Bulletin of Insectology ***64*** *(Supplement): S41-S42, 2011*
ISSN 1721-8861

Development of rapid in-field loop-mediated isothermal amplification (LAMP) assays for phytoplasmas

Jennifer HODGETTS[1], Jenny TOMLINSON[1], Neil BOONHAM[1], Irene GONZÁLEZ-MARTÍN[2], Petra NIKOLIĆ[3], Philip SWARBRICK[4], Egya N. YANKEY[4], Matthew DICKINSON[4]

[1]*The Food and Environment Research Agency, Sand Hutton, York, YO41 1LZ, UK*
[2]*Centre de Recerca i Investigació de Catalunya, S.A., Travessera de Gràcia 108, Entl., 08012 Barcelona, Spain*
[3]*National Institute of Biology, Večna pot 111, 1000 Ljubiana, Slovenia*
[4]*School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, UK*

Abstract

Loop-mediated isothermal amplification (LAMP) is an isothermal amplification technique that can be undertaken with minimal equipment to obtain amplification of target DNA within 30 minutes. A range of assays for specific 16Sr phytoplasma groups, which when combined with rapid DNA extraction techniques can result in detection of the phytoplasma in plant material within 1 hour of sampling was developed. A range of alternative methods are available for detection of the amplification product including incorporation of hydroxyl napthol blue into the reagent mix, agarose gel electrophoresis and real-time detection systems. The advantage of the real-time method is that tubes do not have to be opened, minimising the risk of contamination of samples.

Key words: DNA extraction, LAMP assays, field detection, phytoplasmas.

Introduction

Numerous techniques have been developed for phytoplasma diagnostics, in particular the use of PCR-based methods. These may either be generic, with the use of RFLP analysis or sequencing to assign the phytoplasma to a 16Sr group, or they may involve the use of group-specific primers (Smart *et al*., 1996; Firrao *et al*., 2005). However, it is also important to guard against false negatives during such detection by building an internal control into the diagnostic test to confirm that a negative result is due to a lack of phytoplasmas and not PCR inhibition. More recently, real-time PCR assays have also been developed for both generic and specific phytoplasma detection, and these assays have the advantage of being more easily automated and less labour intensive than conventional PCR, such that appropriate controls can be conducted more easily (Christensen *et al*., 2004; Hodgetts *et al*., 2009; Hren *et al.,* 2007).

However, these assays are also relatively slow compared to Loop-Mediated Isothermal Amplification (LAMP) assays, and require bulky equipment such that they generally have to be conducted in laboratories. Our goal has been to develop a more rapid diagnostic assay for phytoplasmas that can be used to produce a diagnosis within an hour of sampling in the field. Whilst techniques such as lateral flow devices have been used to achieve this for some viral plants diseases, antibody-based techniques have had limited success for phytoplasma diagnostics because of a lack of sensitivity. We have therefore been developing the use of LAMP which, when combined with rapid DNA extraction techniques, has the potential to provide rapid in-field analyses. Here we report some of the specific tests that we have developed to date along with a protocol that we are piloting for rapid in-field diagnostics of phytoplasmas.

Materials and methods

Plant material (healthy and infected) for testing the DNA extraction techniques and LAMP assays were obtained from the University of Nottingham and the Food and Environment Research Agency phytoplasma collections, along with samples of coconut trunk borings from Ghana (kindly provided by J. Nipah), and papaya and wild grass plant material from Ethiopia (kindly provided by B. Bekele). Two methods were used for DNA extraction; an LFD extraction method (Tomlinson *et al.*, 2010) and an alkaline polyethylene glycol DNA extraction method (Chomczynski and Rymaszewski 2006). Primers for the LAMP assays were designed as described in Tomlinson *et al.* (2010) and Bekele *et al.* (2011) based on the 16S-23S intergenic spacer region, and LAMP reactions and detection methods were as previously described (Tomlinson *et al.,*2010; Bekele *et al.*, 2011). In addition, *cox* gene primers were used to confirm that all DNA extractions supported LAMP (Bekele *et al*., 2011).

Results

Primers for LAMP assays were designed against a range of ribosomal groups (16SrI, 16SrII, 16SrIII, 16SrV, 16SrXI, 16SrXII and 16SrXXII) and these primer sequences are listed in previous papers and/or are available from the authors. The different primer sets were tested on a range of DNA strains from our collection, and with the exception of the 16SrXI assay, the primers were group specific i.e. they only amplified DNA samples from the group they were designed to detect. The 16SrXI primers were different in that they also detected 16SrXIV isolates, but did not detect sugarcane whiteleaf/grassy shoot or rice yellow dwarf samples, even though these are also designated as 16SrXI group

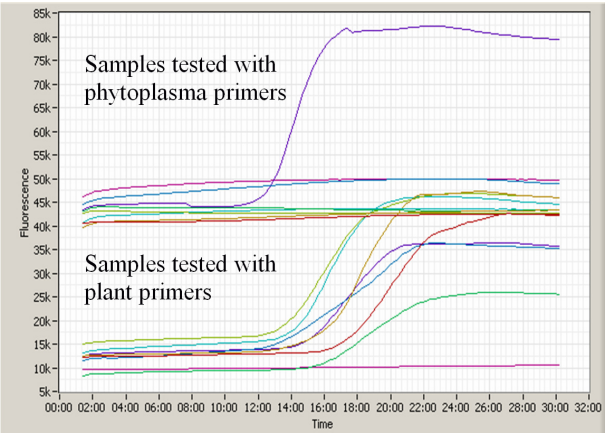

Figure 1. Real-time LAMP profile for seven infected plant material samples and a negative (water) control. The top panel used the 16SrXXII group-specific primers and the bottom panel used the *cox* gene primers. Amplification can be detected as the increase in fluorescence at 12-20 minutes.
(In colour at www.bulletinofinsectology.org)

phytoplasmas. Figure 1 shows the typical results of a LAMP assay using the real-time detection system developed by OptiGene (Horsham, UK), in which seven samples plus a negative (water) control have been tested with the 16SrXXII group phytoplasmas (upper panel) and the same samples tested with the *cox* gene primers (lower panel). The results show that all seven plant samples were amplified within 20 minutes by the plant primers but only the positive control sample was amplified with the 16SrXXII primers.

To compare the DNA extraction methods, samples of periwinkle (*Catharanthus roseus)* leaves, papaya and wild grass leaves and coconut trunk borings were tested using the LFD-based method and the alkaline PEG method. Both methods reliably produced DNA able to support LAMP, though the DNA from the PEG method started to show reduced reliability after storage for more than a week, whilst the LFD DNA was stable for many months.

Discussion

We have developed a protocol for LAMP-based diagnostics for a range of phytoplasmas that can be conducted in the field and used to provide a diagnosis within 1-hour of DNA extraction. The simplest method of DNA extraction is to use the alkaline-PEG method, which involves gently macerating a small amount of plant tissue in the buffer and then using this in the LAMP reaction. The disadvantage of this method is that the DNA cannot be stored reliably long-term, but it is quicker and requires less equipment than the LFD-based method, reducing the likelihood of sample contamination. Having isolated the DNA, the LAMP reactions take only a few minutes to set-up, particularly when field stable lyophilised reagents are used.

Currently we are using the Genie II real-time LAMP reader (Optisense), which is portable and battery operated, enabling amplification within 30 minutes and a visual display of results. Whilst removing the need to open tubes following amplification, it does not integrate DNA extraction. The VITISENS project seeks to develop a hand held device capable of performing extraction, set-up and real-time detection for grapevine phytoplasmas. The device will make a single step homogeneous system from sampling to results, further reducing the risk of sample-to-sample contamination and enabling testing by non-specialists in the field.

Acknowledgements

The work was partially supported by BBSRC/DFID Grant No. BB/F004044/1, Leverhulme/Royal Society Africa Award Grant AA090003 and the EU project 262032 - VITISENS.

References

BEKELE B., HODGETTS J., TOMLINSON J., BOONHAM N., NIKOLIC P., SWARBRICK P., DICKINSON M., 2011.- Use of a real-time LAMP isothermal assay for detecting 16SrII and XII phytoplasmas in fruit and weeds of the Ethiopian Rift Valley.- *Plant Pathology*, 60: 345-355.

CHOMCZYNSKI P., RYMASZEWSKI M., 2006.- Alkaline polyethylene glycol-based method for direct PCR from bacteria, eukaryotic tissue samples, and whole blood.- *BioTechniques*, 40: 454-458.

CHRISTENSEN N. M., NICOLAISEN M., HANSEN M., SCHULZ A., 2004.- Distribution of phytoplasmas in infected plants as revealed by real-time PCR and bioimaging.- *Molecular Plant-Microbe Interactions*, 17: 1175-1184.

FIRRAO G., GIBB K., STRETEN C., 2005.- Short taxonomic guide to the genus '*Candidatus* Phytoplasma'.- *Journal of Plant Pathology*, 87: 249-263.

HODGETTS J., BOONHAM N., MUMFORD R., DICKINSON M., 2009.- Panel of 23S rRNA gene-based real-time PCR assays for improved universal and group-specific detection of phytoplasmas.- *Applied and Environmental Microbiology*, 75: 2945-2950.

HREN M., BOBEN J., ROTTER A., KRALJ P., GRUDEN K., RAVNIKAR M., 2007.- Real-time PCR detection systems for flavescence dorée and bois noir phytoplasmas in grapevine: comparision with conventional PCR detection and application in diagnostics.- *Plant Pathology*, 56: 785-796.

SMART C. D., SCHNEIDER B., BLOMQUIST C. L., GUERRA L. J., HARRISON N. A., AHRENS U., LORENZ K. H., SEEMÜLLER E., KIRKPATRICK B. C., 1996.- Phytoplasma-specific PCR primers based on sequence of the 16S-23S rRNA spacer region.- *Applied and Environmental Microbiology*, 62: 2988-2993.

TOMLINSON J. A., BOONHAM N., DICKINSON M., 2010.- Development and evaluation of a one-hour DNA extraction and loop-mediated isothermal amplification assay for rapid detection of phytoplasmas.- *Plant Pathology*, 59: 465-471.

Corresponding author: Jennifer HODGETTS (email: jennifer.hodgetts@fera.gsi.gov.uk), Fera, Sand Hutton, York, YO41 1LZ, UK.