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Assessment of a new qPCR tool for the detection and identification of the root-knot nematode *Meloidogyne enterolobii* by an international test performance study

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Abstract Rapid and reliable tools for detection and identification of plant parasitic nematodes are needed to prevent the introduction and spread of quarantine nematodes. A fast and simple DNA extraction method for target nematodes in nematode suspensions obtained from soil samples and a new quantitative real-time PCR method (qPCR) for the specific detection, identification and potential quantification of *M. enterolobii* were tested in an inter-laboratory comparison (ring test) to allow for a thorough evaluation of these molecular diagnostic tools. A test performance study involving seven laboratories was conducted to validate the developed protocols and to identify possible difficulties when implemented by diagnostic laboratories or national reference centers. Validation included test performance in terms of accuracy, analytical specificity, analytical sensitivity, repeatability, and reproducibility as defined by European Plant Protection Organization (EPPO) standard PM7/98. All positive and negative results for detection, identification and specificity were consistent between different

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laboratories despite different equipment used. Accuracy of real-time PCR was 100 % because test results and accepted reference values were in agreement. Analytical sensitivity results also matched between laboratories independent of the equipment used. The smallest amount of target DNA tested, two second-stage juveniles of *M. enterolobii* in a background of 500 non-target nematodes, was reliably detected by all labs. In addition, the repeatability and reproducibility of test results between laboratories was 100 %, even at the limit of detection. Thus, the inter-laboratory comparison showed the robustness of the developed methods and confirmed the in-house validation data.

Keywords Real-time PCR · Performance assessment · *Meloidogyne enterolobii* · Sensitivity · Specificity

Introduction

Root-knot nematodes (*Meloidogyne* spp.) are obligate endoparasites on plant roots and affect growth, yield, lifespan and tolerance to environmental stresses of infested plants. Typical symptoms include stunted growth, wilting, leaf yellowing and deformation of plant organs. Crop damage due to root-knot nematodes mostly consists of reduced quantity and quality of yield. While there have been close to 100 species of root-knot nematodes described (Hunt and Handoo 2009), about ten species pose a significant risk to agricultural production systems worldwide. One of those species is *Meloidogyne enterolobii* (syn. *M. mayaguensis*, Karssen et al. 2012) with yield losses of up to 40–65 % (Cetintas et al. 2007; Kiewnick et al. 2008). The organism was first described from Hainan Island, China in 1983 and is a polyphagous species that attacks woody as well as herbaceous plants and thus has many host plants including weeds and cultivated plants (Anonymous 2011, 2014). It has been found on important agricultural crops as well as ornamentals significant for trade (Castagnone-Sereno 2012; Elling 2013; Onkendi and Moleleki 2013; Ye et al. 2013; Wang et al. 2014; Ramirez-Suarez et al. 2014). In addition, *Meloidogyne enterolobii* is considered to be a highly pathogenic and virulent root-knot nematode species able to reproduce on plants carrying resistance genes to other root-knot nematodes, which makes its control extremely difficult (Brito et al. 2007; Kiewnick et al. 2009, 2011). In Europe, *M. enterolobii* was first recorded in South Brittany, France (Blok et al. 2002), but was under eradication and is currently no longer present. However, several interceptions of *M. enterolobii* on imported ornamental plants by the Dutch and German authorities in 2008 and its occurrence on tomato plants in greenhouses in Switzerland (Kiewnick et al. 2008) lead to a pest risk analysis in 2009 (www.eppo.org/) with the result of adding this species in 2010 to the EPPO A2 list containing pests recommended for regulation as quarantine organisms that are locally present in the EPPO region and need special attention by its member countries. One of the main questions raised during the pest risk analysis was the possible source(s) and route(s) of introduction into Europe. The presence of *M. enterolobii* on tomato in greenhouses in Switzerland and two new interceptions of *M. enterolobii* on Bonsai plants received from China noted in France in 2011 and 2014 (Folcher and Le Rheu Cedex 2015, pers. communication) clearly demonstrated that there are pathways for introduction which have to be identified. To ensure that appropriate phytosanitary measures and management strategies can be implemented to protect European agriculture against *M. enterolobii's* increasing emergence and spread due to abundant international commodity exchange and trade, rapid and reliable detection and identification tools are needed.

As root-knot nematode species are difficult to identify based on morphology, and thus specialists are needed, reliable and fast DNA-based detection and identification tools such as conventional PCR (Tigano et al. 2010; Kiewnick et al. 2013; Gamel et al. 2014), quantitative real-time PCR (qPCR) or even DNA-barcoding (Holterman et al. 2012; Kiewnick et al. 2014) can provide accurate diagnosis independent of or in addition to expert knowledge. Real-time PCR assays have successfully been used for identification of other root-knot nematodes including the species *M. chitwoodi* and *M. fallax* (Zijlstra and Van Hoof 2006; Berry et al. 2008; De Weerdt et al. 2011; Agudelo et al. 2011). Recently, a qPCR protocol based on locked nucleic acid (LNA) probes and a fragment of the COI gene sequence of *M. enterolobii* (Kiewnick et al. 2014) was developed and validated (Kiewnick et al. 2015).

To implement new methods as standard assays in laboratories performing analyses for National Plant Protection Organizations (NPPOs), agricultural ministries, or other official agencies, these methods need to be reliable, cost-effective and easy to apply. Therefore, validation of these tests under a range of conditions is recommended and may be a requirement in the future.

As most European laboratories have little or no experience with the identification of *M. enterolobii*, training and participation in test performance studies of new methods are recommended to support the introduction and implementation of new techniques to diagnostic laboratories helping in NPPO's decision making.

In 2006, the EUPHRESCO Phytosanitary ERA-NET was established to coordinate national, transnational and EU-funded research in direct support of the Community Plant Health Regime to prevent the introduction, establishment and spread of regulated and quarantine pests. These pests pose increasing risks to European agriculture, horticulture, forestry and the environment due to increased globalization of trade, climate change and EU expansion (increased pathways). In the framework of the EUPHRESCO-Project (European Phytosanitary Research Coordination; www.euphresco.net/), resources for national plant health inspection services, science programmes and research are available and cooperation among European diagnostic laboratories regarding method testing is encouraged. Previous projects funded have focused on the epidemiology and risk assessment of new emerging plant pests (fruit flies, virus-vectors) or pathogens (viruses, bacteria, phytoplasmas, fungi) and/or on their fast diagnostics using new molecular diagnostic methods such as conventional PCRs, real-time PCR, LAMP (www.euphresco.org/projects/portfolio), or DNA-barcoding (Van de Vossenberg et al. 2013). Projects regarding nematodes included the detection and identification of quarantine species such as the potato cyst nematodes *Globodera rostochiensis* and *Globodera pallida*, the pine wood nematode *Bursaphelenchus xylophilus* and the root-knot nematodes *Meloidogyne chitwoodi* and *Meloidogyne fallax* (www.euphresco.org/projects/portfolio). The project presented here, validated a new molecular assay for the specific detection and identification of *Meloidogyne enterolobii*. The test performance study (TPS) aimed at testing the robustness of a rapid and simple DNA extraction protocol followed by molecular detection/quantification and identification. The qPCR protocol for *M. enterolobii* (Kiewnick et al. 2015) was therefore tested using different equipment, chemistry and personnel on the same samples provided by the TPS organizer. Out-house validation included accuracy, repeatability, and reproducibility testing in addition to verifying in-house validation in terms of analytical specificity and sensitivity.

Materials and methods

Organization of test performance study

Seven European laboratories working on the diagnostics of plant pests and pathogens for their countries participated in this study. During the start-up period laboratories were asked to provide a list of equipment available and methods used in their labs for detection and identification of plant pathogenic nematodes such as *Meloidogyne* species. Sample sets containing 18 blind samples per set (Table 1) were then prepared and provided to the laboratories, together with the protocols for DNA extraction and qPCR. Sample sets included nematode suspensions as well as template DNA. Samples containing nematode suspensions were either analysed directly by laboratories or kept frozen until analysis. Samples containing DNA were stored at −20 °C and analysed within 4–6 weeks after receiving samples. Results from the assays were provided within 8 weeks after the samples had been sent to participating labs.

Nematode isolates and suspensions

As a range of *Meloidogyne* species and other nematode genera from worldwide collections had been used for testing specificity during the development of the qPCR assay (Kiewnick et al. 2015), the number of non-target *Meloidogyne* species in this study was limited to the species *M. hapla*, *M. graminicola*, *M. chitwoodi* and *M. fallax*, provided as DNA samples. In addition, nematode DNA from two other genera, *Nacobbus* and *Globodera*, were chosen for testing cross reactions when used by participating labs (Table 1). Nematode suspensions used as background nematodes to detect the target nematode *M. enterolobii*, were obtained from soil samples collected from three different field sites in Switzerland using the centrifugation flotation technique. Soil A was collected from an organic farm, while soils B and C were obtained from conventional farms. Nematode suspensions from soil A had been previously used for the development and validation of the assay (Kiewnick et al. 2015). For this soil, the full biodiversity spectrum of the background nematode assemblages were determined by pyrosequencing in the context of the Swiss NEMA-BOL project (Kiewnick and Frey 2014). The soil nematode suspension contained nematode species from a range of clades including plant

Table 1 List of blind samples (sample codes) and tube contents (nematode suspensions or DNA, respectively) sent to participating laboratories

Sample codes	Tube content	Source	Analysis/qPCR performance
1	Nematode suspension[a]	Soil A only (control)	Detection
2	Nematode suspension+2 *M. e.*[b] J_2	Soil A + target nematodes (*M. e.*)	Detection
3	Nematode suspension+10 *M. e.*J_2	Soil A + target nematodes (*M. e.*)	Detection
4	Nematode suspension	Soil B only (control)	Detection
5	Nematode suspension	Soil C only (control)	Detection
6	Lysis buffer	Buffer only control (negative amplification control)	Identification
7	Lysis buffer+2*M. e.* J_2	Target nematodes (*M. e.*) (positive amplification control)	Identification
8	Lysis buffer+10*M. e.* J_2	Target nematodes (population Wauwil) (positive amplification control)	Identification
9	*M. e.* DNA diluted 100 x	Wauwil, CH	Linearity and specificity
10	*M. e.* DNA diluted 1000 x	Wauwil, CH	Linearity and specificity
11	*M. e.* DNA diluted 10000 x	Wauwil, CH	Linearity and specificity
12	*M. e.* DNA diluted 100000 x	Wauwil, CH	Linearity and specificity
13	*M. hapla* DNA	Population E-6345, NL	Specificity
14	*M. graminicola* DNA	University of Bonn, GER	Specificity
15	*Nacobus abberans* DNA	FR	Specificity
16	*Globodera rostochiensis* DNA	CH	Specificity
17	*M. chitwoodi* DNA	JKI Münster, GER	Specificity
18	*M. fallax* DNA	CH	Specificity

[a] Non-target background nematodes

[b] *M. e.* = *M. enterolobii* Population Wauwil, CH

parasitic nematodes belonging to genera such as *Rotylenchus, Helicotylenchus, Aphelenchoides, Ditylenchus, Aphelenchus* and the root-knot nematode *M. naasi*. Suspensions from soil sample B contained 50 % *Meloidogyne* J_2 (predominately *M. hapla*), 49 % other plant parasitic and saprophaguous nematode species as well as 1 % *Heterodera* spp. J_2. The suspensions from soil C contained 87 % bacterial and fungal feeders, predatory and onmivorus nematodes; 4 % plant parasitic nematode species and 9 % *Meloidogyne* sp. J_2 (also predominately *M. hapla*). Nematode suspensions were adjusted to 500 nematodes per 1.5 ml tap water, of which some were spiked with 2 or 10 handpicked *M. enterolobii* second-stage juveniles (J_2), respectively (Table 1). The latter juveniles were obtained from a *M. enterolobii* population (Wauwil, CH) reared on tomato plants in the greenhouse. In addition, three suspensions containing only target nematodes in water (no soil or non-target nematode background) and corresponding controls were provided and spiked as described above (Table 1).

Nematode lysis and DNA extraction

In order to successfully implement qPCR assays for detection and identification of *M. enterolobii*, the DNA extraction step (for lysis of nematode specimen and release of DNA) prior to real-time testing is critical and needs to result in sufficient amounts of target DNA. DNA extraction was done from part of the samples provided (Table 1, sample codes 1 to 8) and included a simple lysis buffer protocol, which did not require any other DNA purification steps following extraction (Holterman et al. 2006). Lysis buffer stock solution (×2 concentrated) was sent to each lab with the instruction to add 40 µl Proteinase K (20 mg/ml stock solution) and 10 µl beta-mercaptoethanol prior to DNA extraction.

Sample sets contained seven Eppendorf tubes with nematodes (see above) in approx. 1.5 ml water and one tube with lysis buffer only. In order to minimize sample handling and therefore potential losses of target nematodes, the DNA extraction was performed in the provided Eppendorf tube with no further purification step.

Eppendorf tubes were placed in a centrifuge and nematode suspensions were spun down at 10,000×*g* for 10 min (alternatively 8000×*g* for at least 20 min). After centrifugation, the volume was carefully reduced to 100 μl, then 100 μl of the×2 concentrated lysis buffer was added. After vortexing, the suspension was spun down for 5 s. Eppendorf tubes were then placed on a thermoshaker at 65 °C with a 1 min mixing interval at 300 rpm for 2 h. If no thermoshaker was available, samples were placed in a water bath at 65 °C, but regular vortexing was recommended for complete lysis. After 2 h at 65 °C, tubes were incubated at 99 °C for 5 min to inactivate the proteinase K. After lysis and DNA release, samples were stored at −20 °C until further use. Before using the template DNA for the real-time assay, DNA was diluted 1:4 by transferring 50 μl DNA template to a fresh tube with 150 μl of Milli-Q water.

qPCR assays

Testing new qPCR protocols under different laboratory constellations and by different laboratory personnel is critical for successful out-house validation. Detailed protocols for laboratories using Applied Biosystem or Roche Light Cycler Real-time platforms and appropriate chemistry were provided with the samples (see below). Half of the samples used for qPCR were provided as dried DNA (sample codes 9–18, Table 1), and DNA had to be extracted from the other half (sample codes 1–8, Table 1) by using the lysis buffer as described above. Before performing the qPCR, DNA of nematode suspensions extracted from soil (sample codes 1 to 5, Table 1) and DNA of target nematodes in lysis buffer (sample codes 6–8, Table 1) was diluted as mentioned above. 4 μl of this template DNA solution was then used for qPCR. Tubes labelled DNA (sample codes 9–18, Table 1) contained dry DNA in GenTegra™ DNA tubes (IntegenX Inc, Pleasanton, CA, USA). This DNA was re-suspended in 30 μl of Milli-Q or other molecular grade water before use and 4 μl were used per qPCR reaction. Primers were also sent dry together with the samples, but LNA probes, as well as appropriate master mixes, were ordered by each participating lab separately according to their equipment used. Primers were re-suspended in 140 μl sterile molecular grade water before use.

The qPCR assays producing a 66 bp fragment of the mitochondrial cytochrome oxidase I gene (COI) with the primers Ment 17F (forward)/ Ment 17R (reverse), and the LNAprobe # 17 (Roche Universal ProbeLibrary; cat.no. 04 686 900 001; Roche Applied Science, Mannheim, Germany) were performed according to the method developed previously by Kiewnick et al. (2015). Briefly, qPCR was done in reaction volumes of 20 μl containing 4 μl of template DNA, 900 nM of each primer, 200 nM of probe, 10 μl TaqMan® Environmental PCR Master Mix (Applied Biosystems, Switzerland) for the use on the Applied Biosystems 7500 FAST Real-Time PCR instrument (Applied Biosystems Europe BV) or 10 μl Roche LC480 Probes Master if using the Roche LC480 Light cycler instrument (Roche, Basel, Switzerland). On the ABI 7500 FAST instrument, the baseline was set automatically, and the fluorescence threshold manually at a predetermined value of 0.05 to intersect with the linear part of the amplification curves of all real-time PCR assays, resulting in the final Ct value for each well. Cycling conditions were 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 using the ABI 7500 FAST instrument. On the Roche Light Cycler platform (LC480) cycling conditions were 5 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 1 min at 60 °C, 3 s at 72 °C, and a final cooling step at 40 °C for 10 s. All PCR reactions were performed in duplicate.

Additional samples sets were provided upon request by participating labs to test PCR methods for detection and identification of target nematodes as required by their national regulations. However, these tests were optional and not part of this TPS.

Evaluation of the qPCR performance included testing for detection of target nematodes (sample codes 1–5, Table 1), identification (sample codes 6–8), linearity (sample codes 9–12) and specificity (sample codes 9–18) on target DNA (*M. enterolobii*) dilutions and non-target DNA (other *Meloidogyne* spp. or other nematode genera).

Data interpretation based on in-house validation and setting of qPCR cut-off Ct-values

Previously done in-house validation resulted in detecting one individual target nematode juvenile (J_2) in a background of 1000 other nematodes at a Ct-value of approx. 30. In addition, the limit of detection (LOD) cut-off values for the COI qPCR assay were defined at a Ct-value of 35.2 on Applied Biosystems (ABI) platforms and at a Ct-value of 36.0 on Roche Light Cycler

(LC480) platforms (Kiewnick et al. 2015). These values, known prior to the test performance study, were not provided to participating laboratories in order to keep it as a blind study.

Performance criteria (out-house validation)

The assessment of performance criteria was carried out according to the EPPO standard protocol for test validation PM7/98 (Anonymous 2010). This evaluation included studies for linearity, studies with non-target species to test for cross-reactions, studies at the limit of detection for finding analytical sensitivity, and comparing results of different labs to check for repeatability and reproducibility of test results and finally evaluate the robustness of the developed methods.

Linearity Linearity testing was related to target DNA dilutions (Table 1) and is critical for q-PCR performance. Pure template DNA of *M. enterolobii*, containing 0.1 μg DNA/μl was used to prepare DNA dilutions. Serially diluted DNA samples contained 1.0, 0.1, 0.01, and 0.001 ng DNA/μl. Based on this dilution series, standard curves of *M. enterolobii* were prepared by defining the slope (k) and the y-intercept of the regression line describing the linear relationship between DNA concentration and the corresponding Ct-values.

Analytical specificity (*SP)*, according to PM 7/98 (Anonymous 2010), is defined as performance of a test with regard to cross-reactions with non-target species. It was tested by all laboratories using samples coded 13–18 (Table 1). It is further defined as a test's capacity to obtain a negative result for those samples for which the accepted reference value is negative, and is calculated using the following formula: *SP*=NA / (NA + PD), where NA stands for negative agreement and is the number of all negative sample set results that are in agreement with the accepted reference negative value, while PD stands for positive deviation and is the number of test results that are positive, but were expected negative.

Analytical sensitivity (SE), according to PM 7/98 (Anonymous 2010), is defined as the smallest amount of target, that can be reliably detected (limit of detection). It is also defined as a test's capacity to obtain a positive result for those samples for which the accepted reference value is positive. It is calculated as: *SE*=PA / (PA + ND), where PA stands for positive agreement and ND for negative deviation and is the proportion of test results that are in positive agreement divided by the total number of test results (including negative samples set results whose accepted reference values were positive, i.e. negative results, that were expected positive).

Repeatability This term describes the level of agreement between replicates of a sample tested under the same conditions (percentage of agreement is given for a certain target level, e.g. 100 % for five J_2). Since no duplicate of biological samples were provided, repeatability of the test is based on duplicate qPCR reactions.

Reproducibility Defined as the ability of a test to provide consistent results, when applied to aliquots of the same sample tested under different conditions (time, personal, equipment, location, etc.), it is expressed as the percentage of agreement for a given target level, e.g. 100 % for five J_2.

Results

DNA extraction

DNA extraction with the×2 lysis buffer followed by qPCR was performed with samples (codes 1–8; Table 1) by all laboratories according to the protocols provided by the TPS organizer. Performance of DNA extraction was evaluated based on the results of the qPCR for these samples which were as expected for detection and identification. No differences between laboratories or qPCR platforms were noticed, i.e. all labs provided identical results, in that positive blind samples were tested positive and negative blind samples were tested negative. Thus, results were 100 % in agreement between labs (Table 2).

qPCR results and out-house validation

All qPCR results were 100 % in agreement between laboratories regarding detection, identification, and specificity (Tables 2 and 4) and similar to expected values defined per in-house validation experiments. No differences were found between labs or qPCR platforms used for plus/minus reactions: as expected, positive blind samples were tested positive and negative samples were tested negative (Table 2).

The qPCR assay was evaluated based on calculations of performance criteria defined in PM 7/98

Table 2 Results of the test performance study for all participating labs and their equipment used based on plus/minus reactions of all blind samples in a sample set

Sample code	Content	Analysis	Expected results		Lab 1	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
			ABI	Roche	ABI	Roche	ABI	BioRAD	Roche	BioRAD	Roche	ABI
1	Soil A only	Detection	–	–	–	–	–	–	–	–	–	–
2	Soil A+2 J_2	Detection	+	+	+	+	+	+	+	+	+	+
3	Soil A+10 J_2	Detection	+	+	+	+	+	+	+	+	+	+
4	Soil B	Detection	–	–	–	–	–	–	–	–	–	–
5	Soil C	Detection	–	–	–	–	–	–	–	–	–	–
6	Lysis buffer only	Identification	–	–	–	–	–	–	–	–	–	–
7	Lysis buffer+2 J_2	Identification	+	+	+	+	+	+	+	+	+	+
8	Lysis buffer+10 J_2	Identification	+	+	+	+	+	+	+	+	+	+
9	*M.e.*[a] target DNA 100x dil	Linearity	+	+	+	+	+	+	+	+	+	+
10	*M.e.* target DNA 1000x	Linearity	+	+	+	+	+	+	+	+	+	+
11	*M.e.* target DNA 10000x	Linearity	+	+	+	+	+	+	+	+	+	+
12	*M.e.* target DNA 100000x	Linearity	+/–	+/–	+	+	+	+	+	+	+/–	+
13	*M. hapla*	Specificity	–	–	–	–	–	–	–	–	–	–
14	*M. graminicola*	Specificity	–	–	–	–	–	–	–	–	–	–
15	*N. abberrans*	Specificity	–	–	–	–	–	–	–	–	–	–
16	*G. rostochiensis*	Specificity	–	–	–	–	–	–	–	–	–	–
17	*M. chitwoodi*	Specificity	–	–	–	–	–	–	–	–	–	–
18	*M. fallax*	Specificity	–	–	–	–	–	–	–	–	–	–
Ntc[b]			–	–	–	–	–	–	–	–	–	–

[a] *M. e.* = *Meloidogyne enterolobii* Population Wauwil, CH

[b] Ntc = no template control

+/– = positive/negative reaction

(Anonymous 2010) comparing the number of positive and negative agreements or deviations compared to the status of the sample. All positive and negative results for detection, identification and specificity were 100 % in agreement between different laboratories and equipment using the qPCR assay (Table 4).

The three laboratories using ABI platforms (labs 1, 2, and 7) provided consistent results for target nematode detection and identification samples, despite minor differences in true Ct-values (Fig. 1). Results were as expected, i.e. lower Ct-values with increasing amounts of target DNA (10 vs 2 *M. enterolobii* J_2). Two J_2 juveniles per reaction could be detected at an average Ct-value of 29.7. All values for positive blind samples were well below the pre-defined cut-off Ct-value of 35.2 and thus were tested positive by all labs. Similar results were obtained by the three laboratories using Roche platforms (labs 1, 4, and 6; Fig. 2) with minor differences in Ct-values. DNA from samples with two *M. enterolobii* J_2 yielded an average Ct-value of 30.3 and values for positive blind samples were well below the pre-defined cut-off Ct-value of 36. Laboratories 3 and 5 using Bio-Rad platforms provided similar Ct-values compared to labs using Roche platforms with Ct-values for positive blind samples well below the pre-defined Ct-value of 36 for Roche platforms. As an exception, lab 3 generated Ct-values and standard deviations close to the limit of detection for *M. enterolobii* identification (Fig. 2).

Linearity

The assessment of sample linearity included samples containing different target DNA concentrations (sample codes 9–12, Table 1) and the analysis of data across all eight sample sets. We found an excellent linear relationship between target DNA concentrations and Ct-values

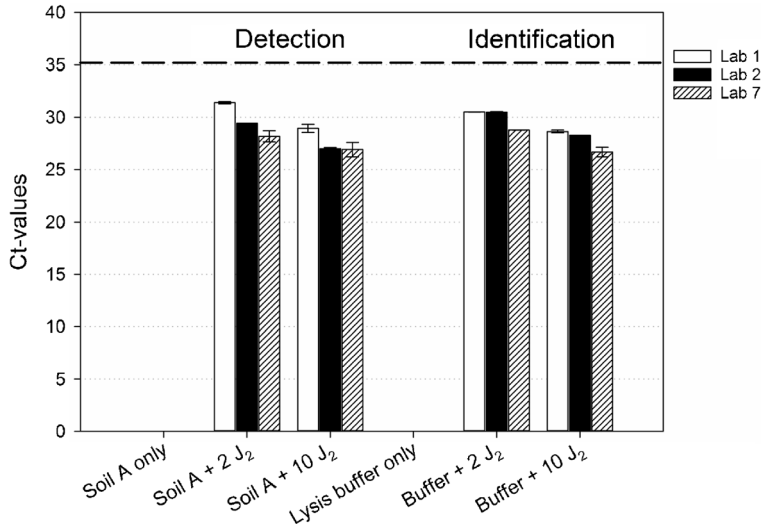

Fig. 1 qPCR results of three laboratories using ABI platforms in the test performance study on samples with suspensions containing 500 non-target nematodes or lysis buffer only spiked with and without 2 and 10 target *M. enterolobii* J_2 used for detection and identification. *Bars* represent mean Ct-values± standard deviation and the predefined cut-off Ct-value of 35.2 (*dashed line*)

(R^2=0.991–1.000) for seven sample sets, whereas only one participating lab provided data with a smaller goodness of fit (R^2=0.781, Table 3). Based on the predefined cut-off Ct-values, BioRAD machines were comparable to Roche Light Cycler machines in that they provided similar Ct-values in the range of 24 to 36, while ABI machines resulted in slightly lower Ct-values (22–33). In any case, DNA was detected in all samples containing target DNA, with the lowest DNA concentration being close to the cut-off Ct lines of 35 for ABI machines and 36 for the Roche Light Cycler and BioRAD machines, respectively.

Analytical sensitivity (= limit of detection, SE) The limit of detection reached by all labs with a Ct-value of 35/36 was therefore in the expected range. The smallest amount of target nematode that was aimed at being reliably detected was two *M. enterolobii* J_2 in 200 µl extraction buffer (Ct≤30) and this level was reached by all participating labs independent of the platform used. Thus, the calculated sensitivity according to PM 7/98 was 100 % for detection, specificity and identification (Table 4).

Analytical specificity (SP) All labs participating in the study detected and identified *M. enterolobii* correctly in blind samples, while none of the six non-target nematode species was tested positive. Thus, analytical specificity was also 100 % confirming the previous in-house validation (Table 4).

Accuracy Defined as sum of positive agreement samples divided by the number of samples for which the

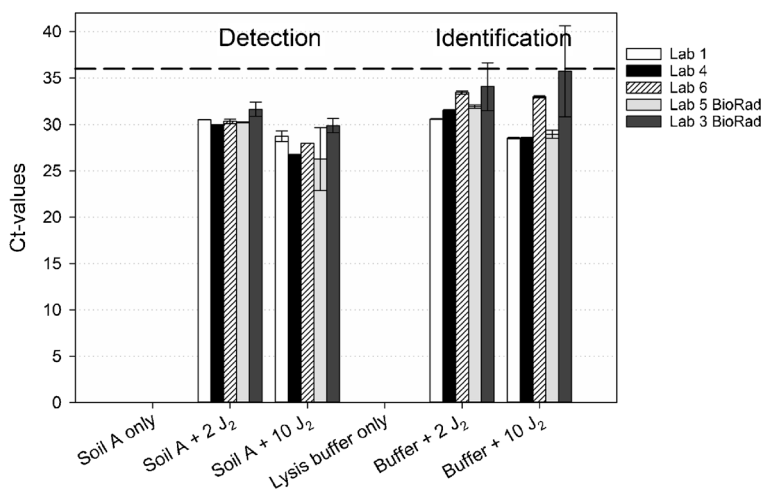

Fig. 2 qPCR results of three laboratories using Roche (lab 1, 4 and 6) and two laboratories using BioRad (labs 3 and 5) platforms in the test performance study on samples with suspensions containing 500 non-target nematodes or lysis buffer only spiked with and without 2 and 10 target *M. enterolobii* J_2 used for detection and identification. *Bars* represent mean Ct-values± standard deviation and the predefined cut-off Ct-value of 36.0 (*dashed line*)

Table 3 qPCR performance criteria based on linearity of test results related to *Meloidogyne enterolobii* target DNA concentrations

Laboratory	Slope (k)	Adjusted R^2	Platform used
1	3.44	0.991	ABI 7500 FAST
2	3.61	0.995	ABI 7900 HT
7	3.32	0.781	ABI 7900 HT
1	3.80	0.991	LC 480
4	3.94	1.000	LC 480
6	3.84	0.991	LC 480
3	3.73	0.998	BioRAD
5	3.66	0.992	BioRAD

k=slope of the linear regression line, R^2 =average square regression coefficient

accepted reference value is positive plus the sum of negative agreement samples divided by the number of samples for which the accepted reference value is negative, divided by 2, was also calculated to be 100 % for detection, specificity and identification between participating laboratories (Table 4).

Repeatability The overall level of agreement between replicates of a sample tested under the same conditions (repeated qPCR reactions) was 100 % for the detection of two *M. enterolobii* J_2 by all laboratories (Table 2). This was in fact the case for all samples including the ones with 10 J_2, or those with DNA of target and non-target nematodes.

Reproducibility The qPCR test provided consistent, i.e. matching results when applied to aliquots of the same sample tested under different conditions (times, persons, equipment, and location). Thus, the calculated agreement for the detection of two juveniles of *M. enterolobii* (given target level previously defined) was 100 % between labs and thus were reproducible.

Discussion

The purpose of the presented TPS was to evaluate the robustness and reliability of a simple lysis buffer protocol for extraction of target nematode DNA together with a newly developed qPCR assay for detection and identification of *M. enterolobii*. Lessons learned from previous test performance studies were to reduce variability among laboratory test results by reducing the number of methods to be tested and by providing most of the materials needed to perform the assays (Van de Vossenberg et al. 2013). We therefore tested only one DNA extraction method (lysis buffer) and one qPCR method in the presented test performance study. In addition, as an outcome of a previous test performance study on *M. chitwoodi* and *M. fallax* detection by real-time and conventional PCR methods (www.euphresco.org/projects/portfolio) it was suggested to provide the range of threshold cut off Ct-values as well as controls at the limit of detection (LOD). We did this by an in-house method validation so that data were

Table 4 qPCR performance criteria and their calculations according to PM 7/98 (Anonymous 2010)

	Performance criteria	Calculation	Detection	Specificity	Identification
M. enterolobii	Negative agreement (NA[a])	Number of negative samples×8 tests	24	56	8
	Positive agreement (PA[b])	Number of positive samples×8 tests	16	32	16
	Negative deviation (ND[c])	Number of negative deviations×8 tests	0	0	0
	Positive deviation (PD[d])	Number of positive deviations×8 tests	0	0	0
	Sensitivity (SE in %)	PA/N+[e]	100	100	100
	Specificity (SP in %)	NA/N-[f]	100	100	100
	Accuracy (%)	[(PA/N+) + (NA/N-)]/2	100	100	100

[a] Negative sample set results for which the accepted reference value was negative (true negatives)

[b] Positive sample set results for which the accepted reference values was positive (true positives)

[c] Sample set results with negative results for which the accepted reference value was positive (false negatives)

[d] Sample set results with positive results for which the accepted reference value was negative (false positives)

[e] N+ = Number of all samples for which the accepted reference value is positive (PA + ND)

[f] N- = Number of all samples for which the accepted reference value is negative (NA + PD)

available prior to sending out samples for the test performance study. These data were not provided to participating partners in order to keep it as a blind study. Another suggestion raised in the previous study that we implemented was to improve reaction conditions of assays by providing most of the solutions needed (buffers, primers), so that efficiency and sensitivity of qPCR performance would be higher. The number of data generated were not too many, though sufficient, and evaluation demonstrated little variability between laboratories using different equipment. No difficulties were encountered or reported by any of the seven participants during or after the test performance study. Furthermore, all participants provided their expertise and knowledge prior to the test performance study which helped to organize the TPS and adjust protocols accordingly.

The lysis buffer protocol for target nematode DNA extraction had been proven effective before (Kiewnick et al. 2015; Rybarczyk-Mydłowska et al. 2012; Vervoort et al. 2012) and worked well for all participating labs. However, sample stability is an important aspect prior to qPCR and should be mentioned here. Samples need to be sufficiently stable to ensure that they will not undergo any significant change throughout the conduct of the inter-laboratory comparison, including storage and transport conditions. Therefore, sample stability was assessed based on the samples containing nematode suspensions (Table 1, sample codes 1–5). We found an overall excellent sample stability over a period of 8 weeks (investigation period) with only a few variations noticed. Immediate DNA extraction of nematode suspensions and DNA extraction after short storage (< 5 days) at 4 °C yielded the same DNA quality for qPCR (data not shown). Longer storage of nematode suspensions at −18 to −20 °C prior to DNA extraction did also provide the same quality of DNA. However, storage of nematode suspensions at 4–6 °C for longer periods before DNA extraction yielded lower amounts of target DNA, i.e. Ct-values were more than two units higher than the cut-off values (data not shown).

The qPCR method performed as expected by in-house validation and results of all labs were identical for detection and identification. The assay was very specific as no cross-reactions were observed with other non-target nematodes including close relatives. Partners could distinguish *M. enterolobii* from other root-knot nematode species (*M. graminicola, M. chitwoodi, M. hapla, M. fallax*) confirming results from previous specificity tests with a greater number of species and populations (Kiewnick et al. 2015). The assay was highly sensitive as all labs were able to detect two juveniles in a suspension of 500 other nematodes obtained from field soil at an average Ct-value of 30.2. The assay was highly repeatable with a high level of agreement between replications of samples tested under the same conditions. The assay provided consistent results, even when tested under different conditions (time, equipment, location, persons), and was therefore highly reproducible between different laboratories. No differences were observed between qPCR platforms used related to results and data interpretation, even though true Ct-values may differ. In addition, the assay was simple to use and can be performed by any plant pathology laboratory equipped with real-time PCR thermocyclers.

In general, data on method performance tests are rare in agricultural disciplines, as opposed to human or animal health sciences (Den Nijs and Van Den Berg 2013). Proficiency tests are more frequently organized than TPS, because proficiency tests evaluate and control the quality of the laboratories' own methods, which is important for labs that obtain or maintain accreditation for official analyses (Antoine et al. 2008; Van Den Berg et al. 2013). However, testing a same method, especially newly developed ones, in inter-laboratory comparisons is important and warranted when discussing harmonizing methods on the EU level or including new methods in EPPO standard protocols for diagnostics of plant pathogens.

In conclusion, the inter-laboratory comparison confirmed the in-house validation data of using the simple DNA extraction protocol followed by the newly developed qPCR tool for detection and identification of *M. enterolobii* and thus will be included in the next revision of the EPPO standard protocol for diagnostics of *M. enterolobii*.

The assay may speed up routine diagnostics in the future, especially when needed for analysis of many samples. In addition to identification, this sensitive assay helps in reliable detection. This will result in faster implementation of phytosanitary measures (e.g. eradication or integrated control strategies) for this problematic organism and thus helps in NPPOs' decision making. Surveys or monitoring studies for the distribution, spread, and survival of *M. enterolobii* or its introduction into EU countries is now also possible without going through taxonomical identification by nematode

morphology. Import controls at ports of entry can be done rapidly and may stop introduction of this potential quarantine species into non-contaminated areas which in turn might improve trade since consignments do not need to be stored for too long.

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