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Review Article

Field and experimental studies on *Dicrocoelium dendriticum* and dicrocoeliasis in northern Spain

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Abstract

The transmission, control and the relationship between *Dicrocoelium dendriticum* and its definitive (sheep and cattle) and intermediate (molluscs and ants) hosts under natural and experimental conditions are described. Eleven species of molluscs and four of ants were found infected with larval *D. dendriticum* in León province, north-west Spain. Infected ants were observed between April and November and in tetania at 7.5–26.9°C. The highest shedding of eggs by sheep and cattle was detected in winter. Two treatments applied in November and January were the most effective. In experimentally infected molluscs, the parasite was not visible under the stereomicroscope, at least until 50 days post-infection (p.i.). The prepatent period in experimentally infected lambs was 49–79 days p.i. The number of eggs per gram increased with the days p.i. and the parasite burden. The aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase, leukocyte and neutrophil values of infected lambs increased, but those of lymphocytes decreased. Using the enzyme-linked immunosorbert assay technique, the IgG antibody response to excretory–secretory and somatic antigens of *D. dendriticum* was positive from day 30 p.i., although the maximum antibody levels were observed on day 60 p.i. The number of worms per lamb ranged between 30 and 2063. Cholangitis and cholangiectasia of the septal bile and hepatic ducts were observed. The best enzymatic systems for adult and larval *D. dendriticum* characterization were lactate dehydrogenase, glucose phosphate isomerase and phosphoglucomutase. Genetic variability of adult *D. dendriticum* was high using the random amplified polymorphic DNA technique.

Introduction

Dicrocoelium dendriticum is a common parasite, responsible for dicrocoeliasis in many countries worldwide. Adult worms live in the liver and gall bladder of many mammalian species, mainly ruminants and occasionally humans (Mohamed & Mummery, 1990), which act as definitive hosts. In Spain, *D. dendriticum* is common in livestock (Manga-González *et al.*, 1991, 2001a; González-Lanza *et al.*, 1993; Cordero-del-Campillo *et al.*, 1994; Ferre *et al.*, 1994). To complete its life cycle this parasite needs to develop inside terrestrial molluscs and ant species, as first and second intermediate hosts, respectively. About 100 snail species have been quoted as natural and experimental intermediate hosts of *D. dendriticum* by different authors. Likewise, at least 21

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Formicidae species, mainly of the genus *Formica*, have been described as susceptible to this parasite in different countries (Manga-González *et al.*, 2001a). The economic and health importance of dicrocoeliasis is mainly due to direct losses caused by liver condemnation and indirect ones such as reduced meat and milk production (Cavani *et al.*, 1982; Karanfilovski, 1983; Jithendran & Bhat, 1996) as a consequence of hepatobiliary pathological alterations (Wolff *et al.*, 1984; Manga-González *et al.*, 2004), and the cost of anthelmintic treatment. Nevertheless, it is not easy to identify the pathogenic effects of dicrocoeliasis as experimental infections are difficult to produce (Otranto & Traversa, 2002, 2003).

The application of efficacious prophylactic and control measures against dicrocoeliasis, which have not been satisfactory thus far, requires a precise early diagnosis, but integrated studies on parasite transmission and experimental dicrocoeliasis are scarce, possibly due to the complexity and length of the life cycle of *D. dendriticum*. Therefore studies on the transmission of *D. dendriticum* and the relationship between this parasite and its definitive (sheep and cattle) and intermediate (molluscs and ants) hosts under natural and experimental conditions were undertaken. Moreover, assays to establish the most appropriate months for the application of strategic treatment have been performed, using the epidemiological data obtained. Currently we are involved in purifying antigenic molecules of the parasites which could serve as targets for diagnosis of and vaccination against dicrocoeliasis. The results of all these studies are reviewed in the present paper.

Epidemiological studies

A range of studies has been undertaken on dicrocoeliasis in the province of León (north-west Spain) and these included: the identification and biology of the molluscs and ants acting as first and second intermediate hosts of *D. dendriticum*; the kinetics of infection and the degree of development of the larval stages they host; the behaviour of infected ants (in tetania) in relation to the time of day and ambient temperature; the transmission period to definitive hosts by detecting infective metacercariae in ants in the field; the shedding kinetics of eggs in cattle and sheep; the influence of environmental factors on the dynamics of molluscan and ant populations, the degree of larval development and infection rates in the definitive and intermediate hosts.

Molluscs and natural infections

Investigations on *D. dendriticum* in León started about 30 years ago, mainly focusing on the taxonomy, distribution and natural infections of terrestrial molluscs as intermediate hosts. The identification of molluscs was carried out microscopically (Gittenberger & Manga-González, 1977; Manga-González & Cordero, 1979; Manga-González, 1983) and using isoelectric focusing techniques in thin-layer polyacrylamide gels (Manga-González & Rollinson, 1986). Later, a helminthological study was carried out on 10,604 specimens of 23 species of Helicidae (Mollusca, Stylommatophora) collected over 12 years in more than 350 villages throughout León province to investigate the life cycle of *D. dendriticum* in the wild (Manga-González, 1987, 1992; Manga-González *et al.*, 2001a). The following 11 mollusc species, collected in 95 sampled villages, were found to be infected with *D. dendriticum* sporocysts in the hepatopancreas: *Cernuella (Cernuella) virgata* (Da Costa) (1.36% infected specimens); *Cernuella* (*Microxeromagna*) *vestita* (Rambur) (5.17%); *Cernuella (Xeromagna) cespitum arigonis* (Schmidt) (2.77%); *Helicella corderoi* Gittenberger & Manga (7.84%); *Helicella itala* (Linnaeus) (5.68%); *Helicella jamuzensis* Gittenberger & Manga (1.03%); *Helicella madritensis* (Rambur) (1.86%); *Helicella ordunensis* (Kobelt) (2.13%); *Cochlicella barbara* (Linnaeus) (0.20%); *Monacha* (*Monacha*) *cartusiana* (Müller) (2.39%) and *Cepaea nemoralis* (Linnaeus) (0.72%). Higher prevalences were obtained in the autumn (in adult and young specimens) and in the spring (in adults) and, overall, the prevalence of infection increased with the age of molluscs. The highest number of daughter sporocysts with germinal masses were found in molluscs in the autumn, while those with mature or nearly mature cercariae were observed in the winter and spring.

Dicrocoelium dendriticum *egg deposition by sheep and cattle*

The kinetics of *D. dendriticum* egg deposition in the faeces of sheep (Manga-González *et al.*, 1991) and cattle (González-Lanza *et al.*, 1993) chosen at random in five localities in the upper and middle Porma river basin (León province, north-west Spain) were undertaken at monthly intervals over a period of one year. The sedimentation and McMaster techniques were used to analyse 5 and 10 g of each sheep and cattle sample, respectively. Of 995 sheep samples examined, 63.6% contained *D. dendriticum* eggs and the number of eggs per gram (epg) varied from 33 to 5340 with a mean ($\bar{x}$) of 323.4 $\pm$ standard error (SE) 18.5. The highest deposition rate was observed in winter, not only because of the prevalence (90.5%) but also because of the epg ($\bar{x} = 726.6 \pm 88.4$). In cattle, *D. dendriticum* eggs were found in 37.64% of 1251 samples examined, and the number of epg ranged from 10 to 1000 ($\bar{x} = 41.65\% \pm 2.73$). The main egg-deposition period was autumn-winter, althought the mean epg values were similar in all months except for a maximum deposition in March.

Life cycle of D. dendriticum

The transmission of *D. dendriticum* was carried out on labelled sheep, molluscs and ants over two consecutive years from June 1987 to September 1989 in Redipollos, in the upper Porma basin (north-east of León province). Molluscs were sampled at random every two weeks from 101 plots measuring 0.5 × 0.5 m, varying in altitude from 1100 to 1400 m. Monthly prevalence and intensity dynamics of natural snail infections with *D. dendriticum* and the extent of larval development were studied. Ants were collected using traps at ground level, directly from ant hills or taking infected ones from in tetania attached to plants. Metacercariae found within each ant were extracted, counted and studied morphologically and

isoenzymatically. The identification of metacercariae found was confirmed by experimental infection of definitive hosts (hamsters and lambs). Moreover, a coprological study of the monthly shedding of *D. dendriticum* eggs was carried out on faeces from 81 labelled sheep, ranging in age from 4 months to 8 years. Thirty-five were 4 to 8 months old, 22 of these were sent out to graze in June 1987 and the remainder in May 1988.

With reference to the molluscan sampling, 27 molluscan species of Gastropoda were identified, belonging to 21 genera, and 13 families of the order Stylommatophora, subclass Pulmonata, except for one species of the genus *Cochlostoma*, which belongs to the family Cyclophoridae, order Mesogastropoda, subclass Prosobranchia. The largest number of molluscan species was found on pasture on limestone. The most abundant species were, in decreasing order, *Helicella itala*; *Helicella corderoi*; *Chondrina kobelti cliendentata* Gittenberger; and *Pyramidula rupestris* (Draparnaud). The abundance and activity of *H. itala* were greater in the spring and autumn, with young specimens being were more abundant in the spring whereas adults were more so in the autumn. *Helicella itala* was mainly found in pasture areas in the spring and sheltering between soil and rock, in moss and other plants in the summer. *Helicella corderoi,* a species endemic to the León mountain region, was mainly observed at the end of autumn and in winter, with adults more predominant in September and November and young ones in May. Active *H. corderoi* were found in October, March and April, and those with an epiphragm predominated in the summer. This species showed a preference for pasture, except in summer, when it preferred soil–rock transition areas. A total of 3568 molluscs were examined, but *D. dendriticum* infection was only detected in 2.98% of 2084 *H. itala* examined and in 1.06% of 852 specimens of *H. corderoi*. The highest prevalence value was detected in *H. itala* in September and in *H. corderoi* in February. Daughter sporocysts with well-developed cercariae predominated in the spring and autumn and prevalence increased with mollusc age and size (Manga-González *et al.*, 2001a).

With reference to the collection of ants, 8133 specimens of 25 species belonging to various genera of the family Formicidae were sampled throughout the year (except in December). Active ants were only seen between March and November, with infected ants being found between April and November. Ants in tetania attached to plants were observed in the surroundings of 29 nests between May and October (ants per nest: 1–9, $\bar{x} = 5$. The species of ants infected with *D. dendriticum* were: *Formica cunicularia* Latreille (1158 examined specimens, 0.69% infected, 2–56 metacercariae per ant); *F. sanguinea* Latreille (234, 1.28%, 2–63); *F. pratensis* Retzius (2030, 4.33%, 1–186); *F. rufibarbis* Fabricius (288, 6.59%, 2–107; moreover 95.39% of 2085 specimens of *F. rufibarbis* collected in tetania in a flat area close to León, contained 1–240 metacercariae). The prevalence of *F. pratensis* collected in tetania was 100% but only one metacercariae settled in the suboesophageal ganglion of each ant. When the behaviour of *F. pratensis* was followed in areas close to three anthills in June and August, ants in tetania were observed between 0600-1410 and 1800-2150 h with a temperature varying from 7.5°C to 26.9°C. Specimens of *F. rufibarbis* collected in the field in tetania survived in the laboratory, and were maintained and fed in artificial nests, for up to 6.5 months (Manga-González *et al.*, 2001a).

In experimental infections, lambs started to shed eggs 2 months after first going out to graze, with the peak of shedding commencing after 4 months. All lambs and sheep eliminated eggs of *D. dendriticum* in the faeces with a mean epg of 244.5 ± 8.10. The highest number of eggs desposited occurred in January–February with a prevalence of 84.17 and in February with a mean epg of 374.65 ± 38.5. The highest values for prevalence and mean epg were observed in the oldest animal group (Cabanas *et al.*, 1989 and unpublished).

Chemotherapy

A chemotherapeutic study was carried out in an ovine flock destined for milk production and naturally infected with *D. dendriticum*. The aim of this study was to determine the most appropriate months for applying strategic treatments to obtain the maximum reduction of *D. dendriticum* egg deposition and, in addition, to decrease pasture contamination with viable eggs. Five groups of sheep, homogeneous in terms of eggs per gram deposited, were established. Each group received one or two treatments with an oral dose of 20 mg kg^{-1} of albendazole in different months. The kinetics of egg deposition was followed for one year. The efficacy of the anthelmintic was evaluated following treatment. The highest reduction of faecal egg shedding was obtained in a group of sheep treated in November and January (Quiroz-Romero *et al.*, 1996 and unpublished).

A further study was undertaken for two years on anthelmintic treatment against *D. dendriticum* in cattle grazing in the eastern mountains of León province. The cattle, belonging to two farms from different villages, were destined for meat production. In each farm different groups of cattle were established, homogeneous with regard to the number of *D. dendriticum* eggs per gram deposited. Various treatments using an oral dose of 20 mg kg^{-1} of netobimin were administered in each group in different months. The kinetics of egg deposition were studied. The efficacy of the anthelmintic was evaluated following treatment (Manga-González *et al.*, 2001b and unpublished).

Experimental studies

In order to further our understanding of the transmission, pathology and diagnosis of *D. dendriticum*, various aspects of experimental dicrocoeliasis were undertaken in both intermediate and definitive hosts.

Molluscan hosts

In investigations on the larval development of *D. dendriticum,* 1448 specimens of *Cernuella (X.) cespitum arigonis* grouped in eight batches were infected. The snails were maintained for 2 days on filter paper in individual Petri dishes, containing egg doses of between 50 and 150 eggs per mollusc. Infected molluscs and controls (470) were kept under controlled laboratory conditions, at 20°C, 50% relative humidity and 7 h daylight per day, until they

were killed following the protocol established for each type of study. In addition, two batches of 150 *Cernuella (C.) virgata* and 70 *Cernuella (M.) vestita* were tested with doses of 60 and 20 *D. dendriticum* eggs per mollusc, respectively, and killed periodically. Microscopic, isoelectric focusing and histological techniques were used to detect larval development. In the case of *C. (X.) cespitum arigonis* (González-Lanza *et al.*, 1997), *D. dendriticum* hatched eggs eliminated in the mollusc faeces were not produced after the fourth day of infection. Little-evolved daughter sporocysts were observed under the stereomicroscope for the first time on day 50 post-infection (p.i.). The proportion of *C. (X.) cespitum arigonis* infected ranged from 17.53% to 75%. Daughter sporocysts with undifferentiated germinal masses were predominant between days 50 and 90 p.i. From 110 to 189 days p.i., sporocysts with cercariae at various stages of development were found, although the emission of slimeballs with cercariae was not observed. In *C. (C.) virgata* and *C. (M.) vestita*, daughter sporocysts of *D. dendriticum* with undifferentiated germinal masses were observed on days 61 p.i. and 62, respectively. Sporocysts with developed cercariae were observed in *C. (C.) virgata* from day 135 p.i. until the end of the experiment (167 p.i.), while only immature cercariae were detected in *C. (M.) vestita* until day 132 p.i. (Manga-González *et al.*, 1998 and unpublished). Unwalled mother sporocysts were observed filling the spaces between several hepatopancreatic lobules in *C. (X.) cespitum arigonis* on days 19 and 42 p.i. (Álvarez-Nogal *et al.*, 1992; Manga-González & Quiroz-Romero, 1999 and unpublished). Moreover, on day 90 p.i. haemocytic infiltration, calcium cell plasma vacuolization, an increase in digestive cell lipofuscines and much parasitic castration in infected molluscs were observed (Gómez *et al.*, 1996).

For the interpretation of data in the field, studies on *D. dendriticum* larval development were carried out in snails infected in the laboratory and maintained in a natural environment. Over 4 years 150 *C. (X.) cespitum arigonis* were tested each month with an individual dose of 50 eggs of *D. dendriticum*. After 48 h in contact with egg-contaminated food, these snails and 50 controls were moved to plots, located in a fenced-in plot of land irrigated with sown sward on the Agriculture Research Station (CSIC, León) experimental farm. Snails were killed periodically and examined under a stereomicroscope to follow the larval parasitic development. The minimum post-infection period to detect larval parasites, for the first time, was 2 months in the experiments begun in June and July, although the maximum period was 9 months in one experiment begun in October. For the observations of daughter sporocysts with well developed, but not mature, cercariae, at least 6 months p.i. were necessary. These types of sporocysts were only found in snails of the experiments begun in the spring (Manga-González *et al.*, 1995 and unpublished).

Mammalian hosts (lambs and hamsters)

Studies on ovine experimental dicrocoeliasis are scarce, largely due to difficulties in obtaining ants with *D. dendriticum* metacercariae. Our study was carried out on 32 Churra breed lambs aged 4 months, which were kept in isolation throughout the experiment on the premises of the CSIC (León). Twelve lambs were each dosed with 1000 *D. dendriticum* metacercariae per animal, whilst another 12 were each dosed with 3000 metacercariae. The remaining eight lambs were used as controls. Half the lambs in each group were slaughtered on day 60 p.i. and the other half on day 180 p.i. The 48,000 metacercariae used for infecting the lambs came from the abdomen of 814 *Formica rufibarbis* ants collected in tetania. Faecal samples were collected from the rectum of the lambs from day 0 p.i., 6 weeks p.i. and then twice weekly until eggs were detected for the first time. From that date collection was fortnightly in the morning and evening until the end of the experiment. Egg counts were obtained by processing 3 g from each sample by sedimentation and McMaster techniques. Blood samples were taken fortnightly from all lambs from day 0 p.i. until slaughter. Sera were analysed for the hepatic marker enzymes: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), lactate dehydrogenase (LDH) and gamma-glutamyl transpeptidase (GGT); and other biochemical parameters: total proteins, albumin, total bilirubin and glucose. In addition, anti-*D. dendriticum* antibody kinetics were followed using an excretory–secretory and somatic antigen via the indirect ELISA technique. The following parameters were analysed in uncoagulated blood samples: erythrocytes, leucocytes, haematocrit, haemoglobin, erythrocyte indices and leucocytic formula. All lambs were weighed on days 0, 60 and 180 p.i. Once the lambs were killed and necropsied, macroscopic studies of the liver, gall bladder and hepatic lymph nodes were carried out and the *D. dendriticum* worms found in the liver and gall bladder were removed, counted and studied. Samples of the hepatic lobes, hepatic bile duct, cystic duct, gall bladder and hepatic lymph nodes were taken and appropriately fixed for the histopathological and ultrastructural studies.

Infected lambs began *D. dendriticum* egg deposition between days 49 and 79 p.i. ($\bar{x} = 59 \pm 1.6$). The mean number of epg was significantly higher in samples taken in the afternoon than in the morning. The rate of epg eliminated by all infected lambs varied between 33.3 and 2266.4 ($\bar{x} = 274.8 \pm 23.8$). In general, the number of epg increased with the days p.i. and the highest mean epg occurred 180 days p.i. In lambs infected with 3000 metacercariae, the mean epg (347.2 ± 42.4) was greater than in those infected with 1000 (194.9 ± 14.4). The percentages of metacercariae established as adult worms were 21.6% and 16.3% in lambs infected with low and high doses, respectively. The worm burden per animal varied between 30 and 437 in the lambs infected with 1000 metacercariae, and 110 and 2063 in those infected with 3,000. A positive correlation was observed between worm establishment and the mean number of epg eliminated throughout the experiment. Worm length in experimentally infected lambs killed on day 60 p.i. ($\bar{x} = 3.7 \pm 0.2$ mm) was smaller than that in worms recovered on 180 days p.i. ($\bar{x} = 5.6 \pm 0.09$ mm) (Campo *et al.*, 2000).

The kinetics of IgG antibodies against excretory–secretory (ES) and somatic (So) antigens (Ag) of *D. dendriticum*, followed a similar pattern throughout the post-infection period in both groups of lambs, but

higher optical density (OD) values were observed in those infected with 3000 metacercariae, except on days 150 and 180 p.i. The cut-off OD value was 0.434 for ES Ag and 0.278 for So Ag and from day 30 p.i. the sera were positive in both groups and antigens, with maximum OD mean values being observed in both groups on day 60 p.i. ($\bar{x} = 1.031 \pm 0.072$, 0.780 ± 0.065, high dose for ES Ag and So Ag, respectively; 0.993 ± 0.083, 0.735 ± 0.059, low dose for each antigen, respectively). Subsequently, OD values decreased slightly with some fluctuations, although they remained high until the end of the experiment 180 days p.i. There was no correlation between the antibody level and parasite burden (González-Lanza *et al.*, 2000).

With reference to the biochemical parameters, only a small increase was detected in the bilirubin (7%) and albumin (3%) values in infected lambs compared with controls. The values of the hepatic enzymes which increased most in comparison with the controls were ALT (48%) and AST (38%) in lambs infected with 3000 metacercariae. Values for AST, ALT and LDH were higher on day 60 p.i. compared with day 180 p.i., whilst the opposite occurred with those for GGT. No clear relationship was observed between worm burden and enzyme values, although the highest ALT, GGT and AST values were observed in the lamb with the most worms (2063) (Manga-González *et al.*, 2004). The highest neutrophil and the lowest lymphocyte mean counts were recorded in infected lambs around day 60 p.i. On the other hand, the highest leukocyte and neutrophil values and the lowest lymphocyte values were detected in the lamb with the highest worm burden.

Hepatic induration was clearly evident in lambs slaughtered at 180 days. Whitish dilated intrahepatic bile ducts were observed, mainly on the visceral surface on the left hepatic lobe of the liver which, in section, corresponded to enlarged and ectasic bile ducts. In lambs infected with 3000 metacercariae, the gall bladder appeared distended and the hepatic lymph nodes were slightly enlarged and of firm consistency. Worms in the septal bile ducts produced hyperplasia, desquamation and necrosis of the epithelial cell lining, goblet cell differentiation and the presence of intraepithelial globule leukocytes. Frequently the lumina of these bile ducts contained some worms producing extensive eroded areas in the epithelial lining. In addition, portal hepatitis and portal, septal and, on occasions, perisinusoidal fibrosis were observed, especially in lambs with large worm burdens. However, no alterations compatible with hepatic cirrhosis were observed. Occasional worms covered by blood were detected in the septal hepatic veins. Multifocal vacuolar degeneration of hepatocytes was observed, which corresponded ultrastructurally to mitochondrial vacuolization. Epithelial necrosis, oedema and lymphatic ectasis were observed in the gall bladder. In the hepatic lymph nodes, hyperplasia of reticular cells stood out in relation to the medullary sinuses, which contained a large number of plasmatic and macrophage cells. The main hepatic/biliary lesion scores were correlated with worm burden. The most severe lesions were observed in lambs infected with 3,000 metacercariae and slaughtered at 180 days p.i. with a heavy worm burden (Manga-González *et al.*, 2004). Studies carried out on the liver and hepatic lymph nodes immunolabelled by the avidin-biotin complex system showed that *D. dendriticum* induced a humoral and cell-mediated local immune response that contributed to the inflammation observed but this appeared not to destroy the worms (Ferreras *et al.*, 2000 and unpublished). The highest weight loss in comparison with controls was observed 60 days p.i. in both groups of infected lambs, although it was slightly more marked in those infected with 3000 metacercariae. The highest reduction (−18%) was detected in the groups of lambs harbouring with 401–600 worms. Nevertheless, no clinical signs were observed (Manga-González *et al.*, 2004).

The effects of experimental dicrocoeliasis on the hepatic oxidative drug-metabolizing system in hamsters (*Mesocricetus auratus*) were determined in animals infected with 40 *D. dendriticum* metacercariae and killed on days 80 and 120 p.i. The pathology was ascertained by detection of the fluke eggs in faeces, increased serum ALT and AST activities, and post-mortem and histological findings. Cytochrome P-450 concentration, aniline hydroxylase activity and ethoxycoumarin O-deethylase activity were significantly decreased in both groups of infected hamsters. Aminopyrine N-demethylase activity and erythromycin N-demethylase activity were only reduced 120 days after infection. This study demonstrates the effects of experimentally induced dicrocoeliasis on hepatic drug-metabolizing enzymes of hamsters, and indicates that the capacity of the liver for handling drugs and xenobiotics may be impaired as a consequence of the infection (Sánchez-Campos *et al.*, 1996).

Isoenzymatic and molecular studies

In order to characterize the different stages of *D. dendriticum*, study their possible genetic variability, establish corresponding isoenzymatic models and select the most appropriate markers for detecting the parasite, the following enzymes were studied: lactate dehydrogenase (LDH), glucose phosphate isomerase (GPI), phosphoglucomutase (PGM), acid phosphatase (AcP), α-glycerophosphate dehydrogenase (α-GPDH), hydroxybutyrate dehydrogenase (HBDH) and malate dehydrogenase (MDH). Isoelectric focusing in thin-layer polyacrylamide gels was used to analyse soluble extracts of 3131 adult worms from the liver of 59 sheep, six cattle and one goat from various parts of north-west Spain. A total of 1608 metacercariae, extracted from 33 *Formica rufibarbis* ants collected in tetania, was also analysed. Likewise, studies were carried out on sporocysts recovered from the hepatopancreas of naturally and experimentally infected snails belonging to *C. (M.) vestita*, *C. (X.) cespitum arigonis* and *H. itala*. The same technique was also used to detect early larval stages of *D. dendriticum* in *C. (X.) cespitum arigonis* experimentally infected in which hatched eggs of the parasite were observed in the faeces. Activity of the enzymes LDH, GPI, PGM, AcP and MDH was observed in adult worms of *D. dendriticum* from sheep, but not of α-GPDH and HBDH. The enzymes LDH, GPI, PGM and AcP showed activity in worms from cattle and LDH, GPI and PGM in worm from goats. LDH, GPI, PGM and AcP were polymorphic enzymes in worms from cattle and sheep.

Worms from goats were also polymorphic for LDH while GPI and PGM were monomorphic. As the activity of MDH was weak and diffuse no enzymatic types were established. No clear differences were observed between the enzyme systems of *D. dendriticum* isolated from animals of the same species but originating from different habitats. The results suggest that the best enzyme systems to characterize adult *D. dendriticum* are LDH, GPI and PGM, as these show sufficient phenotypic variability (Campo *et al.*, 1998). Some models obtained for these enzymes were common amongst adults, metacercariae and sporocysts of *D. dendriticum* or between two of these development stages. In general, a more reduced number of activity bands and a lower band intensity were detected in metacercarial stages than in adults (Manga-González *et al.*, 2001a). The best enzymatic systems for the detection of *D. dendriticum* sporocysts in infected snails were LDH, as it showed no activity in the molluscan tissue, and also GPI, as there was evidence of the parasite 15 days p.i. (Campo *et al.*, 1992a, 1992b and unpublished).

Intra- and interspecies genetic variability in adult *Dicrocoelium dendriticum*, from 20 sheep of different breeds and geographical locations in the province of León, was studied using the random amplified polymorphic DNA (RAPD) technique. The DNA fragments of *D. dendriticum* were amplified using decamer primer oligonucleotides of an arbitrary sequence. Of 23 primers assayed, seven yielded a high number of polymorphic bands, sufficient to differentiate worms. Some primers produced complex and variable amplified DNA models. The intra- and interpopulation genetic variability of *D. dendriticum* adults was analysed by calculating the Jaccard identity coefficients. The corresponding dendrograms were undertaken with the help of the FITCH program (included in the PHYLIP 3.5C Package), in order to discover the relationship between specimens. The genetic variability of *D. dendriticum* seems high and the genetic similarity within individual worm populations is similar to mean values between worms from different sheep. The results suggest that each animal is infected with genetically different parasites from one or more ant populations (Sandoval *et al.*, 1999). On the other hand, in order to clarify the phylogenetic position of *D. dendriticum*, the gene which codifies for the 18S ribosomal RNA from the DNA of adult *D. dendriticum* was cloned using primers of *Schistosoma mansoni*. A product of approximately 2 Kb in the vector pGEM^{+}T was cloned for later sequencing and the sequence sent to the EMBL / GenBank, Accession No. Y11236, in 1997 (H. Sandoval, M.Y. Manga-González, D. Johnston, D. Rollinson, P. García, M. Pérez de la Vega & J. M. Castro).

Antigens of *D. dendriticum*

The isolation, characterization and purification of *D. dendriticum* antigens or their fractions are necessary to carry out specific immunological diagnosis of dicrocoeliasis and obtain vaccine preparations against the parasite that are currently non-existent. However, publications relating to the antigens of *D. dendriticum* are scarce so various studies on this topic have been carried out over the past few years (Oleaga *et al.*, 1999; Robles *et al.*, 2001; Revilla-Nuín *et al.*, 2003, 2004 and unpublished). Protein profiles of the somatic and excretory–secretory antigens of *D. dendriticum* were studied by SDS–PAGE and their specificity confirmed using Western Blot against sera from lambs experimentally infected with *D. dendriticum*, and with other trematode and nematode species. Two immunoreactive bands specific to *D. dendriticum* of approximately 130 and 24 kDa for the somatic extract, and one of 130 and another of 42 for the excretory–secretory extract were detected. Nevertheless, the 130 kDa polypeptide presented much immunogenic specificity (Revilla-Nuín *et al.*, 2003). The 130 kDa band of both types of antigen was then purified by chromatography in gel-filtration (Sephacryl S-300) and ion-exchange (DEAE-Sepharose). According to the results, the 130 kDa protein could be used for immunological diagnosis and protection assays. However, because of the low concentration of this protein, further research is in progress to obtain and purify, using a cDNA library and monoclonal antibodies, sufficient quantities of native and recombinant specific antigens of *D. dendriticum* to develop and improve the immunological diagnostic tests (direct and indirect) for dicrocoeliasis, thereby increasing their specificity and sensitivity, and to check their protective capacity against the parasite.

Discussion

The shedding of *D. dendriticum* eggs in the faeces of ruminants occurs uninterruptedly throughout the year, although the highest values are recorded at the end of the autumn and in the winter. In Germany, the highest elimination of eggs by sheep takes place in the spring and by cattle in the autumn (Kopp, 1975). In Italy, Ambrosi & Principato (1981) observed the highest rate for egg deposition in sheep between February and May. Eggs of *D. dendriticum* have been experimentally proven to resist temperatures between −20°C and −50°C (Boray, 1985). Likewise studies carried out under controlled field conditions in León have shown that egg mortality in faeces exposed in the environment during the hot months of July and August was nearly 100% (Alunda & Rojo-Vázquez, 1983a). Thus in the colder period of autumn–winter, when the greatest egg elimination takes place in the province of León, the survival of *D. dendriticum* eggs is very high. Due to this, pasture contamination by viable eggs is considerable in the spring, when molluscs (mainly the young ones) become active and are very abundant.

Taking into account our own data and those of Del Río (1967) and Alunda & Rojo-Vázquez (1982) the most important species in the epidemiology of dicrocoeliasis in Spain are *C. (X.) cespitum arigonis* in the plateaux and plains and *H. itala* in the mountains, mainly the north. The latter species has also been found naturally infected with *D. dendriticum* in the UK (Tarry, 1969) and Bosnia-Herzegovina (Rozman *et al.*, 1974). When the percentages of molluscs naturally infected with *D. dendriticum* obtained by us are compared with those given by other authors, much variability is observed (Manga-González *et al.*, 2001a). The egg ingestion periods of *D. dendriticum* dependent on mollusc activity, and the survival and

development of the larval parasites are important in the transmission of dicrocoeliasis. Trematode sporocysts require a temperature of over 4°C to mature, with development increasing with rise in temperature (Schuster, 1993). Under controlled field conditions, we confirmed that in hot months larval development within the molluscan host is accelerated. The mollusc species can also influence *D. dendriticum* development as observed by Gómez *et al.* (1996), with a higher prevalence and a more rapid development occurring in *C. (X.) cespitum arigonis* than in *C. virgata*, when tested with the same dose and kept under the same conditions. According to our results, the infection prevalence increased with mollusc age, a fact that has also been reported by Schuster (1993) and Alunda & Rojo-Vázquez (1983b). In general, the highest rate of infection in molluscs occurred in the autumn and spring. Moreover, immature daughter sporocysts (with germinal masses) predominated in adult and young molluscs collected in the autumn, whilst sporocysts with mature or nearly mature cercariae predominated in adults collected in the winter and spring. In the Marmara region (Turkey) the lowest parasite percentages were generally detected at the end of the summer and in the autumn (Kalkan, 1971). In France, Badie (1978) reported that infection of molluscs occurs between the end of one hibernation period and the beginning of the next. Our results seem to indicate that young molluscs, which are more abundant in spring, are infected between the beginning of spring and the beginning of summer. The first infected ones can shed slimeballs with cercariae in the summer or at the beginning of the autumn, whilst those infected later can shed slimeballs (as adults) in the first half of the following year, provided they survive the harsh winter. According to Schuster (1993), in Germany most specimens of *Helicella (H.) obvia* are infected in the autumn of their second year of life, in which the shell sizes are intermediate. In addition, the percentage of molluscs with daughter sporocysts is higher in the spring and the shedding of slimeballs occurs in May and June. In Germany, Hohorst & Lämmler (1962) also observed the first slimeballs shed by *Zebrina (Z.) detrita* at the beginning of May and the last ones in mid-October. The sun affects the appearance, size and consistency of slimeballs in only a few hours and thus influences the viability of the cercariae. Their infective capacity for ants is prolonged at least 18 h, if slimeballs are stored at 4°C, although a very low number of cercariae maintain mobility after 72 h at this temperature (Del Río, 1967).

When slimeballs are ingested by appropriate species of ants, cercariae become metacercariae. One, two or three of them, called 'brainworms', settle in the suboesophageal ganglion of the ant whilst the remainder of the metacercariae are located in the abdomen. Variability in the number of *D. dendriticum* metacercariae per ant (Manga-González *et al.*, 2001a) may be due to the behavioural ecology, size and species of ant infected (Schuster, 1991). Among the infected ant species we have found, we believe that *Formica rufibarbis* and *F. pratensis* are most important in the epidemiology of dicrocoeliasis in Spain and in many other countries (principally Eastern Europe) according to our review (Manga-González *et al.*, 2001a). The importance of ants as second intermediate hosts is mainly due to their abundance, wide distribution and the alteration (tetania) in their behaviour produced by the 'brainworm', especially when temperatures and solar intensity decrease, which makes infection of the definitive hosts by ingestion of the parasitized ants easier. The tetania stage of infected ants happens mainly in the early hours of the morning and late in the afternoon, although on cloudy or warmer days we also detected ants in tetania at the end of the morning and the beginning of the afternoon, as did Spindler *et al.* (1986). The highest temperature at which ants were observed in tetania was 26.9°C (*F. pratensis*) in the mountains and 28°C (*F. rufibarbis*) in the flat area, and this was higher than that reported by Jonlija *et al.* (1972) 21°C, Paraschivescu (1983) 18–20°C, Schuster (1991) below 18°C and Spindler *et al.* (1986) 20°C. Active ants were observed between March and November and infected ones from April to November in those collected from the nest and in tetania between May and October. Authors from other countries have also observed infected ants between the spring and autumn (Dementev & Karabaev, 1968, Kazakhstan; Jonlija *et al.*, 1972, Bosnia-Herzegovina; Badie, 1978, France; Schuster, 1991, Germany). Transmission to definitive hosts only occurs at times when the ants are not hibernating. Nevertheless, some infected ants survive in their nests during the winter, and they are responsible for infection of the definitive host on becoming active at the beginning of spring (Tarry, 1969; Badie, 1978). In the following months and until November, when ant hibernation starts again, the ingestion of ants containing infective metacercariae by the definitive hosts and the number of adult worms of *D. dendriticum* in their liver increase. As a consequence, parasite egg elimination also increases during this period, reaching the highest values at the end of the autumn but mainly in winter.

With reference to strategic treatments, the greatest reduction in *D. dendriticum* egg elimination by naturally infected sheep kept on pasture was recorded in sheep receiving two treatments, one in November and another in January. These results were as expected, when considering the epidemiological data previously described. Hence these months are the most appropriate for administering treatment in northern Spain for maximum prevention of contaminating pastures with viable eggs. In our opinion, this treatment regime is more effective in the long term and differs from the recommendations made by Cordero del Campillo *et al.* (1982) who considered that treatment administered at the end of spring/beginning of summer eliminates a large number of parasites and that it should be repeated in autumn to eliminate the residual population.

According to our results on ovine experimental infection, some worms contain mature eggs at least 49 days p.i. Wolff *et al.* (1984) also detected the presence of mature eggs in worms recovered from lambs on day 50 p.i. We did not detect any differences in the prepatent period between the two groups of lambs infected with different doses or between lambs with different worm burdens, like Chandra (1973) and Tarry (1969). The length of worms recovered 60 days p.i. was slightly greater than that observed by Paraschivescu (1981) for worms obtained from lambs 37 days p.i., while the length of those we recovered 180 days p.i. was less than that recorded by the same author at 161 days p.i. The percentage of worms

which became established in lambs infected with 1000 metacercariae was lower than that obtained by Wolff *et al.* (1984) in lambs infected with 1500 metacercariae and killed 54 days p.i. However, the percentage infection in lambs dosed with 3000 was included among those observed by Wolff *et al.* (1984) in lambs infected with the same dose and killed 70 days p.i., but it was lower than that recorded by Hohorst & Lämmler (1962) in sheep tested with 3905 metacercariae and slaughtered 270 days p.i. The percentage of worms recovered in lambs 2 and 6 months p.i. generally increased, which coincides with the observations of Chandra (1973) and Wolff *et al.* (1984). Differences in the number of parasites recovered may be explained by difficulties in visualizing and extracting worms 2 months p.i., when some may not yet be mature and are small in size. However, only a few hours (up to 24) are needed for parasites to reach the bile ducts of the different host species (Hohorst & Lämmler, 1962; Srivastava *et al.*, 1978; amongst others). The number of eggs eliminated in the faeces of lambs generally increased as the worms did. Calamel & Giauffret (1976) and Rojo-Vázquez *et al.* (1981) had already observed this tendency in naturally infected sheep.

According to our studies, the detection of *D. dendriticum* antibodies took place 19 and 23 days earlier than the detection of eggs in the faeces. Similarly, Ambrosi *et al.* (1980) reported high antibody titres 4–8 weeks before patency. Our data showed that the pattern of IgG antibody responses assayed with ES or So antigens of *D. dendriticum* adults basically did not differ. However, Wedrychowicz *et al.* (1995, 1996) pointed out the particular importance of surface and ES antigens in the host–parasite relationship. In any case an early serological diagnosis of the infection can by made by an ELISA test, although it is necessary to obtain more purified antigens to improve the sensitivity of the assay.

The two doses (1000 and 3000 metacercariae) used produced no alteration in the values of total proteins and glucose and only a very low increase in those of albumin and bilirubin of the lambs infected with *D. dendriticum* compared with those controls, or with uninfected sheep (Desco *et al.*, 1989). The lack of change in the total protein values and the slight increase in albumin levels observed by us coincides with results obtained by Theodoridis *et al.* (1991), who considered that worm burdens of up to 4000 *D. dendriticum* do not cause significant blood or plasma protein loss in naturally infected sheep. However, Gundlach *et al.* (1982) obtained an increase in both parameters, Petrov & Abalikhin (1983) in the proteins up to 4% in lambs infected with 2600 metacercariae, and Scala *et al.* (1991) in albumin in sheep naturally infected with 16–828 *D. dendriticum*. Ranucci *et al.* (1981) detected a slight decrease in albumin in adult sheep grouped according to the *D. dendriticum* worm burden (from 1460 to 40,000 worms). The fact that an increase in the values of ALT and AST enzymes was observed in lambs infected with 3000, compared with controls, agrees with the results of Dementev *et al.* (1978) in lambs infected with 1000 metacercariae or with 200 per day for 90 days. Increased AST and ALT activity was observed in infected lambs, compared with controls, which was far less than that recorded by Sánchez-Campos *et al.* (1996, 2000) in hamsters infected with 40 *D. dendriticum* metacercariae. These authors correlated increased plasma ALT and AST activities with active liver cell necrosis, with hepatocyte destruction being a central element of most forms of liver fibrosis. The LDH values do not follow any clear pattern in the three lamb groups studied by us. Scala *et al.* (1991) reported moderate differences in the LDH isoenzymatic fractions between groups of sheep naturally infected with *D. dendriticum* (16–828 worms, $\bar{x} = 137.78$) compared with control groups. As regards GGT activity, we observed a slight increase in the infected lambs compared with controls, as occurred in some of the sheep studied by Ranucci & Grol-Ranucci (1978) and naturally infected with *D. dendriticum* and *Echinococcus granulosus*. However, Sánchez-Campos *et al.* (1999) observed a decrease in GGT activity in hamsters infected with *D. dendriticum* in comparison with the controls. The increase in the activity of this enzyme seems to be directly related to worm burden, and this was confirmed by Ranucci *et al.* (1981) in naturally infected sheep.

Grossly visible cholangitis and cholangiectasia were a constant feature in all infected groups and were generally similar to those described in experimental (Wolff *et al.*, 1984) and natural (Massoud, 1981; Ranucci *et al.*, 1981) infections in sheep. However, necrotic and haemorrhagic tracts reported by Ranucci *et al.* (1981), as a result of the migration of the worms through the parenchyma, were not observed in our study. The intensity and severity of lesions encountered in this study seem to be directly related to the worm burdens, again confirmed by Ranucci *et al.* (1981) and Jithendran & Bhat (1996) in naturally infected sheep. It is likely that, as previously suggested by Camara *et al.* (1996), mechanical irritation alone, caused by suckers of adult worms, does not induce periductal fibrosis. Other cells, granular globule leukocytes, which have been considered to have similar properties to mucosal mast cells (Huntley *et al.*, 1984), have been reported to be involved in the development of active hepatic fibrosis by displaying fibrosis-promoting factors (Yamashiro *et al.*, 1998). The common presence of these leukocytes in the biliary epithelial cells observed in our infected lambs, are in agreement with previous reports in ovine (Wolff *et al.*, 1984) and caprine (Rahko, 1972) dicrocoeliasis, and would support the hypothesis that these cells could be involved in the development of fibrosis seen in our study. We never observed cirrhosis, in contrast to that found by Prunescu *et al.* (1979) in hamsters infected with *D. dendriticum* and by Jithendran & Bhat (1996) in sheep harbouring natural infections. Cholangitis was observed not only in septal bile ducts, but also in the interlobular bile ducts, in which parasite eggs or flukes were never observed. This suggests that the entire liver tissue is affected by toxic metabolites released by adult flukes (Frank *et al.*, 1984), inducing inflammatory responses, and could explain the hepatocytic damage observed by us, similar to that described in infected hamsters by Sánchez-Campos *et al.* (2000). Positive histochemical staining for acid mucins, concentrated close to the apical surface in epithelial cells of the septal bile ducts, confirmed on electron microscope study, suggests an increase in secretory activity, which has been associated with proliferating cholangitis (Katayama, 1996). Intraepithelial lymphocytes were probably the first immune cells to contact foreign antigens entering from the lumen (Cross & Mercer, 1998). Lesions detected in hepatic

vessels, including the occasional presence of ectopic flukes in hepatic veins, have not been reported previously in ovine experimental dicrocoeliasis. Young flukes migrate directly up the biliary duct system (Otranto & Traversa, 2002) but for unknown reasons, they may also reach the biliary system through the portal route (Srivastava *et al.*, 1978). According to our results, it seems that *D. dendriticum* infections influence animal weight less up until 60 days p.i. than until 180 days p.i. This coincides with the findings of Salimova (1972) in lambs infected with 2500, 5000 and 10,000 metacercariae (all with clinical signs of severe dicrocoeliasis), who considered the weight loss to be more marked until *D. dendriticum* reached sexual maturity. Apart from what has already been stated, no clinical signs were observed in the animals we infected. This could be due to the fact that the lambs harboured less than 1000 worms, a parasite burden which has no clinical significance or economic repercussions (Calamel & Giauffret, 1976; Rojo-Vázquez *et al.*, 1981; and Ambrosi, 1991). Wolff *et al.* (1984) did not observe clinical signs in sheep infected with doses of 3000 metacercariae, which harboured up to 1946 worms.

In conclusion, under the conditions in which this ovine experimental dicrocoeliasis study was carried out, the initial dose is not a good parameter for forecasting worm burdens. The positive relation between the number of eggs per gram eliminated and that of worms recovered increased as the worm burden and period p.i. did. The increased AST and ALT values were significant 60 days p.i., approximately when *D. dendriticum* egg elimination by lambs started, at the same time as the IgG antibody response against excretory–secretory and somatic antigens was highest and when the largest decrease in lamb weight was also detected. The severity of lesions observed in experimental dicrocoeliasis, affecting mainly the biliary system but also hepatocytes, was closely associated with worm burden. On the other hand, as long as molecules which can be used in the immunoprophylaxis of dicrocoeliasis of ruminants are not obtained, chemotherapeutic treatments must be used at the end of the autumn and in winter, as this is the period of maximum egg elimination despite there being no reinfection and a high survival rate. Infection of molluscan hosts then readily takes place in the spring, when they are most abundant and active.

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