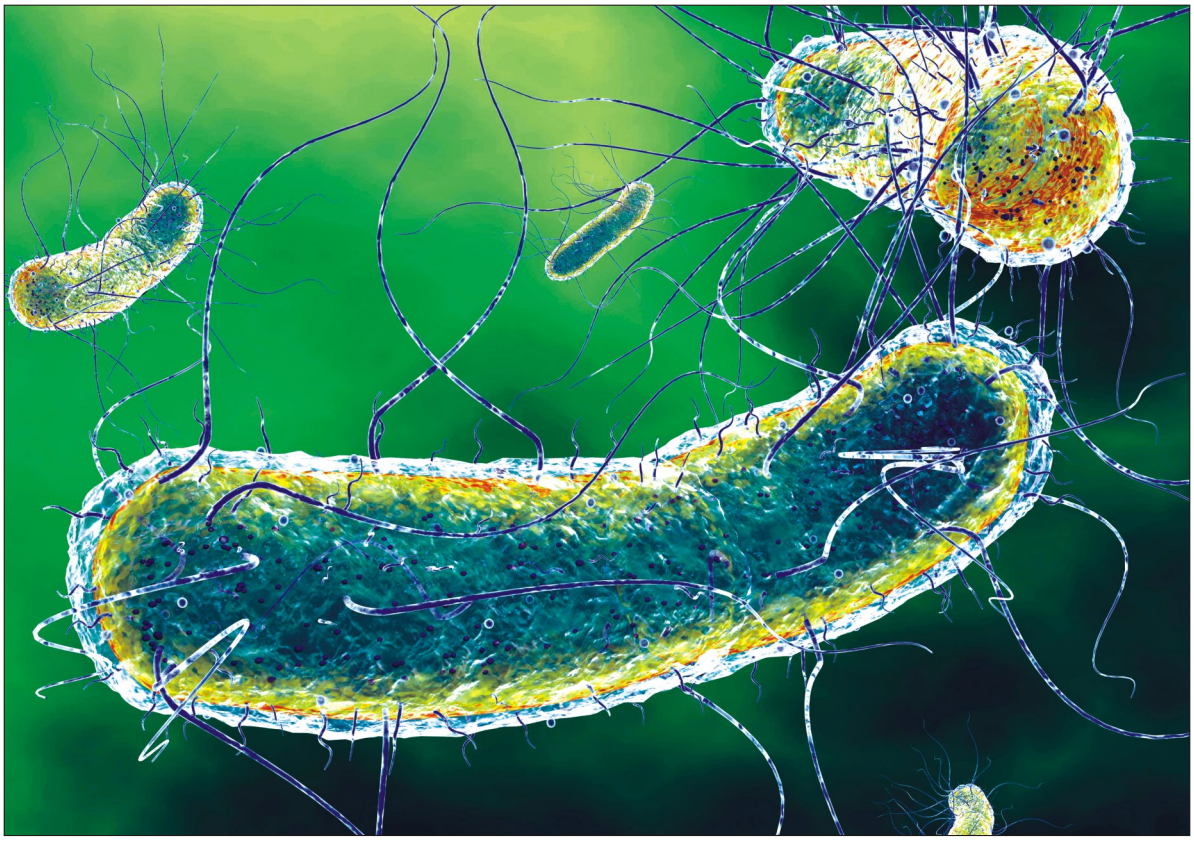

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Research Article
Isolation and Characterization of *Escherichia coli* and *Salmonella* Bacteriophages from Poultry

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Abstract

Background and Objective: *Escherichia coli* and *Salmonella* species are major microbes that badly affect poultry. Various antibiotics are being used to control them and subsequently, antibiotic resistance is increased. Bacteriophages are better alternatives to control resistant *E. coli* and *Salmonella* species. Bacteriophages of choice are expected in the environment of their host bacteria. The present study aims to isolate bacteriophages of *Escherichia coli* and *Salmonella* species from poultry samples. **Materials and Methods:** Poultry litter samples were collected and isolated strains of *E. coli* and *Salmonella* species, were evaluated for their antibiotic resistance pattern and used to isolate the bacteriophages. Poultry litter aqueous suspension was filtered with 0.2 µ syringe filters and used as a phage source. **Results:** Isolated *E. coli* phage is specific to the isolated five strains of *E. coli*, having burst size of 120 plaque forming units per cell, tolerant to salt concentration 0.5-1.5%, temperature 37-40°C, pH 4-8 and found to be a tadpole shaped measuring a diameter of 647 nm and long non-contractile tail of 125 nm. Isolated *Salmonella* phage is specific to the isolated six strains of *Salmonella*, having burst size of 211 PFU per cell, tolerant to salt concentration 0.5-1.5%, temperature 37-40°C, pH 4-8 and found to be tadpole-shaped measuring icosahedral head of 60 nm in diameter and a tail of 32 nm in length. **Conclusion:** The isolated *E. coli* and *Salmonella* phages are specific to only *E. coli* and *Salmonella*, respectively. These phages can be used in poultry to control *E. coli* and *Salmonella*.

Key words: Bacteriophage, *Escherichia coli*, *Salmonella*, antibiotic resistance, phage therapy

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Escherichia coli and *Salmonella* are the major microbes that are affecting the poultry industry adversely. *Salmonella* is a globally distributed food-borne pathogen. *E. coli* infections also cause severe losses to poultry appallingly. There is a considerable need for controlling the effect of these bacteria in the poultry industry. Bacteriophages are viruses that kill bacteria specifically. The use of *E. coli* and *Salmonella* bacteriophages as biocontrol agents has gained significant interest. The bacteriophages are abundant in the source of their specific bacteria. Phages have acquired the interest of researchers due to their host specificity, self-replication and abundance in the environment.

Increased demand for poultry products has forced the overuse of antibiotics and is causing resistant microbial infections. Interest has grown in phage therapy as an alternative treatment. The utilization of bacteriophage to kill resistant bacteria is a bright option for poultry to control diseases. Many bacterial phages are reported to control pathogenic *E. coli* and *Salmonella* in poultry. *Escherichia coli* and *Salmonella* are of main agents causing infections in poultry leading to huge losses to poultry and issues of public health[1]. The *E. coli* infections lead to avian colibacillosis, respiratory diseases, bronchitis[2]. *Salmonella* infections in poultry may cause pullorum disease (*Salmonella* pullorum), foul typhoid (*Salmonella gallinarum*), foul paratyphoid,etc[3]. To control the microbial load in commercial poultry, various antibiotics are being used and it is responsible for the increased emergence of antibiotic-resistant bacteria. Alternative strategies should be attempted to avoid the prevalence of microbial resistance[4-7]. Phage therapy can be a potential alternative to antibiotic treatment and proper control of multi-drug resistant bacteria[8-11]. Bacteriophages are potential therapeutic agents for bacterial diseases because they have high specificity and lysing of target bacteria[12]. In the view of above, the present study was undertaken for the isolation of *E. coli* and *Salmonella* species from different poultry farms and rising bacteriophages against them. Subsequently, their utilization in poultry farms to control *E. coli* and *Salmonella* diseases.

MATERIALS AND METHODS

Sample collection: Samples containing *E. coli, Salmonella* and their bacteriophages are poultry litter and drainage samples collected from 25 poultry farms of Telangana and Andhra Pradesh, India collected during April and May, 2022.

Isolation and identification of *E. coli* and *Salmonella*: Pure *E. coli and Salmonella* strains were isolated from collected poultry samples. The samples were suspended in sterile normal saline and spread on MacConkey agar and *Salmonella Shigella* agar, Himedia, India incubated at 37°C for 24 hrs. Growth on specific media, microscopic morphology, and biochemical tests such as indole test, methyl red test, voges proskauer test, citrate test, TSIA (triple sugar iron agar) test, urease test, dulcitol fermentation test and lysine decarboxylase test were determined[13].

Antibiotic susceptibility test: Antibiotic susceptibility of *E. coli* and *Salmonella* was determined by kirby-bauer disc-diffusion method[14]. Eight different antibiotics were used in these tests ampicillin, norfloxacin, tetracycline, ciprofloxacin, kanamycin, amikacin, streptomycin and amoxicillin obtained from Merck India.

Isolation of the bacteriophages

Bacteriophage enrichment: Bacteriophage enrichment was done by taking 4 mL of 0.2 µ filtered sample water suspension (phage source), 1 mL of 10x luria broth and 1 mL of exponential growing bacteria and incubating at 37°C for 24 hrs. Then the suspension was centrifuged at 15000 rpm for 5 min and filtered through a 0.2 µ syringe filter. The filtrate was mixed with pure culture and overlaid using double agar layered-based plaque assay method[15].

Detection of bacteriophages/plaque assay: In a sterile Eppendorf, 0.2 µ syringe filtered 100 µL of bacteriophage source and 100 µL of exponential bacterial culture were added and incubated at 37°C for 15 min, then it was mixed with 5 mL low melting agar (0.8%) and poured onto a nutrient agar plate. Allowed the low melting agar to solidify for 30 min at room temperature and then plates were incubated inverted at 37°C for 24 hrs.

Purification of phage: Using a sterile scalpel, an isolated plaque was picked from the overlayed nutrient agar plate and suspended with 500 µL of phage buffer and diluted. A dilution was mixed with exponential bacterial culture, incubated and underwent double agar layered-based plaque assay[15]. Individual plaque obtained in this method is selected.

Host inactivation studies: Pure *E. coli* and *Salmonella* were inoculated separately into two flasks containing nutrient broth and incubated at 37°C for 24 hrs. Then the two flasks were infected with 0.2 µ filtered phages and incubated at 37°C with

gentle shaking. The sample was collected from both flask every 1 hr, till 8 hrs consecutively. The hourly samples of both flasks were spread on the nutrient agar plates, respectively for the viability of host cells. The numbers of colonies in the hourly samples were counted by using the colony counter multilab India. The time required to kill 90% of initial cells was measured.

Burst size determination: An isolated plaque was picked into a sterile Eppendorf containing 500 µL of phage buffer and then it was added to 500 µL of bacterial culture in an Eppendorf and 100 µL of the mixture was undergone double-layered agar-based plaque assay[15].

Salt, heat and pH tolerance: Salt tolerance was determined by treating the phage filtrate in successive tubes containing nutrient broth with additional 0.5, 1.5, 2.5, 3.5 and 4.5% salt, incubated at 37°C for 1 hr. Heat tolerance was determined by treating the phage samples at 37, 40, 50, 60, 75 and 85°C for 1 hr. The pH tolerance was determined by treating phage filtrate in consecutive tubes containing nutrient broth of 1 to 10 pH ranges, incubated at 37°C for 1 hr. This bacteriophage filtrate treated with different salt, heat and pH concentrations was overlayed using double layered gar-based plaque assay method[15].

Purification of phages

Phage purification with centrifugation: Phage lysate was made cell-free by centrifuging at 5000 rpm for 10 min and the clear lysate was again centrifuged at 15000 rpm for 5 hrs to precipitate phages. The pellet was suspended in the phage buffer.

Chloroform: The phage lysate was centrifuged at 5000 rpm for 10 min and the cleared phage lysate was taken into phage buffer and treated with 15% chloroform. As the chloroform sediments, the top layer was taken and centrifuged at 5000 rpm for 10 min and the supernatant was filtered through a 0.2 micron syringe filter.

Poly ethylene glycol: By centrifugation of 15000 rpm for 5 min, the cells were removed and the supernatant was collected. The PEG 8000 was added to the supernatant solution to make a 2% concentration and stirred at 4°C overnight to precipitate the bacteriophages. Then the solution was centrifuged at 15000 rpm for 10 min, bacteriophages were collected as pellets and suspended in phage buffer and dialyzed.

Transmission electron microscopy of phages: One drop of the purified phage suspension was placed on a copper grid with carbon-coated Formvar film for 10 min at room temperature. As 4% aqueous phosphotungstic acid was used for staining at pH 7. The sample was air-dried overnight and examined with a Zeiss TEM 900 electron microscope, Carl Zeiss AG it was operated at 50 kV. The phage particles were visualized using the Image SP software V2.5 SYSPROG under the guidance of TRS, Duncelbuch, Moorenweis, Germany and a CCD (charge-coupled device) camera Horiba instruments, Piscataway, New Jersey, United States.

Determination of host range: The host range of obtained phages was determined by *E. coli, Salmonella* and *Campylobacter*. As 1 mL of pure *E. coli*, *Salmonella* and *Campylobacter* were spread on nutrient agar plates, respectively. While 50 µL of phages was sprayed on the nutrient agar plate with pure *E. coli*/ *Salmonella*/ *Campylobacter* culture. These plates were incubated at 37°C for 24 hrs. Then plates were observed for plaques.

Bacteriophage efficacy studies: The plaque formation ability of phages on each bacterial strain or the effectiveness of phage on each bacterial strain was determined by 100 µL of phage and 100 µL of pure isolates of different strains, respectively, mixed with low melting agar overlayed on a nutrient agar plate and incubated at 37°C for 24 hrs. The number of plaques was counted. The highest efficacy was considered in that bacterial strain, where the highest number of plaques was produced.

Statistical analysis: Experiments were repeated three times in triplicates (n = 9) and the average values were provided in the results.

RESULTS

Isolation and identification of poultry *E. coli* and *Salmonella*: Pure *E. coli* was isolated from poultry samples, collected from various parts of Telangana and Andhra Pradesh. The *E. coli* was identified by growth on MacConkey agar, microscopy and biochemical characteristics and the results were presented in Table 1.

Five *E. coli* strains were isolated from different poultry samples. They were identified as *E. coli* based on pink colonies on MacConkey agar and transparent colonies on *Salmonella Shigella* agar. They were gram-negative,

Table 1: Identification of isolated *E. coli* by growth, microscopy and biochemical characteristics

			Biochemical tests			
Strain	Growth on specific media	Microscopy morphology	Indole test	Methyl red test	Vogues-prausker test	Citrate test
E. coli 1	Pink and transparent colonies	Gram-negative rods 1-3×0.4-0.7 µm in size and is arranged in pairs	Positive	Positive	Negative	Negative
E. coli 2	Pink colonies	Gram-negative rods 1.5×0.5 µm in size and It is arranged singly	Positive	Positive	Negative	Negative
E. coli 3	Pink colonies	Gram-negative rods 1×0.4 µm in size and It is arranged singly	Positive	Positive	Negative	Negative
E. coli 4	Pink and transparent colonies	Gram-negative rods 2×0.5 µm in size and It is arranged in pairs	Positive	Positive	Negative	Negative
E. coli 5	Pink and transparent colonies	Gram-negative rods 1.5 x 0.4 µm in size and It is arranged singly and in pairs	Positive	Positive	Negative	Negative

Table 2: Identification of Isolated *Salmonella* by growth, microscopy and biochemical characteristics

		Biochemical tests				
Strain	Growth on specific media	Microscopy morphology	Urease test	Dulcitol fermentation test	Lysine decarboxylase test	TSIA test
Salmonella 1	Transparent colonies on MacConkey agar, black colonies on *Salmonella* Shigella agar	Straight rods, 1.5×3 micrometers	Negative	Negative	Positive	Red slant, yellow butt, H_2S were produced
Salmonella 2	Transparent colonies on MacConkey agar, Black colonies on *Salmonella* Shigella agar	Straight rods, 1.5 × 2.5 micrometers	Negative	Negative	Positive	Red slant, yellow butt, H_2S were produced
Salmonella 3	Transparent colonies on MacConkey agar, Black colonies on *Salmonella* Shigella agar	Straight rods, 1.2×3 micrometers	Negative	Negative	Positive	Red slant, yellow butt, H_2S were produced
Salmonella 4	Transparent colonies on MacConkey agar, Black colonies on *Salmonella* Shigella agar	Straight rods, 1.5×3 micrometers	Negative	Negative	Positive	Red slant, yellow butt and H_2S were produced
Salmonella 5	Transparent colonies on MacConkey agar, Black colonies on *Salmonella* Shigella agar	Straight rods, 1.5×2.5 micrometers	Negative	Negative	Positive	Red slant, yellow butt, and H_2S were produced

rod-shaped and 1-3×0.4-0.7 µm in size. In the biochemical tests, it was indole positive, methyl red positive, vogues prausker test negative and citrate test negative.

Pure *Salmonella* was isolated from poultry samples, collected from various parts of Telangana and Andhra Pradesh. *Salmonella* was identified by growth on *Salmonella* Shigella agar, microscopy and biochemical characteristics and the results were presented in Table 2.

Five *Salmonella* strains were isolated from different poultry samples. They were identified as *Salmonella* based on Transparent colonies on MacConkey agar, black colonies on *Salmonella* Shigella agar. They were gram-negative, Straight rods, 0.7-1.5×2-5 micrometers.

Biochemical tests: It was urease negative, dulcitol fermentation test negative, lysine decarboxylase test positive and in Triple Sugar Iron Agar test specific to *Salmonella* by the formation of red slant, yellow butt and H_2S production.

Antibiotic susceptibility test: Results showed that a high rate of resistance was against ampicillin, amoxicillin and tetracycline, followed by amikacin, norfloxacin, streptomycin, ciprofloxacin, kanamycin. Resistance was observed for all *E. coli* and *Salmonella* strains.

The *E. coli* and *Salmonella* are highly susceptible to ciprofloxacin, kanamycin and streptomycin, whereas they are highly resistant to ampicillin, amoxicillin and tetracycline (Table 3).

Bacteriophage enrichment: Phage enrichment filtrate contained numerous phages and formed plaques of varying sizes specific to *E. coli* and *Salmonella* strains.

Detection of bacteriophages/plaque assay: In the plaque assay, after incubation, bacteriophage plaque formation was determined and plaques were counted as plaque-forming units (PFU). *Salmonella* phages were small and round as in Fig. 1. The *E. coli* phages were large and oval as in Fig. 2.

Fig. 1: Plaque assay of *Salmonella* showing small plaques

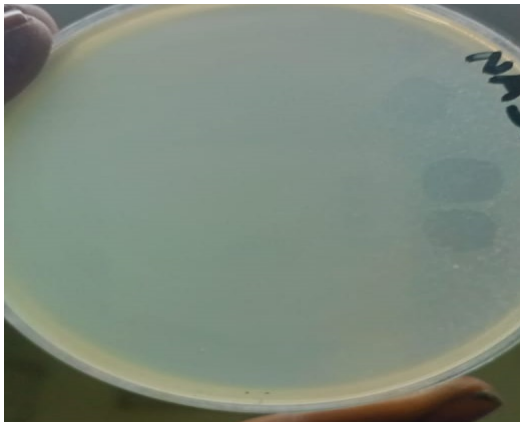

Fig. 2: Phage assay of *E. coli* showing big plaques

Table 3: The antibiotic-sensitivity profile of *E. coli* and *Salmonella* as zone of inhibition (mm) with 100 µg concentration

Bacteria	Strain	Ampicillin	Amoxicillin	Norfloxacin	Streptomycin	Tetracycline	Ciprofloxacin	Kanamycin	Amikacin
E. coli 1	HN1	0.0	05	08	23	02	18	22	03
E. coli 2	HN2	02	15	12	05	00	00	25	18
E. coli 3	HN3	00	00	11	03	05	02	18	09
E. coli 4	HN4	03	06	07	05	05	10	20	07
E. coli 5	HN5	00	02	02	07	10	08	26	05
Salmonella 1	HN6	02	04	06	13	08	12	25	07
Salmonella 2	HN7	00	05	10	08	05	10	26	12
Salmonella 3	HN8	03	03	16	07	04	05	14	11
Salmonella 4	HN9	02	05	10	11	15	15	23	09
Salmonella 5	HN10	02	03	08	09	12	12	24	8

Phage purification: The plaque was purified and used in the plaque assay method which produced plaques specific to *E. coli* and *Salmonella* on nutrient agar plates. The single and isolated plaque was selected for pure phage.

Host inactivation studies: The number of colonies in hourly samples was counted using the colony counter. The viable cell count was more till 1 hr, from the 2nd hr, the number of viable cells started decreasing in descending order. As 90% of *E. coli* cells were inactivated in 5 hrs whereas in *Salmonella* it took 3 hrs only.

Burst size determination: Plaques were observed on the nutrient agar plates. The plaque with the largest burst size

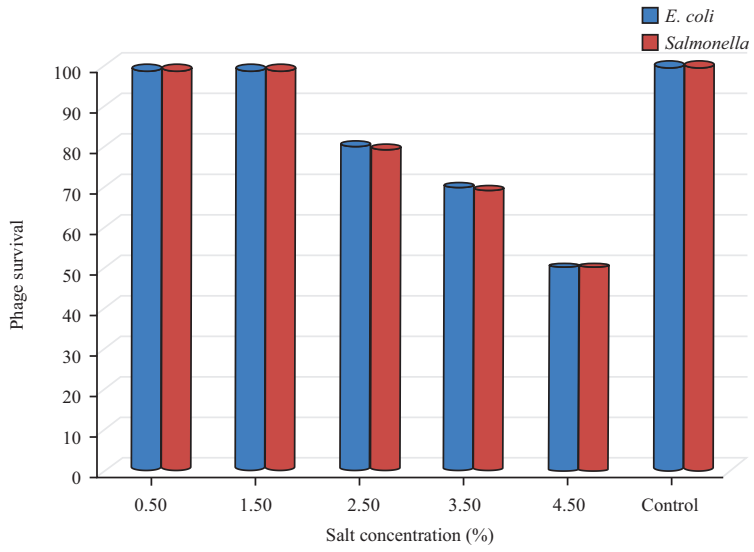

Fig. 3: Tolerance of phages to different salt concentrations

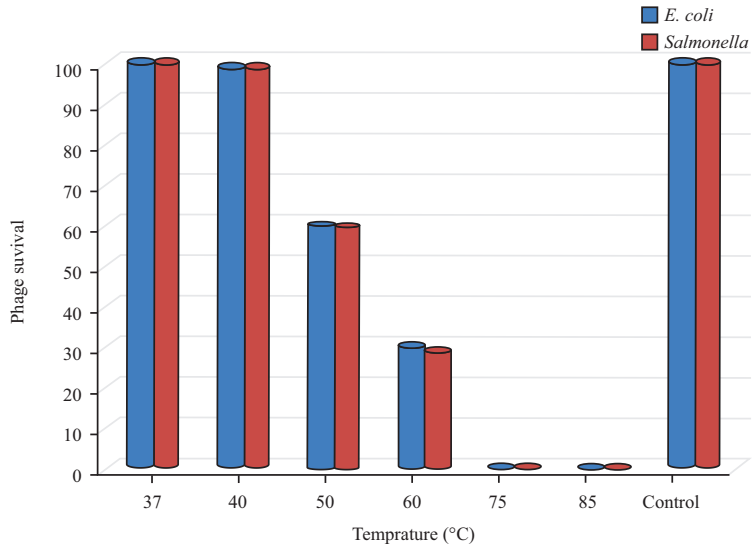

Fig. 4: Tolerance of phages to different temperatures

was the bacteriophage with higher effectivity. Among them, for *E. coli*, lambda phage produced a burst size of 120 PFU per cell and for *Salmonella*, SAL-PG phage produced a burst size of 211 PFU per cell.

Salt tolerance: Phage filtrate was subjected to various salt concentrations of 0.5 to 4.5% (Fig. 3). At salt concentrations of 2.5 to 4.5%, the phages were decreased by 20, 30 and 50%, respectively. The phage filtrate was identical to the control at 0.5 and 1.5%, indicating the ability of phages to survive in a narrow salt concentration range of 0.5 to 1.5%.

Temperature tolerance: Phage filtrate was subjected to various temperatures: 37, 40, 50, 60, 75 and 85°C (Fig. 4). There was no decrease in phages till 40°C. At 50 and 60°C phages were reduced because the number of plaques was decreased consecutively in decreasing order. Phages were not present at 75 and 85°C indicating the non-survival of phages at 75 and 85°C treated for 1hr.

pH tolerance: Phage filtrate was subjected to various pH ranges from 1 to 10 (Fig. 5). The phages were reduced 60, 40 and 30% at 1, 2 and 3 pH, respectively. From pH range 4 to 8,

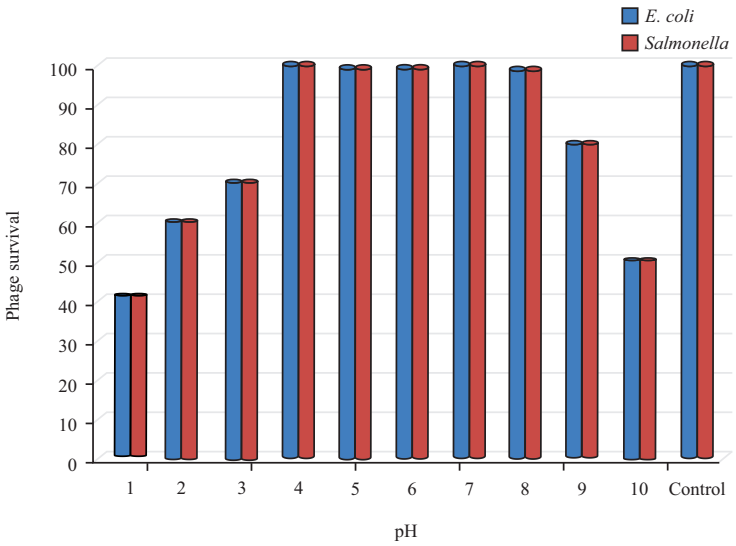

Fig. 5: Tolerance of phages to different pH

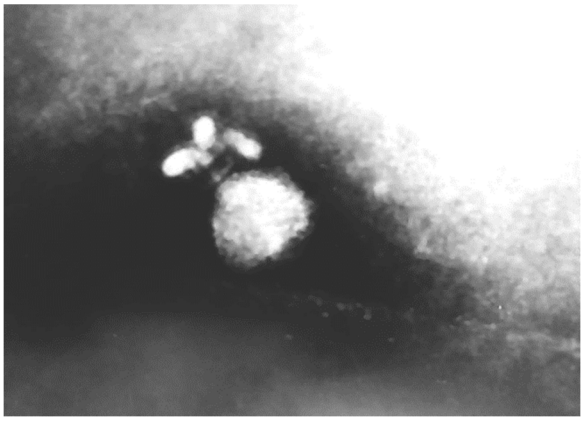

Fig. 6: Transmission electron microscopy images of *Salmonella* SAL-PG phage

the phage filtrate was identical to the control, indicating the ability of phages to survive in a broad range of pH. The phages were reduced by 20 and 50% at 9 and 10 pH, respectively.

Transmission electron microscopy of phages: The *E. coli* phage-phages were like lambda phages in morphological appearance, having an icosahedral head with a diameter of 647 nm and long non-contractile tails of 125 nm as in Fig. 6.

The *Salmonella* phage-phage was SAL-PG phage, with an icosahedral head of 60 nm in diameter and a tail of 32 nm in length as in Fig. 7.

Host ranges: Host ranges were determined using *E. coli, Salmonella* and *Campylobacter.* The SAL-PG bacteriophage infected *Salmonella* isolates only and Lambda phages infected *E. coli* isolates only. The bacteriophages didn't infect *Campylobacter*.

Bacteriophage efficacy studies: Plaque formation efficiency of lambda phages on five *E. coli* strains and plaque formation efficiency of SAL-PG on five *Salmonella* strains was obtained. The *E. coli* phage has infected and lysed all five *E. coli* strains, *Salmonella* phage has killed four of five *Salmonella* strains isolated from poultry samples.

Bacteriophage isolation and purification: The *E. coli* bacteriophages were isolated from poultry samples, phages were like lambda phages in morphological appearance,

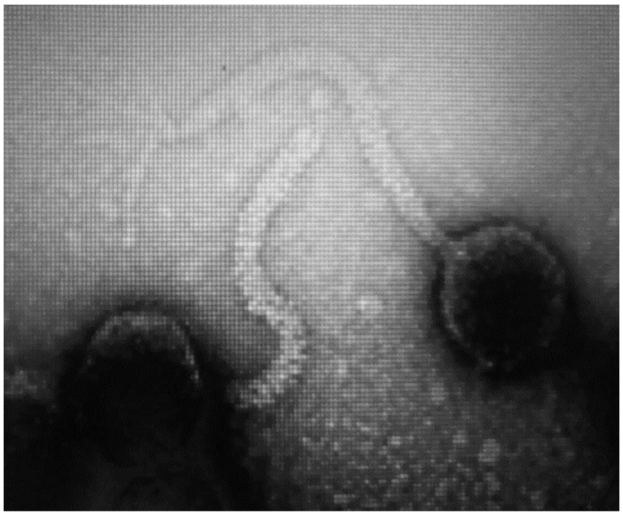

Fig. 7: Transmission electron microscopy images of *E. coli* lambda phage

having an icosahedral head with a diameter of 647 nm and a long non-contractile tail of 125 nm based on TEM. *Salmonella* bacteriophages were isolated from poultry samples. Among them, SAL-PG bacteriophage formed round and big plaques in *Salmonella* species. SAL-PG was selected for *Salmonella* species and lambda phages were selected for *E. coli* species based on host range and clear plaques.

DISCUSSION

Isolated *E. coli* phage is specific to the isolated five strains of *E. coli*, having a burst size of 120 PFU per cell, tolerant to salt concentrations 0.5-1.5%, temperatures 37, 40°C, pH 4-8 and found to be a tadpole-shaped, measuring a diameter of 647 nm and long non-contractile tail of 125 nm. Isolated *Salmonella* phage is specific to the isolated five strains of *Salmonella*, having a burst size of 211 PFU per cell, tolerant to salt concentrations of 0.5-1.5%, temperatures of 37-40°C, pH 4-8 and being found to be tadpole-shaped, measuring an icosahedral head of 60 nm in diameter and a tail of 32 nm in length. The present phages raised against *E. coli* and *Salmonella* are specific to *E. coli* and *Salmonella* respectively, not infecting other bacteria, hence can be a good source for phage therapy as reported by Nilsson[16]. The *E. coli* phage has infected and lysed all five *E. coli* strains, *Salmonella* phage has killed four of five *Salmonella* strains isolated from poultry samples. Bacterial infections in commercial poultry are challenging[17-19]. Uncontrolled usage of antibiotics in poultry is leading to the development of antibiotic-resistant micro flora[20,21]. Bacteriophages offer great potential as an alternative to antibiotics in poultry[22].

Bacteriophages effectively kill resistant bacteria to reduce the prevalence of antibiotic resistance[23-26]. The *E. coli* and *Salmonella* are predominant microbial pathogens of commercial poultry[27]. As bacteriophages and their host bacteria will be present in the same environment, poultry samples were used to isolate *E. coli* and *Salmonella* strains and also to isolate the bacteriophages against this bacteria[28]. *E. coli* and *Salmonella* species were isolated from poultry samples as reported by some researchers[28,29]. The *E. coli* strains are known for the high mortality of chickens and found to be reduced by phages[28,30]. Salmonellosis poses a health threat to farmers and consumers and bacteriophages were found to reduce salmonellosis[31]. The supplementation of *E. coli* and *Salmonella* phages increased the *Lactobacillus* concentration confirming the improved gut ecosystem[32]. Phage specificity for target and lysis of bacteria should be high to prevent non-specific bacterial targeting[32]. As Nillson[16] has reported 20% infectivity in *E. coli* phages and 50% infectivity in *Salmonella* phages. Both the phages are having high infectivity and phage inactivation rates. The 90% of *E. coli cells* were inactivated in 5 hrs, whereas, in *Salmonella* it took 3 hrs only. The inactivation rate was reported 4 and 8 hrs for *E. coli* and *Salmonella*, respectively[33]. The bacteriophages were able to survive in a wide range of pH (4 -8), resistant at 40°C for 1 hr and tolerating up to 1.5% salt as also reported by a researcher[1].

Bacteriophages for specific bacteria can be isolated in the bacterial habitat and can be used against the specific bacteria. The present isolated bacteriophages are specific to the host isolated and hence can be used to control *E. coli* and *Salmonella* spp.

CONCLUSION

Isolated *E. coli* phage is specific to the isolated five strains of *E. coli*, having burst size of 120 plaque forming units per cell, tolerant to salt concentration 0.5-1.5%, temperature 37-40°C, pH 4-8 and found to be a tadpole-shaped measuring a diameter of 647 nm and long non-contractile tail of 125 nm. Isolated *Salmonella* phage is specific to the isolated six strains of *Salmonella*, having burst size of 211 PFU per cell, tolerant to salt concentration 0.5-1.5%, temperature 37-40°C, pH 4-8 and found to be tadpole-shaped measuring icosahedral head of 60nm in diameter and a tail of 32 nm in length. Bacteriophages against *E. coli* and *Salmonella* are highly specific, lytic, tolerant to broad environment conditions and kill specific bacteria in a short duration.

SIGNIFICANCE STATEMENT

As poultry is rich in *E. coli* and *Salmonella* spp. prepared phages can be used in poultry applications. Cocktail of *E. coli* and *Salmonella* bacteriophages may be effective against poultry *E. coli* and *Salmonella*.

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