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Exploring lytic *Salmonella* phages as potential alternatives to antibiotics: Isolation, characterization, and stability assessment of *Jerseyvirus* Ijeoma and *Apdecimavirus* Ayanbimpe

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Abstract:

Background: Typhoid fever, caused by antibiotic-resistant *Salmonella enterica* serovar Typhi (*Salmonella* Typhi), is a significant public health issue, especially in Nigeria, necessitating exploration of alternative treatment strategies such as the use of bacteriophages. Bacteriophages are viruses that specifically infect and replicate within bacteria and play important roles in medicine. The objective of this study is to isolate and characterize two lytic phages against *S*. Typhi, assess their stability under various environmental conditions and determine their potentials as biocontrol agents and alternatives to antibiotics for treatment of *S.* Typhi infections.
Methodology: *Salmonella* Typhi strains previously isolated from stool samples of patients in Jos, Nigeria, were used. Phages were isolated from hospital and poultry wastewater samples using the double-layer agar method. Phage purification and stock preparation were performed using the double-layer agar overlay technique. Stability was assessed by incubating phages at different temperatures, pH levels, and NaCl concentrations. The DNA of the phages were extracted using the DNeasy Blood and Tissue Kit (Qiagen) and the whole genome was then sequenced using the Illumina NextSeq 500 platform. Bioinformatic analysis was conducted using various computational tools.
Results: Two phages, *Jerseyvirus* Ijeoma and *Apdecimavirus* Ayanbimpe, were isolated and characterized. The phages were stable at 4°C-37°C which serves as control and maintained a reasonable titre at 45 and 55° C. Phages were also stable at 0.5% NaCl and pH 8 for at least one hour. *Jerseyvirus* Ijeoma, with a genome size of 41,747 bp and 56 coding sequences (CDS), showed 81% similarity to *Jerseyvirus* SS3e. *Apdecimavirus* Ayanbimpe, with a genome size of 39,601 bp and 48 CDS, exhibited 83% similarity to *Apdecimavirus* AP10.
Conclusion: Both phages demonstrated good stability under various abiotic factors, maintaining reasonable titres in alkaline and high-salt conditions and at elevated temperatures. *Jerseyvirus* Ijeoma and *Apdecimavirus* Ayanbimpe show potential as biocontrol agents and alternatives to antibiotics for treating *S*. Typhi infections. Their stability and tolerance to diverse conditions make them suitable candidates for phage therapy.

Keywords: Lytic Phages, Phage Stability, Typhoid fever, Antibiotic resistance, Phage Genome, *Salmonella* Typhi

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Exploration des phages lytiques de *Salmonella* comme alternatives potentielles aux antibiotiques: Isolement, caractérisation et évaluation de la stabilité de *Jerseyvirus* Ijeoma et d'*Apdecimavirus* Ayanbimpe

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Résumé:

Contexte: La fièvre typhoïde, causée par *Salmonella enterica* sérovar Typhi (*Salmonella* Typhi), une bactérie résistante aux antibiotiques, représente un problème de santé publique majeur, notamment au Nigéria, ce qui rend nécessaire l'exploration de stratégies thérapeutiques alternatives telles que l'utilisation de bactériophages. Les bactériophages sont des virus qui infectent et se répliquent spécifiquement au sein des bactéries et jouent un rôle important en médecine. L'objectif de cette étude est d'isoler et de caractériser deux phages lytiques contre *S.* Typhi, d'évaluer leur stabilité dans différentes conditions environnementales et de déterminer leur potentiel en tant qu'agents de biocontrôle et alternatives aux antibiotiques pour le traitement des infections à *S.* Typhi.
Méthodologie: Des souches de *Salmonella* Typhi précédemment isolées d'échantillons de selles de patients de Jos, au Nigéria, ont été utilisées. Les phages ont été isolés à partir d'échantillons d'eaux usées hospitalières et d'eaux usées d'élevages avicoles par la méthode de la double couche d'agar. La purification des phages et la préparation des stocks ont été réalisées par la technique de la double couche d'agar. La stabilité a été évaluée en incubant les phages à différentes températures, différents pH et différentes concentrations de NaCl. L'ADN des phages a été extrait à l'aide du kit DNeasy Blood and Tissue (Qiagen), puis le génome entier a été séquencé sur la plateforme Illumina NextSeq 500. Une analyse bioinformatique a été réalisée à l'aide de divers outils de calcul.
Résultats: Deux phages, *Jerseyvirus* Ijeoma et *Apdecimavirus* Ayanbimpe, ont été isolés et caractérisés. Les phages étaient stables entre 4°C et 37°C (température servant de contrôle) et ont conservé un titre satisfaisant à 45°C et 55°C. Ils étaient également stables à 0,5% de NaCl et à pH 8 pendant au moins une heure. *Jerseyvirus* Ijeoma, dont le génome mesure 41747 pb et comporte 56 séquences codantes (CDS), présente une similarité de 81% avec *Jerseyvirus* SS3e. L'*Apdecimavirus* Ayanbimpe, dont le génome mesure 39601 pb et comporte 48 CDS, présente une similarité de 83% avec l'*Apdecimavirus* AP10.
Conclusion: Les deux phages ont démontré une bonne stabilité face à divers facteurs abiotiques, conservant des titres satisfaisants en milieu alcalin, en forte salinité et à températures élevées. Le *Jerseyvirus* Ijeoma et l'*Apdecimavirus* Ayanbimpe présentent un potentiel en tant qu'agents de biocontrôle et alternatives aux antibiotiques pour le traitement des infections à *S.* Typhi. Leur stabilité et leur tolérance à diverses conditions en font des candidats prometteurs pour la phagothérapie.

Mots-clés: Phages lytiques, Stabilité des phages, Fièvre typhoïde, Résistance aux antibiotiques, Génome phagique, *Salmonella* Typhi

Introduction:

Typhoid fever is an infectious disease caused by the Gram-negative bacterium, *Salmonella enterica* subspecies *enterica* serovar Typhi (*S*. Typhi). Typically, this infection is contracted through the consumption of contaminated food or water sources (1). Typhoid fever remains a major concern in Nigeria due to urbanization, insufficient water, regional migration, poor waste management, strained health care, and antibiotic overuse, fostering antibiotic-resistant *S.* Typhi (2). Annually, there are approximately 11 million reported cases of typhoid fever globally, leading to over 100,000 fatalities (3). The greatest burden of this disease is observed in low- and middle-income countries (LMICs), where access to clean water and proper sanitation facilities is often limited (4). The effectiveness of treating typhoid fever with antibiotics is progressively diminishing due to the emergence of drug-resistant strains (5). There is, therefore, need to explore alternatives to antibiotics in the control and management of typhoid fevers.

With rise in multi-drug-resistant *S*. Typhi, bacteriophages (phages), with their potent bactericidal activity, host specificity, self-limiting nature, and genetic manipulability, are emerging as promising treatments of bacterial infections in humans and poultry, offering potential alternatives to antibiotics (6). Phages have been used to eliminate *Salmonella* in *Galleria* model (7). A study has shown that *Salmonella* phage reduced the burden of salmonellosis in a mammalian host by inhibiting *S.* Typhi invasion into the liver and spleen tissue (8). In this study, two lytic phages against *S*. Typhi were isolated from hospital and wastewater, their stability in temperature, pH, NaCl and genomic characteristics were reported.

Materials and method:

Bacterial strain isolation and characterization:

In this study, *Salmonella* Typhi strains SO1 previously isolated from Jos by Olorundare et al., (9), and which genome sequence was determined and deposited into the GenBank (Bio Sample/Accession number SAMN168652 06) were used. Approval for this previous study was obtained from the State Emergency Management Agency (SEMA) Plateau State, Nigeria. Informed consent forms and questionnaire were administered to the individuals recruited from the study population (internally displaced camp). All methods were carried out in compliance with the applicable guidelines and regulations.

Bacteriophage isolation protocol:

Sewage samples are always ideal for isolation of phages for enteric bacteria since they contain diverse enteric bacterial hosts

(10). In this study environmental samples were used as source of isolation of phages in the hospital and poultry since isolates are commonly found in this environment and phages are specific and are found where the organisms are present.

The isolation of *Salmonella* phages from hospital and poultry samples was carried out using the standard spot and double-layer agar method (11) with slight modifications. Initially, 50 ml each of hospital and poultry sample were collected and centrifuged at 10,000×g for 10 minutes to eliminate debris. For phage isolation, the supernatant was filtered using a 0.45µm syringe filter. 10ml of filtrate was then mixed with 10ml of double-strength brain heart infusion (BHI) broth supplemented with $CaCl_2$ and an overnight 100 µl of logarithmic growth phase of *S.* Typhi culture was added. This mixture was incubated at 37°C for 48 hours with gentle shaking at 50 rpm. After of incubation, the sample was centrifuged, and supernatant was collected and filtered using a 0.45µm syringe to obtain the phage lysate.

To detect the phages, the double-agar method was employed. Specifically, 100µl of logarithmic growth phase *S.* Typhi grown overnight was added to 5 ml of soft-top agar [Brain Heart Infusion Agar (BHIA) Lifesave Biotech /USA, containing 0.4% (w/v) agar] and then poured onto solid bottom agar [BHIA containing 1.5% (w/v) agar]. The 10 µl phage lysate was spotted on the *S.* Typhi double agar plate and incubated at 37°C for 24 hours. Zones of lysis/clearance on bacterial lawns were observed the next day. Finally, lysed zones were scraped out, transferred to 1 ml sodium magnesium (SM) buffer, and incubated at 4°C for 30 minutes to allow phage particle to diffuse and then filtered through 0.45µm filters.

Plaque purification and phage stock preparation:

The double layer agar overlay technique was employed for the subsequent purification of plaques from two *S.* Typhi phages, namely *Jerseyvirus* Ijeoma and *Apdecimavirus* Ayanbimpe. A single plaque from the phage-agar overlay plate was carefully picked using a standard pipette tip, and the resulting agar plug was expelled into 300 µl of SM buffer (pH 7.0). Afterward, the tubes were incubated at 4°C overnight to allow phage to diffuse. This was followed by centrifugation at 10,000 ×g at 4°C for 10 minutes. The supernatant was then filtered through 0.45µm syringe filters and stored at 4°C until further utilization. The phages were purified three times to obtain single plaques (12).

Phage stability assay:

The stability of *Jerseyvirus* Ijeoma and *Apdecimavirus* Ayanbimpe was evaluated through incubation at different temperatures, pH values, and NaCl concentrations, following a previously established protocol (13) For the assessment of thermal stability, 10^9 pfu/ml of each phage was subjected to incubation at 4°C, 37°C, 45°C, 50°C, and 55°C for 1 hour. Phages at 4°C and 37°C were considered as control since it's the temperature used in the storage of bacteria stock and incubation respectively.

To evaluate their ability to withstand various pH values, SM Buffer solutions with pH levels of 2, 4, 6, 8, 10 and 12 were prepared, adjusting the pH with either 6 N HCl or 6 M NaOH. The phages were suspended in these buffers to a final concentration of 10^9 PFU. The pH solutions were then incubated at 37°C for 1 hour, and the surviving phages were immediately quantified using the spot assay technique and double layer agar overlay plating method, as described previously. Phage suspended in SM buffer at pH 7 was used as control.

To assess phage stability in salt, phage at 10^9 pfu were suspended at different concentrations of NaCl (0.5% 5%, 10%, and 15%). The solutions were incubated at 37°C for 1 hour, and the titre of the phages were determined by spot assay. Phage in SM buffer was used as control. The results are presented as the average of triplicates.

Sequence and bioinformatic analysis:

DNA extraction was performed during the sequencing process using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Following that, a sequencing library was painstakingly created using the Nextera XT DNA Sample Preparation Kit (Illumina) in accordance with the manufacturer's methodology. The whole genome was then sequenced using the Illumina NextSeq 500 platform at the Antimicrobial Resistance Laboratory within the Microbial Genomics Reference Laboratory, CIDMLS, ICPMR, Westmead Hospital (Australia). The raw data underwent trimming through Fastp (14) and assembly using SPAdes v3.13.0 (15) Evaluation of genome quality was conducted using FastQC (v0.11.7) Assembly statistics were generated through BBMap (v38.88) (16), SAM tools (v0.1.8) (17), and QUAST (v5.2.0) (18).

Subsequently, BACterioPHage Lifestyle Predictor (BACPHLIP v0.9.6) (19) was employed to predict the lifestyle (temperate or virulent) of each phage, leveraging a local dataset comprising 634 phage genomes (20). BACPHLIP identifies conserved domains and utilizes them in predicting lifestyle through a random forest classifier. Genome annotation was carried out utilizing Prokka v1.14.6 (21) with PHROGS (Prokaryotic Virus Remote Homologous Groups database) (22) using a previously established method (23), and the results were

visualized with Proksee Genome relatedness was determined through BLAST, and the genome sequence with the highest similarity percentage was selected for downstream analyses to ascertain if the phages were unique. Phage genomes displaying varying degrees of similarity were analyzed using the VIRIDIC (24) web-based version with default settings. Genome visualization was performed using a linear genome plot (20) accessed via https://cpt.tamu.edu/galaxy page

Results:

Isolation of phages:

Two phages were isolated from hospital and poultry sewage specific to *S.* Typhi isolate in Jos, Plateau State, Nigeria. The plaques morphology appears to be large, clear and round on the lawn of *S.* Typhi (Fig 1B), distinct plaques were selected, purified and stored for use.

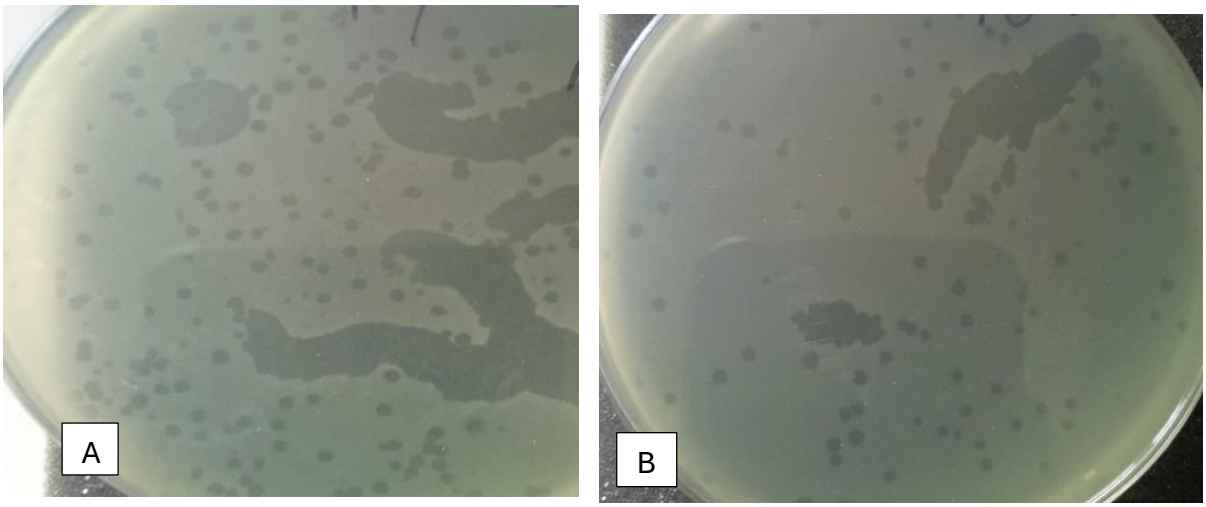

Fig 1: Plaque morphology of *Apdecimavirus* Ayanbimpe on lawn of *Salmonella* Typhi (A); Plaque morphology of *Jerseyvirus* Ijeoma on lawn of *Salmonella* Typhi (B)

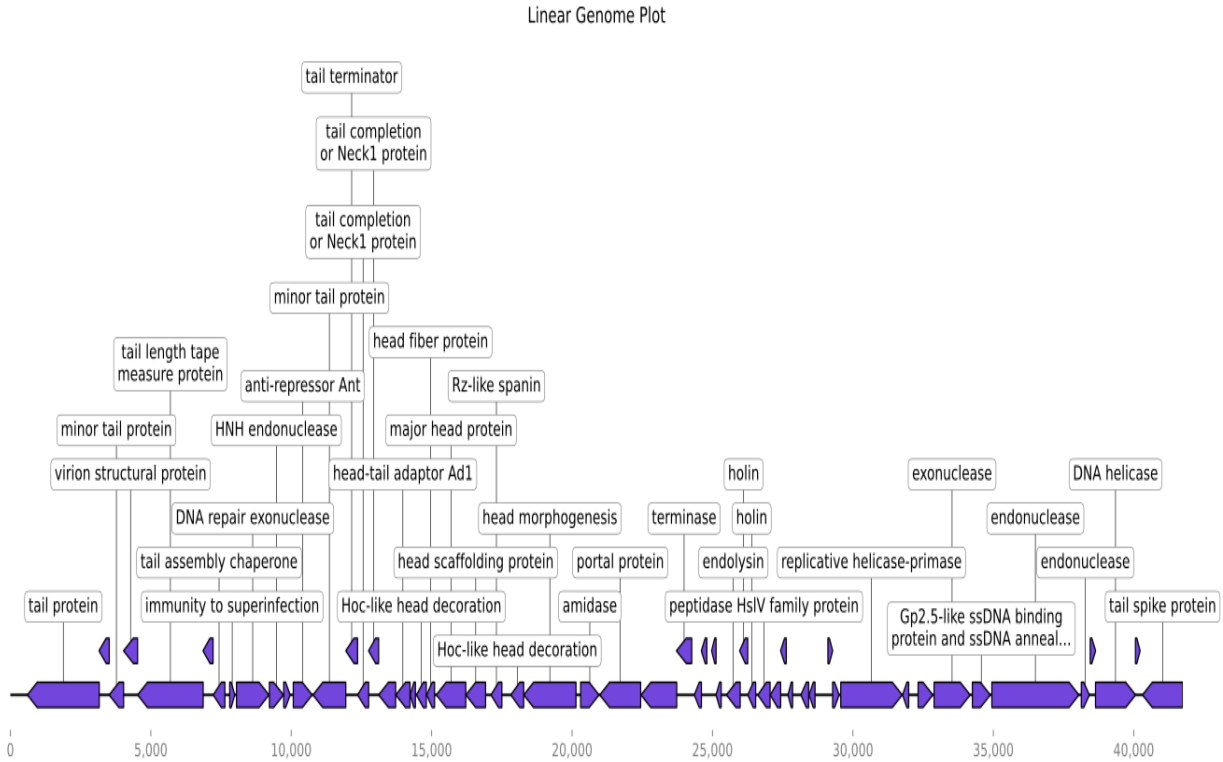

Fig 2: Genome map for *Jerseyvirus* Ijeoma generated using linear genome plot tool

Genomic characterization of phages:

The genome sequence for phage *Jerseyvirus* Ijeoma (Fig 2) isolated from poultry sewage is a double stranded DNA (dsDNA), with a genome size of 41,747 bp, 56 CDS and G+C content 49.99%. The genomic sequence for phage *Apdecimavirus* Ayanbimpe (Fig 3) isolated from hospital sewage is a double stranded DNA (dsDNA) with genomic size of 39,601 bp, 48 CDS and G+C content of 50.72%.

The phylogenic result of the isolated *Salmonella* phages using VIRIDIC showed *Apdecimavirus* Ayanbimpe is 83% similar to *Apdecimavirus* AP10 (KT852574.1) isolated from pig manure in Canada (Fig 4A). *Jerseyvirus* Ijeoma shares 81% similarity to *Jerseyvirus* SS3e isolated from fecal residue in Chile with accession number KR270151 (Fig 4B).

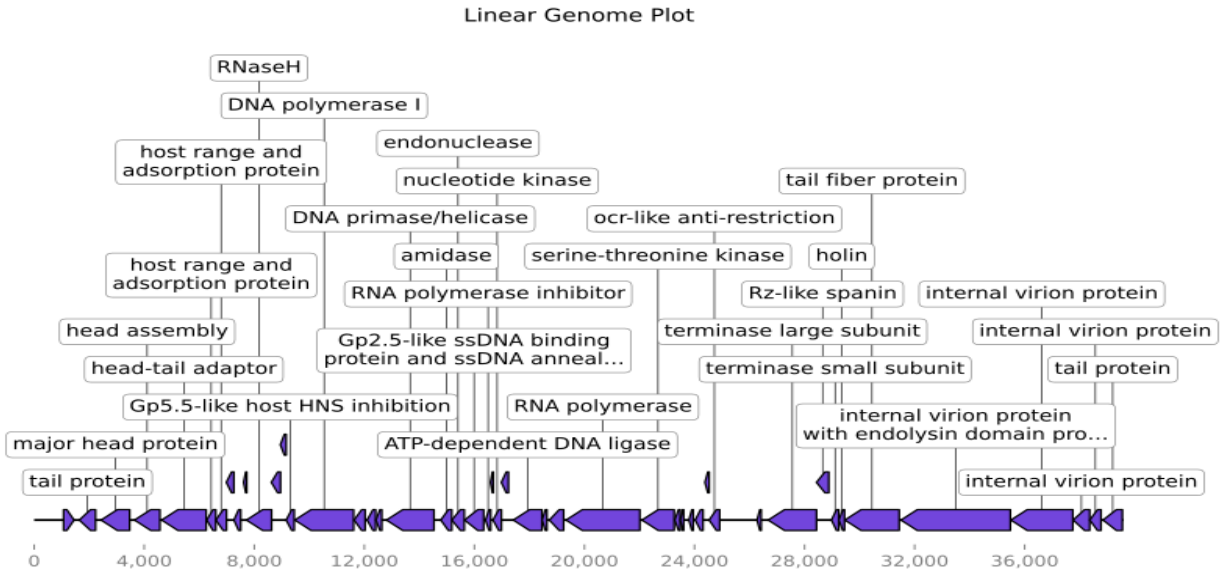

Fig 3: Genome map for *Apdecimavirus* Ayanbimpe generated using linear genome plot tool

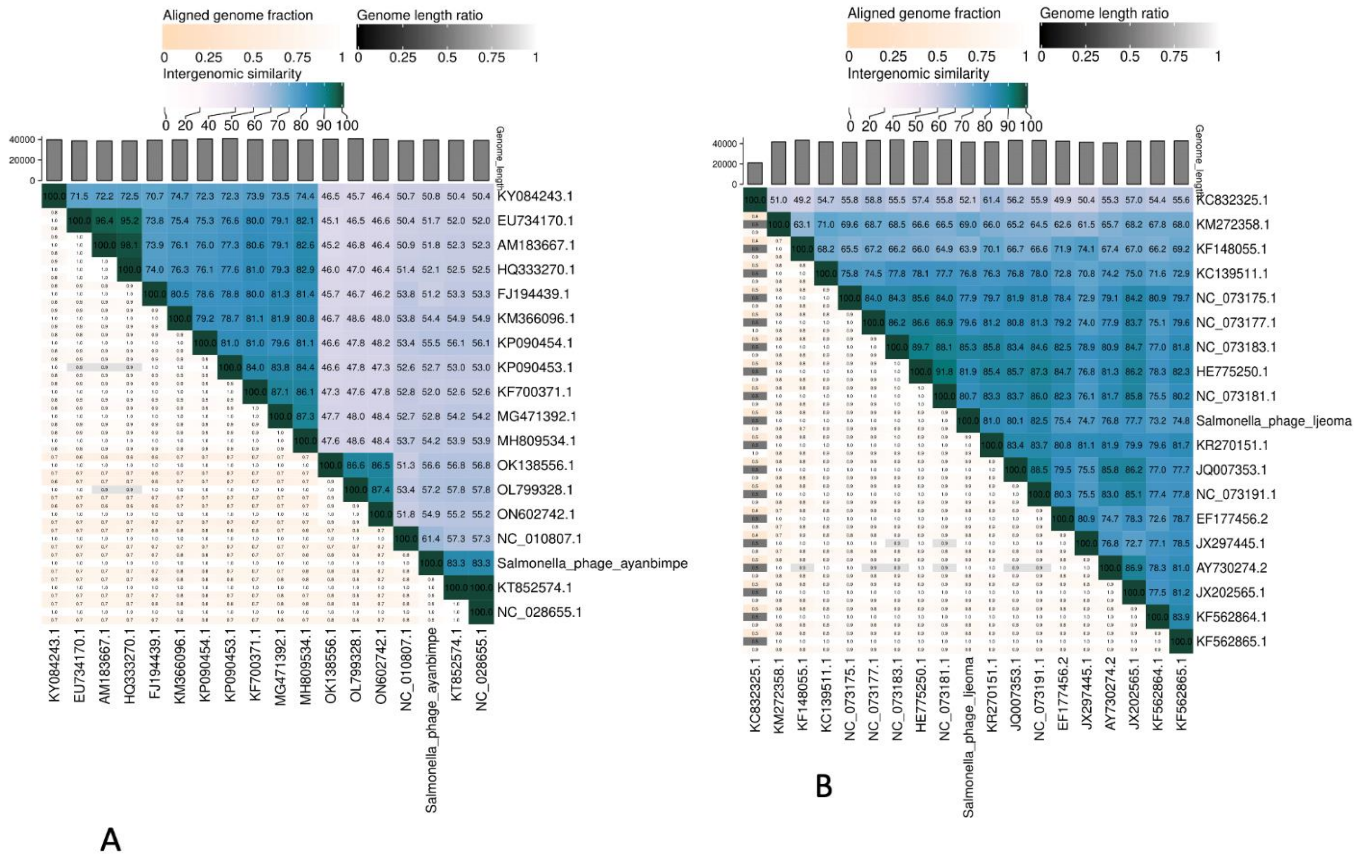

Fig 4 Taxonomic classification of *Apdecimavirus* Ayanbimpe (A) and *Jerseyvirus* Ijeoma (B) using VIRIDIC

Phage stability assay:

The stability of the phages at various abiotic factors (Figs 5,6,7) shows that *Jerseyvirus* Ijeoma exhibited consistent growth with an increase in pH, reaching an optimal growth of 7 log10 PFU/ml. The minimal growth of 2 log10 PFU/ml was observed at pH 2. Conversely, *Apdecimavirus* Ayanbimpe displayed its highest growth at pH 8, reaching about 9 log 10 PFU/ml, and the lowest growth at pH 2, with 5 log10 PFU/ml (Fig 5).

When subjected to varying salt concentrations (Fig 6), *Jerseyvirus* Ijeoma demonstrated its highest growth rate of 9 log 10 PFU/ ml at 0.5% NaCl, 7 log10 PFU/ml at 5% NaCl, and the lowest growth of 3 log10 PFU/ml at 15% NaCl. Similarly, *Apdecimavirus* Ayanbimpe exhibited a parallel pattern, achieving the highest titre of 9 log10 PFU/ml at 0.5% NaCl, 7 log10 PFU/ml at 5% NaCl and the lowest titre of 4 log10 PFU/ml at 15% NaCl. Both phages maintained a high titre at 45°C but experienced a decline at 55°C (Fig 7).

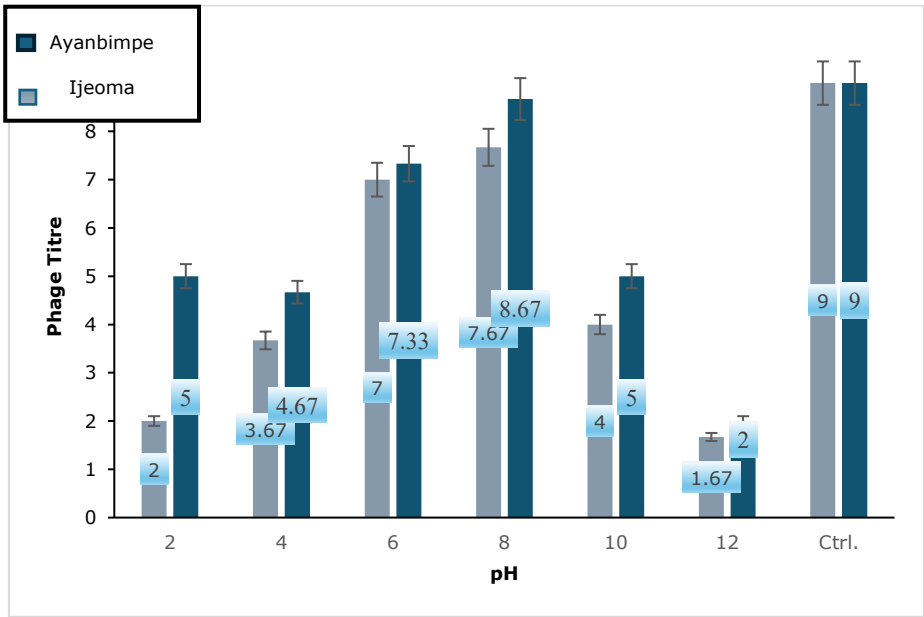

Fig 5: Stability of phages at different pH

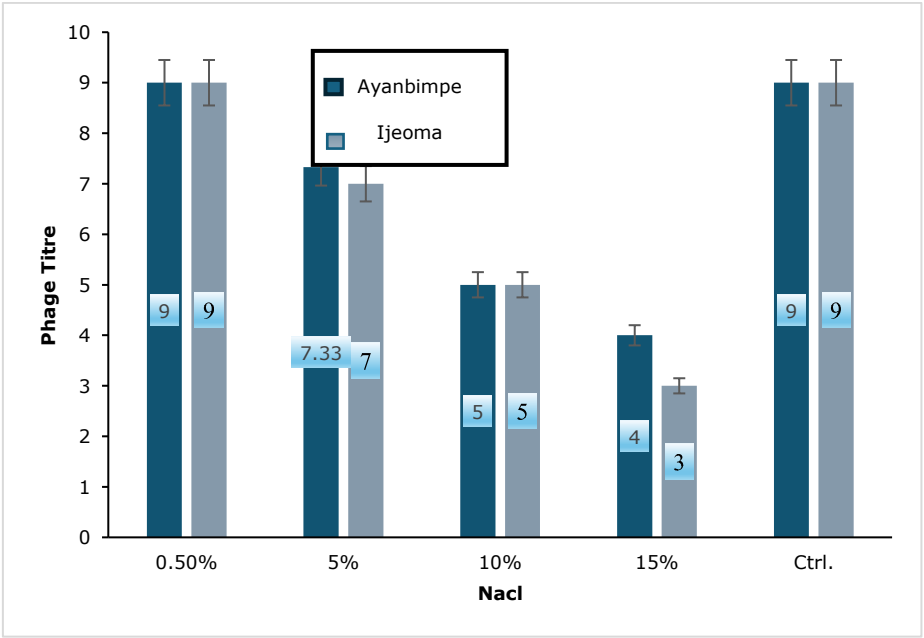

Fig 6: Stability of phages at different NaCl concentrations

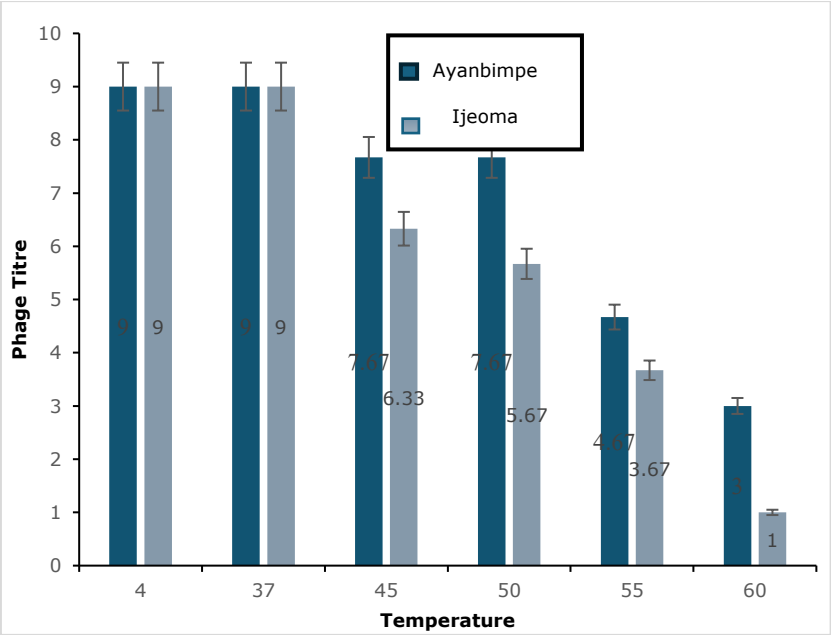

Fig 7: Thermal stability of phages

Discussion:

The two phages were isolated from sewage samples in Jos, Nigeria and were characterized and named as *Jerseyvirus* Ijeoma and *Apdecimavirus* Ayanbimpe according to the binomial nomenclature for phages (25). These phages can be used as a surveillance tool (26). Although the phages are not novel, the predicted life cycle of the phages showed that they are lytic phages which makes them a good potential antibacterial agent and suitable for phage therapy use. In Africa and Asia, the effects of invasive non-typhoidal and typhoidal *Salmonella* infections on public health are especially notable, as they have a major impact on morbidity and death (27).

The burden of typhoid fever is high in low- and middle-income countries (LMICs), including those in these regions, where an estimated 17.8 million cases are recorded annually (28). Particularly in Sub-Saharan Africa, typhoid fever poses a serious threat, with an estimated yearly incidence of more than 100 cases per 100,000 people and a 1% fatality rate (29). The presence of drug resistance *S.* Typhi in Africa makes the need for increased research in phage therapy. Mondal, et al., (8) in their study showed that *S.* Typhi phages increased the survivability of infected mice and effectively reduced bacterial cell numbers, cell culture density, and biofilm formation *in vitro* (30). The stability of phages is significant because it influences the killing activity, and the suitable application can be determined based on their stability (31).

The two phages in our study exhibited good tolerance to alkaline and high-salt conditions, and high stability in low salt concentration (0.5–5%) but increasing the concentration (10-15%) led to gradual reduction in phage activity. Their resilience in alkaline environments enhances their potential as effective biocontrol agents in food and processing settings. In the food industry, phages serve significant roles not only in "starter culture mixes" but also as "in-process agents of concern," particularly in fermentation products (32). Saline stability was observed in studies conducted by Lu and Breidt (33) where high levels of salt, in some cases did not affect the phage titre. Additionally, the phages maintained reasonable titres even at elevated temperatures, their thermal stability qualifies them for growth in elevated temperatures, further expanding their applicability in conditions associated with feverish environments.

Genome analysis helps to determine phage suitability and safety for phage therapy (34) or if it harbors any genes that encode for antimicrobial resistance and virulence genes. Some concerns regarding using phage with harmful genes can be solved by complete genomic characterization of the phage with genome sequencing, annotation of the genome and determining the functions of genes. The knowledge of the functions of the genes will determine the suitability of phage for therapy. In this study, the two phages isolated showed no lysogenic genes or genes that encode virulence factors or antibiotic resistance. This indicates that the isolated phages could be considered genomically safe for further investigations in the control of *S.* Typhi.

Conclusion:

Two lytic *Salmonella* phages from the

Apdecimavirus and *Jerseyvirus* families, respectively, were isolated and examined in this study, and were named *Jerseyvirus* Ijeoma and *Apdecimavirus* Ayanbimpe. These phages showed remarkable stability and tolerance over a broad range of pH and temperature settings, but they lacked virulence, solubility, and antibiotic resistance genes. These results suggest that both phages have potential applications in phage treatment and as tools for the biocontrol of *Salmonella* proliferation. Further studies are recommended to validate the phage efficacy in biological systems *in vitro* and *in vivo* using mice model.

Availability of nucleotides sequences of the phages:

The nucleotide sequences of the genomes of the two phages have been deposited at NCBI GenBank with accession numbers *Apdecimavirus* Ayanbimpe PQ139365 and *Jerseyvirus* Ijeoma PQ139366

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Contributions of authors:

OOO, NEN, LN, and GMA were involved in the study conceptualization; OOO and NEN were involved in the study methodology, and writing/original draft preparation; GMA and LN were involved in the study supervision. All authors read and approved the submitted version of the manuscript.

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No funding was received for the study.

Conflicts of interest:

Authors declare no conflict of interest.

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