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Determination of lytic activity of *coli*-bacteriophage

Davitashvili Magda *, Zuroshvili Lamara and Natsvlishvili Nana

Iakob Gogebashvili Telavi State University, 1, Kartuli Universiteti str., Telavi, 2200, Georgia.

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Abstract

The standard system has been elaborated for the control of lytic activity of the commercial series - active *coli*-bacteriophage and respective stable *E. coli* strains' collection. Special investigations were carried out with an aim to obtain polyvalent bacteriophage, which should comply with the requirements for the standard preparations. While passing of the active phage on the highly reproducible standard strain the coli-bacteriophage phage-lysate was obtained. With an aim to preserve its basic properties the phage-lysate has been lyophilized. The 20% Difco-peptone was used as the stabilizer. The lyophilized bacteriophage completely satisfies the parameter standards for biological preparations: it has high lytic activity, wide range of action, and thermal resistance. These properties are reliably preserved for the period of three years. It was shown experimentally that the lyophilized cultures could be used for evaluation of the lytic activity in bacteriophages. The identical phage-sensitivity was revealed in both lyophilized- and agar-cultures.

Keywords: *E. coli* strains; *Coli*-Bacteriophage; Stabilizers; Lyophilization; Branch Standard Specimen

1. Introduction

Improvement of quality of the biological preparations is one of the most pressing problems of the medical industry. It is obvious that the best efficient means for improvement of the product quality is assessment of its activity in parallel with the standard specimens. For a number of biological preparations a vast experience of standardization has been accumulated and International and National standards have been adopted [1, 2].

In the recent years the nomenclature of curative and disease-preventing bacteriophages has increased enormously, technology of their preparation became more diverse, which leads to more increasing diversity of preparations' quality when they are manufactured in different enterprises. The methods for testing of the manufactured series of bacteriophages, which are stipulated by the official documentation, do not ensure their sufficient unification [3, 4, 5].

Considering the foregoing, it is obvious that presence of the standard specimens of bacteriophages would provide a workable means for manufacturing of commercial series with unified parameters and higher quality. Hence, the goal of our investigation was elaboration and creation of the standard system (the Branch Standard Specimen (BSS) and collection of *Escherichia coli* standard strains) for activity assessment in the manufactured series of bacteriophages [6, 7, 8, 9].

2. Material and methods

Total of 317 enteropathogenic strains of *E. coli*, which were isolated in various geographical zones of the country and donated by the laboratories investigating industrial strains in Tbilisi National Center for Disease Control and Public Health.

* Corresponding author: Davitashvili Magda

The serologic identification of the obtained *E. coli* strains was performed with an aid of diagnostical agglutinating escherichiosis adsorbed group 0 and polyvalent sera, in reaction of agglutination on the glass.

A new *E. coli* phage, isolated from the sewage waters of Kakheti region, served as an initial material for production of the polyvalent bacteriophage [10].

The following stabilizers were used during lyophilization of the phages:

Polyethylenoxide-400 (PEO-400), Polyethylen-glycole-400 (PEG-400), Dimethylsulphateoxide (DMSO) in a concentration of 0.25 M, the saccharose-gelatin (SG) medium (s - 90%, g - 10%) and 20% Difco-peptone.

The cryoprotectants were mixed with the phage filtrate in an even quantities (1:1), except for the SG medium (9:1), and divided into the 1 ml ampoules.

Lyophilization of the phages was made in the TG-15 (Germany) instrument, with preliminary freezing during 24 hours (at -40°C). The total duration, of drying was 48 h, the maximal temperature in the instrument was 35°C. The ampoules were soldered under the vacuum, at 200 μPa.

The content of an ampoule with the dry phage was dissolved in 1 ml of the nutrient broth, then the successive ten-fold dilutions were prepared to the 10^{-9} value, in 4.5 ml of the broth and of 0.5 ml of the previous dilution of the phage.

In the course of drying the *E. coli* strains the sucrose-gelatin (s - 1%, g-10%) medium was used as a protective medium. The concentration of lyophilized microbial mass, dispensed into the 1 ml ampoules, was 10 billion microbial bodies per 1 ml.

Lyophilization of the strains was carried out in LZ-9 apparatus. Regime of lyophilization was as follows: freezing temperature: -40°C, duration - 3 h, maximal temperature in the apparatus +35°C, drying time - 36 h, vacuum at soldering - 200 μPa.

For experimentation the content of ampoules with dry cultures was diluted in 1 ml of the nutrient broth. Concentration of the microbial bodies, after the 10-fold dilution, was 10^9/1 ml. Obtained bacterial suspension (0.03 ml) was added to the test-tubes with diluted bacteriophage [11, 12].

Preliminary inoculation of the cultures on the agar medium was not done.

3. Results and Discussion

With an aim to obtain an active medicinal-preventive *coli*-bacteriophage, which could serve as a basis for creation of standard preparation, the thorough examination of the *E. coli* strains was done. All the strains employed for experimental work had the typical morphologic, cultural and enzyme properties.

For elaboration of the polyvalent *coli*-bacteriophage with high lytic activity, out of the examined cultures 36 strains were chosen as "tutors" for the following serologic types: 054, 0111, 019, 025, 0150, 0124, 0129, 043, 0115, 0127, 0144, 032, 021, 024, 0144, 087, 052, 0160, 074.

Total of 55 specimens of the sewage waters were investigated, out of which active phages against 93% of our *coli*-strains (200 specimens) were isolated.

After selection of the phage races and "tutor"-strains, the polyvalent *coli*-bacteriophage - candidate for BSS - was prepared.

With an aim to increase the stability of the phage, and bearing in mind the requirements of the WHO brought forward to the standard preparations, its lyophilic drying was performed [4, 10]. The choice of the lyophilization regime and the optimal concentration and brand of cryoprotectant was made according to the preliminary experiments, in which the small amounts of phage lysates were dried. Then each variety was investigated by the Appelman method. The initial *coli*-phage filtrate was used as a control. The results of investigation of the phage activity dried in a presence of various stabilizers are presented in Table 1.

Table 1 Lytic activity of the lyophilized *coli*-bacteriophages

NN	Strains of *E. coli*	Bacteriophages' titers				
		Before drying	Following drying in a presence of various protective media			
			20% Difco peptone	PEO-400	PEG-400	"SG"-medium S-90%, G-10%
-	054	10^{-8}	10^{-7}	10^{-7}	10^{-7}	10^{-5}
-	019	10^{-7}	10^{-6}	10^{-6}	10^{-6}	10^{-5}
-	0150	10^{-8}	10^{-7}	10^{-7}	10^{-7}	10^{-6}
5	0150	10^{-8}	10^{-7}	10^{-7}	10^{-6}	10^{-6}
-	0160	10^{-7}	10^{-6}	10^{-6}	10^{-6}	10^{-5}
-	0124	10^{-8}	10^{-7}	10^{-7}	10^{-6}	10^{-5}
8	n.t.	10^{-8}	10^{-7}	10^{-7}	10^{-6}	10^{-5}
-	n.t.	10^{-7}	10^{-7}	10^{-6}	10^{-6}	10^{-5}
-	021	10^{-8}	10^{-7}	10^{-7}	10^{-6}	10^{-5}
0	0124	10^{-7}	10^{-7}	10^{-6}	10^{-6}	10^{-5}

As could be seen from the presented material, the specimens of the bacteriophages, in the presence of the cryoprotectants - 20% Difco-peptone, PEO-400 and PEG-400, completely retained their activity after the lyophilization, while in the presence of SG some decrease of the phage titer was observed. However, some difference was observed after storage of dry phages: those phages, which were dried in the presence of 20% Difco-peptone, did not differ from the initial material, while those lyophilized in the presence of PEO-400, PBG-400 and SG-medium, considerably lost their activity.

Hence, out of above cryoprotectants the 20% Difco-peptone was chosen as the most optimal medium for the *coli*-bacteriophage!

The filtrate of experimental phage was mixed with 20% Difco-peptone in a 1/1 ratio, and then dispensed into 1 ml ampoules. Content of the ampoules was lyophilized according to the regime established in the earlier experiments. The lyophilized *coli*-bacteriophage, proposed here as a BSS, presents a dry mass of white color. The residual humidity did not exceed 1.4%. Variation of the dry substance mass in ampoules was within 0.11-0.14 g range.

Investigation of the lyophilized *coli*-bacteriophage lytic activity has shown its high quality on both broth – 10^{-7} - 10^{-8} (Appelman method) and solid nutrient medium - 5.10^{7} - 6.10^{8} (Grazia method). The preparation titer, according to Appelman, was higher than the minimal value required by official documents (TU 42 No 175-67, for the commercial series of *coli*-bacteriophages, stipulated titer 10^{-5}).

No less important was establishing the range of lytic activity of standard *coli*-bacteriophage specimen against the newly-isolated strains of *E. coli*. The bacteriophage titer in 127 strains of *E. coli* was 10^{-6} – 10^{-7}, in 153 strains – 10^{-5}, and in 37 strains – 10^{-3} -10^{-4} (Appelman titer); the Grazia titers were, respectively, - $5{\cdot}10^{6}$ - $4{\cdot}10^{7}$, $4{\cdot}10^{5}$, $2{\cdot}10^{3}$ - $5{\cdot}10^{4}$. The obtained data allow to claim that presented bacteriophage, which is intended as a standard specimen, possesses high lytic activity against *E. coli* strains and has a wide range of lytic activity.

Investigation of the thermal stability of the manufactured preparation was performed by heating at 60°C, 75°C and 100°C, for one hour. It was found that the phage was highly thermoresistant and even after boiling of the specimens (at 100°C) the activity was preserved in 20%-40% of the phage particles.

Assessment of the phage stability was carried out after storing of the ampoules for several years at 4°C and 37°C. The titers of the preparation following one year of storage did not differ from the initial values and they did not depend on

the temperature regime. Following three years of storage the titer was still higher than the minimal value (10^{-5}) stipulated by the technical proviso for the commercial *coli*-bacteriophages.

Thus, the characterization of the lyophilized *coli*-bacteriophage permits to assess it as a highly active and thermoresistant preparation with wide range of action, contains negligible moisture and is tightly sealed. All the above-mentioned allows to claim that the manufactured phage complies with requirements for standard biological preparations. One of the elements of the standard control system for *coli*-bacteriophage is a set of *E. coli* strains with stable properties. According to a number of investigators the lyophilized strains preserve their morphological, antigenic and virulent properties for a long time. In the present investigation an attempt was made to study the feasibility of employing of lyophilized *E. coli* cultures during titration of the *coli*-bacteriophage by the Appelman method, without preliminary inoculation on the agar media. The analysis of the obtained results has shown the high phage-sensitivity and identity of the *coli*-bacteriophage activity in a course of titration in agar- and lyophilized cultures, during three years of the observation.

4. Conclusion

Therefore, our investigations resulted in preparation of the Standard System (BSS of *coli*-bacteriophage and the collection of standard strains of *E. coli*) for assessment the lytic activity of commercial series of *coli*-bacteriophages, which, in its turn, will provide for increasing of the product quality.

Compliance with ethical standards

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Disclosure of conflict of interest

There is no conflict of interest amongst the authors.

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