Isolation and Characterization of Novel Lytic Bacteriophages Infecting Carbapenem-Resistant Pathogenic Diarrheagenic and Uropathogenic Escherichia Coli

Kenneth Ssekatawa1,2, Ibrahim Ntulume2,3, Denis Karuhize Byarugaba2, Slawomir Michniewski4, Eleanor Jameson5, Eddie M Wampande6, Jesca Nakavuma3

1Department of Science Technical and Vocational Education, Makerere University, Kampala, Uganda; 2Africa Center of Excellence in Materials, Product Development and Nanotechnology (MAPRONANO ACE), Makerere University, Kampala, Uganda; 3Department of Biotechnical and Diagnostic Sciences, Makerere University, Kampala, Uganda; 4Centre for Phage Research, University of Leicester, England, UK; 5Department of Veterinary Pharmacy, Clinical and Comparative Medicine, Makerere University, Kampala, Uganda

Correspondence: Kenneth Ssekatawa; Jesca Nakavuma, Email kseskatzz@gmail.com; kenneth.ssekatawa@mak.ac.ug; jesca.nakavuma@gmail.com

Background: The evolution of antimicrobial resistance has dramatically reduced the efficacy of the first-choice and last-resort antibiotics used to treat E. coli infections. Thus, searching for novel therapeutics to treat and control the emergence of antibiotic resistance is urgent. Therefore, this study aimed to illustrate the lytic effect of phages against carbapenem-resistant pathogenic E. coli.

Methods: Phages were isolated from hospital effluents by the enrichment assay. This was followed by the evaluation of the host range of the phages by the spot assay. The time taken by phages to bind to the host bacterial cells was determined by the adsorption assay. The latent period and burst size were determined using a one-step growth experiment. Phage morphology was determined by the Transmission Electron Microscopy. Molecular characterization of phages was done by whole genome sequencing.

Results: Two phages named UGKSECp1 and UGKSECp2 were isolated from hospital effluents. The phages were lytic with a broad host range. The two phages recorded an average adsorption time of 11.25 minutes, an adsorption rate of 99.3%, a latency period of 20 minutes, and a burst size of approximately 528 phages/infected cell. Phages UGKSECp1 and UGKSECp2 had genome lengths of 167433bp, and 167221bp with 277 and 276 predicted genes, respectively, and no undesirable genes were detected. Phylogenetic analysis revealed the two phages belonged genus Tequatrovirus. TEM micrograph showed that the two phages had a similar morphotype with icosahedral heads and contractile tails; thus, classified as members of the Myoviridae phage family.

Conclusion: The findings demonstrate that the study isolated two novel lytically lytic phages with a broad host range and thus, are candidates for phage-mediated biocontrol.

Keywords: antibiotic resistance, carbapenem resistance, bacteriophages

Background
Pathogenic Escherichia coli has been reported as one of the leading causes of hospital and community-acquired infections globally.1 Diarrhoea is one of the primary causes of ailments and mortality among children under five years in low- and middle-income countries and diarrheagenic E. coli (DEC) pathotypes account for the biggest percentage. E. coli-associated infections outside the gut; for example, meningitis, urinary tract infections (UTI), pneumonia, and septicemia are attributed to extra-intestinal pathogenic E. coli (ExPEC).2 Pathogenic E. coli expresses a multitude of virulence determinants and other factors that enable them to colonize the hosts. Notable examples of DEC include; Enteropathogenic E. coli (EPEC), Enteroinvasive E. coli (IEC), Enteraggregative E. coli (EAEC), Enterotoxigenic E. coli (ETEC), and Diffusely Adherent E. coli (DAEC), Shiga toxin-producing E. coli (STEC).3 The most clinically
significant ExPEC are; (a) uropathogenic *E. coli* (UPEC), which is accountable for causing up to 95% and 50% of community and nosocomial UTIs respectively, and (b) neonatal meningitis *E. coli* (NMEC) which causes meningitis and septicemia. Over the last decade, the acquisition of antibiotic resistance mediated by different mechanisms has been reported. Notable mechanisms of antibiotic resistance include; (a) enzymatic deactivation of the antibiotics by bacterial enzymes expressed by resistance gene located on chromosomes or plasmids, (b) alteration and reduced expression of porins that decreases the permeability of the outer membrane to antibiotics, (c) pumping out of the antibiotics by the bacterium efflux pumps, and (d) acquisition of a new drug target, for example, the penicillin-binding protein (PBP) or modification of the target thereby decreasing the affinity of the target component to antibiotics. Thus, the acquisition of resistance has rendered the first-choice antibiotics used to treat pathogenic *E. coli* infections inefficient. Furthermore, the situation has been complicated by the emergence of resistance to the last-resort drugs such as carbapenems used as therapeutic agents against multi-drug resistant (MDR) bacterial infections. The evolution of MDR strains with limited treatment options constitutes a significant threat to the healthcare sector worldwide. Thus, searching for novel approaches to treat and control the emergence of antibiotic resistance is urgent.

With limited efficacious antibiotics, the use of bacteriophage or phage therapy to manage antibiotic-resistant infections has been reignited. Phages are the most copious entities on earth using the bacterial host machinery to replicate. To infect a host bacterial cell, phages bind to specific receptors on the surface of bacteria. Specificity limits phage infections to only bacteria with conforming receptors where they can bind. Thus, phages are safe to use as they have no impact on mammalian cells and normal microflora. However, extensive studies should be conducted to ensure that the phages used in phage therapies are strictly specific to the target pathogenic bacteria without any effect on the beneficial ones to minimize the risk of dysbiosis. Phages are self-replicating provided that the specific host bacterial cells are present and self-terminating when all the host cells are lysed. Furthermore, phage therapy relies on the capacity of phages to infect and use the host bacteria to produce progeny viruses (replication) that are released through the lysis of the host bacteria. Only obligatory lytic phages are suitable to be applied as bacterial growth inhibitors. Furthermore, phages have been used in compassionate phage therapy to treat patients with unresponsive bacterial infections. Therefore, this study aimed to isolate and characterize phages with broad-spectrum lytic activity against carbapenem-resistant pathogenic *E. coli*.

**Materials and Methods**

**Source of Materials**

**Chemicals and Consumables**

Brain heart infusion broth (Code: 53286–500G) and agar (Code: 70138–500G), tryptic soy broth (Code: 22092–500G) and agar (Code: 22091–500G), bacteriological agar (Code: 05039–50G), calcium chloride (CAS No.:10035–04-8), sodium hydroxide (CAS No.:1310–73-2), hydrochloric acid (CAS No.: 7647–01-0), Chloroform (CAS No.: 67–66-3), gelatin (CAS No.: 9000–70-8), Tris-Cl (Code: SIG52486-1M), magnesium sulfate (CAS No.: 7487–88-9), sodium chloride (CAS No.: 7647–14-5), polyethylene glycol (PEG) 8000 (CAS No.: 25,322–68-3), trypsin (CAS No.: 9002–07-7), DNase I (Code: 11284932001), RNase (Code: 70856–3) and 0.22 µm sterile syringe microfilters (Code: WHA68091102) were obtained from Sigma-Aldrich, South Africa.

**Bacterial Strains**

The study used clinical carbapenem-resistant DEC pathotypes EPEC, S-ETEC, STEC, EIEC, and L-ETEC and UPEC harbouring Pathogenicity island (PAI) IV536 and PAI IICFT073 initially characterized by. All the isolates were subcultured in Brain Heart Infusion Broth (BHIB) and stored at −20°C until further use. *Escherichia coli* O157:H7 was used as the host for the phage isolation.

**Phage Isolation and Proliferation**

*E. coli* phages were isolated using adjusted methods outlined by. Briefly, effluent samples were collected from Mulago National Referral Hospital, Uganda. The samples (50 mL) were centrifuged at 5000 rpm for 15 min to obtain the supernatant.
The supernatant was filtered using a sterile syringe of 0.22 μm pore size microfilters (Whatman® Anotop®) to remove bacterial cells and debris. Twenty millilitres (20 mL) of the filtrate was mixed with 20 mL of double-strength Brain Heart Infusion Broth (BHIB) supplemented with 1M calcium chloride. Two hundred microliter (200 µL) of BHIB containing E. coli cultures (OD₆₀₀ 0.3 (Approx. 10⁸ CFU/mL)) was immediately added to the mixture and incubated at 37°C for 24 h while shaking at 180 rpm. The obtained lysate was centrifuged at 10,000 x g for 10 min. The resultant supernatant was filtered through 0.22 μm sterile syringe microfilters to obtain a crude phage lysate. The spot assay was used to confirm the presence of E. coli phages. Briefly, 4.4 mL of molten agar (0.7%) previously held at 55°C was thoroughly mixed by vortexing with 100 µL of BHIB containing overnight bacterial host cells culture before being poured onto Brain Heart Infusion Agar (BHIA) plates. The overlay was allowed to dry aseptically at room temperature, spotted with 10 µL of the crude phage lysate, and then incubated overnight at 30°C. Distinct plaques on the bacterial lawn were scooped out using sterile bacteriological loops, and each plaque was transferred into 20 mL of BHIB containing overnight E. coli cultures, followed by incubation at 30°C for 24 h. After lysis, the bacteria were inactivated by adding chloroform, followed by performing an overlay assay as described above to purify the phages further. To obtain pure stocks of the isolated phages, each phage underwent three phases of the overlay assay. A pure monoclonal phage was proliferated by performing a double overlay assay whereby 100 µL of each pure clone was thoroughly mixed with 4.4 mL of BHIB with supplemented molten agar (0.7% agar) containing the host bacterial cells at OD₆₀₀ 0.3 (Approx. 10⁸ CFU/mL). The mixture was poured onto the BHIA plate, left to set, and then incubated at 37°C for 24 h. The lysate layer of the overlay was scraped off using a sterile spatula and resuspended in 20 mL sodium magnesium (SM) buffer (2% gelatin, 5 mL; 1 M Tris-Cl pH 7.5, 50 mL; MgSO₄.7H₂O, 2 g; NaCl, 5.8 g and topped with distilled deionized water to 1000 mL). The lysate in SM buffer was filtered through a sterile syringe 0.22 μm filter to remove the bacterial debris, and the obtained filtrate was stored at 4° C until further use.

**Estimation of the Plaque-Forming Units**

The population of phages in a solution was represented as plaque-forming units per millilitre (PFU/mL). A ten-fold serial dilution was performed on a 96-well microtiter plate for each phage isolate. Lawns of the host bacteria were prepared by pouring 4.4 mL of BHIB containing 100 µL of overnight E. coli culture supplemented with 0.7% molten agar onto BHIA plates. The PFU/mL were enumerated by the spot assay in triplicates where 10 µL of SM buffer containing phages at a given dilution was spotted on its respective section of the plate marked with the dilution factor and left to set. The plates were incubated overnight at 37°C, and plaques of each phage were counted. Dilution factors with more than ten clear plaques were considered. The PFU/mL was calculated using the formula below;

\[
PFU/ml = \frac{\text{Number of plaques}}{\text{Volume plated (ml)}} \times \text{dilution factor}
\]

**Phage Purification**

Ten millilitres (10 mL) of the SM buffer containing phages (approximately 10¹⁰ PFU/mL) was centrifuged for 20 min at 10,000 × g. The supernatant was filtered through a 0.22 μm filter, then treated with DNase (1 μg/µL) and RNase (1 μg/µL) and incubated for 1 h at 37 °C to eliminate the bacterial genetic materials. Sodium chloride (1M) and 10% polyethylene glycol (PEG) 8000 were added to the phage suspension and stored at 4 °C overnight. The suspension was centrifuged at 13,000 × g for 30 min at 4 °C to precipitate the phages. Sodium magnesium buffer (2 mL) was used to resuspend the pellet. The concentrated phage suspension was loaded on a glycerol step gradient containing 40% SM buffer and 5% glycerol and subjected to 2 h ultracentrifugation at 15,000 × g and at 4 °C (Beckman ultracentrifuge, SW28 rotor). The phage precipitate was resuspended in SM buffer and kept at 4 °C until further use.

**Determination of the Host Range**

The host range of the isolated phages was examined by the spot assay against 24 bacterial isolates. Two of each carbapenem-resistant pathogenic E. coli, namely; EPEC, S-ETEC, STEC, EIEC, L-ETEC, and UPEC, harbouring PAI IV536 and PAI IICFT073 were used. Furthermore, four uncharacterized carbapenem-resistant Klebsiella pneumoniae, E. coli ATCC 25922, E. coli O157:H7, and Klebsiella pneumoniae ATCC BAA were also used. Briefly, BHIA plates
were divided into four equal zones, each corresponding to the isolated phages using a marker, Figure 1. The marked plates were used to prepare double agar overlay cultures for each isolate. This was followed by pipetting 10 µL of SM containing the phage isolate into the centre of its corresponding zone and incubated at 30°C overnight. To obtain pure phage clones this step was repeated three times. *E. coli* pathotype plates with clear plaques were considered to be susceptible to phage-mediated lysis. Thus, using the level of clarity on the bacterial lawn, the spots were classified as clear (A), turbid (B), or no reaction (O).

**Effect of Temperature, pH, and Trypsin Enzymes on Phage Stability**

The impact of temperature, pH, and gastrointestinal tract (GIT) protein hydrolyzing enzymes on phage stability was examined by adjusting procedures previously described by. To determine the sensitivity of phages to temperature, the phages were incubated at −20, 4, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70°C in a temperature-controlled water bath for 1 h. For the GIT enzyme sensitivity studies, the phages were treated with 143 µg/mL, 500 µg/mL, and 800 µg/mL of trypsin at pH 9. Briefly, 1 mL of Tryptic Soy Broth (TSB) containing 10⁸ PFU/mL of each phage lysate was pipetted into its corresponding Eppendorf tube and incubated at the corresponding temperature for 1 h. Samples of 100 µL were taken off every 10 min in triplicates and mixed with 4.4 mL of TSB containing 10⁸ CFU/mL of the host bacterial cells. The phage viability and concentration were evaluated using the soft agar overlay followed by the plaque assay. The same procedure was repeated for trypsin enzyme stability tests, but the tubes were incubated at 37°C, and aliquots of 100 µL were removed in triplicates at 0, 0.5, 1, 2, 3, 4, 8, 12, and 24 h for phage titer evaluation. For pH stability studies, 1 mL of SM buffer containing 10⁸ PFU/mL of each phage lysate was exposed to different pH conditions ranging from 1 to 14 and incubated at 37°C for 1 h. The phage activity at each pH condition was evaluated in triplicates using the spot assay. For all the stability studies, phage viability and activity were expressed as the ratio of the obtained final phage titer value to initial phage counts.

**Phage Adsorption Period, Latent Period, and Burst Size**

The phage adsorption assay was conducted to find out the time taken by the phages to adsorb to the host bacterial surface. The adsorption assay of the two phages with a broad host range was performed as previously described by Manohar et al but with some adjustments. Briefly, 2 mL of tryptic soy broth (TSB) containing an overnight culture of the host *E. coli* O157:H7 was added to 18 mL of TSB and incubated at 37 °C until OD₆₀₀ 0.3 (Approx. 10⁸ CFU/mL) was attained. The host culture was divided into two using 15 mL falcon tubes to cater for the two phages, and 100 µL of each phage-stock solution (10⁸ PFU/mL) was added to the respective bacterial culture to realise a 0.1 multiplicity of infection (MOI) and incubated at 37°C for 45 min. Aliquots of 100 µL were removed at an interval of 2.5 min in duplicates through the entire duration and diluted in 4.4 mL TSB, and 0.5 mL of chloroform was added to inactivate any bacteria. The samples were
centrifuged at 12,000 X g to sediment the bacteria. The supernatant containing the un-adsorbed phages was filtered through 0.22 µm filters and plated and enumerated using a double agar overlay assay. The percentage of the non-adsorbed phages at each time interval was calculated as the ratio of PFU/mL in the supernatant to the initial PFU/mL. The adsorption curve was plotted using the percentage of the non-adsorbed phage particles. The phage latent period and burst size were determined using a one-step growth experiment as previously outlined by Manohar et al. with minor modifications. Briefly, the bacteria-phage suspension at a MOI of 0.1 was incubated at 37°C to allow the phages to adsorb to the bacterial cells based on the adsorption time determined in the adsorption assay. This was followed by centrifugation of the suspension at 12,000 X g for 5 min, and the pellet containing the infected bacterial cells was immediately transferred into 10 mL of prewarmed (37°C) TSB followed by incubation at 37°C for 120 min. Samples of 100 µL were taken after every 5 min in duplicates. A ten-fold serial dilution was made, and a double overlay assay was executed for each sample to estimate the phage titers. The obtained data was used to plot the one-step growth curve and the latent period was estimated as the time between the adsorbed phage until the release of phage particles. The burst size of the phage was calculated as a ratio of the final free phage particle titers to the initial phage population.

**Transmission Electron Microscopy Analysis**

Transmission electron microscopy (TEM) analysis of the filtered phage lysates was performed by Dr Saskia Bakker from the Advanced Bioimaging Laboratory at the University of Warwick. Briefly, 5 µL of phage stock was applied to a glow-discharged (1 min under vacuum) formvar/carbon-coated copper grids (Agar Scientific Ltd, United Kingdom) and incubated for 90 sec at room temperature. Excess liquid was removed, and a drop of 2% uranyl acetate stain was applied and incubated for 1 min, followed by the removal of excess dye. The staining process was repeated four times, followed by air drying of the grid. Stained phage grids were imaged on a JEOL 2100Plus TEM. Captured images were visualized in ImageJ, and phage sizes were determined as the average of at least ten individual phage particle measurements.

**Phage DNA Isolation and Whole Genome Sequencing**

Phage DNA was extracted using Phage DNA Isolation Kit (Norgen) according to the manufacturer’s protocol. Preparation of short-read libraries and sequencing was carried out by MicrobesNG (Birmingham, UK) using the Illumina platform (2x250bp). Reads were trimmed using Trimmomatic and assembled using SPAdes genome assembler v.2.5.1 software with ‘–only-assembler’ setting. Reconstructed phage genomes were subsequently annotated using Prokka v.1.11 using a custom database of all phage genomes that had previously been extracted from GenBank. Annotated phage genomes were deposited in the European Nucleotide Archive (ENA) as UGKSEcP1 (accession number: OV877085) and UGKSEcP2 (accession number: OV876900).

**Phylogenetic Analysis**

In the first instance, reconstructed phage genomes were analyzed using ViPTree to determine their relationship with the reference phages. This was followed by Blastn comparison of reconstructed phage genomic sequences against the NCBI database of all phages to determine their closest relatives. All known phage genomes belonging to the same viral subfamily were downloaded and re-annotated with Prokka v.1.11 using a previously described database. Phage genomes were searched for either major capsid protein or terminase large subunit with USEARCH v.10.0.240 (Edgar, 2010) using the ‘-evalue 1e-5’ option. Extracted gene nucleotide sequences were aligned using mafft v7.271 followed by phylogenetic analysis performed with IQ-TREE v.1.6.3 using ‘-bb 1000’ option, with models of evolution selected using MODELTEST. Subsequently, analyzed phages along with the phages belonging to the same genus underwent core genome analysis with ROARY using ‘–e –mafft -p 32 –i 90’ settings, followed by the phylogenetic analysis using the concatenated core genes performed with IQ-TEST, as described previously. Average nucleotide identity and phage similarity to determine novel phage genera were performed with VIRIDIC. Resultant phylogenetic trees were visualized using ITOL, Interactive Tree of Life.
Statistical Analysis
Data analysis was done using OriginPro 2021. Comparisons of phage viability and activity under varying pH conditions, temperature, and trypsin enzyme concentrations were performed using One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test. A P value of ≤ 0.05 indicated significant statistical variance.

Ethical Consideration
Ethical Approval No: MHREC1611 was granted by the Research and Ethics Committee for ethical review and approval, at Mulago National Referral Hospital. The Research Ethics Committee of Mulago National Referral Hospital waived the need for informed consent to use already coded archived samples in this study. All the experiments were performed in accordance with the Research and Ethics Committee for ethical review and approval, Mulago National Referral Hospital.

Results
Isolation, Purification, and Amplification of Bacteriophages
Ten (10) lytic *E. coli* phages were isolated using *E. coli* O157:H7 from hospital effluents. The isolated phages were designated as Uganda Kenneth Ssekatawa *E. coli* phages 1 to 10 (UGKSEcP1-UGKSEc10). The phage isolates exhibited plaque morphologies of various sizes ranging from 1 to 2 mm. Phages designated UGKSEcP1 and UGKSEcP2 (20%) showed large plaques; whereas, a large proportion of phages (80%) revealed small plaques on their preferred hosts. All the ten phages revealed clear plaques. After purification and proliferation of phages, the phage titers ranged from $2.2 \times 10^6$ to $6.4 \times 10^{12}$ PFU/mL. UGKSEcP5 had the lowest titer compared to other phages, while phages UGKSEcP1 and UGKSEcP2 had the highest titers.

Host Range Specificity Determination
The host range of the ten *E. coli* phages was evaluated using the spot assay against carbapenem-resistant EPEC, S-ETEC, STEC, EIEC, and L-ETEC DEC pathotypes, UPEC pathotypes harbouring PAI IV536 and PAI IICFT073 (2 isolates each) and four uncharacterised *K. pneumoniae* isolates previously isolated from clinical specimens. In the host range experiments, *E. coli* ATCC 25922, *E. coli* O157:H7, and *Klebsiella pneumoniae* ATCC BAA were also used. The results showed that only two phages, namely UGKSEcP1 and UGKSEcP2, could infect all the *E. coli* pathotypes and *K. pneumoniae* isolates and exhibited clear plaques. However, none of the phages had lytic activity against *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC BAA, Table 1.

Effect of Environmental Conditions Phage Stability and Viability
The effect of pH on the phages was determined by exposing them to varying pH conditions ranging from pH 2 to pH 14 at 37°C for 60 min. The activity of the two phages increased with an increase in the pH up to the neutral. The lowest activity was observed at pH 2 and significantly lower than the highest activity that occurred at pH 7 (P value < 0.05 (P value = 0.0028)). The activity declined considerably (P value < 0.05 (P value = 0.0141)) after pH 10. The thermal stability of phages was assessed by exposing the phages to different temperatures. The viability of two phages was unaffected at low temperatures, and a significant reduction (P value < 0.05 (P value = 0.0034)) was recorded at 50°C. The viability of phages diminished at 55°C. To evaluate the effect of protein-digesting gut enzymes, the two phages were treated with trypsin at a concentration of 143 µg/mL, 500 µg/mL, and 800 µg/mL. The viability of the two phages reduced with increased enzyme concentration for the first four hours and then remained constant. The phage viability of the control was significantly higher than that of phages treated with the enzyme (P value < 0.05 (P value = 0.0002)), Figure 2.

Multiplication Capacity
The replication capacity of UGKSEcP1 and UGKSEcP2 was determined using a one-step growth curve by estimating their adsorption rate, latency period, and burst size, Figure 3. Thus, the least number of free phages was observed after 10 min and 12.5 min for UGKSEcP1 and UGKSEcP2, respectively, but with a similar adsorption rate of 99.3%, a latency period of nearly 20 min, and the burst size of approximately 528 phages/infected cell.
<table>
<thead>
<tr>
<th>Host Bacteria</th>
<th>No</th>
<th>Phage Host range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UGKSEcP1</td>
<td>UGKSEcP2</td>
</tr>
<tr>
<td>EPEC</td>
<td>2</td>
<td>A</td>
</tr>
<tr>
<td>S-ETEC</td>
<td>2</td>
<td>A</td>
</tr>
<tr>
<td>STEC</td>
<td>2</td>
<td>A</td>
</tr>
<tr>
<td>EIEC</td>
<td>2</td>
<td>A</td>
</tr>
<tr>
<td>L-ETEC</td>
<td>2</td>
<td>A</td>
</tr>
<tr>
<td>UPEC-PAI IV36</td>
<td>2</td>
<td>A</td>
</tr>
<tr>
<td>UPEC-PAI IICFT073</td>
<td>2</td>
<td>A</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>4</td>
<td>A</td>
</tr>
<tr>
<td>E. coli ATCC 25922</td>
<td>2</td>
<td>O</td>
</tr>
<tr>
<td>E. coli O157:H7</td>
<td>2</td>
<td>A</td>
</tr>
<tr>
<td>K. pneumoniae ATCC BAA</td>
<td>2</td>
<td>O</td>
</tr>
</tbody>
</table>

**Note:** The level of clarity on the bacterial lawn, the spots classified as clear (A), turbid (B), or no reaction (O).
Genome Analysis and Annotation
Phages’ UGKSEcP1 and UGKSEcP2 reads were assembled into single contigs, 167433bp and 167221bp in length, respectively, with 98.88% average nucleotide identity between them. Their genomes contained 277 and 276 predicted genes, respectively, Supplementary Table 1, Figure 4. UGKSEcP1 genome had 11 tRNAs and 266 coding sequences (CDS), of which 133 were assigned functions and 133 represented hypothetical proteins of unknown functions. Similarly, the UGKSEcP2 genome contained 11 tRNAs and 265 CDS, of which 133 were assigned roles and 132 represented hypothetical proteins. Furthermore, functional annotation revealed that the genomes did not contain bacterial virulence

Figure 2 Effect of temperature, pH, and trypsin enzymes on phage stability. (A) and (B) UGKSEcP1 and UGKSEcP2 viability under different temperature treatment; (C) and (D), UGKSEcP1 and UGKSEcP2 activity under different pH treatment; (E) and (F) UGKSEcP1 and UGKSEcP2 viability after exposure to the GIT concentration range of trypsin.
genes, toxins or toxin-related genes, and genetic determinants of antibiotic resistance. Additionally, no genomic markers such as the integrase gene representing a temperate or lysogenic lifestyle were detected. ViPTree analysis indicated that phages UGKSEcP1 and UGKSEcP2 were related to the reference Myoviridae family phages that infected Gammaproteobacteria based on their total protein content, Figure 5. Blastn analysis showed that both phages were closely related to *Escherichia* phage vB_EcoM_OE5505, *Escherichia* phage vB_EcoM_G29, *Shigella* phage Sf21, and other phages belonging to subfamily Tevenvirinae with >96% Blastn identity. As such, a phylogenetic tree based on the nucleotide sequence of the major capsid protein of phages UGKSEcP1, UGKSEcP2, and all phages belonging to the subfamily Tevenvirinae was constructed, Figure 5. As phages UGKSEcP1 and UGKSEcP2 formed a group with other phages belonging to the genus Tequatrovirus, a subsequent phylogenetic analysis based on UGKSEcP1, UGKSEcP2, and all *Tequatrovirus* phages core genes was performed. The resultant phylogeny showed that phages UGKSEcP1 and

![Figure 3](#)

**Figure 3** Rate of phage adsorption and one-step growth curve. i and ii; Adsorption rate for UGKSEcP1 and UGKSEcP2 respectively, iii and iv; one-step growth curves showing the latency period and burst size of UGKSEcP1 and UGKSEcP2, respectively.
Figure 4 Genomic maps showing some of the predicted genes for the phage isolates. (A) UGKSeCp1 and (B) UGKSeCp2.
UGKSEcP2 formed a separate clade within the genus *Tequatrovirus* and this was confirmed by the results of VIRIDIC analysis, Figures 6 and 7. Phages UGKSEcP1 and UGKSEcP2 had at most 93.5% ANI towards other analyzed phages.

The resultant phylogeny showed that phages UGKSEcP1 and UGKSEcP2 formed a separate clade within the genus *Tequatrovirus* (Figures 6 and 7), sister to that of *Escherichia* phage HY01, which belongs to the already defined by ICTV *Escherichia* virus HY01 species. Therefore, phages UGKSEcP1 and UGKSEcP2 could represent a novel and yet uncharacterised phage species and this was confirmed by the results of VIRIDIC analysis (Supplementary Figure 1). Phages UGKSEcP1 and UGKSEcP2 had at most 93.5% ANI towards other analysed phages (Supplementary Table 2) and, as such, represented a new species within the genus *Tequatrovirus*, as confirmed by VIRIDIC species clustering at 95% ANI cutoff (Supplementary Table 3).

**Morphological Characterization of Phages**

The International Committee on Taxonomy of Virus (ICTV) classification was used to classify phages UGKSEcP1 and UGKSEcP2 based on the three-dimensional structure observed. TEM imaging revealed that the two phage isolates had analogous morphotypes. The representative phages had visible prolate heads (prolate icosahedron) with a mean length and width of $92 \pm 5$ nm, and $66 \pm 2$ nm respectively, and contractile tails of a mean length of $116 \pm 4$ nm, and mean width of $16 \pm 2$ nm, with probable tail fibres, Figure 1E and F.
**Discussion**

E. coli-related infections are caused by pathogenic E. coli classified as DEC and UPEC. To target all the prevalent pathotypes within a geological location, evaluating the phage host range is obligatory when selecting phages intended for phage therapy. Ten phages were used to establish the phage host range. Based on the lytic profiles, plaque size and clarity, the spot assay revealed that only UGKSeCp1 and UGKSeCp2 had the potential to infect all the E. coli pathotypes with extensive clear plaques. The ability of these phages to infect different E. coli pathotypes and K. pneumoniae isolates indicated that they were polyvalent, infecting different strains across species. Similar findings were reported by Montso et al.\(^3\) Bacterial cell surface structures with known functions can also serve as binding sites for phages.\(^3\) Some virulence factors in pathogenic E. coli are surface structural proteins used as binding receptors. In DEC and UPEC isolates, structural virulence factors such as fimbriae are suitable binding receptors for phages with tail fibres. Hence virulence factors can determine phage specificity and host range. Indeed, the UPEC isolates used in the host range study contained PAI IV536 and PAI IICFT073 which harbour the pap gene that encodes the P. fimbriae virulence. Several studies have reported the expression of fimbriae virulence factors in DEC.\(^3\)–\(^7\) Furthermore, Yazdi et al.\(^3\) reported a substantial correlation between virulence factors encoded by the fimbriae adhesin genes pap, sfA, fimH, and afa and bacterial susceptibility to phages. Probably, the K. pneumoniae isolates also expressed the fimbriae adhesion virulence factors. This preliminary data is suggestive to why the UGKSeCp1 and UGKSeCp2 demonstrated lytic activity against all the E. coli pathotypes and K. pneumoniae isolates but could not infect the ATCC isolates. Therefore, further investigations are needed to confirm its validity, reliability, and generalizability.
Figure 7 Phylogenetic analysis of Tequatrovirus genus phages based on the concatenated nucleotide sequence of 105 core genes. Based on the GTR+F+R5 model of evolution with 1000 bootstrap replicates. Phages UGKSEcP1 and UGKSEcP2 are indicated by the green-coloured background. Filled circles denote bootstrap values >70%; size is proportional to the bootstrap value. Branch lengths indicate the number of substitutions per site.
One of the most important aspects to be considered when preparing phages for phage-mediated biocontrol is to examine the physicochemical factors that affect the stability of phages as they determine the phage efficacy and shelf-life. The stability of the two phages under various stress conditions, such as low and high temperatures, acidic and alkaline pH, and the gut protein-digesting enzymes, was studied. Phage viability under storage is affected by temperature, pH, and other environmental conditions. Inactivation of phages due to the loss of the structural elements, capsid proteins, and genetic materials may result when phages are not maintained or used at their favourable physicochemical factors. The trypsin enzyme catalyzes the digestion of proteins in alkaline pH, and its average concentration ranges between 143 µg/mL and 800 µg/mL in the human duodenum. Thus, trypsin can inactivate phages by breaking down the capsid proteins. The viability of phages within the normal range of trypsin concentration was assessed over 24 hours. A reduction in phage viability with an increase in concentration and time was observed but did not exceed 50%. Survival of phages in such extreme conditions is suggestive that UGKSeCp1 and UGKSeCp2 can probably be used to treat and control DEC pathotypes colonizing the mammalian gastrointestinal tract through oral administration. Several studies have reported the effects of pH on phage activity. Low phage activity in highly acidic pH (low phage titers) may be due to loss of affinity to the receptor due to a reversible change in the conformation of the phage shell. Reduced phage activity after pH 10 may be attributed to the disassembly of the phage outer shell due to the deprotonation of the capsid amino acids. Several researchers have reported that high temperatures inactivate phages by proteins and nucleic acids denaturation. Indeed, the viability of the two phages isolated by this study was not significantly affected by low and moderate temperature ranges (−20 to 45 °C). However, phage viability considerably lessened when the temperature was increased beyond 45°C. Different studies obtained similar results; for example, Montso et al., Yazdi et al., and Manohar et al. observed that the phage viability drastically decreased after 1 h of incubations at temperatures of 55°C and above.

The bacteriophages with a high adsorption rate, short latency period, and bigger burst size are more efficacious in lysing bacteria and, therefore, suitable for phage therapy. Accordingly, the rate of phage adsorption, latent period, and burst size significantly affect the outcome of phage-mediated biocontrol and should be estimated when isolating novel phages. In this study, the two bacteriophages characterized exhibited identical growth profiles postulating similar genotypes. The phages recorded a relatively short adsorption time with a high adsorption rate (99.3%), a latency period of 20 min, and a large burst size of 528 phages/infected cell. A similar adsorption velocity of over 99% was previously reported by Yazdi et al. Additionally, a short latency period of 20 min and a large burst size of up to 522 phages/host bacterial cell were observed by Montso et al. among the Myoviridae family phages. Furthermore, the phages demonstrated a broad range of activity against DEC pathotypes EPEC, S-ETEC, STEC, EIEC, and L-ETEC and UPEC harbouring PAI IV536 and PAI IICFT073. Thus, several attributes of healthy growth required for phages to be considered for phage therapy were exhibited by the two phages.

Based on the TEM analysis, the two phage isolates had similar morphotypes. TEM analysis confirmed that phages were tailed; thus, belonging to the order Caudovirales. The representative phage had a visible prolate head (prolate icosahedron) of length: 92 ± 5 nm, width: 66 ± 2 nm and a contractile tail (length: 116 ± 4 nm, width: 16 ± 2 nm) and, as such, was classified as a member of Myoviridae phage family. The morphological characteristics are comparable to those of the E. coli phages reported by other studies. In the Caudovirales order, specificity is determined by the tail fibres as they contain proteins that assist the phages in recognising and binding their specific receptors on the bacterial cell surface and thus restrict the phage host range.

Phages UGKSeCp1 and UGKSeCp2 had statistically similar genome sizes, with 98.88% average nucleotide identity between them. Furthermore, their genomes contained 277 and 276 predicted genes, respectively; each genome had 11 tRNAs, 266 and 265 CDS for UGKSeCp1 and UGKSeCp2, respectively. The genome similarity suggests that phages UGKSeCp1 and UGKSeCp2 were probably reisolates of the same phage. The genome similarity explains why the UGKSeCp1 and UGKSeCp2 demonstrated similar host range, viability, and activity when exposed to different pH values, temperature and trypsin enzyme, adsorption rate, latency period, burst size, and morphology. Based on their protein content, ViPTree analysis showed that the two phages were related to the reference Myoviridae family phages that infect Gammaproteobacteria and in agreement with the TEM morphology analysis. Blastn analysis showed that both phages were closely related to Escherichia phage vB_EcoM_OE5505, Escherichia phage vB_EcoM_G29, Shigella phage Sf21, and other phages belonging to subfamily Tevenvirinae with >96% Blastn identity. Phylogenetic analysis showed that phages UGKSeCp1 and UGKSeCp2 formed an isolated clade within the genus Tequatrovirus, sister to...
Escherichia phage HY01, which belongs to the already defined by ICTV Escherichia virus HY01 species. Thus, phages UGKSEcP1 and UGKSEcP2 could represent novel yet uncharacterized phage species. This was confirmed by the results of VIRIDIC analysis, Figure 4. Phages UGKSEcP1 and UGKSEcP2 had at most 93.5% ANI towards other analyzed phages. As such, represented a new species within the genus Tequatrovirus, as confirmed by VIRIDIC species clustering at 95% ANI cutoff.

The ability of phages to infect, hijack, and utilize the bacterial host cells metabolism to replicate their progeny through lysis of the host cells and release of multiple virions that spread to infect other bacterial cells is the basis of phage-mediated biocontrol and phage therapy. However, following infection of the host cells, lysogenic phages integrate their genetic material into the host genome and thus, exist as prophages within the host cells and all their offspring permanently. Lysogenic phages might harbour undesirable genes such as virulence factors, toxin encoding genes, and antimicrobial resistance genes obtained from other bacterial hosts and thus, can transform the host avirulent and drug-sensitive host bacteria and all their progeny into virulent and antibiotic-resistant strains. In light of the above, it’s a prerequisite to conduct whole genome sequencing and analysis so that only professionally lytic phages are isolated to prevent the horizontal gene transfer of undesirable genes during phage therapy. Indeed, in this study, bioinformatics analysis revealed that UGKSEcP1 and UGKSEcP2 genomes did not harbour any undesirable genes, consequently suitable for phage-mediated biocontrol.

Conclusion
This study successfully isolated two novel lytic bacteriophages UGKSEcP1 and UGKSEcP2 infecting several pathogenic E. coli clinical strains. The phages exhibited a broad-spectrum lytic activity since they were capable of infecting several strains of carbapenem-resistant E. coli such as EPEC, S-ETEC, STEC, EIEC, and L-ETEC DEC pathotypes, and UPEC pathotypes harbouring PAI IV536 and PAI IICFT073. Given the strong broad-spectrum lytic activity, stability at different temperatures, pH conditions, and GIT enzyme concentrations, and the absence of undesirable genes in the genome, the two phages isolated by this study are potential candidates for in vivo treatment of pathogenic E. coli infections.

Abbreviations
DEC, Diarrheagenic E. coli; UTI, Urinary tract infections; ExPEC, Extra-intestinal pathogenic E. coli; EPEC, Enteropathogenic E. coli; EIEC, Enteroinvasive E. coli; EAEC, Enteraggregative E. coli; ETEC, Enterotoxigenic E. coli; DAEC, Diffusely Adherent E. coli; STEC, Shiga toxin-producing E. coli; UPEC, Uropathogenic E. coli; NMEC, Neonatal meningitis E. coli; PBP, penicillin-binding protein; MDR, Multi-drug resistant; PAI, Pathogenicity island; BHIB, Brain Heart Infusion Broth; BHIA, Brain Heart Infusion Agar; PFU, Plaque-forming units; SM, Sodium magnesium; PEG, Polyethylene glycol; TSB, Tryptic soy broth; MOI, Multiplicity of infection; TEM, Transmission electron microscopy; UGKSEcP, Uganda Kenneth Ssekatawa E. coli phages; CDS, Coding sequences.

Data Sharing Statement
Annotated phage genomes were deposited in the European Nucleotide Archive (ENA) as UGKSEcP1 (accession number: OV877085) and UGKSEcP2 (accession number: OV876900).

Ethics and Consent to Participate
Ethical Approval No: MHREC1611 was granted by the Research and Ethics Committee for ethical review and approval, Mulago National Referral Hospital. The Research Ethics Committee of Mulago National Referral Hospital waived the need for informed consent to use already coded archived samples in this study.

Acknowledgments
We are thankful to the Microscopy Unit, School of Life Sciences, University of Warwick for imaging of phages by TEM.
Author Contributions
All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding
The authors declare that this research project was funded by the Africa Centre of Excellence in Materials, Product Development & Nanotechnology; MAPRONANO ACE, Grant No. P151847IDA credit 5797-UG, College of Engineering Design Art and Technology, Makerere University.

Disclosure
The authors declare that they have no competing interests.

References