ISOLATION AND CHARACTERIZATION OF *KLEBSIELLA PNEUMONIAE* BACTERIOPHAGES ENCODING POLYSACCHARIDE DEPOLYMERASES WITH RARE CAPSULE SPECIFICITY

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Bacterial infections caused by antibiotic resistant strains of *Klebsiella pneumoniae* are among the most dangerous threats for the world's public healthcare. Treatment with bacteriophages and/or their derivatives could become one of the alternative methods for therapy of infections caused by *K. pneumoniae*. The study was aimed to isolate from the environment and characterize the capsule-specific *K. pneumoniae* bacteriophages that are useful for therapy and possess the polysaccharide depolymerase genes. Bacteriophages were isolated from the river water samples by enrichment method. The host range of bacteriophages were assessed using the collection of 180 *K. pneumoniae* clinical strains. Bacteriophage whole genome sequencing was performed on the MiSeq platform (Illumina). Four new bacteriophages from different taxonomic groups were isolated and characterized during the study: vB_KpnM_NDO71 (*Vequintavirinae* family), vB_KpnS_MAG26fr (*Casjensviridae* family), vB_KpnS_MDA2066 (*Ackermannviridae* family), and vB_KpnS_PMM-G3 (*Drexlerviridae* family). Bacteriophages vB_KpnM_NDO71, vB_KpnS_MAG26fr, and vB_KpnS_PMM-G3 had a narrow lytic spectrum and lysed all strains with the capsular type of the host: KL45, KL19 or KL28, respectively. Bacteriophage vB_KpnS_MDA2066 showed lytic activity against strains with two different capsular types: KL19 and KL107. Bacteriophages were strictly virulent and contained no integrase genes, potentially dangerous toxin genes or antibiotic resistance determinants. This allows them to be used in therapeutic practice. Receptor-binding proteins represented by polysaccharide depolymerases were predicted for each bacteriophage.

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

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ВЫДЕЛЕНИЕ И ХАРАКТЕРИСТИКА БАКТЕРИОФАГОВ *KLEBSIELLA PNEUMONIAE*, КОДИРУЮЩИХ ПОЛИСАХАРИД-ДЕПОЛИМЕРАЗЫ С УНИКАЛЬНОЙ КАПСУЛЬНОЙ СПЕЦИФИЧНОСТЬЮ

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Бактериальные инфекции, вызываемые устойчивыми к антибиотикам штаммами *Klebsiella pneumoniae*, входят в список самых опасных угроз для мирового общественного здравоохранения. Одним из альтернативных способов терапии инфекций, вызванных *K. pneumoniae*, может стать терапия бактериофагами и/или их производными. Целью работы было выделить из внешней среды и охарактеризовать капсуло-специфичные бактериофаги *K. pneumoniae*, пригодные для терапевтического применения и несущие гены полисахарид-деполимераз. Бактериофаги выделяли из проб речной воды методом накопительных культур. Спектр хозяев бактериофагов оценивали на коллекции из 180 клинических штаммов *K. pneumoniae*. Полногеномное секвенирование бактериофагов выполняли на платформе MiSeq (Illumina). В рамках исследования выделено и охарактеризовано четыре новых бактериофага, принадлежащих к различным таксономическим группам: vB_KpnM_NDO71 (подсемейство *Vequintavirinae*), vB_KpnS_MAG26fr (семейство *Casjensviridae*), vB_KpnS_MDA2066 (семейство *Ackermannviridae*) и vB_KpnS_PMM-G3 (семейство *Drexlerviridae*). Бактериофаги vB_KpnM_NDO71, vB_KpnS_MAG26fr и vB_KpnS_PMM-G3 обладали узким спектром литической активности и лизировали все штаммы с капсульным типом штамма хозяина: KL45, KL19 или KL28 соответственно. Бактериофаг vB_KpnS_MDA2066 проявлял литическую активность в отношении штаммов двух различных капсульных типов: KL19 и KL107. Бактериофаги обладали строго вирулентной природой и не несли в своем составе генов интеграз, а также потенциально опасных генов токсинов и детерминант устойчивости к антибиотикам, что позволяет применять их в терапевтической практике. Для каждого бактериофага предсказаны рецептор-связывающие белки, представленные полисахарид-деполимеразами.

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

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Klebsiella pneumoniae is a Gram-negative non-motile facultative anaerobic bacterium that occurs everywhere in nature and can constitute a part of normal flora in humans and other animals [1, 2]. At the same time, *K. pneumoniae* is the second most common nosocomial pathogen in the world capable of causing numerous infections, such as abscesses, purulent wounds, pneumonia, urinary tract and gastrointestinal tract infections [3]. The Russian study "Marathon 2015–2016" has shown that *K. pneumoniae* strains prevail (47.2%) among all nosocomial strains of Enterobacterales [4]. According to the same study and the map of antibiotic resistance in Russia [5], the share of carbapenem-resistant isolates is 6.9–41.6%, 80.1–90.2% are resistant to III-IV generation cephalosporins, and 6.11% are resistant to colistin. The *K. pneumoniae* strains associated with antibiotic resistance rank third in terms of mortality rate among antibiotic-resistant bacteria [6].

Treatment with bacteriophages and/or their derivatives could become one of the alternative methods for therapy of infections caused by *K. pneumoniae* [7]. Bacteriophages are the most widely spread and abundant group of viruses that are described as natural parasites of bacteria in natural populations [8]. Bacteriophages have been used as antimicrobial agents since early XX century due to their ability to infect and lyse bacterial cells [9]. Phage therapy has a number of advantages, such as the ability to lyse bacteria regardless of their antibiotic resistance, and no side effects to patients, which make it possible to use bacteriophage even for therapy of children and immunocompromised patients [10]. Today, the therapeutic use of bacteriophages is undergoing a rebirth, and the reports of successful treatment cases are more and more often found in the literature [11–13].

Along with the use of bacteriophages, close attention is currently paid to certain phage proteins showing activity against the bacterial surface structures. One such example are polysaccharide depolymerases [14]. These proteins can destroy bacterial capsular polysaccharides, thus providing sensitization of bacteria to antimicrobial drugs and the immune system [15]. Depolymerases usually show narrow specificity limited to certain type of the bacterial capsular polysaccharide [14]. In this regard, the search and description of bacteriophages encoding depolymerases that show activity against a broad range of capsular types of the clinically significant bacteria is an urgent task of the innovative approaches to therapy of infections caused by multidrug-resistant bacteria.

The study was aimed to isolate from the environment and characterize the capsule-specific *K. pneumoniae* bacteriophages that are useful for therapy and contain genes encoding polysaccharide depolymerases.

METHODS

Bacterial strains and their characteristics

The study involved a total of 180 K. pneumoniae clinical isolates collected in 2019–2022 in the Gorbacheva Research Institute of Pediatric Oncology, Hematology and Transplantation (Saint Petersburg, Russia) and the Clinical Hospital № 123 (Odintsovo, Russia), including 12 strains obtained from the State Collection of Pathogenic Microorganisms and Cell Cultures (Obolensk, Russia). Bacterial strains were grown in the lysogeny broth (LB) (Himedia; India) at 37 °C. Species identification was performed by the direct bacterial lysate mass spectrometry profiling in accordance with the previously reported method [16]. Mass spectra were acquired using the Microflex time-of-flight mass spectrometer (Bruker Daltonics; Germany). The flexControl 3.0 and flexAnalysis 3.0 software packages (Bruker Daltonics; Germany) were used for recording, processing and analysis of mass spectra. Species identification was performed in the MALDI Biotyper 3.0 software package (Bruker Daltonics; Germany). The *K. pneumoniae* capsular type was defined by the wzi gene sequencing [17].

Bacteriophage isolation and purification

The river water sample was used as a source of phages. The sample was centrifuged (4000 g, 10 min), and supernatant was filtered through the 0.22 μm filters (Merk Millipore; USA) to remove the bacterial fraction. Equal aliquots (15 mL each) of filtered water and double concentration LB broth were mixed and inoculated with 20 μm of the potential host strain overnight culture. The mixture was incubated overnight at 37 °C on the rocking shaker. The resulting suspension was sterilized by filtration through the 0.22 μm filter, and the filtrate was tested for the presence of bacteriophages by the spot test assay [18]. Phage isolates were purified by triple passage through a single plaque.

Host range determination

The phage host range were defined by the spot test assay [18]. For that 100 μm of the culture of each strain being through the logarithmic phase of growth were added to 5 mL of the unset semi-solid LB agar (0.7% agar) and poured onto Petri dishes containing a thin layer of LB agar (1.5% agar). Testing was performed by applying 5 μL of the bacteriophage serial dilutions onto the fresh lawns of the strains. The Petri dishes were incubated overnight at 37 °C. The bacteriophage lytic activity was defined by the presence of the zones of solid bacterial cell lysis matching the drops by shape. The presence of translucent halo surrounding the lysis zone or a single phage plaque was interpreted as bacteriophage polysaccharide depolymerase activity.

DNA sequencing and analysis

Genomic DNA of the phage was extracted using the standard phenol–chloroform extraction protocol [19]. Sequencing was performed with the MiSeq tool (Illumina; USA) using the MiSeq Reagent Nano Kit v2 (500cycle) (Illumina; USA) in accordance with the manufacturer's recommendations. Genomes were assembled with the SPAdes tool (v.3.14.0). The GeneMarkS online service (ver. 4.32) was used for identification of open reading frames (ORFs) in the genome. The search for tRNA genes was performed with the ARAGORN tool.

The predicted genes were annotated manually using BLASTp, HHPred and InterPro. The absence of genes encoding toxins and antibiotic resistance determinants was confirmed by matching against the databases of the pathogenic bacteria virulence factors [20] and antibiotic resistance genes [21]. The annotated genome sequences of bacteriophages vB_KpnM_NDO71, vB_KpnS_MAG26fr, vB_KpnS_MDA2066, and vB_KpnS_PMM-G3 were deposited in the GenBank database with the numbers OP558001, OP558002, OP558003, and OP558005, respectively.

Phylogenetic analysis involved 62 reference bacteriophage genomes recommended by the International Committee on Taxonomy of Viruses (ICTV). Phylogenetic trees were constructed in the offline version of the ViPTree server (v. 1.1.2) based on the pairwise genetic distanced between the phage genomes [22]. The closest bacteriophage homologues were

defined using the BLASTn algorithm. BLASTp services were used to compare the sequences of certain proteins.

RESULTS

Isolation, morphology and host range of the *K. pneumoniae* bacteriophages

A total of four *K. pneumoniae* bacteriophages were isolated from the water sample collected from the Likhoborka River (Moscow) by enrichment culture method: vB_KpnM_NDO71, vB_KpnS_MAG26fr, vB_KpnS_MDA2066, and vB_KpnS_PMM-G3. The clinical strains with certain capsular types isolated in 2020 were used as host strains: *K. pneumoniae* Kp71 (capsular type KL45), Kp26f (KL19), Kp2066 (KL107), and KpG3 (KL28).

Bacteriophage vB_KpnM_NDO71 formed small (0.5 mm) plaques surrounded by wide (2–4 mm) halos. The plaques formed by bacteriophage vB_KpnS_PMM-G3 were much larger (1–2 mm) and had wide (4–5 mm) halos. Bacteriophages vB_KpnS_MAG26fr and vB_KpnS_MDA2066 formed small (0.5 mm) plaques surrounded by small (1–2 mm) halos (Fig. 1).

Bacteriophage host ranges were assessed on a collection of 180 *K. pneumoniae* strains with known capsular types based on the wzi gene sequence typing. The strains had 31 unique capsular types, among which KL2 (19.4%), KL23 (9.4%), KL39 (8.9%), KL64 (8.9%), and KL20 (6.1%) were the most common.

Bacteriophages vB_KpnM_NDO71, vB_KpnS_MAG26fr, and vB_KpnS_PMM-G3 had a narrow lytic spectrum and lysed all strains with the capsular type of the host strain: KL45 (n = 4; 2.2%), KL19 (n = 6; 3.3%), and KL28 (n = 4; 2.2%), respectively. Bacteriophage vB_KpnS_MDA2066 demonstrated lytic activity against strains with two different capsular types: KL19 and KL107 (n = 7; 3.9%).

Bacteriophage whole-genome sequencing and phylogenetic analysis

Genomes of the studied bacteriophages were represented by double-stranded DNA molecules with the length of 49,477–158,414 bps and G+C content of 44.4–56.1% (Table 1). The number of predicted open reading frames (ORFs) varied between 76 and 236. Some tRNA genes were revealed in bacteriophages vB_KpnM_NDO71 and vB_KpnS_MDA2066 (21 and 7, respectively).

A phylogenetic tree was constructed based on the whole-genome sequences of phages recommended by ICTV in order to define the taxonomic status of bacteriophages (Fig. 2). Bacteriophages vB_KpnM_NDO71, vB_KpnS_MAG26fr, vB_KpnS_MDA2066, and vB_KpnS_PMM-G3 belonged to different phyla and were parts of the clusters formed by members of the Mydovirus, Yonseivirus, Taipeivirus, and Webervirus genera, respectively, of the phylogenetic tree.

According to the results of BLASTn analysis, Klebsiella phage vB_KpnM_KB57 (GenBank KT934943.1; 84% query coverage and 96.49% sequence identity) turned out to be the closest homologue of phage vB_KpnM_NDO71, Klebsiella phage S9a (GenBank ON623732.1; 71% query coverage and 93.88% sequence identity) was most close to phage vB_KpnS_MAG26fr, Klebsiella virus UPM 2146 (GenBank NC_049472.1; 95% query coverage and 98.98% sequence identity) was most close to phage vB_KpnS_MDA2066, and Klebsiella virus UPM 2146 (GenBank NC_049472.1; 95% query coverage and 98.98% sequence identity) was the closest homologue of phage vB_KpnS_PMM-G3.

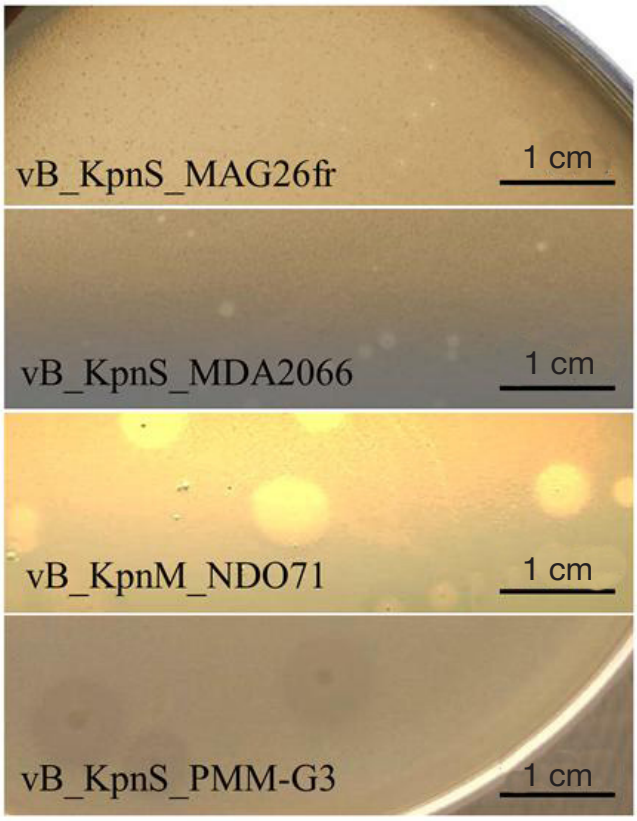

Fig. 1. Morphology of the vB_KpnM_NDO71, vB_KpnS_MAG26fr, vB_KpnS_MDA2066 and vB_KpnS_PMM-G3 phage plaques

Functional analysis of *K. pneumoniae* phages

We successfully predicted the functions of 61 proteins during functional annotation of the vB_KpnM_NDO71 phage genome. The bacteriophage structural organization was typical for rV5-like phages: the phage did not encode RNA polymerase and produced o-spanin as a lytic protein. In contrast to homologue phages Seu621 and VIK251 [23, 24], DNA polymerase gene found in the vB_KpnM_NDO71 phage genome was divided into two reading frames by the gene encoding homing endonuclease.

As for phage vB_KpnS_MAG26fr, the expected functions (structural proteins; enzymes involved in replication, regulation, transcription and translation of DNA; lysis of the host) were attributed to the products of 42 ORFs. Of these 19 were structural proteins of the phages, while the cassette comprising five proteins (o-spanin, component of the inner membrane

Table 1. General characteristics of the genomes of phages vB_KpnM_NDO71, vB_KpnS_MAG26fr, vB_KpnS_MDA2066, and vB_KpnS_PMM-G3

Bacteriophage	Genome size, bp	G+C	ORF	tRNA
vB_KpnM_NDO71	136 566	44,40%	236	21
vB_KpnS_MAG26fr	59 701	56,10%	79	0
vB_KpnS_MDA2066	158 414	46,40%	208	7
vB_KpnS_PMM-G3	49 477	50,10%	76	0

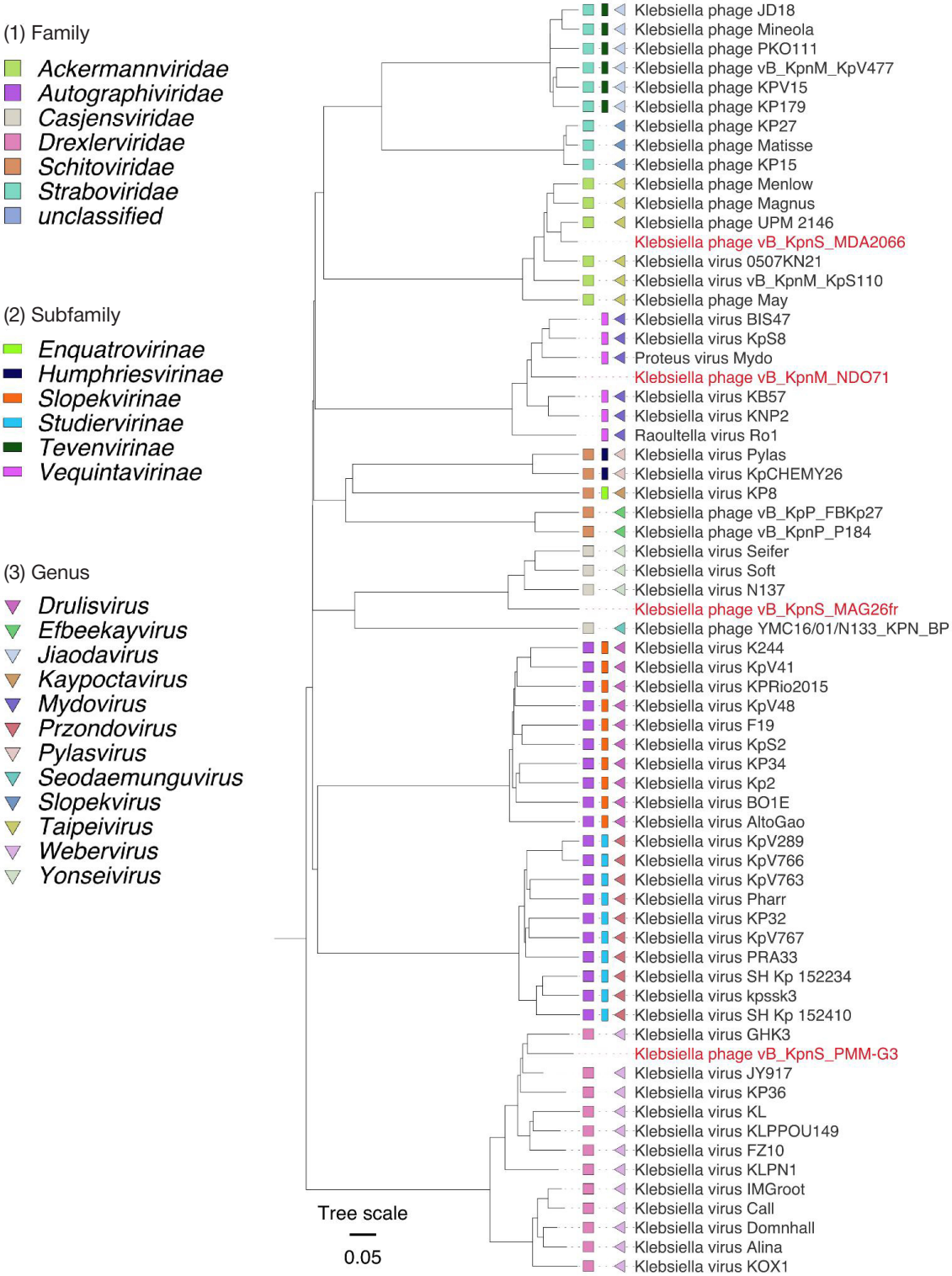

Fig. 2. Phylogeny of the *K. pneumoniae* bacteriophages. The studied bacteriophages are highlighted in red

spanin, endolysin, and two proteins of the choline–anti-choline system) was responsible for the host cell lysis.

Bacteriophage vB_KpnS_MDA2066 encoded 80 proteins assigned the expected functions. Of these 27 were structural proteins, 52 belonged to genes involved in replication, regulation, transcription and translation of DNA. Furthermore, phage encoded one endolysin protein responsible for lysis of the host bacterium.

Of 76 ORFs of the vB_KpnS_PMM-G3 phage, 43 encoded proteins assigned the expected functions, the majority of which were considered structural proteins. The bacteriophage had genome structural organization typical for T1-like bacteriophages and encoded no genes of DNA and RNA polymerases.

Bacteriophage receptor-binding proteins

Five ORFs of the vB_KpnM_NDO71 bacteriophage were annotated as proteins of the phage tail fibers based on the results of functional analysis. It was later found that NDO71_orf047 carried a depolymerase domain represented by pectate lyase 4 (Table 2). The analysis performed using BLASTp showed that this protein shared a high degree of homology

Table 2. Predicted depolymerase domains of phages vB_KpnM_NDO71, vB_KpnS_MAG26fr, vB_KpnS_MDA2066 and vB_KpnS_PMM-G3

ORF	Depolymerase domain	Protein size, aa	Similarity with the closest homologue, %		
			N-terminal domain	Depolymerase domain	C-terminal domain
vB_KpnM_NDO71					
orf047	Pectate lyase 4	597	0	90,8	57,8
vB_KpnS_MAG26fr					
orf055	Family 48 glycoside hydrolase	941	29,4	41,8	50,8
vB_KpnS_PMM-G3					
orf046	Pectate lyase 3	742	98,5	83,5	99,7
vB_KpnS_MDA2066					
orf130	Pectate lyase 3	960	0	38,2	33,5
orf131	Family 28 glycoside hydrolase	766	6,8	73,4	64,7
orf133	Family 28 glycoside hydrolase	660	100	100	100
orf135	Family 28 glycoside hydrolase	721	31,8	67,2	59,6

with the hypothetical proteins of the *K. pneumoniae* prophages (GenBank WP_180812430.1; 89% query coverage and 68.97% sequence identity).

The vB_KpnS_MDA2066 genome also encoded five proteins annotated as proteins of the phage tail fibers. However, in contrast to vB_KpnM_NDO71, four predicted phage tail fiber proteins out of five carried the polysaccharide depolymerase domains: pectate lyase 3 in MDA2066_orf130 and family 28 glycoside hydrolases in MDA2066_orf131, MDA2066_orf133, and MDA2066_orf135. Two phage tail fiber proteins of bacteriophage vB_KpnS_MDA2066 (orf131 and orf135) demonstrated more than 50% homology with the previously reported tail fibers of Klebsiella phage K64-1 that showed specificity for capsular types K30/K69 and KN4, respectively. The tail fiber protein MDA2066_orf130 shared a certain degree of homology (53% query coverage and 34.87% sequence identity according to BLASTp) with the tail fiber protein of the previously reported phage P929 showing lytic activity against strains with the KL19 capsular type (Table 2). The fourth tail fiber protein MDA2066_orf133 was identical to the tail fiber protein of the Klebsiella virus UPM 2146 phage and shared a high degree of homology with similar proteins of the Taipeivirus genus phages (100% query coverage and 99% sequence identity).

The vB_KpnS_MAG26fr phage genome encoded two proteins annotated as proteins of the phage tail fibers. One of these proteins (MAG26fr_orf055) encoded a depolymerase domain represented by the family 48 glycoside hydrolase (Table 2). The Soft bacteriophage distal tail protein (GenBank YP_009851405.1; 100% query coverage and 46.36% sequence identity) was the closest homologue of this tail fiber protein.

Similar to phage vB_KpnS_MAG26fr, the vB_KpnS_PMM-G3 encoded two tail fiber proteins. A closer analysis made it possible to predict a polysaccharide depolymerase domain represented by pectatlyase 3 for one of these proteins (PMMG3_orf046). This protein shared a high degree of homology with the tail fiber protein of the undescribed Klebsiella phage VLCpiD7c (GenBank UVX29830.1; 100% query coverage and 95.15% sequence identity).

DISCUSSION

A total of four bacteriophages capable of lysing K. pneumoniae strains with the capsular types KL19, KL28, KL45 and KL107 were isolated during the study. It should be noted that bacteriophages showing activity against three types were reported for the first time, while phages vB_KpnS_MDA2066 and vB_KpnS_MAG26fr showed activity against strains with the KL19 capsular type, just like the previously reported Klebsiella phage P929 [25].

We have used the whole-genome sequencing, that has been increasingly used to define the phage taxonomic status and structural organization in recent years [26], to describe the genetic features of the phages. Phylogenetic analysis has revealed that the studied phages belong to different genera and families. Furthermore, genome alignment using the BLASTn algorithm has revealed significant differences (> 5%) from genomes of the closest phages, thus allowing the researchers to conclude that the studied bacteriophages are members of new species [26].

Functional analysis of the encoded genes showed that the genome structural organization of the phages was typical for the members of appropriate genera. Bacteriophages were strictly virulent and contained no integrase genes, potentially dangerous toxin genes or antibiotic resistance determinants. This makes it possible to use the phages in therapeutic practice.

The receptor-binding proteins represented by polysaccharide depolymerases were predicted in genomes of all four bacteriophages. No homology with the bacteriophages previously reported in literature was revealed in depolymerases of bacteriophages vB_KpnM_NDO71, vB_KpnS_MAG26fr, and vB_KpnS_PMM-G3. This expands the theoretical knowledge and the possibilities of the future development of the broad-spectrum polysaccharide depolymerase-based drugs. Three tail fibers of the vB_KpnS_MDA2066 bacteriophage (orf130, orf131, and orf135) showed a certain degree of homology with the tail fiber proteins with known specificity: KL19, K30/K69, and KN4, respectively. Meanwhile, the main differences were found in the N-terminus that encoded sites of the tail fiber attachment to other phage proteins. By contrast, a high degree of homology was observed in the region of the predicted enzyme domain and in the C-terminus responsible for substrate recognition [27] (Table 2). It is also worth mentioning that there were no samples with the K30/K69 capsular type in the tested collection of *K. pneumoniae* clinical isolates, and the other typing scheme should be used to define the KN4 type.

The lytic spectrum of the studied phages is limited to four capsular types. Despite the fact that there is a small number of strains with capsular types KL19, KL28, KL45, and KL107 in the tested collection (11.7%), isolates with these capsular types

are associated with nosocomial infections resistant to a wide range of antibiotics, including colistin [28]. Furthermore, two phages show activity against strains with the KL19 capsular type (vB_KpnS_MAG26fr and vB_KpnS_MDA2066 have polysaccharide depolymerase domains of different types), which could potentially result in lower frequency of mutation in case of the combined use.

When developing medications, several bacteriophages with various lytic profiles are usually combined into phage cocktails to increase the therapeutic efficiency. Currently, the following requirements are applied to such cocktails: the bacteriophage titer in the preparation should not be below 10^8 PFU/mL, bacteriophages should be strictly virulent and should not contain potentially dangerous genes, bacteriophages should effectively lyse causative agents of infections [29, 30]. Thus, combining these phages with other capsule-specific bacteriophages has the potential to raise the therapeutic cocktail efficiency to 100%.

CONCLUSIONS

The studied bacteriophages vB_KpnM_NDO71, vB_KpnS_MAG26fr, vB_KpnS_MDA2066, and vB_KpnS_PMM-G3 belong to new species of the well-characterized families and subfamilies and are promising candidates for the development of efficient phage cocktails. In turn, the predicted depolymerases showing activity against rare capsular types KL19, KL28, KL45, and KL107 may be the subjects of further study as potential therapeutic agents.

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