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ORIGINAL ARTICLE

Isolation of new *Klebsiella pneumoniae* phage PSKP16

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ABSTRACT

Background and Objectives: *Klebsiella pneumoniae* is a clinically relevant opportunistic pathogen belonging to the *Enterobacteriaceae* family. It is in the top three bacteria associated with antimicrobial resistance deaths globally, and one of the most dangerous bacteria causing nosocomial infections. Phage therapy offers a potential option for the treatment of drug-resistant bacterial infections.

Materials and Methods: Phage PSKP16 was isolated against *K. pneumoniae*, capsular type K2 (isolated from a wound infection). PSKP16 is a new lytic phage with a *Siphovirus*-like morphology.

Results: PSKP16 is a linear double stranded DNA phage with a GC content of 50% and genome size of 46,712 bp, for which we predicted 67 ORFs. PSKP16 belongs to the genus *Webervirus* and shows high evolutionary proximity to *Klebsiella* phages JY917, Sushi, and B1.

Conclusion: Phage isolation is fast, cheap and efficient, but it requires time and characterization (which adds expense) to ensure that the isolated phages do not pose a health risk, which is essential to safely use phage therapy to treat life-threatening bacterial infections.

Keywords: *Klebsiella pneumoniae*; Bacteriophages; Drug resistance; Infection control; High throughput nucleotide sequencing

INTRODUCTION

Klebsiella pneumoniae can lead to severe urinary tract infections, ulcers, respiratory, and sepsis (1-3). The presence of high antimicrobial resistance (AMR) in *K. pneumoniae*, along with the ability to produce biofilm, has led to a wide range of infections caused by *K. pneumoniae* (4, 5). The lack of effective treatment for *K. pneumoniae* infections has resulted in an increase in costs, hospitalizing, and mortality (3, 6, 7). Today, due to the emergence of wide-spread AMR in *K. pneumoniae* (2, 8, 9) there is an increasing need to find new antimicrobial treatments, such as bacteriophage (phage) based therapies (10-14).

The need for new antimicrobials has fuelled a resurgence in phage isolation and research (14, 15). The discovery of new phages for use in phage therapy leads to different challenges and concerns, including the limited host range of phages compared to antibiotics, phage resistance, lysogenic conversion

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and phage production and purification (15-17). This highlights a need for a ‘‘biobank’’ containing a wide range of characterized phages ready to combat AMR bacteria when the need arises. The characterisation of phages is essential to enable timely, efficient treatment and ensure there are no adverse side effects (18). Therefore, increasing knowledge and understanding in the field of phage therapy, combined with creating a strong phage biobank will be of great help to scientists, clinicians and patients. The purpose of this article is to describe the characteristics of a new bacteriophage, PSKP16, against a clinical strain of *K. pneumoniae*.

MATERIALS AND METHODS

Phage isolation. The host bacteria strain used in this work was *K. pneumoniae* Bou-Ali, capsule type K2, which was isolated from a patient's wound, at Bou-Ali hospital in Qazvin, under *in vitro* conditions and verified using specific monovalent antisera against *K. pneumoniae* and PCR (19). Sewage from Bou-Ali hospital, Qazvin (sampled in 2020) was used as the source to isolate phages. Briefly, a sample of hospital wastewater was centrifuged at 14,000 rpm for 20 min and then passed through a 0.22 µm filter. The filtered hospital wastewater was then added to *K. pneumoniae* cultured in Lysogeny Broth (LB) and incubated at 37°C for 24 hr to enrich for phages. Following 24 hr co-incubation of *K. pneumoniae* and filtered wastewater, the enrichment lacked turbidity due to bacterial growth, indicating the presence of a lytic phage. The cleared enrichment culture was centrifuged; the supernatant was retained and filtered with a 0.22 µm filter to remove debris. The phage-enriched filtrate was serially diluted in LB and used in overlay agar plaque assays.

To carry out the plaque assays, 50 µl of phage enriched filtrate was mixed with 500 µl logarithmic phase (~OD600 0.2) *K. pneumoniae* for 5 min. To this we added 2.5 mL of hand-warm, molten LB agar (0.4%) and mixed. The agar mix was overlayed onto 1 % LB agar plates. These overlay plaque assays plates were left to set, before incubating at 37°C overnight. The overnight plates were observed for plaque formation and single plaques were picked and mixed with 50 µl of LB broth. These plaques were further purified with two further rounds of plaque assay to isolate clonal phages.

Transmission electron microscopy. The purified phage was visualised by transmission electron microscopy (TEM). Briefly, 5 µl of stock phage was applied to a glow-discharged formvar/carbon-coated copper grid and incubated at room temperature for 1.5 min. The grid was blotted, and then a drop of 2% uranyl acetate stain was added and incubated for 1 min then blotted. Staining was carried out four times before air drying the grid. The fully stained grid was imaged on a JEOL 2100Plus TEM. The phage particles were visualized in ImageJ to determine morphology and size. To determine size, PSKP16 phage particles were measured in ImageJ (20) using the measure function.

Genome sequencing and analysis. Phage DNA was extracted using Phage DNA Isolation Kit (Norgen) following the manufacturer’s protocol. Complete phage genome sequencing was performed using Illumina technology by MicrobesNG (Birmingham, United Kingdom), according to the following steps. Genomic DNA libraries were prepared using the Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer’s protocol with the following modifications: input DNA is increased 2-fold, and PCR elongation time is increased to 45 s. DNA quantification and library preparation are carried out on a Hamilton Microlab STAR automated liquid handling system (Hamilton Bonaduz AG, Switzerland). Pooled libraries are quantified using the KapaBiosystems Library Quantification Kit for Illumina. Libraries were sequenced (Illumina sequencers, HiSeq/NovaSeq) using a 250 bp paired-end protocol.

Raw Illumina reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15. *De novo* assembly of the reads was performed using SPAdes version 3.10.1. Genome annotation was carried out with Prokka, version 1.14.6 using the PHROGS database (20).

Analysis of the closest relative and percent identity-based analysis was carried out using INPHARED (21), against all known refseq phage genomes available from NCBI (April 2022), with a distance cutoff of 0.1 (which roughly translates to 0.9 similarities = 90% similarity). Phylogenetic trees were constructed to visualise the similarity of PSKP16 and closely related phages, using the neighbour-joining method, with bootstrap values generated from 10,000 replicates in Mega-X (22). Putative depolymerase genes were identified by carrying out

BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of previously characterized *Klebsiella* phage depolymerase genes (23, 24) against the PSKP16 genome. The genome sequence and associated data for phage PSKP16 were deposited under GenBank accession number OW251746.1, BioProject accession number PRJNA833576, SRA accession number SRR8869225, and BioSample accession number SAMN27615979.

RESULTS

Phage isolation. Phage enrichment and purification from Bou-Ali hospital sewage against *K. pneumoniae* Bou-Ali resulted in the isolation of phage PSKP16.

Phage PSPK16 morphology. Plaque assays of the purified phage PSKP16 resulted in clear plaques 2-3 mm in diameter surrounded by turbid halos that measured 10 mm in diameter (Fig. 1A). PSKP16 produces cloudy halos around the plaques because PSKP16 encodes an enzyme as depolymerase against the *Klebsiella* K2 capsule. TEM images revealed phage particles that showed *siphovirus*-like morphology (Fig. 1B). ImageJ analysis of the phage particles showed had capsids with a width of 60 nm and a tail length of 130 nm.

Genome analysis. INPHARED analysis revealed that phage PSKP16 belongs to the *Webervirus* genus. PSKP16 has a genome size of 46,712 bp with a GC content of 50%. Genome annotation identified 67 ORFs, including a putative depolymerase (Fig. 2). No known toxin or antimicrobial resistance genes were identified from the genome annotation. Genome mapping showed that genes were grouped in the PSKP16 genome according to function (Fig. 2). Genome homology analysis identified the closest related phages

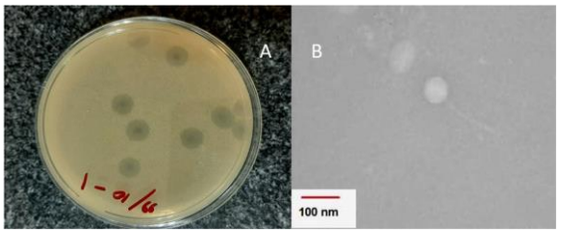

Fig. 1. PSKP16 phage morphology A. plaque morphology on a lawn of *K. pneumoniae* Bou-Ali and B. Transmission Electron Micrograph (TEM) negatively stained with uranyl acetate, with a 100 nm scale bar.

were Sushi, B1, and JY917, from the genus *Webervirus* (Fig. 3). The genome identity comparison of PSKP16 to Sushi, B1, and JY917 was >96%, indicating that PSKP16 is a novel phage of the same species (species cutoff is 95% identity).

The BLAST analysis performed (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of the putative PSKP16 de- polymerase. The analysis showed that PSKP16 pu- tative depolymerase had high identity to previously identified depolymerases from: *Klebsiella* phage RAD2 (96.15%), *Klebsiella* phage LF20 (95.71%), *Klebsiella* phage GHK3 (95.68%), *Klebsiella* phage B1 (95.60%), *Klebsiella* phage MMBB (95.57%) re- spectively. These relationships were reflected in the phylogenetic tree of the depolymerase genes (Fig. 4). All these identified depolymerase genes had a length of 2,724 bp (Table 1).

DISCUSSION

The *K. pnuemoniae*-infecting *Webervirus* PSKP16 was isolated from hospital wastewater and has not previously been described. PSKP16 is a lytic phage that infects *K. pnuemoniae* Bou-Ali, with capsular type K_2.

Phage PSKP16 has a modular genome and contains no genes that are likely to result in harmful side effects, adding to its suitable for phage therapy (14, 22). Interestingly, PSKP16 was able to clear an overnight culture of the clinical *K. pnuemoniae*, indicating it is highly virulent which is a desirable trait for therapy, and not all *Klebsiella* phages are capable of successfully preventing *Klebsiella* regrowth (12). Further testing would be needed to determine if PSKP16 can clear *Klebsiella* efficiently *in vitro*.

Based on the diversity of the polysaccharide components of the capsule, structures and antigens, *K. pneumoniae* can be divided into at least 79 serotypes (25). Previous studies shows that K1 and K2 capsular types are hypervirulent and cause disease (26-28). Because of lacked mannose and rhamnose in these capsular types, they could be recognized by macrophage lectin receptors to induce phagocytosis. Moreover, sialic acid, as an important structural constituent in K1 and K2 capsular polysaccharide, contributes to hyper mucoviscous phenotype, and is thus responsible for the anti-phagocytic activity directly or indirectly (20). Due to the protective

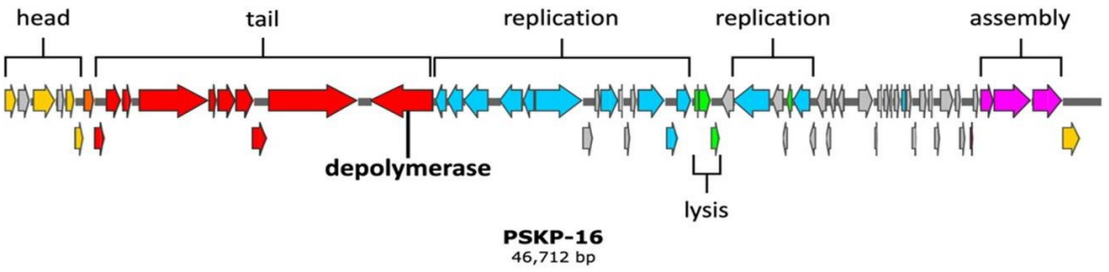

Fig. 2. Genome map of PSKP16 showing gene classifications, produced in Snapgene Viewer. The colour of the ORF relates to their predicted function, genes coloured: Yellow are head associated; Red are tail; Blue are replication; Green are lysis; Pink are assembly and Gray are hypothetical. The putative depolymerase gene is indicated, the final tail fibre gene.

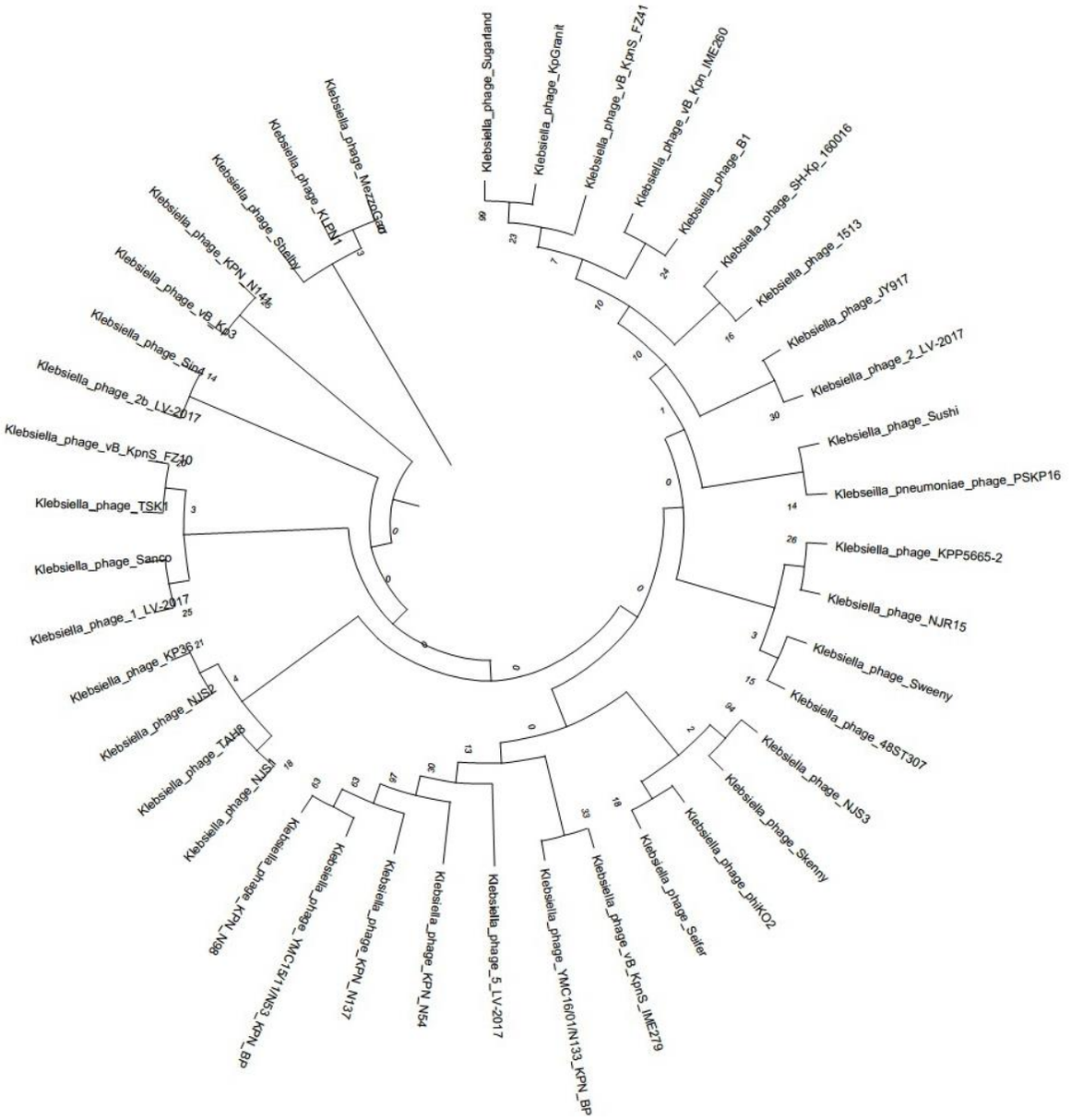

Fig. 3. Phylogenetic tree of known *Weberviruses* and PSKP16 using the neighbour-joining method visualized in Mega-X software. The numbers on the branches represent bootstraps generated from 10,000 replicates.

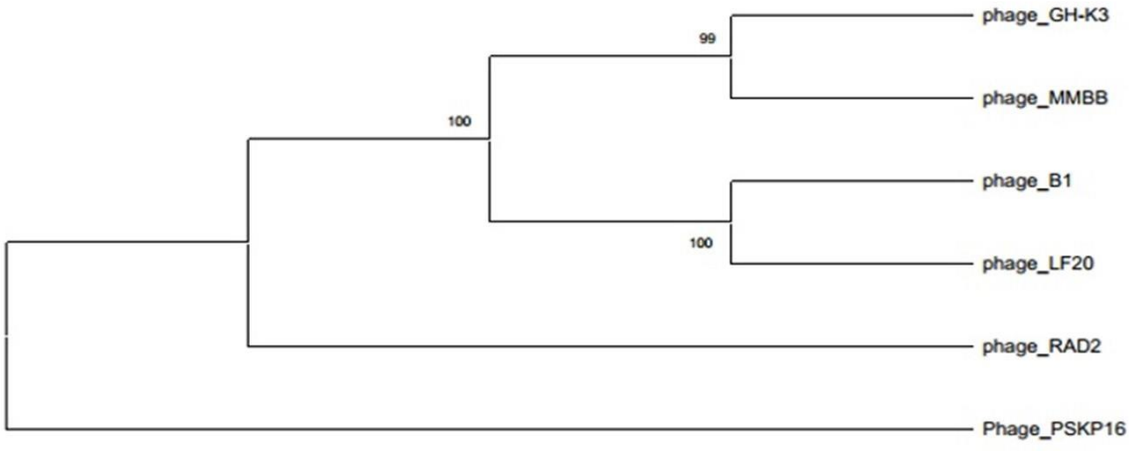

Fig. 4. Phylogenetic tree of the *K. pneumoniae* phages depolymerase belong to webervirus with the highest homology to phage PSKP16 (>95%), using the neighbour-joining method in Mega-X. the number on the branches represent bootstraps generated from 10,000 replicates.

Table 1. Comparison of *Webervirus* depolymerases with high homology to PSKP16 putative depolymerase

Bacteriophage Name	Length (bp)	Depolymerase halo diameter (mm)	Identity to PSKP16 gene (%)	Accession Number
PSKP16	2724	10	100	OW251746.1
RAD2	2724	8	96.15	NC_055956.1
LF20	2724	3-4	95.71	MW417503.1
GH-K3	2724	1-2	95.68	NC_048162.1
B1	2724	6	95.60	MW672037.1
MMBB	2724	3-4	95.57	MT894005.1

capsules of *Klebsiella*, phages that target them frequently encode depolymerases (sugar-degrading enzymes) that target specific *Klebsiella* capsule types (23, 29, 30). The analyses reveal that PSKP16 shows depolymerase activity against *Klebsiella* K_2 capsule as indicated by the halos surrounding phage plaques (31), furthermore sequencing results identified a putative depolymerase gene in PSKP16 genome. Depolymerases enzymes that breakdown the protective capsule of *Klebsiella*, making them more sensitive to antibiotics and the immune system (24, 25). These properties make depolymerases important for phages in the infection and killing of *Klebsiella* and have the potential to be used as antimicrobials in their own right (10, 25, 32). In future studies, projects will seek to identify the function and characteristics of the PSKP16 depolymerase. Phage PSKP16 appears to target clinically relevant K_2 capsule-forming *K. pnuemoniae* with depolymerase activity, making it an excellent candidate for phage-related treatments or raising the possibility of using PSKP16 depolymerase as a therapeutic.

CONCLUSION

Phage isolation is fast, cheap and efficient, but it requires time and characterisation (which adds expense) to ensure that the isolated phages do not pose a health risk, which is essential to safely use phage therapy to treat life-threatening bacterial infections. This study demonstrates the principals involved in isolating a phage for the personalised treatment of a patient's infection and the minimal information that is necessary to ensure the isolated phage is safe and efficient. Furthermore, we have isolated and characterised a new phage suitable for biobank storage for phage therapy.

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