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Advanced Biocontrol of multidrug - resistant *salmonella* isolated from Broiler Farms using Bacteriophage

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ABSTRACT

Salmonellosis is among most widespread bacterial diseases, posing serious concerns for both public health and poultry industry. This study focused on isolating and characterizing bacteriophages to evaluate their potential use as targeted biocontrol agents against pathogenic *Salmonella* serovars. Fourteen *Salmonella enterica* isolates were analyzed in total. Of these, seven were obtained directly from poultry farms, representing a 7% prevalence rate. These included two *S. Typhimurium* isolates and one isolate each of *S. Enteritidis*, *S. Virchow*, *S. Infantis*, *S. Kentucky*, and *S. Derby*. The other seven isolates were supplied by the Serology Unit at the Animal Health Research Institute (AHRI). Comprising *S.* Typhimurium, *S.* Enteritidis, *S.* Kentucky, *S.* Florida, *S.* Infantis, *S.* Gallinarum, and *S.* Shangani. All isolates displayed multidrug resistance upon antibiotic susceptibility testing.
Two lytic bacteriophages, designated Phage 1 (vB_ST_Sph_SW1) and Phage 2 (vB_ST_Pod_SW2), were successfully isolated from sewage samples and were active against *S.* Typhimurium. Transmission electron microscopy (TEM) classified vB_ST_Sph_SW1 under the family *Siphoviridae* while vB_ST_Pod_SW2 classified under the family *Podoviridae*. The bacteriophages demonstrated broad-spectrum lytic capabilities against various *Salmonella* serovars. Specifically, vB_ST_Sph_SW1 was effective in lysing 6 out of the 14 tested isolates (43%), while vB_ST_Pod_SW2 targeted 4 isolates (28.5%). Stability assessments re-

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vealed that both phages remained active across a pH range of 3 to 10 and withstood temperatures up to 50 °C, although their lytic function was lost at 60 °C. These findings suggest that both vB_ST_Sph_SW1 and vB_ST_Pod_SW2 have strong potential as biological alternatives for controlling *Salmonella* infections in poultry production environments.

Introduction

Salmonellosis continues to pose a major challenge in poultry farming, both in terms of economic losses and public health concerns **(Cosby *et al*., 2015).** *Salmonella* species are rod-shaped, Gram-negative bacteria that belong to the *Enterobacteriaceae* family. These organisms are globally recognized as prominent foodborne pathogens. As reported by the European Food Safety Authority (EFSA, 2019), *Salmonella* ranks as the second most common zoonotic agent within the European Union. To date, more than 2,600 serovars of *Salmonella* have been documented, mainly differentiated based on variations in somatic (O) and flagellar (H) antigens **(Sun *et al*., 2022).** These bacteria commonly inhabit the intestinal tracts of poultry, livestock, and companion animals. Transmission typically occurs via ingestion of contaminated food or water, or through direct exposure to infected animals **(Jajere, 2019).**

Salmonella transmission in poultry production occurs through multiple routes, including contaminated feed, infected hatcheries, vertical transmission from breeder flocks to offspring, and during various stages of poultry processing. These pathways can lead to human infection primarily via the fecal–oral route. Among the numerous *Salmonella* serovars, *S. Enteritidis* and *S. Typhimurium* are most frequently associated with poultry-related salmonellosis cases **(Vose *et al*., 2011).** On a global scale, *S. Enteritidis* is considered the leading serovar linked to human salmonellosis **(Bao *et al*., 2015).** Although antibiotics have traditionally played a dual role in poultry production—as therapeutic agents and growth promoters—their extensive use has raised growing concerns **(Roth *et al*., 2019).** Increasing awareness of antimicrobial resistance has triggered international action to reduce antimicrobial use. The transmission of antimicrobial resistance (AMR) genes between bacterial populations often occurs through horizontal gene transfer, making poultry production systems potential hotspots for AMR proliferation **(Agyare *et al*., 2018).** The increasing concern over antibiotic resistance underscores the urgent need for alternative strategies to control bacterial infections in livestock. One such promising approach is bacteriophage therapy. Bacteriophages, or phages, are viruses that specifically infect and kill targeted bacterial species, offering a highly selective method of bacterial control that does not disrupt the beneficial microbiota **(Rahaman *et al*., 2014; Nabil *et al*., 2018).** Phages are generally divided into two categories: lytic and lysogenic. Lytic phages reproduce within the bacterial host and ultimately cause its lysis, whereas lysogenic phages integrate their genetic material into the host genome, replicating passively without destroying the cell **(Kasman *et al*., 2020).** Due to their specificity, strong bactericidal effects, and resilience under various environmental conditions, lytic phages are considered especially advantageous **(Mahony *et al*., 2011).** Their use has been shown to effectively reduce bacterial loads in foods of animal origin, such as poultry meat and eggs **(Atterbury *et al*., 2020).** Overall, phage therapy is gaining attention as a potent alternative to traditional antibiotics, particularly in addressing infections caused by resistant bacterial strains **(Sonalika *et al*., 2020).** Several phage-based products have already been approved and commercialized for managing *Salmonella* infections in poultry farms; for instance, SalmoFREE has been developed specifically for this purpose **(Clavijo *et al*., 2019).** This study reports the isolation of two newly identified lytic bacteriophages—vB_ST_Sph_SW1 and vB_ST_Pod_SW2—were successfully isolated from sewage samples obtained in Giza, Egypt, using *S. Typhimurium* as the host organism. The study focused on assessing their biological characteristics, including lytic activity, thermal stability, and tolerance to varying pH levels. The primary aim was to assess the occurrence and antibi-

otic resistance profiles of *Salmonella* isolates from infected broiler chickens while also exploring the effectiveness of phage therapy as a biocontrol alternative strategy for managing salmonellosis in poultry production environments.

Materials and Methods

Bacterial Isolates:

I. Sample collection and identification

During the period spanning 2023 to early 2024, a total of 100 diseased broiler chickens of varying ages were sampled from five poultry farms located in Giza Governorate, Egypt. From each bird, internal organs such as the spleen, liver, and cecum were carefully excised under aseptic conditions, properly labeled, and immediately placed in ice boxes for transportation to the Serology Unit at the Animal Health Research Institute (AHRI), Dokki, for *Salmonella* isolation. The three organs from each chicken were pooled and processed as a single sample. The isolation and identification of *Salmonella* were carried out following the procedures outlined in ISO 6579:2017. presumptive *Salmonella* colonies were confirmed using a series of biochemical tests, including triple sugar iron (TSI) agar, urea hydrolysis, indole production, and lysine iron agar, following ISO 6579-1:2017 guidelines. Confirmed isolates were subsequently serotyped according to ISO 6579-3:2014, which involves the detection of somatic (O) and flagellar (H) antigens using SIFIN antisera (Berlin, Germany) at the Serology Department of the Animal Health Research Institute (AHRI), Dokki, Giza.

II. In this study, seven *Salmonella* isolates provided by the Serology Unit of the Animal Health Research Institute (AHRI), Egypt, were utilized for bacteriophage isolation, propagation, and evaluation of host range specificity. The isolates were authenticated through a combination of biochemical profiling and molecular identification, using PCR assays targeting the 16S rRNA and *invA* genes, based on the method described by **Mthembu *et al*. (2019).** Once confirmed, the strains were preserved at –80°C to ensure viability for subsequent experimental applications.

Table (1). *Salmonella* isolates provided from serology unit

Serovars of *salmonella* isolates	No.
Salmonella Typhimurium	1
Salmonella Enteritidis	1
Salmonella Florida	1
Salmonella Infantis	1
Salmonella Kentucky	1
Salmonella Shangan*i*	1
Salmonella Gallinarum	1
Total	7

Antimicrobial Susceptibility Testing and MAR Index Calculation

The antimicrobial resistance patterns of the *Salmonella* isolates were determined using the standard disc diffusion technique, in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2020). A total of nine antibiotics representing six distinct antimicrobial classes were selected for analysis. These included: penicillins [amoxicillin/clavulanic acid (AMC, 30 µg)], cephalosporins [cefotaxime (CTX, 30 µg)], aminoglycosides [amikacin (AK, 30 µg), neomycin (N, 30 µg), and gentamicin (CN, 10 µg)], sulfonamides [trimethoprim-sulfamethoxazole (SXT, 25 µg)], quinolones [levofloxacin (LEV, 5 µg) and ciprofloxacin (CIP, 5 µg)], and polymyxins [colistin (CT, 10 µg)]. Following incubation, the diameters of inhibition zones were measured and interpreted in line with CLSI standards (2020).

To assess the degree of resistance, the Multiple Antibiotic Resistance (MAR) index was calculated using the formula:

MAR Index = Number of antibiotics the isolate is resistant to / Total number of antibiotics tested

According to **Christopher *et al*. (2013),** a MAR index ≤ 0.2 typically indicates that the bacterial strain originates from an environment with minimal antibiotic exposure, whereas an index > 0.2 is suggestive of contamination from high-risk sources, where antibiotics are commonly misused **(Akande *et al*., 2019).**

III. Isolation and purification of phages

1. Isolation of Bacteriophages from *Salmonella* (Akhtar *et al*., 2014).

For the purpose of isolating specific bacteriophages, a multidrug-resistant *Salmonella Typhimurium* strain—previously recovered from infected broiler chickens in this study—was employed as the host. The phage screening and enrichment process followed the methodology described by **Akhtar *et al*. (2014).** A total of five sewage samples were collected from poultry farm environments. These samples were centrifuged at 8496 × *g* for 10 minutes to remove solid debris. The resulting supernatants were then passed through 0.22 µm syringe filters to eliminate any remaining bacterial cells, ensuring sterile conditions for downstream phage isolation steps.

The host strain was cultured overnight at 37°C on nutrient agar to obtain isolated colonies. A bacterial suspension was then prepared in peptone water or saline, adjusted to match the turbidity of a 0.5 McFarland standard (approximately 10^8 CFU/mL). A volume of 0.1 ml From the suspension was added to 10 mL of nutrient broth and incubated at 37°C for 3 hrs under shaking conditions to achieve log-phase growth of bacterial culture.

To enrich bacteriophages, a mixture of 4.5 mL of filtered sewage was mixed with 0.5 mL of a log - phase bacterial culture and 0.5 mL of concentrated nutrient broth. Following 24 hrs of incubation at 37°C, five drops of chloroform were added to the mixture to ensure bacterial cell lysis , the suspension was then centrifuged at 8496 × *g* for 10 minutes, and the supernatant was filtered using 0.45 µm syringe filters (Corning) for obtaining the phage-containing lysate.

To detect and enumerate lytic bacteriophages both spot test and plaque assay techniques were employed as described by **(Chang *et al*. 2005),** using Tryptic soy agar plates. Purified phage lysates were subsequently stored at 4°C for future applications.

2. Propagation of bacteriophages

Bacteriophage propagation was performed through three successive rounds of enrichment, the procedures was carried out according to protocol outlined by **(Clokie *et al*. 2009).** In each cycle, the previously obtained phage lysate was used in place of the original sewage sample to further amplify the phage population.

3. Detection of lytic bacteriophages using spot test and plaque assay

To detect the presence of lytic bacteriophages in the enriched filtrates, the spot test method was applied, following the protocol of **Chang *et al*. (2005).** A clear lysis zone on the agar surface indicated phage activity, whereas the absence of such a zone was considered a negative result. Each of the 14 multidrug-resistant *Salmonella* isolates was cultivated separately, and 1 mL of each culture was evenly spread on individual nutrient agar plates. After removing any excess liquid, the plates were allowed to air-dry at room temperature. Then, 10 µL of the enriched phage filtrate was spotted onto the surface of each bacterial lawn and left to dry. Plates were incubated overnight at 37°C. The appearance of distinct, clear zones the next day confirmed the presence of lytic phages, as described by **Rahaman *et al.* (2014).**

Plaque assays were performed according to the method outlined by **Akhtar *et al*. (2014)** to quantify phage particles. Ten-fold serial dilutions of the enriched phage lysates were prepared using 0.1 mL of the original lysate. For each *Salmonella* isolate, a single colony from an overnight culture was transferred into 5 mL of nutrient broth and incubated at 37°C for approximately 3 hours to achieve log-phase growth. Meanwhile, sterile tubes containing 3 mL of 0.5% soft agar were maintained at 45°C in a water bath. Each tube was inoculated with 0.1 mL of diluted phage lysate and 0.5 mL of the corresponding host bacterium (*S. Typhimurium*), gently mixed, and then poured over solidified nutrient agar plates. After the overlay solidified, the plates were incubated at 37°C for 18–24 hours. The following day, plaques—clear zones formed due to bacterial lysis—were counted, and phage concentrations were calculated as plaque-forming units per milliliter (PFU/mL).

4. Determination of Bacteriophage Host Range Using the Spot Test

The host range of the isolated phage cocktail

was assessed using the spot assay technique, following the methodology outlined by **Rahaman *et al.* (2014)**. Testing was conducted individually on each of the 14 multidrug-resistant *Salmonella* isolates one by one. A base layer of Tryptic Soy Agar (TSA) was prepared for each plate, onto which 3 mL of molten double-layer agar containing the test *Salmonella* strain was overlaid. Once solidified, 15 µL of the phage lysate was spotted onto the surface of each plate. After incubation at 37°C overnight, plates were visually inspected for lytic activity. The presence of a clear zone at the site of phage application surrounded by an intact bacterial lawn was taken as evidence of phage infectivity against the tested isolate.

5. Assessment of Phage Efficiency of Plating (EOP)

The Efficiency of Plating (EOP) assay was conducted to confirm the host range of the isolated *Salmonella* phages, as previously suggested by spot test results. This assay followed the plaque assay methodology described by **Vongkamjan *et al.* (2017)** and was applied to all 14 *Salmonella enterica* isolates included in this study. Serial ten-fold dilutions of the phage lysates, ranging from 10^{1} to 10^{10}, were prepared. For each dilution, 100 µL of the phage suspension was mixed with 100 µL of the corresponding host strain (*S. Typhimurium*). The mixture was combined with 3 mL of molten soft agar (prepared using 13 g/L nutrient broth and 28 g/L nutrient agar) and gently poured over pre-solidified tryptic soy agar (TSA) plates. Plates were incubated at 37°C for 24 hours, after which plaque formation was recorded. The number of plaques was used to determine the phage titer, expressed as plaque-forming units per milliliter (PFU/mL).

The Efficiency of Plating was calculated using the following equation:

EOP = (Average phage titer on test strain) / (Average phage titer on original host strain)

EOP values were interpreted using the classification criteria defined by **Manohar *et al.* (2019),** which categorize plating efficiency as follows:

- **High**: EOP ≥ 0.5
- **Medium**: 0.5 > EOP ≥ 0.1
- **Low**: 0.1 > EOP > 0.001

Inefficient: EOP ≤ 0.001

Assessment of Phage Stability under different Temperature and pH conditions

The evaluation of phage stability in response to temperature and ph changes was conducted using the procedures reported by **(Shang *et al.*, 2021).** To assess thermal stability, 2 mL aliquots of phage filtrate dispensed into sterile test tubes and subjected to incubation at different temperatures 4°C, 25°C, 37°C, 42°C, and 50°C for 30 minutes. After incubation, the samples were rapidly cooled under running tap water, and residual phage viability was assessed using the plaque assay method. For pH stability, nutrient broth was adjusted to a range of pH values (from pH 4 to pH 12) using either 0.1 N HCl or 0.1 N NaOH. Phage suspensions were diluted into 9 mL of these pH-adjusted media in sterile test tubes. The mixtures were incubated overnight at 4°C. Residual phage activity was then determined using the standard plaque assay technique as previously described.

Morphological Characterization of Bacteriophages

The structural morphology of phages isolated against multidrug-resistant (*Salmonella*) strains was investigated using transmission electron microscopy (TEM). Phage lysates with high-titer, obtained after two to three rounds of successive propagation. The lysate was purified through centrifugation at 7826 × g for 25 minutes, performed twice, and then filtered using a 0.22 µm syringe filter to remove residual debris. To prepare samples for electron microscopy, A drop of the purified phage lysate was applied to carbon-coated 200 -mesh copper grids and allowed to adsorb. Negative staining was performed using a 2% sodium phosphotungstate solution (pH 7.6). Morphological analysis was carried out using a JEM-2100 transmission electron microscope (TEM) at the National Research Centre.

Results

Isolation and Identification of *Salmonella enterica* Serovars

From a total of 100 diseased broiler chickens sampled from various poultry farms in Giza Governorate, seven *Salmonella* isolates were

successfully recovered, indicating a 7% isolation rate. Biochemical characterization of the suspected colonies showed negative results for the urease test, while positive results were observed for lysine decarboxylase (LDC), citrate utilization, and hydrogen sulfide (H_2S) production tests.

Subsequent serotyping of the confirmed isolates identified seven distinct *Salmonella enterica* serovars: *S. Typhimurium* (2 isolates), and one isolate each of *S. Enteritidis*, *S. Virchow*, *S. Infantis*, *S. Kentucky*, and *S. Derby*.

Table (2). *Salmonella* serovars from diseased broiler chickens

Serovars of *salmonella* isolates	No.
S. Typhimurium	2 isolates
S. Enteritidis	1 isolate
S. Virchow	1 isolate
*S.*Infantis	1 isolate
S. Kentucky	1 isolate
S .Derby	1 isolate
Total	7 isolates

Antimicrobial Susceptibility

All *Salmonella* isolates exhibited considerable resistance to the classes of antibiotics tested in this study.

Table (3). Antibiogram patterns of fourteen *Salmonella* isolates:

Salmonella serovar	No. of strains	Resistance profile*	MAR Index
***S*. Typhimurium**	**3**	CT10, AMC30, N30, CTX30, AK30	0.50%
***S*. Typhimurium**		CT10, AMC30, N30, CTX30, AK30, CN120M	0.60%
***S*. Typhimurium**		CT10, AMC30, N30, CTX30, AK30, SXT25	0.60%
***S*. Enteritidis**	**2**	CT10, AMC30, N30, SXT25, CTX30, CN120M, LEV5, OFX5	0.80%
***S*. Enteritidis**		CT10, AMC30, CTX30, CN120M, LEV5, OFX5	0.60%
***S*. Florida**	**1**	CT10, AMC30, N30	0.30%
***S*. Infantis**	**2**	CT10, AMC30, N30, SXT25, CTX30, CN120M, LEV5, OFX5	0.80%
***S*. Infantis**		SXT25, CTX30, CN120M, LEV5, OFX5	0.50%
***S*. Gallinarum**	**1**	CT10, AMC30, N30	0.30%
***S*. Shangani**	**1**	CT10, AMC30, N30	0.30%
***S*. Kentucky**	**1**	CT10, AMC30, N30, OFX5, CIP5, LEV5	0.60%
***S*. Kentucky**	**1**	AMC30, N30, OFX5, CIP5, LEV5	0.50%
***S*. Derby**	**1**	CT10, AMC30, N30, OFX5, CIP5, LEV	0.60%
***S*. Virchow**	**1**	CT10, CTX30, AMC30, AK30, CN30, SXT25, OFX5, CN120	0.80%

***Antibiotics panel;** amoxicillin/clavulanic (AMC), Cefotaxime (CTX) Amikacin (AK), Neomycin (N), Gentamycin (CN), [Trimethoprimsulfamethoxazole (SXT), Levofloxacin (LEV), ciprofloxacin (CIP), colistin (CT).

Isolation and Characterization of *Salmonella* specific Lytic Bacteriophages

Two phages, designated vB_ST_Sph_SW1 and vB_ST_Pod_SW2, were successfully isolated using spot test and plaque assays. The bacteriophage vB_ST_Sph_SW1 produced small, clear plaques with diameters less than 1 mm, while vB_ST_Pod_SW2 formed medium-sized clear plaques ranging between 1 and 2 mm. Morphological characterization by Transmission electron microscopy (TEM) analysis revealed that *vB_ST_Sph_SW1* belongs to the family **Siphoviridae**, while *vB_ST_Pod_SW2* was classified under the family **Podoviridae**.

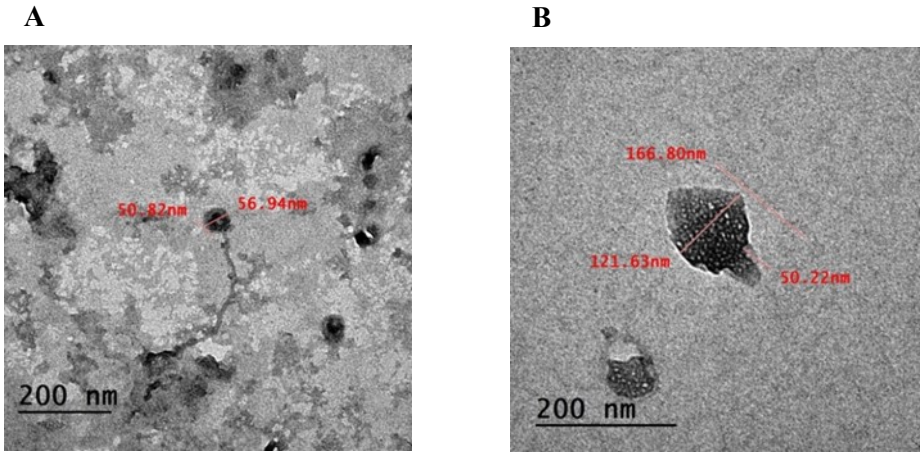

Fig. (1). Morphological characterization of *Salmonella* phages observed using transmission electron microscopy (TEM) following negative staining with 2% sodium phosphotungstate (Na-PTA).A: *vB_ST_Sph_SW1* (Siphoviridae family); B: vB_*ST_Pod_SW2* (Podoviridae family).

Determination of Bacteriophage Host Range

Table (4) illustrates the evaluation of the isolated bacteriophages' host range, tested against 14 *Salmonella* strains encompassing diverse serotypes. The initial screening was performed using the spot test, followed by confirmation through plaque assays. Among the examined strains, only one isolate was susceptible to both phages, as evidenced by the formation of distinct, well-defined clear plaques. The majority of the *Salmonella* isolates displayed resistance to several antibiotics. Importantly, the phages exhibited strong lytic activity against multiple *S. enterica* strains, with Efficiency of Plating (EOP) values reaching or surpassing 0.5, indicating effective replication and host cell lysis.

Table (4). Spot Test Results for *Salmonella* Phage Isolates

***Salmonella* serova**	**Phage 1(vB_ST_Sph_SW1)**	**Phage 2(vB_ST_Pod_SW2)**
***S*. typhimurium**	+++	+++
***S*. typhimurium**	+++	-
***S*. typhimurium**	+++	+
***S*. enteritidis**	-	+
***S*. enteritidis**	+	+++
***S*. Florida**	+	+++
***S*. Infantis**	+	+++
***S*. Infantis**	+	-
***S*.Gallinarum**	+++	+
***S*.Shangani**	+++	+
***S*. kentucky**	+	+
***S*. kentucky**	+	-
***S*.Derby**	+	+
***S*. virchow**	+++	+

clear and strong lytic activity (+++); Moderate lysis (+); No plaque formation observed (−)

Table (5). Efficiency of Plating (EOP) of *Salmonella* Phages against *S.* enterica isolates

Salmonella serovars	Host strain	Phage 1 vB_ST_Sph_SW1	Efficiency of plating (EOP)	Phage 2 vB_ST_Pod_SW2	Efficiency of plating (EOP)
		Average bacteriophage titer ($PFUml^{-1}$)		Average bacteriophage titer ($PFUml^{-1}$)	
	S.* Typhimurium	$145 \times 10^8 \pm 0.00$		$140\times 10^8 \pm 0.00$	
S.* Typhimurium		$145 \times 10^8 \pm 0.00$	1	$140\times 10^8 \pm 0.00$	1
***S.* Typhimurium**		$160 \times 10^8 \pm 0.01$	1.1	0	0
***S.* Typhimurium**		$158 \times 10^8 \pm 0.02$	1.09	$129 \times 10^8 \pm 0.01$	0.9
***S.* Enteritidis**		0	0	$133 \times 10^8 \pm 0.01$	0.95
***S.* Enteritidis**		$135 \times 10^8 \pm 0.01$	0.9	$147 \times 10^8 \pm 0.01$	1.05
***S.* Florida**		$139 \times 10^8 \pm 0.01$	0.95	$150 \times 10^8 \pm 0.01$	1.07
***S.* Infantis**		$140 \times 10^8 \pm 0.01$	0.96	$159 \times 10^8 \pm 0.02$	1.1
***S.* Infantis**		$153 \times 10^8 \pm 0.02$	1.05	0	0
***S.*Gallinarum**		$164 \times 10^8 \pm 0.01$	1.1	$120 \times 10^8 \pm 0.02$	0.85
***S.*Shangani**		$159 \times 10^8 \pm 0.01$	1.09	$110 \times 10^8 \pm 0.01$	0.78
***S.* Kentucky**		$150 \times 10^8 \pm 0.01$	1.03	$100 \times 10^8 \pm 0.01$	0.7
***S.* Kentucky**		$141 \times 10^8 \pm 0.01$	0.97	0	0
***S.D*erby**		$137 \times 10^8 \pm 0.01$	0.94	$115 \times 10^8 \pm 0.01$	0.8
***S.* Virchow**		$160 \times 10^8 \pm 0.02$	1.1	$112 \times 10^8 \pm 0.02$	0.8

All bacteriophage titers were determined in triplicate and reported as the mean ± standard deviation, expressed in plaque -forming units per milliliter (PFU/mL). The Efficiency of Plating (EOP) was calculated as the ratio of the phage titer obtained on each tested *Salmonella enterica* strain to the titer obtained on the reference host strain, *S. Typhimurium*.

Temperature and pH Stability of Isolated Bacteriophages

The recovered bacteriophages *vB_ST_Sph_SW1* and *vB_ST_Pod_SW2* demonstrated notable stability across a wide pH range (pH 3 to 10) following 2 hours of exposure. However, both phages lost infectivity at extreme alkaline conditions (pH >10). In terms of thermal resistance, both phages maintained high lytic activity at temperatures up to 40°C. A noticeable reduction in phage titers was observed after 30 minutes at 50°C. Complete loss of lytic activity occurred after 1 hour of incubation at 60°C, as confirmed by the spot test. Therefore, the thermal inactivation threshold for both phages was determined to be 60°C.

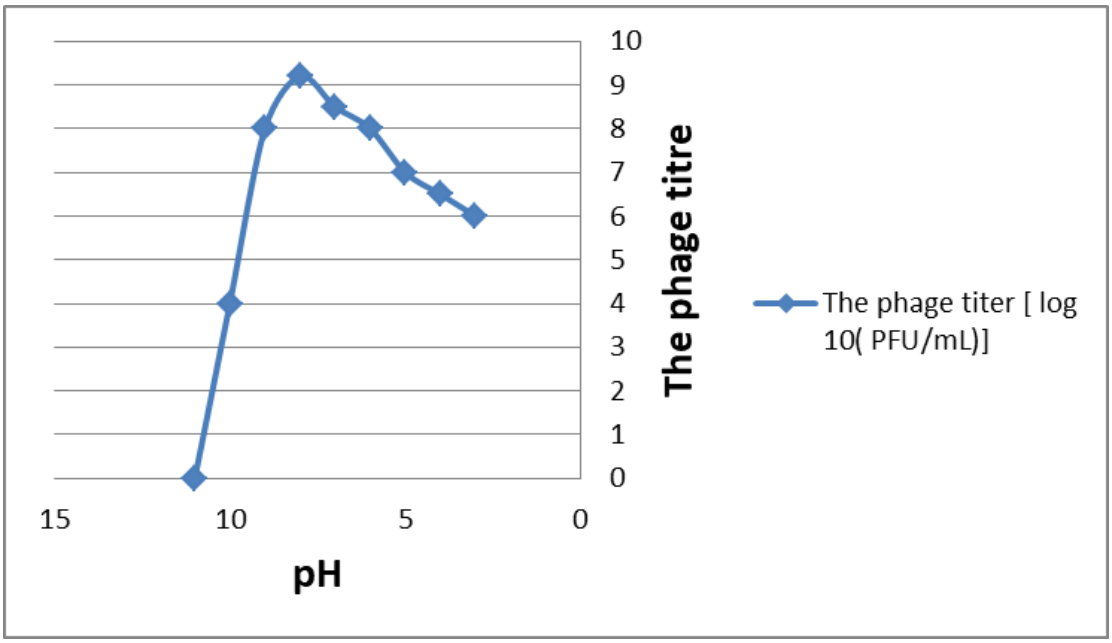

Fig. (2). Effect of pH on the stability of the isolated *Salmonella* bacteriophages

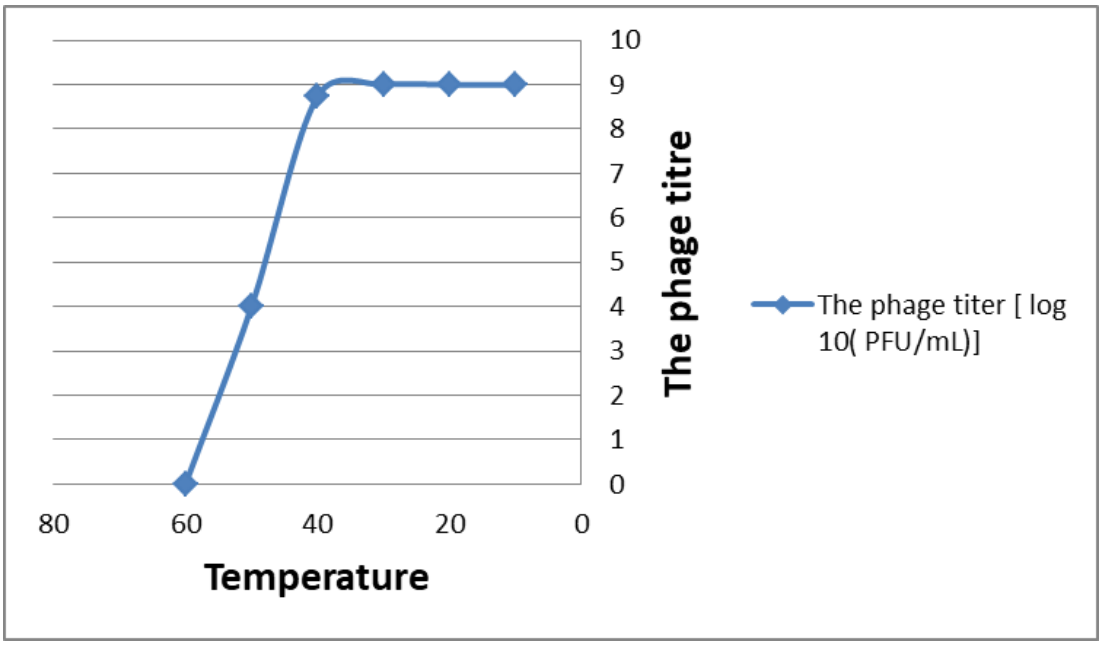

Fig. (3). Thermal effect on the stability of the isolated *Salmonella* phages.

Discussion

Bacteriophages are viruses that infect specific bacterial hosts, and they have emerged as potential alternatives to conventional antibiotics **(Dalmasso *et al.*, 2016)**. In current study, *Salmonella* was isolated with a prevalence of 7%, which is agree with the findings of **(El-Ghany *et al.* 2012),** who recorded a similar isolation rate of 7.03% from diseased broilers in Kalubia Governorate, Egypt. The isolation of *S.* Typhimurium, *S.* Kentucky, and *S.* Enteritidis in this study is consistent with previously reported serovars. Conversely, a notably lower *Salmonella* prevalence of 2.5% was observed in broiler chickens from Kafr Elsheikh Governorate, Egypt, as documented by **Mohamed *et al.* (1999),** a higher incidence rate of 15.4% was observed in broiler chickens by **(Yang *et al.* 2019)**, indicating regional and temporal variability in *Salmonella* prevalence.

Bacteriophages represent the most numerous biological entities on the planet and are typically found in microbe-dense environments, including sewage, animal waste, and soil **(Pires *et al*., 2020).** In this study, bacteriophages targeting *Salmonella* were successfully isolated from sewage samples obtained from poultry farms. The occurrence of these phages in poultry-associated settings, such as broiler house waste, has been previously reported **(Bao *et al*., 2011).** To verify the existence of lytic bacteriophages within the samples, spot assays were performed, aligning with methodologies described in previous research (e.g., **Rahaman *et al*., 2014**). The worldwide emergence of multidrug-resistant (MDR) bacteria primarily driven by excessive antibiotic use has sparked renewed interest in bacteriophage-based therapies. Bacteriophages are increasingly recognized as promising alternative to antimicrobials for controlling resistant infections in medical, veterinary and agricultural settings. Several studies, including that of **(Hur *et al*., 2010),** have highlighting the effectiveness of bacteriophage therapy against MDR pathogens like *Salmonella*, often utilizing virulent phages isolated from natural environments such as sewage and poultry waste.

The improper and excessive use of antibiotics in veterinary practice has been a key factor in the development of (MDR) *Salmonella* strains. Consequently, such MDR isolates are more commonly detected in animals than in human infections **(Briers *et al*., 2014)**. Antimicrobial resistance (AMR) in *Salmonella* serovars presents a growing public health threat, particularly when it involves resistance to critical and high-priority antibiotics such as cephalosporin's (third-generation) or fluoroquinolones **(Velazquez *et al*., 2022)**.This growing challenge highlights the critical need for innovative and efficient strategies to combat bacterial pathogens that have developed resistance to standard antibiotics. Among the most promising solutions are bacteriophages, which have gained attention as viable antibiotic alternatives. They are increasingly being utilized across multiple areas of food production and processing to specifically target and diminish antibiotic-resistant bacterial populations **(Górski *et al*., 2020).** One of the primary challenges in phage-based biocontrol is the typically narrow host range of bacteriophages, as they often display high specificity to individual bacterial strains. However, **(Bielke *et al*., 2007)** emphasized that not all phages are strictly limited to a single host or genus, suggesting the feasibility of selecting phages with broader host spectra. In support of this, **(Mahmoud *et al*., 2018)** found that variety of *Salmonella* serovars, including *S. Typhimurium*, *S. Enteritidis*, *S. Kentucky*, and *S. Typhi*, are susceptible to Siphoviridae phages specifically Salmacey1 and Salmacey2 as evidenced by spot test result. Similarly, the two lytic phages isolated in the present study, vB_ST_Sph_SW1 and vB_ST_Pod_SW2, showed potent lytic activity and a broad host range. Notably, vB_ST_Pod_SW2 exhibited effectiveness toward multiple *Salmonella* serovars, such as *S. Enteritidis*, *S. Typhimurium*, *S. Florida*, and *S. Infantis*. This wide spectrum of activity is in line with previous findings on phages such as vB_SalP_LDW16 and vB_SalM_SW2, which also demonstrated broad lytic potential across diverse *Salmonella* strains **(Cao *et al*., 2022; Saad *et al*., 2023).** The two isolated bacteriophages were efficiently characterized and found to possess desirable properties for biocontrol applications. In particular, stability under varying pH and temperature conditions is essential for effective field use. Both phages maintained stability across pH levels from 3 to 10, indicating compatibility with diverse environmental and food-related conditions **(Sun *et al*., 2022).** In terms of heat tolerance, the phages maintained their lytic potential up to 40 °C, but their activity declined sharply at temperatures above 50 °C. These thermal stability results are consistent with previous findings on *Salmonella* phages such as vB_SenS_SE1, which also showed a marked reduction in viability when subjected to higher temperatures **(Lu *et al*., 2020).**

Conclusion

This study highlights the promising characteristics of the novel identified phages targeting *Salmonella*, vB_ST_Sph_SW1 and vB_ST_Pod_WS2, which demonstrated broad host specificity, potent lytic effects, and notable stability across a range of pH levels and

temperatures. These features suggest their strong potential as biocontrol tools for managing *Salmonella* infections in poultry systems. As antibiotic resistant *Salmonella* cases increase and conventional treatments prove less effective, phages represent an attractive alternative approach. Their targeted action, minimal disruption to beneficial microbiota, and lower risk of resistance development make them well-suited for inclusion in comprehensive disease management strategies. Ultimately, the use of such bacteriophages could contribute significantly to improving food safety and curbing antimicrobial resistance within the poultry sector.

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