Therapy of*Enterobacteriaceae* Infections using their specific Bacteriophages

Abstract: The *Enterobacteriaceae* are a family of bacteria, including many familiar pathogens that cause diarrhoea in humans and animals, such as *Escherichia coli, Salmonella, Shigella, and Klebsiella*. *Enterobacteriaceae* are bacilli (rod-shaped) facultative anaerobes. They ferment sugars to produce lactic acid and other end products. They are usually about 1-5µm in length. Most are motile because of the presence of many flagella; however, a few genera are non-motile. They do not form spores. Most *Enterobacteriaceae* members have fimbriae necessary for the adhesion of the bacterial cells to their hosts. They are economically important and thus a huge concern because they cause deaths in millions of people each year, resulting in a huge concern to curb their infection. There is an urgency to search for replacement therapies against bacteria in the *Enterobacteriaceae* family. This is because of the emergence of drug-resistant bacteria. To find a solution to this traumatic problem, studies have been launched in the areas of bacteriophages and their therapeutic application as a significant replacement to antibiotics. Bacteriophage therapy utilizes a different mechanism in destroying bacteria; hence, it is a better alternative to antibiotics. This paper sheds light on *Enterobacteriaceae* and bacteriophage therapy, as well as the history of bacteriophage therapy and its antibacterial mechanisms.

Comment [M1]: Diarrhoea signs not disease
The Enterobacteriaceae caused enteritis, nephritis, respiratory infection, endocarditis

Comment [M2]: First gram negative bacteria

Keywords: Bacteriophage, phage, *Enterobacteriaceae,* virus, phage therapy, phage formulation.

1. Introduction

Bacteriophages, also known as phages, are a broad group of viruses that infect bacteria and are easily manipulated for use in the area of biotechnology, research, and therapeutics. Phage therapy is recorded to be practiced time immemorial, for example, in France, since 1919, when d'Herelle treated children suffering from severe dysentery at the Hospital des Enfants Malades in Paris [1]. However, early uses of phage therapy were often tricky because of the tedious processes involved and, most importantly, the loss of interest in the use and study of phage therapy as a result of the production of penicillin, which was successfully purified in 1942, and by 1945, became kk available in pharmacies in the United States and Europe. Just at the dawn of the discovery of these antibiotics, they proved to work incredibly well. They cured many ailments, thereby saving many human lives. Nevertheless, since almost a century ago, the story has changed, and scientists are gradually and rapidly losing the fight again. We fear a time when bacterial infections like Septicemia and ventilator-associated pneumonia will not be manageable using antibiotics [2].

The whole world is currently in the middle of a severe problem due to resistance by microbial pathogens. Studies have shown that the core reason for antibiotic resistance is widespread abuse of antibiotics, which could either be the misuse or overuse of them. Current research also reveals that some bacterial bodies have become resistant to antibiotics, especially those produced explicitly for them. The resistance has occurred in many ways and is noted in

different pathogens. Important examples are the worldwide spread of Methicillin-Resistant *Staphylococcus aureus* (MRSA), infection and Vancomycin-Resistant Enterococci (VRE) [3].

It is now mandatory that we collectively agree that we are in a crisis, a critical point in treating infectious diseases. That, no matter how many drugs are formulated, they are no longer able to compete with the speed at which bacteria evolve and grow to defend themselves [4].

Since the onset of its application in 1996, bacteriophages have proven themselves useful in research as antimicrobial agents in the study of molecular biology [4]. From both past and even present research, using bacteriophages show a great sign of reliability [4].

Taking a glimpse at a critical point of view, combining more than one therapeutically beneficial bacteriophage (even with antibiotics) can lead to a meaningful outcome. More accreditation to phages is the fact that it can play the role of the vehicle for vaccines (both DNA & protein), which can help study pathogenic strains of bacteria and detect changes in many different proteins and antibodies. There are successful approaches effective against pathogens when trying to control them biologically in food safety and public health [4]. More so in agriculture and even in the petroleum industry, the use of phage as a bioagent can be very strategic. In human health, phages can be very effective in many diverse ways.

The concise information gathered in the piece by Golkar *et al.,* 2014 has expatiated on the prospects and widespread application accredited to phages in the systematic research fields of medical science and biotechnology.

Firstly, it can be used independently to combat infections by using the phage to lyse the bacterial cell due to its natural ability to do so. Secondly, it can be used as a mixture of more than one phage (a cocktail). This method combats many drug-resistant bacterial infections that refuse to respond to treatment to the latest generations of antibiotics. Thirdly, the versatile nature of phages also enhances the use of antibodies produced against the bacteria on the phage surface. The critical role of phage also extends to food spoilage and treatment of infection caused by bacteria in plants and fruits. Bacteriophage has a broad scope of application scaling from diseases diagnosing (through phage typing), its prevention (phage vaccines), to treatment (phage therapy) [4].

2. Phage Therapy History

In discussing phage therapy, the initial point of discussion would be the discovery of the entity phages itself. We give credit to Hankin, who happened to be the first person who made observations about bacteriophages. His observation was that of a presence of antibacterial activity going on against *Vibro cholerae* far back in 1896 [5]. Credit also goes to Gamelaya, who observed a similar process as he was working with *Bacillus subtilis* [6]. The subsequent discovery was the presence of plagues on *Staphylococcus aureus* cultures prepared by Twort. After much research in 1915, Twort concluded it was a viral infection that led to the formation of those plagues [7]. Three years along the research line, d'Herelle became the first to demonstrate treatment using phages. He successfully treated *Shigella* strains isolated from sick patients suffering from dysentery in his research. When recording his results, he observed small clear zones on the plates. He decided to name them 'bacteriophage'. He did this by merging two words; bacteria and phagein [1]. The second most significant stride

he made was when he firmly stood his ground to the opinion that phages were live viruses and not some sort of 'enzymes' as suggested by many others in the field at that time.

That stride was the beginning of research and studies in the line of phage therapy. People started using phages to treat infections. For instance, d'Herelle's anti-dysentery phage therapy was used to cure a boy and some set of patients who responded very well. Unfortunately, these findings were not adequately documented. The first documentation came in 1921 when Bruynoghe and Masin used a phage to cure a staphylococcal skin infection [8]. After many repeated animal and human tests, many firms like the Parke-Davis Company and Eli Lilly & Company began commercial production of phages against numerous bacterial pathogens. This, however, was after the East European Scientific researchers had recognized a standard dosage of these phages in 1932. The therapy was birthed in China in 1955 when Si *et al*. used bacteriophages to treat *Shigella dysenteriae* [9]. Sadly, phage therapy still has challenges and limitations, especially in narrow host range, less purity, and inconsistency or instability. [10].

3. The Bacteriolytic Mechanism

Bacteriophage therapy has to do with applying phages therapeutically in destroying infectious pathogens of bacteria. When these phages attach to a bacteria, bacteriolysis (the processing of lysing bacterial cells) begins; and this happens in two different host lysis mechanisms, notwithstanding whether they are in line with an endolysin requirement or not. Hence they are two types of bacterial lytic mechanisms. The first mechanism depends on the phage, producing lysozymes with their dsDNA to lyse bacteria, while the second mechanism does not. Examples of lysozyme-dependent phages include phage K and T4, while that independent of lysozyme include phage φX174 with ssDNA [11][12].

a. The Lysozyme-Independent Lysis System

The lysozyme independent lysis system targets the host cell wall synthesis. Bacteriophages in the system lack genes that can encode lysozymes. They lyse the host strains by synthesizing proteins that can hinder the biosynthesis of the host cell wall. When achieved, the host cell will break up (lysis) during cell growth. For instance, *E. coli* ssRNA phage Qβ produces protein A2 and binds to protein MurA, a catalytic enzyme in cell wall formation. This binding hinders catalysis by blocking phosphoenolpyruvate from accessing the active site [13]. Another example is where the ssDNA phage, Phage φX174, encodes a protein E (a membrane protein). This membrane truncates the activities of MraY enzymes (an enzyme that fastens the initial step for the synthesis of peptidoglycan precursor, an essential component of the cell wall), thereby resulting in host cell lysis [14]. After lysis of the cell wall, there is always a dump of large cell debris, leaving the small lesions formed by the host's cell walls [11].

b. Lysozyme-Dependent Lysis System

In this system, the bacteriophages possess dsDNA, which helps to encode lysozymes that lyse host cell walls. These bacteriophages are from the order *Candovirals.* They currently make up about 95% of all the bacteriophages studied [15].

4. Enterobacteriaceae

The class of *Enterobacteriaceae* consists of gram negative bacteria. They are facultatively anaerobic in nature and rod-like in shape. *Enterobacteriaceae* has been implicated in many diseases and infections today, cutting through humans and animals such as poultry and fish. These diseases lead to the death of millions of people in the world every year. They include bacteremia, septic arthritis, lower respiratory tract infections, urinary tract infections, intra-abdominal and ophthalmic

infections. Some drugs have shown efficacy against some of them [12].

Some of the bacteria in this class and their implication include *Klebsiella pneumonia* (implicated pneumonia), *Salmonella enterica* (implicated with gastroenteritis), and *Shigella* strains (implicated with Shigellosis) [16][17] [18]

5. Bacteriophage Therapy targeted against *Enterobacteriaceae*

The treatment of infections by microorganisms, especially bacteria, is done with experimented drugs; however, most bacteria have developed radical resistance to some of the produced medicines [19].

a. Escherichia coli

E. coli is one of the *Enterobacteriaceae* that is causing many diseases. More than any other bacterial species, it has a wide range of diseases [20]. Because of its high infection rate, it is responsible for many diseases and infections in children. According to WHO, acute diarrhoea has killed up to five (5) million children worldwide every year [12].

Treating *E. coli* has not been resolved yet. No specific drug or treatment procedure has been secured yet for its diseases and infections. Back then, oral rehydration played a significant role as a treatment route [21] and even helped save lives. Nevertheless, all the simple measure has not addressed the natural course of the diseases nor enlightened on the underlying potential of anti-bacteria. Due to widespread resistance, people now have less trust in using antibiotics [22].

Recently, phages have been used to treat *E. coli* infections. It has been reported that phages are safer in usage in tackling *E. coli* infections through murine and human tests [23] [24]. Denou *et al.*, in a report, used a T4 coliphage in treating *E. coli* diarrhoea using a combination of both in vitro and in vivo tests. This treatment proves zero adverse effect but instead shows a significant therapeutic effect with no anti-T4 antibodies triggered after one month of observing treatment. Smith *et al.* used phage combination to tackle diarrhoea in young animals, precisely calves, piglets, and lambs [25].

b. Salmonella enterica

Another case to be looked at is *S. enterica*. According to Paterson, 2006, this species is the cause of Salmonellosis in humans, and it inhibits the intestinal tracts of some birds and mammalians [26]. The most available transmission mode in humans is egesting food contaminated with animal faeces. Phage therapy has been found to also work on this class of *Enterobacteriaceae* according to research done by Leverentz *et al.*, 2001[27], Atterbury *et al.*, 2007[28]; and Wall *et al.*, 2010[29].

Leverentz *et al.* tried using phages with melons & apples infected with *S. enterica* found a more positive in melon than the apples (probably because of the low pH in apples which affects phage activities) [27].

c. Klebsiella pneumoniae

K. pneumoniae, a member of this family, is an opportunistic pathogen implicated with intra-abdominal infections, urinary tract, and popularly with pneumonia ([18]. Bacteremia caused by *K. pneumoniae* usually results in significant morbidity and even death among the general population [30]. With the emergence of antibiotic resistance, the treatment of *K. pneumoniae* strains

Comment [M3]: *Sal. enterica and Sal. bongori*

Comment [M4]: In addition to *E. coli*

infections has become even more challenging [18].

Specific phages attack on *K. pneumonia* cells has been observed to control its infection [18]. In an article by Malik *et al*., he used bacteriophage KØ1 in treating third-degree burn wounds of mice administered with a lethal dose of *K. pneumonia* [31]. After treatment, a remarkable decrease in bacterial load was observed in the peritoneal lavage, blood, and lung tissue of mice compared to the control experiment groups. This fall in the microbial count was notable through subcutaneous or intraperitoneal bacteriophage therapy. In curtailing the occurrence of phage-resistant bacteria variants, Gu *et al*. established a systematic approach by making a phage cocktail that consisted of three phages established for *K. pneumoniae* [32]. The phage cocktail significantly reduced the rate of mutation of *K. pneumonia* compared to when used with any single phage and efficiently salvaged *K. pneumonia* bacteremia. Besides, the phage mixture's nominal protective dose was significantly smaller than a single monophage and could protect bacteremic mice from lethal *K. pneumoniae* K7 infection. Also, Hung *et al*. (2011) [33] treated *K. pneumoniae*-induced liver infection by using an isolated phage φNK5. Their results indicated that a single dose of lower than 2×10^8 PFU phages was effective. Through intraperitoneal or intragastric treatment, the mice showed that *K. pneumoniae* was significantly eliminated from the blood and liver tissues compared to those of the control experiments. Their work suggested that the low dose of the phage, φNK5, was an efficient therapeutic agent against *K. pneumoniae*-induced liver infection [33]. Also, the administration of phage showed recommendable protection in infected mice in a short time. The phage was appropriate to rescue *K. pneumoniae*-mediated respiratory infections in the same study. However, the phage treatment was ineffective after a six-hour delay of phage administration following the induction of infection.

It is relevant to pay attention to time during phage therapy. This is because it affects the result and its success. Although there are a few phage therapies for human *K. pneumonia* infection, the studies suggest that bacteriophages or bacteriophage mixtures can modulate the infection caused by *K. pneumonia* [12].

d. Shigella strains

Shigella, a gram-negative rod, is also non-motile and a bacteria that lack capsule. It causes 'Shilgellosis' in humans, posing a severe health challenge, especially in developing countries, and even death [34][17]. As reported still by Phalipon and Sansonetti, 2007, the infectious dose can sometimes be as minute as just 100 bacterial cells to cause infection; taking its contamination through the fecal-oral route, direct person to person contact, via fomites, water, food, or insects.

Four species of Shigella can cause disease in humans; they include *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei*[35].

The legendary d'Herelle was the first person who attempted the treatment of *Shigella* with phages in 1917. He used phages to split *Shigella* strains isolated from several soldier patients with hemorrhagic dysentery [36].

Exciting research was conducted in Tbilisi, Georgia, around 1963 and 1964 to see how effective therapeutic phages can be in

treating bacterial dysentery [37]. Youqiang *et al.* (2015)[12] explain the outcome of this research; thus, a total of 30,769 children between ages six months to seven years were covered in the study. Out of the total number, 17,044 received *Shigella* phages orally, while the rest of the children were not given. The final results showed that the incidence of dysentery was 3.8-fold higher in the group without phage treatment than that of the phage-treated group, indicating the efficiency of phage therapy against *Shigella* strains.

e. Serratia marcescens

S. marcescens is a bacteria with a close attraction for the central nervous system; and meningoencephalitis or a brain abscess implicated with this pathogen has a severe neurologic projection [38]. Also, newly born babies can be infected with *S. marcescens,* most likely when they have immunocompromised systems and low birth weight [39]. Recently we have seen a new development of drug-resistant strains of *S. marcescens* in pediatrics, and this has made the prophylaxis of this bacterium difficult with antibiotics [40].

Two research has again proven how effective phage therapy can be in treating *S. marcescens*. One of these research was conducted as far back as 1967 by Iino and Mitani and another recent one in 2009 by Matsushita *et al*.[41]

In 1967, Iino *et al.* used a phage with a broad host range, phage χ, to lyse 20 of *S. marcescens* strains. However, this phage was only able to affect the strains with flagella which indicated that the possible binding sites (receptors) of phages was somewhere on the flagella [42]

In 2009, Matsushita *et al.* [41] isolated two phages, KSP90 and KSP100, from environmental water that is related to the T4-type phage and phiEco32 phage, respectively [41]. They extensively studied the biological features, DNA features, virion proteins, and phylogenic relationships of these two phages. Their study showed the therapeutic potential of the phages to control *S. marcescens* infection.

6. Therapy for other strains of *Enterobacteriaceae* family

Bacteriophage therapy research is ongoing on other members of the Enterobacteriaceae family. Some include *Edwardsiella* [43], *Proteus* [44] *Erwinia* [45], and *Citrobacter* [46]. These works point to the workability of therapeutic candidates of bacteriophages.

7. Usefulness of Bacteriophage Therapy

There is a need to revisit bacteriophage therapy as an alternative in controlling *Enterobacteriaceae-related* infections[12]. Also, an excellent level of awareness must be done among people, mostly the health workers, if virologists must help reduce the rate at which multi-drug-resistant bacterial strains are growing. Education must be put in place for the general population and health personnel on the coherent and balanced use of antibiotics, regulated sales of over-the-counter antibiotics, and an intentional assessment of the general health system structure.

Secondly, an additional way to solve the multi-resistance problem is to find alternative remedies against drug-resistant pathogens; this is a pressing challenge to contemporary medicine. Both scientists and clinicians alike are looking to find alternative treatment in the form of phage therapy [19].

The curiosity in using phages as therapy has been revitalized in Western countries

due to the ever increase in antibiotic-resistant bacteria. Also, very significantly after the US National Institute of Allergy and Infectious Diseases enlisted phage therapy as one of seven approaches to tackle antibiotic resistance. This therapy is projected to be one of the best alternative treatment plans to control and treat Enterobacteriaceae and other bacterial infections in humans and animals. The application will also reduce food contamination for safe consumption [47].

8. Phage Formulations

Today we have an increasing number of articles discussing about phage therapy yet there is lack of concentration on the formulations types and its effectiveness/effects of each formulation type. Developing Phage formulations can help widen the scope of applications suitable for phage therapy. By bringing up different types of formulations, the mode of delivery can be broadened to suit more specific bacterial infections. It is also important to create long-term studies on the stability of these formulations to avoid detrimental reaction in the treatment process. For us to fully engage the full potential of phage therapy, the above area needs to be attended to[48].

9. Factors affecting Phage Formation

Compared to the storage of phage lysates in the laboratory, the preparation of bacteriophage formulations poses a greater challenge. Normally, phage lysates can be stored long term in conducive conditions but this is not the case for phage formulation as it is always subject to various extreme conditions depending on the application of such phage formulation. From observation, phage formulations prepared as dried, non-liquid forms tend to be more stable over a long time although they can be influenced and affected by different factors example heat which can cause a decline in the titer. Also, during production of some phage formulations, bacteriophage degradation can set in, in the actual process of the production. This is evident in the production processes of phage formulations methods such as freeze-drying and spray-drying [49], [50]. These and more are basic factors one must consideration when creating a phage formulation especially in the aim is to deliver the phage to target bacteria, creating a high level stability and improving phage survival when producing these formulations [51].

10. Methods of Phage Formulations

In producing most common phage formulation, we must encapsulation, because of most of them rely on it. Encapsulation is a term that connote methods (which we will briefly look at) whereby bacteriophage are submerged or surrounded by agents that can improve stability thereby shielding the phage from external environment which may not be favorable for it. Once phagesare encapsulated, they need to be released from the material when needed to target bacterial cells[51].

Encapsulation methods include

a. Emulsification

The bacteriophage or host genus in this method is K *(Staphylococcus)* and the formulation is semi-solid [52]. The benefit of this method is that the material produced is ideal for cream-type treatments and promote absorption when applied topically. However the limitation is that it is difficult to transport/store at large scale, easily prone to bacterial

contamination and can only be stabilized when refrigerated [51].

b. Freeze-Drying

Here the host genus is M13 *(Escherichia)* and can be formulated into a powder [53]. The final product of freeze-drying is easy to store/transport compared to emulsification. It also has high stability after production with different varieties of applications but it is time-consuming, involves a costly process. Mostly, ice crystal formation can decrease phage viability [51].

c. Spray-Drying

In spray-drying, the bacteriophage used is PEV2, PEV40 *(Pseudomonas)* and can be formulated into powder just like the freeze-drying [49]. Also, final product easy to store/transport with high post-production stability and various applications modes. Still though, its process is energy consuming and the temperature can decrease phage viability during process [51].

d. Liposome Entrapment

KP01K2 *(Klebsiella)* is the organism used in preparing this type of formulation and is always formulated into a liquid form[54]. The Liposome entrapment protects phages against in vivo conditions. However there are limitation associated with this method. Encapsulation yield of phages in liposomes are difficult to control, transportation and storage in large scale is also very difficult and needs refrigeration to remain stable [51].

e. Electrospinning

The host genus often used is Felix O1 *(Salmonella)* and is formulated into nanofibers [55].In electrospinning, diverse array of materials can be produced, there is easy deposition of fiber-encapsulated phage onto other substrates but fiber-spinning process can damage phages in the process [51].

11. Importance of Phage Formulations

When a phage is formulated, it can ensure its preservation over a long time in adverse environmental conditions thus making their therapeutic application more effective. Also, formulations ensures mass productions of these phage therapies which can actually be stored easily without drops in phage titer from time to time [51].

Conclusion

Bacteriophage therapy, undoubtedly, is full of efficacy and can be a very reasonable approach to bring back bacterial infections under control. Bacteriophage therapy has more advantages when compared to antibiotics because of the unique nature of bacteriophages. This includes the ability to multiply in numbers, specifically at the host's target site during the bacteria-killing process, and contribute to creating an established phage dose; again, it has a lower cost of agent production [56]. The second advantage is that bacteriophages have a host-specific range and rarely divert from them. By this, it means that they can only face their target bacteria, leaving very little or no effect on the body's normal flora [57]. Also, as expressly narrated by Bentley and Bennett, 2003,phages show little or no toxicity at all compared to antibiotics which can be toxic at times to the flora and environment [58].

However, many factors that can limit bacteriophage as a potential medicine. One of these is the safety problem. All contributing phages required for producing a phage cocktail or mixture need accurate

dissecting or characterization before they can be used clinically for treatment. Fortunately, the intense improved studies in genome sequencing technologies have given us an edge over this challenge. Though good in an aspect, the second troubling challenge is the fact that phages have a narrow host range. However, the newest development of joining more than one phage together to produce cocktails handles that challenge. Thirdly, the nature of phage therapeutic agents can be unstable at times; nevertheless, studies and research are still actively sorted to curb this challenge. The fourth challenge still is the phage-resistance developed by bacteria during their co-evolution with phages. There is a bone of contention about if the same result will surface again in the future, as bacteria will develop multi-phage resistance like antibiotics. This is a significant problem, and future studies and research should focus on this [12].

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