Jordan Journal of Agricultural Sciences, Volume **14**, No.**2** 2018

Detection And Identification of Potato Soft Rot *Pectobacterium Carotovorum* Subsp. *Carotovorum* (Dye1969) by Pcr Using Different Sets Of Primers

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ABSTRACT

Various bacterial species are known to be agents causing soft rot of potatoes. In this study different detection methods were used to investigate these bacteria. Rotted potato samples were collected from different potato growing regions through different seasons. One hundred and thirty one bacterial isolates were identified as *Pectobacterium carotovorum* subspecies *carotovorum* (*Pcc*) by biochemical and physiological tests. Furthermore, the identities of these isolates were identified by PCR analysis of total DNA using different sets of primers and sequencing of representative PCR products amplified with all sets of primers. Primers used were; 16S rDNA universal primer Fd1/Rd1, species-specific primer ExpccF/ExpccR, *rec*AF/*rec*AR and pathogencity gene *pmr*AF/*pmr*AR. The four sets of primers 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*. 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. This study indicated that potato soft rot disease caused by *Pectobacterium carotovorum* subsp. *carotovorum* is widely spread in Jordan and using molecular techniques such as amplification of different regions by using different sets of primers is sensitive and specific for detecting *Pcc* as potato soft rot causal agent.

Keywords: *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*), soft rot of potato, PCR, sequencing, detection.

INTRODUCTION

Potato (*Solanum tuberosum* L.) ranks the second among the economical agricultural crops grown in Jordan, where the area planted with potato in Jordan during the year 2016, was 50860 dunums with total production of 158580 tons ((Statistical Year Book, 2016).

Soft rot is one of the most important diseases of potatoes causing great economic losses in field, and is reported to be caused by various bacterial species of these: *Bacillus* spp., fluorescent *Pseudomonas* spp., *Enterobacter cloacae* and *Erwinia* spp. (Schroeder *et al.*, 2009). However, *Pectobacterium carotovorum* subsp. *carotovorum (Pcc)* (Dye, 1969) is reported as the most common causal agent of bacterial soft rot of potatoes and other commercially important crops belonging to different major vegetable families including cabbage, cauliflower, lettuce, onion, pepper, carrot and potato (Rajeh and Khlaif, 2000; Perombelon, 2002; Bhat *et al.*, 2010a; Monilola and Abiola, 2011).

Different methods are followed in order to detect, identify and differentiate soft rot causing agents to species and subspecies level, of these methods are the traditional methods; microscopy, isolation, biochemical characterization, serological techniques, pathogenicity

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Received on 25/11/2017 and Accepted for Publication on 11/7/2019.

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and bioassay tests. All of these methods are time consuming, insensitive, inaccurate and not suitable for routine work to test large number of samples (Narayanasamy, 2011; Czajkowski *et al*., 2014). Recently, 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 (Kang *et al*., 2003; Czajkowski *et al*., 2014).

The main causal agent of soft rot disease in Jordan, was identified as *Pcc*, of, its detection based on biochemical and physiological traditional techniques. Soft rot of potatoes is a tuber borne disease where the contaminated mother tubers were reported to be the main source of inoculum. Furthermore, this bacterium was found to survive in the soil with population trends that vary with the fluctuation in soil temperature (Rajeh and Khlaif, 2000).

Accordingly, this research was conducted in order to detect and identify the causal agent of potato soft rot isolates obtained from infected potato tubers by PCR technique using four sets of primers and further confirming the identity by sequencing.

MATERIALS AND METHODS

Samples collections

Potato rotted samples were randomly collected including stem and from different potato growing areas in Jordan. The collected samples were labeled and placed in an ice box took to the laboratory for further work.

Pathogen isolation

The rotted tubers and plant tissues were placed in washing jars under tap water to clean them from soil particles; surface disinfected with sodium hypochlorite 0.5% for 3 minutes, rinsed with sterile distilled water (SDW) then dried onto sterile filter paper. Samples of 10 g of plant tissues were cut into small pieces, placed into sterile bottle with 90 ml of SDW, placed on a shaker at 200 rpm at room temperature. After the suspension become homogenized a series of serial dilutions were prepared up to 10^{-3} dilution, then 0.1 ml of the 10^{-3} dilution were spread by a sterile glass rod onto the surface of three Logan's medium plates (Schaad *et al*., 2001). The inoculated plates were incubated at 27±2 ºC checked periodically. Appearance of small, circular bacterial colonies with pink centers within the first 24 hrs. of inoculation was suspected to be *Pcc*, then single colonies were restreaked onto new nutrient agar plates. The obtained bacterial isolates were kept as suspension in SDW at 4º C for further identifications (Fahy and Parsley, 1980).

Pathogen identification

Twenty four hours old cultures of the obtained bacterial isolates were identified based on biochemical and physiological tests as described by Schaad *et al*. (2001). 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, United Kingdom and against negative control, these tests were including: oxidase and catalase reactions, potato soft rot, oxidative fermentative test, ability to grow at 37 ºC, growth in 5% sodium chloride, ability of reducing substances from sucrose, urease production test and acid production from carbohydrates.

Bacterial genomic DNA extraction from bacterial cultures

Bacterial genomic DNA was extracted from 24 hrs. old pure bacterial cultures of one hundred and thirty one bacterial isolates grown on NA media at 27 ºC, 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 over night at 37 ºC, 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, which was designed for purification of total DNA from gram-negative bacteria.

PCR amplification, purification and sequencing

In order to detect the presence of the desired DNA fragments that confirm the presence of *Pcc*, four sets of primers were used as shown in Table 1. Polymerase chain reaction mix contained ;0.13 µl *Taq* polymerase (5U/µl), 1.1 µl (25 mM) $MgCl_2$, 0.5 µl (10 mM) dNTPs mixture, 1.25 µl of (10 µM) of each primers, 5.0 µl of (5X) Crimson *Taq* buffer, 2.0 µl of DNA template and nuclease free water (NFW) to total volume of 25 µl.

Polymerase PCR amplification reactions were performed in a thermal cycler BIORAD T100™ (Biorad, Hercules, CA) using the following protocol and adjusted as needed: Initial denaturation 94 ˚C for 5 min followed by 35 cycles of (94 ˚C for 1 min) for denaturation, (55 ˚C for 1 min) for annealing, and (72 ˚C for 1 min) for extension and a final extension step at72 ˚C for 7 min.

The amplified product was electrophoresed on a 1.5% agarose gel and visualized following staining with ethidium bromide. Chain Reaction mixture and conditions were adjusted for each set of primers.

Products were purified using the Wizard Purification System (Promega, Madison, Wisconsin), the protocol was performed according to the manufacturer's instructions. After purification DNA fragment was 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 that obtained were analyzed doing homology search of PCR products using Basic local alignment searching tool (BLAST) at NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Blast search was performed for both nucleotide and amino acids using BLASTn and BLASTx respectively, according to the amplified region .

RESULTS

Samples collection

Potato soft rot was widely spread throughout different potato growing areas in Jordan during Summer, Autumn and Winter seasons. Two hundreds and five samples were collected from 20 locations including: Amman (Commercial Stores), AR-Ramtha (Torrah/Hallan way and Torrah/Tanqeeh Station), Jordan Valley (Ashshuna Al Janoubiyya/Wadi Al Abiad, Deir Alla, Deir Alla Station, Ghor Kabed, Karameh, North Ghor/Al Sleakhat and Sharhabeel), Ma'an (Modawwarah and Al Quwayra), Madaba (Al Ariesh, Jrainah and Samek) and Mafraq (Mghayyer serhan, Sama serhan and Thoghret El Jobb).

Isolation, identification and characterization of causal agent

Small circular bacterial colonies with pink to red purple centers were developed on the surface of dried Logan's media plates, inoculated with the suspension of the natural diseased potato samples suspected to be infected with soft rot, 24 hrs. after incubation at 27 ºC. The results of biochemical and physiological tests are listed in Table 3.

One hundred and thirty one bacterial isolates were found to be oxidase negative, catalase positive, rotting induced on inoculated potato slices, fermentation of glucose positive, growth developed on nutrient agar plates incubated at 37±2 ºC, and on 5% sodium chloride. Also, all isolates were not able to reduce substances from sucrose, and urease enzyme was produced and able to oxidize the alcoholic sugar and discharge it in the media to acidic reaction.

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.

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, 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 fragments with the universal primers set (Fd1/Rd1) which are commonly used for the detection of bacteria (Fig. 2). The specific set of primers that were designed to bind within *rec*A gene, directed amplification of fragments of about 730 bp (Fig. 3). About 98 isolates out of 131 tested isolates (74.8 %) were tested positively by using *rec*A primer. While, 67 isolates (51 %) showed a 550 bp bands with the specific Expcc set of primers (Fig. 4). On the other hand, only 33 isolates (25.2%) showed band of about 660 bp (Fig. 5) when tested using the specific set of primers *pmr*A (F0145/E2477) (Table 4). 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.

Sequencing analysis

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, and the nucleotide sequence similarity percentage ranged from 96% up to 100%.

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%. 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 similar pattern ,the highest similarity percentage was with *Pcc* strain (Acc. no. CCP50100.2) with percentage of 100%.

Maximum nucleotide similarity (BLASTn) results of sequences for selected *Pcc* Jo-isolates that amplified with Expcc; species-specific set of primer 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. Maximum similarity percentage ranged from 86% to 98%.

Maximum nucleotide similarity (BLASTn) results for selected *Pcc* Jo-isolates amplified with *pmr*A primers set showed percentage similarity ranged from 91% to 100% with *Pcc* strain P603AH1 (Acc. no. JQ278721.1) from Morocco.

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. Result of survey for all potato growing areas during all seasons at different plant growing stages revealed that potato soft rot disease occurred in all surveyed areas including: Amman, AR Ramtha, Jordan Valley, Ma'an, Madaba and Mafraq.

The isolation of bacteria from diseased samples on Logan's media developed small circular bacterial colonies with pink to red purple centers confirms that the isolated bacterium is suspected to be *Pcc*. 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 (Schaad *et al*., 2001), which confirmed the results that *Pcc* is widely spread and the main causal agent of potato soft rot in Jordan (Rajeh and Khlaif, 2000).

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 (De Boer *et al*., 2012). 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 (Ibtihal Abu-Obeid *et al*,. 2018). 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 (Kang *et al*., 2003; Zhu *et al*., 2010; Rahmanifer *et al*., 2012; Kettani-Halabi *et al*., 2013).

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. Where most of *Pcc* Jo-isolates showed high degree of similarity with *Pcc* different strains from the GenBank.

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 ribosomal regions, in general 16S rDNA is amplified and sequenced with universal primers to identify species and subspecies (Zhu *et al*., 2010; Rohinshree and Negi, 2011).

In contrast, the *rec*A gene has been used successfully to compare the relationship among Enteriobactereacae. Sequence analysis of *Pcc* Jo-isolates sequenced on the bases of *rec*A gene indicted that isolates were related to *Pcc*. Our results are in agreement with Rahmanifer *et al*. (2012) and Lee *et al*. (2014) 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), which was 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 test. Same results were obtained by De Boer *et al*. (2012) where the specific primer set for *Pcc* amplify 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 Azadmanesh *et al*. (2013) 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 Kang *et al*. (2003) 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 (Azadmanesh *et al*., 2013). 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 (Kang *et al*., 2003).

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*. The same results have been reported by Kettani-Halabi *et al*. (2013).

Conclusion

This study indicated that using molecular techniques such as amplification of different regions using different sets of primers and DNA sequencing was found to be the most reliable way in specific detection and confirmation of the causal agent of soft rot than other traditional methods. 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*.

Compliance with Ethical Standards

Authors states that:

This research is a part of Ph.D degree thesis conducted in Plant Protection department /Jordan University, and is partly funded from Jordan University and National Agricultural Research Center (NARC).

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

No.	Primer name	Expected product size	Sequence 5'- 3'	Reference
1.	16S rDNA Fd1 Rd1	1530 bp	 CAGAGTTTGATCCTGGCTCAG AAGGAGGTGATCCAGCC	Lane, 1991
2.	Oligonucleotide ExpccF ExpccR	550 bp	GAACTTCGCACCGCCGACCTTCTA GCCGTAATTGCCTACCTGCTTAAG	Kang *et al*., 2003; Mahmoudi *et al*., 2007; Palacio-Bielsa *et al*., 2009
3.	*pmr*A F0145 E2477	660 bp	 TACCCTGCAGATGAAATTATTGATTGTTGAAGAC TACCAAGCTTTGGTTGTTCCCCTTTGGTCA	Kettani-Halabi *et al*., 2013
4.	*rec*A *rec*AF *rec*AR	730 bp	 GGTAAAGGGTCTATCATGCG CCTTCACCATACATAATTTGGA	Rahmanifer *et al*., 2012

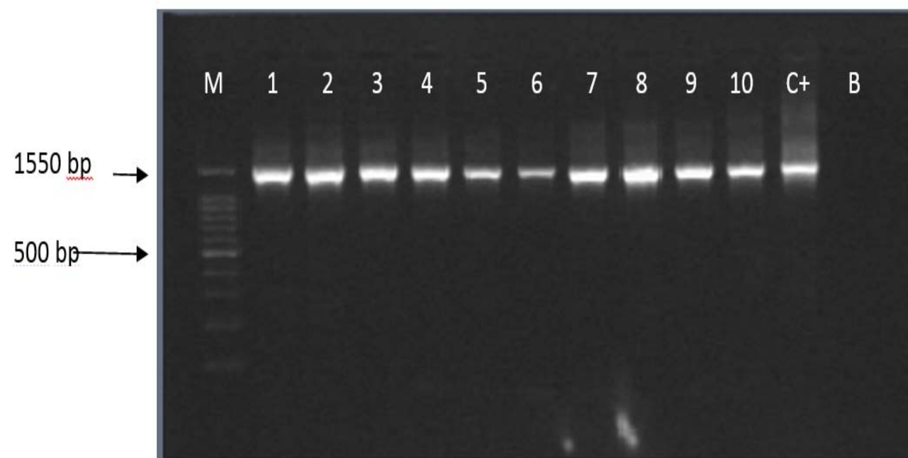

Figure 1. Agarose gel electrophoresis for PCR amplified DNA of *Pectobacterium carotovorum* subsp. *carotovorum* isolates using Fd1 and Rd1 primers with the expected amplified product of 1530 bp. Lane M represents Ladder 100 bp (Gene Direx). Lanes 1-10; isolates Jo-Q16, Q19, A11, A2, Q14, Q21, Q29, A5, Q23, Q27 and Q30, respectively. Lanes C+; Positive control (reference isolate NCPPB312) and B; Buffer (Ibtihal Abu-Obeid *et al*., 2018).

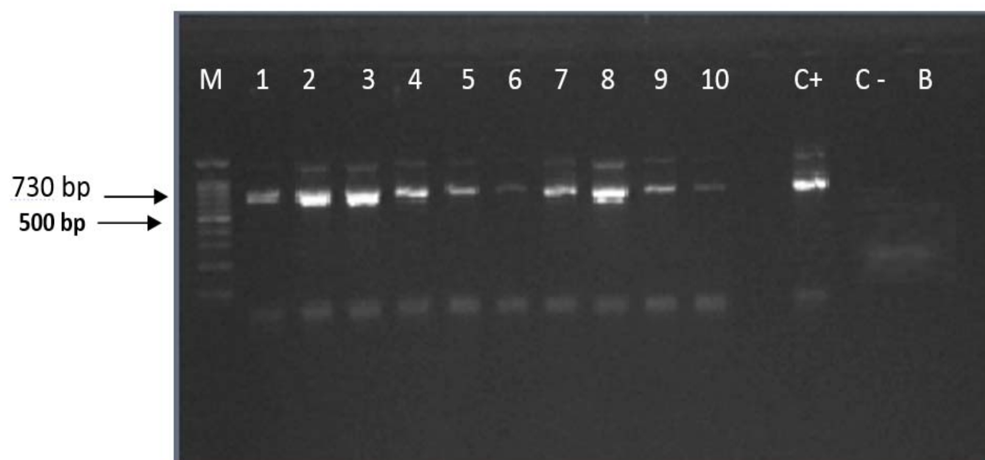

Figure 2. Agarose gel electrophoresis for PCR-amplified DNA of *Pectobacterium carotovorum* subsp. *carotovorum* isolates using *rec*A primers set with the expected amplified product of 730 bp. Lane M represents Ladder 100 bp (GeneDirex). Lanes 1- 10; isolates Jo-Q16, Q19, A11, A2, Q14, Q21, Q29, A5,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

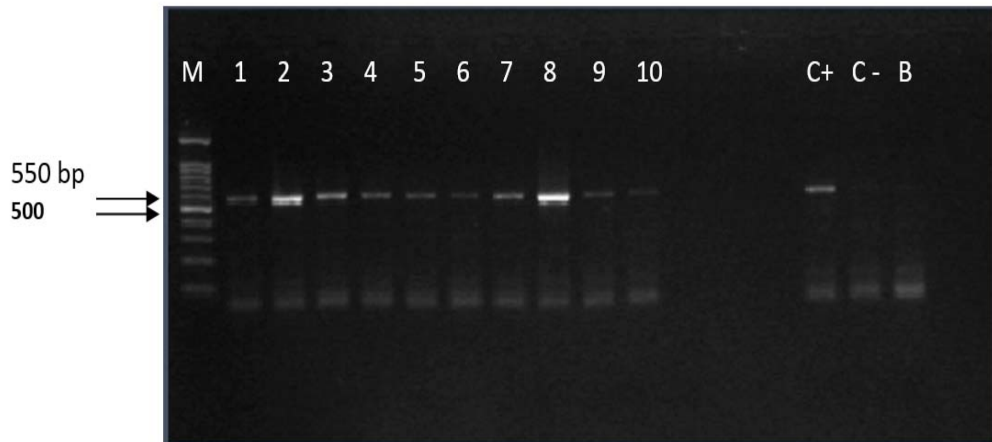

Figure 3. Agarose gel electrophoresis for PCR-amplified DNA of *Pectobacterium carotovorum* subsp. *carotovorum* isolates using EXPCC primers set with the expected amplified product of 550 bp. Lane M represents Ladder 100 bp (GeneDirex). Lanes 1- 10; isolates Jo-Q 16, Q19, A11, A2, Q14, Q21, Q29, A5, Q23 and Q27, respectively. Lanes C+; Positive control (reference isolate NCPPB312), C-; Negative control (*Escherichia coli*) and B; Buffer (Abu-Obeid *et al*., 2017).

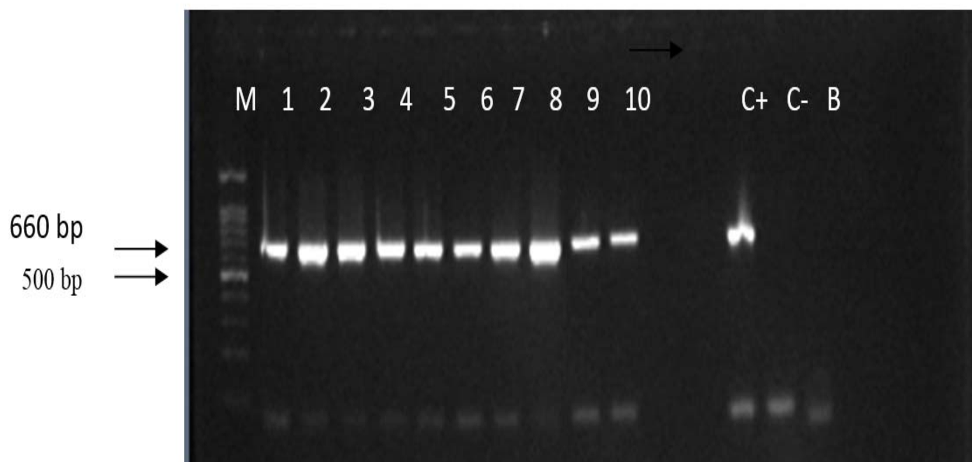

Figure 4. Agarose gel electrophoresis for PCR-amplified DNA of *Pectobacterium carotovorum* subsp. *carotovorum* isolates using *pmr*A primers set with the expected amplified product of 660 bp. Lane M represents Ladder 100 bp (GeneDirex). Lanes 1- 10; isolates Jo-Q16, Q19, A11, A2, Q14, Q21, Q29, A5, Q23 and Q27, respectively. Lanes C+; Positive control (reference isolate NCPPB312), C-; Negative control (*Escherichia coli*) and B; Buffer (Abu-Obeid *et al*., 2018).

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الكشف عن وتعريف بكتيريا العفن الطري على البطاطا *Pectobacterium carotovorum* subspecies *carotovorum* (Dye, 1969) بواسطة تفاعل البلمرة المتسلسل باستخدام العديد من البادئات المتخصصة

ابتهال أبو عبيد[1]،حامد خليف[2]، نداء سالم[3]

ملخص

عرف العديد من الأنواع المختلفة من البكتيريا تسبب مرض العفن الطري على البطاطا. في هذه الدراسة تم استخدام عدة طرق للكشف عن المسبب المرضي للعفن الطري على البطاطا. حيث تم تجميع عينات من نباتات البطاطا المصابة بمرض العفن الطري من مختلف مناطق زراعة البطاطا في الردن وفي مختلف المواسم الزراعية حيث تم عزل وتعريف 131عزلة من البكتيريا *Pectobacterium carotovorum* subspecies *carotovorum* (*Pcc*) باستخدام الفحوص الفسيولوجية و البيوكيميائية .استخدم تفاعل البلمرة المتسلسل بعد عملية استخلاص الحامض النووي الرايبوزي منقوص الاكسجين للعزلات التي تم تعريفها وذلك باستخدام العديد من البادئات المتخصصة في الكشف عن الحمض النووي الرايبوزي منقوص الاكسجين لبكتيريا العفن الطري *Pcc* حيث تم استخدام كل من البادئات التالية (Fd1/Rd1, ExpccF/R, recAF/R and pmrAF/R) : ومن ثم تمت عملية الاستنساخ النووي تلتها عملية تحديد التتابع النووي. تباينت البادئات المختلفة في حساسيتها للكشف عن بكتيريا العفن الطري حيث كانت البادئات recA و pmrA أكثرالبادئات فعالية في الكشف عن بكتيريا *Pcc* . من ناحية اخرى لم تكن البادئة Expcc دقيقة في الكشف عن عزلات البكتيريا *Pcc* حيث كشفت عن 51% فقط من العزلات. دلت نتائج الدراسة الميدانية ان مرض العفن الطري على البطاطا منتشر في مختلف مناطق زراعة البطاطا في الاردن وبينت هذه الدراسة ان استخدام الفحوص الجزيئية مثل التكثير باستخدام تفاعل البلمرة المتسلسل كان دقيقا وحساسا في الكشف عن بكتيريا *Pcc* المسببة للعفن الطري على البطاطا.

الكلمات الدالة: العفن الطري، البطاطا.

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تاريخ استلام البحث 2017/11/25 وتاريخ قبوله 2019/7/11.