ISOLATION AND CHARACTERIZATION OF VIRULENT BACTERIOPHAGES AGAINST *KLEBSIELLA PNEUMONIAE* OF SIGNIFICANT CAPSULAR TYPES

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The growing proportion of antibiotic-resistant *Klebsiella pneumoniae* strains raises challenges to the healthcare system and requires the development of alternative treatment options. Bacteriophage therapy is one of such options. The study was aimed to isolate and describe bacteriophages effective against *K. pneumoniae* strains of clinically significant capsular types. The bacteriophages were isolated from the sewage and river water samples using the enrichment culture technique. The spectrum of lytic activity of the phages was tested on the collection of *K. pneumoniae* clinical isolates (n = 279). The studied bacteriophages lysed 52.8–100% of *K. pneumoniae* strains of respective capsular types: phage VKV295 lysed 100% of strains with the capsular type KL1, SAA231 — 52.8 of strains with KL2, NNK-G4 — 100% of strains with KL39, VSG32 — 66.7% of strains with KL41, NKA196 — 87.5% of strains with KL47, Rappa3 — 87.5% of strains with KL57, PEA128 — 95.5% of strains with KL64, and ChM-G5 — 69.6% of strains with KL102. Whole-genome sequencing and subsequent bioinformatic analysis revealed that the phages belong to the *Autographiviridae* family and are classified into three genera.The lytic spectrum of phages was limited to specific capsular types due to the presence of specific receptor-binding proteins, polysaccharide depolymerases. The isolated bacteriophages were strictly virulent, did not carry harmful genetic determinants, and had a specific host range, making them applicable in therapeutic practice for combating antibiotic-resistant infections caused by *K. pneumoniae*.

Keywords: virulent bacteriophages, *Klebsiella pneumoniae*, antibiotic resistance, polysaccharide depolymerases

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ВЫДЕЛЕНИЕ И ХАРАКТЕРИСТИКА ВИРУЛЕНТНЫХ БАКТЕРИОФАГОВ ПРОТИВ *KLEBSIELLA PNEUMONIAE* ЗНАЧИМЫХ КАПСУЛЬНЫХ ТИПОВ

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В контексте растущей устойчивости к антибиотикам бактериофаги — альтернатива традиционной антимикробной терапии. Терапия бактериофагами — одна из таких альтернатив. Целью исследования были выделение и характеристика бактериофагов, эффективных против штаммов *Klebsiella pneumoniae* клинически значимых капсульных типов. Из проб сточных и речных вод методом накопительных культур было выделено восемь фагов. Определение спектра литической активности фагов проводили на коллекции клинических изолятов *K. pneumoniae* (n = 279). Бактериофаги лизировали 52,8–100% изолятов *K. pneumoniae* соответствующих капсульных типов: фаг VKV295 — 100% изолятов с капсульным типом KL1, SAA231 — 52,8% с KL2, NNK-G4 — 100% с KL39, VSG32 — 66,7% с KL41, NKA196 — 87,5% с KL47, Rappa3 — 87,5% с KL57, PEA128 — 95,5% с KL64 и ChM-G5 — 69,6% с KL102. Их геномы были секвенированы и проанализированы биоинформатически. Фаги принадлежали к семейству *Autographiviridae* и относились к трем родам. Литический спектр фагов был ограничен конкретными капсульными типами вследствие наличия специфичных рецептор-связывающих белков — полисахаридеполимераз. Выделенные бактериофаги были строго вирулентными, не несли вредных генетических детерминант, что позволяет их применять в терапевтической практике для борьбы с антибиотикорезистентными инфекциями, вызванными *K. pneumoniae*.

Ключевые слова: вирулентные бактериофаги, *Klebsiella pneumoniae*, антибиотикорезистентность, полисахарид-деполимеразы

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Klebsiella pneumoniae is a Gram-negative, rod-shaped bacterium belonging to the *Enterobacteriaceae* family. Bacteria of this species are the cause of many human infectious diseases. Pneumonia (inflammation of the lungs) is the best known one, however *K. pneumoniae* can also cause urinary tract infections, bloodstream infections, wound infections, and sepsis [1]. Antibiotic therapy remains the main method to prevent and treat infections caused by *K. pneumoniae*, despite the fact that the share of mulridrug-resistant strains can reach 20–30% [2, 3]. The *K. pneumoniae* infection mortality is as high as 38%, while the annual number of deaths associated with antibiotic resistance is 650,000 people [4, 5].

Bacteriophage therapy is considered to be a simple, safe and highly effective alternative to antibiotics [6]. Bacteriophages are the largest and most common group of viruses; they have been used as antimicrobials since their discovery in the early 20th century. Today, monophages and cocktails of several lytic phages are successfully used for personalized therapy [7–9]. However, commercially available broad-spectrum phage cocktails have limited efficacy [10].

The *K. pneumoniae* bacteriophage efficacy is largely defined by the type of capsular polysaccharide of the host bacterium [11]. The *K. pneumoniae* polysaccharide capsule is a key factor of virulence protecting the bacterium against environmental factors, including host immunity [12]. Today, more than 100 different polysaccharide capsule types are distinguished based on the conventional serological method and the method of sequencing distinct genes of the cps gene cluster, some of them (KL1, KL2, KL8, KL20, KL39, KL41, KL47, KL53, KL57, KL64, KL102 и KL107) are associated with increased virulence and antibiotic resistance [13–17].

The *K. pneumoniae* bacteriophages are adsorbed on the surface of bacteria, they dissolve the polysaccharide capsule with the specialized enzymes, polysaccharide depolymerases, usually found on the phage tail fibers fiberand spikes. Polysaccharide depolymerases possess enzyme activity against certain bond between monosaccharides in the polysaccharide monomer [11].

The study was aimed to isolate and describe bacteriophages capable of lysing *K. pneumoniae* strains of clinically significant capsular types.

METHODS

Bacterial strains and their characteristics

The collection of (n = 279) *K. pneumoniae* clinical isolates was compiled in 2018–2022: 79 strains were obtained from the Raisa Gorbacheva Memorial Research Institute for Pediatric Oncology, Hematology and Transplantation (Saint Petersburg, Russia), 66 from the Sklifosovsky Research Institute for Emergency Medicine (Moscow, Russia), 64 from the collection of the Pediatric Research and Clinical Center for Infectious Diseases of FMBA of Russia (Saint Petersburg, Russia), 58 from the Clinical Hospital № 123 of the Lopukhin Federal Research and Clinical Center of Physical-Chemical Medicine of FMBA of Russia (Odintsovo, Russia); 12 isolates were generously provided by SCPM-Obolensk (Obolensk, Russia).

Bacterial strains were grown using the lysogeny broth (LB) (Himedia; India) at 37°C. Bacterial species were identified by MALDI-TOF mass spectrometry [18]. The fact of the *K. pneumoniae* belonging to certain capsular type was determined by the *wzi* gene sequencing [19].

Bacteriphage isolation and purification

Hospital sewage, from which *K. pneumoniae* strains were isolated, and water of the Likhoborka (Moscow) and Klyazma (Korolev) rivers were used as the sources of bacteriophages.

To eliminate bacterial component, the sample of sewage or river water was centrifuged at 4000 g for 10 min, supernatant was filtered using the 0.22 μm filters (Merk Millipore; USA). Equal amounts (15 mL) of filtered water and double concentration LB broth were combined and inoculated with 20 μL of the overnight culture of the potential bacterial host strain. This mixture was incubated overnight on the shaker at 37 °C. The resulting suspension was sterilized by filtering through the 0.22 μm filter, and the presence of bacteriophages in the filtered liquid was confirmed by the spot test [20]. Isolation and buildup of pure bacteriophage culture was accomplished via three passages through a single plaque.

The study also involved NER40 bacteriophage isolated from the Chermyanka river (Moscow) that was described in the previous paper [21].

Determination of lytic spectrum

The lytic spectra of bacteriophages were defined by the spot test assay [20]. For that 100 μL f the culture of each *K. pneumoniae* strain grown to logarithmic phase (OD_{600} = 0.3) were mixed with 5 mL of semi-solid agar in LB (0.7% agar) and distributed among the Petri dishes with thin layer of agar in LB (1.5% agar). Testing included application of 5 μL of monophage lysates with a titre of 106 PFU/mL to the surface of fresh lawns of the tested *K. pneumonia* strains. Then the Petri dishes were incubated at 37 °C overnight. Lytic activity of bacteriophages was determined based on the presence of the zone of continuous bacterial cell lysis matching the shape of initial drop. The presence of translucent area surrounding the zone of lysis was interpreted as polysaccharide depolymerase activity.

Whole-genome bacteriophage sequencing and bioinformatics data analysis

The phage genomic DNA was extracted using the standard phenol–chloroform extraction protocol [22]. Sequencing was carried out using the MiSeq tool (Illumina; USA) and the MiSeq Reagent Nano Kit v2 (500 cycle) (Illumina; USA) in accordance with the manufacturer's instructions. Genomes were assembled with the SPAdes software (v. 3.14.0). The GeneMarkS online service (v. 4.32) was used to identify open reading frames (ORFs) in the genome. Assessment of tRNA genes was performed with ARAGORN (v. 1.2.41).

Genes were predicted and annotated manually using BLASTp, HHPred, and InterPro. To confirm the lack of genes encoding toxins and antibiotic resistance determinants, comparison with the databases containing virulence factors of pathogenic bacteria [23] and antibiotic resistance genes [24] was performed. The annotated sequences of bacteriophage genomes were deposited in the GenBank database.

Phylogenetic analysis involved 40 reference bacteriophage genomes proposed by the International Committee on Taxonomy of Viruses (ICTV). Phylogenetic trees were constructed based on the bacteriophage complete genomes using the VICTOR tools [25]. The closest homologues among bacteriophages were determined with the BLASTn algorithm. Comparative analysis of distinct protein sequences was accomplished using the BLASTp service. Comparative analysis of complete genomes was performed using the Circoletto tools [26].

RESULTS

Characteristics of *K. pneumoniae* strains

The *wzi* gene nucleotide sequence was determined for all 279 strains of the collection. Comparative analysis of the

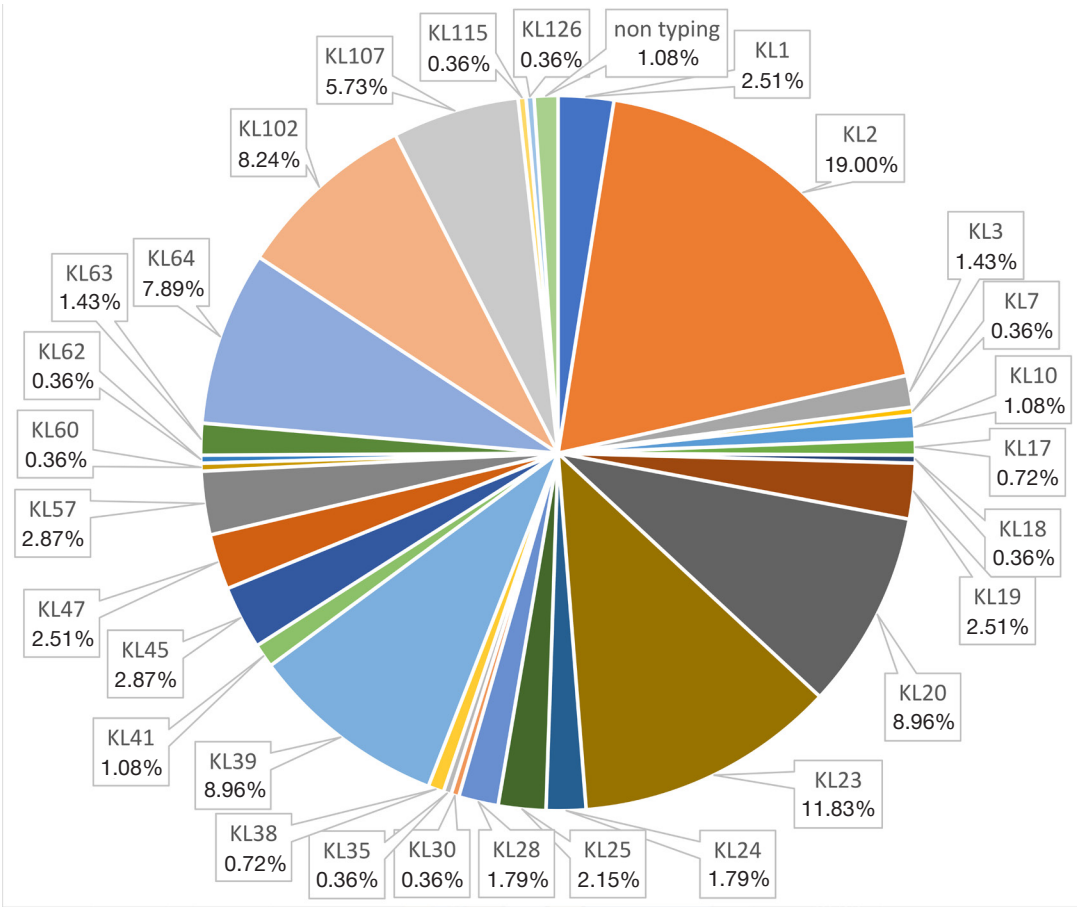

Fig. 1. Diversity of capsular types in the *K. pneumoniae* collection

resulting sequences and the sequences from the Institut Pasteur database made it possible to determine the alleles corresponding to distinct capsular types. A total of 40 unique *wzi* gene allele variants were found, among which 37 were associated with certain capsular types; no associations with the known capsular types were found for three variants (*wzi* 475, *wzi* 493 and *wzi* 163). The collection included 29 different capsular types, among which seven constituted 70% of all isolates: KL2 (19%), KL23 (12%), KL20 (9%), KL39 (9%), KL64 (8%), KL102 (8%), and KL107 (6%) (Fig. 1). The shares of other capsular types, often associated with high virulence, were less than 5%: KL1 — 3%, KL41 — 1%, KL47 — 3%, KL57 — 3%.

Isolation, phenotypic characteristics and lytic spectrum of bacteriophages

A total of eight bacteriophages (VKV295, SAA231, NKA196, NNA-G4, VSG32, Rappa3, PEA128, and ChM-G5) lysing the *K. pneumoniae* strains of eight clinically significant capsular types (KL1, KL2, KL39, KL41, KL47, KL57, KL64, and KL102) were extracted from three sewage samples and two river water samples. Strains of these capsular types constituted 53.05% of the collection.

The majority of bacteriophages formed small, round, transparent plaques (1–2 mm) surrounded by the 1–2 mm halo. Certain bacteriophages (VKV295 and Rappa3) formed larger round, transparent plaques (2–4 mm) also surrounded by halo (Fig. 2, Table 1).

Bacteriophages showed high specificity of the lytic spectrum: each isolated phage was capable of lysing only strains with the same capsular type as the strain, on which the bacteriophage was isolated. All the studied bacteriophages lysed 52.8–100% of strains of certain capsular types (Table 1). The previously described bacteriophage NER40 specifically lysing strains with the capsular type KL2 was included in the study for reference [21].

Whole-genome bacteriophage sequencing and phylogenetic analysis

Complete genomes of phages were assembled and deposited in the NCBI GenBank database (Table 2). The genome size varied between 39058 and 44575 bp, the G + C content was 50.4–54.3%. All phage genomes had terminal repeats sized 167–282 bp on both ends. No tRNA genes were found in the phage genomes, and the number of open reading frames (ORFs) predicted for various bacteriophages was 42–53 (Table 2).

Phylogenetic analysis has shown that all the studied bacteriophages belong to three genera of the family *Autographiviridae* (Fig. 3). Phages VKV295, SAA231, NKA196, and NNA-G4 belong to the genus *Drulisvirus*, Rappa3 and PEA128 are members of the genus *Przondovirus*, while VSG32 and ChM-G5 belong to the genus *Teetrevirus*. According to the BLASTn analysis results, the closest homologues of *Drulisvirus* phages were represented by KpV2883 (GenBank MT682065.1; 90.53% identity) for phage VKV295, vB_KpnP_KpV74 (GenBank NC_047811.1; 88.12% identity) for phage SAA231, and KPPK108.1 (GenBank OK583892.1; 90.56% and 85.03% identity) for NNA-G4 and NKA196. The closest homologues of phages Rappa3 and PEA128 were represented by phages of the genus *Przondovirus* K5-2 (GenBank NC_047798.1; 81.32% identity) and 066037 (GenBank MW042800.1; 86.27% identity), respectively. Homologues of phages *Teetrevirus*VSG32 and ChM-G5 were represented by *Salmonellaphage* phiSG-JL2

Table 1. Microbiological characteristics of bacteriophages

Source of bacteriophages	Bacteriophage	Bacteriophage capsular specificity	Number of lysed strains of certain capsular type	Plaque morphology	Halo, mm
				Plaque, mm	
Sewage of the Clinical Hospital No. 123 of the Lopukhin Federal Research and Clinical Center of Physical-Chemical Medicine of FMBA of Russia	VKV295	KL1	7/7	2	3
	NNA-G4	KL39	25/25	0,5–1	1
Sewage of the Sklifosovsky Research Institute for Emergency Medicine	Rappa3	KL57	7/8	4	3
	VSG32	KL41	2/3	1–2	1–3
Sewage of the Raisa Gorbacheva Memorial Research Institute for Pediatric Oncology, Hematology and Transplantation	PEA128	KL64	21/22	1–2	1
	ChM-G5	KL102	16/23	1	1
Klyazma river	SAA231	KL2	28/53	1	1
Likhoborka river	NKA196	KL47	7/8	1–2	2–3
Chermyanka river	NER40 [21]	KL2	49/53	3–5	2–4

(GenBank NC_010807.1; 84.00% identity) and *Klebsiellaphage* 6998 (GenBank OL362282.1; 90.13 % identity), respectively (Table 2).

Functional annotation and comparative analysis of genomes

All the studied bacteriophages were members of the family *Autographiviridae* and, therefore, had similar genome structure: all genes were located on the leading DNA strand, phages encoded both DNA and RNA polymerases, while genes of nucleic acid metabolism and genes encoding structural proteins formed clusters in the left and right parts of the genome, respectively. Members of this family are virulent phages that carry no integrase genes. The annotated genes of the studied bacteriophages include no genes encoding integrases, antibiotic resistance determinants, toxins or any other known genes that are potentially unfavorable in terms of therapy.

The genomes of phages of the genus *Drulisvirus* carried 51–53 ORFs, among which 22–24 were annotated as genes encoding hypothetical proteins, 12–14 were nucleic acid metabolism genes, 12–13 were genes encoding capsid proteins; there were also three genes responsible for host cell lysis represented by the genes encoding spanin, choline and endolysin following one another.

Each of four phages of the genus *Drulisvirus* carried two genes encoding phage fiber proteins, however, both genes encoded polysaccharide depolymerase domains only in VKV295; in three other phages, a depolymerase domain was found on one fiber out of two only. The fiber genes of phage VKV295 (*orf0043* and *orf0051*) carried glycoside hydrolase family 28 and K1 lyase domains and showed 82.53 and 99.75% identity with the fibers of phage KpV2883 that was considered to be the closest based on BLASTn. In turn, the fiber genes of bacteriophage SAA231 showed 96.18 and 97.57% identity with the closest homologue, phage vB_KpnP_KpV74; the first fiber gene (*orf0044*) carried no depolymerase domain, while the second one (*orf0052*) encoded the glycoside hydrolase family 28 domain. This depolymerase (SAA231_ orf0052) showed 98.1% homology with the earlier reported fiber *orf0053* of phage NER40. Bacteriophage NNA-G4 carried two fiber genes, among which only one (*orf0052*) encoded depolymerase with pectate lyase 3 domain and showed 95.65% identity with the fiber gene of phage VLC5 (GenBank MT197175.1; 74.97% identity). As in NNA-G4, only the second fiber of phage NKA196 (*orf0052*), which showed 99.13% identity with the fiber of phage

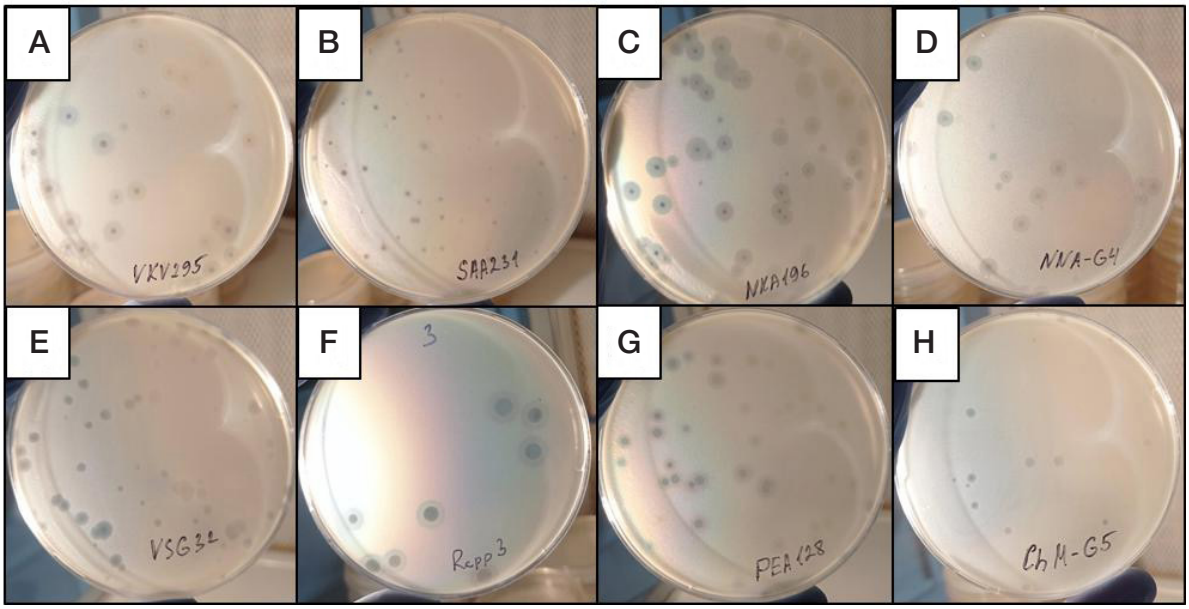

Fig. 2. Plaque morphology of phages VKV295 **(A)**, SAA231 **(B)**, NKA196 **(C)**, NNK-G4 **(D)**, VSG32 **(E)**, Rappa3 **(F)**, PEA128 **(G)**, and ChM-G5 **(H)**

Table 2. Genetic characteristics of bacteriophages

Bacteriophage	GenBank	Taxonomic status	Size, bp	ORF	G + C	Identity with the closest homologue, %
VKV295	OR287807	*Drulisvirus*	42380	51	54.10%	90.53
SAA231	OR287809	*Drulisvirus*	44281	53	54.30%	88.12
NNA-G4	OR287810	*Drulisvirus*	44575	52	53.80%	90.56
NKA196	OR287808	*Drulisvirus*	44083	52	53.90%	85.03
Rappa3	OR287806	*Przondovirus*	40593	42	53.10%	81.32
PEA128	OR287812	*Przondovirus*	40386	47	52.80%	86.27
VSG32	OR287811	*Teetrevirus*	39058	48	50.40%	84
ChM-G5	OR287804	*Teetrevirus*	39235	45	50.90%	90.13

KPPK108.2 (GenBank OK583892.1; 85.03 % identity), carried a depolymerase domain of glycoside hydrolase family 28.

Genus *Przondovirus* was represented by two phages, the genomes of which carried 42–47 ORFs. As a result of the annotation, we managed to predict the functions of 71.2–73.8% of hypothetical proteins. A total of 15–16 nucleic acid metabolism genes, 14–15 structural genes, and two genes responsible for host bacterium lysis represented by class II choline and Rz-like spanin were annotated.

Rappa3 bacteriophage had two fibers (*orf0037* and *orf0038*) containing depolymerase domains represented by pectate lyases 3. The first fiber showed 29.28% identity with the fiber of phage K11 (GenBank NC_011043.1; 81.01% identity), while the second one showed 71.38% identity with the fiber of phage vB_KpnP_KpV767 (GenBank NC_047772.1; 78.09% identity). A single fiber of phage PEA128 showed 99.72% identity with the fiber of phage TUN1 (GenBank HG994092.1; 84.11% identity) and carried the glycoside hydrolase family 28 domain.

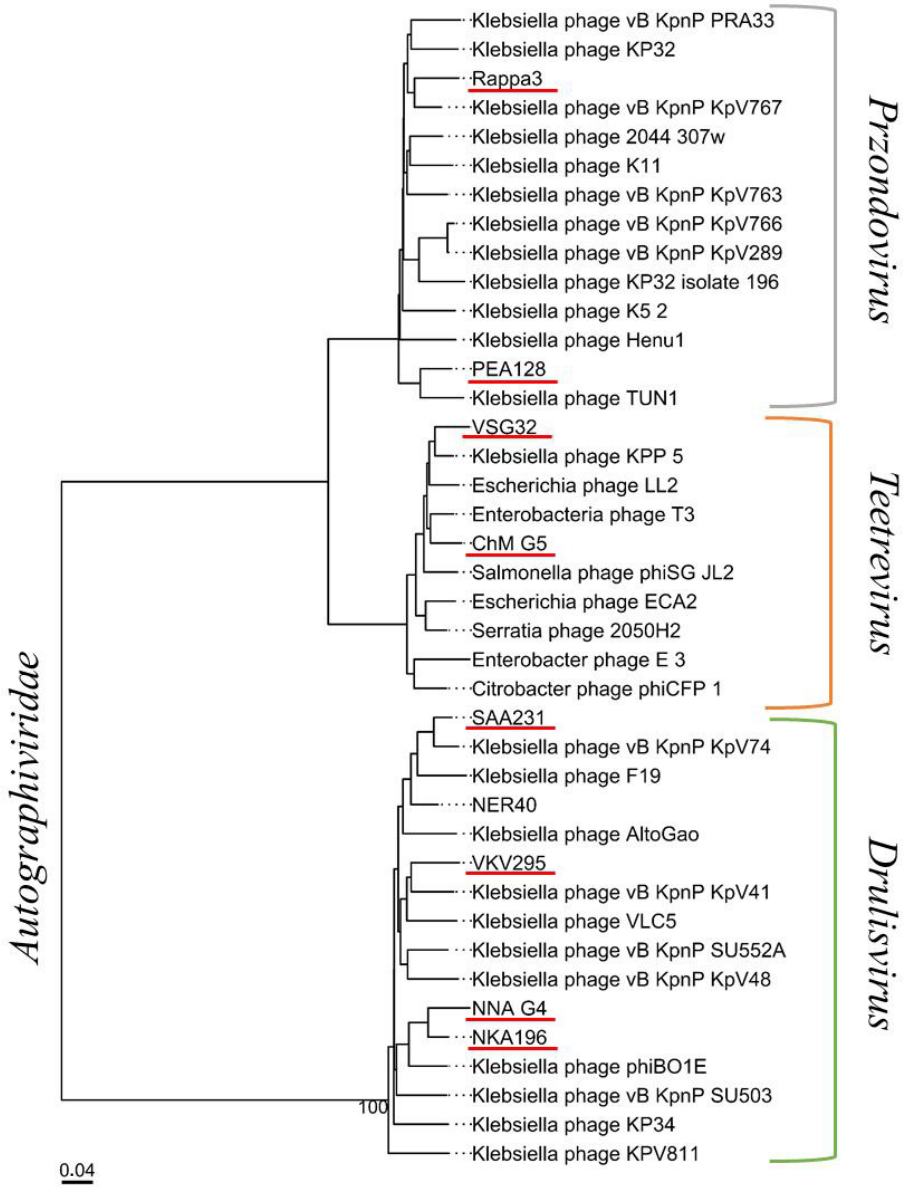

Fig. 3. Phylogeny of the *K. pneumonia* bacteriophages. The studied bacteriophages are highlighted in *red*

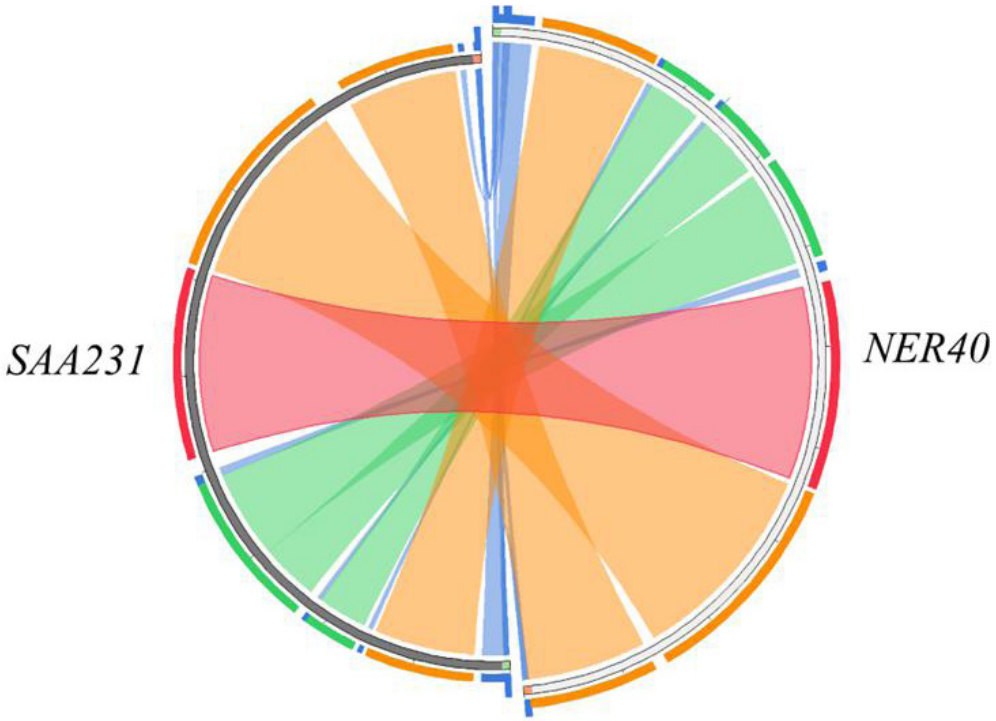

Fig. 4. Comparison of the NER40 and SAA231 phage genomes (percentage of homology is highlighted in *blue* < 25%; *green* < 50%; *orange* < 75%; *red* >75%)

Bacteriophages VSG32 and ChM-G5 of the genus *Teetrevirus* had 48 and 46 ORFs, respectively, among which functions were predicted for 83.33 and 78.26% ORFs: 21 and 19 ORFs encoded nucleic acid metabolism genes, 17 and 15 encoded structural genes. Genes responsible for host lysis were organized in the same manner as in phages of the genus *Przondovirus* and represented by two ORFs encoding choline and Rz-like spanin.

Each of the isolated phages carried one fiber gene encoding the receptor-binding protein. Depolymerase of phage ChM-G5 was represented by the *orf0040* fiber showing 90.41% identity with the fiber of phage 6998 (GenBank OL362282.1; 90.13% identity) and carrying the pectate lyase 3 domain. BacteriophageVSG32 encoded the *orf0042* fiber showing 94.97% identity with the fiber of phage KPP-5 (GenBank MW600722.1; 87.70% identity) and carrying an adhesion domain of indeterminate nature.

DISCUSSION

The *K. pneumoniae* strains taken as host strains have the capsular types associated with nosocomial infections that are difficult to treat due to the presence of antibiotic resistance determinants [3, 16, 27]. These strains are widespread in Russia and neighboring countries, they often carry genes responsible for carbapenem and broad spectrum β-lactam antibiotic resistance, as well as genes responsible for hypervirulence [16, 27]. Strains with the capsular types KL1, KL2, KL39, KL41, KL47, KL57, KL64, and KL102 constitute 53.05% of the collection compiled, which means high relevance of isolating therapeutic bacteriophages against them.

Novel bacteriophages were isolated from sewage of the same hospitals, where the strains of the collection were isolated, as well as from water of the rivers flowing through Moscow. All the isolated bacteriophages formed specific translucent halos surrounding individual plaques, which was a characteristic feature of the presence of receptor-binding proteins represented by polysaccharide depolymerases. This is also confirmed by the narrow range of phage hosts limited to *K. pneumoniae* strains of specific capsular types. Bacteriophages specific for *K. pneumoniae* strains of the capsular types KL1, KL2, KL47, KL57, KL64, and KL102 were earlier described in the literature as members of different taxons. However, to date, only one phage specific for *K. pneumoniae* strains of the capsular type KL39 have been reported; no phages able to specifically lyse *K. pneumoniae* strains of the capsular type KL41 have been reported [28].

The analysis of genomes of the isolated bacteriophages has shown that all phages are members of the family *Autographiviridae* and are more than 5% different from the closest phages presented in the NCBI database, which allows us to say that the isolated bacteriophages are new species of appropriate genera [29]. Despite the differences between complete genomes sufficient for identification of new species, the fiber genes responsible for phage adsorption on the surface of bacteria and largely determining the host range showed higher degree of homology with the earlier reported bacteriophage fibers. Thus, for example, fibers of phages VKV295, SAA231, NKA196, and PEA128 turned out to have 82.53–99.75% homology with the fibers of earlier characterized bacteriophages KpV2883, vB_KpnP_KpV74, KPPK108.2 and TUN1. In contrast, fibers of phages NNA-G4, ChM-G5, VSG32 and Rappa3 were either homologous to bacteriophages with undescribed host specificity, or showed poor (< 75%) homology with the closest fibers of the known phages (based in BLASTp).

Interesting is the fact that our collection includes the earlier reported bacteriophage NER40 (GenBank MZ602146.1) of the genus *Drulisvirus* specific for *K. pneumoniae* strains with the capsular type KL2 [21]. A significant difference between the two bacteriophages was that, while specifically lysing *K. pneumoniae* strains with the capsular type KL2, bacteriophage NER40 showed higher efficiency, 49/53 (90.57%) vs. 28/53 (52.8%) for SAA231. The main differences between genomes of phages NER40 and SAA231 are within the region between 6.5–17.5 kbp, where the genes responsible for life cycle are located, while the genes of adsorption apparatus have shown

high degree of homology (98.1%) (Fig. 3). Given the above, such significant differences in the host ranges can be due to the differences in the success in bypassing bacterial antiphage defense systems, such as restriction modification system and CRISPR. It can be assumed that the genes ensuring successful bypassing of such systems are located in this specific region of the phage genome (6.5–17.5 kbp) and determine the differences in potential therapeutic efficacy.

It is important to note that no potentially undesired determinants have been found in the genomes of isolated bacteriophages, which, along with their phylogenetic position, characterizes them as strictly virulent bacteriophages suitable for antibacterial therapy. In turn, high lytic activity of phages and the presence of polysaccharide depolymerases as receptor-binding proteins make it possible to use both bacteriophages and their derivatives for therapy.

CONCLUSIONS

We have isolated and characterized bacteriophages possessing specific lytic activity against clinically significant *K. pneumoniae* strains of certain capsular types: VKV295 against KL1, SAA231 against KL2, NNK-G4 against KL39, VSG32 against KL41, NKA196 against KL47, Rappa3 against KL57, PEA128 against KL64, ChM-G5 against KL102. The phage genomes were tested for any genes potentially dangerous for therapy (integrases, toxins, antibiotic resistance factors), which means that these phages may be used for treatment.

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