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Phage Therapy for Controlling Antibiotic-Resistant *Salmonella* and *E. coli* in Poultry

Eman A. Hatem[1*], Salma A. Dabash[1], Zeinab R. Bakr[1], Dina K. AboAhmed[1], Hager M. Allam[1], Nourhan M. Temraz[1], Aya G. Mostafa[1], Nada E. Rashad[1], Kaloud A. Tahoun[1], Nada A. Elamy[1], Huda M. Abdelmegid[1], Nourhan S. Emam[1], Nada W. Elsayed[1], Yasmeen G. Zahra[1], Nermen A. Ramadan[1], Mariam M. Khalil[1], Hany S. Ahmed[2]

[1] *Department of Botany and Microbiology, Faculty of Science, Menoufia University, Egypt.*

[2] *Department of Botany and Microbiology, Faculty of Science, Tanat University, Egypt*

*Correspondence
Email: emanhatem69@gmail.com

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ABSTRACT

Even with improvements in food management practices, foodborne illness remains an immense concern. Salmonellosis and other gastrointestinal disorders are caused by contamination with *Salmonella* and *Escherichia coli* infections, particularly in the poultry industry. These illnesses result in millions of fatalities globally. Antibiotic-resistant bacteria have become more prevalent as a result of the overuse of antibiotics and other chemical therapies. **Aims:** Isolation and characterization of bacteriophages against *Salmonella sp.* and *Escherichia coli*, and their efficacy with the combination of antibiotic to minimize the infection of poultry meat with *Escherichia coli* and *Salmonella sp*. Resistance strain of *Salmonella* and *E.coli* used for isolation phages (pSAs, pSC, pSD, pEG). *Salmonella* phages (pS) characterized by having broad host rang and the viability of phage pSAs is increased with increasing pH values and high stability with different temperature degree so (pSD1, pSC) is more stable and active phage at 80°C. *E.coli* phage (pE) characterized by it is sensitivity by temperature degree as thermal inactivation at 60°C. The prefect MOI for *E.coli* phage equal 1 which give high reduction rate with bacteria than combination with antibiotic.

Keywords: *Salmonella sp, Escherichia coli,* Phages, Biocontrol.

1. Introduction

The world's poultry industry is a major supplier of animal protein. Hence, the production and usage of chicken meat have increased over time on a global scale. Foodborne infections are a serious public health hazard that affects both developed and developing nations, putting both people's health and finances at risk. Chicken flesh contamination happens through the manufacturing habitat by air, upward or downward transmission, or within the butchering process. As a result, the microbiological safety of chicken and poultry products is critical *Salmonella sp.* and *Escherichia coli* are two of the most prevalent pathogenic microbes which attack humans via infected chicken flesh, thus detecting their presence even at low levels is critical (Poojari et al, 2022).

E. *coli* and *Salmonella* sp. are bacteria of the Enterobacteriaceae family. Both are rod-shaped Gram-negative bacteria that don't produce spores. Furthermore, they are facultative anaerobes, meaning they may switch between fermentation and aerobic respiration depending on the availability of oxygen (Oludairo *et al*., 2022 and El-Mongy *et al*., 2017). According to many studies, the frequency of *Salmonella* in Egyptian poultry farms ranges from 2.5% in layers to 11.3% in broilers (Guyard-Nicodème *et al*., 2023). Also, In 2016, 98.3% of poultry farms in Egyptian governorates had the

highest *E.coli* prevalence (Abdel-Rahman *et al*,. 2023).

Salmonella spp. causes Salmonellosis, an intestinal infection in humans. Every year, non-typhoidal *Salmonella* (NTS) infections in humans account for 94 million instances of gastroenteritis and roughly 155,000 fatalities (mostly due to serovars *S. typhimurium and S. tnteritidis)* (Majowicz *et al*., 2010). It was discovered that a *Salmonella*-infected egg might contaminate all eggs and chicks during hatching. Enteric fever, a potentially fatal condition affecting the entire body, is thought to be caused by typhoidal *Salmonella* (Gibani *et al.*, 2018). *Salmonella* infection of food products may have a substantial impact on customer demand and producer income. *E. coli* is another prevalent microbial flora of the poultry gastrointestinal system. Some of the illnesses include meningitis, septicemia, urinary tract infection, epidemic diarrhea, endocarditis, yolk sac infection, swollen head syndrome, omphalitis, coli granuloma, cellulitis, and colibacillosis. *E.coli* enteritis (colibacillosis) is a significant illness in the chicken business due to increased mortality and impaired performance.

Enteropathogenic *E.coli* causes acute, profuse and watery diarrhea which rarely becomes persistent (Wilson *et al.,* 2001). Because of the human health implications of *Salmonella* and *E.coli*, most nations have created national monitoring and control programs (Spricigo *et al.*, 2013). Antibiotic treatment of these bacteria has been used to kill the bacteria and alleviate the symptoms of the infection, but several factors limit their effectiveness, including bacterial resistance to commonly used antibiotics, disruption of gut flora as antibiotics not only kill the harmful bacteria causing the infection, but also disrupt the natural balance of the bacteria in the gut, causing gastrointestinal issues and increasing the risk of developing other infections (Loc-Carrillo and Abedon *et al.,* 2011). Phage treatment has become a more viable method for food safety and preservation in recent times (Spricigo *et al*., 2013). Phage treatment, first described in the early 1900s, involves using phages (bacteriophages) that are specialized to certain bacteria to eradicate undesirable microorganisms, such as those linked to infectious diseases (Loc-Carrillo and Abedon *et al.,* 2011). The most common biological agent on the planet, bacteriophages, is known to be present everywhere (Bao *et al*., 2015). These are viruses that can only spread and multiply within bacteria. These viruses possess the unique ability to specifically recognize and infect bacterial hosts, replicating within them and ultimately leading to their lysis and death (Kortright *et al*., 2019). As is the case with all viruses, bacteriophages are mostly restricted to a particular species of bacteria or even individual strains within that species (Kasman and Porter *et al.,* 2022).

Bacteriophages are becoming more and more popular due to their unique properties, which include a decreased susceptibility to antibiotics, the capacity to multiply themselves, and a rapid eradication of bacteria, relatively low cost, easy extraction and low environmental impact and low toxicity because their components are mainly protein and nucleic acid. Phages offer an abundance of benefits and are a potentially useful tool as a biocontrol agent alternative to the antibiotics in an era where antibiotic-resistant bacterial diseases are become more common (Romero-Calle *et al*., 2019; Loc-Carrillo and Abedon *et al.,* 2011). The current project aims to investigate bacteriophage therapy as a substitution biocontrol strategy against infection in poultry caused by *Salmonella* and *E.coli*.

2. Material and methods

2.1. Isolation and purification of *Salmonella Spp.* and *E-coli* from poultry

Samples were enriched in peptone water were incubated at 37 °C for 18 hours. Then, the samples were inoculated on xylose-lysine-deoxycholate (XLD) agar for the selection of *Salmonella spp*. and on MacConkey agar for the selection of *Escherichia coli*. Colorless suspected colonies were inoculated on nutrient agar for purification. Then, it was incubated at 37°C for 24 hours, and biochemical tests such as the TSI and urease tests were used to confirm suspected *Salmonella sp. Escherichia coli* as described by (Mohammed *et al*., 2012) and (Islam *et al.,* 2014)

2.2. Biochemical identification of bacterial isolates

2.2.1. Triple Sugar Iron test

Using an inoculating needle to pick up an isolated colony of TSI agar inoculation by first stabbing through the center to the bottom of the tube and then the lines on the surface of the agar mile, the lid leave loosely and the tube was incubated at 35°C in ambient air for 18 to 24 hours. (Acharya e*t al.,* 2024) and (Macfaddine *et al.,* 2000).

2.2.2. Citrate test

In the absence of fermentable sugar some microbes can use citrate as their energy source if they possess citrate enzyme. Citrate agar was streaked with a needle by using sterile technique and

incubated at 37°C for 24 hours. (Macfaddine *et al*., 2000).

2.2.3. Urease test.

A pure bacterial colony was inoculated into motility indole urease agar. Bacteria migrated away from the original inoculation line 48 hours after incubation indicated a positive result. (Rashid *et al*., 2013).

2.2.4. Gram's Stain

A small colony was streaked on a glass slide and the bacteria were thermally fixed on it. The slide is immersed in violet crystal solution for 1 minute, rinsed briefly under running tap water for a few seconds and air dried. Then it immersed in Lugoand iodine solution for 1 minute, decolored with 95% ethanol for 30 seconds and rinsed again with running tap water and dried air. 0.5% Safranin was smeared for 10 seconds, rinsed under running tap water for a few seconds and air-dried. The glass slide was then checked at 40x and 100x magnification using oil immersion. (Rashid *et al.,* 2013*).*

2.3. Isolation, Purification, propagation and titration of lytic Phages

Samples for bacteriophage isolation were collected from raw sewage (Quesna, Shebin Al-koum, Ashmoon, Menouf, Al-Shuhada). The collected Samples were centrifuged and filtered by using a sterile 0.22 µm syringe filter (Merck Millipore). Then , 20 ml of the filtered supernatant, and 50 µl of each bacterial strain (10^9 CFU/ml) were mixed and incubated overnight at 37 ◦C under aerobic conditions in a shaking incubator at 220 rpm. The incubated mixture was then centrifuged at 10,000×g for 10 min at 4 ◦C and the supernatant was filtered with a sterile 0.22 µm syringe filter. The filtrate was examined for the presence of bacteriophages using Plaque assays with a double-agar. Almost 45 ml of supernatant sewage sample was added to 5 ml nutrient broth (supplemented with 10 mm $CaCl_2$ and 1M MgSO4) and inoculated with 1 ml of early log-phase strain. After incubation at 37 °C for 16–18 h, the medium was centrifuged at 7000 rpm for 10 min. The supernatant was filtered with a 0.22 µm pore size syringe filter and the presence of lytic phage in the sample was demonstrated by the appearance of a clear zone in spot testing on soft layer agar which indicated successful lysis of bacterial cells by phages. The lytic phages were purified by three ways using Agar overlay assay as the method described previously (Akhtar *et al.,* 2014). The high titer phage stocks were prepared by inoculating 100 µl of purified phage filtrate with 1 ml of overnight host bacterial culture into 100 ml New Zealand Casamino Yeast extract medium (NZCYM) (supplemented with 10 mM $CaCl_2$ and 1M MgSO4) and incubated at 37 °C for more than 48 h. The phage titer was determined as plaque-forming units (PFU/ml) using the agar overlay assay. The high titer (109-1012 PFU/ml) phage filtrate was stored at 4 °C. (Sun *et al.,* 2022)

2.3.2. Plaque Assay

Plaque counting is considered the benchmark method for counting phages. The double agar overly assay (DLA) facilities localized interaction between phage and host in a confined environment (petri dish) counting two layers of agar on each other. The bottom layer is prepared with medium sustaining bacterial growth counting 1-15% agar. The top layer contains the same medium with lower concentration of agar (0.4 to 0.6%) commonly mentioned to as soft -agar and it is mixed with the host bacterium and spilled onto the bottom layer consequenting in a so -called lawn. In the top -agar, diffusion permits the bacteria to occupy the lawn completely and phages to bind to the bacteria. Phage samples are placed on the second layer then dried or directly mixed with the soft agar and the bacteria. This is then incubated at the ideal temperature and duration for bacterial growth. (Ács *et al.,* 2020).

2.4. Electron Microscope.

The structure of the phage lysates with higher titer (10^9–10^11 PFU/ml) was analyzed by transmission electron microscopy (TEM), according to the procedure described by (Deveau *et al*., 2006). Briefly, 1 ml of lysate was centrifugated at 4 ◦C for 1 h at 25,000× g. The supernatant was filtrated by syringe filter. The carbon coat Formvar and carbon grid were prepared using 15 µl of purified lysates. Then, the phage preparations were negatively stained with 15 µl of phosphotungstic acid (1% w/v) for 1 min, dried for 5 min at 55 °C, and analyzed using the JEM-2100.

2.5. Characterization of Isolated bacteriophages

2.5.1. Host range of isolated bacteriophage.

The host range of the phage was determined using spoting tests as qualified (sun *et al*., 2022). All strains under studying (S3, S5, S6, E1,E2, *Salmonella typhi* ATCC 14028, *Actinobacteria, Klebsiella, pseudomonas* ATCC 902) were used to define the

host range, each strain was cultivated overnight at 37 °C in shaking incubator and then 100 of each bacterial culture supplemented to 5 ml of semi-solid N.A (0.75% agar) and mixed . The mixture was then spilled onto the surface of a prepared nutrient agar plate. Afterwards, 10µl of phage (108 PFU/ml) with dilution in sterile SM Buffer was spotted on the lawn and the plates were cultivated at 37°C for 12 h. The experiment was conducted three times.

2.5.2. Thermal stability test

Using the methodology outlined by (Pajunen *et al*., 2000). The titer of filtered phages lysate was ranged between 9.1x10^8 to 9.9x10^9 PFU/ml. In sterile eppendorfs, 1ml of phages suspension was incubated in water bath at the following temperature degrees: 40, 50, 60, 70, 80, 90 and 100 ^{o}C. Each one was incubated for 10 min Then cooling under tap water. The residual phage activity was determined by spot assay technique (Silva *et al.,* 2014).

2.5.3. pH sensitivity of phages

Studying the influence of pH degree on phages stability was established by using Nutrient broth medium with different values of pH. The phages lysate were diluted in eppendorf tubes containing 1 ml of liquid medium adjusted to different pH values (3, 4 , 6, 7, 9 , 11 and 13) using 0.1 N HCL and/or 0.1 N NaOH. After incubation of the mixtures for 1hr the residual phage activity was determined by spot assay technique. (Silva *et al.,* 2014*).*

2.6. MIC Test

The minimum inhibitory concentration (MIC) of antibiotics was calculated by the broth micro-dilution assay as described earlier (Kowalska-Krochmal *et al*., 2021). Briefly, 0.5 McFarl and standards were prepared using freshly culture of *Salmonella*. Antibiotic dilutions were prepared in 96-well microtiter plates. The plates' wells showing turbidity were used to obtain inoculum for streaking; then bacterial growth was demonstrated by streaking on a nutrient agar plate (Chaudhry *et al.,* 2020).

2.7. Phage lytic activity and the phage-antibiotic combination against E. coli:

MOI (Multiplicity of Infection) refers to the number of viral particles per bacterial particle. *E.coli* culture was inoculated in LB broth and incubated in a shaking incubator with 120 rpm at 37°C for 8 hours to obtain freshly culture. The pellets of bacteria were suspended in PBS to reach OD600 (0.26 CFU/ml). Different bacteriophages concentrations were utilized (1,10,100), and PBS treatment served as control. They were incubated for 24 hours at 37°C in a shaking incubator with 120 rpm. Bacterial growth was estimated orderly every (2) hr. by recording OD600. To determine the effectiveness of the phage-antibiotic combination, phages were combined with single antibiotic (IPM). Freshly culture *E.coli* (10^8 CFU/ml) was added in the flasks containing 25ml nutrient broth, then incubated at 37°C for 24 hours. After that the phage-antibiotic (1: 1) mixture was added in the same flask, and incubation persisted at 37°C for 24 hours. The MOI of phages was set at (1), and the antibiotic concentration used was 1000 µg/ml. Every 2 hr. measure the growth of the bacteria by estimating OD600. All experiments were performed in triplicates (Bedi *et al.,* 2009).

3.8. Antibiotic Susceptibility Test

Antimicrobial sensitivity test was performed on *Escherichia coli* and *Salmonella sp.* isolates by the disk diffusion method as expressed by (Humphries *et al*., 2021). The test was performed against 11 frequently utilized chemotherapeutic drugs. Amoxicillin (30 mg), chloramphenicol (30 mg), streptomycin (10 mg), gentamicin (10 mg), imipenem (10 mg), Tetracycline (30 mg), cefoxitine (30 mg), Nalidixic acid (30 mg), Ciprofloxacin (10 mg), meropeneme (10 mg) and piperacillin (100 mg) (Humphries *et al.,* 2021).

3. Results

3.1. Isolation and characterization of bacterial isolates

Eight isolates were isolated from poultry. Six of them might be *Salmonella spp.* They were named S1, S2, S3, S4, S5 and S6. The other two of them were *Escherichia coli* namely E1 and E2. All isolates were tested by being grown on XLD, MacConkey and TSI media, and tested by urease and citrate to ensure that isolates were not contaminated. Some biochemical characteristics of isolates were presented in Table(1). All isolates were grown on XLD, six isolates gave red colonies on XLD and colorless colonies on macConkey, while other two turned the media to yellow on XLD and pink colonies on macConkey. On TSI media, six isolates gave red slants with H_2S production, while the other gave yellow slants. All isolates were citrate and urease negative except S1 and S2**.**

Table 1. Biochemical characterization of bacterial isolates**.**

Isolate code	MacConkey agar medium	XLD agar medium	TSI		Citrate test	Urease test
			Color	H_2S Production		
S1	**Colorless**	**Red colony**	**K/A**	+	+	+
S2	**Colorless**	**Red colony**	**K/A**	+	+	+
S3	**Colorless**	**Red colony with black center**	**K/A**	+	**-**	**-**
S4	**Colorless**	**Red colony with black center**	**K/A**	+	**-**	**-**
S5	**Colorless**	**Red colony with black center**	**K/A**	+	**-**	**-**
S6	**Colorless**	**Red colony with black center**	**K/A**	+	**-**	**-**
E1	**Pink**	**Yellow color**	**A/A**	+	**-**	**-**
E2	**Pink**	**Yellow color**	**A/A**	+	**-**	**-**

K=alkaline=red / A=acid=yellow.
(+) : positive test / (-) : negative.

3.1.2. Gram staining

All bacterial isolates showed gram negative with rod shaped under microscope Fig (3).

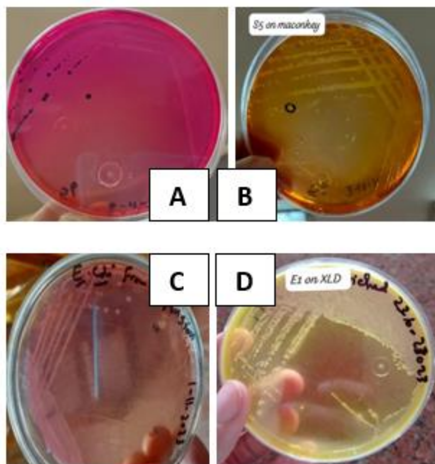

Figure 1. Growth of bacterial isolates on various media, showing distinct colony morphologies.
(A) S5 appears colorless colonies on MacConkey media.
(B) S6 isolate appears red colonies with black center.
(C) E2 colonies appear with pink color on MacConkey.
(D) E1 isolate on XLD media.

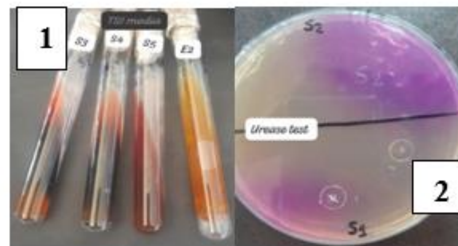

Figure 2. (1)The growth parameter of some bacterial isolates on TSI medium.
(2) Urease test of S1 and S2 isolated.

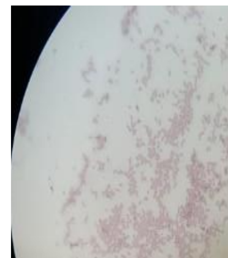

Figure 3. Showed the negative gram stain of isolates under microscope.

4.2 Intrinsic antibiotic resistance

Intrinsic antibiotic resistance of isolates was detected, and the results were shown in Table (2).

Figure 4. Showed the sensitivity of isolates for different antibiotic classes.

According to the results of this test, the isolates E1 and E2 were highly resistance (88.9%) MAR index, the isolates S3 and S4 were highly sensitive (14%) MAR index and the isolates S5 and S6 were mediated between each other (50.0%).

3.3. Isolation of bacteriophage from different Egyptian sewage water

Five samples were used for the *Salmonella* and *E-coli* phage isolation. Out of all the samples, two phages that were isolated were found in the sewage of poultry and other isolated phages was obtained from sewage water samples. Four of them were selected.

3.4. Spot test

Presence of phage was detected for all isolates. The results were showed in Table(3) and Figure(5).

Table 2. Intrinsic antibiotic resistance

Antibiotics (µg/ disc)	Mode of action	Diameter of inhibition zone around each disc(mm) for all isolates					
		S3	S4	S5	S6	E1	E2
AMC(30)	**Inhibit cell wall formation**	-	-	0 (R)	0 (R)	0 (R)	0 (R)
CX(30)	**Inhibit cell wall formation**	0 (R)	0 (R)	0 (R)	10 (R)	0 (R)	0 (R)
IPM(10)	**Inhibit cell wall formation**	30 (S)	27 (S)	-	-	27 (S)	30 (S)
MEM(10)	**Inhibit cell wall formation**	-	-	30 (S)	28 (S)	0 (R)	0 (R)
PI(100)	**Inhibit cell wall formation**	-	-	12 (R)	12 (R)	0 (R)	0 (R)
C(30)	**Inhibit protein synthesis**	28 (S)	25 (S)	31 (S)	33 (S)	0 (R)	0 (R)
CN(10)	**Inhibit protein synthesis**	17 (S)	16 (S)	-	-	-	-
S(10)	**Inhibit protein synthesis**	0 (R)	12 (R)	21 (S)	21 (S)	0 (R)	0 (R)
TE(30)	**Inhibit protein synthesis**	-	-	15.5 (S)	23 (S)	0 (R)	0 (R)
CIP(5)	**Prevent DNA replication**	24 (S)	24 (S)	15 (R)	23 (R)	0 (R)	0 (R)
NA(30)	**Prevent DNA replication**	27 (S)	25 (S)	-	-	-	-
Total S/R		5/2	5/2	4/4	4/4	1/8	1/8
MAR index		14%	14%	50.0%	50.0%	88.9%	88.9%

S= represent sensitive strain. R= resistance strain, - = not detected. AMC = Amoxcillin, C = Chloramphenicol, CN = Gentamicin, CIP = Ciprofloxacin, CX = Cefoxitine, IMP = Imipenem, MEM = Meropenem, NA = Nalidixic acid, PI = Piperacillin, S = Streptomycin, TE = Tetracycline.

Table 3. List of different localities of sewage water and their response on spot test.

Government	Localities	Sewage filtrate code	Formation of lytic area on different isolates			
			S5	S6	E1	E2
Menoufia	Quesna	As	+	+	-	-
		Al	+	+	-	-
	Shebin Al-koum	B	+	-	+	+
	Ashmoon	C	+	+	-	-
		G	-	-	+	+
	Menouf	D1	+	+	-	-
		D2	+	+	-	-
	El-Shohada	E	+	+	-	-

(+): lytic area appeared on spot test
(-): no lytic area appeared on spot test.

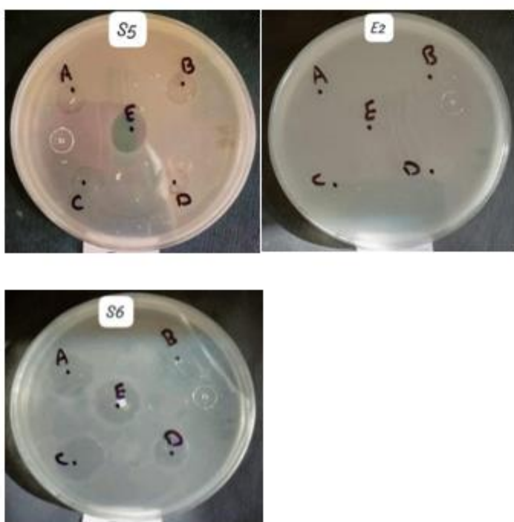

Figure 5. Showed the lytic area on isolates.

3.5. Characterization of bacteriophages:

The bacteriophages isolated from different sewage samples were purified.

3.5.1. Plaque morphology:

Four isolated bacteriophages were distinguished by differences in plaque morphology, appearance, diameter of plaques. Different phages termed as pSAs, pSAl, pSC, pSD1, pSD2, pEG were identified as indicated in Table (4) and Figure(6).

Table (4)

Phages	Plaques morphology	
	Diameter (mm.)	Appearance of plaque
pSAs	3 ±0.3	Clear
pSC	2.56± 0.5	Turbid clear center
pSD1	2.9±0.1	Clear
pEG	Pin point	Clear

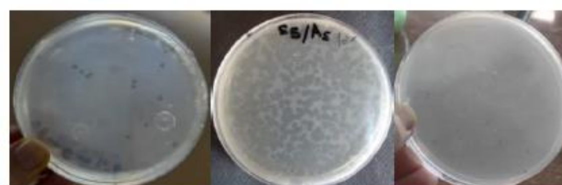

Figure 6. Showed the morphology of plaques

3.5.2. Electron microscopy studies

The purified four phages pSAs, pSC, pSD, pEG were negatively stained with phosphotungstic acid and screened by TEM as shown in Figure(7) to determine their morphological features and dimensions. All phages belonged to Family podovirideae**.**

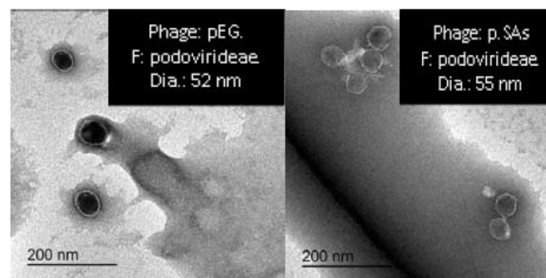

Figure 7. The morphological features and dimensions of some isolated phages

3.5.3. Biological characters of bacteriophages

3.5.3.1. Host-range of bacteriophage

Host range of isolated phages were examined by spotting of bacteriophages suspensions on nine

isolates showed in Table (5). All isolated *Salmonella* phages have broad host range against different strains of *Salmonella spp.*, but phage

(pSE) has short longevity, was not formed lytic area. Phage (pEG) has effect on *E-coli* strain only.

3.5.4. Physiochemical characters of bacteriophages

3.5.4.1.Thermal stability test

The stability of bacteriophage was detected by spotting and shown in Table(6). The results indicated that the thermal inactivation point (TIP) of *Salmonell*a phages at 90°C except the pSAs at 80°C, while coliphage thermal inactivation point (TIP) at 50°C, showed on Table(6) and Figure(8).

3.5.4.2. pH stability:

The stability of bacteriophages was shown in Table(7). The viability of phage pSAs increased with increasing pH values, the viability of phage pEG was increased with decreasing pH values, while the phages pSD1and pSC were affected by highly acidic and alkaline pH. This result showed in Figure(9). **Figure(9).** Showed the result of pH stability

Table 5. Host range.

Bacteria isolates	Source	Spot test to isolated phages					
		pSAs	pSAl	psC	psD	pSE	pEG
S3	Present Study	+	+	+	+	-	-
S5		+	+	+	+	-	-
S6		+	+	+	+	-	-
E1		-	-	-	-	-	+
E2		-	-	-	-	-	+
Salmonella typhi ATCC 14028	ATCC	+	+	+	+	-	-
Klebsiella		-	-	-	-	-	-
Pseudomonas ATCC 9027		-	-	-	-	-	-
Acinobactar		-	-	-	-	-	-

(-) Absence of lytic area.
(+) Presence of lytic area.
(ATCC) American type culture collection.

Table 6. Thermal stability of phages.

tem.	Phages			
	pSAs	pSD1	pSC	pEG
40°C	+	+	+	+
50°C	+	+	+	-
60°C	+	+	+	-
70°C	+	+	+	-
80°C	-	+	+	-
90°C	-	-	-	-
100°C	-	-	-	-

(-) Absence of lytic area.
(+) Presence of lytic area.

Figure 8. Showed the result of thermal stability of phages.

Table 7. pH stability of phages.

pH values	*Phages*			
	pSAs	pSD1	pSC	pEG
3	+	+	+	+++
4	+	++	++	+++
5	+	++	++	++
7	++	+++	+++	++
9	++	+++	+++	++
11	++	++	++	+
12	+++	++	++	+
13	+++	+	+	+

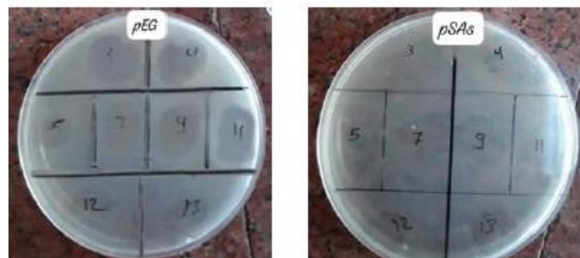

Figure 9. Showed the result of pH stability

Table 8. Effect of the best MOI of phage and MIC of IPM and their combination on bacterial growth.

	Optical density (OD.) Of bacterial growth			
Time hr.	Control	Phage (PEG) MOI=1	Antibiotic (IPM) at MIC	Combination MOI+ MIC
0	0.36± 0.03	0.36± 0.03	0.33± 0.03	0.36± 0.04
1	0.47± 0.03	0.30± 0.01	0.45± 0.03	0.39± 0.04
2	0.68± 0.05	0.25± 0.01	0.64± 0.09	0.27± 0.01
4	0.99± 0.01	0.23± 0.01	0.34± 0.03	0.24± 0.01
6	1.28± 0.06	0.23± 0.26	0.25± 0.05	0.21± 0.00
8	1.45± 0.01	0.23± 0.02	0.32± 0.03	0.22± 0.01
10	1.72± 0.03	0.22± 0.20	0.41± 0.01	0.22± 0.02
12	1.62± 0.07	0.22± 0.19	0.55± 0.04	0.36± 0.02
24	1.66± 0.05	0.32± 0.03	1.06± 0.06	0.58± 0.02

3.5.5. Phage lytic activity and the phage-antibiotic combination against *E. coli*:

The pEG without antibiotic gave the lowest OD , while the antibiotic at MIC gave the highest OD. The results are on Table(8) and shown in curve in Figure(10), the turbidity of bacterial growth was shown in Figure(11).

4.Discussion

Salmonella sp. and *Escherichia coli* are two of the most common pathogens that may infect humans through contaminated chicken flesh, thus detecting their presence even at low levels is critical (Poojari *et al.,* 2022). Phage treatment has become a more viable method for food safety and preservation in recent times (Spricigo *et al*., 2013). Bacteriophages are becoming more and more popular due to their unique properties, which include a decreased susceptibility to antibiotics, the capacity to multiply themselves, and a rapid eradication of bacteria, relatively low cost, easy extraction and low environmental impact and low toxicity because their components are mainly protein and nucleic acid.

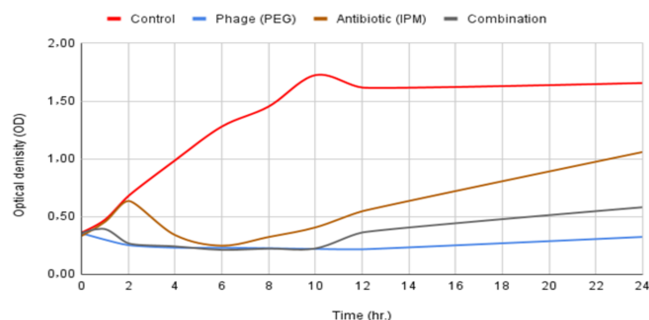

Figure 10. Effect of phage, antibiotic and phage-antibiotic combination on growth curve of *Salmonella* sp.

Figure 11. Difference in bacterial growth turbidity due to the effect of the phage and antibiotic

4.1. Isolation of bacterial isolates

About 8 bacterial samples were gathered from different places in Menoufia governrate in Egypt, one of them was isolated from poultry waste water. Six of them may be *Salmonella* and two of them may be *E.coli*. An previous experimental was carried out on 34 bacterial isolates (23 *E.coli* and 11 *Salmonella*) that were obtained from the stock culture collection from Uganda and Kenya. (Nyachieo *et al.,* 2021).

4.2. Biochemical characterization of bacterial isolates

4.2.1.MacConkey

MacConkey is intended to selectively isolate Gram-negative and enteric bacteria and distinguish them using lactose fermentation. The study showed that E1 and E2 gives pink color as they are Lactose

fermenters. This occurs because the pH indicator turns to pink. S1, S2, S3, S4, S5, S6 are colorless and don't cause pH change, because of the absence of lactose fermentation, they are non-fermenters (Jung and Hoilat, 2022).

4.2.2.XLD agar

To distinguish *Salmonella* from other enter pathogens, utilize XLD agar, because XLD contains sugars including xylose, sucrose, lactose, and lysine, some *Salmonella sp.* produced hydrogen sulphide (H_2S), which caused *Salmonella* isolates to create a red colony with or without a black center. The indicator will be phenol red, and the H_2S indicator will be ferric ammonium citrate in the medium. *Salmonella* can ferment xylose and raise the pH of the medium to an acidic level, but it cannot ferment lactose or sucrose. However, Salmonella's decarboxylation of lysine results in the basic product cadaverine, which balances the acidic pH and causes the process to become alkaline. A yellow colony is produced by *E.coli* isolates because of digesting lactose (MicrobeOnline, n.d.)

4.2.3.TSI Test

Triple Sugar Iron (TSI) agar is used to differentiate between S*almonella* and *Proteus* which have the same red color on XLD agar. We observed that *Salmonella* isolates S1 to S6 gives red (slant), yellow (butt) and H2S gas is produced, while *E.coli (*E1 and E2*)* gives yellow (slant), Yellow (butt) and H2S production .K/A refers to Red/yellow. These colors are due to glucose fermentation only; peptone catabolized. A/A refers to Yellow/yellow. These colors are due to glucose and lactose and/or sucrose fermentation (Austin Community College, n.d.)

4.2.4.Citrate test

The citrate test is used to differentiate between *Klebsi ella* and *E. coli* also between *Salmonella* and *Proteus.* S1 and S2 showed a blue color due to the ability to use citrate as a carbon source, the enzyme citrase hydrolyzes citrate into oxaloacetic acid and acetic acid. After that, the oxaloacetic acid hydrolyzes to produce CO_2 and pyruvic acid. An alkaline chemical is created when CO_2 interacts with the medium's ingredients. The pH indicator (bromothymol blue) turns blue when the pH is alkaline. S3 to S6, E1 and E2 showed no change in the green color of the media as there is no change in pH.

4.2.5. Urease test

The urease test indicates organisms which have the ability to break down urea into ammonia and gaseous carbon dioxide. *Proteus* species can be distinguished from non-lactose fermenters, such as *Salmonella.* S1and S2 gave pink color due to synthesis of urease enzyme and production of ammonia that results in increasing pH and the color alters from yellow (pH 6.8) to the shiny pink (pH 8.2). S3 to S6 didn't show any changes in the color of the media as there is no change in pH (Meghana, 2020).

4.2.6. Gram stain

The isolates appeared red, so they are gram-negative and may be *Salmonella* and *E.coli*. Because the peptidoglycan layer on gram-negative cells is thinner, the addition of ethanol causes the crystal violet to disappear. The counterstain, usually safranin or fuchsine, causes them to become pink or scarlet. This outcome was in line with what discovered (Tripathi and Sapra., 2020).

From the previous biochemical tests it was confirmed that S1 and S2 are *Proteus*, S3 to S6 are *Salmonella,* and E1 and E2 are *E.coli.*

4.3. Intrinsic antibiotic resistance

Different antimicrobial resistance patterns of *Salmonella sp*. and *E.coli* was reported in the current study. Both isolates of *Salmonella* and *E. coli* recorded the greatest resistance to ampicillin, 41% and 71%, respectively, and greatest susceptibility to ciprofloxacin. (Ngai *et* al., 2021).Table(2) shown that the isolates E1 and E2 were extremely resistant as it was 88.9% resistant to various antibiotics. The isolates S5 and S6 were mediated between others (50.0%), and the isolates S3 and S4 were extremely sensitive (14%) according to the MAR index. The sensitivity test and biochemical tests showed that S1 and S2 are proteus that's why they were excluded. S3 and S4 were excluded also due to their high-sensitivity to antibiotics.

4.4. Isolation of bacteriophages

In this study, we have isolated 8 phages of *E.coli* and *Salmonella* from 6 sewage and poultry wastes samples collected from different places in Minoufia governorate which proved the spread of phages everywhere and their isolation from environmental samples is possible especially from the sources where the intended host is present. A previous research has shown Ten phages were isolated from sanitary water drainage characterized.The Phages also showed activity against 23 out of the 24 *Salmonella* serovars

evaluated. Two other phages have also shown activity against *Escherichia coli*. (Bryan *et al.,*2023).

4.5. Characterization of bacteriophages
4.5.1. Host-range of bacteriophage

Phages were used to characterize the host range of two strains of *E. coli*, four strains of *Salmonella*, and three additional bacterial strains since they were reported to be species-specific. The results showed that *Salmonella* phages have a broad host range. *E. coli* phage has a broad host rang. By testing against *Klebsiella*, *Actinobacteria* and *Pseudomonas*, none of the phages demonstrated lytic activity. A previous study has demonstrated that lytic phages can effectively suppress foodborne infections over a broad host range of bacterial strains. (Kumar *et al.,* 2022).

4.5.2. Thermal stability test

Phage inactivation occurs at high temperatures because of denaturation of protein structures and genetic materials. (Zhang *et al.,* 2015). Following 15 minutes of varying temperature heating for each phage, it was found that the yield of pEG bacteriophage was temperature-dependent. pEG bacteriophage could not multiply or lyse *E. coli* after being exposed to 60°C. But after being exposed to 40°C the bacteriophage developed and performed lysis on his host bacterium. Its activity was weak after being exposed to 50°C.High levels of thermal stability were demonstrated by *Salmonella* Phages pSD1 and pSC from 40 °C to 80 °C and the phage pSAs from 40°C to 70°C. *Salmonella* phages shown impressive thermal stability in a prior investigation, with temperatures ranging from 30°C to 70°C. (Esmael *et al.,* 2021).

4.5.3.pH stability

This study has shown the effects of acidic and alkaline environments on phage survivability. We discovered that the four *Salmonella* and *E. coli* phages could live at pH levels between 3 and 13. Significantly less phage viability was seen at the extremely low pH of 3. In a previous study, it was shown that ten *Salmonella* phages could endure in pH ranges of 4 to 10. (Zhang *et al*., 2015).

4.6.Multiplicity of infection (MOI)

From our studies on MOI it was tested that the best MOI for *Salmonella* isolates was 10 and for *E.coli* isolates was 1 .This agrees with the results of previous studies as following. A prior study indicated that *S. typhimurium* was not inhibited by adding low-titer PSDA-2 (MOI = 0.0001 and 0.01) to LB medium at 4°C. The phage's inhibitory effect on *S. typhimurium* was strengthened, and the time required to eradicate *S. typhimurium* was reduced with an increase in titer (MOI = 10). We concluded that adding high titers of phages had a considerably greater antibacterial effect than adding low titers. (Sun *et al.,* 2022).. An earlier study demonstrated phage titers of the *E. coli* strains SWJM-01 and SWJM-02 showed a clear decline from 10 log.10 PFU per mL to 8 log10 PFU/ml as the MOI rose from 0.0001 to 100. When MOI was 1, SWJM-01's phage titer reached its peak. (Hong *et al*., 2023).

4.7. Lytic activity of phage, and phage antibiotic combination against *E.coli*

A prior survey demonstrated the phage-antibiotic synergy (PAS) effect in reduction of MIC and MBC values of antibiotics. Long-term interaction between phages and bacteria is likely to result in the formation of phage-resistant bacterial strains (Malic *et al*., 2021). Through our research ,we found that the combination effect of phage - antibiotics was lower than the effect of phage alone, which does not agree with a pervious study in which the kinetics of synergistic antibacterial activity of the phage and ampicillin revealed that the combination of the phage and sublethal concentrations of ampicillin inhibited the growth of *E.coli* more effectively than antibiotic or phage alone,and the recovery rate of resistant strain was considerably reduced in combination (Moradpour *et al*., 2020).

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