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Molecular Typing of Cyst-Forming Nematodes *Globodera pallida* and *G. rostochiensis*, Using Real-Time PCR and Evaluation of Five Methods for Template Preparation

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Abstract

Globodera pallida and *G. rostochiensis* are two cyst-forming nematodes known to infest potato crops, causing severe economic losses worldwide. In this study, a real-time TaqMan PCR assay was developed and optimized for the simultaneous detection of *G. pallida* and *G. rostochiensis*. The assay's analytical and diagnostic sensitivity and specificity were evaluated using reference isolates. Four different DNA extraction methods and one rapid crude template-preparation procedure were compared in terms of extraction purity, efficiency for PCR applications, utility and cost. Extraction methods A and B included two commercially available kits that utilize silica columns and magnetic beads, respectively. Method C was based on DNA isolation using Chelex resin, and method D was a standard chemistry in-house protocol. Procedure E included the direct use of crude mixture composed of disrupted cysts in Tris–EDTA buffer. The multiplex TaqMan PCR assay successfully discriminated the two nematode species from all reference cyst samples and its recorded diagnostic sensitivity (Dse) and specificity (Dsp) was 100%. On the contrary, in conventional (Co) PCR tests, the overall Dsp and Dse were lower and estimated at 94 and 87% for *G. pallida*, and 97 and 88% for *G. rostochiensis*, respectively. Spectrophotometric results showed that DNA extraction methods A, B and C yielded the purest DNA and gave the lowest mean C_t values as well as the most consistent results in Co PCR. Alternative crude preparation method E resulted in statistically similar and C_t values consistent with those obtained with methods A to C when tested by TaqMan PCR. The developed assay, using crude template-preparation E, allows the simple, accurate and cost-effective testing of a large number of cyst samples and can be applied in surveys and certification schemes.

Introduction

In potato (*Solanum tuberosum*) crops, two species of cyst-forming plant-parasitic nematodes are currently responsible for severe economic losses: *Globodera pallida* (Stone) Behrens and *G. rostochiensis* (Wollenweber) Behrens, commonly refer to as potato cyst nematodes (PCN) (Bendezu et al. 1998). Because of their large damage potential, which often leads to potato plants' death, these nematodes are considered of great phytosanitary importance, because once they infest an agricultural field, it is practically impossible to eradicate, as their eggs can survive inside cysts in the soil for more than 20 years (Philis 1980; Brodie et al. 1993; Fleming and Powers 1998). Both organisms are listed as quarantine pests in Europe according to the EU Council Directive 2007/33/EC, and each member state is enforced to perform annual surveys to identify the PCN incidence and distribution (Anonymous 2007). In Cyprus, both PCN species have been

recorded and associated with major yield losses in potato crops. During the past 10 years, a national PCN eradication programme was initiated to identify the presence of *Globodera* species, to map the infested potato fields and to develop an integrated pest management system.

Precise traditional classification of these nematodes is based on juvenile or on cyst morphology and other phenotypic characters, involving laborious work from specialized taxonomists (Stone 1985; Trudgill 1985). Several molecular-based methods have been developed and used for the detection and differentiation of *G. rostochiensis* and *G. pallida*. These techniques include polymerase chain reaction (PCR) using species specific primers and/or probes (Bullman and Marshall 1997; Fullaondo et al. 1999; Madani et al. 2005, 2008; Nowaczyk et al. 2008; Nakhla et al. 2010), restriction fragment length polymorphism (RFLP), analysis of PCR-amplified genes (Thiery et al. 1997; Fleming et al. 2000; Skantar et al. 2007; S irca et al. 2010), sequencing and phylogenetic analysis (Griffiths et al. 2006; Madani et al. 2010).

In recent years, advanced molecular diagnostic tools, such as real-time PCR, have been used widely for the rapid detection of nucleic acid targets. The 5′-nuclease fluorogenic assay, also known as TaqMan real-time PCR, is a powerful and rapid technique providing increased sensitivity and specificity and thus alleviating the need for post-PCR manipulations, such as gel electrophoresis (Holland et al. 1991). The objective of this study was to develop and evaluate a real-time PCR assay for the simultaneous detection of *G. rostochiensis* and *G. pallida* isolates, which involves a simple template-preparation method from cyst samples, suitable for large-scale applications. For this purpose, four different DNA extraction methods and one crude template-preparation procedure were compared in terms of quantity, purity and PCR suitability, as well as utility and cost.

Materials and Methods

Nematode isolates and reagents

A total of seventeen PCN populations of *G. pallida* and *G. rostochiensis* were provided by the Department of Agriculture and the Cyprus University of Technology national collection, the Agricultural Institute of Slovenia, the Benaki Phytopathological Institute of Greece and the Plant Breeding and Acclimatization Institute (Poland). A genetically similar nematode species (*G. tabacum*) was also included. Enzymes and reagents used for molecular assays were obtained as follows: Platinum® *Taq* DNA Polymerase, dNTP set, Charge-Switch® Micro Tissue Kit and Pure Link™ Gel Extraction system (Invitrogen Life Technologies, Carlsbad, CA, USA), DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Primers and dual-labelled probes were synthesized by Microsynth AG (Balgach, Switzerland).

PCN species discrimination using PCR amplification and DNA sequencing

Discrimination of collected cysts at a species level was performed using a previously described multiplex Co PCR protocol (Fullaondo et al. 1999). To obtain sequencing data from the Cypriot and other PCN species used in this study, primers GLO-F and GLO-R were designed to amplify a 385 bp part of the internal transcribed spacer (ITS) region (Table 1). PCR assays were performed in a final volume of 25 μl containing 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 of each deoxyribonucleoside triphosphates (dNTPs), 1 unit of Platinum® *Taq* PCR DNA Polymerase, a final concentration of 0.5 μM for each primer (GLO-F/GLO-R) and 1 μl of template DNA, which was extracted using the DNeasy Blood and Tissue Kit. The amplification was carried out in a PTC 200 Thermal Cycler (MJ Research, Waltham, MA, USA), and the cycling profile was as follows: 94°C for 4 min followed by 35 cycles segmented in 94°C for 30 s, 55°C for 30 s and 72°C for 45 s, with a final extension step of 72°C for 10 min. PCR products were then separated by electrophoresis on a 2% agarose gel and visualized by staining with ethidium bromide. The amplified PCR fragments from 15 samples were purified using PureLink™ Gel extraction system, and both strands were sequenced using the ABI PRISM BigDye Terminators v3.0 Cycling Sequencing Kit in an ABI automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing data were analysed and deposited in the NCBI GenBank database as the accession numbers of HE795025–HE795041.

Primers and probes design

A multiple alignment of *Globodera* sp. sequences available from the GenBank database, including the sequenced isolates used in this study, was made using the ClustalX program (Thompson et al. 1997) (*G. pallida* Database ID: AJ606687, EF153835, HM159429, GQ294523, DQ847109, FJ212165, EU006704, HQ670270; *G. rostochiensis* Database ID: HM584981, DQ847119, JF907552, GQ294512,

Table 1 Primers and probes used for Co and real-time PCR

Primer name	Primer sequence (5′–3′)	TM	Position	Product size (bp)	Reference
GLOBOFOR	CACATGCCTCCGTTTGTTGT	60	1811–1830[a]	81	This study
ROSTOTAQ	{FAM}- CATATGCCCACTGTGTATGGGCTGGC -{BHQ1}	70	1838–1863[a]		
ROSTOREV	GGCGCTGTCCGTACATTGTT	60	1892–1873[a]		
GLOBOFOR	CACATGCCTCCGTTTGTTGT	60	1814–1833[b]	80	
PALLITAQ	{Cy5}-CACATGCCCGCTATGTTTGGGCTG-{BHQ2}	69	1841–1864[b]		
PALLIREV	GCGCTGTCCATACATTGTTGA	60	1894–1874[b]		
GLO-F	AACCTGCTGCTGGATCATTACC	62	1735–1756[b]	385	
GLO-R	AGCGCAGACATGCCGC	58	2135–2120[b]		
G.rost1	GCAAGCCCAGCGTCAGCAAC	60		315	(Fullaondo et al. 1999)
G.rost2	GAACATCAACCTCCTATCGG	58			
G.pall1	TGTCCATTCCTCTCCACCAG	60		798	
G.pall2	CCGCTTCCCCATTGCTTTCG	61			

[a]GenBank accession no. EU855120.
[b]GenBank accession no. EU855119.

FJ212163, GU084810). Variable regions were identified among the two species, and species-specific primers and probes were manually designed for a TaqMan assay to amplify an 80- and 81-bp fragment for *G. pallida* and *G. rostochiensis*, respectively. The probes were designed to have the Tm values approximately 10°C higher (70°C) than that of the primers (60°C), with their length not exceeding 30 nt and their percentage of GC content higher than 40%. The two probes' (ROSTOTAQ and PALLITAQ) 5 ends were labelled with 6-carboxy fluorescein (FAM) and cyanine 5 (Cy5), whereas their 3 ends with black hole quencher BHQ1 and BHQ2, respectively. The sequences of the primers and probes used are given in Table 1.

Real-time (TaqMan) PCR assay set-up

To determine the optimum primer concentration for the TaqMan PCR assay, a range of primer concentration titration was carried out using 5 × 5 matrix of 600, 500, 300, 100 and 50 nM. The reaction was performed in a final volume of 25 μl containing 12.5 μl of a 2 × Mastermix (100 mM KCl, 40 mM Tris–HCI, pH 8.4, 0.4 μM of each dNTP, 5.5 mM $MgCl_2$ and 1 unit of Platinum *Taq* DNA polymerase), 0.3 μM of each primer, 0.1 μM of each TaqMan probe and 1 μl template DNA. Cycling parameters were the following: 4 min at 94°C followed by 40 cycles of 10 s at 94°C and 30 s at 60°C. The assays were developed and evaluated on the Bio-Rad CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The amount of primer that gave the highest recorded fluorescence and the lowest threshold cycle (C_t; the cycle at which a significant fluorescence occurs) was selected. The capability of multiplexing the two assays was investigated further, and C_ts for a ten-fold dilution series of 1 ng/μl *G. pallida* and *G. rostochiensis* plasmid DNA, amplified using GLO-F/GLO-R primers, respectively, were plotted to yield standard curves and determine the linearity and efficiency of the developed TaqMan PCR assay.

Analytical sensitivity and specificity comparison between Co and real-time PCR assays

The relative analytical sensitivity (Ase) of the TaqMan PCR assay was estimated for single cysts and compared with that of the Co PCR. For that purpose, serial ten-fold dilutions of DNA or crude samples from each preparation method were tested using Co and real-time PCR. The obtained C_t values were compared with the presence and intensity of the gel electrophoresis band after Co PCR amplification. The analytical specificity (Asp) of the assay was also determined for all nematode isolates collected from Cyprus and the other EU countries and compared with the Co PCR reaction.

Diagnostic performance of the real-time and the Co-PCR assay

Diagnostic sensitivity (Dse), diagnostic specificity (Dsp), and accuracy (Acc) of the developed real-time and the Co PCR (Fullaondo et al. 1999) were calculated using DNA extracts from 199 *G. pallida* and 180 *G. rostochiensis* reference cyst samples, using the following formulas (Martin 1984; Jennings et al. 2009; Koskinen et al. 2009; Mattocks et al. 2010).

$$\text{Dse}\% = [\text{number of true positive samples}/(\text{number of true positive samples} + \text{number of false-negative samples})] \times 100.$$

$$\text{Dsp}\% = [\text{number of true negative samples}/(\text{number of true negative samples} + \text{number of false positive samples})] \times 100.$$

$$\text{Acc}\% = [(\text{number of true negative samples} + \text{number of true positive samples})/(\text{number of true negative samples} + \text{number of true positive samples} + \text{number of false-negative samples} + \text{number of false positive samples})] \times 100.$$

Evaluation of five template-preparation methods

Four DNA extraction and one crude template-preparation methods subsequently referred as methods A to E were evaluated using single cyst samples.

Methods A and B were direct applications of two commercially available kits according to the manufacturer's instructions: Qiagen's DNeasy Blood and Tissue kit, which was used as a silica column to bind DNA, and Invitrogen's ChargeSwitch Micro Tissue kit, which utilizes a novel switchable magnetic-based surface technology, depending on the pH of the surrounding buffer, respectively. Method C included a process that involved boiling a cyst sample at 95°C for 5 min in a 10% suspension of deionized water and Chelex® 100 resin (C-7901, Sigma, St. Louis, MO, USA). The suspension was then centrifuged, separating the resin and cellular debris from the supernatant containing the denatured DNA, which was eventually used as template. In method D, DNA was extracted using a modified previously reported protocol that was originally developed and applied for fungal and insect DNA extraction (Cenis 1992; Cenis et al. 1993). In brief, a single cyst was crushed in a 2-ml microfuge tube filled with 50 μl of extraction buffer (200 mM Tris–HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). 50 μl of 3 M sodium acetate, pH 5.2, were added and tubes were placed at −20°C for 30 min. Samples were then centrifuged for 10 min at 5000 × ***g***, and 80 μl of the supernatant was transferred into a clean tube. An equal volume of chloroform/isoamyl alcohol (24 : 1) was added, and samples were centrifuged for 10 min at 10000 × ***g***. Sixty microlitres of the supernatant were transferred in a new tube containing an equal volume of cold isopropanol and incubated at room temperature for 25 min. The DNA was precipitated by centrifugation at 12000 × ***g*** for 20 min, washed with 70% ethanol and air-dried under sterile conditions. DNA pellet was resuspended in 15 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8). Finally, method E involved disrupting the cyst in 80 μl of TE buffer, followed by a centrifugation at 5000 × ***g***. One microlitre from each extract was used as template in the PCR tests.

Cyst disruption was performed using a TissueLyser (Qiagen, Hilden, Germany) programmed to perform 1800 oscillations/min for a total of 6 min. Twenty-five individual cysts from each PCN species were tested in every method, in triplicates.

Comparison of nucleic acid recovery, technical difficulty and cost of template-preparation methods

The quality of DNA extracted by the different methods was estimated using a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Absorbance ratio at 260 and 280 nm was used to assess possible contamination of protein residuals, whereas absorbance ratio at 260 and 230 nm was calculated to indicate contamination of other organic compounds, such as thiocyanates, glycogens and carbohydrates. Relative technical difficulty, cost per sample and time needed were recorded for every method.

Statistical analysis

All data, including the absorbance measurements (260/280 nm and 260/230 nm) and C_t values from the different DNA extraction and alternative preparation methods, were analysed using Least Square Differences (LSD) at the significance level of 0.05. All analyses were performed using the R statistical package (R Development Core Team 2011, www.R-project.org).

Results

Optimization of the TaqMan PCR assay

The TaqMan primers and probes were initially subjected to a primer optimization matrix on extracted DNA from reference samples of *G. pallida* and *G. rostochiensis* species. The concentration of primers and probes that yielded the highest reported fluorescence and the lowest threshold cycle was 300 nM for

primers and 100 nM for fluorogenic probes on both tests (data not shown). Results showed that the two assays were specific and did not react with each other. The C_t values obtained by singleplex and multiplex assays were not different, and both formats gave the same results in terms of detection and differentiation of the two species. Standard curves based on threshold cycles for 10-fold dilution series of DNA were constructed, illustrating the sensitivity and linearity of the technique. The Cts were proportional to the logarithm of the starting template quantity and the TaqMan PCR slopes for the *G. rostochiensis* and *G. pallida* assays, estimated by the formula $E = [10^{-1/\text{slope}}] - 1$, were 99.8%(slope : −3.318) and 97.5% (slope: −3.358), respectively.

Analytical specificity and sensitivity comparison between real-time and Co PCR assays

The analytical specificity of the assays (TaqMan and Co PCR) was evaluated by testing reference *Globodera* spp. from different geographical locations. Results from multiplex TaqMan PCR showed that the developed assay was specific for each organism and no cross-reactivity was observed. Primer pair GLOBOFOR/ROSTREV and probe ROSTOTAQ amplified and produced increased fluorescence only for the *G. rostochiensis* samples, and similarly, primers GLOBOFOR/PALLIREV and probe PALLITAQ reacted only with *G. pallida* isolates. Both target nematodes were detectable in all cyst samples from Cyprus, Greece, Poland, Slovenia and UK, and no positive result was obtained for *G. tabacum* cysts or for the negative controls used (Table 2).

The analytical sensitivity of the real-time and the conventional PCR assay was tested in a direct comparison, using DNA and crude template-preparation extracts, diluted serially. Results showed that the TaqMan PCR end-point detection efficiency for DNA extraction methods A and B was approximately 1000 times higher than that of the conventional PCR (Table 3). At the same time, DNA extraction methods C and D, as well as crude template-preparation E, presented higher analytical sensitivity at least 100 times than Co PCR. Finally, in method E, the recorded gel electrophoresis bands from the PCR amplicon were visible only in undiluted samples, whereas the TaqMan PCR was able to identify the target at a dilution of 10^{-5} (Table 3).

Table 2 Geographical origin of tested nematode isolates and results from conventional (Co) and real-time (TaqMan) PCR

Accession number	Acronym	Nematode species	Origin	Co PCR results[a]		TaqMan PCR results[b]	
				Globodera pallida (798 bp)	*G. rostochiensis* (315 bp)	*G. pallida*	*G. rostochiensis*
HE795025	CY-GPX	*G. pallida*	Cyprus	+	–	20.2	–
HE795026	CY-GPO	*G. pallida*	Cyprus	+	–	21.5	–
HE795027	CY-GPL	*G. pallida*	Cyprus	+	–	19.8	–
HE795028	CY-GPA	*G. pallida*	Cyprus	+	–	19.1	–
HE795029	CY-GPK	*G. pallida*	Cyprus	+	–	18.5	–
HE795034	CY-GRX	*G. rostochiensis*	Cyprus	–	+	–	20.9
HE795035	CY-GRO	*G. rostochiensis*	Cyprus	–	+	–	20.3
HE795036	CY-GRL	*G. rostochiensis*	Cyprus	–	+	–	21.6
HE795037	CY-GRK	*G. rostochiensis*	Cyprus	–	+	–	19.9
HE795030	UK-GP	*G. pallida*	UK	+	–	18.8	–
HE795038	UK-GR	*G. rostochiensis*	UK	–	+	–	19.2
HE795031	PO-GP	*G. pallida*	Polland	+	–	22.6	–
HE795039	PO-GR	*G. rostochiensis*	Polland	–	+	–	22.3
HE795032	S-GP	*G. pallida*	Europe	+	–	19.3	–
HE795040	SL-GR	*G. rostochiensis*	Slovenia	–	+	–	19.7
HE795033	GRE-GP	*G. pallida*	Greece	+	–	22.7	–
HE795041	GRE-GR	*G. rostochiensis*	Greece	–	+	–	23.1
———	SL-GT	*G. tabacum*	Slovenia	–	–	–	–

[a]Reactions were performed according to Fullaondo et al. 1999. Single amplification bands of 798 bp indicate detection of *G. pallida* and single amplification bands of 315 bp indicate detection of *G. rostochiensis*. All tests were performed in duplicate.

[b]Detection of nematode species was determined using the multiplex TaqMan PCR developed in this study. DNA was obtained from single cysts using Qiagen's Blood and Tissue Kit (Extraction method A). The mean threshold cycle (C_t) is presented for each species tested, after running samples in triplicates.

Table 3 Sensitivity comparisons of Co and TaqMan (q) PCR assays for the detection of *Globodera pallida* and *G. rostochiensis* cysts

Dilution series (of single cyst extracts)[a]	Template-preparation methods[b]									
	Method A		Method B		Method C		Method D		Method E	
	Co PCR[c]	qPCR[d]	Co PCR	qPCR	Co PCR	qPCR	Co PCR	qPCR	Co PCR	qPCR
[*G. pallida*]										
10^0	+++	18.7	+++	19.9	+++	20.5	++	24.9	+	21.2
10^{-1}	++	22.3	++	23.1	++	23.7	+/−	28.3	−	24.6
10^{-2}	+	25.5	+	26.4	+/−	27.1	−	31.9	−	27.7
10^{-3}	−	28.9	−	29.9	−	30.4	−	35.5	−	30
10^{-4}	−	32.1	−	33.1	−	33.7	−	38.8	−	33.4
10^{-5}	−	35.2	−	36.3	−	37.4	−	−	−	37.1
[*G. rostochiensis*]										
10^0	+++	19.4	+++	20.3	+++	20.3	++	25	+	21.9
10^{-1}	++	22.7	++	23.4	++	23.7	+/−	28.4	−	25.1
10^{-2}	+	26	+	26.7	+/−	27	−	32.1	−	28.4
10^{-3}	−	29.3	−	29.9	−	30.2	−	35.2	−	31.7
10^{-4}	−	32.5	−	33.2	−	33.5	−	38.6	−	35.0
10^{-5}	−	35.3	−	36.5	−	37.2	−	−	−	38.4

[a]Each cyst extract was subsequently diluted in PCR-grade water.

[b]Method A: DNeasy Kit; Method B: ChargeSwitch Kit; Method C: Chelex resin; Method D: in-house protocol (Cenis et al. 1993); Method E: Crude template preparation (Tris–EDTA buffer).

[c]Each dilution sample was tested in triplicate. Amplification band intensity is presented for each sample (+++ indicates a highly intense amplification band, ++ indicates an intense amplification band, + indicates a visible amplification band, +/− indicates a faint but positive amplification band and − shows no visible band).

[d]The mean C_t is presented after testing five samples in triplicate for each dilution.

Assays diagnostic performance

The developed real-time PCR assay provided correct identification results for all single cyst nematode isolates. No false positive or negative reactions occurred and therefore Dsp and Dse were 100% for all *G. pallida* and *G. rostochiensis* reference isolates. On the contrary, based on the results obtained by Co PCR and the presence of indicative visible bands in gel electrophoresis (both faint and intense), the mean Dsp was estimated at approximately 94–97%, while the mean Dse at 86–87% for *G. pallida* and *G. rostochiensis*, respectively. Therefore, the developed TaqMan PCR assay presented a 100% diagnostic accuracy, whereas the Co PCR accuracy was calculated at 90–93% (Table 4).

Table 4 Comparison of diagnostic performance of real-time and Co PCR, using reference nematode cyst samples

		Globodera pallida	*G. rostochiensis*
Real-time PCR	True Positives	199 (199)[a]	160 (160)
	False Positives	0 (199)	0 (160)
	True Negatives	183 (183)	183 (222)
	False Negatives	0 (183)	0 (222)
	Dse	100%	100%
	Dsp	100%	100%
	Accuracy	100%	100%
Co PCR	True Positives	165 (199)	145 (160)
	False Positives	11 (199)	7 (160)
	True Negatives	178 (183)	215 (222)
	False Negatives	25 (183)	19 (222)
	Dse	87%	88%
	Dsp	94%	97%
	Accuracy	90%	93%

[a]Total number of tested samples is shown in parenthesis.

Evaluation of template purity extracted using different preparation methods

The means of nanospectrophotometer measurement ratios for each extraction method and comparisons between the different procedures derived from statistical analysis are shown in Fig. 1. The absorbance ratio results of 260/280 nm showed that methods C (Chelex isolation method), A (DNeasy Blood and Tissue Kit) and B (ChargeSwitch Micro Tissue Kit) extracted the purest DNA, without any significant differences ($P > 0.05$). Similar results were recorded for the crude template-preparation method E ($P > 0.05$). On the contrary, the in-house DNA isolation method D presented the lowest mean value ($P < 0.05$). According to results for 260/230 nm absorbance ratio,

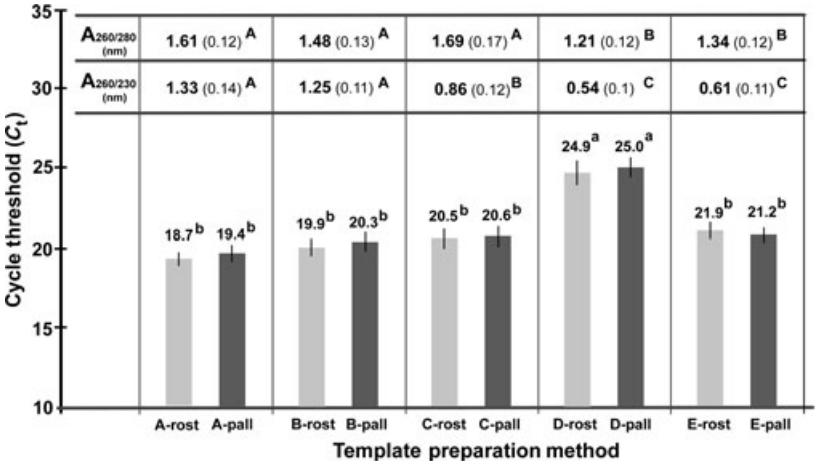

Fig. 1 Comparison of five different template-preparation methods for processing *Globodera rostochiensis* (-rost) and *G. pallida* (-pall) nematode cysts using two spectrophotometer ratios (260/280 and 260/230 nm) and real-time PCR threshold cycle (C_t) results; Standard errors are indicated in parenthesis (n = 25). (A) Blood and Tissue DNeasy kit (Qiagen); (B) ChargeSwitch Micro Tissue Kit (Invitrogen); (C) Chelex-resin isolation; (D) Sodium acetate and chloroform/isoamyl alcohol procedure; (E) Crude preparation in TE. [a,b,c]Comparison of mean values within each line; values with the same superscript do not differ statistically (P > 0.05), whereas values with different superscript show significant differences (P < 0.05).

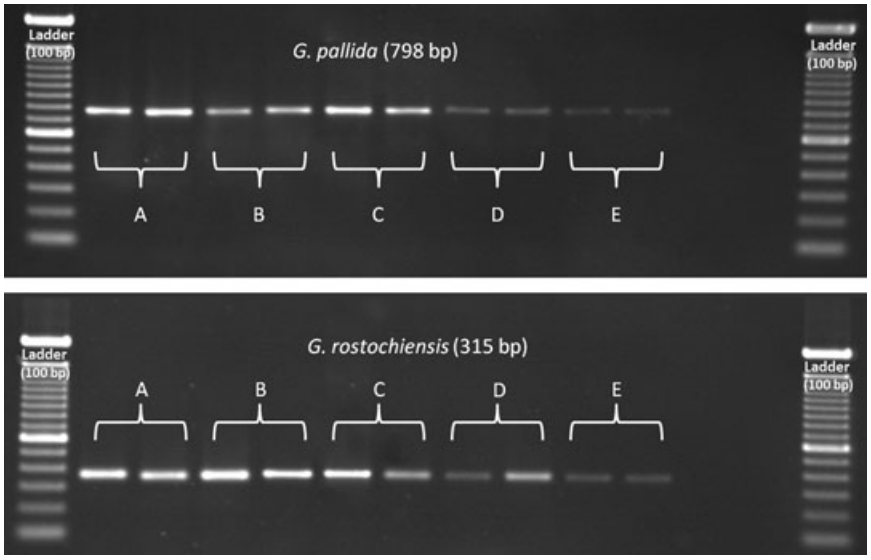

Fig. 2 Gel electrophoresis results from Co-PCR-amplified DNA (up: 798 bp for *Globodera pallida*; down: 315 bp for *G. rostochiensis*) isolated by five methods. (A) Blood and Tissue DNeasy kit (Qiagen); (B) ChargeSwitch Micro Tissue Kit (Invitrogen); (C) Chelex-resin isolation; (D) Sodium acetate and chloroform/isoamyl alcohol procedure; (E) Crude preparation in TE.

methods A and B showed the highest and most desirable values (P > 0.05). Method C presented an overall lower mean value, while methods D and E recorded the lowest values.

Effect of template-preparation methods on TaqMan and Co PCR

Template used from extraction method A presented the lowest mean C_t values. These values were similar to the corresponding mean C_t as recorded for methods B, C and E (P > 0.05) (Fig. 1). C_t results for method D were 5–6 cycles higher than those recorded with procedures A, B, C and E. Co PCR results, as evaluated by gel electrophoresis, showed that DNA template obtained with methods A and B resulted in highly intense bands. Amplification band intensity from templates extracted using methods C to E was not consistent (Fig. 2).

Technical difficulty, time, cost analysis and DNA volume recovery

Comparison of the different template-preparation methods for technical difficulty, time, volume recovery and cost is presented in Table 5. Alternative template preparation (Method E) was the most rapid and inexpensive method. However, DNA in such buffers was not stable for long-time preservation (data not shown). Method C was also quite cheap and easy to perform. Method D was slightly more expensive than methods C and E, but it was considered to be time-consuming. The two commercial kits (A and B) were the most expensive methods, and although yielded high-quality DNA, trained laboratory technicians were needed to complete the protocols effectively.

Discussion

In this study, a real-time PCR assay, based on TaqMan chemistry, was developed and optimized for the simultaneous detection of *G. pallida* and *G. rostochiensis*, two important nematode pathogens that negatively affect the potato industry. The assay was successfully applied for discrimination of isolates from different geographical locations around Europe. To avoid any possible cross-reaction and increase the assay specificity, TaqMan probes were designed to possess at least four nucleotide mismatches against the two species targets. At the same time, primer and probes were designed to prevent amplification of other *Globodera* species, which present a high genetic similarity with PCN. Results showed that no amplification signal occurred for *G. tabacum*, a nematode that is often found in infested potato fields.

Isolation of a sufficient amount of high-quality DNA is essential to PCR applications, and the selection of appropriate DNA extraction methods is a prerequisite for success of such techniques (Fredricks et al. 2005; Dauphin et al. 2009; Psifidi et al. 2010). Unlike other biological tissues, PCN cysts have an extremely small size with a diameter of ~500 μm and an average weight of 60–80 pg (Mulvey and Golden 1983). These cysts represent the matured dead body of a female nematode, in which approximately 300–600 eggs are enclosed under a layer of dried, hardened skin which enables their viability for more than 20 years in soil (Golden 1985). Another important issue is that the amount and quality of eggs, and therefore DNA inside cysts, is variable due to the unknown age of each collected sample. In this work, four different DNA extraction methods and one alternative template-preparation procedure that does not involve nucleic acid isolation were evaluated using *Globodera* sp. cysts. Evaluation criteria included the extract purity, efficiency for PCR downstream applications, labour, utility and cost. The purity of extracted material was assessed by spectrophotometric readings at 260/280 nm and 260/230 nm ratios. The first ratio is indicative of protein contamination with optimum values between 1.8 and 2 (Manchester 1995; Glasel 1997), whereas the second ratio refers to the presence of other contaminants that absorb near 230 nm such as carbohydrates, guanidine salts, EDTA etc., and readings of 1.8–2.2 suggest a high degree of purity (Wilfinger et al. 1997; Sambrook and Russel 2001). The two commercial kits (Methods A and B) showed similar values at both ratios. DNA extract obtained using the Chelex procedure had the least protein contamination from all evaluated methods. However, the low mean 260/230 nm ratio suggests that the DNA was not as pure as the two commercial kits. The standard chemistry, chloroform-based in-house protocol (Method D), resulted in a low-purity DNA extract and both ratios were significantly lower than the other three DNA extraction methods. Remarkably, the

Table 5 Comparison of DNA extraction methods from nematode cysts based on cost, time, difficulty and the potential to be applied using automated equipments

Template preparation method	Cost per test[a]	Process time (h:m)[b]		Technical difficulty	Automation potential[c]
		Minimum	Maximum		
A. DNeasy blood and tissue kit	3	2:05	2:30	++	Yes (QIAcube)
B. ChargeSwitch Micro tissue kit	3	2:15	2:40	++	Yes (MagMax™ Express)
C. Chlelex isolation method	0.5	0:20	0:35	+	No
D. Chlorophorm/sodium acetate	0.75	2:00	2:00	+++	No
E. TE Buffer crude preparation	0.2	0:15	0:25	+	No

[a]Costs for commercially available kits were estimated in euro, excluding VAT.
[b]The minimum processing times reflect extractions from single samples, and the maximum times reflect extractions of 12 samples.
[c]Possibility to perform template preparation extractions using automated, commercially available equipment.

crude extracts obtained by the alternative method E showed statistically similar 260/280 nm ratio values with methods A, B and C, although the organic purity was quite low. In addition, real-time PCR C_t values were analysed to assess the utility of each extract in assay performance. The recorded C_ts were statistically similar for all isolation procedures and ranged between 18.7 and 21.9, except for method D, where the mean C_t was approximately 25. We estimate that this result was due to the presence of increased inhibitors in the DNA extract, as reflected by the low 260/280 nm ratio. Nevertheless, in terms of detection, all methods seem appropriate as the means recorded C_ts for both species were less than 25.

The five extraction procedures were also compared for technical difficulty, speed and cost. Although the two commercial kits provided the best results in terms of DNA purity and C_t detection, they appear to be quite expensive and time-consuming. However, both kits could be used in automated DNA extraction equipment. Method D was cheaper, but it appeared to be laborious and not as effective. Crude sample preparation method E was the simplest, fastest and the cheapest method, followed by the Chelex-resin isolation method. These two methods could streamline the detection of *G. pallida* and *G. rostochiensis* cysts without compromising the reliability of the developed TaqMan PCR assay. The choice among these five extraction procedures is to be made according to the conducted research project, as long-term storage of an extracted sample requires DNA of high purity.

Direct comparisons were carried out between the developed real-time PCR and a previously reported Co PCR assay, to assess the limit of detection for all types of extracts. The TaqMan PCR assay was capable of detecting the two *Globodera* species in preparations diluted up to $1 : 10^5$ for DNA extracts obtained from the commercial kits (Methods A and B), $1 : 10^4$ for extracts from Chelex isolation and TE crude preparation procedures (Methods C and E, respectively) and $1 : 10^3$ for the in-house method D. Therefore, real-time PCR analytical sensitivity was higher than traditional PCR in all kinds of templates used. Even so, higher analytical sensitivity does not guarantee acceptable diagnostic sensitivity (Dse) or specificity (Dsp) (Saah and Hoover 1997). False-positive and/or false-negative reactions could occur because of sample contamination, test failure to detect the target due to reaction inhibition or low initial template quantity. For this purpose, the overall performance of the multiplex TaqMan and Co PCR was investigated using DNA extracted from reference cyst samples. Real-time PCR resulted in 100% Dse (no false positives) and Dsp (no false negatives) for nematode cyst isolates obtained from several collections. On the contrary, Co PCR resulted in lower Dsp and Dse, which were estimated at 94 and 87% for *G. pallida* and 97 and 88% for *G. rostochiensis*, respectively. Therefore, Co PCR was a less accurate method (90–93%) than TaqMan PCR, which showed absolute (100%) accuracy for the detection of these PCN species.

In conclusion, a real-time TaqMan PCR assay was designed and optimized for the simultaneous detection of *G. pallida* and *G. rostochiensis* cyst-forming nematodes. The assay shows an excellent analytical and diagnostic sensitivity and holds much promise as a routine tool for diagnosis of the two pathogens in samples across different countries. The assay can be accomplished either by using total DNA or crude extracts prepared directly from cysts, offering users a reliable, wide-range diagnostic tool.

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