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Application of microbiological quantitative methods for evaluation of changes in the amount of bacteria in patients with wounds and purulent fistulas subjected to phage therapy and for assessment of phage preparation effectiveness (*in vitro* studies)

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ABSTRACT

Purpose: Quantitative microbiological studies may provide important information required for successful phage therapy (PT), however methods for PT monitoring of purulent wounds and fistulas has never been reported before. Therefore our goal was to determine and apply microbiological quantitative methods (MQMs) for monitoring experimental PT.

Methods: Samples from agar plates with growing bacteria were collected using dry and wet sterile compresses, or swabs. After shaking the sample in saline the amount of bacteria in suspension was determined. The method was standardized. The MQM using compress was applied for comparison of in vitro activity of phage preparations with other agents for wound rinsing. The usefulness of this swabbing method was tested in the Phage Therapy Unit for monitoring of experimental PT of patients with chronic wounds or purulent fistulas.

Results: Minimum, maximum and standard deviation values used for standardization of the studied method showed that data repeatability was good; thus the method was used for quantitation of bacteria taken both from plates in vitro and patients samples.

Effectiveness of phage preparations was compared to gentamicin in vitro. Phages were as effective as antibiotics in reducing the amount of bacteria on agar plates, and this effect was not only due to simple mechanical removal of bacteria, but dependent on their antibacterial activity. We have also observed that the results of bacteria quantitation may correlate with the local status of a wound/fistula in a particular stage of PT.

Conclusion: The standardized swabbing method of bacteria quantitation can be used for PT monitoring. Presented MQMs are simple and may help to monitor the therapy process and to decide on its duration, frequency and a kind of the phage applied. They can also be applied in other antibacterial treatment strategies.

Key words: Bacteriophages, Phage preparation, Phage therapy, Wound infection, Purulent fistula

INTRODUCTION

Bacteriophages (phages) are viruses of bacteria that can kill and lyse the bacteria they infect. After their discovery in the early 20th century, phages were used to treat bacterial diseases in people and animals. At present, increasing levels of antibiotic resistance constitute one of the main problems in medicine, so phage therapy (PT) became an alternative way to eliminate bacterial infections [1,2]. Only a few institutions in Eastern Europe continue to study and use phages as

therapeutic agents for human infections (including the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, IIET) [2]. One of the best known centers of bacteriophage study and production of phage preparations for the treatment and prophylaxis of human infections has been the George Eliava Institute of Bacteriophages, Microbiology and Virology in Tbilisi, Georgia. Both centers published many papers on successful clinical application of PT [3-5]. Recently the results of standardized controlled clinical trials on PT safety as well as effectiveness done by other groups were also published [6-9].

The Phage Therapy Unit (PTU) at the IIET has extensive experience in the bacteriophage treatment of patients suffering from chronic bacterial infections in which standard antibacterial treatment was ineffective [5]. Our recent studies have confirmed that phages can be highly effective in treating many different types of bacterial diseases. Specificity of phages and the safety of applying phages make them efficacious antibacterial agents [5,10,11] that may also be less expensive than antibiotic treatment [12].

In the Bacteriophage Laboratory of the IIET that cooperates with PTU, monitoring of the PT is carried out such as phage typing of pathogenic strains isolated from patients. The quantitative monitoring of bacteria during PT has been introduced by the Laboratory since 2009. The justification for these studies comes from our previous observations revealing that a number of patients, although treated with active preparations, did not improve as expected. PTU is an ambulatory care unit, so the best possible and safe way of collecting patients' material was the use of sterile cotton swabs or compresses (as 'stamps').

Typical quantitative methods for determination of the amount of bacteria in wounds are: wet compresses, wet swabs, microscopy (smear of swabs or homogenized tissue obtained from biopsies), Gram staining of wound smears, and others [13-15]. Cotton swabs are usually considered as possible sampling devices, but considered to provide semi-quantitative quantification [13-16]. There is no general agreement as to the results of comparison between wound tissue samples and bacterial swabs. [15,17,18]. Nevertheless, most of these comparative studies were conducted in animal models, which allow for a large number of probes to be performed. These methods, although well accepted and reliable, are difficult to transfer to human patients. Currently, quantitative biopsy is considered as the most reliable method for assessing of infection dynamics, because it allows investigators to determine the number of bacterial cells per gram of infected tissue [19]. Usually, biopsy of infected tissues alone, or pairing of biopsy and swabs, is considered the most reliable. Nevertheless, when taken from various areas of a wound, neither swabs nor biopsy are very likely to give the same results. This probability was estimated at approx. 50% (for both swabs and biopsy) [15]. Additionally, swabs offer a larger, i.e. more complete, spectrum of aerobic bacterial species than in biopsy. However, in a large wound the biodiversity of microorganisms on its surface and underneath may be impossible to detect, whether with swabs or with biopsy [13]. Some bacterial strains are less likely to be detected by biopsy than by swabs [16]. Interestingly, in the case of *Staphylococcus aureus* its detection by cotton swabs on the surface is connected with the presence of these bacteria in the deeper tissue [17]. Of course, technical details of swabbing are important for the result, which also correlates with the tendency for analyzing all samples in the same laboratory [16,17].

Histological methods, although probably the most reliable for monitoring of the infected wound, can be used only in high-specialized health care units and they are neither universal nor common [13]. Biopsy seems to be highly respected, but as an invasive method it requires special conditions, qualified staff and constant accessibility of a patient as probes have to be collected every 3-4 days. Therefore it is often impossible to apply [13].

The methods applied in our laboratory resemble the quantitative methods by Gardner or Levine [18,20]. Some investigators use only the swab method, mostly because of its lower costs, lower risk of additional infection resulting from the procedure and probable negative results of biopsy [21]. In pneumonia, these kinds of microbiological diagnostics were performed with cut histological brushes [22]. An ophthalmic clinic has developed an individual protocol of eye swabbing [23]. Recently, a quantitative method of bacterial count in the purulent discharge taken from the ear using a cotton swab was used as a one of the parameters to test the efficacy of phage application for treatment of chronic *Pseudomonas aeruginosa* otitis in man during controlled clinical trial [6].

The aim of this study is to propose a simple standardized method using swabs and compresses for estimation of the number of viable bacterial cells present in an infected site, and for monitoring the effectiveness of PT during treatment of the purulent wounds and fistulas with the perspective of other microbiological applications.

MATERIALS AND METHODS

Patients

Patients with different chronic bacterial infections were qualified for phage treatment in PTU under the protocol of therapeutic experiment "Experimental phage therapy of drug-resistant bacterial infections, including MRSA infections". The detailed inclusion and exclusion criteria as well as PT protocol was described by Międzybrodzki et al. [5].

Materials

The method was standardized for *Staphylococcus aureus*, from a clinical sample, since *S. aureus* was the most frequent bacterial species infecting patients' wounds. Bacterial isolates

were stored in culture medium supplemented with 25% glycerol at –70ºC. Bacterial cultures were carried out in COS medium (BioMerieux). Plate cultures were used up to 48 h (maximum 5 passages). Quantitation of bacterial number was carried out with Mueller-Hinton Agar 2 (MH2; BioMérieux) and with two different size: thick (the diameter 5 mm) or thin (the diameter 2.5 mm) sterile cotton swabs (CULTIPLAST, LP Italiana SPA) and sterile cotton compresses 25×25 mm^2 or 30×30 mm^2 (Toruń, Poland). Other materials used included sterile 0.9% NaCl for wetting swabs or compresses and sample dilutions, sterile 30 ml PP flasks. All the materials used had been purchased from qualified companies.

Staphylococcus aureus bacteriophages P4/6409, liz/80 and 676/F were derived from the IIET phage collection. These therapeutic phage lysates (produced by the BIOMED Kraków, Poland) were routinely used in the treatment in PTU. They were prepared as according to modified Ślopek's method [5].

Standardization of the method of collecting samples for quantification of bacteria

Staphylococcus aureus 27821 (clinical strain) culture was prepared at 2.0 MF (McFarland scale) that is 9.0×10^7 CFU/ml. This was calculated based on the dilution method. Three independent samples of 2.0 MF were diluted in sequence from 10^{-1} to 10^{-8}. Each sample dilution was applied (0.1 ml) on an MH2 culture plate, and spread. After 18 h incubation bacterial colonies were counted (when there were 30 to 300 colonies per plate). Calculation was done with regard to the dilution and the final value was an average for three samples.

For standardization of the method for quantification of bacteria using compresses twenty MH2 culture plates were covered with *S. aureus* 27821 (2.0 MF), dried at 37ºC (30 min). Ten compresses were soaked with sterile 0.9% NaCl in sterile PP flasks, while ten other were used dry. Both wet and dry compresses were put on the surface of the culture plates previously covered with *S. aureus* for 10 s (the compresses were evened using a sterile plastic inoculation loop). The same person did the sampling in all cases, to ensure the same pressure on the swab or compress was applied (for repeatability of results). Next, compresses were transferred to sterile flasks with 10 ml of 0.9% NaCl (in the case of wet compresses the rest of the saline left after pre-soaking was applied). After 5 min each sample was vortexed twice for 30 s at 1600 rpm, each time. The number of viable bacteria in these saline samples was counted using the dilution method (from10^{-1} to 10^{-6}). Each sample dilution was applied on an MH2 culture plate in volume of 0.1 ml, and spread. After 18 h incubation bacterial colonies were counted (when there were 30 to 300 colonies per plate). Calculation of bacterial number in sample was done with regard to its dilution and the final value was calculated per square centimeter.

For standardization of the swabbing method, *S. aureus* cultures were prepared as described above. Forty plates covered with bacteria were marked with 1 cm markers. Each of 20 thick swabs or 20 thin ones were rolled left and then right at a 1 cm distance (the contact time was 10 s), immersed into 2 ml of sterile 0.9% NaCl, and then processed identically to compresses. Calculation of bacterial number in sample was done with regard to its dilution and the final value was calculated per milliliter.

Comparing antibacterial activity of phage lysates with other typical means for rinsing bacteria off the surface.

Five *Staphylococcus aureus* clinical strains were selected from the IIET Microbial Collection. *Tab. 1* presents MIC (minimal inhibitory concentration) values, for gentamicin, established with E-tests (BioMerieux) by the Microbiological Department of the 4th Military Clinical Hospital in Wrocław. All these strains were susceptible (had the same pattern of sensitivity) to phages P4/6409, liz/80, 676//F used in the experimental therapy prepared by IBSS Biomed S.A. in Cracow, Poland. These three phages were selected for *in vitro* study due to the best activity against strains isolated from wounds in PTU patients. Water and saline were used as controls to rinse the plate surface to determine the effect of mechanical removal of bacteria from the plate.

Five milliliters of suspensions of a selected staphylococcal strain (2.0 MF) were poured on each culture plate, and excess liquid was removed from the plate. Then plates were dried at 37ºC for 30 minutes. Each sample was tested in triplicate. Control plates were not washed with any agent. Other groups were washed with 5 ml of: sterile saline, sterile deionized water, sage infusion (*Salvia officinalis*), gentamicin (3 mg/ml, 10–15 µg/ml = 10×MIC, 2–3 µg/ml = 2×MIC) or phages in different titers as shown in *Fig. 1.* Washing was done by five round moves during 3 s, then the agent was poured out, and the plate's brim was dried on sterile blotting paper. The plates were incubated at 37ºC for 6 h. The number of bacteria was counted on the plates using the standardized compress method (as described earlier). However in non-diluted samples of gentamicin (3 mg/ml), 10^8 PFU/ml of the phage liz/80, 10^8 PFU/ml of the phage 676/F, 1 ml of sample was put on plate instead of 0.1 ml because the number of bacteria on plates was less than 20 colonies. Each test series was conducted for all five *S. aureus* strains shown in *Tab. 1.* Results were presented as an average from three independent experiments.

Table 1. **MIC values for gentamicin in selected *Staphylococcus aureus* strains.**

Clinical bacterial strains	MIC
Staphylococcus aureus 27929	1.0 µg/ml
Staphylococcus aureus 27935	1.0 µg/ml
Staphylococcus aureus 27941	1.0 µg/ml
Staphylococcus aureus 27947	1.5 µg/ml
Staphylococcus aureus 27956	1.5 µg/ml

Collection of samples from patients for bacteriological quantification tests

The thick or thin swab (depending on the diameter of fistula orifice) was put inside the fistula. If the fistula was dry (with no exudate), the swab was soaked with saline before use. Then it was kept for 10 s, moved round slowly to the right 180º and back to its initial position, and immediately processed in the Laboratory. If the swab contact was not complete, which might be caused by the special characteristics of the fistula (e.g. a stricture just behind the orifice of fistula), this information was recorded in the patient's card to enable all the tests for a particular patient to be done in the same way. Before the study started, a physician was trained at performing the method. All staphylococcal strains isolated from the fistulas were typed with phages.

RESULTS

Standardization of the method of collecting samples for quantification of bacteria in wounds and for in vitro studies

Standardization was done with clinical *Staphylococcus aureus* 27821 strain (2.0 MF: 9×10^7 CFU/ml – an average from 3 samples). We compared different variants: dry versus wet compresses; thin versus thick swabs; or dry versus wet. Ten samples were collected for each group. The results are presented in *Fig. 2*.

The method with sterile compresses was selected and devised both for *in vitro* experiments and for use in patients with extensive wounds. The swabbing method was applied to patients with purulent fistulas and small ulcerations.

In vitro studies: comparison of activity of phage preparation with other agents for rinsing bacteria off the surface

The phage activity in this test was compared with an antibiotic (i.e. gentamicin) and other rinsing agents (*Fig. 1)*. The main criterion for choosing gentamicin was its wide use in the topical wound treatment. Comparative use of water and saline for rinsing the surface of plates was used to demonstrate the specific activity of antibacterial agents not only resulting from simple mechanical removal of bacteria.

Our data showed that rinsing the plates with sterile water, sterile saline, or salvia infusion did not result in a substantial decrease in the amount of bacteria compared to the phage suspensions or antibiotic. Washing with the phage preparation (10^8 PFU/ml) or gentamicin (0.3%, as in the pharmaceutical preparation) resulted in a large decrease of bacteria: up to 7 orders of magnitude. Interestingly we have observed that rinsing the plate with three different phages at the same titer of 10^8 PFU/ml resulted in different efficacy properties towards the five staphylococcal strains tested, although they presented the same sensitivity to the phages in the phage typing

Figure 1. **Standardization of the method of collecting samples for quantification of bacteria in wounds. Minimum, maximum, average and SD values are presented; samples from compresses as CFU/cm², samples from swabs as CFU/ml.**

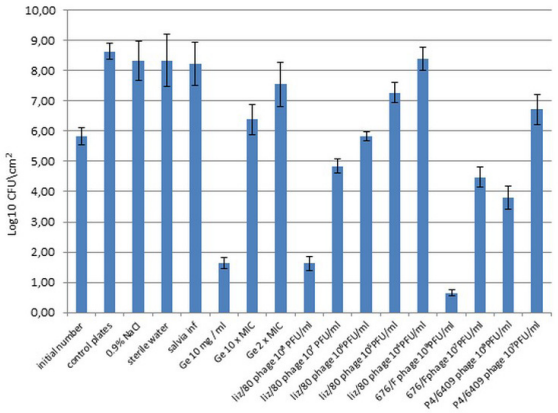
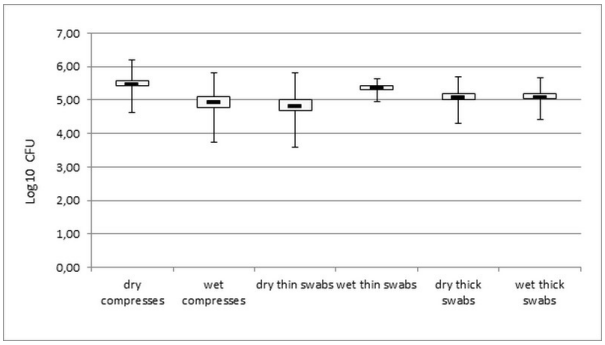


Figure 2. In vitro **comparison of activity of phage preparation with other agents for wound rinsing tested in bacterial culture plates (after 6 h incubation in 37º C).**



procedure, which is routinely applied in our Laboratory. This illustrates the necessity of proper selection of the most active phages. In the same study, we observed the best phage activity with titers of 10^8 PFU/ml and 10^7 PFU/ml (in comparison to 10^4-10^6 PFU/ml). Both observations contribute to important aspects that must be regarded in the PT.

Quantitative microbiological tests during PT

This preliminary study was conducted in the PTU between 2008 and 2010. The results of bacteriological quantification of swabs taken at least once before PT and once during or after PT were available for 10 patients with monoinfection (caused by *S. aureus*, *P. mirabilis*, or *P. aeruginosa*). Patients were monitored as out-patients, under the regimen of therapeutic experiment which is not a controlled clinical trial. They came to the PTU form remote parts of the country at their own expenses which made sampling not always possible in some cases (*e.g.* after the treatment). From this group we selected 6 cases to present in detail (*Tab. 2*). All patients were infected only by *Staphylococcus aureus* – a single bacterial strain was detected. Bacteriophage preparations were applied orally (3 x 10 ml per day) or locally (2-6 ml per dose) as wet compresses or for irrigation of fistulas. Microbiological samples were

Table 2. **Characteristics of six patients subjected to phage therapy (PT) with staphylococcal phages.**

Diagnosis	Right femur infection with a purulent fistula in the postoperative cicatrix: patient 1 Suppurative infection of the hip joint: patient 2 Tibia bone infection with a purulent fistula in the postoperative cicatrix: patient 3 Wound infection after cardiac surgery: patient 4 Tibia bone infection with a purulent fistula in the ankle joint area: patient 5 Facial ulcerations: patient 6			
Pathogen causing infection	*Staphylococcus aureus* (n=6): patients 1-6			
Microbiological sampling site	Fistula (n=5): patients 1-5 Ulceration (n=1): patient 6			
Microbiological sampling method	Swab (n=6): patients 1-6			
Bacteriophage applied	*Staphylococcus aureus* phages, different phages for individual patients, monotherapy (n=6): patients 1-6			
Phage titer[a]	3.65×10^7 PFU/ml – 6.3×10^8 PFU/ml			
Phage application route	Irrigation of the fistula and wet compresses applied on the fistula orifice (n=2): patients 1, and 3 Irrigation of the fistula (n=1): patient 2 Irrigation of the fistula and oral administration (n=1): patient 4 Wet compresses applied on the fistula orifice (n=1): patient 5 Wet compresses applied on the ulcerations (n=1): patient 6			
Phage application frequency	Irrigation of the fistula, 1-2 times daily, (n=1): patients 1-4 Wet compresses, 3-5 times daily, (n=2): patients 5, and 6 Orally, 10 ml 3 times daily, (n=1): patient 4			
Concomitant antibacterial treatment	No other antibacterials applied (n=6): patients 1-6			
Number of bacteria in samples	**Patient number evaluated PT scheme**	**Patient 1** 32 days of PT	**Patient 2** 41 days of PT	**Patient 3** 38 days of PT *break for 63 days* 14 days of PT
	Prior to PT:	1.9×10^3 CFU/ml	3.5×10^2 CFU/ml	2.3×10^3 CFU/ml
	During/after PT:	**Day 32:** 2.6×10^3 CFU/ml	**Day 7:** 5.3×10^2 CFU/ml **Day 42**: 60 CFU/ml	**Day 102:** 1.0×10^2 CFU/ml **Day 115:** 3.0×10^3 CFU/ml
	Patient number evaluated PT scheme	**Patient 4** 42 days of PT *break for 13 days* 22 days of PT	**Patient 5** 33 days of PT *break for 8 days* 33 days of PT	**Patient 6** 32 days of PT *break for 20 days* 55 days of PT
	Prior to PT:	2.0×10^4 CFU/ml	5.0×10^4 CFU/ml	1.5×10^2 CFU/ml
	During/after PT:	**Day 7:** 10 CFU/ml **Day 77:** <10 CFU/ml	**Day 20:** 1.3×10^3 CFU/ml **Day 41:** 1.0×10^3 CFU/ml	**Day 14:** 6.3×10^2 CFU/ml **Day 28:** 1.4×10^2 CFU/ml **Day 81:** 2.0×10^2 CFU/ml
Response to phage treatment[b]	Wound healing (n=1): patient 4 No changes in local status of the fistula (n=2): patients 1, and 3 Clinical improvement (n=3): patients 2, 5, and 6			
Microbiological data for Staphylococcus aureus strains isolated form the patients:				
Sensitivity to phages[c]	Sensitivity to all phages from the test panel (n=4): patients 1, 2, 4, and 6 Sensitivity only to several phages from the test panel (n=2): patients 3, and 5			
Presence of different colony types[d]	2 types with the same phagogram (n=1): patient 1 2 types with different phagogram (n=1): patient 5 3 types with different phagogram (n=1): patient 2 No differences (n=3): patients 3, 4, and 6			
Appearance of phage-resistant strain	Resistance of isolated staphylococcal strain to all phages from the test panel appeared at the end of the second cycle of PT (n=1): patient 3			

[a] *PFU – plaque forming unit*

[b] *In patient 2 the discharge of fistula changed from purulent to serous, and it was associated with improvement of leg mobility and general improvement of well-being, physical activity and reduction of pain observed during PT. In patient 5 the amount of discharge of fistulas gradually decreased, tissue edema and was decreased, and it was associated with reduction of pain. In patient 6 a significant reduction of ulceration dimension had been observed and it was followed by signs of epithelialization.*

[c] *IIET Bacteriophage laboratory test panel is composed with nine Staphylococcus aureus phages.*

[d] *Presence of different colony types prior to the therapy was not associated with appearance of a resistant strain. In samples from patient 3 Staphylococcus aureus strain was without morphological differences.*

collected before and during PT using swabs and the number of bacteria was calculated per 1 ml of the diluent (see: Materials and Methods). All samples from a particular patient were collected in the same way with an identical method. The detailed microbiological characteristic of patients and the results of quantitative microbiological test are presented in *Tab. 2*. A positive effect of PT during evaluated period of PT was observed in 3 cases (patients No. 2, and No. 4-6). In three cases (patients No. 2, No. 4, and No. 5) we could observe decrease in bacterial count in the site of infection (reduction by 1-2 log). In one of them (patient No. 4) wound healing was accompanied by almost complete eradication of bacteria (bacterial titer in the sample dropped from 2.0×10^4 CFU/ml before PT to <10 CFU/ml at the end of treatment).

DISCUSSION

Monitoring of efficacy of treatment against an infection requires an analysis of multiple clinical factors in which microbiological methods are not sufficient. Quantitative methods are only one of many diagnostic aspects. Nevertheless, they are important for antibacterial therapy monitoring [16]. The importance of preparing the detailed method has been noted in numerous articles [15-18,23].

Our studies confirmed that a standardization of simple microbiological quantitative methods using swabs or compresses was possible: minimum, maximum, and SD values (*Fig. 2*) showed that data repeatability was good. Therefore we decided to conduct an *in vitro* study and test the robustness of our methods to determine: the efficacy of phage preparation, gentamicin and other agents used for wound rinsing. Similar studies have been conducted in other scientific units on animal models (e.g. goats or rats with model wounds or fistulas) or technical surfaces covered with bacteria (plastic, cement). In these studies, the sterile rinsing methods were saline, water, Ringer buffer, liquid soap, and various antibiotics (compared). Rinsing was carried out with press-ups, syringes, and pulse-pumps [24-27].

The comparison of phage lysates to other agents for wound rinsing was only possible with the *in vitro* study, which used culture plates covered with bacteria. The mechanical rinsing could not substantially decrease the number of bacteria on a plate lawn, in contrast to phage suspensions or antibiotic, which reduced the number of bacteria by 7 orders of magnitude. We therefore conclude that the activity of the phages tested and a typical antibiotic were comparable. From the data shown in *Fig. 1* it is clear that the efficiency of the removal of bacteria by phage suspension is dramatically increased if the phage concentration is increased from 10^7 to 10^8 PFU/ml. This value is in good agreement with theoretical expectations based on the kinetics of phage adsorption [28-30]. Differences in activity between particular phage preparations were also observed. Two phages, liz/80 phage and 676/F phage, were substantially more effective in decreasing the bacterial number than P4/6409 phage (approx. 3 orders of magnitude).

The Phage Therapy Unit in Wroclaw operates as an ambulatory (out-patient) care center. The results obtained from these *in vitro* studies suggest that the swabbing technique outlined above is a good method of choice for quantitation of bacterial number in fistulas or small wounds. Therefore collection of microbiological samples from patients was done and conducted similarly to the *in vitro* studies. Each patient was treated individually, with an individual schedule: duration of the therapy, number of therapeutic cycles, different phage preparation, administration route, dosage, and daily dose. Individual course of the treatment as well as individual conditions of out-patients resulted in differentiated (between patients) control visits; thus one unified schedule of sampling was not possible. Therefore the sampling time-points were defined as follows: 'prior to the therapy' and 'during/after the therapy' (*Tab. 2*). Phage treatment was conducted mainly externally (compresses, washing of fistulas) as mono-therapy with active phage strains at titer of 10^7–10^8 PFU/ml. Generally we prefer topical mode of phage administration to ensure good phage penetration into the site of infection. In one case, oral phage application was administered due to penetration into wound site not being possible.

One patient was treated orally three times a day with 10 ml of phage preparation. We take into account our Institute's previous experience with the treatment of chronic infections, which shows that in some (chronic) cases improvement can be achieved only as a result of prolonged treatment, therefore our PT protocol is constructed to fit these cases which constitute the significant percentage of PTU patients [5].

We observed that the microbiological data might correlate with clinical status of some patients at a particular moment of the therapy: decrease in the amount of purulent discharge from fistula or wound healing was accompanied by decrease in the number of bacteria in the site of infection. Interestingly microbiological analysis revealed that a phage-resistant bacterial strain was isolated in one patient (*Tab. 2*) during PT. However this may have resulted not from the selection of a phage-resistant strain but from a simple bacterial succession by a new phage-resistant strain in the wound [5].

The data presented herein can be useful for PT development, especially because a large number of patients are treated externally, *in situ*. High titers of phages appear to be necessary for good results of the treatment.

CONCLUSIONS

Our results showed that the method of swabbing with microbiological quantitation can be used to study the amount of bacteria in wounds and microbiological data may correlate with the clinical status of a wound in a particular stage of

antibacterial therapy. However other bacterial strains should be verified for this correlation in further studies on larger groups of patients. The results of microbiological studies, including quantitative methods, offer important practical information for quantifying PT results. They help to evaluate the efficacy of therapy and may have an effect on therapy duration, frequency and kind of phage applied. Presented microbiological quantitative methods are simple and can be used in most microbiological laboratories.

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REFERENCES

1. Duckworth DH, Gulig PA. Bacteriophages: potential treatment for bacterial infections. BioDrugs. 2002;16(1):57-62.

2. Górski A, Miedzybrodzki R, Borysowski J, Weber-Dabrowska B, Lobocka M, Fortuna W, Letkiewicz S, Zimecki M, Filby G. Bacteriophage therapy for the treatment of infections. Curr Opin Investig Drugs. 2009 Aug;10(8):766-74.

3. Kutateladze M, Adamia R. Phage therapy experience at the Eliava Institute. Med Mal Infect. 2008 Aug;38(8):426-30.

4. Kutateladze M, Adamia R. Bacteriophages as potential new therapeutics to replace or supplement antibiotics. Trends Biotechnol. 2010 Dec;28(12):591-5.

5. Międzybrodzki R, Borysowski J, Weber-Dąbrowska B, Fortuna W, Letkiewicz S, Szufnarowski K, Pawełczyk Z, Rogóż P, Kłak M, Wojtasik E, Górski A. Clinical aspects of phage therapy. Adv Virus Res. 2012;83:73-121.

6. Wright A, Hawkins CH, Anggård EE, Harper DR. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant Pseudomonas aeruginosa; a preliminary report of efficacy. Clin Otolaryngol. 2009 Aug;34(4):349-57.

7. Rhoads DD, Wolcott RD, Kuskowski MA, Wolcott BM, Ward LS, Sulakvelidze A. Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. J Wound Care. 2009 Jun;18(6):237-8, 240-3.

8. Bruttin A, Brüssow H. Human volunteers receiving Escherichia coli phage T4 orally: a safety test of phage therapy. Antimicrob Agents Chemother. 2005 Jul;49(7):2874-8.

9. Sarker SA, McCallin S, Barretto C, Berger B, Pittet AC, Sultana S, Krause L, Huq S, Bibiloni R, Bruttin A, Reuteler G, Brüssow H. Oral T4-like phage cocktail application to healthy adult volunteers from Bangladesh. Virology. 2012 Dec 20;434(2):222-32.

10. Miedzybrodzki R, Fortuna W, Weber-Dabrowska B, Górski A. A retrospective analysis of changes in inflammatory markers in patients treated with bacterial viruses. Clin Exp Med. 2009 Dec;9(4):303-12.

11. Fortuna W, Miedzybrodzki R, Weber-Dabrowska B, Górski A. Bacteriophage therapy in children: facts and prospects. Med Sci Monit. 2008 Aug;14(8):RA126-32.

12. Miedzybrodzki R, Fortuna W, Weber-Dabrowska B, Górski A. Phage therapy of staphylococcal infections (including MRSA) may be less expensive than antibiotic treatment. Postepy Hig Med Dosw (Online). 2007 Aug 3;61:461-5.

13. Holder JA. Nonusefulness of quantitative wound cultures. Clin Microbiol Newsletter. 1997; 19(6):45-48.

14. Kowalska-Krochmal B. Znaczenie badan mikrobiologicznych u pacjentow z ranami przewleklymi. Diagnosta Laboratoryjny. 2008 Apr-May;1(16):10-12. Polish.

15. Steer JA, Papini RP, Wilson AP, McGrouther DA, Parkhouse N. Quantitative microbiology in the management of burn patients. I. Correlation between quantitative and qualitative burn wound biopsy culture and surface alginate swab culture. Burns. 1996 May;22(3):173-6.

16. Dow G. Bacterial swabs and the chronic wound: when, how, and what do they mean? Ostomy Wound Manage. 2003 May;49(5A Suppl):8-13.

17. Gardner SE, Frantz RA, Saltzman CL, Hillis SL, Park H, Scherubel M. Diagnostic validity of three swab techniques for identifying chronic wound infection. Wound Repair Regen. 2006 Sep-Oct;14(5):548-57.

18. Levine NS, Lindberg RB, Mason AD Jr, Pruitt BA Jr. The quantitative swab culture and smear: A quick, simple method for determining the number of viable aerobic bacteria on open wounds. J Trauma. 1976 Feb;16(2):89-94.

19. Buchanan K, Heimbach DM, Minshew BH, Coyle MB. Comparison of quantitative and semiquantitative culture techniques for burn biopsy. J Clin Microbiol. 1986 Feb;23(2):258-61.

20. Gardner SE, Frantz RA, Hillis SL, Park H, Scherubel M. Diagnostic validity of semi-quantitative swab cultures. Wounds. 2007; 19(2):31-38.

21. Landis SJ. Chronic wound infection and antimicrobial use. Adv Skin Wound Care. 2008 Nov;21(11):531-40; quiz 541-2.

22. Kirtland SH, Corley DE, Winterbauer RH, Springmeyer SC, Casey KR, Hampson NB, Dreis DF. The diagnosis of ventilator-associated pneumonia: a comparison

of histologic, microbiologic, and clinical criteria. Chest. 1997 Aug;112(2):445-57.

23. Miño de Kaspar H, Kreutzer TC, Aguirre-Romo I, Ta CN, Dudichum J, Bayrhof M, Klauss V, Kampik A. A prospective randomized study to determine the efficacy of preoperative topical levofloxacin in reducing conjunctival bacterial flora. Am J Ophthalmol. 2008 Jan;145(1):136-42.

24. Owens BD, Wenke JC. Early wound irrigation improves the ability to remove bacteria. J Bone Joint Surg Am. 2007 Aug;89(8):1723-6.

25. Bahrs C, Schnabel M, Frank T, Zapf C, Mutters R, von Garrel T. Lavage of contaminated surfaces: an in vitro evaluation of the effectiveness of different systems. J Surg Res. 2003 Jun 1;112(1):26-30.

26. Brown LL, Shelton HT, Bornside GH, Cohn I Jr. Evaluation of wound irrigation by pulsatile jet and conventional methods. Ann Surg. 1978 Feb;187(2):170-3.

27. Conroy BP, Anglen JO, Simpson WA, Christensen G, Phaup G, Yeager R, Gainor BJ. Comparison of castile soap, benzalkonium chloride, and bacitracin as irrigation solutions for complex contaminated orthopaedic wounds. J Orthop Trauma. 1999 Jun-Jul;13(5):332-7.

28. Abedon ST, Thomas-Abedon C. Phage therapy pharmacology. Curr Pharm Biotechnol. 2010 Jan;11(1):28-47.

29. Letarov AV, Golomidova AK, Tarasyan KK. Ecological basis for rational phage therapy. Acta Naturae. 2010 Apr;2(1):60-72.

30. Abedon S. Phage therapy pharmacology: calculating phage dosing. Adv Appl Microbiol. 2011;77:1-40.