

Bacteriophage therapy – Looking back in to the future

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Antibiotics are small molecules that are used to cure severe infection caused by pathogenic bacteria. Antibiotics kill or stop bacterial growth by several ways, as they disrupt bacterial cell wall, plasma membrane, stop DNA replication, transcription, and protein synthesis. There are mainly two types of antibiotics namely broad-spectrum antibiotics that affect a wide range of bacteria and narrow spectrum antibiotics that affect only one or a few types of bacteria. The mode of action of different antibiotics is different depending on chemical nature of antibiotics and bacterial species. Due to excessive use many bacteria have developed resistance to many antibiotics and such bacteria referred as Multi Drug Resistant bacteria. For the treatment of infections caused by these MDR bacterial strains needs to develop a novel alternative antimicrobial agents. Bacteriophage is the virus that infects and kills the bacteria and may be used as an alternative antimicrobial therapy. Bacteriophages are highly specific means they infect only one or a few types of bacteria and non-toxic to human, animals and plants. Bacteriophage therapy is superior to antibiotics because bacteriophage is the only unique class of antimicrobials whose titer increases during the course of therapy that leads to increase in efficacy. Therefore, in this chapter we are highlighting the bacteriophages therapy to be reconsidered as an approach to combat with increasing drug resistance among pathogenic bacteria.

Keywords: Bacteriophage; Phage therapy; Phages typing; Phage nano-diagnostics; Drug resistance

1. Introduction

One of the greatest scientific achievements of the twentieth century was the development and mass production of antibiotics, to cope with increasing incidences of bacterial infections. Over past 60 years antibiotic become most reliable and widely used prescriptions against bacterial diseases. But more frequent and uncontrolled use of this miracle cure, the bacteria start getting resistance against many established antibiotics. This inherent feature of bacteria to develop resistance posed a serious threat to our chemical shield that has been increasingly leaked. World Health Organization (WHO) warned that “the world is on the brink of losing these miracle cures -the antibiotics” and that “in the absence of urgent corrective and protective actions, the world is heading toward a post-antibiotic era, in which many common infections will no longer have a cure and, once again, kill unabated”. Thus it seems unrealistic to depend entirely on antibiotics against bacterial threats, and even the most effective antibiotics have also detrimental effects on endogenous gut microbiome which plays a vital role in human digestion and nutrition. Additionally, it's time now to think beyond the chemicals to seek sustainable and safe alternatives to cope with bacterial infections. The viral predators, ‘bacteriophages’ can be the most suitable and potential alternative to cope with bacterial diseases.

Phage therapy has been visited as one of seven approaches to “achieving a coordinated and nimble approach to addressing antibacterial resistance threats” in a 2014 status report from the National Institute of Allergy and Infectious Diseases (NIAID). That showed the importance of phage therapy as potential tool for fighting against the drug resistant bacteria. However, the success of phage therapy depends on our ability to overcome the limitations of using them as a therapeutic agent (1, 2).

2. Bacteriophages

Bacteriophages are the most abundant organisms on the Earth. They are ubiquitous, obligate intracellular parasites and attack on the host cell, hijack the machinery and finally destroy it. Archaea and cyanobacteria are also attacked by a group of viruses often called as cyanophages (3, 4). Bacteriophages widely occur in, sewage, soil, water and marine water etc. Structurally, most of bacteriophages consist of three parts i.e. head, tail and tail fiber. The head encloses nucleic acid which can be either DNA or RNA but not both. The tail is like a hollow tube through which nucleic acid passes in to the host cytosol during infection and tail fibers help bacteriophages to attach on the bacterial surface. The size of most bacteriophages in general ranges from 22-200nm in length, while the largest bacteriophage known is T4 which is about 200nm long and 80-100nm wide (3). The most important feature of phages is their narrow host range i.e. they kill only specific bacterial strain and that makes them as potential antimicrobial agents. This feature of bacteriophages is very advantageous because unlike broad-spectrum antibiotics, phage can kill specific pathogenic bacteria without affecting the balance of beneficial bacterial microflora (5). However one drawback of this narrow bacteriophage host range is

that, bacteria may develop resistance against bacteriophage. To solve this problem, phage “cocktail” *i.e.* a mixture of different bacteriophages are used that provides a wider antimicrobial range (6). Normally lytic bacteriophages infect and kill specific bacteria and are widely used in therapy because they act on short period of time. Furthermore, the mode of antimicrobial action of bacteriophages is very complex than mechanism of action of antibiotics. Therefore in this chapter we are highlighting the use of bacteriophages as an alternative antimicrobial therapy and also other potential applications of bacteriophages (7-11).

2.1 Life cycle of bacteriophages

The bacteriophages display two types of life cycle *i.e.* lytic cycle and lysogenic cycle. In lytic cycle, bacteriophage attach on bacteria through various cell surface receptors such as lipopolysaccharids (LPS), teichoic acids and various structural proteins (*OmpA*, *C* and *F*) on bacterial cell wall and injects its nucleic acid into the cytosol. Soon after, phage nucleic acid hijack the whole bacterial synthetic machinery that direct synthesis of phage specific *mRNA*, structural proteins and various other components. Later on, different phage components are assembled to form mature viral particles that come out from cell by lysis of the cell wall. Approximately, a total of about 1000 viral particles may be released per cycle (3). In lysogenic cycle, phage nucleic acids get integrate in to the bacterial genome and replicate along with genome and passed on to the daughter cells. The integrated viral genome is called ‘prophage’ and this process is known as lysogeny. Under certain condition like high radiation, change in metabolism, stresses, phage genome gets excised and becomes a lytic phage (3, 12).

3. Historical background of Phage therapy

Bacteriophages were discovered independently by a British microbiologist Frederick Twort in 1915 and Felix de Herelle (13, 14) however, the concept of bacteriophage therapy was introduced by Felix de Herelle in 1920. Many countries like France, Georgia and United States and in Europe there are several phage therapy centers are working, and dealing with various human diseases. The extensive work on phage therapy was carried out between 1920 to 1930 in USA to treat infection caused by *Streptococcus* and *Bacilli*, “Staphylogel” and bacteriophage “gel labeled” preparation were manufactured by Eli Lilly and Company. At the same time, antibiotics were discovered and widely used that causes rejection of bacteriophages as therapeutic agent in many countries include Europe and USA. Many papers have published between 1950 to 1980 that showed benefits of bacteriophage therapy in animal models (7-11). Due to increase antibiotics resistance in bacteria, bacteriophage therapy was “rediscovered” with the work done by Smith and Huggins in 1980s (15). Now treatment of infection by phage was widely used in several countries namely Poland, United States, Europe (Georgia) and Russia (9, 16).

Poland-The Hirszfeld Institute of Immunology and Experimental Therapy in Wroclaw was established in 1954 in which thousand patients were successfully treated with phages. For each patient phages were selected and prepared and treatment was done by specialized physician. The 75% to 100% cure rates for specific infection types were reported. After the successful treatment of severe antibiotic resistance infections, the Institute itself opens a phage therapy center in 2005 (17, 18).

United States-In US, phage therapy was started during 1920s and 1930s and treatment of chronic furunculosis was carried out by the Michigan Department of Health where phage administered subcutaneously (19, 20).

Georgia- George Eliava established George Institute in Tbilisi in 1930s in which phage therapy was started in association of Felix d’ Herelle. One pathogenic bacteria strain was received from the Soviet Union and phages were isolated from different sources, tested and administered against this bacterial strain (19, 21). Particularly the bacteriophage Sb-1 was found to be quite effective in eliminating MRSA infections. The Sb-1 bacteriophage was found to be effective against MRSA strains and in a case study involving a young cystic fibrosis patient infected with MRSA, treatment with the Sb-1 bacteriophage reduced MRSA levels below the limit of detection (22).

Russia- In addition to the above, Russia also play important role in the development of phage therapy. In Russia, diarrheal infection, gas gangrene and also infection of infants and child were successfully treated with phage therapy. Bacteriophages were used in the form of tablets, creams, liquids and Russia is the first country where stable more effective tablet forms of bacteriophage was developed (20, 23).

4. Why we need phage therapy?

The emergence of antibiotic –resistance bacteria has becomes a major problem for clinician over the last 2-3 decades. In United State, antibiotic resistance bacteria infect approximately 2 million people each year in which at least 23,000 people die annually (24). At present, most of the bacteria such as *Acinetobacter baumannii*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecium*, *Klebsiella pneumonia* have becomes multi-drug resistance and cause major healthcare crisis in ICUs (25, 26). The number of newly approved antibiotics have decreases in United State since 1980s and development of new antibiotics becomes unprofitable due to underlying economic condition and probably most of the newly developed antibiotic becomes ineffective soon after their introduction (27, 28). The development of

severe new infectious disease caused by multidrug resistance bacteria that are not cure by antibiotics requires alternatives to classical antibiotics. Due to steadily declined discovery of new class of antibiotics, there is an urgent need to investigate new antimicrobial drugs. Therefore, bacteriophage may be used as therapeutic agent to treat infections caused by multi-drug resistance bacteria (29-31).

5. Advantages of bacteriophage therapy over conventional antibiotics

Bacteriophages are the potential unique therapeutic agents that have many advantages over classical antibiotics (19, 32). Some most important advantages are given in the following table (Table 1). Apart from those generalized advantages there are many specific advantages that the phage therapy has like 'Auto dosing'. Phages during the bacterial-killing process are capable of increasing in number specifically where hosts are located, so phages are intelligent enough to put a desire dose to kill host bacteria *i.e.* we called it auto dosing. Low inherent toxicity, since phages consist mostly of nucleic acids and proteins, they are inherently nontoxic. The relatively narrow host range exhibited by most phages limits the number of bacterial types with which selection for specific phage-resistance mechanisms can occur. Thus there is low pressure of resistance associated with phage therapy. Because phages infect and kill using mechanisms that differ from those of antibiotics, specific antibiotic resistance mechanisms do not translate into mechanisms of phage resistance (32).

Table 1 Advantages of bacteriophage therapy over conventional antibiotics.

SI. No.	Bacteriophages	Antibiotics
1	Bacteriophages are very specific means they targeted specific bacterial strains without affecting the normal beneficial bacterial micro flora of the body.	Most of the antibiotics are broad spectrum .In addition to killing specific pathogenic bacteria; they also destroyed the normal beneficial micro- flora of the body. Thus they disturb microbial balance and may cause severe side effects.
2	When a bacterium becomes resistance to specific bacteriophage then it is very easy to find new bacteriophages against that bacterium.	If a bacterium becomes resistance to an antibiotic ,it is very difficult to develop new antibiotic and this development process is very tedious and take many years
3	Many antibiotics cause allergies in several patients; in this case infections are successfully treated with bacteriophage therapy.	Cure of infection will be very difficult if the patients are allergic to antibiotics.
4	Manufacture process of bacteriophage is very easy and cost effective.	Manufacture process of antibiotics is costly, complex and time taken.
5	Bacteriophages have unique ability to multiply and increase their number at the site of infection thus treatment can be performed with very small dose.	Antibiotics do not accumulated at the site of infection; they are normally metabolized in the body and removed from the body through urine.
6	Multi-drug resistance (MDR) bacteria are easily treated with bacteriophages therapy.	If the bacteria become multi-drug resistance, treatment with antibiotics will be very difficult.
7	Bacteriophages are nontoxic to human thus; there are no severe side effects.	Use of antibiotic cause severe side effects includes allergic reaction, intestinal disorders and several secondary infections.

6. Bacteriophages as antimicrobial

Bacteriophage therapy is widely used to treat severe infections caused by multi-drug resistance pathogenic bacteria in human ,animals and plants and it is now also employ to enhance the shelf –life of meats, vegetables, fruits and stored plant parts (33, 34). Mzia Kutateladze, who heads the scientific council at the Eliava Institute in Tblisi, Georgia, says that antibiotic resistance is driving more Western patients towards phage-therapy clinics in Europe and America. The US National Institute of Allergy and Infectious Diseases in Bethesda, Maryland, now list phages as a research priority to address the increasing drug resistance bacteria (35). Thus there is increasing demand for isolation and characterization of more bacteriophage against major clinical threats (9).

6.1 Bacteriophage isolation, purification and preparations of commercial phage

Bacteriophages can be easily isolated from their natural sources such as water, soil, sewage, fermented product and unprocessed vegetables following standard protocol (36). This is the first step of any phage therapy protocol. After isolation, purification was performed by using filtration, ultracentrifugation, polyethylene glycol method and various forms of chromatography. The above methods normally removed ruptured bacterial ghost cells, uninfected cells and other components of bacteria (30). Several phage preparations were developed by Eli Lilly Company against *Syaphylococci*, *Streptococci*, *E. coli* and for various other infectious pathogenic bacteria for the human use. Important example of commercially obtainable bacteriophage preparations includes Ento-lysate, Staphylo-lysate, Neiso-lysate and Colo-lysate. Diabetic wounds, chronic infections, upper respiratory tract infection, abscesses and various other infections are successfully treated by using these phage preparations (20). D'Herelle's commercial laboratory in Paris

produced at least five phage preparations against various bacterial infections. The preparations were called as Bacté-coli-phage, Bacté-rhino-phage, Bacté-intesti-phage, Bacté-pyo-phage, and Bacté-staphy-phage, and they were marketed by the French company L'Oréal later on. In the 1940s, the Eli Lilly Company (Indianapolis, Ind.) in United States, produced seven phage products for human use, targeted against *Staphylococci*, *Streptococci*, *Escherichia coli*, and other bacterial pathogens. Mainly these preparations contains phage-lysed, sterile cultures of the host bacteria (e.g., Colo-lystate, Ento-lystate, Neiso-lystate, and Staphylo-lystate) or the same preparations in a water-soluble jelly base (e.g., Colo-jel, Ento-jel, and Staphylo-jel). They were used to treat various infections, including abscesses, suppurating wounds, vaginitis, acute and chronic infections of the upper respiratory tract, and mastoid infections.

6.2 Mode of delivery of bacteriophages

The success of bacteriophage-derived therapeutics and biosensors will ultimately rely on suitably robust, reproducible, delivery technologies. Delivery of suitably-engineered phage has permitted isolation of allergens inducing IgE production using high throughput screening technologies (37, 38). Phages can also be engineered to bear target-specific peptides or proteins for biorecognition, and thus may have application in development of novel chemical and biological sensors that may provide quantitative or semi-quantitative data through exploitation of a chemical or biological recognition element (39).

The potential routes of administration of phages include topical, oral, rectal, and parenteral; topical administration to chronic wound infections is the most frequently reported route. In such infections, phage cocktails—combinations of a variety of phages—have been used. One product used at the Eliava Institute is PhageBioDerm, a biodegradable polymer wound dressing impregnated with ciprofloxacin, benzocaine, chymotrypsin, bicarbonate, and 6 lytic phages (Pyophage) with activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus species*, and *Proteus species* (40). Other potential means of topical administration include sprays, aerosols, lozenges, mouthwash, suppositories, bandages, eye drops, and tampons. Intrapleural administration and bladder irrigation are also feasible.

6.2.1 Oral administration of phage

Gastrointestinal infection and systemic infections are successfully treated with oral delivery of phage (41). The main difficulty with phage delivery through this route is that phage can be inactivated in the highly acidic condition of the stomach. To avoid such problem, polymer microencapsulated phages are used that protect phage from inactivation by acid and also enhance efficacy of phages. Other way of neutralizing acidity of stomach is use of sodium bicarbonate or sodium bicarbonate mineral water before administration of phages (38).

6.2.2. Local administration of phage

This is the most successful route of phage administration where phage suspensions directly apply on the infected area (7). In addition to above routes, phage can also be administered to human by intravenously (IV), intaperitonal (IP), intramuscular (IM), and subcutaneous (SC) methods (38).

6.3 Pharmacology and pharmacokinetics of phage therapy

The effect of drug's on the body as well as the body's impact on drugs is known as pharmacology. Pharmacokinetics is explanation of a drug's potential to arrive in the near of specific target cells or tissues which are enough to achieve primary pharmacodynamic effect (42). This explanation is to differentiate in to distribution, absorption, metabolism as well as elimination of drugs. In case of bacteriophage, metabolism represents "inactivation" of phage because host immune system interacts and inactivates the phage or "activation" due to phage replication inside the host body (43). For success of a phage therapy, generation of adequate number of phages is necessary in the immediate vicinity of specific target bacteria that cause pathogen eradication from the body at some adequate rate. Bacteriophage will increase their sufficient number through in situ replication in host body and so-called active treatment. Thus the main goal pharmacologically is to gain sufficient densities of phages in the vicinity of target bacteria that lead to bacterial eradication (44).

6.4 Phage therapy in human

In human, several infectious diseases such as septicemia, wound infections, skin infection, osteomyelitis, urinary tract infections and middle ear infections were successfully treated with phage therapy (21). In many countries like US, phages were widely used for human and animal vaccine preparation. In phage therapy, phages were used in the form of liquid suspension, tablets, creams rinses, aerosols (7, 9). Table 2 shows some of the successful phage therapy done so for against the human infection.

Table 2 Some successful human phage therapy studies

Year	Target organisms	Disease	Success	Details
2002	<i>E. coli</i> Proteus Pseudomonas Staphylococcus	Ulcers and wounds	70%	Healing associated with reduction or elimination of target organisms in 22 patients with ulcers
2001	Proteus Staphylococcus Streptococcus	Burn wounds		Pyophage; Reduced septic complications, better temperature normalization, two-fold reduction of staphylococci and streptococci, and a 1.5-fold Proteus with phage use
1995	<i>E. coli</i> Proteus Staphylococcus	Acute and chronic urogenital inflammation	92%, 84%	92% for marked clinical improvement; 84% for bacteriological clearance
1993	Salmonella Shigella	Salmonellosis		Phages versus combined phages and antibiotics was examined with combination effective but not antibiotics alone
1992	<i>K. ozaenae</i> <i>K. pneumoniae</i> <i>K. rhinoscleromatis</i>			Adapted phages used; treatment reportedly effective;
1991	Enterococcus <i>E. coli</i> <i>P. aeruginosa</i> Proteus Staphylococcus Streptococcus	Infectious allergoses	86%	Phages only,* n = 360, 86% success; antibiotics only, n = 404, 48% success; antibiotics plus phages, n = 576, 83% success
1989	Pseudomonas Staphylococcus	Post-surgical wounds	82%	Cancer patients; treatment was successful in 61% of antibioticonly treatment
1984	Shigella	Dysentery (prophylaxis)		Double-blinded; ca. 10-fold lower incidence of dysentery in phage-treated group
1984	<i>P. aeruginosa</i> <i>S. aureus</i>	Prophylactic		2 times/day for 3–5 days in 27 patients; normalization of microflora in infected sites with IgA production stimulated
1982	Staphylococcus	Infections of the lung parenchyma and pleura	82%	Full recovery seen with phages versus 64% with antibiotics only (n = 117)
1981	<i>E. coli</i> Proteus Dysentery			Immunosuppressed leukemia patients treated with improved results in combination with bifidobacteria
1980	<i>E. coli</i> Proteus Staphylococcus Streptococcus	Lung and pleural infections		Successful phage use in combination with antibiotics
1978	<i>E. coli</i> Proteus	Antibiotic-associated dysbacteriosis	success	Premature/low-birth-rate infants; phages used in combination with bifidobacteria
1978	<i>E. coli</i> Proteus Staphylococcus Streptococcus	S.I.		Improved efficacy using phages selected against bacterial strains isolated from individual patients versus commercial phage preparations
1976	Unknown	Abscessing pneumonia		Multiple treatment approaches including use of phages
1974	Proteus Staphylococcus Streptococcus		92%	Success = elimination of infections

(*n= number of healthy volunteers)

6.5 Phage therapy in animals

Bacteriophages are now widely used for the treatment of infections caused by *Escherichia coli* and *Salmonella* in chickens, calves, pigs and lambs. Smith and Huggins (1983) experimentally proved that, a single dose of phage R is very effective in preventing death in mice due to septicemia caused by *E.coli* which cannot be treated with multiple doses of antibiotics. They subsequently used bacteriophages for the treatment of infections caused by enterotoxigenic *E.coli* in neonatal lambs, calves and pigs (45). The earliest research on the therapeutic efficacy of phages was conducted by Felix d'Herelle, who carried out field studies on fowl typhoid (*Salmonella enterica* subspecies *enterica* serovar Gallinarum) and laboratory studies on dysentery in rabbits (*Shigella dysenteriae*) (19). Subsequent animal studies have shown that phage therapy works in animal systems. Nevertheless, many well controlled experiments with animals suggest that phages can be used therapeutically in animals and have some advantages over antibiotics. In 1943, René Dubos and colleagues (46) at Harvard University (USA) showed that anti-S dysenteriae phage injected intraperitoneally

in mice appeared in the blood stream (and even crossed the brain-blood barrier) but were rapidly cleared by the reticuloendothelial system, particularly the spleen).

6.5 Bacteriophage safety

The description of toxicity of drugs and their impact on non-target tissues comes under the category of secondary pharmacodynamics. Many evidences clearly showed that bacteriophages interact with non-target cells and tissues of human. Several phages are taken up by gastrointestinal tract in to the blood stream and reticulo-endothelial system is also appears to interact with phages. However, interaction of phages with non-target tissues has no major side effect recorded so far (47).

The immunology of phages is not yet known extensively. When the phages delivered by intravenous route, they are quickly removed from circulatory system and predominantly stored in liver and spleen. Many evidences suggested that, the head proteins of bacteriophages interact with certain receptors found on the immune cells which result in cytokines production and humoral immune response while it is unclear that, phage stimulate cell-mediated immune response. One of the most serious problems that have been displayed by phage therapy safety is that some phages (temperate phage) have unique ability to incorporate their genome and modify host bacteria that make them more potential pathogenic thus it is important to avoid temperate phage for therapy (27). The U.S. Food and Drug Administration (FDA) did not have guidelines for reviewing bacteriophage preparations until recently, and those guidelines needs yet to finalize. Earlier FDA-approved clinical uses in the 1970s, 1980s, and 1990s involved phage phi x174, which was administered intravenously for nontherapeutic purposes to patients with Down syndrome, Wiskott-Aldrich syndrome, HIV infections, and other immunodeficiencies. FDA also conducted a safety review of bacteriophages in 1973 after phages were found in several vaccines. Agency officials concluded that the vaccines were safe and allowed their continued use. That first U.S. trial also put lot of concern about developing phage therapies in humans that FDA officials might insist on single phage preparations instead of cocktails. The long-term efficacy of single-phage preparations is questionable, and early commercial failures could hinder development of phage therapy in the United States. Most phage therapy trials in the former Soviet Union and Eastern Europe have involved multivalent phage cocktails, according to published reports (Challenges in Developing Phage Therapies).

7. Improvement of phage therapy through modern techniques

Therapeutic and prophylactic application of bacteriophages are encouraging over conventional medicine, there is an obvious need to develop phage therapy in wide range of applications. Though the first reported discovery of antibacterial-like activity linked to bacteriophages was made in 1896, where Ernest Hanbury Hankin stated that the waters of the Ganga and Jamuna rivers might have restricted outbreaks of cholera (48). Since then due to lack of information regarding basic phage biology and early antibacterial drugs together restricted the development of phage therapy. In recent years due to increased threat of bacterial resistance to drugs and lack of new antibacterial drug development, opened new opportunities to develop phase therapy. Moreover with increased information regarding phage biology and improvements in molecular biology researchers have now progressed to the point where sufficient knowledge acquired to improve phase therapy as next-generation application (49).

7.1 Development of genetically engineered bacteriophages

Genetic engineering helps to improve the phase therapy to overcome many obstacles involved in using wild type phases. While treating gram-negative bacteria cell wall liberates endotoxin which is harmful and cause bacterimia, septicemia etc. To overcome this difficulty, several bacteriophages have now been developed through genetic engineering that are non-replicating and non-lytic. These genetic engineered bacteriophage, encode specific proteins that are deleterious to bacteria with release of only small amount of endotoxin. *Helicobacter pylori* and *Pseudomonas aeruginosa* infection are successfully treated with these genetically engineered and non-replicating bacteriophages (7, 50). Engineered bacteriophage can enhance the killing of antibiotic-resistant bacteria, persister cells, and biofilm cells, reduce the number of antibiotic-resistant bacteria that arise from an antibiotic-treated population, and act as a strong adjuvant for other bactericidal antibiotics (51). Host range of a T7 phage increased by expression of an endosialidase which degrades the K1 capsule present in some *E. coli* strains, thereby allowing the modified T7 to surpass this barrier, enhancing phage adsorption to efficiently infect K1 *E. coli* (52). Cock tail of phages that are genetically modified would be more helpful in addressing a wide variety of bacterial infections that are otherwise resistant to the latest generations of antibiotics (53). SOS gene networks of *E. coli* was inactivated by the use of genetically engineered bacteriophages, enhance the capability of antibiotics such as β -lactams, quinolones, aminoglycosides to works on bacteria quickly and more effectively. Likewise many researchers are using genetic engineering approach to advance the phase therapy

7.2 Genetic engineered bacteriophage as adjuvant

There are many genes in bacterial genome whose products are harmful to human on which antibiotics cannot target directly. Therefore, genetic engineered bacteriophages were developed that have ability to overexpress proteins to target the bacterial gene networks on which the antibiotics cannot work and increase destruction of bacteria by antibiotics. With recent advances of DNA synthesis, synthetic phages were developed and well-suited for incorporating targets, identified via systems biology, in a modular fashion to create effective antibiotic adjuvants. These ever improving technologies will enable large-scale modifications of phage libraries to produce antibiotic-adjuvant phage that target different gene networks and that can be applied with different antibiotic drugs against a wide range of bacteria. Combination therapy which couples antibiotics with engineered antibiotic adjuvant phage is a promising antimicrobial strategy for the future (51).

7.3 Use of phage lytic enzymes

Many bacteriophages possess lytic enzymes such as holins and lysins that have ability to battle against host to survive. They have yet to be exploited. Now new techniques are employed in which bacteriophages are used in combination with phage lytic enzymes that enhance the killing capacity of bacteriophages. All of these enzymes are highly evolved molecules designed for a specific purpose, to quickly destroy the bacterial cell wall, and as such, nanogram quantities of enzyme are sufficient to sterilize a 10^7 bacterial suspension in seconds. Because of the serious problems of resistant bacteria in hospitals, day care centers, and nursing homes, particularly Staphylococci and Pneumococcal, such enzymes may be of immediate benefit in these environments. Thus, we may add phage enzymes to our armamentarium against pathogenic bacteria (54).

8. Other potential applications of bacteriophages

In addition to phage therapy, bacteriophages are also being employed for phage display, phage typing, vehicles for vaccine delivery, targeted gene delivery, bio-control, eradication of biofilm and food industry (55, 56).

8.1 Phage display

Phage display was likely the first phage application as a tool of modern biotechnology (53). The term phage display was first introduced in 1985 (57). Phage display is a unique molecular technique in which exogenous proteins are expressed on the surface of a bacteriophage. In phage display technique, desired gene that encodes the protein is fused with phage coat-protein gene and is displayed on the surface of bacteriophage. Through phage display technique, novel phages are developed, these phages have a variety of applications. *Escherichia coli* filamentous phage M13 is most extensively used in phage display but other phages such as lambda phage of *E.coli* and T7 Phage have also been widely used (56). By the use of phage display, a specific protein or antibody can be purified because specific phages are very easy and cost effective to purify. The various applications of phage display include mapping of epitopes, in vaccine design, in study of protein-protein interaction, in determination of specificity of enzymes and inhibitors, in screening for anticancer peptide and proteins and in screening for receptors (55).

8.2 Phage typing

Bacteriophages are very specific to their host means a bacteriophage especially infect and destroy only one or a few type of bacteria. This specificity of phages for bacterial cells are widely used for the detection of pathogenic bacteria and for bacterial strain typing, a technique known as phage typing (58). In Phage typing the lawn of unknown bacterial strains, is provided with various different phages, when the phages infect and lysed specific bacterial strains clear zones (plaques) appear on the lawn, in this way specific bacterial strains can be easily detected. In addition to phage typing, other techniques are also widely used for the detection of pathogenic bacteria such as use of green fluorescent proteins and use of phages that deliver reporter genes specifically (55, 56).

8.3 Phages as vehicles for vaccine delivery

Bacteriophages have been widely used as vehicles for the delivery of vaccines (59). They are employed in the two ways, one in which phages can be used directly, bearing vaccine antigens display on their surfaces and other in DNA vaccine, the gene or sequences that synthesized vaccine antigen firstly incorporated in to the phage genome then phage would act as vehicles for the delivery of DNA vaccine (56).

8.4 Targeted gene delivery through phages

Bacteriophages have also been widely used as specific gene delivery vectors. The phages employ in this purpose is similar to that for using phages for DNA vaccine delivery. The foreign proteins expressed on the surface of

bacteriophage that enable them targeting specific cell types and after injection, phage coat protein save DNA from degradation, which is a prerequisite for successful gene therapy (56, 59).

8.5 Phages as bio-control agents

Bacteriophages are widely used as bio-control of plant pathogens. Many plant pathogens such as *Xanthomonas pruni* that infect cabbage, peppers, and peaches, *Ralstonia solanacearum* cause disease in Tobacco, *Xanthomonas compestris* cause spots on tomatoes, *Pseudomonas talaasi* cause blotch of mushrooms have been successfully control by bacteriophages (60, 61).

8.6 Eradication of biofilm by phages

Biofilms are aggregation of microorganisms, growing either on living or non-living surfaces and secrete huge amount of extracellular polymers (EPS) that surround them. Environmental conditions stimulate most of the bacteria to form biofilms and biofilms formation is an important strategy of bacterial survival. Biofilms formation cause severe disease in human and is resistance to antibiotics and host immune systems (62). Because biofilms are resistance to antimicrobial drugs, therefore alternative therapy must be needed to control biofilm-associated diseases. Evidences suggest that, bacteriophages efficiently eradicate biofilms (63, 64).

8.7 Phages in food industry

Many pathogenic bacteria have contaminated the foods which result in foodborn disease (65, 66). At present, leading cause of death due to the foodborn pathogens are *Listeria* and *Salmonella* and followed by *E.coli* and *Campylobacter jejunii*. Bacteriophages give effective and safe means to eradicate the food born pathogenic bacteria that contaminate the foods (61).

9. Major obstacles of phage therapy

Although it seems everything in favor of using lytic phages as therapeutics but they are not commonly used to the extent of expectations and sometimes their efficacy is a matter of controversy. One of the most significant factors associated with the efficacy of phage therapy is the paucity of a appropriate placebo-controlled studies. Here is some major obstacle in the application of phage therapy.

1. Heterogeneity and ecology of both phages and bacteria
2. Selection of highly virulent phages against targeted host bacteria
3. Monophage preparations against different bacteria
4. Selective phage cocktail appropriately characterize or titre phage preparations
5. Genetically engineered phages
6. Lateral gene transfer of virulence factors and antibiotic resistance
7. Restriction modification degradation of phage DNA upon infection
8. Resistance mutations in bacterial genes for adsorption, lysogeny and lysogenic conversion
9. Phage-host interactions
10. Phage preparation and characterization *i.e.* to determine the virulence to the target
11. Neutralize gastric pH prior to oral administration
12. Immunogenicity antibodies developed against phage
13. Endotoxins in phage preparation
14. Phage reprogramming

9.1 Rapid clearance of bacteriophages

The quick removal of bacteriophages by liver, spleen and other different filtering organs of reticuloendothelial system lowers the densities of phages to a level, this lower concentration of phages is not sufficient to combat against infections (11).

9.2 Generation of antibodies against bacteriophages

When the phages administered intravenously, humoral immune system activated which result in production of antibodies. These phage specific antibodies rapidly inactivate the phages. This is the major obstacles for phage therapy. However, antibodies are not produced, if local and oral route of administration used. The issue of bacteriophage interactions with the mammalian immune system and its components is still not precisely defined. Evidence increasingly suggests that phages influence mammalian immune responses, including the attenuation of specific and

nonspecific immune reactions, and maintenance of local immune tolerance to gut microorganism-derived antigens. Although innate immunity cannot be entirely separated from its adaptive counterpart, for the purpose of clarity, we will also discuss antiphage immunity according to the mechanism(s) of neutralization and clearance (67).

9.3 Intracellular pathogens

Many pathogens such as *Mycobacterium tuberculosis*, *Salmonella Typhimurium*, *Rickettsia*, and *Chlamydia* etc. are intracellular means they multiply inside the host cells and make pathogens inaccessible by phages. Thus the infections caused by these intracellular bacteria cannot be treated by bacteriophages. Therefore use of bacteriophage is limited in case of intracellular pathogens.

9.4 Narrow host range of phage

The bacteriophage has narrow host range. When patients are infected with multiple bacteria, in this situation bacteriophage therapy will not effective (27).

9.5 Development of resistance

Like human immune system bacteria also have innate immune system (e.g. Restriction –modification (R-M) system and acquired immune system (e.g. CRISPR-Cas system) that cleave and destroy phage DNA entering in to cells. Thus bacteria developed resistance against bacteriophages, which result in failure of phage therapy (68). In addition to above, phage preparations contain bacterial debris and endotoxins, failure to establish scientific proof of efficacy and development of lysogeny limits the use of phage therapy (69, 70).

10. Future directions

There is increasing interest in ‘Phage therapy’ as possible new drug with hope to cope with multi drug resistance bacterial infections. Despite approval of some phage formulation by agencies like FDA, US-FSIS and GRAS in various applications in the field of food and biomedical sectors, there still a lot of concerns associated for their commercial acceptance. If not carrying a variance factor most of phages are supposed to be safe for human beings, but increasing efforts to make recombinant viral particles raises several biosafety concerns over possibility of bringing new genetic traits in bacterial populations. So, new methods must be developed to reprogram the phages without any genetic modifications to possess auxiliary mechanisms for phage adherence/binding and uptake that are critical for plaque formation. Another core area that need more focus in phage therapy is to allow a better selection of lytic phages for reprogramming them. This should be capable of converting naturally occurring wild phages into smart phages with a broader range of host specificity that can overcome a bacterial defense mechanisms. These findings encourage new optimism and a re-evaluation of the potential for phage therapy.

Acknowledgements Authors acknowledging the financial support from Indian Council of Medical Research (ICMR), Council of Scientific and Industrial Research (CSIR), Department of Science and Technology (DST) and University Grant Commission (UGC) New Delhi, India.

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