
Review

The Safety and Toxicity of Phage Therapy: A Review of Pre-Clinical and Clinical Studies

Dan Liu^{1,2#}, Jonas D Van Belleghem^{2#}, Christiaan R. de Vries², Elizabeth Burgener², Qingquan Chen², Robert Manasherob³, Jenny R. Aronson², Derek F. Amanatullah³, Pranita Tamma⁴, Gina A. Suh^{5*}

1 Department of Burns, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, 200025, China

2 Division of Infectious Diseases and Geographic Medicine, Dept. of Medicine, Stanford University School of Medicine, Stanford, CA, 94305, USA

3 Department of Orthopaedic Surgery, Stanford University School of Medicine, Stanford, CA, 94305, USA

4 Department of Pediatrics, Division of Pediatric Infectious Diseases, Johns Hopkins University School of Medicine, Baltimore, Maryland, 21287, USA

5 Division of Infectious Diseases, Mayo Clinic, Rochester, MN, 55905, USA

These authors contributed equally to this work.

* Correspondence to G. A. Suh, Division of Infectious Diseases; 200 First St SW, Rochester, MN, 55905, USA, suh.gina@mayo.edu

Abstract: Increasing rates of infection by antibiotic resistant bacteria have led to a resurgence of interest in bacteriophage (phage) therapy. Several phage therapy studies in animals and humans have been completed over the last two decades. We conducted a systematic review of safety and toxicity data associated with phage therapy in both animals and humans reported in English-language publications from 2008 – 2021. Overall, 69 publications met our eligibility criteria including 20 animal studies, 35 clinical case reports or case series, and 14 clinical trials. After summarizing safety and toxicity data from these publications, we discuss potential approaches to optimizing safety and toxicity monitoring with the therapeutic use of phage moving forward. In our systematic review of the literature, we found few, but no serious, adverse events associated with phage therapy. Comprehensive and standardized reporting of potential toxicities associated with phage therapy has generally been lacking in the published literature. Structured safety and tolerability endpoints are necessary when phages are administered as anti-infective therapeutics.

Keywords: Phage therapy; clinical trials; animal models; safety and toxicity; immune activation.

1. Introduction

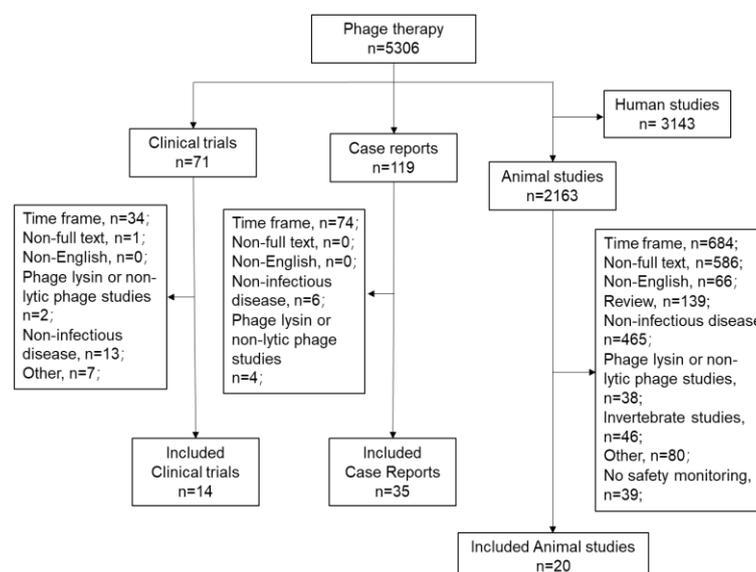
Antibiotic resistance remains an ongoing global threat. The failure to implement widespread stewardship over these precious resources, the resulting spread of antibiotic resistance, and an under-resourced antibiotic pipeline portend the coming of a “post-antibiotic era” [1]. Lytic bacteriophages (phages) 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 [2-4]. While much progress has been made, many questions remain [2, 5-7]. To ensure confidence in the use of phage as anti-infectives, it is critical that the safety of phage therapy is established [8-10].

2. Materials and Methods

Here, we focus on the safety of phage therapy in a review of both clinical trials and in vivo pre-clinical studies. We have limited these efforts to reports published in English language journals between January 1, 2008 and March 28, 2021 available on PubMed. We

excluded the following: (1) non-vertebrate animal studies, (2) reports of phage use other than for anti-infective purposes, (3) phage lysis investigations, and (4) investigations into non-lytic phage. We identified 20 animal studies, 35 case reports/series, and 14 clinical trials that met our eligibility criteria (Figure 1).

Fig 1. Search strategy.



The search terms “phage therapy” or “bacteriophage therapy” were used in PubMed. The study types were defined as “Clinical Trials”, “Case Reports” and “Animal Studies”, respectively. The time frame was defined as January 1, 2008 to March 28, 2021. Full text and English-language articles were included; Non-research articles, reviews, opinion pieces were excluded. Studies involving invertebrates, non-infection diseases, phage lysins, or non-lytic phages were excluded. This resulted in a total of 14 clinical trials, 35 case reports and 20 animal studies included and discussed in this review.

3. Results

3.1 Pre-clinical studies

Of the pre-clinical studies, four studies focused on safety (Table 1). Dufour et al. for example detected the proinflammatory stimulation of phage-induced bacterial lysis and/or the phage particle itself. This murine acute pneumonia model was initiated by intranasal instillation of two *E. coli* strains (536 and LM33) and treated by two phages (536_P1 and LM33_P1; intranasal) with antibiotics as a comparator. The complete blood counts (CBC), lung edema, cytokine level, bacterial and bacteriophage counts were determined. Phage and antibiotics displayed similar endpoints, but phage decreased the bacterial load and corrected the blood cell count abnormalities at a more rapid rate. The rapid lysis of bacteria by phages did not increase the innate inflammatory response compared to antibiotics. Meanwhile, phage 536_P1 promoted a weak increase in antiviral cytokines (IFN- γ) and IL-12 in the lungs, which was not observed in infected animals [11]. Drilling et al. assessed the safety and efficacy of a topically applied cocktail of *S. aureus* specific phage (CTSA) alone and in combination with EDTA for treatment of *S. aureus* biofilms in a sinusitis sheep model. Safety was assessed using histology and scanning electron microscopy (SEM) after 3 days of treatment. No significant signs of inflammation nor toxic effects or damage to mucosal cilia were noted. Furthermore, a significant reduction in biofilm levels were observed with a cocktail of *Staphylococcus aureus*-specific phage (CTSA), EDTA, and CTSA-EDTA treatments indicating that CTSA and EDTA are safe and efficacious for short-term topical application [12]. A long-term study (20 days) by Drilling and colleagues investigated the safety of topical sinonasal flushes with phage cocktail NOV012 (P68 and K710) against *S. aureus* in the same model. General wellbeing, mucosal structural changes and inflammatory load were assessed. With no inflammatory

infiltration or tissue damage within the sinus mucosa observed, the application of NOV012 was found to be safe [13]. Fong and colleagues assessed the safety of a *P. aeruginosa* phage cocktail (CT-PA) in a sinusitis sheep model. After a 7-day biofilm formation period, sheep received frontal trephine flushes of CT-PA twice-daily for 1 week. Blood and fecal samples were collected. Histopathology of frontal sinus, lung, heart, liver, spleen, and kidney tissue was performed. Phage was detected consistently in feces and sporadically in blood and organs. Sinus cilia were visualized using SEM. The authors showed that CT-PA reduced the biofilm biomass significantly. No safety concerns of tissues were noted [14].

16 efficacy studies evaluated safety measures in some capacity, including general health, physical examination, hematology, organ function or immune response (Table S1). In contrast to Dufour's conclusions that phage therapy did not induce an inflammatory response, phage therapy for endocarditis in rats induced by *P. aeruginosa* showed that phage therapy, but not ciprofloxacin, correlated with the significantly increased plasma levels of IL-1 β and IL-6. Because ciprofloxacin is not bacteriolytic, the increase in IL-1 β and IL-6 levels was considered related to phage-induced bacterial lysis [15]. Additional studies have shown that treatment with phage leads to increased anti-phage antibody titers. For example, 170-fold and 50-fold increase of IgG and IgM titers against phage in mice *Vibrio parahaemolyticus* has been observed [16, 17]. Another study looking at *S. aureus* bacteremia in mice showed a 2500-fold and 100-fold of IgG and IgM respectively after intra-peritoneal (IP) phage administration [16, 17]. No adverse effects were reported.

Table 1. Safety monitoring in phage therapy studies.

Reference	Physical Exam			Adverse Events	Distribution	Laboratory Assessment				Immune Response		
	Animal Studies					Hematology	Liver Function	Kidney Function	Electrolytes	Cell Infiltration	Cytokine	Ig Production
Dufour, et al 2019												
Fong, et al, 2019												
Drilling, et al, 2017												
Drilling, et al, 2014												

Reference	Subjective Data	Physical Exam			Adverse Event	Imaging	Distribution	Lab Exam.				Immune Response		
		Case Reports							Hematology	Liver Function	Kidney Function	Electrolyte	System Inflammation	Cytokine
Lebeaux et al., 2021														
Ferry et al., 2020														
Bao et al., 2020														
Cano, et al, 2020														
Rostkowska, et al., 2020														
Doub, et al., 2020														
Rubalski, et al., 2020														
Gainey, et al., 2020														
Aslam, et al., 2019														
Nir-Paz, et al., 2019														
Tkhilishvili, et al., 2019														
Onsea, et al., 2019														
Corbellino, et al., 2019														
Susan, et al., 2019														
Gilbey, et al., 2019														
Law, et al., 2019														
RM, et al., 2019														
Kuipers et al. 2019														
Fish, et al., 2018														
Ferry, et al., 2018														
Hoyle, et al., 2018														
Chan, et al., 2018														
Duplessis, et al., 2019														
LaVergne, et al., 2018														
Ferry, et al., 2018														
Ujmagundze, et al., 2018														
Schooley, et al., 2017														
Zharnia, et al., 2017														
James, et al., 2017														
Fish, et al., 2016														
Fadlallah, et al., 2015														
Rose, et al., 2014														
Khavaldah, et al., 2011														
Krachadze, et al., 2011														
Letskiewicz, et al., 2009														

Reference	Physical Exam			Adverse Event	Imaging	Distribution	Lab Exam.				Immune Response		
	Clinical Trials							Hematology	Liver Function	Kidney Function	Electrolyte	System Inflammation	Cytokine
Leitner, et al., 2020													
Grubb, et al., 2020													
Fabijan, et al., 2020													
Coli, et al., 2019													
Fabovic, et al., 2019													
Gindin, et al., 2018													
McCallin, et al., 2018													
Sarker, et al., 2017													
McCallin, et al., 2013													
Sarker, et al., 2012													
Rhoads, et al., 2009													
Patrick, et al., 2018													
Sarker, et al., 2010													
Wright, et al., 2009													

Dark Blue =have values or result within article; **Grey** = not mentioned within article. "Subjective Data" include important feedback from health volunteers or patients taken during or after phage administration. "Physical Exam" data include general condition like appetite, fever, deaths, weight, symptoms of lethargy, ruffling, hunching or irregular breathing or other signs of systemic illness, and vital sign. "Phage Distribution" refers to phage spread in blood or other organ, except site of

infection. “Adverse Event” refers to any reported adverse effect or adverse events. “Imaging” refers to any imaging test, including ultrasound, x-ray, CT, MRI, etc. “Lab Exam” denotes clinical laboratory testing for Hematology, Liver Function, Kidney Function and Electrolyte. “Immune Response” refers to systemic inflammatory markers, like CRP and ESR, topical or circulating cytokine levels. “Ig Production” refers to testing for immunoglobulins in blood or feces.

3.2 Case Reports

Thirty-five case reports/series of phage therapy were published between 2008 – 2021 (Table S2). Most involved combined use of phage with antibiotics, targeting a variety of pathogens (Figure S2). The conditions treated included cystic fibrosis pulmonary disease, bone/joint infection, pneumonia, bacteremia, urinary tract infection, endocarditis, cardiothoracic surgery-related infections, aorto-cutaneous fistula, necrotizing pancreatitis, skin infection, brain infection, diabetic foot ulcers, corneal abscess, lung transplant-related infection, and intestinal infection. Twenty-seven cases included safety measures (Table S2), including subjective symptom reporting, physical examination, hematologic measurements, liver function, kidney function, electrolytes, imaging and adverse events. Some studies also included additional clinical markers such as erythrocyte sedimentation rate (ESR), c-reactive protein (CRP), cytokine levels, and anti-phage antibody production.

Among these 35 studies, a 72-year-old male with a chronic methicillin-resistant *S. aureus* prosthetic joint infection developed a reversible transaminitis after three intravenous dose of phage prompting discontinuation. The investigators hypothesized that underlying steatosis induced a dysregulated local cytokine response in the macrophages within the liver when challenged with large amounts of phages that needed to be cleared [18]. Another case report involved a 68-year-old diabetic patient with necrotizing pancreatitis with *A. baumannii*. Two days following the intravenous phage administration, the patient’s vasopressor requirements abruptly increased, and phage therapy was temporarily withheld. It was subsequently demonstrated that the clinical deterioration was accompanied by a transient septic episode. Phage therapy was resumed about a week later and the patient’s condition improved [19]. For a 2-year-old male with *P. aeruginosa* bacteremia, phage therapy was withheld due to anaphylaxis-related decompensation, which was attributed to progressive heart failure, although endotoxin release could not be excluded as a contributing factor. Shortly after phage therapy resumed, the patient had clinical improvement [20]. Another case series showed a patient with *P. aeruginosa* induced urinary tract infection (UTI) experienced sudden fever (38.5 °C) and chills on the third day of phage therapy, which was considered to be related to released endotoxins during *P. aeruginosa* lysis. The phage treatment was subsequently stopped. The body temperature normalized 48 h after third generation cephalosporin was prescribed [21]. Moreover, bacterial components and toxins such as endotoxin, have the potential to induce infusion related reactions. For example, a 77-year-old male with a multidrug-resistant *Acinetobacter baumannii* craniectomy site infection developed hypotension 115 minutes after the first dose of phage therapy. As this did not require vasopressors, phage treatment was continued [22]. Another case involving a 15-year-old patient with cystic fibrosis and a *Mycobacterium abscessus* infection reported diaphoresis and flushing, but no fever was observed after IV administration [23].

3.3 Clinical Trials

The first investigation into the bioavailability of oral *E. coli* phage T4, in 2005 involving fifteen healthy humans, did not identify any adverse events [24]. Since 2008 there have been 14 clinical trials of phage therapy (Figure 1) investigating a multitude of bacterial infections (Figure S2). Indications for phage therapy included endocarditis, sepsis, rhinosinusitis, UTI, venous leg ulcers, chronic otitis media, acute bacterial diarrhea, and burn wounds (Table S3). All these trials evaluated safety measures (Table 1). The safety endpoints reported by these trials included subjective data/symptom reporting, physical examination, hematologic measurements, liver function, kidney function, and electrolytes.

Among these trials, a double-blinded, placebo-controlled crossover trial in which healthy adults consumed a commercial cocktail of *E. coli*-targeting bacteriophages for 28 days [25]. The gut microbiota and markers of intestinal and systemic inflammation were examined. The fecal *E. coli* loads reduced, with no significant changes to the microbiota. Short-chain fatty acid production, inflammatory markers, and lipid metabolism were largely unaltered, but there was a small but significant decrease in circulating IL-4 [25]. In another trial, the safety of broad-spectrum cocktail, Eliava Pyophage, was tested by comparing the effects of nasal and oral exposure with a mono-species counterpart and placebo in healthy human carriers of *S. aureus*. Physical examination and clinical chemistries and hematologic studies were analyzed. Fluctuations of body temperature were observed, but none exceeded 38 °C. One subject during Pyophage application had back pain and gastric acidity for 48 h, while two adverse events were noted during monophage application with mild pain in the epigastric region for 6 h, and allergic rhinitis and low-grade fever for 72 h, respectively. None of them were considered to be related to the oral phage treatment by the clinicians [26]. In a rhinosinusitis trial, mild adverse events in six patients, including diarrhea, self-resolved epistaxis, symptoms of upper respiratory tract infection, rhinalgia, oropharyngeal pain or decreased blood bicarbonate level were reported. These were classified as treatment-emergent adverse effects and all resolved without discontinuation of therapy [27] (Table S3).

4. Discussion

4.1 Potential Impact of Phage Therapy

Humans are exposed to phages in the environment and from their microbiomes. Some studies suggest that phage can spread into the blood easily and accumulate in distinct tissues [29-32]. There are indications that phages are taken up by eukaryotic cells and can trigger innate immune pathways [33, 34]. While most of these studies involve temperate, resident phages it is plausible that lytic phages are also able to penetrate eukaryotic cells [35, 36]. Nonetheless, the distribution of phages within the body and their impact on tissues and physiologic processes are largely unknown.

4.2 Impact of Phage on the Microbiome

The human body harbors a vast and complex microbiome that may contribute to both health and disease [37-39]. 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 [40]. A murine model of gut carriage of *E. coli* showed that microbiota diversity was less affected by phage therapy than antibiotics [41]. 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 [25, 42]. A pediatric trial of children with diarrheal disease found that oral coliphages transited safely in children with no adverse effects [43]. 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 [43, 44]. 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.[43].

4.3 Endotoxin Release Associated with Bacterial Lysis

Endotoxin is one of the most potent inducers of the inflammatory cytokine response in Gram-negative bacterial infections [45]. As phages can kill bacteria within minutes, phage therapy can potentially result in rapid and significant endotoxin release [46]. There

have been a few studies reported regarding potential bacterial lysis-related effects, as mentioned above. However, current 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 [47], Staphylococcal enterotoxin B (a potent bacterial superantigen) [48], alpha hemolysin and other exotoxins [49], or lipoteichoic acid (an important cell wall polymer found in Gram-positive bacteria) [50, 51]. Bacterial components and toxins such as endotoxin, that are typically difficult to purify from phage agents have the potential to induce infusion related reactions [52-54]. 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 [55]. 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.

4.4 Impact of Phages on Immune Activation

Phage have been regarded as bystanders that only impact immunity indirectly via effects on the mammalian microbiome [56]. Recently, both *in vitro* [57, 58] and *in vivo* [59, 60] studies verify that phages also impact innate and adaptive immunity directly [61]. However, results related to immune response instigated by phage are inconsistent and their role in phage therapy also unclear. Mathematical models have been developed showing their potential importance in a phage therapeutic setting [62, 63]. Independent of the phage purification strategy, it is often difficult to attribute these immune responses purely to the phage [59, 64].

Phages themselves are immunogenic biological entities that can stimulate adaptive immune responses [65]. Clinical studies in healthy adults, children 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 [24, 25, 42]; However, intraperitoneal phage administered triggered increases in phage-specific IgG and IgM antibody titers [16, 17]. 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 [29, 66, 67]. Currently, antibody production is thought to affect the efficacy of phage therapy; yet their role in 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).

4.5 Safety and Toxicity Associated with Phage Preparations

Tables 2 index the characteristics of phage preparations described in pre-clinical 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. Less 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 69 studies describe the process used to remove viable bacteria from the phage preparation. Although less than 5 units (EU)/kg/hour has been required by the United States Food and Drug Administration (FDA) in clinical phage preparations [68-70], only 15 of the 69 studies reported the level of endotoxin contamination. The bacteria host DNA was reported in only four of the evaluated studies.

Other toxins and contaminations such as lipoteichoic acid, superantigens, or cesium chloride [19, 71] were rarely considered in most studies. Additional quality controls regarding shelf life [26, 71], pH [72, 73], visual appearance [73] are sporadically mentioned. Some phage preparations were developed through commercial production pipelines. Few of these entities reported information regarding phage product manufacturing [14, 74-78]

although some information on production processes and quality controls are available [79].

Table 2. Characteristics of phage preparations used in the phage therapy studies.

Reference	Titration	Characterization		Composition & Purity			
	PFU	Genotype	Protein Profile	Sterility	Endotoxin	Host Cell DNA	Other Toxins
Animal studies							
Dufour, et al. 2019	Dark Blue	Grey	Grey	Grey	Grey	Grey	Grey
Fong, et al. 2019	Dark Blue	Grey	Grey	Grey	Grey	Grey	Grey
Drilling, et al. 2017	Dark Blue	Grey	Grey	Grey	Grey	Grey	Grey
Cao, et al. 2015	Dark Blue	Grey	Grey	Grey	Grey	Blue	Grey
Drilling, et al. 2014	Dark Blue	Grey	Grey	Grey	Grey	Grey	Grey
Chhibber, et al. 2008	Dark Blue	Grey	Grey	Grey	Grey	Grey	Grey
Jongsoo, et al. 2019	Dark Blue	Grey	Grey	Blue	Grey	Grey	Grey
Chang, et al. 2018	Dark Blue	Grey	Grey	Blue	Grey	Grey	Grey
Gelman, et al. 2018	Dark Blue	Grey	Grey	Blue	Grey	Grey	Grey
Cheng, et al. 2017	Dark Blue	Grey	Grey	Grey	Grey	Grey	Grey
Oechslein, et al. 2016	Dark Blue	Grey	Grey	Grey	Grey	Grey	Grey
Galtier, et al. 2016	Dark Blue	Grey	Grey	Grey	Grey	Grey	Grey
Jun, et al. 2014	Dark Blue	Grey	Grey	Grey	Grey	Grey	Grey
Takemura-Uchijama, et al. 2014	Dark Blue	Grey	Grey	Grey	Grey	Blue	Grey
Osanai, et al. 2012	Dark Blue	Grey	Grey	Blue	Grey	Grey	Grey
Pouillot, et al. 2012	Dark Blue	Grey	Grey	Blue	Grey	Grey	Grey
Ľubomíra Tóthová et al. 2011	Dark Blue	Grey	Grey	Blue	Dark Blue	Blue	Grey
Hung, et al. 2011	Dark Blue	Grey	Grey	Blue	Dark Blue	Blue	Grey
Hawkins, et al. 2010	Dark Blue	Grey	Grey	Blue	Grey	Grey	Grey
Sunagar, et al. 2010	Dark Blue	Grey	Grey	Blue	Grey	Grey	Grey
Nishikawa, et al. 2008	Dark Blue	Grey	Grey	Blue	Grey	Grey	Grey
Case Reports							
Lebeaux, et al., 2021	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Ferry, et al., 2020	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Bao, et al., 2020	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Cano, et al., 2020	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Rostkowska, et al., 2020	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Doub, et al., 2020	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Rubalskii, et al., 2020	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Gainey, et al., 2020	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Aslam, et al., 2019	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Nir-Paz, et al., 2019	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Tkhilashvili, et al., 2019	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Onsea, et al., 2019	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Corbellino, et al., 2019	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Susan, et al., 2019	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Gilbey, et al., 2019	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Law, et al., 2019	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
RM, et al., 2019	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Duplessis, et al., 2019	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Kuipers, et al., 2019	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
LaVergne, et al., 2018	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Ferry, et al., 2018	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Fish, et al., 2018	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Ferry, et al., 2018	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Hoyle, et al., 2018	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Chan, et al., 2018	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Ujmajuridze, et al., 2018	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Schoolley, et al., 2017	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Zhvania, et al., 2017	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Jennes, et al., 2017	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Fish, et al., 2016	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Fadlallah, et al., 2015	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Rose, et al., 2014	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Khawaldeh, et al., 2011	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Kvachadze, et al., 2011	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Letkiewicz, et al., 2009	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Clinical Trials							
Leitner, et al., 2020	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Grubb, et al., 2020	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Fabijan, et al., 2020	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Ooi, et al., 2019	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Febvre, et al., 2019	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Gindin, et al., 2018	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
McCallin, et al., 2018	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Sarkier, et al., 2017	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
McCallin, et al., 2013	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Sarkier, et al., 2012	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Rhoads, et al., 2009	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Patrick, et al., 2018	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Sarkier, et al., 2016	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue
Wright, et al., 2009	Dark Blue	Dark Blue	Grey	Blue	Dark Blue	Blue	Dark Blue

Dark Blue =have values or result within article; **Blue** = mentioned, but no values or results published within article; **Grey** = not mentioned. "Titration" is 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 the lipoteichoic acid, superantigens, or CsCl, etc.

4.6 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 [80, 81]. 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 [82], 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 FDA-approved biodegradable polymer often used for drug delivery systems [83-86]. 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 is method is not ideal for large scale phage purification [87].

4.7 Optimization of Safety and Toxicity Monitoring in Phage Therapy

In the animal studies, phage doses were variable, ranging from 10^3 to 10^{12} PFU/ml. None 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 in large animals (e.g., pigs), limiting generalizability to humans. The majority of animal studies utilized intraperitoneal 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 event, laboratory derangements, changes in vital signs, review of systems, and physical examination of subjects [88]. 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 [89]. 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, and information on immune responses should be evaluated prior, during, and after phage therapy. We offer some safety endpoints for consideration in Table 3, which may provide researchers and clinicians guidance on the safety monitoring of phage therapy.

Table 3. 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 [90, 91]. One study utilized a scoring method for assessing physical examination

findings in septic mice treated with phage [92]. Another study in a murine bacteremia model introduced a health assessment score [93]. 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 [28]. Such methods provide an opportunity to improve safety and the application of scales or standardized scoring method 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 [94, 95]. The FDA and the EMA mandate that any modern phage therapy products must be made to GMP standards [96, 97]. Along with GMP, we feel phage preparations should include information on the characteristics of the phages used in pre-clinical 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 4.

Table 4. 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

5. Conclusions

There is substantial support for the development of phage therapy as an adjunct to conventional antibiotics. However, proof that phage therapy is safe and non-toxic in humans will be critical for their ultimate success. While phage therapy has generally been safe and well tolerated in studies to date, a comprehensive understanding of the interactions of phage and human hosts are lacking.

Standardized assessments of safety are essential elements of reports of phage therapy in both animals and humans, although undoubtedly generating these data can be resource-intensive, it is ultimately in the interest of all stakeholders engaged in this field to advance this work.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1. pre-clinical and clinical studies of phage therapy 2008-2021, Figure S2. Pathogens targeted in phage therapy studies. Table S1: Pre-clinical studies of phage therapy, Table S2: Case report in phage therapy, Table S3: Clinical trial of phage therapy,

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