3. MATERIALS AND METHODS

3.1 Study area

Infected ants needed for the experimental infection were collected during late spring (April-May 2002), in eastern Brandenburg (northeastern Germany, see Figure 1), previously identified as *Dicrocoelium* biotopes by SCHUSTER (1991).

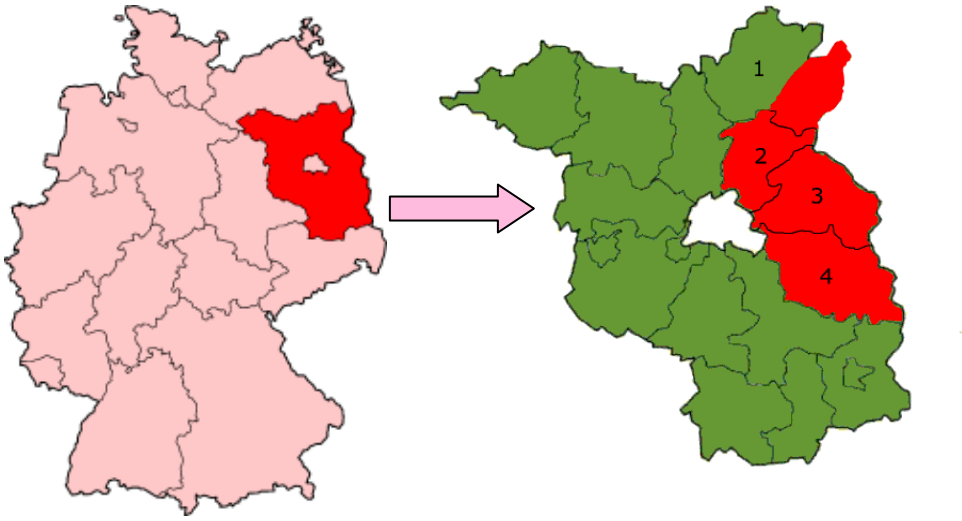

Fig. 1. Localization of Brandenburg in Germany (left) and eastern Brandenburg districts (right) (1: Uckermark; 2: Barnim; 3: Märkisch-Oderland; 4: Oder-Spree).

Eastern Brandenburg was not included in previous investigations into the distribution of dicrocoeliosis in Germany (NÖLLER, 1929; HENKEL, 1931). MIELKE (1969) described the first case of lancet fluke infection in this area, probably due to infected sheep introduced from Thuringia. In eastern Brandenburg, the parasite would have found suitable conditions for its life cycle.

Later this area, the district Frankfurt/Oder of the former German Democratic Republic (GDR), was studied for eight years (1982-1989). Based on the examination of 71600 sheep livers at meat inspection, a distribution map of *D. dendriticum* was drawn (SCHUSTER *et al.*, 1991). The presence of *D. dendriticum* was associated with farms having dry grass biotopes, exposed to south, and extended along geological outcrops formed during the glacial period (moraine), which offer good life conditions for the intermediate hosts. Nowadays many of these areas are cultivated and are grazed mostly

by sheep flocks. This also explains the lower *D. dendriticum* prevalence in cattle in this area (SCHUSTER *et al.*, 1991).

Figure 2. Typical *D. dendriticum* biotope in eastern Brandenburg (Photo: R. Schuster).

Currently the sheep flocks of the former GDR are markedly reduced, and agriculture has undergone many drastic changes. For this reason, the *D. dendriticum* life cycle is now maintained only in the most suitable biotopes, thanks to the resistance of *D. dendriticum* eggs and to the life expectation of infected intermediate hosts (snails and ants), also in long absence of final hosts (SCHUSTER *et al.*, 1991).

The most important species of intermediate hosts in this area are the snail species *Helicella obvia*, as first, and *Formica pratensis* or *F. rufibarbis* as second intermediate host. In my experience, the ants collected *in tetania* belong to the species *F. pratensis* (98% of ants collected).

3.2 Experimental infection

Once the anthills in the biotopes were found, the paralyzed ants were collected from the top of the grass next to the anthills, early in the morning or late in the afternoon, when the temperature was lower than 18°C.

The paralyzed ants were carried to the laboratory in plastic tubes, then euthanized using acetic acid-ethyleter and dissected under stereomicroscope. Metacercariae were isolated, put in normal saline solution and counted. Pools of fresh metacercariae were used to prepare infective doses for experimental infection of lambs.

Six 4-month-old lambs were used for the experimental infection. The lambs were dewormed with albendazole one month before the infection. Two animals were infected with 1000 metacercariae each, two with 2000 and two were kept uninfected as a control. The metacercariae were administered as a trickle dose over a week (150 metacercariae/day) in order to simulate natural infection (LECLIPTEUX *et al.*, 1998). Metacercariae, suspended in physiological solution, were administered orally using a plastic syringe.

The lambs were kept outside, in a paddock of the Veterinary Faculty, Free University Berlin, where no *Dicrocolieum* intermediate hosts were present, for six months.

Blood samples from each animal were taken at days 0, 15, 30 and 45 post infection and then at monthly intervals until day 150 post infection. Serum was separated and stored at -20 °C until used.

Faecal samples were collected once a week directly from the rectum of the animals, starting at day 45 post infection until *D. dendriticum* eggs were first detected. The faecal samples were examined by sedimentation method. Finally, the lambs were slaughtered at six months post infection.

At necropsy, the livers with the gall bladder were isolated, dissected and accurately washed in order to recover the *D. dendriticum* flukes from the bile ducts and the gall bladder. Then only intact flukes were washed five times in washing solution (see appendix) to remove all traces of host blood and bile.

3.3 Enzyme -Linked Immunosorbent Assay (ELISA)

All following procedures (antigen preparation, ELISA settings, SDS-PAGE) were carried out at the Federal Institute for Risk Assessment, in Berlin.

3.3.1 Antigen preparation

Two different kinds of antigens were used, an excretory/secretory (E/S) antigen and a somatic (So.) antigen.

Excretory/secretory antigen

The excretory/secretory antigen was produced according to NÖCKLER (2003).

After washing, the adult worms of *D. dendriticum* were put in 20 ml culture flasks with Dulbecco's modified Eagle Medium (see appendix) at a concentration of 20 worms/ml. Worm vitality was checked under the stereomicroscope. The culture was kept at 37° C for 20 hours. Then the supernatant was removed and centrifuged at 10 000 r.p.m. for 20 minutes.

Once the supernatant was removed, the worm culture was refilled with fresh culture medium. This operation was repeated for three days consecutively.

After centrifugation, the supernatant solution obtained was filtered through a 0.2 µm membrane and dialysed using carbonate buffer (see appendix) through a cellulose tube (cut-off 5000 Da, Zelltrans®) at 4°C for 48 hours. Every 12 hours the carbonate buffer was changed. Then the solution was concentrated in Aquacide II. The protein concentration was estimated by BioRad Protein Assay®, a microtiter method that uses a colorimetric reaction readable with photometer at 570 nm. As standard curve an albumin serial dilutions were used. The antigen solution was then collected in 1 ml Eppendorf® tubes and stored at -20°C.

Somatic antigen

For the preparation of whole-worm So. antigen, adult trematodes were washed several times in washing solution (see appendix). Approximately 200 washed flukes were placed into 10 ml phosphate buffer solution (PBS, see appendix) and sonicated by an ultrasound processor with pulse-type vibration for l min and 3 pulses of 30" each (GONZALEZ-LANZA *et al.*, 2000). Finally, the suspension was centrifuged at 10 000 r.p.m. for 30' and the supernatant fraction collected. After estimating the protein concentration by BioRad Protein Assay (see above), the antigen was stored in 1 ml-aliquots at -20 °C until used.

3.3.2 Setting ELISA parameters

Optimal dilutions of the antigen, serum and conjugate were estimated by checkerboard titration as follow.

3.3.2.1 Titration of the antigen. Flat-bottomed polystyrene microtiter plates were coated with 50 μl/well of decreasing dilutions of antigen (E/S and So. antigen respectively) from line A to line G (see Table 1). For antigen dilution, carbonate buffer was used. Then the plate was incubated for 1 hour at 37° C, and washed once with distilled water, three times with PBS-Tween (see appendix) for five minutes each.

3.3.2.2 Titration of serum. The positive serum sample (150 d.p.i.) was diluted 1:10 in PBS-Tween and titrated from column 1 to column 11, as in the table. The wells of column 12 were coated with pure PBS-Tween. Incubation for 30 minutes at at 37° C followed. The plate was then washed once with distilled water and three times with PBS-Tween (see appendix) for five minutes each.

Table 1. Serial dilution of antigen and sera in an ELISA plate

	Serum dilutions	**1:10**	**1:20**	**1:40**	**1:80**	**1:160**	**1:320**	**1:640**	**1:1280**	**1:2560**	**1:5120**	**1:10240**	PBS-Tween
Antigen dilutions		1	2	3	4	5	6	7	8	9	10	11	12
1:1	A												
1:2	B												
1:4	C												
1:8	D												
1:16	E												
1:32	F												
1:64	G												
Carbonate buffer	H												Blank

3.3.2.3 Conjugate and substrate. As secondary antibody, a peroxidase-conjugated rabbit anti-sheep immunoglobulin (DAKO, Denmark) was used at a concentration of 1:1500, diluted in PBS-Tween. Incubation for 30 minutes at 37° C followed. The plate was then washed once with distilled water, three times with PBS-Tween and once more with distilled water for five minutes each.

Finally the peroxidase substrate was added: 2, 2'azino-bis (3-ethylbenzthiazoline 6 sulfonic acid) was used (ABTS, Sigma), which gives a colorimetric reaction after 20-30 minutes, readable with a photometer (Dynatech Laboratories) at 405 nm.

The checkerboard titration also allows visualisation of non-specific binding interactions between the polystyrene substrate of the plate and the serum (not antigen-coated wells, line H), and between the antigen and the conjugate (where no serum was incubated, column 12). Moreover, the blank well (H12, neither antigen, nor serum incubation) allows verification of whether the plate was well washed between the different steps, and, not less important, if the conjugate reacts with the polystyrene substrate of the plate.

The blank value also allows establishment of the "background effect" of the ELISA test. Non-specific background effects may occur to a varying extent in different ELISA systems. The specific reaction can be calculated subtracting the background extinction value (E_{blank}) from the absolute extinction value of the respective antigen-coated well (E_a). The resulting value is called relative extinction value (E_r) (STAAK *et al.*, 2001).

3.3.2.4 Implementation of ELISA test

Flat-bottomed microtitre plates were coated with *D. dendriticum* antigen (E/S or So) in carbonate buffer (pH 9.6) at 1.7 μg/ml (50 μl/well) and then incubated for one hour at 37°C. After removal of the antigen, the plates were washed once with distilled water and three times with PBS-Tween for 5 min each. Afterwards, 50 μl sheep serum diluted l:100 in PBS-Tween was added to each well and incubated for 30 minutes at 37 °C. Tween-20 is a detergent that prevents antibody binding to the free sites of the polystyrene plates, avoiding non-specific reactions[1].

After a further washing, 50 μl anti-sheep IgG peroxidase conjugate (Dako, Denmark) diluted to l :1500 in PBS-Tween was added to each well. The plates were incubated for 30 minutes at 37 °C and then washed as previously described, with a final destilled water washing. Fifty microliters of peroxidase substrate solution (2,2'-azino-bis (3 -ethylbenzthiazoline-6-sulfonic acid (ABTS, Sigma) were added to each well and the plates were incubated for 15-30 min in darkness at room temperature. The optical

[1] Some other blocking solutions can be used (skimmed milk 5%, albumin, etc.) but in our experience the detergent effect of Tween-20 (0,05%) was sufficient.

density values were read at 405 nm using an automated microplate photometric reader (Dynatech Laboratories) connected to a computer using Mikrowin software (Mikrotech, 1995) to read, export and elaborate the results. Each serum sample was tested in duplicate and the results were expressed as the mean of the OD values obtained.

3.3.2.5 Cut-off definition

In order to compare the results in each microtitre plate two reference values were used: a positive control serum sample from a sheep highly infected with *D. dendriticum* and a blank in which the serum was substituted with PBS-Tween.

In our case, positive control serum was obtained from experimentally infected lambs, whose infection was confirmed at slaughtering. Negative control serum was obtained from 17 *Dicrocoelium*-free lambs that never had access to the outside.

In order to draw diagnostic conclusions out of quantitative ELISA data (optical density), a threshold value (cut-off value, or critical value) was defined. This value is commonly defined as the mean plus three standard deviations of the results observed in the sera of negative control samples (RICHARDSON *et al.*, 1983), when they are normally distributed[2] (GREINER *et al.*, 1995).

3.3.3 Cross-reaction with other parasite infections

Serum samples from sheep infected by other helminths were tested by the ELISA *Dicrocoelium* test to investigate eventual cross-reactions.

3.3.3.1 Cross-reaction with Fasciola hepatica and Paramphistomum spp.

Eleven serum samples of sheep naturally infected by *Fasciola hepatica* and *Paramphistomum* spp**.** were tested with *D. dendriticum* antigens. These sample were taken from sheep of a farm next to Müritz lake, (Mecklenburg, Germany), an area characterised by wet pastures all year-round, where the intermediate host of *F. hepatica*, the snail *Galba truncatula,* has a suitable habitat condition for its survival. Therefore, this area has been classified as *Fasciola* biotope.

Ten sheep were coprologically positive for *F. hepatica* and one for *Paramphistomum* spp.

[2] However it should be borne in mind that, in general, such a determination is not obligatory and a critical evaluation of the application of results in the actual situation has to be made. By using a low cut-off, the test becomes more sensitive, which may be useful to exclude the infection in question or to screen asymptomatic individuals. On the other hand a higher cut off increases the specificity of the test. This is useful when a clinical suspicion has to confirmed or to diagnose a fatal disease (GRINER *et al.*, 1981; GALEN, 1982).

3.3.3.2 Cross-reaction with nematode parasite infection

Seventeen serum samples of sheep experimentally infected with different species of nematode parasites were tested. The serum samples were kindly provided by Dr. S. Rehbein (Merial) and by Dr. C. Bauer (Institute for Parasitology, University of Giessen). Ten of these samples were from lambs infected with a single infection dose of L_3 larvae of *Nematodirus battus, Trichostrongylus colubriformis,* and *Haemonchus contortus*. The remaining seven serum samples were from lambs experimentally infected with four repeated infection doses during two weeks, consisting of different nematode species (*Dictyocaulus filaria*, *Oesophagostomum venulosum*, *Ostertagia ostertagi*, *T. colubriformis*, *Cooperia curticei*, *N. battus*) with a total infection dose of 27000 larvae.

3.4 Enzyme Immune Transfer Blotting (EITB).

The enzyme-linked immunoelectrotransfer blotting technique (EITB) combines the high resolving power of gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the high sensitivity of Enzyme-Linked Immunosorbent Assay (ELISA) to produce an extremely powerful qualitative tool for studying antigen-antibody pairs. Using this procedure, antigens electrophoretically resolved on SDS-PAGE are transferred onto nitro-cellulose membrane or diazo sheets and identified by ELISA methods (TSANG *et al.*, 1983).

In our specific case the application of EITB for the qualitative analysis of *D. dendriticum* E/S and So. antigen was used to assess the results obtained with ELISA test, and in particular:

- to analyse the protein band pattern of the two antigen solutions;
- to study the immune response along the time-course in the experimentally infected lambs;
- to put in evidence eventual non-specific immunological answers of any protein bands with sera from animals infected with other helminths.

As EITB protocols and technical procedures the guidelines by WESTERMEIER (1990) were followed.

3.4.1. Sample treatment

According to TSANG *et al.* (1983) the optimal sample concentration for EITB is 5-10 μg of total protein in 10-100 μl for each sample lane. In our case the initial antigen concentration was too low, especially for the E/S antigen solution, and no band was visible on SDS-PAGE. Therefore, the protocol for protein concentration proposed by Parsch (PARSCH *et al.*, 2001) was adopted. Trichloroacetate (TCA) was added to the antigen solution at a final concentration 20%. The solution was then stored at –20°C for five minutes and at +4°C for 15 minutes. Then the tube was spun at 14 000 r.p.m. for ten minutes, the supernatant removed, and the proteic pellet washed with 200 μl of HCl-acetone (5%) for ten minutes at –20°C.

The protein sample was heated at 95°C for five minutes, and, after cooling, 2 μl of dithiotrheitol (DTT) solution (50 %) were added.[3]

[3] Sample buffer contains, among other compounds (see appendix), sodium dodecyl sulphate (SDS, detergent compound), dithiotrheitol (DTT, reducing compound), bromophenol blue (electrophoresis

3.4.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE was performed using prepared gel slabs (ExcelGel® 2-D Homogeneous 12,5, Pharmacia Biotech). This has the advantage of avoiding the step of acrylamide gel preparation, saving time and improving repeatability. The gel size is 250x110x0.5 mm, with 25 available lanes. A 6% acrylamide stacking zone on the catodic side merges continously into a homogeneous 12,5% acrylamide separation zone. Such gel slabs are designed for horizontal electrophoresis using buffer strips (ExcelGel SDS Buffer Strips, Pharmacia Biotech, NJ) instead of a buffer bath. These polyacrylamide buffer strips contain all the buffers needed for SDS electrophoresis, and are ready to use.

The gel slab was placed on a cooling plate[4] (13°C) of a horizontal electrophoresis unit (Multiphor II Unit, Pharmacia Biotech), and the buffer strips were positioned at cathodic and anodic sides and protein samples charged on the respective lanes. As molecular weight markers, prestained Low Molecular Weight (LMW) SDS Marker Kit (Amersham Biosciences) were used. The LMW band pattern showed 6 bands: 97, 66, 45, 30, 20 and 14 kDa.

Electrodes were connected to a power supply (EPS 600, Pharmacia) with the following run parameters: 600 V, 50mA, for 80 minutes.

When the bromophenol blue marker had reached the anodic side, the power supply was switched off, and the gel taken and stained with 0.02% Coomassie Brilliant Blue in 10% acetic acid solution at 50°C for eight minutes. In order to visualise the proteic bands, decoloration followed in 10% acetic acid solution for one hour.

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colour marker). When the proteins are denatured with excess of SDS, the detergent binds the polypeptide at constant mass ratio (1,4 g SDS per gram polypeptide) and the polypeptide is organized into a rodlike structure. The agent DTT efficiently reduced disulphide bonds that are broken between and within the chains. The bromophenol blue is a coloured compound that migrates in the electric field of electrophoresis and confirm that the migration process has happened.

[4] By cooling, the gel is prevented from dissolving and maintains a suitable consistence; in this way protein bands appear clear. Between the cooling plate and the gel support film some drop of kerosene are placed to allow a good contact for heat transmission.

3.4.3 Blotting

Blotting means transferring macromolecules, in this case proteins, onto a nitro-cellulose membrane[5]. There are several techniques available. We used the Semi Dry Blotting method (KHYSE-ANDERSEN, 1984); this has several advantages if compared to the traditional Tank Blotting (see literature review). Protein transfer from the gel to the nitro-cellulose membrane takes place between two high-circuit-capacity graphite plates, at low voltage and low current intensity (10 V, 200mA), thus no cooling is required. Gels and membranes are wet with buffers between a filter paper sandwich. The filter paper sheets are soaked with different transfer buffers (Cathode buffer, Anode buffer I, Anode buffer II, see appendix)[6], the so called discontinuous buffer system (see literature review), that should guarantee efficient and regular protein transfer, and clear and sharp protein bands.

Procedure. After electrophoresis, the gel is taken from the electrophoresis chamber and equilibrated in Anode buffer II (see appendix). Filter paper sheets are wet with the various transfer buffers (nine sheets with Cathode buffer, six sheets with Anode buffer I and three sheets with Anode buffer II), the nitro-cellulose membrane is wet with Anode buffer II. The gel is removed from the support plastic film and the "sandwich" assembled and positioned between the graphite plates (Nova Blot Kit, Pharmacia Biotech) as showed in Figure 3. Air bubbles are avoided by using a roller.

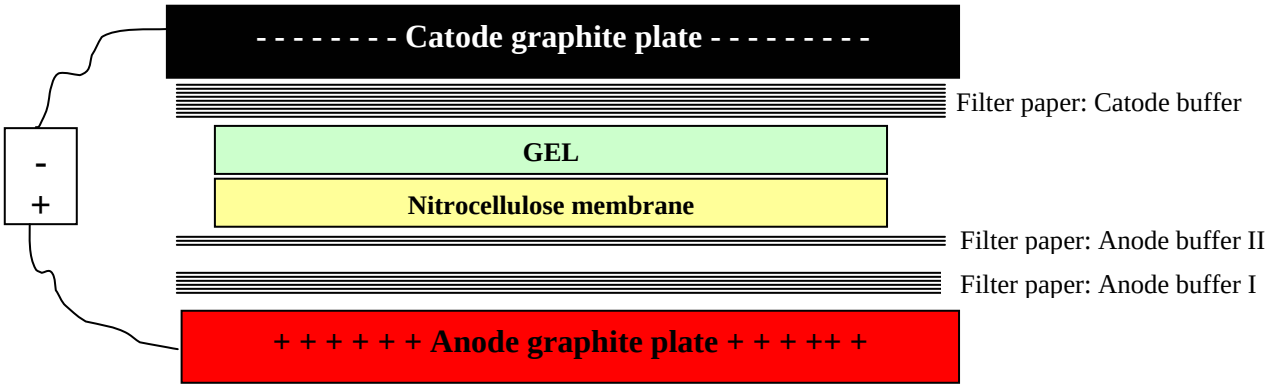


Figure 3. Scheme of the SemiDry blotting *sandwich*.

[5] There are many material used for blotting membranes: nitrocellulose, nylon, polyvinylidenfluorid (PVDF), diazobenzyloxymethyl (DBM) cellulose paper, etc. We preferred nitrocellulose membranes (0,45 μm pore size), because of good binding capacity, semplicity of use and storage and low cost.
[6] All the transfer buffers contain 20% methanol, by means of which the gel does not pour and the binding capacity of nitrocellulose is increased.

Once the sandwich is ready, the graphite plates are connected to a power supply (EPS 600, Pharmacia), run parameters are set (10V, 200 mA) and the transfer was run for one hour. Then the power was switched off, the sandwich was separated, the molecular mass marker side on the nitro-cellulose membrane was marked, and the membrane was soaked in PBS for five minutes. Confirmation of the protein transfer was done by staining the membrane with Ponceau S (see appendix) reversible staining (SALINOVICH *et al.*, 1986), which gives a red coloration of the protein bands (see appendix). The strip of membrane with the molecular mass markers was cut away and stained with Amido Black solution (see appendix).

The Ponceau coloured nitro-cellulose membrane was destained in distilled water-NaOH 0.2M and cut into thin strips (0.5 cm of width).

3.4.4 Immunoassay

Through the immunoassay procedure the antigen-antibody binding reaction between antigenic protein bands and anti-*Dicrocoelium* antibodies was tested.

The nitro-cellulose strips with protein bands were put into "Mini Incubation Trays" (BioRad Laboratories) and soaked in blocking buffer[7] (see appendix) for 30 minutes at room temperature. Afterwards the strips were incubated with 800 μl of the primary antibody solution (the sheep serum samples, 1:100) at 37°C for 45 minutes, using a horizontal shaker.

Three washings followed. The strips were put in a wash tray (BioRad) and washed three times with five ml TRIS washing buffer (see appendix) for 15 minutes each, while shaking.

During the last washing, the secondary antibody solution was prepared. Anti-sheep IgG-alkaline phosphatase conjugate (whole molecule, Sigma) was diluted 1:2000 with conjugate buffer (see appendix) and 800 μl per lane were put in a clean incubation tray.

The strips were removed from the wash tray and incubated with the conjugate solution at 37°C for 30 minutes, while shaking.

Afterwards three washings followed as described above.

[7] The blocking buffer is a solution containing an inert protein (albumin, skimmed milk, gelatine, etc) that can "occupy" the free sites on the membrane, so the antibody solution can bind only to the sites where the antigenic protein is set. In this way non-specific reactions are avoided or at least greatly reduced.

The colorimetric reaction was performed using substrate solution (SIGMA *Fast* BCIP/NBT tablets, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, see appendix) in which the nitro-cellulose strips were incubated for ten minutes at room temperature. The colorimetric reaction was stopped with distilled water, the strips were dried on filter paper and stored in darkness until measurement.

3.4.5 Determination of protein molecular weight

The molecular mass of protein bands was estimated by comparing the migration distance of the sample with that of known molecular mass markers (QURESHI *et al.*1995*).*

A linear relationship exists between the logarithm of the molecular weight of an SDS-denatured polypeptide and its relative mobility coefficient (*Rf).* The *Rf* is calculated as the ratio between the migration distance of a molecule to that of a marker dye-front. A method to determine the relative molecular weight *(M*r) is to plot a standard curve of migration distances vs. $\log_{10}$ molecular weights for known samples (markers), and read off the log*Mr* of the sample after measuring its migration distance on the same gel.

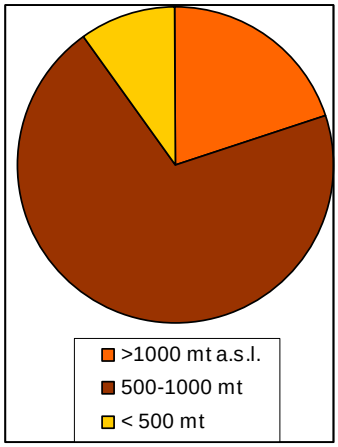
3.5 Field serum samples of sheep flocks from province of Trento (Italy)

Thanks to the collaboration of *Istituto Zooprofilattico Sperimentale delle Venezie*, *Sezione di Trento*, 892 field serum sample from 14 sheep flocks from the province of Trento (northeastern Italy) were available for *Dicrocoelium*-ELISA testing. A preliminary study of serological prevalences of dicrocoeliosis in province of Trento is the aim of this test, since so far there are no data available on this.

The province of Trento is located in the northeastern part of the Alps (see Figure 4).

Figure 4. Geographical position of the province of Trento

The territory of the province of Trento has a surface of 6.233 km², with altitude range between 65 m a.s.l. (Garda lake), and 3764 m a.s.l. (Mt. Cevedale). The percentage of highlands is much bigger than lowlands: 70% of the territory is above 1000 m a.s.l., 20% is between 500 and 1000 m a.s.l. and only 10% is under 500 m a.s.l. (see Figure 5). The many rivers, lakes and forests confer a typical alpine appearance to the territory. Half of the territory is covered by forest, a quarter by pasture, 11% by rock and glaciers and only 3% is urbanised (see Figure 6).



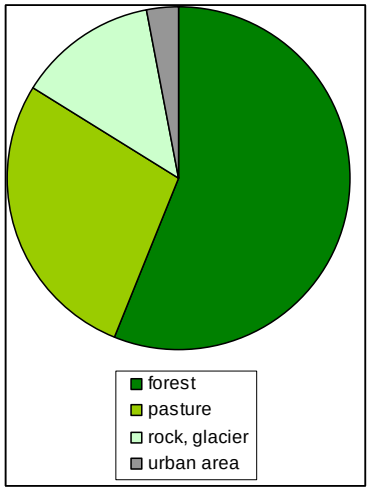


Figure 5. Altitude range in province of Trento. **Figure 6. Land cover types in province of Trento**

The wide altitude range of this region generates a great climatic variety, classified in types, classes, zones, and subzones. Nevertheless, all the climatic types in Trentino reflect the alpine characteristic: the most common type could be ascribed to the humid mesotermic classes (oceanic temperate type), this means without arid season, with dry cold winter and fresh rainy summer (Data source: Provincia Autonoma di Trento).

In the province of Trento there are approximately 19 000 sheep. Sheep flocks are managed both as transhumant flocks, migrating during the summer from the lowlands to alpine pastures, and as stable flocks in the alpine lands, using extensive pastures in the summer.

All these elements, above all the mountainous extensive pastures, represent suitable habitats for intermediate hosts of *D. dendriticum* (ECKERT and HERTZBERG, 1994). Many snail species (e.g. *H. obvia, Bradybaena frumentum, Cochlicopa lubrica*) and several species of ants belonging to the subfamily Formicinae (e.g. *F. pratensis, F. cunilicularia*) are known to occur up to an altitude of 1800-2600 m a.s.l., on mountainous pastures in Austria (which is contiguous to Trentino-Alto Adige), Switzerland and Spain (GEBAUER and HOHORST, 1968; MEIER, 1987; MANGA-GONZALEZ *et al.*, 1991).

There are various reports about the distribution of dicrocoeliosis in Italy (AMBROSI and POLIDORI, 1978; MARTINI *et al.*, 1986; BATTELLI *et al.* 1988; SCALA *et al.*, 1991; CRINGOLI *et al.*, 2002; SÁNCHEZ-ANDRADE *et al.*, 2003) but no data about the

distribution and prevalence of dicrocoeliosis of sheep in the province of Trento have been published to date. In this area dicrocoeliosis was reported in wild ruminants (red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and chamois (*Rupicapra rupicapra*) (BROGLIA and LANFRANCHI, 2002). It must be considered that in the alpine environment in the last decades there has been a marked increase of wild ungulates and of domestic sheep. These phenomena lead to a situation of overlapping of distribution areals of domestic and wild animal populations, a fact that can have severe epidemiological consequences (ROSSI *et al.*, 1997), such as the interspecific transmission of some parasites (BROGLIA, 2001). In the case of dicrocoeliosis, such an event may occur, therefore epidemiological studies on both wild and domestic animals in the alpine environment may contribute to understand better these phenomena.

3.5.1 Serum samples

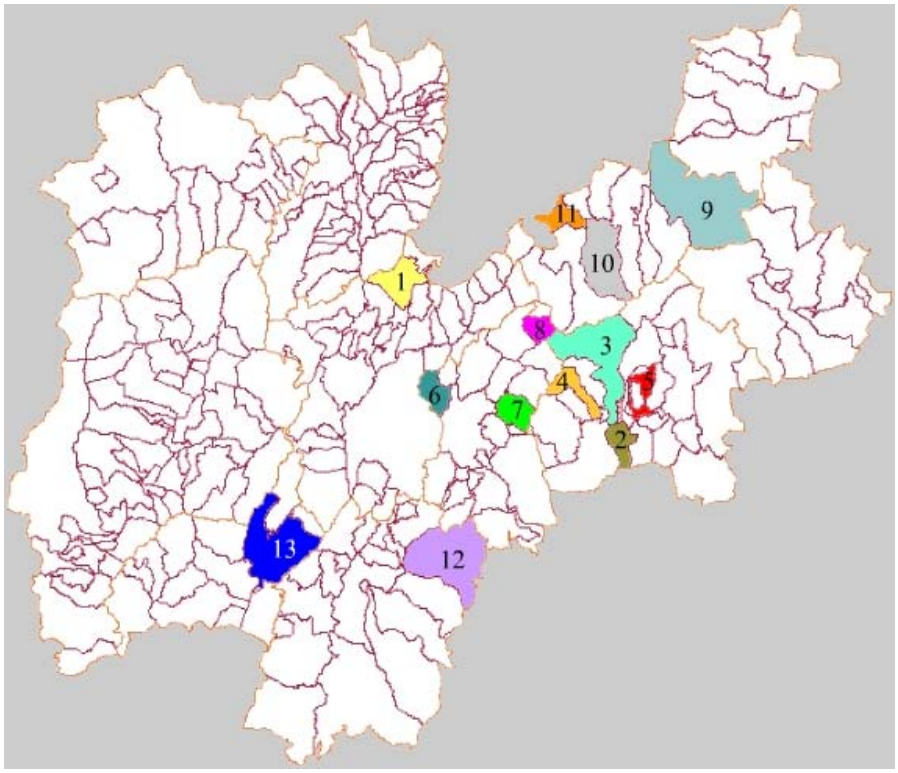
The serum samples were collected by the staff of the *Istituto Zooprofilattico Sperimentale delle Venezie*, *Sezione di Trento*, during the routine test for brucellosis control.

The different origin of the samples is presented in Table 2 (municipalities and districts are indicated).

Table 2. Sheep flocks, municipality, district, and number of serum samples.

	Municipality	**District**	**Nr. of samples**
1	Mezzocorona	Valle dell'Adige	50
2	Castelnuovo	Bassa Valsugana e Tesino	50
3	Telve	Bassa Valsugana e Tesino	50
4	Torcegno	Bassa Valsugana e Tesino	50
5	Strigno	Bassa Valsugana e Tesino	50
6	Civezzano	Alta Valsugana	50
7	Frassilongo (1)	Alta Valsugana	50
7bis	Frassilongo (2)	Alta Valsugana	50
8	Baselga	Alta Valsugana	50
9	Predazzo	Val di Fiemme	242
10	Cavalese	Val di Fiemme	50
11	Carano	Val di Fiemme	50
12	Folgaria	Vallagarina	50
13	Arco	Alto Garda e Ledro	50
Total			**892**

The municipalities are represented in Figure 7.



1 - Mezzocorona
2 - Castelnuovo
3 - Telve
4 - Torcegno
5 - Strigno
6 - Civezzano
7 – Frassilongo (2 flocks)
8 - Baselga
9 - Predazzo
10 - Cavalese
11 - Carano
12 - Folgaria
13 - Arco

Fig. 7. Province of Trento. Municipalities from which serum samples were collected.

Fifty serum samples from each sheep flock were tested, and 242 serum samples from the stable sheep flock in Predazzo (municipality nr. 9 Fig. 7, district: Val di Fiemme), where dicrocoeliosis in wild ruminants was reported previously (BROGLIA and LANFRANCHI, 2002).