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PHYSICO-CHEMICAL PROPERTIES OF SOME *LISTERIA* PHAGES [16]

Yasmer S. Hussein[1], Samar S. El-Masry[1], Abeer A. Faiesal[2], El-Dougdoug Kh.A.[1] and Othman B.A.[1]

1. Agric. Microbiology Dept., Fac. of Agric., Ain Shams Univ., P.O. Box 68, Hadayek Shobra 11241, Cairo, Egypt
2. Basic and Applied Agric. Sciences Dept., Higher Institute for Agric. Co-Operation, Cairo, Egypt.

*Corresponding author: samar_sayed@agr.asu.edu.eg

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ABSTRACT

Listeria monocytogenes is a food borne pathogenic bacteria and caused a dangerous infection of humans. Six lytic bacteriophages specific to *L. monocytogenes* were isolated from irrigation and sewage waters and named ØLG, ØLA, ØLM, ØLD, ØLN and ØLP. The phages were propagated, and then purified by the ultracentrifugation. Morphological properties of *Listeria* phages showed that the phages were tailed phages, varied in their sizes and assigned to be a member of *Siphoviridae* (ØLG, ØLA, ØLM) and *Myoviridae* (ØLN, ØLD, ØLP) families. All *Listeria* phages were highly stable under different temperature conditions and its thermal inactivation point was reached to 80°C. The longevity *in vitro* of the phages was up to 60 days, as well as phages were active at pH values ranging from 4.0 to 12.0. *Listeria* phages did not lose their infectivity after exposure to UV for 90 min at 35 and 53 cm distances. Results of SDS-PAGE showed that phages had 5-6 protein fragments with molecular weights of 66, 45, 37, 35, 33 and 28 kDa distributed among the six phages.

Key words: *Listeria* phages, sewage water, physico-chemical properties, SDS-PAGE.

INTRODUCTION

Listeria monocytogenes is one of the most common bacteria associated with food-borne illness and it is an opportunistic pathogen that causes listeriosis due to its ability to colonize the human gastrointestinal system **(Klumpp and Loessner, 2013).** They also showed that listeriosis is known for pregnant women infection, which later infects the fetus and often results in stillbirth or miscarriage. Eating of contaminated food is the most possible cause of *L. monocytogenes* infection **(Hagens and Loessner, 2014)**. *Listeria* is a psychrotrophic microorganism, Therefore, killed at high temperatures such as 160^{o}F, but able to grow well at lower temperatures including refrigerator temperatures **(Schmid et al 2009).** Foods products like meat, dairy and soft cheeses are frequently contaminated with *L. monocytogenes*. Most countries applied law which required the absence of *L. monocytogenes* from 25 g of a food sample and apparently is a challenge for any detection system **(Yutaka et al 2004).**

Bacteriophages are considered a great weapon against pathogenic bacteria. There are more than 400 phages specific for genus of *Listeria* which have been isolated and characterized **(Lossner and Rees, 2005 and Schmuki et al 2012).** They also showed that most of the phages belongs to Siphoviridae family and only few to family Myoviridae.

This study aims to isolate and characterize *Listeria* phages depending on their physico-chemical properties.

MATERIAL AND METHODS

Listeria monocytogenes source

L. monocytogenes CRIFS LJH391 was obtained from Virology Lab., Agric. Microbiol. Dept., Fac. of Agric., Ain Shams Univ., Cairo, Egypt.

Sources of water samples

Eight samples of Nile and drange water were collected from different recourses such as canals

(El-Sharkawya, Meet Nama, Ismailia, and El-Mariotya), sewage treatment stations (El-Gabal El-asfar sewage, Passous, and Shoubra El-Khaima) and Water drainage system of Agricultural Faculty, Ain Shams University. Samples were directly transferred to the Virology Lab., Agric. Microbiol. Dept., Fac. of Agric., Ain Shams Univ., Cairo, Egypt. The samples were maintained at 4°C until used.

Isolation of *Listeria* virulent phages:

Sterile Erlenmeyer flask 250 ml containing 50 mL of nutrient broth medium (2x) (**Madigan and Martinko (2005)** was inoculated with mixture of 5.0 mL of the tested water or sewage water samples and a volume of inoculated by 5.0 mL of overnight liquid culture of *Listeria* were added to each flask. The inoculated flasks were incubated at 37ºC for 48 hrs under shaking conditions (250 rpm/min). The inoculated cultures were centrifuged at 6000 rpm for 15 min at 4°C. The supernatants were collected into a clean flask. Chloroform was added at rate of 1:10 (v/v) to the supernatants followed by vigorously shaking for 5.0 min and the crude phages lysates were transferred into a fresh tube.

The presence of bacteriophages specific to *Listeria* was detected in the crude phage lysates qualitatively by spot test technique according to the method of **Borrego et al (1987)**. Phages were assayed quantitatively in the samples which gave positive results by the plaque assay method according to **Othman, (1997).**

***Listeria* phage lysates propagation**

Listeria phage lysates were prepared by single plaque isolation (SIP) technique according to the method of **Adams (1959).** SPI was repeated three times until obtaining uniform plaque morphology of *Listeria* phage lysates. Propagation of the phage isolates on their liquid *Listeria* culture was carried out to obtain large amount of high titer *Listeria* phage stock as reported by **Goodridge et al (2001).**

***Listeria* phages purification and concentration:**

The purified propagated *Listeria* phage isolates were concentrated using the differential centrifugation method of **Figrski and Christensen (1974).** Firstly, *Listeria* phage lysates were centrifuged at low speed at 6000 rpm for 15 min at 4°C. Secondly, supernatants were centrifuged using Backman L 7-35 ultracentrifuge at 30000 rpm for 90 min at 4^{o}C. The pellets were re-suspended in 300 µL from CM buffer.

Characterization of purified *Listeria* phages:

To determine shape and size of *Listeria* phage particles were determined according to the method of **Adams (1959)**. Five µL of *Listeria* phage suspension was placed onto 200 mesh cupper coated carbon grid, then examined using a JOEL-JEM 1010 electron microscope (Electron Microscope Unit, Regional Center for Mycology and Biotechnology, Al-Azhar Univ., Cairo, Egypt)**.**

Stability of *Listeria* phages

The effect of temperature on the *Listeria* phages *in vitro* was determined by exposure the phage suspensions to different temperature degrees 30, 40, 50, 60, 70, 80, 90 and 98ºC for 10 min. according to the method of **Basdew and Laing (2014).** 15 µL from each treated *Listeria* phages were assayed qualitatively by the spot test technique **Othman (1997)**.

Two mL of *Listeria* phage suspensions were pipetted in each Eppendorf tube. The tubes were left for 60 days at room temperature (25-30ºC) and refrigerator (4ºC). One tube was taken every 7 days and the phage suspension was assayed qualitatively for its infectivity by the spot test technique (**Othman, (1997)**.

Effect of pH on *Listeria* phages:

The infectivity of *Listeria* phages were evaluated by exposure to adjusted pH values ranged from 4.0 to 12 using 0.1 N HCl or NaOH for 12 and 24 hrs at room temperature according to the method of **Taj *et al.,* (2014)**. After incubation *Listeria* phage lysates were neutralized and the activity was determined by the spot test method as mentioned before.

Sensitivity of *Listeria* phages to UV irradiation

Open Petri dishes each containing five mL of high titer *Listeria* phage suspensions (10^{8} pfu/ mL) were placed at different distances (35 and 53 cm) and times (15, 30, 45, 60, 75, and 90 min) from UV (254 nm) irradiation sources according to the protocol of **Jamalludeen et al (2007**). These, the suspensions of *Listeria* phages were assayed qualitatively by the spot test method. Ten µL of each irradiated suspensions of *Listeria* phages were spotted over double layer agar plates con-

taining the listerial indicator bacteria host, incubated at 37ºC for 16-24 h in upright positions. Then, plates were inspected for lysed spots.

SDS-PAGE analysis of *Listeria* phages:

SDS-PAGE analysis of *Listeria* phages were conducted as described by **Laemmli, (1970)**. *Listeria* phage suspensions were centrifuged at 16000 rpm for 90 min at 4°C and the pellets were collected and re-suspended in 1.0 mL resuspension buffer (1.0 mM NaCl, 5 mM EDTA). One volume of phage suspension was mixed with an equal volume of 2X treatment buffer containing BPB as a tracking dye and boiled in a water bath for 90 s then quickly transferred into ice water and kept until loading into the gel. Eequal amounts of proteins (25 µL) of each sample were loaded in each well, and high range molecular weight protein marker was also loaded into a separate well. Electrophoresis was done at about 50 V (15 mA) for running through stacking gel after that it was 120 v (30 mA) for running through separating gel in 1x Tris/glycine-SDS-running buffer. The gel was stained with Coomassie Brilliant Blue for overnight and destined with acetic acid glacial (**Sambrook et al 1989**).

RESULTS

Isolation of *Listeria* virulent phages from different water sources

Different samples of water and sewage water collected from different locations were used to isolate specific lytic *Listeria* phages. Spot test was successfully used to detect the presence of *Listeria* phages (Qualitatively) in the collected water samples (**Table 1**). Six out of the eight samples gave positive reactions confirmed the presence of *Listeria* phages. The positive samples were also assayed quantitatively using the plaque assay technique and the phages concentrations were noted as shown in (**Table 1**).

Table 1. Qualitative and quantitative assaying of *Listeria* lytic phages in water samples.

Water sources	Locations	Governorates	Phage codes	Qualitative assay (Spot test)	Quantitative assay (pfu/mL)
Canals	El-Sharkawya	El-Kalubia	-	-	0.00
	El-Esmailia	El-Kalubia	-	-	0.00
	Meet Nama	El-Kalubia	ØLN	+	3.0×10^4
	El-Mariotya	Giza	ØLM	+	3.5×10^4
Sewage treatment stations	El-Gabl El-Asfar	El-Kalubia	ØLG	+	4.0×10^4
	Passous	El-Kalubia	ØLP	+	5.0×10^4
	Shoubra Elkhema	El-Kalubia	ØLD	+	3.0×10^5
Water drainage system	Fac. of Agri., Ain Shams Univ.	El-Kalubia	ØLA	+	2.0×10^5

- = no lysis (-ve result). + = lysis (+ve result). Ø: Phage. L.: Listeria. N: Meet Nama. M: El-Mariotya. G: El-Gabl El-Asfar. P: Passous. D: Shoubra El-Khaima.. A: Ain Shams University.

Propagation, purification and plaques morphology of *Listeria* phages

Single plaque isolation was done to obtain pure *Listeria* phage isolate. Only one plaque was picked up from each bacteriophage positive sample and concentrations of *Listeria* phages were determined. *Listeria* phage particles were purified and concentrated using the ultracentrifugation. High titer phage stock of the isolated *Listeria* phages was obtained by phage propagation several times by the liquid listerial culture method. Data in **Table (2)** showed that all the isolated phages have plaques with circular, clear shape with diameter ranged between 2 and 3 mm.

Table 2. Concentrations and plaques morphology of *Listeria* phages.

Phages	Plaque diameters (mm)	Presence of halo*	Plaque shapes	Concentrations (pfu/mL)
ØLA	< 2	+	clear, circular	6×10^6
ØLD	2	+	clear, circular	8×10^6
ØLG	< 2	+	clear, circular	6.5×10^6
ØLM	3	+	clear, circular	5×10^6
ØLN	2	+	clear, circular	6.3×10^7 6.5×10^7
ØLP	2	+	clear, circular	7×10^6

*+ = plaque was surrounded with halo.

Morphology of *Listeria* phages

Electron microscopy of the isolated *Listeria* phages are shown in **Table (3)** and illustrated by **Fig**. **(1)**. Results showed that phage ØLG had isometric head with diameter of 69.2 nm and long noncontractile tail with length of 269.2 nm and width of 15.4 nm, phage ØLA had isometric head with diameter of 80 nm and long noncontractile tail with length of 260 nm and width of 20 nm. Phage ØLM had isometric head with diameter of 61.5 nm and long noncontractile tail with length of 207.7 nm and width of 7.7 nm. Phage ØLN had isometric head with diameter of 100 nm and long contractile tail with length of 156 nm and width of 22 nm. Phage ØLD had an elongated head with diameter of 84.6×92.3 nm and long contractile tail with length of 169.2 nm and width of 23.1 nm. Phage ØLP had isometric head with diameter of 100 nm and long contractile tail with length of 153.8 nm and width of 23.10 nm. Based on the experimental results phages ØLG, ØLA and ØLM were belonging to family Siphoviridae. Phages ØLN, ØLD and ØLP were belonging to family Myoviridae as indicated by tail characters of the phages.

Table 3. Sizes and morphology of *Listeria* phages particles.

Listeria phages	Heads		Tails	
	Sizes (nm)	Morphology	Sizes (nm)	Shapes
ØLA	80×80	Isometric	260×20	Long non-contractile
ØLD	84.6×92.3	Elongated	169.2×23.1	Long contractile
ØLG	69.2×69.2	Isometric	269.2×15.4	Long non-contractile
ØLM	61.5×61.5	Isometric	207.7×7.7	Long non-contractile
ØLN	100×100	Isometric	156×22	Long contractile
ØLP	100×100	Isometric	153.8×23.1	Long contractile

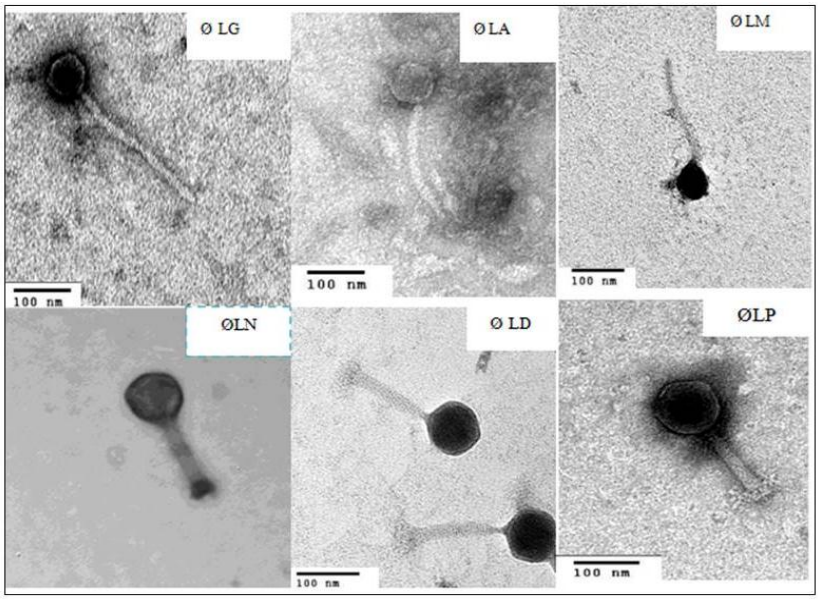

Fig. 1. Electron micrographs of *Listeria* phages (ØLG, ØLA, ØLM, ØLN, ØLD and ØLP) negatively stained with 2% uranyl acetate.

Listeria phages stability

The effect of temperature on *Listeria* phages stability was studied as mentioned in the materials and methods. Results showed that the *Listeria* phages ØLG, ØLN, ØLA, ØLD, ØLM and ØLP were able to lysis the bacterial host up to 80ºC. All *Listeria* phages were inactivated when exposed to thermal treatment at 90 ºC for 10 min. Results of LIV of *Listeria* phages showed that phages were survived and had high stability until 60 days at different temperatures (25-30°C) as well as refrigerator (4°C).

Effect of pH degrees on *Listeria* phages

Results revealed that, all *Listeria* phages didn`t lose their abilities to lyse *L. monocytogenes* cells at pH ranged from 4.0 to 12. Results indicated that the *L. monocytogenes* phages were stable in both of the alkaline and acidic conditions.

Sensitivity of *Listeria* phages to UV radiation

Results showed that *Listeria* phages didn`t lose their infectivity after exposure to the UV irradiation at distances of 35 and 53 cm from irradiation source for 90 min.

SDS-PAGE analysis of purified *Listeria* phages

Results in **Table (4)** and illustrated in **Fig. (2)** showed the presence of variation among the protein patterns of *Listeria* phages determined *via* SDS-PAGE. Moreover, the protein bands were also varied in their densities. Results also showed a number of total protein bands of 10. These bands were representing among the 6 *Listeria* phages with 5 or 6 bands out of the 10 protein bands. This variation was presented in either number of protein bands or molecular weights, which ranged from 28 to 87 kDa. The 10 total bands contained 5 bands representing structural proteins in ØLG with molecular weights of ~87, 70, 45, 37 and 35 kDa. ØLN phage had five structural proteins with molecular weights of ~ 68, 45, 35, 33 and 30 kDa. ØLA phage had 6 bands (structural proteins) with molecular weights of ~ 66, 37, 35, 33, 30 and 28 kDa. ØLD phage had 6 structural proteins with molecular weights of ~ 45, 37, 35, 33, 30 and 28 kDa. ØLM phage had 5 bands (structural proteins) with molecular weight ~ 66, 45, 37, 33 and 28 kDa. ØLP phage had 6 structural proteins with molecular weights of ~ 66, 45, 35, 33, 30 and 28 kDa. Different protein polymorphisms were recorded for 7 out of 10 with percentage 70 %. ØLG and ØLN phages appeared unique protein bands**.**

Table (5): Protein patterns of *Listeria* phages determined by SDS-PAGE analysis.

M_W (kDa) of protein bands	Phages						Types of protein bands*
	ØLG	ØLN	ØLA	ØLD	ØLM	ØLP	
87	+	-	-	-	-	-	U
70	+	-	-	-	-	-	U
68	-	+	-	-	-	-	U
66	-	-	+	-	+	+	P
45	+	+	-	+	+	+	P
37	+	-	+	+	+	-	P
35	+	+	+	+	-	+	P
33	-	+	+	+	+	+	P
30	-	+	+	+	-	+	P
28	-	-	+	+	+	+	P
Total	5	5	6	6	5	6	10

(+): Present. (-): Absent. M= Monomorphic. P= Polymorphic. U= Unique.

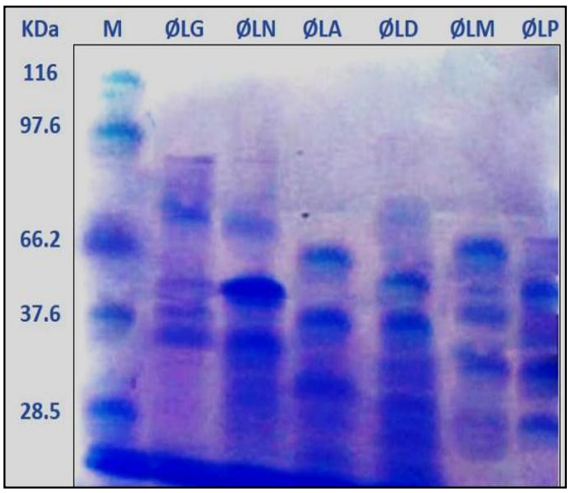

Fig. 2. SDS-PAGE analysis shows the protein patterns of *Listeria* phages (ØLG, ØLN, ØLA, ØLD, ØLM and ØLP).

DISCUSSION

Many water-borne pathogens, e.g. *L. monocytogenes* are zoonotic, they are responsible for infecting both humans and animals **(Bridle, 2013).** *L. monocytogenes* is an opportunistic human path-

ogen, broadly distributed in the environment and transmitted to humans and animals through contaminated foods. It causes listeriosis, it is a severe disease which may possibly result in septicemia, meningitis, encephalitis, or loss of the fetus during pregnancy **(Guenther et al 2009).**

Bacteriophages are viruses that are responsible of infecting bacteria **(Lossner and Rees, 2005)**.

In this investigation, six virulent phages specific to *L. monocytogenes* were isolated from the polluted irrigation and sewage water. Similar study was conducted by **Akhtar et al (2017)** who isolated *L. monocytogenes* phages from raw sewage samples, from water treatment plants. Also, both of **Kim et al (2008) and Arachchi (2013)** isolated *Listeria* phages from sewage and silage.

Listeria phage titers usually determine by the double layer agar technique and obtaining of the phage isolates by picking up single plaques from the plates resulting from the high dilutions of the phage as described by **Arachchi (2013)**. In this study using the same techniques, *Listeria* phages forming clear circular plaques of ≤ 2 to 3 mm in diameter with halo were isolated. Data agree with the results that found by **Arachchi (2013)** who isolated three phages forming plaques with narrow haloes from seafood processing plants.

Electron microscopy examination of negatively stained *Listeria* phage preparations of this investigation showed that ØLG, ØLA and ØLM belonging to family Siphoviridae while the other phages were belonging to family Myoviridae, this was based on the morphology of phage particles. Data of **Klumpp and Loessner (2013)** supported the experimental results. They stated that all *Listeria*-specific phages are members of the *Caudovirales*, featuring the long and noncontractile tails of the Siphoviridae family, or the complex contractile tail of the Myoviridae family.

Different external physical and chemical factors such as: temperature, acidity and salinity change the viability and storage of bacteriophages, which may inactivate the phage through damage of its structural elements (head, tail and envelope), lipid loss, and/or DNA structural changes were reported by **Ackermann et al (2004).** Therefore, some physical characters of *Listeria* phages were studied to determine the effects of some factors on phages.

All isolated *Listeria* phages lost their infectivity after exposure to 90ºC for 10 min. The previous results didn`t agree with that of **Arachchi (2013)** who stated that the three isolated phages in his study were more heat-labile since only LiMN4L survived up to ≈10% at 60°C for 10 min. and the other two phages reduced to non-detectable levels within 10 min. The difference between the tolerances of the phages generally was associated with the environment host from which they are derived.

Also, the previous studies of **Jończyk et al (2011)** investigating phage stability at various temperatures and have suggested that the ability of phages to remain stable in unsuitable temperatures which is varied within and among phage families. In this study, the results of stability of *Listeria* phages at room temperature and refrigerator reveled that *Listeria* phages were high stable and survived for at least 60 days at room temperature (25-30°C) and refrigerator (4°C) and this agree with that of **Arachchi (2013)** who investigated stability of *Listeria* phages during refrigerated storage and found that infectivity of his three phages remained stable for one year.

All *Listeria* phages didn`t lose its ability to lyse *L. monocytogenes* cells at pH ranged from 4.0 to 12.0 .The results indicated that the *Listeria* phages are stable in both of the alkaline and acidic media. Results of this study was differed from that of **Arachchi (2013)** who mentioned that two *Listeria* phages were survived at pH 4.0-10.0 while one phage was survived at pH 4.0-9.0 for 1 h, and this means that the phages isolated in this study were more resistant to pH values than the others and that may be return to the fact that tolerance of bacteriophages was differing between them.

The high stability of the *Listeria* phages across a range of pH, suggested that the *Listeria* phages have the potential to be successfully applied in foods and preparation surfaces which are exposed to acidic and neutral environments, as the *Listeria* phages remain stable and lytic under these conditions **(Nonis, 2016).**

In this work *Listeria* phages were exposed to UV irradiation at two different distances far from the source of UV. *Listeria* phages didn`t lose their infectivity after exposure to UV irradiation. The results agreed with that of **Jończyk et al (2011)** who stated that tailed phages (as *Listeria* phages) were the most stable in contrary conditions. In addition to the phages with a large capsid as *Listeria* phages (100 nm in diameter) survived better than phages with a head 60 nm in diameter.

The results of SDS-PAGE analysis of *Listeria* phages showed that phages had 5-6 protein fragments with molecular weights of 66, 45, 37, 35, 33 and 28 kDa distributed among the six phages.

Data of the present work didn`t agree with **Loessner et al (1994)** who generated individual protein profiles by SDS-PAGE of viral polypeptides. The major structural proteins of *Listeria* phages ranged in size from approximately 15 to 38 kDa. Protein compositions of *Listeria* phages permitted the differentiation of individual phages as well as the recognition and grouping of similar viruses.

The possibility of application of the virulent phages for controlling the *L. monocytogenes* in other experiments was recommended by this study.

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الخصائص الفيزيائية والكيميائية لبعض فاجات الليستريا

[16]

يسمر سيد حسين[1]– سمر سيد المصري[1]– عبير عبدالوهاب فيصل[2]– خالد عبدالفتاح الدجدج[1]– بدوي عبدالسلام عثمان[1]

1. قسم الميكروبيولوجيا الزراعية– كلية الزراعة– جامعة عين شمس– ص.ب 68– حدائق شبرا 11241– القاهرة– مصر
2. قسم العلوم الزراعية الأساسية والتطبيقية– معهد العالي التعاون الزراعي– القاهرة– مصر

*Corresponding author: samar_sayed@agr.asu.edu.eg

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الموجـــــز

تنتقل بكتيريا الـ *Listeria monocytogenes* وهي بكتيريا مسببة للأمراض عن طريق الغذاء وتسبب عدوى خطيرة للإنسان. تم عزل ستة أنواع من الفيروسات التي تصيب الليستريا مونوسيتوجينيس من مياه الري ومياه الصرف الصحي وتم تسميتهم ØLG، ØLA، ØLM، ØLD، ØLN و ØLP . وقد تم إكثار الفاجات وتركيزها عن طريق الطرد المركزى فائق السرعة. وأظهرت الخصائص المورفولوجية لفاجات الليستيريا بأنها من الفاجات ذات الذيل، تختلف فيما بينها في أحجامها وهى إما تكون تابعة لعائلة الـ Siphoviridae أو الـ Myoviridae. واحتفظت فاجات الليستريا بقدرتها على العدوى تحت ظروف درجات الحرارة المختلفة و كانت درجة الحرارة الفاقدة لنشاط الفاجات هى 80 درجة مئوية. كما ظلت الفاجات قادره على إحداث العدوى معمليا لأكثر من 60 يوما. وكذلك كانت نشطة في قيم الأس الهيدروجيني و اللتى تتراوح ما بين 4.0 إلى 12.0. لم تفقد فاجات الليستريا قدرتها على التحلل بعد التعرض للأشعة فوق البنفسجية لمدة 90 دقيقة على مسافات 35 و 53 سم. ثبت أن الفاجات تحتوي على 5 إلى 6 بروتينات هيكلية وتم تقدير أوزانها الجزيئية باستخدام SDS–PAGE. ووجد أن الفاجات تختلف فيما بينها فى بوليمورفيزم بروتيناتها.

الكلمات الدالة: فاجات الليستيري ، مياه الصرف الصحي، خصائص فيزيائية–كيميائية، SDS–PAGE.

تحكيم: ا.د عاطف شكري صادق
ا.د جمال الديدموني محمد