Discrimination of Three Amphistome Species by PCR-RFLP Based on rDNA ITS2 Markers

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ABSTRACT. Three bovine amphistomes, *Calicophoron calicophorum*, *Orthocoelium streptocoelium* and *Homalogaster paloniae*, are common in Japan. This study was carried out to describe ITS2 sequences in the 3 species, and to evaluate a PCR-RFLP technique based on ITS2 sequence for the species identification in single eggs of the parasites. The ITS2 sequences of the three species contained 19 variable sites including one gap. The sequence difference between species was 4.2–5.3%. The three species of amphistomes were identified based on the difference in the restriction sites of *Acc* II on the ITS2 sequence.

KEY WORDS: bovine amphistome, ITS2, PCR-RFLP.

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More than 70 species of amphistomes have been described in cattle host [12], and 3 species, *Calicophoron calicophorum*, *Orthocoelium streptocoelium* and *Homalogaster paloniae* are common in Japan [3]. Although the diagnosis of amphistome infections is carried out by the detection of eggs in fecal examination, it is impossible to identify the species accurately based on morphological characters of the eggs. However, the discrimination of the eggs at species levels is important to make epidemiological surveys and to assess the efficacy of anthelmintics against each amphistome species, for example, since *H. paloniae* is of known as the pathogen causing bovine intestinal paramphistomiasis (gastrodisciasis), not only by young but also adult flukes [9]. Current DNA-based techniques have provided accurate tools for the species identification of parasitic helminths [5, 7, 11]. The second internal transcribed spacer (ITS2) of ribosomal DNA has been shown to be a useful genetic marker in species identification of parasites [2, 8, 10]. Further, in the nematoda of the genus *Strongylus*, the PCR-RFLP technique based on ITS2 markers enabled single eggs to be identified at species levels [2]. ITS2 sequences of amphistomes have not been reported up to date. The purpose of this study is first to describe ITS2 sequence in *C. calicophorum*, *Orthocoelium streptocoelium* and *H. paloniae*, and secondly to evaluate a PCR-RFLP method for the species identification in single eggs from the flukes.

Adult worms were isolated from naturally infected cattle sacrificed in Iwate, Aomori, Akita and Hyogo Prefecture, Japan (Table 1). The parasites were identified morphologically to the species-level and stored at –80°C or in 70% ethanol until needed. Fresh eggs of these parasites (Isolate code: Cc1, Cc2, Os1, Os2, Hp1) were collected from the uterus. Genomic DNA was prepared from single or pooled adults by the followings. The parasites were homogenised in a buffer (50 mM Tris-HCl, 100 mM EDTA, 200 mM NaCl, 0.5% SDS, pH 9.0) with proteinase K (150 µg/ml),

and incubated at 65°C for 4 hr. The DNA was extracted in phenol-chloroform, precipitated in ethanol, and then resuspended in an appropriate volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA solution was treated with RNase A (15 µg/ml). The ITS2 plus primer flanking sequence was amplified by PCR from adult DNA or directly from single eggs. PCR primers used to generate a fragment spanning 5.8S, ITS2 and 28S were the forward, ITS2-F (5'-TGTGTCGATGAAGAGCGCAG-3') and the reverse, ITS2-R (5'-TGGTTAGTTTCTTTTCTCCGC-3'). PCR amplification was performed in a total reaction volume of 50 µl containing template DNA and the following PCR mix: 10x *Taq* buffer, 25 pmol of each primer, 200 µM each of dATP, dCTP, dTTP and dGTP, 2.0 mM MgCl₂ and 2.5 units of *Taq* polymerase (Greiner Japan). The PCR condition was 94°C for 2 min initially, then 30 or 35 cycles of 94°C for 1 min, 53–55°C for 90 sec and 72°C for 1 min, and finally 72°C for 10 min. DNA from single eggs was amplified directly without extraction according to Gasser *et al.* [6].

Table 1. Isolates of three amphistomes species used in the study

Species	code	No. adults	Origin
<i>C. calicophorum</i>	Cc1	1	Iwate
	Cc2	1	Iwate
	Cc3	1	Iwate
	Cc4	1	Aomori
	Cc5	1	Aomori
	Cc6	1	Akita
	Cc7	1	Hyogo
<i>O. streptocoelium</i>	Os1	3	Iwate
	Os2	2	Iwate
	Os3	3	Aomori
	Os4	2	Akita
<i>H. paloniae</i>	Hp1	1	Iwate
	Hp2	1	Iwate

<i>C. calicophorum</i>	1	TGAATTAATGTGAAGTGCATACTGCTTTGAACATCGACATCTTGAACGCACATTGCGGCC
<i>O. streptocoelium</i>	1	TGAATTAATGTGAAGTGCATACTGCTTTGAACATCGACATCTTGAACGCACATTGCGGCC
<i>H. paloniae</i>	1	TGAATTAATGTGAAGTGCATACTGCTTTGAACATCGACATCTTGAACGCATATTGCGGCC
5.8 S ←] [→ ITS2		
<i>C. calicophorum</i>	61	ACGGGTTTTCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCAGACGCCCA
<i>O. streptocoelium</i>	61	ACGGGTTTTCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCAGACGCCCA
<i>H. paloniae</i>	61	ACGGGTTTTCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCAGACGCCCA
* * *		
<i>C. calicophorum</i>	121	AAAAGTCGTGGCTTGGGATCTGCCAGCTGGCGTGATTTCCTCTGTGGTTCGCCACGTGAG
<i>O. streptocoelium</i>	121	AAAAGTCGTGGCTTGGGATCTGCCAGCTGGCGTGATTTCCTCTGTGGTTCGCCACGTGAG
<i>H. paloniae</i>	121	AAAAGTCGTGGCTTGGGATCTGCCAGCTGGCGTGATTTCCTCTGTGGTTCGCCACGTGAG
* * *		
Acc II		
<i>C. calicophorum</i>	181	GTGCCAGATCTATGGCGTTTTCCTAATGTCTCCGGACACAACCGCGTCTTGCTGGTAGCG
<i>O. streptocoelium</i>	181	GTGCCAGATCTATGGCGTTTTCCTAATGTCTCCGGACACAACCGCGTCTTGCTGGTAGTG
<i>H. paloniae</i>	181	GTGCCAGATCTATGGCGTTTTCCTAATGTCTCCGGACACAACCGCGTCTTGCTGGTAGCG
* * *		
<i>C. calicophorum</i>	241	CAGACGAGGGTGTGGCGGTAGAGTCGTGGCTCAGTGAAGTGA-ATGGTAGCACGCTCTG
<i>O. streptocoelium</i>	241	CAGACGAGGGTGTGGCGGTAGAGTCGTGGCTCAATGAAGTGA-ATGGTAGCACGCTCTA
<i>H. paloniae</i>	241	CAGACGAGGGTGTGGCGGTAGAGTCGTGGCTCAGTGAAGTGAATGTATGGTAGCACGCTCTG
* * *		
Acc II		
<i>C. calicophorum</i>	300	CTGTTGTGCCTTTGTTAGTGTAAGTGGTTTGAGATGCTATTGCTGTCCGCGCAATTACGA
<i>O. streptocoelium</i>	300	CTGTTGTGCCTTTGTTAGTGTAAGTGGTTTGAGATGCTATTGCTGTCCGCGCAATTAGGA
<i>H. paloniae</i>	301	CTGTTGTGCCTTTGTTAGTGTAAGTGGTTTGAGATGCTATTGCCGTCCGCCAAATTATGA
* * *		
ITS2 ←] [→ 28 S		
<i>C. calicophorum</i>	360	TCACCTACTATGGTGTTCCTGTACCTGACCTCGGATCAGACGTGAATACCCGCTGAACCTT
<i>O. streptocoelium</i>	360	TCACCTACTGTGGTGTTCCTGTACCTGACCTCGGATCAGACGTGAGTACCCGCTGAACCTT
<i>H. paloniae</i>	361	TCACCCACTGTGGTGTTCCTGTACCTGACCTCGGATCAGACGTGAATACCCGCTGAACCTT
* * *		
<i>C. calicophorum</i>	420	AAGCATATCA 429
<i>O. streptocoelium</i>	420	AAGCATATCA 429
<i>H. paloniae</i>	421	AAGCATATCA 430

Fig. 1. Sequence alignments of ITS2 including partial 5.8S and 28S for three species of amphistomes. Sequences are displayed at 5'-3'. The restricted site of *Acc II* is underlined. Nucleotide difference is indicated by "*". Alignment gaps are indicated by "-".

Briefly, eggs were washed five times in distilled water, and single eggs were pipetted in 6 μ l H₂O into separate 0.2 ml PCR tubes. The tubes were subjected to 3 cycles of freezing (-80°C) and boiling (100°C), and then centrifuged. Forty-four μ l of PCR mix was then added to the tube and subjected to the PCR condition described above. PCR products were electrophoresed in 1% agarose gel with ethidium bromide, then purified using Qiaex gel extraction kit (Qiagen), and sequenced using Cycle sequencing kit (Takara Biomedicals) according to the manufacture's protocol. The same primers employed for primary amplification were used for sequencing. The cycle condition was 95°C for 1 min, then 25 cycles of 95°C for 40 sec, 55°C for 40 sec and 72°C for 40 sec. Each DNA sample was sequenced at least 2 times using both forward and reverse primers. Restriction maps based on the sequence data were constructed using the computer program Genetyx-Mac 6.0.2 (Software Development Co., Ltd.). PCR products amplified from single eggs were digested with 8 and 10 units of endonuclease, *Acc II* (Takara Biomedicals) in a volume of 50 μ l at 37°C for 3 hr. The digested fragments were electrophoresed on 3% agarose

gels, stained with ethidium bromide and photographed.

The sequences (430 bp) of ITS2 including partial 5.8S and 28S of the three amphistomes are shown in Fig. 1. No intra-specific variation in the sequences of each species was detected, though genomic DNAs from adults and eggs were used. Similarly, in the trematodes of the genus *Schistosoma* [4], *Echinostoma* [10] and *Paragonimus* [1], ITS2 sequence has exhibited little or no intra-specific variation. The length of the ITS2 sequence was 284 bp in *C. calicophorum* and *O. streptocoelium*, and 285 bp in *H. paloniae*. The ITS2 sequences contained 19 variable sites including one gap, and the degree of variation was 4.2–5.3% among the three species. The restriction map revealed that 3 amphistomes shared the restriction sites for a number of endonucleases, but they showed difference in the *Acc II* site (Fig. 1). The size of PCR products was estimated as approximately 480 bp for the three amphistomes species (Fig. 2). As expected from the restriction maps, the PCR products digested with *Acc II* showed the equivocal discrimination among the three species. The digested products produced three fragments of approximately 250 bp, 125 bp and 105 bp for *O. streptocoe-*

Fig. 2 PCR-RFLP patterns of the products amplified for three species of amphistomes. The products were digested with endonuclease *Acc* II. Lane 1: *C. calicophorum*; Lane 2: *H. paloniae*; Lane 3: *O. streptocoelium*. Position of DNA marker, Φ x 174 *Hae* III is shown in the right side.

lium and two (250 bp and 230 bp) for *H. paloniae*, while the product for *C. calicophorum* was not digested.

The present study revealed that single eggs of three species of amphistomes could be distinguished by PCR-RFLP based on the ITS2 sequence. Therefore, this PCR-RFLP technique would provide a good tool for the diagnosis and epidemiological study at the species level of bovine amphistomiasis, although further molecular study is needed to clarify ITS2 sequences in the other species of amphistomes

occurring in Japan.

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