

Occurrence of *Bacillus thuringiensis* Bacteriophages in the Egyptian Arid Soil

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Abstract In this study, two phages specific for *Bacillus thuringiensis* were isolated from arid soils. The phages were designated as Bt1 and Bt2. They were propagated by the liquid enrichment method and purified by the polyethylene glycol and dextran sulfate followed by high speed centrifugation, the morphology of purified particles was determined using transmission electron microscopy. Electron micrograph indicated that Bt1 phage has isometric particle, its head diameter is about 140 x 165 nm and contractile tail type, about 117 nm length, while the particle of phage Bt2 has long contractile tail with 206 nm length and its head is isometric with diameter of about 140 x 124 nm, phages appeared to be a member of the *Myoviridae* family based on their structure and particle morphology. The isolated phages have narrow host range, which they able to infect *Bacillus thuringiensis*, *Bacillus subtilis* and *Bacillus lechiformis*, Bt1 and Bt2 viral genomes were digested by *EcoRI*. The genome size was estimated to be ~4.06 kbp for Bt1 phage and ~6.43kbp for Bt2 phage. To our knowledge, this is the first report on the isolation of a bacteriophage specific for *Bacillus thuringiensis* from arid soil in Egypt.

Keywords *Bacillus thuringiensis*, Phage morphology, Purification, Restriction enzyme

1. Introduction

Desert biomes cover 33% of global land masses, and yet the diversity and roles of viruses in these dominant ecosystems remain poorly understood. There is evidence that hot hyper arid desert soils are characterized by high levels of bacterial lysogens and low extracellular virus counts [1]. Research on phages ecosystem ecology in hyper arid desert soils has demonstrated that desert soil viruses are numerous and diverse and carry novel genes whose functions are yet to be determined. Bacteriophages are the most abundant biological entities on the planet; their ability to infect and kill their bacterial hosts makes them key factors in both the evolution of bacteria and the maintenance of ecological balance [2-4]. Research on the virus ecology of soil environments has progressed more slowly and has received proportionally less attention [5-7].

Desert soil microbial ecology research has primarily focused on bacterial communities, which have been shown to be largely responsible for primary production and provision of key ecosystem services [8-11].

Bacillus thuringiensis (Bt) is gram-positive, naturally occurring soil bacterium that produces proteinaceous endotoxins, known as crystal inclusion (cry) protein, which

are highly toxic to insects and pests [12, 13]. More than 60 cry genes, with toxicity spectra extending over several, the natural occurrence of *B. thuringiensis* in soil environments was reported from Egypt [14] and Jordan [15]. However, sensitivity of Bt to lysis by bacteriophages is the most one of complicated to such industry. Lysis can either be caused by exogenous phage infection or be due to strain lysogeny. About 83% of Bt subspecies contain lysogenic phage [16].

Bacteriophages are a diverse group of viruses that infect bacteria and are found in a variety of environments, which include soil, drinking water, food, and so on [17]. It is estimated that the biosphere consists of approximately 10^{31} bacteriophages and archaeal viruses. Bacteriophages are known to proliferate wherever their bacterial hosts exist [18]. Bacteriophages are the most numerous form of life on earth; ten times more numerous than bacteria [19, 20]. They can be found in all environments where bacteria grow: in the Sahara, hot springs, the North Sea, and polar inland waters [21-23]. Phages are detected in ground and surface water, soil, food (e.g. sauerkraut, wine), sewage, and sludge [24-26]. They have also been isolated from humans and animals, for example from feces, urine, saliva, spit, rumen, and serum [27-29].

Bacteria and their viruses (called bacteriophages, or phages), have been found in virtually every ecological niche on Earth. Arid regions, including their most extreme form called deserts, represent the single largest ecosystem type on the Earth's terrestrial surface [30]. This research aims to investigate the presence of bacteriophages specific for

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Bacillus genus specially *B. thuringiensis* in the arid soil because the researches in this field are very few and need the more to add much information about the occurrence of bacteriophages.

2. Materials and Methods

2.1. Soil Samples

Twelve soil samples were collected from different locations of the Egyptian desert as arid soil. Four samples from Giza governorate (Bahariya Oases, Mines, Bowiti, Peace) and eight samples from black desert, white desert, Farafra 1, major general sabih, Abohrirh, Abumankar, Mountains negev 1 and Great sand sea were investigated, and Enumerated for total count of bacteria.

2.2. Source of Bacteria

Different identified species of *Bacillus subtilis*, *B. thuringiensis*, *B. polymyxa*, *B. megaterium*, *B. amyloliquefaciens*, *B. lechiformis* and *B. circulans* were kindly obtained from virology lab., Agricultural microbiology Dept., Faculty of Agriculture, Ain Shams University, Cairo, Egypt. All bacterial cultures were sub-cultured and maintained on nutrient broth and nutrient agar medium.

2.3. Enumeration of total Count of Bacteria

Tryptic soy agar medium was used to enumerate the total count of bacteria in all collected samples by plate count technique. The plates were incubated for 48h at 30°C.

2.4. Preparation of the Crude Phage Lysates

The specific phages for *Bacillus sp.*, were detected in different arid soil samples using the liquid enrichment technique, method of Duff and Wyss [31] modified by Othman [32]. Five grams of soil were added to 50 ml of nutrient broth medium was inoculated with a mixture of different bacillus strains were used (*Bacillus subtilis*, *Bacillus thuringiensis*, *Bacillus polymyxa*, *Bacillus megaterium*, *Bacillus amyloliquefaciens*, *Bacillus lechiformis* and *Bacillus circulans*) in 250 ml Erlenmeyer flask with traces of CaCO₃. Then, flasks were incubated at 30°C for 72 hr with shaking (250 rpm/min). After incubation the cultures were centrifuged at 6000 rpm for 15 min and the supernatant was collected in a clean flask. Chloroform was added with rate of 1:10, followed by vigorously shaking for 5 min. The crude lysate of the phages was obtained and assayed qualitatively and quantitatively.

2.5. Qualitative and Quantitative Assaying of *Bacillus* Phages

Bacillus phages were qualitatively assayed by the spot test technique according to the method of Othman [32]. Quantitative assaying of the phages was carried out by over layer agar technique according to Adams [33] and as

reported by Othman *et al.* [34]. For the quantitative assaying, phage dilutions were made in nutrient broth and plaque assay technique was done.

2.6. Preparation of the Phage Isolates

The different isolates of phages specific for *Bacillus thuringiensis* were performed by a single plaque isolation method based on the plaque morphology. Single characterized plaques of *Bacillus thuringiensis* phages were characterized and picked up, then transferred to flasks containing 10 ml of liquid host culture (10⁸ cfu/ml), followed by incubation at 30°C for 48-72 hr with shaking at 150 rpm using a shaker incubator. The single plaque isolation was repeated three times until a high degree of plaque purity was achieved [35].

2.7. Preparation of High Titer Phage Stock Lysates

A large amount of the phage stocks were obtained by propagation the phage by the liquid propagation method used by Othman *et al.* [34].

2.8. Purification and Concentration of Bacteriophages

Dextran sulfate-polyethylene glycol two phase liquid system was used [34]. To purify and concentrate the bacteriophages of *Bacillus thuringiensis* phage lysates, the weights 222.3, 0.48, 15.8 and 4.2 grams of phage lysate, dextran sulfate 500, polyethylene glycol (PEG 6000) and NaCl, respectively were mixed in a separating funnel to give a mixture containing ratio 6.5%, 0.2% and 1.7% (W/W) of PEG 6000, dextran sulfate and NaCl, respectively. After mixing the funnel was allowed 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 suspended in 2.5 ml of a 1 % (W/W) dextran sulfate solution. Then, 0.15 ml of a 3M KCl was added to each milliliter 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, the phage suspensions were centrifuged at 16000 rpm for 2 h. at 4°C. Then, the supernatants were discarded and the pellets were resuspended in 1.0 ml of saline solution and then assayed.

2.9. Characterization of *Bacillus thuringiensis* Phages

2.9.1. Adsorption of UV

Absorption spectra of purified phages Bt1 and Bt2 were measured at serial wave lengths viz. 220, 240, 260, 280, 300 and 320 nm by 2 nm intervals in a spectrophotometer model UV vis Spectrophotometer UV 9100B, Lab Tech. in Biochemistry Dept., Faculty of Agriculture, Ain Shams Univ.

2.9.2. Electron Microscopy Examination

The isolated phages were examined as described by

Othman *et al.* [35]. A drop of the phage suspension (10^{12} pfu/ml) was placed on 200 mesh copper grids with carbon-coat formvar films, and the excess was drawn off with filter paper. A solution of 2% uranyl acetate was then placed on the grids and the excess drawn off as before. Specimens were examined using JOEL-JEM-1010 electron microscope (Electron Microscope Unite, Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt).

2.9.3. Lytic Pattern of the *Bacillus* Phages

Lysosensitivity of the isolated phages was carried out using the spot test technique as described by Sambrook *et al.* [36]. Plates containing base layers were inoculated with the over layer which containing the tested bacterial hosts (*Bacillus subtilis*, *Bacillus thuringiensis*, *Bacillus polymyxa*, *Bacillus megaterium*, *Bacillus amyloliquefaciens*, *Bacillus lechiformis* and *Bacillus circulans*) separately in a 3 ml of melted semi-solid agar media, 20 μ l of phage isolate was dropped on the surface of plates and then incubated for 24 hr 37°C.

2.9.4. Action of *Eco*RI Restriction Enzyme on *B. thuringiensis* Phages Genome

2.9.4.1. Extraction of Phages DNA

The nucleic acid of the isolated phages was extracted according to the method described by Maniatis *et al.* [37] with minor modification by Compos *et al.* [38]. Fifteen ml of high titer phage suspension were incubated at 37°C for 30 min with DNase and RNase at final concentration of 1 μ g/ml to get rid of contaminating bacterial DNA and RNA.

Phage particles were precipitated by addition of NaCl and polyethylene glycol (PEG6000) to final concentrations of 3 and 5% (w/v), respectively. The mixture was incubated on ice for 30 min and centrifuged at 12,000 rpm for 20 min. The phage pellets were resuspended in 500 μ l of 0.2 M ammonium acetate, 0.001 M Na-EDTA pH 7.5 and heated at 55°C for 20 min an equal volume of buffer saturated phenol was added and mixed by inverting the tube several times. The phases were separated by centrifugation at 13000 rpm for 5 min at room temperature in microcentrifuge. The aqueous phase was transferred to a clean tube and extracted once again with chloroform. The aqueous phase was recovered as described above and transferred to clean tube. The DNA was precipitated by adding 0.1 volume of 3 M sodium acetate pH 5.5 and equal vol. of cold ethanol followed by freezing for 7-8 hrs, the tube was thawed and DNA precipitate was collected by centrifugation at 13,000 rpm for 10 min. The pellet was washed in 70% ethanol, air dried at room temperature, resuspended gently in 30 μ l of TE (10 mM tris-HCl pH 8.0, 1 mM EDTA) and stored at 4°C.

2.9.4.2. Digestion of bacteriophages DNA with restriction enzyme

The reactions were assembled by mixing the components in a sterile 0.5 ml eppendorf (2 μ l 10x buffer, 1 μ g phage

DNA, 3-5 units Restriction enzyme *Eco*RI and complete the total volume to 20 μ l with Sterile deionized water). The reactions were incubated for 2 hrs at 37°C, and then 5 μ l of bromophenol blue dye (450 mM tris borate pH 8.3, 50% (v/v) glycerol, 0.2% (w/v) bromophenol blue) were added and the reactions were heated at 60°C for 5 min to stop the reaction. The digested DNAs were stored at 4°C until ready for analysis by agarose gel electrophoresis. The digested DNA was separated in 1% agarose gel according to Peacock and Dingman [39] in the presence of 1 Kbp DNA ladder (biomol) as molecular weight standard to allow estimation of the DNA size of each bacteriophag. The stained gel was examined using an ultraviolet transilluminator with wavelength of 302 nm. The DNA bands which fluoresced orange were photographed using digital camera.

3. Results and Discussion

3.1. Enumeration of total Count of Bacteria

The total count of bacteria were conducted by plate count technique using Tryptic soy agar medium. Results are shown in the following table (1):

Table (1). Enumeration of bacterial count in soil samples

Location of sample	Bacterial count (cfu/ml)
Bahariya Oases	2.3×10^6
Mines	4×10^6
Bowiti	-
Peace	1.1×10^2
Black desert	2.5×10^3
White desert	1.8×10^3
Farafra 1	3.3×10^4
Major general sabih	4.1×10^4
Abohrirh	6.7×10^4
Abumankar	1.7×10^3
Mountains negev 1	3.3×10^5
Great sand sea	6.1×10^5

It was found that, all soil samples from different locations have a bacterial count except that one from Bowiti area. the highest bacterial count was recorded from soil sample of Bahariya oases (2.3×10^6 cfu/ml), while the lowest count was 1.1×10^2 cfu/ml as a result of sample located from Peace.

3.2. Occurrence of *Bacillus* Phages Different Soils

Twelve different samples from the Egyptian deasert were tested using spot test technique (Fig. 1) for the presence of bacteriophages specific for *Bacillus subtilis*, *Bacillus thuringiensis*, *Bacillus polymyxa*, *Bacillus megaterium*, *Bacillus amyloliquefaciens*, *Bacillus lechiformis* and *Bacillus circulans*. The results (Table 2) revealed the presence of phages specific for *Bacillus subtilis* in samples 1 and 12 (Bahariya Oases and Great sand sea), phages specific for *Bacillus thuringiensis* occurred in samples 1, 2 and 12

(Bahariya Oases, Mines and Great sand sea), phages specific for *Bacillus polymxa* at samples 1 and 11 (Bahariya Oases and Mountains negev 1), phages specific for *Bacillus megaterium* at samples 1 and 12 (Bahariya Oases and Great sand sea) and phages specific for *Bacillus circulans* at samples 9 and 10 (Abohrirh and Abumankar), while no phage was detected for *Bacillus amyloliquefaciens* and *Bacillus lechiformis* at any soil sample as shown in table (2).

Data also indicated that phages specific for *B. thuringiensis* occurred in 25% from the tested soils, followed by phages of *B. subtilis* which occurred in 17% from soils. Results showed that 7 out of 12 soils were free from any phages specific for tested species of *Bacillus*. Because of phages specific for *B. thuringiensis* were more distributed than the others, the titer of the phages specific for *B. thuringiensis* was determined by the plaque assay method

and it was found that the number of phages isolated from Bahariya Oases and specific for *B. thuringiensis* was 12×10^{11} pfs/ml. El-Didamony [40] found that phages specific for *B. thuringiensis* occurred in the soil of Yemen country and this agree with our data. Also, Prigent *et al.* [21] isolated different bacteriophages from the surface sands of Sahara Deserts.

Single characterized plaques of *Bacillus thuringiensis* phages were characterized and picked up, then transferred to flasks containing 3 ml of liquid host culture (10^8 cfu/ml), followed by incubation at 30°C for 48-72 hr with shaking at 150 rpm using shaker incubator. The single plaque isolation was repeated two times until a high degree of plaque purity was achieved. Two phage isolates specific for *Bacillus thuringiensis* were isolated from soil 1 (Bahariya Oases) and designated Bt1 and Bt2, depending upon the first latter of host scientific name.

Table (2). Detection of *Bacillus* phages in arid soils by the spot test technique

Samples	<i>Bacillus</i> species as an indicator host							Remarks
	<i>B. subtilis</i>	<i>B. thuringiensis</i>	<i>B. polymxa</i>	<i>B. megaterium</i>	<i>B. amyloliquefaciens</i>	<i>B. lechiformis</i>	<i>B. circulans</i>	
Bahariya Oases	+	++	+	+	-	-	-	12X10 ¹¹ pfu/ml Phage titer of <i>B. thuringiensis</i>
Mines	-	++	-	-	-	-	-	
Bowiti	-	-	-	-	-	-	-	
Peace	-	-	-	-	-	-	-	
Black desert	-	-	-	-	-	-	-	
White desert	-	-	-	-	-	-	-	
Farafra 1		-	-	-	-	-	-	
Major general sabih		-	-	-	-	-	-	
Abohrirh	-	-	-	-	-	-	+	
Abumankar	-	-	-	-	-	-	+	
Mountains negev 1	-	-	-	-	-	-	-	
Great sand sea	++	++	-	+	-	-	-	

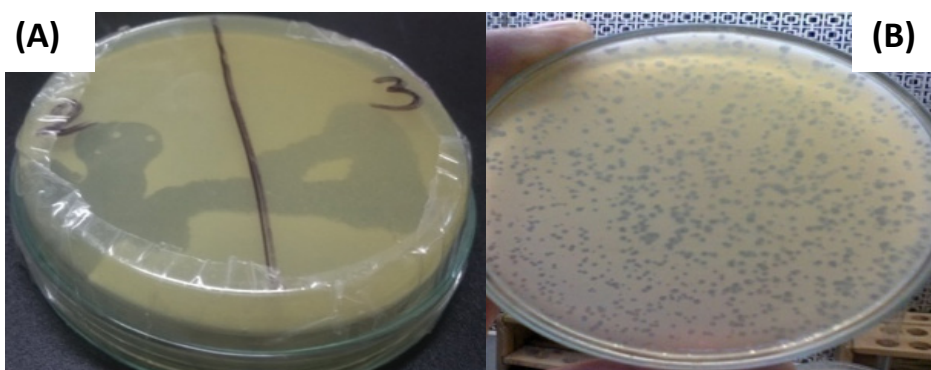


Figure (1). Assaying of the phages specific for *Bacillus* qualitatively by the spot test (A) and quantitatively by the over layer technique (B)

3.3. Preparation of High Titer Phage Lysates

High titer phage stocks were obtained using the liquid culture method as following: Erlenmeyer flasks (250 ml) containing 100 ml of liquid culture medium were prepared; each flask was inoculated with *Bacillus thuringiensis* and the phage lysate was added in a ratio of 1:10 and incubated at 30°C with shaking for 48 hr, after the incubation period, cultures were centrifuged at 6000 rpm for 15 min, then the suspension containing phages was assayed quantitatively and the phage count was 10^7 , then transferred into sterile flasks and stored at 4°C with traces of chloroform.

3.4. Purification of Phages Specific for *Bacillus thuringiensis*

Dextran sulfate-polyethylene glycol system (two phase system) was used [35] the weights 200, 14.2, 0.435 and 3.8 grams of phage lysate, polyethylene glycol (PEG 6000), dextran sulfate 500 and NaCl, respectively were mixed in a separating funnel (Fig 2.). After mixing the funnel was allowed to stand at 4°C overnight. A heavily turbid bottom layer was slowly collected into a clean tube and centrifuged at 2000 rpm for 15 min. The clear top and bottom phases were removed by pipette and the resulted remaining interface "cake" was 2.5 ml for phage 1 and 2 ml for phage 2, then they were suspended in 15 ml of a 1% (W/W) dextran sulfate solution. Then, 1.7 ml of a 3 M KCl was added to each milliliter of suspension; the mixture was allowed to stand for 2 hr at 4°C and centrifuged at 2000 rpm for 10 min.

The phage suspensions were centrifuged at 16000 rpm for 2 hr at 4°C. Then, the supernatants were discarded and the pellets were resuspended in 1.0 ml of saline solution and then assayed. The resulted concentrations of the phages were assayed quantitatively by the plaque assay method and it was 10^{14} pfu/ml for both phages Bt1 and Bt 2. The same method of purification was used for different phages [34, 35].

3.5. Adsorption of UV

Absorption spectra for purified viruses Bt1 and Bt2 phages were measured at serial wave lengths viz. 220, 240,

260, 280, 300 and 320 nm by 2 nm intervals. As shown in table (3), the purified phage particles of phages Bt1 and Bt2 suspended in saline solution have different absorption spectra at ultraviolet rays. Absorption of Bt1 phage at 260 nm was 0.0982, absorption at 280 nm was 0.0852, absorption at 260/280 nm was 1.1525, and absorption at 280/260 nm was 0.8676. Absorption of Bt2 phage at 260 nm was 0.152, absorption at 280 nm was 0.134, absorption at 260/280 nm was 1.134, and absorption at 280/260 nm was 0.8815.

3.6. Electron Microscopy Examination

The particle morphology of the isolated viruses was determined by examination the negatively stained preparations using the transmission electron microscope. Electron micrograph (Fig. 3) shows that Bt1 phage has icosahedral capsid with hexagonal outlines with dimensions of about 140x165 nm and contractile tail type. The tail is about 117 nm length and 34 nm width, while the particle of phage Bt2 has long contractile tail with 206 nm length and 27 nm width. Its head Icosahedral capsid with hexagonal outlines with dimensions of about 140x124 nm.

The classification of bacteriophages depends principally on both morphological and molecular characterization. According to the morphology, 96% of bacteriophages belong to Caudovirales which is subdivided into three families. These families are *Myoviridae* (25%) characterized by icosahedral head and contractile tail with a neck and central tube [41]. The morphological characterization suggest that, the isolated phages could be a members of the *Myoviridae* family based on their structure in electron micrograph. In the Namib Desert soil samples, 20 distinct morphotypes were identified, all members of the *Myoviridae* and *Siphoviridae* families with no apparent *Podoviridae*-like virions [42], while El-Didamony [40] isolated 5 phages active against Bt isolates from Yamen soil. Four phages with long non-contractile tails and hexagonal heads (*Siphoviridae*) and one phage with very short tail and isometric head (*Podoviridae*).

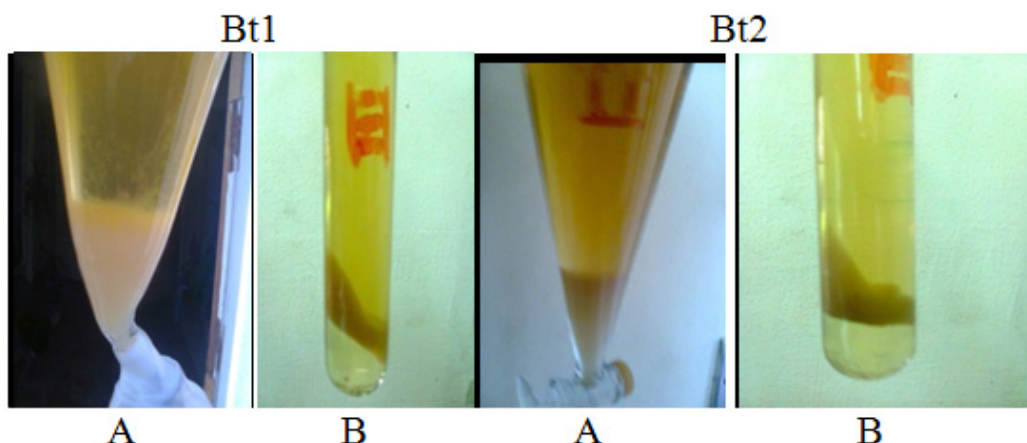
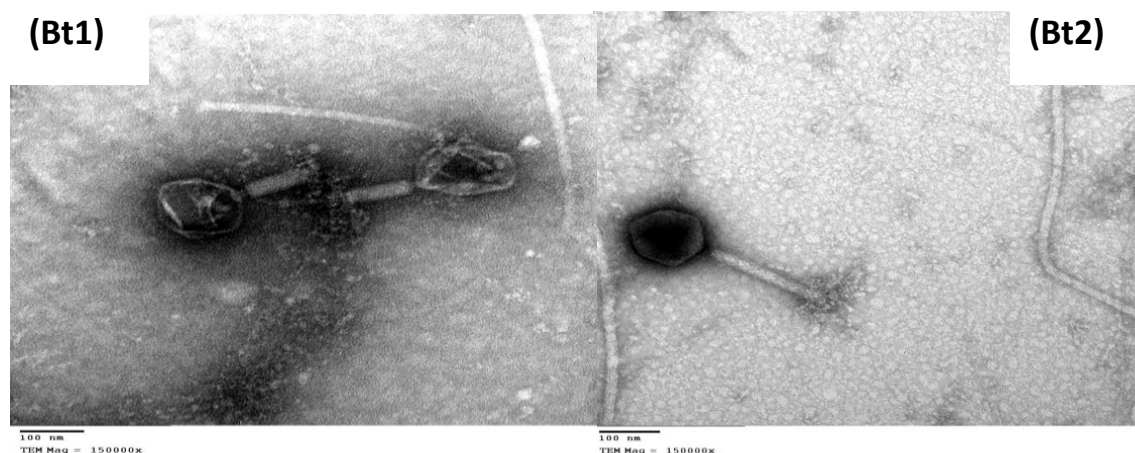


Figure (2). Purification and concentration of phages specific for *B. thuringiensis* by dextran sulphate-polyethylene glycol, two phase system. (A): Turbid precipitates in separating funnel containing the virus particles. (B): Intermediate phase (cake) containing the phage particles

Table (3). UV characteristic of *Bacillus thuringiensis* phages

Phage	A280 nm	A260 nm	Ratio A260/A280 nm	Ratio A280/A260 nm
Bt1	0.0982	0.0852	1.1525	0.8676
Bt2	0.152	0.134	1.134	0.8815

**Figure (3).** Electron micrograph of purified *Bacillus thuringiensis* bacteriophages Bt1 and Bt2 negatively stained with 2% uranyl acetate using TEM (Magnification X-150000)

3.7. Lytic Pattern of the *Bacillus thuringiensis* Phages

Lysosensability of *Bacillus subtilis*, *Bacillus thuringiensis*, *Bacillus polymyxa*, *Bacillus megaterium*, *Bacillus amyloliquefaciens*, *Bacillus lechiformis* and *Bacillus circulans* to the two isolated phages (specific for *B. thuringiensis*) was investigated qualitatively by spot test technique. Data represented in table (4) show that, *B. thuringiensis* phage Bt1 and phage Bt2 both have a narrow host range and able to lyse the main host *Bacillus thuringiensis* and another two bacterial hosts *Bacillus subtilis* and *Bacillus lechiformis*. El-Sayed *et al.* [42] isolated phages specific to *Bacillus cereus* and *Bacillus thuringiensis* strains and mentioned that the phages have narrow host range.

Table (4). Host specificity of the isolated phages BT1 and BT2

Bacterial host		Presence of lysis by spot test	
		Phage BT1	Phage BT2
1	<i>Bacillus subtilis</i>	+	+
2	<i>Bacillus thuringiensis</i>	+	+
3	<i>Bacillus polymyxa</i>	-	-
4	<i>Bacillus megaterium</i>	-	-
5	<i>Bacillus amyloliquefaciens</i>	-	-
6	<i>Bacillus lechiformis</i>	+	+
7	<i>Bacillus circulans</i>	-	-

+ : Bacterial lyses - : No lyses of bacterial cells

3.8. Digestion of Bacteriophages DNA with Restriction Enzyme

The Bt1 and Bt2 phages had a linear double-stranded (ds) DNA because they were susceptible to digestion with restriction enzyme endonucleases, *EcoRI* and subsequently subjected to electrophoretic analyses. As shown in Fig (4), the phage DNA was sensitive to *EcoRI*. The genomes were fragmented into four fragments with size of 1500, 1400, 800 and 510 bp. They were estimated to be 4.210 kbp for Bt1 phage, as determined by combining the fragment sizes obtained with the restriction enzyme used, and 6.480 kbp for Bt2 phage with 4 fragments with size of 3000, 1600, 1350 and 530 pb.

The isolated *Bacillus* phages, in previous studies, possess dsDNA with a size ranged between 18.0 kbp and 158.0 kbp [6, 42-45]. For Sahara Desert surface sand samples, induced phage genomes were estimated to range in size from 45 to 270 kb. On the other hand, in the Namib Desert soil samples, 20 distinct morphotypes were identified, PFGE profiles from Namib soils indicated an average genome size of 55 to 65 kb, with several genomes of up to 350 kb in size [30].

4. Conclusions

In this study two phages specific for *Bacillus thuringiensis* were isolated from arid soil in Egypt, they were assayed qualitatively and quantitatively, propagated on their specific bacteria, purified, morphologically characterized, their host range was studied, and the action of *EcoRI* restriction enzyme on their genomes was studied.

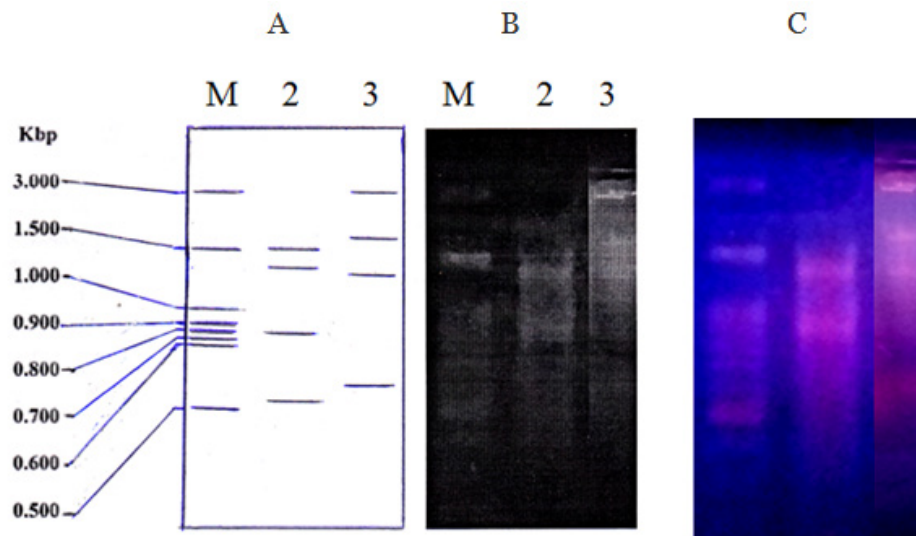


Figure (4). A diagram (A) and a photograph (B) of DNA restriction patterns of bacteriophages Bt1 and Bt2 digested with *EcoRI*. (C) The gel under ultraviolet transillumination. M= Marker DNA

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