Eur J Plant Pathol (2019) 155:71–80
https://doi.org/10.1007/s10658-019-01750-x

Detecting *Spiroplasma citri*: a comparison of PCR methods to be used for quarantine diagnostics

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Accepted: 15 April 2019 / Published online: 3 May 2019

Abstract *Spiroplasma citri*, a plant pathogenic bacterium responsible for citrus stubborn disease in citrus, is listed as a quarantine pest in Europe and in other parts of the world. For this reason, the introduction of this pathogen is prohibited in many countries and post-entry quarantine is required for imported citrus plants. Therefore, reliable and highly sensitive detection methods are required to test plants in post-entry quarantine. This paper presents a study that characterizes the test parameters of a number of PCR protocols for the detection of *S. citri*. First, during intra-laboratory evaluation, we characterized four PCR methods and selected three of them. Second, those three methods were compared in the framework of an inter-laboratory study involving five laboratories. As a result, the real-time PCR developed by Yokomi et al. (*Plant Disease, 92*, 253–60, 2008) targeting the putative gene of the adhesin P58 is recommended for routine use in post-entry quarantine.

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Keywords *Spiroplasma citri* · Detection · PCR · Inter-laboratory study

Introduction

Spiroplasma citri is a species of spiroplasmas in the *Spiroplasmataceae* family. This bacterium does not have a cell wall but is contained by a plasma membrane, is motile, has a distinctive helical morphology and resides in the phloem sieve tubes. *S. citri* is the causal agent of citrus stubborn disease (Saglio et al. 1973). This bacterium is disseminated by infected planting material and by phloem feeding leafhoppers (Nejat et al. 2011). Citrus stubborn disease is preferentially found in hot and dry climate such as the Mediterranean basin and the Middle East. It is also present in parts of the USA, Mexico and northern Africa (EPPO 2018).This disease causes a decline in yields, with abnormally small and malformed fruits (Bové et al. 1988). It affects all citrus, particularly orange trees, but can also infect many species of herbaceous plants, often without apparent symptoms (Nejat et al. 2011).

S. citri is considered as a quarantine pest following the regulation (EU) 2016/2031 of the European council of 26 October 2016 and is also a quarantine pest in other parts of the world. As a result, the introduction in Europe of plants of *Citrus* L., *Fortunella* Swingle, *Poncirus* Raf., and their hybrids, other than fruit and seeds is controlled, among other things, to prevent accidental introduction of the pathogen. In order to meet the regulatory needs, it is necessary to have efficient and reliable detection methods.

In post-entry quarantine, the infected plants are generally asymptomatic, bacterial titer is low and the bacteria are unevenly distributed in the infected plant (Shi et al. 2014). *S. citri* is one of the few plant pathogenic mollicutes to have been cultured. Routine detection of *S. citri* from plants can be implemented by culturing in SP4 or M1A medium followed by examination of the organism for helical morphology and motility by dark field microscopy (Lee and Davis 1983). However, this method is time consuming and generally less sensitive than serological and molecular methods. Enzyme-linked immunosorbent assay (ELISA) using polyclonal antisera is also available for the detection of *S. citri* (Clark et al. 1978). Bové et al. (1987) have found that the sensitivity of ELISA and culture are of the same order of magnitude. However, the tests are not sensitive enough to reliably detect *S. citri* in symptomless trees (Bové et al. 1987) and, in their conditions of use, Bové and collaborators (Bové et al. 1983) pointed out that ELISA is not specific as it produces false positive results with other spiroplasmas of group I.

Currently, detection methods for bacteria are mainly polymerase chain reaction (PCR)-based (Palacio-Bielsa et al. 2009). There are many methods of gene amplification for the detection of *S. citri* targeting the genome of the bacteria itself, or phage-related sequences, such as plectrovirus SpV1, ubiquitously contained in the genome of *S. citri*. At least, 14 different conventional PCR assays targeting different areas of the genome are described including the detection of the 16S rDNA (Gasparich et al. 2004; Lee et al. 2006), the putative adhesin P89 gene (Yokomi et al. 2008), the putative adhesin P58 gene (Yokomi et al. 2008), the integrated virus (Lee et al. 2006), the spiralin gene (Foissac et al. 1996; Yokomi et al. 2008; Khanchezar et al. 2010) and the pE gene (Khanchezar et al. 2012). Real-time PCR allows accurate detection of plant pathogenic bacteria with higher sensitivity levels when compared to conventional PCR (Palacio-Bielsa et al. 2009). Seven real-time PCR assays are described targeting different areas of the *S. citri* genome, including the putative adhesin P58 gene (Yokomi et al. 2008), the integrated SpV1 virus (Wang et al. 2015) and the spiralin gene (Shi et al. 2014; Yokomi and Sisterson 2011). However, there have been no studies to compare the performance of these different methods.

To prevent the spread of the pathogen and disease outbreak, the early detection of latent infections in planting material, especially in the context of post-entry quarantine, is an important principle in plant disease control. In this context, rapid, sensitive and specific techniques to detect *S. citri* are essential. This study aims to evaluate the performance of different PCR methods for the detection of *S. citri* and provide robust validation data for these tests.

Materials and methods

Design of the study

Based on the literature and available validation data, a selection of methods for the detection of *S. citri* was made. The selection criteria were: i) easy and fast to use in routine analysis; ii) high analytical sensitivity; iii) high diagnostic sensitivity; and iv) no false positive results on healthy host tissue. These criteria resulted in selecting mainly real-time PCR protocols but conventional PCRs were kept for the evaluation if they targeted different parts of the *S. citri* genome. During a preliminary study in the Plant Health Laboratory of ANSES (LSV-UBVO), one DNA extract of citrus contaminated by *S. citri* and one DNA extract of healthy citrus were amplified two times with twelve different PCR methods. If unexpected results were obtained (i.e. false negative or false positive results), the related PCR protocols were removed from this study. This preliminary study allowed selection of four different PCR methods for the detection of *S. citri* (data not shown).

Then, in a first step, the LSV-UBVO compared and characterized the analytical specificity, the analytical sensitivity and the repeatability of these four PCR protocols and selected the three most effective. In a second step, an inter-laboratory test performance study (TPS) was conducted to confirm the results and to evaluate the reproducibility of the selected protocols.

Tested methods

For the first step, four different PCR protocols were tested: one conventional and one real-time PCR developed by Yokomi et al. (2008) targeting the P58 putative adhesin multigene of *S. citri*, one real-time PCR developed by Wang et al. (2015) targeting the SpV1-ORF3 integrated virus of *S. citri* and one real-time PCR developed by Yokomi and Sisterson (2011) the spiralin gene of *S. citri*. Details (amplification conditions) are provided on each method in Table 1.

Table 1 Amplification conditions of PCR methods evaluated during the study

Bibliographical references	Targeted area of the genome	Primer pair for *S. citri* detection	Recommended master mix[a]	Concentrations		Amplification [time(s)/T(°C)]			
				Master mix (X)	Primers (nM)	ID[b]	Amplification cycle		N[c]
Yokomi et al. 2008	P58 putative adhesin multigene[d]	P58-6f/P58-4r	Go Taq® Hot start Polymerase (Promega)	1	500	120/95	30/95	30/56 60/72	40
Wang et al. 2015	SpV1-ORF3 integrated virus	Php-orf3-F/Php-orf3-R	*Power* SYBR™ Green PCR Master Mix (Applied Byosystems™)	1	200	600/50	20/95	60/59	35
Yokomi and Sisterson 2011	Spiralin gene[d]	SP209f/SP288r	*Power* SYBR™ Green PCR Master Mix (Applied Byosystems™)	1	200	600/95	20/95	60/59	35
Yokomi et al. 2008	P58 putative adhesin multigene[d]	P58-3f/P58-4r	*Power* SYBR™ Green PCR Master Mix (Applied Byosystems™)	1	800	600/95	15/95	60/61	45

[a] Those master mixes were recommended but the participants were free to use other ones

[b] Initial denaturation

[c] Number of cycles

[d] Those methods were also tested during the TPS

Performance criteria

Performance criteria and validation procedures were established following PM 7/76 (4) and PM7/98 (2) EPPO standards (European Plant Protection Organization 2014a, 2017) and International Organization for Standardization ISO 16140:2003 (2003). In particular, analytical specificity with diagnostic specificity (DSP), diagnostic sensitivity (DSE), analytical sensitivity (ASE), repeatability (DA) and reproducibility (CO) were assessed. The definitions and the calculations of these performance criteria are detailed in Chabirand et al. (2017).

In addition, for each method tested during TPS, a probability of detection was calculated per dilution level, according to the following equation x/n where x corresponds to the number of positive results and n corresponds to the number of results obtained for a given dilution level.

Intra-laboratory characterization

To evaluate DSP, DNA extracts from twenty-one *S. citri* infected plant samples, representative of the diversity of the bacterium, were analyzed two to three times per protocol (Table 2).

To evaluate DSE, fifteen samples consisting of healthy citrus plants known as susceptible to *S. citri*, four closely related *Spiroplasma* species and nine plant pathogens of citrus (Table 3). These samples were analyzed two to three times per protocol.

To evaluate ASE and DA, 10-fold serial dilutions of two *S. citri* strains (16/47.1 and 16/47.3) spiked in DNA extracts of healthy citrus were tested six times each.

Inter-laboratory test performance study

The TPS involved five partner laboratories in four countries (Table 4). For the evaluation of the different methods, the participating laboratories had to test an identical series of blinded samples according to the three working protocols and data reporting sheets provided.

A panel of fifty coded samples of frozen DNA extracts was sent to each participant. The detailed composition of each panel is presented in the Table 5. Preliminary tests aiming to verify the homogeneity and stability of the different samples were performed (data not shown).

From these samples, the TPS participants were to carry out the different methods under the normal working conditions of the laboratory and in the same manner

Table 2 List of *Spiroplasma citri* strains used in the intra-laboratory study

Sample ID	Source of isolation	Geographic origin	Provider
16/08	*Circulifer haematoceps*	Morocco	INRA - UMR 1332
16/09	*Citrus sinensis*	Spain	Bordeaux, France
16/10.1	*Citrus sinensis*	California, USA	
16/10.2	*Citrus sinensis*	California, USA	
16/11	*Circulifer haematoceps*	Corsica, France	
16/12	*Citrus sinensis*	Israel	
16/13	*Citrus sinensis*	Turkey	
16/14	*Citrus sinensis*	Morocco	
16/47.1	*Citrus sp.*	California, USA	ANSES (LSV-UQ)
16/47.2	*Citrus sp.*	California, USA	Lempdes, France
16/47.3	*Citrus sp.*	California, USA	
16/47.4	*Citrus sp.*	California, USA	
16/47.5	*Citrus sp.*	California, USA	
16/47.6	*Citrus sp.*	California, USA	
16/47.7	*Citrus sp.*	California, USA	
16/47.8	*Citrus sp.*	California, USA	
16/47.9	*Citrus sp.*	California, USA	
16/48.2	*Citrus sp.*	California, USA	
16/48.3	*Citrus sp.*	California, USA	
16/64	*Daucus carota*	Spain	University of Valencia
16/65	*Apium gravolens*	Spain	Valencia, Spain

as other samples which are usually analyzed in the laboratory. PCR reagents and controls (positive and negative controls) were not provided by the organizer.

All participants were involved in the evaluation of each method.

Results

Analytical specificity

The analytical specificity of the evaluated methods ranged from 95 to 99% during the intra-laboratory study.

During the intra-laboratory study, the PCR protocols developed by Yokomi et al. (2008) allowed the detection of all the tested isolates of *S. citri* and the diagnostic sensitivity of those protocols was 100%. On the other hand, the diagnostic sensitivity of the other real-time PCR protocols ranged from 92 to 96% (Table 6). Those protocols did not allow the detection of two *S. citri* isolates (16/47.6 and 16/47.7). The diagnostic sensitivity of all tested protocols was 100% on condition of reproducibility (TPS) confirming the ability of the tested protocols to detect a diversity of *S. citri* isolates.

The real-time PCR developed by Yokomi and Sisterson (2011) targeting the spiralin gene was the most specific method with a diagnostic specificity of 100%. The two protocols targeting the P58 putative adhesin multigene of *S. citri* (Yokomi et al. 2008) provided a false positive result with the DNA extract of *Spiroplasma phoeniceum*. The real-time PCR targeting the SpV1-ORF3 integrated virus of *S. citri* repeatedly provided false positive results with the DNA extracts of *S. phoeniceum* and *Spiroplasma kunkelli*. During TPS, with the real time PCR developed by Yokomi et al. (2008), one laboratory returned a false weak positive result with a high Ct value (38.74 cycles) and was obtained in a non-repetitive mode for sample LXX-47. This sample was a DNA extract of *Citrus medica* which did not produce a cross reaction during intra-laboratory testing.

Analytical sensitivity and probability of detection

During the intra-laboratory study, the best analytical sensitivity was obtained with both PCRs targeting the

Table 3 List of DNA samples used as non-target in the intra-laboratory study

Sample ID	Organism	Species of isolation	Provider
Rp	HC[a]	*Eremocitrus glauca*	ANSES (LSV-UQ)
16/49	CMBV *Citrus mosaic virus*	*Citrus jambhiri*	Lempdes, France
16/50	CTV *Citrus tristeza virus*	NA[b]	
16/51	*Candidatus* Liberibacter americanus	NA	
16/52	*Candidatus* Liberibacter africanus	NA	
16/53	*Candidatus* Liberibacter asiaticus	*Citrus sinensis* cv. *Madame vinous*	
16/54	CPsV *Citrus psorosis virus*	*C. reticulata x C. sinensis*	
16/55	SDV *Satsuma dwarf virus*	NA	
16/56	CTLV *Citrus tatter leaf virus*	NA	
16/61.1	HC	*Citrus volkameriana*	
16/61.2	HC	*Citrus sinensis* cv. *Madame vinous*	
16/61.3	HC	*Citrus medica*	
16/61.4	HC	*Citrus aurantium*	
16/61.5	HC	*Citrus aurantifolia*	
16/61.6	HC	*C. sinensis x Poncirus trifoliata*	
41,03	*Xylella fastidiosa*	*Citrus sp.*	ANSES (LSV-UBVO) Angers, France
16/108.1	HC	*Aeglopsis chevalieri*	INRA CIRAD CITRUS BRC,
16/108.2	HC	*Atlanica ceylanica*	San Giuliano, France
16/108.3	HC	*Balsamocitrus dawei*	
16/108.4	HC	*Eremocitrus glauca*	
16/108.5	HC	*Fortunella japonica*	
16/108.6	HC	*Microcitrus australasica*	
16/108.7	HC	*Poncirus trifoliata*	
16/108.8	HC	*Swinglea glutinosa*	
16/03	*Spiroplasma melliferum*	NA	DSMZ
16/04	*Spiroplasma floricola*	NA	Braunschweig, Germany
16/05	*Spiroplasma phoeniceum*	NA	
16/06	*Spiroplasma kunkelli*	NA	

[a] *HC* healthy control, [b] *NA* not available

putative adhesin multigene P58 (Yokomi et al. 2008). Those PCR protocols were able to detect *S. citri* at 100% down to a 1×10^{-2} dilution of DNA extracts of infected plants with *S. citri*. The other real-time PCR protocols were 10 to 100 times less sensitive (Table 6).

Table 4 List of participants involved in the inter-laboratory trials

Institution	Contact	Country
Agriculture Victoria	F. Constable	Australia
Institute of Fruit Tree and Tea Science, National Agriculture and Food Research Organization (NARO)	T. Fujikawa	Japan
Plant Health and Environment Laboratory, Ministry for Primary Industries	R. Taylor	New Zealand
Plant Health Laboratory of ANSES, Unit of Bacteriology, Virology and GMOs (LSV-UBVO)	M. Loiseau[a]	France
Plant Health Laboratory of ANSES, Unit of Quarantine (LSV-UQ)	J.-P. Renvoisé	France

[a] Coordination and organization of the ring test

Table 5 Type of samples used to evaluate performance criteria of methods for detection of *Spiroplasma citri* during TPS

Samples of the panel	Sample ID	Nature	Number of replicates	Performance criteria evaluated
LXX-2 LXX-16	16/47.3	*S. citri* isolate 1	2	DSE
LXX-37 LXX-32	16/47.5	*S. citri* isolate 2	2	DSE
LXX-35 LXX-27	16/47.9	*S. citri* isolate 3	2	DSE
LXX-31 LXX-43	16/47.1	*S. citri* isolate 4	2	DSE
LXX-04 LXX-49	16/47.8	*S. citri* isolate 5	2	DSE
LXX-12 LXX-47	16/61.3	*Citrus medica*	2	DSP
LXX-01 LXX-26	16/61.4	*Citrus aurantium*	2	DSP
LXX-28 LXX-33	16/61.2	*Citrus sinensis* cv Madam vinous	2	DSP
LXX-24 LXX-40	16/54 +16/56 +41.03	Mix of pathogens of citrus (CPsV - *Citrus psorosis virus*; *Xylella fastidiosa*; CTLV *Citrus tatter leaf virus*)	2	DSP
LXX-20 LXX-50	16/04	*Spiroplasma floricola*	2	DSP
LXX-11 LXX-18 LXX-19 LXX-36 LXX-39 LXX-48	16/48.3	*S. citri* diluted at 10^{-1a}	6	ASE, DA, CO
LXX-10 LXX-21 LXX-23 LXX-25 LXX-38 LXX-46	16/48.3	*S. citri* diluted at 10^{-2a}	6	ASE, DA, CO
LXX-14 LXX-15 LXX-17 LXX-22 LXX-42 LXX-44	16/48.3	*S. citri* diluted at 10^{-3a}	6	ASE, DA, CO
LXX-09 LXX-13 LXX-29 LXX-30 LXX-41 LXX-45	16/48.3	*S. citri* diluted at 10^{-4a}	6	ASE, DA, CO
LXX-03 LXX-05 LXX-06 LXX-07 LXX-08 LXX-34	16/48.3	*S. citri* diluted at 10^{-5a}	6	ASE, DA, CO

LXX: number attributed to the participant laboratories; DSE: Diagnostic sensitivity; DSP: Diagnostic specificity; ASE: Analytical sensitivity; DA: Repeatability; CO: Reproducibility

[a] DNA extract of plant infected by *S. citri* in DNA extract of healthy citrus

Table 6 Results for the characterization of the PCR methods for the detection of *Spiroplasma citri*: diagnostic sensitivity, diagnostic specificity, analytical sensitivity, repeatability, reproducibility

Bibliographical references		Wang et al. 2015	Yokomi and Sisterson 2011	Yokomi et al. 2008	
Targeted area of the genome		SpV1-ORF3 integrated virus	Spiralin gene	P58 putative adhesin multigene	
Type of method		SYBR Green real-time PCR			Conventional PCR
Analytical specificity	ILS	95%	96%	99%	98%
	TPS	NE	100%	99%	100%
Diagnostic sensitivity	ILS	96%	92%	100%	100%
	TPS	NE	100%	100%	100%
Diagnostic specificity	ILS	94%	100%	99%	96%
	TPS	NE	100%	98%	100%
Analytical sensitivity	ILS 16/47.1	1×10^{-1}	1×10^{-0}	1×10^{-2}	1×10^{-2}
	ILS 16/47.3	1×10^{-2}	1×10^{-1}	1×10^{-2}	1×10^{-2}
	TPS	NE	1×10^{-2}	1×10^{-3}	1×10^{-3}
Repeatability	ILS	100%	94%	94%	96%
	TPS	NE	Between 72 and 100%	100%	100%
Reproducibility		NE	98%	100%	100%

ILS intralaboratory study, *TPS* test performance study, *NE* not evaluated

The samples corresponding to the two last levels of dilution (1×10^{-4} and 1×10^{-5}) of the target *S. citri* using for TPS cannot be considered as homogenous and/or stable (data not shown), the results of those samples were excluded from data analysis. If the results of these dilutions (1×10^{-4} and 1×10^{-5}) of the target *S. citri* were taken into account, the real-time PCR protocol targeting the putative adhesin multigene P58 (Yokomi et al. 2008) provided a better analytical sensitivity (Fig. 1). Furthermore, the results of analytical sensitivity obtained during the TPS confirmed the results of the intra-laboratory study.

Repeatability and reproducibility

In the first step of the study at LSV-UBVO, the best repeatability was obtained with the real-time PCR targeting the SpV1-ORF3 integrated virus of *S. citri* (Wang et al. 2015). In TPS and among all the laboratories, the best repeatability was obtained by the PCR protocols (Yokomi et al. 2008) targeting the putative adhesin multigene P58 with a 100% repeatability while the real-time PCR (Yokomi and Sisterson 2011) targeting spiralin gene had a repeatability ranging from 72 and 100% depending on the laboratory (Table 6).

The reproducibility of the evaluated methods ranged from 98 to 100%. The best reproducibility is obtained with the PCR protocols targeting putative adhesin multigene P58 (Yokomi et al. 2008) (Table 6).

Discussion

S. citri is a plant pathogen that has been reported to cause a significant economic impact on citrus production (Bové 1986; EPPO datasheet). For this reason, the introduction of this pathogen is prohibited in many countries and post-entry quarantine is required for imported citrus plants. Therefore, reliable and highly sensitive detection methods are required to test plants in post-entry quarantine. This paper presents a study that evaluates the test parameters of a number of PCR protocols for the detection of *S. citri*.

During the preliminary study in the LSV-UBVO, many PCR protocols were removed from further evaluation and the TPS because these protocols provided false negative results from citrus samples containing *S. citri* (data not shown). Surprisingly, false negative results were also detected for the real-time PCR developed by Wang et al. (2015) that target the multicopy prophage gene, SpV1 ORF1, which had showed improved detection of *S. citri* when compared to other *Spiroplasma* genes (Spiralin, P58, SpV1-ORF3) (Wang et al. 2015). SpV1 replicative

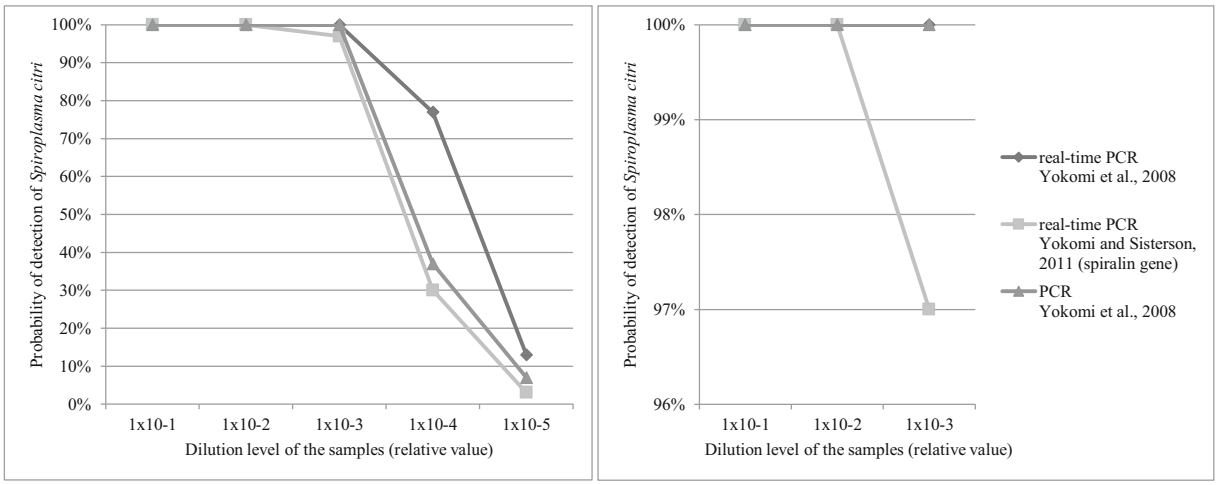

Fig. 1 Probability of detection of the methods evaluated for the detection of *Spiroplasma citri* based on dilution level of a DNA extract of citrus infected by *Spiroplasma citri* in a DNA extract of healthy citrus. On the left, the two last levels of dilution were taken into account even if those samples were considered not homogeneous and/or not stable. On the right, only results with homogeneous and stable samples were included

form (RF) has already shown a structural instability and the primer sequences may not be present in all isolates (Marais et al. 1996). Moreover, Wang et al. (2015) did use different test samples compared to those used in our study. Since such false negative results are unacceptable in testing plants in post-entry quarantine, those methods were not rated.

During our intra-laboratory study, the best performances were obtained with the PCR protocols developed by Yokomi et al. (2008) targeting the putative adhesin multigene P58. The targeted region of the *S. citri* genome allowed the specific detection of all the tested strains. The PCR protocols targeting the SpV1 ORF3 integrated virus (Wang et al. 2015) and the spiralin gene of *S. citri* (Yokomi and Sisterson 2011) produced false negative results in two DNA extracts. However, the high Ct values obtained from those samples with the real-time PCR of Yokomi et al. (2008) indicated that the target concentration was low. The failure of detection of those samples can be due to a problem of analytical sensitivity more than a problem of diagnostic sensitivity. This hypothesis is supported by the fact that some other isolates from the same area (California, USA) were detected by those PCR protocols. The best diagnostic specificity was obtained with the PCR protocol targeting the spiralin gene (Yokomi and Sisterson 2011) with 100% diagnostic specificity. The protocols targeting the putative adhesin multigene P58 (Yokomi et al. 2008) cross reacted with *Spiroplasma phoeniceum*. This was in accordance with the results obtained by Yokomi et al. (2008). *S. phoeniceum* is a spiroplasma isolated from symptomatic periwinkles in Syria between 1983 and 1984 but which since has never been described in a sanitary or epidemic context. As *S. phoeniceum* has never been found in citrus and is potentially a plant infectious microorganism, it's co-detection in post-entry quarantine is not considered a real problem. Likewise, *S. phoeniceum* and *S. kunkelli* were detected by the real-time PCR targeting the SpV1-ORF3 of *S. citri* (Wang et al. 2015). Wang et al. (2015) did not include those spiroplasmas in their study. Both PCRs targeting the putative adhesin multigene P58 (Yokomi et al. 2008) were 10 to 100 times more sensitive than the other PCR protocols.

The second part of the study was designed as a TPS which provides additional value to the validation process as it provides more information on repeatability and reproducibility (EPPO 2014b). As the real-time PCR targeting the SpV1-ORF3 of *S. citri* (Wang et al. 2015) gave the worst diagnostic specificity in the first part of the study, this method was not rated in the TPS. The methods developed by Yokomi et al. (2008), which provided the best performance, were assessed and compared to the PCR protocol targeting the spiralin gene (Yokomi and

Sisterson 2011). This allowed evaluation of a different method targeting another part of the *S. citri* genome. The results of the TPS confirmed the results previously obtained in the intra-laboratory study and showed that the PCR protocols targeting the putative adhesin multigene P58 (Yokomi et al. 2008) were the most efficient methods to detect *S. citri*. However, it also highlighted a risk of micro-contaminations of 2% with the real-time PCR of Yokomi et al. (2008). As for all highly sensitive methods, measures should be taken to avoid this type of contamination. The legitimacy to use these methods for the detection of *S. citri* in post-entry quarantine is reinforced by the high reproducibility (98–100%) obtained during the TPS for the evaluated methods using different reagents and real-time PCR machines.

As real-time PCR is faster and more sensitive than conventional PCR, the real-time PCR targeting the putative adhesin multigene P58 (Yokomi et al. 2008) can be efficiently used in post-entry quarantine. In case of a positive detection and in critical cases, the other evaluated protocols can be used to confirm the results obtained by real-time PCR. For testing laboratories following the standard ISO 17025 (International Organization for Standardization 2017), the methods used should be characterized (point 7.02.2 of the standard). That is why, in a context of quality assurance management system, the study reported in this paper provides essential validation data for the detection of *S. citri*.

Acknowledgments We thank Colette Saillard for taking time to increase our knowledge of *Spiroplasma citri* and detection methods. We gratefully thank Jean-Luc Danet and Ana Alfaro Fernandez for kindly providing us with *Spiroplasma citri* strains and other pathogens. We also thank Jean-Pierre Thermoz for his help to provide with different healthy citrus species.

Author contributions ML: designed the experiments, analyzed and interpreted the data, wrote the paper; RG, MP and IR: performed the experiments; RT, TF, RM, AP: participate to the TPS and revised the manuscript; J-PR: defined the needs and revised the manuscript.

Compliance with ethical standards

Conflict of interest We hereby state that no conflict of interest are included in this manuscript and that no humans or animals were used in the study. All authors agreed to the publishing of this manuscript.

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