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EVALUATING LAMP ASSAYS FOR DETECTION OF PHYTOPLASMAS CLASSIFIED IN DIFFERENT RIBOSOMAL GROUPS

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ABSTRACT:

A new set of loop-mediated isothermal amplification assays was developed for detection of phytoplasmas associated with plant diseases and optimised for rapid laboratory detection. This set of assays was designed based on the leucyl tRNA synthetase gene expressed in most of the phytoplasmas and showing a good level of sequence divergence among strains from different 16Sr groups. This makes it a good target for developing discrete assays that can detect specific phytoplasma 16Sr groups. In this study, unique sets of LAMP assays were designed and validated for seven phytoplasma ribosomal groups (16SrI, 16SrII, 16SrIII, 16SrV, 16SrVI, 16SrX, and 16SrXII). These assays are specific, rapid and as sensitive as the nested PCR assays and the results could be obtained in less than 30 minutes in real-time LAMP assays.

KEYWORDS: 16S-23S ITS region, *leucyl tRNA synthetase* gene, rapid detection.

Phytoplasmas are wall-less, phloem-limited bacterial pathogens that belong to the class Mollicutes. They are associated with many diseases in a wide range of plant species (wild and cultivated), resulting in significant losses in important crops, and economically damaging epidemics worldwide (Hodgetts *et al*., 2009). Phytoplasmas infect major cultivated crops such as many annual crops, fruit trees, grapevines and palms, which makes control of these diseases a priority. The first step for the management of their associated-diseases is their efficient and effective phytoplasma detection. Currently, most detection techniques use PCR and primers to amplify phytoplasma ribosomal DNA after extraction from diseased plants (Hodgetts *et al*., 2009; Bekele *et al.*, 2011). In general, the methods for PCR amplification of DNA and analysis of results require relatively expensive equipment and involve the use of buffers for DNA extraction such as cetyltrimethyl ammonium bromide buffer (CTAB) along with phenol/chloroform and isopropanol, therefore they cannot be performed in the field. However, the loop-mediated isothermal amplification (LAMP) (Notomi *et al.,* 2000) has been applied for both generic and specific phytoplasma detection (Hodgetts *et al*., 2009), which can be used in the field, and which takes less than one hour to be performed. Amongst the advantages of LAMP assays are that they are less prone to inhibition from compounds in DNA extracts, so relatively crude DNA preparations can be used. In addition, in order to avoid post-amplification contamination (Hogenhout *et al.,* 2010) real-time LAMP methods have been developed, which have the same advantages as quantitative PCR over PCR-based diagnostic techniques in that they are closed tube systems. A further advantage of real-time LAMP methods is that equipment has been designed that is lightweight and battery operated so is easy to use in fields and faster when compared to PCR or quantitative PCR. Real-time LAMP can be performed by adding a dye such as SYBR green. It creates a visible colour change that can be tracked or seen by the naked eye, so no need to use the expensive equipment and the measurements are taken more accurately.

A number of protocols for phytoplasma detection assays based on LAMP have been developed (Tomlinson *et al*., 2010a; Bekele *et al.,* 2011; Kogovšek *et al*., 2015). The aims of this study were to design and validate additional LAMP assays for different phytoplasma ribosomal groups. In this study, total of six LAMP primers were used targeting eight distinct regions of phytoplasma DNA to get a higher specific amplification reaction. Moreover, loop-F and loop-B primers are used to accelerate the LAMP reaction (Nagamine *et al*., 2002; Kogovšek *et al*., 2017).

Phytoplasmas belonging to seven 16Sr groups are kept at the University of Nottingham (UK), in periwinkle (*Catharanthus roseus*) (Table 1), and DNA was extracted from these using a cetyltrimethyl ammonium bromide (CTAB) method (Daire *et al.,* 1997). Additional DNA samples were also used for assay validation and are listed in Table 2. For this study, confirmation of the phytoplasma DNA presence in samples and comparative sensitivity assays with LAMP were done by using nested PCR with the phytoplasma universal primer pairs P1and P7 (Deng and Hiruki, 1991; Schneider *et al.,* 1995) followed by R16F2n and R16R2 as described in Gundersen and Lee (1996). Mastermixes were prepared containing 12.5 µl mangomix (MangoTaq™ DNA polymerase, Bioline, USA), 10.5 µl sterile water, 1 µl forward primer (50 ng/µl) and 1 µl reverse primer (50 ng/µl) per PCR reaction. For the nested reactions, PCR amplicons were diluted 1:40 with sterile distilled water and used as templates, an initial denaturing temperature of 94°C for 2 min, followed by 35 cycles of 94°C for 30 seconds, 53°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min were performed. Healthy periwinkle leaves were used as a negative control and PCR products were visualized on 1% agarose gels stained with ethidium bromide.

To obtain sequences for the design of group specific LAMP primers, the leucyl tRNA synthetase gene (*leuS)* was chosen, since previous work had shown a significant numbers of point mutations across the approximately 1,120-bp length of the amplified sequence, and consistent variations in length for phytoplasmas from some ribosomal groups (Abeysinghe *et al.,* 2016). For phytoplasmas where sequences were not already available from previous study (Abeysinghe *et al.,* 2016), nested PCR was done using the universal nested primers for the phytoplasma *leuS* gene described in Abeysinghe *et al.* (2016). The PCR products were then sequenced (MWG, Germany) and compared with sequences in the NCBI database, and compared with sequences of different ribosomal phytoplasma groups mentioned in Abeysinghe *et al.* (2016). LAMP primers were designed for groups 16SrI, 16SrII, 16SrIII, 16SrV, 16SrVI, 16SrX and 16SrXII using the LAMP Designer software from Premier Biosoft http://www.premierbiosoft.com/isothermal/lamp.html, this software provided six LAMP primers for each assay for seven phytoplasma ribosomal groups (loop primers loop-F and loop-B, external primers F3 and B3, and internal primers FIP and BIP) (Table 3).

All LAMP reactions were performed and optimised in 16 well-strips with a locking cap providing a closed system, in a portable Genie II machine (OptiGene Limited, UK). Isothermal amplification reactions were performed in a final volume of 20 µl for each reaction, which contained 15 µl isothermal master mix (OptiGene, ISO-001), 2 µl of LAMP primers mixture (to provide a final concentration of 0.2 µM for the F3 and B3 primers, 1.0 µM for the Loop-F, Loop-B, FIP and BIP primers), 1 µl of template DNA, and 2 µl of sterile distilled water. For the isothermal amplification, all reactions were run at 63 °C for 30 min, followed by an annealing temperature assay from 98 to 80 °C. Results were confirmed by gel electrophoresis in 1% agarose gels.

Initial validation tests were undertaken using the LAMP primers for seven groups of phytoplasmas (16SrI, -II, -III, -V, -VI, -X, and -XII) by using DNA samples listed in Table 1, plus two DNA samples for 16SrXII phytoplasmas from our frozen DNA collection and healthy periwinkle plant as a negative control, this negative control was confirmed to be phytoplasma free by PCR using P1 and P7 primers. The group specific primers only amplified DNA from phytoplasmas in that specific ribosomal group (Table 4), with no cross-reactions to phytoplasmas from other groups, and with amplification times ranging from 09:23 to 22:26 minutes (MM:SS). The annealing temperatures were consistent within the same ribosomal group and provide a useful evaluation parameter in the real-time LAMP protocol, comparable to the melt curve analysis that is a feature of quantitative PCR assay evaluation. To further verify amplifications, samples from the 16SrI group were also checked by gel electrophoresis (Figure 1).

Further tests were performed on the samples listed in Table 2, and confirmed the specificity of the assays. In addition, the evaluation of the sensitivity of the LAMP assays was performed by diluting DNA samples of the 16SrI *Rehmannia glutinosa* phytoplasma (16SrI-B), and comparing the LAMP with PCR assays. Total (plant and phytoplasma) DNA concentrations were determined by NanoDrop as 29.3 ng/µl, and serial dilutions of 1/10, 1/100, 1/1000, 1/10000 and 1/100000 were made. Whilst the direct PCR assay only detected the phytoplasma DNA in the undiluted and 1/10 diluted sample, both the nested PCR and LAMP assay were able to detect the phytoplasma DNA down to the 1/10,000 dilution (Figure 2).

This study has developed and validated LAMP assays for phytoplasma strains enclosed in seven 16Sr groups based on the *leuS* gene. Previous studies had targeted the ribosomal RNA

gene (Tomlinson *et al*., 2010a; Bekele *et al.,* 2011; Kogovšek *et al*., 2015). However, it has often been difficult to find regions that are sufficiently specific for each of the phytoplasma ribosomal groups, because of the relatively well-conserved nature of such sequences Abeysinghe *et al.* (2016) reported that the *leuS* gene has a specific sequence for most groups of phytoplasma, and this has been reflected through the ability to design LAMP assays for phytoplasmas belonging to a range of different ribosomal groups. It should be noted that the study by Kogovšek *et al.* (2015) has suggested that the amplification efficiency of phytoplasma DNA in LAMP assays can vary among different targeted genes.However, in this study, the time needed to amplify phytoplasma DNA for the different assays was usually within 20 minutes, with some assays, such as those for studied phytoplasmas in 16SrIII and 16SrX groups being around 10 minutes. These amplification times were similar to those previously reported for assays based on the 16S-23S rRNA genes Kogovšek *et al.* (2015), despite the *leuS* gene being a single copy gene (Abeysinghe *et al.,* 2016), which indicates that the design of the primers rather than the copy number of the target gene is a key factor in the efficiency of amplification.

Of the assays designed, all appeared to give good specificity for the phytoplasmas belonging to groups they were designed for. In previous studies, it was found that the rRNA assay for 16SrII is not able to detect all strains or subgroups of group 16SrII tested (Bekele *et al.,* 2011). However, based on the strains tested, the *leuS* based assay deteced all strains used in this ribosomal group. Similarly, the 16SrIII assay was able to amplify DNA from several strains belonging to this group with different amplification times. In the studies of Tomlinson *et al.* (2010a), it was found that the rRNA assay for the 16SrXXII-B CSPWD phytoplasamas (Cape Saint Paul Wilt disease), also amplified the closely related Mozambique lethal decline strains

(16SrXXII-A), so it was able to show good specificity and sensitivity at the ribosomal group level.

Results of the detection sensitivity of LAMP assays show that such assays can amplify phytoplasma DNA with dilutions down to 1/10000, however, nested PCR has similar sensitivity, but the LAMP assays provide these results within 30 minutes, whilst nested PCR takes a few hours. Neither LAMP assays nor PCR could amplify DNA from the sample with 1/100,000 dilution, which agrees with the results of Tomlinson *et al.* (2010a). These results show that real-time LAMP assays can be used in rapid, specific and sensitive detection of phytoplasmas and can be a useful alternative closed-tube approach to quantitative PCR assays.

ETHICAL STATEMENT:

This manuscript has not been published partly or in whole elsewhere. Also, all author and co-authors declare that they have no conflict of interest.

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Table 1: List of phytoplasmas in the plant collection held in the School of Biosciences of the University of Nottingham (used in this study).

Phytoplasma name or host plant name	16Sr Subgroup	Source
Rehmannia glutinosa (RG)	I-B	Czech Republic
Strawberry Green Petal (SGP)	I-C	Czech Republic
Ribes in Vinca (RIV)	I-C	Czech Republic
Faba Bean Phyllody (FBP)	II-C	Fera, UK
Soybean Phyllody (SP)	II-C	Rothamsted, UK
Vinca Coconut Phyllody (VCP)	II	Rothamsted, UK
Plum Leptonecrosis (PL) - LNI	III-B	Fera, UK
Peach Western X (PWX)	III-A	USA from Italy
Elm Yellows (EY-C)	V-A	Fera, UK
Brinjal Little Leaf (BLL)	VI-A	Rothamsted, UK
Potato Witches' Broom (PWB)	VI-A	USA from Italy
Apple Proliferation (AP)	X-A	Rothamsted, UK
Napier Grass Stunt (NGS)	XI	Kenya

'*Candidatus* phytoplasma fragariae'	XII	Cordyline, Cornwall UK, provided by P Jones, Rothamsted Research, UK
'*Candidatus* phytoplasma fragariae'	XII	Cordyline, Jersey provided by P Jones, Rothamsted Research, UK

Table 2. List of additional stored DNA samples used in this study

Strain name	**16Sr Group**	**Sample origin**
Chrysanthemum yellows	I-A	Germany, E. Seemüller
Clover phyllody	I-C	UK, P Jones, Rothamsted Research, UK*
Diplotaxis virescence	I-B	Spain, P Jones, Rothamsted Research, UK*
Aster yellows from apricot	I-F	Germany, E. Seemüller
Aster Yyellows	I-L	Germany, E. Seemüller
Atypic aster yellows	I-M	Germany, E. Seemüller
Cleome phyllody	II-A	Thailand, P Jones, Rothamsted Research, UK*
Faba bean phyllody	II-C	Sudan, P Jones, Rothamsted Research, UK*
Tomato big bud	II-D	Australia, provided by P Jones, Rothamsted Research, UK*
Pichris echoides yellows	II-E	Italy, C. Marcone
Cotton phyllody	II-F	Burkina Faso, provided by P Jones, Rothamsted Research, UK*
Peach X disease	III-A	Canada, provided by P Jones, Rothamsted Research, UK*
Euscelidius variegatus	III-B	Italy, L. Carraro
Poinsettia branching factor	III-H	UK, commercially purchased plant
Lethal yellows *Cocus nucifera*	IV-A	USA, N Harrison, University of Florida, USA
Elm witches' broom (EY-C)	V-A	France, P Jones, Rothamsted Research, UK*
"Flavesence dorée"-C	V-C	Italy, Jones, Rothamsted Research, UK*

"Flavesence dorée"--D	V-C	Italy, P Jones, Rothamsted Research, UK*
Rubus stunt	V-E	Italy, C. Marcone
Potato witches' broom	VI-A	USA, P Jones, Rothamsted Research, UK*
Catharanthus phyllody	VI-C	Sudan, P Jones, Rothamsted Research, UK*
Ash yellows	VII-A	USA, W. Sinclair and Griffith
Pigeon pea witches broom	IX	USA, N Harrison, University of Florida, USA
Apple Proliferation	X-A	Germany, E. Seemüller
German stone fruit yellows	X-B	Germany, E. Seemüller
Pear decline	X-C	Germany, E. Seemüller
Apple proliferation	X-A	Apple tree, Slovenia, P Nikolic, NIB, Slovenia
European stone fruit yellows	X-B	Apricot tree, Slovenia, P Nikolic, NIB, Slovenia
Pear decline	X-C	Pear tree, Slovenia, P Nikolic, NIB, Slovenia
Flower stunting	XI-C	Germany, E. Seemüller
'Candidatus Phytoplasma fragariae'	XII-E	Cordyline, Cornwall UK, P Jones, Rothamsted Research, UK*
'Candidatus Phytoplasma fragariae'	XII-E	Cordyline, Jersey provided by P Jones, Rothamsted Research, UK*
Mexican periwinkle virescence	XIII-A	USA, provided by N Harrison, University of Florida, USA
Bermuda grass whiteleaf	XIV-A	Malaysia, collected by M Dickinson
Cape st paul wilt of coconut	XXII-B	Takoradi, Ghana, collected by M Dickinson

*These isolates were maintained by Dr Jones, Rothamsted Research, UK.

Table 3 LAMP primers designed for 16SrI, -II, -III, -V, -VI, -X, and -XII-group phytoplasmas.

16Sr groups	Primers Sequences 5'-3'
Group 16SrI	F3 AAGAGACAATCATCCAGTTGTT B3 CTGTGCCATAATGAGGAAGAA FIP AAAGAGTATTAGGGCGAGTGGTAATG CAAGTTAATTGGATTGGT BIP CGCCTGAACACGAATTAGCCTTGTCT TTGTTGATGTCTCTTTC Loop_F AACTAGGAAGGAGACGATTGC Loop_B CCGAATACCAACAAGCAGTTAA
Group 16SrII	F3 GGAGTAGAGAATTAGCGACTTC B3 AGGATGTTCAGGAGCCAA FIP CAGGATGACCACCACGTTCTGTTGT CCTCAATTAGGTACAGT BIP TTGGCCAGAGTCTATTAAGGAAAG GCATAGTAGTGAAAACAGAC Loop_F AGAAATAACCTCTTCATTCGCT Loop_B AATGGATTGGCAAAACACCG

Group 16SrIII	F3	ATTAACTACTCCAGATCGTTGG
	B3	CGCTTCTTCATAATTAAGACCG
	FIP	CCCGTACCGTAATGAGGCAAAT GCTATTAACTTTGCTAACCAA
	BIP	CCATTATGGCCGTTCCTTATCAG AAATCTGGGTTTCTTCAGGTA
	Loop_F	AATCAGCGATCCAAATAGGGAT
	Loop_B	AGAGATTTTGAATTCGCTCGT
Group 16SrV	F3	TTTCCTTTCCTGTTTTATCAGA
	B3	TGACATCAAATGATACTTATCG
	FIP	AGGATGTTCTGGCGCTAAAACTAG AAGTTTTCACGACTAAACCA
	BIP	TGGTAGTTACGTTTTTCATCCT GAACCAACATAATAGCTCCTGT
	Loop_F	GCGCTTACTCCAAAAATAGTAC
	Loop_B	TTATGTCCTTCCTTCTTATGCT
Group 16SrVI	F3	TTCAAACAAATTGGATAGGCAA
	B3	GCATCATTAGTGGATTCGGA
	FIP	ACACCTTCGACAAAATCTGTTTTG GAAGTTTTTACGACAAAACC
	BIP	TGAACAAAGATCAAACAGGAGTC CGGTACCATAATAAGGCAAT
	Loop_F	AGGGTGTTCAGGAGATAAAACT
	Loop_B	TGGTAGTTATGCTATTCATCCT
Group 16SrX	F3	AGATAGAGTACAAATGGCAACG
	B3	ATCCATTGCGTCCAATGATAA
	FIP	CGCTGGCCGTATAACCTTCAATGT TTCCCTATCCCTCGG
	BIP	ACATCCTTTTGGTTGGGATTCTTTC TAGGATTGTTGCCAGTT
	Loop_F	TGACCTACATGTAAACCTTCGG
	Loop_B	TTACCAGCTGAACAATATGCTT
Group 16SrXII	F3	AGTTTTAGCTACCGAACATGAA
	B3	CGGAACGATTTCCTTAGTAAGA
	FIP	ATCGGCAACCCAAATAGGTATTGAAC AAAGATAAAAGTGGCGTT
	BIP	TGGTACAGGGATTTTGATGGGATG GTTGAATCACTTGAATCATT
	Loop_F	CCATTGCAAGGATTAATAGCGT
	Loop_B	GTTCCCTGCCACGATCAA

Table 4 Validation of primers for different phytoplasma groups. The top numbers are the time (MM:SS) when amplifications started, and the bottom numbers in brackets are the annealing temperature for each sample (°C)

Isolate	Group16Sr	Name of phytoplasma group assay						
		I	II	III	V	VI	X	XII
RG	I	19:26 (84.74°C)	-	-	-	-	-	-
RIV	I	22:26 (84.35°C)	-	-	-	-	-	-
SGP	I	16:56 (84.27°C)	-	-	-	-	-	-
FBP	II	-	16:38 (82.54°C)	-	-	-	-	-
SP	II	-	16:53 (82.39°C)	-	-	-	-	-
VCP	II	-	15:53 (82.41°C)	-	-	-	-	-
LNI	III	-	-	12:42 (84.67°C)	-	-	-	-
PWX	III	-	-	10:57 (85.47°C)	-	-	-	-
EY-C	V	-	-		18:32 (81.15°C)	-	-	-
PWB	VI	-	-	-	-	17:32 (82.24°C)	-	-
BLL	VI	-	-	-	-	22:32 (82.34°C)	-	-
AP	X	-	-	-	-	-	09:23	-

							(84.22°C)	
NGS	XI	-	-	-	-	-	-	-
* *'Candidatus* Phytoplasma fragariae'. Cornwall	XII	-	-	-	-	-	-	13:27 (84.47°C)
*'Candidatus Phytoplasma fragariae'. Jersey	XII	-	-	-	-	-	-	11:42 (84.57°C)

Data are presented as the time of amplification, with the annealing temperature given in parenthesis. - = no amplification. * these are DNA samples from our frozen collection (Table 2). A range of phytoplasma groups was tested with each primer set to confirm that each set can only amplify for the intended group.

LIST OF FIGURES:

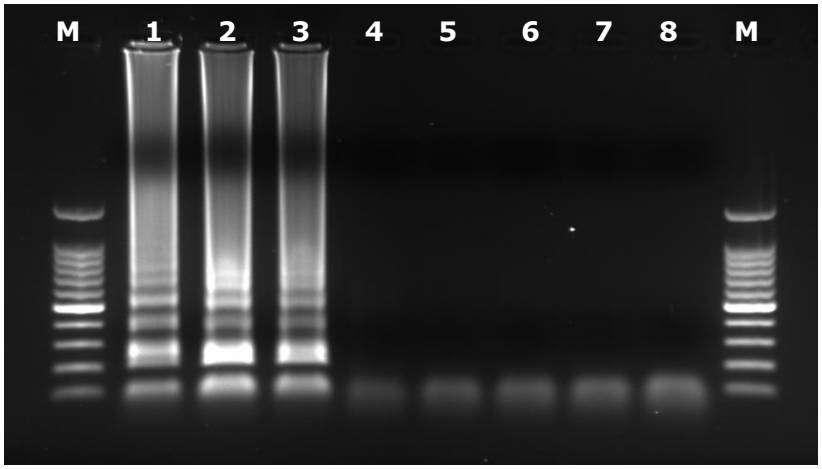

Figure 1: Gel electrophoresis of LAMP products with isolates belonging to different 16rRNA (sub)groups (as abbreviated in Table 1). M-100 bp DNA Ladder (Promega), 1: *Rehmannia glutinosa* (RG), 2: RIV, 3: SGP, 4: SP, 5, LNI, 6: AP, 7: NGS and 8: control (healthy periwinkle plant). Only the first three samples belonging to the ribosomal group 16SrI gave positive results (laddering), confirming the specificity of the 16SrI primers.

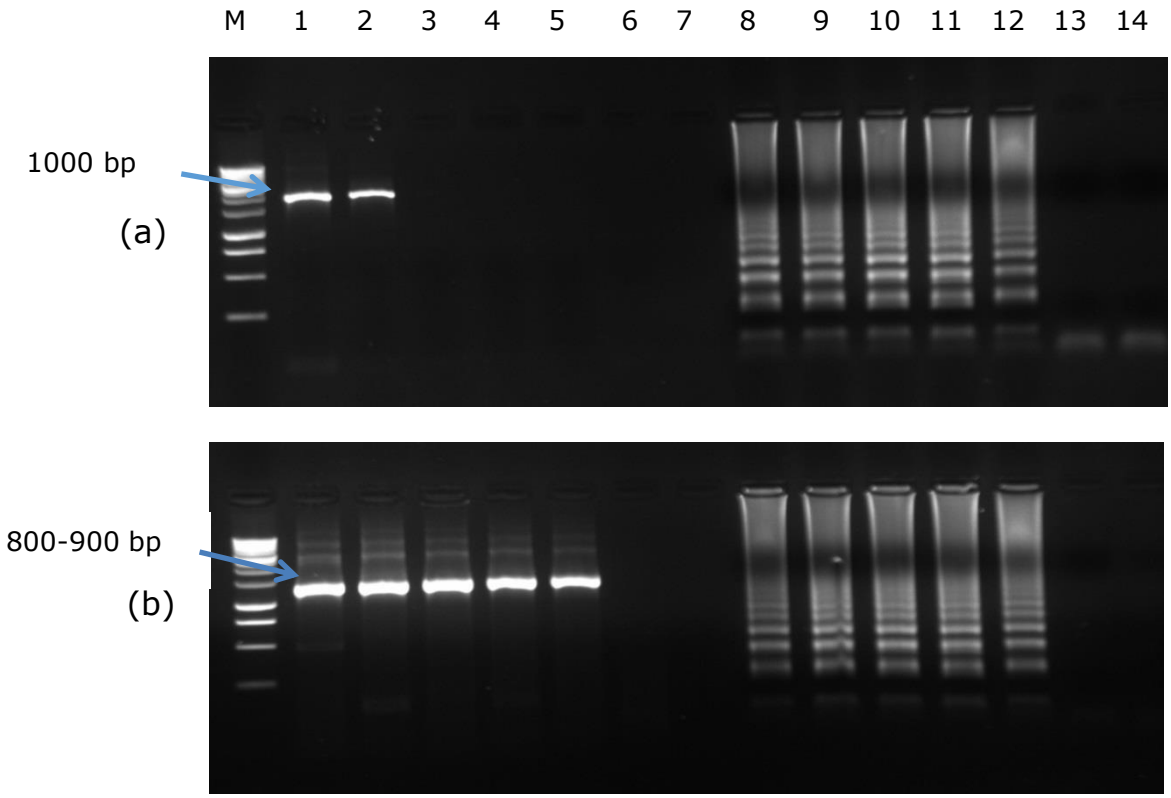

Figure 2: Detection sensitivity comparison of LAMP assays and the conventional PCR using 16S primers. The DNA sample from Reihmannia glutinosa belonging to the ribosomal subgroup 16SrI-B was used for dilutions. Lanes: 1 and 8: undiluted *Rehmannia glutinosa* samples, 2 and 9- 1/10, 3 and 10- 1/100, 4 and 11- 1/1000, 5 and 12- 1/10000, 6 and 13- 1/100000, 7 and 14- negative control (periwinkle healthy plant). Samples 1 to 7 samples were tested by first-round PCR (a) or nested PCR (b) and 8 to 14 by LAMP assays.