Application of Lytic Bacteriophage against Carbapenem Resistant *Klebsiella pneumoniae* biofilms and Cloning of Holin Gene Responsible for Bacterial Lysis

Dipendra Kumar Mandal[1], Rojina Pandey[1], Puja dahal[1],Gun Raj Dhungana[1], Rajindra Napit[2], Gaurav Adhikari[1], Abdul Rehaman Miya[1], Gorkha Raj Giri[1], Rajani Malla[1]

Affiliation1: *Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu,Nepal*, ***Affiliation2:*** *Center for Molecular Dynamics Nepal*

Presenting author: dipendradas@iom.edu.np

Presented to NHRC's

10th National Summit of Health and Population Scientists in Nepal

"Advancing Health and Population Research and Innovations: Achieving SDGs"

April 10-12, 2024, Hotel Hyatt Regency, Jorpati, Kathmandu, Nepal

Presenting by

Dipendra Kumar Mandal (Ph.D. Scholar)
Central Department of Biotechnology,
Tribhuvan University, Kirtipur

Date: April 12, 2024

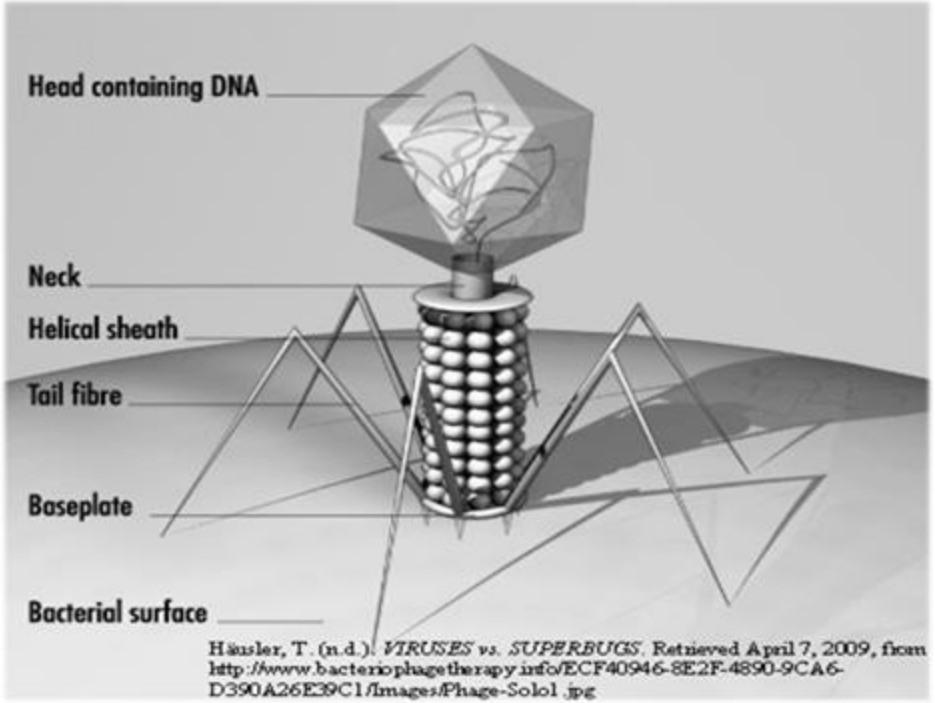
Supervisor

Prof. Dr. Rajani Malla
Central Department of Biotechnology,
Tribhuvan University, Kirtipur, Nepal

Background

Antibiotics and Bacteriophage

- ✓ Carbapenem-resistant *Klebsiella pneumoniae* (CRKP):
  - Global and a significant clinical threat (lack of therapeutics)
- ✓ Worrisome - CRKP strains recently developed resistance
  - Last-line antibiotics - Polymyxin and Tigecycline
- ✓ Antibiotic resistance threat - Inability in breaking the biofilm
  - Urgently need of novel strategies
- ✓ Antibiotic therapeutic choices against CRKP - limited,
  - Ultimate alternative -Phage therapy



Häusler, T. (n.d.). *VIRUSES vs. SUPERBUGS*. Retrieved April 7, 2009, from http://www.bacteriophagetherapy.info/ECF40946-8E2F-4890-9CA6-D390A26E39C1/Images/Phage-Solo1.jpg

- ✓ Phage borne depolymerases degrade biofilm exopolysaccharide matrix
  - Acts as a barrier for antimicrobials

Research Objective

Application of lytic bacteriophage against carbapenem resistant *Klebsiella pneumoniae* causing UTI biofilms

- Characterization of *Klebsiella pneumoniae* causing urinary tract infection
- Isolation of bacteriophage/s against (CRKP) the *Klebsiella pneumoniae* from natural resources
- Morphological, molecular and proteomic characterization of the phage/s.
- Characterization and cloning of *Holin* gene responsible for bacterial lysis
- Activity analysis of the phage for removal of biofilm produced by CRKP causing UTI

Research Plan

WP: Work Plan

WP-1

MDR/Biofilm producing Bacteria

- Isolation of MDR and biofilm producing bacteria causing UTI
- Confirmation of the bacteria (16SrRNA Sequencing)
- Determination of antibiogram and molecular analysis of MDR gene of *Klebsiella pneumoniae* (e.g. NDM etc)

WP-2

Bactriophage against MDR/Biofilm producing Bacteria

- Isolation of lytic bacteriophages against MDR and biofilm producing bacteria causing UTI from natural resources
- Characterization of the isolated phage for pH, thermal stability, growth curve and morphology under TEM
- Analysis for removal of biofilm producing bacterial isolates with phage alone under SEM

WP-3

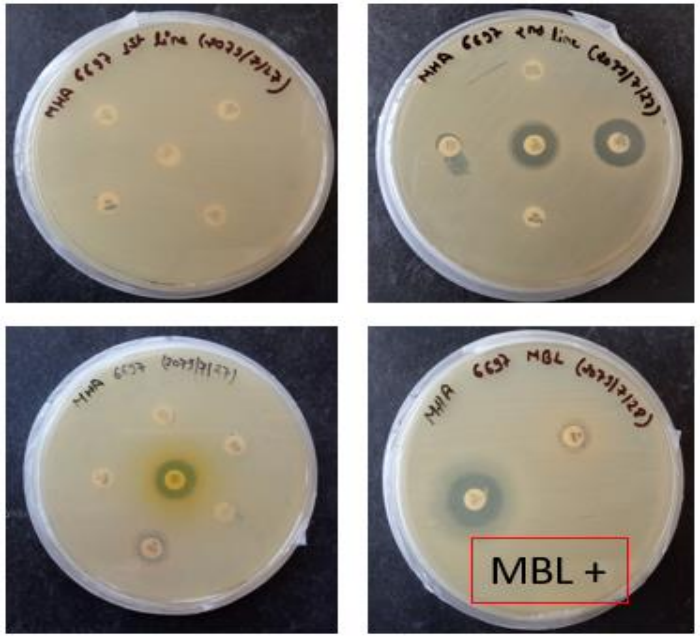
Specific gene and lytic activity of phage

- Isolation and characterization of *Holin* gene responsible for bacterial lysis
- Cloning of holin gene in a compatible vector
- Establishment of a phage bank as a repository of potential therapeutic phages

Results

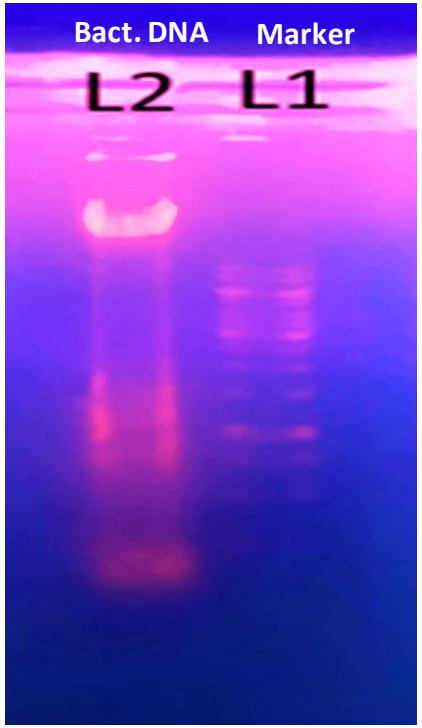
Identified *Klebsiella pneumoniae* by amplifying 16SrRNA and NDM gene

Antibiogram



AST by Kirby-Bauer method and MBL detection by combined disk diffusion method

Molecular identification



K pneumoniae bacterial DNA amplification by PCR

16sRNA

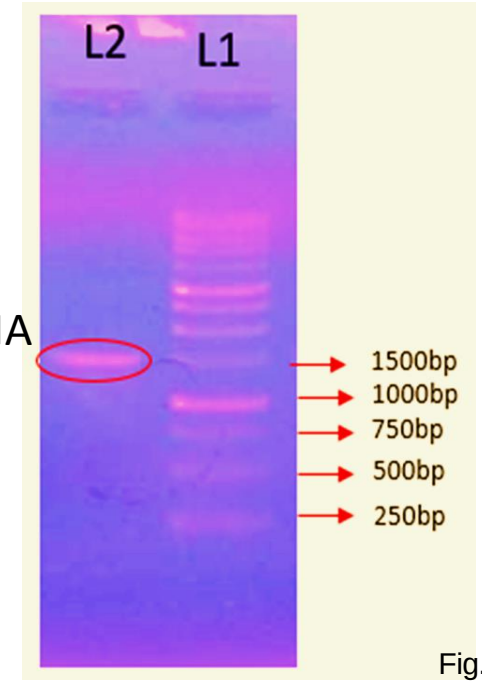


Fig. 16SrRNA PCR

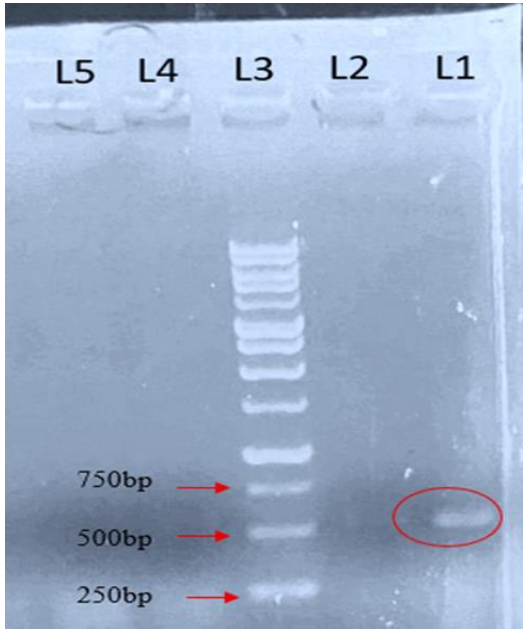
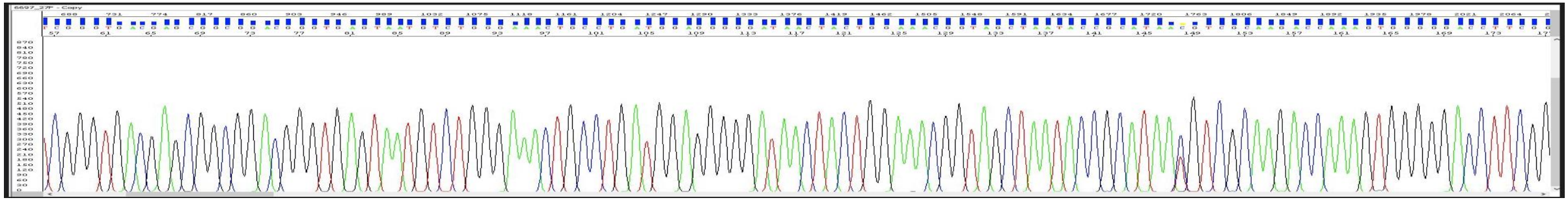


Fig. L1= 6697 bacterial NDM gene, L2=6661 , L3= 1kb DNA Ladder and L4=TU_pse1B

NDM-F:(5' CGGAATGGCTCATCACGATC3')
NDM-R:(5'GGTTTGGCGATCTGGTTTTC3')
621bp

Identified *Klebsiella pneumoniae* by sequencing 16SrRNA amplicon

Chromatogram of *Klebsiella pneumoniae (6697)*



Sequences producing significant alignments

Descriptions | Graphic Summary | Alignments | Taxonomy

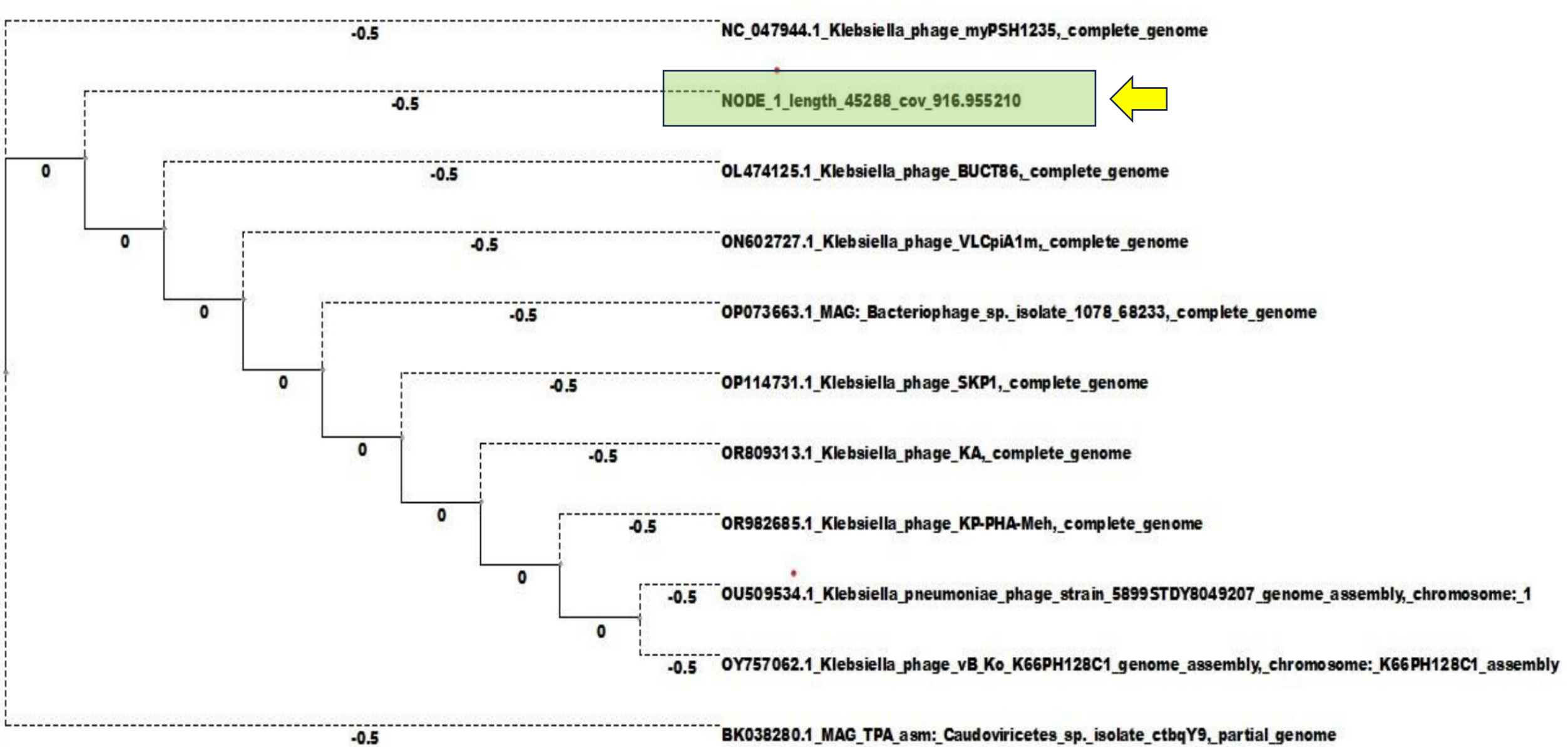
Sequences producing significant alignments Download Manage Columns Show 100

select all *0 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Common Name	Taxid	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Klebsiella pneumoniae strain KLB_MDR_391377 chromosome	Klebsiell...	NA	573	848	6662	28%	0.0	98.95%	5209225	CP133388.1
Uncultured bacterium clone 7-22D47 16S ribosomal RNA gene, partial sequence	uncultur...	NA	77133	848	848	28%	0.0	98.75%	510	AY457787.1
Klebsiella pneumoniae strain FF-66 16S ribosomal RNA gene, partial sequence	Klebsiell...	NA	573	845	845	28%	0.0	98.74%	862	MK918563.1
Klebsiella pneumoniae strain Bckp206 chromosome, complete genome	Klebsiell...	NA	573	843	6662	28%	0.0	98.74%	5126042	CP050845.1
Klebsiella pneumoniae strain Kp8701 chromosome, complete genome	Klebsiell...	NA	573	843	6623	28%	0.0	98.74%	5337408	CP049604.1
Klebsiella pneumoniae strain 2019036D chromosome, complete genome	Klebsiell...	NA	573	843	6605	28%	0.0	98.74%	5369757	CP047336.1
Klebsiella pneumoniae strain QD23 chromosome, complete genome	Klebsiell...	NA	573	843	6612	28%	0.0	98.74%	5803733	CP042858.1
Klebsiella pneumoniae strain 23 chromosome, complete genome	Klebsiell...	NA	573	843	6640	28%	0.0	98.74%	5287221	CP030320.1
Klebsiella pneumoniae strain 11311 chromosome, complete genome	Klebsiell...	NA	573	843	6640	28%	0.0	98.74%	5270063	CP030313.1

Fig. NCBI BLAST result (6697)

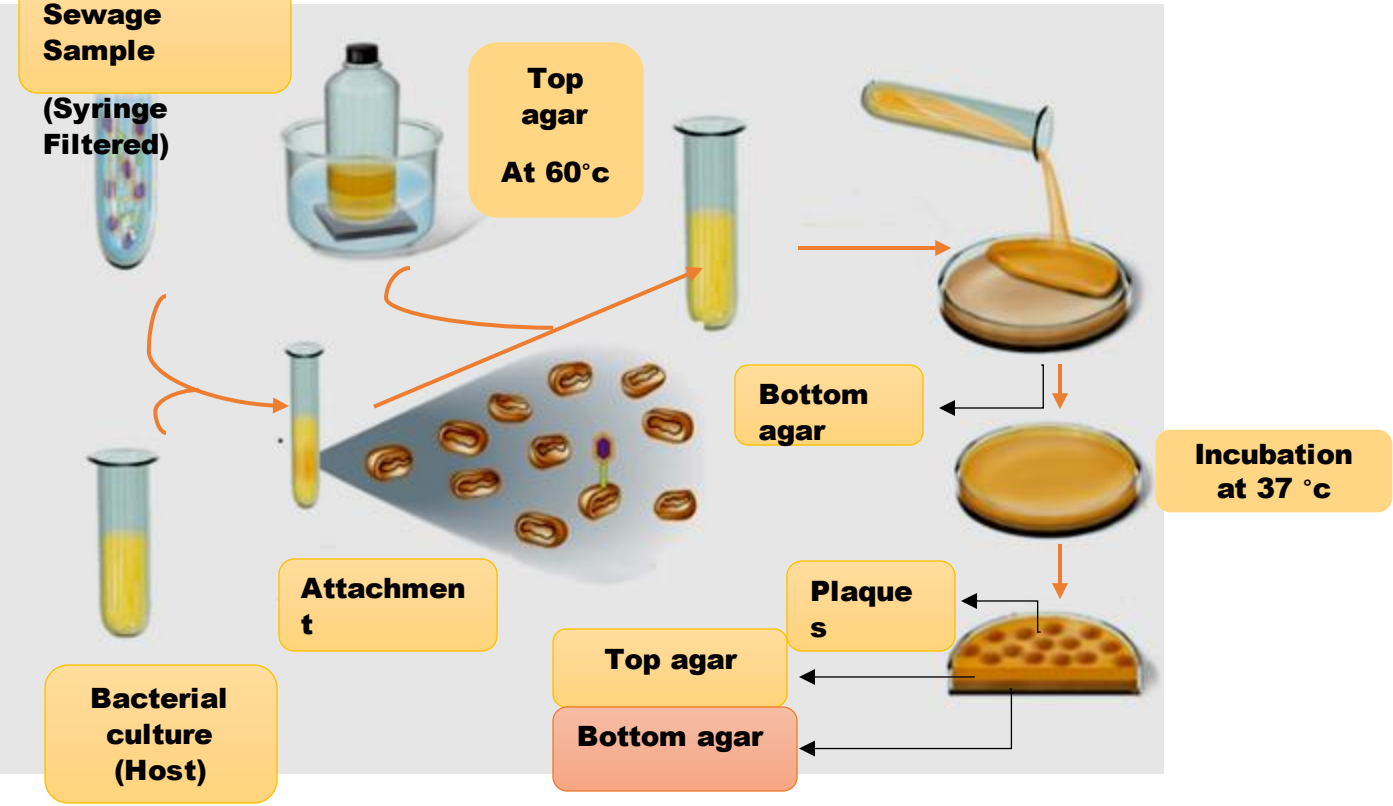
Identified *Klebsiella pneumoniae* and phylogenetic relationship



Methodology

Phage isolation and purification

Double Layer Agar Assay



Ultracentrifuge

- ✓ Double layer agar assay (DLAA) for bacteriophage isolation from different samples.
- ✓ Bacteriophage purification was performed by using the continuous streaking method and ultracentrifuge

Methodology

Phage genome sequencing

Phage genomic DNA extraction will be performed using the QIAamp DNA Mini Kit

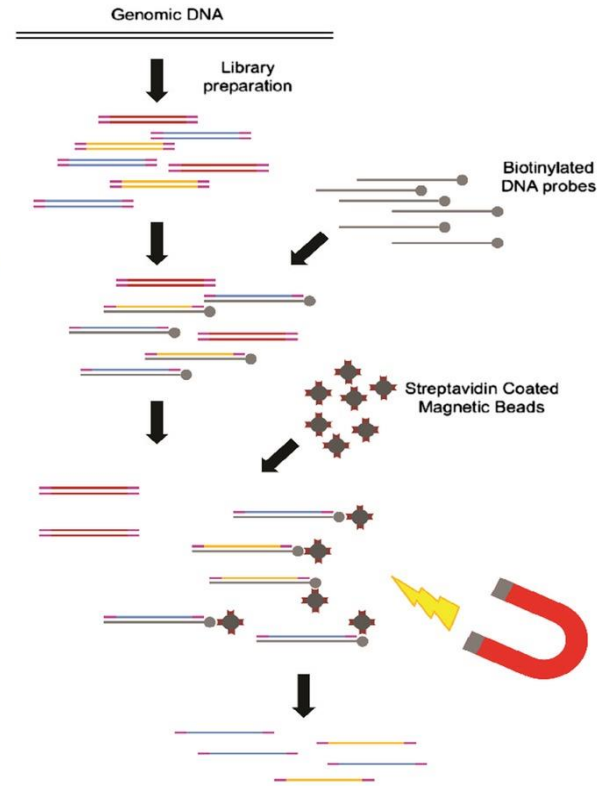
Prior to the extraction of phage DNA, remnants of bacterial DNA will be removed through a DNAse digest for 15min.

A

Sample collection and DNA extraction

B

Library preparation and hybrid-capture

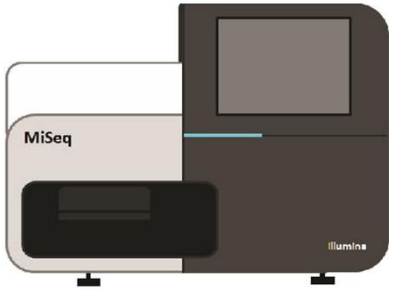


For sequencing, a paired-end library will be generated using the Nextera XT Library Prep Kit, and 2×150 bp reads will be generated using the MiSeq v2 Reagent Kit.

WGS according to the product manual (Nextera XT DNA Sample Preparation Guide)

C

NGS on illumina Miseq platform



D

Bioinformatic Analysis

De novo assembly of reads will be performed via the St. Petersburg genome assembler (SPAdes). Via genome wide BLASTn analysis the most closely related phages deposited in GenBank were searched and the result visualized via BRIG

E

Report

Potential ORFs will be identified using GeneMark.S and annotation will be performed using PHASTER

Methodology

Cloning, Recombinant Protein Expression, and Purification

Phage(ØKp_NEP or 6697)

Phage DNA

PCR/Site Directed amplification of *Holin* gene

Amplicon + Linearized Vector → Ligation → Recombinant Vector

Holin genes transferred into $DH_5\alpha$ (Plasmid cell) Transformation

Plasmid Isolation

Transformation into BL21 Gold (Protein expression)

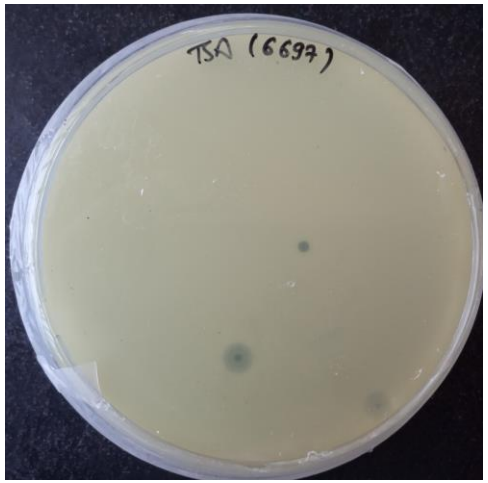
Protein Extraction and Purification (SDS PAGE gel electrophoresis)

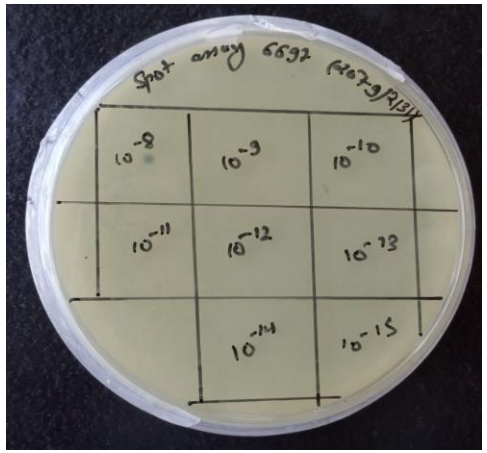
Quantification of protein using BCA TM protein Assay kit (Thermo scientific)

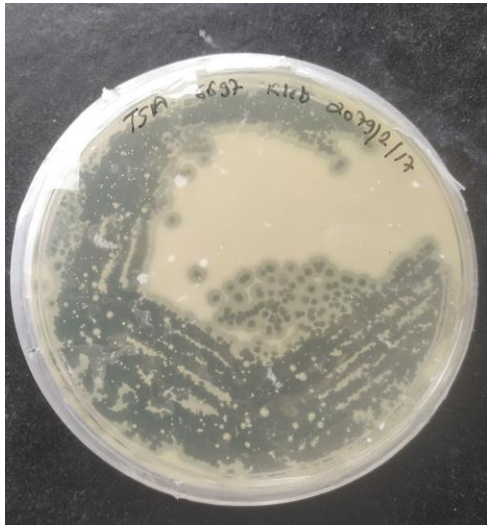
Protein expression either in cell line/ animal inoculation

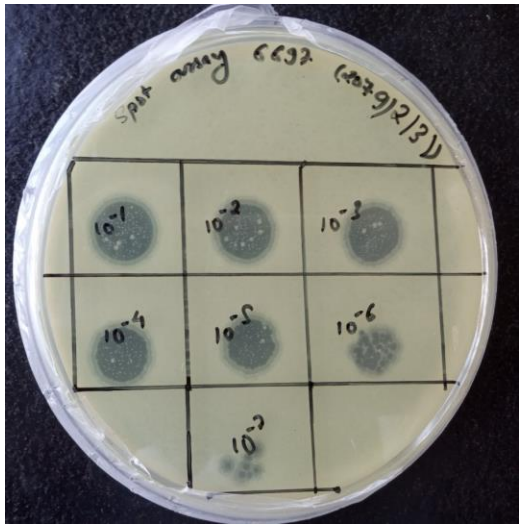
Results

Isolation of phages from different sources







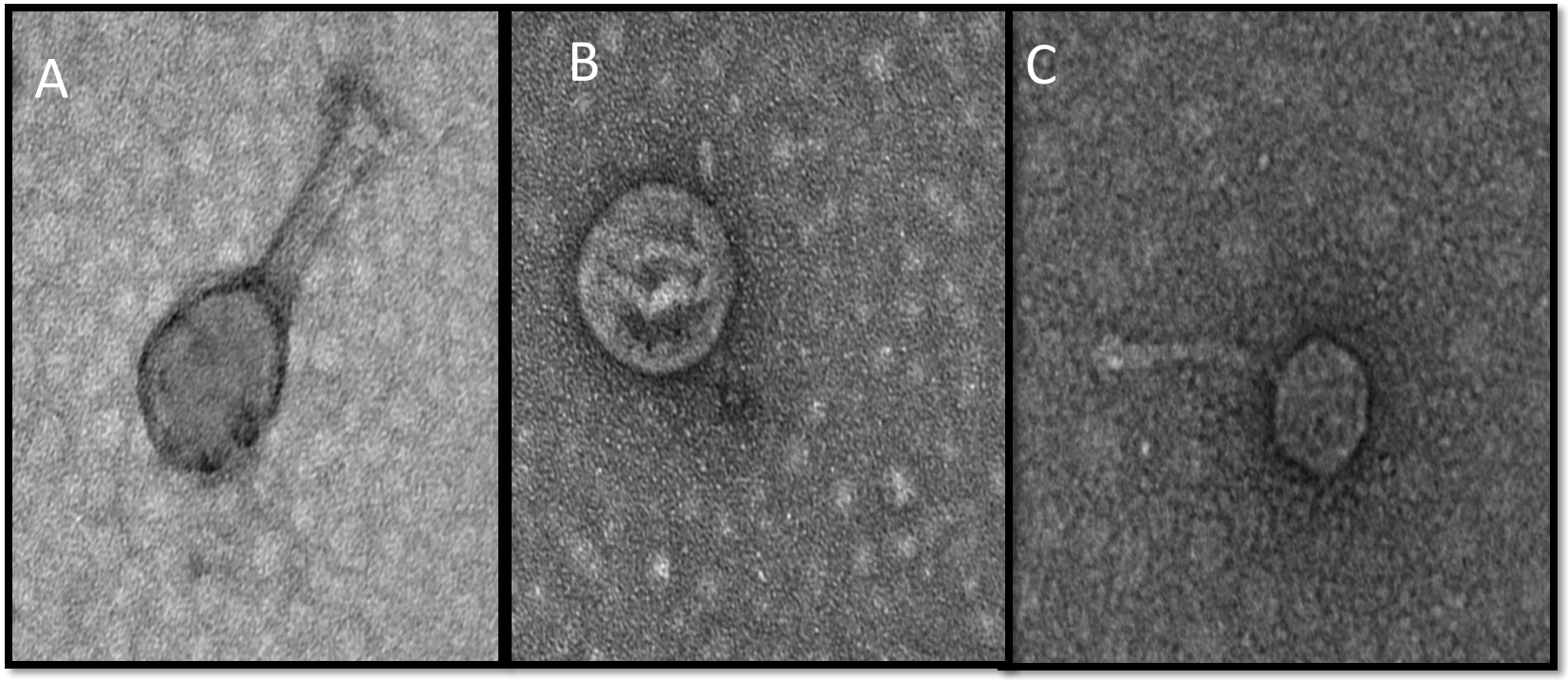


Source:	Kalimati River
Morphology:	Large, bull's eye
Opacity:	Clear
Nomenclature:	Phage 6697 (ØKp_NEP)
Host:	*K. pneumoniae*

ØKp_NEP (*Klebsiella pneumoniae* phage) isolation, purification and spot assay

Results

Morphology under Transmission Electron Microscopy (TEM)

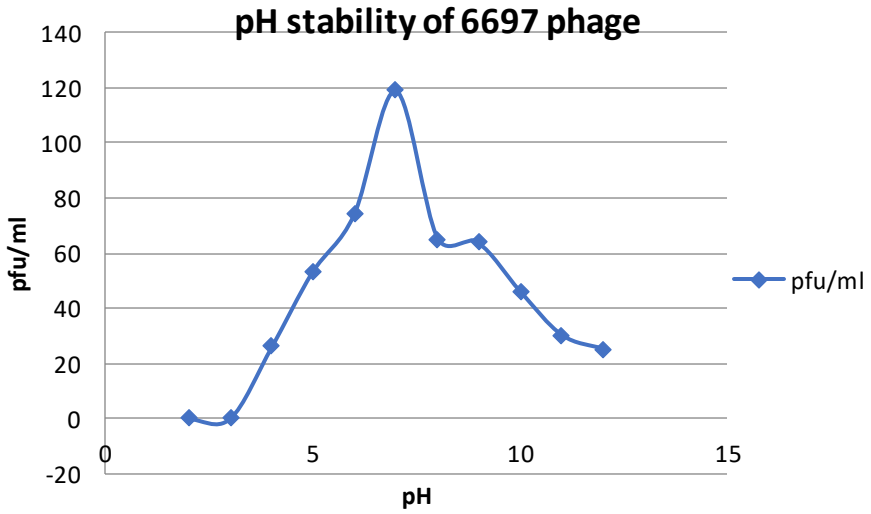


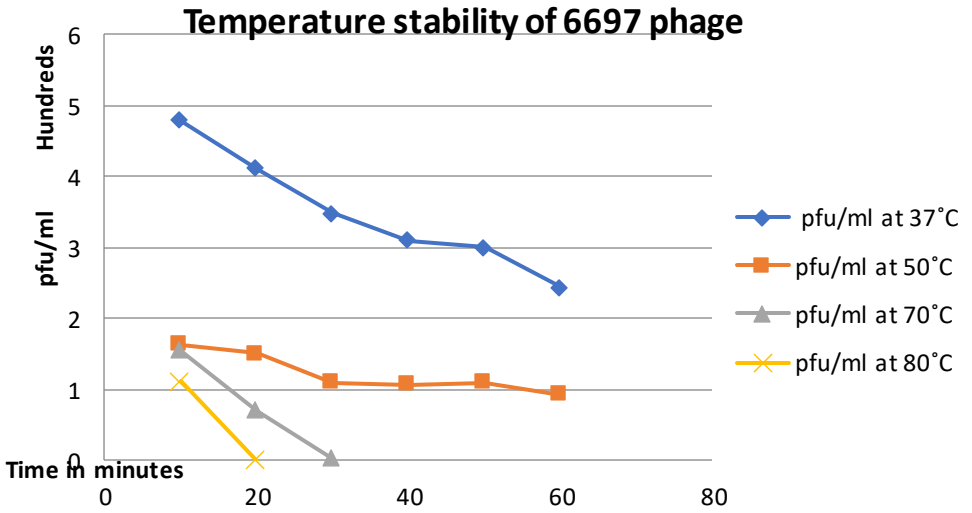
A,B and C are ØKp_NEP or 6697 Phage belongs to the family Myoviridiae of the order Caudovirales

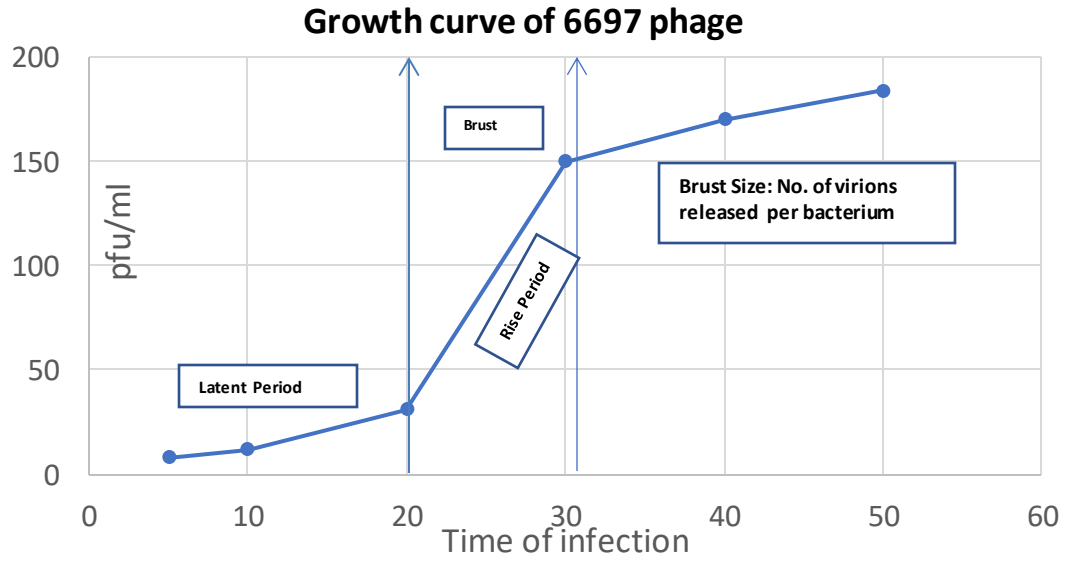
Results

Physiochemical properties of isolated phages

6697: *Klebsiella pneumoniae* phage



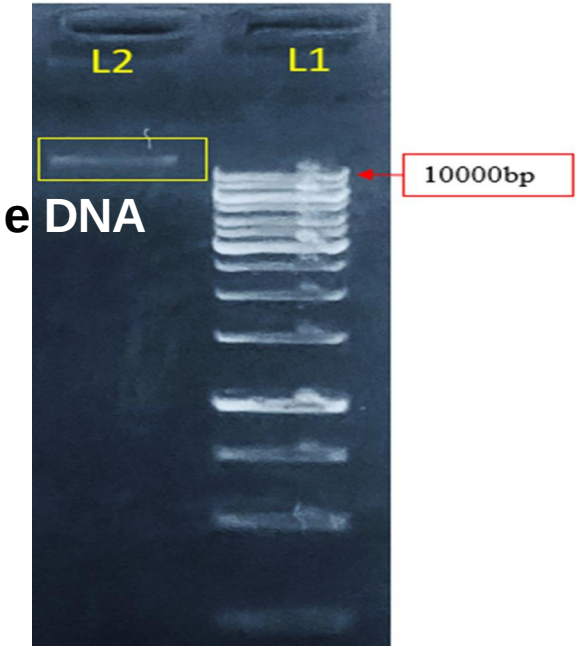


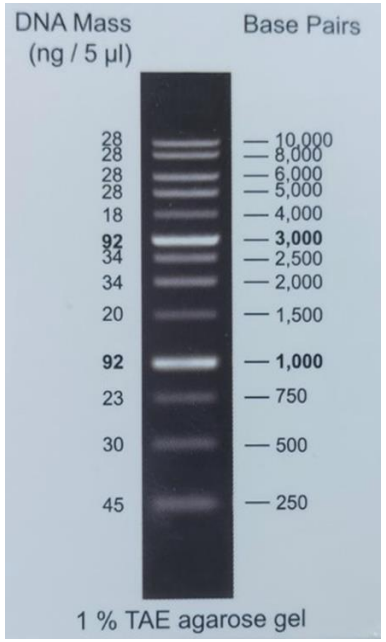


The optimum temperature and pH for the phage was 37˚C and 7 respectively and is highly specific to host strain. Latent period of the phage was 20 minute and burst size 119 virion per bacterium.

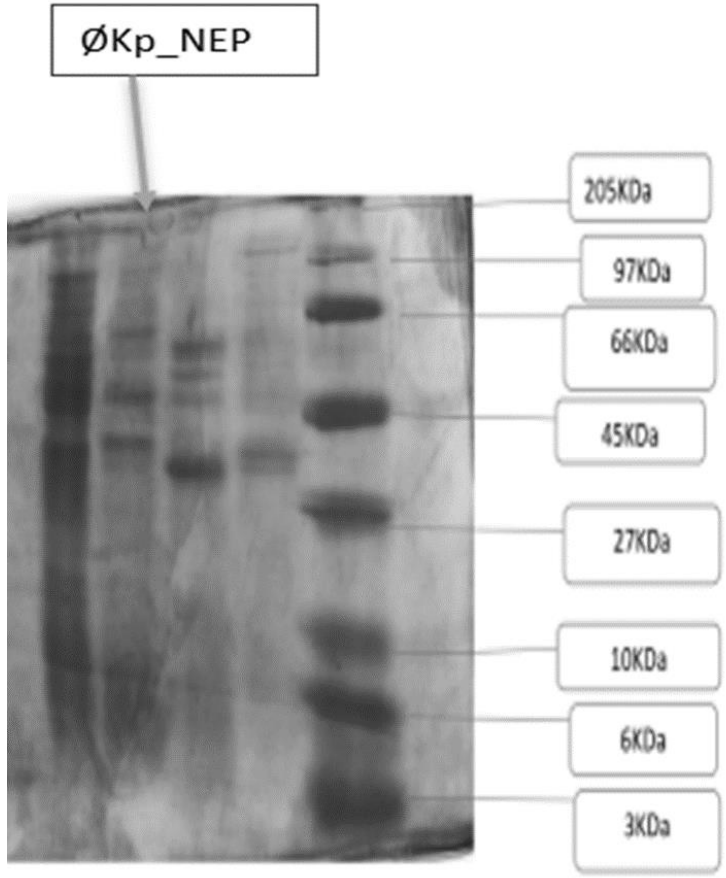
Results

Phage DNA and protein profiling (SDS PAGE)





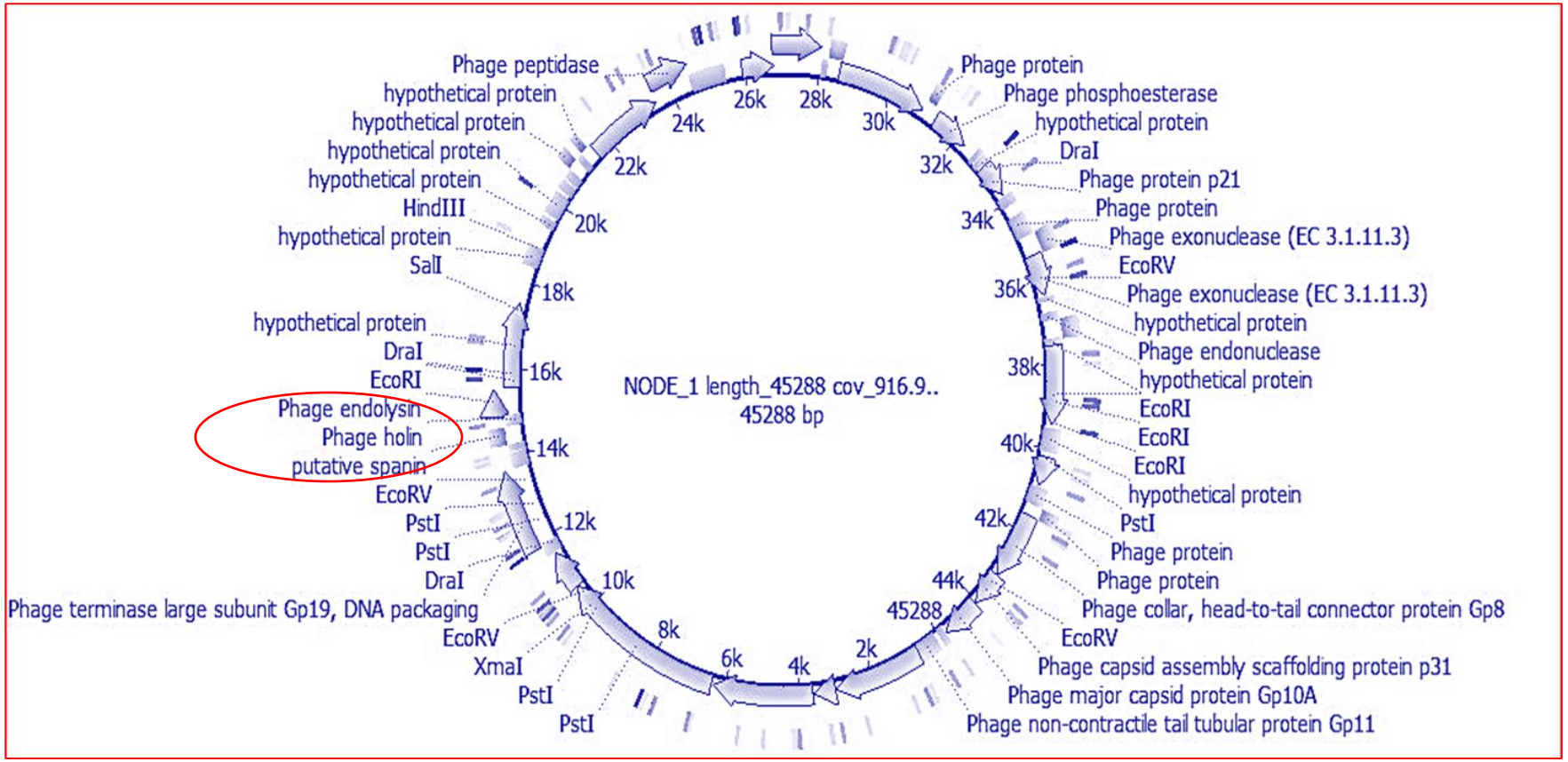
Electrophoresis of phage DNA



Phage protein profile (SDS PAGE)

Results

Whole genome circular view of 6697Ø: *Klebsiella pneumoniae* phage



Length	45,288nt(45kb)
GC Content	53.97%
Molecular weight	27985314.46
Extinction coefficient	720395602l/(mol*cm)
Ug/OD260	38.85
Melting temperature	86.99°C
Open reading frame	90

General Information of phage genome from whole genome sequencing

Results

Isolation and characterization of *Holin* gene responsible for bacterial lysis

Eurofins Genomics (Primer of *Holin*)
RM1: CATATGATTAAGGTGGGAGACATGG
Total base pair: 25
RM2: CAGCAAGCTTGCTTAGTCCTTAAACTCATA
Total base pair: 30

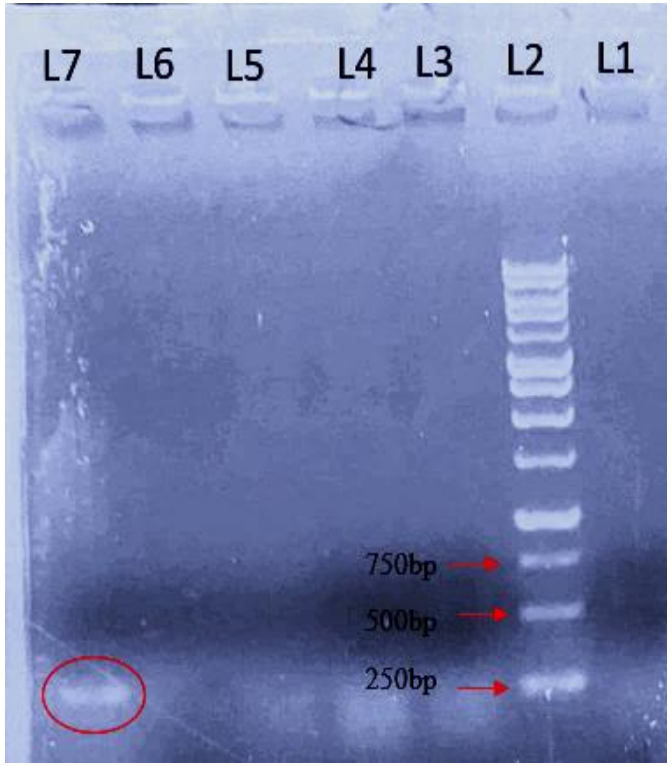


Fig. Holin PCR where as L2=1KB DNA Ladder, L3= 6661, L4= TU_pse1B and L7=6697

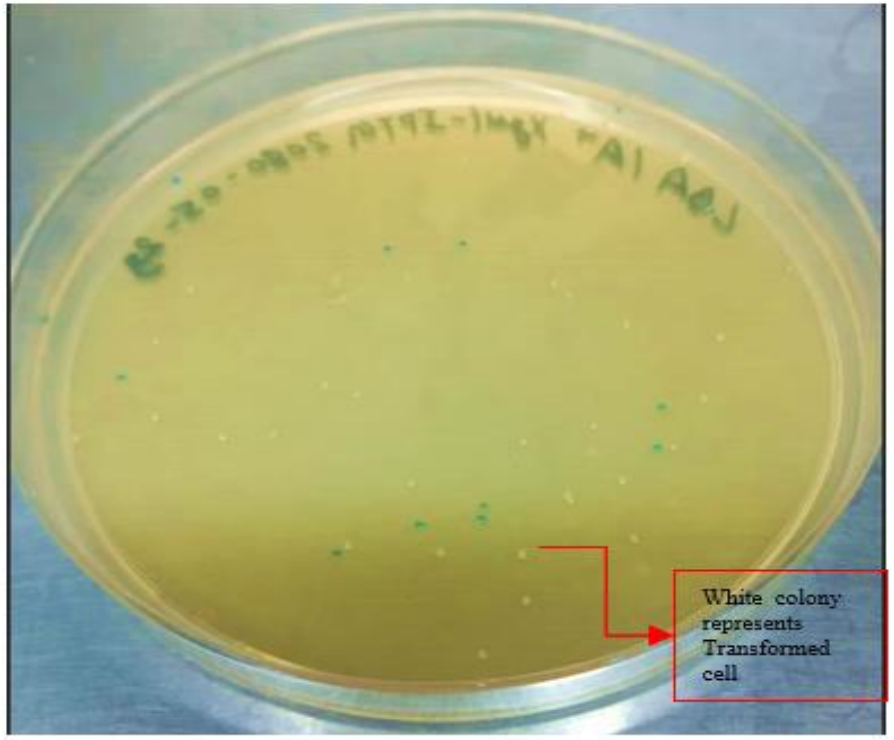
```
14161 ggtgcagcag gatgattaag gtgggagaca tggttgggtc agacctcgct acccgggcag
14221 gtgcagcagt taccggcgct acggtatcag gaggttggtt ggcagagtta atgagctgga
14281 actggagcac tatcagcttc atcactgcga cggtgtgcgc ggtgctaacc ctggcgtgga
14341 atgcgtatta caagcggcgt acattcaagc tcctagagga gcaggcacgt aaggggacta
14401 ttaaatatga gtttaaggac taaggttatt gcggccctca cgggggccac tatgcttggt
```

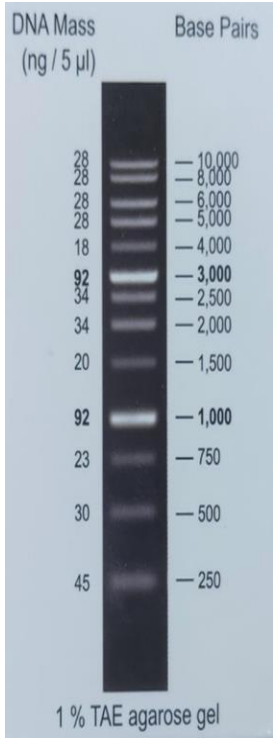
Holin gene (252bp) after sequecncing checked by NCBI

Results

Cloning process of *Holin* gene

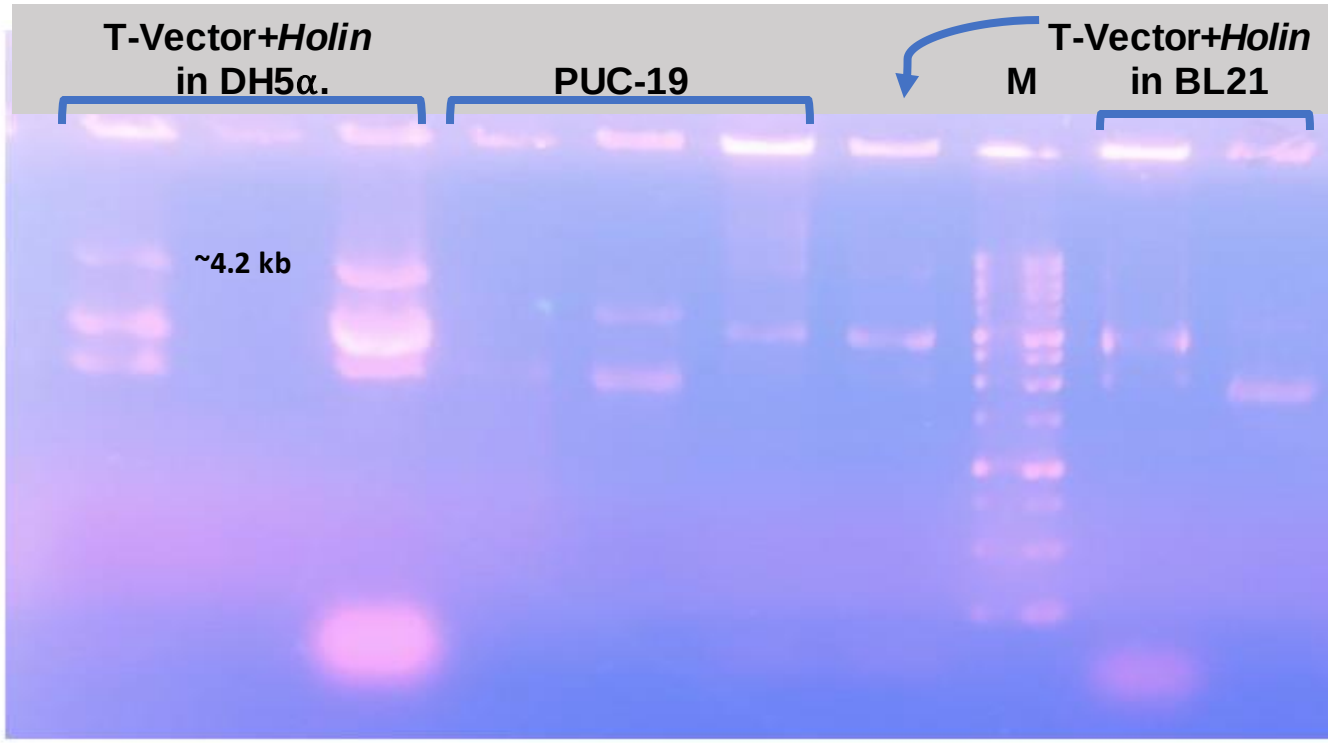
Transformation and Blue-White Screening





Blue white screening with X-GAL (5-Bromo-4-chloro-3-indolyl beta-D-galactopyranoside and IPTG (Isopropyl beta-D-1 Thiogalactopyranoside)

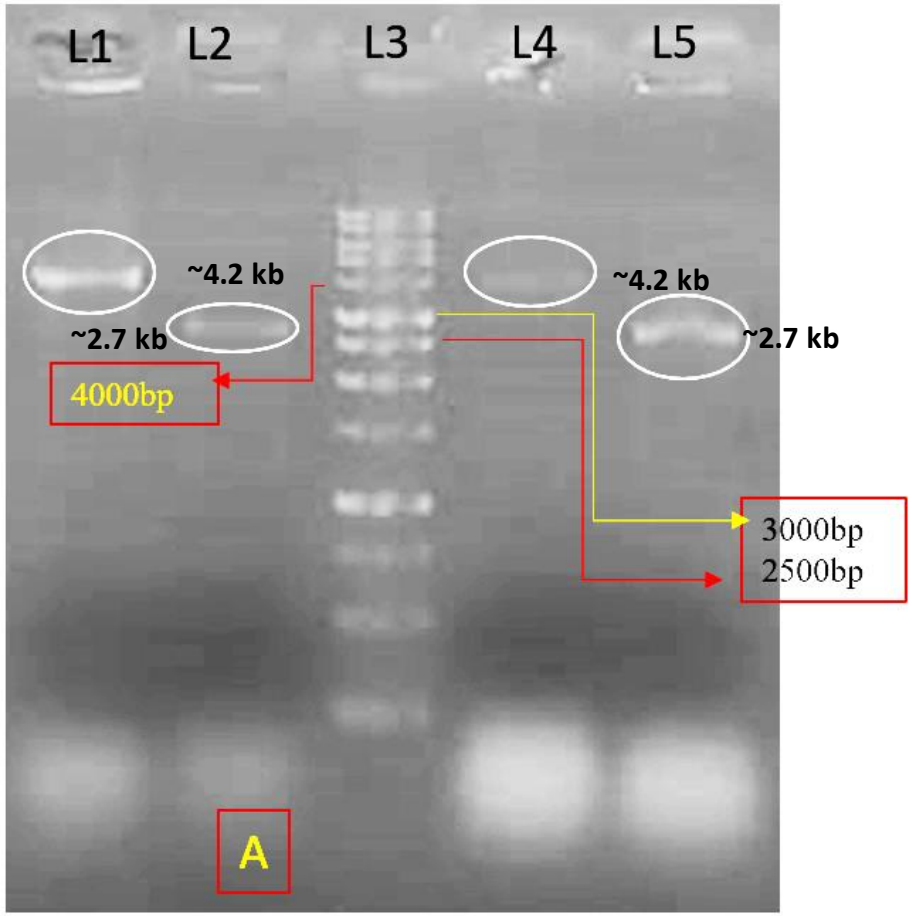
Validation of transformed vector



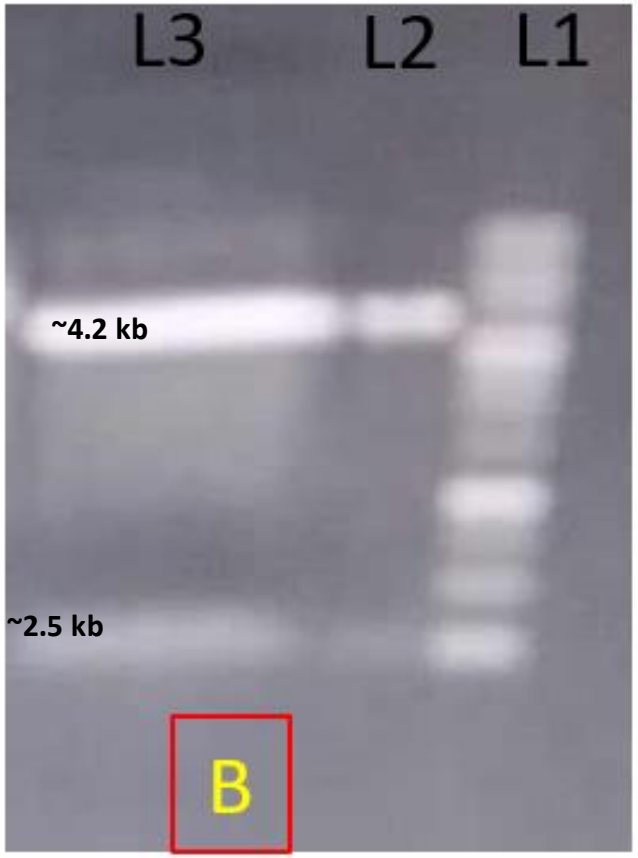
Results

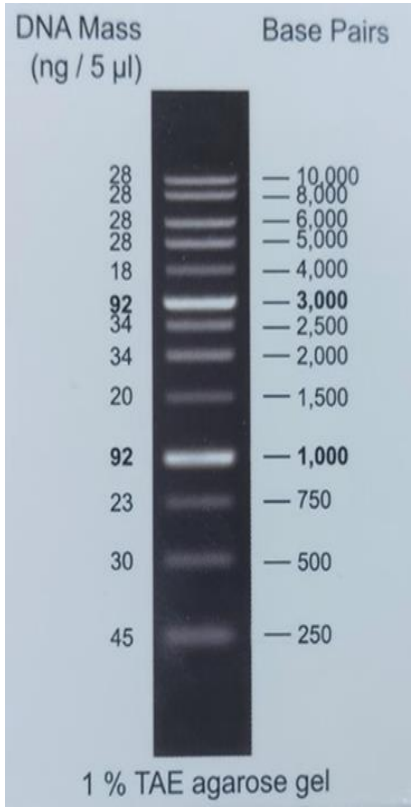
Cloning process of *Holin* gene

Plasmid Extraction after Single Restriction Digestion with HindIII



Double Restriction Digestion with HindIII and NdeI

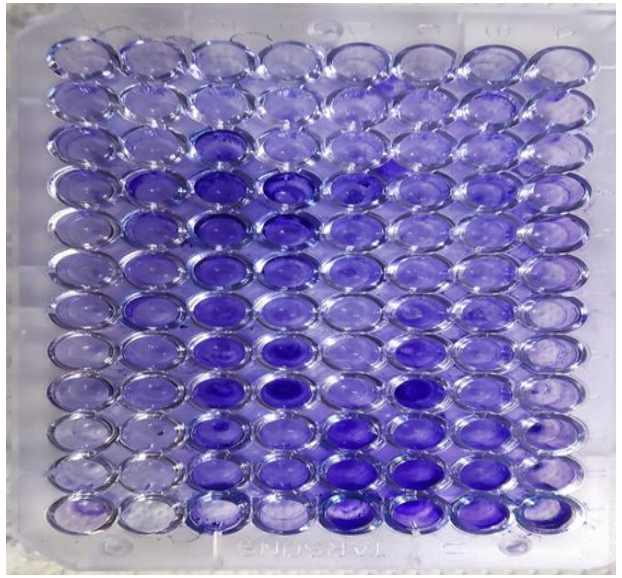


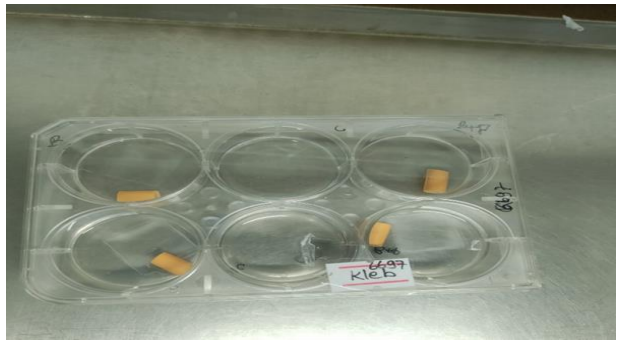


PLAN: Cloned product for sequencing
Ligation, transformation and double restriction digestion will be performed in pET-28b Vector from this cloned product

Results

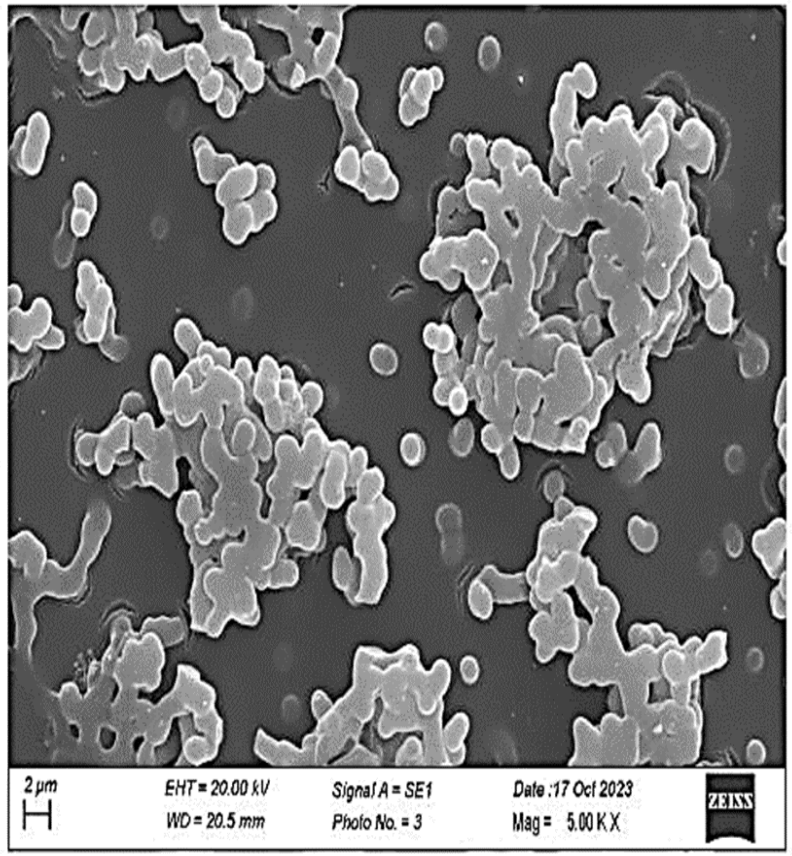
Biofilm identification



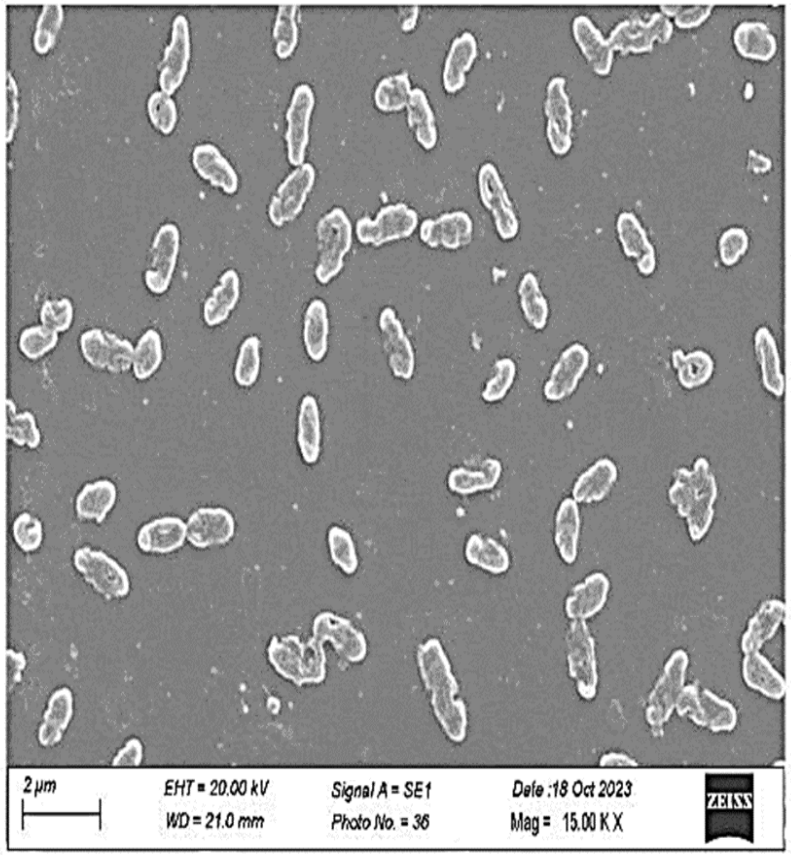


Biofilm observed under SEM

Biofilm without Phage treatment



Biofilm treated with Phage



TEM performed at AIIMS, India

Conclusion

- The phage was found to be stable in different physiological conditions
- The Phage belonged to order Caudovirales and family Myoviridiae
- Phage therapy may be a promising alternative candidate to antibiotics for preventing and controlling infections caused by CRKP biofilms.
- Phage **holin protein** can be next generation antibacterial agent to treat MDR pathogen if further research is carried out in clinical trial.

Acknowledgement

- Prof. Dr. Rajani Malla (Supervisor)
- **Head of Dept. Prof. Dr. Krishna Das Manandhar** and all respected faculty members
    - Mrs. Pragati Pradhan
    - Dr. Gorkha Raj Giri(Co-supervisor)
    - Dr. Jarina Joshi
    - Dr. Smita Shrestha
    - Mr. Rajendra Napit
    - Dr. Guna Raj Dhungana
    - Administrative staff, Lab members of Central Dept. of Biotechnology, TU
    - Sudip Timilsina, Puja dahal, Pragya sapkota, Shobha amagain, Sangharsika Chaudhary, Sushila Neupane and
    - Rojina Pandey, Abdul Rehaman Miya and Gaurav Adhikari

NHRC

UGC

CMDN

RESEARCH INSTITUTE FOR BIOSCIENCE AND BIOTECHNOLOGY

Prajwal Rajbhandari

Dr. Yuvraj Pokharel

SAU SOUTH ASIAN UNIVERSITY

References

1. Mah TF, O'Toole GA: Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 2001, 9: 34-39. 10.1016/S0966-842X(00)01913-2.
2. Zhang R., Dong N., Huang Y., Zhou H., Xie M., Chan E. W., *et al*. (2018). Evolution of tigecycline- and colistin-resistant CRKP (carbapenem-resistant *Klebsiella pneumoniae*) in vivo and its persistence in the GI tract. *Emerg. Microbes Infect.* 7:127. 10.1038/s41426-018-0129-7.
3. Hughes KA, Sutherland IW, Clark J, Jones MV: Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. Microbiology. 1998, 144: 3039-3047. 10.1099/00221287-144-11-3039.
4. Ciacci N., D'Andrea M. M., Marmo P., Dematte E., Amisano F., Di Pilato V., *et al*. (2018). Characterization of vB_Kpn_F48, a newly discovered lytic bacteriophage for *Klebsiella pneumoniae* of sequence type 101. *Viruses* 10:482. 10.3390/v10090482.

Photographs

Mother of Phage in Nepal because she had started the research in phage first time

Thank you