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Case study

BACTERIOPHAGES: AN ANTIBIOTIC ALTERNATIVE

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ABSTRACT
Bacteriophages are the largest known virus group and are the most numerous biological entities in the biosphere which can kill bacteria but are harmless to eukaryotic cells. The efficacy of bacteriophages to prevent and treat human and animal diseases has been shown in both laboratory and commercial field studies, without any adverse effects in the animals. The most serious challenge with the antibiotic is appearance and spread of multidrug-resistant pathogenic bacteria on which phage therapy seems to be promising alternative. This review will briefly describe the general characteristics of bacteriophages, difference between antibiotics and bacteriophages, therapeutic phage preparations, phage therapy and potential problems of phage therapy with their possible solutions.

KEY WORDS: Diseases, antibiotics, resistance, bacteriophages and phage therapy.

INTRODUCTION

Bacteriophages are the most abundant organisms on earth and are ubiquitous in nature. They are commonly referred as “phages”. These are the viruses that infect bacteria. They are present on the surface as well as deep water ecosystems, soil, oral cavity, blood and gut of healthy humans and animals (Sulakvelidze *et al.,* 2001). Some bacteriophages are highly specific attacking only certain bacterial strains which allow them for the treatment of a targeted bacterial infection, while others are quite broad in their host range (Skurnik and Strauch, 2006). In 1896, Ernest Hankin first reported the presence of an unidentified substance in the water of rivers in India which have an antibacterial activity against *Vibrio cholera*. Two years later, Nikolay Fyodorovich Gamaleya a Russian bacteriologist observed a similar phenomenon while working with *Bacillus subtilis*. In 1915, English bacteriologist Frederick Twort re-introduced the subject by advancing the hypothesis that such antibacterial activity could be due to a virus. Finally, the phages were ‘officially’ discovered by Canadian Felix d’Herelle in 1917 (Hermoso *et al.,* 2007). He coined the term “Bacteriophage” and was the first to use bacteriophages for therapeutic purposes (Sulakvelidze *et al.,* 2001). Since phages were discovered in 1915 and 1917 they have been used for treating bacterial infections in humans and animals in Eastern European countries and by former Soviet Union (Payne *et al.,* 2000). During World War II, bacteriophages were successfully used to treat bacterial infections on the battlefield by the former Soviet Union; such treatment undoubtedly saved the lives of numerous soldiers (Duckworth *et al.,* 2002). Before 1940, a variety of infections including sepsis, suppurative wounds, dermatitis, gastroenteritis, osteomyelitis, emphysemas and pneumonia were cured by phage therapy in human. In these experiments, there was no undesirable reaction and the success rate was about 80 percent to 95 percent. After a short period of development of phage therapy, the developmental focus of antimicrobial therapy shifted from phage therapy to chemotherapy since, Alexander Fleming and Gerhard Domagk discovered penicillin and sulfas in 1928 and in 1932, respectively. After this discovery, the numerous drugs with antimicrobial effects have been produced and used as a therapy to treat bacterial infections (Hagens *et al*., 2010; Boriea *et al.,* 2014).

Antibiotics are not only essential for human health but also for the well-being of plants and animals (Jing, 2012). Antibiotics are naturally occurring, semi-synthetic and synthetic compounds with antimicrobial activity that can be applied parenterally, orally or topically (Geidam *et al.,* 2009). Most agricultural livestock receives antibiotics to prevent and treat infections that can spread through farms. Discovery of such antibiotics have saved millions of lives, but indiscriminate use of such antibiotic kills healthy bacteria in the digestive track and they pollute the environment when animal and human excretes these antibiotics. An increase of bacterial resistance to such antimicrobials is now becoming a subject of global concern in human and veterinary medicine (WHO, 2000). Infectious disease experts have warned that now there is a compelling need for totally new classes of antibacterial agents to combat antibiotic-resistant bacteria prophylactically and therapeutically. Phage therapy represents such a ‘new’ class without harmful effects on the ecosystem and human life on which scientists have begun to research (Hermoso *et al.,* 2007; Nath, 2013). It is the time to re-evaluate the potential of phage therapy as a promising agent to control multidrug-resistant bacteria (Masoud *et al.,* 2012). This review will briefly describe the general characteristics of bacteriophages, difference between antibiotics and bacteriophages, therapeutic phage

preparations, phage therapy and potential problems of phage therapy with their possible solutions.

Bacteriophages: General characteristic

Bacteriophages are the largest virus group, therefore; their classification is very necessary to identify novel and therapeutic phages. Many scientists have proved that phages differed in size and are morphologically diverse (Chibani *et al.,* 2004). About 96% phages have a tail, constituting the Order Caudovirales and the three families characterised by contractile, long and noncontractile or short tail and named respectively Siphoviridae, Podoviridae and Myoviridae. Polyhedral, filamentous and pleomorphic phages represent less than 4% of these viruses (Dabrowska *et al.,* 2005). Bacteriophages come in many different shapes and sizes but all share several common structural features i.e. capsid (Head), genomic material, tail (optional), base plate and fibers (optional). The size of the phages ranges from 24-200 nm in length. Head encloses nucleic acid which can be either DNA or RNA but not both. Some phages have a tail attached to the phage head. Phages like T4 have a base plate at the end of the tail, and one or more tail fibers attached to it.

Bacteriophages can follow two different destinations regarding the infection of their host. Lytic (virulent) phages follow the lytic infection cycle wherein one or more phage particles simultaneously adsorb to a single bacterial cell, for this purpose phages can use bacterial capsules, flagella, fimbriae, different parts of lipopolysaccharides (LPS) and many other surface proteins as receptors. After attaching phages inject their genome into the bacterial cell leaving the phage coat outside. Next step in the replication is the transcription of the phage DNA to produce mRNA which is translated to phage proteins. Further, phage coat proteins, other protein components and DNA are produced separately, during this process host DNA degrades. Lastly, the phage components are assembled into mature virions and phages lyse the bacterial cell and active phages get release into the environment.

On the other side, the lysogenic (tempered) phages use the lysogenic pathway, where the phage injects the genome in the bacterial cytoplasm which then integrates (prophage) and replicates as part of the host genome and remains latent for extended period; if the host bacterium faces adverse environmental conditions this prophage may activate and return to the lytic cycle, and later the newly formed phage particles are released after bacterial lysis (Skurnik and Strauch, 2006). There are three reasons to avoid temperate phages as therapeutic agents. First, the genome of temperate phages may contain genes which alter the phenotype of the host cell when a lysogen is established, a process called lysogenic conversion. The second concern is establishment of lysogeny, which causes the lysogen to become immune to superinfection by the same phage or related phages and the third consideration is that many temperate phages are capable of generalized transduction, which is the phage-medated movement of large amounts of bacterial DNA from one host to another (Gill and Hyman, 2010).

Antibiotics versus bacteriophage

Sr. No.	Antibiotics	Bacteriophages
1.	Non-specific mode of action (i.e. active against a wide range of bacteria) therefore, destroys the commensal microflora.	Very specific (i.e. active against particular bacterium) therefore, reduces the possibility of secondary infections.
2.	Multiple side effects, including intestinal disorders, allergies, and secondary infections have been reported	No serious side effects have been reported.
3.	Numerous molecules and repeated administration of the antibiotic is necessary to kill a given bacterium.	Multiple administrations are not required because as long as the target bacterium is present, the phage will be able to reproduce (self-perpetuating).
4.	Antibiotics are expensive to produce.	Bacteriophages are cheap to produce
5.	They are metabolized and eliminated from the body and do not necessarily concentrate at the site of infection.	Replicate at the site of infection and are thus available where they are most needed.
6.	Developing a new antibiotic against antibiotic resistant bacteria is a time- consuming process and may take several years	Selection of new phages against phage-resistant bacteria is a relatively rapid process that can frequently be accomplished in days or weeks.

Therapeutic phage preparations

Samples for the phage isolation are taken wherever the pathogens of interest are abundantly present such as water, soil, plant cuttings, faecal material, sewage effluent, etc. To isolate the phage directly from any biological sample, the sample is first sterilized to remove contaminating cellular microorganisms. The sterilized sample is then directly plated on one or more host strains and further evaluated for the appearance of plaques or prior to plating samples may be concentrated by employing any number of methods including tangential flow filtration, direct-flow ultrafiltration, superspeed centrifugation or even flow cytometry etc. Phages must be characterized to confirm that they are active against any important bacterial target strain and to determine whether they have the properties that are suitable for the intended applications.

On the other hand, instead of sample concentration the samples may be enriched for the phages of interest by culturing the sample in the presence of one or more of the desired bacterial hosts. This step serves amplification of the phage that can infect the prepared bacteria. After enrichment, phages are isolated by simple centrifugation

which separates phages from bacterial hosts. The supernatant is then heated to kill other host cells or bacterial cell debris followed by sterile filtration through a membrane filter. After checking sterility, the purified phage preparations are processed into final products in the form of a liquid, gel, or dried phage preparations. Enriched phage preparations can be stored in the refrigerator until further use. Phage cocktails can be prepared by combining phylogenetically diverse phages into a single phage therapy product for trials (Meaden and Koskella, 2013).

The method of therapeutic phage preparation is very briefly explained in this review but other scientists viz. Gill and Hyman 2010 discussed in detail in their review about the methods of isolation, analysis and identification of phage species for phage therapy. They also discussed the various methods available for the purifying phages as well as considerations of the degree of purification for various applications.

Phage therapy

Phages as therapeutic agents in humans were first used in Paris in 1919 when D'Herelle used oral phage preparations to treat bacterial dysentery and in 1925, he reported treatment of plague by antiplague phages (Pires *et al.,* 2015). Similarly, in veterinary medicine, the first published investigation dates from 1941 (Slanetz and Jawetz, 1941). The efficacy of bacteriophages to prevent and treat animal diseases has been shown in almost all production animals in both laboratory and commercial field studies, without any adverse effects. D'Herelle's commercial laboratory in Paris produced five phage preparations against various bacterial infections (Summers *et al.,* 1999). Therapeutic phages were also produced in the United States. In 1940, the Eli Lilly Company (Indianapolis, Ind.) produced seven phage products for human use. The early strong interest in phage therapy is reflected in the fact that some 800 papers were published on the topic during 1917-1957 (Sulakvelidze *et al*., 2001). The efficacy of phage preparations was controversial after the arrival of the golden era of antibiotics regarding the use of phages due to the paucity of appropriately conducted, placebo-controlled trials. The interest in phage therapy decreased sharply and commercial production of therapeutic phages ceased in most of the Western world (Eaton *et al.,* 1934, Sulakvelidze *et al.,* 2001). Subsequently, phage therapy was rediscovered in the English-language literature starting with the work of Smith and Huggins in the 1980s. The field finally began maturing from those days of the 1990s starting approximately in the year 2000 (Abedon *et al.,* 2011).

The history of phage therapy has been recounted in some depth and from various perspectives in a number of excellent reviews; Abedon *et al.,* (2011) focused on potential of phages to treat bacterial infections, therapeutic applications of phages in veterinary medicine and various aspects of human phage therapy; Borie *et al.,* (2014) reviewed some *in vivo* studies of phage therapy in veterinary medicine, specifically their application as a therapeutic tool in several animal species similarly, Sulakvelidze *et al.,* (2001) emphasized in minireview on some of the major human phage therapy studies performed in Poland and the Former Soviet Union.

Phage therapy limitations and possible solutions

There are so many attributes of phages that would tend to favour a therapeutic response although; applications of phages as an antibacterial agent orally or topically have some potential problems. Lack of understanding of the heterogeneity and mode of action of phages or failure to differentiate between lytic and lysogenic phages may results in the horizontal transfer of bacterial toxin gene, antibiotic resistance gene, etc., by lysogenic phages. The presence of host bacteria in therapeutic phage preparations or contamination of therapeutic phage preparation with endotoxin or exotoxin even in minute concentration can be fatal to the patient. Rapid uptake and inactivation of phages by spleen, liver and other filtering organs of the reticuloendothelial system may reduce the number of phages to a level which is not sufficient to combat the infecting bacteria. The development of phage-neutralizing antibodies is another possible problem which may hamper phage effectiveness in lysing targeted bacteria in vivo.

However, Carlton (1999), Sulakavelidze (2001), Inal (2003), Ghannad and Mohammadi (2012), Adhya *et al.,* (2014) and Gandham (2015) have explained the possible solutions on these problems in their reviews. For therapeutic phage preparation careful selection of lytic phages is most important; therefore either screen the bacteria against a panel of phages, to ensure that the selected phage strains is lytic or develop "multivalent" phages that lyse all or most of the bacterial strains. To ensure that phage preparations do not contain live bacteria (sterile preparation), chemicals like mercurials or oxidizing agents can be used or phage preparations can be heat treated but such treatments may inactivate the phages; in such conditions viability and titre of phages should be determined before using them therapeutically. To remove endotoxin or exotoxin; different methods of purification such as ion exchange chromatography, density centrifugation, banding and other methods of purification can be used to get phage preparation of high purity. To address the issue of removal of phage by reticuloendothelial system "serial passage" method can be used; this is the natural selection strategy where phages having an increased ability to remain in the circulation can be selected for the phage therapy. To overcome the problem of phage-neutralizing antibodies phage cocktails can be used which are having two main purpose one is to overcome bacterial resistance and another is to streamline "real world" clinical therapy (Gill and Hyman, 2010).

An easy way to solve many of the aforementioned problems with "classical" bacteriophage therapy is the use of phage product instead of the whole phages. Bacteriophage endolysin is one of the very promising and novel alternatives. There are two examples of these products. The first one is capsid protein A2 that is derived from the RNA phage Qβ (Bernhardt[a] *et al.,* 2001) and the other example is a lysis protein called E protein of phage φX174 (a kind of DNA phages) (Bernhardt[b] *et al.,* 2001). A2 protein works like penicillin and E protein blocks the bacterial peptidoglycan synthesis pathway. However, we need to develop the technologies for preservation of phage strains; purification of phages; preservation of ready to use phage preparations; safe dosage; safety of phage therapy; human trials and many other unknown ethical issues.

Phage therapy is needed more in the developing world than in western countries. The most important point while experimenting on phage therapy is controlled, double-blinded placebo studies with highly purified, lytic phages should be conducted, and results must be evaluated based on both clinical observations and scrupulous laboratory analysis.

In summary, bacteriophages have a dominant role in controlling bacterial population in the natural environment. In India, there are few study groups which are working in this area. As the technology was born on the banks of the Ganges, India should take the lead to use bacteriophages not only in humans and animals but in agriculture also because bacteriophages have several attributes which make them potentially attractive therapeutic agents. The most important fact with the phage therapy is that the bacteria keep on developing resistance against the phages but the new phages will keep on appearing in the environment and for this reason, we must continuously keep on isolating new local phages for the local strain of the bacteria.

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