

Detection and Identification of Potato Soft Rot *Pectobacterium carotovorum* Subspecies *carotovorum* by PCR Analysis of 16S rDNA in Jordan

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Abstract

Various bacterial species are known to be agents causing soft rot of potatoes. The results of this study showed that potato soft rot is widely spread in different potato planting areas in Jordan. A survey was conducted through the years 2013-2015 to detect potato soft rot disease in Jordan, two hundred and four rotted potato samples were collected from different potato growing areas through different potato growing seasons. One hundred and thirty one bacterial isolates were isolated, purified on selective media and identified as *Pectobacterium carotovorum* subspecies *carotovorum* (*Pcc*) by different biochemical and physiological tests. Furthermore, 131 *Pcc* Jordanian (Jo) isolates were identified by PCR analysis of total DNA extracted from isolates that were biochemically identified as *Pcc* using universal primer Fd1/Rd1. Cloning and sequencing of representative PCR products, amplifying the 16S rDNA region were done. Phylogenetic analysis of the *Pcc* Jo-isolates revealed other than 90% similarity with different reference *Pcc* strains available at the GenBank. Different rot causal agents also were detected by PCR amplification and further sequences. The sequencing data revealed similarities to *Pseudomonas fluorescence*, Enterobacteriaceae genera such as *Enterobacter* spp., *Serratia* spp. and *Klebsiella* spp., in addition to *Pectobacterium carotovorum* subsp. *carotovorum*. This study indicated that using molecular techniques such as amplification of 16S rDNA region is a sensitive and specific method for detecting *Pcc* as potato soft rot causal agent. So far this is the first study where *Pcc* has been identified by using PCR and sequencing approaches in Jordan.

Keywords

Pectobacterium carotovorum subsp. *carotovorum* (*Pcc*), Soft Rot of Potato,

1. Introduction

Potato (*Solanum tuberosum* L.) is one of the most common and important vegetables listed among the five principle and popular crops grown in the world as well as in Jordan for both consumption and export. In 2013, the area planted with potato in Jordan was 34,028.5 dunams, with total production of 103,223.4 tons [1].

Different bacterial diseases have been reported to attack potatoes leading to a high economic loss in yield and quality under favorable environmental conditions. However, the potato soft rot is one of the most important diseases of potatoes causing great reduction in yield resulting in economic losses in the field, during transit, and reported to be caused by various species: *Bacillus* spp., fluorescent *Pseudomonas* spp., *Enterobacter cloacae* and *Erwinia* spp. [2] [3] [4] [5]. However, *Erwinia carotovora* subsp. *carotovora* is reported as the most common causal agent of bacterial soft rot of potato and other commercially important crops [4] [6] [7].

Controlling bacterial diseases are very difficult and efficient control should rely on cultural practices and could benefit from different tools allowing rapid and specific detection of causal agents at low levels of infectivity.

Efficient and cost-effective detection and identification methods are essential to investigate the ecology and pathogenesis of soft rot *Erwinias*. Different methods are followed in order to detect, identify and differentiate soft rot causing bacteria to species and subspecies level, of these methods: microscopy, isolation, biochemical characterization, serological techniques, pathogenicity and bioassay tests. All of these methods are time consuming, insensitive, inaccurate and not suitable for routine work to test a large number of samples [8] [9].

In the last 30 years, Polymerase Chain Reaction (PCR) has been used for specific, rapid detection and identification of pathogen isolates. PCR techniques greatly enhance detection sensitivity, simplicity and rapidity compared with other methods of identification and are based on specific amplification of a target DNA sequence that is unique to a bacterial genome [9] [10] [11].

In Jordan, *Erwinia carotovora* subsp. *carotovora*, recently known as *Pcc*, was identified as the causal agent of soft rot disease of vegetables; its detection and identification was carried out through traditional techniques such as isolation on selective media and biochemical characterization. The pathogen infects and causes disease on a wide variety of hosts belonging to different families of vegetables either in field and storage in different areas including: Jordan Valley and Uplands. Soft rot of potatoes is a tuber borne disease where the contaminated mother tubers were reported to be the main source of inoculum. However, this bacterium was found to survive in the soil with population trends varying with

the fluctuation in soil temperature [12].

Traditional techniques used for detection and identification of the causal agents are time consuming and relatively insensitive. Therefore, there is an urgent need for a sensitive and highly specific technique for rapid detection and identification of *Pcc*.

This research was conducted in order to isolate and identify potato soft rot causal agent by PCR technique.

2. Materials and Methods

2.1. Sample Collections and Bacterial Isolation

A minimum of five potato representative samples was collected from each site, including stem and tubers suspected to be infected with soft rot, grown in the fall and spring seasons and storage facilities from different potato growing areas in Jordan.

The rotted tubers and plant tissues were washed; surface disinfected, rinsed with sterile distilled water (SDW). Approximately 10 g of rotted tubers was cut, placed in sterile bottles, placed on a shaker at 200 rpm at room temperature. After the suspension becomes homogenized a series of serial dilutions were prepared up to 10^{-3} dilution, then 0.1 ml of the 10^{-3} dilution was spread by a sterile glass rod onto the surface of three Logan's medium plates [13]. The inoculated plates were incubated at $27^{\circ}\text{C} \pm 2^{\circ}\text{C}$ checked periodically. Appearance of bacterial colonies with wide, pink centers within the first 24 hours of inoculation was suspected to be *Pcc* [14], and then single colonies were restreaked onto new NA plates. The obtained bacterial isolates were kept in refrigerator for further identifications.

2.2. Identification of the Causal Agent

Twenty four hours old cultures of the obtained bacterial isolates were subjected to biochemical and physiological tests for characterization and identification as described by [13] [15]. The same tests were run against a reference culture of *Pcc* isolate NCPPB312 (National Collection of Plant Pathogenic Bacteria) obtained from Food and Environment Research Agency (*fera*), United Kingdom and against negative control, these tests were:

Oxidase Test, Catalase Test, Potato soft rot, Oxidative fermentative Test (OF), Growth at 37°C , Sodium chloride tolerance test, Reducing substances from sucrose, Urease Production Test, Acid Production from Carbohydrates Test.

2.3. Isolation and PCR Amplification of Genomic

Bacterial DNA was extracted from 24 hours old pure bacterial cultures of one hundred and thirty one bacterial isolates grown on NA media at 37°C , obtained and identified by biochemical and physiological tests as *Pcc* isolates. Pure bacterial colonies were picked with a sterile loop and mixed in 4 ml of nutrient broth media in a sterile and labeled culture tubes (Greiner, Bio-One, GmbH,

Germany) incubated over night at 37°C, with shaking at 150 rpm.

Genomic DNA extraction was done using DN easy Blood and Tissue Kit (Qiagen, Valencia, CA); the protocol was performed according to the manufacturer's instructions. The quantity and quality of the extracted genomic DNA were measured using the spectrophotometer (BioPhotometer plus, Eppendorf, Hamburg, Germany) and DNA was visualized by electrophoresis (BIORAD power PAC300) in 1.0% agarose gel in Tris-Acetate-EDTA (TAE 1X) (Promega, Madison, Wisconsin) buffer stained with ethidium bromide (0.5 µg/ml) (Sambrook and Russell, 2001). The extracted DNA was stored at -20°C for further PCR work.

2.4. Polymerase Chain Reaction (PCR) Amplification

In order to detect the presence of the desired DNA fragments that confirm the presence of *Pcc*, the 16S rDNA sets of primers (Fd1: CAGAGTTTGATCCTGGCTCAG, Rd1AAGGAGGTGATCCAGCC), were used (Lane, 1991). The concentration of each primer was adjusted to 10 pmol/µl by Nuclease Free Water (NFW) and stored at -20°C.

DNA amplification was done according to the conventional method and the PCR reaction mix was in the final reaction volume of 25 µl contained; 5.0 µl 5X Crimson *Taq* buffer, 1.1 µl 25 mM MgCl₂, 0.5 µl 10 mM dNTPs, 0.13 µl 5 U/µl *Taq* polymerase, 1.25 µl 10 µM of each primer and 2.0 µl DNA template finalized to 25 µl by adding 13.77 NFW.

PCR was performed in a thermal cycler BIORAD T100™ (Biorad, Hercules, CA) using the following protocol and adjusted as needed. The reaction involved Initial denaturation (94°C, 5 min) followed by 35 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), extension (72°C, 1 min) with a final extension (72°C, 7 min).

2.5. DNA Sequencing and Phylogenetic Analysis

Clones containing inserts and PCR products were identified and nucleotide sequencing was performed in both directions by MacroGen Korea (Seoul, Rep. OF Korea) or Quintara Biosciences (South San Francisco, CA). The homology search of the cloned sequences or PCR products was performed using Basic local alignment searching tool (BLAST) at the NCBI server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). A Blast search was performed for nucleotide using BLASTn. Evolutionary trees for the data were reconstructed using MEGA [16] and they were inferred by using the Neighbor-Joining (NJ) program of MEGA.

3. Results

3.1. Sample Collection, Identification and Characterization of the Causal Agent

Approximately, two hundreds and four rotted potato samples suspected to be

infected with soft rot disease were collected from fields and storage throughout potato growing areas in Jordan during summer, autumn and winter seasons, during the period extended from November/2013 until July/2015 from 20 locations.

After inoculation tests, observation of different characteristics, biochemical, physiological and nutritional tests; one hundred and thirty one bacterial isolates could be identified as strains of *Pcc*. The reactions of the tested bacterial isolates to the different biochemical, physiological and nutritional tests were identical to the results of the same tests ran against the reference bacterial culture of *Pcc* isolate NCPPB312.

3.2. Detection of *Pcc* Using Polymerase Chain Reaction (PCR)

One hundred and thirty one bacterial isolates yielded a 1530 bp DNA fragments with the universal primers set (Fd1/Rd1) which are commonly used for the detection of bacteria (**Figure 1**).

3.3. Sequencing Analysis

Searching nucleotide data base using a nucleotide query (BLASTn) of all isolates sequences obtained showed high range of similarity to different plant rotting causal agents such as *Pseudomonas fluorescens*, Enterobacteriaceae genera such as *Enterobacter* spp., *Serratia* spp. and *Klebsiella* spp. Some sequences showed high similarity to *Pectobacterium* spp., in addition to the similarity to *Pcc* isolates (**Table 1**).

Maximum nucleotide similarity (BLASTn) results obtained from Jo-isolates that amplified with Fd1 and Rd1 set of primers showed high similarity with different strains of *Pcc* deposited in the GenBank (**Table 2**), and the nucleotide sequence similarity percentage ranged from 96% up to 100%. In addition to similarity with different strains of *Pcc*, some isolates showed similarity with other rotting causal agents such as *Pseudomonas* spp., *Serratia* spp. and *Enterobacter* spp., where the similarity ranged between 94% and 98% (**Table 1**).

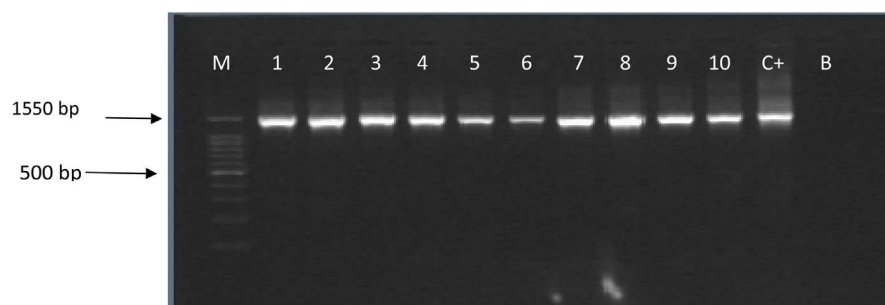


Figure 1. Agarose gel electrophoresis for PCR amplified DNA of *Pectobacterium carotovorum* subsp. *carotovorum* isolates using Fd1 and Rd1 primers with the expected amplified product of 1530 bp. Lane M represents Ladder 100 bp (Gene Direx). Lanes 1-10; isolates Jo-Q16, Q19, A11, A2, Q14, Q21, Q29, A5, Q27 and Q30, respectively. Lanes C+; Positive control (reference isolate NCPPB312) and B; Buffer [17].

Table 1. Homology search (BLAST) results of all sequenced samples.

No.	Primer Set	Isolate	Type of Sequenced sample (Clones/PCR product)*	Maximum similarity hit
1	Fd1 Rd1	M86\1	clones	98% <i>Pseudomonas</i> sp.
2		G20\2		98% <i>Serratia</i> sp.
3		G18\2		98% <i>Enterobacter</i> sp.
4		G29\1		96% <i>Pseudomonas</i> sp.
5		G34\2		99% <i>Pseudomonas</i> sp.
6		G37\4		99% <i>Enterobacter</i> sp.
7		G49\1		97% <i>Pseudomonas</i> sp.
8		G55\1		97% <i>Pseudomonas</i> sp.
9		G60\3		94% <i>Pseudomonas</i> sp.
10		G62\1		97% <i>Pseudomonas</i> sp.
11		G65\3		96% <i>Pseudomonas</i> sp.
12		G70\1		98% <i>Pseudomonas</i> sp.
13		S102\1		96% <i>Pseudomonas</i> sp.
14		R16\3		96% <i>Agrobacterium</i> sp.
15	Fd1 Rd1	G27\4	PCR products	94% <i>P. carotovorum</i>
16		Q111\4		99% <i>Enterobacter</i> sp.
17		R105\4		95% <i>Pcc</i>
18		G18\3		96% <i>P. carotovorum</i>
19		G43\1		97% <i>Enterobacter</i> sp.
20		G70\2		99% <i>Pcc</i>
21		M113\2\4		95% <i>Klebsilla</i> sp.
22		M113\4		95% <i>Pcc</i>
23		R105\4		99% <i>Pseudomonas</i> sp.
24		NCPBP312		99% <i>Pcc</i>

*The type of product that have been sequenced.

3.4. Phylogenetic Analysis

The preliminary analysis for the partial DNA sequences obtained compared with different *Pcc* strains deposited in the GenBank revealed that different isolates from different regions clustered closely to the reference strains used for comparison (Figure 2). Isolates Jo-M2, 113, 86 and Jo-G 70, 18, 43 clustered closely to reference strains; *Pcc* C150 from Germany (Acc. no. J243601.1), strain *Pcc* ECC301901 from Korea (Acc. No. FJ595867.1) and *Pcc* strain NBRC 3830 from Japan (Acc. No. AB680143.1) in addition to the reference strain *Pcc* NCPBP312 (Acc. No. NZ_JQHJ01000042.1) from UK with 100% bootstrap value. Whereas isolates Jo-G37 and Jo-Q111 clustered individually and were closer to the *Pcc* strain Y46 (Acc. No. KP187511.1) from China with bootstrap value of 98%.

Table 2. Maximum nucleotide similarity (BLASTn) between *Pectobacterium carotovorum* subsp. *carotovorum* Jo-isolates amplified with Fd1 and Rd1 set of primers and most closely species/subspecies [17].

No.	Isolate and Accession no. in GenBank	Closely related species/subspecies	E-value	Maximum % similarity	Accession no.
1	Jo-G18 (MF375782)	<i>Pcc</i> strain C142	5e ⁻¹⁷⁵	100%	JF926752.1
2	Jo-G43 (MF375783)	<i>Pcc</i> strain Y46	9e ⁻¹⁶⁹	99%	KP187511.1
3	Jo-G70 (MF375784)	<i>Pcc</i> strain Y46	4e ⁻¹⁷²	100%	KP187511.1
4	Jo-M113 (MF375785)	<i>Pcc</i> strain Y46	4e ⁻¹⁷²	100%	KP187511.1
5	Jo-M86 (MF375786)	<i>Pcc</i> strain Y46	4e ⁻¹⁷²	98%	KP187511.1
6	Jo-G37 (MF375787)	<i>Erwinia</i> spp.ST12	4e ⁻¹⁵⁸	99%	KP405846.1
7	Jo-M2 (MF375788)	<i>Pcc</i> strain Y46	4e ⁻¹⁷²	98%	KP187511.1
8	Jo-G20 (MF375789)	<i>Pcc</i> C1 strain	0.0	96%	CP001657.1
9	Jo-Q111 (MF375790)	<i>Pcc</i> strain KN28216	0.0	99%	GU936999.1
10	Reference strain NCPPB312	<i>Pcc</i> strain Y46	6e ⁻¹⁵⁶	98%	KP187511.1

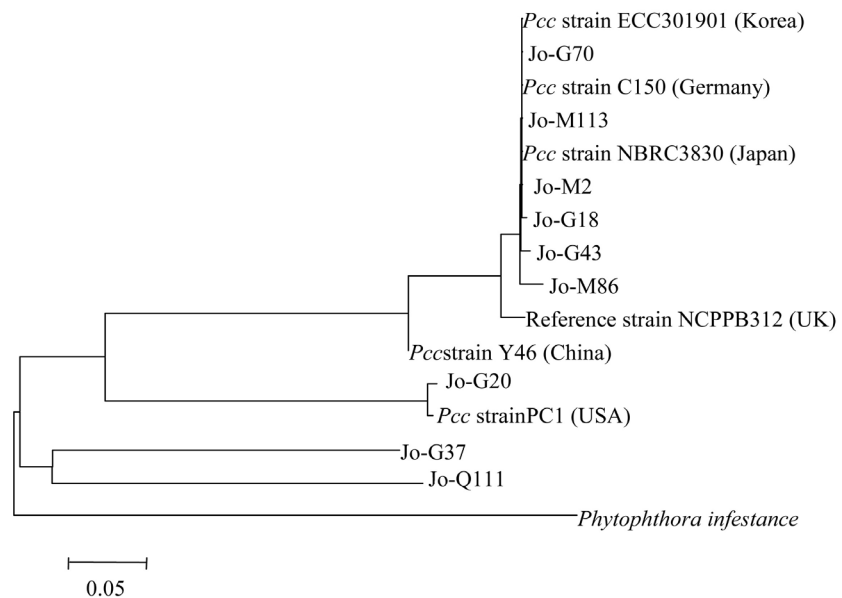


Figure 2. Phylogenetic analysis of nucleotide sequences of *Pectobacterium carotovorum* subsp. *carotovorum* Jo-isolates and reference strains based on 16S rDNA partial gene sequences. The generation of the tree was conducted in MEGA program. The branching pattern was generated by the Neighbor-Joining method; stability of the tree was assessed by 1000 bootstrap replication. *Phytophthora infestans* (Acc. No. ES466728.1) was used as out group.

Moreover, the individual isolate Jo-G20 clustered with the reference strain *Pcc* C1 (Acc. No. CP001657.1) from USA with 100% bootstrap value. *Phytophthora infestance* (Acc. No. ES466728.1) from USA was used as out group because the 16S rDNA primers set can detect all bacterial strains.

4. Discussion

Seed potatoes have been imported into Jordan with reports of increasing incidence and dispersal of important bacterial potato diseases in different potato growing regions. Field trips to all potato growing areas during all seasons of the study at different plant growing stages revealed that potato soft rot disease occurred in all surveyed areas.

The number of species and subspecies of *Pectobacterium* has been increased over recent years and, as a result, their identification and differentiation by classical microbiological tests have become challenging. It has become more difficult to make accurate identification based on biochemical tests alone because phenotypic characteristics vary among strains of same species and subspecies [18]. However, identification of Jordanian potato soft rot isolates using traditional methods; biochemical tests which are usually used for identification of *Pcc* at species level, results indicated that *Pcc* was the causal agent of the disease, but our findings later on, indicated that these tests were not highly accurate when compared to molecular methods. Compared with different DNA sequence analysis used in this study, biochemical tests were able to identify most isolates but misidentified others.

Sequence analysis identified in addition to *Pcc* other bacterial genera that could be associated with potato soft rot. Our findings indicated different bacterial causal agents that would be associated with *Pcc* and causing rot diseases such as *Pseudomonas fluorescens*, different Enterobacteriaceae genera such as *Enterobacter* spp., *Serratia* spp., and *Klebsiella* spp., whereas these species belonging to these genera cause rots and were reported by different studies [2] [3] [4] [6] [18] [19]. Therefore, utilization of molecular data will be required since morphological or biochemical characters may be less robust.

In this study, PCR was carried out for all DNA extracts of bacterial soft rot isolates that were confirmed by biochemical tests as *Pcc*, the result of PCR for 131 isolates tested detected the presence of the desired DNA fragments of 1530 bp using the 16S rDNA set of primers, (Fd1 and Rd1) compared with the positive DNA extract of the reference strain of *Pcc* NCPPB312 which gave a product size of 1530 bp, The 16S rDNA sequences are conserved with stable copies and its analysis is discriminative than other ribosomal regions, and in general 16S rDNA is amplified and sequenced with universal primers to identify species and subspecies [20] [21].

Consequently, the identity of *Pcc* causing soft rot of potato in Jordan was confirmed by phylogenetic analysis of 16S rDNA from variety of phytopathogenic bacteria. Jo-isolates tested from different regions of Jordan clustered together

with *Pcc* 16S rDNA sequences presented in the GenBank. Results support the classification of *Pcc* as a species or subspecies distinct from *Phytophthora infestance* strain which was used as out group.

In our homology search; BLASTn of Jo-isolates sequenced on the bases of the 16S rDNA, detected high similarity with different reference strains at the GenBank and was closely related to the sequences of different bacterial rotting causal agents such as; *Pseudomonas* spp., *Bacillus* spp., *Serratia* spp. and *Enterobacter* spp.. In fact the Fd1 and Rd1 primers that were used in this study are general primers which can detect different bacterial causal agents [22], but could be used as a preliminary step in bacterial identification.

Interestingly, the *Pcc* Jo-isolates from different regions didn't cluster distinctively according to their locations and this would be attributed to the fact that the source or origin of potato seeds in Jordan is mainly from one source and the importing companies are few, which distribute seeds to all agricultural regions (personal communication with MOA). On the other hand, a study done by [23] and confirmed by [20] indicated that *Pectobacterium* can easily spread by different agents such as air currents and may travel long distance in the atmosphere and surface water in addition to contaminated stored seeds [24].

In majority of cases, genotypically isolates were more closely related to one another than those isolated from different geographical regions. However, the similarity between groups of isolates isolated from different regions suggested the same genetic origin of these isolates.

5. Conclusions and Recommendations

Soft rot disease is widely common in different potato growing areas in Jordan and the results of biochemical and physiological tests confirmed that the main causal agent of soft rot in Jordan is *P. carotovorum* subsp. *carotovorum*. While using the PCR primer pair Fd1/Rd1 was found to be reliable in detection and identification of all soft rot Jordanian isolates of *Pcc*, DNA sequencing was found to be the most reliable way in specific detection and confirmation of the causal agent of soft rot.

On the other hand, more studies need to be implemented in order to study soft rot disease etiology and epidemiology.

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