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Isolation and characterization of three bacteriophages infecting *Erwinia amylovora* and their potential as biological control agent

Wessam Hassan[1], Osman Ahmed[2,3], Rasha E. Hassan[2], Sahar A. Youssef[1] and A. A. Shalaby[1*]

Abstract

Background Fire Blight, incited by *Erwinia amylovora*, is one of the most damaging pear and apple diseases in the world. Fire blight was introduced to Egypt in the 1960 and threatens the Egypt's costs for pear industry. Currently, Phage therapy is considered to be secured biological method for controlling plant bacterial diseases. This investigation aimed to isolate and identify molecularly for bacteria causing fire bright disease. As well as isolation and identification bacteriophages via spot and plaque assay techniques from pear fire blight lesions and soil. On the other hand, bacteriophages were identified based on plaque morphology, virion morphology, physical characters, profile of DNA restriction and protein.

Results Pathogenicity test revealed that healthy seedlings and pear fruits were responsive to fire blight *E. amylovora*. Considering the relatively wide host range and greatest protein and genetic variability, using restriction enzyme pattern, the three diversity phage isolates named, EAP1, EAP2 and EAP3 showed a lack of diversity out of five were fatherly characterized. The phages confirmed the close relation of EAP1, EAP2 to Siphoviridae (hexagonal head and long flexible non-contractile tail) and EAP3 to Myoviridae (icosahedral head and contractile tail). The phages retained higher lytic competence of 90.4; 92.68 and 95.25% for EAP1, EAP2 and EAP3, respectively. The phages were stable at strong alkaline (pH 10) 2% salt solution conditions and UV spectra. While EAP3 phage revealed the hexagonal head and very short tail that belongs to Myoviridae family. Bacteriophages were characterized by digestion of the phage DNA with three restriction endonucleases and were placed into three groups based on the patterns. Bacteriophages were 9 used for reducing bacterial infection populations and severity on pear. In a bioassay, the biocontrol of *E. amylovora* was evaluated using disks of immature pear fruit. On the pear disk surface, bacterial exudate was considerably suppressed by all phage isolates. According to measurements of the bacterial population still present on the disk surface, phage therapy could reduce it by up to 97%. Bacteriophages reduced pear fire blight disease severity on pear fruit trails.

Conclusion The results indicated that bacteriophage isolates may demonstrate variable reactivity against *E. amylovora*. Bacteriophages reduced pear fire blight disease severity on pear fruit trials. The results indicated that bacteriophage isolates may demonstrate variable reactivity against *E. amylovora*.

Keywords Pear, Fire blight, Bacteriophage, RAPD and biocontrol

*Correspondence:
A. A. Shalaby
aaashalaby@gmail.com
Full list of author information is available at the end of the article

Background

Pear (*Pyrus communis* L.) is popular with consumers for its distinct scent, delicate aroma, sweetness, and crispness. It is a common fruit found in temperate regions, has a delicate flavor that is pleasant, and is well-liked by people. According to (Agricultural Statistics Institute 2019) pear cultivated area is 13,439 fadden produced 68,407 tons. Production of pears varied from one orchard to another and from year to year. This difference may be the result of a variety of circumstances, such as inadequate fruit set brought on by fire blight, rootstock, and insufficient chilling hours. Fire blight disease is the most serious bacterial disease to affect pome fruit trees and other plants of the Rosaceae family globally, which is brought on by the bacterium *Erwinia amylovora* (Burill) Winslow et al. (1920). The native of this disease is in North America, and in the 1950s it was transferred to Europe. In 1964, it was initially discovered in Egypt. Early in April 1982 and 1983, orchards in several areas of northern Egypt were devastated by symptoms like fire blight, flower, and twig blast of pears. Despite the record set by the fire blight epidemic, the circumstances needed for fire blight breakouts, including rain, are not present. In most governorates, the disease spread quickly and caused significant economic losses in pear-growing areas (Khamis et al. 2018). The disease was gradually discovered in many regions of Egypt, reaching pear orchards and causing serious damage in nearly all Nile delta districts. As a result of the ineffectiveness of the control measures used in Egypt and the subsequent failure to control the disease, growers began to remove pear orchards and replace them with other lucrative and less problematic crops to help growers manage the disease and implement preventative measures (Ashmawy et al. 2015).

Bacteriophages are the largest group of viruses, utilizing species in the eubacteria and archaebacteria as hosts. As viruses, they are dependent on the biological processes of a host cell to complete their life cycle because they lack a metabolism of their own. The genome of a phage can be made of either single-stranded or double-stranded DNA or RNA, and it is encased in a proteinaceous coat (Ackermann and Dubow 1987). Several researchers have used bacteriophages to combat bacterial infections with varying degrees of effectiveness (Schnabel and Jones 2001a, b). Plaque morphology, restriction fragment pattern, and electron microscopy (EM) morphotype have all been used to classify bacteriophages. To identify structural proteins, mass spectrometry (MS) with MALDI-TOF technology was used. Five *E. amylovora* phages had their whole genomes sequenced. Phage fEra103 (Accession No. EF160123; Vandenbergh and Cole (1986), fEa1h and fEa100 (Müller et al. 2011b) are member of the Podoviridae with a short-tailed, while phages fEa21-4 (Accession No. EU710883, Lehman et al. 2009) and fEa104 (Müller et al. 2011b) belong to the Myoviridae with a long contractile tail structure. The EPS depolymerase, which the Podoviridae incorporate into their coat (Bernhard et al. 1996), breaks down the pathogen's amylovora capsules and makes *E. amylovora* vulnerable to plant defense systems (Kim and Geider 2000).

Apple and pear fire blight symptoms were lessened by the enzyme produced in plant cells under control of the strong 35S promoter (Flachowsky et al. 2008). Bacteriophage applications for the management of fire blight have been described (Schnabel and Jones 2001a, b). Our focus has been on reducing the symptoms of fire blight in tissue, such as apple blooms and pear slices, and meeting the growth needs of *E. amylovora* phages. Additional difficulties arise when using bacteriophages to manage fire blight, which is an airborne disease as opposed to one that is soil-borne. One of these is bacteriophage's capacity to sustain large populations on tree aerial parts. Many studies have noted that phages have been shown to be sensitive to both desiccation and ultraviolet radiation (Ritchie and klos 1979), and they may be stabilized by the presence of organic matter or the charged particles found in soil (Williams et al. 1990).

Aim of this study was to identify and characterize *E. amylovora* strains isolated from diseased pear from Egypt. Isolation and identification of the virulent bacteria *E. amylovora* bacteriophages; determination of them according to plaque morphology, thermal inactivation point, sensitivity to Ultra violet light, the optimum pH of each phage isolate, host specificity as well as size and morphology of phage particles and determine the biological effectiveness of the *E. amylovora* bacteriophages isolated from pear in Egypt to control the fire blight disease under the natural conditions.

Methods

Isolation and purification of a causal bacterium

Isolation trials were carried out using infected pear (*Pyrus communis*), tissues (blossoms, leaves and stems with cankers) as well as twig pieces with well-developed symptoms. The plant material was collected from pear orchards located in Kafr El-Dawar, Beheira Governorate, Egypt, during 2019 season according to (Mitrev and Kostadinovska 2016). A loop full of the resulting suspension was streaked on 5% sucrose nutrient agar (SNA) plates (Billing et al. 1961). Single colonies observed after 48 h of incubation at 25 °C were subsequently purified using nutrient agar, 5% sucrose nutrient agar (SNA) medium (Billing et al. 1961) and King's medium B (King et al. 1954). Morphology of the bacterial isolates, including cell and colony morphology, arrangements and motility, was investigated by light microscopy. Tests including

Gram reaction, oxidase and catalase tests, growth at 36–39 °C, growth in 5% NaCl and nitrate reduction tests were carried out according to the protocols described by Jones and Geider (2001). Two identified *E. amylovora* isolates namely (EaI and EaII) were kindly provided from Biological control Unit, Plant Pathology Res. Institute, Agricultural Research Center (ARC), Giza, Egypt. Local bacterial strains *i.e., Pseudomonas* sp., *Xanthomonas* sp., *Bacillus* sp., *Serratia liquefaciens* and *E. amylovora*), identified according to 16 srRNA sequencing, were obtained from Agricultural Microbiology Dept., Fac, Agric., Ain Shams Univ. Cairo, Egypt were used during this experiment.

Pathogenicity test

Immature pear fruits and shoots were inoculated with different bacterial isolates as described by Beer and Rundle (1983) with some modifications. The immature fruits were surface sterilized with 70% ethanol and cut in transverse slices (1 cm) thick. For each isolate, three slices were placed on a sterile moist filter paper in a sterile plastic dish. To each slice, 50 µl of bacterial suspension at a concentration of approximately 1×10^8 CFU ml/G1 was added at the center of each slice. The slices were maintained under humid conditions at 27 °C for 5 days.

Molecular identification of *E. amylovora*

DNA extraction from bacterial cells

All bacterial isolates were cultured overnight in nutrient medium at 28 °C with constant shaking at 200 rpm. Two ml of culture cells (10^8 cfu/ml TSB) were liquated into sterile eppendorf tube and the bacterial genomic DNA was extracted according to Llop et al. (1999). The DNA concentration and purity was examined by examining the O.D. 260/280 and by separating the DNA on 1% agarose gel.

PCR detection using 16S r RNA gene

The Full length of 16S rRNA gene (1550 bp) for all bacterial isolates was amplified for all bacterial isolates using two universal primers 16SrRNA. P0 (5`-GAAGAGTTT GATCCTGGCTCAG-3′) and 16SrRNA. P6 (5`-CTA CGGCTACCTTGTTACGA-′3) (Gutell et al. 1994). The PCR amplification was carried out in a total volume 25 µl containing 1 µl of template DNA, 5 µl 10.0×PCR reaction buffer, 2.5 µl 50 Mm MgCl, 2.5 µl 4 mM dNTPs, 10 pmol of each primer and 1 unit, 0.2 µl promega Taq DNA polymerase 5.0U/µl and 11.8 µl Sterile Mili Q water. Amplifications were performed with a Bio-Rad PTC-100 Thermal Cycler (Bio-Rad, USA). The PCR program was applied as follows: initial denaturation at 95 °C for 5 min.; 34 cycles of 95 °C for 1 min.; annealing at 53 °C for 1 min. and extension at 72 °C for 1 min. a final extension step at 72 °C for 10 min. PCR products were separated on agarose gel electrophoresis using 1% (w/v) agarose in 0.5×TBE buffer. (Shoeib et al. 2016). The amplified products of 16S rRNA gene (1500 bp) were purified using Gene JET™ PCR Purification Kit (Fermentas). The products were sequenced at Macrogen Ltd., Korea by use ABI 3730xl DNA sequences. Sequences obtained by automated DNA sequencer were analyzed and compared to Nucleotide sequence accession numbers i.e., AJ010485.1, X83265.1, CP024970.1, CP064855.1, FN434113.1, FN666575.1, CP055227.2, FP236842.1, FN392235.1 and NR_041970.1 for *E. amylovora* collected from GenBank.

Source of bacteriophage

Pear cuttings taken from the aerial portions of trees which exhibited symptoms typical of fire blight and soil sample were collected from sites in and around Behera Governorate, Egypt.

Isolation of bacteriophages

Effective Bacteriophage against isolated *E. amylovora* was isolated from collected infected pear tissues and soil according to the method of Othman and Shamloul (1997).

Detection of temperate bacteriophages

To detect the presence of prophage in *E. amylovora*, liquid culture was exposed to U.V irradiation (240 nm) at distance of 60 cm for 5–30 min., ascending 5 min. then temperate phage was assayed qualitatively according to Ambroa (2020).

Detection of virulent bacteriophage

The virulent phage was detected by spot test as described by Hammad and Dora (1993). Bacteriophage was isolated and purified using the single plaque isolation technique as described by Kiraly et al. (1970).

Stability of bacteriophage

For studying the physical stability of lytic phage isolate, spot test was used for determined to thermal inactivation point according to Basdew and Laing (2014) and Longevity in vitro according to Yoshida et al. (2006). Also, stability of the isolated phage to different pH value according to Taj et al. (2014) by exposure the phages for overnight to 5, 6, 7, 8, 9 and 10 degrees of pH that adjusted by 0.1 M of NaOH or HCl. Then the pH was adjusted to the optimum degree (7.5) and the infectivity of phages was assayed qualitatively and quantitatively.

Bacteriophage assaying

Detection of bacteriophages isolated from soil and plant was assayed qualitatively using the spot test according to Borrego et al. (1987).

Quantitative assaying

Titration of concentration of the isolated bacteriophages from plant and free soil was assayed using the plaque assay technique according to Lillehaug (1997).

Selection of lytic phage isolates

The individual phages were selected using single plaque isolation method (SPI) according to Othman et al. (2008). Different types of plaques were picked out and transferred to about 500 µl CM buffer and stored for overnight at 4 °C. Then, the single plaque isolated was repeated for 3 times for each isolate.

Purification of bacteriophage isolates

Isolates of phages were purified according to Kiraly et al. (1970) using single plaque isolation technique.

Phage particle morphology

The isolated phages were observed by transmission electron microscope (Joel, Model GEM 1010) at 50 kv, according to Hayat and Miller (1990). 0.5% uranyl acetate pH 4.5 was used to stain grids (Stacey et al. 1984). The grid was examined at 50 kv in a Philips CM10 Transmission Electron Microscope with an accelerating voltage of 80 kV in Faculty of Agriculture Research Park (FARP), Cairo University, Egypt using negative staining with to determine the shape and size of the particles.

UV spectrophotometric properties of the purified phage

UV absorbance of the purified phages was determined according to Setlow and Boyce (1960). The purified phages were red on UV spectrophotometer (Orion Aquamate 8000 UV/VIS) from 200 to 300 nm with interval wavelength 10 nm and the spectrophotometric data (ratio of A_{260}, A_{280}, A_{260}/A_{280}, A_{280}/A_{260}) were record.

Host rang specificity

Host specificity of each phage was estimated via the spot test. Each isolate of *E. amylovora* phages was tested against different type of bacteria as mentioned by (Gill et al. 2003).

Genotypic identification of bacteriophages

DNA extraction

The total nucleic acids were extracted from purified phages using extraction method according to Dellaporta et al. (1983) was used. The concentration of the extracted DNA was determined using spectrophotometer (Orion AquaMate 8000) at 260 nm and 320 nm according to equation: DNA concentration (µg/ml) = (reading at 260–320 nm) × dilution factor × 50. Total DNA were used in PCR and RFLP assays.

PCR detection

Primer sequences specific for the bacteriophage PEa1-A and PEa1-B (Jones et al. 1998) were used. Primer sequences were 5′-AATGGGC ACCGTAAGCAGT-3′ for PEa1-A and 5′-TAATGGGTATGATAGAAGGCA GAC-3′ for PEa1-B. Primers were expected to amplify a 304 bp product. Reactions were run in a total volume 25 µl containing 1 µl of template DNA, 5 µl 10.0 × PCR reaction buffer, 2.5 µl 50 Mm MgCl, 2.5 µl 4 mM dNTPs, 10 pmol of each primer and 1 unit, 0.2 µl promega Taq DNA polymerase 5.0U/µl and 11.8 µl Sterile Mili Q water. Amplifications were performed with a Bio-Rad PTC-100 Thermal Cycler (Bio-Rad, USA) under the following conditions: 95 °C for 2 min for the first cycle, followed by 30 cycles at 95 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s, 30 s.

Restriction fragment length polymorphisms (RFLPs)

Bacteriophage DNA was digested with *Eco*RI, *Hind*III, and *Bam*HI (Promega), according to the supplier's instructions and using 0.5–1 g of DNA, 3 U of enzyme, and 0.1 mg/ml acetylated bovine serum albumin per 50-reaction mixture. Samples were digested with *Eco*RI, *Hind*III, and *Bam*HI overnight at 37 °C Fragments were separated on a 1% agarose gel in Tris–acetate-EDTA and stained in 1 g of ethidium bromide/ml (Elise and Jones 2001).

SDS-PAGE electrophoresis analysis

The phage proteins were analyzed using SDS-PAGE according to Laemmli method (Laemmli 1970). Purified phage solution was precipitated with four volumes of ice cold acetone. After centrifugation (16,000×*g* 20 min, 4 °C), the pellet was air-dried and re-suspended in the buffer. Briefly, 50 µl of sample was added to 10 µl of loading buffer and boiled for 10 min. Separation was carried out in 10% resolving gel (Tris–HCl buffer with pH 8.8) and 4% polyacrylamide in Tris–HCl buffer with pH 6.8 was used as a stacking gel. Electrophoresis was carried out in standard Tris–glycine chamber buffer at a constant current of 100 mA. A molecular weight standard (Perfect TM Color Protein Ladder, EurX, PL) with a molecular weight range from 7 to 240 kDa was used in the electrophoretic separation, after which the gels were stained with Coomassie blue (Sigma, Ge). The stained electropherograms were analyzed with a densitometer in Quantity One software (Bio-Rad, Ge) (Müller et al. 2011a).

Mature pear slice bioassays

Mature pear fruit slice (*Pyrus communis* L. cv. Le-Conte) were used to evaluate the biological control activity of phage isolates by screening for the presence or absence of bacterial exudate (ooze), and also by quantifying the bacterial population remaining on the slice surface after treatment with phage. Forty-five mature pear fruits were collected from local market. Pears were surface disinfected by soaking in NaoCl 0.5% solution for 3 min and aseptically sliced latitudinally to a thickness of 2–3 cm and diameter of slices 15 cm were cut using a sterilized knife (the skin and the pith of the fruit). Slices with the same treatment were placed in five separate Petri dishes. Slices were placed on 9 cm qualitative Whatman No. 1 filter paper disks moistened with tap water in 9 cm diameter Petri dishes in order to randomize variations caused by individual differences between fruits. The plates containing inoculated Slices were incubated at 27 °C in closed plastic bins lined with wet paper towels to maintain a high RH. Bacteriophage suspensions were always made and inoculated on the same day in order to ensure accurate titers (EAP1 (10^8 PRU/ml), EAP2 (10^9 PRU/ml) and EAP3 (10^7 PRU/ml) and their cocktail combinations (10^{10} PRU/ml). Phage was suspended in CM. In all cases phage isolates were only evaluated against their corresponding bacterial propagation host. One treatment for each of the bacterial strains was bacteria plus sterile PB amended with 7 mM MgS04, which served as a positive control. Slices without inoculation served as negative controls. The experimental design Petri dish was divided. Disease severity index was used in the evaluation of mature pear fruit slice in bioassay experiments. A rating of O = no necrosis, 1 = incomplete necrosis of the slice, 2 = one-quarter complete necrosis of slice, 3 = one-half complete necrosis of slice, 4 = Three -quarter complete necrosis of slice, 5 = Complete necrosis of the slice. Rating scale modified from Pusey (1997). Experiment treatments, Control Healthy, Control Infected bacteria, Infected Bacteria and treated with EAP1, Infected Bacteria and treated with EAP2, Infected Bacteria and treated with EAP3, Infected Bacteria and treated with cocktail phage and Infected Bacteria and treated with streptomycin (Akremi et al. 2020).

Results

Bacteria isolation and identification

Eleven bacterial isolates were isolated from infected pear trees materials as stated in materials and methods. Distinct colonies that possessed typical morphological characteristics of *E. amylovora* were purified. High level of homogeneity among isolates was observed where colonies were typically white, domed, shiny, mucoid (levan type) with radial striations and possessed dense flocculent center. All isolates (three were oxidase-negative, catalase-positive and consisted of gram-negative motile rods. They were able to ferment glucose without gas formation and did not reduce nitrate. Moreover, all the isolates did not grow at 36–39 °C and were not able to grow in the presence of 5% NaCl. Based on the aforementioned morphological and biochemical tests, all the bacterial isolates possessed the characteristics of *E. amylovora*. The pathogenic bacteria were identified based on potential symptomatology, pathogenicity test, morphological characteristics and molecular detection of 16sRNA gene.

Symptomatology

Symptoms illustrated in (Fig. 1), indicated the presence of *E. amylovora.* The most types of infection detected were cast or shoot blight, cankers of branches, trunk and root blight. Infected pear trees acquired the typical appearance of burnt trees. The symptoms appeared on trees as brown or black, typically deformed, shepherd's crook-like shoots when compare to the healthy tree. Flower blight was more common on pear trees. Infected flowers became brown. Spur blight leaves on the spur became necrotic, frosty and black. Twig lesion begins at the growing tips of the shoots and moves down to the

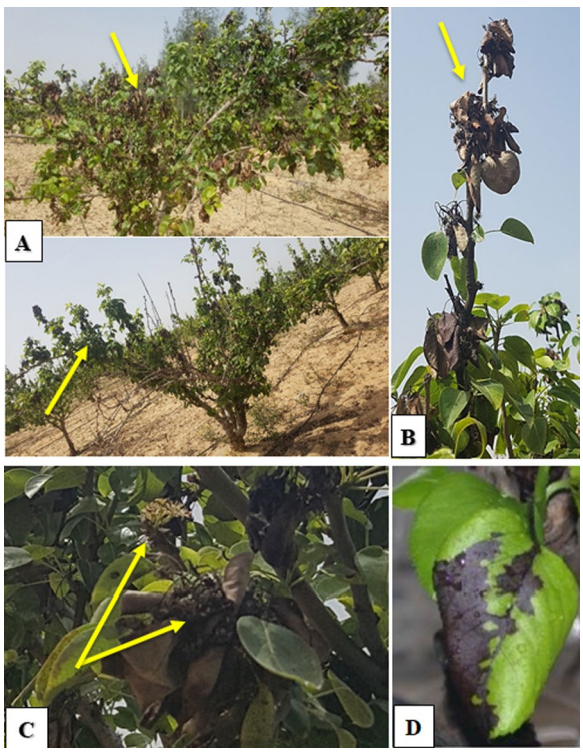

Fig. 1 Fire blight Symptoms: **A**, **B**—necrotic flowers and leaves; **C**, **D**—necrotic shoot

old parts of twig. Damaged twigs appear turning dark brown or black. Damaged leaves remain attached to dead branches. The end of the branch may be bent, resembling the bend of a shepherd or a candy cane.

Pathogenicity test

All the tested bacterial isolates showed typical symptoms of the disease upon infection to unripe pear fruits such as necrosis accompanied by oozes, brownish and blackish colorization of the fruits. In vitro, the test was successful with each pear showing intense reaction and typical symptoms after 5–14 days. The pears were pollinated and necrotic spots were widespread as shown in Figs. 2 3 and 4. In vivo plants inoculated with 5×10^7 cells/ml suspension of bacterial isolate showed different levels of infection. The shoots showed an intense reaction and typical symptoms were formed after 5–14 days as shown in (Plate, 2).

Morphological character of *E. amylovora*

Morphological characterizations of the isolated *E. amylovora* were detected by electron microscopes showed rod shape, gram negative, non-spore forming, short-rods, non-spore former, and gram negative and motile. Also, under the transmission electron microscope using negative staining with phosphotungestic acid appeared as short rod with length of 1.8 micron with peritrichous flagella as shown in (Fig. 3).

Genotypic characterization of *E. amylovora* using 16S rRNA gene and sequence analysis

16S rRNA gene sequence was used to study the classification and identification of *E. amylovora* isolates, using universal primers P0 and P6 consequently, using polymerase chain reaction (PCR). Approximately 1550 bp region of the 16S rRNA gene was amplified for all *E. amylovora* isolates (Fig. 4). High similarity, but in varying proportions, was observed during the sequence comparison between Egyptian isolate EAEgW1 (Accession No. Quary_48847) of *E. amylovora* of this study and other *E. amylovora* accession numbers provided from the GenBank and the highest similarity was observed between the Egyptian isolate and the German isolate (AJ010485.1) with 99% identity (Fig. 5).

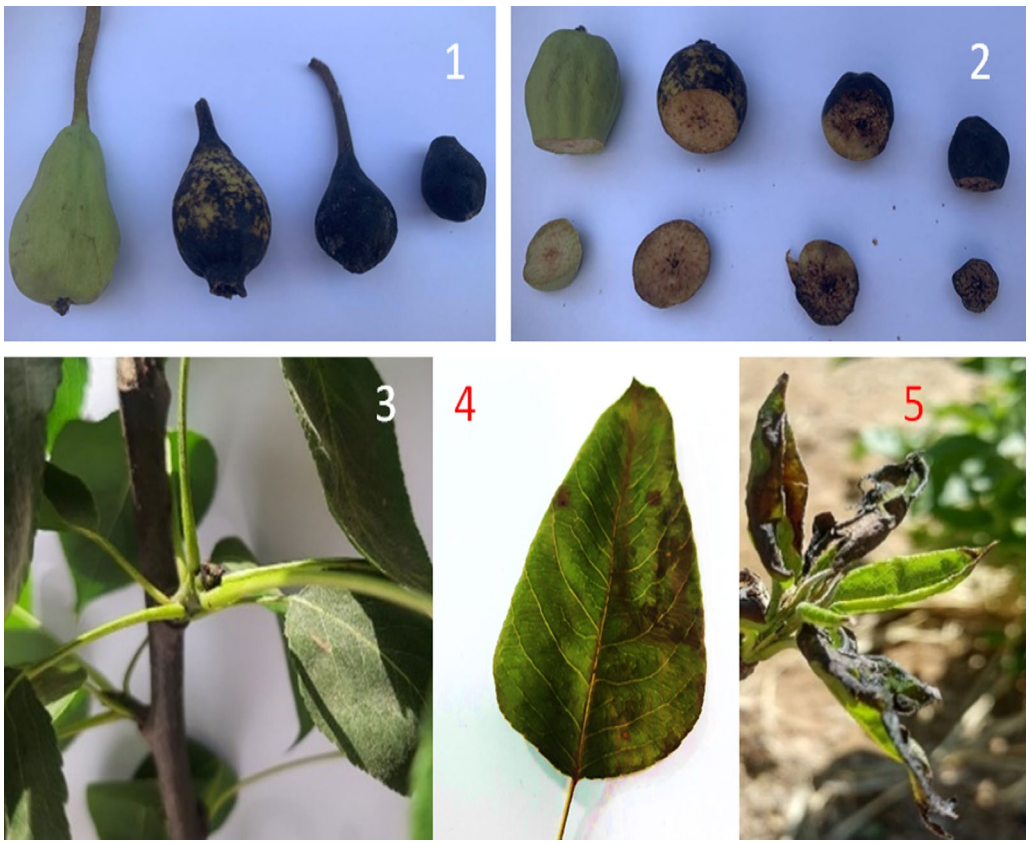

Fig. 2 Immature pear fruitlets showing blackened area after inoculation with *Erwinia amylovora* and immature pear fruitlet inoculated by sterile distilled water as a negative control (for left)

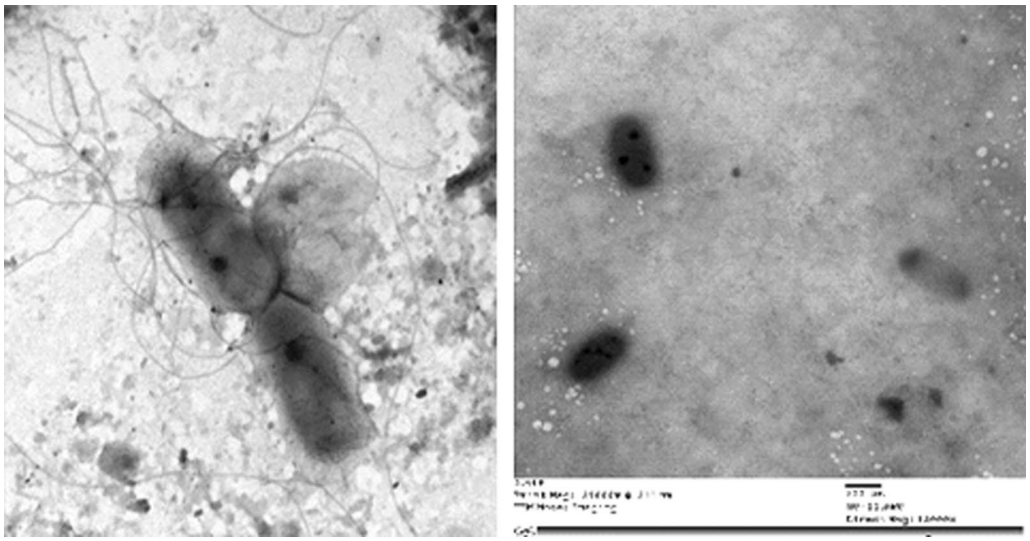

Fig. 3 Micrograph of isolated bacteria using TEM. The isolated bacterium is short rod with 1.35 micron in length and has peritrichous flagella. Photograph with scale bar

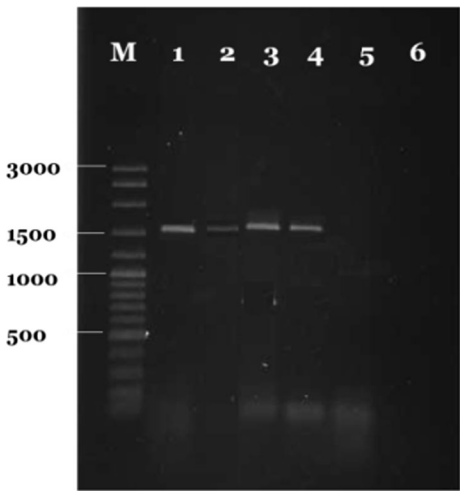

Fig. 4 16S rRNA-PCR amplification of *Erwinia amylovora* isolates. Lane M, 1 bp DNA ladder (Gibco BRL); lanes 1, 2, 3, E. amylovora isolates, Lane 4, positive control

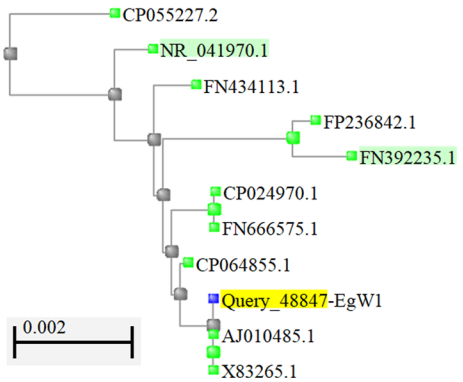

Fig. 5 Phylogenetic tree of the Egyptian isolate EA1 of *Erwinia amylovora* in this study and other *E. amylovora* isolates reported in the GenBank revealed that, the isolate EA1 is high similarity with German isolate (AJ010485.1) with 99% identity

Detection and isolation of specific lytic bacteriophages for *E. amylovora*

Bacteriophages specific for *E. amylovora* were enriched from infected pear trees and from free soil obtained from nursery's pear, respectively, by the liquid enrichment method to prepare the original crude phage suspensions occurrence of the specific bacteriophages in the crude phage suspensions was detected qualitatively by the spot test method using *E. amylovora* as indicator hosts.

Plaque morphology

Plaque technique was done for quantitative assaying of the bacteriophages of *E. amylovora* present in the ordinal crude phage lysates or suspensions by 10^7 pfu/ml and also to obtain single phage isolates by the single plaque isolation method, which single plaques differ in their morphology (size, shape and clearness) picked up to represents different phage isolates for *E. amylovora*. Different phages were isolated, using single plaque isolation method from the resulting double layer agar plats technique. The isolated phages for *E. amylovora* are named *E. amylovora* phage 1 (EAP1), which isolated from infected trunk and *E. amylovora* phage 2 (EAP2) and *E. amylovora* phage 3 (EAP3), which isolated from

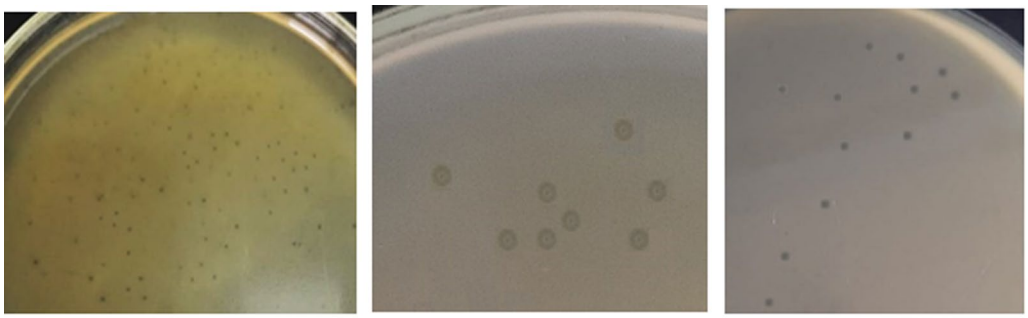

Fig. 6 Morphology of plaques of *Erwinia amylovora* from single plaque isolation. Plaques are clear, circular as pin point in EAP1, clear in center, circular with halo zone 2 mm in diameter in EAP 2 and EAP3 is clear, circular with about 0.5 mm in diameter

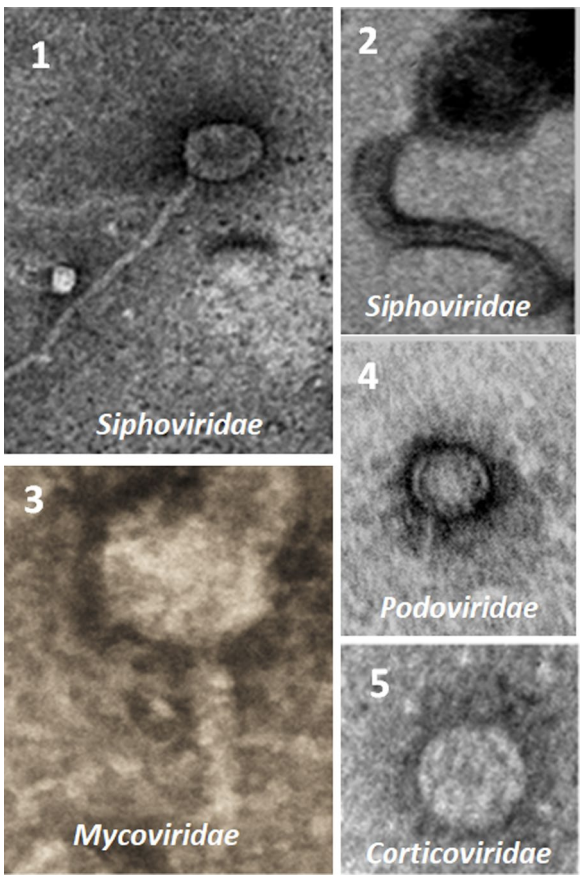

Fig. 7 Electron micrographic visualization of three types of *Erwinia amylovora* phages (1 and 2) Siphoviridae, (3) Mycoviridae, (4) Podviridae and (5) Corticoviridae, negatively stained with uranyl acetate. Magnification bar = 100 nm

soil. Plaques were isolated according to their morphology as shown in (Fig. 6). The isolated plaques showed that plaque EAP1 was clear, circular as a pin point, plaque EAP 2 was clear in center, circular with halo zone 2 mm in diameter and plaque EAP3 was clear, circular with about 0.5 mm in diameter. The diameter of plaques was measured using paint.net program.

Table 1 Host range of four selected *Erwinia amylovora* phages revealed can the ability for lysis using Spot test

Bacterial strains	Isolated phages			
	AMP1	AMP2	AMP3	AMP4
Erwinia amylovora-S	+	+	+	+
E. amylovora-P	+	+	+	+
E. amylovora-Ph	+	+	+	+
E. amylovora EaI	+	+	+	+
E. amylovora EaII	+	+	+	+
Pectobacterium carotovorum	–	+	–	+
Xanthomonas axonopodis	–	–	–	–
Pseudomonas sp.	–	–	–	–
Enterobacter sp.	–	+	+	+

Susceptible (+); Resistant (–)

Phage particle (virion) morphology

The examination of bacteriophages negatively stained by 2% uranyl acetate under TEM showed that EAP1 was assigned to the Siphoviridae family by hexagonal head with 66.5 nm in diameter and long flexible non-contractile tail with about 289.1 nm in length (Fig. 7). On the other hand, the Myoviridae family examination of EAP2 showed that it was characterized by icosahedral head of 80 nm diameters, and contractile of 110.42 nm length. EAP3 phage with hexagonal head with 57.5 nm in diameter and a very short tail with about 33 nm in length that belongs to Podoviridae family and in temperate Phage EATP, the particles had an isometric head with 57.6 nm in diameter without tail that belongs to Corticoviridae family.

Table 2 UV absorbance for *Erwinia amylovora* phages

Purified phages	A maximum (nm)	A minimum (nm)	$^{A}260/^{A}280$	$^{A}280/^{A}260$	Conc. (ng/µl
EAP1	250	235	1.35	0.53	672.6
EAP2	260	230	1.6	0.62	421.3
EAP3	255	235	1.2	0.84	212.7
EAP4	245	235	1.06	0.54	146.6

Host range assay

Spot test performed to evaluate the host range of the four selected phages revealed that all phages have the ability for lysis of different *E. amylovora* strains and *Enterobacter* sp. (Table 1). Interestingly, only AMP2 and AMP4 displayed a lytic activity against *Pectobacterium carotovorum* and Ph2; Ph4 and Ph4 displayed a lytic activity against *Enterobacter* sp. No phages exhibited activity against tested strains of *Pseudomonas* sp., *Xanthomonas axonopodis. Bacillus* sp. and *Serratia liquefaciens*. Local identified bacterial strains according to 16 srRNA sequencing and obtained from Agricultural Microbiology Dept., Fac, Agric., Ain Shams Univ. *E.* amylovora S *E. amylovora*, Two *E. amylovora* isolates (EaI and EaII) were kindly provided by Biological Control Unit, Plant Pathology Res. Institute, ARC, Giza, Egypt. Selected phages revealed that all phages can infect different *E. amylovora* including (Ph1; Ph2; Ph3 and Ph4).

Table 3 Phage stability for thermal inactivation point (TIP)

Phages	TIP	Pfu	
		Initial	Final
EAP1	65	6×10^{10}	4
EAP2	98	7×10^{10}	2
EAP3	55	4×10^{9}	6
EPTP	50	5×10^{9}	9

Absorption spectrum of *E. amylovora* phages

Spectrophotometric properties of *E. amylovora* phages (The absorbance of phages) were taken at 5 nm distance intervals of the whole range of wavelength (220–300 nm). The obtained data are presented in (Table 3). Data showed that *E. amylovora* phages EAP1, EAP2, EAP3 and EATP had maximum absorbances at 250, 260, 255 and 245 nm, respectively. While the minimum absorbance recorded 235, 230, 235 and 235 nm, respectively (Table 2).

Thermal inactivation point (TIP)

Suspension of *E. amylovora* phages EAP1, EAP2, EAP3 and EATP were heated for 10 min at different degrees of temperatures (from 30 to 100 with intervals 2 °C). Suspensions were cooled immediately and assayed by the spot test. Results showed that the thermal inactivation point for *E. amylovora* phages were 54, 98, 52 and 50 nm for EAP1, EAP2, EAP3 and EATP, respectively, as shown in (Table 3).

Effect of pH values on *E. amylovora* phages

Stability of *E. amylovora* phages in different pH values (5, 6, 7, 8 and 9) was tested. Results presented in (Table 4) showed that phage EAP1 was inhibited completely in pH 5 and pH 6 and affected partially by pH 7, pH8 and pH 9 which its 3.5×10^{4} pfu/ml with reduction rate 6.2 log in pH 7, 6×10^{7} pfu/ml with reduction rate 3 log in pH8 and 5×10^{5} pfu/ml with reduction rate 5.07 log in pH 9 comparing with the control (6×10^{10} pfu/

Table 4 Effect of pH values on *Erwinia amylovora* phages

Phages	pH																			
	Control			pH5			pH6			pH7			pH8			pH9			pH10		
	Pfu*	Log		Pfu	Log		Pfu	Log		Pfu	Log		Pfu	Log		Pfu	Log		Pfu	Log	
		pfu	R*		pfu	R*		pfu	R*		pfu	R*		pfu	R*		pfu	R*		pfu	R*
EAP1	6×10^{10}	10.7	0	0	–	–	1×10^{3}	3	7.3	9×10^{7}	7.9	2.8	2.7×10^{7}	7.4	3.3	2×10^{5}	5.3	5.4	30	1.4	9.2
EAP2	7×10^{10}	10.8	0	3×10^{2}	2.5	8.3	2×10^{4}	4.3	4.0	7×10^{9}	9.8	1.0	5×10^{8}	8.7	2.1	4×10^{6}	6.6	4.2	2×10^{2}	2.3	8.5
EAP3	4×10^{9}	9.6	0	0	–	–	–	–	–	3×10^{6}	6.5	3.1	3.5×10^{4}	4.5	5.1	2×10^{3}	3.3	6.3	10	1	8.6
EAP4	5×10^{9}	9.7	0	0	–	–	–	–	–	6×10^{6}	6.7	3.0	5×10^{4}	4.7	5.0	1×10^{2}	2.0	7.7	0	–	–

*pfu: plaque form unit

*R: Log ratio of reduction

ml). EAP2 lost the activity in pH 5 completely and partially with rate of 7.7 log with 1×10^3 pfu/ml in pH 6, 4.2 log with 2.7×10^6 pfu/ml in pH 7, 1 log with 5×109 pfu/ml in pH 8, 3.7 log with 9.8×10^6 pfu/ml at pH 9 comparing to the control (5×10^{10}). phage EAP3 was inhibited partially at pH 5, pH 6, pH 7and pH 9 which its activity reduced with rate of 7.7 log with 8×10^2 pfu/ml in pH 5, 5.7 log with 7×10^4 pfu/ml in pH6, 3.2 log with 2.4×10^7 pfu/ml in pH 7 and 5 log with 4×10^5 pfu/ml in pH 9 comparing to control (4×10^{10} pfu/ml). The results showed also the phages differed in their stability when treated with different pH values and it is sensitive to acidity more than alkalinity. *E. amylovera* phages were exposure to freezing followed by thawing 4 times and their activity was assayed quantitatively by double agar layer technique and data were recorded it in Table 5. Data showed that phages EAP1 and EAP2 titer discarded after 3 times of freezing and thawing while EAP3 and EATP Phage decreased after 2 times. The obtained results showed that phage EAP2 is the most stable on if it exposed to freezing and thawing were identified as related to PEa1 using PCR produced similar restriction patterns.

Longevity in vitro for *E. amylovora* lytic phages

Phages of *E. amylovora* (EAP1, EAP2 and EAP3) in crude sap was divided at 12 eppendorf tubes (each contained 1 ml of the phage suspension) and left at room temperature for 90 days. The phage's activity was examined by the spot test technique weekly to determine their longevity in vitro. Results along the 90 days indicated that the phages still active and could be lyses their main bacterial host.

PCR detection of phages

Using the primers specific for phages EAP1, EAP2 EAP3 and EAP4 produced about 300 bp PCR product, indicating relatedness to phage EAP1 (Fig. 8).

Restriction fragment length polymorphisms (RFLP)

DNA was extracted from the 4 surviving bacteriophage isolates, collected from the field, were placed into four groups based on the patterns obtained by digestion of the bacteriophage DNA with three restriction endonucleases. All of the phages which were identified as related to PEa1 using PCR produced similar restriction patterns (Fig. 9 and Table 5).

Phage proteins profile

SDS-PAGE was applied to analyze the proteins contends corresponding to four phages (Fig. 4). The phages, EMP1; EMP2; EMP3 and EMP4 had 11; 12; 9 and 7 proteins contents with a molecular weight ranged from 224 to 10 kDa, respectively. The four *E. Amylovora* phages revealed variability in proteins profile; number of protein contents and polymorphism. The four *E. amylovora* phages showed four common proteins with (28.57% Monomorphic) and 10 specific proteins with 71.42% Polymorphic (Fig. 10 and Table 6).

Mature pear slice bioassays

Phage efficiency tests on unripe pear slices

Biological control activity of phage isolates was determined by bacterial exudate (ooze) on slice and disease severity using mature pear fruit slices. Phage treatments reduced the symptom severity of fire blight than positive

Table 5 Bacteriophage isolates placed into groups based on digestion of phage DNA with four restriction endonucleases

Fragments' size bp	EcoR I				Hind III				BamH I				Polymorphism
	Ph1	Ph2	Ph3	Ph4	Ph1	Ph2	Ph3	Ph4	Ph1	Ph2	Ph3	Ph4	
1500	–	–	–	–	–	–	+	–	–	–	–	–	Unique
1300	–	+	+	–	–	–	–	–	–	–	–	–	Polymorphic
1200	+++	–	–	–	–	–	–	–	–	++	+	+	Polymorphic
1000	–	–	–	–	–	++	+	–	–	–	–	–	Polymorphic
900	++	–	+	++	–	–	–	–	–	–	++	–	Polymorphic
800	–	–	–	–	–	–	–	–	+	–	–	–	Unique
700	–	–	–	–	+	+	+	+	–	–	–	–	Monomorphic
600	–	+	–	–	–	–	–	–	–	–	–	–	Unique
400	–	–	–	–	–	–	–	–	+	+	+	+	Monomorphic
300	–	++	+	+	–	–	–	–	–	–	–	–	Polymorphic

– = not detected (no amplified Fragment), + = barely visible Fragment, + + = visible amplified Fragment,

Polymorphic = Specific fragment

Monomorphic = Common fragment

Unique fragment = Genetic marker

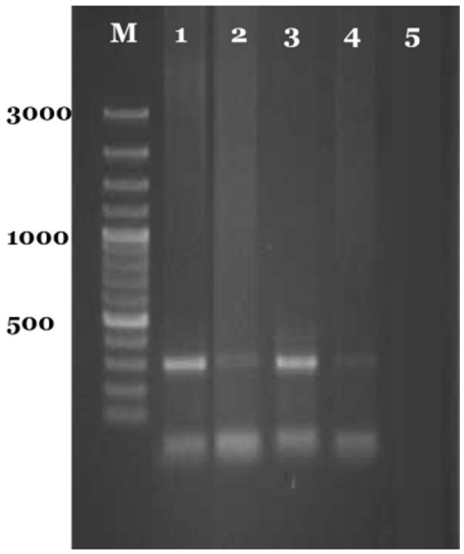

Fig. 8 Agarose gel electrophoresis of PCR products amplified using primers PEa1-A and PEa1-B, designed to amplify about. 300 bp product from phage EAP1, M: 100 bp DNA ladder as size standard (MBI Ferments); PEa1 as positive control

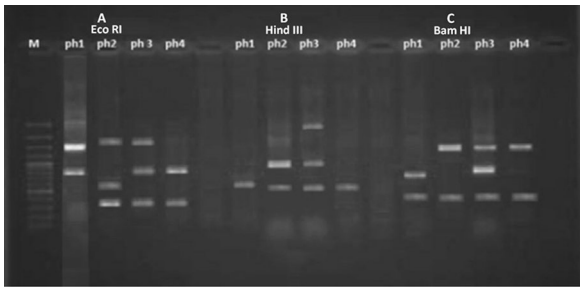

Fig. 9 Agarose gel electrophoresis of restriction analysis of DNA from *Erwinia amylovora* phages EAP1, EAP2 and EAP3and EAP3, and (**A**) *Eco*RI restriction digestion analysis, (**B**) HindIII restriction digestion analysis and (**C**) BamHI restriction digestion analysis of phage DNA. Lane M, 1-kb Ladder

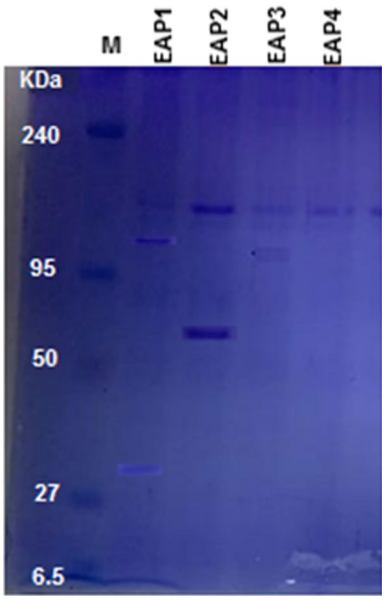

Fig. 10 Electrophoretic profiles (SDS-PAGE) of phages of the family Myoviridae specifc for *Erwinia amylovora*. M-Perfect™ Color Protein Ladder, EurX, with a mass range from 7 to 240 kDa, Line 1—phage induced from EAP1; Line 2—phage induced from EAP2; Line 3—phage induced from EAP3 and Line 4—phage induced from EAP4

controls. Also, the antibacterial effect of phages was markedly active than that conferred by streptomycin sulfate in all experiments. Based on these results phage treatments proved to be more efficient compared to antibiotic. Data presented in (Table 7 and illustrated in Figs. 11 and 12) indicated that the infected *E. amylovora* pear fruit slices showed severe symptoms complete bacterial exudate (ooze) on slice 100% with 90% disease severity. Phage isolates (EAP1, EAP2, EAP3 and cocktail phage) showed reduction of bacterial antibiotic streptomycin. exudate (ooze) by 62.739, 74.089, 44.309 and 77.287%, respectively, with 34, 16, 44 and 12 disease severity than with antibiotic streptomycin 74.723% bacterial exudate (ooze) and 14% disease severity. It was

Table 6 Protein profile and polymorphism among four *Erwinia amylovora* phages

Phages MW (KDa)	EAP1	EAP2	EAP3	EAP4	Polymorphism
224	+	+	–	–	Polymorphic
195	–	+	+	+	Polymorphic
176	++	++++	++	+	Polymorphic
152	+	+	+	–	Polymorphic
125	++	–	–	+	Polymorphic
82	–	++	+	–	Polymorphic
73	+	+	–	–	Polymorphic
70	+	+	+	+	Polymorphic
60	–	++++	+	–	Polymorphic
45	+	+	+	–	Polymorphic
32	+	+	+	+	Polymorphic
25	+	+	–	–	Polymorphic
18	++	–	–	–	Polymorphic
10	+	+	+	+	Polymorphic
Total					Polymorphic

Monomorphic = common bands 42.8% (–) No band

Polymorphic = specific bands 21.4% (+) Low density

Unique = Genetic marker 35.8%

(++) moderate density (+++) high density

found that cocktail phage showed more effect. On the other hand, the antibacterial effect of phages was markedly lower than that conferred by streptomycin sulfate in all experiments. Based on these results phage treatments proved to be more efficient on cultivar Le qunte as compared to cultivar pineapple.

Table 7 Reduction in rotting necrosis spots diameter and disease severity on infected *Erwinia amylovora* Slices fruits pear and treated with streptomycin and phages compared to control ones

Treatments	*Rating index							Phage infectivity		
	No. of inf. slices	0	1	2	3	4	5	% Inf	**% red	% DS
Control healthy	0	0	0	0	0	0	0	0	0	0
Control infected bacteria	10	0	0	0	1	3	6	100	0	90
Infected bacteria										
EAP1	6	4	1	1	2	2	0	60	62.739	34
EAP2	4	6	1	2	1	0	0	40	74.089	16
EAP3	8	2	0	4	2	2	0	60	44.309	44
Cocktail phage	2	8	0	0	2	0	0	20	77.287	12
Streptomycin	3	7	0	2	1	0	0	30	74.723	14

*Rating index: O = no necrosis, 1 = incomplete necrosis of the slice, 2 = one-quarter complete necrosis of slice, 3 = one-half complete necrosis of slice, 4 = Three -quarter complete necrosis of slice, 5 = Complete necrosis of the slice

**% reduction = Calculated mean of Diameter of rotting (Cm) from 10 replicates

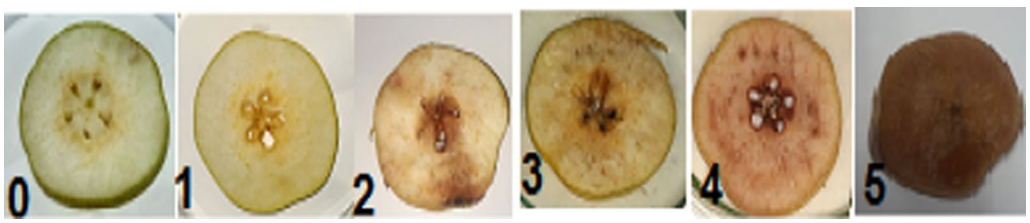

Fig. 11 Disease severity index used in the evaluation of mature pear fruit slice in bioassay experiments. A rating of O = no necrosis, 1 = incomplete necrosis of the slice, 2 = one-quarter complete necrosis of slice, 3 = one-half complete necrosis of slice, 4 = Three-quarter complete necrosis of slice, 5 = Complete necrosis of the slice. Rating scale

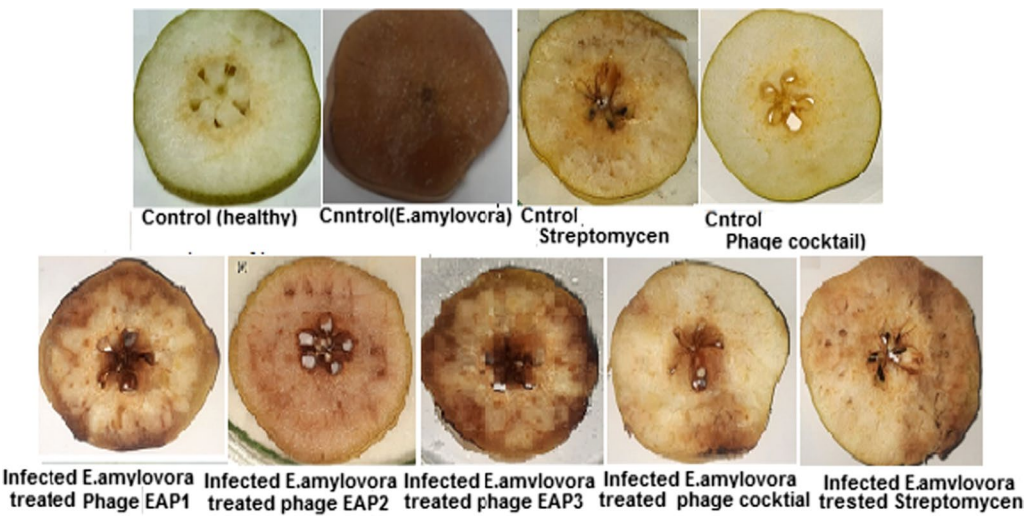

Fig. 12 Slices of fruits pear infected with *Erwinia amylovora* and treated with streptomycin and phages showing reduction rot spots diameter compared with control ones

Discussion

In recent years, trees of pear have been seriously affected by fire bright disease caused by a pathogen *Erwinia amylovora*. In this study, pear orchards at Kafr El Dawar, El-Behera governorate was subjected to seasonal examination for two successive seasons (2019–2020). The symptoms appeared on trees as brown or black, typically deformed, shepherd's crook-like shoots. Flower blight was more common on pear trees. Infected flowers became brown. Spur blight leaves on the spur became necrotic, frosty and black twig lesion begins at the growing tips of the shoots and moves down to the old parts of twig. Damaged twigs and leaves appear turning dark brown and leaves remain attached to dead branches implied the presence of *E. amylovora* (Khamis et al. 2018). Eleven bacterial isolates were isolated from infected plant materials and soil. Purified colonies were those that had the typical morphological features of *E. amylovora*. Colonies were typically white, domed, shiny, mucoid (levan type) with radial striations, and had dense flocculent centers, indicating high levels of homogeneity among isolates (Ashmawy et al. 2015). All of the isolates (three of them) were gram-negative motile rods that lacked oxidase and catalase. They failed to reduce nitrate and were able to ferment glucose without producing gas. Additionally, none of the isolates were capable of growing in the presence of 5% NaCl or between 36 and 39 °C. All of the bacterial isolates possessed the characteristics for *E. amylovora* based on the morphological and biochemical tests indicated above (Ashmawy et al. 2015). When infected with immature pear fruits, all bacterial isolates showed the disease's typical symptoms, including necrosis with oozes as well as browning and blackening of the fruits. When immature pear fruits were inculcated with the identified fire blight isolates, the fruit's infected areas appeared water soaking at the edges of the wounds they had been caused, and eventually became a dark brown color. When Sticky fluid oozed out at the site of inoculation within 4–5 days of inoculation kept in a moist chamber at a temperature ranging from 25 to 27 °C, all isolates were capable of inducing typical fire blight symptoms from the pathological point of view. Also, when tested on immature green fruits of pear plants, all isolates were able to cause typical fire blight symptoms, confirming their identity as *E. amylovora*. These findings are consistent with that reported by Bereswill et al. (1997). The 16S rRNA gene's 1550 bp region was amplified for all bacterial isolates using the universal primers P0 and P6, and the amplicons were then submitted for further examination. At Sigma Company, the automated DNA sequencer ABI PRISM model 310 was used to sequence the purified 1550 bp region of the 16S rRNA gene. The following partial sequence was obtained for two Egyptian isolates Ea1. *E. amylovora* was identified at the molecular level in the present work by sequencing the 16S rRNA and aligning it with sequences found in the GenBank database. And the identification of *E. amylovora* was successfully accomplished using the 16S rRNA sequencing method. Ashmawy et al. (2015) achieved similar results. From pear tissues exhibiting fire blight symptoms, bacteriophages that may infect *E. amylovora*, the pathogen responsible for the disease, were isolated. Four of the isolated phages were isolated from liquid bacterial culture, and three were chosen to treat the pear fruits. It can be concluded that under in vitro conditions, the phages used in this work were quite effective at getting rid of *E. amylovora* on pear fruit. Therefore, these bacteriophages may potentially be utilized as a biological fire blight control (Schwarczinger et al. 2011). Based on the aforementioned findings, many characteristics were used in this study to classify the phage isolates of *E. amylovora*. The optimum pH, thermal stability, UV sensitivity, host specificity, morphology, and size of the phage particles must all be investigated collectively to provide significant differences between the phage isolates tested. No single method of phage characterization is adequate in itself for classification. These results confirmed those created previously by Zeller (2006). Obtained phage isolates produced plaques on the soft agar layer containing the test bacteria with diameters ranged from 0.5 to 7.1 mm. The present plaques halo had a diameter between 0.1 and 5.0 mm in the different isolates; the morphology of plaques isolated in the present study was an important characteristic of phages reported in detail by Gill et al. (2003). The *E. amylovora* phages isolated in Egypt belonged to the order Caudiovirales and to the Podoviridae and Myoviridae families, respectively, based on their morphotypes C1 and A1. Egyptian phage head diameter was similar to findings from the literature (Müller et al. 2011a). Podoviridae-related isolates had a head diameter of 60 nm, while Myoviridae-related isolates had a head diameter of around 70 nm. The isolate that has previously been described in the literature is a member of the Family Siphoviridae, according to Meczker et al. (2014).

Analysis of the phage isolates induced from bacteriophage isolates showed slight variation in protein profiles in SDS-PAGE electrophoresis. The isolated bacteriophages were all determined by the morphological study done under an electron microscope to be members of the Myoviridae, siphaloviridae, and podoviridae families. Analysis of the protein profiles of phages in SDS-PAGE electrophoresis can show which characteristics (such as morphology, protein or DNA composition, genome structure, or restriction endonuclease pattern) are more conserved than others and which will develop depending on the type of bacteria they replicate on, as demonstrated

in a study by Jarvis et al. (1993). Very similar protein profiles were obtained for 4 phages induced from phage strains of serotypes EAP1, EAP2 and EAP3 in the case of the protein profiles shown in SDS-PAGE electrophoresis. These results confirmed earlier by Urban-Chmiel et al. (2015).

The antibacterial activity of phage isolates and their triple combinations, which had previously been evaluated on flowers, was also examined on fruit slices of several pear cultivars. When compared to other treatments, it was observed that phages reduced the severity of fire blight symptoms. On the other hand, in every trial, the antibacterial effect of phages was significantly less than that provided by streptomycin sulfate. According to these results, phage treatments were shown to be more effective on the cultivars "Le Conte" than on the cultivar "pineapple." According to these results, phage treatments on the cultivars "Le Conte" were shown to be more effective than those on the cultivar "pineapple." It is important to note, however, that phage treatments of pear slices were significantly less effective than those of flowers, as reported by Nagy et al. (2012).

Conclusion

This work represents the first stages in the development of a biological control agent against *E. amylovora* based on bacteriophages. Future research will benefit greatly from the identification and characterization of the three phage isolates presented in this study. This will enable more rapid phage classification when they are isolated and allowed for additional study of ecology and spread of the phages of *E. amylovora*. Because certain isolated phage types have host ranges that are restricted to specific strains of *E. amylovora*, caution must be used in assessing the biological control activity of these phages and in the selection of possible bacterial hosts for future phage isolations. Designing future biological control systems will be greatly aided by the discovery that some *E. amylovora* phages can also infect strains of *E. amylovora*. On the other hand, phage isolates showed notable biological control action against *E. amylovora* in the pear slides bioassay experiment, although it is yet unknown whether these results apply to field circumstances. It is highly exciting that the phages in an improved system can practically tenfold lower bacterial population on the surface of pear slides.

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Author contributions
WH: implementation of the work plan. OA and REH: participating in designing of the work & interpretation of data. SAY: participate in developing the work plan and follow up on its implementation and data analysis. AAS: collecting results and writing the manuscript with some help from others. All authors participated in developing the work plan and follow up the implementation and read and approved the final manuscript.

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All data are available in the manuscript and the materials used in this work are of high transparency and grade.

Declarations

Ethics approval and consent to participate
All of us agreed to all concerned regulations Consent of publication, also we agree to publish this scientific paper in the EJBPC. All procedures performed in studies are in accordance with the ethical standards of the institutional and/or national research committee. We further declare that no animal was harmed during this study. Consent for publication Informed consent was obtained from all individual participants included in the study.

Consent for publication
All authors have consent for its publication.

Competing interest
All authors declare that they have no competing interest.

Author details
[1]Virus and Phytoplasma Research Department, Plant Pathology Research Institute, ARC, Giza, Egypt. [2]Biochemistry Department, Faculty of Science, Ain Shams University, Cairo, Egypt. [3]Biotechnology Programe, Institute of Basic and Applied Sciences, Egypt-Japan University of Science and Technology, New Borg El-Arab City, Egypt.

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