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Detection and transmission of Carrot torrado virus, *a* novel putative member of the *Torradovirus* genus

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Abstract

A new *Torradovirus* tentatively named Carrot torrado virus (CaTV) was an incidental finding following a next generation sequencing study investigating internal vascular necrosis in carrot. The closest related viruses are *Lettuce necrotic leaf curl virus* (LNLCV) found in the Netherlands in 2011 and Motherwort yellow mottle virus (MYMoV) found in Korea in 2014. Primers for reverse transcriptase-PCR (RT-PCR) and RT-qPCR were designed with the aim of testing for the presence of virus in plant samples collected from the field. Both methods successfully amplified the target from infected samples but not from healthy control samples. The specificity of the CaTV assay was also checked against other known carrot viruses and no cross-reaction was seen. A comparative study between methods showed RT-qPCR was the most reliable method, giving positive results in samples where RT-PCR fails. Evaluation of the Ct values following RT-qPCR and a direct comparison demonstrated this was due to improved sensitivity. The previous published *Torradovirus* genus specific RT-PCR

primers were tested and shown to detect CaTV. Also, virus transmission experiments carried out suggest that unlike other species of the same genus, Carrot torrado virus could be aphid-transmitted.

1. Introduction

The genus *Torradovirus*, within the family *Secoviridae* was first described to place two new viruses, *Tomato torrado virus* (ToTV) and *Tomato marchitez virus* (ToMarV) (Sanfaçon *et al*., 2009, Verbeek *et al*., 2007, Verbeek *et al*., 2008). More recently, new species have also been proposed as members of the genus: Tomato chocolàte virus (ToChV), Tomato chocolàte spot virus (ToChsV), *Lettuce necrotic leaf curl virus* (LNLCV), Motherwort yellow mottle virus (MYMoV) and Cassava torrado-like virus (CsTLV) (Verbeek *et al*., 2010, Batuman *et al*., 2010, Verbeek *et al*., 2013a, Seo *et al*., 2014, Carvajal-Yepes *et al*). ToTV is considered the type species of the genus, it was first found in Spain in 2004 and since then, isolates have been described all around the world (Van der Vlugt *et al*, 2015). Analysis of its genome structure indicates there are two (+)ssRNA of ~7.7kb (RNA1) and ~5.2kb (RNA2) (Verbeek *et al*., 2007, Budziszewska *et al*., 2008). RNA1 contains one open reading frame (ORF) and has coding regions for the protease (Pro), helicase (Hel) and RNA-dependent RNA polymerase (RdRp). RNA2 has two ORFs: The function of ORF1 is still unclear whilst ORF2 encodes three coat proteins (Vp35, Vp26 and Vp23) and the movement protein (MP). Specific primers were designed for the detection of the ToTV (Pospieszny *et al*., 2007) and two generic primer sets, Torrado-1F/Torrado-1R and Torrado-2F/Torrado-2R, were also designed for the detection and identification of all the other viruses in the genus (Verbeek *et al*., 2012). Additional sets of primers have been recently developed to detect ToTV isolates from different sources (Herrera *et al*., 2015). Previous studies showed that torradoviruses are transmitted by the whiteflies *Trialeurodes vaporariorum, Trialeurodes abutilonea* and *Bemisia tabaci,* suggesting also that they are transmitted in a semi-persistent and stylet-borne manner (Amari *et al*., 2008, Barajas-Ortiz *et al*., 2013, Verbeek *et al*., 2013b). However, experiments with LNLCV and whiteflies using the same acquisition and inoculation periods did not lead to virus transmission suggesting another vector species could be responsible (Verbeek *et al*., 2013).

Internal necrosis has been seen in carrots (*Daucus carota*) in the UK for at least ten years and has been associated with virus infection (Fox, 2011). Recent analysis using next generation sequencing

(NGS), found that *Carrot yellow leaf virus* (CYLV) was strongly associated with the development of internal necrosis symptoms in carrots (Adams *et al*., 2014). Several novel viruses were also identified in that study including a new torrado virus tentatively named as Carrot torrado virus (CaTV) (KF533719 and KF533720). Analysis of sequence data showed the closest related virus in the genus *Torradovirus* was LNLCV (Adams et al., 2014). The acronym CaTV for Carrot torrado virus was later proposed in order to avoid confusion with *Carrot tristeza virus* (CTV) (van der Vlugt *et al*., 2015).

This study describes the development of a rapid and reliable molecular detection method for the identification of the first *Torradovirus* found in carrots, Carrot torrado virus, using reverse-transcriptase (RT)-PCR and RT-qPCR and describes the first report of aphid transmission of one member of the *Torradovirus* genus.

2. Methods

2.1. Source of samples

Carrot leaves exhibiting a range of foliar symptoms (interveinal chlorosis, generalised chlorosis, tip reddening and tip necrosis) and asymptomatic leaves were taken from Elveden Estate Field, Thetford, Norfolk, UK (Latitude 52.3656, Longitude -0.56407). Weed samples from the Apiaceous family, assumed to be more likely infected with carrot viruses, were also taken from the margins of Bratleys Field, Stamford Bridge, Yorkshire, UK (Lat. 53.9992, Long. -0.8855) and Sutton Park field, Sutton on the Forest, Yorkshire, UK (Lat. 54.0577, Long. -1.0518). ToTV infected plants were kindly supplied by the Plant Protection Service in the Netherlands for comparative testing.

2.2. Nucleic Acid extraction

Carrot leaves and weed samples were extracted using the Kingfisher® mL system (Thermo labsystems) following the method described in Mumford *et al*., 2002 and 2003. Leaf material was

ground in lysis buffer and centrifuged at 13000 rpm for 1 min. Samples were then loaded into the instrument and the extraction protocol was followed as described. RNA was eluted in 200 µl of molecular grade water and stored at -20 °C.

2.3. Conventional RT-PCR assay setup

Two pairs of CaTV primers were designed using Primer Express 2 (Applied Biosystems) for a RT-PCR assay according to the sequencing data obtained by Adams *et al*., (2014) (table 1). The primers designed to RNA1 amplify a fragment of 262 bp and RNA2 primers a fragment of 299 bp. The extracted sample (1 µl) was added to a 24 µl reaction mix, containing Verso™ 1-Step RT-PCR ReddyMix™ Kit (Thermo Scientific) and 400 mM of each primer. Assays were carried out in a Bio-Rad C1000^{TM} thermal cycler (Bio-Rad laboratories) and PCR conditions consisted of 45 min at 48 °C for cDNA synthesis, 2 min at 94 °C, then 40 cycles of, 30 sec at 94 °C, 1 min at 56°C and 1 min at 68 °C and a final extension step for 6 min at 68 °C. These conditions followed the method described in Verbeek *et al*. 2012, but primer annealing temperature of 56 °C was chosen for CaTV when a gradient primer annealing temperature test was performed on a CFX96 Touch Thermal cycler (Bio-Rad laboratories) for optimization. PCR products were separated using a 1.8 % agarose gel (130 V) stained with ethidium bromide, visualized in a UV transilluminator. Products were purified using the QIAquick® PCR Purification kit (Qiagen) before being sent for sequencing. Generic *Torradovirus* genus assays were performed following the method described in Verbeek *et al*., (2012).

2.4. One step reverse-transcriptase (RT)-qPCR assay

2.4.1. Development of a RT-qPCR assay for CaTV detection

Primers and probes for the real-time assay were designed using Primer Express 2 (table 1). The 5'- and 3'- ends of the probes were labelled with the reporter dye FAM (6-carboxyfluorescein) and quencher dye TAMRA (tetra-methylcarboxyrhodamin). A concentration of 300 nM of each primer and 100 nM of probe were used in each 25 µl reaction and same conditions were used for both RNAs.

Reactions were carried out in 96 well plates using the ABI 7900 (Applied Biosystems). Positive controls consisting of a CaTV sample obtained by NGS and negative controls consisting of healthy leaf material and water were used to validate the results. RT-qPCR cycling conditions were: 10 min at 55 °C for the reverse transcription followed by 8 min at 95 °C and then 40 cycles of 10 sec at 95 °C and 1 min at 60 °C. Results were analysed using the SDS 2.4 Software (Applied Biosystems). A threshold cycle (Ct) value below 40 was considered as a positive result and was fixed by default parameters of the Software.

2.4.2. Validation experiments

The efficiency of the new assays for both RNAs was measured using serial 10-fold dilutions (from 1 to 10^{-8}) of total RNA from plant infected extracts quantified using the Nanodrop ND-1000 Spectrophotometer (Labtech). Samples were chosen from a pool of positive samples previously tested using RT-qPCR and all the dilutions were tested in duplicate in the same run. Standard curves were generated using the Ct values obtained and the logarithm of the dilution and regression coefficient represented. Specificity of the real-time test was assessed using a panel of carrot and other viruses. Analytical sensitivity was also compared to a RT-PCR assay comparing RT-qPCR Ct values with band intensity of the PCR product after gel electrophoresis.

2.5. Transmission experiments

Infected leaves were ground in potassium phosphate buffer, pH 7.7., mixed with cellite and inoculated onto leaves of ten plants of *Nicotiana benthamiana, Anthriscus cerefolium* (chervil) and *Daucus carota* (carrot). Plants were kept in the green house with a 12 hours photoperiod and an average temperature between 18-20 °C. Five mock inoculated plants of each species were also used as controls and kept in the same conditions. All plants were assessed weekly for symptom development and tested for virus presence using RT-qPCR from random leaves.

Cavariella aegopodii and Myzus persicae collected from a CaTV infected field plant were collected and cultured on chervil and carrot plants in a glasshouse (20°C, 60% RH, L16h:D 8h with

supplemental lighting). Aphids were then used to inoculate 3x50 trays of healthy cotyledon stage *N.benthamiana*, chervil and carrots. Healthy plants of each species were also used as negative controls. Each aphid was transferred onto an individual indicator plant and covered with a plastic tube (30 mm dia. x 110 mm) to prevent it from escaping. The tubes were removed after 24h and the aphids killed by spraying the plants with Bug Clear Ultra (0.05gl-1 acetamiprid, The Scotts Company (UK) Ltd), following the manufacturer's instructions. The plants were grown on in a plant growth room (20 °C, 60 % RH, L16h:D 8h) for three weeks when they were tested for virus presence using RT-qPCR.

3. Results

3.1. Diagnostic performance of RT-PCR

Comparison of primer sequences to sequences in Genbank using BLAST did not indicate significant homology with any other species except the targets. Assays for RNA1 and RNA2 were evaluated using extracts of carrot leaf samples from the field. Amplification products were analysed using agarose gel electrophoresis and results showed a single amplicon of the desired size for both primer sets, 262 bp for RNA1 and 299 bp for RNA2 (figure 1a). RT-PCR products were purified and sequenced confirming CaTV virus presence. No PCR products were amplified from the healthy or water controls and primer pairs did not produce non-specific amplicons.

Specific *Torradovirus* genus primers developed by Verbeek et al., (2012) were evaluated for the detection of CaTV and compared with the species specific assays. As expected the Generic set of primers detected an amplicon of 514 bp from CaTV and 515 from ToTV (figure 1b). The CaTV assay developed did not cross react with ToTV, similarly, the ToTV assay did not amplify CaTV. Comparative analysis of the sequences using MEGA 6 did not indicate cross-reaction with LNLCV or MYMoV, non-tomato Torradoviruses and the closest related viruses to CaTV.

3.2. Detection of CaTV by RT-qPCR

Each primer sequence was compared to published sequence information available and no cross-reaction was found (NCBI, BLAST). The RT-qPCR assay was evaluated using samples collected from the field on an ABI 7900 HT system (Applied Biosystems). Amplification curves gave Ct values between 15.98 and 33.19 for RNA1 and 17.02 and 32.69 for RNA2, indicating virus presence in the samples. A test designed to detect the cytochrome oxidase gene sequence (COX) of the plant was also used as an internal control. Detection of one of the two RNAs was considered as a positive result. Negative controls consisting of RNA extracts from healthy plants extracts and water were used and no amplification was detected.

To test the specificity of the assays, a range of UK field isolates affecting carrots, related and unrelated viruses were used: ToTV (*Tomato torrado virus*), CYLV (*Carrot yellow leaf virus*), CRLV (*Carrot red leaf virus*), CRLaV (*Carrot red leaf associated virus*), CMoV (*Carrot Mottle virus*), CtCV1 (Carrot closterovirus 1), PYFV (*Parsnip yellow fleck virus*), CMV (*Cucumber mosaic virus*), SLRV (*Strawberry latent ringspot virus*), TBRV (*Tomato black ring virus*) and PVY (*Potato virus Y*). Each assay was run in triplicate for every virus and no amplification was found between the species tested or the healthy or negative controls for RNA1 and RNA2 (table 2). For RNA1, a Ct value of 38.12 and 39.9 was obtained for CYLV and CMoV respectively in one of the replicates. Those samples were tested again and no amplification was seen.

3.3. RT-qPCR assay validation and sensitivity comparison with RT-PCR

In order to assess the efficiency of the assays, standard curves for RNA1 and RNA2 were generated with 10-fold serial dilutions of total RNA (plant + virus) from a pool of positive samples to determine the maximum dilution detected. For each dilution, two replicates with 1 µl of total RNA were prepared in a 25 µl well. Results showed standard curves with 0.9932 and 0.9956 regression coefficients (R^2) for RNA1 and RNA2 respectively (figure 2a). Both assays were assessed following the guideline described in OEPP/EPPO Bulletin PM7/98 (2), 2014, (table 3). Repeatability tests were performed for both RNAs with the lowest level of dilution detected reliably in the sensitivity assays and six replicates of each sample were tested. Ct values between 33.91 and 35.17 were obtained with a standard deviation (SD) of ± 0.46 for RNA1 and between 32.15 and 32.63 with a SD of ± 0.19 for

RNA2 were obtained. No differences were seen either when tested by two different operators in two different 7900 HT systems.

Diagnostic sensitivity of the RT-qPCR assay was also compared to RT-PCR. Results showed that the developed RT-qPCR was more sensitive than the conventional method detecting dilutions ranging between 19 ng and 1.9 pg and detecting levels of dilution up to 10^{-5} for RNA1 and 10^{-4} for RNA2 (figure 2b), 1000 and 10^{4} times more sensitive than the conventional method which detected levels of dilution of 10^{-1} in both RNAs (figure 2c). Further comparisons were made between both methods using a pool of 45 samples collected from the field. For RNA1, RT-qPCR detected 23 positive samples (51.1%) while RT-PCR detected virus presence in a total of 20 samples (44.4%) (data not shown). When tested for RNA2, RT-PCR gave positive results in 27 of the samples (60%) in comparison with the 31 positives obtained by RT-qPCR (68.8%) (figure 2d).

3.4. CaTV transmissionMechanical transmission experiments with *N. benthamiana*, chervil and carrots resulted in one symptomless *N. benthamiana* CaTV infected plant. Weed samples surrounding carrot fields consisting of 30 cow parsley (*Anthriscus sylvestris)*, and 19 hogweed (*Heracleum sphondylium),* were collected and tested to look for possible sources of CaTV infection in carrots but all tested negative for the virus (data not shown). Aphid transmission experiments with *M. persicae* and *C. aegopodii* resulted in some symptomatic chervil and *N benthamiana* plants when tested by RT-qPCR. Transmission rates of 35.3% and 12.7% were found in chervil and tobacco plants respectively with *M. persicae* . Lower transmission rates were achieved when using *C.aegopodii* in both plant species (table 4). Carrot to carrot transmission of 10% and 2.7% was also seen with *M. persicae* and *C. aegopodii* respectively. Virus was successfully transmitted from infected Chervil to healthy carrots using *M. persicae* and 2% of the plants tested positive for the virus. All the negative controls consisting of healthy carrot, N. benthamiana and chervil plants were also used for each experiment and tested negative for CaTV.

4. Discussion

CaTV was firstly detected in the UK in 2013 in a study investigating the agent responsible of the development of necrotic symptoms in carrots (Adams *et al*., 2014). In order to detect CaTV infected

plants from the field and study its transmission, RT-PCR and RT-qPCR assays were developed and optimized. Both methods are routinely used in diagnostic laboratories for the detection of pathogens. In this study both methods successfully amplified CaTV RNA1 and RNA2 in infected samples collected from the field. Neither assay cross-reacted with ToTV, the first Torradovirus found and type member of the genus, indicating the specificity of the assays. Several studies had been performed previously using RT-PCR and two generic primer sets were developed for the detection of all the viruses of the Torradovirus genus (Verbeek *et al*., 2012). These sets of primers were evaluated and the detection of CaTV RNA1 and CaTV RNA2 using the same cycling conditions was confirmed. Assays were subjected to the EPPO validation international standard method and all the requirements were met. Validation experiments using RT-qPCR were carried out and it was seen to detect virus levels up to 1.9 pg. In specificity assays, samples with Ct values between 36 and 40 were re-tested to confirm if there was cross-reaction or low level contamination due to late amplification of some random samples. Re-testing confirmed there was no cross-reaction with any of the species tested. Similar results were obtained when the assays were performed by different people, different days in different 7900 HT systems indicating the reproducibility and repeatability for both RNAs and the robustness of the developed assays. RT-qPCR method gave positive results in field samples where RT-PCR failed and further comparison between both methods indicated this was due to improved sensitivity.

Some samples were found to be only positives for RNA1 but negative for RNA2 and vice-versa when tested using RT-PCR or RT-qPCR, describing possible replication differences between RNAs when infection takes place in the host. The performance of the tests for both RNAs is recommended to avoid any false negative result.

CaTV was successfully inoculated and transmitted to healthy *N. benthamiana* and Chervil plants but symptom description was not possible due to infection with *Carrot red leaf virus* too. Previously studies suggested that tomato infecting *Torradoviruses* were transmitted by different whiteflies species (Amari *et al*., 2008, Verbeek *et al*., 2013b). However, aphid transmission experiments developed in the glasshouse with CaTV, indicated that *M. persicae* could be the natural vector of the virus to carrots. Equally, LNLCV did not result in transmission when experiments with whiteflies were carried out suggesting there could be another different species involved (Verbeek *et al*., 2013). These

results might conclude the possibility that non-tomato infecting Torradoviruses could be transmitted by different species than tomato infecting *Torradoviruses*. Back transmission experiments also showed the virus can be transmitted between members of the Apiaceae family. Additional studies are currently being carried out with *M. persicae* in order to establish virus acquisition and virus inoculation periods.

In 2008, ToTV was found in weed species from *Amaranthaceae*, *Caryophyllaceae*, *Chenopodiaceae*, *Cruciferae*, *Malvaceae*, *Polygonaceae*, and *Solanaceae* families (Alfaro-Fernandez *et al*., 2008) indicating they could act as reservoir hosts for this virus before their transmission to tomatoes by aphids. However, limited surveys carried out in 2014 and 2015 with weeds from several species, 59 Hogweed (*Heracleum sphondylium*), 27 Cow Parsley (Anthriscus sylvestris), one Hemlock (*Conium maculatum*) and three Rough chervil (*Chaerophyllum temulum)* did not identify any alternative host for CaTV suggesting that infection could come from another different source. Due to the limited number of weed samples tested, further studies need to be carried out in order to establish possible sources of infection in carrots with CaTV.

The study describes the development of a new, reliable, and sensitive RT-qPCR method for the detection of CaTV and the first report of aphid transmission of a member of the Torradovirus genus. However, additional studies are also currently being carried out evaluating CaTV host range, transmission and further weed testing. Symptom development and incidence of this virus in the UK will also be assessed in order to obtain a complete characterization of this new finding.

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Tables

Table 1

RT-PCR and RT-qPCR CaTV RNA1 and RNA2 primers used during the study.

	Primer	Sequence (5'-3')	Location (nt)
RT-PCR	**CaTVPCR 1F**	TCAATCAGTATTAAGCGAGGAATGG	2742 - 2762
	CaTVPCR1R	CCTCAATGGGCTTGTAATGA	2985 – 3004
	CaTVPCR 2F	TGTGCAACCACGAGGAATACA	3942 – 3962
	CaTVPCR 2R	GATGCCTCATAGCAAACTGTCAT	4219 - 4241
RT-qPCR	**CaTV-1F**	CCGTTGTTATTCGTCTTCCTCAA	2819 – 2841
	CaTV-1R	TGGATGATTGTAAATACTGCACCAT	2918 - 2942
	CaTV-1P	FAM-TTCAGAGGTGTTTACGTGAGATCGGGATG-TAMRA	
	CaTV-2F	TTACAAAGACTACTGGTGATCGTGACTT	2654 – 2681
	CaTV-2R	ATTCGTACAAACCCACCTCAAAG	2730 - 2752
	CaTV-2P	FAM-AGAGTTGGAAATGATGCAACCCATGATAGC-TAMRA	

Table 2

CaTV RT-qPCR assay specificity was tested against ToTV, carrot viruses and non-target viruses*.

	CaTV	ToTV	CYLV	CRLV	CRLaV	CMoV	CtCV1	PYFV	CMV	SLRV	TBRV	PVY	HC
RNA1 Ct	15.98 ± 0.0 (3/3)	-	38.12 (1/3)**	-	-	39.9 (1/3)**	-	-	-	-	-	-	-
RNA2 Ct	17.47 ± 0.14 (3/3)	-	-	-	-	-	-	-	-	-	-	-	-

*ToTV (Tomato torrado virus), CYLV (*Carrot yellow leaf virus*), CRLV (*Carrot red leaf virus*), CRLaV (*Carrot red leaf associated virus*), CMoV (*Carrot Mottle virus*), CtCV1 (Carrot closterovirus 1), PYFV (*Parsnip yellow fleck virus*), CMV (*Cucumber mosaic virus*), SLRV (*Strawberry latent ringspot virus*), TBRV (*Tomato black ring virus*), PVY (*Potato virus Y*).

** Samples tested negative when assay was repeated, indicating there was no cross-reaction with any other viruses.

Table 3: Validation results obtained for CaTV RNA1 and RNA2 assays following the criteria described in OEPP/EPPO Bulletin PM 7/98 (2).

	CaTV RNA1	CaTV RNA2
Sensitivity		
Ct value obtained for the smallest amount of target detected reliably	36.48 ± 0.66	33.6 ± 1.46
Specificity		
Cross reacts with	-	-
Repeatability		
Calculated % of agreement for a low concentrated sample (10^{-5} dilution)	100%	100%
Ct	34.33 ± 0.46 (33.91-35.17)	32.43 ± 0.19 (32.15-32.63)
Reproducibility		
Calculated % of agreement for a low concentrated sample (10^{-4} dilution)	100%	100%
Operator 1:		
7900 HT system 1 Ct	34.93 ± 0.49	33.34 ± 0.23
7900 HT system 2 Ct	34.77 ± 0.55	33.38 ± 0.83
Operator 2:		
7900 HT system 1 Ct	34.97 ± 0.44	31.99 ± 0.11
7900 HT system 2 Ct	34.66 ± 0.40	31.91 ± 0.27

Table 4

Results of transmission experiments using *Cavariella aegopodii* and *Myzus persicae* aphid species from chervil and carrot to three blocks of 50 *Nicotiana benthamiana*, Chervil and carrot plants.

Source	Species	*M. persicae*		*C. aegopodii*	
		Positives	**% transmission**	**Positives**	**% transmission**
Carrot (*D. carota*)	*N. benthamiana*	10/50 2/50 7/50	12.7	0/0 0/0 0/0	0
	Chervil	18/50 13/50 23/50	35.3	3/50 0/50 4/50	4.7
	Carrot	5/50 6/50 4/50	10	1/50 2/50 1/50	2.7
Chervil (*A.cerefolium*) back transfer	Carrot	1/50 2/50 0/50	2	-	-

Figure legends

Figure 1: RT-PCR products for RNA1 (266 bp) and RNA2 (299 bp) using CaTV specific primers (a). CaTV and ToTV gel bands obtained when CaTV, ToTV and Torradovirus genus sets of primers were used (b).

Figure 2: RT-qPCR standard curves and linear regression coefficients (R^2) for RNA1 and RNA2 using serial 10-fold dilutions of total RNA with primers described in table 1 (2a). Amplification plots obtained using RT-qPCR (2b). Gel bands obtained with CaTV serial diluted samples using RT-PCR (2c). Comparative test between RT-qPCR and RT-PCR using a pool of 45 samples collected from the field (figure 2d).

Figure 1

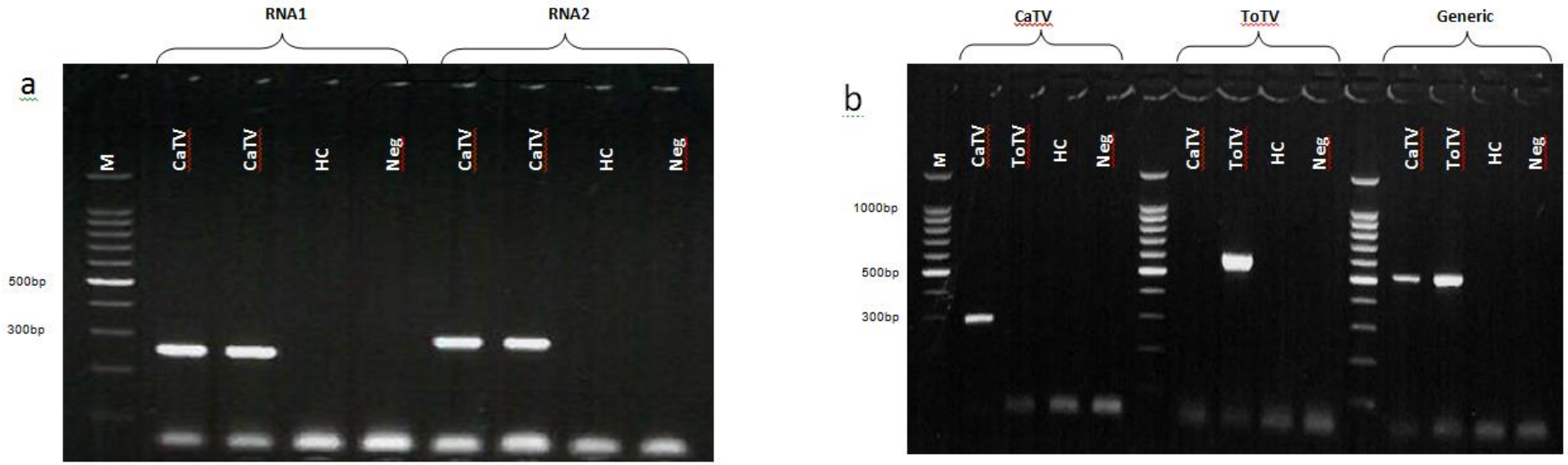
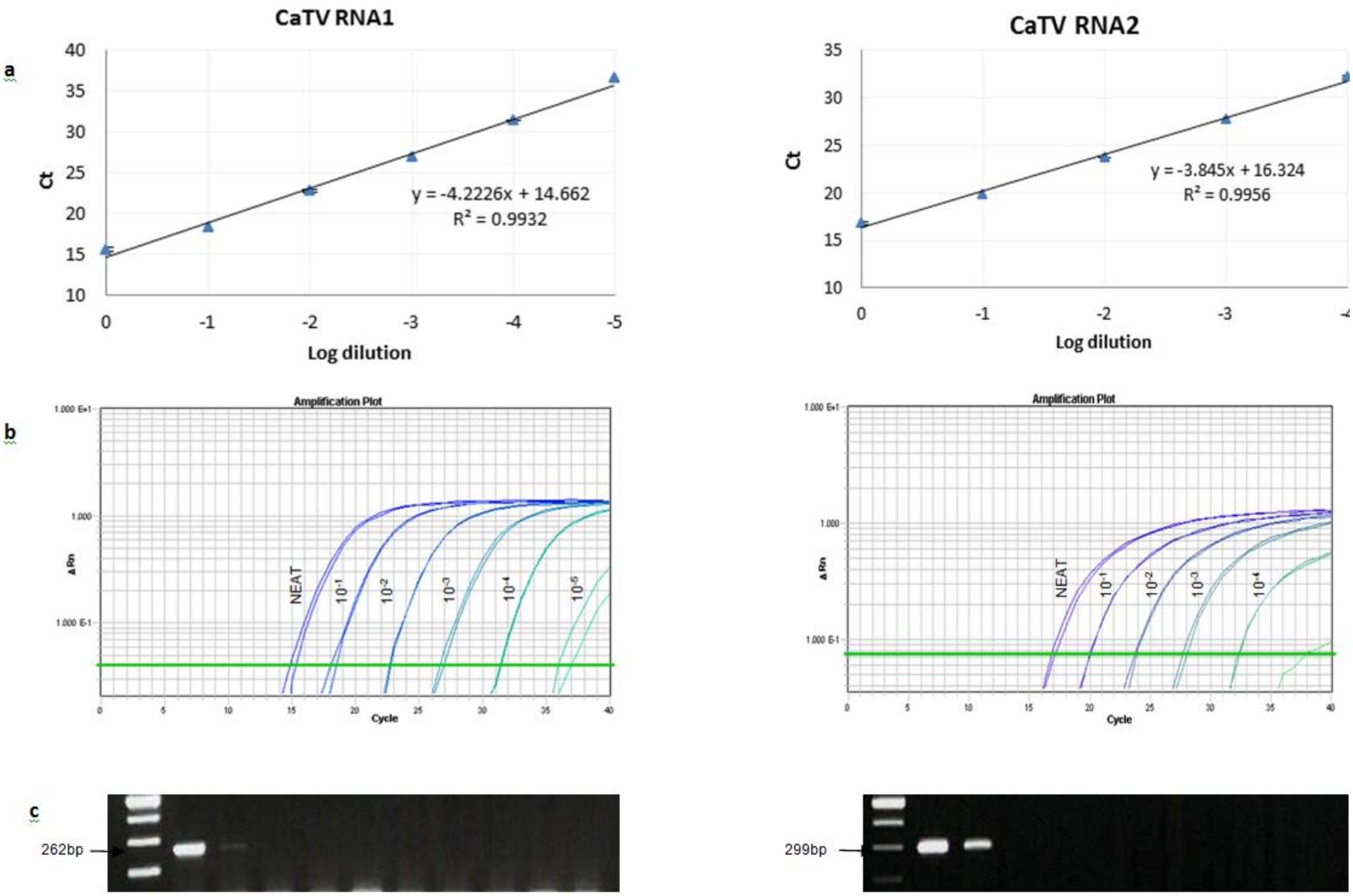

a
RNA1
RNA2
M
CaTV
CaTV
HC
Neg
CaTV
CaTV
HC
Neg
500bp
300bp
b
CaTV
ToTV
Generic
M
CaTV
ToTV
HC
Neg
CaTV
ToTV
HC
Neg
CaTV
ToTV
HC
Neg
1000bp
500bp
300bp

Figure 2


a
CaTV RNA1
Ct
Log dilution
y = -4.2226x + 14.662
R² = 0.9932
CaTV RNA2
Ct
Log dilution
y = -3.845x + 16.324
R² = 0.9956
b
Amplification Plot
ΔRn
Cycle
NEAT
10-1
10-2
10-3
10-4
10-5
Amplification Plot
ΔRn
Cycle
NEAT
10-1
10-2
10-3
10-4
c
262bp
299bp

d

		RT-PCR		
		+	**-**	**Total**
RT-qPCR	**+**	27	4	31 (68.9%)
	-	0	14	14 (31.1%)
	Total	27 (60%)	18 (40%)	45 (100%)