Reduced Efficacy of Ivermectin Treatments in Gastrointestinal Nematode Infections of Grazing Cattle in Japan

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ABSTRACT. Fecal egg count reduction tests (FECRT) and larval migration inhibition tests (LMIT) were conducted to assess the efficacy of ivermectin (IVM) against gastrointestinal nematodes on 2 cattle farms in northern Japan in 2009 and 2010. Twelve to 20 calves on each farm were treated topically with 0.5 mg IVM/kg 2 (Farm 2) or 4 times (Farm 1) during the grazing season (May–October). On Farm 1, fecal egg count (FEC) reduction at 14 days post-treatment ranged from 16 to 87% in 2009 and from 24 to 96% in 2010, with relatively low reductions in August and October (16–53%). Conversely, IVM treatment on Farm 2 reduced FEC by 97% in September 2009. Larvae obtained from fecal cultures and identified by PCR-RFLP analysis revealed that the dominant species on both farms prior to IVM administration was *Cooperia oncophora*. In 2009, the FEC reduction of *C. oncophora* on Farm 1 decreased from 85% in May to 56% in August. In 2010, the reduction in *C. oncophora* in August was 28%. In the LMIT using larvae collected from the fecal cultures on Farm 1 in May and August 2009, the EC_{50} value of IVM in *C. oncophora* in August (0.892 μg/m*l*) was 3 times higher than that in May (0.296 μg/m*l*). The results of the LMIT corroborated the FECRT data, indicating the presence of IVM-resistant *C. oncophora* on Farm 1, at least in August. This is the first report of IVM-resistant nematodes in Japanese cattle.
KEY WORDS: anthelmintic resistance, cattle, *Cooperia*, ivermectin, Japan

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Gastrointestinal nematodes in grazing cattle are important causative agents of disease and are responsible for considerable economic losses around the world. The high efficacy and broad-spectrum nature of the anthelmintic agent, ivermectin (IVM), has meant that it is widely used to control nematode infections in livestock. However, increased IVM resistance in bovine nematodes, primarily among members of the genus *Cooperia*, has been reported in countries, such as New Zealand [24], United Kingdom [3], Argentina [1, 10], Brazil [20], Germany, Belgium and Sweden [7], the United States [11] and Australia [15, 19]. Demeler *et al.* [8] reported that monitoring anthelmintic resistance is a key component of nematode control in the livestock industry.

In Japan, several species of gastrointestinal nematodes, including *Cooperia* spp., infect cattle [18], and the control of nematode infections has been highly dependent upon IVM treatment for the last 25 years. Nonetheless, no surveys of IVM resistance in bovine nematodes have been conducted to date. The present study therefore examined the efficacy of IVM treatment against bovine gastrointestinal nematodes in Japan using the fecal egg count reduction test (FECRT) and the larval migration inhibition test (LMIT).

MATERIALS AND METHODS

Animals and treatment: Two farms rearing Japanese Shorthorn cows in Aomori Prefecture in northern Japan were surveyed from May 2009 to October 2010. Ivermectin pour-on formulation has been used on both farms for nematode control since before the start of this survey. Farm 1 routinely used the drug 3 or 4 times a year during the grazing season over the previous 4 years, but a detailed history of the anthelmintic regime was not available for Farm 2. Farm 1 had 16 and 20 calves aged between 9 and 10 months at the beginning of the experiment in 2009 and 2010, respectively, while Farm 2 had 12 animals aged between 10 and 14 months in 2009. The calves on Farm 1 were kept on separate pastures with their mothers after parturition until late autumn, before being housed indoors during winter and then used for this study in the following spring. None of the calves were treated with anthelmintics before the start of the experiment. Conversely, the calves on Farm 2 that were used in this experiment were obtained from several of the surrounding farms, and their previous management histories (e.g. anthelmintic treatment) were unknown. The calves on both farms were grazed on pastures from May to October in this survey.

The animals were treated with anthelmintic agents 2 or 4 times during the grazing season. The calves on Farm 1 were treated at weeks 2, 8, 15 and 25 post-turn out (May, June or July, August and October, respectively), while those on Farm 2 were treated at the beginning of pasturage (May)
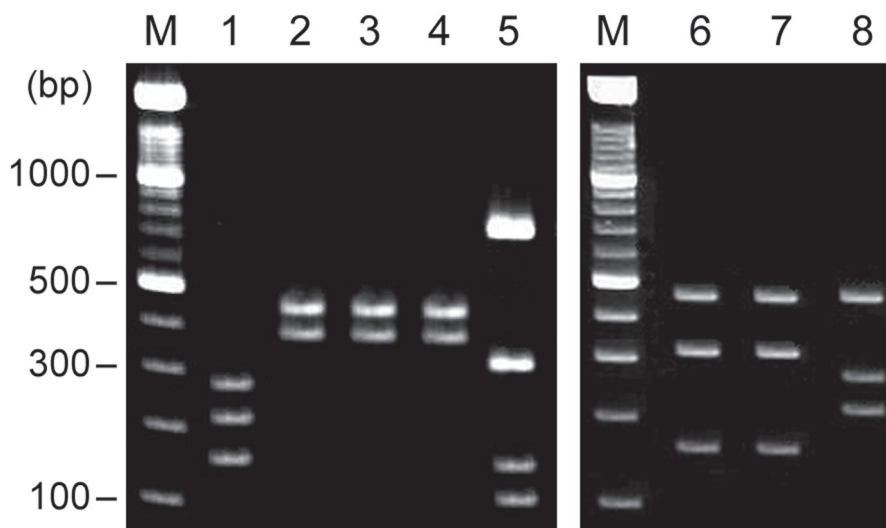
Fig. 1. PCR-RFLP analysis of ITS rDNA digested with the endonuclease *Hinf* I to show interspecific differences in restriction patterns. Lane 1, *Cooperia punctata*; lanes 2-4, *C. oncophora*; lane 5, *Ostertagia* ostertagi; lanes 6 and 7, *Mecistocirrus digitatus*; lane 8, *Oesophagostomum radiatum* and lanes M, 100 bp ladder marker.

and at 20 weeks post-turn out (September). Individual calves were marked with ear tags, weighed or measured with a girth tape, and treated topically with 0.5 mg IVM/kg bodyweight (Ivomec® Topical, Merial Japan, Tokyo, Japan).

Fecal egg count reduction test (FECRT): Fecal samples were obtained per rectum from individual animals at days 0 and 14 post-treatment and immediately processed for strongyle egg counts and larval culture. Fecal egg counts (FEC) were performed on each sample (4 g) using a sugar centrifugal flotation technique [14]. The obtained egg counts were divided by 4 to give the number of eggs per gram (EPG) of feces.

The RESO software program recommended by the World Association for the Advancement of Veterinary Parasitology was used to calculate the reduction in fecal strongyle egg count as a percentage [4]. The reductions were calculated as follows: $100 \times (1 - $ Day 14 mean FEC/Day 0 mean FEC).

Positive fecal samples with strongyle counts greater than 20 EPG were used for larval culture. Nematode eggs obtained using the sugar centrifugal flotation technique were washed with water and centrifuged for 3 min at $1,160 \times g$ before being mixed with a small amount of fecal fluid obtained from cattle that had not been treated with anthelmintics (parasite eggs and debris were removed by centrifugation for 10 min at $1,670 \times g$). The solution was plated on 2.5% Bacto Agar medium and incubated at 25°C for 7 days. Each culture was then flooded with water, and infective third stage larvae (L3) were collected under a dissecting microscope.

Genomic DNA isolated from 20 to 30 of the larvae in each sample (i.e. a total of 70 to 150 L3 were collected for each sample date) was used to identify the obtained larvae to species by PCR-RFLP analysis [17]. The internal transcribed spacer region (ITS) was amplified by PCR, and polymor-

phisms of the *Hinf* I restriction enzyme sites were analyzed to identify larvae to species (Fig. 1). In addition, the nucleotide sequences of amplified products were determined and compared for species identification. These data were used to calculate the species-specific percentage of fecal egg count reduction (FECR).

Larval migration inhibition test (LMIT): On Farm 1, the strongyle-positive fecal samples obtained in 2009 from 10 and 8 calves at weeks 2 and 15 post-turn out (day 0 post-treatment), respectively, were used for the LMIT. The nematode eggs obtained from each sample were cultured to obtain infective L3 larvae, equal numbers of which were collected from each culture to produce a pool of L3 larvae for testing. The LMIT was performed following the methods of Gatongi *et al.* [12] and Wagland *et al.* [23]. Briefly, a commercial formulation of IVM (Ivomec® Injection, Merial Japan, Tokyo, Japan) was serially diluted with dimethylsulfoxide (DMSO) to give final concentrations ranging from 0.1 to 30 µg IVM /m*l* which were then used for this experiment. About 250 exsheathed L3 larvae were pre-incubated in test solutions at 23°C for 3 hr. The larvae, which were suspended in the test solutions, were then transferred to larval migration inhibition (LMI) tubes and allowed to migrate through a 20-µm mesh sieve at the open bottom of each tube, into the well of a 48-well plate at 23°C for 17 hr. After incubation, the LMI tubes were removed from the plate, and the migrated larvae in each well were recovered and counted. All of the tests were performed in triplicate, and each included a negative control (1% DMSO solution). The recovered larvae were then identified by PCR-RFLP analysis, and the species-specific percentage of larvae that had been inhibited was calculated as follows: $100 \times (1 - $ number of migrated larvae in test solution/number of migrated larvae in negative control).

Table 1. Fecal egg count reduction (FECR) of strongyles after treatment of cattle with ivermectin pour-on

Farm No.	Year	Month	$N^{a)}$	FEC Day 0 mean epg (min–max)	FECR%[b]
1	2009	May	16	47 (10–129)	87 (74–93)
		June	16	70 (12–227)	76 (40–91)
		August	16	185 (6–666)	53 (0–79)
		October	16	64 (1–207)	16 (0–69)
	2010	May	20	155 (67–261)	96 (92–98)
		July	20	24 (1–98)	81 (53–92)
		August	20	141 (4–489)	24 (0–63)
		October	20	50 (3–200)	42 (0–81)
2	2009	May	12	ne[c]	–
		September	12	21 (2–71)	97 (94–99)

a) Number of animals. b) The 95% confidence intervals are given in parentheses. c) Not examined.

Table 2. Fecal egg count reduction (FECR) of *Cooperia oncophora* (C. o.), *C. punctata* (C. p.) and *Ostertagia ostertagi* (O. o.) after treatment of cattle with ivermectin pour-on

Farm No.	Year	Month	$N^{a)}$	Larval culture Day 0 C. o.	C. p.	O. o.	Others	FECR%[b] C. o.	C. p.	O. o.	Others	Larval culture Day 14 C. o.	C. p.	O. o.	Others
1	2009	May	16	77%	8%	14%	1%[c]	85 (71–92)	86 (72–93)	98 (96–99)	86 (72–93)	88%	9%	2%	1%[c]
		August	16	65%	12%	23%	1%[d]	56 (0–81)	0 (0–30)	100	100	60%	40%	0%	0%
	2010	August	20	86%	5%	9%	0%	28 (0–65)	0 (−29–0)	100	–	81%	19%	0%	0%
2	2009	September	12	57%	7%	28%	9%[c]	e)	e)	e)	e)				

a) Number of animals. b) The 95% confidence intervals are given in parentheses. c) *Mecistocirrus digitatus*. d) *Oesophagostomum radiatum*. e) Percent reduction not calculated because of insufficient larvae at day 14.

The dose-response data were analyzed using a non-linear regression (four-parameter logistic equation, SigmaPlot®), and the effective concentration of IVM required to inhibit the migration of 50% of the larvae (EC_{50}) was estimated.

RESULTS

Fecal egg count reduction test: The mean EPG values and FECRT results for strongyles from the 2 farms in 2009 and 2010 are shown in Table 1. The mean EPG value for each test at day 0 ranged from 21 to 185 and was relatively high on Farm 1 in August. The FECR observed on this farm at day 14 post-treatment ranged from 16 to 87% in 2009 and 24 to 96% in 2010, with the smallest reductions (≤53%) observed in August and October of both years. On Farm 2, IVM treatment reduced FEC of strongyles by 97% in September 2009.

The species-specific FECRT results for Farm 1 in May and/or August and for Farm 2 in September are shown in Table 2. The predominant species prior to treatment was *C. oncophora*, with smaller numbers of *C. punctata*, *Ostertagia ostertagi* and other nematodes (*Mecistocirrus digitatus* and *Oesophagostomum radiatum*) also observed in each test. In 2009, the FECR of *C. oncophora* and *C. punctata* observed on Farm 1 at day 14 post-treatment decreased from 85% and 86% in May to 56% and 0% in August, respectively. The

reductions in August 2010 were also very small, with efficacies of 28% (*C. oncophora*) and 0% (*C. punctata*) recorded. The efficacy against *Os. ostertagi* was 98 to 100% in all tests. The species-specific FECR on Farm 2 in September 2009 was not calculated due to insufficient numbers of larvae being obtained at day 14 post-treatment.

The results of the LMIT for Farm 1 in 2009 are shown in Fig. 2. In May and August, the FECR of strongyles in the calves used for the LMIT was 86% and 26%, and the FECR of the dominant species, i.e., *C. oncophora*, was 84% and 31%, respectively. The LMI percentages obtained for the strongyle larvae exhibited dose-response trends in May and August, and dose-dependent efficacy was also observed in *C. oncophora*. The EC_{50} value obtained for *C. oncophora* in August (0.892 µg/ml, 95% confidence interval (CI) 0.763–0.995 µg/ml) was 3-fold higher than the value observed in May (0.296 µg/ml, 0.268–0.324 µg/ml). The 95% CI of the EC_{50} values for *C. oncophora* did not overlap, indicating that these differences were significant.

DISCUSSION

In the present study, the FECR of strongyles after treatment with IVM pour-on on Farm 1 ranged from 16% to 96% in 2009 and 2010. According to Coles *et al.* [4], anthelmintic
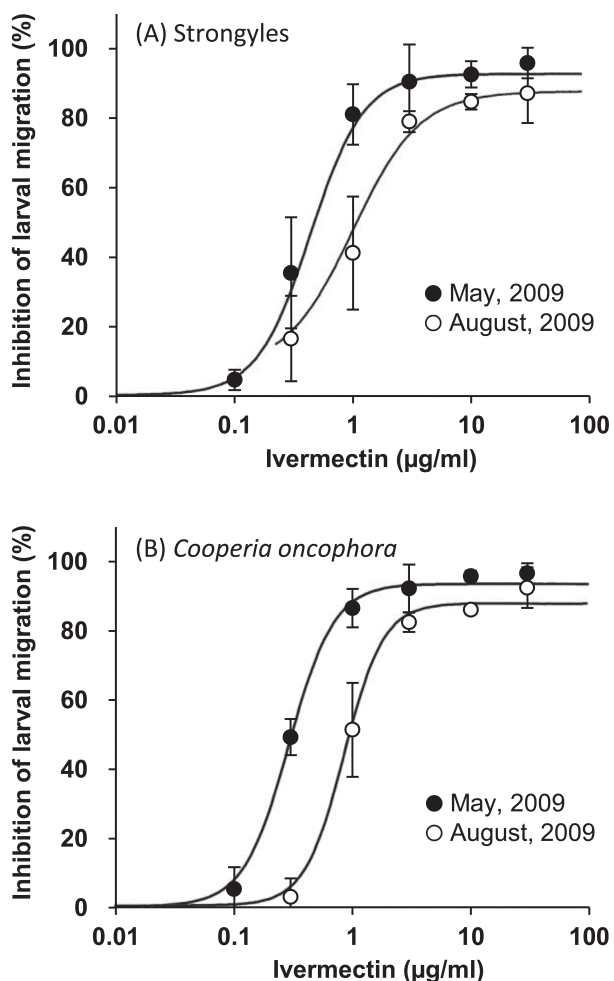
Fig. 2. Dose-response curves of LMIT for strongyle larvae obtained from Farm 1 in May and August 2009 using ivermectin. Curves for (A) mixed species L3 and (B) *C. oncophora* L3 identified using PCR-RFLP analysis.

resistance is confirmed, if the percentage reduction in egg count is less than 95% and the 95% CI is less than 90%. In addition, Soutello *et al.* [20] considered that resistance was indicated if FECR was less than 90% when FEC were low and the number of animals per test group was small. Thus, the very low efficacy observed for IVM against the strongyles on Farm 1 after June or July suggests the development of IVM resistance. Conversely, the relatively high IVM efficacies observed in May indicate the presence of IVM-susceptible nematodes in the calves that were placed in their own pastures after birth.

The species composition of the larvae observed prior to treatment in this study was typical for grazing cattle in northern Japan, with *C. oncophora* being dominant, and *C. punctata, Os. ostertagi, M. digitatus* and *Oe. radiatum* also being present. Similarly, *C. oncophora* is widespread in many countries, and most instances of IVM resistance in gastrointestinal nematodes of cattle are attributed to this nematode

species [7, 9, 10, 16]. In these reports, IVM resistance in *C. oncophora* (6–83% reduction in FEC) was observed in calves treated with the pour-on formulation of the drug in New Zealand. The species-specific FECR due to IVM treatment on Farm 1 was 28–85% for *C. oncophora*, which suggests that the nematodes are resistant to IVM. On the other hand, IVM administration in calves was effective against *O. ostertagi*, even though IVM resistance in this species has been reported in several countries [7, 19, 21, 22]. However, it is possible that the percentage reductions calculated using pre- and post-treatment egg counts in this survey may have been under-estimated in instances where the calves were more heavily infected with *Cooperia* spp. (predominant nematodes prior to treatment) in August 2009 and 2010 in Farm 1, as the prepatent periods of *Cooperia* spp. are relatively short; 17–22 days for *C. oncophora* [13] and 11–16 days for *C. punctata* [2]. Consequently, we also performed *in vitro* LMIT to confirm IVM resistance in the nematode parasites, especially in *C. oncophora*.

According to Wagland *et al.* [23], the *in vitro* LMIT is useful for detecting nematode resistance to anthelmintics that affect the nervous system of nematode worms. Demeler *et al.* [5] reported that the LMIT successfully differentiated between susceptible and IVM-resistant *C. oncophora* isolates from cattle; the EC_{50} value of IVM-resistant nematodes was significantly higher than that of susceptible nematodes. Furthermore, the EC_{50} value for IVM in the LMIT correlates well with the FECR of nematode parasites after IVM treatment in cattle [6]. In the present study, although a LMIT was used to evaluate the IVM resistance of mixed-species larvae recovered from the cattle on Farm 1 in 2009, the species-specific LMI data obtained using molecular markers revealed that the EC_{50} value of *C. oncophora* was significantly higher in August than in May. In addition, the results of the LMIT corroborated the data obtained by the FECRT, indicating the presence of IVM-resistant *C. oncophora* on Farm 1, at least in August, in this study. This is the first report of IVM-resistant nematodes in Japanese cattle.

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