Strategic control of *Dicrocoelium dendriticum* (Digenea) egg excretion by naturally infected sheep

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ABSTRACT: The aim of this study was to determine the most appropriate months for applying albendazole (ABZ; oral suspension dose 20 mg/kg body weight) to sheep naturally infected with *Dicrocoelium dendriticum* and kept at pasture, in order to reduce parasite egg shedding to a minimum, mainly during the cold months and, as a result, decrease pasture contamination by viable eggs. Five animal groups (G), homogeneous as regards the number of eggs per gram (EPG) in faeces, were established. The treatment months were: G1, November and January; G2, November and February; G3, November and April; G4, January; and G5 (control), April. Ten samplings (S1-S10) were carried out every 35 to 45 days to collect faecal samples from the rectum of each animal in the five groups. The sedimentation technique and McMaster egg counting chambers were used to analyze the faecal samples. Due to the effect of albendazole (ABZ) treatments, the five groups behaved differently with regard to EPG reduction and the percentage of samples positive for *D. dendriticum* eggs. Using the Kruskal-Wallis test, statistically significant differences ($P < 0.05$) were observed between the EPG values obtained in G5 and the rest of the groups from November to May, but not from May onwards. The biggest reduction in egg excretion was obtained in G1, mainly in the cold period when elimination is highest and egg survival greatest, so G1 gave the best result, followed by G2, G4, G3 and finally G5 in descending order.

Keywords: dicroceliosis; trematoda; strategic treatment; albendazole; ovine; Spain

Dicroceliosis, caused by *Dicrocoelium dendriticum* (Digenea, Dicrocoeliidae), is a hepatic parasitic disease of clinical and financial significance in ruminant breeding, which causes direct losses due to confiscation of parasitized livers (Jithendran and Bhat, 1996), and indirect losses due to hepatobiliary alterations produced by the parasites and the costs associated with anthelminthic treatments (Wolff et al., 1984; Otranto and Traversa, 2002, 2003; Manga-Gonzalez et al., 2004; Ferreras et al,. 2007). This Digenea occurs very frequently in ruminants from the Iberian Peninsula (Cordero-del-Campillo et al., 1994) and in those of various other countries in Europe, America, Asia and North Africa. A wide range of species of land molluscs and ants, which act as first and second intermediate hosts, respectively, intervene in the complex life cycle of *D. dendriticum*, in addition to the domestic and wild mammals which are the definitive hosts (Manga-Gonzalez et al., 2001). Due to the complex-

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ity of the cycle and the low parasitic specificity of *D. dendriticum* in relation to its hosts, it is not easy to apply prophylactic and control measures. Currently the most effective method is livestock anthelminthic treatment, although it is unsatisfactory (Eckert and Hertzberg, 1994). The administration of efficacious chemotherapeutic control measures requires a good knowledge of dicrocoeliosis epidemiology in the area, as well as the use of an appropriate anthelminthic.

The studies carried out on the control of dicrocoeliosis are mostly critical tests for evaluating the effectiveness of anthelminthics, but are not administered strategically on the basis of epidemiology. In spite of there being various chemotherapeutic compounds available for treatment of dicrocoeliosis (Rojo-Vazquez et al., 1989; Ambrosi et al., 1995; Otranto and Traversa, 2002; Senlik et al., 2008), none are effective against the juvenile and immature stages of *D. dendriticum*, or, if they are effective, as is diamphenethide, the dose is high (240 mg/kg: 93–95% efficacy) and serious side effects appear after administration (Stratan, 1986).

The efficacy of albendazole (ABZ) (Valbazen, registered by Pfizer), one of the compounds most frequently tested against *D. dendriticum* and only effective against adult parasites, varies between 82.43% and 99.6% according to the dose and administration route (Himonas and Liakos, 1980; Tharaldsen and Wethe, 1980; Cordero-del-Campillo et al., 1982; Theodorides et al., 1982; Corba and Krupicer, 1992; Schuster and Hiepe, 1993; Corba et al., 1994), although doses of 15 and 20 mg/kg are those most often recommended by different authors. According to the study carried out by Skalova et al. (2007), the effect of mouflon (*Ovis musimon*) dicrocoeliosis on the activities of biotransformation enzymes and albendazole metabolism in liver manifested itself as only mild changes in ABZ hepatic transformation, so undesirable alterations in ABZ pharmacokinetics are not expected. From this point of view, the use of ABZ in the therapy of dicrocoeliosis in mouflon can be recommended. Moreover, the authors pointed out that as mouflon and domestic sheep are phylogenetically two forms of identical species, the experimental data found in one can also be used for the other. Lamka et al. (2007) treated adult female mouflons infected with *D. dendriticum* with a single dose of ABZ (30 mg/kg of body weight; oral route). The ABZ administration was very effective because the faecal EPG values decreased rapidly and was very low by the 7th day after treatment. Moreover, only a few dead fluke adults and low egg content were found in the livers and bile. Concerning the modulation of biotransformation enzyme activities involved in ABZ metabolism, the highest inductive effect of ABZ was detected on cytochrome P4501A (CYP1A) activities. In hepatic and intestinal microsomes, the velocity of albendazole sulfoxide (ABZ.SO) formation was unaffected, although a shift in ratio of individual ABZ.SO enantiomers was observed. Moreover, the formation of the pharmacologically inactive albendazole sulfone was significantly accelerated in both the liver and intestine of ABZ treated animals. The authors pointed out that the increase in ABZ deactivation could facilitate the development of anthelmintic resistance in parasites. They also mentioned that, although a single ABZ dose is therapeutically effective, its potential to induce CYP1A should be taken into account for controlling helminthoses. Cvilink et al. (2009) did not observe sulphoreduction of albendazole sulfoxide (ABZ.SO) in their experiments *in vivo* and *in vitro* with *D. dendriticum*. So it appears that reverse metabolism of ABZ.SO does not occur in lancet flukes. Complete knowledge of drug metabolism in helminths is necessary so that control of parasitic infection, including dicrocoeliosis, becomes more effective.

According to several studies carried out on dicrocoeliosis epidemiology, shedding of *D. dendriticum* eggs with ruminant faeces occurs without interruption throughout the year in the province of Leon (Spain). The highest values are recorded in the cold period, that is, at the end of autumn and in winter (Manga-Gonzalez et al., 1991, 2007; Gonzalez-Lanza et al., 1993; Manga-Gonzalez and Gonzalez-Lanza, 2005). At these times survival of *D. dendriticum* eggs in the province of León is high, due to the low temperatures (Alunda and Rojo-Vazquez, 1983), so pasture contamination by viable eggs is significant in spring, when the molluscs are abundant and active. In addition, ants generally hibernate between November and March (Manga-Gonzalez et al., 2001), so livestock are not re-infected during this period.

Taking into account the epidemiological data, the aim of this study was to determine the most appropriate months for applying ABZ strategic treatments in sheep naturally infected with *D. dendriticum* and kept at pasture in order to reduce *D. dendriticum* egg shedding by the definitive hosts, mainly during the cold months to a minimum, and

as a result, decrease pasture contamination by viable eggs.

MATERIAL AND METHODS

The study began in October 1993 on a flock of approximately 600 Churra, Assaf and Churra × Assaf, dairy sheep on a farm in Grulleros (altitude 787 m) in the lower basin of the river Bernesga, 10 km south of the city of Leon (NW Spain). The climate is continental within the Mediterranean-Atlantic transition, with cold winters and hot summers (Figure 1). The flock was subjected to a daytime shepherding system over an area of several km^2 and housed at night.

At the start of the experiment samples of faeces were collected from the rectum of 240 animals in the flock between 8 and 12 a.m. on the same day. In order to count the *D. dendriticum* eggs per gram (EPG) in the faeces, 3 g of each sample was processed individually using the sedimentation technique. First of all about 45 glass balls were placed in a 120 ml bottle and 42 ml water were added. Immediately the 3 g of faeces were put in the bottle and this was closed and shaken until all the faecal matter was broken down. The mixture was passed through a wire mesh screen with an aperture of 0.15 mm and the strained fluid caught in a bowl. The debris left on the screen were discarded. The fluid was passed to a one litre capacity sedimentation cup, stored for one hour and then the supernatant was decanted. The cup was filled up again, stored for sedimentation for one hour and the supernatant was again decanted. The fluid obtained was passed to a 60 ml sedimentation cup, then homogenised with a Pasteur pipette and carefully poured into each square (0.15 ml) of a Mc Master chamber (see photograph in Thienpont et al., 1979) to count the number of eggs in both squares under the optical microscope. After further stirring a second sample was taken out and poured into the other chamber. If one egg was found after checking the four squares of the two samples using the Mc Master chamber (0.15 + 0.15 + 0.15 + 0.15 = 0.6 ml), then considering that the 3 g of faeces were in 60 ml fluid, the number of eggs per gram was 33.33 (in Table 1 the decimals were eliminated to reduce space). In this way 63.7% of the animals were positive for *D. dendriticum*. For the treatment studies 179 *D. dendriticum*-positive animals were divided into five groups (G), with 37 in G1, 39 in G2, 38 in G3, 32 in G4 and 31 in G5. The groups were homogenous in terms of EPG, as no statistically significant differences were detected among them on carrying out the Kruskal-Wallis test (Daniel, 1984). Each animal was individually given one or two treatments with an oral suspension dose of 20 mg/kg of ABZ [[5-(propylsulphoxi)-1H-benzimidazol-2-yl] carbamate methyl ester; registered by Pfizer as Valbazen] in different months. The treatments were administered as follows: G1, in November 1993 and January 1994; G2, in November 1993 and February 1994; G3, in November 1993 and April 1994; G4, in January 1994; G5, in April 1994. The last group (G5) was considered a control (C), because the animals were not treated in autumn and winter, when the highest *D. dendriticum* egg shedding by sheep occurs in the province of Leon (Manga-Gonzalez et al., 1991).

Ten samplings (S1–S10) were carried out every 35 to 45 days (a period slightly shorter than the prepatent one; Campo et al., 2000) to collect faeces samples from the rectum of each animal in the five groups. The first sampling (S1) was carried out in November 1993 and the rest in the following months in 1994: January (S2); February (S3); April (S4); May (S5); July (S6), August (S7), September (S8), October (S9) and November (S10).

The faecal samples were taken and processed individually as previously described. The data obtained from coprological examinations allowed us to calculate the percentage of animals that eliminated eggs and the number of EPG shed by each group and in each sampling. Mean EPG ± standard error (SE) and the range of minimum and maximum EPG were obtained considering both the positive and negative samples as a whole and the positive ones alone. The EPG reduction percentages in each of the four G1, G2, G3 and G4 *vs* G5 (control) were calculated using the formula given by Greenberg et al. (1998). The efficacy of the ABZ was measured by the "extensity effect" (EE) (percentage reduction in the number of animals excreting eggs in the group) and the "intensity effect" (IE) (percentage reduction in the egg excretion in the group) calculated with the corresponding formula used by Eckert et al. (1984), considering the group itself on the treatment day as the control.

The EPG values obtained in the different samplings carried out in the same group were compared using the Friedman test (Daniel, 1984) and, when statistically significant differences were detected, the Nemenyi (Zar, 1996) was applied. The compari-

son between EPG values obtained in the same sampling from the five groups of animals was carried out using the Kruskal-Wallis test, and when statistically significant differences were detected the Nemenyi was also applied. The Kruskal-Wallis test was also used to compare the EPG values obtained in the five groups during Period-1 (November 1993–May 1994) and also during Period-2 (July–November 1994). The analyses were carried out using the Statistical Analysis System (SAS) program (Cody and Smith, 1991).

RESULTS

Group 1. Treatments in November and January

The percentage of animals that eliminated *D. dendriticum* eggs (Figure 2) was 100% at the beginning of the experiment (November, S1), decreased to 20% in April (S4) and then increased again, with some oscillations, to 88.9% the following November (S10).

When the positive and negative samples were considered as a whole (Table 1, Figure 2), the highest value of the EPG mean (164.6 ± 55.0) was detected in S10, followed by S1 and S2, and the lowest value (16.0 ± 7.4) was obtained in S4. Using the Friedman test, statistically significant differences were detected between the EPG values obtained in S1 and S2 ($P < 0.05$) and between S1 and the rest of the samplings ($P < 0.01$), except S10. The same statistical differences were also observed between the EPG value obtained in S10 and the rest of the samplings, except those of S1. On the other hand, when only the positive samples were taken into account, the EPG mean values in the ten samplings exceeded those shown in Table 1, and the highest value was obtained in November 1994 (179.1 ± 56.8). The extensity (EE) and the intensity (IE) effects were 64.2% and 37%, respectively, for the November treatment and 38.8% and 68.5% for the January treatment.

Group 2. Treatments in November and February

At the beginning of the experiment (S1) 100% of the animals eliminated *D. dendriticum* eggs. The lowest percentage was observed in May (S5) (30%) and then increased again until it reached 88.2% in S10 (see Figure 2).

When the positive and negative samples were considered as a whole (Table 1, Figure 2), the highest *D. dendriticum* EPG mean (160.8 ± 46.5) was detected in S10, followed by S1 and S2, while the

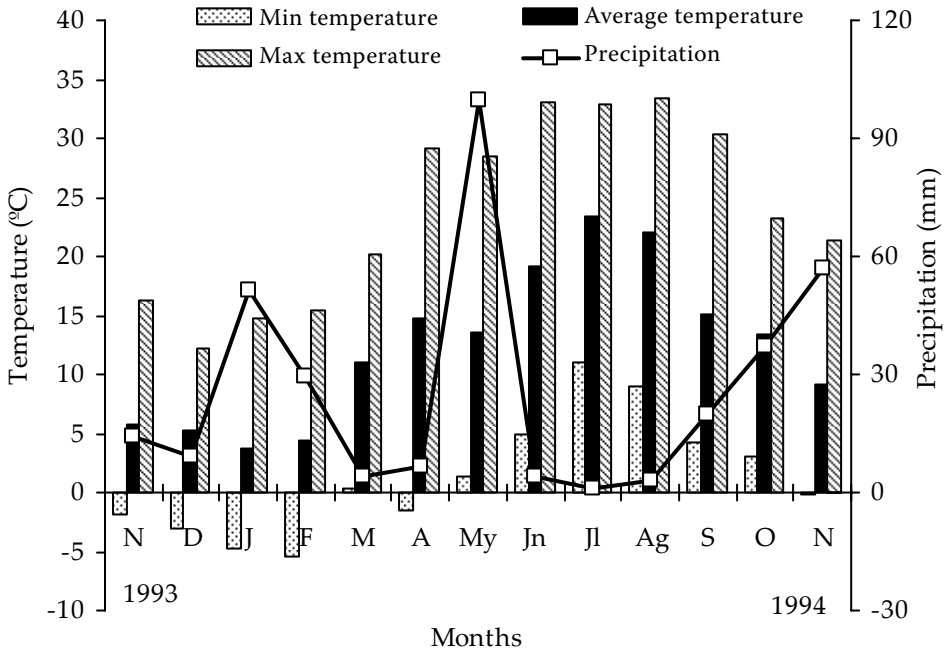

Figure1. Maximum, minimum and average monthly temperature and precipitation values obtained throughout the sampling period

Table 1. Mean ($\bar{x}$) ± standard error (SE) and range values of *D. dendriticum* eggs per gram (EPG) obtained in each sampling and animal group when the positive and negative samples were considered as a whole

Sampling/ Month-Year	EPG Group 1		EPG Group 2		EPG Group 3		EPG Group 4		EPG Group 5 (control)	
	$\bar{x}$ ± SE	range	$\bar{x}$ ± SE	range	$\bar{x}$ ± SE	range	$\bar{x}$ ± SE	range	$\bar{x}$ ± SE	range
S1/Nov-93	133.8 ± 17.9	33–500	132.5 ± 17.3	33–500	122.8 ± 15.5	33–367	108.3 ± 12.4	33–267	110.2 ± 12.4	33–267
S2/Jan-94	84.8 ± 19.6	0–366	80.8 ± 17.1	0–433	79.6 ± 12.0	0–300	113.7 ± 15.9	0–267	216.3 ± 55.1	0–1249
S3/Feb-94	27.1 ± 9.0	0–167	71.9 ± 14.1	0–267	85.8 ± 14.8	0–267	29.6 ± 7.8	0–167	117.2 ± 16.2	0–267
S4/Apr-94	6.9 ± 3.0	0–67	19.3 ± 6.3	0–133	55.3 ± 16.7	0–500	29.0 ± 8.7	0–167	81.1 ± 22.2	0–366
S5/May-94	16.0 ± 7.4	0–181	16.7 ± 5.2	0–100	28.5 ± 6.3	0–133	22.6 ± 9.7	0–233	22.8 ± 7.2	0–100
S6/Jul-94	23.0 ± 9.0	0–200	28.4 ± 9.0	0–200	20.8 ± 6.1	0–133	27.4 ± 10.4	0–233	25.9 ± 6.9	0–67
S7/Aug-94	22.2 ± 4.8	0–67	48.6 ± 25.9	0–567	61.0 ± 16.0	0–433	19.6 ± 7.2	0–133	72.9 ± 31.2	0–467
S8/Sep-94	17.2 ± 4.3	0–67	28.0 ± 7.4	0–100	23.0 ± 5.8	0–100	24.6 ± 7.3	0–100	48.9 ± 13.3	0–167
S9/Oct-94	38.0 ± 12.0	0–167	46.5 ± 13.7	0–133	26.1 ± 14.5	0–200	31.1 ± 10.3	0–167	51.8 ± 28.9	0–233
S10/Nov-94	164.6 ± 55.0	0–767	160.8 ± 46.5	0–667	143.6 ± 25.1	0–500	135.0 ± 25.3	0–400	144.0 ± 27.1	33–367

The ABZ treatments were administered as follows: Group 1 = in November and January; Group 2 = in November and February; Group 3 = in November and April; Group 4 = in January; Group 5 (control) = in April

lowest was detected in S5. Using the Friedman test, statistically significant differences ($P < 0.01$) were observed between the EPG values obtained in S1 and S10 and those obtained in S2 and S3 ($P < 0.05$) and the rest of the sampling ($P < 0.01$), except between S1 and S10. When only the positive samples were taken into account, the EPG mean values exceeded those shown in Table 1, and the highest mean EPG value was obtained in the last sampling (182.2 ± 50.2). The extensity (EE) and intensity effects (IE) were 70.5% and 38.0%, respectively, for the November treatment, and 50.8% and 73.1% for the February one.

Group 3. Treatments in November and April

The highest percentage of animals that eliminated eggs (100%) was obtained in S1 and decreased to its lowest value (33.3%) in July (S6). The percentage then underwent some oscillations and finally reached 96.2% in the last sampling (S10), (Figure 2).

When the positive and the negative samples were considered as a whole (Table 1, Figure 2), the highest EPG mean value (143.6 ± 25.1) was observed in S10 and the lowest (20.8 ± 6.1) in S6. Using the Friedman test, statistically significant differences were observed between the EPG values obtained in S1 and S10 and those obtained in S2 and S3 ($P < 0.05$) and the rest of the samplings ($P < 0.01$), except between S1 and S10. When the positive samples were considered alone, the EPG mean values exceeded those shown in Table 1, and the highest EPG mean was obtained in S10 (148.6 ± 24.4). The extensity (EE) and the intensity effect (IE) were 81% and 34.7%, respectively, for the November treatment and 20.0% and 49.3% for the April one.

Group 4. Treatment in January

The percentage of animals that eliminated *D. dendriticum* eggs (Figure 2) was 100% at the beginning of the experiment (S1), decreased to 36% in S5 and then increased again, with some oscillations, to 80% in S10.

Concerning the EPG mean values obtained in positive and negative samples as a whole (Table 1, Figure 2), the highest value (135.0 ± 25.3) was detected in S10, followed by S2 (113.7 ± 15.9). The mean values were low from then until S10, and were at their lowest in August (S7) (19.6 ± 7.2). By applying the Friedman test, statistically significant differences ($P < 0.01$) were detected between the EPG values obtained in S1, S2 and S10 and those of the rest of the samplings, except between S1, S2 and S10. When the positive samples were considered alone, the EPG mean values exceeded those

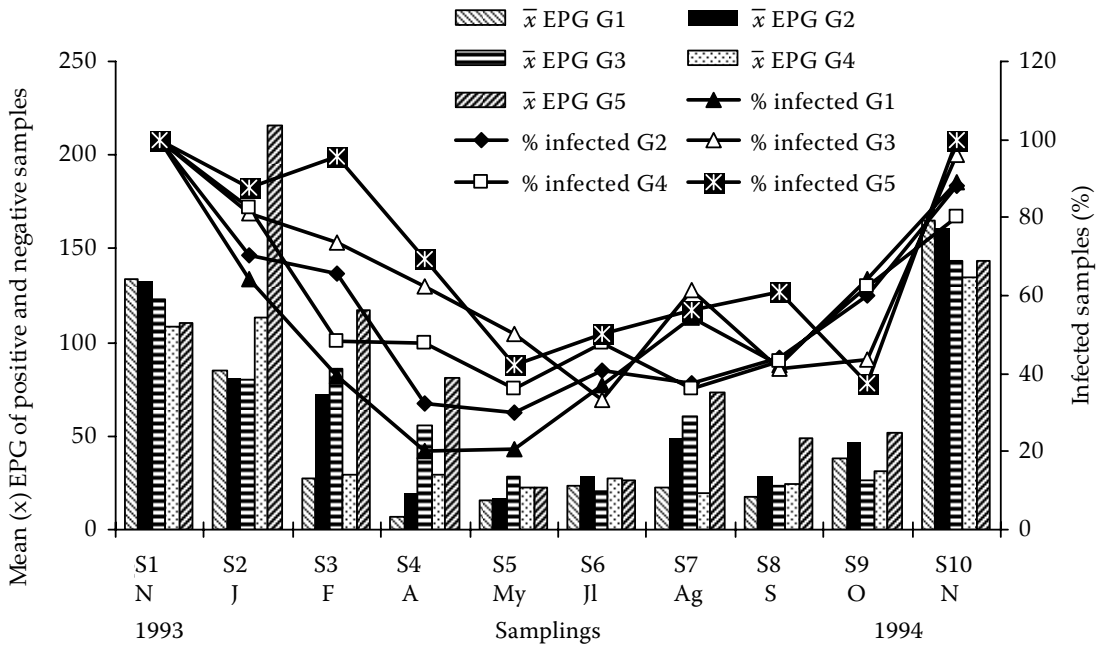

Figure 2. Percentage (%) of faeces samples infected by *D. dendriticum* eggs in each sampling and animal group (G). Mean ($\bar{x}$) values of *D. dendriticum* eggs per gram (EPG) obtained in each sampling and animal group (G), when the positive and negative samples were considered as a whole. The ABZ treatments were administered as follows: G1, in November and January; G2, in November and February; G3, in November and April; G4 in January; and G5 (control), in April

shown in Table 1 (except in S10 because they were the same) and the highest EPG mean was obtained in S10 (168.7 ± 25.1). The EE and the IE of the treatment applied in January were 41.4% and 73.9%, respectively.

Group 5. Treatment in April

The percentage of animals that eliminated eggs was 100% in November (S1) and continued to be high until April, when the treatment was applied. The lowest percentages were detected in May (42.2%) and October (37.5%: this low percentage could be due to the fact that only nine animals could be examined). At the end of the experiment in S10, 100% of the animals eliminated eggs in their faeces (Figure 2).

When the positive and negative samples were considered as a whole (Table 1, Figure 2), the EPG mean values were high between November (S1) and April (S4), although the highest mean EPG value was detected in January (216.3 ± 55.1). The lowest EPG mean (22.8 ± 7.2) was obtained in May (S5). At the end of the experiment the EPG mean value (144.0 ± 27.1) was higher than that of the first sampling (110.2 ± 12.4). Using the Friedman test, statistically significant differences were detected between the EPG value obtained in S1, S2, S3 and S10 and those of S4 ($P < 0.05$) and the rest of the samplings ($P < 0.01$), except between S1, S2, S3 and S10. On the other hand, when only the positive samples were taken into account, the EPG mean values were superior to those shown in Table 1 (except in S1, S3 and S10 in which the values were the same), and the highest mean (247.2 ± 60.1) was obtained in January. The extensity (EE) and intensity (IE) effects of the treatment applied in April were 39.4% and 71.9%, respectively.

Comparison between groups

In order to discover which of the five treatment models were the more appropriate to decrease egg excretion, mainly from November to April, when the control group (G5) was treated, the EPG elimination (Table 1) in each sampling was compared amongst the five groups. Using the Kruskal-Wallis test, statistically significant differences were observed between the EPG values obtained in January (S2): between G5 and those of G1, G2, G3 ($P < 0.01$) and

G4 ($P < 0.05$). In February (S3), between: G5 and those of G2, G3 ($P < 0.05$), G1 and April (S4) G4 ($P < 0.01$); in S4, between: G5 and G1, G2, G4 ($P < 0.01$).

When the EPG elimination was grouped in two periods, that is from November to May (Period-1) and from then to the next November (Period-2), the EPG mean was calculated for each group and period as well as the corresponding percentage of EPG reduction vs. G5 (control) in both periods. Using the Kruskal-Wallis test, statistically significant differences ($P < 0.05$) were detected in the EPG values between the control group G5 and each of the other groups in Period-1, but not in Period-2.

DISCUSSION

According to our information, there are no publications on strategic control of *D. dendriticum* which take the epidemiological model into account, only some specific data on critical tests for evaluating the effectiveness of anthelminthics which we will refer to later.

First of all, we must point out that the results obtained in the control group (G5) from November to April corroborated those previously obtained by us on the kinetics of *D. dendriticum* egg excretion in our region (Manga-Gonzalez et al., 1991, 2007; Gonzalez-Lanza et al., 1993). That is, the most important period for *D. dendriticum* egg excretion as regards the number of animals that eliminate eggs and EPG shedding in faeces is the end of autumn and winter, when temperatures are low. This type of temperature favours *D. dendriticum* egg viability in the field, as the eggs are more resistant to low temperatures than high ones (Alzieu and Ducos de Lahitte, 1991; Alunda and Rojo-Vazquez, 1983). Experiments have shown that they can withstand temperatures as low as –20°C to –50°C (Boray, 1985). So, taking into account the transmission model of *D. dendriticum* in our region (Manga-Gonzalez et al., 2001; Manga-Gonzalez and Gonzalez-Lanza, 2005), the high contamination of pastures which occurs during autumn and winter facilitates viable egg ingestion by the molluscs, which start to be active and are very abundant in spring. The molluscs infected at the beginning of this period could shed slimeballs with cercariae at the end of summer and during autumn, whilst those infected later can shed slimeballs the following year, beginning in spring, if they survive the harsh winter. The cercariae ingested together with the slimeballs by ants will have become infected metacercariae for the definitive hosts. This will allow the parasite cycle to be completed when the ants are ingested by ruminants on grazing, during the ants' active period between March and November. The ingestion of the infective metacercariae (contained in the ants) by the definitive hosts and the number of *D. dendriticum* adult worms in the liver of the animals increase with the ants' activity period. As a consequence of this, egg excretion reaches its highest values in January-February, that is, about two months after the last ingestion of the infected ants before hibernation starts.

Due to the effect of ABZ treatments, the five groups behaved differently regarding the positive sample percentage for *D. dendriticum* eggs and the EPG of the positive and negative samples considered as a whole, during the 10 samplings. In view of the results obtained for both parameters, if two treatments are administered, then the best regime for the conditions in Leon (Spain) is that used in G1 (November and January), that is treatment in November, when ant hibernation starts (Manga-Gonzalez et al., 2001) to eliminate the adults worms, since the anthelminthic used does not act against juvenile stages. The treatment is repeated in January, when, without reinfection, most of the metacercariae ingested by the animals until November have become adults capable of shedding eggs. These treatment dates are more appropriate for decreasing egg excretion just when it is at its highest, that is, at the end of autumn-winter (Manga-Gonzalez et al., 1991, 2007). It must also be remembered that *D. dendriticum* eggs are more resistant to low temperatures than high ones and that their viability is high from September to June in León province, whilst mortality is almost 100% in July and August (Alunda and Rojo-Vazquez, 1983). Therefore, if pasture contamination by viable eggs is reduced to a minimum in autumn and winter, infection of the intermediate host molluscs will be prevented in spring, when they become very active and abundant (Manga-Gonzalez, 1987; Manga-Gonzalez et al., 2001).

According to the results of this study, the second regime recommended for applying two treatments would be to administer one in November and another in February, as done with G2, since treatment efficacy in February is even higher than in January. This is because all the worms should be mature in February, according to the prepatent period (49–76 days) obtained in experimental infections

by Campo et al. (2000). In spite of this, treatment in February prevents pasture contamination to a lesser extent than the January one as it allows juvenile worms, not affected by the November treatment, to mature and eliminate eggs for longer, at a time when elimination is higher (see G5, control, which was not treated until April). It must be borne in mind that ABZ efficacy against *D. dendriticum* is not 100% (Himonas and Liakos, 1980; Tharaldsen and Wethe, 1980; Cordero del Campillo et al., 1982; Theodorides et al., 1982; Corba and Krupicer, 1992; Schuster and Hiepe 1993; Corba et al., 1994), and also that there might possibly be some degree of resistance by the parasite to treatment with ABZ or a delay in the prepatent period, as Boray (1990) and Overend and Bowen (1995) have reported for another parasite.

All the above makes it easy to deduce that administering the second treatment in April, after the November one (as with G3), is not recommended as it does not prevent pasture contamination by viable eggs eliminated during the cold months by worms maturing after November. In addition, elimination fell naturally from March (see G5, control) until autumn (Manga-Gonzalez et al., 1991, 2007), in spite of the fact that the animals could be reinfected by infected ants which survived the winter (Tarry, 1969; Badie, 1978) and later by others reinfected the same year after hibernation (Manga-Gonzalez et al., 2001). It must also be considered that egg viability falls as temperatures rise, with possible 100% mortality in the hottest summer months (Alunda and Rojo-Vazquez, 1983).

If we consider the groups to which only one treatment was administered, that treated in January (G4) presented better behaviour in positive sample reduction and mean *D. dendriticum* EPG than the group treated in April (G5), although elimination fell naturally in the latter, as previously reported by Manga-Gonzalez et al. (1991, 2007), and as has been seen in the results obtained in this study in the control group (G5). Therefore the high value of the intensity effect of the April treatment in G5 (which was not treated during the autumn-winter period when pasture contamination is at its highest) cannot only be attributed to the anthelminthic.

On considering all the groups receiving both one and two administrations, the treatment in January, either after the November one (G1), or alone (G4), is the most effective against *D. dendriticum* as it reduces egg excretion to the greatest extent and also does this during the period when its viability in the field is highest due to the low temperatures. However, the April treatment, administered after the November one (G3), or alone (G5), is the least suitable. The treatment regimes applied to G1, G2, G3 and G4 permitted a significant reduction in *D. dendriticum* EPG elimination in comparison with G5 (control group, treated in April) during Period-1 (November to May), but not from May onwards.

The results obtained corroborate our hypothesis, based on epidemiological studies carried out in Leon (Manga-Gonzalez, 1987; Manga-Gonzalez et al., 1991, 2001, 2007), and coincide to a certain extent with what was reported by Schuster and Hiepe (1993) who chose to do their studies (in Germany) on the efficacy of ABZ, luxabendazole and netobimin against *D. dendriticum* in winter due to the selective effect of the (pro) benzimidazoles, mainly on mature worms. They obtained reductions in the worm load of 92.9% to 94%, with doses of 15 and 20 mg/kg ABZ, respectively. Tharaldsen and Wethe (1980) studied the reduction in *D. dendriticum* egg excretion by sheep administered two ABZ treatments (10–12 mg/kg) one week apart at the start of stabling in November. According to the coprological analyses carried out weekly during the stabling period, egg excretion dropped by 90%, which exceeds our figure with the three treatments administered in November. This could be due to the fact that a larger percentage of the worms were already adults when stabling started in November. Tharaldsen and Wethe's experiments (1980) were carried out in Norway, which is colder than Spain, so ant hibernation begins earlier than in Leon.

Cordero del Campillo et al. (1982) tested the efficacy of ABZ in sheep naturally infected with *D. dendriticum* in the province of Leon (Spain). They carried out three types of experiments on two groups of treated sheep and a control group for each experiment. The first experiment started at the end of March 1978 and tested a 7.5 mg/kg dose of ABZ and two doses of 7.5 mg/kg one week apart. The second experiment started at the beginning of November 1978 and used two doses of 7.5 mg/kg 15 days apart and two doses of 10 mg/kg seven days apart. The third experiment started in mid-May 1979 and tested a dose of 10 mg/kg and two doses of 7.5 mg/kg administered seven days apart. The authors state that general egg excretion followed a similar profile in the three tests, although the 10 mg/kg dose reduced egg excretion more than the 7.5 mg/kg one and the effect of repeating the dose was less in the first and third

tests and greater in the second. As regards the parasite load observed at autopsy, the lowest worm reduction was obtained in the animals treated at the end of March (82.43–85.54%), whilst the highest was observed in the group administered two 10 mg/kg doses seven days apart at the beginning of November (94.05%). It can be deduced from the results obtained by Cordero-del-Campillo et al. (1982) that worm reduction was lower when the treatments were administered at the end of March and in mid-May than in November.

Himonas and Liakos (1980) studied the reduction of *D. dendriticum* worms in naturally infected sheep by administering ABZ at a dose of 15 mg/kg (intraruminal) and 20 mg/kg (intraruminal and oral) and obtained reductions of 99.6% and 98.2%, respectively. The authors state that the experiments were carried out at the end of November 1978 and beginning of June 1979, but the paper does not state clearly which experiments were done at each time, so in our opinion it is impossible to reach any conclusions for comparison with the results obtained in this study.

Corba and Krupicer (1992) studied the efficacy of intraruminal albendazole boluses in sheep naturally infected with *D. dendriticum*. The anthelminthic efficacy was assessed by coprological tests carried out during the autumn pasture and comparison of worm number collected at the necropsy of 22 animals (11 treated and 11 untreated) at the end of the experiment. The mean of eggs per gram decreased significantly during week 2, and it was nearly negative between weeks 4–12. The efficacy of ABZ, when the treated animals were killed in December, was 91.8%. Nevertheless, a small number of parasites were found in all livers from the treated group. In our opinion these parasites which remain alive are important for egg pasture contamination, mainly in winter, as we have explained above.

CONCLUSIONS

The information obtained in this study suggests that, when the climate is continental within the Mediterranean Atlantic transition, using a dicrocoelicide only effective against adult parasites, like ABZ, the chemotherapy control strategic model that reduces *D. dendriticum* egg shedding most in the cold period, when elimination is highest and when egg survival is greatest, is the control model administered to G1. That is, treatment at the beginning of November (when ant hibernation starts) to eliminate the adult worms, and treatment in January when, without reinfection (because ant hibernation finishes about the end of March), most of the metacercariae ingested by the animals until November have become adults capable of shedding eggs. The next most appropriate control models, in descending order, are those with treatments in November and February (G2), in January (G4), and in November and April (G3). However, the control model with only one treatment in April (G5) is the least effective and appropriate, because it does not avoid the highest egg excretion during the end of autumn and winter months. Moreover, egg excretion falls naturally from spring and in the next few months the viability of the eggs is reduced by the temperature increase.

Due to the complexity and length of the *D. dendriticum* life cycle the beneficial effect of the treatments cannot be measured immediately, but rather in the long term, so effective control of the parasite requires repeated strategic treatments over several years to reduce contamination of pastures with eggs and the infection rate in both the definitive (ruminants) and intermediate hosts (molluscs and ants).

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