Introduction

General Introduction

The passage of liquid or loose stools three or more times a day or more frequent passage of stool than normal can be defined as diarrhoea. This disease can be caused by a variety of bacterial, viral and parasitic organisms (WHO, 2013). There are many organisms responsible for the disease, among them Rotavirus is the leading cause of acute diarrhoea and accounts for 40% of childhood diarrhoeal disease. Other pathogens contributing to the disease are E. coli, Shigella, Campylobacter, Salmonella and V. cholerae. Among protozoans, Cryptosporidium is the major contributor. Even though V. cholerae is considered as the major cause it actually occurs in adults than in children. Diarrhoea can be fatal and leading cause of death during natural calamities and disasters. In 1994 it has been estimated that 50,000 deaths occurred in the first month among the 5,00,000 to 8,00,000 Rwandan refugees around Goma. The outbreak was cited to be occurring due to scarcity of water added along with the malnutrition. Usually this disease spreads from a person to the other through fecal-oral route. Most of the diarrhoeal pathogens gets transmitted in similar ways, they take the fecal-oral transmission. Especially drinking of fecal contaminated water and food are the main source of transmission.

Different forms of acute childhood diarrhoea

The childhood diarrhoeal disease can be classified into three types, where all the types are fatal/life threatening.
**Acute watery diarrhoea**

Major example is Cholera where a significant water loss in a short period and dehydration of the infected individual is the main characteristic which lasts up to several hours/days. The pathogen for this type of diarrhoea is *E. coli* and *V. cholerae*.

**Bloody diarrhoea**

Generally diarrhoea is referred as dysentery. This is characterised by visible blood in the stool as there will be damage in the intestine and nutrient loss in the infected person. *Shigella* is the major causative agent of bloody diarrhoea.

**Persistent diarrhoea**

Extended period of diarrhoea lasting even up to 14 days is referred to as persistent diarrhoea. Malnutrition in children with an illness will be more susceptible to this disease. Persistent diarrhoea is generally related to the HIV infection. Children are at greater risk due to this life threatening illness. As the child’s body has a greater proportion of water, the loss of water from their body leads to fatal effect during diarrhoea (UNICEF and WHO, 2009).

Diarrhoea is a major disease affecting children under the age of two. It kills 1.5 million children annually and is the second largest leading cause of death in children. There have been approximately two billion diarrhoeal cases every year (WHO, 2013). In India, diarrhoea is the third major cause of death among child below the age of 5. It amounts to 13% of death among child below the age of 5. Diarrhoea leaves the body without water and essential salts. The mortality in diarrhoea is mainly due to loss of water and micronutrients. The pathogen *Campylobacter* is a common cause of bacterial diarrhoea, but the bacterial infections by *Salmonella*, *Shigella* and some strains of *Escherichia coli* are the frequent causative agents for diarrhoea (Viswanathan *et al.*, 2009).
Figure 1. Distribution of causes of death among children aged under five years and within the neonatal period, 2004


Figure 2. Distribution of causes of death among children aged under five years and within the neonatal period, 2004

<table>
<thead>
<tr>
<th>Distribution of causes of deaths in children under-5 (2010)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pneumonia</strong></td>
</tr>
<tr>
<td><strong>Prematurity</strong></td>
</tr>
<tr>
<td><strong>Diarrhoea</strong></td>
</tr>
<tr>
<td><strong>Birth asphyxia</strong></td>
</tr>
<tr>
<td><strong>Other diseases</strong></td>
</tr>
<tr>
<td><strong>Neonatal sepsis</strong></td>
</tr>
<tr>
<td><strong>Congenital anomalies</strong></td>
</tr>
<tr>
<td><strong>Injuries</strong></td>
</tr>
<tr>
<td><strong>Measles</strong></td>
</tr>
<tr>
<td><strong>HIV/AIDS</strong></td>
</tr>
<tr>
<td><strong>Malaria</strong></td>
</tr>
</tbody>
</table>

WHO Report - Country Health Profile - India. (Source - http://www.who.int/gho/countries/ind.pdf)
**Enterobacteriaceae**

Most of the bacterial pathogens responsible for diarrhoea come under the bacterial family Enterobacteriaceae. This family is comprised of various species responsible for different clinical symptoms namely typhoid fever, dysentery and plaque. The Enterobacteriaceae is Gram negative, motile, facultative anaerobic rod bacteria. This family is largely found in the intestinal tracts of humans and animals.

**Salmonella enterica**

*Salmonella* is a genus of bacteria which is the major causative agent of food borne illness throughout the world. *Salmonella* enterica is classified into seven subspecies and sub classified into 2000 serovars based on their O and H antigens. *Salmonella* serovars typhi and paratyphi A, B and C cause Typhoid Salmonellosis (Ananthanarayan and Paniker, 2005, Park, 2007, Zaki and Karande, 2011). It invades through the intestine and enters the lymphatic tissue. In initial stages, it spreads through lymph and later through blood. A generalized sepsis is common in the later stages of the disease where as in enteric Salmonellosis it is restricted to the gastrointestinal tract (Kayser and Bienz, 2005).

Kauffman-White scheme is usually used for different serovars of the *Salmonella* sp. based on their O and H antigen. *Salmonella* genus was considered to have two species *Salmonella enterica* and *Salmonella bongori* until recently (Usera et al., 2003). The *S. enterica* is divided into six subspecies namely *S. enterica* subsp. enteric (I), *S. enterica salmæ* (II), *S. enterica* subsp. arizonae (III), *S. enterica* subsp. diarizonae (IIIb), *S. enterica* subsp. houtenae (IV) and *S. enterica* subsp. indica (VI) (Usera et al., 2003). The *Salmonella bongori* which was considered as the subspecies V was later considered as the new separate species (Reeves et al., 1989, Usera et al., 2003). The third species were added to the *Salmonella* genus and is called as
Salmonella subterranean in 2005 (Tindall et al., 2005). A recent report states that there are totally 2541 serovars in the genus of Salmonella (Usera et al., 2003).

Table -1. Kauffman - White Scheme of serovars of Salmonella

<table>
<thead>
<tr>
<th>Group</th>
<th>Serovar</th>
<th>O antigens</th>
<th>H antigens</th>
<th>Phase 1</th>
<th>Phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Paratyphi A</td>
<td>1, 2, 12</td>
<td>A</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Schottmuelleri (syn. Paratyphi B)</td>
<td>1, 4, (5), 12</td>
<td>B</td>
<td>1, 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Typhimurium</td>
<td>1, 4, (5), 12</td>
<td>I</td>
<td>1, 2</td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>Hirschfeldii (syn. Paratyphi C)</td>
<td>6, 7, (Vi)</td>
<td>c</td>
<td>1, 5</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>Choleraesuis</td>
<td>6, 7</td>
<td>(c)</td>
<td></td>
<td>1, 5</td>
</tr>
<tr>
<td>D1</td>
<td>Newport</td>
<td>6, 8</td>
<td>e, h</td>
<td></td>
<td>1, 2</td>
</tr>
<tr>
<td></td>
<td>Typhi</td>
<td>9, 12, (Vi)</td>
<td>D</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enteritidis</td>
<td>1, 9, 12, (Vi)</td>
<td>g, m</td>
<td>(1, 7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dublin</td>
<td>1, 9, 12, (Vi)</td>
<td>g, p</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Gallinarum</td>
<td>1, 9, 12</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Panama</td>
<td>1, 9, 12</td>
<td>I, v</td>
<td></td>
<td>1, 5</td>
</tr>
<tr>
<td>E1</td>
<td>Oxford</td>
<td>3, 10</td>
<td>A</td>
<td></td>
<td>1, 7</td>
</tr>
</tbody>
</table>

In Typhoid Salmonellosis, Salmonella bacterium attaches to the cells in the jejunum. It invades the host system using the system’s endocytosis mechanism in subserosa by macrophages and moves into the mesenteric lymph nodes. It proliferates and disseminates through lymph and blood. During the second stage of infection, the in focus is on the spleen, liver, bone marrow, bile ducts, skin and Peyer’s patches. (Kayser and Bienz, 2005).

The symptoms of typhoid Salmonellosis starts with fever for the entire I week, followed by leucopenia, bradcardia, swelling of the spleen and abdominal roseola. In the beginning of the third week, diarrhoea joins with other symptoms (Kayser and Bienz, 2005).
The diagnosis for the typhoid Salmonellosis largely depends on the method 1. Isolation of the pathogen from stool. 2. By using specific antisera. In the later method, the titre of the agglutinating antibodies against the O and H antigens will be determined which is widely mentioned as WIDAL test. For treatment, antibiotics/anti-infective agents are generally preferred as the disease induces a generalized sepsis (Kayser and Bienz, 2005).

**Multidrug resistant typhoid**

Salmonella typhi strains are resistant to the first line recommended drugs chloramphenicol, ampicillin and co-trimoxazole and are considered as Multidrug resistant typhoid fever (MDRTF) (Zaki& Karande, 2011). Multidrug resistant typhoid emerged around 20 years back and spreads worldwide which increases the risk of increasing the mortality rate due to the disease (Gupta, 1994, Kato *et al.*, 2007, Kumar and Gupta, 2007, Memon *et al.*, 1997, Parry *et al.*, 2007, Saha *et al.*, 1999). This multidrug resistant typhoid fever is an added burden for the developing countries health sector. Hence, an efficient method is required to control the Multidrug resistant typhoid. With the emergence of MDRTF, bacteriophages are believed to be the life saver. The treatment of MDRTF seems to be a nightmare at present as antibiotics remains useless. Bacteriophages which can evolve along with its host and being a strong predator for bacterium will certainly give an upper hand in treating the bacterial diseases.

**Bacteriophage**

Bacteriophage or phage is a virus that infects bacteria and is believed to be the most abundant and most diverse organism on the earth. Krueger (1936) stated “Bacteriophages is a generic term including a large group of agents which share in common the ability to produce dissolution of growing bacterial cultures (lysis), they
all possess the curious property of regenerating themselves during her contact with growing susceptible bacteria”. Phages are particulate particles which can pass through bacteria retaining filters. Bacteriophages vary in size and shapes, it occurs from 20 to 300 nm in size, hence for visualization of phages electron microscope is essential. The phages are considered as the most diversely occurring entity in the world. They have been isolated from a wide variety of environments including seawater, soil, sewage, food products and even in feces. The phages vary from each other based on their natural host, host range and other similar characteristics. Phages are even characterized based on their RNA & DNA content (Moat et al., 2003). Phage therapy was first reported for treating a patient with dysentery by Felix d’Herelle in 1919 (Summers and Yale University Press., 1999).

Frederick W. Twort (1915) is believed to be the Pioneer in the field of Bacteriophages. He started the scientific research in the field of bacteriophages. Later Felix d’ Herelle in 1917 from Paris observed an antagonistic property to microbes that resulted in the lysis of the bacterium in discrete patches and he called them “Plaques”. He conceived that they are invisible microbes as Ultraviruses that invade bacteria and multiply at their expenses and he termed them as Bacteriophages. The direct visualization of phages by electron microscope beginning in the early 1940’s support the findings of d’ Herelle who advocated that the phages are particulate particles capable of producing discrete plaques on dilution. After examining phages in the Electron Microscope in 1940, the phages were accepted as viruses that infect bacteria. In 1921 d’ Herelle carried out experiments to study the phage prophylaxis properties against Bacillus gallinarium natural infection in chicken. This early study was the initial step towards the usage of phages as the natural curing agents for infectious diseases (Kutter and Sulakvelidze, 2004).
Classification of Phages

The important parameters used to classify bacteriophages include their morphological characteristics and their nucleic acid properties. There exists more than one type of classification. The other parameters used to classify the phages are their host range and immunological relationships. The phage genetic materials may vary among each other, either it may be DNA or RNA, though most of the well known of them are double stranded DNA (Prescott et al., 2002). The ICTV classification of viral taxonomy includes 3 orders, 61 families, 214 genera and more than 3600 species, in addition to the 20 floating genera mostly of plant viruses.

Morphology

Phages are highly heterogeneous in their structural, physicochemical and biological properties. Virions may occur in the nature as tailed, polyhedral, filamentous and pleomorphic. Most of the phages contain double stranded DNA and some of them contains genetic materials in the form of Single stranded DNA, Single stranded RNA or double stranded RNA. Based on the morphology and genetic material, the phages are classified as Caudovirales (tailed phages). The Caudovirales are further classified into Myoviridae (consisting of contractile tail with sheath and a central tube), Siphoviridae (with long, non-contractile tail) and Podoviridae (with short, non-contractile tail) (Kutter and Sulakvelidze, 2004).

The tailless phages include only 190 known viruses of which less than 4% of them belong to the known viruses. This structure varies from polyhedral, filamentous to pleomorphic phages (Kutter and Sulakvelidze, 2004).

The genetic material, size of the phages varies from each other. Few phages contain genomes even in the order of few thousand bases. The phage with a larger genome size known was with 480,000 base pairs which equal the size of a bacterium.
In the Caudovirales - the tailed phages, the virion genetic material is double stranded DNA. The genetic material shares 50% of the total mass of the phage particle and the protein shares the remaining 50% of the mass in Caudovirales. The virions are usually made of many copies of specific protein or two. Corners are made of pentamers and each side is made up of hexamers.

The Caudovirales has three main families. The three main families are defined by their tail morphologies. *Siphoviridae* are with long, flexible tail which accounts for 60% of the characterized phages; 25% characterized are *Myoviridae* with double-layered, contractile tails; and 15% are *Podoviridae* with short, stubby, tails (Kutter and Sulakvelidze, 2004).

The tailless phages are classified into ten families. They are differentiated based on their shape (Rods, Spherical, Lemon shaped or Pleomorphic) (Kutter and Sulakvelidze, 2004).

**Phage ecology**

Discovery of very large numbers of phage particles from the oceans introduced phage ecology as an inevitable branch of Marine Microbiology. Phages are present abundantly in the surface and oceanic waters around the world. Phages have been detected in surface waters, ocean depths, ice and sediments. The exploration of the marine phages leads to the hypothesis that the phages may be largely unexplored biological treasures in the biosphere. The total number of phages estimated in the earth was approximately around $10^{30}$ to $10^{32}$. Phages are found even in the most extreme environments on the earth along with their host bacterium. (Kutter and Sulakvelidze, 2004). Breitbart et al., (2002) estimated around 400 to 7000 types of phages present in 100 litres of marine waters. He also suggested that the human gut
harours hundreds of different types of phages in it. The phage host interaction
determines the phage ecology.

**Life cycle of Phages**

Generally phages can be widely classified into two categories based on their
lifestyle - Virulent or temperate. Virulent phages carry out a lytic life cycle, multiply
at the cost of the death of the host. The phage adsorbs to the surface of the host cell
membrane and injects its genetic material into the host cells. The injected genetic
material hijacks the host metabolic machinery for making more phages. Within
minutes after injection, the host cells get lysed releasing many new phage virions. The
events taking place during a lytic infection can be explained by the experiment called
One step growth curve. The method was developed by Max Delbrick and Emory
Eillis in 1939.

**Lytic Cycle**

The lytic cycle can be divided into phases - latent period which occurs
immediately following the addition of the phages or initial phase of the infection
process. In these phases, there will be no release of the phages. The initial segment of
the latent period is called as the eclipse period as the virions are detectable before
infection but are now eclipsed. This phase will be followed by a rise period and burst
where the host cell rapidly get lysed and release the infant phages (Prescott *et al.*, 2002). The lytic cycle of a phage can be explained in detail based on the various
processes happening during the infection.

**Adsorption to the host cell and penetration**

The phages attaches randomly to the surface of the host cells using the
specific surface receptors. The nature of the receptors varies among phages. The host
specificity of the phages is highly dependent on the receptors. For example, the T-
even phage uses its tail fibre to make contact with the host cells. The base plate settles down on the surface. Conformational changes occurs between the base plates and the sheath. The tail cylinder shortens and the central tube is pushed into the cell wall of the host. The DNA is extruded from the head through the tail tube and into the host cells (Prescott et al., 2002).

**Synthesis of Phage Nucleic Acids and Protein**

The injected DNA halts the synthesis of host DNA, RNA and protein. The host RNA polymerase starts to synthesise phage mRNA which is called as early mRNA. This early mRNA codes for proteins and enzymes required to hijack the host system. Some early virus specific enzymes degrades the host DNA to nucleotides, thereby simultaneously halting host gene expression and providing raw material for virus DNA synthesis. The expressions of the gene of the phages are tightly regulated as the genes are arranged precisely. Genes with related function are clustered together. T4 DNA shows terminal redundancy, the long repeated copies of the phage genome are called as concatemers. During assembly, the concatemers gets cleaved (Prescott et al., 2002).

**Assembly of the Phage Particles**

The assembly of the T4 phage is a complex process. The late mRNA directs the synthesis of the phage structural protein, phage assembly, helping protein which does not participate in the structure of the virion and proteins involved in cell lysis. The structural protein required for the prophages will be synthesized. The base plate will be constructed using 15 gene product, later the tail will be formed on it with the sheath assembled. The prohead will be constructed separately and gets assembled with the tail. The replicated DNA will be packed in the procapsid. The tail fibers attach to it finally completing the assembly of the prophages (Prescott et al., 2002).
Release of Phage Particles

There are several T4 genes involved in the lysis of the host bacterium cells to release the prophages. Two major proteins Endolysin and Holin play the crucial role in this process. The endolysin break open the cell wall peptidoglycan whereas the holin makes lesions on plasma membrane. This process stops the respiration of the cells and facilitates endolysin to act on the cell wall which break up the cell wall and release the phages (Prescott et al., 2002).

Lysogeny

In temperate phages, the injected genetic material gets integrated into the phage chromosomes and become quiescent which is referred to as prophages. The viral genome does not take control of its host system, but replicates along with the host and copies itself to be transferred to the next generation. In rare cases, the lysogenic phage genetic material can be maintained as plasmids in the host cells. The lysogenic phages can produce phages and lyse the host under suitable environmental conditions. The process of conversion of a lysogenic phage into lytic is referred to as Induction (Prescott et al., 2002).

Evolution of Bacteriophages

It is estimated that there are greater than $10^{30}$ bacteriophages on the earth, making them as the most abundant and highly diverse biological entity (Ashelford et al., 2003). The environment permits the phages to replicate rapidly as they are highly specific to the host bacterium (Brabban et al., 2005). There are typically $10^7$ tailed phage particles per millimeter in coastal sea water (Wommack and Colwell, 2000). The phages are five to ten fold excess when compared to the total number of bacterium present in the biosphere. Arguably tailed phages are considered to be ancient. The phages are believed to be evolved even before the divergence of three
forms of the life. Phages evolve rapidly and constantly by recombination to exchange DNA. Apart from tailed phages, different types of phages exist in the nature. The life style, structure and genetic material vary from each other. Even though the phages vary in their physiological and biochemical properties and function, there remains evidence that some of these phage groups share common ancestry with other phages, and eukaryotic virus. The similarities between the phages/virus suggest that they should have shared a common ancestor. These properties of similarities can be explained by the hypothesis that the phages/ virus ancestor should acquire these properties when they infect organism as their host prior to the divergence of the three forms of life. Another hypothesis explains that each group of the virus should have evolved independently in each domain of the life which might have evolved at the subsequent time (Hendrix, 2002).

**Application of Phages**

The emergence of the multidrug resistance pathogens, killing five million people annually emphasis the need of an alternative to antibiotics (Inal, 2003). The application of the phages to treat infectious diseases was first taken up during 1920. *Vibrio cholera* infection was the first disease treated using bacteriophages. The phage therapy to demonstrate the possibility of using phages to cure infectious diseases was widely studied using animal models. *E. coli* systemic infection in rats, calves and lambs were successfully eradicated using the phages (Smith and Huggins, 1982, 1983, Smith *et al.*, 1987b). Similarly the diarrhoeal disease caused by *S. typhimurium* was treated successfully in chicks using Phages (Berchieri *et al.*, 1991). *Pseudomonas* infection in the skin grafts in burned rabbits was prevented using Phages. *E. coli* phage isolated from the sewage was used to treat chicken septicemia and meningitis like infection in chicken. This phage attaches to the K1 capsular antigen and are able
to multiply in the blood (Barrow et al., 1998a). To treat *Helicobacter pylori*, a recombinant filamentous phage was prepared and the pretreated *H. pylori* were orally administered to mice which reduced the infection significantly (Cao et al., 2000). Thirty one patients were treated for suppurative skin infection caused by *E. coli, Klebsiella, Proteus, Pseudomonas* and *Staphylococcus* of which 23 cases showed improvement (Cislo et al., 1987).
Outbreaks of Multidrug resistant bacterial pathogens impose great threats in treating infectious diseases. The phages which have a heritage in treating bacterial diseases now emerge as a frontrunner among the alternative medicines available to treat MDR pathogens. The current research work was designed to identify a better phage candidate to treat Salmonellosis among the diversity of *Salmonella* phages available across the different geographical regions of the State, Tamil Nadu. The research focuses on the following objectives:

- To collect sewage samples across different geographical regions of Tamil Nadu
- To isolate *Salmonella* specific phages from the collected sewage samples
- To characterize the physico chemical makeup of the isolated phages
- To check the stability of the phages under different physico-chemical environments
- To record the interactions of the phages with the human gastric environment
- To explore the possible ways to increase the survival capacity of the phages in the stomach acidic environment
- To study the toxicity exhibited by the phage in *in-vivo* conditions
- To explore any other possible applications of the isolated phages