



# Implications of Bacteriophage- and Bacteriophage Component-Based Therapies for the Clinical Microbiology Laboratory

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**ABSTRACT** Treatment of bacterial infections is increasingly challenged by resistance to currently available antibacterial agents. Not only are such agents less likely to be active today than they were in the past, but their very use has selected for and continues to select for further resistance. Additional strategies for the management of bacterial illnesses must be identified. In this review, bacteriophage-based therapies are presented as one promising approach. In anticipation of their potential expansion into clinical medicine, clinical microbiologists may wish to acquaint themselves with bacteriophages and their antibacterial components and, specifically, with methods for testing them. Here, we reviewed the literature spanning January 2007 to March 2019 on bacteriophage and phage-encoded protein therapies of relevance to clinical microbiology.

**KEYWORDS** bacteriophages

Following the early therapeutic application of bacteriophages (phages) in France in the 1930s, industrial investment in phage production took place across Europe, the former Soviet Union, and the United States (1). The popularity of human phage therapy waned in Western countries with the advent of antibiotics; as chemicals, antibiotics were easier to test in the clinical laboratory and administer than were bacteriophages. At the time, there was also an underdeveloped understanding of phage biology and a lack of standardized *in vitro* methods to assess bacteriophage activity, alongside a lack of clinical trial methodologies to evaluate them; what clinical data were available yielded inconsistent results (1). Today, phages continue to be used clinically in parts of Eastern Europe as well as the former Soviet Union (e.g., Poland, the Republic of Georgia) (1–4) and are also used in agronomy and food processing (5), as well as in veterinary medicine (6). With the current challenge of antibiotic resistance, the case for human phage therapy in Western medicine has been reopened. In addition, the potential use of phage components as antibacterial agents is being evaluated. While there are no U.S. Food and Drug Administration (FDA)-approved bacteriophages or phage component products available for human clinical application in the United States, some are being administered on an expanded-access basis, with others being used in clinical trials.

Clinical microbiologists have historically used phages for phage typing to differentiate between bacterial strains as part of outbreak investigations, though with the advent of molecular techniques, including, most recently, whole-genome sequencing, younger clinical microbiologists may be unfamiliar with phage-based testing. In anticipation of the potential expansion of phage therapy into clinical medicine, clinical microbiologists may wish to acquaint themselves with bacteriophages and their antibacterial components and specifically with methods for testing them. Here, we review

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the literature spanning January 2007 to March 2019 on bacteriophage and phage-encoded protein therapy of relevance to clinical microbiology.

Bacteriophages are a clade of bacteriolytic viruses of individual sizes ranging from 20 to 200 nm that infect their hosts, typically manifesting strain- or species-level specificity (7, 8). More than 6,000 phages have been characterized to date, with genome sizes ranging from a few thousand to 480,000 nucleotides or more (7). Residing in close association with bacteria, phages are found throughout the biosphere, including in bodies of water and sewage, and in and on humans and animals, totaling an estimated  $1 \times 10^{31}$  virions and outnumbering bacterial cells by 10-fold (9). Given their abundance and diversity, it is not surprising that most are as yet undescribed. Phage bioprospecting efforts are ongoing; this typically involves adding target bacteria in a concentrated nutrient broth to a solid (e.g., soil) or liquid (e.g., water) sample, followed by incubation for several hours (10) and then assessment for phage using the double-overlay plaque assay described below (see Fig. 2), with isolated phages then being purified by serial plating. Thirteen (as many as 19, by some estimates) phage families are distinguished by morphology, the presence or absence of an envelope, as well as the size and composition of their genome (single-stranded or double-stranded DNA or RNA) (11).

That “phages are nature’s version of precision medicine” manifests advantages and disadvantages to their therapeutic application (12). While their specificity may minimize off-target effects on commensal bacteria compared to antibiotics, this same property may limit their empirical administration by requiring testing of the bacterium with which a particular patient is infected before selecting the specific phage to treat that patient, requiring the availability of laboratory testing and potentially engendering treatment delays. The narrow spectrum of individual phages may, however, be overcome by the concurrent use of combinations of phages in phage cocktails (12).

Although bacteriophages have traditionally been classified as lytic or lysogenic, a more precise terminology is strictly lytic and temperate (13) (Fig. 1). Temperate phages enter bacteria and become incorporated into the host genome, assuming a quiescent prophage form for a period of time, and may sometimes extrude progeny phages in the absence of lysis (7). Prophages may also be excised from the genome in a process known as spontaneous induction or following cellular stress (7). Phage excision may occur precisely or may result in the splicing and packaging of adjacent bacterial genes within newly synthesized virions, followed by host lysis. Many phages are capable of lytic and temperate cycles, with the switch between them being governed by such factors as the intracellular concentrations of second messenger molecules, extracellular phage density, and environmental resources (14). Temperate phages may encode virulence (including toxin) genes, preclude subsequent phage infection (including lytic phages), and promote bacterial fitness. Interestingly, Sweere et al. recently demonstrated that Pf4 phage infecting *Pseudomonas aeruginosa* sourced from human wounds contributed to chronic infection by binding Toll-like receptor 3, reducing phagocytosis and tumor necrosis factor secretion (15). Strictly lytic phages engage only the lytic cycle; that is, they enter their hosts, seize control of the bacterial replication machinery, and express proteins that destabilize the cell envelope, resulting in immediate bacteriolysis and the dissemination of new virions for subsequent infection. Because they effect temporally constrained cell death, are not frequent vectors for horizontal gene transfer, and are not known to interact with eukaryotic cells, strictly lytic phages are typically used for phage therapy. Still, generalized transduction of host genetic material may occur with strictly lytic phages as a consequence of the inadvertent encapsulation of host genes within newly produced viral capsids (7, 68).

The clinical use of phage protein components, including endolysins (described below), depolymerases (further categorized as hydrolases [or polysaccharases], endosialidases, endorhamnosidases, and polysaccharide lyases [16]), and holins (enzymes which insert holes into bacterial cell membranes), has recently been considered (17). The use of phage components eliminates the risk of genomic integration and gene transduction, while featuring ease of dosing, quality control, and, potentially, storage. In addition, phage protein components may have more predictable activity against

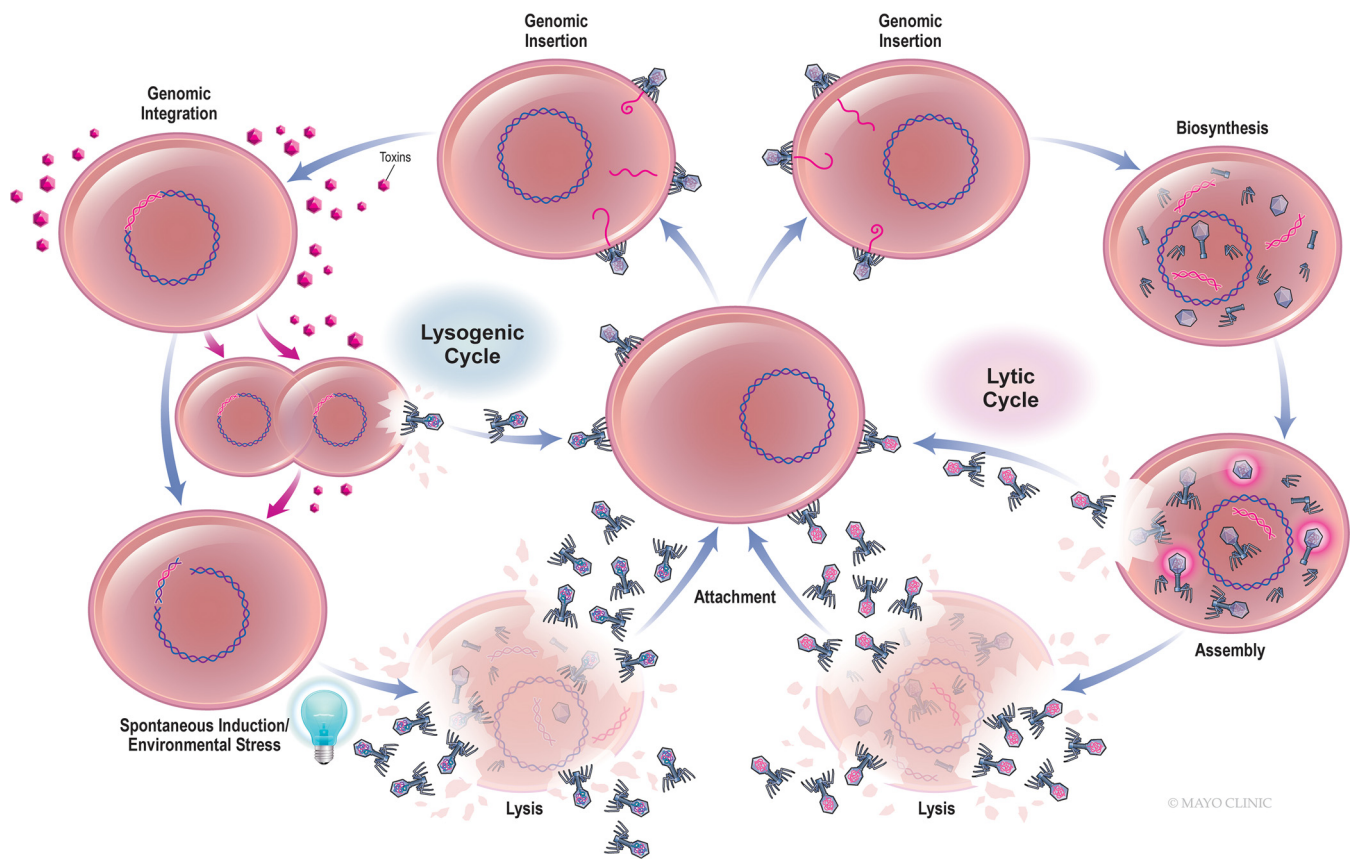


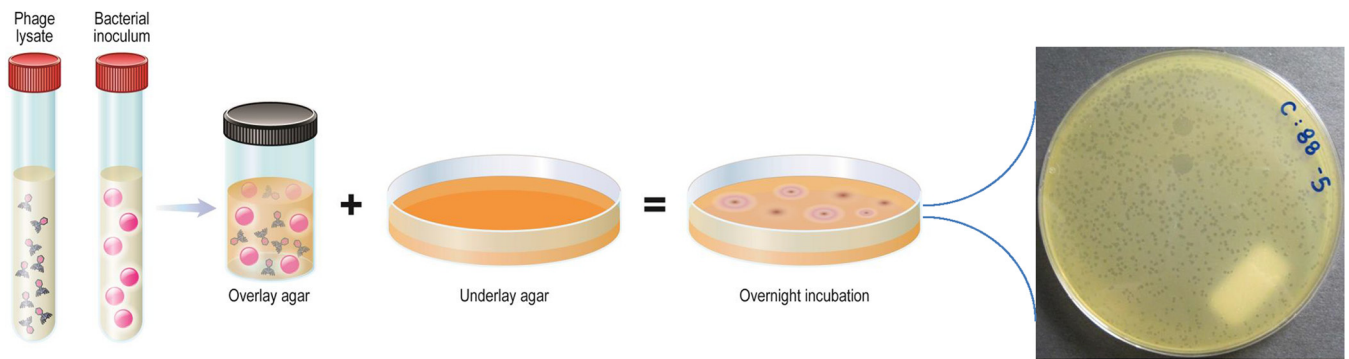
FIG 1 Bacteriophage life cycle.

strains of targeted species than phages. The drawbacks of lysin therapy include abbreviated circulation due to human host proteases and the absence of endogenous viral amplification associated with conventional phage therapy, in addition to immunogenicity, abrogating the future efficacy of the same phage protein component (18).

Endolysins, alternatively known as lysins or peptidoglycan hydrolases, hydrolyze bacterial cell walls, with those targeting Gram-positive bacteria generally being composed of an enzymatically active domain and a cell wall binding domain and with those targeting Gram-negative bacteria lacking the latter (19). Endolysins are classified on the basis of which murein bond that they cleave (20). There are two phage proteins in clinical trials at present, tonabacase (N-Rephasin SAL-200; iNtRON Biotechnology) (21) and exebacase (CF-301; ContraFect Corporation) (22), both of which are endolysin formulations specific to *Staphylococcus aureus*; tonabacase cleaves amide and peptide bonds, while exebacase cleaves amide bonds (23–25).

Adoption of phage therapy into human medicine would likely have implications for the clinical microbiology laboratory if the performance of phage susceptibility testing (PST) were to be required to select a phage or phage combinations for individual patients' isolates and to assess whether resistance to particular phages or phage combinations has been selected for in cases of individual patient treatment failure. Testing the susceptibility of patient isolates to fixed-composition phage cocktails could be considered, with screening against individual phages from a phage library being performed only in cases that are not susceptible to generic cocktails (26).

If an individualized phage therapy approach were to be adopted, health care facilities would need not only to have access to PST but possibly also to engage in phage preparation. What role clinical laboratories versus pharmacies or other entities might play in the preparation of individualized phage formulations for patient administration, as is customary in some other countries and a paradigm that is discussed



**FIG 2** Double-overlay plaque assay. A combination of amplified phage and host bacterium in cooled, molten agar supplemented with divalent cations (e.g.,  $\text{CaCl}_2$ ,  $\text{MgSO}_4$ ) is poured over solid agar and the medium is incubated overnight. Quantifiable clearings (plaques) in the bacterial lawn indicate the presence of infectious phage. At least two different plaque morphologies are observed in the example photograph, illustrating an experiment involving coculture of *Salmonella enterica* serotype Typhimurium and phages obtained from municipal sewage. (© Mayo Clinic).

further below, remains to be determined (27). Once matched with the target bacteria, phages would need to be amplified to obtain viral titers sufficiently high for human administration. This entails incubation of the phage with its bacterial target, followed by purification for pyrogen removal. Care needs to be taken to avoid the use of lysogenized bacteria for phage amplification, lest genes carried by integrated temperate phages encoding bacterial resistance or toxins become inadvertently intermingled with lytic virions. Since physical phage isolation using centrifugation and filtration does not remove all bacterial components (e.g., endotoxin), methods for removal of these need to be applied, using monovalent cations (28) or elution filters and phase separation by 1-octanol, dialysis against ethanol and NaCl and speed vacuuming (29), for example.

No standard method for PST exists. A common approach is the double-overlay plaque assay or a modification thereof (including the spot plate method, used for screening high numbers of phage-bacterium combinations), in which known concentrations of phage and bacteria are combined with semisoft agar and incubated, with the presence or absence of plaques denoting bacterial susceptibility or a lack thereof, respectively (Fig. 2). Another method is the cross-streak method, in which phage lysate is plated across an agar plate and bacteria are plated in perpendicular fashion, with susceptibility being inferred based on the zone of inhibition size (30). Merabishvili et al. described the use of large square petri dishes with 2% Luria broth agar inoculated with the target bacteria in horizontal strips, air dried, and then spotted with  $5 \mu\text{l}$  of  $10^7$ -PFU/ml suspensions of the individual bacteriophages (65). Following incubation for 16 to 18 h at  $37^\circ\text{C}$ , the degree of phage activity is classified as confluent lysis, opaque lysis, semiconfluent lysis, several plaques, or negative. These approaches can be time- and labor-intensive and may not be user-friendly in clinical laboratories. The OmniLog system (Biolog, Haywood, CA) has been adapted for PST by assaying various phage concentrations and bacteria in the presence of a tetrazolium dye in microtiter plates. If bacterial growth occurs, cells respire and reduce the tetrazolium dye to induce a color change; conversely, cell lysis secondary to phage infection abrogates host respiration which is colorimetrically apparent. This method also serves as the basis of the Host Range Quick Test, which is capable of screening a single bacterial sample against as many as 5,000 bacteriophages in less than 18 h (Adaptive Phage Therapeutics, Gaithersburg, MD). While the technology is not commercially available, it may portend the phage screening capacity of clinical laboratories in the future.

An unanswered question pertaining to PST regards the need for the establishment of positive, intermediate, and negative phage infectivity thresholds, as is standard for interpretation of the MICs of traditional antibiotics. The convention in phage research is to measure the multiplicity of infection (MOI), that is, the ratio of phages to bacteria. This metric may be challenging to apply in phage therapy because one would not

necessarily know how many bacteria are being treated, that number could change over time, and some bacteria may be more or less accessible to phage than others (31). Additionally, unlike traditional antibiotics, phage can amplify in the host, such that the dose increases *in vivo*, the very attractiveness of phage therapy. In the end, phage will likely be delivered as a specific number of phages; how that delivery will take place is the subject of ongoing studies and may vary depending on the site and type of infection. This, too, could influence the interpretation of results of laboratory studies on phage activity. Further, it is likely that phage will be applied in challenging-to-treat infections, which, in some cases, will involve biofilms. As such, methods for determining phage antibiofilm activity, which is not even standardized for traditional antibiotics, may be needed.

In contrast to PST, testing for susceptibility to phage-encoded proteins is more similar to conventional antibacterial susceptibility testing. For example, a standard or modified broth microdilution method is used for susceptibility testing of tonabacase (21) and exebacase (32), respectively, with the modification comprised of the addition of 0.5 mM DL-dithiothreitol and 25% horse serum to cation-adjusted Mueller-Hinton broth (33). Quality control ranges for exebacase are being developed by the Clinical and Laboratory Standards Institute (34). As a result of the use of broth microdilution, lysin susceptibility testing should be amenable to automation on commercial susceptibility testing instruments (35).

Exercising proper storage and maintenance is as crucial as selecting a phage(s) active against the target bacteria toward successful phage therapy. Given the proteinaceous composition of viruses, shear forces or variations in the temperature and pH to which phages are subjected during handling may impact titers as a result of protein misfolding or denaturation. Their shelf life may vary by bacteriophage species, as a result of phage size and morphology (29, 36–39). The storage methods utilized in the Republic of Georgia, where phage therapy is used clinically, have included freeze-drying, freezing, refrigeration, and short-term ambient storage (Marina Tediashvili, personal communication), though some studies suggest that phase changes, including freezing or drying, may result in drops in titer, at least among certain phages (37). These effects may be abrogated by the addition of excipients, such as skim milk (37, 40). Phage viability over long-term storage appears to be maximized at 4°C, –80°C, or –196°C (41). It is likely that conditions for ideal stability will need to be evaluated for each phage considered for clinical use. Lysins, in contrast to whole phages, may exhibit stability at a wider range of temperatures and pHs (22).

Whether or not phages will ultimately be adopted into the therapeutic armamentarium in Western medicine is unknown. Case studies of phage therapy continue to be added to the published literature (Table 1). Patey et al. note that the generally positive results reported from isolated applications stand in contrast to the negative results of some clinical trials (Table 2) (42). The routes of phage administration and dosing intervals, as well as the diversity of target bacteria and infection types, portend that the investigational use of phage therapy will likely continue for some time in the United States. PST methods ought to be standardized in order to guide studies and enable results between studies to be compared and observations generalized and extrapolated.

Phages may be used as a complementary approach to currently available antimicrobial strategies rather than a replacement (42). As viruses, they and their components may be recognized by the immune system and eliminated via innate and/or adaptive defenses before reaching sites of infection, and their use may also compromise the future use of similar species in the same patient. Phages are also 6-fold larger than small-molecule antibiotics, limiting their distribution and ability to diffuse across some physiological membranes, such as the blood-brain barrier, and, subsequently, their utility to treat certain types of infection (3, 43). Finally, their host specificity requires identification of the pathogen prior to treatment, such that empirical therapy may be challenging (43, 44).

How phage therapy would be regulated in the United States is presently unclear,

**TABLE 1** Human case reports and case series involving phage and phage lysin therapy published between January 2007 and March 2019

Bacterium	Clinical presentation	Phage(s) administered	Method of phage administration	Outcome	Reference(s)
<i>Acinetobacter baumannii</i>	68-yr-old man, necrotizing pancreatitis	φPC cocktail (AC4, C1P12, C2P21, C2P24), φIV cocktail (AB-Navv1, AB-Navv4, AB-Navv71, AB-Navv97), φIVB cocktail (AB-Navv71, AbTP3φ1)	Intracavitary and intravenous administration of approximately 10 <sup>9</sup> PFU/dose alongside multiple antibiotics	Recovery	50
	77-yr-old man, craniectomy site infection	1 proprietary phage	Intravenous administration of 2.14 × 10 <sup>7</sup> PFU/ml in 4 ml lactated Ringer solution every 2 h for 8 days	Withdrawal of treatment and death	51
<i>Enterococcus faecalis</i>	3 men (32–45 yr old), chronic bacterial prostatitis	Assorted lytic phages (from the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences) screened against prostatic secretion isolates	Twice daily intrarectal administration of 10 ml of 4.5 × 10 <sup>7</sup> to 2.8 × 10 <sup>8</sup> PFU/ml for 28–33 days	Recovery	52, 53
Multiple-antibiotic-resistant bacteria	62 patients, genitourinary tract infections, prostatitis, bone infections, upper or lower respiratory tract infections, or skin or soft tissue infections	MS-1 cocktail, OPMS-1 cocktail, monovalent <i>Staphylococcus aureus</i> phages (unspecified), monovalent <i>Enterococcus faecalis</i> phages (unspecified), monovalent Gram-negative bacterial phages (unspecified)	Oral, oral and local, intrarectal, or local bacteriophage (10 <sup>6</sup> –10 <sup>9</sup> PFU/ml) twice or three times daily for 12 wk or more at dosing volumes of 10 ml for oral and intrarectal administration (volume unspecified for local administration)	Favorable response in 40–55% of patients	54
<i>Pseudomonas aeruginosa</i>	76-yr-old man, infected, fistulated aortic graft	OMKO1	10 ml of 1 × 10 <sup>7</sup> PFU/ml of intrafistular phage and ceftazidime	Recovery	55
	61-yr-old man, pressure sores and septicemia	BFC1 cocktail (PNM, 14/1, ISP)	Topical (50 ml 10 <sup>9</sup> PFU/ml every 8 h) and intravenous (50 μl 10 <sup>9</sup> PFU/ml/6 h) for 10 days	Blood cultures negative, but local infection persisted and patient died 4 mo later due to unrelated infection	56
	67-yr-old woman, urinary tract infection	Pyophage 051007 (proprietary 6-phage cocktail)	Bladder irrigation of 20 ml containing 2 × 10 <sup>7</sup> PFU twice daily for 10 days with concurrent colistin and meropenem and subsequent meropenem	Recovery	57
	2-yr-old boy, bacteremia	Proprietary 2-phage cocktail	Intravenous administration of 3.5 × 10 <sup>5</sup> PFU every 6 h for 3 days alongside meropenem, tobramycin, aztreonam, colistin, and polymyxin B	Recovery	58
<i>Staphylococcus aureus</i>	65-yr-old woman, bacterial keratitis	SATA-8505	Intravenous, intranasal, and intraocular administration over 4 wk (dose unspecified)	Recovery	59
	6 men (44–92 years old), diabetic foot ulcers	Sb-1	Weekly tissue debridement and topical phage treatment (0.1–0.5 ml of 10 <sup>7</sup> –10 <sup>8</sup> PFU/ml)	Recovery	60

**TABLE 2** Phage and phage lysin therapy in human clinical trials from January 2007 to March 2019

Yr of trial start	Trial registration no.	Reference	Phage/lysin formulation (investigator/sponsor)	Bacteria or bacterium targeted	Treatment indication	Trial phase	Outcome
2006	NCT00663091	61	WPP-201 cocktail (Southwest Regional Wound Care Center)	<i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i>	Infected venous leg ulcers	I	No adverse effects observed
2009	NCT00937274	62, 66	T4 coliphage cocktail: AB2, 4, 6, 11, 46, 50, 55, JS34, 37, 98, D1.4 (Nestlé)	<i>E. coli</i>	Dysentery	I/II	No adverse effects or benefit noted; study discontinued prematurely
2009	EudraCT 2004-001691-39	63	Biophage-PA (Biocontrol Ltd.)	<i>P. aeruginosa</i>	Otitis externa	I/II	No adverse effects noted; at 42 days posttreatment, the phage-treated cohort exhibited reduced median bacterial abundance compared to placebo group
2015	NCT02116010	67	PhagoBurn (Pherecydes Pharma)	<i>P. aeruginosa</i> , <i>E. coli</i>	Infected burn wounds	I/II	Fewer adverse effects noted in phage versus antibiotic treatment arms; median time to sustained bacterial reduction comparable between treatment groups by day 7; study prematurely discontinued
2017	NCT03089697	NA <sup>a</sup>	SAL200 (Tonabacase; iNtRON Biotechnology)	<i>S. aureus</i>	Bacteremia	II	Ongoing
2017	NCT03163446	64	Exebacase (CF-301; ContraFect Corp.)	<i>S. aureus</i>	Bacteremia, including endocarditis (single intravenous dose added to standard-of-care antibiotics)	II	Well tolerated; higher clinical response rate compared to antibiotics alone in methicillin-resistant <i>S. aureus</i> - but not methicillin-susceptible <i>S. aureus</i> -infected subgroups
2017	NCT03140085	NA	Pyophage (Tzulakidze National Center of Urology)	<i>Enterococcus</i> species, <i>E. coli</i> , <i>Proteus mirabilis</i> , <i>Staphylococci</i> , <i>streptococci</i> , <i>P. aeruginosa</i>	Urinary tract infections	II/III	Results pending (Thomas Kessler, personal communication)
2019	NCT03808103	NA	EcoActive (Intralix, Inc.)	Adherent invasive <i>E. coli</i> (AIEC)	Exacerbation of inflammation in Crohn disease secondary to AIEC	I/II	Recruitment ongoing
2019	NCT02664740	NA	PhagoPied (Pherecydes Pharma)	<i>S. aureus</i>	Diabetic wounds	I/II	Not yet recruiting

<sup>a</sup>NA, not applicable.

with some questioning whether this can be done within the existing drug and device approval infrastructure of the U.S. Food and Drug Administration, founded on the concept of drugs as small molecules of fixed composition (42, 44, 45). Subjecting the production and oversight of therapeutic phage to current good manufacturing practice and evaluation within conventional randomized clinical trials will be associated with significant cost and incite process development inefficiencies (e.g., in a phage cocktail, each phage as well as the final cocktail may require FDA approval; the composition of off-the-shelf phage products might require updating at regular intervals to avoid resistance and to maintain clinical relevance to current pathogens), culminating in approval delays (42, 44, 46, 47). Some countries in which phages are used therapeutically operate under different regulatory guidelines; for example, Belgium uses magistral preparations, the adoption of which some favor on a broader scale (27, 46). By this course, phage is prescribed by a clinician, and the corresponding formulation is produced by an associated pharmacy using master phage stocks that are quality controlled by an accredited laboratory (27). Regulation will also depend upon the level at which oversight of phage treatment is controlled: individual patients, hospitals, local or national governments, or some combination thereof (48, 49). While offering a critique or suggestions for the regulation of phage therapy is beyond the scope of this review, clinical microbiologists should be aware of the possibilities receiving consideration as well as their predicted impact on laboratory practice.

Although more translational research must be completed before the clinical implementation of bacteriophage therapy is feasible, it is not too early to consider how this antibacterial strategy may shift current treatment paradigms. Such a shift will likely impact the clinical microbiology laboratory, upon which the burden of PST, reporting parameters, and possibly phage storage (at least for *in vitro* testing) will rest.

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