Rapid Detection of *Xanthomonas citri* pv. *fuscans* and *Xanthomonas phaseoli* pv. *phaseoli* in Common Bean by Loop-Mediated Isothermal Amplification

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Abstract

A single loop-mediated isothermal amplification (LAMP) assay was developed for specific detection of both pathogens that cause bacterial blight in common bean, *Xanthomonas phaseoli* pv. *phaseoli* (Xpp) and *Xanthomonas citri* pv. *fuscans* (Xcf). The objective was to provide a simple, easy-to-use, specific, and sensitive method to investigate the presence of one or both pathogens in plant material and seeds for routine diagnosis. The detection limits for both pathogens were 10 CFU/ml for cell suspensions and 1 fg of DNA, whereas in conventional PCR, the primers detected up to 10⁵ CFU/ml and 1 ng of DNA. Specificity was confirmed by testing DNA from bean leaves, other *Xanthomonas* species, common fungal and bacterial bean pathogens, and bacteria from the leaf microbiota. The method was tested with bean leaves inoculated with Xpp, and the

pathogen could be detected from 4 h up to 15 days postinoculation, even before disease symptoms were visible. When the method was applied to bacterium detection (Xpp or Xcf) in seed lots from infected plants, the bacterium detection rate was 100% (24 of 24). The pathogens were detected in seeds incubated for just 1 h in saline solution (0.85%), reducing the time needed for bacterium detection. The LAMP assay could be useful as a tool in bean bacterial blight management. Rapid and sensitive detection of bacteria in bean seed lots would reduce the risks of planting highly contaminated seeds in environments favorable to blight multiplication.

Keywords: common bacterial blight, prokaryotes, field crops, oilseeds and legumes, pathogen detection, *Phaseolus vulgaris* L

Common bacterial blight (CBB) is among the most destructive diseases of common bean, causing major losses in production, mainly in the rainy season when the environmental conditions (humidity and high temperature) are favorable for disease development and the spread of the pathogen (Vieira 1983). The disease is caused by four different genetic lineages belonging to two species within the genus *Xanthomonas* (Alavi et al. 2008; Schaad et al. 2005, 2006). The fuscans lineage and two nonfuscans lineages (NF2 and NF3) belong to the *Xanthomonas citri* pv. *fuscans* (Xcf) species (formerly *Xanthomonas fuscans* subsp. *fuscans* and *Xanthomonas axonopodis* pv. *phaseoli* group 9.6 by rep-PCR). The nonfuscans lineage (NF1) belongs to the *Xanthomonas phaseoli* pv. *phaseoli* (Xpp) species (formerly *Xanthomonas axonopodis* pv. *phaseoli* group 9.4 by rep-PCR) (Constantin et al. 2016; Rademaker et al. 2005).

The disease has been reported for the first time in Brazil in the state of Pará, and its causal agent was described as *Xanthomonas campestris* pv. *phaseoli* (Robbs 1954). The variant *fuscans* was later described in the state of São Paulo (Paradela Filho et al. 1967). The bacterium can survive and remain viable for periods of 5 to 15 years in bean seeds (Marques et al. 2005), and being carried in seeds is an important means of disease transmission over short and long distances. The disease is found worldwide, and it is the target of quarantine measures in many European countries, where it is not endemic. Thus, the

movement of contaminated seeds should be considered as a target to prevent pathogen spread worldwide.

According to the literature, different methods have been developed for diagnosis of CBB, including the use of semiselective media (Clafflin et al. 1987; Tebaldi et al. 2007), sorology (Maringoni et al. 1994), biochemical tests, and specific PCR. The specific primers X4c/X4e developed by Audy et al. (1994) amplify a 730-bp fragment of DNA that allows detection and identification of both fuscans and nonfuscans lineages. The primer pair Xf1/Xf2 was selected for specific detection of the fuscans lineage, and they amplify a DNA fragment of 450 bp from all fuscans isolates tested (Toth et al. 1998).

Based on the sequences of virulence-associated genes *avrBsT* and *xopL*, Boureau et al. (2013) developed two pairs of primers (Am1F/R and Am2F/R), which when used in conjunction with the primers X4c/X4e, composed a multiplex reaction. This would allow the differentiation of pathogenic and nonpathogenic isolates, decreasing the risk of false positives. The authors suggest that pathogenicity tests should be carried out for those results that are ambiguous.

The loop-mediated isothermal amplification (LAMP) assay was described by Notomi et al. (2000), and it became a tool for pathogen detection, including several species of the genus *Xanthomonas* (Hodgetts et al. 2015; Jun-hai et al. 2015; Lang et al. 2014; Palacio-Bielsa et al. 2015; Wang and Turechek 2016). In contrast to PCR, LAMP amplifies DNA at a single and constant temperature, and therefore, it does not require thermal cycler equipment, reducing costs and minimizing the time for pathogen detection.

LAMP assays require the *Bst* DNA polymerase enzyme (with strand displacement activity) and a set of four to six primers that hybridize in six to eight distinct regions of the target sequence, conferring high specificity. The amplification, in general, is completed under isothermal conditions between 60 and 65°C, resulting in large quantities of amplified product in a short period (20 to 60 min) (Jun-hai et al. 2015; Larrea-Sarmiento et al. 2018).

As the reaction progresses, the turbidity can be observed by using magnesium pyrophosphate, a by-product of the amplification reaction, which is directly related to the amount of amplified product (Arroyo et al. 2008). It is also possible to perform the visual

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detection by adding intercalating DNA dyes, such as ethidium bromide, SYBR Green, and propidium iodide, or indicators of metal ions, such as hydroxynaphthol blue and calcein. DNA intercalators must be added after the reaction and require ultraviolet (UV) illumination for visualization, and metal ion indicators are difficult to discern with the naked eye. When low concentrations of buffer are used in the reaction, a significant change from an initial alkaline pH to a final acidic pH makes it possible to add pH-sensitive indicator dyes directly in the reaction for visualization (Tanner et al. 2015). The addition of pH-sensitive indicator dyes before amplification also prevents aerosol contamination, which may result in false positives. This is a common problem when the amplification is verified by gel electrophoresis (Huang et al. 2017).

Considering that both species (Xpp and Xcf) cause similar symptoms and damage to the common bean, the objective of this work was to develop an LAMP method for the specific detection of the causal agents of bacterial blight in symptomatic and asymptomatic bean plants as well as seed lots originating from CBB-infected plants under field conditions.

Materials and Methods

Bacterial isolates and DNA isolation. The bacterial isolates used in this study were retrieved from the Collection of Multifunctional Microorganisms from Embrapa Arroz e Feijão, Brazil. Xpp and Xcf isolates were grown in 523 culture medium (Kado and Heskett 1970). Their identity was confirmed by specific PCR with X4c/X4e primers (Audy et al. 1994) and Xf1/Xf2 (Toth et al. 1998) and pathogenicity assays on the susceptible bean cultivar BRS Ártico using the scissor method (Rava et al. 1988).

DNA was isolated from pure bacterial cultures using the Wizard Genomic DNA Purification Kit (Promega) following the manufacturer's instruction. DNA was quantified using NanoDrop 2000/c Spectrophotometers (Thermo Fisher Scientific).

Conventional PCR. The reaction was performed using the QIAGEN multiplex PCR Kit in a final volume of 10 µl containing 5 µl of the master mix, 1 µl of Q solution, 0.1 µM each primer (Xf1/Xf2 and X4c/X4e), and 1 µl of bacterial cell suspension (10⁷ CFU/ml). The PCR program consisted of an initial denaturation of 95°C for 15 min followed by 37 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min and a final extension at 72°C for 10 min. Subsequently, the PCR products (5 µl) were separated by electrophoresis in 1.5% agarose gel in TBE (Tris-borate/EDTA) 1× buffer stained with GelRed 2x, viewed under UV light, and photo-documented via a digital system (L-PIX Loccus).

Primer design and LAMP protocol. The sequence amplified by the X4c/X4e primers (Audy et al. 1994) was located in the plasmid pA complete sequence (accession number CP021013.1) and used to develop the LAMP primers. The PrimerExplorer V5 software (Eiken Chemical Co. Ltd.) was used for the design of four LAMP primers: two internal (FIP and BIP) and two external (F3 and B3) primers. The sequences of the selected primers are described in Table 1.

The concentration of the primers was optimized, and the reactions were conducted according to the protocol established by Tanner et al. (2015), containing 2× buffer solution [10 mM (NH₄)₂SO₄, 10 mM KCl, 8 mM MgSO₄, 1.4 mM dNTPs, and 0.1% (vol/vol) Tween-20, pH 8.6 to 8.8 adjusted with 1 M KOH], 200 µM neutral red dye, pH 7.8, 0.2 µM F3 and B3 (each), 1.6 µM FIP and BIP (each), 8 U of *Bst* DNA polymerase large fragment (Biolabs; New England), and 1 µl of lysed bacterial cell suspension (10⁷ CFU/ml) or DNA (10 ng/ml). The bacterial cells were lysed at 95°C for 5 min before PCR.

To find the optimal amplification temperature, which was 63°C, the reaction was carried out in a thermocycler (BioRad Thermal Cycler) with a temperature gradient that ranged from 55 to 65°C for 1 h. Negative controls containing nuclease-free water were included in each assay, and the positive controls were DNA from two pathogenic isolates from common bean crops in Brazil, BRM 25302 (Xpp) and BRM 25304 (Xcf). The neutral red dye (Vetec) added

in the reaction allowed the amplification to be monitored by observing color change to shades of pink for the positive samples and yellow for the negative samples (Alhassan et al. 2016; Poole et al. 2017).

Specificity and sensitivity of the LAMP assay. To determine the specificity of the LAMP protocol, the primers were initially tested with 18 isolates of Xpp and 27 isolates of Xcf collected from many different geographical regions of Brazil (Table 2) and later tested with different species of the genus *Xanthomonas* (*Xanthomonas gardneri*, *Xanthomonas axonopodis* pv. *manihotis*, *Xanthomonas axonopodis* pv. *glycines*, and *Xanthomonas campestris* pv. *campestris*), other bacterial and fungal pathogens of common bean (*Curatobacterium flaccumfaciens* pv. *flaccumfaciens*, *Colletotrichum lindemuthianum*, *Fusarium solani*, *Fusarium oxysporum* f. sp. *phaseoli*, *Macrophomina phaseolina*, and *Pseudocercospora griseola*), DNA extracted from bean leaves, and the epiphytic microbial population present in bean leaves. Healthy bean leaves were collected randomly from plants growing in the greenhouse and an experimental field trial. The leaves were immersed in sterile water (10 ml) and macerated, and from this extract, 1 µl was used directly in the LAMP reactions.

To determine the minimum detectable concentration, cell suspensions of BRM 25302 (Xpp) and BRM 25304 (Xcf) isolates were prepared in water, and the concentrations were adjusted to 10⁸ CFU/ml followed by 10-fold serial dilutions up to 10 CFU/ml. Suspensions were then incubated at 95°C for 5 min for cell lysis. The limit of detectable DNA was also determined with 12 dilutions of DNA from isolate BRM25302 at 100 ng/µl. Each dilution was used in LAMP and conventional PCR with X4c/X4e primers to compare the sensitivity of the two techniques. To confirm the results, 12 replicates of each of the five lower dilutions (cells or DNA) were used.

Bacterial detection in symptomatic and asymptomatic inoculated bean leaves. To evaluate the potential of LAMP to detect latent infections, 10-day-old bean plants of the susceptible BRS Ártico were inoculated by the scissor method (Rava et al. 1988) with a 10⁷ CFU/ml cell suspension of the BRM 25302 isolate. Two leaves per plant were collected randomly at 4 h and 1, 2, 3, 4, 5, 10, and 15 days postinoculation (dpi). Leaves of healthy plants were collected before inoculation and used as negative controls. For each leaf, fragments of ~0.5 mm² were removed from the inoculated area and macerated in microtubes containing 200 µl of sterile water at room temperature. An aliquot of 1 µl was used directly in the LAMP reactions.

LAMP detection of *Xanthomonas* spp. in bean seeds. A seed lot of cultivar BRS Esplendor was obtained from an experimental field planted at unfavorable climate conditions for CBB and showing no disease symptoms. A standard seed health test according to the International Rules for Seed Testing (ISTA) was performed to detect the presence of Xpp and Xcf. One thousand seeds were soaked in 0.85%

Table 1. The sequences of the primers used in the PCR and loop-mediated isothermal amplification (LAMP) assays

Method and primer	Sequence (5' → 3')	Reference
PCR		
X4c	GGCAACACCCGATCCCTAAACAGG	Audy et al. 1994
X4e	CGCCGGAAGCAGCATCCTCGAAG	Audy et al. 1994
Xf1.2	ACGCAAGACCCATCGTCATTC	Toth et al. 1998
Xf2.2	ATGGCTCAAGGAAAACTTTCAGG	Toth et al. 1998
LAMP		
F3	TCCATACCTTCGGTCAACGA	This study
B3	GCACCTCTTCGAGGATCGT	This study
FIP	TAGGCTCGGCCGCTCACTATCGTT TTGAACGCCACCTGCTTTTCCT	This study
BIP	CAGCTTCCACACCAGCAGCATGTT TTCGGCGGAAGGATGAC	This study

saline solution (2 ml/g) for 16 h at 4°C followed by a serial dilution of 1-ml aliquots of the seed-soaking extract, a colony count in agar medium, and identification by specific PCR as previously described (Grimault et al. 2014). Each dilution of the seed extract was plated four times.

Table 2. Identification, species, and genetic lineage of bacterial isolates used to evaluate the specificity of the loop-mediated isothermal amplification (LAMP) assay for detection of *Xanthomonas phaseoli* pv. *phaseoli* and *Xanthomonas citri* pv. *fuscans*

Strains	Species	Genetic lineage	LAMP results
BRM 25297	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 25302	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 25306	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 25307	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 25311	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 25313	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 25314	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 25319	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 25320	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 25321	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 25327	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 25328	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 25329	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 25333	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 25342	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 48903	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 48904	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 48905	<i>X. phaseoli</i> pv. <i>phaseoli</i>	NF1	+
BRM 32182	<i>X. citri</i> pv. <i>fuscans</i>	NF2	+
BRM 32192	<i>X. citri</i> pv. <i>fuscans</i>	NF2	+
BRM 32203	<i>X. citri</i> pv. <i>fuscans</i>	NF2	+
BRM 32185	<i>X. citri</i> pv. <i>fuscans</i>	NF2	+
BRM 32197	<i>X. citri</i> pv. <i>fuscans</i>	NF2	+
BRM 32201	<i>X. citri</i> pv. <i>fuscans</i>	NF2	+
BRM 48898	<i>X. citri</i> pv. <i>fuscans</i>	NF2	+
BRM 48906	<i>X. citri</i> pv. <i>fuscans</i>	NF2	+
BRM 48899	<i>X. citri</i> pv. <i>fuscans</i>	NF3	+
BRM 48900	<i>X. citri</i> pv. <i>fuscans</i>	NF3	+
BRM 48901	<i>X. citri</i> pv. <i>fuscans</i>	NF3	+
BRM 48902	<i>X. citri</i> pv. <i>fuscans</i>	NF3	+
BRM 25304	<i>X. citri</i> pv. <i>fuscans</i>	Fuscans	+
BRM 25308	<i>X. citri</i> pv. <i>fuscans</i>	Fuscans	+
BRM 25310	<i>X. citri</i> pv. <i>fuscans</i>	Fuscans	+
BRM 25316	<i>X. citri</i> pv. <i>fuscans</i>	Fuscans	+
BRM 25343	<i>X. citri</i> pv. <i>fuscans</i>	Fuscans	+
BRM 25347	<i>X. citri</i> pv. <i>fuscans</i>	Fuscans	+
BRM 25351	<i>X. citri</i> pv. <i>fuscans</i>	Fuscans	+
BRM 48912	<i>X. citri</i> pv. <i>fuscans</i>	Fuscans	+
BRM 48913	<i>X. citri</i> pv. <i>fuscans</i>	Fuscans	+
BRM 25359	<i>X. citri</i> pv. <i>fuscans</i>	Fuscans	+
BRM 48912	<i>X. citri</i> pv. <i>fuscans</i>	Fuscans	+
BRM 48913	<i>X. citri</i> pv. <i>fuscans</i>	Fuscans	+
BRM 48914	<i>X. citri</i> pv. <i>fuscans</i>	Fuscans	+
BRM 48915	<i>X. citri</i> pv. <i>fuscans</i>	Fuscans	+
BRM 48916	<i>X. citri</i> pv. <i>fuscans</i>	Fuscans	+
BRM 51627	<i>X. campestris</i> pv. <i>campestris</i>	—	—
BRM 51556	<i>X. axonopodis</i> pv. <i>manihotis</i>	—	—
BRM 51557	<i>X. axonopodis</i> pv. <i>glycines</i>	—	—
BRM 51628	<i>X. gardneri</i>	—	—
BRM 14933	<i>Curtobacterium flaccumfaciens</i> pv. <i>flaccumfaciens</i>	—	—
BRM 15124	<i>Pseudocercospora griseola</i>	—	—
BRM 7447	<i>Colletotrichum lindemuthianum</i>	—	—
BRM 31143	<i>Fusarium solani</i>	—	—
BRM 14990	<i>Fusarium oxysporum</i> f. sp. <i>phaseoli</i>	—	—
BRM 32281	<i>Macrophomina phaseolina</i>	—	—

After the healthy status of the seeds was confirmed, a subsample was artificially inoculated by spraying a bacterial suspension (10^8 CFU/ml) of the BRM 25302 isolate over the seeds until they were completely covered. The seeds were left to dry under laminar flow. The sensitivity of the LAMP assay for detecting levels of contamination of 0.1 and 1% (1:999 and 10:990, respectively) was tested. The seeds were soaked in saline solution for 16 h, and a 1-ml aliquot of the extract was centrifuged for 10 min at 12,000 rpm; the pellet was resuspended in 100 µl of sterile water, and an aliquot of 1 µl was used for the LAMP reactions.

To verify LAMP efficiency compared with the standard ISTA method, seed lots of 24 cultivars/lineages of common beans showing high incidence of CBB were used. One pathogen-free lot was used as a negative control. Each seed lot was subjected to the conventional ISTA detection method, and then, an aliquot of each crude extract was used for LAMP. In all assays, a positive control, which was a bacterial suspension of isolate BRM 25302, and a water negative control were included.

To reduce the time needed for detection, samples of 1,000 bean seeds were soaked in saline solution for 1 and 4 h, 1 ml of solution was centrifuged, and the pellet was resuspended in water (100 µl). Aliquots of 1 µl were used for the LAMP reactions.

Results

Specificity and sensitivity of the LAMP assay. The LAMP reaction conditions (temperature and time) were optimized for 63°C for 60 min. The limits of LAMP detection were 10 CFU/ml for cell suspensions and 1 fg for DNA (Fig. 1), which were confirmed with 12 replicates of each of the five lower dilutions (Supplementary Figs. S1 and S2). Conventional PCR, with primers X4c/X4e (Audy et al. 1994) (Table 1), was less sensitive, detecting up to 10^5 CFU/ml and 1 ng of DNA. Because the same results were obtained for both species, only the results obtained for Xpp are presented.

The specificity of the LAMP assay was tested with other pathogens associated with common bean, bacteria isolated from bean leaves, bean DNA, and other species of the genus *Xanthomonas*. Only samples containing DNA from Xpp developed a pink coloration after the reaction. Samples containing only water, bean DNA, and DNA and cells from other pathogens or microorganisms showed a yellow color after the reaction, indicating that there was no DNA amplification (Fig. 2).

Bacterial detection in symptomatic and asymptomatic inoculated bean leaves. To demonstrate its applicability, the LAMP method was tested with samples collected from inoculated bean leaves before and after disease symptoms were visible. The leaves began to show symptoms 4 dpi (Table 4); however, the presence of bacteria was detected by LAMP at all evaluation times from 4 h after inoculation until the end of the evaluation time at 15 dpi. This demonstrated the potential of the LAMP assay for the detection of the CBB pathogens in latent infections (Fig. 3).

LAMP detection of *Xanthomonas* spp. in bean seeds. The LAMP assay was tested with 24 bean seed lots collected from an experimental field with bacterial blight. The rate of pathogen detection was 100% with the LAMP method. Compared with the ISTA standard method, the same seed lots showed a lower detection rate of 18 of 24 (75%) (Supplementary Table S1). Pathogen detection by LAMP was achieved even when the time needed for seed soaking in saline was reduced from 16 h (ISTA method) to 1 or 4 h. The LAMP assay also detected contamination rates of 1.0% (10 of 1,000 seeds) and 0.1% (1 of 1,000 seeds) (Fig. 4).

Discussion

The diagnosis of CBB in common bean is based on a set of complementary methods, such as recognition of symptoms, use of semi-selective medium for bacterial isolation (Claflin et al. 1987; Tebaldi et al. 2007), biochemical tests (Ryu 1940; Schaad et al. 2001), pathogenicity tests (Rava et al. 1988), and molecular methods based on PCR (Audy et al. 1994; Boureau et al. 2013; Toth et al. 1998), which are often time consuming. In this study, an LAMP assay was

developed for rapid detection of Xpp and Xcf in leaves and seeds of common beans.

LAMP assays can be used for the detection of plant pathogens, with numerous advantages in relation to other techniques. LAMP reactions show high sensitivity and speed, and the results can be seen with the naked eye with the addition of dyes, without the need for postamplification procedures, such as gel electrophoresis.

All parameters for detection were optimized, and the assay can be successfully performed using a water bath or any another heating device at a constant temperature of 63°C. The neutral red dye allowed the visualization of the reactions, which were pink for the positive samples and yellow for the negative ones; these results were confirmed by quantitative measurements with a DR2800 spectrophotometer (Hach) (Supplementary Table S2). It is important to emphasize that it is preferable to add contrast dyes before the amplification and that detection should be performed in a closed tube to reduce the risk of cross-contamination by aerosols, producing false positives.

In the sensitivity tests, the LAMP assay showed a detection limit of 1 fg of target DNA or 10 CFU/ml bacterial suspension, lower than the limits of conventional PCR (1 ng and 10⁵ CFU/ml, respectively) (Table 3). It is possible that this high sensitivity is because of the target sequence being repetitive and present in both the plasmid and the chromosome (Audy et al. 1994; Gilbertson et al. 1989), which gives a larger amount of the target sequence per cell. Similar sensitivity levels were reported for other plant pathogenic bacteria, such as *Erwinia amylovora* (Bühlmann et al. 2013), *Ralstonia solanacearum* (Lenarčič et al. 2014), and *Xanthomonas albilineans* (Dias et al. 2018). The specificity of the LAMP assay was validated with other *Xanthomonas* species, seven other pathogens of common bean, and the leaf epiphytic population, which showed that it was highly specific to the target organisms.

Sensitive and specific detection of pathogens is very important for disease diagnosis, prediction, and control. The developed LAMP assay allowed the bacterium to be detected in symptomatic and latent

Fig. 2. Specific detection of *Xanthomonas phaseoli* pv. *phaseoli* (Xpp) and *Xanthomonas citri* pv. *fuscans* (Xcf) by loop-mediated isothermal amplification. **A**, N, negative control; Xag, *Xanthomonas axonopodis* pv. *glycines*; Xam, *Xanthomonas axonopodis* pv. *manihotis*; Xcc, *Xanthomonas campestris* pv. *campestris*; Xg, *Xanthomonas gardneri*. **B**, Cff, *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*; Cl, *Colletotrichum lindemuthianum*; Fop, *Fusarium oxysporum* f. sp. *phaseoli*; Fs, *Fusarium solani*; Mp, *Macrophomina phaseolina*; Pg, *Pseudocercospora griseola*. **C**, Lanes 1 to 5, microbiota from bean leaves (greenhouse); bean, DNA extracted from bean leaves. **D**, Lanes 1 to 12, microbiota from bean leaves (field).

¹ Conventional PCR with specific primers X4c/X4e (Audy et al. 1994).

Fig. 1. Loop-mediated isothermal amplification (LAMP) and conventional PCR sensitivity evaluated by serial dilution of **A**, cell suspension (10⁸ to 1 CFU/ml) and **B**, DNA (10 ng to 0.1 fg) from *Xanthomonas phaseoli* pv. *phaseoli* BRM 25302 isolate. N, negative control.

infections, confirming the presence of the bacterium on the leaf tissue before the onset of the first symptoms. This demonstrates the effectiveness of the method for detecting the pathogen present in the plant tissue even at low concentrations, which may be applied for field samples.

Symptoms of CBB in the field are often visible on the leaves, and they are easy to recognize. However, when the seeds are contaminated, they rarely show any symptoms, making it difficult to test seed samples for the presence of bacteria with efficiency and precision. The method recommended by the ISTA allows for bacterial quantification, which is an advantage; however, it is time consuming and requires a skilled technician for bacterial isolation and identification. The LAMP assay developed in this study made it possible to detect the bacterium in the seeds with just 1 h of seed immersion in saline followed by 1 h of amplification by LAMP.

Seed contamination levels as low as 0.1% (that is, 1 contaminated seed in 999 healthy seeds) were detected by LAMP. When the method was tested in different samples of contaminated seeds, detection was 100% (24 of 24) against 75% (18 of 24) detection by the standard ISTA method. Similar detection rates were found by Lang et al. (2014) using specific LAMP primers for *Xanthomonas oryzae* pv. *oryzae*. The bacterium was detected in 100% of the evaluated samples, and a 0.5% detection rate in 5 g of seeds was obtained.

Six seed lots were negative for the ISTA method but positive for LAMP. The ISTA method is culture based; thus, it only detects viable and culturable cells. Several *Xanthomonas* species, such as *X. campestris* and *Xanthomonas citri* pv. *citri*, may enter the viable but not culturable (VBNC) state (Del Campo et al. 2009; Ghezzi and Steck 1999; Oliver 2005). It is not known whether the CBB pathogens may become unculturable. In this VBNC state, Xpp and Xcf cells would have been detected by LAMP but not by the standard culture-based method. However, the detection of DNA from unviable bacterial cells would also explain the positive result for LAMP and negative result for the ISTA method. This would represent a disadvantage of the LAMP assay. The use of DNA intercalants, such as propidium monoazide (PMA), has been proposed for LAMP and real-time PCR detection of foodborne and waterborne pathogens (Banihashemi et al. 2012) as well as plant pathogenic bacteria (Han et al. 2018; Temple et al. 2013). PMA binds to the chromosomal DNA of dead bacterial cells and therefore, blocks DNA amplification by PCR. If the VBNC state occurs in Xcf and Xpp from a commercial bean seed lot, then it is likely that the bacterial population can be underestimated by a culture-based method, which would result in false negatives. In that case, a modification would also be necessary in the LAMP assay to ensure that only DNA from viable cells is amplified.

Table 3. Limit of detection of *Xanthomonas phaseoli* pv. *phaseoli* cells by loop-mediated isothermal amplification (LAMP) and conventional PCR

Method ^a	Bacterial cell suspensions (CFU/ml)							
	10 ⁷	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰
LAMP	+ ^b	+	+	+	+	+	+	–
Conventional PCR	+	+	+	–	–	–	–	–

^a Assays were repeated three times with the same results.

^b +, positive reaction; –, negative reaction.

Table 4. Detection of *Xanthomonas phaseoli* pv. *phaseoli* in symptomatic and asymptomatic common bean leaves before and after inoculation

	Days postinoculation								
	–1	0	1	2	3	4	5	10	15
LAMP ^a	–	+	+	+	+	+	+	+	+
Symptoms	–	–	–	–	–	+	+	+	+

^a LAMP, loop-mediated isothermal amplification.

Some advantages of the LAMP method developed in this study are that it can be performed with minimum sample preparation, that there is no need for bacterial DNA extraction, and that the crude extract of seeds or leaves can be used directly in the reactions (Supplementary Fig. S3). Several studies demonstrate that LAMP tolerates higher levels of certain inhibitors and that sensitivity is less affected by the various plant compounds, allowing the DNA purification step to be omitted (Francois et al. 2011; Kaneko et al. 2007; Tomlinson et al. 2013).

The LAMP assay with visual detection designed for Xpp and Xcf shows simplicity, rapidity, sensitivity, and specificity as advantages. It could be potentially useful for screening bean seed lots, which would reduce the risk of introducing the pathogens in areas with environmental conditions favorable to their multiplication. Consequently, it would facilitate the decision to use preventive and control measures before the multiplication and dissemination of the pathogen(s) in the field.

Fig. 3. Detection of *Xanthomonas phaseoli* pv. *phaseoli* by loop-mediated isothermal amplification in symptomatic and asymptomatic bean leaves after inoculation. The reactions were repeated twice. N, negative control; P, positive control; tube 1, 5 days postinoculation (dpi); tube 2, 4 dpi; tube 3, 3 dpi; tube 4, 2 dpi; tube 5, 1 dpi; tube 6, 1 day before the inoculation; tube 7, 10 dpi; tube 8, 15 dpi.

Fig. 4. Detection of *Xanthomonas phaseoli* pv. *phaseoli* by loop-mediated isothermal amplification in bean seed lots. **A**, Seeds maintained in saline solution for 1 h. N, negative control; P, positive control; tubes 1, 2, and 3, contaminated seeds; tube 4, healthy seeds. **B**, Seeds maintained in saline solution for 4 h. Tubes 1, 2, and 3, contaminated seeds; tube 4, healthy seeds. **C**, Seeds maintained for 1 h in saline solution. Tubes 1 to 7, different lots of contaminated seeds. **D**, Healthy common bean seeds were mixed with seeds artificially inoculated with *X. phaseoli* pv. *phaseoli* at different levels of contamination in 1,000 seeds. Tubes 1 and 2, 1% contamination (10 of 990); tubes 3 and 4, 0.1% contamination (1 of 999); tubes 5 and 6, 0% contamination (0 of 1,000).

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