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Detection and Differentiation of *Potato Virus Y* Strains from Potato Using Immunocapture Multiplex RT-PCR

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Abstract Potato virus Y (PVY) is a serious problem for the seed potato industry in the United States. The maximum allowable infection level of PVY in certified seed potatoes is 2 % and is substantially lower in early generations of seed production. Moreover, recent emergence of genetically recombinant and serologically different strains of Potato virus Y has led to the development of diagnostic procedures to determine strain identity and to detect mixed strain infections more easily, sensitively and accurately. In the studies reported here a protocol for the detection of single or mixed PVY infections in potato incorporates the advantages of enzyme-linked immunoassay (ELISA) and multiplex reverse transcriptase PCR (RT-PCR). The viral particles from plant sap were enriched by ELISA and then lysed by heating to release the viral RNA for the reverse transcriptase. The cDNA product was used as template for the detection of infection by multiplex PCR eliminating the need for RNA extraction and handling. The sensitivity tests conducted indicate that the immunocapture reverse transcriptase PCR was more sensitive in detecting PVY in infected plant sap than multiplex RT-PCR or ELISA alone while retaining the ability to differentiate strains of PVY that can infect potato in the United States. The immunocapture multiplex RT-PCR described will be particularly useful for seed certification and diagnostic laboratories as a confirmatory test in conjunction with ELISA.

Resumen El virus Y de la papa (PVY) es un problema serio para la industria de producción de semilla de papa en los Estados Unidos. El nivel máximo de infección permitido para el PVY en semilla de papa certificada es 2 %, y es substancialmente más bajo en generaciones más tempranas en la producción de semilla. Más aun, el surgimiento reciente de recombinantes genéticas y de variantes serológicamente diferentes del virus Y de la papa ha conducido al desarrollo de procedimientos de diagnóstico para determinar la identidad de variantes y para detectar infecciones de variantes mixtas más fácilmente, tanto en sensibilidad como en precisión. En los estudios que aquí se reportan, el protocolo para la detección de infecciones simples o mezcladas de PVY en papa incorpora las ventajas de la serología con enzimas conjugadas (ELISA) y la PCR de transcriptasa reversa múltiple (RT-PCR). Las partículas virales de la savia de la planta fueron enriquecidas con ELISA y después lisadas por calentamiento para liberar el ARN viral para la transcriptasa reversa. El producto de cADN se usó como molde para la detección de la infección por PCR múltiple eliminando la necesidad de extracción y manejo del ARN. Las pruebas de sensibilidad que se hicieron indican que la inmunocaptura de transcriptasa reversa de PCR fue más sensible en la detección de PVY en la savia de planta infectada que la PCR-RT múltiple o ELISA solas, mientras retenía la habilidad de diferenciar variantes de PVY que pueden infectar a la papa en los Estados Unidos. La inmunocaptura múltiple de PCR-RT descrita será particularmente útil para la certificación de semilla y laboratorios de diagnóstico como una prueba de confirmación junto con la ELISA.

Keywords Reverse transcriptase · Polymerase chain reaction · Pathogen detection · Diagnostics

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Introduction

Potato virus Y (PVY) belongs to the genus *Potyvirus* which is considered the largest group of plant viruses. PVY is a

serious pathogen infecting several important crop species belonging to the Solanaceae family including potato, tomato, pepper and tobacco.

Classically, PVY has been grouped into three main clusters of parental strains, PVY^{O}, PVY^{N} and PVY^{C} based on the host hypersensitive response, genome sequence and/or serological properties (Singh et al. 2008), although variants of PVY strains have emerged due to recombination and mutation.

The three mostly studied recombinants of PVY are PVY^{NTN} (Glais et al. 2002; Nie and Singh 2002; Nie and Singh 2003a, b; Crosslin et al. 2002), PVY^{N}-Wilga and $PVY^{N:O}$ (Chrzanoska 1991; Crosslin et al. 2002; Nie et al. 2004) and are known to induce a veinal necrosis in tobacco. PVY^{NTN} strain causes the potato tuber necrotic ringspot disease (PTNRD). A number of $PVY^{N:O}$ isolates are also capable of causing an atypical PTNRD symptom (Piche et al. 2004).

A variant of PVY^{NTN} strain PVY-12 was reported to have four recombinant junctions with two recombinant points at both HC-Pro/P3 and 6 K2/NIa (Hu et al. 2009). This strain induced the PTNRD in potato but the genomic sequence was found to be quite different from other PVY^{NTN} strains (Chikh Ali et al. 2007). Two variants of PVY^{NTN}, namely NE11 (Lorenzen et al. 2008) and L26 (Hu et al. 2009) have been reported. More recently, isolate PVY^{O}-O5 was discovered during a survey study in North America (Karasev et al. 2010). PVY^{O}-O5 reacted positively with PVY^{N}-specific MAb but was found to be a variant within PVY^{O}, most likely due to a single amino acid substitution within the capsid protein (Karasev et al. 2010).

The high degree of genetic variability in PVY has resulted in the emergence of different strains with wide host ranges causing mild to severe mosaic symptoms in potato. The detection of recombinant PVY strains in certified seed potatoes is made difficult by the fact that many potato cultivars do not express disease symptoms in the field or under controlled conditions (Lorenzen et al. 2008; McDonald and Singh 1996). Since the control of PVY in potato is largely dependent on the unambiguous detection of the virus during visual field inspections conducted as a part of the seed certification process, there is a need to differentiate PVY strains among the many potato cultivars that vary widely in their ability to express disease symptoms. A rapid, accurate and easy diagnostic procedure that can be widely used to test potato plants would aid effective management of the disease by improving detection during seed certification procedures.

Enzyme-linked immunosorbent assay (ELISA) is routinely used to detect PVY (Singh et al. 1993). ELISA is rapid but unable to distinguish among the necrotic strains (McDonald and Singh 1996; Nie and Singh 2002) and the possibility of escape in the detection of a recombinant PVY isolate has been reported when using strain specific monoclonal antibodies (Singh et al. 2003). In addition, false positive reactions can occur due to genome recombination (Singh 1992) or the misidentification of PVY strain type can occur due to amino acid substitutions within the capsid protein (Karasev et al. 2010). As a result, a wide range of molecular methods have been developed for detection and strain differentiation including uniplex (Boonham et al. 2002; Schubert et al. 2007), three primer PCR (Moravec et al. 2003), PCR-RFLP (Glais et al. 1996), tagging of viral transcripts (Rosner and Maslenin 2003), AmpliDet RNA assay (Szemes et al. 2002) and SNaPshot assay (Rolland et al. 2008). A multiplex PCR is also available which is able to characterize the major PVY strains in mixed infections with a single assay (Chikh Ali et al. 2010; Lorenzen et al. 2006).

Although there have been a number of reports of detecting mixed viral infections by immunocapture-RT-PCR (IC-RT-PCR) assay in plants and insects (Ptacek et al. 2002; Vigano and Stevens 2007), none of the previous studies have used the number of PVY strains currently known to be in existence today. The sensitivity of using ELISA coupled with RT-PCR has been demonstrated to be greater than just total nucleic acid extraction due to the immunocapture enrichment of samples prior to RT-PCR (Kogovsek et al. 2008). The purpose of the studies reported here was to develop a simple, rapid and sensitive diagnostic method to characterize and differentiate strains of PVY in single or mixed infections in potato plants at an early stage without having to purify RNA. A comparative study was conducted to detect individual and mixed infections of PVY strains in the potato cultivar, Red Lasoda, using three different detection methods of ELISA, RT-PCR and IC-RT-PCR. To improve overall utility, IC-RT-PCR was developed using two different previously described multiplex PCR assays (Ali et al. 2010; Lorenzen et al. 2006).

Materials and Methods

PVY Strains and Plant Inoculations The PVY isolates representing the different strains used in this study were originally provided to us as from other laboratories or were collected from commercial potato fields, characterized and archived in our laboratory as previously described (Lorenzen et al. 2008; Lorenzen et al. 2006; Piche et al. 2004). For this study PVY^{O}, $PVY^{N:O}$, NA-PVY^{NTN}, EU-PVY^{NTN} and PVY^{N} strains were revived by inoculating them onto tobacco var. Samsun NN (Piche et al. 2004) (Table 1). Successful inoculation was verified by RT-PCR and ELISA and subsequently leaves were lyophilized using a bench-top Freeze dryer (Vertis) for further inoculations and laboratory studies.

To determine and compare the detection sensitivity of all three assays, ELISA, IC-RT-PCR and RT-PCR the virus-free plants of the cv Red Lasoda plants were inoculated with either

Table 1 Potato host variety, source, location and symptoms in tobacco var. Samsun NN and potato tuber (var. Red Lasoda) of Potato virus Y (PVY) field isolates

Type	Isolate code	Source	Variety	Location	Tobacco symptoms	Tuber symptoms
PVY^{O}	PVY^{O}-139	Rudra Singh	Tobacco	Canada	Mosaic	None
$PVY^{N:O}$	Y01OR-1	Our Lab[a]	Alturas	ND	Veinal Necrosis	Necrotic
PVY^{N}	Mont N	Jim Lorenzen	NA	ID	Veinal Necrosis	None
$NAPVY^{NTN}$	Tu 660	Rudra Singh	Tobacco	Canada	Veinal Necrosis	PTNRD
$EUPVY^{NTN}$	Y03MN-2	Our Lab[a]	Alturas	MN	Veinal Necrosis	PTNRD

NA Not Available, [a] isolate collected by or requested by one of the authors (N. C. Gudmestad) from commercial field in the year 2001–2003, characterized and archived in our laboratory collection

single PVY strains or mixed infections comprising of various strain combinations such as PVY^{N}-PVY^{O}; $PVY^{N:O}$-PVY^{O}; EU-PVY^{NTN}-$PVY^{N:O}$; EU-PVY^{NTN}-PVY^{O}; PVY^{N}-$PVY^{N:O}$; EU-PVY^{NTN}-PVY^{N}; PVY^{N}-NA-PVY^{NTN}; NA-PVY^{NTN}-$PVY^{N:O}$; EU-PVY^{NTN}-NA-PVY^{NTN}; and finally a mixed infection involving all the five strains together. Each PVY strain or mixed strain combination was inoculated onto six potato plants 2 weeks post-emergence and the experiment was performed twice. The inoculated plants were placed in the greenhouse in a completely random design and monitored for symptom development on a daily basis. At 3 weeks post inoculation, leaves from each inoculated plant were collected in individual bags and processed for laboratory assay to confirm successful virus infection.

Potato leaflets were crushed in liquid nitrogen using sterilized mortars and pestles and freeze-dried before long term storage at −80 °C freezer. For immediate testing with ELISA, 0.1 g of freeze dried samples were macerated in 100 μl general extraction buffer (0.13 % Sodium Sulfite, 2 % polyvinylpyrrolidone, 0.02 % sodium azide and 2 % Tween-20 in 1X PBST, pH adjusted to 7.4).

ELISA and IC-RT-PCR A triple antibody sandwich (TAS) ELISA kit was used to perform the assay according to manufacturer's protocol (Agdia, Elkhart, IN). This kit consists of a polyclonal general PVY antibody capturing reagent and a group of PVY specific monoclonal antibodies as detecting agent known to detect all known strains of PVY in foliage of inoculated plants. The capturing antibody (stock concentration of 0.1 mg/ml), alkaline phosphatase enzyme conjugate and detecting antibody (4C3) were diluted with carbonate coating buffer and ECM buffer at a working concentration of 1:200. ELISA tests on leaf samples of inoculated plants were performed at 2, 3 and 4 weeks post-inoculation. A plant was considered to be infected if the leaf extract gave an absorbance value at 405 nm (Bio-Tek Instruments) greater than two times the corresponding blank consisting of only general extraction buffer after an incubation of 1 h at ambient temperature. The plate was washed at least four times with PBS-T after reading the absorbance.

IC-RT-PCR was performed directly in the ELISA plate. In each well to be tested, 20 μl PCR grade, nuclease-free water was added and the plate was incubated in a water bath at 70 °C for 15 min. After the incubation, 15 μl of RT-PCR mixture consisting of M-MLV RT 1X buffer, 2 mM dNTP, 20 U RNase Inhibitor (Promega), 0.25 μg Random Primers (Promega) and 200 U of M-MLV Reverse Transcriptase (Promega) was added to each well. The plates were placed in a humid chamber and incubated for 1 h at 42 °C. Following the incubation the plates were heated briefly at 75 °C for 5 min and then placed on ice till further use.

Multiplex RT-PCR Total RNA was extracted from approximately 100 mg of freeze dried leaves using the SV Total RNA extraction kit (Promega). The reverse transcription and the multiplex PCR were performed as previously described (Ali et al. 2010; Lorenzen et al. 2006). Briefly, for the first multiplex PCR assay using primers previously described, 4 μl of total RNA extract was diluted with 6 μl of PCR grade nuclease free water (Teknova Inc., CA) and incubated at 65 °C for 8 min and then chilled on ice for 3 min. To this denatured RNA extract, 15 μl of reverse transcriptase mixture consisting of M-MLV RT 1X buffer, 2 mM dNTPs, 20 U RNase Inhibitor (Promega), 0.25 μg Random Primers (Promega) and 200 U of M-MLV Reverse Transcriptase (Promega) was added to a final volume of 25 μl and incubated at 42 °C for 1 h. This incubation was followed by a final incubation at 95 °C for 3 min to end the reaction. The cDNA (2 μl) product was added to a 23 μl PCR reaction mixture, consisting of 1X Go-taq DNA polymerase buffer, 0.2 mM dNTPs, 0.12 μM of each of the eight primers (Lorenzen et al. 2006) and 1U of Go Taq DNA polymerase (Promega). A touch-down PCR program was applied as previously reported (Lorenzen et al. 2006).

The reverse transcription step described above was performed to generate cDNA for another multiplex assay using the multiple primers described previously by Ali et al. (2010). The cDNA solution (2 μl) was added to a reaction mix consisting of 1X Go-taq DNA polymerase buffer, 0.2 mM dNTP, twelve primers and 1U of Go Taq DNA

polymerase (Promega) as to have a 25 μl of total volume. All primers were used at a final concentration of 0.3 μM except for S5585m and o6400, which were used at a concentration of 0.6 μM. The amplification was performed using a PCR program previously described (Ali et al. 2010). The PCR products were resolved in a combination of 0.8 % agarose (VWR) and 1 % Synergel (Diversified Biotech, MA) and then visualized with a fluorescent imager (Kodak). GelStar nucleic acid gel stain (Lonza, ME) was incorporated in the gel for the fluorescence.

Detection Sensitivity of ELISA, Immunocapture RT-PCR and Conventional RT-PCR Infected leaves from potato plants inoculated with PVY^{O}, a mixture of PVY^{O} with EU-PVY^{NTN} and mixture of all the five strains as described previously, were collected in sterile bags at 3 weeks post-inoculation. The initial detection sensitivity studied was performed using macerated leaf tissue. In this study 0.04 g of infected leaf tissue was homogenized in 1 ml of extraction buffer. Further dilution of 0.02 g, 0.01 g, 0.05 g and 0.025 g of leaf tissue was achieved by carefully weighing and homogenizing leaf tissue in 1 ml of general extraction buffer. The concentration of ground tissue ranged from 0.04 % to 0.0025 % (w/v).

Detection sensitivity studies were also performed by macerating infected tissue (0.5 g) in 500 μl of general extraction buffer. The leaf sap (diluted 1:2 in extraction buffer) was transferred to a sterile micro-centrifuge tube. The sap was diluted serially ten-fold with general extraction buffer. Dilutions with buffer up to 1:20,000 were used for further sensitivity studies.

RNA was extracted (SV Total RNA extraction, Promega) from the leaf sap and leaf tissue dilutions. ELISA, IC-RT-PCR and multiplex RT-PCR as described by Lorenzen et al. (2006) were performed on the dilutions as previously described.

Potential Application of IC-RT-PCR In addition to the controlled experiments using manually inoculated potato plants leaves in the greenhouse, IC-RT-PCR was also performed on ELISA plates received from other laboratories to evaluate the utility of this method using "real-world" samples. All samples were evaluated using multiplex IC-RT-PCR with PCR primers previously described (Ali et al. 2010; Lorenzen et al. 2006). The cooperating laboratories performed ELISA (Agdia, Inc.) using either potato sprout or leaf tissue to detect the presence of PVY in certified seed or commercial potato fields.

The laboratories were instructed to wash the plates at least four times after obtaining absorbance values and then to seal each plate thereafter (Acetate Plate Sealer, Thermo Electron Corporation, MA). Approximately 290 sprout and leaf samples were examined by IC-RT-PCR following submission of ELISA plates from diagnostic laboratories.

Results

Plant Inoculation Potato plants were monitored for visual symptoms starting 1 week after inoculation. Symptom development was generally apparent late in the second to third week post-inoculation. Inoculations with single strains of PVY in both potato and tobacco plants resulted in 100 % infection, with every plant inoculated displaying typical symptoms of PVY. With mixed infections of PVY strains, the success rate of infection varied from 66.6 % to 100 %. Infected plant leaves were confirmed for the presence of PVY strains by both ELISA and multiplex RT-PCR as previously described.

IC-RT-PCR and Conventional RT-PCR IC-RT-PCR was performed on five different strains, PVY^{O}, $PVY^{N:O}$, PVY^{N}, EU-PVY^{NTN}, NA-PVY^{NTN} as well as on all mixtures using primers and profiles as previously described (Ali et al. 2008, 2010; Lorenzen et al. 2006). The bands obtained using the primers of Lorenzen et al. (2006) were as expected with 689 bp and 267 bp for PVY^{O}; 398 bp and 328 bp for PVY^{N}; 689 bp and 181 bp for $PVY^{N:O}$; 452 bp and 181 bp for EU-PVY^{NTN} and 328 bp for NA-PVY^{NTN} (Fig. 1a). In comparison, the bands observed using primers of Ali et al. (2008, 2010) were 853 bp and 532 bp for PVY^{O}; 853 bp, 633 (faint) and 441 bp for $PVY^{N:O}$; 633 bp (faint) and 398 bp for PVY^{N}; 1,307 bp, 633 bp and 441 bp for EU-PVY^{NTN} and 1,307 bp for NA-PVY^{NTN} (Fig. 1b). An additional band of 633 bp (faint) for both $PVY^{N:O}$ and PVY^{N} was apparent while using the PVY strains in this study. This band was not described for $PVY^{N:O}$ and PVY^{N} amplicons in the original description of these multiplex primers (Ali et al. 2010).

In a mixture of all the five PVY strains, NA-PVY^{NTN} remained undetected in both the IC-RT-PCR and conventional RT-PCR. This was primarily due to the primer design to yield an amplicon for NA-PVY^{NTN} of 328 bp which is masked by the presence of two bands, 328 bp and 398 bp for the PVY^{N} strain in the multiplex (Lorenzen et al. 2006). Similarly, in the multiplex performed with Ali et al. (2008, 2010), the amplicon for NA-PVY^{NTN} is a single band of 1,307 bp, which gets masked in a mixed infection of all five strains or in a mixed infection with EU-PVY^{NTN}.

Detection Sensitivity of ELISA, IC-RT-PCR and Conventional RT-PCR Detection sensitivity of ELISA, and conventional RT-PCR was compared to that of IC-RT-PCR using primers described by Lorenzen et al. (2006). The sensitivity of IC-RT-PCR was comparable to ELISA and RT-PCR in the detection of individual or mixed PVY infections in potato when ground leaf tissue was used. All detection assays were capable of detecting PVY infections in plant tissue sample (w/v) dilutions of 0.0025 % (results not shown).

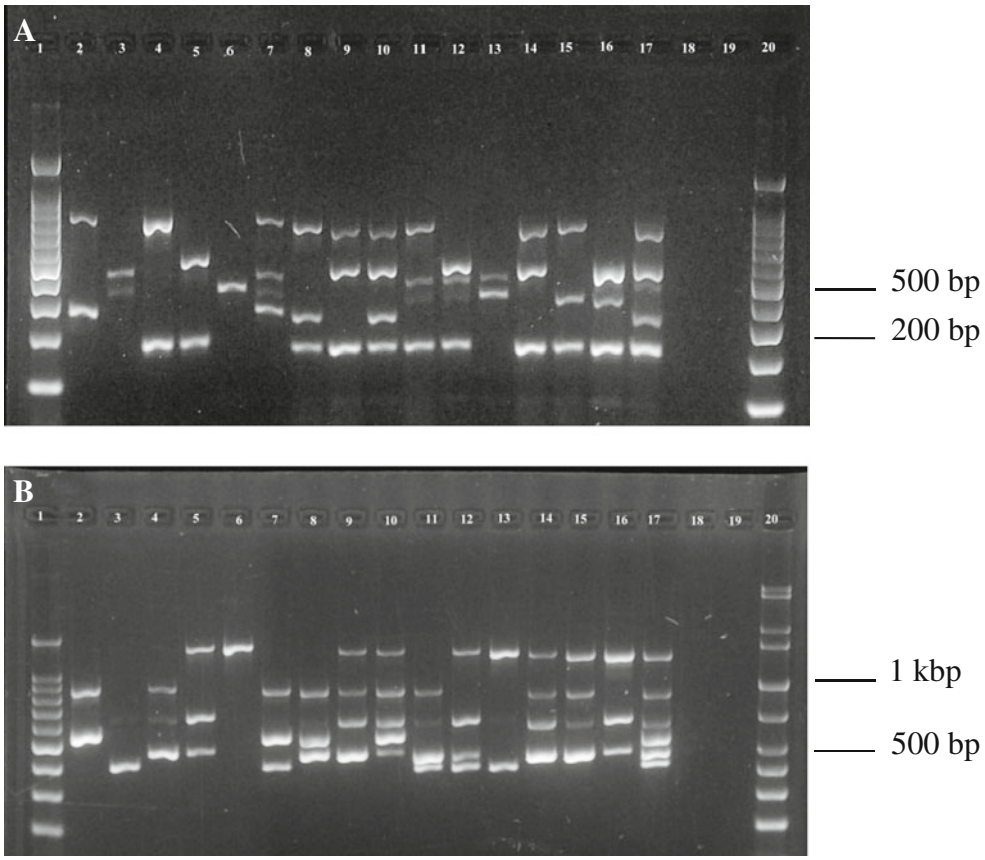

Fig. 1 IC-RT-PCR with Lorenzen et al. (**a**) and Ali et al. (**b**) post ELISA on the same plate. Lane 1, 100 bp DNA plus ladder and; Lane 2, PVY^{O}; Lane 3, PVY^{N}; Lane 4, $PVY^{N:O}$; Lane 5, EU-PVY^{NTN}; Lane 6, NA-PVY^{NTN} Lane 7, mixed infection of PVY^{N} and PVY^{O}; Lane 8, mixed infection of $PVY^{N:O}$ and PVY^{O}; Lane 9, mixed infection of EU-PVY^{NTN} and $PVY^{N:O}$; Lane 10, mixed infection of EU-PVY^{NTN} and PVY^{O}; Lane 11 mixed infection of PVY^{N} and $PVY^{N:O}$; Lane 12, mixed infection of EU-PVY^{NTN} and PVY^{N}; Lane 13, mixed infection of PVY^{N} and NA-PVY^{NTN}; Lane 14, mixed infection of EU-PVY^{NTN} and $PVY^{N:O}$; Lane 15, mixed infection of NA-PVY^{NTN} and $PVY^{N:O}$; Lane 16, mixed infection of EU-PVY^{NTN} and NA-PVY^{NTN}; Lane 17, mixed infection involving all the five strains; Lane 18, healthy plant and Lane 19, water blank; Lane 20, 100 bp DNA plus ladder in (**a**) and (**b**) and 1Kb DNA plus ladder (Qiagen) in (**c**) and (**d**). The PCR products were electrophoresed in 0.8 % Agarose gel+1 % Synergel

Since the end point for sensitivity was not obtained with all three methods using ground leaf dilutions, further studies were conducted using ten-fold serial dilutions of infected leaf sap. ELISA was capable of detecting PVY strains in the sap dilutions of 1:200, similar to that observed for RT-PCR (Table 2) using RNA extracted from the same leaf sap. However, the expected bands of all the PVY strains were visible in dilutions of 1:2,000 with IC-RT-PCR (Table 2) performed in ELISA plates despite an absorbance value (at 405 nm) of 0.05 which was below the positive threshold value of 0.2 and too low for a positive detection of PVY. This indicates that the level of sensitivity of IC-RT-PCR is at least 10 times higher than in ELISA and RT-PCR.

Potential Application of IC-RT-PCR The IC-RT-PCR using Lorenzen et al. (2006) multiple primers was used successfully to characterize different strains of PVY with absorbance values as low as 0.05 (Fig. 2). IC-RT-PCR characterized, approximately 58 % $PVY^{N:O}$, 39 % PVY^{O} and 2 % NA-PVY^{NTN} from ELISA plates that were used to test tuber sprout tissues. When IC-RT-PCR was performed on the ELISA plates that used leaf tissue from certified seed potato fields, approximately 96 % were found to be $PVY^{N:O}$ and 4 % PVY^{O}. There was no evidence for the presence of newer recombinant strains since both assays were in agreement with PVY strain classification based on the expected bands (Ali et al. 2010; Lorenzen et al. 2006).

Discussion

One of the most important strategies in managing losses due to PVY in potato industry is to certify potato seed with low or no virus levels. Foliar symptoms caused by some of the PVY strains, including the tuber necrosis strains, are very mild or cause no foliar symptoms in some potato cultivars making visual detection of PVY

Table 2 Comparison of data for ELISA, IC-RT-PCR and RT-PCR using serial ten-fold dilution of PVY infected leave sap

Sample	Dilution (v/v)[d]	ELISA	IC-RT-PCR	RNA-RT-PCR
O[a]	1:2	+	+	+
O	1:20	+	+	+
O	1:200	+	+	+
O	1:2000	−	+	−
O	1:20000	−	−	−
O+NTN[b]	1:2	+	+	+
O+NTN	1:20	+	+	+
O+NTN	1:200	+	+	+
O+NTN	1:2000	−	+	−
O+NTN	1:20000	−	−	−
O+N+NO+NAN[c]+NTN[d]	1:2	+	+	+
O+N+NO+NAN+NTN	1:20	+	+	+
O+N+NO+NAN+NTN	1:200	+	+	+
O+N+NO+NAN+NTN	1:2000	−	+	−
O+N+NO+NAN+NTN	1:20000	−	−	−

The PVY strains used for the sensitivity testing were PVY^{O} [a], mixture of PVY^{O} and EU-PVY^{NTN} [b], mixture of PVY^{O}, PVY^{N}, $PVY^{N:O}$, NA-PVY^{NTN} (NAN)[c]and EU-PVY^{NTN} (NTN)[d]. The infected leaf was homogenized in 1:2 (w/v) the extraction buffer. The leaf sap thus obtained was diluted ten-fold (v/v) [d]. + and − indicates positive and negative reactions respectively

during inspections of seed potato fields more difficult (Lorenzen et al. 2008; Nie et al. 2004). While ELISA is used routinely to detect PVY it does not effectively discriminate among the different PVY strains (Karasev, et al. 2010; McDonald and Singh 1996; Nie and Singh 2002) and, therefore, is unable to differentiate tuber necrosis-causing strains from more innocuous strains. The development of a diagnostic assay that can simultaneously detect PVY in seed potatoes while also characterizing the strain present would be a useful tool for certified seed potato and commercial potato growers alike.

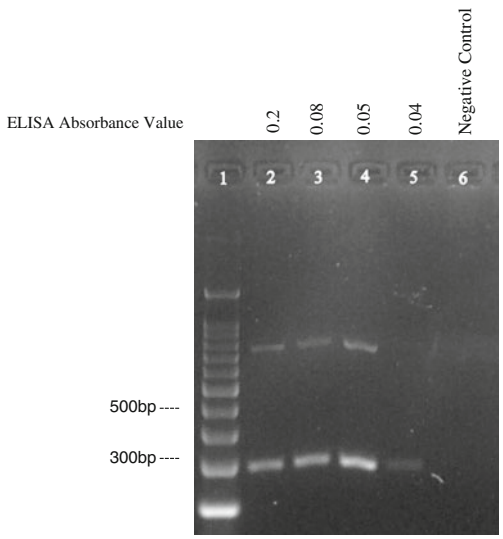

Fig. 2 IC-RT-PCR on samples with very low ELISA absorbance values. The absorbance values are mentioned in the parenthesis. Lane 1, 100 bp DNA plus ladder; Lane 2, $PVY^{N:O}$ (0.2); Lane 3, $PVY^{N:O}$ (0.08); Lane 4, $PVY^{N:O}$(0.05); Lane 5, $PVY^{N:O}$ (0.04) and Lane 6, healthy plant. The PCR products were electrophoresed in 0.8 % Agarose gel+1 % Synergel

In recent years there has been a considerable research and development in detection and differentiation of PVY strains by various molecular methods (Ali et al. 2010; Kogovsek et al. 2008; Lorenzen et al. 2006; Ptacek et al. 2002; Rolland et al. 2008; Schubert et al. 2007; Xu et al. 2005). However, few of these studies have included the number of PVY strains used in the studies reported here and most of the methods developed are directed at improving detection and differentiation for virological research studies. The preparation and storage of total RNA from plant cells requires extreme care and caution, particularly in a non-research laboratory setting such as in a diagnostic laboratory. The presence of nucleases in the plant cell, RNase in the laboratory setting, and DNA carried over from the plant cells during the isolation procedure, can hinder RT-PCR in downstream procedures. Due to the expense and challenges involved in using RT-PCR with total RNA isolation, many diagnostic laboratories in universities and seed certification facilities are not equipped to perform these molecular detection methods (K. Kinser, personal communication). The use of the IC-RT-PCR method described here circumvents these issues effectively.

It has been well documented that immunocapture followed by the detection of viruses using RT-PCR is a versatile, sensitive and robust diagnostic technique (Ptacek, et al. 2002; Vigano and Stevens 2007). The application of this hybrid method of virus detection in plants is particularly useful in species or tissues that inhibit the PCR reaction and hence, molecular detection. The biggest advantage of using this method is to avoid the extraction of high quality RNA required for molecular methods including the most recent microarray-based detection systems. IC-RT-PCR assay is expected to be more specific because of an antibody capture followed by the specificity rendered by the multiplex RT-PCR.

However, the most important advantage of using this assay described here is being able to detect single or mixed PVY virus infections in the same plates that have been used for the ELISA test, thus providing complementary molecular results to the serological results. Moreover, with so many strains involved, it is more convenient and economical to use multiplex RT-PCR instead of several individual RT-PCR reactions that have been previously described (Boonham, et al. 2002; Glais et al. 1996; Kogovsek et al. 2008; Moravec et al. 2003; Nie and Singh 2003a, b; Ptacek, et al. 2002; Schubert et al. 2007). With these objectives in mind, a sensitive and rapid method of detection and differentiation of various single and mixed PVY strains was developed using the primers to perform the multiplex RT-PCR (Lorenzen et al. 2006). The results indicated that the IC-RT-PCR detected all the strains successfully and more sensitively than either ELISA or RT-PCR. Although we observed that NA-PVY^{NTN} (328 bp) was masked by the presence of PVY^{N} (328 bp and 398 bp) in the case of multiplex with Lorenzen primers (2006) and by the presence of EU-PVY^{NTN} (1,307 bp, 633 bp and 441 bp) when multiplex PCR was performed using Ali et al. primers (2008, 2010), this could be resolved by designing a different primer for NA-PVY^{NTN}, yielding an amplicon of different size. However, in reality, very few mixed PVY infections among potato samples were found during our studies and in general PVY^{N} incidence is very low (Gudmestad and Mallik, unpublished) so this apparent liability is unlikely to be an issue when used in diagnostic laboratories.

We also observed that IC-RT-PCRs using both sets of multiplex primers were able to detect and characterize PVY strains, even with ELISA absorbance values as low as 0.05, well below the positive ELISA threshold commonly used by diagnostic laboratories. The attribute of the IC-RT-PCR was found useful as it confirmed a "true positive" after an ELISA was performed. This was an unexpected result and found to be particularly useful when ELISA tests are performed on tuber sprout tissues which normally yield a high background absorbance with healthy tissue (W. Schrage, personal communication). In addition, IC-RT-PCR was also found to be more sensitive than RT-PCR using total RNA in detecting all PVY strains with an approximate 10-fold increase in sensitivity. This should also be useful when using IC-RT-PCR to detect PVY in potato cultivars that do not express the disease and may suppress virus titer (Draper et al. 2002). The combination of the post-ELISA treatment with another multiplex PCR utilizing different set of multiple primers for the differentiation of PVY strains (Ali et al. 2008; Ali et al. 2010) will also be useful as an additional method to rule out the possibility of the presence of newer mixed and recombinant strains. Although there were additional bands observed while detecting $PVY^{N:O}$ and PVY^{N} when using previously described primers (Ali et al. 2008; Ali et al. 2010), this may be due to different isolates of PVY present in North America than those used in the original study.

Both multiplex PCR assays were utilized in IC-RT-PCR format to detect PVY strains in post-ELISA plates submitted by other diagnostic laboratories. The procedure identified $PVY^{N:O}$ as the most prevalent strain followed by PVY^{O} and in some cases NA-PVY^{NTN} but more importantly, strain identification was identical using both assays. The results show that the IC-RT-PCR protocol developed and optimized in this study is a rapid yet sensitive method to characterize and differentiate isolates of PVY in single or mixed infections in potato and should be amenable for use in any diagnostic laboratory.

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