Safety, Toxicity of Phage Therapy

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Lytic bacteriophages (phages) have been known to be a potential antibacterial agent for over a century since their first formal discovery and application as a treatment against human bacterial infections in the 1920s. Though subsequent success of antibiotics had quelled investigations into phage as potential anti-infectives, increasing antibiotic resistance has hastened the reemergence of interest in phage therapy. Despite attractive advantages, including widespread prevalence, activity against multidrug-resistant (MDR) bacteria, high specificity, and activity against biofilms, phage therapy is still not considered a mainstream treatment due to several obstacles. First, the lack of reliable data regarding its safety and efficacy in clinical settings. Second, appropriate regulatory guidelines specific to phage therapeutics have not been developed. Finally, the pharmaceutical and biotech industries have not yet developed economical and scalable production processes for widespread adoption of phage therapy. While much progress has been made, many questions remain, such as the safety and toxicity of phage preparations.

Keywords: phage therapy ; clinical trials ; safety and toxicity ; immune activation

1. Potential Impact of Phage Therapy

Humans are exposed to phages in the environment and from their microbiomes. Some studies have suggested that phages can spread into the blood easily and accumulate in distinct tissues [1][2][3][4]. There are even indications that phages are taken up by eukaryotic cells and can trigger innate immune pathways [5][6]. While most of these studies involve temperate, resident phages it is plausible that lytic phages are also able to penetrate eukaryotic cells [7][8]. Nonetheless, the distribution of phages within the body and their impact on tissues and physiologic processes are largely unknown.

1.1. Impact of Phage on the Microbiome

The human body harbors a vast and complex microbiome that may contribute to both health and disease [9][10]. The impact of phage therapy on this flora is unclear. In addition, phages are being explored as potential microbial modifiers in infected and microbiota-imbalanced gut disease [11]. A murine model of gut carriage of *E. coli* showed that microbiota diversity was less affected by phage therapy than antibiotics [12]. Two other clinical trials of healthy adults and children also indicated that coliphage reduced the target organism in feces without any considerable change in microbiota composition [13][14]. A pediatric trial of children with diarrheal disease found that oral coliphages transited safely in children with no adverse effects [15]. Additionally, clinical trials with healthy adults and children who ingested coliphage, which targets *E.coli*, showed that fecal phage detection was dependent on the oral dose. No intestinal amplification was detected, suggesting there is passive transit of coliphages through the gut [15][16]. Sarker et al. demonstrated that phage passed through the intestine of healthy people largely passively. Possible adverse effects are limited to the physical presence of virion particles, not to infectious viruses replicating and killing target bacteria. Only when the phage meets its target within patients harboring high numbers of the target *E. coli*, and the mucosal integrity is compromised by the diarrhea pathology, is there concern for the undesirable effects of phage therapy [15].

1.2. Endotoxin Release Associated with Bacterial Lysis

Endotoxin is one of the most potent inducers of the inflammatory cytokine response in Gram-negative bacterial infections [17]. As phages can kill bacteria within minutes, phage therapy can potentially result in rapid and significant endotoxin release [18]. There have been a few studies reported regarding potential bacterial lysis-related effects, as mentioned above. However, comprehensive data on the release of endotoxin and its effects are rarely reported and are inconsistent. Endotoxins and other bacterial components that could be present in phage preparations are typically overlooked. These include bacterial DNA [19], Staphylococcal enterotoxin B (a potent bacterial superantigen) [20], alpha hemolysin and other exotoxins [21], or lipoteichoic acid (an important cell wall polymer found in Gram-positive bacteria) [22][23]. Bacterial components and toxins such as endotoxin, which are typically difficult to purify from phage agents, have the potential to induce infusion-related reactions [24][25][26]. These reactions include hypersensitivity and cytokine release syndromes.

Symptoms can include flushing, alterations in heart rate and blood pressure, dyspnea, bronchospasm, back pain, fever, urticaria, edema, nausea, and rash [27]. Endotoxin release and infusion-related reactions can be difficult to distinguish, but the presence of these bacterial components should be quantified and documented in phage preparations nonetheless.

1.3. Impact of Phages on Immune Activation

Phages have been regarded as bystanders that only impact immunity indirectly via effects on the mammalian microbiome [28]. Recently, both in vitro [29][30] and in vivo [31][32] studies verified that phages also impact innate and adaptive immunity directly [33]. However, results related to immune response instigated by phages are inconsistent and their role in phage therapy is also unclear. Mathematical models have been developed showing their potential importance in a phage therapeutic setting [33][34]. Independent of the phage purification strategy, it is often difficult to attribute these immune responses purely to the phage [31][35].

Phages themselves are immunogenic biological entities that can stimulate adaptive immune responses [36]. Clinical studies in healthy adults, as well as children with acute bacterial diarrhea, showed no detectable phage in the blood stream nor any increase in IgG, IgM, IgA, and sIgA [37][38][39]; however, when administered via intraperitoneal administration, phage triggered increases in phage-specific IgG and IgM antibody titers [40][41]. Phage antibody production may therefore depend on the route of phage administration. In addition, the antibody production was also dependent on the phage type and application time [42][43][44]. Currently, antibody production is thought to affect the efficacy of phage therapy; yet their role in the safety of phage therapy is unclear. Data regarding phage-induced immune responses, including inflammatory cytokine production and antibodies, are an underexplored area and are generally lacking in the studies we reviewed here (**Table 1**).

2. Potential Contaminants from Bacterial Components within Phage Preparations

Besides phages themselves, additional components can influence phage safety and toxicity. The major bacterial component that could instigate pro-inflammatory reactions are endotoxins, major components of the cell wall of Gram-negative bacteria that are highly immunogenic in humans [45]. Other potential bacterial components that could be present in phage preparations and are typically overlooked are Staphylococcal enterotoxin B, a potent bacterial superantigen [20], alpha hemolysin and other exotoxins [21], lipoteichoic acid, an important cell wall polymer found in Gram-positive bacteria [22][23], and bacterial DNA [19]. The presence of these bacterial components needs to be quantified and documented in phage preparations.

3. Potential Chemical Contaminants from Phage Preparation and Purification

Currently there are three major strategies employed regarding the purification of phages. Cesium chloride (CsCl) is often used to obtain high density and high purity phage preparations [26][46]. However, CsCl is typically removed from phage preparations prior to clinical administration as it can be toxic to cells in high concentrations. The most frequently attributed effects of CsCl intoxication are gastrointestinal distress, hypotension, syncope, numbness, or tingling of the lips [47], although a different isotype of CsCl is used in density gradients for phage purification.

Another method of phage purification involves polyethylene glycol (PEG). PEG is an United States Food and Drug Administration-approved biodegradable polymer often used for drug delivery systems [48][49][50][51]. Fortunately, PEG has a high molecular weight and readily undergoes renal clearance leading to a safe toxicity profile and tolerability when used in the phage purification process.

A third method is filtration. Anion exchange is a more controlled purification of phage; however, this method is not ideal for large scale phage purification [52].

4. The Current Safety and Toxicity Monitoring Associated with Phage Preparations

Table 1 indexes the characteristics of phage preparations described in animal and clinical studies. These characteristics include the phage protein profile, sterility, endotoxin levels, and bacterial DNA levels.

In our review of the literature, data on phage preparations were frequently absent. Almost all studies offered the phage concentration (PFU/mL) directly. Fewer than 40% of the studies reported genotype information. Protein profiles showing

the difference between proteins from phage or bacterial origin were mentioned in only 10% of the studies. Twenty-four of the 66 studies described the process used to remove viable bacteria from the phage preparation. Although fewer than 5 units (EU)/kg/hour are required by the FDA in clinical phage preparations [53][54][55], only 14 of the 66 studies reported the level of endotoxin contamination. The bacterial host DNA was reported in only four of the evaluated studies.

Other toxins and contaminations such as lipoteichoic acid, superantigens, or cesium chloride [43][56] were rarely considered in most studies. Additional quality controls regarding shelf life [43][57], pH [58][59], visual appearance [59], were sporadically mentioned. Some phage preparations were developed through commercial production pipelines. Few of these entities reported information regarding phage product manufacturing [60][61][62][63][64][65], although some information on production processes and quality controls was available [66].

Table 1. Characteristics of phage preparations used in the phage therapy studies.

Reference	Titration	Characterization		Composition & Purity			
Animal Studies	**PFU**	**Genotype**	**Protein Profile**	**Sterility**	**Endotoxin**	**Host Cell DNA**	**Other Toxins**
Dufour et al., 2019 [67]							
Fong et al., 2019 [60]							
Drilling et al., 2017 [68]							
Drilling et al., 2014 [69]							
Chhibber et al., 2008 [70]							
Jongsoo et al., 2019 [71]							
Chang et al., 2018 [65]							
Gelman et al., 2018 [72]							
Cheng et al., 2017 [73]							
Oechslin et al., 2016 [74]							
Galtier et al., 2016 [12]							
Jun et al., 2014 [41]							
Takemura-Uchiyama et al. 2014 [75]							
Osanai, et al. 2012 [76]							
Pouillot, et al. 2012 [77]							
Ľubomíra Tóthová et al. 2011 [78]							
Hung, et al. 2011 [79]							
Hawkins, et al. 2010 [80]							
Sunagar, et al. 2010 [40]							
Nishikawa, et al. 2008 [81]							
Case Reports							
Lebeaux et al., 2021 [82]							
Ferry et al., 2020 [83]							
Bao et al., 2020 [84]							
Cano et al., 2020 [85]							
Rostkowska et al., 2020 [86]							
Doub et al., 2020 [87]							
Rubalskii et al., 2020 [88]							

Reference	Titration	Characterization		Composition & Purity			
Animal Studies	**PFU**	**Genotype**	**Protein Profile**	**Sterility**	**Endotoxin**	**Host Cell DNA**	**Other Toxins**
Gainey et al., 2020 [37]							
Aslam et al., 2019 [38]							
Nir-Paz et al., 2019 [89]							
Tkhilaishvili et al., 2019 [90]							
Onsea et al., 2019 [42]							
Corbellino et al., 2019 [91]							
Susan et al., 2019 [61]							
Gilbey et al., 2019 [62]							
Law et al., 2019 [63]							
RM et al., 2019 [43]							
Duplessis et al., 2019 [92]							
Kuipers et al., 2019 [93]							
LaVergne et al., 2018 [94]							
Ferry et al., 2018 [58]							
Fish et al., 2018 [95]							
Ferry et al., 2018 [96]							
Hoyle et al., 2018 [97]							
Chan et al., 2018 [98]							
Ujmajuridze et al., 2018 [39]							
Schooley et al., 2017 [56]							
Zhvania et al., 2017 [99]							
Jennes et al., 2017 [100]							
Fish et al., 2016 [101]							
Fadlallah et al., 2015 [102]							
Rose et al., 2014 [103]							
Khawaldeh et al., 2011 [104]							
Kvachadze et al., 2011 [105]							
Letkiewicz et al., 2009 [106]							
Clinical Trials							
Leitner et al., 2020 [107]							
Grubb et al., 2020 [11]							
Fabijan et al., 2020 [108]							
Ooi et al., 2019 [109]							
Febvre et al., 2019 [13]							
Gindin et al., 2018 [110]							
McCallin et al., 2018 [57]							
Sarker et al., 2017 [15]							

Reference	Titration	Characterization		Composition & Purity			
Animal Studies	**PFU**	**Genotype**	**Protein Profile**	**Sterility**	**Endotoxin**	**Host Cell DNA**	**Other Toxins**
McCallin et al., 2013 [14]							
Sarker et al., 2012 [16]							
Rhoads et al., 2009 [59]							
Patrick et al., 2018 [111]							
Sarker et al., 2016 [112]							
Wright et al., 2009 [64]							

Dark Blue = Values or result reported within article; Blue = reported, but no specific values or results published within article; Grey = not reported. “Titration” refers to the phage concentration offered by “PFU”. “Genotype” refers to the genetic information, such as the accession number or sequence information of phage. “Protein profile” refers to protein composition of phage; “Sterility” refers to the specific bacterial colony in phage preparation. “Endotoxin” refers to the concentration of endotoxin; “Host cell DNA” refers to the host bacterial DNA; “Other toxins” denotes lipoteichoic acid, superantigens, or CsCl, etc.

5. Optimization of Safety and Toxicity Monitoring in Phage Therapy

In animal studies, phage doses were variable, ranging from 10^3 to 10^{12} PFU/ml. None of them defined the median effective dose (ED50), lethal dose for 50% (LD50), or the therapeutic index (TI), a quantitative measurement of the relative safety of a drug that compares the amount of a therapeutic agent that causes the therapeutic effect to the amount that causes toxicity, of the phage preparations. Effects of phage therapy on pregnancy, growth, and development were not described. Additionally, data were mostly limited to rodents and not large animals (e.g., pigs), limiting generalizability to humans. The majority of animal studies utilized IP injection, analogous to IV administration typically used in human studies but challenging to draw direct comparisons.

Clinical safety data analysis and evaluation of new drugs often includes reporting of adverse events, laboratory derangements, changes in vital signs, reviews of systems, and physical examinations of subjects [113]. Biological products such as cytokines, antibodies, and recombinant proteins typically report their immunogenicity. The incidence and consequences of neutralizing antibodies and potential adverse events related to the combination of antibody formation and their adverse reactions were evaluated as well [114]. Including an analysis of the immunogenicity of phages should therefore be an important part of both animal studies as well as case reports. Our review of the phage literature demonstrates the paucity of these data. We believe assessments of safety and toxicity ought to be incorporated into all clinical and preclinical studies of phage therapy, independent of the FDA and the European Medicines Agency (EMA) regulation. Ideally, publications reporting on the safety of phage therapy should include information on the general health of participants, adverse events, chemistry, and hematologic testing data., Information on immune responses should be evaluated prior, during, and after phage therapy. In **Table 2**, we offer some safety endpoints for consideration that may provide researchers and clinicians guidance on the safety monitoring of phage therapy.

Table 2. Safety endpoints in phage therapy study to be considered.

Safety Monitoring	Safety Endpoints
General assessment	**Vital signs; physical exam; subjective symptoms**
Labs—Chemistry	**Liver function; kidney function; electrolytes; glucose; CRP**
Labs—Hematology	**CBC with differential; ESR**
Pharmacology	**Absorption; distribution; excretion; metabolism endpoints (e.g., LE50, ED50, TI)**
Immune Response	**Non-specific and specific immune responses (e.g., DC, inflammatory factor level; phage specific antibodies)**

Abbreviations: Erythrocyte sedimentation rate (ESR); C-reaction protein (CRP); CBC: Complete Blood Count; WBC: White blood cells; DC: CBC with differential; BPC, Blood platelet count; LE50, Lethal Dose 50; ED50, Median Effective

Dose; TI: Therapeutic Index.

Comprehensive assessments of safety will likely benefit from standardization of safety monitoring. Objective methods of assessment have been employed in some clinical trials, such as gastrointestinal questionnaires or a Visual Analogue Scale (VAS) to assess pain [110][64]. One study utilized a scoring method for assessing physical examination findings in septic mice treated with phage [72]. Another study in a murine bacteremia model introduced a health assessment score [73]. A recent clinical trial applied the National Cancer Institute Common Terminology Criteria for Adverse Events (version 4) to assess the frequency and severity of adverse events during phage treatment [107]. Such methods provide an opportunity to improve safety and the application of scales or standardized scoring methods would better facilitate inter-study comparisons.

In the United States, the Center for Biologics Evaluation and Research (CBER) at the FDA is the main regulatory body overseeing investigational phages [115][116]. The FDA and the EMA mandate that any modern phage therapy products must be made to GMP standards [117][118]. Along with GMP, we feel phage preparations should include information on the characteristics of the phages used in animal studies and clinical studies, including their morphology, genetics, and protein profile, as well as the composition of the phage preparations, including the levels of bacterial contaminants and other impurities. Documentation of the sterility of the phage preparations is necessary. A clear description of the methods used to propagate and purify the phage preparations ought to be provided. These toxicity endpoints are summarized in **Table 3**.

Table 3. Characteristics of phage preparation to be considered.

Phage Parameters	Phage Preparation Measurements
Identify	**Morphology**
Potency	**Titer**
Sequencing	**Genotype; Protein profile**
Bacterial contaminants	**Viable bacteria; Endotoxin; Enterotoxin B; Bacterial DNA**
Other impurities	**CsCl**
Others	**Sterile; PH; shelf time; suspended buffer; osmotic pressure**

The morphology, titration and genomic description of the used phage, including the genome sequence as well as a complete annotation of the proteins encoded in the genome. The presence of both bacterial remnants, endotoxin level, bacterial DNA, as well as potential presence of toxic components of the purification method itself; Sterility, suspended buffer, pH stability, temperature range and shelf life should be denoted.

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