4. RESULTS

4.1. Experimental infection

4.1.1. Collection of ants

In Table 3 the results for ants collected *in tetania* are reported. Most of the ants collected belonged to the species *F. pratensis* (98%) (Figure 8).

Table 3. Prevalence, intensity, infection range and total metacercariae in collected ants.

Date (year 2002)	N. collected ants	Prevalence %	Mean burden	St.dev.	Infection range	Total N. metacercariae
15th april	-		-			-
18th april	-		-			-
22nd april	1	100	-			54
7th may	52	96.15	59.60	54.01	0-308	3098
15th may	37	97.30	46.30	38.13	0-180	1714
18th may	21	100	56.60	35.43	20-140	1189
Total	111	97.30	55	45.95	0-308	6055

Fig. 8. Ants *in tetania* (*F. pratensis*) on grass collected early in the morning in eastern Brandenburg (Photo A. Broglia)

The distribution of metacercariae (represented in discrete burden classes) in the paralyzed ants in our sample is shown in Figure 9.

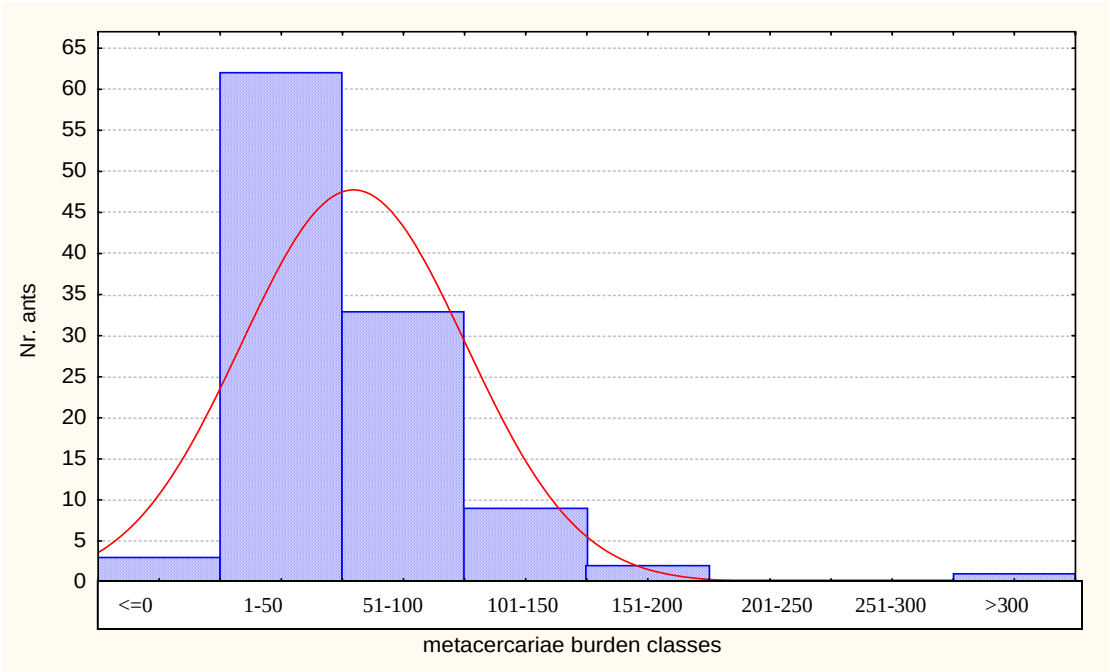

Figure 9. Metacercariae distribution classes in ants collected *in tetania*

As seen in Figure 9 most of ants have a metacercarial burden ranging from 1 to 100. Only one specimen was infected with more than 300 metacercariae.

4.1.2. Establishment rate

In Table 4 the relationship between infection dose and adult fluke burden in infected lambs is reported. In all infected animals, adult *D. dendriticum* flukes were recovered in liver bile ducts and in the gall bladder (see Figure 10). Pathological lesions in the liver were scant (see Figure 11). The two control animals were negative for flukes at necropsy.

Table 4. Number of recovered flukes at necropsy and relationship with infection dose.

Lamb nr.	Infection dose	Adults flukes (liver)	Adults flukes (gall bladder)	Total adult flukes recovered	Establishment rate %
A1	1000	150	2	152	15.2
A2	1000	260	-	260	26.0
B1	2000	750	40	790	39.5
B2	2000	800	-	800	40.0

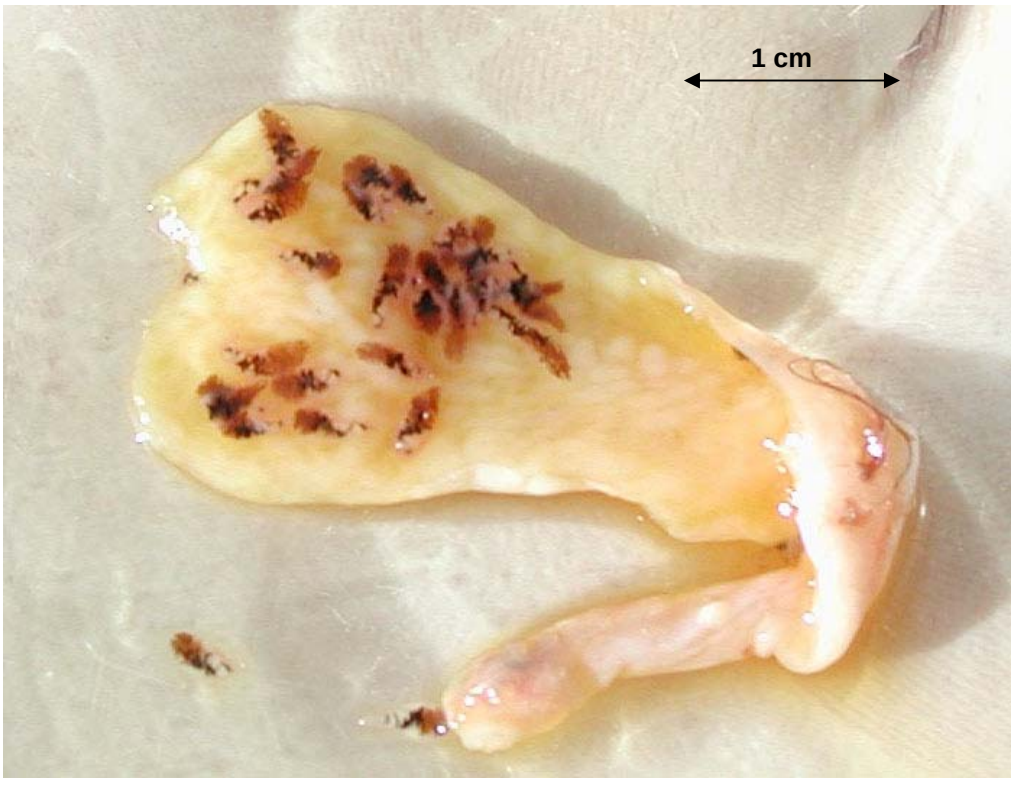

Figure 10. Dissected gall bladder of sheep experimentally infected with *D. dendriticum* (infection dose: 2000 metacercariae). Many *D. dendriticum* adults were present (Photo: A. Broglia).

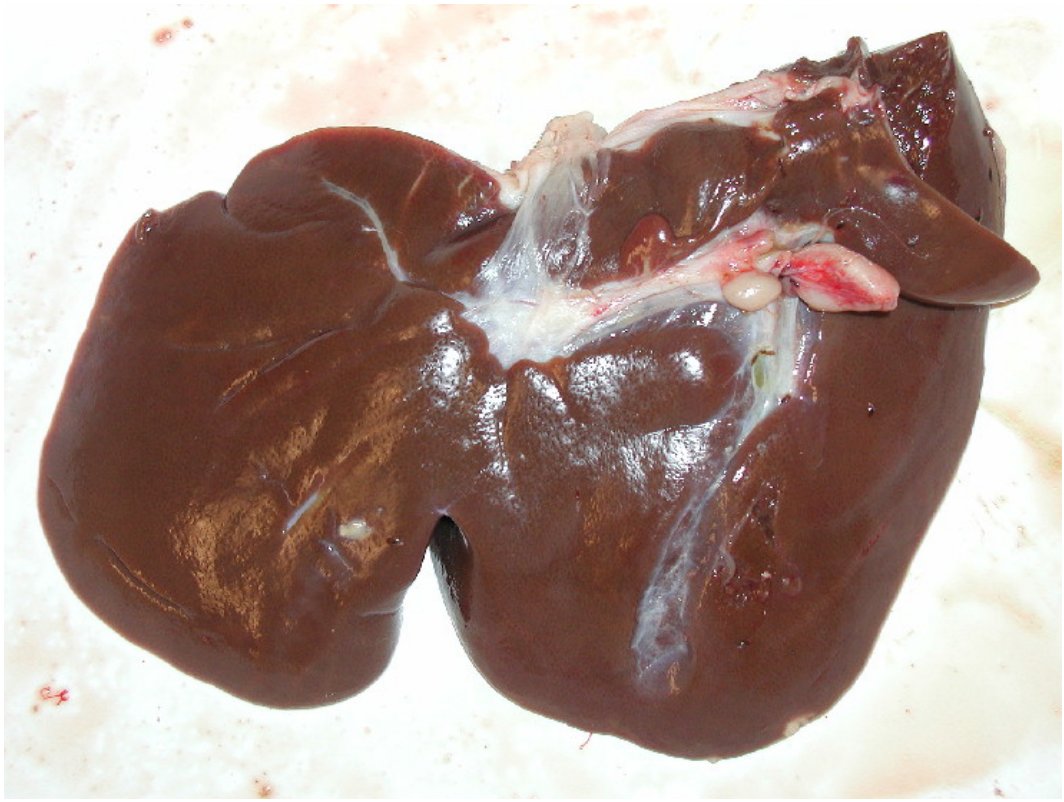

Figure 11. Liver of sheep experimentally infected with *D. dendriticum* (infection dose: 2000 metacercariae). Gall bladder has been removed. (Photo: A. Broglia)

4.2. ELISA test

4.2.1. Determination of protein concentration of antigen solution

The protein concentration was estimated by BioRad Protein Assay, a microtitre method that uses a colorimetric reaction readable by photometer at 570 nm:

- Excretory/secretory (E/S) antigen protein concentration: 26 μg/ml;
- Somatic (So.) antigen protein concentration: 600 μg/ml.

4.2.2. Antigen and serum dilution

Through the checkerboard titration, the following optimal dilutions for antigens and serum were estimated:

- Optimal antigen dilution:
    - E/S antigen optimal dilution: 1:15 (final concentration: 1.7 μg/ml);
    - So. antigen optimal dilution: 1:100 (final concentration: 6 μg/ml);
- Optimal serum dilution: 1:100.

4.2.3. Cut-off definition

As described in chapter “Materials and methods”, cut-off was defined as the mean value plus three standard deviations of the optical density (OD) values observed in the negative control samples. In Table 5, OD values of the negative serum samples tested with E/S and So. *D. dendriticum* antigens are reported. The cut-off value set for E/S and So. antigens were OD: 0.319 and OD: 0.411 respectively (Table 5).

Table 5. OD values in negative serum samples.

Nr. lambs	OD - E/S antigen	OD – So. antigen
1	0.292	0.285
2	0.258	0.278
3	0.146	0.271
4	0.129	0.056
5	0.126	0.115
6	0.108	0.133
7	0.124	0.029
8	0.078	0.044
9	0.075	0.123
10	0.113	0.156
11	0.125	0.161
12	0.123	0.086
13	0.122	0.118
14	0.097	0.135
15	0.213	0.314
16	0.112	0.138
17	0.085	0.101
Mean	**0.137**	**0.150**
St.dev.	**0.061**	**0.087**
Cut-off	**0.319**	**0.411**

4.2.4. Temporal dynamics of IGg response of experimentally infected lambs

In all the infected lambs, *D. dendriticum* eggs were detected 58 days after the infection, and the lambs remained coprologically positive during the whole period of study (150 days). Lamb B (1000 metacercariae) died at day 50 post infection (d.p.i.), so only the first four OD values are available for this lamb.

Immune response to E/S antigen

In the Fig. 12 the time-course dynamics of the immunological response against E/S antigen is reported, according to infection dose (lambs A and B: 1000 metacercariae; lambs C and D: 2000 metacercariae). The horizontal line indicates the cut-off value (OD= 0,319).

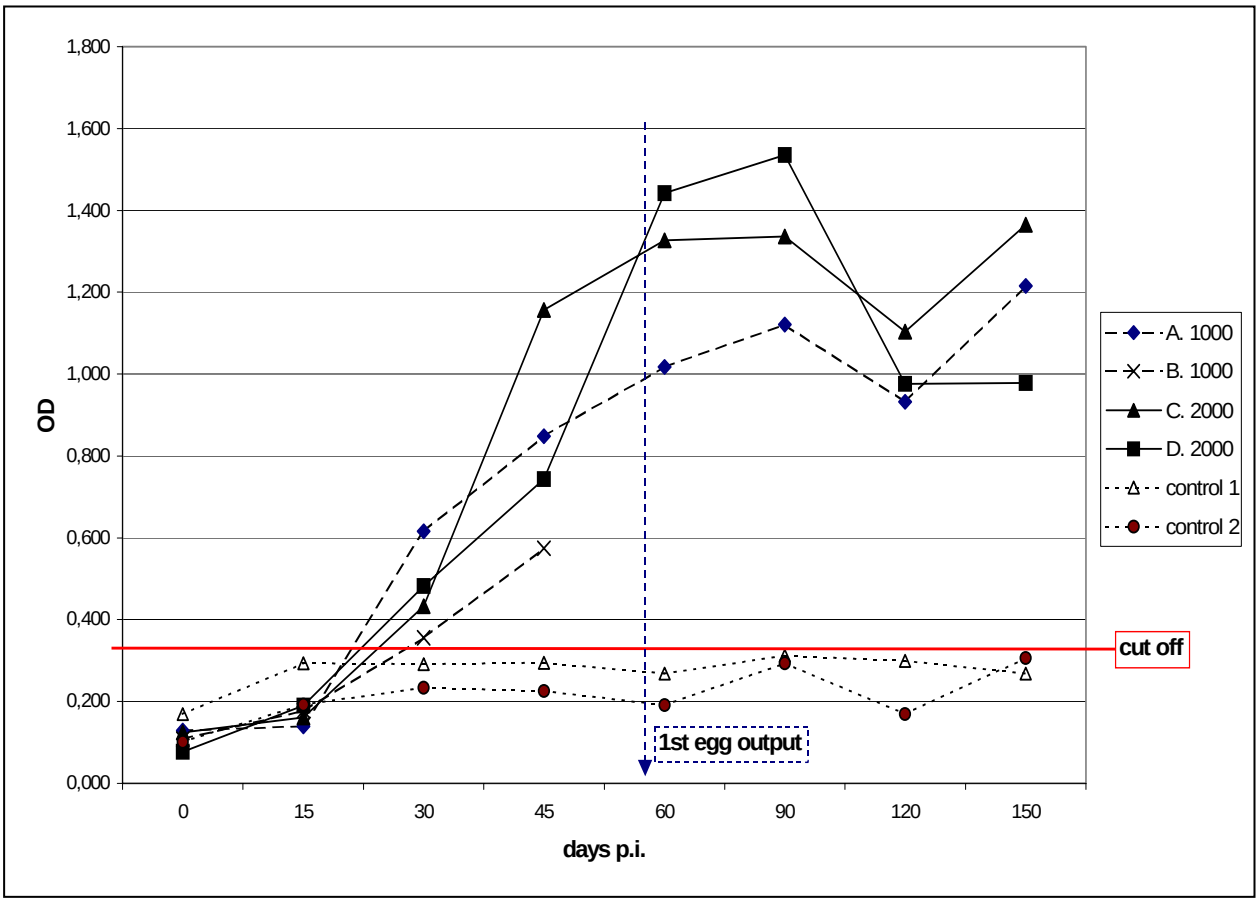

Figure 12. OD values of IGg antibodies against *D. dendriticum* E/S antigen throughout the experimental infection.

All the infected lambs became serologically positive starting from 30 d.p.i. The maximum OD value (OD: 1.536, see appendix) was reached by lamb D (infection dose: 2000 metacercariae) at 90 d.p.i. The antibody titre increased sharply in the lambs

infected at higher dose until 60 d.p.i., whereas in the lamb infected at lower dose (1000 metacercariae) the titre increased more gradually. However, in all the lambs, although some fluctuations were observed, the titres remain positive until the end of the study (150 d.p.i.). The two control animals were negative for the whole period observed.

Immune response to So. antigen

In the Figure 13 the time-course dynamics of the immunological response against So. antigen is reported, according to infection dose (lambs A and B: 1000 metacercariae; lambs C and D: 2000 metacercariae). The horizontal line indicates the cut-off value (OD= 0.411).

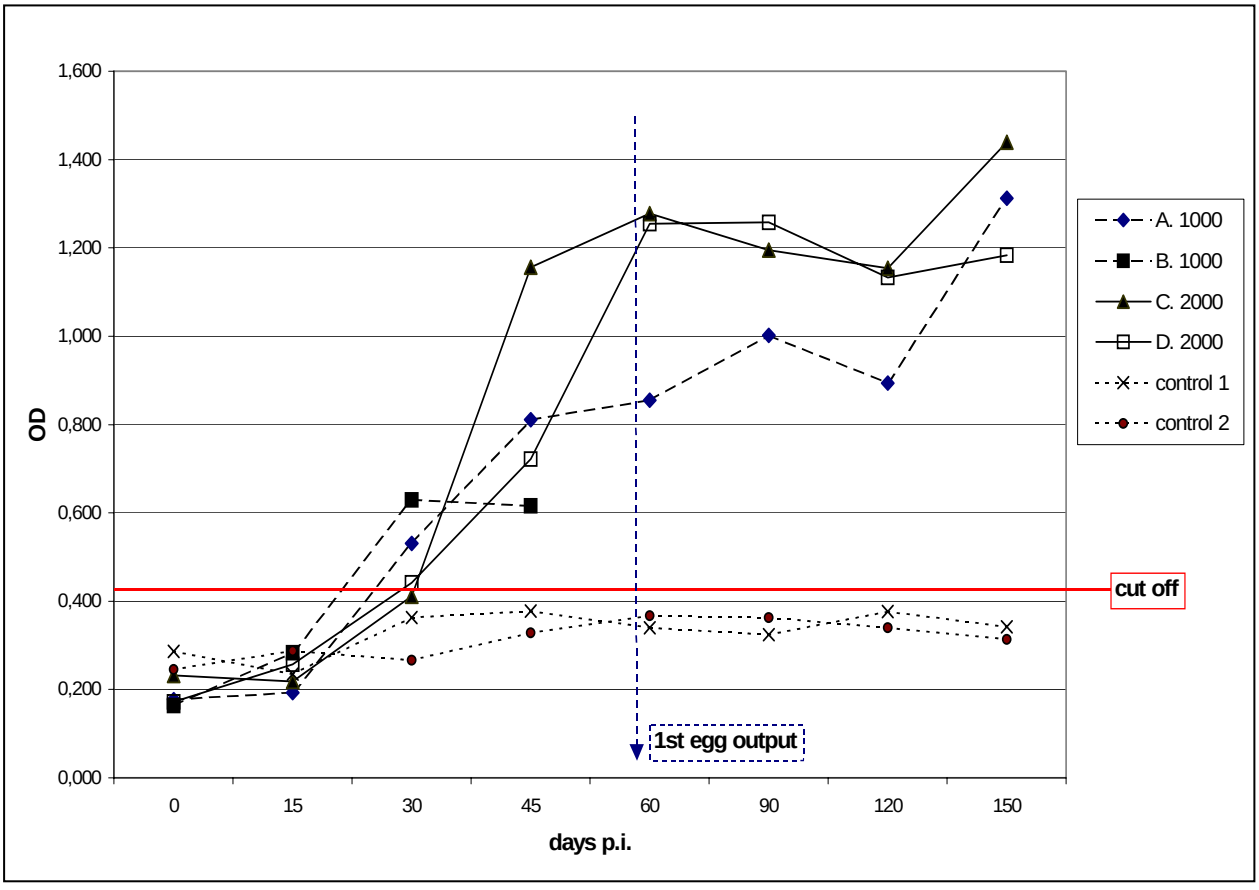

Fig. 13. OD values of IGg antibodies against *D. dendriticum* So. antigen throughout the experimental infection.

Using So. antigen, the infected lambs at lower infection dose were clearly positive starting from 30 d.p.i., whereas the titres of lambs C and D (higher infection dose, 2000 metacercariae) were still doubtful at 30 d.p.i., increased later but reached then higher levels. The maximum OD value (OD: 1.439, see appendix) was reached in

lamb C (infection dose: 2000 metacercariae) at 150 d.p.i. In the lambs C and D (higher infection dose, 2000 metacercariae) the antibody titre increased until 60 days p.i., then it remained at the same level, with some slight fluctuations, until the end of the experiment. In lamb A infected at a lower dose (1000 metacercariae) the titre increased constantly during the whole period of study. After 45 d.p.i., in all the lambs the antibody titres remained positive until the end of the study (150 d.p.i.). The two control animals were negative for the whole period considered.

4.2.5. Cross-reaction with other helminth infections

Serum sample from sheep infected with other helminths were tested by ELISA for dicrocoeliosis, in order to investigate possible cross-reactions.

4.2.5.1 Cross-reaction with *F. hepatica* and *Paramphistomum* spp.

In Table 6 OD values and coprological analysis results of serum sample from sheep infected by *F. hepatica* and *Paramphistomum* spp (Müritz lake, Mecklenburg Vorpommern, Germany) are reported.

Table 6. OD values and results of coprological analysis of sheep infected with *F. hepatica* and *Paramphistomum* spp.

Sheep	Coprological analysis	OD values E/S Ag	OD values So. Ag
s1	++ *Paramphistomum*	0.175	0.245
s2	++*F. hepatica*	0.325	0.472
s3	++ *F. hepatica*	0.247	0.343
s4	negative	0.198	0.245
s5	++ *F. hepatica*	0.688	0.710
s6	++ *F. hepatica*	0.344	0.289
s7	++ *F. hepatica*	0.409	0.395
s8	++ *F. hepatica*	0.063	0.213
s9	++ *F. hepatica*	0.165	0.096
s10	++ *F. hepatica*	0.228	0.188
s11	++ *F. hepatica*	0.323	0.421

In Figure 14 the OD values of the serum samples of sheep infected with *F. hepatica* and *Paramphistomum* spp. are reported.

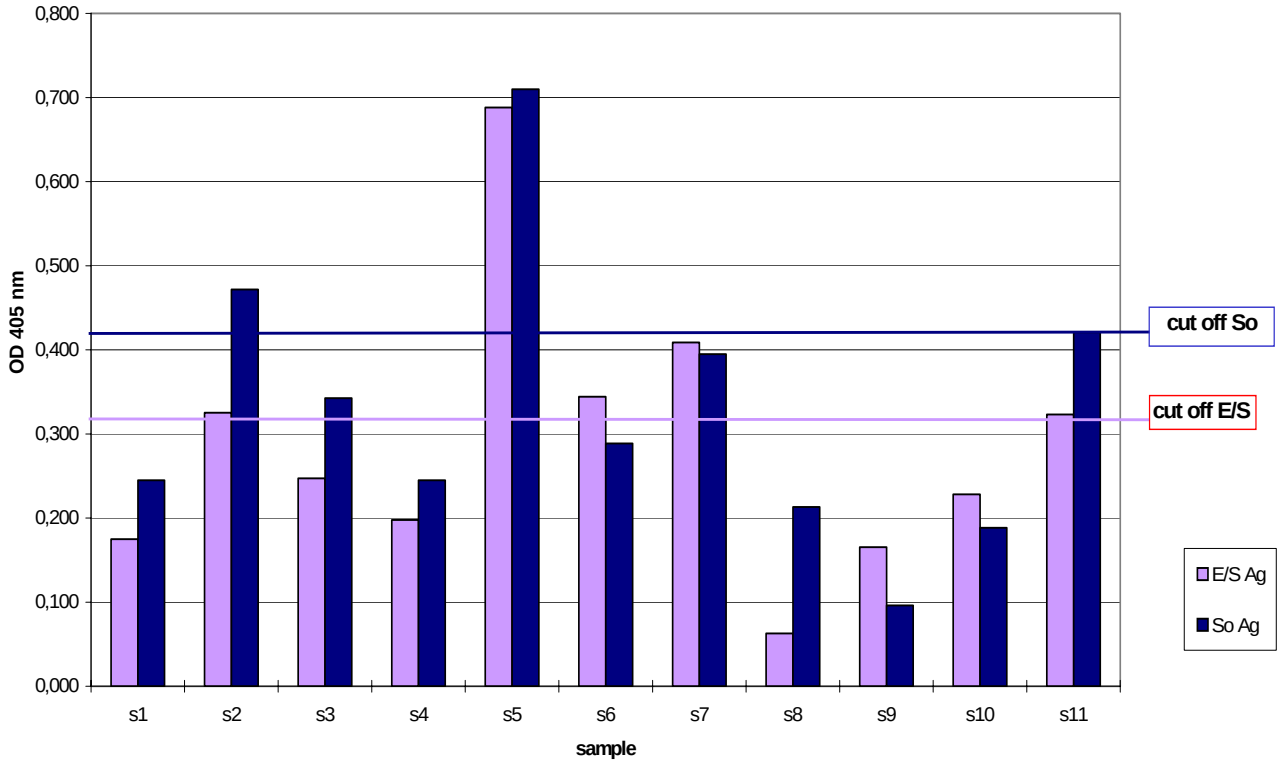

Figure 14. OD values of sera from sheep infected with *F. hepatica* and *Paramphistomum* spp. with relative cut-off values.

Cross-reactions against E/S antigen were seen in the animals s2, s5, s6, s7, s11; samples s2, s5 and s11 cross-reacted with So. antigen.

4.2.5.2. Cross-reaction with nematode parasite infections

Single infection dose

Ten serum samples from lambs experimentally infected with a single dose of infective larvae of *Nematodirus battus*, *Trichostrongylus colubriformis* and *Haemonchus contortus* were tested for cross-reactions against E/S and So. antigen of *D. dendriticum* (Figure 15).

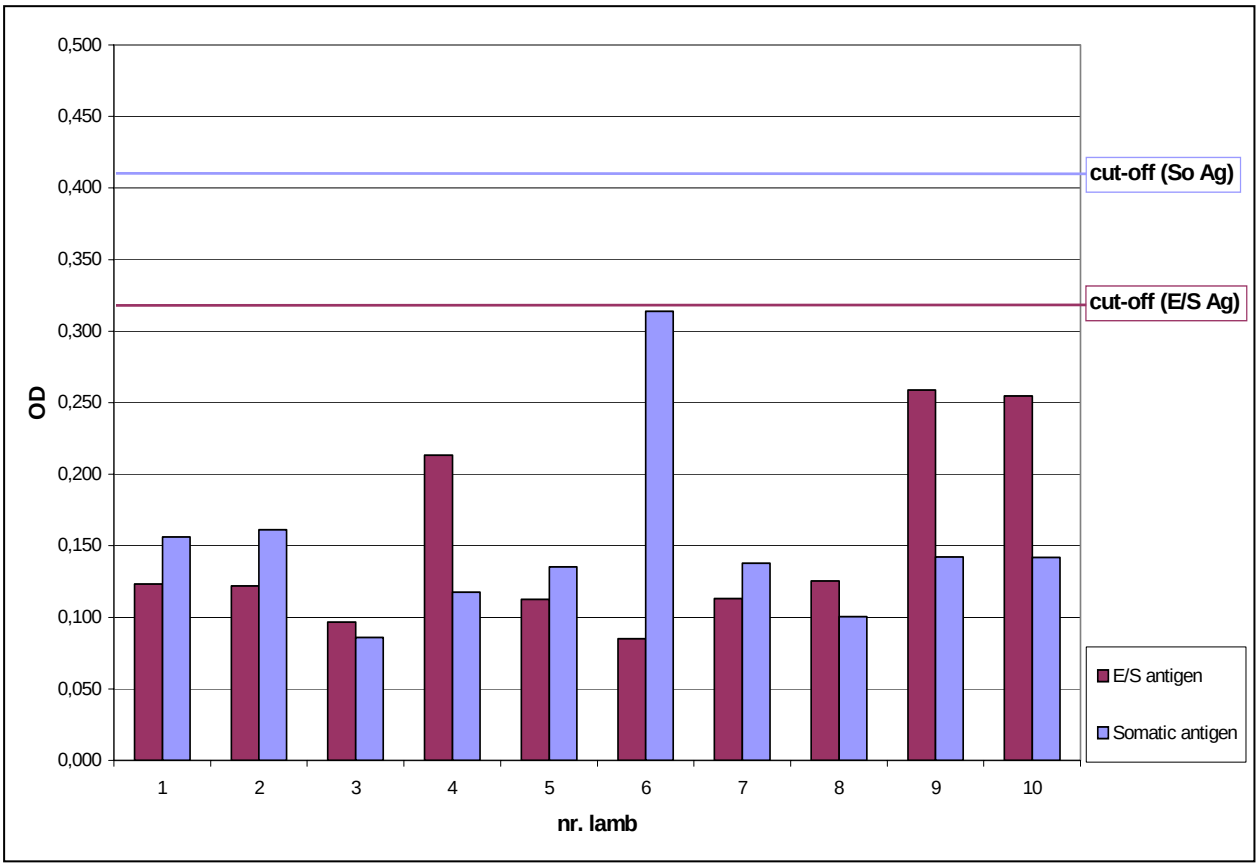

Figure 15. OD values of antibody response of lambs infected with *N. battus, T. colubriformis, H. contortus* against E/S and So. antigen of *D. dendriticum.*

No sample showed cross-reactions either with E/S antigen or with So. antigen.

Repeated infection dose

Seven serum sample from lambs experimentally infected with repeated infection doses of different nematode species (*Dictyocaulus filaria*, *Oesophagostomum venulosum*, *Ostertagia ostertagi*, *T. colubriformis*, *Cooperia curticei*, *N. battus*, with a total infection dose of 27 000 larvae) were also tested with E/S and So. antigen of *D. dendriticum* (Fig.16).

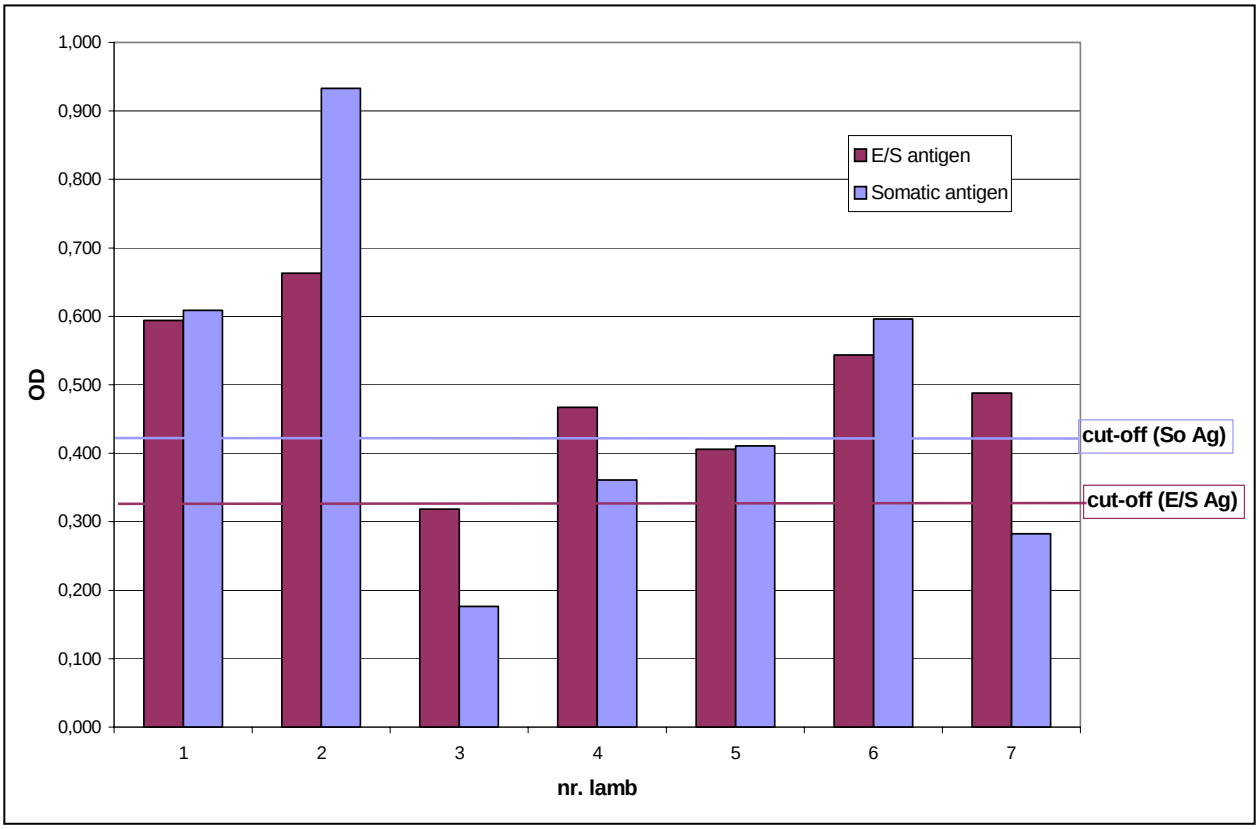

Figure 16. OD values of antibody response of lambs infected with repeated dose of different nematode species L3 against E/S and So. antigens of *D. dendriticum*

As shown by Figure 16, most of the tested samples showed cross-reactions with both antigens used, in particular sample 1, 2 and 6.

4.3. Enzyme Immune Transfer Blotting (EITB) (Western Blot)

4.3.1. Proteic fractions of *D.dendriticum* antigens by SDS-PAGE.

The optimal concentration for electrophoresis was determined by sample titration (QURESHI, *et al.*, 1995). For the E/S antigen the best sample concentration was 8 μg of protein dissolved in 20 μl per lane; the proteic pellet obtained from 1 ml solution (initial concentration of E/S antigen solution: 26 μg/ml) was diluted in 65 μl sample buffer (see appendix) at a final concentration of 400 μg/ml.

For the So. antigen (initial concentration: 600 μg/ml), the optimal concentration was established at 5 μg in 15 μl per lane. The proteic pellet obtained from 1 ml initial solution was dissolved in 1.8 ml sample buffer.

Through SDS PAGE, the proteic fractions of E/S and So. antigen were separated on a acrylamide gel sheet and stained with Coomassie Blue. Banding patterns are shown in Figure 17.

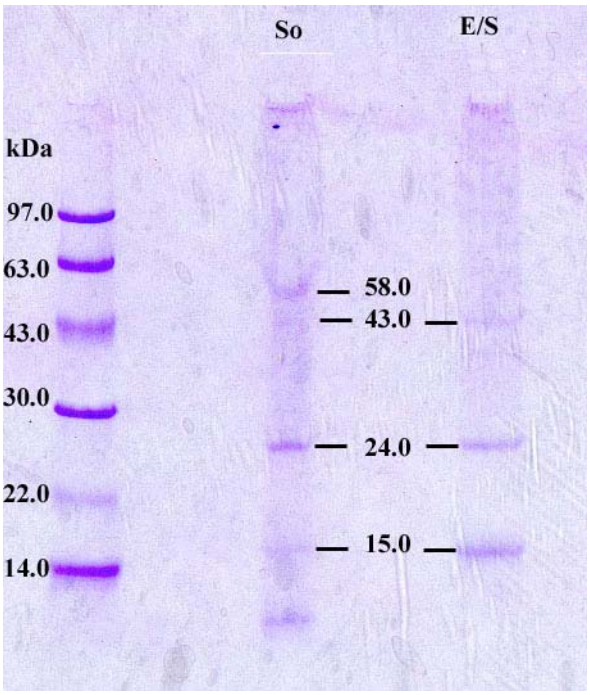

Figure 17. Pattern of proteic fractions of E/S and So. antigen of *D. dendriticum* by SDS-PAGE.

Proteic bands for both types of antigen were clustered in the molecular mass range of 58-15 kDa. One cluster of bands of So. antigen was observed between 58 and 43 kDa, whereas a more defined band was observed in the E/S antigen banding pattern

at around 43 kDa of molecular weight range. Two identical clusters in the mass range of 24 and 15 kDa were observed for both antigen solutions.

4.3.2. Antibody response dynamics in experimentally infected lambs.

Sera from *D. dendriticum*-experimentally infected animals reacted mostly with E/S antigen at a range of 43 kDa, starting from 60 days after infection. Reaction of sera with somatic proteins was at the same molecular mass range, only more evident, probably due to a higher protein concentration. At heavier molecular weight range (100-150 kDa) there was also a diffuse reaction, more evident in the somatic extracts immuno-precipitates (Fig 19). No reaction at all was detected at molecular mass range less than 43 kDa. Immunoblot patterns are shown in Figure 18 and Figure 19.

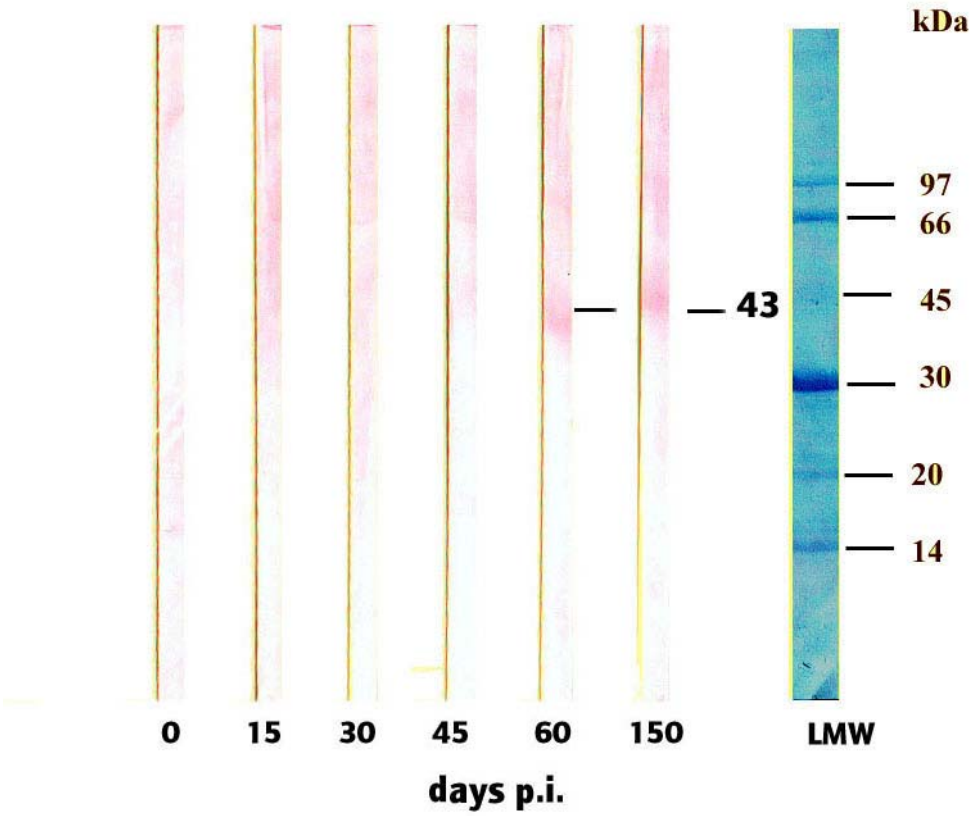

Figure 18. Immunoblot of *D. dendriticum* E/S antigen with sera of experimentally infected lambs (infection dose: 2000) through the study period. (0-150 d.p.i: sera obtained at *n* days post infection).

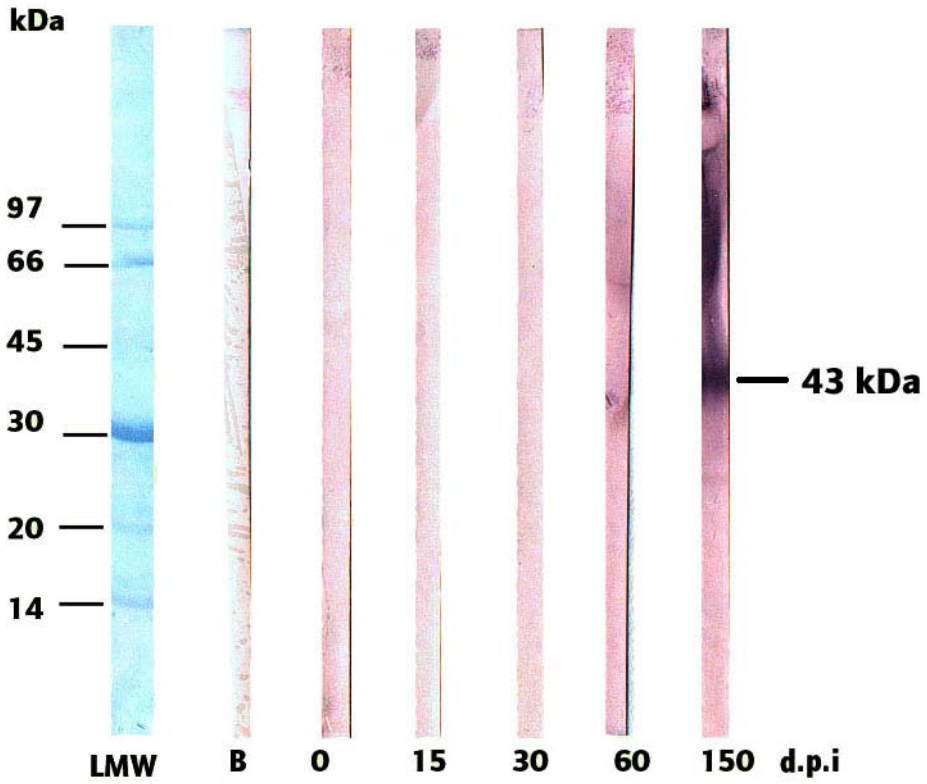

Figure 19. Immunoblot of *D. dendriticum* So. antigen with sera of experimentally infected lambs (inf. dose: 2000) during the study period.
(B: Blank; 0-150 d.p.i.: sera obtained at *n* days post infection).

4.3.3. Comparative immunoblot with sera of sheep infected with *F. hepatica*

Cross-reactions were detected from the immunoprecipitates of *D. dendriticum* E/S antigens with field sera of *F. hepatica*-infected sheep.

In particular, cross-reactions were observed in three samples at the molecular mass range of 43 kDa. The results are shown in Figure 20. The diffuse band at molecular weight range between 50 and 100 kDa was present in the lane nr. 2.

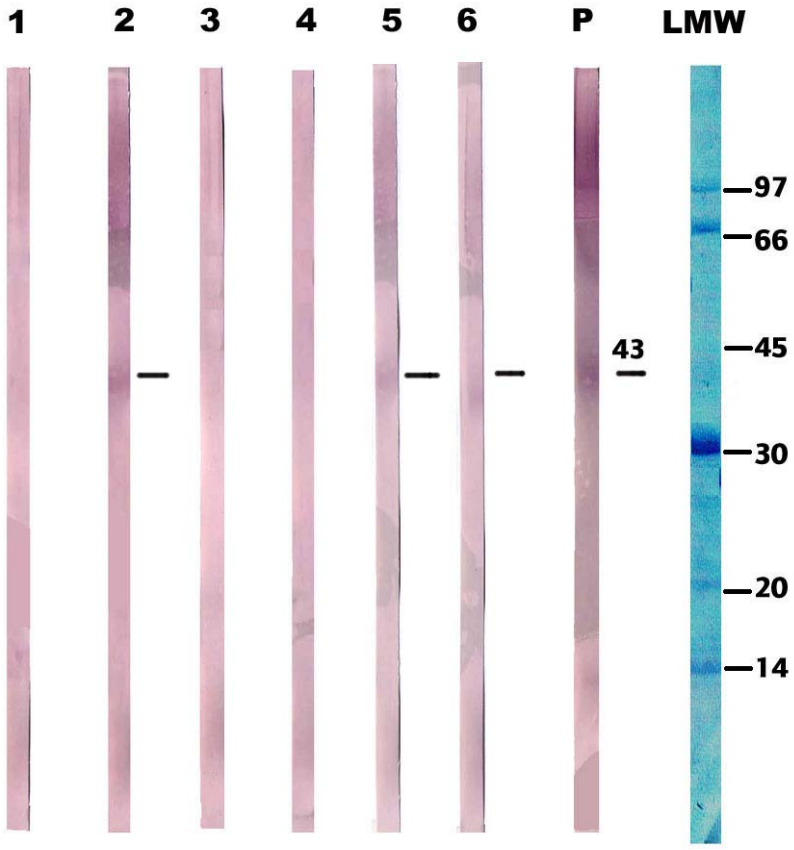

Figure 20. Immunoblot of *D. dendriticum* E/S antigen with field sera of sheep infected with *F. hepatica* (lane 1-6) and sera of lambs infected with *D. dendriticum* (lane P, inf. dose: 2000 metacercariae).

4.3.4. Comparative immunoblot with sera of lambs infected with others nematodes.

Immunoblot of *D. dendriticum* antigens with sera from experimentally infected lambs with different species of nematodes *(T. colubriformis, N. battus*) did not show any cross-reactions. The results are shown in Figure 21.

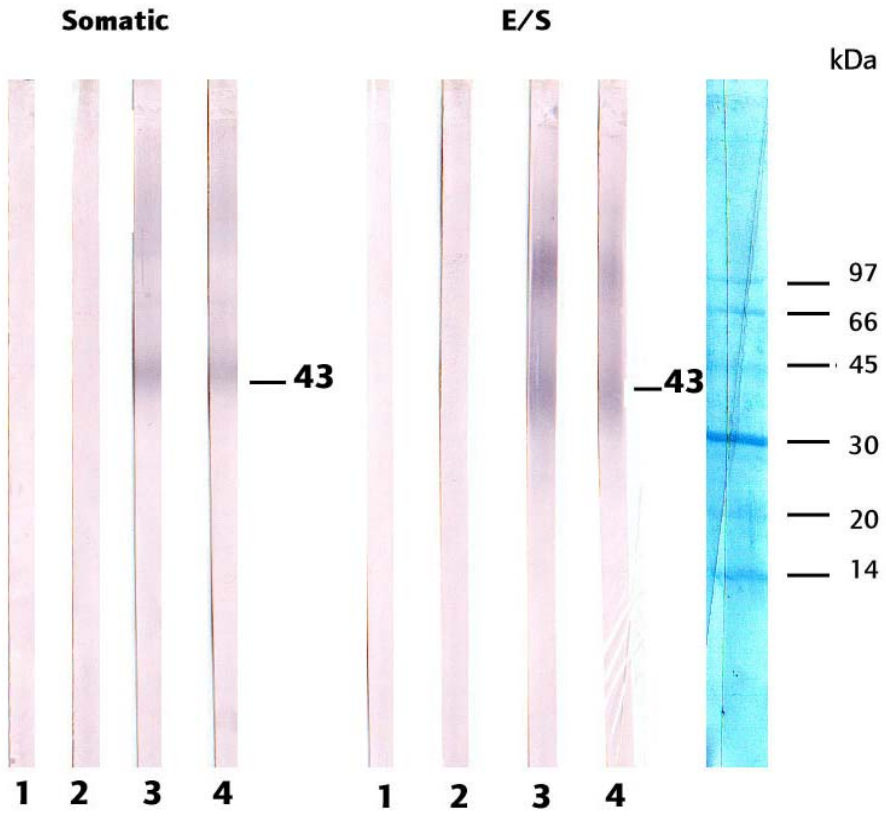

Figure 21. Immunoblot of *D. dendriticum* E/S and So. antigen with sera of lambs infected with *T. colubriformis* (lane 1) and *N. battus* (lane 2) and sera of lambs infected with *D. dendriticum* (lane 3, inf. dose: 2000; lane 4, inf. dose: 1000).

4.4. Experimental screening of dicrocoeliosis in sheep flocks in northeastern Italy (province of Trento)

By means of the ELISA test, 892 serum samples from sheep flocks in the province of Trento were tested with E/S and So. *D. dendriticum* antigen. The results of the essay (seroprevalence) are reported in Table 7, Figure 22 and Figure 23.

Table 7. Seroprevalence rates of dicrocoeliosis (E/S and So. antigen) in sheep flocks in the province of Trento (north eastern Italy).

Sheep flock	**Municipality**	**Nr. of samples**	**Seroprevalence (%)** E/S antigen	**Seroprevalence (%)** So. antigen
		Total: 892	**Mean: 91%**	**Mean: 91%**
1	Mezzocorona	50	94%	97%
2	Castelnuovo	50	100%	98%
3	Telve	50	100%	100%
4	Torcegno	50	100%	100%
5	Strigno	50	72%	88%
6	Civezzano	50	94%	82%
7	Frassilongo (1)	50	100%	98%
7	Frassilongo (2)	50	100%	100%
8	Baselga	50	80%	98%
9	Predazzo	242	87%	87%
10	Cavalese	50	98%	98%
11	Carano	50	94%	100%
12	Folgaria	50	92%	80%
13	Arco	50	68%	52%

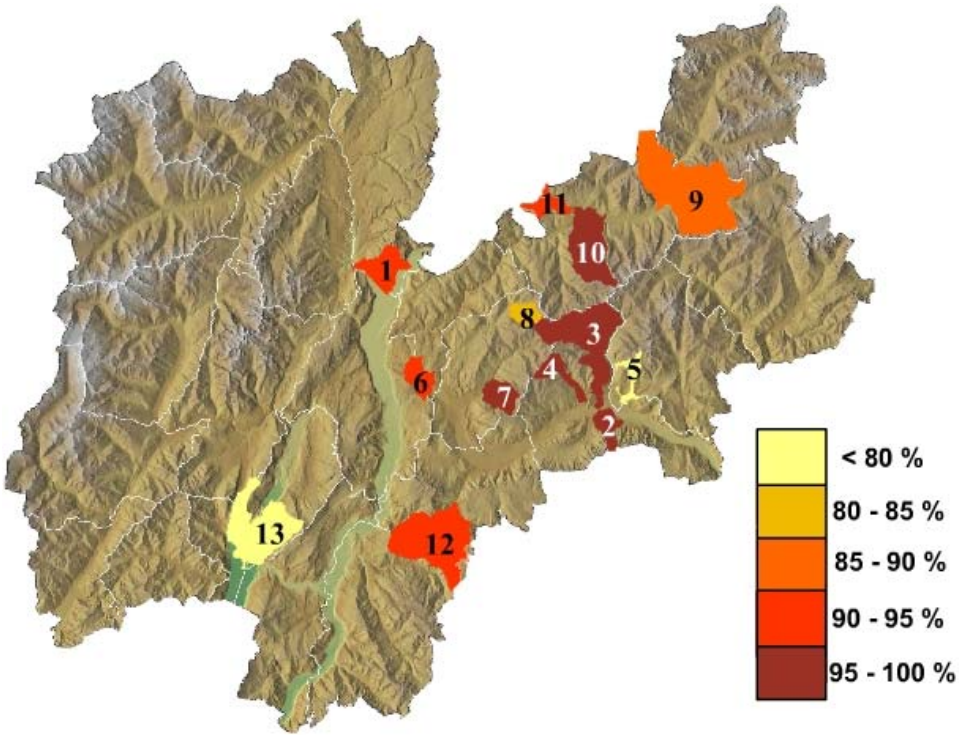

Figure 22. Seroprevalence rates of dicrocoeliosis (E/S antigen) in sheep flocks in the province of Trento (northeastern Italy)[7].

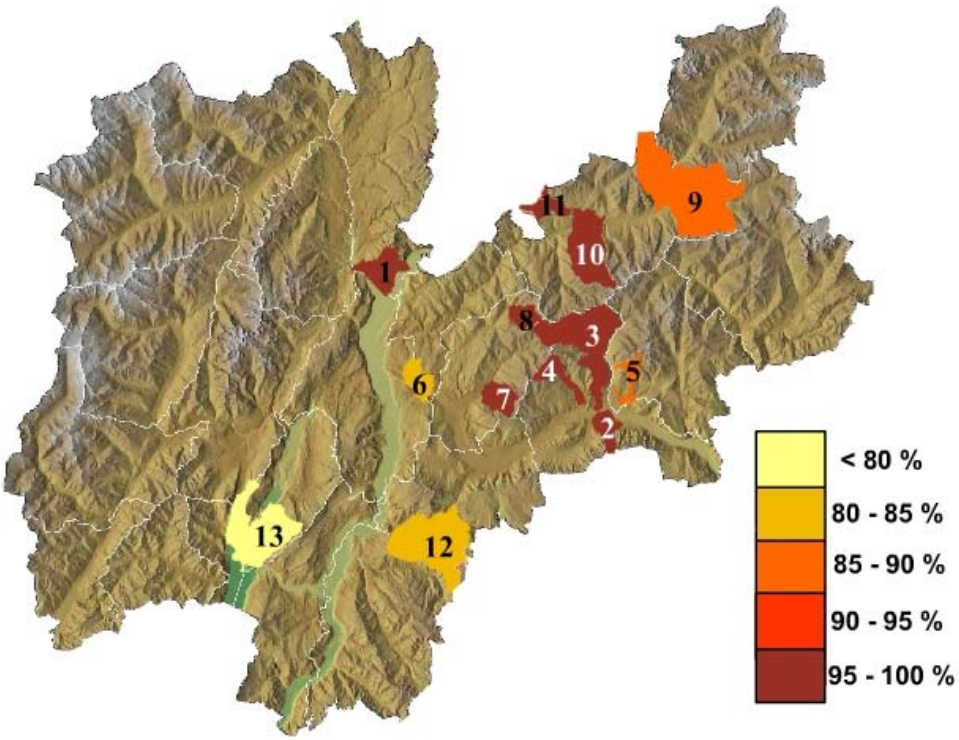

Figure 23. Seroprevalence rates of dicrocoeliosis (So. antigen) in sheep flocks in the province of Trento (northeastern Italy)[7].

[7] The numbers on the maps from 1 to 13 refer to the municipalities reported in Table 7.

4.4.1. Detection of *D. chinensis* (syn *D. suppereri)* in Italy

In the municipality of Predazzo (province of Trento), an infection with *D. chinensis* (classified by HINAIDY (1983) as *D. suppereri* syn. *orientalis*) has been described in a male red deer (*Cervus elaphus*) shot in the hunting season of 2001 (Figure 24). Even if it is a sporadic detection, this event could assume an epidemiological value because *D. chinensis* is an exotic species, originating from eastern Siberia, originally described in musk deers (*Moschus moschiferus*) by SUDARIKOV and RYJIKOV (1951).

Probably this trematode species was imported to Europe by sika deer (*Cervus nippon*) from Japan; it was first detected in a muflon (*Ovis ammon musimon*) by HINAIDY in Austria (1983). Then this parasite species managed to cross the Alps.

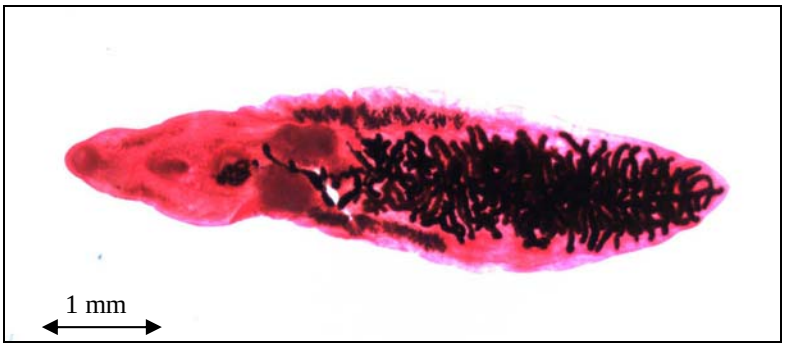

Figure 24. An adult specimen of *D. chinensis* found in a male red deer (province of Trento) (Photo: A. Broglia)

D. chinensis differs from the most common species *D. dendriticum* in some morphological characteristics (Fig. 25): a) presence of shoulders, b) symmetrical position of the testes, c) the cirrus sack does not reach the ventral sucker, d) the vitellaria extend into the testicular field (HINAIDY, 1983).

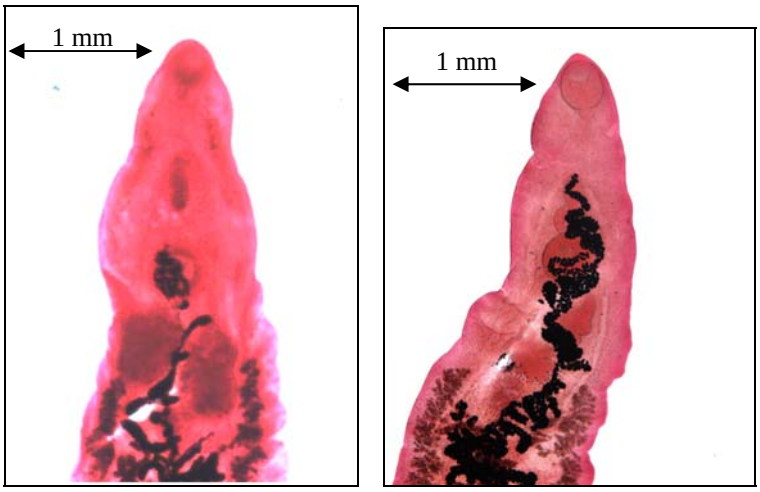

Figure 25. Detail of the cranial end of *D. chinensis* (left) and *D. dendriticum* (right) (Photo: A. Broglia).