Physiological properties of indigenous lytic bacteriophages as monophage suspension and cocktail against poultry-adapted typhoidal *Salmonella* variants

Rida Haroon Durrani[1†], Ali Ahmad Sheikh[1*†], Masood Rabbani[1], Muti-ur-Rehman Khan[2], Muhammad Ilyas Riaz[1], Muhammad Anas Naeem[1], Salman Ashraf Chattha[1], Aleena Kokab[1], Munazzah Maqbool[1], Muhammad Athar Abbas[3], Naila Siddique[3]

[1] Institute of Microbiology, Faculty of Veterinary Sciences, University of Veterinary and Animal Sciences, Lahore, Pakistan; [2] Department of Pathology, Faculty of Veterinary Sciences, University of Veterinary and Animal Sciences, Lahore, Pakistan; [3] National Reference Laboratory for Poultry Diseases, Animal Sciences Institute, National Agricultural Research Council, Islamabad, Pakistan.

Article Info	Abstract
	The emergence and spread of multidrug resistance among pathogens of the agro-food sector is increasing at an alarming rate, which has directed attention to the search for alternative to antibiotic therapy. The present work studied the physiological and population dynamics of lytic bacteriophages against avian-adapted *Salmonella*. Out of 28 positive samples, four bacteriophage isolates (SalØ-ABF37, SalØ-RCMPF12, SalØ-MCOH26, SalØ-DNLS42) were selected based on their ability to clearly lyse bacterial test strains. The isolates propagated were active against closely related D1 serotypes, i.e., *S.* Enteritidis and *S.* Typhimurium, with no heterologous activity against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 23235. Each of the monophage suspension and cocktail efficiently suppressed the bacterial count from exponential increase in comparison to the untreated bacterial control. The bacterial turbidity was recorded as 0.244 at λ_{600} during 400 min of co-incubation, in contrast to bacterial control showing $\lambda_{600} = 0.669$. The latent period was recorded to be 25, 35, 25 and 30 for SalØ-ABF37, SalØ-RCMPF12, SalØ-MCOH26 and SalØ-DNLS42, with 73.00, 97.00, 132 and 75.00 PFU cell^{-1}, respectively. The highest lytic activity was seen at 37.00 ℃ - 42.00 ℃, with phage particle count being fairly stable at pH 3.00 - 9.00. Each of the isolates possessed dsDNA by being resistant to RNase A. The current study concludes that lytic phages are promising alternative to combat multidrug resistant superbugs. The physiological characterization and bacterial growth inhibition are important parameters in standardization of phage therapy.

Introduction

In the last five decades, the poultry industry has substantially expanded to meet the demands of the growing global population with an average of 314.20 kg per capita consumption of poultry meat.[1] The universal success of poultry meat owes to its inexpensive availability, consumers propensity for convenience, nutritional properties and absence of religious taboos.[2] Despite playing a pivotal role in the provision of a sustainable food supply, the sector is still facing unprecedented challenges from diseases that have a significant impact in terms of productivity and public health. The scenario is further exacerbated by the excessive and imprudent use of antibiotics and the spread of multidrug resistance against bacterial pathogens, particularly in developing countries where basic biological interventions and hygienic measures are limited.[3,4] Avian salmonellosis is a highly infectious disease and is among the most prevalent bacterial diseases affecting Pakistani poultry sector. *Salmonella* spp. display a unique phenotype and can generate hybrid plasmids which lead to the positioning of the majority of the gene cassettes around these plasmids conferring them an ability to resist the choice of drugs.[5] The increasing antibiotics resistance will result in an annual increase of death toll from currently 0.70 million to 10 million along with economic losses of 100 trillion USD by 2050.[6] Rapid surge in antibiotic resistance has directed attention to search for alternatives, [7] and there is a renewed interest in the pre-and post-harvest application of lytic bacteriophages.

*Correspondence:

Ali Ahmad Sheikh. PhD

Institute of Microbiology, Faculty of Veterinary Sciences, University of Veterinary and Animal Sciences, Lahore, Pakistan

E-mail: ali.ahmad@uvas.edu.pk

† These authors have contributed equally to this manuscript.

Current study was designed to study the physiology and growth kinetics of lytic bacteriophages against host-specific *Salmonella*. This study highlights the important aspects of physiology such as growth kinetics, lytic spectra, host range, thermal tolerance, stability under acidity and alkalinity. The study additionally compared the efficacy of monophage suspensions with a phage cocktail, with a reduction in bacterial number as a presumptive factor to use the cocktail *in vivo*.

Materials and Methods

Bacterial host strains and growth conditions. The bacterial strains used in the present study were host-adapted non-motile typhoidal strains from *S. enterica* subsp*. enterica* serovar Gallinarum biovar Gallinarum and Pullorum. These strains were procured from Poultry Research Institute, Rawalpindi. The strains were reference typed by polymerase chain reaction based on *rat*A gene and serovar identifier region (*SIR*) at National Reference Laboratory for Poultry Diseases (NRLPD), Islamabad.

Isolation and enrichment of bacteriophages. The sewage effluents from poultry units were utilized because they serve as the rich source for the isolation of *Salmonella* bacteriophages.[8] A total of 50 samples were collected in 500 mL sterile media bottles and were transported to the Diagnostic Laboratory, Central Laboratory Complex, University of Veterinary and Animal Sciences, Ravi Campus. A volume of 25.00 mL of sample was withdrawn from the bottle and centrifuged (Spectra 6C; Rays Technologies, Lahore, Pakistan) at 15,000 g for 15 min to precipitate the remaining debris. The supernatant was filtered through 0.45 µm polyvinyl difluoride (PVDF) syringe filter (Millipore Millex® GV, Merck, Bedford, USA). The filtrates were enriched in 5X trypticase soy broth (TSB; Oxoid, Basingstoke, UK), having equal volumes (1:1) of filtrate and log phase (OD_{600} = 1.00) bacterial broth cultures. The mixtures were incubated for 24 hr at 37.00 °C with shaking at 120 rpm. Following day, the mixtures were centrifuged at 9,000 g for 15 min and the supernatant was filtered through a 0.22 µm syringe filter. Spot assays were performed to screen samples for the presence of bacteriophages in the filtrate by spotting 10.00 µL of filtrate on bacterial lawn seeded with 200 µL of the log phase bacterial culture on trypticase soy agar (TSA; Oxoid). After incubation at 37.00 °C for 24 hr, plates were observed for a visible zone of clearance *i.e.,* spot.[9]

Titration, purification and propagation of bacterio-phages. Phage titration was measured using the double agar overlay method by serially diluting (10-fold) phage suspensions. Then, 100 µL of phage suspension and 200 µL of log-phase bacterial broth culture (OD_{600} = 1.00) were added to 0.75% soft agar (Oxoid) at 50.00 °C in conical tubes and the mixture was poured on TSA plates with an overnight incubation at 37.00 °C. Each plaque was subjected to a minimum of three successive rounds of purification by plaque assay.[10] Plaques were pulled out with the help of a sterile micropipette tip and propagated by transferring in log phase (OD_{600} = 1.00) host strains with supplementation of 30.00 µL 1.00 mM $CaCl_2$ (Sigma-Aldrich, Gillingham, UK) and 1.00 mM $MgCl_2$ (Sigma-Aldrich). The mixture was filtered through a 0.22 µm syringe filter.

Host range profiling. The host range of the isolated bacteriophages was tested against the already identified and confirmed isolates of *S.* Typhimurium (4), *S.* Enteritidis (4), *S.* Gallinarum (3), *S.* Pullorum (1), *Escherichia coli* ATCC 25922 (1) and *Staphylococcus aureus* ATCC 23235 (1). The TSA plates were seeded with 100 µL of bacterial inoculum of the above-mentioned pathogens and spotted with 10.00 µL of phage lysate followed by overnight incubation at 37.00 °C.[11]

Effect of temperature and pH on viability. The thermal stability of bacteriophages was monitored by incubating the bacteriophage suspensions in test tubes at temperatures starting from 37.00 to 80.00 °C for an hour in a pre-heated water bath. The titre of bacteriophages was measured by double agar overlay method. Similarly, for pH stability bacteriophages were mixed in 1X TSB in 15.00 mL conical test tubes with pH ranging from 3.00 to 6.00 (sodium acetate buffers; Sigma Aldrich), 7.00 to 9.00 (phosphate-buffered saline) and 10.00 to 11.00 (Tris-Cl buffer; Sigma Aldrich) for 30 and 60 min. The titre was measured by double agar overlay method.[12]

Profiling of lytic spectra. For the assessment of *in vitro* lytic potential 1.00 mL of overnight incubated bacterial culture (× 10^8 CFU) was added to five flasks containing 50.00 mL of TSB. Four of the flasks contained 1 mL of bacteriophage lysate at a multiplicity of infection (MOI) of 100 while the fifth flask served as bacterial control with no phages. Flasks were incubated at 37.00 °C for 8 hr with shaking at 120 rpm. The absorbance was recorded after every 2 hr, post-inoculation via spectrophotometer (Specord 200 Plus; Analytik Jena GmbH, Jena, Germany) at 600 nm wavelength.[13]

Assessment of adsorption rate and mass action kinetics. For the assessment of adsorption rate previously described methods with minor modifications were used. The bacteriophage was added to overnight incubated bacterial cultures at MOI = 100 and incubated with shaking (120 rpm) for 10 min at 37.00 °C. To aid the adsorption process, 1.00 mM $CaCl_2$ and 1.00 mM $MgCl_2$ were added to the mixture. Post-incubation samples were drawn and centrifuged at 7,000 g for 5 min. The unabsorbed phages were measured using the double agar overlay method following centrifugation.[13] The partially infected cells were re-suspended in 100 mL of pre-warmed TSB and incubated at 37.00 °C in a shaking incubator at 120 rpm. After every 10 min, for up to 60 min, the sample was withdrawn and assessed for phage titer using the double agar overlay method.[14,15]

The single-step growth kinetic assay for the bacterio-phages was performed to estimate the latent period and burst size. The host bacterial strain was incubated at 37.00 °C to reach the mid-log phase (OD_{600} = 0.40 - 0.60), after which it was supplemented with bacteriophage at MOI = 100 and the mixture was incubated at 37.00 °C for 10 min. After pre-incubation, the mixture was centrifuged at 13,000 *g* for 5 min and the pellet obtained was re-suspended in 100 mL of pre-warmed TSB and incubated at 37.00 °C with shaking (120 rpm). At every 10-min interval (up to 60 min), a sample was withdrawn and assessed for phage titer using double agar overlay method.[11,12]

Extraction of bacteriophage genomic material. The nucleic acid extraction was executed as reported by Zhang *et al.* and Kwon *et al.* with little modifications. The TSB containing the bacteriophage was centrifuged at 15,000 *g* for 30 min. The supernatant was filtered through a 0.22 μm PVDF syringe filter and concentrated through Amicon Ultra Centrifugal Filters (Ultracel-3K; Merck). Bacterio-phage filtrate was first treated with 1.60 μL DNase I (RNase free – 1000 U mL^{-1}; Thermo Fischer Scientific, Waltham, USA) supplemented with 12.50 μL $MnCl_2$ (Thermo Fischer Scientific) and Ribonuclease A (DNase free – 10.00 mg mL^{-1}; Thermo Fischer Scientific) followed by 1 hr incubation at 37.00 °C. After incubation, 40.00 μL of 0.50 M EDTA, 20.00 μL proteinase K (Recombinant grade – 10.00 mg mL^{-1}; Thermo Fischer Scientific, Waltham, USA) and 50.00 μL of 10.00% sodium dodecyl sulfate (Merck) was added to the pre-treated bacteriophage aliquots and the solution was incubated overnight at 56.00 °C. An equal volume of (PCI) phenol: chloroform: isoamyl alcohol (25:24:1; Merck) was mixed with broth to remove unwanted proteins and the mixture was centrifuged at 15,000 *g* for 15 min. The aqueous phase was collected and mixed with 1.00 mL of 95.00% chilled ethanol and 50.00 μL of 3.00 M sodium acetate (Merck). The mixture was centrifuged at 13,000 *g* for 15 min and the supernatant was discarded. Pellet was washed with 500 μL of 70.00% ethanol and re-suspended in 50.00 μL of deionized water. The extracted nucleic acid was resolved and visualized using 1.00% agarose gel electrophoresis (prepared in 1X TAE buffer; Thermo Fischer Scientific). The digestion of extracted phage genome was executed with RNase A,

DNase I and S1 Nuclease (100 U mL^{-1}; Thermo Fischer Scientific) to identify the nature of the genome and visualized by resolving in 1.00% agarose 1X TAE buffer (Tris-acetate EDTA; Thermo Fischer Scientific™) and staining gel with ethidium bromide (10.00 mg mL^{-1}; UltraPure™ Invitrogen).[11,16]

Restriction digestion of bacteriophage genome. FastDigest *Eco*RI (Thermo Fischer Scientific) and Fast-Digest *Hind*III (Thermo Fischer Scientific) were used for restriction digestion of extracted viral genome. A total of 50.00 μL of the reaction mixture was prepared at room temperature consisting of 10.00 μL of bacteriophage DNA, 5.00 μL of FastDigest Green buffer (Thermo Fischer Scientific), 5.00 μL of enzyme and 30.00 μL of nuclease-free water. The digested fragments were resolved using 6.00% polyacrylamide gel in TAE buffer and visualized under UV transilluminator (Trans Lum™ Solo TU1002; Biotop, Beijing, China).[9,17]

Results

The spot assay for the initial screening of isolates can be seen in (Fig. 1A). All four monophage suspensions (SalØ-ABF37, SalØ-RCMPF12, SalØ-MCOH26, SalØ-DNLS42) consistently achieved titers of 1.00×10^9 PFU yielding four different plaque morphologies as shown in (Fig. 1B, C, D, and E). The bacteriophage isolates showed a steady titre of 1.69×10^9 PFU mL^{-1} and 3.60×10^9 PFU mL^{-1} against *S.* Typhimurium and *S.* Enteritidis, respectively. No activity against *E. coli* ATCC 25922 and *S. aureus* ATCC 23235 was observed which indicates that bacteriophages were strictly adapted to closely related serotypes of poultry origin.

The absorbance of bacterial control at 0 hr was preset to have a cell concentration of $\log_{10}$ 8.18 CFU mL^{-1}. The cell concentration peaked at 10th hr with $\log_{10}$ 10.47 CFU mL^{-1} in bacterial control with λ_{600} = 0.669. The lowest optical density was recorded for the cocktail at λ_{600} = 0.244 at 400 min of co-incubation in comparison to bacterial control with λ_{600} = 0.669. The measurements obtained through absorbance were converted to $\log_{10}$ CFU in mL followed by calculation of $\log_{10}$-reduction. A stable reduction in bacterial population by monophage suspensions and cocktail can be seen in Table 1.
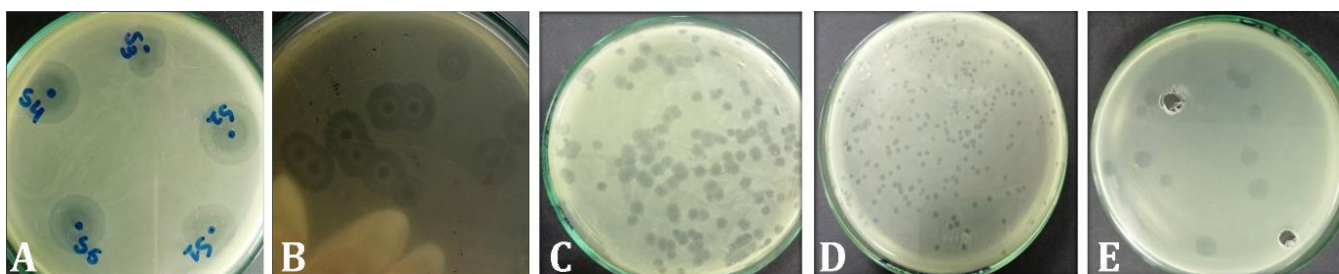
Fig. 1. A) The sewage effluent samples tested positive for presence of phages in spot assay showing zone of clearance, **B)** Double-halo plaques of SalØ-ABF37 (greater than 5.00 mm), **C)** Single plaques of SalØ-RCMPF12 with dense center spot, **D)** The 1.00 mm pin-head sized plaques of SalØ-MCOH26, **E)** Clear 2.00 mm plaques of SalØ-DNLS42.

Table 1. Log$_{10}$ reduction in bacterial incidence in monophage versus phage cocktail; (Sal = Salmonella; Ø = phage).

| Time (min)	Control 1.	Control 2.	SalØ_ABF37	SalØ-RCMPF12	SalØ-MCOH26	SalØ-DNLS42	Quad cocktail
120	8.29	8.25	2.53 ± 0.02	2.68 ± 0.02	2.58 ± 0.02	1.31 ± 0.02	2.72 ± 0.02
240	8.40	8.38	2.58 ± 0.02	2.72 ± 0.03	2.60 ± 0.02	1.58 ± 0.02	2.89 ± 0.03
300	9.16	9.15	2.63 ± 0.03	2.61 ± 0.02	2.63 ± 0.02	1.71 ± 0.02	2.81 ± 0.03
360	10.34	10.12	2.61 ± 0.03	2.66 ± 0.02	2.61 ± 0.02	1.39 ± 0.02	2.69 ± 0.02
400	10.47	10.36	2.66 ± 0.02	2.70 ± 0.03	2.66 ± 0.02	1.89 ± 0.02	2.66 ± 0.02

The growth kinetics experiments help in the verification of latent time and burst size which are important parameters in selection of candidate phages for therapeutic purpose. The monophage suspension of SalØ-ABF37 showed a latent time of 25 min with 73.00 PFU CFU^{-1} as the first burst, as can be seen in Figure 2A. The isolate maintained a steady latent state of approximately 20 min, at which the phage particle count increased to log$_{10}$ 7.73 PFU mL^{-1} but after 25 min the count was measured to be log$_{10}$ 9.94 PFU mL^{-1} whereas SalØ-RCMPF12 had a latent time of 35 min as shown in Figure 2A with 97.00 PFU CFU^{-1} as first progeny released at phage titre log$_{10}$ 10.02 PFU mL^{-1}. Isolates SalØ-MCOH26 and SalØ-DNLS42 showed a latent time of 25 and 30 min with 132 and 75.00 PFU CFU^{-1} as burst size as shown in Figure 2B. The SalØ-DNLS42 reached a growth plateau after 40 min as can be seen (Fig. 2B).

The bacteriophages *viz.* SalØ-ABF37, SalØ-RCMPF12, SalØ-MCOH26, SalØ-DNLS42 were stable at refrigerated (4.00 °C) and ambient (25.00 °C) temperatures in liquid (*i.e.,* sterile TSB) with phage particle count at log$_{10}$ 10^9 PFU mL^{-1}. All four isolates were well-tolerant up to 49.00°C without a decrease in the titre. At 50.00 °C isolate SalØ-DNLS42 showed 0.73 log$_{10}$ reduction with log$_{10}$ 8.77 PFU

Fig. 2. Population growth kinetics. **A)** Growth curve of *Salmonella* phages SalØ-ABF37 and SalØ-RCMPF12, **B)** Growth curve of *Salmonella* phages SalØ-MCOH26 and SalØ-DNLS42, with phage titre plotted (*y*-axis) against latent time and burst size.

mL^{-1}, with the rest of three isolates SalØ-ABF37, SalØ-RCMPF12, SalØ-MCOH26 being relatively stable at phage particle count of log$_{10}$ 10^9 PFU mL^{-1}. At 54.70 °C SalØ-DNLS42 showed another 1.06 log$_{10}$ reduction (90.00%) after which its activity was diminished. The isolates SalØ-ABF37, SalØ-RCMPF12, SalØ-MCOH26 showed tolerance up till 65.70 °C with log$_{10}$ 8.42, log$_{10}$ 7.54 and log$_{10}$ 7.93 PFU mL^{-1}, respectively. Beyond 65.70 °C the counts were significantly dropped with negligible phage titre. When concocted as a cocktail, phage particle counts were stable until 65.70 °C with log$_{10}$ 8.83 PFU mL^{-1}.

The SalØ-ABF37 isolate showed log$_{10}$ 0.93 and log$_{10}$ 1.25 reduction at 30 - 60 min, respectively. The titre determined for SalØ-ABF37 were log$_{10}$ 8.14 and log$_{10}$ 6.89 PFU mL^{-1} at 30 and 60 min, respectively. SalØ-RCMPF12 showed a decline with a 1.73 log$_{10}$ reduction in the first half (30 min) with a further 0.91 log$_{10}$ reduction at 60 min. The titre for SalØ-RCMPF12 was recorded as log$_{10}$ 7.48 and log$_{10}$ 6.57 PFU mL^{-1} at 30 and 60 min, respectively. SalØ-MCOH26 quickly declined in comparison to fellow isolates within first half (30 min). A 2.19 log$_{10}$ reduction was seen at 30 min which was followed by an additional 1.09 log$_{10}$ reduction upon completion of 60 min. The titre determined for SalØ-MCOH26 was recorded to be log$_{10}$ 6.98 and log$_{10}$ 5.89 PFU mL^{-1} at 30 and 60 min, respectively. SalØ-DNLS42 was relatively stable than SalØ-MCOH26 with a decline of 1.66 log$_{10}$ at 30 min, with an added log$_{10}$ reduction of 1.24 at 60 min. The titre for SalØ-DNLS42 showed log$_{10}$ 7.35 and log$_{10}$ 6.11 PFU mL^{-1} at 30 and 60 min, respectively. SalØ-DNLS42 and SalØ-RCMPF12 were relatively comparable in behavior upon exposure to 2.00 pH. When concocted as a cocktail, phage particle count remained stable during first with 1.07 log$_{10}$ reduction, later, 1.29 log$_{10}$ reduction was recorded at 60 min.

The isolate showed greater stability at pH 4.00 in contrast to 2.00 with a negligible decline in log$_{10}$ phage particle count during double agar overlay. At pH 7.00 there was no effect on bacteriophage concentration as both individual suspensions and cocktail. Incubation with pH 10.0 also greatly influenced the stability and tolerance profile of individual suspensions and cocktail. A collective decline between 1.79 - 2.27 log$_{10}$ can be seen for individual or monophage suspensions and cocktail as well. A further decline of 1.11 log$_{10}$ and 1.24 log$_{10}$ was recorded for SalØ-ABF37 and SalØ-MCOH26 which was followed by a decay in SalØ-RCMPF12 (2.00 log$_{10}$) and SalØ-DNLS42 (2.09 log$_{10}$). A decline of 0.90 log$_{10}$ was recorded upon completion of 60 min.
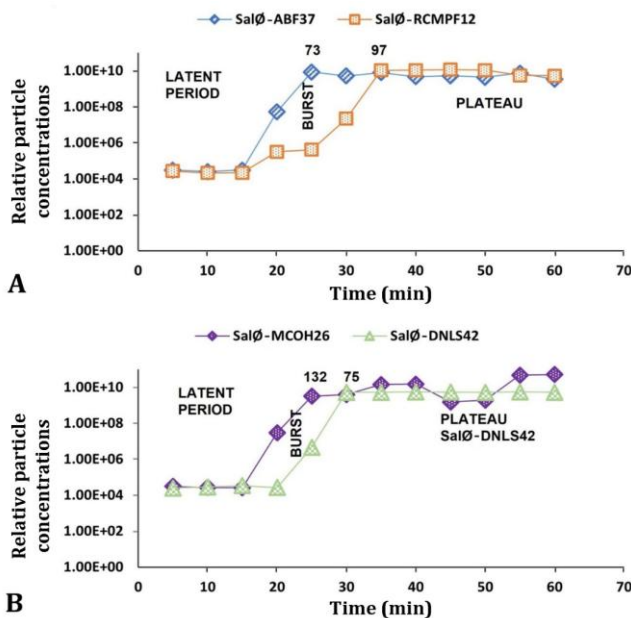
The isolates SalØ-ABF37, SalØ-RCMPF12, SalØ-MCOH26, SalØ-DNLS42 were found to be resistant to RNase A and S1 Nuclease with sensitivity to DNase I enzyme. For SalØ-ABF37, the *Eco*RI produced seven fragments and *Hind*III produced five fragments. For SalØ-RCMPF12, *Eco*RI produced eight fragments and *Hind*III produced seven fragments. *Eco*R1 and *Hind*III produced five fragments for SalØ-MCOH26 isolate. For SalØ-DNLS42, *Eco*RI produced three fragments and *Hind*III produced seven fragments. All the isolates produced restriction fragments of varying lengths against the standard 1 kB extended ladder showing that all isolates were different from each other as can be seen in Figure 3.
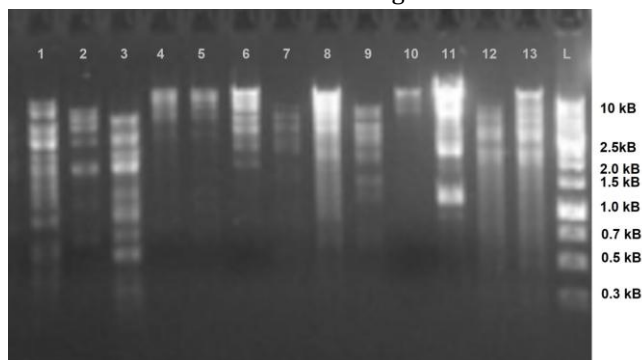
Fig. 3. Restriction analysis of bacteriophage DNA with *Eco*R1 and *Hind*III. Lanes 1, 2: digested DNA of SalØ-ABF37; lanes 3, 6: digested DNA of SalØ-RCMPF12; lanes 8, 9: digested DNA of SalØ-MCOH26, and lanes 12, 13: digested DNA of SalØ-DNLS42 followed by 1.00 kB extended ladder. Lanes 4, 5, 7, 10, and 11 are not part of this study.

Discussion

Irrational use of antibiotics as a surrogate to hygiene, exposing birds to sub-therapeutic antibiotic concentrations and antibiotic resistance often remains under-reported. The situation has become more intricate with the emergence of multi-drug resistant bacteria.[18,19] Bacterio-phages have emerged as an alternative solution. They are the most diverse and ubiquitous entities in the biosphere, superseding all other organisms in number highlighting their origin dating back to the Pre-Cambrian era, the earliest period of earth's history that spanned about 4.50 billion years ago.[20] Previously anti-*Salmonella* bacterio-phages have been isolated from litter and sewage effluents which attest their presence in an environment containing the targeted host bacterial population,[21,22] and have been demonstrated to control the *Salmonella* infection in animals.[23] To our knowledge, this is the first study that involves isolation and preliminary *in vitro* assessment of biological parameters of bacteriophages against typhoidal biotypes from serovar Gallinarum, associated with significant production and monetary loss in Pakistan.

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.[11] *Salmonella* bacteriophages like LPST153 have been reported with broad host ranges of over 65.00% and have been used as a biocontrol agent for surface decontamination of chicken meat.[24] Out of 14 strains, four bacteriophage isolates were active against 12 of the strains including four strains of *S.* Typhimurium, four strains of *S.* Enteritidis, three strains of *S.* Gallinarum and one strain of *S.* Pullorum. Overall, bacteriophages showed lytic activity in over 85.00% of bacterial strains tested. The results of our study agree with those of Bao *et al.,* in which they tested the bacteriophages PSPu-95 and PSPu-4-116 against 18 *Salmonella* spp. and two strains of enterotoxigenic *E. coli*. They reported that PSPu-95 had a lytic effect on 17 out of 20 strains used in the study accounting for 85.00% covered while PSPu-4-116 had a lytic effect on 14 strains accounting for 70.00% coverage: most notably against enterotoxigenic *E. coli* K88.[9]

A study conducted by Rahaman *et al.* demonstrated the host range of isolated bacteriophages against nine isolates of *Salmonella* spp., one enterotoxigenic strain of *E. coli*, *P.multocida* and *H. paragallinarum*. Their bacteriophage isolate showed a wide host range against *Salmonella* isolates with lytic activity observed in 77.77% of strains tested; no activity was observed against *E. coli*, *P. multocida* and *H. paragallinarum*.[8]

The bacteriophage isolates cited in the present study were tolerant to moderately high temperatures over 60.00˚C and remained viable between pH 3.00 - 9.00. A similar study by Hong *et al.* reported the thermal and pH stability of their three bacteriophage isolates (namely ST4, L13 and SG3) against *S.* Gallinarum in which ST4 showed remarkable efficacy *in vitro* and was most stable at pH 5.00 - 8.00 while L13 was most stable around pH 9.00 and SG3 was most stable around pH 11.0. They also reported a decrease in phage viability above 45.00 ˚C.[25] The study of Bao *et al.* agrees with the present study in which they measured the viability of *S.* Enteritidis bacteriophages at different temperatures. The two isolates PA13076 and PC2184 were stable between 30.00 - 50.00 ˚C for 30 min and 60 min, followed by 2 log reductions for PA13076 and slight reductions in PC2184. PA13076 was not viable at 70.00 ˚C whereas PC2184 lost viability at 80.00 ˚C after 60 min. The titers were relatively stable at 6.00 - 11.00 pH and declined dramatically under lower conditions.[9]

In vitro assessment of the lytic spectrum of the bacteriophages, the cocktail suppressed the bacterial activity for up to 8 hr. The results of the current study are supported by the study of Hungaro *et al.* in which they demonstrated that bacterial concentrations exposed to their phage isolates were reduced in 2 to 4.5 hr depending upon the conditions of the experiment like the multiplicity of infection, temperature, concentration of bacterial host population and bacteriophage concentration.[26] The study

of Hamza *et al.* is also in agreement with our study as they reported that the SA bacteriophage isolates repressed bacterial growth for up to 8 hr, however; even after 24 hr, the bacterial growth in the mixture treated with bacteriophage was less than the untreated control.[12]

Adsorption is *sine qua non* to initiate the infection cycle through a successive interaction between the binding proteins of bacteriophage particles and the receptors that are present on the bacterial surface. Apart from the initiation of infection cycle, this step is significant as the presence or absence of host receptors plays a significant role in the host range of a phage. The bacteriophage isolates from the present study showed greater than 50.00% adsorption within 10 min and after 15 min adsorption increased to 95.00%. These results indicate a strong ligand-receptor relationship and specificity between the phages and the bacterial strains tested by Rahaman *et al.* also cited greater than 95.00% adsorption within 15 min intervals.[8] The shorter latent period accompanied by higher burst size is the ultimate goal to harness the full potential of bacteriophages in therapeutic applications. In the present study average latent time was recorded at 20 min with the burst sizes observed as 76.00 to 88.00 PFU per lysed cell. Bao *et al.* also reported results that are concordant with our study stating that the average latent period for PSPu-95 and PSPu-4-116 was also of short duration (i.e., 20 min) yielding burst sizes of 77.50 and 86.00 PFU CFU^{-1}, respectively.[9] Santos *et al.* also supported the study with somewhat similar results demonstrating 58.00 PFU CFU^{-1} in 30 min.[14] Hong *et al.* cited the burst sizes for their isolates as 1,670, 80.00 and 28.00 PFU CFU^{-1} around 30 min for ST4, L13 and SG3, respectively.[25]

All candidates from this 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*. Upon restriction, the extracted genome of SalØ-DNLS42 with *Hind*III yielded seven bands of various sizes and digestion with *Eco*R1 yielded five bands. Overall size of the restriction fragment of SalØ-DNLS42 exceeded 35.00 kbp in size. The study of Bao *et al.* also reported similar results where restriction digestion showed that bacteriophage isolates PSPu-95 and PSPu-4-116 had genomes of 58.30 kbp and 45.20 kbp in length.[9] The study of Hyuk-Joon Kwon *et al.* also reported that the T7-like bacteriophage isolate ØSG-JL2 (lytic against) *Salmonella Gallinarum*) was found to contain 38, 815 bp.[11] Another study by Zhang *et al.* reported that the SJ2 isolate used in their study contained a genome of 152, 460 bp.[16] Similar results have been reported in the literature for many characterized *Salmonella* bacteriophages[27,28]

In conclusion, the present study provides a 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 a novel cocktail to be used as an effective alternative to antibiotics for prophylaxis and treatment against avian salmonellosis.

Acknowledgments

Conflict of interest

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

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