Bulletin of Insectology **64** (Supplement): S281-S284, 2011
ISSN 1721-8861

European interlaboratory comparison and validation of detection methods for '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma prunorum' and '*Candidatus* Phytoplasma pyri': preliminary results

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Abstract

A working group was established in the frame of EUPHRESCO Phytosanitary ERA-NET Project aimed to compare and validate diagnostic protocols for the detection of '*Candidatus* Phytoplasma prunorum', '*Ca*. P. mali' and '*Ca*. P. pyri' in fruit trees. Four molecular protocols were submitted to interlaboratory trials starting from extracted DNAs prepared in four laboratories. The tested molecular protocols consisted in universal and group-specific real time and conventional nested PCR assays. A good agreement among laboratories was obtained and high values of diagnostic sensitivity were revealed for all tested protocols. The preliminary analysis of the results also highlighted some diagnostic specificity problems that require further investigations.

Key words: ringtest, '*Candidatus* Phytoplasma prunorum', '*Candidatus* P. mali' and '*Candidatus* P. pyri'.

Introduction

In the competitiveness of agricultural products the phytosanitary quality is of increasing importance and harmonized protocols had taken an active role in the agricultural food chain.

In this context a working group was established in the frame of EUPHRESCO Phytosanitary ERA-NET Project aimed to compare and validate diagnostic protocols for the detection of '*Candidatus* Phytoplasma prunorum', '*Ca*. P. mali' and '*Ca*. P. pyri' in fruit trees. These phytoplasmas are agents of relevant diseases inducing severe crop losses in fruit trees and particularly, the last two phytoplasmas are included in the EPPO A2 List of quarantine pests.

Protocols based on conventional and real time PCR for the detection of above mentioned phytoplasmas were selected and submitted to ringtest trials performed in 22 European laboratories. Validation data (diagnostic sensitivity and specificity) were calculated and statistically analysed according with the UNI CEI EN ISO/IEC 17025.

In the present paper the preliminary results of the collaborative studies are reported.

Materials and methods

Design of the study

The ringtest program was scheduled from April 2010, for exchange of procedures, to February 2011 for laboratories trials and statistical analysis.

The 22 participant laboratories (table 1) analysed an identical series of 30 blind samples following the provided working protocols. The involvement of each participant laboratory in the ringtest is reported in table 2. In the analysis of the results all the participant laboratories are reported with anonymous number.

Table 1. List of participants involved in the interlaboratory trials.

Institution	Contact person	Location
AGES	Helga Reisenzein	AT
CRA-W	Stephen Steyer	BE
ILVO	Kris de Jonghe	BE
CLPQ	Zhelju Avramov	BG
ACW	Santiago Schaerer	CH
State Phytosan. Admin.	Gabriela Schlesingerova, Hana Orsagova	CZ
JKI	Bernd Schneider	DE
Aarhus Univ.	Mogens Nicolaisen	DK
DAR	Ester Torres, Joan Bech	ES
IRTA	Assumpcio Batlle, Amparo Laviña	ES
UPVLC	Isabel Font	ES
CRA-PAV	Graziella Pasquini, Luca Ferretti	IT
Phytosanitary Lab. Lombardy	Marica Calvi	IT
University of Bologna	Samanta Paltrinieri, Assunta Bertaccini	IT
BIOFORSK	Dag-Ragnar Blystad, Sonja Sletner Klemsdal	NO
PPS	Linda Kox, Jeanette Teunisse, Bart van de Vossenberg	NL
Main Inspect. of Plant Health	Ewa Hennig, Justyna Moszczynska	PL
INRB/L-INIA	Esmeraldina Nascimento Agostinho de Sousa, Eugenia Andrade	PT
UKSUP	Lubomir Horvath, Michaela Hudecoba	SK
NIB	Marina Dermastia, Natasa Mehle	SI
GDAR	Nursen Ustun, Aydan Kaya	TR
FERA	Adrian Fox, Anna Skelton	GB

Table 2. Numbers of laboratories involved in each tested protocol.

Number of labs involved	Nested PCR	Specific qPCR (Nikolic *et al.*, 2010)	Universal qPCR (Christensen *et al.*, 2004)	Universal qPCR (Hodgett *et al.*, 2009)	Plasmid
6	X	X	X	X	X
8	X	X	X	X	
2	X	X	X		
4	X				
1	X		X	X	
1	X	X		X	

To avoid manipulation of living quarantine organisms, the samples to be tested were constituted by extracted DNAs. Standards of a cloned P1/P7 fragments from '*Ca*. P. mali' and '*Ca.* P. pyri' in concentration from 10^7 to 10^1 were also included in the trials.

In order to standardize the experiments Taq DNA polymerase and real time master mix brand were specified and Taqman probes were supplied by the organizer.

Samples

A series of 30 samples, target (symptomatic and asymptomatic infected plants) and non-target (healthy plants and closely related bacteria) have been selected to perform all experiments (table 3).

Table 3. List of tested samples and their origin.

N°	Specie	Sanitary status	Origin
1	apple	healthy	JKI
2	apple	'*Ca*. P. mali'	JKI
3	apple	'*Ca*. P. mali'	JKI
4	apricot	'*Ca*. P. prunorum'	CRA-PAV
5	apricot	'*Ca*. P. prunorum'	CRA-PAV
6	plum	healthy	DAAM
7	pear	healthy	JKI
8	plum	healthy	DAAM
9	pear	'*Ca*. P. pyri'	JKI
10	extracted DNA	bacteria	FERA
11	extracted DNA	bacteria	FERA
12	apple	'*Ca*. P. mali'	JKI
13	pear	'*Ca*. P. pyri'	JKI
14	extracted DNA	bacteria	FERA
15	apple	'*Ca*. P. mali'	JKI
16	apple	'*Ca*. P. mali'	JKI
17	extracted DNA	bacteria	FERA
18	pear	'*Ca*. P. pyri'	JKI
19	apple	healthy	JKI
20	pear	'*Ca*. P. pyri'	JKI
21	plum	healthy	DAAM
22	pear	'*Ca*. P. pyri'	JKI
23	extracted DNA	bacteria	FERA
24	extracted DNA	bacteria	FERA
25	pear	healthy	JKI
26	apricot	'*Ca*. P. prunorum'	CRA-PAV
27	pear	healthy	JKI
28	apricot	'*Ca*. P prunorum'	CRA-PAV
29	pear	healthy	JKI
30	plum	'*Ca*. P. prunorum'	DAAM

From each tested sample the total DNA was extracted from midribs using CTAB protocol (Doyle and Doyle, 1990) in four laboratories (DAAM, JKI, CRA-PAV, FERA) and sent to each partner as dried DNA. All the laboratories were asked to re-suspend it in 500 µl of distilled water.

In all tested protocol undiluted and tenfold diluted extracted DNA from each sample was used as template.

Tested protocols. Four molecular protocols were submitted to interlaboratory trials:

1. AP group specific nested PCR (nested PCR);
2. real time for the specific detection of '*Ca*. P. mali', '*Ca.* P. pyri' and '*Ca.* P. prunorum' (Nikolic *et al.*, 2010) (specific qPCR);
3. real time PCR for the universal detection of phytoplasmas (Christensen *et al*., 2004) (universal qPCR-C);
4. real time PCR for the universal detection of phytoplasmas (Hodgetts *et al*., 2009) (universal qPCR-H).

Nested PCR (1)

The protocol is based on a direct PCR using the universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995), followed by a group specific nested PCR performed with the primer pairs fO1/rO1 (Lorentz *et al*., 1995). Direct and nested PCR were performed in a 25 µl mixture containing: 1X Green GoTaq reaction buffer, 200 µM dNTPs (Promega), 0.4 µM of each primer, 0.625 U of GoTaq DNA polymerase (Promega), 1 µl of extracted DNA for direct PCR and 1 µl of P1/P7 amplicons diluted 1:30 for nested PCR (for 50 µl mixture: 2 µl DNA/diluted PCR product).

The cycling parameters included an initial denaturation step at 94°C for 2 min, followed by 36 and 38 cycles, for direct and nested PCR, respectively: 1 min at 94°C (denaturation), 1 min at 55°C and 50°C, for direct and nested PCR, respectively (annealing), 2 min at 72°C (extension) and a final extension step at 72°C for 8 min.

Specific qPCR (2)

The protocol consisted in a TaqMan real time PCR (qPCR) employing three specific FAM-MGB probes (AP-P, ESFY-P and PD-P) in separate reactions, using chemicals and amplification conditions reported in Nikolic *et al*. (2010).

Universal qPCR-C (3)

The protocol consisted in a TaqMan qPCR employing a FAM-TAMRA phytoplasma universal probe using chemicals and amplification conditions reported in Christensen *et al*. (2004).

Universal qPCR-H (4)

The protocol consisted in a TaqMan qPCR employing a VIC-TAMRA phytoplasma universal probe using chemicals and amplification conditions reported in Hodgetts *et al*. (2009). All the participants included their own positive and negative template controls. By qPCR, all samples were tested in duplicate. COX or human 18S rRNA (Applied Biosystem) was used as endogenous quality control of DNA extraction. qPCR were performed in 10 or 25 µl reactions.

Processing of the result data

The participants were asked to provide only '+' or '-' results for nested-PCR. Ct values for each replicate were asked for qPCR protocols, specifying threshold and baseline (manual or automatic).

The following parameters were calculated, using the R statistical framework (2010), to analyze the result:

- Agreement between laboratories - measured by calculation of the Kappa coefficient (Fleiss *et al*., 2003) and interpreted as reported in Landis and Koch (1977). The output included also significance tests for the kappa index (the null hypothesis is a zero kappa value).
- Diagnostic sensitivity (SE) - an estimation of the ability of the method to detect the target.
- SE=100xTP/(TP+FN) (table 4);
- Diagnostic specificity (SP) - an estimation of the ability of the method not to detect the non-target.
- SP= 100xTN/(FP+TN) (table 4).

If a laboratory had more than 10% of no-coincident replicates or more than 10% of unexpected differences between undiluted and diluted sample for one method, all the results obtained were omitted in the statistical analysis.

Table 4. Parameters for calculation of diagnostic specificity and sensitivity.

TP – true positive	positive detected from positive expected
FN - false negative	negative detected from positive expected
FP - false positive	positive detected from negative expected
TN - true negative	negative detected from negative expected

Results

All the participant laboratories carried out analysis on all DNA samples according to provided protocols. Results obtained with endogenous controls included in real time PCR protocols confirmed the good quality of all extracted DNAs. Analytical sensitivity obtained from standards of the cloned P1/P7 fragments from '*Ca*. P. mali' and '*Ca*. P. pyri' is reported in table 5. No relevant differences were observed among the protocols.

Agreement among laboratories. Fleiss' kappa index showed a 'almost perfect' agreement for all tested protocols (table 6), and p-values were almost 0. Diagnostic sensitivity and diagnostic specificity. SE and SP were calculated for each protocol and for each laboratory and mean values are reported in table 6. SE values resulted high for all protocols, ranging from 99.3% to 100%, whereas the specificity ranged from 93.8% to 99.7%.

Table 5. Analytical sensitivity of tested protocols calculated using serial dilutions of P1/P7 cloned fragment from '*Ca.* P. mali' and '*Ca.* P. pyri'. The analysis was not performed for universal qPCR-H, that amplify a region different from 16S-23S.

Laboratories	Nested-PCR		Specific qPCR		Universal qPCR-C	
	'*Ca*. P. mali'	'*Ca*. P. pyri'	'*Ca*. P. mali'	'*Ca*. P. pyri'	'*Ca*. P. mali'	'*Ca*. P. pyri'
1	n.t.	n.t.	10^1	10^1	10^1	10^2
8	n.t	n.t	10^1	10^1	10^1	10^1
11	n.t.	n.t.	n.t.	10^1	10^1	10^1
12	10^3	10^1	n.t.	n.t.	10^1	10^2
13	n.t	n.t	10^1	10^1	10^1	10^1
16	10^1	10^1	n.t	n.t	n.t	n.t

Table 6. Diagnostic sensitivity (SE), Diagnostic specificity (SP) and agreement values calculated by the Fleiss' kappa index for each tested protocol.

Method	Number of laboratories considered	Mean		Kappa index*
		SE	SP	
Nested-PCR	20	99.3	97.7	0.940
Universal real time-C	10	100.0	96.0	0.926
Universal real time-H	12	99.4	97.2	0.945
Specific qPCR – '*Ca*. P. pyri'	12	100.0	99.7	0.980
Specific qPCR – '*Ca*. P. mali'	12	100.0	98.7	0.924
Specific qPCR – '*Ca*. P. prunorum'	13	100.0	93.8	0.840

*, Kappa values interpreting: <0 poor agreement; 0.00-0.20 Slight agreement; 0.21-0.40 Fair agreement; 0.41-0.60 Moderate agreement; 0.61-0.80 Substantial agreement; 0.81-1.00 Almost perfect agreement (Landis and Koch, 1977).

Discussion

The results obtained in the interlaboratory trials showed that all four tested protocols resulted sensitive. The robustness of the protocols was also supported by the agreement levels for the different participants using different thermo cycler.

Nevertheless, diagnostic specificity values resulted affected by some unexpected results that open important questions and make necessary further investigations. Particularly, some non-target samples (plants assumed as healthy and phytoplasma related bacteria) gave positive results in different experiments and laboratories. In case of related bacteria the positive reactions can be explained as 'laboratory contamination', whereas in case of samples assumed as negative this result indicates the necessity to establish if the samples are 'true negative' or if they have a low titre of phytoplasma, detectable only by highly sensitive techniques.

Finally, in this ringtest the DNA extraction step have not been taken into consideration because living quarantine pathogens have to be manipulated as reported in CE 95/44 directive. Therefore the results are only related to the reliability of the amplification procedures, even if the nucleic acid extraction should be considered a critic step in phytoplasmas detection.

All the questions and problems derived from the preliminary analysis of the results have induced the participants to implement the experiments and further trials are in progress.

Acknowledgements

This work was carried out in the frame of EUPHRESCO NC Project 2° round in collaboration with WG1 of COST Action FA0807 "Integrated Management of Phytoplasma Epidemics in Different Crop Systems".

The EUPHRESCO FruitPhytoInterlab Group would like to thank Jennifer Hodgettes (FERA, UK) and Miguel Calvo (University of Barcelona, Spain) for statistical analysis.

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