Characterization of adult *Dicrocoelium dendriticum* by isoelectric focusing

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Abstract

Water soluble extracts of 3131 adult specimens of *Dicrocoelium dendriticum* from cattle, sheep and goats, mainly from León province, were analysed by isoelectric focusing in thin-layer polyacrylamide gels. Activity of the following enzymes was studied: lactate dehydrogenase (LDH, EC 1.1.1.27), glucose phosphate isomerase (GPI, EC 5.3.1.9), phosphoglucomutase (PGM, EC 2.7.5.1), acid phosphatase (AcP, EC 3.1.3.2), α-glycerophosphate dehydrogenase (α-GPDH, EC 1.1.1.8), hydroxybutyrate dehydrogenase (HBDH, EC 1.1.1.30) and malate dehydrogenase (MDH, 1.1.1.37). Five distinct enzyme types were recognized for LDH (pH range 6.30–7.13), GPI (pH 6.13–6.80) and PGM (pH 6.20–6.60) whereas AcP showed three different patterns (pH 5.70–5.92). Weak and diffuse activity was detected for MDH (pH 4.8–6.2) and no activity was observed for α-GPDH and HBDH. In general, little phenotypic variation was observed between worms recovered from a single host, between those from hosts of the same species and between those from hosts of different species, although some enzyme types were found in some animals but not others. Nevertheless, it must be taken into account that most parasites came from sheep and also from a relatively small area in north-west Spain.

Introduction

The liver fluke *Dicrocoelium dendriticum* (Rudolphi, 1819) Looss, 1899 is a little known parasite responsible for the disease dicrocoeliosis in sheep and cattle in various parts of the world. The life cycle of *D. dendriticum* involves several species of terrestrial molluscs as the first intermediate host, various species of ants as the second intermediate host and a wide range of mammals, principally ruminants, as definitive hosts.

Recent studies on the transmission of *D. dendriticum* to its definitive (Manga-González *et al.*, 1991a; Campo *et al.*, 1993b; González-Lanza *et al.*, 1993) and intermediate hosts (Manga-González, 1987, 1992; Manga-González *et al.*, 1991b) have provided further insights into the complexity of the life cycle.

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The economic importance of this helminthiasis has been indicated by several authors (Panasyuk *et al.*, 1972; Cavani *et al.*, 1982) but its control is unsatisfactory (Eckert & Hertzberg, 1994), possibly because of the genetic variability of the parasite and its complex life cycle.

Thus a greater understanding of the genetic diversity of *Dicrocoelium dendriticum* would be very useful, since many helminths, which are morphologically similar, show marked epidemiological differences including infectivity, pathogenicity, immunogenicity and drug sensitivity (Rollinson *et al.*, 1986). Enzyme electrophoresis is a technique which has been used successfully to determine the relationships between species of parasites and their molluscan intermediate hosts and which has allowed a valuable insight into genetic variation occurring within a species (e.g. Rollinson & Southgate, 1985) as well as providing diagnostic characters appropriate for species identification (Avise, 1974; Ferguson, 1980; Oxford

Table 1. Number of *Dicrocoelium dendriticum* adults recovered from different hosts and studied conjointly (from 10 to 100 worms) and individually for each enzyme system.

Host	Infection	Origin of animals	No. animals Examined	No. *D. dendriticum* adults examined conjointly and individually for: LDH	GPI	PGM	AcP	α-GPDH	HBDH	MDH
Cattle	Natural	Bustillo de Cea*	1	36	67	12	13	–	–	–
		Villabrágima**	6	27	21	15	–	–	–	–
Sheep	Natural	Ambasaguas de Curueño*	3	8	8	5	–	–	–	–
		Matadeón de los Oteros*	1	75	75	100	–	–	100	100
		Benavente market***	8	138	58	183	113	–	–	5
		León market*	7	46	75	41	32	4	11	416
		Oteruelo de la Vega*	2	–	15	–	–	–	–	–
		Palazuelo de Torío*	2	–	11	2	2	–	–	–
		Redipollos*	1	26	24	4	4	–	–	–
		Ribaseca*	1	3	6	–	3	–	–	–
		Santa Olaja de la Ribera*	11	54	39	19	6	4	–	–
		Toledo****	2	85	9	41	43	–	–	–
Sheep	Experimental	CSIC Experimental Center*	22	429	424	–	–	–	–	–
Goats	Natural	León market*	1	19	42	30	3	–	–	–

Province of: * León; ** Valladolid; *** Zamora; **** Toledo.

& Rollinson, 1983; Manga-González & Rollinson, 1986; Johnson & Hoberg, 1989; Lee & Zimmerman, 1993). Some studies have been carried out on adult *D. dendriticum* using the technique of horizontal electrophoresis in polyacrylamide gels (León *et al.*, 1986, 1988, 1989).

In view of the lack of research using the technique of isoelectric focusing in thin-layer polyacrylamide gels, to study the characterization and the genetic variability of the *D. dendriticum*, we considered it advisable to investigate worms from the livers of some ruminant species from various parts in Spain and to identify the more appropriate enzyme markers which might be of value in strain differentiation.

Materials and methods

Water soluble extracts of 3131 adult specimens of *D. dendriticum* were analysed to study the activity of the following enzymes: lactate dehydrogenase (LDH), glucose phosphate isomerase (GPI), phosphoglucomutase (PGM), acid phosphatase (AcP), α-glycerophosphate dehydrogenase (α-GPDH), hydroxybutyrate dehydrogenase (HBDH) and malate dehydrogenase (MDH). Some worms were recovered from naturally infected livers of different breeds and crossbreeds of cattle (Friesian; Parda × Asturian), sheep (Assaf; Awassi; Castellana; Manchega; Churra; Churra × Assaf; Churra × Awassi; Churra × Manchega) and goats, the number and origin of which are shown in table 1. Other worms were obtained from two groups of 11 Churra lambs each (table 1), experimentally infected with a dose of 1000 and 3000 metacercariae per animal, respectively. Some of the animals were killed 2 months post-infection (p.i.) and the others 6 months p.i. The metacercariae were obtained from 814 naturally infected *Formica rufibarbis* in a tetanic state collected in the field from a 30 km^2 area to the south of León city.

Adult worms of *D. dendriticum* were washed in a physiological solution before being stored in liquid nitrogen until enzymatic analysis was carried out. Sample preparation was as specified by Manga-González & Rollinson (1986). The LKB Multiphor system of electrofocusing in thin-layer polyacrylamide gels (0.7 mm thickness) was used and the gels prepared according to the LKB 2117 Multiphor II Electrophoresis System Laboratory Manual (Anon., 1986).

Table 1 shows the total number of worms examined as pooled samples (from 10 to 100 worms) and individually, whilst tables 2 to 5 show the number of individuals examined for each enzyme. In order to determine within which pH range there was activity for the enzymes studied, gels with the following pH ranges were used: 3.5–9.5; 4–6; 4–7.5; 5–7; 5–8. Each sample used consisted of 10 to 100 worms. Once the gradient was known, pH 5–8 gels were used for LDH, GPI and PGM activity studies, pH 4–6 for AcP and pH 3.5–9.5 for α-GPDH, HBDH and MDH. In this case, each *D. dendriticum* specimen was analysed individually. Isoelectric focusing was carried out as described by Wright & Rollinson (1979) and De Bont *et al.* (1994).

The partial frequency (%) for each enzyme type according to the animal species from which worms were recorded and to its infection type was calculated. In the same way, total frequency (%) for each enzyme type of the total of *D. dendriticum* samples examined from the three species of ruminants was obtained.

In order to test whether the percentage of partial frequency of each enzyme type in the different hosts was significant, the two percentage equality test (Sokal & Rohlf, 1969) was used. The chi-square test (χ^2) was used to detect possible positive associations between the different enzyme types of LDH and GPI found only in worms from experimentally infected sheep.

The Hedrick (1971) similarity index was used to evaluate the similarity between different worm populations analysed for LDH, GPI, PGM and AcP (the parasites found in each individual animal were considered as one population). This index, applicable to phenotypic data, was used to determine if worms from an animal can be grouped by species, locality or breed. The matrixes obtained were utilized to produce phenograms by means of the UPGMA method (Sneath & Sokal, 1973).

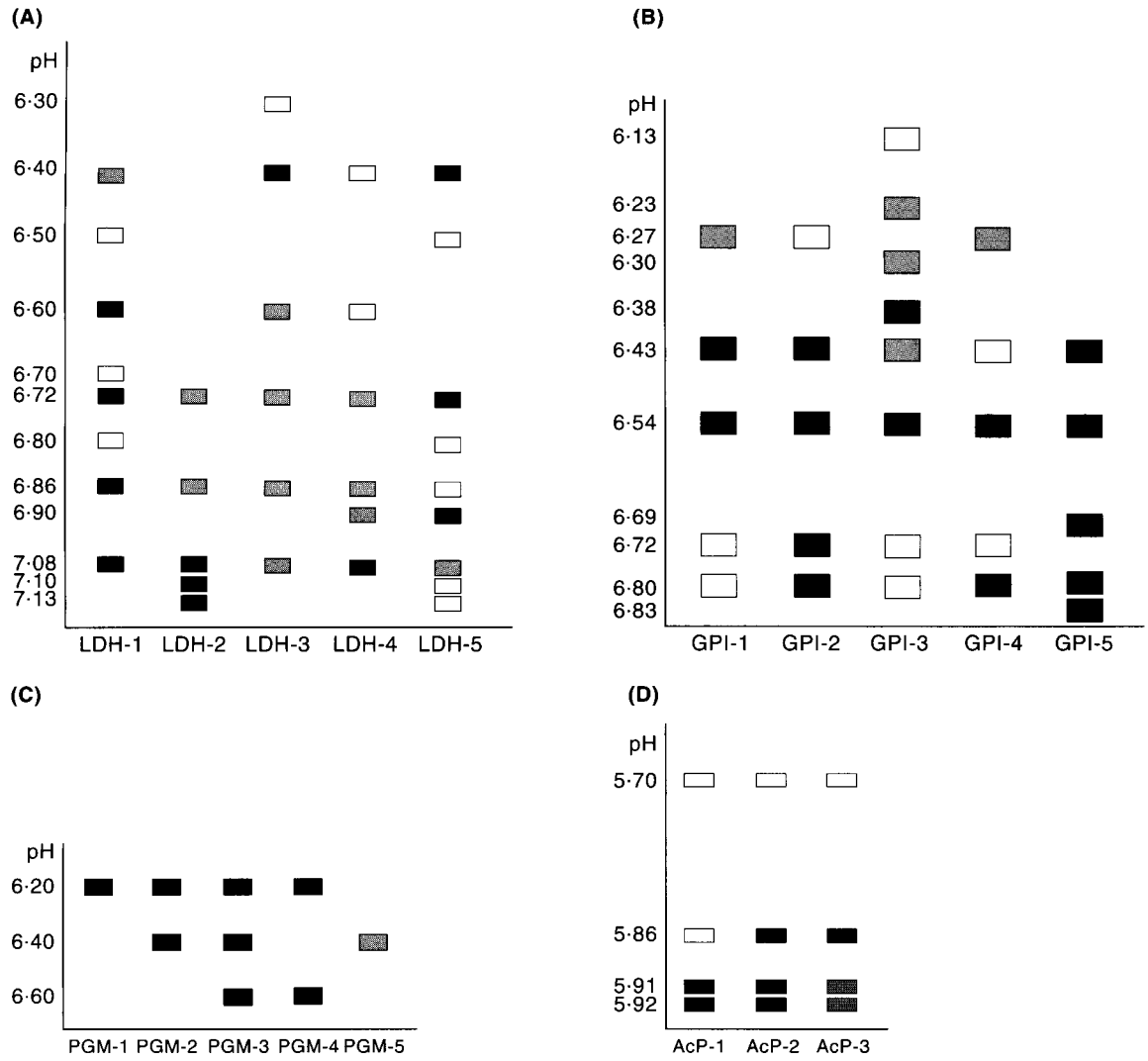

Fig. 1. Diagramatic representation of LDH (**A**), GPI (**B**), PGM (**C**) and AcP (**D**) enzyme types found in adult *Dicrocoelium dendriticum*: ■ Strong bands; ▨ weak bands; □ very weak bands.

Results

All bands of enzyme activity were scored in order to establish isoenzymatic models. They were classified as strong, weak and very weak bands. The results presented here are only the ones obtained for each enzyme on analysing individual adult worms.

LDH

The bands of LDH activity in all adult *D. dendriticum* worms analysed were observed within a pH range of 6.30 to 7.13 (figs. 1A, 2A). Five enzyme types were detected with a decreasing order of frequency (table 2) in all animals: LDH-1, LDH-3, LDH-4, LDH-5 and LDH-2. The five types were found in worms from sheep, but LDH-5 was not recorded in those from cattle, nor LDH-2 in those from goats. Worms from naturally and experimentally infected sheep showed the highest frequency for LDH-3 and LDH-1 and the lowest for LDH-5 and LDH-2, respectively. In worms from cattle and goats LDH-1 was the most frequent enzyme type, while LDH-2 and LDH-5 were the lowest for the first and the second hosts, respectively. The highest frequency for LDH-1 and LDH-2 was detected in cattle, for LDH-3 in naturally infected sheep and for LDH-5 in goats.

When pairs of the partial frequency values were compared for each enzyme type, using the two percentage equality test, statistically significant differences were detected ($P \leq 0.05$) for: (i) LDH-1 and LDH-3 between worms from cattle and naturally infected sheep (t = 5.98, t = 5.58, respectively); (ii) cattle and experimentally infected sheep (t = 3.29); and (iii) naturally and experimentally infected sheep (t = 3.30). Moreover, for LDH-2 statistically significant differences were observed between cattle and naturally infected sheep (t = 2.39), and cattle and experimentally infected sheep (t = 2.77).

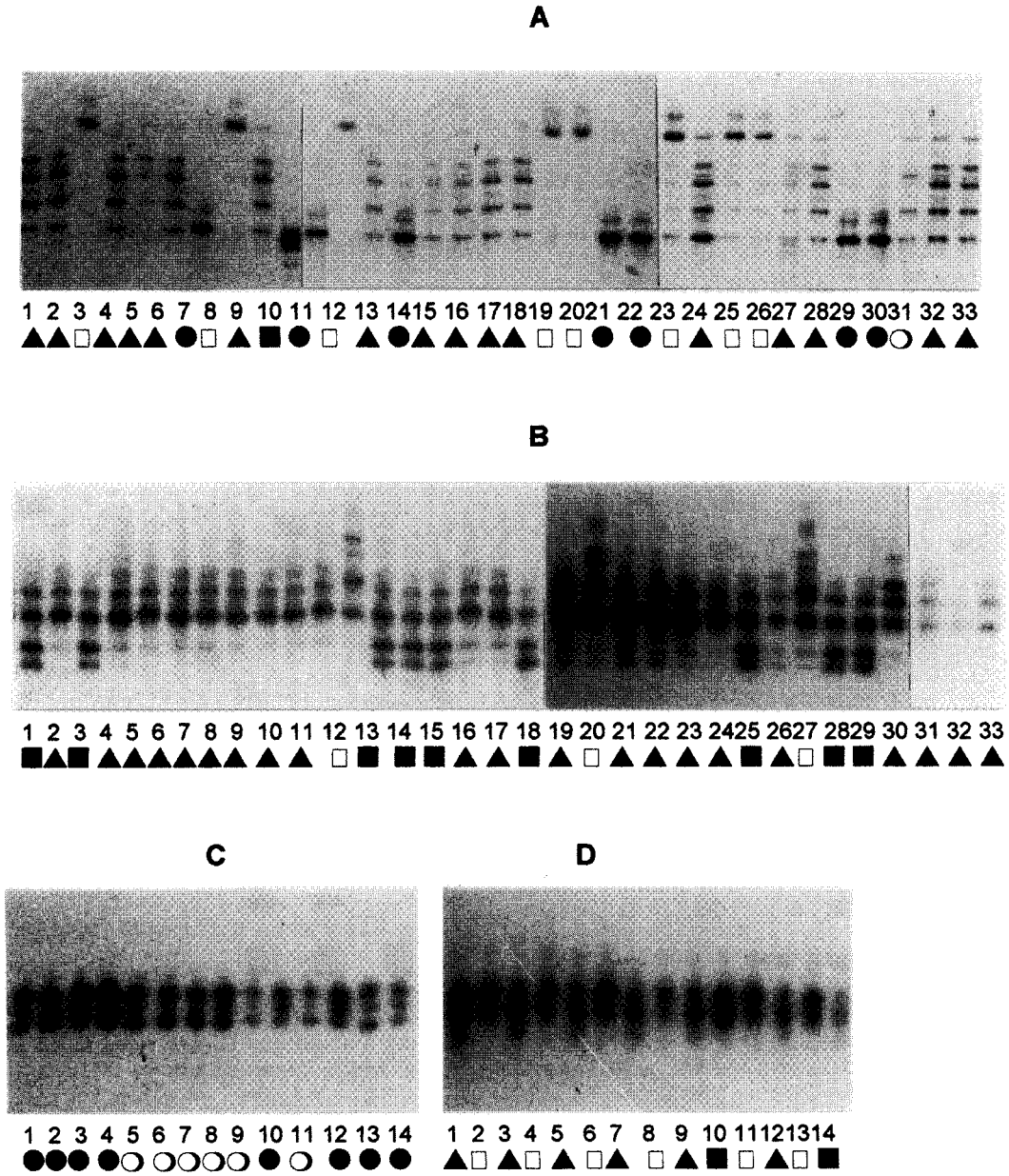

Fig. 2. Examples of enzyme patterns obtained from adult *Dicrocoelium dendriticum*. **A**: LDH found in worms from cattle (samples 1–10), sheep (11–22) and goats (23–33); ▲ LDH-1, ■ LDH-2, □ LDH-3, ● LDH-4, ○ LDH-5. **B:** GPI found in worms from cattle (samples 1–5), sheep (6–30) and goats (31–33); ▲ GPI-1, ■ GPI-2, □ GPI-3, ● GPI-4. **C:** PGM found in worms from sheep (1–14); ● PGM-4, ○ PGM-5. **D:** AcP found in worms from sheep (1–14); ▲ AcP-1, ■ AcP-2, □ AcP-3.

Enzyme activity from non-infected hepatic tissue obtained from sheep was observed in only one band at pH 6.2.

GPI

The enzyme activity of each adult *D. dendriticum* from different hosts indicated that five enzyme types were identified with bands of activity within the 6.13 to 6.80 pH range (figs 1B, 2B). The frequency of the five enzyme types (table 3) in worms in all host animals studied was, in decreasing order: GPI-1, GPI-2, GPI-3, GPI-5 and GPI-4. Worms from goats were monomorphic, with type GPI-1, although only six worms from this host were examined. In worms from cattle, GPI-3 and GPI-4 were not detected; moreover, GPI-1 and GPI-5 were the most and the least frequent, respectively. In worms from sheep, GPI-4 and GPI-5 were not observed, while the frequency of GPI-1 was the highest and that of GPI-3 the lowest. In *D. dendriticum* from experimentally infected sheep, GPI-5

Table 2. LDH enzymatic types from *Dicrocoelium dendriticum* adults recovered from different hosts.

Host	Infection	No. *D.dendriticum* adults examined individually	Adult number and frequency (%) with each enzyme type				
			LDH-1	LDH-2	LDH-3	LDH-4	LDH-5
Cattle	Natural	62	47 (75.80%)	4 (6.45%)	5 (8.06%)	6 (9.67%)	0
Sheep	Natural	162	64 (39.50%)	1 (0.61%)	68 (41.97%)	27 (16.66%)	2 (1.23%)
Sheep	Experimental	426	233 (54.69%)	2 (0.46%)	118 (27.69%)	59 (13.84%)	14 (3.28%)
Goats	Natural	15	8 (53.33%)	0	3 (20.0%)	3 (20.0%)	1 (6.66%)
Total		665	352 (52.93%)	7 (1.05%)	194 (29.17%)	95 (14.28%)	17 (2.55%)

Table 3. GPI enzymatic types from *Dicrocoelium dendriticum* adults from different ruminant species.

Host	Infection	No. *D. dendriticum* adults examined individually	Adult number and frequency (%) with each enzyme type				
			GPI-1	GPI-2	GPI-3	GPI-4	GPI-5
Cattle	Natural	58	29 (50.0%)	25 (43.10%)	0	0	4 (6.89%)
Sheep	Natural	108	62 (57.40%)	45 (41.66%)	1 (0.92%)	0	0
Sheep	Experimental	424	278 (65.56%)	139 (32.78%)	6 (1.41%)	1 (0.23%)	0
Goats	Natural	6	6 (100.0%)	0	0	0	0
Total		596	375 (62.91%)	209 (35.06%)	7 (1.17%)	1 (0.16%)	4 (0.67%)

Table 4. PGM enzymatic types from *Dicrocoelium dendriticum* adults recovered from different hosts.

Host	Infection	No. *D. dendriticum* adults examined individually	Adult number and frequency (%) with each enzyme type				
			PGM-1	PGM-2	PGM-3	PGM-4	PGM-5
Cattle	Natural	17	5 (29.41%)	4 (23.52%)	0	0	8 (47.05%)
Sheep	Natural	52	9 (17.30%)	8 (15.38%)	20 (38.46%)	15 (28.84)	0
Goats	Natural	15	15 (100.0%)	0	0	0	0
Total		84	29 (34.52%)	8 (9.52%)	12 (14.28%)	20 (23.80%)	15 (17.85%)

was absent; furthermore, GPI-1 and GPI-4 showed the highest and the lowest frequency, respectively. The highest frequency for GPI-1 was detected in goats and for GPI-2 in cattle. The GPI-3, GPI-4 and GPI-5 percentage values were low, the first two types only being observed in sheep and the third in cattle.

When the partial frequency values were compared, for each enzyme type, using the two percentage equality test, statistically significant differences were detected ($P \leq 0.05$) for GPI-1 between worms from: (i) cattle and experimentally infected sheep (t = 2.26); (ii) cattle and goats (t = 3.66); (iii) naturally infected sheep and goats (t = 97.14) and (iv) experimentally infected sheep and goats (t = 87.40).

Three activity bands were observed in the 6.89 to 6.98 pH range in non-infected hepatic tissue from sheep.

PGM

The activity bands of the five PGM types detected in *D. dendriticum* from the three ruminant species were observed within a 6.20 to 6.60 pH range (figs 1C, 2C). From the results for worms from all animals examined (table 4), PGM-1 was the most frequently recorded form, followed by PGM-4, PGM-5, PGM-3 and PGM-2. In worms from naturally infected sheep, PGM-5 was not detected, while PGM-3 and PGM-2 were the most and the least frequent, respectively. In *D. dendriticum* from cattle, PGM-3 and PGM-4 were absent, with the frequency of PGM-5 the highest and that of PGM-2 the lowest. In worms from goats, all enzyme types were absent, except PGM-1. The highest frequency for PGM-1 was observed in goats and for PGM-2 in cattle. PGM-3 and PGM-4 were detected only in naturally infected sheep and PGM-5 only in cattle.

When the partial frequency values were compared for each enzyme type, using the two percentages equality test, statistically significant differences were detected ($P \leq 0.05$) for PGM-1 between worms from: (i) cattle and goats (t = 5.63); and (ii) naturally infected sheep and goats (t = 7.79).

Three activity bands within the 6.80 to 7.47 pH range were observed in non-infected hepatic tissue from sheep.

AcP

The activity bands of this enzyme in all adult worms analysed were detected in the 5.70 to 5.92 pH range (figs 1D, 2D). Three types of AcP were detected and their frequency (table 5) in decreasing order was: AcP-2, AcP-3 and AcP-1. In worms from cattle, AcP-2 was not detected, and AcP-1 was the most frequent type encountered. In those from naturally infected sheep the most frequent type was AcP-2 and the least AcP-1. The frequency of AcP-1 was higher in cattle than in sheep whilst the converse occurs for AcP-3.

Table 5. AcP enzymatic types from *Dicrocoelium dendriticum* adults recovered from cattle and sheep.

Host	Infection	No. *D. dendriticum* adults examined individually	Adult number and frequency (%) with each enzyme type		
			AcP-1	AcP-2	Ac-P3
Cattle	Natural	8	7 (87.50%)	0	1 (12.50%)
Sheep	Natural	51	10 (19.60%)	24 (47.05%)	17 (33.33%)
Total		59	17 (28.81%)	24 (40.67%)	18 (30.50%)

When the partial frequency values were compared for each enzyme type, using the two percentages equality test, statistically significant differences were detected ($P \leq 0.05$) for AcP-1 between worms from cattle and naturally infected sheep (t = 3.94).

By means of χ^2 test statistically significant associations were not detected between different enzyme types of LDH and GPI studied. The similarity index of Hedrick produced results showing no relationship between phenotypic similarity for LDH, GPI, PGM and AcP and species, breed and origin of the parasite animal hosts. Nevertheless, these results are preliminary so further worms from different breeds and localities should be analysed.

MDH

This enzyme, which was only studied in *D. dendriticum* specimens from sheep (table 1), showed enzyme activity between pH 4.8 and 6.2 but it was too weak and diffuse to permit detailed interpretation.

α-GPDH and HBDH

No enzyme activity for α-GPDH or HBDH was observed in worms studied from sheep (table 1), although only eight specimens were examined for α-GPDH. However, three α-GPDH activity bands were observed between pH 7.10 and 7.35 in non-infected hepatic tissue from sheep.

Discussion

In the present study, 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 first four and the first three enzymes mentioned also showed activity in worms from cattle and goats, respectively.

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. Due to the fact that the activity of MDH was weak and diffuse no enzymatic types were established.

As there are no previous studies on *D. dendriticum* using the isoelectric focusing in thin-layer polyacrylamide gel technique, it is useful to compare our results with those obtained by León *et al.* (1986, 1988, 1989). On analysing LDH enzyme activity in *D. dendriticum* from cattle, sheep and goats, the number of bands we detected when taking all the different enzyme types into account was greater than that reported by León *et al.* (1986), and even when only those of the highest intensity were considered.

Most enzyme polymorphism for LDH was recorded in *D. dendriticum* from sheep, with five enzyme types detected, whilst only four were observed in worms from cattle and goats, and three of them were common. The absence of enzyme types in cattle (LDH-5) and goats (LDH-2) could be due to the smaller number of individuals analysed in these two groups. This result does not agree with that of León *et al.* (1986), who stated that the LDH enzyme profiles in *D. dendriticum* from cattle and sheep were similar but different from those in worms from goats. When AcP enzyme activity was analysed, four bands were detected in *D. dendriticum* from cattle and sheep. However, as the intensity of one of them was always very weak, our results seem to agree with those of León *et al.* (1989) in worms from sheep and goats.

On the other hand, two AcP enzyme types common to *D. dendriticum* isolated from cattle and sheep livers were observed, although one more type was detected in the latter. This, together with the different frequency with which these types were observed, shows the existence of certain differences in the enzyme profiles of *D. dendriticum* from both ruminant species. A similar result in the case of those from sheep and goats, was obtained by León *et al.* (1989).

Although we detected MDH enzyme activity in *D. dendriticum* from sheep, we do not consider it an appropriate enzyme system for characterizing this parasite, as it was not possible to clearly distinguish activity bands. Greater resolution was obtained by León *et al.* (1988) who observed two *D. dendriticum* bands from sheep and goats. It is interesting to compare the LDH, GPI, PGM, AcP, MDH and α-GPDH enzyme activity in *D. dendriticum* with that of other digenean species, using the same technique. According to Campo *et al.* (1993a) LDH has no activity in adults of *Fasciola hepatica* from sheep and cattle, but α-GPDH does. This is contrary to what happens in *D. dendriticum*. These authors also detected GPI, PGM and AcP activity in *F. hepatica*.

Our results suggest that the best enzyme systems to characterize adult *D. dendriticum*, using the technique of isoelectric focusing in thin-layer polyacrylamide gels, are LDH, GPI and PGM as they are those that show sufficient phenotypic variability. Moreover, the bands of their activity are well-defined and situated within a pH range different to those in hepatic tissue of control lambs. This will be useful to distinguish the activity of this parasite from that of the liver, as was reported by León *et al.* (1986) for LDH. Our appraisal of these enzymes seems to coincide, in general, with what has been observed by

different authors in other species of Digenea, mainly the genus *Schistosoma* (Ross, 1976; Southgate *et al.*, 1985).

No clear differences were observed between the enzyme systems of *D. dendriticum* isolated from animals of the same species but originating from different habitats. However, it should be pointed out that the habitats all occurred in the región of Castile-León, with one exception.

The significant differences between percentages for the different enzyme types in each animal group is of interest. This may be a reflection of the sampling or may reflect small differences between parasites circulating in different hosts. For example, six of the enzyme types (LDH-5, GPI-3, GPI-4, PGM-3, PGM-4 and AcP-2) were found in parasites from sheep but were not observed in those from cattle.

The differences observed by us in the *D. dendriticum* enzyme activity could be induced by the definitive host, as already quoted for *Fasciola hepatica* by Lee *et al.* (1992). Nevertheless, in the case of *D. dendriticum* the above mentioned enzymatic differences could also be due to the different species of molluscs (Manga-González, 1992) and ants (Manga-González *et al.*, 1991b) used by the parasites as first and second intermediate hosts, respectively.

Further work is required to determine the variability of isolates on a larger geographical scale. Enzyme markers should prove to be a useful tool for determining infection in naturally infected snails.

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