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BACTERIOLOGICAL AND MOLECULAR STUDY OF *E. AMYLOVORA* BACTERIA THAT CAUSES A DISEASE OF FIRE BLIGHT IN SOME ECONOMIC CROPS

Ali Hameed Mezher and Ali Abd- Rahim Al Nashi
Department of Biology, College of Education, University of Al-Qadisiya, Iraq
E-mail : ah145167@gmail.com

Abstract

The study included the collection of 40 samples of plants infected with fire blight disease caused by infection *E. amylovora* was isolated and the diagnosis of 20 strains of these bacteria, using the tests of phenotypic, microscopic and biochemical tests were bacteria gram-negative, rod-shaped, facultative anaerobic, moving, positive tests for catalysis, red methyl, Hydrolysis of Gallatin and motility test, but negative tests of oxidase, indole, and vogue Proskauer. The *E. amylovora* strains grew in general media all the strains were fermented with lactose sugar on the macConkey Agar media and gave a pink color to the media . In addition, eight strains were selected and molecularly identified using PCR and 16SrRNA primer. Genetic detection of pathogenic virulence factors was carried out in the *E. amylovora* strains, most of which had pathogens. Gene ratios were the cause of the disease (DspA/E, Hrp , Eps-ams A, c-di-GMP) (100%, 100%, 50%, 62.5%) respectively are important factors of this type, which are encoded for various processes such as inhibiting the calos in host cells, Necessary for disease events, encryption of secretion of different enzymes, and the formation of biofilms.

Keywords **:** *E. amylovora*, Fire blight, Economic crops, Virulence factors

Introduction

Erwinia amylovora belongs to the Enterobacteriaceae family, a pathogenic bacteria of many plants. Cells are characterized by rod shape, Gram-negative, facultative anaerobic is not sporadic, cell dimensions (0-3 × 1-3) micrometers are formed in single cells or in pairs and sometimes in short chains, Animated by oceanic whips. These bacteria are characterized by colonies of a distinct color and the colony takes a round shape that is limited to nutritious oxides (Paulin, 2000).

The first pathogenic bacteria known to infect the plant has been shown to be the cause o, devastating necrosis for apples, pears and pink plants (Eastgate, 2000). *E. amylovora* is genetically linked to several important human and animal pathogens such *as Escherichia coli, Yersinia pestis, Yersinia enterocolitica, Salmonella enterica* and *Shigella flexneri*. Primary infections of *E. amylovora* occur during the flowering season and the buds, or when the fruits are formed (Vanneste, 2000).

The pathogen travels from primary injury sites through the tissue, and from it to other parts of the infected plant (Bogs *et al*., 1998). Symptoms appear as sticky droplets on the fruit and cracking on the branches and leaves, leading to characteristic pathological symptoms also called shepherd sticks (Agrios, 2005). These bacteria are considered satisfactory pathogens for a large number of plant specimens. It has been observed that most pathogenic strains isolated from apples are also pathogenic to pears, too many Rosaceae, and to Prunus. On the other hand, it was observed that strains isolated from the mulberry family were not infectious to apple and pear plants (Momol & Aldwinckle, 2000). Some researchers divided *E. amylovora* strains into two major groups according to their host type. Spiraeoideae-infecting strains infect a large number of apple species such as apples, pears, hawthorns, and quail. And the other Rubus strains, which affect the berry and blackberry plants (Braun & Hildebrand, 2005).

These bacteria have caused blistering on plants that belong to the Rosacea family. The story of this serious disease began to develop when the disease spread from its original location in the Hudson Valley in New York, the United States of America through North America, the Pacific Coast, Europe, and the Middle East over the past years to reach 55 countries in the world (Bonn & Van der Zweit, 2000).

This bacterial disease is more dangerous to apples and pears, caused by bacteria *E. amylovora* (Mansfield *et al*., 2012). The spread of this disease can lead to the destruction of orchards and thus stop production for many years. The outbreak of the disease caused heavy losses throughout the world between 1994 and 1999. The bacterial pathogen invaded many countries, especially Romania and Italy. As a result, 500000 fruit trees (Calzolari *et al*., 1999). The risk of firefighting is increasing because of its continuous spread across continents to new areas such as Australia and Finland. Moreover, the rapid spread of the fire has resulted in the total destruction of orchards, since no pesticides can control the spread of the disease (Vanneste, 2000). Begins the initial infection of bacterial invasion of parts of the first flowering, and then enter the plant through the secretary cells called nectarrthodes, in flowering plants such as pear, secondary infection blight fireworks in all plant members occur as the start of the infected tissue as a result of infection of flowers. And the wounds of an important role in the entry of the pathogen and the events of injury. It can also produce blight the root of the apple by entering the pathogen with the water and move to the inner part and the top of the tree (Thomson, 2000). Bacteria also have the ability to move from primary infection areas such as flowers or infected areas to the main parts, such as branches and roots, to internal tissues such as wood and bark, and to prevent the flow of fluids inside the vessels, causing tissue damage and decay (Vanneste, 2000).

E. amylovora causes a rift in different parts of the plant showing its symptoms on flowers, leaves or fruits. Flowering flowers lead to their wilting with different colored colors such as green, gray and black, then tissue necrosis occurs and the dead flowers die on the plant. Under high wet conditions, Infected with droplets of bacterial exfoliation (Steiner, 2000). As for the flap of the leaves, it appears quickly on the young leaves in the developing summit, and the leaves that are found in the buds are infected with black color and then

tissue necrosis occurs. Sporadic patches may also appear on the old leaves with full withering and stunting (Johnson, 2000). In the early infection, when the fruit is immature, the infected fruit remains small, becomes deformed, withered, but remains attached to the tree. In the later infection, the infected fruit remains fresh and does not change color (Johnson,2000). The *E. amylovora* strains are characterized by numerous ferment factors that cause serious injury to many plants, which have the ability to move quickly and spread within the members of plant species as in apple trees and pears when environmental conditions are favorable. This pathogen also moves quickly through the wood vessels to reach the flowers, buds, and roots causing disease (Koczan *et al*., 2011).

It has been shown that there are different types of virulence systems for these bacteria to infect the host plants, notably EPS amylovoran, Hrp secretion and T3SS (Mann *et al.*, 2013). Biofilm, As well as receptors delivered by Hrp, Hrp/(T3SS), amylovoran and external polysaccharides, are considered to be key factors (Oh & Beer, 2005).

Hrp-T3SS also encrypts the *DspA / E* receptor protein by urging the infected plant cells to respond and suppress defenses from the cell wall (DebRoy *et al*., 2004). In addition, DspA / E interacts with the kinases of receptor kinases to work within the host plant cells (Meng *et al*., 2006). Along with Hrp-T3SS, *E. amylovora* has a large number of excretory systems, T1SS, T2SS, T3SS, inv / spa and T6SS. However, T1SS and T2SS do not possess inv/spa, which inhibits bacterial pathogenesis in the fruit of apple trees or immature pears (Zhao *et al*., 2009).

Materials and Methods

Collection of samples

Forty samples of the aerial parts of the plants of the infected plants were collected from the *E. amylovora* virus by 20 samples of apple plants and 20 samples of pear plants. During the period from 2018/11/1 to 31/1/2019 from different nurseries of Babil governorate, With a sharp knife clean and placed in clean plastic bags and sterile and transferred the same day to the microbiology research laboratory in the Department of Life Sciences, Faculty of Education, University of AL. Qadisiyah and conducted tests required in isolation and diagnosis

Culture of samples

The infected parts of the plant were washed with clean water to remove the impurities. They were sterilized with 5% sodium hypochlorite solution and for 1 min followed by washing with sterile distilled water three times and the plant, parts were stripped with sterile filter leaves. A piece of tissue was taken 0.5 cm from each model and planted in the center of nutritious broth Nutrient broth At the rate of three replicates for each model, incubated samples were incubated at 27 °C for 48 h. The bacterial culture of the liquid farm was then spread over the agar Nutrient medium in the Petri dishes by the loop. The dishes were incubated at 27 °C for 24 hours. The different colonies were selected and replanted on medium Nutrients (Shibani, 2000)

Isolation and diagnosis

Phenotype test

Untreated bacterial isolates were planted on the agar Nutrient to examine colony characteristics in terms of shape, color, nature of the edges, size and height of the colony (Holt *et al.*, 1994).

Microscopy test

Use a microscope to examine the bacterial cells where published smear on a glass slide and set the flame passes three times quickly and then dye gram stain.

Biochemical tests

Biochemical tests such as indol, oxides, catalase, red-methyl, gelatin, and vox Proscar, and the use of API-20E to confirm the diagnosis of Enterobacteriaceae were isolated. Work according to instructions from the French manufacturer BioMerieux.

Molecular diagnosis of *E. amylovora* and pathogenic virulence factors

A number of genetic foci were used to diagnose *E. amylovora* genetically and determine virulence factors based on the NCBI-Gen bank and the Primer3plus primer program. They were processed by the Korean company Macrogen as in Table 1 and a polymerase reaction technique (PCR) was used in the molecular detection of bacteria, For the disease.

Table 1: primers used to diagnose bacteria and virulence factors

Primers	Sequence		Amplicon	Code
16SrRNA gene *E. amylovora*	F	CTTGCTCTTGGGTGACGAGT	543bp	NC_013961.1
	R	CAGGTTAAGCCCGGGGATTT		
Hrp gene	F	GGAACCCTGTCAACGCCAAA	501bp	U97504.1
	R	AAGCTTCAGCATGGCTTTCAG		
Eps-amsA gene	F	AAGTCGGTCAGTTCGAGCAG	598bp	X77921.2
	R	CGAACAATCTCCTGCTGGGT		
c-di-GMP	F	CCTGTTATCCCTACGGTCGC	606bp	NC_012917.1
	R	CGCCCAATCACATCCGTTTC		
DspA/E	F	ATCAGATGAGGCAGGGGAGT	563bp	AF271717.1
	R	TGCTGGATCCGGATTTTGCT		

PCR analysis

The DNA was extracted from eight *E. amylovora* strains using several genomic DNA extraction kit processed by the American Generaid Company. Extraction was carried out according to the company's instructions. In summary, 1 mL was injected into the center of the liquid Hinton Muller and placed in 5, 1 ml sterile and then transfer to the centrifuge at 10000rpm for an accurate duration, add 200 µl of GT lysis buffer solution and mix, incubate at room temperature for 10 minutes, add 200 µl of GB Buffer solution and incubate, add 200 µl of ethyl alcohol, Dispose of the dissolved solution of the decomposing cells, add 200 µL of Buffer W1 solution DNA sequestration The residue was then discarded and then a guest of 600 µl of the washing solution containing the absolute washing alcohol was removed to remove the fat and then returned to the central centrifuge for the purpose of disposing of the precipitate and then dried and stored in the thermal -20c DNA, NanoDrop Spectrophotometer for detection and measurement of nucleic acid concentration DNA was detected by determining the DNA concentration and purity by reading the absorbance at a wavelength of 280-260 nanometers. The polymerase chain reaction mixture was prepared using the AccuPower® PCR PreMix Processed by a company Bioneer Korean According to the company's instructions as in Table 2, was an

examination of the interaction of polymerase chain using a Thermocycler PCR as Table 3, was an electrical migration using gel agarose by 1% as a result of reading polymerase chain reaction PCR product. The sample was loaded with PCR and placed in the gel. The measurement ladder 2000-100bp DNA ladder was used to measure the PCR output and place in the first hole. After the loading process, the agarose gel was immersed using the 1X TBE Buffer solution and the transfer lid was closed. Transfer with 100 V and 80 mA for 1 hour After the migration, the PCR-containing gel was tested using the UV light source to determine output with the unit of measurement.

Table 2 : Prepare PCR master mix

Volume	PCR master mix	
5µL	DNA template 5-50ng	
1µL	F. primer	Primers (10pmol)
1µL	R. primer	
13µL	PCR water	
20µL	Total	

Table 3 : Polymerization reaction program used to amplify the genes used in the study

PCR Step	Temperature	Time	Repeat
Initial denaturation	95˚C	5min	1
Denaturation	95˚C	30sec.	30 cycle
Annealing	58˚C	30sec	
Extension	72˚C	1min	
Final extension	72˚C	5min	1
Hold	4˚C	Forever	-

Results and Discussion

Isolation and Diagnosis

The results showed that the number of positive samples showing bacterial growth was 20 samples distributed in 10.10 apple and pear samples, respectively. The percentage of samples showing growth in apples was 50% and pears were 50%. These results were consistent with Al Mashhadani (2011) results for the study of the *Erwinia* spp bacterial species that cause mild rotting and pyelonephritis.

Bacteria were diagnosed based on phenotypic, microscopic and physiological and physiological tests performed on bacterial isolates. The results of the phenotypic examination showed that colonies growing on the center of the small, irregular, brown-colored wafers were brown and creamy glistening and the rim was round. The colonies were pink, being fermented with lactose sugar, and by microscopic examination, the bacterial cells appeared under a microscopic microscope and were moved by oceanic vesicles, negative to a non-corpus chromosome. These results coincided with Al Mashhadani (2011) On the bacterial species of *Erwinia* spp isolated from infected plants. The results of the biochemical tests showed that the bacteria were positive for the Indole test, the catalase, the consumption of citrate, the red-type test, the ability to analyze the gelatin, and the negative for the oxides and the Fox Brooks. These results were consistent with El-habbak & Refaat (2019)

Testing with API20E

Bacterial diagnosis has been confirmed by using this system because it is characterized by its speed in detecting bacteria. It is special for the diagnosis of the Gram-negative bacteria and the intestinal family in general without the use of planting circles and time-consuming in the preparation process and it is characterized by the fact that it does not cause pollution or reduce the percentage of pollution during the transplant process. as Table (4) If the results show that the diagnosed species are *E. amylovora* bacteria, as shown in Fig. 1

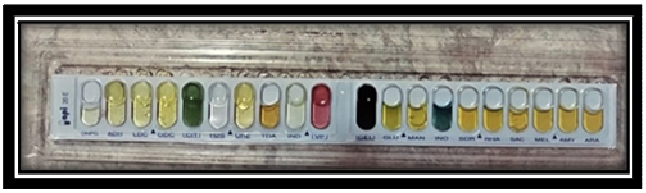

Fig. 1 : API20E used to diagnose *E. amylovora*

Table 4 : System of API20E used in the diagnosis of *E. amylovora*

Reaction	Test
Colorless (–)	ONPG
Yellow (–)	ADH
Yellow (–)	LDC
Yellow (–)	ODC
green (–)	CIT
Colorless (–)	H2S
Yellow (–)	URE
Yellow(–)	TDA
Colorless (–)	IND
Pink (+)	VP
black pigment (+)	GEL
Yellow(+)	GLU
Yellow(+)	MAN
Blue (–)	INO
Yellow(+)	SOR
Yellow(+)	RHA
Yellow(+)	SAC
Yellow(+)	MEL
Yellow(+)	AMY
Yellow(+)	ARA

Molecular diagnosis of *E. amylovora* bacteria

Results of the amplification of the 16SrRNA gene *E. amylovora* showed that all tested isolates of E. amylovora were positive for testing with a size of 625bp and 100% as in Fig.(2). These results were consistent with Dardouri *et al* (2017) for bacteria *E. amylovora*.

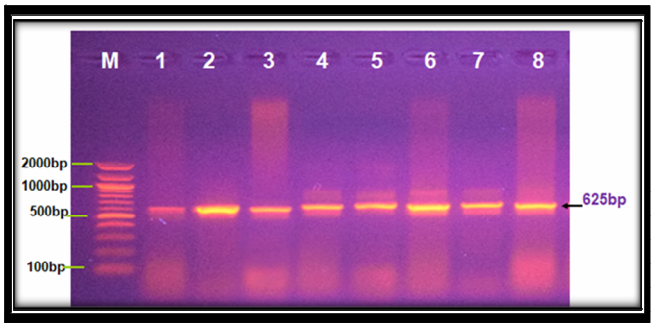

Fig. 2: A deportation electrophoresis agarose gel and containing the results of the examination of the special PCR to investigate the gene 16S rRNA gene in the *E. amylovora* isolates. Where M: Marker 2000-100bp and numbers from 1-8 represent positive isolates for testing with a length 625bp.

Genetic detection of virulence factors with *E. amylovora*

The results of gene screening in 8 isolates of *E. amylovora* showed that all Hrp isolates with a 501bp size and 100%, one of the factors responsible for plant pathogenesis, are the *Hrp* genes of the Harpin family's response genes that encode the secretion system of The third type is necessary for the virility of the bacteria. It has the role of causing bacterial infections on the plants by controlling the enzymes necessary for the events of the infection on the plant cells. The bacteria use the *Hrp* path of the third type T3SS responsible for the pathway of enzymatic secretions. The importance of *Hrp* is attributed to being responsible for the process. Transport Protein Avr and alkaline deposits into the cells and in sensitizing the sensitivity of the host plants in the infection and the removal of the causative agents of the callus (Boureau *et al*., 2011). The *Eps -amsA* was 50% of the isolates and the length was 598bp Figure 2 shows that external sugars (EPS) play a key role in bypassing the host plant's defense system, blocking the vascular system, protecting bacteria from water and nutrient loss during dry conditions (Ordax *et al*., 2010). Studies have also shown that EPS is an important component in the formation of biofilms by *E. amylovora* bacteria, which enables them to stick to the walls of plant cells in many plant species (Koczan *et al*., 2011). Most of the isolates were c-di-GMP with 62.5% and only 5 isolates and with a 606 bp output. In Figure (3), c-di-GMP is a second reporter known to regulate various cellular processes, Motor, and phobia. The c-di-GMP concentration inside cells adversely affects diguanylate cyclase enzymes and phosphodiesterase enzymes. C-di-GMP positively regulates amylovoran secretion in *E. amylovora* bacteria, which increases the formation of the biomembrane and regulates the movement of bacteria. Although the secretion of amylovoran and the formation of the biomembrane are important for bacterial colonization of tissue and the development of systemic infection, the results coincided with the results of Edmunds *et al* (2013) for studying the role of *c-di-GMP* in the development of *E. amylovora* disease on host plants. As for the gene *DspA / E*, the results of the genetic test showed that all isolates were carrying the gene and by 100%. And a length of 563bp as in Fig. (4). The effectiveness of this gene is attributed to the fact that it acts as a key killer of cell death and participates in the generation of oxidative stress on host plants and is directly related to plant injury and injury. It has been shown to be a protein zone associated with the *Hrp* genes that represent the structure of the third secretion system T3SS and *DspA / E* Claus deposits are suppressed on host plants by preventing the deposition of substances within plant cells to facilitate the entry and regulation of the necessary enzymes. This gene is regulated by HrpL (DebRoy *et al*., 2004; Oh *et al*., 2005). The results were consistent with the results of Aksoy *et al.* (2017). In their study, they showed that the *E. amylovora* strains of *E. coli* For the *dspA / E* gene region through the PCR technique used for molecular detection.

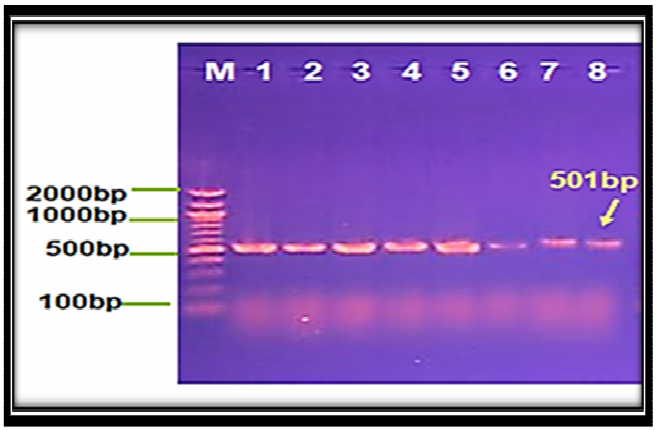

Fig. 3 : A deportation electrophoresis agarose gel and containing the results of the examination of the special PCR to investigate the gene Hrp gene. Where M: Marker representations 2000-100bp. The numbers from 1-8 represent positive isolates of *E. amylovora* with a length of 501bp.

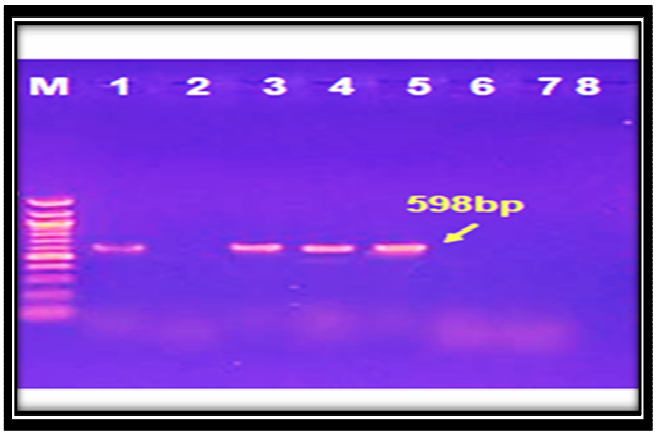

Fig. 4 : A deportation electrophoresis agarose gel and containing the results of the examination of the special PCR to investigate gene Eps-amsA gene. Where M: Marker 2000-100bp The numbers from. 1, 3,4,5 represent positive isolates of *E. amylovora* with a length 598bp.

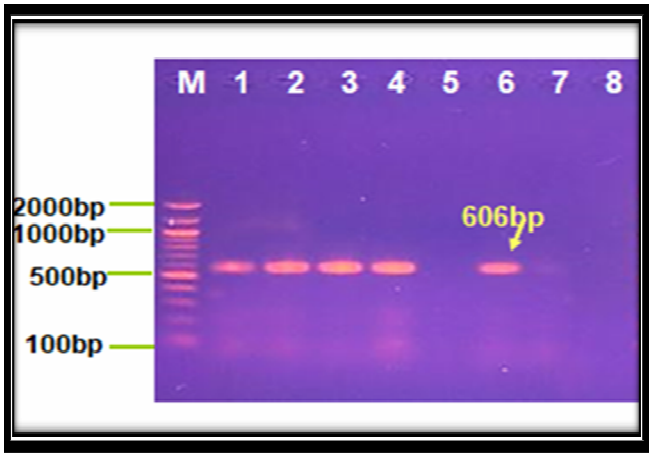

Fig. 5 : A deportation electrophoresis agarose gel and containing the results of the examination of the special PCR to investigate gene c-di-GMP gene Where M: Marker 2000-100bp The numbers from1,2,3,4,6 represent positive isolates of *E. amylovora* with a length 606bp.

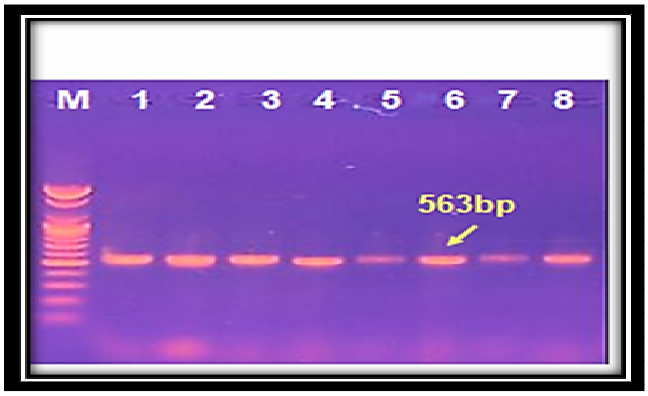

Fig. 6 : A deportation electrophoresis agarose gel and containing the results of the examination of the special PCR to investigate gene DspA/E gene Where M: Marker 2000-100bp The numbers from1-8 represent positive isolates of *E. amylovora* with a length563bp.

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