Loop-Mediated Isothermal Amplification of Specific Endoglucanase Gene Sequence for Detection of the Bacterial Wilt Pathogen Ralstonia solanacearum

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Abstract
The increased globalization of crops production and processing industries also promotes the side-effects of more rapid and efficient spread of plant pathogens. To prevent the associated economic losses, and particularly those related to bacterial diseases where their management relies on removal of the infected material from production, simple, easy-to-perform, rapid and cost-effective tests are needed. Loop-mediated isothermal amplification (LAMP) assays that target 16S rRNA, flfC and egl genes were compared and evaluated as on-site applications. The assay with the best performance was that targeted to the egl gene, which shows high analytical specificity for diverse strains of the betaproteobacterium Ralstonia solanacearum, including its non-European and non-race 3 biovar 2 strains. The additional melting curve analysis provides confirmation of the test results. According to our extensive assessment, the egl LAMP assay requires minimum sample preparation (a few minutes of boiling) for the identification of pure cultures and ooze from symptomatic material, and it can also be used in a high-throughput format in the laboratory. This provides sensitive and reliable detection of R. solanacearum strains of different phylotypes.

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Introduction
Ralstonia solanacearum is a Gram-negative soil-borne betaproteobacterium that is the causal agent of bacterial wilt disease, or potato brown-rot disease [1,2]. Ralstonia solanacearum is listed as one of the most important plant pathogens [3], as its host range covers more than 450 plant species that belong to over 50 plant families [2], the majority of which are members of Solanaceae and Musaceae [4]. Among these, there are a number of economically important crops, including potato, tomato, eggplant, tobacco and pelargonium [5,6], and therefore an effective diagnostic system for use during the production and importation of these plants is needed [7].

The exceptional host range and evolution of R. solanacearum reflect the heterogeneous nature of this ‘species complex’ [8]. Based on phylogenetic grouping and deeply separated evolutionary lineages, R. solanacearum is divided into four phylotypes that reflect its geographic isolation and spatial distances: phylotypes I and II are composed of Asian and American strains, respectively, phylotype III members are of African origin, and phylotype IV isolates originate from Indonesia, Japan, and Australia, and include R. syzigii and blood disease bacteria (BDB). These phylotypes are further subdivided in sequevars that are based on differences in the sequences of a portion of the endoglucanase (egl) gene [9,10]. Strains of R. solanacearum phylotype IIB, sequevar 1 (historically known as race 3 biovar 2) are of particular interest to Europe and North America, as they cause potato infections in temperate climates that can result in high economic losses [5]. Therefore, R. solanacearum is recognized as a quarantine pest in most countries, and by regional Plant Protection Organizations [11,12].

The importance of infected propagative material of ornamental plants was shown by an outbreak of R. solanacearum in North America [7]. Due to the increased trade and/or movements of various host plants, in combination with changes in climatic conditions, there is an increased risk of the introduction and establishment of non-European R. solanacearum strains in both the environment and in greenhouse production facilities. This is exemplified by introduction of infected pelargonium and geranium cuttings through their importation [13–15] and the occurrence of other cold-tolerant strains [16]. In Europe, reports have demonstrated that R. solanacearum can survive in waterways at low temperatures in northern countries, and can cause infection of host plants after irrigation with contaminated water [17].

Several diagnostic methods are currently available to detect R. solanacearum. Serological techniques rely on the detection of bacterial components using antibodies [18], while molecular techniques are based on the detection of bacterial DNA [19,20], or RNA of viable bacterial cells [21]. To control the spread of R.
Ilan, a rapid and more user-friendly test is needed that can be easily deployed on-site. Available on-site tests also include serological and molecular tests, such as real-time PCR with the portable SmartCycler (Cepheid, Sunnyvale, CA).

Our aim was to develop a loop-mediated isothermal amplification (LAMP) assay [22] that can be performed in a high-throughput format in the laboratory or can be used with a portable device based on real-time detection of *R. solanacearum* strains. Ideally, this would cover all four of the *R. solanacearum* phylootypes from its various host plants. The LAMP assays developed herein that target the 16S rRNA gene and the endoglucanase (egl) gene are compared to an optimized version of a previously published LAMP assay that targets the flagellar subunit βC [23]. The best of these assays, the egl LAMP assay, is adapted for on-site performance with a portable device, and thus for real-time visualization. This is also compared to the real-time PCR assay developed by Weller and coworkers [19], which is routinely used for diagnostics in our laboratory.

**Methods**

**Sample preparation**

**Bacterial isolates.** The LAMP assays were evaluated on 114 bacterial strains that included 88 *R. solanacearum* 'species complex' strains that represent all four of the phylootypes: phylootypes I (20), IIA (17), IIB (24), III (15), and IV (8). Four strains with undetermined phylootypes were also included. In addition, the LAMP assays were evaluated on 15 non-target strains, which included potentially cross-reacting reference strains (as listed in European Union Council Directive 2000/29/EC) [24], plus 13 other pathogens from selected *R. solanacearum* hosts (for full listing and additional information, see Table S4 in File S1).

The bacterial strains were grown on YPGA agar medium (0.5% yeast extract, 0.5% peptone, 1% glucose and 1.2% agar) and incubated at 28 °C for two days. A single colony of each was then suspended in 10 mM phosphate-buffered saline (PBS; pH 7.2) to a final concentration of 10^6 cells/mL, which was standardized by turbidity measurements (DEN-1B McFarland Densitometer, Biosan). The bacterial suspensions were incubated at 95 °C for 30 min in a thermal block, to lyse the cells and release their DNA. Then 2.5 mL of this solution was tested in the LAMP reaction.

**Preparation of plant material.** Plants and plant parts were inoculated with *R. solanacearum* to produce the necessary plant material that mimicked naturally infected symptomatic plants.

Tomato plants at the third true leaf stage were inoculated with pure cultures of *R. solanacearum* (NCPPB 1453 or NCPPB 4156) using a sterile needle. The plants were incubated at 90% relative humidity under a regimen of 16 h light (3000 lux) at 26 °C and 8 h darkness at 23 °C. When the plants showed wilting symptoms (2–4 days after inoculation), the bacterial ooze at the site of inoculation was collected using a sterile plastic inoculation loop, and suspended in 100 μL sterile water. For additional confirmation, after a week of incubation, the plant stems above the inoculation point were cut into pieces and tested using the LAMP assay. Artificially infected potato tubers were: (i) stabbed at the stolon end using an inoculation needle dipped in an *R. solanacearum* colony, and then incubated at 28 °C and 70% humidity for 2–3 weeks; or (ii) collected from artificially infected plants (two months old potato plants of Fontane variety were infected by irrigation with 10^6 cells/mL of *R. solanacearum* strain LMG 9576 and tubers were then collected after three months). Infected tubers were cut into halves and the bacterial ooze was collected with a sterile plastic inoculation loop and suspended in 100 μL sterile water.

The ooze (2.5 μL) of both, artificially inoculated tomato plants and potato tubers was tested in the LAMP reactions as described below.

Infected and healthy potato tuber extracts used for testing latent *R. solanacearum* infection were prepared using official EC procedure [24], by cutting out small cores of vascular tissue from the stolon end of each of a sample of 200 tubers, and comminuting these in 30 mL 50 mM phosphate buffer (pH 7.0), with an overnight incubation (shaking: maximum, 16 h). The supernatant was then decanted into a 50-mL centrifuge tube and centrifuged at 7000 × g for 15 min at 6 °C. The pellet was resuspended in 1 mL 10 mM phosphate buffer (pH 7.2), and the DNA was isolated as described below. The DNA (2.5 μL) isolated from the extract was then tested in the LAMP reactions.

Tuber extracts of potato cultivars Désirée and Bella Rosa were spiked with a 10-fold dilution series of *R. solanacearum* NCPPB 4156 (phylotype IIB), which ranged from 10^9 to 1 cell/mL, followed by DNA extraction. The isolated DNA from the spiked potato tuber extracts was then used for determination of analytical sensitivity.

Extracts of healthy plants of other hosts (tomato (4), eggplant (3), pelargonium (6) and *Solanum dulcamara* (6)) were prepared by cutting approximately 0.1 g of the plant stems into pieces and comminuting them in 4 mL 10 mM PBS (pH 7.2). The liquid part was removed and 100 μL was used for DNA extraction.

**DNA extraction.** For conventional DNA extraction, 100 μL of the extracts from each potato sample or from healthy plants of tomato, eggplant, pelargonium and *S. dulcamara* were included, using magnetic-bead-based QuickPick SMI, Plant DNA kits (Bio-Nobile) with the KingFisher mL system (Thermo Labsystem), according to Pirc et al., 2009 [25], with a minor modification (440 μL lysate used for purification).

The resuspended ooze from the artificially infected tomato plants and potato tubers were incubated at 95 °C in a thermal block for 5 min and 2 min, respectively, to lyse the cells and to release the DNA. Then 2.5 μL of this solution was tested in the LAMP reaction.

Furthermore, different incubation times of the homogenate at 95 °C were tested with pure bacterial cultures: 2, 5, 10, 15, 20 and 30 min, and these were compared with the time needed to obtain positive results from symptomatic test material.

**The LAMP assay**

The performance of the egl LAMP assay is described in the following section. Readers are referred to Supplementary Materials for descriptions of the 16S rRNA LAMP assay (Table S1 in File S1) and the optimized βC LAMP assay (Table S2 and Table S3 in File S1).

**Design of the LAMP primers.** All of the available sequences of the *R. solanacearum* endoglucanase genes were collected at the time of analysis, from the NCBI database, available at http://www.ncbi.nlm.nih.gov/genbank/. These were aligned using the muscle alignment algorithm and the Molecular Evolutionary Genetics Analysis v5 (Mega 5) software [26]. The consensus (98% conservation) of the most conserved part of the alignment, identical to sequence GenBank accession number DQ657595, was then used for the design of the LAMP primers, using the LAMP Designer software (Premier Biosoft, Palo Alto, CA). The standard BLAST algorithm (standard nucleotide BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for each primer set with the default settings, to check for specificity against the whole database and for cross-reactivity with other nontarget sequences, including bacterial and plant sequences. Using this strategy, three primer sets that target the endoglucanase (egl) gene were designed, all of which satisfied the required parameters described by Notomi.
et al. [22]. After preliminary sensitivity and specificity assessments on a set of 14 isolates that represented all of the R. solanacearum phytophones (data not shown), one primer set was chosen for further validation (Table 1).

**LAMP reactions.** The LAMP reactions were performed in single tubes in 25 μL total reaction volume, containing 2.5 μL sample (boiled bacterial suspension or DNA isolated from plant extract), 12.5 μL Isothermal Master Mix (Optigene Ltd., Horsham, UK), and the primer mix that consisted of all six primers, at the final concentrations as indicated in Table 1. The egl LAMP reactions were run at 60 °C for 30 min. All of the reactions for evaluation were performed in a SmartCycler instrument (Cepheid, Sunnyvale, CA), using a negative control (distilled water) and a positive control (R. solanacearum strain GMI100 endoglucanase precursor (egl) gene (GenBank accession number DQ657595) sequence).

**Positioning criteria and running-time determination.** The criteria for positive signals were based on two parameters: \( t_P \) (time optimal and sufficient to detect all of the R. solanacearum melting curves). \( t_P \) is the temperature at which the amplification products melt, which is however distinguishable based on their fluorescence. \( t_P \) is the temperature at which the amplification products melt into two single-stranded DNA molecules, and this is expressed in °C.

The running time of the egl LAMP assay was determined based on the sensitivity test results from the R. solanacearum strain that showed the lowest rate of positivity \( (t_P) \) at the limit of detection for each sample. Based on these data, the reaction running time was optimal and sufficient to detect all of the R. solanacearum-positive samples (Table S4 in File S1). This approach supports optimum assay performance by allowing target detection in samples where the bacterial concentration is at the limit of detection, while preventing the appearance of eventual signals from nonspecific amplification, which is however distinguishable based on their melting curves.

**Real time PCR**

All of the samples tested with the egl LAMP assay were also analyzed by real-time PCR using the primers RS-I-F and RS-II-R that flank the 16S rRNA region, as suggested for broad-range detection of all R. solanacearum biovars [19]. The real-time PCR reactions were performed in triplicate on an ABI PRISM 7900 HT Sequence Detection system (Applied Biosystems), using the following universal cycling conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, and 1 min at 60 °C, using standard mode. The reaction volumes of 10 μL contained, as final concentrations: 900 nM primers (Eurofins MWG Operon), 200 nM probe (Eurofins MWG Operon), 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), and 2 μL sample DNA. The real-time PCR results are given in \( C_q \) (quantification cycle): the real-time PCR cycle at which the fluorescence exceeded the threshold value [27].

**Confirmational methods**

For the determination of health status of the potato tuber extracts, the diagnostics followed EC Directive 2000/29/EC [24]; immunofluorescence microscopy was used as a screening test with the IACR-PS-278 anti-R. solanacearum (biovar 2) polyclonal rabbit primary antibody (Rothamstead Research) [28], with a goat anti-rabbit FITC-labeled secondary antibody (Sigma F6005). Tuber extracts with positive results in the immunofluorescence were further analysed using PCR (primers Rs-1-F, Rs-1-R; [29]), isolation on semi-selective SMSA medium, immunofluorescence, and PCR on pure cultures, and pathogenicity testing in tomato plants cv. Moneymaker. In addition, negative tuber extracts in immunofluorescence were further tested with real-time PCR that targeted the 16S rRNA gene using a broad range R. solanacearum probe [19].

**Analytical specificity assays**

Analytical specificity was determined on boiled suspensions of \( 10^8 \) cells/mL (egl LAMP assay) or \( 10^9 \) cells/mL (real-time PCR), standardized by turbidity measurements (DEN-1B McFarland Densitometer, Biosan). Comparisons with real-time PCR were carried out as described above.

The cross-reactivity of the LAMP assays with the potato plant tissue and its microflora was assessed by testing DNA isolated from potato tuber extracts that had previously tested negative using other methods (immunofluorescence and/or real-time PCR). The following cultivars were included: Adora (2), Agria (1), Aladin (1), Anuschka (1), Arrow (2), Bella Rosa (1), Bistra (1), Carlingford (3), Carrera (1), Cheric (1), Desirec (2), Fiana (1), Frisia (2), Jelly (3), Marbel (7), Mirra (1), Plata (1), Sante (3), Silvana (1), Sora (3), Soraja (1), two samples of an unknown cultivar, and two samples of a new hybrid (Table S5 in File S1). In addition, DNA isolated from extracts of the following healthy plant tissues were tested: tomato (4), eggplant (3), pelargonium (6) and S. dulcamara (6). The health status of these plant extracts was confirmed by real-time PCR for broad range detection of R. solanacearum, and by immunofluorescence microscopy, as described above.

**Analytical sensitivity assays**

Analytical sensitivity was evaluated on pure bacterial cultures diluted in distilled water. A \( 10^9 \) cells/mL suspension of a representative strain for each phytophyme was prepared. This
suspension was then used for the preparation of 10-fold dilution series on which the analytical sensitivity was evaluated in triplicate with the LAMP assay, and compared to real-time PCR, as described above.

In addition, the analytical sensitivity of the LAMP assay in the potato tuber samples was determined on spiked potato extracts prepared as described above. The *R. solanacearum* concentrations in tuber extracts ranged from $10^6$ to 1 cell/mL. DNA was extracted from 100 µL samples and analyzed with real-time PCR and LAMP assays, as described above.

For the purpose of this study, the limit of detection was determined for each technique, which was defined as the lowest dilution where two out of three and one out of three reactions were positive in LAMP and real-time PCR, respectively.

**Results**

**Performance of the LAMP assays**

Three LAMP assays are described and compared in this study: the in-house developed LAMP assays that target the 16S rRNA and endoglucanase (egl) genes, and the previously developed LAMP assay that targets *fliC*, which was optimised for real-time performance and made more rapid by the addition of another loop primer. A summarized evaluation scheme and the criteria for choosing the LAMP assays with better performances are presented in the Supplementary Materials (Figure S1).

All three LAMP assays successfully amplified the DNA of the NCPPB 3997 *R. solanacearum* isolate, as shown on Figure 1. However, due to cross-reactivity with DNA from healthy potato extracts, the 16S rRNA LAMP assay was not suitable for diagnostic testing, and this was therefore removed from further evaluation. The optimized *fliC* LAMP assay showed a better performance than the 16S rRNA LAMP assay in terms of specificity, sensitivity, and speed, but it did not detect several economically important *R. solanacearum* strains, and this was therefore not selected for on-site application. The egl LAMP assay was the fastest of these tested LAMP assays, and the time to result when tested at $10^8$ cells/mL ranged from 12 min to 20 min for the majority of the isolates (Table S4 in File S1). Based on the speed and the above-mentioned criteria, the running time for the egl LAMP assay was set to 30 min.

**Analytical specificity assays**

The analytical specificity was tested on pure bacterial cultures. The egl LAMP assay detected all of the isolates that belonged to the phylotypes I, IIA, IIB, and III, and four out of eight strains currently classified into phylotype IV. The phylotype IV strains that were not detected included one *Ralstonia syzygii* strain (CFBP6447), both of the BDB strains tested, and *R. solanacearum* strain RUN14/ACH732, which is genetically distinct from the other phylotype IV strains [30].

The analytical specificity of the egl LAMP assay was compared to that of the real-time PCR assay that was used to detect a broad range of *R. solanacearum* isolates [19]. This latter assay detected all of the tested *R. solanacearum* strains, as well as *R. syzygii* and the BDB strains, with the notable exception of the same phylotype IV *R. solanacearum* RUN14 strain that was not detected by the egl LAMP assay (Table S4 in File S1). Real-time PCR, but not egl LAMP, also detected the non-target *Ralstonia mannitolilytica* strain (CFBF6737).

To examine potential cross-reactivity with, e.g., soil microflora or plant DNA, the DNA samples isolated from healthy potato extracts (as confirmed by immunofluorescence with the sensitivity of $10^4$ cells/mL for biovar 2 *R. solanacearum*) were tested with the egl LAMP assay and by real-time PCR. The egl LAMP assay showed positive signals of amplification for four of the tested healthy potato samples (Table S5 in File S1). However, the melting curve analysis of the positive signals showed significantly different melting temperatures (difference of 1°C or more) compared to the melting curve defined for the true positive signals obtained by egl LAMP. Based on the defined criteria of positivity (Table S4 and Table S2 in File S1), these data were classified as negative, and did not cause problems in the interpretation of the results.

In contrast, four samples of potato tuber extracts that were negative using the official detection methods which are immunofluorescence microscopy and PCR [29] gave false-positive results in real-time PCR. Cross-contamination was excluded using
appropriate negative and positive controls for DNA extraction and amplification.

In addition, to test the on-site applicability, bacterial ooze from seven symptomatic tubers was tested. All of these samples showed positive signals, with tₚ of 18.8 min (±1.88 min) and Tᵥ of 93.2 °C (±0.07 °C), both of which are characteristic of the R. solanacearum endoglucanase amplicon.

Melting curve analysis

Although the egl gene is present in all of the R. solanacearum strains, the geographical separation and consequent separation of the evolution of the R. solanacearum species resulted in specific nucleotide substitutions in the egl gene sequence; this allows the differentiation of the phytopathotypes. From the results of our specificity assessment of the egl LAMP assay, R. solanacearum can be divided into two groups based on the melting temperatures observed for the LAMP products (Figure 2). The Tᵥ of LAMP products obtained in the amplification of strains that belong to phylotypes I (Asia) and III (Africa) were 94.6 °C (±0.2 °C) and 94.5 °C (±0.4 °C), respectively, whereas Tᵥ of the LAMP products of the strains of phytopathotypes IIA and IIB (America) were 93.8 °C (±0.2 °C) and 93.7 °C (±0.2 °C), respectively. This is in agreement with the reported phylogenetic studies: phytopathotypes IIA and IIB are closely related compared to phytopathotypes I and III [30]. Phytopathotype IV is a very heterogeneous group, and it was tested with a limited number of strains [8], and the Tᵥ fell into the same range: 94.1 °C (±0.3 °C). Overall, the range of Tᵥ found with the egl LAMP assay for R. solanacearum was between 93.1 °C and 94.9 °C, thus defining a criterion for LAMP signal acceptability (true positive).

Analytical sensitivity assays

The sensitivity was first evaluated on pure bacterial cultures diluted in distilled water, using a representative strain for each phytypathotype (Table 2). The egl real-time LAMP assays showed a sensitivity limit of 10⁴ cells/mL (25 cells per LAMP reaction) when tested on strains belonging to phytopathotypes I and III, and a sensitivity limit of 10⁵–10⁶ cells/mL for strains from phytopathotypes IIA, IIB and IV (Table 2).

In addition, the sensitivity of the egl LAMP assay was evaluated in spiked plant extracts, to simulate real plant samples and to confirm that the level of sensitivity is not influenced by substances co-extracted with the DNA from the plant tissues. To test a worst case scenario, a strain with poor LAMP performance (NCPPB 4156) was tested. The sensitivity for the spiked potato extract was estimated to be 10⁵ CFU/mL.

On-site application

To offer an egl LAMP assay that is suitable for application outside the laboratory, a quick and simple extraction protocol (boiling of samples) of symptomatic potato tubers was combined with LAMP, using the portable Genie II equipment. In general, the longer boiling times resulted in shorter times to the signal of the LAMP reactions (Table S7 in File S1). A boiling time of 2 min at 95 °C was repeatedly successful for the confirmation of tuber infections (six tubers tested). Positive signals using the egl LAMP assay had a tₚ of 18.4 ±1.07 min and a Tᵥ of 92.1 ±0.11 °C, which were characteristic for R. solanacearum when tested on the Genie II apparatus. Testing symptomatic tomato plants (infected with R. solanacearum strains NCPPB 4153 and NCPPB 4156) showed comparable results (data not shown). Taken together, these data gave good indications that the test can be easily performed on-site, with reliable results obtained.

Discussion

Our investigation focused on the development of a molecular assay for rapid testing of symptomatic material. The availability of portable equipment for on-site performance that allows LAMP amplification to be followed in real-time was another reason for this study.

Of the three assays compared here, the LAMP assay that targets the egl gene appears to be optimal for testing symptomatic material, in terms of specificity, sensitivity and time needed to complete the analysis (Tables 2, 3 and Tables S4, S5, S8 in File S1).

This egl LAMP assay covers all of the tested R. solanacearum strains from phytopathotypes I, II and III, and most of the tested R. solanacearum strains from phytopathotype IV. Most importantly, all of the strains within the four phytopathotypes that have been reported to be found in potato were correctly detected. In addition, the egl LAMP assay confirmed its excellent specificity when tested on carefully chosen nontarget samples.

The proposed egl LAMP assay presents further advantages in terms of detection. It detects all of the bacteria from clonal phytopathotype IIB and the genetically more diverse phytopathotype IIA [30]. Therefore, the egl LAMP assay is a relatively sustainable detection test for the phytopathotype IIB strains, which includes previously designated race 3 biovar 2 isolates, which are classified as a quarantine isolates in European Union and Canada [31]. Moreover, the egl LAMP assay is not restrained to the strains that are currently causing economic losses, but can also detect all of the strains from phytopathotype I, the phytopathotype that has the biggest evolutionary potential and also the largest host range [30]. As only three R. solanacearum phytopathotype IV strains were tested (including the genetically distinct RUN14/ACH732 strain), it is difficult to forecast how efficient the egl-specific LAMP assay will be for the detection of strains belonging to this phytopathotype. However, a recent genomic comparison revealed that strains classified into phytopathotype IV R. solanacearum, R. syzygii and the BDB strains show high levels of
evolutionary homology and might actually be considered as species distinct from R. solanacearum [32].

In terms of the time of the analysis, the egl LAMP assay is also the most rapid of these three assays, as only a 30-min reaction is necessary to complete the detection of all of the R. solanacearum strains.

Although the sensitivity of the egl LAMP assay (10⁴–10⁵ cells/mL) is lower than that of the real-time PCR (10²–10⁴ cells/mL) [19], its level of sensitivity is suitable for reliable confirmation of the presence of R. solanacearum in symptomatic potato samples. Moreover, data obtained in our laboratory show that latent infections of potato tubers usually contain bacterial concentrations of 10⁶ CFU/mL or higher (Table S5 in File S1). This suggests that the sensitivity of the egl LAMP assay may be sufficient for testing of latent infections.

Given the good specificity of this egl LAMP assay when tested on pure cultures, and that there were no false positives when compared to real-time PCR, we propose this assay to be validated for the confirmation of results that have been obtained using other diagnostic methods, such as immunofluorescence, selective isolation, and PCR. In addition to melting temperatures, the sequencing of the LAMP products provides further information on the phylogenetic identity of the isolates. Based on partial cds of the endoglucanase (egl) gene of a subset of R. solanacearum strains, several nucleotides provide good indications of the phylogenetic position of isolates; relative to the R. solanacearum strain CFBP 4599.

Table 2. Analytical sensitivity of the egl LAMP assay.

| Concentration (cells/mL) | GBBC 1172 (Phylotype I) | RUN 30 (Phylotype IIA) | GBBC 729 (Phylotype IIB) | LMG 2296 (Phylotype III) | RUN 71 (Phylotype IV) |
|--------------------------|--------------------------|------------------------|--------------------------|--------------------------|------------------------|
| 10⁸                      | tp (min) 12.7 ± 0.38     | tp (min) 15.8 ± 1.59   | tp (min) 12.9 ± 1.07     | tp (min) 12.2 ± 0.06     | tp (min) 19.4 ± 1.67   |
| 10⁷                      | tp (min) 14.2 ± 1.81     | tp (min) 16.5 ± 2.73   | tp (min) 14.7 ± 1.76     | tp (min) 13.8 ± 1.56     | tp (min) 18.8 ± 1.90   |
| 10⁶                      | tp (min) 17.0 ± 3.03     | tp (min) 18.1 ± 3.97   | tp (min) 16.3 ± 1.86     | tp (min) 15.7 ± 2.03     | tp (min) 19.3 ± 2.44   |
| 10⁵                      | tp (min) 18.1 ± 3.13     | tp (min) 23.3a         | tp (min) 17.7 ± 2.41b    | tp (min) 17.4 ± 2.02     | tp (min) 21.3a         |
| 10⁴                      | tp (min) 20.8 ± 3.99     | tp (min) 26.3a         | tp (min) 21.3a           | tp (min) 20.5 ± 2.28     | -                      |
| 10³                      | -                        | -                      | -                        | -                        | -                      |
| 10²                      | -                        | -                      | -                        | -                        | -                      |
| Tₘ                       | 94.1 ± 0.10              | 93.5 ± 0.27            | 93.2 ± 0.08              | 94.1 ± 0.14              | 93.8 ± 0.25            |

Data are means (±SD), calculated from three independent runs.

*: negative result (absence of signal).

a: detected once out of three replicates.
b: detected twice out of three replicates.

Table 3. Performance characteristics of the LAMP assays compared with real-time PCR.

| Assay characteristic | 16S rRNA LAMP | Modified flic LAMP | egl LAMP | Real-time PCR |
|---------------------|---------------|--------------------|---------|---------------|
| Target gene         | 16S rRNA      | Flagellar subunit  | Endoglucanase | 16S rRNA     |
| Running temperature (°C) | 65            | 65                 | 60       | Cycling       |
| Running time* (min)  | 60            | 40                 | 30       | 90            |
| Analytical sensitivity in watera (cells/mL) | ND**          | 10⁻⁵–10⁶         | 10⁻⁴–10⁶  | 10⁻²–10⁴     |
| Analytical sensitivity in tuber extractb (CFU/mL) | 10⁻⁴–10⁴      | 10⁻⁴              | 10⁵       | 10⁻¹–10³     |
| 10⁸                  | 31.0 ± 0.21   | 16.5 ± 0.70        | 19.8 ± 0.41 | 24.2 ± 0.03  |
| 10⁷                  | 34.1 ± 1.52   | 22.5 ± 5.00        | 20.2 ± 5.01 | 27.7 ± 0.08  |
| 10⁶                  | 43.7 ± 3.91   | /                  | /        | 31.5 ± 0.40  |
| 10⁵                  | 49.6          | /                  | /        | 39.8 ± 1.38  |
| 10⁴                  | /             | /                  | /        | /             |
| 10³                  | /             | /                  | /        | /             |
| 10²                  | /             | /                  | /        | /             |
| 10¹                  | /             | /                  | /        | /             |
| 10⁰                  | /             | /                  | /        | /             |

Running time: time needed to complete the test with reliable detection of all samples above the limit of detection.

Analytical sensitivity was tested on a pure culture suspension of a single strain representing each phylotype (see Table 2). Analytical sensitivity differs between phylotypes. More detailed results are presented in Table 2 (egl LAMP assay) and Table S3 in File S1 (modified flic LAMP assay) and Table S6 in File S1 (real time PCR).

Analytical sensitivity tested on DNA isolated from spiked tuber extracts (with DNA of strain NCPPB 4156 that belongs to phylotype IIB) was assessed on two serial dilutions.

**ND: Non-determined. Analytical sensitivity in spiked plant extract was not tested with the 16S rRNA LAMP assay because of cross reactivity observed; therefore validation was not completed for this assay.
The herein described endoglucanase (egl) specific real-time LAMP assay allows specific detection of *R. solanacearum* strains belonging to all four phylotypes. The assay is performed with a portable instrument that is fully suitable for on-site extraction and testing at the port of entry of plant material. It allows simple manipulation, fast analysis, and easy interpretation of the results. Its practicability has already been shown on-site, combined with boiling as a simple DNA extraction step. In addition, the assay can easily be implemented in laboratories in a high-throughput format, using their own real-time PCR instruments. Given its sensitivity level, this generic *R. solanacearum* detection assay is proposed for use as a detection tool for the presence of this bacterium in symptomatic plant material, although it can also be used as a rapid confirmation tool for pure bacterial colony identification.

**Supporting Information**

**Figure S1** Scheme of evaluation of different LAMP assays and the decision scheme for choosing LAMP with best performance. (DOCX)

**Figure S2** Alignment of partial cds of the endoglucanase (egl) gene of a subset of *R. solanacearum* strains. (JPG)

**File S1** Supporting information that includes: Table S1: Primers used in the 16S rRNA LAMP assay. Table S2: Primers used in the modified fliC LAMP assay. Table S3: Analytical sensitivity of the modified fliC LAMP assay in different *R. solanacearum* phylotypes. Table S4: Bacterial strains tested with LAMP targeting 16S rRNA, fliC and egl compared to real-time PCR. Table S5: Evaluation of LAMP performance on potato extracts (diagnostic samples). Table S6: Analytical sensitivity of the real-time PCR for the different *R. solanacearum* phylotypes. Table S7: Incubation times at 95 °C before the assays, and the following time to positivity and time taken for the egl LAMP assays with infected potato tuber. Table S8: Summary of the egl LAMP validation. (DOCX)

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**Author Contributions**

Conceived and designed the experiments: RL, DM, MP, PL, MR, TD. Performed the experiments: RL, MP, PL. Analyzed the data: RL, DM MP, PL, MR, TD. Contributed reagents/materials/analysis tools: RL, DM MP, PL, MR, TD. Wrote the paper: RL, DM, MP, PL, MR, TD.

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