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RESEARCH ARTICLE

APPLICATION OF A REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (RT-LAMP) AND REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) FOR THE DETECTION OF CUCURBIT YELLOW STUNTING DISORDER VIRUS (CYSDV) IN EGYPT.

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Abstract

Cucumbers and squash affected by *Cucurbit yellow stunting disorder virus* (CYSDV) show severe yellowing symptoms in the field and greenhouse-grown plants in Egypt and are heavily infested by *Bemisia tabaci* Genn.. Symptoms start as an interveinal chlorosis on the older leaves and intensify as leaves age. In order to detect CYSDV reverse transcription-polymerase chain reaction (RT-PCR) techniques were developed that target either the coat protein gene in RNA2 or p22 in RNA1. Electrophoretic analysis of the RT-PCR amplification revealed the primers amplified products of 756 bp for the coat protein ORF and 560 bp for p22. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) was also developed and proved to be a rapid method with high specificity and efficiency under isothermal condition using a set of six specifically designed primers that recognize six distinct sequences on the target gene p22.RT-LAMP was reliable for diagnosis of CYSDV-infected leaf samples and insect vectors from the field in 60 min. RT-LAMP has the potential to replace PCR due to its simplicity, rapidity, specificity, sensitivity and cost-effectiveness without the need for specialized equipment.

Introduction

Cucurbit yellow stunting disorder virus (CYSDV) is one of the most destructive pathogens affecting field and greenhouse-grown cucurbit plants in tropical and subtropical regions worldwide (Tzanetakis *et al.*, 2013). Typically, yellowing disease caused by CYSDV-infection is associated with a loss of photosynthetic capability, often characterized by interveinal yellowing or reddening of leaves, chlorotic mottle, leaf brittleness, reduced plant vigour, yield reductions, and early senescence, depending on the host plant affected. CYSDV has recently become a significant production threat throughout cucurbit production regions in the Middle East, the Mediterranean Basin (Lecoq and Desbiez, 2012), North Africa, and Southern Europe, as well as the Canary Islands, the Southern United States, Mexico, and Central America (Bananej *et al.*, 2013). CYSDV is grouped in the genus Crinivirus, which includes the whitefly-transmitted members of the family *Closteroviridae* (Wintermantel *et al.*, 2009; Fidan *et al.*, 2012; Abrahamian *et al.*, 2015). CYSDV particles range from 825 to 900 nm in length, and the CYSDV genome consists of bipartite molecules of single-stranded (ss) RNA of positive (+) polarity designated RNAs (RNA1 and RNA2) which are 9.1 and 8 kb respectively, with genome organization similar to other criniviruses (Celix *et al.*, 1996; Aguilar *et al.*, 2003).

The most common methods for laboratory diagnosis of CYSDV include serological methods and nucleic acid-based techniques utilizing polymerase chain reaction (PCR), which have been used to detect and compare between coat protein genes of CYSDV-strains spread worldwide (Rubio *et al.*, 1999; Abou-Jawdah *et al.*, 2000; Abrahamian *et*

al., 2013). Agranovsky (1996) and Livieratos *et al.* (1999) showed that the closteroviruses were distinguished by the presence of a heat shock protein 70 (HSP70) homolog gene and a diverged coat protein duplicate (CPd) gene, as well as the formation of characteristic membranous vesicles in the cytoplasm of infected phloem cells. More recently, cloning and complete genome sequencing of RNA1 and RNA2 of CYSDV and its closely related viruses has been used for analysis of coat protein genes and other regions (Open reading frames "ORFs") in the genomic structure (Livieratos *et al.*, 1999; Rubio *et al.* 2001; Aguilar *et al.*, 2003; Marco and Aranda 2005; Livieratos *et al.*; 2004; Lee *et al.*, 2011; Tzanetakis *et al.*, 2013 and Yanhong *et al.*, 2013).

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay is being increasingly used as an alternative to PCR for rapid detection and typing of emerging human and animal diseases (Notomi *et al.*, 2000; Lu *et al.*, 2012 and Neeraja *et al.*, 2015). LAMP has proved to be useful in detecting plant pathogenic agents and has the potential to replace PCR due to its simplicity, rapidity, specificity, sensitivity and cost-effectiveness without the need for specialized equipment (Zhou *et al.*, 2012; Sasaya 2015 and Kil *et al.*, 2015). For this reason, the present study was undertaken to develop improved methods to detect CYSDV using RT-LAMP. Additionally, two RT-PCR techniques were designed to detect and evaluate any sequence differences in two different regions of the CYSDV genome, ORF4 (gene p22) in RNA1, and ORF10 (coat protein gene "CP") in RNA2.

Materials and methods

Source of the virus:-

The CYSDV isolate used in this study was originally obtained from cucumber plants cv. El-Safa heavily infested with *B. tabaci* Genn. and grown in the experimental station at the fields and greenhouse of the Faculty of Agriculture, Cairo University. The plants were exhibiting yellowing and interverinal chlorotic spots on lower leaves which coalesced to give a bright yellow colour, while leaves slightly curled downward, remaining turgid and becoming brittle. The isolated virus was purified biologically through several passages of viruliferous whiteflies as described by Abdel-Salam *et al.* (1999) (data of purification, antiserum production and serological tests in press). The CYSDV isolate was maintained by whitefly inoculation on squash and cucumber plants.

Molecular detection of CYSDV RNAs by reverse transcription- polymerase chain reaction:-

Two different RT-PCR approaches were used to detect CYSDV RNAs, by the amplification of the coat protein gene in RNA2 and the p22 gene in RNA1, as well as Loop-mediated isothermal amplification (LAMP):

(a) Reverse-transcription polymerasechainreaction (RT–PCR):-

In this context firstly, RT–PCR was applied to amplify the coat protein gene in RNA2 as described by Livieratos *et al.* (1999) with some modifications as follows: RNA was extracted from squash leaf tissue using total RNA mini (Plant) Kit (Geneaid, co. Cat. # RP050) according to the manufacturer's instructions, and was used as the template for RT-PCR. Specific oligonucleotide primers CYSDVCP1f (5′ AAT AGC ATG CAA TGG CGA GTT CGA GTG AGA 3′) and CYSDVCP2r (5′ AAT TCT GCA GTC AAT TAC CAC AGC CAC CTG 3′) corresponding to both ends of the CYSDV CP gene were used and RT was performed in a reaction mixture (25 µl) consisting of 12.5 µl of 2x master mix (Promega), 1.5 µl of CYSDV CP1f primer, 1.5 µl of CYSDVCP2r primer, 5 µl RNA template and 0.5 µl of AMV reverse transcriptase (Promega). The mixture was incubated at 45℃ for 45 min. PCR was performed in a 25 µl reaction containing 3 µl of the synthesized cDNA , 2.5 µl 10x PCR buffer,1.25 µl of each primer. The mixture was incubated first at 94℃ for 3 min, followed by 30 cycles at 94℃ for 90 sec, 53℃, 72℃ for 1.5 min, and by a final cycle at 72℃ for 7 min. RT-PCR products were fractionated by electrophoresis in 1.5% agarose gels in 0.5x TBE buffer and visualized after ethidium bromide staining. A DNA fragment of ~756bp was detected for the CYSDV genome. No cDNA products were amplified from healthy plants.

Secondly for the p22 gene, the primers published by Marco *et al.* (2003) were used with some modifications as follows: specific oligonucleotide primers CYSDV Ma 156f (5′ GAA GAA TTC CAG GCA AGG 3′) and CYSDV Ma129r (5′ TCA CAT CAT CAA TCC AAA AG 3′) corresponding to both ends of the CYSDV p22 gene were used. RT–PCR was performed in a reaction mixture (25 µl) of 12.5 µl of 2x master mix (Promega), 1.5 µl of CYSDV Ma 156f primer, 1.5 µl of CYSDV Ma 129r primer, 5 µl RNA template and 0.5 µl of AMV reverse transcriptase (Promega). The mixture was incubated at 45℃ for 45min. PCR was performed in a 25 µl reaction containing 3 µl of the synthesized cDNA, 2.5 µl 10x PCR buffer, 1.25 µl of each primer. The mixture was incubated first (an initial denaturation step) at 94℃ for 5 min, followed by 35 cycles at 94℃ for 35 sec., 52℃ for 35sec., 72℃

for 1.5 min, and by a final cycle (an extension step) at 72℃ for 3 min. RT-PCR products were fractionated by electrophoresis in 1.5% agarose gels in 0.5x TBE buffer and visualized after ethidium bromide staining. A DNA fragment of ~560bp was detected for CYSDV infected material and no DNA products were obtained from healthy plants.

(b) Reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay:-

The potential target regions were selected by aligning CYSDV sequences from GenBank, and the RT-LAMP primers were designed from conserved region of each serotype using the Primer Explorer version 4 software (Eiken Chemical Co., Tokyo, Japan). A set of six primers for the viral p22 gene comprising two outer (F3 and B3), two inner (FIP and BIP), and two loop primers (FLP and BLP) that recognize eight distinct regions on the target sequence was designed. The primer sets used in this study are listed in Table (1).

Table 1. LAMP primers based on the CYSDV p22 gene

F3	TTCTTCACCACTGAGACTTTC
B3	TTGTTGTAAGCCAAATTCGC
FIP (F1c+F2)	ACATCGGTACACACCCATCAAAAATGCAGAGTGTTGGAGTAG
BIP (B1c+B2)	CGTGGCTGACTTCTCGTTAGGTCGTCAATTCTTCCATTCGT
LoopF	CTCAACTCTCGTGGTTGGAA
LoopB	TTACCGTGCAACCTGAGG

Assay protocol for RT-LAMP amplification:-

The LAMP reactions were performed in a total 25 µl reaction volume containing 50 pmol each of the primers FIP and BIP, 5 pmol each of the outer primers F3 and B3, 25 pmol each of loop primers FLP and BLP in a 2X reaction mixture having 20mM Tris-HCl, pH 8.8, 10mM (NH_4) $2SO_4$, 8mM $MgSO_4$, 10mM KCl, 1.4 mMdNTPs, 0.8 M betaine, 0.1% Tween-20, 8 units of the *Bst* (*Bacillus stearothermophilus*) DNA polymerase (large fragment; New England Biolabs Inc., Beverly, MA, USA) and 2 µl of cDNA template. Infected samples and positive, as well as negative controls were included in each run , and precautions to prevent cross -contamination were observed . Reactions were performed at 60℃ for one hour.

Visual detection using naked eye or by fluorescence:-

In order to facilitate application of LAMP assay in the field, monitoring of amplification can also be carried out with naked eye inspection either in the form of visual turbidity or visual fluorescence. Concerning visual fluorescence, the tube containing the amplified products can also be better visualized in the presence of fluorescent intercalating dye viz; ethidium bromide, SYBR Green I, Calcein, etc. by illuminating with a UV lamp. In practice, usually the visual inspection for amplification is performed through observation of colour changing. When SYBR Green I dye was added 1µl at a 1,000x concentration (Invitrogen, USA) to 25 µl of the RT-LAMP products, its color will be changed from orange to green, while the product for a negative reaction remains orange.

Analysis of gel electrophoresis:-

Then the products of RT- LAMP were analyzed by gel electrophoresis. Ten µl aliquot of LAMP amplified products are electrophoresed on 1.5% agarose gel in 0.5% Tris-borate buffer followed by staining with ethidium bromide and visualization on a UV trans illuminator at 302 nm.

Results and discussion:-

Symptomatology of the virus:-

In Egypt, *Cucurbit yellow stunting disorder virus* (CYSDV) has been considered as a major threat and devastating for cucurbit crops either grown in the fields- or greenhouses. All cucurbit plant species exhibited interveinal chlorosis on the middle leaves, usually progressing extensive interveinal yellowing on older leaves and the veins remain green. In both of naturally infected and whiteflies inoculated plants, symptoms generally first appear 3–4-weeks after infection, and are most apparent on the older areas of the plant, but no symptoms near the apex (Figs.1-4). Such described symptoms are similar to symptoms induced by CYSDV-infection on cucurbits species as have been described worldwide (Abou-Jawdah *et al.*, 2000; Desbiez *et al.*, 2000; Louro *et al.*, 2000; Marco *et al.*, 2003; Yakoubi1 *et al.*, 2007 and Lecoq and Desbiez, 2012) and other crops-infecting criniviruses (Qiao *et al.*, 2011; Dong *et al.*, 2012 and Tzanetakis *et al.*, 2013).

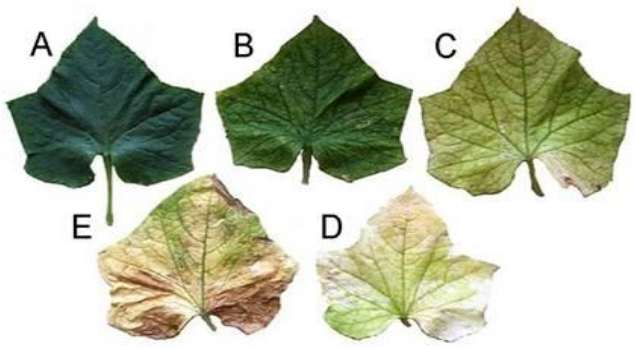


Fig.1. Typical symptoms of CYSDV on naturally infected cucumber plants compared with health plants. A. Healthy leaf. **B.** Interveinal chlorosis and green spots. **C.** Interveinal chlorosis develop prominent yellowing sectors. **D.** Complete yellowing of all the leaf laminas expect for the veins. **E.** large necrotic sectors expect the veins which remain green.

Fig. 2. CYSDV naturally infected squash plants exhibited symptoms of interveinal chlorosis and green veins.

Fig. 3. Whiteflies inoculated cucumber plants reacted with yellowing and green veins after 3-4 weeks.

Fig. 4. Whiteflies inoculated squash plants reacted with yellowing and green veins after 3-4 weeks.

Molecular detection of CYSDV:-
(a). Reverse Transcription-Polymerase Chain Reaction (RT- PCR):

In the present study, RT-PCR was used for the detection of the CYSDV by two different techniques as the following:
The first technique involved detection of the RNA2 common region of the coat protein (CP). Data obtained in Fig. (5-A) shows an RT-PCR amplified product size of 756 bp in the RNA extracted from CYSDV-infected tissues. Negative control includes RT-PCR on extracts from healthy plants. These results indicate that RT-PCR amplification of the complete CYSDV coat protein (CP) gene from viral RNA, using virus-specific CP oligonucleotide primers. Although, the juvenile leaf (near the apex) appear no symptoms it gave positive results. This result might be due to the high levels of hormones in the phloem cells, which reflect a defect of these cells in young leaves for the support of the replication or the movement of closteroviruses. Similarly, same results were obtained by Livierators *et al.* (1999); Rubio *et al.* (2001); Marco *et al.* (2003) and Lee *et al.* (2011). Conversely, Qin *et al.* (2013) in their studies on complete genome sequences of SPCSV showed that RNA1 lacks the p22 gene and contains others four ORFs, while RNA2 contains eight ORFs included coat protein region. Further, they mentioned that the isolates from China showed that SPCSV isolates display a high degree of sequence conservation similar to closely relate the genus *Crinivirus*.

The second technique was designed to detect p22 gene (The putative protein encoded by ORF 4; nt 8324 to 8902) which exist in genomic structure RNA1 with specific oligonucleotide primers *i.e.*, CYSDV Ma156f (5′ GAA GAA TTC CAG GCA AGG 3′) and CYSDV Ma129r (5′ TCA CAT CAT CAA TCC AAA AG 3′) corresponding for both ends of the CYSDV p22 gene. Electrophoresis of RNA extracted from infected squash samples revealed the

presence of CYSDV p22 gene with an apparent size of ~560bp in samples of RNA extracted from CYSDV-infected tissues and in the positive samples, while no results were observed in case of RNA extracted from healthy tissues (Fig. 5-B). The obtained results confirmed the efficacy of specific oligonucleotide primers used in detecting the expected size of p22 gene in CYSDV RNA1. This result is resembling those obtained by Aguilar *et al.* (2003) working on CYSDV and others related viruses *i.e.*, *Lettuce infectious yellows virus* (LIYV) and *sweet potato chlorotic stunt virus* (SPCSV), who revealed that the putative protein encoded (p22 gene; nt 8324 to 8902) is 192 amino acids and has a predicted molecular mass of 22.4 kDa. Also, p22 has a similar size to the proteins encoded from the most 3′-terminal genes of the RNA1 of SPCSV and LIYV, and the *Beet yellows virus* (BYV) genome. All these proteins share some sequence similarity which suggests that they could have some functional equivalence in the above mentioned related viruses. Thus, the approach of detection p22 gene aimed to use all the available primers in detecting another region in the genomic structure RNA1 to insure the presence of the virus in the infected tissues. As well as the comparison between RT-PCR in detecting the p22 gene and the following technique named as RT-LAMP in their time required and equipments, simplicity, specificity and sensitivity.

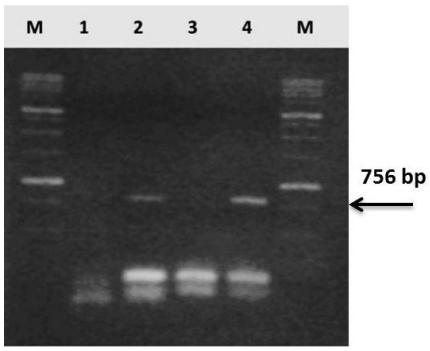
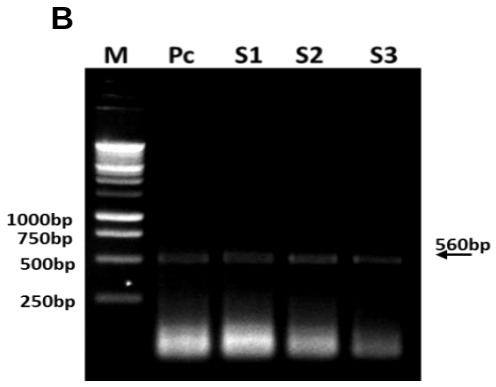


Fig.5. **A**) .Agarose gel electrophoresis of (RT-PCR) amplified products obtained from total RNA extracts of squash using CYSDV-specific primers Lanes 1- healthy squash, 2-positive samples of squash, 3- old infected leaves, 4-young infected leaves and Lanes M- marker (base pair) are shown on the left & right.



B) Agarose gel electrophoresis of (RT-PCR) products for the detection of CYSDV RNA in squash plants. Lane Pc positive sample. Lanes S1, S2, S3 of RNA extracted from CYSDV-infected squash plants. Lane, M marker (base pair) is shown on the left.

(b) Reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay:-

Primer design for RT- LAMP:-

We developed a new loop-mediated isothermal amplification (LAMP) method to detect p22 gene of CYSDV in one step. Total RNA extracted from each sample was added directly to the RT-LAMP reaction mix for LAMP. The total amplification time was limited to 60 min/60℃ in one step. After elapsed time of the reaction, the tubes were removed from the machine and examined immediately under daylight without spinning down the reaction mixture. SYBR Green I dye was added to the post-amplification mixture, and the positive samples changed to green color, while the negative control samples remained orange (Fig.6. A). The above mentioned result reveals that addition of SYBR Green I to the RT-LAMP amplified products may be judged according to the different colors under day light and add up to the advantage of its accuracy compared with RT-PCR. Moreover, immediately monitoring results of RT-LAMP amplified products confirm the simplicity of this technique compared with the complicated processing in RT-PCR.

On the other hand, when the amplified products were subjected to gel electrophoresis, all the observed patterns of RT-LAMP positive reactions were identical to the ladder, while pattern was not appeared in case of negative (healthy) samples (Fig.6. B). Result indicates that the designed primers in this investigation in the target virus RT-LAMP were able to amplify the target genes of CYSDV. The RT-LAMP assays could detect all of the samples shown to be positive by RT-PCR. Also, the ability of the six primers utilized in RT-LAMP to recognize all the six target regions (F1, F2, F3, B3, B2, and B1) confirms the specificity of RT-LAMP should be higher than that of the conventional RT-PCR method. Further, the specificity of RT-LAMP could also be evaluated by digestion of the

amplification products. Using the endonuclease site included in the target sequence of the RT-LAMP reaction, the specificity of amplification could be determined by the size of the fragments obtained by digestion with restriction enzymes.

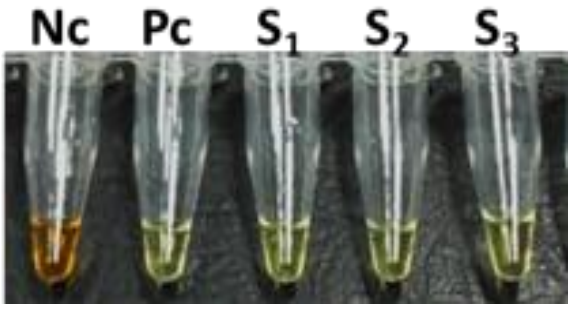


Fig.6. A) Visual detection of amplified LAMP products using SYBR green I. The color changes from orange (negative reaction) to green (positive reaction). NC-negative control, Pc-positive sample, S_1-CYSDV-infected cucumber, S_2- CYSDV-infected squash, S_3- viruliferous whitefly.

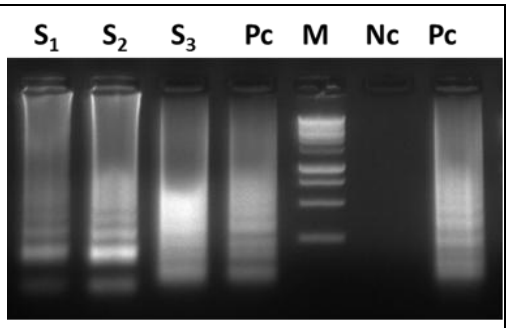


Fig.6. B) Agarose gel electrophoresis of CYSDV specific RT-LAMP assay products on a 1.5% agarose gel. Lanes S_1, S_2, S_3 RT-LAMP amplification products, lane Pc-positive RT-LAMP amplification product, lane M 5-100-bp DNA ladder (Sigma Genosys, Japan) lane- Nc, negative control without target RNA, lane Pc-positive RT-LAMP amplification.

In comparison between the two different molecular techniques (RT-PCR & RT-LAMP) used in the present study, the obtained results revealed although that RT-PCR assay is considered the most popular molecular diagnostic method, but RT-PCR has inherent disadvantages *i.e.* requires an expensive thermocycler, insufficient specificity and low amplification efficiency and is relatively time consuming because of its longer amplification time, as well as an additional visualization step on agarose gels involving the use of the mutagen ethidium bromide. In contrast, the newly developed technique (RT-LAMP) has several advantages such (i) rapidity, accuracy, specificity in amplification of the target gene is completed after approximately 60 min/60℃ and one step, (ii) high efficiency when using six primers to recognize six distinct regions on the template DNA, (iii) avoiding the need for thermocycler and agarose gel electrophoresis as well as the UV light which is irritating to the eyes, (v) Visual detection depending on colour changing and can be exclude the additional visualization step on agarose gels involving the use of the mutagen ethidium bromide. Due to the above mentioned advantages of RT-LAMP considered as a more reliable, alternative and can be adopted technique not only in plant viral diagnosis but also in the epidemiological studies.

The aforementioned results confirm those obtained by Nie (2005) and Almasi *et al.* (2013) who revealed that RT-LAMP proved to be a high sensitivity and specificity compared with other nucleic acid-based methods such as RT-PCR in detecting *Potato virus Y* and *Potato leaf roll virus*. Also, they stated that the rapidity of RT-LAMP comparing with other assays based upon PCR and DAS-ELISA requires 3h and 2 days, respectively. Furthermore, Silgo and Bhat (2014) in their works on *Banana bract mosaic virus* mentioned that the detection limit for RT-LAMP was up to 100 times that for conventional RT-PCR and on a par with that for RT-PCR. Also, they showed that the RT-LAMP is less expensive in terms of equipment required and more sensitive. Further, they found that it is possible to detect the products of RT-LAMP through direct observation by looking for turbidity or for green fluorescence gave it simplicity and rapidity than RT-PCR. All the abovementioned results confirming those obtained by Zhao, *et al*. (2012) working on tobacco viruses (*Cucumber mosaic virus* (CMV), *potato virus-Y* (PVY), *tobacco etch virus* (TEV), *tobacco mosaic virus* (TMV) and *tobacco vein banding mosaic virus* (TVBMV); Liu *et al.* (2010) *tobacco mosaic virus* (TMV); Zhou *et al.* (2012) working on *Southern rice black-streaked dwarf virus*; Kil, *et al.* (2015) working on *Tomato chlorosis virus* (ToCV) and Sasaya (2015) working on rice viruses. In addition to the detection of plant pathogenic agents, several investigators previously used RT-LAMP for rapid detection and typing of emerging human and animal diseases (Notomi *et al.*, 2000; Lu, *et al.*, 2012; Mahony, *et al.*, 2013; Neeraja, *et al.*, 2015).

In conclusion, RT-LAMP as an innovative technique proved to be reliable for virus diagnosis of CYSDV-infected samples from the field in 60 min., has the potential to replace PCR due to its simplicity, rapidity, specificity, sensitivity and cost-effectiveness without the need of specialized equipment. Also, it has potential for detection of CYSDV in the vectors, the management of viral diseases and the detection of plant pathogens in the imported plant materials (seeds, tubers and transplants).

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