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Experimental selection for ivermectin resistance in *Ostertagia ostertagi* in cattle

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Abstract

Recent reports of suspected ivermectin (IVM) resistance in *Ostertagia ostertagi* have highlighted the need for research into the mechanisms of IVM resistance. However, there are no reports of resistant field isolates of *O. ostertagi*, which have been characterized for molecular research. Therefore, an anthelmintic susceptible *O. ostertagi* population was selected for IVM resistance by repeatedly exposing the population to subtherapeutic and therapeutic levels of IVM over 10 generations. In each selection round, a group of calves was infected with the progeny of the previous IVM-selected *O. ostertagi* population. In the last selection round a therapeutic IVM dose (0.2 mg/kg BW) only reduced the faecal egg counts by 57% and 65% on days 7 and 14 after treatment, respectively. In contrast, the therapeutic IVM dose was 100% effective at eliminating the parental IVM-susceptible isolate.

Keywords: *Ostertagia ostertagi*; Nematode; Anthelmintic resistance; Macrocyclic lactones; Ivermectin; Selection

1. Introduction

The introduction of ivermectin (IVM) in 1981, a macrocyclic lactone (ML) which has excellent broad-spectrum activity against nematodes and several parasitic arthropods, opened new markets and new management options for parasite control. However, the intensive and frequent use of these anthelmintics has resulted in the development of ML resistance in several hosts (Geary, 2005; Kaplan, 2004; McKellar and Jackson, 2004). In small ruminants, anthelmintic-resistant nematodes are already a serious problem worldwide (Jackson and Coop, 2000). IVM resistance was first reported in *Haemonchus contortus* and later in *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* (Kaplan, 2004). Reports of ML resistance in nematodes of cattle have been less common, and the general belief is that resistance is not yet an important issue in this host (Kaplan, 2004). However, reports of ML-resistant nematodes in cattle are emerging in several parts of the world and resistance will probably become more widespread. In recent years, ML resistance in *Cooperia* spp. has become increasingly common in Brazil (Echevarria and Pinheiro, 1999), Argentina (Anziani et al., 2001; Fiel et al., 2001; Mejia et al., 2003), New Zealand (Familton et al., 2001; Loveridge et al., 2003; Vermunt et al., 1996), the US (Gasbarre et al., 2004) and the UK (Coles et al., 1998, 2001). To date, the first cases of suspected emerging IVM resistance in *O. ostertagi* are reported in New Zealand (Mason and McKay, 2006; Waghorn et al., 2006) and Argentina (Suarez and Cristel, 2007). These reports have

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not yet been confirmed with controlled field studies. In the light of these recent findings ML resistance in *O. ostertagi* might be more common than currently recognized. Therefore, it is important to investigate the increase in resistance alleles before ML resistance in *O. ostertagi* is widespread. Due to the lack of sensitive tests, the detection of anthelmintic resistance is currently not possible at an early stage. The development of sensitive molecular tests requires knowledge of the mechanisms of ML resistance, but no resistant field isolates of *O. ostertagi* has been characterized for molecular research. For that reason, the aim of this study was to experimentally select a laboratory IVM-resistant *O. ostertagi* isolate in cattle. In the near future, this IVM-resistant isolate, derived from a parental IVM-susceptible isolate, will be used to examine the mechanisms of ML resistance in *O. ostertagi* in cattle.

2. Materials and methods

2.1. Parasite isolates

The anthelmintic susceptible parental *O. ostertagi* population was initially isolated from a commercial Belgian dairy farm in 1987 and stored in liquid nitrogen until 1993. Thereafter the population was maintained in the laboratory by passage through helminth-free calves.

2.2. Experimental trial design

An anthelmintic susceptible *O. ostertagi* population was selected for IVM resistance by repeatedly exposing the population to subtherapeutic and therapeutic levels of IVM over 10 generations. For each trial helminth-free Holstein-cross-breed calves between 5 and 8 months old were orally infected with 50,000 infective larvae (2× 25,000 L3 in 10 ml tap water). The calves were fed maize silage and hay ad libitum and had free access to drinking water.

In the first selection round sixteen helminth-free calves, infected with the parental IVM-susceptible *O. ostertagi* isolate (IVMS), were randomized over four groups of four animals based on their egg counts on day 25 post-infection. The animals were weighted twice (days 24 and 25 post-infection) with a balance calibrated with an accuracy of 1 kg. On day 25 after infection each group of calves was treated subcutaneously with a different subtherapeutic IVM dose (0.1, 0.05, 0.025 and 0.0125 mg/kg Ivomec Merial®) to assess the efficacy of IVM against the IVMS isolate. The IVM dose was calculated based on the mean weight of all the animals on days 24 and 25 post-infection. Blood samples were collected from each calf on 2, 5 and 10 days post-treatment to determine the IVM plasma levels. Faecal egg counts (FEC) were performed on day 25 after infection and weekly (on 7, 14, 21 days) after IVM treatment. To obtain the infective third-stage larvae (L3) for the next generation, the total faecal output of the group of calves which gave the desired egg count reduction (<95% FEC reduction for the first selection round) was collected from days 3–21 post-treatment and cultured. Infective L3 were collected using baermannisation. The L3 progeny of the first five and the eight selection rounds was passaged through a helminth-free calf to produce sufficient infective larvae for the sequential selection round. For each sequential selection round, a group of calves was infected with the progeny of the previous IVM-selected *O. ostertagi* isolate (2× 25,000 L3 in 10 ml tap water) (Table 1). The IVM doses gradually increased from 0.0125 to 0.2 mg/

Table 1
Overview of the sequential selection rounds: the number of calves per selection round, the IVM doses, area under the curve (AUC value) of the IVM plasma concentration, the percentage reduction in faecal egg counts (FEC) on days 7, 14 and 21 post-treatment

Selection round	Number of calves	IVM dose (mg/kg BW)	AUC of IVM plasma (ng day ml^{-1}) (±S.D.)	Reduction in FEC on day 7 (%) (range)	Reduction in FEC on day 14 (%) (range)	Reduction in FEC on day 21 (%) (range)
1	4	0.0125	13.7 (±1.9)	94.9 (93–98)	88.2 (71–94)	93.1 (86–98)
2	6	0.025	30.7 (±9.9)	94.5 (79–100)	93.4 (79–100)	86.8 (79–100)
3	5	0.05	74.3 (±33.5)	100 (100–100)	99.5 (98–100)	100 (100–100)
4	4	0.075	52.9 (±20.0)	76.1 (50–100)	70.0 (0–100)	56.9 (0–100)
5	4	0.1	87.3 (±42.1)	99.2 (98–100)	99.3 (98–100)	99.1 (98–100)
6	3	0.1	86.8 (±35.7)	70.5 (25–96)	65.9 (25–91)	81.3 (50–99)
7	3	0.1	98.1 (±19.6)	89.0 (71–98)	88.8 (70–98)	81.5 (50–98)
8	5	0.2	111.1 (±48.8)	90.0 (50–100)	79.5 (0–100)	ND[a]
9	6	0.2	252.9 (±58.2)	87.8 (50–100)	91.6 (75–100)	ND[a]
10	6	0.2	149.4 (±34.9)	57.2 (0–92)	65.4 (0–94)	ND[a]

[a] In the last three selection rounds (0.2 mg/kg BW IVM) the calves were euthanized on day 17 to collect the adult *Ostertagia ostertagi* from the abomasum.

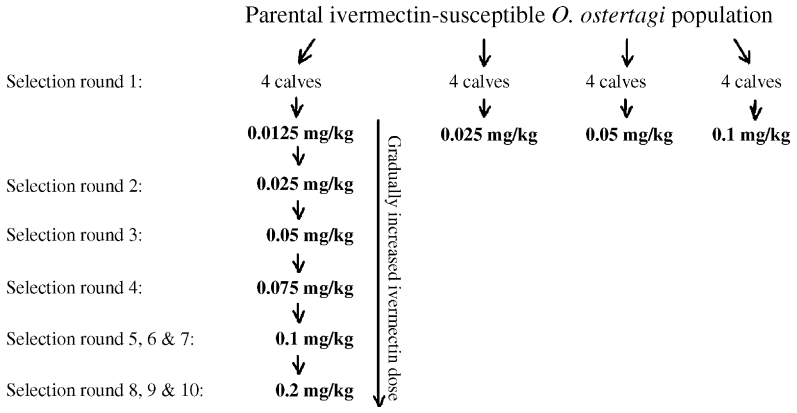

Fig. 1. Schematic diagram of the experimental selection for IVM resistance in *Ostertagia ostertagi*.

kg body weight (BW) over ten generations (Fig. 1) until an efficacy of <90% with the therapeutic dose (0.2 mg/kg BW) was reached.

In the last selection round a controlled efficacy test was conducted. Twelve helminth-free calves were randomly divided in two groups. One group was infected with the parental IVMS isolate and one with the progeny of the IVM-resistant (IVMR) isolate from selection round nine. The two groups were treated subcutaneously with the therapeutic IVM dose on day 25 post-infection to assess the efficacy of IVM against the IVMS and IVMR isolates. The IVM dose was calculated based on the mean weight of all the animals on days 24 and 25 post-infection. Faecal samples and blood samples were collected as described above. Individual faecal cultures were made on day 25 post-infection and days 7 and 14 post-treatment. The two groups of calves were euthanized on day 17 after treatment and the *O. ostertagi* were collected from the abomasum.

2.3. Pharmacokinetic analysis

In each selection round the IVM levels of all the calves were analyzed in plasma samples collected on days 2, 5 and 10 after IVM treatment. All the blood samples were centrifugated at 3000 × *g* for 15 min and plasma was stored at −20 °C until analysis. The IVM plasma concentrations were determined by high performance liquid chromatography (HPLC) with automated solid phase extraction and fluorescence detection according to a previously described method (Alvinerie et al., 1993; Lifschitz et al., 2000). The limit of quantification of the IVM analysis method was 0.1 ng/ml and the coefficient of variation was 3.5%. The areas under the concentration–time curves (AUC) for IVM in plasma were calculated by the linear trapezoidal rule and expressed as ng day ml^{-1}. A Spearman rank correlation test was used to assess the relationship between the administered IVM dose and the bovine plasma concentration of IVM (AUC). Probability (*P*) values <0.01 were considered to indicate significant correlations.

2.4. Efficacy of ivermectin

The faecal egg output was determined using a modified McMaster technique (Thienpont et al., 1979) combined with the Cornell-Wisconsin technique (Egwang and Slocombe, 1981) with a sensitivity of 12.5 eggs per gram (EPG). The percentage efficacy of the IVM treatment was assessed by the new individually based faecal egg count reduction test (FECRT) proposed by Cabaret and Berrag (2004). This method is based on an individual evaluation of the egg counts before and after treatment without untreated controls and where each calf serves as its own control. The equation of the formula used to determine the percentage reduction in FEC is: $i\text{FECRT} = (1/n)\sum 100(1 - [T_{i_2}/T_{i_1}])$, where T_{i_2} is post-treatment and T_{i_1} is pre-treatment egg count in host *i* from a total *n* calves. IVM resistance was defined as a less than 95% reduction in FEC post-treatment (Coles et al., 1992, 2006). As noted above, the relationship between the IVM efficacy and the bovine plasma concentration of IVM (AUC) was assessed. Probability (*P*) values <0.05 were considered to indicate significant correlations.

In the last selection round the IVM efficacy was also assessed by the reduction in larval counts and by the worm counts of the treated IVMS- and IVMR-infected calves. To assess the reduction in larval counts 1.5% of the larval cultures (day 25 post-infection, days 7 and 14 post-treatment) of each calf was counted and the same formula as for the FECRT was used. The two groups of calves were euthanized on day 17 after treatment.

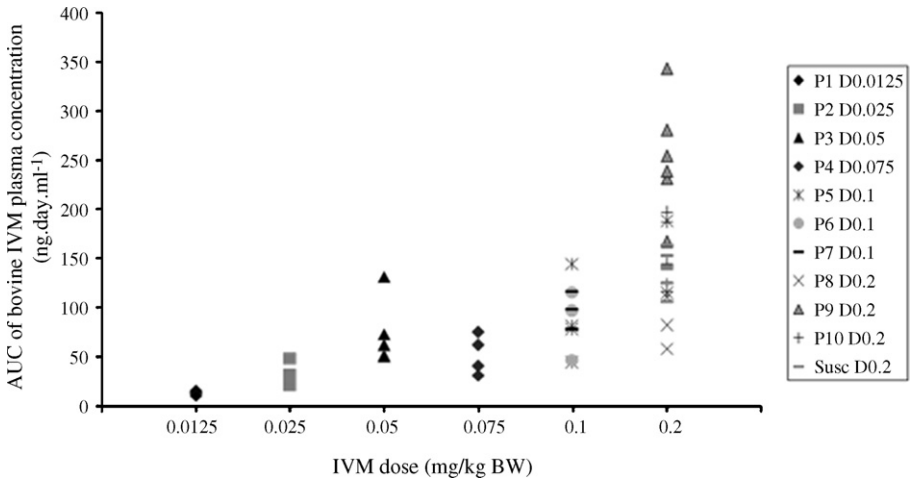

Fig. 2. The individual bovine plasma concentration of ivermectin after subcutaneous administration.

Necropsy, abomasal washing and abomasal digests (HCl–pepsin) were done according to standard techniques (Ritchie et al., 1966; MAFF, 1986). The total *O. ostertagi* worm burdens were estimated by counting 2% of the abomasal washings and digests of each calf.

3. Results

In the first selection round, the treatment with the 0.1 and 0.05 mg/kg IVM against the IVMS isolate was 100% effective on days 7, 14 and 21 post-treatment. *O. ostertagi* eggs were only recovered after treatment with the two lowest IVM doses. The 0.025 mg/kg IVM reduced the FEC by 98%, 99% and 100% on days 7, 14 and 21, respectively. The reduction in FEC with 0.0125 mg/kg BW IVM dose was 95%, 88% and 93% on days 7, 14 and 21, respectively. The larvae that survived the 0.0125 mg/kg BW IVM dose were used for further selection (Table 1).

The treatment efficacies on days 7, 14 and 21 in the sequential selection rounds are summarized in Table 1.

In the 10th selection round a controlled efficacy test was conducted. The treatment with the therapeutic IVM dose (0.2 mg/kg BW) was 100% effective against the IVMS isolates since no eggs were detected in the faeces on days 7 and 14 after treatment and no worm burdens in the abomasum were found in the treated IVMS animals. For the IVMR isolate, IVM reduced the FEC by 57% and 65% at days 7 and 14 post-treatment, respectively. All the treated IVMR-infected calves had *O. ostertagi* worm burdens ranging from 50 to 4800, with a geometric mean of 590. The efficacy of IVM against the IVMR isolate, based on the pre- and post-treatment larval counts was 89% and 59% on days 7 and 14 post-treatment, respectively. In contrast, the reduction in larval counts for the IVMS isolate was 100% confirming the IVM-susceptibility of the parental IVMS *O. ostertagi* isolate.

The mean AUC values for the IVM plasma concentration are presented in Table 1. A significant correlation was observed between the administered IVM doses and the IVM plasma concentrations ($R = 0.844$, $P < 0.01$) in the 10 passages. However, large individual variations in the IVM plasma concentrations were present within the same treatment doses (Fig. 2). In the selection rounds with the therapeutic treatments the mean AUC value for IVM varied from 111.1 to 252.9 ng day ml^{-1} for the IVMR-infected calves (Table 1). In the IVMR-infected calves of the controlled efficacy test a significant positive correlation ($R = 0.771$, $P < 0.05$) was demonstrated between the IVM plasma concentration (AUC value) and IVM efficacy on day 7 post-treatment. However, no significant correlation was found on day 14 post-treatment ($R = 0.714$, $P > 0.05$). The IVMS-infected calves treated with the therapeutic IVM dose had a mean AUC value for IVM of 138.3 ng day ml^{-1} and showed a similar individual variability (S.D. 2 ± 0.4).

4. Discussion

In the present study an IVM-susceptible *O. ostertagi* population was selected for IVM resistance by repeatedly exposing the population to subtherapeutic and therapeutic levels of IVM over 10 generations. While IVM was 100% effective against the susceptible IVMS *O. ostertagi* isolate, the efficacy against the resistant IVMR *O. ostertagi* isolate was 57% and 65% on days 7 and 14 post-treatment, respectively. These results were supported by the adult worm burdens.

O. ostertagi were recovered post-treatment in the abomasum of IVMR-infected calves, but no adult worms were found in IVMS-infected calves.

Surprisingly, a large individual variability in IVM efficacy (0–92%) was demonstrated in the IVMR-infected calves in selection round 10. Several studies have described a strong relationship between the efficacy of ML's and the drug concentration (Lanusse and Prichard, 1993; Baggot and McKellar, 1994). Furthermore, the plasma concentration is closely related to the IVM concentration in the target tissues such as the abomasum (Lifschitz et al., 2000; Lespine et al., 2005). Therefore, it is possible that the individual variability in efficacy in the last selection round is caused by the individual variability in IVM concentrations. In contrast, despite a similar variability in the AUC value for IVM in the IVMS-infected calves, treatment with the therapeutic dose was 100% effective in all the calves. Although a significant correlation was observed between the administered IVM doses and the IVM plasma concentrations, large variations in plasma concentration were present within the same treatment doses. A similar pharmacokinetic variability in the IVM plasma profiles has also been observed in other studies (McKellar and Benchaoui, 1996; Lanusse et al., 1997) and can be attributed to differences in breed, the site of IVM injection (muscle or fat tissue), the extent and rate of absorption, availability of the drug, metabolism and body composition, diet intake and body condition (Hennessy and Alvinerie, 2002).

Despite the fact that IVM resistance can be induced in *O. ostertagi*, as shown in the present experiment, there are very few reports on IVM resistance in *O. ostertagi* in the field. The basic population biology of *O. ostertagi* and the selection pressure are likely to play an important role in this (Coles, 2002). The most important reason for the slow development of anthelmintic resistance in cattle is considered to be the lower treatment frequency needed for adult cow, compared to ewes, and the consequent presence of a source of refugia. The selective treatment of only first year calves and the natural resistance of adult cows to nematodes have resulted in a very low level of treatment. For that reason the refugia consist primarily of susceptible worms from untreated second year and older animals. In addition, infective *O. ostertagi* larvae can overwinter in large numbers on the field, which results in a large refugium in the spring and therefore resistance is likely to develop much slower compared to sheep nematodes such as *H. contortus*. Moreover, under usual field conditions, adult *O. ostertagi* worms only survive for a relatively short period (25–50 days) and produce less egg compared to *C. oncophora* in cattle and *H. contortus* in sheep. Therefore *O. ostertagi* worms that survive the treatment could have a relatively small advantage over susceptible worms. In contrast, adult *H. contortus* in sheep can persist for many months giving a large advantage to resistant worms to produce the next generation (Coles, 2002). In addition, the IVM concentration that *O. ostertagi* experiences in the abomasal mucosa is higher than in the intestine, the target site of *C. oncophora* (Lifschitz et al., 2000). This is likely to be the reason why *C. oncophora* is the dose-limiting species (Coles, 2002). It was therefore expected that *C. oncophora* would be the first genus showing ML resistance in cattle (Coles, 2002).

On the other hand, the apparent lack of anthelmintic resistance in *O. ostertagi* can also be explained by the lack of sensitive detection tests (Coles, 2002). To date, the detection of anthelmintic resistance in field surveys is mainly based on the *in vivo* FECRT (Coles et al., 1992). Martin et al. (1989) demonstrated that the FECRT only detects benzimidazole (BZ) resistance when the frequency of the resistance alleles is greater than 25% in a population. Although the genetic basis of ML resistance and consequent the detection level is different from BZ resistance, the FECRT most likely detects IVM resistance only when the frequency of the resistance alleles is high and significant treatment failure occurs. The lack of sensitive tests means that the detection of anthelmintic resistance is not possible at an early stage and we are not able to follow the spread of resistance alleles. However, the development of sensitive and reliable molecular diagnostic tests requires knowledge of the mechanisms of resistance and this is currently lacking. Therefore a good starting point for further investigation would be to compare the molecular background of the IVM-susceptible parental isolate and the laboratory IVM-resistant *O. ostertagi* isolate in order to understand better the mechanisms of IVM resistance in *O. ostertagi*. Candidate genes that have been associated with ML resistance in other trichostrongylid nematodes will be analysed in *O. ostertagi.*

One should also take into account that a laboratory-selected isolate not necessarily reflects a resistant field *O. ostertagi* isolate. Different selection protocols can select for different resistance phenotypes and, presumably, genotypes (Gill et al., 1998). It is likely that selection with doses below the recommended dose rate tends to reveal all the potential resistance-associated genes, but fails to distinguish which gene might have the largest effect in field-selected resistance (Prichard, 2001; Gilleard and Beech, 2007). Another issue that needs to be considered is the possibility that different

isolates of *O. ostertagi* may have different mechanisms of resistance. It will therefore be important to validate results obtained with the laboratory-selected isolate against resistant isolates from the field.

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