Successful treatment of antibiotic resistant poly-microbial bone infection with bacteriophages and antibiotics combination

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Abstract

A patient with a trauma-related left tibial infection associated with XDR *Acinetobacter baumannii* and MDR *Klebsiella pneumoniae* was treated with bacteriophages and antibiotics. Tissue healing and eradication of positive cultures were rapidly observed. As a result, the patient's leg did not have to be amputated and he is undergoing rehabilitation.

Keywords. Bacteriophage, Phage, Phage therapy, antibiotic resistance, Orthopedic infections

Resistant bacteria pose a major medical challenge, leading to morbidity, mortality and significant economic costs [1]. Recently, therapy with bacteriophages (phages), viruses which lyse their bacterial targets, have been given intravenously to patients infected by multidrug resistant bacteria [2]. They appear to be safe and effective, even in attacking biofilms [3].

Lytic phages were described in 1917 by Felix d'Herelle and since then they have been used in clinical practice in Eastern Europe [4]. In spite of this, well controlled trials of phages, with or without antibiotics, are limited [5]. Recently phages have been used to treat a small number of patients suffering from fulminant bacterial infections [6-9] and a phase 1/2 study of phage in *Pseudomonas* burn infections has been completed [10].

Here, we describe a patient with bacterial osteomyelitis who had extensively drug resistant (XDR) *Acinetobacter baumannii* (*Ab*) and multi-drug resistant (MDR) *Klebsiella pneumoniae* (*Kp*) infections. These were successfully treated with a combination of bacteriophages and antibiotics. A phage-resistant *Ab* mutant developed *in vitro*, but fortunately, not in the patient, and we could quickly isolate a new lytic phage to combat it. This shows the potential flexibility of phage treatments.

METHODS

Isolation of Pathogens

Bacterial strains were isolated from deep tissue biopsies and wound swab samples. Bacterial isolates were identified by MALDI-TOF (VITEK MS, BioMerioux). Antibiotic sensitivity was tested using the VITEK 2 system (BioMerioux) and validated using the E-test (BioMerioux).

Screening and preparation of phages for treatment

The pathogenic isolates were screened at the Naval Medical research Center and Adaptive Phage Therapeutics labs for phages with lytic activity as was previously described (Figure S1-3 and [7]). For more details, see Supplementary Methods [S1].

Bacteria, phages, antibiotics

The bacteria studied here and their accession numbers are *A. baumannii Ab*KT722 (sequence accession number: RXIN00000000) and *K. pneumoniae Kp*KT1 which were isolated from the patient and *Ab*KT722res (RXIM00000000), an *Ab*KT722 ϕAbKT21phi3 phage resistant derivative isolated in the lab. The phages used for treatment (ϕAbKT21phi3; MK278859 and ϕKpKT21phi1; MK278861) were obtained from the Naval Medical Research Centre phage bank. ϕAbTZA1 (MK278860) vs *Ab*KT722res was isolated from sewage but not used for treatment. The antibiotics employed were Colistin (Colistimethate sodium, Rafa laboratories) and Meropenem (Anfarm, Hellas, Greece). For *in vitro*

studies, bacteria were grown in LB at 37 ^{0}C, and phages were propagated as previously described [11]. When mixtures of different bacteria were used, they were plated on MacConkey agar plates in which colony color differentiated between the two (see also Supplementary Methods S1).

DNA sequencing and analysis

Bacterial and phage genomes were sequenced and analyzed as previously described [11] (Supplementary Methods S1).

Ethics approval

The experimental treatment protocol and informed consent agreement were approved by the Hadassah-Hebrew University Medical Center ethics committee and the Israeli Ministry of Health. The treatment was given after the patient gave informed consent.

RESULTS

The case

A 42-year-old man was admitted to the trauma unit of Hadassah-Hebrew University Medical Center after a motor vehicle accident. His injuries included bilateral Grade IIIA open fractures of the lower extremities: a left bicondylar tibial plateau fracture with compartment syndrome and a right distal femoral fracture. He was treated initially with external fixation, irrigation, and debridement plus left leg fasciotomies. A few days later, the external fixations were converted to internal fixations after open reduction of both fractures. Nine days after his admission, an infection of his right proximal tibia was detected and MDR *Kp* was isolated from the wound. Six weeks after admission, an infection of his left tibia with XDR *Ab* (Table S1, designated *Ab*KT722) and an MDR *Kp* (Table S1, *KP*KT1) was found. During his long course of treatment, he underwent serial irrigations and debridements (I&D), and placement of a cement spacer due to bone loss of the lateral tibial condyle. The patient was also treated with a prolonged course of antibiotics: 6 weeks of Piperacillin/tazobactam (4.5 gr tid) initially followed by an 8 week course of Meropenem (1gr tid) and high dose Colistin (2 million units tid). Afterwards, he had secondary bone grafting (iliac crest tricortical autograft) and fixation of his left tibial plateau, local irrigation, debridement and coverage with a lateral rotational gastrocnemius flap (LGF) to fill a soft tissue deficit. Despite the aggressive I&Ds and flap coverage, the wound broke down (Figure 1A) and XDR *Ab*KT722, resistant to carbapenems, and colistin, was again cultured from the. Although the infections could not be eradicated with antibiotics, his inflammatory markers were close to normal--a WBC of 7-8x10^{9}/L with normal differential, and CRP of 1.5 mg/dL (norm up to 0.5) probably due to the chronicity of infection. Because of the highly resistant pathogens and failure of the antibiotic and surgical

treatments, an above the knee amputation was recommended, but the patient refused. At this point (7 months after his initial admission) phage therapy was considered as a last resort.

Phage therapy

The patient received a combination of phage ϕAbKT21phi3 and ϕKpKT21phi1 targeting both the *Ab* and *Kp* strains, along with IV meropenem (2 gr tid) and colistin ($4.5x10^6$ units/ bid), which were found to be highly effective *in-vitro* (Figures 1B-E and Supplemental figures S1 – S4). One ml of each phage ($5x10^7$ PFU/ml) was administered IV tid, over 35 minutes. The endotoxin concentrations were 35 EU/ml for ϕKpKT21phi1 and 5 EU/ml for ϕAbKT21phi3. The first 3 doses were given in the Intensive Care Unit to monitor adverse events. No deleterious effects of phage therapy were observed during or after the treatments which were given for 5 days. Since *Ab* was isolated from the healing wound at the end of the 1st treatment course, a 2nd treatment course was given for additional six days one week later.

The first signs of wound recovery (Figures 1A and S5), graft healing, and elimination of subtle chronic bone pain in the patient's left leg were noted within few days after the initiation of phage therapy. During an 8-month post-treatment follow-up period, no positive cultures for either *Ab* or *Kp* were obtained from any site. The patient's wound has closed with no secretions (Figure 1A and S5) and his pain has disappeared. Blood, stool, urine and saliva samples were analyzed for the presence of active phages against the patient's bacterial isolates but none were found. PET-CT imaging of his legs, 2 months after the therapy, suggested that active inflammatory processes are present in both of his lower extremities. Of note, 3 months post-treatment the patient had a methicillin sensitive *Staphylococcus aureus* infection of right femur that was successfully treated with 6 weeks of IV cefazolin.

AbKT722 and KpKT1 mutuality

*Kp*KT1 could not grow in the presence of meropenem (Figure 1D). However, when mixed with *Ab*KT722 its growth recovered (Figure 1E), most probably due to the carbapenemase that *Ab*KT722 produces (Supplemental Table S2). Moreover, when they were cultured together, the 2 bacteria were relatively resistant to antibiotics. It was only when both of the phages and all 3 antibiotics were given together that growth of both bacteria ceased (Figure 1E).

Isolation of a phage-resistant bacterium and a new phage that attacks it

Fortunately, no phage-resistant *A. baumannii* emerged in the patient described above. However, during our *in-vitro* experiments, such a mutant did evolve (designated *Ab*KT722res, accession: RXIM00000000). Comparing sequences of *Ab*KT722 and *Ab*KT722res revealed alterations in 37 genes (Table S3). While

non is an obvious phage adhesion target, we speculate that a surface adhesin and an EpsG family protein (Pfam: PF14897) encoding a glycosyl-transferases might account for ϕAbKT21phi3 insensitivity.

Within a few weeks we succeeded in isolating a new phage from sewage water, termed ϕAbTZA1, which effectively killed *Ab*KT722res (Figure 1F) as well as *Ab*KT722, albeit, less efficiently (Figure 1G). Genome sequencing and electron microscopy (Figure 1H) revealed that ϕAbKT21phi3 belongs to the Podoviridae family, and ϕAbTZA1, like many other phages reported to be efficient for phage therapy, belongs to the Myoviridae family. Bacteriophage genome analysis did not reveal any putative toxins and antimicrobial resistance genes.

DISCUSSION

Phages are currently emerging as potential treatments for multidrug resistant bacterial infections. Our case illustrates this, but it is unique in some regards. We treated our patient with two phages directed at two different bacterial species, *Ab* and *Kp*. We suggest that these bacteria cooperated with one another to produce antibiotic- and phage- resistance, and that the combination of antibiotics and phages was more effective than either treatment modality alone. Osteomyelitis heals slowly and can relapse months or years after the initial infection. Eight months after completion of the combined treatment with antibiotics and phage, the wound remains closed and dry. Nonetheless, it is too early to know whether the infection has resolved completely. Yet, it is clear that phage treatment allowed the patient's wound to close so that he could be discharged from the hospital after almost 7 months as an inpatient. In addition, we have shown that if a phage-resistant mutant had emerged, a new phage could be isolated in a few weeks.

In the setting of orthopedic implant persistent infections, bacteriophages have the potential of penetrating biofilms [12]. Thus, when it is desirable to retain an implant, phage therapy has the potential to promote healing without implant removal. Many questions remain to be answered. What might be the right dose of phage and schedule of administration to use? Would local administration be helpful? How long should patients be treated? Should bacteria be tested for phage susceptibility in the presence and absence of potential antibiotics? Could phage that is directed against a single bacterium help eliminate infections caused by multiple co-dependent pathogens? Obtaining answers to these questions should pave the way for personalized phage treatments of infectious diseases.

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Potential conflicts of interest

M. H. and B. B. have a patent pending, "Bacteriophage Compositions and Methods of Selection of Components against Specific bacteria," Application No.: 15/628,368, which was filed on June 20, 2017. M. B. is CMO of Adaptive Phage Therapeutics, Inc. and has equity in the company. G. M. reports personal fees from Adaptive Phage Therapeutics, Inc., during the conduct of the study. R. S. is an uncompensated member of the AmpliPhi Biosciences Scientific Advisory Board and reports personal fees from Pfizer, outside the submitted work.

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FIGURE LEGENDS

Figure 1.

A. The progress of the patient wound prior to and after phage treatment. Before treatment, the flap edges did not heal well with dehiscence and evisceration. Left Panel). Two weeks after treatment the wound completely healed and no dehiscence and evisceration of flap was noted even with probing. (Middle panel) and 5 months after treatment complete healing of wound was observed (Right panel).

B – C. Growth curves of *Ab*KT722 (B) and *Kp*KT1 (C), grown separately or in the presence of a relevant phage preparation (ϕAbKT21phi3 or ϕKpKT21phi1, respectively) at multiplicity of infection (MOI) of ~100, with or without the addition of the antibiotics colistin (70,000 IU) and meropenem (5 μg/ml).

D – E. CFU counts at the endpoint of the experiments shown in B-C, of the bacterial strains grown individually (D) and as a mixture (E), following treatment. Note that for the bacterial mixtures (E) both ϕAbKT21phi3 and ϕKpKT21phi1 were added. The mixed bacteria were plated on MacConkey agar plates which differentiate between *Ab* and *Kp* colonies (Figure S4).

F–G. *Ab*KT722res is resistant to ϕAbKT21phi3 but not to the newly isolated phage ϕAbTZA1 (F), which is also able to also kill, but less effectively, the original strain *Ab*KT722 (G).

H. A TEM picture of both *A. baumannii* phages (left and middle panels), showing that they are distinct from each other, and of ϕKpKT21phi1 (right panel).

Figure 1