FIRST INTERNATIONAL PROFICIENCY TESTING FOR LABORATORY PERFORMANCE FOR DETECTION OF *XYLELLA FASTIDIOSA*

Giuliana Loconsole
Researcher at the University of Bari (Italy)

Loconsole G., Olivier V., Chabirand A., Poliakoff F.,
Essaki S., Potere O., Boscia D., Saponari M.

efsa European Food Safety Authority
UIB Universitat de les Illes Balears
European Commission
PONTE Pest Organisms Threatening Europe
XF ACTORS
Euphresco

WHAT IS A PROFICIENCY TEST ?

A way to evaluate and assess the performance and competence of 1 or more laboratories:

- standardized samples with known status regarding the presence of the target pathogen(s) sent out to **participating laboratories;**
- laboratories use **their own methods, equipment and reagents to perform the tests;**
- the Organizer(s) analyzes the results and provides a **report detailing all participants' results in confidential manner** together with actual sample status.

AIM/OBJECTIVE OF A PROFICIENCY TEST

- Help for the laboratory to **improve its quality**
- Used by customers or regulatory bodies for the **selection of qualified laboratories**
- An affordable means to the verification of the **laboratory's capabilities and the accuracy of analysis.** Laboratory can determine, whether imprecision or bias is the **reason for its inaccuracy**
- **Corrective actions** to achieve a better performance

EU-XF- PT-2017-02:
Proficiency testing for the evaluation of molecular and serological diagnosis of *Xylella fastidiosa*

Organizers

UNIVERSITÀ DEGLI STUDI DI BARI ALDO MORO
DISSPA – DIPARTIMENTO DI SCIENZE DEL SUOLO, DELLA PIANTA E DEGLI ALIMENTI

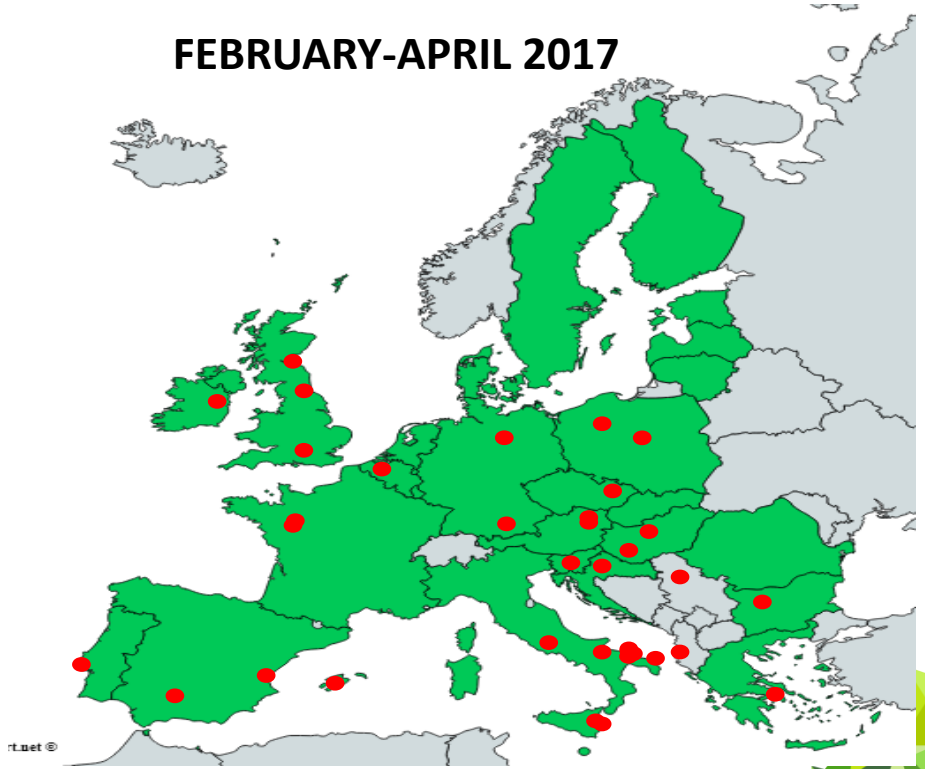
IPSP
Istituto per la Protezione Sostenibile delle Piante
Consiglio Nazionale delle Ricerche

Supported by anses

(organized in accordance with EPPO 7/122 and ISO/IEC 17043 guidelines)

18 EU/non-EU Countries
35 participating laboratories
identified by an anonymous alphanumeric code to ensure results confidentiality

FEBRUARY-APRIL 2017



EU-XF- PT-2017-02:
Proficiency testing for the evaluation of molecular and serological diagnosis of *Xylella fastidiosa*

OBJECTIVE

- evaluate the performance (efficiency and accuracy) of laboratories involved in the diagnosis of *Xylella fastidiosa*, by serological (ELISA) and molecular assays (PCR, qPCR) on a panel of blind samples

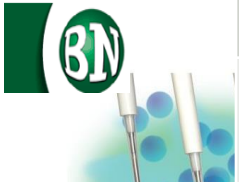
- An educational training for those laboratories that had never approached the detection of *X. fastidiosa* using some of the protocols tested in this PT

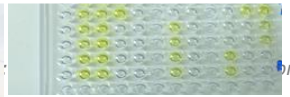
TIMELINE OF THE EU-XF- PT-2017-02

Preparation of samples, storage at -20°C	13-17 February 2017
Shipment	20-24 February 2017
Homogeneity tests	13-15 February 2017
Stability tests	Molecular tests on 10-15 April, ELISA tests on 27 April 201
Diagnostic test performed and result sent to organizer	by March 27 2017
Preliminary report	May 5, 2017
Discussion of the report during the the meeting of the EPPO Panel on Diagnostic in Bacteriology	May 30, 2017
final report	**end of July 2017**

DIAGNOSTIC PROCEDURES PERFORMED

* Protocols supplied to support no-experience labs





DNA extraction with	Taqman PCR Harper N. Lab	End point PCR Minsavage N. Lab
CTAB	20	25
Mericon food kit (QIAGEN)	17	22
Quick pick plant kit (BIONOBILE)	12	9
Dneasy plant minikit (QIAGEN)	4	6
ELISA tests	**Loewe N. 9 lab**	**Agritest N. 11 lab**

European

roblem

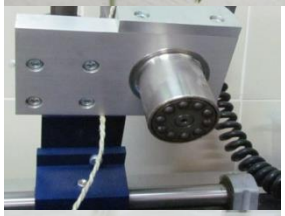
PANEL OF EXPERIMENTAL SAMPLES

Spiked plant sap from olive leaf petioles prepared depending on methods

with X. f. subsp. pauca strain CoDiRO (CFBP8402)

ELISA

Number of Samples	Replicate	Concentration (cfu/mL)	Expected result
1	Rep 1	5.10E+06	Positive
2		5.10E+05	Positive
3		5.10E+04	Positive
4		Healhy	Negative
5	Rep2	5.10E+06	Positive
6		5.10E+05	Positive
7		5.10E+04	Positive
8		Healhy	Negative
9	Rep3	5.10E+06	Positive
10		5.10E+05	Positive
11		5.10E+04	Positive
12		Healhy	Negative
13	Lure	+/-	



qPCR & PCR

Number of Samples	Replicate	Concentration (cfu/mL)	Expected result
1	Rep 1	10E+06	Positive
2		10E+05	Positive
3		10E+04	Positive
4		Healhy	Negative
5	Rep2	10E+06	Positive
6		10E+05	Positive
7		10E+04	Positive
8		Healhy	Negative
9	Rep3	10E+06	Positive
10		10E+05	Positive
11		10E+04	Positive
12		Healhy	Negative
13	Lure	+/-	

HOMOGENEITY AND STABILITY

-Assessed for all the diagnostic methods included in the PT
-Performed on 3 replicates for each artificially contaminated sample and 3 replicates of the *Xylella*-free sample
- Stability tests conducted once all laboratories had completed their tests (after 1 month)

Based on the analysis of :
- the quantitative (Cq values, ΔCq, SD, OD_{405} values) results
- qualitative (positive/negative) results

all the samples were considered to be SUFFICIENTLY HOMOGENOUS AND STABLE for qPCR, ELISA and PCR and SUITABLE to evaluate the lab – performance

ANALYSIS OF THE RESULTS

1. Qualitative results

Definition of the parameters adapted from ISO 16140

Laboratory Results	Assigned value	
	Positive	Negative
Positive	PA= positive agreement	PD= positive deviation
Negative	ND= negative deviation	NA= negative agreement
Undetermined (if any contradictory or unclear results are obtained)	ND= negative deviation	PD=positive deviation

Example for a laboratory

Sample	Assigned value	Labortory result	N. PA, NA, ND ,PD
A	+	+	PA
B	+	+	PA
C1	+	-	ND
C2	+	-	ND
C3	+	+	PA
D	+	+	PA
E	+	+	PA
F1	+	+	PA
F2	+	-	ND
F3	+	-	ND
G	-	-	NA
H	-	-	NA
I	-	-	NA
J	-	-	NA
K	-	-	NA
L	-	-	NA
M	-	und	PD
N	-	-	NA
O	-	-	NA
P	-	-	NA

1. Qualitative results

Performance criteria	Definition	Calculation
Accuracy (AC)	Closeness of agreement between the laboratory result and the assigned value	AC= $(N_{PA}+N_{NA})/N$
Sensitivity (SE)	Closeness of agreement between the laboratory result and the assigned value for samples for which the assigned value is positive	SE= $N_{PA}/N+$
Specificity (SP)	Closeness of agreement between the laboratory result and the assigned value for samples for which the assigned value is negative	SP=$N_{NA}/N-$
Repeatibility (DA)	Closeness of agreement between independent test results obtained under conditions of repeatability, i.e. independent test results obtained by the same method, on identical test samples in the same laboratory, by the same operator, using the same equipment, within a short period of time	DA denotes the percentage chance of obtaining the same result (positive, negative or indeterminate) from two identical samples analyzed in the same laboratory

The proficiency was expressed as percentage, with 100% being the highest performance level (Chabirand et al., 2014)

2. Quantitative results

- quantitation cycles: recorded for qPCR assays
- Absorbance OD_{405} values: record for the ELISA test

CATEGORIZATION OF THE LABORATORIES BASED ON THEIR PERFORMANCE

Based on the values (%) recovered for the “accuracy” the laboratories were categorized as:

Lab categorization	level of accuracy
highly proficient	100%
proficient	90-100% (1 PD, 1 ND)
non-proficient	<90% (>1 PD, > 1 ND)

The declaration of conformity to the PT assigned to “highly proficient” and “proficient” labs

QUALITATIVE RESULTS OF THE MOLECULAR TESTS

PERFORMANCE CRITERIA RECOVERED IN THE DIFFERENT LABORATORIES FOR qPCR (Harper *et al.*, 2010)

Performance parameters and criteria	DNA extraction methods					
	CTAB	MERICON Food	Quick pick			DNeasy plant minikit
	N. Lab	N. Lab	N. Lab			N. Lab
	20/20	17/17	10/12	1/12	1/12	4/4
N. of PA	9	9	9	9	5	9
N. of NA	3	3	3	2	3	3
N. of ND	0	0	0	0	4	0
N. of PD	0	0	0	1	0	0
Sensitivity	100%	100%	100%	100%	56%	100%
Specificity	100%	100%	100%	67%	100%	100%
Repeatability	100%	100%	100%	89%	89%	100%
Accuracy	**100%**	**100%**	**100%**	**92%**	**67%**	**100%**
CATEGORY	**Highly proficient**	**Highly proficient**	**Highly proficient**	**Proficient**	**Non-Proficient**	**Highly proficient**
Conformity	**YES**	**YES**	**YES**	**YES**	**NO**	**YES**

Performed manually , using a magnetic pipet

PERFORMANCE CRITERIA RECOVERED IN THE DIFFERENT LABORATORIES FOR PCR (Minsavage *et al.*, 1994)

Performance parameters and criteria	DNA extraction methods													
	CTAB			MERICON Food		Quick pick					DNeasy plant minikit			
	N. LAB (tot. 25)			N. LAB (tot. 22)		N. LAB (tot. 9)					N. LAB (tot. 6)			
	23	1	1	21	1	5	1	1	1	1	3	1	1	1
N. of PA	9	8				9	8				9			
N. of NA	3	3				3	3				3			
N. of ND	0	1				0	1				0	4	3	3
N. of PD	0	0				0	0				0	0	0	0
Sensitivity	100%	89%				100%	89%	67%	56%	33%	100%	56%	67%	67%
Specificity	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Repeatability	100%	89%	89%	100%	100%	100%	89%	100%	89%	100%	100%	89%	100%	78%
Accuracy	100%	92%	67%	100%	75%	100%	92%	75%	67%	50%	100%	67%	75%	75%
Category	Highly profic.	Profic	Non-Prof.	Highly profic.	Non-profic.	Highly profic.	Profic	Non-Profic.	Non-Prof.	Non-Prof.	Highly Profic.	Non-Profic.	Non-Profic.	Non-Profic.
Conformity	YES	YES	NO	YES	NO	YES	YES	NO	NO	NO	YES	NO	NO	NO

L28 failed to detect Xf in the rep10^4 CFU/ml regardless the method used for the DNA extraction (non efficient PCR reagents)

Performed manually, using a magnetic pipet or rack

Detection failed in the rep10^4 CFU/ml

QUALITATIVE RESULTS OF THE ELISA TESTS

PERFORMANCE CRITERIA RECOVERED IN 13 LABORATORIES FOR ELISA TESTS USING TWO DIFFERENT COMMERCIAL KITS

Performance parameters and criteria	N. LAB (tot.11) KIT AGRITEST				N. LAB. (tot. 9) KIT LOEWE	
	4	1	1	5	6	3
N. of PA	9	7	7	6	9	6
N. of NA	3	3	3	3	3	3
N. of ND	0	2	2	3	0	3
N. of PD	0	0				
Sensitivity	100%	78%				
Specificity	100%	100%	100%	100%	100%	100%
Repeatability	100%	89%	89%	100%	100%	100%
Accuracy	100%	83%	83%	75%	100%	75%
Category	Highly proficient	Non-proficient	Non-proficient	Non-proficient	Highly proficient	Non-proficient
Conformity	YES	NO	NO	NO	YES	NO

Accuracy for the ELISA tests lower than Accuracy values obtained using the molecular tests, ND recorded for the samples 5x10^4 CFU/ml.

considering only the results obtained for samples containing 5x10^6 CFU/ml and 5x10^5 CFU/ml, all laboratories were proficient with an accuracy of 100%

OVERVIEW ON THE PERFORMANCE OF THE LABORATORIES

Number and percentage of laboratories and considered "conformed/not conformed to the PT" for each method

Status of the laboratories	Diagnostic protocols									
	CTAB		MERICON		QUICK PICK		DNeasy plant		ELISA	
	qPCR	PCR	qPCR	PCR	qPCR	PCR	qPCR	PCR	Agritest	Loewe
CONFORM (Highly proficient and proficient)	20 100%	24 96%	17 100%	21 95%	11 92%	6 67%	4 100%	3 50%	4 36%	6 67%
NON-CONFORM (Non-proficient)	0	1	0	1	1	3	0	3	7	3
Total number of laboratories	**20**	**25**	**17**	**22**	**12**	**9**	**4**	**6**	**11**	**9**

COMMENTS:

1. **qPCR assays:** Despite the use different methods of extraction and different qPCR master mixes, **the totality of the laboratories that performed the detection of *X.f.* resulted proficient**, only 1 one exception

2. **PCR assays: highest number of non-proficient lab when using the Quick Pick kit (Bionobile)** for the extraction of the DNA, as consequence of the use of the manual magnet pipet as alternative to an automated platform, and to the fact that some laboratories were not used and trained to use this specific kit.

3. **Lower sensitivity of ELISA tests compared to molecular tests:** in this specific PT, several parameters may have influenced the performance of the laboratories: (i) use of different plates, (ii) different volume of samples loaded into the plates, (iii) use of in-house prepared buffers (iv) artificially contaminated samples, different from fresh infected plant samples.

CONCLUSION ON EU-XF- PT-2017-02

1. this PT provided **a good overview on the laboratory performance for the diagnostics** currently used in the EU/Mediterranean countries **for the detection of *Xylella*** in the plant samples.

2. The results indicated that using the most sensitive and the most widely adopted diagnostic protocol (i.e. qPCR) **the laboratories's performance was very satisfactory.**

3. At the same time **useful insights** were obtained **to achieve a better performance for the unsatisfactory laboratories**, i.e. select different protocol for DNA extraction, different reagents and amplification conditions.

ADDITIONAL INSIGHTS

A TEST PERFORMANCE STUDY (TPS) WAS CONDUCTED BASED ON THE ANALYSIS OF THE RESULTS OF THE MOLECULAR ASSAYS

Evaluation of the performance of the molecular diagnostic methods using the results obtained by laboratories that performed proficiently in PT

METHODS	CTAB		MERICON		QUICK PICK	
	qPCR	PCR	qPCR	PCR	qPCR	PCR
DECLARED CONFORMITY in the PT (N. lab)	20	24	17	19	11	6

TPS: ANALYSIS OF THE RESULTS

Performance criteria	Definition	Calculation
Accuracy (AC)	Closeness of agreement between the laboratory result and the assigned value	AC= $(N_{PA}+N_{NA})/N$
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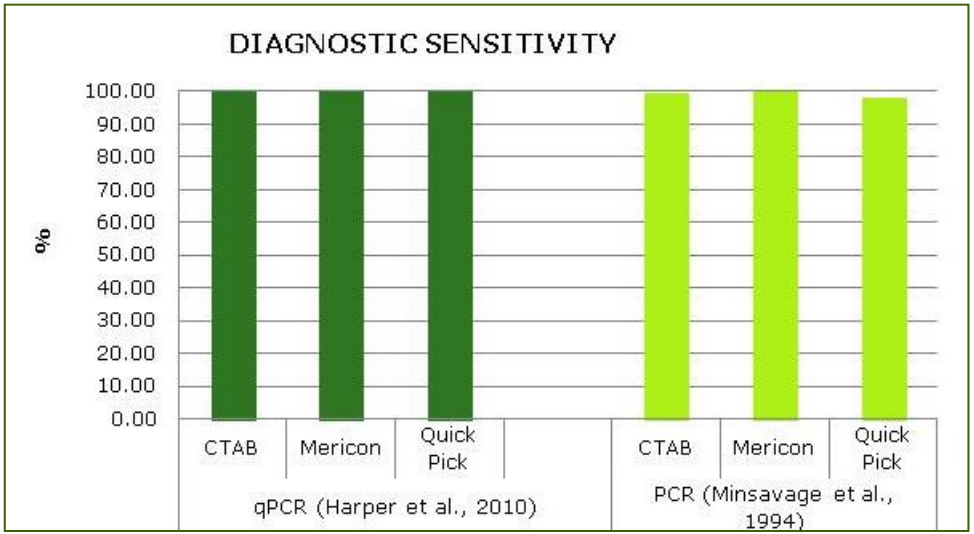
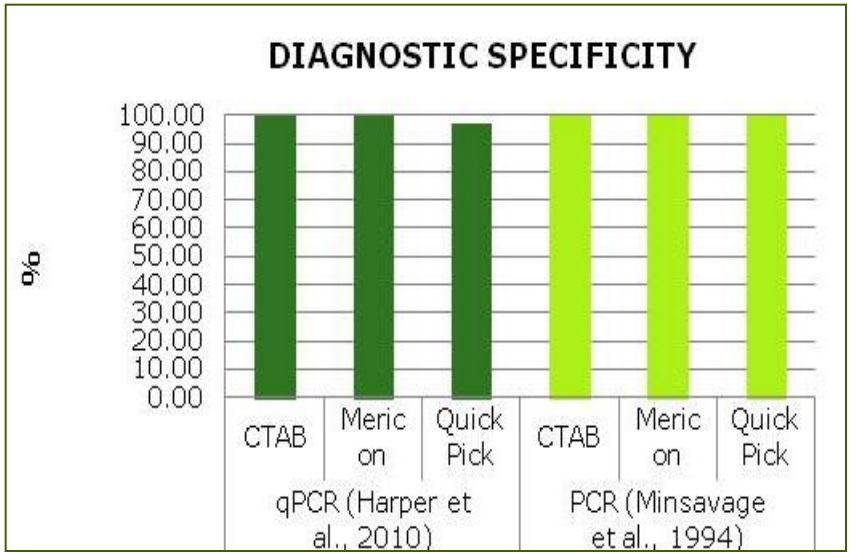
Reproducibility	as the ability of a test to provide consistent results when applied to aliquots of the same sample tested under different conditions (time, persons, equipment, location, etc)	based on the number of interlaboratory pairs of same results/total number of interlaboratory pairs.

Analysis included also the quantitative results expressed as Cq values for qPCR

TPS: RESULTS OF qPCR AND PCR ASSAYS

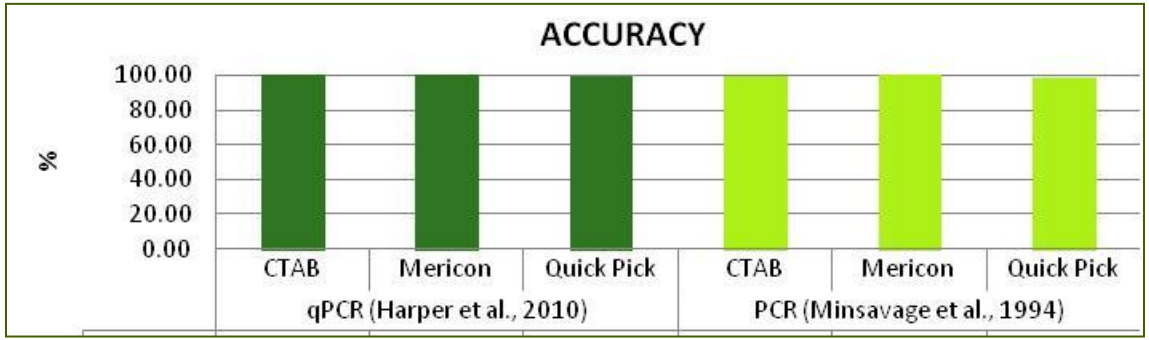
Performance criteria calculated using the results obtained in qPCR and PCR assays using the DNA extracts prepared following 3 different extraction protocols (CTAB, Mericon food kit, Quick Pick)

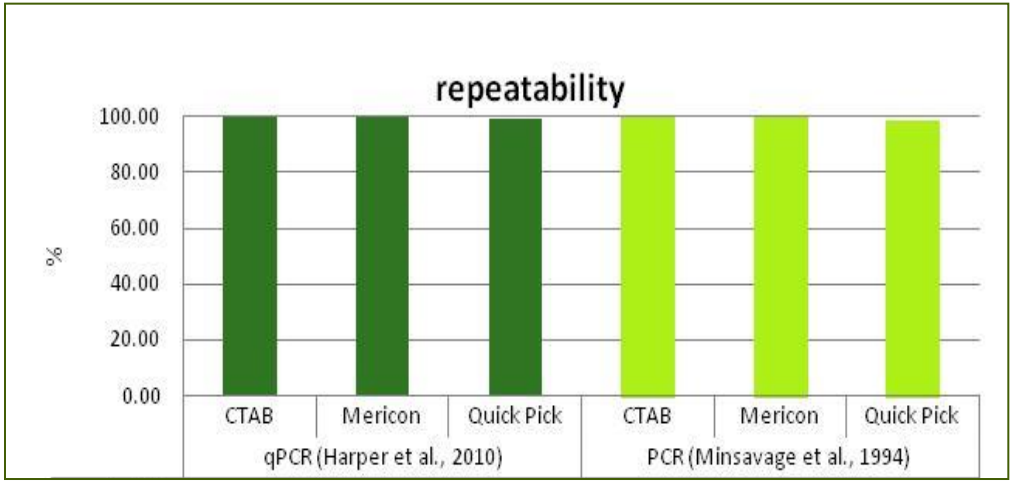


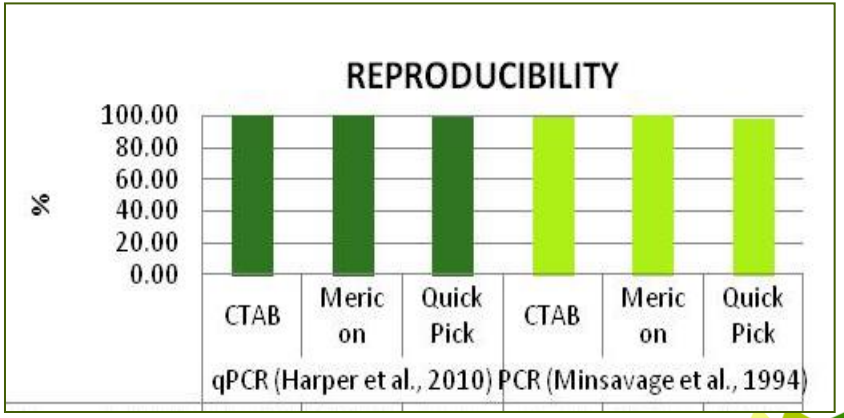


qPCR and PCR assays consistently resulted in performance values of sensitivity, specificity, accuracy, repeatability and reproducibility in the range 97-100%.

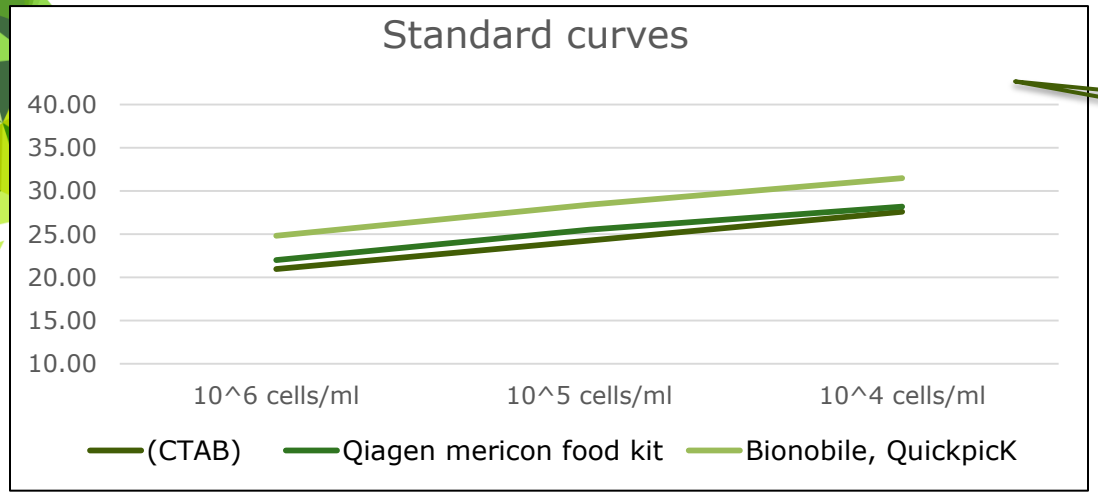
Performance criteria calculated using the results obtained in qPCR and PCR assays using the DNA extracts prepared following three different extraction protocols (CTAB, Mericon food kit, Quick Pick)





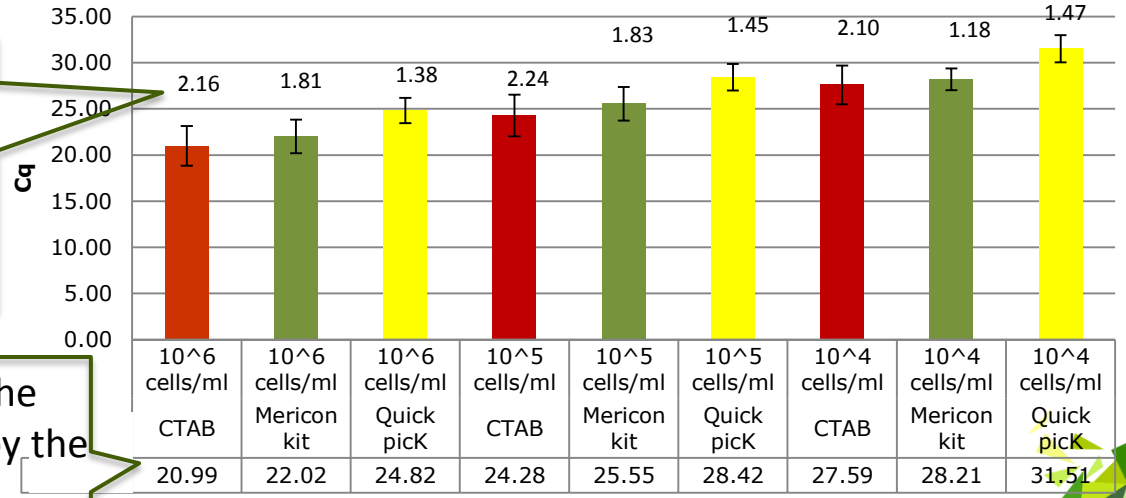


TPS: QUANTITATIVE RESULTS OF QPCR



-ΔCq among the dilutions = expected value of "3" (approximately)
-qPCR reaction efficiency: 90%-110% (optimal)

The overall SD among the Cq values recovered in the different laboratories are affected by the use of different qPCR conditions (amplification master mixes, reaction volumes, etc.)



The Lowest Cq values obtained with the DNA recovered using CTAB followed by the Qiagen Mericon Food kit

TPS: CONCLUSION

1. Despite the use of different amplification conditions and master mix, by simulating a **TPS among the proficient labs, optimal performance values (ranging from 97 to 100%)** were obtained confirming the robustness and reproducibility of the molecular methods tested

2. **Robustness (PM 7/76) of the molecular diagnostic tests (extraction procedures and amplification protocols) evaluated in this PT,** and currently being the most common used protocols, confirming their suitability for the diagnosis of *X. fastidiosa* in plant materials

ONGOING ACTIVITIES ON INTERLABORATORY VALIDATIONS

IPSP Istituto per la Protezione Sostenibile delle Piante
Consiglio Nazionale delle Ricerche

November 2017

UNIVERSITÀ DEGLI STUDI DI BARI ALDO MORO
DISSPA – DIPARTIMENTO DI SCIENZE DEL SUOLO, DELLA PIANTA E DEGLI ALIMENTI

TEST PERFORMANCE STUDY

Molecular detection of *Xylella fastidiosa* through quantitative real time PCR assays

Objective

-Interlaboratory comparison of the performance and the accuracy of different qPCR assays:

a) Real-time PCR based on the primers/probe designed by Li et al., 2013, with MGB/standard TaqMan probe
b) Francis et al., 2006 Using SYBR green/TaqMan probe [EPPO, PM 7/24 (2)]
c) Real-time PCR based on the primers/probe designed by Harper et al., 2010 (erratum 2013) [EPPO, PM 7/24 (2)]

- on the DNA extracts prepared in the framework of the Proficiency Test EU-XF- PT-2017-02
- 5 different qPCR assay formats will be tested
- 14 EU/non-EU labs involved

ONGOING ACTIVITIES ON INTERLABORATORY VALIDATIONS

October-November 2017

Interlaboratory test for validation of diagnostic procedures for the detection of *Xylella fastidiosa on vectors*

DNA extraction methods and molecular methods [EPPO standard PM7/24 (2)]

3 protocols for the preparation of the samples followed by molecular detection

- real time **LAMP** *developed by Yaseen et al. (2015)*
- DNA extraction using **CTAB** and **QuickPick™ SML Plant DNA kit (Bio-Nobile**), followed by **real-time PCR methods**: [Harper *et al., 2010, erratum 2013 / Harper et al., 2010 erratum 2013 duplexed with Ioos et al., 2009) / Francis et al., 2006 (TaqMan) and LAMP (Yaseen et al; 2015)*]

spiked insect macerate obtained with *Xf* free *Philaenus spumarius* → Collected from Xf free-areas

Naturally infected *Philaenus spumarius* →

THANKS TO

EPPO SECRETARIAT
and
EPPO Panel on Diagnostic in Bacteriology

F. Poliakoff, V. Olivier, A. Chabirand

All the 35 participating laboratories