Detection of local *Erwinia* Isolates Causing Diseases in Potato by Using DNA Amplification by Polymerase Chain Reaction Technique (PCR)

Maitham J. Mohammed and Ehab D. Selman
Department of Biotechnology, College of Science, Baghdad University, Baghdad-Iraq.
E-mail: maialjalali@yahoo.com.

Abstract

Seventy samples of infected potato's tubers were collected from Baghdad city during the period from September 2011 to February 2012. These samples were collected from Alradwaniyah potato's store/ Albyaa market and from Awrad Alnahar potato's store in Abu ghraib and two bacterial strains of *Erwinia* were obtained from Plant Protection department/ College of Agriculture/ Baghdad University. The Crystal Violet Pectate (CVP) used as a selective- diagnostic medium to isolate and purify *Erwinia.* The strains and isolates of *Erwinia* were characterized according to the morphology and microscopic characteristics, along with the biochemical tests and API 20E tests. The *Erwinia* isolates have a positive reaction for Catalase, Raffinose and Cellobiose and a negative reaction for Oxidase and Sorbitol. By using Reagent Genomic DNA Kit method for Total DNA extraction, during electrophoresis process, the results showed that some of selected isolates and the two standard strains of *Erwinia* contained at least two bands of small plasmid DNA. The identification was confirmed by using DNA amplification by polymerase chain reaction technique (PCR) and *Erwinia carotovora* subsp. *carotovora* specific primer such as (Ec001), the selected local isolates of *Erwinia* and *Erwinia carotovora* subsp. *carotovora* ATCC 15713 potato's pathogen had a positive reaction (one band 312 bp) for the former primer.

Keywords: *Erwinia carotovora* subsp. *carotovora*, biochemical test, primer Ec001, PCR.

Introduction

The most potatoes (*Solanum tuberosum*) were grown and consumed in Europe, North America and countries of the former Soviet Union. Since the 1990s, there has been a dramatic increase in potato production and demand in Asia, Africa and Latin America, where the output rose from less than 30 million tons in the early 1960s to more than 165 million tons in 2007 [4].

The Food and Agriculture Organization of the UN data showed that in 2005, the developing world's potato production exceeded that of the developed world. China is now the biggest potato producer, and almost a third of all potatoes are harvested in China and India. Asia and Europe are the world's major potato producing regions, accounting for more than 80% of world production in 2007 [4].

Since 2010, there has been a significant increase in production in most of the major potato producing countries like China, India, United States, and Europe. Asia consumes almost half of the world's potato supply. More than a billion people worldwide eat potatoes and the heartiest potato eaters are Europeans [4].

The predominant species involved in the *Erwinia* disease complex is *Erwinia carotovora*. It is divided into several subspecies, two of which are important on potato. The subspecies *atroseptica* and *carotovora* are the important potato pathogens and possess an array of pectolytic enzymes as pathogenicity factors. *Erwinia chrysanthemi* also produces pectolytic enzymes but differs significantly from *Erwinia carotovora* and warrants status as a separate species [2].

There is a real need for development of effective and rapid methods of identification and isolation of plant pathogenic bacteria (Schaad 1979). Rapid and accurate diagnosis is essential for successful treatment and control of diseases. A diagnostic technique of *Erwinia* spp. includes the use of both traditional and modern molecular techniques. The modern molecular techniques such as DNA amplification by polymerase chain reaction (PCR) are used to help to investigate the disease, for example, they can be used broadly as method to differentiate bacteria based on the composition of their biological molecules such as proteins, fatty acids, carbohydrates and nucleic acids [5].

Aims of the Study

Because of the importance of *Erwinia* spp. as a plant pathogenic bacteria and can use the PCR Technique as a rapid and accurate diagnosis with the primer (Ec001) to identify the locally selected isolates of *Erwinia carotovora* subsp. *carotovora* from potato samples compared with standard strain *Erwinia carotovora* subsp. *carotovora* (potato's pathogenic or non pathogenic) then early detection of the pathogen erwinias strains especially the latest infection to prevent the disease incidence or to prevent further spread and subsequent disease outbreaks, we suggested this study.

Material and Methods

Bacterial isolates and strains

Presumptive the twenty local *Erwinia spp.* isolates EM (1-20) from several locations in Baghdad during the period from September 2011 to February 2012. These samples were collected from Alradwaniyah potato's store/ Albyaa market and from Awrad Alnahar potato's store in Abu ghraib and two types strains of *Erwinia carotovora* subsp. *carotovora* ATCC 15713 potato's pathogen and ATCC 495 potato's nonpathogen were obtained from Plant Protection department/ College of Agriculture/ Baghdad University. These local *Erwinia* isolates and strains were used in this study.

Isolation procedure

The local *Erwinia* spp. isolates were isolated from rotting potato tuber or stem with typical symptoms of soft rot (Infected tubers typically develop a watery soft rot accompanied by an offensive odor) [16], employing a standard procedure [10].

Media and culture conditions

The selective-diagnostic Crystal Violet Pectate (CVP) medium [1, 14] was used for the isolation of *Erwinia*. The characteristic of the bacteria were deep cup growth- like cavities or pits. Brain Heart Infusion (BHI) Broth was used for DNA isolation (according to Geneaid company instructions). The *Erwinia* isolates and strains were maintained on potato dextrose agar (Oxoid Ltd, London, UK). The cultivation was done at 30 °C for 24 to 48 hs depending on the analysis performed.

Pathogenicity test

The pathogenicity of the *erwinias* was examined by two methods. The first one was by using PDA medium [8]. The second method included inoculated potato tuber's slices by using the best six local *Erwinia* isolates inoculation from previous PDA experiment [12, 11].

Phenotypic characterization

The micromorphology of the local erwinias isolates (cell morphology, arrangement and motility) was investigated by light microscopy. Gram reaction, the presence of oxidase disc and catalase, growth at 30 °C [10]. The fermentation tests by using Sorbitol, Raffinose and Cellobiose discs (HiMedia Laboratories; 10]. The biochemical characterization of the strains was carried out by miniaturized system- API 20E. The API strips were inoculated with 24 hs old cultures.

Isolation of DNA

Chromosome and plasmid DNA were isolated and purified by a Reagent Genomic DNA Kit [Geneaid Corporation] according to the manufacture's instruction.

DNA amplification by polymerase chain reaction (PCR)

The specific DNA primers for *Erwinia* subspecies were used to amplify a targeted *Erwinia* DNA fragment enzymatically by polymerase chain reaction (PCR), the primer Ec001: F (5′-CGGTTACGATCAGCGTCTCG-3′) and R (5′-GATGTGCCGATGCCGATAC-3′) were derived from a DNA probe specific to (*Erwinia carotovora* subsp. *carotovora*) and used to amplify DNA with slight modifications. The master mix was used in a standard PCR reaction assay which was employed a one cycle of 5 min pre denaturation step at 94 °C and 30 sec of annealing temperature according to the primer which was at 62 °C and 30 sec of extension at 72 °C and 30 or 35 cycles of 30 seconds denaturation at 94 °C, 5 min extension at 72 °C and 10 min hold at 10 °C. Test strains were served as positive controls [3].

Electrophoresis

PCR products were separated on a 1.0% Agarose gel at 50 volt after 75 min- 90 min. and stained with ethidium bromide [3], and photographed under UV light.

Results and Discussion

Isolation and initial characterization

The erwinias were isolated from infected potato. Distinct colonies that possessed the morphology characteristic of *Erwinia spp.* (deep cups- like cavities or pits, white circular, convex, smooth and entire margin. After 4-5 days, the colony was appeared resemble a fried egg, with a blue); the result is compatible with [7] and [10] and which were oxidase- negative, this reaction was observed later than 60 seconds at 25- 30° C (purple coloration) which means the reaction was negative and the isolate were *Erwinia* spp., the result was compatible with [10], catalase-positive by formation of the gas bubbles, which indicated a positive reaction, that would mean the isolates were as *Erwinia* spp., the result was compatible with [10] and consisted of Gram-negative, non-motile rods were picked after cultivation at 30 °C for 24 hs.; the result is compatible with [13]. After the purification procedures, all erwinias isolates were screened for fermentation of suger using Sorbitol, Raffinose and Cellobiose discs then the twenty local erwinias isolates EM (1-20) were selected on the base of this screening; (5 from Awrad Alnahar potato's store, 4 from Alradwaniyah potato's store and 11 from Albyaa Market) that was according to [10]/ (HiMedia Laboratories).

Pathogenicity tests

The twenty local isolates of *Erwinia* spp. were tested by using PDA medium [8], the best 6 local *Erwinia* isolates (EM4, EM5, EM7, EM8, EM12 and EM18) were determined according to the growth rate and the growth spreading on the tuber's slices., which was the evidence of the isolates activities. Pectolytically active isolates were recognized by the appearance of soft rotted which were scattered on the inoculated tuber's slices. Initiation and rate of rotting in potato tissue occurs when free water covers the potato tissue surface, the temperature is above the minimum required for growth of the pathogen, and physiological factors such as high water potential are present to favor infection, the inoculating of potato tissue caused by a number of different enzymes, jointly or independently, therefore the best *Erwinia* isolate has a high level of production of the extracellular plant cell wall- degrading enzymes and then a high efficiency to infect the potato, the result was compatible with [12, 11].

Biochemical characteristics

Several biochemical tests were done by using NA or CVP medium to characterize *Erwinia* isolates according to [10]/ (HiMedia Laboratories). The results were interpreted after 24 hs.- at 30° C, it was found that local *Erwinia* isolates have a positive reaction for Raffinose and Cellobiose and a negative reaction for Sorbitol, which means that all isolates were as *Erwinia carotovora* subsp *carotovora, Erwinia carotovora* subsp *atrosiptica or Erwinia chrysanthemi*, the yellow coloration appeared while the acid and gas produced during the fermentation which that means the reaction was positive and if the colour did not change then that indicates that the reaction was negative, the result was compatible with [10]/ (HiMedia Laboratories) that revealed that tested isolates belongs to *Erwinia* spp.

In addition, several biochemical tests were done to characterize *Erwinia carotovora* subsp *carotovora strains via the LGC Standards office in United* Kingdom. Therefore, the ATCC had supplied the biochemical tests for *Erwinia carotovora* subsp *carotovora* ATCC 15713 & ATCC 495) [19, 6] (Table (1)).

Table (1)
Biochemical tests of the Erwinia carotovora subsp carotovora strains.

Biochemical Test	*Erwinia carotovora subsp carotovora ATCC 15713& ATCC 495*
Aesculinhyd rolysis	+
Gelatin	+
Lipase	_
Pectin	+
Indole	_
Nitrate reduction	+
Nitrite reduced	_
Catalase	+
Oxidase	_
Gluconate oxid	+
Beta-galactosidase	+
L-arabinose	+
Cellobiose	+
Dulcitol	_
Fructose	+
D-glucose	+
Glycerol	_
M-inositol	+
Lactose	+
D-mannitol	+
Raffinose	+
Salicin	+
D-sorbitol	_
Sucrose	+
Trehalose	+
D-xylose	+

To confirm the biochemical results, the API 20E strips were used for *Enterobacteriaceae* identification containing 12 tests was performed [15, 9].The results were interpreted after 24 hs.-at 37°C, which revealed that tested strains belongs to *Erwinia* spp..

Total DNA extraction

By using Reagent Genomic DNA Kit method for Total DNA extraction (according to Geneaid company instructions), during electrophoresis process between 30-60 minutes, the result revealed that the selected isolates (EM4, EM5, EM7, EM12 and EM18) have bands for chromosomal DNA and no plasmids bands however the isolate EM8 and the standard strains (ATCC 15713& ATCC 495) were containing one band of chromosomal DNA and two bands of small DNA plasmids as compared with local isolate *Pseudomonas aeruginosa*, as shown in Fig.(1)/ (according to Geneaid company instructions), concluded that this method is not appropriate to isolate the DNA plasmids from the Erwinia local isolates or may be attributed to the low DNA concentration.

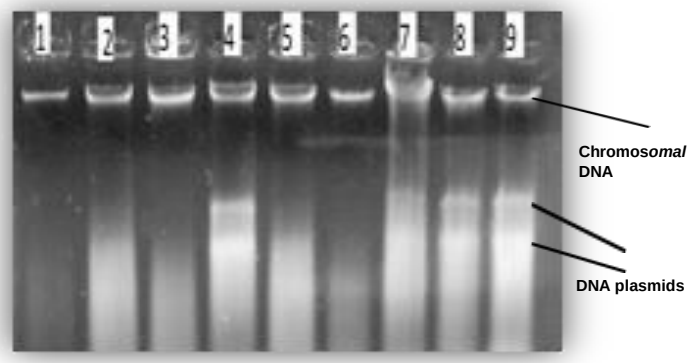

Fig. (1) Total DNA Extraction by using (Reagent Genomic DNA Kit).
1= EM4/ 2=EM5/ 3=EM7/ 4=EM8/ 5=EM12/ 6=EM18/ 7= Pseudomonas aeruginosa/ 8= Erwinia carotovora subsp. carotovora ATCC 495/ 9= Erwinia carotovora subsp. carotovora ATCC 15713 at 70 volts/ after 1 hour.

Measurement of DNA Purity and Concentration

The method was described by [17]. In order to measure the purity and the concentration of the isolated DNA, UV/VIS spectrophotometer OPTIZEN POP (MECASYS Company/ KOREA) need to be used, the results appeared that the *Erwinia* local isolates EM8, EM12 had a high DNA purity approximately 1.927 but the isolate EM7 was 1.489 (Table (2)).

Table (2)
The DNA Purity and Concentration.

DNA sample	*Concentration (µg/ ml)*	*Purity*
1	14.050	1.873
2	66.750	1.527
3	74.250	1.489
4	46.350	1.927
5	64.450	1.927
6	26.550	1.825
7	46.400	1.600
8	69.950	1.898

1= EM4/ 2=EM5/ 3=EM7/ 4=EM8/ 5=EM12/ 6=EM18/ 7= Erwinia carotovora subsp. carotovora ATCC 495/ 8= Erwinia carotovora subsp. carotovora ATCC 15713.

DNA amplification by polymerase chain reaction (PCR)

The specific DNA primers for Erwinia subsp. were used to amplify a targeted Erwinia DNA fragment enzymatically by polymerase chain reaction (PCR), the primer (Ec001) which have a sequence F(5′-CGGTTACGATCAGCGTCTCG-3′) and R (5′-GATGTGCCGATGCCGATAC-3′) were derived from a DNA probe specific to *Erwinia carotovora* subsp. *carotovora* and used to amplify DNA, during the electrophoresis process within one and half hour, the results showed that five selected isolates of *Erwinia* (EM4, EM5, EM8, EM12 and EM18) and the strain *Erwinia* carotovora subsp. *carotovora* ATCC 15713 (potato's pathogenic) had a positive reaction (one band) 312bp for the former primer (Ec001) as compared with ladder DNA RTU 1500 bp and appeared as *Erwinia carotovora* subsp. *carotovora* (potato's pathogenic), this result was compatible with [3] but the sixth *Erwinia* isolate EM7 and *Erwinia carotovora* subsp. *carotovora* ATCC 495 (potato's nonpathogenic) had a negative reaction (no band), maybe this isolate was nonpathogenic for potato or another *Erwinia* species (Fig.(2)).

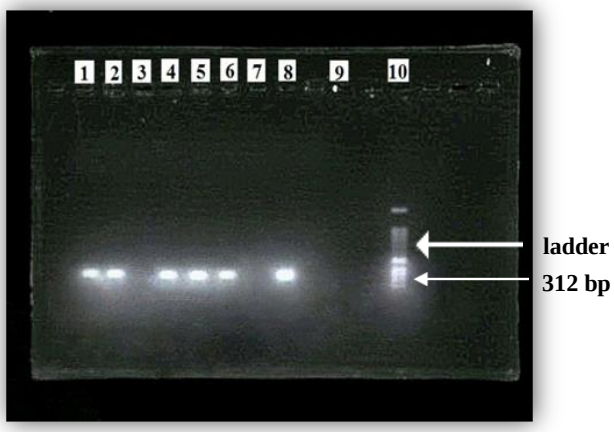

Fig. (2) DNA amplification by polymerase chain reaction (PCR) by using primer Ec001F & Ec001R.
1= EM4/ 2=EM5/ 3= EM7/ 4=EM8/ 5=EM12/ 6=EM18/ 7= Erwinia carotovora subsp. carotovora ATCC 495 -nonpathogenic for potato / 8= Erwinia carotovora subsp. carotovora ATCC 15713 –pathogenic for potato/ 9= blank/ 10= 1500 bp DNA ladder RTU/ Gene Dire X-USA/ at 50 volt / after 75 min- 90 min.

References

[1] Cuppels, D.; Kelman, A. "Evaluation of selective media for isolation of soft-rot bacteria from soil and plant tissue''; Phytopathology. 64, 468- 475, **1974**.

[2] De Boer, S. H. "Prospects for control of potato diseases caused by pectolytic Erwinias'' in; Zehnder, G. W.; Powelson, M. L.; Jansson, R K.; Raman, K. V. (eds). "Advances in Potato pest biology and management'' **;** APS Press, St Paul, Minnesota; pp 136-148; **1994**.

[3] Degefu, Y.;Virtanen, E.; Vayrynen, T. "Pre-PCR Processes in the Molecular Detection of Blackleg and Soft Rot Erwiniae in Seed Potatoes''; Journal of phytopathology. 157, 370- 378, **2009**.

[4] FAOSTAT data, "The Top 5 Potato Producing Countries''; Potato World, Commodity report, **2012**.

[5] Farber, J. M. "An introduction to the Hows and Whys of molecular typing''; Journal of Food Protection. 59, 1091- 1101, **1996**.

[6] Gardan, L.; Gouy, C.; Christen, R.; Samson, R. "Elevation of three subspecies of *Pectobacterium carotovorum* to species level:

Pectobacterium atrosepticum sp. nov., *Pectobacterium betavasculorum* sp. nov. and *Pectobacterium wasabiae* sp. Nov''; International Journal of Systematic and Evolutionary Microbiology. 53, 381-91, **2003**.

[7] Hyman, L. J.; Sullivan, L.; Toth, I. K.; Perombelon, M. C. M. "Modified crystal violet pectate medium (CVP) based on a new polypectate source (Slendid) for the detection and isolation of soft rot erwinias''; Potato Research. 44, 265- 270, **2001**.

[8] Lelliott, R. A.; Stead, D. E. "Methods for the diagnosis of bacterial diseases of plants''; Blackwell Scientific Publications; Oxford, **1987**.

[9] Mergaert, J.; Verdonck, L.T.; Kersters, K.; Swings, J.; Boeufgras, J. M.; De Ley, J. "Numerical taxonomy of *Erwinia* species using API systems''; Journal of General Microbiology. 130, 1893- 1910, **1984**.

[10] Perombelon, M .C. M.; Van der Wolf, J. M. "Methods for the detection and quantification of *Erwinia carotovora* subsp. *atroseptica* (*Pectobacterium carotovorum* subsp *atrosepticum*) on potatoes''; a laboratory manual, **2002**.

[11] Perombelon, M. C. M.; Kelman, A. "Ecology of the soft rot erwinias''; Annual Review of Phytopathology. 18, 361- 387, **1980**.

[12] Perombelon, M. C. M. "Factors affecting the accuracy of the tuber incubation test for the detection of contamination of potato stocks by *Erwinia carotovora*''; Potato Research., 22, 63- 68, **1979**.

[13] Perombelon, M. C. M. "The Prokaryotes''; Second Edition., pp 2899- 2921, **2006**.

[14] Perombelon, M. C. M.; Burnett, H. "Two modified crystal violate pectate (CVP) media for detection, isolation and enumeration of soft rot Erwinias''; Potato Research., 34, 79- 81, **1991**.

[15] Prescott, H. "Laboratory exercises in microbiology''; 5ed, the McGraw−Hill, **2002**.

[16] Rich, A. E. "Potato diseases''; Academic Press. New York., pp 238, **1983**.

[17] Sambrook, J.; Fitgah, E.; Maniatis, T. "Molecular cloning; A laboratory Manual''; 3rd ed.; Cold spring; Harbor Laboratory; New York, **2000**.

[18] Schaad, N. W. "Serological identification of plant pathogenic bacteria''; Annual Review of Phytopathology. 17, 123- 147, **1979**.

[19] Verdonck, L.; Mergaert, J.; Rijckaert, C.;Swings J.; Kersters, K.; De ley, J. "Genus *Erwinia*: Numerical Analysis of Phenotypic Features''; International Journal of Systematic and Evolutionary Microbiology. pp 4-18, **1987**.

الخلاصة

جمعت سبعون عينة من درنات البطاطا المصابه من مدينة بغداد خلال الفترة من أيلول ٢٠١١ إلى شباط ٢٠١٢. وقد أخذت هذه العينات من الأماكن التالية والتي شملت: مخزن البطاطا في الرضوانية، مخزن أوراد النهار للبطاطا في أبي غريب، سوق البياع، وتم الحصول على سلالتين من بكتريا *Erwinia* من قسم وقاية النبات/ كلية الزراعة/ جامعة بغداد. أستخدم وسط الكريستال البكتيت البنفسجي Crystal Violet Pectate (CVP) كوسط لعزل وتنقية بكتريا *Erwinia*.

وصفت السلالات والعزلات من بكتريا *Erwinia* وفقا لمظهرها وخصائصها المجهرية جنبا إلى جنب مع الاختبارات الكيموحيويه والاختبارات API 20E.

أظهرت نتائج الاختبارات الكيموحيوية أن بكتريا *Erwinia* موجبة لأختبار Catalase،Raffinose، Cellobiose وسالبة لأختبار Oxidase و Sorbitol وبأستخدام طريقة Reagent Genomic DNA Kit وجد بأن بعض العزلات الحلية لبكتريا *Erwinia* والسلالتين تحوي على الاقل حزمتين صغيرتين من الدنا البلازميدي وتم تحديد هوية على مستوى تحت نوع للعزلات المحليه من *Erwinia* بواسطته ومن خلال تقنية تفاعلات تضاعف سلسلة الدنا و بادئ حمض نووي متخصص على مستوى تحت النوع لبكتريا *Erwinia* وهو Ec001 وأظهرت النتائج أن العزلات المحلية من بكتريا *Erwinia* والسلالة القياسية *Erwinia caratovora* subsp. *caratovora* ATCC 15713 والممرضه لنبات البطاطا بأنها أيجابية بأرتباطها بالبادئ المذكور سابقا وظهور حزمه واحدة (312bp).