WWJMRD 2024; 10(09): 16-18
www.wwjmrd.com
International Journal
Peer Reviewed Journal
Refereed Journal
Indexed Journal
Impact Factor SJIF 2017: 5.182 2018: 5.51, (ISI) 2020-2021: 1.361
E-ISSN: 2454-6615

Maia Elizbarashvili
G. Eliava Institute of Bacteriophages, Microbiology and Virology Gotua str. 3, 0160 Tbilisi, Georgia.

Tamar Suladze
G. Eliava Institute of Bacteriophages, Microbiology and Virology Gotua str. 3, 0160 Tbilisi, Georgia.

Marina Darsavelidze
G. Eliava Institute of Bacteriophages, Microbiology and Virology Gotua str. 3, 0160 Tbilisi, Georgia.

Sophio Gunia
G. Eliava Institute of Bacteriophages, Microbiology and Virology Gotua str. 3, 0160 Tbilisi, Georgia.

Marine Dadunashvili
G. Eliava Institute of Bacteriophages, Microbiology and Virology Gotua str. 3, 0160 Tbilisi, Georgia.

Correspondence:
Marina Darsavelidze
G. Eliava Institute of Bacteriophages, Microbiology and Virology Gotua str. 3, 0160 Tbilisi, Georgia.

Characterization of virulent phages of *Klebsiella pneumoniae*.

Maia Elizbarashvili, Tamar Suladze, Marina Darsavelidze, Sophio Gunia, Marine Dadunashvili

Abstract
During this study six *Klebsiella pneumoniae* phages: vB_Kp6, vB_Kp8, vB_Kp4, vB_Kp4/1, vB_Kp19, vB_Kp19/1 were selected and studded. Based on TEM investigation was determined that phages belong to the *Caudovirales* order. Phages vB_KP6 and vB_KP8 belonged to the *Siphoviridae* family and phages vB_KP4, vB_KP4/1, vB_KP19 and vB_KP19/1 – to *Myoviridae* family. The range of activity of phages against *K. pneumoniae* - 92 and *K. oxytoca* - 9 strains were revealed that phages vB_Kp6 and vB_Kp8 were distinguished by the highest activity rate. Their range of activity was 60% and 59%, respectively. Based on the obtained results the biological properties (single reproduction cycle, frequency of formation of phage-resistant mutants, stability of lysis in the liquid area) and the influence of environmental factors (temperature and pH) of these two phages were investigated. As a result of the conducted work, these phages vB_Kp6 and vB_Kp8 can be recommended for creation a therapeutic-prophylactic phage preparation against Klebsiella infection.

Keywords: *K. pneumonia* bacteriophage; phage genome; virion morphology; phage therapy.

Introduction

Microorganisms of the family *Enterobacteriaceae* are the dominant, natural representatives of the human and animal microbiome. The rapid increase in the number of antibiotic-resistant pathogens of this family is a serious economic problem today.

Klebsiella pneumoniae, a member of the family *Enterobacteriaceae*, is a commensal bacterium. It is ubiquitous in the environment. *K. pneumoniae* is a natural inhabitant of the gastrointestinal tract microbiome of healthy humans and animals. [1]

In this population, the colonization with the microorganism ranges from 19 to 88%. Colonization is considered a potential reservoir of Klebsiella infections, and the risk of these infections in colonized individuals is four times higher than in non-colonized individuals. [2]

K. pneumoniae is an opportunistic pathogen that can cause a wide range of infections such as urinary and respiratory tract, wound and soft tissue infections, meningitis, sepsis. In recent years, the pathogen is one of the leading etiologic agent of nosocomial infections in immunocompromised individuals, neonates, and the elderly.[3]

The ability of *K. pneumoniae* to form biofilms promotes colonization of the gastrointestinal, respiratory, and urinary tracts and the development of invasive infections, resulting in increased antibiotic resistance, which in turn reduces the effectiveness of treatment. [4]

Intensive use of broad-spectrum antibiotics increased Klebsiella carrier state, development of multidrug-resistant strains characterized by high virulence and rapid spreading ability. Currently, *K. pneumoniae* is showing a high resistance to a wide-ranging spectrum of drugs including beta-lactam antibiotics, fluoroquinolones, and aminoglycosides. The reduced effectiveness of antimicrobial therapy for the treatment of Klebsiella associated infections, made relevant search for alternative therapies. Such alternative can be considered bacteriophages, which have a long history of use in therapy. [5]

Phage therapy is being used to control pathogenic bacterial infections especially multiple antibiotic-resistant bacterial infections and as potential anti-inflammatory and immunomodulatory agent. Their therapeutic potential in medicine to control MDR pathogens

is due to their specificity and potency in inducing lethal effects in the host bacterium by cell lysis. In the context of therapeutics, only virulent phages can be used. Strictly virulent phages can attack particular bacterial strains and contribute to lytic infection associated with metabolic disturbance and cell lysis, which decreases the number of bacterial cells found in the infected human host to a level that presents no danger or harm to the organism. [6,7]

The aim of our study is to select active virulent phages against multiple antibiotic- and phage resistant strains of *K. pneumonia* and to study their basic biological properties for obtaining a polyvalent phage that can be used for development of phage preparation.

Materials and Method

Bacterial strains

During this study were used 92 *K. pneumoniae* and 9 *K. oxytoca* bacterial strains: 50 strains were from Phage and Strain Collection Laboratory; 20 strains - from Molecular Biology Laboratory and 31 strains - from Microbial Ecology Laboratory (EIBMV). Strains were isolated from different clinical samples (feces, urine, sputum). *K. pneumoniae* strains were propagated on the LB broth and agar at 37°C.

Bacteriophages

40 primary phage isolates active against *Klebsiella spp.* from Phage and Strain collection Laboratory of EIBMV were used during this study, from which biological properties of 4 phages - vB_KP4, vB_KP6, vB_KP8, vB_KP19 - were studded. For propagation and testing of phages, LB media were used. Main phage biological properties were studded by standard methods. [8]

Results and Discussion

Lytic activities of 40 primary phage isolates against 50 strains of *K. pneumoniae* were tested. Out of them 27 revealed activities against them. Effectiveness of phages ranged from 2% to 46%. For further studies, 4 primary phage isolates with a wide range of action were selected - KP4, KP6, KP8, KP19. They were lysed 28% to 46% of the strains. All 50 strains of *K. pneumoniae* were tested on phage content, and as a result was determined that only 6 strains were contained phage.

The titer of phages obtained as a result of adaptation and concentration on the host strain varied from $5x10^9$ - $1x10^{11}$ PFU/mL. At the next stage, from the phages KP4, KP6, KP8, KP19 (by selection of negative colonies of one morphological type and series of propagation on the relevant hosts) 6 pure lines were obtained: vB_Kp6, vB_Kp8, vB_Kp4, vB_Kp4/1, vB_Kp19, vB_Kp19/1. The titer of phages ranged from $4x10^9$ to $2x10^{10}$ PFU/mL.

The range of activity of Klebsiella phages against *K. pneumoniae* - 92 and *K. oxytoca* - 9 strains was determined. Phages vB_Kp6 and vB_Kp8 were distinguished by the highest activity rate. They caused lysis of both *K. pneumoniae* strains and *K. oxytoca* strains, and their range of activity was 60% and 59%, respectively. The range of activity of phages vB_Kp4, vB_Kp4/1, vB_Kp19 and vB_Kp19/1 against Klebsiella strains ranged from 16% to 32%. It should be noted that unlike vB_Kp6 and vB_Kp8 phages, they showed activity only against *K. pneumoniae* strains.

Based on plaque morphology, phages vB_Kp6 and vB_Kp8 are polymorphic. From phage vB_Kp4 were obtained two phages with different plaques. vB_Kp4 had plaques in size with 5 mm, with a large transparent center and a turbid zone, and vB_Kp4/1 - 1 mm plaques with transparent colonies. Also, two different plaque morphology phages were obtained from phage vB_Kp19 phage. vB_Kp19 - 4 mm plaques with a bright center and a turbid zone and vB_Kp19/1 - with transparent colonies of 1 mm in size.

Based on TEM investigation was determined that phages belonged to the *Caudovirales* order. Phages vB_KP6 and vB_KP8 belong to the *Siphoviridae* family. They were characterized by a head size of 78.3x78.3 nm and a long non contractile tail - 226x13 - 239x13 nm in length, respectively. Phages vB_KP4, vB_KP4/1, vB_KP19 and vB_KP19/1 belonged to the morphological family of *Myoviridae* phages. The head size of the phages ranged from 56.5x56.5 nm to 69.6x69.6 nm, and the tail size from 70x22 nm to 117x21.7 nm.

Restriction analysis of vB_KP6 and vB_KP8 phages with EcoRV, Sau3AI, PvuII and SspI endonucleases determine that phages are slightly different from each other. Same enzymes showed that these phages are different from other vB_Kp4, vB_Kp4/1, vB_Kp19 and vB_Kp19/1 phages. KP6 phage DNA was not hydrolyzed by help of KpnI. Restriction of phages with EcoRV, Sau3AI, PvuII and SspI endonucleases revealed that phages vB_Kp4, vB_Kp4/1, vB_Kp19 and vB_Kp19/1 do not differ from each other, while treatment with KpnI shows a slight difference between them.

Based on the obtained results (range of action, restriction analysis) two phages - vB_KP6 and vB_KP8 - were selected for further research. The biological properties of these phages (single reproduction cycle, frequency of formation of phage-resistant mutants, stability of lysis in the liquid area) and the influence of environmental factors (temperature and pH) were investigated.

Studying of a one-step growth cycle of phage multiplication, determined that the adsorption time for vB_KP6 and vB_KP8 phages was 12 minutes. Adsorption efficiency for vB_KP6 phage was 96%, and for vB_KP8 phage - 94.5%. The latent period for both phages was 50 minutes, and the burst size for vB_KP6 and vB_KP8 phages were 90.5 - 85 PFU per infected cell, respectively.

The stability of phages in the liquid culture was determined using suspensions with initial titer of $8x10^8$ - $4.2x10^8$ PFU/mL. The titer of both phages was 10^{-6} at room temperature after 18 h. and after 7 h. of incubation at 37°C.

It was found that investigated phages revealed a low frequency of formation of phage-resistant mutants in relation to the host strain. For phage vB_KP8 frequency of formation of phage-resistant mutants was $1.3x10^{-5}$ and for vB_KP6 phage - $4.5x10^{-6}$. Also was determined that the resistant mutants formed against vB_KP6 and vB_KP8 phages (after triple generation) still maintain their resistance to the mentioned phages.

The sensitivity of Klebsiella phages to increasing temperature at 10-, 30-, and 60-minute intervals determined that at 40°C the titer of vB_KP6 and vB_KP8 phages slightly decreased compared to their titer at 37°C - $2.8x10^8$ - $4.2x10^8$PFU/mL. At 50°C, the phage titer decreased significantly and at 60°C after 10 minutes no viable phage particles were observed in case of both phages.

Influence of acid and alkaline condition revealed that the titer of vB_KP6 and vB_KP8 phages is unchanged after

exposure to pH6 and pH8 for 60 minutes. At pH4 and pH10, the titer of vB_KP6 and vB_KP8 phages decreased and consisted of $2.8x10^4$ - $4.2x10^5$PFU/mL. No viable phage particles were observed at pH 2 and pH 12.

Based on the conducted research, it was established that vB_KP6 and vB_KP8 phages are characterized by high lytic activity, wide range of action, stable lysis and low mutation rate. They were completely inactivated after 10 minutes of exposure to 60°C, and the phages remained stable at pH6 and pH8.

A mixture of two phages - vB_KP6 and vB_KP8 - was obtained with titer $3×10^8$ PFU/mL. The stability of phage mixture in liquid culture was 10^{-6} at 37°C after 7 hours incubation. The range of activity of the phage mixture against 101 strains of *K. pneumoniae* and *K. oxytoca* determined that it did not exceed 60%. The frequency of formation of phage resistant mutants of the mixture was low - $2.3x10^{-6}$.

As a result of the work, active virulent phages against *K. pneumoniae* were selected and characterized, which can be used to create a therapeutic-prophylactic phage preparation.

Conclusion

Klebsiella pneumoniae is a clinically important pathogen causing a variety of antimicrobial resistant infections in both community and nosocomial settings. Bacteriophage therapy is considered a primary option for the treatment of drug-resistant infections. In this regard phages vB_KP6 and vB_KP8 with high lytic activity, wide range of action, stable lysis and low mutation rate can be considered for development of phage preparation.

References

1. Warren P. Herridge, Preetha Shibu, Jessica O'Shea, Thomas C. Brook, and Lesley Hoyles. Bacteriophages of Klebsiella spp., their diversity and potential therapeutic uses. J Med Microbiol. 2020 Feb; 69(2): 176–194.
2. Claire L Gorrie, Mirjana Mirceta, Ryan R Wick, David J Edwards, Nicholas R Thomson, Richard A Strugnell, Nigel F Pratt, Jill S Garlick, Kerri M Watson, David V Pilcher, Steve A McGloughlin, Denis W Spelman, Adam W J Jenney, Kathryn E Holt. Gastrointestinal Carriage Is a Major Reservoir of Klebsiella pneumoniae Infection in Intensive Care Patients. Clin Infect Dis 2017 Jul 15;65(2):208-21.
3. Shon AS, Bajwa RPS, Hypervirulent RTA. hypermucoviscous Klebsiella pneumoniae: a new and dangerous breed. Virulence. 2013;4:107–118.
4. ADi Domenico EG, Cavallo I, Sivori F, Marchesi F, Prignano G, Pimpinelli F, Sperduti I, Pelagalli L, Salvo Di F, Celesti I, Paluzzi S, Pronesti C, Koudriavtseva T, Ascenzioni F, Toma L, De Luca A, Mengarelli A, Ensoli F. Biofilm production by carbapenem-resistant Klebsiella pneumoniae significantly increases the risk of death in oncological patients. Front Cell Infect Microbiol 10:561741. 2020.
5. Yanping Li, Suresh Kuma, Lihu Zhang. Mechanisms of Antibiotic Resistance and Developments in Therapeutic Strategies to Combat Klebsiella pneumoniae Infection. Infection and Drug Resistance. 2024:17 1107–1119.
6. Mohamed S. Fayez, Toka A. Hakim, Bishoy Maher Zaki, Salsabil Makky, Mohamed Abdelmoteleb, Kareem Essam, Anan Safwat, Abdallah S. Abdelsattar & Ayman El-Shibiny, Morphological, biological, and genomic characterization of Klebsiella pneumoniae phage vB_Kpn_ZC2 Virology Journal volume 20, Article number: 86 (2023).
7. Mehrdad Mohammadi, Mahmood Saffari & Seyed Davar Siadat. Phage therapy of antibiotic-resistant strains of Klebsiella pneumoniae, opportunities and challenges from the past to the future Folia microbiologica, Volume 68, pages 357-368.
8. Adams.M. Bacteriophages, 1961.