Detection of *Xylella fastidiosa* by colorimetric LAMP and droplet digital PCR assays

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Background

Xylella fastidiosa (Xf) is a Gram-negative bacterium belonging to *Xanthomonadaceae* family, colonizing the xylem vessel of over **600 plants** and the foregut of the insect vectors. In 2013, the entry of Xf subsp. pauca (Xfp) ST53 in Salento Peninsula (Apulia, Italy) represented the first confirmed outdoor outbreak of this exotic bacterium in Europe, leading to the dramatic epidemic of olive quick decline syndrome (OQDS) in Italy.

The detrimental impacts of the infections entail that, at European level and in several countries worldwide, the bacterium is categorized as **quarantine** and **priority pest**, with the consequent adoption of mandatory preventive and containment measures. Preventive strategies rely on early detection of infected plants, followed by the quick removal of these sources, reducing risks for further spread or for the establishment of new foci. Surveillance programs rely on molecular assays which are currently the only official recognized diagnostic tests at European level. In this work, we aimed to extend the panel of validated molecular tests by developing a novel format of Loop-mediated isothermal amplification (**LAMP**) and by implementing the Droplet digital polymerase chain reaction (**ddPCR**) protocols. Both approaches can complement the qPCR assays, which remain the golden standard and the most common used test for Xf detection. LAMP can represent a rapid and simple **point-of-care testing** (**POCT**) assay to be used for screening tests at port of entry or prior to move large lots of propagating materials. While ddPCR can support the assessment of the Xf status in samples yielding inconclusive results by qPCR.

LAMP is a low-cost and portable diagnostic assay that provides nucleic acid amplification in a short time (15-60 min) under constant temperature (from 60° to 70°C) producing stable amplicons. The strand displacement activity and the higher tolerance to inhibitors of the *Bacillus stearothermophilus* (**Bst**) DNA polymerase, allows for testing crude extracts, obtained directly with an alkaline extraction buffer, facilitating the chemical disruption of plant tissues overcoming the need for DNA denaturation by heating and the purification phases.

ddPCR is an advanced and powerful technology that allows an accurate detection and absolute quantification of the nucleic acid present in the samples, even when the target is quite low. The sampleis tested through the generation of thousands independent nanoliter-sized droplets that act as individual PCR test tubes, where amplification can take place. Partitions with an amplified fluorescent product are considered positive, whereas those without fluorescence are considered negative.

Methods

Crude Plant Extracts Preparation

Approximately **50-60 mg** of thin slices (0.3-0.5 mm length) of stem or leaf midribs/petioles recovered from olive twigs were soaked in 500 µL **extraction buffer** (EB) [**NaOH 0.4M**; Sodium diethyldithiocarbamate (**Na-DIECA**) **2%**; Polyvinylpyrrolidone (**PVP 25-30k**) **1%**] for 15 min at room temperature to lyse the bacterial cells and allow the release of target DNA molecules. An aliquot of 50 µL was then neutralized by mixing an equal volume of **neutralization buffer** (NB) [**HCl 0.4 M** and **NaCl 50 mM**]. Finally, 10 µL of the neutralized sap were diluted 1:10 in sterile water to dilute plant debris and contaminants inhibiting or reducing the efficiency of the amplification. Colorimetric LAMP (cLAMP) and ddPCR assays were then set up using this crude alkaline sap, employing previously validated primer [**1**].

cLAMP assay and device

The cLAMP assay was performed in a final volume of 25 µL, containing 12.5 µL **Bst 2.0 WarmStart® DNA Polymerase 2x Master Mix** (New England Biolabs), 2.5 µL LAMP primer mix (0.2 µM of primer F3/B3, 1.6 µM of primers FIP/BIP, 0.4 µM of primers LF/LB), 1 µL of crude sap, sterile water up to a final volume and, 25 µL of mineral oil to cover the LAMP reaction mix. A healthy control (a crude alkaline extract from a Xf-free olive tree) and a no-template control (NTC) were also included. The reaction mixture was incubated for **30 min** at **65°C** in a 3D-printed **portable incubator** device recharged by a power bank, connected via Bluetooth to a smartphone or tablet. The Phenol red indicator contained in the WarmStart® master mix allowed for the immediated visual detection based on the color transition of the reactions.

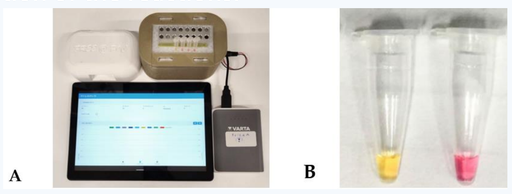


Figure 1. (**A**) Overview of the 3D-printed portable thermal block device. The plastic box contains a heating metal block, set to operate at 65 °C for 30 min. The device is powered by a portable power bank and connected via Bluetooth to a tablet. (**B**) The positive reaction (amplified DNA) is visualized by the color change from pink (negative signal) to yellow (positive signal).

Optimization of the Droplet Digital PCR Assay

ddPCR reaction were set up using both, purified DNA extracts and crude sap preparations. Experimental conditions included the optimization of the primers/probe concentration, the starting amount of the samples recovered from five plant matrices (*O. europaea*; *V. vinifera*; *P. dulcis; N. oleander*; *C. sinensis)* and insect (*P. spumarius),* and the number of cycles. The reaction mix (20 µL) and 70 µL of droplet-generating oil were added to a cartridge and loaded onto an Automated Droplet Generator. The water-in-oil droplets (40 µL) were carefully transferred to a 96-well PCR plate and placed in a Thermal Cycler for end-point PCR. Amplification reactions were performed with the cycling parameters optimized by Dupas *et al*., 2019 [**2**], with the number of cycles increased to 45 with a temperature ramp rate of 2°C/s.

Figure 2. Schematic illustration of a typical ddPCR workflow

The analytical sensitivity (detection limit; **LoD**) of both assays were assessed using 10-fold dilutions of both the Xfp bacterial suspension and the target region rimM cloned as recombinant plasmid DNA.

Results

Diagnostic Sensitivity of Xfp cLAMP Assay

The LoD achieved by cLAMP was **10^2 CFU/mL** for the bacterial culture and **0.9 pg/µL** for *rimM*-plasmid DNA (equivalent to 169.2 target copies/µL) [**3**].

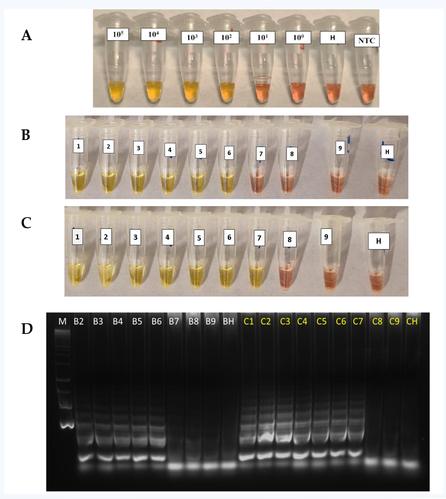


Figure 3: Healthy crude sap spiked with 10-fold serial dilution of (A) bacterial pure culture from 10^5 to 10 CFU/mL (H: Healthy control; NTC: NoTemplate Control) and (B–C) two replicates of purified plasmid DNA dilutions in healthy alkaline olive sap (from 90 ng/µL [tube 1] to 0.9 fg/µL [tube 9]). (D) Amplified products of the panel B (in white) and C (in yellow) were analysed by gel electrophoresis. M: Molecular weight; B2–B9: 9 ng/µL to 0.9 fg/µL; C1–C9: 90 ng/µL to 0.9 fg/µL; BH and CH: Healthy controls.

Diagnostic Sensitivity of Xfp ddPCR Assay

The best primers/probe concentration were found to correspond to 600/300 nM while the purified DNA amounts were 4 µL and 6 µL respectively for the five plant matrices analysed and insect samples and, 2 µL for olive crude DNA extract. The LoD of ddPCR by ten-fold dilution of plasmid DNA in the olive crude alkaline sap was determined to be **169.2 copies/µL** corresponding to 3384 target copies in 20 µL/reaction (**Figure 4**).

LoD were determined to be **4.30×10^{-1}** and **5.06×10^{-1} copies/µL** for purified DNA of the bacterial suspension and the plasmid respectively, corresponding to 8.60×10^{0} and 1.01×10^{1} copies per 20 µL/reaction (**Figure 5**) [**4**].

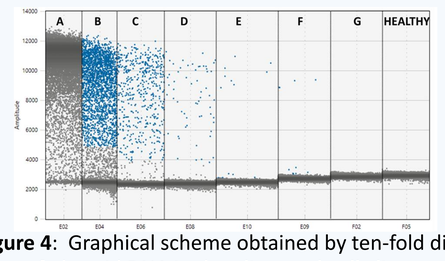


Figure 4: Graphical scheme obtained by ten-fold dilution of plasmid DNA in the olive crude alkaline sap.

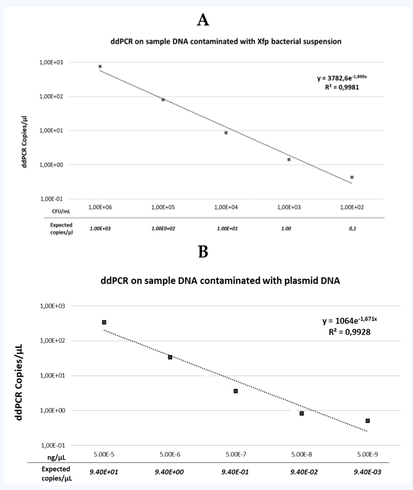
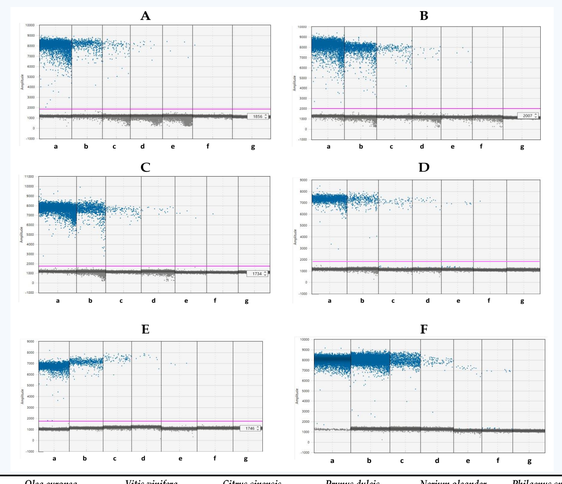


Figure 5: Linear regression of the ddPCR generated using 10-fold dilution of bacterial suspension (A; from 1.00×10^6 to 1.00×10^1 CFU/mL) and *rimM* plasmid DNA (B; from 50 fg/µL to 5 ag/µL.).



Dilution range	*Olea europea*		*Vitis vinifera*		*Citrus sinensis*		*Prunus dulcis*		*Nerium oleander*		*Philaenus spumarius*	
CFU/mL	*copies/µL*	*Replicates**	*copies/µL*	*Replicates**	*copies/µL*	*Replicates**	*copies/µL*	*Replicates**	*copies/µL*	*Replicates**	*copies/µL*	*Replicates**
1,00E+06	6,09E+02	2/2	7,97E+02	2/2	3,74E+02	2/2	6,62E+02	2/2	2,21E+02	2/2	4,99E+03	2/2
1,00E+05	1,08E+02	5/5	2,00E+02	2/2	5,43E+01	2/2	1,28E+02	2/2	3,90E+01	2/2	7,73E+02	2/2
1,00E+04	4,36E+01	5/5	1,98E+01	2/2	7,79E+00	2/2	1,33E+01	2/2	6,37E+00	2/2	1,20E+02	2/2
1,00E+03	5,10E+00	11/11	2,55E+00	12/12	1,08E+00	10/10	3,24E+00	10/10	2,02E+00	8/8	8,33E+00	8/8
1,00E+02	2,40E+00	14/14	4,74E-01	12/12	2,43E-01	9/10	3,92E-01	6/10	9,22E-01	7/8	2,02E+00	8/8
1,00E+01	1,20E+00	4/7	n.d.**		n.d.**		n.d.**		n.d.**		9,22E-01	4/8

*Replicates: Number of positive replicates/number of replicates analyzed.
**n.d.: Not detectable.

Figure 6: Comparison of the different LoDs obtained in the five plant spiked matrices and in the insect vector. Blue dots represent positive droplets. Gray dots represent the negative droplet background, with no amplification. (**A**): *O. europaea*; (**B**): *V. vinifera*; (**C**): *P. dulcis*; (**D**): *N. oleander*; (**E**): *C. sinensis*; and (**F**): *P. spumarius*. On x-axis: dilution of Xf suspension reported in wells a (10^6 CFU/mL) to f (10 CFU/mL). Well g: NIC (negative internal control), specific for each matrix; on the y-axis: amplitude value.

Table 1: Mean concentrations were estimated in copies/µL, as measured by ddPCR in each serial dilution of the spiked plant and insect matrices.

Validation of cLAMP and ddPCR on Field-Grown Olive and Insect Samples

Template: Olive crude DNA extract

qPCR Cq value	cLAMP positives / Nr analysed plants (per Cq value)	Diagnostic sensitivity %	Diagnostic specificity %
18-21	22/28	78.57	/
22	24/29	82.76	/
23	21/26	80.77	/
24	16/29	55.17	/
25	19/39	48.72	/
26	14/29	48.28	/
27	13/28	46.43	/
28	3/9	33.33	/
29-32	7/27	25.93	/
33-37	3/11	/	72.73
N/A	6/27	/	77.78
Total	**148/282**		

qPCR Cq value	ddPCR positive / Nr. analysed plants (per Cq value)	Diagnostic sensitivity %	Diagnostic specificity %
18-21	9/10	90.00	/
22	7/9	77.78	/
23	12/12	100.00	/
24	8/10	80.00	/
25	12/14	85.71	/
26	13/15	86.67	/
27	10/13	76.92	/
28-32	9/22	40.91	/
NA	1/10	/	90.00
Total	**81/115**	/	

Template: Purified DNA

Olive samples grouped based on the Cq values obtained in qPCR				ddPCR		
Group	Cq values	N. of samples	qPCR result	Positive	Negative	Total
(i)	27-29	N. 10	Positive	10	0	10
(ii)	30-32	N. 9	Positive	9	0	9
(iii)	33-34	N. 15	Undetermined	13	2	15
			Total	**32**	**2**	**34**

Insect samples grouped based on the Cq values obtained in qPCR				ddPCR			
Group	Cq values	N. of samples	qPCR result	Positive	Negative	Undetermined	Total
(i) 36	23-32	N. 11	Positive.	11	0	0	11
(ii)	33-36	N. 13	Undetermined	8	3	2	13
(iii)	>36	N. 3	Negative	0	3	0	3
			Total	19	6	2	27

Performance of cLAMP (**Table 2**) and ddPCR (**Table 3, 4** and **5**) for the detection of Xfp in naturally infected olive samples and insects already assessed by qPCR assay. All samples were grouped according their Cq value.

Conclusion

The work herein presented demonstrates that cLAMP and ddPCR can effectively implement the panel of molecular tests currently adopted for the official diagnostic control of Xf. With the cLAMP protocol herein described being suitable for rapid on site checks, while ddPCR because of its higher LoD can successfully support the detection of the bacterium in samples with low populations which remain undetectable with other molecular approaches.

References

1) Harper S.J. *et al*., *Phytopathology* **2010,** 100
2) Dupas E. *et al*., *J. Microbiol. Methods* **2019**, 162, 86–95
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Acknowledgements

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