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Comparison of conventional and novel molecular diagnostic methods for detection of *Xylella fastidiosa* from insect vectors

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Summary. The efficiency of three diagnostic methods, i.e. PCR, real-time PCR and LAMP, for detection of *Xylella fastidiosa* (*Xf*) genomic DNA from *Philaenus spumarius* (*Ps*) and *Neophilaenus campestris* (*Nc*) insect vectors was evaluated using three total nucleic acids (TNA) extraction methods (EM). In addition, a new real-time LAMP technology, Fluorescence of Loop Primer Upon Self Dequenching-LAMP (FLOS-LAMP), originally developed for human virus diagnoses, was optimized and assessed for detection of Xf in insect vectors. EM1 consisted of entire insects heated in an extraction buffer (EB) containing Tris-EDTA and TRITON-X100. In EM2, TNAs were extracted only from excised heads of insects, and heated again in the EB of EM1. EM3 consisted of grinding entire insects, heads and bodies recuperated from EM2, with a CTAB buffer. The molecular analyses conducted on 100 specimens of Ps and 50 of Nc, collected from a Xf-infected olive orchard (Lecce province, Italy), showed that 29% of specimens (40 Ps and four Nc) were positive to the presence of Xf. The comparison between the three methods revealed that EM3 is the most efficient for extracting Xf-genomic DNA from insect vectors, of which 44 specimens were positive for Xf in each of the diagnostic methods used, including the newly optimized FLOS-LAMP assay. In general, the real-time PCR and LAMP assays were more competent than the conventional PCR for detection of Xf in insect vectors, independently from the EM used. The newly optimized FLOS-LAMP technique had a detection limit of 1 fg μL^{-1} of Xf-genomic DNA, compared to the 10 fg μL^{-1} for conventional LAMP. The high sensitivity of the FLOS-LAMP was evident through the greater number of overall Xf-infected insect vectors detected (60%), compared to those for LAMP (45%,), real-time PCR (28%) and PCR (10%). FLOS-LAMP, being a more sensitive and specific assay, together with EM3, were the most appropriate approaches for an accurate detection of Xf in insect vectors.

Keywords. *Philaenus spumarius*, *Neophilaenus campestris*, PCR, real-time PCR, LAMP, FLOS-LAMP

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INTRODUCTION

Xylella fastidiosa (*Xf*) is a xylem-inhabiting, vector-borne, Gram-negative polyphagous plant pathogenic bacteria, which causes important diseases on many crops (Denancé *et al.*, 2019). In Europe, *Xf* has been reported firstly in Italy and subsequently in France, Spain, Portugal and Germany (outbreak eradicated), where three different subspecies of the pathogen have been found (EFSA, 2019). In Italy, *Xf* is associated with the Olive Quick Decline Syndrome (OQDS), causing leaf scorch; extensive dieback and death of plants (Martelli *et al.*, 2016). In nature, the short-distance transmission of *Xf* occurs through xylem-feeding insects, such as sharpshooter leafhoppers (family *Cicadellidae*) and spittlebugs (family *Aphrophoridae*) (Redak *et al.*, 2004). In the Apulia region (south of Italy), the meadow spittlebug *Philaenus spumarius* L. (*Ps*), *P. italosignus* Drosopolous & Remane (*Pi*) and *Neophilaenus campestris* (Fallen) (*Nc*) had been reported as vectors of *Xf*, transmitting the bacterium from infected to uninfected plants (Elbeaino *et al.*, 2014; Bucci ,2018; Cavalieri *et al.*, 2019). Furthermore, *Ps* has been recognized as the major vector involved in the spread of *Xf* infections among olive trees in the Apulia region, being the most abundant and prevalent insect vector species (Cavalieri *et al.*, 2019).

The diagnostic methods used to detect *Xf* in infected plant material and/or insect vectors include serological (DAS-ELISA, DTBIA) and molecular (PCR, real-time PCR and LAMP) techniques; and molecular techniques are recommended exclusively for detection of the pathogen in insect vectors (EPPO PM7\24[4], 2019). Molecular techniques are the most widely used due to their high throughput potential for detecting *Xf*-genomic DNA in plants and\or insect vectors. However, their application is hampered by: (i) low concentrations of bacterial DNA in infected sources, (ii) adequate protocols for extracting pure DNA, (iii) host microbiome-related DNA similar to that of *Xf*, (iv) heavy reliance on indirect detection methods including non-specific dyes, (v) on site application (laboratory, field, inspection points), (vi) techniques with laborious handling, and (vi) high costs.

To cope with these constraints, the present study aimed to compare PCR, real-time PCR and LAMP assays, and to evaluate their efficiency and sensitivity for detection of *Xf* in *Ps* and *Nc*. Application of the recently developed technique, Fluorescence of Loop Primer Upon Self Dequenching-LAMP (FLOS-LAMP) was also evaluated. This technique relies on direct detection, whereby a labeled loop probe quenched in its unbound state, fluoresces only when bound to its target (Gadkar *et al.*, 2018). This new approach of using labeled fluorescent primers in the LAMP assay offers increased sensitivity and specificity in the detection of *Xf*, which could cope with the constraints mentioned above. These evaluation trials were conducted on three different total nucleic acids (TNA) extraction methods (EM) of *Xf*-genomic DNA from insect vectors.

MATERIALS AND METHODS

Collection of infected plant material and insect vectors

In September 2018, 24 samples of scorched leaves were collected from olive trees situated in, and nearby to, the '*De Donno*' *Xf*-affected orchard (Gallipoli, Lecce province, Italy). This orchard was also screened for the presence of *Ps* and *Nc* insect vectors. Insects were manually trapped using a sweeping net, which was passed over the olive canopy and ground vegetation. Individual insects were stored in a solution of 95% ethanol and were brought to the laboratory for species identification. The classification and nomenclature of captured insects were based on key taxonomic factors described in Elbeaino *et al.* (2014). During the identification, only *Ps* and *Nc* specimens were retained and were stored at -20°C for the molecular analyses.

Extraction of total nucleic acids from plants and insect vectors

Bacterium DNA was extracted from leaf tissues of infected olive plants following the CTAB protocol (2% hexadecyl trimethyl-ammonium bromide, 0.1 M Tris-HCl pH 8, 20 mM EDTA and 1.4 M NaCl) (Hendson *et al.*, 2001). Briefly and for each sample, a 0.3 g piece of fresh leaf midrib and petiole was homogenized with 2 mL of CTAB buffer, using an automated hammer. Extracted sap was incubated at 65°C and then chloroform treated. TNA was precipitated with 0.6 volume of cold 2-propanol and resuspended in 120 µL of sterile water. Samples were used in various molecular assays as positive controls. TNAs were extracted from *Ps* and *Nc* insects by three different EM that were performed as the following:

(i) EM1: Insects were each rinsed with sterile water, dried on a tissue paper, and then immersed in 200 µL of extraction buffer (EB) containing 1× TE (10mM Tris-HCl, 1mM EDTA-Na_2, pH 8.0) and 0.5% TRITON-X100. The mixture was then incubated for 5 min at 94°C, followed by chilling on ice. A 25 µL volume of TNA from the EM1 was stored at -20°C for further molecular analysis. The remaining 175 µL containing the TNAs

in suspension, and not the insect, were added to the TNAs obtained from EM2 and all were precipitated in one plastic tube with cold 2-propanol at 10 000 g for 20 min. Through this operation, 87.5% of the TNA (175 µL of 200 µL of EB) obtained from each insect in EM1 was recovered; and by adding it to that extracted in EM2, the total amount of TNA that should normally be extracted in EM2 would not be compromised. Consequently, the comparison of the different diagnostic tests applied on the TNA models of each EM would be valid.

(ii) EM2: The heads of individual adult insect specimens were excised from the bodies, as reported in Elbeaino *et al.* (2014). Each insect body was stored in a plastic tube for further manipulation in EM3. Each excised head was added to 200 µL of EB and heated as described in EM1. A 25 µL volume containing the TNA was also stored separately for subsequent molecular assays. The remaining 175 µL were further precipitated with the TNAs obtained during EM3.

(iii) EM3: the head and body of each insect, recovered from EM1 and EM2, was ground in a mortar and pestle containing 500 µL of CTAB and carborundum particles. The extract was incubated at 65°C for 10 min and subsequently treated with chloroform for further purification. The supernatant was centrifuged at 8,000 g for 5 min, and then precipitated together with the 175 µL TNAs from EM2 with 0.6 volume of cold 2-propanol. The recovered TNAs were resuspended in 30 µL of sterile water. TNA samples from various methods were used in different molecular assays.

PCR, real-time PCR, LAMP and FLOS-LAMP

The TNA samples obtained from each insect using the three methods were subjected to PCR, real-time PCR and LAMP assays for the detection of *Xf*. Conventional PCR has been performed on TNA of olive plants to identify infected samples to be used as *Xf*-positive controls in the different molecular procedures. PCR reactions were performed using primers RST31/33, widely used in the detection of different *Xf* subspecies (Minsavage *et al.*, 1994), in a 1× amplification buffer in a final volume of 25 µL containing 2.5 µL of TNA, 0.2 mM of dNTPs, 0.2 µM of each primer and 1.25 U of DreamTaq™ DNA polymerase (Thermofisher). PCR cycles were as follows: 95°C for 1 min followed by 40 cycles of (95°C for 30 s, 55°C for 30 s, 72°C for 45 s) and a final step of 72°C for 5 min. All reactions were submitted to electrophoresis in 1.2% TAE agarose gels.

Real-time PCR was performed as described by Harper *et al.* (2010, *erratum* 2013), in 20 µL reaction volumes containing 10 µL of the SsoAdvanced ™ Universal Probes Supermix (BioRad), 0.3 µM *Xf*-forward (*XF*-F) and *Xf*-reverse (*XF*-R) primers, 0.1 µM of labeled *XF*-P probe and 2.5 µL of TNA. Thermocycling conditions were as follows: 95°C for 6 min, followed by 40 cycles of 94°C for 10 s and 62°C for 40 s. Reactions were conducted in a CFX96 thermocycler (BioRad,). A cycle threshold (Ct) value below 35 was scored as a positive result.

LAMP assays were carried out using Enbiotech's LAMP system® (Yaseen *et al.*, 2015). Reactions were carried out in 25 µL of final volume using 2.5 µL of TNA and 22.5 µL of LAMP Mix, at 65°C for 30 min in a CFX96 thermocycler.

The FLOS-LAMP approach developed in this study was based on three sets of primers; namely outer, inner and loop, used in LAMP for the detection of *Xf* (Harper *et al.*, 2010 *erratum* 2013). The B3, BIP and LB in, respectively, the outer, inner and loop primers categories, were appropriate to be labeled by substituting the internal thymine (T) residue at the 3' terminus of the primer with the fluorescein (FAM) fluorophore (Table 1). The criteria followed for the exact T residue to which the fluorophore can be attached were those reported in Gad-

Table 1. Six primers of three categories (outer, inner and loop) used in the FLOS-LAMP assay. The internal thymine residues (**T**) at the 3' terminus of primers for each category (B3, BIP, LB) were fluorescein-labeled.

Primer	Sequence (5'-3')	Binding sites on *Xf* genome
Outer		
Xf-F3	CCGTTGGAAAACAGATGGGA	(106,676–106,694)
Lab*Xf*-B3	GAGACTGGCAAGCGTT**T**GA	(106,884–106,865)
Inner		
Xf-FIP	ACCCCGACGAGTATTACTGGGTTTTTCGCTACCGAGAACCACAC	(106,788–106,862)
Lab*Xf*-BIP	GCGCTGCGTGGCACATAGATTTTTGCAACCTTTCCTGGCA**T**CAA	(106,773–106,695)
Loop		
Xf-LF	TGCAAGTACACACCCTTGAAG	(106,824–106,844)
Lab*Xf*-LB	TTCCGTACCACAGA**T**CGCT	(106,753–106,735)

kar *et al.* (2018), *i.e.* (i) presence of cytosine or guanine residue at the terminal 3' end, (ii) T residue at the second or third position from this 3' end, and (iii) one or more G nucleotides flanking the T residue. Different combinations of labelled primers were tested in the FLOS-LAMP assays. PCR reactions were performed in a 25 µL of final volume containing 2.5 µL 10× Isothermal amplification Buffer, 5 µM of $MgSO_4$, 1.4 mM dNTPs mix with 0.06 µM of *XF*-F3/*XF*-B3 outer primers, 0.12 µM pf *XF*-LF/*XF*-LB loop primers, 0.5 µM of *XF*-FIP/*XF*-BIP inner primers, 2,400U of *Bst* 3.0 DNA Polymerase (New England Biolabs) and 2.5 µL of DNA sample. The thermocycler used for FLOS-LAMP was a BioRad CFX96.

Sensitivity of FLOS-LAMP

In order to determine the detection limit of the newly FLOS-LAMP, optimized for the detection of *Xf*, 10-fold serial dilutions of 10 ng μL^{-1} of DNA extracted from a pure culture of *Xf*-ST53 strain were conducted and subjected to FLOS-LAMP assay, following the conditions and cycles described above.

RESULTS

Identification of Ps and Nc

In total, 233 adult specimens of *Ps* and 141 of *Nc* were identified. One hundred *Ps* and 50 *Nc* specimens were randomly selected and used in the molecular analyses. The large number of *Ps* captured from the ground vegetation and canopies of olive trees in the affected orchard reflect the high population density of this species in the Apulian environment, compared to that of *Nc*. This predominance of *Ps* was also recorded in previous epidemiological studies of these two species in the Apulia region (Ben Moussa *et al.*, 2016; Cavalieri *et al.*, 2019).

PCR, real-time PCR, LAMP

PCR assays conducted on olive leaf samples showed that 22 plants (out of 26 tested) were infected with *Xf*. This high proportion of infections was expected, since *Xf* is widespread in the Gallipoli location. PCR assays conducted on TNAs from the three EM detected *Xf* only in aliquots obtained from the EM3, for which 40 specimens of *Ps* and four of *Nc* were positive for presence of *Xf* (Table 2).

The real-time PCR gave negative results when applied to TNA extracts from EM1; whereas those from EM2 and EM3 were more appropriate for amplifying *Xf*-genomic DNA (Figure 1). Twenty-four specimens of *Ps* and three of *Nc* were found with *Xf* in EM2; whereas 40 *Ps* and four of *Nc* positive specimens were detected when TNAs from EM3 were used (Table 2).

LAMP assays showed differential positive reactions when applied on TNAs from EM1, EM2 and EM3 (Figure 1). For *Ps*, this assay detected 16, 34 and 40 *Xf*-infected insects from, respectively, EM1, EM2 and EM3. For *Nc*, only one, two and four *Xf*-infected specimens were detected, respectively, from EM1, EM2 and EM3. The proportions of infections detected with LAMP, using our conditions, were 11% from EM1, 24% from EM2 and 29 % from EM3.

FLOS-LAMP

Among the outer, inner and loop functional categories defined for the six-primer LAMP, the *XF*-LB primer identified only in the loop category in FLOS-LAMP of the *Xf* genomic DNA. Positive fluorescence signals were gener-

Table 2. Comparative analyses between PCR, real-time PCR, LAMP and FLOS-LAMP assays, applied on TNAs extracted by three different methods (EM1, EM2 and EM3) from 100 *Philaenus spumarius* (*Ps*) and 50 *Neophilaenus campestris (Nc*) specimens, for detection of *Xylella fastidiosa* (*Xf*).

Insect species	PCR			Real-time PCR			LAMP			FLOS-LAMP		
	EM1	EM2	EM3	EM1	EM2	EM3	EM1	EM2	EM3	EM1	EM2	EM3
Xf-positive *Ps*	0	0	40	0	24	40	16	34	40	34	36	40
Xf-positive *Nc*	0	0	4	0	3	4	1	2	4	3	3	4
Ps-, *Nc*-*Xf* positive	0	0	44	0	27	44	17	36	44	37	39	44
Infection %	0	0	29.3	0	18	29.3	11.3	24	29.3	24.6	26	29.3
Detection %	0	0	100	0	61.3	100	38.6	81.8	100	84	88.6	100
Mean detection %		9.7			27.7			45			60.3	

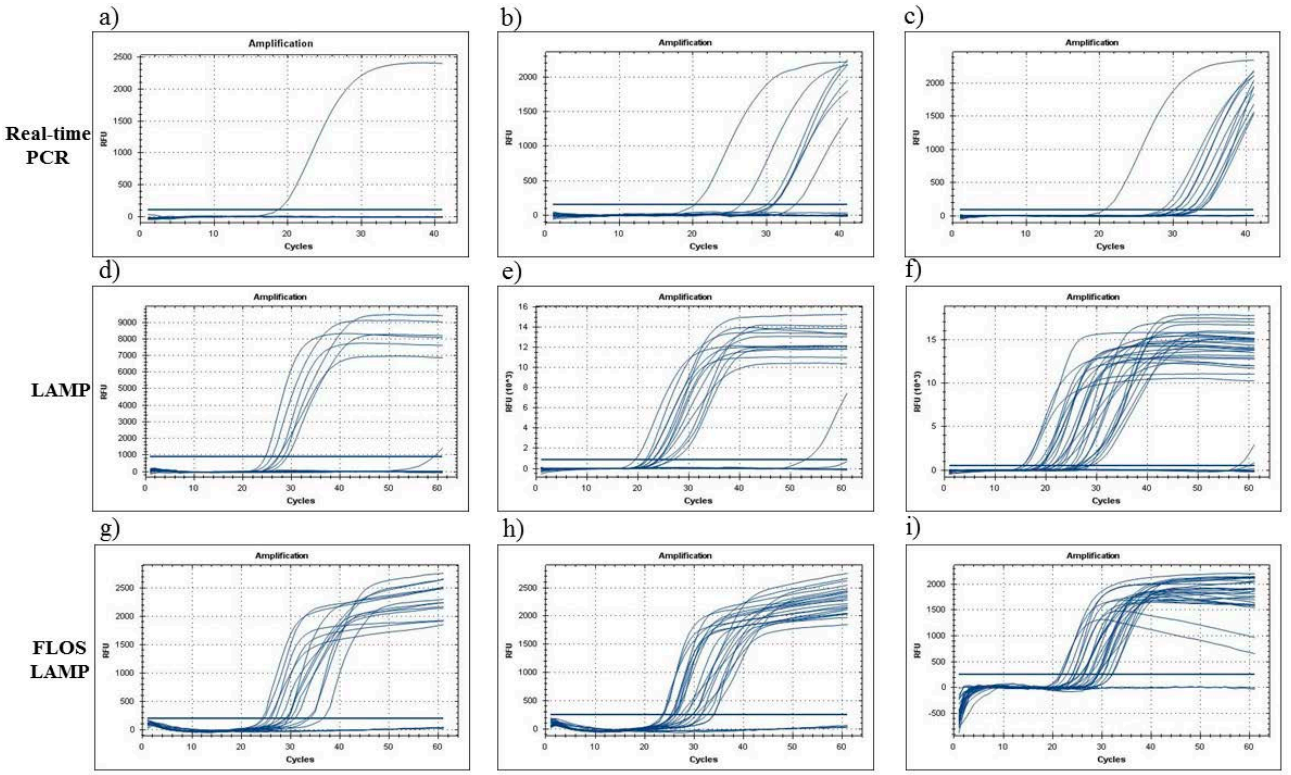

Figure 1. Differential responses of real-time PCR, LAMP and FLOS-LAMP assays to *Xylella fastidiosa* (*Xf*) infections in *Philaenus spumarius* and 50 *Neophilaenus campestris* specimens, using TNA extracted with three different methods (EM1, EM2 and EM3) for the same insects. *Xf*-positive reactions increased when TNA from EM1, EM2 and EM3, and from real-time PCR to FLOS-LAMP were used. A cycle threshold (Ct) values below 35 were scored as positives.

ated when the FAM-labeled LB probe was tested against the templates. No signals were detected from FAM-labeled FIP and B3 primers functioning as probes. This indicated that the *XF*-LB self-quenched FLOS probe bound and fluoresced only in the presence of *Xf*-genomic DNA target. The FLOS-LAMP confirmed the PCR results, successfully amplifying all samples found PCR-positive to *Xf*, without producing non-specific reactions from healthy plants.

Of the 150 insects analyzed, 34 *Xf*-infected *Ps* were identified using EM1, 36 using EM2 and 40 were identified using EM3. Three *Nc* positive specimens were detected using EM1 and EM2, and four positives were detected using EM3 (Figure 1). Based on the FLOS-LAMP approach, the proportions of infection were 256% using EM1, 26% using EM2 and of 29% using EM3 (Table 2). These results reflected the different sensitivity and specificity of the two approaches. Overall, FLOS-LAMP applied to samples from EM1, EM2 and EM3 detected greater numbers of *Xf*-infected insect vectors, thus identifying 60% of the *Xf*-positive samples. The LAMP assay identified 45% of the positive samples, with increments of 13% for EM1 and 2% for EM2.

Detection limit of FLOS-LAMP

FLOS-LAMP gave high levels of sensitivity by amplifying all dilutions greater than 1 fg μL^{-1}. Compared to the sensitivity of the other techniques tested here, FLOS-LAMP was 10 times more sensitive than conventional LAMP. The detection limit of LAMP is reported to be 10 fg μL^{-1} for *Xf*-genomic DNA (Yaseen *et al.*, 2015), and 100 times more than that of real-time PCR (Harper *et al.*, 2010, *erratum* 2013). The sensitivity of FLOS-LAMP was demonstrated by the large number of positive samples detected from EM1 (37 *Xf*-infected *Ps* and *Nc* compared to 17 in LAMP) and EM2 (39 *Xf*-infected *Ps* and *Nc* compared to 36 in LAMP).

DISCUSSION

Detection of *Xf* in infected plant material is conventionally carried out using serological (ELISA) and molecular (PCR, real-time PCR and LAMP) diagnostic techniques. When versatility, precision and sensi-

tivity are required, preferential differences occur with application of these approaches. Serological assays have been widely adopted to detect *Xf* in infected plant material but not for detection in insect vectors. The preferential use of some methods is conditioned by the nature of this pathogen. Localization of the pathogen in host xylem tissues makes extraction difficult. Uneven distribution within plants can miss infected tissues with high bacterium concentrations. Latent infections due to the low bacterium concentrations can be less than minimum detection limits for some techniques. Most importantly, some techniques must be validated for not previously identified insect vectors of *Xf*. Therefore, we aimed to compare, validate and refine conventional and new diagnostic methods for the detection of *Xf* in *Ps* and *Nc* vectors of the pathogen. In our conditions, the EM3, *i.e.* grinding whole insects using the CTAB protocol, was the most suitable for extracting enough *Xf*-genomic DNA to be detectable using conventional and new molecular techniques, including the FLOS-LAMP assay here optimized. This was confirmed by identification of the greatest number of *Xf*-infected insects (44 specimens) in all four diagnostic techniques using TNA extracts from EM3.

Among the three EM tested here, EM1 and EM2 were the simplest to perform and least expensive, as few reagents were needed for their preparations. However, their outcomes remain precarious when used in different diagnostic techniques to detect *Xf* in insect vectors. In contrast, EM3 was the most efficient for bacterial DNA extraction but required greater effort than the other two extractions.

In general, the real-time PCR and LAMP techniques were more efficient than the conventional PCR for detection *Xf* in the insect vectors, independently from the EM used. Furthermore, when applied on TNAs from EM2, real-time PCR was more sensitive (18% infection), identifying 24 *Xf*-infected *Ps* and three infected *Nc*, compared with no detection using PCR. The failure of the real-time PCR to detect *Xf* in TNAs from EM1, and of the PCR when extracts from EM1 and EM2 were used, was probably due to the low concentration of bacterial DNA. The real-time PCR was therefore more sensitive than the PCR, and detected *Xf*-infected samples in extracts from EM2 with greater bacterial DNA concentration. However, and as expected, the LAMP method had high sensitivity, detecting 17 *Xf*-infected specimens in TNAs from EM1, while the other techniques did not detect the pathogen. The FLOS-LAMP technique gave even greater sensitivity than the LAMP assay, particularly when applied on TNAs from EM1, with 37 positive specimens detected compared with 17 from LAMP and none from real-time PCR and conventional PCR. This superiority was tested for detection of 1 fg μL^{-1}l of *Xf*-genomic DNA compared to that reported for conventional LAMP (10 fg μL^{-1}) (Yaseen *et al.*, 2015). Another advantage of the FLOS-LAMP was the reduction of non-specific reactions observed in the conventional LAMP assays after a Ct value >35 (Figure 1). This, often-generated misinterpretation of results, for whether those insect vectors should be considered as negative to the presence of *Xf* or positive with low *Xf*-genomic DNA concentrations, resulting in a greater Ct value. These artifacts of LAMP reactions, probably caused by the use of non-specific dyes, are overcome using self-quenching fluorogenic probes, in a direct detection approach. This increases the specificity of LAMP reactions.

In terms of costs, LAMP and FLOS-LAMP are inexpensive when the various components of the reactions are managed in the laboratory without the need to purchase commercial kits. Under our conditions, the estimated costs for LAMP and FLOS-LAMP were, respectively, approx. 0.2 and 0.25 Euro per sample, compared with an average of 10 Euro per sample required by diagnostic companies. The only disadvantage of the FLOS-LAMP, similarly to LAMP, is high sensitivity, which is a disadvantage when dealing with very small DNA contaminations, leading to false positive reactions that would be irrelevant in other techniques (PCR, ELISA).

This study has demonstrated that EM1 and EM2 are not suitable for extracting enough amounts of *Xf*-genomic DNA for amplification using any of the techniques here tested. The FLOS-LAMP technique was found, in our conditions, to be more sensitive and specific than conventional LAMP. We recommend that FLOS-LAMP be used for testing of *Ps* and *Nc* insect vectors for the presence of *Xf* in infested regions.

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