Isolation and Whole Genome Sequencing of a novel Salmonella lytic bacteriophage

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Abstract

Salmonella is one of the leading causes of foodborne diseases in the world. In this study, *Salmonella* killing bacteriophage was isolated using the double-layer agar overlay technique and characterization of the phage genome was done. The morphology of phage was studied using Transmission Electron Microscopy. Whole-genome sequencing of phage DNA was performed in the Miseq platform. The genomic DNA library was prepared using the NexteraXT library prep kit method using the NexteraXT index kit. Paired-end sequencing of 2×251 cycles read length; the run was performed using the Miseq V3 kit. Phage formed clear plaques on *S. enteritidis* in the double agar overlay technique. The electron microscopy revealed that the phage belonged to Myoviridae family. The assembly produced a draft genome of length 161329 bp with a GC content of 44.425% and coverage of 104x. Rapid annotation subsystems technology yielded 333 coding sequences of which 285 proteins were hypothetical and 48 were assigned functions that included DNA ligase, capsid protein, endolysin, and other proteins. A pairwise comparative analysis showed closest genome as *Salmonella* Phage SSE-121. The study on the phylogenetic tree obtained showed a very close relationship with *Salmonella* Phage SSE-121 genome with a distance score of 0.1 disclosing it to be a new member of the SSE121 virus.

Introduction

Salmonella is recognized as one of the four global causes of diarrhoeal diseases (WHO, 2020). According to Global Burden of Disease estimation in 2017, non-typhoid *Salmonella enterocolitis* resulted in 95.1 million cases, 50771 deaths, and 3.10 million disability-adjusted life years -DALYs (GBD, 2017). *Salmonella* causes 1.35 million infections, 26.500 hospitalizations, and 420 deaths each year in the United States according to the center for diseases control and prevention (CDC, 2020).

Over the past decade, India emerged as the world's major consumer of antibiotics. In India infectious disease burden is high and the irrational use of antibiotics to cure these infectious diseases resulted in antimicrobial resistance in these infectious agents (Kumar et al., 2013). This reason leads to a search for a new treatment to control bacteria (Boeckel et al., 2014). The use of bacteriophages merged as a novel tool in the area of food preservation and safety (EFSA, 2009). Virus infecting bacteria are bacteriophages. These phages act as natural antimicrobials and already proved their effectiveness in controlling pathogens in food products. Such phages have received GRAS (Generally Recognised as Safe) status from USFDA.

In this study, an attempt was successful to isolate *Salmonella* killing lytic phage and to characterize genetic information phage genome NINP13076 for further study. Information on the genetic information of phage will be useful in the development of broad host range phage control of multiple foodborne pathogens.

Materials And Methods

Bacterial cultures

For this study reference culture of *Salmonella enteritidis* was procured from American Type Culture Collection (ATCC-13076). Tryptic Soy broth (HIMEDIA, Mumbai) media was used to grow this culture and maintained at 4^{o}C.

Isolation – Double Layer Agar Overlay Technique

Raw sewage sample (40ml) was collected aseptically in a sterile container (HIMEDIA, Mumbai) from Hyderabad Municipal sewerage treatment plant. To this sample, 5 ml of 10X nutrient broth and 5 ml of exponential phase culture of *S. enteritidis* were added. Flask was incubated at 37^{0}C for 24 h in a shaker incubator. After incubation 10 ml of culture was centrifuged at 2000 rpm for 5 min. The supernatant was taken in a syringe fitted with a 0.22 µ filter. The filtrate was collected in a microcentrifuge tube and stored at 4^{0}C. The filtrate (100 µl) and exponential phase culture of *Salmonella* (100 µl) are added to 3 ml of molten agarose (0.7%). Mixed contents gently and poured this mixture onto the pre-warmed agar plate. The mixture was spread evenly on the surface of the plate. Plates were allowed to cool down. Plates were incubated in an inverted position at 37^{0}C for 24 h. Plates were observed for plaques after incubation. The total number of plaques forming units (PFU) per ml of sample was calculated by using the formula number of plaques divided by Volume plated and dilution factor.

Phage Purification

This process was carried out by picking a single plaque with a sterilized pipette tip followed by serial purifications and amplifications from the host *Salmonella enteritidis* (ATCC13076).

Phage Host Spectrum Study

To perform this experiment, a spot test assay and agar well diffusion assay was done. For spot test assay 1 ml of exponential phase culture of *S. enteritidis* was cultured on nutrient agar by spread plate method. Filtrated supernatant of phage (10 µl) was spotted on the agar and allowed for air drying. The plates were incubated at 37^{0}C for 24 h and observed for the clear zone (lytic spot) formation over the bacterial lawn. Agar diffusion assay was performed by making a well with cork borer in a nutrient agar pre-inoculated with *Salmonella*. Phage filtrate (10 µl) was added to the well and the plates are incubated at 37^{0}C for 24 h. After incubation, the plates were observed for the zone of inhibition.

Host Range Assay

Spot test assay was performed to determine the host range. Reference cultures of *Salmonella* (*Salmonella abony*-NCTC 6017, *Salmonella arizonae*-ATCC 13314, *S. typhi*-MTCC 733, *Salmonella poona*-

NCTC 4840, *S. enteritidis*-ATCC 13076, *Salmonella* spp- MTCC 1162) were procured from the National Collection of Type Cultures (NCTC), Microbial type culture collection (MTCC), and American Type Culture Collection (ATCC) to conduct this experiment.

Phage Morphology

TEM (Transmission Electron Microscopy) was used to obtain images of isolated phage at an acceleration of 80KV. A small drop of phage filtrate was loaded on a carbon-coated copper mesh grid. Negative staining phage filtrate was done by using 1% phosphotungstic acid.

Phage DNA isolation

Isolation of phage DNA was done using a phage DNA isolation kit (NORGEN, CANADA). For DNA isolation, phage filtrate (1 ml) was taken in a microcentrifuge tube and treated with DNase (10 µl). Phage filtrate was obtained by plucking individual plaque and subsequently inoculated to nutrient broth containing *Salmonella* exponential culture. After overnight incubation (37^0C for 24 h in a shaker incubator) and centrifugation (2000 rpm for 5 min) followed by filtration, the filtrate was obtained. Incubate this at 75^0C for 45 min in a water bath. Lysate buffer (500 µl) was added to this and vigorously vortexed for 10 sec. Proteinase K (4 µl) was added and incubated at 55^0C for 15 min. Again this solution was incubated at 65^0C for 15 min. Isopropanol (320 µl) was added to the lysate and vortexed. Centrifugation was done in a collection tube for 1 min at 8000 RPM. The flow-through was discarded from the collection tube. Once again centrifugation step was repeated and added 400 µl of ethanol as wash solution and again centrifuged for 1 min at 8000 RPM. This particular step was repeated twice. Elution buffer (75 µl) was added and centrifuged for 1 min at 8000 RPM. Phage DNA concentration was measured using nanodrop. The purified DNA sample was used for genome sequencing.

Whole-genome Sequencing

Whole-genome sequencing of phage DNA was performed in the Miseq platform (Illumina, USA). The genomic DNA library was prepared using the NexteraXT library prep kit (Illumina, USA) method using NexteraXT index kit and standard protocol provided by the manufacturer. The concentration of the DNA library was quantified in Qubit (ThermoScientific, USA) and the average library size was measured in Bioanalyzer (Agilent Technologies, Germany). Paired-end sequencing of 2×251 cycles read length; the run was performed using Miseq V3 kit (Illumina, USA). Upon completion of the sequence run, raw data fastq files for forward and reverse reads were carried forward for computational analysis.

Genome assembly and analysis

The raw reads were processed for QC using Trimmomatic v0.36 (Bolger et al., 2014). The reads that passed QC were assembled *de novo* using Unicycler v0.4.8 (Wick RR et al., 2017) assembler specific for

Illumina short-reads. The assembly quality was checked with Quality Assessment Tool for Genome Assemblies (QUAST) v5.0.2 (Gurevich A 2013). Annotation of the genome was carried out by Rapid Annotation Subsystems Technology (RAST) tk which uses a k-mer based search method against CoreSEED (Brettin T et al., 2015). If the K-mer search of FIGfam didnt yield anay results a BLAT and BLASTP search of non-redundant genus-specific protein databases for the organism's genus was employed at cutoff of e-value < = 1e-5 and a percent identity > = 50%. A Phylogenetic tree was built for the Capsid sequence using GTRGAMMA model of RAxML v8.2.4 with 1000 bootstrapping value (Stamatakis A 2014). A comapartive study was carried using NCBI Pairwise Sequence Comparison (PASC) tool (Bao Y et al., 2014). A circular genome map for the assembled genome was developed using CGView (Grant JR 2008). Using ViPTree a whole-genome phylogeny was built by comparing against reference viral genomes obtained from Virus-Host DB of dsDNA category (Nishimura Y et al., 2017). GenBank Accession Number for the deposited sequence is MZ326168.

Results

Salmonella specific phage was isolated from sewage and designated as NINP 13076. Phage formed clear plaques on *S. enteritidis* in double agar overlay technique. Freshly prepared phages were used for TEM. The TEM images revealed that *Salmonella* specific NINP 13076 exhibits isometric heads and a flexible tail indicating members of Myoviridae family (Fig. 1a & b). In spot test assay, an inhibition zone was observed at the place where phage filtrate supernatant was added indicating that isolated phage is specific to *Salmonella enteritidis* (Fig. 2). The host range assay showed that the isolated phage was effective against *Salmonella enteritidis, Salmonella arizonae*, and *Salmonella poona. Salmonella* specific phage showing larger plaque and higher transparency was chosen for genome sequencing.

The Phage genome assembly was carried-out step by step. As a first step, the raw reads obtained from the Illumina sequencing machine were subjected to Quality Control (QC). The QC was carried out using trimmomatic with parameters of phred score 15 and sliding window of 4:20 with trimming 20 bases at both ends and also removing adapter sequences. About 95% of reads were retained after the trimming process. The paired-end reads which passed QC were *de novo* assembled using Unicycler with default parameters. Unicycler uses SPAdes to assemble short-reads obtained from Illumina. It assembles based on various k-mer sizes and produces graph. Finally, it automatically selects the best graph based on minimum contig count with no dead ends. The assembly produced a draft genome of length 161329 bp with a GC content of 44.425% and coverage of 104x. After the assembly, it was quality-checked using Quality Assessment Tool for Genome Assemblies (QUAST). The QUAST analysis identified 51 contigs. For gene calling and annotation, the assembly was submitted to the Rapid Annotation Subsystems Technology tool kit(RASTtk). RASTtk yielded 333 coding sequences of which 285 proteins are hypothetical proteins and 48 were assigned functions which included DNA ligase, capsid protein, endolysin, and various other proteins. Including tRNA count of 21 and 8 repeat regions, the total genomic features added to 362 (Table 1, Supplementary Table 1). A comparative study using the NCBI PASC tool showed that the assembled genome is most similar to Salmonella virus SSE121 with a similarity percentage of 76.25%. A circular genome map was built using CGview. The outer ring shows the

annotated proteins' functions with ORF content and also the level of similarity with the *Salmonella* Phage SSE-121 genome (Fig. 3). A maximum-likelihood tree for the Salmonella phage NINP13076 phage genome built using proteomic viral genomes in ViPTree against dsDNA of all genomes shows that the genome is closely related to *Salmonella* Phage SSE-121 (Supplementary Fig. 1). And using the same ViPTree maximum-likelihood tree for the Salmonella phage NINP13076 phage genome built using proteomic viral genomes only Myoviridae family and gamma proteobacteria host group for the sake of image clarity (Fig. 4).

Table 1
Phage genome assembly features

Genome details	Genome features
Genome Length	161329bp
GC content	44.425%
CDS	333
Hypothetical proteins	285
Proteins with functional assignments	48
tRNA	21
repeat_region	8
Genomic features	362
Coverage depth (X)	104

A phylogenetic analysis was carried out to study its evolutionary relationship with other Phage genomes. The capsid gene sequence was BLAST searched against the nucleotide nr database. The top 10 hits were selected and a multiple-sequence alignment was built using ClustalW. The output was saved in phylip format. A Maximum-Likelihood tree was built using MSA obtained with the GTRGAMMA model and bootstrapping of 1000 using RaxML. The tree obtained showed a very close relationship with the *Salmonella* Phage SSE-121 genome with a distance score of 0.1(Fig. 5). The genome was compared for similarity with the *Salmonella* Phage SSE-121 genome by a pair-wise BLAST alignment. The pair-wise alignment showed the similarities covering the whole genome. The genome sequence has been submitted to NCBI and an accession number has been given. The GenBank Accession Number for the deposited sequence is MZ326168.

Discussion

Bacteriophages have been recognized as a promising green technology to control foodborne pathogens (Moye et al., 2018). Selection of candidate phages against foodborne pathogen is he crucial step and is essential for the successful application for food safety. In this study, the phage isolated from sewage

against *Salmonella enteritidis* was given the name NINP13076. This phage was selected for sequencing because of its broad host range and strong lytic activity. In a similar kind of study four phages were isolated from sewage targeting pathogenic bacteria *S. newport* [16].

In our study, the assembly of phage NINP13076 produced a genome of length 161329 bp with a GC content of 44.425% and coverage of 104x. In a study the sequence of whole genome vB_SnwM_CGG4-1 linear dsDNA with 159,878 bp and a GC content of 36.9% (El-Dougdoug et al., 2019). NINP13076 is a circularly permuted linear dsDNA. Maximum numbers of isolated phages have linear ds DNA genomes and belong to the order *Caudovirales* (Hatfull and Hendrix 2011). NINP13076 genome shares similarities with the *Salmonella* phage SSE-121 genome revealing it to be a new member of the SSE121 virus genus. Similar kind of study where the CGG4-1 genome shared 79% and 80% overall nucleotide similarity to *Salmonella* phages STML-198 and S16 indicating a new member of the S16 virus genus (Marti et al., 2013). Another study showed wksl3 phage related to SETP3, SE2,vB_S4nS-Emt1, and SS3e based on homology in terms of genome size and sequence identity (De Lappe et al., 2009; Tiwari et al., 2012; Turner et al., 2012).

RAST study yielded 333 coding sequences of which 285 proteins are hypothetical proteins and 48 were assigned functions which included DNA ligase, capsid protein, endolysin and various other proteins. A similar type of study on annotated genes of CGG4-1 phage did not show homology to any available virulence and lysogenic gene sequences in the NCBI database. This important criterion of phages helps in biocontrol and therapeutic applications (Hagens and Loessner 2010; Monk et al., 2010).

The study on the phylogenetic tree obtained showed a very close relationship with *Salmonella* Phage SSE-121 genome with a distance score of 0.1. A similar study done on *Salmonella* Jumbo-Phage pSal-SNUABM-04 showed that the BLAST search revealed that the phage is related to the *Machinavirus* group with more than 96% similarity. Genetic analysis of the same phage revealed that the phage is having 239,626 base pair genome with 280 putative open reading frames, 76 of which have predicted function and 195 of which have none (Kwon et al., 2021). The genome sequence of *Salmonella* bacteriophage SS3e revealed a linear dsDNA sequence of 40793 bp having 58 open reading frames which are similar to *Salmonella* phages SETP13 and MB78 (Kim et al., 2012).

Study on genomic and Proteomic Characterization of the Broad-Host-Range Salmonella Phage PVP-SE1 showed that 46% of encoded proteins are unique to the phage and genomic sequence shares homology with 140 proteins of *E. coli* (Santos et al., 2011).

Conclusion

NINP13076 genome shares similarities with the *Salmonella* phage SSE-121 genome revealing it to be a new member of the SSE121 virus genus which belongs to the Myoviridae group. The lytic component of the phage genome was identified and the work on the synthesis of lytic enzymes by recombinant technology for commercial application toward food preservation is in progress.

Declarations

Funding

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Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Data availability

All the data generated or analyzed in the current study are available with the corresponding author.

Author Contributions

Naveen Kumar R: Conceptualization, Investigation, Methodology, Ashok Kumar: Acquisition and analysis of data, Tamilazagan: Analysis of sequencing data, Virendra Panpatil: DNA isolation and analysis, Venkatesh K: Interpretation of data, Gauthami Guda: Collection and analysis of data, Uday Kumar P: Project administration, Ranganathan UD: Concept and revision of manuscript Hemalatha R: Resources, Supervision, Sudip Ghosh: Writing, review and editing, Ramalingam B: Validation and editing.

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Figures

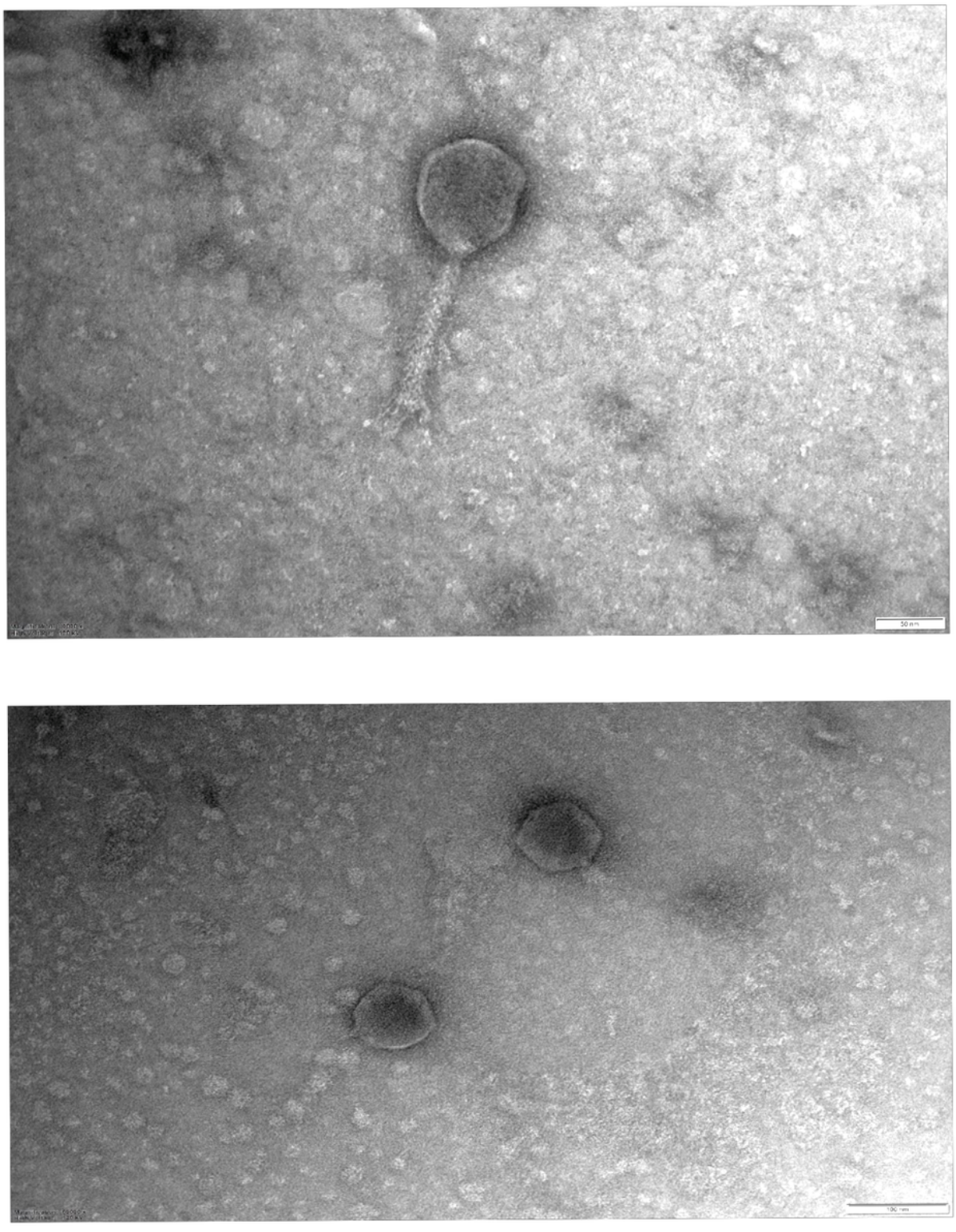

Figure 1

a & b. TEM (Transmission Electron Micrographs) of *Salmonella* specific bacteriophages (100000-260000 magnification) showing the icosahedral shaped head

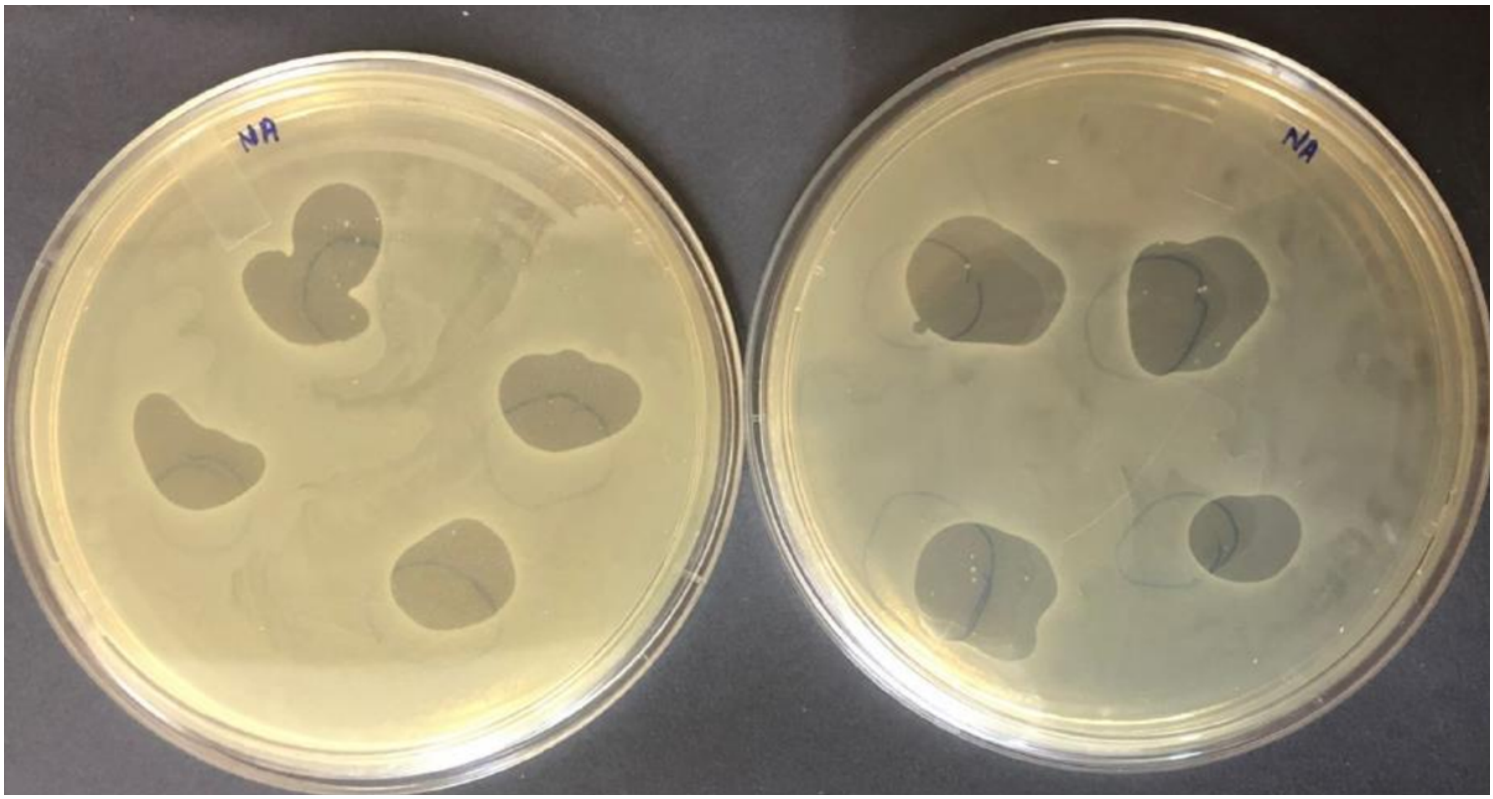

Figure 2

Cleared zones formed by phages on the bacterial lawn in Spot Test Assay

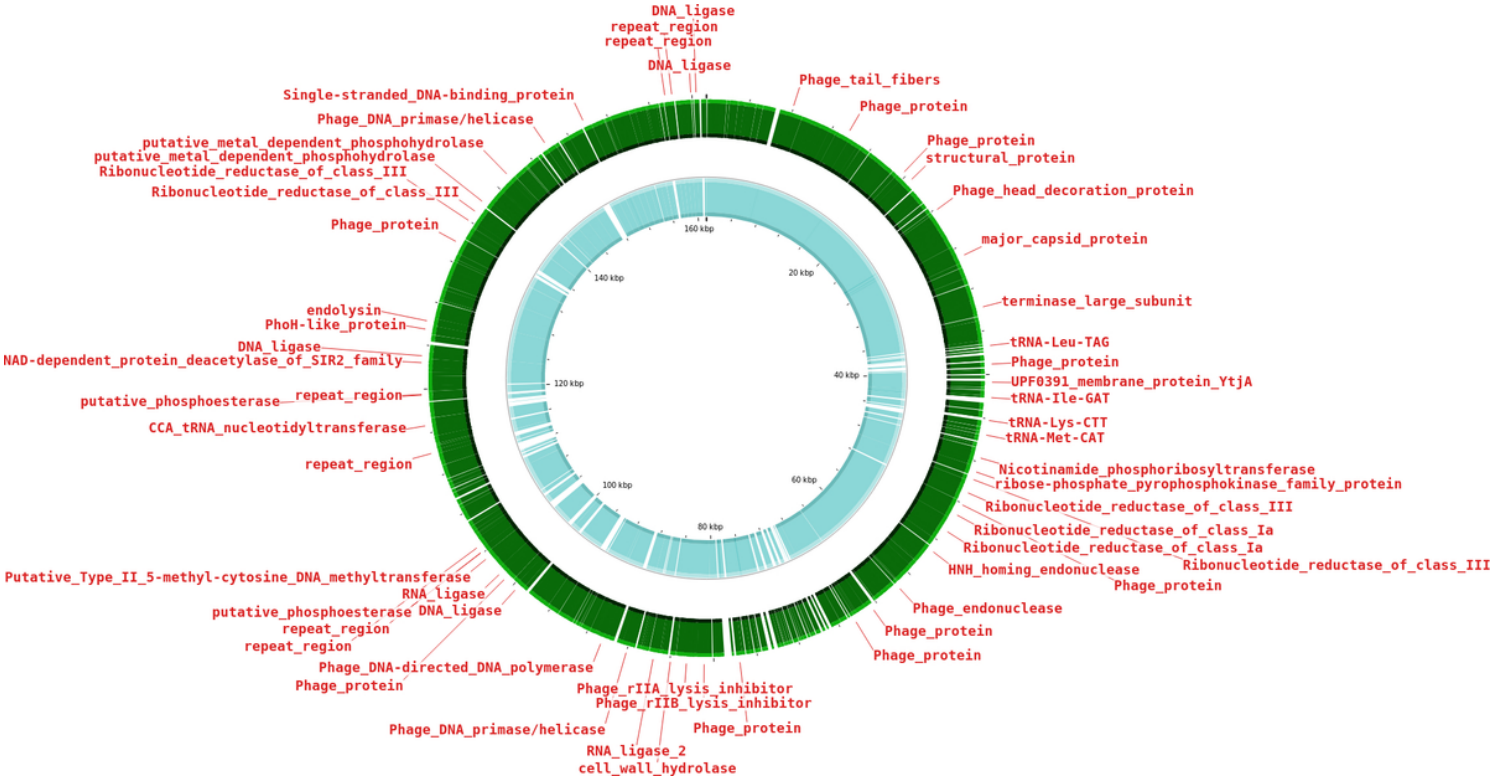

Figure 3

Circular genome view of Phage. The inner ring shows the GC of the genome, the middle ring GC skew of both strands, and the outer ring shows ORFs with strand orientation and functional annotation.

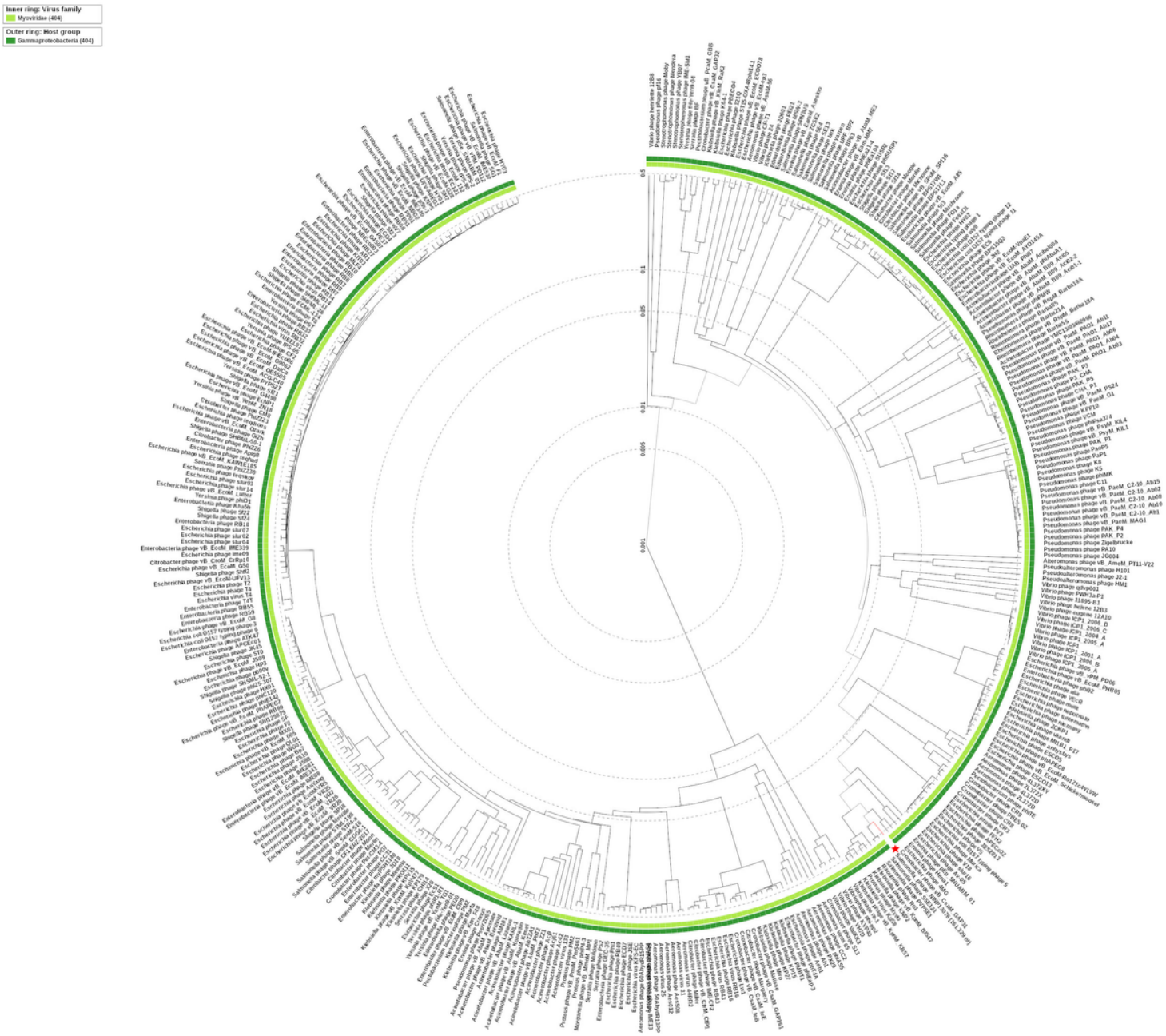

Figure 4

A maximum-likelihood tree for the *Salmonella* phage NINP13076 genome was built using proteomic viral genomes in ViPTree against dsDNA of only the Myoviridae family and Gammaproteobacteria host group for the sake of image clarity

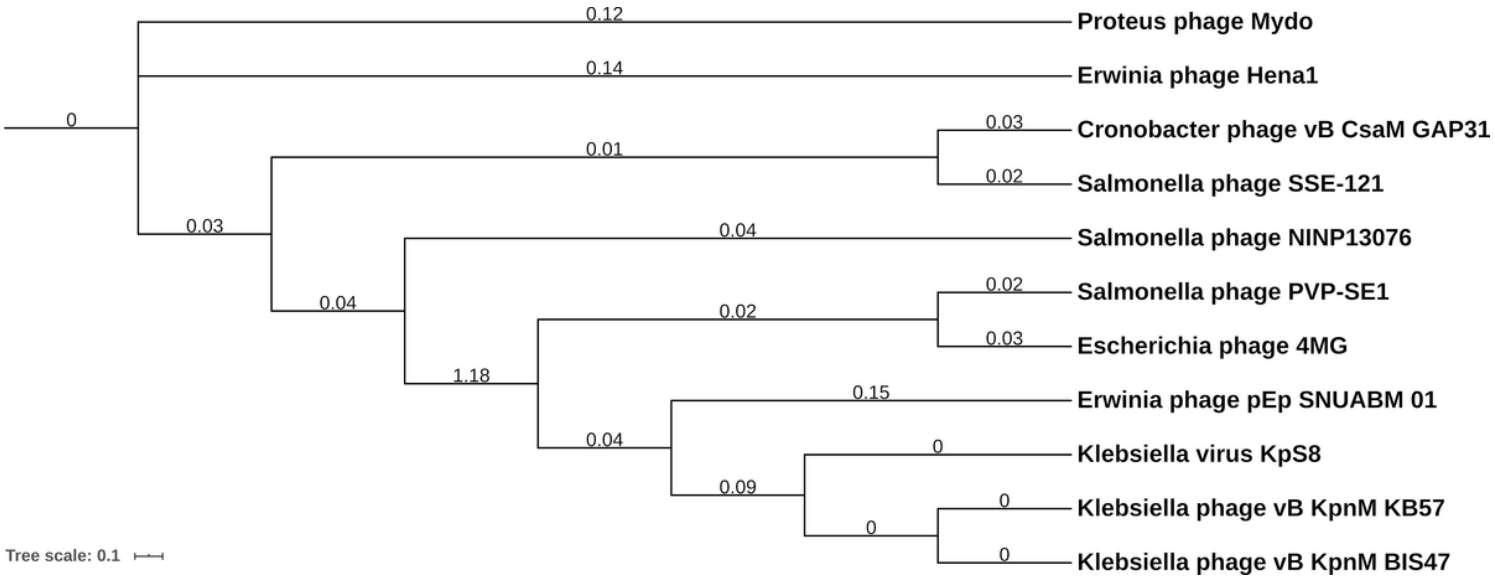

Figure 5

The Maximum-Likelihood tree shows a close relationship with *Salmonella* phage SSE-121 genome

Supplementary Files

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- SupplementaryTable1.xls
- Supplementaryfigure1.jpg