

MOLECULAR GENETIC CHARACTERIZATION OF THREE NEW *KLEBSIELLA PNEUMONIAE* BACTERIOPHAGES SUITABLE FOR PHAGE THERAPY

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The *Klebsiella pneumoniae* bacterium is capable of causing the broad range of human nosocomial infections associated with antibiotic resistance and high mortality. Virulent bacteriophage therapy is one of the promising alternatives to antibiotic treatment of such infections. The study was aimed to isolate virulent bacteriophages effective against the relevant clinical *K. pneumoniae* strains, and to perform the molecular genetic characterization of these phages. Bacteriophages were isolated from the river water samples using the enrichment method. The whole-genome sequencing was performed on the MiSeq platform (Illumina). Three novel *K. pneumoniae* bacteriophages belonging to families *Autographiviridae* (vB_KpnP_NER40, GenBank MZ602146) and *Myoviridae* (vB_KpnM_VIK251, GenBank MZ602147; vB_KpnM_FRZ284, GenBank MZ602148) have been isolated and characterized. On the collection of 105 *K. pneumoniae* clinical strains, it has been found that bacteriophages vB_KpnP_NER40 and vB_KpnM_VIK251 have a narrow lytic spectrum (22% and 11%), which is limited to strains of the capsular types K2 and K20 respectively. In contrast, bacteriophage vB_KpnM_FRZ284 has a broad lytic spectrum (37%), causing the lysis of strains with different types of capsular polysaccharide. The phages are strictly virulent and have no genes encoding integrases, toxins or pathogenicity factors in their genomes. Genes of depolymerases, encoding the potential receptor binding proteins, have been found in the genomes of the capsular-specific bacteriophages vB_KpnP_NER40 and vB_KpnM_VIK251. The cocktail of three bacteriophages has lysed about 65% of the studied collection of *K. pneumoniae* strain and is potentially applicable for therapeutic purposes.

Keywords: virulent bacteriophages, *Klebsiella pneumoniae*, antibiotic resistance, phage therapy, depolymerases

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МОЛЕКУЛЯРНО-ГЕНЕТИЧЕСКАЯ ХАРАКТЕРИСТИКА ТРЕХ НОВЫХ БАКТЕРИОФАГОВ *KLEBSIELLA PNEUMONIAE*, ПЕРСПЕКТИВНЫХ ДЛЯ ПРИМЕНЕНИЯ В ФАГОВОЙ ТЕРАПИИ

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Бактерия *Klebsiella pneumoniae* способна вызывать широкий спектр внутрибольничных инфекций человека, ассоциированных с антибиотикорезистентностью и высокой смертностью. Одна из перспективных альтернатив применению антибиотиков для лечения таких инфекций — терапия вирулентными бактериофагами. Целью работы было выделить из внешней среды вирулентные бактериофаги, эффективные против актуальных клинических штаммов *K. pneumoniae*, и дать их молекулярно-генетическую характеристику. Бактериофаги выделяли из проб речной воды методом накопительных культур. Полногеномное секвенирование бактериофагов выполняли на платформе MiSeq (Illumina). Выделено и описано три новых бактериофага *K. pneumoniae*, принадлежащих к семействам *Autographiviridae* (vB_KpnP_NER40, GenBank MZ602146) и *Myoviridae* (vB_KpnM_VIK251, GenBank MZ602147; vB_KpnM_FRZ284, GenBank MZ602148). На коллекции из 105 клинических штаммов *K. pneumoniae* установлено, что бактериофаги vB_KpnP_NER40 и vB_KpnM_VIK251 обладают узким спектром литической активности (22% и 11%), ограниченными штаммами с капсульным типом K2 и K20 соответственно. Бактериофаг vB_KpnM_FRZ284, напротив, имел широкий спектр литической активности (37%), вызывая лизис штаммов с различным типом капсульного полисахарида. Фаги обладают строгой вирулентной природой и не несут в составе генома гены интеграз, токсинов или факторов патогенности. В составе геномов капсулоспецифичных бактериофагов vB_KpnP_NER40 и vB_KpnM_VIK251 обнаружены гены деполимераз, кодирующие потенциальные рецепторсвязывающие белки. Коктейль из трех бактериофагов лизировал около 65% штаммов исследуемой коллекции *K. pneumoniae* и потенциально применим в терапевтических целях.

Ключевые слова: вирулентные бактериофаги, *Klebsiella pneumoniae*, антибиотикорезистентность, фаготерапия, деполимераза

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Klebsiella pneumoniae is a Gram-negative, non-motile, facultative anaerobic bacterium, widely spread in the environment. Microorganisms of this species have traditionally been regarded as commensals, they can be found on human skin, in the gastrointestinal and respiratory tract [1]. This is the second most common nosocomial pathogen in the world capable of causing a wide range of infections, such as abscesses, purulent wounds, septicemia, pneumonia, infections of urinary tract and gastrointestinal tract [2].

Inappropriate use of antibiotics all over the world resulted in emergence and spread of drug resistant bacteria. Among the members of the genus *Klebsiella*, the isolates, carrying genes encoding extended spectrum beta-lactamases or carbapenemase-encoding genes, are the most dangerous [3]. According to the antibiotic resistance map of Russia, 30–60% of nosocomial isolates can be resistant to carbapenems, and 60–80% can be resistant to the third generation cephalosporins [4].

As a consequence of the crisis caused by dissemination of resistant bacteria, searching for new approaches to antimicrobial therapy is especially relevant. Virulent bacteriophage (phage) therapy is one of the promising alternatives [5]. Phages are natural antagonists of bacteria in wild populations, which are capable of quick selective lysis of pathogens, including the *K. pneumoniae* isolates associated with antibiotic resistance. Phage therapy has been used in clinical practice since the early 20th century. No significant side effects of phage therapy have been detected throughout history [6]. To date, the phage therapy safety and efficiency have been confirmed by mammal and *Galleria mellonella* models, as well as by clinical data [7, 8]. The narrow host range of distinct phages is the natural limitation of this approach. As a result, phage cocktails of active bacteriophages targeting various bacterial species have to be used to combat the undefined pathogen [9].

The host range of *K. pneumoniae* phages strongly correlates with the capsular polysaccharide (CPS) type [10]. To date, at least 130 CPS types have been described. CPS is the key virulence factor protecting the bacteria against the human immune system and the effects of some antibiotics [11]. Strains, characterized by the increased CPS expression, are often more virulent, and belong to the hypervirulent *K. pneumoniae* group [12].

The majority of *K. pneumoniae* phages encode depolymerases: the enzymes capable of breaking down the polysaccharide capsule through glycosidic bond cleavage [10, 13]. The diversity of phage depolymerases is of particular interest, since studying the enzymes may lead to developing the new class of antimicrobial agents. It has been shown that phage depolymerases can accelerate inactivation of bacteria with the serum *in vitro* and significantly increase the survival rate in murine and *Galleria mellonella* larvae models [14, 15]. Moreover, phage depolymerases can be used for the express microbial capsular type identification or the bacterial biofilm destruction [16].

The study was aimed to isolate the virulent bacteriophages, active against the relevant clinical *K. pneumoniae* strains, perform bacteriophage characterization, and assess the viability of using the bacteriophages as the agents for phage therapy.

METHODS

Bacterial strains and their characteristics

The collection of 105 *K. pneumoniae* strains was compiled in 2018–2019 at the Clinical Hospital № 123 of the Federal

Research and Clinical Center of Physical-Chemical Medicine of the FMBA of Russia. All bacteria were grown in the lysogeny broth (LB) (Himedia; India) at 37 °C. The bacteria were identified by MALDI-TOF mass spectrometry as described previously [17]. Antimicrobial susceptibility was tested by the disk-diffusion method in accordance with the guidelines issued by the Clinical and Laboratory Standards Institute [18].

Molecular genetic characterization of bacterial strains

Genomic DNA was extracted using the DNA-Express kit (Lytech; Russia) following the manufacturer's instructions. Multilocus sequence typing (MLST) of *K. pneumoniae* strains was performed by sequencing of seven housekeeping genes in accordance with the standard scheme as described previously [19]. The capsular type was defined by the *wzi* gene Sanger sequencing [20]. Amplification of genes, included in the molecular genetic typing schemes, was carried out in the TETRAD DNA ENGINE (MJ Research; USA). Sanger sequencing was performed with the 3730 DNA Analyzer (Thermo Fisher Scientific; UK).

Bacteriophage isolation and purification

Three *K. pneumoniae* strains, Kp40, Kp25-1 and Kp284, were used as the hosts for bacteriophage isolation. Phages vB_KpnP_NER40, vB_KpnM_VIK251 and vB_KpnM_FRZ284 were isolated from the water samples taken from the Chernyanka River in accordance with the previously reported method [21]. The 15 mL river water sample was centrifuged at 10,000 g for 15 min; supernatant was filtered through the 0.22 µm membrane sterile syringe filter (Millipore; USA). The filtered supernatant and 0.2 mL of the host strain culture being in the logarithmic growth phase (OD 600 nm = 0.3) were combined with 15 mL of the double concentration LB broth and incubated overnight while stirring (200 rpm) at 37 °C to enrich the phage fraction. Then the culture was centrifuged at 10,000 g for 15 min and filtered through the 0.22 µm filters. The resulting lysates were serially diluted in LB, the phage titer was determined by the method of agar layers according to Grazia in order to detect and isolate individual phages [22]. Monoisolates were obtained by sequential (three-fold) extraction from the distinct phage plaque.

Lytic spectrum determination

The lytic spectrum of the phages was defined by the spot test assay with the use of 105 *K. pneumoniae* strains. The 100 µl aliquot of the culture of each strain being in the logarithmic growth phase (OD 600 nm = 0.3) was added to 5 mL of semi-solid agar (0.6%), which was later applied on the bottom agar layer to form the top layer. Phage lysate, 5 mL with a titer of 10⁶ pfu/mL, was applied drop by drop onto the fresh bacterial lawn and left to dry until completely absorbed. The results were assessed after the overnight incubation at 37 °C by the presence of lysis zones at the sites of the applied bacteriophage drops. In case of solid lysis zone or sporadic phage plaques, the bacterial strain was considered sensitive to bacteriophage.

Whole genome sequencing of bacteriophages and bioinformatic data analysis

The total DNA isolation was performed using the standard phenol-chloroform extraction protocol as described previously [23]. Sequencing was carried out with the use of the MiSeq



Fig. 1. Plaque morphology for the vB_KpnP_NER40, vB_KpnM_VIK251 and vB_KpnM_FRZ284 bacteriophages

tool and the MiSeq Reagent Nano Kit v2 (500 cycle) (Illumina; USA) following the manufacturer's instructions.

Genomes were assembled with the SPAdes software (v.3.14.0). Open reading frames (ORFs) were predicted using GeneMarkS (version 4.32), Phast and VGAS. The tRNAScan-SE (University of California Santa Cruz, USA) and the ARAGORN (Murdoch University, Australia) software tools were used to predict the presence of transfer RNA (tRNA) in the genome. The expected functions of proteins, encoded by distinct ORFs, were predicted in the manual mode with the use of BLASTp, HHPred, Phast and InterPro. The search for genes encoding toxins and other factors in the bacteriophage genomes was performed using the pathogenic bacteria virulence factor databases [24]. Matching with the Antibiotic Resistance Gene Database was carried out in order to identify the antibiotic resistance determinants [25]. Phylogenetic analysis was performed using the amino acid sequences of RNA polymerase (for phages of *Autographiviridae* family) and terminase large subunit (for phages of *Myoviridae* family) following the guidelines issued by the International Committee on Taxonomy of Viruses (ICTV) (<https://talk.ictvonline.org/taxonomy>). Phylogenetic tree was constructed by the Genome-BLAST distance phylogeny method on the VICTOR web-server.

RESULTS

Isolation and phenotypic characteristics of three new *Klebsiella pneumoniae* phages

The Kp40 strain of *K. pneumoniae*, belonging to the sequence type (ST) 395 and capsular type K2, was used as a host strain for the vB_KpnP_NER40 phage. The Kp25-1 strain of *K. pneumoniae*, belonging to the ST268 and capsular type K20, was used as a host for isolation of vB_KpnM_VIK251 bacteriophage. The third strain of *K. pneumoniae*, Kp284, of the rare sequence type ST1655 and the undefined capsular type, was selected as a host strain for the vB_KpnM_FRZ284 phage. *K. pneumoniae* strains used as the host strains were resistant to three or more than three classes of antibiotics, including meropenem.

Bacteriophage vB_KpnP_NER40 formed large (3–5 mm) phage plaques, surrounded by wide halos. Plaques formed by the vB_KpnM_VIK251 phage were smaller (2–3 mm), and were surrounded by small (1–2 mm) halo. Phage vB_KpnM_FRZ284 formed small (1–2 mm) plaques with no halo (Fig. 1). According to literary sources, hazy halo surrounding the phage plaque is associated with the supposed bacteriophage-encoded depolymerase activity [26].

Table 1. Lytic spectrum of phages vB_KpnP_NER40, vB_KpnM_VIK251 and vB_KpnM_FRZ284

Capsular type	Total number of strains	Number of strains lysed by vB_KpnP_NER40 (% of the total number)	Number of strains lysed by vB_KpnM_VIK251 (% of the total number)	Number of strains lysed by vB_KpnM_FRZ284 (% of the total number)
K1	3	0	0	1 (33,3%)
K2	25	23 (92%)	0	4 (16%)
K7	1	0	0	1 (100%)
K19	4	0	0	2 (50%)
K20	17	0	12 (70,6 %)	2 (11,8%)
K23	6	0	0	3 (50%)
KL24	3	0	0	3 (100%)
KL25	2	0	0	0
KL38	1	0	0	0
KL39	14	0	0	8 (57,1%)
KL45	5	0	0	4 (80%)
K47	2	0	0	0
K57	6	0	0	3 (50%)
K60	1	0	0	0
KL62	1	0	0	1 (100%)
KL63	2	0	0	0
KL64	9	0	0	5 (55,6%)
KL107	1	0	0	1 (100%)
KL108	1	0	0	0
wzi475	1	0	0	1 (100%)

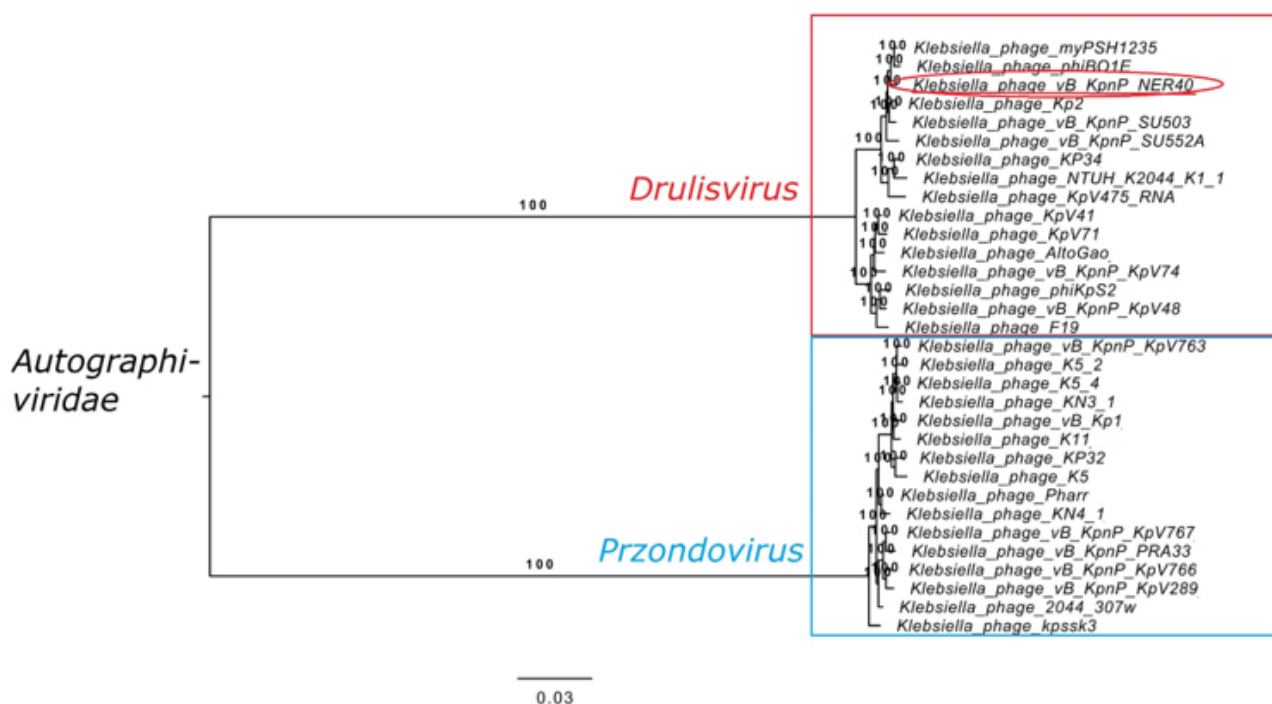


Fig. 2. Phylogeny of the *Autographiviridae* phage RNA polymerase amino acid sequences

Lytic spectrum of bacteriophages

The lytic spectrum of the studied phages was defined using the collection of 105 *K. pneumoniae* strains. According to the results of the *wzi* gene sequencing, *K. pneumoniae* strains had a total of 20 unique capsular types. The most common capsular types were as follows: K2 ($n = 25$; 23.8%), K20 ($n = 17$; 16.2%), KL39 ($n = 14$; 13.3%) and KL64 ($n = 9$; 8.6%).

Bacteriophages vB_KpnP_NER40 and vB_KpnM_VIK251 had a narrow lytic spectrum, which was limited to strains having one capsular type (Table 1). Phage vB_KpnP_NER40 lysed 23 out of 25 strains of the capsular type K2, whereas phage vB_KpnM_VIK251 lysed 12 out of 17 strains with the capsular type K20. By contrast, phage vB_KpnM_FRZ284 showed lytic activity, not limited to a single capsular type. The phage lysed 39 strains from the collections having 14 different capsular types (Table 1).

Analysis of bacteriophage whole-genome sequencing results

The whole-genome sequencing with subsequent data analysis was performed for bacteriophages vB_KpnP_NER40, vB_KpnM_VIK251 and vB_KpnM_FRZ284. Annotated genome sequences were deposited into the NCBI GenBank database under the numbers MZ602146 (vB_KpnP_NER40), MZ602147 (vB_KpnM_VIK251) and MZ602148 (vB_KpnM_FRZ284).

Genome of vB_KpnP_NER40 phage was represented by double-stranded DNA and had the size of 42,674 bp. The G + C content was 54.3%. Bioinformatic analysis revealed 53 ORFs with a total length of 39,659 bp (Table 2). Analysis of the genome nucleotide sequence using the BLASTn algorithm has shown that phage vB_KpnP_NER40 belongs to the *Autographiviridae* family. In order to verify the phylogenetic position of the phage, a phylogenetic tree was constructed based on the RNA polymerase amino acid sequences of the phages belonging to the *Autographiviridae* family, recommended by ICTV. Based on the phylogenetic analysis, phage vB_KpnP_NER40 belongs to the *Drulisvirus* genus of the *Autographiviridae* family, and seems

the most closely related with the phage phiBO1E (GenBank KM576124.1; 87% query cover and 92.05% sequence identity according to BLASTn) (Fig. 2).

The vB_KpnM_VIK251 phage genome was a linear dsDNA molecule with a length of 141,994 bp and the G + C content of 44.6%. A total of 242 open reading frames and 23 sequences encoding tRNAs were identified. According to BLASTn, the phage matched the *Myoviridae* family and seemed similar to the *Mydovirus* genus members.

The vB_KpnM_FRZ284 phage genome was also represented by dsDNA. According to BLASTn, it belonged to the *Myoviridae* family. However, it seemed much more similar to the phages of the *Jiaodavirus* genus. The phage had a length of 166,376 bp and the G + C content of 39.6%, it encoded 274 open reading frames and 16 tRNAs (see Table 2).

To clarify the phylogenetic position of the *Myoviridae* phages, the tree was constructed based on the amino acid sequences of the terminase large subunit of phages vB_KpnM_VIK251 and vB_KpnM_FRZ284, and phages, recommended by ICTV (Fig. 3). According to phylogenetic analysis, phage vB_KpnM_VIK251 belonged to the *Mydovirus* genus and was most closely related with the phage vB_KpnM_KB57 (GenBank KT934943.1; 87% query cover and 96.71% sequence identity according to BLASTn). Phage vB_KpnM_FRZ284 matched the phages of the *Myoviridae* family, genus *Jiaodavirus*, and seemed closely related with KPN5 (GenBank MN101229.1, 93% query cover and 97.23% sequence identity according to BLASTn) (see Fig. 3).

Functional annotation of *K. pneumoniae* phages

When performing annotation of the phage vB_KpnP_NER40, we managed to predict the functions of 25 proteins encoded by the genome. The vB_KpnP_NER40 phage structural organization was typical for T7-like phages and was characterized by the presence of phage DNA and RNA polymerases, as well as of the lysis cassette composed by spanin-, holin- and endolysin-encoding genes located next to each other [27]. No genes encoding tRNAs, known determinants of antibiotic

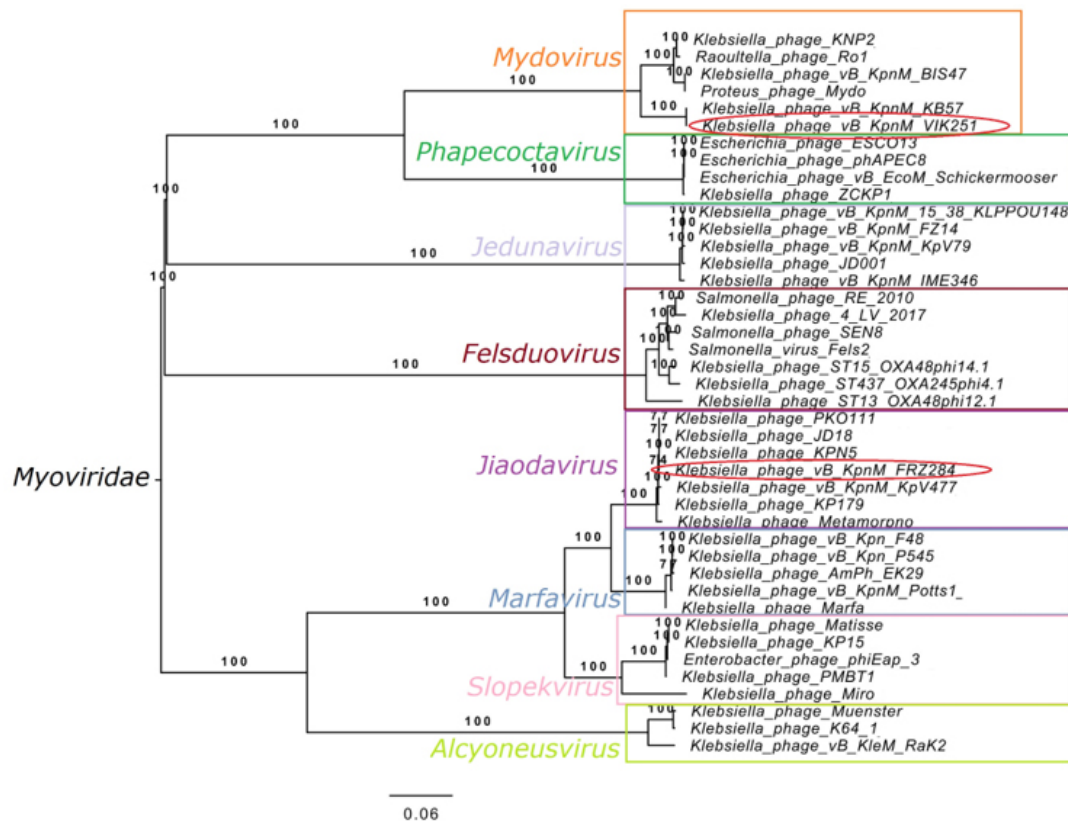


Fig. 3. Phylogeny of the Myoviridae family bacteriophage terminase large subunit amino acid sequences

susceptibility, integrases or toxins were found in the genome.

The vB_KpnP_NER40 phage genome encoded two proteins determining the host specificity: NER40_00045 and NER40_00053. The first protein, NER40_00045, has the length of 318 amino acids. It is the highly conserved protein for phages of the *Drulisvirus* genus. The second protein, NER40_00053, shows a high degree of homology with the tail fibers of six *K. pneumoniae* phages (QE50388.1, CAD5239035.1, QJ52632.1, YP_009789295.1, YP_009792408.1, YP_009006074.1; 96% query cover and 98.42–97.52% sequence identity according to BLASTp). Moreover, this protein encodes the potential pectate lyase domain possessing a beta helical structure, typical for the phage-derived depolymerases.

For the vB_KpnM_VIK251 phage, the expected function (structural proteins, enzymes involved in DNA replication, regulation, transcription and translation, lysis of the host) could be attributed to the products of 145 open reading frames. No genes were found, the function of which was somehow associated with the phage lysogenic cycle. At least two genes encoding proteins homologous to the phage fibers (VIK251_00041 and VIK251_00046) were found in the genome of this phage.

The vB_KpnM_FRZ284 bacteriophage encodes 126 proteins, which could be assigned the expected function. No genes encoding integrases, toxins or any other pathogenicity factors, ruling out the therapeutic use of the phage, were found in the genome. The receptor binding proteins of this phage are represented by four proteins (FRZ284_00009, FRZ284_00012, FRZ284_00098 and FRZ284_00101) homologous to the well known proteins of the *Jiaodavirus* phage fibrils.

DISCUSSION

Multidrug-resistant strains of *K. pneumoniae* of the sequence types 395 and 268 and the capsular types K2 and K20

were used as the host strains for bacteriophage isolation. Such isolates are widespread, and are often associated with nosocomial outbreaks, caused by carbapenem-resistant and hypervirulent strains in Asia and Europe [28, 29].

Three new lytic phages of *K. pneumoniae* were isolated within the framework of searching for possible alternative antimicrobial therapeutic agents. Phages vB_KpnM_VIK251 and vB_KpnM_FRZ284 belonged to different genera of the Myoviridae family. Phage vB_KpnP_NER40 belonged to the Autographiviridae family. Phages vB_KpnP_NER40 and vB_KpnM_VIK251 were markedly capsule-specific (K2- and K20-specific respectively) and lysed 70–90% strains of appropriate capsular type. In turn, vB_KpnM_FRZ284 lysed 36% strains of the collection regardless of the capsular type.

Bacteriophage vB_KpnP_NER40 encoded depolymerase comprised in the receptor binding protein, which determined host specificity. Depolymerase of phage vB_KpnP_NER40 was homologous to several phage receptor binding proteins, including the described above K2-specific depolymerase of phage KpV74 (NC_047811.1, 96% query cover and 98.2% sequence identity according to BLASTp) [14].

Looking at the vB_KpnM_VIK251 bacteriophage genome more closely, one finds out that VIK251_00052 gene encodes a protein with unknown function, however, the N-terminal region of this protein shows a high degree of homology with the tail fibril of the phage vB_KpnM_Seu621 (QO168629.1), carrying the receptor binding depolymerase domain. Other regions of this protein encode the pectate lyase domain possessing a beta helical structure, which could be the proof of this hypothetical protein belonging to the tail fibril machinery, responsible for recognition of host receptors and depolymerase activity of this phage. The putative vB_KpnM_VIK251 phage depolymerase has no homologous proteins deposited in the NCBI database, which is a matter of great interest due to the potential of depolymerases as possible therapeutic agents targeting *Klebsiella* infections [15].

Table 2. General characteristics of vB_KpnP_NER40, vB_KpnM_VIK251 and vB_KpnM_FRZ284 phage genomes

Bacteriophage	Genome, bp	G + C, %	ORF	Family	Genus
vB_KpnP_NER40	42 674	54,3	53	<i>Autographiviridae</i>	<i>Drulisvirus</i>
vB_KpnM_VIK251	14 1994	44,6	242	<i>Myoviridae</i>	<i>Mydovirus</i>
vB_KpnM_FRZ284	16 6376	39,6	274	<i>Myoviridae</i>	<i>Jiaodavirus</i>

To date, the mechanism of adsorption of the third phage, vB_KpnM_FRZ284, is unknown. However, broad lytic spectrum makes this phage promising for the further investigation aimed at potential expansion of the lytic spectra of other phages using the engineering approach.

When discussing the possibilities for treatment of infectious diseases caused by *K. pneumoniae*, it should be pointed out that the cocktail of the studied phages lyses 65% of the strain collection, which makes them comparable with the commercial phage-based products comprising several dozen bacteriophages in terms of efficiency [30].

CONCLUSIONS

Bacteriophages vB_KpnP_NER40, vB_KpnM_VIK251 and vB_KpnM_FRZ284, isolated from environmental sources, have a great potential as the alternative antibacterial agents targeting the antibiotic resistant *K. pneumoniae*. Strict host specificity of phages vB_KpnM_VIK251 and vB_KpnP_NER40, as well as the capability of breaking down capsular polysaccharides and the broad lytic spectrum of phage vB_KpnM_FRZ284 make the studied phages the prospective candidates for developing highly efficient phage cocktails.

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