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RESEARCH NOTE

Development of a rapid, accurate, and field deployable LAMP-CRISPR-Cas12a integrated assay for *Xylella fastidiosa* detection and surveillance

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Abstract

Xylella fastidiosa is an aggressive plant pathogenic bacterium of significant quarantine concern. Accurate and reliable detection tools are essential to minimise the risk of the pathogen's spread and for outbreak control, as limited post-infection management strategies are possible. Here, we report the development of a specific and potentially field-deployable assay combining a pre-existing Loop-Mediated Isothermal Amplification (LAMP) assay and a Cas12a-based DNA Endonuclease-Targeted (DETECTR) Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) trans reporter for *X. fastidiosa* detection. The LAMP-CRISPR-Cas12a integrated assay detected the amplified target region of the *X. fastidiosa* specific *rimM* gene at the low femto-molar range within 10 min of initiation. The assay detected varied *X. fastidiosa* sub-species in a range of naturally infected and economically relevant host material, with no non-target amplification recorded. The results show integration of LAMP with CRISPR-based detection is a specific, sensitive and a potentially field-adaptable strategy for the detection of *X. fastidiosa* and has the potential for further operationally focused improvements.

Keywords *Xylella fastidiosa* · CRISPR · Cas12a · LAMP · Field detection

Xylella fastidiosa (Wells et al. 1987) is a gram-negative bacterial plant pathogen vectored by xylem-feeding insects, namely sharpshooters (Cicadellidae: Cicadellinae) and spittlebugs (Cercopidae) (Overall and Rebek 2017) and has spread among global production regions by movement of infected propagative material (Berisha et al. 1998; Amanifar et al. 2014; Rapicavoli et al. 2018; Trkulja et al. 2022). With ability to infect at least 88 plant families (Baldi and La Porta 2017; Baldassarre et al. 2023; EFSA et al. 2022) the pathogen is a direct threat to environmental health and global food security, with severe economic impacts expected in grapevine, olive, and citrus, among many others. Although pre-symptomatic infection is transmissible, initial symptoms may be indistinguishable from abiotic stress, increasing the likelihood of spread before identification of pathogen presence. With extremely limited effective treatment strategies (Baldassarre et al. 2023), early and sensitive detection of the pathogen is the most effective intervention strategy.

The efficacy of a single molecular assay to deliver rapid, reliable, and robust detection is often limited by the large breadth of plant host matrices, ability to remain Xylella specific with high sensitivity, or the ability to translate across field-based surveillance or laboratory environments using non-symptomatic and symptomatic tissues. Thus, several Xylella diagnostic tools have been developed and are often used in combination to diagnose pathogen presence, the gold standard of which is a qPCR targeting the *rimM*

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gene region (Harper et al. 2010; EPPO 2019; IPPC 2018). Although less sensitive than qPCR (Waliullah et al. 2019), a LAMP assay to the same Xylella specific *rimM* gene target has shown operational capability, and advancement of this protocol and other isothermal linked techniques with varied genomic targets have been successfully developed (Yaseen et al. 2015; Aglietti et al. 2019; Luchi et al. 2023; Waliullah et al. 2019).

Recently the capability of incorporating isothermal amplification with CRISPR/Cas systems to develop specific and sensitive rapid diagnostic tools to detect bacterial and viral plant pathogens has been shown (Broughton et al. 2020; Wheatley et al. 2021).

Here, we report the outcomes for a *X. fastidiosa* detection assay combining a pre-existing LAMP assay with CRISPR/Cas12a, with potential to further develop and optimise the method into a rapid-answer field-deployable tool. In our LAMP-CRISPR-Cas12a integrated assay, LAMP-amplified DNA binds to the target RNA sequence of the guide RNA (gRNA) in the gRNA/Cas12a complex and transforms the Cas12a protein into non-specific endonuclease to cleave the single-stranded reporter. Cleavage of the reporter produces a fluorescent signal that is readable using detection platforms such as Genie® (OptiGene, UK).

To create the Xylella CRISPER-Cas12a detector system, species specific LAMP primers (Harper et al. 2010) (Supplementary data: Table 1) were used to isothermally amplify *X. fastidiosa rimM* followed by binding of gRNA (5'-uaauuucuacuaaguguagauGCUACCGAGAACCAC ACC-3') which was designed based on the sequence of LAMP amplified 16S region. The target specific CRISPR RNA (crRNA) region of the gRNA was designed to anneal with the LAMP Forward Inner Primer (FIP) following a protospacer adjacent motif (PAM) site (TTTC).

To test the sensitivity and specificity of gRNA, synthetic double stranded (dsDNA) oligonucleotides were created with the Synthetic Positive Template (SPT) dsDNA oligonucleotide, designed to include the gRNA binding site of the target strand (SPT_TS) (Supplementary data: Table 1). Synthetic Negative Template (SNT) was designed with five nucleotide substitutions from the positive template, located after the PAM site. Substitutions after the PAM site were designed for insufficient binding of gRNA to SNT for an intentionally reduced signal. The PAM site sequence (TTTC) in the non-target strands (SPT_NTS and SNT_NTS) and its reverse and complementary sequence (AAAG) in target strands (SPT_TS and SNT_TS) are highlighted in yellow (Supplementary data: Table 1). Single-stranded DNA (ssDNA) fluorescent reporters (56-FAMN/TTATT/3IABkFQ,) were designed as per methods described within He et al. (2020). The reporters consisted of a FAM (Fluorescein) reporter separated from a fluorescence quencher (FQ) by a short TTATT sequence. All primers, synthetic controls, fluorescent reporters, and gRNA were obtained from Integrated DNA Technologies, (IDT) and listed in supplementary data: Table 1.

To create a reaction assay, LbCas12a-crRNA complexes were pre-assembled by incubating 1 µM LbCas12a (M0653S, New England Biolabs; NEB, USA) and 1.25 µM crRNA (IDT, USA) at room temperature (as per methods described in He et al. 2020) prior to the addition of the fluorescent reporter and target DNA. Each reaction assay contained 2 µl of NEBuffer (2:1) (B7202S, NEB, USA), 13 µl of nuclease-free water (ThermoFisher Scientific, Australia), 50 nM ssDNA reporter, 2 µl of LbCas12a-crRNA complexes (50 nM LbCas12a and 62.5 nM crRNA), and 2 µl of pre-amplified target DNA. The mixture was incubated at 37 °C for 120 min in a Genie®II portable isothermal amplification and fluorometer instrument (OptiGene, United Kingdom; UK) for detection. Fluorescence readings were measured as a 'relative fluorescence unit' (RFU) and occurred every 2 min.

The reaction assay was optimised using 50 nM SPT, and within 10 min a significantly higher fluorescence ($P < 0.05$) was observed compared to the related SNT and background. A 1000 RFU was considered an appropriate positive detection cut-off boundary as no negative or non-target controls produced a signal above this fluorescence value.

To compare the fluorescence signal intensities between stand-alone LAMP (Harper et al. 2010) and LAMP-CRISPR-Cas12a integrated assays, a dilution series (range from 15.5 nM to 155 aM) of purified 16S target DNA template, amplified from DAFF sample *X. fastidiosa* isolate *X6A* (*X. fastidiosa* sub-sp. *multiplex* in *Olea europaea* leaf material), diluted in nuclease-free water, was tested with both assays and the development of fluorescence signals were observed over time (Fig. 1).

The standalone LAMP assay was run for 30 min, based on European and Mediterranean Plant Protection Organization (EPPO) assay recommendations (EPPO 2018) and although detection activity was shown to occur rapidly after initiation, the LAMP-CRISPR-Cas12a integrated assay was run for 120 min to analyse possible late-stage off-target activity. No late-stage activity was shown, with total peak fluorescence approximately seven-fold higher than recorded for standalone LAMP.

The maximum fluorescence intensity of ~ 55 000 RFU was observed from the highest concentration (15.5 nM) of target DNA using standalone LAMP assay. As target concentration reduced, stand-alone LAMP continually produced lowered fluorescent intensities over increasing time with a LOD at 1.5 fM at approx. 25 min, with inconsistent detection occurring at lowered concentrations (Fig. 1A).

The LAMP-CRISPR-Cas12a assay showed ~ 50 000 RFU for all concentrations until the LOD at 1.5 fM, however, this signal was recorded within 10 min (Fig. 1B). Both assays record a similar sensitivity, however, the increased total signal intensity of LAMP-CRISPR-Cas12a assay for reducing

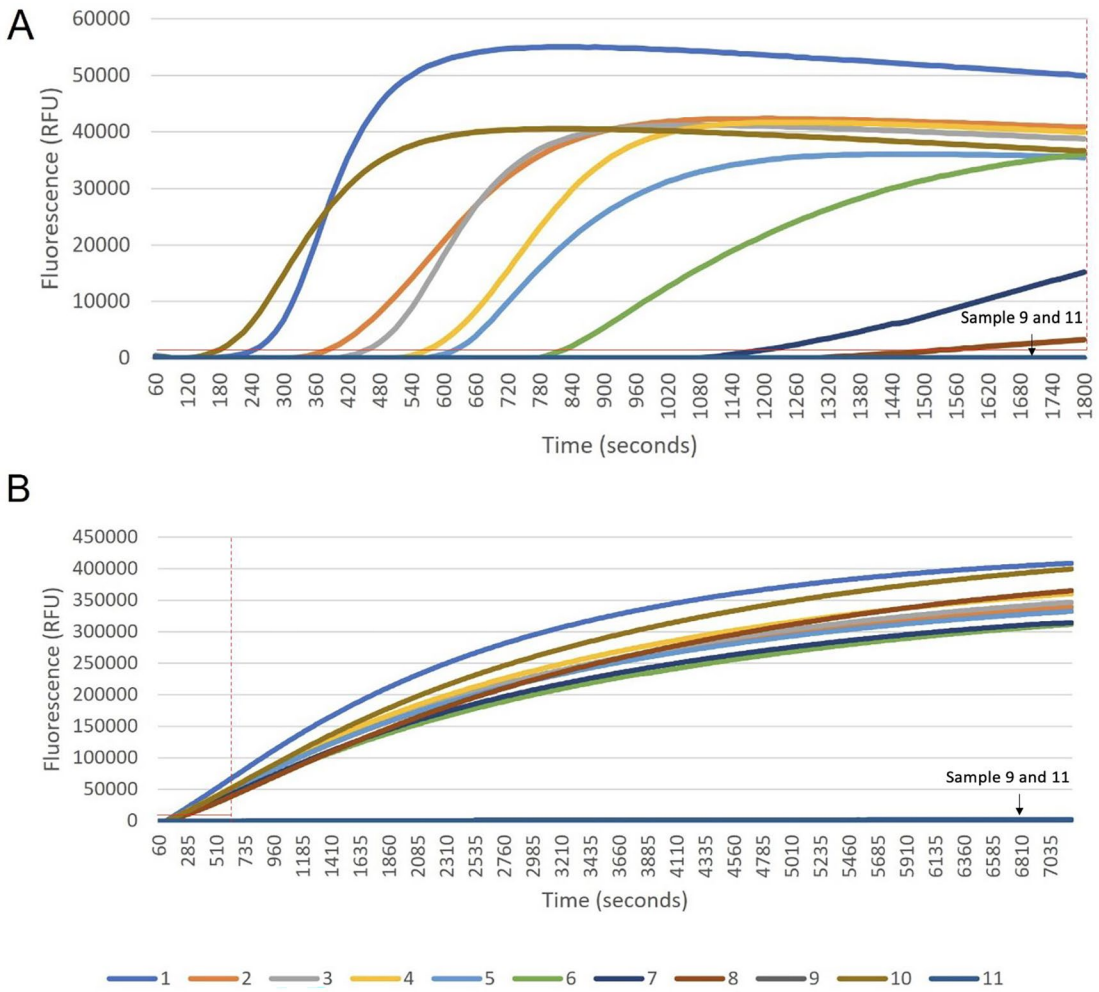


Fig. 1 Fluorescence signals in *X. fastidiosa* LAMP (**A**) and LAMP-CRISPR-Cas12a integrated (**B**) assays across serially diluted concentrations of *X. fastidiosa* target DNA template. The integrated fluorescence spectra of three technical replicates are shown. Varying concentrations of target DNA (*X. fastidiosa*) were analysed: 1. 15.5 nM; 2. 1.55 nM; 3. 155 pM; 4. 15.55 pM; 5. 1.55 pM; 6. 155 fM; 7. 15.55 fM; 8. 1.55 fM; 9. 155 aM; 10. Positive control; 11. NTC. Red dotted lines show the positive/negative cut-off

concentration samples has shown increased diagnostic decision interpretation over a reduced timescale compared to LAMP. This is a direct result of isothermal amplification and then detection by the detector complex, where samples are entering the detection phase of the assay at higher initial concentration, rather than a continual isothermal amplification of the target with continual detection phase, where low concentration samples have reduced signals. Thus, the LAMP-CRISPR-12a offers a higher increasing decision-making capability though increased signal performance and reducing diagnostic call timeframe. This may increase the utility of the LAMP-CRISPR-Cas12a assay for surveillance events, especially for very low titre infections.

We then used the LAMP-CRISPR-Cas12a assay to analyse DNA from *X. fastidiosa* infected plants, non-infected plants, and a limited selection of non-target bacterial isolates. *X. fastidiosa* positive samples were supplied as genomic DNA (gDNA) extracts from historical samples of Xylella infected plant tissue.

The original infection titre, symptomatic nature, and DNA extraction method of the historical samples are unknown – with samples stored at -80 °C for approximately 10 years. The samples offer examples of natural infection of the *X. fastidiosa* sub-species *fastidiosa*, *sandyi*, and *multiplex* within economically relevant host tissue, namely *Vitis vinifera, Nerium oleander, Olea sp., and Citrus sinensis.* Historical samples were pre-qualified for amplification using a COX DNA assay (Weller et al. 2000) and as Xylella positive using qPCR (Harper et al. 2010). Due to the limited availability and paucity of natural Xylella infected material available to this study, samples 5 and 6 were not assessed by qPCR. For qPCR assays, a 20 µl master-mix containing 2 µL template DNA, 1 X GoTaq probe qPCR Master Mix (A610A; Promega, Australia), nuclease-free water (ThermoFisher

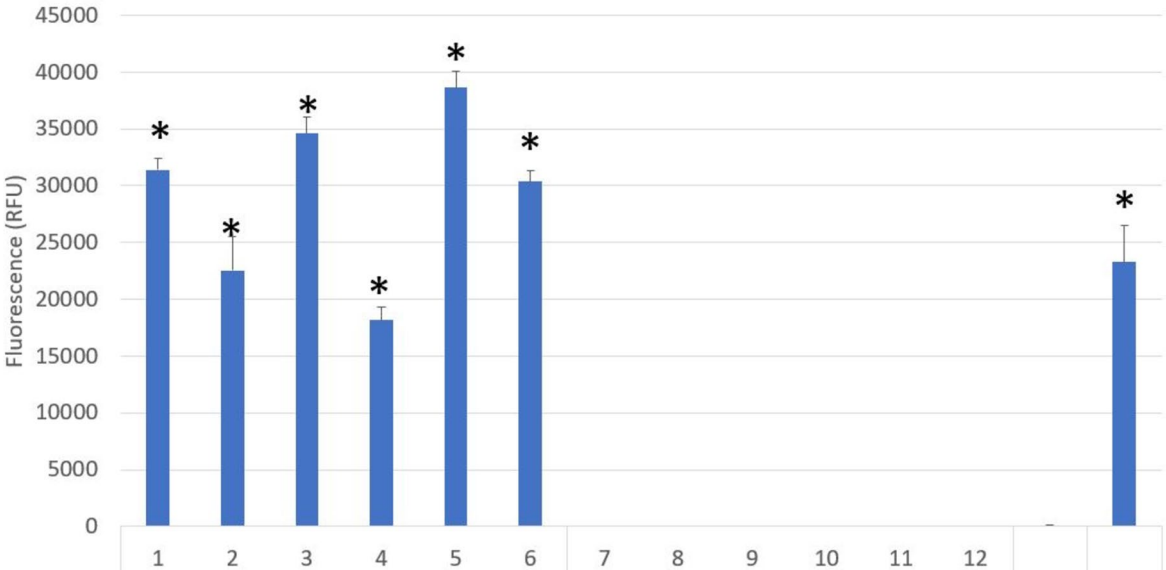
Scientific, Australia), 5% 10 mg/ml Bovine Serum Albumin, 0.5 µM forward primer (XF-F; IDT, USA), 0.5 µM reverse primer (XF-R; IDT, USA) and 0.25 µM probe (XF-P; IDT, USA). Cycling conditions were 95 °C for 2 min followed by 37 cycles of 95 °C for 15 s and 60 °C for 1 min. Ct values below 37 were considered positive. The assay was performed using Mic qPCR Cycler (Bio Molecular Systems, Australia) and independently on a CFX-96 qPCR platform (Bio-Rad, USA).

Historical samples were assessed by the LAMP-CRISPR-Cas12a system. Prior to Cas12a detection pre-amplification of the target region of the *X. fastidiosa rimM* gene was performed using LAMP with each 25 µl reaction containing; 1 × isothermal master-mix (OPG-ISO-DR001; OptiGene, UK), 1 µM of inner primers (FIP/BIP; IDT, USA), 0.2 µM of outer primers (F3/B3; IDT, USA), 0.5 µM of loop primers (LF/BF; IDT, USA), nuclease-free water (ThermoFisher Scientific, Australia), and DNA template. After an incubation at 65 °C for 30 min, 2 µl of purified LAMP product was used as the target DNA for LbCas12a detection in the LAMP-CRISPR-Cas12a assay.

Host tissue negative control gDNA was extracted using plant leaf material using Maxwell RSC PureFood GMO and Authentication kit (AS1600; Promega, Australia) following the manufacturer's instructions. No non-target amplification was recorded from these samples by CRISPR-Cas12a system (Fig. 2).

Negative bacteria of plant genera previously showed *rimM* specificity were used at high DNA concentrations to act as a negative control of high non-target bacterial DNA, with a focus of potential inhibition or non-target fluorescence assessment. No non-target detection was observed from non-target bacteria tested in this study (Fig. 2).

The LAMP-CRISPR-Cas12a integrated assay successfully detected *X. fastidiosa* in DNA samples from naturally infected DNA samples sourced for this study (Fig. 2). Although we did not have complete records of the exact sample extraction techniques for each of the historical samples, samples were shown to pass DNA quality control assessment



	1	2	3	4	5	6	7	8	9	10	11	12	Neg	Pos
qPCR	+	+	+	+	n/a	n/a	-	-	-	-	-	n/a	-	+
Ct (n=3)	26.61	28.45	32.91	29.20	n/a	n/a	40+	40+	40+	40+	40+	n/a	40+	25.98

Fig. 2 Detection of *X. fastidiosa* DNA from naturally infected plant materials using the LAMP-CRISPR-Cas12a integrated assay and the corresponding qPCR outcomes. DNA extracted from infected plant materials, uninfected plant materials and untargeted bacterial species were used to test the assay: 1. *Vitis vinifera* infected with *X. fastidiosa* subsp. *fastidiosa*; 2. *Nerium oleander* infected with *X. fastidiosa* subsp. *sandyi*; 3. *Olea sp.* Infected with *X. fastidiosa* subsp. *multiplex*; 4. Unknown plant species infected with *X. fastidiosa*; 5. *Citrus sinensis* infected with *X. fastidiosa* subsp. *pauca*; 6. Unknown plant species infected with *X. fastidiosa subsp. fastidiosa*; 7. Uninfected *V. vinifera*; 8. Uninfected *Olea sp*; 9. *Pseudomonas fluorescens* DUS1-29; 10. *Pantoea agglomerans* DUS1-2; 11. *Janthinobacterium sp.* DUS1-33; 12. *Clavibacter michiganensis* (QDAF isolate); Neg. negative control (H_20); Pos. synthetic positive target (SPT). Asterisks indicate significant differences ($P < 0.05$) from negative control. Statistical significance analysis was performed in Microsoft Excel using ANOVA. Tested qPCR samples are shown as mean Ct for three sample replicates, with a cut-off at Ct 37. qPCR Samples with Ct + 37 are considered negative

using NanoDrop™ and Qubit fluorometer, and each historical sample was confirmed as Xylella positive using the methods of LAMP, qPCR (Harper et al. 2010) and our protocol, fulfilling the EPPO requirements of two diagnostic tests to assess pathogen presence. Although the symptomatic level of the historical samples used in this study cannot be confirmed, LAMP-CRISPR-CAS12A positive diagnostic detection matched clearly positive Ct values (ranging of ~ 26–33) returned from samples with natural infection titres (Fig. 2).

This data supports the use of LAMP-CRISPR-Cas12a integrated assay to specifically and selectively detect the diagnostic target. However, as our positive sample set was limited to historical collections, further assessment of assay specificity using infected material from a wide range of host tissues and selectivity via inclusion of uninfected expected host material and closely related bacterial genera – namely *Stenotrophomonas* spp. and *Xanthomonas* spp. – would provide further data toward validation of our tool. In support of our sample selection and workflow decisions, the Xylella *rimM* gene tarted region has previously been assessed as suitably reliable assay target in a high diversity of operationally relevant host tissue types and infection titre loads (Waliullah et al. 2019).

Sensitive and specific detection of *X. fastidiosa* is critical to outbreak control and management, as infected plants can be asymptomatic or show similar symptoms to abiotic stresses or nutrient deficiencies. Although several Xylella detection tools are available, per-test price, reagent accessibility, user experience, and surveillance scale are all logical barriers that are potentially reduced by a new tool, so irrespective of the operational context, access to reliable information can be achieved. As performance of the assay can occur without the need for a thermocycler and highly temperature sensitive reagents, movement of the assay into field-like scenarios are likely future outcomes. Inclusion of Xylella detection capability in insect vectors is possible (Rodrigues et al. 2003) and would be of value towards future development of the LAMP-CRISPR-Cas12a assay as a versatile and reliable surveillance tool. As too would be compatibility with bioassays and non-electronic detection indicators (Ivanov et al. 2021) or the use of evolved Cas systems (Aman et al. 2020; Mustafa and Makhawi 2021).

The overlap in LAMP-CRISPR-Cas12a and qPCR data suggests the sensitivity of the LAMP-CRISPR-Cas12a assay as suitable for use on Xylella positive material. We note the need for further assessment against validation criteria defined by EPPO (2021) or other similar standards, however the technique presented here holds promise as a suitable tool. Thus, we consider this data as evidence for use of LAMP-CRISPR-Cas12a based assays for the detection natural Xylella infections, including three sub-species, in economically relevant hosts.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13313-023-00954-4.

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Data availability The authors confirm that the data used to support the findings in this study is provided in the article and its supplementary material. Raw data that supports the findings of this study may be made available on request.

Declarations

Conflict of interest The authors have no conflict of interest to declare that are relevant to this article.

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