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PCR-based Species-Specific Amplification of ITS of *Mecistocirrus digitatus* and Its Application in Identification of GI Nematode Eggs in Bovine Faeces

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ABSTRACT. Differential diagnosis of *Mecistocirrus digitatus* infection relies on morphological examination of either eggs in faecal samples or L3 larvae developed *in vitro*. Technical limitations hinder the practicability of these approaches. Hence, in order to develop a specific diagnostic measure for *M. digitatus* infection, we determined the sequence of the internal transcribed spacer (ITS) of its ribosomal DNA (rDNA) and designed primers for PCR-based species-specific amplification of the ITS to differentiate between *M. digitatus* and other common gastrointestinal (GI) nematode species. The newly designed primers amplified a single specific 520 base pair (bp) fragment from the *M. digitatus* ITS, and its detection limit was as low as 0.001 *n*g. Further, this sensitivity suggested that the specific fragment could be amplified even from a unicellular egg that collected directly from uteri of an adult *M. digitatus* female. In fact, we designed a method that employs a small piece of a cover slip and a filter paper by which we could differentially amplify a PCR fragment from a unicellular egg. The reliability of the specific PCR assay was also demonstrated with 10 oval samples that collected from bovine faeces by using sugar flotation method. These data suggested that the specific PCR assay of the ITS region of *M. digitatus* rDNA could be useful for the identification of GI nematodes.

KEY WORDS: cattle, gastrointestinal nematode, ITS, *Mecistocirrus digitatus*, PCR.

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Gastrointestinal (GI) nematodes of domestic ruminants are of major veterinary importance due to their high prevalence, ubiquitous distribution and the pathological consequences of infection and the associated economic production losses [1, 3, 18]. In Japan, the major GI nematode species in cattle are *Ostertagia ostertagi, Mecistocirrus digitatus* and *Trichostrongylus axei* which are found in the abomasum; *Strongyloides papillosus* and *Cooperia* and *Nematodirus* species which are found in the small intestine; and *Oesophagostomum radiatum* found in the large intestine [7, 15, 16, 23]. Of these species, *M. digitatus*, *O. ostertagi* and *Cooperia oncophora* are considered the most prevalent in cattle; in particular, the infection rate of *M. digitatus* was high at approximately 30–50 % [7,16]. This nematode is commonly described as a large stomach worm, namely, a trichostrongylid nematode, and is an important blood-sucking nematode present in the abomasums of cattle. This parasite may cause mucosal inflammation, haemorrhage, ulcers and necrosis in the abomasums. It appears to be confined mainly to Asian countries; however, it has also been found in Central America, Egypt and Russia [6, 7, 13, 21, 22]. The movement of its hosts for agricultural purposes has resulted in the global spread of this parasite.

Generally, ruminants are concurrently infected with more than one species of GI nematodes, each having a different pathological effect on the host. It has been a difficult task to eradicate GI nematodes from grazing ruminants due to the variation in host susceptibility to the parasite, the wide distribution of nematodes in nature and the presence of wild ruminants. An effective method is required for the control of nematodes by reducing their infection rate and their transmission to the host in order to protect cattle from production losses. Strategies for the control of nematodes by using anthelmintic drugs should be devised based on the quantitation and identification of species. The identification of GI nematode species is primarily based on the morphology of their eggs in faeces and the infective third-stage larva (L3) developed *in vitro*. Since all nematode eggs have similar morphological characteristics, identification of species level is not possible through microscopic observation. The average length of an *M. digitatus* egg is 95–122 μm [6] and it is either of the same size or larger than the other GI nematode eggs. The differentiation of *M. digitatus* eggs from those of other GI nematodes requires experienced investigators. Furthermore, the microscopic identification of L3 in faecal cultures is not only time-consuming but also requires experienced microscopists. In addition, the results are not always reliable due to subtle differences in the morphological characteristics [8].

Recently, DNA techniques have been widely used to identify parasite species and to assess the genetic diversity among parasite populations. Ribosomal DNA (rDNA) is a useful target since it is abundant in an organism [4], thus enabling the development of highly sensitive techniques. Internal transcribed spacer (ITS) sequences have been a popular choice because the ITS region is one of the most variable nuclear loci. Several reports have described the ITS of rDNA as a useful target for the identification of parasites [12, 14, 20, 24, 25]. The use of such markers in PCR-

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based assays has allowed the identification of single eggs and larvae at the species level [2, 19, 25]. Precise and rapid identification of parasite species infecting the host would greatly assist the development of control programs and reduce unnecessary drug treatment. However, no study has examined the DNA of *M. digitatus*. The present study aimed to determine the species-specific sequences and primers of *M. digitatus*. Further, it aimed to detect *M. digitatus* eggs among other GI nematode eggs by using species-specific PCR of *M. digitatus*.

MATERIALS AND METHODS

Parasites: Adult *M. digitatus* (from Holstein-Friesian cows in Japan and buffaloes in the Philippines) and *O. ostertagi* and *C. oncophora* (from Holstein-Friesian cows in Japan) were collected from the abomasums and small intestine at necropsy, washed repeatedly in physiological saline and preserved in 70% ethanol until DNA extraction. Male specimens were identified by the morphological characteristics of the copulatory bursa and spicules [6]. The infective third-stage larvae (L3) of *M. digitatus*, *O. radiatum* and *Trichostrongylus* species (from Holstein-Friesian cows in Japan) and *Haemonchus* species (from Black beef cattle in Japan) were collected from faecal culture, identified up to the genus level based on morphological criteria and preserved at 4°C until DNA extraction. *S. papillosus* egg (from sheep in Japan) was collected from faeces and preserved at –30°C until DNA extraction.

Collection of eggs from faeces and faecal egg count: Five samples of fresh bovine faeces were collected from a farm infected with *M. digitatus* for several years; five fresh faeces samples were also collected from a farm that was not infected by *M. digitatus* for several years. The eggs were recovered from the faeces by using a sugar flotation technique (specific gravity: 1.27). A fresh faeces sample weighing 10 g was suspended in 60 m*l* water, and the suspension was filtered through a coarse strainer to remove any large particles. A 15 m*l* aliquot of the filtrate was transferred into 2 test tubes and centrifuged at 800 rpm for 10 min. The supernatant was discarded and the precipitate was resuspended in a sugar solution and centrifuged at 1,500 rpm for 10 min. One of the test tubes was allowed to stand for 20 min. Subsequently, the top 500 μl of the faecal suspension was transferred into a new 2 m*l* tube, and 1.5 m*l* of double-distilled water was added; this solution was then centrifuged at 20,000 g for 5 min. The supernatant was discarded and the precipitate was stored at –30°C as the oval sample until DNA extraction. A cover slip was placed on the fluid surface of the solution in the other test tube and it was allowed to stand for 20 min. Subsequently, the cover slip was removed and placed onto a glass slide; based on the morphology of the eggs, the number of eggs per 10 g of faeces was counted microscopically [6]. Eggs measuring more than 110 μm were counted as *M. digitatus*.

Extraction of DNA from nematodes and oval samples: Genomic DNA was isolated from 10 male *M. digitatus* worms and from a worm, L3 or egg of the other nematode species by using DNeasy™ Tissue Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. Genomic DNA was extracted from 10 oval samples by using the QIAamp® DNA Stool Mini Kit (Qiagen) in accordance with the manufacturer's protocol. The DNA was eluted with 10–50 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at –20°C.

Sequencing of the M. digitatus rDNA: The *M. digitatus* partial 18S to partial 28S rDNA region was amplified using the following primers: forward primer 18SF: 5'-GAGAGGACTGCGGACTGCTGTATCG-3' that was designed from the 18S rDNA sequence of *Caenorhabditis elegans* (GenBank accession number X03680) and the reverse primer NC2: 5'-TTAGTTTCTTTTCCTCCGCT-3' that is the conserved region of the 28S rDNA fragment, as described by Gasser *et al.* [9]. PCR amplification was performed in a 25 μl reaction mixture containing 10× *Ex Taq*™ buffer, 2.5 mM dNTP mix, 25 mM $MgCl_2$, 0.15 U Takara *Ex Taq*™ (Takara, Shiga, Japan), 0.5 μM of each primer, and 2 μl of the DNA extract. The PCR program was run as follows: 1 cycle at 95°C for 10 min, 35 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec; this was followed by a final extension at 72°C for 10 min. The PCR products were detected on a 2% agarose gel, and purified using Qiaquick™ spin columns (Qiagen). The nucleotide sequences were determined by an automated DNA sequencer (ABI PRISM® 310 Genetic Analyzer; Applied Biosystems, U.S.A.) using the primers 18SF and NC2 in separate reactions with a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham, Piscataway, U.S.A.). The sequences were aligned using the ClustalW method. The 5' and 3' ends of ITS1 and ITS2 were determined by comparison with the rDNA sequence of *C. elegans*.

Design of species-specific primers and PCR amplification: Based on the sequences obtained from *M. digitatus*, 2 primers were designed for species-specific amplification of a 520 base pair (bp) fragment between the ITS1 and ITS2 regions (Md1: 5'-TGTTTATGCAAAGTTCCTATCACGTTTAATG-3' (forward) and Md2: 5'-ATTGATCTGTTGATCTAAAATGGGATGTATCCT-3' (reverse)). The specificity of these primers was confirmed by comparing each primer sequence with those of other GI nematodes obtained from GenBank (Table 1). Further, the availability of these primers was verified using those of the genomic DNA of adult *M. digitatus* from Japan and the Philippines;

Table 1. GenBank number of GI nematode species in the ITS region

Species	ITS1	ITS2
Ostertagia ostertagi	AF044933	X86027
Cooperia oncophora	AB238687	X83561
Oesophagostomum radiatum	AF344881	AJ006149
Trichostrongylus axei	Y15875	X78065
Haemonchus contortus	AF044937	X78803
Haemonchus placei	AF044929	X78812
Nematodirus helvetianus	AJ251570	Y14013

the L3 of *M. digitatus*; 1 DNA sample each from an adult, L3 or egg of the other GI nematodes, namely, *O. ostertagi, C. oncophora, O. radiatum, S. papillosus, Trichostrongylus* and *Haemonchus* species; a mixture of DNA extracts from all the nematode species; and the genomic DNA of the host liver since *M. digitatus* is a blood-sucking parasite.

For the determination of the sensitivity of the species-specific primers of *M. digitatus*, the genomic DNA of an adult worm and a unicellular egg of *M. digitatus* from Japan were used in the PCR. The concentration of the genomic DNA was determined photometrically based on optical density (OD_{260}). A serial dilution of the *M. digitatus* genomic DNA was used in the PCR, and 10, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005 and 0.0001 *n*g quantities of genomic DNA were added to the PCR. A unicellular *M. digitatus* egg was collected directly from uteri of an adult female. The egg was transferred onto a glass slide by using a pipette under microscopic control. A cover slip was pressed onto the glass slide to destroy the egg and a filter paper was used to absorb the egg solution. PCR amplification was performed by directly adding this filter paper instead of the DNA template into the reaction mixture. Finally, the genomic DNA of 10 oval samples was used for the species-specific PCR to confirm *M. digitatus* infection.

All amplifications were carried out in a 25 μl reaction mixture, and species-specific primers were used. The PCR programs were performed by changing the annealing temperature to 60°C and the number of cycles to 40. PCR of the oval samples was performed using 3 μl of the DNA extract. No template control was included. The amplification products were separated on 2% agarose gels and visualized by staining with ethidium bromide.

RESULTS

The PCR product of *M. digitatus* rDNA between the partial 18S and partial 28S regions was obtained on an agarose gel as a single band of approximately 970 bp (Fig. 1). The GC content of the amplified rDNA region was 34.7%–35.2%. The length of the ITS1 was either 421 or 422 bp, while that of the ITS2 was either 257 or 259 bp. The ITS1 and ITS2 of *M. digitatus* sampled from the respective areas of Japan and the Philippines were identical in sequence and length. The *M. digitatus* rDNA sequences from Japan and

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                                                                18S←][→ITS1
MdJ  TCGCAATGGCTTGAACCGGGTAAAAGTCGTAACAAGGTATCTGTAGGTGAACCTGCAGATGGATCATCGTTGAAACCTTTGACAAAAGGTCACTTTGATT  100
MdP  ....................................................................................................  100

MdJ  ACGAGAAACCAACAGCTATTTTTATGACTTTGTCGTGAAAGTTGGAAGTATCATCCCCGTTAAAGCTCTATTATATGAGGTGTCTATGTATAACATGAGT  200
MdP  ..................................A.................................................................  200

                  Md1→
MdJ  CGTTCAAGAGTGGCGGCTATGATTGTTTATGCAAAGTTCGTATCACGTTTAATGATAGTTGAGCTTTAGACTTAATAAGTATTGCTAAAATACTGCCTCA  300
MdP  ..........................................................A.........................................  300

MdJ  TGGTTTTATAATGGTGGTTAACTATGAGATATATCACTTATTCAAGTGTTCTCTCTAATAC-TCCTCTATCCGAACCTTATGAACCGGAGAACCTTAATG  399
MdP  ..................................................T.....C.....................................  400

                                                                                    ITS1←][→5.8S
MdJ  ATCATTTATAATGACGCCATTATAAAAAAAAAAAAAATTATTTATTTTTATAGATTACAGATTGTGTCACTGATTTGTGTCACATTTATCGATTAGCTTTA  499
MdP  ....................................................................................................  500

MdJ  GCGATGGATCGGTTGATTCGCGTATCGATGAAAAACGCAGCTAGATGCGTTATTTACCACGAATTGCAGACGCTTAGAGTGGTGAAATTTTGAACGCATA  599
MdP  ....................................................................................................  600

                                                5.8S←][→ITS2
MdJ  GCGCCGTTGGGTTTTCCCTTCGGCACGTCTGGTTCAGGGTTGTTAACGGTTTCACTACAATGTGGCTAATTATAACATTGTTTGTCAAATGGTATTCATC  699
MdP  ....................................................................................................  700

                                                ←Md2
MdJ  CTTTTCATAAAGGATACATCCCATTTTAGATCAACAGATCAATTTATGCAACGTGATATCAATGTTGTGTAAAAGAACATTAATATTTCAGAATGATATG  799
MdP  ..................................................T.................................................A  800

MdJ  AATATA--TTGCCTATACTATTTAAGTATACTCAAGGAAATAAATTTGAGTTAAATTTAAATAGGGACATGAATGGCAATAATTTCATTTATCATCTCTA  897
MdP  ......TA..............................C.....................................................  900

 ITS2←][→28S
MdJ  TAATGCAACCTGAACTCAGGCGTGATTACCCGCTGAACTTAAGCATATCATTTAGCGGAGGAAAAGAAA  966
MdP  ..........................................................................  969
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Fig. 1. Alignment of the 18S to 28S sequence (5'–3') of *M. digitatus* rDNA. (–) : aligment gap. (.) : same base as MdJ. MdJ and MdP was indicated Japan or the Philippines sample of an adult *M. digitatus*. The species-specific primer of *M. digitatus* was indicated Md1, Md2 and under lines. These sequences have been deposited in the GenBank and DDBJ Nucleotide Sequence Databases (accession numbers: AB222059 and AB222060, respectively).

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ITS1  (5'–3')
Md1  ----TGTTTATGCAAAGTTCCTATCACGTTTAATG----
MdJ  TGAT.............................ATAG
MdP  TGAT.............................ATAG
Oo   TGAT....C..........C..TC.AA.GGT..GTTA
Co   TGAT....C....G.......C.AT..TA.ATTG.TTGA
Or   TGAT....GTACATT.....GCG.TTACGC.G.GCTGCT
Ta   TRAT....C....G.......C....ATGA.GG.TGAGC
Hc   TGAT....C....G..........TTGA.GG.TGAGC
Nh   TGAT.CG.CG.A.........C..CTA.CG.GG.CGAGC

ITS2  (5'–3')
Md2  ----AGGATACATCCCATTTTAGATCAACAGATCAAT----
MdJ  ATAA.............................TTAT
MdP  ATAA.............................T...TTAT
Oo   TATTGT....AT.......CC..T....G.ATATG.AATGC
Co   TGTTTT..C.A.....G..A...T.S..G.ATAAC..ATGC
Or   ATGCTCA..GATC.TCG..C...G...GA.CCCT.T.GCAA
Ta   TATTGT....AT..........T.T..G.ATAAT.CATGC
Hc   CTTTTA..C.AT........C..T....G.ACAT.TACATG
Nh   TGTTRCAGCGA........-C.AG.G..G.A.AT.TGCAAC
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Fig. 2. Sequence comparison (5'–3') with GI nematode species of the species-specific primer (Md1 and Md2) region. (–) : aligment gap. (.) : same base as primer of *M. digitatus*. MdJ: Japan sample of *M. digitatus*, MdP: the Philippines sample of *M. digitatus*, Oo: *O. ostertagi*, Co: *C. oncophora*, Or: *O. radiatum*, Ta: *T. axei*, Hc: *H. contortus*, Nh: *N. helvetianu*s.

the Philippines reported in this paper are available in the GenBank database under the accession numbers AB222059 and AB222060, respectively.

The intraspecific variation in the sequence of the *M. digitatus* ITS region between samples obtained from Japan and the Philippines was due to the insertion of a C at position 362 in ITS1 and the insertion of a TA repeat at position 807 in ITS2. Six nucleotide transitions were also found in the rDNA at positions 135, 264, 351, 740, 800 and 836. The partial 18S rDNA, 5.8S rDNA and the partial 28S rDNA showed no sequence variation.

Two species-specific primers were designed based on the ITS1 and ITS2 sequences of *M. digitatus* rDNA (Fig. 1) and their specificity was confirmed by comparing them to the sequences of 6 other GI nematode species (Fig. 2). The size of the specific amplification product was 520 bp. The specificity of the primers was demonstrated by the presence of a single band obtained by the agarose gel electrophoresis of the PCR product that was amplified from genomic DNA of *M. digitatus* adults (from Japan and the Philippines) and L3 (Japan) (Fig. 3, Lanes 1–3). Non-specific amplification was absent in the PCR as demonstrated in reaction and by using a separate or mixture of DNA from adult *O. ostertagi* and *C. oncophora*, L3 of *O. radiatum*, *Trichostrongylus* and *Haemonchus* species and an *S. papillosus* egg (Fig. 3, Lanes 4–10). Further, we observed that the host liver DNA was not amplified in the PCR (Fig. 3, Lane 12). Further, when the DNA extracted from *M. digitatus* and other GI nematodes was mixed, specific amplification of *M. digitatus* was detected (Fig. 3, Lane 11).

The genomic DNA of adult *M. digitatus* was serially diluted for the determination of the detection limit. A weak signal was visible even with 0.001 *n*g of genomic DNA (Fig. 4). The filter paper used to absorb the solution from the destroyed unicellular egg was used directly in the PCR assay. The PCR result clearly indicated a specific *M. digitatus* band obtained by the agarose gel electrophoresis of the PCR product (Fig. 5).

Ten oval samples from bovine faeces were used to compare the results of the faecal egg counts, larva culture and

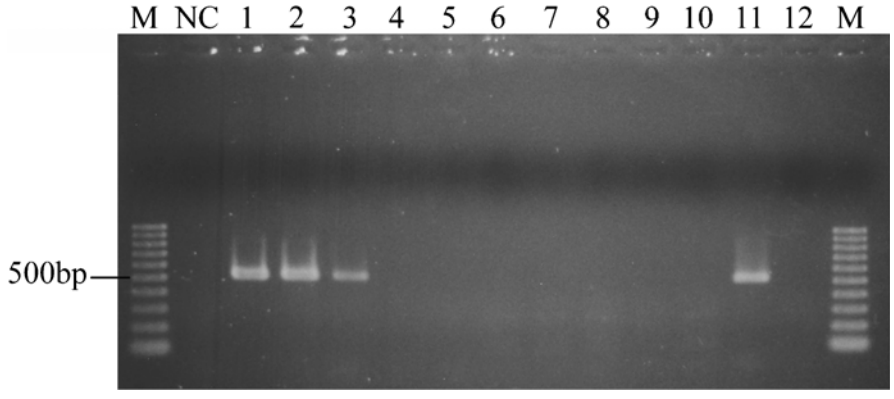

Fig. 3. Species specificity of new designed primers for *M. digitatus* rDNA. PCR was performed with Md1 and Md2 using each DNA extract. Lane 1: Japan sample of *M. digitatus*. Lane 2: the Philippines sample of *M. digitatus*. Lane 3: L3 of *M. digitatus*. Lane 4: *O. ostertagi*. Lane 5: *C. oncophora*. Lane 6: *O. radiatum*. Lane 7: *S. papillosus*. Lane 8: *Trichostrongylu*s sp.. Lane 9: *Haemonchus* sp.. Lane 10: a mixture of DNA extracts from 6 GI nematodes except *M. digitatus*. Lane 11: a mixture from 7 GI nematodes. Lane 12: bovine liver. Lane M: 100 bp marker. Lane NC: no template control.

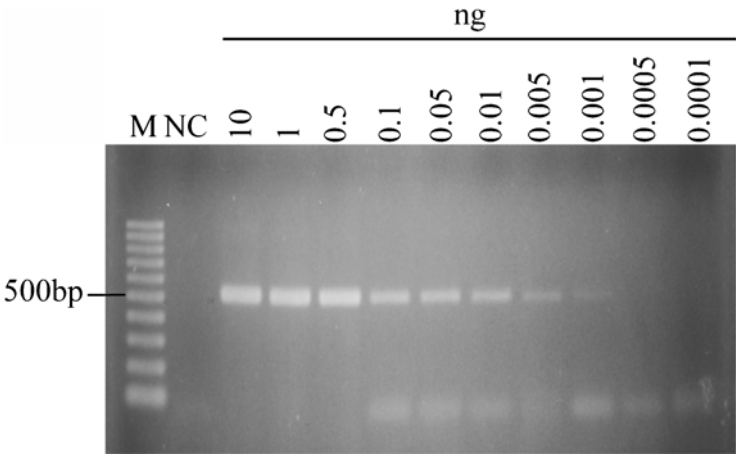

Fig. 4. Sensitivity of the specific amplification to detect adult *M. digitatus*. Quantities of 10, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005 and 0.0001 *n*g of genomic DNA diluted in double-distilled water were used as template. Lane M: 100 bp marker. Lane NC: no template control.

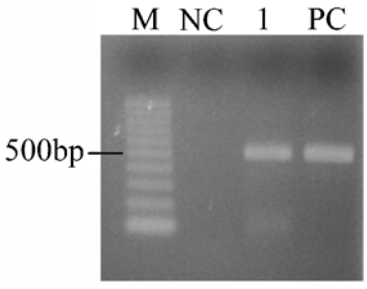

Fig. 5. Detection of PCR amplification by using destroyed a unicellular egg. Lane 1: a filter paper absorbing the destroyed egg solution. Lane M: 100 bp marker. Lane NC: no template control. Lane PC: positive template control (DNA extract of *M. digitatus*).

PCR assay of the faecal samples (Table 2, Fig. 6). When *M. digitatus* was detected by the faecal egg count, the species-specific amplification of *M. digitatus* could be obtained in the PCR assay. Further, when the faecal egg count was negative, non-specific amplification was absent in the PCR assay.

DISCUSSION

Intraspecific variation was detected in the rDNA sequences of *M. digitatus* sampled from Japan and the Philippines (Fig. 1). The insertion of 1 base in ITS1 and 2 bases in ITS2 were recorded in the samples from the Philippines. Further, a difference was recorded at 4 positions in both the sequences. However, the differences at 5 positions in ITS1 and 6 positions in ITS2 comprise only 0.7% of the intraspecific variation. This level of ITS sequence variation was also observed among individuals of the same species [2, 10, 17]. For instance, Heise *et al*. reported a comparison between the ITS2 sequences of 8 GI nematode species found in ruminants [11]. The degree of intraspecific variation observed ranged from 0.0–2.6%. The ITS sequence of *M. digitatus* was compared with that of *Haemonchus contortus* and *H. placei*. This is because all 3 organisms are similar with regard to the effects of their infection and belong to the *Haemonchinae*. The ITS1 sequence of *M. digitatus* (Japan sample: 421 bp and the Philippines sample: 422 bp) was longer than those of *H. contortus* and *H. placei* (404 bp long in both species; Table 1). Similarly, the ITS2 sequence of *M. digitatus* (Japan sample: 257 bp and the Philippines sample: 259 bp) was longer than those of *H. contortus* and *H. placei* (231 bp long in both species; Table 1). The magnitude of sequence variation in ITS1 between *M. digitatus* (Japan sample and the Philippines sample) and *Haemonchus* species was 15.4% and 15.7%, respectively. The magnitude of sequence variation in ITS2 was 20.9% between the *M. digitatus* sampled from Japan and *H. contortus*, and between the *M. digitatus* sampled from the Philippines and *H. placei*, 21.3% between the *M. digitatus* sampled from Japan and *H. placei*, and 20.4% between the *M. digitatus* sampled from the Philippines and *H. contortus*. The GC content of the ITS of *M. digitatus* was 32.0%, the ITS1 sequence in the Philippines sample and 32.5% in the Japan sample and 25.9% ITS2 sequence in the Philippines

Table 2. Comparison of the detection of *M. digitatus* from oval samples by two different methods

Method	Species	Oval samples									
		1	2	3	4	5	6	7	8	9	10
Egg count[a)]	GI nematodes[b)]	40	420	308	60	636	604	3076	4	8	20
	M. digitatus	24	36	304	48	50	ND[c)]	ND	ND	ND	ND
PCR assay[d)]	*M. digitatus*	+	+	+	+	+	–	–	–	–	–

a) Indicated by the egg number per 10 g of faeces. Eggs were recovered from the faeces using a sugar flotation technique. Eggs measuring more than 110 μm were counted as *M. digitatus*.
b) Containing the egg number of *M. digitatus*.
c) ND: Not detected.
d) (+) : Species-specific amplification of *M. digitatus* was detected. (–) : Not detected.

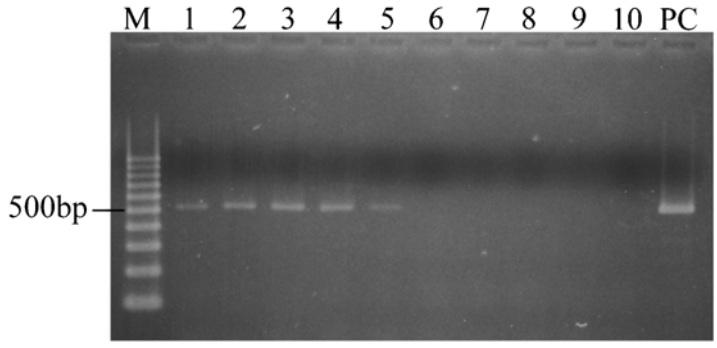

Fig. 6. PCR amplification of genomic DNA of oval sample isolated from bovine faeces. Lanes 1–10: oval samples. Lane M: 100 bp marker. Lane M: 100 bp marker. Lane PC: positive template control (DNA extract of *M. digitatus*).

sample and 26.9% in the Japan sample. These values were lower than those of *H. contortus* and *H. placei* (ITS1: 39.4% and 39.6%, respectively; ITS2: 33.3% and 33.8%, respectively). These data indicated that ITS is suitable for the differential detection of *M. digitatus*.

To detect species-specific amplification of *M. digitatus*, we designed the new primers Md1 and Md2, and evaluated their sequence using *M. digitatus* and 6 other GI nematode species (*O. ostertagi, O. radiatum, C. oncophora, T. axei, H. contortus* and *N. helvetianus*) that are frequently found in cattle (Fig. 2). The 2 primers sequences of *M. digitatus* were clearly different from the sequence of the other GI nematode species and thus, suggestive of specificity. Moreover, when species-specific PCR was performed with genomic DNA of the nematode species, the species-specific products were obtained by using the genomic DNA of *M. digitatus* isolated from an adult (from Japan and the Philippines), an L3 in faecal culture and a mixture of genomic DNA from other GI nematode species, including *M. digitatus*, without reacting these with the genomic DNA of other GI nematodes (Fig. 3). PCR using the species-specific primers of *M. digitatus* was performed to determine the efficacy of the species-specific identification at the larval and adult stages. Identification of cultured larvae is a standard procedure for diagnosing an infection. However, it is an unreliable method when applied to quantitation in the egg stage since several factors affect egg viability and development [5]. With the availability of species-specific primers, the laborious and time-consuming morphological differentiation of larvae can be supplemented with a molecular tool that can also be used for L3 genotyping. The specificity of amplification indicates that the species-specific primers could be used to differentially diagnose an *M. digitatus* infection from those caused by the more prevalent genera of GI nematodes.

The sensitivity of the PCR assay was determined by a serial dilution of genomic DNA from adult *M. digitatus* sampled from Japan. This PCR revealed a detection level of 0.001 *n*g at which a weak signal was obtained (Fig. 4). An improvement in the method enabled differential PCR to be performed even for a unicellular egg collected from an adult female; it also enables PCR analysis of the solution of a destroyed egg onto a filter paper without DNA extraction (Fig. 5). These results suggest that the PCR assay can detect the specific band even if there is only 1 egg in the oval sample from bovine faeces.

Positive PCR signals were obtained for the oval samples in which *M. digitatus* was detected by the faecal egg counts in bovine faeces from a farm infected by *M. digitatus* for several years. No specific amplification was obtained in oval samples from the farm not infected by *M. digitatus* for several years. Further, faeces contain a number of components that can inhibit the PCR; therefore, effective removal of these inhibitors is critical to achieve the desired result. In this study, the *M. digitatus* infection could be efficiently detected by the PCR assay. It was possible to isolate the egg DNA of *M. digitatus* suitable for PCR by removing the impurities by purification of the oval samples by using the sugar flotation method. Compared to the time-consuming identification of L3 from faecal cultures or eggs from faeces, the species-specific PCR was a rapid and reliable method.

In conclusion, we developed a PCR assay using species-specific primers to distinguish *M. digitatus* from common GI nematode species found in cattle, and we demonstrated that it is applicable to faeces-derived egg DNA. This DNA technique will be a major advantage for epidemiological studies and population biology, particularly in Asian countries. In the present study, efforts towards the sequencing of *M. digitatus* rDNA were made for the first time, and the practicability of the PCR assay for the diagnosis of an *M. digitatus* infection was discussed.

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