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Pseudomonas aeruginosa ventricular assist device infections: Findings from ineffective phage therapies in five cases

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ABSTRACT (245 words)

Background

Left ventricular assist devices (LVAD) are increasingly used for management of heart failure; infection remains a frequent complication. Phage therapy has been successful in a variety of antibiotic refractory infections and is of interest in treating LVAD infections.

Methods

We performed a retrospective review of four patients that underwent five separate courses of intravenous (IV) phage therapy with concomitant antibiotic for treatment of endovascular *Pseudomonas aeruginosa* LVAD infection. We assessed phage susceptibility, bacterial strain sequencing, serum neutralization, biofilm activity, and shelf-life of phage preparations.

Results

Five treatments of 1-4 wildtype virulent phage(s) were administered for 14-51 days after informed consent and regulatory approval. There was no successful outcome. Breakthrough bacteremia occurred in 4 of 5 treatments. Two patients died from the underlying infection. We noted a variable decline in phage susceptibility following 3 of 5 treatments, 4 of 4 tested developed serum neutralization, and prophage presence was confirmed in isolates of two tested patients. Two phage preparations showed an initial titer drop. Phage biofilm activity was confirmed in two.

Conclusions

Phage susceptibility alone was not predictive of clinical efficacy in *P. aeruginosa* endovascular LVAD infection. IV phage was associated with serum neutralization in most cases though lack of clinical effect may be multifactorial including presence of multiple bacterial isolates with varying phage susceptibility, presence of prophages, decline in phage titers, and possible lack of biofilm activity. Breakthrough bacteremia occurred frequently (while the organism remained

susceptible to administered phage) and is an important safety consideration.

INTRODUCTION

Heart failure affects more than 64 million people worldwide.[1] Mechanical circulatory support devices have been increasingly utilized for managing heart failure that is refractory to medical therapy, with increasing numbers supported on left ventricular assist devices (LVAD) either as a bridge to heart transplantation or as destination therapy.[2]

One main complication of prolonged LVAD support is device related infection, occurring in about a third of LVAD recipients, and associated with a high degree of morbidity and mortality.[2,3] *Pseudomonas aeruginosa* is a very common etiologic agent, and frequently is multidrug resistant (MDR).[3] In general, once infected, LVAD recipients remain on long term antibiotics for suppression as cure of the infection is not possible without device removal.[3] Recent successful outcomes with bacteriophage (phage) therapy (viruses that directly infected and lyse their bacterial host) in MDR and antibiotic recalcitrant infections has prompted phage use for LVAD infections as well.[4] Large gaps in knowledge remain, which have contributed to continued treatment heterogeneity in approach, formulation, and clinical investigation. Moreover, relatively few phages have been shown to be effective in the clinical management of device related infection.[4–10]

In this study, we carried out a retrospective analysis of four patients with MDR *P. aeruginosa* LVAD endovascular infections that were treated with five distinct courses of phage therapy and concomitant antibiotics. None of the treatments were successful and we investigate potential causes of treatment failure including phage, bacteria and patient factors.

METHODS (further details in Supplement):

Regulatory aspects

All patients were treated after obtaining informed consent and approval to proceed from regulatory authorities. Two patients (Cases 1, 2.1, 2.2) were treated at the University of California San Diego hospital (San Diego, CA) under compassionate single use authorizations from the Food and Drug Administration and institutional review board (IRB) approval from the Human Research Patient Protection committee (IRB #200163). Two patients (Cases 3, 4) were treated at the Schnieder Childrens Hospital (Petach Tikva, Israel) and Sheba Medical Center (Ramat-Gan, Israel) respectively with informed consent and approval from the local IRB and Israeli Ministry of Health.

Antibiogram

Bacterial isolates were tested by the relevant clinical microbiology laboratories at each hospital using VITEK® 2 (BioMérieux) for most antibiotic susceptibility data and the disk diffusion method for ceftolozane/tazobactam.

Phage susceptibility testing

Clinical *P. aeruginosa* isolates were cultured and all treatment phages were confirmed to have lytic activity by one of the following methods: 1) double agar overlay or spot titer method to visualize plaques on agar lawns as previously described, or) time-kill kinetic curves.[11–14]

Anti-biofilm testing

Phage anti-biofilm activity was carried out on Case 3's bacterial isolate using both a static biofilm model in 96-well microplate as previously described[15] and a dynamic model in which biofilm was grown in a chamber connected to a peristaltic pump with circulating bacterial culture to which phage was added after 24 hours as previously described.[16]

Phage-antibiotic synergy

PASA16 (used in Cases 3 and 4) was confirmed to have synergy with co-treatment antibiotic via the checkerboard assay using the patient's bacterial isolates.[17]

Serum phage neutralization

To assess whether the patient's serum contained phage-neutralizing antibodies, serum was diluted between 1:5 to 1:640 in PBS and incubated with phage serum between 1:1 to 1:10 for 30 min up to 24 hours. Samples were then titrated to count viable plaques after incubation using soft agar overlay method.

Whole genome sequencing of bacterial isolates (detailed in Supplement).

RESULTS:

Four patients received five separate courses of antibiotic and phage combination therapy. Table 1 summarizes the clinical course of each case, along with details regarding phages used and Figure 1 depicts a visual timeline of the duration of phage therapy, concomitant antibiotics used, and episodes of breakthrough bacteremia. All phages were sequenced prior to therapeutic use and lacked antibiotic resistance genes as well as genes denoting lysogenic potential such as integrase and repressor genes (data not shown). Table 2 summarizes the key investigational aspects of each phage course that may have contributed to a failed outcome. Supplement Table 1 depicts antibiotic susceptibility profiles of all *P. aeruginosa* isolates.

Case 1

This was a 60- year-old male with LVAD placement in 2013 complicated by *P. aeruginosa* bacteremia and device infection since March 2017 leading to recurrent bacteremia, persistent drainage from the driveline and sternotomy sites, and multiple hospitalizations. He was treated with several prolonged courses of intravenous (IV) antibiotics (piperacillin-tazobactam, cefepime, meropenem) and surgical debridement. He eventually received a 42-day course of three lytic phages PaBAP5ϕ3, PaMTAE8ϕ1, and PaMTAE8ϕ3 (details in Table 1) with concomitant antibiotics. Pre-phage blood cultures on Day1 were negative; however, blood cultures on Days7 and 13 both grew *P. aeruginosa.* These new isolates had different antibiotic susceptibility patterns leading to antibiotic change to ceftazidime on Day14. Blood cultures on Day17 were negative but Day19 grew *P. aeruginosa* again. The patient remained afebrile, hemodynamically stable, and overall asymptomatic other than chronic driveline drainage. Imaging was negative for an abscess and transesophageal echocardiogram was negative for vegetations. His central line was changed but Day22 blood cultures remained positive. Eventually, bacteremia cleared on a combination of ceftazidime-avibactam and aztreonam and the patient remained on systemic antibiotics until heart transplant approximately seven months later; surgical cultures were positive for *P. aeruginosa*.

Phage susceptibility: Baseline blood and driveline *P. aeruginosa* isolates as well as breakthrough bacteremia isolates on Days 7 and 13 remained susceptible to the phage cocktail (Figure 2).

Serum Neutralization: At the time this patient was treated in 2017 we did not routinely save serum samples. In this case, only the Day17 sample was tested and revealed reduction in viable PFU against all three phages used to treat the patient -75.7% reduction in PaBAP5ϕ3, 57.6% reduction in PaMTAE8ϕ1, and 53.7% in PaMTAE8ϕ3 (Supplement Table 2A). Both hazy and clear plaques were noted with 2 of 3 phages (PaBAP5ϕ1, PaBAP5ϕ3). Additionally, there were no plaques observed when plating 100 ul of serum with the *P. aeruginosa* host strain.

Bacterial isolate sequence, assembly, and annotation: Comparison of one pre- and one breakthrough isolate (GD1-2) genomes revealed that they were identical (Supplement Table 3). These isolates encoded more genes associated with antimicrobial resistance than did the reference strain (186 versus 49, Supplement Table 4).

Case 2.1

An 82-year-old male with an LVAD placed in 2017; his course was complicated with *P. aeruginosa* recurrent bacteremia, driveline infection, and multiple hospitalizations over the next two years. He was admitted with *P. aeruginosa* bacteremia and IV 2-phage cocktail (PAK_P1 and E217) along with piperacillin-tazobactam was initiated. (Table 1, Figure 1). He also received a single intraoperative dose of the same phage cocktail at the site of infection following surgical debridement on Day 8; surgical cultures were positive for *P. aeruginosa*. After 15 days of the PAK_P1 and E217 combination, the E217 was discontinued and PAK_P1 was continued as monotherapy for 11 days as we found that the Day 8 driveline pseudomonal isolate was resistant to E217 (but remained susceptible to PAK_P1; the blood isolate from Day 4 was unfortunately not tested)). A new phage PAK_P5 was added to the ongoing PAK_P1 on Day 28 and the new combination was continued through Day 50 of phage treatment. Weekly wound cultures following surgery were negative and phage/antibiotic were stopped after 50 days. By this time, the previous sinus tract site had healed with resolution of drainage. One week after stopping treatment, the patient was readmitted with septic shock, recurrent *P. aeruginosa* bacteremia and an LVAD-related abscess. New purulent drainage from the previous sinus tract grew *P. aeruginosa.* However, the isolate exhibited a different antibiotic susceptibility pattern than before (Supplement Table 1). He subsequently had two more admissions with similar clinical presentations.

Phase susceptibility: Five baseline isolates were collected from the patient's driveline (PaD1, 2, 3, and 5) and blood (PaD4) starting 52 days before the first course of phage. PAK_P1 was highly lytic against all, PAK_P5 showed intermediate activity, and E217 was inactive against 3 of 5 isolates at baseline (Figure 3A, Supplement Table 5). Phylogenetic analysis based on whole genome single nucleotide polymorphisms (SNPs) confirmed the presence of three clades (Figure 2B).

After eight days of IV phages, *P. aeruginosa* driveline isolate (PaD6) exhibited a fourth pattern of susceptibility with PAK_P1 and PAK_P5 showing high activity and E217 inactive (Figure 2A). This prompted PAK_P1 only treatment from Days16-27, and addition of PAK_P5 for the remainder of the course. Phage susceptibility of post-phage blood (PaD7), driveline (PaD8), and sternum (PaD9) cultures displayed reduced susceptibility to PAK_P1 and PAK_P5. Although all three isolates belonged to the same clade as the originating PaD1 though PaD9 diverged significantly from PaD1 (Figure 2B).

Stability of the clinical phage preparations: Undiluted PAK_P1 and E217 stock solutions remained stable when titered after 31 days (PAK_P1 5.78 x 10^{10} PFU/mL, E217 1.89 x 10^{11} PFU/

mL). However, the diluted patient dose samples contained 7.58×10^5 PFU/mL PAK_P1 and 1.63×10^8 PFU/mL E217 which was a 4-log loss of PAK_P1 and a 1-log loss of E217 at point of administration. Titration on Day69 revealed that phages did not decay as significantly after the first titer drop. Stability of PAK_P5 was not tested.

Case 2.2

The same patient as Case 2.1 was readmitted for the third time with recurrent *P. aeruginosa* bacteremia approximately three months after the first phage therapy course. Bacteremia was cleared on ceftolozane/tazobactam, ciprofloxacin, and tobramycin. A second IV course of a 4-phage cocktail PPM3 was administered for 36 days (Table 1). On Day29 of phage treatment, he had an aspiration event, septic shock, fever, and multisystem organ failure. While still on phage, his blood cultures were now positive for *P. aeruginosa*. Due to overall poor prognosis, the patient transitioned to comfort care and passed.

***Phage susceptibility*:** New custom phage cocktail, PPM3 (phages Epa11, Epa17, Epa22, Epa39), commenced 105 days after the end of the first phage course (Case 2.1). These phages were chosen from a library of seven *Pseudomonas* phages screened for lytic activity on the initial PaD1-9 isolates and two additional sternum isolates PaD10-11. (Figure 3A, Supplement Table 6). All four phages were mostly active against all 11 isolates, except PaD4 which was resistant to all PPM3 phages. On the contemporary isolates PaD10-11, Epa11 and Epa39 were highly lytic, Epa22 showed variable activity, and Epa17 only moderate activity (Figure 3A). PaD10-11 were in the clade with PaD1 and most closely related to PaD9 (Figure 3B). PaD4 also belonged to the same clade but showed divergence from the cluster of PaD9-11. Variability in phage susceptibilities to individual phages, including PaD4, did not appear to correlate with timing of phage therapy.

After 36 days of PPM3 treatment, two new breakthrough bloodstream isolates, PaD12-13, exhibited an inversed susceptibility pattern (Figure 3A). Although both isolates were still susceptible to all four phages, phages Epa11, Epa22, and Epa39 had reduced efficiency of plating (EOP) while the Epa17 exhibited increased EOP. Phylogeny showed that PaD12-13 had diverged from the contemporary isolates PaD9-11 (Figure 3B). Likewise, in Case 2.1, isolates during phage treatment evolved reduced susceptibility towards phages and antibiotic.

Biofilm activity of the phage: PPM3 was not tested specifically against the patient isolates but has known anti-biofilm activity when tested against a laboratory *P. aeruginosa* isolate (Supplement Figure 2).

Serum neutralization: We did not see any serum neutralization for three of the phages used in this case (Epa11, Epa 22, Epa39) from Days1-30 of PPM3 administration. Minimal neutralization was seen with Epa17 at Day30 (Supplement Figure 3).

Stability of the clinical phage preparations: Total (4-phage) titers on days 3, 14 and 60 were 6.0 $\times 10^8$, 2.0 $\times 10^8$ and 5.5 $\times 10^4$ PFU/mL respectively. Thus, a one-log decrease was noted at day 3, remained stable at Day14 and had a 4-log drop by 2 months.

Isolate sequence, assembly and annotation of Cases 2.1 and 2.2: All 13 bacterial isolates from this patient were strains of the same sequence type (a novel sequence type by a traditional MLST scheme for *P. aeruginosa* most similar to ST-690) and closely related to each other. However, there were genomic differences between isolates that had some correlation with differences in phage susceptibilities (Supplement Tables 7, 8). The genomes of isolates PaD1-9 contained two prophages that were predicted in all 9 strains.

Case 3

A 10-year-old female with a genetic cardiomyopathy was admitted with cardiogenic shock and underwent placement of a Berlin heart Excor VAD in January 2019 as a bridge to transplant. In August 2019, she developed recurrent and almost persistent *P. aeruginosa* bacteremia attributed to endovascular LVAD infection. She was treated with several IV antibiotics including ceftazidime, ciprofloxacin, piperacillin-tazobactam and eventually due to increasing drug resistance, with ceftolozane/tazobactam and amikacin. In September 2019, she developed an intracranial hemorrhage requiring ventriculoperitoneal shunt. While on antibiotics, she developed fever and recurrent *P. aeruginosa* bacteremia and was started on IV monophage PASA16 [18,19] twice daily plus meropenem. During phage and antibiotic treatment, she had fever spikes every 3-4 days along with altered consciousness, without shunt malfunction or elevated intracranial pressure; however, blood cultures remained negative. After 33 days on phage and antibiotic combination, the patient clinically worsened with daily fever and worsening consciousness, but multiple blood cultures remained negative, C-reactive protein was normal, and meningoencephalitis was excluded. Phage and meropenem were stopped on Day51 due to concern for inflammatory/allergic reaction to either the antibiotic or phage. The patient remained febrile and three days after stopping the phage and meropenem, blood cultures were again positive for MDR *P. aeruginosa.* This isolate had similar antibiotic susceptibilities as the pre-phage isolate. Due to poor neurological status, persistent *P. aeruginosa* infection, and failure to thrive, the patient transitioned to palliative care and passed.

Phage susceptibility: Baseline bacterial isolates, SH1-2 were susceptible to PASA16 when tested by plaque assay and growth kinetics (Figure 4A, 4B). The activity of PASA16 was also tested in the presence of sub-inhibitory concentrations of various antibiotics (Figure 4C). Based on these results, PASA16 and meropenem combination was chosen for clinical use. Two additional *P. aeruginosa* isolates were collected after the end of phage therapy, SH3-4; both had reduced phage plaquing denoting reduced phage susceptibility.

Serum Neutralization: Baseline serum did not neutralize phage; however, serum on Days7, 12, 19 demonstrated almost undetectable phage recovery indicating serum neutralization (Supplement Table 2B).

Biofilm activity: PASA16 demonstrated reduction of SH1-2 biofilms in vitro using static and dynamic models (Figures 4E-F).

Isolate sequence, assembly and annotation: Isolates SH1, SH2 and SH4 were identical on sequencing and SH3 showed 98% identity with them. Regarding lysogens, spontaneous plaques were observed in the cultures of SH1, SH3 and SH4 but we could not observe them on SH2. PHASTER (https://phaster.ca/) analysis detected 5 putative lysogens in the genome of the strains, but no induction was observed.

Stability of the clinical phage preparations (Case 3, 4): Stability of PASA16 was tested after storage for a year in -80C and no reduction in titer was observed. Moreover, PASA16 was later used in several other treatments and in all cases titers remained stable.[19]

Case 4

A 52-year-old male was admitted in June 2021 with cardiogenic shock requiring extracorporeal membrane oxygenation and then LVAD**.** Two months later purulent discharge from the LVAD driveline was positive for *P. aeruginosa* which was treated with ceftazidime for 6 weeks though the patient had recurrent episodes of driveline drainage that was treated with ciprofloxacin or ceftazidime based on antibiotic susceptibility data. In August 2022, the patient developed persistent *P. aeruginosa* bacteremia. He was treated with several antibiotics including ciprofloxacin, piperacillin/tazobactam, ceftazidime, meropenem and gentamicin without clearance of the bloodstream. In November 2022, PET/CT scan demonstrated infection along the driveline and around the pump itself, including the deep cannula of the device. In April 2023, IV monophage PASA16 and ceftazidime co-therapy was initiated for 2 weeks. Patient became

bacteremic again on day 12 of phage therapy and thus therapy was not continued. Phage PASA16 susceptibility on the initial infection isolates (August 2022) and PASA16 synergy with ceftazidime, was used to determine combination formulation.

Phage susceptibility: Baseline bacterial isolate, C393 (August 2022) was susceptible to PASA16 when tested by plaque assay and growth kinetics (Figure 5A, 5B). The activity of PASA16 was also tested in the presence of sub-inhibitory concentrations of various antibiotics (Figure 5C). Based on these results, PASA16 and ceftazidime combination was chosen for clinical use. Additional *P. aeruginosa* isolate, C442, was collected after the end of phage therapy. This isolate had increased phage susceptibility as demonstrated by plaque assay (Figure 5A).

Serum Neutralization: Baseline serum neutralized phage by 2 logs; however, serum on Day8 demonstrated undetectable phage recovery indicating complete serum neutralization (Figure 5E).

Isolate sequence, assembly and annotation: Sequencing was not performed on isolates C393 and C442

DISCUSSION

Interest in phage therapy for antibiotic recalcitrant infections is growly rapidly and current interest far outstrips availability of phage.[5] The current literature has a preponderance of successful cases and unsuccessful cases are not published.[4] In this paper, we describe the clinical course of four patients with *P. aeruginosa* LVAD endovascular infections that were treated with five separate courses of phage therapy and explore reasons for unsuccessful outcomes.

Infections occur in up to a third of LVAD recipients and commonly are due to *Staphylococcus aureus* and *P. aeruginosa*.[3] In general, once the device is infected, targeted antimicrobial therapy is usually long-term as antibiotics alone cannot resolve the biofilm based infection; over time patients may develop increasing antimicrobial resistance as well as superinfections from other organisms. All our cases had persistent MDR pseudomonal LVAD infection associated with recurrent bacteremia and hospitalization. Several case reports demonstrate cure of cardiac device infections with phage and antibiotic combination: these include two cases of localized *P. aeruginosa* driveline infections without vascular infection,[8,9] a case of MDR *Klebsiella pneumoniae* LVAD pump and driveline infection (the device was exchanged in this case),[20] *S. aureus* LVAD-related abscess with drainage and local application of phage,[21,22] *S. aureus* cardiovascular implantable electronic device infection (device explanted),[21] and a case of *S. aureus* LVAD infection associated with driveline infection, sternal osteomyelitis and bacteremia

(device removed during transplant).[10] Only one case of *P. aeruginosa* driveline infection that failed phage therapy has been published.[21] We are unaware of successful outcomes of phage therapy in the setting of LVAD endovascular infections marked by recurrent/ persistent bacteremia due to *P. aeruginosa.*

In this study, we investigated each case to try and pinpoint reasons for the unsuccessful outcome, though no single etiology was readily observed. All patients had longstanding device infection associated with bacteremia with infection duration ranging between months to years. All received IV phage in addition to systemic antibiotics. Only one case underwent debridement and intra-operative local phage application. The infected devices remained in situ for all cases in our series, as opposed to two previously published successful cases of LVAD bacteremia in which the device was removed (though neither was due to *P. aeruginosa*).[10,20] Other successful LVAD phage publications have all been local device infections *without* bacteremia.

Baseline phage susceptibility testing was performed in all cases. For the second patient (Case 2.1, 2.2), the individual phages were active against a majority of baseline isolates though not all, with some phages showing intermediate susceptibility pattern. However, in Cases 1, 3 and 4, phages were active against all baseline isolates (though isolate collection was not as extensive). Cases 2.1 and 2.2 clearly had several *P. aeruginosa* variants at baseline though all appeared within the same MLST lineage and potentially reflected prolonged antibiotic pressure as the infection had been present for a few years. The breakthrough bacterial isolate in Case 2.1 was resistant to 1 of 3 phages (conversely remained susceptible to the other 2 phages) in the cocktail being used; breakthrough isolates during phage therapy in Cases 2.2 and 3 showed reduced plaquing denoting reduced susceptibility; however, the breakthrough isolate in Case 4 actually had improved phage susceptibility. In Case 1, breakthrough isolates remained susceptible to the phage cocktail as a whole but individual phages were not tested. Of note, there are no clear definitions of what constitutes “susceptible”, “intermediate”, and “resistant” for phage and this hinders our interpretations of in vitro results and subsequent phage choices, especially for development of personalized therapies.[23] Within these limitations, it does not seem that bacterial resistance to phage was responsible for clinical failure in these 5 phage treatments though “less” susceptible isolates were noted at time of infection relapse in some cases.

In vitro studies demonstrate that synergy as well as antagonism is possible with various phage and antibiotic combinations and inclusion in baseline assessment when considering phage therapy is recommended.[23–26] Cases 3 and 4 had baseline antibiotic and phage synergy testing performed which assisted in using a synergistic combination for treatment; however this was not

associated with a successful outcome.

We planned to treat each patient with a phage concentration of at least 10^9 PFU/mL or higher. However, stability testing of the clinical preparations demonstrated a modest drop in titers in Cases 2.1 and 2.2. Case 1 was not tested and titers of PASA16 in Cases 3 and 4 were maintained. Thus, it is unclear if lower than planned phage concentration impacted outcomes in Cases 2.1 and 2.2. Of note, the actual delivered phage concentration used in published case studies is unknown as stability testing results have not been reported and so the threshold concentration for a successful clinical outcome is not clear.

Four phage treatments were complicated by development of bacteremia while on phage (associated with septic shock in Cases 2.1 and 2.2). Development of bacteremia after initiating phage occurred within the first week in two cases; we hypothesize that this could potentially be related to release of pathogens within the bloodstream as the device biofilm is rapidly degraded by phage, as the isolates were mostly susceptible to the administered phages. This would be an important safety point moving forward in the treatment of vascular device infections; it has not been described in non-vascular infections treated with phage. Another possibility is that the phage selectively eradicated a few specific isolates quickly from the many present at baseline allowing for "less" susceptible isolates to take over the resulting ecological niche. Phage biofilm activity was only confirmed in the setting of Case 3, though PPM3 (used in Case 2.2) has demonstrated antibiofilm activity against a laboratory *P. aeruginosa* isolate. Given that these were all biofilm based infections, we recommend assessment of biofilm activity against patient isolates prior to development of personalized phage cocktails in a standardized fashion.[23] Development of bacteremia after phage initiation in endovascular infection is an important safety consideration.

Phages elicit an immune response which has been described with several modes of administration including enteral, nebulization and IV.[27–29] At least in one published case, serum neutralization of administered phage was associated with worsening clinical status [27] though this has not been seen in other cases in which successful clinical outcomes occurred despite development of serum neutralization.[6,28] We noted complete serum neutralization in 4 of 5 treatments; this may have been an important element impacting effectiveness of phage therapy in our patients and for endovascular infections in general. As noted, serum neutralization did not develop in response to PPM3 used in Case 2.2; thus, phages that preferentially do not lead to serum neutralization may be preferred in the setting of endovascular infection (though this one issue alone may not be sufficient for clinical success).

Sequencing also noted the presence of prophages in the bacterial isolates from 2 patients (though not assessed in the others) and this potentially may have impacted treatment outcome as well. Previous studies demonstrate that in vitro induction of filamentous Pf4 prophages in *P. aeruginosa* can lead to a change in antibiotic susceptibility pattern of the organism,[30] enhance biofilm formation,[31] trigger a maladaptive local immune response impairing bacterial clearance,[32] and can confer a competitive advantage against phage superinfection.[33] In our series, it is unknown if presence of prophages in the patient isolates impacted clinical outcomes.

In summary, we describe the clinical course of five phage treatments for MDR *P. aeruginosa* endovascular LVAD infections. Unfortunately, therapy failed in all cases, and we posit that this may be due to a combination of factors including serum neutralization, presence of multiple bacterial isolates at baseline with varying phage susceptibility patterns, development of reduced phage susceptibility in some cases, presence of prophage, and lack of testing for anti-biofilm activity. Additionally, assessment of phage titers of the administered product and phage-antibiotic synergy may be informative for future cases. There may be other factors that we have not investigated that are pertinent to the success of phage therapy in this specific clinical situation. Lastly, we note an important safety concern with development of bacteremia after phage initiation.

GenBank Accession numbers:
PAK_P1 # KC862297.1, PAK_P5 # KC862301.1, E217_# MF490240, EPa11: MT108727.1; EPa17: MT108728.1; EPa22: MT108729.1; EPa39: MT118303.1

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Biswajit Biswas: Navy Work Unit # A1417. Dr. Biswas has a patent "Bacteriophage compositions and methods of selection of components against specific bacteria" US patent #10357522, which was licensed before.
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Daniel Gelman: none

Ronen Hazan: none

FIGURE LEGENDS

Figure 1. Timeline depicting phage duration, onset of positive blood cultures and antibiotic therapy in five cases of multidrug resistant *Pseudomonas aeruginosa* left ventricular assist device infections.

Figure 2. Susceptibility of *Pseudomonas aeruginosa* isolates from Case 1 to phages used in the treatment of the patient using the Biolog method. This consisted of inoculation of standardized bacterial suspensions with bacteriophages individually and in combination in 96-well microtiter plates incubated at 37°C in a Biolog® machine for 24 hours. Bacterial respiration led to a reduction of the tetrazolium dye leading to a color change which is depicted as relative units of bacterial growth.

Figure 3: Antimicrobial susceptibility and relatedness of *Pseudomonas aeruginosa* isolates for Cases 2.1 and 2.2. (A) Phage and antibiotic susceptibility of isolates PaD1-13. Susceptibility was determined by spotting 4μL of 10^9 PFU from a library of *Pseudomonas* phages to determine the three candidate virulent myoviruses with the highest activity PAK_P1, PAK_P5, and E217 The isolate ID indicates the order of isolation. The source, day, and timeline of sample collection are listed, with day 0 being the start of first course phage administration (Case 2.1). Phage susceptibility was tested using efficiency of plating (EOP) for both Cases 2.1 and Case 2.2 phages. Phage susceptibility is indicated as sensitive (blue), partial clearing as intermediate (yellow), no plaquing as resistant (red), or not determined (white). Antibiotic susceptibility was determined using VITEK® 2 in the clinical microbiologic laboratory. (B) Phylogenetic tree assembled with the complete genome sequence of case originating isolate PaD1 and short read sequences of PaD2 to 13. Branch colors indicate clades and lengths indicate relative evolutionary distance.

Figure 4. Phage susceptibility testing for Case 3. A) Plaque morphologies of baseline *Pseudomonas aeruginosa* isolates, Sh1 and Sh2. B) Growth curves of baseline Sh1, Sh2, and combined culture of the two strains in 1:1 ratio as affected by the phage. Graphs are average of 3 replicates and standard deviations (SD) are shown. C) Growth curves of Sh1 and Sh2 combined culture in the presence of various sub-inhibitory levels of antibiotics: ciprofloxacin (cipro), gentamicin (genta), meropenem (mero). Graphs are average of 3 replicates and SD are shown. D) Plaque morphologies of post-phage *Pseudomonas aeruginosa* isolates, Sh3 and Sh4. E-F) Effect of PASA16 on the strains SH1 and SH2 which were isolated before the treatment, in biofilm setting in two assays; CFU count in a 96-well plate static model (E) and biomass detection by crystal violet staining in a flow model (F). * denotes differences with P-value < 0.05.

Figure 5. Phage susceptibility testing for Case 4**.** A) PFU of phage PASA16 on the *Pseudomonas aeruginosa* isolates, C393 (left panel) and C442 (right panel). As a positive control for PASA16 efficacy served the strain PA14 B) Growth curves of C393 alone and with

PASA16 phage. Graphs are average of 3 replicates and standard deviations (SD) are shown. C) Growth curves of C393 in presence of combinations of PASA16 and various antibiotics in concentration of their MIC and 0.1 MIC. Graphs are average of 3 replicates and SD are shown. D) CFU count of the cultures presented in C, at the endpoint of experiment E) Effect of patient's serum on the PFU of PASA16 pre- and post- treatment.

REFERENCES:

1. Savarese G, Becher PM, Lund LH, Seferovic P, Rosano GMC, Coats AJS. Global burden of heart failure: a comprehensive and updated review of epidemiology. Cardiovasc Res 2023;118(17):3272–87.

2. Molina EJ, Shah P, Kiernan MS, et al. The Society of Thoracic Surgeons Intermacs 2020 Annual Report. Ann Thorac Surg 2021;111(3):778–92.

3. Tran H, Aslam S. Ventricular Assist Devices, ECMO, and Cardiac Support Devices: Challenges in the Bridge to Transplant [Internet]. In: Morris MI, Kotton CN, Wolfe CR, editors. Emerging Transplant Infections: Clinical Challenges and Implications. Cham: Springer International Publishing; 2021 [cited 2023 Jan 8]. p. 1475–94.Available from: https://doi.org/10.1007/978-3-030-25869-6_59

4. Suh GA, Lodise TP, Tamma PD, et al. Considerations for the Use of Phage Therapy in Clinical Practice. Antimicrob Agents Chemother 2022;66(3):e0207121.

5. Aslam S, Lampley E, Wooten D, et al. Lessons Learned From the First 10 Consecutive Cases of Intravenous Bacteriophage Therapy to Treat Multidrug-Resistant Bacterial Infections at a Single Center in the United States. Open Forum Infect Dis 2020;7(9):ofaa389.

6. Ramirez-Sanchez C, Gonzales F, Buckley M, et al. Successful Treatment of Staphylococcus aureus Prosthetic Joint Infection with Bacteriophage Therapy. Viruses 2021;13(6):1182.

7. Cano EJ, Caflisch KM, Bollyky PL, et al. Phage Therapy for Limb-threatening Prosthetic Knee Klebsiella pneumoniae Infection: Case Report and In Vitro Characterization of Anti-biofilm Activity. Clin Infect Dis 2021;73(1):e144–51.

8. Mulzer J, Trampuz A, Potapov EV. Treatment of chronic left ventricular assist device infection with local application of bacteriophages. Eur J Cardiothorac Surg 2020;57(5):1003–4.

9. Tkhilaishvili T, Merabishvili M, Pirnay J-P, et al. Successful case of adjunctive intravenous bacteriophage therapy to treat left ventricular assist device infection. J Infect 2021;83(3):e1–3.

10. Aslam S, Pretorius V, Lehman SM, Morales S, Schooley RT. Novel bacteriophage therapy for treatment of left ventricular assist device infection. J Heart Lung Transplant 2019;38(4):475–6.

11. Kropinski AM, Mazzocco A, Waddell TE, Lingohr E, Johnson RP. Enumeration of bacteriophages by double agar overlay plaque assay. Methods Mol Biol 2009;501:69–76.

12. Luong T, Salabarria A-C, Edwards RA, Roach DR. Standardized bacteriophage purification for personalized phage therapy. Nat Protoc 2020;15(9):2867–90.

13. Sergueev KV, Filippov AA, Farlow J, et al. Correlation of Host Range Expansion of Therapeutic Bacteriophage Sb-1 with Allele State at a Hypervariable Repeat Locus. Appl Environ Microbiol 2019;85(22):e01209-19.

14. Henry M, Biswas B, Vincent L, et al. Development of a high throughput assay for indirectly measuring phage growth using the OmniLog(TM) system. Bacteriophage 2012;2(3):159–67.

15. Müsken M, Di Fiore S, Römling U, Häussler S. A 96-well-plate–based optical method for the quantitative and qualitative evaluation of Pseudomonas aeruginosa biofilm formation and its application to susceptibility testing. Nat Protoc 2010;5(8):1460–9.

16. Nielsen MW, Sternberg C, Molin S, Regenberg B. Pseudomonas aeruginosa and Saccharomyces cerevisiae Biofilm in Flow Cells. JoVE (Journal of Visualized Experiments) 2011;(47):e2383.

17. Łusiak-Szelachowska M, Międzybrodzki R, Drulis-Kawa Z, et al. Bacteriophages and antibiotic interactions in clinical practice: what we have learned so far. Journal of Biomedical Science 2022;29(1):23.

18. Alkalay-Oren S, Yerushalmy O, Adler K, et al. Complete Genome Sequence of Pseudomonas aeruginosa Bacteriophage PASA16, Used in Multiple Phage Therapy Treatments Globally. Microbiol Resour Announc 2022;11(4):e0009222.

19. Onallah H, Hazan R, Nir-Paz R, et al. Refractory Pseudomonas aeruginosa infections treated with phage PASA16: A compassionate use case series. Med 2023;S2666-6340(23)00225-8.

20. Hope KD, Tunuguntla HP, Puri K, et al. Bacteriophage Therapy for Treatment of a Multi-Drug Resistant VAD-Specific Infection in a Pediatric Patient. The Journal of Heart and Lung Transplantation 2022;41(4):S510–1.

21. Tkhilaishvili T, Potapov E, Starck C, et al. Bacteriophage therapy as a treatment option for complex cardiovascular implant infection: The German Heart Center Berlin experience. The Journal of Heart and Lung Transplantation 2022;41(5):551–5.

22. Rojas SV, Junghans S, Fox H, et al. Bacteriophage-Enriched Galenic for Intrapericardial Ventricular Assist Device Infection. Antibiotics (Basel) 2022;11(5):602.

23. Gelman D, Yerushalmy O, Alkalay-Oren S, et al. Clinical Phage Microbiology: a suggested framework and recommendations for the in-vitro matching steps of phage therapy. Lancet Microbe 2021;2(10):e555–63.

24. Gu Liu C, Green SI, Min L, et al. Phage-Antibiotic Synergy Is Driven by a Unique Combination of Antibacterial Mechanism of Action and Stoichiometry. mBio 2020;11(4):e01462-20.

25. Schooley RT, Biswas B, Gill JJ, et al. Development and Use of Personalized Bacteriophage-Based Therapeutic Cocktails To Treat a Patient with a Disseminated Resistant Acinetobacter baumannii Infection. Antimicrob Agents Chemother 2017;61(10):e00954-17.

26. Van Nieuwenhuyse B, Van der Linden D, Chatzis O, et al. Bacteriophage-antibiotic combination therapy against extensively drug-resistant Pseudomonas aeruginosa infection to allow liver transplantation in a toddler. Nat Commun 2022;13(1):5725.

27. Dedrick RM, Freeman KG, Nguyen JA, et al. Potent antibody-mediated neutralization limits bacteriophage treatment of a pulmonary Mycobacterium abscessus infection. Nat Med 2021;27(8):1357–61.

28. Terwilliger A, Clark J, Karris M, et al. Phage Therapy Related Microbial Succession Associated with Successful Clinical Outcome for a Recurrent Urinary Tract Infection. Viruses 2021;13(10):2049.

29. Łusiak-Szelachowska M, Zaczek M, Weber-Dąbrowska B, et al. Phage neutralization by sera of patients receiving phage therapy. Viral Immunol 2014;27(6):295–304.

30. Gavric D, Knezevic P. Filamentous Pseudomonas Phage Pf4 in the Context of Therapy-Inducibility, Infectivity, Lysogenic Conversion, and Potential Application. Viruses 2022;14(6):1261.

31. Secor PR, Sweere JM, Michaels LA, et al. Filamentous Bacteriophage Promote Biofilm Assembly and Function. Cell Host Microbe 2015;18(5):549–59.

32. Sweere JM, Van Belleghem JD, Ishak H, et al. Bacteriophage trigger antiviral immunity and prevent clearance of bacterial infection. Science 2019;363(6434):eaat9691.

33. Schmidt AK, Fitzpatrick AD, Schwartzkopf CM, et al. A Filamentous Bacteriophage Protein Inhibits Type IV Pili To Prevent Superinfection of Pseudomonas aeruginosa. mBio 2022;13(1):e0244121.