Journal of Microbiological Methods 112 (2015) 36–39

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Note

Evaluation of a real-time PCR and a loop-mediated isothermal amplification for detection of *Xanthomonas arboricola* pv. *pruni* in plant tissue samples

Ana Palacio-Bielsa [a,*], Pablo López-Soriano [b], Andreas Bühlmann [c], Joop van Doorn [d,1], Khanh Pham [d], Miguel A. Cambra [e], Isabel M. Berruete [a], Joël F. Pothier [f], Brion Duffy [f], Antonio Olmos [b], María M. López [b]

[a] Centro de Investigación y Tecnología Agroalimentaria de Aragón, Av. Montañana 930, 50059 Zaragoza, Spain
[b] Instituto Valenciano de Investigaciones Agrarias, Ctra. de Moncada a Náquera km 4,5, 46113 Moncada, Valencia, Spain
[c] Plant Protection Division, Agroscope Changins-Wädenswil ACW, 8820 Wädenswil, Switzerland
[d] Applied Plant Research, Wageningen University & Research Centre, Prof. van Slogterenweg 2, 2161 DW, The Netherlands
[e] Centro de Sanidad y Certificación Vegetal, Av. Montañana 930, 50059 Zaragoza, Spain
[f] Environmental Genomics and Systems Biology Research Group, Institute of Natural Resource Sciences, Zurich University of Applied Sciences, 8820 Wädenswil, Switzerland

ARTICLE INFO

Article history:
Received 21 January 2015
Received in revised form 9 March 2015
Accepted 9 March 2015
Available online 11 March 2015

Keywords:
Xanthomonas arboricola pv. *pruni*
Diagnostic assays
Real-time PCR
LAMP

ABSTRACT

Operational capacity of real-time PCR and loop-mediated isothermal amplification (LAMP) diagnostic assays for detection of *Xanthomonas arboricola* pv. *pruni* was established in a ring-test involving four laboratories. Symptomatic and healthy almond leaf samples with two methods of sample preparation were analyzed. Kappa coefficient, sensitivity, specificity, likelihood ratios and post-test probability of detection were estimated to manage the risk associated with the use of the two methods.

Xanthomonas arboricola pv. *pruni*, causal agent of bacterial spot disease of stone fruits and almond, is affecting a wide range of *Prunus* species worldwide (EPPO, 2006, 2014). It is regulated as a quarantine organism by the European Union phytosanitary legislation (Anonymous, 2000 and amendments), and by the European and Mediterranean Plant Protection Organization (EPPO) (EPPO, 2003).

Recently, real-time PCR (Palacio-Bielsa et al., 2011) and loop-mediated amplification (LAMP) (Bühlmann et al., 2013) have been developed for detection and identification of *X. arboricola* pv. *pruni*. However, estimation of diagnostic parameters for such tests is not available, and our aim was to estimate them in a ring-test performed in four European laboratories. These new diagnostic assays were evaluated to provide critical data for making informed decisions regarding appropriate guidelines for analysis of samples from surveys and to improve routine diagnostics.

Samples of healthy almond leaves (cv. Guara) and leaves naturally infected with *X. arboricola* pv. *pruni* were collected. Approximately 1 g of fresh weight tissue samples were prepared by washing (washates) or slightly crushed with a pestle (comminuted) in 15 ml of sterile distilled water (pH 6.54) and incubated for 15 min or 5 min at room temperature, respectively. Before sending the samples to each of the European laboratories involved in the ring-test, the presence of *X. arboricola* pv. *pruni* was verified by real-time PCR (Palacio-Bielsa et al., 2011) and isolation on yeast-peptone-glucose agar (YPGA) medium (Ridé, 1969) supplemented with 250 mg l^{-1} cycloheximide (Sigma-Aldrich, St. Louis, USA). After incubation at 25 °C colonies morphologically resembling *X. arboricola* pv. *pruni* were confirmed by real-time PCR. Healthy samples were similarly analyzed. Isolation added confidence to interpreting molecular results and determination of hypothetical false positives.

Samples sent to the different laboratories were heat-treated (100 °C, 10 min), divided into aliquots of 1 ml and conserved at −80 °C until shipment. Samples were coded before sending to ensure a double-blind test. All the reagents for real-time PCR (Quantimix Easy Probes kit, Biotools, Madrid, Spain; primers Xap-2 F/Xap-2R; TaqMan® probe Xap-2P, Applied Biosystems, Life Technologies, NY, USA) and LAMP

* Corresponding author.
E-mail addresses: apalaciob@aragon.es (A. Palacio-Bielsa), plopez@ivia.es (P. López-Soriano), andreas.buehlmann@acw.admin.ch (A. Bühlmann), joop.vandoorn@wur.nl, j.vandoorn@enzazaden.nl (J. van Doorn), khanh.pham@wur.nl (K. Pham), mcambra@aragon.es (M.A. Cambra), imberruete@aragon.es (I.M. Berruete), poth@zhaw.ch (J.F. Pothier), dufy@zhaw.ch (B. Duffy), aolmos@iva.es (A. Olmos), mlopez@ivia.es (M.M. López).
[1] Present address: Seed Technology, Enza Zaden Seed Operations BV. Haling 1E, 1602 DB Enkhuizen, The Netherlands.

http://dx.doi.org/10.1016/j.mimet.2015.03.005
0167-7012/

Table 1
Ranges of inter-laboratory agreement of real-time PCR and LAMP for the detection of *Xanthomonas arboricola* pv. *pruni*.

Sample preparation[a]	Real-time PCR[b]	LAMP[b]
Washates (undiluted)	0.87 ± 0.25–1.00 ± 0.25	1.00 ± 0.25–1.00 ± 0.25
Washates (1:10 dilution)	1.00 ± 0.25–1.00 ± 0.25	1.00 ± 0.25–1.00 ± 0.25
Washates (1:100 dilution)	0.75 ± 0.24–1.00 ± 0.25	0.48 ± 0.21–0.84 ± 0.25
Comminuted DNA (undiluted)	0 ± 0–1 ± 0.25	0 ± 0–0.73 ± 0.25
Comminuted DNA (1:10 dilution)	0.87 ± 0.25–1.00 ± 0.25	0.37 ± 0.19–1.00 ± 0.25
Comminuted DNA (1:100 dilution)	0.87 ± 0.25–1.00 ± 0.25	0 ± 0–0.50 ± 0.24

[a] Washates, washed tissue without DNA extraction; comminuted, DNA extraction (Llop et al., 1999) of crushed tissue performed before real-time PCR or LAMP.
[b] Cohen's Kappa index ± standard deviation.

(Isothermal Master Mix, Optigene, Horsham, UK; forward F3 and reverse B3 outer primers; FIP and BIP inner primers; forward loopF and reverse loopR loop primers), sterile distilled water (pH 6.54), and detailed protocols were provided.

Each laboratory tested washed tissues with no DNA extraction (washates) versus DNA extracts (Llop et al., 1999) from crushed leaves (comminuted) and their 1:10 and 1:100 dilutions. Negative controls containing no template DNA were tested (washed tissue samples, DNA extracts from healthy leaves and master-mix-only). *X. arboricola* pv. *pruni* strain ISPaVe B4 was included as a positive control. Duplicates of controls and samples were subjected simultaneously to real-time PCR and LAMP assays performed as described in Palacio-Bielsa et al. (2011) and Bühlmann et al. (2013). Different thermocyclers were used in participating laboratories, including SmartCycler® on FAM channel (Cepheid, Sunnyvale, USA); LightCycler® 480 (Roche Diagnostics, Indianapolis, USA); Step One Plus (Applied Biosystems, Foster city, USA); Genie® II (Optigene, Horsham, UK) and Lisse Mx3000P qPCR System (Stratagene, Santa Clara, USA).

Diagnostic parameters were analyzed according to Olmos et al. (2008). Inter-laboratory agreement for both techniques was evaluated using Cohen's Kappa index (CKI) (Cohen, 1960), which indicates the proportion of agreement beyond that expected by chance. The benchmarks of Landis and Koch (1977) were used to categorize the CKI (values <0.00 is poor agreement, 0–0.2 is slight agreement, 0.21–0.40 is fair agreement, 0.41–0.60 is moderate agreement, 0.61–0.80 is substantial agreement, and 0.81–1.00 is almost perfect agreement). The McNemar χ^2 test (McNemar, 1947) was used to detect bias effect, which affects CKI (Feinstein and Cicchetti, 1990). If the McNemar χ^2 test was significant, the Bias-Adjusted Kappa (BAK) index was applied to correct possible bias effects (Byrt et al., 1993). Sensitivity was defined as the proportion of true positives that were correctly identified (number true positive/number of infected samples) (Altman and Bland, 1994a). Specificity was defined as the proportion of true negatives that were correctly identified (number of true negative/number of healthy samples) (Altman and Bland, 1994b). Unilateral 95% confidence intervals (CI) were calculated for the global estimation of both parameters for each assay. These confidence intervals included the real value of the corresponding parameter in 95% of trials.

Results of this ring-test demonstrated a near perfect inter-laboratory agreement for real-time PCR and LAMP assays when undiluted and 1:10 dilutions of washates were analyzed, with a CKI ranging from 0.87 to 1.0 (Table 1). However, when 1:100 dilutions were analyzed LAMP was less accurate, with a CKI ranking from 0.48 to 0.84 for LAMP compared with 0.75 to 1.0 for real-time PCR (Table 1). Similarly, when undiluted DNA extracts were analyzed LAMP was slightly less accurate, with a CKI ranging from 0 to 0.73 for LAMP compared to 0 to 1.0 for real-time PCR. Real-time PCR analysis of 1:10 and 1:100 dilutions of DNA extracts gave a CKI ranging from 0.87 to 1.0. LAMP analysis was less accurate, with a CKI ranging from 0.37 to 1.0 for 1:10 dilutions and from 0 to 0.5 for 1:100 dilutions (Table 1).

Estimated sensitivity of real-time PCR in this ring-test was very high, with values of 0.98 for washates and 0.89 for DNA extracts from comminuted tissue samples, respectively (Table 2). Sensitivity of LAMP was lower, with values of 0.86 for washates and 0.58 for DNA extracts (Table 2). The differential sensitivity and detection limits observed in this ring-trial contrast with Bülhmann et al. (2013), who reported a lower detection limit and essentially equivalent sensitivity for LAMP and real-time PCR. This discrepancy is likely due to different sample preparations used by Bühlmann et al. (2013) (i.e., boiled comminuted tissues) and Palacio-Bielsa et al. (2011) (i.e., DNA extracts from comminuted tissues). Indeed, Palacio-Bielsa et al. (2011) observed a reduction in sensitivity for real-time PCR when comminuted tissues were analyzed without prior DNA extraction. This was attributed to inhibitory plant compounds. Estimated specificity for real-time PCR was 0.99 irrespective of sample treatment. Estimated specificity for LAMP was 1.00 and 0.99 for tissue washates and DNA extracts, respectively (Table 2).

Likelihood ratios (Deeks and Altman, 2004) based on ratio between sensitivity and specificity for real-time PCR and LAMP were also estimated (Table 2). Positive likelihood ratios above 10 and below 0.1 are considered to provide strong evidence to rule in or rule out diagnosis. In the case of positive likelihood ratios all developed methods with different sample preparations was over 10, indicating that positive results by any method are reliable. In the case of negative likelihood ratios, only the real-time PCR using washates as template was below 0.1, indicating that the negative results by this method are the most reliable.

In order to estimate and manage the risk associated with the use of the methods in any prevalence of infection, post-test probabilities (Deeks and Altman, 2004) were calculated for single methods and combinations of two methods (Table 3). In the case of low prevalences, the most confident single method for the negatives, with less false negative results is real-time PCR using washates, followed by real-time PCR using DNA extracts from comminuted samples and LAMP using washates. LAMP assay using communited samples had the higher negative likelihood ratio and a negative result should be confirmed by another test. However, in the case of positive likelihood ratios, the best method was LAMP using washates, which implies that a positive result is a true positive, followed by real-time PCR using washates, comminuted samples and LAMP using comminuted samples. In the case of higher prevalences any method was reliable in the positive results, although only real-time

Table 2
Sensitivity, specificity and likelihood ratios of real-time PCR and LAMP methods used with different sample preparations.

Method[a]	Sample preparation[a]	Sensitivity				Specificity				Likelihood ratio	
		Value	SE[b]	Low CI[c]	Up CI[d]	Value	SE[b]	Low CI[c]	up CI[d]	Positive	Negative
Real-time PCR	Washates	0.98	0.01	0.96	1.00	0.99	0.004	0.98	1.005	98	0.02
	Comminuted DNA	0.89	0.02	0.85	0.94	0.99	0.004	0.98	1.00	89	0.11
LAMP	Washates	0.86	0.02	0.79	0.92	1.00	0	1.00	1.00	∞	0.14
	Comminuted DNA	0.58	0.03	0.49	0.67	0.99	0.006	0.97	1.01	58	0.42

[a] Washates, washed tissue without DNA extraction; comminuted, DNA extraction (Llop et al., 1999) of crushed tissue performed before real-time PCR or LAMP.
[b] Standard error.
[c] Lower limit of the 95% confidence interval.
[d] Upper limit of the 95% confidence interval.

Table 3
Probability of infection scores for results of real-time PCR and LAMP diagnostic methods used with different sample preparations.

Probability of infection	Method (sample preparation)[a]	Percentage									
Pre-test probability (prevalence)		1	10	20	30	40	50	60	70	80	90
Post-test probability											
1 diagnostic method	Real-time PCR(W) +	49.75	91.59	96.08	97.67	98.49	98.99	99.32	99.56	99.75	99.89
	Real-time PCR (W) −	0.02	0.22	0.50	0.85	1.32	1.96	2.91	4.46	7.41	15.25
	Real-time PCR (CS) +	47.34	90.82	95.70	97.45	98.34	98.89	99.26	99.52	99.72	99.88
	Real-time PCR (CS) −	0.11	1.21	2.68	4.50	6.83	9.91	14.16	20.42	30.56	49.75
	LAMP (W) +	100	100	100	100	100	100	100	100	100	100
	LAMP (W) −	0.14	1.53	3.38	5.66	8.54	12.28	17.36	24.62	35.90	55.75
	LAMP (CS) +	36.94	86.57	93.55	96.13	97.48	98.31	98.86	99.27	99.57	99.81
	LAMP (CS) −	0.42	4.46	9.50	15.25	21.88	29.58	38.65	49.49	62.69	79.08
2 diagnostic methods	Real-time PCR(W) + LAMP(W) +	100	100	100	100	100	100	100	100	100	100
	Real-time PCR(W) − LAMP(W) −	0	0.03	0.07	0.12	0.19	0.28	0.42	0.65	1.11	2.46
	Real-time PCR(W) + LAMP(W) −	12.17	60.39	77.43	85.47	90.14	93.21	95.37	96.97	98.21	99.20
	Real-time PCR(W) − LAMP(W) +	100	100	100	100	100	100	100	100	100	100
	Real-time PCR(CS) + LAMP(CS) +	98.12	99.83	99.92	99.95	99.97	99.98	99.99	99.99	100	100
	Real-time PCR(CS) − LAMP(CS) −	6.05	41.48	61.46	73.22	80.96	86.45	90.54	93.71	96.23	98.29
	Real-time PCR(CS) + LAMP(CS) −	27.41	80.6	90.33	94.12	96.14	97.39	98.25	98.87	99.34	99.70
	Real-time PCR(CS) − LAMP(CS) +	6.05	41.48	61.46	73.22	80.96	86.45	90.54	93.71	96.23	98.29
	Real-time PCR(W) + Real-time PCR(CS) +	98.88	99.90	99.95	99.97	99.98	99.99	99.99	100	100	100
	Real-time PCR(W) − Real-time PCR(CS) −	0	0.02	0.05	0.09	0.15	0.22	0.33	0.51	0.87	1.94
	Real-time PCR(W) + PCR(CS) −	9.82	54.5	72.94	82.21	87.79	91.51	94.18	96.18	97.73	98.98
	Real-time PCR(W) − Real-time PCR(CS) +	1.77	16.51	30.80	43.27	54.27	64.03	72.75	80.60	87.68	94.12
	LAMP(W) + LAMP(CS) +	100	100	100	100	100	100	100	100	100	100
	LAMP(W) − LAMP(CS) −	0.06	0.65	1.45	2.46	3.77	5.55	8.11	12.06	19.04	34.61
	LAMP(W) + LAMP(CS) −	100	100	100	100	100	100	100	100	100	100
	LAMP(W) − LAMP(CS) +	7.58	47.43	67.00	77.68	84.41	89.04	92.41	94.99	97.01	98.65
	Real-time PCR(W) + LAMP(CS) +	98.20	99.83	99.93	99.96	99.97	99.98	99.99	99.99	100	100
	Real-time PCR(W) − LAMP(CS) −	0.01	0.09	0.21	0.36	0.56	0.83	1.24	1.92	3.25	7.03
	Real-time PCR(W) + LAMP(CS) −	29.37	82.06	91.14	94.64	96.48	97.63	98.41	98.97	99.40	99.73
	Real-time PCR(W) − LAMP(CS) +	1.16	11.42	22.48	33.21	43.61	53.70	63.50	73.02	82.27	91.26
	Real-time PCR(CS) + LAMP(W) +	100	100	100	100	100	100	100	100	100	100
	Real-time PCR(CS) − LAMP(W) −	0.02	0.17	0.38	0.66	1.02	1.52	2.26	3.47	5.80	12.17
	Real-time PCR(CS) + LAMP(W) −	11.18	58.06	75.70	84.23	89.26	92.57	94.92	96.67	98.03	99.12
	Real-time PCR(CS) − LAMP(W) +	100	100	100	100	100	100	100	100	100	100

[a] W, washed tissue (washates); CS, DNA extracts from comminuted samples.

PCR using washastes is recommended because of the values of the negative likelihood ratios. For instance, in 60% of prevalence a negative result by LAMP using comminuted samples implies that around 38% of negatives are true positives, or 14% using real-time PCR with comminuted samples. Real-time PCR using washates had the less false negative results as single method in 60% of prevalence with around 3% of false negatives. The combination of two methods clearly improves the diagnostic accuracy being the best combination testing a sample with real-time PCR and LAMP using washates as template.

In conclusion, according to these ring-test results, real-time PCR assay using almond leaves washes was slightly more accurate than LAMP assay for detection of *X. arboricola* pv. *pruni*. However, practical advantages of LAMP would be simplicity and speed of the reaction, returning test results in 15 min. Specificity results were very good in both cases. Although further assays using different hosts would be necessary in order to evaluate their efficiency in other types of samples, either real-time PCR or LAMP are appropriate for using in diagnosis of *X. arboricola* pv. *pruni* affording a flexibility depending upon limiting conditions (maximum sensitivity, time and cost per test, high-throughput demand, etc.).

Acknowledgments

This research was supported by the Instituto Nacional de Investigaciones Agrarias (INIA) (project RTA2011-00140-C03-03), the EU FP7 KBBE Projects Q-Detect and DROPSA. The work was conducted within the European Science Foundation COST Action 873, COST Action FA1104, EU FP7 ERA-NET EUPHRESCO II (project ERA26-XAPDIAG-CITA), and CYTED FRUT-SAN (project 112RT0441) research networks. The first and seventh authors thank the Agricultura, Ganadería y Medio Ambiente of Aragón Government and CITA convenant for contracts. We thank J. Peñalver, C. Morente, R. Collados and M. L Palazón for their valuable technical assistance, and M. Scortichini for providing the reference bacterial strain.

References

Altman, D.G., Bland, J.M., 1994a. Diagnostic tests 1: sensitivity and specificity. BMJ 308, 1552. http://dx.doi.org/10.1136/bmj.308.6943.1552.

Altman, D.G., Bland, J.M., 1994b. Diagnostic tests 2: predictive values. BMJ 309, 102. http://dx.doi.org/10.1136/bmj.309.6947.102.

Anonymous, 2000. Council directive 2000/29/EC of 8 May 2000 on protective measures against the introduction into the community of organisms harmful to plants or plant products and against their spread within the community. Off. J. Eur. Communities L169 (43), 1–112.

Bühlmann, A., Pothier, J.F., Tomlinson, J.A., Frey, J.E., Boonham, N., Smits, T.H.M., Duffy, B., 2013. *Genomics-informed design of loop-mediated isothermal amplification for detection of phytopathogenic Xanthomonas arboricola* pv. *pruni at the intraspecific level*. Plant Pathol. 62, 475–484. http://dx.doi.org/10.1111/j.1365-3059.2012.02654.x.

Byrt, T., Bishop, J., Carlin, J.B., 1993. Bias, prevalence and kappa. J. Clin. Epidemiol. 46, 423–429. http://dx.doi.org/10.1016/0895-4356(93)90018-V.

Cohen, J., 1960. A coefficient of agreement for nominal scales. Educ. Psychol. Meas. 20, 37–46. http://dx.doi.org/10.1177/001316446002000104.

Deeks, J.J., Altman, D.G., 2004. Diagnostic tests 4: likelihood ratios. BMJ 329, 168–169. http://dx.doi.org/10.1136/bmj.329.7458.168.

EPPO, 2003. Data sheets on quarantine organisms. *Xanthomonas arboricola* pv. *pruni* (available online). http://www.eppo.org/QUARANTINE/BACTERIA/Xanthomonas_pruni/XANTRP_ds.pdf.

EPPO, 2006. EPPO standards PM 7/64. (1) Diagnostics *Xanthomonas arboricola* pv. *pruni*. Bull. OEPP/EPPO Bull. 36, pp. 129–133

EPPO, 2014. PQR-EPPO database on quarantine pests (available online). http://www.eppo.int.

Feinstein, A.R., Cicchetti, D.V., 1990. High agreement but low kappa: I. The problems with two paradoxes. J. Clin. Epidemiol. 43, 543–549. http://dx.doi.org/10.1016/0895-4356(90)90158-L.

Landis, J.R., Koch, G.G., 1977. The measurement of observer agreement for categorical data. Biometrics 33, 159–174.

Llop, P., Caruso, P., Cubero, J., Morente, C., López, M.M., 1999. A simple extraction procedure for efficient routine detection of pathogenic bacteria in plant material by

polymerase chain reaction. J. Microbiol. Methods 37, 23–31. http://dx.doi.org/10.1016/S0167-7012(99)00033-0.
McNemar, Q., 1947. Note on the sampling error of the difference between correlated proportions or percentages. Psychometrika 12, 153–157.
Olmos, A., Bertolini, E., Capote, N., Cambra, M., 2008. An evidence-based approach to plum pox virus detection by DASI-ELISA and RT-PCR in dormant period. Virol. Res. Treat. 1, 1–8.
Palacio-Bielsa, A., Cubero, J., Cambra, M.A., Collados, R., Berruete, I.M., López, M.M., 2011. Development of an efficient real-time quantitative PCR protocol for detection of *Xanthomonas arboricola* pv. *pruni* in *Prunus* species. Appl. Environ. Microbiol. 77, 89–97. http://dx.doi.org/10.1128/AEM. 01593-10.
Ridé, M., 1969. Bactéries phytopathogènes et maladies bactériennes des végétaux. In: C.V.M. (Ed.), Les Bactérioses et les Viroses des Arbres Fruitiers. Bourgin Ponsot, Paris, France, pp. 4–59.