Multiplex Real-Time PCR Assays for the Identification of the Potato Cyst and Tobacco Cyst Nematodes

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ABSTRACT

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TaqMan primer-probe sets were developed for the detection and identification of potato cyst nematodes (PCNs) *Globodera pallida* and *G. rostochiensis* using two-tube, multiplex real-time polymerase chain reaction (PCR). One tube contained a primer-probe set specific for *G. pallida* (pale potato cyst nematode) multiplexed with another primer-probe set specific for *G. rostochiensis* (golden potato cyst nematode). A second tube consisted of the *G. pallida*-specific primer-probe set multiplexed with a primer-probe set specific for *G. tabacum* (the morphologically similar tobacco cyst nematode). This internal transcribed spacer rDNA-based system was specific for the *Globodera* spp. of interest and successfully identified several populations of PCN. This rapid, sensitive, and specific quantitative PCR assay presents a useful tool for PCN regulatory response and management programs.

The pale potato cyst nematode *Globodera pallida* (Stone, 1973) Behrens, 1975 and the golden potato cyst nematode *G. rostochiensis* (Wollenweber, 1923) Behrens, 1975 are regulated pathogens of potato (*Solanum tuberosum*) in the United States. Previously, the distribution of *G. pallida* in North America was limited to Newfoundland, Canada but, in 2006, *G. pallida* was discovered in northern Bingham County, ID (7). The presence of this nematode represents a significant threat to the U.S. potato industry; therefore, accurate identification of *Globodera* spp. found in North America is essential for rational regulatory decisions to eradicate and prevent the spread of potato cyst nematodes (PCNs).

G. pallida and *G. rostochiensis* are pathogens of potato (*S. tuberosum*) and other closely related species in the family *Solanaceae*. The tobacco cyst nematode, *G. tabacum* (Lownsbery and Lownsbery, 1954) Behrens, 1975 is a third species of *Globodera* that can parasitize members of the *Solanaceae* family. Although it is not a known pathogen of potato, the *G. tabacum* complex contains three subspecies that, in nature, parasitize solanaceous weeds (*G. tabacum virginiae*) and both tobacco and tomato (*G. tabacum tabacum* and *G. tabacum solanacearum*) (2,25). Within North America, *G. tabacum* is found in the states of Virginia, Connecticut, Massachusetts, and North Carolina, as well as in Mexico and Quebec, Canada (4,13,14,25). The morphological similarity between *G. tabacum* and *G. pallida* can make identification based on these features alone very difficult (2,6,15–17). This complication led to the development of species-specific molecular assays to separate *G. pallida*, *G. rostochiensis*, and *G. tabacum* (23).

In order to determine the extent of *G. pallida* infestation in the United States, federal, state, and local authorities have initiated voluntary potato field surveys and monitoring programs in Idaho and other potato-producing states. Processing of soil samples to extract the nematodes and preliminary morphological characterization of suspect PCN samples is typically performed by state or regional facilities. Isolated nematode cysts suspected of being PCN are typically sent to United States Department of Agriculture (USDA) confirmation laboratories at Beltsville, MD for final identification based on both morphometric characterization and established molecular assays, including conventional polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis (18,23). These tools are capable of identifying PCNs (*G. pallida* and *G. rostochiensis*) and distinguishing PCNs from the tobacco cyst nematode *G. tabacum*. One disadvantage of the current identification scheme is that the entire process is rather labor intensive and takes a minimum of 2 days to complete. Thus, the rationale for this study was to develop a fast and reliable confirmation method, particularly for use by diagnostic laboratories that need to provide results as quickly as possible.

Real-time, quantitative PCR (qPCR) has gained acceptance for pathogen identification due to its improved speed, sensitivity, reproducibility, robustness and the reduced risk of carryover contamination compared with conventional PCR (11). Several qPCR assays have been developed for simultaneous detection of *G. pallida* and the sugarbeet cyst nematode *Heterodera schachtii* (12) as well as for identification of *G. pallida* and *G. rostochiensis* (1,3,22). These assays are rapid and specific but do not detect *G. tabacum*. In this study, we present, for the first time, the development of multiplex TaqMan qPCR for the simultaneous detection and identification of the two known PCN species and the morphologically similar tobacco cyst nematode. Because our molecular diagnostic laboratory receives nematode specimens already isolated from soil or plant material, we developed and optimized our molecular diagnostic tool for use on these isolated nematode specimens.

MATERIALS AND METHODS

Nematode materials. Three established *Globodera* spp. populations were used for the development of the multiplex TaqMan qPCR. *G. pallida* cysts that had been previously collected from infested fields in Idaho were kindly provided by Eoin Davis (USDA Animal and Plant Health Inspection Service PPQ, Idaho Falls, ID). *G. rostochiensis* cysts were kindly provided by Dr. Xiaohong Wang (USDA Agricultural Research Service, Ithaca, NY) and *G. tabacum solanacearum* cysts were kindly provided by Dr. Jon Eisenback (Virginia Tech University, Blacksburg, VA). Specimens from other populations of plant parasitic nematodes of *Globodera* spp., *Heterodera* spp., *Meloidogyne* spp., *Nacobbus aberrans*, and *Pratylenchus penetrans*

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were used for validation of the qPCR (Table 1).

DNA extraction. Two alternative methods were used for nematode DNA extraction. For the "single-worm smash" extraction method, each juvenile was visualized with a Nikon SMZ645 dissecting microscope (Nikon, Melville, NY) and mechanically disrupted with an eye-knife in 20 µl of nematode extraction (NE) buffer (10 mM Tris [pH 8.2], 2.5 mM $MgCl_2$, 50 mM KCl, 0.45% Tween 20, proteinase K at 60 µg/ml, and 0.0005% gelatin) (26). To reduce the potential of cross contamination during PCR analysis, this step was performed in a separate location from the molecular diagnostic laboratory. Alternatively, the "rapid nematode-DNA" extraction method was developed for rapid processing of samples obtained for PCN detection, as part of the federal and state surveys. Individual cysts or juveniles in 50 µl of double-distilled water were loaded into 2.0-ml screwcap microtubes (Sarstedt, Inc., Newton, NC). This is normally the way samples (from federal and state surveys) are received after morphological identification. Equal volumes of 2× NE buffer were added to the samples, then stored at –80°C for at least 30 min. Frozen nematodes were thawed and ground thoroughly using the Mini-Beadbeater-1 (BioSpec Products, Inc., Bartlesville, OK) with 5 mm of glass beads (approximately 30 s); then, 2.4 µl of proteinase K was added to each tube. Tubes were incubated for proteinase K treatment at 60°C for 60 min, followed by deactivation of the enzyme by incubation at 95°C for 15 min. The nematode extracts were used directly in sequence-specific multiplex qPCR and conventional PCR. DNA extractions from single, 10, 50, or 100 juveniles (or eggs from a cyst) or 1 cyst of each of three *Globodera* spp. (*G. pallida, G. rostochiensis*, and *G. tabacum*) in a minimum of three replicates were used for validation of the assay. Nematode DNA extracts (2 µl) prepared by either extraction method were used for each qPCR, and 5 µl of the extract was used for each conventional PCR.

Primer and probe design. *Globodera* spp. rDNA sequences (including multiple sequences for individual species) were downloaded from GenBank (http://www.ncbi.nlm.nih.gov). The sequences (Table 2) were then aligned using DNAMAN Sequence Analysis Software (version 6.0.3 95; Lynnon Corporation, Vaudeuil-Dorion, Quebec, Canada). Internal transcribed spacer (ITS)1 region sequences that are conserved within each species were identified visually and used to design three new species-specific primer sets and two new TaqMan probes (Table 3). We developed a set of primers (PITSpf and PITS4) and a TaqMan probe (GFAMp) for the specific detection of *G. pallida*, and also developed another set of primers (PGrtf and Prostor) and a TaqMan probe (GTETp) for the specific detection of *G. rostochiensis*. For the specific detection of *G. tabacum*, we again utilized the GTETp TaqMan probe and developed a third primer set (PGrtf and PITSt3mr). Primers and TaqMan probes were analyzed using OligoAnalyzer (version 3.1; http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/) prior to synthesis by Integrated DNA Technologies, Inc. (Coralville, IA).

Real-time PCR protocol. TaqMan qPCR assays were performed in a SmartCycler II (Cepheid, Sunnyvale, CA) in a 25-µl reaction volume. We tested each sample in two tubes (tube A and tube B). Tube A (Fig. 1) contained the *G. pallida*-specific set (primer PITSpf, primer PITSp4, and the FAM-labeled TaqMan probe GFAMp) multiplexed with the *G. rostochiensis*-specific set (primer PGrtf, primer Prostor, and the TET-labeled TaqMan probe GTETp). In tube B (Fig. 1), we used the same *G. pallida*-specific set for the detection of *G. pallida*; however, we multiplexed it with the *G. tabacum*-specific set (primer PGrtf, primer PITSt3, and the TET-labeled TaqMan probe GTETp). The components for each multiplex assay in 25 µl were 200 nM each primer, 100 nM each probe, 6.0 mM $MgCl_2$, 240 µM (each) dNTPs, 1× PCR buffer, and 1 unit of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). The cycling parameters included an initial denaturation step at 95°C for 120 s followed by 40 cycles at 95°C for 1 s and 58°C for 40 s. Each run contained one positive control (for each species) and one negative control.

Conventional PCR protocol. Amplifications of ITS1 and ITS2 were performed on each nematode sample to confirm the DNA template quality for all populations included in this study. Negative controls containing water instead of DNA template were included with each experiment. PCR reactions contained 0.2 µM each for primers TW81 (9) and AB28 (8), 5 µl of nematode DNA extract, 200 µM (each) dNTPs, 1.5 mM $MgCl_2$, 1.5 units of Platinum Taq DNA Polymerase (Invitrogen), and supplied enzyme reaction buffer at 1× in a total volume of 50 µl. The Biometra TGradient cycling parameters included an initial denaturation step of 95°C for 120 s; followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 90 s; and finished with one cycle at 72°C for 5 min. An 8-µl aliquot of each PCR reaction was analyzed by electrophoresis on 1% agarose in 1× Tris-acetate-EDTA. Gels were stained with ethidium bromide and visualized by UV illumination. Gel images were captured with a ChemiImager (Alpha Innotech, San Leandro, CA).

Establishment of standard curves. Conventional PCR products from three samples each of *G. pallida, G. rostochien-*

Table 1. Average cross-threshold (Ct) values for single juveniles extracted using the single-worm smash method

		Ct value			
		Tube A		Tube B	
Species	Origin	FAM	TET	FAM	TET
Globodera pallida	Idaho, United States	30.01	0	29.73	0
	Switzerland	27.49	0	27.80	0
	Peru[a]	26.84	0	27.09	0
	Peru[b]	27.16	0	27.14	0
	England, United Kingdom	26.21	0	26.62	0
	Netherlands	26.67	0	27.08	0
	Chile	28.73	0	28.87	0
G. rostochiensis	New York, United States	0	30.33	0	
	British Columbia, Canada	0	31.74	0	0
	British Columbia, Canada	0	33.03	0	0
	York, United Kingdom	0	32.89	0	0
G. tabacum solanacearum	Dinwiddie Co., Virginia, United States	0	0	0	28.75
	Richmond, Virginia, United States	0	0	0	27.17
G. tabacum tabacum	Connecticut, United States	0	0	0	27.47
	Virginia, United States	0	0	0	29.16
	Warner Co., Virginia, United States	0	0	0	29.12
	Virginia, United States	0	0	0	28.04
Globodera sp.	Oregon, United States	0	0	0	0
Heterodera avenae	Idaho, United States	0	0	0	0
H. glycines	Maryland, United States	0	0	0	0
H. lespedezae	Egypt	0	0	0	0
M. chitwoodi	Oregon, United States	0	0	0	0
M. hapla	Hawaii, United States	0	0	0	0
M. naasi	Oregon, United States	0	0	0	0
M. arenaria	Virginia, United States	0	0	0	0
Nacobbus aberrans	Peru	0	0	0	0
Pratylenchus penetrans	Wisconsin, United States	0	0	0	0

[a] Pathotype P4A.
[b] Pathotype P5A.

sis, and *G. tabacum* were excised from agarose gels, purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), and cloned into the pCR4-TOPO plasmid vector (Invitrogen). Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen) and the cloned DNA fragments were sequenced in both directions using the M13 forward (–20) and M13 reverse primers. Sequencing was performed at the University of Maryland Biotechnology Institute, Center for Biosystems Research (College Park, MD). Sequences were compared with sequences in GenBank of other *Globodera* populations. The BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to determine the level of nucleotide similarity with other *Globodera* spp. The plasmids pGp, pGr, and pGt were selected for *G. pallida, G. rostochiensis*, and *G. tabacum*, respectively. To evaluate the amplification efficiency, assay accuracy, and low detection limits of the qPCR methods, an absolute standard curve was established for each of the three nematode species using the respective plasmid. The standard curves were based on 10-fold serial dilutions prepared in water from 1.00×10^7 copies per microliter of initial plasmid concentration.

RESULTS

DNA extraction efficiency. In the traditional single-worm smash extraction method, specially trained personnel physically separate juveniles with the aid of a dissecting microscope and slice individuals to liberate the DNA for extraction. In contrast, the rapid nematode-DNA extraction method was carried out in a closed-tube system using disposable microcentrifuge tubes and disposable glass beads. DNA extraction from 12 samples required about 2 h with no need for personnel trained in the manipulation of single nematodes or use of a microscope. The rapid nematode-DNA extraction method yielded high-quality DNA suitable for PCR amplification from *G. pallida*, *G. rostochiensis*, and *G. tabacum* (Fig. 2). The PCR-amplified DNA bands were comparable with those produced from DNA extracted using the single-worm smash method. The yield and quality of DNA extracts were readily monitored by the cross threshold (Ct) values of the multiplex qPCR. The Ct value is inversely related directly to the amount of the PCR product and, therefore, to the original amount of the nematode target DNA present in the PCR reaction. The lower the Ct value, the higher the nematode target DNA concentration in the nematode DNA extract. Of the 20 µl of total DNA extracted from a single juvenile (using either the single-worm smash or the rapid nematode-DNA extraction methods), 2 µl yielded stable qPCR Ct values, averaging 27.81, 31.99, and 28.28 for the tested populations of *G. pallida*, *G. rostochiensis*, and *G. tabacum*, respectively (Tables 1 and 4).

Primer and probe specificity. The consensus sequences of *G. pallida*, *G. rostochiensis*, and *G. tabacum* were aligned and analyzed. Based on the conserved nucleotide regions, a primer pair and a TaqMan probe were designed for the detection of each species (Table 3; Fig. 3). The specificity of each assay was evaluated in multiplex qPCR assays using DNA extracts of *G. pallida*, *G. rostochiensis*, *G. tabacum*, and other nematode species (Tables 1 and 4). As expected, each primer/probe set proved specific to each target, and non-template negative controls gave 0.00 Ct values. None of the three primer/probe sets cross-reacted with other selected nematode species evaluated in this study (Table 1), including *H. avenae, H. glycines, H. lespedezae, M. chitwoodi, M. hapla, M. naasi, N. aberrans, P. penetrans*, and an unusual *Globodera* population of unknown host status from Oregon. The *G. pallida* primer/probe set, specific for *G. pallida*, reacted positively with all populations of

Table 2. List of sequences for GenBank accession numbers of the nematode species and populations used in the development of primers and probes during this study

Species	Location	Accession no.
Globodera pallida	United States	EF153835
	…	EF153836
	…	EF153837
	Canada	FJ212165
	…	GQ294522
	…	GQ294523
	…	GQ355975
	Peru	AF016866
	…	AF016868
	Chile	EF153834
	Argentina	DQ097514
	United Kingdom	DQ847109
	…	AF016869
	Spain	AF016871
	Romania	AF016870
	Ukraine	AJ606687
	New Zealand	EF622533
G. rostochiensis	United States	EF153839
	United Kingdom	EF153840
	Russia	DQ847117
	…	DQ847119
	…	DQ847120
	Japan	AB207271
	Australia	EF622522
	…	EF622523
	…	EF622525
	…	EF622528
	…	EF622532
G. tabacum solanacearum	United States	DQ847114
	…	DQ847115
	…	EF153841
G. tabacum tabacum	United States	EF153842
	…	DQ847116
	Japan	AB207272
G. tabacum virginiae	United States	DQ847112
	…	DQ847113
G. achilleae	Slovenia	AY599498
G. artemisiae	China	AF274415
G. hypolysi	Japan	AB207273
G. millefolii	Estonia	AF161004

Table 3. Sequence of TaqMan primers and probes for amplification of rDNA internal transcribed spacer I region of *Globodera* spp.

Primer or probe name	5′ to 3′ Sequence
PITSpf[a]	ACGGACACATGCCCGCTA
PITSp4[b]	ACAACAGCAATCGTCGAG
GFAMp[a]	FAM/ACATGAGTGTTGGGGTGTAAC/BHQ-1
PGrtf[a]	TCTGTGCGTCGTTGAGC
Prostor[c]	CGCAGACATGCCGCAA
GTETp[a]	TET/CGCAGATATGCTAACATGGAGTGTAG/BHQ-2
Ptab-rt2f[a]	TCGTTGAGCGGTTGTTGC
PITSt3mr[d]	AGCGCAGATATGCCGCG

[a] Developed for this study.
[b] Bulman and Marshall primer (5).
[c] Modification of the primer PITSr3 (5).
[d] Modification of the primer PITSt3 (23).

pale potato cyst nematode tested, including those from the United States, Peru, Chile, the United Kingdom, Switzerland, and Netherlands. This set did not cross react with other tested *Globodera* spp. (*G. rostochiensis*, *G. tabacum*, or the *Globodera* sp. from Oregon). The *G. rostochiensis* set, specific for *G. rostochiensis*, reacted positively with golden potato nematode populations from Canada, the United States, and the United Kingdom and did not cross react with other tested *Globodera* spp. (*G. pallida*, *G. tabacum*, and the Oregon *Globodera* sp.). Finally, the *G. tabacum* set, specific for *G. tabacum*, reacted positively with *G. tabacum tabacum* populations from Virginia and Connecticut and with a *G. tabacum solanacearum* population from Virginia. Similar to the *G. pallida* and *G. rostochiensis* sets, this set did not cross-react with any of the other tested *Globodera* spp. (Table 1).

Assay sensitivity. To evaluate the analytical detection sensitivity, four standard curves were established using the ITS1-cloned plasmid DNA (Fig. 4). As expected from their primer and probe design, all three qPCR reactions for *G. pallida, G. rostochiensis*, and *G. tabacum* had very high assay accuracy ($R^2 > 0.99$), with no significant differences found among them. All three PCR reactions also showed very high assay precision, as indicated by low standard deviations of the mean Ct values (standard deviation ranging from 0.03 to 0.33). The PCR amplification efficiency calculated from the slope of the standard curves (10) was 98.93, 86.51, and 88.54% for *G. pallida, G. rostochiensis*, and *G. tabacum*, respectively. Based on their standard curves for absolute quantification, the analytical low detection limit was 1 to 5 copies of their target ITS1 templates per reaction. Based on average Ct values (Table 4) and the use of 2 µl of DNA extract (of 20 µl total), the average ITS1 copy number per single juvenile calculated from standard curves was 6.97×10^4, 8.34×10^2, and 7.44×10^4 copies for *G. pallida, G. rostochiensis,* and *G. tabacum*, respectively. There were no significant differences in the FAM Ct values for *G. pallida* between the two tests in tube A and tube B, indicating that no significant influences on assay sensitivity or performance of the *G. pallida* set by the *G. rostochiensis* set in tube A or by the *G. tabacum* set in tube B occurred in the multiplex qPCR reactions.

Two-tube assay utility. The two-tube assay developed in this study allowed the detection and identification of *G. pallida*, *G. rostochiensis*, and *G. tabacum* simultaneously. Both PCN species were detected and identified using the multiplex reaction in tube A (*G. pallida* was detected using FAM, whereas *G. rostochiensis* was detected using TET reporting dye; Table 4). Using the multiplex reaction in tube B, *G. pallida* was detected using the FAM reporting dye, whereas *G. tabacum* was detected using TET.

Each sample of *G. pallida* twice tested positive with reporting dye FAM in both tube A and tube B of the two-tube system (Tables 1 and 4). Using single juveniles of

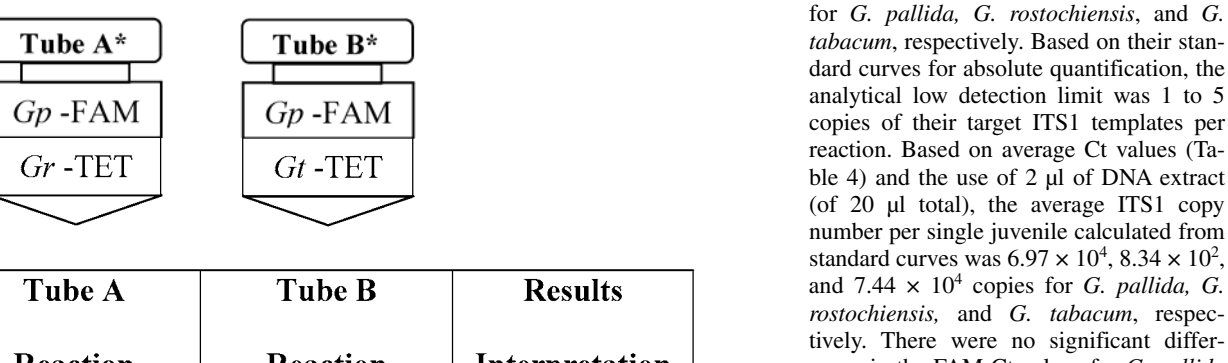

Fig. 1. Multiplex quantitative polymerase chain reaction illustration displaying the use of two tubes for identification of three *Globodera* spp. *Gp-FAM = *Globodera pallida*-specific primer pair and TaqMan probe with FAM reporter dye; Gr-TET= *G. rostochiensis*-specific primer pair and TaqMan probe with TET reporter dye; Gt-TET= *G. tabacum*-specific primer pair and TaqMan probe with TET reporter dye

Table 4. Average cross-threshold (Ct) values for nematode samples extracted using rapid nematode-DNA extraction method

	Ct value			
	Tube A		Tube B	
Species, sample description[a]	FAM	TET	FAM	TET
Globodera pallida				
1 cyst	23.62	0	23.84	0
100 juveniles	23.05	0	22.93	0
50 juveniles	25.16	0	25.3	0
10 juveniles	26.43	0	26.28	0
1 juvenile	29.89	0	29.78	0
G. rostochiensis				
1 cyst	0	24.59	0	0
100 juveniles	0	23.07	0	0
50 juveniles	0	25.93	0	0
10 juveniles	0	26.43	0	0
1 juvenile	0	30.33	0	0
G. tabacum solanacearum				
1 cyst	0	0	0	24.33
100 juveniles	0	0	0	22.19
50 juveniles	0	0	0	24.70
10 juveniles	0	0	0	25.40
1 juvenile	0	0	0	28.75

[a] Intact cysts were examined using a microscope to insure that they contained eggs or juveniles before DNA extraction. Other cysts were cracked open in molecular-biology-grade water and the exact numbers of juveniles required were transferred to microcentrifuge tubes for DNA extraction.

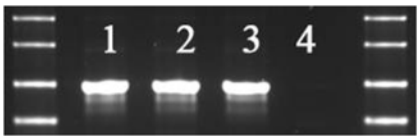

Fig. 2. Agarose gel electrophoresis displaying conventional polymerase chain reaction (PCR) reaction of *Globodera* spp. using the general primers TW81 and AB28. DNA was extracted using the rapid nematode-DNA extraction method. Outer lanes, BioMarker Ext DNA ladder (BioVentures, Inc., Murfreesboro, TN); lanes 1–3, PCR-amplified approximately 980-bp DNA fragments representing the ribosomal internal transcribed spacer (ITS)1 and 2 of *Globodera* spp.; lane 1, *Globodera pallida*; lane 2, *G. rostochiensis*; lane 3, *G. tabacum*; lane 4, nontemplate negative control.

G. pallida populations from Idaho, Switzerland, Peru, England, Netherlands, or Chile, the average FAM Ct value was 27.81. The average FAM Ct values for the Idaho population were 29.84, 26.36, 25.23, 22.99, and 23.73 for single, 10, 50, and 100 juveniles and 1 cyst, respectively (Table 4). All *G. rostochiensis* samples tested positive with the reporting dye TET in tube A. The average TET Ct value was 31.99 for the single juveniles tested from four populations (Table 1). For the *G. rostochiensis* population from New York, the average TET Ct values were 30.33, 26.43, 25.93, 23.07, and 24.59 for single, 10, 50, and 100 juveniles and 1 cyst, respectively (Table 4). All *G. tabacum* populations tested positive with the reporting dye TET in tube B. The average TET Ct value was 28.28 for single juveniles tested from six populations (Table 1). The Richmond, VA population TET Ct average values were 28.75, 25.4, 24.7, 22.19, and 24.33 for single, 10, 50, and 100 juveniles and 1 cyst, respectively.

DISCUSSION

The main focus of the PCN eradication program in the United States has been on detection of the pale potato cyst nematode (*G. pallida*) and limiting spread of the golden potato cyst nematode (*G. rostochiensis*). As part of this effort, federal and state agencies have conducted field surveys of potato-growing acreage to determine the extent of possible PCN infestations. In some cases, the poor condition of the specimens obtained from field samples has precluded easy identification of the nematodes based on cyst or juvenile morphology. Based on morphological characterization, it can be extremely challenging to differentiate *G. pallida*, a potato cyst

A ***G. pallida* detection**

	Primer PITSpf		*Probe GFAMp*		*Primer PITSp4*
Gpal cons	**ACGGACACATGCCCGCTA**	xxx	**ACATGAGTGTTGGGGTGTAAC**	xxx	**CTCGACGATTGCTGTTGT**
Gpal Arg	ACGGACACATGCCCGCT**G**	xxx	ACATGAGTGTTGGGGTGTAAC	xxx	**G**TCGAC**A**ATTGCTG**C**TGT
Gros cons	ACGGACA**Y**ATGCCC**R**CT**G**	xxx	ACATGAGTGTTGGGGT**R**TAAC	xxx	**GC**CGACGATTGCTG**C**TGT
Gtab cons	ACGGACACATGCCCGCT**G**	xxx	ACATGAGTGTTGGGGTGTAAC	xxx	**GC**CGACGATTGCTG**CTNT**
Gach	ACGGACACATGCCCG**T**TG	xxx	ACAAGAGTTTTGGGGTGT**T**AC	xxx	**GC**CGACGATTGCTGT**CA**T
Ghyp	ACGGACACATGCCCG**T**TG	xxx	ACAAGAGTTTTGGGGTGT**T**AC	xxx	**GC**CGACGATTGCTGT**C**GT
Gart	ACGGACACATGCCCG**T**TG	xxx	ACAAGAGTTTTGGGGTGT**T**AC	xxx	**GC**CGACGATTGCTGT**C**GT
Gmil	ACGG**G**CACA**G**GCCC**TAC**G	xxx	**G**CA**CT**AGT.TTGGGG**C**G**CT**AC	xxx	**GC**CG.....GCTG**C**TG**C**
Glob cons	ACGG**R**CA**Y**A**K**GCCC**DHYR**	xxx	**R**CA**HK**AGT**K**TTGGGG**YRYW**AC	xxx	**SY**CG**ACRATT**GCTG**YYNY**

B ***G. rostochiensis* detection**

	Primer PGrtf		*Probe GTETp*		*Primer Prostor*
Gpal cons	TCTGTGCGTCGTTGAGC	xx	CG**SB**G.ATATGCT**G**ACATGGAGTG..TAG	xx	**CR**GCGGCATRTCTGCG
Gpal Arg	TCTGTGCGTCGTTGAGC	xx	CGC**G**G.ATATGCT**G**ACATGGA.TG..TA**T**	xx	**CA**GCGGCATGTCTGCG
Gros cons	**TCTGTGCGTCGTTGAGC**	xx	**CGCAG.ATATGCTAACATGGAGTG..TAG**	xx	**TTGCGGCATGTCTGCG**
Gtab cons	TCTGTGCGTCGTTGAGC	xx	CGC**G**G.ATATGCTAACATGGAGTG..TA**N**	xx	**CC**GCGGCATATCTGCG
Gach	TCTGTGCGTCGTTGAGC	xx	CGC**T**G.ATATGCT**G**ACATGGAGTG**TG**TAG	xx	T**G**GCGGCATGTC**G**GCG
Ghyp	TCTGTGCGTCGTTTGA**G**	xx	CGC**T**G.ATATGCT**G**ACATGGAGTG**TG**TAG	xx	T**G**GCGGCATGTC**G**GCG
Gart	TCTGTGCGTCGTTGAGC	xx	CGC**T**G.ATATGCT**G**ACATGGAGTG**TG**TAG	xx	T**G**GCGGCATGTC**G**GCG
Gmil	TCTGTGCGTCGTTGAG**T**	xx	CG**TG**G**G**A**C**ATA**C**TG**A**TG**T**GTA**AG**TG**T**TG**T**	xx	**CA**GCGG**T**ATGTCTG**T**G
Glob cons	TCTGTGCGTCGTTGAG**B**	xx	CG**BN**G.A**Y**ATR**C**TR**AYR**TG**K**A**RKK**..T**RN**	xx	**YD**GCGG**Y**ATR**T**C**K**G**Y**G

C ***G. tabacum* detection**

	Primer PGrtf		*Probe GTETp*		*Primer PITSt3mr*
Gpal cons	TCTGTGCGTCGTTGAGC	xx	CGS**B**G.ATATGCT**G**ACATGGAGTG..TAG	xx	**R**GCGGCAT**R**TCTGCGC**Y**
Gpal Arg	TCTGTGCGTCGTTGAGC	xx	CGCGG.ATATGCT**G**ACATGGA.TG..TAT	xx	**A**GCGGCAT**G**TCTGCGCT
Gros cons	TCTGTGCGTCGTTGAGC	xx	CGC**A**G.ATATGCTAACATGGAGTG..TAG	xx	**T**GCGGCAT**G**TCTGCGCT
Gtab cons	**TCTGTGCGTCGTTGAGC**	xx	**CGCGG.ATATGCTAACATGGAGTG..TAK**	xx	**CGCGGCATATCTGCGCT**
Gach	TCTGTGCGTCGTTGAGC	xx	CGC**T**G.ATATGCT**G**ACATGGAGTG**TG**TAG	xx	**G**GCGGCAT**G**TCGGCGTT
Ghyp	TCTGTGCGTCGTTTGAG	xx	CGC**T**G.ATATGCT**G**ACATGGAGTG**TG**TAG	xx	**G**GCGGCAT**G**TCGGCGTT
Gart	TCTGTGCGTCGTTGAGC	xx	CGC**T**G.ATATGCT**G**ACATGGAGTG**TG**TAG	xx	**G**GCGGCAT**G**TCGGCGTT
Gmil	TCTGTGCGTCGTTGAG**T**	xx	CG**T**GG**G**A**C**ATA**C**TG**A**TG**T**GTA**AG**TG**T**TG**T**	xx	**A**GCGG**T**ATGTC**T**G**T**GC**T**
Glob cons	TCTGTGCGTCGTTGAG**B**	xx	CG**BN**G.A**Y**ATR**C**TR**AYR**TG**K**A**RKK**..T**RN**	xx	**N**GCGG**Y**ATR**T**C**K**G**Y**G**YY**

Fig. 3. TaqMan quantitative polymerase chain reaction target sequences (lines in bold) aligned with sequences from other *Globodera* spp. (sequence accession numbers listed in Table 2). **A,** *Globodera pallida* detection. **B,** *G. rostochiensis* detection. **C,** *G. tabacum* detection. Gpal cons = *G. pallida* sequence consensus (16 sequences, representing populations from the United States, Canada, the United Kingdom, New Zealand, Spain, Romania, Peru, Chile, and Ukraine); Gpal Arg = *G. pallida* sequence from Argentina; Gros cons = *G. rostochiensis* sequence consensus [11 sequences, representing populations from the United States, the United Kingdom., Russia, Japan, and Australia); Gtab cons = *G. tabacum* sequence consensus (*G. tabacum solanacearum* from the United States); *G. tabacum tabacum* from the United States and Japan; *G. tabacum virginiae* from the United States; Gach = *G. achilleae* sequence from Slovenia; Ghyp = *G. hypolysi* sequence from Japan; Gart = *G. artemisiae* sequence from China; Gmil = *G. millefolii* sequence from Estonia; Glob cons = consensus sequence. Bases that were ambiguous or mismatched the probe sequences are indicated in bold for nontarget species. Y = C,T; R = A,G; W = A,T; S = C,G; K = G,T; D = G,T,A; B = C,G,T; H = A,C,T; and N = A,G,C,T.

nematode, from *G. tabacum*, the closely related tobacco cyst nematode. The existence of three *G. tabacum* subspecies of overlapping morphology further complicates morphological identification. Although molecular diagnostic assays capable of distinguishing *G. pallida, G. rostochiensis*, and *G. tabacum* have been developed recently (23), these conventional PCR assays take 1 to 2 days to complete.

A number of qPCR assays have been developed for *Globodera* spp. detection but none of these studies have included all species of regulatory concern in Canada, United States, Mexico, and Central America. Bates et al. (3) employed melting-peak analysis of ITS PCR products, generated with the Bulman-Marshall primers (5) and detection with SYBR Green I fluorescent dye to identify the relative proportion of *G. pallida* and *G. rostochiensis* within mixed-cyst samples. Subsequent work by Quader et al. (22) focused on improving DNA extraction methods to give more consistent PCR using a commercial kit rather than phenol-chloroform extraction, and the associated qPCR assay also employed SYBR Green I dye for detection of ITS PCR products. Their study demonstrated that a broad geographic range of *G. rostochiensis* populations was successfully detected by those primers, focusing on species of primary concern in Australia and New Zealand. A TaqMan assay using ITS-based primers and probes was also developed to distinguish *G. rostochiensis*, a pest of regulatory concern in Europe, from *G. artemisiae*, which is not a parasite of potato (19).

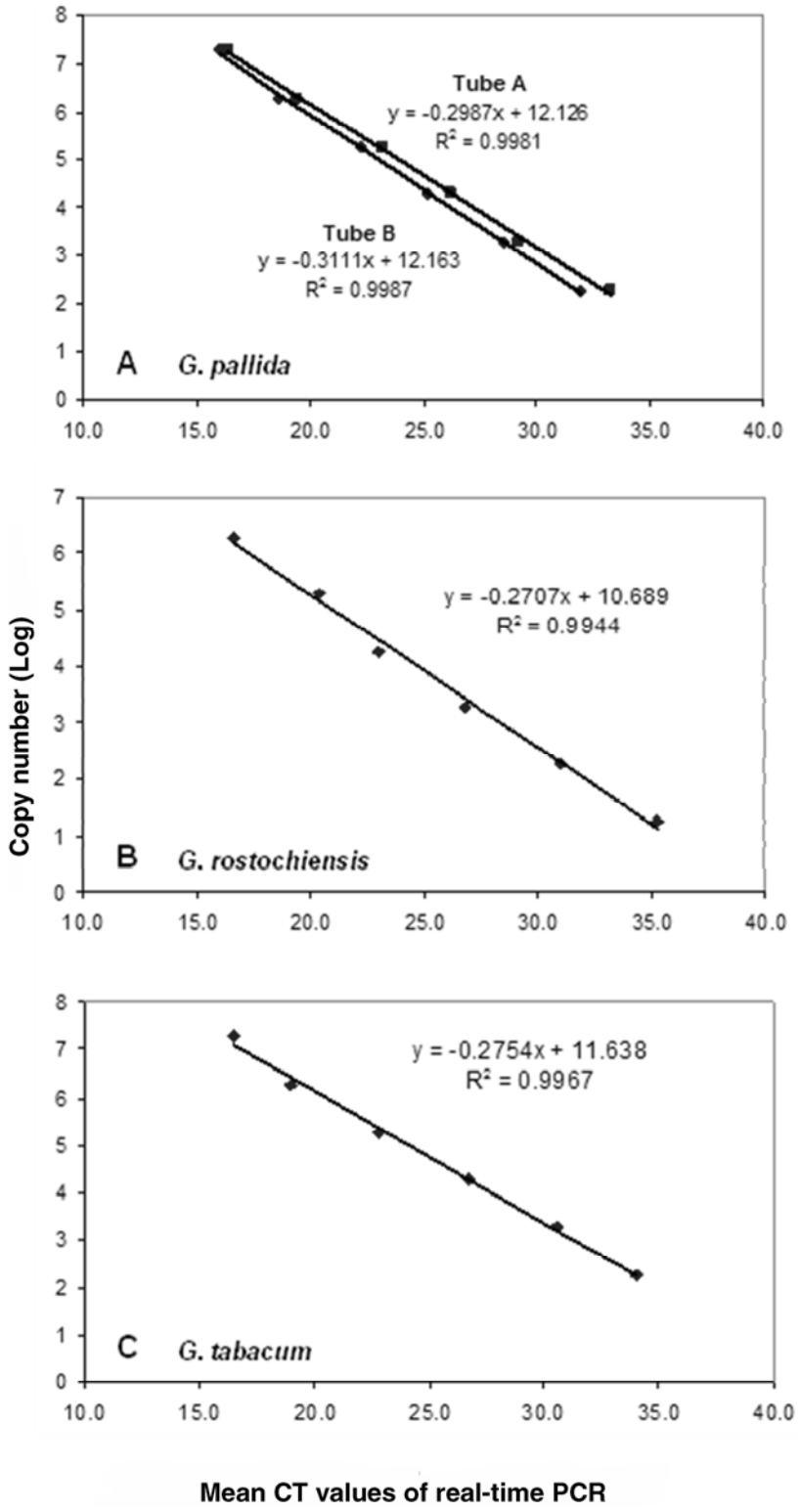

Fig. 4. Standard curves of the quantitative polymerase chain reaction (qPCR) for detection of **A,** *Globodera pallida*; **B,** *G. rostochiensis*; and **C,** *G. tabacum*. Standard curves were established on the mean cross-threshold (Ct) values of 10-fold serial dilutions of template-cloned plasmid DNA in triplicates in tube A for *G. pallida* and *G. rostochiensis* and tube B for *G. pallida* and *G. tabacum*, in the two-tube system of multiplex qPCR.

In this study, the combination of rapid nematode-DNA extraction with multiplex qPCR resulted in a robust detection and identification protocol available for wide-scale surveys and for the diagnostic confirmation of PCN-suspect samples. Molecular identification of PCN suspect samples was completed within about 4 h from the time of delivery to the diagnostic laboratory because we eliminated the need to use a microscope or perform and analyze conventional PCR. Furthermore, the use of qPCR assays minimized laboratory exposure to concentrated PCR products, a potential source of contamination that can confound accurate diagnostics.

As predicted from the primer design alignments, *G. pallida* was successfully identified using the specific PITSpf forward primer paired with the species-specific PITSp4 (5) reverse primer. One *G. pallida* sequence from Argentina contained one mismatch in PITSpf and three mismatches in PITSp4. Reaction of the *G. pallida*-specific primers with this population could not be tested directly, because the Argentina population was not available. However, subsequent to the timing of this study, an unnamed *Globodera* population from Oregon was found to have ITS sequences that are highly similar to the Argentina population and also contain mismatches to the *G. pallida* primer set (*not shown*). As expected, qPCR results from this primer set with the Oregon population were negative, as they were with the other *Globodera* spp. tested. Although the population was not available to be tested directly, ITS sequences from the as-yet-unvalidated species *G.* "*mexicana*" (GenBank accession numbers EU006707,

EU006708, and EU006709) were compared with the primer and probe sequences. Except for one base mismatch with pITSp4, the *G.* "*mexicana*" sequences matched the *G. pallida*-specific primer/probe combination.

In combination with the common forward primer PGrtf, the reverse primer Prostor (a modification of the Bulman & Marshall PITSr3 primer; 5) was used for the specific detection of *G. rostochiensis*, while the specific reverse primer PITSt3mr (a modification of primer PITS3; 23) was used for the specific detection of *G. tabacum*. The GTETp probe was designed to anneal to *G. rostochiensis*, *G. tabacum*, and *G. pallida* but not to *G. achilleae*, *G. hypolysi*, *G. artemisiae*, and *G. millefolii* because the target sequences of these latter *Globodera* spp. each have two nucleotides extra in the target sequence of the probe. As predicted, by combining the gap in the probe and the 3′ end specificity of the reverse primers, both *G. rostochiensis* and *G. tabacum* were successfully detected and identified using the *G. rostochiensis*- and *G. tabacum*-specific sets, respectively. These primer sets also reacted negatively with the Oregon population.

Primer/probe sets that were specific to *G. pallida*, *G. rostochiensis*, or *G. tabacum* showed promising assay sensitivity, yielding positive mean Ct values between 27 to 32 when using 2 µl of the single juvenile DNA extracts from the three nematode species. The analytic low detection limits for the three primer/probe sets were as low as one to five copies of the DNA template per reaction. In addition, the qPCR sensitivity remained the same in the multiplex assay compared with the singleplex qPCR assay. As expected, the qPCR Ct values dropped linearly and accordingly with the increase of the number of juvenile nematodes used per extraction. The multiplex qPCR yielded Ct values around 24 for single cysts of the three *Globodera* spp. evaluated.

The two-tube multiplex qPCR system developed in this work could detect, identify, and quantify even a single juvenile of the three *Globodera* spp. most likely to be encountered during North American surveys for PCN. It is realistic to expect that the future may bring new diagnostic challenges as additional PCN populations are described both morphologically and molecularly. *G. pallida* originating from the cordillera of South America is of particular interest, considering the genetic diversity of those populations relative to the majority of European and North American populations (20,21,23,24). It is expected that development of PCN diagnostic protocols will continue to evolve as information about new populations is revealed.

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