Characterization and genome analysis of novel phage vB_KpnM _Bp5 infecting Klebsiella pneumonia

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Abstract

Background With the incidence of antibiotic resistance reaching crisis point, it is imperative to find alternative treatments for multidrug-resistant infections. Using phage for pathogen control might be a promising treatment option to combat bacterial resistance. Results In this study, a lytic phage, designated vB_KpnM _Bp5, was isolated from pig faecal sample in Nanning, Guangxi province of China, and classified as a member of the family Muscle virus based on electron microscopy analysis. A one-step growth curve of the phage at the optimal MOI revealed that the latent time was 40 min and the burst size was 24 PFU/cell, indicative of good lysis capacity. Whole genome sequencing showed that phage vB_KpnM _Bp5 had a small dsDNA genome of 43872 bp. BLASTn analysis showed that it shared 94.06% identity (94% genome coverage) with Klebsiella phage vB_KpnP_SU552A of complete genome idefix. RAST genome analysis showed that the phage had 50 ORFs due to its small genome size, and the number of functional proteins was consistent with other phages. To evaluate the therapeutic effect of Klebsiella pneumoniae infection in mice, the results showed that phages provided vB_KpnM _Bp5better protection. Conclusion The phage vB_KpnM _Bp5 had the characteristics of broad host spectrum, strong environmental adaptability, short incubation period, large outbreak amount, and can cure the mouse model infected by Klebsiella pneumoniae. These findings suggested that phage vB_KpnM _Bp5 could be considered a potential therapeutic or prophylactic candidate against Klebsiella pneumonia infection.

Background

Klebsiella pneumoniae is generally considered as a pathogen with the propensity for acquiring antibiotic resistance [1]and could cause lots of infections including pneumonia, urinary tract infections, sepsis, and soft tissue infections. *K pneumoniae* is widely spread in farming environments, especially livestock and poultry farms. It is also a significant nosocomial pathogen due to its resistance to antibiotics [2,3]. In addition, as a result of increasing international mobility, antibiotic-resistant *K pneumoniae* tends to disseminate rapidly through different countries as well as evolves into real pandemics [4]. *K pneumoniae* has spread rapidly and becomes endemic in several countries (e.g. USA [5], China, Israel [6], Italy [7] and Colombia [8-10],Portugal [11],Greece [12]). It is now a major cause of healthcare-associated infections correlated with high morbidity and mortality rates [10]. Relevant study has shown that pathogenic bacteria developed resistance much faster than the development of new antibiotics [13].

Using phage for pathogen control is an alternative treatment strategy to fight against bacterial antibiotic resistance [14]. Phages are natural viruses that infect bacteria, and exist as the most abundant biological entities in the biosphere [15]. Phages only kill their corresponding host bacteria and rely on host bacteria for their reproduction. Bacterial antibiotic resistance has no effects to impede phage therapy on bacterial infections. Hence phage plays an important role in the application of antimicrobial pathogens in the fight.

The host range of a bacteriophage is usually narrow. In addition, phage-resistant mutant bacteria arise almost instantly upon exposure to a phage, which severely limits the therapeutic use of phage on bacterial infections. To overcome this hurdle, a cocktail consisting of several different phages is

frequently used to reduce the evolution of resistant bacteria and to maintain highly lytic efficacy [16]. Accordingly, it is necessary to isolate and characterize new phages. In the current study, we isolated lytic phage vB_KpnM _Bp5, which showed activity against *K pneumoniae*, from farm sewage and carried out genome sequencing and analysis of its biological characteristics.

Results

Phage isolation and morphology

To isolate bacteriophages with specific lytic activity against *K pneumoniae*, various environmental samples were collected from sewage taken from pig farms. A new lytic bacteriophage, named as vB_KpnM _Bp5, was isolated. The phage plaque was clean and bright, with neat edge and no halo ring, which showed typical lytic phage characteristic (Fig 1a). Electron microscopy showed that the phage tubular tail was approximately 56 nm in length, and the head was 53 nm in diameter (Fig. 1b). According to the provisions of the International Classification Committee of Viruses, the phage was assigned to the family *Caudovirales*, *Myoviridae*.

Host specificity

To test whether vB_KpnM _Bp5 could lyse human *K.peumoniae* isolates, the host range of phage vB_KpnM _Bp5 was examined on 34 *K pneumoniae* strains isolated from fetal swabs and urine collected in a hospital in Nanning, Guangxi Zhuang autonomous region. The result showed that vB_KpnM _Bp5 exhibited an ability to produce plaques in 2 human isolates, respectively, *K pneumoniae* strains GX L63 and GX L28. (Table 1). It indicated that phage Bp5 had a host range of both pig and human isolates.

Table 1 The *K.peumoniae* used for host range determination

Strain	Region of isolation	Infection by Phage Bp5	Strain	Region of isolation	Infection by Phage Bp5
GX L15	NanNing,GuangXi	-	GX 20-1	NanNing,GuangXi	-
GX L40	NanNing,GuangXi	-	GX RS3	NanNing,GuangXi	-
GX L44-1	NanNing,GuangXi	-	GX L22	NanNing,GuangXi	-
GX L27	NanNing,GuangXi	-	GX L59	NanNing,GuangXi	-
GX L13	NanNing,GuangXi	-	CX L30	NanNing,GuangXi	-
GX SL39	NanNing,GuangXi	-	GX L3-1	NanNing,GuangXi	-
GX 08	NanNing,GuangXi	-	GX 21	NanNing,GuangXi	-
GX L63	NanNing,GuangXi	+	GX L68	NanNing,GuangXi	-
GX SL26	NanNing,GuangXi	-	GX L48	NanNing,GuangXi	-
GX L18	NanNing,GuangXi	-	GX L67	NanNing,GuangXi	-
GX L28	NanNing,GuangXi	+	GX L55	NanNing,GuangXi	-
GX 13	NanNing,GuangXi	-	GX L34	NanNing,GuangXi	-
GX 49	NanNing,GuangXi	-	GX L11	NanNing,GuangXi	-
GX L01	NanNing,GuangXi	-	GX L03	NanNing,GuangXi	-
GX 06-1	NanNing,GuangXi	-	GX L45-1	NanNing,GuangXi	-
GX L56	NanNing,GuangXi	-	GX L02	NanNing,GuangXi	-
GX 20-2	NanNing,GuangXi	-	GX 54-2	NanNing,GuangXi	-

One-step growth

An one-step growth experiment was conducted to determine the latent time period and burst size of phage vB_KpnM _Bp5 (Fig.2a).The assays revealed a latent period, defined as the time interval between the adsorption and the beginning of the first burst,the results showed that the incubation period was about 5min, and the outbreak period was about 40min. The burst size was calculated as the ratio of the final number of free phage particles to the number of infected bacterial cells during the latent period, and was determined to be 24 plaque-forming units (PFU)/cell for phage vB_KpnM _Bp5.

Thermal and pH stability

When heated to 50 °C, The phage titer of vB_KpnM _Bp5 decreased rapidly with the increase of water temperature (Fig.2b). When water bath temperature reached 80 °C, no living phages were detected, which showed high temperature affected the activity of phages. According to the result, phages could maintain the stable activity when temperature heated to 30 ~ 50 °C. In addition, Phage vB_KpnM _Bp5 exhibited stable activity at pH 4.0-10.0 (Fig.2c).Moreover, phage titer declined sharply with the enhancement of acid or alkalinity.

Bacteriolytic activity

The bacteriolytic activity of phage vB_KpnM _Bp5 was tested on an early exponential phase culture of *K pneumoniae* (Fig.2d). The absorbance (OD_{600}) of the phage-infected culture dropped rapidly compared with the uninfected control from 1 to 5 h post infection. Although the bactericidal effect exhibited slight differences with changes in the MOI (multiplicity of infection), both 1 MOI and 0.001 MOI phage-infected culture showed significantly ($p<0.01$) inhibited bacterial growth after 2 h. The result showed using MOI=1 phage to infect culture, the absorbance decreased rapidly and remained at a very low level ($OD_{600}<0.1$), while using MOI=0,001 phage to infect, the absorbance first raised then descended. It indicated that the phage bacteriolytic activity would increase with the rising of its concentration.

Evaluation of phage therapy

To evaluate the effect of phage therapy on *K.pneumoniae* infected mice, the minimum lethal dose (MLD_{100}) of *K pneumoniae* was detected as 4.0×10^7cfu/ piece. Each mice were infected with *K.pneumoniae* at the MLD. At the same time, phage had a good therapeutic effect, the advance treatment group and the current treatment group showed that the protection rate of phage on mice was up to 100.00%. Meanwhile, the protection rate of phage on mice in the delayed treatment group was only 60.00%. All mice in the bacterial infection group died, and the mice in the control group did not die (Fig.3).

Phage vB_KpnM _Bp5 complete genome sequencing was conducted using next-generation sequencing (NGS). A total of about 5,304,348 reads were detected and 99.33% of reads were high quality reads. The genome was assembled using A5-miseq v20150522 and SPAdesv3.9.0 assembler. To be specific, the complete genome length of vB_KpnM _Bp5 was 43872bp, and 94.93% of the whole reads were matched onto the complete genome. The distribution of each base was the same as, the average GC content was 53.90%.

Genome overview

BLASTn analysis showed that vB_KpnM _Bp5 shares 94.06% identity (94% genome coverage) with a *K pneumoniae* phage vB_KpnP_SU552A of complete genome (KP708986.1) identify. The RAST annotation results indicated that the genome contained only 52 open reading frames (ORFs), and that ATG was used as the start codon for all 52 ORFs. Based on the homology comparisons, 52 ORFs were assigned significant similarity (E value B 1E-4; Table2) to other proteins in the GenBank database. The nucleic acid coding sequences ranged in length from 153 to 3699 bp, corresponding to protein sequence lengths of 51–1232 aa. Together, the ORFs accounted for a total of 40473 bp, with a gene density as high as 92.25%.

Table 2 ORF analysis of the vB_KpnM _Bp5 genome

	Hit_name	Hit_description	length	Identity	value
	YP_009204790.1	hypothetical protein SU552A_01 [*Klebsiella* phage vB_KpnP_SU552A]	71	98.59%	1.50E-31
	YP_009188313.1	hypothetical protein [*Klebsiella* phage Kp2]	190	95.79%	3.10E-95
	YP_009199887.1	hypothetical protein SU503_03 [*Klebsiella* phage vB_KpnP_SU503]	74	85.14%	8.30E-36
	YP_009302709.1	hypothetical protein kpv71_05 [*Klebsiella* phage KpV71]	77	76.62%	3.50E-29
	YP_009188747.1	hypothetical protein kpv41_05 [*Klebsiella* phage KpV41]	87	97.33%	1.70E-36
	YP_009006027.1	hypothetical protein F19_07 [*Klebsiella* phage F19]	59	94.92%	8.10E-26
	YP_003347658.1	hypothetical protein [*Klebsiella* phage KP34]	69	95.65%	5.60E-34
	APZ82721.1	hypothetical protein kpv74_09 [*Klebsiella* phage vB_KpnP_KpV74]	655	88.71%	0.00E+00
	APZ82722.1	putative peptidase *[Klebsiella* phage vB_KpnP_KpV74]	348	97.70%	2.30E-205
	AIT13582.1	hypothetical protein BO1E_0013 [*Klebsiella* phage phiBO1E]	154	53.25%	2.90E-35
	No hits				
2	YP_009188325.1	DNA primase/helicase [*Klebsiella* phage Kp2]	261	98.08%	2.10E-145
3	YP_009204805.1	putative DNA helicase [*Klebsiella* phage vB_KpnP_SU552A]	426	99.52%	9.20E-241
4	YP_009188759.1	hypothetical protein kpv41_17 [*Klebsiella* phage KpV41]	51	98.04%	5.20E-21
5	YP_009204807.1	putative DNA polymerase [*Klebsiella* phage vB_KpnP_SU552A]	796	98.99%	0.00E+00
6	YP_009199903.1	hypothetical protein SU503_19 [*Klebsiella* phage vB_KpnP_SU503]	182	88.46%	9.90E-91
7	YP_009188331.1	hypothetical protein [*Klebsiella* phage Kp2]	73	98.46%	4.40E-33
8	YP_009204809.1	hypothetical protein SU552A_20 [*Klebsiella* phage vB_KpnP_SU552A]	344	98.84%	1.10E-202
9	APZ82733.1	hypothetical protein kpv74_21 [*Klebsiella* phage vB_KpnP_KpV74]	77	95.31%	5.80E-25
0	YP_009204811.1	large tegument protein [*Klebsiella* phage vB_KpnP_SU552A]	277	97.47%	8.60E-145
1	YP_009188766.1	hypothetical protein kpv41_24 [*Klebsiella* phage KpV41]	84	97.62%	5.50E-36
2	YP_009280692.1	hypothetical protein kpv475_23 [*Klebsiella* phage KpV475]	92	96.74%	1.10E-45
3	YP_009188768.1	hypothetical protein kpv41_26 [*Klebsiella* phage KpV41]	123	97.54%	3.60E-60
4	APZ82738.1	hypothetical protein kpv74_26 [*Klebsiella* phage vB_KpnP_KpV74]	53	96.23%	6.90E-16
5	YP_009204817.1	hypothetical protein SU552A_28 [*Klebsiella* phage vB_KpnP_SU552A]	53	100.00%	7.40E-23
6	YP_009204818.1	putative 5'-3' exonuclease [*Klebsiella* phage vB_KpnP_SU552A]	322	99.69%	2.10E-192
7	APZ82743.1	putative HNH endonuclease [*Klebsiella* phage vB_KpnP_KpV74]	153	58.73%	2.60E-35
8	YP_009098366.1	putative endonuclease [*Klebsiella* phage	140	82.31%	2.20E-55

		NTUH-K2044-K1-1]			
9	YP_009204821.1	putative polynucleotide kinase/phosphatase [*Klebsiella* phage vB_KpnP_SU552A]	164	98.78%	4.40E-90
0	YP_009204822.1	hypothetical protein SU552A_33 [*Klebsiella* phage vB_KpnP_SU552A]	104	100.00%	4.30E-54
1	AOT23867.1	DNA-dependent RNA polymerase [*Klebsiella* phage KP-Rio/2015]	822	98.18%	0.00E+00
2	AIT13609.1	hypothetical protein BO1E_0040 [*Klebsiella* phage phiBO1E]	146	99.32%	2.00E-78
3	YP_003347631.1	hypothetical protein [*Klebsiella* phage KP34]	87	100.00%	1.30E-35
4	YP_009204826.1	head-tail connector protein [*Klebsiella* phage vB_KpnP_SU552A]	531	98.87%	9.10E-294
5	YP_009204827.1	putative scaffolding protein [*Klebsiella* phage vB_KpnP_SU552A]	280	99.64%	4.50E-151
6	AOT23872.1	capsid protein *[Klebsiella* phage KP-Rio/2015]	339	97.05%	4.10E-191
7	YP_009280706.1	hypothetical protein kpv475_37 [*Klebsiella* phage KpV475]	60	96.67%	4.00E-20
8	YP_009280707.1	putative tail tubular protein A [*Klebsiella* phage KpV475]	186	99.46%	4.90E-101
9	YP_009188784.1	putative tail tubular protein B [*Klebsiella* phage KpV41]	786	98.98%	0.00E+00
0	YP_009204832.1	putative internal virion protein B [*Klebsiella* phage vB_KpnP_SU552A]	195	97.95%	1.60E-94
1	YP_009188357.1	hypothetical protein [*Klebsiella* phage Kp2]	894	99.11%	0.00E+00
2	YP_009199928.1	putative internal core protein [*Klebsiella* phage vB_KpnP_SU503]	1232	98.62%	0.00E+00
3	APZ82760.1	putative tail fiber protein [*Klebsiella* phage vB_KpnP_KpV74]	602	90.00%	2.40E-67
4	No hits				
5	YP_009199930.1	putative DNA maturase A [*Klebsiella* phage vB_KpnP_SU503]	100	94.12%	8.00E-35
6	YP_009188791.1	putative DNA maturase B [*Klebsiella* phage KpV41]	618	99.19%	0.00E+00
7	YP_003347646.1	hypothetical protein [*Klebsiella* phage KP34]	124	98.37%	3.60E-60
8	AOT23882.1	hypothetical protein KPRIO2015_44 [*Klebsiella* phage KP-Rio/2015]	60	96.67%	6.10E-21
9	YP_003347648.1	hypothetical protein [*Klebsiella* phage KP34]	134	99.25%	5.50E-62
0	YP_009006072.1	Putative holin protein F19_49 [*Klebsiella* phage vB_KpnP_SU552A]	83	98.00%	8.00E-111
1	YP_009204842.1	putative endolysin [*Klebsiella* phage vB_KpnP_SU552A]	202	95.05%	1.50E-99
2	YP_009098386.1	putative HNH endonuclease [*Klebsiella* phage NTUH-K2044-K1-1]	153	94.74%	6.80E-85

Note: “No hits” indicates no significant hits detected for a particular amino acid sequence

Functional ORF Analysis

Analysis of protein sequences using BLASTp and RAST revealed that Structural protein ORF20 and ORF36, with a length of 299 and 377 bp, respectively, were the major tegument proteins. While ORF34 appeared to be phage head-tail connector proteins. In addition, ORF35, as a scaffold protein, had a supporting and protective effect on the overall structure of phage. ORF38-39 were associated with Tail tubular protein A (TTPA), these were structural tail proteins of *K pneumoniae* bacteriophage. The gene clusters encoding these proteins played a significant role in phage infection and adsorption of host bacteria. Specifically, seven proteins were involved in the replication and regulation of phage DNA. ORF12-13, were likely to be a DNA primase/helicase protein. ORF 15 encoded DNA polymerase protein with a length of 796 bp was detected. Meanwhile, ORF 26-28 had the functional characteristics of endonuclease, played a vital role in Phage DNA replication regulation. ORF31 showed the greatest identity to phage DNA-dependent RNA polymeras proteins. As the phage genome was small, no relevant genes coding for DNA topoisomerases, or transcriptional regulators were identified. ORF50 was shown to be a holin, a protein that perforates the bacterial cell membrane, while ORF51 (endolysin of phage) was associated with phage cleavage. Therefore, phage vB_KpnM _Bp5 might mediate host cell lysis by simultaneously expressing both proteins. A detailed description of the ORFs and their identified functionality was shown in Fig 4.

Evolutionary Analysis

Genome BLAST results illustrated that the vB_KpnM _Bp5 genome showed very high similarity with other current NCBI published genomes in the aspects of complete genome (Table 3). To illustrate the evolutionary relationship between vB_KpnM _Bp5 and all of the other known *K pneumoniae* phage representative strains, their multiple alignments were performed based on their complete genomes (Fig.5). The Genetic evolution tree showed that vB_KpnM _Bp5 was on the same branch as *K pneumonia* phage vB_KpnP_SU552A, and closely related to the *K pneumoniae* phage genome, such as vB_KpnP_KpV41□KP -Rio_2015□myPSH1235 et al.

Table 3 vB_KpnM _Bp5 genome alignment result using BLAST program

ssion	Description	Max.score	Total score	E.value	Query Cover (%)	Identity (%)
)8986.1	*Klebsiella* phage vB_KpnP_SU552A, complete genome	27855	56643	0.0	86	94.06
54103.1	*Klebsiella* phage vB_KpnP_KpV41, complete genome	25531	52717	0.0	82	92.99
13938.2	*Klebsiella* phage KP34, complete genome	25130	48598	0.0	82	92.31
76124.1	*Klebsiella* phage phiBO1E, complete genome	24640	48990	0.0	84	90.91
56662.1	*Klebsiella* phage KP-Rio/2015, complete genome	24221	50772	0.0	83	91.70
11991.1	*Klebsiella* phage vB_KpnP_KpV475, complete genome	23363	48647	0.0	81	90.81
66550.1	*Klebsiella* phage vB_KpnP_KpV71, complete genome	22692	54214	0.0	84	94.17
37949.2	*Klebsiella* phage phiKpS2, complete genome	19867	46571	0.0	82	90.84
)8985.1	*Klebsiella* phage vB_KpnP_SU503, complete genome	13538	49058	0.0	83	93.78
57886.1	*Klebsiella* phage vB_Kp2, complete genome	13381	38967	0.0	75	87.63

Discussion

With the advent of molecular biology, we were now better able to understand the co-evolved relationships between phage and their hosts, which could facilitate individualized treatments by using phages for bacterial infection [17].These advent of molecular biology gave us greater insights on how to most effectively use bacteriophage as potential therapeutic agents. Therefore, the purpose of this study was to analyze the biological characteristics and complete genome of phage, so as to provide valuable knowledge and insight for single phage or cocktail therapy.

Recently, a patient with cystic fibrosis with a disseminated *Mycobacterium* abscessus infection was treated with a three-phage cocktail following bilateral lung transplantation, and achieve good therapeutic results [18-20]. Fortunately, the fact that phage therapy for infection has shifted from scientific research to clinical use and achieved the desired results, which would open up a new phase of therapy for multi-drug resistance (MDR) infections.

In this study, we successfully isolated a novel lytic phage from farms sewage using a clinical multidrug-resistant *K pneumoniae* strain as an indicator. The phage was named vB_KpnM _Bp5 and classified as a member of the family *Muscle* virus based on electron microscopy analysis. Bacteriophage was used to treat *K pneumoniae* infection in mice. The results showed that the number of viable bacteria in the phage treated mice was significantly reduced compared with that in the PBS group. It was worth mentioning that the delayed treatment group only provided 60% protection, which might be mainly related to the slight differences in the phage replication rate and bacterial proliferation. In addition, it was worth noting

that phage vB_KpnM _Bp5 have a certain host range for both animals and humans. This feature provided the possibility for phage treatment of infection in clinical application.

RAST genome analysis showed that the phage had only 52 ORFs due to its small genome size. It was interesting that the number of functional proteins was consistent with other phages. Unfortunately, only five proteins fall into the category of well-defined functions, and most of the rest fall into the category of hypothetical or putative proteins. For example, ORF20 and ORF36 encoded structural proteins that provide a stable backbone for phage life-sustaining activities. ORF 15 and ORF 26-28 were involved in DNA replication and regulation, etc. Therefore, the whole genome sequence of vB_KpnM _Bp5 was of great significance in elucidating the functions of various genes.

Because the need to control MDR infections was urgent, phages were being newly studied as potential antibiotic alternatives. Phages were able to perform the function of phagocytic bacteria, mainly because it could undergo two different life cycles: the holing cycle and the endolysin cycle [21]. In this study, ORF 50 and ORF 51 were defined as regions encoding holin and endolysin .Phage endolysins accumulated in the host cell cytoplasm during phage development [22], until holins suddenly induce formation of holes in the cytoplasmic membrane [23].These holes provided a pathway for endolysin release to the cell wall that was rapidly cleaved, leading to cell burst [24].Therefore, the holin function had the crucial role of defining the time of lysis, which was fundamental for phage fitness. By clearly localized the enzymes of holin and lyase genes, it could provide a feasible idea for exploring the phage lysis mechanism.

Meanwhile, further exploration was required to improve the application of phage vB_KpnM _Bp5 as antibacterial agents in animals. For example, increased phage concentration might improve the therapeutic effect of the treatment, a cocktail including single phage and several other potential therapeutic phages might broad the lytic spectrum and reduce the possibility of phage resistance. Overall, the study of phages for using in antibacterial therapies was still in its infancy, meant that there was much room to explore alternative approaches and develop novel phage-based therapies. In summary, our study on the vB_KpnM _Bp5 provided new information, which from biocharacteristic analysis and whole genome sequencing. It could provide the basis of developing phage therapy of *K pneumoniain* fections.

Conclusion

The phage vB_KpnM _Bp5 had the characteristics of broad host spectrum, strong environmental adaptability, short incubation period, large outbreak amount, and could cure the mouse model infected by *K pneumoniae*. These findings suggested that phage vB_KpnM _Bp5 could be considered a potential therapeutic or prophylactic candidate against *K pneumonia* infection.

Methods

K. peumoniae strain J5 was isolated from farm sewage. A total of 34 *K. pneumoniae* strains were isolated from stool swabs and urine collected from a hospital in Nanning, Guangxi province. All bacterial strains were routinely cultivated at 37°C using lysogeny broth (LB).

Phage isolation and purification

The conventional double-layered agar method was used to isolate phages and enumerate plaque forming units (PFUs)[25]. *K. pneumoniae* was routinely used as the host bacterial strain for phage isolation. Plaque morphologies were observed after 12 h -post-incubation. Bacterial cultures in the exponential phase were inoculated with sewage effluent collected from a pig farm in Nanning, Guangxi, China. The mixture were initially incubated for 12-16 h at 37 ℃. Then it was centrifuged for 10 min at 12000 rpm, and filtered through a 0.25 μm pore size membrane filter to become the original liquid containing phage. The filtered phage lysate will be concentrated for 10 times dilution and join the corresponding host bacterium, using the method of double layer tablets after observing plaque growth situation at 37 ℃ for 12 h. Uniform size plaque was screened and titer was determined. Phages were stored at 4℃ for further experiments.

Electron microcopy

The purified phage sample was loaded onto a copper grid followed by negative staining with 2 % Phosphotungstic acid (PTA, 2% w/v) staining and drying. Phage sizes were calculated by means of at least 3 measurements.

Host range analysis

The host range of the phage was determined by spot test[26]. Lysis zone or plaque formation was monitored upon application of 10 μl of phage lysate, adjusted to contain 1>10^7 PFU. ml^{-1}, by spotting assay.

One-step growth

One-step growth experiments were carried out by a modification of methods described elsewhere [27,28].The logarithmic phase mixed with an overdose of phage host (MOI≈10), 37 ℃ warm bath after 15 min 12000 rpm centrifugal 1 min, discard supernatant and wash with LB to precipitate .Then, suspended in LB that had been pre-warmed to 37 °C, followed by incubation at 37 °C. Samples were taken at 10 min intervals (up to 90min) and immediately diluted, and phage titers were then determined by double-layered agar plate method.

Thermal and pH stability

For thermos-stability testing, samples of the isolated phage were incubated at 30, 40, 50, 60, 70, 80℃, and aliquots were taken after 30 and 60 min to be titer by the double-layer agar method. For pH stability testing, samples of the isolated phage were mixed in a series of tubes containing SM of different pH

values (2.0–11.0, adjusted using NaOH or HCl), incubated for 2 h at 37 °C, and then titer by the double-layer agar plate method [29,14].

Bacteriolytic activity

The host bacteria were cultured overnight and diluted to 1× 10^8CFU/ml. The host bacteria control group was added with 1.5 ml bacterial solution and 1.5ml culture solution. The experimental group was added with bacterial solution and phage suspension of different concentrations according to MOI=1 and 0.001, respectively. Three parallel measurements were made for each MOI, once every 1hand for 5 h.

Animals, diets and experimental design

Animal protocols were conducted on the basis of the regulations of Institutional Animal Experimental Ethical Inspection Form of Guangxi University, Nanning, China (GXU2019-062). The SPF mice were purchased from Experimental Animal Center of Guangxi Medical University. According to the standards committee, the survival mice were euthanized by cervical dislocation.

Assess the virulence of host bacteria

To evaluate the virulence of host bacteria in vivo with the minimum lethal dose, that was, the minimum dose that could cause individual poisoning death of a group of experimental animals. Six groups of SPF (Specific pathogen Free) Kunming mice (aged 6weeks) with six mice per group. Intraperitoneal injection of different doses of bacterial fluid (3.2×10^8、1.6×10^8、8×10^7、4×10^7、2×10^7cfu/ mouse) was performed. The control group was intraperitoneally injected with the same amount of sterile PBS, and the death of the mice was observed after a period of time. The survival mice were euthanized by cervical dislocation.

Phage therapy in the mouse infection model

To evaluate the therapeutic safety and efficacy of the bacteriophage in vivo, 5 groups of SPF Kunming mice (aged 6weeks) with five mice per group. Divided as follows: group 1, advance treatment group (phage-treatment before infection 1h); group 2, current treatment group (phage-treatment concurrent infection) ;group 3, delayed phage-treatment groups(phage-treatment groups after infection 1h); group 4, bacteria-only-infection mouse group; group 5, blank treatment (PBS) mouse group. Briefly, postinfection phage-treatment mouse groups used in the experiments were immunized 2×10^8 pfu/phage by the intraperitoneal (i.p.) route. Each group was monitored for mortality for 7 days. The number of bacteria living was counted in mice using the phage therapy in two hours later. The survival mice were euthanized by cervical dislocation.

Bacteriophage genome extraction

Phage gDNA was isolated from purified phages (procedure described above) and extracted by an alkaline lysis method[30]. After the phage pellet was suspended in SM buffer, sodium dodecyl sulfate (SDS) (final

concentration, 0.5%) and proteinase K (final concentration, 50μg/ml) were added. DNA/RNA enzyme action 37 °C, 30 min. The mixture was vortexed thoroughly and incubated at 56°C for night. An equal volume of phenol–chloroform (1:1) was added to remove the proteinaceous material. The extraction was repeated twice, and the DNA was precipitated according to ethanol precipitation procedures. The pellet was dissolved in 30 μl of distilled water, and the isolated nucleic acids were separated using 0.8 % agarose gel electrophoresis, stained with ethidium bromide, and analyzed under a UV light. After digestion, electrophoresis of the samples in 0.8 % agarose containing ethidium bromide (1 μg/ml) was performed. A pyrosequencing approach (Sangon Biotech Shanghai Co., Ltd.), using a Genome Sequencer Illumina System was applied, and the high-quality filtered reads were assembled into a complete genome sequence using velvet. ORFs were predicted with RAST (http://www.rast.nmpdr.org).

Abbreviations

K pneumonia: Klebsiella pneumonia; PFUs plaque-forming units; MOI: multiplicity of infection; $MLD_{100:}$ minimum lethal dose; NGS: next-generation sequencing; RAST: Rapid Annotation using Subsystem Technology; ORFs: open reading frames; BLASTp : Protein to Protein BLAST; TTPA: Tail tubular protein A; NCBI: National Center for Biotechnology Information; MDR: multi-drug resistance; LB: lysogeny broth; cfu: colony-forming unit; PTA: Phosphotungstic acid; SPF: Specific pathogen Free; SM: sterilized sodium chloride-magnesium sulfate; SDS: sodium dodecyl sulfate; UV: Ultraviolet Rays

Declarations

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Consent for publication

Not applicable

Authors' contributions

Authors' contributions XYW conceived and designed the experiments and critically evaluated the manuscript. CLG carried out the data analysis and wrote the manuscript. CZ isolated and identified the phage and carried out the experiments. CZ and CLG are co-first authors of the article. DYW extracted the phage nucleotide. XL analyzed the phage sequences. HBS and CHH conducted the sequencing experiments. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no conflict of interest.

Availability of data and materials

The data were presented in the main manuscript and available to readers.

Ethics approval and consent to participate

All animal experiments followed the regulations of the Institutional Animal Experimental Ethical Inspection Form of Guangxi University, Nanning, China (GXU2019-062).

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Figures

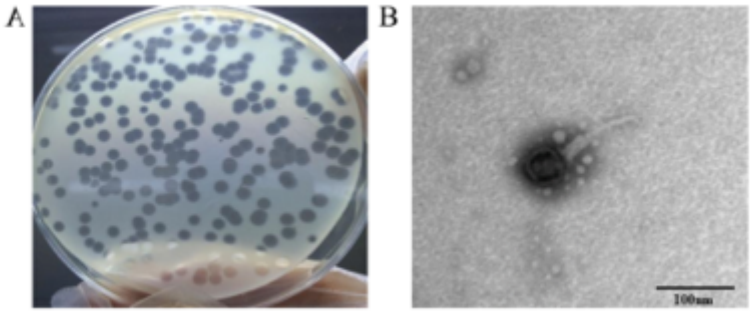


Figure 1

Morphology of phage vB_KpnM _Bp5. Phage vB_KpnM _Bp5 plaques (a).Morphology of bacteriophage vB_KpnM _Bp5 as revealed by transmission electron microscopy (b).

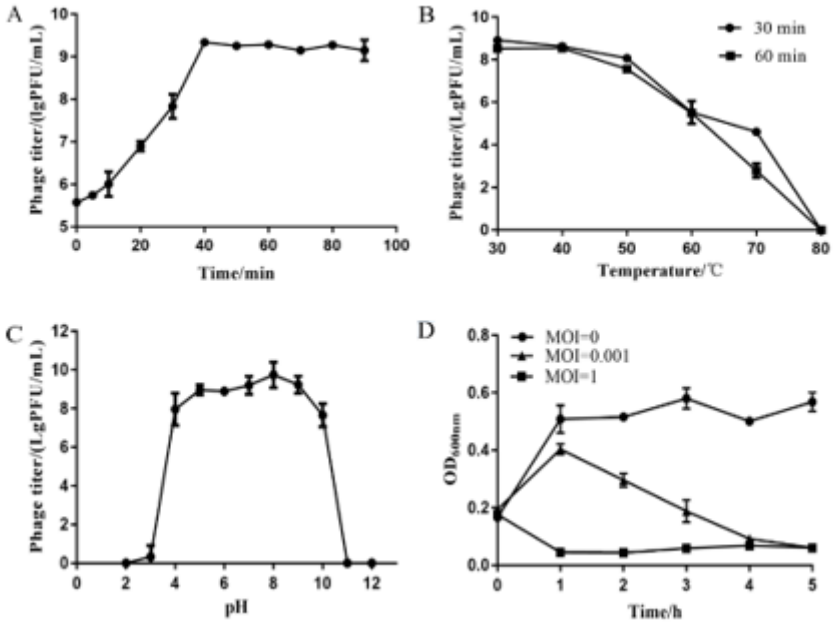
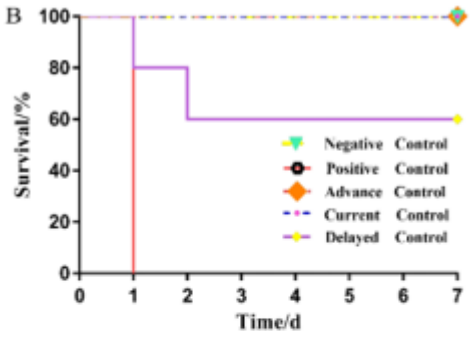


Figure 2

Biological characteristics of phage vB_KpnM _Bp5.One-step growth curve (a),thermostability (b),pH stability (c),and bacteriolytic activity of phage vB_KpnM _Bp5 on Klebsiella pneumonia J5□d□.Values represent mean ± SEM.

A

Item	Number of live bacteria/one	mortality
1	3.2×10^{8}	5/5
2	1.6×10^{8}	5/5
3	8.0×10^{7}	5/5
4	4.0×10^{7}	5/5
5	2.0×10^{7}	3/5
Control	PBS	0/5



C

Item	Number of infected bacteria/n	Number of phage therapy/n	Number of live bacteria after treatment (h)				
			2	4	6	8	12
Negative Control	0	0	0	0	0	0	0
Positive Control	4×10^{7}	0	1.78×10^{7}	5.3×10^{7}	5.3×10^{7}	7.9×10^{8}	/
Advance Control	4×10^{7}	2×10^{8}	0	0	0	0	0
Current Control	4×10^{7}	2×10^{8}	0	0	0	0	0
Delayed Control	4×10^{7}	2×10^{8}	8.04×10^{6}	4.15×10^{7}	2.85×10^{4}	0	0

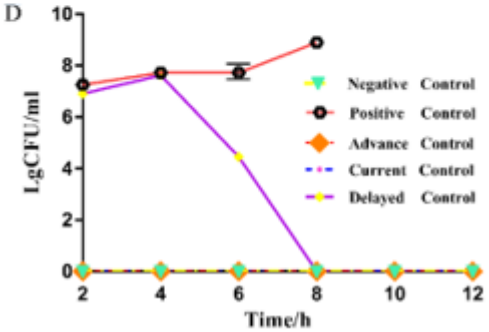


Figure 3

Assess the virulence of host bacteria and phage vB_KpnM _Bp5 therapy in the mouse infection model. Phages provided better protection, and the number of viable bacteria in mice in both the advance treatment group and the concurrent treatment group decreased significantly. In contrast, the positive control group both died

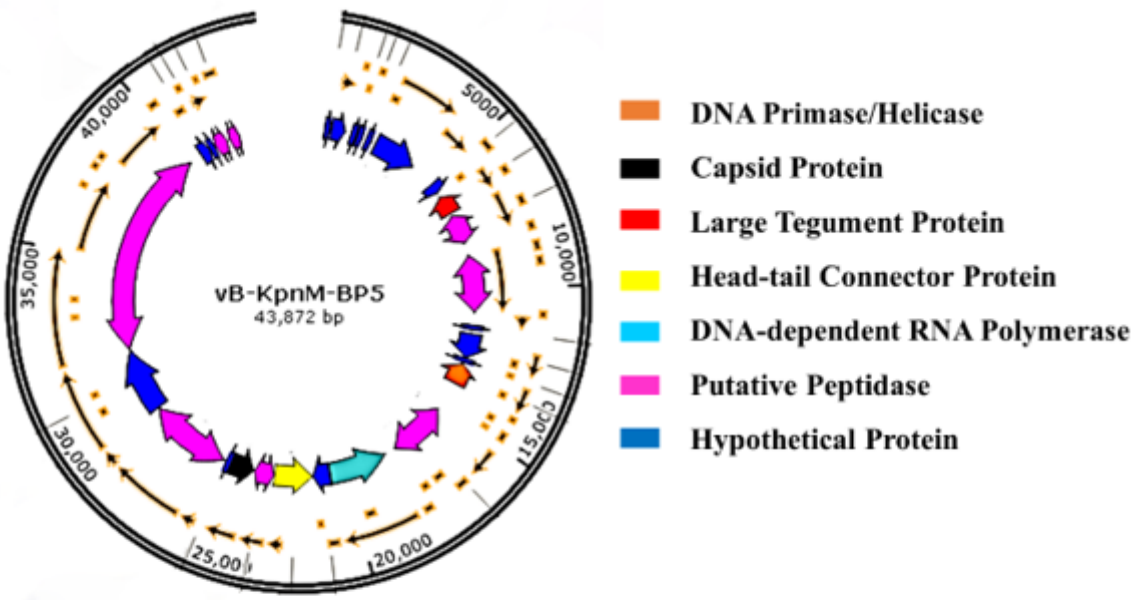


Figure 4

Genome map of vB_KpnM _Bp5, the linear genome of vB_KpnM _Bp5 is depicted in a circularized format. Comparative analysis of these two phages was conducted using Easyfig, with nucleotide identity above 90%. Well conserved segments are paired by shaded regions,and arrows indicate the direction of transcription for the predicted ORFs.

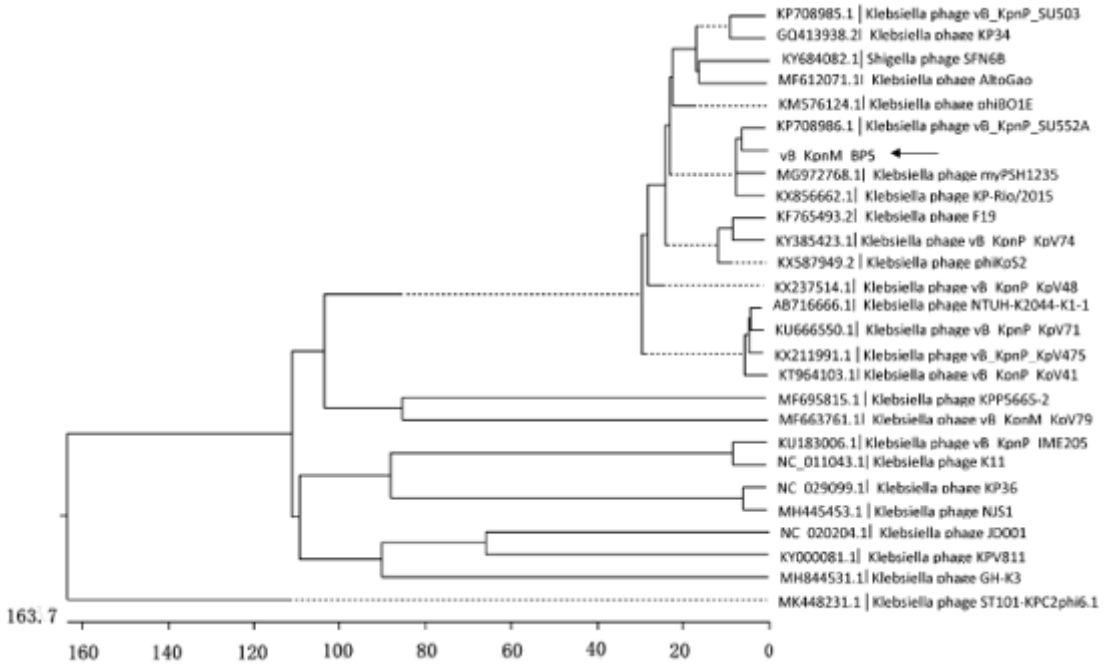


Figure 5

Phylogenetic tree of phage vB_KpnM _Bp5 .The sequences of 27 reference phages with the experimentally identified packaging strategy were selected based on previously published studies.The phages isolated in this study are in Black arrow.

Supplementary Files

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