CHARACTERISTICS OF THE VIABILITY AND ACCORDANCE WITH THE TAXONOMIC STATUS OF THE LYOPHILIZED SAMPLES OF MUSEUM STRAINS OF ESCHERICHIA COLI ISOLATED IN 1946-1959 YEARS

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The many years of existence of microorganism collections in museums of many countries of the world have shown the great significance of their research for the study of microorganisms' evolution laws, the microbiological monitoring of the infectious diseases agents, production of novel diagnostic, therapeutic and prophylactic agents, as well as for the development of the medical science as a whole. [1, 2]. Effective conservation of microorganisms with the aim of long-term storage of strains in collections without changes in morphological, physiological and genetic properties is ensured with the methods that allow the shift of the vegetative forms of bacterial cells to the state of anabiosis. The most widespread among them are the deep cryogenic freezing of the microorganisms or their drying from frozen (lyophilized) or liquid (L-drying) state [3, 4].

The museum of microorganisms of the State Establishment "Mechnikov Institute of Microbiology and Immunology of the National Academy of Medical Sciences of Ukraine" has one of the oldest microorganisms collections in Europe that exist from the moment of the Institute foundation (May 1886 y.). The main collection funds were formed since the year 1915, new strains were constantly added in different years and at present there are more than 4000 lyophilized microorganisms strains in the collection.

Some researchers have established that lyophilization allows supporting the strains of the collection fund without losing the viability for a prolonged time period (more than 50 years) [5, 6], but studies devoted to the efficacy of conservation of microorganisms of different species are extremely important, as they represent a significant theoretical and practical investment into the preservation of the species diversity of the microflora [7].

The aim of the study was to test the viability and species-specific properties of the *E. coli* museum strains that were stored in the lyophilized state for a prolonged time period.

Materials and methods. The objects of the study were 30 lyophilized samples of the 22 strains of *E. coli* from Microorganisms museum of the SE "IMI NAMS" that were preserved in the collection for 14 to 61 years. The provision of the strains is represented in the table 1. Lyophilized cultures were restored with by dilution of the ampule content with 1,0 ml nutrient broth and the seeding of the microbial suspension from 10-times dilutions onto the agar-based nutritive media (blood agar, Endo medium); the viability was determined by the colony forming units (CFU/ml) count. During the evaluation of the survival parameters of the collection bacterial cultures it was taken into account that for preparation of the lyophilized samples biomaterials suspensions with the microorganism content no lower than 10^9 CFU/ml were used. Re-identification of the microbial cultures was carried out with the use of the API system produced by «Bio-Merieux», France (ID 32 GN – for enterobacteria identification).

Table 1. Provision of the studied *E. coli* strains

№ п/п	Strain name	Inv. number	Place and year of isolation	Where was the strain from, year of arrival
1.	*E. coli* 637	01003	IMI[1], Kharkiv, 1956 y.	IMI[1], Kharkiv, 1956y.
2.	*E. coli* 321	01005	IMI[1], Kharkiv, 1957 y.	IMI[1], Kharkiv, 1957 y.
3.	*E. coli* 26	01009	IVS[2], Moscow, 1957 y.	IVS[2], Moscow, 1957 y.
4.	*E. coli* 561	01011	IMI[1], Kharkiv, 1957 y.	IMI[1], Kharkiv, 1957 y.
5.	*E. coli* 168	01012	IMI[1], Kharkiv, 1957 y.	IMI[1], Kharkiv, 1957 y.
6.	*E. coli* 126	01014	Bulgaria, 1957 y.	SISC[6], 1957 y.
7.	*E. coli* Cigleris	01015	Kopenhagen, 1958 y., Kaufman collection	SISK[6], 1959 y.
8.	*E. coli* 4932-53	01016	Kopenhagen, 1958 y., Kaufmann collection	SISK[6], 1959 y.
9.	*E. coli* Canioni	01017	Kopenhagen, 1958 y., Kaufmann collection	SISK[6], 1961 y.
10.	*E. coli* O86, B17	01018	Sweden, 1946 y.	SISK[6], 1959 y.
11.	*E. coli* 537-52	01019	Kopenhagen, 1958 y., Kaufmann collection	SISK[6], 1959 y.
12.	*E. coli* 113-3	01020	Place of isolation unknow	SISK[6], 1958 y.
13.	*E. coli*	01021	IEM (Ek)[3], 1946 y.	SISK[6], 1959 y.
14.	*E. coli* 408 «Новгородская»	01022	IVS (S-P)[4], 1947 y.	SISK[6], 1959 y.
15.	*E. coli* F 103	01023	Kopenhagen, 1958 y., Kaufmann collection	SISK[6], 1959 y.

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16.	*E. coli* 27	01024	Hungary, 1955 y.	SISK[6], 1959 y.
17.	*E. coli* 26	01025	Hungary, 1955 y.	SISK[6], 1961 y.
18.	*E. coli* 145	01026	IMEH (M)[5], 1958 y.	SISK[6], 1959 y.
19.	*E. coli* 28	01028	Hungary, 1955 y.	SISK[6], 1959 y.
20.	*E. coli* 3219-54	01029	Kopenhagen, 1958 y., Kaufmann collection	SISK[6], 1961 y.
21.	*E. coli* 10244	01030	IMI[1], Kharkiv, 1959 y.	IMI[1], Kharkiv, 1963 y.
22.	*E. coli* O55	01030	Kopenhagen, 1953 y., Kaufmann collection	IEID[7] (K), 2004 y.

Notes: 1- Mechnikov Institute of Microbiology and Immunology, Kharkiv; 2-Institute of Vaccines and Serums, Moscow; 3- Institute of Epidemiology and microbiology, Ekaterinburg; 4- L. Pasteur Institute of Vaccines and Serums, St. Petersburg; 5- Institute of microbiology, epidemiology and hygiene, Moscow; 6- L. O. Tarasevich State Institute of standardization and control; 7- L. V. Gromaschevsky Institute of epidemiology and infectious diseases.

The phenotypic intra-strain heterogeneity of the population was evaluated according to the index dissociation parameter, that reflects the percentage (%) of the certain colony forms (S-, R-, D-, M- forms) from the total count.

The statistical analysis of the obtained data as carried out with the help of parametric statistic method with the use of computer programs Microsoft Excel 2007, STATISTICA 6.0. The confidence level of the differences was evaluated with the help of the Student criteria. The establishment of the relationships between variables was carried out with the help of correlation analysis.

Results and discussion

The viability of the samples from 1957, 1958, 1961, 1964, 1967, 1971, 1973, 2002 and 2004 years of lyophilization was completely restored. The third of the samples from 1959, 1963, 1966, 1970, 1972 years of lyophilization and all samples prepared in 2003 were found to be unviable. Taken together, there were (66,7±8,6) % viable *E. coli*. samples among all samples studied in course of the experiment. Therefore, 20 samples of *E. coli* strains were used for the further study.

It was established that the quantity of the colonies on the solid nutritive media in the restored strains varied in the range from 10^4 to 10^9 CFU/ml, the survivability parameter average was (26,7±4,6) %. It should be noted that the 100% preservation of the growth properties that corresponds to the quantity of viable cells 10^9 CFU/ml was observed in 2 (10,0±3,5) % strains, those were strains of *E. coli* 637 (01003) and *E. coli 561* (01011) from 1972 and 1970 years respectively. The lowest survivability values that were 0,001 % and 0,1 % respectively were observed in *E. coli* Cigleris (01015), from 2004 year of lyophilization and *E. coli* 113-3 (01020), from the 2002 year of lyophilization. In the majority of strain samples the survivability values were between 5,0 % and 50,0 %. During the statistical analysis of the results there were no correlation between the quantity of CFU and the length of the storage period (r=0). For example, in the strain *E. coli* 27 (01024), isolated in Hungary in the year 1955 and in the strain *E. coli* 321 (01005), isolated in Ukraine in 1957 year that were stored in lyophilized state for more than 60 years, the quantity of viable cells after re-cultivation was near the maximal levels and was equal to $5x10^8$ CFU/ml. It should be noted that the results obtained are in accordance with the reports of other researches. For instance, according to the data from M. B. Kupletska et al. [6], lyophilization allows to store the strains of different taxonomic groups for more than 50 years with the high content of viable cells (10^6 -10^9 CFU/ml) in samples.

The study of the morphological and tinctorial properties of the restored *E. coli* strains has shown that the cells had a rod-like form, stained Gram-negative, and were arranged separately, in pairs or in groups in the samples

The growth of microorganisms in the liquid nutritive media (meat infusion broth) was characterized by diffuse obfuscation that was accompanied by the formation of weakly expressed sediment on the bottom of the test tube and delicate film on the surface of the broth on the second day of cultivation.

On the Endo medium, (70,0±10,2) % formed red colonies with metallic sheen, (15,0±9,7) % – raspberry-colored colonies without metallic sheen and the rest of the strains formed colonies of the rose color. Almost half of the studied strains were characterized by hemolytic phenotype in case of growth on blood agar, and strains with α-hemolytic activity were dominant (62,5±17,1) %.

In case of growth of the experimental strains on the solid nutritive media the variability in sizes and forms of the colonies was observed: small, 1-2 mm in diameter – D-form, medium (3-5 mm), voluminous, smooth, with smooth edges – S-form; big, flat, rough - R-form; mucous colonies – M-form. It was established that the specific weight of strains without featured of colonial dissociation (S-form) constituted only (10,0±3,5) %, the overall majority of the strains (90,0±3,8) % were characterized by dissociation into the different colonial and morphological variants. The quantity of the strains with the monomorphic phenotype of R-form and M-from constituted (5,0±2,9) % each, D-form – (10,0±3,5) %. Population polymorphism was observed in the rest of the studied strains – the presence of the S and R- form colonies, while the specific weight of the strains with the S – form prevalence was (64,3±12,8) %, R-form – (35,7±12,8) %. The index of dissociation values (ID) of the microbial population in the studied Escherichia strains varied from 10,0 % to 90,0 %. During the statistical analysis of the data the correlation between the dissociation index and the length of the storage of the sample in the lyophilized state was established (r>0,95).

The observed colonial polymorphism of the studied *E. Coli* strains, in our opinion, is caused by the

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adaptation to the stressful conditions and contributes to the survival of the bacterial population during the long-term storage in the lyophilized state. According to the opinion of some researchers [8], the heterogenous system ensures the greater resistance to the influence of negative factors and the distribution to the different colonial forms, as the variant of directed reconstruction of the bacterial population, leads to the increase of the species survival boundaries. This is the biological value of the dissociation process.

According to the results of the re-identification in the majority of samples, in (90,0±3,5) % of the samples the species identity was in accordance with the strain passport. Two strains that according to the overall results of biochemical tests did not correspond to their initial identification were the exception: the strain *E. coli* 168, inv. № (01012), that was isolated in the year 1957 in Kharkiv IMI (Kh) was identified as *Klebsiella pneumoniae*; the biochemical profile of the strain *E. coli* 537-52, inv. № (01019) from the Kaufmann collection (Kopenhagen, Denmark), isolated in 1958 corresponded to the *Enterobacter aerogenes* profile. In our opinion, the established taxonomic differences could be related to the fact that in the middle of the previous century the biochemical identification criteria of E. coli were different from the rules of the modern microbiological systematics. Moreover, before publication of the first edition of the "Confirmed bacterial names list" in 1980 (Skerman et al., 1980) the same bacteria could have different names.

The results of re-identification using API system ID 32 GN for the enterobacteria identification shows the concordance of the biochemical properties with the *Escherichia coli* typical profile for 18 (90,0±3,8) % of the studied strains (Bergey's manual, 2009) [9].

Conclusions

1. The restoring of lyophilized cultures of *E. coli* of 1946-1959 yy. of isolation has established the viability of the majority of samples, the viability values were in the range of 0,001 % - 100,0 %.
2. It was established that the majority (90,0±3,8)% of restored strains populations were characterized by dissociation for different colonial and morphological variants.
3. Based on the identification results, the taxonomic status of two studied strains was established and corrections were made in their passports.

The perspectives of further studies

It is planned to continue the studies further and analyze the antibiotic resistance in the collection *E. coli* strains that were isolated in the different historical periods of antibiotic use.

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Effective microorganisms' conservation with the aim of long-term storage of the strains in the collection without changes in the morphological, physiological and genetical properties is provided by methods that allow the shift of the vegetative forms of bacterial cells into the anabiosis state. The most widespread among them is the lyophilization method. The museum of microorganisms of the State Establishment "Mechnikov Institute of microbiology and immunology of the National Academy of Medical Sciences of Ukraine" has one of the oldest bacterial collections in Europe that consists of more than 4000 lyophilized samples of microorganisms strains. **The aim** of the study was the testing of viability and species specific properties of the museum strains of *E. coli*, that were preserved in lyophilized state long-term. **Materials and methods.** The objects of the study were the 30 lyophilized samples of the 22 strains of *E. coli.* Lyophilized cultures were restored through dilution of the ampule content in

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the 1,0 ml nutritive broth and seeding of the microbial suspension from the 100-times dilutions onto the agar-based media (blood agar, Endo medium); the viability was determined based on the quantity of the colony forming units (CFU/ml). Re-identification of the microbial cultures was carried out with the use of API system produced by «Bio-Merieux», France (ID 32 GN – for enterobacteria identification). The phenotypic intra-strains heterogeneity of the population was evaluated by dissociation index that reflects the ratio (%) of the certain colony forms (S-, R-, D-, M- forms) compared to the total count. The statistical analysis was carried out based on the parametric statistic methods with the use of Microsoft Excel 2007 and STATISTICA 6.0 computer programs. **Results and discussion.** There were (66,7±8,6) % of the total amount of *E. coli* strains participating in the experiment that were able to be restored, and therefore those samples were selected for further studies. It was established that the quantity of the colonies on the solid nutritive media in the restored strains varied from 10^4 to 10^9 CFU/ml, the average survivability parameter was (26,7±4,6) %. During the statistical analysis of the results no correlations between the CFU count and the storage term were established (r=0). It was established that the majority of the re-cultivated strains (90,0±3,8) % was characterized by dissociation into different colonial and morphological variants. The dissociation index (ID) values in the microbial populations of the studied Escherichia strains varied in the range of 10,0 % to 90,0 %. The statistical analysis of the data has established the presence of correlation between the dissociation index and term of storage of the sample in the lyophilized state (r>0,95). The established colonial polymorphism of the studied strains of *E. coli*, in our opinion, is caused by adaptation to the stressful conditions and leads to the increase of the survivability of the bacterial population in course of the long-term storage in the lyophilized state. According to the re-identification results, the majority (90,0±3,5) % of the samples corresponded to the data indicated in the strains passport, except two strains that did not correspond to their initial identification based on the total of biochemical tests. **Conclusions**. The restoring of the lyophilized cultures of *E. coli* from 1946-1959 yy. of isolation the majority of samples was found to be viable and the viability varied in the range of 0,001 % to 100,0 %. It was established that the populations (90,0±3,8) % of restored strains were characterized by dissociation into different colonial and morphological variants. Based on the re-identification results of *E. coli* the correct identification of strains was carried out and corrections were put in their passports. **Further studies perspectives.** It is planned to study the sensitivity to antibiotics of the collection strains of *E. coli*, isolated in the different periods of antibiotic use.

Key words: lyophilized samples, museum *E. Coli* strains, viability, taxomic status.

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