Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant Acinetobacter baumannii infection

Running head: Successful Bacteriophage Therapy

Robert T. Schooley, M.D.1,*, Biswajit Biswas, Ph.D.2,3,*, Jason J. Gill, Ph.D.4,5, Adriana Hernandez-Morales, M.S.6, Jacob Lancaster3, Lauren Lessor5, Jeremy J. Barr, Ph.D.7,15, Sharon L. Reed, M.D.1,8, Forest Rohwer, Ph.D.7, Sean Benler, Ph.D.3, Anca M. Segall, Ph.D.7, Randy Taplitz, M.D.1, Davey M. Smith, M.D., M.A.S.1, Kim Kerr, M.D.1, Monika Kumaraswamy, M.D.1, Victor Nizet, M.D.9,10, Leo Lin, Ph.D.8, Melanie D. McCauley, M.D.1, Steffanie A. Strathdee, Ph.D.1, Constance A. Benson, M.D.1, Robert K. Pope, Ph. D.11, Brian M. Leroux11, Andrew C. Picel, M.D.12, Alfred J. Mateczun, M.D.2, Katherine E. Cilwa, Ph.D.14, James M. Regeimbal, Ph.D.2, Luis A. Estrella, Ph.D.2, David M. Wolfe, Ph.D.2, Matthew S. Henry, M.S.2,3, Javier Quinones, M.S.2,3, Scott Salka13, Kimberly A. Bishop-Lilly, Ph.D.2,3, Ry Young, Ph.D.5,6, Theron Hamilton, Ph.D.2

1Department of Medicine, University of California, San Diego, La Jolla, CA; 2Biological Defense Research Directorate, Naval Medical Research Center, Frederick, MD; 3Henry M. Jackson Foundation, Bethesda, MD; 4Department of Animal Science, Texas A&M, College Station, TX; 5Center for Bacteriophage Technology, Texas A&M AgriLife Research and Texas A&M University College Station, TX; 6Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX; 7Department of Biology, San Diego State University, San Diego, CA; 8Department of Pathology, University of California, San Diego, La Jolla, CA; 9Department of Pediatrics, University of California, San Diego, La Jolla, CA; 10Skaggs School of Pharmacy & Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA; 11National Biodefense Analysis and Countermeasures Center, Frederick, MD; 12Department of Radiology, University of California, San Diego, La Jolla, CA; 13AmphiPhi Biosciences, San Diego, CA; 14Advanced Surgical Imaging Program, Department of Regenerative Medicine, Naval Medical Research Center, Silver Spring, MD; 15Monash University, School of Biological Sciences, Melbourne, Australia

*These authors contributed equally.

Address correspondence to: rschooley@ucsd.edu
ABSTRACT

Widespread antibiotic use in clinical medicine and the livestock industry has contributed to the global spread of multidrug-resistant (MDR) bacterial pathogens, including Acinetobacter baumannii. We report on a method used to produce a personalized bacteriophage-based therapeutic treatment for a 68-year old diabetic patient with necrotizing pancreatitis complicated by a MDR A. baumannii infection. Despite multiple antibiotic courses and efforts at percutaneous drainage of a pancreatic pseudocyst, the patient deteriorated over a four-month period. In the absence of effective antibiotics, two laboratories identified nine different bacteriophages with lytic activity for an A. baumannii isolate from the patient. Administration of these bacteriophages intravenously and percutaneously into the abscess cavities was associated with reversal of the patient’s downward clinical trajectory, clearance of the A. baumannii infection, and a return to health. The outcome of this case suggests that the methods described here for the production of bacteriophage therapeutics could be applied to similar cases and that more concerted efforts to investigate the use of therapeutic bacteriophages for MDR bacterial infections are warranted.

INTRODUCTION

Increased antibiotic resistance is an important global issue. The World Health Organization (WHO), the Centers for Disease Control (CDC), the National Institutes of Health (NIH), the Gates Foundation, and other entities have tried to draw public attention to the growing crisis. The CDC has termed the present time the “post-antibiotic” era (1) because resistance abounds to almost every available antibiotic, and multidrug resistant...
infections are increasingly more common. This problem stems from a variety of factors including widespread agricultural use of antibiotics, inappropriate prescription of antibiotics, a decrease in the number of new antibiotics entering the market, and the increased positive selection of multidrug resistance when gained through the natural prokaryotic exchange of genetic material (reviewed in (2)).

The ESKAPE pathogens are a group of commonly MDR organisms at the heart of the antibiotic resistance crisis. As one of the ESKAPE pathogens, *Acinetobacter baumannii* is a frequently isolated organism from infections in clinical settings and has been of particular concern to active duty military service members injured in combat (3). *A. baumannii* is a Gram negative, naturally competent organism that is highly adept at acquiring and maintaining multiple genetic elements encoding antimicrobial resistance determinants (4-6). The growing impact of MDR pathogens has increased initiatives to discover infectious disease therapeutics with novel mechanisms of action (7, 8). Recently, as part of a collaboration between the U.S. Army and the U.S. Navy, we rescued mice from a MDR *A. baumannii* wound infection using a mixture of natural phages that were selected specifically for their synergistic activity against the infecting *A. baumannii* strain (9). Even with these positive results there are relatively few studies of phage efficacy and/or safety in the current medical literature (relevant examples include (10-18)). In addition to demonstrating that phage treatment can be efficacious when a panel of phages are isolated from the environment against the particular strain causing the infection, the *A. baumannii* study also demonstrated that the surviving *A. baumannii* had decreased virulence as measured in the *Galleria mellonella* (wax worm) model (9). This decreased virulence of phage-resistant *A. baumannii* was correlated with decreased capsule
production, as measured by Raman spectroscopy (9). In the current study, a bacterial isolate was provided from a patient suffering from necrotizing pancreatitis complicated by a MDR *A. baumannii*-infected pancreatic pseudocyst. In two independent laboratories, the patient’s initial isolate was used to screen previously-isolated phages and to select new phages for incorporation into phage mixtures to make specific phage cocktails. Food and Drug Administration (FDA) authorization to administer the cocktails as an Emergency Investigational New Drug (eIND) was obtained and the patient’s condition improved dramatically following phage therapy. Herein, we present our investigation of certain key aspects of the phage therapy in vivo, such as cocktail development, pharmacokinetics, immune response, and phage resistance. Despite an eventual rise of phage resistance, an iterative process of phage cocktail formulation resulted in resolution of the patient’s infection. Interestingly, the phage resistant phenotype that arose over time was associated with increased antibiotic sensitivity when phage and antibiotics were simultaneously administered.

**MATERIALS AND METHODS**

*A. baumannii clinical isolates.* *A. baumannii* clinical isolates were cultured from multiple drains, peritoneal fluid, and respiratory secretions of the patient. The three isolates used in this study were designated TP1, TP2 and TP3 for the first, second, and third temporal variants, respectively. *A. baumannii* isolates originally used to harvest natural phages from various environmental samples were genetically diverse and obtained from the Navy’s Wound Infections Culture Collection, originally received from the Army’s Multidrug-Resistant Organism Repository and Surveillance Network.
Selection of therapeutic phages. Phages used for this treatment were selected and prepared for clinical use by two different groups. Phages provided by the Biological Defense Research Directorate (BDRD) of the Naval Medical Research Center (AB-Navy1, AB-Navy4, AB-Navy71, AB-Navy97 and AbTP3Φ1) were isolated from various environmental samples using routine isolation techniques, as previously described (19).

Briefly, A. baumannii clinical isolates from the Navy’s Wound Infections Culture Collection were used to isolate and store pathogen-specific phages. Following isolation, the phages were triple plaque-purified on their respective host bacterium. Finally, small-scale phage amplifications on their corresponding host bacterium were performed to prepare the A. baumannii specific phage library which was subsequently stored at 4°C until required. The growth of A. baumannii clinical isolates TP1, TP2, and TP3 in the presence of phage was evaluated via spot testing and also in a BioLog® imaging system (BioLog®, Hayward, CA) (20, 21). In this case, one covered 96-well plate was used per phage strain and incubated at 37°C for 24 hours, with bacteria-only positive wells and test wells with a single phage added at a multiplicity of infection (MOI) of approximately 100. The phage candidates that showed the strongest antibacterial activity as measured by this assay were selected for inclusion in the therapeutic cocktails: AB-Navy1, AB-Navy4, AB-Navy71, AB-Navy97 against strain TP1 and, later, AbTP3Φ1 against strain TP3.
At the Center for Phage Technology (CPT), ten *A. baumannii* phages from the CPT collection and 30 more solicited from multiple academic, clinical and corporate sources were screened for activity on TP1 by spot assays using crude lysates. A phage from AmpliPhi Corporation, AC4, was found to form plaques on lawns of TP1. Another 100 environmental samples available at the CPT were screened by enrichment, yielding three new isolates (C1P12, C2P21 and C2P24) that plated on TP1.

**Propagation and purification of therapeutic bacteriophages.** Large scale bacteriophage amplification of each BDRD bacteriophage isolate was performed in two steps before purification. First, initial amplifications (via a plate lysate method) of the phage were used to inoculate 3.6 L large-scale liquid lysates (10). These lysates were centrifuged at 10,000 g for 20 minutes to remove bacterial debris, vacuum filtered through 0.22 μm filters, then concentrated using a Millipore Pelican 2 cassette 300K MWCO tangential flow filtration system to a volume of approximately 0.5 L. Following concentration, the culture medium was replaced with PBS via diafiltration. The resulting lysate was further concentrated via diafiltration to a final volume of 0.2 L prior to collection. Finally, the concentrated bacteriophage mixture was purified using CsCl isopycnic gradient centrifugation as previously described (10), and dialyzed in PBS to remove cesium chloride. Purified bacteriophages were combined into a four-bacteriophage cocktail designated ΦIV. After the patient’s *A. baumannii* isolate became insensitive to the ΦIV bacteriophage cocktail, a second bacteriophage cocktail designated ΦIVB was prepared by combining AB-Navy71 from the original cocktail with a newly isolated bacteriophage (AbTP3Φ01) that was capable of forming plaques on lawns of *A. baumannii* TP3.
At the CPT, large scale propagation and purification of phages C1P12, C2P21, C2P24, and AC4 was performed using plate lysates grown from single plaques to inoculate 1 L logarithmic cultures of host at an input MOI of $<10^2$ in the presence of 5 mM MgSO$_4$. The infected cultures were grown until the onset of lysis, as measured by OD$_{550}$, at which time sodium citrate was added to a final concentration of 10 mM. The infected culture was aerated until lysis was complete. Lysates were cleared by centrifugation (6,000 x g, 40 min, 4 ºC) and sterilized by filtration through 0.22 µm membranes. Bacteriophages were concentrated by centrifugation at 6,000 x g for 10 h at 4 ºC and phage pellets were gently re-suspended in 10 ml of DPBS and sterilized again by filtration. The sterilized phage suspensions were subjected to ultrafiltration and 1-octanol extraction to remove lipopolysaccharide (LPS) as previously described (22). In most cases, this final treatment was performed at the Laboratory for Viral Information at San Diego State University. The four phages were combined to comprise a cocktail (ΦPC) that was used for intracavitary administration. Phage were introduced into each cavity every 6 hours.

**Efficiency of plating of selected bacteriophages on clinical isolates.** To evaluate the killing efficacy of each phage on clinical isolates, a dilution series of bacteriophage preparation was spotted on a bacterial lawn to observe plaque formation (28). Briefly, 100 µl of an overnight culture of each *A. baumannii* isolate was used to individually inoculate 2.5 ml of 0.7% molten top agar (temperature 50ºC). The inoculated agar was spread onto tryptic soy (TS) agar plates. Top agar was allowed to cool at room temperature, then 10 µl aliquots of 10-fold serial dilutions of each selected bacteriophage...
were spotted on the plate surface. All spots were allowed to fully absorb into the top agar and plates were incubated at 37°C for 24 h in a humidified chamber for plaque formation.

Electron microscopy. The *A. baumannii* phages were grown in their corresponding host by standard procedures and purified via CsCl density gradient centrifugation. The phage preparation was fixed using a solution of 4% paraformaldehyde and 1.0% glutaraldehyde and spread onto electron microscope grids, negatively stained with uranyl acetate, and imaged in an FEI Tecnai T12 transmission electron microscope.

Endotoxin assays in phage preparations. Endotoxin levels of each ΦIV cocktail were estimated using the Endpoint Chromogenic LAL Assay QCL-1000, and ΦPC endotoxin levels were determined by the PyroGene Recombinant Factor C Assay (Lonza, Walkersville, MD), according to manufacturer’s directions.

The MDR *A. baumannii* infection and pathophysiology of the patient. The patient, a 68-year old diabetic man, developed gallstone-induced acute pancreatitis. An abdominal CT scan revealed a pancreatic pseudocyst (Supplemental Figure 1). The pseudocyst was drained through two pigtail cystgastrostomy tubes and a pancreatic stent was placed. Cultures of the pseudocyst aspirate grew *Candida albicans* and MDR *A. baumannii*. The patient received courses of vancomycin, meropenem, colistin, and tigecycline. Subsequent culture from the pseudocyst fluid grew *A. baumannii* resistant to cephalosporins, meropenem, gentamicin, amikacin, trimethoprim/sulfamethoxazole, tetracycline, ciprofloxacin and colistin. Susceptibility testing revealed synergy between
colistin and azithromycin against the MDR *A. baumannii*, and treatment with these two antibiotics was initiated on day 36 after initial infection. MDR *A. baumannii* was repeatedly isolated from multiple abdominal drains, the patient’s clinical condition further deteriorated, and on day 51 he developed respiratory failure and hypotension requiring intubation, fluid resuscitation, pressors and ultimately transfer to the ICU. Rifampin was determined *in vitro* to provide added antibiotic synergy against the MDR *A. baumannii* and it was therefore included in his regimen. During the remainder of the second and third months of hospitalization, his clinical course further deteriorated. He developed emphysematous cholecystitis and he became increasingly delirious with declining renal function and increasing leucocyte counts. Cultures of multiple drains, peritoneal fluid and respiratory secretions all produced MDR *A. baumannii*. Renal and hepatic function worsened. By day 108, the patient was on multiple pressors and unresponsive, with a plasma creatinine of 3.68 mg/dl. Due to the unavailability of any additional effective antimicrobial agents, an eIND Application was submitted to the FDA requesting authorization to treat his uncontrolled *A. baumannii* infection with a combination of phages.

**Treatment with phage.** Phage therapy was initiated on day 109 (after initial infection) with the installation of a cocktail containing four of the anti-*A. baumannii* phages (~10⁹ pfu/dose) described previously as ΦPC through percutaneous catheters draining the pseudocyst cavity, the gall bladder and a third intra-abdominal cavity. Intracavitary instillations of this cocktail were continued at 6 – 12 hourly intervals. After 36 hours of initiation of intracavitary instillations of the bacteriophage cocktail, bacteriophage
therapy was intensified and broadened through intravenous administration of an additional bacteriophage cocktail (~$10^9$ pfu/dose) consisting of the Navy’s four anti-
*baumannii* bacteriophages (described previously as ΦIV). Since this was well tolerated, IV bacteriophage therapy was repeated 12 hours later and then at increasingly frequent intervals over the next two days to reach a dosing frequency of every 2 hours. Azithromycin, colistin, and rifampin were discontinued; meropenem and fluconazole were continued. However, two days following initiation of intravenous phage therapy, patient’s pressor requirements abruptly increased and bacteriophage therapy was temporarily withheld. The meropenem dose was increased and intravenous catheters were changed. It was subsequently demonstrated that the clinical deterioration was accompanied by a transient septic episode with *B. thetaiodonicron* that were felt to have arisen from his pancreas. On day 115, the patient’s *A. baumannii* isolate was found to be susceptible to minocycline (3 μg/ml), which was added to patient’s treatment regimen. Intracavitary and intravenous bacteriophage therapy was resumed on days 116 and 118, respectively. Subsequently the combinations of intracavitary and intravenous therapy with the ΦPC and ΦIV cocktails, respectively, were continued (generally at 6 – 8 hourly intervals) until day 167. When reduced phage susceptibility of serial isolates of the patient’s *A. baumannii* was demonstrated in vitro, a third bacteriophage cocktail consisting of one new bacteriophage and one of the initially selected phages (designated ΦIVB) was developed to effectively target the phage-resistant bacterial isolate and administered during the last two weeks of therapy. With ongoing clinical improvement minocycline and meropenem were discontinued on day 168 and 179, respectively.
Combinations of intracavitary and intravenous bacteriophage therapy were continued for total of 59 days.

**Pharmacokinetic studies.** Plasma and serum samples were collected after bacteriophage therapy and filtered through 0.22 μm Corning Spin-X® filters. An aliquot of each filtrate was further diluted 1:10 and 1:100 in SM buffer and 10 μl of each concentration of plasma/serum dilutions were mixed with 100 μl of *A. baumannii* of the TP1 culture at OD 0.5. The bacteriophage-bacterial mixtures were incubated at 37°C for 20 minutes before plating via soft agar overlay (23). Plates were incubated at 37°C overnight in a humidified chamber and bacteriophage plaques were counted the next day.

**Raman spectroscopic analysis of *A. baumannii* strains.** *A. baumannii* isolates were examined for modifications in cellular and extracellular composition via Raman Spectroscopy using a Kaiser Rxn1 PhAT probe 830 nm system (Kaiser Optical Systems, Inc., Ann Arbor, MI, USA) and 1 mm diameter excitation spot size as previously described (9). Prior to spectral acquisition each sample was obtained from LB agar plates and directly transferred to an aluminum foil covered disposable weigh dish for spectral collection. Dark subtracted and intensity-corrected spectra were each acquired using 5 second acquisitions, 10 accumulations, and the cosmic ray removal feature selected for a total laser exposure of 100 seconds. Each sample was assayed in three locations, one spectrum per location. Per sample, the three localized spectra were examined to ensure steady sample hydration and spectral consistency across locations. Once verified, spectra were averaged per sample, truncated to 600-1800 cm⁻¹, baseline subtracted using a sixth-
order polynomial fitting routine, and normalized to the methyl/methylene scissoring band at 1445 cm$^{-1}$. (See Supplemental Figure 2 for a schematic.)

Capsule staining. 

$A. \text{baumannii}$ isolates were examined for potential capsule expression using crystal violet staining. For each isolate, a loopful of bacteria in PBS was used to smear a glass slide and then allowed to air dry. Each slide was stained with crystal violet for 1 minute, rinsed with 20% copper sulfate, and dried prior to imaging at 100X magnification under oil immersion using an Olympus BX51TRF microscope equipped with an Olympus DP72 camera (Olympus Corporation, Waltham, MA).

RESULTS

$A. \text{baumannii}$ susceptibility to bacteriophages. The clinical course of the patient, a 68-year old diabetic patient who developed necrotizing pancreatitis complicated by a MDR $A. \text{baumannii}$ infected pancreatic pseudocyst, is illustrated in Figure 1. Due to his rapidly deteriorating condition and the inability to control his infection with antibiotics, a bacteriophage therapy process was embarked upon as an eIND. A subset of 98 $A. \text{baumannii}$-specific lytic bacteriophages (out of 200 total) that were previously harvested from environmental sources by NMRC-BDRD were screened for activity against the three clinical isolates in this study (Figure 2). This subset of 98 phages was initially selected for screening because previous work had demonstrated that, collectively, these bacteriophages are highly active against a broad range of MDR $A. \text{baumannii}$ isolates (data not shown). Within 18 hours of receipt of the first clinical isolate, selection of an appropriate therapeutic cocktail for the original isolate, named TP1, was achieved via an
OmniLog® based time-kill assay system (20, 21). The bacterial isolates from this study are summarized in Table 1. The assay revealed that most of the phage screened have almost no discernable effect against this clinical isolate. However, phage Abϕ5, Abϕ6, and Abϕ73 were able to inhibit growth for up to six hours. Phage Abϕ66 and Abϕ68 were able to inhibit growth for 12 hours. Most notably, phages AB-Navy1, Abϕ2, Abϕ3, AB-Navy4, AB-Navy71, and AB-Navy97 were able to inhibit growth for over 20 hours. Phage AB-Navy1, AB-Navy4, AB-Navy71, and AB-Navy97 were used in the phage cocktail based on previous host range analysis (data not shown) that indicated that each phage was different from the others. TP1 growth inhibition effects were more pronounced when a cocktail of these bacteriophages was used as compared to the individual bacteriophages used separately (Figure 3).

Initial screening of 37 A. baumannii-specific bacteriophages against the TP1 isolate at the Center for Phage Technology (CPT) at Texas A & M University revealed that one of these bacteriophages, AC4 (originally obtained from AmpliPhi Corporation) inhibited TP1 growth. Subsequently, three more bacteriophages (C1P12, C2P21 and C2P24) were identified after additional screening of 100 environmental samples available at the CPT. The capability of these three new bacteriophage isolates (C1P12, C2P21 and C2P24) to inhibit TP1 growth was similar to that of bacteriophage AC4 (Figure 3). The individual bacteriophages used in this study are detailed in Table 2 and the composition and administration of the bacteriophage cocktails are detailed in Table 3. With continued treatment, in vitro susceptibility studies of serial A. baumannii isolates demonstrated stepwise selection of resistance to the eight phages present in the original therapeutic cocktails. Representative data related to the antimicrobial activity of the bacteriophages
comprising the ΦIV and ΦPC cocktails over the initial three weeks of therapy are presented in Figure 3 a-c and e-g, respectively. It was determined retrospectively on day 19 of phage treatment by use of the OmniLog® based time-kill assay system that by day 116 (which was the 8th day of bacteriophage therapy), each of the phages had lost activity individually and in their respective original mixtures against the A. baumannii isolates that emerged in the presence of the phages. In other words, the TP3 isolate was found in vitro to be resistant to both of the two original bacteriophage cocktails. Therefore, an additional phage, AbTP3Φ01, was selected for its activity against TP3, and combined with one of the original phages (AB-Navy71) to produce a third phage cocktail, called ΦIVB (Table 3), which was then administered to the patient.

**Endotoxin level in bacteriophage preparation.** As potential residual endotoxin from the bacterial host cells could be harmful to the patient, each separate bacteriophage cocktail was assessed for residual endotoxin using a commercial assay. Average endotoxin levels of bacteriophage cocktails ΦPC, ΦIV and ΦIVB were 2.4x10^3 EU/ml, 5.89x10^3 EU/ml and 1.64x10^3 EU/ml respectively. Therefore, each bacteriophage cocktail preparation was diluted accordingly in lactated Ringer’s solution to meet the FDA recommended endotoxin limitation for intravenous application of 5 EU/kg body weight/hour.

**Outcome of bacteriophage therapy.** The patient’s prognosis was grave when bacteriophage therapy was first initiated with cocktail ΦPC through percutaneous catheters draining the pseudocyst cavity, the biliary cavity and a third intraabdominal
During this time the patient was unresponsive to commands and had developed renal failure with a creatinine of 3.68 mg/dL. Over the next 36 hours his clinical condition was stable but he remained comatose, intubated, and on three pressors with worsening renal and hepatic function. In view of his ongoing critical clinical condition and since it was clear that his *A. baumannii* infection included anatomic sites well beyond the intraabdominal cavities, an additional systemic administration of the bacteriophage therapy was instigated through intravenous administration of a new bacteriophage cocktail (ΦIV). The intravenous bacteriophage administration was well tolerated and was repeated at increasingly frequent intervals over the next two days. His pressor requirements diminished and he abruptly awoke from his coma and became conversant with his family for the first time in several weeks. It was then noted that a recent *A. baumannii* isolate was susceptible to minocycline and that antibiotic was added to his regimen four days after the initial administration of cocktail ΦPC. Over the ensuing three weeks, the course remained complex but he generally demonstrated ongoing improvement on all fronts. His mental status continued to improve and he was fully conversant and lucid. He was weaned off the ventilator, his pressors were gradually weaned and discontinued, and his renal function gradually improved. Bacteriophage therapy was continued for an additional eight weeks, during which time he demonstrated continued clinical improvement. All drains were removed and he was discharged home on day 245. He has subsequently returned to work.

**Emergence of bacteriophage-resistant bacteria during bacteriophage therapy.**

During the course of treatment, the bacteriophages used in this study were examined for
Consequently, it was discovered that an *A. baumannii* isolate, TP3, which was isolated eight days after initiation of bacteriophage therapy, was resistant to both of the initially used bacteriophage cocktails (ΦPC and ΦIV). Therefore, TP3 was used to rapidly (within 72 hours) select for additional bacteriophages with lytic activity against this isolate. Using conventional bacteriophage enrichment techniques, an additional bacteriophage, AbTP3Φ01, was isolated from raw sewage that inhibited growth of isolate TP3. Interestingly, this effect was enhanced when combined with bacteriophage AB-Navy71 from the original ΦIV cocktail (Figure 3d). The activity of the ΦIV cocktail was tested in combination with different concentrations of minocycline to determine whether the bacteriophage cocktail affected the activity of sub-MIC concentrations of the drug. As shown in Figure 3h, although the bacteriophage cocktail itself had lost activity against the organism, the bacteriophage appeared to prevent the outgrowth of bacteria with enhanced minocycline resistance after extended culture.

**Bacteriophage pharmacokinetics.** To better understand the pharmacokinetics of intravenous administration of therapeutic bacteriophages, we examined the titer of active phage in plasma samples after IV administration of the ΦIVB phage cocktail. We found bacteriophage concentrations of 18,000 pfu/ml in plasma five minutes after an IV bolus of 4x10^9 pfu of bacteriophages. These levels fell over the 6-hour dosing interval (Figure 4). An *in vitro* study of phage inactivation in plasma was subsequently conducted using plasma collected 90 days following cessation of phage therapy. The study demonstrated that the titer of each phage in the ΦIVB phage cocktail as well as phage AbTP3Φ1...
declined at a more rapid rate than when suspended in the patient’s plasma than in normal saline. This supports the possibility that phage neutralization by plasma might be one of the contributors to the decay of phage activity in plasma in the pharmacokinetic studies. (Supplemental material).

Morphological characteristics of bacteriophages and the hosts. Electron micrographs of the ΦIV and ΦPC cocktail bacteriophages indicated that these bacteriophages display a short tail with well-defined head structure. Morphologically these eight bacteriophages are all consistent with the Myoviridae family of bacteriophage, whereas bacteriophage AbTP3Φ1 is comparatively small and consistent with the Podoviridae family (Figure 5).

In addition, we sought to investigate the morphological characteristics of the bacterial isolates and some phenotypic differences observed among them. First, following cultivation of the patient’s isolates in the laboratory, a ‘sticky’ phenotype was observed only for TP3. As organisms absorbed water a mucinous overlayer was formed. Second, Raman spectroscopy was conducted on TP1, TP2, and TP3 with the purpose of examining alterations in the molecular composition of bacterial isolates during the evolution of phage resistance. Mean Raman spectra of each isolate are shown in Figure 6a. Raman spectra are dominated by polysaccharide and protein contributions. The Raman spectral band at 979 cm\(^{-1}\) previously reported to be associated with capsule related colony morphology and phage susceptibility in AB5075 strains (9) was not observed in any of the interrogated strains suggesting alterations to capsule structure in the encapsulated TP strains. When comparing spectra of TP1 (capsulated, susceptible to first cocktail), TP2 (capsulated, reduced susceptibility to first cocktail) and TP3
(unencapsulated, resistant to first cocktail), the intensity within the 1030-1380 cm$^{-1}$ region is significantly increased for TP2 and TP3 spectra and only minor alterations in relative peak intensities are observed across all three strains. For bacterial isolates, the 1030-1380 cm$^{-1}$ spectral region is composed of contributions from both proteins and polysaccharides. There are no observed changes within the Amide I region (1600-1700 cm$^{-1}$), a spectral region sensitive to changes in protein contribution and secondary structure and not associated with polysaccharides (24, 25). The marked increase in peak intensity within 1030-1380 cm$^{-1}$ can therefore be attributed to increased polysaccharide content in TP2 and TP3 samples. Loss of the bacterial capsule in TP3 with observed increase in polysaccharide content suggests increased production of extracellular polymeric substance in response to phage treatment and may contribute to the transition in phage susceptibility observed from TP1 to TP3. Finally, given these results, we performed capsular staining of TP1, TP2, and TP3 isolates. Upon staining and microscopy TP1 and TP2 were both found to exhibit a typical capsule, whereas TP3 did not (Figure 6b-d and Supplemental Figures 3-5), consistent with these Raman spectroscopy data interpretations.

**DISCUSSION**

Here we demonstrated the successful application of a novel approach to the preparation of personalized therapeutic bacteriophage cocktails to rescue a patient from a life-threatening MDR *A. baumannii* infection. This case supports further study of the use of
Phage therapy in treating patients suffering from MDR bacterial infections with limited therapeutic options. As with any uncontrolled clinical observation, there are a number of important caveats; primarily, we cannot exclude the possibility that reversal of his clinical deterioration was unrelated to the phage therapy. However, after an inexorably downhill clinical course over the prior three months to the point that discussions about clinical futility had been initiated, a clear turning point was observed within 48 hours of starting intravenous bacteriophage therapy. The potential interplay between the bacteriophages used in his therapy and minocycline is also complex. His A. baumannii infection rapidly became resistant to colistin and tigecycline early in his course of treatment, but activity of minocycline was maintained for several weeks when it was added to his bacteriophage therapy five days after bacteriophage therapy was initiated. We were able to demonstrate an additive in vitro activity between the bacteriophages and sub-inhibitory concentrations of minocycline when used in combination against a bacteriophage resistant A. baumannii isolate. Additive or synergistic activity has been previously demonstrated both in vitro and in animal model systems between bacteriophages and traditional antibiotics (for example see (9, 18, 26-28) and references therein). Of particular relevance is a recent paper by Oechslin, et al, describing the interaction between phage and antibiotics in a Pseudomonas aeruginosa endocarditis infection model which describes unprecedented practical evidence for the idea that the most significant benefits of phage therapy will emerge synergistically and that the emergence of phage resistant bacteria can be avoided or delayed through combination therapy. It is worth noting the significantly different pharmacokinetic data between our study and the Oechslin report. Where phage clearance was on the order of hours in the rat...
model, it was on the order of minutes in our human patient. Differences between the two studies could contribute to the differences observed. In particular, phage circulation longevity was measured in uninfected rats rather than in the presence of infection; the P. aeruginosa cocktail had twelve phages, ours had four; and the phage-titer to body weight ratio differed greatly - 1x10^{10} pfu/mL was injected into a small rat body; 5x10^9 total phages was injected into much larger human body. It is also possible that differences in plasma clearance kinetics reflect antibody-mediated neutralization or other clearance mechanisms that were operative in our patient that were not present in the rat model. A fuller understanding of peripheral blood phage kinetics in humans will require more systematic studies in additional patients.

Bacterial mutation to phage resistance has also been associated with significant fitness costs for the bacterium. Surface features such as capsule, lipopolysaccharide, or pili may be used as bacteriophage receptors but can also be pathogenicity factors, and their loss may result in attenuated virulence. Although A. baumannii was not immediately cleared from abscess cavity drainage or bronchial washings, strains with substantially reduced susceptibility to the phages administered subsequently emerged. This strongly suggests that the A. baumannii population evolved in response to selection pressure exerted by the phages. In fact, we observed differences in colony morphology among these isolates, which could possibly be due to differences in capsular production and a loss of virulence, consistent with previous results obtained from phage treatment in a mouse model of A. baumannii infection (9). Results obtained from Raman spectroscopy and capsular staining are in support of this idea, with Raman spectroscopy showing subtle
differences among all 3 isolates, and what could potentially be extracellular polymeric
substance associated with TP3 and capsular staining indicating lack of capsule around
TP3 cells. It has been shown in other studies that culturing A. baumannii with antibiotics
below the minimum inhibitory concentration can result in differences in capsular
production (29). The lack of capsule on TP3 cells may also contribute to the enhancement
of their phage-antibiotic synergy possibly because antibiotics may more easily penetrate
TP3’s outer membrane (Figure 3h). Although A. baumannii with putative reduced fitness
could be isolated from open abscess cavities and bronchial washings for some time after
initiation of phage therapy, the only time phage was documented in his bloodstream after
therapy was initiated was in association with migration of his cholecystostomy tube into
his hepatic parenchyma on day 127. The bacterial isolates from this study, as well as the
bacteriophages used in treatment, are currently being characterized at the genetic level to
facilitate our understanding of the mechanisms involved in the loss of capsule, the
enhancement of antibiotic synergy, and the rise of bacteriophage resistance during the
course of this patient’s treatment with phages.

A number of concerns have been raised about the potential toxicities and the
practicality of bacteriophage therapy for MDR bacterial infections. However, in this
particular case, we overcame these hurdles and did not observe any discernible adverse
clinical events. For example, concerns have arisen about the possibility that an
accelerated lysis of Gram-negative bacterial pathogens could release clinically significant
levels of endotoxin (30, 31). A parallel concern can be made for administration of some
antibiotics or any other alternative treatment that could lead to rapid, widespread cell
lysis. While the patient’s clinical instability at the time therapy was initiated made it
difficult to detect all but the most dramatic deleterious clinical effects of phage therapy,

no adverse effects of phage administration were evident in association with either the
intracavitary or IV phage administrations.

It has also been posited that phages could facilitate the transfer of genetic
elements conferring drug resistance and pathogenicity among bacteria; however, by
producing phage on the same bacterial isolate already present in the patient, the risk of
introducing exogenous genetic information conferring increased virulence or antibiotic
resistance is minimized. The inherent specificity of bacteriophage to the species if not
strain level minimizes the potential of horizontal gene transfer, compared to more
promiscuous plasmid conjugation or the uptake of exogenous DNA in naturally
transformable hosts. Counter to the concerns for increased antibiotic resistance following
phage therapy, in the current case we also consistently observed decreased resistance to
antibiotics after phage therapy. Additionally, the narrow bacterial host range of
bacteriophages can also be a potential advantage in the treatment of MDR organisms as
their specificity would be less likely to perturb the commensal microbiome of the patient.

Since A. baumannii phages have narrow host ranges it was necessary to identify
phages capable of propagating on the TP1 strain isolated from the patient. Through a
labor-intensive enterprise, two laboratories were each able to independently identify,
propagate and purify four bacteriophages with lytic activity directed at the isolate within
ten days of receiving the strain. A. baumannii isolates characterized after the start of
bacteriophage therapy had reduced susceptibility to the initial bacteriophages. Emergence
of bacteriophage-resistant populations of A. baumannii was likely delayed by the use of
combinations of bacteriophages (see Figure 3f for example). When the resistant bacteria
were isolated, it was possible to identify a “second generation” bacteriophage that was active against the bacteriophage-resistant *A. baumannii* strain to counter the emergence of phage-resistant subpopulations. The specificity of anti-bacteriophage systems makes it unlikely that broad anti-bacteriophage mechanisms will arise, as opposed to the spread of multi-drug resistant efflux pump mechanisms in response to conventional broad-spectrum antibiotic therapy. Finally, other reported benefits of bacteriophage therapy include an ability to exhibit synergy with or restore susceptibility to conventional antimicrobial agents, as observed in this case, and the potential ability to disrupt biofilms (27, 28).

Many questions remain about the clinical potential of bacteriophage therapy for serious MDR organisms. Future studies should focus on delineating and optimizing safety; pharmacokinetics and pharmacodynamics; multiplicity of infection and valency; efficiency of bacteriophage identification and deployment; modes of administration; and methodologies for monitoring emergence of bacteriophage resistance once therapy is initiated. In addition, the impact of phage on biofilms and on the microbiome of the host is of interest, as is how best to manage concomitant antimicrobial therapy. These trials will face the same challenges posed by those who seek to evaluate novel small molecule antimicrobial agents in the treatment of patients with severe multidrug resistant infections. Nonetheless, while it is clear that administering phage as a monotherapy is unlikely in cases of serious illness, the increasing threats posed by MDR bacterial pathogens and the slow progress in the development of novel classes of traditional antimicrobial agents, clinical trials focusing on delineating the extent to which bacteriophage-based therapeutics could be used on their own as a last resort or as an adjunct to traditional antibiotics are warranted.
Acknowledgements and disclaimers

The views expressed in this manuscript are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, the Department of Defense, nor the U.S. Government. TH, DW, LE, and JR are military service members of the U.S. Government. This work was prepared as part of their official duties. Title 17 U.S.C. §105 provides that ‘Copyright protection under this title is not available for any work of the United States Government.’ Title 17 U.S.C. §101 defines a U.S. Government work as a work prepared by a military service member or employee of the U.S. Government as part of that person’s official duties. RY, JG, JL, LL, and AH-M were supported by funding for the Center for Phage Technology from Texas A&M AgriLife and Texas A&M University. RKP and BML were funded under Contract No. HSHQDC-15-C-00064 awarded by the Department of Homeland Security (DHS) Science and Technology Directorate (S&T) for the operation and management of the National Biodefense Analysis and Countermeasures Center (NBACC), a Federally Funded Research and Development Center. The views and conclusions contained in this document are those of the authors and should not be interpreted as necessarily representing the official policies, either expressed or implied, of the DHS or S&T. In no event shall DHS, NBACC, S&T or Battelle National Biodefense Institute have any responsibility or liability for any use, misuse, inability to use, or reliance upon the information contained herein. DHS does not endorse any products or commercial services mentioned in this publication.
Table 1: Bacterial isolates from this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isolation date</th>
<th>Site of isolation</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP1</td>
<td>March 10, 2016</td>
<td>Pancreatic drainage of patient</td>
<td>Capsulated, susceptible to ΦIV</td>
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<tr>
<td>TP2</td>
<td>March 21, 2016</td>
<td>Pancreatic drainage of patient</td>
<td>Capsulated, reduced susceptibility to ΦIV</td>
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<td>TP3</td>
<td>March 23, 2016</td>
<td>Pancreatic drainage of patient</td>
<td>Unencapsulated, resistant to ΦIV</td>
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Table 2

<table>
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<tr>
<th>Phage</th>
<th>Activity against</th>
<th>Source</th>
<th>Isolated from</th>
<th>Family</th>
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<tr>
<td>AB-Navy1</td>
<td>TP1</td>
<td>US Navy phage library</td>
<td>Sewage water</td>
<td>Myoviridae</td>
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<tr>
<td>AB-Navy4</td>
<td>TP1</td>
<td>US Navy phage library</td>
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<td>AB-Navy71</td>
<td>TP1</td>
<td>US Navy phage library</td>
<td>Sewage water</td>
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<td>TP1</td>
<td>US Navy phage library</td>
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<td>AC4</td>
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<td>AmpliPhi Corporation, CA</td>
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<td>C1P12</td>
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<td>Texas A &amp; M University</td>
<td>Environmental sample</td>
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Table 2: Phages used in this study.
<table>
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<tr>
<th>Phage cocktail</th>
<th>Composition</th>
<th>Route of administration</th>
<th>Timing of administration</th>
<th>Therapeutic dose</th>
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<tr>
<td>ΦPC</td>
<td>AC4, C1P12, C2P21, C2P24</td>
<td>Intracavitary - through percutaneous catheters draining the pseudocyst cavity, the gall bladder and a third intra-abdominal cavity</td>
<td>18 weeks beginning day 109</td>
<td>NA*</td>
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<td>ΦIV</td>
<td>AB-Navy1, AB-Navy4, AB-Navy71, AB-Navy97</td>
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<td>16 weeks beginning day 111</td>
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<td>ΦIVB</td>
<td>AB-Navy71, AbTP3Φ1</td>
<td>intravenous</td>
<td>2 weeks beginning day 221</td>
<td>$5 \times 10^9$ pfu</td>
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*Used for intracavitary washes

Table 3: Composition and details of administration of each phage cocktail.
Figure 1: Clinical course before and during the initial phase of bacteriophage therapy.
Positive blood cultures are depicted above the graphic data, whereas antibiotic and phage administration are indicated below.

Figure 2: Screening of *A. baumannii* phage library against TP1 clinical isolate. A subset of 98 phages from the Navy phage library were individually tested against the *A. baumannii* TP1 strain using the OmniLog system (BioLog®, Hayward, CA) as in (20, 21). Briefly, growth of bacteria or lack of growth due to lysis from phage infection was monitored every 15 minutes via a redox chemical reaction employing cellular respiration as a universal reporter, where cellular respiration from growth reduces a tetrazolium-based dye and produces a color change. If the growth is weakly positive or is negative, then respiration is slow or absent and so little to no color change is observed. The results of this assay after 20 hours are summarized here, where the color gradient indicates duration of bacterial growth inhibition.

Figure 3: Activity of bacteriophage cocktails. Activity of cocktails ΦPC and ΦIV against serial isolates of *A. baumannii* isolated from intraabdominal drains before bacteriophage therapy (strain TP1, panels 3a and 3e), and four days after initiation of antibiotic therapy (Strain TP2, panels 3b and 3f), and eight days after initiation of bacteriophage therapy (Strain TP3, panels 3c and 3g). Panel d demonstrates the derivation of a second generation bacteriophage cocktail directed at the TP3 *A. baumannii* strain, a mixture of AB-Navy71 and AbTP3Φ1 against this strain. Panel h demonstrates the additive activity of the ΦIV bacteriophage cocktail (10⁷ pfu) and a sub-lethal concentration of minocycline (0.25 μg/mL) against *A. baumannii* strain TP3. The IC₅₀ of *A. baumannii* strain TP1, TP2 and TP3 to minocycline were 1, 2 and 4 μg/mL respectively.

Figure 4: Bacteriophage titer from plasma samples during bacteriophage therapy. Plasma sample collected five minutes prior to and following administration of 5x10⁹ pfu of bacteriophage via intravenous injection indicated that bacteriophage titers in systemic circulation increase rapidly from 0 pfu/ml to 1.8x10⁴ pfu/ml. Bacteriophage titer dropped to 4.4x10³ pfu/ml by 30 minutes, 3.3x10² pfu/ml by 60 minutes, and 20 pfu/ml by 120 minutes post injection. Plasma samples collected six hours following initial injection contained no detectable bacteriophage titer (limit of bacteriophage detection was 20 pfu/ml).

Figure 5: Transmission electron micrographs of *A. baumannii*-specific bacteriophages. Electron micrographs of the ΦIV cocktail phages showed large prolate myobacteriophage morphology (a-d) and short small podophage morphology (e). Bacteriophage AbTP3Φ1, isolated against strain TP3, is a small podophage. Panels a-e: AB-Navy1, AB-Navy4, AB-Navy71, AB-Navy97 and AbTP3Φ1, respectively.

Figure 6: Investigation into morphological characteristics of *A. baumannii* isolates. The three *A. baumannii* isolates’ apparent morphological differences were examined by a) Raman spectroscopy using a PhAT Probe 830 nm system and in b-d, by capsule staining with crystal violet. Capsule staining of isolate TP1 is shown in b, of TP2 is shown in c, and of TP3 is shown in d.
REFERENCES


Therapy of Acute Bacterial Diarrhea With Two Coliphage Preparations: A Randomized Trial in Children From Bangladesh. EBioMedicine 4:124-137.


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Bacterial growth inhibition scale:

- 0 hours
- 6 hours
- 12 hours
- 20 hours
Erratum for Schooley et al., “Development and Use of Personalized Bacteriophage-Based Therapeutic Cocktails To Treat a Patient with a Disseminated Resistant Acinetobacter baumannii Infection”

Robert T. Schooley,a Biswajit Biswas,b,c Jason J. Gill,d,e Adriana Hernandez-Morales,f Jacob Lancaster,a Lauren Lessor,a Jeremy J. Barr,a,h Sharon L. Reed,a,h Forest Rohwer,g Sean Benler,g Anca M. Segall,g Randy Taplitz,a Davey M. Smith,a Kim Kerr,a Monika Kumaraswamy,a Victor Nizet,i Leo Lin,j Melanie D. McCauley,a Steffanie A. Strathdee,a Constance A. Benson,b Robert K. Pope,b Brian M. Leroux,b Andrew C. Pichel,l Alfred J. Mateczun,b Katherine E. Cilwa,n James M. Regeimb, Luis A. Estrella,b David M. Wolfe,b Matthew S. Henry,b,c Javier Quinones,h,c Scott Salka,m Kimberly A. Bishop-Lilly,b,c Ry Young,a,d Theron Hamiltonb

Department of Medicine, University of California, San Diego, La Jolla, California, USAa; Biological Defense Research Directorate, Naval Medical Research Center, Frederick, Maryland, USAa; Department of Animal Science, Texas A&M University, College Station, Texas, USAa; Center for Phage Technology, Texas A&M AgriLife Research and Texas A&M University, College Station, Texas, USAa; Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas, USAa; Department of Biology, San Diego State University, San Diego, California, USAa; Department of Pathology, University of California, San Diego, La Jolla, California, USAa; Department of Pediatrics, University of California, San Diego, La Jolla, California, USAa; Skaggs School of Pharmacy & Pharmaceutical Sciences, University of California, San Diego, La Jolla, California, USAa; National Biodefense Analysis and Countermeasures Center, Frederick, Maryland, USAa; Department of Radiology, University of California, San Diego, La Jolla, California, USAa; AmpliPhi Biosciences, San Diego, California, USAa; Advanced Surgical Imaging Program, Department of Regenerative Medicine, Naval Medical Research Center, Silver Spring, Maryland, USAa; Monash University, School of Biological Sciences, Melbourne, Australiaa

Volume 61, no. 10, e00954-17, 2017, https://doi.org/10.1128/AAC.00954-17. Materials and Methods, 2nd paragraph, 2nd sentence: “Phages provided by the Biological Defense Research Directorate (BDRD) of the Naval Medical Research Center (AB-Navy1, AB-Navy4, AB-Navy71, AB-Navy97, and AbTP3ϕ1) were isolated from various environmental samples by using routine isolation techniques, as previously described (9)” should read “Phages provided by the Biological Defense Research Directorate (BDRD) of the Naval Medical Research Center (AB-Navy1, AB-Navy4, AB-Navy97, and AbTP3ϕ1) were isolated from various environmental samples by using routine isolation techniques, as previously described (9), except for one phage (AB-Navy71, also known as phage vB_Ab-M-G7, DSM strain no. 25639), which was purchased from Liebniz Institute DSMZ (Germany).”