Parasitol Res (2012) 110:2083–2087
DOI 10.1007/s00436-011-2753-1

SHORT COMMUNICATION

Characterization of a multidrug resistant *Teladorsagia circumcincta* isolate from Spain

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Received: 29 December 2010 / Accepted: 1 December 2011 / Published online: 17 December 2011

Abstract The aim of this work was to know the anthelmintic resistance (AR) status of a Spanish sheep flock infected by gastrointestinal nematodes (GIN) and the possible cross resistance among anthelmintics of the macrocyclic lactones (ML) family. The Faecal Egg Count Reduction Test (FECRT) was carried out to check the efficacy of albendazole (Zodalben®), levamisole (LEV) (Endex®) and an oral formulation of ivermectin (IVM) (Oramec®), at the recommended dose rates. Then, the study was extended to check the cross resistance between drugs of the ML family: injectable IVM (Ivomec®), oral moxidectin (Cydectin®), injectable moxidectin (Biodectin®) and doramectin (Dectomax®), at the recommended dose rates. The GIN species were identified after faecal cultures in all groups. The FECRT showed the resistance of a *Teladorsagia circumcincta* isolate against LEV (39–58%), IVM (88–92%) and doramectin (85%). This study is the first report to confirm the side resistance between these MLs, which belong to the avermectin chemical group, in a Spanish sheep flock. The in vitro efficacy of LEV and IVM was measured by the Larval Feeding Inhibition Assay (LFIA) using the IC_{50} measurement (concentration needed to inhibit the ingestion of 50% L1). The values of the multidrug resistant isolate were 0.25 μg/ml for LEV and 3 ng/ml for IVM. Both results were higher than the values obtained with a susceptible isolate, which could be indicative of AR. However, further research examining the response of a greater range of susceptible and resistant nematodes isolates should be carried out to establish a discrimination threshold.

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Introduction

Infection by gastrointestinal nematodes (GIN) in sheep is widespread around the world, and its importance is due to the production losses caused such factors as lower milk yield and decreased weight gain (Holmes 1993). To date, drugs are used generally as the only method to control the infection and against GIN. Most of the available anthelmintics for sheep fall into three main families: benzimidazoles (BZs), imidazothiazoles (IMs) and macrocyclic lactones (MLs), although since 2009 two new anthelmintic families have been introduced in the market, monepantel and derquantel (Kaminsky et al. 2008; Little et al. 2010). However, the inappropriate and abusive use of the most common drugs is one of the main factors in the development of anthelmintic resistance (AR) (Prichard 1994). During the 1960s, the first report of AR appeared, and from that moment this phenomenon started spreading, principally BZ resistance (Prichard et al. 1980). Today, it has become a threat to small ruminant production in many countries. A recent survey conducted in the northwest of Spain, in particular León province, reported AR cases in 12.7%, 34.6% and 15.7% of sheep flocks against BZs, IMs and MLs, respectively, after the Fecal Egg Count Reduction Test (FECRT) (Álvarez-Sánchez et al. 2006). Moreover, cross resistance, against two anthelmintic families, were described in 7% of flocks in the same survey. Another study carried out in Galicia (Spain) showed that 18% and 3% of sheep flocks were resistant against BZs and MLs, respectively;

however, multiple resistance was not found in any of the flocks sampled (Díez-Baños et al. 2008).

The aim of this work was to know the resistance status in a sheep flock, and the possible cross resistance among anthelmintics of the ML family.

Materials and methods

A FECRT was carried out in a 125-Assaf sheep flock located in León province, Northwest of Spain, to detect any possible AR. The data on anthelmintic management were collected using a questionnaire to obtain information about the flock, number of hours grazing per day, treatment frequency and the drugs last used. Previously, faecal samples from 20 randomly selected sheep were taken to estimate the parasitic infection level of the flock. According to the criteria established to conduct a FECT in a farm, the arithmetic mean of GIN eggs per gram (epg) in faeces should be higher than 150. After confirmation, 45 animals were selected and divided into three groups to check the efficacy of albendazole (Zodalben®, Laboratorios Calier), levamisole (LEV) (Endex®, Novartis) and oral ivermectin (IVM) (Oramec®, Merial), at the recommended doses. The study was extended to check the cross resistance between drugs of ML family. With this objective, four additional groups of 15 sheep each were treated with injectable IVM (Ivomec®, Merial), oral moxidectin (Cydectin®, Fort Dodge), injectable moxidectin (Biodectin®, Fort Dodge) and doramectin (Dectomax®, Pfizer), at the recommended doses. To confirm the resistance level against LEV, the FECRT was repeated again in the following season.

Nematode egg counts were calculated on the treatment day, day 0, and 10–14 days post-treatment (pt) following a modified McMaster method sensitive to greater than 15 epg of faeces.

The GIN species were identified after faecal cultures of faeces taken on day 0 and 10–14 days pt of each group (Roberts and O'Sullivan 1950). The morphological study of at least 100 L3, per faecal culture, was carried out following MAFF's keys (1986).

The FECRT as well as the lower limit for a 95% confidence interval was calculated according to the recommendations of World Association for the Advancement of Veterinary Parasitology (WAAVP) (Coles et al. 1992). When the FECRT value is less than 95% and the lower limit is less than 90%, the flock should be considered resistant. If only one criterion is described, the flock must be classified as borderline, but when none is shown, the flock is susceptible. The FECRT percentage was calculated according to the following formula:

$$\text{FECRT\%} = (\text{Arithmetic mean epg day } 0 - \text{Arithmetic mean epg days} + 10 - 14)/\text{Arithmetic mean epg day } 0 \times 100$$

The Larval Feeding Inhibition Assay (LFIA) was also carried out to calculate in vitro the effectiveness of LEV and IVM, measuring the concentration needed to inhibit the ingestion of *Escherichia coli* of 50% L1 (IC_{50}) after the drug administration. IVM and LEV have been used conventionally in the LFIA as the representative drugs to measure the IM and ML efficacies in vitro. The technique was based on the assays described by Álvarez-Sánchez et al. (2005) but with slight modifications. Nematode eggs were recovered from faeces collected on day 0 of the study (previous treatment) to recover L1. The concentration of L1 was adjusted to provide between 120 and 150 L1/100 μl. Seven dilutions, ranging from 0.04 to 2.58 μg/ml for LEV (Sigma) and from 0.1 to 20 ng/ml for IVM (Oramec®, Merial), were tested in duplicate. Into a 1.5-ml Eppendorf tube were added 50 μl of each anthelmintic dilution or PBS as control, 100 μl of water containing 120–150 L1 and 1,340 μl PBS. After incubation for 2 h at 23°C, 10 μl of *E. coli* labelled with fluorescein-5-isothiocyanate (FITC) (Sigma) was added to each tube, and after that tubes were incubated again for 2 h at 23°C. To eliminate the remaining fluorescence, L1 were washed three times with PBS by centrifugation at 13,000 rpm for 2 min. L1 were reconstituted with 200 μl PBS and added to a well of a 48-well plate. Fed larval counts, by means of the observation of intestinal fluorescence, were carried out through a fluorescence inverted microscope (Olympus CK40). Logarithm of doses against the percentage of fed larvae was plotted and the IC_{50} for each anthelmintic was calculated (Sigma Plot 5.05). The feeding assay was also carried out with a susceptible *Teladorsagia circumcincta* isolate to compare the dose–response curves with both anthelmintics.

Results

The anthelmintics used by the farmer during the past 2 years were a combination formulation of triclabendazole and LEV (Endex ®), for GIN and fasciolosis control, twice a year, and injectable IVM solution (IvomecF®) to oestrosis control, at one treatment per year. All sheep included in this study were grazing for an average of 5–6 h/day in communal pastures, close to the farm, during at least 2 months before the treatment.

In the preliminary sampling with 20 animals of the flock, the mean of the epg was 713, so we proceeded to carry out the study. The results of the efficacy of each drug are shown in Table 1. LEV, IVM and doramectin had efficacies were

Table 1 Efficacy of each anthelmintic according to the FECRT

Drug	Mean (±STD) day 0	Mean (±STD) day +10–14	FECRT (%)	Lower limit	Classification
Albendazole	796 (±613)	4 (±9)	99.5	97.5	Susceptible
Levamisole	330 (±367)	138 (±208)	58.2	−26.9	Resistant
Oral Ivermectin	225 (±170)	18 (±35)	92.0	74.5	Resistant
Inject. Ivermectin	415 (±205)	48 (±42)	88.4	79.7	Resistant
Inject. Moxidectin	337 (±317)	4 (±7)	98.8	96.1	Susceptible
Oral Moxidectin	488 (±469)	14 (±29)	97.1	88	Borderline
Inject.Doramectin	168 (±95)	25 (±38)	85.1	62.7	Resistant
Levamisole (confirmation)	208 (±202)	126 (±130)	39.4	−4.2	Resistant

lower than 95%, which indicates different degrees of AR, with LEV being the anthelmintic with the lowest efficacy.

The identification of L3 recovered from faeces on day 0 was 85% *T. circumcincta* and 15% *Trichostrongylus colubriformis*. L3 from faeces collected after 10–14 pt were all identified as *T. circumcincta*.

After LFIA, the IC_{50} results with the field population were 0.25 μg/ml for LEV and 3.37 ng/ml for IVM, both indicative of AR according to the thresholds suggested by Álvarez-Sánchez et al. (2005). With the susceptible *T. circumcincta* isolate, the IC_{50} was 0.12 μg/ml for LEV and 2.08 ng/ml for IVM (Figs. 1 and 2).

Discussion

Due to the increase of AR development, the efficacy of the available drugs should be reviewed before controlling the infection in sheep flocks. Up to date, several in vivo and in vitro methods have been described to detect AR. The in vitro techniques should be faster and more accurate than the FECRT but must be validated with in vivo results. In this assay, we have reported multiple resistances against LEV and MLs by means of the FECRT and also by an LFIA which was previously optimized to obtain a better vision of larvae. Previously, the LFIA technique has been carried out with monospecific isolates to compare the resistance status of *T. circumcincta*, *Haemonchus contortus* and *T. colubriformis* to LEV and MLs (Sheriff et al. 2002; Álvarez-Sánchez et al. 2005). On the other hand, a hookworm population of *Ancylostoma canimum* was tested to detect resistance to pyrantel by means of this assay (Kopp et al. 2008). Nowadays, the LFIA is also being using to study the potential use of new essential oils against *H. contortus* and *Trichostrogylus* spp (Katiki et al. 2011) as well as tannins against *C. oncophora* and *O. ostertagi* (Novobilský et al. 2011) because of their nematocide effect. Under natural and mixed GIN infections, this in vitro test has been carried out to detect AR in sheep flocks to MLs with successful correlations with the results of the FECRT (Díez-Baños et al. 2008).

Álvarez-Sánchez et al. (2005) compared the IC_{50} with IV resistant and susceptible monospecific populations and reported that the mean values were 1, 1.05 and 1.1 ng IV/ml for susceptible *T. circumcincta*, *Trichostrongylus vitrinus* and *T. colubriformis* isolates, respectively. The authors suggested that an IC_{50} higher than 2.5 ng IV/ml would be likely associated with resistance. In the same study, they also tested the IC_{50} with LEV resistant and susceptible monospecific isolates, and consequently mean values varied

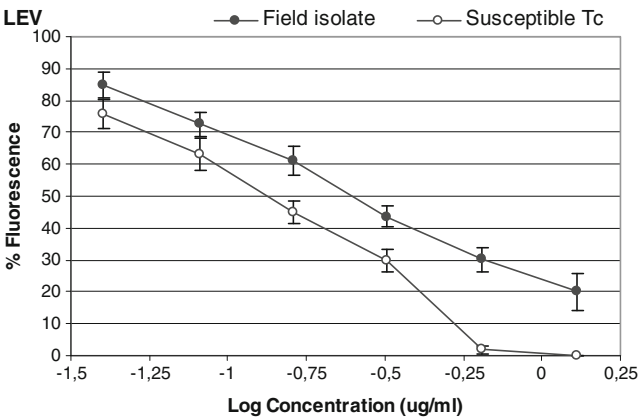

Fig. 1 Dose–response curve for LEV

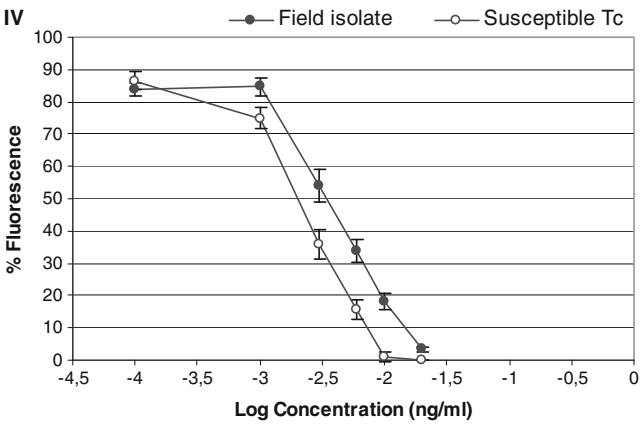

Fig. 2 Dose–response curve for IVM

between 0.11 and 0.12 μg LEV/ml for the same susceptible isolates. Although more validations are necessary, the threshold for LEV was suggested to be 0.2 μg LEV/ml for these species. In the present study, the animals were infected by a population constituted by *T. circumcincta* (85%) and *T. colubriformis* (15%). According to the prior description, the IC_{50} for both species were very similar and since *T. circumcincta* was the prevalent species, this population was considered a monospecific isolate to carry out the LFIA.

Firstly, we showed the presence of a double resistance against LEV and oral IVM in a flock infected mainly with *T. circumcincta*. These findings were confirmed with further in vivo and in vitro test at the same flock and with an IVM injectable formulation. The side resistance between MLs belonging to the avermectin chemical group was observed between IVM and doramectin since they are closely related. This study is the first report to confirm side resistance between these drugs in a Spanish sheep flock. However, in other countries such as Brazil, both resistances were already described (da Cruz et al. 2010), in combination with a lack of efficacy of other drugs even in an area new to sheep farming.

On the other hand, we were not able to confirm the cross resistance of IVM with other ML, such as moxidectin, which belong to the group of milbemycins. Moxidectin presents a longer persistence of anthelmintic action compared to IVM because of the differences in potency, physicochemical properties and pharmacokinetic behaviour between them. Indeed, moxidectin is much more lipophilic than ivermectin and is mainly stored in fat (Alvinerie et al. 1998). The efficacy of this drug has been shown against different GIN species in sheep (Martínez-Valladares et al. 2010). Our results are in accordance with previous trials where moxidectin was effective in sheep against IVM resistant isolates of *T. circumcincta* (Traversa et al. 2007) or of *Haemonchus contortus* (Craig et al. 1992; Borgsteede et al. 2007). The higher drug potency (Njue et al. 2004) and the lower affinity by glycoprotein-P (Lespine et al. 2007) of moxidectin compared to IVM may explain the higher efficacy obtained against resistant *T. circumcincta* in the current trial. Moxidectin could be an alternative anthelmintic in case of avermectin resistance to control GIN in sheep but since they share a common mechanism, resistant IVM isolates are likely to become also resistant to moxidectin, after a frequent use. Ardelli et al. (2009) have demonstrated that while avermectins and milbemycins have some similar effects on *Caenorhabditis elegans*, there are differences in transcriptional profiles of genes coding for ligand-gated chloride channel subunits. Also recently, a field study in sheep described a failure on the persistence of moxidectin in a *T. circumcincta* isolate resistant to BZs, IMs and MLs (Sargison et al. 2010).

On the other hand, we found slight differences in moxidectin efficacy, depending on the formulation used: oral or injectable. In general, treatment of sheep with MLs, administered subcutaneously, produces higher concentrations of MLs in plasma for a longer period (Alvinerie et al. 1998). Because the oral moxidectin is borderline between susceptibility and resistance, we suggested to the farmer a limited use of this drug and always to use it in alternation with a BZ. Another option when the AR is already established in the farm is the use of new drugs that have recently been developed against GIN in sheep: monepantel and derquantel, with the latter in a combination with an ML, abamectin. These drugs were shown to be effective in case of multi-resistant nematode populations in sheep; therefore, they are a promising alternative against AR (Kaminsky et al. 2010).

The results obtained by FECRT have been confirmed by the LFIA in several occasions (Álvarez-Sánchez et al. 2005; Díez-Baños et al. 2008) and also in the present study. However, further research examining the response of a greater range of susceptible and resistant nematodes isolates should be carried out to establish a discrimination threshold.

Acknowledgements This study has been funded by Fort Dodge Veterinaria, S.A. Madrid and by the national project INIA-MEC, RTA2006-00183-C03-02. The work of Martínez-Valladares M. has been supported by a postdoctoral Jae-Doc contract from Consejo Superior de Investigaciones Científicas (CSIC).

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