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Comparison of Different Sets of Primers Used in Detection and Identification of Potato Soft Rot *Pectobacterium carotovorum* subsp. *carotovorum (DYE1969)*

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Authors' contributions

This work was carried out in collaboration between all authors. Author IAO designed the study, wrote the protocol, and write the first draft of the manuscript. All the authors managed the analyses of the study read and approved the final manuscript.

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ABSTRACT

Two hundred and four rotted potato samples were collected from different Jordanian potato growing areas and commercial stores. The isolated bacteria were subjected to morphological observation, biochemical and physiological identification as well as PCR. Using different sets of primers, and sequence analysis, 131 bacterial isolates were identified as *Pectobacterium carotovorum subspecies carotovorum (Pcc).* Primers used; universal primer 16S rDNA Fd1/Rd1, species-specific primer ExpccF/ExpccR, *rec*AF/*rec*AR and pathogencity gene *pmr*AF/*pmr*AR. The results of our study showed that the four sets of primers varied in their specificity in detection, where *rec*A and *pmr*A sets of primer were found to be more efficient in detecting *Pcc*. On the other hand, Expcc sets of primer were not so specific in detecting *Pcc* isolates where it was able to detect only 51% of the isolates. In conclusion, our results revealed that molecular techniques using the specific sets of primer was more sensitive and specific in detecting *Pcc* as potato soft rot causal agents.

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Keywords: Sets of primers; amplification; PCR; sequencing.

1. INTRODUCTION

Potato (*Solanum tuberosum* L.) is considered one of the economic vegetables grown in Jordan for both consumption and export where the area planted with potato in Jordan during the year 2013 was 3402,85 ha, with total production of 103223 tons [1].

Different bacterial diseases have been reported to attack potato leading to a high economic loss in yield and quality, including: brown rot (*Ralstonia solanacearum*), common scab (*Streptomyces scabies*), ring rot (*Clavibacter michiganensis* subsp. *sepodonicus*), black leg (*Pectobacterium carotovorum* subsp. *atrosepticum*) and soft rot *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*). However, potato soft rot is one of the most important diseases of potato, causing great economic losses in the field, and is reported to be caused by various bacterial species: *Bacillus* spp., fluorescent *Pseudomonas* spp., *Enterobacter cloacae* and *Erwinia* spp. [2-5]. *Pcc* is reported as the most common causal agent of bacterial soft rot of potato and other commercially important crops [4,6-7]. It is considered among the factors contributing to yield losses and among the most prevalent and destructive bacterial diseases that is difficult to control [8-9].

Soft rot disease caused by *P. carotovorum* subsp. *carotovorum* (Dye, 1969) has a wide host range spreading in tropical and subtropical regions infecting vegetable species, including cabbage, cauliflower, lettuce, onion, pepper, carrot and potato [10-11].

Soft rot is a seed-born disease, where the contaminated potato seed tubers are considered as the major source of inoculum. A range of traditional and modern techniques were followed for the detection of the pathogen and vary in the degree of sensitivity, simplicity and rapidity for large scale use [12, 4].

The methods followed in order to detect, identify and differentiate soft rot causing bacteria to species and subspecies level are; 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 large number of samples [13-14]. 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 [12,14,15].

In Jordan, *Pcc* was identified as the causal agent of soft rot disease of vegetables, where its detection and identification was carried out through traditional techniques such as isolation on selective media and biochemical characterization [10].

Accordingly, this research was conducted through the years 2013 to 2015 in order to isolate and identify the causal agents of potato soft rot, from different potato growing areas and facilities surveyed in Jordan. Secondly, detection and identification of potato soft rot causal agents isolates was obtained by PCR technique using four different sets of primers, confirming the identity by sequencing and further to compare these sets of primers in specificity and sensitivity in *Pcc* detection.

2. MATERIALS AND METHODS

2.1 Isolates and Media

More than 200 potato samples were randomly collected during the years 2013 to 2015 from different potato growing areas in Jordan. All isolates were isolated obtained from rotted tissues using Logan's medium plats incubated at 27±2°C. The bacterial isolates were kept as a suspension in SDW at 4°C for further identifications.

2.2 Biochemical and Phenotypic Tests

Bacterial colonies with small circular colonies with pink centers on Logan's medium plates [16] within the first 24 hours of inoculation were suspected to be *Pcc* [17].

Biochemical and physiological tests used in this study were: oxidase and catalase reactions, potato soft rot, oxidative fermentative test, ability to grow at 37°C, growth in 5% sodium chloride, ability to reduce substances from sucrose, urease production test and acid production from carbohydrates (lactose, sucrose) as described by [16] and [8]. The same tests

were run against a reference culture of *Pcc* isolate NCPPB312.

2.3 Bacterial Genomic DNA Extraction from Bacterial Cultures

Bacterial genomic DNA was extracted from 24 hrs old pure bacterial cultures of 188 bacterial isolates grown in NA media at 27°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 and incubated overnight at 37ºC, with shaking at 150 rpm.

Genomic DNA extraction was done using DNeasy Blood and Tissue Kit (Qiagen, Valancia, CA); the protocol was performed according to the manufacturer's instructions.

Extracted DNA was detected by electrophoresis in 1.5% agarose gel and in Tris – acetate–EDTA (TAE) buffer, visualized following staining with (0.5 µg/ml) ethidium bromide. DNA was stored at - 20ºC until used.

2.4 PCR Amplification, Purification and Sequencing

In order to detect the presence of DNA fragments that confirm the presence of *Pcc*, four sets of primers were used. PCR amplification reactions were performed in a thermal cycler BIORAD T100™ (Biorad, Hercules, CA) using the protocols and conditions as shown in (Table1) and adjusted for each set of primers as needed.

The amplified products were electrophoresed on a 1.5% agarose gel and visualized following staining with ethidium bromide.

The products were purified using the Wizard Purification System (Promega, Madison, Wisconsin) using the protocol according to the manufacturer's instructions.

After purification, DNA fragments were sequenced in both directions in Macrogen Korea (Seoul, Rep. of Korea) or Quintara Biosciencs (South San Francisco, CA). The same sets of primers were used for both amplification and sequencing.

The DNA sequence data obtained were analyzed doing a homology search of PCR products 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 both nucleotide and amino acids using BLASTn and BLASTx respectively, according to the amplified region [18].

3. RESULTS

3.1 Sample Collection

Two hundred and four rotted potato samples suspected to be infected with the soft rot disease were collected from 20 locations throughout potato growing areas in Jordan during Summer, Autumn and Winter seasons. Soft rot was widespread in all regions surveyed.

3.2 Isolation, Identification and Characterization of Causal Agent

After inoculation, observation of morphological characteristic and biochemical tests of all isolates, 188 bacterial isolates could be identified as of *Pcc*. The reactions of the tested bacterial isolates to the different biochemical, and physiological tests were identical to the results of the same tests ran against the reference bacterial culture of *Pcc* isolate NCPPB312.

3.3 Detection of *Pcc* Using Polymerase Chain Reaction (PCR)

The DNA extracts of 188 different isolates biochemically identified as *Pcc* were of good quality and quantity; distinctive bands were detected when DNA extracts ran in 1.0% agarose gel. Furthermore, the spectrophotometer readings indicated that the DNA concentrations ranged between 450-600 ng/µl. and the 260/280 ratio ranged between 1.8 and 2.0.

One hundred and thirty one bacterial isolates yielded a 1530 bp DNA fragment with the universal primers set (Fd1/Rd1) (Fig. 1). The specific set of primers that were designed to bind within *rec*A gene, directed amplification of fragments of about 730 bp (Fig. 2). Ninety eight isolates out of 131 tested isolates (74.8%) were tested positive by using *recA* primer, while, 67 isolates (51%) showed a 550 bp band with the specific Expcc set of primers (Fig. 3). On the other hand, only 33 isolates (25.2%) showed a band of about 660 bp (Fig. 4) when tested using the

specific set of primers *pmr*A (F0145/E2477). This showed that all primers sets including Fd1/Rd1, recAF/recAR, ExpccF/ExpccR and *pmr*A (F0145/E2477) amplified only the target DNA with the expected product size of 1530 bp, 730 bp, 550 bp and 660 bp, respectively.

3.4 Sequencing Analysis

Searching nucleotide data base using a nucleotide query (BLASTn) and amino acid query (BLASTx) of all isolates sequences obtained by different primers sets (Fd1/ Rd1, *recA* and Expcc) showed a high range of similarity to different plant rotting causal agents, according to the set of primers used, such as *Pseudomonas fluorescens*, and to Enterobacteriaceae genera such as *Enterobacter* spp., *Serratia* spp. and *Klebsilla* spp. Some sequences showed high similarity to *Pectobacterium* spp., in addition to the similarity to *Pcc* isolates.

Maximum nucleotide similarity (BLASTn) results obtained from the sequenced Jo-isolates that amplified with the 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 88% and 98%.

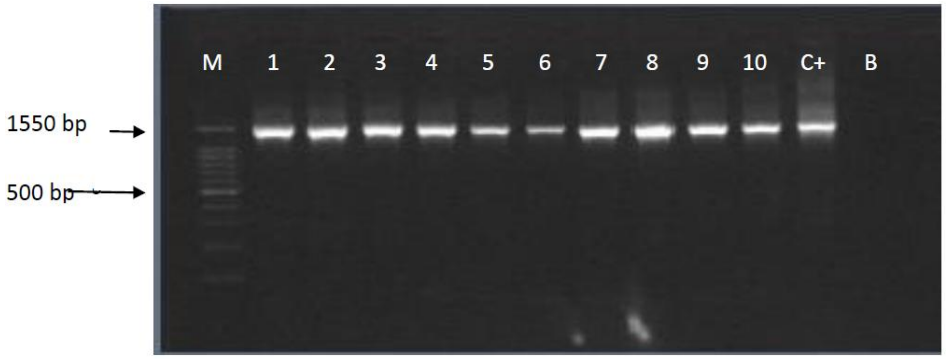

Fig. 1. Agarose gel electrophoresis for PCR amplified DNA of *Pectobacterium carotovorum* subsp. *carotovorum* isolates using Fd1 and Rd1 primers with the expected amplified product of 1530 bp. Lane M represents Ladder 100 bp (Gene Direx). Lanes 1-10; isolates Jo-Q16, Q19, A11, A2, Q14, Q21, Q29, A5, Q23, Q27 and Q30, respectively. Lanes C+; Positive control (reference isolate NCPPB312) and B; Buffer

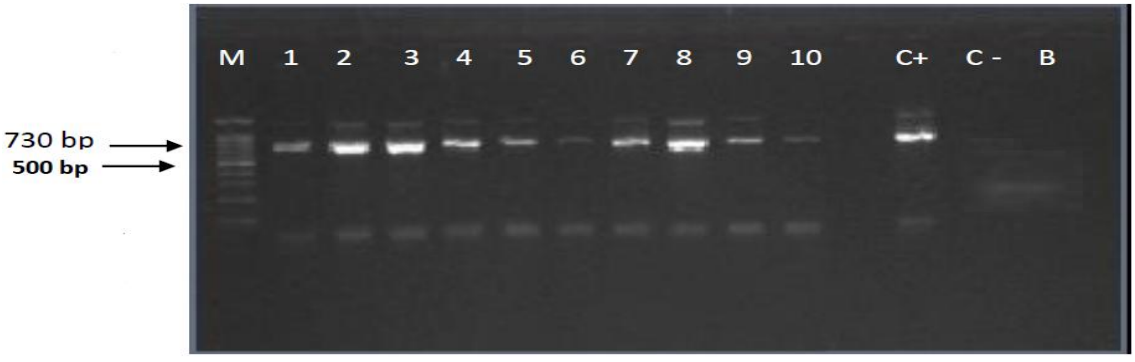

Fig. 2. Agarose gel electrophoresis for PCR-amplified DNA of Pectobacterium *carotovorum* subsp. *carotovorum* isolates using *rec*A primers set with the expected amplified product of 730 bp. Lane M represents Ladder 100 bp (GeneDirex). Lanes 1- 10; isolates Jo- Q16, Q19, A11, A2, Q14, Q21, Q29, A5, Q23 and Q27, respectively. Lanes C+; Positive control (reference isolate NCPPB312), C-; Negative control (*Bacillus* sp.) and B; Buffer

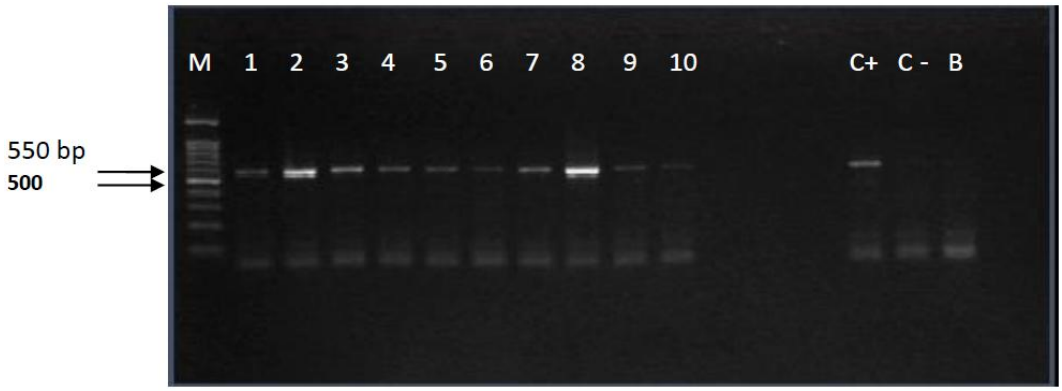

Fig. 3. Agarose gel electrophoresis for PCR-amplified DNA of *Pectobacterium carotovorum* subsp. *carotovorum* isolates using EXPCC primers set with the expected amplified product of 550 bp. Lane M represents Ladder 100 bp (GeneDirex). Lanes 1- 10; isolates Jo-Q 16, Q19, A11, A2, Q14, Q21, Q29, A5, Q23 and Q27, respectively. Lanes C+; Positive control (reference isolate NCPPB312), C-; Negative control (*Escherichia coli*)and B; Buffer * [19]

BLASTn; maximum nucleotide similarity for sequences of selected *Pcc* Jo-isolates that were amplified with *rec*A primer set, query sequences reveled high similarity with different closely related *Pcc* sequences deposited in the GenBank, where maximum percentage similarity ranged from 82% to 100%, closely related *Pcc* strains. Accession numbers are in Table 3. Maximum percentage similarity for most *Pcc* Jo-isolates was with *Pcc* ATCC 15713 *rec*A strain (Acc. no. AY2647991) from Poland. Whereas, maximum amino acid similarity (BLASTx) analysis was done for the same isolates, and results showed a similar pattern, the highest similarity percentage was w ith *Pcc* strain (Acc. no. CCP50100.2) (Table 4).

Maximum nucleotide similarity (BLASTn) results of sequences for selected *Pcc* Jo-isolates that amplified with Expcc; species-specific set of primer, are presented in Table 5. Searching results showed that most of *Pcc* Jo-isolates showed high similarity with the strain *Erwinia carotovora* subsp. *carotovora* (Acc. no. AF046928.1) from Korea and with *Pcc* strain PC1 (Acc. no. CP001657.1) from USA. The maximum similarity percentage ranged from 86% to 98%, some *Pcc* Jo-isolates showed high similarity with the reference strain *Pectobacterium carotovorum* subsp. *odoriferum* (Acc. no. CP009678.1) from China, whereas, their maximum similarity percentage ranged from 85% to 98%.

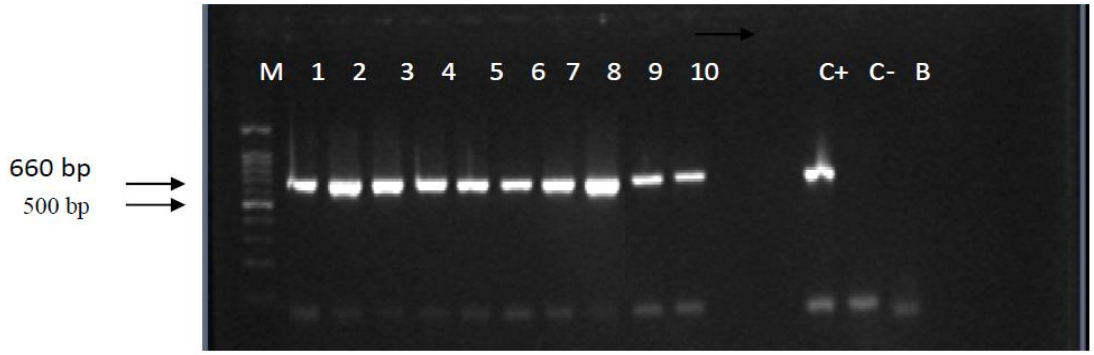

Fig. 4. Agarose gel electrophoresis for PCR-amplified DNA of *Pectobacterium carotovorum* subsp. *carotovorum* isolates using *pmr*A primers set with the expected amplified product of 660 bp. Lane M represents Ladder 100 bp (GeneDirex). Lanes 1- 10; isolates Jo-Q16, Q19, A11, A2, Q14, Q21, Q29, A5, Q23 and Q27, respectively. Lanes C+; Positive control (reference isolate NCPPB312), C-; Negative control (*Escherichia coli*) and B; Buffer

Table 1. Primers sequence used for detection of *Pectobacterium carotovorum* subsp. *carotovorum*

No.	Primer name	Sequence 5'- 3'	Note	References
1.	16S rDNA		16S rDNA	[20]
	Fd1	CAGAGTTTGATCCTGGCTCAG		
	Rd1	AAGGAGGTGATCCAGCC		
2.	Oligonucleotide		Reported to	[12]
	ExpccR	GCCGTAATTGCCTACCTGCTTAAG	be Specific	[21-22]
	ExpccF	GAACTTCGCACCGCCGACCTTCTA	for *Pcc*	
3.	*pmr*A			[23]
	F0145	TACCCTGCAGATGAAATTATTGATTGTTGAAGAC	*pmr*A gene	
	E2477	TACCAAGCTTTGGTTGTTCCCCTTTGGTCA		
4.	*rec*A			[24]
	F	GGTAAAGGGTCTATCATGCG	*rec*A gene	
	R	CCTTCACCATACATAATTTGGA		
5.	INPCCF	TTCGATCACGCAACCTGCATTATC	Nested PCR	[12]
	INPCCR	GGCCAAGCAGTGCCTGTATATCC	after amplification with Expcc	

Table 6 demonstrates the maximum nucleotide similarity (BLASTn) results for selected *Pcc* Jo-isolates amplified with *pmr*A primers set. Maximum percentage similarity with closely related species/subspecies ranged from 91% to 100% with *Pcc* strain P603AH1 (Acc. no. JQ278721.1) from Morocco.

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 of different plant growing stages revealed that the potato soft rot disease occurred in all surveyed areas including: Amman, AR Ramtha, Jordan Valley, Ma'an, Madaba and Mafraq. Studies and reports indicated spreading of soft rot bacterial diseases in potato growing areas in Jordan [10].

The isolation of bacteria from diseased samples on Logan's media developed small circular bacterial colonies with pink to red purple centers means that the isolated bacterium is suspected to be *Pcc* [17]. The reactions of the bacterial isolates obtained from diseased potato samples collected from different locations of the study to the biochemical tests were found to be similar and identical to the reaction of the reference culture of *Pcc* (NCPPB312) to the same tests [16], which confirmed the results that *Pcc* is the main causal agent of potato soft rot in Jordan [10].

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

No.	Isolate	Closely related species/subspecies	E-value	Maximum % similarity	Accession no.
1	Jo-G18	*Pcc* strain C142	$5e^{-175}$	100%	JF926752.1
2	Jo-G43	*Pcc* strain Y46	$9e^{-169}$	99%	KP187511.1
3	Jo-G70	*Pcc* strain Y46	$4e^{-172}$	100%	KP187511.1
4	Jo-M113	*Pcc* strain Y46	$4e^{-172}$	100%	KP187511.1
5	Jo-M86	*Pcc* strain Y46	$4e^{-172}$	98%	KP187511.1
6	Jo-G37	*Erwinia* spp.ST12	$4e^{-158}$	99%	KP405846.1
7	Jo-M2	*Pcc* strain Y46	$4e^{-172}$	98%	KP187511.1
8	Jo-G20	*Pcc* C1 strain	0.0	96%	CP001657.1
9	Jo-Q111	*Pcc* strain KN28216	0.0	99%	GU936999.1
10	Reference strain NCPPB312	*Pcc* strain Y46	$6e^{-156}$	98%	KP187511.1

Table 3. Maximum nucleotide similarity (BLASTn) between *Pectobacterium carotovorum* subsp. *carotovorum* jo-isolates amplified with *rec*A set of primers and most closely species/subspecies

No.	Isolate	Closely related species/subspecies	E-value	Maximum % similarity	Accession no.
1	Jo-Q16	*Pcc* ATCC 15713 *rec*A	0.0	100%	AY264799.1
2	Jo-Q19	*Pcc* ATCC 15713 *rec*A	0.0	100%	AY264799.1
3	Jo-A11	*Pcc* ATCC 15713 *rec*A	0.0	99%	AY264799.1
4	Jo-A2	*Pcc* ATCC 15713 *rec*A	0.0	99%	AY264799.1
5	Jo-Q14	*Pcc* ATCC 15713 *rec*A	0.0	99%	AY264799.1
6	Jo-Q 21	*Pcc* ATCC 15713 *rec*A	0.0	99%	AY264799.1
7	Jo-Q29	*Pcc* ATCC 15713 *rec*A	0.0	100%	AY264799.1
8	Jo-A5	*Pcc* ATCC 15713 *rec*A	0.0	100%	AY264799.1
9	Jo-Q23	*Pcc* ATCC 15713 *rec*A	0.0	100%	AY264799.1
10	Jo-Q27	*Pcc* ATCC 15713 *rec*A	0.0	100%	AY264799.1
11	Jo-Q30	*Pcc* ATCC 15713 *rec*A	0.0	99%	AY264799.1
12	Jo-R83	*Pcc* ATCC 15713 *rec*A	0.0	89%	AY264799.1
13	Jo-R123	*Erwinia* spp.	$4e^{-125}$	82%	AY208918.1
14	Jo-G68	*Pcc* IRICA5 strain	$1e^{-76}$	90%	KJ634056.1
15	Reference strain NCPPB312	*Pcc* ATCC 15713 *rec*A	0.0	97%	AY264799.1

Table 4. Maximum amino acid similarity (BLASTx) between *Pectobacterium carotovorum* subsp. *carotovorum* jo-isolates amplified with *rec*A set of primers and the most closely related species/subspecies

No.	Isolate	Closely related species/subspecies	E-value	Maximum % similarity	Accession no.
1	Jo-Q16	recombinase A *Pcc*	$5\ e^{-125}$	100%	CCP50100.1
2	Jo-Q19	recombinase A *Pcc*	$5\ e^{-125}$	100%	CCP50100.1
3	Jo-A11	recombinase A *Pcc*	$1\ e^{-113}$	100%	CCP50100.1
4	Jo-A2	recombinase A *Pcc*	$5\ e^{-125}$	100%	CCP50100.1
5	Jo-Q14	recombinase A *Pcc*	$5\ e^{-125}$	100%	CCP50100.1
6	Jo-Q21	recombinase A *Pcc*	$6\ e^{-125}$	100%	CCP50100.1
7	Jo-Q29	recombinase A *Pcc*	$2\ e^{-118}$	99%	CCP50100.1
8	Jo-A5	recombinase A *Pcc*	$5\ e^{-125}$	100%	CCP50100.1
9	Jo-Q23	recombinase A *Pcc*	$5\ e^{-125}$	100%	CCP50100.1
10	Jo-Q27	recombinase A *Pcc*	$5\ e^{-125}$	100%	CCP50100.1
11	Jo-Q30	recombinase A *Pcc*	$2\ e^{-116}$	98%	CCP50100.1
12	Jo-R83	recombinase A *Pcc*	$7\ e^{-112}$	99%	CCP50100.1
13	Jo-R123	recombinase A *Pcc*	$3\ e^{-113}$	100%	CCP50109.1
14	Reference strain NCPPB312	recombinase A *Pcc*	$1\ e^{-52}$	99%	CCP50100.1

The number of defined species and subspecies of *Pectobacterium* has 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 the same species and subspecies [25]. However, identification of Jordanian potato soft rot isolates using traditional methods such as biochemical tests which are usually used for identification of *Pcc* at species level 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.

Detection of the *Pcc* Jo-isolates using PCR amplified with different sets of primers, differed according to the set of primers used, whereas all of the DNA extracts of 131 isolates were detected using the universal primer 16S rDNA; Fd1and Rd1 primers set, and 74.8% of the isolates gave bands with 730 bp when

amplified with *rec*A primers set and 51% of the isolates gave positive reaction when amplified with Expcc set of primers with expected size of 555 bp, while only 25.2% gave the desired bands of 660 bp when PCR was run using *pmr*A set of primers which confirm the results obtained in different studies [12,23,24,26].

Consequently, the identity of *Pcc* causing soft rot of potato in Jordan was confirmed by sequencing analysis of 16S rDNA, *rec*A gene, Expcc species specific and *pmr*A gene. Most of the *Pcc* Jo-isolates showed high degree of similarity with different *Pcc* strains from the GenBank.

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 indicated the presence of the desired DNA fragments of 1530 bp using the 16S rDNA set of primers, (Fd1and Rd1). The 16S rDNA sequences are conserved with stable copies and its analysis is discriminative than other primer pairs used in this study. In general 16S

Table 5. Maximum nucleotide similarity (BLASTn) between *Pectobacterium carotovorum* subsp. *carotovorum* jo-isolates amplified with Expcc set of primers and the most closely related species

No.	Isolate	Closely related species/subspecies	E-value	Maximum % similarity	Accession no.
1	Jo-G59	*Pco*	5 e^{-42}	89%	CP009678.1
		Ecc	2 e^{-35}	86%	AF046928.1
2	Jo-S97	*Ecc*	2 e^{-26}	97%	AF046928.1
3	Jo-Q16	*Pco*	2 e^{-79}	98%	CP009678.1
		Ecc	8 e^{-30}	94%	AF046928.1
		Pcc PC1 strain	1 e^{-157}	92%	CP001657.1
4	Jo-Q19	*Pco*	4 e^{-82}	98%	CP009678.1
		Ecc	8 e^{-10}	94%	AF046928.1
		Pcc PC1 strain	2 e^{-155}	92%	CP001657.1
5	Jo-A11	*Pco*	3 e^{-133}	98%	CP009678.1
		Ecc	4 e^{-122}	94%	AF046928.1
		Pcc PC1 strain	2 e^{-155}	92%	CP001657.1
6	Jo-A2	*Pco*	3 e^{-133}	98%	CP009678.1
		Ecc	4 e^{-122}	94%	AF046928.1
		Pcc PC1 strain	2 e^{-155}	92%	CP001657.1
7	Jo-Q14	*Pco*	3 e^{-133}	97%	CP009678.1
		Ecc	4 e^{-122}	93%	AF046928.1
		Pcc PC1 strain	2 e^{-155}	91%	CP001657.1
8	Jo-Q16	*Pco*	3 e^{-133}	97%	CP009678.1
		Ecc	4 e^{-122}	93%	AF046928.1
		Pcc PC1 strain	7 e^{-157}	91%	CP001657.1
9	Jo-Q29	*Pco*	1 e^{-127}	98%	CP009678.1
		E cc	2 e^{-116}	94%	AF046928.1
		Pcc PC1 strain	4 e^{-147}	92%	CP001657.1
10	Jo-A5	*Pco*	7 e^{-125}	98%	CP009678.2
		Ecc	9 e^{-115}	94%	AF046928.2
		Pcc PC1 strain	2 e^{-155}	92%	CP001657.1
11	Jo-Q23	*Pco*	3 e^{-133}	97%	CP009678.2
		E cc	4 e^{-122}	93%	AF046928.2
		Pcc PC1 strain	2 e^{-155}	90%	CP001657.1
12	Jo-Q27	*Pco*	3 e^{-133}	98%	CP009678.2
		Ecc	4 e^{-122}	94%	AF046928.2
		Pcc PC1 strain	3 e^{-148}	91%	CP001657.1
13	Jo-Q30	*Pco*	5 e^{-126}	85%	CP009678.2
		E cc	7 e^{-115}	88%	AF046928.2
14	Reference strain NCPPB312	*Pco* BC S7	9 e^{-154}	98%	CP009678.1
		Ecc	1 e^{-131}	94%	AF046928.2
		*Pcc*1 PC1 strain	2 e^{-120}	91%	CP001657.1

Data in this table have been published by Abu-Obeid et al. [19]

Table 6. Maximum nucleotide similarity (BLASTn) between *Pectobacterium carotovorum* subsp. *carotovorum* jo-isolates amplified with *pmr*A set of primers and the most closely related species/subspecies

No.	Isolate	Closely related species/subspecies	E-value	Maximum % similarity	Accession no.
1.	Jo-Q16	Morocco *Pcc* strain P603AH1	0.0	100%	JQ278721.1
2.	Jo-Q27	Morocco *Pcc* strain P603AH1	2 e^{-152}	91%	JQ278721.1
3.	Jo-Q30	Morocco *Pcc* strain P603AH1	8 e^{-148}	96%	JQ278721.1
4.	Jo-Q19	Morocco *Pcc* strain P603AH1	0.0	99%	JQ278721.1
5.	Jo-A11	Morocco *Pcc* strain P603AH1	0.0	100%	JQ278721.1
6.	Jo-A2	Morocco *Pcc* strain P603AH1	0.0	98%	JQ278721.1
7.	Jo-Q14	Morocco *Pcc* strain P603AH1	0.0	94%	JQ278721.1
8.	Jo-Q21	Morocco *Pcc* strain P603AH1	0.0	99%	JQ278721.1
9.	Jo-Q29	Morocco *Pcc* strain P603AH1	0.0	96%	JQ278721.1
10.	Jo-A5	Morocco *Pcc* strain P603AH1	0.0	99%	JQ278721.1
11.	Jo-Q23	Morocco *Pcc* strain P603AH1	0.0	99%	JQ278721.1

rDNA is amplified and sequenced with universal primers to identify species and subspecies [26-27].

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 were 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 Fd1and Rd1 primers that were used in this study are general primers which can detect different bacterial causal agents [20], but could be used as a preliminary step in bacterial identification.

In contrast, the *rec*A gene has been used successfully to compare the relationship among Enteriobactereacae. Sequence analysis of *Pcc* Jo-isolates sequenced for the *rec*A gene indicted that isolates were related to *Pcc*. Our results are in agreement with [24] and [28] who found that PCR amplification of *rec*A gene was a good tool to group different strains of potato soft rot.

By using the species-specific set of primers (ExpccF/ExpccR), designed to test specificity of *Pcc* isolates, only 67 isolates out of 131 isolates from different potato growing regions which represent about 51% of tested isolates, yielded the expected 550 bp product size. Specificity of PCR with this set of primers was more limited because they also amplified the expected 550 bp product from some isolates identified as species other than *Pcc* and did not amplify DNA from other isolates that were identified as *Pcc* on the basis of biochemical tests. Similar results were obtained by [25] where the specific primer set for *Pcc* amplified the expected size of strains identified as *P. wasabiae* and did not amplify other strains biochemically identified as *Pcc*.

Our results also are in agreement with previous finding of [29] where none of the 12 Iranian *Pcc* tested isolates produced the 550 bp products in PCR in contrast to two standard *Pcc* isolates that produced the desired bands and they found that these two isolates could not be identified by PCR using *Pectobacterium* subsp. specific primers. Also [12] found that only genomic DNA of 29 strains of *Pcc* out of 54 bacterial strains which equal to about 54% yielded the expected 550 bp amplified product following PCR with Expcc specific primers. These results could be related to genomic differences between; either Jordanian or Iranian *Pectobacterium* agents and other subspecies of *Pectobacterium* in different regions of the world [29]. Whereas, the Species-specific Expcc was generated from the nucleotide sequence of a *Pcc* specific universal rice primer (URPs) PCR product, although URPs were developed from repetitive sequences in the rice genome that have been used to fingerprint genomes of diverse organisms [12].

Homology search results for all sequenced Jo-isolates on the bases of Expcc set of primers showed high similarity percentage with *Pc* subsp. *odoriferum* (*Pco*), and this is contrary t o of [12] who reported that RFLP-PCR specific primers were able to differentiate *Pcc* strains among other subspecies. *Pectobacterium carotovorum* subsp. *odoriferum*, was reported as a typical

Pc subsp. *atrosepticum* strain pathogenic to only chicory that and produce an odorous volatiles [30]. Consequently it differs from *Pcc* that is known to be a widely distributed pathogen with a broad host range [12]. For all of the above this confirms that all our isolates are *Pcc* rather than *Pco*.

Using specific primers for *pmr*A gene, isolates produced a 666 bp PCR product were sequenced and analyzed using maximum nucleotide similarity (BLASTn). Results confirmed these isolates as *Pcc*. The *Pcc* Jo-isolates were strongly differentiated from other *Pectobacterium* responsible for disease on potato including *Pca*. Similar results have been reported by [23].

5. CONCLUSION

As a conclusion, different from the results of this study, using molecular techniques such as amplification of different regions using different sets of primers is a sensitive and specific method for detecting *Pcc* than other traditional methods. Furthermore, different sets of primers used varied in their specificity in detection soft rot pathogens, where *rec*A and *pmr*A sets of primer were found to be more efficient in detecting *Pcc*. DNA sequencing was found to be the most reliable way in specific detection and confirmation of the causal agent of soft rot. Taken together, the different rotting causal agents detected in this study, it was difficult at this stage to define if these causal agents are a major soft rot causal agent or that are only associated with *Pcc* soft rot. More work is needed for studying soft rot disease in Jordan.

COMPLIANCE WITH ETHICAL STANDARDS

Authors states that:
This research is a part of a big thesis conducted in order to achieve the PhD degree from the Plant Protection Department /Jordan University, and is partly funded from Jordan University and National Center for Agricultural Research and Extension (NCARE). Some data concerning using the specific EXPCC set of primer have been used and published by (Abu- Obeid et al., 2017).

The research is implemented on potato tubers and does not involve human's participants or/and animals.

All authors agree and looking forward to publish this paper in this journal.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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