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LAMP assay and rapid sample preparation method for on-site detection of flavescence dorée phytoplasma in grapevine

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In Europe the most devastating phytoplasma associated with grapevine yellows (GY) diseases is a quarantine pest, flavescence dorée (FDp), from the 16SrV taxonomic group. The on-site detection of FDp with an affordable device would contribute to faster and more efficient decisions on the control measures for FDp. Therefore, a real-time isothermal LAMP assay for detection of FDp was validated according to the EPPO standards and MIQE guidelines. The LAMP assay was shown to be specific and extremely sensitive, because it detected FDp in all leaf samples that were determined to be FDp infected using quantitative real-time PCR. The whole procedure of sample preparation and testing was designed and optimized for on-site detection and can be completed in one hour. The homogenization procedure of the grapevine samples (leaf vein, flower or berry) was optimized to allow direct testing of crude homogenates with the LAMP assay, without the need for DNA extraction, and was shown to be extremely sensitive.

Keywords: flavescence dorée, homogenization, loop-mediated isothermal amplification, on-site application, validation

Introduction

Several taxonomically unrelated phytoplasmas from at least 10 ribosomal subgroups cause grapevine yellows diseases (GY) with nearly identical symptoms (Constable *et al.*, 2003). Flavescence dorée (FD) is the most severe of the GYs and is currently widespread in many vine-growing regions of France and Italy, with outbreaks in Slovenia, Portugal and Serbia and a few recorded occurrences in Spain, Switzerland and Austria (EPPO, 2014). The causal agent of FD is a phytoplasma (FDp), which, based on 16S rDNA sequence similarities, belongs to 16SrV subgroups C and D (Lee *et al.*, 2004). FDp is listed in the EU2000/29 Council Directive on Harmful Organisms and the EPPO A2 quarantine list of pests, and the destruction of diseased stocks, plants with symptoms and surrounding plants, as well as the control of its vector *Scaphoideus titanus*, is mandatory. Therefore, a method for rapid detection of FDp is urgently needed to speed up decision-making and limit the spread of the pathogen either in plants moving in trade or in the field.

The detection of phytoplasmas is difficult due to their uneven distribution within the host and low titre, which can be affected by the season. However, it was recently demonstrated that before symptoms develop in certain grapevine tissues, namely leaf midribs and flowers, the FDp concentration may be high enough for its detection using a suitable technique (Prezelj *et al.*, 2012). Additional problems associated with FDp detection include a laborious DNA extraction procedure (Fig. 1). Currently, the most accurate and reliable detection of FDp is based on various molecular approaches. PCR-based methods, including nested and multiplex PCR that amplify either ribosomal or non-ribosomal phytoplasma DNA (Daire *et al.*, 1997; Clair *et al.*, 2003), RFLP methods using different restriction enzymes on those PCR products (Lee *et al.*, 1998; Angelini *et al.*, 2001; Marzachi *et al.*, 2001; Martini *et al.*, 2002) or sequencing, have been developed for distinguishing the subgroups of FDp. More recently, quantitative real-time PCR (qPCR)-based assays have been developed for FDp -specific detection (Bianco *et al.*, 2004; Hren *et al.*, 2007; Mehle *et al.*, 2013a). Although the sensitivity and specificity of these diagnostic assays are sufficiently high when they are properly applied, the procedures are time-consuming, require expensive laboratory equipment and cannot be performed in the field because of the lack of convenient portable instruments.

Recently, a loop-mediated isothermal amplification (LAMP) method (Notomi *et al.*, 2000) has been developed. It circumvents the real-time PCR sensitivity to inhibitors (Francois *et al.*, 2011) present in plant extracts (Boonham *et al.*, 2004) and its isothermal nature provides it with the potential to be deployed in the field (Tomlinson *et al.*, 2010b). Because of its speed, robustness and simplicity, the use of LAMP is gaining popular-

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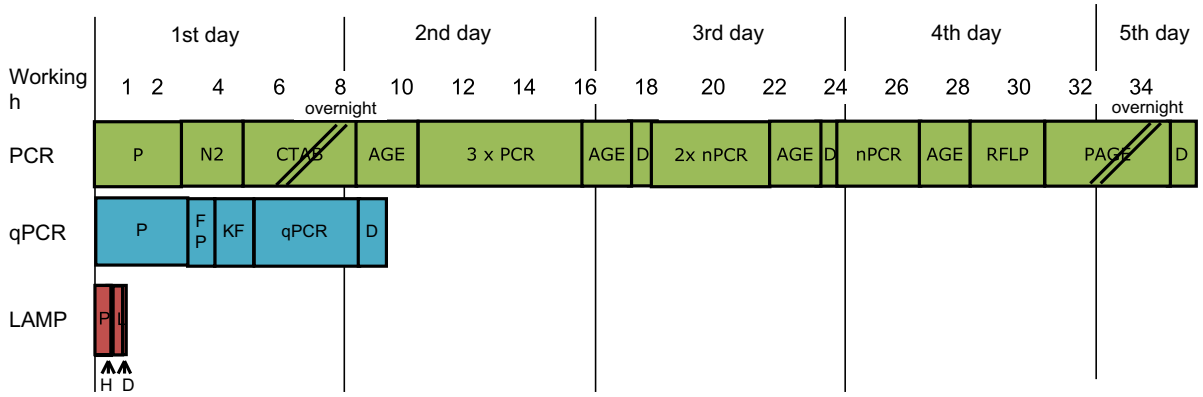

Figure 1 Comparison of time needed for FDp detection with different methods. P, sample preparation; FP, fast prep homogenization; KF, KingFisher DNA extraction; qPCR, real-time polymerase chain reaction; D, data analysis; N2, homogenization in liquid nitrogen; CTAB, CTAB extraction; AGE, agarose gel electrophoresis; PCR, polymerase chain reaction; nPCR, nested PCR; RFLP, restriction fragment length polymorphism; H, homogenization; L, LAMP.

ity for diagnostics in human medicine (Parida *et al.*, 2008) and, more recently, in plant health, including phytoplasma detection (Tomlinson *et al.*, 2010a; Bekele *et al.*, 2011; Hodgetts *et al.*, 2011).

In this work the development of a rapid detection protocol for FDp in grapevine using LAMP is reported, together with a technique for on-site plant material homogenization in place of DNA extraction. The whole procedure was tested and validated according to the EPPO standards and following MIQE guidelines (Bustin *et al.*, 2010).

Materials and methods

Plant material, phytoplasma isolates and other pathogens

Samples used for testing and validation of the LAMP assay and on-site homogenization were collected over the 2006–2012 growing seasons. Leaf and stem veins were excised and flowers and berries were picked from plants with symptoms and stored at −20°C or −80°C for further analysis. The status of the plants/vectors (FD infected, FD non-infected) was confirmed by qPCR (Hren *et al.*, 2007).

More than 100 grapevine field samples were included in the study (Table 1), including FDp infected and healthy leaf, berry, flower and stem samples of cultivars Barbera, Cabernet Sauvignon, Chardonnay, Kerner Kraljevina, Malvazija, Modra Frankinja (syn. Blaufränkisch), Pinot blanc, Pinot noir, Rebula, Refošk (syn. Refosco d'Istria), Renski rizling (syn. Weißer Riesling), Rizvanec (syn. Müller-Thurgau), Rumeni muškat (syn. Muscat blanc de Frontignan), Sauvignon, Scheurebe, *Vitis riparia* × *Vitis berlandieri*, white wild *Vitis vinifera*, Zelen, Zweigelt and Žametna črnina. For some samples the cultivar was not determined. FDp subgroups were determined by RFLP analysis and/or sequencing (Mehle *et al.*, 2010).

In addition, the following samples were also included in the testing (Table 1): samples of *Alnus glutinosa*, *Clematis vitalba*, *Orientus ishidae* (collected from alder trees) and *Scaphoideus titanus* (collected from grapevines); samples of FD phytoplasmas, classified into different subgroups, from the INRA phytoplasma collection; phytoplasma strains from other 16Sr groups, originating from the NIB, University of Bologna, Rothamsted Research and University of Udine collections; various plant pathogenic bacterial strains and one fungus from the collections at NIB, Fera, the National Collection of Plant Pathogenic Bacteria (NCPPB, York, UK), the Agricultural Institute of Slovenia (KIS, Ljubljana, Slovenia) and Instituto Valenciano de Investigaciones Agrarias (IVIA); and uncharacterized bacterial isolates from extracts of leaves from various grapevine cultivars grown on nutrient agar (NA; Difco) or YPGA (yeast extract 7 g, proteose peptone 5 g, glucose 10 g, agar 15 g, distilled water 1 L, pH 7·0).

Apple, pear, peach and plum tree leaf and root phloem were included in the comparison of efficiency of the homogenization buffer used prior to DNA extraction.

Plant material homogenization and DNA extraction

For DNA extraction 1 g of plant material was homogenized in 15-mL tubes filled with sand (Matrix A; MP Biomedical) and five ceramic spheres (0·64 cm diameter) using FastPrep-24 (MP Biomedical) for 40 s at 5 m s^{-1}. Lysis buffer (2 mL) (QuickPick SML Plant DNA kit; Bio-Nobile) or ELISA buffer (264 mM Tris, 236 mM Tris-HCl, 137 mM NaCl, 2% PVP K-25, 2 mM PEG 6000, 0·05% Tween 20, pH 8·2) was added to the plant material prior to homogenization. DNA extraction was based on magnetic particles using a KingFisher machine (Thermo Scientific) and QuickPick SML Plant DNA kit (Bio-Nobile), and was carried out as described in Mehle *et al.* (2013b). Alternatively 0·3 g of plant material was extracted using the CTAB method (Doyle & Doyle, 1990). Undiluted DNA was tested with the LAMP assay, whilst DNA was diluted 10-fold (in water) prior to qPCR testing.

On-site homogenization was first optimized on grapevine berries (3–5 berries), where different buffers and homogenization approaches were used and compared. Selected homogenization approaches were also tested on leaf veins (two veins from five leaves) and flowers (five small flower clusters). ELISA and Na-acetate (50 mM Na acetate buffer, pH 5·5, 50 mM NaCl, 30 mM ascorbic acid) buffers, water and pure berry juice were used in the experiments. For all approaches 2–5 mL (for leaf veins) or 10 mL (for berries) of buffer was used.

Three different methods for sample homogenization were tested. (i) A syringe was used for pressing the berry juice from the berries. The juice was added to different buffers or tested directly (no buffer added). The same samples were also boiled

Table 1 Sources, infected and uninfected hosts of different phytoplasma groups and subgroups used to evaluate specificity of 23S rRNA LAMP assays and results of testing

Host	Tissue type	Phytoplasma (acronym)	16Sr group	Source of DNA[a]	No. of samples	Tp (min ± SD)	Tmelt (°C ± SD)
Catharanthus roseus	Leaf	Aster yellows (AY)	16SrI-B	Plant, University of Udine	1	–	–
C. roseus	Leaf	Crotalaria saltiana phyllody (FBPSA)	16SrII	Plant, Rothamsted Research	1	–	–
C. roseus	Leaf	Western X-disease (WX)	16SrIII-A	Plant, University of Udine	1	–	–
C. roseus	Leaf	Elm yellows (EY-C)	16SrV-A	Plant, University of Bologna	1	+(10·15)	85·60
C. roseus	Leaf	Elm yellows (EY-1)	16SrV-A	Plant, University of Udine	1	+(6·97)	85·53
C. roseus	Leaf	FD70	16SrV	DNA, INRA	1	+(13·28)	85·65
Vicia faba	Leaf	FD-C	16SrV	DNA, INRA	1	+(8·00)	85·60
Vitis vinifera	Leaf	FD-D	16SrV	Plant, field sampling	23	+(15·6 ± 5·0)	85·4 ± 0·3
V. vinifera	Berry[b]	FD-D	16SrV	Plant, field sampling	2	+(16·2 ± 0·5)	85·4 ± 0·0
V. vinifera	Stem phloem	FD-D	16SrV	Plant, field sampling	1	+(32·6)	85·25
V. vinifera	Leaf	FD-C	16SrV	Plant, field sampling	6	+(15·2 ± 5·6)	84·8 ± 0·6
V. vinifera	Leaf	FD (type not determined)	16SrV	Plant, field sampling	9	+(15·9 ± 3·7)	85·5 ± 0·4
V. vinifera	Berry[c]	FD (type not determined)	16SrV	Plant, field sampling	8	+(15·0 ± 4·6)	85·2 ± 0·5
V. vinifera	Leaf	FD (type not determined)	16SrV	Plant, field sampling, homogenate testing	18	+(26·5 ± 6·5)	85·1 ± 0·2
V. vinifera	Berry[c]	FD (type not determined)	16SrV	Plant, field sampling, homogenate testing	8	+(24·3 ± 6·9)	85·1 ± 0·2
V. vinifera	Flower	FD (type not determined)	16SrV	Plant, field sampling, homogenate testing	1	+(41·3)	84·2
Alnus glutinosa	Leaf	FD70, FD-C, ALY mix	16SrV	Plant, field sampling	3	+(11·1 ± 3·1)	84·6 ± 1·0
Orientus ishidae	Individual	FD-D, type not determined	16SrV	Insect, field sampling	2	+(10·1 ± 5·2)	85·6 ± 0·0
Scaphoideus titanus	Individual	FD-D	16SrV	Insect, field sampling	1	+(11·2)	84·0
Clematis vitalba	Leaf	FD-C	16SrV	Plant, field sampling	8	+(13·7 ± 0·9)	84·5 ± 0·5
C. roseus	Leaf	Potato witches' broom (PWB)	16SrVI	Plant, University of Bologna	1	–	–
C. roseus	Leaf	Brinjal little leaf (BLL)	16SrVI	Plant, Rothamsted Research	1	–	–
C. roseus	Leaf	Apple proliferation (AP15)	16SrX-A	Plant, University of Udine	1	–	–
C. roseus	Leaf	European stonefruit yellows (ESFY)	16SrX-B	Plant, University of Udine	1	–	–
Pyrus	Leaf	Pear decline (PD)	16SrX-C	Plant, field sampling	1	–	–
C. roseus	Leaf	German stone fruit yellows (GSFY)	16SrX	Plant, University of Bologna	1	–	–
Pennisetum purpureum	Leaf	Napier grass stunt (NGS)	16SrXI	Plant, Rothamsted Research	1	–	–
C. roseus	Leaf	Stolbur (StolSE)	16SrXII-A	Plant, University of Udine	1	–	–
V. vinifera	Leaf	Bois noir (BN)	16SrXII	Plant, field sampling	12	–	–
V. vinifera	Leaf	Unknown pathogenic bacteria, similar to *Xylophilus ampelinus*		Plant, field sampling	2	–	–
V. vinifera	Leaf	*X. ampelinus*		Plant, field sampling	1	–	–
V. vinifera		*X. ampelinus*		NCPPB3026[d]; NCPPB2217[d]	2	–	–
V. vinifera		*Xanthomonas campestris* pv. *viticola*		NCPPB2475[d]	1	–	–
Prunus persica		*Xylella fastidiosa*		ICPB50032[d]	1	–	–
Prunus		*X. fastidiosa*		ICPB50039[d]	1	–	–
V. vinifera		*X. fastidiosa*		ICPB50047[d]	1	–	–

(continued)

Table 1 (continued)

Host	Tissue type	Phytoplasma (acronym)	16Sr group	Source of DNA[a]	No. of samples	Tp (min ± SD)	Tmelt (°C ± SD)
V. vinifera		*Agrobacterium vitis*		KIS Av 13-2[d]	1	–	–
V. vinifera		*A. vitis*		IVIA 339-2[d]	1	–	–
Not determined		*Agrobacterium tumefaciens*		IVIA C58[d]	1	–	–
V. vinifera		*A. tumefaciens*		NCPPB100[d]	1	–	–
Not determined		*Agrobacterium rhizogenes*		IVIA K84[d]	1	–	–
V. vinifera		*Pseudomonas syringae*		NCPPB2805[d]	1	–	–
P. persica		*Bacillus subtilis*		Culture, Fera	1	–	–
V. vinifera	Leaf	Non-identified bacterial isolates		Culture, NIB	30	–	–
Not determined		*Alternaria alternata*		Culture, Fera	1	–	–
V. vinifera	Leaf	FDp negative plants		Plant, field sampling	13	–	–
V. vinifera	Leaf	FDp negative plants		Plant, field sampling	35[e]	–	–
V. vinifera	Leaf/berry	FDp negative plants		Plant, field sampling, homogenate testing	2/2	–	–
C. vitalba	Leaf	FDp negative plant		Plant, field sampling	2	–	–
A. glutinosa	Leaf	FDp negative plant		Plant, field sampling	1	–	–
O. ishidae	Individual	FDp negative insect		Insect, field sampling	1	–	–
S. titanus	Individual	FDp negative insect		Insect, field sampling	1	–	–

Sources of phytoplasma infected plant material are given as surnames of individuals mentioned in the Acknowledgements; phytoplasma and bacteria from field sampling are stored in the collection at NIB and were collected in 2006–2012.

"–" indicates no amplification; ALY, alder yellows; Tp, time to positive; Tmelt, melting temperature

[a]Isolated DNA was used, only samples marked as homogenates were tested without previous DNA extraction.

[b]Berries were collected in August.

[c]Berries were collected in June.

[d]Bacterial collection.

[e]Three pools of samples were tested (prepared from 20 samples which were randomly selected) from a group of 35 healthy samples.

for 10 min and tested afterwards. (ii) Berries, leaf veins and flowers were homogenized manually with vigorous shaking for 2 min in tubes filled with stainless steel beads (5 mm diameter), sand/quartz (0·2–0·8 mm) and buffer. (iii) An automated approach for homogenization, using the Ultra-Turrax Tube Drive (UTTD; IKA) device with tubes filled with stainless steel beads (5 mm diameter) and sand/quartz (0·2–0·8 mm) was also tested. Samples were homogenized for 1 min at maximum settings. The latter procedure was used for testing of field samples. All homogenates (10-times diluted) were tested with 16S rRNA or 23S rRNA LAMP assays (see below).

For determination of the minimal number of berries needed for testing, berries were cut into four pieces and mixed to obtain a homogenous starting material. Four pieces were randomly selected from the pool and put into one sample representing one berry. Similarly, eight and 12 pieces were put together for two and three berries, respectively. The berries were then homogenized manually with beads/sand in Na-acetate buffer and the homogenates were tested with 16S rRNA and 23S rRNA LAMP assays.

The percentage of FDp positive berries within one berry cluster was evaluated. Fourteen randomly selected separate berries were homogenized as described above and tested with the 16S rRNA assay. The experiment was repeated on three berry clusters originating from different grapevine plants. The ratio of FDp positive berries (out of the 14 randomly tested) was used as a measure of overall berry cluster phytoplasma infection. With this information hypergeometric distribution was used in order to determine the probability of detecting an FDp positive sample when testing one, two or more randomly selected berries within a cluster (Table S1).

LAMP primer design and reactions

A LAMP assay was designed to the 16S rRNA region by identifying regions of sequence suitable for primer design from sequence alignments described previously (Hodgetts *et al.*, 2008), which included a range of phytoplasmas from diverse 16Sr groups with the addition of other publicly available sequences from phytoplasmas and a range of other bacteria. Primers for the 16S rRNA were designed without the use of software. A second FD assay was also used, designed to the 23S rRNA, and available in kit form from OptiGene Ltd (http://www.optigene.co.uk). Primers for the 16S rRNA assay were synthesized by either Integrated DNA Technologies or Eurofins MWG Operon. All LAMP reactions were performed in single tubes, 8-well strips or 96-well plates in a 25 μL reaction volume, containing 1 or 5 μL of sample DNA or 10-times diluted plant homogenate, 2× isothermal master mix (OptiGene), 0·2 μM F3 and B3 primers, 2 μM FIP and BIP primers and 1 μM F-loop and B-loop primers. LAMP reactions were performed in a GenieII (OptiGene) or in a Roche LC480 instrument. For LAMP product annealing temperature determination (Tm), the samples were heated to 98°C and then cooled to 80°C, fluorescence was detected in real-time (on the FAM channel for the Roche LC480) and the annealing temperature recorded.

LAMP assay optimization

To determine the ideal conditions for amplification, LAMP assays were run at 60, 62, 65 and 67°C. The temperature at which the fastest positive signal was obtained and where the reactions gave specific amplification, was selected.

Validation of the LAMP 23S rRNA assay

Analytical sensitivity of the 23S rRNA LAMP assay was evaluated by testing FDp positive DNA diluted in DNA extracted from healthy grapevine midribs. Three-fold dilutions were prepared and analysed with LAMP and qPCR assays specific for FDp. The experiment was repeated three times and each time the samples were analysed in triplicate with each method. Analytical specificity of the 23S rRNA LAMP assay was investigated by testing different FDp types (FD70, FD-D and FD-C), bois noir phytoplasma, other phytoplasmas, bacteria and fungi (Table 1). Diagnostic sensitivity and specificity were evaluated by testing FDp infected samples and samples of healthy hosts with LAMP and qPCR assays (Table 1). Selectivity of the 23S rRNA LAMP was evaluated by testing samples from various grapevine cultivars, hosts and tissues (Table 1). In addition, DNA extracted from different grapevine cultivars was spiked with FDp DNA; repeatability and reproducibility of the assay was evaluated by analysing at least three replicates of DNA sample with various FDp DNA concentrations. The reproducibility was also tested by performance of analyses on two to nine different days with freshly prepared reaction mix, by two different operators and on two different devices (GenieII and Roche LC480), where one or more parameters, e.g. operator, device, day, were changed per repetition.

Validation of direct homogenate testing using LAMP 23S rRNA assay

The analytical sensitivity of the method incorporating the direct homogenate technique was tested using 23S rRNA LAMP. Infected grapevine leaf vein material was diluted in healthy grapevine leaf vein material in a three-fold serial dilution series. Homogenates were prepared using the UTTD and FastPrep devices. From the resulting homogenates DNA was extracted as previously described (Mehle *et al.*, 2013b), with the exception of the lysis buffer (QuickPick SML Plant DNA kit), which was replaced with the ELISA buffer. ELISA buffer was shown to be as efficient as a lysis buffer for homogenization of leaf veins (grapevine, *Clematis*, peach, plum and pear tree) and root phloem (apple tree) and preparation of their DNA for extraction using the QuickPick SML Plant DNA kit and KingFisher (Table S2). The experiment was repeated three times. Homogenates and DNA were tested with LAMP and qPCR assays respectively. Dilution curves were prepared from a dilution series of FDp infected homogenate in homogenate from healthy grapevine material and were used for evaluation of diagnostic sensitivity (Table 1; Fig. 2).

Quantitative real-time PCR (qPCR) assays

Results obtained from LAMP testing were compared to a qPCR assay performed as described by Hren *et al.* (2007) and Mehle *et al.* (2013a). For the comparison of homogenization efficiency, when different buffers (Lysis and ELISA buffer) were used prior to DNA extraction, the qPCR assay described by Nikolić *et al.* (2010) was used. The relative FDp DNA concentration in grapevine homogenate and in DNA samples was estimated by qPCR via analysis of dilution curves prepared from FDp infected material (Table 2), with the assumption that one to three copies are present in the last dilution giving positive results (Hren *et al.*, 2007). For determination of homogenization efficiency and for confirmation of the success of DNA extraction the presence of the host (plant or insect) 18S rRNA gene was tested.

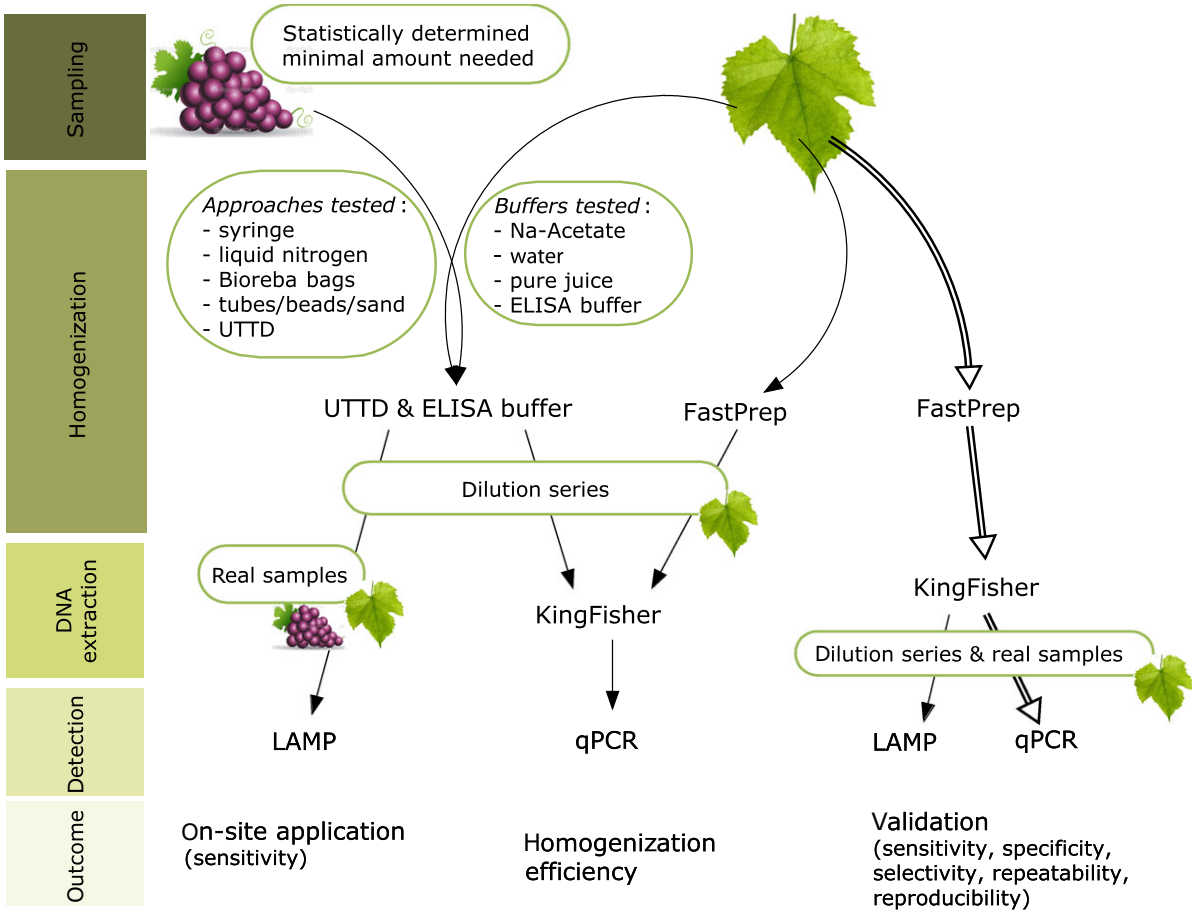

Figure 2 Overview of the generally used and the newly developed process for FDp detection in grapevine samples. Double arrows indicate generally used protocol and single arrows show the process of development, optimization and validation of the on-site procedure for FDp testing by 23S rRNA LAMP assay. UTTD, Ultra-Turrax Tube Drive.

Table 2 Sensitivity of the 23S rRNA LAMP assay compared with qPCR, on representative serial dilutions of flavescence dorée phytoplasma (FDp) DNA

Plant DNA dilution	Estimated FDp DNA copy number	qPCR (C_q ± SD)	LAMP (Tp ± SD)
1×	729–7290	24·94 ± 0·21	+(11·7 ± 0·2)
10×	243–729	28·89 ± 0·31	+(12·7 ± 0·3)
30×	81–243	30·19 ± 0·18	+(15·4 ± 1·8)
90×	27–81	31·99 ± 0·18	+(15·1 ± 0·8)
270×	9–27	34·38 ± 0·67	+(23·7 ± 9·8)
810×	3–9	35·37 ± 1·30	–
2430×	1–3	36·46 ± 0·11	–
7290×	0	–	–

Tp, time to positive (min). SD, standard deviation calculated from three measurements.

Results

In this study, a 16S rRNA LAMP assay was designed for the detection of FDp in grapevine samples. Following optimization, the performance of the assay was compared to the commercially available FD assay based on the 23S rRNA gene (OptiGene). The best performing LAMP assay (23S rRNA) was validated in accordance with the EPPO recommendations (Table 3). In preparation of the manuscript, MIQE précis guidelines were followed and the data is given in Table S3.

Primer design and evaluation of the LAMP assays

A set of primers was designed to the 16S rRNA (Table 4), where regions of sequence specific for 16SrV phytoplasmas were identified. The performance of 16S rRNA and 23S rRNA LAMP assays, in terms of time to positive reaction (Tp), specificity and sensitivity, was evaluated by testing samples with different amounts of FDp and FDp-negative plant samples. The 23S rRNA LAMP assay was found to perform best at 62°C and the 16S rRNA assay at 65°C, where Tp was the shortest and the sensitivity was the highest (data not shown). For sensitivity comparisons, serial dilutions of FDp DNA in water were tested with both assays. The 16S rRNA LAMP assay showed 10-times higher sensitivity when

Table 3 Validation of 23S rRNA LAMP assay according to the EPPO standards

Performance criteria	Result	Verification method
Analytical sensitivity (DNA)	9–27 copies of FDp DNA (1:270 dilution of FDp DNA)	3 experiments with 8 serial dilutions of DNA were performed. Maximum dilution of FDp DNA that was detected was 1:270.
Analytical sensitivity (homogenate)	9–27 copies of FDp DNA (1:81 dilution of FDp infected homogenate)	3 experiments with at least 6 serial dilutions of plant homogenate were performed. Maximum dilution of FDp DNA that was detected was 1:81.
Analytical specificity	100% accurate[a]	No. of targets analysed: 65 + 2 (EY)[b]. No. of non-targets analysed: 123 (for details see Table 3).
Selectivity	There was no impact observed of different hosts, grapevine cultivars or tissues on the test results.	FD was confirmed using LAMP in 12 different grapevine cultivars, either in berries or leaf veins, and also in *C. vitalba*, *A. glutinosa*, *O. ishidae* and *S. titanus*[c]
Repeatability	High FDp conc: 100% (29 pos/29 repeats) Medium FDp conc: 100% (12 pos/12 repeats) Low FDp conc: 81% (22 pos/27 repeats)	At least 3 replicates of DNA sample with low (8 samples with <81 copies of FDp DNA), medium (4 samples with 81–729 copies of FDp DNA) and high (5 samples with >729 copies of FDp DNA) concentration of FDp were analysed.
Reproducibility	High FDp conc: 100% (3 pos/3 repeats) Medium FDp conc: 100% (2 pos/2 repeats) Low FDp conc: 100% (8 pos/8 repeats)	2 replicates of DNA sample with low (8 samples with <81 copies of FDp DNA), medium (2 samples with 81–729 copies of FDp DNA) and high (3 samples with >729 copies of FDp DNA) concentration of FDp were analysed. Analyses were performed on 2–9 different days, by two different operators and two different devices.
Diagnostic sensitivity (DNA)	100%	No. of targets analysed with LAMP and qPCR: 52 FDp infected samples (38 grapevine leaf vein, 8 *C. vitalba*, 3 *A. glutinosa*, 1 *S. titanus* and 2 *O. ishidae* samples)
Diagnostic sensitivity (homogenate)	100%	No. of targets analysed with LAMP and qPCR: 27 FDp infected grapevine samples
Diagnostic specificity	100%	No. of non-targets analysed with LAMP and qPCR: 53 FDp non-infected samples (48 grapevines, 2 *C. vitalba*, 1 *A. glutinosa*, 1 *O. ishidae* and 1 *S. titanus*)

Conc, relative concentration of the FDp DNA estimated from the dilution series.
[a]Calculated from the ratio between the number of correct results and the number of all results.
[b]23S rRNA LAMP is specific to 16SrV phytoplasmas including EY-phytoplasma. Detailed description of the validation is in the text.
[c]*Clematis vitalba, Alnus glutinosa, Orientus ishidae, Scaphoideus titanus.*

compared with the 23S rRNA assay (data not shown). Nevertheless, cross reactivity of the 16S rRNA LAMP assay in FDp negative samples was observed, presumably due to reagent-borne DNA contamination (16 positive reactions out of 68 negative controls), and the 23S rRNA LAMP assay was selected for further validation.

Sensitivity of the 23S rRNA LAMP assay

The sensitivity of the 23S rRNA LAMP assay was compared to that achieved using the qPCR assay specific for FDp (Hren *et al.*, 2007; Table 2). The analytical sensitivity of the 23S rRNA LAMP assay was estimated to be between 9 and 27 FDp copies in a reaction, which is a nine-times lower sensitivity than that of the qPCR. All positive reactions were observed before 33 min of amplification (Table 1), therefore a run reaction time of 40 min is sufficient to achieve a result.

To determine diagnostic sensitivity of the 23S rRNA LAMP assay, grapevine leaf vein, *C. vitalba*, *A. glutinosa*, *O. ishidae* and *S. titanus* diagnostic samples with different levels of FDp infection were selected. The amount of FDp DNA in the samples was estimated with qPCR, where lower C_q values represent higher FDp DNA quantities. With the 23S rRNA LAMP assay it was possible to detect FDp in all tested samples (Table 1; Fig. 3). The trend line on the chart shows high correlation between C_q values and Tp values ($R^2 = 0{\cdot}58$) indicating the semiquantitative nature of the LAMP assay.

Table 4 LAMP primers for 16S rRNA gene. Forward and backward inner primers (FIP, BIP), outer primers (F3, B3) and loop primers (FL, BL) were designed

Primer name	Sequence (5′–3′)
16S-F3	CGTGTCGTGAGATGTTAGGTTAAG
16S-FL	ACCATTACGTGCTGGCAACTAG
16S-FIP	TATCCCCACCTTCCTCCAATGTTTAAT TCTAAAACGAACGCAACCCC
16S-B3	CGCGATTACTAGCGATTCCAG
16S-BL	GCTACAAACGTGATACAATGGCTA
16S-BIP	TCAAATCATCATGCCCCTTATGATCT GGCAGACTTCAATCCGTACTGAGACTA

Specificity

Analytical specificity of the 23S rRNA LAMP assay was first evaluated by *in silico* analysis, which predicted a high specificity to 16SrV phytoplasmas including FD (data not shown). Furthermore, FD70, FD-C and FD-D

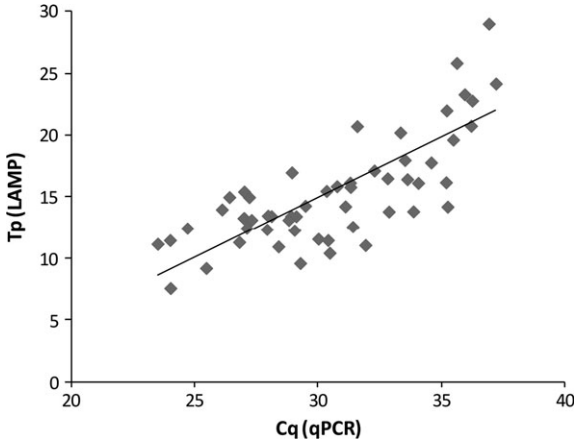

Figure 3 Diagnostic sensitivity of the 23S rRNA LAMP. Time to positive (minutes, Tp) for LAMP and cycles to positive (qPCR, C_q) are given on *y*- and *x*-axis, respectively. Samples with different amounts of FDp (represented with different C_q) were tested with LAMP assay. Trend line shows a positive correlation (R^2 = 0·58) between Tp and C_q values.

phytoplasma types were tested with the 23S rRNA LAMP assay and no difference in the specificity was observed (Table 1). Phytoplasma DNA from other 16Sr groups, bacterial and fungal isolates and healthy hosts were tested and in no cases did the 23S rRNA LAMP assay give positive reactions.

Annealing temperature (Tm) analysis of the LAMP product showed that all signals, obtained in the case of FDp infected samples, were specific. The Tm for the specific amplicon ranged from 84·0 to 85·0°C, when samples were analysed on the GenieII, and from 84·9 to 86·9°C when samples were analysed on the Roche LC480 machine (Table 1).

Selectivity

The influence of different types of tissue or host plant material on the detection of FDp using LAMP was evaluated. First, samples from infected grapevine plants of various cultivars were tested and when the results were compared to qPCR, no differences were observed. This observation was confirmed by analysis of DNA extracted from 12 different cultivars that were spiked with equal amounts of FDp DNA (data not shown). The influence of different tissue types, plant hosts or insect material on the LAMP reaction was tested by analysing FDp DNA in grapevine berries, leaf and stem veins, in *C. vitalba* and *A. glutinosa* and the insect vectors *O. ishidae* and *S. titanus* (Table 1). All the results obtained with the 23S rRNA LAMP were in accordance with the results of qPCR.

Repeatability and reproducibility

Repeatability and reproducibility of the assay were evaluated by analysing several replicates of DNA sample with various FDp DNA concentrations. The assay was shown to give repeatable and reproducible results. Results were 100% reproducible when tested with different devices on different days and with different reaction mixes (Table 3). When testing replicates of the same sample with high and medium concentrations of the FDp DNA the assay was shown to be 100% repeatable. At lower concentrations the detection of the FDp DNA by the 23S rRNA LAMP assay varied, which can be attributed to stochastic effects in target copy distribution in replicates (Hren *et al.*, 2007).

On-site application

Sampling

The amount of the starting material required for successful FDp DNA detection was investigated. One grapevine berry homogenized manually with beads/sand and the Na-acetate buffer approach was shown to be sufficient for detection with the LAMP assay (Table S4). However, analysis of separate berries from a berry cluster and statistical evaluation showed that not all berries were FDp positive (Table S1a) and that there is a low probability that the correct infected berry will be selected and tested (Table S1b). When five berries are selected for testing, the probability of having at least one FDp positive berry is at least 95% in each of the three tested berry clusters (Table S1c). The probability of having at least one positive result (i.e. FDp infected berry) when one or two berries were randomly selected from each of the three clusters was also tested and found to be 95 and 99%, respectively (Table S1d,e).

Sample preparation

When comparing different homogenization methods for berries, manual shaking of tubes filled with metallic beads and sand was found to be the most efficient procedure, because FDp was detected in all samples, regardless of the buffer used (data not shown). The tubes with beads/sand were also shown to be efficient for homogenization of leaf veins and flowers, and FDp DNA could be directly detected with the LAMP assay in homogenates of all tissues tested (Table S4).

Comparison of different buffers revealed that ELISA and Na-acetate buffers were similarly efficient, because 6/7 and 7/7 berry samples were positive when homogenized in ELISA or Na-acetate buffer, respectively (Table S4). Testing pure berry juice homogenized with the beads/sand approach gave positive results only for 3/7 samples. ELISA buffer was selected for further testing and optimization because it is compatible with the QuickPick Plant DNA kit, used for extraction of DNA, and can therefore be used for the DNA extraction from grapevine leaf veins.

To ease the manual homogenization and provide uniform results, the Ultra-Turrax Tube Drive (UTTD) device was tested. The results showed that this method is suitable for homogenization of leaf veins, berries and flowers (Table S4). The efficiency of the UTTD-assisted homogenization was compared to the FastPrep homogenization

Table 5 Efficiency of homogenization with Ultra-Turrax Tube Drive (UTTD) and with FastPrep and comparison of sensitivity between in-lab procedure (FastPrep/DNA) and on-site procedure (UTTD/homogenate)

		FastPrep		UTTD		UTTD
		Extracted DNA		Extracted DNA		Homogenate
		qPCR (C_q ± SD[a])		qPCR (C_q ± SD[a])		LAMP (Tp)
Dilution	FDp DNA copy no.	IC	FD	IC	FD	FD
3×	243–729	16·7	27·9 ± 0·1	20·6	31·6 ± 0·3	+(21·1)
9×	81–243	16·8	29·5 ± 0·2	20·5	33·1 ± 0·3	+(27·3)
27×	27–81	17·0	31·4 ± 0·6	20·6	34·7 ± 0·2	+(25·0)
81×	9–27	17·5	32·9 ± 0·7	20·6	–	+(19·1)
243×	3–9	17·6	34·4 ± 0·3	20·8	–	–
729×	1–3	17·4	34·8 ± 1·0	20·7	–	–
2187×	0	17·5	–	20·9	–	–
6561×	0	16·8	–	20·8	–	–

FDp DNA copy number was estimated from the dilution curve. Efficiency of homogenization approaches was compared by analysing the presence of internal control (IC, 18S rRNA gene) and detection of FDp DNA (FD) by qPCR.
Tp, time to positive (min). –, no amplification.
[a]Standard deviation (SD) was calculated only for FD amplicon.

procedure that is used for grapevine samples (Mehle *et al.*, 2013b) by analysing different concentrations of infected plant material or homogenate, diluted with healthy plant material or homogenate, respectively. On the basis of detection of the 18S rRNA gene internal control by qPCR it was estimated that homogenization with UTTD was approximately 10-times less efficient than with FastPrep (Table 5). When comparing the efficiency of the homogenization in terms of detection of FDp by qPCR, the UTTD-assisted homogenization was found to be at the most 27-times less efficient then homogenization with FastPrep.

Sensitivity of on-site grapevine testing method

For on-site application of the FDp testing protocol using the LAMP assay, the sample preparation procedure was simplified and shortened by omitting the DNA extraction step. Crude homogenate was found to be suitable to be used directly in LAMP testing (Table 5). Analytical sensitivity of the whole procedure developed for the on-site detection (UTTD homogenization, direct homogenate testing with LAMP assay) was compared to the standard in-lab FDp detection procedure (FastPrep homogenization, KingFisher-assisted DNA extraction and qPCR analysis; Fig. 1). The on-site procedure was shown to be suitable for detection of FDp DNA in samples where as low as 9–27 copies of FDp DNA are present (Table 5). Diagnostic sensitivity was evaluated by testing crude homogenates prepared from FDp infected grapevine leaf veins and was shown to be 100% (Table 3). Berry samples collected in June, when the lowest amount of the FDp DNA is expected (Prezelj *et al.*, 2012), were tested as well and FDp was confirmed in all berry homogenates (Table 1). Selectivity of the direct homogenate testing was also evaluated by testing other grapevine tissues and various cultivars, and the results were in accordance with qPCR results.

Validation data

The 23S rRNA LAMP assay for testing FDp DNA presented here was validated according to the EPPO standard (EPPO, 2014). In addition, specificity of the on-site testing of crude homogenate was evaluated (Table 3).

Discussion

A LAMP assay for detection of the quarantine organism FDp was validated in accordance with the EPPO procedures (EPPO, 2010) and was shown to be suitable for testing of grapevine and other host plant samples and vectors. In addition, the overall procedure of testing was developed and optimized for on-site application.

Analytical and diagnostic sensitivity, specificity, selectivity and repeatability of the 23S rRNA LAMP assay were evaluated (Table 3). The analytical sensitivity of the LAMP assay was shown to be nine times lower than that of the qPCR method (Hren *et al.*, 2007); previous work has shown that conventional nested PCR (often used for FDp detection) is approximately 1000-times less sensitive than qPCR. By inference this would suggest that the LAMP approach is more sensitive than nested PCR. The diagnostic sensitivity of the 23S rRNA LAMP assay was determined to be 100%, which is supported by the detection of the FDp in all tested samples (Table 1; Fig. 3).

Three FD phytoplasma types are described at the moment, namely FD70, FD-C and FD-D, where FD-D and FD-C are prevalent types on grapevine and *C. vitalba*, respectively (Mehle *et al.*, 2011). All three FDp types were detected with the 23S rRNA LAMP assay showing its high specificity. Moreover, the assay did not react with BN phytoplasma, which also occurs at high levels in vineyards and causes symptoms indistinguishable from FDp infected plants (Constable *et al.*, 2003); nor did it detect phytoplasmas from the seven other 16Sr groups

tested. No cross-reactivity was observed with the other fungal and bacterial strains tested, including grapevine epiphytic bacteria, which could be present on the grapevine leaves. The 23S rRNA LAMP did not react with DNA extracted from healthy host plants (grapevines, *C. vitalba*, *A. glutinosa*) or vectors (*O. ishidae* and *S. titanus*). The specificity of the amplification can be assessed by analysis of the Tm of the amplified DNA, which was consistent in different samples from various sources. The annealing temperature for the LAMP product was consistent from reaction to reaction. The variation in Tm observed between the instruments used in this study can be explained by different approaches for Tm determination by anneal and melt curve in GenieII and Roche LC480, respectively (Lenarčič *et al.*, 2012). Nevertheless, the difference always remained the same when using any of the individual machines, and the variability between machines did not affect the final result.

Due to its speed and isothermal amplification, the 23S rRNA LAMP assay can be applied for in-laboratory use as well as for on-site detection, because it allows use of simple, portable, battery-powered equipment, e.g. GenieII (OptiGene), which is affordable to small on-site laboratories. Interpretation of the final result is easy and does not require any intensive data analysis. The LAMP-based analysis can therefore be established in small laboratories at production sites, customs, ports, etc. The whole procedure of FDp testing, from sampling, sample preparation to the final analysis, was optimized for on-site diagnostics.

The sampling procedure is one of the most crucial steps in the process of pathogen detection and identification. In the case of FDp detection, uneven distribution in plant material poses the most significant issue (Baric *et al.*, 2008). Therefore, it is advisable that the tissue is taken from at least three different shoots on the plant. In the case of testing leaf veins, 1 g of material is needed for reliable diagnostics, which equates to approximately two veins from each of five leaves. Berries present a good source of FDp for on-site testing, because they are highly infected (Prezelj *et al.*, 2012) and easier to homogenize manually. However, not all of the berries in the berry cluster were found to be positive and the statistical analysis showed that for reliable detection with the LAMP 23S rRNA assay preferably two randomly selected berries from three berry clusters should be tested.

Due to limitations in equipment availability, hard lignified leaf veins and low phytoplasma titre, homogenization is the most demanding task for applying in field conditions. Two homogenization procedures were selected as preferred for on-site use, namely manual shaking of tubes and Ultra-Turrax Tube Drive (UTTD)-assisted homogenization in tubes filled with metallic beads and sand. Both procedures were efficient for homogenization of leaf veins, berries and flowers. Comparison of the on-site applicable homogenization method to the FastPrep-assisted homogenization, used in the in-lab DNA extraction protocol (Mehle *et al.*, 2013b), revealed higher efficiency of the latter; however, this is not suitable for fast on-site analysis. Therefore, a new simplified procedure was developed where the DNA extraction step was omitted, meaning that crude extract/homogenate can be directly used for analysis. Even though FastPrep was more efficient in homogenization, it was found not to be suitable for crude extract testing by the LAMP assay using current parameters (see Material and Methods). Nevertheless, optimization of the FastPrep-assisted homogenization could improve sample preparation and fulfil the needs for direct LAMP testing and for the DNA extraction process.

Lower analytical sensitivity of the on-site procedure for FDp testing (UTTD-assisted homogenization, homogenate testing with LAMP assay) was observed in comparison to the in-lab procedure (FastPrep, DNA extraction, qPCR). This can be mostly attributed to lower sensitivity of the LAMP assay in comparison to qPCR (Table 2). The results indicate that direct homogenate testing has an advantage over the DNA extraction procedure, because the DNA extraction procedure involves dilutions of the FDp DNA, which lowers the amount of target DNA in the final sample. Nevertheless, diagnostic sensitivity of the on-site procedure was shown to be 100%, because all tested samples, originating from different tissues and cultivars and collected at different times in the season, were positive.

By simplifying and shortening the time for analysis, the whole FDp detection method became fast, efficient and inexpensive. When comparing the time needed for detection of FDp in grapevine samples, the LAMP-based procedure was approximately 10 times faster than the qPCR-based method (Fig. 1). One hour is needed from sampling to the final result with LAMP, whereas almost 10 hours are needed for the qPCR-based method (Mehle *et al.*, 2013a), which is semi-automated (Mehle *et al.*, 2011). Furthermore, the classical nested PCR-based approach (EPPO, 2007) is even more time-consuming and demands a whole working week to reach a final result.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1 Hypergeometric distribution calculations for determination of the probability of FDp detection.

Table S2 Comparison of ELISA buffer and Lysis buffer homogenization efficiency by qPCR.

Table S3 Checklist of MIQE précis with details on parameters associated with LAMP 23S rRNA assay.

Table S4 Homogenization buffer selection and determination of the minimal number of berries needed for positive result.