Evaluation of phage cocktail efficacy for controlling infections caused by pathogenic *Escherichia coli* in *in vivo* experiments

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Abstract. Colibacillosis is a common veterinary infection that causes huge economic losses to the livestock and poultry industry. Uncontrolled use of antibiotics has led to the emergence and spread of multidrug resistant bacteria and has reduced the effectiveness of standard treatment regimens for infectious diseases. This stimulated the renewed interest in bacteriophages among scientists since the beginning of the 21st century. Bacteriophages are widespread in nature and accompany bacteria in every environment they colonize, including the microbiota of warm-blooded animals. The purpose of the research was to study the therapeutic activity of a cocktail created on the basis of bacteriophages isolated in the territory of the Republic of Kazakhstan. The therapeutic activity of the phage cocktail was determined through experimental infection of chickens with a pathogenic strain of *E. coli*. The *in vivo* experiments showed that the administration of the bacteriophage cocktail enabled 86% of the chickens to survive after being infected with lethal doses of pathogenic *E. coli*. They also revealed that the frequency of phage cocktail administration did not affect its efficacy. Thus, the investigated phage cocktail is a promising candidate for the creation of biotechnological antibacterial agents on its basis for safe and effective treatment of poultry against colibacillosis.

1 Introduction

Escherichia coli is a symbiotic agent that can acquire certain virulence traits and cause a wide range of diseases in domestic animals, birds and humans. Based on the presence of these traits and the site of infection, these strains have been classified into 11 pathotypes [1, 2]. Strains pathogenic for livestock and poultry can cause significant damage to agriculture by causing mass diseases among livestock, resulting in reduction of body weight gain or even death. Significant financial expenses are incurred in the course of treating the sick animals, but both the disease itself and the therapeutic measures reduce the quality of the final product and, in addition to the financial losses, may create health risks for the consumer [3].

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The causal relationship between pathogenic strains of *Escherichia coli* and disease states in various bird species was known more than a century ago [4], but these strains have never received a special status. Today, systemic diseases of poultry caused by pathogenic strains of *E. coli* are called avian colibacillosis. Outbreaks of colibacillosis can cause considerable economic losses to poultry farms worldwide. In the presence of predisposing factors such as a weakened or compromised immune system and the presence of virulence factors of the strain, the disease can occur in the form of acute sepsis in chickens aged 1–10 days. Colibacillosis can be controlled with antimicrobial agents. A wide variety of antimicrobials are used in poultry production in most countries [5, 6] not only to treat the disease but also to prevent it in order to continuously increase growth and productivity [7]. However, uncontrolled use of antibiotics has dramatically increased the incidence of resistance among bacterial pathogens in animals and humans, resulting in less effective treatment of bacterial infections and an increased risk of mortality. The problem of spreading antibiotic resistance has already reached such a scale that according to WHO forecasts, by 2050 humanity will no longer have effective means of fighting bacterial infections, and infectious diseases will regain the primacy among the causes of mortality as at the time before the discovery of antibiotics [8]. The excessive and uncontrolled use of antibacterial preparations in livestock production has increased the likelihood of antimicrobial resistance not only in pathogenic but also in commensal microflora. Concerns about antimicrobial resistance of bacteria in poultry production are complemented by human health concerns associated with the presence of antibiotic residues in food products of animal origin, such as milk, meat and eggs [9]. Due to the spread of antibiotic resistance and restrictions on the use of antibiotics themselves, there is a need to find and utilize alternative methods to combat bacterial infections. Bacteriophages are best suited for this role, and with the necessary research, phage therapy may become an effective means of combating antibiotic-resistant bacterial infections [10-12]. Given the high level of specificity of bacteriophages, it is common in the world practice to use a mixture of different bacteriophage strains (a phage cocktail) in phage therapy, as it significantly increases the efficacy of phage therapy and the range of its application.

The aim of this research was to study the therapeutic activity of phage cocktail in experimental infection of chickens with a pathogenic strain of *E. coli*.

2 Materials and methods

The study used a previously archived avian pathogenic *E. coli* (APEC) isolated from diseased chickens with signs of escherichiosis collected between 2020 and 2021 from private farms in Almaty Oblast in Kazakhstan. The bacterial isolate F.H.pr. Uz was confirmed as an *E. coli* using the Biolog GEN III biochemical test panel, according to the manufacturer's protocol (Biolog, USA). The presence of virulence genes most commonly associated with avian pathogenic *E. coli* were determined by PCR [13]. *E. coli* isolate was stored at 4°C in the collection of the Research and Production Center of Microbiology and Virology.

The cocktail against the pathogenic *E. coli* strain F.H.pr.Uz was composed of phage lysates containing in equal ratio 10 lytic bacteriophages isolated on *E. coli* strains belonging to different phylogroups (B1, B2 and D). The bacteriophages used included representatives of all three major morphotypes of the *Caudoviricetes* class. The total titer of the phage cocktail was 10^8 viral particles per 1 ml.

The therapeutic activity of phage cocktail was studied by experimental infection of healthy 1-day-old chickens (Leghorn breed) with a lethal dose of the pathogenic *E.coli* strain F.H.m/l Uz. A 0.5 ml bacterial suspension with a turbidity index of 1 McF was injected into the abdominal cavity. After that, the chickens were intraperitoneally injected

with the phage cocktail once, twice and three times; a single therapeutic dose of the cocktail was 0.5 ml per chicken; the titer of viral particles in the cocktail was 10^8 per 1 ml. As a result, 3 experimental groups of chickens were formed: the first group of chickens was given the cocktail 1 time, the second group of chickens was given the cocktail 2 times, and the third group of chickens was given the cocktail 3 times after being infected with the *E. coli*. The time intervals for double and triple administration were 24 hours. Thus, 3 experimental groups and 2 control groups were formed (7 chickens in each group), with the control groups of chickens (not exposed to phage cocktail) kept in a separate room. All three experimental groups of chickens were infected with the pathogenic strain *E. coli* F.H.pr. Uz., after which, depending on the group, single, double and triple therapeutic doses of phage cocktail were administered. The first control group of chickens was infected with the pathogenic strain of *E. coli* F.H.pr.Uz., but instead of the phage cocktail, chickens of this group were injected intraperitoneally with sterile phosphate buffered saline (placebo). The second control group was not infected and was only injected with sterile phosphate buffered saline (placebo). The chickens were observed for 5 days after the infection, during which time feed (commercial grower feed) and water were unlimited. Therapeutic activity was assessed by the number of surviving chickens.

Statistical processing of the obtained results and plotting of graphs were performed in Microsoft Excel. The unpaired Student's t-test was used to determine the difference between experimental and control samples. The obtained data were expressed as mean values ± standard deviation (SD). P values less than 0.05 were considered statistically significant.

All protocols in this study were agreed upon and approved by the Local Ethical Commission of the Research and Production Center for Microbiology and Virology, Almaty, Kazakhstan (LEC No. 02-12-107 dated 26.07.2021, Appendix B), in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revision 1996).

3 Results and discussion

As a result of the experiments performed, it was shown (Figure 1) that on the second day after experimental infection of chickens with lethal doses of *E. coli* F.H.pr.Uz, in the control group, where placebo was administered instead of phage cocktail, the mortality rate was 86%, whereas in the experimental groups receiving therapeutic doses of phage cocktail, the survival rate was 100%. After 5 days of observation, it was found that 86% of chickens survived in the groups of 1-fold and 3-fold administration of phage cocktail, 60% survived in the group of 2-fold administration, all chickens survived in the control group that got only placebo, and 14% of chickens survived in the group that was infected with pathogenic *E. coli* but did not receive the phage cocktail.

Immediately after the discovery of bacteriophages, Felix d'Hérelle began to study the possibility of using them to treat bacterial infections. He conducted laboratory studies on the use of bacteriophages against rabbit dysentery (*Shigella dysenteriae*) and successful field trials on the treatment of chicken typhoid fever (*Salmonella enterica* subspecies enterica serovar Gallinarum) with phages [14]. Further studies on the antibacterial activity of bacteriophages have shown high efficacy against foodborne pathogens such as *Escherichia coli*, *Campylobacter* and *Salmonella* [15, 16]. Thus, the pioneering work of Smith and Huggins experimentally proved that a single dose of phage R effectively prevented mortality in mice with sepsis caused by an *E. coli* resistant to higher doses of an antibiotic. Continuing their research, they demonstrated the efficacy of phage therapy in the treatment of newborn lambs, calves and pigs for infections caused by enterotoxigenic *Escherichia coli* [17, 18].

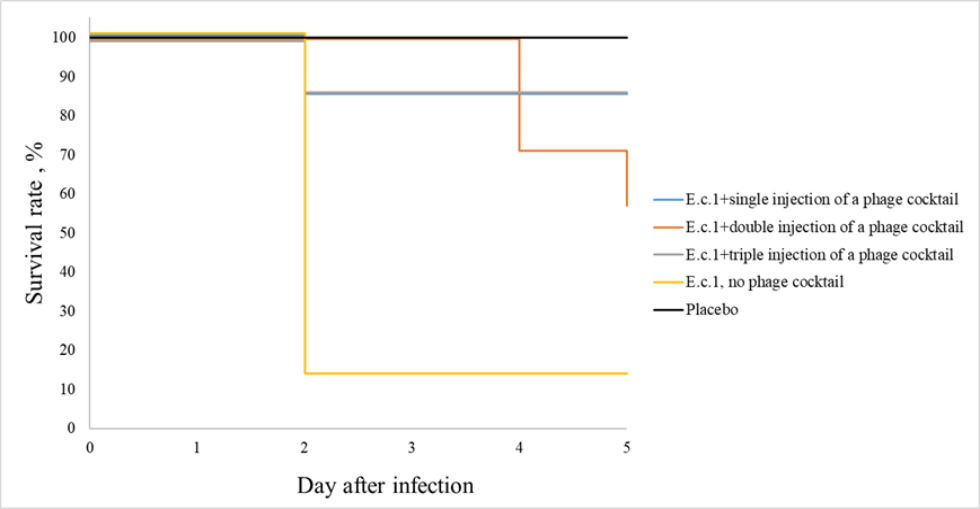

Fig. 1. Survival rate of chickens during experimental infection with pathogenic strain of *E. coli* 1 (strain F.H.pr.Uz) followed by therapeutic use of a phage cocktail

Huff et al. demonstrated the efficacy of using a mixture of investigational phages with different titers to reduce mortality in chickens with colibacillosis [19].

In our work, we investigated the therapeutic potential of a phage cocktail containing a mixture of isolated bacteriophages capable of lysing pathogenic strains of *E. coli*. As a result, it was found that therapeutic application of the phage preparation by intraperitoneal injection significantly increases the survival rate of chickens in experimental infection with pathogenic strains of *E. coli*. Moreover, a single injection of the phage cocktail demonstrated maximum efficacy, while an increase in the frequency of phage administration did not lead to an increase in the number of surviving chickens. This fact can be explained by the high titer of bacteriophages, which is sufficient for rapid elimination of the target bacteria at a single injection. Some studies have demonstrated that injection of phage preparations is the most effective route of administration compared to oral administration. Injection of phage preparations also ensures their faster delivery to the focus of infection. Thus, active phages can be detected in the blood circulation within the first five minutes after their injection [20 -22]. In experiments on protection of mice from lethal septicemia caused by *Pseudomonas aeruginosa* it was shown that in comparison with intramuscular or subcutaneous administration, intraperitoneal administration of the investigated bacteriophages resulted in faster accumulation of high phage titers in blood, which contributed to the efficacy of phage therapy [23].

4 Conclusion

The emergence and dissemination of drug-resistant pathogens that have evolved new mechanisms of antimicrobial resistance continues to limit our ability to treat common infections in both human and veterinary medicine. A particularly alarming trend is the rapid spread of so-called “superbugs” - bacteria that have multiple or complete resistance to currently existing antibiotics. In this regard, there was an urgent need to search for alternative ways to combat bacterial infections that would allow overcoming the phenomenon of antibiotic resistance. These searches have led to the revival of phage therapy and, as a consequence, to new studies into possible uses of bacteriophages as

supplements or alternatives to antibiotics. Various studies show that bacteriophages have antimicrobial activity, are effective in combating bacterial infections not only in vitro but also in vivo, and are a promising alternative to antibiotics. It should also be noted that phage therapy is a harmless, highly specific and effective method of combating pathogenic bacteria; moreover, bacteriophages easily multiply and persist for a long time.

Our experiments showed that the investigated phage cocktail is a promising candidate for the creation of antibacterial agents on its basis for safe and effective treatment of poultry colibacillosis. Such preparations are especially relevant for infections caused by antibiotic-resistant forms of bacteria. However, given the high specificity to hosts, there is a need for constant search for new strains of bacteriophages, as creation and preservation of an extensive collection of bacteriophages will allow to promptly respond to outbreaks of bacterial infections by using lytically active strains of phages. Also, an extensive collection of phages will make it possible to develop phage cocktails that can most effectively fight bacterial infections in each specific case.

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