IDENTIFICATION AND ANALYSIS OF CRISPR/CAS SYSTEMS STRUCTURES IN THE GENOMES OF ANTIBIOTIC-RESISTANT STRAINS OF *KLEBSIELLA PNEUMONIAE*

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ABSTRACT

Background. *Klebsiella pneumoniae belongs to a group of opportunistic bacteria that can form multiple resistance to antibiotics and transmit it to various types of bacteria through horizontal gene transfer. These studies examine the structural and functional diversity of CRISPR/Cas systems that protect bacteria from foreign DNA. Their analysis using the example of antibiotic-resistant strains of Klebsiella pneumoniae will demonstrate their resistance to certain bacteriophages, which will make it possible to develop approaches to the treatment of complex infectious diseases caused by these microorganisms by creating targeted phage therapy.*

The aim of the study. *To perform a bioinformatics analysis of the identified structural components of CRISPR/Cas systems for screening bacteriophages through CRISPR cassette spacers using the example of antibiotic-resistant strains of Klebsiella pneumoniae.*

Materials and methods. *The article analyzed 29 full-genome sequences of Klebsiella pneumoniae, in the genome of which the structures of CRISPR/Cas systems and antibiotic resistance genes were determined (according to NCBI). To achieve this goal, using software modeling methods, a search was made for Cas genes and CRISPR cassettes, and their structural and functional characteristics were given.*

Results. *Using bioinformatic search algorithms in the genome of antibiotic-resistant strains, functionally active CRISPR/Cas systems with the presence of one or two CRISPR cassettes and belonging to Type I Subtype IE were identified. Groups of resistant strains with identical spacer composition of CRISPR cassettes have been identified. A phylogenetic analysis was carried out confirming their common origin. By analyzing the spacer sequences of CRISPR cassettes, the spectrum of diversity of phages of bacteria of the genus Klebsiella, Salmonella, belonging to the same family Enterobacteriaceae, was determined. Thus, information was obtained about the bacteriophages that are targeted by the action of CRISPR systems of Klebsiella pneumoniae strains that have antibiotic resistance.*

Conclusions. *Analysis of the functional and structural features of the CRISPR/Cas systems of antibiotic resistant Klebsiella pneumoniae strains made it possible to obtain information about their evolutionary history and about the bacteriophages against which their action is directed, that is, about their phage resistance. The approach used in this study may serve as the basis for the creation of personalized phage therapy.*

Key words: *Klebsiella pneumoniae, spacer, antibiotic resistance, CRISPR/Cas-system, bacteriophage, protospacer, bioinformatics*

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ИДЕНТИФИКАЦИЯ И АНАЛИЗ СТРУКТУР CRISPR/CAS-СИСТЕМ В ГЕНОМАХ АНТИБИОТИКОРЕЗИСТЕНТНЫХ ШТАММОВ *KLEBSIELLA PNEUMONIAE*

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РЕЗЮМЕ

Обоснование. *Klebsiella pneumoniae относится к группе бактерий-оппортунистов, обладающих способностью формировать множественную антибиотикорезистентность и передавать её разным видам бактерий путём горизонтального переноса генов. Данные исследования посвящены изучению структурного и функционального разнообразия CRISPR/Cas-систем, защищающих бактерии от инородной ДНК. Их анализ на примере антибиотикорезистентных штаммов Klebsiella pneumoniae продемонстрирует их устойчивость к определённым бактериофагам, что позволит разработать подходы в лечении сложных инфекционных заболеваний, вызванных данными микроорганизмами, путём создания таргетной фаговой терапии.*

Цель исследований. *Выполнить биоинформатический анализ выявленных структурных компонентов CRISPR/Cas-систем для скрининга отбора бактериофагов через спейсеры CRISPR-кассет на примере антибиотикорезистентных штаммов Klebsiella pneumoniae.*

Материалы и методы. *В статье проанализированы 29 полногеномных последовательностей Klebsiella pneumoniae, в геноме которых были определены структуры CRISPR/Cas-систем и гены антибиотикорезистентности (по данным NCBI). Для решения поставленной цели с помощью программных методов моделирования произведён поиск Cas-генов и CRISPR-кассет, дана их структурная и функциональная характеристики.*

Результаты. *При помощи биоинформационных алгоритмов поиска в геноме антибиотикорезистентных штаммов были определены функционально активные CRISPR/Cas-системы с наличием одной или двух CRISPR-кассет и относящиеся к Type I Subtype IE. Определены группы резистентных штаммов, обладающие идентичным спейсерным составом CRISPR-кассет. Проведён филогенетический анализ, подтверждающий их единое происхождение. Путём анализа спейсерных последовательностей CRISPR-кассет определён спектр разнообразия фагов бактерий рода Klebsiella, Salmonella, относящихся к одному семейству Enterobacteriaceae. Таким образом, была получена информация о бактериофагах, на которые нацелено действие CRISPR-систем штаммов Klebsiella pneumoniae, обладающих антибиотикорезистентностью.*

Заключение. *Анализ функциональных и структурных особенностей CRISPR/Cas-систем антибиотикорезистентных штаммов Klebsiella pneumoniae позволил получить информацию об их эволюционной истории и о бактериофагах, против которых направлено их действие, то есть об их фагоустойчивости. Использованный в данный исследованиях подход в дальнейшем может послужить основой для создания персонифицированной фаготерапии.*

Ключевые слова: *Klebsiella pneumoniae, антибиотикорезистентность, CRISPR/Cas-система, спейсер, протоспейсер, бактериофаг, биоинформатика*

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INTRODUCTION

Antibiotics are one of mankind's greatest achievements. With their invention, previously fatal diseases became curable. Nevertheless, the formation of microorganism resistance to antibacterial drugs is one of the most serious problems of the health care system today [1]. The prevalence of antibiotic-resistant strains is increasing worldwide [2–6]. Infectious diseases caused by these pathogens are characterized by a longer course, adherence to numerous complications, increased disability and mortality [7–9]. As a result, the economic costs associated with the increase in the duration and cost of treatment of patients in hospital significantly increased [10, 11]. In February 2017, the World Health Organisation (WHO) published a list of bacterial pathogens that require new treatment approaches as a consequence of their high virulence and antibiotic resistance. *Klebsiella pneumoniae* belongs to this group. It is a Gram-negative opportunistic pathogen causing various infectious diseases including urinary tract infections, bacteremia, pneumonia and abscesses [12, 13]. The worldwide spread of Klebsiella pneumoniae clones producing extended-spectrum beta-lactamases (ESBL) and *Klebsiella pneumoniae* carbapenemase (KPCs) has become a serious threat to healthcare facilities, as they are a major cause of life-threatening nosocomial infections [14]. The widespread prevalence of strains with multiple antibiotic resistance necessitates the search for alternative methods to combat them. WHO has initiated numerous campaigns to combat antibiotic resistance. Epidemiologic surveillance of microbial resistance to antimicrobial agents has been launched [1]. New approaches are being created in the treatment of infectious diseases that reduce or completely replace the use of antibiotics [15]. Against this background, there is a renewed interest in the use of bacteriophages for the treatment of diseases of bacterial origin in medical practice. One of the real tools of effective control is the recently discovered CRISPR/Cas system in bacteria, which protects bacteria from alien mobile genetic elements. Bacteria are capable of acquiring and integrating genetic elements into their own genome [16]. In this manner, they preserve the genetic record of previous attacks by foreign nucleic acids in CRISPR arrays. These arrays consist of short and conserved repetitive sequences called repeats, which are strategically placed between unique sequences called spacers. They are being integrated by specialised Cas proteins into the CRISPR array during infections by nucleic acid invasion [17–20]. Adaptive immunity of prokaryotes against foreign genetic elements is achieved through the formation of effector complexes of RNA-oriented endonucleases, which have the ability to detect and incise foreign DNA previously integrated into the CRISPR array upon secondary infection [17, 21]. Thus, the study of the peculiarities of the structure and functioning of CRISPR/Cas systems of Klebsiella pneumoniae strains with antibiotic resistance will provide an opportunity to work out approaches for the development of targeted phage therapy in the treatment of complex infectious diseases.

THE AIM OF THE STUDY

Perform bioinformatics analysis of the identified structural components of CRISPR/Cas systems for screening bacteriophages through CRISPR cassette spacers using antibiotic-resistant *Klebsiella pneumoniae* strains as an example.

MATERIALS AND METHODS

The study was performed at the Laboratory of Molecular Virology and Biotechnology of the Research Institute of Biomedical Technologies of Irkutsk State Medical University. The system design of the conducted research is presented in Figure 1.

In these studies, 50 full-genome sequences of *Klebsiella pneumoniae* were randomly identified in the genome of which CRISPR/Cas systems were revealed by a bioinformatic method. The object of the study was 29 out of 50 full genome sequences of *Klebsiella pneumoniae*, downloaded from GenBank database, in the genome of which antibiotic resistance genes were revealed (according to NCBI (National Center for Biotechnology Information)). To address this goal, the developed bioinformatics software algorithm was used to search for CaS genes and CRISPR cassettes, and their structural and functional characteristics were obtained.

Software modeling methods were used to search for CRISPR/Cas systems and *Cas* genes: Macromolecular System Finder (MacSyF, ver. 1.0.2) with the auxiliary packages makeblastDB (ver. 3.0) and HMMER (ver. 2.2.28). CRISPR cassettes in genomes were detected and analyzed using online services of available programs: CRISPRCasFinder [22] and CRISPROne [23]. To search for phages, the decoded spacer sequences in FASTA format were uploaded to the CRISPRTarget online application. Only those strains in whose genome CRISPR/Cas-systems were determined according to the results of all used programs were considered in this study.

Phylogenetic trees were constructed and aligned using the MEGA X program using the nearest neighbor joining method (NJ) with bootstrap topology statistical significance analysis (number of replicates – 500) and using the Maximum Composite Likelihood genetic distance model. To "root" the tree, a strain of another *Escherichia coli* species (NC 000913.3) was added to the sample of test organisms and constituted an outgroup.

RESULTS AND DISCUSSION

Twenty-nine out of 50 strains with CRISPR/Cas system present in the genome and possessing antibacterial drug resistance genes (according to NCBI) served as the object of the study. Of the 29 strains tested, 55.2 % ($n = 16$) had *Klebsiella pneumoniae* carbapenemase activity, 3.4 % ($n = 1$) each were resistant to rifampicin and trimethoprim-sulfamethoxazole and 11 (37.9 %) strains had multiple antibiotic resistance.

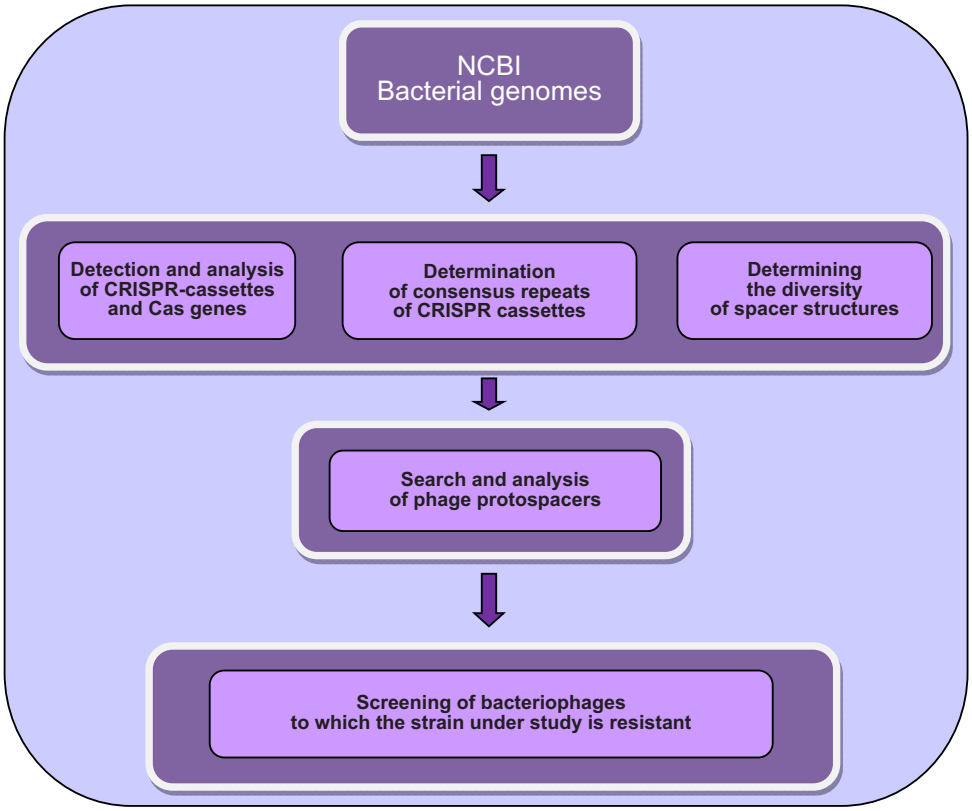

FIG. 1.
Scheme of systematic research design

FIG. 2.
Arrangement of CRISPR cassettes and Cas genes of the Type I Subtype IE system in the genome of Klebsiella pneumoniae strains (data from GenBank: NZ_CP011624.1, NZ_CP006798.1)

Using several bioinformatic search programs, the presence of one or two CRISPR cassettes was revealed in the CRISPR/Cas systems of the strains studied. In all cases, a complete set of *Cas* genes characteristic of Type I Subtype IE systems (*cas2*, *cas1*, *cas5*, *cas7*, *cas6*, *cse2gr11*, *cas8*, *cas3*) was revealed next to the cassettes, which indicates the functional activity of CRISPR/Cas systems of the studied bacteria (Fig. 2).

In all CRISPR cassettes examined, the number of revealed spacers was 1629. Among them, 498 spacers were non-repeatable, and 276 spacers had repeats at two or more CRISPR loci. No spacer repeats were recorded within the cassettes. The number of spacers in the cassettes ranged from 4 to 64. The total number of spacers was greater in strains containing one cassette compared to strains containing two cassettes.

Two types of consensus sequences of CRISPR cassette repeats were observed during the study. This may indicate the presence of a certain type of CRISPR system (in our case it is Type I Subtype IE) and its rather stable operation, since these repeats are recognized by Cas endonuclease proteins, the action of which is aimed at cutting and destruction of the target (Fig. 3).

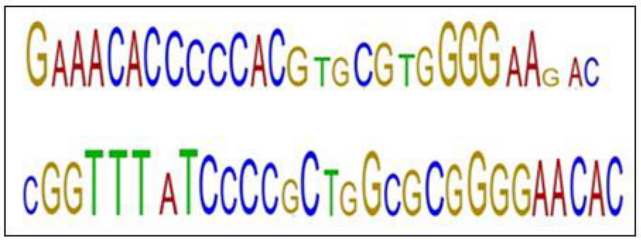

FIG. 3.

Nucleotide sequence of CRISPR consensus repeats of antibiotic-resistant Klebsiella pneumoniae strains

Screening of CRISPR cassette spacer sequences of the strains under study showed their complete correspondence to the protospacers of phages of bacteria of the family *Enterobacteriaceae* of the genus *Klebsiella* (Table 1). It is important to note that for most spacers, there was no complete correspondence between the protospacers of phages from known databases.

In the analysis it was found that in CRISPR cassettes of the studied strains (NZ_CP013322.1) there was a correspondence of the site of one spacer (6) to protospacers of several phages of bacteria of the same family, and correspondence of several spacers (52, 55) of one strain (NZ_CP017934.1) to protospacers of the same phage. This may indicate that during evolution, bacteria have adapted to acquire only those phage DNA spacers that are efficiently recognized by their effector complex, thus enhancing the protective effect of CRISPR/Cas systems.

Therefore, the full spectrum of bacteriophages detected through spacer sequences of CRISPR cassettes of antibiotic-resistant *Klebsiella pneumoniae* strains with access to their complete genome in GenBank was determined. Considering the principle of operation of bacteria CRISPR/Cas-systems, it can be assumed that when encountering these phages, they will be destroyed by Cas-nucleases provided that a part of its genome corresponds to the sequence of the spacer in the CRISPR-cassette of bacteria. This approach can be used in the future as a basis for the creation of personalized phagotherapy.

Next, groups of bacteria possessing identical CRISPR spacer cassettes were revealed by analyzing antibiotic-resistant strains. The first group consisted of two strains possessing a different combination of *Klebsiella pneumoniae* carbapenemase genes (NDM-1, OXA-48, OXA-181) that were associated with other resistance determinants, including extended-spectrum β-lactamases and ArmA methylase encoding resistance to aminoglycosides. So, they were defined as pan-resistant, as they possessed multiple antibiotic resistance. Among the antibiotic-resistant bacteria, five strains were revealed. It should be noted that these strains, having similar spacer composition, were isolated in different regions of the world (South Korea, China (Shanghai)) and at different times (2013, 2014) (Table 2).

The second group consisted of three strains with multiple antibiotic resistance isolated in the same hospital (Greece) but at different times (2011, 2013). They were identified as intrahospital strains. One CRISPR cassette was identified in each of their genomes. Furthermore, two strains had the same spacer cassette composition represented by 24 spacers (Table 3).

The third group consisted of eight *Klebsiella pneumoniae* carbapenemase-producing strains isolated from patients treated at a hospital in Lower Saxony, Germany, where a nosocomial outbreak caused by *Klebsialla pneumoniae* was reported. These strains were found to have one CRISPR cassette each in the genome, consisting of 35 spacers that were completely identical to each other. Also included in this group were three other strains with similar spacer cassette composition but isolated in different regions of the world (Arab Emirates, USA, Singapore) and at different times (2015, 2016, 2014).

The similarity of the spacer composition of cassettes in the genomes of the strains of the represented groups may indicate their common origin. It can be assumed that as a result of widespread circulation, strains genetically changed but retained the CRISPR cassette structure, while in hospital conditions (in-hospital outbreaks) they exchanged genetic information, acquiring new properties but also retaining the CRISPR cassette structure. To confirm this point, a phylogenetic analysis of the strains under study was performed based on the 16S rRNA nucleotide sequence (Fig. 4).

Phylogenetic analysis revealed that strains with similar spacer composition of CRISPR cassettes in the studied groups are closely related, as they are located on branches having the same ancestor (one node). At the same time, the strain (NZ_CP012745.1), which is a member of group 2 and has an individual spacer composition of CRISPR cassettes, has no common ancestor with the other representatives of this group.

TABLE 1

DIVERSITY OF SPACERS AND THEIR CORRESPONDING PROTOSPACER PHAGES IN THE GENOMES OF ANTIBIOTIC-RESISTANT *KLEBSIELLA PNEUMONIAE* STRAINS

Strain	Cassette/spacer	Bacteriophage	GenBank access number
NZ_CP008929.1	1/6	*Klebsiella* phage ST13-OXA48phi12.4	MK422450
NZ_CP009208.1	1/2	*Klebsiella* phage ST512-KPC3phi13.1	MK448235
NZ_CP009208.1	1/2	*Klebsiella* phage ST11-VIM1phi8.1	MK448233
NZ_CP009208.1	2/4	*Klebsiella* phage ST147-VIM1phi7.2	MK448232
NZ_CP009208.1	2/4	*Klebsiella* phage 1 LV-2017	KY271401
NZ_CP012426.1	1/3	*Klebsiella* phage ST101-KPC2phi6.1	MK448231
NZ_CP012743.1	1/12	*Klebsiella* phage ST11-OXA245phi3.2	MK416010
NZ_CP012744.1	1/12	*Klebsiella* phage ST11-OXA245phi3.2	MK416010
NZ_CP012745.1	1/3	*Klebsiella* phage ST101-KPC2phi6.1	MK448231
NZ_CP012745.1	1/22	*Klebsiella* phage YMC1601N133_KPN_BP	MF476925
NZ_CP012745.1	1/25	*Klebsiella* phage 2b LV-2017	KY271395
NZ_CP013322.1	1/6	*Klebsiella* phage ST512-KPC3phi13.1	MK448235
NZ_CP013322.1	1/6	*Klebsiella* phage ST101-KPC2phi6.1	MK448231
NZ_CP013322.1	1/6	*Klebsiella* phage ST11-VIM1phi8.1	MK448233
NZ_CP013322.1	1/6	*Klebsiella* phage 2 LV-2017	KY271396
NZ_CP013322.1	1/6	*Klebsiella* phage 2b LV-2017	KY271395
NZ_CP015382.1	1/3	*Klebsiella* phage ST101-KPC2phi6.1	MK448231
NZ_CP015500.1	1/3	*Klebsiella* phage ST101-KPC2phi6.1	MK448231
NZ_CP015500.1	1/22	*Klebsiella* phage YMC1601N133_KPN_BP	MF476925
NZ_CP015500.1	1/25	*Klebsiella* phage 2b LV-2017	KY271395
NZ_FO834906.1	1/10	*Klebsiella* phage ST11-VIM1phi8.2	MK448234
NZ_CP022127.1	2/6	*Klebsiella* phage ST13-OXA48phi12.4	MK422450
NZ_CP017934.1	1/53	*Klebsiella* phage ST405-OXA48phi1.2	MK416007
	1/55	*Klebsiella* phage 1 LV-2017	KY271401
	1/22	*Klebsiella* phage ST101-KPC2phi6.3	MK416017
	1/36	*Klebsiella* phage 2b LV-2017	KY271395
	1/52	*Klebsiella* phage ST101-KPC2phi6.3	MK416017
	1/52	*Klebsiella* phage ST147-VIM1phi7.2	MK448232
	1/52	*Klebsiella* phage 1 LV-2017	KY271401
	1/18	*Klebsiella* phage ST16-OXA48phi5.1	MK416013
	1/18	*Klebsiella* phage 5 LV-2017	KY271399
NZ_CP018140.1 NZ_CP018686.1 NZ_CP018695.1 NZ_CP018701.1 NZ_CP018707.1 NZ_CP018713.1 NZ_CP018719 NZ_CP017985.1	1/3	*Klebsiella* phage ST101-KPC2phi6.1	MK448231
NZ_CP018140.1 NZ_CP018686.1 NZ_CP018695.1 NZ_CP018701.1 NZ_CP018707.1 NZ_CP018713.1 NZ_CP018719 NZ_CP017985.1	1/14	*Klebsiella* phage YMC1601N133_KPN_BP	MF476925

TABLE 1 (*continued*)

NZ_CP018140.1 NZ_CP018686.1 NZ_CP018695.1 NZ_CP018701.1 NZ_CP018707.1 NZ_CP018713.1 NZ_CP018719 NZ_CP017985.1	1/17	*Klebsiella* phage 2b LV-2017	KY271395
NZ_CP018458.1	2/6	*Klebsiella* phage ST13-OXA48phi12.4	MK422450
NZ_CP019047.1	2/11	*Klebsiella* phage 2 LV-2017	KY271396
NZ_CP019047.1	2/5	*Klebsiella* phage ST11-OXA245phi3.2	MK416010
NZ_CP019047.1	2/13	*Klebsiella* phage ST101-KPC2phi6.1	MK448231
NZ_CP019047.1	2/12	*Klebsiella* phage ST16-OXA48phi5.2	MK448230

TABLE 2

SPACER COMPOSITION OF CRISPR CASSETTES OF THE FIRST GROUP OF PAN-RESISTANT *KLEBSIELLA PNEUMONIAE* STRAINS

No. of strain in GenBank	NZ_CP014004.1	NZ_CP012753.1
Place and year of strain isolation	China, Jiangxi 2014	South Korea 2013
Cassette 1	TGCCTCCAATGCAATCACCGGCCTGCTAACCGG	TGCCTCCAATGCAATCACCGGCCTGCTAACCGG
	CGTGTCGAAGCGCACCTCGTAGCCGAGCCAGTC	CGTGTCGAAGCGCACCTCGTAGCCGAGCCAGTC
	CGTCATCAGCGCCTTGTTCCAGCGGCGACCACC	CGTCATCAGCGCCTTGTTCCAGCGGCGACCACC
	TCCAGTCGTCGTAGTCCTCGGTAATGTCCTCGA	TCCAGTCGTCGTAGTCCTCGGTAATGTCCTCGA
	TATCGTGCAGAGTCACAACCTGACGGGATTATC	TATCGTGCAGAGTCACAACCTGACGGGATTATC
	TCGTGCATGGTGAGGATTCTACAGTCGCACCAT	TCGTGCATGGTGAGGATTCTACAGTCGCACCAT
	TACCTCCCGGCGTCCGCGCCAGGGCGATCACGTG	TACCTCCCGGCGTCCGCGCCAGGGCGATCACGTG
	CCTGCAGCTGGCCGTCGAGCTGACGGATGCCGG	CCTGCAGCTGGCCGTCGAGCTGACGGATGCCGG
	TTCATCACGTGTGAGCGGATTTGGCTCTATCCT	TTCATCACGTGTGAGCGGATTTGGCTCTATCCT
	TATCATCCCTATCGCGCAGCACTTCGACGGCGA	TATCATCCCTATCGCGCAGCACTTCGACGGCGA
	TACCGCCGCGATACTGGCAGTTTTCAGCTGAAT	TACCGCCGCGATACTGGCAGTTTTCAGCTGAAT
	TCCCGCTGGGTAAGCAATATATAACATTTGCAG	
	TTTAACATTCTGAAAGTGCAATTTTTGAGGCTC	
	TTAAGGAGGGGCGCCATGGAGCCCGTATTGATT	
Cassette 2	CAGCGGCGGCGGTAACGCCGCCAGGAGCAACCT	CAGCGGCGGCGGTAACGCCGCCAGGAGCAACCT
	CACCGATCTGCGCCAGCTGGGTGAGACGATGAC	CACCGATCTGCGCCAGCTGGGTGAGACGATGAC
	TACACTCAAGAAAACAAAATCTCAGGTTGATAC	TACACTCAAGAAAACAAAATCTCAGGTTGATAC
	TGGAAGGCGCGATTTGGAGATAGAGCAGCATGA	TGGAAGGCGCGATTTGGAGATAGAGCAGCATGA
	CTCGCACAGCATCGCCCGGATCCGCTTCCACGC	CTCGCACAGCATCGCCCGGATCCGCTTCCACGC
	CACCCGCGTTTTCGAAAGGGATGGCGGCTATGT	CACCCGCGTTTTCGAAAGGGATGGCGGCTATGT
	CGACGGGGCAGGTTTACGTCTACCCGGGCAGGG	CGACGGGGCAGGTTTACGTCTACCCGGGCAGGG
	CATACCAGTCTCCGCCGCGGTCGTACTCAATAT	CATACCAGTCTCCGCCGCGGTCGTACTCAATAT
	TCCGCCGTTTAATCGCGGTGATGATATCCGGCA	TCCGCCGTTTAATCGCGGTGATGATATCCGGCA
	GGAATCCACGACGCGCCGTACCAGCGCGGGCATTCGTTCT	TGGAATCCACGACGCGCCGTACCAGCGCGGGCATTCGTTCT

Note. Identical spacers are highlighted in color.

TABLE 3

SPACER COMPOSITION OF CRISPR CASSETTES OF THE SECOND GROUP OF RESISTANT *KLEBSIELLA PNEUMONIAE* STRAINS

No. of strain in GenBank	NZ_CP012743.1	NZ_CP012744.1	NZ_CP012745.1
Place and year of strain isolation	Greece 2011	Greece 2013	Greece 2013
Cassette 1	CATTTCATAGTGATTCGACTATTTAATTAACA	CATTTCATAGTGATTCGACTATTTAATTAACA	GAGCAGGCACCCGCCGCAACGACGAAGAGCGC
	AGTTCACGACAGGCAAAGTCTTTACGGTATGC	AGTTCACGACAGGCAAAGTCTTTACGGTATGC	AAATCAGCCAGCACCACGATTCTGGGAAATTT
	TCTGCTGGTTACAGGAGAAAAAAATGATTGGT	TCTGCTGGTTACAGGAGAAAAAAATGATTGGT	ACAGGCTTACCCGTATTGAGACGGTTGCTGAA
	TTATTTCTAAACTAAGTTTTGTTTCATGCAGT	TTATTTCTAAACTAAGTTTTGTTTCATGCAGT	GAAACCCCATCAGATGACCCTCCCCATGTTGGC
	TTTTTTTGACGAAGACGGCAACGAGTTAGAAG	TTTTTTTGACGAAGACGGCAACGAGTTAGAAG	TTGCCTGGTCTGCTTGGTGATGATCCGTGGTA
	GTCTCTGCCAGTTTTACCTGCTCAGCGGATAA	GTCTCTGCCAGTTTTACCTGCTCAGCGGATAA	TACAGAACGACTGAGGCGGCGTGTATTGCATA
	CTGACAGCTGGCGCTAACCCGTCGTTTATCGC	CTGACAGCTGGCGCTAACCCGTCGTTTATCGC	GATCTTAACTCTATTGCCAATGGCGCAATTCA
	CGTGAGCATCTGGGCATCTTCAGTGATAGCGT	CGTGAGCATCTGGGCATCTTCAGTGATAGCGT	GGCGATGCGCGCTCTGCTGGCTATCGGTAAAA
	TATTTTTGAGATGATGGATTGTTGCACACCAG	TATTTTTGAGATGATGGATTGTTGCACACCAG	AATGCAGCAACCGGCAAATATATCGCCGGTAA
	CGCTTCTCGGCTTCTCTGAATTTATCCGCCCA	CGCTTCTCGGCTTCTCTGAATTTATCCGCCCA	GGGCTGCCGCACGCCTGGGACGAGTCGAGCCC
	ATCCCGACCCGCTCCTCCAGAGCGAATACGAT	ATCCCGACCCGCTCCTCCAGAGCGAATACGAT	CCGCAATAACAAAAATAAATGAGGGTTAAAGT
	TGCTTTATGGCAAATAAGAGAGGATATAACCA	TGCTTTATGGCAAATAAGAGAGGATATAACCA	GTAAATGGGAATGAGTAGAAGAGCGTCATTGG
	GCGTCCGCCTCCCAAATAGCCAGGCTGTTATT	GCGTCCGCCTCCCAAATAGCCAGGCTGTTATT	CCCCCGCGCACATGCTTAAACGCGCTATCACG
	TAAATCCCAGCTGGTTTGACGTAGTGGCACTG	TAAATCCCAGCTGGTTTGACGTAGTGGCACTG	GGCATCTGTTGTGTAATGTTGAGTTTTTTTCA
	CAATGAAGGCTTATAAGGGTGAAGAGAGCAGG	CAATGAAGGCTTATAAGGGTGAAGAGAGCAGG	CAGGTTAAACATGTAAAAAATGACCGTCGCCG
	CAGTCGGTAACGGCTGGGCGTGACCTCAAAGC	CAGTCGGTAACGGCTGGGCGTGACCTCAAAGC	CACATTGCCCGGTCTGAAAAGTATTTGAAAAT
	GTGCCATTTTTATTTTGCTTAAAATAAATATC	GTGCCATTTTTATTTTGCTTAAAATAAATATC	TCCGCACAGTCAAACGCTCCAGACACCAACCC
	CCTATTGTGTTCAGTCGTAACAGCTGAATCAG	CCTATTGTGTTCAGTCGTAACAGCTGAATCAG	CCGGAACACCACCAGTAACAGCTACTGTAGGC
	GGCGCGGCGGAATAACCACTTTATGAGCAGGT	GGCGCGGCGGAATAACCACTTTATGAGCAGGT	TGACCCTGTTGATTTTGTTCCAGGTAATACGT
	GCGACCATGCCGTAGTCTTCAATGACGTAATC	GCGACCATGCCGTAGTCTTCAATGACGTAATC	TTAACCTCGTCGTTCTGGTTTCCGCCCAGGAT
	CCCCGTCGTCATTCGCGCATTCTGCGCACAGA	CCCCGTCGTCATTCGCGCATTCTGCGCACAGA	GAACCTGAATTCGAAGGGTGGGTCATCCTTCC
	TAACAGGGCCATTTTCCCGGGTTGCCAGACGA	TAACAGGGCCATTTTCCCGGGTTGCCAGACGA	GGACCCCGAGCGACCCGGTCACCCTCCGACCT
	TCACCCTCGTAGCCGATACCACTTTCGCGCAG	TCACCCTCGTAGCCGATACCACTTTCGCGCAG	CCGTCGAACGGCGGTTATATCCATCTTGAGTC
	GGGTTCACTTGGGTGAAACTGAACTAACT	GGGTTCACTTGGGTGAAACTGAACTAACT	ACCGATCCCACAATTGCGGCGGTTGAGATTGA

The detection of similar spacers in CRISPR cassettes of different strains may thus indicate phylogenetic relationships between strains. This technique can be used in epidemiologic analysis of outbreaks caused by antibiotic-resistant strains of *Klebsiella pneumoniae*.

Screening of bacteriophages by spacer sequences of CRISPR cassettes of strains from the above groups is presented in Table 1, with the exception of two pan-resistant strains from the first group (NZ_CP014004.1 and NZ_CP012753.1), as no complete correspondence of spacers in the composition of their cassettes to protospacers from known databases was revealed. The analysis of the spacer sequences, however, resulted in the highest correspondence to the protospacers of phages of *Klebsiella* and *Salmonella* bacteria belonging to the same family Enterobacteriaceae (Table 4).

Of significance, the CRISPR cassettes of the strains studied revealed the correspondence of one of the spa-

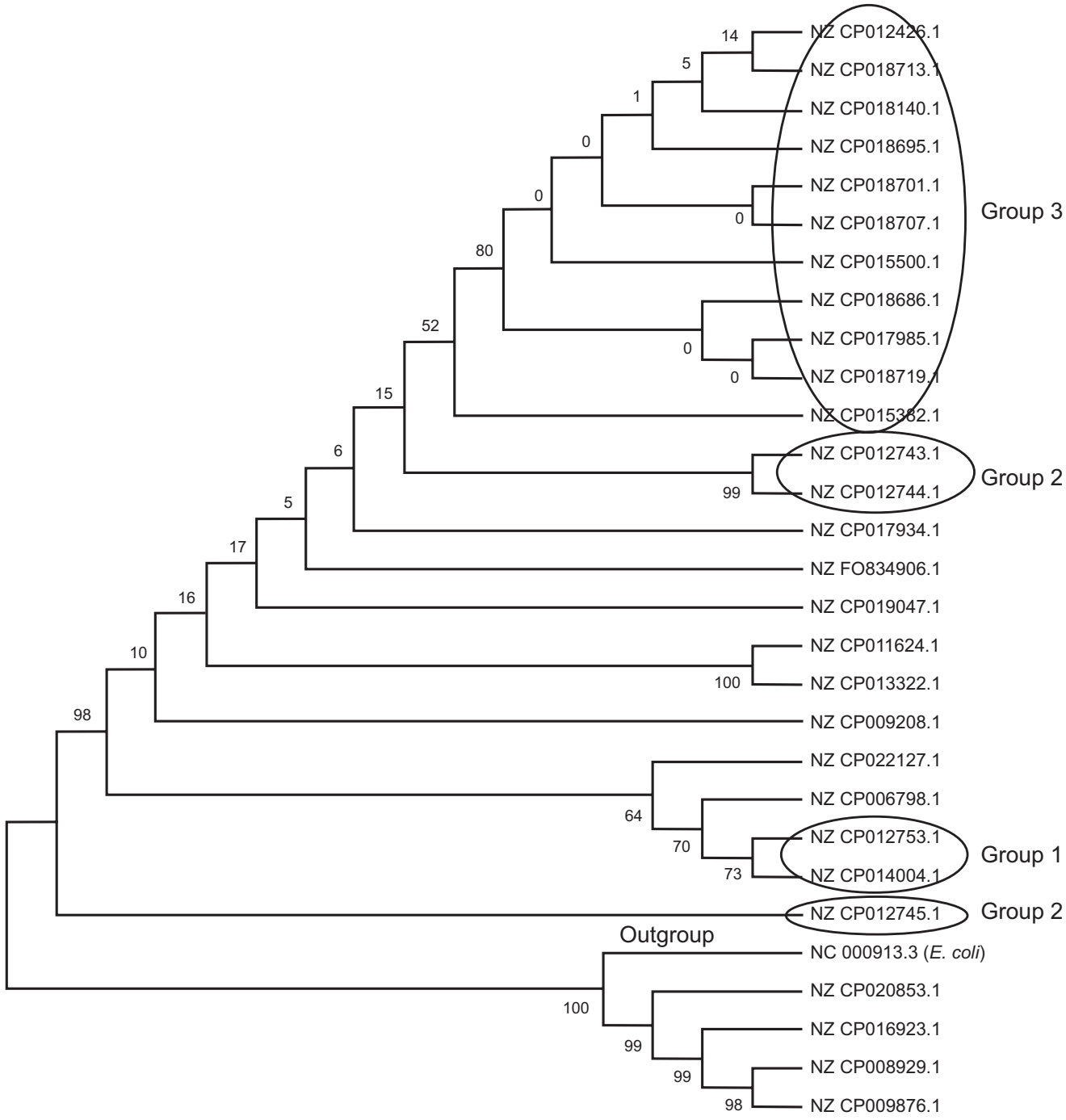

FIG. 4.

Phylogenetic tree based on the 16S rRNA marker of antibiotic-resistant Klebsiella pneumoniae strains (Maximum Composite Likelihood model of genetic distances was used)

cers to protospacers of several bacterial phages belonging to the same family. This may indicate the conservativity of the acquired new spacers from phage DNA regions. In this way, the bacterium "one spacer" protects itself against multiple phages. The presence of this mechanism increases the efficiency of the protective action of CRISPR/Cas systems.

The study of the identified bacteriophages revealed that all *Klebsiella* phage are prophages of *Klebsiella pneumoniae* bacteria belonging to isolates which are part of clonal group ST 307 (according to NCBI data) producing *Klebsiella pneumoniae* carbapenemase (Table 5). The ST 307 genome has been demonstrated to encode genetic features that may provide an advantage

TABLE 4

DIVERSITY OF BACTERIOPHAGES CORRESPONDING TO IDENTICAL SPACERS IN THE FIRST GROUP OF *KLEBSIELLA PNEUMONIAE* STRAINS WITH MULTIPLE ANTIBIOTIC RESISTANCE

GenBank strain access number	Nucleotide sequence of the spacer	Bacteriophage	GenBank bacteriophage access number	Number of nucleotide substitutions
NZ_CP014004.1 NZ_CP012753.1	TTCATCACGTGTGAGCGGATTTGGCTCTATCCT	*Klebsiella* phage 6 LV-2017	KY271400	2
NZ_CP014004.1 NZ_CP012753.1	TGCCTCCAATGCAATCACCGGCCTGCTAACCGG	*Klebsiella* phage ST101-KPC2phi6.1	MK448231	1
NZ_CP014004.1 NZ_CP012753.1	CGTCATCAGCGCCTTGTTCCAGCGGCGACCACC	*Salmonella* phage FSL SP-062	KC139634	2
		Klebsiella phage ST512-KPC3phi13.1	MK448235	1
		Klebsiella phage ST11-VIM1phi8.1	MK448233	1
NZ_CP014004.1 NZ_CP012753.1	TCCAGTCGTCGTAGTCCTCGGTAATGTCCTCGA	*Klebsiella* phage ST101-KPC2phi6.1	MK448231	1
		Klebsiella phage 2b LV-2017	KY271395	1
		Klebsiella phage 2 LV-2017	KY271396	2

TABLE 5

LIST OF IDENTIFIED BACTERIOPHAGES IN THE FIRST GROUP OF *KLEBSIELLA PNEUMONIAE* STRAINS WITH MULTIPLE ANTIBIOTIC RESISTANCE

Bacteriophage	Bacteriophage Access Number (GenBank)	Source of bacteriophage isolation	Location of bacteriophage isolation
Klebsiella phage 6 LV-2017	KY271400	*Klebsiella pneumoniae* ST307	Италия, Рим
Klebsiella phage ST101-KPC2phi6.1	MK448231	*Klebsiella pneumoniae* ST101-KPC2	Испания, Ла-Корунья
Salmonella phage FSL SP-062	KC139634	Молочная ферма	Нью-Йорк, США
Klebsiella phage ST512-KPC3phi13.1	MK448235	*Klebsiella pneumoniae* ST512-KPC3	Испания, Ла-Корунья
Klebsiella phage ST11-VIM1phi8.1	MK448233	*Klebsiella pneumoniae* ST11-VIM1	Испания, Ла-Корунья
Klebsiella phage ST101-KPC2phi6.1	MK448231	*Klebsiella pneumoniae* ST101-KPC2	Испания, Ла-Корунья
Klebsiella phage 2b LV-2017	KY271395	*Klebsiella pneumoniae* KH43 – клон *Klebsiella pneumoniae* ST307	Италия, Рим
Klebsiella phage 2b LV-2017	KY271396	*Klebsiella pneumoniae* H151440672 – клон *Klebsiella pneumoniae* ST307	Италия, Рим

in adaptation to the hospital environment and human host. All ST 307 isolates are encapsulated and therefore have a higher resistance to complement-mediated eradication. All this ensures the spread and prevalence of strains with this genetic sequence worldwide [24]. It is known that prophages remain latent in the genome for several cell divisions, but under the influence of external factors they change into virulent forms and lysate the bacterial cell. In summary, the antibiotic-resistant strains under study have a genetic memory of these bacteriophages in the form of spacers of their CRISPR cassettes.

This analysis of the strains under study showed a rather wide spacer composition of their CRISPR cassettes, diversity of bacteriophages detected through spacer sequences, which makes it possible to suggest that they possess not only antibiotic resistance, but also resistance to many bacteriophages. Consequently, a personalized approach to the integrated selection of antibiotics and bacteriophages will potentially help to address the treatment of diseases caused by these strains.

CONCLUSION

The results of these studies provided information about the structure of CRISPR/Cas systems in the genomes of antibiotic-resistant *Klebsiella pneumoniae* strains and their functional features. The revealed diversity of spacer sequences indicates the evolutionary history and adaptive capabilities of these strains. In this case, the detection of similar spacers in CRISPR cassettes of different strains may indicate phylogenetic relationships between strains. The study of phage-bacteria interaction patterns based on spacer and protospacer sequences makes it possible to determine the resistance and sensitivity of strains to specific bacteriophages. In this way, the putative resistance of antibiotic-resistant strains to specific phages was determined. The mechanism of studying CRISPR/Cas systems in bacterial genomes using bioinformatics analysis will in the future provide more complete information about the properties of causative agents, their evolution and the selection of highly specific bacteriophages.

Conflict of interest

The authors of this article declare no conflicts of interest.

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REFERENCES

1. World Health Organization. *Global action plan on antimicrobial resistance.* Geneva: WHO; 2015.

2. Webale MK, Wanjala C, Guyah B, Shaviya N, Munyekenye GO, Nyanga PL, et al. Epidemiological patterns and antimicrobial resistance of bacterial diarrhea among children in Nairobi City, Kenya. *Gastroenterol Hepatol Bed Bench*. 2020; 13(3): 238-246.

3. Yangzom T, Tsering DC, Kar S, Kapil J. Antimicrobial susceptibility trends among pathogens isolated from blood: A 6-year retrospective study from a tertiary care hospital in East Sikkim, India. *J Lab Physicians*. 2020; 12(01): 3-9. doi: 10.1055/s-0040-1712814

4. Zalewska A, Wilson J, Kennedy S, Lockhart M, MacLeod M, Malcolm W. Epidemiological analysis of antimicrobial resistance in *Staphylococcus epidermidis* in Scotland, 2014–2018. *Microbial Drug Resistance*. 2021; 27(4): 485-491. doi: 10.1089/mdr.2019.0502

5. Mouiche MMM, Moffo F, Akoachere J-FTK, Okah-Nnane NH, Mapiefou NP, Ndze VN, et al. Antimicrobial resistance from a one health perspective in Cameroon: A systematic review and meta-analysis. *BMC Public Health*. 2019; 19(1): 1135. doi: 10.1186/s12889-019-7450-5

6. Melese A, Genet C, Andualem T. Prevalence of Vancomycin resistant enterococci (VRE) in Ethiopia: A systematic review and meta-analysis. *BMC Infect Dis*. 2020; 20(1): 124. doi: 10.1186/s12879-020-4833-2

7. Cassini A, Plachouras D, Monnet D. Attributable deaths caused by infections with antibiotic-resistant bacteria in France – Authors' reply. *Lancet Infect Dis*. 2019; 19(2): 129-130. doi: 10.1016/s1473-3099(19)30004-0

8. Phodha T, Riewpaiboon A, Malathum K, Coyte P. Annual relative increased in inpatient mortality from antimicrobial resistant nosocomial infections in Thailand. *Epidemiol Infect*. 2019; 147: 133. doi: 10.1017/s0950268818003436

9. Pessoa e Costa T, Duarte B, João AL, Coelho M, Formiga A, Pinto M, et al. Multidrug-resistant bacteria in diabetic foot infections: Experience from a Portuguese tertiary centre. *Int Wound J*. 2020; 17: 1835-1839. doi: 10.1111/iwj.13473

10. World Health Organization. *Report on surveillance of antibiotic consumption: 2016–2018 early implementation.* Geneva: WHO; 2018.

11. Shrestha P, Cooper B, Coast J, Oppong R, Do Thi Thuy N, Phodha T, et al. Enumerating the economic cost of antimicrobial resistance per antibiotic consumed to inform the evaluation of interventions affecting their use. *Antimicrob Resist Infect Control*. 2018; 7(1): 98. doi: 10.1186/s13756-018-0384-3

12. Brauberg CA, Palacios M, Miller VL. Klebsiella: A long way to go towards understanding this enigmatic jetsetter. *F1000Prime Reports*. 2014; 6: 64. doi: 10.12703/P6-64

13. Khaertynov KhS, Anokhin VA, Nikolaeva IV, Semenova DR, Lyubin SA, et al. Klebsiella neonatal sepsis. *Medical Bulletin of the North Caucasus*. 2016; 11(1): 82-86. (In Russ.). [Хаертынов Х.С., Анохин В.А., Николаева И.В., Семенова Д.Р., Любин С.А., и др. Клебсиеллезный неонатальный сепсис. *Медицинский вестник Северного Кавказа*. 2016; 11(1): 82-86]. doi: 10.21508/1027-4065-2019-64-5-176-182

14. Wyres KL, Holt KE. Klebsiella pneumonia population genomics and antimicrobial-resistant clones. *Trends Microbiol*. 2016; 24: 944-956. doi: 10.1016/j.tim.2016.09.007

15. Makabenta J, Nabawy A, Li C, Schmidt-Malan S, Patel R, Rotello V. Nanomaterial-based therapeutics for antibiotic-resistant

bacterial infections. *Nature Reviews Microbiology*. 2021; 19: 23-26. doi: 10.1038/s41579-020-0420-1

16. Mojica FJM, Díez-Villaseñor C, García-Martínez J, Soria E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol.* 2005; 60: 174-182. doi: 10.1007/s00239-004-0046-3

17. Nuñez JK, Kranzusch PJ, Noeske J, Wright AV, Davies CW, Doudna JA. Cas1-Cas2 complex formation mediates spacer acquisition during CRISPR-Cas adaptive immunity. *Nat Struct Mol Biol.* 2014; 21: 528-534. doi: 10.1038/nsmb.2820

18. Nuñez JK, Harrington LB, Kranzusch PJ, Engelman AN, Doudna JA. Foreign DNA capture during CRISPR-Cas adaptive immunity. *Nature*. 2015; 527: 535-538. doi: 10.1038/nature15760

19. Jackson SA, McKenzie RE, Fagerlund RD, Kieper SN, Fineran PC, Brouns SJJ. CRISPR-Cas: Adapting to change. *Science*. 2017; 356: eaal5056. doi: 10.1126/science.aal5056

20. Mojica FJM, Díez-Villaseñor C, García-Martínez J, Almendros C. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology.* 2009; 155: 733-740. doi: 10.1099/mic.0.023960-0

21. Stepanenko LA, Dzhioev YuP, Zlobin VI, Borisenko AYu, Salovarova VP, Arefieva NA, et al. Development of screening approaches of highly specific bacteriophages based on bioinformatic analysis of CRISPR-Cas structures of Corynebacterium diphtheriae systems. *Proceedings of Universities. Applied Chemistry and Biotechnology.* 2021; 11(2): 216-227. (In Russ.).[Степаненко Л.А., Джиоев Ю.П., Злобин В.И., Борисенко А.Ю., Саловарова В.П., Арефьева Н.А., и др. Разработка подходов скрининга высокоспецифичных бактериофагов на основе биоинформационного анализа структур CRISPR-Cas систем *Corynebacterium diphtheriae*. *Известия вузов. Прикладная химия и биотехнология*. 2021; 11(2): 216-227]. doi: 10.21285/2227-2925-2021-11-2-216-227

22. CRISPRCasFinder. URL: https://crisprcas.i2bc.paris-saclay.fr/CrisprCasFinder/Index [date of access: 22.08.2023].

23. CRISPRone. URL: https://omics.informatics.indiana.edu/CRISPRone/ [date of access: 22.08.2023].

24. Villa L, Feudi C, Fortini D, Brisse S, Passet V, Bonura C, et al. Diversity, virulence, and antimicrobial resistance of the KPC producing *Klebsiella pneumoniae* ST307 clone. *Microbial Genomics*. 2017; 3. doi: 10.1099/mgen.0.000110

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