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Research Article
Isolation and Characterization of *Pseudomonas aeruginosa* and its Virulent Bacteriophages

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Abstract

Background and Objective: *Pseudomonas aeruginosa* is a free living bacterium in widely different areas such as plants, soil, water and other moist locations. It is pathogenic to plants and humans. *P. aeruginosa* causes several disease symptoms to plants such as wet rot and curved leaves. The virulent bacterial viruses of *P. aeruginosa* were found to be of widespread occurrence in nature and isolated from widely different sources. Bacterial viruses were applied to control pathogenic bacteria in different fields and successfully. Therefore, this work aimed to study the different characteristics of *P. aeruginosa* lytic phage isolates. Moreover, the bio-control of *P. aeruginosa* by lytic phage isolates was also studied. **Material and Methods:** Different physical and molecular characteristics were assayed and determined of *P. aeruginosa* lytic bacteriophages. Also, the effect of phage isolates on *P. aeruginosa* as a bio-control under lab condition was studied. **Results:** *Pseudomonas aeruginosa* pathogenic bacterium was isolated from a sewage water sample. Two lytic bacteriophages specific to *P. aeruginosa* were isolated from same sewage water sample and designated Pa1 and Pa2. Both phage isolates (Pa1 and Pa2) found to be stable in 90°C and different pH low and high levels. The total count of *P. aeruginosa* decreased after 48 h in broth treated with lytic phages. RAPD-PCR amplification was indicated that the two phage isolates (Pa1 and Pa2) are belonging to two different phage types. **Conclusion:** The results of this study indicated that both lytic phage isolates could be used as a biological control agents against the plant pathogen *P. aeuroginosa*.

Key words: *Pseudomonas aeruginosa*, virulent bacteriophages, sewage water, RAPD-PCR, bio-control

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pseudomonas aeruginosa is a free living bacterium in widely different areas such as plants, soil, water and other moist locations. It is pathogenic to plants and humans[1,2]. Moreover, it is stated that *Pseudomonas aeruginosa* is widely distributed in nature, it has been found in external environmental conditions surrounding dairy animals such as air, sewage, soil, grass[3,4].

Pseudomonas aeruginosa causes several disease symptoms to plants such as wet rot and curved leaves. A bacterial leaf spot disease of tobacco caused by *P. aeruginosa* was detected in Philippines[5] and China[6].

Also, in California, 2 types of *P. aeruginosa* were found in other plants, type B-7 was found in tomato leaves and type S was found in a celery plant[7].

The virulent bacterial viruses of *P. aeruginosa* were found to be of widespread occurrence in nature and isolated from widely different sources such as sewage water samples[4,8,9] as well as soil and vegetable materials[10].

Bacterial viruses were applied to control pathogenic bacteria in foodstuffs, industrial environments and successfully used as phage therapy against some animal diseases[8,11-13]. Moreover, in United States bacteriophages were applied to certain meat products since August, 2006 to control *Listeria monocytogenes*[14].

Random amplified polymorphic DNA (RAPD) technique is a powerful approach and accessible genotyping method can be used to classify *P. aeruginosa* isolates[15-17].

On the basis of the above mentioned information, the lytic phages of *P. aeruginosa* could be used to control the plant diseases caused by this bacterium.

Therefore, this study aimed to study the physical and molecular characteristics of lytic bacteriophages specific to *P. aeruginosa.* Also, study the effect of phage isolates on *P. aeruginosa* as a biocontrol under lab condition.

MATERIALS AND METHODS

The study was carried out at Virology Laboratory, Department of Agric. Microbiology, Faculty of Agriculture, Ain Shams University, Cairo, Egypt (from April, 2018-March, 2019).

Source of bacteria: *Pseudomonas aeruginosa* was isolated from a sewage water sample collected after primary treatment from Sarpium forest site -Ismailia Governorate, Cairo, Egypt.

Serial dilutions of the sewage water were prepared as described by Goto and Enomoto[18]. Plates containing MacConkey agar medium were inoculated with the prepared dilutions (10^{-4} and 10^{-5}) and incubated at 37°C for 24-48 h. A single colony was transferred onto slant surface of MacConkey agar and incubated at 37°C for 48 h. The bacterial isolate was identified in Sohag University, Egypt, using Biolog[19] and was found to be *Pseudomonas aeruginosa*.

Detection of prophage in the isolated *P. aeruginosa*: *Pseudomonas aeruginosa* was tested for lysogeny using U.V. irradiation (240 nm) as described by Prinsloo[20]. To induce temperate phages, liquid culture of *P. aeruginosa* (24 h old) was prepared in nutrient broth medium[21].

Ten milliliter of the prepared liquid culture were placed in a petri plate and exposed to UV irradiation (240 nm) at distance of 60 cm from the germicidal UV lamp. One milliliter of this culture was withdrawn every 5 up to 30 min and kept in Eppendorf tube. After overnight incubation at 37°C, bacterial cells were precipitated by centrifugation at 5000 rpm and the temperate phage was qualitatively assayed in the supernatant using the spot test[22].

Isolation and enrichment of *P. aeruginosa* lytic phages: The liquid enrichment technique[22] was carried out to isolate virulent phages specific to *P. aeruginosa* from the collected sewage water sample. Nutrient broth medium[21] was used to grow the bacterial host (*P. aeruginosa*).

Detection of phages: Bacteriophages were detected using the spot test as described by Adams[22].

Bacteriophages purification: The single plaque isolation (SPI) technique was used as described by Kiraly *et al.*[23] to obtain pure single phage isolates.

High titer phage suspension: High titer suspensions of *P. aeruginosa* bacteriophages were prepared using the nutrient broth medium for enrichment technique as described by Sambrook *et al.*[24]. Titers of the prepared phage suspensions were estimated using the method described by Kiraly *et al.*[23].

Physical properties of phages:

- **Different pH level for phage infection:** The optimum pH level for infection of *P. aeruginosa* with each of the isolated bacteriophages was estimated as described by Hammad *et al.*[25]

- **Stability of phage to UV irradiation:** The stability of phages to UV irradiation with different periods for infection of *P. aeruginosa* was estimated as described by Hammad *et al*.[25]
- **Thermal inactivation point:** The thermal inactivation point of each isolated bacterial virus of *P. aeruginosa* was estimated according to Hammad *et al*.[25]
- **Electron microscopy:** Size and morphology of the purified particles using Backman L 7-35 ultracentrifuge at 30.000 rpm for 1.5 h at 4°C of virulent phage isolates were estimated as described by Hayat and Miller[26] and Stacey *et al*.[27]. Phosphotungstic acid (pH 6.8) was used for negative staining of the isolated phage particles. The stained Phage particles were examined using transmission electron microscopy (Joel, Model GEM 1010) in Sohag University, Sohag

Effect of phages on *P. aeruginosa* as a biocontrol: Under lab condition experiment was carried out to study the effect of presence of Pa1 and Pa2 phages on the efficiency of *Pseudomonas aeruginosa* as a biocontrol. Twelve flasks (250 mL) were filled with 100 mL broth media for each one and sterilized. The flasks were divided into 4 groups, each group comprised 3 flasks. The groups were subjected to the following treatments:

- First group were inoculated with 5 mL of liquid culture (33×10^6 CFU mL^{-1}) *P. aeruginosa* and 5 mL of Pa1 lytic phage (43×10^8 PFU mL^{-1})
- Second group was inoculated with 5 mL of liquid culture (33×10^6 CFU mL^{-1}) *P. aeruginosa* and 5 mL of Pa2 lytic phages (36×10^8 PFU mL^{-1})
- Third group was inoculated with 5 mL of *P. aeruginosa* and 5 mL of Pa1+5 mL of Pa2 lytic phages
- Fourth group was inoculation with 5 mL of liquid culture (33×10^6 CFU mL^{-1}) *P. aeruginosa* only

Total count of *Pseudomonas aeruginosa* was estimated of each group after 48 h. from inoculation with each treatment by using the dilution plate technique according to Clark *et al*.[28].

Molecular characterization:

- **Extraction of bacteriophages DNA:** Genomic DNA of each bacteriophage isolate specific to *P. aeruginosa* was extracted as described by Maniatis *et al*.[29]
- **Determination of DNA concentration:** The quality of the DNA extract was estimated by 0.8% agarose gel electrophoresis using 1X TBE (Tris-Borate-EDTA) buffer. Ethidium bromide was used to stain Gels. The genomic DNA concentration was estimated by measuring (UV-1601 UV/VIS Japan spectrophotometer) the optical density at 260 nm. The DNA quality was estimated using the 260/280 nm ratio and gel electrophoresis
- **Digestion of bacteriophages DNA with *Hin*dIII restriction enzyme:** The reactions were assembled by mixing the following components in a sterile 0.5 mL Eppendorf vial: 1 µg bacteriophage DNA, 3 units of *Hin*dIII restriction enzyme (enzynomics), 4 µL enzyme buffer and sterile deionized water to a final volume of 20 µL

The reaction mixtures were incubated for 2-3 h at 37°C. Five µL of bromophenol blue dye stop [450 mM tris borate pH 8.3, 50 mM EDTA, 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 reactions. The digested DNA was stored at 4°C and analyzed in 1% agarose gel using 1X TBE (Tris-Borate-EDTA) buffer for 1.5 h at 90 V as described by Peacock and Dingman[30].

- **PCR reaction and amplification condition:** PCR amplification was carried out using two random 10 mer arbitrary primers, OPH-01 and OPH-02 were supplied by Bio Basic Inc, Canada. Whereas, 3 SCoT primers, SCoT-7, SCoT-8 and SCoT-9 were obtained from iNtRON Biotechnology, Inc, Korea with GC content of 60 % were also used. Sequences of the used RAPD and SCoT primers were presented in Table 1

The PCR amplification was conducted according to Williams *et al*.[31] using 2X PCR Master mix solution [(i-Taq™) iNtRON Biotechnology] in 10 µL reaction volume contained 1 µL (40 ng) genomic DNA as a template, 5 µL of 2X PCR Master mix solution [(i-TaqTM) iNtRON Biotechnology], 1 µL of primer (10 pmol μL^{-1}) and 3 µL double distilled water. The reaction mixtures were over-laid with 20 µL of mineral oil/sample. The PCR amplification was carried out in a DNA

Table 1: Names and nucleotide sequence of the used primers

Primer names	Primer sequence 5'→ 3'
OPH-01	GGTCGGAGAA
OPH-02	TCGGACGTGA
SCoT-7	ACAATGGCTACCACTGAC
SCoT-8	ACAATGGCTACCACTGAG
SCoT-9	ACAATGGCTACCACTGCC

Thermal Cycler (Cetus, Perkin Elmer) programmed for a first denaturation step of 5 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min annealing temperature at 30°C for RAPD primers and at 50°C for SCoT primers, 1.5 min for extension at 72°C, followed by a final 7-min elongation step at 72°C and then held at 4°C until the tubes were removed.

The amplification products were separated by a horizontal gel electrophoresis unit using 1.5 % agarose gel for RAPD and SCoT markers. Gels were then run for 1.5 h at current 90 V. Bands were documented using Benchtop UV-transilluminator and photographed using photo Doc-It™ imaging system. The molecular size of the amplified products was determined against 1 Kb (+) DNA ladder (enzynomics). Data were created from photographs of gels and analyzed using Gel Analyzer 2010a program.

RESULTS

Isolated bacterium: The isolated bacterium formed 2-3 mm in MacConkey agar medium plates, flat and smooth, colonies with regular margin. This bacterium was identified in Sohag University, Egypt and was found to be *Pseudomonas aeruginosa*.

Temperate and lytic phages of *P. aeruginosa*: *Pseudomonas aeruginosa* was treated with UV irradiation (240 nm) to induce temperate phages. No temperate phages were detected.

Two lytic bacteriophages specific to *P. aeruginosa* were isolated from a sewage water sample collected from sewage treatment plant. The spot test was used for detection of phages in the collected sample. As shown in Fig. 1 this test indicated that phages of *P. aeruginosa* were found to be common in the collected sample.

Purification of phages: The single plaque isolation (SPI) technique was used to obtain pure phage isolates of *P. aeruginosa*. As shown in Fig. 2 the *P. aeruginosa* specific phages produced different plaques morphologies. The diameters of different plaques were (1 and 3 mm). The plaques were circular single and clear in appearance. These two phage isolates were designated as Pa1 and Pa2.

High titer phage suspensions: The titer of each isolate in one hundred ml was estimated to be 43×10^8 and 36×10^8 for Pa1 and Pa2, respectively.

Physical properties of phages

Different pH level for phage infection: The stability of both phage isolates was estimated with different pH levels (pH 4-12) as shown in Table 2. Both phage isolates produced lysed spots at all pH tested levels. Both phage isolates formed the widest lysed spots at pH 6.

Sensitivity to ultraviolet irradiation: As shown in Table 3, the UV irradiation at wave length of 254 nm. was able to inactivate the two isolated phages (Pa1 and Pa2) after exposure for 45 min.

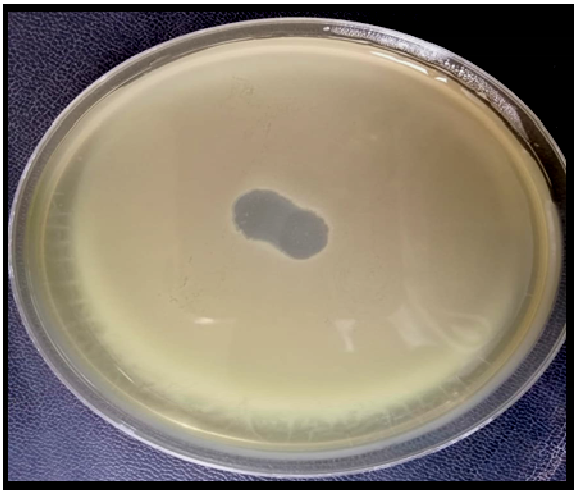

Fig. 1: Lysis of *Pseudomonas aeruginosa* spotted with lytic phage lysate

Table 2: Stability of *P. aeruginosa* bacteriophages (Pa1 and Pa2) at different pH levels

	pH levels								
Phage isolates	4	5	6	7	8	9	10	11	12
Diameter of the lysed spots (mm)									
Pa1	6.5	7.6	7.7	6.8	6.8	6.8	6.8	6.6	5.0
Pa2	6.8	6.8	7.1	6.7	6.6	6.6	6.4	6.3	4.7

Thermal inactivation point of the phage isolates: Data presented in Table 4 showed that both bacteriophages Pa1 and Pa2 specific to *P. aeruginosa* were inactivated at 90°C for 10 min.

Morphological properties of phage particles: The two phage isolates (Pa1 and Pa2) specific to *P. aeruginosa* were found to be of head and tail phages (Fig. 3). Both phage isolates possess long non-contractile tail. Therefore, these two phage isolates could be classified under order Caudovirales, Family Siphoviridae. As shown in Table 5 these two phage isolates were found to be different in their head diameter as well as in tail length and width.

Effect of Pa1 and Pa2 phages on *P. aeruginosa* as a biocontrol: Data presented in Fig. 4 indicated that, the numbers of *P. aeruginosa* were determined after 48 h from inoculation with each treatment. Data showed that, the total count of *P. aeruginosa* decreased after 48 h in broth treated with treated by bacteria+Pa1+Pa2 phages, bacteria+Pa1 phage and bacteria + Pa2 phage respectively, when compared with inoculation with *Pseudomonas aeruginosa* only.

Molecular characterization

Concentration and purity of genomic DNA: DNA concentration of Pa1 and Pa2 phage isolates were 983.43 and 795.19 μg mL^{-1}, respectively. DNA purity of Pa1 and Pa2 phage isolates was verified by agarose gel electrophoresis and calculated as the ratio of A260/A280 and found to be 1.91 and 1.89 for Pa1 and Pa2, respectively.

Restriction patterns of the isolated DNA: The DNA of each phage isolate (Pa1 and Pa2) was extracted and digested with the restriction enzyme *Hin*dIII. As shown in (Fig. 5).

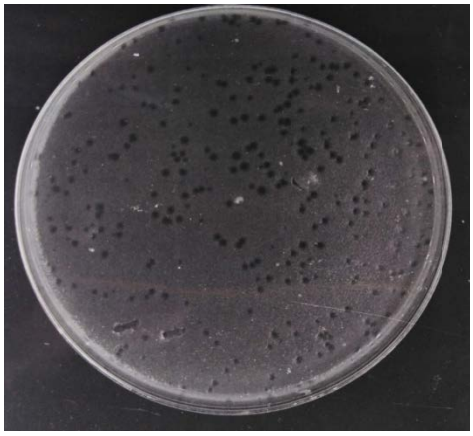

Fig. 2: A Petri dish containing different single plaques resulting from lytic bacteriophages specific to *Pseudomonas aeruginosa*

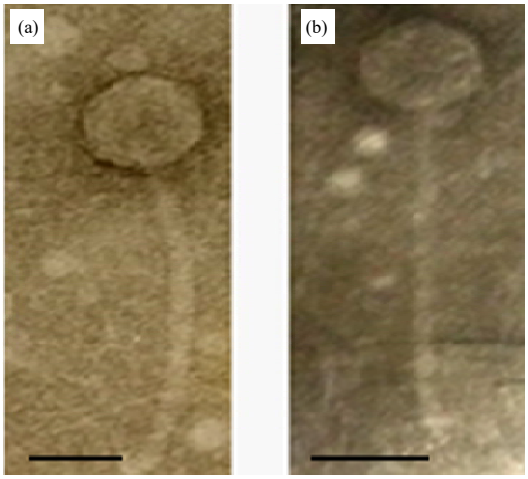

Fig. 3(a-b): Electron micrographs of lytic phages (a) Pa1 and (b) Pa2 infected *P. aeruginosa* stained with phosphotungstic acid
Magnification bar = 50 nm

Table 3: Effect of UV irradiation (254 nm) on the 2 *P. aeruginosa* specific phages

Phage isolates	Exposure time (min)																
	5	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
Pa1	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Pa2	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-

+: Lysis, -: No lysis

Table 4: Thermal inactivation points of bacteriophages Pa1 and Pa2 specific to *P. aeruginosa* exposed to 40-95°C for 10 min

Phage isolates	Temperature									
	50	55	60	65	70	75	80	85	90	95
Pa1	+	+	+	+	+	+	+	+	-	-
Pa2	+	+	+	+	+	+	+	+	-	-

+: Lysis, -: No lysis

Table 5: Dimensions of bacteriophages Pa1 and Pa2 specific to *P. aeruginosa*

Bacteriophages	Head diameter±SD (nm)	Tail	
		Length±SD (nm)	Width±SD (nm)
Pa1	62±2	168±3	8±2
Pa2	57±3	177±2	7±3

SD: Standard deviation, (the recorded values represent the average of 5 replicates)

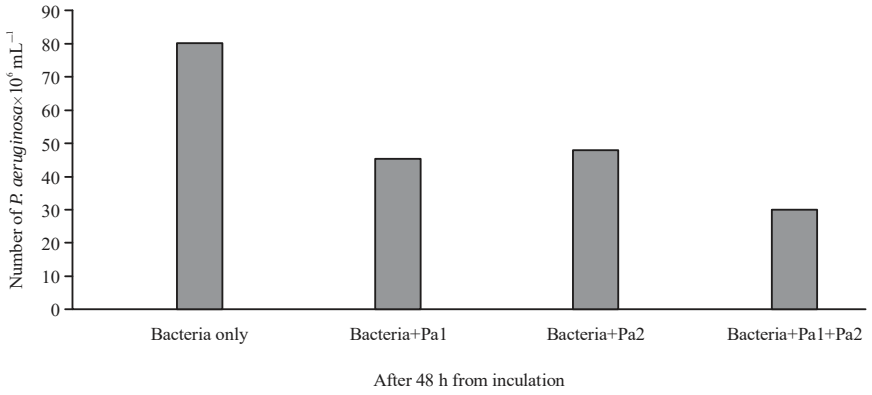

Fig. 4: Total number of *Pseudomonas aeruginosa* in broth media after 48 h with different treatments
Bacteria only: *Pseudomonas aeruginosa*, Pa1: Pa1 Phage, Pa2: Pa2 Phage

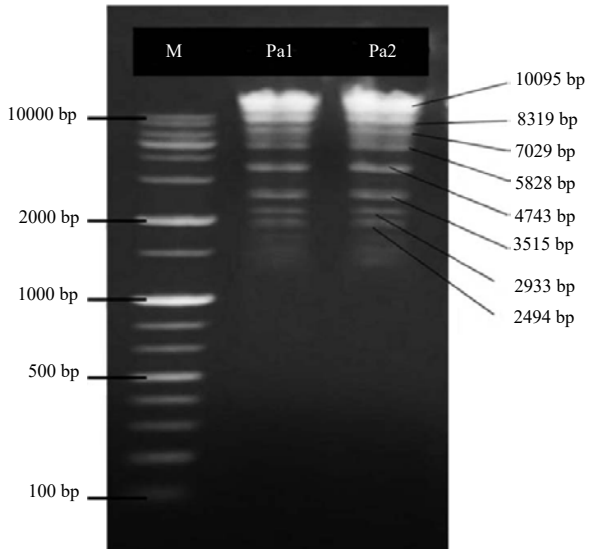

Fig. 5: Electrophoresis of *Hin*dIII DNA restriction fragments of phage isolates Pa1 and Pa2 specific to *P. aeruginosa*
M: 1 Kb (+) DNA ladder

Digestion of Pa1 and Pa2 bacteriophage DNAs with *Hin*dIII produced eight fragments with the same molecular sizes ranging from 2.494-10.095 kbp.

Genotyping and polymorphism using RAPD and SCoT-PCR analysis: RAPD and SCoT-PCR analysis were used to study the genetic diversity of DNA products of the two *pseudomonas aeuroginosa* virulent phages (Pa1 and Pa2) under study. Four primers succeeded to generate reproducible polymorphic DNA products with different sizes with each primer and one primer succeeded only with the Pa1 virulent isolate. The total amplified products were 49 DNA fragments for the 2 virulent isolates, with an average of 9.8 bands/primer (Table 6 and Fig. 6).

Thirty seven bands were found to be unique polymorphic bands for the two virulent phage isolates with all primers

Table 6: Molecular size and polymorphism percentage using RAPD and SCoT markers

Primer names	Molecular size range (bp)	Total number of amplified bands	Polymorphic bands	Percentage of polymorphic loci
OPH-01	170-4066	12	8	66.67
OPH-02	314-696	4	3	75.00
SCoT-7	286-4111	11	9	81.82
SCoT-8	205-1291	8	8	100.00
SCoT-9	251-4971	14	9	64.29
Total		49	37	75.51
Mean		9.8	7.4	

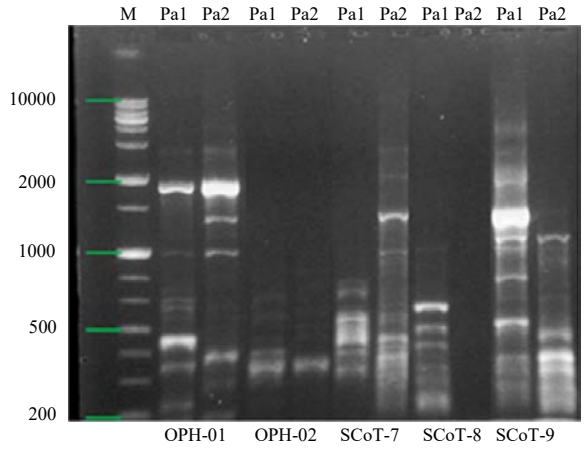

Fig. 6: DNA fingerprints of RAPD and SCoT amplification products generated by OPH-01, OPH-02, SCoT-7, SCoT-8 and SCoT-9 primers for Pa1 and Pa2 phage isolates of *P. aeuroginosa*
M: 1 Kb (+) DNA ladder

(Table 6) with an average of 7.4 polymorphic bands/primer. The total percentage of polymorphism was 75.51%. The sizes of the amplified fragments were ranged from 170-4971 bp. with all used primers.

For OPH-01 primer, eight DNA unique bands were observed, five of them with Pa1 phage isolate and three with Pa2 isolated phage. These sizes ranged from 170-4066 bp. While, for OPH-02 primer, only Pa1 phage isolate exhibited 3 unique DNA bands. The band sizes ranged between 314-696 bp. Regarding SCoT-7 primer, the results showed that 3 unique DNA bands were recorded to Pa1 phage isolate and 6 unique bands were detected with Pa2 phage isolate. The molecular sizes of these bands were between 286-4111 bp. For SCoT-9 primer, eight DNA unique bands were appeared only with Pa2 phage isolate and only one DNA unique band was appeared only with Pa1 phage isolate. The sizes of these bands were in the range of 251-4971 bp.

On the other hand, SCoT-8 primer did not show any response with Pa2 but succeeded to generated eight unique DNA bands only with Pa1 phage isolate. The sizes of the generated bands ranged between 205-1291 bp.

DISCUSSION

In this study *Pseudomonas aeruginosa* was isolated from a sewage water sample after primary treatment collected from Sarpium forest site-Ismailia Governorate, Cairo, Egypt.

The isolated *P. aeruginosa* was tested for lysogeny using UV irradiation (240 nm). No temperate phages were detected.

Lytic bacteriophages specific to *P. aeruginosa* were successfully isolated from the collected sewage water sample. Two phage isolates specific to *P. aeruginosa* were picked and designated Pa1 and Pa2. Similarly results were obtained by Kumari *et al.*[4], Filali *et al.*[32], Alsaffar and Jarallah[33], El Didamony *et al.*[34] and Elmaghraby *et al.*[35] isolated bacteriophages specific to *P. aeruginosa* from sewage water collected from different locations.

Both phages were found to be tolerant to alkaline and acidic reactions. The optimum pH for infection was found to be the same (pH 6) for both phage isolates (Pa1 and Pa2). The 2 lytic phages exhibited the same sensitivity to UV irradiation was found to be 40 min at wave length 245 nm. Bacteriophages Pa1 and Pa2 specific to *P. aeruginosa* were inactivated at 90°C for 10 min. These results are in agreement with those obtained by Elsharouny[36].

The electron micrographs of the two phage isolates (Pa1 and Pa2) indicated that both phages were of head and tail type. The particle dimensions of these two phage isolates were found to be different in their head diameter as well as in tail length and width, this may indicate that these two phages are belonging to Caudovirales, Family Siphoviridae. These results are in agreement with those obtained by Garbe *et al.*[37].

The obtained data, it was found that the isolated virulent phages (Pa1 and Pa2) specific for *Pseudomonas aeruginosa* were efficiently active in reduction the number of the bacterium when compared with inoculation with *Pseudomonas aeruginosa* only after 48 h from inoculation. This result is in agreement with those of Lim *et al.*[38], Hassan[39] and Carstens *et al.*[40] when evaluated the efficacy of complex phage cocktail against *Pectobacterium carotovorum* subsp. *carotovorum* as a biocontrol agent in potato plant.

To confirm if *P. aeuroginosa* phage isolates (Pa1 and Pa2) belong to 1 or 2 different types, two molecular genetic techniques (i.e., restriction pattern and PCR analysis) were carried out. The DNA of each phage isolate (Pa1 and Pa2) was extracted and digested with the restriction enzyme *Hin*dIII. The two phage isolates (Pa1 and Pa2) exhibited the same DNA restriction pattern. This result indicated that the two virulent phage isolates specific to *P. aeuroginosa* contained linear double strand (ds) DNA as a viral genome while restriction enzymes are active only on dsDNA molecules. This result is in agreement with those of Grose and Casjens[41] and Comeau *et al*.[42].

In this study, RAPD and SCoT-PCR analysis of the 2 virulent *P. aeuroginosa* phage isolates DNA, showed that among the 49 amplified DNA fragments, 37 bands were unique polymorphic. The sizes of amplified fragments ranged from 170-4971 bp. Generally, RAPD and SCoT-PCR results strongly confirmed that the 2 *P. aeuroginosa* phage isolates (Pa1 and Pa2) are belonging to two different phage types. So, it could be classified under order Caudovirales, Family Siphoviridae since, both possess long non-contractile tail. Similar results were obtained by Comeau *et al*.[42], Gutierrez *et al*.[43] and Winget and Wommack[44], who evaluated 26 different bacteriophages infecting different bacterial strains using RAPD-PCR.

Based on the obtained results, can be recommend in the future to use the phage as a safe effective biological control agents against the plant pathogen *P. aeuroginosa*.

CONCLUSION

In this study, different physical, molecular characteristics and the effect of phage isolates on *P. aeruginosa* as a biocontrol under lab condition were applied. These two phages are belonging to Caudovirales, Family Siphoviridae. Based on the obtained results, found that both phages are highly stable. Also, both phages in this study could be used as biological control agents against the plant pathogen *P. aeuroginosa*.

SIGNIFICANCE STATEMENT

This study confirmed that lytic phage can be use as a effective agent against the plant pathogen *P. aeuroginosa*. The study contributes to using the phages as biological control under environmental condition.

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