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Can phage effectively treat multidrug-resistant plague?

Andrey A. Filippov,* Kirill V. Sergueev and Mikeljon P. Nikolich
Bacterial Diseases Branch; Walter Reed Army Institute of Research; Silver Spring, MD USA

The spread of natural or weaponized drug-resistant plague among humans is a credible high consequence threat to public health that demands the prompt introduction of alternatives to antibiotics such as bacteriophage. Early attempts to treat plague with phages in the 1920s–1930s were sometimes promising but mostly failed, purportedly due to insufficient knowledge of phage biology and poor experimental design. We recently reported the striking stability of plague diagnostic bacteriophages, their safety for animal use, propagation in vivo and partial protection of mice from deadly plague after a single injection of phage. In this addendum we reflect on that article, other recent publications and our unpublished data, and discuss the prospects of phage therapy against plague.

Keywords: *Yersinia pestis*, multidrug-resistant plague, bacteriophages, phage safety, pharmacokinetics and pharmacodynamics, plague therapy

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*Correspondence to: Andrey A. Filippov; Email: andrey.filippov@amedd.army.mil

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Multidrug-Resistant Plague: Urgent Need for New Therapies

Yersinia pestis, the cause of plague, is considered the most devastating bacterial killer in the history of mankind. Plague still poses a serious public health problem. There are 2,000–4,000 cases of human plague every year globally and this is on the increase. Plague is a severe infection resulting in 60–100% mortality without antibiotic therapy and is fatal even for 4–60% of patients that receive intensive antibacterial treatment. Owing to easy aerosol dissemination and high lethality of pneumonic infection, *Y. pestis* is classified as a category A biothreat agent.[1] A phenomenon of high concern is the isolation of three multidrug-resistant strains of *Y. pestis*, from patients and from a wild rodent, that include an isolate resistant to all antimicrobials recommended for plague treatment and prophylaxis.[2,3] In at least two strains, the resistance genes are encoded on conjugative plasmids, one of which was shown to transfer to *Y. pestis* at a high frequency in the flea, suggesting a high probability of the emergence of new drug-resistant strains.[2] Natural multidrug-resistant strains of *Y. pestis*, or those engineered by bioterrorists, could cause epidemics of deadly plague with no effective therapeutic solutions.

Thus, new alternatives to antibiotics in the treatment of plague are urgently needed. These should include the utilization of lytic bacteriophages. The first attempt to treat plague with phage was performed as early as 1925 in four patients with bubonic plague, by direct injection of a lytic phage suspension into buboes. All the patients recovered in several days.[4] However, further plague phage therapeutic studies in the 1920s–1930s offered conflicting results, possibly caused by ignorance of phage biology and improper laboratory practices.[5] After the advent of chemotherapy and the first success of plague treatment with a sulfonamide in 1938,[6] the interest in phages as potential anti-infectives was lost for about 60 years. Inspired by multiple successful phage therapeutic trials against various infections,[7,8] we recently tested several phages lytic for *Y. pestis* as potential plague therapeutics[9–11] and observed up to 40% recovery of mice injected with phage from fatal plague infection and a marked extension of time to death in nonsurvivors.[11] Below, we comment on that article[11] and some of our unpublished data, and discuss possible ways to increase the therapeutic efficacy of phages against emerging drug-resistant plague.

Phages Lytic for *Y. pestis* and Future Plague Therapeutic Cocktails

Table 1 lists 11 bacteriophages highly active against *Y. pestis* that belong to four groups: T7, T4, T1 and P2, whose genomes have been sequenced to date. The genome sequences of ϕA1122,[12] L-413C,[17] Yep-phi,[15] and PY100[16] were published. We recently sequenced the genomes of Pokrovskaya, Y, R, d'Herelle-m, PST and ϕJA1 (unpublished data). Sequence analysis showed that the Pokrovskaya, Y, R and d'Herelle-m phages turned out to be sequenced earlier under different designations (**Table 1**). It is important that no genes that were potentially toxic for warm-blooded animals were found in the well-annotated phage genomes.

Phages Pokrovskaya,[9,10] L-413C,[9,10,13,17] ϕA1122,[9,10,12,13] and Yep-phi[15] are species-specific and widely used for *Y. pestis* identification and plague diagnosis. The Y and ϕJA1 phages were also shown to be *Y. pestis*-specific.[10] R, d'Herelle-m and PST are considered pseudotuberculosis diagnostic phages but they are also active against *Y. pestis*.[9,10] Pokrovskaya, L-413C, ϕJA1, ϕA1122, Y and d'Herelle-m display low efficiencies of plating on *E. coli* at 37°C[10] and therefore they seem not to affect normal microflora in animals and humans. The receptor for ϕA1122 was identified in *Y. pestis* LPS inner core.[9,14] We detected six more cell surface receptors for other phages capable of lysing *Y. pestis*; nine phages were shown to have seven receptors, mostly in different parts of the LPS core[9] (**Table 1**). Using these phages in a therapeutic cocktail would help to prevent cross-resistance: *Y. pestis* mutants resistant to a certain phage would be still susceptible to the others.[18] The potential problem of therapeutic phage resistance was further addressed by testing the virulence of spontaneous and site-directed phage-resistant mutants of *Y. pestis*. The majority of them were shown to be attenuated, thus such mutants should be eliminated by the immune system without risk of impeding the efficiency of phage therapy.[9] The resistance problem is not applicable to the ϕA1122 phage because we could not isolate any spontaneous ϕA1122-resistant mutants; such mutations should arise at extremely low frequency, < 10^{10} per cell per generation.[9] Thus, there is a battery of highly lytic phages promising for the formulation of plague therapeutic cocktails.

Phage Stability and Safety

For further tests as potential plague anti-infectives,[11] we selected phages ϕA1122 and L-413C (**Table 1**). They were purified by double CsCl gradient ultracentrifugation[19] and single overnight dialysis against 1,000 volumes of phosphate-buffered saline (PBS) and were stored in PBS supplemented with gelatin (BSG buffer).[20] Since phage viability is critical for therapeutic efficacy, it is important to test and maintain their stability.[7] It is well-known that phage stocks in special storage buffers are stable for years.[19] We showed that ϕA1122 and L-413C suspensions in BSG buffers suitable for therapeutic use are surprisingly stable: they did not reduce their viable titers for at least 27 mo at +4°C.[11]

Phage ϕA1122[12] and L-413C[17] genomes displayed no toxin-coding genes but it was important to test potential cytotoxicity and acute toxicity of phage preparations, because the phages were propagated on *Y. pestis* producing endotoxin and several other toxic substances.[1] No cytotoxicity was determined for mouse macrophages, human monocytes or hepatocytes, using as high multiplicity of infection as 10,000:1.[11] Moreover, we observed a moderate but stable and reproducible increase in viability of each cell line in response to ϕA1122 and L-413C (unpublished data). The ϕA1122 phage also showed a lack of acute toxicity for mice after intraperitoneal injection.[11] Therefore, two plague diagnostic phages demonstrated a high stability and safety for animal studies.

Do Phages Target the Plague Bacterium Inside Macrophages?

Since a critical phase of plague infection is survival and multiplication of *Y. pestis* inside phagocytes,[21] we investigated if phage can gain entry into macrophages and kill *Y. pestis* there. A recent publication claimed that phage adsorbed to *Staphylococcus aureus* cells can be engulfed by mouse macrophages and lyse the phagocytosed bacteria.[22] However, the experiments were designed so that gentamicin was added to a macrophage culture after bacterial infection to kill extracellular bacteria for only 1 h, then the antibiotic was removed, cells were incubated

Table 1. Bacteriophages lytic for *Y. pestis*—potential components of therapeutic cocktails

Bacteriophage	Group	Cell wall receptor*	Reference(s) and/or sequence No.
Pokrovskaya (YepE2, YpP-G)	T7	HepII/HepIII	9, 10; NC_011038; JQ965702
φA1122	T7	KdoI/KdoII	9–14; NC_004777
Y (YpP-Y)	T7	HepI/Glc	9, 10; JQ965700
R (YpP-R)	T7	Beyond LPS core	9, 10; JQ965701
d'Herelle-m (YpsP-G)	T7	ND†	10; JQ965703
Yep-phi	T7	ND	15; HQ333270
Berlin	T7	ND	NC_008694
PST	T4	HepII/HepIII	9,10
φJA1*	T4	KdoI/KdoII	9,10
PY100	T1	ND	16; AM076770
L-413C	P2	GlcNAc	9–11, 13, 17; NC_004745

*Sugar residues of *Y. pestis* lipopolysaccharide (LPS) critical for phage receptor structure are presented. †ND, not determined.

without it for 3 h before phage infection and 45 h after phage infection.[22] We suppose that under these conditions, there was a possibility of survival of *S. aureus* outside macrophages followed by phage propagation and reduction of overall bacteria due to killing by the phage. We tested both ϕA1122 or L-413C suspensions and each phage adsorbed to *Y. pestis* cells for potential uptake by mouse macrophages and subsequent intracellular lysis of *Y. pestis* inside macrophages but modified the experimental design, keeping gentamicin in the medium at all times after bacterial infection and phage inoculation, to make sure that all extracellular bacteria were killed.[11] Under these conditions, we did not observe any intracellular bactericidal effect of the phages. This could happen due to difficulties for phage to find and target intracellular bacteria or because of phage inactivation. Anyway, our data suggest that phages ϕA1122 and L-413C can kill *Y. pestis* mostly in extracellular matrix, blood and body fluids but not inside mammalian cells.

Phage Pharmacokinetics, Pharmacodynamics and Therapy of Plague

Bacteriophage ϕA1122 was selected for further animal trials in BALB/c mice[11] because of its higher lytic potential and a lack of phage resistance mutations. Since intraperitoneal (IP) phage administration is very efficient[8] and intramuscular (IM) route can provide even higher phage concentrations in blood and organs,[23] we compared ϕA1122 pharmacokinetics by IP and IM routes. Phage concentrations in spleen and liver suspensions and blood were one log higher after a single IP than after IM injection. When using the IP route, live phage titers in blood, liver and spleen dropped in 96 h by six, four and half orders of magnitude, respectively.[11] More rapid reduction of titers of *Pseudomonas aeruginosa* phages in blood in comparison with liver and spleen was observed earlier.[23,24]

Phage ϕA1122 pharmacodynamics was tested for three days, using subcutaneous injection of 10,000 median lethal doses (LD_{50}) of *Y. pestis* followed after 1 h by IP injection of 5×10^9 of phage particles; the same doses of the bacteria and phage were administered separately to control mice.[11] The experiment demonstrated ϕA1122 propagation in mice infected with *Y. pestis*, significant reduction of *Y. pestis* by the action of ϕA1122 in liver and total clearance of blood and spleen. But when using a similar design with additional IP injection of ϕA1122 in the same dose on day 3 and extending the time of observation up to five days, we found increase in numbers of live bacteria on day 5 in liver, as well as emergence and rapid growth of *Y. pestis* on day 4 in spleen and on day 5 in blood (unpublished data). It was previously shown that *Y. pestis* cells start intensively growing within splenic $CD11b^+$ macrophages on day 3 postinfection; days 4 and 5 are characterized by a rapid increase in bacterial numbers and escape of bacteria into the extracellular milieu.[21] The rise of *Y. pestis* cell numbers in spleen and liver suspensions despite high concentration of ϕA1122 seems to reflect disconnection between the bacteria and phage: phage particles reside in extracellular matrix, gradually diminishing in numbers, while *Y. pestis* cells rapidly multiply inside splenic and hepatic cells.

Phage ϕA1122 treatment of bubonic plague in mice was performed by a single IP injection of the phage in two doses (5×10^8 and 5×10^9 live particles) 1 h after subcutaneous challenge with 0; 1; 10; 100; 1,000; and 10,000 LD_{50} of *Y. pestis*. A therapeutic effect was observed with both phage doses in significant extension of mean time to death (MTD) and survival of some mice. The most effective result at both phage doses (survival of 20–40% mice and 84% extension of MTD in nonsurvivors) was observed after the challenge with 10^3 LD_{50} of *Y. pestis*.[11] This result should be considered highly encouraging, taking into account that all previous phage therapy trials of systemic infections in mice were performed against relatively low virulence bacteria, e.g., *P. aeruginosa, S. aureus*, *Vibrio vulnificus* (LD_{50} = 10^6–10^9 cells),[7,8,20,22–24] while we utilized phage for the treatment of fulminant infection with *Y. pestis* (LD_{50} for mice = 2 bacteria; MTD at 1 LD_{50} = 5.8 d, at 10^4 LD_{50} – 3.6 d).[11] Using a single IP injection of the ϕA1122 phage, we observed a therapeutic efficiency comparable with the results of 14- to 25-fold administration of a high dose of antibiotic.[25,26]

Future Prospects: How to Improve Phage Therapeutic Efficacy against Plague

Since bacteremia is an important stage of plague[1,21] and phage concentration first decreases in blood,[11] a promising way to enhance the efficiency of plague therapy would be to apply intravenous administration (up to once or twice a day) in order to provide higher concentrations of phage in the blood. One more possible approach is the selection of long-circulating phage mutants.[27] The sentinel phages persisting in blood for 10–14 d could rapidly lyse *Y. pestis* cells released from phagocytes and prevent massive one-step bacterial lysis that could result in septic shock.[1] A very efficient means of application in the case of bubonic plague could be administration of phage directly into the bubo[4] or several subcutaneous injections around the bubo. Phages other than ϕA1122 may be more efficacious. For example, Pokrovskaya has an even higher lytic ability than ϕA1122 and L-413C has a much higher burst size.[13] It is tempting to speculate that some phages can get inside macrophages and be efficient intracellular killers of *Y. pestis*, in contrast to ϕA1122 and L-413C.[11] Phages of different groups in a therapeutic cocktail could synergize due to diverse tropism for different organs and body fluids. We think that mouse models are non-optimal for testing phage therapy against plague, because the infectious process is too rapid and it is difficult to achieve 100% inactivation of *Y. pestis*, while just one to two surviving bacterial cells can kill a mouse. Rat and guinea pig models could provide much better results due to higher lethal doses of *Y. pestis* and longer durations of infection. It should be noted that the lethal doses of *Y. pestis* administered by aerosolization are much higher than those by subcutaneous infection: LD_{50} in mice for the same *Y. pestis* strain that we used (CO92) was 2×10^4 bacteria,[25] and thus intranasal or intravenous administration of phages is supposed to be efficient against experimental pneumonic plague in mice. Therefore, there are clear prospects for developing and using phages as

a new therapy against multidrug-resistant plague.

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