1987; Silja *et al.,*

2010) and to determine the potential application of employing both *in vitro* and *in vivo* assays.

Specific objectives included the following:

1. **To isolate *Salmonella* specific lytic phages**
2. **To characterize the isolated phages**
3. **To investigate the potential of phages as biocontrol agents**

Chapter 2

REVIEW OF LITERATURE

2.1 *Salmonella*

Salmonella a Gram-negative facultative rod-shaped bacterium belongs to the proteobacterial family *Enterobacteriaceae.* It is trivially known as the "enteric" bacteria.

The genus *Salmonella* was named after Daniel Elmer Salmon, an American veterinary pathologist. But the actual discoverer of the type bacterium (*Salmonella enterica* var. *choleraesuis*) was Theobald Smith. Dr. Salmon was the administrator of the United States Department of Agriculture (USDA) research program at that time, and thus the organism was named after him by Smith (Schultz, 2008).

Salmonella nomenclature is complex and evolving. Currently, the nomenclature system used at the Center for Disease Control and Prevention (CDC) for the genus *Salmonella* is based on recommendations from the World Health Organisation (WHO) collaborating centre. The genus *Salmonella* consists of only two species, *S. bongori* and *S. enterica*, with the latter being divided into six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*. *Salmonella enterica* is one of the most common causes of food poisoning in humans (Rabsch *et al*., 2001). Within *S. enterica* subsp. I (*S. enteric* subsp. *enterica*), the most common O-antigen serogroups are A, B, C1, C2, D and E. Strains within these sero-groups cause approximately 99% of *Salmonella* infections in human and warm-blooded animals (Uzzau *et al*.,2000).

The scheme used worldwide for serological identification of *Salmonella* serovars was first proposed by White and expanded by Kauffman (Minor and Popoff, 1987). The list of 2600 *Salmonella* serotypes is maintained and annually updated by the WHO collaborating centre for reference and research on *Salmonella* at the Pasteur Institute, Paris, France (Grimont and Weill, 2007). The Kauffman White scheme (KW) is based on the antigenic structure of *Salmonella* serotypes (Helmuth, 2000). The typical format for a serotype formula is: Subspecies [space] O antigens [colon] Phase 1 H antigen [colon] Phase 2 H antigen.

By latest convention, names are retained only for subspecies *enterica* serovars and these names are no longer italicized. The first letter is a capital letter "*S*" followed by the serovar names of subspecies *enterica* (e.g. Typhimurium) (Brenner *et al*., 2000; Molbak *et al.*, 2006).

The principal habitat of the *Salmonella* is the intestinal tract of human and animals (Ohl and Miller, 2001). Some species are ubiquitous. Other species are specifically adapted to a particular host. The principal clinical syndromes associated with *Salmonella* infection are enteric (typhoid) fever and gastroenteritis (Miller and Pegues, 2000). Enteric fever is a protracted systemic illness that results from infection with the exclusively human pathogens, *S.* Typhi and *S.* Paratyphi. In contrast, the many nontyphoidal *Salmonella* strains, such as *S.* Enteriditis and *S.* Typhimurium, infect a wide range of animal hosts, including poultry, cattle, pigs and human (Ohl and Miller, 2001).

2.1.1 *Salmonella enterica* serovars associated with food poisoning

The two serovars most frequently reported as causative agents responsible for infection are *S.* Typhimurium and *S.* Enteritidis. Historically, *S.* Typhimurium was among the most common serovar isolated from poultry across many countries from the 1950s to the late 1970s (Baumler *et al*., 2000), but was overtaken by *S.* Enteritidis, the most common serovar isolated from poultry by the mid 1980s to date (Guard-Petter, 2001; Poppe, 2003).

CDC has reported that the leading cause of death due to food borne bacterial diseases are pathogenic *Listeria* and *Salmonella*, followed closely by *E. coli* O157:H7 and *Campylobacter jejuni*, and consequently studies on the use of phages against pathogenic bacteria in food systems have focused mainly on these pathogenic organisms (CDC 2005, 2007a and b, 2008a and b). In addition to the infections and pathogenesis, the appearance of *Salmonella* isolates resistant to multiple antibiotics, complicates an already difficult situation escalating the pressing need to develop effective alternatives (Boyle *et al.,* 2007). There are reports on increased *Salmonella* colonization of intestines in chicken treated with selected antibiotics, possibly due to reduction of normal bacterial flora in the gastrointestinal tract that serve as a natural barrier to *Salmonella* infection (Manning *et al.*,1992, 1994). In addition, antibiotic resistant *Salmonella* have been recovered from experimentally challenged birds treated with antibiotics (Kobland *et al.*, 1987; Gast *et al.*, 1988).

Caecal colonisation of chicken is considerably important to the epidemiology of *Salmonella enterica* serotype Enteritidis infection in both man and animal hosts. The caecum was shown to be the site of colonisation by *Salmonella* during infection of chicken (Fanelli *et al*., 1971) and the contamination of carcasses and eggs by *S.* Enteritidis is alleged to originate predominantly from caecal and cloacal contamination (Keller *et al*., 1995).

2.1.2 *Salmonella* pathogenesis

Serovar Enteritidis can colonize both human and chicken. In human, the infection can manifest as a non-bloody diarrhoea with abdominal pain, nausea, vomiting and fever. The primary route of *Salmonella* infection in human and other animal species is the fecal-oral transmission of the organism. The estimates of the number of organisms required to cause disease are quite variable, ranging from about 30 to more than 10^9 infectious organisms (Morgan *et al.*, 1994; Vought and Tatini, 1998). This variability in infectious dose is in part due to the food matrix contaminated with *Salmonella* and intrinsic factors of the infecting organisms (Giannella *et al.*, 1972, 1973; Blaser and Newman, 1982).

In the course of an infection, *Salmonella* first colonize the intestine and penetrate the intestinal barrier (Frost *et al.*, 1997), where invasion of intestinal mucosal cells including M cells is mainly conferred by the type-III-secretion system (TTSS) located on *Salmonella* Pathogenicity Island (SPI)-1 (Baumler *et al.*,1996; Penheiter *et al.*,1997; Marcus *et al.*, 2000). The TTSS structural genes encode proteins that can form a needle-like structure which appears to partly resemble the structure of bacterial flagella (Kubori *et al.*, 1998). When *Salmonella* adheres to a target cell, this needle-like structure is assumed to form a channel with its base anchored in the cell wall and its tip puncturing the membrane of the host cell. Through this channel, several bacterial proteins can be secreted into the cellular cytoplasm. These injected proteins trigger host cell signaling events that cause local rearrangement of the actin cytoskeleton leading to cell membrane ruffling and the active uptake of bacteria by the host cell (Francis *et al.*, 1993; Foster, 1995; Zhou *et al.*, 1999). The invasion of epithelial cells elicits the production of the pro-inflammatory cytokines (Eckmann *et al.*, 1993), which

stimulate the influx of polymorphonuclear leukocytes into the infected mucosa. Via this route, bacteria can reach the Peyer's patches (Penheiter *et al.,* 1997). The bacteria are then engulfed by macrophages which carry them to systemic sites through the lymphatic system. In the mouse liver, *Salmonella* can be found inside macrophages (Richter-Dahlfors *et al.,* 1997). During this invasive process, *Salmonella* secrete a heat-labile enterotoxin that precipitates a net efflux of water and electrolytes into the intestinal lumen manifesting in diarrhoea.

Infections of adult chicken with serovar Enteritidis are generally symptomless. These symptomless carriers are difficult to trace which leads to problems in the control of the infection in a chicken flock (Lumsden *et al.,* 1991). *Salmonella* infections in chicken are mostly caused by the intake of contaminated food, although vertical transmission has been described, where the bacteria are directly transferred from the mother to the offspring via the egg (Gast and Beard, 1990; Shivaprasad *et al.,* 1990; Humphrey *et al.,* 1991). In newly hatched chicken, serovar Enteritidis can cause diarrhea and septicemia with invasion and infection of a variety of internal organs including liver, spleen, peritoneum, ovaries and oviducts (Gast and Beard, 1990). When the animals become infected by this serovar, extensive interstitial oedema of the lamina propria and the submucosa can be observed within one day of infection, followed by a rapid influx of granulocytes and macrophages (Desmidt *et al.,* 1996). Once the bacteria have reached the macrophages it is disseminated to the different organs. In this regard, the course of serovar Enteritidis infection in young chicken resembles that in susceptible human.

2.1.3 *Salmonella* control measures: chemical, physical and biological

Preventing *Salmonella* from contaminating food during the farm-to-table process remains challenging. Some of the control measures adopted include

combinations of temperature and pressure on sprays with or without bactericides at different levels, steam with or without vacuum (Davidson *et al.*, 1985). A 52% reduction in *Salmonella*-positive samples was reported using steam for 20 seconds on retail portions of poultry meat. Variability in success depending on portion of meat treated and exposure time limitations due to organoleptic effects are the major drawbacks (Davidson *et al.*, 1985). Gamma irradiation as an effective non-specific means of reducing bacterial counts was studied (Bruhn, 1995), the major advantages of which include penetrating ability with minimal effects on the final product. However, installation of irradiation equipments and containment facilities is expensive and likely cost-prohibitive for small processors (Bruhn, 1995). High voltage electricity and oscillating magnetic fields are also very effective, but the cost of implementing these methods would be very high (Corry *et al.*, 1995). Application of hydrostatic pressure to meat foods has been studied to check the effectiveness in reducing pathogenic bacteria (Ananth *et al.*, 1998; Alpas *et al.*, 1999), where suspensions of *Salmonella* were treated with 345 MPa pressure for 5 minutes at 25°C using a hydrostatic unit. Reductions of 5.45 from 8.34 log10 were obtained, depending on the serotype of *Salmonella* treated. Chemical treatments employed include treatment with carbon dioxide (Baker *et al.*, 1986). *S.* Typhimurium growth on experimentally contaminated ground chicken was found to be suppressed using 80% CO_2 with storage at 2°C for 5 days. Mullerat *et al.* (1994) found dose-dependent *Salmonella*-reducing efficacy with Salmide®, a sodium chlorite disinfectant, alone and in combination with other agents on experimentally-contaminated broiler drumstick skin. They observed a significant 0.7 $\log_{10}$ reduction with 54 mM Salmide®. A reduction of 2.52 $\log_{10}$ was achieved with this dose in combination with ethylenediaminetetraacetic acid (EDTA) and a 1.74 $\log_{10}$ reduction resulted when Salmide® was combined with sodium lauryl sulfate (Mullerat *et al.*, 1994). The efficacy of a 0.5% lactic acid/0.05% sodium benzoate wash to reduce bacterial load in experimentally contaminated wing drumettes with *S. enterica* serovars Dublin, Enteritidis and Typhimurium was

reported (Hwang and Beuchat,1995). Trisodium phosphate (TSP) is one example of a pre-chill chemical application currently used by the poultry industry to reduce bacterial pathogens. Ten percent TSP applied in a pressurized spray can achieve statistically significant reduction of *Salmonella* on experimentally contaminated chicken skin when compared to tap water-treated controls (Wang *et al.*, 1997). However, others have questioned the efficacy of this application because of pH changes that result from the treatment. Bianchi *et al.* (1994) reported a significant decrease in experimental contamination with both *S.* Typhimurium and *S. arizonae* using a peroxidase-catalyzed chemical dip. The dip also contained citrate buffer, sodium iodide and sodium carbonate. Biological control measures employed include application of bacteriocins. Nisin, a heat-stable peptide produced by *Lactobacillus lactis* is an example of bacteriocin which is capable of retarding growth of *Salmonella* (Stevens *et al.*,1991). Researchers have reported a 3-$\log_{10}$ reduction of *Salmonella* Typhimurium on experimentally-contaminated chicken skin when nisin was added to packaging film (Natrajan and Sheldon, 1995).

Another biological approach to the control of *S.* Enteritidis is the use of specific lytic bacteriophages which have many advantages over the several methods discussed above.

2.2 Bacteriophages

Bacteriophages, commonly known as phages, are natural viral pathogens of bacteria. They are considered as the most abundant and ubiquitous life-like entity in both marine environment (Bergh *et al.*, 1989) and terrestrial soil environments (Ashelford *et al.*, 2003; Srinivasiah *et al.*, 2008). The claims for the discovery of these wonderful entities have been subject to plenty of speculations and controversies. Interestingly it was from India, that the existence of

bacteriophages was first reported. Ernest Hankin, a British bacteriologist working in India reported the existence of unidentified substance (which passed through fine porcelain filters and was heat labile) responsible for marked activity against the bacterial pathogen *Vibrio cholerae* in the waters of the Ganges and Yamuna rivers in 1896 (Hankin, 1896). After almost 20 year Frederick Twort, a medically trained bacteriologist from England reported a similar phenomenon and put forth the hypothesis that it may have been due to among other possibilities, a virus (Twort, 1915). But the credit of officially discovering phages goes to Felix d'Herelle, a French-Canadian microbiologist at the Institute Pasteur in Paris (d'Herelle, 1917). The term "bacteriophage" was proposed by d'Herelle which comes from the words "bacteria" and "phagein" which in Greek means to eat or devour.

Since their discovery, phages have become the topic of interest to many researchers worldwide. Initial research was more concentrated on defining its nature but soon its potential in deciphering the secrets of life was sensed by the scientific community, which ultimately aided in the development of molecular biology (Duckworth, 1987). With more recent advances in technology, there has been a revival of interest in phage research as more information is gathered concerning their biology, their ecological role and their natural diversity (Wommack and Colwell, 2000). Phage research in more recent years has revealed their dramatic impact on the ecology of our planet (Suttle, 1994; Wommack and Colwell, 2000), their influence on the evolution of microbial populations (Chibani-Chennoufi *et al.*, 2004) and their potential applications (Sulakvelidze *et al.*, 2001). Phage-derived proteins are currently being used as molecular machines (Smith *et al*., 2001), diagnostic agents (Schuch *et al*., 2002), therapeutic agents (Loeffler *et al*., 2001*;* Nelson *et al*., 2001; Schuch *et al*., 2002) and for drug discovery (Liu *et al*., 2004).

2.2.1 Classification of phages

Initial classification of bacteriophages was based merely on host specificity (Nelson, 2004). The advent of electron microscopy enabled scientists to classify phages based on their morphology (Luria *et al*., 1943). Molecular characters of phages like size and type of nucleic acid (single stranded DNA, double-stranded DNA and single-stranded RNA) were also taken into consideration with the advances in the field of molecular biology (Thomas and Abelson**,** 1966). Present day classification by International Committee on Taxonomy of Viruses (ICTV) is derived from the scheme proposed by Bradley (1967) using gross morphology and nature of their nucleic acid. It includes six basic morphological types, exemplified by phages T4, λ, T7, φX174, MS2 and fd. Over the years many new families were added and currently it includes one order, 17 families and three "floating" groups (Ackermann, 2007, 2009). Classification criteria adopted by ICTV is based on polythetic species concept, meaning that a species is defined by a set of properties that may or may not all be present in a given member (Regenmortel, 1990). The ICTV classification method as described above is currently being reevaluated, since it ignores the vast amount of available genome sequence data which can occasionally cause contradiction in the classification criteria adopted (Ceyssens, 2009) and also because new phages are discovered daily and the ICTV lags in its classification schedule (Ackermann, 2007).

Table.2.1 Overview of phage families

Shape	Nucleic acid	Family	Genera	Example	Members
Tailed	dsDNA (L)	*Myoviridae*	6	T4	1,320
		Siphoviridae	7	λ	3,229
		Podoviridae	4	T7	771
Polyhedral	ssDNA (C)	*Microviridae*	4	φX174	40
	dsDNA (C, S)	*Corticoviridae*	1	PM2	3?
	dsDNA (L)	*Tectiviridae*	1	PRD1	19
	dsDNA (L)	SH1∗		SH1	1
	dsDNA (C)	STIV∗		STIV	1
	ssRNA (L)	*Leviviridae*	2	MS2	39
	dsRNA (L, M)	*Cystoviridae*	1	φ6	3
Filamentous	ssDNA (C)	*Inoviridae*	2	M13	67
	dsDNA (L)	*Lipothrixviridae*	4	TTV1	7
	dsDNA (L)	*Rudiviridae*	1	SIRV-1	3
	dsDNA (C, S)	*Plasmaviridae*	1	L2	5
	dsDNA (C, S)	*Fuselloviridae*	1	SSV1	11
	dsDNA (L, S)	-	1∗∗	His1	1
	dsDNA (C, S)	*Guttaviridae*	1	SNDV	1
	dsDNA (L)	Ampullaviridae∗		ABV	1
	dsDNA (C)	Bicaudaviridae∗		ATV	1
	dsDNA (L)	Globuloviridae∗		PSV	1

C Circular; L linear; M multipartite; NC nucleocapsid; S supercoiled; _ no name ; ∗Awaiting classification (adapted from Ackermann, 2009)

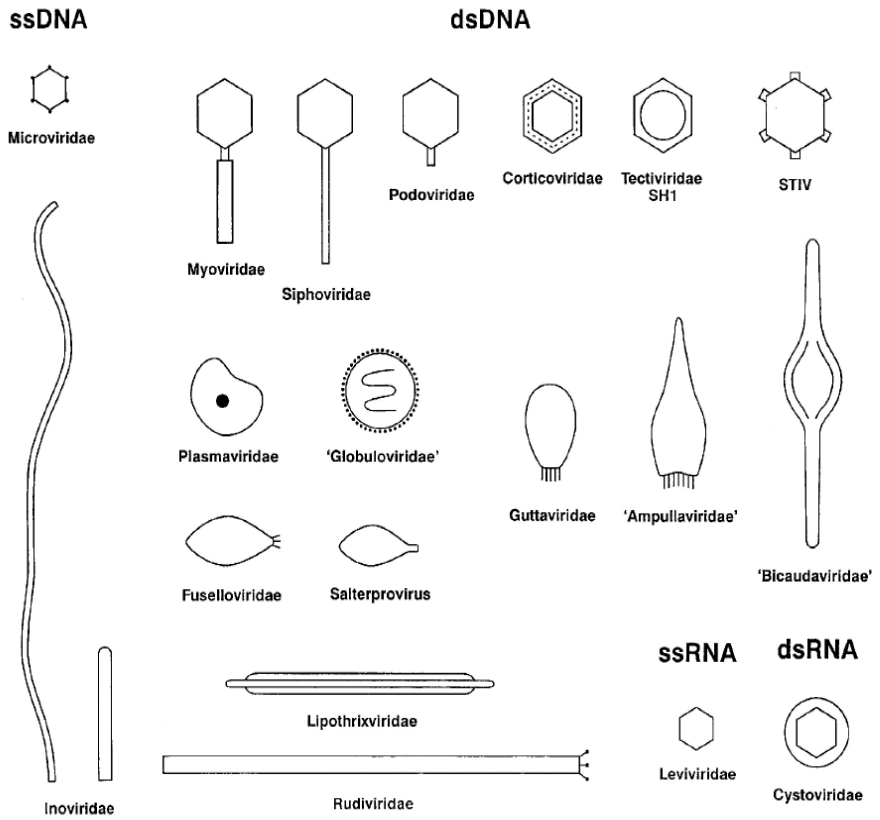


Fig.2.1 Phage morphotypes (figure adapted from Ackermann, 2009)

2.2.2 Lifestyles of bacteriophage

Like any other virus, phages also require the help of specific host for their multiplication/propagation. In the absence of a host they remain as inert particles with their genetic material safely packed inside the protective protein covering. Adsorption of phage on to the host is the first and the most important step in phage multiplication and it is via their tail tips that they recognize the receptor on the host surface. The receptor material on host cell can either be carbohydrate, protein, lipopolysaccharides (LPS) or flagella. This attachment is weak and reversible and is followed by irreversible binding to a bacterium, mediated by one or more of the

components of the base plate. The phage genome is introduced into the bacterial cytoplasm either by the contraction of the sheath or by weakening the components of the bacterial envelope by digestive enzymes (Fischetti *et al.,* 2006). Nucleic acid is the only phage component that actually enters the cell. Entrance of the phage genome into the cell starts the infection period and the productive cycle ends either with phage-induced bacterial destruction (lysis) or with an extruding or budding of maturing phage virions (chronic release) across the bacterial cell envelope (in a manner that does not lead to immediate bacterial death). Infections that result in phage release can be described as productive. Alternatively, phage infections can enter a latent state (lysogeny or pseudolysogeny) or can fail to infect successfully (phage restriction or abortive infection).

2.2.2.1 Lytic infection

Entrance of the phage genome into the cell begins the infection period. The first stage is eclipse period during which infectious phage particles cannot be found either inside or outside the bacterial cell. The phage nucleic acid takes over the host biosynthetic machinery, whereby phage specified mRNAs and proteins are made. There is an orderly expression of phage directed macromolecular synthesis. Early mRNA codes for early proteins that are necessary for phage DNA synthesis and for shutting off host DNA, RNA and protein biosynthesis. In some cases the early proteins actually degrade the host chromosome. The late proteins are the structural proteins that comprise the phage as well as the proteins needed for lyses of the bacterial cell. Synthesis of late mRNA and late proteins takes place only after phage DNA synthesis. Once this is over, phage nucleic acid and structural proteins are assembled and accumulated within the cell which is followed finally by the release of hundreds of new phages by bacterial cell lysis (Pelczar *et al.,* 1988).

2.2.2.2 Lysogenic infection

In the lysogenic cycle, the phage genome is integrated into the bacterial genome, wherein it multiplies cooperatively with the host bacteria without destroying it, and consequently the phage genome is passed to all bacterial progeny by cell division. Bacteria that integrate the phage genome into their own are known as lysogens; they are resistant to infection by phages that are genetically related to previously lysogenized phages (Campbell, 1996). Some lysogenic phages have toxic genes in their genome.

The first step in lysogeny after entering the bacterial cytoplasm is the circularization of the single stranded ends by base pairing the cohesive ends to produce circular molecule. This is followed by site-specific recombination that occurs between a particular site on the circularized phage DNA and on the host chromosome mediated by phage coded enzyme. The result is the integration of the phage DNA into the host chromosome. The phage genome is repressed by phage encoded protein called repressor, which binds to a particular operator site on the phage DNA, shutting off transcription of almost all phage genes except that of the repressor. Prophages remain dormant until the lytic cycle is induced by number of physical or chemical agents, such as mitomycin C, hydrogen peroxide, UV radiation, temperature and pressure (Jiang and Paul, 1996; Weinbauer and Suttle , 1999). This process is called induction.

2.2.2.3 Pseudolysogenic infection

Pseudolysogeny is a form of phage-host cell interaction, where the nucleic acid of the phage resides within the bacterial cell in an unstable, inactive state. Pseudolysogeny occurs mainly when host is exposed to starvation (Williamson *et al.,* 2001). When proper nutrients are added, the pseudolysogenic state is resolved

resulting in either the establishment of true lysogeny or the initiation of the lytic production of progeny virions (Ripp and Miller, 1997). Pseudolysogeny provides a means by which phage can maintain themselves in the large numbers observed in what would appear to be hostile habitats (Bergh *et al.,* 1989; Paul *et al.,* 1991; Hennes and Suttle, 1995).

2.2.2.4 Chronic or continuous infection

A chronically infecting phage can release progeny into the extracellular environment without terminating its infection, i.e. phages are extruded across the host cell envelope continuously without destroying either the infected bacterium or the phage multiplication (Abedon, 2008). The intracellular chronic cycle has been best described for filamentous phages of *E. coli* (Maniloff *et al*., 1981; Calender and Inman, 2005). The extrusion process driven by phage-encoded proteins located in the cell envelope preserves the integrity of the cell membrane of the host (Snyder and Champness, 2003).

2.2.2.5 Restricted infection

Passive or active bacteria-mediated death of infecting phage occur via restriction endonucleases. Here phage does not get a chance to replicate and produce progeny (Abedon, 2008). Restriction endonucleases fragment the double stranded phage DNA by cleaving at specific sites, which are then further degraded by other endonucleases thereby preventing infection by effectively destroying the foreign DNA introduced by phages. Approximately one quarter of all known bacteria possess Restriction Modification (RM) systems and about one half of those have more than one type of system. The methylation of the bacterial genome by methyltransferase confers protection from its own endonucleases as methylated

sites are not recognized by the endonucleases (Arber and Linn 1969; Enikeeva *et al*., 2010).

2.2.2.6 Abortive infection

Various mechanisms enable bacteria to resist bacteriophage infection. One such strategy includes the abortive infection (Abi) systems. Abi systems provide population protection by promoting "altruistic suicide" of an infected bacterium (Forde and Fitzgerald, 1999). This decreases the number of progeny phage particles, limiting their spread to other cells thereby allowing the bacterial population to survive (Chopin *et al*., 2005). The majority of Abis have been found on plasmids of Gram-positive lactococcal strains (Chopin *et al*., 2005), but a few have been found in Gram-negative species, including *Escherichia coli*, *Vibrio cholerae* and *Shigella dysenteriae* (Smith *et al*.,1969; Chowdhury *et al*.,1989; Snyder,1995)

The most common types of life style is depicted in the Fig.2.2.

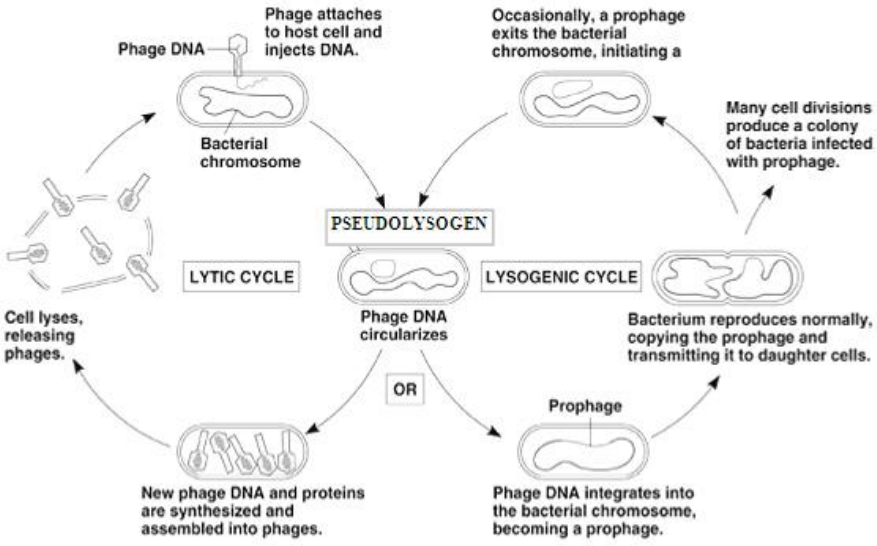


Fig.2.2 Lifestyle of bacteriophage (Copyright ©Pearson Education, Inc, publishing as Benjamin Cummings)

2.2.3 Mechanism of bacteriolysis by phages

The first step in phage infection is adsorption to the protein or sugar receptor on the bacterial surface. Phages absorb only to specific bacterial species or specific strains. Phage therapy can therefore eradicate target bacteria without disturbing the normal flora. After adsorption, phage DNA is injected into the bacterial cytoplasm, the DNA is replicated and these multiple copies of DNAs are packed into the capsid, which is constructed *de novo* during the late stage of phage infection. Descendant phage particles are completed by the attachment of a tail to the DNA-filled head.

The progeny phage production requires compromising the structural integrity of the murein sacculus, which forms a tough, dynamic meshwork that surrounds and confers the shape on the cell (Holtje and Glauner, 1990). It is carried out by the coordinated action of two proteins, holin and endolysin (lysin) encoded by the phage genome. Holin proteins form a "hole" in the cell membrane, enabling lysin to reach the outer peptidoglycan layers (Young, 1992). Lysin is a peptidoglycan-degrading enzyme (peptidoglycan hydrolase). At least four different muralytic activities are found in phage endolysins: transglycosidases and lysozymes that attack the glycosidic bonds linking the amino-sugars in the cell wall, amidases and endopeptidases that attack the amide and peptide bonds of the oligopeptide cross-linking chains (Young, 1992). Although the endolysins, like the T4 E lysozyme, the T7 gp 3.5 amidase and the lamda R transglycosylase are small globular proteins, the recently identified endolysins possess multiple muralytic activities and greater molecular masses≥60kDa (Navarre *et al*., 1999). The mechanism of bacteriolysis by phage is depicted in Fig 2.3.

The released descendant phages infect neighbouring bacteria in quick succession. Even though the initial phage number is less than that of the bacteria,

it exceeds that of bacteria after several generations, eventually lysing the entire bacterial population (Matzusaki *et al.*, 2005).

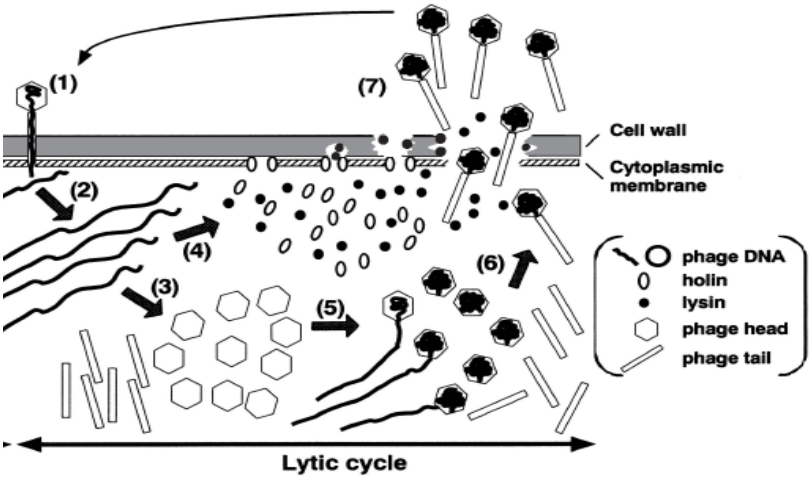


Fig.2.3 Mechanism of bacteriolysis by phage (figure adapted from Matsuzaki *et al*., 2005)

All phages resort to lysis of the host cell for release and dispersion of progeny as explained above except filamentous phage (Young *et al*., 2000) and small single stranded DNA or RNA phages (Matsuzaki *et al*., 2005).

Filamentous phage particles do not form in the cytoplasm; rather they are continually extruded or secreted across the bacterial membranes as they are assembled, without causing cell lysis. They do not kill their host and the infected cells continue to grow and divide indefinitely while producing phage (Model and Russel, 1988).

Some small phages such as ΦX174 and Qβ, which have single stranded DNA and RNA respectively, do not have the genes for holin or lysin proteins (Bernhardt *et al*., 2000a; 2000b; 2001). They accomplish lysis by producing a protein that inhibits cell wall biosynthesis. Their inhibitory gene products are known as "protein antibiotics". The ΦX174 gene product, *gpE* inhibits MraY

which catalyzes the formation of the first lipid-linked murein precursor while Qβ gpA2 inhibits MurA which catalyzes the first step in the murein biosynthesis pathway (Bernhardt *et al*., 2000a; 2000b; 2001).

2.2.4 Bacteriophages and therapy

The potential of phages as a therapeutic agent was first identified and put into test by Felix d'Herelle. He used phages to successfully treat a 12-year-old boy with severe dysentery. He also succeeded in treating three other patients with the same disease (Sulakvelidze *et al.*, 2001). During his stay in India he worked on phage therapy of plague at the Haffkine Institute, Bombay, where he treated several patients affected with cholera and bubonic plague (Summers, 1999). d'Herelle commercialized bacteriophage preparations as therapy for infectious diseases. These products—Bacté-coli-phage, Bacté-rhino-phage, Bacté-intesti-phage, Bacté-pyo-phage and Bacté-staphy-phage were marketed by the Societé Francaise de Teintures Inoffensives pour Cheveux (Safe hair dye company of France; now L'Oréal) (Sulakvelidze *et al.*, 2001). Success with phage therapy was repeated by many other researchers worldwide. Unfortunately, most of this study was conducted without proper characterization of phage (lytic or lysogenic), their host range etc. In addition, clinical investigations in the 1920s and '30s were carried out without proper standards or control. Furthermore, the discovery of antibiotics during the Second World War, served to marginalize the use of phage therapy in most western countries (Fischetti *et al.*, 2006). Nevertheless phages continue to be used therapeutically together with or instead of antibiotics in Eastern Europe and in the former Soviet Union (Alisky *et al.*, 1998). Major institutes involved in phage research are Eliava Institute of Bacteriophage, Microbiology and Virology (EIBMV) of the Georgian Academy of Sciences, Tbilisi, Georgia and the Hirszfeld Institute of Immunology and Experimental Therapy (HIIET) of the Polish Academy of Sciences, Wroclaw, Poland

(Sulakvelidze *et al.*,2001). However with the emergence of bacterial multi drug resistance, western scientific community is now seriously considering phage therapy for combating bacterial diseases that are inoperable by antibiotics as reviewed by Barrow and Soothill, 1997, Alisky *et al.*, 1998 and Fischetti *et al.*,2006. Works related to phage therapy started reappearing in the English literature with the work of Smith and Huggins in the 1980s (Smith and Huggins 1982; Smith and Huggins 1983; Smith *et al.*, 1987a; Smith *et al.*, 1987b). But the western phage therapy renaissance gained momentum only in the 1990s, as access was increasingly gained to the rich trove of Soviet and Polish work (Abedon *et al.*, 2011). Presently various laboratories worldwide and a handful of small pharmaceutical companies are shifting their attention towards phages as therapeutic and biocontrol agent (Thiel, 2004). Recently in the United States, a FDA approved phase I clinical trial was conducted. No safety concerns were found (Kutter *et al.*, 2010). A British phage therapy company conducted a phase I/IIa clinical trial in chronic otitis which gained approval through the UK Medicines and Healthcare products Regulatory Agency (MHRA) and the Central Office for Research Ethics Committees (COREC) ethical review process (Kutter *et al.*, 2010). Furthermore several companies have successfully developed phage-based products with Environmental Protection Agency (EPA), USDA and FDA approval. Products targeting *Listeria monocytogenes* represent one of the first examples of phage cocktails to obtain Generally Recognized As Safe (GRAS) status from the FDA (Lu and Koeris, 2011). These products are designed to be used as sterilizing agents on processed foods (ListShield™ and LISTEX™ P100). Several other similar products are in various stages of development against other bacterial pathogens including *Escherichia coli* strains (O157:H7) and *Salmonella enterica* (Abuladze *et al*., 2008).

2.2.4.1 Advantages of phage therapy over antibiotic

Majority of the phages have a narrow host range (Ackerman *et al.*, 1978). High host specificity is the most significant criteria of a phage when considered as a biocontrol agent, as only the targeted bacterial species are attacked by the phage leaving behind the beneficial microflora unharmed (Kutter and Sulakvelidze, 2005). Whilst, most of the antibiotics used are broad spectrum, killing not only the targeted bacteria but also the harmless natural microflora leading in turn to secondary infections by opportunistic bacteria (Chernomordik, 1989). Moreover, the resistance developed towards a particular antibiotic is never limited to the targeted bacteria, but may well be acquired by all other related bacteria (Sulakvelidze, 2001) by horizontal gene transfer. Bacteria try to acquire resistance against phages by altering the phages receptor sites on their cell surface. But as these receptors sites are important in conferring virulence, any alteration in these vital sites may in turn lead to attenuation of virulence or may even affect their viability (Cislo *et al.*, 1987). Even if bacteria do overcome all the above said factors and acquire resistance, natural selection aids phages to overcome the phage-resistant bacterium (Sulakvelidze *et al.*, 2001).

Unlike antibiotics, repeated administration is not required as phages are living entitles, that multiply until their host (bacteria) is present (Inal, 2003). Since phages consist mostly of nucleic acids and proteins, they are inherently nontoxic (Skurnik *et al*., 2007; Abedon and Abedon, 2010; Kutter *et al*., 2010). However, phages can interact with the immune system, at least potentially resulting in harmful immune responses, although there is little evidence for this and therefore this is actually not a cause for concern during phage treatment (Alisky *et al*., 1998; Carlton, 1999; Górski *et al*., 2007; Kutateladze and Adamia, 2010). Furthermore, administration of phages may have only minor side effects due to the liberation of endotoxins from the bacterial cell lysis (Cislo *et al.*, 1987, Slopek *et al.*, 1987).

Such effects may also be observed when antibiotics are used (Prins *et al.*, 1994). Another important aspect is the lack of cross-resistance with antibiotics. Because phages infect and kill using mechanisms that differ from those of antibiotics, specific antibiotic resistance mechanisms do not translate into mechanisms of phage resistance. Consequently, phages can readily be employed to treat antibiotic-resistant infections (Alisky *et al*., 1998; Carlton, 1999; Górski *et al*., 2007; Skurnik *et al*., 2007; Kutter *et al*., 2010). Another advantage is that phages like antibiotics, can be versatile in terms of formulation development, e.g. being combined with certain antibiotics (Alisky *et al*., 1998; Kutter *et al*., 2010). They are also versatile in terms of application in form of liquids, creams or impregnated into solids, to name a few, in addition to being suitable for most routes of administration (Carlton 1999; Górski *et al*., 2007; Kutateladze and Adamia, 2010; Kutter *et al*., 2010). Different phages furthermore can be mixed as cocktails to broaden their properties, typically resulting in a collectively greater antibacterial spectrum of activity (Goodridge, 2010; Kutateladze and Adamia 2010; Kutter *et al*., 2010).

Unlike broad-spectrum chemical antibiotics, phages have a low environmental impact when discarded, as they are composed predominantly of nucleic acids and proteins (Abedon and Abedon, 2010). They possess relatively narrow host ranges (Hyman and Abedon, 2010) and therefore will at worst have an impact only on a small subset of environmental bacteria (Ding and He, 2010). Phages not adapted to degradative environmental factors, such as sunlight, desiccation or temperature extremes can be rapidly inactivated. Finally, developing new antibiotics is a time-consuming process, taking several years and involving millions of dollars (Silver and Bostian, 1993). Isolating novel phages which are active against pathogens are relatively simpler and costs of discovery (isolation) and characterization are economical (Skurnik *et al*., 2007).

2.2.4.2 Hurdles in phage therapy and possible solutions

Bacterial cell lysis due to antibiotic and phage therapy may release endotoxins, a component of the outer membrane in Gram-negative bacteria, which may lead to general pathological aspects of septicaemia (Slopek *et al*., 1983).

One strategy for avoiding cellular disintegration is the use of nonlytic filamentous phage as a delivery vehicle for genes encoding proteins that are toxic when synthesised in the cytoplasm of the host (Hagens *et al*., 2004). In the study, export protein gene of the *P. aeruginosa* filamentous phage Pf3 was replaced with a restriction endonuclease gene. This rendered the Pf3 variant (Pf3R) non-replicative and concomitantly prevented the release of the therapeutic agent from the target cell. Although the mutant phage could not multiply in *P. aeruginosa* cells, the restriction endonuclease expressed by the injected phage DNA digested the host genomic DNA and consequentially killed the bacteria with minimal release of endotoxin *in vitro* (Hagens *et al*., 2004).

A phage display technique was also shown to tackle this problem in the pathogen *Helicobacter pylori* (Cao *et al.,* 2000). A modified filamentous phage M13, which expressed a coat protein fused with part of an antibody specific to an antigen on the cell surface was constructed. The modified M13 did not multiply in *H. pylori*, but suppressed its growth *in vitro*. Macrophages then quickly cleared the phage-filled bacterial ghosts (Fischetti *et al*., 2006).

A second type of approach relied on whole phage as transport vehicles for delivery of chemically linked antibiotics to target bacteria. Yacoby *et al*., (2007) in their work reported success of antibody-targeted phage nanoparticles to carry a large payload of the antibiotic chloramphenicol connected through the aminoglycoside neomycin and demonstrated complete growth inhibition toward

the pathogens *Staphylococcus aureus*, *Streptococcus pyogenes* and *Escherichia coli.*

Bacteriophages, just as any other foreign proteins, tend to be rapidly cleared from the circulation when applied. This problem was first recognized in a study in non-immune germ-free mice (Geier *et al*., 1973), where it was discovered that the phages were rapidly cleared by the spleen, liver and other filtering organs of the reticulo-endothelial system, when injected in high titers. To overcome that, a serial passage technique was developed in mice to select for phage mutants able to remain in the circulatory system for longer periods of time. By this approach the long-circulating mutants of *Escherichia coli* phage A and of *Salmonella* Typhimurium phage P22 were isolated (Geier *et al*., 1973). A study also demonstrated that the long-circulating mutants have greater capability as antibacterial agents than the corresponding parental strain in animals infected with lethal doses of bacteria (Merril *el al*., 1996). Drug-delivery technologies, such as polymer-based coatings can also enhance systemic phage delivery and reduce phage inactivation and clearance (Kim *et al*., 2008).

Escherichia coli strains that produce the K1 polysaccharide capsule have long been associated with pathogenesis. This capsule is believed to increase invasiveness, allowing the bacteria to avoid phagocytosis, inactivation by complement and physically blocks infection by T7, a phage that recognizes LPS as the primary receptor. This defensive mechanism of bacteria prevents the use of phage as biocontrol agent. Enzymatic bacteriophages can be engineered to degrade barriers to phage adsorption (Scholl *et al*., 2005).

Formation of bacterial biofilms is crucial in the pathogenesis of many clinically important infections and is difficult to eradicate because they exhibit resistance to antimicrobial treatments, removal by host immune systems and

phages. It has been demonstrated that bacteriophage engineered to express a biofilm-degrading enzyme can successfully disrupt the structure of bacterial biofilms, thereby overcoming this problem (Lu and Collins, 2007).

Phages are basically species-specific antibacterial agents (Hagens and Loessner, 2007), therefore can be targeted far more specifically than most antibiotics to particular bacteria resulting in much less damage to the body's normal microbial balance. Although an advantage, this extremely refined host specificity enables phage to attack only one species or even a single bacterial strain. However broad range within the genus is necessary for phage and usually it is difficult to find a phage with broad host range within the genus. Another aspect of phage mediated biocontrol is the emergence of phage-resistant mutants of pathogens rendering phage ineffective just like antibiotics becoming ineffective against antibiotic resistant bacteria. Thus limited phage host ranges and the evolution of phage-resistant cells is a major hurdle in the successful use of phage as biocontrol agent. Phage cocktails are a major solution on offer for addressing both these issues. Another solution is the use of endolysin. Endolysins are double-stranded DNA bacteriophage encoded peptidoglycan hydrolases produced in phage-infected bacterial cells toward the end of the lytic cycle (Young, 1992). They reach the peptidoglycan through membrane lesions formed by holins. They cleave the peptidoglycan thereby inducing bacterial cell lysis and enabling progeny virions to be out (Loessner, 2005; Young *et al*., 2000). The potential of lysins to digest the cell wall (especially in Gram-positive bacteria) when applied from outside bacterial cells has enabled their utility as substitute antibacterial (Borysowski *et al*., 2006). The inability of bacteria to gain resistance against endolysin is because a cell has to change its wall structure to become lysin resistant, which is a difficult task. Indeed, even after 40 cycles of exposing bacteria to a particular lysin, resistant strains did not develop (Fischetti, 2008).

The capability of a partially purified lysin to kill bacteria was first reported way back in 1959 (Freimer *et al*., 1959). However, the ability of lysin as an antibacterial has been demonstrated only recently (Nelson *et al*., 2001). *In vivo* efficacy of lysin treatment using mice challenged by *Streptococcus pyogenes* (Nelson *et al*., 2001), *S. pneumoniae* (Loeffler *et al*., 2001; Jado *et al*., 2003; Loeffler *et al*., 2003; Loeffler and Fischetti, 2003), *Bacillus anthracis* and group B *Streptococcus* (Cheng *et al*., 2005) has been examined. But in Gram negative bacteria, the peptidoglycan layer surrounded by the outer membrane is impermeable to these macromolecules (Vaara, 1992) making them endolysin resistant (Loessner, 2005). However some endolysins are capable of killing Gram-negative bacteria despite the presence of the outer membrane by means of their C-terminal membrane-active peptide sequences (Dùring *et al*.,1999; Orito *et al*., 2004). The ability of the C-terminal region of *Bacillus amyloliquefaciens* phage endolysin on *Pseudomonas aeruginosa* lysis via the permeabilization of the outer membrane was proved (Orito *et al*., 2004).

2.2.4.3 Safety of bacteriophages

The contribution of bacteriophages to the pathogenicity of their bacterial hosts began to be uncovered as early as 1927, when Frobisher and Brown discovered that non-toxigenic Streptococci exposed to filtered supernatants of toxigenic Streptococcal cultures acquired the ability to produce scarlatinal toxin (Frobisher and Brown, 1927). This was followed by many reports with similar content as reviewed by Boyd and Brüssow (2002). Genome sequencing can help to rule out the use of bacteriophages containing undesirable genes encoding toxins or integrases (Denou *et al*., 2009). The safety of bacteriophage preparations is also a matter of concern. However, purification techniques have evolved considerably in the last few years and standard procedures may eventually be developed,

improving the homogeneity and safety of these preparations (Courchesne *et al*., 2009; Merabishvili *et al*., 2009; Gill and Hyman 2010; Kramberger *et al*., 2010).

2.2.5 *Salmonella* phages

Based on a compilation of bacteriophage names assembled and supplemented by a scan of the scientific literature up to 2005, some 170 *Salmonella* phages have been named (Kropinski *et al*., 2006). Among the large tailed viruses of the order Caudovirales (Ackermann, 1998), 44 *Myoviridae* phages, 65 *Siphoviridae* phages and 63 *Podoviridae* phages have been listed. These are grouped based on their phylogenic relatedness deduced using a phage proteomic tree approach (Kropinski *et al*., 2006) analogous to that of Rohwer and Edwards (2002) method. The "Phage Proteomic Tree," which is based on the overall similarity of completely sequenced phage genomes, places phage relative to both their near neighbours and all other phage included in the analysis. This method groups phage into taxa that predicts several aspects of phage biology and highlights genetic markers that can be used for monitoring phage biodiversity. The phage proteomes were compared by researchers using CoreGenes (Zafar *et al*., 2002) a BlastP algorithm employing a cutoff score of 100 to ensure identification of homologs.

CoreGenes analysis revealed that the *Salmonella* phages fall into five groupings viz. P27- like, P2-like, lambdoid, P22-like and T7-like and include three outliers i.e., ε15, KS7 and Felix O1. The P27 group is represented only by ST64B; the P2 group contains SopEΦ, Fels-2 and PSP3; the lambdoid *Salmonella* phages include Gifsy-1, Gifsy-2 and Fels-1. The P22-like viruses include P22, ST104, ES18, ε34 and ST64T. The only member of the T7-like group is SP6 (Kropinski *et al*., 2006).

2.2.5.1 *Salmonella* phage groups

2.2.5.1.1 P27- like *Salmonella* phages

P27 like phages belong to group C in Bradley's morphological classification (Bradley, 1967), with a hexagonal head symmetry and a short, noncontractile tail terminating in a base plate (Vieu *et al*., 1965). The P27 group is only represented by ST64B.

2.2.5.1.1.1 ST64B

Phage ST64B, like phage ST64T, was isolated from *S. enterica* serovar Typhimurium DT 64 strain 2558 (Mmolawa *et al.,* 2002). Except for the lack of a tail structure, this phage is morphologically identical to ST64T and possesses a genome of 40.1 kb (51.3% G+C). Its genomic layout is very similar to that of coliphage lamda. The DNA packaging, capsid and tail genes of ST64B are most closely related (and collinear) to those of ΦP27, a shiga toxin-carrying siphovirus (Recktenwald and Schmidt, 2002) and the serotype-converting *Shigella flexneri* phage V that is a member of the *Podoviridae*. This phage is defective in that it cannot propagate (Allison *et al.,* 2002).

2.2.5.1.2 P2-like *Salmonella* phages

The P2-like phages are temperate members of the *Myoviridae* and include coliphages P2 and 186, *Pseudomonas* phage ΦCTX, *Haemophilus* phages HP1 and HP2 and *Salmonella* phages SopEΦ, Fels-2 and PSP3.

2.2.5.1.2.1 SopEΦ

SopEΦ is a temperate P2-like *sopE*-encoding phage (SopEΦ) derived from *S.* Typhimurium strain DT204 upon induction with mitomycin C. They

infect sensitive *Salmonella* strains in a FhuA, TonB-independent manner. It carries the type III effector protein SopE that binds to and transiently activates eukaryotic RhoGTPases (Rudolph *et al.,* 1999). Experiments using a derivative of SopEΦ implicate lysogenic conversion as an important mechanism facilitating horizontal transfer of type III-dependent effector proteins (Mirold *et al.,* 1999).

2.2.5.1.2.2 Fels-2

The Fels-2 phage has a head 55 nm in diameter and a contractile tail 110 nm×20 nm (Yamamoto, 1969). The prophage genome is 33.7 kb (52.5% G+C) and appears to be bounded by 47 bp direct repeats. As a prophage, it is in the genome of *Salmonella* Typhimurium LT2. It was demonstrated that the Fels-2 prophage could recombine with the morphologically unrelated P22 phage to give rise to F22 that is morphologically and serologically related to Fels-2 but carries the P22 genes (Yamamoto and McDonald, 1986).

2.2.5.1.2.3 PSP3

Phage PSP3 was originally isolated from *Salmonella potsdam* but can lysogenize *E. coli.* DNA of phage PSP3 contained both circular and linear molecules and is about 31 kb. Linear PSP3 DNA molecules possess single-stranded cohesive termini (cos). Sequencing of the fragment anticipated to contain cos revealed a 19-base sequence identical to cos of phage 186. Of the 107 bp to the right of cos, 94 were identical in 186 DNA (88% similarity) and of the 370 bp to the left, 229 were identical (62% similarity) (Bullas *et al*., 1991).

2.2.5.1.3 Lambdoid group *Salmonella* phages

Three complete lambda-related prophages belonging to the family *Siphoviridae* (Gifsy-1, Gifsy-2 and Fels-1) have been identified in the sequenced *Salmonella* genomes (Yamamoto, 1967 and 1969; Figueroa-Bossi *et al.*, 1997, 2001).

2.2.5.1.3.1 Gifsy-1

This 47.8 kb prophage of *Salmonella enteric* serovar Typhimurium is mitomycin C and UV-inducible (Slomiński *et al.*, 2007). The capsid measures approximately 60 nm in diameter and possesses a flexible tail approximately 133 nm in length. The surface receptor for this phage and for Gifsy-2 is *OmpC* and therefore, these phages propagate most easily on rough mutants of *Salmonella* wherein this outer membrane protein is surface exposed rather than obscured by a coating of LPS (Ho and Slauch, 2001). Gifsy-1 carries a number of potential virulence modulating genes including *gogB.*

2.2.5.1.3.2 Gifsy-2

This is a 45.5 kb of prophage of *Salmonella enteric* serovar Typhimurium (Bossi and Figueroa-Bossi, 2005). Induction results in the release of a siphovirus with a head diameter of approximately 55 nm. Gifsy-2 also carries a range of potential virulence determinants, two of which-*gtgA* and *sodC1* (periplasmic superoxide dismutase) have been implicated in the host pathogenesis (Ho *et al*., 2002).

2.2.5.1.3.3 Fels-1

Induction of *S.* Typhimurium LT2 led to the discovery of two serologically and morphologically different phages (Fels-1 and Fels-2) (Yamamoto, 1969). Fels-1 at 41.7 kb is clearly a member of the lambdoid *Siphoviridae*. It is integrated between the host genes *ybjP* and STM0930 and carries two potential virulence genes: *nanH* (neuraminidase) and *sodC3* (superoxide dismutase) (Figueroa-Bossi *et al*., 1997, 2001).

2.2.5.1.4 P22-like *Salmonella* phages

P22-like phages are identified by their prominent base plates with six spikes. Phage heads often show capsomeres. Phages are temperate and associated with *S.* Typhimurium. They are known for their transducing and converting ability. P22-like phages are common in salmonellae and *Proteus* bacteria and have also been reported in marine vibrios (Ackermann and DuBow, 1987). The P22-Like phage genus is proposed to include *Salmonella* phages P22, ST104, ES18, ε34 and ST64T (Kropinski *et al*., 2006).

2.2.5.1.4.1 P 22

It is one of the best-studied bacterial virus. P22 is a temperate dsDNA phage, whose morphology puts it in the formal *Podoviridae* group. It was originally isolated after induction in 1952 of a *Salmonella* Typhimurium lysogen that was from either Sweden or Chile. Many studies have suggested that P22, despite its morphology, is a member of the lambdoid family (Kropinski *et al*., 2006).

2.2.5.1.4.2 ST104

Mitomycin C treatment of *S. enterica* serovar Typhimurium DT104 resulted in the induction and isolation of phage ST104, which based on its close homology to phage P22 and the lack of a gene for a tail tape measure protein, is a member of the *Podoviridae* (Tanaka *et al*.,2004). Its genome is 41.4 kb (47.3% G+C) and it encodes at least 65 proteins, a high percentage of these are homologous to proteins of ε34, P22 and ST64T (Kropinski *et al*., 2006).

2.2.5.1.4.3 ES18

Originally isolated in 1953, bacteriophage ES18 is a temperate, broad host range, generalized transducing virus of O antigen-containing (smooth) and O antigen-lacking (rough) strains of *S. enterica* (Kuo and Stocker, 1970; Le and Chalon, 1975). Morphologically, this phage possesses an isometric head 56 nm in diameter and a long tail (121 nm×12 nm), making it a member of the *Siphoviridae* (Casjens *et al*., 2005). As such, it differs from other transducing phages in this genus which require smooth LPS. The cellular receptor for ES18 is FhuA, the outer membrane protein involved in ferrichrome transport (Killmann *et al*., 2001).

2.2.5.1.4.4 *ε34*

Bacteriophage *ε34* has an isometric head 62.5 nm in length, a neck 11 nm in width and a short tail 4.4 nm in width by 5.5 nm in length and is characterized by its ability to infect, lysogenize and seroconvert *S. enterica* serovar Anatum *ε* 15 lysogens. The sequence of the genome of this bacteriophage was recently completed. *ε34* has a 43.0 kb genome, with 47.3% G+C. It showed considerable overall sequence and spatial similarity to P22 (Greenberg *et al*., 1995).

2.2.5.1.4.5 ST64T

This mitomycin C induced phage, along with ST64B was from *S. enterica* serovar Typhimurium DT64 and like P22 is a generalized transducer with a capsid 50 nm in diameter (Mmolawa *et al.,* 2003). It is currently classified as a P22-like virus by National Center of Biotechnology Information (NCBI). It's genome is 40.7 kb with a G+C content of 47.5%. It is also a serotype-converting phage possessing homologs of the P22 *gtrABC* operon. Lysogenization by this phage is probably also responsible for converting *S.*Typhimurium phage type (DT) 9 to 64, 135 to 16 and 41 to 29 (Mmolawa *et al.,* 2002).

2.2.5.1.5 T7-like *Salmonella* phages

Coliphage T7 is one of the best-studied virulent (lytic) bacteriophages and one of the first to have been completely sequenced (Dunn and Studier, 1983). One of the interesting features of this group of phages is the under-representation of certain common restriction sites—for example, both T7 and SP6 are similar in mass and mol% GC content but show no DNA homology, lack sites for *Bam*HI, *Pst*I, *Sac*I, *Sac*II, *Sal*I, *Sma*I and *Sph*I (Kropinski *et al.,* 2006). The only T7-like *Salmonella* phage is SP6.

2.2.5.1.5.1 SP6

SP6 has a 43.8 kb (47.2% G+C) genome (Dobbins *et al*., 2004, Scholl *et al*., 2004). Although NCBI currently lumps it with "unclassified T7-like viruses" using (Chen and Schneider, 2005) criteria, it is a T7-like phage described as belonging to "an estranged subgroup of the T7 supergroup" showing the closest relationship to coliphage K1-5 (Scholl *et al.,* 2004).

2.2.5.1.6 *Salmonella* phages that are Orphans or outliers
2.2.5.1.6.1 Felix O1

Originally known as phage O1, this virus is a member of the *Myoviridae* (Ackermann and DuBow, 1987*)*, originally isolated by Felix and Callow (Felix and Callow, 1943). This phage lysed well over 99% of *Salmonella* strains it was tested on (Kallings, 1967*)*. It has been widely used as a diagnostic test for this genus and is an excellent candidate for use in bioremedial and therapeutic applications. DNA of the phage was found to be a double-stranded linear molecule of about 80 kb. 11.5 kb has been sequenced and the % A + T content is 60% in this region. There are relatively few restriction endonuclease cleavage sites in the native genome and clones show that this is due to their absence rather than modification (Kuhn *et al*., 2002).

2.2.5.1.6.2 KS7

The 40.8 kb genome of this phage has been recently sequenced by a group at Korea National Institute of Health, Laboratory of Enteric Infections, in Seoul (South Korea) (NC_006940). Although classified as an "unclassified bacteriophage" at NCBI (Kim *et al*., 2005), the data entry suggests that this is a member of the P22-like viruses.

2.2.5.1.6.3 εs15

ε 15 is a short tailed dsDNA bacteriophage infecting *S. enterica* serovar Anatum. Its genome contains 39,671 base pairs with 49 open reading frames (Jiang *et al.,* 2006). The receptor for phage ε15 is the O antigen of *S. enterica* serovar Anatum that belongs to serogroup E1, expressing serotype factors 3 and 10. The structure of the phage was revealed by cryoelectron microscopy and

proteomics (Jiang *et al.,* 2006). The latter analysis also revealed that the virus particles contain capsid protein (gp*7*), which is partially proteolytically processed and may also be, in part cross linked to a 12-subunit portal complex (gp*4*), six bulbous tailspikes (gp*20*) and a tail hub probably composed of gp*15*, gp*16* and gp*17*(Jiang *et al.*,2006).

2.2.5.2 Therapy using *Salmonella* phages

Interestingly *Salmonella* phages were the first phages examined for their ability to prevent and treat bacterial infections in various settings. Felix d'Herelle , was the pioneer who initiated the use of *Salmonella* bacteriophages isolated from chicken. He treated birds experimentally infected with *Salmonella* Gallinarum. It was a very small-scale study but the results were promising. Later he successfully used it for treatment of infected chickens that caused cessation of the epidemic (Summers, 1999).

d'Herelle's early phage therapy studies were followed by many studies checking the potential of *Salmonella* phages to prevent and treat salmonellosis. Fisk reported that by injecting antityphoid phage, mice were strongly protected from subsequent infections (Fisk, 1938). This was followed by several studies giving promising results. With the advent of antibiotics, combined with the lack of knowledge of the mechanisms involved in phage mediated bacterial cell death, the focus of the western world research turned away from phage therapy. But with the emergence of antibiotic resistance, phages are now seriously being reconsidered as an alternative. *Salmonella* is one among many several other pathogens, against whom phages are considered as biocontrol agents.

In the former Soviet Union, *Salmonella* phages have been effectively used to prevent salmonellosis in children (Kiknadze *et al*., 1986). HIIET in Poland have

also reported clinical applications for *Salmonella* phages. The institute has used its large collection of bacteriophages to treat various bacterial infections in several hospitals in Poland ever since its establishment in 1952. The most commonly targeted bacterial pathogens included *S. aureus, P. aeruginosa* and *E. coli*, but phages lytic for *Salmonella* were also employed successfully to treat human infections (Slopek *et al*., 1987). Presently therapeutic *Salmonella* bacteriophages are commercially produced by at least one company in Russia: ImBio currently manufactures several phage-based therapeutics, including a *Salmonella* phage cocktail ("Bacteriophagum salmonellae gr.ABCDE liquidum") targeting approximately 10 different *Salmonella* serotypes (http://home.sinn.ru/~imbio/ Bakteriofag.htm).

Boury (2005) has tested the ability of a well-known *Salmonella* bacteriophage Felix 01 and two recently isolated phages (HL03 and HL18), to reduce the *Salmonella* Typhimurium burden in orally challenged, susceptible mice. He found out that there was significant reduction in the *Salmonell*a burden in the treated mice when the phages were given at a 1:10, 1:50 and 1:100 MOI when compared to the bacterial challenge dose. These data indicated that pathogen can be potentially limited during transport and lairage of swine prior to slaughter by phage application.

A study was conducted to test the efficacy of *Salmonella* specific bacteriophages and competitive exclusion to reduce *Salmonella* colonization in experimentally infected chickens, wherein a "cocktail" of distinct phage (i.e., phage showing different host ranges and inducing different types of plaques on *Salmonella* Typhimurium cultures) was developed. The results indicated a protective effect of both *Salmonella*-specific bacteriophages and a defined competitive exclusion product against *Salmonella* colonization of experimentally infected chickens. These findings encourage further work on the use of phages as

an effective alternative to antibiotics to reduce *Salmonella* infections in poultry (Toro *et al.*, 2005).

Atterbury and coworkers reported that phages administered in antacid suspension to birds experimentally colonized with specific *Salmonella* host strains, effectively reduced the bacterial count. Even though the phage resistance was observed, the authors concluded that the selection of appropriate bacteriophages and optimization of both the timing and method of phage delivery were key factors in the successful phage-mediated control of salmonellae in broiler chickens (Atterbury *et al.*,2007).

In another study, to investigate the effect of the method of delivery on the efficacy of phage therapy, three phages were used to reduce *S.* Enteritidis colonization in experimentally infected 10 day old chicken. The chicken were treated by coarse spray or drinking water with a cocktail of the three phages 24 hours before challenge with *S.* Enteritidis. Bacterial count was taken from 20 day old chicken. It was found out that the phage treatment, either by aerosol spray or drinking water, significantly reduced *Salmonella* infection in poultry (Borie *et al.*, 2008).

2.2.5.3 *Salmonella* phages for improving food safety

A possible and novel application of bacteriophages, wherein they are directly applied onto food products or onto environmental surfaces in food processing facilities, in order to reduce the levels of food borne bacterial pathogens in food has recently generated increased interest. But is case of *Salmonella* it is not an easy task. About 95% of natural strains of *S.* Typhimurium, for example, reportedly contain temperate phages as prophages (Schicklmaier *et al.*, 1998), which may prevent super-infection, by similar phages (Porwollik and

McClelland, 2003). Cocktails comprising multiple phages and specificities may be required to overcome this problem (McLaughlin *et al.,* 2006).

Lytic phages specific for *Salmonella* have reduced experimental *Salmonella* contamination in a variety of sources. Many of the experiments conducted by different scientists to evaluate the effectiveness of specific phages in killing *Salmonella* have given very promising results. Details of some of the above said experiments are discussed. The first report on this approach with *Salmonella* was recently published where the authors studied the ability of phages to reduce experimental *Salmonella* contamination of fresh-cut melons and apples stored at various temperatures (Leverentz *et al*., 2001).

Pao and coworkers too evaluated the potential of bacteriophages to control *Salmonella* in sprouting seeds in a study, where two phages (Phage-A, capable of lysing *S.* Typhimurium and *S.* Enteritidis and Phage-B, capable of lysing *S.* Montevideo) were isolated and characterized as members of the *Myoviridae* and *Siphoviridae* families. A log1.37 CFU suppression of *Salmonella* growth was achieved by applying Phage-A on mustard seeds. The mixture of Phage-A and Phage-B caused a log1.50 CFU suppression of *Salmonella* growth in the soaking water of broccoli seeds (Pao *et al*., 2004).

Reducing *Salmonella* contamination in poultry is of major importance to prevent the introduction of this microorganism into the food chain. Salmonellae may spread during storage time (shelf life) whenever pre harvest control fails or post-harvest contamination occurs. Therefore, preventive measures should also be included in the post-harvest level of poultry production to control salmonellae. A study conducted where chicken skin samples were experimentally contaminated by immersing whole legs (thighs and drumsticks) in a suspension of *Salmonella* Enteritidis phage type 4 (SE PT4) on the slaughter day. This followed by one

exposure to three wild *Salmonella*-lytic bacteriophages, previously isolated from feces of free range chickens showed a significant reduction in the bacterial count. These findings suggest that the use of bacteriophages may reduce SE PT4 in chicken skin (Fiorentin *et al*., 2004). In another study by the same author, bacteriophages isolated from free-range chickens were tested as a therapeutic agent for reducing the concentration of *Salmonella enterica* serovar Enteritidis phage type 4 (*S.* Enteritidis PT4) in caeca of broilers. The study also revealed that treated birds had lower colony-forming units of *S.* Enteritidis PT4 per gram of caecal content (Fiorentin *et al*., 2005).

Another recent study showed that applying Felix O1 or its mutant possessing increased *in vitro* lytic activity against *S*. Typhimurium strain DT104, onto chicken frankfurters experimentally contaminated with the bacterium, reduced its concentration by approximately 1.8 and 2.1 CFU logs, respectively, compared to phage-untreated control frankfurters (Kuhn *et al.*, 2002).

Lytic bacteriophages, applied to chicken skin that had been experimentally contaminated with *Salmonella enterica* serovar Enteritidis or *Campylobacter jejuni* at a multiplicity of infection (MOI) of 1, increased in titer and reduced the pathogen numbers by less than 1 $\log_{10}$ unit. Phages applied at a MOI of 100 to 1,000 rapidly reduced the recoverable bacterial numbers by up to 2 $\log_{10}$ units over 48 h. When the level of *Salmonella* contamination was low (< $\log_{10}$ 2 per unit area of skin) and the MOI was 10^5, no organisms were recovered. By increasing the number of phage particles applied (i.e., MOI of 10^7), it was also possible to eliminate other *Salmonella* strains that showed high levels of resistance because of restriction, but to which the phages were able to attach (Goode *et al*., 2003).

A combinatorial approach of bacteriophages and their lysins, together with natural antimicrobial substances has also been employed as evidenced from recent publications, where a combination of *Enterobacter asburiae* JX1, an organism with antagonistic activity against *Salmonell*a and a *Salmonella*-specific phage cocktail was shown to be effective against *Salmonella javiana* associated with tomatoes (Jianxiong *et al.*, 2009).

The application of a combination of antagonistic bacteria and lytic bacteriophages to control the growth of *Salmonella* on sprouting mung beans and alfalfa seeds was recently reported. The biocontrol preparation was effective at controlling the growth of *Salmonella* under a range of sprouting temperatures (20 to 30^{o}C) and was equally effective at suppressing the growth of *Salmonella* on sprouting alfalfa seeds (Ye *et al.*, 2010).

2.3 *Caenorhabditis elegans* as model organism for infection and phage prophylaxis

Genetic analysis of host–pathogen interactions has been hampered by the lack of genetically tractable models of such interaction. Ideally, a eukaryotic host for which a complete genome sequence is available such as *Caenorhabditis elegans* and or *Drosophila melanogaster*, would be a model genetic organism.

The experimental advantages associated with *C. elegans* include simple growth conditions and a rapid generation time. The transparent body allows clear observation of internal organs. Experimental study of *C. elegans* was started more than half a century ago by Dougherty, who with his colleagues Hansen, Nigon, and Nicholas, promoted its use as a model organism (Ankeny, 2001). Work with *C. elegans* only really took off when it was chosen by Brenner as a genetically tractable model for the study of development and behavior (Brenner, 1974). Over

the last 20 years, the number of groups working with worms has grown constantly and there are now probably close to 4000 researchers world-wide, all using the same strain of *C. elegans*, 'N2 Bristol'(Ewbank , 2002).

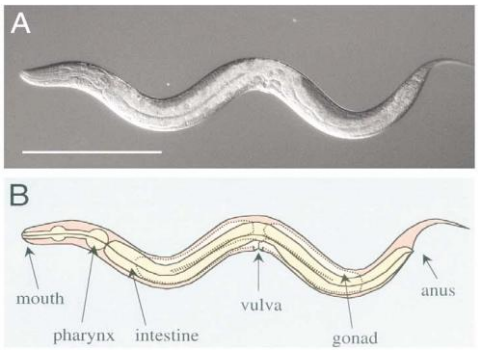


Fig 2.4. Basic anatomy of *C. elegans*. A. Photomicrograph of a young adult hermaphrodite worm. The scale bar corresponds to approximately 250 μm. B. Schematic representation of the worm in A, showing the position of the major organs (figure adapted from Edwards and Woods, 1983)

The nematode *Caenorhabditis elegans* has also been used as animal model system to study bacterial pathogenesis. Pathogens that are reported to be relatively virulent to *C. elegans* include certain strains of *P. aeruginosa* (Tan and Asubel, 2000), *Serratia marcescens* (Pujol *et al.*, 2001) and *Burkholderia paseudomallei* (O'Quinn *et al*. 2001). It establishes intestinal infections and provokes obvious signs of sickness: locomotory problems, distension of the intestine and cell lysis. These are followed by the precocious death of the worms (Ewbank, 2002).

If a pathogen is less virulent and does not provoke such obvious signs, such as is the case with *Shewanella massilia*, the life span of individual worms can be measured and compared with that of worms cultivated on non-pathogenic

bacteria. The Gram-positive bacterium *Microbacterium nematophilum* falls into a further category as it does not provoke a lethal infection. Rather the bacteria adhere to the cuticle of the worm, just behind the anus and provoke a pronounced swelling (Hodgkin *et al*., 2000).

Salmonella enterica serovar Typhimurium, a pathogen seemingly well adapted to its intracellular lifestyle in mice is also capable of infecting and killing *C. elegans* (Aballay *et al.*, 2000, Labrousse *et al.*, 2000). Unlike in the murine host, throughout the course of the infection, the bacteria remain extracellular. Certain *S.* Typhimurium mutants however, that are less virulent in the mouse, including *phoP/phoQ* (Aballay *et al*., 2000) and mutants that affect acid tolerance (Labrousse *et al.*, 2000) are also attenuated for their virulence in the worm.

Several genes needed for virulence in mammals are also required for pathogenesis in *C. elegans* (Aballay *et al.*, 2000; Labrousse *et al.*, 2000; Ernst *et a*l., 2001; Tenor *et al.*, 2004), implying that the invasion and proliferation of serovar Typhimurium in the host intestine depend on mechanisms common to both the nematode and mammals. This makes *C. elegans* a relevant model for determining the infectivity and fitness of antibiotic-resistant bacteria during a host infection.

C. elegans is reportedly also infected by *S.* Enteritidis and *S.* Pullorum (Aballay *et al.*, 2000; Aballay and Ausubel, 2001). *C. elegans* has been used as animal model system to test phage protection against infection by *Salmonella* Enteritidis and *S. Pullorum* using bacteriophage prophylaxis assay (Santander and Robeson, 2007) and it was found that pre-treatment with bacteriophage resulted in enhanced survival of *C. elegans* of nematodes when challenged with these bacterial pathogens.

2.4 National status of bacteriophage research

There are only a very few ongoing research works on bacteriophages in India. The National Centre for aquatic animal health (NCAAH), Cochin University, has been working on vibriophages for several years now. The focus of their research is on biocontrol of *Vibrio* pathogens using vibriophages in prawn larvi culture and study phage ecology in hatchery and estuarine systems. National Institute for cholera and enteric diseases (NICED), Kolkata, has been working mostly on *Vibrio* and a little on their phages. Dr. Sarkar and his group successfully developed a phage typing scheme for V. *Cholerae* 01 biotype ElTor in 1993. Subsequently, he developed the phage typing scheme for *Vibrio cholerae* O139. It operates as a reference laboratory and receives 1000 to 1500 strains per year from 30-40 institutions in India and abroad for biotyping, serotyping and phage typing and is serving the nation by sending the results from time to time. Cloning and sequencing of phage DNA, phage therapy in animal model and surveillance studies in aquatic environments is also carried out by their team. The Centre for environmental management of degraded ecosystem, University of Delhi has been working on the development of ecological restoration technologies using plants and their associated microbes where the contribution of soil-borne bacteriophages to its success are of immense value.

A commercial biotechnology company in India (*GangaGen* Biotechnologies Pvt Ltd - Bangalore), is involved in the development of proprietary products for the prevention and treatment of bacterial infections, particularly infections resistant to antibiotics, through the application of contemporary molecular and clinical sciences which also includes phage-based therapies.

Chapter 3

MATERIALS AND METHODS

3.1 Isolation of *Salmonella*

3.1.1 Sample collection and preparation

The intestinal contents from the broiler chicken, collected from the retail markets in Ernakulam, Kerala were used as a source for isolating *Salmonella*. The same samples were also used for the isolation of *Salmonella* specific phages. All the samples were collected in sterile polythene bags, sealed well and brought to the laboratory in an icebox within 2 hours of sampling for further processing.

3.1.2 Screening of *Salmonella*

The Food and Drug Administration (FDA) guidelines in the Bacteriological Analytical Manual (BAM) (Andrews *et al.*, 2007) were used for the isolation of *Salmonella*.

3.1.2.1 Pre-enrichment

Pre-enrichment helps in reviving bacteria which are sub lethally injured and in low numbers. The presence of other bacteria as well as components of the food sample also may hinder growth and recovery of *Salmonella*. Pre-enrichment in a nonselective medium such as lactose broth allows repair of cell damage, dilutes toxic or inhibitory substances and provides a nutritional advantage to *Salmonella* over other bacteria.

For pre-enrichment, 25 g of sample was weighed aseptically, added to 225ml sterile lactose broth (Appendix-1), mixed thoroughly and incubated for 60 ± 5 minutes at 28°C±2°C. Adjusted the pH wherever necessary to 6.8 ± 0.2 and incubated the sample mixtures at 35°C for 24 ± 2 hours.

3.1.2.2 Selective enrichment

Tetrathionate broth and Rappaport–Vassiliadis medium (Appendix-1) were used for the selective enrichment of *Salmonella* host.

Tetrathionate Broth: It is used as a selective enrichment for the cultivation of *Salmonella* that may be present in small numbers and therefore have to compete with intestinal flora. The selectivity of the medium depends on the ability of thiosulphate and tetrathionate in combination to suppress commensal coliform organisms. Tetrathionate is formed in the medium upon addition of the iodine and potassium iodide solution. Organisms containing the enzyme tetrathionate reductase will proliferate in this medium. Bile salts, a selective agent, will suppress the coliform bacteria and inhibit Gram positive organisms. Calcium carbonate neutralizes and absorbs toxic metabolites.

Rappaport–Vassiliadis medium: It is also used for the selective enrichment for the cultivation of *Salmonella*. Enzymatic casein digest is the carbon and nitrogen source for general growth requirements in Rappaport-Vassiliadis broth. Magnesium chloride raises the osmotic pressure in the medium and Potassium dihydrogen phosphate acts as a buffer. Malachite green oxalate is inhibitory to organisms other than *Salmonella.* The low pH of the medium, combined with the presence of Malachite green oxalate and Magnesium chloride aids in selection of the highly resistant *Salmonella*.

Procedure

0.1 ml each of pre-enriched cultures were transferred to 10 ml tetrathionate broth (Hi Media, Mumbai, India) and Rappaport–Vassiliadis broth (HiMedia, Mumbai, India). Tetrathionate broth was incubated at 35 ± 2.0°C for 24 ± 2 hours, while Rappaport–Vassiliadis broth was incubated at 42 ± 0.2°C for 24 ± 2 hours.

3.1.2.3 Selective plating

After selective enrichment, a loopful of culture was streaked onto xylose–lysine–deoxycholate (XLD) agar (Difco, USA), Hektoen enteric (HE) agar (Difco, USA) and Bismuth sulfite (BS) agar (Appendix-1) and incubated at 35°C for 24 hours.

Xylose – lysine–deoxycholate agar: It is a selective and differential medium used for isolating *Salmonella*. Lactose, sucrose and xylose are the fermentable carbohydrates present in XLD agar and phenol red is the pH indicator. Sugar fermentation lowers the pH, registered by phenol red indicator by changing color to yellow. Most gut bacteria, including *Salmonella*, can ferment xylose to produce acid. After exhausting the xylose supply *Salmonella* colonies will decarboxylate lysine, elevating the pH once again to alkaline and mimicking the red *Shigella* colonies. *Salmonellae* metabolize thiosulfate to produce hydrogen sulfide, which leads to the formation of colonies with black centers and allows them to be differentiated from the similarly colored *Shigella* colonies. Sodium deoxycholate is added to inhibit Gram positive growth and to retard the growth of many strains of coliforms. Other *Enterobacteria* such as *E. coli* will ferment the lactose and sucrose present in the medium to an extent that will prevent pH reversion by decarboxylation and acidify the medium turning it yellow.

Hektoen Enteric Agar: It is a selective and differential medium designed to isolate and differentiate members of the species *Salmonella* and *Shigella* from other *Enterobacteriaceae*. Bile salts and the dyes bromothymol blue and acid fuchsin inhibit the growth of most Gram positive organisms. Lactose, sucrose, and salicin provide fermentable carbohydrates to encourage the growth and differentiation of the enterics. Sodium thiosulfate provides a source of sulfur. Ferric ammonium citrate allows the visualization of hydrogen sulphide production by reacting with hydrogen sulfide gas to form a black precipitate.

Salmonella reduce sulfur to hydrogen sulfide and will produce black colonies or blue-green colonies with a black center on HE Agar.

Bismuth sulfite agar: It is used for the selective isolation of *Salmonella*. Brilliant green and Bismuth sulfite largely inhibit the accompanying bacterial flora. Iron (III) sulfate is an indicator for hydrogen sulphide produced by H_2S positive *Salmonella*. This reaction causes a brown or black precipitate on the medium and a black or green metallic colony. Surrounding medium is usually brown at first, but may turn black in time with increased incubation, producing the halo effect.

Isolated typical single colonies were picked, repeatedly purified on Nutrient agar (NA) plates (Appendix-1), sub- cultured, stocked and used for further characterization.

Salmonella from the Microbial Genetics Laboratory culture collections, Department of Biotechnology, Cochin University of Science and Technology, and the *Salmonella* cultures kindly donated by Dr. A. A. M. Hatha, Department of Marine biology, Microbiology and Biochemistry, Cochin University of Science and Technology, were also used as host for isolating specific lytic phages.

3.1.3 Identification of *Salmonella*

The identity of all the cultures used as host for isolating phages were confirmed biochemically and serologically.

3.1.3.1 Triple Sugar Iron Agar test

This test aids in differentiating members of family *Enterobacteriaceae*. Triple Sugar Iron agar (TSI) contains three sugars (dextrose, lactose and sucrose), phenol red for detecting carbohydrate fermentation and ferrous ammonium sulfate for detection of hydrogen sulfide production (indicated by blackening in the butt of the tube). Carbohydrate fermentation is indicated by the production of gas and a change in the color of the pH indicator from red to yellow. To facilitate the detection of organisms that only ferment dextrose, the dextrose concentration is one-tenth the concentration of lactose or sucrose. The small amount of acid produced in the slant of the tube during dextrose fermentation oxidizes rapidly, causing the medium to remain red or revert to an alkaline pH. In contrast, the acid reaction (yellow) is maintained in the butt of the tube because it is under lower oxygen tension. After depletion of the limited dextrose, organisms with the ability to utilize the lactose or sucrose will begin to do so. To enhance the alkaline condition of the slant, free exchange of air must be permitted by closing the tube cap loosely.

An alkaline slant-acid butt (red/yellow) indicates fermentation of dextrose only. An acid slant-acid butt (yellow/yellow) indicates fermentation of dextrose, lactose and/or sucrose. An alkaline slant-alkaline butt (red/red) indicates dextrose or lactose were not fermented (non-fermenter). Cracks, splits, or bubbles in medium indicate gas production. A black precipitate in butt indicates hydrogen sulfide production.

The test was conducted using overnight cultures from NA slants. TSI slants (Appendix-1) were inoculated by stabbing the butt and streaking the surface of the medium. Slants were examined after incubation at 35ºC for 24 hours. The cultures showing red- alkaline slant, yellow -acid-butt, with hydrogen sulphide production (blackening of the medium), typical of *Salmonella* in TSI, were considered positive.

3.1.3.2 Lysine Iron Agar test

Salmonella species can be identified by demonstrating decarboxylation or deamination of lysine and H_2S production on Lysine Iron agar (LIA). Dextrose is incorporated into the medium at 0.1% concentration. Organisms that ferment dextrose produce acid, often accompanied by gas production demonstrated as bubbles or cracks in the medium. Organisms which decarboxylate lysine revert the medium to the alkaline range by producing alkaline by-products , demonstrated by a purple color throughout the medium. Organisms that do not decarboxylate lysine will produce an acid reaction in the butt, resulting in a yellow color. The slant may remain alkaline due to oxidative decarboxylation of proteins, peptides, and amino acids in the medium.

The test was conducted using overnight cultures from NA slants. LIA slants (Appendix-1) were inoculated by stabbing the butt and streaking the surface of the medium. Slants were examined after incubation at 35ºC for 24 hours. The cultures showing purple color throughout the medium with hydrogen sulphide production were considered positive.

3.1.3.3 Indole production

Some bacteria can produce indole from tryptophan using the enzyme tryptophanase. Production of indole is detected by Kovac's reagent (isoamyl alcohol, para-dimethylaminobenzaldehyde in concentrated hydrochloric acid). Indole reacts with the aldehyde in the reagent to give a red color. An alcoholic layer concentrates the red color as a ring at the top.

Tryptone medium (Appendix-1) was inoculated with an overnight culture from NA slants and incubated at 35ºC for 24 hours. After the incubation, 0.2-0.3 ml Kovac`s reagent was added. *Salmonella* test negative for indole production (lack of deep red color at surface of broth).

3.1.3.4 Urease production

The urease test is mainly performed to differentiate *Salmonella* (urease negative) and *Proteus* (urease positive).

Urease producing bacteria hydrolyze urea, splitting it into ammonia and carbon dioxide, making these two products available for bacterial use. The test for urease production relies on phenol red, a pH indicator present in the medium. Phenol red is yellow in color at pH 6.8, but the ammonia released from the hydrolysis of urea, causes the pH to rise above 8.1, causing it to turn pink. Organisms that are unable to synthesize urease do not produce ammonia, and therefore the yellow color of the urea broth remains unchanged.

The test bacteria were inoculated into the Urea broth (Appendix-1) incubated at 35ºC for 24 hours. No color change of the medium is characteristic of *Salmonella.*

3.1.3.5 Simmons Citrate Agar test

Simmons Citrate Agar is an agar medium used as a differential medium for the identification of enteric bacteria, using citrate as the sole source of carbon and ammonium ions as the sole nitrogen source. The medium contains bromothymol blue as an indicator. Organisms able to utilize the citrate grow well on the surface of the medium and alkalinize the medium changing it from green to blue. Poor or no growth is observed if the organism does not utilize citrate, and medium remains unchanged.

The test bacteria were inoculated into the Simmons citrate slant (Appendix-1), incubated at 35ºC for 24 hours. Blue color of medium was taken as indication of citrate utilization. *Salmonella* are citrate positive.

3.1.3.6 Carbohydrate fermentation tests

Cultures segregated as *Salmonella* after the primary biochemical reactions were further confirmed by secondary screening involving carbohydrate fermentation tests (lactose, sucrose, dulcitol and salicin) with phenol red as indicator. Peptone medium (Appendix-1) with 1% carbohydrate was used to determine fermentative ability of the test organism, i.e. its ability to utilize the carbohydrate and produce acid. The capability of the test organism to ferment the supplied sugar, with consequent acid production, will change the color of the medium from red to yellow. *Salmonella* ferments only dulcitol.

The broth was inoculated with a small amount of culture growth from the TSI slant, incubated at 35°C and observed for color change after 24 hours up to 48 hours.

Isolates confirmed as *Salmonella*, were purified and maintained as stock cultures employing 3 methods, viz., paraffin oil overlay, semisolid agar method and glycerol stock.

Paraffin overlay method: Nutrient agar was prepared in sterile glass vial. A single colony was inoculated onto the same and incubated at 37°C for 24 hours. Sterile paraffin oil was added to the vials to cover the medium, capped with sterile rubber stoppers and was stored in a cool, dark place, at room temperature until use.

Semisolid/soft agar method: Semisolid nutrient agar (0.8% agar) made in 5 ml test tubes, were inoculated with overnight bacterial culture. After 24 hour growth at 37°C, the tubes were plugged with sterile rubber plugs and were stored at room temperature until use.

Glycerol stocking: The bacterial isolates were grown in nutrient broth. After 24 hour growth at 37°C, the culture broth were stored in 15% (final) glycerol at $^{-}$80°C for long term storage; and at $^{-}$20°C for use as working stock.

3.1.4 Serological agglutination test

3.1.4.1 Polyvalent somatic (O) test

The bacterial isolates after the primary and secondary screening underwent a serological agglutination test. A loopful of the culture from 24-48 hour TSI slant was emulsified with 2 ml 0.85% saline on a clean glass slide. Added 1 drop of *Salmonella* polyvalent somatic (O) antiserum (Difco, USA) and mixed the culture suspension for 1 minute by back-and-forth motion. Any degree of agglutination resulting after this was considered to be positive.

For final confirmation the isolates were serotyped at the National *Salmonella* and *Escherichia* Centre, Kasuali, Himachal Pradesh, India.

3.1.5 Identification of *Salmonella* phage host by partial 16S rRNA gene sequence analysis

The *Salmonella* strain that was successfully infected by the phage during isolation process was characterized by 16S rRNA gene sequence analysis.

3.1.5.1 Template preparation for polymerase chain reaction (PCR) (Ausubel *et al*., 1987)

Log phase culture (2 ml) was taken in sterile microfuge tube and centrifuged (Sigma, 3K30, Germany) at 5000g for 10 minutes at 4ºC. The supernatant was discarded and the cell pellet was blot dried. The pellet was resuspended in 875 µl of Tris EDTA (TE) buffer (pH: 8) (Appendix-3). 5 µl Proteinase K (Sigma- Aldrich) (10mg/ml) and 100 µl of 10% SDS (Appendix-3) was added, mixed gently and incubated at 37ºC for 1 hour. Equal volume of phenol-chloroform mixture (1:1) was added to this, centrifuged at 12000g (Sigma, 3K30, Germany) for 10 minutes at 4ºC, and then the supernatant was transferred to another sterile tube using sterile cut tip. The extraction with phenol-chloroform was repeated three times. To this supernatant, 0.1 volume of 5 M sodium acetate (pH 5.2) (Appendix-3) and double volume of ice-cold isopropanol was added. This was kept at -20ºC for about 1 hour and centrifuged at 12000 g for 10 minutes at 4ºC. The DNA pellet obtained was washed with 70% ethanol, air dried and dissolved in 50 µl TE buffer (pH8) (Appendix-3). The concentration of the genomic DNA was estimated spectrophotometrically and appropriate dilutions (~80 -100 ng) were used as template for PCR reactions.

3.1.5.2 Polymerase chain reaction

Molecular identification of the host *Salmonella* strain was done by PCR using a primer pair for 16S rDNA. A portion of the 16S rRNA gene (1.5kb) was amplified from the genomic DNA (Table.3.1). Products after PCR amplification were subjected to sequencing, followed by homology analysis.

Table.3.1. Primers used to amplify 16S rRNA gene

Sequence	Amplicon	Reference
16SF 5' AGTTTGATCCTGGCTCA 3'	**1500** bp	(Shivaji *et al*., 2000)
16SR 5' ACGGCTACCTTGTTACGACTT 3'	„	(Reddy *et al*., 2002a; Reddy *et al*., 2002b)

PCR Mix composition

10X PCR buffer	2 µl
2 mM each dNTPs (Chromous Biotech, India)	2 µl
Forward primer (10 picomoles) (Sigma Aldrich)	2 µl
Reverse primer (10 picomoles) (Sigma Aldrich)	2 µl
*Taq*DNA polymerase (1U/µl) (Sigma-Aldrich)	1 µl
Template DNA (50 ng/µl)	3 µl
$MgCl_2$ (Sigma-Aldrich)	1.2 µl

Sterile distilled water to a final volume of 20 µl

Amplifications were carried out in a Thermal Cycler (BioRad MJ Mini Gradient) using the following program: a hot start cycle of 94ºC for 5 minutes, then 30 cycles of 94ºC for 1 minute, annealing at 56ºC for 1 minute and extension at 72ºC for 90 seconds, ending with a final extension step of 72ºC for 10 minutes.

Aliquots (5µl) of the amplified products were analyzed by electrophoresis on 1.2 % agarose gels in Tris Acetate EDTA (TAE) buffer (Appendix -3). Gel was stained with ethidium bromide (0.5 mg/ml) for 20 minutes and viewed on a UV transilluminator and image captured with the help of Gel Doc system (Syngene, UK).

3.1.5.3 DNA sequencing

Nucleotide sequences of the PCR amplicon of 16S rRNA gene were determined by the ABI XL DNA analyzer, using the big dye Terminator kit (Applied Biosystems, USA) at SciGenom Cochin, India Ltd. The identity was determined by comparing the sequences obtained with the gene sequences available in the Genbank database using Basic Local Alignment Search Tool (BLAST) software (Altschul *et al.*, 1980) at NCBI site. (http://blast.ncbi. nlm.nih.gov). The sequence was deposited in the Genbank database and accession number was obtained.

3.2 Bacteriophage isolation

3.2.1 Sample preparation

3.2.1.1 Direct method

The intestinal contents from the broiler chicken were homogenized in sterile physiological saline and thoroughly mixed. This homogenate was centrifuged at 4000 g for 10 minutes at 4ºC, filtered through 0.22 µm membrane filter (Millipore, USA) to make them bacteria-free and this filtrate was screened for the presence of phage.

3.2.1.2 Enrichment method

As a part of the enrichment process, a portion of the crude lysate was mixed with the host bacteria (in log phase), incubated at 37ºC for 12 hours, after which it was made bacteria-free by centrifugation and filtration as described in section 3.2.1.1. This enrichment step was intended to amplify the phage counts that may otherwise be undetected due to low number.

3.2.2 Double agar overlay method

The lysate was then assayed according to the double-agar overlay method of Adams (1959) with modification. The logarithmic phase cells (1 ml) of the host bacterial strains in nutrient broth were mixed with 1 ml of the serially diluted lysate and were incubated at 37ºC in a water bath (Scigenics, Chennai, India) for 1 hour. After incubation, 3 ml of sterile soft agar (nutrient broth containing 0.8% agarose) was added to this, mixed well and was immediately overlaid on nutrient agar plates. The plates were incubated for 16 hours at 37ºC. Phage-free cultures (containing only bacterial host) and host-free cultures (containing only phage) were used as controls. A sample was scored positive for phages when plaques were observed on the bacterial lawn in the plates.

The host *Salmonella* for which phage could be isolated was used as a representative strain for phage isolation and propagation throughout this study. The identity of the host was confirmed both by serotyping and 16S rRNA gene sequencing and analysis as described under section 3.1.4 and 3.1.5, respectively.

3.2.3 Tetrazolium staining

Tetrazolium staining helps to improve phage plaque visibility against the backdrop of the bacterial growth, whereby each plaque can be observed as a sharp, clear area against the intense red background produced by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to the insoluble formazan by the bacterial cells. (Pattee, 1966). The petri plates with plaques were flooded with 10ml of trypticase soy broth (HiMedia, Mumbai, India) containing 0.1% 2, 3, 5 - triphenyltetrazolium chloride (TTC) (HiMedia, Mumbai). After incubation at 37^{o}C for 20 minutes, the broth was poured off and the plaques were observed.

3.3 Phage purification

A single plaque was picked from the plate with a sterile tooth-pick, introduced into 3 ml of a log phase culture of the *Salmonella* host in nutrient broth, and was incubated at 37ºC in an environmental shaker (Orbitek, Scigenics, India) at 120 rpm for 12 hours. This was then centrifuged at 10000g (Sigma, 3K30, Germany) followed by filtration through 0.22 µm membrane (Millipore, USA). The lysate obtained was used for double agar overlay. This procedure was repeated 6 times, until uniform sized plaques were obtained on the plate.

3.4 Large scale production of phage lysate

The plates prepared as described in the section 3.3., showing infective centers at the rate of 10^{10} plaque forming units (PFU)/ml, were washed with SM buffer(Appendix -2) using the following method.

The plates with uniform sized plaques were overlaid with 10 ml of SM buffer and were incubated overnight at 4ºC, with gentle rocking so that phages

could easily diffuse into the buffer. The phage suspension was recovered after incubation from all the plates and pooled. Chloroform was added to this pooled mixture to a final concentration of 5 % (v/v), mixed well using a vortex mixer and incubated at room temperature for 15 minutes. The cell debris were then removed by centrifugation at 5000 g for 10 minutes (Sigma, 3K30, Germany) and the supernatant was transferred to sterile polypropylene tube. Chloroform was added to a final concentration of 0.3 % (v/v) and this was stored at 4ºC until use. The titer of this lysate was noted after serial dilution (Sambrook *et al.*, 2000).

3.5 Phage concentration

Phage was concentrated using Polyethylene glycol (PEG) 6000 as described by Sambrook *et al.*(2000). Briefly 1% (v/v) of a overnight culture of the host bacteria was transferred to 200 ml nutrient broth (Himedia, Mumbai, India), and incubated at 37ºC for 3.5 hours in an environmental shaker at 100 rpm (Orbitek, Scigenics, India). Phage was added at a multiplicity of infection (MOI) of 0.2 and the incubation at 37ºC was continued at 100 rpm for 12-15 hours. This broth was then centrifuged at 10000g for 20 minutes (Sigma, 3K30, Germany), the supernatant was collected and filtered through 0.22 µm membrane filter (Millipore, USA). DNase I (Bangalore Genei) and RNase (Bangalore Genei) was added, to a final concentration of 1 µg/ml each, and incubated at room temperature for 30 minutes. Solid NaCl was added to a final concentration of 1 M and dissolved by stirring with a sterile glass rod. This mixture was kept in ice for 1 hour, followed by centrifugation at 11000g for 10 minutes at 4ºC. Solid PEG 6000 (SRL, India) was added to the supernatant at a final concentration of 10% (w/v), dissolved by slow stirring on a magnetic stirrer at room temperature. This was then kept in ice overnight, followed by centrifugation at 11000 g for 10 minutes at 4ºC. The supernatant was discarded completely, while the pellet was resuspended in 5ml of phosphate buffered saline (PBS) (Appendix-2). PEG and

cell debris were removed from the phage suspension by the addition of an equal volume of chloroform, vortexing for 30 seconds, followed by centrifugation at 3000 g for 15 minutes at 4ºC. The aqueous phase containing the phage particles were recovered and stored at ⁻20ºC.

3.6 Maintenance and storage of phage

Phage lysate for long term storage was maintained as stock cultures employing 2 methods, viz., storage at 4ºC as such and as glycerol stock.

Phage lysate obtained after large scale production (Section 3.4.) was stored in sterile 40ml polypropylene screw-cap tubes at 4ºC until use.

Nutrient broth containing 50% glycerol was mixed with filtered phage lysate in a sterile microfuge tube (1.5ml capacity) and the mixture was frozen at ⁻80ºC, until use.

3.7 Characterization of phage

Salmonella specific lytic phages that exhibited excellent and consistent bacterial cell lysis capacity were chosen for further characterization.

3.7.1 Morphological analysis by Transmission Electron Microscopy (TEM)

One drop of high titer phage sample was spotted onto a carbon-coated TEM grid, allowed to settle for 2-3 minutes and excess of sample was removed by blotting. A drop of 2% uranyl acetate (pH 7.0) was placed on the spot, allowed to react for 2-3 minutes and the excess stain drained off by touching a blotting paper strip to the edge of the grid. The grids were dried for 3 hours, examined and

photographed using a Transmission Electron Microscope (Model JOEL JEM-100X) and TEM (Model JOEL JEM 100SX) operated at 80 kV at Indian Institute of Horticulture Research (IIHR) Hesaragatta, Bangaluru and Cancer institute, Chennai, respectively. Phage morphology was observed from the micrographs.

3.7.2 Determination of optimal multiplicity of infection

Multiplicity of infection (MOI) is the ratio of phage particles to host bacteria. It is calculated by dividing the number of phage added (volume in ml x PFU/ml) by the number of bacteria added (volume in ml x colony forming units/ml). Optimal MOI was determined according to Lu *et al*. (2003). Briefly, bacteria were infected at different MOI (0.01, 0.1, 0.5, 1, 5 and 10 PFU/ml) and were incubated at 37 ºC for one hour. At the end of the incubation period, the mixture was centrifuged (Sigma, 3K30, Germany) at 8000 g for 10 minutes and supernatant was passed through 0.22 µm membrane filter (Millipore, USA). The lysate was then assayed to determine the phage titre employing the double agar overlay method described previously. Phage-free cultures (containing only bacterial host) and host-free cultures (containing only phage) were used as controls. All assays were performed in triplicates. The MOI giving maximum yield was considered as optimal MOI.

3.7.3 Phage adsorption

The first step in the growth of bacteriophage is its attachment to susceptible bacteria. This process is called adsorption.

The adsorption studies were carried out as per Lu *et al*. (2003). Log phase culture of host was infected using the optimal MOI of the phage and incubated at 37ºC. Aliquots of 5 ml were sampled at 0, 5, 10, 15, 20, 25, 30, 35, 40 and 45

minutes time intervals after infection. All the samples drawn were immediately filtered through 0.22 µm membrane filter (Millipore, USA). The phage titer was determined using double agar overlay method after appropriate dilutions. All plating was done in triplicates and appropriate controls were maintained. The percentage of phage adsorption was calculated as follows: [(control titer – residual titer)/control titer] X 100% (Durmaz, 1992). The phage titer observed at time zero was considered as the control titer.

3.7.4 One step growth curve

The construction of the one-step growth curve of a phage is important as it helps in the calculation of the growth kinetics parameters like latent period, rise period and the burst size. Latent period is the time elapsed between the moment the host culture is infected with phage to the moment the first bacterial cells are lysed. The rise period is the time span starting from the end of latent period until all phages are extra cellular. Burst size is the average number of progeny phage particles produced per infected bacterium. It is calculated as follows: (final PFU-initial PFU)/ number of infected bacterial cells (Adams, 1959).

One step growth curve experiment was performed according to the protocol of Capra *et al*. (2006). Mid log phase culture of the host *Salmonella* (200 ml) was harvested by centrifugation at 9000g for 10 minutes and resuspended in 1/5 of the initial volume (40 ml) of pre -warmed nutrient broth. The phage was added at the optimal MOI, allowed to adsorb for 15 minutes at 37ºC, followed by harvesting of phages by centrifugation at 10000g (Sigma, 3K30, Germany) for 5 minutes and resuspension in 200 ml nutrient broth. This was incubated at 37ºC.

Samples were taken at 10 minutes intervals (up to 2 hours) and immediately titered by the double agar overlay method. Assays were carried out in

triplicate and appropriate controls were maintained. The graph was plotted with log of PFU/ml against time. The latent period, the rise period and the burst size of the phage were calculated from the one step growth curve.

3.7.5 Influence of physical and chemical parameters on phage viability

Physical and chemical parameters have a critical role in maintaining phage viability. Hence the effect of different physical and chemical parameters like temperature, pH, NaCl and different sugars on phage viability was studied.

3.7.5.1 Effect of temperature on phage viability

The influence of temperature on phage viability/propagation was studied following the protocol of Lu *et al*., (2003). 900 µl of sterile distilled water was pre-heated to temperatures ranging from 50ºC to 100ºC, followed by the addition of 100 µl of phage sample (10^{10} PFU/ml) to these pre-heated tubes. The tubes were maintained at these temperatures for varying intervals, i.e., 15 seconds, 30 seconds, 1 minute, 2 minutes and 3 minutes. After incubation these phage containing tubes were immediately placed in ice. Samples were assayed using double agar overlay method to determine the number of surviving plaque PFU. All plate assays were done in triplicates and appropriate controls were maintained. The counts of surviving phage were expressed as PFU/ ml and plotted against temperature values.

3.7.5.2 Effect of NaCl on phage viability

In order to study the influence of NaCl on phage viability, NaCl solutions of varying molar concentrations such as 0.1 M, 0.25 M, 0.5 M, 0.75 M, 1 M, 2 M and 3 M were prepared in deionised water. Phage sample was added (10^{10} PFU

/ml), incubated for 30 minutes at 37ºC (Capra *et al*., 2006), then diluted and assayed with the mid-log phase host for surviving phage particles by the double agar overlay method. The plaques obtained were counted. All plate assays were performed in triplicates and appropriate controls were maintained. The result was expressed as PFU/ml and plotted against concentration of NaCl.

3.7.5.3 Effect of pH on phage viability

Influence of pH on phage viability was evaluated by incubating the phages in suitable buffers of different pH, ranging from 2-13(Capra *et al*., 2006). Hydrochloric acid-potassium chloride buffer was used for the studies at pH 2, while the citrate – phosphate buffer system was used for pH 3 to 6; Phosphate buffer for pH 7, Tris (hydroxymethylamino methane buffer system for pH 8 and 9; carbonate – bicarbonate buffer for pH 10 and 11; Sodium hydroxide - Potassium chloride buffer (pH 12 and 13) (Appendix-2). The phage was added (10^{10} PFU /ml) to 10 ml of sterilized buffer solutions, incubated at 37ºC for 30 minutes and assayed with the mid log phase host using double-layer agar plate method to determine the surviving PFU. The plate assay was done in triplicates and appropriate controls were maintained. The viable phage particles were counted. The results were expressed as PFU/ ml and plotted against the values of pH.

3.7.5.4 Effect of sugars on phage viability

Effect of various sugars like arabinose, dextrose, galactose, fructose, maltose, mannitol, mannose, lactose, rhamnose, ribose and xylose on phage viability was studied following Capra *et al*. (2006). Sugars were added at a final concentration of 500 mM/1000 ml to each phage sample(10^{10} PFU/ml). The mixture was incubated at 37°C for 30 minutes, diluted adequately and assayed with the mid log host cells for plaques by the double agar overlay method to

determine the surviving phage particles. The plate assay was done in triplicates and appropriate controls were maintained. The results were compared with titre of control samples without the sugars and then expressed as a percentage of phage inactivation.

3.7.6 Influence of physical and chemical parameters on phage adsorption

Phage adsorption is critical for causing phage infection in bacteria. All factors having an influence on phage adsorption, also affect phage infection. Hence the effect of various physical and chemical parameters on phage adsorption was studied.

3.7.6.1 Effect of temperature on phage adsorption

The adsorption of phages on the host *Salmonella* was determined at temperatures of 0, 10, 20, 30, 37, 40, 45 and 50°C. The methodology was adopted from Capra *et al*. (2006). Briefly, exponentially growing host culture ($O.D_{600}$ = 1) was centrifuged and resuspended (approximately 10^8 CFU/ ml) in nutrient broth (pH 8). Phage was added at optimal MOI and was incubated, each at the different temperatures mentioned above for 30 minutes. After centrifugation at 12000 g for 5 minutes (Sigma, 3K30,Germany) at 4°C, the supernatant after appropriate dilutions were assayed for unabsorbed phages employing double agar overlay method. All plating was done in triplicates and appropriate controls were maintained. The phage titer was compared with the control titer. The results were expressed as percentages of adsorption and plotted against temperature.

3.7.6.2 Effect of NaCl on phage adsorption

Influence of different concentrations of NaCl on adsorption was investigated following Capra *et al.* (2006). Exponentially growing host culture was added to nutrient broth with concentrations of NaCl ranging from 0.1, 0. 25, 0.5, 0.75 and 1 M NaCl, and infected with optimal MOI of phages and incubated at 37°C for 30 minutes for adsorption. The mixture was then centrifuged at 10000g for 5 minutes at 4°C (Sigma, 3K30, Germany) to sediment the phage adsorbed bacteria. The supernatant was then assayed for unabsorbed free phages employing double agar overlay method and the counts were compared with the titre of the control which contained no NaCl in nutrient broth. All plating was done in triplicates and appropriate controls were maintained. The results were expressed as a percentage of adsorption and plotted against NaCl concentration.

3.7.6.3 Effect of pH on phage adsorption

Exponentially growing *Salmonella* host culture was centrifuged at 10000g for 10 minutes (Sigma, 3K30, Germany) at 4°C, and the cells resuspended in nutrient broth adjusted to the desired pH. Adsorption was determined at the pH values ranging from 2 to 13. Phage was added at an optimal MOI, incubated at 37°C for 30 minutes for adsorption, centrifuged at 10000g for 5 minutes, the supernatants assayed to determine surviving PFU employing double agar overlay method and was compared to control (Capra *et al* ., 2006). All experiments were performed in triplicates and appropriate controls were maintained. The results were expressed as a percentage of adsorption and plotted against pH values.

3.7.6.4 Effect of calcium ions on phage adsorption and propagation

The influence of calcium ions on phage adsorption and propagation was determined following (Lu *et al*., 2003). The protocol involved adding 10 ml of exponentially growing *Salmonella* host culture to 100 ml nutrient broth, and incubation for 3.5 hours at 120 rpm. 10 ml each of this mid log phase host culture was added to five, 15 ml McCartney bottle. Appropriate volumes of filter sterilized 1M $CaCl_2$ (Millipore, USA) solution were added to the host aliquots to make 0, 1, 10, 20, and 30 mM concentrations. After the final volume was adjusted to 15 ml with sterile distilled water, each tube was infected with the phage at optimal MOI. All tubes were incubated at 37ºC for 2 hours. 1 ml aliquots were drawn and centrifuged at 10000 g for 10 minutes (Sigma, 3K30, Germany) at 4°C. The supernatants were serially diluted and assayed using double agar overlay method to determine plaque formation. The phage titer was determined employing double agar overlay method for the medium with and without $CaCl_2$. All plating was done in triplicates and appropriate controls were maintained. The results was expressed as PFU/ ml and plotted against $CaCl_2$ concentrations.

3.7.7 Effect of optimized physicochemical parameters on phage propagation

The cumulative effect of all the parameters optimized under section 3.7.2, 3.7.3, 3.7.5 and 3.7.6 was studied. Phage lysate was added at its optimum MOI to mid log phase host cells grown in nutrient broth (pH adjusted to 8 with 10 mM $CaCl_2$ and optimum NaCl concentration). The incubation temperature was set at 40°C. One step growth curve experiment was repeated as described in section 3.7.4. Aliquots were sampled at 10 minutes intervals, mixed with mid log phage host cells grown in nutrient broth (pH adjusted to 8 with 10 mM $CaCl_2$ and 0.25 M NaCl), followed by incubation for 30 minutes and was immediately titered by double agar overlay method. All plating was done in triplicates. Appropriate

controls were maintained. The graph was plotted with log of PFU/ml against time. The latent period, the rise period and the burst size of the phage were calculated.

3.7.8 Propagation of phage under nutrient depleted states of the host cell

Phages intended for use as a biocontrol agent have an added advantage when they have the capability to infect host under stationary as well as various nutrient deprived conditions. Therefore the ability of the phages to infect host under different nutrient deprived conditions was studied.

3.7.8.1 Preparation of log- and stationary-phase, starved- and nutrient-depleted cultures

Host cells grown as overnight cultures at 37°C were used for the stationary phase cell infection experiments. A fresh 6 hour culture was used for exponential phase host cell infection experiments. The starved host cells were prepared by resuspending cell pellet obtained after centrifugation of a 6 hour old culture, in an equal volume of physiological saline and incubating it for 24 hours at 37°C (Kadavy *et al.*, 2000). Nutrient depleted cultures were prepared by growing the bacteria to log phase in nutrient broth, and the cells harvested by centrifugation were resuspended in appropriate starvation suspension media, like minimal media (Appendix-2) without carbon, minimal media without phosphate and minimal media without ammonium chloride (nitrogen source) (NystrÖm *et al*., 1992). In all the cases except for stationary phase, the $O.D_{600}$ of cultures was adjusted to 0.5 ($1X10^5$ colony forming units (CFU)/ml) using respective medium prior to the addition of the phage. Phages were introduced into each of these cultures at a low multiplicity of infection (MOI) of 0.1, incubated at 37°C for 24 hours and plated (double agar overlay method) to observe the efficacy of phage

multiplication under various nutrient deprived conditions. All plating`s was done in triplicates and appropriate controls were maintained.

3.7.8.2 Statistical analysis

Statistical evaluations by ANOVA, followed by Students–Newman–Keul Test using GraphPad InStat (version 2.04a, San Diego, USA) computer program.

3.7.9 Bacteriophage genome analysis

3.7.9.1 Phage DNA isolation

Phage DNA extraction was carried out as previously described (Sambrook *et al.*, 2000). Briefly, 1 ml of the PEG 6000 concentrated phage suspension was incubated at 56°C for one hour with proteinase K at a final concentration of 50 µg/ml and SDS at a final concentration of 0.5%. After incubation, the digestion mix was cooled to room temperature and extracted first by adding equal volume of phenol equilibrated with 50 mM Tris (pH 8.0). The digestion mix in the tube was inverted gently many times until a complete emulsion was formed. The phases were separated by centrifugation at 3000 g for 5 minutes at room temperature (Sigma, 3K30, Germany). The aqueous phase was transferred to a clean tube using wide-bore pipette, and then extracted with 50:50 mixtures of equilibrated phenol: chloroform, followed by a final extraction with equal volume of chloroform. Double volume of ethanol and sodium acetate (pH 7) (Appendix 3) to a final concentration of 0.3 M was added to the extract followed by incubation at room temperature for 30 minutes. After incubation, the precipitated DNA was collected by centrifugation at 10000g for 5 minutes at 4°C. The supernatant was discarded and DNA was dissolved in Tris- EDTA (TE) buffer (pH 7.6) (Appendix-3). DNA was run on 1 % agarose gel. Gel was stained with ethidium bromide and visualized in UV light. The image of the gel was captured using gel documentation system (Syngene, UK).

3.7.9.2 Restriction analysis

The restriction pattern of the phage DNA was studied using the enzyme, *Bam* HI. (Fermentas, USA). Enzyme digestions were performed as recommended by the manufacturer. For digestion, each 20µl digestion solution containing approximately 1 µg of bacteriophage DNA and 1U of the restriction enzyme in reaction buffer was incubated for 1 hour at reaction temperature as the protocol prescribed for the enzyme. Restricted fragments were separated by agarose (1.2%) gel electrophoresis. Gel was stained with ethidium bromide and visualized in UV light. The image of gel was captured using gel documentation system (Syngene, UK).

3.7.9.3 Phage genome size determination by Pulsed-Field Gel Electrophoresis (PFGE)

Phage DNA was run in 1% agarose gel SeaKem® Gold Agarose, (Pulsed-field grade) in 0.5 X TBE buffer (Appendix-3) and electrophoresis was carried out in a CHEF DRII PFGE system (Bio-Rad, USA). The gel was run at 4.5 volts/cm, using ramped pulse times from 5 to 120 seconds for 18 hours at 15°C. Bacteriophage lambda DNA 1000 kb (NewEngland BioLabs, USA) were used as molecular weight marker. The gel was stained with ethidium bromide and photographed. The molecular weight was calculated using Quantity One® software (BioRad, USA).

3.7.9.4 Screening for virulence /virulence related genes in the bacterial host and in phage

PCR based method was adopted to screen for the presence of 12 virulence genes on the phage and host genome viz. - *invE/A, slyA, pho P/Q, ttrC, mgtC, spi4R, sopE, gog B, sod, gtg E* and *agfA.* The details of the primers used are as

given in Table.3.2. Multiplex PCR was performed with set 1 containing *slyA, invE/A* and *agfA* primers , set 2 containing *ttrC , mgtC* and *phoP/Q* primers,set 3 containing *spi4R, sod and gtgE* primers and set 4 containing *sopE* and *gog B* primers in Bio Rad MJ Mini™ Gradient Thermal Cycler.

Table.3.2 List of primers used to screen for the virulence and virulence related genes in the phage and host genome by PCR

Virulence gene	Sequence 5`-3`	Function	References
invE/A	**F - TGCCTACAAGCATGAAATGG** **R- AAACTGGACCACGGTACAA**	Invasion to the host	Stone *et al.*,1994
slyA	**F–GCCAAAACTGAAGCTACAGGTG** **R - CGGCAGGTCAGCGTGTCGTGC**	Production of cytilysin	,,
pho P/Q	**F – ATGCAAAGCCCGACCATGACG** **R–GTATCGACCACCACGATGGTT**	Resistance within macrophages	,,
ttrC	**F- GTGGGCGGTACAATATTTCTTTT** **R –TCACGAATAATAATCAGTAGCGC**	Vital for tetrathionate metabolism	Sara *et al*., 2006
mgtC	**F- TGACTATCAATGCTCCAGTGAAT** **R–ATTTACTGGCCGCTATGCTGTTG**	Survival within macrophages and growth in low Mg^{2+} environments	,,
spi4R	**F -GATATTTATCAGTCTATAACAGC** **R –ATTCTCATCCAGATTTGATGTTG**	Intra-macrophage survival and may also contribute to toxin secretion	,,
sopE	**F - CCGTGGAACGATTGACTG** **R- AGCCATTAGCAGCAAGGT**	Type III effecter involved in invasion	Susanne *et al*., 1999
gog B	**F - GCTCATCATGTTACCTCTAT** **R -AGGTTGGTATTTCCCATGCA**	Lambdoid prophage gene	Donatella *et al*., 2004
sod	**F -TATTGTCGCTGGTAGCTG** **R –CAGGTTTATCGGAGTAAT**	Protects from products of phagocyte NADPH-oxidase and nitric oxide synthase of host cell	,,
gtg E	**F -AGGAGGAGTGTAAAGGT** **R –GTAGAACTGGTTTATGAC**	Lambdoid prophage gene	,,
agfA	**F-TCCGGCCCGGACTCAACG** **R –CAGCGCGGCGTTATACCG**	Binding to fibronectin of host	Doran *et al*., 1993

PCR Mix composition

10 X PCR buffer	2 µl
2 mM each dNTPs (Chromous Biotech, India)	2 µl
Forward primer (10 picomoles) (Sigma Aldrich)	2 µl
Reverse primer (10 picomoles) (Sigma Aldrich)	2 µl
Taq DNA polymerase (1U/µl) (Sigma-Aldrich)	1 µl
Template DNA (50 ng/µl)	3 µl
$MgCl_2$ (Sigma-Aldrich)	1.2µl

Sterile distilled water to a final volume of 20 µl

Amplifications were carried out in Thermal Cycler (BioRad MJ Mini Gradient) using the following program: a hot start cycle of 94°C for 5 minutes, then 30 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 2 minutes, ending with a final extension step of 72°C for 5 minutes. Aliquots (5 µl) of the amplification products were analyzed by electrophoresis on 1.2 % agarose gels. Gel was stained with ethidium bromide and visualized in UV light. The image of gel was captured using gel documentation system (Syngene, UK).

3.7.9.5 Identification of phages based on Major Capsid Protein (MCP) gene analysis

3.7.9.5.1 Designing of primers and PCR

The three pairs of primers were designed from conserved stretches of major capsid protein (MCP) of reported *Salmonella* phages available in the NCBI database and they are listed in Table.3.3. Primer designing was done using bioinformatics tools like ClustalW and Primer3 software. Products after PCR amplification were subjected to sequencing, followed by homology analysis.

Table.3.3 Primers designed for major capsid protein (MCP) amplification by PCR and their estimated amplicon size

Primer	Sequence of primers	Amplicon size	Reference
MCPF1	**AGACCAGATCCAGCAGTCC**	866	*Jeena *et al.*, 2012a
MCPR1	**AGCGGTAGTCCTCAACCAC**		
MCPF2	**AAGACCAGATCCAGCAGTCC**	861	”
MCPR2	**AGTCCTCAACCACATAGGCTTC**		
MCPF3	**GAACTGTCCAGCAAGTTCACC**	851	”
MCPR3	**GGGTTGTCGATAACTGAACG**		

MCPF: major caspid protein forward primer
MCPR: major caspid protein reverse primer

PCR Mix composition

10 X PCR buffer	2 µl
2 mM each dNTPs (Chromous Biotech, India)	2 µl
Forward primer (10 picomoles) (Sigma Aldrich)	2 µl
Reverse primer (10 picomoles) (Sigma Aldrich)	2 µl
Taq DNA polymerase (1U/µl) (Sigma-Aldrich)	1 µl
Template DNA (50 ng/µl)	3 µl
$MgCl_2$ (Sigma-Aldrich)	1.2µl

Sterile distilled water to a final volume of 20 µl

Amplifications were carried out in BioRad MJ Mini Gradient Thermal Cycler using the following program: an initial denaturation at 94°C for 5 minutes, then 30 cycles of 94 °C for 1 minute, 58°C for 1 minute and 72°C for 2 minutes, ending with a final extension step of 72°C for 10 minutes. 5 µl of the amplification product samples were analyzed by electrophoresis on 1 % agarose

gels. Gel was stained with ethidium bromide and visualized in UV light. The image of gel was captured using gel documentation system (Syngene,UK).

3.7.9.5.2 Sequencing

The amplicon was sequenced and compared with the sequences in Genbank, by BLAST programme (Altschul *et al*., 1980) to determine its identity. The sequence was deposited in the Genbank database and accession number was obtained for the submission.

3.7.9.5.3 Multiple sequence alignment and phylogenetic tree construction

Based on the BLAST analysis of obtained sequences and the maximum similarity shown towards a particular bacteriophage family, sequences available from the NCBI databases of some of the already reported phages of that same particular family were selected for multiple sequence alignment and phylogenetic tree was constructed. The tree was drawn employing Neighbour-joining method (Saitou and Nei, 1987). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Sequences were aligned using BioEdit version 5.0.6 software (Hall, 2001). The evolutionary distances were computed using the Tajima-Nei method (Tajima and Nei, 1984) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted using MEGA5 (Tamura *et al*., 2011).

3.7.10 Phage structural protein analysis

3.7.10.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) of purified phage proteins was performed under both non reducing and

reducing conditions for evaluating the nature of the phage capsid protein using vertical slab electrophoresis (Genei, Bangalore, India) by the method of Laemmli as adopted by Sambrook *et al*.(2000). Low molecular weight protein marker (Bio –Rad, USA) was used as standard and molecular weight was determined using Quantity One Software (Bio-Rad, USA).

3.7.10.1.1 Non reductive SDS –PAGE
3.7.10.1.1.1 Procedure

The gel plates were cleaned and assembled. Resolving gel (10%) was prepared by mixing 10 ml of acrylamide: bis-acrylamide (30:0.8), 3.75ml of resolving gel buffer stock, 300µl of 10% SDS and 15.95 ml of water followed by 100µl of ammonium persulfate solution (10%) and TEMED (15 µl) (Appendix - 4). The mixture was immediately poured into the cast and a layer of water was added over the gel and allowed to polymerize for at least one hour. Water layer was poured out after polymerization. The stacking gel (2.5%) was prepared by combining 2.5 ml of 30:0.8 acrylamide: bis-acrylamide solution, 5ml of stacking gel buffer stock, 200µl 10% SDS and 12.3 ml of distilled water, followed by 100µl of ammonium persulfate and 15 µl of TEMED (Appendix 4). The stacking gel was then poured into the gel assembly, above the resolving gel and the comb was immediately inserted. Gel was allowed to polymerize for 30 minutes, placed in the electrophoresis apparatus and upper and lower reservoirs filled with reservoir buffer (Appendix-4) and was pre run for 1 hour at 80V.

3.7.10.1.1.2 Phage sample preparation

Sample was prepared by mixing 100 µl of 1X sample buffer for non-reductive SDS-PAGE (Appendix-4) with concentrated phage lysate. 25 µl of this sample and 5µl low molecular weight marker mix was loaded on to the gel and

run at 80V. The current was increased to 100V, when the dye front entered the resolving gel. The run was stopped when the dye front reached 1 cm from the lower end of the plate, the gel was removed and stained.

3.7.10.1.2 Reductive SDS –PAGE
3.7.10.1.2.1 Procedure

Procedure followed for reductive SDS poly acrylamide gel electrophoresis was essentially same as that of non reductive SDS-PAGE which is as described in section 3.7.10.1.1.1**.** 100 µl of 1 X sample buffer for reductive SDS-PAGE (Appendix-4) was added to the concentrated phage lysate, mixed well, boiled for 5 minutes in a water bath and cooled to room temperature. 25 µl of this sample and 5 µl of low molecular weight marker mix were loaded onto the gel, electrophoresed and stained.

3.7.10.2 Silver staining

The gel was fixed for 30 minutes in fixing solution 1, followed by incubation in fixing solution 2 for 15 minutes. This gel was washed 5 times in water for duration of 5 minutes each**.** Sensitized the gel in freshly prepared sensitizer for 1 minute and washed in water twice for 2 minutes each. The gel was then incubated in staining solution for 25 minutes at 4°C, washed twice for duration of 1 minute each and then incubated in developing solution until the bands appear. To prevent over staining, the gel was treated for 10 minutes in sodium EDTA (Appendix-4), washed in water twice for duration of 2 minutes each. The image of gel was captured using gel documentation system (Syngene, UK).

3.7.11 Comparison of LPS from phage sensitive and resistant mutants

3.7.11.1 Isolation of phage resistant strains of S49 and cross-lysing

An exponential phase culture of the host *Salmonella* was infected with the phages. All the infections were made at MOI of 10. The host-phage mix were incubated at 37ºC for 1 hour and plated onto nutrient agar. The resistant colonies obtained were isolated. The identity of the resistant mutants was confirmed employing biochemical tests as described in section 3.1.3. Capability of phages to cross-lyse the phage-resistant mutants of each other was carried employing double agar overlay method.

3.7.11.2 LPS micro-extraction using proteinase K digestion

Lipopolysaccharides (LPSs) are a family of bacterial glycolipids which consist of a lipid that has been designated lipid A and a carbohydrate component of variable length. LPS is embedded in the external layer of the bacterial outer membrane and is linked through 2-keto-3-deoxyoctulosonic acid to a carbohydrate component that extends into the surrounding environment (Luderitz *et al*., 1966). LPS was extracted (Hitchcock, 1984) from the parental as well as phage resistant strains in order to compare the variations in the pattern displayed.

3.7.11.2.1 Procedure

The colonies of the host *Salmonella* grown on nutrient agar medium were picked with a sterile swab and suspended in 10 ml of cold phosphate-buffered saline (PBS, pH 7.2) to a $O.D_{650}$ of 0.4. It was centrifuged for 1.5 minutes at 14000g (Sigma, 3K30, Germany). The pellet was solubilized in 50µl of lysing buffer (Appendix-4) and then heated at 100ºC for 10 minutes. 25 µg of proteinase

K in 10µl of lysing buffer was added to each boiled lysate to digest the bacterial proteins, incubated at 60ºC for 60 minutes. 2 µl of this preparation was directly loaded for acrylamide gel electrophoresis.

3.7.11.3 LPS Acrylamide Gel Electrophoresis

3.7.11.3.1 Procedure

LPS Acrylamide Gel Electrophoresis was performed using vertical slab electrophoresis (Bangalore Genei, India) following the procedure of Apicella, (2008). 14% resolving gel was prepared by combining 18.45 ml of 30% acrylamide, 7.9 ml of resolving buffer and 12.57 ml of distilled water followed by 300µl of ammonium persulfate solution(10%) and TEMED (10 µl /50 ml gel solution) (Appendix-4). The gel was overlayed with distilled water and allowed to polymerize for 2 hour. The stacking gel was prepared by combining 2 ml of 30% acrylamide, 2ml of stacking buffer and 15.6 ml of distilled water followed by 200µl of ammonium persulfate, 200µl of 10% SDS and 10 µl of TEMED (Appendix-4). The layer of distilled water over the resolving gel was removed, the stacking gel was poured and the comb immediately inserted. The set up was allowed to polymerize for at least 1 hour. The gel was placed in the electrophoresis apparatus, the upper and lower reservoirs filled with reservoir buffer and was pre run for 1 hour at 80 V. The prepared samples were then loaded to the gel and were run at 80V until the dye front entered the resolving gel, then the current was increased to 100V. The run was stopped when the dye front reached 1 cm from the lower end of the glass plate. The gel was removed from the cast and silver stained.

3.7.11.4 Silver staining for LPS gels (Tsai and Frasch, 1982)

The gel was fixed in a fixing solution (Appendix-4) overnight, rinsed thrice with distilled water, transferred to a separate dish, followed by three additional washes with distilled water with agitation for 10 minutes each. The gel

was then transferred to a separate dish with freshly prepared staining reagent (Appendix-4) with shaking for 10 minutes, followed by transfer to a separate dish, rinsed thrice with distilled water, transferred again to a clean dish and added fresh formaldehyde developer (Appendix-4). Reaction was stopped by rinsing the gel in water once the bands were developed. The image of the stained gel was captured using gel documentation system (Syngene,UK).

3.7.12 Host range studies

The host range of the phage was assessed on the basis of its ability to form plaques on *Salmonella* strains present in the microbial genetics lab culture collection. A total of 96 cultures were tested including the standard strains, *Salmonella* Typhimurium (National Collection of Industrial Microorganisms (NCIM) 2501), *Salmonella* Abony (NCIM 2257), *Escherichia coli* (NCIM 2343), *Klebsiella pneumoniae* (NCIM 2957) *Pseudomonas aeruginosa* (NCIM 2863), *Bacillus cereus* (NCIM 2155), *Staphylococcus aureus* (NCIM 2127) and *Proteus vulgaris* (NCIM 2027). All the strains used in the study were grown at 37ºC in nutrient broth (HiMedia, Mumbai, India). The phage lysate was added to the cultures which were in their exponential phase, incubated for 1 hour and plated using the double agar overlay method. All plating was performed in triplicates. The plates were incubated at 37ºC and were observed for plaques.

3.8.1 Bioassay using experimentally dosed cooked chicken cuts

A simple experimental design as per Goode *et al.* (2003) and Bigwood *et al.* (2008) was used to test the biocontrol ability of the phages. The chicken cuts

collected from a local poultry farm were cut into 2 cm X 2 cm square surface area pieces, having a mean weight of 5g. Samples were cooked by autoclaving (121 ºC for 15 minutes at 15 lbs) and were experimentally contaminated by 10^3 CFU/ml of *Salmonella* by carefully pipetting on to the surface of the meat. It was then allowed to dry and was divided into four groups as shown in the Table.3.4.

Table.3.4 Phage bioassay using dosed cooked chicken-cuts

Group	**Experimental groups - cooked chicken cuts dosed with**
Group-1	*Salmonella* strain S 49 (control)
Group-2	*Salmonella* strain S 49 and ΦSP-1
Group-3	*Salmonella* strain S 49 and ΦSP-3
Group-4	*Salmonella* strain S 49, ΦSP-1 and ΦSP-3

The application of bacteriophages for the above experimental design also included using a low multiplicity of infection (MOI=10) and a high MOI (MOI=1000). Phages were also applied by carefully pipetting lysate onto the surface of the spiked chicken cut. The experiment was carried out at three different incubation temperatures i.e. 4ºC, room temperature (28± 0.5^{o}C) and 37ºC. *Salmonella* enumerations were done every 24 hours for 72 hours. For enumeration, each chicken sample was washed by agitating well in 5 ml of phosphate buffered saline pH 7 (Appendix-2). One ml of this suspension was withdrawn and was quantified on Mac Conkey agar plates after appropriate serial dilution. All plating was done in triplicates.

3.8.1.1 Statistical analysis

Statistical evaluations were done by ANOVA followed by Students–Newman–Keul Test using GraphPad InStat (version 2.04a, San Diego, USA) computer program.

3.8.2 Bioassay using model organism - *C. elegans*

Caenorhabditis elegans is a free-living, transparent, non-parasitic soil nematode (roundworm), about 1 mm in length. It has been used as a model system due to ease of manipulation and availability of detailed knowledge of its biology to study bacterial pathogenesis.

3.8.2.1 *C. elegans* strain and maintenance

The nematode *C. elegans* Bristol N2 wild type (kind courtesy of Dr Sandhya P. Koushika, National Centre for Biological Sciences, Bangaluru**)** was used for the study. It was propagated and maintained in modified Nematode Growth Medium (NGM) agar (Appendix-5) at 20ºC. Medium size plates (60 mm diameter) were used for general strain maintenance and small plates of 35 mm diameter were used for the bioassay. *E. coli* strain OP50 was used as the food source (Brenner, 1974). *E. coli* OP50 is a uracil auxotroph whose growth is limited on NGM plates. A limited bacterial lawn is desirable because it allows for easier observation of the worms. A starter culture of *E. coli* OP50 was prepared by aseptically inoculating a Luria Bertani broth (Appendix-1), and incubated overnight at 37ºC. This overnight culture was used in seeding NGM plates. The

worms were observed under a simple dissecting stereomicroscope equipped with a transmitted light source.

3.8.2.2 Long term storage of *C. elegans*

Stiernagle Soft Agar Freezing Solution was used for long term storage of *C. elegans* (Stiernagle, 2006). The procedure used was as follows: 5-6 small NGM plates containing freshly starved L1-L2 animals (Appendix-6) were washed with 0.6 ml of S Buffer (Appendix-5). The liquid was then collected in a covered sterile test tube and was place in ice for 15 minutes. Equal volume of soft agar freezing solution (Appendix-5) was added to the test tube and was mixed well. Aliquoted 1 ml of mixture into 1.8 ml cryovials and placed in ˉ80°C freezer overnight. A scoop of frozen mixture from one vial was retrieved from the lot as a tester to check how well the worms survived the freezing.

3.8.2.2.1 Thawing of frozen *C. elegans*

After removing the vial from ˉ80°C freezer, 250 µl of the frozen solution was removed from the vial with the help of a small sterile spatula, while it was still in the frozen condition. The solution was placed on a NGM plate with *E. coli* OP50 lawn. If the freezing was successful, worms wiggling after just a few minutes can be observed. Vial with the remaining worms were returned to ˉ80°C freezer as quickly as possible.

3.8.2.3 Bacterial infection and phage prophylaxis assay

The experiment was designed to study the bacterial infection in *C. elegans* Bristol N2 wild type and the role of phage in controlling the infection consisted of six groups as given in Table.3.5.

Table 3.5 Experimental setup for *Salmonella* infection and phage prophylaxis assay

Groups	***Step-1: infection*** **C. *elegans* on NGM agar *with***	***Step-2: C. elegans* from *step-1* *transferred* to NGM agar *with***
Group-1	*Salmonella* Typhimurium (NCIM 2501, as positive control for pathogen mediated death of *C. elegans*)	*E. coli* OP50
Group-2	*E. coli* OP50 (negative control)	*E. coli* OP50
Group-3	*Salmonella* strain S 49	*E. coli* OP50
Group-4	*Salmonella* strain S 49	*E. coli* OP50+ΦSP-1
Group-5	*Salmonella* strain S 49	*E. coli* OP50+ΦSP-3
Group-6	*Salmonella* strain S 49	*E. coli* OP50+ΦSP-1and ΦSP-3

C. elegans killing assays were conducted as per Aballay *et al.* (2000). *Salmonella* strains and *E.coli* OP 50 grown overnight in Luria Bertani (LB) Broth (Appendix-1) and were spread on to the center of NG agar media in 3.5 cm diameter plates. The plates were incubated at 37°C for 2–12 hours and allowed to equilibrate to room temperature. 15 to 20 worms (L-4 stage) (Appendix-6) were placed on each of these plates and were incubated at 25°C for 24 hours. After incubation, the nematodes from plates of group 1, 2 and 3 were transferred to NGM plates seeded with *E. coli* OP 50. Nematodes from plates of group 4 and 5 are transferred to plate containing *E. coli* OP 50 lawn in which ΦSP-1 and ΦSP-3 are incorporated ($1X10^{10}$ PFU) respectively. Worms from group 6 plates are transferred onto plates with *E. coli* OP 50 lawn incorporated with ($5X10^{8}$ PFU) of ΦSP-1 and ΦSP-3 each. All the plates were again incubated at 25°C for 24 hours. After incubation worms were transferred to fresh *E. coli* OP 50 plate every 24 hours for the next 4 days of the assay or until no more progeny were evident. This was done in order to avoid losing track of the original worms due to crowding by reproduction. Worm mortality was scored for 10 days, and a worm was considered

dead when it failed to respond to touch. Worms that died as a result of getting stuck to the wall of the plate were excluded from the analysis. The experiments were conducted in triplicates.

3.8.2.3.1 Statistical analysis

The time taken for 50% of the nematodes to die (time to death 50, TD_{50}) was calculated using the PRISM (version 5.04) computer program using the equation: Y = Bottom + (Top – Bottom)/ (1 + 10^ ((LogEC50 – X)*Hill Slope)), where X is the logarithm of days and Y is the average of dead worms. The data represents the mean ± the standard error.

3.8.2.4 Microscopy of *C. elegans*

Agarose pads were prepared on microscope slides using 2% (w/v) agarose. 1-2 µl drop of M9 buffer (Appendix 5) containing 25 mM Sodium azide (NaN_3) was placed onto the center of the agar pad. NaN_3 anesthetizes the worms, thereby preventing their movement. Worms to be viewed were transferred into the drop with the help of a worm pick. Once the worm was anesthetized, a cover slip was gently placed on top and observed under the microscope.

Chapter 4

RESULTS

4.1 Isolation and identification of *Salmonella*

The isolation of *Salmonella* specific lytic phage requires the presence of suitable *Salmonella* host. In this study, attempts were made to isolate suitable host bacteria from the intestinal contents of 18 samples of broiler chicken collected from the retail markets in Ernakulam, Kerala.

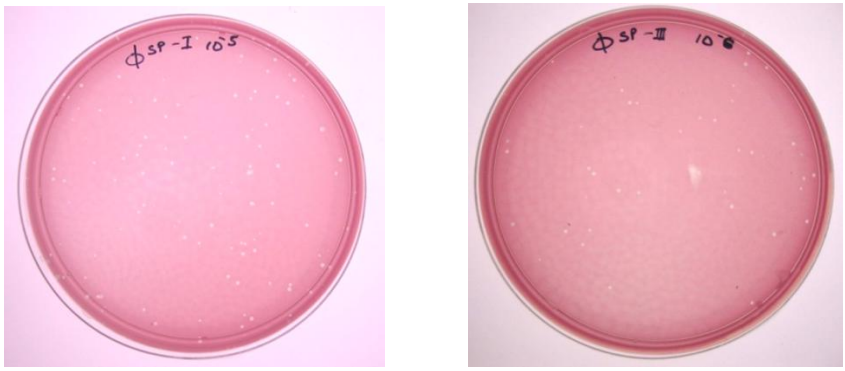
25 isolates of *Salmonella* were segregated based on their biochemical characteristics. The isolates which showed red- alkaline slant, yellow- acid butt with hydrogen sulphide production on TSI agar medium, purple slant with hydrogen sulphide production in LIA agar medium, were indole negative, urease negative, citrate positive and which fermented dulcitol were segregated as *Salmonella,* as these were standard biochemical results for *Salmonella*. These isolates, along with the other *Salmonella* cultures as mentioned previously in section 3.1.2 were used as host for isolating specific lytic phages.

The *Salmonella* strain, designated as S49 which could successfully act as a host in isolation of three bacteriophages was further characterized by serotyping and was identified as *Salmonella* Enteritidis 9, 12: g, m:- at the National *Salmonella* and *Escherichia* Centre, Kasuali, Himachal Pradesh. The 16S rRNA gene sequence analysis of S49 by BLAST showed maximum sequence similarity with *Salmonella enterica* subsp. *Enterica* and the sequence was submitted to GenBank (Accession Number: S49 : HQ268500).

4.2 Bacteriophage isolation

Salmonella strain S49 was the host bacterium that helped to isolate three lytic bacteriophages from the intestinal contents of broiler chicken,namely ΦSP-1, ΦSP-2 and ΦSP-3. All three phages were obtained only via the enrichment protocol. Of the three, only ΦSP-1 and ΦSP-3 consistently exhibited excellent bacterial cell lysis capability and were therefore selected for further study.

ΦSP-1 and ΦSP-3 were purified by repeated plating and picking of single isolated plaques from the lawns of *Salmonella* S49 plates, and produced small, clear plaques indicating their lytic nature. The tetrazolium stained plates showing plaques formed by phage ΦSP-1 and ΦSP- 3 on bacterial lawn of *Salmonella* S49 is as presented in Fig 4.1a and b.



(a) (b)

Fig 4.1. Tetrazolium stained plates showing plaques formed by phage on bacterial lawn of *Salmonella* S49 (a) ΦSP-1 (b) ΦSP- 3.

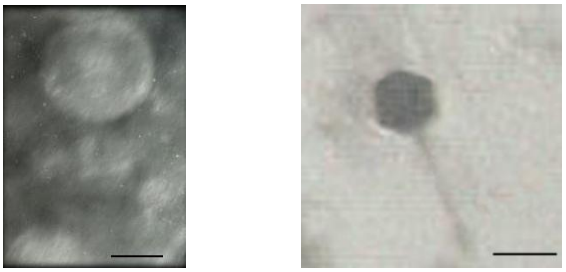
4.3 Concentration of ΦSP-1 and ΦSP-3

ΦSP-1 and ΦSP-3 were concentrated up to 1 X 10^{10} PFU/ml using PEG precipitation and these phage concentrates prepared in large quantities were used for all further studies.

4.4 Characterization of phage

4.4.1 Morphological analysis by TEM

Transmission Electron Microscopy is routinely used in the morphological characterization of phage. The TEM elucidated morphology has great significance, as it forms the basis for the classification of bacteriophages.



(a) **(b)**

Fig 4.2. Transmission Electron micrograph image of phage stained with 1% uranyl acetate (a) ΦSP-1 (bar represents 20nm) (b) ΦSP-3 (bar represents 60nm).

The TEM picture of ΦSP-1 revealed identical hexagonal/ isomeric head outline, indicating their icosahedral nature, with an extremely short tail, which is characteristic of family *Podoviridae* (Fig 4.2 a). The diameter of the head was 44.71 ± 0.62 nm and the length of the tail was 15.88 ± 0.83 nm. The electron micrograph of ΦSP-3 on the other hand clearly showed bacteriophage with a hexagonal head (53.77 ± 0.38nm) having a long non- contractile tail (123.66 ± 0.32nm), which are typical morphological features of family *Siphoviridae* (Fig 4.2 b). The phage sizes were determined from the average of 3 independent measurements (mean ± standard deviation).

4.4.2 Optimal multiplicity of infection

Multiplicity of infection, which is defined as the ratio of virus particles to that of the host cells is an important criterion for the large scale production of bacteriophages, due to its significant impact on phage titre. With *Salmonella* strain S49 as host, the optimal MOI of ΦSP-1 was five phages per bacterium and that of ΦSP-3 was one phage per bacterium. These optimal MOI resulting in the highest phage titre under standard conditions were used in all subsequent large scale phage production of ΦSP-1 and ΦSP-3 respectively, unless otherwise specified.

4.4.3 Phage adsorption

The adsorption curve of ΦSP-1 and ΦSP-3 are shown in Fig 4.3. For ΦSP-1 adsorption nearing 100% was achieved after 30 minutes of exposure to the host bacteria, where as for ΦSP-3 it took only 25 minutes to achieve the same.

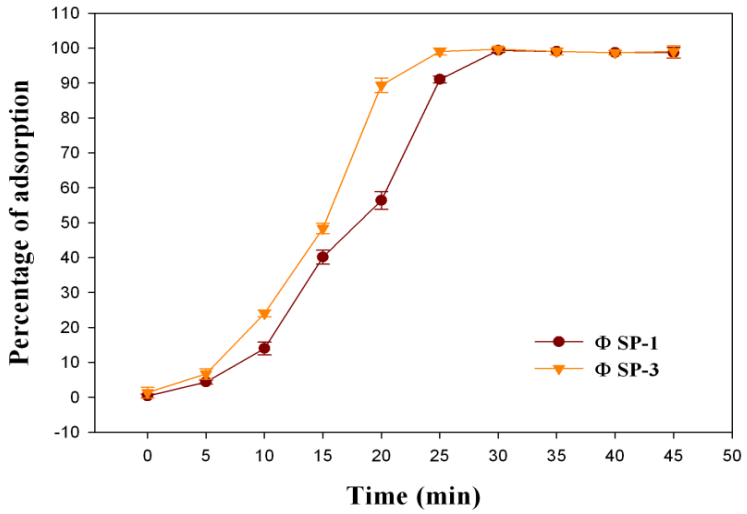


Fig 4.3. Adsorption curves of ΦSP-1 and ΦSP-3

4.4.4 One step growth curve

The one step growth curve helped in understanding the growth kinetics parameters like latent period, rise period and the burst size of the bacteriophages under study. The one step growth curve of ΦSP-1 and ΦSP- 3 is as given in Fig 4.4. These experiments were performed at 37°C, with an MOI of five for ΦSP-1 and one for ΦSP-3.

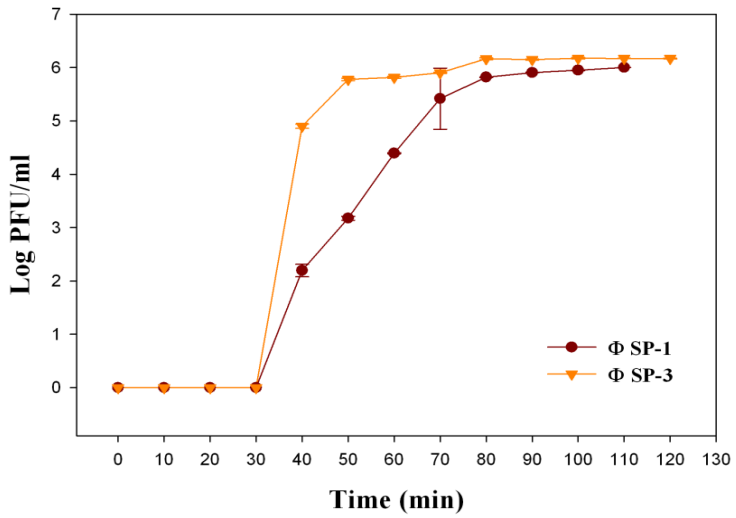


Fig 4.4. One step growth curve of ΦSP- 1[1] and ΦSP-3. ([1]Data presented is adapted from Jeena *et al.*,2012b).

The calculated latent period of ΦSP-1 was approximately 30 minutes, the rise period as 55 minutes, and the burst size was 44 phages per bacterial cell. The multiplication period reached a plateau at about 90 minutes after infection with ΦSP-1 (Fig 4.4).

The one step growth curve of ΦSP-3(Fig 4.4) clearly showed the latent period and the rise period to be about 30 minutes each. The calculated burst size

was 60 phages per bacterium. It was noticeable from the results that ΦSP-3 has a larger burst size and a shorter generation period than that of ΦSP1.

4.4.5 Influence of various physical and chemical parameters on viability / propagation and adsorption of ΦSP-1 and ΦSP-3

4.4.5.1 Effect of temperature on viability

The effect of temperature on the viability of ΦSP-1, investigated by heat treatment at different temperatures, over varying time intervals is represented in Fig 4.5.

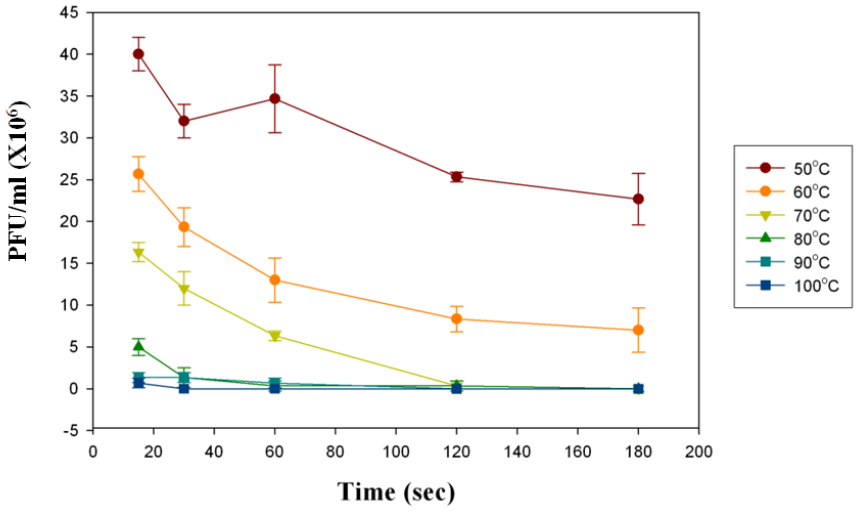


Fig 4.5. Effect of temperature on viability of ΦSP-1 (Data presented is adapted from Jeena *et al*., 2012b).

It is evident that phage viability was drastically reduced by exposure to high temperatures. Viable PFU of ΦSP-1 were highest when exposed to 50°C for 3 minutes. At 60°C viable PFU were reduced by more than 50% at the end of 3 minutes when compared to the count at 50°C, whereas complete viability was lost at the end of 2 minutes at 70°C. Although exposure to 80°C was fatal over an exposure period of 3 minutes, there were nevertheless a few survivors at the end of

15 seconds. ΦSP-1 failed to survive when exposed to 90°C and 100°C, even for a few seconds. In all cases, phage count steadily decreased with increase in exposure time at different temperatures.

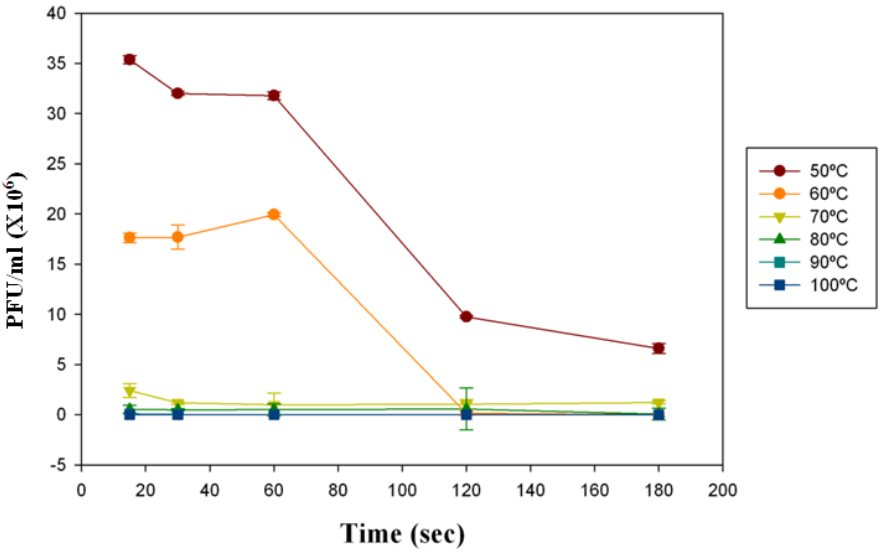


Fig 4.6. Effect of temperature on viability of ΦSP-3

The influence of temperature variation on viability of phage ΦSP-3 is as depicted in Fig 4.6. Viability was drastically reduced to a few PFU/ml at 70ºC, while exposure to temperatures above 70ºC even for a few seconds, was fatal for phage ΦSP-3 as there were no survivors. However, considerable survival was noted at 60ºC. The number of viable PFUs were highest when exposed to 50ºC, but that was only for a relatively shorter period (up to 60 seconds) when compared to that ΦSP-1.

It may be noted that ΦSP-1 was much tolerant to higher temperatures than ΦSP-3 and showed greater survival under the experimental conditions.

4.4.5.2 Effect of NaCl on viability

The effect of varying concentration of NaCl on the viability of the phage Φ SP-1 and ΦSP-3 is as shown in the Fig 4.7.

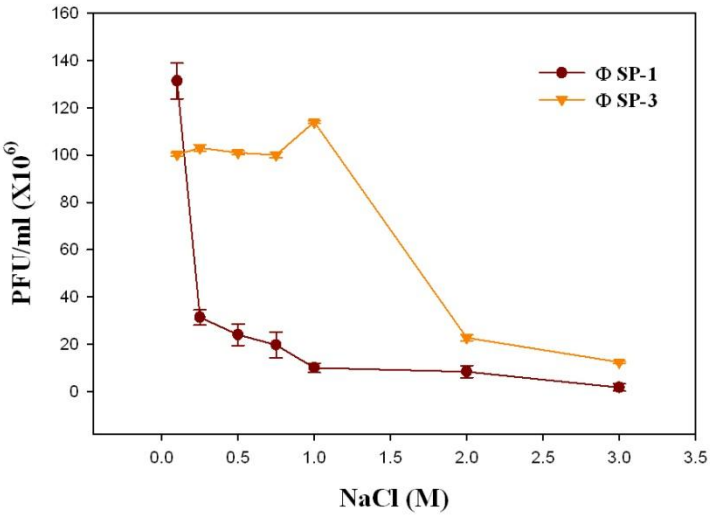


Fig 4.7. Effect of NaCl on viability of phage ΦSP-1[1]and ΦSP-3 ([1]Data presented is adapted from Jeena *et al.*,2012b).

It is apparent that for ΦSP-1**,** the optimum concentration of NaCl required for phage survival was 0.1M (Fig 4.7). There was considerable reduction in viability of ΦSP-1 at concentrations higher than 0.1M NaCl and the phages did not survived concentrations beyond 1M NaCl.

The study on viability of ΦSP-3 in the presence of varying concentration of NaCl revealed 1M NaCl as optimal for phage survival. The presence of higher concentrations of NaCl caused a decline in the viability of ΦSP-3 as observed from Fig 4.7.

4.4.5.3 Effect of pH on viability

The viability of the phages was studied over a pH range of 2 – 13. Fig 4.8. elucidates the effect of pH on viability of ΦSP-1 and ΦSP-3**.**

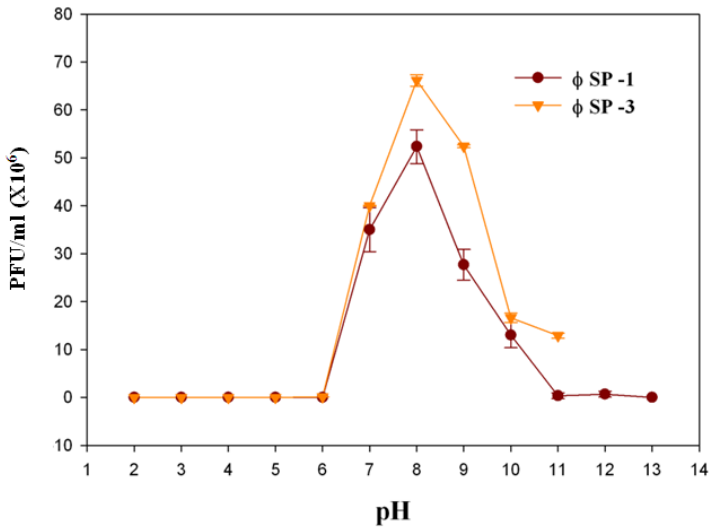


Fig 4.8. Effect of pH on viability of phage ΦSP-1[1] and ΦSP-3 ([1]Data presented is adapted from Jeena *et al*., 2012b).

From the figure, it is evident that pH 8 is optimum for survival of ΦSP-1. Viability was observed even at pH as high as 10, although in small numbers. At the acidic pH of 6 and below and at pH above 11, ΦSP-1 did not survive.

The optimum pH for ΦSP-3 viability was found to be 8. It was clear from the figure that ΦSP-3 also could not survive at acidic pH ranging from 2 to 6. Both ΦSP-1 and ΦSP-3 had poor tolerance to acidic pH conditions, but showed greater survival at alkaline pH. Even though both phages shared similar pH

affinities, ΦSP-3 survived better under these conditions which was evident from the higher PFUs at all higher pH studied, starting from pH 7.

4.4.5.4 Effect of sugars on phage viability

The effect of sugars on the viability of phage ΦSP-1 and ΦSP-3 was studied using sugars - xylose, ribose, mannose, mannitol, dextrose, arabinose, fructose, galactose, rhamnose, maltose and lactose , each at a final concentration of 500 mM/l, is as shown in Fig 4.9 and 4.10.

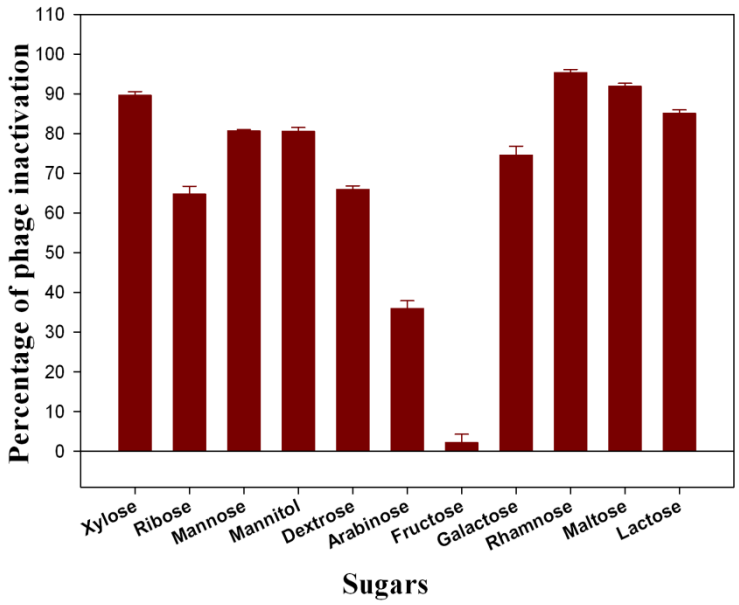


Fig 4.9. Effect of sugars on viability of ΦSP-1[1] ([1]Data presented is adapted from Jeena *et al*., 2012b).

The influence of eleven different sugars on ΦSP-1 viability is depicted in Fig 4.9. Sugars like rhamnose, maltose and xylose resulted in drastic inactivation of ΦSP-1 by as much as 95%, 92% and 89% respectively. Phage inactivation by lactose was 85%. Mannose and mannitol were able in inactivate about 80% of phages, while galactose, dextrose and ribose caused 74%, 65% and 64 %

inactivation respectively. Arabinose caused only 35 % inactivation. Fructose had the least inhibition capacity with only 2% inactivation, compared to control.

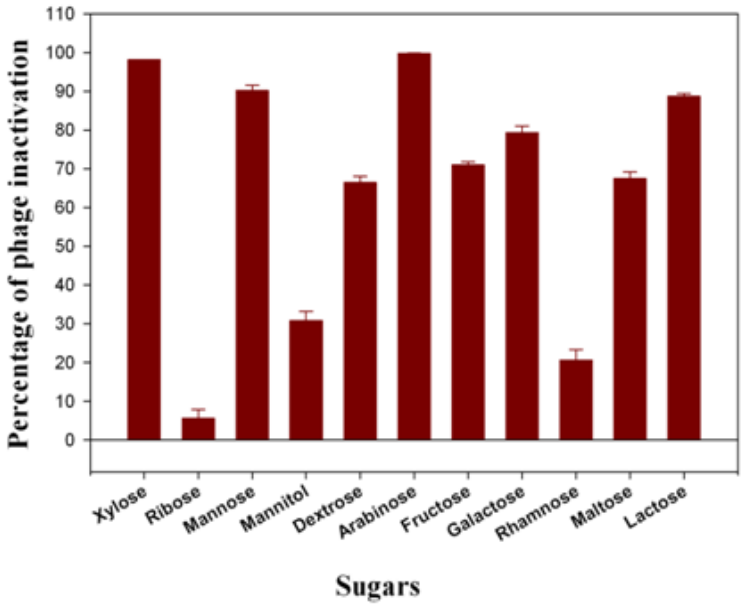


Fig 4.10 Effect of sugars on viability of ΦSP-3

The influence of sugars on ΦSP-3 viability is depicted in Fig 4.10. Arabinose (99%) followed by xylose (98%) was found to be the most influential in inactivating ΦSP-3. Presence of sugars like mannose (90%), lactose (88%) and galactose (79%) also significantly inactivated the phage. Fructose, maltose and dextrose caused 71%, 67% and 66% phage inactivation respectively. The inhibition capacity exhibited by mannitol (30%) and rhamnose (20%) was comparatively low. Ribose (5%) showed the least inhibitory capacity among the sugars studied.

4.4.5.5 Effect of temperature on adsorption

The effect of different temperatures ranging from 0ºC to 50ºC on the adsorption of ΦSP-1 and ΦSP-3 are as represented in Fig4.11.

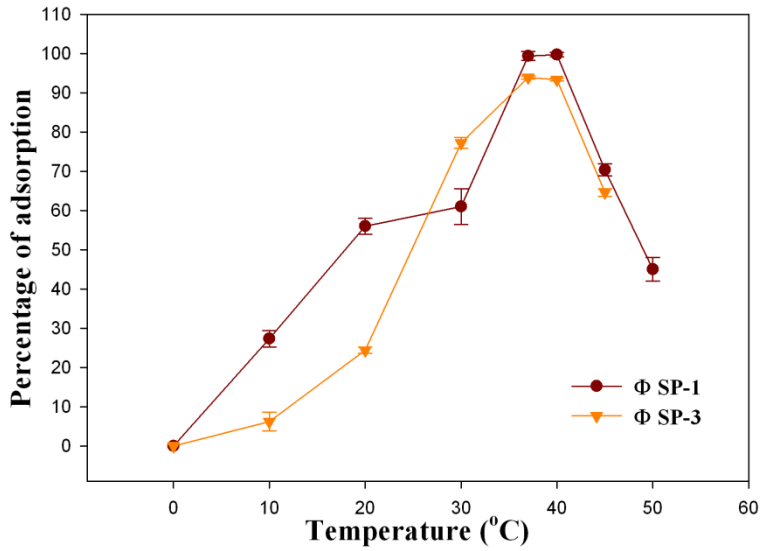


Fig 4.11. Effect of temperature on adsorption of ΦSP-1[1] and ΦSP-3 ([1]Data presented is adapted from Jeena *et al.*, 2012b).

In case of ΦSP-1, maximum adsorption was observed at 37ºC and 40ºC, and these temperatures were therefore considered optimal for ΦSP-1. There was no observable adsorption at 0 ºC, but there was a steady increase in adsorption as the temperature was raised to 10ºC. It was interesting to note that moderate adsorption occurred at higher temperatures i.e., 70% adsorption at 45ºC and 45% adsorption at 50ºC.

Similarly for ΦSP-3,maximum adsorption nearing 100% was observed at 37ºC and at 40ºC, while 80% adsorption was observed at 30ºC . Ability to

successfully adsorb on to the bacterial surface at higher temperatures, albeit at lower percentiles was shown by both ΦSP-1 and ΦSP-3.

4.4.5.6 Effect of NaCl on adsorption

The influence of sodium chloride on adsorption by ΦSP-1and ΦSP-3 is as represented in Fig 4.12.

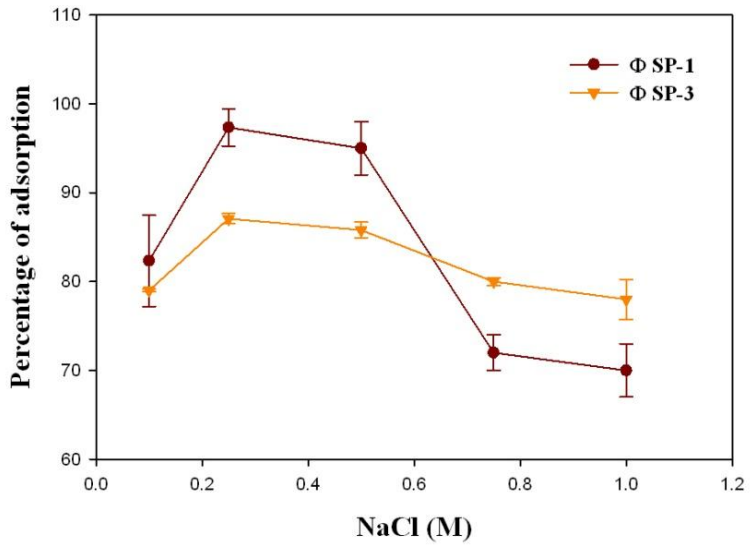


Fig 4.12. Effect of NaCl on adsorption of phage ΦSP-1[1] and ΦSP-3 ([1]Data presented is adapted from Jeena *et al.*, 2012b).

Optimal NaCl concentration for maximal adsorption was found to be 0.25M for ΦSP-1 beyond which the efficacy dropped. Adsorption was observed to take place even at concentration of 1M NaCl, although at comparatively lower levels. The percentage of adsorption dropped to 70% at 1 M NaCl concentration. 82% adsorption was observed at a concentration of 0.1M NaCl.

In case of ΦSP-3, 0.25M concentrations of NaCl favoured adsorption. Maximum adsorption of 87% was observed at 0.25M sodium chloride. Adsorption continued to occur at higher concentration of NaCl i.e. 0.75M and 1M (80 and 78% respectively). The ability of ΦSP-3 to successfully adsorb on exposure to a wide range of NaCl concentration was apparent, unlike ΦSP-1.

4.4.5.7 Effect of pH on adsorption

The influence of pH on the adsorption of ΦSP-1 and ΦSP-3 to S49 is as shown in Fig 4.13.

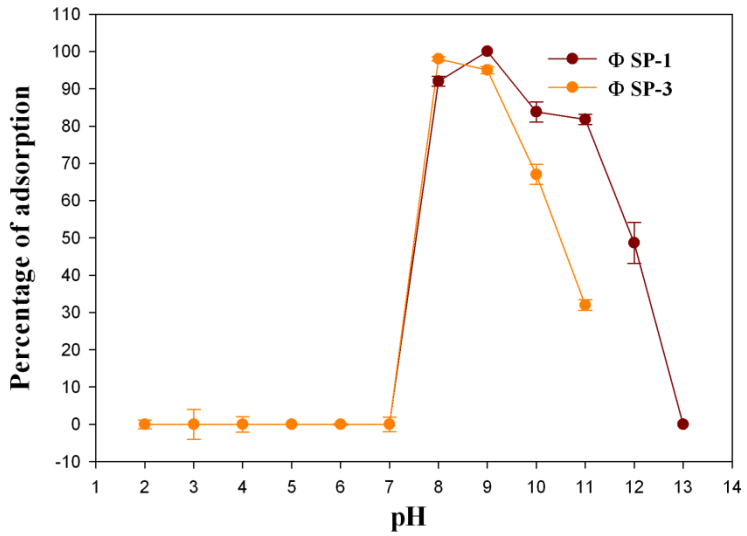


Fig 4.13. Effect of pH on adsorption of ΦSP-1[1] and ΦSP-3 ([1]Data presented is adapted from Jeena *et al*., 2012b).

From the Fig 4.13, it is evident that 100% adsorption of ΦSP-1 was recorded at pH 9, while adsorption greater than 80% was observed over the pH range of 8 to 11. Optimal pH for maximal adsorption was pH 9, above and below this pH the efficacy decreased and 0% adsorption was recorded below pH 8.

In the case of ΦSP-3, pH 8 was observed to be optimum, giving maximum adsorption (98%), closely followed by pH 9, at which 95% of phages were successfully adsorbed on the bacterial host. Less than 40% of the phage ΦSP-3 adsorbed at pH 11. Adsorption was not observed at pH ≤ 7.

The pH optima for maximal adsorption for both ΦSP-1 (pH 9) and ΦSP-3 (pH 8) were different, although they were both in the alkaline pH range. pH ≤ 7 was detrimental in both cases, with no observed adsorption.

4.4.5.8 Effect of $CaCl_2$ on adsorption and propagation

The propagation of Φ SP-1 and Φ SP-3 in the presence of varying concentration of $CaCl_2$ is as depicted in Fig 4.14.

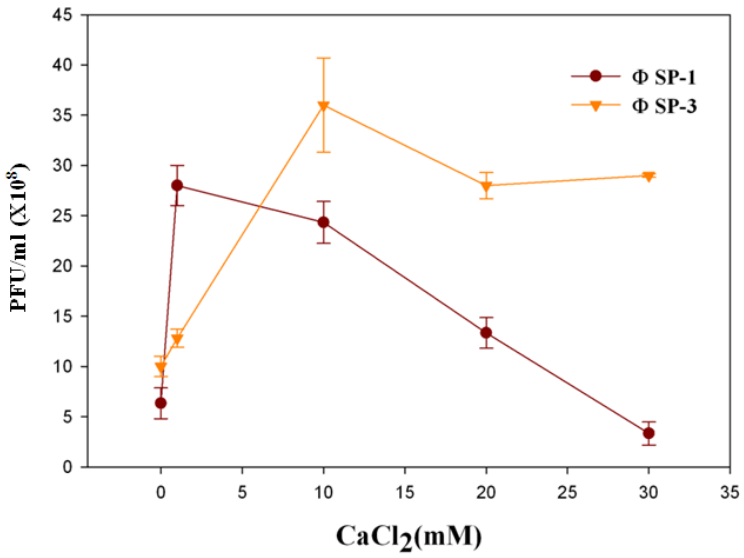


Fig 4.14. Effect of $CaCl_2$ on propagation of phage ΦSP-1[1] and ΦSP-3 ([1]Data presented is adapted from Jeena *et al.*, 2012b).

In the case of ΦSP-1, propagation was optimum at 1mM $CaCl_2$, closely followed by 10mM $CaCl_2$ concentration.

On the other hand, 10mM of $CaCl_2$ was found to be optimum for ΦSP-3. For ΦSP-3, the decline in the number of viable viral particles was gradual beyond 10mM $CaCl_2$, unlike ΦSP-1 where a drastic dip in viral count was observed.

4.4.6 Cumulative effect of optimized parameters on propagation of ΦSP-1 and ΦSP-3

The phages were propagated under optimized parameters viz. section 3.7.2. , 3.7.5., and 3.7.6, and this affected an increase in the phage titre of both ΦSP-1 and ΦSP-3, as evidenced from Fig.4.15 and 4.16 respectively.

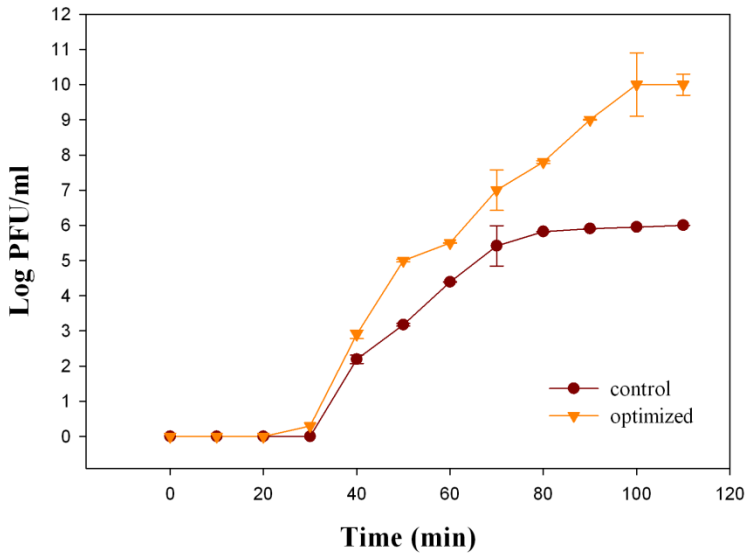


Fig 4.15. Effect of optimized parameters on propagation of ΦSP-1

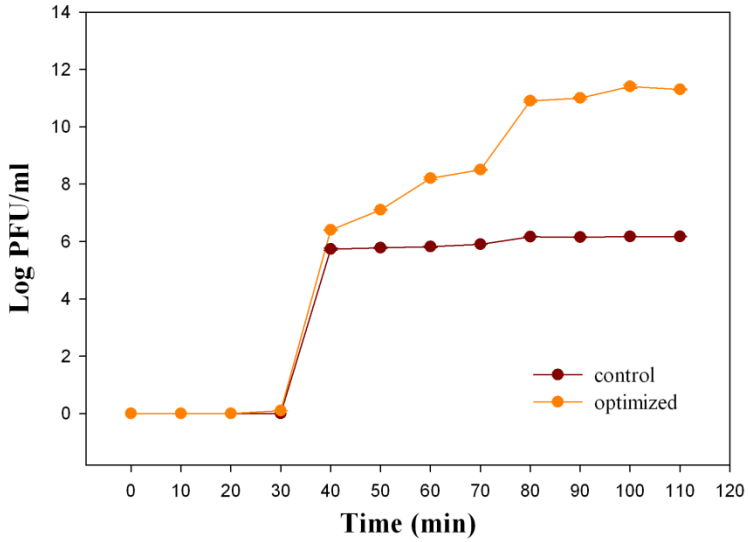


Fig 4.16. Effect of optimized parameters on propagation of ΦSP-3

Under optimized conditions, the latent period remained the same (30 minutes) for ΦSP-1 and ΦSP-3. A longer rise period of 70 minutes was observed in case of ΦSP-1 and the burst size increased from 44 to 73 phages per bacterial cell. For ΦSP-3 the rise period increased to 50 minutes. Burst size was raised from 60 to 110 phages per bacterial cell.

4.4.7 Propagation of ΦSP-1 and ΦSP-3 under nutrient depleted state of host cell

The ability of ΦSP-1 and ΦSP-3 to infect host under different nutrient deprived conditions as depicted in Fig 4.17 and 4.18 respectively.

ΦSP-1 was able to multiply under multiple nutrient starved states ($\log_{10}$ 2.60 ± 0.43^{a} PFU/ml). In all other conditions except when S49 host cells were in

logaritmthic phase, ΦSP-1 failed to multiply (Fig4.17). (*Value represent mean ± standard deviation, [a] p<0.001 when compared to logarithmic phase).

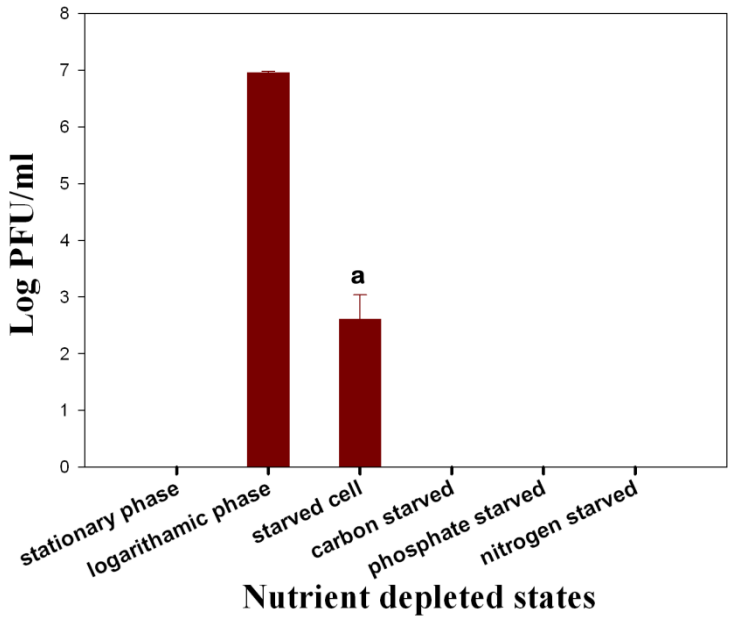


Fig 4.17. ΦSP-1 propagation in nutrient deprived conditions of the host cell S49
([a] indicates p< 0.001 when compared to logarithmic phase).

The propagation ΦSP-3 on host bacterial strain *Salmonella* S49 under various nutrient limited conditions exhibited significant outcome (Fig 4.18). ΦSP-3 multiplication was maximum when the host was in the logarithmic phase (log_{10}9.81± 0.10 PFU/ml) as depicted in Fig 4.18. ΦSP-3 also infected host under stationary phase, although in low numbers (log $_{10}$0.51 ±0.07 PFU/ml) and was able to multiply even under multiple nutrient starved states, as evidenced by a significant level of increase in phage titre, log_{10}4.61±0.15[a] PFU/ml. However, under phosphate starved condition, ΦSP-3 failed to infect its host and multiply. Nevertheless, successful propagation of ΦSP-3 in the host S49 under carbon limiting conditions (log_{10} 3PFU/ml)[a] and nitrogen starved state (log $_{10}$2.81±0.13 PFU/ml)[a] was exhibited. In all cases, except under stationary phase, phages were

able to outnumber the host. (*Values represent mean ± standard deviation,[a] $p<0.001$ when compared to stationary phase).

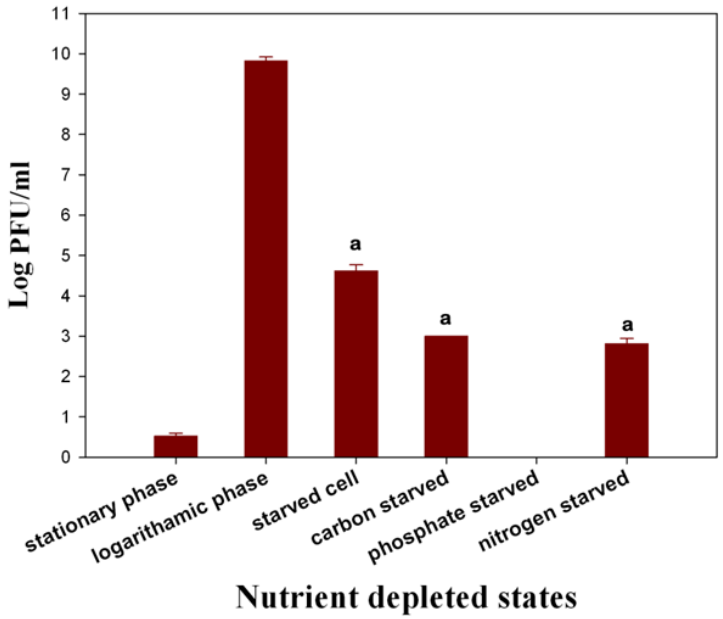


Fig 4.18 ΦSP-3 propagation in nutrient-deprived condition of host cell S49.([a] indicates $p< 0.001$ when compared to stationary phase, data presented is adapted from Jeena *et al.*, 2012b).

4.4.8 Bacteriophage genome analysis

4.4.8.1 Phage DNA isolation

The genomic DNA of ΦSP-1 and ΦSP-3 was isolated and was visualised by agarose gel electrophoresis as single band (Fig 4.19).

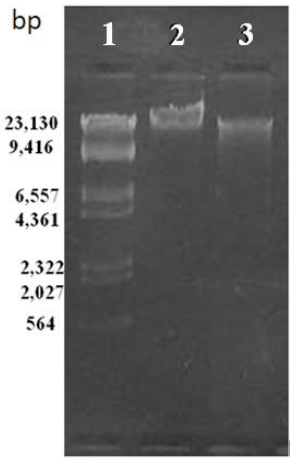


Fig 4.19. Agarose gel (1%) electrophoresis of phage DNA. Lane 1: Lambda DNA / *Hind* III Digest, Lane 2: ΦSP-1 DNA and Lane 3: ΦSP-3 DNA.

4.4.8.2 Restriction analysis

The restriction pattern of the phage DNA revealed the susceptibility of phage genome to the restriction endonuclease *Bam* HI. The result is as shown in Fig. 4.20.

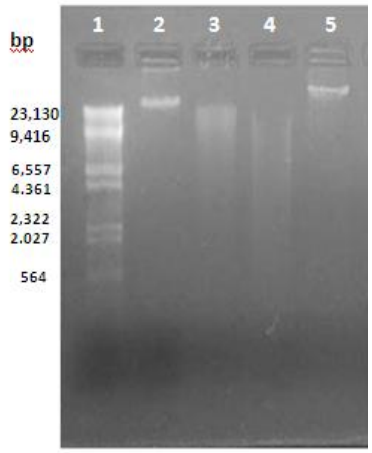


Fig. 4.20. Restriction analysis of phage DNA. Lane 1 : Lambda DNA/*Hin*d III Digest , Lane 2: uncut ΦSP-1 DNA ; Lane 3: *Bam*HI digest of ΦSP-1 DNA, Lane 4: *Bam*HI digest of ΦSP-3 DNA and Lane 5: uncut ΦSP-3 DNA.

The nature of the genome of both the phages ΦSP-1 and ΦSP-3, was identified as double stranded DNA on the basis of its sensitivity to digestion by restriction endonuclease. The double stranded nature of the phage DNA places both Φ SP-1 and ΦSP-3 under the order *Caudovirales*.

4.4.8.3 Pulsed Field Gel Electrophoresis (PFGE) of phage DNA

The genome of phages ΦSP-1 and ΦSP-3 were visualised after Pulsed-Field gel electrophoresis and is as shown in the Fig 4.21.

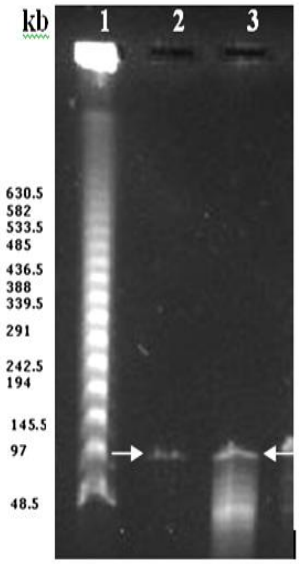


Fig 4.21.Pulsed-field gel electrophoresis of phage DNA. Lane 1: lambda DNA 1000 kb concatamers , Lane 2: ΦSP-1 DNA[1] and Lane 3: ΦSP-3 DNA[2] ([1]Data presented is adapted from Jeena *et al*.,2012b, [2]Data presented is adapted from Jeena *et al*., 2012a).

The genome size of ΦSP-1 was determined to be 86 kb and that of ΦSP-3 as 88.43 kb, by comparing it with the 1000kb ladder using Quantity One® software (BioRad, USA).

4.4.8.4 Virulence gene profiling

A set of eleven virulence genes common among *Salmonella* Enteritidis were selected for screening (Table 3.2). The multiplex PCR screening method used to screen for virulence genes in the genome of the bacterial host *Salmonella* Enteritidis strain S49, indicated the presence of the following virulence genes: *invE/A* (457bp) , *afgA* (261bp), *slyA* (700bp), *phoP/Q* (299bp), *ttrC* (920bp) and *mgtC* (655bp) confirming its pathogenicity (Fig 4.22).

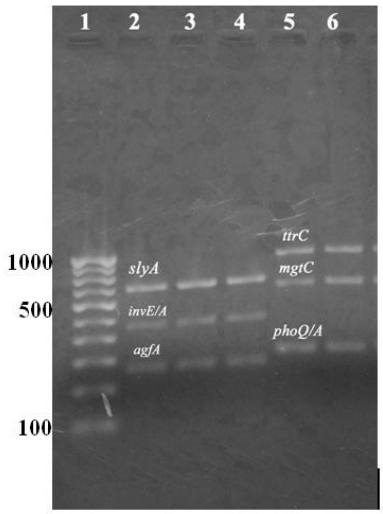


Fig 4.22 Multiplex PCR amplification for screening of virulence genes in host *Salmonella* S49. Lane 1: 100 bp ladder; Lane 2: *slyA* ,*invE/A and afgA* -positive control (*S.* Typhimurium); Lane 3&4: S49 with *slyA, invE/A and afgA*; Lane 5: *ttrC* , *mgtC and phoP/Q* -positive control (*S.* Typhimurium); Lane 6: S49 with *ttrC* , *mgtC and phoP/Q.*

All the sequences obtained were deposited in Genbank and accession numbers obtained (Accession Number: HQ260701, HQ260703, HQ260704, HQ260706, HQ260707, HQ260708 and HQ260709). The multiplex PCR indicated the absence of all these virulence genes in the genome of the *Salmonella* phages, ΦSP-1 and ΦSP-3.

4.4.8.5 Identification of phages based on MCP gene sequence analysis

The three sets of primers were designed from conserved stretches of major capsid protein (MCP) of reported *Salmonella* phages and are as described in Table 3.3. These designed primers were successfully used in amplification of MCP in both ΦSP-1 and ΦSP-3 by multiplex PCR. The agarose gel electrophoresis of the PCR product for MCP gene in ΦSP-1 and ΦSP-3 is as shown in Fig 4.23 a and b.

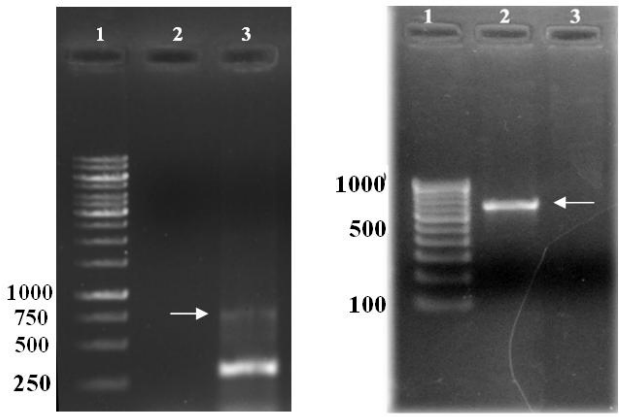


Fig 4.23.Agarose gel electrphoresis of the PCR product for MCP gene in *Salmonella* specific bacteriophage (a) ΦSP-1 and (b) ΦSP-3[1] (a) Lane 1: 1 kb ladder; lane 2 : negative control and lane 3: amplicon of MCP gene from ΦSP-1 (b) Lane 1: 100 bp ladder, lane 2: amplicon of MCP gene from ΦSP-3 and lane 3: negative control. ([1]Data presented is adapted from Jeena *et al*., 2012a)

Amplicons of about 750bp were obtained for both ΦSP-1 and for ΦSP-3 in PCR with the designed MCP primers.

These amplicons obtained were sequenced and the sequences obtained were submitted to GenBank (ΦSP-1: Accession Number JQ638925; ΦSP-3: Accession Number JQ638926). Based on the MCP amplicon sequences, the relatedness of Φ SP-1 and ΦSP-3 with other *Salmonella* phages were studied by constructing a phlyogenetic tree as depicted in Fig 4.24 and Fig 4.25 respectively.

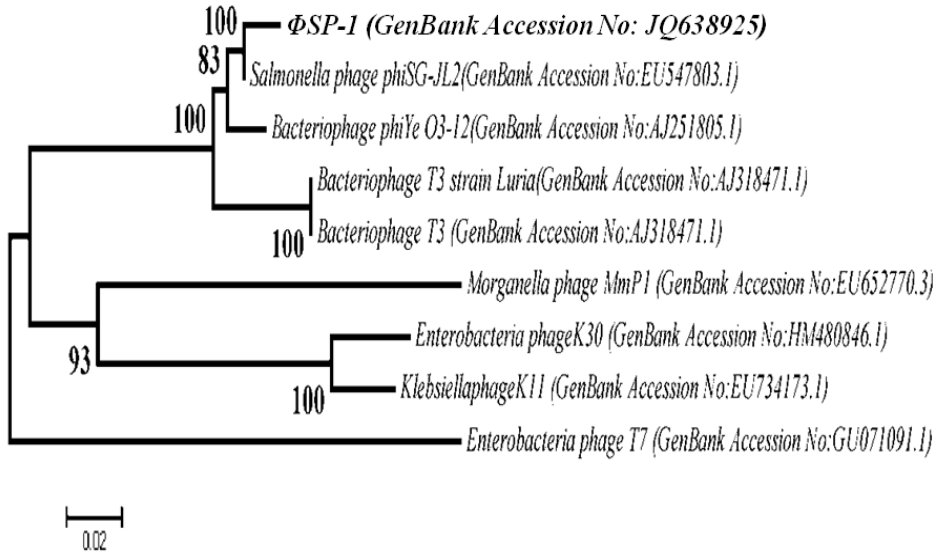


Fig 4.24. Phylogentic tree of ΦSP-1 based on MCP gene sequences. The optimal tree with the sum of branch length = 0.60 is shown. The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates (Felsenstein, 1985). Bar = 0.2 % sequence divergence.

BLAST analysis of MCP gene sequence obtained from ΦSP-1 revealed maximum similarity (93%) towards *Salmonella* phage phiSG-JL2 (GenBank Accession No: EU547803.1), which is a T7 -like phage. The lineage of *Salmonella*

phage phiSG-JL2 is *Caudovirales; Podoviridae; Autographivirinae*; T7-like viruses.

Phylogenetic tree of ΦSP-1 was constructed using reported phage of the *Podoviridae* family, which includes *Salmonella* phage phiSG-JL2 (GenBank Accession No : EU547803.1), Bacteriophage phiYe O3-12(AJ251805.1), Bacteriophage T3 strain Luria(AJ318471.1), Bacteriophage T3 (AJ318471.1), *Enterobacteria* phageK30 (HM480846.1), *Klebsiella* phageK11 (EU734173.1), *Enterobacteria* phage T7 (GU071091.1) and *Morganella* phage MmP1 (EU652770.3). ΦSP-1 was found to have maximum similarity towards *Salmonella* phage phiSG-JL2 (GenBank Accession No: EU547803.1). The phylogenetic tree of ΦSP-1 based on the MCP gene sequence is depicted in Fig 4.24.

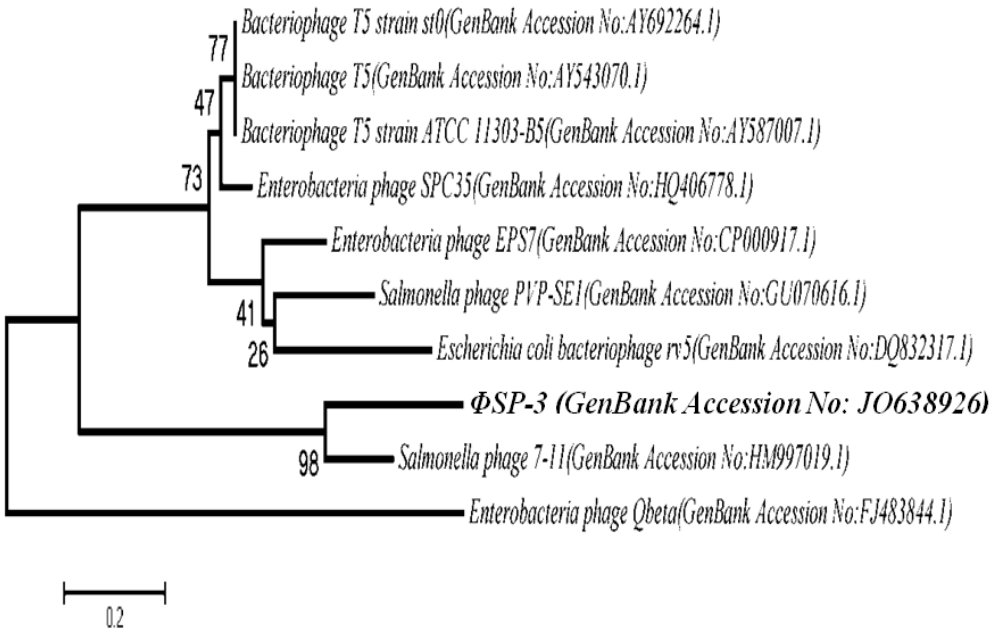


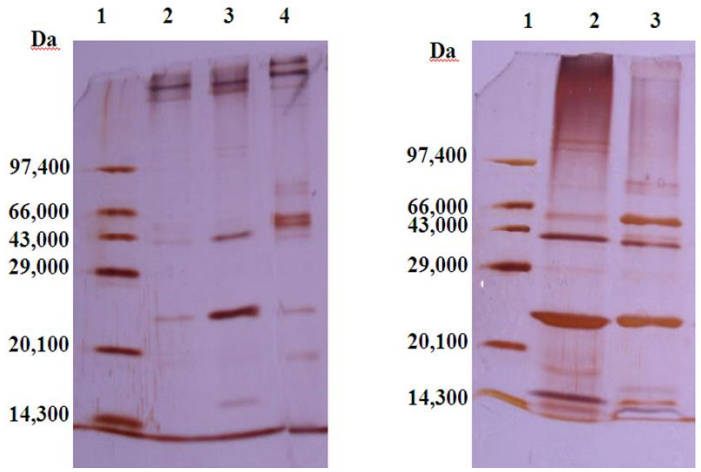
Fig.4.25. Phylogentic tree of ΦSP-3[1] based on MCP gene sequences. The optimal tree with the sum of branch length = 3.05 is shown. The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates (Felsenstein, 1985). Bar = 2 % sequence divergence ([1]Data presented is adapted from Jeena *et al*.,2012a).

BLAST analysis of MCP amplicon sequence from ΦSP-3 revealed maximum similarity (95%) towards Enterobacteria phage SPC35 (GenBank Accession No: HQ406778.1), whose lineage is : *Caudovirales; Siphoviridae;* T5-like viruses; unclassified T5-like viruses).

Other reported *Siphoviridae* phages include Enterobacteria phage SPC35 (GenBank Accession No:HQ406778.1), Bacteriophage T5 strain st0 (AY692264.1), BacteriophageT5 (AY543070.1), Bacteriophage T5 strain ATCC 11303-B5 (AY587007.1), Enterobacteria phage EPS7 (CP000917.1), *Salmonella* phage PVP SE1 (GU070616.1) *Escherichia coli* bacteriophage rv5 (DQ832317.1), *Salmonella* phage 7-11 (HM997019.1) and Enterobacteria phage Qbeta (FJ483844.1). The MCP gene sequences for these phages were therefore selected for the phylogenetic analysis involving ΦSP-3. Fig 4.25 shows the phylogenetic tree of ΦSP-3. ΦSP-3 was found to be closely related to the phage *Salmonella* 7-11 (GenBank Accession No:HM997019.1) in this analysis.

4.4.9 Structural protein analysis

To compare the structural protein of the phages, the protein profile of ΦSP-1 and ΦSP-3 were analysed by SDS-PAGE under non reducing and reducing conditions (Fig 4.26 a and b). Molecular weights of the proteins were determined using Quantity One® software (BioRad, USA).



(a) **(b)**

Fig.4.26. SDS PAGE of phage proteins of ΦSP-1 and ΦSP-3 under (a) non reducing and (b) reducing conditions. (a) lane 1: marker; lane 3: ΦSP-1 and lane 4: ΦSP-3. **(b)** lane 1: marker; lane 2: ΦSP-1 and lane 3: ΦSP-3

Under non reducing conditions, ΦSP-1 profile showed a total of seven bands on the gel, with four prominent bands, whose molecular size was calculated as 215520 Da , 202252 Da, 43000 Da and 23947 Da (Fig 4.26 a : lane 3).

For ΦSP-3 under non-reducing conditions, eleven protein bands were observed, of which the bands with molecular weights- 258714 Da , 235196Da, 60210 Da and 54094 Da, were the prominently visualized on the gel (Fig 4.26 a: lane 4).

The SDS-PAGE gel for ΦSP-1 under reducing conditions , showed a total of 8 bands. The prominent bands were of 119740 Da, 108855 Da , 53697 Da, 38967 Da, 22590 Da and 16953 Da (Fig 4.25 b: lane 2).

On the other hand, for ΦSP-3, the molecular weights of the prominent bands observed under reducing conditions were calculated as 48051 Da, 36274 Da and 22499 Da (Fig 4.26 b: lane 3).

4.4.10 Comparison of LPS from phage sensitive and resistant mutants

4.4.10.1 Isolation of phage resistant strains of S49 and cross-lysing

Phage resistant strains of S49 were isolated, purified and their identity was reconfirmed by biochemical tests. Phages were found capable of cross-lysing the phage-resistant S49 mutants.

4.4.10.2 Comparison of phage resistant mutant strains of S49 to the parental strain by LPS profiling

LPS was isolated from S49 and its phage resistant mutants and the LPS PAGE was carried out.

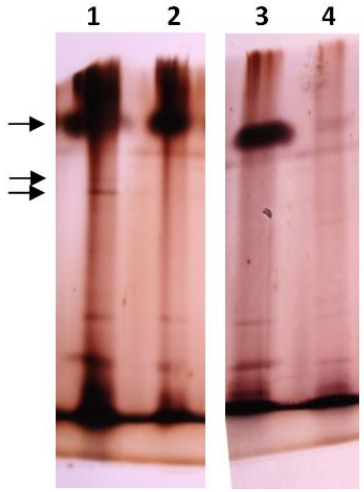


Fig 4. 27. LPS pattern of the parental strain *Salmonella* S49 and the phage-resistant host strains. Lane 1: parental *strain Salmonella* S49; lane 2: ΦSP-1 resistant mutant-1; lane 3: ΦSP-3 resistant mutant-1; lane 4: ΦSP-3 resistant mutant-2.

The lipopolysaccharide (LPS) is one of the major structural molecules of the Gram negative outer membrane (Nikaido and Nakae, 1979). This glycolipid consists of three structural and functional domains: lipid A, core and O antigen . The O-chain region is composed of repeating subunits of differing chain lengths. Polysaccharides chains with the same chain length co-migrate in SDS-PAGE gels

to form a ladder pattern with high molecular weight O-chain region seen in the upper half of SDS PAGE gel. Bacteria which contain LPS that lacks the O-antigenic side chain are referred to as rough owing to their colony morphology, whereas bacteria which have this LPS component are referred to as smooth.

The LPS profile of the parental strain *Salmonella* S49 and the phage-resistant mutants are as shown in Fig 4.27**.** The parental strain is smooth strain with the typical bimodal distribution O-specific polysaccharide (lane 1). All the mutants (lane 2, 3 and 4) exhibited marked difference from the parental strain LPS. Bands in lane 1 indicated by the arrows where found missing in mutant strains. ΦSP-1 resistant mutant-1 and ΦSP-3 resistant mutant-1 in lane 2 and 3 respectively, exhibited absence of bands corresponding to the medium length chain of O- chain as indicated by the arrows. ΦSP-3 resistant mutant-2 in lane 4 exhibited the typical rough LPS pattern with the high molecular weight O-chain missing as indicated by the arrow.

4.4.11 Host range studies

31 *Salmonella* Enteritidis strains including the standard strain *Salmonella enterica* subsp. *enterica* serovar Abony and the clinical strain *S.* Typhi were found to be sensitive to ΦSP-1 in the host range study conducted. But bacteria belonging to the other genera including, the close relative *E.coli* were resistant to phage ΦSP-1 infection. On the other hand, out of the 96 strains tested, ΦSP-3 was able to infect 33 *Salmonella* Enteritidis strains, including *Salmonella* Abony and *S.* Typhi. Bacteria belonging to the other genera including, the close relative *E.coli* were resistant to phage ΦSP-3 infection also. The list of strains sensitive towards the *Salmonella* bacteriophages ΦSP-1 and ΦSP- 3 are indicated in Table.4.1. These include strains that had been previously identified by biochemical tests and serotyping (Siju M V, unpublished data).

Table 4.1. Strains of *Salmonella* sensitive to ΦSP-1 and ΦSP-3

Sl.	Culture	Identity	ΦSP - 1	ΦSP - 3
1	S1	*S.*Typhimurium	✔	✔
2	S19	*S.*Typhimurium	✔	✔
3	S32	*S.*Typhimurium	✔	✔
4	S33	*S.*Typhimurium	✔	✔
5	S37	*S.*Weltevreden	✔	✔
6	SAL27	*Salmonella* sp.	✔	✔
7	SAL29	*S.*Enteritidis	✔	✔
8	SAL 31	*S.*Enteritidis	✔	✔
9	SAL32	*S.*Typhimurium	✔	✔
10	SAL33	*S.*Enteritidis	**X**	✔
11	SAL35	*S.*Typhimurium	✔	✔
12	SAL36	*Salmonella* sp.	✔	✔
13	SAL37	*Salmonella* sp.	✔	✔
14	SAL38	*Salmonella* sp.	✔	✔
15	SAL40	*Salmonella* sp.	✔	✔
16	SAL41	*S.*Typhimurium	✔	✔
17	SAL42	*S.*Typhimurium	✔	✔
18	SAL43	*S.*Enteritidis	✔	✔
19	SAL44	*S.*Typhimurium	✔	✔
20	SAL45	*S.*Enteritidis	✔	✔
21	SAL46	*S.*Enteritidis	✔	✔
22	SAL47	*S.*Enteritidis	✔	✔
23	SAL48	*S.*Typhimurium	✔	✔
24	SAL50	*S.*Enteritidis	✔	✔
25	SAL2	*S.*Typhimurium	✔	✔
26	SAL12	*Salmonella* sp.	✔	✔
27	SAL14	*Salmonella* sp.	✔	✔
28	SAL25	*Salmonella* sp.	✔	✔
29	SAL33	*S.*Enteritidis	✔	✔
30	SAL27	*Salmonella* sp.	✔	✔
31	SAL 72	*S.*Typhimurium	**X**	✔
32	*S.* Abony	*Salmonella* sp.	✔	✔
33	*S.* Typhi	*Salmonella* sp.	✔	✔

✔ : Indicates lysis by phage; **X** : indicates insensitive to phage infection (no cell lysis)

4.5.1 Bioassay using experimentally dosed cooked chicken cuts

The effect of phage activity on *Salmonella* in chicken cuts at 4°C at low MOI is represented in Fig 4.28. Values represent mean ± standard deviation, [a] p<0.001 when compared to control.

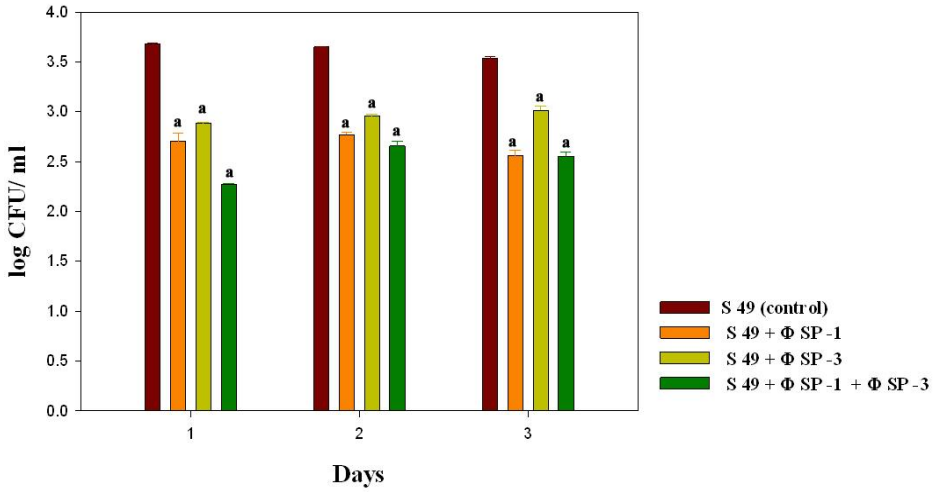


Fig4.28. Effect of bacteriophage on experimentally dosed cooked chicken cuts at low MOI at 4 °C. ([a] p<0.001 when compared to control)

The bacterial count in the untreated group (control) on all three days of the experiment was $\log_{10}3.68\pm0.03$, $\log_{10}3.65\pm0.01$ and $\log_{10}3.53\pm0.04$ CFU/ml respectively.

In the ΦSP-1 treated group, the application of the phage at low MOI brought the bacterial count, down to $\log_{10} 2.70\pm0.04^{a}$ CFU/ml on day 1, while on day 2, it was $\log_{10} 2.77\pm0.03^{a}$ CFU/ml. The final sampling at the end of 72 hours revealed the reduction of CFU to $\log_{10} 2.55\pm0.05^{a}$ CFU/ml in this group. Similar results were obtained with the ΦSP-3 treated chicken cuts as well. The application of ΦSP-3 affected a reduction in the bacterial count on day1($\log_{10} 2.88 \pm0.03^{a}$ CFU/ml) day 2 ($\log_{10}2.95\pm0.06^{a}$ CFU/ml) and day 3 (3.01 ± 0.03^{a} CFU/ml). The

application of ΦSP-1 and ΦSP-3 as a cocktail (group 4) was most effective in bringing down the bacterial load. Bacterial count was reduced to $\log_{10}$ 2.27 ± 0.02[a] on day 1, $\log_{10}$ 2.65± 0.01[a] CFU/ml on day 2 and $\log_{10}$ 2.55± 0.01 on day 3.

The effect of phage at high multiplicity of infection at 4°C on *Salmonella* S49 in cooked chicken cuts is presented in Fig 4.29, Values represent mean ± standard deviation, [a] p<0.001 when compared to control.

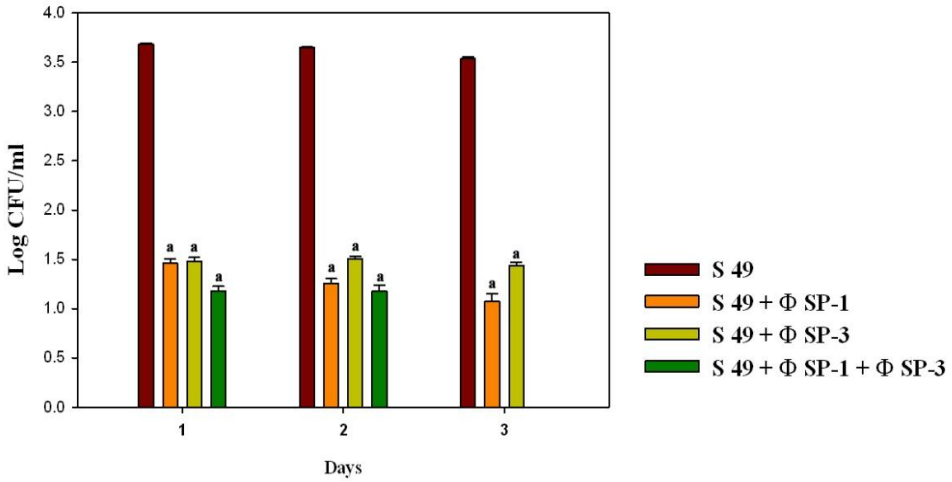


Fig 4. 29. Effect of bacteriophage on experimentally dosed cooked chicken cuts at high MOI at 4 °C([a] p<0.001 when compared to control)

The bacterial count in the untreated group was $\log_{10}$3.68±0.03, $\log_{10}$3.65±0.01 and $\log_{10}$3.53±0.04 CFU/ml on day 1, 2 and 3 respectively, while the application of ΦSP-1 at high MOI reduced the bacterial count to $\log_{10}$ 1.46±0.07[a], $\log_{10}$ 1.25±0.02[a] and $\log_{10}$ 1.07±0.04[a] CFU/ml respectively. Similar results were observed for ΦSP-3 also. Bacterial plate count dropped to $\log_{10}$ 1.47± 0.06[b] , $\log_{10}$ 1.50± 0.05[b] and $\log_{10}$ 1.43±0.03[a] CFU/ml on day 1,2 and 3 repectively. Application of ΦSP-1 and ΦSP-3 as cocktail restricted the bacterial growth to $\log_{10}$1.17+ 0.21[a] on day 1 and 2 and on day 3 bacterial level dropped to beyond detectable level.

The efficacy of phage to reduce *Salmonella* load in cooked chicken cuts at low MOI at room temperature is presented in Fig 4.30. Values represent mean ± standard deviation, [a] p<0.001 when compared to control.

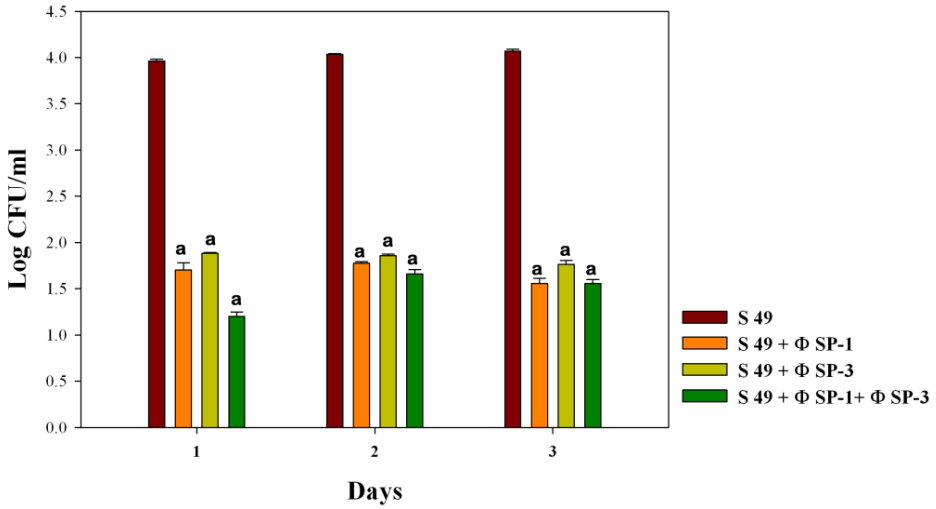


Fig 4. 30.Effect of bacteriophage on experimentally dosed cooked chicken cuts at low MOI at room temperature ([a] p<0.001 when compared to control)

The mean *Salmonella* plate count was $\log_{10}$ 3.96±0.01, $\log_{10}$ 4.03±.07 and $\log_{10}$4.06±0.02 CFU/ml in untreated control on first, second and third day respectively, but with the application of ΦSP-1, it was lowered to $\log_{10}$ 1.70±0.07[a], $\log_{10}$1.77±0.01[a] and $\log_{10}$1.55±0.05[a] CFU/ml respectively. Bacterial count was recorded as log $_{10}$1.88±0.05[a], $\log_{10}$1.85±0.01[a] and $\log_{10}$ 1.76±0.04[a] CFU/ml on day 1, 2 and 3 respectively when ΦSP-3 was applied. The bacterial count was the lowest when both phages were applied together, an outcome quite similar to those obtained for experiments conducted at 4^{o}C. Effective reduction was observed in total plate count $\log_{10}$1.20±0.04[a], $\log_{10}$1.65±0.05[a], and $\log_{10}$ 1.55±0.04[a] CFU/ml on day 1, 2 and 3 respectively.

The efficacy of phage to reduce *Salmonella* loads in cooked chicken cuts at high multiplicity of infection at room temperature is presented in Fig 4.31. Values represent mean ± standard deviation, [a] p<0.001 when compared to control.

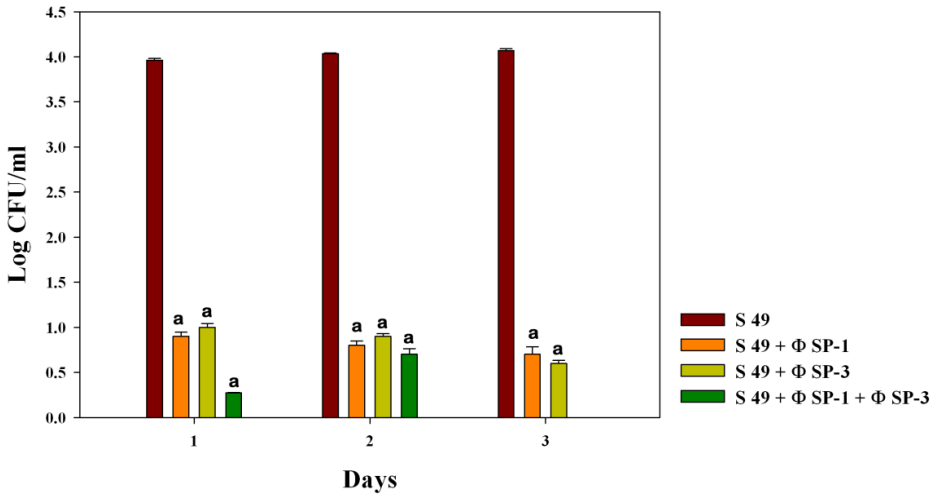


Fig 4. 31. Effect of bacteriophage on experimentally dosed cooked chicken cuts at high MOI at room temperature ([a] p<0.001 when compared to control)

The mean *Salmonella* plate count was $\log_{10}$ 3.96±0.01, $\log_{10}$ 4.03±.07 and $\log_{10}$4.06±0.02 CFU/ml in untreated control on first, second and third day respectively. Application of ΦSP-1 restricted the count to $\log_{10}$0.90±0.04[a], $\log_{10}$ 0.80±0.04[a] and $\log_{10}$0.70±0.08[a] CFU/ml, while the application of ΦSP-3 reduced the bacterial count to log $_{10}$1.00 ±0.04[a], $\log_{10}$0.90±0.02[a] and $\log_{10}$ 0.60±0.03[a] CFU/ml on day 1, 2 and 3 respectively. The bacterial recovery when ΦSP-1 and ΦSP-3 were applied together was $\log_{10}$0.27±0.01[a] and $\log_{10}$0.7±0.06[a] CFU/ml on day 1 and day 2 and was beyond detectable level on day 3.

It was noted that despite the increase in bacterial count at room temperature when compared to the count at 4°C, the phages individually and in the cocktail helped to lower the bacterial count.

Effect of phage activity on *Salmonella* recovery at 37°C at low and high MOI (Fig.4.32 and Fig.4.33) showed a pattern similar to that at 4°C and room temperature. Values represent mean ± standard deviation, [a] $p<0.001$ when compared to control.

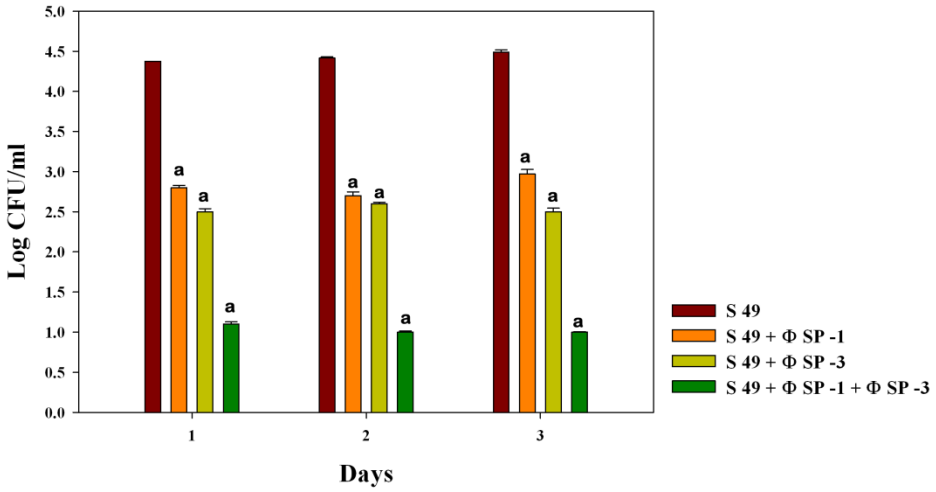


Fig 4. 32. Effect of bacteriophage on experimentally dosed cooked chicken cuts at low MOI at 37°C ([a] $p<0.001$ when compared to control)

Salmonella growth was highest at 37°C when compared to that at 4°C and room temperature as was expected, nevertheless the phage activity brought down the bacterial count. In untreated control the *Salmonella* plate count was $\log_{10}$ 4.37±0.04, $\log_{10}$ 4.41±0.01 and $\log_{10}$ 4.48±0.02 CFU/ml on day 1, 2 and 3 respectively. With the application of ΦSP-1 at low MOI (Fig 4.32), the count reduced down to $\log_{10}$ 2.8 ± 0.02[a] , $\log_{10}$ 2.70±0.04[a] and $\log_{10}$ 2.96±0.05[a] CFU/ml on day 1, 2 and 3 respectively. The *Salmonella* count in the presence of ΦSP-3 on day 1, 2 and 3 was $\log_{10}$ 2.50±0.03[a], $\log_{10}$2.60±0.01[a] and $\log_{10}$2.50±0.04[a] CFU/ml respectively. The mean *Salmonella* count on day 1 was $\log_{10}$ 1.10±0.02[a] CFU/ml and on day 2 and 3 it was $\log_{10}$1.0± 0.02[a] and 1.0± 0.00[a] CFU/ml respectively, when the two phages were applied simultaneously.

Effect of phage activity on *Salmonella* recovery at 37°C at high MOI is depicted in Fig 4.33. Values represent mean ± standard deviation, [a] p<0.001 when compared to control.

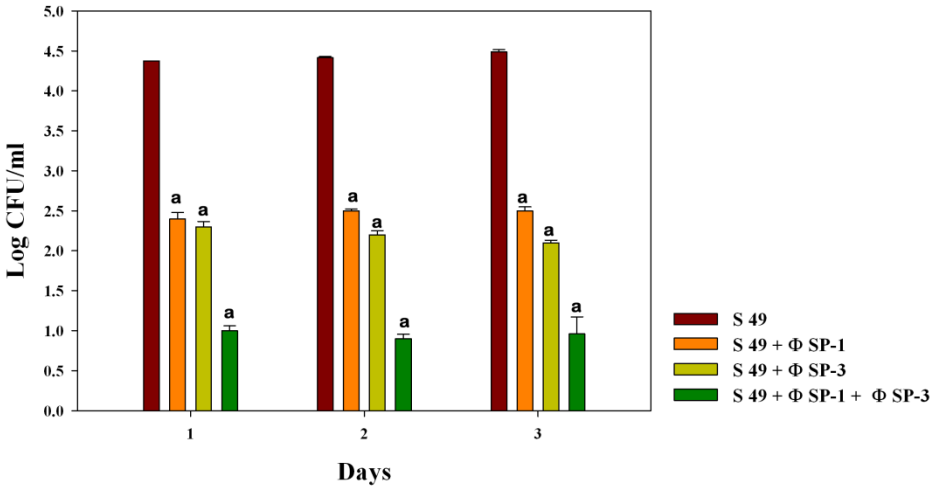


Fig 4. 33. Effect of bacteriophage on experimentally dosed cooked chicken cuts at high MOI at 37°C ([a] p<0.001 when compared to control)

In untreated control the *Salmonella* plate count was $\log_{10}4.37\pm0.04$, $\log_{10}4.41\pm0.01$ and $\log_{10}4.48\pm0.02$ CFU/ml on day 1, 2 and 3 respectively. With the application of ΦSP-1 the bacterial recovery was $\log_{10}2.40\pm0.07^{a}$, $\log_{10}2.50\pm0.02^{a}$ and $\log_{10}2.50\pm 0.04^{a}$ CFU/ml on day 1, 2 and 3 respectively. The *Salmonella* count in the presence of ΦSP-3 on day 1, 2 and 3 at high MOI dropped to $\log_{10}2.30\pm0.06^{a}$, $\log_{10}2.20\pm0.05^{a}$ and $\log_{10}2.10\pm0.03^{a}$ CFU/ml respectively. The maximum impact of phages in biocontrol of *Salmonella* was observed when they were applied together at high MOI. The bacterial count was $\log_{10}1.00\pm0.06^{a}$, $\log_{10}0.90\pm0.05^{a}$ and $\log_{10}0.96\pm0.21^{a}$ CFU/ml on day 1, 2 and 3 respectively.

The performance of the phages in reducing the burden of *Salmonella* in the cooked chicken cuts was found to be promising, at low and high MOI and at

all the different temperatures tested. Phage cocktail was found to be more effective than the individual phages in bringing down the bacterial count.

4.5.2 Bioassay using *C. elegans*

4.5.2.1 Bacterial infection and phage prophylaxis assay

The nematode *Caenorhabditis elegans* was used as a model system to study the bacterial pathogenesis of *Salmonella* strain S49 and to test the ability of the bacteriophages ΦSP-1 and ΦSP-3 to confer protection against the bacterial infection. Fig 4.34 shows the outcome of the phage prophylaxis assay. It was observed that the phages ΦSP-1 and ΦSP-3 had a positive influence in increasing the life span of the infected worm. The figure represents the percentage mortality of *C. elegans* over a span of 10 days when exposed to the pathogen S49 and the influence of the bacteriophages individually and in combination, in maintaining the life span of worms to near normal levels when compared to the untreated (control) worms fed on *E. coli* OP50.

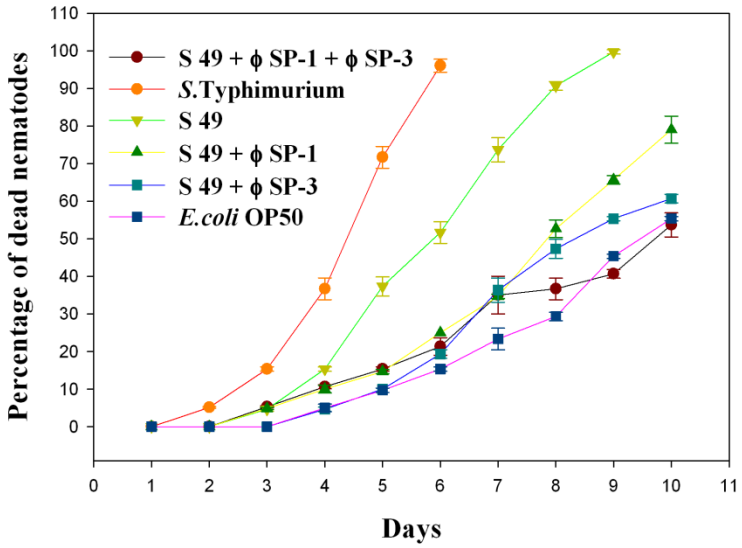


Fig 4. 34. Phage prophylaxis assay *in C. elegans* infected by *Salmonella* strain S49

The time required for the mortality of 50% of the nematodes (TD_{50}) was calculated from the results obtained using the PRISM (version 5.04) computer program. TD_{50} was calculated in three independent experiments and values were represented in mean ± standard error. The time required for 50% of nematodes to die when fed on an *E. coli* OP50 lawn was 9.48 ± 0.34 days. TD_{50} of the nematodes when infected with the known pathogen S. Typhimurium was 4.33± 0.12 days, while it was 5.85±0.20 days when the nematodes were fed with *Salmonella* strain S49, indicating the pathogenicity of this strain.

The outcome of the phage prophylaxis experiments was promising. When phages were applied, their ability to control the *Salmonella* infection by cell lysis, helped in reducing the mortality rate of infected worms. TD_{50} of infected worms in presence of ΦSP-1 was calculated as 7.91± 0.26 days and in presence of ΦSP-3 it was 8.29 ± 0.33 days. Maximum protection was conferred by phages when they were applied together, whereby the TD_{50} reached to near control levels of 9.29± 0.26 days.

4.5.2.2 Microscopy of *C. elegans*

As the nematodes *C. elegans* are transparent organisms, microscopy of the healthy as well as the infected worms were performed to observe and confirm the pathogenesis in the infected worms when compared to the healthy ones. The normal morphological as well as anatomical features of a healthy adult nematode are shown in Fig 4.35.

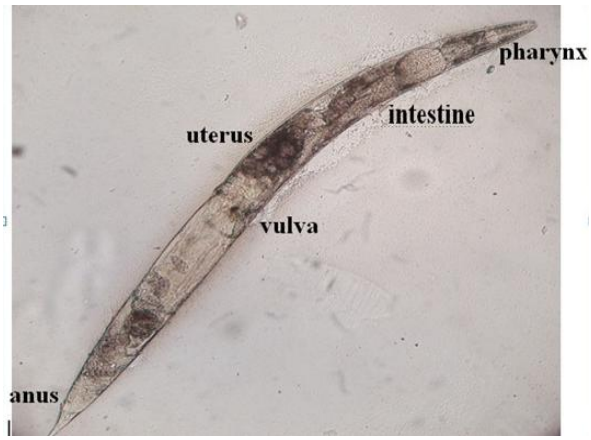


Fig 4.35. Micrograph of a healthy *C. elegans* fed on *E. coli* OP 50

Fig.4.36 shows the micrograph of an infected *C. elegans* fed on *Salmonella* strain S49, taken on 4th day of infection. Distension of the pharyngeal region as well as intestinal lumen from the terminal bulb to the anus is evident.

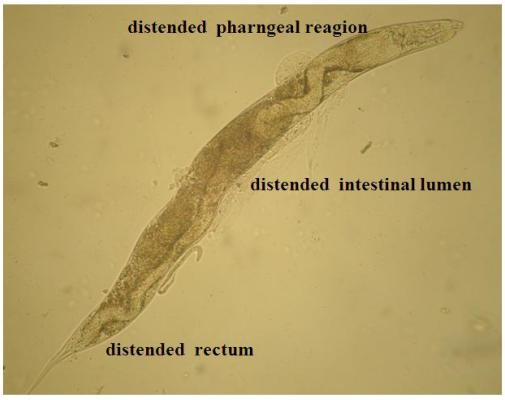


Fig.4.36 Micrograph of *C. elegans* infected by *Salmonella* strain S49

Another notable observation in the infected nematodes was 'bagging'. Bagging is a condition in which the adult nematodes are unable to lay eggs, leading to the internal hatching of the eggs. Live larval worms actively moving

inside the moribund adult could be observed (Fig 4.37). Arrow indicates the live larval forms hatched inside the adult.

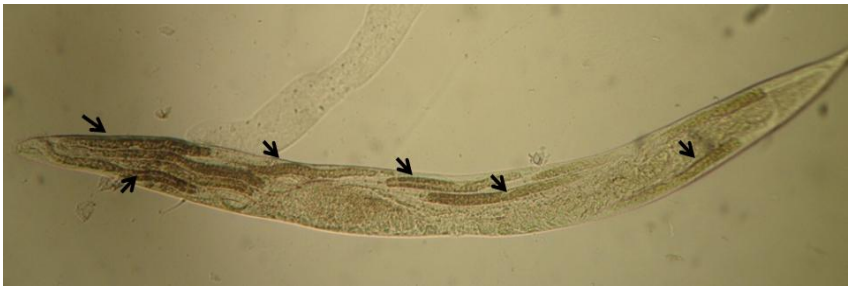


Fig 4.37. "Bagging" phenomenon in *C. elegans* infected *by Salmonella* strain S49

The *Salmonella* specific lytic bacteriophage, ΦSP-1 and ΦSP-3 isolated from the intestinal content of broiler chicken, were characterized and identified during this study. Their potential as a useful and effective bio control agent was proved *in vitro* employing experimentally dosed chicken cuts and *in vivo* using *C. elegans* as a model organism.

Chapter 5

DISCUSSION

Salmonellae are Gram-negative bacteria, known worldwide as the causative agents of gastroenteritis, bacteremia and enteric fever (Fiorentin *et al.*, 2004; Hardy, 2004). The incidence of Salmonellosis is higher in industrialized and developing countries (Majowicz *et al.*, 2010) and there have been periodic reports of *Salmonella* from a variety of sources from India, where it is endemic (Gupta and Verma,1989; Hatha and Lakshmanaperumalsamy, 1997; Tankhiwale *et al*., 2003; Suresh *et al.*, 2006; Singh *et al*., 2010; Harsha *et al.*, 2011). Majority of human gastroenteritis has been attributed to the consumption of *Salmonella* contaminated eggs and chicken meat (Fiorentin *et al.*, 2004). The wide use of antibiotics utilized as therapeutics and as growth promoting agents in animal husbandry (Donoghue, 2003), has enhanced the emergence of antibiotic resistant bacterial strains among farm animals, which in due course reach human (Threlfall, 2000). This has in turn enhanced the prevalence of clinical infections with multiple antibiotic resistance strains (Oliveira *et al.*, 2005).

The prospects of lytic phages as biocontrol agents against pathogenic bacteria are being reconsidered worldwide with the surfacing of multiple antibiotic resistances (Sulakvelidze *et al.*, 2001). Recent review also hint at the potential of phages as alternatives to antibiotics (Greer, 2005) and for phage therapy (Thiel, 2004; Skurnik and Strauch, 2006).

Phages are natural viral pathogens of bacteria. They are the most abundant entities in the biosphere, with total estimated numbers ranging from 10^{30} to 10^{32} (Kutter and Sulakvelidze, 2005). *Salmonella* is one among many bacterial

pathogens against which phages are experimented as therapeutic agents (Kropinski *et al.,* 2006). Numerous bacteriophages specific to *Salmonella* have been isolated and/or identified as part of host genome sequencing projects. Even though the intimate roles played by phage in *Salmonella* ecology may complicate their use in biocontrol (McLaughlin *et al.,* 2006), several studies conducted in this direction have given promising results (Goode *et al.*, 2003; Pao *et al.*, 2004; Boury, 2005; Fiorentin *et al.,* 2005 ; Atterbury *et al.,* 2007; Filho *et al.*, 2007).

5.1 Isolation of *Salmonella* and *Salmonella* specific lytic phages

Bacteriophages being natural viral pathogens of bacteria co-exist with their hosts, sharing the same ecological niches (Goyal *et al.,* 1987; Silja *et al.,* 2010). *Salmonella* Enteritidis is by far the most common serotype isolated from laying hens (De Buck *et al.*,2004) and human infection with this pathogen has been attributed mainly to poultry and poultry-derived products (Fiorentin *et al.,* 2004; Vasudevan *et al.*,2005; Woo, 2005). Thus the rationale of the present study was to search for specific lytic phages where it was most likely to be found i.e. from the intestinal contents of chicken - the same environment where their host *Salmonella* is known to reside (Ricke, 2003; Harvey *et al.*,2011). Hence, chicken caecum which is known to be the primary colonization site for *Salmonella* (Allen-Vercoe and Woodward, 1999; Amit-Romach *et al.,* 2004) was used for phage isolation.

The *Salmonella* strain, designated as S49 successfully played host in the isolation of three lytic phages. The identity of this *Salmonella* strain S49 was confirmed by 16S rRNA sequence analysis as *Salmonella enterica* subsp. *enterica* (Jeena *et al.,* 2012a). The use of 16S rRNA gene sequencing is one of the easiest methods for identifying bacteria (Janda and Abbott, 2007). But as there are reports of 16S rRNA gene sequencing not being foolproof (Boudewijns *et al.,*

2006), the identity of the host strain was reconfirmed as *Salmonella* Enteritidis 9, 12: g, m:- by Serotyping at the National *Salmonella* and *Escherichia* Centre, Kasuali, Himachal Pradesh, India. The *Salmonella* serotyping system is probably the best phenotypic bacterial typing system ever developed. The serotyping scheme developed by White and Kauffmann in 1920 was based on the discovery of flagellar H- antigen, the somatic O -antigen and the phase-shift in the H-antigen (Molbak *et al*., 2006). The Kauffmann-White method used worldwide, is considered the gold standard for identification of *Salmonella* serotypes (González, 2010).

Phages were isolated employing double agar overlay method of Adams, (1959). The samples were used directly as well as after enrichment with the host strain *Salmonella* S49. The direct protocol for isolation did not yield any bacteriophages. It was only after proper enrichment with the host, that phages could be isolated. The viscous nature of the intestinal contents of chicken may reduce the chances of phage-bacteria collision and possibly be one of the reasons for the undetectable levels of phages without proper enrichment (Joerger, 2003). A large number of morphologically different plaques were obtained even in a single sampling (Jeena *et al*., 2012b). The three phages, namely ΦSP-1, ΦSP-2 and ΦSP-3 isolated by the enrichment process were differentiated based on their plaque morphology. Two phages which exhibited excellent bacterial cell lysis capacity were ΦSP-1 and ΦSP-3, and were therefore studied further. All results henceforth will be discussed with respect to these two phages.

The two *Salmonella* phages ΦSP-1 and ΦSP-3 consistently produced small, clear and round plaques with well-defined edges. As concentration and purification of virus particles are prerequisites for structural and functional characterization of phages (Boulanger, 2009), ΦSP-1 and ΦSP-3 were purified and concentrated before further characterization. Concentration was done employing

PEG-NaCl precipitation method was as described by Sambrook (2000). The efficiency of this method is almost independent of phage concentration and is therefore useful in order to concentrate even phage lysates with very low titer (Yamamoto and Alberts, 1970). This mild, but fast procedure allows a 100-fold phage concentration, even after low speed centrifugation with negligible loss of infectivity (Boulanger, 2009).

Morphological, physicochemical and molecular characterization of the *Salmonella* specific bacteriophages ΦSP-1 and ΦSP-3 were carried out employing a variety of experiments.

5.2 Morphological characterization of ΦSP-1 and ΦSP-3

The morphological features of bacteriophages greatly aid in their classification (Ackermann, 2009). Therefore, transmission electron microscopy was employed to aid in this morphological analysis.

The transmission electron micrograph of ΦSP-1 revealed isometric head with an extremely short tail. The head was 44.71 ± 0.62 nm in diameter and the tail 15.88 ± 0.83 nm long. The short tail, their isometric morphology, as well as their size are the characteristic morphological features, typical of members belonging to family *Podoviridae* according to ICTV. A study on phages used by laboratory of enteric pathogens (Health Protection Agency, London, UK) for phage-typing, reported the average head diameter to be 62.5 nm and the tail length to be 13nm for phages belonging to *Podoviridae* family (Lappe *et al.*, 2009). Even though ΦSP-1 has morphological dimensions slightly smaller than this reported average, other similar small *Podoviridae* bacteriophages have also been previously reported (Kropinski *et al*., 2006). *Salmonella* phage M infecting

S. Typhi reportedly had a 49-54 nm diameter head and a 15-20 nm long tail (Bliznichenko *et al.,* 1972).

The micrograph of ΦSP-3 exhibited morphological traits, typical of family *Siphoviridae* according to ICTV. It had a hexagonal head 53.77 ± 0.38 nm in diameter with a 123.66 ± 0.32 nm long tail. In the study on phages used by laboratory of enteric pathogens (Health Protection Agency, London, UK), the reported the average head diameter for phages of family *Siphoviridae* is 62.5 nm with 120nm long tail (Lappe *et al.,* 2009). Other reported phages from family *Siphoviridae* with similar morphological dimensions as that of ΦSP-3 are phage ST104b with a 131 ± 17 nm long tail and a head of diameter of 59 ± 4 nm and phage ST 104b with a 128nm long tail and a head diameter of 59 ± 5 nm (O'Flynn *et al*., 2006).

The morphotypes exhibited by both ΦSP-1 and ΦSP-3 have been previously reported in association with genus *Salmonella*. There are prior reports on 63 *Salmonella* phages belonging to family *Podoviridae* and on 65 *Siphoviridae Salmonella* phages (Kropinski *et al*., 2006).

5.3 Physicochemical characterization of ΦSP-1 and ΦSP-3

For a fruitful phage- host interaction, several critical factors need to come into play simultaneously. One of the first and important factor is the careful deduction of the optimal MOI. MOI is the ratio of the phage particles to the infected bacteria (Adams, 1959). MOI giving maximum yield per infection is considered as the optimal MOI. Determination of optimal MOI is important as too many phages attaching to a single bacterial cell can cause cell lysis, even before the infection process can yield progeny (lysis from without). On the other hand, if

only a few bacteriophages are used for infection, it may be difficult to detect or measure the response being tested (Abedon, 2011).

The optimal MOI of ΦSP-1 was five phages per bacterium and that for ΦSP-3 was one phage per bacterium. These optimal MOI resulting in highest phage titre under standard conditions were used in all subsequent large-scale phage production of ΦSP-1 and ΦSP-3 using *Salmonella* host strain S49.

Phage adsorption on to the susceptible host is the second significant factor affecting the successful phage- host interaction. Adsorption is described as the first step of phage infection and may be defined as the attachment of phage particles to bacterial surfaces so that phage and bacteria can sediment together (Adams, 1959). The phage extracellular search is a time of free diffusion that delays the onset of virus attachment (Shao and Wang, 2008). Careful determination of the time taken by the phages to adsorb onto to the host cell is of paramount importance, as it may serve in later experiments for accurate characterization of the phage. While it took 30 minutes for 100% adsorption by ΦSP-1, the same was achieved by ΦSP-3 in 25 minutes.

Once the phage genetic material is taken up into the host cytoplasm, infection commences, the duration varying as a function of phage genes, bacterial physiology and a host of other factors. The details of intra cellular kinetics of virus growth have been modeled for several phages (Srinivasan and Rangan, 1970; Gáspár *et al*., 1979; Buchholtz and Schneider, 1987; Rabinovitch *et al*., 1999).

The one step growth experiment developed by Ellis and Delbruck (1939) depends on the production of synchronous phage infection. A typical curve from the experiment is triphasic, with an initial latent phase when the number of infectious centers is constant, followed by a rise period, and then a plateau,

indicating little variation. The average number of phage particles released per infected cell (burst size) can also be calculated from the curve.

The one step growth curve to study the growth kinetics of ΦSP-1 and ΦSP-3, used log phase host S49 cells at 37ºC. These two *Salmonella* bacteriophages showed a comparable latent period of 30 minutes. The rise period of ΦSP-1 was relatively longer at 55 minutes when compared to that of ΦSP-3, where it was of 30 minutes. The burst size for ΦSP-1 was calculated to be 44 phages per bacterial cell, which was smaller than that for ΦSP-3, which was 60 phage particles per bacterial cell.

The growth kinetics of both phages shared a similar pattern with the well studied *Salmonella* specific lytic phage, Felix 01 (Kuhn *et al.,* 2002). The rise period of Felix 01 is 40 minutes. The burst size of ΦSP-1 and ΦSP-3 was also well within the range as that of Felix 01, which averaged to around 56 phage particles per cell. However, the results obtained can only be used/evaluated with caution as the phage multiplication kinetics may vary, depending on the physiological state of the host cell (You *et al.*,2002) as well as on the environmental conditions which will vary dramatically in natural milieu, compared to the highly controlled lab conditions (Hadas, 1997). In addition, it is suggested that the timing of phage-induced host cell lysis may be subject to a host quantity- and host quality-dependent selection. Phages will have a shorter latent period when either host density is high or host quality is good (Abedon, 1989; Wang *et al*., 1996).

Various environmental properties such as temperature and the chemical makeup of the phage-host ecology have a substantial influence (Schlesinger, 1932; Stent, 1963) not only on the phage viability, but also most importantly on phage adsorption, a very crucial step in phage infection (Capra *et al.,* 2006). Optimal host and growth conditions must be carefully studied and selected for the

production of each bacteriophage candidate for application as biocontrol agents (Sillankorva *et al*., 2010).

In this work, the influence of both physical and chemical parameters on phage viability/propagation and phage adsorption was studied. The parameters studied include temperature, pH, salinity, presence of calcium ions and sugars. The affect of each parameter and the knowledge about the phage growth dynamics in varying ecological conditions can be exploited during their future intended use as a therapeutic agent. Furthermore it will also help in optimization of the large scale phage propagation process in the lab conditions (Jeena *et al.*, 2012a).

Response of phages on exposure to varying temperatures is considered as a key model for understanding the ability of the organism under question to adapt to novel environments (Johnston and Bennett, 1996). The influence of temperature variation on ΦSP-1 viability revealed that this phage was able to withstand exposure to temperatures as high as 80ºC, without affecting phage viability. But the viability of phage ΦSP-3 was drastically reduced to a few PFU/ml at even 70ºC, above which there were no survivors. The ability of these two *Salmonella* specific phages to survive temperatures above 70ºC and/or more is one of the many desirable traits for consideration, especially in its function as biocontrol agent in surface pasteurization of poultry foods where hot water is applied for a short period of time (Jeena *et al.*, 2012a).

Optimal concentration of NaCl in phage preparation is known to confer protection to phages especially against high temperature (Krueger and Fong, 1937) and is therefore a very crucial factor affecting phage viability. Taking this into consideration, the viability of phage ΦSP-1 in the presence of varying concentration of NaCl was studied in order to find the NaCl concentration optima. It was observed that the ΦSP-1 was highly stable at a minimal concentration of

sodium chloride (i.e., 0.1M) beyond which viability decreased drastically; whereas 1M NaCl was found to be optimum for ΦSP-3. The marked difference in NaCl optima is not unforeseen as previous findings (West and Kelly, 1962) had shown that the free phages differ in their NaCl tolerance.

Excellent viability and stability was shown by the phages ΦSP-1 and ΦSP-3 exposed to pH ranging from 7-10. Maximum number of phage particles survived at pH 8, with phages showing viability even at higher pH (up to 12) though in few numbers. The affinity of ΦSP-1 for the alkaline environment is easily explained, since they were isolated from intestinal contents, where the pH normally is 8 or higher in caecum (Siragusa *et al.*, 2008). This property can be exploited in future in their possible use as a biocontrol agent in the form of feed incorporates to reduce *Salmonella* load in live poultry.

Capsular polysaccharides of Gram negative bacteria directly involve in phage host interaction (Deveau *et al*., 2002) and hence the influence of various sugars on ΦSP-1 and ΦSP-3 viability was studied. Sugars like rhamnose, maltose and xylose effectively reduced the ΦSP-1 viability. It can be inferred that these sugars and/or their analogues may have a key role as phage receptors on the host surface outer membrane, as their presence in the host phage medium during the adsorption stage effectively inhibited the process (Jeena *et al.*, 2012b). Rhamnose was reported to be a determinant of a phage receptor in *Lactobacillus casei* (Monteville *et al.*, 1994). Bacterial phage inactivation by free sugars like D-glucosamine, D-mannose and L-rhamnose has been previously demonstrated (Patel and Roa, 1983). The possibility of phage 2 receptors in lipopolysaccharide containing L-rhamnose, D-glucosamine, and (or) D-glucose, or a structurally related molecule has also been suggested (Francisco and Pasquale, 1974). Arabinose followed by xylose was most influential in inactivating ΦSP-3. This

may be relevant considering that 4-Amino-4-deoxy- arabinose has been previously reported as a constituent in *Salmonella* LPS preparation (Volkc *et al.*, 1970).

Temperature dependant phage resistance has been reported earlier (Kim and Kathariou, 2009) and in order to study that aspect the influence of different temperatures on the rate of adsorption of the phages ΦSP-1 and ΦSP-3 on to their host S49 was studied. Maximum percentage or nearing 100% adsorption was observed for both the phages ΦSP-1 and ΦSP-3 at 37^{o}C and 40^{o}C i.e., at temperatures corresponding to the avian body temperature, which ranges in between 40^{o}C and 42^{o}C. There was no visible adsorption at 0^{o}C, but it steadily increased as the temperature was raised to 10 ^{o}C, an attribute seen as desirable for a biocontrol agent for application in cold storage of food products (Jeena *et al.*, 2012a).

The ionic environments in which the phage and host interact have a profound influence on the irreversible phage adsorption on host surface (Adams, 1959). As early as in 1923, it was reported that bacteria grown in salt-free Witte's peptone resisted lysis by phage, but that the addition of sodium chloride or calcium chloride resulted in phage infection followed by bacterial cell lysis (da Costa, 1923). The presence of electrolytes in the phage- bacteria growth medium has a profound effect on the adsorption of phage to host cell. It was demonstrated by Lisbonne and Carrere (1923), that phage and bacteria mixed in salt-free peptone could readily be separated by centrifugation, but in the presence of salts, the phage speedily became attached to the bacteria and were therefore not separated by centrifugation.

Optimal NaCl concentration for maximal adsorption of ΦSP-1 on host S49 was 0.25M, beyond which the efficacy dropped. In case of ΦSP-3 also, 0.25M concentration of NaCl was found to be favorable. Unlike ΦSP-1, ΦSP-3

adsorbed onto host cells at higher concentrations of NaCl also. Certain enzymes produced by phage tail ends have a pivotal role in host cell adsorption (Adams and Park, 1956; Park, 1956; Murphy, 1957; Richmond, 1959; Eklund and Wyss, 1962). It is conceivable that such a cell-wall lysing enzyme may have a high electrolyte requirement for optimal activity. However, the results are met with skepticism as the effects of different levels of electrolytes on different phages vary; it is the specific property of the phages, not that of the host that decides the effects (West and Kelly, 1962).

The enzyme lysozyme, located in the phage tail, aids in weakening the bacterial cell wall during the phage adsorption process. It is through this weakened cell wall, that the phage tail fiber injects the bacteriophage DNA into the bacterial cell (Archana, 2007). Finding the optimum pH for this enzyme activity is important as pH can interfere with lysozyme or protein coat, thereby preventing phage attachment to the receptor sites of the host cell (Leverentz *et al.,* 2001; Leverentz *et al*., 2004). The optimum pH range for bacteriophage viability and adsorption is between 5 and 8 (Adams, 1959). ΦSP-1 and ΦSP-3 exhibited a similar pattern. 100% adsorption by ΦSP-1 was recorded at pH 9, while that by ΦSP-3 was at pH 8. This affinity shown by ΦSP-1 and ΦSP-3 towards a slightly alkaline environment may be due to their caecal origin.

Propagation and Adsorption is not only dependent on the presence of specific receptors on the cell surface but can also depend on the presence of certain cations in the media (Watanabe and Takesue, 1972). The calcium requirement for successful phage-host interaction varies from phage to phage (Brodetsky and Romig, 1965). As phages usually require higher concentration of divalent cations like calcium, effect of varying concentration of $CaCl_2$ on phage adsorption was studied. Numerous studies have proved the positive influence of calcium on phage- host interaction (Norman and Nelson, 1952; Shafia and

Thompson, 1964; Watanabe and Takesue, 1972). In this study, ΦSP-1 propagation or viability was optimum at 1mM as well as at 10 mM $CaCl_2$. At higher concentrations of 20 mM and 30 mM $CaCl_2$, ΦSP-1 PFU dropped significantly, indicating the deleterious effects on plaquing efficiency. On the other hand, 10mM of $CaCl_2$ was optimum for Φ SP-3.

The data collected from the above experiments revealed the physico chemical affinities of ΦSP-1 and ΦSP-3. The optimized parameters led to an increase in the titres of these phages. The phage burst size increased from 44 to73 phages per bacterial cell for ΦSP-1 and that for ΦSP-3 rose from 60 to 110 phages per bacterial cell. An increase in rise period was also observed in both, whereas the latent period remained the same. Both burst size and the phage generation time are controlled by the phage latent period, with greater burst sizes associated with longer latent periods but shorter generation times associated with shorter latent periods (Abedon *et al*., 2001). Even though in the present study the latent period remained the same, an overall increase in phage generation time was observed under optimized conditions, which ultimately resulted in an increase in burst size.

5.4 Infection of host under nutrient deprived states by ΦSP-1 and ΦSP-3

The physiological state of the host is an important factor for successful phage host interaction (Chibani-Chennoufi *et al.,* 2004; Capra *et al.,* 2006). The capability of *Salmonella* to survive under diverse stress environments (Foster and Spector, 1995) makes it a difficult target for phages. The standard protocol followed for isolation of phages involved using host in exponential growth phase (Adams, 1959), and hence the isolated phages were capable of propagating only in fast growing host cells. Bacteria can be maintained in the log-phase only when there is no nutrient limitation, which can be achieved only under laboratory

conditions (Robb and Hill, 2000). In natural environments though, bacteria exist as "long-term stationary-phase cultures" where a set of stress response genes and metabolic pathways are essential for survival (Finkel, 2006). These stress conditions are experienced in the lab set up when the culture reaches stationary phase (Chibani-Chennoufi *et al.,* 2004). Thus, it can be inferred that phages infecting stationary phase bacteria can well infect bacteria in the natural conditions. The ability to infect host under stationary as well as nutrient deprived conditions confer an added advantage on phages intended for use as a biocontrol agent. There are only a few reports on this aspect. The first case of phage infection in stationary phase was reported in α 3, a phage infecting *Achromobacter* (Woods, 1976). Another was regarding a *Pseudomonas* phage that successfully infected host cells that were starved for 5 years (Schrader *et al.,* 1997). Infection of MS2 virus on glucose, sulphur and nitrogen starved cells of *Escherichia coli* resulted in production but no progeny release (Propst-Ricciuti, 1976).

Under various nutrient deprived conditions, even though ΦSP-1 performed poorly in this study, ΦSP-3 produced excellent results. ΦSP-3 multiplication was maximum when host was in logarithmic phase (PFU: $\log_{10}$ 9.81± 0.10) as was expected. Successful phage infection yield maximum number of progeny, when phages were added to the bacterial host growing in optimal conditions (Jeena *et al.,* 2012b); a not so frequent situation in the natural environment (Lenski, 1988). Physiological state of the host, characterized by levels and activities of host cellular functions, plays a pivotal role in phage infection and propagation (You *et al*., 2002). The infection and propagation of a phage on a susceptible bacterial host can be modulated with alterations in growth medium under laboratory conditions (Hedén, 1951; Hadas *et al*., 1997). Bacterial cells entering into stationary phase undergo substantial changes in cell morphology, including metabolism and surface characteristics (Kjelleberg *et al*., 1987) that may negatively interfere with phage infection (Sillankorva *et al*., 2004).

However, in this study, ΦSP-3 was able to infect host under stationary phase, although in low number. Restrictions in nutritional factors are known to limit the phage propagation (Miller and Day, 2008), but ΦSP-3 was able to multiply even under multiple nutrient starved states as evidenced by a significant level of increase in phage titre, $\log_{10}$ 4.61± 0.15 when compared to stationary phase (p<0.001). Bacteria grown in carbon - starved condition are reported to defy phage infection (Marcin *et al*., 2007), but ΦSP-3 was able to infect the host strain S49 (PFU: $\log_{10}$ 3) (p<0.001) even under such a nutrient- deprived state. However under phosphate- starved condition ΦSP-3 failed to infect and therefore multiply. The inability to multiply under phosphate starved conditions can be attributed to the higher nucleic acid to protein ratio in viruses (Bratbak *et al.,* 1993). Nitrogen starvation results in alteration of bacterial cell surface hydrophobicity (Borges *et al*., 2008) that can temporarily make the bacteria resistant to phage infection (Sijtsma *et al.,* 1990). This may be the reason for the failure of ΦSP-1 in this study and most other phages to infect host under N-limiting conditions. However, ΦSP-3 was able to successfully propagate in the host S49 (PFU: $\log_{10}$ 2.81± 0.13) (p<0.001when compared to stationary phase) even under nitrogen limiting conditions. There are only few reports on phages competent to infect their host under both nutrient- rich and nutrient- deprived conditions (Chibani-Chennoufi *et al.,* 2004). This characteristic of ΦSP-3 is a distinctive quality required to be an effective, successful biocontrol agent.

5.5 Molecular characterization of ΦSP-1 and ΦSP-3

The nature of the phage ΦSP-1 and ΦSP-3 genome was identified as double stranded DNA on the basis of its sensitivity to digesion by restriction endonuclease – *Bam* HI. The double stranded nature of their DNA places both the phages under the Order *Caudovirales*.

The genome size of ΦSP-1 was determined to be 86 kb by PFGE. This genome size is unusual among other reported bacteriophages of *Podoviridae* where genome sizes range between 40 to 42 kilobases. *Salmonella* phage ST104, a member of *Podoviridae* has a genome size of 41.4 kb (Tanaka *et al.,* 2004),while in phage ST64B, another *Salmonella* phage, the genome size was 40.1 kb (Mmolawa *et al.,* 2003). The genome size of ΦSP-1 was almost double that of the reported *Podoviridae* phages.

The genome size of ΦSP-3 as determined by PFGE was 88.43 kb. This size is slightly below the reported genome size of *Siphoviridae* phages, which is 121 kb (Wang *et al.,* 2005), and T5-like coliphage SPC35 (*Siphoviridae*), which is 118 kb in size (Kim and Ryu, 2011). The two phages reported in this study have genome sizes unlike those reported for their respective families, an indication of the diversity of *Salmonella* phages.

5.5.1 Virulence profile of host and phage

Virulence in *Salmonella* requires the coordinated expression of complex arrays of virulence factors that allows the bacterium to evade the host's immune system. Each host environment possesses several distinct chemical and physical properties, such as temperature, pH, osmolarity, and nutrient availability. The pathogen also confronts the various components of the host innate immune system ranging from the anatomical barrier functions of the epithelial cells to the microbicidal activities of the antimicrobial peptides, complement and phagocytes. Sensing these changing surroundings and responding with coordinated programs of gene expression, the pathogen is provided is an adaptive advantage in each new host environment (Ohl and Miller, 2001). Thus the presence of several virulence genes in the pathogen can be used as an indication of its competence in infecting humans.

Acquisition of virulence factors by bacterial pathogens, via mobile genetic elements like bacteriophages, is a common affair. In the process, phages convert a non-pathogenic strain to a virulent form or to a strain with increased virulence (Boyd and Brüssow, 2002). Hence, whenever a whole phage preparation intended for use as biocontrol agent is envisaged, the confirmation of the absence of any associated virulence genes in their genome is important in order to prevent the possibility of horizontal gene transfer of virulence genes during the phage- host interactions.

Eleven virulence genes common among *Salmonella* Enteritidis were screened in the current study in the host *Salmonella* strain S49 and its two phages. The multiplex PCR screening method indicated the presence of the following virulence genes: *invE/A*(457bp*), afgA*(261bp), *slyA*(700bp), *phoP/Q*(299bp), *ttrC*(920bp) and *mgt C* (655bp) in the genome of the bacterial host *Salmonella* Enteritidis strain S49, proving beyond doubt the pathogenic nature of this bacteria. The same screening also confirmed the absence of these virulence genes in the ΦSP-1 and ΦSP-3 genome. Although full genome sequencing is necessary to completely negate the possibility of these two phages harboring other virulence genes, this outcome takes both these phages a step forward towards their candidature as biocontrol agents against *Salmonella* Enteritidis (Jeena *et al.*, 2012b).

5.5.2 Identification of ΦSP-1 and ΦSP-3 based on MCP gene sequence analysis

After the discovery of phages, taxonomic classification of phages has gone through several epochs. In the 1920s and 1930s, it was observed that different phage types, or "races," displayed bacterial host specificity; the field of phage typing was thus established (Evans, 1934), establishing an initial

classification system for phage. The advent of the electron microscope allowed phage biologists to measure the physical size of the phage and the length of the tail fibers, allowing the determination of the phage capsid symmetry (Luria *et al.*, 1943) and giving rise to taxonomy based on morphotypes in the 1940s and 1950s. During the 1960s, the advances in biochemical methods, allowing isolation of nucleic acids from phage, helped to resolve both genome size and type (single stranded DNA, double-stranded DNA, single-stranded RNA, double-stranded RNA), adding further information to augment the phage taxonomic schemes (Thomas and Abelson, 1966). It is generally agreed that future phage classifications must reflect genomic data as a primary component. In bacteria, this is easily accomplished by examining the conserved 16S ribosomal genes (Nelson, 2004) and analyzing them.

A clear candidate gene for taxonomic identification of bacteriophage would be the major capsid gene (Lawrence *et al.,* 2002). While other genes dictate phage lifestyle (tail genes affect host range, integrases and replicases affect modes of reproduction etc.), the major capsid gene could be viewed as defining the bacteriophage lineage; it has even been proposed that phages could have evolved as a consortia of genes that increased their collective fitness by allowing more efficient packaging of themselves, including the capsid gene (Hendrix *et al.*, 2000).

The three sets of primers designed from conserved stretches of major capsid protein of reported *Salmonella* phages (Jeena *et al*., 2012b), were successfully used in the amplification of MCP gene in both Φ SP-1 and Φ SP-3 by multiplex PCR giving amplicons of ~750bp as predicted. The BLAST analysis of the MCP sequences revealed the maximum similarity of ΦSP-1 towards *Salmonella* phage phiSG-JL2 (EU547803.1) which is a T7 phage, belonging to family *Podoviridae*. T7-like viruses have short non- contractile tails, being

members of the family *Podoviridae* (Maniloff and Ackermann , 1998) The TEM picture of ΦSP-1 showed similar morphological features, adding support to its molecular claim to family *Podoviridae*. Coliphage T7 is one of the best-studied virulent (lytic) bacteriophages and also one of the first to have been completely sequenced (Kropinski *et al*., 2006). ΦSG-JL2, a lytic bacteriophage infecting *Salmonella enterica* serovar Gallinarum is another T7 phage, a relatively new discovery (Kwon *et al.,* 2008).

The 750 bp amplicon sequence of Φ SP-3 obtained by PCR amplification with the MCP primers, when compared with the Genbank database entries showed maximum similarity (95%) towards Enterobacteria phage SPC35, which is a T5-like phage (HQ406778.1). T5 phages are members of the family *Siphoviridae* with characteristic long flexible non-contractile tail attached to an isometric icosahedral capsid containing the double-stranded DNA genome (Effantin *et al.,* 2006). More than 60% of the tailed phages belong to family *Siphoviridae* (Chagot *et al.,* 2011). The TEM analysis of Φ SP-3 clearly elucidating its morphotype, also up holds this identification and classification. San 2, 12 (Ackermann and Gershman, 1992), G5 (Slopek and Krzywy, 1985) and Φ1 (Bradley and Kay, 1960) are some of the reported T5-like *Salmonella* phages. ΦSP-3 was found to be closely related to the phage *Salmonella* 7-11 from the constructed phylogram. *Salmonella* 7-11 is an enterobacterial phage belonging to the order *Caudoviridae* and its genome closely shares proteins similar to T5 phage which is evident from its full genome sequence analysis and the phylogram (HM997019.1) (Jeena *et al*, 2012b).

Similar approach for identifying and classifying phages was employed by earlier workers to identify and group T4 phages isolated from diverse marine environments (Filèe *et al.,*2005), where designed PCR primers targeting gene *g23*, which encodes the major capsid protein in all of the T4-type phages were used .

Recently degenerate primers targeting the capsid gene of the myoviruses were designed and used to identify and examine molecular diversity of *Clostridium difficile* myoviruses (Nale *et al.,* 2012). Lactococcal bacteriophages were also detected and identified by specific primer designed using the major capsid protein gene (*mcp*) as the target (Labrie and Moineau, 2000).

5.6 Structural proteins of ΦSP-1 and ΦSP-3

The structural protein composition the two *Salmonella* phages were analyzed by SDS-PAGE under reducing and non reducing conditions to further characterize them. Four major structural proteins and a few minor proteins were detected during the analysis of the two phages under non reducing condition. Under reducing conditions, ΦSP-1 had 8, while ΦSP-3 had 3 prominent protein bands. The banding pattern of these two phages revealed the differences between them. Protein profiling can be used as a molecular signature tag of a phage, helping in identifying as well as differentiating it from other phages, as phages even within the same family tend to have different structural proteins owing to differences in phage specificity (Shivu *et al.,* 2007) as evidenced by the lack of comparable protein profile among *Salmonella* phages belonging to *Podoviridae* family. However, the banding pattern was comparable to that of known *Podoviridae* phages infecting bacteria of other genera, for example ΦKMV, a lytic phage infecting *Pseudomonas aeruginosa* (Lavigne *et al.,* 2006) and ϕIBB-PF7A infecting *Pseudomonas fluorescens* (Sillankorva *et al.*,2008). But in case of ΦSP-3, the banding pattern showed a close resemblance with that of two *Siphoviridae Salmonella* phages, ES18 (Casjens *et al.,* 2005) and Vi phage E1 (Pickard *et al.,* 2008).

5.7 LPS pattern of strain S49 and phage resistant mutants of S49

Bacteria have a capacity to counteract phages by undergoing mutation to become resistant towards the particular phage. Many studies have proved that presence of virulent bacteriophages drive rapid bacterial evolution by imposing a strong selection for phage-resistant bacteria (Bohannan and Lenski, 1999; Buckling and Rainey, 2002; Brockhurst *et al*., 2003). Similar phenomenon was observed in the present study as well, where *Salmonella* strain S49 developed bacteriophage resistant mutants against both the phages ΦSP-1 and ΦSP-3.

One of the key steps in bacterial infection is the binding of the viral particle to the outer membrane of the bacterium. For many strains of bacteria and phage, such a binding event requires specific interactions between the phage tail and the bacterial receptors (Schwartz, 1980). In most of the cases, bacteria themselves undergo mutation so as to alter the bacterial cell surface where the receptor site for phage attachment is present, to gain resistance from their attack. Considering the general importance of the bacterial cell surface as a virulence factor, phage-resistant variants of a virulent organism would not be pathogenic (Almeida *et al*., 2009).

Different parts of LPS function as receptors for a number of phages in many different genera. For example, LPS is known to act as a receptor for bacteriophages T3, T4, and T7 in *E. coli*, for T2 and T4 in *Shigella dysenteriae*, for Sf6 in *Shigella flexneri*, for P22 in *Salmonella enterica* serovar Typhimurium, for K139 in *Vibrio cholerae* and for ΦYeO3-12 and ΦR1-37 in *Y. enterocolitica* (Lindberg, 1973; Prehm *et al*., 1976; Krüger and Schroeder, 1981; al-Hendy *et al.*, 1991; Baxa, 1996; Chua *et al*.,1999; Nesper *et al*., 2000; Pajunen *et al*., 2000; Kiljunen *et al.*, 2005; Pinta , 2010).

The role of LPS in phage adsorption was also made clear in the current work, where the effect of sugars on phage adsorption was studied. LPS is a major constituent of the outer membrane of Gram-negative bacteria (Nikaido and Nakae, 1979). Some of its functions include a role in the outer membrane permeability barrier (Nikaido, 1979), resistance to phagocytosis (Rest *et al*., 1977; Robertson *et al*., 1979), resistance to serum (Schneider *et al*., 1982), and as a receptor for adsorption of some bacteriophages (Rapin and Kalckar, 1971).

LPS of the parental strain *Salmonella* S49 and the phage-resistant mutants were analyzed to investigate the correlation between LPS and phage resistance. Comparison of the LPS profile of the bacteriophage resistant mutants of S49 to the parental strain S49, showed a clear difference in LPS pattern. The parental strain had the typical smooth type LPS, i.e. LPS with long O-specific polysaccharide. Even though two of the resistant forms (ΦSP-1 resistant mutant-1 and ΦSP-3 resistant mutant-1) retained typical bimodal distribution of O-antigen polysaccharides corresponding to the smooth phenotype (Santander and Robeson, 2007) some of the bands corresponding to medium length O- polysaccharides were missing in the LPS from the resistant forms.

ΦSP-3 resistant mutant-2 exhibited a much more visible difference in LPS profile exhibiting the typical rough phenotype profile where the high molecular weight O- polysaccharide is absent (Santander and Robeson, 2007). Visible variation in LPS profile of resistant mutant forms is indicative of LPS being the possible site of mutation in *Salmonella* strain S49 for gaining resistance against phages.

However the emergence of resistant mutants is not as detrimental as emergence of antibiotic resistance in virulent pathogenic bacteria. Phage-resistance has been negatively correlated with some other bacterial life-history

traits, such as growth efficiency (Lenski and Levin, 1985; Lenski, 1988; Bohannan and Lenski, 1999; Brockhurst *et al*., 2004) and motility (Heierson *et al.,* 1986; Paruchuri and Harshey, 1987; Brockhurst *et al.,* 2005), all of which are important traits for bacterial infection and pathogenicity. This hypothesis is supported by several studies where phage-resistance has been shown to correlate with lowered pathogenicity in *Serratia marcescens* (Flyg *et al*., 1980), *Bacillus thuringiensis* (Heierson *et al*., 1986) and *Salmonella enterica* serovar Enteriditis (Santader and Robeson, 2007). In addition, it has been demonstrated recently that rough mutants of *Salmonella*, which appear to be the phage-resistant mutants selected most frequently, are much less able to colonize the alimentary tract of poultry (Craven, 1994; Turner *et al.,* 1998). Furthermore, the ability of the phages in this study to cross-lyse each others bacteriophage resistant mutants, also indicates the candidature of these phages as biocontrol agents, as this characteristics portrayed by these phages, will effectively help in backing up each other whenever phage resistant mutants arise.

5.8 Host range of ΦSP-1 and ΦSP-3

Specificity is the primary requisite of a phage to be used as a biocontrol agent. Even though *Salmonella* bacteriophages are generally host specific and often infect only one bacterial species or only one serotype within a species (Welkos *et al*., 1974), there are several reports on phages that productively infect a range of bacterial species crossing the genus barrier (Jensen *et al*., 1998). *Salmonella* phage that infects multiple genera is also common (Bielke *et al*., 2007).

As *Salmonella* phages infecting many genera are not so rare, the host range of ΦSP-1 and ΦSP-3 was investigated. ΦSP-1 exhibited the ability to infect 31 *Salmonella* Enteritidis strains belonging to different serotypes, including the

standard strain *Salmonella* Abony and clinical strain *Salmonella* Typhi, but were unable to infect bacteria belonging to other genera including its close relative *E. coli,* indicating its narrow host range (Jeena *et al.,* 2012b). ΦSP-3 could infect 33 strains of *Salmonella* belonging to different serotypes, but was unable to infect other genera, including *E. coli* (Jeena *et al.,* 2012a).

For a biocontrol agent to be effective, it should not infect non targeted beneficial bacterial population, but has to be genus specific and at the same time, have the capability of infecting different strains within a genus. This is especially true of *Salmonella* where large numbers of pathogenic strains exist within the genus. The broad host range within the genus and the ability to specifically infect and cause lysis of only *Salmonella* Enteritidis make both these *Salmonella* specific lytic phages, ΦSP-1 and ΦSP-3 desirable candidates as biocontrol agent.

5.9 *In vitro* and *in vivo* bioassay for biocontrol

The potential of virulent phages as specific biological control agents for bacterial pathogens have been studied by many workers. The effect of lytic, S*almonella* specific phages on reducing *Salmonella* numbers in experimentally contaminated fresh-cut melons stored at various temperatures has been pointed out, where phage mixture reduced *Salmonella* populations by approximately 3.5 log on honeydew melon slices stored at 5 and 10 degrees C and by approximately 2.5 log on slices stored at 20 degrees C, which is greater than the maximal amount achieved using chemical sanitizers (Leverentz *et al.,* 2001). Lytic phages were also shown to reduce *Salmonella* colonization in broiler chickens, wherein three phages exhibiting broad host range were given in antacid suspension to birds which were experimentally infected and two phages were able to significantly reduce caecal colonization of the pathogen (Atterbury *et al.,* 2007). Similar results were reported where phages isolated from poultry and human sewage sources

were able to reduce *Salmonell*a contamination in chickens both *in vitro* and *in vivo* conditions (Filho *et al.,* 2007). Work by Toro *et al*. (2005) indicated a protective effect of both *Salmonella*-specific phages in combination with a competitive exclusion product, against *Salmonella* colonization of experimentally infected chicken. Application of bacteriophages at high MOI on chicken carcass surface skin proved to eliminate bacterial contamination efficiently (Goode *et al.,* 2003). The potential of phages in controlling *Salmonella* was revealed in experimentally contaminated sprout seeds (Pao *et al.,* 2004). The efficacy of orally administered bacteriophages in reducing the concentration of *Salmonella* Enteritidis in caecal contents of broilers has also been proved (Fiorentin *et al.,* 2005). Experiments with the well characterized lytic bacteriophage Felix 01, proved their capability in reducing multidrug resistant *Salmonella enterica* subsp. *enterica* serovar Typhimurium burden in mice (Boury, 2005). The potential of phages as alternatives to antibiotics (Greer, 2005) and for phage therapy (Thiel, 2004; Skurnik and Staunch , 2006) has been well reviewed.

The outcome of the application of the *Salmonell*a specific lytic phages ΦSP-1 and ΦSP-3 in the *in vitro* bioassay for biocontrol, where cooked chicken cuts were experimentally contaminated with *Salmonella,* was extremely promising. When compared to the control, phages proved to be effective control agents in terms of their lethality at all temperatures studied. The efficacy of phages as a biocontrol agent was enhanced when used as a cocktail as they complemented each other in cell lysis. Rapid growth of *Salmonella* was observed during the initial stages of propagation at room temperature as well as at 37^{o}C. This was expected as these conditions are perfect for the growth and multiplication of the pathogen. Even under these circumstances, which were conducive for the pathogenic bacteria, the phages could efficiently cut down the number of viable bacteria. Storage at 4ºC helped to keep bacterial multiplication

down and the presence of phages further brought the bacterial count to near zero level.

Multiplicity of infection was found out to be another critical factor determining the effectiveness of phages as biocontrol agents. From the present study it was obvious that presence of phages in high multiplicity of infection provided better protection as it quickly lysed the host cell, not giving sufficient time for emergence of bacteriophages insensitive mutants from the surviving bacteria. A similar result was also reported by Goode *et al.* (2003) where phages applied at high MOI rapidly reduced the recoverable bacterial numbers. In conclusion, these data provided evidence for the effectiveness of lytic phages to limit the growth of pathogenic *Salmonella* in processed poultry foods.

In order to study the effectiveness of phages in combating the bacterial pathogen *in vivo*, *C. elegans* was chosen as the model organism. *C. elegans* is a small, free-living soil nematode that feeds primarily on bacteria. Its usefulness as a model organism is due to its genetic tractability, rapid generation time, ease of propagation, transparent body, a well-defined cell lineage map and a fully sequenced genome that contains a large number of vertebrate orthologues (Portal-Celhay and Blaser, 2012). Several human pathogens, including *Pseudomonas aeruginosa, Salmonella* Typhimurium, *Serratia marcescens, Staphylococcus aureus, Vibrio cholerae*, and *Burkholderia pseudomallei*, kill *C. elegans* when supplied as a food source (Tan *et al.,* 1999; Aballay *et al*., 2000; Kurz and Ewbank, 2000; Labrousse *et al.,* 2000). Different strains of *Salmonella* like *S.* Typhimurium as well as other *Salmonella enteric* serovars including *S.*Enteritidis and *S.*Dublin were also effective in killing *C. elegans* (Aballay *et al.,* 2000) and it was demonstrated that *S. enterica* could establish a persistent infection within the gut of the nematode to achieve full pathogenicity (Labrousse *et al.,* 2000).

In the present study *Salmonella* strain S49 identified as *Salmonella* Enteritidis was also seen to infect and reduce the longevity of the worm. The experiment as set up as per Aballay *et al.*(2000). The time required for 50% of nematodes to die (time to death 50; TD_{50}) when fed on a S49 lawn at 25°C was determined to be 5.85±0.20 days, whereas the time required for 50% of nematodes to die when fed on an *E. coli* OP50 lawn was 9.48 ± 0.34 days. The time required for 50% of nematodes to die in presence of *S.* Typhimurium, a known pathogen used as a positive control was 4.33± 0.12 days. These results obtained from the pathogenesis of S49 in *C. elegans* was found comparable with that of Aballay *et al.,* 2000, who had reported a TD_{50} of 5.3± 0.8 for *S.* Enteritidis and a TD_{50} of 9.9 ± 0.9 days when fed with *E. coli* OP50.

The effect of infection by strain S49 in *C. elegans* was evident upon microscopy of the infected nematodes. The most apparent manifestation of pathogenicity was the dilation of the nematode intestine. This is a typical symptom shown by *C. elegans* infected by pathogenic *Salmonella* (Aballay *et al.,* 2000). The distension of the intestine may be due to clumping of pathogenic microorganisms accumulated within and probably the primary cause of early deaths in the worm (Dhakal *et al.,* 2006). Normally, the pharyngeal grinder of the worm efficiently disrupts the *E. coli* on which it feeds and essentially no intact bacterial cells are found within the intestinal lumen. However, virulent bacterial strains like *Vibrio vulnificus* or *Vibrio cholerae* have been shown to accumulate in both the pharynx and in the lumen of the worm intestines (Dhakal *et al.,* 2006; Vaitkevicius *et al.,* 2006).

Another observed pathogenicity associated with *Salmonella* S49 infection is the “bagging” effect, a condition in which the worms laden with eggs, which in some cases hatched internally, causes a phenotype called “bag of worms”

(Thomsen *et al.,* 2006). Bagging is suggested to be caused by a weakening of the infected worm, rendering it incapable of laying eggs normally (Sifri *et al.,* 2003).

The ability of the bacteriophages ΦSP-1 and ΦSP-3 to confer protection against the bacterial infection by S49 was also proved by this study. When the nematodes were exposed to lawns of *E. coli* OP50 containing the prophylactic phages, after their prior exposure to the bacterial pathogens, they showed significantly less mortality compared to the untreated controls. The longevity of the nematode reached back to near control levels, with phages as a cocktail exhibiting maximum impact. With the phage cocktail treatment, the TD_{50} of infected worms was recorded as 9.29± 0.26 days. These results proved beyond doubt, the capability of ΦSP-1 and ΦSP-3 as prophylactic agents, in effecting a successful and sustainable biocontrol of *Salmonella* Enteritidis *in vivo*.

This work was envisaged to understand the potential of phages as biocontrol agents. During the course of this study, two potent lytic bacteriophages, ΦSP-1 and ΦSP-3 capable of infecting *Salmonella* Enteritidis, exhibiting excellent properties like high stability, specificity, absence of virulence genes, sturdiness under various physical and chemical exposures and ability to infect the host under various nutrient limited conditions, were isolated. The promise of ΦSP-1 and ΦSP-3 as biocontrol agents was proved by the *in vitro* study using experimentally dosed cooked chicken cuts and *in vivo* study using *C. elegans*. This work is of special significance, as *Salmonella* is a tough foe to combat especially in this age of antibiotic resistance.

Chapter 6

SUMMARY

Salmonella were screened using standard methodologies from various environmental samples including chicken caecum. *Salmonella* strains, which were previously isolated and stocked in the lab, were also included in this study as host, for screening *Salmonella* specific lytic phages. The *Salmonella* strain in this study designated as S49 which helped in phage propagation by acting as host bacteria was identified as *Salmonella enterica* subsp. *enterica* by 16S rRNA gene analysis and serotyping.

A total of three *Salmonella* specific phage named as ΦSP-1, ΦSP-2 and ΦSP-3 were isolated from chicken intestine samples via an enrichment protocol employing the double agar overlay method. ΦSP-1 and ΦSP-3 showing consistent lytic nature were selected for further study and were purified by repeated plating after picking of single isolated plaques from the lawns of *Salmonella* S49 plates. Both the phages produced small, clear plaques indicating their lytic nature. ΦSP-1 and ΦSP-3 were concentrated employing PEG-NaCl precipitation method before further characterization.

Morphological characterization of ΦSP-1 by Transmission Electron Microscopy revealed identical hexagonal/ isomeric head outlines, with an extremely short tail, characteristic of family *Podoviridae*. Micrograph of ΦSP-3 indicated a hexagonal head and long non- contractile tail, which are typical morphological features of family *Siphoviridae*.

The basic growth properties of phage necessary to deduce a productive phage- host interaction is the optimal MOI and time taken for phage to adsorb on bacterial. Optimal MOI of ΦSP-1 was five phages per bacterium and for ΦSP-3 it was one. Adsorption studies showed 100% adsorption of ΦSP-1 and ΦSP-3 after 30 minutes and 25 minutes of exposure, respectively. These optimized parameters were used in all subsequent large scale phage production of ΦSP-1 and ΦSP-3.

From the construction of the one step growth curve of ΦSP-1, the latent period was calculated to be approximately 30 minutes and the rise period to be 55 minutes, while the burst size was 44 phages per bacterial cell. The one step growth curve of ΦSP-3 indicated that the latent period and rise period was 30 minutes each and the burst size was 60 phages per bacterium.

The physical and chemical nature of the environment in which the phage-host interaction (propagation and adsorption) takes place is considered important as the precise knowledge about these factors help in determination and fine tuning of the physical and chemical aspects which in turn support better production and characterization of these little entities. The parameters studied included temperature, pH, NaCl, sugars and $CaCl_2$.

Exposure to high temperatures ranging from 50ºC to 100ºC drastically reduced ΦSP-1 viability, although it was able to withstand exposure to temperatures as high as 80ºC. In comparison, ΦSP-1 was much tolerant of higher temperatures than ΦSP-3 and showed greater survival under the experimental conditions, which also clearly showed that exposure to temperatures above 70ºC proved fatal for ΦSP-3. Ability to survive under higher temperatures is a desirable trait for a biocontrol agent in surface pasteurization of poultry foods, where hot water is applied for a short period of time. The optimum concentration of NaCl required for ΦSP-1 survival was 0.1M and that for ΦSP-3 was 1M NaCl. The

study also showed that pH 8 was optimum for survival of ΦSP-1 and ΦSP-3, a property due to their primary niche – the chicken intestine. The phage propagation study also revealed that sugars like rhamnose, maltose and xylose resulted in drastic inactivation of ΦSP-1 by as much as 95%, 92% and 89%, respectively. In case of ΦSP-3, the presence of arabinose (99%) and xylose (98%) caused phage inactivation. These results are indicative of the roles of these sugars and/or their analogues as phage receptors on the host surface outer membrane, evidenced by effective inhibition of the phage- host interaction.

The optimum temperature resulting in 100% adsorption was 37^{o}C and 40^{o}C for both the phages. These temperature optima for ΦSP-1 and ΦSP-3 correspond to the temperature of avian body, the source from which they were isolated. Maximum adsorption for ΦSP-1 and ΦSP-3 occurred at 0.25M NaCl. But unlike ΦSP-1, ΦSP-3 absorption continued to take place at higher concentrations of NaCl .

Adsorption was determined at the pH values ranging from 2 to 13. The pH optimum for maximal adsorption of phages was observed to be in the alkaline pH range. pH optima for adsorption for ΦSP-1 was pH 9 and that for ΦSP-3 was pH 8. The calcium ions at high and low concentrations influenced phage propagation. 1 mM $CaCl_2$ closely followed by 10 mM $CaCl_2$ was optimum for ΦSP-1 adsorption, whereas 10 mM $CaCl_2$ was optimum for ΦSP-3.

The cumulative effect of all the optimized parameters gave a much better yield of phages as evidenced by the considerable increase in phage burst size. In the case of ΦSP-1, the burst size increased from 44 to 73 phages per bacterial cell and that in case of ΦSP-3 increased from 60 to 110 phages per bacterial cell. A longer rise period was observed for both the phages (70 minutes for ΦSP-1 and 50

minutes for ΦSP-3). However, the latent period remained the same for both the phages used in the study.

Phages intended for use as a biocontrol agent have an added advantage when they have the capability to infect host under stationary as well as in various nutrient deprived conditions. Ability to thrive in adverse condition is a desirable trait for a biocontrol agent to be more effective. ΦSP-1 was able to multiply only under multiple nutrient starved states where as ΦSP-3 exhibited capacity to infect host even under stationary phase, multiple nutrient starved states, carbon starved and nitrogen starved conditions. ΦSP-3 failed to multiply only under phosphate starved conditions.

The susceptibility of both phage genomes to restriction digestion by the endonuclease *Bam*HI is indicative of the double stranded nature of DNA in their genomes. This data along with the morphological analysis helped in placing both *Salmonella* phages ΦSP-1 and ΦSP-3 in the order *Caudovirales* (double stranded tailed DNA phages).

The genome size of ΦSP-1 was determined to be 86 kb and that of ΦSP-3 to be 88.43kb by Pulsed-Field Gel Electrophoresis of phage DNA. PCR screening of selected virulence /virulence related genes- *invE/A, slyA, pho P/Q, ttrC, mgtC, spi4R, sopE, gog B, sod, gtg E* and *agfA* was carried out in the host S49 and phages. PCR screening indicated the absence of the tested virulence /virulence related genes in the phage genome, thus negating any chance of transfer of these genes from phage to host. The presence of the virulence genes, *inv*E/A, *afg, slyA, pho P/Q, ttrC and mgtC* in the host strain *Salmonella* S49 indicated the pathogenic nature of this bacterium.

Three sets of primers, targeting major coat protein genes in the phage genome, were designed using ClustalW and Primer3. Molecular level identification of phages were done by sequencing of the amplicons obtained employing these designed primers, followed by construction of the phylogenetic tree using BIOEDIT and MEGA.5 software. ΦSP-1 showed maximum similarity to *Salmonella* phage phiSG-JL2, a known member of the family *Podoviridae*, while ΦSP-3 was closely related to the phage *Salmonella* 7-11, a member of the *Siphoviridae* family.

The analysis of phage proteins by SDS-polyacrlamide gel electrophoresis, both reductive and non reductive, confirmed the differences in the protein components of these two phages belonging to two different families.

Bacteriophage resistant mutant emerge whenever phage-host interaction takes place. LPS profiling using LPS acrylamide gel electrophoresis was used to compare the LPS of bacteriophage resistant mutants and the parental strain. A significant difference in the LPS pattern of the mutants and sensitive parental strain, indicates the role of LPS in phage adsorption. As mutations in LPS has a detrimental effect on bacterial viability, the emergence of resistance is not as serious as emergence of antibiotic resistance. Furthermore, the ability of phages to cross-lyse each other's resistant mutant host, will help in fighting resistance when applied together.

Host Range studies revealed that both the phages have a wide host range within the *Salmonella* genus. ΦSP-1and ΦSP-3 were able to infect 31 and 33 strains of *Salmonella* belonging to different serotypes respectively. Wide host range within the genus is considered to be a desirable quality of phage as a biocontrol agent.

The evaluation of phages as biocontrol agent was done *in vitro* using experimentally contaminated chicken cuts and *in vivo* by the animal model study.

Bioassay using experimentally contaminated chicken cuts confirmed the potential of both phages as biocontrol agents. Promising results were obtained. Phage cocktail was found to be more effective when compared to other combinations and control as they complemented each other in cell lysis. Phages proved to be better in terms of their lethality at all the temperatures studied. Bacteriophage prophylaxis assay in *C. elegans* gave promising results. Treatment of S49 infected *C. elegans* by phages increased their longevity which was comparable to control. Maximum protection was conferred when the two phages were administered together. Microscopy of the healthy and infected nematodes aided in demonstrating the pathogenicity of the *Salmonella* strain S49.

Chapter 7

CONCLUSION

In 1930, Felix d'Herelle wrote "…the actions and reactions are not solely between these two beings, man and bacterium, for the bacteriophage also intervenes; a third living being and hence, a third variable is introduced" (d'Herelle, 1930).

With the ever increasing incidence of antibiotic resistance, humanity is pushed back towards the pre-antibiotic era where simple bacterial infections were deadly. Indiscriminate use of antibiotics is the major perpetrator of this problem. The scientific world is therefore looking for effective alternatives to counteract this imminent crisis.

Felix d'Herelle`s third variable is one among those that are considered as potential alternative candidates to solve this problem. Salmonellae are one of the leading causes of foodborne disease worldwide. Applying *Salmonella*-specific bacteriophages to various foods and food preparation surfaces may provide a natural means for significantly reducing *Salmonella* contamination of various foods, thereby considerably improving public health.

Therefore, the focus of present study was to isolate, characterize and verify the efficacy of lytic bacteriophages against the robust pathogen *Salmonella*, capable of surviving under various hostile conditions. Two phages, ΦSP-1 and ΦSP-3, belonging to two families, *Podovoridae* and *Siphoviridae* were isolated. The two phages exhibited some excellent properties including their high stability, genus specificity, absence of virulence genes and sturdiness under various

physical and chemical exposures, making their candidature promising as a biocontrol agent. In addition to this, one of the key findings of the study was the ability of ΦSP-3 to infect its host under various nutrient-limited conditions. There are only few reports on phages competent to infect their host under both nutrient-rich and nutrient-deprived conditions. This quality ΦSP-3 is an added advantage and an important criterion for an effective biocontrol agent. The prospective nature of these two phages as an efficient biocontrol agent was also established by the *in vitro* study using experimentally dosed cooked chicken cuts and the *in vivo* study using *C. elegans* as the animal model.

As the effectiveness of phage cocktails in conferring better protection was evident from the present study, isolating more numbers of *Salmonella* specific lytic phages from specific localities will aid in devising formulations with better biocontrol competency as individual phages with diverse properties will complement each other in combating the pathogen. In addition, whole genome of phages under study should be sequenced. This analysis will allow complete characterization of the phages, as genome analysis will improve our understanding of the phage characterization, propagation, adsorption as well as give an insight into the virulence genes, if any that they carry; all factors supremely important for their consideration in phage therapy and biocontrol.

Chapter 8

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APPENDIX – 1

Nutrient Medium

Peptone	5g
Sodium chloride	5g
Beef extract	1g
Yeast extract	2g

Suspended 1.3g of media (Himedia, Mumbai, India) in 100ml of distilled water. Mixed well, autoclaved at 15 lbs pressure for 15 minutes and cooled to 50-55°C. When used as solid agar medium, 2.0% agar (w/v) was added to the medium for agar plate preparation. Final pH-7.4±0.2.

Lactose Broth

Peptone	5g
Beef extract	3g
Lactose	5g
Distilled water	1000ml

Suspended 13g of media (Himedia, Mumbai, India) in 1000ml of distilled water. Mixed well and dispensed 225 ml portions into 500 ml Erlenmeyer flasks, autoclaved at 15 lbs pressure for 15 minutes. Final pH- 6.9 ± 0.2.

Tetrathionate Broth base

Polypeptone	5g
Bile salts	1g
Calcium Carbonate	10g
Sodium Thiosulfate	30g
Distilled water	1000ml

Suspended ingredients in 1 liter distilled water, mixed and heated to boiling. Cooled to less than 45°C. Added 20 ml of Potassium iodide solution into it (Potassium iodide-5g, resublimized iodine - 6g in 20 ml distilled water). Dispensed as desired into tubes and used immediately. Final pH- 8.4 ± 0.2.

Rappaport-Vassiliadis

Tryptose	4.59g
Papaic digest of soyabean meal	4.50g
Sodium chloride	7.20g
Mono-Potassium phosphagte	1.44g
Magnesium chloride	36g
Malachite green	0.036g

Suspended 49.2 grams of media (Himedia, Mumbai, India) in 1000ml distilled water. Heat to dissolve the medium completely. Dispensed as desired into tubes and sterilze by autoclaving at 10lbs pressure (121^{o}C) for 10 minutes. Final pH 5.2±0.2.

Triple Sugar Iron Agar

Peptic digest of animal tissue	2g
Beef extract	3g
Yeast extract	3g
Lactose	10g
Sucrose	10g
Glucose	1g
Ferric citrate	0.3g
Sodium chloride	5g
Sodium thiosulphate, pentahydrate	0.3g
Phenol red	0.024g
Agar	12g

Suspended 64.51 grams of media (Himedia, Mumbai, India) in 1000 ml distilled water. Heated to boiling, to dissolve the medium completely. Mixed well and distributed into test tubes. Sterilized by autoclaving at 15 lbs pressure (121°C) for 10 minutes. Allowed the medium to set in sloped form with a butt about 1 inch long. Final pH - 7.4±0.2.

Xylose Lysine Deoxycholate Agar

Yeast extract	3g
L-Lysine	5g
Lactose	7.5g
Sucrose	7.5g
Xylose	3.5g
Sodium chloride	5g
Sodium deoxycholate	2.5g
Sodium thiosulphate	6.8g
Ferric ammonium citrate	0.8g
Phenol red	0.08g
Agar	15g

Suspended 56.68 grams of media (Himedia, Mumbai, India) in 1000 ml distilled water. Heated with frequent agitation, until the medium boiled.

Transferred immediately to a water bath at 50°C. After cooling, poured into sterile Petri plates. Final pH -7.4±0.2.

Hektoen Enteric Agar

Proteose peptone	12g
Yeast extract	3g
Lactose	12g
Sucrose	12g
Salicin	2g
Bile salt mixture	9g
Sodium chloride	5g
Sodium thiosulphate	5g
Ferric ammonium citrate	1.5g
Acid fuchsin	0.1g
Bromo thymol blue	0.065g
Agar	15g

Suspended the ingredients in 1 liter distilled water and mixed. Heated with frequent agitation, to boiling. Transferred immediately to a water bath, at 50°C. After cooling, poured into sterile Petri plates. Final pH -7.5±0.2.

Bismuth Sulfite Agar

Peptic digest of animal tissue	10 g
Beef extract	5g
Dextrose	5g
Disodium phosphate	4g
Ferrous sulphate	0.3g
Bismuth sulphite indicator	8g
Brilliant green	0.025g
Agar	20g

Suspended 52.33 grams of media (Himedia, Mumbai, India) in 1000 ml distilled water. Heated to boiling to dissolve the constituents completely. Prepared plates on previous day, and stored in dark. Final pH - 7.7±0.2.

Tryptone broth

Tryptone	1.5g
Sodium Chloride	0.5g
Distilled Water	100ml

Suspended ingredients in 100ml distilled water, mixed and heated to boiling. Sterilized by autoclaving at 15 lbs pressure (121°C) for 10 minutes and plated.

Carbohydrate Fermentation test

Peptone	10 g
NaC1	5 g
Beef extract (optional)	1 g
Phenol red	0.018 g
Distilled water	1 litre
Carbohydrate*	

*Suspended either 5 g dulcitol, 10 g lactose, or 10 g sucrose in this basal broth. Dispensed 2.5 ml aliquots into culture tubes. Sterilized by autoclaving at 15 lbs pressure (121°C) for 10 minutes. Final pH-7.4 ± 0.2.

Christensens' Urea agar

Peptone	1.0g
Sodium Chloride	5 g
Monopotassium phosphate	2g
Glucose 0.1%	1g
Phenol Red	0.012g
Agar	15g

Suspended all the ingredients except urea in 900 ml water (basal medium). Autoclaved for 15 minutes at 121°C. Cooled to 50-55°C.

Urea	20g
Distilled Water	100ml

Filter-sterilized urea was added aseptically to cooled basal medium and mixed well. Dispensed in to sterile tubes and converted to slants with 2 cm butt and 3 cm slant. Final pH- 6.8±0.2.

Lysine Iron Agar

Peptic digest of animal tissue	5g
Yeast extract	3g
Dextrose	1g
L-Lysine	10g
Ferric ammonium citrate	0.50g
Sodium thiosulphate	0.04g
Bromocresol purple	0.02g
Agar	15g

Suspended 34.56 grams of media (Himedia, Mumbai, India) in 1000 ml distilled water. Heated to boiling to dissolve completely. Dispensed into tubes and

sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cooled the tubes in slanted position to form slants with deep butts. Final pH- 6.7±0.2.

Simmons Citrate Agar

Magnesium sulphate	0.2g
Ammonium dihydrogen phosphate	1g
Dipotassium phosphate	1g
Sodium citrate	2g
Sodium chloride	5g
Bromothymol blue	0.08g
Agar	15g

Suspended 24.28 grams of media (Himedia, Mumbai, India) in 1000 ml distilled water. Heated to boiling to dissolve completely, mixed well and distributed into tubes. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes and made slants. Final pH-6.8±0.2.

Luria Bertani Broth

Casein enzymic hydrolysate	10g
Yeast extract	5g
Sodium chloride	10g

Suspended 25 grams of media (Himedia, Mumbai, India) in 1000 ml distilled water. Heated to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Final pH- 7.5±0.2.

Tryptone Soya Broth

Pancreatic Digest of Casein	17g
Papaic digest of soyabean meal	3g
Sodium chloride	5g
Dipotassium hydrogen phosphate	2.5g
Dextrose (Glucose)	2.5g

Suspended 64.51 grams of media (Himedia, Mumbai, India) in 1000 ml distilled water. Heated to boiling to dissolve the ingredients completely. Mixed well and distributed into test tubes. Sterilized by autoclaving at 15 lbs pressure (121°C) for 10 minutes. Final pH -7.3 ± 0.2.

APPENDIX – 2

SM buffer

NaCl	-	5.8 g
$MgSO_4.7H_2O$	-	2.0 g
1 M Tris HCl (pH 7.5)	-	50 ml
2% gelatin	-	5.0 ml

Ingredients were dissolved and was made up to 1 litre with milliQ water and autoclaved at 15lbs for 20 minutes and stored at 4°C until use.

Phosphate Buffered Saline (PBS)

NaCl	-	8.0 g
KCl	-	0.2 g
Na_2HPO_4	-	1.44 g
KH_2PO_4	-	0.24 g

Ingredients were dissolved in 800 ml of distilled water, pH adjusted to 7 with 1N HCl. The volume was made up to 1 litre with distilled water, autoclaved at 15lbs for 20 minutes and stored at room temperature until use.

Hydrochloric acid- potassium chloride buffer (pH 2)

Solution A: 0.2 M KCl
Solution B: 0.2 M HCl

Mixed 50 ml of solution A with 10.6 ml of solution B and made up to 200ml with distilled water.

Citrate buffer (pH 3 - 6)

Solution A: 0.1 M Citric acid
Solution B: 0.1 M sodium citrate

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to make up volume to 200 ml and then filter sterilized.

Desired pH	Solution A (ml)	Solution B (ml)
3	46.5	3.5
4	33.0	17
5	20.5	29.5
6	9.5	41.5

Phosphate buffer (pH 7)
Solution A: 0.2 M NaH_2PO_4
Solution B: 0.2 M Na_2HPO_4

Mixed 39 ml of solution A with 61 ml of solution B and the volume was made up to 200ml with distilled water, followed by filter sterilization.

Tris (hydroxymethylamino methane buffer system (pH 8 and 9)
Solution A: 0.2 M Tris buffer
Solution B: 0.2 M HCl

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 ml and filter sterilized.

Desired pH	Solution A (ml)	Solution B (ml)
8	50	26.8
9	50	5

Carbonate – bicarbonate buffer (pH 10 and 11)
Solution A: 0.2 M Na_2CO_3
Solution B: 0.2M $NaHCO_3$

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 ml and filter sterilized.

Desired pH	Solution A (ml)	Solution B (ml)
10	27.5	22.5
10.7	45.0	5

Sodium hydroxide - Potassium chloride buffer (pH 12 and 13)
Solution A:0.2 M KCl
Solution B:0.2M NaOH

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 ml and then filter sterilized.

Desired pH	Solution A (ml)	Solution B (ml)
12	50	12
13	50	132

Minimal media

5X minimal media	-	20ml
Distilled water	-	80ml
1 M $MgSO_4$	-	0.2 ml
Autoclaved and added		
20 % glucose	-	2 ml
1 M $CaCl_2$	-	0.01 ml

Minimal media (5X concentrate)

$Na_2HPO_4.7H_20$	-	6.4 g
KH_2PO_4 (dibasic)	-	1.5g
NaCl	-	0.25g
NH_4Cl	-	0.5g

Ingredients were dissolved in 80 ml distilled water. Adjusted the volume to 100ml with distilled water and sterilized by autoclaving at 15lbs for 20 minutes before use.

APPENDIX – 3

TE buffer

1M Tris-Cl	-	10ml
500mM EDTA (pH 8.0)	-	2ml

1M Tris-HCl

Tris base	-	60.57 g
Deionised water	-	500ml

Adjusted to desired pH using concentrated HCl

0.5M EDTA

EDTA	-	18.6 g
Deionised water	-	100ml

50X TAE Buffer

Tris base	-	121 g
Glacial acetic acid	-	28.6ml
0.5M EDTA pH 8.0	-	50 ml

Deionised water added to make volume to 500ml.

1X TAE Buffer

50X TAE buffer	-	10mL
Deionised water	-	490ml

10X TBE

Tris base	-	108g
Boric acid	-	55g
0.5M EDTA (pH 8.0)	-	20ml

Deionised water added to make volume to 1000ml.

0.5X TBE Buffer

10X TBE buffer	-	50mL
Deionised water	-	1000ml

3M Sodium acetate (pH 5.2, 7.0)

Sodium acetate.$3H_2O$	-	408.3 g
Distilled water	-	800 ml

pH adjusted to 5.2 with glacial acetic acid. Dilute acetic acid was used to adjust the pH to 7.0. Adjusted the volume to 1 liter with distilled water. Sterilized by autoclaving.

APPENDIX – 4

Polyacrylamide Gel Electrophoresis

Stock acrylamide solution (30:0.8)

Acrylamide (30%)	-	60.0 g
Bis-acrylamide (0.8%)	-	1.6 g
Distilled water	-	200.0 ml

Filtered through Whatman No. 1 filter paper and stored in amber colored bottle at 4°C.

Stacking gel buffer stock (0.5M Tris-HCl, pH 6.8)

Tris buffer	-	6 g in 40 ml distilled water

Titrated to pH 6.8 with 1M HCl (~ 48 ml) and made up to 100 ml with distilled water. Filtered through Whatman filter paper No. 1 and stored at 4°C.

Resolving gel buffer stock (3M Tris-HCl, pH 8.8)

Tris buffer	-	36.3 g

Titrated to pH 8.8 with 1M HCl (~ 48 ml) and made up to 100 ml with distilled water. Filtered through Whatman No. 1 filter paper and stored at 4°C.

Reservoir buffer for SDS-PAGE (pH 8.3)

Tris buffer	-	3.0 g
Glycine	-	14.4 g
SDS	-	1.0 g

Dissolved and made up to 1L with distilled water. Prepared as 10X concentration and stored at 4°C.

Sample buffer for Reductive SDS-PAGE (1 X)

SDS-PAGE sample buffer (2X)	-	1.0 ml
50% Sucrose in distilled water	-	0.6 ml
Distilled water	-	0.4 ml

SDS-PAGE sample buffer (2X)

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol (optional)	-	10% (v/v)
SDS	-	2%
Dithiothreitol	-	0.1M
Bromophenol blue	-	0.01%

Sample buffer for Non-reductive SDS-PAGE(1X)

Sample buffer for Non-reductive SDS-PAGE (2X)	-	1.0 ml
50% Sucrose	-	0.4 ml
Distilled water	-	0.6 ml

Sample buffer for Non-reductive SDS-PAGE (2X)

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol (optional)	-	10% (v/v)
SDS	-	2%
Bromophenol blue	-	0.01%

SDS (10%) - 1 g in 10 ml distilled water

Sucrose (50%) - 5 g in 10 ml distilled water

Autoclaved at 121° C for 15 minutes and stored at 4°C until use.

Protein Markers for SDS-PAGE

Low molecular weight marker mix (BioRad, USA) was used. The standards were diluted at a ratio of 1: 20 in SDS reducing sample buffer and heated at 95^{o}C for 5 minutes. 5 µl of the marker was loaded on to the gel. The composition of the marker mix is as given below.

Components		**MW (daltons)**
Phosphorylase b	-	97,000
Serum Albumin	-	66,200
Ovalbumin	-	45,000
Carbonic anhydrase	-	31,000
Trypsin inhibitor	-	21,500
Lysozyme	-	14,400

SDS Reducing Sample Buffer (prepared immediately before use)

ß-mercaptoethanol	-	25 µl
Stock Sample Buffer	-	475 µl

Stock Sample Buffer (stored at room temperature)

Distilled water	-	4.8 ml
0.5M Tris-HCl (pH 6.8)	-	1.2 ml
Glycerol	-	1.0 ml
10% (w/v) SDS	-	2.0 ml
0.1% (w/v) Bromophenol blue	-	0.5 ml

Silver staining

Fixing solution 1	-	50ml methanol and 5ml acetic acid in 45ml water
Fixing soution 2	-	50 ml methanol in 50ml water
Sensitizer	-	Sodium thiosulfate (20mg/100ml)
Staining solution*	-	Silver nitrate (200mg/100ml)

Developing solution*

Sodium carbonate (anhydrous)	- 3g/100ml
Formaldehyde	- 25µl/100ml
Sodium EDTA solution	- 1.4 g/100ml

* Mixed and prepared fresh before use.

LPS microextraction lysing buffer

SDS	-	2%
β mercaptoethanol	-	4%glycerol
1 *M* Tris–Cl (pH 6.8)		
Bromophenol blue	-	A pinch

LPS Acrylamide Gel Electrophoresis

Stock acrylamide solution (30:0.8)

Acrylamide (30%)	-	29.2 g
Bis-acrylamide (0.8%)	-	0.8 g
Distilled water	-	100.0 ml

Filtered and stored in amber colored bottle at 4°C until use.

Resolving gel buffer stock (1.88M Tris-HCl, pH 8.8)

Tris buffer - 22.78 g in 70 ml distilled water

Titrated to pH 8.8 with concentrated HCl and made up to 100 ml with distilled water. Filtered with Whatman No. 1 filter paper and stored at 4°C until use.

Stacking gel buffer stock (1.25 M Tris-HCl, pH 6.8)

Tris buffer - 15.12 g in 70 ml distilled water

Titrated to pH 6.8 with concentrated HCl and made up to 100 ml with distilled water. Filtered with Whatman No. 1 filter paper and stored at 4°C until use.

Reservoir buffer, Tris–glycine buffer (pH 8.3)

Tris buffer	-	24 g
Glycine	-	115.2 g
SDS	-	8.0 g

Dissolved and made up to 8 litre with distilled water.

Silver staining

1. Fixing solution - 40% ethanol , 5% acetic acid and 0.9% periodic acid
2. Staining solution*
   - 20% Silver nitrate - 5 ml
     add silver nitrate drop-wise)
   - 0.1N NaOH - 28 ml
   - concentrated NH_4OH - 2.1 ml
   - Distilled water - 115ml
3. Developing solution *
   - anhydrous citric acid - 50 mg
   - 37% formaldehyde - 0.5 ml
   - Distilled water - 200ml

* Mixed and prepared fresh before use.

APPENDIX – 5

Nematode Growth Medium

NaCl	-	3g
Agar	-	17g
Peptone	-	3.5g
Distilled water	-	975 ml

Autoclaved and cooled the flask in 55°C water bath for 15 minutes and added

1 M $CaCl_2$	-	1ml
Cholesterol in ethanol (5 mg/ml)-		1ml
1 M $MgSO_4$	-	1ml
1 M KPO_4 buffer	-	25 ml

Swirled to mix well before use.

1 M KPO_4 buffer (pH 6)

KH_2PO_4	-	108.3 g
K_2HPO_4	-	35.6 g
Distilled water	-	1000ml

Soft Agar Freezing Solution

NaCl	-	0.58 g
KH_2PO_4	-	0.68 g
Glycerol	-	30 g
1 M NaOH	-	0.56 ml
Agarose	-	0.4 g

Distilled water to 100 ml. Sterilized by autoclaving at 15lbs for 20 minutes.

S Buffer

0.05 M K_2HPO_4	-	129 ml
0.05 M KH_2PO_4	-	871 ml
NaCl	-	5.85 g

M9 Buffer

Na_2HPO_4	-	6 g
KH_2PO_4 (dibasic)	-	3 g
NaCl	-	5g
1 M MgSO4	-	1 ml

Dissolved in 800 ml distilled water. Adjusted the volume to 1000ml with distilled water and sterilized by autoclaving at 15lbs for 20 minutes.

APPENDIX – 6

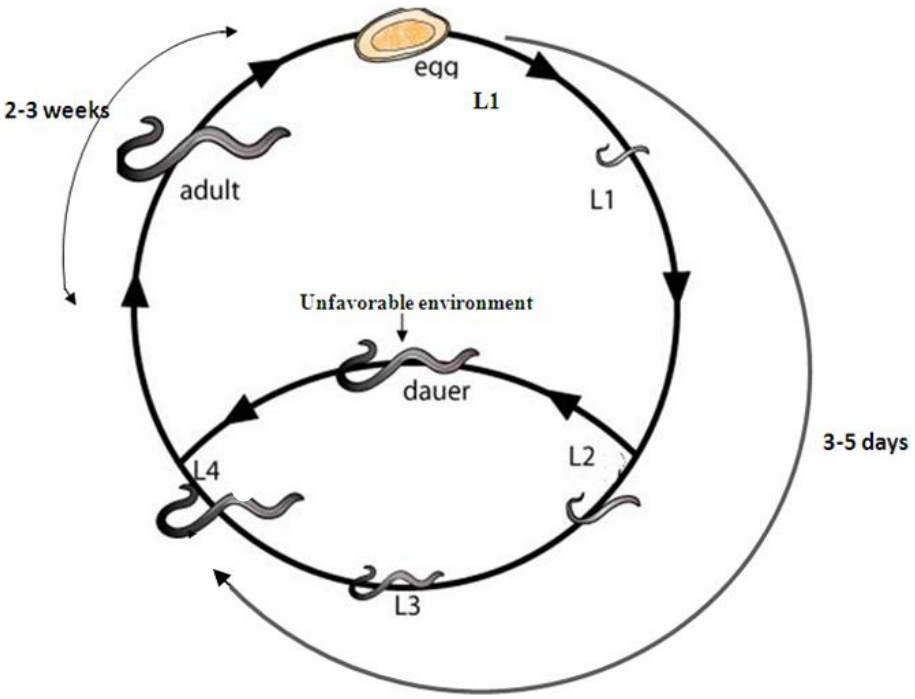

Fig 9. 1. Life cycle of *C. elegans* (figure adapted from http://www.scq.ubc.ca/genetic-studies-of-aging-and-longevity-in-model-organisms - with modification)

The life cycle of *C. elegans* is comprised of the embryonic stage, four larval stages (L1-L4) and adulthood. The end of each larval stage is marked with a molt. Under favorable environments, *C. elegans* will develop rapidly to reproductive maturity, but in unfavorable environments, animals will arrest at the dauer diapause, a larval stage geared for survival. Dauer larvae are thin and can move but their mouths are plugged and they cannot eat. When animals reach adulthood, they produce about 300 progeny each. They live a total of up to 3 weeks depending on the temperature.

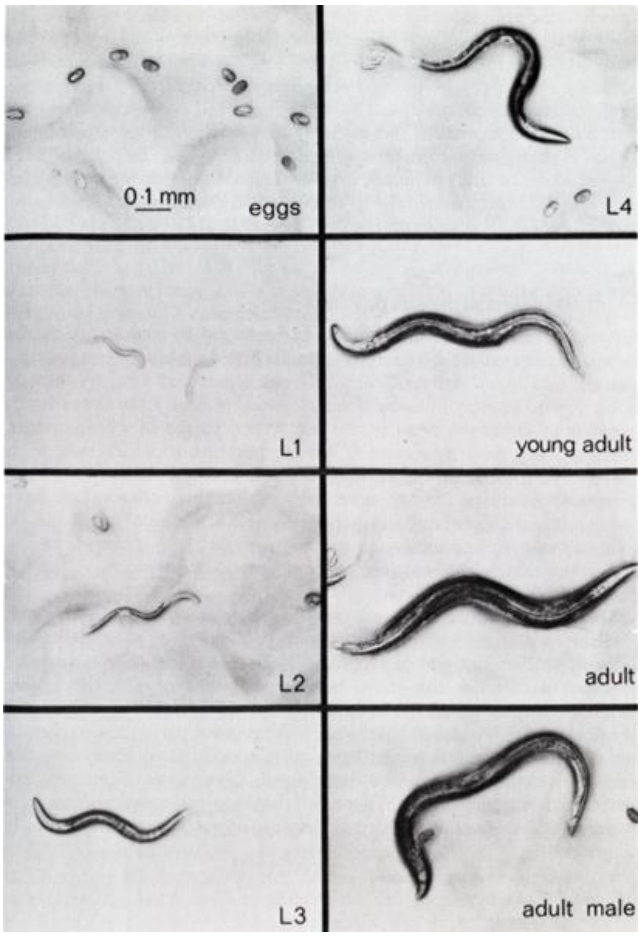

Fig 9. 1. Life stage of *C. elegans* – Microscopic features. Adapted from George L. Sutphin and Matt Kaeberlein at University of Washington, Seattle. (http://openwetware.org/wiki/BISC_219/2009:_Mod_1_*C.elegans*_ General_Information).

LIST OF PUBLICATIONS

a) Peer Reviewed

1. **Jeena Augustine**, Linda Louis, Siju M Varghese, Sarita G Bhat and Archana Kishore, 2012, Isolation and partial characterization of ΦSP-1, a *Salmonella* specific lytic phage from intestinal content of broiler chicken. Journal of Basic Microbiology, DOI 10.1002/jobm.201100319.

2. **Jeena Augustine**, Siju M Varghese and Sarita G Bhat, 2012, ΦSP-3, a *Salmonella* specific lytic phage capable of infecting host under nutrient deprived states. Annals of Microbiology, DOI 10.1007/s13213-13012-10485-13219.

3. Siju M Varghese , **Jeena Augustine** and Sarita G Bhat, 2012, Rapid sensitive detection of low number of *Salmonella* in water by Loop-mediated isothermal amplification, Advanced Biotech 11(12),28-30.

b) Full paper in proceedings of National Symposium

4. **Jeena Augustine** , Siju M Varghese, Sartia G Bhat and Hatha AAM, 2011, Phenotyping, genotyping and virulence gene profiling of two *Salmonella* strains isolated from chicken gut, Proceedings of 2 day National symposium on "Emerging trends in Biotechnology" conducted by Department of Biotechnology, CUSAT, 1st and 2nd September, 2011. **ISBN number: 978-93-80095-30-1.**

5. Linda Louis, **Jeena Augustine** and Sarita G Bhat, 2011, Prevalence of virulence genes among non-01 *Vibrio Cholerae* isolated from marine environment. Proceedings of 2 day National symposium on "Emerging trends in Biotechnology" conducted by Department of Biotechnology, CUSAT, 1st and 2nd September, 2011. **ISBN number: 978-93-80095-30-1.**

c) Extented abstracts in proceedings of National Seminar

6. **Jeena Augustine**, Linda Louis, Mridula VG and Sarita G Bhat, 2011, *Salmonella* phages as biocontrol agents to reduce *Salmonella* in experimentally contaminated chicken cuts, Proceedings of UGC sponsored two day national seminar on " Emerging trends in biopharmaceuticals" organized by St. Mary's College, Thrissur.

7. Smitha S, **Jeena Augustine**, Raghul Subin S and Sarita G Bhat, 2011, Antimicrobial protein from *Bacillus licheniformis* isolated from Cochin backwaters, Proceedings of UGC sponsored two day national seminar on " Emerging trends in biopharmaceuticals" organized by St. Mary'sCollege, Thrissur.

c) Poster presentations in National/International Symposium/Conferences

8. **Jeena Augustine** and Sarita G Bhat, 2010, *Salmonella phages :* an.alternative to antibiotics in poultry farms. National level technical symposium– "Bioconcorrenza`10"organized by Centre for Plant Molecular Biology, TNAU, Coimbatore.

9. Linda Louis, **Jeena Augustine** and Sarita G Bhat, 2009, Isolation, purification and partial characterization of *Vibrio* phages from mangrove ecosystem. Book of abstracts of MECOS 09, Cochin, Kerala. International symposium on Marine Ecosystems challenges and Opportunities, Cochin.

10. **Jeena Augustine** and Sarita G Bhat, 2008, *Salmonella* phage: potential candidate for ecofriendly biocontrol, Proceedings of BIOCAM 2008, Cochin, Kerala. International conference on biodiversity conservation and management, Kochi.

d) Genbank Submissions

1. Genbank Accession Number - HQ268499 - *Salmonella enterica* subsp. *enterica.* strain S37 - 16S ribosomal RNA gene, partial sequence – **Jeena Augustine**, Siju M Varghese, Raghul Subin S, AAM Hatha, Sarita G Bhat.

2. Genbank Accession Number - HQ268500 - *Salmonella enterica* subsp. *Enterica.* strain S49 - 16S ribosomal RNA gene, partial sequence, **Jeena Augustine**, Siju M Varghese, Raghul Subin S, AAM Hatha, Sarita G Bhat.

3. Genbank Accession Number - HQ260701 – Virulence gene , tetrathionate reductase complex, subunit C (*ttrC)* from S49 , partial sequence, **Jeena Augustine**, Siju M Varghese, Raghul Subin S, AAM Hatha, Sarita G Bhat.

4. Genbank Accession Number - HQ260702 – Virulence gene , surface presentation of antigens protein (*spam)* from S49 , partial sequence, **Jeena Augustine**, Siju M Varghese, Raghul Subin S, AAM Hatha, Sarita G Bhat.

5. Genbank Accession Number - HQ260703 – Virulence gene , Aggregative Fimbriae (*agfA*) from S49, partial sequence, **Jeena Augustine**, Siju M Varghese, Raghul Subin S, AAM Hatha, Sarita G Bhat.

6. Genbank Accession Number - HQ260704 – Virulence gene , invasion protein (*InvE*) from S37, partial sequence, **Jeena Augustine**, Siju M Varghese, Raghul Subin S, AAM Hatha, Sarita G Bhat.

7. Genbank Accession Number - HQ260705 – Virulence gene , invasion protein (*InvE*) from S49, partial sequence, **Jeena Augustine**, Siju M Varghese, Raghul Subin S, AAM Hatha, Sarita G Bhat.

8. Genbank Accession Number - HQ260706 – Virulence gene , Regulatory system (*pho P/Q*) from S37, partial sequence, **Jeena Augustine**, Siju M Varghese, Raghul Subin S, AAM Hatha, Sarita G Bhat.

9. Genbank Accession Number - HQ260707 – Virulence gene , Regulatory system (*pho P/Q*) from S49, partial sequence, **Jeena Augustine**, Siju M Varghese, Raghul Subin S, AAM Hatha, Sarita G Bhat.

10. Genbank Accession Number- HQ260708 – Virulence gene , Transcriptional regulator(*slyA)* from S37, partial sequence, **Jeena Augustine**, Siju M Varghese, Raghul Subin S, AAM Hatha, Sarita G Bhat.

11. Genbank Accession Number- HQ260709 – Virulence gene, Transcriptional regulator (*slyA)* from S49 , partial sequence, **Jeena Augustine**, Siju M Varghese, Raghul Subin S, AAM Hatha, Sarita G Bhat.

12. Genbank Accession Number - GU904006 - *Vibrio* sp. BTTC27 16S ribosomal RNA gene, partial sequence, Raghul SS, Sarita BG, Smitha S, **Jeena Augustine**, Siju VM, Vijaya A, HelvinV.

13. Genbank Accession Number- GU904004 - *Vibrio* sp. BTTN18 16S ribosomal RNA gene, partial sequence, Raghul SS, Sarita BG, Smitha S, **Jeena Augustine**, Siju VM, Vijaya A, HelvinV.

14. Genbank Accession Number- GU904005 - Vibrio sp. BTTC10 16S ribosomal RNA gene, partial sequence Raghul SS, Sarita BG, Smitha S, **Jeena Augustine**, Siju VM, Vijaya A, HelvinV.

15. Genbank Accession Number- HM635765 - *Salmonella enterica* subsp. enterica serovar Typhi strain STY1 16S ribosomal RNA gene, partial sequence, Siju MV, **Jeena Augustine**, Raghul SS, TobyA, Sarita GB.

16. Genbank Accession Number- HM030818 - *Bacillus subtilis* subsp. *subtilis* strain BTFK101 16S ribosomal RNA gene, partial sequence, Smitha S, **Jeena Augustine**, Sarita BG.