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 (GY) 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; Mehele et al., 2013). 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., 2010). Because of its speed, robustness and simplicity, the use of LAMP is gaining popular-

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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. Blafränkisch), Pinot blanc, Pinot noir, Rebula, Refosk (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 Zametna crnina. For some samples the cultivar was not determined. FDP subgroups were determined by RFLP analysis and/or sequencing (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
| Host                               | Tissue type | Phytoplasma (acronym)              | 16Sr group | Source of DNA* | No. of samples | Tp (min ± SD) | Tmelt (°C ± SD) |
|------------------------------------|-------------|-----------------------------------|------------|---------------|---------------|---------------|----------------|
| Catharanthus roseus                | Leaf        | Aster yellows (AY)                | 16Sr-I-B    | Plant, University of Udine | 1             | –             | –              |
| C. roseus                          | Leaf        | Crotonia saitiana phylloyd (FBPSA) | 16Sr-II    | Plant, Rothamsted Research | 1             | –             | –              |
| C. roseus                          | Leaf        | Western X-disease (WX)            | 16Sr-III-A  | Plant, University of Udine | 1             | –             | –              |
| C. roseus                          | Leaf        | Elm yellows (EY-C)                | 16Sr-V-A    | Plant, University of Bologna | 1             | +(10.15)      | 85.60          |
| C. roseus                          | Leaf        | Elm yellows (EY-1)                | 16Sr-V-A    | Plant, University of Udine | 1             | +(6.97)       | 85.53          |
| C. roseus                          | Leaf        | FD70                              | 16Sr-V      | DNA, INRA      | 1             | +(13.28)      | 85.65          |
| Vicia faba                         | Leaf        | FD-C                              | 16Sr-V      | DNA, INRA      | 1             | +(8.00)       | 85.60          |
| Vitis vinifera                     | Leaf        | FD-D                              | 16Sr-V      | Plant, field sampling | 23            | +(15.6 ± 5.0) | 85.4 ± 0.3     |
| V. vinifera                        | Berry*      | FD-D                              | 16Sr-V      | Plant, field sampling | 2             | +(16.2 ± 0.5) | 85.4 ± 0.0     |
| V. vinifera                        | Stem phloem | FD-D                              | 16Sr-V      | Plant, field sampling | 1             | +(32.6)       | 85.25          |
| V. vinifera                        | Leaf        | FD-D                              | 16Sr-V      | Plant, field sampling | 6             | +(15.2 ± 6.6) | 84.8 ± 0.6     |
| V. vinifera                        | Leaf        | FD (type not determined)          | 16Sr-V      | Plant, field sampling | 9             | +(15.9 ± 3.7) | 85.5 ± 0.4     |
| V. vinifera                        | Berry*      | FD (type not determined)          | 16Sr-V      | Plant, field sampling | 8             | +(15.0 ± 4.6) | 85.2 ± 0.5     |
| V. vinifera                        | Leaf        | FD (type not determined)          | 16Sr-V      | Plant, field sampling | 18            | +(26.5 ± 6.5) | 85.1 ± 0.2     |
| V. vinifera                        | Berry*      | FD (type not determined)          | 16Sr-V      | Plant, field sampling | 8             | +(24.3 ± 6.9) | 85.1 ± 0.2     |
| V. vinifera                        | Flower      | FD-C                              | 16Sr-V      | Plant, field sampling | 1             | +(41.3)       | 84.2           |
| Alnus glutinosa                    | Leaf        | FD70, FD-C, ALY mix               | 16Sr-V      | Plant, field sampling | 3             | +(11.1 ± 3.1) | 84.6 ± 1.0     |
| Orientus isihade                   | Individual  | FD-D, type not determined          | 16Sr-V      | Insect, field sampling | 2             | +(10.1 ± 5.2) | 85.6 ± 0.0     |
| Scaphoideriae titanus              | Individual  | FD-D                              | 16Sr-V      | Insect, field sampling | 1             | +(11.2)       | 84.0           |
| Clematis vitalba                   | Leaf        | FD-C                              | 16Sr-V      | Plant, field sampling | 8             | +(13.7 ± 0.9) | 84.5 ± 0.5     |
| C. roseus                          | Leaf        | Potato witches’ broom (PWB)       | 16Sr-VI     | Plant, University of Bologna | 1             | –             | –              |
| C. roseus                          | Leaf        | Brinjal little leaf (BLL)         | 16Sr-VI     | Plant, Rothamsted Research | 1             | –             | –              |
| C. roseus                          | Leaf        | Apple proliferation (AP15)         | 16Sr-X-A    | Plant, University of Udine | 1             | –             | –              |
| C. roseus                          | Leaf        | European stonefruit yellows (ESFY) | 16Sr-X-B   | Plant, University of Udine | 1             | –             | –              |
| Pyrus                              | Leaf        | Pear decline (PD)                 | 16Sr-X-C    | Plant, field sampling | 1             | –             | –              |
| C. roseus                          | Leaf        | German stone fruit yellows (GSFY) | 16Sr-X     | Plant, University of Bologna | 1             | –             | –              |
| Pennisetum purpureum               | Leaf        | Napier grass stunt (NGS)          | 16Sr-XI     | Plant, Rothamsted Research | 1             | –             | –              |
| C. roseus                          | Leaf        | Stoilur (StoSE)                   | 16Sr-XII-A  | Plant, University of Udine | 1             | –             | –              |
| V. vinifera                        | Leaf        | Bois noir (BN)                    | 16Sr-XII    | Plant, field sampling | 12            | –             | –              |
| V. vinifera                        | Leaf        | Unknown pathogenic bacteria, similar to Xylophilus ampelinus | | Plant, field sampling | 2             | –             | –              |
| V. vinifera                        | Leaf        | X. ampelinus                      | 1           | –             | –             | –             | –              |
| V. vinifera                        | Leaf        | X. ampelinus                      | NCPPB3026d, NCPPB2217d | – | – | – |
| V. vinifera                        | Leaf        | Xanthomonas campestris pv. viticola | NCPPB4245d | – | – | – |
| Prunus persica                     | Leaf        | Xylella fastidiosa               | ICPB50032d  | – | – | – |
| Prunus                             | Leaf        | X. fastidiosa                    | ICPB50039d  | – | – | – |
| V. vinifera                        | Leaf        | X. fastidiosa                    | ICPB50047d  | – | – | – |

*(continued)*
Table 1 (continued)

| Host                        | Tissue type | Phytoplasma (acronym)          | 16Sr group | Source of DNA*                      | No. of samples | Tp (min ± SD) | Tmelt (°C ± SD) |
|-----------------------------|-------------|--------------------------------|------------|------------------------------------|----------------|---------------|-----------------|
| V. vinifera                 | Leaf        | Non-identified bacterial isolates |            | Culture, Fera                      | 30             | –             | –               |
| Not determined              | Leaf        | Alternaria alternata           |            | Culture, Fera                      | 1              | –             | –               |
| V. vinifera                 | Leaf        | FDp negative plants           |            | Plant, field sampling              | 13             | –             | –               |
| 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
*Isolated DNA was used, only samples marked as homogenates were tested without previous DNA extraction.
*Berries were collected in August.
*Berries were collected in June.
*Bacterial collection.
*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 designed, used to detect 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 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 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 Nikolic 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.

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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 compared to the commercially available FD assay based on the 23S rRNA gene (OptiGene).

**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...
Diagnostic specificity 100% No. of non-targets analysed with LAMP and qPCR: 53 FDp

Diagnostic sensitivity (DNA) 100% No. of targets analysed with LAMP and qPCR: 52 FDp infected

Conc, relative concentration of the FDp DNA estimated from the dilution series.

Selectivity There was no impact observed of different hosts, grapevine cultivars or tissues on the test results.

Repeatability High FDp conc: 100% (29 pos/29 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 (6 samples with >729 copies of FDp DNA) concentration of FDp were analysed.

Reproducibility High FDp conc: 100% (3 pos/3 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%

Diagnostic specificity (homogenate) 100%

Diagnostic specificity 100%

Analytical specificity of the 23S rRNA LAMP assay was estimated to be 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 Cq values represent higher FDp DNA quantities. With the 23S rRNA LAMP assay it was possible to detect FDp in all tested samples (123 for details see Table 3). The trend line on the chart shows high correlation between Cq and Tp values (\(R^2 = 0.58\)) indicating the semiquantitative nature of the LAMP assay.

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 | 3 experiments with 8 serial dilutions of DNA were performed. Maximum dilution of FDp DNA that was detected was 1:270. |
| (homogenate) | 1:270 dilution of FDp DNA | 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% accuratea | No. of targets analysed: 65 + 2 (EY)b. No. of non-targets analysed: 123 |
| Selectivity | There was no impact observed of different hosts, grapevine cultivars or tissues on the test results. |
| Repeatability | High FDp conc: 100% (29 pos/29 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 (6 samples with >729 copies of FDp DNA) concentration of FDp were analysed. |
| Reproducibility | High FDp conc: 100% (3 pos/3 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 grapevine samples |
| (homogenate) | 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) |
| Diagnostic specificity | 100% | |

Conc, relative concentration of the FDp DNA estimated from the dilution series.

aCalculated from the ratio between the number of correct results and the number of all results.

b23S rRNA LAMP is specific to 16SrV phytoplasmas including EY-phytoplasma. Detailed description of the validation is in the text.

cClematis vitalba, A. glutinosa, O. ishidae, Scaphoideus titanus.

dFDp 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.

eComparing 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 Cq 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 Cq and Tp values (\(R^2 = 0.58\)) indicating the semiquantitative nature of the LAMP assay.

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
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. isibidae and S. titamis (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.
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.

*Standard deviation (SD) was calculated only for FD amplicon.

Valiation 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 grapevines 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 support 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. vitis, 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. titanius). 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 (Lenarcic 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 fulfill 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.