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Development and evaluation of a loop-mediated isothermal amplification assay for detection of *Erwinia amylovora* based on chromosomal DNA

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Abstract A reliable and rapid pathogen detection protocol that utilizes loop-mediated isothermal amplification (LAMP) was developed for detection of *Erwinia amylovora*, the casual agent of fire blight. The six LAMP primers applied were derived from the highly conserved fragment of the chromosomally *amsH* gene. Despite the proposed LAMP as well as nested PCR presenting equal values of sensitivity (2×10^1 CFU/ml or more) for pure cultures, as compared with conventional PCR (2×10^3 CFU/ml), both methods were together superior. The specificity assay also showed that the LAMP protocol is species-specific for detection of *E. amylovora* even in inter-species analysis. Meanwhile, when all 208 naturally infected samples were examined, the specificity value of LAMP was 84%, while conventional and nested PCR could detect only 59% and 73% of the whole collection. Significantly, an independent behaviour versus host plant as well as each strain origin was also observed regarding the current LAMP method as well as other two PCR-based methods. All the results, overall, indicated that the LAMP offers an interesting novel and convenient assay format for the quick and specific chromosomal detection and diagnostic tool of recognition of *E. amylovora* and therefore presents an alternative to PCR-based assays.

Keywords Conventional PCR · Nested-PCR · *Erwinia amylovora* · Fire blight · LAMP

Introduction

Erwinia amylovora (Enterobacteriaceae) causing fire blight, the most important threat to pome fruit production (i.e., apple, pear, and quince) globally, was first described in the late 1790s, originated in North America, and then has undergone a rapid spread to many countries throughout the world (Bonn and van der Zwet 2000). The damage of this pathogen generally covers a wide range of Rosaceae (primarily Maloideae), ranging from ornamental and native forest species important in rural economies to cultural heritage and landscape ecosystems (Llop et al. 2000; Duffy et al. 2005). Control of fire blight, like other diseases caused by plant-pathogenic bacteria seems to be

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somehow complicated and accordingly requires precise and reliable detection methods to discriminate the causal organism *E. amylovora* (Palacio-Bielsa et al. 2009).

In this context, however, several primary approaches were first established (van Belkum 2003) all of which unfortunately were time-consuming, wearisome, of low sensitivity and had also some cross-contamination troubles. Hence, to overcome shortcomings of the classical diagnostic methods, a large number of rapid and sensitive techniques developed, the majority of which were based on amplifying DNA sequences (Palacio-Bielsa et al. 2009). Meanwhile, with the advent of a novel and high specific based-PCR method, so-called real-time PCR, the creation of high-throughput screening in a short time was made widely possible (De Bellis et al. 2007; Pirc et al. 2009; Svircev et al. 2009). Even though, this method has been considered as a reliable molecular-based instrument, like other molecular approaches it is occasionally accompanied by some drawbacks (e.g., cost and availability). It is believed that isothermal-based methods particularly loop-mediated isothermal amplification (LAMP) can be extremely helpful without having the limitations of current diagnostic tools (Mori and Notomi 2009).

LAMP commonly is a one-step amplification reaction that amplifies a target DNA sequence with high sensitivity and specificity under isothermal conditions (Notomi et al. 2000). The mechanism of the LAMP reaction can be explained in three steps: an initial step, a cycling amplification step as well as an elongation step. LAMP employs a *Bst* DNA polymerase with strand-displacement activity, along with two inner primers (FIP, BIP) and outer primers (F3, B3) which recognize six separate regions within a target DNA(Notomi et al. 2000). The LAMP assay has high specificity, because the amplification reaction occurs only when all six regions within a target DNA are correctly recognized by the primers. Meanwhile, several methods have been reported to examine the LAMP products: measuring the increase in turbidity derived from magnesium pyrophosphate formation to conclude increase in amplified DNA concentration; producing green or red colour (as positive and negative symptoms, respectively) when the fluorescent dye such as SYBR Green is added to the mixture; and finally the existence of ladder-like bands on agarose gel (Mori et al. 2001; Niessen and Vogel 2010). LAMP has attracted a lot of attention as a potentially fast, truthful, and cost-effective novel nucleic acid amplification method. As a result, more than 500 reports have been recorded to evaluate the LAMP efficiency, for example in recognizing bacterial, viral, fungal and parasitic diseases worldwide (Parida et al. 2008; Mori and Notomi 2009; Fu et al. 2011; Temple and Johnson 2011).

It is noticeable that a number of PCR-based methods have been generally utilized so far in identification of *E. amylovora*, either based on pEA29 (Bereswill et al. 1992; McManus and Jones 1995; Llop et al. 2000; Temple and Johnson 2011) or chromosomal DNA (Bereswill et al. 1995; Taylor et al. 2001; Mohammadi et al. 2009; Gottsberger 2010). Nonetheless, in the previous detection assays for *E. amylovora* which were on the basis of the pEA29 plasmid, some strains have been proved susceptible to be lost or absent in about 1% to 4% of *E. amylovora* field populations (Llop et al. 2006; Brennan et al. 2002; Mohammadi et al. 2009; Gottsberger 2010; Temple and Johnson 2011), and could accordingly lead to some unbiased errors in the final results. Instead, to avoid such inevitable cases, here, all six LAMP primers were consequently designed based on the chromosomal DNA (i.e., *amsH* gene). In the following, the performance ability of the current LAMP protocol was compared with the detection power of both conventional and nested PCR regarding identification of *E. amylovora*. Finding a diversity pattern of pEA29 in *E. amylovora* strains originated from Iran was the last objective of this research.

Materials and methods

Bacteria strains and DNA extraction

A collection of standard bacterial strains containing 18 *E. amylovora* strains and several species of bacteria confirmed by biochemical, carbohydrates and virulence tests for identification of *E. amylovora* isolates (data not shown) were employed to estimate the specificity test (Table 1). Furthermore, in order to assess the performance of two PCR methods and LAMP assay, about 208 symptomatic plant samples, were used. This collection was obtained from various plant tissues (e.g., flowers, shoots, leaves, fruits, and limbs) belonging to apple, pear and quince cultivars

Table 1 Bacteria used in this study and their relevant characteristics

Species and strain(s)	Host plant	Origin	Reference
	Erwinia amylovora		
Ea 273 ATCC 49946	Apple	USA	ATCC[a]
Ea K1	Apple	Karaj-Iran	SPII[b] (2004)
Ea K2	Pear	Karaj-Iran	SPII[b] (2004)
Ea S1	Apple	Karaj-Iran	SPII[b] (2004)
Ea Z1	Apple	Zanjan-Iran	SPII[b] (2004)
Ea Z2	Pear	Zanjan-Iran	This study
Ea 1	Wild pear	Loristan-Iran	This study
Ea 2	Wild pear	Loristan-Iran	This study
Ea 3	Apple	Loristan-Iran	This study
Ea 6	Quince	Semnan-Iran	This study
Ea 9	Apple	Loristan-Iran	This study
Ea 11	Quince	Loristan-Iran	This study
Ea 12-1	Pear	Loristan-Iran	This study
Ea 12-2	Pear	Loristan-Iran	This study
Ea 16	Pear	Ghazvin-Iran	This study
Ea 32	Quince	East Azerbaijan-Iran	This study
Ea 33-2	Quince	Semnan-Iran	This study
Ea 36	Apple	East Azerbaijan-Iran	This study
Pantoea agglomerans (Erwinia herbicola) IRIPP Abp2	Pear	Karaj-Iran	IRIPP[c] (2010)
Pectobacterium carotovorum (*Erwinia carotovora*) ECC	Potato	Netherland	Yao et al. (1995)
Pseudomonas syringae ATCC 11355	Apple	USA	ATCC[a]
P. fluorescens CHAO	–	France	Meyer et al. (1992)
Salmonella enteritidis ATCC 49223	–	USA	ATCC[a]
Klebsiella pneumonia ATCC 75388	–	USA	ATCC[a]
Escherichia coli O157: H7 ATCC 35150	–	USA	ATCC[a]

[a] American Type Culture Collection, Manassas, USA

[b] Seed and plant improvement institute, Karaj, Iran

[c] Iranian Research Institute of Plant Protection, Tehran, Iran

originating from different regions of Iran, during spring and summer of 2009 and 2010. For preparing samples, the same method of Gorris et al. (1996) was used: 100 µl of each dilution and other standard bacterial routinely cultivated on Luria-Bertani agar (LB) or LB agar medium and incubated at 28°C for 48 h. In the following, total genomic DNA of each standard strain was isolated by lysis of bacterial pellets from 1 ml of broth culture, incubated overnight in DNA extraction buffer, purified with phenol–chloroform–isoamyl alcohol (25:24:1) and precipitated with isopropyl alcohol (Llop et al. 1999, 2000; Schaad et al. 2001), DNA isolated from each strain was lastly eluted in 100 µl of elution buffer and stored at −20°C prior to further assessment. The numbers of *E. amylovora* in final suspension of each culture were pelleted, washed with water, and adjusted to 2×10^7 CFU/ml ($OD_{600\ nm}=1$), determined by plating dilutions on to LB agar. Dilutions series were prepared in water, and 2 µl of each dilution was used for LAMP, conventional and nested. Sensitivity threshold experiments were performed three times. Even though the LAMP method should be as simple as possible even in DNA extraction, since here conventional and nested PCR were used, both of which strongly depend on quality and quantity of genomic DNA, a Plant Mini Kit (*Qiagen*) was accordingly utilized to avoid probable errors. It is noticeable that our current LAMP protocol exhibited a positive response even without DNA extraction.

Conventional PCR

Since the primer pair named Ea71(1) and Ea71(2) (Table 2), of Taylor et al. (2001), has been proved to have enough potential in producing more reliable results, this primer pair was accordingly selected for detection of *E. amylovora*. PCR amplification was carried out in a Bio-Rad thermocycler. The amplification was done in a 50 μl volume containing 2.5 μl of 10X buffer (Tris-HCl (pH 8.3) and KCl) 1.5 mM $MgCl_2$ (CinnaGen Co., Iran), 0.5 μM of primer, 0.2 mM of dNTPs (CinnaGen Co., Iran), 2 U of *Taq* DNA polymerase (CinnaGen Co., Iran), and 2 μl template DNA. Amplification was performed with the following PCR profile: 3 min at 95°C (1 cycle); 35 cycles of 20 s at 94°C, 20 s at 60°C, 60 s at 72°C and 5 min at 72°C for final extension. PCR products were visualized by staining with ethidium bromide after electrophoresis on 1% agarose gel. Finally, using a UV transilluminator equipped with a video, a photo of each gel containing PCR fragments (expected size 187 bp) of all strains of *E. amylovora* was provided for further processing.

Nested PCR

Nested PCR was performed using external primers named AJ75 and AJ76 with annealing temperature of 72°C which lie within 844 bases of the fragment from the 29-kb plasmid pEA29 amplified and also internal primers PEANT1 and PEANT2 produce amplification products at 56°C. Here, the protocol of (Llop et al. 2000) was employed with some minor modifications using external and internal primers (Table 2). Moreover, the final volume of each PCR reaction was 50 μl with the following reagents: 2.5 μl of 10X buffer (Tris-HCl (pH 8.3) and KCl) 3 mM $MgCl_2$, 0.2 mM of dNTPs (CinnaGen Co., Iran), 0.5 μM each of external primers AJ75 and AJ76 and 1.5 μM each of internal primers PEANT1 and PEANT2, 3 U of *Taq* polymerase (CinnaGen Co., Iran) and 2 μl template DNA. Each reaction consisted of denaturation step of 94°C for 4 min followed by 25 cycles of 94°C for 30 s and 72°C for 1 min. This first round of PCR amplification was followed in the same thermocycler by a second denaturation step of 94°C for 4 min and 40 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s. Then, PCR products were visualized after electrophoresis on 1% agarose gels. Lastly, all amplicons of the expected size (391 bp) were detected for all strains of *E. amylovora*.

Design of primers for LAMP

LAMP specific primers were designed using the Primer Explorer V4 software (http://primerexplorer.jp/e/).

Table 2 Details of primers used in this study

Primers	Primer sequence (5′-3′)	Target	Amplicons (bp)	References
	Conventional primers (nt)			
Ea71(1)	CCTGCATAAATCACCGCTGACAGCTCAATG	Hypothetical Protein	187	Taylor et al. (2001)
Ea71(2)	GCTACCACTGACGCTCGAATCAAATCGGC			
	Nested primers (nt)			
AJ75[a]	CGTATTCACGGCTTCGCAGAT	pEA29	844	McManus and Jones (1995)
AJ76[a]	ACCCGCCAGGATAGTCGCATA			
PEANT1[b]	TATCCCTAAAAACCTCAGTGC	pEA29	391	Llop et al. (2000)
PEANT2[b]	GCAACCTTGTGCCCTTTA			
	LAMP primers (nt)			
AMSHFIP	CCACCAGCGGCATTAATGGCATTTTTAACTTCAGGTCAGCAAGCG	*amsH*	148	This study
AMSHBIP	GCAGACTGGCGCAATGTGGTTTTTCAGAGCCTGTAGGGAAACAG			
AMSHF3	ACGTAACTGGCGAAGTGAC			
AMSHB3	TGATTTTGCGACGGGTCAC			
AMSHLF	AGGATAGTCAGGGGGACGTTG			
AMSHLB	GCTAACGCATGACGGACGC			

[a] External primers

[b] Internal primers

Based on a conserved fragment of the *amsH* gene that belongs to an *ams* cluster genes, which includes 12 genes (*amsA* to *amsL*) organized in a large cluster, (Vanneste 2000) (GenBank accession number X77921), an entire set of four primers recognizing six distinct regions in the target sequence were designed, including outer primers (F3 and B3), and inner primers (FIP and BIP). It is noticeable that in order to accelerate LAMP reaction as well as reduce the time, additional loop primers (LF and LB) were utilized (Table 2). Figure 1 presents the position of these primers within the nucleotide sequence of the *amsH* gene.

Optimization of LAMP reaction conditions

The LAMP reaction was conducted as described by Notomi et al. (2000), with some minor modifications. The LAMP assay was carried out in a 25 µl reaction mixture containing 2.5 µl 10X ThermoPol Buffer (New England Biolabs) 1.2 mM dNTPs (CinnaGen Co., Iran), 6.0 mM $MgSO_4$, 1 M betaine (Sigma-Aldrich), 1.6 µM each of FIP and BIP, 0.2 µM each of F3 and B3 and 0.4 µM each of LF and LB primers, 2 µl template DNA, and finally 8 units of the *Bst* DNA polymerase large fragment (New England Biolabs). The mixture was incubated at 63°C for 45–60 min using a Thermo Block (simple heating block); the mixture, in the following, was heated at 80°C for 5 min to terminate the reaction, and finally all LAMP products were separated on 1.5% (w/v) agarose gel for confirmation of the LAMP products.

Real-time LAMP

To confirm LAMP results, a Real-time LAMP reaction was also performed for all standard strains of *E. amylovora*. Each reaction consisted of 25 µl: 2.5 µl 10 X ThermoPol Buffer (New England Biolabs), 1.2 mM dNTPs (CinnaGen Co., Iran), 6.0 mM $MgSO_4$, 1 M betaine (Sigma-Aldrich), 1.6 µM each of FIP and BIP, 0.2 µM of each F3 and B3 and 0.4 µM of each LF and LB primers, 8 units of *Bst* DNA polymerase large fragment (New England Biolabs), 0.8 µM SYBR® Premix Ex Taq™ II (Perfect Real TIME, TAKARA Bio Co, LTD, RR081A) and 2 µl template DNA. PCR amplification was accomplished in a real time thermocycler (Roto Gene CR6000).

Detection and confirmation of LAMP products

The LAMP amplification products were first inspected by visual detection (turbidity). Then, about 0.5 µg ethidium bromide/ml, (Sigma) was added to each tube and positive products were identified when a yellow colour was detected under a UV transilluminator. Likewise, 0.8 µM SYBR® Premix Ex Taq™ II dye was added to 10 µl LAMP products, after a short vortex, each reaction was examined under a UV transilluminator (302 nm) to produce green and red colours as positive and negative evidence, respectively. Finally, the products were monitored using agarose gel electrophoresis stained with ethidium bromide. To confirm amplified LAMP products of the correct DNA target, PCR amplification was performed using two outer primers, F3 and B3, to amplify the LAMP products. The PCR assay was carried out in 25 µl reaction mixture containing 2.5 µl of 10X buffer (Tris-HCl (pH 8.3) and KCl), 1.5 mM $MgCl_2$, 0.2 mM of dNTPs (CinnaGen Co., Iran), 0.5 µM each of F3 and B3, 2 U of *Taq* DNA polymerase (CinnaGen Co., Iran), and 0.5 µl LAMP products. Initial denaturation was conducted at 94°C for 3 min, followed by 35 cycles of denaturation (60 s at 94°C), annealing (60 s at 57°C) and extension (60 s at 72°C). About 5 µl of LAMP products and PCR were subjected to 3% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light. Using a 100-bp DNA ladder, the expected length of the PCR amplification products was identified (e.g., 148 bp), implying the fact that this

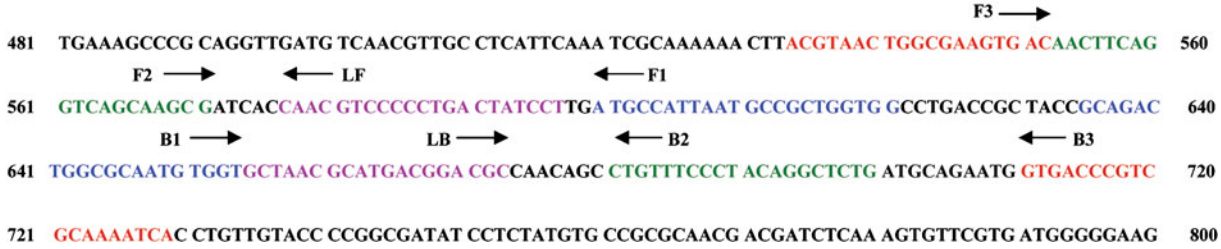


Fig. 1 Nucleotide sequences of targets for primers in the LAMP assay on *E. amylovora* (Ea7/74) *amsH*, (GenBank: X77921)

segment belongs to the genome of *E. amylovora*. Moreover, to verify the specificity of the LAMP reaction, the amplified product was purified by ethanol precipitation and digested with a restriction enzyme *Hga*I (Fermentas) at 37°C for 30 min before electrophoresis on 3% agarose gel.

Detection of plant tissues-derived *E. amylovora*

Different infected parts of apple, pear and quince originating from three geographical areas of Iran (see the above) were used to compare the detection power of the three mentioned diagnostic method regarding *E. amylovora* (Table 3). In Iran, because of the suitable conditions for horticultural activities, a large area of land, particularly in the north, west and centre have been allocated to plant fruit trees. Similarly to other regions worldwide, in Iran the majority of gardens have been affected by different bacterial and fungal diseases like fire blight, often accompanied by negative effects on annual yield of orchards (Abdollahi, unpublished data). Accordingly, these orchards were used to determine whether our current LAMP protocol could recognize different isolates of *E. amylovora*, which have been reported in ample orchards located in Iran. Both sample preparation and DNA (genomic and plasmid) extraction were performed using the above mentioned methods. To avoid or reduce probable errors, all LAMP and PCR analyses followed by DNA extraction of each sample were repeated twice, and in all assays 2 μl template DNA (100 ng/μl) was used.

Results

Detection and confirmation of LAMP product

To examine current LAMP protocol, several successive steps were performed: LAMP products were first identified using visually observations in tubes containing white magnesium pyrophosphate precipitate leading to an acceptable cloudy white colour pattern (Fig. 2a). A clear sharp solution with yellow ochre colour was then observed as 0.5 μg ethidium bromide/ml was added (Fig. 2b). Augmenting 0.8 μM SYBR® Premix Ex Taq™ II to 10 μl LAMP products was the third step, resulted in green and red colour for positive and negative LAMP PCR reactions, respectively (Fig. 2c). All LAMP amplicons as ladder-like band patterns were finally visualized after running on 1.5% agarose gels (Fig. 2d). Meanwhile, to confirm LAMP reaction specificity, PCR was used to amplify these LAMP products, an expected 148 bp fragment was obtained (Fig. 3). The LAMP amplified products were then purified with ethanol precipitation and digested with a restriction enzyme (*Hga*I) at 37°C for 30 min before electrophoresis on 3% agarose gel. In addition,

Table 3 Performances of conventional, nested and LAMP assays in detecting *E. amylovora* in naturally infected pears, apples and quinces from different Iranian locations

Plant species	Location	Samples tested[a]	Positive samples		
			No (%)		
			Conventional PCR	Nested PCR	LAMP
Apple	Karaj	21	12 (57%)	16 (76%)	19 (90%)
pear		24	15 (62%)	16 (67%)	20 (83%)
Quince		23	13 (57%)	18 (78%)	19 (83%)
apple	Ghazvine	22	12 (55%)	17 (77%)	18 (82%)
Pear		23	12 (52%)	18 (78%)	20 (87%)
Quince		25	16 (64%)	15 (60%)	23 (92%)
apple	Zanjan	24	15 (62%)	16 (67%)	20 (83%)
pear		22	13 (59%)	18 (81%)	18 (81%)
Quince		24	14 (58%)	17 (71%)	18 (75%)
Total	–	208	122 (59%)	151 (73%)	175 (84%)

[a] Randomly collected from various commercial orchids

the result of digestion DNA component fragments, with a restriction enzyme (*Hga*I), were approximately about 126 and 22 bp, in good agreement with the predicted sizes from the expected DNA (Fig. 3). Real-time LAMP reaction used in this study was performed by measuring fluorescence derived from SYBR® Premix Ex Taq™ II, which binds to the DNA, using a real-time thermal cycler. The generation of a standard curve confirmed that amplification could begin between 18 and 34 min (data not shown).

Both nested PCR products (Fig. 2e) and conventional PCR products (Fig. 2f) were electrophoresed on 1% agarose gels containing ethidium bromide, the same materials with the same order were used. In conventional PCR, as expected for of all strains of *E. amylovora*, PCR fragments with the same expected allelic size (i.e., 187 bp) were detected. Contrary to the conventional PCR, even though in nested PCR, surprisingly a reasonable size variation ranging from 391 bp to 490 bp was detected (Fig. 2e), showing a high level of pEA29 plasmid-based diversity in Iran. In addition, 4 out of 208 samples (nearly 2%) were surprisingly plasmid-free, which were confirmed then by biochemical tests (data not shown). These results were consistent with the previous studies (Brennan et al. 2002; Llop et al. 2006; Mohammadi et al. 2009; Gottsberger 2010). For instance, in the study of Brennan et al. (2002), among 65 used samples, 4 strains had no plasmid, while Llop et al. (2006) could introduce two strains free of plasmid. In Iran, the same phenomenon was reported first by Mohammadi et al. (2009) who observed just one strain (i.e., EaIrn 37) with no plasmid. More recently, Temple and Johnson (2011), introduced a novel pEA29 plasmid-based LAMP protocol for detection of *E. amylovora* in the U.S., similarly 1% of the used samples had no plasmid. All the results evidently show that using pEA29 plasmid-based assays may lead to some ambiguous results; alternatively it is advisable to use chromosomal-based LAMP which seems to provide more reliable results.

Sensitivity of the LAMP assay

Both quality and quantity of DNA template may have a dramatic influence on the results of each PCR method. To determine sensitivity value of the LAMP assay together with other two PCR methods, a seven-dilution series (2×10^0 to 2×10^7 CFU/ml) of standard *E. amylovora* 273-DNA were prepared, dilutions series were prepared in water, the numbers of *E. amylovora* in final suspension of each culture were pelleted, washed with water, and adjusted to 2×10^7 CFU/ml ($OD_{600\ nm}=1$), determined by plating dilutions on to LB agar, 2 μl of each dilution was used for LAMP, conventional and nested. Sensitivity threshold experiments were performed three times. Our results, interestingly, indicated that both LAMP (Fig. 4a), and nested PCR (Fig. 4b) can equally produce reliable products even under lower DNA concentrations (2×10^1 CFU/ml or more), whilst the third one, conventional PCR, requires higher level of DNA (at least 2×10^3 CFU/ml). All of the above results indicate that detection of *E. amylovora* by the LAMP assay and nested PCR is approximately 100 times more sensitive as compared with conventional PCR (Fig. 4c).

Specificity of LAMP detection

To test whether the LAMP assay have enough potential to detect different strains of bacteria, in addition to all *E. amylovora*, we also used several standard species of bacteria including *E. herbicola*, *E. carotovora*, *Pseudomonas fluorescens*, *P. syringae, Salmonella enteritidis, Klebsiella pneumonia* and *Escherichia coli* O157 (see Table 1). Likewise, to verify LAMP consequences, both conventional PCR and nested PCR assay were also accomplished as described above. Surprisingly, regardless of all the *E. amylovora*, there were no distinguishable bands concerning other mentioned species as well as the negative control, indicating that this process is clearly species-specific for *E. amylovora* in the three mentioned methods.

Comparison of LAMP with conventional and nested PCR assays

To assess the detection ability of the LAMP and other two PCR methods in naturally-infected plant material (described previously), 208 samples were employed. Among three methods, generally, LAMP assay showed the highest power of detecting the pathogen in all symptomatic samples (Table 3). In fact, conventional and nested PCR detected 59% and 73% of positive samples, respectively while, by the use of LAMP method the specificity value reached to 84% of positive samples. In order to justify this phenomenon, it is believed that the natural inhibitors like phenol can seriously diminish the efficiency of the

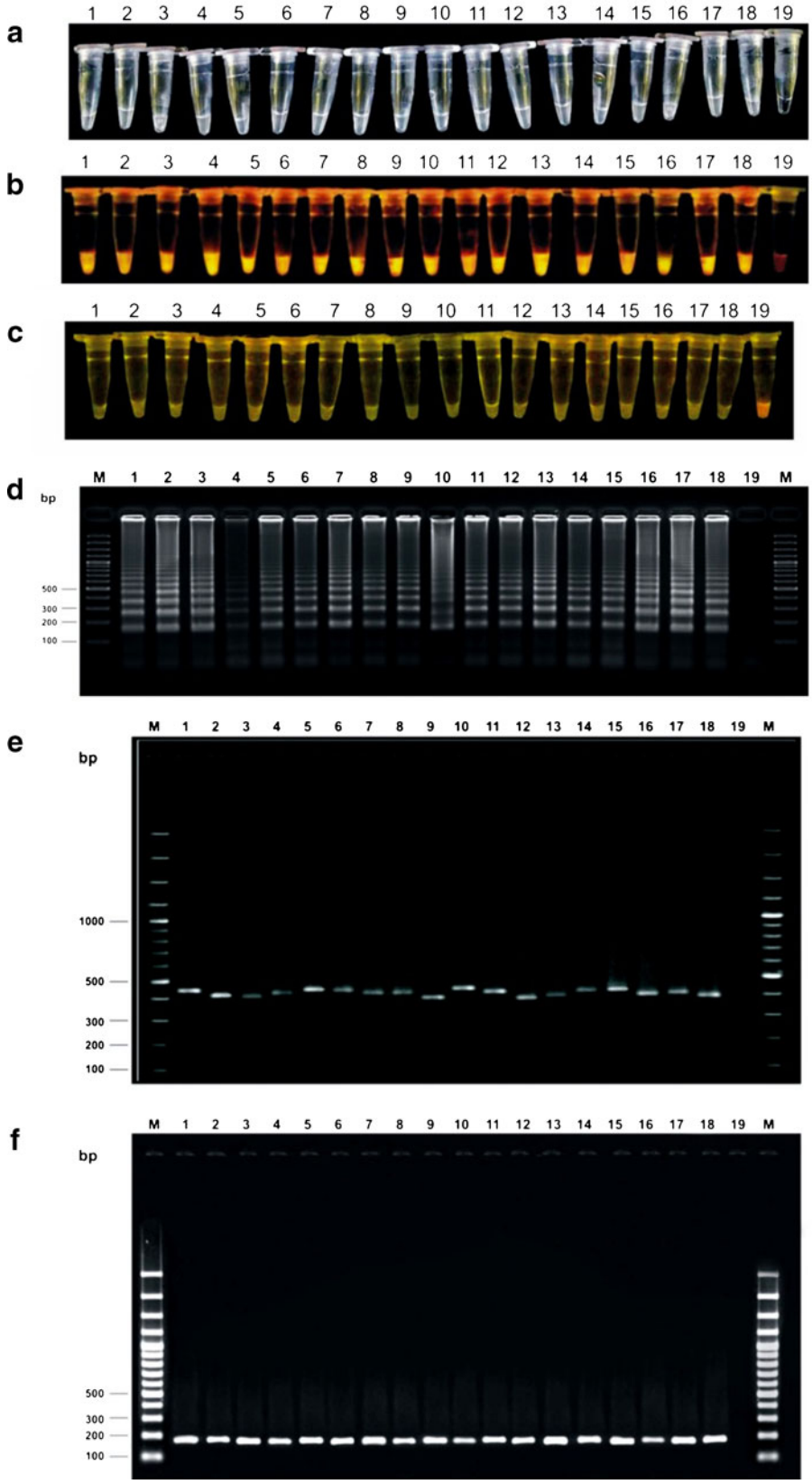
reaction. As a result, a negative reaction should be repeated several times which is not cost-effective and sometimes due to the cost is impossible. Interestingly, these inhibitors have no significant effects on the LAMP reaction (Kaneko et al. 2007), as the same observations were detected in this study. Furthermore, as shown in Table 3, the specificity value of LAMP ranged from 81% (for Zanajn pear samples) to 90% (for Karaj apple samples), but these differences seem to be statistically insignificant. These results indicate that LAMP technology is independent of the host plant or the origin of each strain. The same finding was also reported in the study of Gottsberger (2010) who employed 439 plant and bee samples to evaluate the performance ability of three different PCR methods. In the study of Llop et al. (2000), however, when analyzing symptomatic and asymptomatic plant material, in both positions, the number of positive samples detected by the developed nested PCR was the highest relative to both conventional PCR and two-tube nested PCR. We again point out that our current LAMP protocol is much easier, more efficient, sensitive (in infected natural samples) and most surprisingly applied in the field phase, which particularly the last item is impossible concerning nested and conventional PCR.

On the other hand, during visualizing of amplified products of the conventional and nested-PCR reactions, a number of nonspecific DNA amplicons were also detected. It is argued that these non-specific amplicons are false positive data as the same results were reported for example in the study of (Bereswill et al. 1992; Llop et al. 2000; Mohammadi et al. 2009). On the subject of nested-PCR, the proportion of false positives amplified in the first round was remarkably higher than the second denaturation step, which is mainly because of the presence of large-size fragments in the first step. Even so, no false positive bands were detected for the LAMP assay here, in the LAMP assay the same products or even false negatives may occur for example if the plant materials infected by a specific pathogen were directly analyzed (Niessen and Vogel 2010). Besides, degraded template DNA may also inhibit successful PCR amplification, leading to make both nonspecific and nonamplified DNA fragment(s) of interest. It is noticeable that even though *amsH*-based primers for conventional PCR be may accompanied by false positives (Powney et al. 2011), since in the LAMP assay four primers detect six regions of the target gene, no false positive band could be observed.

Fig. 2 Details of three different steps of visualization of LAMP analysis, (**a**) Left to right: tube1 to tube18, consist of Ea1, Ea2, Ea3, Ea6, Ea9, Ea11, Ea12-1, Ea12-2, Ea16, Ea32, Ea273, Ea33-2, Ea36, EaZ1, EaZ2, EaK1, EaK2 and EaS1 as a cloudy white color and tube 19 negative reaction (distill water) as a clear liquid, respectively; (**b**) tube1 to tube19 like above with containing EB that produce yellow ocher color for positive tubs and pale red color for negative reaction (distill water); (**c**) and tube1to tube19 like above with containing 0.8 μM SYBR® Premix Ex TaqTM II that followed by observing green color for positive tubes and red color for negative one (distill water). Electrophoresis pattern of three methods LAMP, nested PCR and conventional PCR. (**d**) A banding pattern of LAMP analysis separated on a 1.5% agarose gels: Lane 1 to 18 consist of Ea1, Ea2, Ea3, Ea6, Ea9, Ea11, Ea12-1, Ea12-2, Ea16, Ea32, Ea273, Ea33-2, Ea36, EaZ1, EaZ2, EaK1, EaK2 and EaS1, Lane 19, labeled as blank lane, containing negative control (distill water), and finally, size markers positions are indicated in lane M (100 bp sequencing ladder); (**e**) nested PCR banding pattern analysis separated on a 1% agarose gels: obtaining from amplifying pEA29 fragments using PEANT1 and PEANT2 internal primers Lane 1 to 19 and lane M like above; (**f**) conventional PCR products analysis separated on a 1% agarose gels: Lane 1 to 19 and lane M like above

Discussion

Plant diseases continue to affect annual yield of a large number of plant species around the world, most of the time leading to large-scale damage economically. As one case, *E. amylovora* isolates have been able to diminish remarkably the production of some fruit trees including apple, pear, quince, etc. (Bonn and van der Zwet 2000). Meanwhile, for prevention strategies to protect crops, the availability of cost-effective, fast, reliable and simple screening methods with high throughput characteristics is required. Over the last decades, consequently, several nucleic acid-based amplification methods for recognition of *E. amylovora* infection have been developed, including conventional PCR (Bereswill et al. 1992, 1995; Taylor et al. 2001; Kokoskova and Mraz 2005), nested PCR (McManus and Jones 1995; Llop et al. 2000), real-time PCR (De Bellis et al. 2007; Mohammadi et al. 2009; Pirc et al. 2009; Svircev et al. 2009; Gottsberger 2010). Each technique can be accompanied by some advantages and disadvantages. A collection composed of 208 symptomatic plant samples collected from different geographical areas of Iran were selected to make a comparative analysis of three routine detection procedures followed by introducing the best diagnostic method(s). The current study actually is the first one about discriminating *E. amylovora* strains of Iran by


a
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
b
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
c
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
d
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 M
bp
500
300
200
100
e
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 M
bp
1000
500
300
200
100
f
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 M
bp
500
300
200
100

chromosomal DNA-based methods and by now only one investigation has been accomplished in the U.S. but only on the basis of the pEA29 Plasmid (Temple and Johnson 2011). This is a key factor at least since some strains of *E. amylovora* can continue their survival without the plasmid. In fact, as a result of the intermediate copy number of plasmid pEA29, primers derived from the plasmid will be more sensitive than chromosomal primers and might be preferred to screen for low levels of the fire blight pathogen. To support this idea, Mohammadi et al. (2009) noted that some strains of *E. amylovora* from Iran appear to have no plasmids. The lack of this plasmid has been also determined in the U.S. (1%, Temple and Johnson 2011; 3–6% of isolates from the Pacific Northwest, V. O. Stockwell, unpublished data,). To examine this hypothesis, the plasmids of all 208 plant samples were isolated and analyzed as described in laboratory manuals (Mohammadi et al. 2009). Four samples (nearly 2%) surprisingly had no plasmid without any interference in amplifying LAMP products, while no band was observed when the same samples were employed in the nested PCR reactions. This accordingly demonstrates that a substitution of pEA29-encoded functions by another plasmid may not be required for natural survival of the plasmid-free pathogen (Llop et al. 2006). In addition, chromosome-derived LAMP primers can identify *E. amylovora*, even when the pathogen has no pEA29 plasmid. As a whole, the current chromosomal-

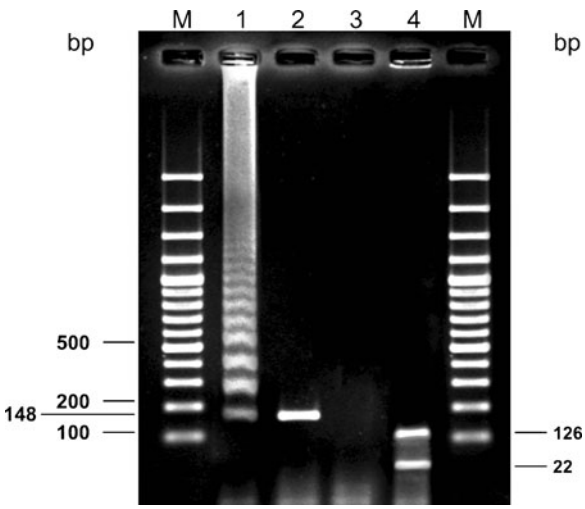


Fig. 3 Confirmation of LAMP products; Left to right: lane 1, product of the LAMP reaction; lane 2, PCR product after amplified these LAMP products, an expected 148 bp fragment was obtained; Lane 3, negative reaction (distilled water) of PCR amplification; lane 4, products of the LAMP reaction were digested by the enzyme *Hga*I and digested products were analyzed in a 3% agarose gel containing ethidium bromide to evaluate the primer specificity, DNA component fragments, approximately about 126 and 22 bp were obtained; lane M, DNA size Marker (100 bp; Fermentas)

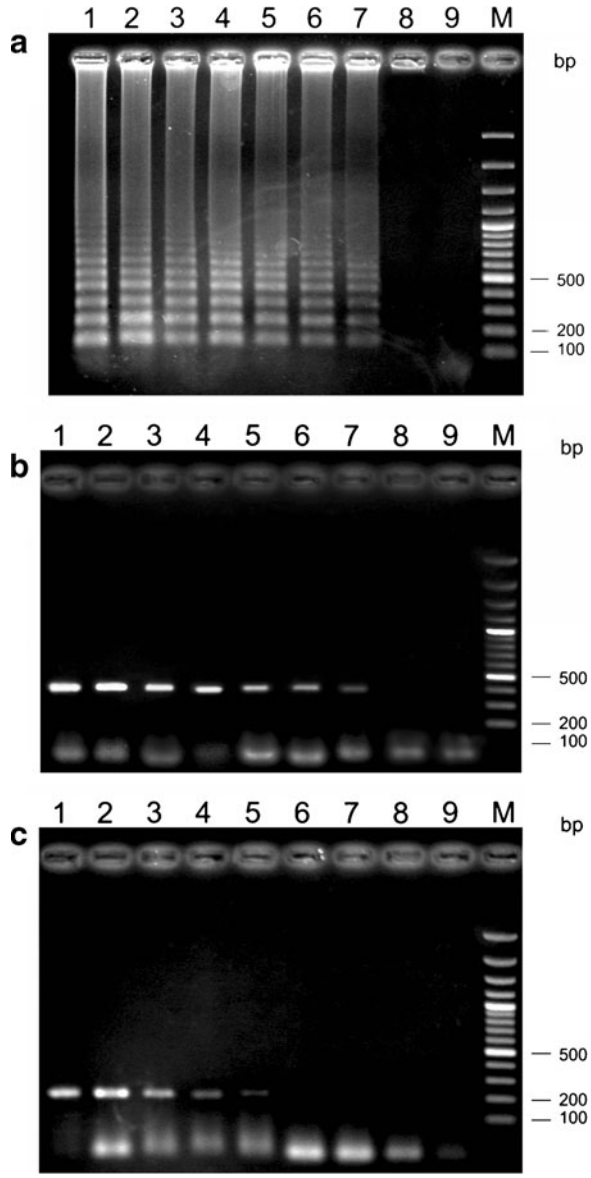


Fig. 4 Comparative analysis of the sensitivity of LAMP, conventional PCR and nested PCR, using a seven dilution series of *E. amylovora* 273-DNA as template; Left to right: Lanes 1–8, LAMP, conventional PCR and nested PCR 2×10^7, 2×10^6, 2×10^5, 2×10^4, 2×10^3, 2×10^2, 2×10^1 and 2 CFU/ml, respectively; Lane 9, negative distill water, Lane M, DNA size Marker (100 bp); (a) Electrophoresis analysis of LAMP; (b) nested PCR and; (c) conventional PCR products reaction. All products were run on 1.5% agarose gels and stained with ethidium bromide

based LAMP protocol seems to have much more potential in detection of different strains of *E. amylovora* versus the first one.

The performances of both conventional and nested PCR were also assessed and compared with the detection power of current LAMP assay. Overall, the behaviour of these three diagnostic techniques on each PCR reaction with different DNA concentrations known as sensitivity value followed a systematic trend: conventional PCR sensitivity appears to be slightly dependent on the higher DNA concentrations (at least 2×10^3 CFU/ml or more), whereas LAPM and nested PCR could easily create reliable amplicons even under lower concentrations of template DNA (i.e., 2×10^1 CFU/ml). On the other hand, the rate of sensitivity detection by naked-eye inspection based on colour change was slightly lower than the electrophoresis-based approach. Nevertheless, considering the time-consuming procedure of the LAMP against conventional PCR (3.5 h vs. 45 min) and nested PCR (5.5 h vs. 45 min), the LAMP by the naked-eye inspection is undoubtedly superior to the other two mentioned techniques. Anyway, in the study of Temple and Johnson (2011) on detection of different strains of *E. amylovora*, this parameter was 10^2 CFU/ml. Llop et al. (2000) showing the same sensitivity value for nested PCR more than conventional PCR. Surprisingly, in a LAMP-based research versus conventional PCR to distinguish *Schistosoma japonicum*, the former was 10000 times more sensitive than the latter (Xu et al. 2010). It is also noticeable that although LAMP and nested-PCR had the same sensitivity, LAMP specificity was far higher. This perhaps arises from the fact that nested-PCR is commonly based on plasmids, and surprisingly some *E. amylovora* strains not only in Iran (Mohammadi et al. 2009) but also elsewhere (Brennan et al. 2002; Llop et al. 2006; Gottsberger 2010), may continue their life without the pEA29 plasmid, while the main principle of LAMP primers strongly depends on the chromosomal DNA (*ams* cluster genes). Accordingly, our new LAMP protocol can be applicable not only to recognize *E. amylovora* even in field phases, but it can also cover strains lacking the pEA29 plasmid or strains showing possible variation in other less conserved genomic areas.

In conclusion, the sensitivity value of the proposed LAMP protocol to recognize *E. amylovora* was equal to that of nested-PCR, the high performance of this technique will be revealed if its specificity is also taken into account. On the other hand, our current LAMP protocol is on the basis of chromosomal DNA of bacteria, it can be consequently more useful for the specific detection and identification of all *E. amylovora* isolates as well as strains with no pEA29 plasmid. In addition, since LAMP methodology does not require expensive equipment such as a PCR machine, and its results can also be visually observed by the appearance of white and green colouration in positive responses, it is accordingly extremely suitable for example in detecting a wide range of destructive diseases in laboratories and particularly under field conditions.

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