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Detection of Pectobacteria Causal Agents of Potato Soft Rot in North Western Provinces of Iran

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Abstract

Various bacteria belonging to *Enterobacteriaceae* are known to be agents causing blackleg and soft rot in potato. In this study, a rapid and specific detection method was used to investigate bacteria in some northwestern provinces of Iran (Zanjan, Kordestan and eastern Azarbaijan). 26 strains were selected for the study that had been identified in previous studies as representative pathogens, they were as follows: 14 *Pectobactrium carotovorum subsp carotovorum*, 7 *Dickeya chrysanthemi* and 5 *Pectobactrium atrosepticum*. Primers Y1/Y2, Expccf/r, ADE1/2 and Eca1f/r, published as specific primers for the pathogens. They were tested to determine if they could be used for specific amplification of DNA in the strains. The expected specific fragment was not amplified in many strains or several non-specific bands, some close to those expected fragments were amplified. Specific primers for amplification of the DNA of pathogens were designed by sequencing the ITS region from representative strains and a PCR test was developed. In the PCR test a 300 bp fragment was amplified from all strains collected from the provinces. In specificity tests, no PCR products were obtained from other bacteria belonging to other genera.

Keywords: 16S-23S rDNA intergenic transcribed spacer; *Pectobactrium carorovorum* subsp *carotovorum; Dickeya chrysanthemi; Pectobactrium atrosepticum*

Introduction

Potato is the second most important crop after cereals in northwestern Iran because of favorable climatic conditions meaning that it can be cultivated throughout the region. However, soft rot caused by bacteria belonging to *Entrobacteriaceae* is among the main factors contributing to yield loss in this area.

Recently, bacteria belonging to the genus *Erwinia*, which causes soft rot in various plants, were grouped into the genus *Pectobacterium* and *Dickeya* [1] and the main virulence determinants of these genous are the pectolytic enzymes secreted through the type II secretion system. There are two species *P. carotovorum* and *D. chrysanthemi* that are particularly damaging to potato crops worldwide. The former is a major pathogen in temperate regions, while the latter is the most damaging in warmer climates. *P. carotovorum* has been divided into five sub species based on biochemical, physiological and pathogenicity characteristics from which two subspecies *carotovorum* and *atrosepticum* are able to cause symptoms on potato both during the vegetative cycle of the crop and in stored tubers [2]. Also a new *Pectobacterium* subspecies, *P. carotovorum* subsp. *brasiliensis* was recently described in Brazil and later found in the United States, Israel, and South Africa [3].

As a first step in this study of the disease in northwestern Iran, pathogens present in potato crops grown for consumption and seed tubers were collected and identified as *P. c. subsp. carotovorum, P. c. subsp. atrosepticum* and unusual strains of *P. carotovorum* [4-6].

The major source for primary inoculum of these bacteria in potato crops is infected/infested seed tubers. Harmful bacteria can survive in soil and can be transferred by irrigation water therefore, it is important to detect the pathogen in seed potatoes and other inoculum sources to eliminate the disease [7].

Traditionally biochemical and physiological tests have been used for identification and differentiation of *Pectobacterium* isolates [8], however traditional techniques of bacterial isolation and characterization are time consuming and relatively insensitive because of the high level of saprophytes in the samples [9]. Serological techniques using both polyclonal and monoclonal antibodies have also been used [10] and current identification methods based on biochemical and phenotypic characterization are neither rapid nor accurate [2]. However, diversity of isolates in serological properties does not allow for reliable and accurate identification and there are high serologically heterogeneity and cross-reactions in this technique [11]. As an alternative, PCR-based methods have been used for specific and rapid detection and identification of pathogens isolates, it is notable that 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 [9,12] (Table 1). Also, since *Pectobacterium carotovorum* subsp. *carotovorum* and subsp. *atroseptica* can affect potato tubers with another pathogenic fungi and bacteria(*Phytophthora infestans, Phytophthora erythroseptica, Pythium ultimum* and *Fusarium sambucinum*), Atallah and Stevenson, developed a methodology to detect and quantify of the five aforementioned diseases from whole potato tubers, using real time quantitative polymerase chain reaction [13].

Recently, *Pectobacterium* taxa are distinguished and classified with Multi-locus Sequence Typing (MLST) [14].

While potato soft rot caused by *Pectobacterium* spp. is considered to be a serious problem in northwestern Iran, an efficient and reliable

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Primer name	Target gene	Product length
Y1 / Y2	pectate lyase-encoding pel gene belonging to the pelY family	420bp
EXPCCR/ EXPCCF	primers generated from a URP-PCR fingerprinting derived polymorphic band	550bp
ADE1/ADE2	pectate lyase-encoding pel gene belonging to the pelADE family	420bp
ECA1f/ECA2r	derived from a DNA probe specific to E. carotovora subsp. atroseptica	690bp

Table 1: Target genes and length of the amplicons of PCR primers which are used in identificational studies of *pectobacterium* ssp.

method to detect and identify pathogens in pure culture or plant material has been lacking. Therefore, this study was conducted to estimate the efficiency of available PCR-based procedures to detect Iranian isolates and to develop a molecular PCR-based method to detect and distinguish pathogens rapidly and accurately.

Materials and Methods

Bacterial strains

Bacterial strains used in this study and their sources are listed in table 1. Twenty-six strains of the pectolytic *Pectobacterium* and *Dickeya* were obtained as described by [5,6,15]. Two standard strains were obtained from ICMP and to assess the specifity of DNA-based methods two isolated bacteria, one *Klebsiella pneumonia* and the other *Escherichia coli* (naturally occurring around potato tubers) were provided from pasture institute of Iran, Tehran.

All bacterial strains were cultured at 27°C on nutrient agar medium (Bayer) for 2 days.

Preparation of bacterial DNA

Genomic DNA was extracted and purified using modified extracting method described by Yap et al. [10]. DNA was stored at -20°C until required.

Detection of bacteria with published specific primers

Detection of bacteria with primers Y1 and Y2, specific for *P. carotovorum* subspecies was tested. A primer set was obtained from sigma (Germany). The primer set used in this study was able to detect all *P. carotovorum* subspecies except *P. carotovorum* subsp *betavasculorum*, which is not pathogenic on potato. Each reaction (50 µL), as described by [11] contained 0.2 U taq polymerase (Cinnagen), 5 µL of PCR buffer 10X, 3 µL $MgCl_2$ (25mmol L^{-1}), 4 µL(1 mmol L^{-1}) each of desoxy nucleotides dATP, dCTP, dGTP, dTTP, 0.2 µmolL^{-1} final concentrations of each primer and 5 µL of DNA extract. The protocol modified from [9] and PCR amplification was carried out in Eppendorf thermal cycler and was programmed as follows: An initial denaturation step at 94°C for 5 min, 1 min at 94°C, 1 min at 65°C, and 1 min and 30 s at 72°C for 34 cycles, and final extension step one cycle at 72°C for 7 min.

Detection of bacteria with primers EXPCCF and EXPCCR, specific for *P. carotovororum* subsp *carotovorom, P. carotovorum* sub sp *carotovorum* was amplified with a set of primers EXPCCF and EXPCCR as described by [12], synthesized by Bioneer (England). Amplification was performed in a final volume of 50 µL PCR mixture containing 5 µL 10X PCR buffer, 0.5 µL each of desoxy nucleotides dATP, dCTP, dGTP, dTTP, 0.5 µmol (2.5 U)Taq polymerase and 1 µL (100 ng) primers. PCR amplification was performed in an Eppendorf thermal cycler (Germany), using the following program [12] one cycle of 4 min at 94°C as initial denaturation step, 30 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C and one cycle as final extension for 7 min at 72°C.

Detection of bacteria with primers ECA1f/ECA2r, specific for *P. atrosepticum*. PCR amplification of *P. atrosepticum* genomic DNA was done using primers ECA1f/ECA2r as designed by De Boer and Ward [8]. PCR amplification was carried out in an Eppendorf thermal cycler. The PCR mixed (20 µL) consist of 0.5 µM Mg^{++}, 100 µM each of desoxy nucleotides dATP, dCTP, dGTP, dTTP, 0.5U Taq polymerase, 2 µL 10X PCR buffer, 0.5 µM each of primers and 1 µL DNA. The PCR was run for 5 min at 95°C in the first cycle, 40 cycles of 30s at 94°C, 45s at 62°C, 45 s at 72°C and for the final step, 8 min at 72°C.

Detection of bacteria was done with primers ADE1/ADE2, specific for *D. chrysantemi*. Genomes of *D. chrysantemi* were amplified using ADE1/ADE2 primers obtained from Bioneer (England). The protocol was as follows: The PCR mixture per reaction (50 µL) contained, 100 ng DNA, 200 µM each of desoxy nucleotides dATP, dCTP, dGTP, dTTP, 2.5U Taq polymerase, 2 µL 10X PCR buffer, 100 pmol each of primers and 1 µL DNA. The PCR was run for 5 min at 94°C in the first cycle, 25 cycles of 1 min at 94°C, 2 min at 72°C and for the final step, 8 min at 72°C [16].

16S-23S rDNA spacer region amplification and sequencing

Genomic DNA of all isolated bacteria were amplified using primers 1491f and L1r from conserved regions in the flanking of 16S -23S rDNA transcribed spacer region as described by [17]. Reaction conditions for a 50 µL PCR were: 2 mM $MgCl_2$, 0.16 µM each of desoxy nucleotides dATP, dCTP, dGTP, dTTP, 0.4 µM each primer, 2.5 U Taq polymerase, 5 µL 10X PCR buffer and 2 µL genomic DNA. PCR amplifications were carried out in an Eppendorf thermal cycler as follows: 94°C for 2 min as an initial step followed by 31 cycles of 94°C for 45 s, 64°C for 45 s and 72°C for 1.5 min and finally 72°C for 10 min.

For all samples, 5 µL of PCR products were analyzed on 1.5% agarose gels in TAE buffer and stained with ethidium bromide (0.5 µg/mL) electrophoresd at 35V/cm for 1 h.

After amplification two representative isolates Pcc and Pa were chosen for sequencing of the ITS region. DNA fragments were obtained by the 'band stab' amplification technique, in which resolved PCR products, based on the 1491f and L1r primer were recovered directly from an agarose gel with a pipet tip after electerophoresis and then reamplified [18]. PCR products were purified using a DNA Kit as described by the manufacturer (Roche, Germany). DNA sequencing was performed in both directions and the sequencing reactions were carried out by the Seqlab Company, Germany. The chromas software (Technelysium Pty Ltd, Qld, Australia) was used for editing and regenerating the sequences. Alignment and comparition were performed with ClustalX and GeneDoc programs available on the Bioanformatics Net database. The sequences were compared with sequences in the GenBank, EMBL, DDBJ and PDB databases, using the basic Alignment Search Tool (BLAST). Sequence data from the small and large subunits 16S-23S rDNA transcribed spacer region of Iranian strains Azs52(Pca), Azl52(Pca), Azl2(Pcc), Azs2(Pcc), have been deposited in Genbank az accession numbers HM130661, HM130662, HM130663 and HM130664 respectively.

Sequence data analysis and design of specific PCR primers for the pathogen

Comparisons of sequences of ITS, polymerase chain reaction and sequencing of the ITS were carried out as described by [17]. The

multiplex sequence alignment consisted of 37 sequences from 16S-23S intergenic transcribed spacer region: 23 from the small ITS of the 16S-23SrDNA and 14 from the large ITS of the 16S-23S rDNA region. The sequence alignment of the small ITS region reaveled the presence of a single copy of the $tRNA^{Glu}$ gene and a single copy of the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes in the large region. Sequences used in this study were retrieved from Genbank or taken from published reports [17]. After comparing large and small subunits of the ITS, one forward primer named S1-F (5'-ACC TGT AAG AAC CTG CCT-3') and another primer as reverse named S1-R (5'-CAG TGT GTC GTT TCA ATT T-3') were designed on the basis of differences between small ITSs (Figure 1).

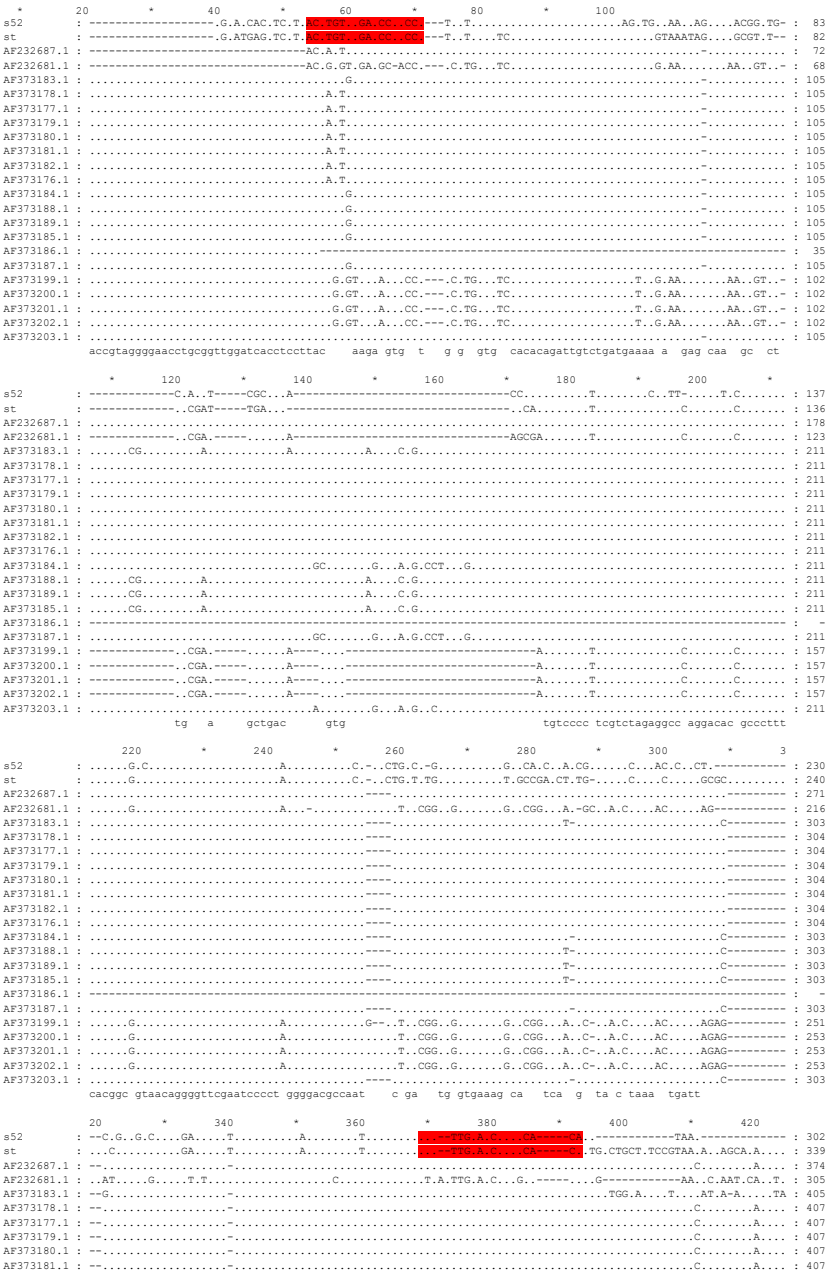

Figure 1: ITS sequencing used to design primers specific for PCR amplification of potato soft rot pathogen rDNA. S52:*Pectobacterium carotovorum* subsp *atrosepticum* (Genbank, HM130661), S2: *P. carotovorum* subsp *carotovorum* (Genbank, HM130664), Pca1: *Pectobacterium carotovorum* subsp *atrosepticum* (Genbank, AF232687), Dch2: *Dickeya chrysanthemi*(Genbank, AF232681), Pca3: *Pectobacterium carotovorum* subsp *atrosepticum* (Genbank, AF373183), Pca4: *Pectobacterium carotovorum* subsp *atrosepticum* (Genbank, AF373178), Pca5: *Pectobacterium carotovorum* subsp *atrosepticum* (Genbank, AF373177), Pca6: *Pectobacterium carotovorum* subsp *atrosepticum* (Genbank, AF373179), Pca7: Pectobacterium carotovorum subsp *atrosepticum* (Genbank, AF373180), Pca8: *Pectobacterium carotovorum* subsp *atrosepticum* (Genbank, AF373181), Pca9: *Pectobacterium carotovorum* subsp *atrosepticum* (Genbank, AF373182), Pca10: *Pectobacterium carotovorum* subsp *atrosepticum* (Genbank, AF373176), Pcb11: *Pectobacterium carotovorum* subsp *betavascolorum* (Genbank, AF373184), Pcc12: *Pectobacterium carotovorum* subsp *carotovorum* (Genbank, AF373188), Pcc13: *Pectobacterium carotovorum* subsp *carotovorum* (Genbank, AF373189), Pcc14: *Pectobacterium carotovorum* subsp *carotovorum* (Genbank, AF373185), Pcc15: *Pectobacterium carotovorum* subsp *carotovorum* (Genbank, AF373186), Pcc16: *Pectobacterium carotovorum* subsp *carotovorum* (Genbank, AF373187), Dch17: Dickeya chrysanthemi (Genbank, AF373199), Dch18:Dickeya chrysanthemi (Genbank, AF373200), Dch19: *Dickeya chrysanthemi* (Genbank, AF373201), Dch20: *Dickeya chrysanthemi* (Genbank, AF373202), Dch21: *Dickeya chrysanthemi* (Genbank, AF373203).Sequences of the primers are shaded and arrows indicate the direction of the priming of the primers.

PCR cycling conditions

The reaction mixture in a 50 µL volume containing 2 mM $MgCl_2$, 0.16 µM each of desoxy nucleotides dATP, dCTP, dGTP, dTTP, 1 µL, 1 µM of each primer, 0.5 µL(2.5 U) Taq polymerase, 5 µL 10X PCR buffer and 4 µL genomic DNA. The reaction was carried out with the following program: initial denaturation in 94°C for 5 min followed by 25 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 45 s and at the end 72°C for 5 min. The amplification was carried out in an Eppendorf thermal cycler (Germany).

PCR specificity tests

To assess the specificity of the designed primers, PCR was carried out with DNA from 27 *Enterobacteriaceae* ssp strains as epiphytic bacteria collected from soil around tubers, for better comparisons two isolates belonging *Klebsiella* and *Escherichia* genera were examined.

Results

CR tests with specific primers

P. carotovorum –specific PCR.A 434-bp DNA fragment was not amplified from any strains of *P. atrosepticum* and Dickeya chrysanthemi. Produce was observed in five *P. carotovorum* subsp *carotovorum* strains, of which two of them were standard isolates. In test *P. carotovororum* subsp *carotovorom* –specific PCR, none of the 12 strains of Iranian Pcarotovorum subsp carotovorum produced amplicons in PCR in contrast to the 550-bp amplicon obtained with two standard Pcc isolates. Using *P. atrosepticum* specific primers (ECA1f and ECA2r) in PCR, many fragments were amplified from DNA of all Iranian *P. atrosepticum* strains whereas a 690-bp amplicon must be obtained using these primers. None of *D. chrysanthemi* isolates could amplify 420-bp amplification products using ADE1 and ADE2 specific primers (Table 2).

Using the 1491f/L1r consensus primers, individual isolates within *D. chrysanthemi, P. carotovorum* sub sp *carotovorum* and *P. atrosepticum* generated several different banding patterns with ITS-PCR (Figure 2). These various banding patterns showed higher levels of diversity than within other species or subspecies. Phenotypic and genotypic technique confirmed this level of diversity [9,19].

PCR specificity and sensitivity tests

All 26 strains of these pathogenic bacteria were tested with the designed primer and all Iranian strains produced PCR product (300-bp fragment), no PCR amplicon were obtained from test strains or other bacterial genus (Figure 3).

Discussion

The rRNA genes are essential for the survival of all organisms. Ribosomal RNA genes evolve slowly and are highly conserved but it has

Strain	Isolated from	Geographic origin	Species/sub species	Primers Y1and Y2[c]	Primers Expccf and Expccr[d]	Primers ECA1f and ECA2r[e]	PrimersADE1 And ADE2[f]	Primers S1f and S2r(designed)[g]
CA3	Cabbage	Zanjan	*P.c.carotovorum*	+	-			+
E2	Potato	Ijrood	*P.c.carotovorum*	-	-			
+								
B5	Potato	Zanjan	*P.c.carotovorum*	-	-			+
D8	Potato	Kheir abad	*P.c.carotovorum*	-	-			+
D34	Potato	Kheir abad	*P.c.carotovorum*	-	-			+
Ecc6	Potato	Azarshahr	*P.c.carotovorum*	-	-			+
Ecc10	Potato	Azarshahr	*P.c.carotovorum*	-	-			+
Ecc16	Potato	Ajabshir	*P.c.carotovorum*	-	-			+
Ech21	Potato	Azarshahr	*P.c.carotovorum*	+	-			+
Ech25	Potato	Azarshahr	*P.c.carotovorum*	-	-			+
Ech33	Potato	Sarab	*P.c.carotovorum*	-	-			+
Ecc42	Potato	Torkamanchay	*P.c.carotovorum*	-	-			+
Eca52	Potato	Sarab	*P.atrosepticum*	-	-	-		+
Eco57	Carrot	Sarab	*P.atrosepticum*	+	-			+
Ecc62	Carrot	Bostan abad	*P.c.carotovorum*	-	-			+
Ecc74	Carrot	Bostan abad	*P.c.carotovorum*	-	-			+
62	Potato	Dehgolan	*P.atrosepticum*	-	-	-		+
34	Potato	Dehgolan	*P.atrosepticum*	-	-	-		+
49	Potato	Dehgolan	*P.atrosepticum*	-	-	-		+
40	Potato	Ghorve	*P.c.carotovorum*	-	-			+
unnamed	Potato	Ghorve	*P.c.carotovorum*	-	-			+
44	Potato	Ghorve	*P.c.carotovorum*	-	-			+
Ecc11523	-	ICMPa	*P.c.carotovorum*	+	+			-
Ecc55990	-	ICMP	*P.c.carotovorum*	+	+			-
Hd	Potato	Tajarg	*D.chrysanthemi*	-	-		-	-
Hd3	Potato	Tajarg	*D.chrysanthemi*	-	-		-	-
Hd1	Potato	Tajarg	*D.chrysanthemi*	-	-		-	-
Hv	Potato	Tajarg	*D.chrysanthemi*	-	-		-	-
E.c	-	IPIb	*E.coli*					-
K	-	IPI	*Klebsiella pneumonia*					-

Table 2: List of bacterial strains tested in this study and their origins and detection of Iranian pectobacterium with primers Y1 and Y2, EXPCCF and EXPCCR, ECA1f and ECA2r, ADE1and ADE2 and designed primers S1f and S2r.

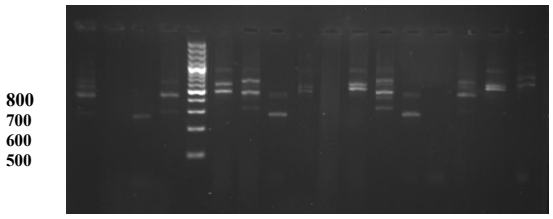

Figure 2: ITS-PCR amplification patterns of *Pectobacterium* and *Dickeya* on agarose gel. Lane1, *P. carotovorum* subsp *carotovorum* strain 74;2, *P. carotovorum* subsp *carotovorum* strain 42;3, *P. carotovorum* subsp *carotovorum* strain 40;4, *P. carotovorum* subsp *carotovorum* strain Ecc10;5, L, molecular weight markers (bp) (100 bp, Cinnagen, Tehran, Iran);6, *P. carotovorum* subsp *carotovorum* strain E2;7, *P. atrosepticum* strain Eca52;8, *D. chrysanthemi* strain Hd5;9, *P. carotovorum* subsp *carotovorum* strain Ech33;10, *P. carotovorum* subsp *carotovorum* strain Ecc62;11, *P. carotovorum* subsp *carotovorum* (Type strain;Ecc11523);12, *P. carotovorum* subsp *carotovorum* strain CA3;13, *D. chrysanthemi* strain Hd3;14, *P. atrosepticum* strain49;15, *P. carotovorum* subsp *carotovorum* Ecc16;16, *P. carotovorum* subsp *carotovorum.*

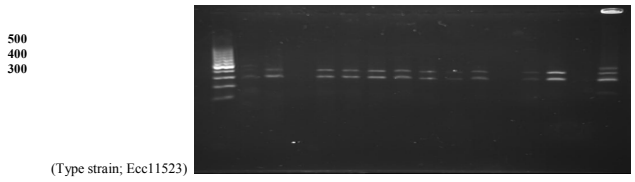

Figure 3: Amplification of DNA from *Pectobacterium carotovorum* isolates using assay designed in this study. The figure shows products of the PCR run on an agarose gel: Lanes: L, molecular weight markers (bp) (100 bp, Cinnagen, Tehran, Iran); 1, *P. carotovorum* ssp. *carotovorum* strain CA3;2, *P. carotovorum* subsp *carotovorum* strain E2;3, *P. carotovorum* subsp *carotovorum* (Type strain);4, *P. carotovorum* subsp *carotovorum* strain B5;5, *P. carotovorum* subsp *carotovorum* strain Ecc16;6, *P. carotovorum* subsp *carotovorum* strainEcc42;7, *P. carotovorum* subsp *carotovorum* strainEcc10;8, *P. carotovorum* subsp *carotovorum* strainEcc62;9, *P. carotovorum* subsp *carotovorum* strain Ecc6; 10,P. atrosepticum strainEca52;11, *P. carotovorum* subsp *carotovorum* (standard isolate Ecc55990)12, *P. atrosepticum* strainEco57;13, *P. carotovorum* subsp carotovorum strainEcc42;14, *P. carotovorum* subsp *carotovorum* strain Ech25;15, *P. carotovorum* subsp *carotovorum* strain Ecc74.

been considered that the nucleotide sequences of the rRNA intergenic spacer regions are not as essential as the rRNA genes themselves and consequently not as highly conserved [20]. These properties made them a suitable site for developing specific PCR primers that can differentiate similar bacteria.

Using high sequence variation of ITSs between pectobacterium subspecies and Iranian pectobacteria causal agents of potato soft rot, two primers were designed from the variation region of small ITS for specific PCR detection of Iranian pectobacteria causal agents of potato soft rot. Thus a rapid and highly specific method has been developed for the detection of pectobacteria causal agents of potato soft rot in northwestern provinces of Iran.

Genomic DNA from all Iranian isolates from potato previously identified as *Pectobactrium atrosepticum, Pectobacterium carotovorum subsp carotovorum* and *Dickeya chrysanthemi* on the basis of physiological and serological techniques were specifically amplified using these designed primers, however these isolates could not be identified by PCR using *Pectobacterium* subspecies specific primers.

We can not exactly explain the failure of amplification of the Iranian pectobacterium agents other than to consider variation in the pectate genes [9,16]. However sizes and sequences of the large and small ITS regions of Iranian isolates were also similar to those of other P.carotovorum subspecies and D.chrysanthemi and the size of the produced bands were between 200-800 bp [17].

No PCR products were observed in standard isolates and type strains using the PCR assay with the Iranian pectobacteium primer set. It is notable that the physiological and biochemical propertie [21-23] but not for the subspecies of Iranian isolates. This failure might be related to genomic differences between Iranian pectobacterium agents and other subspecies of pectobacterium.

However, the sensitivity of the designed primer was not tested and the actual number of pectobacterium required for causing of symptoms of disease on potato tubers determines the minimum required sensitivity of the detection method [24]. The specifity of primers was assessed by amplifying DNA of all Iranian isolates and no amplicons were obtained from other bacteria that existed near the tubers. It must be noted that the specifity of the designed primers tested with 27 saprophytic bacteria, isolated around infected tubers and none of them produced the expected band. These results confirm that a delicate method has been developed using S1f/S2r primers.

In summary our results strongly suggest that Iranian pectobacterium agents of potato soft rot strains are different from other pectobacterium and that a PCR-based assay based on the 16S-23S transcribed spacer region was developed to detect them.

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