Zagazig Journal of Applied Microbiology and Biotechnology 423-434

Zagazig J. Agric. Res., Vol. 43 No. (2) 2016

http:/www.journals.zu.edu.eg/journalDisplay.aspx?Journalld=1&queryType=Master

ISOLATION AND PARTIAL CHARACTERIZATION OF TWO BACTERIOPHAGES VIRULENT TO *Enterobacter cloacae*

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ABSTRACT

Enterobacter cloacae is a potential bacterial pathogens in aquatic water . In this study, two lytic phages designated as Ec02 and Ec03 infectious to *Enterobacter cloacae* were isolated from agricultural irrigation water. Morphological analysis by transmission electron microscopy revealed that both phages belongs to *Myoviridae* family. Phage Eco2 had an isometric head 92 nm in diameter with a long contractile tail 125 nm in length, while phage Ec03 had a head of about 75 nm in diameter with a long contractile tail 92 nm in length. Both phages were shown to be virulent to different *Enterobacter* sp isolates except *Enterobacter aerogenes* (57), and had no effect on other tested bacteria. One- step growth curves revealed that the latent period, rise periods and burst size of phages Ec02 and Ec03 were (20, 20 min) , (30,40 min) and (146, 94 PFU/ infected cell), respectively . Both phages were thermal stable and were tolerant to a wide range of pH. These results suggest that both phages have a high potential for phage application to control *Enterobacter cloacae*.

Key words: *Enterobacter cloacae*; lytic bacteriophage, *Myoviridae*; TEM.

INTRODUCTION

Enterobacter spp., the third leading cause of respiratory tract, nosocomial and hospital-acquired blood stream infections, are Gram-negative infectious bacterial human pathogens (CDC, 1999; Piagnerelli *et al*., 2000). The two most common *Enterobacter* spp., causing human infections are *E. aerogenes* and *E. cloacae*, responsible for 90–99% of *Enterobacter* infections. These two species have been included together in almost all clinical studies of *Enterobacter* infections (Marcos *et al*., 2008). These pathogenic bacteria exhibit high resistance to commonly used antibiotics, including vancomycin, and have become a serious global clinical problem and are usually predominant in hospitals causing complicated secondary infections (Bornet *et al*., 2000 ; Anggakusuma *et al.,* 2009). However, management of these infections is often complicated by multiresistance to antibiotics associated with *E. cloacae* (Fernández *et al.,* 2011).

Bacteriophages are characterized by their ability to selectively infect a bacterial host belonging to a single strain of a genus or species. Since the preantibiotic era, the use of virulent phages to combat bacterial infections has been suggested for diseases such as cholera, diphtheria, bubonic plague and anthrax (Summers, 2001). Bacteriophage (phage) therapy is one of several potential therapeutic approaches against bacterial infections. Several papers have been published on the use of phages as specific antimicrobial agents (Abedon *et al*., 2011; Burrowes *et al.*, 2011; Chan *et al*., 2013). For therapeutic purposes, virulent or lytic bacteriophages are highly desirable due to their ability to kill target host cells.

Thus, in this study, bacterial isolates of *Enterobacter cloacae* were identified and used as target cells to screen lytic phages from aquatic samples. A two lytic phages (designated Ec02 and Ec03) specifically infecting *Enterobacter cloacae* isolates were isolated and characterized. Their basic biological features

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including host specificity, morphology, one-step growth, adsorption rate and effect of two physical factors on these phages were investigated.

MATERIALS AND METHODS

Bacterial Strains

The host bacteria were originally isolated from agricultural irrigation water and wastewater samples from a surface raw water sources from 6 different sites in Sharkia Governorate. These samples were collected during one year period between June 2013 and May 2014.

The water samples were serially diluted, spread on LB (Luria-Bertani medium) agar plates and incubated at 37°C for 24 hr. Representative colonies were picked and transferred onto LB plates for further purification. The bacterial isolates were identified and confirmed as *Enterobacter cloacae* in CMW: Microbial Resource Center, Faculty of Agriculture, Ain Shams University. Stock cultures were stored in LB broth containing 20% glycerol at -20°C. *E. cloacae* strain was selected as the host bacterium for further characterization of the isolated phages.

Isolation, Propagation, and Purification of *Enterobacter cloacae* Phages

Procedure of phage isolation was conducted as previous described by Wommack *et al*. (2009). Aliquots (20 ml) of water samples were centrifuged at a low speed (1,000 rpm) for 10 min to precipitate debris, and the supernatants were filtered through 0.45µm membrane filters (Gelman Sciences, Inc., Ann Arbor, Mich).The filtrate was added to a fresh bacterial culture in LB broth and incubated at 37°C for 18 hr. Chloroform (final volume, 0.5%) was added to the supernatant to kill any unlysed bacteria. The culture was centrifuged at 4,000 rpm for 10 min, and the supernatants were filtered through 0.45µm membrane filters. The filtrated supernatant was used to check the presence of lytic phages by the double- layer method (Yang *et al*., 2010) using LB agar as the culture medium. The plates were incubated at 37°C for 18 hr. A single discrete plaque was picked and put into a log phase culture of *Enterobacter cloacae*. After being incubation at 37°C for 18 hr., the isolated phages were purified by five successive single-plaque isolation with sterile pasture pipette until homogenous plaques were obtained. Phage stocks were stored at 4°C with 1% chloroform for further studies.

Determination of the Lysis Spectrum of the Isolated Bacteriophages

The host range of the isolated phages was determined according to Jamalludeen *et al*. (2007) with some modifications. *Enterobacter* phage stocks used in this test were obtained by propagating the phages on the respective isolates of *Enterobacter* originally used for phage isolation. Overnight cultures of 11 bacterial isolates (6 isolates of *Enterobacter* spp. and five isolates of *Aeromonas* spp.) were used in seeding double layered agar plates. Such plates were spotted with *Enterobacter* phage suspensions which contained 10^5 to 10^6 PFU/ml when tested against their original host. Plates were spotted with suspension of each phage isolate. After incubation for 18-24 hr., at 37°C, plates were examined for lysis at sites where the drops had been applied.

Transmission Electron Microscopy (TEM)

Morphology of purified phage particles was examined by transmission electron microscope of negatively stained preparations. Ten µl of phage particles (˜5x1010PFU/ml) was spotted onto a 400 mesh-size Formvar carbon-coated copper grid, stained with 1% potassium phosphotungstate (PTA at pH 7.0) and then examined by TEM Lab FARP (Faculty of Agriculture Research Park- Cairo University). Based on their morphology, phages were identified and classified according to the guidelines of the International Committee on Taxonomy of Viruses (Fauquet *et al.*, 2005).

Adsorption Rate and One-Step Growth

Procedures for adsorption rate were conducted by standard method of Karumidze *et al*. (2013). Briefly, filtered lysates ($1.0x10^7$ PFU/ml of the tested phages) was added to a log-phase culture of the host ($1.0x10^8$ CFU /ml), mixed with mid-exponential host cells at multiplicity of infection (MOI) of 0.01. The mixture was incubated at 37C°, at 3 min intervals for 15 min, samples were periodically drawn, diluted (100- fold) in ice-cold LB broth containing 10% chloroform and centrifuged at 4000 rpm for 5 min. The supernatant was filtered through Millipore membrane filter (0.45 µm). Unabsorbed phage particles were assayed. The adsorption rate constant was determined by the equation: *Log p / PO = - (1/2.3) KNT* (Hyman and Abedon 2009) where *P* and *Po* are ending and starting phage densities, respectively, *K* is the phage adsorption constant, *N* is the bacterial density and *t* is that time over which one desires to have phage adsorption to taken place.

One-step growth curves, were performed as described by Pajunen *et al.* (2000) and Sillankorva *et al*. (2008) with some modifications. The culture of mid-exponential phase was harvested by centrifugation (4000 rpm for 10 min) and the cells were resuspended in 5ml fresh LB medium (2x 10^8 ml). Five ml of phage suspension were added in order to have a MOI of 0. 01 and phages were allowed to adsorb for 10 min. at 37ºC. The mixture was then centrifuged as mentioned above. The pellet was resuspended in 10ml of LB medium. A sample was taken every 10 min over a period of 1 hr., diluted and plated on plates. The numbers of phages per ml versus minutes of incubation were plotted and the latent period, rise period, and burst size were counted after overnight incubation at 37C°.

pH and Thermal Stability Test

Resistance to different pH values at 37C° was carried out as previously described by Verma *et al*. (2009). Briefly, the pH of the LB was adjusted with either 1 M HCl or 0.5 M NaOH to obtain a pH ranging from 3 to 12. A total of 100 µl of bacteriophage suspension (4x10^8or 4x10^9 PFU/ml) for Ec02 or Ec03, respectively, was inoculated into 5 ml of pH-adjusted medium. After incubation for 1 hr., at 37°C, the surviving phage particles were counted immediately using the double-layer method. Thermal stability of phage at different temperatures (50, 60 and 70°C) was determined by incubating the phage suspensions at the indicated temperature for 60 min at pH 7 in LB plates medium in water bath, diluted and assayed for infectivity. The dilutions were then plated on LB plates and incubated at 37°C overnight. One hundred microliter of phage dilution was removed before an exposure to different temperatures and plated as a control. The surviving phages were then calculated.

Effect of Different Storage Temperatures on Phage Infectivity

The stability of the two phages isolated in the previous experiment was determined at various storage temperatures. The filterates were incubated at ambient temperature (22± 2°C), refrigerator (4°C) and at freezer (-20 °C) for various periods. Samples were withdrawn at different period namely: 1, 3, 7, 14, 21, 30 days for the ambient temperature storage, and every two months for the 4ºC and at -20°C. The loss in phage infectivity was assayed using double layer technique (Adams, 1959).

RESULTS AND DISCUSSION

Isolation of *Enterobacter cloacae* Strains and their Lytic Bacteriophages

Enterobacter cloacae strains were isolated from six different aquatic samples. Two phage isolates lytic to *E. cloacae* were isolated from the same aquatic samples and designated as Ec02 and Ec03. Host specificity of each phage isolate was determined by spotting every phage suspension containing about 10^5 to 10^6 PFU/ml on double layer plates previously cultured with bacterial isolates as indicated in Table 1. The host range of the isolated bacteriophages Ec02 and Ec03 toward some bacterial isolates were determined by detecting their efficiency against two strains of *Enterobacter* spp. (*E. cloacae and E. aerogenes*) and 4 different bacterial isolates isolated from river water. Also, one strain of *Aeromonas salmonicida* sub sp. *pectinolytica* as well as *Aeromonas* spp. isolated from agricultural irrigation water and sewage water were tested for their sensitivity to Ec02 and Ec03. Results in Table 1 show that 5 *Aeromonas* spp. were found to be resistant to the two phages used in this investigation. It is apparent that among the 11 isolates used, all *Enterobacter* isolates used in this study were sensitive to the two phages examined except *Enterobacter aerogenes* which was resistant to both phages (Ec02 and Ec03). These different patterns of lysis could reflect heterogencity in *Enterobacter* spp. and *Aeromonas* spp. population and for genetic diversity amongst the phage isolates.

Table 1. Bacterial susceptibility to two *Enterobacter* phages isolated from some aquatic sources

Bacterial host	Water sources	Phage	
		Ec 02	Ec 03
***Aeromonas*. *salmonicida ss pectinolytica* (6)**	Sw	R	R
***Aeromonas* spp. (38)**	Sewage	R	R
***Aeromonas* spp. (43)**	Sewage	R	R
***Aeromonas* spp. (44)**	Sewage	R	R
***Aeromonas* spp. (56)**	Sewage	R	R
***Enterobacter* spp. (28)**	Sw	Sc	St
***Enterobacter* spp. (29)**	Sw	St	St
***Enterobacter* spp. (46)**	Sw	Sc	Sc
***Enterobacter* spp. (51)**	Sw	St	St
***Enterobacter aerogenes*(57)**	Sw	R	R
***Enterobacter cloacae* (58)**	Sw	Sc	Sc

R, resistant; S, susceptible; c, clear plaque; t, turbid plaque, Sw, Surface water.

These results are in agreement with those obtained by Verthe *et al*. (2004), who found that Phage UZ1 was infectious only to *E. aerogenes* BE1 LMG 22092. No plaque formation was observed on the other strains tested : *E. aerogenes* BE2 LMG 22093, *E. aerogenes* ATCC 13048, *E. cloacae* LM W011 LMG 22094, *E. coli* ESBL 111 LMG 22095, and *E.coli* ESBL 112 LMG 22096. On the other hand, El Didamony *et al*. (2015) found that the host range of the isolated bacteriophages against 13 *Pseudomonas aeruginosa* strains isolated from patients showed that both phages (ϕ PSZ1 and ϕPSZ2) could infect all tested *P. aeruginosa* strains used as host in this study indicating a quite broad host range of these phages.

Morphology of Lytic Bacteriophages

The purified particles of the two phage isolates (Ec02 and Ec03) were negatively stained with 1% potassium phosphotungstate (PTA at pH 7.0), and then examined by TEM. Both phages (Ec02 and Ec03) were found to be belongs to the *Myoviridae* family whose members typically exhibited an icosahedral head and a contractile tail with spikes, these phages showed a hexagonal isometric heads accompanied by long tubular tails. The results presented show also that the particle of phage Ec02 was composed of an isometric head 92 nm in diameter with a tail of 125 nm long and 16 nm wide with a base plate (Distal tail Knob) 33 nm wide to which no spike was attached (Fig. 1). While phage Ec03 has an isometric head with 75 nm in diameter and a long contractile tail of a diameter 27nm and length 92 nm with a distal tail konb (base plate like structure) 16 x 27 nm with spikes 20 nm length (Fig. 2). On the other hand, the capsomers were not visible on negatively stained particles. The tails were consisted of a contractile sheath and a sided base plate provided with a few tail pins (Fig. 2) but no spikes were detected with phage Ec 02. While the core of the tail of this phage (Ec02) was shown very clearly (Fig. 1). Also, the head of the two phages under this investigation were appeared to be separated from the sheath by a neck. The lengths of these necks were 8 and 7nm of phages Ec02 and Ec03, respectively. Phages of the *Myoviridae* family have been frequently isolated from fresh mammalian feces and are associated with the lytic effect in *E. coli* and *Salmonella* spp. (Buckling and Rainey, 2002; Abedon *et al*., 2003; Carey-Smith *et al*. 2006). Also, Kim *et al*. (2010) isolated SP18 (phage specific to *Shigella sonnei*). SP18 phage

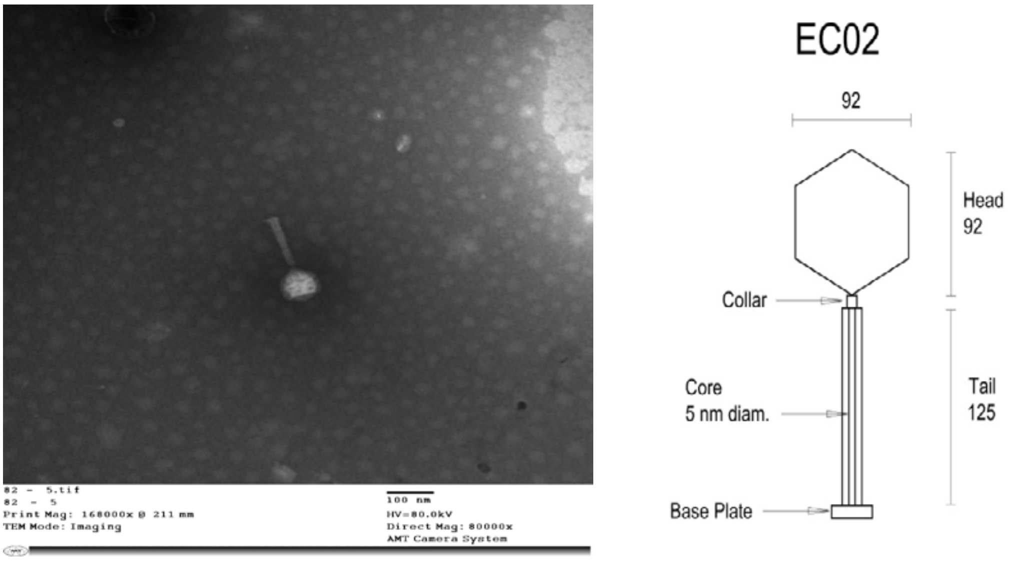


Fig. 1. Schematic diagram and electron micrograph of negatively stained phage Ec02. The bar represents 100 nm.

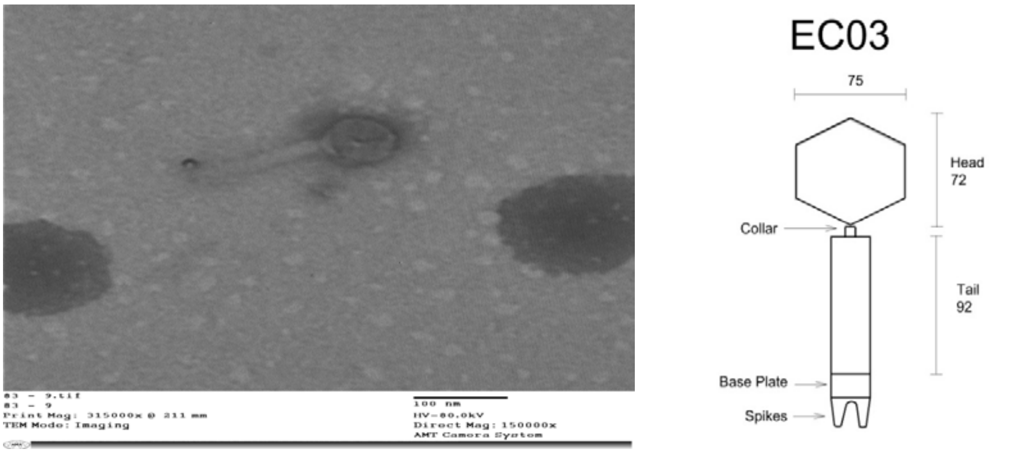


Fig. 2. Schematic diagram and electron micrograph of negatively stained phage Ec03. The bar represents 100nm

was examined by transmission electron microscopy. The hexagonal head diameter of SP18 was 81×110 nm and the dimensions of the contractile tail (short and long tails in TEM figures) with fibers were 50- 110 nm in length and 23-25 nm in width. Based on its morphology, phage SP18 likely belongs to the family *Myoviridae*, whose members typically exhibit an icosahedral head and a contractile tail with fibers .Whereas Vinod *et al*. (2006) examined the phage infectious to *Vibrio harveyi*. The examined phage has a head measuring about 40–45 nm diameter with hexagonal outline, and a non-contractile tail of diameter 7 nm and length 60 nm and was therefore identified as Siphovirus on the basis of morphology.

Adsorption Rate and One-Step Growth Curve

The adsorption rates in ml/min were $2.0x10^{-9}$ and 3.1 $x10^{-9}$ ml/min for phages of Ec02 and Eco3, respectively (Table 2). These data show that the adsorption rate constant (k) of phage Ec02 was lower than that of phage Ec03. Concerning the time required to achieve 50%

Table 2. General characteristics of two phages specific against *Enterobacter cloacae*

Characteristics	Phage Ec02	Phage Ec03
Plaque appearance	Clear	Clear
Family	*Myoviridae*	*Myoviridae*
Head diameter (nm)	92	75
Tail length (nm)	125	92
Tail width (nm)	16	27
Adsorption rate (ml/min)	2.0×10^{-9}	3.1×10^{-9}
Latent period (min)	20	20
Rise period (min)	30	40
Burst size (PFU/cell)	146	94

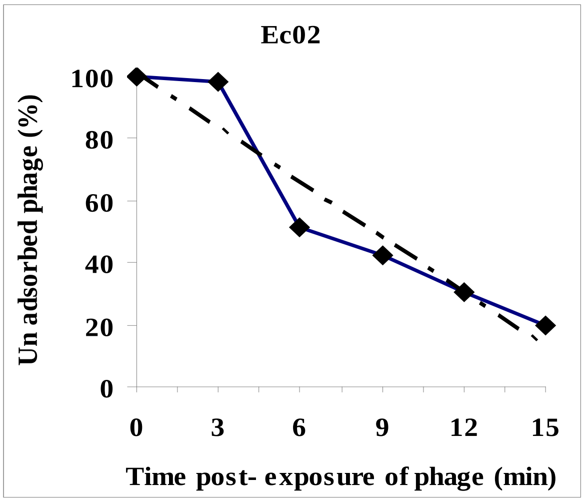
adsorption, phage Ec02 was faster than the other one and higher than 50% of the adsorbed particles was shown after 9 min. On the other hand, phage Ec03 exhibited an adsorption rate slower than that of Ec02 hence its adsorption percentage was less than 50% giving 45.5% after 9 min. (Fig. 3). These values were similar to that obtained by Kasman *et al* (2002) who pointed out that coli phage T4 which recognizes several hundred receptor sites per cell displays a K of 2.4×10^{-9} ml/min. Also, these results are comparable very well with those found by Sillankorva *et al*. (2008) who reported that when phage φ IBB- PF7 infected the host bacterial cell of *Pseudomonas fluorescens* appeared to have two adsorption phases, a very rapid adsorption into host during the first 5 min is followed by a slower rate of attachment after 5 min.

A one-step growth experiment revealed that the latent period and burst size of phage Ec02 and Ec03.were 20 min for both phages (Ec02 and Ec03), and the burst size for phage Ec02 was 146, while the burst size of phage Ec03 was 94 PFU/ infected cell (Table 2 and Fig. 4). Short latent period, as described by Abedon *et al*. (2001), can be associated with short generation times, as observed in this study, which fluctuated from 35 to 40 min. This might be a specific property of the phage- host system. In addition, the length of the latent period, depends on the specific phage growth rate, the physiological conditions, host, incubation conditions, medium, and temperature (Abedon *et al*., 2003).

pH and Thermal Stability

pH values

The stability of phages under different thermal and pH conditions was investigated based on the survival rate examination of the two phages under this investigation after treatment. One of the two phages (Ec02) under this investigation was stable between pH 4 and 10, and the survival rates of this phage remained with 44% at pH 4 and 34% at pH 10 from the original titre at pH 7 which was 4×10^{8} PFU/ ml. Concerning the stability of phage Ec03 at different values of pH, phage lost 55% of its survivals at pH 6. Also the inhibition of acidic side on this phage was detected since no phage particles were found at pH < 5 (Fig 5). By contrast phage Ec02 was survived up to pH 10 with a reduction in the phage titre reached 5 log decrease from the original titre at pH 7 (4×10^{8} PFU/ml) corresponding to 6-log decrease with phage Ec03 from the titre at pH 7 (4×10^{9} PFU/ml). Previous studies, in this respect, showed that pH stability of phages varied depending on strains (Karumidze *et al*., 2013 ; Yu *et al*., 2013). The survival of phages in a broad pH range is rarely reported for the *Enterobacter* phages except what it was reported by Verthe *et al*. (2004) and recently by Mishra *et al*. (2012) who studied the stability of phages UZ1 and F20 in different pH values.



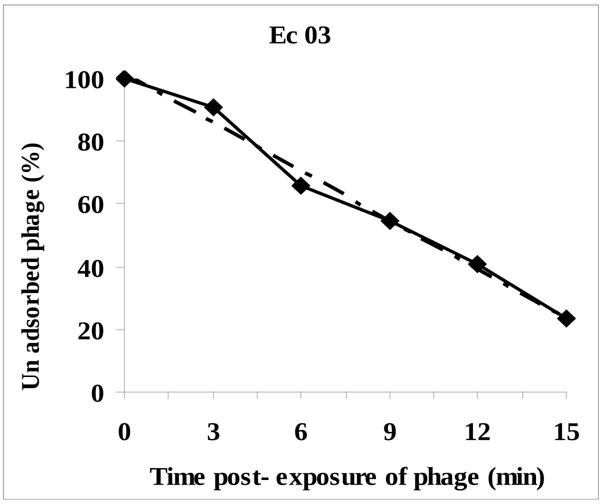
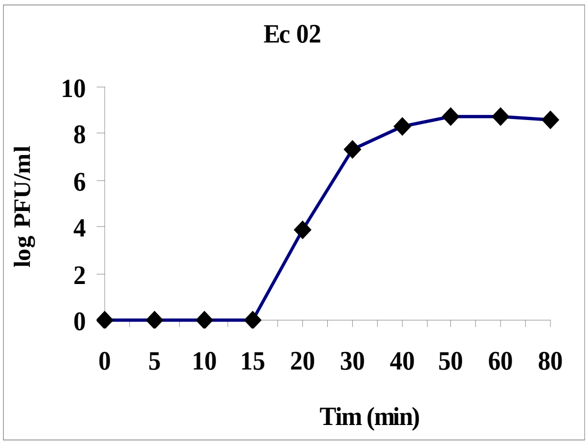


Fig. 3. Adsorption of phages Ec02 and Eco3 to *Enterobacter cloacae*



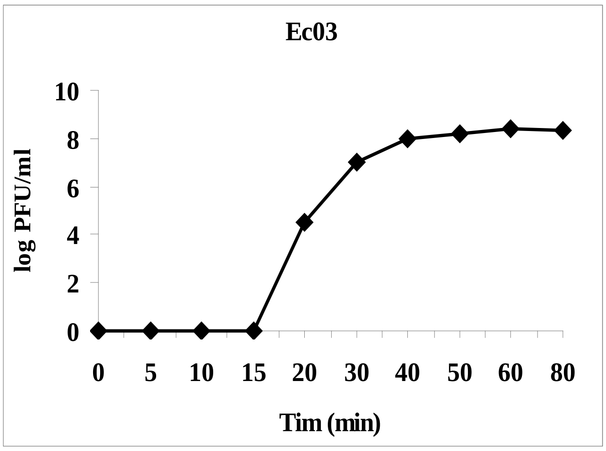


Fig. 4. One-step growth curves of phages Ec02 and Eco3 on *Enterobacter cloacae*

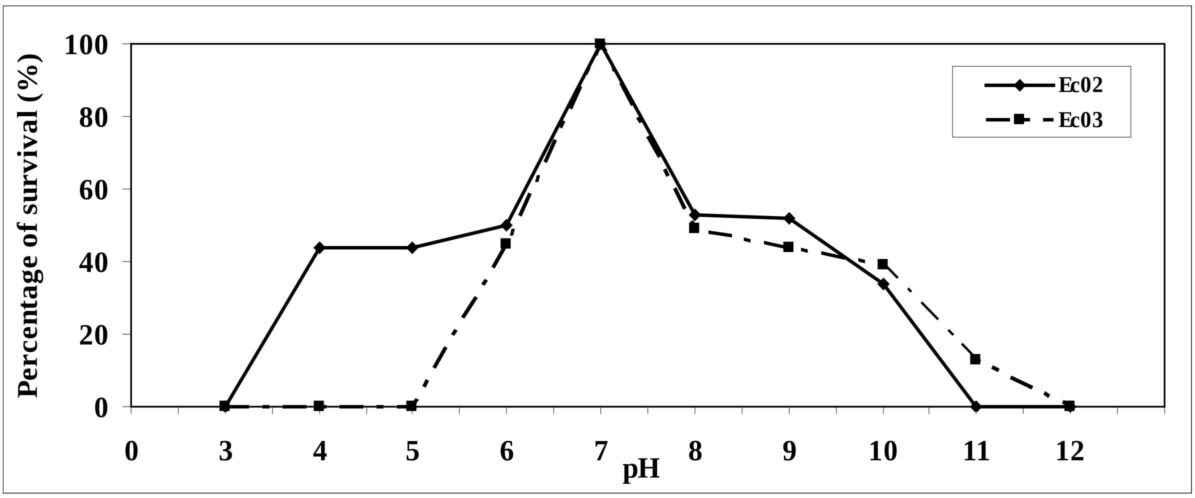


Fig. 5. Effect of pH values on *Enterobacter* phages (Ec02 and Ec03) survival

Thermal stability

The two phages under this investigation were different in thermal stability (Fig. 6). The survival rates of Ec02 and Ec03 phages were 66% and 52% after incubation at 50°C for 1 hr., respectively. However, the survival rate of Ec02 was less than that of Ec03 after incubating phages at 60°C for 1 hr., giving 30% and 47% from the original survival, respectively. These results are similar to those obtained by Han *et al*. (2014) who stated that nearly 100% of phage φPA-HF17 remained alive after 30 min and 60 min at 50°C. However, the number of viable phages decreased from 10^7 PFU/ml to both 10^6 PFU/ml after 30 min and 4.5 $x10^6$ PFU/ml after 60 min at 60°C, respectively. Both phages were unstable at 70°C since more than 85% of phage lost their infection capability in 20 min and 50 min at 70°C for Ec02 and Ec03, respectively. The same trend was found by Yu *et al*. (2013) who found that five phages belonging to family *Siphoviridae* were unstable at 70°C and the phages lost their infectivity to *Vibrio* strains closely related to *Vibrio owensii.*

As talking on storage period, when phage (Ec02) was stored at 4°C an initial loss of its activity was observed after 6 months. Whereas the other phage (Ec03) lost its activity after 4 months (Fig. 7a). On the other hand, when stored at -20°C phage Ec02 lost its infectivity after four months. While phage Ec02 lost its infectivity after two months. (Fig. 7b). On the contrary, phage Ec03 was more sensitive than Ec02 since it lost its activity between 2-4 months. Data in (Fig.8) show that *Enterobacter* phages were more stable at refrigerator and freezer conditions than at ambient temperature. The titre of both phages (Ec02 and Ec03) was lost after 21 days. The same trend was observed by Mishra *et al.* (2012) who found that no significant loss in phage titre (phage F20 of *E. aerogenes*) was observed after 6 months at temperatures below 4°C. In addition, Olson *et al*. (2004) recommended a 4 °C as an optimum temperature for short (no longer than 40 days) phage storage.

Finally, *Enterobacter* phages were more stable at refrigerator and freezer conditions than at ambient temperature, in which the titre of both phages (Ec02 and Ec03) was lost after 21 days. All of these characteristics have implications for the use of these phages as a stable antimicrobial agent for the treatment of *Enterobacter cloacae* infections.

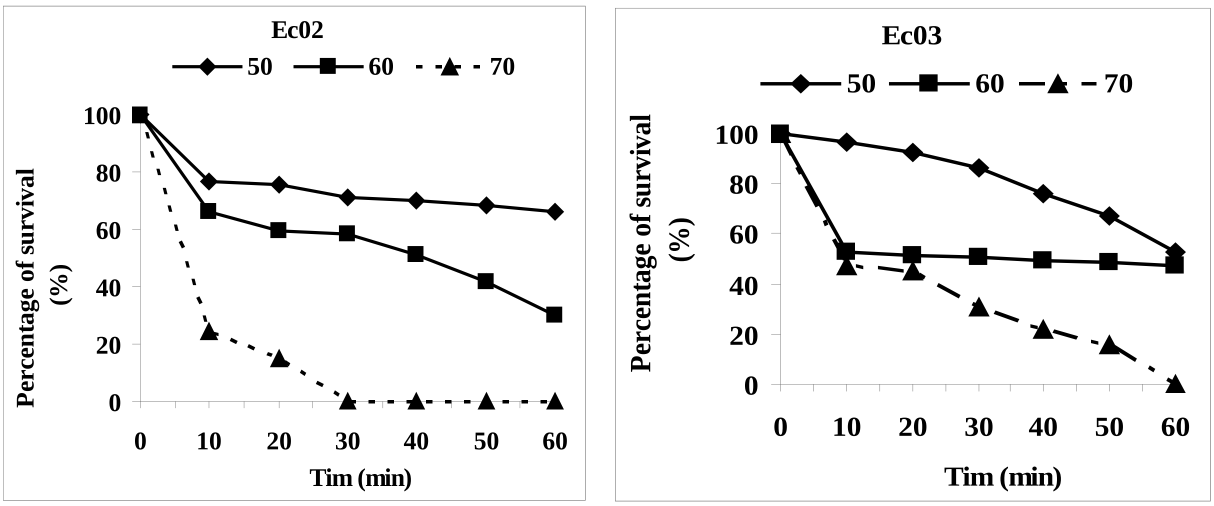


Fig. 6. Thermal inactivation of *Enterobacter* phages Ec02 and Ec 03

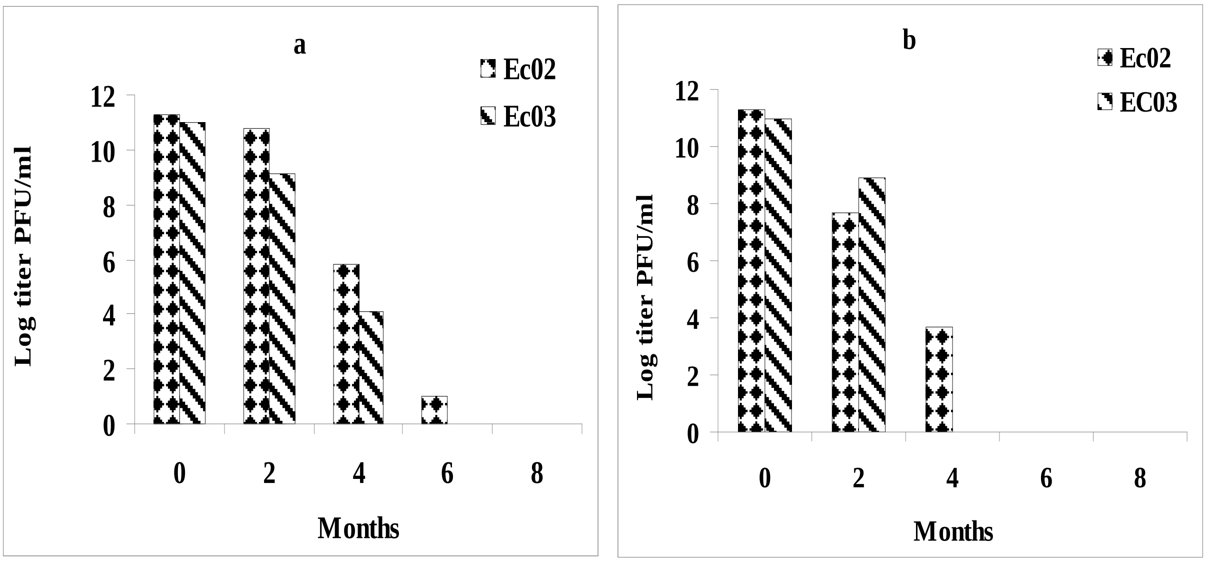


Fig. 7. The effect of storage period on the maintenance of phage suspension at a-refrigerator (4°C) and b- freezer conditions (-20°C)

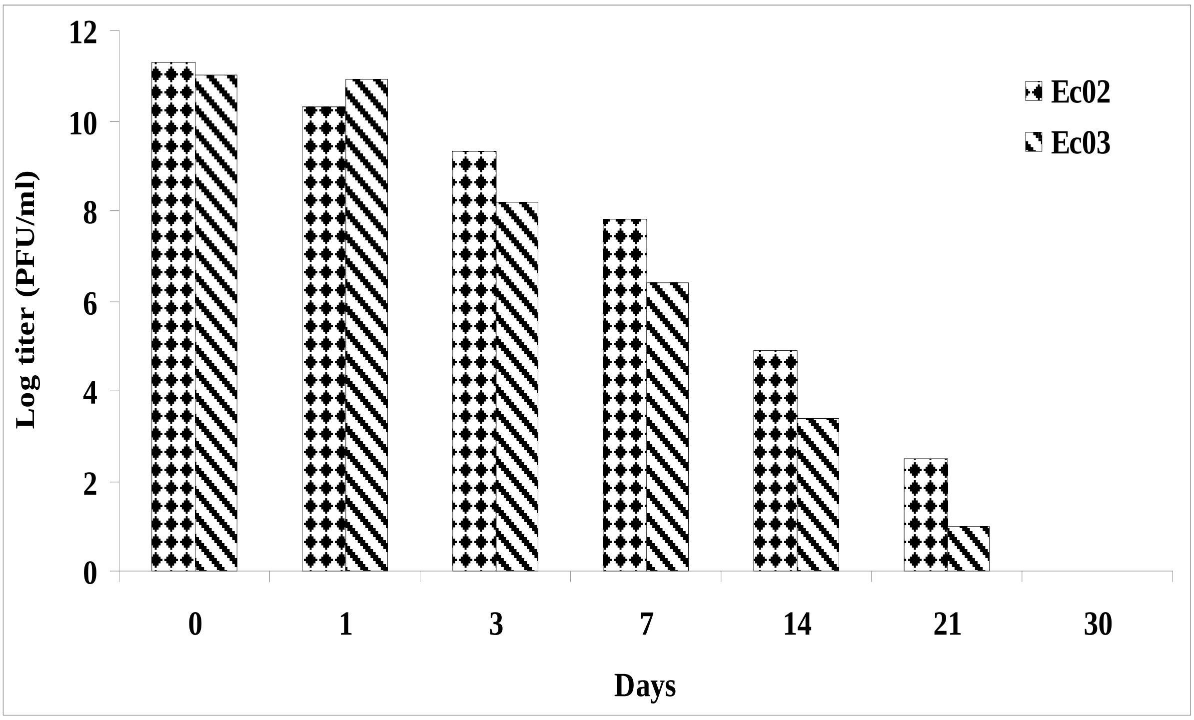


Fig. 8. The effect of storage at ambient temperature (22±2°C) on the infectivity of *Enterobacter* phages Ec02 and Ec03

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عـــزل وتـوصيـف جـــزئـــي لاثنيـــن مـــن البكتيـــريـــوفـــاجـــات الضـــارية لبكتريا الانتيروباكتر كلوأسي

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تعتبر الانتيروباكتر كلوأسى من المسببات البكتيرية المرضية القوية في الوسط المائي، في هذه الدراسة تم عزل أثنين من الفاجات المحللة للانتيروبكتر كلواسي والتي تم تسميتها بـ Ec02، Ec03 والمعزولة من مياه سطحية تستخدم في الري، وباستخدام الميكروسكوب الإلكتروني لمعـــرفة الشكل المورفولوجـــي لكلا الفاجين ظهـــر أنهمـــا يتبعان عائلـــة الـ *Myoviridae* ، حيث كانت أبعاد الرأس للفاج الأول Ec02 بقطر ٩٢ نانوميتر وذيل طوله ١٢٥ نانومتير بينما كان الفيروس الثاني Ec03 بقطر ٧٥ نانوميتر للرأس وذيل متقبض بطول ٩٢ نانوميتر، وأظهر الفاجين قدرة إصابة تحليلية لعدد مختلف من عزلات الانتيروباكتر ما عدا الانيتروباكتر ايروجينز وفي نفس الوقت ليس لها تأثير على غيرها من البكتيريا المختبرة. كما أظهرت منحنيات النمو ذو الخطوة الواحدة ان فترة الحضانه كانت ٢٠ دقيقه لكلا الفاجين ومرحلة الصعود ٣٠ دقيقه لفاج Ec02، ٤٠ دقيقه لفاج Ec03 أما حجم الانفجار فكان لفاج Ec02 ١٤٦، وكان لفاج Ec03 ٩٤ جزئ فيرسي لكل خلية مصابة، وكان كلا الفاجين ثابتين حرارياً ومقاومين لمدى واسع من الـ pH، من هذه النتائج يمكن اقتراح أن كلا الفاجين يمكن استخدامهما بكفاءة في المجال التطبيقي لمقاومة الإصابة بالانتيروباكتر كلوأسي.

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