Use of live phages for therapy on a background of co-evolution of bacteria and phages

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This article discusses the prospects and necessary preconditions for wide application of bacteriophages in therapy and prevention of infections. Standard procedures which are necessary to ensure of permanent and safe use of phage therapy everywhere for a long time are discussed. Special attention is given to consideration of principal differences in use of antibiotics and bacteriophages. This includes differences in a range of infections and pathogens, in methods of application, in the need for so-called preclinical testing for phages, in the regional character of phage therapy, in the necessity of organizing of a system for permanent estimation of the activity of phage preparations with purpose to bypass arising of phage resistant mutants. The possible negative role in use of live phages in veterinary and as antiseptics is also discussed. Some probable hypothetical scenarios as result of arising and mass accumulation of multiply phage-resistant pathogens are briefly discussed to stress the importance to remember in the process of usage of phage therapy about the critical role of phages in bacterial evolution.

Keywords: Phages, bacteria, co-evolution, therapy.

INTRODUCTION

During the last decade there were a large number publications in scientific and popular press about necessity to return to use bacterial viruses (bacteriophages) for treatment and prevention of bacterial infections resistant to antibiotics. Most of publications are very optimistic and, indeed, real successes` were achieved in some cases, mostly because of enthusiastic physicians. However, these success stories are limited to only few hospitals in the world.

The idea of Félix d'Herelle to use bacterial viruses in the treatment of bacterial infections ("phage therapy") resulted from the discovery of bacterial viruses in 1917. He found an infectious substance which was able to kill pathogenic bacteria in feces of patient recovering from bacillary dysentery. Félix d'Herelle called it “bacteriophage" (βακτηριοφάγος) (from the Greek "φάω", meaning “eat”). Thus, the discovery of bacteriophages occurred during a natural and lucky (both for the scientist and the first confirmed patient) interaction of bacterial virus and pathogen.

“Félix d'Herelle style phage therapy” - as direct application of live phages isolated from environment, with consideration only for their antibacterial activity, - was used with variable success in Western countries until the introduction of antibiotics into medical practice. Our present knowledge of bacteriophages indicates that the basic reasons for frequently unsuccessful application of phages were a general lack of knowledge of their nature and diversity. Nevertheless, that kind of phage therapy is up to present time still in use in some countries (Russia, Poland, Georgia).

Phage therapy attracted new and widespread attention in the West after the emergence and increasing frequency of diseases caused by multiple drug antibiotic-resistant (MDR) pathogens. Frequently it was presented as a possible alternative to antibiotics. Such view is reflected in newspaper publications, television films, a large numbers of patents, the appearance of specialized phage companies, and numerous review papers on the history of phage therapy, its remarkable potential and the necessity to introduce it into medical practice as soon as possible (Stone, 2004; Merril et al., 2003; http://www.intralytix.com; Chibani- Chennoufi S. et al.,

2004). And now virtually in every, even basic study of bacteriophages as biological subjects, the idea of practical application of phages is dominating as the main rationale for the necessity of their study. I am sure that even if the necessity in the use of phages in F. d'Herelle style therapy would disappear, the significance of fundamental studies of bacterial viruses will not only remain, but arise (especially in relation with growing understanding of their role as potential participants in evolution of the life on the planet Earth).

In many Western publications, phage therapy is described as procedure which is inexpensive, completely safe, simple in application and suitable for treatment of any disease caused by pathogenic bacteria (Kutter et al. 2010; Courchesne et al. 2009; Ceyssens et al., 2009; http://www.professorpatents.com).

This creates a distorted view on the problem for the society as a whole. For example, all publications, which underline the safety and high efficiency of live phage therapy, mentioned the experience of Russia, Poland, and Georgia. However, there is no answer for the obvious question - *why in the above-mentioned countries where phage therapy is officially permitted and specialized enterprises produced therapeutic phages even in the era of antibiotic therapy, nevertheless, commercial phage mixtures had not become yet the dominant anti-infective therapeutic medicine?*

The purpose of this article is to try to answer to these and other questions and to estimate the prospects and possible final results of practical use of live phages in therapy of humans, livestocks and as antiseptics too, while taking in account the active participation of phages in the evolution of bacteria. We are not discussing here in details basic knowledge about phage' biology, evident benefits of phage therapy in humans or use of phages in veterinary. Interested reader can find such data in fresh numerous excellent reviews (Gorski et al.,2009; Duggan et al.,2010; Morello et al.,2011; Connerton et al., 2011; Patel et al., 2011; Maura and Debarbieux, 2011; Gross ,2011; Abedon, 2011; Moradpour and Ghasemian, 2011)

Reasons for the appearance of multi-drug resistant (MDR) bacterial pathogens.

What are the real reasons for emergence of MDR bacterial pathogens? Frequently among such reasons are mentioned the misuse of antibiotics (prescription without real need, inappropriate dosage, use for too long time, administration without appropriate control and accessibility of antibiotics in drugstores without prescriptions), incorrect use of antibiotics in industrial rising of animals for food (Ananda, 2010). It is only part of the truth. The right answers to the question assess to estimate the possible outcomes of future live phage therapy (unless, of course, the latter will grow as rapidly as antibiotic therapy). Soil microorganisms produceantibiotics of different structures and with different mechanisms of action. For instance, B-lactam antibiotics (as penicillin) inhibit bacterial cell wall synthesis, macrolides (as erythromycin), tetracyclines and aminoglycosides inhibit different steps in protein synthesis. Thus, MDR bacteria manifest concomitantly various mechanisms of resistance.

Microorganisms produce the products with antibiotic properties to compete with other microorganisms, including bacterial species, for favorable niches in environment. As result of such competition had arisen soil MDR bacteria (as an example, MDR strains of *Providencia* which could survive in the soil or in intestines of insects, for instance larvae of oil flies living in asphalt seeps (Kadavy et al., 2000), in animal manure (Chander et al., 2006). Thus, it is most probable that non pathogenic bacterial species with gene cassettes carrying genes for antibiotic resistance existed long before appearance of Homo sapiens.

The explosive spread of MDR strains was caused by the simultaneous action of many factors. In my opinion, the most important among them are lifestyle changes of modern society, including:

1) a strong tendency towards urbanization with a simultaneous reduction of rural population and, as result, a quantitative decrease in good quality foods for cities. This was the reason for the appearance of industrial enterprises for mass production of food (broiler and egg-producing factories, closed content of industrial animals). Products of such factories become basic sources for acute intestinal infections in humans (frequently distributed by supermarkets or fast food places);

2) the overcrowded hospitals and low levels of hygienic conditions in them support the introduction and maintenance of nosocomial MDR pathogens;

3) an increase of mobility due to air travel also contributes to rapid transfer of dangerous infections from endemic areas and their spread throughout the world. This is seen in the recent spread of cholera, and strains with genes coding NDM1 ("cosmetic migration") (Karthikeyan et al., 2010);

4) an increase of biological mass of *Homo sapiens* above a certain threshold value leads to high population density; this, in turn, increases the exchange of bacterial strains within a population and frequency of infections. Immediate consequence is permanent modifications of pathogenic islands in bacterial genomes and a general tendency towards increasing of pathogenicity and virulence for known pathogens, as well as acquisition of pathogenic and virulent properties by the previously nonpathogenic bacteria (Chapalain et al.,2008; Madi et al., 2010; Lombardi et al., 2002; Yomoda et al., 2003). Sometimes the impression arises that the species *Homo sapiens* is converting its home - Planet Earth- into an analogue of a Petri dish, the "Petri Globe", and himself

into the analogue of a nutrient medium.

Thus, the introduction of antibiotics as therapeutics on the background of such transformations in structure of human society is the direct cause for the emergence of MDR pathogens. It is evident that introduction and widespread of phage therapy against the preservation of these conditions will lead to arising of multiple phage resistant (MPR) strains of pathogens. Some such MPR strains may be found now (for instance, among *Pseudomonas aeruginosa* isolates in cases of cystic fibrosis) (personal observation). In principle, such strains could arise even in absence of directed phage therapy, for example, during the frequent outbreaks of acute intestinal infectious or due to permanent nosocomial infections, because phages are very common everywhere. Nevertheless, the permanent use of phages for therapy will create directed conditions for selective pressure and for quick accumulation of MPR strains.

Participation of bacteriophages in bacterial evolution

(a) Canonical interactions

In majority of publications discussing the positive sides of phage therapy, there is no mention that all phages are powerful participants in bacterial evolution. From the time of their discovery, the relation of bacteriophages to bacteria has long been considered as the relation of typical predators to prey. Such considering of phages as simple predators, which is the basis for current phage therapy, is obsolete. The successful multiplication of bacteriophages requires physiologically active bacteria. Plenty of evidence indicates that phages and bacteria are in state of co-evolution. Bacterial evolution involves a variety of genetic elements and mechanisms, including plasmids, different transposable elements, insertion sequences, and interaction with bacterial viruses of all kinds (virulent, temperate and filamentous). This is the reason for the necessity of more careful consideration of different possible events in result from wide introduction of phage therapy.

Described up to now bacterial viruses constitute two groups – of tailed and filamentous viruses (such subdivision has no relation to classification and introduced and used only for convenience in description of different structures and developmental strategies). Viruses of these groups have basic differences in structure of genome and capsid, in adsorption and introduction of genome into cells, in intracellular development. These two groups of phages interact with bacteria through three canonical ways - lytic development, temperate cycle (lysogenization) and chronic infection.

As result of infection of bacterial cells by true virulent phages, cells will be lysed with release of phage progeny.

Canonical temperate phage has a choice: to kill bacteria with liberation of progeny or accomplish lysogenization. In course of lysogenization genome of phage enters into special state - "prophage" when the expression of most genes is turned off with negative regulator of transcription (repressor). Some of prophages are integrated into bacterial chromosome, while other ones can exist as plasmids, which division is synchronized with cell division. Some prophage' genes avoid repression and manifest itself in change of the features of lysogenic cells. That phenomenon – lysogenic conversion- may change such properties of cells as surface antigens, toxicity, and other features related with increase of pathogenicity and virulence. This is one reason why the use of natural temperate phages is considered undesirable in phage therapy.

Temperate phages, whose genomes in the prophage state are integrated into the bacterial chromosome, can transfer the fragments of the bacterial chromosome which are adjacent to the sites of their integration (specialized transduction), and they could conduct generalized transduction as well. Temperate bacteriophages are actively involved into construction of pathogenic islands in bacterial genomes (Faruque and Mekalanos, 2003; Miao.and Miller, 1999; Tinsley et al; 2006). Temperate transposable phages, being integrated into conjugative plasmids, migrate within a large group of bacteria (Kaplan et al., 1988; Gorbunova et al., 1985; Krylov, 1996; Plotnikova et al.,1982; Plotnikova et al.,1983). All such events are examples of horizontal genetic transfer (HGT), which is one of the basic processes in bacterial evolution. Finally, a special kind of interaction with bacterial cells is typical for filamentous viruses (commonly called as "phages"). Such viruses are described for different bacterial species. Until recently it was thought that these phages cause only a chronic infection, not accompanied by destruction of bacteria. But sometimes (as “supervirulent mutants”) they can lyse bacteria and produce plaques, typical for phages, which use lytic cycle for multiplication (Kuo et al., 1994).

Filamentous phages of gram-negative bacteria use bacterial pili for adsorption (Rakonjac et al., Online journal at http://www.cimb.org). Development of various filamentous phages may differ. Phages of one group after the infection immediately produce replicative circular genomes, which expression leads to replication and the formation of proteins needed for maturation. The other group of filamentous phages shows some resemblance with classical temperate phages. After introduction of their genome into cell, they form a double-stranded genomic DNA, which integrates into bacterial chromosome and exist in unexpressed condition under control of repressor. Induction stimulates replication and expression of the phage genome but without its excision. Phage' single-stranded DNA genome is leaving cell with concomitant covering with protein shell.

Filamentous bacterial viruses can convert commensal strains into mortally dangerous pathogens (Bille, et al., 2005). We will consider such possible effects in relation with phage therapy later. But even true virulent phages can participate in HGT (in form of general transduction) as a consequence of packing of parts of bacterial genome of previous hosts into capsids. Presence in recipient cells of plasmids capable to block intracellular phage development can contribute for such event. Polyvalent phages (which use as hosts bacteria of different species) are contributing to interspecies recombination.

Availability of genome sequencing and annotation permits to make a preliminary assessment for a newly isolated phages: phages can be considered as lytic when genes for integrase or/and for repressor were not found in their genomes. However, use of this criterion has its subjective and objective limitations.

1). Here is an example of subjective reason. There are clearcut and understandable differences in mentalities of practicing doctors (with their evident purpose to cure patients with use of any effective phage mixture) and of molecular biologists (with their knowledge about danger of HGT as result of use temperate phages). Thus, in spite of the presence genes, coding integrase and/or repressor, such phages, being unique in their host-range, sometimes can be placed as virulent derivatives into phage' therapeutic mixtures as the only possibility to save human life.

2). Annotation of phage's proteins relies on their similarity with previously described proteins. But usually significant number of alleged products of new phage's species has no analogs among previously described proteins. Thus even positive results of phage genome annotation cannot guaranty the total safety in interaction of phage and particular sensitive bacterial pathogen.

3). An important reason is that a limited number of lytic phage types which are available now for each of pathogens may be not enough to apply phage therapy for a long time.

Additional difficulties in choice of therapeutic phages may arise because of our limited knowledge on behaviour of phages in real places of infections.

I think there is a sense to consider here also some known non-canonical modes of interaction of phages and bacteria, the contribution of such interactions in the evolution of bacteria, and possible influence on phage therapy.

(b) Psedolysogenic conditions and phage interactions

Studies of bacteriophages in laboratories usually are accomplished under conditions which are optimal for bacterial host growth. But interactions of bacteria and phages in place of infection may occur in other conditions. To identify such possible deviations in the in teraction of phage and bacteria before introduction of a phage into therapeutic mixture, it is necessary to conduct simulation studies under different conditions (for instance, in presence of different prophages and plasmids, mixed infections with different virulent phages, proposed partners in therapeutic mixtures, infections with different multiplicity, viscosity and pH, the availability of nutrients, with use of different clinical isolates etc.). Importance of such studies is confirmed with the existence of particular types of phage development which differ from the canonical lytic, temperate and chronic infections. Frequently such special situations are described under the common name of "pseudolysogeny", although there may be differences in their essence (and in reasons too).

Common feature for the determination of non-canonical interactions between phages and bacteria as pseudolysogenic ones is non-stable state of the phage-bacteria interactions. Sometimes it may be revealed as heterogeneity of bacterial population - the presence in it simultaneously infected and non-infected bacteria, and difficulties in isolation of stable lysogens, as well as of pure cultures without phages. Such features may arise in case of two fundamentally different situations. In such case "pseudolysogeny" has evident populational reason caused by presence in the population and competition of different kinds of bacterial cells, sometimes mutants, to varying degrees capable to support phage' development. The other situation can be designated as "true weak (unstable) lysogeny". It arises as a consequence of specific phage development in each infected cell of homogeneous population. Sometimes here is also possible arising of secondary mutant bacterial clones with changed relation to phage' infection. Let us consider some different examples of pseudolysogeny. The extent of study of such interactions is different and sometimes it is impossible to make final conclusions about all details.

a) Frequent reasons for establishment of pseudolysogeny and its maintenance are changes of cultural conditions on the background of specific bacterial and phage genotypes. For instance, infection of marine bacterium *Listonella pelagia* with phage phiHSIC produces extremely unstable lysogens. Analysis of chromosomal DNA preparations suggested the presence of a chromosomal integrated prophage. The prophage was not induced by mitomycin, but shows an extremely high level of spontaneous induction. That transition from lysogeny to lytic development was more frequent in high-salinity nutrient artificial seawater media with high aeration. The lowest titers of phage (less level of induction) were produced under low salinity or rate of aeration. Thus, generally speaking, good conditions for bacterial growth stimulated lytic phage' development and bad growth conditions were favorable for establishment

lysogeny (Williamson et al., 2001; Williamson and Paul. 2006).

b) Similar results were received in study with opportunistic bacteria *P. aeruginosa* slowly growing in presence of its phage in chemostat continuous culture model. As cells became more starved, the frequency of pseudolysogens increased but when nutrients are supplied to the bacterium, the pseudolysogens resolve into either true lysogeny or active production of virions. Authors consider pseudolysogeny as specific strategy for bacteriophages to survive periods of starvation of their host organisms (Ripp and Miller, 1998).

c) Transition of lysogenic cells from active multiplication into stationary phase also can stimulate high frequency of phage loss, as it has been found in study of gram-positive bacteria *Propionibacterium acnes* (cause of acne vulgaris and post-surgical infections of the skin), with a group of its closely related temperate inducible phages. Bacterial clones surviving such event were susceptible to new infection by the same phages. Most likely the phage genome in this case was not integrated into the bacterial chromosome (Lood and Collin, 2011).

d) Bacteria may acquire capability for lysogeny as result of mutation. Phage VHS1 produces clear plaques on its host *Vibrio harveyi* (strain VH1114). Bacterial clones were isolated which could be lysogenized. But such lysogens produced many cured cells containing no more phage DNA after approaching the stationary growth phase. The cured bacteria had an inheritable difference from initial strain what proves that their properties were caused with mutation (Khemayan et al., 2006).

e) Sometimes pseudolysogeny may result from several different genetical modifications as in a phage as well as in a bacterial genome. Neurotoxins C and D of *Clostridium botulinum* are encoded by genes of bacteriophages. In an earlier study it was shown that serial subcultures of bacteria initially infected with phages encoding the toxins frequently leads to a rapid loss of toxin production, even in the absence of specific antiphage serum. At the same time, in certain combinations of phages and bacteria toxigenicity persisted during long passages, although nontoxigenic variants of bacteria were segregating too. However, the segregants revealed sensitivity to phage infection and toxin production after infection (Oguma,1976).

This latter case has been subjected to more detailed study. The genome of converting phage c-st was sequenced and annotated. As it turned out c-st has the largest known temperate phage genome (185,682 base pairs) with terminal repeats and as a prophage it is present in a circular plasmid state. Plasmid prophages require special mechanisms to ensure their stable inheritance in lysogens. Phage c-st also encodes special proteins necessary to resolve the plasmid multimer, partition, and segregation, but it is possible that their activity is not enough to ensure the stable inheritance of c-st as plasmid. Besides, there is other important reason for the plasmid instability. A remarkable feature of the c-st genome is presence of IS elements (12 copies, including 7 of structurally intact elements, and 5 truncated or degenerated ones). The presence of such number of IS has been completely unexpected for a viable phage. Recombination between these multiple elements is additional reason for the plasmid prophage instability. This case of pseudolysogeny is typical weakened lysogenic state (Sakaguchi et al., 1995).

But pseudolysogenic condition is possible not only for bacteria, infected with phages having obvious features of temperate phages, but for generally recognized virulent phages. Let us compare two cases described for large bacteriophages, T4 of *E. coli* and phiKZ of *P. aeruginosa* (Krylov and Zhazykov, 1978; Krylov et al., 1984; Burkal'tseva et al., 2002; Krylov et al., 2004; Pleteneva.er al., 2009), because phages of both groups are used in commercial therapeutic blends.

The development of virulent phage T4 was observed under various growth rates in carbon-limited chemostat. T4 forms pseudolysogens not only in cells with totally inhibited growth, but in growing host cells too. As it has been shown earlier (Herriott and Barlow, 1957), active *E. coli* cells may be killed after contact with phage ghosts without DNA injection. But, considering survival cells in suppressed state even after infection with intact phage, it may be suggested that lethal effect of phage tail after contact with cell surface requires active participation of bacterial cell. According to data received, function of gene r1 has an important role in optimization of phage development in condition of slow growth and in establishment and maintenance of pseudolysogeny. On the other hand, r-system genes of T-even phages participates in control of lysis inhibition (Doermann, 1948) and in postinfection modification of cell wall (Garen, 1961; Krylov, 1970). It is not clear now if that case may be considered as pseudolysogeny. Phage T4 has no genes with repressor-like activity and it is not clear how long cells infected with T4 can survive in such condition (Los et al., 2003).

Pseudolysogeny in the case of phiKZ-like phages of various species (phiKZ, EL) has completely different features. These phages in one step growth cycle experiment, after infection of sensitive cells with multiplicity of infection (m.o.i.) in range 1-5 particles, have characteristics of virulent phages – all infected cells were killed with liberation of not very high number of phage particles (Krylov and Zhazykov, 1978). It was found that significant increase of m.o.i. of sensitive bacteria with phiKZ-like phages leads eventually to a special condition, when bacterial cells, infected with large numbers of phages continue to division with formation of colonies which can grow for several days in Petri dishes. As result, these phages produce huge amounts of progeny

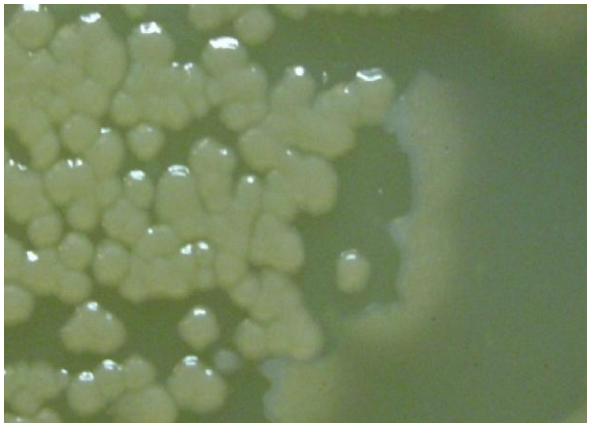

Figure 1: shows an appearance of the pseudolysogenic growth of *P.aeruginosa* PAO1 infected with phiKZ

particles, which by many times exceeds phage concentrations in case of low m.o.i. All phiKZ-like phages that were sequenced up to now do not encode “standard” DNA-polymerases (Mesyanzhinov et al., 2002; Hertveldt et al., 2005). Nevertheless, a very unusual DNA polymerase activity encoded in genomes of phiKZ-like phages has been found A.Cornelissen (Catholic University of Leuven, Belgium, personal communication). It is possible that in conditions which arise after infection of cells with high multiplicity, phage development is turned off for a while. Precise mechanism for the such effect of m.o.i. is not clear yet, but it is evident, that such phages in their "natural" wild type genome condition can lead to HGT (because pseudolysogenic cells can support development of other phages, including temperate ones).It is one of the reasons why phiKZ-like wild type phages are not desirable components of phage mixtures. We have isolated their virulent variants, which can kill cells in pseudolysogenic condition. Their use prevents the possibility of HGT in case phiKZ- like phages (Krylov et al., 2010; Pleteneva et al., 2010; Krylov, S. et al. 2011)

Figure.1 shows an appearance of the pseudolysogenic growth of *P.aeruginosa* PAO1 infected with phiKZ-like phages and their mutants in conditions of high m.o.i. Careful consideration of the picture gives some new ideas about the general properties and behavior of infected cells.

First of all, material taken of the “blue” (opalescent) initial growth gives arising of highly viscous colonies. The reason of such change is not known yet. But one of the possible explanations may be conversion of infected bacteria. Growth of phage sensitive colonies can be seen after incubation for several days around of initial zone of viscous growth. Such colonies again are able to give opalescent growth of phage on their border. This means that the sensitive bacteria and phage were able to move a certain distance from the original planting. As phage particles are incapable for active movement, it is evident, the phage was transported by bacteria. Apparently, such bacterial carriers were resistant to phage killing activity. The mechanism of this temporary resistance is not elucidated yet. This may be activity of repressor, blocking of intracellular development of phage and / or disturbance of the mechanism of an injection without influence on phage adsorption on the cell surface (phage as a "rider"). Only a portion of the runaway bacteria survive and be released from the phage. The remaining bacteria are killed in the process of escape, as evidenced by a high concentration of phage in the area of evasion. Unconditional evidence of true but unstable lysogeny in this case is the selection of mutants with properties similar to those of virulent mutants of temperate phages. Interestingly, that phiKZ and EL behave like phages with repressors of different specificity. At the same time, in the case of these phages, there is an additional mechanism of growth inhibition displayed by phage phiKZ against pseudolysogens for phage EL (Pleteneva et al., 2010) It may be possible that pseudolysogenic condition for cells infected with phiKZ-like phages at high m.o.i. has a biological sense. In the absence of phage-coded DNA-polymerase it leads to great increase of phage production. The T- even virulent phages of *Escherichia coli* have developed another mechanism, delay of lysis ("lysis inhibition") after reinfection (Doermann, 1948) which substantially increases the yield of phage from the single bacteria.

Examples for phage-phage interactions

The above example of dominance of phage phiKZ in mixed infections with EL shows that even in choice between related phages for phage therapy it is necessary to take into attention the possibilities of mutual inhibition. The same is true in case of temperate phages. Transition of temperate phages genomes in prophage state or their

lytic growth may depend on such conditions, as presence of plasmids or other lytic phages or prophages otherwise affecting lysogeny. As some examples – inhibition of growth of transposable phage B39 in *P. aeruginosa* cells with D3112 prophage, or increase the frequencies for lysogeny after infection with transposable immB39 phages in presence of plasmid RMS163 (Gerasimov et al., 1984; Freizon et al., 1989). Such interactions occur also between unrelated phages of different species which manifest activity in bacteria of different species (Kopylova et al., 1988).

There are also several impressive examples for studies of phage's interactions in model and in real (non-laboratory) conditions. For instance with use of the model system of two genetically identical *Bordetella* strains that differ only in that one is the carrier of phage and the other is susceptible to the phage *Bordetella* were studied effects caused with bacteriophage infection. It was found that lysogenic strain had the competitive advantage. But that advantage conferred by the lysogenizing phage may be revealed only in the presence of phage sensitive strain (Joo et al., 2006). In the other system (with *Campylobacter jejune* and its phages) it has been found that cells of one of *Campylobacter jejune* genotype overgrow another one on the background of bacteriophage infection.

In real conditions phage sensitive strains are more active in colonization of broiler chickens in comparison with phage resistant strain. But with the addition of lytic bacteriophage the phage resistant strain become dominated. It was shown that bacterial interstrain recombination *in vivo* can generate genome diversity in *C. jejuni* and that bacteriophage in such condition helps to dominate phage resistant strain (Scott et al.,2007). Thus, since the use of "natural" phages can become a source for serious problems, each new phage proposed for phage therapy should be carefully studied, and not only with genome sequencing and annotation, but with subsequent compulsory detailed physiological study. It may be suggested that similar situations with competing phage strains will arise in hospitals in case of active use of phage therapy. And although some of the considerations relating to the possible properties of future multiple phage-resistant mutants (MPR strains) can be regarded now as a brave speculation, nevertheless the properties of some natural MPR species make us to think twice (see below).

The current status of phage therapy

The current applications of phage therapy can only be seen as a demonstration of possibilities, but not as a standard therapeutic technique. The most complete study of phage therapy as an independent method of treatment has been carried out in Wroclaw, Poland, in the laboratory formerly led by Prof. S. Slopek (now chaired by Prof. A.Gorski) (Slopek et al., 1953 a, b; Slopek et al., 1985;a,b,c; Slopek et al., 1987; Zimecki et al., 2009; Letkiewicz. et al., 2010). . Even now, this work is only a tantalizing demonstration of possibilities (see below in details).

And the fact itself that physicians continue to prescribe, and patients have to buy expensive antibiotics of new generations is most convincing evidence that modern condition of phage therapy is not very reliable to rely on it in case of serious bacterial infections. It is necessary to recognize that for practicing physicians "therapy" is such method of treatment which must be available for use in any certified medical facility (clinic, hospital, maternity wards), located in any place (large city, small town or even in a village with an ambulatory). Obviously, for such access, is not enough to have just official permission for use of phages in therapy, some specific system measures are necessary. There is no such well elaborated system in case of phage therapy.

Let us try to imagine compulsory conditions which are necessary to use bacteriophage treatment in terms of practitioner: 1) presence in any hospital large enough phage collection (or simple accessibility to such collection); 2) possibility for rapid choice of necessary specific phages in such collections; 3) possibility to receive necessary phage or mixture of phages in a sufficient for the treatment quantity as quick as possible; and, finally, 4) possibility to replace the first mixture of phages for other one in case of arising of phage resistant variants. And it is crucial that all this must be carried out as a standard set of procedures by which doctors and their colleagues in clinical laboratories must be trained, but not as a certain magic skill.

Let's make an attempt to understand what is holding back the development of real phage therapy.

Start of phage therapy in the Soviet Union.

It is useful at first to remind on origin and use of phage therapy in Soviet Union and in Russia now. The phage therapy in Soviet Union started with two visits of Felix D'Herrelle in Soviet Union and his active work in city Tbilisi at 1930th years. D'Herrelle has donated not only his knowledge, his phages but even basic equipment for the work. It was the time of fight against different epidemics after period of First World War, two Revolutions and Citizen War. Thus the phage therapy has been placed in right place at precisely right time to prove its efficiency.

However, phage therapy could have remained just new curious method of treatment, if there has not been taken a decision on the organization of a decentralized system of counter epidemic organizations. This decision was forced due to the impossibility of organizing such work

only from the center. Thus, next extremely important point in support of phage therapy in USSR has been the organization of several Scientific Institutes with primary purpose to fight local infections. Being situated in different regions of Soviet Union (Moscow, Nizhniy Novgorod, Perm, Tbilisi, Saratov, Ufa, Khabarovsk) this system has covered entire country.

A constant exchange of bacteriophages between these institutes significantly helped in work and often has replaced the need to look for new phages in the natural conditions. Great importance phage therapy has played during the war of 1941-1945, taking into account huge number of infected wounds under the field conditions and frequent intestinal infections.

The Institutes with their own production facilities have become a system, responsible for the monitoring of local epidemic situation and for preparation of vaccines, antiserums and bacteriophages to fight such local infections. The existence of such system (and not general backwardness, as sometimes you can hear) is main reason why phage therapy in Soviet Union was not totally forgotten (as in the West) after introduction of antibiotics into medical practice.

Phage therapy in the Russia now.

Nevertheless, after start of antibiotics production in The Soviet Union the significance of phage therapy here has considerably decreased. So now, not every doctor can use phages for treatment (in spite of their availability). Moreover, far from all doctors even know about the possibility of phage application (I know this from conversations with my medical institute friends).

But, it is important that fortunately, production capacities are preserved, selection of new phages and their introduction into the production continues and that some doctors continue to use phages in their practical work. They have accumulated good experience for the use of phage therapy in the surgery, urology, and epidemiology (Perepanova et al., 1995; Akimkin et al., 1998; Lazareva et al., 2001; Gabriélian et al., 2004; Aslanov et al.,2003). It should be noted, however, that in Russia even specialists who frequently use phage therapy, do not publish their results each time (in comparison with today's flow of publications related with phage therapy in the West) and often their results can only be found in Internet. For example, a group of no-named doctors cites the treatment of urological infections ("Medicinskaya kartoteka" № 6,1998, http://medi.ru/doc/6280613.htm): "phage therapy was used mainly for treatment of chronic infectious-inflammatory urological diseases: chronic cystitis, chronic pyelonephritis, chronic prostatitis, urethritis, suppuration wounds, in some cases of acute purulent-septic condition of the patients - in total for 46 patients. For the treatment were used liquid bacteriophage preparations active against pseudomonas, proteus, coli, staphylococcus and combined pyobacteriophage containing all these phages. Phages used topically as: through drains into the bladder (50 ml 1-2 times a day), wound (10-20 ml) in renal pelvis (5-7 ml) and inside (daily dose of 100 ml day for 30 minutes before eating). Treatment course is 7-10 days. Satisfactory clinical tests have been received already for 2-4 days of treatment with bacteriophages: a decrease in symptoms of intoxication, dysuria, lower body temperature, improving the intestine condition (especially in children). The overall bacteriological efficacy was more than 84%, clinical - more than 92%. Clinical efficacy of phage therapy almost comparable with results in the control group of patients treated with modern antibiotics - fluoroquinolones". It is clear that such information may be interesting for urologists worldwide.

Thus, the basis for sufficiently rapid revival of phage therapy at the new level is preserved in Russia (provided it meets necessary requirements for used phages). The fact is that level of study of phages, included in commercial mixtures, does not correspond to the contemporary understanding of their safe use. Their studies in essence are limited with estimation of lytic activity with periodical replacement of some phages for more active ones. This process for some reason has obtained unsuccessful name as "adaptation". It is impossible to understand, what precisely it means in sense of origin of phages. It may be understood as selection of mutants with increased spectrum of lytic activity or as the introduction of a phage of some other species. First case may be sometimes preferable (it may be a mutant of well studied phage). But introduction of a phage belonging to a new species will require its detailed study. Sometimes this difference can have vital importance. In absence of deep studies of commercial phages arise some contradictory propositions. For instance, up to now there is no clear cut evidence which confirm penetration of phages into blood stream after per oral introduction. Some people consider that it is proved (Weber-Dabrowska et al., 1987), but in other publication such penetration and wide distribution through the circulatory system was not found (Denou et al.,2009). We observed a patient who was infected with hospital strain *P. aeruginosa* after operational removal of kidney stones. This particular strain was resistant to all available commercial phage preparations, being sensitive only to phage Lin21 (Lin21 is phiKZ-like phage from a phage typing collection of Lindberg). Preparation of phage Lin21 purified in CsCl gradient and in high concentration has been taken per os by patient and two collaborators of laboratory (it was not control for safety – just to taste the preparation and look for possible transit of phage through renal barrier) once a day for a week (after neutralization of acidic stomach contents with alkaline water). We could not find the phage in the urine of patient and both

volunteers. Nevertheless, the final result was positive (substantial reduction of *P. aeruginosa* cells concentration for a time was observed). The size of pores in healthy kidney is no more than 6 nanometers. The diameter of the Lin21 capsid is 120 nm (Khrenova et al., 1984). .We have no reasonable explanation for such temporary effect (after a week bacterial concentration has returned to initial size) (the one of the possibilities is activation of innate immunity mechanism). Difference in results of independent authors may be explained with difference in dimensions or other features of phages, with physiological states of kidneys or with plenty of other reasons. With other kinds of introduction of phage (intraperitoneal, subcutaneous or intramuscular) there were found phage deliveries to blood, spleen, and liver. Thus, authors consider that efficacies of phage therapy may greatly depend on way of phage inoculation, with the best effect (at least in the murine infection assays) after intraperitoneal administration) (McVay et al., 2007).

Phage preparations produced in Russia

It may be interesting, that all wonderful studies of phages, which were carried out since the mid-40s for about 30 years and which are now basis of contemporary biology, have passed past practical phage therapy. But it should be noted that some of phages active for instance against such species as *Escherichia coli, Pseudomonas aeruginosa, Salmonella sps*., etc., which were investigated in details in basic science have close relatives in existing phage therapeutic preparations.

The assortment of phage products and principles of their compositions are similar ones for different producers: they are polyvalent mixtures of anti-dysentery, anti -salmonella, anti coli- proteus, anti-staphylococcus, anti-pseudomonas aeruginosa, anti- *Klebsiella pneumoniae* and anti-streptococci phages.

Some preparations, as Pyobacteriophage and Intesti, contain phages active on bacteria of different species to enlarge lytic spectrum of final mixture against purulent and intestinal infections, caused with *P. aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae, Salmonellas, Shigella*, *Proteus* etc. Such complex preparations are useful in case when epidemiologists suspect start of mass food poisoning, and there is no time to wait for identification of particular pathogen. Behind of idea for such “blind” use is reliance that in such mixture may be a phage active against pathogen which was the reason of mass diarrhea.

Such preparations can be made in tablet form which is the most convenient for quick distribution and use. Frequently such blind application of polyvalent phage preparations gives good effect and it is recognized in the regulations of State Sanitary service in Russia. Here is as example a paragraph from “Sanitary- epidemiological Rules(http://www.mnogozakonov.ru/catalog/date/2002/3/22/19809/) (22 March, 2002. N 13), "СП 3.1.1.1117-02:" **4.9.1.** In the event of group centers of AEI (acute enteric infections) in organized collectives of children and adults, as well as the population be ‘’*prevented by specific bacteriophages, depending on the type (species) of the selected agent in accordance with the instructions on the drug.**"***

Although phage therapy in principal can be used in case of many different bacterial infections, nevertheless now it is limited with infections, having two common features: a) they are have limited localization on a "surface" (skin, mouth, enteric epithelium, ears, eyes, burn infections, bladder), where phages may immediately contact with pathogens and where result of phage therapy can be observed visually and b) a work with such pathogens in laboratory for the selection of active phages and their industrial production do not require special conditions for safety of personnel. Thus, the basic infections which are now priorities for live phage therapy:

1). Acute intestinal infections with *Shigella sps., Salmonella enteritidis , Campilobacter pylori, Campilobacter jejuni, E. coli, Yersinia enterocolitica.* They are extremely frequent everywhere and can cause in some person’s life dangerous threats. According to data of WHO at 2005 the number of deaths from acute intestinal infections was near 1, 5 millions (Buzby and Roberts, 2009). Near 70 % of such illnesses have as their reason the use of infected food stuffs.

2). Nosocomial infections, as post operational infections in surgical departments and in burn centers with *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, *Burkholderia cepacia*, *Streptococcus epidermidis, Staphylococcus aureus*, including methicillin-resistant strains (MRSA). MRSA gives a high level of deaths and many patients after leaving hospital become carriers of the pathogen. It is possible to conjecture that selection and studies of other phages, active against dangerous infections as anthrax, plague etc., are in progress in specially equipped laboratories in different countries.

But let us repeat: all cases of successful use of phage therapy with live phages up to now even in such limited number of infections may be considered only as *demonstration of possibilities**.*** There is no mass use of phage therapy what is necessary to consider it as real medicinal procedure. To overcome that difference it will be necessary to accept some special organizational measures. Comparison of phage therapy and use of antibiotics will help to understand the differences which may be reasons for such limited use of phage therapy. It is necessary also to mention them just to overcome some generally accepted opinions about phage therapy as extremely cheap and simple medicine. Let us compare different aspects in use of phages and antibiotics just to find the weak points for introduction of phages as potential substitutes of antibiotics.

Comparison of therapy with live phages and antibiotics

Before to compare the use of antibiotics and phages in therapy let us consider the safety of antibiotics and phages per se. Antibiotics, being substances with complicated chemical structure, frequently cause side harmful effects. It may depend on the wrong use (including dosage) or arise as result of specific reaction (for example, allergy) of patients. It is the basic reason why each substance with antibacterial properties must be carefully studied before official permission will be granted for use it as medical preparation. Thus, pre-clinical and clinical tests are compulsory, expensive, time consuming but absolutely necessary ones. On the other hand, bacteriophages are natural components. They are distributed everywhere and permanently can be isolated from different sources, including food, water etc. Numerous tests have shown absence of their toxicity after peroral consumption or application to infected wounds. Thus, considering the compulsory local application of phages (only in such case it is possible to be sure that phage particles will meet with sensitive bacteria) there is no need for complex and lengthy pre-clinical and clinical trials.

(1) Efficiency.

a). The good antibacterial efficiency of live phage therapy has been well documented in excellent Polish studies fulfilled in Wroclaw under guidance of Professors S.Slopek and A.Gorski. These good results were obtained *only as consequence of careful selection of right phages for each isolated pathogen.* In the years 1981-1986 bacteriophage therapy was applied in 550 cases of suppurative bacterial infections. Positive results were obtained in 508 cases (92.4%). In 38 cases (6.9%) a transient improvement was observed and in 4 cases (0.7%) phage treatment proved ineffective. Considering that majority of patients (518 cases) were resistant to antibiotic treatment, the results of phage therapy may be regarded as extremely good (Slopek et al., 1987, c). . It is an example of what kind results can be achieved in conditions of standard clinics with the good selection and supply of active phages against different pathogens.

b). Efficiency of phage therapy is directly related with capability of active phages to reach infected place in human body. The direct introduction of phages (even highly purified) in blood stream in case of septicemia may be deadly dangerous (the reaction on phage proteins or residual bacterial endotoxins). On the other hand there are experiments where it has been shown that active life time for phage introduced into blood stream is not too long, but for the virulent mutant of phage lambda long persisting variant was received (Merrill and Adhya, 1996). I could not find similar publications concerning phages actually in use for therapy. In any case it is obvious that question about possibility of introduction of live phages into blood stream requires further studies. In case there will be found positive results it will greatly increase the range of infections for use of phage therapy.

c) Antibiotics are more simple in their use than phages and may be used in such cases where phages useless. But in the fight with antibiotic resistant pathogenic strains phage therapy is priceless and is able to save millions of human lives.

(2) Regional specificity in use of phage therapy.

Other important difference of phage therapy from antibiotic therapy is its regional specificity. A group of physicians from Saint-Petersburg Medical Academy have checked sensitivity of *P. aeruginosa* strains isolated in local surgical department to phage composition “Pyobacteriophage” which was produced in Ural region. And not more than 48% of strains were sensitive. Such level is absolutely unacceptable for serious medical use. And only after special selection of additional phages capable to lyse strains in this particular hospital the efficiency of phage therapy could be raised up to acceptable level about 82 % (Aslanov et al., 2003). Thus, in principle regional specificity of phage therapy can be overcame with its rational organization.

(3). Production.

In difference from antibiotics, the polyvalent phage mixtures *cannot be produced for a long time without adapting of their composition to changes of pathogenic microflora in local hospitals.* And it must make quite frequently because, as a rule, resistant mutants to phages are arising very frequently. But exchanges of phages in the mixtures usually occur just once or twice a year (spring is the best time for such "phage safari"). It means that at least during a half of year plants are producing the same preparations without adapting it to the regional change of nosocomial pathogens (and hospitals situated nearby can have different set of local pathogens!).

(4). A good phage bank is a compulsory condition for permanent phage therapy.

Phage bank is central establishment to support real phage therapy with live phages for a long time. Existence of such bank will ensure continuous use of phage therapy and necessary speed in its application in hospitals.

The basic purposes for phage bank are: 1)

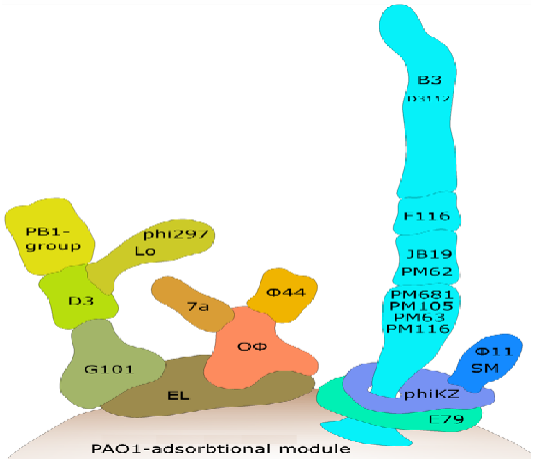

Figure 2: Rational organization of phage therapy

Accumulation of phages to have some buffer set of phages to prevent regional specificity and to adapt mixtures for the real needs in hospitals; 2) To supply hospitals with small samples of different phages to help clinicians and laboratory in quick selection of appropriate phages; 3) To store all phages which certified for their safety. 4) To be a place to gather and store unique phage gene sequences which could be useful in the development of future therapies, for instance, AMPs (Anti-Microbial Peptides) based on use of phage products capable to kill pathogenic bacteria. The phage bank deposits must be available for all hospitals and producers.

The understanding for the necessity of phage banks as precondition for introduction of real phage therapy is now recognized internationally - for instance, German Collection of Microorganisms (DSMZ) is ready to be “A home for Phages” (the proposition made at the 2010 “Viruses of Microbes” Congress, Paris).

It is evident that such phage bank must cooperate with laboratories in different countries engaged in isolation and studies of phages active against different pathogens. Such internationalization will contribute for high efficiency of phage therapy and converting it into continuous process of curing new infections (for instance, such as caused with bacteria carrying NDM1-coding gene).

(5). Safety of phage therapy

Bacteriophages itself have no immediate toxicity. But safety of their application in phage therapy – i.e. safety of their infection –must be proven. It suggests use of all available studies for potential “therapeutic” phages, including electron microscopy, DNA restriction analysis, genome sequencing with annotation and comparisons with data base, evidence against real temperate state or its potential possibility and absence of functions which promotes conversion into toxigenity, horizontal genetic transfer events, and other phenogenetic studies in different conditions.Sometimes it is possible to hear dogmatic statements about the complete safety of phage therapy on the ground that in practical use of phages up to now there were no negative effects. Apparently, this may be expected in some extent only in sense of immediate safety of patients, and there is not considered possibility of inheritable modification of bacteria due to the HGT by incorrectly selected phages. Such negative effects can be manifested not immediately, but in another time, another place and, moreover, in other people (in use of antibiotics for a long time also there were no problems!). But the physician should be aware of such possibility, in particular, to be ready for arising of phage resistant nosocomial pathogens. It is necessary to stress that successful and safe in prospect *phage therapy can be used only in hospital conditions*. This point may cause some doubts but it is necessary to insist on this idea. Full success in curing with phage therapy is hardly achievable without permanent collaborative work of physician and clinical laboratory. Substitution of phages which will be frequently necessary in course of long treatment can be done only in case of such collaboration. But determine the time for arise of phage-resistance in pathogens is possible only in hospital conditions.

The proposed interactions in rational organization of phage therapy are presented on Figure 2.

Some of the necessary steps in development of such full cycle of phage therapy have been created in its earlier period in Soviet Union but total scheme never been in action. Now in Russia only regional phage selection and production are conserved. But some of necessary elements were lost or are not accomplished yet.

1). After disruption of network of Institutions which produce commercial phage mixtures the common pool of therapeutic phages has been transformed into private

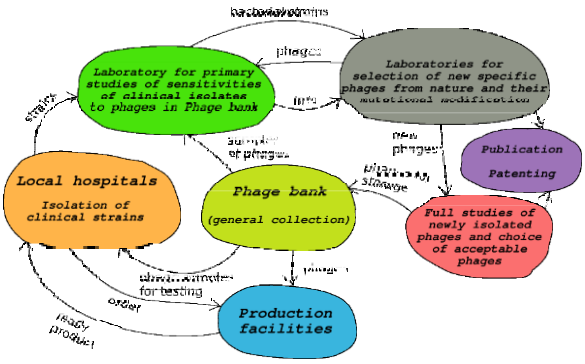

Figure 3: A part of formal scheme for phage adsorption receptors in *P.aeruginosa* PAO1

united phage production facilities and there is a possibility to organize a good united phage bank.

2). Distribution of commercial phage mixtures has lost its regional character - you can buy in different places of country phage mixtures prepared in any of regional centers against their local pathogens. Nevertheless, even in Moscow sometimes phage mixtures in drugstores are unavailable or are presented in limited assortment.

3). There is use of phage therapy in hospital conditions but it is not compulsory. Phage preparations (as antibiotics too) can be acquired without prescriptions. Such uncontrolled phage therapy in home conditions may be permanent source for arising and distribution of phage resistant strains. Especially popular is use of phage preparations at home conditions among mothers for curing intestinal disturbances in babies. Usually results are positive, but sometimes such mothers mention regional specificity of phage mixtures!

Practically only a few enthusiasts in Russia can make a choice of active phages to specific pathogens and therefore bacteriophage treatment in Russia is limited to a few large hospitals (N. V. Sklifosovsky Institute of Emergency Medicine, Moscow Institute of Urology, Burdenko's Military hospital - all in Moscow, Mechnikov's Medical Academy St. Petersburg, some regional hospitals as burn center in Chelyabinsk, etc.)

Phages as antiseptics? Phages in veterinary? Phages on the diner table?

Up to now there were discussed problems of phage therapy in human society. But some "extremists" are ready to use *the same phages* for other purposes. For instance, it has been shown that after use of phages for treatments of wards, operational rooms, there is decrease in frequency of hospital infections (Kutter et al., 2010).Yes, it is expected consequence of such treatment. But let us remember that nosocomial infections are not especially connected with the particular hospital. They become local hospital infections being transferred here with infected patients. Thus, as it was shown in other study (Aslanov et al., 2003) the use of phages for direct treatment of patients leads to comparable decrease in frequency of post operational infections in hospital. Practical doctors observe some periodicity in change of nosocomial pathogens in hospital. Thus there arise a new important question - is it for good or for bad to use phages as antiseptics? May be such “phage as antiseptic” ideas arise as consequence of general opinions that phage production is extremely cheap, that phages are “natural enemies” of bacteria and that phage potential for a bacterial species is limitless. Some people think that Mother Nature can be an endless source of different phages. But in reality not only phage potential of a bacterial species is limited one, but diversity in host ranges of a phage species is also limited. To understand that it is enough to look on phage data base of NCBI. There are plenty of phages with sequenced genomes which belong to the same species and have only minor differences.

Study of species composition of real commercial mixtures made earlier during our work for the classification of *P.aeruginosa* phages (Krylov et al.,1993) revealed only two species (from the Eliava Institute collection, marked as PTB) which were not represented in phage typing Lindberg's collection and in our laboratory collection. Earlier phage therapeutic mixture for *P. aeruginosa* contained virulent phages of 4-5 unrelated species. Recently we have found that in two different commercial mixtures made in different regions are included phages of just two different species (Burkal’tseva, et al., 2011). It is evident that variability in lytic spectra of these phages is not infinite and in course of phage therapy mutants will arise with multiple phage resistance (which frequently is caused by specificity of phage adsorption on bacterial surface and which is evident from the formal scheme for phage adsorption receptors in P.aeruginosa PAO (Figure 3) (Pleteneva, et al., 2009).

The second similar idea to use live phages as antiseptics is proposition to sprinkle ready-to-eat food with phage mixture (approved by FDA of USA) (http://www.intralytix.com).

And the third proposition of such kind is to use bacteriophages in veterinary practice, industrial farming of chickens, pigs, and cattle. As a matter of fact reason of the most cases of acute intestinal infections cases is consumption of infected food products (eggs, meat of birds, etc.) (Cutler et al., 2010). According to United States Department of Agriculture (USDA) livestock and poultry produce 335 million tons of manure per year, what is one of basic source of drug resistant pathogens for people (Chander et al., 2006). Really the situation in industrial production of animal food is very serious. The basic purpose for use of phages is not to cure animals but to diminish pathogenic bacterial concentration in the meat product and apply phages immediately before to slaughter and then to treat raw meat product with phage (Loynachan et al., 2004; Loynachan and Harris, 2005; Wall et al., 2010).

It is impossible to choose special therapeutic phages to fight these drug resistant pathogens only for animals or only for humans. More than half of industrial chickens are infected with *Campylobacter jejuni* and *Salmonella enterica,* which are commensals for birds but highly pathogenic for humans. Thus such use of phages in veterinary or as antiseptics after some time will inevitably create difficulties for use of similar phages in treatment of human infections. In course of work in the frame of EC project ("PhageVet") in our lab has been isolated phage phi 2/2 with wide host range on S.enterica strains (Pleteneva et al., 2010) , but later it was renamed (Santos et al., 2010). Changing the names of phages and their mutants must be forbidden (it can introduce difficulties in future comparative studies). Such phage could be very useful as component of therapeutic anti-salmonella mixtures for humans. But it is evident that use of this phage for treatments of usually highly infected broiler chicken houses will quickly make it useless for human patients.

A compromise solution?

The compromise solution could be separation in use of phages in accordance with infection agents. Indeed, as rule main intestinal infections of modern society have their direct or indirect origin from industrial food animal production. Recent withdrawal from sale of millions of chicken eggs which caused mass salmonellosis in the USA, multiple cases of summer 2011 infection in Europe with strain of *E. coli, causing hemolytic-uremic syndrome (HUS)* or "Ural-broiler" incident in Russia (http://uralpress.ru/reviews/uralbroyler-reshaet-mushinuyu-problemu) testify that industrial carrying out of cattle-breeding production with unsufficient level of sanitary will become constant problem for any highly urbanized society. Reduction in the expenditures for desinfection of waste in animal food production (chicken, pigs) and lowering of sanitary standards on all levels are leading to serious increase in incidence of intestinal infections of various etiologies in people. Possible partial decision of the problem (which is disputable with different points of view) is to limit use of active phage therapy in cure of human infections (nosocomial purulent infections in hospitals, intestinal infections) or for preventive use in special cases (see later). Treatment of agricultural animals practically has no sense - some bacterial species, being pathogenic for humans, are harmless components of intestinal microflora in animals. Thus use phages against intestinal pathogens as antiseptics in food production will accelerate emergence of multiple phage resistant (MPR) strains.

There is extremely low probability that total refusal from the above mentioned applications can be possible without state interferences. But without such refusal the use of phage therapy to cure human intestinal infections (and phage preventive use too) will become practically impossible and senseless).

Some speculations on possible properties of future MPR pathogens.

It is evident that appearance of MPR bacterial strains will be inevitable result of introduction of phage therapy. Emergence and subsequent spread of MPR strains may have more deep consequences than spread of MDR strains. Multiple antibiotic resistance had created significant problems for practical medicine in the treatment of infections, but had not led immediately to increase of pathogenicity and / or virulence. But different outcome is possible in case of arising and the massive spread of MPR strains. Which properties could be awaited in future for MPR pathogens? At least two scenarios are possible.

a) External bacterial cell structures (LPS for gram-negatives or teichoic acids for gram positives) are active participants in adhesion and invasion of pathogens and are considered as pathogenic factors (Raetz and Whitfield, 2002). Different LPS are obligatory for phage adsorption on gram-negatives. After loss or modification of LPS bacteria can become less pathogenic. It may be estimated as a good result (may be even as a step in retroevolution of modern pathogens into free living bacteria) (but with a possibility for further degeneration into unpleasant L-forms). It is known that mutants of gram-negative enteric bacteria, surviving treatment with the virulent phages, had lost the O-antigen and have a shortened LPS (Kudva, et al., 1999).

b) Let us consider another, purely hypothetical

possibility, taking as example two species of *Neisseria, N. gonorrheae* and *N. meninghitidis.* First one is recognized pathogen. The other, *N. meninghitidis* is relatively harmless common inhabitant of human nasopharinx, but in some conditions it becomes the cause of epidemic meningitis. Bacterial cells of both species *Neisseria* have instead of usual LPS its shortened variant, designated as LOS - lipooligosaccharide. In addition, treatment *N. gonorrhoeae* with pyocin leads to the selection of pyocin-resistant mutants, which acquire further changes in LOS (John et al., 1991),

Both species may be considered as natural MPR at least because attempts to isolate active *Caudata* (with tail) bacteriophages were unsuccessful (Felps, 1967) . On the contrary, for natural commensal *Neisseria*, active phages are described (Steinberg et al., 1976). Nevertheless, genomic sequences of different bacteriophages - lambdoids, transposons or filamentous bacterial viruses were found in different strains of pathogenic *Neisseria,* what can be result of recent lateral genetic transfers (Piekarowicz et al., 2007). Some of the acquired phage genes in genomes of pathogenic *Neisseria* are coding for surface-associated antigens, which induce production of bactericidal antibodies or can regulate the expression of some bacterial genes (Masignan et al., 2001) , but bacteria also regulates expression of foreign phage genes. Thus, HGT of phage' genes interferes in bacterial evolution even in the case where bacterial hosts have no their own specific phages.

Sensitivity of *N. gonorrhoeae* cells for pyocin (John et al.,1991) and finding in some of defective phages a sequence homologous with DNA of *P. aeruginosa* phage F116 (Piekarowicz et al., 2007), may be an indirect evidence that there is an open channel for HGT between even such distant bacterial species.

In the case of pili-specific filamentous bacterial viruses there is another situation. It is known that a prerequisite for adherence of meningococci to nasopharyngeal mucosal surfaces and colonization of exclusive human host, is the presence of type IV pili. And obligatory condition into conversion of commensal *N. meningitides* into pathogen is infection with filamentous bacterial virus whose genome insert itself into bacterial chromosome (Bille et al., 2005; Piekarowicz. et al., 2006). *Neisseria* specific filamentous viruses (Nf) have their own genetical system for integration into bacterial chromosomes of both species (Kawai et al., 2006). Eleven intact copies of Nf were located at different loci in the four genomes of strains of both *Neisseria species*. The filamentous virus controls also its own protein (secretin) which is necessary for mature virus to leave infected bacterium. The secretin observed in EM is cylindrical tube with an internal diameter of about 8 nm, enough to accommodate filamentous phage (diameter of 6,5 nm) (Linderoth et al.,1997).

Thus, the possible modification of bacteria with products of filamentous viruses is the immediate reason for conversion of *Neisseria meningitidis* into form which is insensitive to human immunity system. And further spreading of phage among the population may promote arising of new epidemic clones of *N. meningitidis.*

Plenty of different species among nosocomial pathogens have pili of IV type. Thus, in case of the irreversible loss of LPS and change it for LOS with subsequent infections with filamentous phages there could arise situation at least formally similar with *Neisseria* situation. For instance, *Yersinia pestis* is another bacterial pathogen where is no confirmed data on existance of active virulent phages, and where are evidences for negative role of filamentous viruses (Gonzalez, et al.,2002; Derbise et al., 2007). Saprophyte bacterial species can acquire pathogenicity after introduction into them genes of pathogenic species (Pereira et al., 2011).

It is possible that some of so-called attenuated laboratory strains (which can be used in industry for therapeutic phages production) may restore their pathogenicity after HGT related with phage infection.

It is clear, that second scenario is extremely undesirable because it has unpredictable consequences. Thus, one of the most important points before wide introduction of phage therapy with live phages have to become careful studies of MPR mutants (at least of bacterial species which are now consider as real objects of therapy with live phages). In this regard, of particular interest to determine is there a specific relation between loss of sensitivity to tailed phages and choice of such MPR variants as preferential niches for filamentous phages - it is one of several problems here to solve. Prospects to use phages as a source for valuable antibacterial products in phage therapy of human beings are promising (as example, lytic phage products, bacteriocins) (Fischetti, 2010; Kageyama et al., 1996; Briers et al., 2006; Kozlov et al., 2010; Sozhamannan, et al., 2008; Yoong et al., 2006).

More than 500 phage genomes have been decoded now. But functions of gene products are known not more than for a half of revealed ORFs. Some of ORFs could code antimicrobial peptides (AMP). Nevertheless, the use of these future phage derived antibiotics must be as careful as use of live phages. Hardly Mother-Nature (Evolution) who is responsible for placing such ORFs in phage genomes had a second thought about usefulness of them for the modern humankind. The similar care should be in use of phage lytic enzymes. Their uncontrolled use can lead to the disturbances of natural microflora in the human body and development of yeasts and fungae. In this regard, lytic enzymes do not differ

from antibiotics.

GENERAL CONCLUSIONS

It is evident that phage therapy is incapable to substitute totally antibiotics in therapy of most infections, nevertheless, in some cases therapeutic or preventive use of phages may be priceless. But each time it is necessary to follow specific conditions. Let us mention such cases and conditions.

1) Minimal requirement for phages selected to be used in phage therapy is classification up to species with generally accepted procedures (EM, RELP, estimation of DNA homology and annotaion of sequenced genome). It is extremely desirable to develop new approaches to find real functions encoded in genomes of therapeutic phages for which annotations just mention "hypothetical protein" ("Devil hides in the trifles"). It may be done through development of genetical and deep phenogenetical research of phages (realization of genotype in real conditions). It is necessary to control and evaluate frequencies of phage-resistant mutants of different types and their properties to avoid unexpected effects.

2) Additional studies are necessary for understanding phage propagation in human organism and penetration of phages into blood stream, as possibility to develop long living therapeutic phages.

3) Implementation of real phage therapy with alive phages requires organization of large and interconnected banks of phages in different countries for storage and, when it is necessary, quick exchange with phages.

4) There are two open front lines for immediate use of phage therapy.

a) To prevent arising of large epidemics of intestinal infections and their localization it is necessary to accumulate beforehand large collections of virulent bacteriophages against isolated pathogens and of their future phage resistant variants. It means permanent work in laboratory conditions with selection of new phage resistant pathogens and phages (natural, mutant or genetically engineered) capable to overcome such resistance. To localize epidemic it is necessary to keep in storage of most active phages and apply them immediately among the group of people in place of spreading infection. It is evident that the last will be possible only in case of successful work of epidemiologist in finding source of infection.

b) To prevent hospital infections it is necessary at first to enhance the sanitary standards in clinics, hospitals, maternity homes. In spite of compulsory requirement for permanent training on infection control frequently considerable proportion of medical personnel have no necessary knowledge. Most frequent among hospital infections are (approximate data): wound infections- 25 %, urinary tract infections - 22 % and pneumonia - 21 %. Conventional transmission mechanisms are different (airborne, fecal-oral, contact-household and as a result of surgical operations). As a rule each hospital has its own set of local bacterial strains, causing such infections and frequently the strains are resistant to different antibiotics. Here are some bacterial species which are most frequent cause of hospital infections: *P.aeruginosa*, *S.aureus*, *K.pneumoniae*, *E.coli*. Commercial mixtures of different specific phages are frequently useful in therapy of hospital infections, but it is necessary permanently check bacterial sensitivity to phages and substitute preparations after arising of resistant mutants. The last requirement (control of sensitivity) is true also in case use of phage therapy for curing of local infections, caused with MDR bacterial strains.

5) Real success in use of well-characterized phages in hospital conditions (with detailed publications in medical journals) will be the best (and obligatory one) stimulus for real recognition of phage therapy in medical society. Otherwise that very promising direction of anti-infective therapy is doomed to be used only by its fanatically loyal supporters. And it will be very sad.

This review is an attempt to show for all specialists, interested in promoting phage therapy, that phage therapy can become generally accepted medicinal procedure only in case of specific preconditions and that very careful and professional attitude at all levels to phage therapy is necessary. With use of bacteriophages we intervene into course of natural evolution. And only in case there is no other way to help patient, such intervention may be justified.

ACKNOWLEDGEMENTS

I express my deep gratitude to Professors H.-W. Ackermann, R.Lavigne, L.Kulakov for their attentive reading of the artickle and extremely useful comments. The work of the author is supported with grant of Russian Foundation for Basic Research № 11-04-00270-a.

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