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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2022.433.443

Research Article
Morphogenesis and Genetic Diversity of Some Virulent Phages Specific for *Bacillus velezensis*

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Abstract

Background and Objective: Phosphorus (P) is one of the most limiting nutrients for plant growth. Phosphorus deficiency is limiting crop production in many agricultural soils worldwide. The application of phosphorus solubilizing bacteria (PSB) to soils can replace or partially reduce using of inorganic P fertilizers. A bacteriophage, or phage, is a virus that infects a bacterial cell, taking over the host cell's genetic material. The four phages were propagated, purified, studied for the morphological properties, finally studying the genetic diversity. **Materials and Methods:** Obtained, examined the efficiency and identification of bacteria for solubilizing phosphorus. Isolation, studying the properties and studying genetic diversity. **Results:** Four virulent phages (Bv_1, Bv_2, Bv_3 and Bv_4) specific for *Bacillus velezensis* were isolated from the Egyptian soil. The *Bacillus* phages were purified by alternative low and high-speed centrifugation methods. Electron micrographs showed that phages appeared to be a member of the *Siphoviridae* family based on their structure and particle morphology (the particles have a head and long non-contractile tail). Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique was performed to determine the properties of viral proteins. It was found that the Bv_1 virus had five structural proteins, while Bv_2 and Bv_3 virus had eight structural proteins and finally, the Bv_4 virus had ten structural proteins. The purity and quantity of isolated DNAs were determined spectrophotometrically. Data showed that the concentration of Bv_1 DNA was 0.75 µg, Bv_2 DNA and Bv_3 DNA was 0.60 µg and finally Bv_4 DNA 0.55 µg μL^{-1}. The analysis of genetic material of *B. velezensis* phages was determined based on both the ISSR-PCR technique and the effect of restriction enzymes. Data showed different amplification patterns with all phages. **Conclusion:** The bacteriophages of *B. velezensis* were isolated from soil, propagated, purified, study some of its properties.

Key words: Morphogenesis, genetic diversity, bacteriophages, virulent phages, solubilizing phosphorus, *Bacillus velezensis*, electron microscopy, SDS-PAGE, restriction enzymes, ISSR-PCR

Citation: Alaa, M.B., A.M. El-Ahdal, B.A. Othman and S.S. El-Masry, 2022. Morphogenesis and genetic diversity of some virulent phages specific for *Bacillus velezensis*. Pak. J. Biol. Sci., 25: 433-443.

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Bacillus velezensis is an aerobic gram-positive rod-shaped bacterium is found mainly in soil[1]. This species was initially proposed as the type strain of *B. amyloliquefaciens* subsp. *plantarum* before it was recognized as *B. velezensis*[2]. Many strains of *B. velezensis* are root-associated which are used as plant growth promoters and phosphate-solubilizing bacteria (PSB)[3]. When using sustainable phosphate fertilizers, phosphate-solubilizing microorganisms play an imperative role in supplementing plants with phosphorus. Using of phosphate solubilizing microorganisms (PSM) have been recognized as an alternative method to chemical phosphate fertilizers and provides the available forms of Phosphate to plants[4-6].

Bacteriophages (phages) are the type of viruses that can infect and lyse bacterial cells. Phages have the absolute necessity to infect host cells to multiply and survive. Phages have nucleoid acid (DNA or RNA) that is encapsulated in a protein coat. Two types of the life cycle of phages are found, the first one is the lytic cycle and the second one is the lysogenic cycle. In the Lytic cycle, phages kill bacteria through a multiple-step process and the bacterial cells are destroyed resulting in the release of numerous phage particles. Lytic phages are responsible for controlling bacteria in different environments[7].

The study of protein and nucleic acid is reflected as one of the most important methods used to study the chemical properties of phages. Till now, phages of *B. velezensis* had little study and therefore study aims to shed light on the differences between the isolated virulent bacteriophages specific for *B. velezensis* based on morphogenesis, DNAs and protein contents and genetic diversity.

MATERIALS AND METHODS

Study area: The study was carried out at the Laboratory of Virology, Department of Microbiology, Faculty of Agriculture, Ain Shams University, Cairo, Egypt from February, 2019 to November, 2021.

Source of *B. velezensis* isolate: *Bacillus velezensis* bacterial isolate was kindly obtained from the Department of Microbiology, Faculty of Agriculture, Ain Shams University, Cairo, Egypt

Confirmation of the bacterial isolate by sequencing of 16s rRNA: A slant of pure bacterial culture was sent to macrogen (908 world meridian venture centre, No. 60-24, Gasandong, Geumchun-gu, Seoul 153-781, Korea) for determining the nucleotide sequences of 16s rRNA according to Zhang *et al.*[8]. About 16S rRNA genes were amplified using the following universal primers as shown in Table 1.

Table 1: Primers used for determining the nucleotide sequences of 16s rRNA for *B. velezensis*

Primer names	Types	Sequences
785F	Universal	GGATTAGATACCCTGGTA
907R	Universal	CCGTCAATTCMTTTRAGTTT
518F	Universal	CCAGCAGCCGCGGTAATACG
800R	Universal	TACCAGGGTATCTAATCC

Sequencing analysis: Analysis of the obtained sequences was done by BLASTN 2.2.24 T software (http://www.ncbi.nlm.nih.gov/blast/) for determining the genotype of the *B. velezensis* isolate. The sequence that presented the lowest e value and maximum identity was reflected as the genotype.

Source of bacteriophages of *B. velezensis*: Tryptic soy broth medium (Oxoid, UK) (50 mL) was inoculated with 5 g of sieved free soil and 5 mL overnight mixture culture of *B. velezensis* (10^8 CFU mL^{-1}) then incubated at 30°C for 72 hrs. using a shaking incubator (BOECO, German). Samples were centrifuged at 6000 rpm for 15 min (U-320R BOECO, German). The crude phage suspensions were prepared and examined for the presence of the specific lytic phages according to Krasowska *et al.*[9].

Propagation of the isolated phages: Phage lysates were prepared by the single plaque isolation (SPI) technique according to Krasowska *et al.*[9]. The SPI was repeated three times till obtaining uniform plaque morphology of *B. velezensis* phages. Propagation of the phage isolates on liquid *B. velezensis* culture was done according to Goodridge *et al.*[10], to obtain large scale production of the phages.

Purification and concentration of the phages: Purification of the propagated *B. velezensis* phages was done by alternative low (3000 rpm min^{-1}) (BOECOU-320, German) and high (30000 rpm min^{-1}) speed centrifugation (Beckman L7-35, USA) according to Krasowska *et al.*[9].

Determination of size and shape of *B. velezensis* phages: Electron microscopy examination of the prepared purified phage samples was performed using transmission electron microscope JEOL (JEM 2100, USA) high resolution at the Nanotech for photo electronics, Dreamland, Gate3, 6th October, Giza, Egypt after negative staining with 2% (w/v) Uranyl acetate (Sigma, USA) (pH 4.9). Electron micrographs were taken at an accelerating voltage of 60 kV to determine the size and shape of the examined phage particles[11].

Determination of the protein pattern of *B. velezensis* phages: The molecular weight of the main virus proteins was determined by the SDS-polyacrylamide gel electrophoresis technique according to Walker[12]. Gels were formed between two glass plates (16×16 cm separated by 1.0 mm thick Teflon spacer) (Biorad, UK). About 25 µL of viral proteins samples were loaded in each well. Electrophoresis was done in the presence of a high range molecular weight protein marker at about 100 and 200 V for running through stacking and separating gel, respectively. Gels were stained with 0.1% Coomassie Brilliant Blue G-250 (Sigma, USA) and destained in destaining solution according to Sambrook and Russell[13] and results were analyzed.

Extraction of viral genomes: To extract the phages' genomes, the method described by Campos *et al.*[14] was used. At the final step, the pellets were washed in 70% ethanol (Sigma, USA) and air-dried at room temperature. Then suspended gently in 30 µL of TE buffer (Sigma, USA) and stored at 4°C up to use. The purity and quantity of the extracted viral DNAs were spectrophotometrically determined using UV-Vis spectrophotometer (Orion Aquamate 8000, USA). The obtained optical density (OD) values at 260 nm wavelength were recorded.

Agarose gel electrophoresis: Agarose gel electrophoresis was carried out according to Walker[12] using the tris-borate EDTA buffer (TBE) (Difco, USA). A stock of 10× TBE contains 890 mM tris base, 890 mM boric acid, 25 mM EDTA (Difco, USA) pH 8.3 was set. The DNA samples containing markers were prepared by mixing 10 µL of the DNA solution with 3 µL of loading dye containing 450 mM tris borate pH 8.3, 50 mM EDTA, 50% (v/v) glycerol and 0.2% (w/v) bromophenol blue (Difco, USA). Electrophoresis (Cleaver Scientific, MSMAXI 10, UK) was conceded at 60 V for 3 hrs. About 1 µg mL^{-1} of ethidium bromide (Sigma, USA) was used to stain the gel for 30 min and another 30 min was used for destaining using 1 L of distilled water. The gel was examined using an ultraviolet transilluminator at 302 nm (Cleaver Scientific, electrophoresis Translaminator, CSLUVTS xxx, UK). The DNA bands were photographed and the obtained data were analyzed (Cleaver Scientific micro DOC compact Gel documentation system DI-HD-220, UK).

Digestion of phages DNAs using restriction enzymes: The procedure was carried out according to Abo-Senna[15]. Two microliters of 10× buffer, 1 µg of phage DNA and 3-5 units of restriction enzyme (*Eco*RI, *Hind III* and *Bam*) (Sigma, USA) were mixed and the total volume was completed to 20 µL with sterile deionized water. The mixture was incubated for 2 hrs at 37°C. The digested DNAs were stored at 4°C until being ready for analysis using the agarose gel electrophoresis technique. The 1.2% agarose gel was used to separate the digested DNA according to Lee *et al.*[16] in the presence of a 1 Kbp DNA ladder (RTU). Using an ultraviolet transilluminator to examine the stained gel (Cleaver Scientific, electrophoresis Translaminator, CSLUVTS xxx, UK).

Table 2: Sequences and annealing temperature of primers for ISSR

Primers	Sequence	Annealing temperature (°C)
1	5'-AGAGAGAGAGAGAGAGYC-3'	53
2	5'-AGAGAGAGAGAGAGAGYG-3'	
3	5'-ACACACACACACACACYT-3'	
4	5'-ACACACACACACACACYG-3'	
5	5'-GTGTGTGTGTGTGTGTYG-3'	
6	5'-CGCGATAGATAGATAGATA-3'	
7	5'-GACGATAGATAGATAGATA-3'	
8	5'-AGACAGACAGACAGACGC-3'	
9	5'-GATAGATAGATAGATAGC-3'	
10	5'-GACAGACAGACAGACAAT-3'	

Inter Simple Sequence Repeat (ISSR) PCR: Ten primers were used to amplify the extracted DNA samples. The code names, sequences and annealing temperature of the used primers are shown in Table 2. The annealing temperature for all used primer was 53°C. Mixture of 25 µL as a total volume containing (0.4 mM dNTPs, 3 mM Mg Cl_2, 30 mMKCl, 10 mMTris (pH 8.3), 1X buffer primer ONA (25 pmol) and Taq DNA polymerase) (Sigma, USA). Amplification was carried out in a DNA thermal cycler Thermocycler MJ Research (PTC-200, Montreal, Canada) under the following condition, Initial denaturation for 5 min at 95°C, followed by 40 cycles for 1 min at 94°C denaturations, annealing at 43°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 5 min was used for amplification. About 25 µL of amplified products were mixed with 3 µL loading buffer (10 m MTris –HCl, pH 7.6) 0.03% bromophenol blue, 0.03% xylene cyanol F F, 60% glycerol 60 mM EDTA) 1.3% agarose gel (Sigma, USA) was used to separate the extracted DNA. Then stained with 0.5 µg mL^{-1} ethidium bromide in 1× TBE buffer (89 mMTris-borate, 89 mM boric acid , 2 mM EDTA, pH 8.3) (Sigma, USA). Visualization of gel was done under an ultraviolet transilluminator (Cleaver Scientific, electrophoresis Translaminator, CSLUVTS xxx, UK). The DNA fragments sizes were determined by comparing with the 1 kb DNA ladder marker according to Al-Qurainy *et al.*[17].

ISSR data analysis: The obtained data of ISSR-PCR analysis was entered separately in a computer file as a binary matrix where '1' for the presence of DNA band and '0' for the absence of a band for each primer in each individual. The number of monomorphic and polymorphic amplified products produced by each primer was recorded. The genetic diversity was calculated for all viral samples and subjected to cluster analysis to produce a dendrogram based on Jaccard's similarity Co-efficient by unweighted pair group method (UPGMA) using PAST programme version 2.17 c described by Yadav *et al*.[18].

RESULTS

Identification and confirmation of *B. velezensis* strain: The identification and confirmation of the *B. velezensis* isolate were done using the 16S rRNA sequencing technique. Table 3 showed that, after determination of the nucleotide sequences of the 16S rRNA gene, a partial sequence of 1407nts was obtained and recorded in the Gene bank under the accession number LC634434. The identities percentage between *B. velezensis* and the different eight *Bacillus* strains recorded in the Gene bank was done. Results revealed that the identities percentages ranged from 92.38-99.64% between *B. velezensis* strains and the isolated *B. velezensis* (LC634434). Whereas, the highest identities percentage were 99.64% between the isolated *B. velezensis* (LC634434) and *Bacillus velezensis* (CP054714.1) with query covering 100%. The same identities percentages (99.64%) was recorded between the isolated *B. velezensis* (LC634434) and *Bacillus velezensis* (MT573877.1) but the query cover was 98%. On the other hand, the lowest identities percentages were 92.38% between the isolated *B. velezensis* (LC634434) and *Priestia megaterium* (FR715572.1) with query covering 92%.

The phylogenetic tree of the *B. velezensis* strain based on the 16 s rRNA gene illustrated by Fig. 1 showed that *Bacillus* strains befallen in two clusters. Cluster one contains *B. megaterium* isolate 11E and *B. megaterium* strain 21:1. While cluster two contains the isolated *B. velezensis* (Query_2562), *Bacillus subtilis* subsp. Subtilis, *Bacillus amyloliquefaciens* strain NBRC 15535, *Bacillus siamensis* strain KCTC and *Bacillus velezensis* strain KKLW.

Isolation and detection of *Bacillus velezensis* phages: Sixteen soil samples were collected from different sites (8 from Qalubiah, 3 from Giza, 2 from Behira, 1 from Bani- soif, 1 from Menofiah and 1 from Ismailia governorates Egypt. All samples were tested qualitatively by the spot test and quantitatively by the plaque assay technique for the presence of lytic *Bacillus velezensis* phages. Table 4 and Fig. 2 show that, lytic phages specific for the target bacteria were found in all samples. Concentrations or titers of the isolated phages were ranged from 1.23×10^{8}-4.24×10^{9} PFU mL^{-1}. The phage isolated from Ismailia governorate showed a single clear and

Table 3: Sequences producing significant alignments of *Bacillus* isolate under investigation compared to different *Bacillus* species

Description	Scientific names	Query_2562 Cover (%)	Identities (%)	Accessions
Bacillus velezensis strain Y17W 16S ribosomal RNA gene, partial sequence	*Bacillus velezensis*	98	99.64	MT573877.1
Bacillus velezensis strain KKLW chromosome, complete genome	*Bacillus velezensis*	100	99.64	CP054714.1
Bacillus siamensis strain KCTC 13613 16S ribosomal RNA gene, partial sequence	*Bacillus siamensis*	100	99.57	KY643639.1
Bacillus amyloliquefaciens strain NBRC 15535 16S ribosomal RNA, partial sequence	*Bacillus amyloliquefaciens*	100	99.42	NR_041455.1
Bacillus subtilis subsp. subtilis str. 168 genome	*Bacillus subtilis* subsp. subtilis str. 168	100	99.35	CP019662.1
Bacillus megaterium partial 16S rRNA gene, isolate 11E	*Priestia megaterium*	94	93.28	HE965432.1
Bacillus megaterium partial 16S rRNA gene, strain 21:1	*Priestia megaterium*	92	92.38	FR715572.1

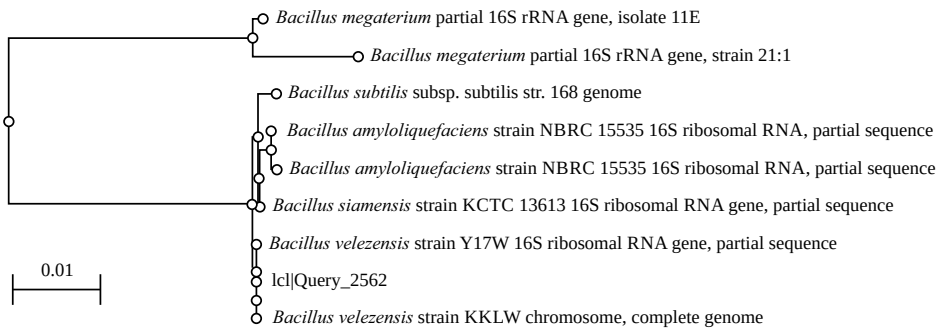

Fig. 1: Phylogenetic tree of the isolated *B. velezensis* (Query_2562) compared with different *Bacillus* species

circular plaque with a diameter of about 2 mm and with a clear centre. The phage isolated from the Menofiah governorate showed the same plaque morphology but with a diameter of about 3 mm. But the phages that were isolated from Bani-soif samples presented large circular, clear plaques of 4 mm and with the opaque area. Finally, the phages that were isolated from the Qalubiah sample presented small circular plaques with a diameter of about 2 mm. These plaques were picked up and propagated using the host liquid culture (10^8) CFU mL^{-1} to obtain biologically purified phage lysates.

Propagation, purification and concentration of *B. velezensis* phages: To obtain biologically pure phage isolate, the single plaque isolation method was done. The high titer of the isolated phage stock was taken by phage propagation several times by the liquid culture method. Table 4 showed that the titer of phages after the propagation were 10^8, 10^8, 10^8 and 10^9 PFU mL^{-1} for Bv_1, Bv_2, Bv_3 and Bv_4 phages, respectively. But the titers were 10^{10}, 10^{11}, 10^{11} and 10^{11} PFU mL^{-1} for Bv_1, Bv_2, Bv_3 and Bv_4 phages after the purification as described above in materials and methods.

Morphological properties morphogenesis of *Bacillus velezensis* phages: Electron micrographs were obtained after the electron microscopy to determine the size and shape of the isolated phages. Table 5 and Fig. 3a-d show that isolated phages have an isometric head with a diameter of 65.88, 61.48, 80.02 and 71.38 nm and non-contractile tail with a length of 264.65, 241.68, 241.15 and 237.66 nm for Bv_1, Bv_2, Bv_3 and Bv_4 phages, respectively. Based on the morphogenesis, all the isolated phages are belonging to the family siphoviridae.

Nomenclature of the isolated phages specific for *B. velezensis*: The nomenclature of the isolated lytic phages specific for *B. velezensis* was designed depending upon three main criteria to preceded by VB which referred to the bacterial virus, bacterial host abbreviation name and the viral family abbreviation which is indicated by the viral morphology illustrated by electron microscope and finally the simple abbreviation of common name in letter/number designated specifically for the laboratory depending upon the working researchers, therefore the names of the isolated phages according to the above mentioned are:

- 1: VB-BVS-AE1
- 2: VB-BVS-AE2
- 3: VB-BVS-AE3
- 4: VB-BVS-AE4

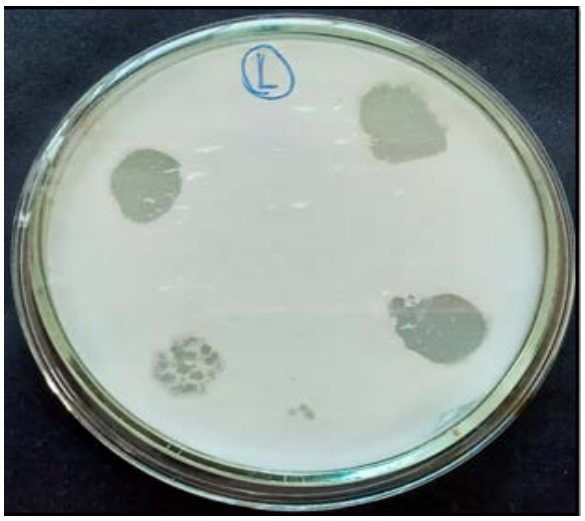

Fig. 2: Detection of *Bacillus velezensis* phage by the spot test

Table 4: Qualitative and quantitative assaying of the isolated *Bacillus velezensis* phages

Samples	Sample location	Qualitative assaying	Quantitative assaying
1	Behira	+	2.32×10^8
2	Behira	+	3.12×10^8
3	Ismailia	+	1.21×10^8
4	Menofiah	+	2.24×10^8
5	Bani-soif	+	1.23×10^8
6	Giza	+	2.56×10^8
7	Qalubiah	+	4.24×10^9
8	Giza	+	3.22×10^8
9	Giza	+	1.54×10^8
10	Qalubiah	+	2.45×10^8
11	Qalubiah	+	3.13×10^8
12	Qalubiah	+	3.67×10^8
13	Qalubiah	+	1.23×10^8
14	Qalubiah	+	2.11×10^8
15	Qalubiah	+	1.67×10^8
16	Qalubiah	+	2.49×10^8

+: Means that positive result of the phage presence

Table 5: Morphological properties of *Bacillus velezensis* phages

	Head		Tail		
Phage	Shape	Diameter of the head (nm)	Shape	Length (nm)	Expected family
Bv_1 (VB-BVS-AE_1)	Isometric-cubic	65.88	Non-contractile	264.65	Siphoviridae
Bv_2 (VB-BVS-AE_2)	Isometric-cubic	61.48	Non-contractile	241.68	
Bv_3 (VB-BVS-AE_3)	Isometric-cubic	80.02	Non-contractile	241.15	
Bv_4 (VB-BVS-AE_4)	Isometric-cubic	71.38	Non-contractile	237.66	

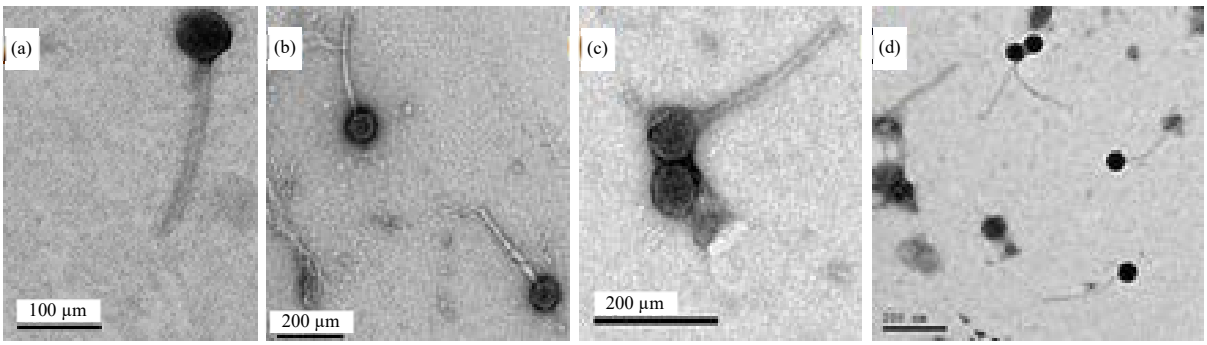

Fig. 3(a-d): Electron micrographs of *B. velezensis* phages stained with 2% uranyl acetate show the morphogenesis of the isolated *B. velezensis* phages, (a) Bv_1, (b) Bv_2, (c) Bv_3 and (d) Bv_4

Table 6: Relationship between molecular weight (MW) of *B. velezensis* phages and retardation factor (R_f)

Lane 1 (Bv_1)			Lane 2 (Bv_2)			Lane 3 (Bv_3)			Lane 4 (Bv_4)			Lane 5 (Marker)		
Band	Rf	MW	Band	Rf	MW	Band	Rf	MW	Band	Rf	MW	Band	Rf	MW
1	0.284	67	1	0.31	61	1	0.269	71	1	0.374	49	1	0.02	250
2	0.32	58	2	0.382	48	2	0.3	63	2	0.413	44	2	0.114	130
3	0.41	44	3	0.421	43	3	0.377	48	3	0.541	34	3	0.205	100
4	0.504	36	4	0.52	35	4	0.495	36	4	0.659	30	4	0.278	70
5	0.54	34	5	0.537	34	5	0.526	35	5	0.776	27	5	0.4	55
6	0.636	30	6	0.575	32	6	0.56	33				6	0.673	35
7	0.704	29	7	0.637	30	7	0.651	30				7	0.799	25
8	0.727	28	8	0.81	27	8	0.803	27				8	0.987	15
9	0.78	27												
10	0.955	26												

Table 7: DNA contents of the isolated phages

Phage	Concentration (µg mL^{-1})
Bv_1	0.75
Bv_2	0.60
Bv_3	0.60
Bv_4	0.55

Electrophoretic properties of the viral proteins: One of the chemical characteristics of phages includes protein patterns. SDS-polyacrylamide gel electrophoresis was used to study the protein profiles of *B. velezensis* phages. The molecular weights of phages proteins were determined compared with marker proteins using Gel Analyzer V19.1. Figure 4 shows the Bv_1 phage had five structural proteins with molecular weights known and extrapolated of 49, 44, 34, 30 and 27 KDa. The Bv_2 phage had eight structural proteins with molecular weights known and extrapolated of 61, 48, 43, 35, 34, 32, 30 and 27 KDa. Also, Bv_3 phage had eight structural proteins but with molecular weights known and extrapolated of 71, 63, 48, 36, 35, 33, 30 and 27 KDa. Finally, the Bv_4 phage had ten structural proteins with molecular weights known and extrapolated of 67, 58, 44, 36, 34, 30, 29, 28, 27 and 26 KDa. Quantitative protein bands and retardation factor (R_f) of *B. velezensis* phages were analyzed with Gel Analyzer V19.1. Table 6 shows that there was an inverse relationship between the molecular weight of bands of all isolated phages and R_f.

Determination of DNA contents of the isolated phages: The quantity of the isolated phage DNAs was determined spectrophotometrically and the optical densities (OD) values at 260 nm are shown in Table 7.

Digestion of bacteriophages DNAs with restriction enzymes: The type of nucleic acid in the isolated *B. velezensis* phages was a linear double-stranded (ds) DNA because they were susceptible to digestion with two endonucleases restriction enzymes. Table 8 and Fig. 5 approved that the DNA of the isolated phages are sensitive to *Eco*RI, *Hind III*, but using *Bam* doesn't show any effects on the DNA of all isolated phages. The genomes were fragmented into 3, 2, 1, 2 and 2, 3, 3, 5 total amplified bands (TABs) with *Eco*RI and *Hind III*, respectively. But the genomes were not fragmented with the *Bam* restriction enzyme.

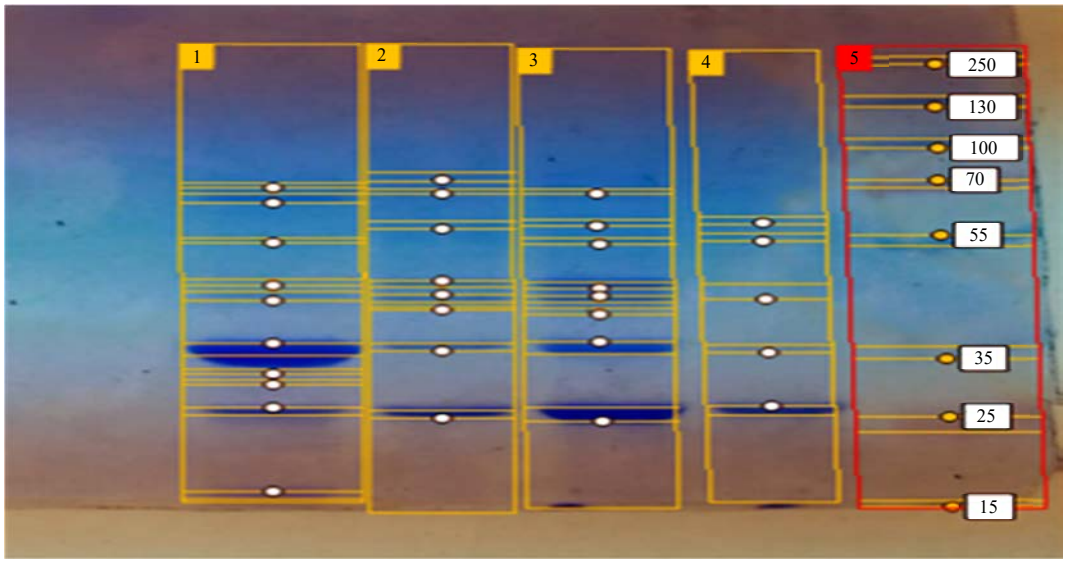

Fig. 4: Protein patterns of *B. velezensis* phages as determined by 12% SDS- PAGE
Lane 1: Bv_1, Lane 2: Bv_2, Lane 3: Bv_3, Lane 4: Bv_4 and Lane 5 M: Marker protein

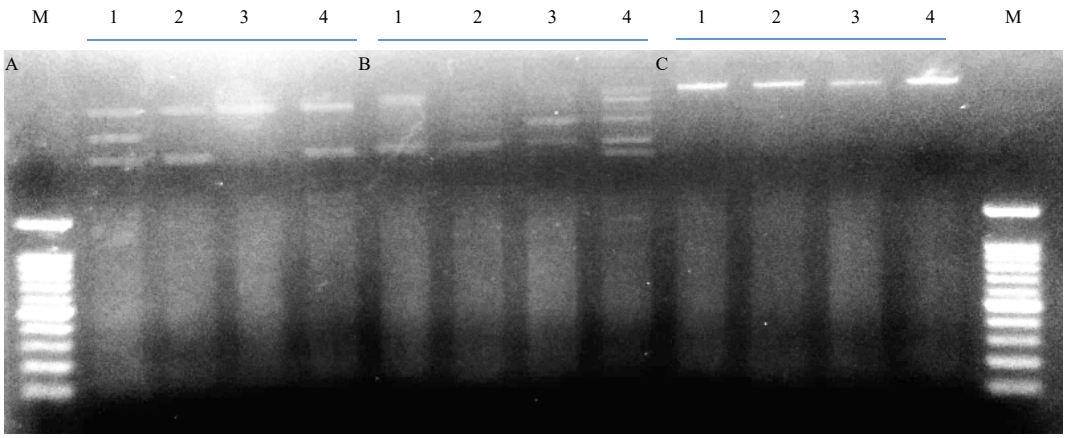

Fig. 5: Restriction enzymes pattern on 1.2% agrose gel of *Bacillus velezensis* phages
1: Bv_1 phage, 2: Bv_2 phage, 3: Bv_3 phage, 4: Bv_4 phage, A: *EcoR*$_1$, B: *Hind III*, C: *Bam* and M: Marker

Table 8: Total amplified bands of *B. velezensis* phages as revealed by Restriction Enzymes

Restriction	Bv_1 DNA				Bv_2 DNA				Bv_3 DNA				Bv_4 DNA			
Enzyme	TAB	MP	PM	Unique	TAB	MP	PM	Unique	TAB	MP	PM	Unique	TAB	MP	PM	Unique
ECORI	3	1	1	1	2	1	1	0	1	0	1	0	2	1	1	0
HIND III	2	0	2	0	3	1	2	0	3	1	2	0	5	1	2	2
BAM	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

TAB: Total amplified band, MB: Monomorphic band and PB: Polymorphic band

ISSR-PCR analysis: Table 9 and Fig. 6a-d represent that the ten used primers differ from each other in the total number of amplified bands of each phage. Total amplified bands (TABs) ranged between 3-14 bands with all used primers (from A-J). The best and highest primers were primer A, G and H with sequence of 5'-AGAGAGAGAGAGAGAGYC-3', 5'-GACGATA GATAGATAGATA-3' and 5'-AGACAGACAGACAGACGC-3', respectively. The lowest primer was primer J with the sequence of 5'-GACAGACAGACAGACAAT-3' which separate 3 TABs with all isolated phages, 1 TAB with Bv_2, Bv_3 and Bv_4, Where separate 0 TAB in Bv_1. All used primers were produced unique bands that responsible for genetic diversity between

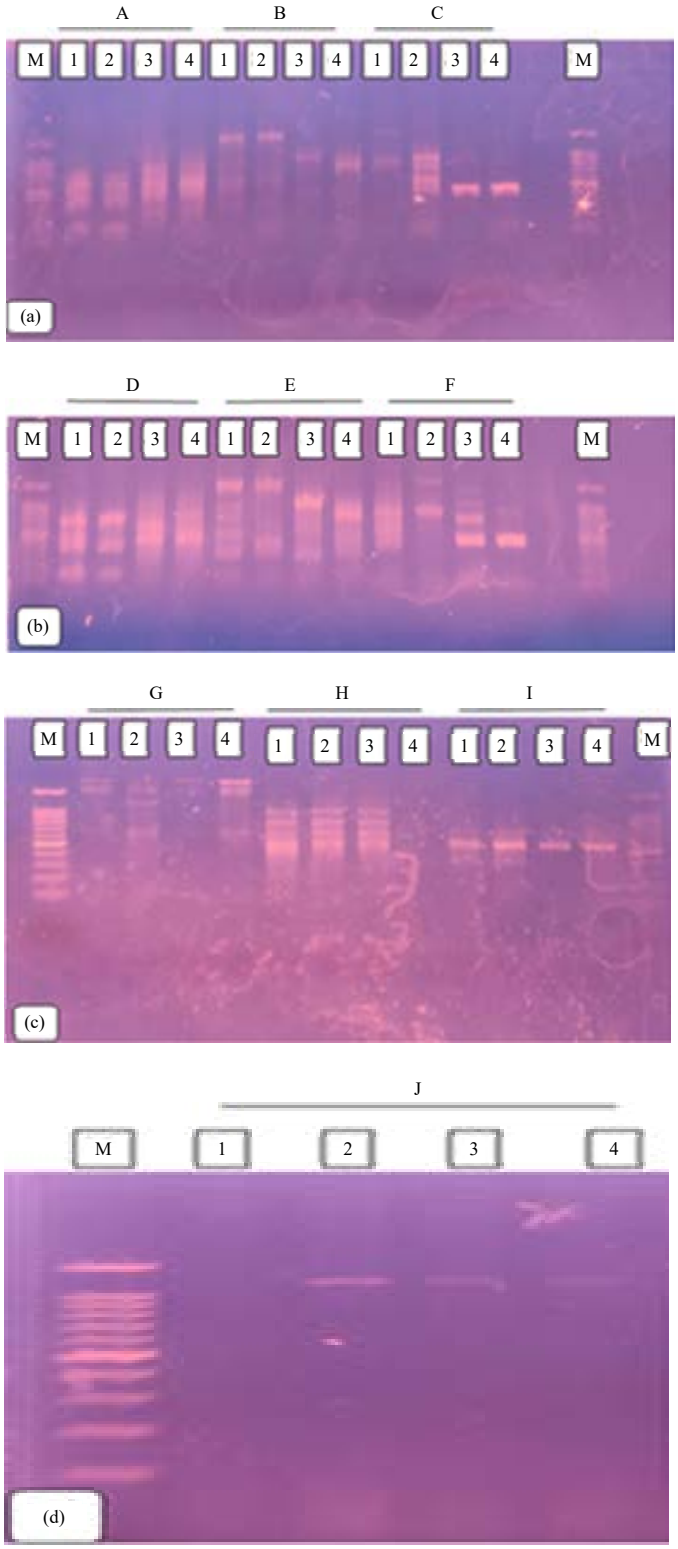

Fig. 6(a-d): ISSR-PCR pattern on 1.2 % agrose gel of *Bacillus velezensis* phages , (a) *B. velezensis* phages with primers (A, B, C), (b) *B. velezensis* phages with primers (D, E, F), (c) *B. velezensis* phages with primers (G, H, I) and (d) *B. velezensis* phages with primers (J)

Lane 1: Bv_1, Lane 2: Bv_2, Lane 3: Bv_3, Lane 4: Bv_4 and Lane M: Marker DNA 100 bp

Table 9: Total amplified bands of *B. velezensis* phages as revealed by ISSR-PCR technique

		Bv_1				Bv_2				Bv_3				Bv_4			
Primers	Sequence of nucleotides	TAB	MP	PM	Unique	TAB	MP	PM	Unique	TAB	MP	PM	Unique	TAB	MP	PM	Unique
A	5'-AGAGAGAGAGAGAGAGYC-3'	4	3	1	0	4	3	1	0	3	2	1	0	3	1	1	1
B	5'-AGAGAGAGAGAGAGAGYG-3'	4	3	0	1	2	2	0	0	1	1	0	0	2	2	0	0
C	5'-ACACACACACACACACYT-3'	2	1	0	1	4	1	0	3	1	1	0	0	1	1	0	0
D	5'-ACACACACACACACACYG-3'	4	3	1	0	3	2	1	0	2	1	1	0	2	1	1	0
E	5'-GTGTGTGTGTGTGTGTYG-3'	5	5	0	0	2	2	0	0	2	2	0	0	1	1	0	0
F	5'-CGCGATAGATAGATAGATA-3'	2	2	0	0	2	1	0	1	4	3	0	1	2	2	0	0
G	5'-GACGATAGATAGATAGATA-3'	3	1	2	0	6	1	2	3	2	0	2	0	3	1	2	0
H	5'-AGACAGACAGACAGACGC-3'	4	4	0	0	5	5	0	0	5	5	0	0	0	0	0	0
I	5'-GATAGATAGATAGATAGC-3'	2	1	1	0	3	1	1	1	1	0	1	0	2	1	1	0
J	5'-GACAGACAGACAGACAAT-3'	0	0	0	0	1	1	0	0	1	1	0	0	1	1	0	0
	TABs	30				32				22				17			

TAB: Total amplified band, MB: Monomorphic band, PB: Polymorphic band

Table 10: Similarity percentage between *B. velezensis* phages revealed by ISSR PCR techniques

0	Bv_1	Bv_2	Bv_3	Bv_4
Bv_1	1	0.75	0.612	0.5
Bv_2	0.75	1	0.53	0.454
Bv_3	0.612	0.53	1	0.54
Bv_4	0.5	0.454	0.54	1

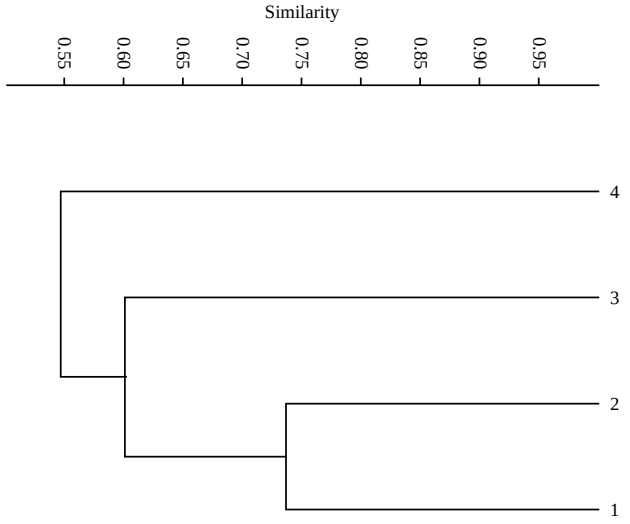

Fig. 7: Pattern of the phylogenetic tree of *B. velezensis* phages revealed by ISSR- PCR technique

viruses, exclusion primers D, E, H and J don't represent any unique bands with all isolated phages. In Bv_4 one of the unique bands is produced by primer A only. But in Bv_1, two unique bands can produce by primer B and C only. Total amplified bands (TAB) were 30, 32, 22 and 17 for Bv_1, Bv_2, Bv_3 and Bv_4, respectively, with all used primers. But total unique bands were 2, 8, 1, 1 for Bv_1, Bv_2, Bv_3 and Bv_4, respectively.

Table 10 and Fig. 7 revealed that the similarity percentage ranged from 45.4-75% between the isolated phages. The maximum genetic similarity was (75%) between Bv_1 and Bv_2. When the minimum genetic similarity was (45.4%) between Bv_4 and Bv_2. Cluster analysis was done to estimate the level of polymorphism between *B. velezensis* phages. The generated dendrogram based on ISSR-PCR data analysis grouped the phages into three main clusters (Fig. 7). In this grouping, Bv_1 and Bv_2 were in separated clusters while Bv_3 was in the separated cluster and Bv_4 in another cluster.

DISCUSSION

The traditional identification methods of bacterial isolates based on the phenotypic properties are generally. Comparison of the bacterial 16S rRNA gene sequence has appeared as an ideal genetic technique[19]. The identification of the obtained bacterial isolate was made by the 16s rRNA sequencing method, the results showed that a partial sequence of 1407 nts of *B. velezensis* isolate was achieved and documented in the Gene bank under the accession number LC634434. By comparing the identities percentage of the obtained sequences of 16S rRNA genes of the eight bacterial strains on the Gene bank, logical data were obtained and the percentages ranged from 92.38-99.64% between *Bacillus velezensis* and other strains recorded in the Gene bank. Four phages specific for *Bacillus velezensis* named VB-BVS-AE_1, VB-BVS-AE_2, VB-BVS-AE_3 and VB-BVS-AE_4 were isolated from different locations in Egypt depending upon the plaque morphology by SPI technique. The obtained data agree with Krasowska *et al.*[9], who isolated *Bacillus* bacteriophages from the soil.

Large amounts with a high titer of target isolated phages were prepared in this study by the liquid propagation method. Some investigators used the liquid propagation method to propagate their isolated bacteriophages[20], whereas others prepared the high titer phage lysates from the plates resulting from the plaque assay method and representing confluent and semi-confluent lysis[20].

After high titer phages lysates had been obtained, all isolated phages were purified using the differential centrifugation technique. The cycles of low and high-speed centrifugation were repeated three times to concentrate remove undesired impurities. Some authors used the same method to purify their phages[9]. The titers of phages after propagation were ranged between 10^8-10^9 PFU mL^{-1}. Though titers of phages were ranged between 10^{10}-10^{11} PFU mL^{-1} after propagation and purification.

To determine the size and shape of all isolated phages, an examination by TEM was done. Electron micrographs showed that the four isolated phages appeared to be members of family *Siphoviridae* based on particle morphology. The obtained data agree with Othman *et al.*[21].

Proteins patterns of the isolated phages were electrophoretically achieved and it was found that the Bv_1 phage showed five structural proteins with molecular weights ranging between 27-49 KDa while the Bv_2 phage had eight structural proteins with molecular weights ranging between 27-61 KDa. Also, the Bv_3 phage had eight structural proteins with molecular weights ranging between 27-71 KDa and finally, the Bv_4 phage showed ten structural proteins with molecular weights ranging between 27-67 KDa. As revealed by Krasowska *et al.*[9] phages SIOΦ, SUB and SPO specific for *Bacillus* had structural proteins with molecular weight 52, 31 and 45 kDa for SIOΦ, SUB and SPO phages, respectively. Whereas, phage AR showed at least 14 structural proteins and revealed that it produces three major protein bands at molecular masses around 42, 37 and 31 kDa.

When the phage DNA concentration was determined it was found that the DNA concentrations were 0.75, 0.60, 0.60 and 0.55 µg for Bv_1, Bv_2, Bv_3 and Bv_4, respectively. Authors repeated that the ratio of OD values for DNA at 260/280 nm was more or less similar (1.7-1.9)[22].

Restriction endonucleases enzymes *Eco*RI, *Hind III* and *Bam* were used to digest phages DNAs, it was found that the phages DNAs were sensitive to *Eco*RI and *Hind III* whereas, *Bam* didn't show any effect on the phage DNAs. The genomes were fragmented into 3, 2, 1, 2 and 2, 3, 3, 5 total amplified bands (TAB) with *Eco*RI and *Hind III* with Bv_1, Bv_2, Bv_3 and Bv_4, respectively while the phage genomes didn't fragment with *Bam*. This obtained data agree with Abo-Senna[15], who revealed that the genomes of Bt1 and Bt2 phages were fragmented into four fragments with the size of 1500, 1400, 800 and 510 bp.

In the ISSR-PCR technique, the phages DNAs were amplified by ISSR-PCR by microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18 bp). Around 10-60 fragments from multiple loci are generated concurrently, separated using gel electrophoresis and scored as the presence or absence of fragments of a particular size according to the previous reports[23]. One of the main advantages of ISSRs is that no sequence data for primer edifice are necessary. Because the analytical procedures contain PCR, only low quantities of template DNA are essential. Moreover, ISSRs are haphazardly distributed in the genome. Because of the multilocus fingerprinting profiles achieved, ISSR analysis can be useful in studies including genetic identity, parentage, clone and strain identification and taxonomic studies of narrowly related species. In addition, ISSRs are reflected beneficial in gene mapping studies[23]. The analysis based on ISSR-PCR technique allowable the estimation of the genetic material of *B. velezensis* phages. Using of ten ISSR primers gave different amplifications patterns obtained that, Primers 5'-AGAGAGAGAGAGAGAGYC-3', 5'-GACGATAGATAGATAGATA-3' and 5'-AGACAGACAGACAGACGC-3' produced the highest total number of amplified bands. On the other hand, Primer 5'-GACAGACAGACAGACAAT-3'generated has the lowest total number of amplified fragments. The maximum genetic similarity was 75% between Bv_1 and Bv_2, 61.2% between Bv_1 and Bv_3 and 50% between Bv_1 and Bv_4. While the lowest value of genetic similarity was 45.4% between Bv_4 and Bv_2.

CONCLUSION

In this investigation, four phages specific for *B. velezensis* were isolated from Egyptian soil and signed as Bv_1, Bv_2 and Bv_3 and Bv_4. The four phages were propagated, purified, studied for their morphological properties. Finally studying the genetic diversity by both restriction enzymes technique and ISSR-PCR technique for all phages were done and results revealed that the four phages are different between them.

SIGNIFICANCE STATEMENT

This study revealed the effect of some virulent phages on *B. velezensis* as a solubilizing phosphorus bacteria. Which is reflected in the process of solubilizing phosphorus in the Egyptian soils. Thus affecting the crops, for which phosphorus is an essential factor, as in the oil crops. This study will help the researcher to find different ways to protect the solubilizing phosphorus bacteria from phages attacks. Especially the field of study of the effect of phages on *B. velezensis* has not been studied sufficiently and research on this area are limited and possibly other combinations, may be arrived at.

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