NATIONAL INSTITUTE OF BIOLOGY

FINAL REPORT ON THE 'NIB Proficiency Test Round 2018-01: PROFICIENCY TEST FOR THE MOLECULAR AND/OR SEROLOGICAL DETECTION OF *Erwinia amylovora*

The proficiency test was organized in 2018 by the National Institute of Biology, Depatment of Biotechnology and Systems Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia. This report is distributed by the organizer to the participants of the proficiency test. Reproduction of this document is permitted only in full.

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1 Background

Proficiency test is a way in which competence of laboratories is assessed and demonstrated. In proficiency testing standardized samples are prepared with known status regarding the presence of harmful organisms. These are sent out to participating laboratories that analyse them using their own methods, equipment and reagents and send results back to the organizer. Organizer analyses the results and provides a report detailing all participants' results in confidential manner together with actual sample status.

2 Organisation of the proficiency test

This proficiency test covered molecular and/or serological detection of *Erwinia amylovora*, providing each participant with seven (7) samples of deactivated plant extracts. The test was organised following guidelines (PM 7/122 (1), EPPO, 2014) and with the following timeline:

- registration: August 25 – September 14, 2018.
- samples sent out in the week of October 22-26, 2018 (all samples were sent out October 23, 2018).
- deadline for submitting results and reporting: December 14, 2018.
- reports on performance sent out by February 15, 2019.

In total, 27 laboratories participated in the proficiency test. The laboratory codes assigned to each participant are confidential. Participants are listed in Annex I Participants.

3 Study Materials

Bacterial strains

An *Erwinia amylovora* strain (NIB Z 903) isolated in 2007 from a symptomatic *Malus domestica* cv. Gloster in Slovenia and previously confirmed as positive, was used to prepare bacterial suspensions in phosphate buffer saline buffer (10 mM PBS, pH = 7.2). The bacterial suspensions were inactivated by heating at 70 °C for 10 min, immediately cooled on ice and mixed with plant extract as described below.

Preparation of plant material and samples

Two plant extracts were prepared from healthy *Malus domestica* twigs sampled in a Zona Protecta area of Slovenia where symptoms of fire blight were not observed yet and latent testing did not detect latent infections with *Erwinia amylovora*. Leaves form twigs were first removed and twigs surface sterilized with 70% ethanol and let dry. From twigs external bark was removed using sterile scalpel and underneath tissue was sampled. Collected plant material was weighed and covered with 10 mM PBS with glycerol (9:1) buffer (4,5 mL of buffer

per 0,2 gram material was added). The extracts were incubated for 20 minutes and then plant material was removed and extracts stored frozen (< -15 °C) before further preparation and testing. The samples were tested for *E. amylovora* via enrichment-real-time PCR (EPPO PM 7/20 (2)).

To prepare the samples for proficiency test first plant extracts were inactivated by heating at 70°C for 15 min in approximately 30 mL volumes, and immediately cooled on ice. Three (3) positive samples were prepared for each participant of the proficiency test. Samples were prepared by mixing inactivated bacterial suspensions with inactivated plant extracts from twigs to target concentrations. The target concentrations in samples were selected in such a way to represent high target concentrations, all above the theoretical and reported analytical sensitivity of various molecular and serological methods of detection of symptomatic fire blight plant material. The target concentrations were 10^7 and 10^8 target copies per mL, corresponding to equal number of cells when the target is present in single copies per genome. All mixtures were vortexed for 30 seconds and shaken at room temperature on orbital shaker (100 rpm) for minimum 30 minutes before aliquoting them in 500 µL volumes in DNA LoBind Tubes (Eppendorf 022431021). Samples were stored < -15 °C before further analyses and distribution. Additionally, four (4) negative samples were prepared for each participant. The negative samples consisted of two inactivated plant extracts used for preparation of positive samples.

Real-time PCR

DNA from extracts was extracted using QuickPick™ SML Plant DNA Kit (Bio-Nobile, Turku, Finland) and MagMax™ Express-96 Deep Well Magnetic Particle Processor (Applied Biosystems). 100 µL of each extract was mixed with 400 µL lysis buffer and 25 µL of proteinase K, incubated for 30 min at 65°C and centrifuged at 6000 g for 1 min. Lysate (440 µL) was transferred to plate 1 of a MagMax™ Express-96 Deep Well Magnetic Particle Processor. Plates contained; 20 µL of MagaZorb™ Magnetic Particles and 500 µL binding buffer (plate 1), 800 µL wash buffer (plates 2 and 3), 100 µL elution buffer (plate 4). The instrument program in MagMax™ Express-96 Deep Well Magnetic Particle Processor was with following parameters: binding time in plate 1, 3 x 1 min release plus 2 min binding; wash in plate 2 15 s; wash in plate 3, 15 s; elution in plate 4, 10 min.

Real-time PCR assay was used to assess the homogeneity and stability of the samples, and included real-time PCR assay developed by Pirc *et al*. (2009) targeting *amsC* gene of *E. amylovora*.

All of the qPCR reactions were performed in triplicate on a ViiA™ 7 Real-Time PCR System (Life Technologies) using the following universal cycling conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, and 1 min at 60 °C, and using standard temperature ramping mode. The reaction volumes of 10 µL contained, as final concentrations, 900 nM primers, 200 nM FAM/BHQ1 probe (Integrated DNA Technologies), 1×TaqMan Universal PCR Master Mix (Life Technologies), and 2 µL sample DNA. The qPCR data were analysed using the Viia7™ Software v. 1.2.4 (Life Technologies) with automatic baseline and a manually selected threshold of 0,1. Positive and no template controls were used in each run. The qPCR data are given as minimum and maximum Cq values (i.e. qPCR quantification cycle) and as the average Cq values together with the associated coefficient of variation (CV) value.

Lateral flow device and immunofluorescence

Lateral flow device AgriStrip (Bioreba) was used to assess the serological stability of the samples before and after heat deactivation, and over time. Integrity of cells and the influence of heat deactivation on serological tests was checked additionally using immunofluorescence with *Erwinia amylovora* antiserum of Prime Diagnostics.

Assigning reference values to samples with digital PCR (dPCR)

Digital PCR using reagents developed by Pirc *et al*. (2009) targeting *amsC* gene of *E. amylovora*, was used to determine the concentration of the target copy numbers in samples. Digital PCR was performed on QX100TM Droplet Digital™ PCR system (Bio-Rad) as described previously (Dreo *et al*., 2014). The sample volume analysed in each reaction was 8 µL. The software package provided with the dPCR system (QuantaSoft™ Software, verison 17.4.0917, Bio-Rad) was used for data acquisition. A minimum of 10.000 accepted droplets per reaction was required for the reaction to be considered valid. A fixed manual global threshold discriminating between negative and positive droplets was set at 4000. A reaction was interpreted as positive if the number of positive droplets was ≥3. Positive and no template controls were used in each run.

Unless stated otherwise, the assigned values (target concentrations) for samples were determined as an average concentration of three sample aliquots per each concentration level, each in triplicates (Table 1).

Table 1: Concentration levels of samples as determined with digital PCR using primers and probes designed by Pirc et al. (2009; amsC assay). *Cps = target DNA copies, CV = coefficient of variation.*

Sample ID	cells/mL*		log(cps/mL extract)		Concentration level [cells/mL]
	Min - Max	Average ± CV	Min - Max	Average ± CV	
Ea 1**	0	0	0	0	0
Ea 2**	0	0	0	0	0
Ea 3**	0	0	0	0	0
Ea 4**	0	0	0	0	0
Ea 5	193375000 - 449225000	313416250 ± 0,33	8,2 - 8,6	8,4 ± 0,02	3xE8
Ea 6	28262500 - 34807500	30642500 ± 0,12	7,4 - 7,5	7,4 ± 0,01	3xE7
Ea 7	285600000 - 395675000	331712500 ± 0,14	8,4 - 8,5	8,4 ± 0,01	3xE8

** the concentration is expressed as cells/mL of extract and is calculated from the concentrations (copies/µL of DNA), taking into account the DNA extraction process.*

*** samples Ea 1, Ea 2 and Ea 3, Ea 4 are aliquots of the same starting material respectively.*

Homogeneity and stability testing

For homogeneity testing ten randomly selected aliquots of samples prepared for proficiency test were selected for each concentration level. From selected samples, DNA was extracted according to procedure above, and tested in three technical repeats (wells) each using real-time PCR assay (Pirc *et al*. (2009) targeting *amsC* gene), analysing 2 µL of DNA in each reaction. The results were in concordance with the true values for all samples and concentration levels with coefficients of variations below 2% (Table 2).

Table 2: Real-time PCR results of homogeneity testing of samples. *Homogeneity testing was done with real-time PCR assay developed by Pirc et al. (2009) targeting amsC gene. Ten aliquots per concentration level and spiked plant extract were tested in three technical repeats (wells) each. Cq = cycle of threshold. CV = coefficient of variation, NA = not applicable.*

Concentration level [cells/mL]	Spiked plant extract	Sample ID	Min(Cq) - Max(Cq)	Average(Cq) ± CV
Ea amsC probe-primer set (Pirc et al. 2009)				
0	D729/18	Ea 1	neg (45)	NA
0	D729/18	Ea 2	neg (45)	NA
0	D778/18	Ea 3	neg (45)	NA
0	D778/18	Ea 4	neg (45)	NA
3xE8	D729/18	Ea 5	17,5 - 18,1	17,84 ± 0,010
3xE7	D729/18	Ea 6	21,6 - 22,1	21,78 ± 0,007
3xE8	D778/18	Ea 7	17,2 - 18,6	18,12 ± 0,018

Short term and long term stability of the samples were tested with real-time PCR developed by Pirc *et al*. (2009) targeting *amsC* gene, and with lateral flow devices AgriStrip (Bioreba). Short-term stability was tested after mimicking conditions during transport by incubating three randomly selected aliquots of samples of each concentration level for one week at different temperatures (< -15°C, 2-8 °C, and 25 °C) in the dark. Long-term stability was tested on aliquots stored < -15 °C for 5 and 10 weeks, the latter corresponding to the deadline for reporting results. Samples were stable in all cases (Tables 3 - 6).

Table 3: Results of short-term stability testing of aliquots stored at different temperatures for 1 week. *Three aliquots per concentration level and spiked plant extract, were tested in three technical repeats (wells) with real-time PCR assays developed by Pirc et al. (2009) targeting amsC gene, after one week of incubation at temperature below -15 °C, 2-8 °C and 25 °C. Cq = cycle of threshold, CV = coefficient of variation, NA = not applicable.*

Concentration level [cells/mL]	Spiked plant extract	Sample ID	T < -15 °C		2 - 8 °C		25 °C	
			Min(Cq) - Max(Cq)	Average(Cq) ± CV	Min(Cq) - Max(Cq)	Average(Cq) ± CV	Min(Cq) - Max(Cq)	Average(Cq) ± CV
Ea amsC probe-primer set (Pirc et al. 2009)								
0	D729/18	Ea 1	neg (45)	NA	neg (45)	NA	neg (45)	NA
0	D729/18	Ea 2	neg (45)	NA	neg (45)	NA	neg (45)	NA
0	D778/18	Ea 3	neg (45)	NA	neg (45)	NA	neg (45)	NA
0	D778/18	Ea 4	neg (45)	NA	neg (45)	NA	neg (45)	NA
3xE8	D729/18	Ea 5	17,1 - 17,6	17,33 ± 0,010	17,1 - 17,2	17,17 ± 0,002	16,9 - 17,6	17,22 ± 0,016
3xE7	D729/18	Ea 6	20,4 - 20,6	20,51 ± 0,004	20,3 - 20,6	20,44 ± 0,005	20,4 - 20,6	20,45 ± 0,003
3xE8	D778/18	Ea 7	17,1 - 17,4	17,22 ± 0,007	17,2 - 17,5	17,34 ± 0,006	17,0 - 17,6	17,24 ± 0,013

Table 4: Results of short-term stability testing of aliquots stored at different temperatures for 1 week. *Three aliquots per concentration level and spiked plant extract, were tested in one technical repeat with lateral flow device AgriStrip (Bioreba) after one week of incubation at temperatures below -15 °C, 2-8 °C and 25 °C.*

Concentration level [cells/mL]	Spiked plant extract	Sample ID	T < -15 °C		2 - 8 °C		25 °C	
			15 min	30 min	15 min	30 min	15 min	30 min
0	D729/18	Ea 1	neg	neg	neg	neg	neg	neg
0	D729/18	Ea 2	neg	neg	neg	neg	neg	neg
0	D778/18	Ea 3	neg	neg	neg	neg	neg	neg
0	D778/18	Ea 4	neg	neg	neg	neg	neg	neg
3xE8	D729/18	Ea 5	pos	pos	pos	pos	pos	pos
3xE7	D729/18	Ea 6	weak pos	weak pos	weak pos	weak pos	weak pos	weak pos
3xE8	D778/18	Ea 7	pos	pos	pos	pos	pos	pos

Table 5: Results of long-term stability testing of sample aliquots stored at temperature of < -15 °C. Three aliquots per concentration level and spiked plant extract, were tested in three technical repeats (wells) with real-time PCR assays developed by by Pirc et al. (2009) targeting amsC gene, after 1, 5 and 10 weeks. Cq = cycle of threshold, CV = coefficient of variation, NA = not applicable.

Concentration level [cells/mL]	Spiked plant extract	Sample ID	Week 1		Week 5		Week 10	
			Min(Cq) - Max(Cq)	Average(Cq) ± CV	Min(Cq) - Max(Cq)	Average(Cq) ± CV	Min(Cq) - Max(Cq)	Average(Cq) ± CV
Ea amsC probe-primer set (Pirc et al. 2009)								
0	D729/18	Ea 1	neg (45)	NA	neg (45)	NA	neg (45)	NA
0	D729/18	Ea 2	neg (45)	NA	neg (45)	NA	neg (45)	NA
0	D778/18	Ea 3	neg (45)	NA	neg (45)	NA	neg (45)	NA
0	D778/18	Ea 4	neg (45)	NA	neg (45)	NA	neg (45)	NA
3xE8	D729/18	Ea 5	17,1 - 17,6	17,33 ± 0,010	17,3 - 17,6	17,47 ± 0,008	16,8 - 17,5	17,17 ± 0,011
3xE7	D729/18	Ea 6	20,4 - 20,6	20,51 ± 0,004	20,5 - 20,7	20,66 ± 0,003	20,6 - 21,0	20,78 ± 0,007
3xE8	D778/18	Ea 7	17,1 - 17,4	17,22 ± 0,007	17,5 - 17,7	17,56 ± 0,005	17,2 - 17,4	17,28 ± 0,005

Table 6: Results of long-term stability testing of sample aliquots stored at temperature of < -15 °C. *Three aliquots per concentration level and spiked plant extract were tested in one technical repeat with lateral flow device AgriStrip (Bioreba) after 1, 5 and 10 weeks.*

Concentration level [cells/mL]	Spiked plant extract	Sample ID	Week 1		Week 5		Week 10	
			15 min	30 min	15 min	30 min	15 min	30 min
0	D729/18	Ea 1	neg	neg	neg	neg	neg	neg
0	D729/18	Ea 2	neg	neg	neg	neg	neg	neg
0	D778/18	Ea 3	neg	neg	neg	neg	neg	neg
0	D778/18	Ea 4	neg	neg	neg	neg	neg	neg
3xE8	D729/18	Ea 5	pos	pos	pos	pos	pos	pos
3xE7	D729/18	Ea 6	weak pos	weak pos	weak pos	weak pos	weak pos	weak pos
3xE8	D778/18	Ea 7	pos	pos	pos	pos	pos	pos

4 Distribution of samples

Samples were sent on dry ice with exception to countries where shipment on dry ice is not allowed. Those samples were sent with ice packs. Most of the participant received the samples in two days. Eleven days was maximum time for delivery.

5 Reported results

In total, 28 laboratories registered for the proficiency test for *Erwinia amylovora*. One participant withdrew from the proficiency test after realizing the isolation on plates is not possible due to sample preparation (heat deactivation of target bacteria). The total number of participants used for evaluation is 27.

Of the 27 participants, 11 (41 %) performed and reported on both serological and molecular tests while 16 (56 %) of participants relied on molecular tests only.

Majority of participants (25/27, 93 %) reported the results on time. Two participants reported results after the deadline, one on the 17.12.2018 and one on 14.2.2019, as indicated in the results tables (deadline was 14.12.2019). While late submissions are automatically regarded as non-conforming result we have nevertheless included the results in the overall analysis.

Results, as reported, are summarized in Tables 7-9.

6 Evaluation of the results

Taking into account serological test only, all results of all participants (both at sample and participant level) were in concordance with the true qualitative values (Table 7).

Using molecular tests all the participants correctly identified all positive samples however, three participants obtained false positive results (Table 8; Figure 1). Of these, two used only molecular tests and reported non-conforming overall result for two samples.

One participant used both molecular and serological tests and concluded a correct overall result for the false positive sample in their molecular tests. Overall, two participants reported false positive results for two samples each (Table 9).

Table 7: Results reported for serological tests and the corresponding conformity levels.

		Conformity at sample level							Conformity at participant level			
Sample		**Ea 1**	**Ea 2**	**Ea 3**	**Ea 4**	**Ea 5**	**Ea 6**	**Ea 7**	**Number of conforming**	**Number of non-conforming**	**% Conforming**	**% Non-conforming**
Expected result		**neg**	**neg**	**neg**	**neg**	**pos**	**pos**	**pos**				
Concentration		**0**	**0**	**0**	**0**	**3xE8**	**3xE7**	**3xE8**				
ID of participant	**1**	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	14	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	16	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	21	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	22	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	23	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	30	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	32*	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	37	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	41	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	43	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
Number of participating		11	11	11	11	11	11	11				
Number conforming		11	11	11	11	11	11	11				
Number of non-conforming		0	0	0	0	0	0	0				
% Conforming		100%	100%	100%	100%	100%	100%	100%				
% Non-conforming		0%	0%	0%	0%	0%	0%	0%				

**late submission. Participant ID 32 reported results on December 17th 2018 (deadline for submission was December 14th 2018).*

Table 8: Results reported for molecular tests and the corresponding conformity levels.

	Conformity at sample level							Conformity at participant level			
Sample	**Ea 1**	**Ea 2**	**Ea 3**	**Ea 4**	**Ea 5**	**Ea 6**	**Ea 7**	**Number of conforming**	**Number of non-conforming**	**% Conforming**	**% Non-conforming**
Expected result	**neg**	**neg**	**neg**	**neg**	**pos**	**pos**	**pos**				
Concentration	**0**	**0**	**0**	**0**	**3xE8**	**3xE7**	**3xE8**				
ID of participant 1	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
2	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
4	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
5	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
9	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
11	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
13	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
14	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
15	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
16	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
21	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
22	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
23	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
24	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
26*	pos	neg	neg	pos	pos	pos	pos	5	2	71%	29%
28	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
29	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
30	pos	neg	pos	neg	pos	pos	pos	5	2	71%	29%
31	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
32*	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
33	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
35	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
36	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
37	neg	neg	neg	pos	pos	pos	pos	6	1	86%	14%
40	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
41	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
43	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
Number of participating	27	27	27	27	27	27	27				
Number conforming	25	27	26	25	27	27	27				
Number of non-conforming	2	0	1	2	0	0	0				
% Conforming	93%	100%	96%	93%	100%	100%	100%				
% Non-conforming	7%	0%	4%	7%	0%	0%	0%				

**late submission. Participant with ID 26 reported results on February 14th 2019 and participant with ID 32 reported results on December 17th 2018 (deadline for submission was December 14th 2018).*

Table 9: Overall results reported for detection of Erwinia amylovora in test items based on serological and/or molecular tests.

		Conformity at sample level							Conformity at participant level			
Sample		**Ea 1**	**Ea 2**	**Ea 3**	**Ea 4**	**Ea 5**	**Ea 6**	**Ea 7**	**Number of conforming**	**Number of non-conforming**	**% Conforming**	**% Non-conforming**
Expected result		**neg**	**neg**	**neg**	**neg**	**pos**	**pos**	**pos**				
Concentration		**0**	**0**	**0**	**0**	**3xE8**	**3xE7**	**3xE8**				
ID of participant	1	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	2	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	4	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	5	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	9	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	11	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	13	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	14	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	15	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	16	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	21	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	22	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	23	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	24	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	26*	pos	neg	neg	pos	pos	pos	pos	5	2	71%	29%
	28	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	29	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	30	pos	neg	pos	neg	pos	pos	pos	5	2	71%	29%
	31	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	32*	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	33	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	35	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	36	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	37	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	40	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	41	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
	43	neg	neg	neg	neg	pos	pos	pos	7	0	100%	0%
Number of participating		27	27	27	27	27	27	27				
Number conforming		25	27	26	26	27	27	27				
Number of non-conforming		2	0	1	1	0	0	0				
% Conforming		93%	100%	96%	96%	100%	100%	100%				
% Non-conforming		7%	0%	4%	4%	0%	0%	0%				

**late submission. Participant with ID 26 reported results on February 14th 2019 and participant with ID 32 reported results on December 17th 2018 (deadline for submission was December 14th 2018).*

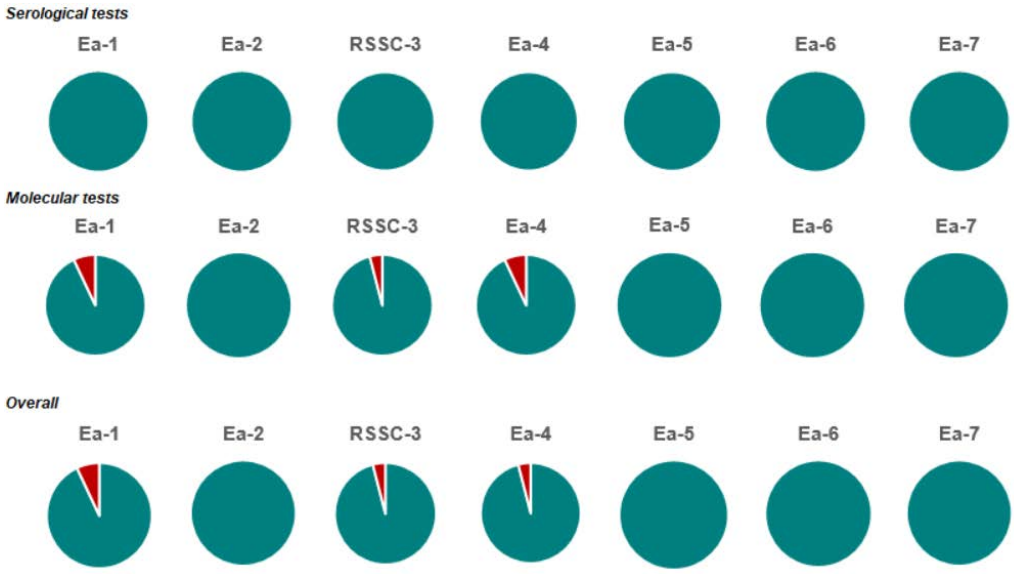

Figure 1: Ratio of conforming (green) and non-conforming (red) results at sample level separately for serological, molecular tests and overall result. *False positive results were observed for molecular tests.*

7 Tests used

A range of different tests were used by the participants. Among these the most commonly used were real-time PCR by Pirc *et al*. (2009), real-time PCR by Gottsberger (2010) and immunofluorescence test employing different antisera (Table 10). In the case of real-time PCR described by Pirc *et al*., (2009) the information is often missing on which test of the two described in the publication was used.

Participants used 1 (11, 41 %), 2 (8, 30 %), 3 (5, 19 %) or 4 tests (3, 11 %) to report the results. The number of tests is also related to the participation to serological and/or molecular part of the proficiency test.

The participants obtaining false positive results used PCR test (Stöger *et al*. (2006)) and real-time PCR (Pirc *et al*., 2009) however, it is unlikely that the issue is with the tests themselves as they were used by other participants obtaining conforming results i.e. by 2 and 14 other participants for the two tests, respectively. It may be worth noting that one of the participants reporting false positive results using real-time PCR (Pirc *et al*., 2009) mentioned the use of multiplex PCR. As the test was not described as multiplex it seems further modification must have been done and may have had adverse effect on its performance. One participant reported results of molecular tests as neg/pos/neg, depending on the sample preparation (with or without DNA extraction); this result was interpreted as positive in the results tables and is thus one of the false positive results.

A likely cause of false positive results is the high concentration of the target bacteria in samples which was chosen to reflect naturally contaminated symptomatic samples. This exacerbated the risk of cross-contamination of samples during handling in participating laboratories.

Table 10: Tests reported by the participants.

Test	No of participants employing the test	% of participants employing the test
Imunofluorescence (3x Loewe poly.antiser.(07369), 2x PlantPrint Diagnostics S.L., 1x Primer diagnostics antisera Eam (108) IgG, 1x Rapid Biotech (Todi Perugia, Italy) #320_2_18)	7	26%
LFD (Agristrip Bioreba)	2	7%
ELISA (Kit for Enrichement DAS-ELISA, Gorris *et al*, 1996)	1	4%
Pirc *et al*., 2009	15	56%
Gottsberger *et al*., 2010	9	33%
Llop *et al*., 2000	4	15%
Stöger *et al*. (2006)	3	11%
PCR Taylor *et al*., 2001	3	11%
Gottsberger modified by Obradovic	3	11%
Bereswill *et al*., 1992	2	7%
Obradović *et al*., 2007	1	4%
real-time PCR unspecified	1	4%
Salm & Geider, 2004	1	4%

8 Conclusions

Overall results of the proficiency tests indicate that concentrations of *Erwinia amylovora* as expected in symptomatic plant material are readily detected by a range of serological and molecular tests. Indeed and as expected, the issue is rather the risk of cross-contamination of samples because of high target concentrations.

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Annex I Participants

Altogether, 27 participants from 20 countries took part in the proficiency test (Figure 2). Participants are listed in alphabetical order and their shipping addresses below.

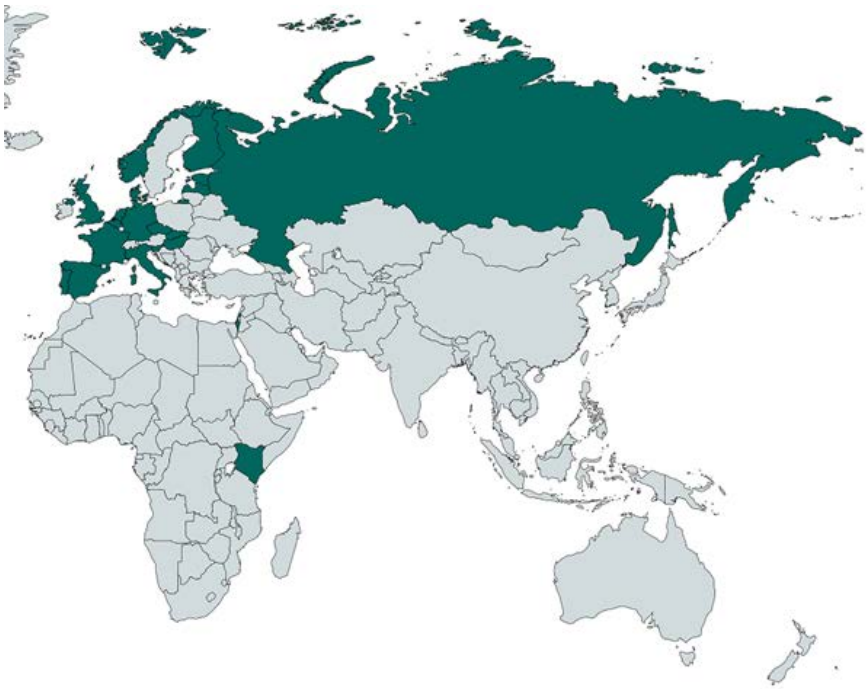

Figure 2: Countries where participating laboratories are situated are coloured in green. Map was created wmapchart.net.

List of participants

Agricultural Research Centre, Laboratory of Plant Health and Microbiology, Teaduse 4/6, 75501 Saku, Estonia

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Central Control and Testing Institute in Agriculture (UKSUP), Department of Diagnostics, Osada 281, 044 57 Haniska pri Kosiciach, Slovak Republic

"Central Control and Testing Institute in Agriculture, Department of Molecular Biology, Matúškova 21, 833 16 Bratislava, Slovak Republic"

Danish Veterinary and Food Administration, Søndervang 4, 4100 Ringsted, Denmark

DARP - Generalitat de Catalunya, Laboratory for Agriculture and Plant Health of Catalonia, IRTA 2 Building - UdL Agriculture Campus, Alcalde Rovira Roure Av., 191, 25198 Lleida, Spain

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