Stability of *Salmonella typhimurium* Bacteriophage to Some Physical and Chemical Factors

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Abstract

A lytic bacteriophage specific for *Salmonellatyphimurium* was isolated from sewage water and designated St-1. The isolated phage formed identical clear circular single plaques, without halo of 3 mm in diameter. Electron microscopy of *Salmonella* bacteriophage particles (St-1) indicated that the phage particle has an isometric head of about 69.6 nm in diameter and non contractile tail of 243.5 nm in length and 17.4 nm in width. The *Salmonella* bacteriophage (St-1) has a narrow host range since, it was infectious to *Salmonella typhimrium* ATCC25566 and *S. typhimurium* MM11 among the eight bacterial isolates tested belonging to family enterobacteriacae. The thermal inactivation point of the isolated phage was found to be 76 ºC. *Salmonella* bacteriophage (St-1) survived for 7 days at 4, 25, 37, 42 and -20 ºC. The virus lost its ability to lyse *salmonella* cells at pH 4, 5, 6, 10, 11 and 12 while it was infectious only at pH 7, 8 and 9.The virus lost its infectivity after exposure to UV for 50 min. at distance of 53 and 70 cm from the UV source. The bacteriophage (St-1) was inactivated in presence of NaCl at concentration higher than 15%; sodium benzoate at concentration of 0.5%; potassium sorbate at concentration of 1.0 % and citric acid at concentration of 1.0% as well as in presence of 5% of either Sodium hypochlorite or SDS. The phage DNA was resistant to digestion with PvuI, BamHI, XhoI and EcoRI. The genome size of the phage was estimated to be 18 kbp.

Key words: *Salmonella typhimurium*, bacteriophage, physical properties, biology, morphology, stability, Restriction enzymes.

INTRODUCTION

Salmonellae are enterobacteriacae that are widely distributed in the environment and include more than 2,000 serotypes. They are the most predominant pathogenic bacteria in wastewater and they cause typhoid and paratyphoid fever and gastroenteritis. This pathogen produces an endotoxin that causes fever, nausea and diarrhea and may be fatal if not properly treated by antibiotics (Bitton 1994). Species implicated in food contamination are *S. paratyphi* and *S. typhimurium*. These species can grow readily in contaminated foods and cause food poisoning (Cabadaj et al. 1995). Species such as *S. typhimurium* and *S. enteritidis* cause gastroenteritidis, which is characterized by diarrhea and abdominal cramps (Sahlström 2003).

Bacterial viruses (bacteriophages) might be used to fight pathogenic bacteria either therapeutically or in decontamination of food and water supplies (Merril et al., 1996). Phages have been proposed as natural antimicrobial agents to fight bacterial infections in humans, in animals or in crops of agricultural importance (Chen and Griffiths, 1996; Wommack and Colwell, 2000). Salmonella bacteriophages were isolated, propagated, purified, and characterized by many authors **(Nutter *et al.*, 1970; Higgins *et al.*, 2005; Kanjana, 2007 and shin *et al.*, 2012)**.

This work was carried out to study the occurrence of bacteriophages specific for salmonella in sewage water. In addition characteristics of the phages; e.g. plaque morphology, host range, particle size and morphology termal inactivation point as well as stability of the phages to different pH levels, some preservative agents and detergents were also studied.

Materials and Methods:

Source of bacteriophages

Sewage water Samples were collected from drainage system of Fac. of Agric., Ain Shams Univ.; El-Ayat, Giza Governorate and Shoubra EL-Kheima sewage water treatment plant. The obtained samples were taken in 250 ml sterile amber glass bottles and directly transferred to the Virology Lab., Agric. Microbiol. Dept., Fac. of Agric., Ain Shams Univ. in refrigerated container and then maintained at 4 ^{o}C to be used as a source of bacteriophages. Isolation of Salmonella bacteriophages was carried out within 12 hr from sampling.

The used bacterial strains:

Salmonella typhimrium ATCC 25566, ***Salmonella typhimurium*** MM11 and *E. coli* MM24T were kindly supplied by American Type Culture Collection (ATCC).

Escherichia coli NRRL3008, *E. coli* NRRL25922 and *Shigella flexneri* CCM4421 were provided by Microbiological Resources Center, Cairo Mircen, Fac. of Agric., Ain Shams Univ., Cairo, Egypt.

E. coli strainsB and H1B1D1 were obtained from Virology Lab., Agric. Microbiol. Dept., Fac. of Agric., Ain Shams Univ.

Isolation of *Salmonella* bacteriophages from sewage water:

The liquid enrichment technique was used to isolate the phages of *Salmonella* as described by Barnet (1972). A hundred ml of nutrient broth medium (Allen 1959) in 250 ml Erlenmeyer flask were inoculated with 10 ml of the tested sewage water and 10 ml 24 hr old liquid culture of each *Salmonella* strain. The flasks were incubated at 37 ºC for 48 hr with shaking (250 rpm/min). After incubation the cultures were centrifuged at 6000 rpm for 15 min and the supernatant was collected into a clean flask, chloroform was added at rate of 1:10 followed with vigorously shaking for 5 min. The crude lysate of the phages were obtained and assayed qualitatively and quantitatively according to **(Kanjana , 2007)**.

Detection of bacteriophages :

Bacteriophages were detected in the prepared crude lysate using the spot test technique in double layer agar plates . Purification of the phages was carried out using single plaque isolation technique as described by **Othman *et al.* (2008).**

Preparation of high titer phage suspension:

Liquid culture enrichment technique was used as described by **Othman *et al.* (2008**) to prepare high titer cyanophage lysate.

Purification and concentration of *Salmonella* bacteriophages from sewage water:

Dextran sulfate-polyethylene glycol two phase liquid system was used (Othman et al., 2008) to purify and concentrate the bacteriophages. Dextran sulfate 500, polyethylene glycol (PEG 6000) and NaCl, were mixed in a separating funnel to give a mixture containing ratios 5, 0.2 and 1.7 % (w:w:w), respectively. After mixing, the funnel wasallowed to stand at 4°C

overnight. A heavily turbid bottom layer was slowly collected into a clean tube and centrifuged at 2000 rpm for 10 min. The clear top and bottom phases were removed by pipette and the remaining interface "cake" was resuspended in 1 % (w:w) dextran sulfate solution. Then 0.15ml of a 3M KCl was added to each ml of suspension, the mixture was allowed to stand for 2 hr, at 4°C and centrifuged at 2000 rpm for 10 min. After centrifugation, the supernatant containing phages was obtained and dialyzed against saline solution at 4°C for 48 hr. The phage suspensions were concentrated by centrifugation at 15000 rpm for 2 hr at 4°C, and the supernatants were discarded and the pellets were re-suspended in a small amount of saline solution and then maintained at -20 °C.

Electron Microscopy of *Salmonella* bacteriophages

A drop of the resuspended pellet was placed on 200 mesh formvar-coated grid and allowed to settle for 1 min. The excess liquid was removed with a filter paper wick. Grid was stained with 1 % uranyl acetate for 15 seconds. The grid was air dried and examined in JOEL-JEM-1010 electron microscope (Electron Microscope Unite, Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo).

Extraction of Salmonella phage DNA and restriction enzymes digestion:

DNA **of** Salmonella phage was extracted and purified as described by Maniatis et al. (1982). the extracted DNA was digested with four different restriction endonucleases (PvuI, BamHI, XhoI and EcoRI). After the enzymatic digestion, the DNA fragments were separated by electrophoresis at 100 V in a 1.0 % agarose gel stained with ethidium bromide (0.5 µg/ml) in Tris-boric acid-EDTA buffer, in a Bio-Rad agarose gel electrophoresis system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). One kbp DNA ladder was applied to the same gel to allow estimation of the size of each phage DNA fragment.

Biological properties of *Salmonella* bacteriophage:

Host specificity:

Double agar layer plates were prepared. Each of the eight bacterial strains under study was used as indictor host in individual plates. The surface of every plate was spotted with drops of the isolated phage suspension. After incubation for 24-30 hrs, at 30˚C, plates were inspected for lysis of bacterial lawn at the sites where spots had been applied **(Sambrook *et al.*, 1989)** .

Physical properties of *Salmonella* bacteriophages:

Thermal inactivation point:

Thermal inactivation point of *Salmonella* bacteriophages*in vitro* was carried out by exposure the phage to different degree of temperature 30, 40, 50, 60, 70, 72, 74, 76, 78, 80, 90 and 98ºC for 10 min using controlled water bath and then directly cooled under the tap water. The treated phage was assayed qualitatively by the spot test **(Othman, 1997)**.

Storage stability of *Salmonella* bacteriophages:

The infectivity of *Salmonella* phage was examined by spottingphage lysates daily after incubated at different temperatures -20, 4 and 37 ºC for 14.

Stability of *Salmonella* bacteriophages to different pH levels:

The activity of phages to survive at different pH levels from 1 to 11 using 0.1 M HCl or NaOH over 16 hr at 37ºC was evaluated by the method of **(Jamalludeen *et al.*, 2007**).

Stability of *Salmonella* bacteriophage to UV radiation:

Fifty microliters of the bacteriophage suspension were spread inside a Petri dish using a fine pipette and then were exposed at 35, 53 and 70 cm distance from the UV source for 30, 40, 50 and 60 min and to study the effect of distance from UV source on bacteriophage activity the spot test.

Effect of some preservative agents on *Salmonella* bacteriophages:

Salmonella bacteriophage particles were preservedat different concentrations of sodium chloride (5, 10, 15, 20, 25, 30, 35 and 40%) andsodiumbenzoate; potassium sorbate; and citric acid at 0.05, 0.1, 0.5 and 1.0 % for 24 hr and then assayed qualitatively for their infectivity.

Effect of detergent stability of *Salmonella* bacteriophages:

The effect of detergent as inhibitor agent to *Salmonella* bacteriophages was carried out in different concentrations (1, 2, 3, 4 and 5 %) of sodium hypochlorite andsodium dodecyl sulfate. Bacteriophage particles were exposed for 24 hr and the bacteriophage activity was assayed qualitatively.

Results and Discussion:

Occurrence of lytic bacteriophage specific for *Salmonella* in sewage water samples:

It is well known that bacteriophages are of widespread occurrence and are usually readily isolated from areas which contain the appropriate bacterial host. *Salmonellae* are enterobacteriacae that are widely distributed in the environment. They are the most predominant pathogenic bacteria in wastewater (**Venglovský, *et al.*,2005)**.

In this study the spot test was used for detection of salmonella bacteriophages. The obtained results (Figure 1 and Table 1) indicate that lytic phages of salmonella were found to be common in three sewage water samples among the four collected ones. Similarly, Ryan (1972) isolated *Salmonella* spp. from sewage water and **Kanjana (2007)** isolated lytic bacteriophages of *Salmonella typhimrium* from a sewage treatment plant.

A B

Figure (1):(A) Spot test showing lysis of the bacterial lawn caused by virulent bacteriophage specific for *Salmonella typhimurium* and (B) Single plaques of the isolated phage of identical morphology.

Table (1): Presence of lytic bacteriophages specific for *Salmonella typhimrium* in the collected sewage water samples.

Indicator Bacteria	**Sewage water samples**			
	1	2	3	4
***Salmonella typhimrium* ATCC25566**	-	+	+	+
***Salmonella typhimurium* MM11**	-	+	+	+

1: Sewage water collected from from drainage system of Faculty of Agriculture, Ain Shams University.
2: Sewage water collected from from drainage system of El-Ayat, Giza Government.
3 and 4: Sewage water collected from Shoubra EL-Kheima sewage treatment station.
+ = Lysis - = No lysis

It is assumed that each plaque has originated from the progeny of a single phage particle. Moreover, shape, size and outline of theplaques are characteristic of the phage strain. Therefore, single plaque isolation technique was used to obtain pure phage isolates. As shown in Figure (1-B) the isolated phage formed identical clear circular single plaques, without halo of 3 mm in diameter.

Since, the formed single plaques were identical in their appearance morphology, this may indicate that the isolated phages belong to one type of phage. The isolated phage was designated **St-1** .

Particle size and morphology of Salmonella bacteriophage (St-1):

Electron microscopy of *Salmonella* bacteriophage particles (St-1) revealed the phage particle has an isometric head of about 69.6 nm in diameter and non contractile tail of 243.5 nm in length and 17.4 nm in width (Fig. 2).The phage was found to be belonging to family Siphoviridae as indicated by the presence of a long tail and the absence of contractile sheath.Similarly, **Heringa *et al.* (2005); Santos *et al.* (2010); Shin *et al.* (2012)** and **Bigwood, *et al.* (2009)** showed that the phages of *Salmonella* are spermatozoid shape and vary in their dimensions. Some phages have isometric heads with diameter of 66 nm and long flexible non contractile tail with length of 157 nm and 17 nm width.

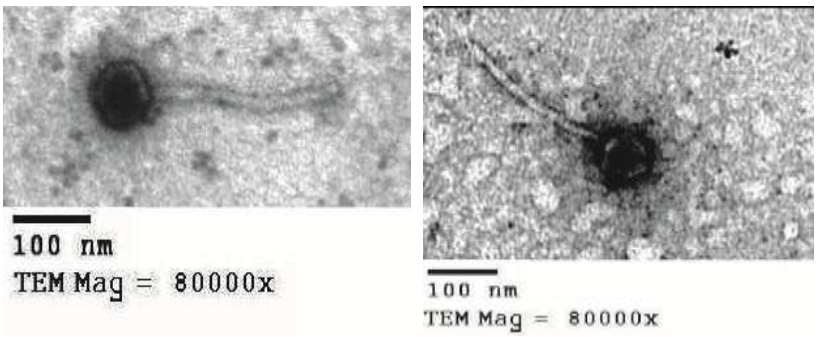

Fig. (2): Electron micrographs of purified *Salmonella* bacteriophage, negatively stained with 1 % uranyl acetate (Magnification = 80000 X).

Host range of salmonella bacteriophage (St-1)

The host range of a particular bacteriophage may be narrowly restricted within a bacterial species, or it may cross wider taxonomic boundares to the species of the same family .
Since salmonellas are members of the family *Enterobacteriaceae***(Pelczar *et al.*, 1993)**, it was of a particular interest to study the susceptibility of other members of family *Enterobacteriaceae* (*e.g.Escherichia coli*and*Shigella flexneri*) to the isolated phage (St-1).

The isolated salmonella phage (St-1) was tested against each of the tested eight bacterial strains of family*Enterobacteriaceae*.

Host specificity of *Salmonella* bacteriophage (St-1) was determined qualitatively by the spot test against eight bacterial strains belonging to Entirobacteriaceae. The obtained data presented in Table (2) indicate that, *Salmonella* bacteriophage (St-1) has a narrow host range since, it was infectious to *Salmonella typhimrium* ATCC25566 and *S. typhimurium* MM11 among the eight bacterial strains tested. No lysis was observed when the isolated phage was tested against either *Escherichia coli* or *Shigella flexneri*. Such results indicate that the phage isolate (St-1) has a host range restricted within the species of genus *Salmonella*. Similarly**, Kanjana (2007)** found that *Salmonella* phage formed clear zones of lysis only on *S. typhimrium* DMS5784.

Table (2): The host specificity of salmonella bacteriophage (St-1).

Indicator bacteria	**Salmonella phage (St-1)**
***Salmonella typhimrium* ATCC25566**	+
***S. typhimurium* MM11**	+
***Shigella flexneri*CCM4421**	-
***Escherichia coli* MM24T**	-
***E. coli* strainB**	-
***E. coli* strain H1B1D1**	-
***E. coli* NRRL3008**	-
***E. coli* NRRL25922**	-

+ = Lysis - = No lysis

Effect of some physical and chemical factors on *Salmonella* bacteriophage (St-1):

Knowledge of the effect of the chemical and physical factors on viruses is of a great interest for two reasons. It is important to know: (1) How to inactivate viruses when the object is to eliminate them. (2) How to preserve the viruses when the object is to avoid loss of infectivity.

Thermal inactivation point

The particles of the isolated *Salmonella* bacteriophage (St-1) were exposed to different temperature degrees (from 30 to 98 ºC) for 10 min to determine the viral thermal inactivation point, and the results indicated that, the virus lost its infectivity at 80 ºC for 10 min. To determine the thermal inactivation point exactly, further experiment was done by exposing the viral particles to different temperature degrees with two degree intervals (70, 72, 74, 76, 78 and 80 ºC). As shown in Figure (3) the thermal inactivation point was found to be 76 ºC. Similar results were obtained by **Adams (1959)** whoreported that, inactivation of coliphages takes place between 60 and 75 ºC depending on the surrounding medium. **Kanjana (2007)** alsofoundthat inactivity of STP *Salmonella* phage decreased after treatment at 70 ºC.

Longevity *in vitro*

Salmonella bacteriophage (St-1) survived for 7 days at 4, 25, 37, 42 and -20 ºC, as shown in (Fig. 4), the virus remained infectious without any decrease in its infectivity upto 7 day.

Effect of pH levels

Infectivity of *Salmonella* bacteriophage (St-1) was tested at different pH values using spot test. The obtained results (Table 3) indicate that, the virus lost its ability to lyse *salmonella* cells at pH 4, 5, 6, 10, 11 and 12 while it was active only at pH 7, 8 and 9. **Kanjana (2007)** reported that, the coliphages inactivated after the treatment with pH 2 and 3, and however the STP phage remained infectious after treatment with pH 4-11.

Effect of UV radiation

The Effect of UV radiations on the infectivity of *Salmonella* phage (St-1) was tested after exposure to UV radiation for 30, 40, 50 and 60 min at distance of 35, 53 and 70 cm from the UV source. The virus lost its activity after exposure for 50 min at distance of 53 and 70 cm from the UV source. Moreover, the phage lost its infectivity after exposure to UV for 30 min at distance of 35 cm from the source of UV.

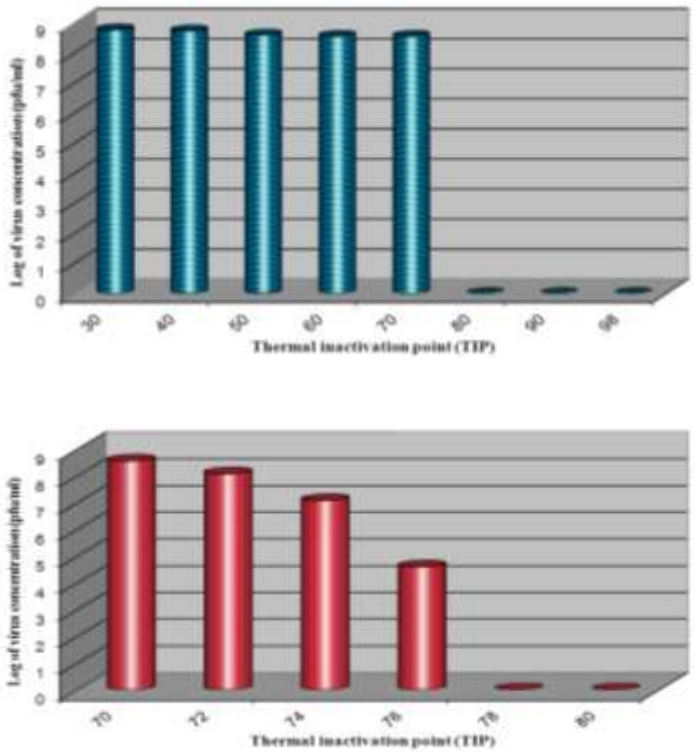

Figure (3): Effect of temperature on *Salmonella* bacteriophage (St-1).

Effect of some preservative agents on *Salmonella* bacteriophages:

Since bacteriophages might be used to fight pathogenic bacteria in food products which may contain preservative agents (*e.g.* sodium chloride, sodium benzoate, potassium sorbate and citric acid) it is of a particular interest to find out the effect of these preservative agents on the infectivity of the bacteriophages.

The infectivityof *Salmonella* bacteriophage particles (St-1) were tested at different concentrations of sodium chloride (NaCl), sodium benzoate, potassium sorbate and citric acid for 24 hr. As shown in figure (4) the bacteriophage (St-1) was found to be infectious in presence of NaCl at concentration up to 15%, and inactivated completely at concentration exceed 15% NaCl.

In presence of sodium benzoate, the infectivity of salmonella phage (St-1) did not affect at concentrations of 0.05 to 0.1% and inhibited completely at concentration of 0.5% (Fig. 4).

In presence ofpotassium sorbate, no inhibition in infectivity of the phage was detected at concentration of 0.05 to 0.1 % .Doubtful inhibition was noticed at 0.5 %, and the virus was inhibited completely at the concentration of 1.0 %(Fig. 4) .

As Shown in Figure (4) the phage was inhibited at concentration of 1.0% citric acid. Whereas, citric acid at concentrations of 0.05 and 0.1% has no effect on the infectivity of salmonella phage (St-1).

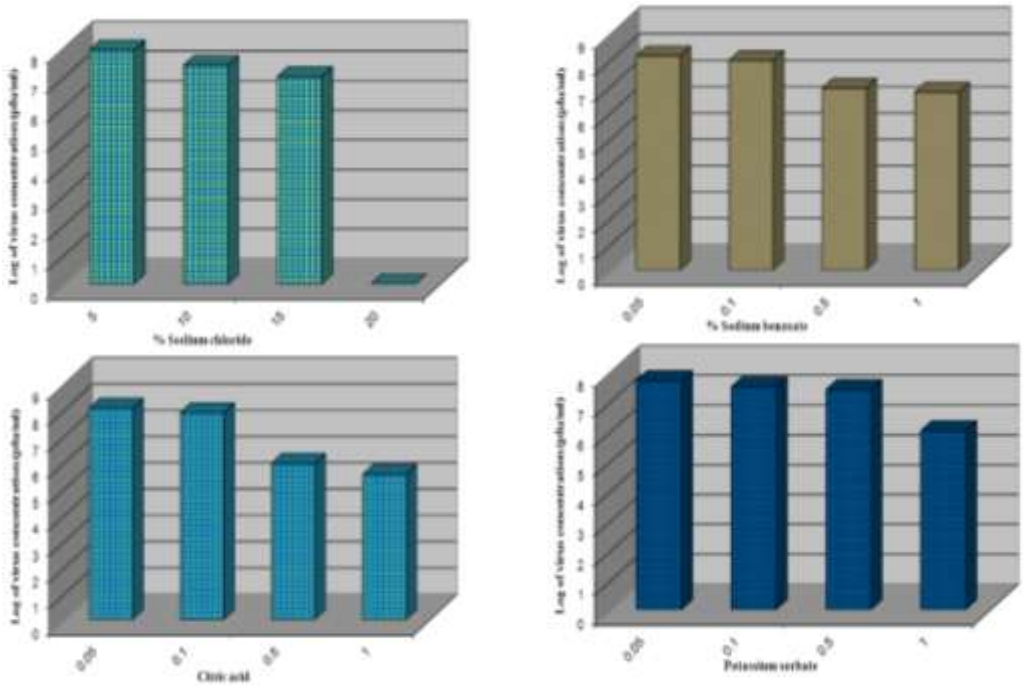

Figure (4): Effect of some preservative agents on Salmonella bacteriophage (St-1).

Effect of some detergents on *Salmonella* bacteriophage:

Effect of Sodium hypochlorite and Sodium dodecyl Sulphate (SDS) on *Salmonella*bacteriophage activity was examined, as shown in Figure (5) The inactivation of the virus was occurred at concentration of 5 % for both Sodium hypochlorite and SDS.

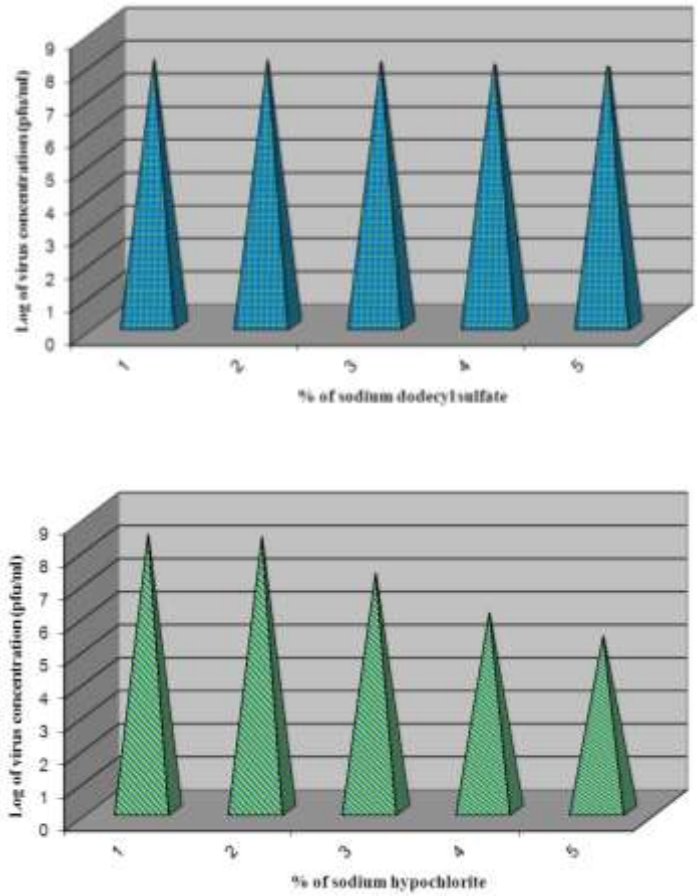

Figure (5): Effect of some detergents on Salmonella bacteriophage (St-1).

Restriction analysis of phage DNA:

The DNA of salmonella phage (St-1) was isolated and separately digested with each of PvuI, BamHI, XhoI and EcoRI. The digested DNA was analysed by electrophoresis on 1% agarose gel. The results in Figure (6) indicate that DNA of *Salmonella* bacteriophage was resistant to digestion by the used restriction enzymes (PvuI, BamHI, XhoI and EcoRI). In order to establish that this resistance to the used enzymes was not due to the presence of impurities in the DNA leading to inhibition of the enzymes. Several independent samples of DNA were isolated and subjected to further purification. None of these DNA

samples exhibited susceptibility to any of the used enzymes. The resistance of the phage DNA to digestion with the used restriction enzymes may be due to that the phage DNA carries additional methyl groups which block the degradative enzyme action **(Brown, 1987 and Kęsik-Szeloch *et al.*, 2013).** According to Fig. (6) the genome size of the phage was estimated to be 18 kbp.

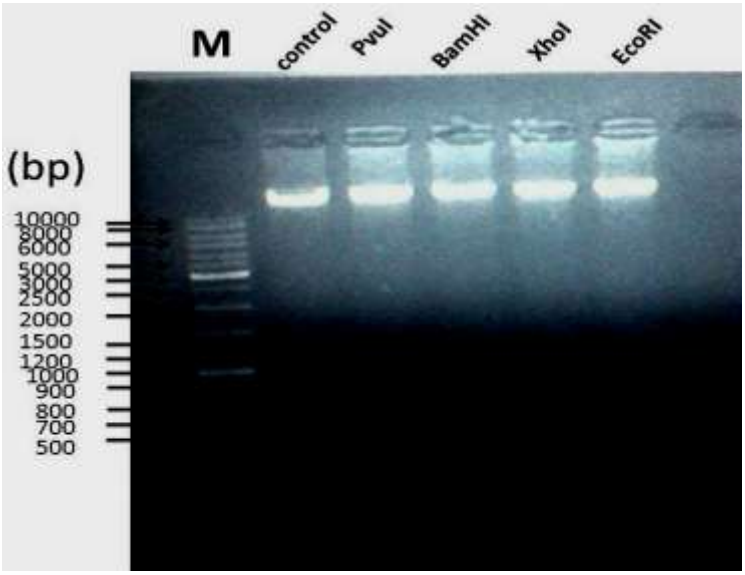

Figure (6): Restriction enzyme digests of *Salmonella* phage DNA.

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