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Scientific Research

Common Preconditions for Safe Phage Therapy of *Pseudomonas aeruginosa* Infections

V. Krylov, E. Pleteneva, O. Shaburova, M. Bourkaltseva, S. Krylov, E. Chesnokova, O. Polygach

Mechnikov Research Institute for Vaccines & Sera, RAMS, Moscow, Russia
Email: krylov.mech.inst@mail.ru

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Abstract

One of the approaches to the treatment of infections caused by multiply-(MDR) or extra drug-resistant (XDR) pathogenic strains may be application of bacterial viruses (bacteriophages)—phage therapy. Results of a long, but quite limited use of phage therapy in several Eastern European countries, as well as experiments on animal models in Western countries support the possibility to use phage therapy. However, given the role of phages in the evolution of bacterial pathogens, it is necessary to discuss and evaluate negative consequences of mass introduction of phage therapy, as the measures necessary for its safe use. We discuss some actions in case of transiting to world-wide use of phage therapy with purpose to prolong the active life of phage therapy and to diminish possible complications.

Keywords

Phage Therapy, Safety Measures, *Pseudomonas aeruginosa*

1. Introduction

Bacteria of *Pseudomonas aeruginosa* species have very high capability for adaptation and survival in different conditions [1] [2]. They belong to the most frequent opportunistic pathogens causing different hospital infections in people with weakened immune systems, infections of open wounds of different origins and are the inevitable component of the lung microbiota in cystic fibrosis (CF, frequent hereditary disease Caucasians) patients. Prolonged use of a variety of antibiotics to eradicate *P. aeruginosa* has been the reason for arising of MDR (multiple drug resistant) and XDR (extra drug resistant) strains *P. aeruginosa* which put serious problems

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for practical medicine.

Transition of *P. aeruginosa* infection into chronical state in CF patients greatly worsens the prognosis. As a possible approach, a return to the use of bacterial viruses is offered, bacteriophages—phage therapy (PT). There is no doubt of possibility to use specific phages proved by long time use of PT in Russia and Georgia from the 30-es of last age in treatment of infected wounds or hospital infections. There are plenty of fresh publications on bacteriophages, the history of phage therapy and its possible use in practice, in the case of MDR or XDR-strains [3]-[12].

2. *P. aeruginosa* Virulent ("Lytic") Bacteriophages

It is generally accepted that one of the most important advantages of phage therapy is relative simplicity in isolation of new natural or mutant phages for substitution of those ones which have lost their activity after arising of phage resistant bacterial variants. Abundance of different phage species active on *P. aeruginosa* [13] with more than 80 different phages with sequenced genomes available in NCBI database [14] confirms the possibility for choosing the active phages.

But it is necessary to take into attention that phage therapy in countries of Eastern Europe has really a limited application. Thus, there is deep concern that the global introduction of phage therapy in real hospital practice could contribute to the emergence of new, more dangerous diseases because bacteriophages are natural motors in the evolution of bacteria. Development of new approaches in studies of bacterial and phage genomes has revealed close interactions of them in evolutionary process, sometimes with unfavorable final result for humankind. Virulent ("lytic") and temperate phages are capable to transfer separate bacterial genes or pathogenic islands, between different bacterial strains (horizontal gene transfer—HGT), thus creating new highly dangerous pathogens [15]-[18].

Thus, the earlier statements that phage therapy is a simple, cheap procedure, absolutely safe for patient and harmless to the environment and natural microflora can not be more an argument taken without careful considerations. It is evident that the phage therapy can not be complete alternative of antibiotics. Its use is restricted mainly to pyogenic surface infections (infected wounds, urological and gynecological infections, infections of the ear, throat and nose, intestinal infections and abscesses-after their opening and emptying), and, possibly, lung infections in CF patients.

Apparently, right now, before the widespread introduction of phage therapy into medical practice it is necessary to discuss two problems and to look for their solutions.

Firstly, whether PT could be used for a long time, and secondly, how to prevent in course of its use the arising of bacterial strains with features of the so-called "emerging diseases"? Results of practical application in clinical and ambulatory conditions in several countries of Eastern Europe and new experiments with use of animal models support the necessity for its further development and adaptation to new more strict medical standards [19]-[24].

Up to recent time the therapeutic phage mixtures were estimated only on the basis of their lytic spectrum. But now their detailed study, including not only the genome sequencing and annotation, but careful studies of phage-bacterium interactions should be considered as the necessary and sufficient condition for choice of phages for therapy. Phage genomes studies would exclude the use of the phages whose genomes encode such undesirable products as toxins, transposases, repressor proteins, etc. But, unfortunately, it still does not guarantee the safety of phages. Genomes of all newly isolated natural phages contain plenty of genes coding gene products with functions which can not be predicted as result of comparison with existing databases. These genes can control some yet unknown pathogenicity factors. Thus, each phage offered for therapy must be studied in interaction with different strains of sensitive bacteria under different conditions.

Uncontrolled use of multicomponent mixtures of unstudied phages may lead to arising of new pathogenic strains. The resulting strains may be similar to epidemic variants of *P. aeruginosa* which have arisen in CF departments as result of possible interactions of numerous different phages and bacterial strains in the lungs of CF patients [18]. Unlike conventional opportunistic strains of *P. aeruginosa*, such epidemic strains exhibit aggression, displacing the usual strains of *P. aeruginosa*, and at the same time, are highly pathogenic and virulent, what can be cause of pneumonia and death of healthy (non CF) people. As it turned out, the reason for this is the activity of genes in new prophage pathogenic islands, which absent in the *P. aeruginosa* PAO1 laboratory strain (which, although is also a clinical isolate of 60-es, does not cause infections in healthy humans).

In **Table 1** are mentioned 10 species of virulent phages active on *P. aeruginosa* (presented in NCBI, June 2014) for which are available some results of phenogenetic studies.

There are several phage species which are almost compulsory components of real therapeutic mixtures (our unpublished results). The most of the currently used phage mixtures include different phiKZ-like phages [25]-[30]. Bacteria in biofilms of *P. aeruginosa* after multiple infection with phiKZ-like phages enter pseudo lysogenic state and produce large amounts of mucous material obstructing access of phage particles to the bacteria in the wound [29]. Such unusual behavior could not be predicted after the sequencing and annotation phage genomes [27] [28] (**Figure 1**). We have isolated specific mutants of phiKZ-like phages where this feature has been lost [30]. Such mutants should replace wild-type phiKZ-like phages in therapeutic mixtures.

KMV-like phages [31] [32] are the other permanent components of different phage mixtures. Their valuable features are short latent period, good yields and in addition the capability to infect and produce phage in culture of old bacteria in biofilm (which are usually resistant to phages of other species).

Among the other well-studied and perspective phages active on *P. aeruginosa* which usually can be found in commercial mixtures are PB1-like phages [33] [34].

In search for new *P. aeruginosa* therapeutic phages there were isolated several phages with good natural lytic activity, inability to stably lysogenize bacterial cells, but exhibiting unusual features. Thus, genome of phage Luz24 is more than 70% identical to the phage genome of temperate phage PaP3 [35] [36]. Despite of the first

Table 1. Representatives for species of *P. aeruginosa* virulent phages with sequenced genomes (presented in NCBI, June 2014).

№	Species	Family	Number of phages	Genome sizes min - max	№	Species	Family	Number of phages	Genome sizes min-max
1	phiKMV	Podoviridae	14	42,954 - 43,548	6	N4	Podoviridae	2	72,544 - 74,901
2	PB1	Myoviridae	10	64,427 - 66,616	7	119x	Podoviridae	2	43,365 - 43,783
3	PaP1	Myoviridae	6	91,175 - 93,198	8	YuA	Syphoviridae	2	58,663 - 61,167
4	KPP10	Myoviridae	4	88,097 - 88,322	9	phiKZ	Myoviridae	1	280,334
5	Luz 24/PaP3	Podoviridae	4	45,503 - 45,625	10	EL	Myoviridae	1	211,215

(a) (b)

Figure 1. (a) Appearance of *P. aeruginosa* colonies in pseudolysogenic state after phiKZ phage multiple infection; (b) The different growth of phiKZ-like phage EL of wild type (top) and its virulent mutant (bottom).

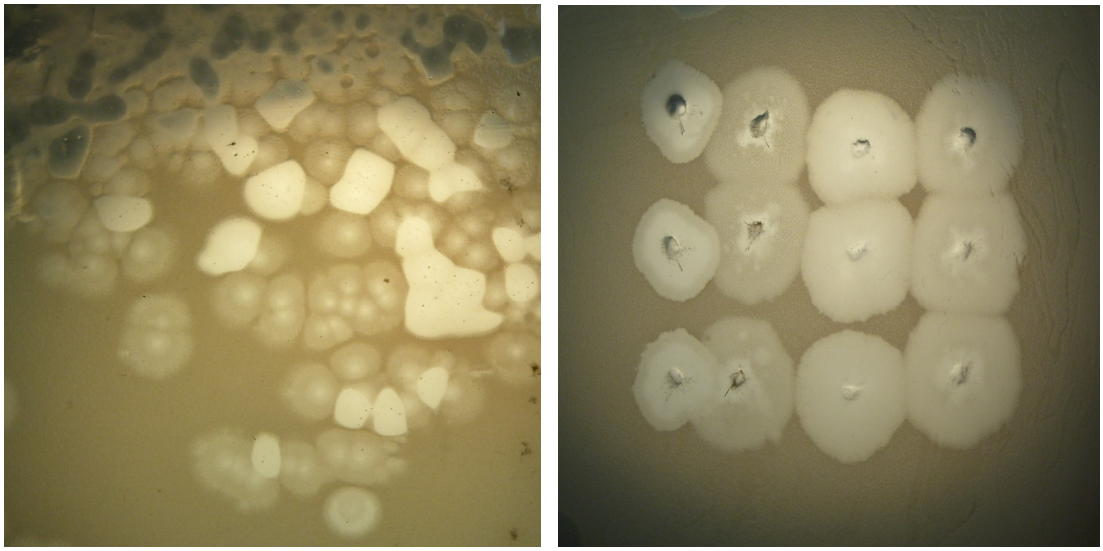
opinion that PaP3 is a temperate one, repressor genes were not found in genomes of both mentioned phages. Thus such phages are regarded as acceptable and good for phage therapy. Nevertheless the phages TL and CHU, closely related with Luz24, reveal a genetic instability being grown on some clinical isolates (**Figure 2**) or specific mutants of standard host strain PAO1 (not shown). The possible reason for the instability is not evident yet.

The phages of other species also reveal some specific features, which may be important in therapeutic use. For example, lytic phage phiPMG1 (closely related with temperate phage D3) [37] [38], is capable to lyse some KMV-resistant mutants. Thus, phiPMG1 could be considered as a component for inclusion in the lytic mixture but however, a comparison of the phiPMG1 phage genome with existing database shows sequences identical to the sequences in D3-like pathogenic island of epidemic strain *P. aeruginosa* LESB58 (**Figure 3**). Obviously, the procedure used now to extend phage lytic activity will select phages which are similar with phiPMG1 with unpredicted final results. Likewise, the same is true for a lytic phage YMC01/01/P52 PAE BP (GenBank: JX403939.1) [39] which is closely related with temperate phage phi297 (GenBank: HQ711984) [40] [41] and has been selected by its ability to lyse a specific antibiotic resistant clinical strain.

Thus, it seems that the the best approach is use of multivalent phage mixtures, because it is not burdensome in the clinic (does not require a preliminary study on the phage sensitivity of pathogenic strain). But their use being acceptable for example, in the treatment of infected wounds, nevertheless can not be considered as universal or safe enough in other cases.

3. Possible Consequences in Use of Phage Therapy

Wide introduction of phage therapy in everyday medical practice (what may be expected in the near future even in case of CF) will increase the extent of HGT, will accelerate bacterial evolution and can lead to arising of *P.*



(a) (b)

Figure 2. Growth of phage CHU on the lawn *P. aeruginosa* 8 - 20 isolate strain. (a) Plating of a single plaque; (b) Pickup plating from different types of plaques. The bacterial strain 8 - 20 is kindly provided by Prof. C. Pourcel.

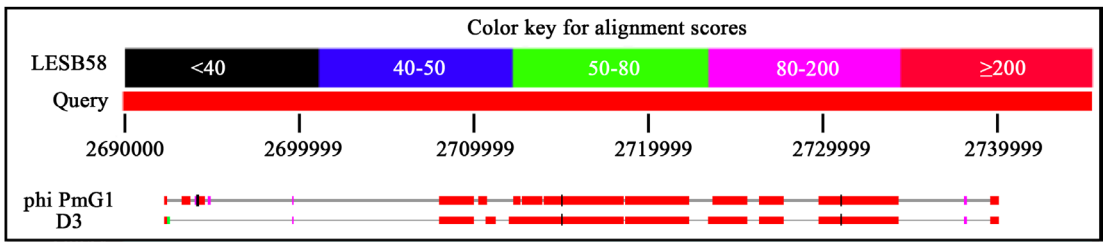


Figure 3. NCBI BLAST alignment of *P. aeruginosa* LESB58 epidemic strain genome fragment (in the region of DNA homology) against phiPMG1 and D3 bacteriophages genome sequences.

aeruginosa epidemic strains in general hospitals. It can be argued that only lytic phages will be used in therapeutic phage mixtures. Yes, of course. However, lytic phages will accomplish general transduction. Moreover, some of the lytic phages have relatedness to temperate phages or carry genes that are functionally similar to the phage repressor genes of temperate phages (see before). Furthermore, phage therapy will be conducted in respect of clinical strains of *P. aeruginosa*, which often contain the same prophages, whose unusual activity was the reason for arising of epidemic variants. Thus, introduction of phage therapy in a real hospital could recreate the same algorithm which led to the emergence of epidemic strains in CF condition. It's hard to say how long it will take.

Interest to phage therapy stimulates search and study of new *P. aeruginosa* phages and some of them increase our knowledge on possible evolutionary events. For example, in [42] has been described a chimeric phage, whose genome have arisen as a result of natural recombination of temperate D3-like phage of *P. aeruginosa* and *Pseudomonas putida* phage AF. Another unusual phage displaying a high level of relationship with transposable phage D3112, as it turned out, can infect *P. aeruginosa* and *Staphylococcus aureus* (!!!) [43]. Naturally, at first glance it seems promising to use such phage in therapy. But it is only at first glance. The use of such phage in complex wound microbiota can lead to unpredictable consequences due to interspecies exchanges between unrelated pathogens. We consider may be it will be better to avoid such actions and accomlish a deep study of such unusual phages.

4. Discussion

What should be taken to prevent such scenario, to avoid *P. aeruginosa* epidemics in hospital conditions and to ensure long and safe use of phage therapy? We propose to discuss the necessity of special organizational preventive and mandatory measures in relation with full introduction of phage therapy into hospital conditions. But one can object-basically such measures already exist as measures to prevent the spread of epidemics. It is right. Their strict application intends to exclude (in principle) the existence of hospital pathogens, however, this is not happening. That means that their compliance is not enough. Indeed, slight air flows or some smallest violations of the existing accepted anti-epidemic measures could lead to undesired phage distribution. And, sure, there is no place for propositions to use phages as antiseptics in hospital conditions [44].

The following measures can be proposed for discussion as obligatory ones in good phage therapy practice (GPTP):

Public relation level

To stop any public promotion of phage therapy preparations as a kind of panacea.

Laboratory level

1) Search for phages which can be propagated on standard laboratory strains (to avoid HGT).

2) Development of *in vitro* in-depth physiological studies of bacteriophages in different conditions simulating real infection (for instance, presence of resident prophages and plasmids, different multiplicity of infection, viscosity, presence of other microbiota components and its phages).

3) Genomes of all phages proposed for inclusion in therapeutic mixtures should be sequenced and annotated to exclude phages coding at least known unacceptable products and properties.

4) Compare adsorption specificity of phages included into the same mixture to increase time interval before arising of multi-phage resistant bacterial strains.

Production level

To give doctors capability to choose the optimal approaches for phage therapy, depending on the specific conditions that it is necessary to produce different sets of phage preparations, including:

1) Sets of individual phages with different spectra of lytic activity for personalized use in clinical settings, including the cases of primary lung infections in CF patients.

2) Traditional broad-spectrum mixtures for use in cases of wound and hospital infections.

Distribution level

To stop distribution of phage therapeutic mixtures without medical prescriptions. Introduce direct delivery and sale of phage preparations in hospitals which are using phage therapy.

Hospital level

The use of phage therapy requires specific conditions. Each hospital participating in use of phage therapy should organize special "Phage Hospital Ward" (PHW), which is safely isolated to prevent accidental spread of

bacteriophages to the other parts of hospital. The PHW should be equipped for quick and reliable disinfection with chemicals and UV irradiation. Personnel hospital clothing must be sterilized after a single use in PHW. Permanent monitoring of the possible spread of phages outside of PHW is compulsory condition for use of PT. Any case of nosocomial *P. aeruginosa* pneumonia (in surgery departments, wound or burn centers, blocks for intensive therapy) must be carefully analyzed to exclude the possible arising of epidemic strain as the reason. Patients with non evident origin pneumonia should undergo treatment in conditions of strict isolation to prevent dissemination of possible epidemic strain.

International cooperation

Exchange of phages and mixtures of phages, additional joint studies, elaboration of common standards for phage therapeutics.

Road by walking!

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