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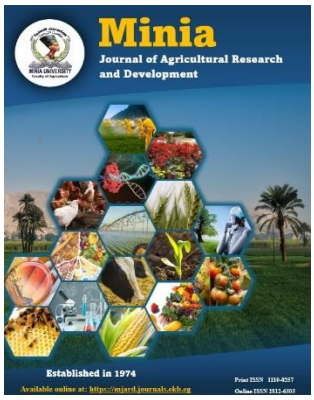

Characteristics of Bacteriophages specific to salt tolerant *Azotobacter* sp. in Minia Soils

Gehad R. A. Osman , Adel M.M. Hammad , Samir A. Haddad , Maha O. A. Omar

Agricultural Microbiology Department, Faculty of Agriculture,
Minia University, Minia, 61715, Egypt.

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ABSTRACT

Five isolates of Azotobacter were isolated from different locations in Minia Governorate - Egypt and designated Az1, Az2 Az3, Az4 and Az5. Az3 was found to be most tolerant isolate to NaCl. Bacterial viruses of Azotobacter (Az3) were isolated from soil samples collected from Minia Governorate. Twelve single plaques of Azotobacter phages, were isolated. Due to the similarity of the phage isolates in their optimum pH sensitivity to UV (260 nm), thermal inactivation point and host range the phages of Azotobacter were classified into groups A and B. On the basis of electron microscope study, Azotobacter phages of every group were found to be similar in their size and morphology. Generally, the obtained results indicated that, the twelve isolates of Azotobacter phage belong to two types of phage. These two phage types were named ∅Az 1, ∅Az 2.

Keywords: *Azotobacter*, Bacteriophages, thermal inactivation point, , host range.

INTRODUCTION

Azotobacter spp. are commonly used as biofertilizers, not only for their activities in fixing nitrogen but also for their ability to release phytohormones such as gibberellinic and indolic nature, that stimulate growth of plants, absorption of nutrients and enhancement of the photosynthesis process **(Fathy, 2004).**

The presence of *Azotobacter* phages is probably one of the crucial environmental factors that affect the activities of these bacteria. The harmful feffect of bacteriophages' on *Azotobacter* in the cultivated soils was proved by **Hammad *et al*. (1995); Hammad (1998 & 1999); Zayed (1998)** and **Fathy (2004).**

Efforts were made for characterization and identification of *Azotobacter* phages according to morphology of their plaques and particle morphology and size. However,

*** Corresponding author: Samir A. Haddad**
E-mail address: samir.mohamed@mu.edu.eg

the tested phages and given details were too too limited.

This study aims at characterizing *Azotobacter* phages, which were found in Minia soils on the basis of plaque morphology, host range, thermal stability, sensitivity to ultraviolate light, the optimum pH as well as phage particles' size and morphology.

MATERIALS AND METHODS

1- The used bacteria:

Five isolates of *Azotobacter* were randomly isolated from five samples of soil obtained from Minia Governorate, using Ashby's liquid medium **(Abdel-Malek and Ishac, 1968),** as described by **Ali (2003).** The pure isolates were stored at 4^{o}C.

Selection of the most tolerant Azotobacter isolate to NaCl

Fifty ml of nutrient broth were put in each Erlenmayer flasks (100 ml)**.** The medium was salinized at concentrations of 0, 1, 2, 3, 4 and 5%with NaCl. Each flask was inoculated with 1 ml liquid culture of *Azotobacter* isolate (10^8 cfu/ml). Flasks were incubated at 30 -33 h. for 48hrs, then growth was estimated as optical density, at 608 nm wave length. The most tolerant isolate was selected.

2- Isolation of *Azotobacter* phages:

Using **Adams (1966)** technique liquid enrichment was used to isolate phages of *Azotobacter*.

3- Phage detection

The spot test was carried out in agar double layer plats as described by **Adams (1966)** for detection of phages.

4- Purification of bacteriophages:

The single plaque isolation technique was adopted as described by **Kiraly *et al.* (1970)** to obtain pure single phage isolates. Every plaque was transferred into eppendorf tube each containing 500 µl of SM medium. These single isolates of phages were maintained over 200 µl of chloroform at 4 $^{\circ}$C**.**

5- Preparation of high titer phage suspension:

The high titer phage suspension **was prepared using** agar double layer plates method as described by **Maniatis *et al*. (1982)**

6- Titre estimation:

Titer of the phage suspensions was determined according to **Kiraly *et al.* (1970).** Titer was calculated as pfu/ml (plaque forming unit /ml).

7- Characterization of isolated phages

Effect of different pH levels :

The isolated phages were subjected to different pH levels (*i.e.* 4.0, 5.0, 6.0, 7.0, …..upto12) for 60 min at 30°C. The optimum pH for every isolate of phages was estimated as mentioned by **Saad (2018)**

d- Sensetivity to ultraviolet irradiation:

Petri dishes containing 5 ml of each phage suspension were exposed to ultraviolet (260nm) at 20 cm distance from germicidal UV lamp. After 10, 20, 30, 40, 50, 60, 70, 80 and 90 min, the infectivity of every phage suspension was tested via spot test on double agar layer plates

b- Thermal inactivation point:

Thermal inactivation point of each single phage isolate was determined as described by **Saad (2018).**

a- Host specificity of phage isolates:

The infectivity of each phage isolate was tested for the five isolates of *Azotobacter*

(indicator bacteria) in individual plates using the spot test as described by **Saad (2018)**.

f- Electron microscopy:

Using 0.5% uranyl acetate (pH 4.5), each phage isolate was stained negatively

by as described by **Aharchi (1992)** and examined in a transmission electron microscope (Joel, Model GEM 1010) at 50 kv.

RESULTS AND DISCUSSION

The most tolerant Azotobacter isolate to salinity

As shown in **Table (1),** the highest growth for any of the tested *Azotobacter* isolates was recorded in the normal medium with no added salt (control). Whereas, in the salinized media, the growth was gradually inhibited with increasing the salt concentration. Moreover, Azotobacter isolate (Az3) exhibited the highest tolerance to salinity among the five isolates tested. Therefore, Azotobacter isolate (Az3) was selected as the most tolerant isolate to NaCl. **Ferreras *et al.*(2006)** reported that high concentrations of salt may have a depressive effect on bacteria due to osmotic stress and .direct toxicity.

Table (1): Growth of *Azotobacter* isolates at different concentration of NaCl estimated as optical density (608 nm) .

Azotobacter Isolaes	NaCl concentration (%)					
	0	1	2	3	4	5
Az1	32.8	16.6	9.3	9.3	4.4	3.4
Az2	37.8	16.7	6.88	6.88	4.6	3.8
Az3	65.3	20.81	17.8	17.8	11.3	6.8
Az4	44.3	14.7	11.3	11.3	3.2	2.7
Az5	37.07	15.3	9.4	9.4	4.0	3.5

Bacteriophages of *Azotobacter*:

Figure (1) proved that bacteriophages of *Azotobacter* were found to be common in the soils from where the samples had been collected (different locations in Minia Governorate). It is known that bacterial viruses are of widely distributed in areas that include the suitable bacterial host. Presence of bacteriophages specific to *Azotobacter*, may reflect the predominance of *Azotobacter* in the soils of Minia Governorate. Similarly, **Othman (1979)** and **Fathy (2004)** reported that *Azotobacter* phages were commonly found in the Egyptian soils.

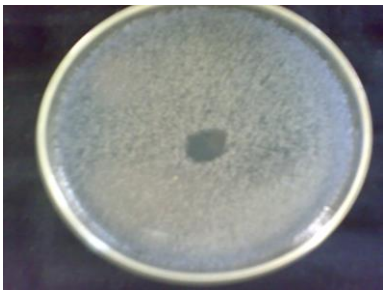

Figure (1): Detection of bacteriophage using spot test.

Bacteriophages purification:

The plate in Figure (2) contains single plaques. It is known that every plaque has developed from a single phage particle (Mohamed *et al.*, 2023), therefore, to purify phages the single plaque isolation technique was used. Therefore, twelve single plaques of *Azotobacter* bacteriophages were picked and kept as single phage isolate, each was morphologically different. Barnet (1972) and Mohamed *et al.* (2023) found that the bacterial viruses of similar plaque had the same morphological type.

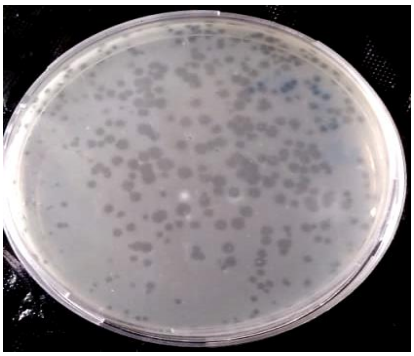

Figure (2): Single plaques of *Azotobacter* phages,

Since the twelve single isolates of *Azotobacter* phages were different in their morphology, it is expected that every phage isolate is considered one type. *i.e.* the phage isolates might be 12 types of phages. To confirm this explanation, these phages were subjected to study more characteristics.

3- Phage suspension's Titer:

Fifty ml of phage suspension were prepared for every *Azotobacter* phage. The titers ranged from 3.6×10^{11} pfu/ml to 8.3×10^{12} pfu/ml. These high phage concentrations was expected, since a single plaque of 2 mm in diameter contains about 10^7 and 10^9 phage particles **(Gunsalus & Stanier, 1960).**

4- Characterization of the isolated phages:

a- Determination of the optimum pH:

As shown in **Table (2)** at any pH tested, the phage isolates formed lysed spots. These data indicate that the isolated *Azotobacter* phages are acidic and alkaline tolerant. Similarly, **Roslycky *et al*. (1962); Challaghan *et al*. (1969); Hammad & Ali (1999)** stated that bacterial viruses were stable at pH 5 to 12.

Table (2): Effect of pH levels on *Azotobacter* phages.

No. of Phage	pH								
	4	5	6	7	8	9	10	11	12
	Lysed spots diameters (mm.)								
1	10.5	12.9	13.5	16.2	18.1	15.0	12.8	11.4	9.1
2	10.2	11.9	14.0	15.0	16.3	13.1	13.2	10.9	9.2
3	11.5	12.8	13.7	14.9	16.0	12.9	12.8	10.3	9.5
4	12.4	13.2	13.8	12.1	16.2	14.0	13.0	12.7	8.9
5	10.0	11.0	13.5	17.05	19.0	12.7	10.5	9.0	05.2
6	9.9	10.0	11.3	10.6	13.7	10.5	10.0	9.2	08.4
7	08.9	11.6	12.4	11.2	15.3	6.8	03.9	00	00
8	06.2	9.8	12.0	14.9	17.0	13.1	07.2	04.5	00
9	05.9	12.7	15.0	13.8	17.8	12.1	11.0	05.0	03.5
10	9.0	12.1	13.0	11.0	13.9	07.2	04.4	00	00
11	11.0	12.0	14.1	11.9	15.4	11.0	9.4	9.5	00
12	10.3	12.8	13.5	10.9	14.9	10.1	09.7	07.1	05.3

At pH 8 all phage isolates (12 phage isolates of *Azotobacter*) exhibited spots wider than those at any other pH. *i.e.* pH 8 is the optimum all phages.

Based on the obtained data different expectations are likely:

1) It is possible for the 12 isolated phages of *Azotobacter* to be one phage type because the optimum pH is the same (pH 8) for all phage isolates.

2) It is possible for the isolated 12 phages to be belonging to different phage types and all have the same optimum pH.

Any of these two hypotheses is likely. To accept or dismiss any of the hypotheses mentioned above, more characteristics were studied.

b- Effect of U.V. irradiation

Data in **Table (3)** showed that U.V. (260 nm) inactivated the isolated phages at different times of exposure. Therefore, the isolated phages of *Azotobacter* were classified in two groups (A and B). Each group contained number of phages that inactivated after the same time of exposure to U.V.

Group A contained isolates of phages No. 1, 2, 3, 5, 8 and 9 which inactivated after 50 min. exposure to U.V. at 260 nm. Group B comprised isolates of phages No. 4, 6, 7, 10, 11 and 12 that inactivated after exposure for 70 min.

Table (3): Effect of U.V. (260 nm) on phages of *Azotobacter* .

Phage group	No. of Phage	Time of exposure (min.)								
		10	20	30	40	50	60	70	80	90
A	1	+	+	+	+	-	-	-	-	-
	2	+	+	+	+	-	-	-	-	-
	3	+	+	+	+	-	-	-	-	-
	5	+	+	+	+	-	-	-	-	-
	8	+	+	+	+	-	-	-	-	-
	9	+	+	+	+	-	-	-	-	-
B	4	+	+	+	+	+	+	-	-	-
	6	+	+	+	+	+	+	-	-	-
	7	+	+	+	+	+	+	-	-	-
	10	+	+	+	+	+	+	-	-	-
	11	+	+	+	+	+	+	-	-	-
	12	+	+	+	+	+	+	-	-	-

Based on the obtained data, two hypotheses can be expected:

1) The twelve *Azotobacter* phages are likely to be two phage types. *i.e*. phages of *Azotobacter* in group A represent only one type of phage since they exhibited the same response to U.V. radiation.
2) Since the isolated phages were morphologically different in their plaques, the phages of each group may be belong to different types of phages but exhibited the same response to U.V. radiation. Actually, further studies were conducted to confirm one of these hypotheses.

c- Thermal inactivation point

Hegazi *et al.* (1980) stated that incubation of two phages of *Azotobacter* at 70°C and 80°C for 15 min. resulted in complete inactivation of phages.

Due to the similarity in the thermal stability **(Table 4)**, *Azotobacter* phages were grouped into two groups (A and B). Every group contained the phages that exhibited the same thermal inactivation point.

Azotobacter phages of group A (No. 1, 2, 3, 5, 8 and 9) after exposure to 95°C for 10 min. were completely inactivated. Whereas, *Azotobacter* phages of group B (isolates No. 4, 6, 7, 10, 11 and 12) were inactivated at 85°C for 10 min..

Table (4): Thermal inactivation points of *Azotobacter* phages .

Phag group	Phage No.	Temperature (°C)									
		50	55	60	65	70	75	80	85	90	95
A	1	+	+	+	+	+	+	+	+	+	-
	2	+	+	+	+	+	+	+	+	+	-
	3	+	+	+	+	+	+	+	+	+	-
	5	+	+	+	+	+	+	+	+	+	-
	8	+	+	+	+	+	+	+	+	+	-
	9	+	+	+	+	+	+	+	+	+	-
B	4	+	+	+	+	+	+	+	-	-	-
	6	+	+	+	+	+	+	+	-	-	-
	7	+	+	+	+	+	+	+	-	-	-
	10	+	+	+	+	+	+	+	-	-	-
	11	+	+	+	+	+	+	+	-	-	-
	12	+	+	+	+	+	+	+	-	-	-

Interestingly, the two phage groups of *Azotobacter* that classified based on U.V sensitivity **(Table 3)** were found to be the same as those classified based on the thermal stability **(Table 4).** Such results show that the phages of every group are belong to a single phage type. This is an expectation and it needs to be confirmed by additional characterization means.

d- Host range:

The host range of every isolate of phage was tested using spot test. The twelve phages of *Azotobacter* were tested against each of the five *Azotobacter* isolates.

Data in **Table (5)** indicated that two host ranges for *Azotobacter* phages were observed for the 12 isolates of phages. Therefore, the isolated phages of *Azotobacter* were grouped into two groups (A and B). Each group comprised number of phages that showed the same host range.

Azotobacter phages in group A (No. 1, 2, 3, 5, 8 and 9) were infectious to three *Azotobacter* isolates (Az1, Az3 and Az4) among five isolates tested. In addition, phages in group B (phage isolates No. 4, 6, 7, 10, 11 and 12) were infectious to two *Azotobacter* isolates (Az2 and Az3), among the five isolates tested. *i.e.* any of the isolated phages of *Azotobacter* was infectious to more than one bacterial isolate.

Table (5): Host range of *Azotobacter* phages

Phage group	Phage No.	*Azotobacter* isolates				
		Az1	Az2	Az3	Az4	Az5
A	1	+	-	+	+	-
	2	+	-	+	+	-
	3	+	-	+	+	-
	5	+	-	+	+	-
	8	+	-	+	+	-
	9	+	-	+	+	-
B	4	-	+	+	-	-
	6	-	+	+	-	-
	7	-	+	+	-	-
	10	-	+	+	-	-
	11	-	+	+	-	-
	12	-	+	+	-	-

The capability of phage to lyse bacteria depends on the absence or presence of surface receptors on the bacterial cell for phages adsorption **(Barnet, 1972)**.

The two groups of *Azotobacter* phages that grouped based on host specificity were found to be the same as those divided on the basis of thermal stability and U.V sensitivity. These data may indicate that the phages of every group are belonging to a single type of phages. *i.e.* the twelve *Azotobacter* phages are two phage types. To confirm these results further characterizations were conducted, *e.g.* size and morphology of phage particles.

e- Electron microscope study:

As shown in Figure (**3**), phage isolates of *Azotobacter* were of tail and head types. **Kowalski *et al.* (1974); Hammad (1989 and 1993) and Mohamed *et al.* (2023)** isolated phages of the tail and head types specific to different bacterial hosts.

Table (6): Dimensions*of bacteriophages (twelve isolates) specific to *Azotobacter*.

Phage group	Phage No.	Head diameter ± SD (nm)	Tail	
			Length ± SD (nm)	Width ± SD (nm)
A	**1**	47 ± 2	140 ± 3	8 ± 2
	2	49 ± 3	142 ± 4	9 ± 2
	3	46 ± 3	141 ± 2	8± 3
	5	50 ± 2	139 ± 3	9 ± 2
	8	45 ± 3	140 ± 2	7 ± 3
	9	47 ± 4	142± 3	8 ± 2
B	**4**	55±3	103±2	12±3
	6	57 ± 2	104 ± 3	13 ± 2
	7	54 ±4	102± 3	11 ± 4
	10	54± 2	104± 2	12± 2
	11	53± 3	103± 3	12± 3
	12	52±3	105±2	13±3

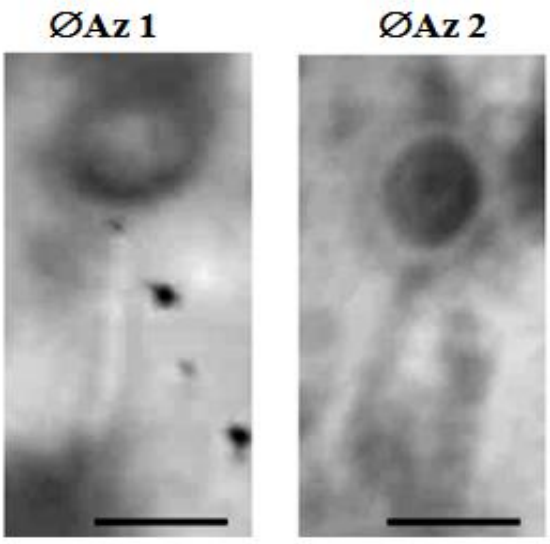

Figure (3): Electron micrographs of *Azotobacter* phages.

Magnification bar = 50 nm

As shown in **Table (6)** phage isolates of *Azotobacter* showed some variation in their dimensions. phage isolates No. 1, 2, 3, 5, 8 and 9 had the smallest head. Whereas, the largest head was found in phage isolates No. 4, 6, 7, 10, 11 and 12. Moreover, the tail length of the isolated *Azotobacter* phages varied from phages of group A and group B.

The longest tails were found in phages of group A, whereas, the shortest ones were detected in phages of group B. The phages of both group (A and B) were found to have contractile tails, According to ICTV, the bacteriophages of both groups (A and B) belong to *Order Caudovirales* and classified under *Family Myoviridae,* since they have long contractile tails.

Interestingly, the phages of every group which classified based on thermal stability, U.V. sensitivity and host specificity were similar in their morphology. The differences in dimensions of tail and head of the phages of each group are are not statistically significant and within the standard deviation. On the basis of the obtained dimensions, phages of group A were found to be statistically different from the phages of group B. Since the phages in each group showed similar thermal stability, U.V. sensitivity and host range, the phages in each group may be belong to one phage type. *i.e.* the twelve phage isolates of *Azotobacter* are two types of phages. The phages of group A and B were named ∅Az 1 and ∅Az2, respectively.

Generally, on based on the obtained data it can be concluded that, the plaque morphology is not an accurate technique to identify and classify the bacteriophages. This because in this study, the twelve phage isolates of *Azotobacter* were found to be belong to two types of phage, although each of the twelve phage isolates was isolated with different plaque morphology. *i.e.* plaques of different morphologies can be formed by a single phage type. This is not surprising since, **Kowalski *et al.* (1963); Barnet and Vincent (1971)** reported that there are many factors which may affect the morphology of plaques of a single phage type. These factors are nutrient medium composition and agar concentration, temperature of incubation, age of the indicator bacteria, osmotic shock and presence of host debris. Therefore, the plaque morphology should not be used for phage identification and classification, whereas, it can be used as a rapid technique to purify the mixed phages.

Moreover, the optimum pH was found to be not an accurate technique as well to identify and classify the bacteriophages. pH 8 is the optimum pH for all phage isolates, in spite of the phage isolates tested were not a single phage type. *i.e.* the two types of *Azotobacter* phages have the optimum pH 8. Such result was expected because it is well known that the medium which give rapid growth of the host bacteria is satisfactory for phage multiplication. *i.e.* the host bacteria (*Azotobacter*) which were used in this study were isolated from alkaline soils (soils collected from Minia Governorate). Therefore, the isolated bacteria are well adapted to the alkaline conditions which give rapid growth of these bacteria and consequently, provide satisfactory conditions for phage multiplication. **Roslycky *et al.* (1962)** reported that tolerance of phages of *Agrobacterium* to alkaline conditions up to pH 11, is probably linked to the ability of *Agrobacterium* to grow at up to pH 12.

Phages of each type exhibited the same response to U.V. at 260 nm. This may indicate that U.V. (260 nm) could be used to differentiate between the phage types.

The thermal inactivation point of the phages revealed that the phage isolates which belonging to one type of phages had the same thermal stability. Such results may indicate that the thermal inactivation point is an accurate feature which could be used to differentiate between phage types. The thermal stability was used as a characteristic of phage isolates by many investigators **(Aharchi, 1992; Hammad, 1993; Hammad**

***et al.*, 1995; Othman, 1997; Hammad & Ali, 1999).**

The host range of the isolated phages of *Azotobacter* was found to be the same for phages of each type. *i.e*. the host range is a diagnostic character for each phage type, and it can be used to identify and classify the phages. Many investigators used the host range to differentiate between phage types, *e.g.* **Kankila and Lindstrom (1994); Hammad and Ali (1999).**

Francki (1973) stated that because there are unknown factors that may affect size of particle during preparative process, it is difficult to make valid comparisons between published morphometric data. Therefore, morphological similarities do not necessarily imply a close relationship between phages, but because in this study, phages of every type showed similarity in the other characteristics (thermal stability, U.V. sensitivity and host range), the electron microscope study can be used to confirm the results of the other characters.

Finally, based on the above mentioned information, different features were used all together in this work to compare and classify the phage isolates of *Azotobacter*. No individual method for characterizing phages is in itself sufficient for complete classification or identification, but these characteristics (thermal stability, sensitivity to U.V., host range and electron micrographs) must be conducted all together to give clear differences between the phage isolates tested.

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الملخص العربى

خصائص الفيروسات البكتيرية المتخصصة على الازوتوباكتر المتحملة للملوحة فى اراضى المنيا

جهاد ربيع ، عادل محمود حماد ، سمير أحمد حداد ، مها عمر عبداللطيف
قسم الميكروبيولوجيا الزراعية – جامعة المنيا - مصر

تم عزل خمس عزلات من بكتيريا Azotobacter من مواقع مختلفة في محافظة المنيا - مصر وتم تسميتها Az1 و Az2 و Az3 و Az4 و Az5. وجد أن Az3 هي العزلة الأكثر تحملاً لـ NaCl. تم عزل الفيروسات البكتيرية المتخصصة على Azotobacter (Az3) من عينات التربة التي تم جمعها من محافظة المنيا .تم عزل اثني عشر عزلة من الفاجات المتخصصة على .Azotobacter نظرًا لتشابه عزلات البكتيريوفاج في درجة الاس الهيدروجينى الامثل والحساسية للأشعة فوق البنفسجية (٢٦٠ نانومتر) ونقطة التثبيط الحراري والمدى العوائلى، تم تقسيم عزلات البكتيريوفاج إلى مجموعتين (المجموعة أ و ب) .بناءً على دراسة المجهر الإلكتروني .وجد أن فاجات كل مجموعة متشابهة في حجمها وشكلها .بناءً على النتائج التي تم الحصول عليها، يمكن أن نستنتج أن عزلات البكتيريوفاج الاثنتي عشرة المتخصصة على بكتيريا Azotobacter تنتمي إلى نوعين من الفاجات تم تسمية هذين النوعين∅ Az 1 و ∅Az 2 .