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Diversity of lytic bacteriophages against XDR *Klebsiella pneumoniae* sequence type 16 recovered from sewage samples in different parts of the world

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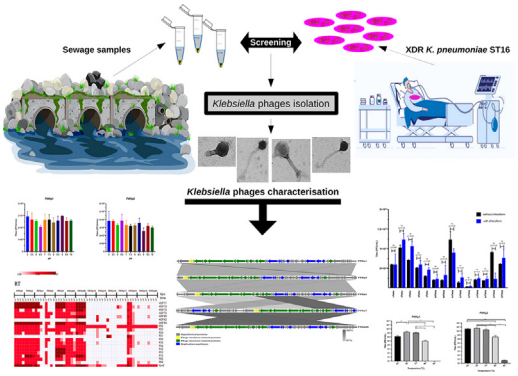
HIGHLIGHTS

- Sewage samples from different parts of the World were used to isolate lytic *K. pneumoniae* ST16 phages.
- Phages isolated from Brazil demonstrated better lytic activity against our *Klebsiella* ST16 collection.
- The phages showed dsDNA and belonged to five different families.
- Phages demonstrated high lytic activity against clinical strains at various temperatures.
- The viruses demonstrated high biotechnological potential.

GRAPHICAL ABSTRACT



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ABSTRACT

Bacteriophages (phages) are viruses considered to be natural bacterial predators and widely detected in aquatic environments. Sewage samples are an important source of phage isolation since high density and diversity of bacterial cells are present, due to human, animal and household fluids. This study aims to investigate and characterise phages against an extremely drug-resistant (XDR) lineage, *Klebsiella pneumoniae* ST16, using sewage samples from different parts of the World. Sewage samples from Brazil, Bangladesh, Saudi Arabia, Thailand and the United Kingdom were collected and used to investigate phages against ten *K. pneumoniae* ST16 (hosts) recovered from infection sites. The phages were microbiological and genetically characterised by double-agar overlay (DLA), transmission electron microscopy and Illumina WGS. The host range against *K. pneumoniae* belonging to different sequence types was evaluated at different temperatures by spot test. Further phage characterisation, such as efficiency of plating, optimal phage temperature, and pH/temperature susceptibility, were conducted. Fourteen lytic phages were isolated, belonging to *Autographiviridae, Ackermannviridae*, *Demerecviridae*, *Drexlerviridae*, and *Myoviridae* families, from Brazil, Bangladesh, Saudi Arabia and Thailand and demonstrated a great genetic diversity. The viruses had good activity against our collection of clinical *K. pneumoniae* ST16 at room temperature and 37 °C, but also against other important *Klebsiella* clones such as ST11, ST15, and ST258. Temperature assays showed lytic activity in different temperatures, except for

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PWKp18 which only had activity at room temperature. Phages were stable between pH 5 and 10 with minor changes in phage activity, and 70 °C was the temperature able to kill all phages in this study. Using sewage from different parts of the World allowed us to have a set of highly efficient phages against an *K. pneumoniae* ST16 that can be used in the future to develop new tools to combat infections in humans or animals caused by this pathogen.

1. Introduction

The one health approach recognises that the health of people is closely related to the health of animals and our shared environment (Mackenzie and Jeggo, 2019). The environment itself plays a significant role in the spread of clinically relevant antimicrobial resistance (Singer et al., 2016). This is because antibiotic resistant organisms are commonly carried in human and animal digestive systems and as such residential, farm and industrial waste waters (sewage), are a source of environmental contamination (Rolain, 2013; Francino, 2016; Kraemer et al., 2019). Resistant bacteria can then re-enter the food system from the environment via contaminated drinking water and foods (Hendriksen et al., 2019; Fouz et al., 2020; García-Aljaro et al., 2019). Sewage is also a rich source of bacteriophages due to abundance of bacterial strains and is commonly employed to isolate relevant bacteriophages (Muniesa and Jofre, 1998; Jurczak-Kurek et al., 2016).

Bacteriophages (also called phages) are viruses with the ability to infect bacteria and are the most abundant organisms on Earth (Doss et al., 2017). Typically, bacteriophages can present two lifestyles: lytic and lysogenic. Lytic phages are known by their high virulence since after bacteriophage infection, the phage starts its replication using bacterial machinery and new virion particles are assembled resulting in bacterial cell lysis (Salmond and Fineran, 2015). After lysis, the projeny viruses are available to infect new bacterial cells (usually cells belonged to the same lineage) with this cycle continuing until bacterial eradication or a bacteria-phage mutual relationship is established (Clokie et al., 2011; Salmond and Fineran, 2015). Lysogenic phages (also called temperate phages) insert their genomes onto the bacterial chromosome (prophages) where they exhibit a period of dormancy until exogenous stimuli induce the activation of the prophage and, consequently, start its lytic cycle (Salmond and Fineran, 2015). Due to the insertion on bacterial chromosomes, lysogenic phages can be associated with the spread of antimicrobial resistance, virulence genes and genomic islands, contributing to bacterial pathogenicity and evolution (Salmond and Fineran, 2015).

Besides the emergence of antimicrobial resistance pathogens in nosocomial settings, we are facing increasing reports of MDR bacterial samples from rivers, coastal regions, sewage and other environments (Taylor et al., 2011; Rowe et al., 2016; Hendriksen et al., 2019). These locations may also be a rich source of natural predators (phages) against micro-organisms such as *Escherichia coli* and *Klebsiella pneumoniae*, well known pathogens that commonly exhibit high resistance rates to antibiotics. *K. pneumoniae* is a WHO bacterial priority pathogen and as such has been the subject of several phage studies. This is especially for well-established lineages of *Klebsiella* considered high-risk clones (e.g., ST11, ST258) (Tan et al., 2019; Li et al., 2021). We have a growing problem in Brazil with *Klebsiella pneumoniae* sequence type (ST) 16, an emergent extremely drug resistant (XDR) lineage, that has also been reported in several countries causing human and animal infections (Espinal et al., 2019; Andrey et al., 2020; Zhang et al., 2021; Nguyen et al., 2021). The increasing prevalence of these bacterial strains encouraged us to look for bacteriophages that could be used to reduce the prevalence of these strains in the environment as well as an alternative to antimicrobial use.

Here, we report the microbiological and genomic characterisation of lytic *Klebsiella* phages targeting *K. pneumoniae* ST16 recovered from sewage samples from distinct geographic locations. We explore their differences and the benefits of having multiple bacteriophages sources for future phage applications.

2. Material and methods

2.1. Investigation, isolation and amplification of lytic bacteriophages using sewage samples

For this study, 56 *K. pneumoniae* isolates belonging to ST16 with capsular type (KL51) were selected: 29 previously described in recent publications from Brazil (n = 19) (Andrey et al., 2020) and Italy (n = 10) (Espinal et al., 2019) and 27 new isolations from Brazil (n = 25), Bangladesh (n = 1), and Vietnam (n = 1). Based on clonal profiles, ten of these were initially selected as hosts for the phage investigation step (Table S1).

Sewage samples were collected across Brazil (11 sites), Bangladesh (8 sites), England (4 sites), Wales (4 sites), Kazakhstan (2 sites), Saudi Arabia (2 sites) and Thailand (6 sites) in 2019, including general and hospital sewage (Fig. S1). Briefly, 100 mL of sewage was collected in 250 mL sterile screw cap Schott glass bottles and kept at room temperature (RT) until processed. In laboratory, samples were centrifuged at 5000 rpm for 10 min; the supernatant was filtered on 0.45 μm membranes and kept at 4 °C until use. For bacteriophage investigations, 100 μL of filtered sewage was inoculated into 3 mL of the 10 selected *K. pneumoniae* ST16 hosts in log phase (Table S1) and duplicate samples of each bacteria/filtered sewage suspension were incubated overnight at RT (first replicate) and 37 °C (second replicate), respectively. In this study, 20-22 °C is the range of temperature considered as RT. Post incubation, the suspensions were centrifuged at 6000 rpm for 5 min, supernatants were serially diluted and then 100 μL of the dilutions (10^{-3} to 10^{-6}) were mixed with 100 μL of the bacterial host. Subsequently, selected dilutions were mixed with 3 mL of Lysogeny Broth (LB) agar 0.5% and pooled on LB agar (1.5%) plates. Plates were dried for 15 min at RT and incubated at RT and 37 °C overnight. Isolated phage plaques were picked and propagated for three consecutive days to ensure the presence of a single population of each isolated plaque. Phage stock solutions were filtered, titred by double-layer agar methodology and stored at 4 °C.

2.2. DNA extraction, genome sequencing, annotation and genomic analysis

Phage DNA was isolated from phage lysate stock solutions at a concentration higher than 10^9 PFU/mL, as previously described, with minor modifications (Jakočiūnė and Moodley, 2018). Briefly, 900 μL of phage lysate was initially treated with RNAse (20 mg/mL) and DNAseI (1 U/μL) and then incubated at 37 °C for 2 h to degrade residual bacterial DNA and RNA. Thereafter, both enzymes were inactivated by EDTA (20 mM) at RT for 10 min, followed by the addition of 2.5 μL of proteinase K (20 mg/mL) and incubation for two hours at 55 °C. DNA purification was performed using DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's recommendations (Jakočiūnė and Moodley, 2018). DNA concentration was assessed by Qubit™ dsDNA HS Assay Kit using Qubit 4 Fluoromenter (Thermo Fisher Scientific). Genomic DNA libraries were constructed using the Nextera XT kit (Illumina), according to manufacturer's instructions. Paired-end sequencing was performed using the Illumina MiSeq platform (MiSeqReagent V3 Kit; 2 × 300 cycles). Raw sequence reads were trimmed using TrimGalore, and the genomes were de novo assembled into contigs using SPAdes (3.9.0) with pre-defined kmers set (Bankevich et al., 2012).

Automatic annotation was performed by PROKKA followed by manual validation using UniProtKB (https://www.uniprot.org/). Phage temperate behaviour was investigated by the Domingo-Calap and colleagues (2020) approach, and the presence of antimicrobial resistance genes by

Resfinder 4.1 (https://cge.cbs.dtu.dk/services/ResFinder/) and CARD (https://card.mcmaster.ca/), insertion sequences by ISFinder (https://isfinder.biotoul.fr/), virulence encoding-genes by VFDB (http://www.mgc.ac.cn/VFs/main.htm) and UniProtKB. Phage taxonomy and the relation with other described bacteriophages was performed by VIPtree and BlastN using the GenBank database. Genomic organization and comparisons were performed using Easyfig.

2.3. Transmission Electron microscopy (TEM)

Phage lysates (>10^9 PFU/mL) were submitted to transmission electron microscopy (TEM). The viral particles were mounted on a 300 mesh formvar/carbon-coated TEM grid floated on droplets, negatively stained with uranyl acetate 2% (*w/v*) and incubated at room temperature until dry. Pictures were taken using Hitachi HT7800 at 100 kV and images captured with Radius software (EMSIS GmbH, Germany) (Bils and Hall, 1962).

2.4. Host range analysis against K. pneumoniae ST16

A spot test methodology was used to check the activity of the newly identified bacteriophages against the collection of 56 *K. pneumoniae* ST16 isolates and also to understand if the different phage concentrations could interfere in phage susceptibility. Briefly, one hundred microliters of an overnight culture was mixed in 3 mL of LB agar 0.5% and poured on LB agar plates. Once dried, 2 μL of each phage was spotted on agar surfaces at four different phage concentrations (10^7, 10^6, 10^5, and 10^4 PFU/mL) and incubated overnight at RT and 37 °C. On the following day, the presence of phage activity was evaluated by the presence of an inhibition zone.

2.5. Host range analysis against K. pneumoniae non-ST16

Non-ST16 *K. pneumoniae* isolates (n = 103) recovered from human and animal sources (Andrey et al., 2020; Hassan et al., 2021), were also evaluated to determine the phage collection's activity against other *K. pneumoniae* strains. For this assay, phage concentration of 10^7 PFU/mL was evaluated at RT and 37 °C. The results were interpreted as described above.

2.6. Phage optimal temperature

The optimal temperature of each bacteriophage was assessed at 22 °C, 30 °C, 37 °C, 40 °C, and 45 °C: A single colony of each host was incubated in 20 mL of LB broth at 37 °C under agitation until it reached an OD_{600} ~ 0.20 ± 0.02. Two milliliters of this culture were transferred to new tubes and infected individually with each bacteriophage at a final multiplicity of infection (MOI) relation of 0.01. Immediately after infection, the tubes were incubated at their respective temperatures for 20 h. After incubation, the culture was transferred to 2 mL tubes, centrifuged at 8000 rpm for five minutes, and then serial diluted on SM Buffer (10^{-1} until 10^{-10}). The concentration of each propagation test was assessed by double-layer agar methodology. For each bacteriophage infection, three independent biological replicates were performed.

2.7. Efficiency of plating

The efficiency of plating (EOP) was determined by double-layer agar methodology for all susceptible *K. pneumoniae* ST16 isolates based on the procedure described by Khan Mirzaei and Nilsson (2015), with some modifications. All isolates were grown overnight to stationary phase (~5 × 10^9-1 × 10^{10} CFU/mL) and 100 μL of bacteria suspension was added to 100 μL of each phage stock solution at (10^1 to 10^7) PFU/mL followed by vortexing. This mixture was left at room temperature for 20 min and then mixed with 3 mL of 0.5% LB agar, poured over LB agar plates and incubated for 20 h at the optimal phage temperature previously established. Thus, the number of plaques produced by each infection was recorded and EOP was estimated by the ratio between the number of plaques obtained in each tested *K. pneumoniae* isolate relative to the original host.

2.8. Bacteriophage stability analysis

Stability studies were conducted on 14 *Klebsiella* phages at different temperatures, pHs and in the presence and absence of chloroform. For thermal stability test's, each phage stock solution was diluted in SM Buffer to 1–5 × 10^8 PFU/mL and then incubated for one-hour at 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C and 80 °C. After incubation, the viability of each phage solution was assessed by the double-agar overlay method and compared with those kept at room temperature. The stability against different pH's was tested by diluting phage stock solutions to 1–5 × 10^8 PFU/mL on SM buffer adjusted with HCl (2 M) and NaOH (2 N) to reach the range 5–10 (0.5 variation). Phage lysates were incubated for one hour at different pHs and then titred at 37 °C. Susceptibility to chloroform was evaluated by mixing 1:1 phage lysates (1–5 × 10^8 PFU/mL) and chloroform 100% (Merck KGaA, Darmstadt, Germany) followed by one-hour incubation. The titration was performed as described above.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 8.4.3, San Diego, CA, USA). One-way ANOVA followed by multiple comparison tests were used to compare phage optimal temperature, efficiency of plating, and pH susceptibility. Multiple comparison tests applied in each analysis are described in their respective figure legends. Difference on chloroform susceptibility was evaluated by Two-tailed *t*-test. *P*-values of <0.05 were considered statically significant.

3. Results

3.1. Identification of Klebsiella phages

Twenty-two *Klebsiella* phages were isolated after screening sewage samples, of which n = 3 were from Bangladesh, n = 14 were from Brazil, n = 1 from Saudi Arabia, and n = 4 from Thailand. Interestingly, although detected in these four countries, *K. pneunoniae* ST16 phages were not found everywhere, since all the isolations were concentrated in individual locations. In Brazil, for instance, samples were collected across all the geographic Brazilian regions, but only samples from São Paulo were positive for these types of phages. No bacteriophages against *K. pneumoniae* ST16 were identified in sewage samples from the United Kingdom (England and Wales) or Kazakhstan. Interestingly, 80% of bacteriophages were isolated when the propagation step was performed at RT.

3.2. Genomic and morphological characteristics

Twenty-two phages were submitted to WGS and initially analysed according to their similarity by multiple alignment (ClustalW method), generating an identity matrix. Some of these phages were highly similar (>99.9%) and were categorized as the same bacteriophage. After this initial step, 14 phages were moved forward for further characterisation. These phages were identified as linear double-stranded DNA phage belonging to five different families: i) family: *Autographiviridae*, genus: Drulisvirus (PWKp1, PWKp2, PWKp3, PWKp7, and PWKp9B); ii) family: *Ackermannviridae*, genus: Taipeivirus (PWKp5); iii) family: *Demerecviridae*, genus: Sugarlandvirus (PWKp9S); iv) family: *Drexlerviridae*, genus: Webervirus (PWKp14, PWKp15, PWKp17, PWKp19, and PWKp20); v) family: *Myoviridae*, sub-family: *Tevenvirinae* (PWKp16 and PWKp18).

Based on the geographical location where these bacteriophages were isolated, all Drulivirus, Sugarlandvirus, and Taipeivirus were recovered from Brazil, whilst Drexlerviruses and Myoviruses were detected

across Bangladesh (PWKp17, PWKp18, and PWKp19), Saudi Arabia (PWKp14), and Thailand (PWKp15, PWKp16, and PWKp20). A proteomic tree demonstrated high amino acid synteny among the Authographiviruses, as well as the Drexlerviruses with bacteriophages contained in the VIPtree database. Viruses belonging to these two families were closely related to other *Klebsiella* viruses, while the remaining phages also demonstrated close relationships with other Enterobacterales bacteriophages such as *Escherichia*, *Edwardsiella*, and *Salmonella* phages (Fig. 1; Fig. S2). Except for the Autographiviruses and Drexlerviruses, that were grouped with Podoviruses, the taxonomy of the remaining viruses corresponded with those obtained by initial blastN on NCBI database. The genome size range was based on each bacteriophages' family: ~46Kb (*Autographiviridae*), 49 ~ 50Kb (*Drexlerviridae*), 109Kb (*Demerecviridae*), 157Kb (*Ackermannviridae*), and 170Kb (*Myoviridae*). As expected, viruses with smaller genomes showed a compact genetic machinery composed of genes related with bacteriophage structure, replication, lysis and hypothetical proteins (Figs. S3-S7). Several tRNA-encoding genes were detected in our larger viruses (Demerecvirus, Ackermannvirus, and Myoviruses), ranging from 6 (PWKp5) to 23 (PWKp9S). The complex endolysin-holin-spanin was detected in all analysed bacteriophages, except for PWKp9S that showed a cell wall hydrolase instead of endolysin. Although the complex endolysin-holin-spanin was frequent, each bacteriophage family showed a distinct spanin, such as Rz1A in all Drexlerviruses and I and O spanin on Myoviruses. Interestingly, a RIIab system, responsible for inhibition of bacteria lysis by the bacteriophages was detected on PWKp5, PWKp16, and PWKp18. No lysogenic behaviour was detected among the studied *Klebsiella* phages (Figs. S3-S7).

Bacteriophage morphological features were assessed on double-agar overlay and by TEM (Fig. 2; Fig. S8). The lytic plaque morphology varied based on each bacteriophage family, e.g. Autographiviruses and Drexlerviruses demonstrated large size plaques and the presence of inhibition halos, typical of genomes encoding depolymerases.

3.3. Host range analysis against Klebsiella pneumoniae isolates

The 14 *Klebsiella* phages were tested against a collection of 56 *K. pneumoniae* ST16 capsular type KL51 isolates at both RT and 37 °C. In general, bacteriophage activity was concentration dependent as some phages were unable to inhibit the bacterial growth at concentrations below 10^6 PFU/mL (Fig. 3). Interestingly, the Autographiviruses recovered from Brazil (PWKp1, PWKp2, PWKp3, PWKp7, and PWKp9B) demonstrated a broad host range against our collection, including *K. pneumoniae* strains from Italy, Bangladesh, and Vietnam (Fig. 3). Although the Autographiviruses had high genomic similarity, subtle differences in the *K. pneumoniae* phage susceptibility were observed, including higher activity of PWKp9B. The same finding was also apparent in viruses belonging to the Drexlerviruses, where PWKp17 exhibited more activity at 37 °C as compared with other viruses of the same genus. Some of our viruses had a narrow host range (e.g., PWKp14, PWKp15, PWKp16, and others). However, they were active against some bacterial isolates that were poorly inhibited by the Autographiviruses (e.g., Kpn8, Kpn32, Kpn36, VNV8). The phage PWKp18 showed a broad activity range only when tested at RT but not at 37 °C. The number of strains infected by each bacteriophage is displayed in Supplemental Table 2.

Among the 103 *K. pneumoniae* non-ST16 isolates tested, our phage collection was active against 50 (48.5%) isolates, which included other nosocomial important lineages such as ST11_KL64 (n = 7), ST15_KL113 (n = 1), ST258_KL107 (n = 18), and ST442_KL107 (n = 5) (Fig. 4). *K. pneumoniae* isolates with sequence or capsule types of less clinical significance were also inhibited by these phages (Fig. 4; Table S3). This activity was mainly driven by the "non-specific activity" of PWKp9S, PWKp15, and PWKp18 (Table S3). These results were also useful to determine that the capsular type KL51 was not a determinant controlling the host range of our bacteriophages. Eight ST231_KL51 isolates were tested and six of them were inhibited mainly by PWKp5, PWKp9S, and PWKp9B (Fig. 4; Table S3).

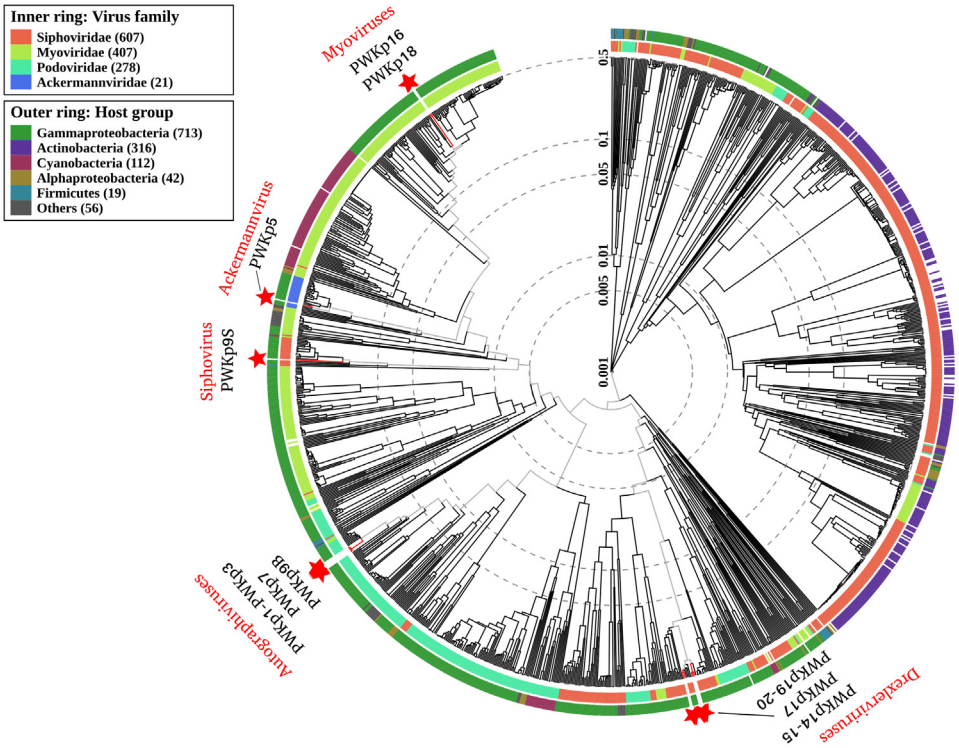

Fig. 1. Circular phylogenetic tree based on proteomic analysis generated by VIPTree. Red stars represent *Klebsiella* phages detected in this study. Beside our 14 *Klebsiella* phages, this tree includes 1313 dsDNA viruses deposited on VIPTree database, classified by their family (inner ring) and their respective hosts taxa (outer ring). A more detailed phylogenetic tree with all labels can been seen in Fig. S2.

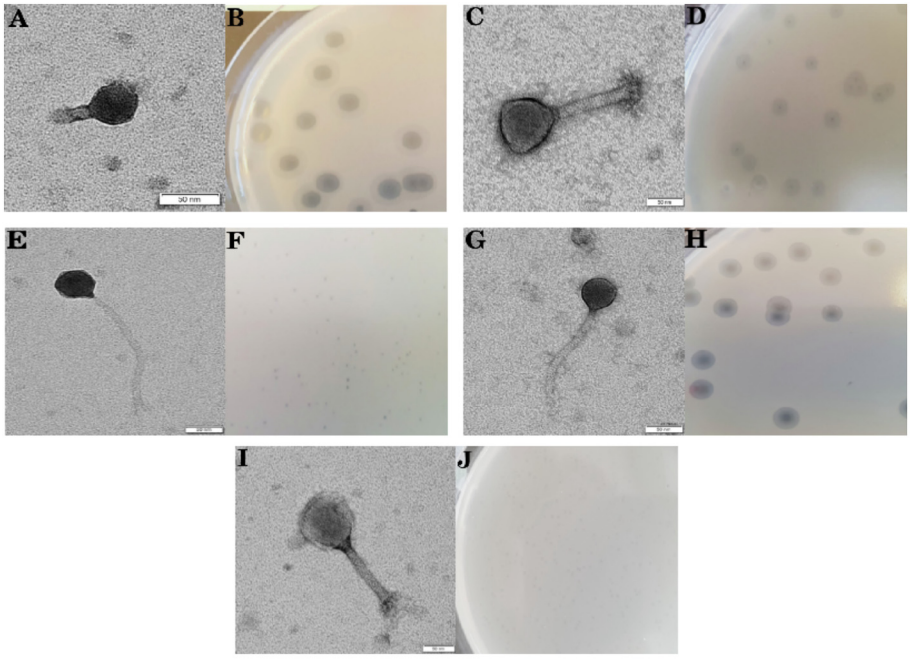

Fig. 2. Transmission electron microscope (TEM) images and bacteriophages' plaques of representative viruses. Scale bars in the TEM images represent 50 nm and the pictures of viruses' plaques were taken after 24 h incubation at the best phage temperature in LB double-layer agar (1.5%–0.5%). A TEM image of *Autographiviridae* (*Drulisvirus*) PWKp1, B plaque morphology of PWKp1 observed in this study. C TEM image of *Ackermannviridae* (*Taipeivirus*) PWKp5, D plaque morphology of PWKp5 observed in this study. E TEM image of *Demerecviridae* (*Sugarlandvirus*) PWKp9S, F plaque morphology of PWKp9S observed in this study. G TEM image of *Drexlerviridae* (*Webervirus*) PWKp14, H plaque morphology of PWKp14 observed in this study. I TEM image of *Myoviridae* sub-family *Tevenvirinae* PWKp16, J plaque morphology of PWKp16 observed in this study. Further images of all bacteriophages isolated in this study can be found in Fig. S7.

3.4. Optimum bacteriophage temperature

The broad activity of our *Klebsiella* phage collection against *K. pneumoniae* ST16 determined by host range at RT and 37 °C was an initial clue that these bacteriophages can work at different temperatures. Surprisingly, except for PWKp18 that only showed activity at RT, all other bacteriophages were active at 22 °C, 30 °C, 37 °C, and 40 °C (Fig. 5). Ten bacteriophages showed activity at 45 °C, with emphasis on PWKp14, PWKp15, PWKp19, and

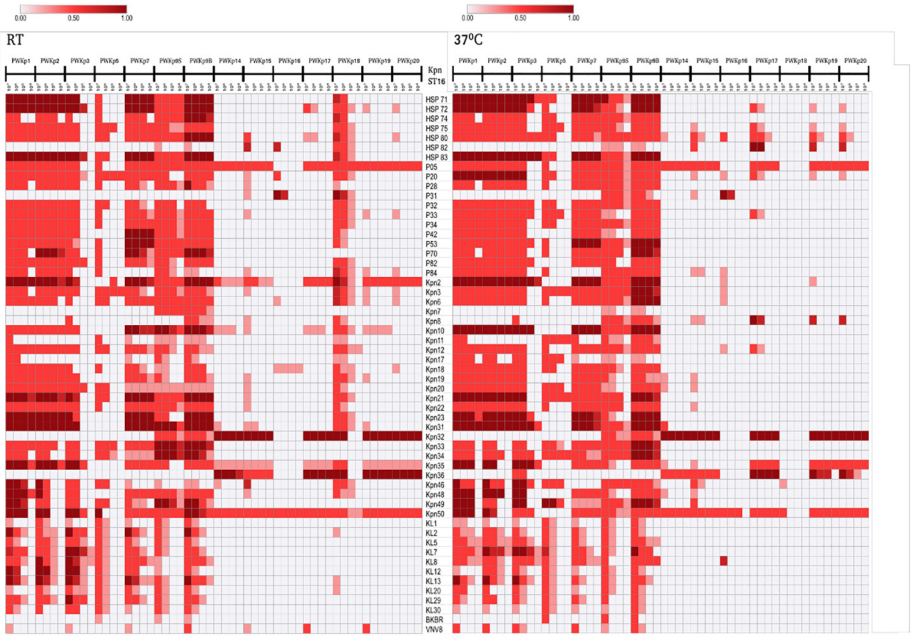

Fig. 3. A heat map to represent the *Klebsiella* phages host range against *K. pneumoniae* ST16 capsular type KL51 at RT (A) and 37 °C (B). Phage susceptibility was assessed by spot-test using four phage concentrations (10^7 to 10^4 PFU/mL). Regarding the results interpretation, we attributed different values (1, 0.75, 0.5, 0.25, or 0) based on inhibition pattern obtained after each spot test to represent the results by heat map: (1) full inhibition with no growth of resistant colonies; (0.75) inhibition with growth of few phage-resistant colonies in the inhibited zone; (0.5) partial inhibition with growth of a bacteria layer in the inhibited zone; (0.25) weak inhibition; (0) absence of inhibition. The scales of colours used for the results representation are in the top of both heat maps, showing as the darker the shade of red, the greater the phage inhibiting activity.

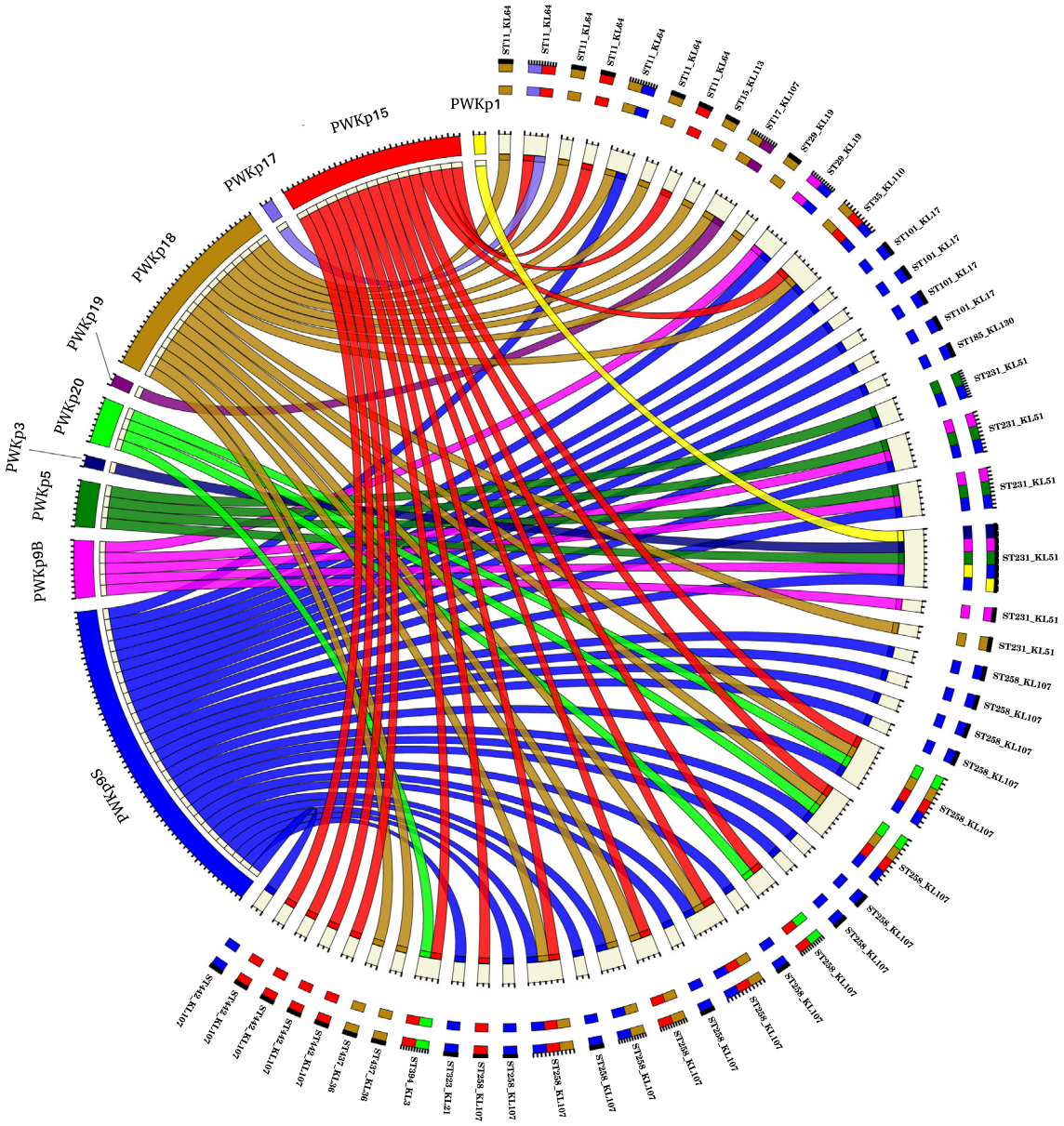

Fig. 4. A chord chart to represent the *Klebsiella* phages host range against non-ST16 *K. pneumoniae* with different capsular types. Inhibition relationships are demonstrated by connections among phages and *K. pneumoniae* isolates with no differentiation between inhibition temperatures. Further details about temperature of inhibition are demonstrated in Table S2.

PWKp20 (Drexlerviruses) that showed titers higher than 10^8 PFU/mL when amplified at this temperature. We also determined optimal bacteriophage temperature for each one of our viruses by recording the temperature responsible for the highest titer after 24 h of amplification. Among Autographiviruses, 30 °C was usually the optimal temperature. However, except for PWKp2 ($p = 0.01$) that showed a significant difference, no statistical variation was observed when 30 °C and 37 °C were compared (Fig. 5). In contrast, increasing the temperature of incubation to 40 °C-45 °C had significant impact on some Autographiviruses (Fig. 5). The Taipeivirus (PWKp5) and Sugarlandvirus (PWKp9S) showed optimal temperatures of 37 °C and 30 °C, respectively, with PWKp9S having significant reduction of activity with temperature variation (Fig. 5). All Drexlerviruses had 37 °C as the optimal temperature. However, no significant difference was observed when compared with 22 °C, 30 °C, and 40 °C, demonstrating high temperature stability. The decrease of Drexlerviruses activity was statistically significant for PWKp15 and PWKp20 at 45 °C. The two isolated Myoviruses (PWKp16 and PWKp18) showed a completely different optimum temperature profile (37 °C for PWKp16 and 22 °C for PWKp18) and were the phages most impacted by temperature variations (Fig. 5).

3.5. Efficiency of plating and stability studies

EOP results demonstrated a discrepancy in the number of infected strains by host-range when compared with productive infection by EOP analysis for the following phages: PWKp14, PWKp15, PWKp16, PWKp17, PWKp19, and PWKp20. For these phages, the number of inhibited isolates by host range was always higher when compared with the productive infection evaluated by EOP tests (Table S2). The EOP values distribution showed a great variation of bacteriophage efficiency, suggesting a heterogeneous collection of bacterial isolates regarding susceptibility to phages (Fig. 6). The proportion of isolates with high productive infection (EOP ≥ 0.5) ranged from 2% (PWKp2, 1/49) to 72.2% (PWKp9S, 39/54) and with

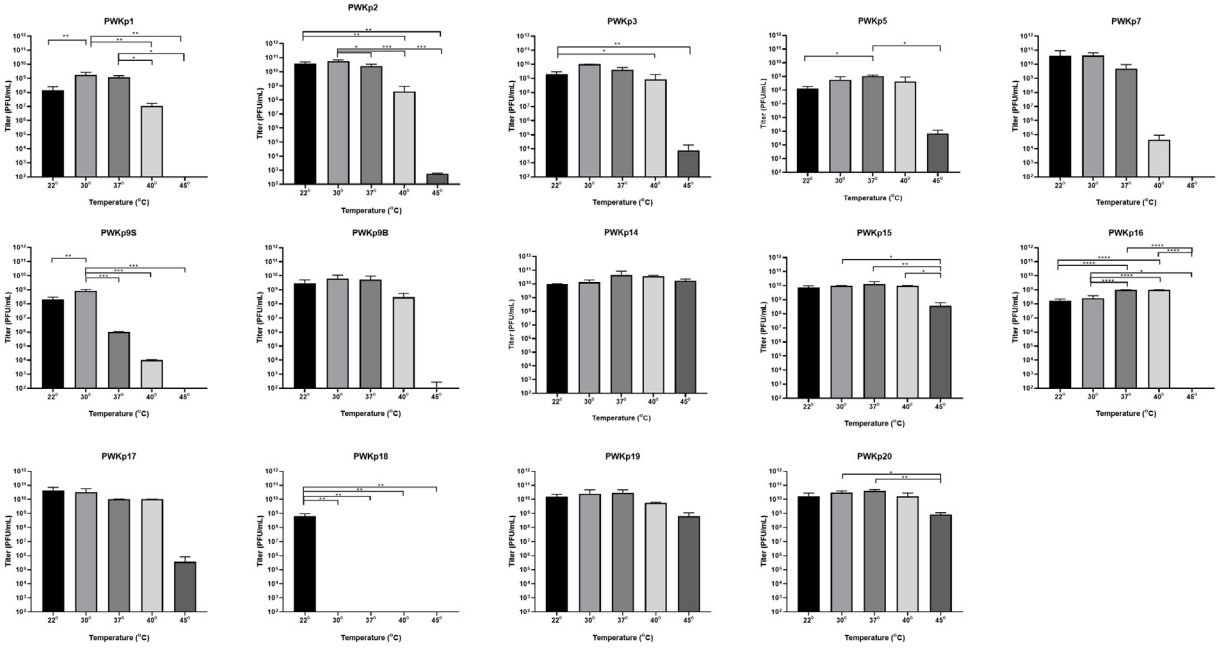

Fig. 5. *Klebsiella* phages optimum temperature. Values plotted in this graph are means and error bars represent the SD ($n = 3$). The difference in the bacteriophages titer at different temperatures were tested using non-parametric ANOVA (Tukey's multiple comparisons tests), * indicates $0.01 < p$ value < 0.05, ** indicates $0.001 < p$ value < 0.01, *** indicates $0.0001 < p$ value < 0.001, **** indicates p value < 0.0001.

inefficient production ranging from 0% (PWKp18, 0/40) to 71.4% (PWKp15, 5/7) (Table S2). In general, the viruses PWKp1, PWKp7, PWKp9B, and PWKp18 showed the best EOP results since more than 50% of our bacterial collection had high or medium productive infection against the tested phages. Corroborating our previous observation, Kurskal-Wallis's analysis revealed significant difference among the mean of the 14 EOP groups ($p < 0.0001$), with Dunn's multiple comparative tests showing greatest statistical difference for PWKp2 vs. PWKp1 ($p < 0.0001$), PWKp7 vs. PWKp2 ($p < 0.0001$), PWKp9S vs. PWKp2 ($p < 0.0001$), PWKp9B vs. PWKp2 ($p < 0.0001$). EOP values for Bangladeshi, Italian, and Vietnamese isolates were lower as compared to Brazilian isolates.

For temperature susceptibility we assumed 10% viability as a cut-off to determine a critical influence of temperature in the phage survival (Fig. S9). PWKp9S demonstrated a high susceptibility to temperature changes, with a decrease of more than 90% when tested at temperatures above 50 °C. On the other hand, PWKp5 was the most heat stable phage since only temperatures above 65 °C were able to reduce its viability below 10%. For the remaining viruses (Autographiviruses, Drexlerviruses and Myoviruses),

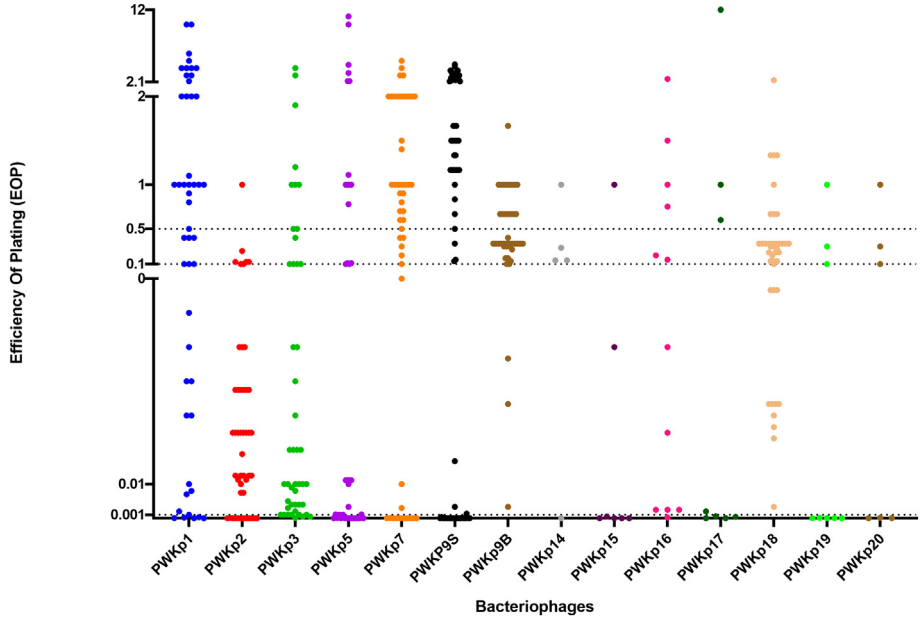

Fig. 6. Distribution of Efficiency of Plating (EOP) values obtained for the fourteen phages against their respective susceptible *K. pneumoniae* ST16 isolates. Each EOP value was estimated by the ratio between the number of plaques obtained in each tested *K. pneumoniae* isolate relative to the original host. For interpretative purposes, EOP ≥ 0.5 indicated high productive infection, $0.1 \leq$ EOP < 0.5 indicated moderate productive infection, $0.001 <$ EOP < 0.1 indicated low productive infection, and EOP ≤ 0.001 inefficient infection.

60 °C and 65 °C were considered critical temperature for their viability (Fig. S9). All Autographiviruses and PWKp20 were stable at the range of tested pH and did not demonstrate any significant variation in their bacteriophage titer. The Myoviruses (PWKp16 and PWKp18) and the Drexlerviruses (PWKp17 and PWKp19) demonstrated the greatest variations against different pHs (Fig. S10). All viruses were stable to chloroform (1:1 vol/vol) after one-hour incubation with no significant difference in the bacteriophage titer (Fig. S11).

4. Discussion

The rapid emergence of antibacterial resistance in key pathogens has meant that new approaches to treat infections are urgently needed. Bacteriophages or phage-related products represent a promising approach and are currently high on the list of options behind direct-acting small molecules and antibodies/vaccines (Theuretzbacher et al., 2020). For this reason, sewage or sewage-contaminated samples are an important source of research for bacteriophage isolation with activity against clinically important bacterial strains, especially MDR lineages (Muniesa et al., 2011; Aghaee et al., 2021). Our work here characterises diverse bacteriophages with lytic activity against a MDR collection of *K. pneumoniae* ST16 capsular type KL51 recovered from sewage samples.

The 14 fully characterised bacteriophages in this study were of diverse sequence and morphology and were members of five different families (Fig. 1). During this study, phages belonging to *Autographiviridae* and *Drexlerviridae* families were the most abundant (10/14) and were geographically related to Brazil and South Asia (Bangladesh and Thailand), respectively. Viruses belonging to *Autographiviridae* had the best activity against our *Klebsiella* collection (Fig. 3), including *K. pneumoniae* ST16 recently isolated in Brazil from several nosocomial infections (Andrey et al., 2020). This corroborates with the principle of biogeography which describes the fact that individual prokaryotes and their specific viruses can be endemic or rare in different locations (Martiny et al., 2006; Thurber, 2009; Hannigan et al., 2018). Phages in turn are naturally able to modulate the host population in an environment by a delicate relationship established between them and their hosts (Clokie et al., 2011). Thus, the increase in the number or efficiency of phages against a certain micro-organism will be observed when that organism is abundant in a particular environment. The abundance of *K. pneumoniae* ST16 phages is related to the increasing reports of human infections caused by this lineage in Brazil and South Asia (Andrey et al., 2020; Raro et al., 2020; Nguyen et al., 2021; Boonyasiri et al., 2021).

Typically, phage with good clinical utility would have a large host range, be stable and able to function at different temperatures (Casey et al., 2018; Fernández et al., 2019). The phages discovered during this study meet these criteria, providing different possibilities of applications. Besides being effective against *K. pneumoniae* ST16, 10/15 phages were also active against *K. pneumoniae* from other STs and capsular types from human and animal sources (Fig. 4; Table S2). For example, the bacteriophages PWKp9S and PWKp15 could also be used against *K. pneumoniae* ST258, an important nosocomial pathogen found worldwide and recently described causing animal infections (Tzouvelekis et al., 2012). Although less frequent we also detected phage activity against *K. pneumoniae* isolates recovered from arthropods which could be an interesting alternative to combat the spread the ARG among animals (Hassan et al., 2021). The reliable activity of many of our bacteriophage at a wide temperature range (20 °C-45 °C) also suggests that they would be applicable for different biocontrol uses such as human systemic and gut decolonisation as well as environmental surface decontamination and use in animals with higher body temperatures such as chickens (D'Accolti et al., 2018; Nobrega et al., 2016). This is pertinent since *K. pneumoniae* ST16 has already been described as causing animal infection and colonising hospital sinks in China (Zhang et al., 2021; Feng et al., 2020).

We also noticed a relationship between the countries where sewage samples were positive for *K. pneumoniae* ST16 phages (Bangladesh, Brazil, Saudi Arabia, and Thailand) and optimum lysis temperatures. Viruses from Asia demonstrated lytic activity at higher temperatures than Brazilian phages (Fig. 5). It is well known that Bangladesh, Saudi Arabia, and Thailand have high temperatures, especially in the summer, which can favor this activity of bacteriophages over a wide range of temperature. PWKp14 a Saudi phage, demonstrated the level of activity up to 45 °C, without any statistical difference among the temperatures evaluated (Fig. 5). The phage PWKp18, recovered from Bangladesh, was the only phage without activity at temperatures below than 30 °C, which could be attributed to the diversity of phages present in the same environment.

Isolation of bacteriophages from sewage samples used in this study using Italian or Asian *Klebsiella* ST16 isolates as hosts was unsuccessful. As demonstrated by our EOP analysis, not every bacterial isolate can be a good host (Fig. 6). Subtle changes in host susceptibility can be due to point mutations or changes in expression of the host's bacteriophage receptor as well as restriction enzyme modification systems and the CRISP-Cas system adaptive immune system (Broniewski et al., 2020). However, even with these limitations one of the key successes of this study was the isolation of a highly effective set of bacteriophages against a non-clonal collection of clinical isolates from different countries.

5. Conclusion

As new reports emerge indicating the spread of MDR *K. pneumoniae* ST16 (Nguyen et al., 2021; Boonyasiri et al., 2021) phage-based approaches are an attractive way to combat these micro-organisms without antibiotics. Thus, the isolation and identification of bacteriophages against MDR lineages are needed. By using sewage samples from different geographic regions, we isolated and characterised 14 lytic *Klebsiella* phages with activity against *K. pneumoniae* ST16. Although numerous studies have demonstrated the ability to detect bacteriophages in oceans, soil, or sewage treatment plants. This study served as a starting point for the development of an efficient phage cocktail with clinical potential against *K. pneumoniae* ST16 (Martins et al., 2022). This study reinforces sewage samples as a rich font for bacteriophages against pathogens of medical importance, although the viruses were only found in places with increasing reports of *K. pneumoniae* ST16 infections.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.156074.

Data availability

Genomic sequences of the fourteen phages have been deposited in GenBank under accession numbers: MZ634338-MZ634351

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CRediT authorship contribution statement

Conceived and designed the experiments: W.M.B.S.M., A.C.G., M.T.
Performed the experiments: W.M.B.S.M., J.C., K.S., E.P., M.L.
Provided sewage samples and bacterial strains: W.M.B.S.M., K.S., B.H., P.P.D., R.M., E.A.M, A.C.G, M.T.
Analysed the data: W.M.B.S.M., M.T.
Secured funds and provided resources: A.C.G., M.T.
Writing of the original manuscript: W.M.B.S.M.
Edition of the manuscript: K.S., E.P., M.T.

All authors contributed extensively to the work presented in this paper. All authors discussed the results and implications and commented on the manuscript at all stages.

Declaration of competing interest

A.C.G. has recently received research funding and/or consultation fees from Cristália, Enthasis Therapeutics, InfectoPharm, Eurofarma, Pfizer, MSD, and Zambon. Other authors have nothing to declare.

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