Isolation, characterization, and genomic analysis of vB_Pae_HMKU_23, a lytic bacteriophage against *Pseudomonas aeruginosa* from clinical bovine mastitis

Aslantaş, Ö.,[1,a(*)], Bayirli Nacaroğlu, M.,[2,b], Karaynir, A.[3,c] and Süheyla Türkyilmaz, S.[4,d]

ORCIDs: [a]0000-0003-0407-8633; [b]0000-0001-6120-0071; [c]0000-0003-0511-0869; [d]0000-0002-1363-4534

[1] Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Microbiology, TR-31060 Hatay-Türkiye

[2] Hatay Mustafa Kemal University, Institute of Health Sciences, TR-31060 Hatay-Türkiye

[3] Aydın Adnan Menderes University, Recombinant DNA and Recombinant Protein Center (Redprom), TR-09010 Aydın-Türkiye

[4] Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Microbiology, TR-09010 Aydın-Türkiye

* **Corresponding author:** Prof. Özkan Aslantaş, Phone: +90 3262458545/115 23; Mobile phone: +90 533 641 22 36; Fax: +90 326 245 57 04; E-mail: ozkanaslantas@yahoo.com, aslantas@mku.edu.tr

ABSTRACT

Pseudomonas aeruginosa is a nosocomial pathogen that can cause severe infections in humans and animals. The emergence of multidrug resistance (MDR) among different species of bacteria make phage therapy an attractive alternative for managing infections. In this study, a novel phage, Pseudomonas phage vB_Pae_HMKU_23, was isolated from urban sewage obtained from a wastewater treatment facility. Phylogenetic analyses revealed that HMKU_23 was a member of *Schitoviridae* family and Litunavirus genus. The HMKU_23 is a virulent phage as no genes encoding lysogeny, toxins or antibiotic resistance were identified. The therapeutic potential of phage HMKU_23 was investigated and revealed that approximately 66.7% (20/30) of *P. aeruginosa* strains, isolated from clinical bovine mastitis cases, were lysed by HMKU_23. In contrast, HMKU_23 did not lyse other Gram negative and Gram positive bacteria suggesting its specificity of infection. Collectively, the genetic and biological characteristics of HMKU_23 make it a potential candidate for phage therapy against *P. aeruginosa* infections.

Keywords: Bovine mastitis; Gene annotation; Phage; *Pseudomonas aeruginosa;* Whole-genome sequencing

INTRODUCTION

Mastitis is one of the important diseases affecting dairy farming that causes significant economic losses for farmers due to a decrease in milk yield and quality, veterinary expenses and increased labor costs ands early culling due to chronic mastitis (1). *Staphylococcus* spp. and *Streptococcus* spp. are the most prevailing Gram positive agents, generally associated with various form of bovine mastitis cases (2). However, an increase in the frequency of infections caused by Enterobacterales (e.g. *Escherichia coli*) and *Pseudomonas aeruginosa* has been reported (2-4).

P. aeruginosa is a Gram-negative opportunistic pathogen that is widely found in nature, on the skin and intestines of humans and animals, causing acute and chronic infections (5). Clinical mastitis, whether appearing sporadically or as outbreaks, is one of the most encountered acute caused by *P. aeruginosa* in dairy cows (6). The overuse and misuse of antibiotics for the purposes of the prevention and treatment of mastitis cases has led to the emergence and spread of multiple drug-resistant (MDR) strains among mastitis pathogens including *P. aeruginosa* (7). This has made treatment of mastitis cases very challenging, and

led to the search of new alternative therapies for prophylaxis and control of bacterial infections (8). Among the alternative therapy methods, the most promising are bacteriophages (phage) and phage-derived products (e.g. endolysins, depolymerases) (9). These can be used specifically to treat multidrug-resistant (MDR) bacteria related infections, either alone or supplemented with antibiotics (10). Except for a few minor side effects reported related to endotoxins released from bacteria following therapeutic phage therapy *in vivo*, phages have several potential advantages over antibiotics such as specificity, self-limiting capability, auto-dosing, easy to isolate, low rate of resistance development, and making 'phage cocktail' to increase the spectrum phages' activity (11).

Currently, despite phages specific to *P. aeruginosa* having been isolated worldwide, there has been little focus on phages isolated from Türkiye. Furthermore, although many isolated phages share 83-97% nucleotide identity, they exhibit large variations in several phenotypic properties, such as their host range or efficient host lysis (12). Phages isolated from one country/region may not effectively kill bacteria from other countries/regions. Therefore, there has been a tendency to focus on locally isolated phages. In addition, it is important to isolate new phages to accumulate information for the development of effective phage cocktail therapy.

In this study, a novel Pseudomonas phage, designated vB_Pae_HMKU_23 (referred to herein as HMKU_23), was isolated from a wastewater treatment plant, and subsequently tested and characterized against *P. aeruginosa* strains from clinical cases of bovine mastitis.

MATERIALS AND METHODS

Ethical Statement

Since the isolates used in the study were from author's previous studies, the study doesn't require ethical approval.

Bacterial strains and culture conditions

In the current study, a panel of 30 *P. aeruginosa* isolated from clinical bovine mastitis was included in the study to determine the lytic capacity of bacteriophages. The non-*P. aeruginosa* bacteria was chosen as negative controls to show the specific lytic activity of bacteriophages. The complete list of bacteria used in this study is provided (Table 1). Bacterial strains were routinely cultured at 37°C in TSB (Tryptic Soy Broth) (Germany, Darmstadt) or on TSA (Tryptic Soy Agar) (Germany, Darmstadt) media. The soft agar media used for phage enrichments, purification and enumeration was prepared from TSB, supplemented with agar (0.5% agar) and calcium chloride (5 mM).

Phage isolation and purification

A sewage sample taken from Hatay Metropolitan Municipality Wastewater Treatment Plant (WWTP) was used as a bacteriophage isolation source. The isolation and enrichment of phages were performed following the method described by Karaynir *et al.* (13). Initially, 10 ml of sewage sample was centrifuged at 5000 × g for 10 minutes at 4°C, and the supernatant was filtered through 0.22 µm syringe filters and transferred to a new sterile tube. To screen the filtrate for phages, 10 µl of the filtered supernatant was spotted onto the indicator *P. aeruginosa* ST3 strain on the top agar and then incubated at 37°C overnight and examined to see whether the lytic zone formed. In addition, for phage enrichment, 100 µl of the filtered phage source and 100 µl of overnight-grown indicator bacteria were mixed and inoculated into 10 ml of TSB, then incubated overnight at 37°C. Following the incubation, the enriched liquid medium was centrifuged for 10 min at 5000 x g at 4°C, and passed through a 0.22 µm filter. The phage enrichment process was repeated as described above.

Purification of isolated phages

The isolated phages were purified using the double layer agar method (single plaque isolation) [13]. Saline Magnesium (SM) buffer [(100 mM NaCl, 8 mM $MgSO_4.7H_2O$, 50 mM, Tris-HCl (pH 7.5) and 0.01% w/v gelatin)] was used to make serial dilutions of the phage lysate. 100 µl of each dilution and 100 µl of indicator bacteria was mixed in 4 mL of soft agar (TSA; 0.5% agar), vortexed, and slowly poured onto TSA petri dishes supplemented with $CaCl_2$. Following solidification of the media, the media was incubated overnight at 37°C. Then, phage plaques were selected from the media with a sterile pipette tip based on their morphology and size, and the selected phage plaques and 100 µl of overnight indicator bacteria were added to sterile tubes containing fresh TSB, and the culture was incubated overnight at 37°C. Then, the culture was centrifuged (+4°C, 5000 × g, 10 min) and the

supernatant was filtered through 0.22 µm pore size syringe filters. This process was repeated at least four times for final validation for purified phages.

Later, bacteriophage titration was performed as previously described by Yang *et al*. (14) according to the double-layer agar method. Briefly, 100 µl of phage and 100 µl of host bacteria were mixed in 4 ml of soft agar and poured onto petri dishes containing TSA. After overnight incubation of the plates at 37˚C, the plaque forming unit (pfu/mL) of the phage was determined.

Lytic effect of bacteriophages on clinical *P. aeruginosa* isolates

Lytic spectra of phages was determined by the spot test method using 30 clinical *P. aeruginosa* isolates (15). Briefly, 100 µl of each bacterial culture was mixed in 4 ml of soft agar and poured onto TSA petri dishes. Once the medium has solidified, 10 µl of phage lysate (10^{10} pfu/mL) was dropped onto the indicated *P. aeruginosa* isolates. The medium was incubated overnight at 37˚C and evaluated for the presence or absence of lytic zones.

Determination of the host spectrum of *P. aeruginosa* phages

Host range analysis was performed to determine the lytic spectrum of the phage HMKU_23. For this purpose, 9 bacterial strains consisting of reference and clinical bacterial isolates were used. Each overnight bacterial culture (100 µl) was mixed with 4 ml of soft agar and poured onto separate petri dishes containing TSA. The susceptibility of the bacterial strain and the lytic activity of the phage were determined using the spot test method (13).

DNA extraction from bacteriophages and restriction fragment length polymorphism (RFLP) analysis

The isolation of phage DNA was performed as previously described by Ali *et al.* (16) with some modifications. Briefly, the suspension of purified phage (10^{10} pfu/ml) was centrifuged (+4˚C, 120 min, 15000 ×g). SM buffer [(100 mM NaCl, 8 mM $MgSO_4.7H_2O$, 50 mM Tris-HCl [pH: 7.5] and 0.01% w/v gelatine)] and DNase at a final concentration of 1 µg/ml were added to the bacteriophage pellet obtained as a result of centrifugation, and incubated at 37˚C for 45 minutes. For thermal inactivation of DNase, the mixture was incubated at 70˚C for 15 minutes. Proteinase K was added to the DNase-treated bacteriophage suspension with a final concentration of 20 µg/ml and incubated at 56˚C for 45 minutes to degrade the viral capsid proteins and extract the viral DNA. To purify the DNA, phenol:chloroform:isoamyl alcohol (25:24:1) extraction followed by ethanol precipitation method was applied. For RFLP analysis, phage DNA was digested with *EcoRI* and *HindIII* restriction endonuclease enzymes, and then the DNA was run on a 1% agarose gel at 100 V for 30 minutes. Size of DNA fragments was predicted using the Lambda-*PstI* marker.

Sequence analysis and annotation of phage genomes

The paired-end library of the phage genome was prepared from the isolated phage DNAs using the Nextera DNA Prep Library Prep Kit (Illumina, San Diego, CA) and sequenced at 2×150 on the Novaseq600 (Illumina Inc., San Diego, California, USA) platform. The quality of the raw data was evaluated using FastQC v0.11.9 (Babraham Bioinformatics, UK) and low-quality bases, adapters and primers were removed using the Trimmomatic v0.36 program (17). Reads were assembled using MEGAHIT v1.2.9 (18), and possible coding sequences were predicted using RASTtk v1.073 annotation pipeline (19), followed by manual verification using HMMER (https://www.ebi.ac.uk/Tools/hmmer/) (20), and online BLASTp search against the NCBI Non-redundant Protein Database (21). The presence of tRNA was analyzed using tRNAscan-SE (22). Additionally, the presence of the 16S rRNA gene was investigated using the BLASTn database against 16S ribosomal RNA sequences (bacteria and archaea) (23). The Phage AI (https://phage.ai/) was employed to predict the lifestyle of phage as either lytic or temperate (24). The presence of possible antibiotic resistance, pathogenicity and virulence genes on the phage genome was investigated using VirulenceFinder 2.0 and ResFinder 4.1 (25,26). The phylogenetic tree was constructed based on the amino acid sequences of the large terminase subunit of phages. The neighbor-joining method (using the Poisson model consisting of 1000 bootstrap replicates) was used to create phylogenetic tree using a MEGA version 11.0 (27). Additionally, VipTree 3.3 was used to build the viral proteomic tree (28).

RESULTS

Isolation of bacteriophage DNA and determination of DNA restriction profiles

Initially, two phages were purified according to their plaque morphology, and their genomic DNA was extracted (Fig. 1). RFLP determination was performed on phage DNA stocks using *HindIII* and *EcoRI* restriction enzymes. The two phage RFLP patterns showed multiple restriction sites by *HindIII* and *EcoRI*, and the restriction digestion profiles were indistinguishable (Fig. 2). Based on the RFLP analysis, the two phages were considered to be the same phage. Following the plaque isolation and phage propagation, the titer value of the phage were calculated and found to be 2.33×10^{19}.

Host range analysis of Pseudomonas phage vB_Pae_HMKU_23

The lytic activity of the isolated HMKU_23 phage on homologous species was examined on 30 clinical *P. aeruginosa* isolates and one reference strain (*P. aeruginosa* PAO1) (Table 1). It was found that HMKU_23 phage was effective against 20 (66.7%) of the tested 30 isolates. To test for the host spectra of the phage on heterologous strains belonging different bacterial species, 9 clinical isolates and 4 reference strain other than *P. aeruginosa* were used. Phage HMKU_23 did not lyse any of the heterologous bacterial isolates tested (Table 1). Infectivity of the phage on bacterial strains was categorized as 10^0 (-), 10^0-10^4 (+), 10^4-10^8 (++) and $>10^8$ (+++) (Fig. 3).

Genome characterization

Genome analysis of phage HMKU_23

The de novo genome assembly revealed presence of a single contig. The genome size of HMKU_23 was 72480 bp with a total 54.9% G+C content. The genome sequence of the phage was deposited in GenBank under the accession number OR988063.1. Annotation of the phage genome with RASTtk v1.073 and BLASTP revealed the presence of 92 putative open reading frames (ORFs). The majority (71.7%) of encoded proteins (66 ORFs) were hypothetical, and 26 (28.3%) were functional. The presence of numerous hypothetical proteins in the phage genome clearly indicated that the phage carries many genes that are yet to be elucidated, and their func-

Table 1: List of the bacterial isolates used in determining the lytic activity and host spectra of the HMKU_23

List No.	Species	Isolates	Infectivity
1	*Pseudomonas aeruginosa*	ST1	+
2	*Pseudomonas aeruginosa*	ST2	+++
3	*Pseudomonas aeruginosa*	ST3*	+++
4	*Pseudomonas aeruginosa*	ST4	++
5	*Pseudomonas aeruginosa*	ST5	+
6	*Pseudomonas aeruginosa*	ST6	-
7	*Pseudomonas aeruginosa*	ST7	+
8	*Pseudomonas aeruginosa*	ST8	-
9	*Pseudomonas aeruginosa*	ST9	+
10	*Pseudomonas aeruginosa*	ST10	-
11	*Pseudomonas aeruginosa*	ST11	+++
12	*Pseudomonas aeruginosa*	ST12	+
13	*Pseudomonas aeruginosa*	ST13	-
14	*Pseudomonas aeruginosa*	ST14	+
15	*Pseudomonas aeruginosa*	ST15	-
16	*Pseudomonas aeruginosa*	ST16	-
17	*Pseudomonas aeruginosa*	ST17	-
18	*Pseudomonas aeruginosa*	ST18	+
19	*Pseudomonas aeruginosa*	ST19	+
20	*Pseudomonas aeruginosa*	ST20	-
21	*Pseudomonas aeruginosa*	ST21	-
22	*Pseudomonas aeruginosa*	ST22	+
23	*Pseudomonas aeruginosa*	ST23	-
24	*Pseudomonas aeruginosa*	ST24	+++
25	*Pseudomonas aeruginosa*	ST25	+
26	*Pseudomonas aeruginosa*	ST26	+++
27	*Pseudomonas aeruginosa*	ST27	+
28	*Pseudomonas aeruginosa*	ST28	+
29	*Pseudomonas aeruginosa*	ST29	++
30	*Pseudomonas aeruginosa*	ST30	+
31	*Pseudomonas aeruginosa*	PAO1	+
32	*Escherichia coli*	ATCC 25922	-
33	*Staphylococcus aureus*	ATCC 45300	-
34	*Salmonella* Typhimurium	ATCC 14028	-
35	*Staphylococcus epidermidis*	Clinical isolate	-
36	*Klebsiella pneumonia*	Clinical isolate	-
37	*Acinetobacter baumanni*	Clinical isolate	-
38	*Proteus vulgaris*	Clinical isolate	-
39	*Proteus mirabilis*	Clinical isolate	-
40	*Listeria monocytogenes*	Clinical isolate	-
41	*Enterobacter* aerogenes	Clinical isolate	-
42	*Enterococcus faecium*	Clinical isolate	-
43	*Citrobacter freundii*	Clinical isolate	-

* Indicator host

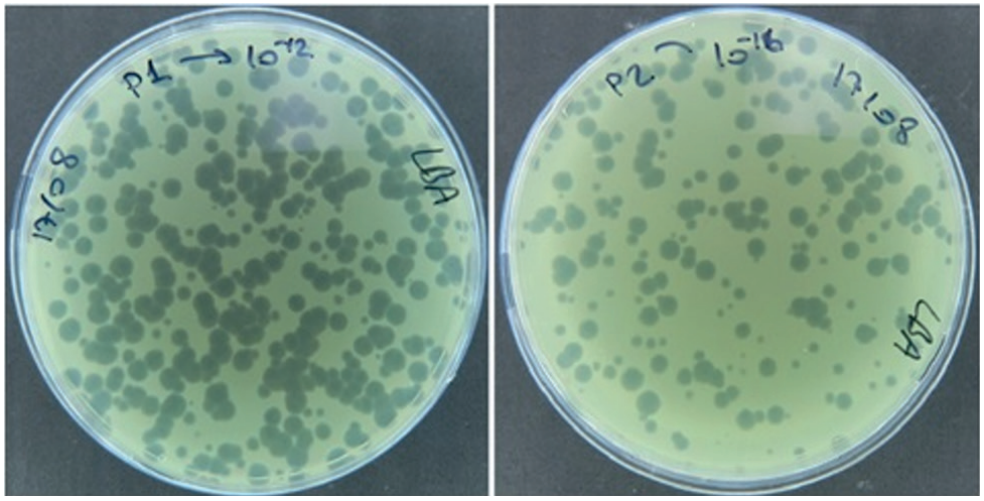

Figure 1: Double-layer agar plates showing homogen plaques after four purifications of plaque and determination of phage titer.

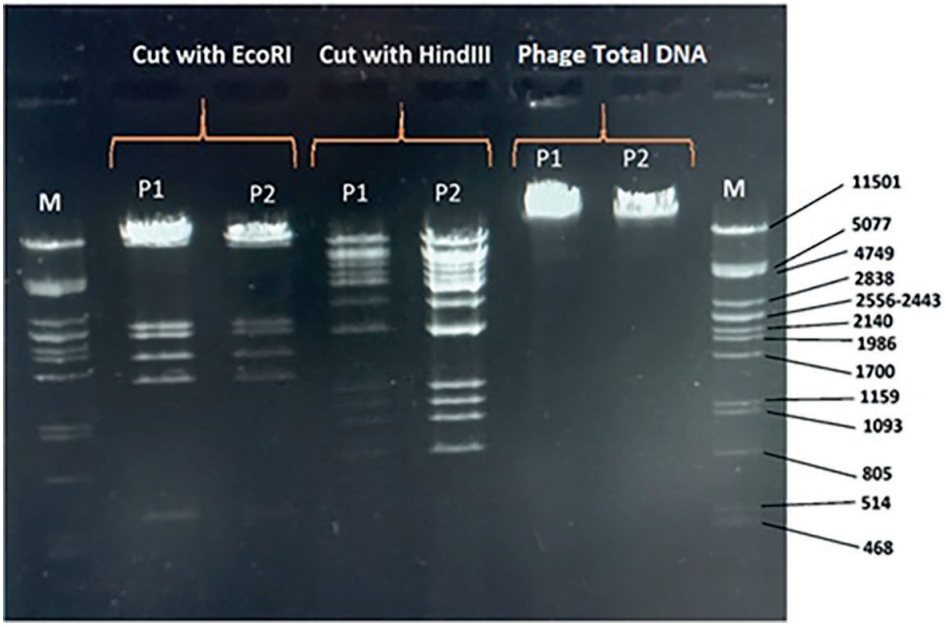

Figure 2: Bacteriophage DNA isolation and restriction profiles. **(A)** Band profiles of phage DNAs after cutting with *EcoRI* and *HindIII* restriction enzymes. **(B)** Genomic DNA of phages. **M**: Lambda-PstI marker, **P1–P2**: Phages purified according to plaque morphology.

tions are yet to be understood. The HMKU_23 phage had the modular genomic architecture as seen in most dsDNA phages (29) and functionally categorized proteins were classified into 7 groups including DNA packaging, RNA replication, DNA replication, structural proteins, lysis inhibitor, nucleic acid metabolism, and lysis were identified (Fig. 4).

The small terminase and large terminase subunit formed the DNA packaging group. The small terminase is essential for initiating packaging, which recognizes viral DNA and brings it to the large terminase for the initial cleavage. The large terminase subunit had an ATPase activity to provide energy for packaging and a nuclease activity for packaging initiation and termination (30). The presence of two different RNA polymerase of phage genome including a large virion-associated RNA polymerase (vRNAP) and DNA-dependent RNA-polymerase suggests that phage HMKU_23 has its own transcriptional machinery, functioning independently of the host RNA polymerase. The RNA polymerase of the virion has been reported to be injected synchronously with

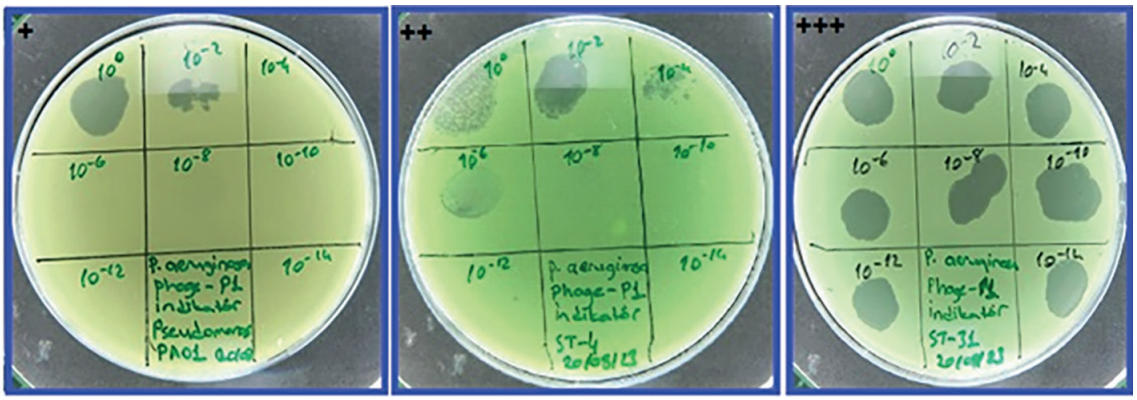

Figure 3: Infectivity of the phage on bacterial strains with spot test

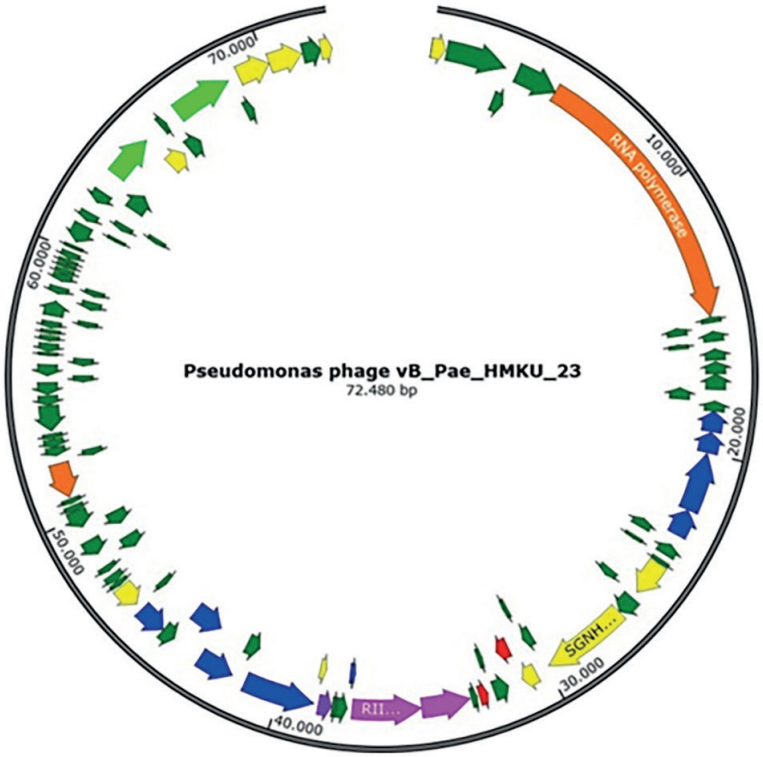

Figure 4: The genome map of phage HMKU_23. The genes encoding proteins were classified into groups and colored according to their functions. These groups are phage DNA packaging (bright green), RNA replication (orange), DNA replication (blue), structural proteins (yellow), lysis inhibitor (pink), nucleic acid metabolism (purple) and lysis (red). Hypothetical proteins are shown in green. The figure was created with Snapgene viewer 5.3.2

the viral DNA upon phage infection and to perform transcription of early, middle, and late genes (31). The presence of phage-encoded DNA polymerase I, DNA helicase, DNA primase, ATPase, Sak4-like ssDNA annealing protein and single strand DNA binding protein indicated that phage HMKU_23 elongates dsDNA independently of the host replication machinery (32).

Nucleic acid metabolism groups contained deoxycytidylate deaminase. Lysis group contained Rz-like spannin and holin. In general, a holin-lysin lysis system exists in double-stranded DNA phages to accomplish host lysis. During the reproduction cycle, phage-encoded holin accumulates and forms pores in the membrane, leading to an increased access for lysins to degrade peptidoglycan in bacterial cell walls (30). In this study, no putative endolysin could be predicted in silico, suggesting a novel type of endolysin or lysis mecha-

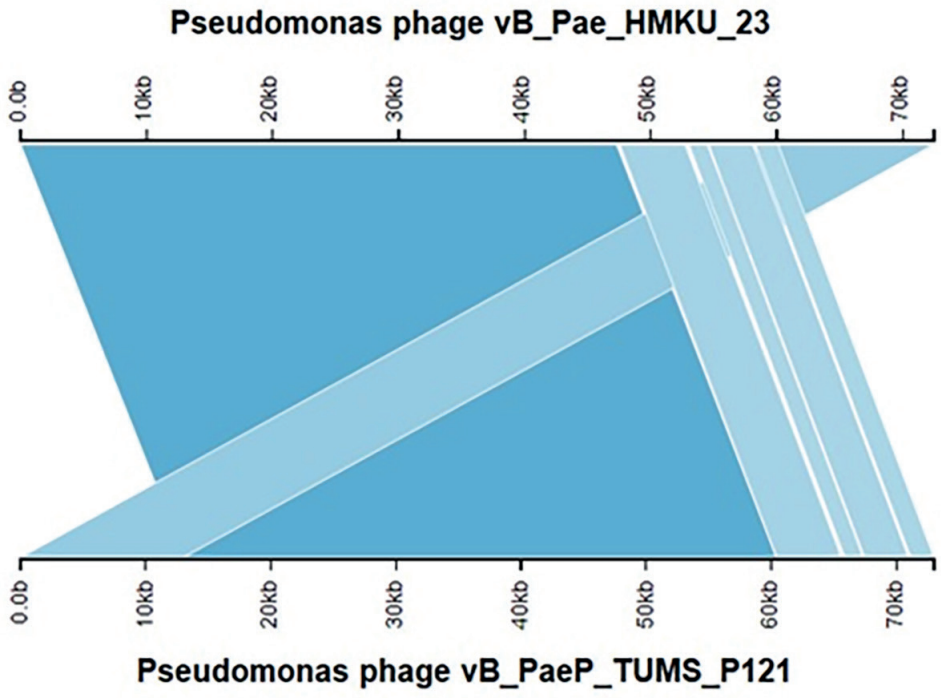

Figure 5: The genome comparison of Pseudomonas phage vB_Pae_HMKU_23 and Pseudomonas phage vB_PaeP_TUMS_P121. The genome coverage and identity are 95.42% and 98%, respectively. The darker blue indicates higher identity.

nism. Therefore, future research is needed targeting candidate lysin genes near possible holins. The phage HMKU_23 also carried RIIA- and RIIB-lysis inhibitor proteins, which play a role in the delay in host lysis resulting in a large increase in the phage burst size, providing HMKU_23 with a competitive advantage over others (34).

Another striking feature is the presence of a gene encoding ferrodoxin (Fd) electron carriers. Fds are thought to redirect the energy harvested from light to phage encoded oxido-reductases that enhance viral fitness (35).

The structural protein group consisted of tail length tape measure protein, tail fiber protein, SGNH hydrolase domain containing tail fiber, major capsid protein and virion structural protein. Remarkably, no genes encoding toxins, antimicrobial resistance-related functions, tRNA or genes encoding for lysogeny-associated proteins like integrase, excisionase, recombinase, and repressor genes were identified supporting the notion that phage HMKU_23 genome is a virulent phage and can be used for therapeutic purposes. The lifestyle of the phage was found virulent with 100% probability with Phage AI tool.

Based on BLASTN analysis, Pseudomonas phage vB_PaeP_TUMS_P121 (coverage: 98%, identity: 95.42%), Pseudomonas phage vB_Pae_TUMS_P11 (coverage: 99%, identity: 95.28%), and Pseudomonas phage vB_PaeS_TUMS_P81 (coverage: 98%, identity: 95.24%) were found to be the most similar phages (Fig. 5). Similarly, VIRIDIC analysis showed that Pseudomonas phage vB_PaeP_TUMS_P121 has the highest similarity (94%) with Pseudomonas phage vB_Pae_HMKU_23 (Fig. 6).

Phylogenetic analysis

Phylogenetic trees were constructed using *Pseudomonas* phages and other phages. Firstly, the amino acid sequences of the large terminase subunit of HMKU_23, and the selected phages were aligned with MUSCLE. In the Fig. 7, the constructed tree is shown. Secondly, Viptree analysis was carried out to construct the proteomic tree based on genome-wide sequence similarities. Circular and rectangular trees were generated and the location of phage HMKU_23 were examined (Fig. 8). The two constructed phylogenetic trees obtained by different methods gave the same results. The analyses indicated that phage HMKU_23 is a member of *Schitoviridae* family and Litunavirus genus.

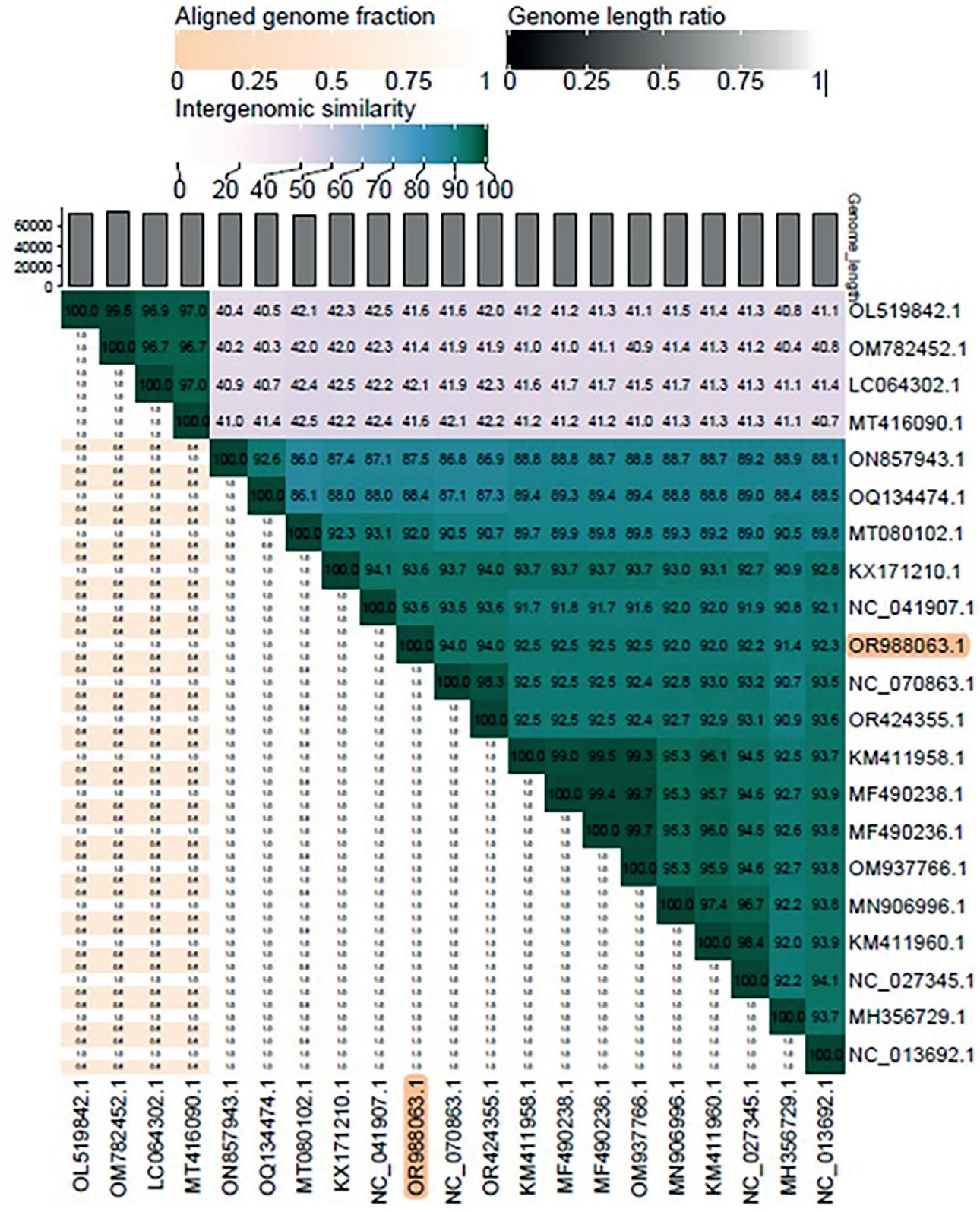

Figure 6: VIRIDIC analysis of Pseudomonas phage vB_Pae_HMKU_23. The right part shows the intergenomic similarity between the 21 genomes, including Pseudomonas phage vB_Pae_HMKU_23 (OR988063.1) colored with orange

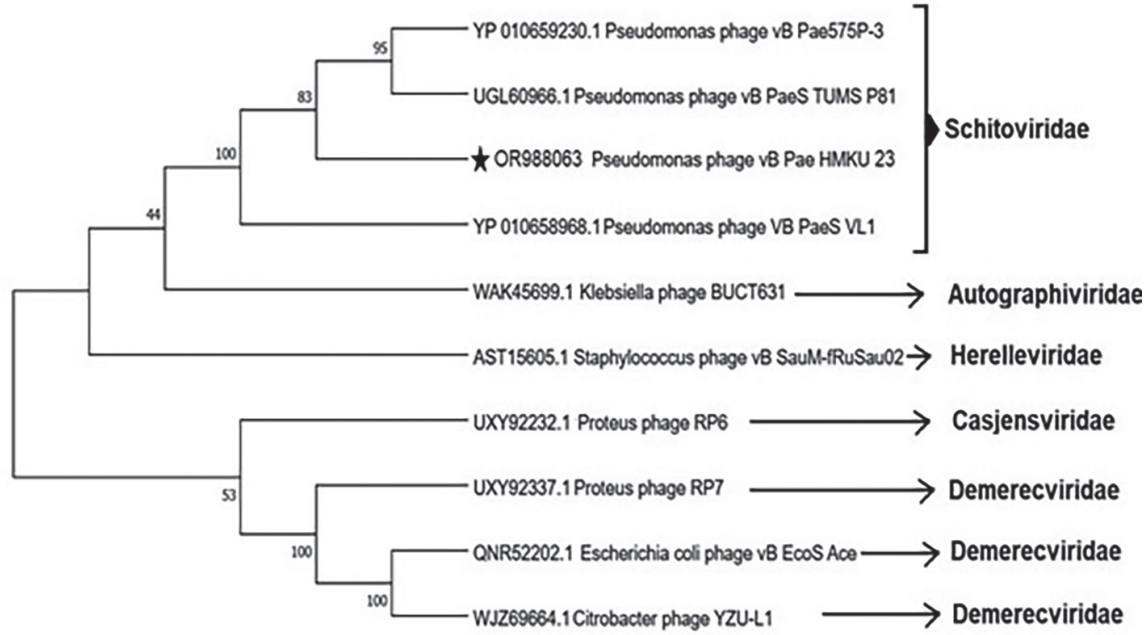

Figure 7: Phylogenetic tree based on the amino acid sequence of the terminase large subunit showing the position of Pseudomonas phage vB_Pae_HMKU_23 (marked with star).

DISCUSSION

P. aeruginosa is a notorious opportunistic pathogen, due to its metabolic versatility, high intrinsic and acquired antibiotic resistance, a large repertoire of virulence factors, and the ability to adapt to a variety of environments (36). The emergence of MDR *P. aeruginosa* is a serious challenge worldwide, leading the need to find new alternatives to control infections caused by these resistant bacteria. The phage therapy has also the advantages over antibiotics such as not affecting the host microbial flora and being host-specific as well. The phage therapy is, therefore, a promising therapeutic alternative to treat infections caused by MDR bacteria (37).

This study provides information about the isolation and characterization of a novel phage, HMKU_23, from wastewater. Based on the predicted gene products, phage HMKU_23 is strictly lytic and free from integrases, virulence factors, toxins, and antimicrobial resistance genes, making it a potentially attractive phage as a therapeutic agent. Hence, genome characteristics indicate the potential of phage HMKU_23 as a candidate for therapeutic value against *P. aeruginosa* infections. There is a need for the isolation and characterization of new phages to be used in the preparation of phage cocktails in order to increase the activity spectrum and depth of action of phages in clinical applications. The majority, 71.7% of the HMKU_23 of genome consisted of hypothetical proteins. The functional annotation of phage genes is a common problem in phage studies. Therefore, careful phage annotation is of great importance for phage therapy (38).

Availability of Data and Materials

The authors declare that data supporting the study findings are also available from the corresponding author on reasonable request.

Funding Support

This study was funded by the Scientific Research Fund of Hatay Mustafa Kemal University with a grant number of 23.GAP.009.

Competing Interests

The authors declared that there is no conflict of interest.

Congress-Symposium Information

The authors declare that the study has not been presented previously in any congress or symposium

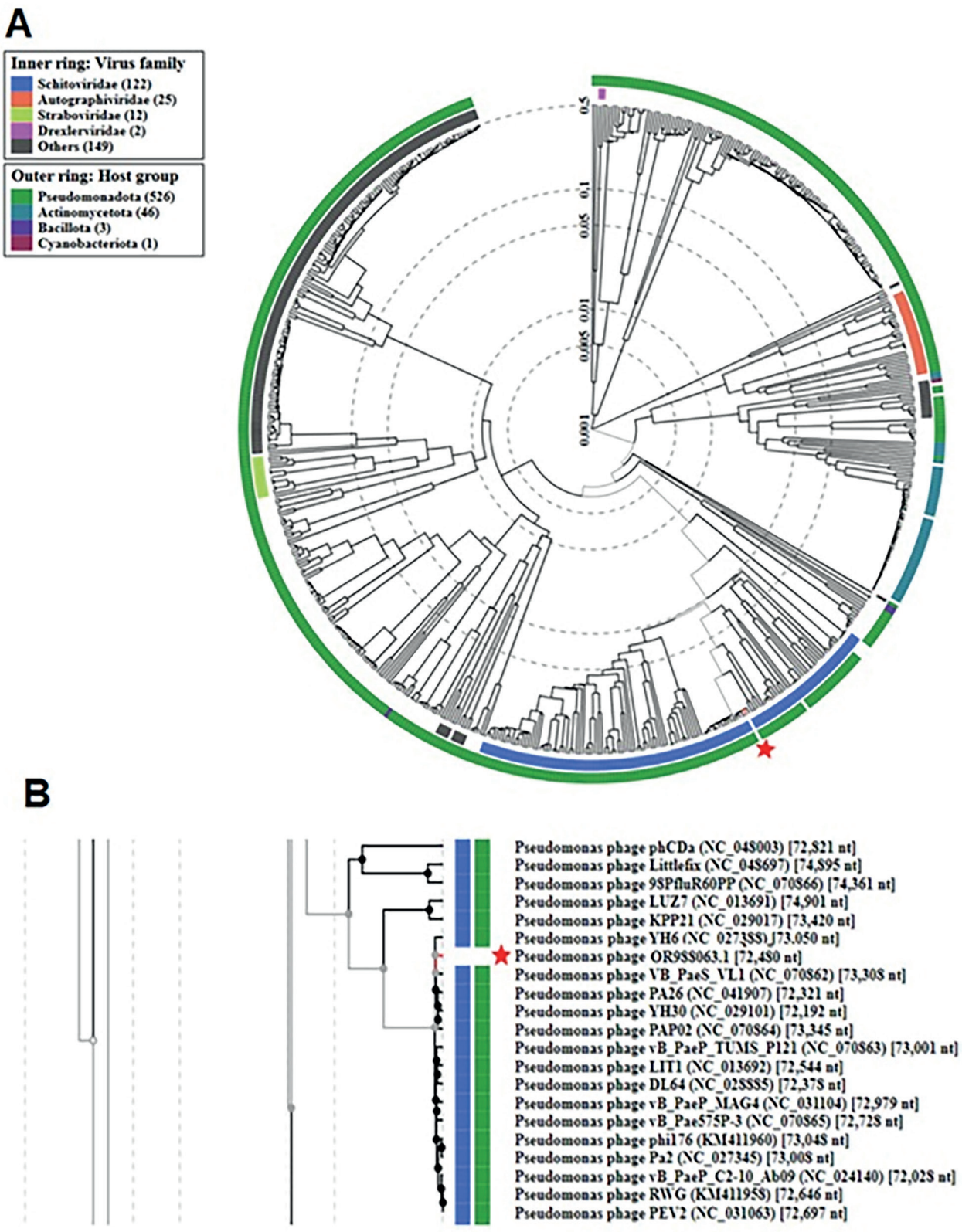

Figure 8: VipTree analysis of phage HMKU_23. (A) Pseudomonas phage vB_Pae_HMKU_23 were represented with star. The outer ring indicates hosts of the phages, and the inner ring indicates the family to which the phages belong. The taxonomic relationship for vB_Pae_HMKU_23 and their relatives showed that HMKU_23 belongs to the family Schitoviridae and genus of Litunavirus

Author Contributions

ÖA planned, designed, and supervised the research procedure, MBN, ST and AK performed all microbiological and molecular experiments, ÖA performed bioinformatic analyses, and ÖA wrote the manuscript. All authors have read and approved the manuscript.

REFERENCES

1. Klaas, I.C. and Zadoks, R.N.: An update on environmental mastitis: challenging perceptions. Transbound. Emerg. Dis. 65(S1):166-185, 2018.
2. Keane, O.M.: Symposium review: Intramammary Infections-Major pathogens and strain-associated complexity. J. Dairy Sci. 102(5):4713-4726, 2019.
3. Kawai, K., Shinozuka, Y., Uchida, I., Hirosa, K., Mitamura, T., Watababe, A., Kuruhara, K., Yuasa, R., Sato, R., Onda, K. and Nagahata, H.: Control of Pseudomonas mastitis on a large dairy farm by using slightly acidic electrolyzed water. Anim. Sci. J. 88(10):1601-1605, 2017.
4. El Garch, F., Youala, M., Simjee, S., Moyaert, H., Klee, R., Truszkowska, B., Rose, M., Hocquet, D., Valot, B., Morrissey, I., de Jong, A. and VetPath Study Group: Antimicrobial susceptibility of nine udder pathogens recovered from bovine clinical mastitis milk in Europe 2015-2016: VetPath results. Vet. Microbiol. 245: 108644, 2017.
5. Wood, S.J., Kuzel, T.M. and Shafikhan, S.H.: *Pseudomonas aeruginosa*: infections, animal modeling, and therapeutics. Cells. 12(1):199, 2023.
6. Aslantaş, Ö., Türkyılmaz, S., Keskin, O., Güllü Yücetepe, A. and Büyükaltay, K.: Molecular characterization of *Pseudomonas aeruginosa* isolated from clinical bovine mastitis cases. Kafkas Univ. Vet. Fak. Derg. 28(6):747-759, 2022.
7. Cheng, W.N. and Han, S.G.: Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments - A review. Asian-Australas J. Anim. Sci. 33(11):1699-1713, 2020.
8. Sharun, K., Dhama K, Tiwari R, Gugjoo MB, Yatoo MI, Patel SK, Pathak M, Karthik K, Khurana, S.K., Singh, R., Puvvala, B., Amarpal Singh, R. and Singh, K.P.: Advances in therapeutic and managemental approaches of bovine mastitis: a comprehensive review. Vet. Q. 41(1):107-136, 2021.
9. Atshan, S.S., Hamat, R.A., Aljaberi, M.A., Chen, J.S., Huang, S.W., Lin, C.Y., Mullins, B.J. and Kicic, A.: Phage Therapy as an alternative treatment modality for resistant *Staphylococcus aureus* infections. Antibiotics (Basel). 12(2):286, 2023.
10. Hibstu, Z., Belew, H., Akelew, Y. and Mengist, H.M.: Phage Therapy: A different approach to fight bacterial infections. Biologics. 16:173-186, 2022.
11. Opperman, C.J., Wojno, J.M. and Brink, A.J.: Treating bacterial infections with bacteriophages in the 21st century. S. Afr. J. Infect. Dis. 37(1):346, 2022.
12. Ceyssens, P.J., Brabban, A., Rogge, L., Lewis, M.S., Pickard, D., Goulding, D., Dougan, G., Noben, J.P., Kropinski, A., Kutter, E. and Lavigne, R.: Molecular and physiological analysis of three *Pseudomonas aeruginosa* phages belonging to the "N4-like viruses". Virology, 405: 26-30, 2010.
13. Karaynir, A., Salih, H., Bozdoğan, B., Güçlü, O. and Keskin, D.: Isolation and characterization of brochothrix phage ADU4. Virus Res. 321:198902, 2022.
14. Yang, H., Liang, L., Lin, S. and Jia, S.: Isolation and characterization of a virulent bacteriophage AB1 of *Acinetobacter baumannii*. BMC Microbiol. 10:131, 2010.
15. Melo, L.D., Veiga, P., Cerca, N., Kropinski, A.M., Almeida, C., Azeredo, J. and Sillankorva, S.: Development of a phage cocktail to control *Proteus mirabilis* catheter-associated urinary tract infections. Front. Microbiol. 7:1024, 2016.
16. Ali, S., Karaynir, A., Salih, H., Öncü, S. and Bozdoğan, B.: Characterization, genome analysis and antibiofilm efficacy of lytic Proteus phages RP6 and RP7 isolated from university hospital sewage. Virus Res. 326:199049, 2023.
17. Bolger, A.M., Lohse, M. and Usadel, B.: Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 30(15):2114-2120, 2014.
18. Li, D., Liu, C.M., Luo, R., Sadakane, K. and Lam, T.W.: MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics. 31 (10):1674-1676, 2015.
19. Brettin, T., Davis, J.J., Disz, T., Edwards, R.A., Gerdes, S., Olsen, G.J., Olson, R., Overbeek, R., Parrello, B., Pusch, G.D. and Shukla, M.: RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. Sci. Rep. 5(1):1-6, 2015.
20. Finn, R.D., Clements, J. and Eddy, S.R.: HMMER web server: interactive sequence similarity searching. Nucleic Acids Res. 39:W29-W37, 2011.
21. Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S. and Madden, T.L.: NCBI BLAST: a better web interface. Nucleic Acids Res. 36 (Suppl-2):W5-W9, 2008.
22. Lowe, T.M. and Chan, P.P.: tRNAscan-SE On-line: integrating search and context for analysis of transfer RNA genes. Nucl. Acids Res. 44(W1): W54-W57, 2016.
23. Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K. and Madden, T.L.: BLAST+: architecture and applications. BMC Bioinf. 10(1):1-9, 2009.
24. Tynecki, P., Guziński, A., Kazimierczak, J., Jadczuk, M., Dastych, J. and Onisko, A.: PhageAI-bacteriophage life cycle recognition with machine learning and natural language processing. bioRxiv, 2020.
25. Bortolaia, V., Kaas, R.S., Ruppe, E., Roberts, M.C., Schwarz, S., Cattoir, V., Philippon, A., Allesoe, R.L., Rebelo, A.R., Florensa, A.F. and Fagelhauer, L.: ResFinder 4.0 for predictions of phenotypes from genotypes. J. Antimicrob. Chemother. 75(12):3491-3500, 2020.
26. Malberg Tetzschner, A.M., Johnson, J.R., Johnston, B.D., Lund, O. and Scheutz, F.: In silico genotyping of *Escherichia coli* isolates for extraintestinal virulence genes by use of whole-genome sequencing data. J. Clin. Microbiol. 58 (10): e01269-20, 2020.
27. Tamura, K., Tao, Q. and Kumar, S.: Theoretical foundation of the Real Time method for estimating divergence times from

variable evolutionary rates. Mol. Biol. Evol. 35 (7):1770-1782, 2018.
28. Nishimura, Y., Yoshida, T., Kuronishi, M., Uehara, H., Ogata, H. and Goto, S.: ViPTree: the viral proteomic tree server. Bioinformatics. 33 (15): 2379-2380, 2017.
29. Comeau, A.M., Bertrand, C., Letarov, A., Tétart, F. and Krisch, H.M.: Modular architecture of the T4 phage superfamily: a conserved core genome and a plastic periphery. Virology. 362(2):384-96, 2007.
30. Sun, S., Gao, S., Kondabagil, K., Xiang, Y., Rossmann, M.G. and Rao, V.B.: Structure and function of the small terminase component of the DNA packaging machine in T4-like bacteriophages. Proc. Natl. Acad. Sci. USA. 109(3):817-822, 2012.
31. Labrie, S., Vukov, N., Loessner, M.J. and Moineau, S.: Distribution and composition of the lysis cassette of Lactococcus lactis phages and functional analysis of bacteriophage ul36 holin. FEMS Microbiol. Lett. 233:37-43, 2004.
32. Hutinet, G., Besle, A., Son, O., McGovern, S., Guerois, R., Petit, M.A., Ochsenbein, F. and Lecointe, F.: Sak4 of phage HK620 Is a recA remote homolog with single-strand annealing activity stimulated by its cognate SSB protein. Front. Microbiol. 9:743, 2018.
33. Kamilla, S. and Jain, V.: Mycobacteriophage D29 holin C-terminal region functionally assists in holin aggregation and bacterial cell death. FEBS J. 283:173-190, 2016.
34. Lerdsittikul, V., Thongdee, M., Chaiwattanarungruengpaisan, S., Atithep, T., Apiratwarrasakul, S., Withatanung, P., Clokie, M.R.J. and Korbsrisate, S.: A novel virulent Litunavirus phage possesses therapeutic value against multidrug resistant *Pseudomonas aeruginosa*. Sci. Rep. 12(1):21193, 2022.
35. Campbell, I.J., Olmos Jr, J.L., Xu, W., Kahanda, D., Atkinson, J.T., Sparks, O.N., Miller, M.D., Phillips Jr, G.N., Bennett, G.N. and Silberg, J.J.: *Prochlorococcus* phage ferredoxin: structural characterization and electron transfer to cyanobacterial sulfite reductases. J. Biol. Chem. 295 (31):10610-10623, 2020.
36. Qin, S., Xiao, W., Zhou, C., Pu, Q., Deng, X., Lan, L., Liang, H., Song, X. and Wu, M.: *Pseudomonas aeruginosa*: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. Signal Transduct. Target Ther. 7(1):199, 2022.
37. Shariati, A., Noei, M. and Chegini, Z.: Bacteriophages: The promising therapeutic approach for enhancing ciprofloxacin efficacy against bacterial infection. J. Clin. Lab. Anal. 37(9-10): e24932, 2023.
38. Grigson, S.R., Giles, S.K., Edwards, R.A. and Papudeshi, B.: Knowing and Naming: Phage Annotation and Nomenclature for Phage Therapy. Clin. Infect. Dis. 77(Suppl 5):S352-S359, 2023.