Potato J (2016) 43 (2): 125-137

SYBR GREEN BASED DUPLEX RT-QPCR DETECTION OF A BEGOMOVIRUS, *TOMATO LEAF CURL NEW DELHI VIRUS-(POTATO)* ALONG WITH *POTATO VIRUS X* AND *POTATO LEAFROLL VIRUS* IN POTATO

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ABSTRACT: SYBR green based RT-qPCR assay coupled with melting curve analysis was developed for the detection of *Tomato leaf curl New Delhi virus-potato* (ToLCNDV-[potato]), *Potato virus X* (PVX), and *Potato leafroll virus* (PLRV). The assays were standardized using four sets of primers for each target and selecting best primer set, optimizing primer concentration and cycle conditions. The amplification of products were verified by specific melting peaks for each targets *viz*., ToLCNDV-[potato], PLRV, PVX and *ef-1 α* gene of potato. Then, duplex RT-qPCR assays were developed to detect these viruses along with elongation factor 1-*α* (*ef-1 α*) gene as plant internal control and to detect ToLCNDV-[potato] along with PVX and PLRV. In duplex RT-qPCR assay, a melting peak specific to ToLCNDV-[potato] was observed along with melting peak of other virus/ *ef-1 α* gene. The assays could detect up to 20 copies/µl using serially diluted plasmids harbouring the targets and up to 0.025 fg of total RNA from infected plant tissues. Sensitivity of duplex RT-qPCR assay was comparable to singleplex RT-qPCR assays. The developed assays could consistently detect these viruses in field samples and was more sensitive than conventional RT-PCR.

KEYWORDS: Melting curve analysis, mixed infection, ToLCNDV-[potato], PVX, PLRV

INTRODUCTION

Infection of potato by a begomovirus (Family, *Geminiviridae*); *Tomato leaf curl New Delhi virus-potato* (ToLCNDV-[potato]) leads to apical leaf curl disease and severe seed degeneration particularly in susceptible varieties. Symptoms appear as curling/ crinkling of apical leaves with distinct mosaic symptoms due to primary infection (Usharani *et al.,* 2004) and in case of secondary infection, the entire plant show severe leaf curling and stunting symptoms (Sohrab *et al.,* 2013). Initially, sporadic incidence of the disease was reported in 1996 at Hisar in Haryana (Lakra, 2003), later severe infection was observed in western UP and other parts of northern India (Chandel *et al.,* 2010; Saha *et al.,* 2014). About 40–75% infection was recorded in cultivars grown in the Indo-Gangetic Plains of India (Venkatasalam *et al.,* 2005). This virus is also reported from other parts of the country (Jeevalatha *et al.,* 2012a; Jeevalatha *et al.,* 2013). Currently, it is one of the most important viral diseases of potato in India. Severe infection (40-100 %) results in heavy yield losses in susceptible varieties particularly in Indo-Gangetic plains and Hisar area of India (Lakra, 2002; Venkatasalam *et al.,* 2005). Significant decrease in size and number of tubers (Lakra, 2003) with a marketable yield loss as high as 50% (Lakra, 2002) is reported. Since the virus spreads through seed tubers (Venkatasalam *et al*. 2011), it is critical

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to maintain quality of seed tubers through effective diagnostic tools. Diagnostic protocols like nucleic acid spot hybridization (NASH), polymerase chain reaction (Jeevalatha *et al.,* 2013; Venkatasalam *et al.,* 2011; Sridhar *et al.,* 2016) and rolling circle amplification-polymerase chain reaction (RCA-PCR) assays (Jeevalatha *et al.,* 2014) have been reported for the detection of ToLCNDV-[potato].

Multiple virus infections are common in potato. During surveys and testing of field samples we could observe infection of potato by ToLCNDV-[potato] along with other potato viruses including *Potato virus X* (PVX) and *Potato leafroll virus* (PLRV). PVX is the type member of the *Potexvirus* genus of the family *Alphaflexiviridae* of plant viruses and it is a rod-shaped filamentous virus with a worldwide distribution in all potato-producing countries. PLRV is another most prevalent viral disease of potato in India and it is the type species of the genus *Polerovirus*, in the family *Luteoviridae*, a group of phloem limited plant viruses. ToLCNDV-[potato], a DNA virus, whereas PVX and PLRV are RNA viruses and there is no report of simultaneous detection of these viruses in a single tube using real-time RT-PCR.

Real-time PCR assays have been developed to detect and quantify several plant RNA viruses (Bester *et al.,* 2012; Mortimer-Jones *et al.,* 2009; Osman *et al.,* 2013) and also DNA viruses (Abrahamian and Abou-Jawdah, 2013; Johnson *et al.,* 2014). It is more attractive due to its rapidity and increased sensitivity compared to serological assays and PCR. SYBR green based assays are more advantageous in large scale testing since it does not require costly probes as compared to TaqMan probe based assays. SYBR green based qPCR assays are non-specific as this dye binds to any double stranded DNA and this drawback is overcome by performing a melting curve analysis to identify and confirm specific PCR amplicon. SYBR green based qPCR assay coupled with melting curve analysis has been successfully used for the detection and quantification of several plant viruses (Bester *et al.,* 2012; Jeevalatha *et al.,* 2015; Johnson *et al.,* 2014). However, there are no reports on qPCR assay for specific detection and quantification of ToLCNDV-[potato] in potato. With an aim to detect this virus along other RNA viruses of potato, initially singleplex RT-qPCR assay was developed and validated to detect isolates collected from different geographical locations. Then we developed duplex RT-qPCR assay to detect ToLCNDV-[potato] along with elongation factor 1-α (ef1-α) gene of potato which served as internal control. Internal controls were used to avoid false negative results which confirm successful DNA/RNA extractions and PCR assay. Simultaneous detection of more than one virus in single tube will save time, labour and also cost of testing. Hence, in this study we report SYBR green based RT-qPCR assay coupled with melting curve analysis for the detection of ToLCNDV-[potato] and also novel duplex RT-qPCR protocols for simultaneous detection of ToLCNDV-[potato], a DNA virus along with two other RNA viruses, PVX and PLRV.

MATERIALS AND METHODS

Source of virus isolates and primers

Pure cultures of ToLCNDV-[potato], PVX and PLRV maintained on potato plants and virus free tissue culture raised micro plants were used as positive and negative controls, respectively for optimization of RT-PCR and RT-qPCR assays. Isolates of ToLCNDV-[potato] (82), PVX (53) and PLRV (30) collected during 2010-2013 were tested for initial validation of singleplex RT-qPCR assays. Samples collected from different potato growing states of India i.e. Uttar Pradesh, Punjab, Haryana, Bihar, Meghalaya, Rajasthan, Madhya Pradesh,

Gujarat, West Bengal, Odisha, Maharashtra and Tamil Nadu were used for validation of the duplex RT-qPCR assays. The primers specific to ToLCNDV-[potato], PVX and PLRV reported by Jeevalatha *et al.* (2012b) and Jeevalatha *et al.* (2013) were used for RT-PCR assays (**Table 1**). The RT-qPCR primers were designed from the conserved regions of coat protein (CP) of the viruses of this study using a pile up of all sequences available in GenBank as well as sequences of the virus strains used locally either using software Primer 3 Input version 0.4.0 or designed manually and the properties were checked by software, Oligonucleotide properties calculator (http://www.basic.northwestern.edu/ biotools/ oligocalc.html).

RT-PCR and duplex RT-PCR detection of ToLCNDV-[potato], PVX and PLRV

Total RNA was extracted using Spectrum™ Plant Total RNA kit (Sigma-Aldrich, Missouri, USA) following the manufacturer's instructions, digested with DNase I and the integrity of the RNA was checked by running total RNA on a gel. First strand cDNA was synthesized using Revert aid™ first strand cDNA synthesis kit (Fermentas, USA) following manufacturer's instructions. RT-PCR assays were carried out as per the standardized protocol using CP gene specific primers (Jeevalatha *et al.*, 2012b; Jeevalatha *et al.*, 2013).

Duplex RT-PCR reactions were carried out in 20 µl reaction volume containing 2.5 µl of cDNA, 2.0 µl of 10X PCR buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM $MgCl_2$), 1.0 µl of 2 mM dNTPs, 0.5 µl each of 10µM forward and reverse primers of ToLCNDV-[potato] (CP primer was used to detect ToLCNDV-[potato] along with PVX. While, replicase primer specific to ToLCNDV-[potato] was used to detect along with PLRV; **Table 1**), 0.5 µl each of 10µM forward and reverse primers of PVX/PLRV, 0.5 µl of 1.5 U *Taq* DNA polymerase (GeNei, Bangalore, India) and 12.0 µl of sterile double distilled water. The temperature profile of the PCR cycle was: pre-incubation at 94°C for 5 min leading to 35 cycles of melting at 94°C for 1 min, annealing at 62°C for 1 min and synthesis at 72°C for 1 min followed by an extension of 72°C for 10 min. The assays were carried out in a GeneAmp PCR system 9700 (Applied Biosystems). The amplified products were analyzed by electrophoresis in a 1% agarose gel in 1X TAE (0.04 M Tris–acetate, 1 mM EDTA, pH 8) buffer at 100 volt for 1 hr.

SYBR green-based RT-qPCR assay

The SYBR Green-based RT-qPCR assays were carried out in a step one plus real-time PCR machine (Applied Biosystems). Four pairs of primers for each target ToLCNDV-[potato], PVX and PLRV were designed from

Table 1. Primers used for RT-PCR assay.

Primer Name	Polarity	Sequence	Product size	gene target
LCV-CP-FP1	Sense	5′-AAAGTCATGTGTGTTAGTGATGTTACC-3′	491 bp	Coat protein
LCV-CP-RP1	Antisense	5′-TAGAAATAGATCCGGATTTTCAAAGTA-3′		
LCrep1F	Sense	5′-TCCGCATATCCATGTTCTCA-3′	306bp	Replicase gene
LCrep2R	Antisense	5′-TCCTTTGGGGCTAATTCCTT-3′		
PVX-CP-FP1	Sense	5′-TCAACTACCTCAACTACCACGAAA-3′	566 bp	Coat protein
PVX-CP-RP1	Antisense	5′-GTAATCTTCACAAAGGCAGCAGTT-3′		
PLRV-CP-FP1	Sense	5′-CTAACAGAGTTCAGCCAGTGGTTA-3′	492 bp	Coat protein
PLRV-CP-RP1	Antisense	5′-CGGTATCTGAAGATTTTCCATTTC-3′		

the conserved region of CP genes using Primer Express 3.0 software and used in optimization to select one pair of primers for each target. Primer pairs were selected such that their amplicons show different melting peaks in melting curve analysis (**Table 2**) and have same annealing temperatures. Experiments were conducted to optimize the singleplex RT-qPCR assay with different concentrations of primers (100-500 nM final concentrations) and cycle conditions with a range of annealing temperatures (60 to 64°C) and extension times (30 to 60 s). Each optimized 20µl reaction mixture contained 10µl of power SYBR green PCR master mix, 0.5µl each of 10µM forward and reverse primers, 2 µl of cDNA templates and 7µl of nuclease free water. Optimized cycling conditions were as follows: 95°C for 10 min followed by 40 cycles consisting of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. After 40 amplification cycles, melting curve analysis was carried out to verify the amplicons by melting temperatures. The temperature range for the melting curve analysis was from 60°C to 95°C, raising by 0.3°C each step.

Duplex RT-qPCR assays with ef1-α gene

Duplex RT-qPCR assays were standardized to detect ToLCNDV-[potato]/PVX/PLRV along with *ef1-α* gene of potato using selected primer pairs of each target virus along with *ef1-α* gene specific primer, EF-1α-F/EF-1α-R (Nicot *et al*., 2005) or EF-1α-F2/EF-1α-R2 (**Table 2**). Different primer concentrations (100nM to 500nM final concentrations) and quantity of 2x master mix (10-20 µl) were tried in order to determine the optimum reaction mixture. Annealing/extension temperatures and times were also optimized to get the successful amplification of both the targets. Optimized reaction mixture contained 15µl of power SYBR green PCR master mix, 0.5µl each of 10µM virus specific forward and reverse primers, 0.3 µl each of 10µM *ef1-α* gene forward and reverse primers, 2 µl of cDNA/ templates and 11.4 µl of nuclease free water. Cycling conditions were as follows: 95°C for 10 min followed by 40 cycles consisting of 95°C for 30 s, 60°C for 30 s and 72°C for 40 s. The temperature range for the melting curve analysis was from 60°C to 95°C, raising by 0.3°C each step.

Optimization of duplex RT-qPCR assays

Duplex RT-qPCR assay was standardized to detect ToLCNDV-[potato] along with PVX/ PLRV. The cDNAs prepared from single infections were mixed for developing the assay. The selected primer pairs of targets ToLCNDV-[potato] and PVX/PLRV were used in different

Table 2. Primers used for RT-qPCR assays.

Primer	Polarity	Sequence	Product size (mean±S.D.)	Product Tm	RT-qPCR efficiency	R^2
LCCP-1F	Sense	5′-GGATGCGAAGGTCCTTGTAA-3	165 bp	79.62±0.45	94.075 %	0.99
LCCP-2R	Antisense	5′-CCATATCTTCCCCAGCACAT-3′				
XQFP2	Sense	5′-GCTAACTGGCAAGCACAAGGTT-3	208 bp	83.42±0.67	86.259 %	0.98
XQRP1	Antisense	5′-CATCTAGGCTGGCAAAGTCGTT-3′				
LRCP1F	Sense	5′- GAGGCTCAAGCGAGACATTC-3′	299 bp	81.85±0.30	88.62 %	0.95
LRCP5R	Antisense	5′-CATCCGCGCTTGATAAGTTT-3′				
EF1-α-F	Sense	5′-ATTGGAAACGGATATGCTCCA-3′	101 bp	78.87±0.15		
EF1-α-R	Antisense	5′-TCCTTACCTGAACGCCTGTCA-3′				
EF1-α-F2	Sense	5′-TGCTGTCAAGTTTGCTGAGATCTT-3′	68 bp	76.10±0.80		
EF1-α-R2	Antisense	5′-GCTCCTTCTCAAGTTCCTTACCT-3′				

concentrations and optimum concentration of primer and cycle conditions were selected. Each 30µl reaction mixture contained 15µl of power SYBR green PCR master mix, 0.5µl each of 10µM ToLCNDV-[potato] specific forward and reverse primers, 0.2 µl each of 10µM PVX specific forward and reverse primers/0.4 µl each of 10µM PLRV specific forward and reverse primers, 2 µl of cDNA/ templates (mixture of cDNAs) and remaining nuclease free water. Cycling conditions were as follows: 95°C for 10 min followed by 40 cycles consisting of 95°C for 30 s, 60°C for 30 s and 72°C for 40 s. The temperature range for the melting curve analysis was from 60°C to 95°C, raising by 0.3°C each step.

Assay specificity, sensitivity and reproducibility

Specificity of the assay was established by carrying out melting curve analysis and agarose gel electrophoresis of the qPCR product. The CP genes of ToLCNDV-[potato], PVX and PLRV were amplified, cloned in pJET1.2 vector and sequenced. The recombinant plasmids were quantified in a Nanodrop 2000 spectrophotometer (Thermoscientific, Leon-Rot, Germany) in triplicates and copy number was calculated in accordance to Jeevalatha et al. (2015). The 10-fold serial dilutions of plasmids harbouring the CP genes of ToLCNDV-[potato], PVX and PLRV containing 2×10^9 to 2×10^1 copies of plasmids were used in qPCR assay. Standard curve was prepared by plotting the C_q values against the amount of plasmid copy number. Reproducibility and repeatability of the developed assays (singleplex and duplex) was determined using three different dilution series. The intra-assay variations were calculated by analyzing each dilution series in triplicates within a single run. For inter-assay variations, each dilution series was analyzed in triplicates in two separate runs on different days. The intra-assay and inter-assay variations were calculated, by determining the mean, standard deviation (SD) and co-efficient of variation (CV). The sensitivity of the assay was determined by using total RNA from samples containing single infection of each virus. The total RNAs was serially diluted in sterile distilled water to have a range of 25 ng to 0.025 fg of infected plant RNA. The cDNAs were prepared from these dilutions after mixing with 200 ng of total RNA from healthy plant. These samples were chosen from a pool of infected samples after verification by RT-qPCR. The sensitivity of both singleplex and duplex RT-qPCR assays were compared with RT-PCR assays using the same total RNA dilutions. The assays were also validated by testing samples from different parts of the country.

RESULTS AND DISCUSSION

During the past one and half decade, the incidence of whitefly transmitted geminivirus, ToLCNDV-[potato] has increased tremendously in Indo-Gangetic plains of India due to the rapid expansion of whitefly populations and transmission of the virus through seed tubers. It is understandable that virus management strategies in vegetatively propagated crop like potato demands healthy vegetative propagation material and it is seed tuber in case of potato. Hence, there is a need for development of sensitive diagnostic tools for the detection of ToLCNDV-[potato], particularly in potato seed production system. PCR and RCA assays have been employed for the detection of ToLCNDV-[potato] (Jeevalatha *et al.,* 2013; Jeevalatha *et al.,* 2014). Real-time PCR has been successfully used for the detection and quantification of other begomoviruses, with significantly greater specificity and sensitivity (Abrahamian and Abou-Jawdah, 2013; Papayiannis *et al.,* 2010; Perefarres *et al.,* 2011; Rodriguez-Negrete *et al.,* 2014). However, there are no reports on qPCR protocols specific to ToLCNDV-

[potato] detection. Generally qPCR assay is used to quantify DNA viruses. But in order to simultaneously detect ToLCNDV-[potato], a DNA virus along with other RNA viruses (PVX and PLRV) we have developed singleplex and duplex RT-qPCR assays and validated using field samples.

Singleplex RT-qPCR assays and specificity

Among four sets of primers used in standardization of RT-qPCR, one set of primer for each target was selected based on melting curve peaks (T_m) of amplicons and also sensitivity and reproducibility (**Table 2**). The primer sets were selected in such a way that the amplicons can be visibly differentiated into two different peaks and also their annealing temperatures are common. The specific melting peaks of amplicons of selected primer pairs were 79.62±0.45 for ToLCNDV-[potato] with primer set LCCP-1F and LCCP-1R, 81.85±0.30 for PLRV with primer set LRCP1F and LRCP5R and 83.42±0.67 for PVX with primer set XQFP2 and XQRP1 (**Fig. 1**). These primers could detect different isolates of the viruses collected from different parts of the country which indicates the robustness of the developed RT-qPCR assays for application in routine testing. Slight differences in the *T*m values produced in different isolates of the same virus were likely caused by presence of mutations in their nucleotide sequences. The melting peaks were specific for the viruses tested and the assays could consistently detect these viruses in field samples and was more sensitive than RT-PCR. Two sets of primers were selected for internal control gene (*ef-1 α*) in order to use in duplex RT-qPCR detection of PLRV/PVX and ToLCNDV-[potato], respectively and the melting peaks of amplicons were 78.87±0.15 and 76.10±0.80 (**Fig. 1**).

The linear regression curve was plotted with the mean C_T (average of three) values

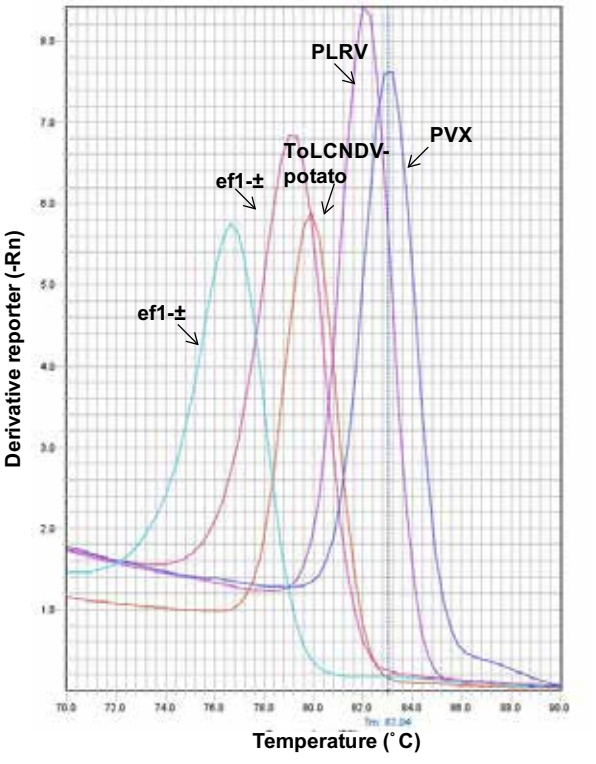

Fig. 1. Melting curve analysis showing the melting peaks of amplicons of different targets, ToLCNDV-[potato], PVX, PLRV and ef-1 α.

on the Y-axis and copy number of plasmid on the X-axis. The curve showed linear regression relationship with co-efficient of determination (R^2) of 0.99 and a slope of y= -3.473x + 44.166 for ToLCNDV-[potato], R^2 of 0.98 and a slope of y = -3.702x + 42.676 for PVX and R^2 of 0.95 and a slope of y= -3.629x + 42.709 for PLRV (**Fig. 2**). This result indicates a linear relationship of the C_T values within the dilution range used and could be used to calculate the viral titres with acceptable reliability, as in previous reports (Johnson *et al.,* 2014). The efficiency of the RT-qPCR assays using selected primer pairs were 94.075% for ToLCNDV-[potato], 86.259% for PVX and 88.62% for PLRV. The optimized assays proved specific for the viruses of interest, as no cross-reaction against a panel of other potato viruses was detected.

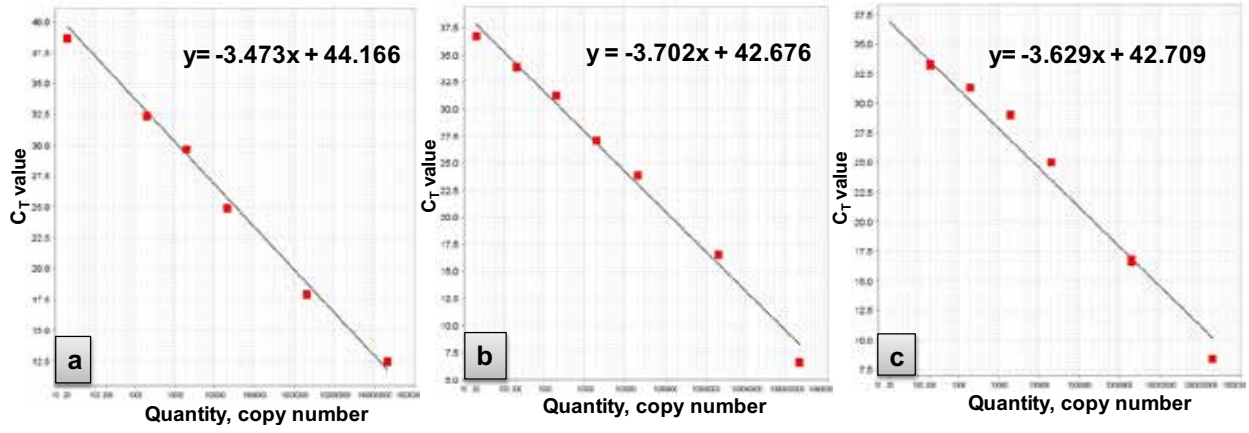

Fig. 2. Standard curve for RT-qPCR amplification of CP gene of ToLCNDV-[potato] (a), PVX (b) and PLRV (c) using 10-fold serial dilutions of plasmids harbouring the targets.

Duplex RT-qPCR assays

Two different primer pairs specific to *ef-1α* gene were used i.e. one for RT-qPCR detection of ToLCNDV-[potato] and another for RT-qPCR detection of PVX and PLRV. An additional melting peak at Tm 76.10±0.80 for *ef-1α* gene was obtained along with 79.62±0.45, specific to ToLCNDV-[potato]. While a melting peak at Tm 78.87±0.15 was obtained along with specific melting peaks of PVX and PLRV. Presence of either 76.10±0.80 or 78.87±0.15 in all healthy and infected samples confirmed the consistent amplification of internal control during validation. In duplex RT-qPCR assay detection of ToLCNDV-[potato] with PVX and PLRV, a melting peak at Tm 79.62±0.45, specific to ToLCNDV-[potato] was observed along with melting peak of other virus (PVX-83.42±0.67; PLRV-81.85±0.30) which indicated the amplification of both the targets.

Mixed infections are common in potato in which two or more viruses occur together in a single plant (Jakeli and Megrelidze, 2014; Li *et al.*, 2013). Under seed production system, the micro plants are tested for major potato viruses individually which is time consuming and also costly. In this context, multiplex real-time RT-PCR protocols are cost effective and time and labour saving. Simultaneous detection of two or more potato viruses in a single tube using TaqMan based real-time RT-PCR has been reported earlier (Agintodan *et al.*, 2007; Mortimer-Jones *et al.*, 2009). However, SYBR green based real-time PCRs does not involve combinations of costly probes and dyes with distinct emission wavelengths for simultaneous detection and differentiation of targets in single reaction tube, which reduces the cost per reaction and adds up an advantage over TaqMan based real-time PCRs particularly when it is applied for large scale testing. In addition, SYBR green I real-time PCRs coupled to melting curves analysis provides a reliable method to discriminate between different viral strains based on genomic sequence variability (Varga and James, 2005). Variations of 1-3°C in the Tm values of the amplicons generally allow the differentiation between viral species or strains through melting curves analysis (Papp *et al.*, 2003). In our study, we could distinctly identify two different virus species with a variation of 2-4°C in Tm values. Sensitivity and reproducibility was better with more difference in Tm values as the case with duplex RT-qPCR

detection of ToLCNDV-[potato] and PVX. While ambiguities in duplex RT-qPCR assay detection of ToLCNDV-[potato] and PLRV might be due to lesser difference in Tm values and competition between the primer pairs.

Sensitivity of singleplex and duplex RT-qPCR assays

To determine the sensitivity of the RT-qPCR, total RNA extracted from single infections, was sequentially (tenfold) diluted (25 ng to 0.025 fg) and cDNA was synthesized along with known amount of healthy RNA (200 ng) and was tested in triplicate. The detection limits of the assays were 0.025 fg of total RNA from infected plant tissues for all the three target viruses (**Table 3**). The developed quantitative RT-qPCR assays were 100-1000 times more sensitive than the conventional RT-PCR. RT-PCR detected ToLCNDV-[potato] from dilutions ranging between 25 ng and 0.025 pg total RNA, while RT-qPCR amplified from dilutions up to 0.025 fg RNA. The detection limit using serially diluted plasmids harbouring the targets (2×10^9 to 2×10^1) for RT-qPCR assays were 2×10^1 copies /reaction for all three target viruses. Whereas for RT-PCR assay, the detection limit was 2×10^4 copies/ reaction for ToLCNDV-[potato], 2×10^2 copies/ reaction for PVX and 2×10^3 copies/reaction for PLRV, respectively.

The sensitivity of duplex RT-qPCR assay was not influenced by duplexing and was as sensitive as singleplex RT-qPCR assay in case of duplex RT-qPCR detection of ToLCNDV-[potato] and PVX. Both of these viruses could be detected up to 0.025 fg of RNA and distinct melting peaks were observed as shown in **Fig. 3**. However, the sensitivity of duplex RT-qPCR assay for ToLCNDV-[potato] and PLRV was influenced and in initial dilutions i.e. from 25 ng to 0.25 pg amplification of PLRV was favoured with only small shoulder peak for ToLCNDV-[potato]. In remaining dilutions (0.025 pg to 0.0025 pg), melting peak of ToLCNDV-[potato] was favoured while 0.25fg to 0.025fg amplification of only ToLCNDV-[potato] was observed indicating the effect of narrow melting temperature difference and also competition between primer pairs (**Fig. 3**). This increased sensitivity could be explained by the ability of qPCR methods to detect lower viral loads in samples which has been previously demonstrated for different plant viruses (López-Fabuel *et*

Table 3. Comparison of sensitivity of singleplex/duplex RT-qPCR assays with singleplex/duplex RT-PCR assays.

Concentration of total RNA	ToLCNDV-[potato]		PVX		PLRV		ToLCNDV-[potato] + PVX		ToLCNDV-[potato] + PLRV	
	RT-PCR	RT-qPCR	RT- PCR	RT-qPCR	RT-PCR	RT-qPCR	RT-PCR	RT-qPCR	RT-PCR	RT-qPCR
25 ng	+	+	+	+	+	+	++	++	++	++
2.5 ng	+	+	+	+	+	+	++	++	++	++
0.25 ng	+	+	+	+	+	+	++	++	++	++
0.025 ng	+	+	+	+	+	+	++	++	++	++
0.0025 ng	+	+	+	+	+	+	++	++	++	++
0.25 pg	+	+	+	+	+	+	++	++	++	++
0.025 pg	+	+	+	+	+	+	++	++	++	++
0.0025 pg	-	+	+	+	-	+	-	++	-	++
0.25 fg	-	+	-	+	-	+	-	++	-	+-
0.025 fg	-	+	-	+	-	+	-	++	-	+-

(+) – Positive (-)- Negative (++)-Positive for both viruses (+-)- Positive for ToLCNDV-potato and Negative for PLRV

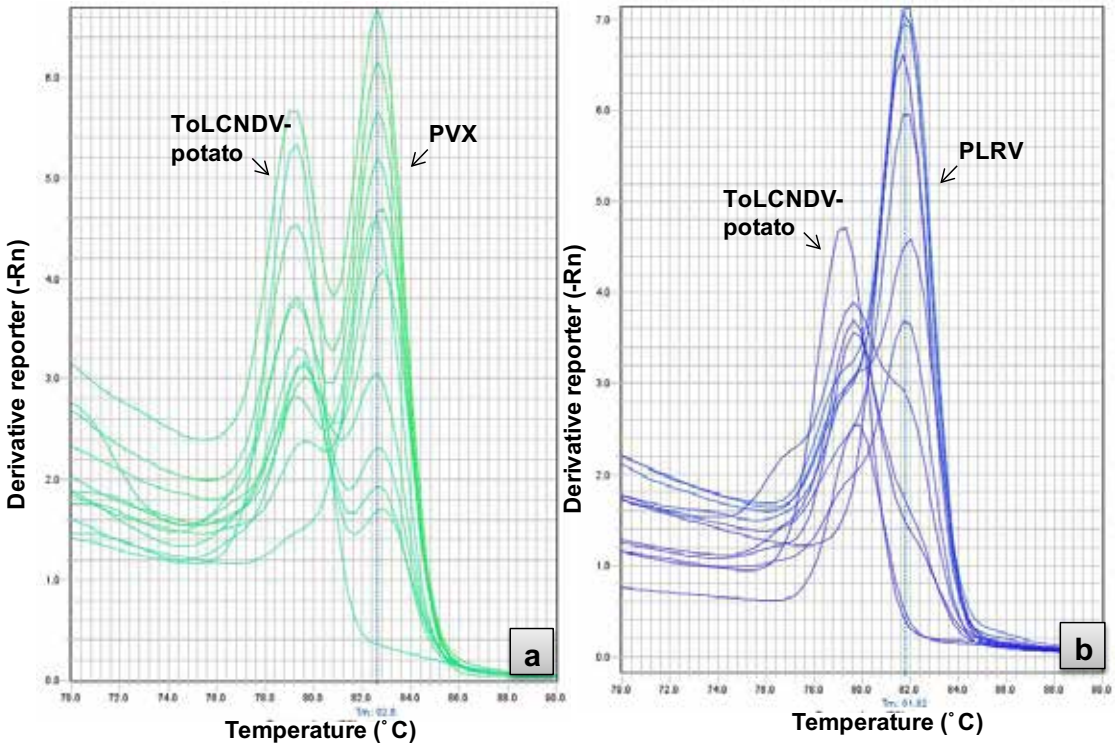

Fig. 3. Sensitivity analysis of duplex RT-qPCR assay using RNA dilutions- Melting curve analysis showing the melting peaks of amplicons (a) ToLCNDV-[potato] and PVX (b) ToLCNDV-[potato] and PLRV.

al., 2013; Osman *et al.*, 2013; Papayiannis *et al.*, 2010). Similarly, Abrahamian and Abou-Jawdah (2013) also reported that the qPCR assay was 1000 times more sensitive than conventional PCR and could detect *Squash leaf curl virus* in 18 fg of total nucleic acid and 30 genomic units.

Intra and inter-assay variability of singleplex and duplex assays

Experimental intra- and inter-assay variability data obtained were found acceptable. For singleplex RT-qPCR of ToLCNDV-[potato], PVX and PLRV, the CV% of the intra-assay variation was in the range of 0.23 to 1.19%, 0.14 to 1.37% and 0.19 to 1.48%, respectively. For duplex RT-qPCR of ToLCNDV-[potato] & PVX and ToLCNDV-[potato] & PLRV, the CV% of the intra assay variation was in the range of 0.12 to 1.41% and 0.22 to 1.54%, respectively. The CV% of inter-assay variation was found to be 0.17 to 2.07%, 0.17 to 2.43% and 0.09 to 2.11% for singleplex RT-qPCR of ToLCNDV-[potato], PVX and PLRV, respectively. While, it was 0.25 to 2.05% and 0.03 to 1.07% for duplex RT-qPCR assay of ToLCNDV-[potato] & PVX and ToLCNDV-[potato] and PLRV, respectively. These results suggest that the developed assays are highly reproducible. The reproducibility and repeatability of the assays demonstrated by intra and inter-assay variation studies is an important parameter supporting the wide use of this RT-qPCR assays in potato seed production system. The mean replicate C_T values of the singleplex and duplex assays were similar, and this justifies using the standard curves to quantitate viral copy numbers for both assays (Mortimer-Jones *et al.*, 2009).

Application of the RT-qPCR assays for testing of field samples

The RT-qPCR assays were tested with a collection of eighty two isolates of ToLCNDV-[potato], fifty three isolates of PVX and thirty isolates of PLRV. It was observed that the assays could successfully detect all the isolates of the viruses. In validation experiment with field samples, the assay could detect the viruses in few field samples identified as negative using ELISA/RT-PCR assay (**Fig. 4**). These results show the improved sensitivity of the developed RT-qPCR assays over RT-PCR assay. Similar observations were made by several researchers whose reports support that real-time PCR assays are more sensitive than RT-PCR assay in detection of plant viruses (Mortimer-Jones *et al.*, 2009; Lopez-Fabuel *et al.*, 2013; Johnson *et al.*, 2014). The duplex RT-qPCR assays were validated to detect mixed infections of these viruses in field samples (**Fig. 5**). Among the 122 field samples tested, 43 samples were infected with both ToLCNDV-[potato] and PVX, while 16 and 59 samples were found infected with ToLCNDV-[potato] and PVX, respectively (**Table 4**). Out of 58 samples, only 3 samples were found infected with both ToLCNDV-[potato] and PLRV, while 30 and 23 samples were found infected with ToLCNDV-[potato] and PLRV, respectively (**Table 5**). Absence of melting peaks in remaining samples confirmed that these samples were free from infection with these viruses.

The novelty of the study is the simultaneous detection of ToLCNDV-[potato], a DNA virus along with two other RNA viruses, PVX and PLRV using RT-qPCR assay. This is the first report of RT-qPCR detection of ToLCNDV-[potato] along with other RNA viruses. Two melting peaks observed in duplex assays were obtained through extensive optimization of primer concentrations, annealing and extension temperature and time. During validation of duplex RT-qPCR assay, it was observed that the occurrence of mixed infection of ToLCNDV-[potato] along with PVX was about 35% of the tested samples. Whereas it was only 0.05% of samples that were found infected with both ToLCNDV-[potato] and PLRV.

CONCLUSION

Sensitive and specific SYBR green based

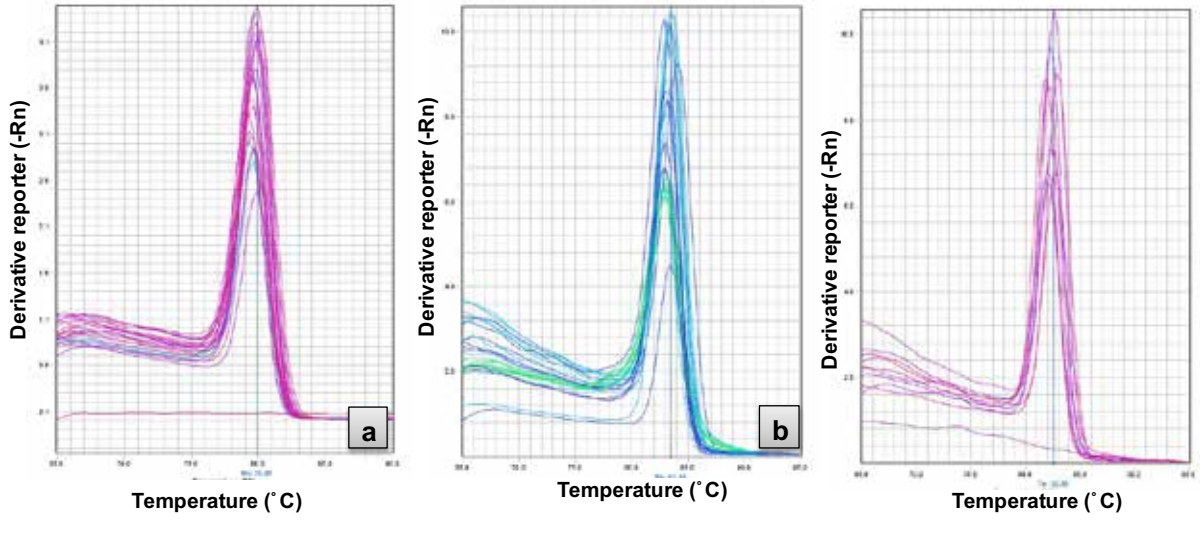

Fig. 4. RT-qPCR detection of ToLCNDV-[potato] (a), PVX (b) and PLRV (c) in field samples.

Table 4. Duplex RT-qPCR detection of ToLCNDV-[potato] and PVX in field samples.

S. No.	Location of samples	Number of positive samples		Negative for both viruses		Total samples
		ToLCNDV-[potato] + PVX	ToLCNDV-[potato]	PVX		
1.	Modipuram, UP	6	-	23	1	30
2.	Bhuvaneshwar, Odisha	6	-	-	-	6
3.	Kota, Rajasthan	-	4	2	-	6
4.	Shillong, Meghalaya	-	-	4	-	4
5.	Hisar, Haryana	10	-	-	-	10
6.	Jalandhar, Punjab	2	6	7	3	18
7.	Pune, Maharastra	-	2	-	-	2
8.	Ludhiana, Punjab	5	1	9	-	15
9.	Patna, Bihar	7	3	1	-	11
10.	Kufri, Himachal Pradesh	-	-	4	-	4
11.	Kanpur, UP	7	-	9	-	16

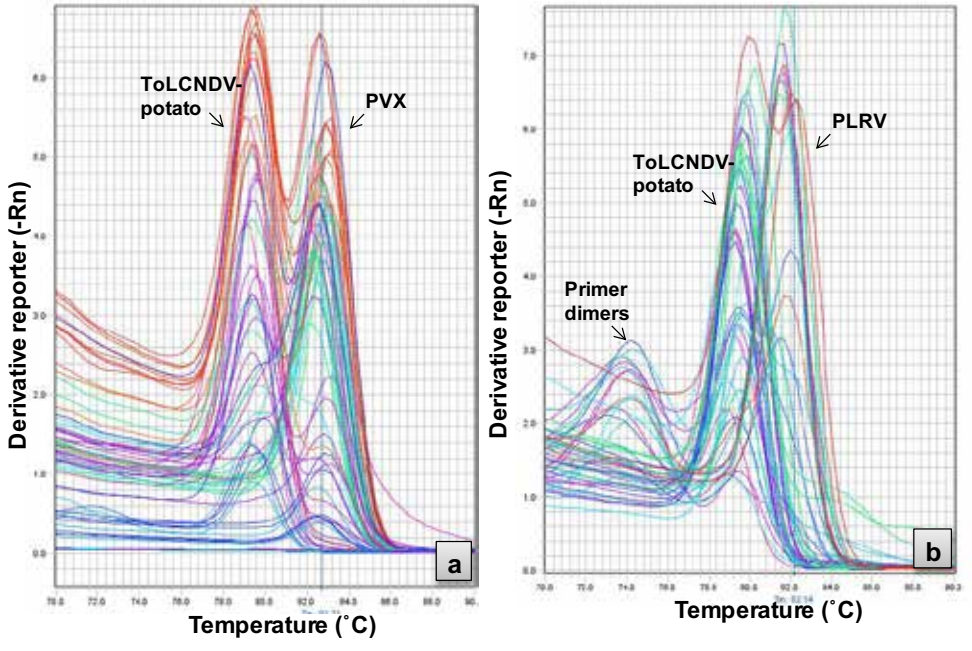

Fig. 5. Duplex RT-qPCR detection of ToLCNDV-[potato] & PVX (a) and ToLCNDV-[potato] & PLRV (b) in field samples.

RT-qPCR assays for the simultaneous detection of ToLCNDV-[potato] and PVX/ PLRV have been developed and evaluated using quite a lot of samples collected from different parts of the country. The results of the study support that the developed assays can be successfully used to detect these viruses in mixed infections. The use of duplex RT-qPCR assays in detection of these viruses in potato seed production system particularly in testing of mother culture plants will not only ensure the health of potato seeds but also saves time, labour and cost involved in testing.

Table 5. Duplex RT-qPCR detection of ToLCNDV-[potato] and PLRV in field samples.

S. No.	Location of samples	Number of positive samples		Negative for both viruses		Total samples
		ToLCNDV-[potato] +PLRV	ToLCNDV-[potato]	PLRV		
1.	Modipuram, UP	-	7	1	-	8
2.	Bhuvaneshwar, Odisha	-	2	2	-	4
3.	Kota, Rajasthan	-	3	1	-	4
4.	Shillong, Meghalaya	-	-	2	1	3
5.	Hisar, Haryana	-	5	-	-	5
6.	Jalandhar, Punjab	1	-	3	-	4
7.	Gwalior, MP	2	1	1	1	5
8.	Ooty, Tamil Nadu	-	-	6	-	6
9.	Patna, Bihar	-	-	1	-	1
10.	Kufri, Himachal Pradesh	-	-	4	-	4
11.	Pune, Maharastra	-	2	-	-	2
12.	Kanpur, UP	-	10	2	-	12

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MS received: 25 June April 2016; Accepted: 14 October 2016