Review
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Study of bacterial susceptibility to antibiotic and phage combinations: a literature review

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Abstract

The aim of the review is to describe existing laboratory methods for determining the sensitivity of bacteria to a combination of antibiotics and bacteriophages. However, more and more often there are scientific papers in which their combined action is described as synergism. The mechanisms of this phenomenon have not been fully studied, but it has been proven that not only virulent but also moderate phages can enter into synergy with antibiotics, allowing the minimum inhibitory concentration of the antibiotic to be reduced several times. Since synergy cannot yet be empirically predicted, microbiological laboratories use various *in vitro* methods, most of which are labor-intensive. The development of a new technique that can be introduced into the daily practice of microbiological laboratories is relevant.

Keywords: *resistance, susceptibility, antibiotic, bacteriophage, synergy*

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Определение чувствительности бактерий к комбинации антибиотиков и фагов: обзор литературы

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Аннотация

Цель обзора — дать описание существующих лабораторных методов для определения чувствительности бактерий к комбинации антибиотиков и бактериофагов.

Бактериофаги до сих пор рассматриваются некоторыми исследователями как альтернатива антибиотикам. Но всё чаще встречаются научные работы, в которых их совместное действие описывается в виде синергизма. Механизмы этого явления до конца не изучены, однако доказано, что в синергию с антибиотиками могут вступать не только вирулентные, но и умеренные фаги, позволяя снизить минимальную подавляющую концентрацию антибиотика в несколько раз. Поскольку синергию эмпирически пока предсказать невозможно, в микробиологических лабораториях используют различные методы *in vitro,* большинство из которых являются трудоёмкими. Актуальна разработка новой методики, которая может быть внедрена в ежедневную практику микробиологических лабораторий.

Ключевые слова: *резистентность, чувствительность, антибиотик, бактериофаг, синергия*

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Источник финансирования. Авторы заявляют об отсутствии внешнего финансирования при проведении исследования.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Introduction

In recent years, the problem of resistance of microorganisms to antibiotics used in medicine has become increasingly urgent, and the widespread emergence of pathogens resistant to them is of concern to clinicians all over the world. Among etiologically significant bacteria, the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.) is distinguished, which is characterized by a variety of antimicrobial resistance mechanisms. In May 2024, the World Health Organization published an updated list of antibiotic-resistant bacterial pathogens posing the greatest threat to human health. Depending on the necessity for the development of new antimicrobial drugs and new treatment options, microorganisms are categorized into priority groups. *A. baumannii* resistant to carbapenems and microorganisms belonging to the order *Enterobacterales*, including producers of extended-spectrum beta-lactamases, are classified as critically high-priority. High-priority pathogens include *Salmonella* spp. and *Shigella* spp. resistant to fluoroquinolones, *E. faecium* resistant to vancomycin, *P. aeruginosa* resistant to carbapenems, *Neisseria gonorrhoeae* resistant to third-generation cephalosporins and/or fluoroquinolones, and methicillin-resistant *S. aureus*. The medium priority level includes *Streptococcus* group A and *S. pneumoniae* resistant to macrolides, *Haemophilus influenzae* resistant to ampicillin, *Streptococcus* group B resistant to penicillin[1]. In Russia during the year 2017, the Strategy for the prevention and spread of resistance for the period up to 2030 was introduced, which provides for the introduction of modern methods to study the mechanisms of its formation, monitoring of its spread and ways of containment. Special importance and attention is given to ESKAPE pathogens in "Sanitary and Epidemiological Requirements 3.3686-21" as the main pathogens of infections associated with the provision of medical care[2].

Given the growing resistance of bacteria to chemical medicines, there is a necessity to introduce alternative approaches to the treatment of diseases caused by them. Instead of antibiotics, different authors suggest using probiotics, microbial enzymes, bacteriocins, bacteriophages and their lysins, synthetic phages, vaccines, serums and other biologics [1–6].

The most promising in this list are phages – bacterial viruses, because they do not have a toxic effect on the cells of the macroorganism and do not suppress immunity, so there are practically no contraindications for their prescription. At the same time, they have a narrowly targeted effect and do not cause negative changes in the composition of the human microbiota. Unlike other antimicrobial drugs, bacteriophages are able to overcome the bacterial immunity developed to them using several strategies. Compared to β-lactam antibiotics, which cause microbial cell death within 3 h, bacterial lysis by phages can occur in less than 10 min. However, unlike antibiotics, the action of bacteriophages does not lead to cumulative accumulation of endotoxin when destroying Gram-negative bacteria [7].

The only Russian manufacturer of medicinal bacteriophages is Microgen, which produces more than 14 unique drugs. Today, the market offers bacteriophages active against not only ESKAPE pathogens, but also against pathogens of diarrheal diseases — shigellosis, salmonellosis, escherichiosis. Medicines based on phages are produced either as combinations drugs — against several genera of bacteria, or as monotherapeutics specific against a particular type of pathogen. It should be noted that in Russia the use of bacteriophages is enshrined in regulatory documents, while most countries in Europe and Asia, Australia and the USA have only recently started to develop documents regulating the use of phages [8, 9].

Most studies have shown high efficacy and safety of tested phages, including those against priority bacterial pathogens [10]. Phage therapy without antibiotics has led to success against vancomycin-resistant enterococci, methicillin-resistant staphylococci (MRSA and MRSE) [11]. In rare cases, antagonism phenomena have been described when antibiotic and bacteriophage are administered together [10]. Therefore, before their administration, the sensitivity of a particular

[1] List of priority bacterial pathogens. URL: https://www.who.int/ru/news/item/17-05-2024-who-updates-list-of-drug-resistant-bacteria-most-threatening-to-human-health (date of access: 05.08.2024).

[2] Resolution of the Chief State Sanitary Doctor of the Russian Federation dated 28.01.2021 No. 4 "On Approval of Sanitary Rules and Norms 3.3686-21 'Sanitary and Epidemiological Requirements for the Prevention of Infectious Diseases'".

strain to antimicrobial agents should be determined. In Russia, the determination of bacterial sensitivity to bacteriophages is regulated by methodological recommendations for the rational use of bacteriophages[3], while sensitivity to antibiotics is regulated by clinical guidelines[4]. This raises the urgent question of determining the sensitivity of bacteria to the combination of antibiotics and phages in microbiological laboratories.

The aim of the review is to describe the existing laboratory methods for the combined determination of bacterial sensitivity to antibiotics and bacteriophages.

The combined effect of phage and antibiotics was first described by Neter and Clark in 1944 using *S. aureus* and penicillin as an example. In 2004, there were results of experiments on a chicken model devoted to studying the interaction of phage and enrofloxacin against *Escherichia coli* by Huff et al., and a few years later, A.M. Comeau and his research group conducted *in vitro* testing and noticed that subinhibitory concentrations of certain antibiotics can affect the production of virulent phages infecting *E. coli*. The authors named this phenomenon Phage-Antibiotic Synergy (PAS). For a long time, the mechanism of synergy remained unknown, until electron microscopy was used to study bacterial cultures treated with antibiotics and phages. It was discovered that chemical medicines which disrupt peptidoglycan synthesis lead to elongation of bacterial cells, which promotes phage replication and possibly its active attachment to the bacterium due to an increase in the cell wall surface area [12–14].

The PAS phenomenon has been extensively studied in many laboratories, resulting in evidence of synergism for various combinations of phages with antibiotics of different pharmacological groups. However, the methods used to evaluate these interactions are still not unified, so the approaches of various researchers have significant differences. The simplest way out of the situation is to borrow the method used to study the interaction of different classes of antibiotics, since combination antimicrobial therapy is administered to patients with bacteremia, pneumonia, surgical infection, and patients with septic shock in intensive care units. To date, 4 methods have been described by which synergy of chemical medicines can be assessed *in vitro*: the checkerboard method; combined testing of the bactericidal effect of several antimicrobial agents; E-test; analysis of the bacterial death graph depending on the time of antibiotic action, also known as time-kill assays [15]. Among the available methods of synergism determination, time-kill assays are the gold standard [16, 17], which was first used to confirm the synergism of phage and antibiotics[5]. Interactions detected *in vitro* are calculated and interpreted as synergistic, additive, indifferent or antagonistic depending on whether the antibacterial activity of the drugs in combination is greater, equivalent or less than the activity of the drugs used separately.

Broth microdilutions

In this method, 96-well plates are used in which wells are co-cultured with a broth suspension of bacteria, antibiotic and phage. The phage activity and the minimum inhibitory concentration (MIC) of the antibiotic are studied beforehand, since their sub-inhibitory concentrations are used for synergy studies. The result is evaluated by measuring growth kinetics by optical density (OD) using a spectrophotometer or by bacterial metabolism after staining with tetrazolium, which changes color in response to cellular respiration. Evaluation of the result with a real-time instrument allows to determine the time taken for partial inhibition, to detect late lysis and resumption of bacterial growth. However, it is impossible to infer bacterial viability from the OD alone and to distinguish dead (not yet destroyed bacteria) from live bacteria. Additional staining eliminates the error and allows detection of only metabolically active (live) bacteria. On the one hand, this method makes it possible not only to test any combinations of antibiotics and bacteriophages, but also to change their concentrations. On the other hand, it should be taken into account that the use of a single concentration of antibiotic (half of the previously known MIC) and phage (below the lysing concentration according to Appelman) does not always allow us to draw a conclusion about their interaction and reveal a pattern. At the same time, using a more labor-intensive method, combining several concentrations of antibiotic and phage, it is possible to find those combinations of two antimicrobial agents in which their synergy will be observed [18, 19]. Some researchers have achieved the PAS phenomenon even when the antibiotic was diluted 4, 10 and 100 times the MIC, and 100 and 1000 times the initial concentration of the phage [20].

In some cases, to study synergy, it is possible to use a bacteriophage lysing a bacterial strain of at least 3+, with the antibiotic taken in two concentrations: the MIC and half of the MIC. In case of resistance to the bacterial phage, the antibiotic is added in the maximum permissible concentration [19].

With the use of automated systems, this method allows the construction of sinograms in real time, studying the concentrations of antibiotics and bacteriophage titer. The instrument reads the absorbance value

[3] Rational use of bacteriophages in therapeutic and anti-epidemic practice: Methodological recommendations. Moscow;2022.

[4] Russian recommendations "Determination of sensitivity of microorganisms to antimicrobial agents". Smolensk;2024.

[5] International Organisation for Standardization. Susceptibility testing of infectious agents and evaluation of performance of antimicrobial testing devices. 2019;Part 1.
URL: https://iso.org/standard/70464.html

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from each well as a separate parameter and converts the data into a heat map representing the percentage of bacterial reduction. As a rule, sinograms can be divided into three parts: the area of antibiotic action, the area of bacteriophage action and the area of their joint action, by which it is possible to assess the effect of their interaction (PAS). The use of this method allows visualizing the effectiveness of the combination and selecting the optimal concentration of antibiotic and phage. An additional advantage of this method is the ability to simulate what is happening in the human body when adding biological fluids to the wells [21].

To simplify this technique, I. Nikolic et al. proposed the checkerboard method, which is used to study the interaction of 2 chemical medicines [22]. For more reliable results, the method is implemented in an automated version. The choice of dilution depends on the lytic activity of the phage and the MIC of the antibiotic, so these parameters should be determined in advance before the test. Antibiotic dilutions are added to the wells of a sterile flat-bottom plate from left to right to create a twofold serial decreasing concentration gradient in the range of 8-0.125 of the MIC. A two-fold serial decreasing gradient of phage concentration in the same range is created in the wells from top to bottom, after which a suspension of the test microorganism is added to the plate. The inhibitory concentrations of the antibiotic and phage allow the calculation of the fractional inhibitory concentration index (FIC) using the following formula:

$$\Sigma FIC = \frac{MICac}{MICa} + \frac{MICbc}{MICb},$$

where MICac — MIC of antibiotic combined with bacteriophage, μg/mL; MICa — MIC of antibiotic, μg/mL; MICbc — MIC of bacteriophage combined with antibiotic, MICb — MIC of bacteriophage, μg/mL.

The following results indicated that:

- FIC < 0.5 — synergy (combination of compounds increases the inhibitory activity of one or both compounds);
- FIC = 0.5–4.0 — no interaction (the combination has no increase in MIC due to the additive effect of both compounds);
- FIC > 4 — antagonism (combination of compounds increases MIC) [22, 23].

Broth microdilutions, although considered to be more reliable tests, are more complicated than the use of a solid medium. They require working with large volumes under aseptic conditions, preliminary determination of the MIC and lytic activity of the bacteriophage, and special equipment for continuous bacterial counts at short intervals throughout the day. In the absence of a spectrophotometer, OD measurement can be replaced by quantitative seeding from wells after a day of incubation, which makes this method less accurate and increases labor costs and the turnaround time by at least a day [20, 24]. The described approaches are not standardized in the Russian Federation, and they require a lot of time for staging, which has a limitation for determining the effects of PAS – joint administration of antibiotic and bacteriophage in practical laboratory conditions.

Use of nutrient dense media

Double-layer agar method

The effect of PAS against uropathogenic *E. coli* strain (UPEC) on a dense nutrient medium was first described by A.M. Comeau et al. [25]. They noticed that phage plaques were significantly larger around some antibiotic disks overlaid on the medium seeded in depth with the tested uropathogenic *E. coli* strain and bacteriophage. The authors hypothesized that a sublethal dose of β-lactam antibiotics stimulates phage activity. The results were further confirmed by adding antibiotics at different concentrations to a mixture of *E. coli* and phage, which were all poured together into semi-liquid agar: phage formed small plaques without cefotaxime and large plaques in the presence of the antibiotic at a concentration of 50 ng/mL. When the concentration of antibiotic was further increased, it completely inhibited the growth of the bacterium and the result of phage action could not be studied due to continuous lysis.

The simplicity of the described methodology allowed other researchers to conduct similar experiments using different bacterial strains, phage and antibiotic drugs, combining phages with bacteria or bacteria with an antibiotic in agar, and placing antibiotic disks or bacteriophage drops, respectively, on the surface of the solidified layer [26–28].

E-test

The gradient diffusion method can be used to determine synergy. There are two modifications of this technique. In the first variant, two strips impregnated with antibacterial drugs are placed perpendicularly to each other on a Petri dish seeded with the test culture, intersecting at the MIC level for each antibiotic. Much like with the checkerboard method, the interpretation of the synergy of the E-test is based on the calculation of the FIC index. In the second variant of the test, a strip with the antibiotic is placed on a lawn culture in a Petri dish, after one hour the strip is removed and replaced with a phage-impregnated strip. As a control, a second dish is used in which the antibiotic and bacteriophage strips are overlaid and not in contact with each other. Synergy is defined as a decrease in MIC by at least three 10-fold dilutions, indifference — as a decrease in MIC by at least two 10-fold dilutions, antagonism — as an increase in MIC by three or more 10-fold dilutions [15].

Disk-diffusion method

In this variant, a bacterial culture (0.5 McF) with bacteriophage (10^8 PFU/mL) is incubated for a day be-

fore the classical disk-diffusion method, after which a daily culture on a dense medium is obtained. The daily culture without pre-incubation with phage is used as control. Determination of antibiotic and phage synergy by this method is difficult because the diameter of growth retardation around the disk with antibiotic changes insignificantly [29]. The disadvantages of the method also include the double consumption of standard disks due to the use of controls.

Conclusion

The analysis of available sources shows that there are currently no available and reproducible methods to determine the interaction between bacteriophages and antibiotics in routine laboratory practice. When comparing known methods, it is not possible to obtain their 100% correlation; the coincidence varies from 44 to 88% when comparing time-kill assays with the checkerboard method, from 63 to 75% – when comparing time-kill assays with the E-test and about 90% – when comparing the E-test with the checkerboard method. Most of the studies propose an author's method without comparison with the existing ones, and use only one species and strain of microorganism as a test strain. At the same time, phage and antibiotic interactions depend not only on the selected drugs, but also on the test strain within the same species. Studies have shown that even predictions derived from artificial intelligence and machine learning require double-checking in the laboratory before treatment [15]. And although putative mechanisms of synergistic action of phages with antibiotics that either do or do not induce SOS repair have been described6, to answer the question whether phages can be combined with antibiotics to treat an infection caused by a particular strain, *in vitro* testing must be performed each time. To determine the sensitivity of bacteria to the combination of antibiotics and phages, all virulent bacteriophages should be included in the study, even if the bacteria are initially insensitive to them, since the restoration of strain sensitivity to phages in the presence of antibiotics and the manifestation of synergy of 2 drugs have been described. One of the new areas of research is the study of mechanisms of joint action of antibiotics and moderate phages, which have always been considered as an insurmountable obstacle to therapy. Synergy has already been described in 7 antibiotic groups with moderate bacteriophage [30].

One of the key objectives of the microbiology laboratory is to provide reliable information on the use of antimicrobial agents, including their combinations, for the treatment of infectious diseases. The methods by which a laboratory assesses sensitivity to antibiotics and bacteriophages individually are highly standardized and reproducible. It is this reproducibility that allows laboratories to obtain comparable results. Given that it is impossible to predict empirically the interaction between antibiotic and phage, and the combination of bacteriophages and antibiotics can cause both positive and negative shifts in chemopreventive MIC changes, it is necessary to develop the simplest possible methodology with a clear protocol and accessible equipment that can be implemented in any microbiology laboratory.

[6] A bacterial defense system that is activated in response to DNA damage or inhibition of replication and triggers a complex chain of defense reactions. SOS (save our souls) is an international distress signal in radiotelegraphic communication using Morse code.

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