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New sensitive and fast detection of *Little cherry virus 1* using loop-mediated isothermal amplification (RT-LAMP)

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ABSTRACT

Little cherry virus 1 (LChV-1) belongs to the genus *Velarivirus*, family *Closteroviridae*, is an economically important pathogen affecting mainly cherry around the world emphasizing the impetus for its efficient and accurate on-site detection. This study describes the development of a reliable diagnostic protocol of LChV-1 based on a one-step reverse-transcription loop-mediated isothermal amplification (RT-LAMP). The protocol detects LChV-1 isolates in less than 10 min by fluorescence monitoring using a mobile detection device and is most optimal when performed at 67 °C. Sharp melting curves and unique melting temperatures (Tm) were obtained for the positive samples. Both the RT-LAMP and classical RT-PCR methods are capable of specifically detecting LChV-1 in infected leaf tissues. In addition, the RT-LAMP has remarkable advantages in comparison to RT-PCR. It is at least hundred fold more sensitive, significantly faster (allowing on-field leaf-to-result diagnostic) and efficient at minimal cost. In conclusion, this innovative RT-LAMP approach can contribute to the implementation of sustainable integrated management strategies for detection of LChV-1 in commercial orchards or for horticultural research stations. It is also suitable for decision support in phytosanitary epidemiological programs.

1. Introduction

Little cherry disease (LChD) is an economically important viral disease associated with two distinct viruses, namely *Little cherry virus 1* (LChV-1) and *Little cherry virus 2* (LChV-2). LChD has a considerable impact on fruit yield and quality in commercial sweet (*Prunus avium* L.) and sour cherry (*Prunus cerasus* L.) production areas mostly located in the North American Pacific Northwest and in Europe (Jelkmann et al., 2008; USDA-NASS, 2017). *Little cherry virus 1* is a member of the newly described genus *Velarivirus* of the family *Closteroviridae* (Al Rwahnih et al., 2012). The second virus, LChV-2, is another member of the family *Closteroviridae* but belongs to the genus *Ampelovirus* (Jelkmann et al., 2008). The first reports of Little cherry disease occurred in 1933 in the Kootenay Valley of British Columbia in Canada, and in other parts of North America (Theilmann et al., 2002a,b). Nevertheless, both *Little cherry virus 1* and *-2* have only been identified as separate viruses in 1997 and 2001, respectively (Jelkmann et al., 1997; Rott and Jelkmann, 2001). Both viruses occur worldwide and are associated with massive yield losses on cherry (Keim-Konrad and Jelkmann, 1996; Katsiani et al., 2015). Several other *Prunus* species, including the ornamental flowering cherry (*P. serrulata* L.), can be infected - often latently and asymptomatically - by both viruses. In recent years, the reported incidence of LChD in sweet cherry orchards and other *Prunus* species has increased and indicates that LChV-1 is more widespread than LChV-2. LChV-1 transmission occurs through exchange and propagation of infected plant material or by direct grafting, although other mechanisms of transmission have been suggested. No insect vector has yet been identified for LChV-1 but LChV-2 is known to be transmitted in a semi-persistent mode by at least two species of mealybugs (Hemiptera, *Pseudococcidae*): the grape mealybug (*Pseudococcus maritimus* Ehrhorn) and the apple mealybug (*Phenacoccus aceris* Signoret). Typical symptoms of LChD are premature reddening or bronzing of leaves, development of small fruits, uneven ripening and tasteless fruits. Conspicuous leaf symptoms are often absent on most cherry cultivars grown in North America and Europe. LChV-1 has also been reported and characterized in other *Prunus* natural host species such as plum, peach and apricot without inducing any obvious symptoms revealing both its high intra-host and -species diversity (Matic et al., 2007, 2009; Katsiani et al., 2015; Tahzima et al., 2017; Safarova et al., 2017; Katsiani et al., 2018a).

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Table 1
List of organisms used for optimization of LChV-1 RT-LAMP detection from infected and healthy host plants (RNA and crude plant material), and non-target *Prunus* associated microbial organisms using the loop-mediated isothermal amplification (LAMP) and conventional polymerase chain reaction (RT-PCR) based on RdRp and CP genomic regions.

Virus Isolate and Organisms	Plant host	Geographical Origin	Date of Collection	Reference ID	RT-LAMP Detection	RdRp RT-PCR Detection[a]	CP RT-PCR Detection[b]
Velarivirus							
LChV-1 B2	*Prunus avium*	Belgium	05/2015	GBVC_LChV1_022	Positive	Positive	Positive
LChV-1 19	*Prunus avium*	Belgium	04/2016	GBVC_LChV1_086	Positive	Positive	Positive
LChV-1 21	*Prunus avium*	Belgium	06/2016	GBVC_LChV1_087	Positive	Positive	Positive
LChV-1 33	*Prunus avium*	Belgium	06/2016	GBVC_LChV1_088	Positive	Positive	Positive
LChV-1 44	*Prunus avium*	Belgium	09/2016	GBVC_LChV1_089	Positive	Positive	Positive
LChV-1 DCP435	*Prunus avium*	Belgium	09/2015	GBVC_LChV1_074	Positive	Positive	Positive
LChV-1 JD2	*Prunus avium*	Belgium	04/2017	GBVC_LChV1_062	Positive	Positive	Positive
LChV-1 TAK_B	*Prunus avium*	Belgium	03/2017	GBVC_LChV1_068	Positive	Positive	Positive
LChV-1 P1	*Prunus domestica*	Belgium	03/2017	GBVC_LChV1_029	Positive	Positive	Positive
LChV-1 No2ISTO	*Prunus avium*	Greece	2015	HG792414	Positive	Positive	Positive
LChV-1 GR_55-3	*Prunus avium*	Greece	2015	HG792407	Positive	Positive	Positive
LChV-1 GR	*Prunus domestica*	Greece	2015	HG792420	Positive	Positive	Positive
LChV-1 G15-3	*Prunus avium*	Greece	2015	LN794218	Positive*	Positive	Negative
LChV-1 JONSRT1	*Prunus avium*	Jordan	09/2017	GBVC_LChV1_103	Positive	Positive	Positive
LChV-1 JONSRT2	*Prunus domestica*	Jordan	09/2017	GBVC_LChV1_104	Positive	Positive	Positive
LChV-1 NZPHEL	*Prunus avium*	New Zealand	2017	GBVC_LChV1_105	Positive	Positive	Positive
LChV-1 40854	*Prunus avium*	Switzerland	02/2015	40854	Positive	Positive	Positive
LChV-1 40856	*Prunus avium*	Switzerland	02/2015	40856	Positive	Positive	Positive
LChV-1 V2356	*Prunus avium*	Germany (JKI)	03/2015	JX669615	Positive	Positive	Positive
Grapevine Leafroll-associated virus (GRLaV-7)	*Vitis vinifera*	Switzerland	02/2016	40855	Negative	Negative	Negative
Ampelovirus							
LChV-2 LC5	*Prunus avium*	Canada	2015	AF333237	Negative	Negative	Negative
LChV-2 TAK_B**	*Prunus avium*	Belgium	03/2017	GBVC_LChV1_068	Positive	Positive	Positive
Closterovirus							
Citrus tristeza virus (CTV)	*Citrus sp.*	Spain	–	GBVC CTV_04	Negative	Negative	Negative
Beat yellows virus (BYV)	*Beta vulgaris*	Germany	–	DSMZ PV0981	Negative	Negative	Negative
Barley yellow dwarf virus (BYDV)	*Hordeum vulgare*	Belgium	–	GBVC_BYDV_01	Negative	Negative	Negative
Capillovirus							
Cherry virus A (CVA)	*Prunus avium*	Belgium	2016	GBVC_CVA_01	Negative	Negative	Negative
Ilarvirus							
Prunus necrotic ringspot virus (PNRSV)	*Prunus cerasus*	Germany	–	GBVC PNRSV_01	Negative	Negative	Negative
Potyvirus							
Plum pox virus (PPV)	*Prunus domestica*	Germany	2014	GBVC_PPV_07	Negative	Negative	Negative
Nepovirus							
Tobacco ringspot virus (TRSV)	*Phaseolus vulgaris*	U.S.A	–	DSMZ PV0236	Negative	Negative	Negative
Tomato ringspot virus (ToRSV)	*Pelargonium sp*	Denmark	–	DSMZ PV0049	Negative	Negative	Negative
Cherry leafrol virus (CLRV)	*Vitis vinifera*	Germany	–	DSMZ PV0797	Negative	Negative	Negative
Bacteria							
Pseudomonas syringae	*Prunus avium*	Belgium	2014	GBBC 1987	Negative	Negative	Negative
Pseudomonas morsprunorum	*Prunus cerasus*	Belgium	2015	GBBC 3047	Negative	Negative	Negative
Agrobacterium tumefaciens	*Prunus sp.*	Belgium	–	LMG 167	Negative	Negative	Negative
Fungi							
Monilinia laxa	*Prunus domestica*	Netherlands	–	CBS 489.50	Negative	Negative	Negative
Monilinia fructigena	*Malus pumila*	Netherlands	1996	CBS 101502	Negative	Negative	Negative
Botrytis cinerea	*Malus sp.*	Belgium	2006	PCF 260	Negative	Negative	Negative
Cladosporium herbarium	*Solanum tuberosum*	Belgium	2017	–	Negative	Negative	Negative

References Material and Collections. GBVC and GBBC - ILVO Virus and Bacteria collections, respectively, Merelbeke, Belgium. JKI - Julius Kühn Institute collection, Neustadt, Germany. CBS – Fungal collection. Centraalbureau voor Schimmelcultures, Utrecht, The Nederlands. DSMZ – Leibniz Institute – German Collection of Microorganisms and Cell Cultures.

* This highly divergent isolate was detected only occasionally, and at a minimum dilution of 1/100.

** This isolate is mix- infected with LChV-1 and LChV-2.

[a] Bajet et al. (2008).

[b] Glasa et al. (2015).

As for other viruses and phytoplasma (*e.g.* Western X disease, developing similar leaf symptoms on cherry) infecting *Prunus* trees, the early and accurate detection of the causal viral pathogen is essential and required for on–field phytosanitary certification programs to prevent outbreaks and improve the disease control. Nevertheless, the lack of obvious symptoms of disease caused by LChV-1 hampers efficient diagnosis. Moreover, other evident reasons are making detection a challenging process: (i) in RT-PCR, plant extracts contaminants from *Prunus* frequently affect RNA quality and inhibition of subsequent tests (Candresse et al., 1998); (ii) in all these tests, high variability in virus concentrations interferes with the analytical sensitivity of the test, making LChV-1 detection fastidious (Korschineck et al., 1991; Demeke and Adams, 1992; Nassuth et al., 2000; Li et al., 2013); (iii) in molecular tests, inclusiveness is hindered by the relatively high level of

variability between isolates of LChV-1. A fast, cheap and reliable detection technique for LChV-1 is therefore essential. Readily published detection methods of LChV include enzyme-linked immunosorbent assay (ELISA), reverse transcription recombinase polymerase amplification (RT-RPA), RT polymerase chain reaction (RT-PCR), and real-time RT-PCR (qRT-PCR) (Eastwell et al., 1996; Vitushkina et al., 1997; Eastwell and Bernardy, 2001; Theilmann et al., 2002a, b; Mekuria et al., 2014; Glasa et al., 2015; Katsiani et al., 2015, 2018b).

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a detection method that amplifies nucleic acids under isothermal conditions (Notomi et al., 2000). It is cost effective and user-friendly and can be performed on-site to detect pathogens in plant health field surveys (Boonham et al., 2008; Harper et al., 2010; Tomlinson et al., 2010; Lu et al., 2015; Przewodowska et al., 2015). RT-LAMP is robust and highly efficient, its specificity being modulated by four primers with two additional optional loop primers to speed up amplification (Nagamine et al., 2002; Iseki et al., 2007; He and Xu, 2011). The final products from RT-LAMP have stem-loop structures of various sizes that can either be visualized as a ladder pattern of bands on a DNA agarose gel or can be detected in vitro after SYBR Green I or SYTO™9 staining and visualized under UV, visible light or fluorescence measurements (Francois et al., 2011; Shen et al., 2014; Notomi et al., 2015). Several LAMP and RT-LAMP assays have previously been developed to detect plant viruses belonging to *Comovirus*, *Potyvirus*, *Geminivirus*, *Ilarvirus*, *Tospovirus*, *Crinivirus* and *Closterovirus* as well as viroids (Candresse et al., 1998; Fukuta et al., 2003a,b, 2013; Boubourakas et al., 2009; Zhao et al., 2010; Zhang et al., 2011; Wei et al., 2012; Lenarčič et al., 2013; Walsh and Pietersen, 2013; Wang et al., 2014; Przewodowska et al., 2015; Okuda et al., 2015; Silva et al., 2015; Budziszewska et al., 2016; Warghane et al., 2017).

Although still considered as golden standard, RT-PCR detection methods have some intrinsic drawbacks and disadvantages, namely the fact that all existing protocols are inappropriate for on-site testing as they all require expensive equipment, sophisticated laboratory setup and highly skilled laboratory staff. Hence, this publication describes the development and application of a new cost-effective one-step RT-LAMP procedure for on-site, rapid, sensitive and specific LChV-1 detection.

2. Materials and methods

2.1. RNA extraction, plant materials and virus isolates

During the growing seasons of 2014–2016, an intensive survey was conducted across Belgium to monitor the incidence and spread of *Little cherry virus 1* and *2* in sweet and sour cherry (*Prunus avium* L.), flowering cherry (*Prunus serrulata* L.) and plum (*Prunus domestica* L.) trees (Tahzima et al., 2017). Leaves originating from various symptomatic and asymptomatic host plants were collected in at least five different orchards where LChV-1 was prevalent and processed in two different ways. First, total RNA was isolated from cambial scrapings of midrib leaf samples using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Belgium) according to the manufacturer's instructions. The RNA quality and quantity were assessed using a Nanodrop ND-1000 spectrophotometer (ThermoFisher Scientific, Waltham, USA). Additionally, total RNA from virus-free (virus tested) healthy greenhouse plants was used as negative control. The final concentration of total RNA was adjusted using a Nanodrop to approximately 50 ng μl^{-1} with RNase-free water. Second, crude leaf extracts were prepared by cutting out 1 cm of leaf midrib from LChV-1 infected trees exhibiting either obvious or no symptoms. These samples were immediately mechanically bead-disrupted in OptiGene lysis buffer (OptiGene Ltd, Horsham, UK) according to the manufacturer's instructions and subsequently tenfold serially diluted in nuclease-free Milli-Q® water (Merck Millipore, Massachusetts, USA). The RNA and crude leaf extracts were stored at -70 °C. The LChV-1 isolates and the other plant pathogens of *Prunus* spp. trees used in the validation experiments are detailed in Table 1.

2.2. RT-PCR based LChV-1 detection

To detect LChV-1 from asymptomatic and symptomatic plant material, conventional RT-PCR was performed according to the conditions described previously. Briefly, cDNA was prepared from tenfold diluted total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Temse, Belgium) according to the manufacturer's instructions. Amplification was carried out using primer pair LCUW7090 (forward, 5′-GGTTGTC CTCGGTTGATTAC-3′) and LCUWc7389 (reverse, 5′-GGCTTGGTTCCA TACATCTC-3′) (Bajet et al., 2008), targeting a 300-bp fragment of the RNA-dependent polymerase (RdRp) gene. A 456-bp fragment of the coat protein (CP) gene was amplified with the primer set 1LC_12776 F (5′-TCAAGAAAAGTTCTGGTGTGC-3′) and 1LC_13223R (5′-CGAGCTAG ACGTATCAGTATC-3′) (Glasa et al., 2015). The PCR reactions were carried out in a total volume of 25 µl of PCR mixture containing 10 µM primers, 2.5 µl 10x FastStart™ Taq DNA Polymerase reaction mix, 0.2 µl of FastStart Taq DNA polymerase (5U μl^{-1}) (Roche, Mannheim, Germany), 2 µl of 10x diluted cDNA and nuclease-free Milli-Q water in a ABI9700 GeneAmp Thermal Cycler (Applied Biosystems, Foster City, CA, USA) under the following PCR thermal conditions for the RdRp PCR: 4 min at 94 °C for initial denaturation; 40 cycles of 30 s at 94 °C, 30 s at 48 °C, 1 min at 72 °C; 5 min final extension at 72 °C. For the CP gene, reaction times were as follow; 4 min at 94 °C for initial denaturation and 40 cycles of 30 s at 94 °C, 30 s at 51.5 °C, 1 min at 72 °C; and 5 min at 72 °C for final extension. Target-specific amplification was visualized by high-resolution capillary electrophoresis with a Qiaxcel Advanced system (Qiagen, Antwerp, Belgium) according to the manufacturer's instructions or by agarose gel electrophoresis using 2% agarose gel in 0.5 x TAE-buffer, stained with 0.06 µl ml^{-1} Midori Green Advanced Stain (Nippon Genetics Europe, Düren, Germany) and a fluorescence camera under EPI Blue light (470 nm) following the manufacturer's instructions (Azure Biosystems Inc., Dublin, CA, USA).

2.3. Design and optimization of RT-LAMP reaction

The LChV-1 conserved genomic region ORF1b, coding for RdRp gene composing the replication polyprotein, was selected as amplification target for primer design. The nucleotide sequence, which spanned a 300-bp fragment of the RdRp gene region from geographically distinct LChV-1 isolates and from closely related *Closteroviridae* species, was downloaded from GenBank (NCBI accession numbers JX669615, EU049867, EU049868, EU049869, EU049871, EU04987, EU049873, EU049874, FR748229, HG792393, HG792394, HG792396, HG792398, HG792399, HG792418, HG792419, HG792420, KM921658 and Y10237). These sequences were aligned to identify conserved LChV-1 genomic sub-regions using MEGA 6 software (Tamura et al., 2011). From this alignment, a consensus sequence was determined and the most conserved and LChV-1-specific genomic regions were used for primer design (Primer Explorer V5 software (https://primerexplorer.jp/lampv5/index.html, Eiken Chemical Co, Ltd, Tokyo, Japan). The designed primers were also submitted to BLASTn analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) as additional *in-silico* quality control. Primers were synthesized at Sigma-Aldrich (Overijse, Belgium). The primer sequences and positions of the RT-LAMP primers are shown in Table 2.

All final RT-LAMP assay reactions were performed in a single tube containing a total volume of 25 µl using a Genie® II thermocycler (OptiGene Ltd, Horsham, UK). The reaction mixture consisted of 1.6 µM of primers LChV1_FIP and LChV1_BIP, 0.2 µM of primers LChV1_F3 and LChV1_B3 and 0.6 µM of primers LChV1_LF and LChV1_LB, 15.8 µl of Isothermal Mastermix ISO-004 (OptiGene Ltd, Horsham, UK). Three microliters of 10x diluted target template and nuclease-free Milli-Q water were added. To investigate the optimal isothermal conditions, the RT-LAMP reaction containing all primers was performed at 60 °C, 63 °C, 65 °C, 66 °C, 67 °C and 68 °C with total RNA and leaf crude extracts as template in a Genie® II instrument (OptiGene Ltd, Horsham, UK). In

Table 2
Oligonucleotide specific primer sequences used for RT-LAMP reaction (Nucleotide position refers to the nucleotide sequence of LChV-1 based on GenBank® accession JX669615).

Primer ID	D escription	Length	Sequence (5′–3′)	5’	3’
LChV1_ F3	External forward primer	19 nt	CGAGTTACCTTGCTTGGAA	42	60
LChV1_B3	External backward primer	20 nt	TAGACGAAACTGCTAACAGG	230	249
LChV1_FIP	Forward inner primer (FIP1_RC + FIP2)	47 mer	TCCTTATAGGTAAAGGTGAACGCTTTTGATCTGCTTTTAACCCCGA	–	–
LChV1_BIP	Backward inner primer (FIB1_RC + FIB2)	50 mer	GCAGACCAGATACTTGGAAACAAGTTTTAGACTCAATGAAGATCTTTCG	–	–
LChV1_LF	Forward loop primer	25 nt	AGGTATCTTACAATTAACGGCCTGA	61	79
LChV1_LB	Backward loop primer	24 nt	ATCTAAACGTAACTTTGCTGCACC	208	229

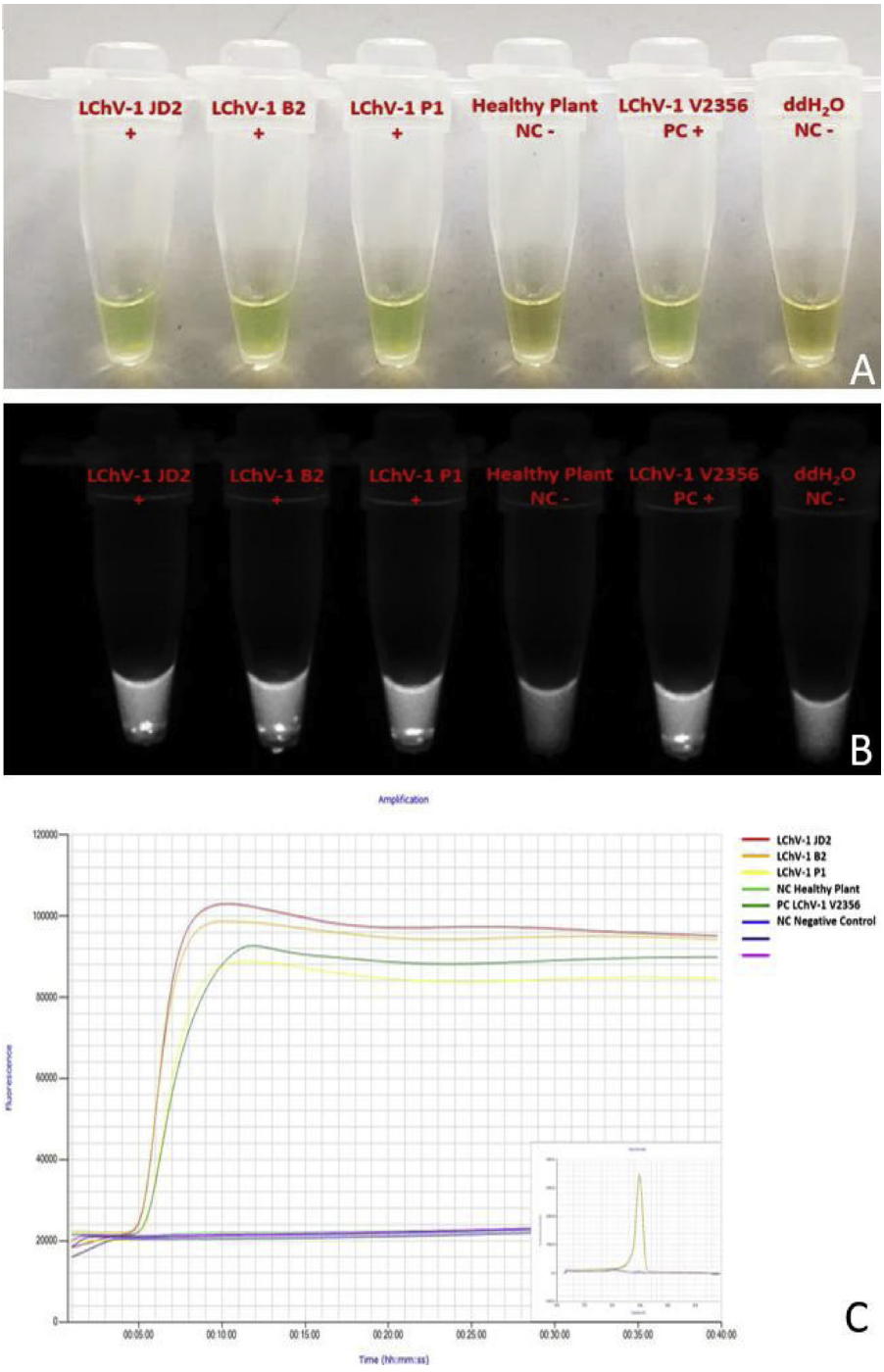

Fig. 1. LChV-1 Loop-mediated isothermal amplification reactions. SYTO™9 staining dye showing the light-green color change observed with LChV-1-positive samples under white light **(A)** and under fluorescent light **(B)**. Ampliplot of LAMP assay showing amplification and Tm results for the same isolates **(C)**. PC = Positive control. NC = Negative controls in which no amplification occurred show a dark brown color (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

addition, 0.2 µl of AMV (100U) RT-001 (OptiGene Ltd, Horsham, UK) was added according to the manufacturer's instructions to accelerate the RT reaction and improve the sensitivity. Colorimetric reactions were also performed with addition in the mastermix of 0.05 μl^{-1} per reaction SYTO™9 green fluorescent dye following the manufacturer's instructions (Invitrogen, Thermofisher Scientific, Merelbeke, Belgium) for direct visualization in the reaction tubes. Positive samples were immediately identifiable due to their bright olive green color. All RT-LAMP reactions were carried out under following conditions: 3 min preheat at 45 °C, 40 min by 67 °C, and finally an annealing run from 98 °C to 70 °C at a ramp rate of 0.05 °C s^{-1}.

2.4. Analytical specificity of the RT-LAMP reaction

The analytical specificity of the LAMP reaction was evaluated using 50 µg μl^{-1} total RNA or DNA of other cherry infecting non-target organisms extracted from infected plants or pure cultures, respectively (Table 1). Total RNA or DNA extracted from those viruses and *Prunus* associated organisms was used as a template in the RT-LAMP and RdRp based RT-PCR assays with their respective LChV-1-specific primer set (Bajet et al., 2008 and Glasa et al., 2015). Total RNA from healthy plants and nuclease-free Milli-Q water were used as negative (matrix) control and technical control, respectively. The analytical specificity of the RT-LAMP of both detection protocols was tested three times independently and assessed on cherry leaf samples collected on field-grown cherry and plum trees from different origins (Table 1).

2.5. Analytical sensitivity of the RT-LAMP reaction

The analytical sensitivity of the RT-LAMP assay was evaluated using total plant RNA and crude leaf extracts. The analytical sensitivity was compared to the analytical sensitivity of the RT-PCR protocol described above using Genie® II (OptiGene Ltd, Horsham, UK) and ABI9700 GeneAmp Thermal Cycler (Applied Biosystems, Foster City, CA, USA), respectively. To compare the sensitivity of both protocols, serial tenfold dilutions of extracted RNA were amplified three times independently. Total RNA from healthy plants and nuclease-free Milli-Q water were used as negative (matrix) control and technical control, respectively. The amplified RT-LAMP products and RT-PCR products were loaded and visualized as described previously.

3. Results

3.1. Extraction of RNA and validation of infection status by RT-PCR

Two readily published diagnostic test confirmed the presence of all LChV-1 isolates used in our study. This detection was achieved from RNA extracts using conventional RT-PCR (Bajet et al., 2008 and Glasa et al., 2015, see Table 1).

3.2. Set up of the RT-LAMP assay

The initial evaluation of this assay was carried out on a selection of representative LChV-1 samples using the described RT-LAMP primer set with loop primers (Table 2) from extracted total RNA or crude leaf extract. This fast and simple extraction from leaves represents a major advantage of our RT-LAMP assay reducing the risks of carry-over contamination. The optimal incubation temperature of the RT-LAMP reaction was 67 °C (Fig. 1A and B). Furthermore, the typical ladder-like pattern of a RT-LAMP reaction for LChV-1 could be observed (results not shown). All subsequent RT-LAMP validation reactions were therefore performed at 67 °C A positive fluorescence signal was obtained within approximately 6–20 min (Fig. 1C and A). After addition of 0.5 U μl^{-1} of AMV-Reverse Transcriptase, significant improvement of the time to signal was observed (Fig. 2A and B). Therefore, all subsequent RT-LAMP assays were performed with AMV-Reverse Transcriptase. The speed of our assay was accelerated by using six primers, making this assay one of the fastest plant virus RT-LAMP assays reported so far. Our results, based on amplification plots of serially diluted LChV-1 infected samples, revealed that the target RdRp gene of most LChV isolates can be rapidly and effectively amplified after the first 5 min (Fig. 2B–D and Table 1).

3.3. Evaluation of sensitivity

The pathogen (LChV-1) was not robustly detected in undiluted RNA of selected samples (Fig. 2B and C), but was reliably detected for dilutions 10^{-1} to 10^{-6}. The comparison of this RT-LAMP assay with available RdRp and CP-RT-PCR tests has been performed on a selection of 3 different isolates from different origins (Suppl. material S1). The limit of detection was 10 to 1000 times lower, compared to the RT-PCR methods of Glasa et al. (2015) and Bajet et al. (2008), respectively. The RT-PCR of Glasa et al. (2015), however, is targeting the more variable CP region which may explain the limit of detection differences, whereas both LAMP method and the Bajet et al. (2008) RT-PCR are targeting the RdRp region. Direct detection on crude leaf extracts of the same samples gave the same reliable results for the 10^{-1} to 10^{-4} dilutions in the RT-LAMP procedure (Fig. 2C), whereas the detection limit of the compared conventional one-step RT-PCR was approximately 10^{-3}, or 100 times less. All negative matrix and no-template controls never showed a positive signal. These data suggest that the detection limit of this assay is at least comparable to the classical LChV-1 RT-PCR. When using diluted crude leaf extract, the new RT-LAMP assay remains more sensitive than RT-PCR. This significant improvement makes this LChV-1 RT-LAMP test ideal for use as a first-line screening assay because LChV-1 infected plants usually contain low concentrations of LChV-1, sometimes below the RT-PCR detection limit. Furthermore, an additional feature of our developed assay is that while the RT-PCR did not always detect LChV-1 due to certain factors such as seasonal variations, mainly during end of spring and summer, as already reported (Katsiani et al., 2018b) and the woody plant matrix (*Prunus* sp.), these shortcomings were not experienced in our RT-LAMP assay. The new assay allowed sensitive robust detection within different *Prunus* matrixes and at different times in the growing season (Table 1).

3.4. Validation of specificity

Our *in silico* BLASTn analysis of the synthetized RT-LAMP primers confirmed the lack of homology with sequences from other members of the family *Closteroviridae*. The RT-LAMP primers sequences designed in this study were also compared with the corresponding genomic region of a large collection of representative LChV-1 isolates from all known phylogenetic groups (Suppl. material S2). Owing to the high degree of genetic variability of LChV-1, all published primers currently used exhibit mismatches with a few LChV-1 isolates, as reported in other studies (Katsiani et al., 2018b). Our LChV-1 RT-LAMP primers, although representing the best-fit comprise, likewise displayed some occasional mismatches with highly divergent isolates (*i.e.* G15.3, UW2), yet without hindering reliable specific and accurate detection of a wider range of isolates. Taking into consideration the *in silico* predictions at the time of assay design and our optimized amplification conditions to circumvent false negative amplification, these primers are expected to enable consistent detection polyvalence of most LChV-1 isolates from different geographic origin and host plants (*i.e.* G55.3, No2ISTO, V2356, GR, LChV-1.073) as shown in Table 1.

To assess the specificity of LChV-1 RT-LAMP assay, the optimized procedure was used on a spectrum of *Prunus* associated organisms including non-European LChV-1 isolates, LChV-2 and other *Closteroviridae*, and additional stone fruit viruses as well as bacteria and fungi. When using the Genie® II instrument, the RT-LAMP amplification plot displayed a unique melting curve and specific corresponding melting temperature (Tm) for LChV-1 isolates (Fig. 2D and Table 3),

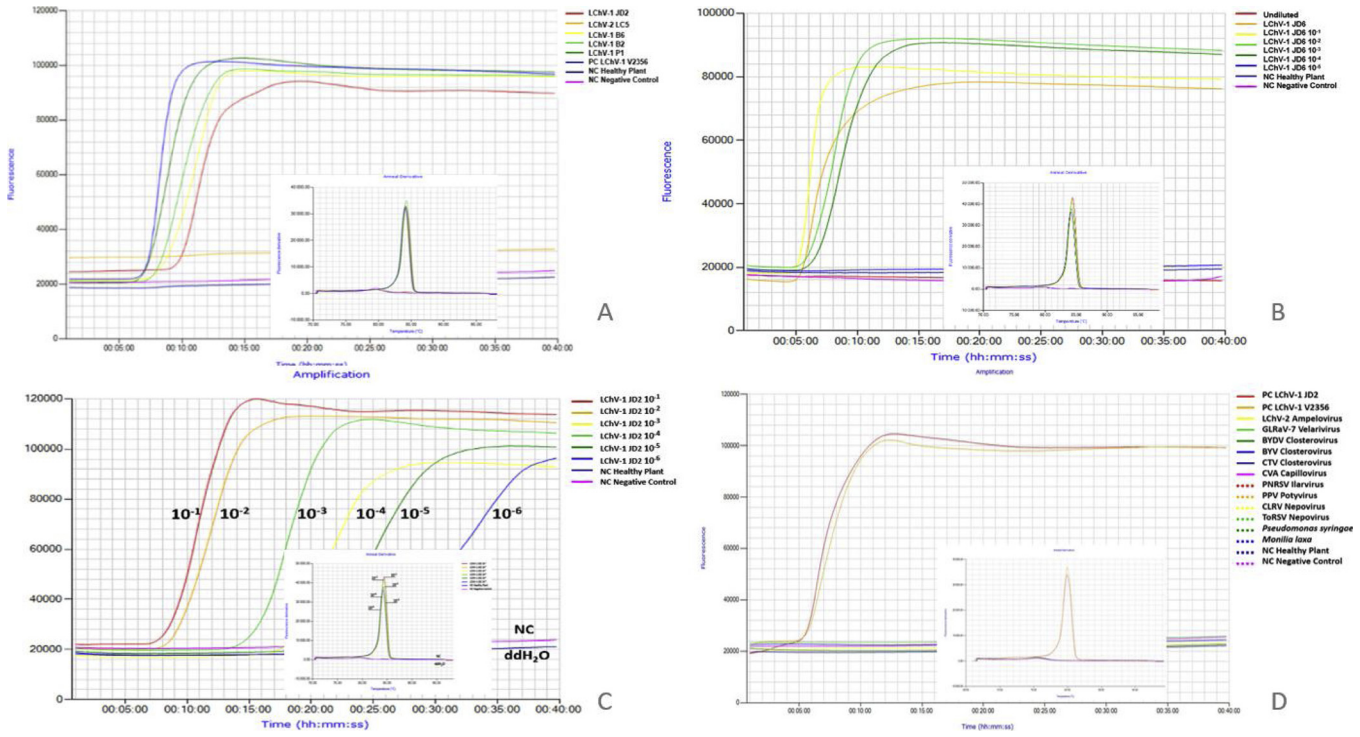

Fig. 2. LChV-1 Loop-mediated isothermal amplification of 5 LChV-1 isolates and 1 LChV-2 (LC5) RNA samples without AMV. PC = LChV-1 V2356. NC = Healthy *P. avium* RNA extract and Milli-Q H_2O **(A)**. LChV-1 loop-mediated isothermal amplification of serially diluted LChV-1 (JD6) infected leaf crude extract. NC = Healthy *P. avium* Leaf extract and Milli-Q H_2O **(B)**. Sensitivity – Amplification plot of LChV-1 loop-mediated isothermal amplification of serially diluted LChV-1 (JD2) RNA. Inner Plot: Sensitivity – Melting temperature (Tm) plot of LChV-1 loop-mediated isothermal amplification of serially diluted LChV-1 (JD2) RNA. NC = Healthy P. avium RNA extract and Milli-Q H2O **(C)**. Specificity – Results of LChV-1 loop-mediated isothermal amplification of LChV-1 and non-LChV-1 *Prunus* associated organisms. PC = LChV-1 V2356. NC = healthy *P. avium* RNA extract and Milli-Q H_2O **(D)**.

allowing the assessment of the high specificity of the amplification product. All LChV-1 isolates from purified RNA and from crude leaf extracts from diverse LChV-1 infected host plants were detected in less than 10 min, whereas all non LChV-1 RNA or DNA samples, including the negative controls, were not detected. This result indicated that the set of sequence-specific primers were specific for detecting LChV-1 under the fixed conditions thereby reinforcing their 100% inclusiveness for the tested isolates. There was no evidence of false amplification in the RT-LAMP assay for all samples considered negative by RT-PCR. The RT-PCR of LChV-1 virus-infected samples showed specific bands, whereas the negative controls showed none. No amplification products were observed for RNA or DNA isolated from non-target viruses or other organisms (results not shown).

3.5. On-site use of LChV-1 RT-LAMP

The specific detection of LChV-1 in field samples showed that our on-site RT-LAMP protocol was successful. The robustness and speed of our LChV-1 RT-LAMP test, together with its lower susceptibility to inhibition, even for crude extracts, constitute valuable advantages for the *in-situ* use of our assay. This prompted us to assess whether we could evaluate the LChV-1 RT-LAMP results by means of a simple colorimetric assay. After adding SYTO™9, the RT-LAMP products could be immediately detected and visually evaluated. These were even more prominent when visualized under ultraviolet light (UV). The detection stability of our assay was not affected when assessed using tenfold dilutions of RNA or crude leaf extracts (Fig. 1A). No false LChV-1 RT-

Table 3
Comparison of detection using specific LChV-1 RT-LAMP and RT-PCR. Melting temperature (Tm) values are means of at least three replicates (with their respective standard deviations).

Virus Isolate	Type of Sample		ABI9700 PCR[a]	GENIE® II LAMP	
			Gel Electrophoresis	Time	Tm
LChV-1 JD2	Symptomatic	Leaf	Positive	06 min 41s ± 0.22	83.72 °C ± 0.25
LChV-1 IWT21	Symptomatic	RNA	Positive	07 min 16s ± 0.04	83.79 °C ± 0.23
LChV-1 DCP435	Symptomatic	RNA	Positive	06 min 12s ± 0.06	83.94 °C ± 0.11
LChV-1 No2ISTO	Symptomatic	RNA	Positive	08 min 36s ± 0.003	83.70 °C ± 0.007
LChV-1 No2ISTO	Symptomatic	Leaf	Positive	12 min 35s ± 0.06	83.16 °C ± 0.23
LChV-1 JONSRT1	Asymptomatic	RNA	Positive	08 min 30s ± 0.001	83.71 °C ± 0.14
LChV-1 NZPHEL	Symptomatic	Leaf	Positive	07 min 40s ± 0.01	83.11 °C ± 0.05
LChV-1 JD6	Asymptomatic	RNA	Positive	06 min 29s ± 0.006	83.85 °C ± 0.13
LChV-1 JD6	Asymptomatic	Leaf	Positive	05 min 55s ± 0.09	83.37 °C ± 0.35
LChV-1 P1	Asymptomatic	Leaf	Positive	10 min 22s ± 0.02	83.42 °C ± 0.28
Prunus avium	Healthy Plant	RNA/Leaf	Negative	Not detected	Not detected
Prunus domestica	Healthy Plant	RNA/Leaf	Negative	Not detected	Not detected

[a] Bajet et al. (2008).

LAMP amplification was noted in non LChV-1 infected samples, or samples containing non-target organisms, allowing our assay to be interpreted visually and in real-time, without any specific equipment and in complete concordance with the other assays. The robustness and repeatability of the LChV-1 RT-LAMP method were assessed with diverse isolates and plant tissue samples from different geographic regions. All isolates where positive based on amplification and gel electrophoresis. It is suggested that, despite variations probably due to known factors caused by stochastic distributions of nucleic acids, RNA lability due to storage (Ellison et al., 2006), the implemented RT-LAMP assay has good repeatability and stability.

4. Discussion

Plant health organizations are being urged to develop and implement rapid and robust on-field pathogen detection tools, in order to reduce the time needed for plant testing as well as the costly consequences of possible delay during certification and shipment of horticultural materials. In addition, *in-situ* fast detection methods are an important focus for the deployment of control measures, especially for quarantine pathogens (Boonham and the PORT CHECK consortium, 2007). RT-LAMP assays have been used for the molecular detection and diagnosis of viral diseases and other plant pathogens, including bacteria and fungi (Okuda et al., 2005; Tomlinson et al., 2010). The RT-LAMP method has the advantages of high specificity and fast amplification efficiency yielding accurate results from limited starting material and without misdiagnosis and expensive infrastructure (Notomi et al., 2000; Mori et al., 2001; Nagamine et al., 2002; Fukuta et al., 2003a,b, Boonham et al., 2008; Tomita et al., 2008; Park et al., 2013). The stakeholders' needs and the recent LChV-1 outbreaks (Glasa et al., 2015; Wang et al., 2014; Tahzima et al., 2017; Safarova et al., 2017; Fiore et al., 2018) underline the importance of striving to timely on-field diagnosis of this pathogen. In this respect, our highly sensitive RdRp-based RT-LAMP assay provides immediate stable visual detection of LChV-1 within 6 min on average, as compared to the 90–180 min required by most popular LChV-1 RT-PCR assays performed under stringent thermal cycling conditions following RNA extraction in the laboratory (Bajet et al., 2008; Glasa et al., 2015; Katsiani et al., 2015, 2018a,b). This emphasizes the very short detection time as an important feature of the RT-LAMP method. LChV detection by RT-PCR requires RNA extraction, which makes it sensitive to performance under field conditions. Nevertheless, our straightforward procedure is still significantly faster (< 15 min) than standard RT-PCR (180 min) or even real-time RT-PCR (40 min). These findings regarding sensitivity are also in correspondence with other previous plant virus and viroids RT-LAMP methods (Nie, 2005; Varga and James, 2006; Park et al., 2013; Shen et al., 2014; Silva et al., 2015; Budziszewska et al., 2016; Zhao et al., 2016).

A likely constrain for this detection method is that, in general, the RT-LAMP reaction can be contaminated due to aerosolization; however, this can be easily overcome using simple pre- and post-LAMP amplification axenic measures. The proposed optimized easy-to-use RT-LAMP procedure enables robust diagnostics, based on a direct single reaction tube assay with crude matrix extract, on a wide and representative spectrum of LChV-1 isolates, therefore achieving improved sensibility and specificity compared to numerous RT-PCR protocols, and in correspondence to previous observations of other plant viruses RT-LAMP assays (Shen et al., 2014; Fan et al., 2015). This represents a major advantage as it simplifies the procedure and significantly minimizes the risks of carry-over contamination, one of the main hazards when performing on-field testing (Lenarčič et al., 2013; Lu et al., 2015).

In conclusion, LChV-1 currently represents a serious threat and exerts a worldwide economic impact to cherry production and its related species worldwide. Development of this new accurate and sensitive RT-LAMP assay represents a rapid and cost-effective, simple and efficient alternative tool to robustly screen LChV-1 infection for diagnosing propagation material and trees in commercial orchards by optimizing the resources needed for quarantine diagnostics. This is the first publication describing the development and implementation of a leaf-to-result RT-LAMP assay that reliably detects LChV-1. It can be extended as a key prospective measure for predicting possible LChD outbreaks by generating molecular epidemiological information and is also decisive to support detection of LChV-1 in potential emerging vectors. This method is therefore suitable for both commercial horticulture and research facilities to speed up diagnostic and increase throughput for complementary use in integrated pest management programs targeting the little cherry disease.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2018.12.019.

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