RESEARCH ARTICLE

Development of PCR Detection System of Bacteriophages Pr 4 UGSHA, E7 ULSAU and Ye5 ULSAU

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Abstract

Development of PCR detection system of bacteriophages *Proteus* phage (Pr 4-UGSHA), *Enterobacter* phage (E7-ULSAU) and *Yersinia* phage (Ye5-ULSAU) are presented. Phylogenetic tree of concordance of their genetic organization against each other was built which was between 24 to 31%. It was determined that specific fragment for *Proteus* phage (Pr 4-UGSHA) is in the genome region of 3700-4500 bps. Highly specific fragments typical only for *Enterobacter* phage (E7-ULSAU) and *Yersinia* phage Ye5-ULSAU in studied genomes were not found, however, we didn't find regions during sharing of PCR that will allow detecting the only genome of a specific group. Two regions were established, their parallel determination was specific for *Enterobacter* phage and for *Yersinia* phage. In the BLAST system for parallel determination of genome fragments specific for groups of studied bacteriophages were determined. Finally, primer systems for PCR typing of bacteriophages were developed. They allowed to carry out identification of bacteriophages *Proteus*, *Enterobacter* and *Yersinia*, referring to specific groups in material obtained out of environment samples and pathological material without detachment of pure growth in screenings of specified groups during detection of genome fragment in 125 bps in size over the range of 3700-4500 bps of DNA *Proteus* phage (Pr 4-UGSHA); sizes of 294 and 431 bps in size over the range of 17500-18000 and 26500-27500 bps of DNA *Enterobacter* phage (E7-ULSAU) and 226 and 85 bps in size over the range of 2000-2500 and 24100-24300 bps of *Yersinia* phage (Ye5-ULSAU).

Introduction:

As per the available literature, we can confirm that in the small intestine of young cattle of agricultural animals and poultry in farming, unfortunate in gastrointestinal diseases, bacteria *Proteus* spp., *Enterobacter* spp. and *Yersinia enterocolitica* were reported in about 20-50 % cases (Shakhov *et al.*, 2014; Khaptseva *et al.*, 2015; Bottone *et al.*, 2015; Brenner *et al.*, 2015; Takahashi *et al.*, 2017; Hamilton *et al.*, 2018). Development in environmentally safe and effective therapeutic for diagnosis, treatment and prophylaxis of bacterial infections, caused by bacteria *Proteus* spp., *Enterobacter* spp. and *Yersinia enterocolitica*, includes searching and selection of specific bacteriophages, on basis of which we can construct new biopreparation (Sboychako *et al.*, 2012; Nifontova *et al.*, 2015; Clokie *et al.*,

2018; Flake, 2019). For the past 20 years quick growth of number and variety of strains of pathogenic microorganisms stable to low- molecular antibiotics, stimulated the search of complementary therapies and control of bacterial infections. For maximum effective and scientifically based appliance of bacteriophages in medicine, veterinary, agriculture and aquacultures, their deep study, systematization on gene-level and also high degree of purification of applied phage preparations were always felt necessary (Chugunova *et al.*, 2016, Feoktistova *et al.*, 2017; Vasilyev *et al.*, 2017). Bacteria viruses, which number is far outweighed, the total quantity of bacteria can become a perfect specific tool to fight against bacterial infections as none of them is harmful to the health of a human. A large quantity of these viruses is found in nature,

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in intestinal tracts, and in bacterial places. It is known that under the influence of lysogenic bacterial crops by inducer (for example by mitomycin and ultraviolet irradiation), phage production grows to a great degree, thus by applying this method we can reduce bacteriophage, then during study of its spontaneous production (Drulis-Kawa *et al.*, 2015; Perez Sepulveda *et al.*, 2017; Lin *et al.*, 2017). Available literature suggests number of methods of bacteriophage detachment out of environmental medium and similarly is described as direct method of detachment from studied material, detachment by enrichment method (without underseeing) and (with underseeing), detection on solid broth by Otto method and by Bilayered method by Gratia (de Cássia Oliveira *et al.*, 2017; Singh, 2018). These are long and material intensive studies, requiring high level of skill. Their end does not guarantee the achievement of positive results. That is why method development, allowing on the setting stage to get the right information on the presence or on absence in analyzed objects of sought bacteriophage. It is a relevant objective, allowing to enlarge the collection of phages by means of reduction of work time with one test during detachment and possibility of analysis of some tests simultaneously at material economy and labor-saving.

The present study was aimed to develop the system of PCR - detection of bacteriophages *Proteus* phage (Pr 4 - UGSHA), *Enterobacter* phage (E7- ULSAU) and *Yersinia* phage (Ye5- ULSAU)..

Methodology:

Research object is virulent bacteriophage Ye5-ULSAU, characterized by the following properties: plague- forming units are transparent, without zones of incomplete lysis 1.0 ± 1.5 cm; lytic activity -titer by Gratia- $1.5 \pm 0.1 \times 10^{10}$ BFU/ml, titer by Appelmann- 10^6 ; specific for cultures, identified as *Yersinia enterocolitica*; cultivating at the temperature of 58°C during 30 min. leads to inactivation.

Bacteriophage E7-ULSAU was used, which has the following characteristics, plague-forming units- transparent, round-shaped 3.5 ± 0.5 mm; lytic activity- titer by Gratia- $2.0 \pm 0.2 \times 10^{10}$ BFU/ml, titer by Appelman - 10^9 ; specific for cultures identified as *Enterobacter* spp.; cultivating at the temperature of 65°C during 30 min. leads to inactivation.

Bacteriophage Pr- 4 UGSHA was detached in 2017 out of objects of the outside environment- diameter of plague forming unit was 0.5 ± 0.1 mm, titer by Gratia- $1.3 \pm 0.2 \times 10^9$ BFU/ml, titer by Appelman- 10^8 , stable to the effect of trichloromethane for 15 min. and specific for cultures *Proteus mirabilis* and *Proteus vulgaris*.

Materials: 5-fold liquid with xylene cyanol, 7.5 mM MgCl₂ (LLC «Interlabservice» Cat.# 861), mixture of deoxyribonucleated-triphosphate (dNTP). Water liquid of deoxy-nucleotide triphosphate with a concentration of 1.76 mM each. (LLC «Interlabservice», Cat.# R3-1), mixture of type-

specific primers by 10 pcM each (Research and Production company «Liteks», Moscow); Taq DNA-Polymerase (5 ea./mcl) (Promega, USA., Cat.# 5001), agarose («Helikon» LOT 2308B504) or analogue, Tris-HCl, («Amresco» Cat.# Am-O234-0.5) or analogue, triton X-100, («Amresco» Cat.# Am-O694-1.0) or analogue, boric acid Helikon (Cat.# H-0202-0.5, (acs.), EDTA-Na, («Amresco» Cat.# Am-O105-0.1) or analogue, NaCl («Helikon» H-1401-1.0 (c.p.), 0.1 %-ethidium bromide («Amresco», Cat.# Am-O492-1.0), molecular weight marker, (LLC «Interlabservice», Cat.# MDNA-100bp), DNA purifier from agarose gel «QIAquick Gel Extraction», QIAGEN, Germany), sequencing kit, kit for DNA detachment «AmplyPrime DNA-sorb-?», («InterLabService», Moscow), microcentrifuge for 12000 r./min. for glass tubes with the volume of 1.5 ml and 0.5 ml (Eppendorf Minispin, Germany), automatic pipette aids for 20 mcl, 100 mcl and 1000 mcl (ThermoScientific), polypropylene tubes for 0.2 ml, 0.5 ml and 1.5 ml (Axygen, the USA), interchangeable head to automatic pipette aids (Axygen, the USA), amplification for PCR carrying («MaxyGene», AXYGENT Scientific, the USA), mixing tool «Vortex» (Biosan, Latvia), dry block heater (Termit, DNA Technology, Moscow), filter conductor Millex 0.22micron (PVDF) (Millipore, Ireland), genetic analyser («Applied Biosystems 3130XL», Applied Biosystems, the USA), kit of component for clearing sequencing mixture («BigDye Terminator kit 3.1», Applied Biosystem, the USA). Primers (F1_C2, F2_C2, F3_C2, F3_C2, R1_C2, R2_C2, R3_C2, F1_B, R1_B, F1_PODO, R1_PODO, terS-F, terS-R, mcp-F, mcp-R, mtp-F, mtp-R, tmp-F, tmp-R) were synthesized in LLC «LITEX». Production chemicals of «Fermentas» and «Alpha ferment».

For PCR layout (in real-time mode) except primers fluorescent oligonucleotides probe is necessary, carrying a fluorescent tag. In Blast system selection of optimum tags was carried for bacteriophages and their main properties were determined. Further experiments with the use of PCR and special equipment were carried- amplification with Real-Time detection (in this project DT-prime-5 (DT-96), DNA-Technology, RF). DNA was denaturated at 95°C for 1 min. Then 35 circles were obtained, including DNA denaturation at 95°C for 10 sec, primer annealing at 60°C for 20 sec. Detection was obtained on each of the cycles at 60°C by Fam channel.

Comparison of collected genomes of bacteriophages with genomes of known annotated bacteriophages was made with the help of algorithm blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and database of nucleotide sequences of NCBI (National Centre for Biotechnology Information, the USA). Visualization of alignment of our collected genomes with known genomes, we carried with the use of software BLAST Ring Image Generator (BRIG).

Results and Discussion:

For the purposes of possible typification of detached bacteriophages GroupWise we built a phylogenetic tree of concordance of their genetic organization between each other (Fig.-1, Table 1-2).

Figure-1: Figure-1: Phylogenetic tree of concordance of genetic organisation *Proteus* phage (Pr 4 - UGSHA), *Enterobacter* phage (E7 - ULSAU) and *Yersinia* phage (Ye5 - ULSAU)

Table-1: Concordance of genome *Proteus* phage (Pr 4 - UGSHA), *Enterobacter* phage (E7 - ULSAU) & *Yersinia* phage (Ye5 - ULSAU)

	<i>Yersinia_p</i>	<i>Proteus_p</i>	<i>Enterob._p</i>
<i>Yersinia_phage</i>	39210	12274	10032
<i>Proteus_phage</i>	12274	44580	10596
<i>Enterobacter_phage</i>	10032	10596	36030

Table-2: Relative concordance of genomes *Proteus* phage (Pr 4 - UGSHA), *Enterobacter* phage (E7 - ULSAU) & *Yersinia* phage (Ye5 - ULSAU)

	<i>Yersinia_p</i>	<i>Proteus_p</i>	<i>Enterob._p</i>
<i>Yersinia_phage</i>	100%	31%	
<i>Proteus_phage</i>	28%	100%	24%
<i>Enterobacter_phage</i>			100%

As concordance between genomes *Proteus* phage (Pr 4 - UGSHA), *Enterobacter* phage (E7 - ULSAU) and *Yersinia* phage (Ye5 - ULSAU) was from 24 to 31%, their organization into a general group was not reasonable. Thus, we developed systems for PCR - typing of bacteriophage groups according to their phylogenetic kinship. On the basis of sequence data we built phylogenetic trees of DNA- concordance of

Figure-2: Phylogenetic tree of DNA concordance *Proteus* phage (Pr 4 - UGSHA) by possible analogues

Figure-3: Phylogenetic tree of DNA concordance *Enterobacter* phage (E7 - ULSAU) by possible analogues

Figure-4: Phylogenetic tree of DNA concordance *Yersinia* phage Ye5-ULSAU by possible analogues

#	Identification	Cu.Fam	Cu.Hex	Res.
A1	Sample-1	13.4		+
A2	Sample-2	13.9		+
A3	Sample-3	16.8		+
B2	Sample-4			-
D7	Sample-5			-
D8	K			-

Figure-5: Amplification of specific fragments during DNA identification of *Proteus* phage: specimen 1 - Pr 4 - UGSHA, specimen 2 - Pr 6 - UGSHA, specimen 3 - Pr 7 - UGSHA

Figure-6: Amplification of specific fragments during identification of DNA *Enterobacter* phage in regions 17500-18000 bps and 26500-bps specimen 1 – E3 - ULSAU, specimen 2 – E4 - ULSAU, specimen 3 – E6 - ULSAU, specimen 4 – E7 - ULSAU

Figure-7: Amplification of specific fragments during identification of DNA *Yersinia* phage in regions 2000-2500 bps and 24100-24300 bps: specimen 1 – Ye 5 - ULSAU, specimen 2 – Ye2 - ULSAU, specimen 3 – Ye1 - ULSAU, specimen 4 – Ye 4 - ULSAU

Proteus phage (Pr 4 – UGSHA), *Enterobacter* phage (E7–ULSAU), *Yersinia* phage (Ye5- ULSAU) by possible analogue (Fig.-2-4).

Further, we determined fragments for the development of primer systems for the purposes of genetic identification and determination of typeable group affiliation of bacteriophages, active towards *Proteus* spp., *Enterobacter* spp. and *Yersinia enterocolitica*. It was established that specific fragment for *Proteus* phage (Pr 4– UGSHA) is in the genome region 3700-4500 bps. After synthesis of primers, we conducted the experiments (Fig.-5) on indication determined by genome fragments *Proteus* phage Pr 4–UGSHA, allowing to carry screening of bacteriophage presence directly within a material without detachment and

clean up steps.

Highly specific fragments, typical only for *Enterobacter* phage E7 – ULSAU and *Yersinia* phage Ye5 - ULSAU in studied genomes were not found, however, regions were discovered, during co-use of PCR for which it allows to detect the only genome of the given group. Regions of 17500-18000 bps and 26500-27500 bps, which simultaneous discovery is typical only for *Enterobacter* phage, two regions of 2000-2500 bps and 24100-24300 bps are established, which simultaneous detection is typical only for *Yersinia* phage.

In BLAST system 2 primer systems for simultaneous determination of genome fragments, specific for bacteriophage groups active towards *Enterobacter* and 2 primer systems for bacteriophages, specific for bacteria - *Yersinia enterocolitica*.

Further, the experiments were conducted (Fig.-6) on an indication of determined genome fragments *Enterobacter* phage E7 – ULSAU and *Yersinia* phage Ye5 - ULSAU (Fig.- 7) (essential presence of fragments, flanked by two primer systems), which allow carrying the analysis of studied material on bacteriophage presence without stages of detachment and clearance.

Further, we determined the fragment for primer system development for the aims of genetic identification and determination of typeable implement to the group of bacteriophages, active towards *Yersinia* spp. Highly specific fragment, specific only for *Yersinia* phage Ye5-ULSAU in the genome was not found, however, there are regions, for which during PCR co-use will allow detecting the only genome of the given group.

Conclusion:

As the result of conducted research we developed primer systems for PCR typing of bacteriophages *Protes*, *Enterobacter* and *Yersinia* groups, which allow to determine bacteriophage presence, referring to special groups in material, received from the environmental samples and pathological material without detachment of clear crop. This characteristic together with high specificity, special for all types of molecular-genetic studies (Conrotto *et al.*, 2008; Krylov *et al.*, 2011; Chugunova *et al.*, 2016; Cahill, 2017; Rowczenio *et al.*, 2018), allows to use these systems during screenings of bacteriophages of listed groups during detection of genome fragments of 125 bps in size in region 3700-4500 bps. of DNA *Proteus* phage (Pr 4 – UGSHA); 294 and 431 bps in size in regions 17500-18000 and 26500-27500 bps in complance of DNA *Enterobacter* phage (E7–ULSAU) and 226 and 85 bps in size in regions 2000-2500 and 24100-24300 bps respectively *Yersinia* phage (Ye5 - ULSAU).

References:

- Bottone, E.J., Bercovier, H. & Mollaret, H.H. (2015): *Yersinia*. In: **Bergey's Manual of Systematics of Archaea and Bacteria**. Pub. By: John Wiley & Sons, Inc.

- Brenner, D.J. & Farmer, J.J. (2015): *Enterobacteriaceae*. In: **Bergey's Manual of Systematics of Archaea and Bacteria**. Pub. By: John Wiley & Sons, Inc.
- Cahill, J.L. (2017): **Molecular, Genetic, and Biochemical Analysis of Phage Lambda Lysis**. Ph.D. dissertation of Texas A & M University.
- Chugunova, O.Y. & Tatarnikova, N.A. (2016): Study of proteic phage properties. *Perm Agricul. Vestnik*, 3(15):108-122.
- Clokie Martha, R.J. & Kropinski, A.M. (2018): **Bacteriophages. Methods and Protocols, Vol.-3**. Lavigne. Pub. By: Humana Press, US. P. 311.
- Conrotto, P. & Souchelnytskyi, S. (2008): Proteomic approaches in biological and medical sciences: principles and applications. *Exp. Oncol.*, 30(3):171-180.
- de Cássia Oliveira, V., Arruda, C.N.F., Monteiro, R.M., Silva-Lovato, C.H., Paranhos, H.F.O. & Watanabe, E. (2017): Bacteriophage Isolation from Denture Biofilm. *Revista Prevenção de Infecção e Saúde*, 3(2):34-38.
- Drulis-Kawa, Z., Majkowska-Skrobek, G. & Maciejewska, B. (2015): Bacteriophages and phage-derived proteins application approaches. *Current Med. Chem.*, 22(14):1757-1773.
- Feoktistova, N.A., Vasilyev, D.A. & Zolotukhin, S.N. (2017): Study of biological properties of bacteriophages Proteus. *Vestnik of Ulyanovsk State Agricul. Acad.*, 3(39):99-105.
- Flake, P. (2019): **Further understanding of bacteriophages that infect the bacterial family Enterobacteriaceae**. Undergraduate Honors Theses. Brigham Young University, US.
- Hamilton, A.L., Kamm, M.A., Ng, S.C. & Morrison, M. (2018): Proteus spp. as putative gastrointestinal pathogens. *Clin. Microbiol. Rev.*, 31(3):e00085-17
- Khaptseva, O.J., Khaptsev, Z.Y., Faust, Y.A., Larionova, O.S. & Sherbakov, A.A. (2015): About effect of acidulent on calves' gastrointestinal microflora. *Scientific life*, 2:103-109.
- Krylov, S.V., Pleteneva, E.A., Burkaltseva, M.V., Shaburova, O.V., Miroshnikov, K.A., Lavin, R., Kornelissen, A. & Krylov, V.N. (2011): Genome instability of phage EL *Pseudomonas aeruginosa* species: study of mutants, showing virulence. *Genetics*, 47(2):183-189.
- Lin, D.M., Koskella, B. & Lin, H.C. (2017): Phage therapy: an alternative to antibiotics in the age of multi-drug resistance. *World J. Gastrointest. Pharmacol. Ther.*, 8(3):162-173.
- Nifontova, V.V. & Chugunova, O.Y. (2015): Obtainment of bacteriophages and their appliance in veterinary. *Vestnik Perm Scientific Cen.*, 2:54-59
- Perez Sepulveda, B., Redgwell, T., Rihtman, B., Pitt, F., Scanlan, D. J. & Millard, A. (2016): Marine phage genomics: the tip of the iceberg. *FEMS Microbiol. Lett.*, 363(15):158.
- Rowczenio, D.M., Pathak, S., Arostegui, J.I., Mensa-Vilaro, A., Omoyinmi, E., Brogan, P., Lipsker, D., Scambler, T., Owen, R., Trojer, H., Baginska, A., Gillmore, J.D., Wechalekar, A.D., Lane, T., Williams, R., Youngstein, T., Hawkins, P.N., Savic, S. & Lachmann, H.J. (2018): Molecular genetic investigation, clinical features, and response to treatment in 21 patients with Schnitzler syndrome. *Blood*, 131(9):974-981.
- Sboychako, V.B., Zakharenko, S.M., Finogeev, Y.P. & Krumgolts, V.F. (2012): Epidemiology, clinic and laboratory diagnosis of bacterial and micosal disease. *Treat. Prophyl.*, 3:77-81.
- Shakhov, A.G., Sashnina, L.Y., Fedosov, D.V., Yerina, T.Y. & Alekhin, Y.N. (2014): Formation of intestinal microbiocenosis at calves with digotrophy syndrome in preweaning period. *Agricul. Biol.*, 2:105-111.
- Singh, V. (2018): Bacteriophage-Mediated Biosensors for Detection of Foodborne Pathogens, pp. 353-384. In: Singh, J. Sharma, D., Kumar, G. & Sharma, N.R. (eds.) **Microbial Bioprospecting for Sustainable Development**. Pub. by: Springer Nature Singapore Pte Ltd.
- Takahashi, H., Saito, R., Miya, S., Tanaka, Y., Miyamura, N., Kuda, T., & Kimura, B. (2017): Development of quantitative real-time PCR for detection and enumeration of Enterobacteriaceae. *Int. J. Food Microbiol.*, 246:92-97.
- Vasilyev, D.A., Feoktistova, N.A. & Zolotukhin, S.N. (2017): Detachment and study of biological properties of bacteria Proteus species. *Vestnik Ulyanovsk State Agricul. Acad.*, 2 (38):70-76.