Successful First Use of a Phage Endolysin for Treatment of Chronic Pelvic Pain Syndrome/Chronic Bacterial Prostatitis

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INTRODUCTION

Chronic prostatitis (CP) is an inflammatory condition of the prostate associated with pain and urinary symptoms occurring either with recurrent bacterial infection (chronic bacterial prostatitis [CBP]) or in the absence of evidence of bacterial infection (chronic pelvic pain syndrome [CPPS])¹,²,³,⁴,⁵. CBP may manifest symptoms such as dysuria, localized pain in the perineum, suprapubic region or lower back, and sexual dysfunction including erectile dysfunction and ejaculatory discomfort¹,⁵. In addition, as previously mentioned, a positive culture from expressed prostatic secretions is indicative of CBP⁶,⁷. It is estimated that chronic prostatitis (combined CBP and CPPS) effects approximately 2-10% of the male population worldwide¹,²,⁸,⁹, with a high rate (50%) of recurrence¹,²,⁸. Some studies suggest that 35 to 50% of men are affected by CP at some point in their lives¹⁰. In the United States, this accounts for almost 2 million patient visits annually¹⁰. When molecular methods (PCR) were applied diagnostically to cases that were previously determined (by cultural methods) to lack any evidence of bacteriuria or
prostate-localized uropathogens (i.e., originally diagnosed as CPPS), 16S rDNA was detected in prostate biopsies from 77% of these cases, indicating that in fact, most of the CP cases had evidence of bacterial infection, and therefore were in actuality CBP\textsuperscript{9,11}. This suggests that a larger proportion of CP is CBP.

A wide variety of bacterial species have been isolated from cases of CBP\textsuperscript{12}. Prominent among these are species of the Gram-negative family, *Enterobacteriaceae* (*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Acinetobacter spp.*, *Citrobacter spp.*)\textsuperscript{3,12}, and the Gram-positive Enterococcal Genus (*Enterococcus faecalis*, *Enterococcus faecium*)\textsuperscript{3,7,12,13}. Enterococcal infections (*E. faecalis* and *E. faecium*) present particularly challenging clinical management problems. These organisms are eminently hardy, surviving great extremes of temperature, pH, and environmental osmolality\textsuperscript{14,15}, and can survive long periods of starvation and desiccation\textsuperscript{16}. Furthermore, many strains of these species exhibit multidrug resistance properties\textsuperscript{17,18,19}.

*E. faecalis*, a species that had been considered to be a ubiquitous commensal organism, found in nature in the soil, water, and the alimentary tracts of mammals\textsuperscript{14}, is now recognized as a major opportunistic pathogen associated with a wide variety of medical and dental illnesses\textsuperscript{20}. *E. faecalis*-associated diseases include infections of the bloodstream, endocardium, abdomen, biliary tract, burn wounds, intravascular catheters as well as the urinary tract\textsuperscript{21}. These organisms are among the most frequent causes of nosocomial infections\textsuperscript{20,22,23}, and are the third leading cause of infectious endocarditis, accounting for approximately 20% of all cases\textsuperscript{24,25}.
Complicating management of these infections has been the emergence of drug-resistance among these organisms. Strains of *E. faecalis* resistant to β-lactam antibiotics as well as to high concentrations of aminoglycosides first appeared in the 1980s\textsuperscript{26,27}, leaving vancomycin as the only reliable remaining treatment option for these infections. However, in 1988, vancomycin-resistant *Enterococci*, (VRE), including *E. faecalis*, were reported\textsuperscript{28}. Therefore, until the subsequent introduction of some of the more recently developed antimicrobials, such as linezolid and synercid, these VRE were the first multidrug-resistant (MDR) organisms to become resistant to all available antimicrobial drugs. Subsequently, there has been evidence that strains of *E. faecalis* have even become resistant to these drugs as well\textsuperscript{29,30}.

The emergence and increasing prevalence of MDR bacteria has prompted the search for alternatives to antibiotics to treat these infections. One alternative approach to conventional antibiotic strategies for treating bacterial infections involves the biological control of bacterial diseases by means of viruses (bacteriophage/phage therapy). The idea of using viruses to control bacterial infections has been considered for more than 100 years\textsuperscript{31,32,33}. This bacteriophage-based strategy for bacterial infection control may take two forms: In one, infectious, virulent bacterial viruses are used to infect and subsequently lyse susceptible pathogenic bacteria. This approach has been explored in several eastern European countries for many years, with claims of highly successful outcomes\textsuperscript{34}. However, since the development of antibiotics, the idea of bacteriophage therapy in western countries has largely been ignored. Now, with the rise of antibiotic-resistant strains in many bacterial species, there is renewed interest in this strategy for
control of bacterial infections\textsuperscript{33,34,35,36,37}. Several recent \textit{in vivo} studies reported successful implementation of phage therapy (using infectious bacteriophages) in modifying bacterial infections by \textit{Acinetobacter baumannii}, \textit{Escherichia coli, Enterococcus faecium, and Pseudomonas aeruginosa} in animal model systems\textsuperscript{38,39,40,41}. In this regard it is significant to note that in a study reported by Smith and Huggins\textsuperscript{42}, a single intramuscular (IM) dose of phage was more effective in protecting mice from normally lethal IM or intracerebral injections of \textit{Escherichia coli} or \textit{Salmonella enterica}, than multiple IM injections of antibiotics such as tetracycline, ampicillin, chloramphenicol, or trimethoprim plus sulphafurazol. Furthermore, several recent clinical case reports demonstrated the efficacy of bacteriophage therapy in recalcitrant human infections\textsuperscript{43,44,45,46,47}.

A second approach in which bacteriophages may be employed to control bacterial infection involves the use of purified, bacteriolytic, phage gene products to cause the lysis and elimination of infecting pathogenic bacteria. For most bacteriophages, the final stage in a productive infective cycle consists of the lysis of the host cell and the release of the progeny virions. This bacteriolyis of the infected cell is mediated by one or more lysins; phage-encoded hydrolytic enzymes (endolysins) that degrade bacterial cell walls by attacking the major bonds in the peptidoglycan polymer. However, if the lysins are applied to a population of susceptible bacterial cells, digestion of the cell wall proceeds from its external surface, resulting in the lysis and death of the cell\textsuperscript{48}. The existence of bacterial cell wall hydrolases of bacteriophage origin has been known since 1957 when Krause demonstrated that there was a “labile lytic factor in phage lysates”, and that this factor was capable of lysing and killing the bacteria from which the factor was produced\textsuperscript{49}. 
However, the potential for therapeutic utility for these lysins was not recognized until 2001 when Nelson et al\textsuperscript{50} showed that mice could be protected from mucosal colonization and infection by Group A Streptococci by the application of a phage lysin. Since then, there have been numerous studies on lysins, detailing their biochemical and biological characteristics, as well as, in some cases, their protective efficacy against infections in \textit{in vivo} animal models (for reviews see references 48,51,52,53,54).

Previously, the Stevens laboratory isolated a bacteriophage from an infected root canal that infects strains of \textit{E. faecalis}\textsuperscript{55}. Sequencing and annotation of the phage genomic DNA permitted the identification of a gene that was predicted to code for the phage endolysin\textsuperscript{56}. Cloning and expression of the putative endolysin gene resulted in the production of a protein whose bacteriolytic activity confirmed the identity of the cloned gene\textsuperscript{57}. The purified protein [designated open reading frame (ORF) 28 endolysin] exhibited remarkably potent lytic activity against many strains of \textit{E. faecalis} including many vancomycin-resistant strains. It was, in fact, more potent, in terms of minimal inhibitory concentration (MIC) than vancomycin against vancomycin-sensitive \textit{E. faecalis} strains\textsuperscript{58}. These \textit{in vitro} data suggested the potential for the therapeutic use of the ORF28 endolysin for \textit{E. faecalis} infections. Here we present a case of chronic bacterial prostatitis in which a bacteriophage endolysin was successfully used to treat and mitigate infection and clinical symptoms.

**PRESENTATION OF THE CASE**

A 39-year-old Slovakian man was referred to a neurologist in October 2016 for two months of developing neuropathic pain in the perineum, which radiated to the scrotum
and the entire anogenital area. The pain began to develop following repeated cold stimuli in the fall of 2016. The patient described several types of pain: pain in the perineum was dominant, radiating to the rectum, scrotum, and penis. Pain behind the pubic bone was also present. At the beginning of the disease, the pain was paroxysmal and neuralgiform, later it was continuous. In the objective neurological findings, significant hyperalgesia of the entire anogenital area was present. The condition was concluded as chronic pelvic pain syndrome. The patient underwent a battery of examinations aimed at clarifying the origin of the pain. The urologist found an enlarged and painful prostate. Ultrasonography and subsequent magnetic resonance confirmed prostatitis. In order to assess prostatitis, ejaculate and expressed prostatic secretion were cultured repeatedly, which confirmed an infection of *Enterococcus faecalis* with high sensitivity to Unasyn (Fig 1, Micro 1). At that time, according to the NIH Chronic Prostatitis Symptom Index (NIH-CPSI)\textsuperscript{59}, the patient reported a pain score of 11 out of 21, a urinary symptom score of 5 out of 10, a quality-of-life impact score of 9 out of 12, and a total score 25, with higher scores indicative of worse outcomes. (In comparison, mean scores for pain, urinary symptoms and quality of life from a cohort of CBP patients were $8.7\pm5.7$, $4.1\pm3.1$, and $6.7\pm3.6$ respectively). The severity of the CBP can be classified as mild (0-9 points), moderate (10-18 points) or severe (19-31 points) according to the NIH-CPSI score\textsuperscript{59}.

The patient’s course of treatment is illustrated in the timeline shown in figure 1. The patient was first-line treated with Unasyn for two weeks with satisfactory clinical improvement and bacteriological eradication of enterococcus (Fig 1, Micro 2). However, the clinical symptoms of chronic pelvic pain and chronic prostatitis persisted. These included all the symptoms described above, but in a milder intensity. The average CPSI
after treatment was 16 points. Over the next three years, the patient had at least three exacerbations a year, when the CPSI reached 26 or more points, and the presence of *E. faecalis* was always confirmed during flares (Fig 1, Micro 3-7). After treatment with Unasyn or quinolones for two weeks, there was a clinical improvement and bacteriological negativity (Fig 1, Micro 8). The average CPSI was around 16 points in remission stages. Unasyn was the drug of choice for several reasons: 1. excellent safety, 2. usual high sensitivity of enterococcus, 3. very good effectiveness *in vivo*, 4. the patient tolerated quinolones very poorly. Beside the antibiotic therapy, the patient was treated with complex supplementary therapy for CBP (silodosin 4mg once a day, serenoa rapens, quercetin 400mg twice a day) with no significant effect on relieving the symptoms.
Fig 1. Timeline of the most relevant attacks, diagnostic procedures, microbiological results (dark blue), antibiotic therapies (dark grey), *E. faecalis* vaccination (light blue), phage therapy (magenta), endolysin therapy (red), and other supportive therapies (green). “Micro” refers to microbiological analysis determining the presence (*E. faecalis*+) or absence (*E. faecalis*−) of detectable *E. faecalis* infection.
In March 2019, the patient again had prostatitis caused by *E. faecalis*, which showed good sensitivity to Unasyn (MIC mg/l AMP 1, VAN 2, CIP 0.5U, TET ≥16, GEN 128, Fig 1, Micro 9). The patient was re-treated with Unasyn in the usual dose, but after a week of therapy the symptoms did not subside. Unasyn was changed to Avelox, which showed equally good sensitivity *in vitro*. After fourteen days of treatment, tachycardia and severe headaches occurred and Avelox was discontinued. After three days of withdrawal, the difficulties returned completely. Re-culture of ejaculate and expressed prostatic secretion again showed *E. faecalis* with practically the same MIC values (Fig 1, Micro 10). The patient was given Unasyn, i.v., in a total dose of 4.5g/day. The therapy lasted 21 days with complete relief of the difficulties. After three weeks of withdrawal, the difficulties returned again. The culture of the ejaculate and prostatic secretion carried out for the third time after massage again showed probably the same strain of *E. faecalis*, but with higher MIC mg/l values (AMP 2, VAN 4, CIP 1U, TET ≥16, GEN 128, Fig 1, Micro 11).

Based on these results, biofilm infection appeared to be the most likely explanation for disease recurrence. Biofilm eradication requires long-term treatment with high doses of antibiotics. In the next course of treatment, Linezolid 600mg twice a day was chosen in combination with Ampicillin 2g every 6 hours for the first week. In the second week, the combination of Linezolid 600mg twice a day with Fosfomycin 8g every 8 hours was continued. For the third and fourth weeks, he received monotherapy with Fosfomycin 8g every 8 hours. After this treatment, the disease subsided and did not recur for a year. During this period the patient underwent treatment by enterococcal vaccine from his own strain of enterococcus.
In June 2020, there was another recurrence of the disease in the sense of urgency and pelvic pain. Culture of the ejaculate and prostatic secretion after the massage again showed *E. faecalis* with an MIC of AMP 2. Furthermore, the clinical microbiology laboratory reported that the *E. faecalis* isolates were resistant to a variety of antibiotics including Oxacillin, Cefoxitin, Gentamicin and Tetracycline (Fig 1, Micro 12). The patient was started on treatment with Unasyn 750 mg twice a day. After a temporary improvement, the condition worsened from the fifth day of treatment. Potential biofilm formation was considered as the main cause of Unasyn's failure. Due to the intolerance to quinolones and the previous eradication of the pathogen with fosfomycin, the drug of choice was Fosfomycin 8g three times a day intravenously for 21 days, followed by Fosfomycin 3g per day orally for 7 days, i.e. a total of 28 days. After this treatment, the difficulties subsided. After two weeks, however, the difficulties recurred and again, enterococcus was cultivated from ejaculate (Fig 1, Micro 13). Considering that the patient received the highest possible doses of Fosfomycin for 21 days, and was intolerant to quinolones, we proceeded to a combination of bacteriophages (Sekstafag 20ml rectally twice a day) and Unasyn 750mg twice a day *per os* for two weeks without any clinical effect. Furthermore, a new bacteriophage cocktail against patient’s own strain in a concentration of $10^9$pfu/ml, with convincing *in vitro* activity, had been prepared in the Science Park Bratislava. The cocktail contained three newly isolated phages (vB_Efa_VP14, vB_Efa_VP15, vB_Efa_VP16) belonging to the genus Efquatrovirus (data not shown). Each of them had a unique host specificity and efficiently lysed the patient's strain. The patient continued to use this cocktail for the next two weeks, 10 ml
twice a day applied rectally, without any clinical effect. In the September 2020 patient started to take Linezolid 600mg twice a day for 21 days.

The lack of improvement of the patient’s condition prompted a search for an alternative therapy to the antibiotic treatments previously employed. In Sept 2020 a request was sent from Slovakia to the Stevens laboratory at Temple University, Philadelphia, for an E. faecalis-specific bacteriophage or a phage-based lytic enzyme that could be capable of degrading an E. faecalis biofilm. The laboratory had both: Previously, a genetically engineered derivative of E. faecalis phage (ϕEf11) had proven in in vitro testing to infect many strains of E. faecalis, reduce the populations of E. faecalis cultures, and drastically disrupt E. faecalis biofilms. However, the genetically modified phage [ϕEf11/FL1C(Δ36)PrnA] possessed a nisin-dependent promoter (PrnA) that required the presence of nisin, as a cofactor for activation. While this was advantageous for controlling phage activity in in vitro experimental conditions, it would not be suitable for in vivo, clinical application. However, a ϕEf11 phage gene that codes for an endolysin had been cloned and expressed. The purified endolysin protein (ORF28 lysin) exhibited rapid and profound lysis of cells of most E. faecalis strains, including those that were antibiotic-resistant. The E. faecalis strain isolated from the CBP patient was sent to the Stevens laboratory for sensitivity testing against the ϕEf11 phage ORF28 endolysin. Spot testing of dilutions of the purified endolysin on lawns of the CBP E. faecalis isolate revealed that this strain was indeed extremely sensitive to the lytic action of the lysin (Fig. 2).
Considering the pronounced lysin-sensitivity of the *E. faecalis* strain isolated from the patient, it was not unreasonable to entertain the possibility that the lysin might have a beneficial effect in controlling the patient’s infection. Consequently, Temple University’s IRB was consulted concerning the appropriateness of the clinical application of the phage lysin. The Temple IRB officers, in turn, consulted with the FDA to determine whether there were any regulatory issues in terms of the lysin’s clinical use. It was determined that due to the fact that the lysin was to be used in Slovakia and not in the USA, it “…was not an FDA issue” and the US FDA did not “…have purview over the activity”. Furthermore, because the lysin was to be used “for treatment-not research” we were informed that the IRB did not need “…to be further involved in this matter”. In terms of the regulatory requirements of Slovakia, we were informed that (1) bacteriophages and their products are considered as an alternative to antibiotics and (2) the clinical use of bacteriophages and their products is solely governed by the expert opinion of the attending physician or consultant. In this case, the expert opinion of an infectious diseases specialist was obtained, concluding that the patient was suffering from “…a biofilm infection which can no longer be eradicated with current antibiotics.” and that “…therapy with bacteriophage lysine (sic) as a last resort in the treatment of refractory bacterial prostatitis”. Therefore,

Fig. 2. Spot testing sensitivity of *E. faecalis* strain (587A2) from the CBP patient to purified phage ORF28 lysin. Dilutions of purified lysin (original concentration = 800 μg/ml) spotted onto a soft-agar lawn of *E. faecalis* 587A2. Lytic zones observed after overnight incubation @ 37° C. Numbers towards center of plate indicate the concentration (μg/ml) of ORF28 applied in each section of the plate. Lytic zone observable down to lysin concentration of 0.4 μg/ml.
with no additional regulatory requirements to be satisfied, purification of the phage lysin was initiated. SDS-PAGE analysis of the affinity-purified ORF28 lysin revealed a single protein band with a molecular mass of 46.1 kDa, which is the predicted size of the ORF28 lysin protein (Fig. 3). The patient was fully informed about potential risks and benefits of the treatment and signed an informed consent and approved the publication of his course of treatment.

The sterile, purified lysin preparation was sent to Slovakia in November of 2020 however at that time, the patient was already asymptomatic due to linezolid therapy. During the year 2021 the patient experienced several attacks of prostatitis usually treated with Unasyn and Levofoxacin respectively. Furthermore in 2021, the patient underwent whole exome sequencing (WES) analysis in the Department of Medical Genetics, Medical
University of Warsaw, Poland. WES revealed a heterozygous variant in *MBL2* gene (hg38, chr10:g.052771482-G>A, NM_000242.3: c.154C>T/ p.(Arg52Cys), rs5030737), which refers to D-allele and HYD haplotype (patient’s genotype A/D and haplotype HYA/HYD). The p.(Arg52Cys) variant is described as “pathogenic” according to ClinVar database (Accession: RCV000015426.29) in relation to Mannose-binding lectin (MBL) deficiency (MIM#614372). Subsequent immunological examination showed a reduced level of MBL to 375 ng/ml (normal value more than 2880).

By November of 2021, the prostatitis worsened, with more intense symptoms in terms of urgencies, nycturia and pelvic pain, and culture of the ejaculate again confirmed *E. faecalis* (Fig 1, Micro 14). For this, the patient began applying probiotics consisting of lactobacilli and the above-mentioned bacteriophage cocktail (10⁹ pfu/ml) against his own strain, which he continued for the next two weeks, 20ml rectally twice a day, however the symptoms continued to worsen. The patient was then again put on Unasyn/ampicillin-sulbactam (750mg twice a day orally) and the condition began to improve, however after 4 days, the patient experienced a severe allergic reaction consisting of whole body itching and exanthema. This necessitated the discontinuation of the Unasyn treatment and its replacement by Fosfomycin 3g once a day orally. Again, a similar severe allergic reaction (whole body itching and exanthema) ensued, and the Fosfomycin also had to be discontinued. At this point the patient applied the phage endolysin preparation, which had been stored refrigerated since its arrival (prior laboratory studies demonstrated that the lysin was extremely stable, and could retain its activity for several years, if kept refrigerated⁵⁷). The preparation was diluted 1:5 in saline, and two doses of 5ml were applied rectally 12 hours apart. After the administration of the two doses, the symptoms
subsided, but the patient did not have additional doses available, so he could not continue the lysin therapy.

During the year 2022, the condition fluctuated, in July 2022 there was a significant recurrence, and culture of the ejaculate again confirmed *E. faecalis* (Fig 1, Micro15). Total CPSI score at this point was 24 (a pain score of 7, a urinary symptom score of 8, and a quality-of-life impact score of 9). In this case, additional lysin received from the Stevens laboratory was immediately applied in the same dose (1ml of lysin diluted 1:5 in saline and applied 5ml rectally every 12 hours for 7 days) with significant clinical improvement and bacteriological eradication of enterococcus. The total CPSI score at this point was 6 (a pain score of 0, a urinary symptom score of 3, and a quality-of-life impact score of 3. After completing the treatment, culture of the ejaculate was sterile, and the expressed prostatic secretion contained only coagulase-negative staphylococci (Fig 1, Micro 16). The patient reported no untoward reactions during or after the lysin treatment with any of the administrations.

**DISCUSSION**

Chronic prostatitis (chronic pelvic pain syndrome and chronic bacterial prostatitis) is a chronic recurrent disease with a very complex etiology and pathogenesis, which significantly reduces the quality of life of patients. The biggest mystery still remains to answer the question of why only some men develop the disease and others never experience it in their lifetime, while leading the same life. The effort to find an answer to this question is very modest in the literature. However, its clarification is of key importance for prevention, therapy, and prognosis for each individual patient. Predisposing factors
are usually hidden immunological deficits, which are manifested by increased susceptibility of the urogenital tract to bacterial invasion. More than 80% of all chronic prostatitis begins as a more or less symptomatic urethritis with a transition to prostatitis and with delayed or insufficient treatment with its chronification and transition to chronic pelvic pain. *E. faecalis* accounts for more than two-thirds of CBP cases. Its natural property is the formation of a biofilm in acini or around prostatic calculi, which protects it from the impact of antibiotics. Repeated administration of antibiotics is associated with a high risk of cumulative toxic effect and allergic reactions. Therefore, the search for alternative approaches is the right way forward. Immunotherapy and alternative antimicrobial therapy are becoming the main directions, mainly in cases of recurrent or refractory CBP. Endolysin therapy is a novel strategy to combat otherwise untreatable infection. Next, the diagnosis of the underlying causes of chronic prostatitis plays a key role. As a rule, these are congenital or acquired immunodeficiencies. From this point of view, a thorough search for the primary cause of the disease and its discovery can lead to the cure of the patient, or to the mitigation of the course of the disease.

In our article, we present a case report of a 39-year-old man with chronic pelvic pain on the basis of chronic recurrent prostatitis. The case report points to the torpidity of the disease, the chronic relapsing nature of the disease and the failure of antibiotic therapy. In terms of diagnostics, whole exome sequencing played a key role, which revealed the MBL2 gene polymorphism with genotype A/D instead of wild type AA and subsequent immunologic testing confirmed MBL2 deficiency. Considering this, the search for alternative treatment options and an individual approach to each patient should be part of the treatment. From the point of view of alternative strategies, the following can be
stated: Bacteriophages in any form did not show any effect. The cause is probably very problematic penetration of the bacteriophages into the prostate. Immunotherapy has potential, but it needs to be "tailored" for each patient. We perceive the most significant impact on the course of the disease was due to the application of bacteriophage endolysin rectally, which resulted in the elimination of enterococcus and a long-term asymptomatic period.

This report demonstrates the utility and efficacy of bacteriophage endolysin therapy in treating recalcitrant infections; particularly those due to drug-resistant bacteria or in cases where effective antibiotic treatment is precluded due to serious adverse reactions. Even though the lysin showed excellent efficacy against enterococcus in vitro, the most important question we asked ourselves was whether it can penetrate through the rectal mucusa and reach the prostatic tissue and acini. Based upon our clinical findings, a dose at least above the MIC was reached, which permitted the eradication of the chronic recurrent infection. Evidence of penetration of the endolysin into the prostate tissue after rectal application remains a significant question. We also do not know whether endolysin passes into the ejaculate. In the ejaculate it could be possible to demonstrate the endolysin by means of antibodies, mass spectrometry, or microbiologically through antimicrobial activity of the ejaculate. We intend to provide such evidence in future research. Antibodies against the endolysin are not yet available, and tissue proteases could possibly degrade the endolysin.

Antibiotic therapy is the conventional standard of care for treatment of chronic bacterial prostatitis. In the present case, conventional therapy using antibiotics, was ineffective.
due to the development of resistance to many antibiotics by the \textit{E. faecalis} strain causing the infection, and the intolerably severe allergic reactions caused by the antibiotics that were used. Another potential impediment to antibiotic treatment is the biofilm nature of prostate infections. That biofilms can form within the infected prostate was shown by Nickel and Costerton\textsuperscript{64} and Arakwa et al\textsuperscript{65}, and it has been well established that biofilm bacterial populations are relatively resistant to the antimicrobial effects of antibiotics\textsuperscript{66}.

Phage therapy has been proposed as a useful alternative to antibiotics. In this regard, several studies have reported the successful use of phage therapy in treating cases of CBP\textsuperscript{44,47,67}. A personalized bacteriophage cocktail that was active (\textit{in vitro}) against the patient’s own strain of \textit{E. faecalis} was used in treating the patient’s infection. In light of the prior reports of successful phage therapy in treating chronic bacterial prostatitis, it is not clear why this was not effective in the present case. A possible explanation is the insufficient access of the phages to the prostate by rectal application of the preparation.

The efficacy of the ORF28 lysin for controlling this patient’s \textit{E. faecalis} infection was presumably due to the ability of the lysin to lyse and kill the infecting organisms. Previous \textit{in vitro} data demonstrated that the ORF28 lysin caused rapid and profound lysis of sensitive \textit{E. faecalis} strains\textsuperscript{57}, and that the patient’s \textit{E. faecalis} strain was shown to be sensitive to the lysin. Thus, it was not unreasonable to anticipate a beneficial effect of the lysin on the patient’s \textit{E. faecalis}-infected prostate, provided that the lysin could gain access to the infected prostate. Anatomic studies disclose that the rectal venous plexus/hemorrhoidal plexus communicates with the prostatic venous plexus via the vesical venous plexus\textsuperscript{68}. This could provide an entrée for rectally-applied lysin to the
prostate. In animal studies involving mice and rabbits, rectally-applied bacteriophages could be detected in the circulation within minutes\textsuperscript{69}. Therefore, the positive outcome that we observed is consistent with both the activity of the phage lysin, and its potential availability to the site of infection.

**METHODS:**

**Phage cocktail preparation:**

The three phages were isolated from wastewater samples collected from different wastewater treatment plants in the Bratislava region on a bacterial strain isolated from a patient. Ten milliliters of wastewater, sterilized by passage through a 22 μm filter, was mixed with the same volume of twofold concentrated Trypticase Soy Broth medium and 200 μL of overnight bacterial culture. The inoculated mixture was cultivated overnight at 37 °C by shaking. Single phage clones were obtained through three repeated isolations from single plaques on double agar followed by precipitation in 10% PEG6000 and 1 M NaCl and subsequent ultracentrifugation in CsCl gradient. The visible phage band was collected (~1.5 mls), and each phage sample was dialyzed against 1 liter of SM buffer (100 mM NaCl; 8 mM MgSO\textsubscript{4}; 50 mM Tris-HCl, pH 7.5; 0.002% gelatin) four times for a minimum of six hours each. This yielded 2 mls of phage with titers of 10\textsuperscript{11} – 10\textsuperscript{12} PFU/ml. For cocktail preparation, 1ml samples were combined to form a three-phage cocktail, each at 10\textsuperscript{9} PFU/ml. The isolated phages were preserved long-term in SM buffer at 6 °C for no more than 12 months, however the cocktail batches were prepared monthly.

**Phage lysin production and purification:**
The production and purification of the phage lysin was accomplished as described previously\textsuperscript{57}. In brief, our laboratory’s previous work on the ORF28 lysin resulted in the creation of an *E. coli* BL21/DE3 strain harboring a recombinant pGEX4T2 expression vector containing the ORF28 lysin gene linked to a glutathione S-transferase (GST) affinity tag. The plasmid also featured an ampicillin resistance marker and an isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG)-inducible *tac* promotor. Expression of the linked ORF28 lysin and GST genes to produce an ORF28-GST fusion protein, was then induced by growing the transformed *E. coli* strain in the presence of IPTG. A sonic extract (SE) was made from the induced *E. coli* culture. The SE was applied to a glutathione resin affinity column, and, after nonadsorbed SE material was eluted, the column was extensively washed with buffer to further remove any non-bound material. The ORF28 lysin-GST fusion protein (bound to the glutathione of the column via the GST) was specifically desorbed from the column by the addition of a buffer containing glutathione. The process was repeated 4 times until only two protein bands (representing the ORF28-GST fusion protein and the GST protein alone) could be seen by SDS-PAGE analysis of the desorbed material. The ORF28 lysin protein was recovered from the ORF28-GST fusion protein by reapplying the purified fusion protein to the affinity column and digesting the bound fusion protein with thrombin to cleave the thrombin-sensitive linkage between the ORF28 lysin and the GST protein. The liberated ORF28 protein was then eluted from the column and collected. The homogeneity of the affinity-purified ORF28 protein was evaluated by SDS-PAGE analysis. As a final step in the purification process, the electrophoretically-homogeneous ORF28 lysin preparation was passed through a
sterilizing filter. The final purified lysin preparation had a protein concentration of 0.8mg/ml.

**Spot testing lysin activity**

Spot testing was used to examine the activity of the ORF28 lysin against the *E. faecalis* strain isolated from the patient. 0.1 ml of an overnight culture of *E. faecalis* strain 587A2 grown in Brain Heart Infusion (BHI) Broth was inoculated into 3 ml of molten soft agar (BHI broth containing 0.7% agar). This was poured into plates over a layer of BHI agar (1.5%) and allowed to solidify and dry for approximately 15 minutes. Drops (3 μl) of dilutions of an ORF28 lysin suspension were then applied to the surface of the solidified soft agar layer. The drops were allowed to dry into the soft agar layer, and the plates incubated overnight at 37° C. The plates were then examined for clear zones where the drops were originally placed, indicating the lytic activity of the lysin against the *E. faecalis* strain.

**Whole exome sequencing (WES) analysis:**

(Department of Medical Genetics, Medical University of Warsaw, Poland). A library was prepared using the Human Core Exome Kit (Twist Bioscience, South San Francisco, CA, USA), according to manufacturer’s instruction, and paired-end sequenced (2 × 100 bp) on a NovaSeq 6000 platform (Illumina, San Diego, CA, USA). Bioinformatic analysis of raw WES data and variants prioritization were performed as previously described.

REFERENCES


