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# Whole genome sequencing of bacteriophage NINP13076 isolated against *Salmonella enteritidis*

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ABSTRACT

*Salmonella* ranks among the prominent etiological agents responsible for foodborne illnesses on a global scale. Within the scope of this investigation, a bacteriophage capable of eliminating *Salmonella enteritidis* was isolated using the double-layer agar overlay technique. The phage's morphological characteristics were elucidated through the application of Transmission Electron Microscopy. The genomic DNA of the phage underwent complete sequencing utilizing the MiSeq platform, with library preparation executed through the NexteraXT library prep kit method accompanied by the NexteraXT index kit. Paired-end sequencing was performed over 2 × 251 cycles read length, employing a Miseq V3 kit within the Illumina MiSeq system. Notably, the phage manifested conspicuous plaques upon *S. enteritidis* when subjected to the double agar overlay technique. NINP13076 displayed a 22-min latency period with a calculated average burst size of 53 PFU/cell. Phages exhibited resilience to the diverse pH conditions, manifesting no discernible impact on their viability over a storage duration of up to one week. storage at temperatures of 4 °C, 26 °C, and 37 °C demonstrated minimal effects on the phage population, with no statistically significant alterations observed. Genome assembly yielded a draft genome encompassing 161,329 base pairs with a GC content of 44.4 % and achieved coverage at a depth of 104x. Phylogenetic tree analysis unveiled a highly proximate relationship with the *Salmonella* Phage SSE-121 genome, demonstrating a distance score of 0.1 and signifying its classification as a novel member within the SSE121 virus group.

## 1. Introduction

*Salmonella* is acknowledged as one of the principal etiological agents of global diarrheal diseases [1]. In line with the 2017 global burden of disease assessment, non-typhoidal *Salmonella enterocolitis* was responsible for an estimated 95.1 million cases, leading to 50,771 fatalities and incurring 3.10 million disability-adjusted life years (DALYs) [2]. Within the United States, *Salmonella* is accountable for 1.35 million infections, 26,500 hospitalizations, and 420 mortalities annually, as reported by the Centers for Disease Control and Prevention [3].

Over the past decade, India has ascended as the predominant consumer of antibiotics globally. This rise is attributed to the heightened prevalence of infectious diseases in India, coupled with the unwarranted use of antibiotics, which has fostered antimicrobial resistance within these pathogenic agents [4]. This scenario has prompted a quest for novel strategies to combat bacterial infections [5]. The utilization of

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bacteriophages has emerged as an innovative modality within the domain of food preservation and safety [6]. Bacteriophages, viral entities infecting bacteria, serve as intrinsic antimicrobial agents and have demonstrably proven their efficacy in the control of foodborne pathogens. Notably, these phages have attained Generally Recognized as Safe (GRAS) status from the United States Food and Drug Administration (USFDA).

The deployment of whole-genome sequencing has become imperative to ascertain the absence of genes encoding established bacterial virulence determinants and potential immunoreactive allergenic components when utilizing bacteriophages as antibacterial agents. Additionally, genome sequencing plays a pivotal role in unraveling various other salient biological attributes and the intricate molecular-level intricacies of the bacteriophage replication cycle [7].

In this particular investigation, we endeavored to isolate a lytic phage with the capacity to eliminate *Salmonella* and meticulously characterize the genetic content of phage genome NINP13076 for subsequent in-depth examination. The information gleaned from the genetic makeup of the isolated phage holds substantial promise for the development of a versatile, broad-spectrum phage capable of mitigating the presence of foodborne pathogens.

## 2. Materials and methods

### 2.1. Bacterial cultures

In this study, the *Salmonella enteritidis* culture used for the host was obtained from the American Type Culture Collection (ATCC-13076). The culture was grown in Tryptic Soy broth media from HIMEDIA, Mumbai, and was kept at a temperature of 4 °C. Additionally, the culture stocks were preserved in a solution containing 50 % Glycerol.

### 2.2. Isolation – double layer agar overlay technique

A 40 ml sample of untreated sewage was aseptically collected in a sterile container manufactured by HIMEDIA, Mumbai, from the municipal sewage treatment plant in Hyderabad. The sample was subjected to centrifugation at 1600*g* for 25 min and filtration was done through a disposable 0.22 μm pore-size filter. To this sample, 5 ml of double-strength nutrient broth and 5 ml of an actively growing culture of *S. enteritidis* in its exponential phase were introduced. The mixture was then incubated at 37 °C for 24 h within a shaker incubator.

Following incubation, a 10 ml portion of the culture was centrifuged at 2000 rpm for 5 min, and the resulting supernatant was drawn into a syringe equipped with a 0.22 μ filter. The filtered liquid was collected into a microcentrifuge tube and stored at 4 °C. The diluted filtrate (100 μl) was combined with 100 μl of the exponential phase culture of *Salmonella*. This mixture was then blended carefully with 3 ml of molten 0.7 % agarose.

The contents of all three components were thoroughly mixed and poured onto a preheated agar plate. The mixture was evenly spread over the surface of the plate and allowed to cool. The plates were subsequently inverted and incubated at 37 °C for 24 h. After incubation, the plates were inspected for the presence of plaques. The total number of plaque-forming units (PFU) per milliliter of the original sample was calculated using the formula: the number of plaques divided by the volume plated and the dilution factor.

### 2.3. 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). This process was done by the method followed by Kumar et al. [8].

### 2.4. Phage host spectrum study

1 ml of an actively growing culture of *S. enteritidis* in its exponential phase was spread onto a nutrient agar plate using the spread plate method. Subsequently, a 10 μl filtrate of the phage was spotted onto the agar and allowed to air dry. The plates were then incubated at 37 °C for a duration of 24 h, during which they were observed for the formation of clear zones, also known as lytic spots, over the bacterial lawn.

The spot test assay was conducted to assess the host spectrum. Reference cultures of various Salmonella strains, including *Salmonella abony* (NCTC 6017), *Salmonella arizonae* (ATCC 13314), *S. typhi* (MTCC 733), *Salmonella poona* (NCTC 4840), *S. enteritidis* (ATCC 13076), and *Salmonella* spp. (MTCC 1162), were obtained from reputable sources such as the National Collection of Type Cultures (NCTC), the Microbial Type Culture Collection (MTCC), and the American Type Culture Collection (ATCC) to facilitate the execution of this experiment.

### 2.5. Phage morphology

TEM (Transmission Electron Microscopy) was used to obtain images of isolated phage at an acceleration of 80 KV. A small drop of phage filtrate ($10^8$ PFU/ml) was loaded on a carbon-coated copper mesh grid. Negative staining phage filtrate was done by using 1 % phosphotungstic acid. This methodology was done by the method followed by Kumar et al. [8].

### 2.6. One-step growth curve

The standard one-step growth curve analysis of bacteriophage NINP13076 was conducted at 37 °C following the methodology outlined by Ellis and Delbruck [9]. *Salmonella* enterica serovar *Enteritidis* (S. enteritidis) strain ATCC-13076 served as the host organism, and phage NINP13076 was introduced into the bacterial culture at a multiplicity of infection (MOI) of 0.01. The latency period and the burst size were then calculated.

### 2.7. Stability of Salmonella phages at different pH and temperatures

The experimental design involved taking 100 μl of phage lysate (with an initial concentration of $10^7$ PFU/ml) and introducing it to 900 μl of buffer solutions at different pH levels (pH 5, 7, and 10). Subsequently, the mixtures were incubated at 37 °C for specified time intervals, namely 1 h, 4 h, 8 h, 24 h, and 1 week. Post-incubation, serial dilutions were prepared for each treatment, and 100 μl of each dilution was combined with 100 μl of an overnight Salmonella culture. This amalgamation was then incubated at 37 °C for 15 min. Following this, the agar overlay technique was implemented on tryptic soy agar (TSA) plates to quantify the phage titre.

Additionally, the impact of temperature on the isolated phages was investigated by incubating 1 ml of the phage lysate (initially at $10^7$ PFU/ml) at different temperatures (4 °C, 26 °C, 37 °C, and 50 °C) for varying durations, specifically 1 day, 1 week, and 2 weeks. After each temperature treatment, the agar overlay technique was executed to ascertain the phage titer. All experiments were conducted in triplicate to ensure the reliability and reproducibility of the results.

### 2.8. Phage DNA extraction

Phage DNA isolation was carried out utilizing a phage DNA isolation kit from NORGEN in Canada. For the DNA isolation process, 1 ml of the phage filtrate was placed into a microcentrifuge tube and treated with 10 μl of DNase. The DNA extraction was performed using a starting DNA concentration of 105 PFU/ml. The phage filtrate was obtained by selecting individual plaques and subsequently introducing them into nutrient broth containing an actively growing *Salmonella* culture. After an overnight incubation period (at 37 °C for 24 h in a shaker incubator)

and subsequent centrifugation at 2000 rpm for 5 min, followed by filtration, the filtrate was obtained. This filtrate was then incubated in a water bath at 75 °C for 45 min. Subsequently, 500 μl of lysate buffer was added to the solution, and it was vigorously vortexed for 10 s. Proteinase K (4 μl) was introduced, and the mixture was incubated at 55 °C for 15 min, followed by another incubation at 65 °C for an additional 15 min. To this lysate, 320 μl of isopropanol was added and vortexed. Centrifugation was performed in a collection tube for 1 min at 8000 rpm, with the flow-through being discarded. This centrifugation step was repeated, and 400 μl of ethanol was added as a wash solution before another round of centrifugation for 1 min at 8000 rpm. This washing step was repeated twice. Finally, 75 μl of elution buffer was added and centrifuged for 1 min at 8000 rpm. The concentration of the phage DNA was measured using a nanodrop, and the purified DNA sample was subsequently employed for genome sequencing.

### 2.9. Whole-genome sequencing

The whole-genome sequencing of phage DNA was conducted on the Miseq platform from Illumina in the United States. To prepare the genomic DNA library, the NexteraXT library prep kit by Illumina, along with the NexteraXT index kit and the standard protocol provided by the manufacturer, were utilized. The concentration of the DNA library was determined using a Qubit instrument from Thermo Scientific in the USA, and the average library size was assessed with a Bioanalyzer by Agilent Technologies in Germany.

The sequencing process involved paired-end sequencing with 2 × 251 cycles read length, utilizing a Miseq V3 kit on the Illumina MiSeq system in Illumina's California, USA facility. Following the completion of the sequence run, the raw data in fastq format for both forward and reverse reads were used for subsequent computational analysis.

### 2.10. Genome assembly and analysis

The raw reads underwent quality control processing, involving the removal of low-quality bases and the elimination of any adapters from clean reads. This was accomplished using Trimmomatic v0.36 [10]. Subsequently, the reads that met the quality control criteria were subjected to de novo assembly using the Unicycler v0.4.8 [11] assembler, specifically designed for Illumina short-read data. The quality of the assembly was assessed using the Quality Assessment Tool for Genome Assemblies (QUAST) v5.0.2 [12]. Genome annotation was performed using the Rapid Annotation Subsystems Technology (RAST) toolkit, which employs a k-*mer*-based search method against CoreSEED [13]. If the k-mer search within FIGfam did not yield any results, a BLAT and BLASTP search was conducted against non-redundant genus-specific protein databases for the organism's genus, with a cutoff of e-value ≤ 1e-5 and a percent identity ≥50 %.

A phylogenetic tree was constructed based on the major Capsid protein DNA sequence, spanning from position 29407 to 28394, utilizing the GTRGAMMA model of RAxML v8.2.4, and 1000 bootstrapping values were employed for robustness assessment [14]. A comparative study was carried out using the NCBI Pairwise Sequence Comparison (PASC) tool [15]. To visually represent the genome, a circular genome map was generated using CGView [16]. For the creation of a whole-genome phylogeny, ViPTree was used, comparing it against reference viral genomes obtained from the Virus-Host DB within the dsDNA category [17]. The GenBank Accession Number for the deposited sequence is MZ326168.

## 3. Results

A specific *Salmonella* phage was isolated from sewage and designated as Salmonella phage NINP13076. This phage formed clear plaques on *S. enteritidis* using the double agar overlay technique. TEM images of freshly prepared phages revealed that Salmonella-specific NINP13076 has isometric heads, resembling members of the Myoviridae family (Fig. 1). In a spot test assay, an inhibition zone appeared where the phage filtrate supernatant was added, indicating that this phage specifically targets *Salmonella enteritidis*, *Salmonella arizonae*, and *Salmonella poona* (Fig. 2). Phage NINP13076 displayed a 22-min latency period with a calculated average burst size of 53 PFU/cell. The *Salmonella*-specific phage with larger plaques and higher transparency was selected for genome sequencing.

The isolated *Salmonell*a phages were subjected to storage in distinct buffers with varying pH values (pH 5, 7, and 9). Remarkably, the phages exhibited resilience to the diverse pH conditions, manifesting no discernible impact on their viability over a storage duration of up to one week. A parallel investigation was undertaken to evaluate the influence of temperature on the isolated phages. Notably, storage at temperatures of 4 °C, 26 °C, and 37 °C demonstrated minimal effects on the phage population, with no statistically significant alterations observed. However, exposure to an elevated temperature of 55 °C for a storage period of 1 day resulted in an approximate 2-log reduction in the NINP13076 phage population (Fig. 6).

The process of phage genome assembly involved several steps. Firstly, raw reads from Illumina sequencing were subjected to quality control (QC). Using trimmomatic with parameters of phred score 15 and a sliding window of 4:20, 20 bases were trimmed from both ends, and adapter sequences were removed. Approximately 95 % of reads were retained after trimming. Paired-end reads that passed QC were then de novo assembled using Unicycler with default parameters. Unicycler employs SPAdes for assembling short reads from Illumina, generating graphs based on various k-mer sizes, and automatically selecting the best graph based on minimal contig count with no dead ends. The assembly produced a draft genome with a length of 161,329 bp, a GC content of 44.425 %, and 104x coverage. To ensure quality, the assembly was assessed using the Quality Assessment Tool for Genome Assemblies (QUAST), which identified 51 contigs.

For ORF prediction and gene annotation, the assembly was submitted to the Rapid Annotation Subsystems Technology toolkit (RASTtk). RASTtk identified 333 coding sequences, including 285 hypothetical proteins and 48 with assigned functions, such as DNA ligase, capsid protein, endolysin, and various other proteins. The genomic features, including 21 tRNA and 8 repeat regions, totaled 362 (Table 1, Supplementary Table 1).

A comparative study using the NCBI PASC tool revealed that the assembled genome shares the highest similarity with Salmonella virus SSE121, at 76.25 %. A circular genome map was generated using CGView, with the outer ring displaying the annotated proteins' functions, ORF content, and similarity with the *Salmonella* Phage SSE-121 genome (Fig. 3). A maximum-likelihood tree for the Salmonella phage

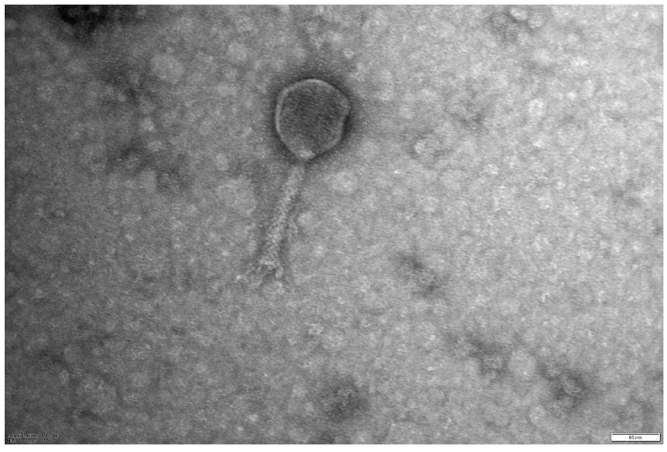

**Fig. 1.** Transmission Electron Micrographs of *Salmonella* specific bacteriophages (100000–260000 magnification).

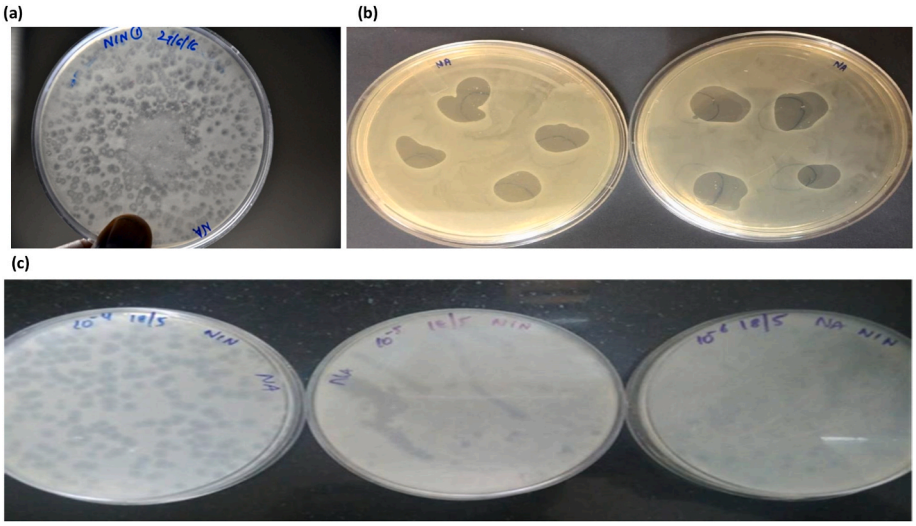


**Fig. 2.** (a). A lawn of *Salmonella* containing plaques formed by lytic phages (region of cell destruction) (b). Cleared zones formed by phages on the bacterial lawn in Spot Test Assay (c). Bacteriophage titer test showing a decrease in PFUs (Plaque Forming Units) with an increase in dilution.

**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 |

NINP13076 genome was constructed using proteomic viral genomes in ViPTree, showing its close relationship to *Salmonella* Phage SSE-121 (Supplementary Fig. 1). Additionally, a maximum-likelihood tree for the Salmonella phage NINP13076 genome was constructed using proteomic viral genomes from the Pseudomonadota host group for image clarity (Fig. 4).

Phylogenetic analysis was performed to study the evolutionary relationship with other phage genomes. The capsid gene sequence was BLAST searched against the nucleotide nr database, and the top 10 hits were selected for multiple-sequence alignment using ClustalW. The resulting alignment was saved in phylip format, and a maximum-likelihood tree was built using the GTRGAMMA model with 1000 bootstrappings via RaxML. The tree demonstrated a close relationship with the *Salmonella* Phage SSE-121 genome, with a distance score of 0.1 (Fig. 5). The genome sequence has been submitted to NCBI, and an accession number has been assigned. The GenBank Accession Number for the deposited sequence is MZ326168.

## 4. Discussion

Bacteriophages have gained recognition as a promising green

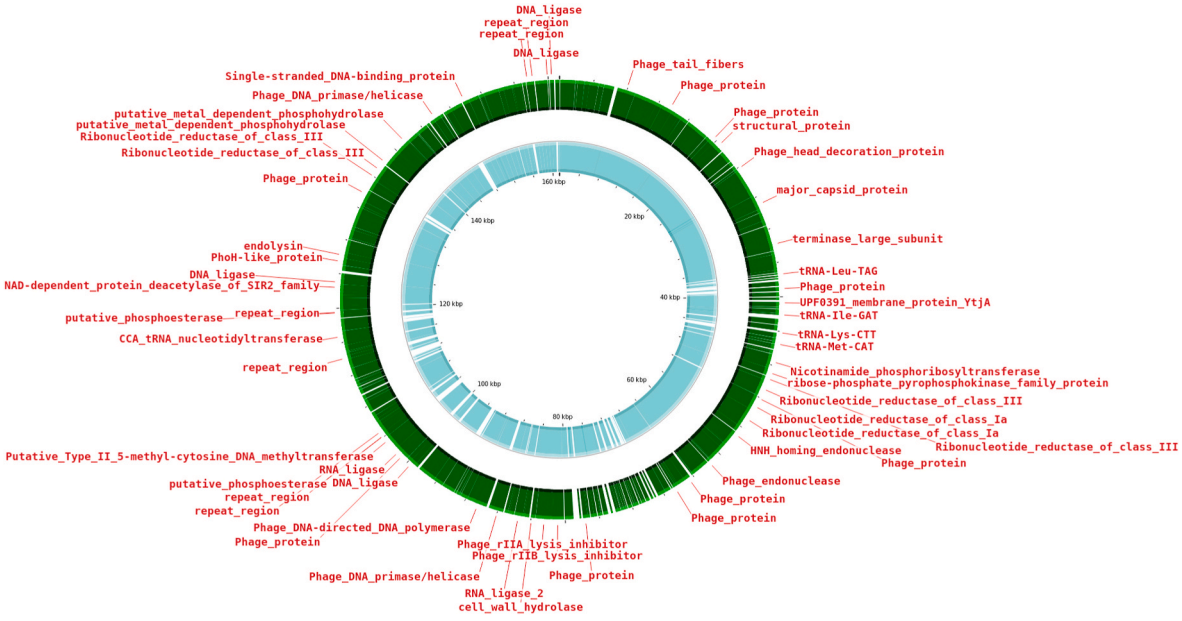


**Fig. 3.** Circular genome view of Phage. The inner ring shows the GC of the genome, the middle ring GC skew of both strands and the outer ring shows ORFs with strand orientation and functional annotation.

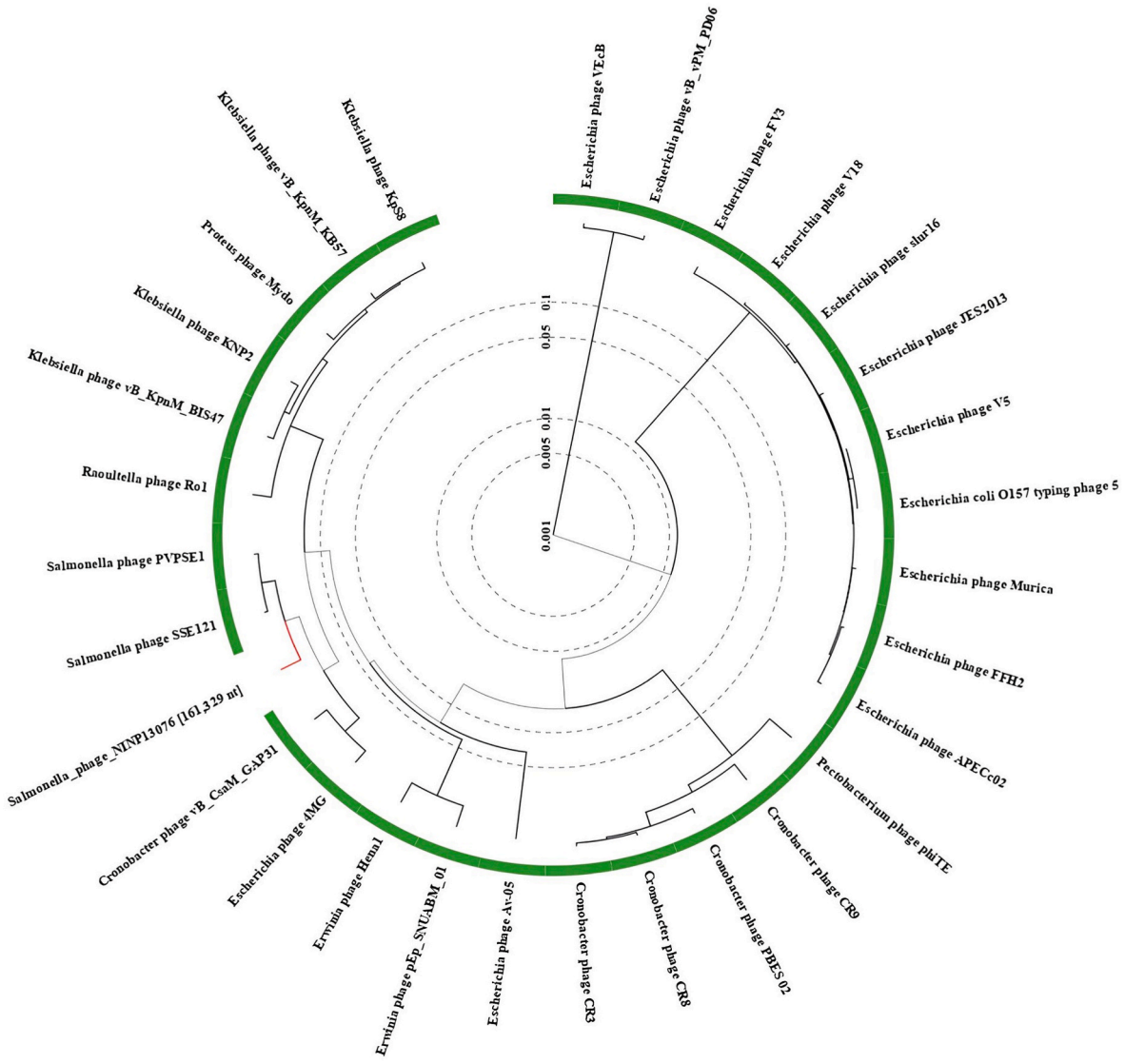


**Fig. 4.** A maximum-likelihood tree for the *Salmonella* phage NINP13076 genome was built using proteomic viral genomes in ViPTree against dsDNA of only the Vequintavirinae family and Gammaproteobacteria host group for the sake of image clarity.

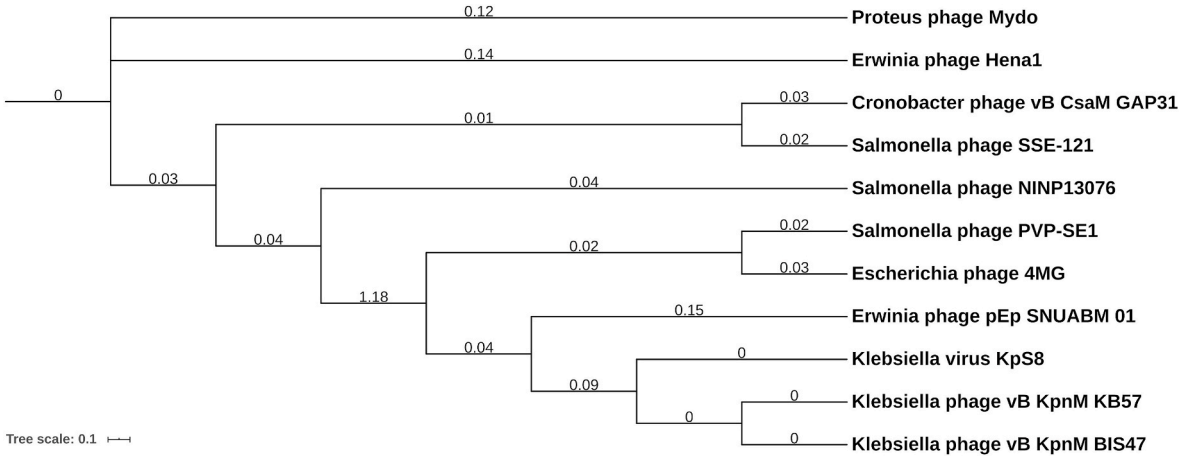


**Fig. 5.** The Maximum-Likelihood tree shows a close relationship with *Salmonella* phage SSE-121 genome.

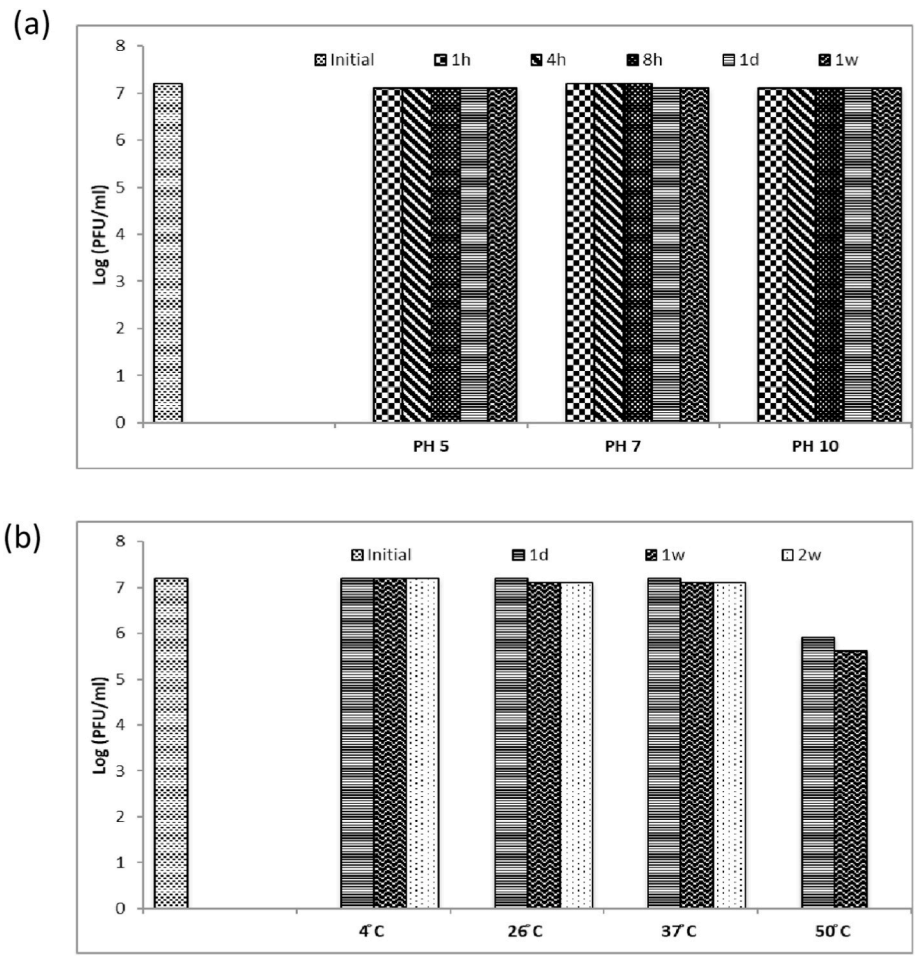


**Fig. 6.** Stability of Salmonella killing phages stored at different pH (a) and temperatures (b).

technology for the control of foodborne pathogens [18]. The selection of suitable phages targeting foodborne pathogens is a critical step essential for ensuring the success of food safety applications. In our research, the phage isolated from sewage targeting *Salmonella enteritidis* was named *Salmonella* phage NINP13076. This particular phage was chosen for sequencing due to its wide host range and strong lytic activity. A similar study involved the isolation of four phages from sewage, which were designed to target pathogenic bacteria *S. newport* [19].

In our study, the assembly of *Salmonella* phage NINP13076 produced a genome with a length of 161,329 bp, a GC content of 44.425 %, and a coverage of 104x. In a comparable study, the whole genome sequence of vB_SnwM_CGG4-1 linear dsDNA resulted in a genome with a length of 159,878 bp and a GC content of 36.9 % [20]. Notably, NINP13076 possesses a circularly permuted linear dsDNA, which aligns with the prevalent observation that most isolated phages have linear dsDNA genomes within the order Caudovirales [21]. The NINP13076 genome exhibits similarities with the *Salmonella* phage SSE-121 genome, suggesting it belongs to the SSE121 virus genus. A similar investigation of the CGG4-1 genome showed 79 % and 80 % overall nucleotide similarity with *Salmonella* phages STML-198 and S16, implying a new member of the S16 virus genus [20] (Marti et al., 2013). Another study revealed the relationship of the wksl3 phage to SETP3, SE2, vB_S4nS-Emt1, and SS3e, based on genome size and sequence identity [22–24].

RAST analysis identified 333 coding sequences in the e, encompassing 285 hypothetical proteins and 48 proteins with assigned functions, including DNA ligase, capsid protein, endolysin, and various others. In a similar study examining annotated genes of the CGG4-1 phage, no homology was found with available virulence and lysogenic gene sequences in the NCBI database. This aspect is crucial for the potential biocontrol and therapeutic applications of phages [25,26].

*Salmonella* phages have been noted to employ various receptors on the surface of *Salmonella* bacteria, including outer membrane protein C (OmpC). These phage tail fibers exhibit notable specificity in recognizing and binding to OmpC on the surface of *Salmonella* bacteria. These tail fibers, often equipped with receptor-binding proteins or domains, are responsible for the initial attachment of the phage to the host cell. The receptor-binding proteins or domains within the tail fibers engage in specific interactions with OmpC, facilitating the initiation of the infection process [27]. Lipopolysaccharides (LPS), complex molecules found on the outer membrane of Gram-negative bacteria, such as *Salmonella*, are composed of lipid A, core oligosaccharide, and O-antigen polysaccharide. LPS plays a vital role in bacterial physiology and interactions with the environment. In the context of *Salmonella* phage infection, specific phages have been reported to target distinct structures, particularly the core oligosaccharide regions. However, these LPS-related receptors may vary among different *Salmonella* phages and strains. It is important to note that the assumptions regarding how this phage utilizes its tail fibers to recognize these LPS regions require validation [28].

The phylogenetic tree analysis in our study revealed a close relationship with the *Salmonella* Phage SSE-121 genome, with a distance score of 0.1. In a related investigation, *Salmonella* Jumbo-Phage pSal-SNUABM-04 was found to have over 96 % similarity to the Machinavirus group based on a BLAST search. Genetic analysis of the same phage showed a genome of 239,626 base pairs with 280 putative open reading frames, 76 of which had predicted functions, while 195 had none [29]. The genome sequence of *Salmonella* bacteriophage SS3e, on the other hand, featured a linear dsDNA sequence of 40,793 bp with 58 open reading frames, sharing similarities with *Salmonella* phages SETP13 and

MB78 [30].

A study involving the genomic and proteomic characterization of the Broad-Host-Range *Salmonella* Phage PVP-SE1 revealed that 46 % of encoded proteins are unique to the phage, and its genomic sequence exhibits homology with 140 proteins from E. coli [3]."

## 5. Conclusion

The phage isolated in the present study demonstrated robustness against a spectrum of pH and temperature variations, emphasizing its capacity to endure diverse environmental conditions. The genome of *Salmonella* phage NINP13076 displays similarities with the *Salmonella* phage SSE-121 genome, classifying it as a novel member of the SSE121 virus genus, which is part of the Vequintavirinae group.

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## CRediT authorship contribution statement

**Naveen Kumar R:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Ashok Selvaraj:** Supervision, Software, Formal analysis, Data curation. **Tamilzhalagan Sembulingam:** Validation, Software, Methodology, Formal analysis. **Virendra Panpatil:** Validation, Software, Resources, Formal analysis. **Kaliaperumal Venkatesh:** Writing – review & editing, Validation, Resources, Formal analysis. **S.D.G. Gowthami:** Validation, Investigation, Formal analysis, Data curation. **Uday kumar Putchaa:** Writing – review & editing, Visualization, Validation, Supervision, Resources. **Uma Devi Ranganathan:** Validation, Supervision, Resources, Investigation, Formal analysis. **Hemalatha Rajkumar:** Writing – review & editing, Visualization, Supervision, Resources, Project administration. **Sudip Ghosh:** Writing – review & editing, Validation, Supervision, Software, Resources, Methodology. **Ramalingam Bethunaickan:** Writing – review & editing, Validation, Supervision, Software, Resources, Data curation.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Data availability Data will be made available on request.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.egg.2024.100223.

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