Running title: Physio-Kinetics of Native *Salmonella* Phages

**Title: “Physio-Kinetics of Indigenous Bacteriophages against *Salmonella enterica* subsp. *enterica* serovar *Gallinarum* biovariants *Gallinarum* and *Pullorum* ”**

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**Statement of Novelty**

In quest to combat the adverse effects of antibiotic resistance in agro-food animals there is a renewed interest in therapeutic application of lytic bacteriophages. This study highlights important aspects of physiology such growth kinetics, lytic spectra, polyvalency, thermal tolerance, acidic and alkaline stability indicating that these indigenous isolates with shorter eclipse and larger burst size can be used as monophage suspension or as cocktail to combat multidrug resistant avian *Salmonella*.

**Abstract**

Emergence and spread of multidrug resistance among pathogens of agro-food sector is increasing at alarming rate has directed attention to search for alternative to antibiotic therapy. The present study highlights the physiological characterization and population kinetics of indigenous bacteriophages against poultry specific *Salmonella*. Sewage effluent samples (***n***= 50) collected from commercial poultry production units showed 56% positive samples in screening. Four isolates (*viz.* SØ-10, SØ-12, SØ-16, SØ-28) presented different plaque morphologies and were propagated to consistent titres of 109 PFU. Greater than 50% of adsorption within 10 minutes was recorded for monophage suspensions (*viz.* SØ-10, SØ-12, SØ-16, SØ-28) leading to an optimal lysis time of 30 minutes on average with a higher burst size up to 132 PFU CFU- for SØ-28. The lytic spectrum was observed for monophage suspension and cocktail separately, with cocktail showing highest reduction 0.104 O.D600 at 8th hour post infection. The monophage suspensions (*viz.* SØ-10, SØ-12, SØ-16, SØ-28) demonstrated polyvalency by successfully infecting *S. enterica* serovars including *S. Enteritidis* and *S. Typhimuirum*. Monophage suspensions (*viz.* SØ-10, SØ-12, SØ-16, SØ-28) were observed to have optimal activity up to 70oC as well as marked tolerance at higher acidity and alkalinity levels. Summing up the results from nucleic acid analysis the monophage suspensions (*viz.* SØ-10, SØ-12, SØ-16, SØ-28) showed to possess dsDNA with fragment sizes exceeding 35 kb. Hence, as suggested indigenous lytic phages are promising alternative to combat multidrug resistant avian *Salmonella* both as monophage suspensions or as cocktail after comprehensive experimental evaluation.

**Keywords:** Bacteriophages, *Salmonella*, Optimal Lysis Time, Plaque, Multidrug Resistance

**Introduction**

The impact of certain highly prevalent bacterial diseases in agro-livestock sector is quite significant in terms of productive and public health levels. The scenario is further complicated by alarmingly high use of antibiotics and spread of multidrug resistance in bacterial pathogens has directed attention in quest to search for alternatives ([Fernández *et al.* 2018](#_ENREF_5)). With the substantial expansion in last five decades, poultry industry is one of the worldwide sectors that utilize antibiotics, particularly in developing countries where basic biological interventions and hygienic measures are limited ([Żbikowska *et al.* 2020](#_ENREF_18)).

Salmonellosis and poultry have been intricately linked since the commercialization of industry to meet the demands of growing global population with an average of 314.2 kg/capita consumption of poultry meat used across the globe (OECD – FAO 2020). The universal success of poultry meat owes to its inexpensive availability, consumers propensity of convenience, nutritional properties and absence of religious taboos ([Petracci *et al.* 2019](#_ENREF_13)). World Health Organization has declared *Salmonella* as “priority pathogen” as the emerging multidrug resistance in this notable pathogen displays a unique phenotype and their ability to generate hybrid plasmids leading to majority of the gene cassettes positioned around these plasmids that confer them the ability to resist choice of drugs ([Castro-Vargas *et al.* 2020](#_ENREF_4)).

Keeping in view, the present study was designed to study the physiology and growth kinetics of lytic bacteriophages against poultry adapted *Salmonella*. For this purpose, mass – action kinetics, lytic spectra, host-range profile, thermal tolerance and pH stability were used as indicative parameters for ideal candidates. The study additionally compared the efficacy of monophage suspensions with cocktail with reduction in bacterial number as presumptive factor to use cocktail *in vivo*.

**Materials and Methods**

**Bacterial Host Strains and Growth Conditions:** The bacterial strains used in present study were host-adapted non-motile typhoidal strains from *Salmonella enterica* subsp*.* *enterica* serovar *Gallinarum* biological variants *Gallinarum* and *Pullorum*.The strains were characterized via conventional and molecular techniques at National Reference Laboratory for Poultry Diseases.

**Media:** All media were from Oxoid, UK.

**Isolation and Enrichment of Bacteriophages:** For isolation of bacteriophages, effluent samples (***n*** = 50) from commercial poultry units were collected. Subsequently, 25ml of effluent was centrifuged at 15000 x *g* for 15 minutes and filtered through 0.45μm PVDF syringe filter (Millipore Millex® GV). Enrichment was performed in 5X trypticase soy broth by mixing equal volumes (1:1) of log phase (OD600 = 1.0). Following overnight incubation, mixtures were centrifuged at 9000 × *g* for 15 min and filtered through 0.22μm PVDF syringe filter (Millipore Millex® GV). Spot assay was carried for initial screening ([Hamza *et al.* 2016](#_ENREF_7)).

**Titration, Purification and Propagation of Bacteriophages:** Phage titration was measured using double agar overlay method by serially diluting (10-fold) phage suspensions. 100 µL of phage suspension and 200 µL of log phase hosts were added to 0.75% soft agar (50°C) in conical tubes and mixture was poured on trypticase soy agar plates with overnight incubation at 37°C. Plaques were pulled out with the help of sterile micropipette tip and propagated by transferring in log phase (OD600 = 1.0) host strains with optional supplementation 30µL 1 *m*M CaCl2 and 1 *m*M MgCl2 and were incubated overnight at 37°C with shaking. Subsequently, mixture was filtered through 0.22μm syringe filter. Each isolate was subjected to minimum three successive rounds of this experiment purified plaques ([Shende *et al.* 2017](#_ENREF_17)).

**Mass – Action Kinetics:** This experiment was carried with 10 minutes adsorption time. Bacteriophage with MOI of 10 ratio was added to infect the overnight grown bacterial culture (O.D600 0.5) and incubated at 37°C. To aid the adsorption process 1 *m*M CaCl2 and 1 *m*M MgCl2 were added in the mixture. Samples were drawn post incubation and were centrifuged at 7000 x *g* for 5 minutes. The unabsorbed phages were measured using double agar overlay plaque assay following centrifugation ([Sadekuzzaman *et al.* 2018](#_ENREF_15)).

The partially infected cells were re-suspended in 100 ml of pre-warmed trypticase soy broth and incubated at 37oC in shaking incubator at 120 rpm. After every 10 minutes, for up to 60 minutes, the sample was withdrawn and assessed for phage titer using double agar overlay method ([Santos *et al.* 2014](#_ENREF_16)).

**Profiling of Lytic Spectra:** For the assessment of *in vitro* lytic potential, 1ml of overnight incubated bacterial culture (108 CFU/ ml) was added in five flasks containing 50ml trypticase soy broth. Four of the flasks contained 1ml of bacteriophage lysate at 100 multiplicity of infection (MOI) while two flasks served as “bacterial control” (for each biovariant). Flasks were given incubation at 37oC with shaking at 120 rpm. The optical density was recorded via spectrophotometer (Specord 200 Plus, Analytikjena, Germany) at 600nm wavelength after every 2 hours post inoculation in comparison to bacterial controls ([Hamza *et al.* 2016](#_ENREF_7)).

**Host Range Profile:** The host sensitivity bacteriophage suspensions was tested against confirmed strains of Salmonella *Typhimurium*, Salmonella *Enteritidis*, Salmonella *Gallinarum*, Salmonella *Pullorum*, *Escherichia coli* ATCC 25922 and ATCC 23235 *Staphylococcus aureus* ([Rahaman *et al.* 2014](#_ENREF_14)).

**Effect of Temperature, Acidity and Alkalinity on Viability:** The monophage suspensions and cocktail were inoculated in pre-warmed trypticase soy broth and subjected to a preset range of temperature (37, 42, 50, 60, 70 and 80oC) up to 60 minutes. The viability at refrigerated (4oC) and ambient temperature (25oC) were also measured via double agar overlay. Similarly, for pH stability monophage suspensions and cocktail were mixed in 1X trypticase soy broth in test tubes having different sets of pH ranging from 3 to 6 (sodium acetate buffers), 7 to 9 (phosphate buffered saline) and 10 to 11 (Tris-Cl buffer) for 60 minutes and the titer was measured by double agar overlay method ([Hong *et al.* 2013](#_ENREF_8)).

**Nucleic Acid Analysis:** Crude lysates were centrifuged at 15000 x *g* for 15 minutes and filtered through 0.22 µm PVDF syringe filter (Millipore Millex® GV) followed by concentration with Amicon Ultra Centrifugal Filters (Ultracel-3K) MERCK. Filtrate was first treated with DNase I (RNase free – 1000 U/ml) and Ribonuclease A (DNase free – 10 mg/ml) followed by incubation at 37oC for 30 min. Following incubation, proteinase K (Recombinant grade – Thermo Fischer Scientific™) and 0.5% SDS was added in pretreated aliquots and was set to overnight incubation at 56oC. The removal of unwanted proteins was performed by Phenol: Chloroform: Isoamyl alcohol (25:24:1) (MERCK). Equal volume of PCI was mixed with aliquots followed by centrifuged at 15000 x *g* for 15 minutes. Add isopropanol to aqueous phase, followed by second centrifugation at 15000 x *g* for 15 minutes. Wash precipitated nucleic acid with 70% ethanol. Re-suspend pellet in sterile deionized distilled water or TE buffer. The extracted genome was digested with RNase A, DNase I and S1 Nuclease (100 U/ml Thermo Fischer Scientific™) to identify the nature of genome. The nucleic acid extracted was visualized using 1% agarose gel electrophoresis.

Two enzymes FastDigest *Eco*RI (Thermo Fischer Scientific™ FD0274) and FastDigest *HindIII* (Thermo Fischer Scientific™ FD0504) were used according to manufacturer’s instructions. A total of 50 µL of reaction mixture was prepared at room temperature which consisted of 10 µL of genome of bacteriophage with 5 µL of FastDigest Green Buffer, 5 µL of enzyme and 30 µL of nuclease free water. The digested fragments obtained were resolved by using 1% agarose gel stained with ethidium bromide ([Parra *et al.* 2016](#_ENREF_12)).

**Statistical Analysis**

Single Factor ANOVA was applied with 95% confidence interval (α = 0.05) to compare lytic spectra (optical turbidity reduction), temperature, acidic and alkaline viability and tolerance data between monophage suspensions and cocktail.

**Results**

The spot assay for initial screening of isolates can be seen in Fig. 1. All four monophage suspensions (*viz.* SØ-10, SØ-12, SØ-16, SØ-28) consistently achieved titres of 109 PFU yielding four different plaque morphologies *viz.* plaques with double halo, plaques with single halo and dense center spot, small spots and clear medium sized plaques with no center spots as shown in Fig. 2.

The adsorption assay showed that more than 50 per cent of the (>50%) bacteriophages were successful in adsorbing to the surface of the targeted host within 10 minutes. The latent time period of candidate mono-suspensions (*viz.* SØ-10, SØ-12, SØ-16, SØ-28) elapsed after 30 minutes with first burst (Filial 1) with sizes recorded as 73, 97, 75 and 132 PFU CFU-1 for SØ-10, SØ-12, SØ-16, SØ-28, respectively, as shown in mass – action kinetics graph (Fig 3a and 3b).

The lytic spectrum assay holds a significant inter-relation towards phage therapy as sustained reduction in the bacterial numbers is of major interest in therapeutic applications. Statistical analysis demonstrated a significant difference (*P =* 0.00) between monophage suspensions and cocktail. The lowest value for optical density was recorded for cocktail (OD600 = 0.104) in comparison to the bacterial control as shown in (Fig. 4). The individual lytic spectra values for monophage suspensions and cocktail against uninfected test cultures are given in Table 1.

Host range profile of phage mono-suspensions against notable *Salmonella* serovars is shown in Table 2. The bacteriophage isolates maintained a steady titer of 1.43 x 109 PFU and 3.60 x 109 PFU against *Salmonella Typhimurium* and *Salmonella Enteritidis*, respectively (Fig. 5). All isolates lacked heterologous activity against *Pasteurella multocida* ATCC 43137and *Staphylococcus aureus* ATCC 23235.

Statistical analysis demonstrated a significant difference (*P =* 0.00) between monophage suspensions and cocktail. Temperature chart outlines the successful viability of monophage suspensions and cocktail up to 60oC with no marked reduction in log10 PFU. At higher temperatures, 70oC and 80oC a marked decrease was observed in the viability and tolerance of monophage suspensions and cocktail as shown in Fig. 6. The monophage suspensions and cocktail were relatively stable at pH 3 – 9 by showing no significant decrease in the titres (Fig. 7). Statistical analysis demonstrated non-significant difference (*P =* 0.99) between monophage suspensions and cocktail suggesting that monophage suspensions and cocktail.

It was observed that genome of the selected bacteriophage isolates possessed dsDNA as genome because it remained undigested after treatment with RNase A (marked by lane A), and it was not cleaved with S1 nuclease (marked by lane S1). The restriction digestion with *Hind*III yielded 7 bands of various sizes and *Eco*R1 yielded 5 bands summing up the restriction fragment size of the isolate to be greater than 35 kbp (Fig. 8).

**Discussion**

In this research work, four bacteriophages were isolated from effluents procured from commercial poultry production units, which were specifically assessed for their physiological properties *in vitro* as potential biological control against poultry adapted *Salmonella* typhoidal strains i.e., Salmonella enterica *subsp.* enterica serovar *Gallinarum* biovar *Gallinarum* and Salmonella enterica *subsp.* enterica serovar *Gallinarum* biovar *Pullorum*. Anti-*Salmonella* bacteriophages have been isolated from litter and sewage effluents which proves their presence in environment that contains target host bacterial population ([Bielke *et al.* 2007](#_ENREF_2); [Borie *et al.* 2008](#_ENREF_3)). They are the most diverse and ubiquitous entities in the biosphere, superseding all other organisms in number highlighting their origin dating back to Pre-Cambrian era, the earliest period of earth’s history that spanned about 4.5 billion years ago ([Ackermann. 2011](#_ENREF_1)). To our knowledge, this is the first study that involves isolation and preliminary *in vitro* assessment of biological parameters of bacteriophages against typhoidal biovars from serovar *Gallinarum* that is associated with significant production and monetary loss at Pakistan, a developing country, where irrational use of antibiotics as a surrogate to hygiene, exposing birds to sub-therapeutic antibiotic concentrations and antibiotic resistance, often remains under-reported.

Adsorption is *sine qua non* in initiation of infection of target bacterial host through successive interaction between predator and prey. The data generated from present study suggests that 10 – minutes adsorption led to swift adsorption of more than 50% bacteriophage particles on host surface membrane. Following adsorption, the latent period leads to formation of new viral progeny and finally to lysis of bacterial cell (burst). The shorter latent period accompanied by higher burst size is ultimate goal to harness full potential of bacteriophages in therapeutic applications. In this study, the average latent period spanned around 30 minutes, with burst sizes of 73, 97, 75 and 132 PFU CFU- for SØ-10, SØ-12, SØ-16, SØ-28, respectively. Their burst size was close to ⱷSG-JL2 which was 100 PFU CFU- which showed much shorter latent period between 10 – 15 minutes ([Hyuk-Joon Kwon *et al.* 2013](#_ENREF_9)). A past study cited the burst sizes for their bacteriophages as 1670, 80 and 28 PFU CFU- around 30 minutes for ST4, L13 and SG3, respectively ([Hong *et al.* 2013](#_ENREF_8)).

Host specificity or host range is an important biological parameter as it serves as the rate-limiting step due to their host sensitivity and specificity, when considering bacteriophages suitable for therapeutic applications ([Hyuk-Joon Kwon *et al.* 2013](#_ENREF_9)). In our study, all four candidate bacteriophages are polyvalent and showed steady host range against all twelve notable paratyphoidal and typhoidal strains except for ATCC 43137 *Pasteurella multocida* and ATCC 23235 *Staphylococcus aureus*. Known *Salmonella* bacteriophages like LPST153 have been reported with broad host ranges of over 65% and has been used as a biocontrol agent for surface decontamination of chicken meat ([Islam *et al.* 2020](#_ENREF_10)).

Effects of pH and thermal stability are critical parameters when optimal activity and proper handling of bacteriophages is concerned for therapeutic applications. This standardization is particularly helpful in optimizing therapy as well as the viability of bacteriophages under harsh conditions. All four isolates showed optimal pH activity (i.e., 3 – 9) and thermal stability (above 60oC), as this would help them in their survival through alimentary tract of chickens making them as potential biological control substitutes. A similar study from literature agrees with our work, stating that the viability of their bacteriophages suspensions decreased over 45oC and maximum activity at pH 5 – 8. Another study reported two bacteriophages used against Salmonella *Enteritidis* showed that their isolates were relatively stable between 30 – 50oC for 30 and 60 minutes, with no viability at 70oC and 80oC after an hour (Hong *et al.* 2013; Bao *et al.* 2015).

All candidates from the present study possessed double stranded DNA as their genome, indicating that candidates have the possibility of belonging to any of the three tailed families in *Caudovirales*. Similar results have been reported in literature for many characterized *Salmonella* bacteriophages ([Fiorentin *et al.* 2004](#_ENREF_6); [Ngangbam *et al.* 2012](#_ENREF_11)). To control maximal output with therapeutic applications, the smaller genome size might decrease lessen the risks associated with long chromosomal DNA fragment which may have the possibility of carrying virulence genes into the animal body. The anticipated size of all four candidate particles was recorded greater than 35kb but less than 50kbp, much smaller than known *Salmonella* bacteriophage Felix 01 with an estimated size of 80kbp. The genome sizes were much close to ⱷSG-JL2 with estimated genome size of 38, 815bp and SJ2 with 152, 460bp ([Hyuk-Joon Kwon *et al.* 2013](#_ENREF_9); [Zhang *et al.* 2015](#_ENREF_19)).

**Conclusion:**

Conclusively, present study provides baseline criterion for investigating native bacteriophage isolates against poultry adapted typhoidal field strains in Pakistan. Subsequently, these bacteriophages can be used as monophage suspensions or concocted as novel cocktail as an effective alternative to antibiotics for prophylaxis and remedy against avian salmonellosis. Moreover, it will reduce the economic and production losses due to this highly prevalent disease of poultry industry.

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**Author’s Contributions**

Conceptualization of idea AAS and MR; experimental methodology, RHD and MRK; technical laboratory analysis, RHD, AK and MM; sampling, SAC; manuscript preparation and statistical analysis, RHD and MIR, reference typing of bacterial strains, RHD, MAA and NS.

**Data Availability**

The data of the study would be available on fair request to corresponding author.

**Ethical Approval**

Not applicable in this study.

Conflict of Interest

The authors of this manuscript have declared no potential conflict of interest.

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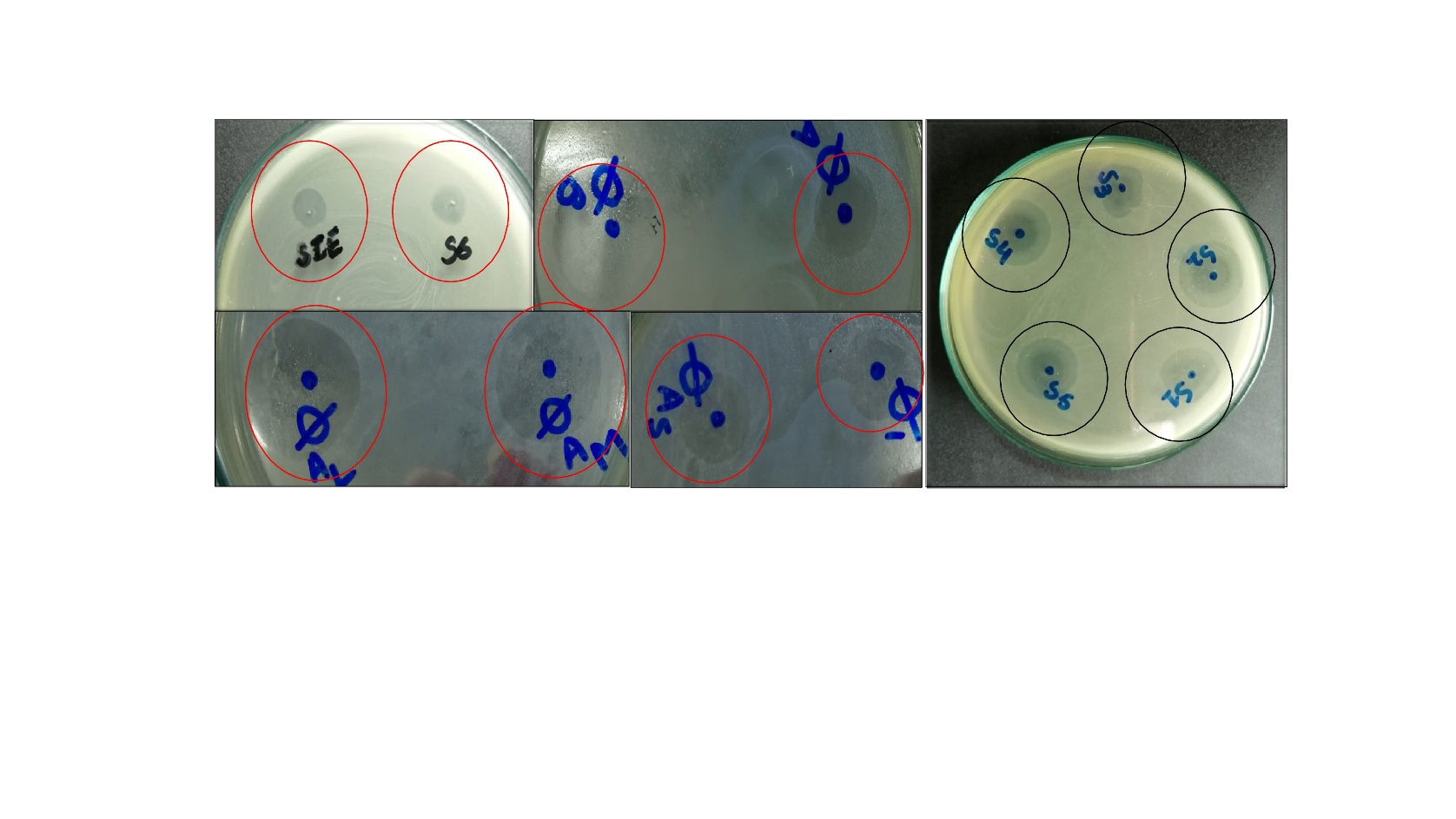


Fig. 1: Zone of clearance/spot (encircled) showing inhibition of bacterial lawn on trypticase soy agar plate for initial screening

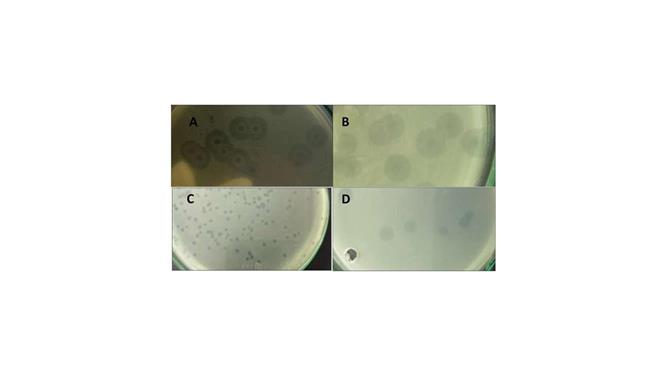


Fig. 2: Plaque morphologies of candidate bacteriophage isolates (A) double halo (B) dense center spot (C) small pinhead size (D) clear medium size

Fig. 3a: Graphical representation depicting single step growth kinetics with first burst recorded at 30 minutes for both candidate isolates with log10 PFU titer reaching 9.58 and 9.38 for SØ-16 and SØ-10, respectively, for 60 minutes

Fig. 3b: Graphical representation depicting single step growth kinetics with first burst recorded at 30 minutes for both candidate isolates with log10 PFU titer reaching 9.34 and 8.69 for SØ-12 and SØ-28, respectively, for 60 minutes

Fig. 4: Graphical representation of monophage suspensions and cocktail showing steady lytic spectrum of cocktail against bacterial control (red/orange trendline) with continuous decline up to 8 hours

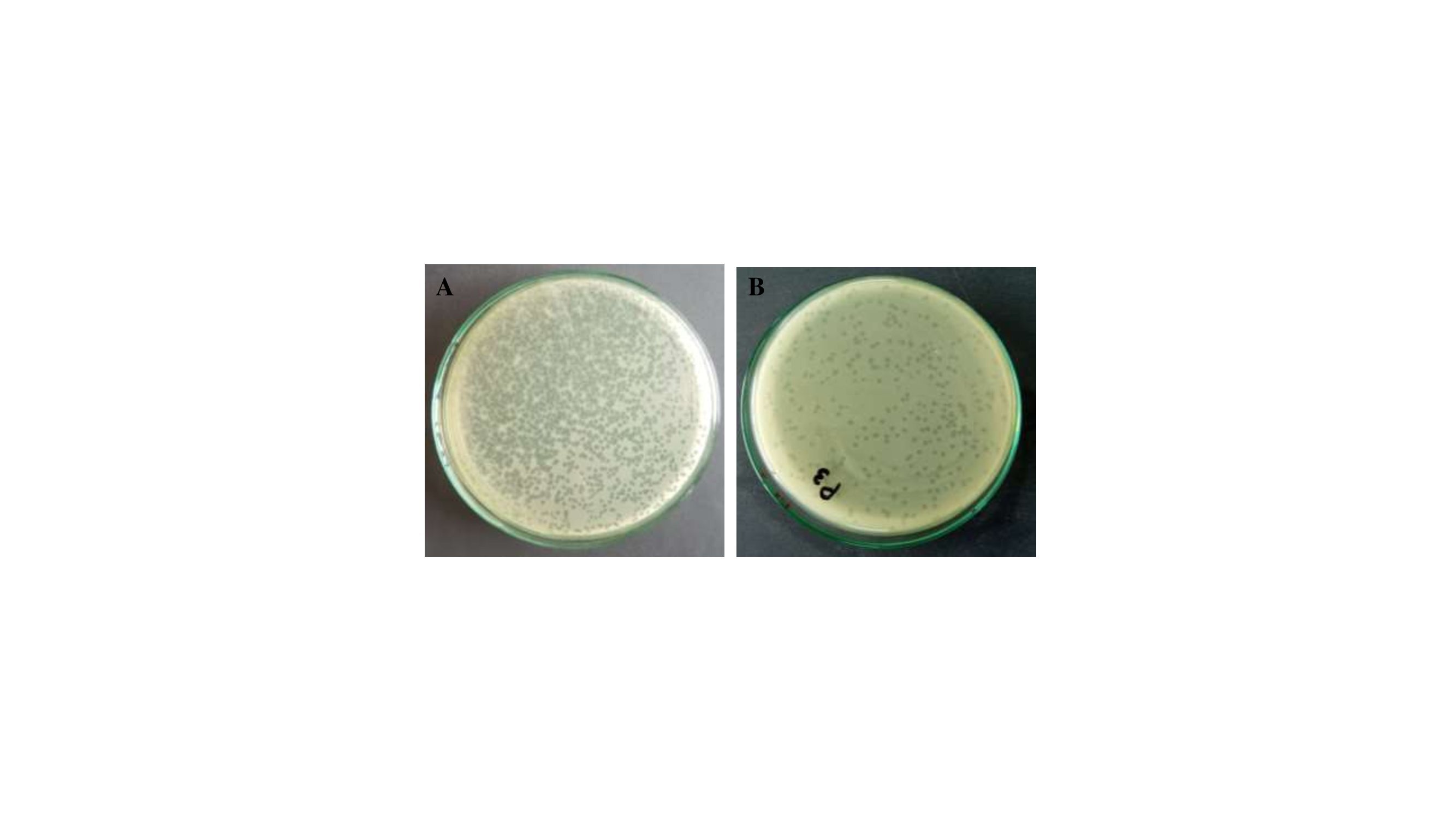


Fig. 5: Host susceptibility spectrum with consistent titer reaching 109 PFU/ml against paratyphoidal *Salmonella* serovars of public importance (A) *Salmonella* *Enteritidis* (B) *Salmonella Typhimurium*

Fig. 6: Graphical representation of thermal stability of monophage suspensions with Log10 values on *y* -axis plotted against temperature at *x* – axis with steady titers

Fig. 7: Acidic and alkaline tolerance of monophage suspensions with Log10 values on *y* -axis plotted against temperature at *x* – axis with steady titers



Fig. 8: Lane “L” represents 1kb extended ladder, S1 lane represents the DNA fragments undigested with S1 nuclease indicating the double stranded nature of genome, “A” lane represents undigested DNA of SØ-28 with RNase A indicating it contains DNA based genome , “2” lane represents digestion of SØ-28 with *Hind*III yielding 7 bands, lane “3” represents digestion of SØ-28 with *Eco*R1 yielding 5 bands

Table 1: Measurement of Optical Density (OD600) of Bacterial Controls, Bacteriophage Suspensions and Cocktail

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Time (hrs.) | Bacterial Control | | Cocktail | SØ-10 | SØ-12 | SØ-16 | SØ-28 |
| **SGal-1788** | **SPul-1880** |
| 0 | 0.5 | 0.5 | 0.501 | 0.504 | 0.507 | 0.506 | 0.502 |
| 2 | 1.0 | 0.9 | 0.320 | 0.421 | 0.419 | 0.399 | 0.399 |
| 4 | 1.1 | 1.1 | 0.211 | 0.338 | 0.330 | 0.320 | 0.327 |
| 6 | 1.4 | 1.7 | 0.122 | 0.289 | 0.299 | 0.299 | 0.288 |
| 8 | 1.8 | 1.8 | 0.104 | 0.201 | 0.227 | 0.207 | 0.214 |

Table 2: Host Range Profile for Bacteriophage Isolates

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| No. Assign. | Strain | SØ-10 | SØ-12 | SØ-16 | SØ-28 |
| SGal-1788 | *Salmonella Gallinarum* | + | + | + | + |
| SGal-1877 | *Salmonella Gallinarum* | + | + | + | + |
| SGal-1878 | *Salmonella Gallinarum* | + | + | + | + |
| SGal-1879 | *Salmonella Gallinarum* | + | + | + | + |
| SPul-1880 | *Salmonella Pullorum* | + | + | + | + |
| SPul-10.23 | *Salmonella Pullorum* | + | + | + | + |
| STy-1 | *Salmonella Typhimurium* | + | + | + | + |
| STy-2 | *Salmonella Typhimurium* | + | + | + | + |
| STy-3 | *Salmonella Typhimurium* | + | + | + | + |
| SE-1 | *Salmonella Enteritidis* | + | + | + | + |
| SE-2 | *Salmonella Enteritidis* | + | + | + | + |
| SE-3 | *Salmonella Enteritidis* | + | + | + | + |
| ATCC 25922 | *Escherichia coli* | - | - | - | - |
| ATCC 23235 | *Staphylococcus aureus* | - | - | - | - |

1(+) clear spot in inoculated indicating activity (-) no spot, 2ATCC = American Type Culture Collection