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APPLICATION OF *SALMONELLA* PHAGE COCKTAIL TO CONTROL *SALMONELLA* TYPHIMURIUM *IN VITRO*

[53]

Yasmer S. Hussein; Kh. A. El-Dougdoug and B.A. Othman
Agricultural Microbiology Dept. Fac. of Agric., Ain Shams Univ., Cairo, Egypt

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ABSTRACT

Members of the genus *Salmonella* are among the microbial pathogens associated with dangerous infection. The increased of *Salmonella* spp. antibiotic resistance has propelled the need of alternate therapeutic methods or strategies. Bacteriophage forms one of these alternate strategies. Six lytic bacteriophages infecting *Salmonella* Typhimurium were isolated from sewage drain water. Phages were purified biologically by single plaque assay and concentrated using the ultracentrifugation. The phage isolates were named Ø SM, Ø SF, Ø SG, Ø SP, Ø SA and Ø SD. Morphological characters of *Salmonella* phages showed that all the phage isolates belong to family *Myoviridae*. All phage isolates were highly stable at room temperature, storage at refrigerator temperature and had thermal inactivation point ranged from 90 to 98 °C. Phages were stable at pH conditions ranging from pH 4 to 12. Phages did not lose their infectivity after exposure to UV for 90 min. at 35 cm and 53 cm distance. The host range of the isolated phages was found that the phages had narrow host range. Phage cocktail with different MOI was used to control *Salmonella* Typhimurium *in vitro*. Data revealed that addition of phage cocktail at MOI 10 reduced the *Salmonella* cells with rate 98.2 % after 4 hrs. from addition.

INTRODUCTION

Salmonella spp. belong to family *Enterobacteriaceae* and widely distributed in nature and often found in the intestinal tract of warm-blooded animals and humans,where they asymptomatically colonize and multiply **(Newell & Fearnley, 2003 and Doyle & Erickson, 2006).** More than 2,500 serotypes of *Salmonella* exist and the most prevalent and important *Salmonella enterica* serotypes reported worldwide are Enteritidis and Typhimurium. These are responsible for 99 % of Salmonellosis in humans and warm-blooded animals. The most common symptoms of *Salmonella* infection is non-bloody diarrhea and abdominal cramps **(Bell and Kyriakides, 2002).** Due to foodborne infections by *Salmonella* are obtained through ingesting contaminated food or water, **(Abd El-Aziz, 2013)** detected high incidence of *Salmonella* Typhimurium , one of the most frequently isolated serovars from food borne outbreaks throughout the world, in retail raw chicken meat and giblets in Egypt.

Bacteriophages are considered an effective weapon against pathogenic bacteria. As a result of development resistant bacteria against antibiotics because of their repeated usage, bacteriophages are used as a safe alternative to control pathogenic bacteria **(Abramia et al 2016)**. In addition, offer a great advantage over antibiotics. First, bacteriophages are specific and target only the pathogens of interest, so the normal gut microflora are not affected. Second, bacteriophages are self-replicating in the bacterial host and lyse bacteria. **(Connerton and Connerton, 2005).**

This paper aims to investigate the incidence of lytic bacteriophages specific for *Salmonella* in sewage water and study the posibility of their application for controling the *Salmonella in vitro*.

MATERIALS AND METHODS

Source of the bacteria

Salmonella enterica subsp. *enterica* serovar Typhimurium ATCC25566 was obtained from Microbiological Resources Center, Cairo Mircen, Fac. of Agric., Ain Shams Univ., Cairo, Egypt.

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Sources of bacteriophages

Eight different sewage drain water samples were collected as described in **Table (1).**The obtained samples were taken in sterile glass bottle of 250 ml capacity and directly transferred to the Virology Lab., Agric. Microbiol. Dept., Fac. of Agric., Ain Shams Univ. The samples were maintained at 4 °C until needing for isolation of the phages.

Isolation of *Salmonella* virulent phages

Erlenmeyer flask 250 ml containing 50 ml of nutrient broth medium was inoculated with 5 ml of overnight liquid culture of *Salmonella* and 5 ml of the sewage drain water sample. The flasks were incubated at 37 ºC for 48 hrs. with shaking (250 rpm/min). After incubation the cultures were centrifuged at 6000 rpm for 15 min at 4°C. The supernatant was collected into a clean flask. Chloroform was added at rate of 1:10 to the supernatant followed by vigorously handle shaking for 5 min. to remove any bacterial debris and small contaminated bacteria. The supernatant was collected as a crude phage lysate by obtaining the upper layer and removing the white lower layer according to **(Borrego et al 1987)**.

Detection of *Salmonella* phages

The presence of bacteriophages specific for *Salmonella* was detected in the crude phage lysates qualitatively by the spot test according to (**Borrego et al 1987).** Bacteriophages were assayed quantitatively in the positive samples by the plaque assay method according to **(Adams, 1959)**.

Preparation of the phage lysates

Phage lysates were prepared by single plaque isolation technique **(Othman, 1997).** Based on the morphological shape of plaques characters, different plaques were picked up and added to 3ml of the liquid culture of *Salmonella.* The cultures were incubated at the optimum conditions. The cultures were centrifuged to obtain the progeny phages. The process was repeated at least three times until obtaining uniform plaque morphology.

Propagation of *Salmonella* phages

After obtaining the phage lysates, single plaque isolation was done, a large amount of high titer phage stock was obtained by propagation the phage isolates on their liquid *Salmonella* culture according to **(Goodridge et al 2001).**

Purification and concentration of *Salmonella* phages

The propagated *Salmonella* phage isolates were concentrated using the ultracentrifugation method as following: the phage lysates were centrifuged at low speed 6000 rpm for 15 min. at 4°C to precipitate bacteria , and the supernatants were transferred to clean centrifuge tubes . The supernatants were centrifuged using Backman L 7-35 ultracentrifuge at 30000 rpm for 90 min. under cooling, then supernatants were carefully decanted and pellets were re-suspended in 300 µl CM buffer (6ml/L 1M Tris buffer; 2.5g/L $MgSO_4.7H_2O$; 0.735g/L $CaCl_2$; 0.05g/L gelatin; pH 7.5), transferred into sterilized tube **(Figrski and Christensen, 1974)**.

Characterization of the isolated *Salmonella* phages

Microscopic examination

One drop of *Salmonella* phage suspension was placed onto 200 mesh carbon coated cupper grid and allowed to adsorb for approximately 20 min. The excess liquid was removed with filter paper wick. The grids were negatively stained with 2 % (w/v) phosphotungstic acid for 90 seconds and left for drying, and then examined using a JOEL-JEM 1010 electron microscope (Electron Microscope Unit, Regional Center for Mycology and Biotechnology, Al-Azhar Univ., Cairo, Egypt) **(Othman, 1997).**

Thermal inactivation points (TIP)

Thermal inactivation point of *Salmonella* phage isolates *in vitro* was carried out by exposure the phage suspension to different temperature degrees 30, 40, 50, 60, 70, 80, 90 and 98 ºC for 10 min using controlled water bath and then directly cooled using ice water. 15 µl from each treated phage was assayed qualitatively using the spot test according to **(Basdew and Laing, 2014).**

Storage of *Salmonella* phages

The infectivity of *Salmonella* phages were examined qualitatively by spot test weekly after incubation at room temperature for 60 days and at 4°C for 60 days.

pH stability

The infectivity of phages to be active at different pH levels was evaluated by exposure the phage suspensions to adjusted pH values ranged from 4 to 12 using 0.1 M HCl or NaOH for 12 and 24 hrs at room temperature according to **(Taj et al 2014)**. After incubation of phage lysates were neutralized and the activity was determined by spot test method at 37°C for 24 h.

UV irradiation

Stability of the isolated phages to different distances and times to UV irradiation was assayed qualitatively after exposure to UV wave length (254 nm) for 15, 30, 45, 60, 75 and 90 min. at distance of 35 cm and for 30, 60 and 90 min. at distance of 53 cm from the UV lamp according to **(Feisal et al 2013)**.The infectivity of the treated particles was determined, qualitatively.

Host range pattern

Agar double layer plates were used for host range assay. Each of the available bacteria belonging to *Salmonella* spp. were used as indicator bacteria in individual plates. The surface of every plate was spotted with drops of the isolated *Salmonella* phage lysates. After incubation for 24 hrs, plates were examined for clearance at the sites where the drops of phage had been applied as performed by **(Rattanachaikunsopon and Phumkhachorn, 2012).**

Controlling *Salmonella* pathogen *in vitro*

The bacterial challenge test was done with some modifications according to **(Kocharunchitt et al 2009)** using broth culture from *Salmonella* Typhimurium in an exponential phase to obtain an optical density at 600 nm, OD_{600}, of 0.560 (~10^7cfu/ml). Then, the broth culture was diluted to obtain 10^6cfu/ml. The cocktail of phage lysates (consisting of six phage isolates) was added to the broth culture to obtain different multiplicity of infection (MOI) 0.01, 0.1, 10 and 100. The experiment was done *in vitro* as follows: the previous mixture (2-replicates re-incubated at 25°C for 12 h). Two-replicates of bacterial cultures (containing only bacteria) and two-replicates of bacteriophages cultures (containing only bacteriophages) used as a negative control, tryptone soy broth was used instead of phage or bacteria in control treatments. Bacterial broth and streptomycin mixture was used as a positive control.

For counting *Salmonella*, aliquots of the treatment were taken and serially diluted in buffer peptone water, and 1 ml from dilutions was counted using pour plate count method on Salmonella Shigella agar (S.S. agar) medium **(Oxoid, 2006)**. After incubation at 37°C for 24 h, *Salmonella* colonies were counted visually and used to calculate *Salmonella* numbers (cfu/ml in experiment samples). To count bacterial numbers to assess the changes in their titer, samples were taken at 0, 4, 8 and 12 hours (at the end of this experiment).The percent of bacterial reduction was done by the following formula: Percent reduction = (A – B) × 100 / A , where A is the number of viable microorganisms before treatment and B is the number of viable microorganisms after treatment. To count phage cocktail numbers to assess the changes in their titer, samples were taken at 0 , 4,8 and 12 hours (at the end of this experiment) like bacteria, then centrifuged at 6000 rpm / 15 min., precipitated bacteria by chloroform using 1:10 ratio and kept in the refrigerator for 2 hours at 4°C. Then phage titer was determined by using plaque assay technique. Control samples were taken at 0 time and every 4 hours in bacteria but at 0 time and the end of the trial in phage.

RESULTS AND DISCUSSION

Incidence of *Salmonella* lytic phages in water

The primary nature habitat of *Salmonella* spp. is the intestinal tract of warm-blooded animals and humans. *Salmonella* spp. are also widespread in soil and water, as a result of their occurrence have been detected in irrigation water as a potential preharvest source of vegetables contamination by *Salmonella* **(Ijabadeniyi et al 2011)**. *Salmonella* Typhimurium phage was isolated from sewage water from Shoubra EL-Kheima, Elkalubia, Egypt **(Feisal et al 2013)**. It is well known that where over you find the bacterial host, you find its bacteriophage. So different sewage drain water were collected to detect and isolate specific lytic *Salmonella* phages. Data in this investigation obtained by the spot test **(Fig. 1 A)** revealed that 6 samples out of 8 samples gave positive results as shown in **(Table 1).**

Lytic phage isolates

All bacteriophage positive samples were assayed quantitatively using the plaque assay technique .Then, single plaque isolation was done to obtain pure *Salmonella* phage isolate according to **(Othman, 1997).** Only one plaque was picked up from each bacteriophage positive sample. The results was recorded in **(Table 2)** and illustrated in **(Fig 1B),** showed that all isolated phages have plaques with circular, clear shape with diameter ranged between 1 and less than 1 mm except phages Ø SG and Ø SD which have circular turbid plaque. Data agreed with **(De Lappe et al 2009)** who obtained also small (≤ 1 mm) circular plaques, belonged to *Myoviridae* but with an exception his plaques were without halo.

After single plaque isolation was done, 200 ml of high titer phage stock was obtained by phage propagation several times the on its liquid bacterial culture. Then, The *Salmonella* phage particles were purified and concentrated using the ultracentrifugation.

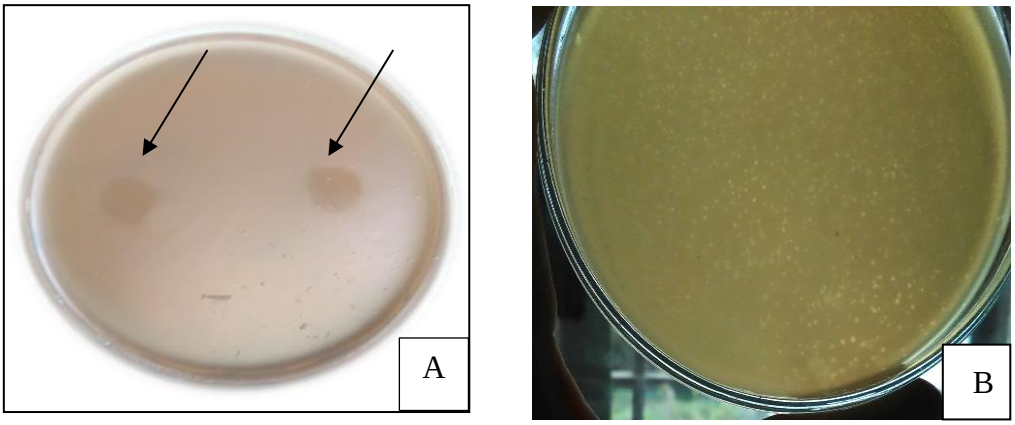

Fig. 1. (A) Spot test showing the bacterial lysis through the bacterial lawn caused by virulent bacteriophage specific for *S.* Typhimurum.

(B) Single plaques of *S.* Typhimurum phage showing identical morphology of the original plaques.

Table 1. Detection of *Salmonella* lytic phages in sewage drain water by the spot test

Location	***Salmonella* phage assaying**	
	Qualitatively	**Quantitatively pfu/ml**
El-Esmailia canal, Mostorod, Elkalubia, Egypt	(-)	-
El-Maryotya canal, Giza ,Egypt	(+)	2.5×10^5
Fac. of Agriculture, Shoubra Elkhema, Elkalubia, Egypt	(+)	5×10^4
Fac. of Agriculture, Shoubra Elkhema, Elkalubia, Egypt	(-)	-
Fac. of Agriculture, Shoubra Elkhema , Elkalubia, Egypt	(+)	7×10^5
El-Gabl El-Asfar - El-Khanka, Elkalubia, Egypt	(+)	5×10^5
Passous. Elkalubia, Egypt	(+)	4.3×10^4
Shoubra Elkhema station of sewage water, Elkalubia, Egypt	(+)	4×10^5

- = no lysis (-ve result) + = lysis (+ve result)

Table 2. Plaque morphology of the isolated *Salmonella* phages

Phages*	Diameter (mm)	Presence of halo	Plaque shape
Ø SM	<1	+	clear, circular
Ø SF	<1	+	clear, circular
Ø SG	1	+	turbid, circular
Ø SP	<1	+	clear, circular
Ø SA	<1	+	clear, circular
Ø SD	<1	+	turbid, circular

* Ø SM= sewage drain water sample from El-Maryotya canal
* Ø SF= sewage drain water sample from poultry farm, Fac. of Agriculture, Shoubra Elkhema.
* Ø SG= sewage drain water sample from El-Gabl El-Asfar - El-Khanka.
* Ø SP= sewage drain water sample from Passous.
* Ø SA= sewage drain water sample from Fac. of agriculture, Shoubra Elkhema.
* Ø SD= sewage drain water sample from Shoubra Elkhema station of sewage water.
+ plaque was surrounded with halo

The six isolated *Salmonella* phages named Ø SM, Ø SF, Ø SG, Ø SP, Ø SA and Ø SD (The 1st letter means phage, the 2nd letter means *Salmonella* and the 3rd letter means the first of isolation source).

Characterization of *Salmonella* phages

Morphological characters

As shown **(Table 3)** and illustrated by **(Fig. 2),** examination of the isolated *Salmonella* phages showed that phage Ø SM has a head with diameter of 109.1 × 109.1 nm and long contractile tail with length of 145.5 nm and width of 27.3 nm , Ø SF has a head with diameter of 77 × 92.3 nm and long contractile tail with length of 153.8 nm and width of 23.1 nm , Ø SG has a head with diameter of 107.1 × 107.1 nm and long contractile tail with length of 164.3 nm and width of 21.4 nm, Ø SP has a head with diameter of 92.9 × 92.9 nm and long contractile tail with length of 157.1 nm and width of 21.4 nm. Ø SA has a head with diameter of 109.1 × 100 nm and long contractile tail with length of 163.6 nm and width of 18.18 nm and Ø SD has a head with diameter of 118.2 × 127.3 nm and long contractile tail with length of 172.7 nm and width of 27.3 nm. The phages assigned to family *Myoviridae* as indicated by the presence of a long tail and occurrence of contractile sheath. All *Salmonella* phages reported belong to the order *Caudovirales* (tailed phages) and represent three families: the *Siphoviridae*, *Podoviridae* and *Myoviridae*. Data agreed with **(Turner, 2012 and Thung et al 2017)**.

Table 3. Morphological characters (head and tail diameters) of *Salmonella* phages:

Phage	Size (nm)	
	Head	Tail
Ø SM	109.1 × 109.1	145.5 × 27.3
Ø SF	77 × 92.3	153.8 × 23.1
Ø SG	107.1 × 107.1	164.3 × 21.4
Ø SP	92.9 × 92.9	157.1 × 21.4
Ø SA	109.1 × 100	163.6 × 18.18
Ø SD	118.2 × 127.3	172.7 × 27.3

Physical properties

Various external physical: temperature, acidity and salinity were affected on the viability and storage of bacteriophages. It can inactivate the phage through damage of its structural elements (head, tail, and envelope), lipid loss, and/or DNA structural changes **(Ackermann et al 2004).**

Thermal inactivation point

The particles of *Salmonella* phage were exposed to different degrees of temperature (range between 30 to 98 ºC) for 10 min, then cold immediately under the ice water to determine the thermal inactivation point. Results indicated that the thermal inactivation point of the phages Ø SM, Ø SF, Ø SG, Ø SP, Ø SA and Ø SD was 98 ºC, 98 ºC, 98 ºC, 90 ºC, 90 ºC and 90 ºC respectively, it was also indicated that the phages Ø SM, Ø SF and Ø SG were more stable after thermal treatment at 98 ºC for 10min., while phages Ø SP, Ø SA and Ø SD lost their infectivity. Phages Ø SP, Ø SA and Ø SD were still had the ability to lyse *Salmonella* after thermal treatment at 90 ºC for 10 min. The previous results proved that all *Salmonella* phages are thermal tolerant agreed with **(Turner, 2013)** who mentioned that bacteriophages infecting serovars of *Salmonella enterica* showed identical thermal tolerance profiles and still can be detectable after incubation at 70 ºC for 120 minutes.

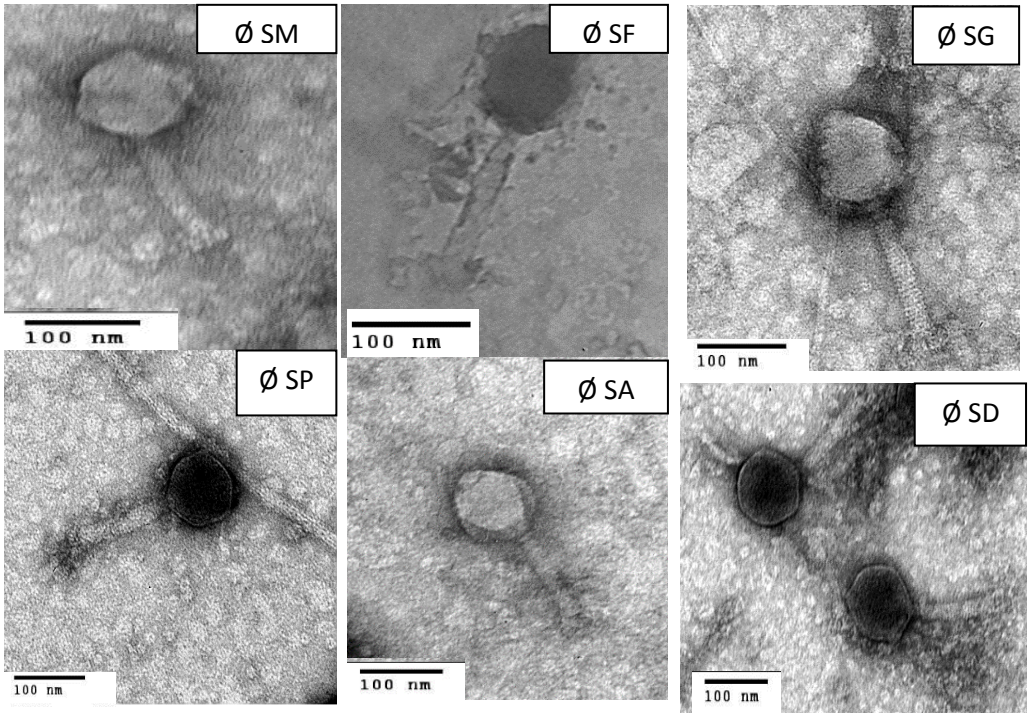

Fig. 2. Electron photograph of *Salmonella* phages (Ø SM, Ø SF, Ø SG, Ø SP, Ø SA and Ø SD) negatively stained with 2% phosphotungstic acid by TEM.

Storage of *S.* Typhimurium phages

The aim of this trial was to assay the infectivity of phage particles in preserved food products stored at room temperature and refrigerator. It was found that *Salmonella* phages were survived for at least 60 days at room temperature and 4 ºC, when the viral infectivity was assayed qualitatively weekly by the spot test. The *Salmonella* phages have high stability until 60 days at different temperature degrees at room temperature and 4 ºC. Data agreed with **(Ngangbam and Devi, 2012)** who studied the effect of different temperatures on the viability of *Salmonella* phages during storage and the titer of his phage which had good a storage stability , was almost unaltered during 14 days of storage at 37 °C, 4 °C, and –20 °C.

Acid and alkaline stability

Infectivity of the isolated *Salmonella* phages was tested at different pH values. The viral infectivity was determined qualitatively by the spot test technique. The obtained results revealed that, all *Salmonella* phages didn`t lose its ability to lyse *S.* Typhimurium cells at pH ranged from 4 to 12. The results indicate that the *S.* Typhimurium phages are stable in both the alkaline media and acidic ones. Data agreed with **(Kanjana, 2007)** who reported that, STP *Salmonella* phage lost its inactivity and didn`t detect after the treatment with pH 2 and 3, and however the STP phage remained infectious after treatment with pH 4-12.

UV irradiation stability

Salmonella phage particles were exposed to UV irradiation at 2 different distances far from it. They are at 35 cm from the UV source for 15, 30, 45, 60, 75 and 90 min., and 30, 60 and 90 min. at 53 cm distance from the UV source. *Salmonella* phages didn`t lose their infectivity after exposure at the different distances for the different times. The results didn`t agree with **(Feisal et al 2013)**, but **(Jończyk et al 2011)** stated that tailed phages like *Salmonella* phages were the most stable in adverse conditions. In addition to phages with a large capsid (100 nm in diameter) survive better than phages with a head 60 nm in diameter.

Host range

Six bacterial strain, serotypes and isolates belonging to *S.* Typhimurium ,*S.* Paratyphi B and *S.*

Typhi were tested against the infection with the isolated *S.* Typhimrium phages. Host specificity of the bacteriophage was assayed qualitatively by the spot test. Results as shown in **(Table 4)** indicate that, the isolated bacteriophages were able to lyse most of them. This result means that, the isolated *Salmonella* phages have a restricted host range. Results agreed with **(Tiwari et al 2013)** who determined host range of phage SE2, *Salmonella* strains of several serovars and non-Salmonella strains were used. Phage SE2 showed a strong lytic effect to all tested *Salmonella* isolates, Enteritidis and Gallinarum It did not form any plaque to other *Salmonella* serovars and non-Salmonella bacteria (*E. coli*, *K. pneumonia* , *P. aeruginosa*, *A. baumannii* and *S. aureus*).

Controlling of *Salmonella* by its specific phages

In vitro experiments were performed to evaluate the ability of *Salmonella* phage cocktail (mixture) to lyse *S.* Typhimurium. When phage cocktail was added to the host at MOI 0.01, numbers of the host cells were reduced after 4 hrs from the addition by 83.33 %, after 8 hrs by 89.44% and after 12 hr by 95 %.When phage cocktail was added the host at MOI 0.1, numbers of the host cells were reduced after 4 hrs after the addition by 83.93 %, after 8 hrs by 93.85 % and after 12 hr by 98.80 %. When phage cocktail was added to the host at MOI 10, numbers of the host cells were reduced after 4 hrs from the addition by 98.20%, after 8 hrs by 99.93 % and after 12 hr by 99.99 %.When phage cocktail was added to the host at MOI 100, numbers of the host cells were reduced after 4 hrs from the addition by 99.14 %, after 8 hrs by 99.99% and after 12 hr by 99.99 % as shown in **(Table 5).** The reduction of *Salmonella* numbers were also accompanied by increases in phage counts 4 hrs after the phage cocktails addition, suggesting that the phage replicated. Streptomycin inhibited the growth of *Salmonella* when was used as a positive control.

Table 4. Host specificity of the isolated *Salmonella* phages

Phages / Host specificity	ØSM	Ø SF	Ø SG	Ø SP	Ø SA	Ø SD
S. Typhimurium* NCTC12023/ ATCC 14028	+	+	+	+	+	+
S. Typhimurium LT2**	+	+	+	+	+	+
S. Typhimurium U288**	-	-	-	-	-	-
S. Typhimurium DT160**	+	+	+	+	+	+
S. Paratyphi B isolate*	+	+	+	+	+	+
S. Typhi isolate**	-	-	-	-	-	-

* Bacterial cultures were obtained from Agric. Microbiol. Dept., Fac. of Agric., Ain Shams Univ.

** Bacterial cultures were obtained from Animal Health Research Institute

Table 5. Effect of different phage multiplicity of infection on the growth of *Salmonella in vitro*

Time hr.	MOI 0.01			MOI 0.1			MOI 10			MOI 100		
	Control	Treated		Control	Treated		Control	Treated		Control	Treated	
	cfu/ml	cfu/ml	% reduction	cfu/ml	cfu/ml	% reduction	cfu/ml	cfu/ml	% reduction	cfu/ml	cfu/ml	% reduction
0	5×10^6	5×10^6	-	4.5×10^6	4.3×10^6	-	4×10^6	4×10^6	-	4×10^6	4.1×10^6	-
4	6×10^7	1×10^7	83.33 %	5.6×10^7	9×10^6	83.93 %	5×10^7	9×10^5	98.20 %	5×10^7	4.3×10^5	99.14 %
8	7.1×10^8	7.5×10^7	89.44 %	6.5×10^8	4×10^7	93.85 %	6.1×10^8	4.5×10^5	99.93 %	6.1×10^8	4.7×10^4	99.99 %
12	8.0×10^9	4×10^8	95 %	7.5×10^9	9×10^7	98.80 %	7×10^9	1×10^5	99.99 %	7×10^9	5×10^3	99.99 %

The results indicated that improving efficacy of *Salmonella* numbers reduction by phages was done by raising the MOI and the optimal used MOI was 10 which achieved the target about reducing the cells by 98.20 % after 4 hr. The results agreed with **(Goode et al 2003)** who used 10^2 up to 10^7 MOI on contaminated chicken skin and found that the reduction of *Salmonella* numbers increased according to increasing of MOI.

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