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In-vitro evaluation and efficacy of bacteriophage isolated from commercial poultry farms against *Salmonella typhimurium*

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Abstract

Avian Salmonellosis impacts the economy and public health, with chicken products being a major cause of gastroenteritis. Hygiene, immunization and medicines are all used as control techniques. Bacteriophages provide a safe, targeted alternative. In the present study *in vitro* evaluation of bacteriophages were done against *Salmonella typhimurium*. Lytic effect of bacteriophages isolated from poultry sludge was checked on culture of *S. typhimurium*. Stability study was checked at range of temperature and pH. The phages were stable at temperature (30.00 - 50.00 ˚C) and pH (5.00 - 9.00) where best activity was seen at 37.00 ˚C and pH 7.00. *In vitro* lytic activity was done at (optical density 600 nm) after exposure to bacterial host at different intervals. Multiplicity of Infection of 1.00 was used to check lytic activity of phages which indicated phages were potent enough to infect bacterial cells within their growth cycle. The percentage of unadsorbed phages was determined by bar chart analysis. The genome of three phages was treated with DNase I where they all were sensitive. Later the nucleic acid of phages was digested by restriction endonucleases (*EcoRI* and HindIII) both of the enzymes produced various restriction sites with different band. The present study proved that the application of bacteriophages *in vitro* into bacterial system *i.e., S. typhimurium* was an attractive method in diminishing infection in commercial poultry thus providing exceptional results that could be used on a large scale.

Introduction

The Salmonella is gram-negative and member of the family *Enterobacteriaceae* that are potentially pathogenic to humans. They are intestinal intracellular pathogens of many mammalians host as well as birds, reptiles, amphibians and even plants. Non-typhoidal *Salmonella* causes an estimated 1.40 million cases of salmonellosis.[1] *Salmonella* genus is divided into two species: *S. bongori* and *S. enteric.* Fifty serogroups are based on somatic antigens while 2,400 serovars are based on flagellar antigen.[2] A small number of serovars infect humans and majority belong to *S. enterica* ssp. I. Two types of diseases in humans occur due to *S. enterica* ssp. I by intake of contaminated food or water: Gastroenteritis which is mainly caused by *S. enterica* serovar *Typhimurium* and *S. enterica* serovar *Enteritidis* (typhoid). *S. enterica* serovar *Typhimurium* belongs to serogroup B and is a prototroph with vast host range including both warm- and cold-blooded animals.[3]

Serovars that are pathogenic to birds are categorized into three main categories (*e.g. S. enterica* subsp. *enterica* serovar *Typhimurium* and *S. enterica* subsp. *enterica* serovar Enteritidis) causing paratyphoid infections are non-host adaptive species.[3] Poultry is particularly prone to paratyphoid infections and is often major source of foodborne illnesses in human being via ingestion of contaminated meat or eggs. The remaining two biovars, being genetically similar, are highly host-adaptive include *S. enterica* subsp. *enterica* serovar Gallinarum biovar *Gallinarum* (*S. gallinarum*) causing fowl typhoid and *S. enterica* subsp. *enterica* serovar Gallinarum biovar *Pullorum* (*S. pullorum*) causing pullorum disease commonly termed as 'white bacillary diarrhea'[3] *S. typhimurium* is majorly involved in eggs and egg products based foodborne illness. This bacterium colonizes into gut

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flora for prolonged time and leads to egg contamination via horizontal route of infection. *S. typhimurium* and *S. enteritidis* have ability to invade into reproductive organs.[4] Crucial factors which are involved to cause the pathogenicity invade in cells of intestinal epithelium, survive intracellular in infected macrophages and suppress the immune system and its response.[5]

Poultry and their products are mainly involved in spreading Salmonellosis worldwide.[6] *Salmonella* being highly specific with narrow host range, such as chickens, have little public health importance. These organisms are responsible for considerable economic loss in poultry production. Effective monitoring at slaughter and processing plants is needed to restrict these pathogens.[7] To control *Salmonella* infection in chicken and in food items a multifactorial approach has been established through control measures. Irrespective of control measures, *Salmonella* is a persistent concern for both poultry and humans.[8]

Excessive use of antibiotics in poultry is leading to antimicrobial resistance strains of *Salmonella*. Resistance at human side through antibiotic residues in poultry derived products is, thus, creating eloquent public health issues. These challenges have pushed scientists to design progressive, effective and improved control strategies.[8] One of the most captivating alternative, which has gained prominence is the use of bacteriophages.[9] Bacteriophages are known to be the simplest and most abundant organisms on planet earth that can be found part of both commensal flora and environmental ecosystem.[10]

Bacteriophages are interested bio-control agent nowadays due to its self-replicating, target specificity and wide host range, and considered to be an excellent replacement of antibiotics.[11] Bacteriophages are used as potent specie in controlling pathogens of human and veterinary concern due to emergence of antimicrobial resistance in past few decades.[12] Use of virulent bacteriophages as a biocontrol are providing sustainable remedies and to circumvent issues related to development of multidrug- resistant bacteria.[13] To minimize the *Salmonella* infection in poultry and risks involved in transmission to humans there should be proper understanding of bacteriophages. Using lytic bacteriophages to decrease colonization of *Salmonella* at early stages of production in poultry will be the operative preventive strategy to limit transfer of infection.[14-16] Bacteriophages pose several advantages to reduce *Salmonella* colonization in chickens. Many research studies have recommended the use of bacteriophages as a substitute in reducing *Salmonella* on chicken carcass, eggs and other poultry derived products.[17,18]

The phages used should not carry genes that cause pathogenicity or increase the pathogenic index of their specific targets.[19] Phages being host specific and safe for human being is an excellent step toward the control of biological entities that are mainly affecting the poultry-based economy and helping in reduction of diseases related to poultry, thus, their transmission into human food chain. The present study was aimed to investigate the *in vitro* efficiency of bacteriophages against *S. typhimurium* as well as their stability, lytic activity and genetic features in order to identify their potential for lowering infections in commercial poultry.

Materials and Methods

Sample collections. Reference strain of *S. typhimurium* (ATCC 14028) was purchased and was revived at Central Lab Complex, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki, Pakistan. For the isolation of bacteriophages, 100 mL sewage samples (n = 4) were collected from various poultry farms located in the vicinity of Lahore city and were transported sterile to lab at 4.00 ˚C for characterization of bacteriophages.

Sample processing. The ATCC strain of *S. typhimurium* was revived by growing it on Salmonella and Shigella (SS) agar and confirmed by "*spy*" gene polymerase chain reaction. Commercially available kit Gene JET Genomic DNA purification kit (Thermo Scientific, Waltham, USA) for extraction was used. Gene amplification was done using reaction mixture 25.00 µL (10.00 µL of Master mix Thermo Scientific DreamTaqTM Green PCR, 2.00 µL DNA Template, 11.00 µL Nuclease free water, 1.00 µL Forward primer, and 1.00 µL Reverse primer) and primers Fp (5'TTGTTCA CTTTTTACCCCTGAA 3') and Fr (5'CCCTGACAGCCGTTAG ATATT 3') under standard PCR conditions.

Isolation of bacteriophage. The sewage samples were labeled as AZ1, AZ3, AZ2 and P4. These samples were centrifuged at 3,900 rpm for 15 min and filtered via syringe filter (0.22 um; 0459 Millex-HV filter unit). Equal volume of the filtrate and culture of *S. typhimurium* (log phase; 18 hr) were mixed and incubated in shaking incubator (SI900R Robus Technologies, Karachi Pakistan) at 37.00 ˚C for 24 hr. After incubation, the mixture was centrifuged at 3,900 rpm for 15 min and filtered through 0.22 um syringe filter. This prepared lysate was used for further confirmation by spot test and plaque assay.[20]

Detection, enumeration and purification of bacteriophage. Presence of bacteriophages in sample was determined by spot assay.[21] Phage suspension showing positive spots against *S. typhimurium* were further purified by plaque assay.[22]

Determination of phage host range. Lytic activity of bacteriophages against various bacterial hosts was checked. A total six bacterial isolates, including *S. pullorum*, *S. gallinarum*, *Escherichia coli*, *Campylobacter jejuni*, *Shigella* and *Proteus* were used in this assay. Purified bacteriophage suspension (10.00 µL) was tested against six bacterial isolates using spot assay. The plates were

analyzed for clear zone of bacterial lysis after 24 hr incubation at 37.00 ˚C.

Thermal and pH stability. Thermal stability of phages was determined by exposing the phage lysate at various temperatures. Bacteriophage lysate (1.00 mL) in Tryptic Soy Broth (TSB) as placed at temperatures 30.00, 40.00, 50.00, 60.00, 70.00 and 80.00˚C for 1 hr. After exposure, the Plaque forming units (PFU) of bacteriophage was calculated. Similarly, for pH stability of the phages was determined by adjusting the pH of phage lysate with NaOH of HCl to obtained 3.00, 5.00, 7.00 and 9.00 pH. The tubes were incubated for 2 hr at 37.00 ˚C. After incubation, PFU were calculated following Plaque Assay.[23]

***In vitro* evaluation of lytic activity of bacteriophages.** For evaluation of lytic activity of phages, multiplicity of infection (MOI) was calculated following formula:

$$MOI = \frac{PFU\ of\ virus\ used\ for\ infection}{number\ of\ bacterial\ cells}$$

The TSB was prepared and poured 100 mL in 2 sterilized conical flasks with labeled as test and control. In both flasks, 1.00 mL of bacterial culture in log phase (1.00 × 10^8 colony-forming unit *per* mL with optical density (OD) 0.40 MacFarland) was added. In test flask, 1.00 mL of phage, with 1.00 MOI equal to 1.00 × 10^8 PFU *per* mL was added while 1.00 mL sterile normal saline was added in control flask. The flasks were placed on shaker for shaking at 120 rpm at 37.00 ˚C. 2.00 mL of material from each flask was collected before shaking was started which served as blanks. The OD values, after every 2 hr interval and up to 24 hr by spectrophotometer (Specord 200, Analytik Jena, Jena, Germany).The OD values were recorded to evaluate the lytic activity of bacteriophages by reducing the bacterial turbidity.[24]

Electron microscopy of bacteriophages. Purified bacteriophages were grown to a high titer (10^{12} PFU/mL) in tryptone soya broth and submitted National Institute of Biotechnology and Genetic Engineering (NIBGE). Samples were mounted on copper grids, washed with ammonium acetate (0.1 M, pH 7.0), and negatively stained with 5.00% uranyl acetate. They were then observed using a JEOL JEM 1010 Transmission Electron Microscope at 100 kV.

One step growth of bacteriophages. Overnight bacterial culture in TSB was centrifuged at 10,000 rpm for 5 min, the supernatant was discarded and pellet was collected. The pellet was suspended in 1.00 mL TSB. Bacterial suspension was mixed with equal volume of phage (1.00 MOI) and incubated at 37.00 ˚C for 10 min. After incubation, it was centrifuged at 20,000 *g* for 40 sec to separate unbound phages. Supernatant was discarded and pellet was resuspended in Luria-Bertani medium (LB) broth (100 mL) and incubated at 37.00 ˚C for 55 min in shaking incubators. Sample (1.00 mL), from the mixture, was collected after every 5 min and processed for plaque assay by inoculating it onto the TSA plates. The plates were incubated for 24 hr at 37.00 ˚ in incubator (New Brunswick, Hamburg, Germany) and PFU were calculated. The graph obtained after PFU counting indicated single step growth curve.[25]

Phage adsorption rate. An equal volume of phage suspension was added to mid log phase of bacterial culture (1.00 × 10^8 colony-forming unit *per* mL) with OD adjusted at 0.40. For better adsorption to bacterial system 67.00 µL of each 1.00 mM $CaCl_2$ and 1.00 mM $MgCl_2$ were added to the phage-bacterial suspension. The mixture was given incubation for 60 min at shaking (180 rpm) at 37.00 ˚C. Samples were collected at 0, 5, 10, 20, 30, 40, 50, and 60 min of incubation. The collected samples were centrifuged at 7,000 *g* for 5 min and the supernatant was checked for unadsorbed phages by agar overlay method.The percentage of unadsorbed phages was interpreted by bar chart.[26]

Nucleic acid extraction. Nucleic acid extraction for purified phages was performed by phenol chloroform isoamyl alcohol method.

Restriction digestion. The genome of phages (nucleic acids) was first digested by DNase I solution (RNase free 1,000 U *per* mL Thermo Fischer Scientific, Waltham, USA) and later treated with two enzymes FastDigest EcoR1 (Thermo Fischer Scientific) and FastDigest HindIII (Thermo Fischer Scientific) to observe the restriction patterns of the isolated bacteriophages as *per* manufacturer's instruction. All reaction mixture solutions were mixed in microfuge tube by gentle spinning at room temperature. The tubes were incubated at 37.00 ˚C for 10 min. Bands were checked on 1.25% agarose gel 2.00 µL) of DNA ladder (GeneRuler™ 1.00 kb; Thermo Scientific) was poured into the respective well followed by addition of treated phage samples with the enzymes EcoR1 and HindIII (8.00 µL) into their corresponding wells. Voltage of 80.00 v was given for 45 min and later the gel was viewed under ultraviolet illuminator.

Results

Growth of *S. typhimurium* on Salmonella and Shigella (SS) agar. This agar is selective and differential media for the growth of Salmonella as it contains bile salts, sodium citrate and brilliant green that inhibit the growth of various gram-positive bacteria, coliforms and proteus while allowing Salmonella to produce colonies. Ferric Citrate and thiosulphate allow detection of hydrogen sulfide that provides black centered colonies in case of *S. typhimurium*. Therefore, typically *S. typhimurium,* being gram negative, lactose non fermenter gave dark black colored colonies covering the entire surface of SS agar plate as shown in Figure 1A.

Polymerase chain reaction. The PCR results further confirmed the *S. typhimurium* by targeting universal primer *16srRNA* showing the size of *S. typhimurium* up to 401 bp mentioned in Figure 1B.

Isolation and characterization of bacteriophages. Four fresh samples were collected from the sewage water. Three (n = 3) isolates (Fig. 1C to 1F) showed positive lytic zones on the media plate labelled as AZ1 with 0.50 mm diameter with well-defined edges, AZ2 with 1.50 mm lytic zone and AZ3 with generally larger zones of 2.00 mm diameter.

Phage host range. The titrated phages were spotted against *S. gallinarum, S. pullorum* and certain members of family Enterobacteriaceae including *E. coli, C. jejuni, Enterococcus faecalis, Shigella* and *Proteus*. Phages showed positive lytic zones among closely related Salmonella serovars including *S. gallinarum* and *S. pullorum*. Purified phages also showed positive spot for *Shigella*, while phages did not produce positive lytic zones against *E. coli, C. jejune, E. faecalis* and proteus as shown in the Table 1. The results indicated that bacteriophages were very specific toward their host or closely related members of the family. It could also be assumed that bacterial receptor modification failed the virus to adsorb on the particular host surface. Also, some resistance genes were encoded in each bacterial system that unable the bacteriophages to cope with host range.

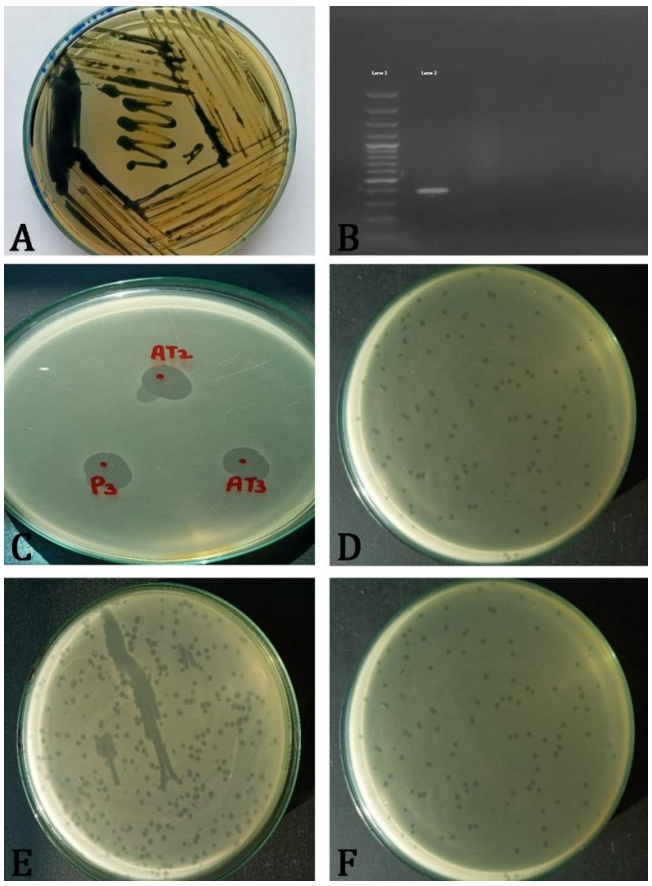

Fig. 1. A) Typical black colored colonies of *S. typhimurium* seen on S.S agar, **B)** Polymerase chain reaction product showing, lane 1: 100 bps DNA ladder and lane 2: 16srRNA gene of *S. typhimurium* band size 401bp, **C)** Spot test for the presence bacteriophages, **D)** AZ1 phage, **E)** AZ2 phage, and **F)** AZ3 phage titration by plaque assay.

Thermal stability. The isolated phage showed titer in decreasing order when treated at 50.00 and 60.00 ˚C. The titer become zero at 70.00 and 80.00 ˚C when kept for one hour due to extreme temperature at which bacteriophages failed to survive in the media, whereas, a significant titer was seen at temperature ranging between 30.00 to 40.00 ˚C. This temperature was considered a suitable temperature for phages to produce lytic activity and the optimal growth was seen at 37.00 and 40.00 ˚C (Table 2).

pH sensitivity. Results of phage suspension, treated with wide range of pH from 3.00 to 9.00, showed that phages were unable to grow at extremely low pH from 2.00 to 3.00, while gradually increasing pH from 4.00 to 9.00, preferably a good titer of bacteriophages was recorded up to 2 hr. The results showed that extreme acidic or basic conditions made phages difficult to grow and multiply in their respective host while pH 7.00 was found ideal for multiplication of the bacteriophages (Table 2).

***In vitro* evaluation of lytic activity of bacteriophages**. Bacterial log reduction was determined using 1.00 MOI of phage samples treated against *S. typhimurium* by adjusting OD at 600 nm after every 2 up to 24 hr. The lytic activity of bacteriophages showed that after 24-hr incubation, OD values are calculated (Table 3).

One step growth of bacteriophages and phage adsorption rate. Growth curve was evaluated by exposure of bacterial culture with purified phages in order to experience generation time, latent period and burst sizes of the phages. Bacteriophage titer was recorded by plaque assay and plotting a graph. The graph showed an average 10 to 15 min for latent period for AZ1 phage with burst size of 25.00 PFU *per* cell, where phage AZ2 showed a latent period of 25 min with a burst size of 29.00 PFU *per* cell and AZ3 had a burst size of 33 PFU *per* cell with a latent time of approximately 30 min (Fig. 2 and Table 4).

Table 1. Bacteriophages host range.

Bacterial host	Spot test	Plaque forming units *per* mL
***Salmonella gallinarum* (ATCC 9184)**	Positive	1.47×10^{10}
***Salmonella pullorum* (ATCC 9120)**	Positive	1.50×10^{9}
Escherichia coli	Negative	-
***Campylobacter jejni* (ATCC 33291)**	Negative	-
***Enterococcus faecalis* (ATCC 19433)**	Negative	-
Shigella	Positive	1.20×10^{10}
***Proteus vulgaris* (ATCC 6380)**	Negative	-

Table 2. Thermal stability and pH stability of all isolated bacteriophages.

Temperature (˚C)	PFU *per* mL			pH	PFU *per* mL		
	AZ1	AZ2	AZ3		AZ1	AZ2	AZ3
30.00	2.50×10^7	5.00×10^7	4.10×10^7	3	0	0	0
40.00	1.50×10^8	2.00×10^8	1.60×10^8	5	5.00×10^7	1.10×10^7	3.50×10^7
50.00	1.10×10^7	1.50×10^7	1.40×10^7	7	1.40×10^8	1.80×10^8	1.50×10^8
60.00	1.00×10^2	0.50×10^2	1.20×10^2	9	1.10×10^7	1.60×10^7	1.10×10^7

PFU: Plaque forming units; AZ 1: Amina Zaib isolate 1; AZ 2: Amina Zaib isolate 2; AZ 3: Amina Zaib isolate 3.

Table 3. Mean optical density showing lytic activity of bacteriophages AZ1, AZ1, AZ3. The data shown are the mean of three replicates.

Time (hr)	Phage + *Salmonella typhimurium*			Bacterial control
	AZ1	AZ2	AZ3	
2	0.11	0.08	0.08	0.3
4	0.11	0.09	0.09	0.55
6	0.11	0.27	0.1	0.71
8	0.23	0.34	0.1	0.83
10	0.36	0.42	0.26	0.93
12	0.44	0.50	0.36	1.14
14	0.58	0.62	0.45	1.35
16	0.71	0.71	0.54	1.44
18	0.82	0.82	0.63	1.52
20	0.92	1.09	0.75	1.65
22	1.18	1.23	0.88	1.74
24	1.33	1.37	1.02	1.88

AZ 1: Amina Zaib isolate 1; AZ 2: Amina Zaib isolate 2; AZ 3: Amina Zaib isolate 3.

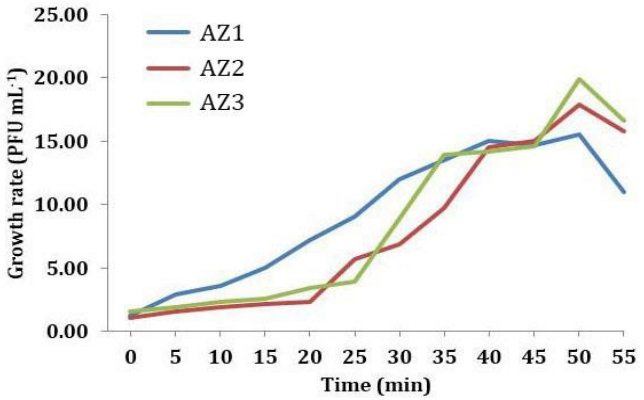

Fig. 2. One step growth curve for phage AZ1, AZ2 and AZ3 (AZ 1: Amina Zaib isolate 1; AZ 2: Amina Zaib isolate 2; AZ 3: Amina Zaib isolate 3); PFU: Plaque forming units.

Table 4. Values for one step growth curve for phage AZ1, AZ2 and AZ3.

Time (min)	AZ1	AZ2	AZ3
0	1.20×10^5	1.10×10^6	1.60×10^8
5	2.90×10^5	1.60×10^7	1.90×10^7
10	3.60×10^6	1.90×10^7	2.30×10^7
15	5.00×10^6	2.20×10^7	2.60×10^9
20	7.20×10^7	2.30×10^8	3.40×10^7
25	9.10×10^8	5.70×10^8	3.90×10^8
30	12.00×10^9	6.90×10^{10}	8.90×10^8
35	13.50×10^8	9.70×10^9	13.90×10^8
40	15.00×10^9	14.50×10^8	14.20×10^9
45	14.70×10^9	15.00×10^9	14.60×10^9
50	15.50×10^9	17.90×10^9	19.90×10^{10}
55	11.00×10^{10}	15.80×10^8	16.60×10^9

AZ 1: Amina Zaib isolate 1; AZ 2: Amina Zaib isolate 2; AZ 3: Amina Zaib isolate 3.

Phage adsorption rate. As obvious from the graphical representation (Fig. 3) that in first 0 to 5 min the percentage of unadsorbed phages is generally high above 80.00% whereas it gradually decreases as the time elapsed after 20 min of incubation the percentage of unadsorbed phages was less than 20.00% proving that the binding of the virus to the host cell nearly took not more than 20 min and was permanent. The results of percentage of adsorbed phages were contrary to the unadsorbed bacteriophages.

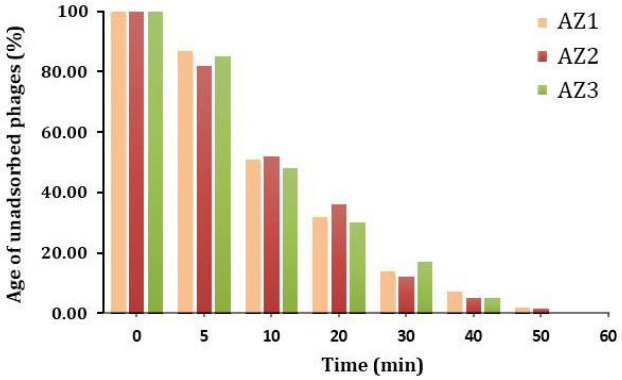

Fig. 3. Adsorption Rate indicating percentage of unadsorbed phages in different times. (AZ 1: Amina Zaib isolate 1; AZ 2: Amina Zaib isolate 2; AZ 3: Amina Zaib isolate 3).

Restriction digestion. Bacteriophages were sensitive to DNase I (1,000 U *per* mL) showing that their genome were DNA based. Whereas, when treated with restriction enzymes, FastDigest EcoR1 and FastDigest *HindIII* produced bands at various sites indicating that the genome of the phages had certain restriction sites (Fig. 4).

This elaborated the restriction digestion where L represented the 1.00 kb DNA ladder and lane 1 and 2 showed the restriction digestion of phage AZ1 by EcoR1 and Hind*III*, respectively. Lane 3 and 4 showed the AZ2 phage digestion by EcoR1 and Hind*III* accordingly and lane 5 and 6 showed digestion of phage AZ3 by EcoR1 and Hind*III*, respectively.

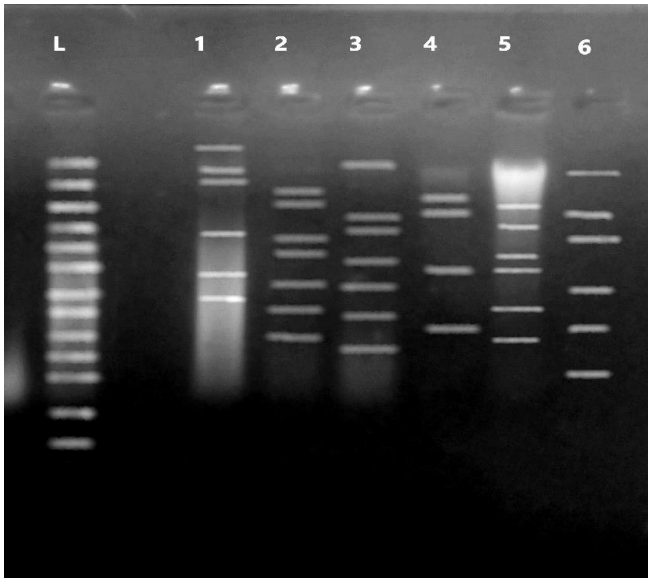

Fig. 4. Restriction digestion by EcoR1 and Hind*III* enzymes. L: 1.00 kb DNA, lanes 1 and 2: Phage AZ1 digested by (EcoR1 and Hind*III*), lanes 3 and 4: Phage AZ2 by (EcoR1 and Hind*III*), Lanes 5 and 6: Phage AZ3 digested by (EcoR1 and Hind*III*).

Discussion

Globally *Salmonella* infection has caused massive economic and health issues. Salmonellosis is a major cause for food-borne illness. Direct contamination from poultry farms and poultry meat and other products are the biggest source of infection which ultimately enters into human food chain.[27]

Multiple cases of Salmonellosis are increasing day by day and especially poultry industry is facing crippled economy. In order to overcome the present situation researchers are moving toward better farm management protocols, vaccination programs and disinfection methods to decelerate the loss. Poultry meat is considered to be the highest consumption meat worldwide, however, still facing hurdles by regular loses. Antibiotics have generally caused resistance among human being and animals.[28]

A study conducted in Portugal stated that on an average non typhoidal salmonellosis caused 93.8 million illnesses and 155,000 yearly worldwide, among which Center for Disease Control and Prevention (CDC) Reports 1.00 million illnesses in USA every year compared to other food borne illness. Thus *Salmonella* infection is thought to be at top to cause gastroenteritis.[29]

Other approaches including antibiotics have created more hindrances due to resistance. Since from past 20 years no new antibiotic has been synthesized and it has created a dilemma worldwide to combat such disorder.[30] Food borne Salmonellosis includes infection by *S. typhimurium* and *S. enteritidis* thought to be majorly transmitted by poultry meat, poultry products and thus induces severe illness.[31]

Health workers and scientists believe in other alternatives such as prebiotics, probiotics and mass level vaccination programs to mask up the disease. Bacteriophage treatment is thought to be cheap and yet admirable approach against infection in poultry.[32] *Salmonella typhimurium* was able to produce dark black centered colonies on the SS agar indicating its presence on its specific medium. In addition to this present study using universal primers the "spy" gene of *S. typhimurium* was targeted resulting into 401 bp size.[33] Phage isolation and titration were performed to understand that each phage is different from other one and to obtain single type of plaques formed by the following phages. In the present study after the isolation only three among total four phages (n = 4) produced positive spots against the *S. typhimurium.* Knowing that AZ1 having almost 0.50 mm diameter, AZ2 with 1.50 mm lytic zones and AZ3 with larger 2mm diameter size with well-defined edges. The following study was in accordance to a study conducted in Republic of Korea.[34] Enumeration of each phage was done by agar overlay method with approximately of countable range of PFU *per* mL.[35]

In the present study host range was evaluated by the identified phages. Tested against seven bacterial strains including *S. Pullorum, S. Gallinarum, E. coli, C. jejuni, E. Faecalis, Shigella* and *Proteus* accordingly. Among these bacterial strains *S. gallinarum, S. pullorum and Shigella* were able to give positive results while all three phages showed no lytic zones against other bacterial cultures. The following results can be interpreted as that bacteriophages are highly specific towards its host and differences occurred in host range due to loss of bacterial receptor toward the bacteriophages or certain bacterial resistance mechanisms.[35]

After the exposure of different temperature provided in the medium containing phages the following results showed that all of the bacteriophages were highly stable at temperature 37.00 ˚C (100%) and survived from a temperature range from 30.00 to 50.00 ˚C. While a little titer lowered at 60.00 ˚C but growth of phages was observed. Moving further at higher temperatures like 70.00 and 80.00 ˚C for 1 hr showed no sound results. Bacteriophage number was decreased all of a sudden due to extreme temperatures. Many studies concluded that physical parameters are utmost importance in stability of the bacteriophages which includes temperature and pH. Bacteriophages which are capable to survive with a wide

range of pH and temperature are significant in reduction of Salmonella colonization in birds.[36]

For the pH sensitivity, the purified phages were administered at a broad range of pH ranging from 3.00 to 9.00 for approximately 2 hr. Our results supported that extremely low pH *i.e*., 2.00 and 3.00 were highly lethal for the growth of bacteriophages in any system. Whereas as the pH increased in ascending order, phages exhibited stable growth from pH 5.00 to 9.00 where 7.00 was optimal for the survival of bacteriophages. The resilient behavior of bacteriophages to survive at vast range of pH suggested that no physiochemical factors of hosts could disturb the effects of phages. The current research findings was in accordance with study described.[37]

In vitro evaluation of lytic activity of bacteriophages against *S. typhimurium* was treated with our isolated phages with intervals of time in accordance with the OD. The isolated phages were tested against the *S. typhimurium* using a MOI of 1.00. Where AZ1 showed decrease in bacterial growth up to 6 hours and AZ2 for almost 4 hours. Isolate AZ3 showed bacterial reduction for 12 hr. Bacterial turbidity in the media was generally low when treated with all the three phages compared to the control tube. However, the OD values started increasing after 24 hr. However, even after 24 hr the bacterial titer treated with phages was low compared to the control. It might indicate the OD increased after 24 hr due to many dead bacteria or the resistance ones (Table 4). A study also indicated that at start the bacterial number might be high due to the gap between concentration of host and bacteriophage, however, as the time increased gradually the titer of phage was increased due to multiplication and penetration into the host cells, as a result salmonella number was decreased and was under control.[38]

In the recent study, one step growth curve was observed for the bacteriophages to determine their latent period, adsorption time and burst sizes where all of the phages (AZ1, AZ2 and AZ3) generally showed a short latent period between 15, 25, 30 min with burst sizes ranging from 25.00, 29.00 and 33.00 PFU *per* cell, respectively. It is considered that the length of latent period of the desired phages depended upon host, physiochemical properties, medium in which they grew, and incubation period. A short latent period with short generation time and large burst sizes are indications that the purified bacteriophage population can be used as a strong antimicrobial therapy.[39]

In this study adsorption rate of phages were determined by bar chart which showed that the initial 0 to 5 min accounted for more than 80.00% of unadsorbed phages to the host cell while as the time increased above 20 min it was seen that the percentage of unadsorbed phages was less than 20.00%. This series of events evaluated that the binding of phage to host cell was an irreversible process and the results of adsorbed phages were refractive to unadsorbed phages that in initial 0 to 5 min the percentage of adsorbed phages was generally less than 20.00%, whereas, more than 50.00% of bacteriophages adsorbed to the host bacterium in 10 to 20 min. 100% of adsorption was seen in complete hour.[26]

In the present study all the potent bacteriophages were subjected to DNA extraction. Many studies promote phenol chloroform method for the extraction of crude DNA samples.[40] In this study after the treatment of phages with DNase I genome of all three phages were sensitive to DNase I showing that all of the nucleic acid was DNA based. Phage genome was digested by restriction endonucleases *(EcoR1* and Hind*III*), respectively, which showed that genome of all three phages (AZ1, AZ2 and AZ3) had various restriction sites for these enzymes and being able to be digested by these restriction enzymes.[41] Several studies reported that sizes of phage samples isolated from sewage water should be determined by following restriction digestion mechanism for classification. Mostly bacteriophages infecting *Salmonella* species belongs to order Caudovirales following three families as *Siphoviridae*, *Podoviridae* and *Myoviridae* which comprises of contractile tailed phages and among total isolated phages so far 96.00% accounts for tailed phages that are involved in reduction of bacterial illnesses.[42]

Phage cocktail is more powerful substitute to eliminate the *Salmonella* infection in birds efficiently. Many researchers believe that more than one phage used becomes potent enough to reduce the growth of bacterial strain up to certain hours. A study by Mariann Landsberger and colleagues described that a single phage when infected bacteria it attacked the CRISPR mechanism thus leading to weakened immune system as a result other phage in a cocktail attack synergistically. As a result, they overwhelmed the pathogenicity of the host and shows effective results. It is however depicted that application of phages is a compelling strategy to overcome Salmonella infection.[43]

The current study proved that exaggerated use of antibiotics and antimicrobials have caused immense resistance in the bacterial systems including Multi Drug Resistance (MDR) Extensive Drug Resistance (XDR) etc. To overcome the situation more suitable and substantial approach to minimize the *S. typhimurium* infection in poultry could be achieved by the administration of potent nontoxic use of bacteriophages. It was demonstrated that by incorporation of cocktail of bacteriophages, they were efficient enough to produce exceptional results *in vitro*.

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Conflict of interest

The author declares that they have no known competing financial interests or personal relationship that could have appeared to influence the work reported in this paper. This research did not receive any specific grant from funding agencies in the public, commercial, or not for profit sectors.

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