INTRODUCTION
**Vibrio cholerae**

Bacteria that colonize the intestine, invade intestinal epithelial cells, and produce one or more toxins are important etiologies of diarrheal disease in both industrialized and developing countries.

Diarrheal diseases, including cholera, are the leading cause of morbidity and the second most common cause of death among children under 5 years of age globally. Cholera is characterized by a severe watery diarrhea caused by toxigenic *Vibrio cholerae*, which colonizes the small intestine and produces an enterotoxin, cholera toxin. Cholera is a severe diarrheal disease caused by the bacterium *Vibrio cholerae* [Bhowmick *et al.*, 2009]. Cholera is a clinical-epidemiologic syndrome caused by *Vibrio cholerae*, usually of serogroup O1. In its severe form, cholera gravis, the clinical disease is characterized by the passage of voluminous stools of rice water character that rapidly lead to dehydration hypovolemic shock, acidosis, and death can result in adults, as well as in children, if prompt and appropriate treatment is not initiated. The distinctive epidemiologic features of cholera are its tendency to appear in explosive outbreaks, often starting in several distinct foci simultaneously, and its propensity to cause true pandemics that progressively affect many countries in multiple continents over the course of many years.

Serogroups other than O1 and O139 are entitled as *Vibrio cholerae* non-O1, non-O139, or nonagglutinating vibrios (NAGs); such serogroups have more than 200 somatic (O) antigens [Yamai *et al.*, 1997] and typically lack CT- and TCP-coding.
genes. Toxigenic and nontoxigenic NAGs have caused numerous diarrhea outbreaks in India and other countries, including Haiti [Hasan et al., 2012, Onifade et al., 2011]. In non–CT-producing NAGs, other virulence factors such as heat-stable enterotoxin (Stn), hemolysin (HlyA), repeat in toxin (RTX), and type 3 secretion systems (TTSS) have major roles in causing infections [Chatterjee et al., 2009].

Cholera continues to be a re-emerging and devastating disease in the field of diarrhoeal diseases. According to World Health Organization (WHO) in the year 2013 total 47 countries from all over the world stated cholera cases. A total of 1, 29,064 cases including 2102 deaths were reported in 2012. In 2013, India has reported 6008 cases including 56 deaths [WER et al., 2014]. In this context it can be mentioned that recent shocking epidemic situations in Haiti (2010), The Democratic Republic of Congo (DRC) (2011) and Sierra Leone (2012) reveals again the deadly nature of Vibrio cholerae.

**History of cholera**

Cholera likely has its origins in the Indian subcontinent being prevalent in the Ganges delta since ancient times [Sack DA et al 2004]. John Snow was the first to identify the importance of contaminated water in spreading cholera disease. Cholera brought to daylight by Filippo Pacini, in 1854 during the Asiatic Cholera Pandemic of 1846-63. A histological examination of the intestinal mucosa of cholera patients led to the discovery of cholera.

Cholera has been seen as endemic in southern Asia and parts of Africa and Latin America, where seasonal outbreaks occur widely and are particularly associated with poverty and poor sanitation. It journeys along the great paths of human intercourse, never going faster than people travel, and generally much more slowly. The disease first spread by trade routes (land and sea) to Russia in 1817, then to Western Europe, and from Europe to North America. Seven times deadliest effects of cholerae has been repeated since 1816. Till now recently an outbreak in Nov 2010 in Haiti is the indication of its fatality.

Every year almost 3-5 million cholera cases have been reported throughout the world with a severe fatality rate of 2-3 % [www.who.int/mediacentre/factsheets/fs107/en/index.html]. Vibrio cholerae has been isolated
habitually from many aquatic milieus throughout the world, often in association with plankton, plants, invertebrates, and fish, and there are some reports of its occurrence in water birds, seals, and diseased farm animals [Alexander et al., 1998, Islam et., 1990, Islam et al., 1993, Kaysner et al., 1987, Lipp et al., 2003, Reddacliff et al., 1993, Visser et al., 1999]. *Vibrio cholerae* was first isolated as the cause of cholera by Italian anatomist Filippo Pacini [Bentivoglio et al., 1995].

**Vibrio cholerae as a causative agent**

*Vibrio cholerae* O1 serogroup is prevalent in most of the cases of pandemic cholera. The first six recorded cholera pandemics occurred between 1817 and 1923 and were caused by *Vibrio cholerae* O1 serogroup strains of the classical biotype [Michelle et al., 2001]. In 1961 El'Tor biotype emerged to cause the 7th pandemic, eventually resulting in the global elimination of classical biotype strains as a cause of disease. This replacement raises the question about the evolution of *Vibrio cholerae* as a human pathogen and environmental organism. During the period of 1817 to 1961 six pandemics were recorded in the history of cholera. *Vibrio cholerae* El Tor biotype O1 is the sole responsible agent for the seventh pandemic and current pandemics which started in 1961. *Vibrio cholerae* is differentiated serologically on the basis of the O antigen of its lipopolysaccharide (LPS) The O1 serogroup survives as two biotypes, classical and El Tor; antigenic determinants allow further differentiation into two major serotypes Ogawa and Inaba. A third serotype was found to be Hikojima which is very rare and unstable in nature. Until late in 1992 it was believed that only O1 strain had epidemic potential, with isolation of non-O1 strains being restricted to sporadic cases. Late in 1992, large outbreaks of cholera-like disease occurred in southern and eastern India and southern Bangladesh [Albert et al., 1993] with subsequent spread into other parts of Asia. The causative agent was a toxigenic strain of the previously unidentified serovar O139. A distinctive epidemiological feature of cholera is its appearance in a regular seasonal pattern in areas of endemic infection and in explosive outbreaks often starting in several distinct foci simultaneously indicating a possible role of environmental factors in triggering the epidemic process [Glass et al., 1982].
Vibrio cholerae strain was appeared in southern India through unprecedented genesis, presently known as Vibrio cholerae O139 [Ramamurthy et al., 1993]. A switchover to O139 serogroup was well-defined as a result of a multigene substitution in the O antigen-coding region of a progenitor O1 El Tor strain [Waldor et al., 1994]. Although, Vibrio cholerae belonging to serogroup O1 and O139 are sole responsible for epidemic and pandemic situation, acute diarrhoea caused by Vibrio cholerae non-O1 and non-O139 serogroups such as O10 and O12 have been reported earlier [Dalsgaard et al., 1995, Rudra et al., 1996].

**Ecology of Vibrio cholerae**

Vibrio cholerae has been regarded as a member of a group of organisms whose major habitats are aquatic ecosystems [Colwell RR et al., 1992]. Although, Vibrio cholerae is part of the normal, free-living bacterial flora in riverine and estuarine areas, non-O1
and non-O139 strains are more commonly isolated from the environment than are O1 and O139 strains. Moreover, outside of areas of epidemic infection and away from areas that may have been contaminated by cholera patients, environmental isolates of *Vibrio cholerae* O1 have been found to be mostly CT negative [Nair et al., 1988]. It seems logical that the natural habitat for toxigenic *V. cholerae* O1 and O139, which in most cases produce various factors necessary for colonization of the mammalian intestine, is likely to be the gastrointestinal tract. In view of the available information on the epidemiology of cholera, the lysogenic conversion by a bacteriophage encoding CT, and the survival and enrichment of *Vibrio cholerae* under in vivo and in vitro conditions, it is apparent that the ecosystem for *Vibrio cholerae* should have a number of components [Sarkar et al., 1993]. These include the bacterium, the aquatic environment, CTXØ and other unidentified genetic elements involved in the transfer of virulence genes, and the intestinal environment of the host population [Sarkar et al., 1999]. It has been postulated that under stress conditions the vibrios are converted to a viable but nonculturable (VBNC) form that cannot be recovered by standard culture techniques and that such VBNC forms are able to cause infection and can revert to the culturable form [Colwell RR et al., 1992].

**Epidemiology**

Cholera has been categorized as one of the reemerging infections diseases [Franco et al., 1994] threatening many developing countries. It is generally accepted that seven distinct pandemics of cholera have occurred since the onset of the first pandemic in 1817 [Pollitzer et al., 1959]. Except for the seventh pandemic, which originated on the island of Sulawesi in Indonesia [Kamal et al., 1974], the pandemics arose in the Indian subcontinent, usually the Ganges delta, and spread to other continents, affecting many countries and extending over many years [Chambers et al., 1938, Laval et al., 1989]. The second pandemic of cholera reached the British Isles in the early 1830s, and fundamental epidemiological observations by John Snow on the waterborne transmission of cholera were made in London between 1847 and 1854 during the late second and the third pandemics [Snow et al., 1855]. During the third pandemic (1852 to 1859), cholera was rampant in the United States, and toward the end of the fourth pandemic cities and towns along the Mississippi, Missouri, and Ohio rivers experienced cholera [Billings et al., 1975]. The fifth
pandemic extensively affected South America; it caused large epidemics in many countries and was characterized by high mortality in Argentina, Chile, and Peru [Laval et al., 1989]. The sixth pandemic (1899 to 1923) extensively involved populations in the near and middle East and the Balkan Peninsula. Cholera remained virtually confined to south and Southeast Asia from the mid-1920s until the onset of the seventh pandemic in 1961. The sixth pandemic and presumably the fifth pandemic were caused by *Vibrio cholerae* of the classical biotype. The seventh pandemic is the most extensive of the pandemics in geographic spread and in duration, and the causative agent is *Vibrio cholerae* O1 of the El Tor biotype. The pandemic, which began in 1961 on the island of Sulawesi in Indonesia, spread to other islands, including Java, Sarawak, and Borneo, and then to the Philippines, Sabah, and Taiwan, thereby affecting the entire Southeast Asian archipelago by the end of 1962. During 1963 to 1969, the pandemic spread to the Asian mainland and affected Malaysia, Thailand, Burma, Cambodia, Vietnam, India, Bangladesh, and Pakistan. Soon after El Tor cholera reached Pakistan, outbreaks were reported in Afghanistan, Iran, Iraq, and nearby republics within the Soviet Union. By 1970, El Tor cholera had invaded the Arabian Peninsula, Syria, and Jordan, and a limited outbreak occurred in Israel [Cohen et al., 1971]. The seventh pandemic reached sub-Saharan West Africa in the early 1970s and caused explosive outbreaks, resulting in high mortality rate, mainly due to a lack of background immunity in the population and inadequacies in the health care infrastructure [Goodgame et al., 1975].

In late 1992, epidemic cholera was reported in Madras and other places in India and in Southern Bangladesh [Ramamurthy et al., 1993]. Although the clinical syndrome was typical of cholera, the causative agent was a *Vibrio cholerae* non-O1 strain, which was later sero grouped as O139 [Shimada et al., 1994]. The epidemic continued through 1993, and *Vibrio cholerae* O139 spread throughout Bangladesh and India and neighboring countries. Outbreaks or cases due to *Vibrio cholerae* O139 have since been reported in Pakistan, Nepal, China, Thailand, Kazakhstan, Afghanistan, and Malaysia [Chongsa-nguan et al., 1993, Swerdlow et al., 1993].

**Molecular Epidemiology**

Several new typing methods paves the way for studies of the epidemiology of *Vibrio cholerae* on a larger global perspective [Cook et al., 1983, Wachsmuth et al., 1991,
Chen et al., 1991]. These techniques include the analysis of restriction fragment length polymorphisms (RFLPs) in different genes. The use of gene probes to study RFLPs in the ctxAB genes and their flanking DNA sequences, which are part of a larger genetic element (CTX element), indicated that the U.S. Gulf Coast isolates of toxigenic Vibrio cholerae are clonal and that they are different from other seventh-pandemic isolates [Kaper et al., 1982]. RFLPs in conserved rRNA genes have also been used to differentiate Vibrio cholerae strains into different ribotypes. Analysis of toxigenic El Tor strains by multilocus enzyme electrophoresis has also been used to group the El Tor strains into major clonal groups. The clones seem to reflect broad geographical and epidemiological associations. Comparative analysis of the El Tor strains of Vibrio cholerae O1 and the epidemic O139 strains suggested that the O139 strains are related to El Tor strains and were derived from them by possible genetic changes in the serotype-specific gene clusters [Wachsmuth et al., 1994]. Numerical analysis of ribotype patterns [Faruque et al., 1995] has also revealed that Vibrio cholerae strains belonging to the non-O1 non-O139 serogroups diverge widely from the O1 and O139 Vibrio cholerae strains. Different ribotypes often showed different CTX genotypes resulting from differences in the copy number of the CTX element and variations in the integration site of the CTX element in the chromosome [Faruque et al., 1997]. These studies indicated that there had been a continual emergence of new clones of toxigenic Vibrio cholerae which replaced existing clones, possibly through natural selection involving unidentified environmental factors and immunity of the host population.

**Cholera Toxin and pathogenesis**

The existence of a toxin responsible for the symptoms of cholera was first proposed by Robert Koch. Vibrio cholerae produces a variety of extracellular products that have deleterious effects on eukaryotic cells. The massive, dehydrating diarrhea characteristic of cholera is induced by cholera enterotoxin, also referred to as CT or choleragen. In 1959, S. N. De and N.K. Dutta demonstrated that an outpouring of fluid resulted when culture filtrates or lysates of Vibrio cholerae were introduced into the rabbit intestinal tract [De et al., 1959; Dutta et al., 1959].
Fig 3: Cholera pathway, showing the specific proteins involved. A1 subunit shown binding with the five Bs.
The genes for CT, the most important virulence factor of *Vibrio cholerae*, have long been thought to be encoded in the chromosome of the bacterium. In 1996, Waldor and Mekalanos reported that these genes are actually encoded in the genome of a newly identified bacteriophage, CTXØ, and that the phage genome is integrated into the bacterial chromosome as a prophage [Thompson et al., 2004].

Purification of the toxin was done by Finkelstein and LoSpalluto. CT consists of five identical B subunits and a single A subunit, and neither of the subunits individually has significant secretogenic activity in animal or intact cell systems.

The receptor for CT is the ganglioside GM1. The interaction between CT and the receptor occurs via the B subunit. The mature A subunit is proteolytically cleaved to yield two polypeptide chains A1 and A2. Potential models of activation include (i) A1 translocation through the membrane with diffusion through the cytoplasm to the adenylate cyclase; (ii) A1 modification of brush border membrane Gs, which then diffuses to the basolateral membrane to activate adenylate cyclase; and (iii) endocytosis of CT with delivery of active A1 in the endosomal membrane to the basolateral adenylate cyclase. A1 subunit of toxin A enters the cytosol and activates the Gs protein which leads to increased adenylate cyclase activity through transferring ADP-ribose moiety of NAD to a specific arginine residue in the Gsa protein, resulting in the activation of adenylate cyclase and subsequent increases in intracellular levels of cAMP. cAMP activates a cAMP-dependent protein kinase, leading to protein phosphorylation, alteration of ion transport, and ultimately to diarrhea [Kaper et al. 1995].

**Diversity in *Vibrio cholerae* strains depending upon cholera toxin**

*Vibrio cholerae* typically contains a prophage that carries the genes encoding the cholera toxin. In recent years, new pathogenic variants of *Vibrio cholerae* have emerged and spread throughout many Asian and African countries. This variants show mixture of both classical and El Tor strains. These variants may be called as atypical El Tor strains. These variants are typed as a) Matlab variants, b) Mozambique variants, c) Altered El tor variants and d) hybrid El Tor variants.

a) The Matlab variants are naturally occurring El Tor variants isolated between 1991 and 1994 in Matlab [Nair et al., 2002] which can not be typed as either classical or El
Tor. MT variants harboured \( \text{rstR}^\text{cla} \) and several characteristics of \( \text{CTX}\phi^\text{Cla} \). Based on the phenotypic and genotypic variations Matlab strains further divided into three types [Safa et al., 2006].

b) The Mozambique variants were reported in 2004 from the country Mozambique, in East Africa. The \textit{Vibrio cholerae} strains of these variant showed phenotypic characteristics of the El tor biotype. However, the \( \text{ctxB} \) and \( \text{rstR} \) genotyping explored the nature of classical biotype in this El Tor strains. These variants contain two tandem copies of the prophage whose sequence was almost identical to that of the typical \( \text{CTX}\phi^\text{Cla} \) [Das et al., 2007; Lee et al., 2006].

C) Altered El Tor variant isolated before 2001 in Bangladesh carried the alleles of seventh pandemic and Latin America epidemic strains and producing CT of the typical El Tor biotype. Remarkably, \( \text{CTX}\phi \) isolated from atypical El Tor strains isolated from Vietnam in 2007 – 2008 contains both \( \text{ctxB} \) allele from classical biotype worldwide and US Gulf coast El Tor strains with a combination of \( \text{rstR}^\text{El} \) [Nguyen et al., 2009].

d) Hybrid El Tor strains isolated between 1991 and 2004 from Asia and Africa, other than India. Investigation of this strains revealed that several of this strains carry both \( \text{rstR}^\text{Cla} \) and \( \text{rstR}^\text{El} \), indicating the presence of two different copies of \( \text{CTX}\phi \), either as a tandem array or located on different chromosomes [Safa et al., 2008].

**Phenotypic characterization of \textit{Vibrio cholerae} strains**

**Serotyping**

The O1 serogroup is alienated into three antigenic forms called Inaba, Ogawa, and Hikojima. The O antigen of \textit{Vibrio cholerae} O1 comprises of three factors designated A, B, and C; The differences among the subtypes is largely quantitative; Ogawa strains produce the A and B antigens and a small amount of C, while Inaba strains produce only the A and C antigens [Sakazaki et al., 1992]. Specific Inaba and Ogawa sera are prepared by absorption with the other subtype. The Hikojima subtype contains all three factors, thereby reacting with both Inaba and Ogawa antisera. The Hikojima subtype is rare and unsteady and is not familiar by some authorities who
would report cultures as Inaba or Ogawa, depending on which serum causes the strongest reaction [Kelly et al., 1991]. *Vibrio cholerae* O1 strains have been revealed to swing between Inaba and Ogawa [Sack et al., 1969]. This interconversion is usually irreversible in the laboratory and occurs more frequently in the direction of Ogawa to Inaba [Manning et al., 1994].

**Biotyping**

*Vibrio cholerae* O1 strains have been divided into two biotypes, classical and El Tor. Isolates from the sixth pandemic were solely of the classical biotype. Until the seventh pandemic, isolates of the El Tor biotype (named after the El Tor quarantine station in the Sinai, where it was first isolated in 1905) were associated only with sporadic diarrhea [Pollitzer et al., 1959]. However, the mainstream isolates of the seventh pandemic have been of the El Tor biotype.

It was found that, except for isolates from the early 1960s, most isolates of *Vibrio cholerae* El Tor are nonhemolytic [Bagchi et al., 1993]. Notable exemptions to the recent trend of nonhemolytic El Tor strains are the U.S. Gulf Coast isolates, which are strongly hemolytic [Bagchi et al., 1993]. Amongst additional standards, biotyping by bacteriophage susceptibility is restricted to a very rare reference centers, leaving the Voges-Proskauer reaction, polymyxin B susceptibility, and hemagglutination of chicken erythrocytes as the most beneficial eccentricities for biotyping.

In case of molecular biology approaches generally utilizes an oligonucleotide probe derived from sequences within the gene encoding the haemolysin to differentiate between the biotypes [Alm et al., 1990]. A second approach to biotyping has recently been described; it utilizes differences in DNA sequence among genes encoding the TCP from classical and El Tor strains [Keasler et al., 1993]. By using multiplex PCR with primers derived from the tcpA gene sequence, classical strains gave amplicons of different sizes from those seen with El Tor isolates. In the next level investigation of restriction fragment length polymorphisms (RFLPs) in rRNA genes has also been reported to differentiate classical and El Tor strains [Koblavi et al., 1990, Popovic et al., 1993].
Phage typing

The specificity of phages for bacterial cells empowers them to be used for the typing of bacterial strains and the recognition of pathogenic bacteria. Phage typing is also known as the use of sensitivity patterns to specific phages for accurately classifying the microbial strains. Among different typing techniques phage typing is one of the important typing tools for bacterial identification. The advantage of this technique is the high specificity towards the bacterial community. A particular phage or group of phages will adsorb to specific site and different phages will adsorb to different sites. Different research studies on phages have reported diverse morphology of cholera phages [Gratia et al., 1922; Asheshov et al., 1924; Burnet et al., 1933; Asheshov et al., 1933]. In the year 1957 Mukerjee et al. first identified four groups of vibriophages against *Vibrio cholerae* classical O1 El Tor strains by their lytic pattern. On the basis of sensitivity phage sensitivity patterns, five different phage types were found for *Vibrio cholerae* O1 classical biotype strains. *Vibrio cholerae* group IV typing phage φ149 is lytic for all classical strains, but all El tor strains are resistant to it. This observation led to the development phage typing schemes to differentiate classical and El Tor biotypes of *Vibrio cholerae* [Mukerjee et al., 1957; Mukerjee 1963a]. Bacteriophage typing scheme has been successfully used to distinguish the classical and El Tor biotypes; the typing phage of Mukerjee [Mukerjee et al., 1963] and the FK phage of Takeya et al. [Takeya et al., 1981] have been used primarily for this purpose. Later, an internationally accepted phage typing scheme was formulated by Basu and Mukerjee in 1968, includes five phages (I, II, III, IV, and V) by which *Vibrio cholerae* O1 biotype El Tor strains can be differentiated into six different phage types [Basu and Mukerjee et al., 1968].

As a matter of fact it has been observed that most of the *Vibrio cholerae* O1 El Tor strains slipped to phage type 2 or 4 and most of the *Vibrio cholerae* strains left untyped. The multiple typing schemes are limited by the fact that two or three phage types account for up to 80% of all *Vibrio cholerae* O1 strains examined [Rowe et al., 1992]. This increasing numbers of untypable strains restrict the older one and leads the way to cultivate a new phage typing scheme for *Vibrio cholerae* O1 biotype El Tor strains. In a response to the strong need a highly efficient typing scheme was reported.
This scheme comprises of 10 typing phage. This new scheme separated 1,000 *Vibrio cholerae* strains into 146 phage types. In 1992, a new and highly pathogenic serogroup of *Vibrio cholerae* was reported known as O139 [Ramamurthy *et al.*, 1993] was reported. The newly evolved *Vibrio cholerae* serotype was later reported from different parts of India as well as Nepal, Bangladesh, Burma, Thailand, Malaysia, Saudi Arabia, Pakistan and China. In 1996 this virulent serotype of *Vibrio cholerae* strikes again which defines its presence in nature was not a onetime event. This finding also disclose about the potential nature of the serotype to sustain its life in nature. So, to identify the strains properly a new architecture of phage typing scheme was developed for *Vibrio cholerae* O139 [Chakrabarti *et al.*, 2000]. These typing schemes are widely accepted throughout the world.

One remarkable application of bacteriophage typing is the use of lysogenic phage as DNA hybridization probes to discriminate strains on the basis of chromosomal integration locates. This technique was used to endorse other studies displaying that the U.S. Gulf Coast isolates are clonal and also exhibited that some nontoxigenic strains from the Gulf Coast are correlated to the toxigenic strains isolated from patients in this area [Goldberg *et al.*, 1983]. The susceptibility of O1 strains to two bacteriophages known as phage IV and phage V is investigated by this test. Classical *Vibrio cholerae* strains are sensitive to phage IV and resistant to phage V. Inversely, typical El Tor strains are typically resistant to the former and sensitive to the later. Besides, O1 strains either sensitive or resistant to both the phages have been reported [Safa *et al.*, 2008].

The sensitivity of the detection would be increased if the phages bound to bacteria are detected by specific antibodies [Watson *et al.*, 1965]. For the recognition of unknown bacterial strain its lawn is provided with different phages, and if the plaque (clear zones) appears then it means that the phage has grown and lysed the bacterial cell, making it easy to identify the definite bacterial strain. There are certain other methods which can be engaged to detect pathogenic bacteria such as the use of phages that can transport reporter genes (e.g. lux) specifically [Kodikara *et al.*, 1991] or using green fluorescent protein, [Funatsu *et al.*, 2002] that would express after infection of bacteria. Dual phage technology is another method of phages in discovery of bacteria,
in which phages are used to detect the binding of antibodies to specific antigens [Sulakvelidze et al., 2005]. Phage amplification assay can also be used to detect pathogenic bacteria [Stewart et al., 1989]. The technique has most extensively been used for the detection of *Mycobacterium tuberculosis*, *E. coli*, *Pseudomonas*, *Salmonella*, *Listeria*, and *Campylobacter* species [Bary et al., 1996].

**Drug resistance**

In recent years, emergence of multidrug-resistance *Vibrio cholerae* is a serious clinical problem in the treatment and containment of the disease cholera. Resistance to traditional antibiotic therapy is most apparent in bacterial diarrheal diseases, respiratory tract infections, and hospital-acquired infections; important examples include *Streptococcus pneumoniae*, vancomycin-resistant *Enterococci*, multidrug resistant *Salmonella*. These deadly pathogens often fail to answer the conventional treatment [Goldstein et al., 1999; Shetty et al., 2000, Byers et al., 2001; Gold et al., 2001; Lepage et al., 1984; Escribano et al., 2006 Cebrian et al., 2006].

All the classical *Vibrio cholerae* strains isolated in the middle of 1988 and 1989 Bangladesh were resistant to tetracycline, whereas strains belonging to the El Tor biotype were sensitive to the drug. Almost after a epoch, tetracycline-resistant El Tor strains appeared in Bangladesh in 1991 and in Tehran in [Pourshafic et al., 2000]. In 1995, emergences of *Vibrio cholerae* O1 El Tor strains resistant to nalidixic acid were reported in Southern India.

On the other hand, all the O1 isolates from Tanzania and Rwanda were resistant to tetracycline, which is a drug of choice against cholera [Urassa et al., 2000]. Several strains of *Vibrio cholerae* O1 strains isolated between 1992 and 1997, and in 2011 in Kolkata exhibited resistance to tetracycline, as well as ampicillin, chloramphenicol, co-trimoxazole, neomycin, streptomycin, and emerging resistance to nalidixic acid [Bhattacharya et al., 2011; Mitra et al., 1996]. Antibiotic susceptibility tests of *Vibrio cholerae* O1 strains isolated from Trivandrum Alleppey, and, Kottayam, in 2000 and from East Delhi amongst 2004 and 2006 showed resistance to nalidixic acid, and/ or neomycin and/or streptomycin, respectively, and sensitivity to tetracycline suggested existence of different R-types of *Vibrio cholerae* strains in different locations [Sabeena et al 2001; Das et al., 2008].
After the extensive cholera outbreaks caused by \textit{Vibrio cholerae} O139 since 1992, \textit{Vibrio cholerae} O1 El Tor strains reemerged in 1994 as the predominant cause of cholera in the Indian continent. Surprisingly, El Tor strains isolated before the O139 outbreak, the re-emerged El Tor strains, such as the initial O139 isolates, were resistant to trimethoprim furazolidone, chloramphenicol, sulfamethoxazole, and streptomycin [Yamamoto \textit{et al.}, 1994]. The analogous genes were located in an Integrative Conjugative Element. The SXT is a 62 kb conjugative, self-transmissible integrating element encoding resistance to sulfamethoxazole, trimethoprim, chloramphenicol and streptomycin [Walder \textit{et al.}, 1996]. This element was first identified in the freshly emerged O139 serogroup of \textit{Vibrio cholerae} in 1992 [Walder \textit{et al.}, 1996]. Although variations were prominent in recent \textit{Vibrio cholerae} O139 isolates from India, which contain SXT but validated variable resistance to streptomycin but sensitivity to sulfamethoxazole and trimethoprim [Sinha \textit{et al.}, 2002; Yam \textit{et al} 1994].

The universal spread of multidrug resistant bacteria and the lack of new antibiotics are now leading to a global restoration of interest in phage therapy [Taubes \textit{et al.}, 2008]. A recent outbreak in South India reported about multidrug resistant \textit{Vibrio cholerae} strains [Balaji \textit{et al.}, 2013; Roy \textit{et al.}, 2012]. \textit{Vibrio cholerae} has been found to be resistant to one of the most favoured drug of choice that is Tetracyclin; it is also evident that few strains of \textit{Vibrio cholerae} are resistant to even third generation antibiotic, Cephalosporin [Ghosh \textit{et al.}, 2011]. However, multidrugs resistant \textit{Vibrio cholerae} strains are reported from all over the world [Quilici, \textit{et al.}, 2010; Dalsgaard \textit{et al.}, 1999; Tran \textit{et al.}, 2012]. Acquisition of antibiotic resistance genes across genera and species is mediated through horizontal and lateral gene transfer. Plasmids, conjugative transposons, and integrons are all vehicles for the acquisition of resistance genes [Hall \textit{et al.}, 1993; Hall \textit{et al.}, 1995; Recchia \textit{et al.}, 1995; Sundstrom \textit{et al.}, 1998; Waldor \textit{et al.}, 1996].

**Role in mobile genetic elements in drug resistance**

The mobile genetic elements such as transposons, conjugative plasmids, integrons and ICEs harbouring drug-resistance genes have been revealed to be mostly responsible for the fluid nature of drug resistance genes in bacteria including \textit{Vibrio cholerae} O1 and O139 serogroups [Amrita \textit{et al.}, 2003; Burrus \textit{et al.}, 2006]. Integrons are gene-
capturing systems incorporating exogenous open reading frames by site-specific recombination. This is facilitated by a tyrosine recombinase, converting the open reading frames to functional genes by confirming their correct expression [Mazel et al 2006]. The employment of exogenous genes is the most effective means by which bacterial species can endure various environmental challenges, including exposure to antimicrobial compounds. Later, superintegrons, and numerous gene cassettes concomitant with integrons in the genomes of environmental bacterial species define the importance of these elements clearly in flourishing antibiotic resistance.

The first ICE detected in Vibrio cholerae O139 strains was the SXT element [Waldor et al., 2006]. The SXT carried by Vibrio cholerae O1 strains (designated ICEVchInd1) varied from those of Vibrio cholerae O139, which possess a trimethoprim resistance determinant distinct from other antibiotic resistance genes [Burrus et al., 2006]. Afterwards, SXT has been perceived with or without drug resistance genes among Vibrio cholerae isolated from different parts of the world like Mexico, Laos, Vietnam, Mozambique and South Africa [2]. SXT elements display substantial variation with respect to drug resistance genes, suggesting genetic rearrangement in these elements. Vibrio cholerae isolated before the emergence of the O139 serogroup rarely possessed SXT, indicating that SXT may have not been an integral part of the Vibrio cholerae genome.

Why bacteriophage is the answer of drug resistance?

There are several reasons which explain why bacteriophage is the prime choice against multi drug resistant bacteria. Here, are some points which are foremost reasons

There are numerous differences between bacteriophages and antibiotics. First, phages are the most abundant living individuals on the planet and are natural enemies of bacteria; nevertheless, they also reproduce naturally and, consequently, are ‘ecologically pure’. Most antibiotics are synthetic or semi-synthetic in their nature. Second, the host range of phages is much narrower than that of antibiotics: most phages are definite to one bacterial species and many are only able to lyse a few specific strains within a species. The advantage of a narrow range is that the phage does not disturb normal body microflora, whereas broad-spectrum antibiotics destroy
all bacterial cells autonomously of whether they are pathogenic. Third, widespread application of antibiotics might distress the human organism itself; most presently used wide-spectrum antibiotics often have unadorned side effects. By contrast, no side effects of phage therapy have been described regardless of decades-long use for human therapy [Chanishvili, et al., 2009; Slopek et al., 1983; Slopek et al., 1984]. Lastly, the ability to proliferate in the occurrence of host bacterial cells makes phages self-regulating tools. The absorption of an antibiotic introduced into the human organism declines with time (natural drug clearance from body), whereas phages endure to grow, decreasing as soon as bacterial cells are eradicated.

**Bacteriophages**

Bacteriophages are the most numerous life forms on earth. The discovery of bacteriophages in the early 20th century has been endorsed to Twort and d’Herelle [Soothill et al., 1992]. Bacteriophages are very common in the gastrointestinal tract and, composed with their bacterial hosts, are an important component of gut flora [Merril et al., 1974; Ashelford et al., 2000; Gorski et al., 2003].

![Diagrammatic representation of a bacteriophage.](image)

Bacteriophages are bacterial viruses that play a intense role in the evolution of their host. Whole genome sequencing of bacteria has discovered that phages contribute significantly to sequence diversity and can regulate the pathogenicity of the host. Phages are ten times more frequent in the environment than bacteria, making them the most abundant ‘life’ forms on Earth, with an estimated $10^{32}$ bacteriophages on the
planet [Brussow et al., 2005]. They are most recurrently accessible from aquatic environments but are generally found wherever bacteria exists and have co-evolved with bacteria over the 3–4 billion years during which life has existed on Earth.

Bacteriophages do not infect eukaryotic cells but specifically target bacteria. This specificity is highly sophisticated and each phage will merely attack one species or in some cases a single strain of bacterium. There is diversity in their morphological types of bacteriophage. Usually, most of the bacteriophages consists of head (or capsid) is a protein shell often in the shape of an icosahedron; this comprises of viral genome that usually make up of double-strand (ds) DNA. The tail may or may not be a contractile structure and to this are coupled typically six tail fibres containing receptors at their tips that recognise attachment sites on the bacterial cell surface.

The life cycle of a typical bacteriophage is either lytic or lysogenic. Lysogenic phages are of less clinical importance compare to the lytic phages which are widely used worldwide as a therapeutic agent. The bacteriophages encounters its host through random wave and attaches via specific receptor locations that may be any one of a wide variety of cell surface components, including protein, oligosaccharide, teichoic acid, peptidoglycan and lipopolysaccharide [Lenski et al., 1988]. In some cases the attachment sites might be present on the cell capsule, flagella or even conjugative pili. Primarily, the attachment is reversible but then becomes irreversible and is completed by transfer of phage genetic material into the host cell. Inoculation of the phage genome into the bacterial cell again can occur by a variety of mechanisms depending on the morphology of the virus but often involves contraction of the tail and creation of a hole within the bacterial cell wall. The viral genome is then transcribed by host cell RNA polymerase, producing early mRNA and the metabolic machinery of the bacterium, has been taken over by redirecting its metabolic processes to manufacture of new virus components. Some of the bases present on the phage DNA are chemically altered to confirm protection against attack by cellular restriction and nuclease enzymes.

**Surveillance of vibriophages**

Bacteriophages can be detected in almost every biological entity as because they are the most abundant biological organism found in nature. Appearance of vibriophages in
environmental water (like sewage water, river and ponds) has been considered to be related to the presence of *Vibrio cholerae*. Surveillance of vibriophages in water bodies signifies the possible outbreak of cholera in near future [Das *et al.*, 2009]. In countries where cholera is endemic, *Vibrio cholerae* O1 bacteriophages (ie. vibriophages) have been perceived in sewage water and served as strain markers [Almeida *et al.*, 1992] and for typing of *Vibrio cholerae* classical, O1 and O139 strains [Chakrabarti *et al.*, 2000]. In countries where cholera displays a seasonal behaviour characterized by fluctuations in incidence environmental surveillance can play an important role in cholera control. Asymptomatic infection with *Vibrio cholerae* O1 occurs much more often than do active cases and surveillance by identifying *Vibrio cholerae* O1 bacteria and vibriophages in sewage water may be a feasible means of predicting outbreaks of cholera before a significant number of cases emerge. It has been reiterated that the presence of vibriophages in the sewage water is related to the number of cholera cases. A study performed in South America preferred the use of presence of phages in sewage water as a potential forecaster of outbreaks of cholera disease [Madico *et al.*, 1996].

**Animal models for *Vibrio cholerae***

Different animal models have been developed to study cholera (Richardson *et al.*, 1994, Sack *et al.*, 1986). There are numerous limitations in producing human like natural infection with *Vibrio cholerae* O1 in animals. A few of these animal models were able to explore significant information relevant to human disease.

A simple technique used to study the secretogenic response with respect to cholera toxin is the rabbit ligated ileal loop, first developed by De and colleagues [De *et al.*, 1959] in India. Later, another model was invented with sealed intestinal tract in adult mouse model [Richardson *et al.*, 1984] in which the ano-rectal canal is sealed with cyanoacrylate ester glue. However, infant mice have also been used in the past to study diarrhea prompted by *Vibrio cholerae* strains and are very convenient in studying the colonization potential of strains in competitive colonization assays [Freter *et al.*, 1981].

Both infant mouse model and infant rabbit model [Dutta *et al.*, 1959], was inadequate in mimicking human infection by the fact that the animals are susceptible to infection.
only for a comparatively short time after birth. The most accepted adult animal model is the RITARD (removable intestinal tie-adult rabbit diarrhea) model [Spira et al., 1981]. This model uses temporary slip knot tie of the small bowel that is subsequently removed 2 h after inoculation of live Vibrio cholerae O1 proximal to the tie. Successful colonization and immunization of adult rabbits by oral inoculation can be performed with administration of tincture of opium to induce hypoperistalsis [Cray et al., 1983]. Lethal watery diarrhea can also be induced in an adult dog model [Sack et al., 1969], although this model is rarely used.

Very promising results have been obtained by many researchers regarding the application of phage therapies in animal models [O’Flynn et al., 2004]. Different animal models have been used in cholera research but the true fact is no individual animal model reproduces all the characteristics of human diseases [Richardson et al., 1994]. A variation of nutritional, chemical, and antibiotic treatments allows the colonization of adult rabbits by Vibrio cholerae, but such manipulations may not be physiologic. In case of suckling mice model Vibrio cholerae can infect suckling animals but their immature host defences and reduced intestinal floras is often fatal and is a poor model to study the development of immune responses [Butterton et al., 1996]. In contrast, adult mice model would convey a mature immune system for assessing the immune responses. Mouse model can perform as a more versatile model than the rabbit because of the availability of different genetic backgrounds of mice as well as ease of handling compare to other animals. Different therapeutic experiments establish the importance of mouse model in the field of research [Maura et al., 2012; Capparelli et al., 2010; Sunagar et al., 2010].

**In vivo survival test of bacteriophages in the absence of host bacteria**

Bacteriophages infiltrate to the blood and other tissues very spontaneously upon their administration by different routes. They can multiply at sites of bacterial infection. Moreover, they retain full biological activity at those sites. Bacteriophages are considered to be safe, economical, self-replicating and effective bactericidal weapon [Bradbury et al., 2004].
Animals could diverge in their response to the oral phage administration by about two orders of extent. In rabbits and mice, bacteriophages were spotted in the circulation only few minutes after administration [Hoffmann et al., 1965]. It has been suggested earlier that phage therapy only needs to decline the viable numbers of infecting bacteria to a level where the host defences can take care of the remaining bacteria [Levin et al., 2004].

**Bacteriophage interaction in the gastrointestinal tract**

It has been proved earlier that bacteriophages reproduce rapidly and efficiently in *Escherichia coli* infected small intestine. During the initial 24 h after treatment, high phage counts were observed in the faeces and declined with the number of bacteria in the calves [Smith and Huggins et al., 1983]. It was interesting that the diarrhoea was also restricted by only $10^2$ phage particles scattered on the litter in the calf rooms. Besides, protection was also attained by keeping calves in the uncleaned rooms that had earlier been engaged by animals treated with phages [Smith et al., 1987b]. Earlier, research studies proved the presence of orally applied phages in the blood and urinary tract of humans [Weber-Dabrowska et al., 1987] and mice [Hoffmann et al., 1965]. Individually, the feeding and gastric lavage of animals with bacteriophages ensued in asymmetrical but steady retrieval of bacteriophages from the blood [Keller and Engley et al., 1958]. The titre was generally highest at initial minutes but the values can diverge individually within a wide range. Animals could differ in their response to the oral phage application by about two orders of magnitude [Hoffmann et al., 1965]. In case of rabbits and mice, model, bacteriophages were perceived in the circulation only few minutes after administration into the rectum [Hoffmann et al., 1965; Sechter et al., 1989]. In fact, the time of intestinal wall permeation can be extremely short. In some mice, phages were already observed in tail-tip blood 10 s after the phage administration.

It has been postulated that few numbers of phage particles could penetrate straight into portal blood, and translocation to the blood occurred via the regional lymphatic system [Hildebrand et al., 1962] but Keller and Engley (1958) explored that phages could be detected in the blood within 5 min of gastric delivery in mice. This advises that significant numbers of bacteriophages can penetrate into the circulation by diffusion rather than via the lymphatic system.
Unquestionably, the neutralization of stomach acid (e.g. aluminium/magnesium hydroxide, calcium/magnesium carbonate) prior to bacteriophage administration is an essential aspect for bacteriophage viability in gastrointestinal tract. Many phages are subtle to a low pH environment [Smith et al., 1987a]. They can rather be absorbed in the higher or deeper sections of the intestine [Hoffmann et al., 1965]. It was proved that decrease in the number of phages occur at pH ranging from 2 to 7. Yet, the sensitivity to low pH values depends on the species of individual bacteriophage. Some bacteriophages were revealed to be comparatively acid resistant in vitro and in vivo, but for other bacteriophages neutralization of gastric acid might be essential for their survival [Smith et al., 1987; Vijayashree et al., 1999].

**Bacteriophages in blood stream**

Blood permeation is the important factor in treating bacterial infection in animals. Several evidence has shown that phages can penetrate into the blood stream. Intravenous (i.v.) injection of phages allows a rapid and direct introduction of bacteriophages into the blood circulation and their spread throughout the system [Bogozoova et al., 1991]. Likewise, intraperitoneal (i.p.) injections are very effective in introducing phages into the circulatory system. In this case, bacteriophage titres usually reflect the primary dose and relative diminution of the phage titre in the animal body. So, the peritoneum-blood infiltration appears to be very fast and facile [Bogozoova et al., 1991; Merril et al., 1996]. One of the important indirect evidence of bacteriophage infiltration to the blood is their capability to cure septicaemia in animals after i.p. injection (Merril et al. 1996). Similarly, intramuscular injections can also defend animals from lethal bacterial infections (Smith and Huggins 1982; Barrow et al. 1998). Intranasal administration of bacteriophages to mice resulted in their occurrence in the blood and internal organs within 24 h, even in the central nervous system (Bogozoova et al. 1991; Frenkel and Solomon 2002; Carrera et al. 2004). This was also followed by an immune response to bacteriophage antigens [Delmastro et al., 1997]. It was proved in mice that intravaginal and intrauterine inoculation of bacteriophages can lead the phage presence in the blood [Georgakopoulos et al., 1968].

It was found earlier that highest bacteriophage titre is typically observed in mice within 24 h after administration, but it was also reported within only few minutes
Bacteriophage concentration in lung, kidney, spleen, liver and brain were significant 3 h after phage application although it was approximately 100 times lower than that of the blood [Keller and Engley et al., 1958; Bogozova et al., 1991]. Bacteriophages applied through the murine peritoneum can be noticed in the brain as early as 1 h after injection and reach their concentrated titre within 2 h. Moreover, intramuscular injections of an suitable phage were very effective in the treatment of mice (Dubos et al. 1943; Smith and Huggins et al., 1982) and chickens (Barrow et al., 1998) with an experimental bacterial encephalitis or meningitis. In these animals, more phage particles were noted in the brain than at other sites. The maximum concentration of bacteriophages reached in 12–18 h, and its level was much greater than the injected phage concentration (Dubos et al., 1943). These experiments explored that bacteriophages are capable to cross the blood–brain barrier.

Bacteriophages were isolated in samples of faeces or straight from the intestine of mice and fish as early as 3 h after injection. Although, recovery ended within approximately 12 h in mice and 10 h in fish [Keller and Engley et al., 1958; Nakai and Park et al., 2002].

The most important message from these experiments is, the time of bacteriophage persistence in an animal’s body and their titre in a particular organ strongly determined on the absence or presence of susceptible bacteria.

**Immune system response against bacteriophage**

Bacteriophages comprises of DNA or RNA and a protein (lipido-protein) coat. The coat consists of a typically several hundred molecules of protein molecules. So, it’s natural to occur an immunogenic response against individual phages subjected to phage therapy. It is fact that naturally occurring bacteriophages are able to induce humoral immunity. However, the immunogenicity may differ for particular bacteriophages: for some phages no antibodies were produced, but for some phages the existence of anti-phage antibodies was very common [Smith et al., 1987].

After oral administration immunogenic effect against bacteriophages was also observed in patients treated with phage against *Staphylococci, Klebsiella, Escherichia, Proteus and Pseudomonas*. The study showed that the anti-phage
antibodies were significantly higher after phage therapy than before it (Kucharewicz-Krukowska and Slopek et al., 1987). It has been seen earlier that in calf serum antiphage antibodies represent itself after an oral administration of bacteriophages (Smith et al., 1987). In clinical microbiology, humoral response to phage phiX174 has been used for three decades as a measure of T helper cell-dependent antibody production (Fogelman et al., 2000; Shearer et al., 2001). In case of fish no antiphage antibodies were perceived after oral treatment with bacteriophages and after intramuscular injections (Nakai and Park et al., 2002).

Bacteriophages not only provoke humoral immunity but also interact with the innate immune system. It has been proved in nonimmune, germ-free mice (the animals had never been previously inoculated with bacteria or bacteriophages, so antibodies did not affect the results), bacteriophages were progressively cleared by the spleen, liver and other filtering organs of the reticulo-endothelial system (Geier et al., 1973; Carlton et al., 1999; Merril et al., 2003). It was proved, when no phage particles could be detected in the blood, they could be recovered in the spleen and liver (but not in the lung, kidney or brain) (Keller and Engley et al., 1958). It was experimentally defined for at least 12 days in rabbits (Reynaud et al., 1992). It was also established in mice that bacteriophages were rapidly phagocytized by the liver Kupffer cells and that this clearance pathway seemed to be the most important one. Bacteriophages inoculated intravenously to mice gathered mainly in the liver (12 times more than in the spleen), but then the bacteriophage titre reduced rapidly than in other organs. Splenic macrophages were also proved to be able to inactivate bacteriophages, but their action was four times slower than the degradation by Kupffer cells (Inchley et al., 1969). The studies of the role of the spleen and the liver in anti-phage antibody production showed the predominant role of the spleen in this process [Inchley and Howard et al., 1969]. Bacteriophages are also capable to pass through the renal filter. The presence of bacteriophages in urine samples was noticed in humans [Weber-Dabrowska et al., 1987], mice [Keller and Engley et al., 1958] and rabbits [Reynaud et al., 1992].

**Bacteriophages vs eukaryotic cells:**

It is obvious that bacteriophages can penetrate higher organisms so freely; bacteriophages are able to make direct contact with eukaryotic cells. Bacteriophages are an exceptionally specific class of viruses that live on bacteria. Determinants of
their capsids bind to specific molecules on the surfaces of their target bacteria. It is proved that bacteria without the specific receptor usually cannot be attacked. It is commonly believed that bacteriophages cannot infect the cells of organisms more complex than bacteria, because of major dissimilarities in cell-surface molecules and in key intracellular machinery that is essential for phage replication [Kutter et al., 1997; Lorch et al., 1999]. It has been shown earlier that bacteriophages can be re-engineered by a phage-display technique for the artificial infection of mammalian cells. In an experiment a gene for the adenovirus penton base protein was inserted into a phage genome. The protein is originally involved in the attachment of adenoviruses to integrin receptors, internalization of viral particles, and release of the capsid from the endosome. Hence, internalization of the re-engineered phages was observed. However, no phage reproduction in mammalian cells was detected [Di Giovine et al., 2001]. There are some sporadic reports that show the capability of bacteriophages to interact with (but rather not infect) mammalian cells. Probably the first report in phage related study, that showed phage can gather in cancer tissue and inhibit the growth of tumours [Bloch et al., 1940]. Next it was demonstrated that phages bind cancer cells in vitro and in vivo and attach to the plasma membrane of lymphocytes [Kantoch et al., 1958; Kantoch and Mordarski et al., 1958; Wenger et al., 1978].

An ability of bacteriophages to influence the metabolism of human fibroblasts in tissue culture was postulated by Merril [Merril et al. 1972]. Gorski et al. advanced a hypothesis concerning the molecular basis of such interactions: at least some phages (e.g. T4) have in their structure proteins comprising a KGD (Lys-Gly-Asp) tripeptide motif, which is a ligand for the b3 integrins on cells. Accordingly, binding of T4 to the membrane of cancer cells was observed in confocal and electron microscopy as well as in direct binding tests. The effect of this binding was observed in murine and human melanoma and lung cancers. Anti-b3 antibodies and b3 synthetic ligands were eliminating this binding. It was also observed that the bacteriophage T4 and its substrain HAP1 (selected in vitro for higher affinity to melanoma cells) significantly inhibit experimental lung metastasis of murine B16 melanoma [Gorski et al., 2003; Switala-Jelen et al., 2003].
Recent significant phage therapy clinical trials

Recent phage therapy experiments with oral T4-like phage cocktail application to healthy adult volunteers from Bangladesh. No adverse events were explored by self-report, clinical examination, or from laboratory tests for liver, kidney, and hematology function. Besides, no effect of oral phage administration was seen on the fecal microbiota composition with respect to bacterial 16S rRNA from stool [Sarker et al., 2012]. In another study, a safety analysis of a Russian phage cocktail by oral application in healthy human subjects showed that the trial did not associate adverse effects with oral phage exposure [McCallin et al., 2013]. In an evaluation of a bacteriophage treatment for infection, ten dogs were included with chronic *Pseudomonas aeruginosa* otitis. Each received, directly into the auditory canal of one ear, a single dose of a topical preparation each of 6 bacteriophage strains, active against *P. aeruginosa*. The bacteriophage counts had risen from the administered dose and the bacterial counts were decreasing. No treatment related inflammation or other adverse events were detected during the trial period. The results show that administration of this topical bacteriophage mixture leads to lysis of *P. aeruginosa* in the ear without apparent toxicity and that it has potential to be a convenient and effective treatment for *P. aeruginosa* otitis in dogs [Hawkins et al., 2010].

In a study, chronic venous leg ulcers (VLUs) patients were treated for 12 weeks with either a saline control or bacteriophages targeted against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*. The positive outcome of the disease is that, no significant difference (p>0.05) was determined between the test and control groups for frequency of adverse events, rate of healing, or frequency of healing. Besides, it explored no safety concerns with the bacteriophage treatment. Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial [Rhoads et al., 2009].

In a research study systematical experiments with *E. coli* [Chibani-Chennoufi et al., 2004] phages were conducted. A series of *E. coli*-specific phages were isolated from different sources and were then applied against a collection of *E. coli* strains, pathogenic EPEC and EHEC strains. Altogether four phages related to the well characterized T4 coliphage that revealed the widest and complementary infection range among the strains were selected for the mouse experiments. The bacteriophages
were administered orally by gradually increasing doses of phages mixed in the drinking water. At different time intervals, lactose-positive colonies were counted from the stools, nevertheless, only a minor reduction in the numbers of bacteria was seen (counts dropped from $10^{6.2}$ to $10^{5.7}$). Thus the survival of the bacteria in the gut during the phage passage could only be enlightened by some physiological reasons that prohibited phage-induced lysis. In next phase, axenic mice were infected with a single *E. coli* strain and then were given phages in the drinking water. In the contrary, in axenic mice the phage titres in the stools increased in one day from the $10^7$/ml in the drinking water to $10^{10}$/ml in the stool, at the same time numbers of *E. coli* in the stools dropped from $10^8$ to $10^4$. Again the recovered colonies were sensitive to the phages suggesting that they have resided in gut sites protected from phage.

In 2002, vancomycin-resistant *Enterococcus faecium* (VRE)-specific phages isolated from raw sewage were inoculated in VRE infected mouse model. It explored first that intraperitoneally (i.p.) 45 min post infection administered phage was capable to rescue mice from VRE bacteremia and that the rescue was accompanied with a significant decrease in bacterial numbers in blood. They also concluded that phage administration before 5 h post infection still fully rescued the mice while treatment delayed beyond 5 h rescued only some of the mice [Biswas et al., 2002].

In a human experiment with bacteriophages, Paisano et al. (2004) showed that *Enterococcus faecalis* infected human dental roots could reduce the viability of the bacteria in root canals when different phage:bacteria ratios were used.

In a small trial of oral phage use, fifteen healthy adult volunteers received in their drinking water a lower *Escherichia coli* phage T4 dose ($10^3$PFU/ml), a higher phage dose ($10^5$ PFU/ml), and placebo. Fecal coliphage was detected in a dose-dependent way in volunteers orally exposed to phage. All volunteers receiving the higher phage dose showed fecal phage 1 day after exposure; this occurrence was only 50% in subjects receiving the lower phage dose. No fecal phage was visible a week after a 2-day course of oral phage administration. Oral phage administration did not cause a reduction in total fecal *E. coli* counts. In addition, no considerable phage T4 replication on the commensal *E. coli* population was revealed. No opposing events related to phage application were reported [Bruttin et al., 2005].
In a study, application of a well-defined and quality controlled phage cocktail, active against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, on colonized burn wounds within a modest clinical trial showed no adverse events, clinical abnormalities or changes in laboratory test results that could be related to the application of phages were observed [Rose *et al.*, 2014].

*Pseudomonas aeruginosa* displays multiple mechanisms of resistance, including efflux pumps, antibiotic modifying enzymes and limited membrane permeability. The primary reason for the exploration of novel therapeutics for *P. aeruginosa* infections is the deteriorating efficacy of conventional antibiotic therapy. No serious or irreversible side effects of phage therapy have been described. Five newly purified *P. aeruginosa* phages have been characterized as potential candidates for use in phage therapy. The results of these preliminary investigations specified that the newly isolated bacteriophages may be considered for use in phagotherapy [Kwiatek *et al.*, 2014].

From various aspects of human therapy and nonclinical settings are reported [Sulakvelidze and Kutter *et al.*, 2005; Brüssow *et al.*, 2007; Górski *et al.*, 2007, 2009; Kutter 2009; Kutter *et al.*, 2010; Abedon *et al.*, 2011]. Phage treatment in human eyes, ears and nose via inhalation was applied regularly at the Eliava Institute in Tbilisi for years [Kutter *et al.*, 2010; Abedon *et al.*, 2011]. Freshly, phages have been recommended to be included in a nebulizer to treat bacterial lung infections in cystic fibrosis patients [Golshahi *et al.* 2008] or to be used as a respirable powders for the treatment of different pulmonary infections [Matinkhoo *et al.*, 2011]. The first controlled clinical trial of a therapeutic phage inoculum was conducted in 2009 and showed efficacy and safety in chronic otitis targeting antibiotic-resistant *Pseudomonas aeruginosa* [Wright *et al.*, 2009]. A year later, in an evaluation of a phage treatment for chronic otitis infection in dogs, the results show once more that administration of this topical phage mixture leads to lysis of *P. aeruginosa* in the ear without apparent toxicity and that it has potential to be a convenient and effective treatment for *P. aeruginosa* otitis [Hawkins *et al.*, 2010].

Phages have been tremendously effective at treating a number of bacterial infections in controlled animal studies, especially as a biocontrol agent in the prevention of food-borne illnesses, due to its target specificity, rapid bacterial killing and ability to
self-replicate [Smith et al., 1987; Biswas et al., 2002; Hawkins et al., 2010]. Phages have the prospective to treat bacterial infections afflicting animals and in particular to prevent fatal *Escherichia coli* respiratory infections in broiler chickens [Huff et al., 2002a, b, 2003a, b]. Virulent antigen-specific phages have been used in a challenge to control *E. coli* O157:H7 in batch culture. Loc-Carrillo et al. (2005) and Wagenaar et al., (2005) reported that phage therapy (biocontrol) reduces *Campylobacter jejuni* colonization of broiler chickens. Several studies have also addressed the use of phages to reduce *Campylobacter* and *Salmonella* concentrations on poultry [Goode et al., 2003; Atterbury et al., 2007; Kittler et al., 2013].

**Regulatory approvals of bacteriophages**

The research directed for the treatment or prevention of human infections first caught the world’s thoughts and which today is the primary motivation of the field of phage therapy [Biswas et al., 2002; Merril et al., 2006; Hawkins et al., 2010; Kittler et al., 2013]. However, the regulatory requirements for bacteriophages as live drugs are still challenging [Potera et al., 2013] and their uses might not encompass to life-threatening infections. The recent USFDA (2006) approval of Listeria-specific phage preparations for food additives has unlocked the door to new applications of these natural drugs. It is established that phages only infect and lyse bacterial cells and are harmless to mammalians (USFDA 2006). This has eventually led to the development of a phage related product which received regulatory approval from the FDA in 2011, as a natural antimicrobial for use in agro-food industry as GRAS and by US-FSIS as safe for use in animals [Sillankorva et al., 2012; Klumpp and Loessner et al., 2013]. In general, although the safety of phages has been toughly suggested by human phage therapy, it should be noted that some phages, notably when in the form of lysogens (prophages), have been documented as important contributors to bacterial virulence, or as vectors in horizontal gene transfer through transduction [Verheust et al., 2010].

Recent information’s concerning studies being conducted and/or ongoing trials with the crucial purpose of experimental studies with the aid of phages, human patients with various types of infections can be obtained from www.clinicaltrial.gov and http://www.clinicaltrial.gov/ct2/results?term=phage+therapy&Search=Search
[Parracho et al., 2012]. The national and international regulatory agreement and regulations being employed can be obtained from Parracho et al. (2012).

**Efforts towards cocktail optimization**

Several studies have suggested different techniques to improve the phage cocktails being technologically advanced. A phage cocktail procedure was developed based on *Staphylococcus* phage K, including multiple passages on previously phage-resistant strains to enrich for broad host-range spontaneous phage mutants [Kelly *et al.*; 2011]. Phage resistance systems were identified by these authors in 29 *S. aureus* strains [Hyman *et al.*, 2010; Labrie *et al.*, 2010]. Of these, 24 had restriction modification mechanisms, three had an adsorption inhibition mechanism and for two, the underlying resistance mechanisms were not identified. Six of the most potent phage derivatives, along with the original phage K, were selected to make up the phage cocktail, which was then verified against a panel of *S. aureus* strains to approve the breadth of their combined spectrum of activity.

Similarly, Gu *et al.* developed a phage cocktail by isolating phages using both wild-type bacteria and phage-resistant variants as hosts [Gu *et al.*, 2012]. Their three-phage cocktail was tested for efficacy by treating mice suffering following a lethal dose of *Klebsiella pneumoniae*. A single intraperitoneal dose applied 1 h postbacterial inoculum resulted in 100% recovery, which was reproducible for a delay of 3 h if a higher phage dose was inoculated. *In vitro*, the phage cocktail was established to lyse 88% of *K. pneumoniae* strains verified. Using a more molecular approach to explore phage receptors, in a case of *Yersinia pestis*, site-directed mutagenesis and transcomplementation was applied to nine phages [Filippov *et al.*, 2011]. They recognized six receptors for eight of the phages in the lipopolysaccharide core, postulating that a combination of these phages could be articulated into a therapeutic cocktail. Testing in mice showed that bacteria that had mutated to develop resistance against these engineered phages had become attenuated, resulting in a higher 50% lethal dose and longer survival times. However, other associated methods with genetically or chemically modifying phages for phage therapy were discussed by Goodridge *et al.*, 2010 and Verbeken *et al.*, 2012.
Focus on cocktail vibriophages as a potential therapeutic agent

There are many encouraging therapeutic studies in animal models [Burrowes et al., 2011; Debarbieux et al., 2010; McVay et al., 2007]. It has been shown that oral administration of bacteriophages was effective in the therapy of septicaemia and other bacterial infections in humans [Dabrowska et al., 2000]. The ability of bacteriophages to penetrate higher organisms determines the potential phage activity in antibacterial treatment. Higher organisms are widely exposed to bacteriophages, which penetrate them quite freely.

Phage therapy for cholera was established as a one of the useful mechanism for the treatment of cholera patients because bacteriophage are capable of eliminating large number of bacterial cells, thus dippin the burden of the pathogenic effect and, ultimately, transmission and symptoms of the disease. The first experiment of phage therapy for cholera, that used high doses of bacteriophage (100–200 phage per vibrio) confirmed the rapid clearance of cholera bacterium but the phage were not able to finishing many cycles of replication and amplification [Summers et al., 2001]. When bacteriophage therapy was performed on cholera infected patients in a hospital and compared with tetracycline treatment and with fluid replacement alone as a control, it was observed that very high-dose phage therapy was comparable to tetracycline in reducing the shadding of vibrios in stools; this reduction, however, did not transform into overall clinical improvement (i.e., shorter duration of diarrhea and more rapid recovery) of the patients [Marcuk et al., 1971]. Different problems were found that complicated the evaluation of phage therapy in cholera; first were the discovery of the variety in serotypes of vibrios and the varying susceptibility of these bacteria to the phages employed; second was the rapid transport of the ingested phages through gastrointestinal tract of cholera patients. Although there are several reports which showed the efficacy of bacteriophages in the treatment of severe cases of cholera [Marcuk et al., 1971]. The impetus for considering bacteriophage-mediated regulation as a strong weapon against pathogens would be the fact that phage-resistant strains rarely occur in a natural habitat.

Studies on phages of Vibrio cholerae O1 have been of historical importance. The phages had been used formerly for the confirmatory diagnosis of Vibrio cholerae O1 infection. Vibriophages are still being used for the distinction between classical and
El Tor biotypes of *Vibrio cholerae* O1. In case of cholera our own previous in the rabbit ileal loop (RIL) model it was reported that phage administration did not have a prophylactic effect because it did not reduce the number of challenge bacteria and the fluid accumulation ratios in this model [Sarkar *et al.*, 1996]. The negative outcome of the RIL model prompted us to perform this study in RITARD model, rabbit oral model and in mice oral model, where phage efficacy was determined against experimental *V. cholerae* infections from various aspects. Indisputably, any aspects of the phage-vertebrates interaction are motivating and significant for further applications of bacteriophages.