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ISOLATION AND CHARACTERIZATION OF BACTERIAL VIRUSES SPECIFIC *TO BACILLUS MEGATERIUM*

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ABSTRACT

This study was conducted to isolate and characterize bacteriophages specific to *Bacillus megaterium*. Seven single lytic phage isolates specific to **B. megaterium** were successfully isolated from rhizosphere soil of maize plants, growing in the Experimental Farm of Faculty of Agriculture, Minia University, Minia, Egypt. The phages were characterized on the basis of the optimum pH for viral infection, thermal inactivation point, sensitivity to UV radiation, host specificity, as well as particle size, and morphology. The isolated phages exhibited tolerance to alkaline and acidic reactions. All phage isolates were found to be of head and tail types. On the basis of the differences in the studied characteristics of these phages, the seven phage isolates were divided into four groups (A, B, C and D). Phages of each group exhibited the same features (i.e. the optimum pH for infection, thermal inactivation point, sensitivity to UV radiation, host specificity, as well as phage particle size and morphology). Accordingly, the seven phage isolates are belonging to four phage types. These four phage types were designated ∅Bm1, ∅Bm2, ∅Bm3 and ∅Bm4.

Key words: *Bacillus megaterium*, bacteriophage, UV radiation, rhizosphere.

INTRODUCTION

Due to alkalinity of some soils, the soluble forms of phosphate fertilizer applied to such soils, rapidly converted to a complex of precipitated form of $Ca_3(PO_4)_2$ **(**Rajan *et al*., 1996; Zayed, 1998 and Hammad, 1999). Some soil microorganisms particularly *B. megaterium* play an

important role in supplying the growing plants with available forms of phosphorus by producing organic acids and CO_2 which increase the soil acidity and convert the insoluble forms of phosphorus into soluble ones. The use of these bacteria as a biofertilizer in the alkaline soils is required to increase the availability of soil phosphorus. For such economically important microorganisms (*i.e.* phosphate dissolving microorganisms), knowledge of factors influencing the maintenance and activities of these desired bacteria in the soil is of a particular interest. Presence of bacteriophages is likely to be one of the most important environmental factors influencing the maintenance and activities of such useful bacteria, since; the depressive effect of bacteriophages specific to phosphate dissolving bacteria in the cultivated soils was reported by Fathy (2004) and El-Balkhi (2006). It is well known that bacteriophages are of widespread occurrence and are usually found in soils which contain the appropriate bacterial host. Therefore, presence of phages in the soils may explain the observation concerning biofertilization failure on several plants grown at different localities, even when different local or foreign inocula of high efficiency were used.

Efforts were made to characterize and identify the bacteriophages of phosphate dissolving bacteria on the basis of plaque morphology and particle size and morphology (Fathy, 2008 and Farahat, 2016). However, the number of the studied phages was too few and the details given are too limited

This work aimed to study the occurrence of bacteriophages specific to *B. megaterium* in Minia soil. Moreover, the different characteristics of the isolated phages (*i.e.* optimum pH for infection, thermal inactivation point, sensitivity to UV radiation, host range, particle size, and morphology) were also studied to characterize and differentiate the phage isolates.

MATERIALS AND METHODS

1- Source of Bacteriophages: A soil sample was randomly collected from the rhizosphere of maize plants, growing in the Experimental Farm of the Faculty of Agriculture, Minia University, Minia, Egypt, to be used as a source of bacteriophages.

2- P-dissolving bacteria: An efficient isolate of phosphate dissolving bacteria (*Bacillus megaterium*) was obtained from the microbial collection of Dept. Agric. Microbiology, Fac. Agric. Minia University.

3- Isolation of Bacteriophages: The liquid enrichment technique of Adams (1966) was used to isolate phages specific to *B. megaterium* from the collected rhizosphere soil sample as described by Barnet (1972).

a- Detection of bacteriophages: The spot test was used for detection of bacteriophages of *B. megaterium* as described by Adams (1966).

b- Purification of bacteriophage isolates: The single plaque isolation technique was used to obtain pure single phage isolates

as described by Kiraly *et al*. (1970).

c- Preparation of high titer phage suspension: Agar double layer plates method described by Maniatis *et al.* (1982) was used to prepare the high titer phage suspension for each single phage isolate as described by Hammad and Dora (1993) and Farahat (2016).

d- Titer Estimation: Titer was estimated using the method described by Kiraly *et al.* (1970). From the phage suspension, a series of tenfold dilution was prepared in sterile eppendorf vials. The dilutions were prepared by measuring 90 µl of SM medium (Maniatis *et al.,* 1982) into each vial. Ten µl of phage suspension were added to the first vial and mixed, then 10 µl from the first vial were transferred into the second one and so on, until the last vial. After dilution, 200 µl of indicator bacterial suspension were placed in each vial. The contents of each tube were shaken and transferred to a sterile test tube containing 3 ml of melted nutrient agar semi-solid medium (0.7% ager), which had been prepared before and kept at 50-55 °C. Each tube was shaken separately, and the contents were poured onto previously prepared solid medium plates, then they were incubated at 30-33°C for 24 h. The formed plaques were counted and the titer was calculated and expressed as plaque forming unit (pfu)/ml.

4- Characterization of bacteriophages

a- The optimum pH level: Nine eppendorf tubes each containing 1 ml SM media with various pHs (*i.e.* 4 up to 12) were prepared. The pH was adjusted with NaOH (0.1 N) and HCl (0.1 N). Individual plaques for each single isolate of phages were transferred to the prepared tubes (plaque/tube). Tubes were incubated at 30°C for 60 min. then 10µl from each tube were spotted over double agar layer plates (three replicates), containing *Bacillus megaterium*, followed by incubation at 30-33ºC for 24-48 h. Diameters of the lysed spots were measured. The average values of the replicates was calculated.

b- Thermal inactivation point: Ten eppendorf tubes each containing 1 ml of high titer phage suspension of each single phage isolate were prepared. Tubes were heated in water baths adjusted at 40,

45, 50, 55, 60, 65, 70, 75, 80, 85, 90 and 95°C for 10 min, then cooled under tap water. After heat treatment 10µl from each tube was spotted over double agar layer plates containing the *Bacillus megaterium* as indicator bacteria. Plates were inspected for lysed spots after 24-48 h incubation at 30-33°C.

c- Sensitivity to ultraviolet irradiation: Five ml of high titer phage suspension of each single phage isolate were put in dishes placed at distance of 20 cm from UV lamp of 254 nm wave length. Ten µl of each irradiated phage suspension were spotted over double agar layer plates that containing the *Bacillus megaterium* as indicator bacteria, after 10, 20, 30, 40, up to 90 min. exposure to

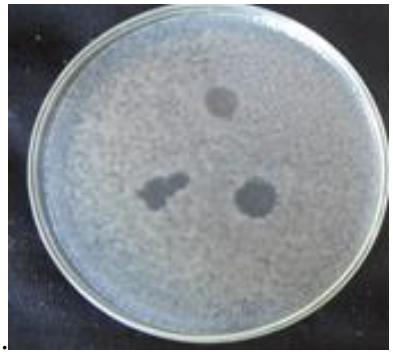

Figure (1): A bacterial lawn of *B. megaterium*, spotted with drops of the prepared phage lysate and incubated for 24-30 h at 30 °C. The lysed spots are clearly seen.

Purification of phages

The single plaque isolation technique was used to obtain pure phage isolates of *B. megaterium*. As shown in Fig. (2) the phages specific to *B. megaterium* formed single plaques of different morphologies. Seven single plaques morphologically different were selected and kept as pure phage isolates. The isolated phages formed circular single plaques of 1 to 3 mm in diameter and clear in appearance.

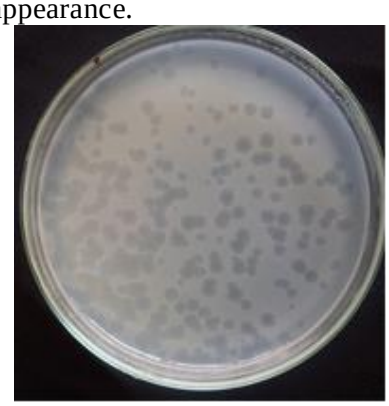

Figure (2): A plate containing single plaques of bacteriophages specific to B. *megaterium*. The differences in morphology of the single plaques are clearly seen.

UV irradiation. Plates were inspected for lysed spots after incubation for 24-48 h at 30-33°C.

d- Host range Assay: The host range of each phage isolate was determined using the spot test technique. Each of the phage isolates of *Bacillus megaterium* was tested against the isolated root nodule bacteria and the available strains.

e- Electron microscope examination: The electron microscope grids were prepared to examine each of the isolated phages as described by Hayat and Miller (1990) and stained by 0.5% uranyl acetate pH 4.5 (Stacey *et al*., 1984). The grids were examined at 50 kv in transmission electron microscope (Joel, Model GEM 1010) in Sohag University, Sohag, Egypt.

RESULTS

Occurrence of bacteriophages specific to *B. megaterium* in the collected soil sample:

Bacteriophages specific to *Bacillus megaterium* were successfully isolated from the soil sample collected from rhizosphere soil of maize plants. The spot test was used for detection of phages in the collected rhizosphere soil sample. As shown in Figure (1) the spot test indicates that phages of *Bacillus megaterium* were found to be common in the collected soil sample.

The high titer phage suspensions: One hundred ml of high titer phage suspension were prepared for each phage isolate of *B. megaterium*. The titers of the prepared suspensions of the seven phage isolates specific to *B. megaterium* were ranged from $3.4x10^{10}$ to $6.4x10^{12}$ pfu/ml.

Characteristics of the isolated phages: The different characteristics of the seven phage isolates of *B. megaterium* were studied to find out if these phage isolates are different types or similar.

a- The optimum pH for phage infection: The infectivity of the seven phage isolates of *B. megaterium* was studied at various pH levels (pH 4-12). As shown in Table (1), all phage isolates formed lysed spots at any pH level up to pH 11. No lysed spots were formed at pH 12, with the exception of phages No. 4 and 6, which formed lysed spots at pH 12. On the basis of the obtained results, the phage isolates were divided into four groups. Group (A) comprised phage isolates No. 1 and 3, which formed the widest spots at pH 8. Group (B) contained phage isolates No. 4 and 6, that formed the widest spots at pH 7. In addition, group (C) comprised one phage isolate No. 2 which formed the widest spots at pH 9. Group (D) contained phage isolates No. 5 and 7 formed the widest spots at pH6.

Table **(1):** Stability of bacteriophages specific to *B. megaterium* to different pH levels.

Phage group	phage number	pH levels								
		4	5	6	7	8	9	10	11	12
A	1	1.32	1.60	1.76	1.80	2.26	1.61	1.52	1.29	-
	3	1.30	1.65	1.75	1.63	2.34	1.42	1.25	1.04	-
B	4	1.35	1.41	1.62	1.91	1.72	1.50	1.41	1.12	0.94
	6	1.22	1.82	1.91	2.51	1.83	1.34	1.80	1.45	1.35
C	2	1.34	1.41	1.56	1.73	1.96	2.35	1.83	1.21	-
D	5	1.20	1.83	2.53	1.93	1.50	2.22	1.71	1.14	-
	7	1.51	1.75	2.41	1.66	1.46	1.34	1.20	1.02	-

The underlined numbers: Optimum pH

b- Thermal inactivation point of the phage isolates:

Data presented in Table (2) indicate that the seven phage isolates of *B. megaterium* were grouped into four groups (A, B, C and D). Group (A) comprised two isolates (isolates No. 1 and 3) of the same thermal inactivation point (80°C). Whereas, group B contained two isolates (No. 4 and 6). The thermal inactivation point of these two phage isolates was found to be 95°C. The thermal inactivation point of of Group (C), which comprised one phage isolate (No. 5) was found to be 85°C. Group (D) contained two phage isolates (No. 5 and 7) of the same thermal inactivation point (90°C).

Table (2): Thermal inactivation points of bacteriophages specific to *B. megaterium*.

.Phage group	Phage number	Temperature (°C)									
		50	55	60	65	70	75	80	85	90	95
	1	+	+	+	+	+	+	-	-	-	-
A	3	+	+	+	+	+	+	-	-	-	-
	4	+	+	+	+	+	+	+	+	+	-
B	6	+	+	+	+	+	+	+	+	+	-
C	2	+	+	+	+	+	+	+	-	-	-
	5	+	+	+	+	+	+	+	+	-	-
D	7	+	+	+	+	+	+	+	+	-	-

+ = Lysis - = No lysis

c- Sensitivity to Ultraviolet Irradiation: As shown in Table (3), the UV radiation at wave length of 254 nm inactivated the isolated phages at different exposure times. Accordingly, the isolated phages of *B. megaterium* were divided into four groups (A, B, C and D). Group (A) comprised two isolates (isolates No. 1 and 3), which were inactivated after 70 min exposure to UV radiation. Whereas, group (B) which comprised two phage isolates (isolates No. 4 and 6) was inactivated after exposure to UV for 30 min. Phage isolate No. 2, which formed group (C) was inactivated after exposure to UV for 50 min. In addition, phage isolates No. 5 and 7 of group (D) were inactivated after 20 min. exposure to UV radiation.

Table (3): Effect of UV radiation (wave length 254 nm) on bacteriophages specific to *B. megaterium*.

Phage group	Phage number	Exposure time (minute)									
		10	20	30	40	50	60	70	80	90	100
A	1	+	+	+	+	+	+	+	-	-	-
	3	+	+	+	+	+	+	+	-	-	-
B	4	+	+	+	-	-	-	-	-	-	-
	6	+	+	+	-	-	-	-	-	-	-
C	2	+	+	+	+	+	-	-	-	-	-
D	5	+	+	-	-	-	-	-	-	-	-
	7	+	+	-	-	-	-	-	-	-	-

+ = Lysis - = No lysis

D- Host range of the isolated phages: Each of the seven phage isolates of *B. megaterium* was tested against four different bacillus species. As shown in Table (4), all phage isolates were infectious to *B. megaterium* (the main host). Whereas, none of the seven phages was infectious to the other tested bacillus species with exception of phage No. 2 which was infectious to *B. ceureus*.

Table (4): The host range of the phage isolates specific to *B. megaterium*.

Phage group	Phage number	Bacillus species			
		B. megaterium	*B. subtilius*	*B. Ploymexa*	*B. ceureus*
	1	+	-	-	-
A	3	+	-	-	-
	4	+	-	-	-
B	6	+	-	-	-
C	2	+	-	-	+
	5	+	-	-	-
D	7	+	-	-	-

+ = Lysis - = No lysis

e- Size and morphology of phage particles: Bacteriophage isolates specific to *B. megaterium* were negatively stained and examined by electron microscope. All phage isolates were found to be of head and tail type (Figure 3). As shown in Table (5), on the basis of the phage particle dimensions the phage isolates No. 1 and 3 were found to be similar in their head diameters as well as in length and width of their tails. Therefore, these two phage isolates formed one group (group A). Moreover, phages No. 4 and 6 were also classified in another group (group B), since they exhibited similar dimensions. Moreover, group C contained only one phage isolate (phage No. 2). In addition, phages No. 5 and 7 were also classified in group D. phages of each group exhibited similar particle dimension.

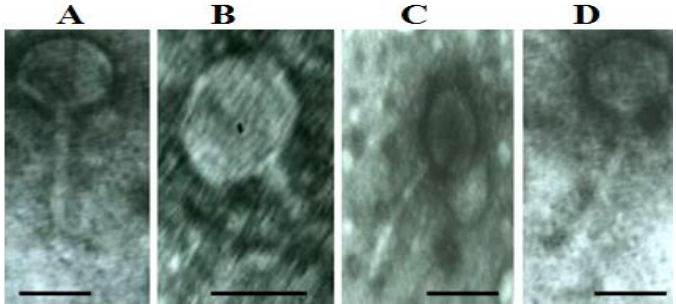

Figure (3): Electron micrographs of negatively stained phage particles specific to *B. megaterium* represent phages of group A, B, C and D. Magnification bar = 50 nm

Table (5): Dimensions of bacteriophage particles specific to *B. megaterium*.

Phage group	Phage No.	Head diameter ± SD (nm)	Tail	
			Length ± SD (nm)	Width ± SD (nm)
A	1	63 ± 2	125 ± 2	12 ± 2
	3	61 ± 3	123 ± 3	11 ± 3
B	4	75 ± 3	53 ± 2	15 ± 2
	6	76 ± 2	51 ± 3	14 ± 2
C	2	50± 3	168± 3	8± 3
D	5	55± 4	165± 3	12± 2
	7	57± 2	163± 4	10± 3

SD = Standard deviation

DISCUSSION

Bacteriophages of *B. megaterium* were successfully isolated from the rhizosphere of maize plants, growing in the Experimental Farm of the Faculty of Agriculture, Minia University, Minia, Egypt, and were found to be common in the soil from where the soil sample had been taken. Similar results were obtained by Zayed (1998); Fathy (2004) and Elmaghraby *et al*. (2015), who isolated phages of *B. megaterium* from rhizosphere soils of different plants.

Since it is assumed that each plaque has originated from the progeny of a single phage particle (Kiraly, *et al.,* 1970 and Elmaghraby *et al*., 2015). The single plaque isolation technique was used to purify phages. Seven single plaques of phages specific to *B. megaterium* having different morphologies were picked and kept as single pure phage isolates. The isolated phages of *B. megaterium* formed circular single plaques of 1 to 3 mm in diameter and clear in appearance.

It is commonly believed that the shape, size and outline of the plaques are characteristic of the phage strain (Marei and Elbaz, 2013; Elmaghraby *et al*., 2015). Barnet (1972) reported that the isolates of *Rhizobium trifolii* phages of the same morphological type had similar plaque characteristics. Moreover, Elsharouny (2007) isolated different phage types specific to *Azotobacter spp.* and *Azospirillum spp.* Each phage type formed single plaques of similar morphology.

One hundred ml of high titer phage suspension was prepared for each phage isolate of *B. megaterium*. The titers of the prepared phage suspension were ranged from $3.4x10^{10}$ to $6.4x10^{12}$ pfu/ml. Such high concentrations of phages are not surprising, since a single plaque of 2 mm in diameter may contain between 10^7 and 10^9 recoverable phage particles (Fathy, 2008; Elsharouny, 2007 and Elmaghraby *et al*., 2015).

Since the single plaques of the seven phage isolates of *B. megaterium* were morphologically different, it was expected that each single phage isolate represents one phage type. *i.e.* the isolated phages are likely to be belonging to seven different phage types. In order to assess this expectation, the different characteristics of the isolated phages were studied.

The infectivity of the seven phage isolates of *B. megaterium* was studied at various pH levels (pH 4-12). All isolated bacteriophages were found to be tolerant to alkaline and acidic reactions. Similar results were obtained by Elsharouny (2007). The optimum pH for infection was studied for each phage isolate. On the basis of the optimum pH, the isolated phages were classified into four groups. Phages of each group exhibited the same optimum pH.

Since the optimum pH for the phage isolates of each group was found to be the same, these results may indicate that: 1- The

phage isolates of each group are belonging to one phage type. 2- The phage isolates of each group may be different phage types and all have the same optimum pH. Elsharouny (2007) isolated different phage types specific to *Azotobacter* and *Azospirillum* and all had the same optimum pH (pH 8). Moreover, Fathy (2008) stated that the optimum pH of different phage types of root nodule bacteria was found to be the same (pH8). Therefore, further characterizations for the phage isolates are needed to be carried out to accept or dismiss any of the above mentioned hypotheses.

The thermal inactivation point of each phage isolate was estimated. According to the thermal inactivation point results, the seven phage isolates were divided to four groups. Phage isolates of each group were found to have the same thermal inactivation point.

On the basis of the above mentioned information, different hypothesis could be possible: **1**- The phage isolates of each group are likely to be one phage type. Hammad (1993) and Hammad and Ali (1999) stated that the different phage types of *B. japonicum* exhibited different thermal inactivation points. Moreover, Abo-Sinna (2004) reported that the thermal inactivation points of four phages of *B. subtilis* ranged between 50-80°C. Elsharouny (2007) reported that phages of either *Azotobacter* or *Azospirillum*, which are belonging to one phage type, were found to have the same thermal inactivation point. 2- Since the phage isolates were different in their plaque morphology, each phage group may comprised phage isolate belonging to more than one phage type but all exhibited the same thermal inactivation point and the same optimum pH. To accept one of the above hypothesis further studies were carried out.

Sensitivity of the isolated phages of *B. megaterium* to UV radiation (at wave length of 254) was studied. According to the sensitivity to U.V. radiation, the seven phage isolates were divided into four groups. Interestingly, the four phage groups of *B. megaterium*, which were divided on the basis of the optimum pH and the thermal inactivation point, were found to be the same as those classified on the basis of the sensitivity to UV radiation. Such results may indicate that the phages of each group may represent a single phage type. Similarly, Elsharouny (2007) found that phage isolates of either *Azotobacter* or *Azospirillum*, which belonging to one phage type, were found to have the same sensitivity to UV radiation.

To confirm this explanation, additional characterizations for the isolated phages were carried out, *e.g*. the host range as well as the particle size and morphology of each phage isolate.

Barnet (1972) stated that the ability of phage particle to lyse a bacterial strain is dependent upon the presence of certain micro-molecules on the surface of the cells, *viz* surface receptors for bacteriophage adsorption. Each of

the seven phage isolates of *B. megaterium* was tested against four different bacillus species. All phage isolates were infectious to *B. megaterium* (the main host). Whereas, none of the seven phages was infectious to the other tested bacillus species with exception of phage No. 2 which was infectious to *B. ceureus*. On the other hand, Hammad and Ali (1999) studied the host range of 60 phage isolates specific for *B. japonicum*. Based on host ranges the 60 phage isolates were classified into 4 phage groups. Each group comprised the phage isolates of the same host range. Kankila and Lindstrom (1994) studied the host range of 11 bacteriophages specific to *R. leguminosarum* bv *trifolii* using 32 *Rhizobium* strains. They concluded that three morphological types were found among the phage isolates. Type 1 phages were able to lyse all bacterial strains tested whereas type 2 and 3 exhibited narrower host range. Bacteriophage isolates specific to *B. megaterium* were negatively stained and examined by electron microscope. All phage isolates were found to be of head and tail type. Similarly, Elmaghraby *et al*. (2015) isolated phages specific to *B. megaterium* of head and tail type. Interestingly, phages of each group (divided on the basis of the thermal inactivation point, sensitivity to UV radiation and optimum pH) were found to be morphologically similar. The differences in head and tail dimensions of the phages of each group are within the standard deviation and are not statistically significant. Francki (1973) reported that since there are a number of unknown factors able to affect particle size during various preparative procedures, it is difficult to make valid comparisons between published morphometric data. Accordingly, morphological differences do not necessarily imply differences in phages of each group but because these phages of each group showed the same optimum pH, the same thermal inactivation point and the same sensitivity to UV radiation, the phages of each group must be belong to a single phage type.

Therefore, on the basis of the above mentioned information, it can be concluded that the phages of each group represent one phage type. *i.e.* the seven isolated phages of *B. megaterium* were found to be belonging to four phage types. The four phage types of *B. megaterium* were designated Bm1, Bm2, Bm3 and Bm4 for phages of group A, B, C and D, respectively.

CONCLUSION

Generally, in this study different characteristics were studied all together to differentiate and classify the isolated phages of *B. megaterium*. No single technique for characterizing phages is in itself sufficient for complete identification or classification, but these features (the optimum pH, thermal inactivation point, sensitivity to UV radiation, host range and electron microscopy) must be studied all together to differentiate between

the isolated phages.

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عزل وتوصيف الفيروسلت البكتيرية المتخصصة على بكتيريا ***Bacillus megaterium***

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قسم الميكروبيولوجيا الزراعية – كلية الزراعة – جامعة المنيا

فى هذه الدراسة تم عزل سبعة عزلات من الفيروسات البكتيرية المتخصصه على بكتيريا *Bacillus megaterium* من تربة منطقة جذور نبات الذره النامى فى المزرعة البحثية لكلية الزراعة جامعة المنيا. اظهرت الفيروسات المعزولة تحمل لظروف الحموضة والقلوية.

تم دراسة درجة تركيز ايون الهيدروجين الامثل لحدوث الاصابة بهذه الفيروسات ودرجة التثبيط الحرارى والحساسية للاشعة فوق البنفسجية والمدى العوائلى ومورفولوجيا وابعاد الجزيئات الفيروسية للفيروسات التى تم عزلها.

اعتمادا على الاختلافات فى الخصائص التى تم دراستها فقد قسمت السبعة عزلات من الفيروسات الى اربع مجموعات. تضمنت كل مجموعة الفيروسات ذات الخصائص المتشابهه.

وبناء على ذلك فقد خلصت الدراسة الى ان السبعة عزلات من الفيروسات تنتمى الى اربع انواع مختلفة حيث تم تسميتهم ∅Bm1, ∅Bm2,∅Bm3 و∅Bm4