Bacteriophage: A novel tool for the eradication of biofilm

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Abstract

Biofilms are bacterial communities that live in association with biotic or abiotic surfaces and enclosed in an extracellular polymeric substance. Their formation on both biotic and abiotic surfaces, including human tissue and medical device surfaces, pose a major threat causing chronic infections. In addition, current antibiotics and antiseptic agents have shown limited ability to completely remove biofilms. In this review, the authors provide an overview on the formation of bacterial biofilms and its characteristics, burden and evolution with phages. Moreover, the most recent possible use of phages and phage-derived enzymes to combat bacteria in biofilm structures is elucidated. From the emerging results, it can be concluded that despite successful use of phages and phage-derived products in destroying biofilms, they are mostly not adequate to eradicate all bacterial cells. Nevertheless, a combined therapy with the use of phages and/or phage-derived products with other antimicrobial agents including antibiotics, nanoparticles, and antimicrobial peptides may be effective approaches to remove biofilms from medical device surfaces and to treat their associated infections in humans.

I. Introduction

A. Bacteriophage

In 1915, Twort discovered bacteriophages as an unidentified molecule that inhibit bacterial growth. In 1917, D'Herelle was the first to isolate and characterize phages and also the phage therapy against few typhoid induced by *Salmonella Gallinarum*in chickens, was developed by him [1]. Positive outcomes of the use of bacteriophages in fighting bacterial infection have come up with the development of research on the potential use of viruses that demolish bacteria in the treatment of diseases both in humans and in animals [2].

Bacteriophages are a group of viruses, that are most abundant entities on earth. Their life cycle is strictly associated with bacterial cell. Bacteriophages are also known as bacterial parasites or viruses because of the absence of the cell structure and enzyme systems required for food uptake, protein synthesis or building of new particle, and as a deficient organism can only replicate in a live cell. The common occurrence of bacteriophages is a remarkable factor facilitating their accession and characterization of their aptness for opposing bacterial infections. Phages are isolated from natural environments which includes human and animal waste, waste water, natural water bodies, soil, food products and other microorganisms [3]. The genetic material encapsulated in these bacterial viruses in the form of either DNA or RNA, enclosed in a protein shell [4]. Phages can be divided into three groups in terms of DNA structure: those containing DNA in the form of a double helix, those with a single strand of DNA and phages containing RNA. Mostly known bacteriophages have a genome that consists of double stranded DNA. On the basis of capsid symmetry two types of bacteriophages are differentiated they are isometric (polyhedral) and helical (spiral) [5].

- **Phage replication cycle:**

Replication of bacteriophage and eukaryotic viruses are alike in numerous ways. Both require adsorption, penetration, replication of nucleic acids, formation of virions and their liberation from the host cell. Bacteriophages are specifically linked with a specific bacterial strain and unveil strong bactericidal activity against Gram-positive and Gram-negative bacteria. Some bacteriophages exhibit specific affinity for single types of bacteria, while others have a broad range of activity. The specificity and range of activity of bacteriophage is determined by the presence of receptors situated on the surface of bacterial cells, amidst which we can differentiate LPS fragments, fimbriae and other surface proteins [6].

Other than filamentous phages, most of the phages have polyhedral capsid. This capsid is attached to a tail consisting of fibres, these fibers are used for the attachments to receptors or bacterial cell surface [7]. Phages contaminate bacteria and can proliferate in two possible ways; lytic life cycle and lysogenic life cycle.

- **Lytic cycle:** Lytic cycle, which is a feature of virulent phages, consists of adsorption, which involves adherence to the bacterial cell, and binding of phage protein to formerly recognized receptors on the cell surface of bacteria, such as teichoic and lipoteichoic acid for Gram-posotive or lipopolysacchride (LPS) for Gram-negative bacteria [8].
- **The penetration phase:** This phase consists of the rupture of the cell wall of the bacteria by the bacteriophage enzymes and insertion of the genetic material into the host cells.
- **Eclipse phase:** Next phase if the eclipse phase, which involves the replication of nucleic acid and proteins comprising the structural part of the capsid, while replication of the bacterial DNA is subdued. This is accompanied by the formation and maturation of the bacteriophage, lysis of the bacterial cell and the liberation of daughter phages. These daughter phages are then capable of infecting other cells [9].

T1 and T4 are the examples of bacteriophages that undergo lytic cycle.

- **Lysogenic life cycle:**

This cycle involves the integration of the genetic material of the bacteriophage with the bacterial chromosome and replication as part of the bacterial DNA, resulting in the appearance of a prophage [6]. When the phage is living inside the chromosome it is known as a prophage and is replicated along with the bacterial genome during cell replication. A few times, prophage may encode virulence genes, which can be horizontally transferred from one bacterium to another by the process called transduction [10]. Bacteriophage with a lysogenic cycle includes λ *Escherichia coli*; Mu, with activity against *E. coli, Salmonella, Citrobacter and Erwinia*; MM1 S. *pneumoniae;* and φ11 *S. aureus* [11].

There are several different pathways of bacteriophage infection which include chronic infections, pseudolysogeny and abortive infection, which depends on environmental factors and the type of bacterial cell. Not all the results into the death of the bacterial cell and replication of phage particles. In many cases daughter virions are produced without introduction of lysis of the bacterial cells, and thus the viral particles are not released outside the cell [9]. If any harsh conditions like ultraviolet (UV) radiations is introduced then the prophage will disappear via lysis of bacteria.

Phages are essentially found everywhere and are known to infect >140 bacterial genera and can be regarded as the most abundant biological entities with estimation of 10^{31} phage particles in the world [12].

B. Biofilm: Formation, dispersal and the risk of dissemination.

The formation of a Biofilm consists of various different phases such as reversible attachment, irreversible attachment, colonization, maturation and dispersion. Micro-organisms living inside a biofilm have distinct mechanisms that enable initial surface attachment, the development of a colony formation and ecosystem, and successive separation from the biofilm. The attachment of micro-organisms to a surface can be accelerated by factors such as increased shear forces, bacterial mobility, and electrostatic interactions between the microorganism and surface. In a state of 'reversible attachment' there is thought to be equilibrium between attached and free-floating microorganisms. However, there are traits of the microbial cell surface that facilitate the attachment procedure to the surface, inclusive of flagella, pili, fimbriae and glycocalyx [13]. In terms of microbial attachment to medical devices, the adhesion of bacteria to biomaterials through cell surface and biomaterial-surface interactions has been outlined. For example, staphylococcal species exhibit cell surface proteins, specifically staphylococcal surface protein-1 and -2 (SSP-1 and SSP-2), confined on the cell surface on a fimbria-like polymer and associated with the adherence of *S. epidermidis* to polystyrene [14]. In addition, the capsular polysaccharide/adhesin has a part to play in the adherence of clinical isolates of coagulase-negative staphylococci to biomaterials [15]. Moreover, the protein autolysin (AtlE) in *S. epidermidis* has been connected with the adhesion of this microorganism to a polymer surface; this protein grant not only the potentiality to adhere to a polystyrene surface but also the capability to bind to vitronectin, thus signifying a role for cellular adhesion to plasma protein-coated polymer surfaces during the upcoming stages of bacterial adherence.

- **Molecular Signalling of Biofilm:**

As the cell populace density of the maturing biofilm varies, the gene expression of cells within the biofilm is managed by a process known as quorum sensing (QS). Through this system, bacteria release chemical indicators called autoinducers, which are constitutively produced and increase in concentration as the density of the biofilm increases. Some integral factors that are required for the survival of micro-organisms within biofilm are lined up by physiological processes which include motility, sporulation and release of virulence factors, all these changes take place when the concentration of the auto inducers reaches a critical threshold which further leads to modifications in gene expression. Molecules called acyl-homoserine lactones and oligopeptide molecules are released by gram negative bacteria and gram-positive bacteria respectively [16]. It has been reported that, *Pseudomonas aeruginosa* biofilm are linked with N-(3-oxo-dodecanoyl)-L-homoserine QS molecules. Additionally, it has been studied that N-(3-oxo-dodecanoyl)-L-homoserine QS molecule is responsible to suppress the host immune responses and increase in *Pseudomonas aeruginosa* biofilm virulence property [17].

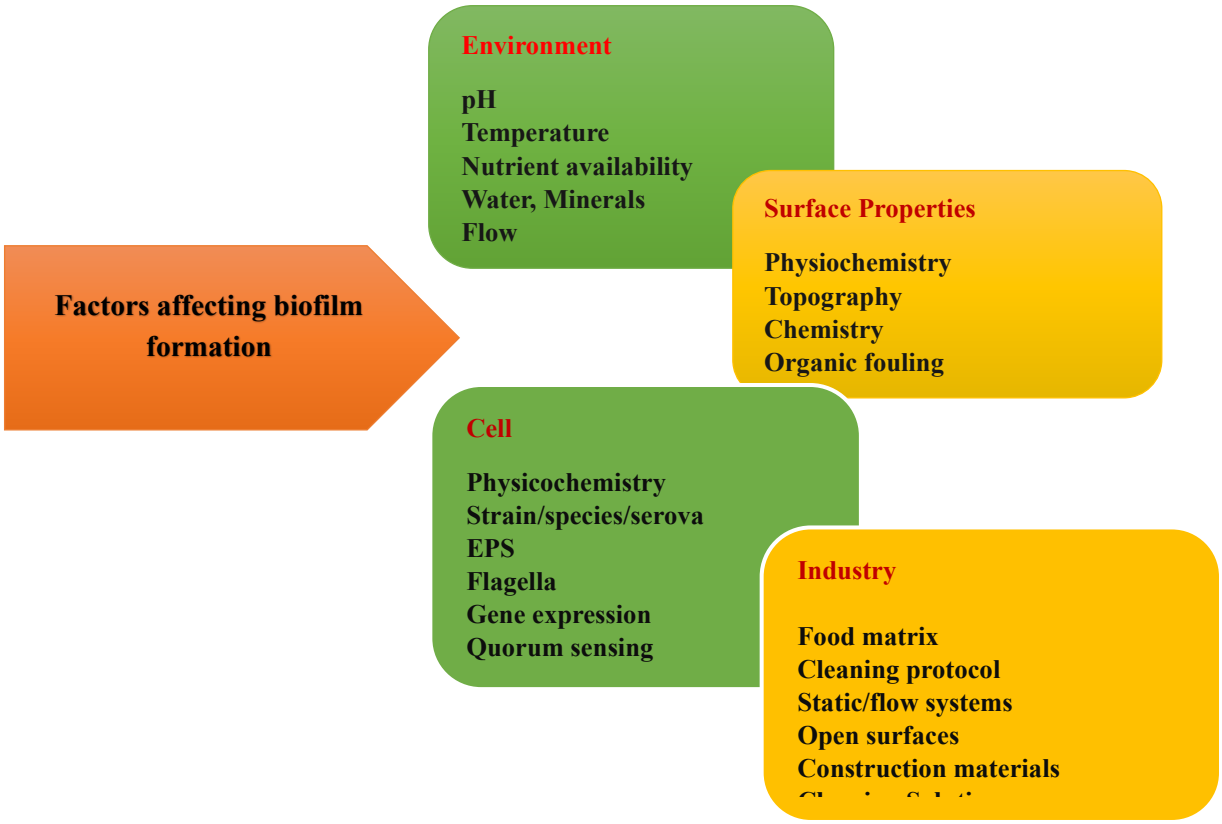
- **Dispersal or detachment of bacteria from a biofilm and the risk of dissemination**

A severe ultimatum give rise to the host, when parts of the biofilm sheds and are capable to form colonies on new sites, this approach is known as biofilm dispersal which further stimulates distribution within the host (Donelli, 2006). Biofilm dispersal is a process that takes place towards the end of the biofilm life cycle, during this phase the cells that were once part of a complex, relatively static, slow growing micro-populace within the biofilm becomes distinguished, frequently highly motile micro-organisms [18]. It is important to note that these diffused cells are different from bacteria that slough off from the biofilm and are truly specialized cells, furthermore these dispersed cells are capable to initiate biofilm growth by attaching to new surfaces.

An intracellular molecule cyclic-di-GMP (c-di-GMP) has been reported to control the transitions from biofilm to planktonic phenotypes. Furthermore, it has been studied that the reduction in intracellular c-di-GMP can lead to dispersal in some microorganisms. This

intracellular molecule act as a secondary messenger molecule within a biofilm and is responsible for intracellular mechanism of dispersal [19]. Biofilm dispersal cannot only affect the motile micro-organisms but can also affect non-motile micro-organisms. In the case of *S. aureus*, not only has the repression of the agr-related QS regulatory gene been shown to play a role in biofilm formation, but its activation has also been reported to induce the release of *S. aureus* cells from the biofilm. Some miscellaneous factors such as changes in nutrients, temperature and oxygen level, initiate dispersal process. Sometimes micro-organisms inside the biofilm, with the help of chemical signals such as acylhomoserine lactones, diffusible fatty acids and peptides can also influence dispersal [20].

II. Factors influencing biofilm formation



- **Biofilms and Health care associated infections**

Now a days indwelling medical devices such as urinary catheters, central venous catheters, endotracheal tubes (ETTs), intrauterine devices, prosthetic heart valves, peritoneal dialysis nephrostomes and other indwelling devices are becoming vital tools in the clinical management of hospitalized patients. The use of these indwelling medical devices on hospitalized patients has been increasing day by day, they are applied to more than 25% of hospitalized patients. The risk factors related to microbial infections and the formation of biofilm reaches to pinnacle in higher morbidity and mortality rates among hospitalized patients. These risk factors are directly related with the duration of the embedded medical device in a hospitalized patient, the longer the duration the more the patient is at risk of biofilm formation [13].

Hospital care associated infections (HCAIs) can be developed by number of risk factors such as prolonged hospitalization, immune-compromised patient (following chemotherapy, transplant patient for instance and those with inherited disease), invasive surgery and home wound management (HPA, 2012a). The most broadly communicated origin of HCAIs is the hospital 'superbug' methicillin-resistant *S. aureus* (MRSA), a common cause of septicaemia or bacteraemia in clinical devices. Though number of agents are responsible

for the formation of biofilm on medical devices like fungi, parasites, viruses and prions, but bacteria are the most common for the HCAIs (HPA, 2012b). The transmission of micro-organism to the medical devices may happen through the patient's or healthcare worker's skin, contaminated water or other external environmental sources. A very small number of micro-organisms leads to the contamination of the medical devices, which is further transferred to the device in question. Although a wide range of micro-organisms has been studied regarding medical device- associated infections, but the micro-organisms that are commonly linked with the formation of biofilm on medical devices and are extensively accepted as a major origin of HCAIs are *S. epidermidis* and *S. aureus* [14].

Micro-organisms such as *A. baumannii*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* have been recognized as multidrug -resistant Gram-negative bacteria and are becoming more widespread in long-term care facilities and acute care hospitals. In actuality, these species are frequently the reason of biofilm-based HCAIs, including catheter–associated urinary tract infection (CAUTI) [21].

III. Phage therapy

In 1919, phages were first used as a therapeutic agent in human, that was the time when they were just discovered [22]. Phage therapy started back in 1896, when the existence of antibacterial activity against *Vibrio Cholera* the causative agent of cholera was first reported by Ernest Hankin, cholera was considered one of the deadliest peril humans had faced. Frederick Twort, in 1915 hypothesized that antibacterial activity could be due to the phage, but he did not follow his discovery therefore in 1917, bacteriophages were discovered by Fe'lix d'He'relle. In 1925, d'He'relle drew attention towards phage therapy by reporting the treatment of plague (four types) by anti-plague phages. The Eliava institute in Tbilisi Georgia is considered the pioneer in this regard where phage therapy is extensively studied and applied [23].

A. Phages as antibacterial agents:

Phages are prominent candidates for exploitation as antibacterial agents as they are the natural killers of bacteria. Phages have multiple intrinsic characteristics which make them delightful candidate for such applications. Phages cannot incorporate their DNA into the genome of eukaryotic cells or can replicate in such cells. They are highly specific in their bactericidal potential. Some phages are strain specific and they normally target a single bacterial species, which means that there is little or no effect on the natural microbiota of the patient/animal. This is a powerful advantage over the many broad range anti-microbials (including antibiotics) which are commonly used now a days. Besides, there have been no reports of harmful effects on eukaryotic cells by purified phages. Phages are non toxic in nature because humans are exposed to phages in the natural environment every day without any adverse side effects. Phages multiply in the presence of the target bacteria and are not able to multiply in its absence and thus ultimately get eliminated from the human/animal which indicates that the phages are considered to be self-dosing. Phages also have the aptness to lyse bacteria present in a biofilm, mucous membrane and suppurative wounds. These are bacterial niches, which are unsuitable for treatment with antibiotics [24].

B. Other potential applications of bacteriophages:

- **Phage display:** In 1985, the concept of phage display was first introduced by Smith. Phage display was possibly the first phage applied as a tool of modern biotechnology. Phage display is a unique molecular technique used for synthesizing polypeptides with novel characteristics. In this technique, the DNA that encodes the protein is fused with phage coat protein gene and the desired protein is expressed on the surface of the phage particle or bacteriophage. The most extensively used phage in phage

display technique is filamentous phage M13 of *Escherichia coli,* other than this, phages like lambda and T7 phage have also been widely used in phage display system [4].

For the purpose of screening and isolation of peptides, phage libraries can be used, these peptide molecules are highly specific and which have affinity for target proteins. These highly specific peptides can be applied in drug designing as reagents for interpretation of molecular recognition and it also reduces the limitations for receptors. These peptides then further can be used as therapeutic agents by inhibiting the interaction between receptor-ligand or acting as agonist [25].

Other than this, phage display can be used for various applications which include mapping of epitopes, in vaccine design, in study of interaction between protein-protein, in determination of specificity of enzymes and inhibitors, screening for anticancer peptide and protein and in the screening for receptors [26].

- **Phage typing:** Bacteriophage infect and demolish only one or a few types of bacteria, which makes them highly specific to their host. This specificity of phage is very helpful and are widely used for bacterial cells which allows them to be used for the typing of bacterial strain, technique known as phage typing and for the detection of pathogenic bacteria [27]. In phage typing, specific phages use sensitivity patterns for precisely identifying the microbial strains. If the phages, that are bound to bacteria are detected by specific bacterial antibodies then the sensitivity of the detection could be increased [28]. For the purpose of detection of unknown bacterial strain the lawn of unknown bacterial strains is provided with various different phages, once the phages starts infecting and lysing the specific bacterial strains formation of a plaque (clear zone) appears on the lawn, which makes it easy to identify the specific bacterial strain. Additionally, there are certain other techniques that can be employed are also widely used for the detection of pathogenic bacteria for example use of phages that deliver reporter genes (eg. Lux) specifically or use of green fluorescent proteins that would express after infecting the bacteria [4].
- **Phages used as vehicle for the delivery of vaccines:** Bacteriophages have been widely used as vehicles for the delivery of vaccines. Bacteriophages can be employed in two different ways. Firstly, phages can be used directly, in which they transport the vaccine antigen that is displayed on their surface. Secondly, in DNA vaccine, in this the gene that is responsible for the synthesis of vaccine antigen, is first incorporated into the phage genome then this phage would act as a vehicle for the delivery of DNA vaccines. Phages can be constructed using phage display technique, that further would display the antigenic peptide on their surface [4]. In some studies, whole phage particles that displayed antigenic peptides have been used as vaccines in animal models.
- **Phages used in targeted gene delivery:** Bacteriophages have been potentially and widely used as specific gene delivery vectors [29]. The phages that are used for the purpose of targeted gene delivery is similar to that for using phages for DNA vaccine delivery in which the DNA inside is protected by phage coat. This phage coat protects the DNA from degradation after it has been injected. The ability of phages to display foreign proteins on their surfaces allows them to target specific cell types which is essential for successful gene therapy. For the purpose of display targeting and processing molecules on the surfaces of phages, phage display and artificial covalent conjugation is widely used [30].
- **Phages therapy in plants:** For the elimination of plant pathogens, bacteriophages have been widely used as a bio-control. Phage mediated bio-controls of plant pathogens such as *Xanthomonas pruni* associated with the infection of cabbage, peppers

and peaches, *Ralstonia solanacearum* cause infection in tobacco. They have been successfully used against *Xanthomonas campestris* cause spots on tomatoes and against *Pseudomonas talaasi* which cause blotch of mushrooms [31].

Application of phage therapy in plants is still in the early stages. For the use of phage therapy, new phages are being discovered and constantly been utilized in many different ways. Treatment of diseased crop with the help of phage therapy has provided promising results, including the treatment of some antibiotic resistant infections involved in bacterial blight on soyabeans [32]. Number of phages have been approved by the United States Food and Drug Administration (FDA) for use on crops meant for human consumption. In order to control, bacterial pathogens in environmental, medical settings, and food processing and also in the expectation of reducing product loss and production cost, many companies and organizations are concentrated on the discovery, isolation and marketing of bacteriophage products [33].

- **Phage therapy in animals:** Bacteriophages are widely used for the treatment and elimination of infection in chickens, claves, pigs and lambs, infections caused by *Escherichia coli* and *Salmonella*. In 1983, experiment performed by Smith and Huggins proves that, a single dose of phage R is very effective in preventing death in mice due to septicemia caused by *E. coli*, this infection cannot be treated with multiple doses of antibiotics. They eventually used bacteriophages for the treatment of infections caused by *E. coli* (entero toxigenic) in neonatal lambs, calves and pigs [34]. Animal studies have shown that phage therapy works in animal system. However, many experiments with animals, recommended that the therapeutic application of phages in animals have some advantages over antibiotics.
- **Phages in food industry:** Foodborne diseases are the contamination of food that are caused by many pathogenic bacteria. Currently, food borne pathogens like *Listeria* and *Salmonella*, followed by *E. coli* and Campylobacter *jejuni* are the major cause of death. Bacteriophages can be used effectively and safely to eradicate the food born pathogenic bacteria that contaminate the food. For example, *Listeria* phage P100 (under the commercial name Listex P100) was developed to eradicate biofilms present in processed meat products and on factory working surfaces, and has already been authorized in the United States by the Department of Agriculture (Fister *et al*., 2016). Other commercial bacteriophages products have been targeted for other pathogenic species as for example *S. enterica* or *E. coli* (Salmofresh^{TM} and ScoShield^{TM}, respectively) [35].
- **Eradication of biofilm by phages:** Formation of biofilm is an important strategy that is adopted by the bacteria for the survival purpose. During this strategy, microorganisms secrete huge amount of extracellular polymer. Microorganisms' aggregates to form biofilm either on living or non-living surfaces. Normally, environmental conditions stimulate most of the bacteria to form biofilm. In humans, formation of biofilms causes severe diseases and the biofilm is resistant to antibiotics and host immune system. An alternative therapy must be needed to control biofilm-associated diseases, since biofilms are resistance to anti-microbial drugs [36].

Nevertheless, phages are furnished with enzymes (eg. EPS deloplymerase) outside of the capsid that degrade the extracellular polymeric substance and disseminate bacterial biofilms, allowing the phage to enter the bacteria embedded within the EPS matrix. Once the lytic cycle is completed, the phage progeny is released, propagate the dispersal of the biofilm through the removal of biofilm embedded within bacteria in following layers. For the penetration of dense biofilms, high doses of antibiotics are typically required to observe any

inhibition of bacterial growth, yet complete eradication of biofilm is rare and at the end of antibiotic treatments re-growth of colonies begin [37].

In one of the studies, Gabisoniya found that the implementation of phages on *in-vitro* colonies of the pathogen *P. aeruginosa* not only prevented additional biofilm formation but it also degraded existing biofilm. Biofilm formed on the surface of the medical devices by *L. monocytogenes P. aeruginosa,* and *Staphylococcus epidermidis* have been eliminated via phage treatment [38].

These discoveries are highly admissible to the problem of persistent infections caused by implanted medical devices such as catheters, lenses, and prostheses where biofilm formation is common.

IV. Advantages of phage therapy

Bacteriophages are natural, potential and unique agents that have many therapeutic advantages over classical antibiotics. Bacteriophages are capable in regulating bacterial population by inducing bacterial lysis. Bacteriophages are known to be active against both Gram-positive and Gram-negative bacteria including those bacteria that are multi drug resistant pathogen in the environment [39]. Bacteriophages have some of the most desirable properties and important advantages that make them enthralling applicant for tackling antibiotic resistance in bacteria. Some of the most important advantages are given as follows:

- Bacteriophages are environmentally friendly.
- Isolation and identification of suitable bacteriophage for the phage therapy is rapid, relatively easy and cost effective.
- Bacterial resistance against phage develops about ten times slower than the resistance to that of antibiotic [40].
- Under very harsh environmental conditions, phages tend to replicate continuously and they remain infective until the host bacterial population density has been significantly reduced [41].
- Once the bacteria get lysed by the lytic phage it will not be able to regain its viability; by contrast antibiotic therapy, in which the targeted bacteria might not get killed, which facilitate the development of antibiotic resistance [42].

Apart from these generalized advantages there are some specific advantages that the phage therapy have, they are as follows:

- Phages are considered to self-dosing or auto-dosing, because phages are capable of increasing in number in the presence of host bacteria (depending on the host population density) and are not able to multiply in its absence and thus ultimately gets eliminated from the human/animal.
- Bacteriophages are highly specific for their host cell is another advantage of phage therapy over antibiotics. They target specific bacterial strain without affecting the normal beneficial bacterial micro flora of the body, due to this property the chance of secondary infections is reduced.
- Low inherent toxicity, phages are inherently non-toxic, since phages consist mostly of nucleic acids and proteins. Most of the phages exhibit relatively narrow host range which limits the number of bacterial types with which selection for specific phage-resistance mechanism can occur. Thus, the chances of resistance associated with phage therapy is low. Because when phages infect and kill bacteria, the mechanism they use is different from those of antibiotics, specific antibiotic resistance mechanism do not translate into mechanism of phage resistance.
- Bacteriophages are non-toxic to humans, thus there are no severe side effects and is suitable for use in humans, since phage do not infect eukaryotic cells [40].

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